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Purification and cDNA cloning of the AdoMet-binding subunit of the human mRNA (N^6-adenosine)-methyltransferase

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ABSTRACT

The methylation of internal adenosine residues in eukaryotic mRNA, forming N^6-methyladenosine (m^6A), is catalyzed by a complex multicomponent enzyme. Previous studies suggested that m^6A affects the efficiency of mRNA processing or transport, although the mechanism by which this occurs is not known. As a step toward better understanding the mechanism and function of this ubiquitous posttranscriptional modification, we have shown that HeLa mRNA (N^6-adenosine)-methyltransferase requires at least two separate protein factors, MT-A and MT-B, and MT-A contains the AdoMet binding site on a 70-kDa subunit (MT-A70). MT-A70 was purified by conventional chromatography and electrophoresis, and was microsequenced. The peptide sequence was used to design a degenerate oligodeoxynucleotide that in turn was used to isolate the cDNA clone coding for MT-A70 from a HeLa cDNA library. Recombinant MT-A70 was expressed as a fusion protein in bacteria and was used to generate anti-MT-A70 antisera in rabbits. These antisera recognize MT-A70 in HeLa nuclear extracts by western blot and are capable of depleting (N^6-adenosine)-methyltransferase activity from HeLa nuclear extract, confirming that MT-A70 is a critical subunit of (N^6-adenosine)-methyltransferase.

Northern blot analysis reveals that MT-A70 mRNA is present in a wide variety of human tissues and may undergo alternative splicing. MT-A70 cDNA probe hybridizes to a 2.0-kilobase (kb) polyadenylated RNA isolated from HeLa cells, whereas it hybridizes to two predominant RNA species (approximately 2.0 kb and 3.0 kb) using mRNA isolated from six different human tissues. Analysis of the cDNA sequence indicates that it codes for a 580-amino acid protein with a predicted MW = 65 kDa. The predicted protein contains sequences similar to consensus methylation motifs I and II identified in prokaryotic DNA (N^6-adenosine)-methyltransferases, suggesting the functional conservation of peptide motifs. MT-A70 also contains a long region of homology to the yeast protein SPO8, which is involved in induction of sporulation by an unknown mechanism.

Keywords: N^6-methyladenosine; nucleoside modification; posttranscriptional processing

INTRODUCTION

Methylation of the N^6-amino group of internally located adenosine residues occurs in mRNA of all higher eukaryotes and in the RNA of viruses that have a nuclear phase in their life cycle (for review, see Narayan & Rottman, 1992). N^6-methyladenosine (m^6A) does not occur randomly, but is found only within specific sequences matching the degenerate consensus RRm^{6}ACH (where R = purine, H = C, A, or U) (Schibler et al., 1977; Wei & Moss, 1977; Nichols & Welder, 1981). Although m^6A is present at an average of 1–3 residues per mRNA in mammalian cells when total polyadenylated RNA is analyzed, its distribution among individual mRNA is not random. Examples of m^6A abundance are approximately 1 residue in bovine prolactin mRNA (Horowitz et al., 1984), 3 residues in mouse dihydrofolate reductase mRNA (Rottman et al., 1986), and from 1 to 15 residues per RNA in SV40, adenovirus 2, Rous sarcoma virus, and influenza virus (Sommer et al., 1976; Beemon & Keith, 1977; Canaani et al., 1979; Narayan et al., 1987). Some cellular mRNAs, including bovine growth hormone and mouse globin,

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do not contain detectable m^{6}A (Perry & Scherrer, 1975; Horowitz et al., 1984), suggesting that this modification is not an absolute requirement for mature mRNA function, but more likely serves some regulatory role. m^{6}A is also present in snRNA, tRNA, and rRNA molecules, however, the methylated residues in these RNAs do not fall within sequences that match the conserved consensus determined for mRNA methylation (Bjork et al., 1987; Maden, 1990; Shimba et al., 1995). Furthermore, it recently has been demonstrated that the enzyme responsible for U6 snRNA methylation is distinct from the mRNA N^6-adenosine methyltransferase (m^{6}A-MT) (Shimba et al., 1995).

Determining the functional significance of m^{6}A in mRNA has proven to be difficult. Mutation of subsets of the methylation sites in RSV did not lead to any measurable effects on steady-state RNA levels, viral replication, or infectivity (Kane & Beemon, 1987; Csepany et al., 1990). Attempts to block m^{6}A formation in cellular messages have exploited the use of methylation inhibitors. These studies have shown that decreased levels of m^{6}A correlate with a delay in the appearance of newly transcribed mRNA in the cytoplasm and an accumulation of unspliced pre-mRNA in the nucleus (Stoltzfus & Dane, 1982; Finkel & Groner, 1983; Camper et al., 1984; Carroll et al., 1990). The interpretation of these experiments is complicated, however, by the pleiotropic effects of such inhibitors on other methyltransferases. Little is known about the regulation of m^{6}A-MT activity in various cell types or developmental stages. It was reported recently by Tuck (1992) and Tuck et al. (1996) that m^{6}A-MT activity in a variety of cell types is increased as a result of cellular transformation. It is not known whether the amount of m^{6}A is increased in transformed cells in mRNA in general or in specific transcripts. However, this finding remains intriguing and suggests that m^{6}A may play an interesting role in mRNA metabolism.

A more direct analysis of m^{6}A function requires the availability of the cDNA encoding this methyltransferase. We have therefore set out to purify and clone m^{6}A-MT from HeLa cells. Our initial characterization and partial purification of the enzyme revealed that m^{6}A-MT is surprisingly complex, consisting of multiple protein components (Bokar et al., 1994). Two of these, MT-A (M_r = 200 kDa) and MT-B (M_r = 875 kDa), are required for in vitro methylation of a bovine prolactin mRNA-derived sequence. MT-A, in turn, is comprised of at least two subunits, and one of these (MT-A70) contains the S-adenosylmethionine-(AdoMet-) binding site. In this study, we have purified MT-A70 and have isolated a full-length cDNA derived from its mRNA. Antibodies generated against recombinant MT-A70 are able to specifically deplete nuclear extracts of m^{6}A-MT activity, verifying that the cDNA codes for a critical component of the m^{6}A-MT complex. The availability of this clone should allow studies of the developmental and tissue-specific regulation of m^{6}A-MT, and will make possible the exploration of the function of mRNA methylation by specifically decreasing expression of this critical methyltransferase subunit through antisense or gene knockout studies.

RESULTS

Purification of the AdoMet-binding subunit of HeLa mRNA m^{6}A-MT

Previously, we reported partial purification of three components that appeared necessary for methyltransferase activity in vitro, and designated them MT-A1 (M_r = 30 kDa), MT-A2 (M_r = 200 kDa), and MT-B (M_r = 875 kDa) (Bokar et al., 1994). In more recent experiments, the presence of factor MT-A1 has not been required for maximal activity. The reason for this discrepancy is not known, but may be due to a difference in the HeLa cells used (commercially grown), or to subtle differences in the preparative purification procedure, which utilizes larger volumes of material over a significantly shorter time. Therefore, further characterization of MT-A1 has not been pursued and our nomenclature has been simplified to reflect this change. The factor previously designated MT-A2 is now referred to simply as MT-A. The requirement for both MT-A and MT-B, as well as the chromatographic properties of MT-A and MT-B, are otherwise as reported previously. Figure 1A illustrates the sequence of chromatographic steps found to be optimal for the highest purification of MT-A. A representative activity profile is shown for the further fractionation of fraction QS-A on the Superose 6 column (Fig. 1B). The elution of MT-A activity coincides with that of the β-amylase standard with M_r = 200 kDa. Further fractionation of fraction Sup6-A on the heparin Sepharose column is also shown (Fig. 1C). As shown previously, methyltransferase activity is detected only when aliquots of column fractions are supplemented with MT-B (partially purified, fraction QS-B).

Aliquots of the heparin affinity fractions were subjected to UV crosslinking with [^{3}H-*methyl*]AdoMet, followed by precipitation and SDS-PAGE (Fig. 1D). A predominant ^{3}H-labeled protein with an M_r = 70 kDa is present in fractions 7–9, which correlates with the elution of MT-A activity. A 70-kDa AdoMet-crosslinked protein also co-eluted with MT-A activity when fractions were analyzed from the gel filtration and cation exchange columns (data not shown). These results suggest that the 200-kDa MT-A is a multimeric protein that contains a 70-kDa AdoMet-binding subunit hereafter referred to as MT-A70. The composition of the remaining portion of MT-A is not known, nor is the stoichiometry of MT-A70 within MT-A known. Based on the co-elution of MT-A70 with MT-A activity, fractions 7–9 were pooled, concentrated, and separated by

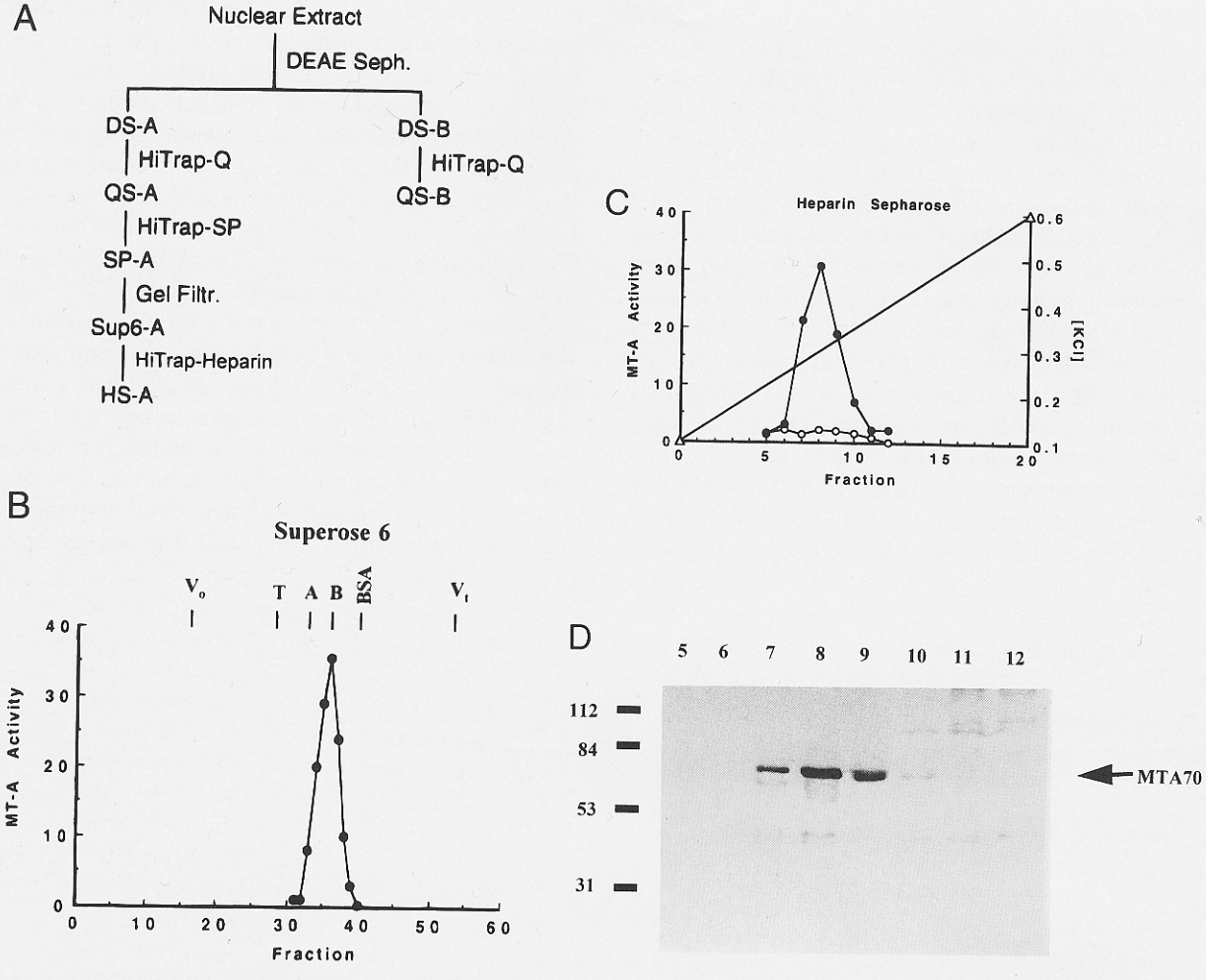

FIGURE 1. Purification of MT-A70. **A:** Summary of chromatographic steps utilized in the partial purification of the two components, MT-A and MT-B, required for m^{6}A-MT activity. **B:** Representative activity profile of the Superose 6 (gel filtration) fractions. Aliquots of the fractions shown were assayed using supplementation with an aliquot of fraction QS-B. In the absence of supplementation, no activity above background was routinely observed. V_0, excluded volume; V_t, total included volume; T, thyroglobulin, M_r = 669,000; A, apoferritin, M_r = 443,000; B, β-amylase, M_r = 200,000; BSA, bovine serum albumin, M_r = 66,700. **C:** Representative activity profile of the heparin Sepharose (HiTrap-heparin) column fractions. Closed circles represent the activity of aliquots of the fractions when assayed with supplemented QS-B; open circles are without QS-B. The KCl concentration units are molar. **D:** [^{3}H-*methyl*]AdoMet-UV crosslinking. Aliquots of the same fractions assayed in C were crosslinked to [^{3}H-*methyl*]AdoMet as described in Materials and Methods, separated by 8% SDS-PAGE, transferred to PVDF membrane, and fluorographed. The 70-kDa band that co-elutes with MT-A activity is shown (MT-A70).

preparative SDS-PAGE, with UV-crosslinked aliquots run in parallel lanes. After electrophoresis, the proteins were transferred to PVDF membrane and the crosslinked lanes were excised and fluorographed. The remainder of the membrane was stained with Coomassie blue. A well-resolved protein band that co-migrated with the crosslinked protein (not shown) was excised from the stained membrane, subjected to digestion with trypsin, HPLC separation, and peptide micro-sequence analysis (W.S. Lane, Harvard Microchemistry Facility). The estimated yield of protein was 3 μg (~40 pmol) or ~$1/10^{-5}$ of the starting material. The yield of enzymatic activity, and therefore the overall degree of purification, could not be estimated because the final purification step depended upon denaturation of MT-A.

Isolation and characterization of cDNA pMT-A70

Peptide sequences were obtained from three peptides, PT60, PT63, and PT66, which ranged in length from 10 to 16 amino acids. A mixture of oligodeoxynucleotides, with 144-fold degeneracy and length of 20 nt, corresponding to a region of peptide PT60, was synthesized (Fig. 2A) because this peptide region contained a stretch of amino acid sequence of high confidence as judged by Harvard Microchemistry and had the least codon degeneracy. This degenerate oligodeoxynucleotide was then used to isolate clones from a HeLa cell plasmid cDNA library using the Genetrapper cDNA Positive Selection System (Life Technologies). The degenerate oligodeoxynucleotide shown in

Figure 2A was coupled to biotin, and was hybridized with single-stranded cDNA-containing plasmids derived from a HeLa cell cDNA library (HeLa pCMV-SPORT, Life Technologies). The plasmids that were selected by solution hybridization and binding to streptavidin-linked paramagnetic beads were then repaired to double-stranded plasmids, transformed into ElectroMax DH10B cells, and plated. More than 2,000 transformed colonies were screened by hybridization to the same degenerate oligodeoxynucleotide labeled with T4 polynucleotide kinase and [γ-^{32}P]-ATP. Approximately 10% of the colonies were positive by hybridization, and 10 of these were analyzed further by restriction analysis and partial sequencing of their plasmids. The inserts ranged from 1 to 2 kb in length, and all 10 clones contained identical seqences at their 3′ termini. The plasmid with the longest cDNA insert was selected for further analysis by complete sequencing, and is referred to as pMT-A70. Nucleotide sequencing was performed at least twice on each strand using a combination of conventional dideoxynucleotide sequencing and automated sequencing (ABI Prism). All three peptides obtained directly from the protein match the predicted cDNA translation product with a very high degree of fidelity (Fig. 2B). Peptide sequence PT60 matches at 15 of 16 residues (Glu 15 is redundant). Peptide sequence PT63 matches at 13 of 15 residues, given that the leucine at position 10 and the unidentified amino acid predicted at position 11 are not coded for by the cDNA. Peptide sequence PT66 matches at 14 of 16 residues. A histidine residue at position 8 matches the secondary peptide sequence at that postion, and a threonine is coded for at position 13, whereas an arginine was detected at that position in the peptide. This second discrepancy could be explained by either an error in peptide sequencing, or a

A **Probe Sequence:**

PT60 subsegment -	glu	ile	tyr	gly	met	ile	glu
Oligodeoxynucleotide -	GAR	ATH	TAY	GGN	ATG	ATH	GA
Actual cDNA sequence -	GAA	ATC	TAT	GGC	ATG	ATT	GA

B **PT60:**

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
peptide:	ser	thr	ser	his	lys	pro	asp	glu	ile	tyr	gly	met	ile	glu	[glu]	[arg]
cDNA seq:	ser	thr	ser	his	lys	pro	asp	glu	ile	tyr	gly	met	ile	glu	...	arg

PT63:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
peptide:	ser	asp	ser	pro	val	pro	thr	ala	pro	leu	-	[ser]	(gly)	-	(pro)
cDNA seq:	ser	asp	ser	pro	val	pro	thr	ala	pro	...	...	ser	gly	gly	pro

PT66:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
peptide 1°:	gly	leu	leu	gln	asp	asp	ala	ala	pro	thr	leu	val	(arg)	[tyr]	[ala]	[asp]
peptide 2°:								his					-			
cDNA seq:	gly	leu	leu	gln	asp	asp	ala	his	pro	thr	leu	val	thr	tyr	ala	asp

FIGURE 2. Sequence of MT-A70-derived peptides and cDNA. **A:** Gel-purified MT-A70 was subjected to trypsin digestion, reverse-phase HPLC, and peptide microsequencing (Harvard Microchemistry). The amino acid sequence from peptide PT60 and the corresponding degenerate oligodeoxynucleotide sequence used for the GeneTrapper solution hybridization screen are shown. The oligodeoxynucleotide has 144-fold degeneracy. The corresponding nucleotide sequence from pMT-A70 shown encodes the predicted peptide sequence. **B:** Amino acid sequence obtained from all three sequenced tryptic peptides (PT60, PT63, and PT66) is shown aligned with the peptide sequence predicted by the cDNA nucleotide sequence. The degree of confidence in the peptide sequence is as follows: X = high, [X] = probable/reasonable, (X) = possible/low. At some positions, a secondary sequence was obtained (peptide 2°). This is shown only where the secondary sequence and not the primary sequence matches the cDNA predicted sequence. **C:** Entire pMT-A70 cDNA nucleotide sequence and translation (GenBank accession number pending). The first 49 nt (−119 to −71) of the 5′-untranslated region are not included in pMT-A70, but are derived from the sequence of the subcloned 5′-RACE product. Regions that match the three sequenced peptides (PT60, 63, and 66), the putative consensus methylation motifs (CM I and II), and the putative nuclear localization signals (NLS 1 and 2) are indicated. (*Figure continues on facing page.*)

C

```
                                        -119
                                          cattttccggttagccttcggggtgtccgcgtgagaatt -80

ggctatatcctggagcgagtgctgggaggtgctagtccgccgcgccttattcgagaggtgtcagggctgggagactagg  -1

ATG TCG GAC ACG TGG AGC TCT ATC CAG GCC CAC AAG AAG CAG CTG GAC TCT CTG CGG GAG   60
met ser asp thr trp ser ser ile gln ala his lys lys gln leu asp ser leu arg glu   20
                                    NLS 1

AGG CTG CAG CGG AGG CGG AAG CAG GAC TCG GGG CAC TTG GAT CTA CGG AAT CCA GAG GCA  120
arg leu gln arg arg arg lys gln asp ser gly his leu asp leu arg asn pro glu ala   40

GCA TTG TCT CCA ACC TTC CGT AGT GAC AGC CCA GTG CCT ACT GCA CCC ACC TCT GGT GGC  180
ala leu ser pro thr phe arg ser asp ser pro val pro thr ala pro thr ser gly gly   60
                          PT63

CCT AAG CCC AGC ACA GCT TCA GCA GTT CCT GAA TTA GCT ACA GAT CCT GAG TTA GAG AAG  240
pro lys pro ser thr ala ser ala val pro glu leu ala thr asp pro glu leu glu lys   80

AAG TTG CTA CAC CAC CTC TCT GAT CTG GCC TTA ACA TTG CCC ACT GAT GCT GTG TCC ATC  300
lys leu leu his his leu ser asp leu ala leu thr leu pro thr asp ala val ser ile  100

TGT CTT GCC ATC TCC ACG CCA GAT GCT CCT GCC ACT CAA GAT GGG GTA GAA AGC CTC CTG  360
cys leu ala ile ser thr pro asp ala pro ala thr gln asp gly val glu ser leu leu  120

CAG AAG TTT GCA GCT CAG GAG TTG ATT GAG GTA AAG CGA GGT CTC CTA CAA GAT GAT GCA  420
gln lys phe ala ala gln glu leu ile glu val lys arg gly leu leu gln asp asp ala  140
                                                PT66

CAT CCT ACT CTT GTA ACC TAT GCT GAC CAT TCC AAG CTC TCT GCC ATG ATG GGT GCT GTG  480
his pro thr leu val thr tyr ala asp his ser lys leu ser ala met met gly ala val  160
          NLS 2

GCA GAA AAG AAG GGC CCT GGG GAG GTA GCA GGG ACT GTC ACA GGG CAG AAG CGG CGT GCA  540
ala glu lys lys gly pro gly glu val ala gly thr val thr gly gln lys arg arg ala  180

GAA CAG GAC TCG ACT ACA GTA GCT GCC TTT GCC AGT TCG TTA GTC TCT GGT CTG AAC TCT  600
glu gln asp ser thr thr val ala ala phe ala ser ser leu val ser gly leu asn ser  200

TCA GCA TCG GAA CCA GCA AAG GAG CCA GCC AAG AAA TCA AGG AAA CAT GCT GCC TCA GAT  660
ser ala ser glu pro ala lys glu pro ala lys lys ser arg lys his ala ala ser asp  220

GTT GAT CTG GAG ATA GAG AGC CTT CTG AAC CAA CAG TCC ACT AAG GAA CAA CAG AGC AAG  720
val asp leu glu ile glu ser leu leu asn gln gln ser thr lys glu gln gln ser lys  240

AAG GTC AGT CAG GAG ATC CTA GAG CTA TTA ATT ACT ACA ACA GCC AAG GAA CAA TCC ATT  780
lys val ser gln glu ile leu glu leu leu ile thr thr thr ala lys glu gln ser ile  260

GTT GAA ATT CGC TCT CGA GGT CGG GCC CAA GTG CAA GAA TTC TGT GAC TAT GGA ACC AAG  840
val glu ile arg ser arg gly arg ala gln val gln glu phe cys asp tyr gly thr lys  280

GAG GAG TGC ATG AAA GCC AGT GAT GCT GAT CGA CCC TGT CGC AAG CTG CAC TTC AGA CGA  900
glu glu cys met lys ala ser asp ala asp arg pro cys arg lys leu his phe arg arg  300

ATT ATC AAT AAA CAC ACT GAT GAG TCT TTA GGT GAC TGC TCT TTC CTT AAT ACA TGT TTC  960
ile ile asn lys his thr asp glu ser leu gly asp cys ser phe leu asn thr cys phe  320

CAC ATG GAT ACC TGC AAG TAT GTT CAC TAT GAA ATT GAT GCT TGC ATG GAT TCT GAG GCC 1020
his met asp thr cys lys tyr val his tyr glu ile asp ala cys met asp ser glu ala  340

CCT GGC AGC AAA GAC CAC ACG CCA AGC CAG GAG CTT GCT CTT ACA CAG AGT GTC GGA GGT 1080
pro gly ser lys asp his thr pro ser gln glu leu ala leu thr gln ser val gly gly  360

GAT TCC AGT GCA GAC CGA CTC TTC CCA CCT CAG TGG ATC TGT TGT GAT ATC CGC TAC CTG 1140
asp ser ser ala asp arg leu phe pro pro gln trp ile cys cys asp ile arg tyr leu  380
                                    CM II

GTC GTC AGT ATC TTG GGC AAG TTT GCA GTT GTG ATG GCT GAC CCA CCC TGG GAT ATT CAC 1200
val val ser ile leu gly lys phe ala val val met ala asp pro pro trp asp ile his  400

ATG GAA CTG CCC TAT GGG ACC CTG ACA GAT GAT GAG ATG CGC AGG CTC AAC ATA CCC GTA 1260
met glu leu pro tyr gly thr leu thr asp asp glu met arg arg leu asn ile pro val  420

CTA CAG GAT GAT GGC TTT CTC TTC CTC TGG GTC ACA GGC AGG GCC ATG GAG TTG GGG AGA 1320
leu gln asp asp gly phe leu phe leu trp val thr gly arg ala met glu leu gly arg  440

GAA TGT CTA AAC CTC TGG GGG TAT GAA CGG GTA GAT GAA ATT ATT TGG GTG AAG ACA AAT 1380
glu cys leu asn leu trp gly tyr glu arg val asp glu ile ile trp val lys thr asn  460

CAA CTG CAA CGC ATC ATT CGG ACA GGC CGT ACA GGT CAC TGG TTG AAC CAT GGG AAG GAA 1440
gln leu gln arg ile ile arg thr gly arg thr gly his trp leu asn his gly lys glu  480

CAC TGC TTG GTT GGT GTC AAA GGA AAT CCC CAA GGC TTC AAC CAG GGT CTG GAT TGT GAT 1500
his cys leu val gly val lys gly asn pro gln gly phe asn gln gly leu asp cys asp  500

GTG ATC GTA GCT GAG GTT CGT TCC ACC AGT CAT AAA CCA GAT GAA ATC TAT GGC ATG ATT 1560
val ile val ala glu val arg ser thr ser his lys pro asp glu ile tyr gly met ile  520
                          PT60

GAA AGA CTA TCT CCT GGC ACT CGC AAG ATT GAG TTA TTT GGA CGA CCA CAC AAT GTG CAA 1620
glu arg leu ser pro gly thr arg lys ile glu leu phe gly arg pro his asn val gln  540
                                    CM I

CCC AAC TGG ATC ACC CTT GGA AAC CAA CTG GAT GGG ATC CAC CTA CTA GAC CCA GAT GTG 1680
pro asn trp ile thr leu gly asn gln leu asp gly ile his leu leu asp pro asp val  560

GTT GCA CGG TTC AAG CAA AGG TAC CCA GAT GGT ATC ATC TCT AAA CCT AAG AAT TTA tag 1740
val ala arg phe lys gln arg tyr pro asp gly ile ile ser lys pro lys asn leu      579

aagcacttccttacagagctaagaatccatagccatggctctgtaagctaaacctgaagagtgatatttgtacaatagc 1819
tttcttctttatttaaataaacatttgtattgtaaaaaaaaaaaaaa  1866
```

FIGURE 2C.

single base change (G to C) in the cDNA sequence at position 437. The cDNA length is 1,873 bp, including 70 nt of 5′-untranslated sequence and a 112-nt 3′-untranslated region (Fig. 2C). An open reading frame encodes a 580-amino acid protein with a deduced molecular weight of 65 kDa, in close agreement with the expected M_r = 70 kDa. In order to ensure that the entire amino-terminal coding region was included in pMT-A70, 5′-RACE (Life Technologies) was performed. A single amplification product was obtained, subcloned, and sequenced. This yielded an additional 49 nt of 5′ sequence, but did not alter the predicted translation initiation codon (Fig. 2C, nt −119 through − 70). Inspection of the complete 5′-untranslated region reveals that there are no additional upstream ATG sequences, and there is one in-frame stop codon beginning at nt −108. Comparison of the sequence surrounding the predicted ATG start codon to the Kozak consensus sequence (Kozak, 1996) reveals that the very highly conserved adenosine at position −3 is present, as is the preferred cytosine at position −5.

Expression of recombinant MT-A70

The entire coding sequence of pMT-A70 was subcloned into a six-histidine-tag expression construct (ProEX HT, Life Technologies). Upon treatment with IPTG, a protein with M_r = ~75 kDa (not shown) was induced and was purified with a nickel-NTA affinity resin. Purified recombinant protein (6H-MT-A70) was used to immunize rabbits, and the resulting antisera from three separate animals tested contained anti-6H-MT-A70 immunoreactivity by both ELISA and western blot (not shown). Western blot analysis shows that the antisera recognize a 70-kDa protein in both HeLa nuclear extract and partially purified fractions of MT-A (Fig. 3).

In order to verify that the 70-kDa immunoreactive protein is in fact MT-A70, and represents a critical component of m^{6}A-MT, two types of experiments were performed. In the first, western blots of column fractions confirm that the 70-kDa immunoreactive protein co-purifies precisely with MT-A70 activity using high-

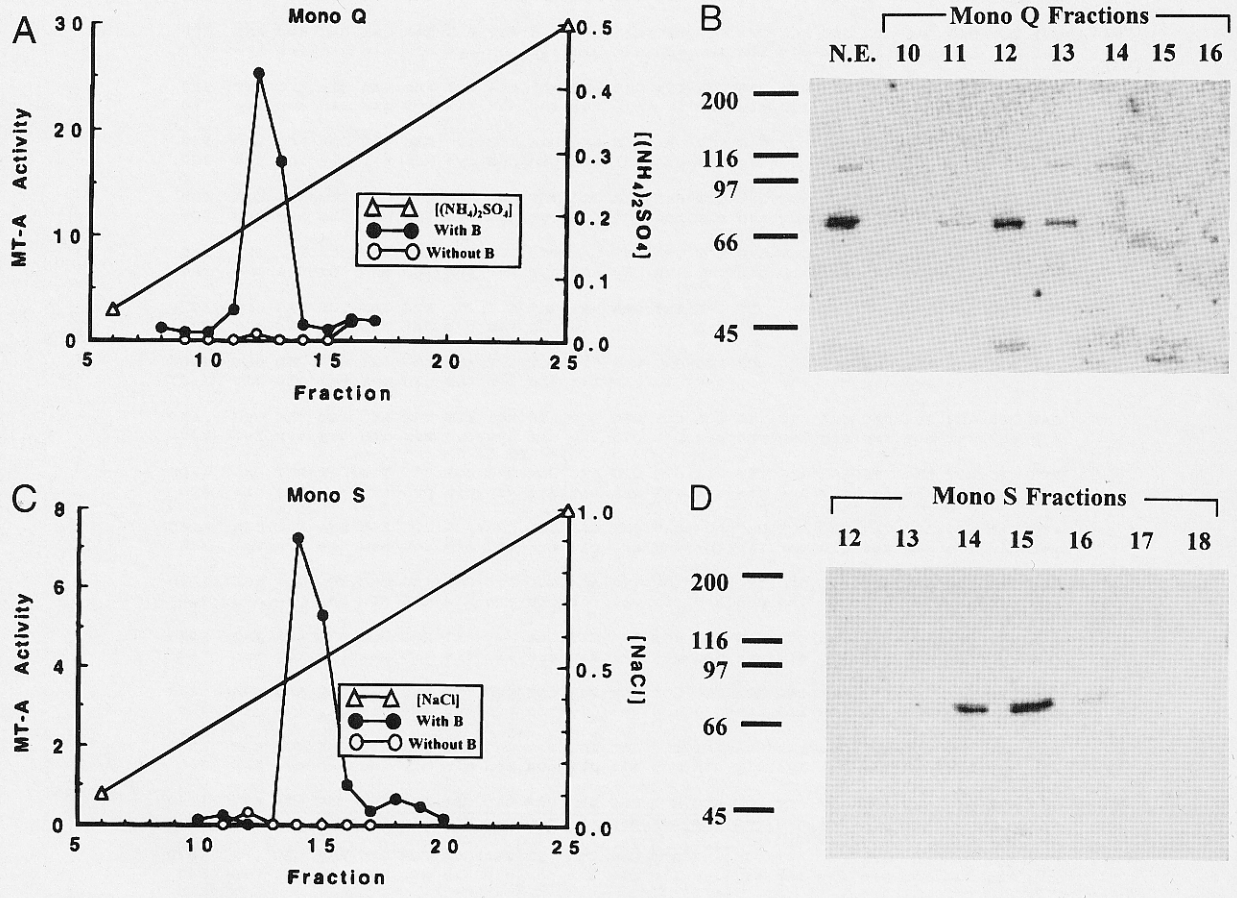

FIGURE 3. Anti-MT-A70 immunoreactive protein co-purifies with MT-A activity. **A:** HeLa nuclear extract (10 mg protein) was fractionated by Mono Q chromatography, and proteins were eluted with a linear gradient of $(NH_4)_2SO_4$. One-milliliter fractions were collected. Aliquots of fractions (2 μL) were assayed for methyltransferase activity with and without supplementation with an aliquot of fraction QS-B. **B:** Western blot of nuclear extract or aliquots of the MonoQ fractions assayed above. Primary antibody was a 1:10^4 dilution of rabbit antiserum 12622. Secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG. **C:** Fractions 12 and 13 were pooled and further fractionated using a Mono S column, eluted with an NaCl gradient, and 1-mL fractions were collected. Assays were performed as in A, except 5 μL of each fraction was used. **D:** Western blot of Mono Q fraction 12 and aliquots of the Mono S fractions was performed as in B.

resolution anion and cation exchange chromatography. In an abbreviated partial purification procedure, nuclear extract was fractionated on a Mono Q (HR5/5) column using a linear gradient from 0.05 M $(NH_4)_2SO_4$ to 0.5 M $(NH_4)_2SO_4$. Aliquots of each fraction were analyzed for methyltransferase activity (Fig. 3A), and also by western blot (Fig. 3B). In the absence of supplemented MT-B, no methyltransferase was detected, but, when fractions were assayed with MT-B, a peak of activity elutes in fractions 12 and 13. Aliquots of the same fractions were also analyzed by western blot using antiserum 12622. A 70-kDa immunoreactive protein co-elutes with MT-A activity in fractions 12 and 13 (Fig. 3B). Mono Q fraction 12 was then further fractionated on a Mono S (HR5/5) column using a linear gradient of 0.1–1.0 M NaCl. Again, MT-A activity and the 70-kDa immunoreactive protein co-elute precisely (Fig. 3C,D).

Antiserum 12622 was next tested for its ability to immunodeplete m^{6}A-MT activity from HeLa nuclear extract. An aliquot of antiserum 12622 or of the preimmune serum from the same animal was incubated with protein-A agarose beads, followed by extensive washing of the beads to remove other serum proteins. The antibody-bound beads were then treated with a protein crosslinking agent and further washing. Aliquots of HeLa nuclear extract were passed over these beads, reloading the flow-through twice. The unbound fraction was assayed for methyltransferase activity (Fig. 4). A decrease in activity of 90% was observed in the sample treated with the anti-MT-A70 antibody-linked beads relative to the control (preimmune) beads.

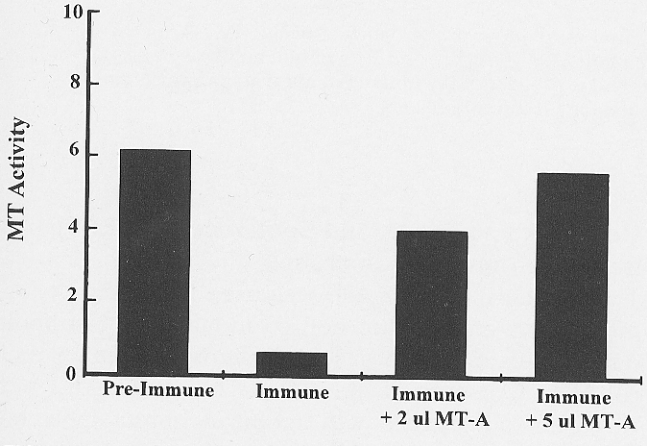

FIGURE 4. Methyltransferase activity immunodepletion. Protein A–agarose beads were incubated with antiserum 12622 or preimmune serum. After washing to remove unbound serum proteins, the beads were treated with a protein crosslinking agent and equilibrated in buffer A. HeLa nuclear extract was passed over the antibody-linked beads and the unbound fractions were assayed for methyltransferase activity. The sample treated with the antiserum-linked beads was also assayed with supplementation with aliquots of partially purified MT-A. Assays were performed in duplicate, with the means reported.

In order to demonstrate that the diminution of methyltransferase was not due to a nonspecific inactivation of the extract, a reconstitution experiment was performed. Partially purified MT-A (fraction 12 from the Mono Q column shown in Fig. 3A) was added to the immune-depleted nuclear extract. Two- and 5-μL aliquots restored activity to 65% and 92% of the control level, respectively. The partially purified MT-A assayed alone had no detectable methyltransferase activity (data not shown). This result indicates that antiserum 12622 is able to specifically deplete MT-A activity from the nuclear extract. Taken together with the fact that a single immunoreactive protein co-elutes with MT-A activity on multiple chromatography columns, the immunodepletion experiment demonstrates that the 70-kDa immunoreactive protein is MT-A70, and that this protein is a critical component of m^{6}A-MT.

Purified recombinant protein (6H-MT-A70) was also tested for its ability to UV crosslink with [^{3}H-*methyl*]AdoMet, as well as for methyltransferase activity both alone and with MT-B supplementation. The recombinant protein was not active in either of these assays (data not shown). Recombinant 6H-MT-A70 was also tested for its ability to restore methyltransferase to the immunodepleted nuclear extract. Again, no methyltransferase activity above the baseline of the depleted nuclear extract alone was detected (data not shown). These negative results may be explained by a number of possibilities discussed later.

Northern blot analysis of MT-A70 expression

Northern blot analysis of polyadenylated RNA isolated from HeLa cells and from a panel of human tissues was performed (Fig. 5). Random primed probe was prepared using the pMT-A70 cDNA insert as template. A single mRNA was detected with a length of approximately 2,000 nt in HeLa cells, in close agreement with the predicted cDNA length of 1,971 nt (lanes 1 and 2). A human multiple tissue northern blot (MTN II, Clontech, Inc.) was hybridized with the same probe. Unexpectedly, two RNA species were detected in polyadenylated RNA isolated from eight human tissues (lanes 3–10). In each tissue, an mRNA with approximate length of 2.0 kb was detected, similar to that observed in HeLa cell mRNA. A second RNA was detected in all tissues, with an approximate length of 3.0 kb. The relative intensities of these two bands varied both within each tissue and between tissues. The exact mobility of the individual bands also varied somewhat between tissues, over a fairly narrow range of 100–200 nt. The blot was stripped and rehybridized to a probe specific for β-actin. Although there are differences in the intensity of the β-actin mRNA signal, these differences do not parallel the distribution seen with the pMT-A70 probe. Furthermore, the differences in mobility of the individual bands seen with the pMT-

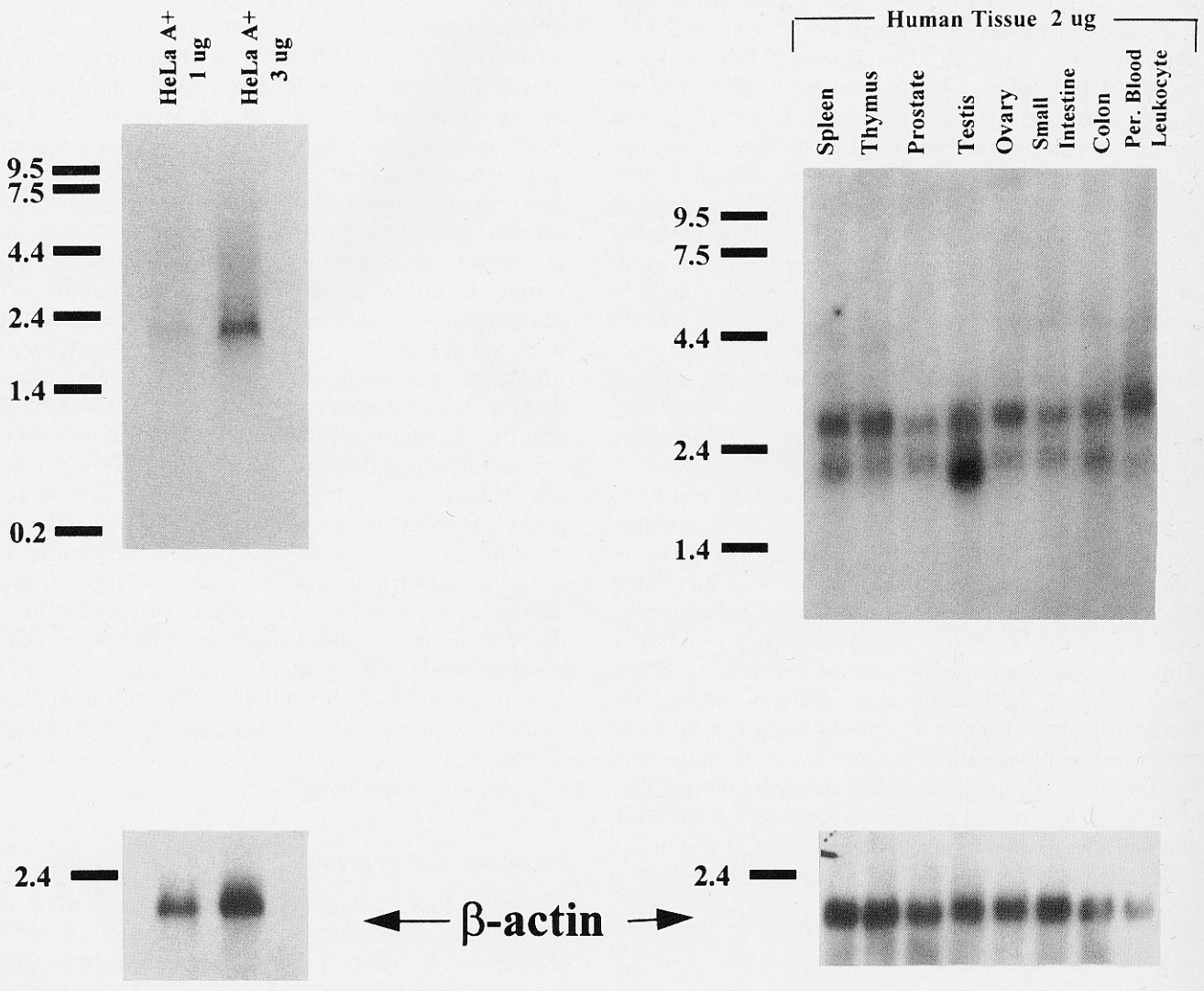

FIGURE 5. Northern blot analysis. ^{32}P-labeled probe was prepared by random primer labeling of the pMT-A70 cDNA insert, and was hybridized to blots containing 1 and 3 μg of HeLa poly-A+ RNA (left) and 2 μg of human tissue poly-A+ RNA (right). Blots were washed using high stringency conditions and exposed to X-ray film overnight with an intensifying screen. Blots were stripped and rehybridized with a probe specific for β-actin mRNA.

A70 probe were not apparent for the β-actin mRNA signal, indicating that these differences are not simply due to an electrophoresis anomaly. The presence of two discrete bands of very different size can result from two possibilities. There may be another gene with sequence homology to pMT-A70 that is expressed in all of the tissues tested that cross-hybridizes with the MT-A70 derived probe. Alternatively, two or more different transcripts may arise due to alternative splicing from a single pre-mRNA transcribed from the gene that codes for MT-A70. The heterogeneity of the sizes within the large and small transcripts between tissues may also arise due to alternative splicing, or may be due to differences in poly-A tail length, or to both.

GenBank and Expressed Sequence Tag (EST) database homology searches

The sequence of pMT-A70 was used to search for homologous genes in the nonredundant (nr) GenBank database using the basic local alignment search tool (BLAST) (Altschul et al., 1990). This search did not reveal any other genes with significant homology when performed using the nucleotide sequence, indicating that pMT-A70 is not a previously reported gene. However, a BLAST search using the predicted amino acid sequence of MT-A70 resulted in the identification of two genes with significant homology. The first, M. Mun1 methyltransferase ($p = 0.005$), is a DNA N^6-adenine-specific methyltransferase isolated from a mycoplasma species. Three homologous regions of 23–30

amino acids were identified containing 47–61% conservation and 37–43% amino acid identity (Fig. 6A). Homologous regions encompass methyltransferase consensus motifs I and II that have been shown to be highly conserved among 69 prokaryotic DNA methyltransferases (Timinskas et al., 1995). Both the relative order and spacing of these sequences is preserved between M. Mun1 methyltransferase and MT-A70. This observation is consistent with the identification of MT-A70 as the SAM-binding subunit of an N^6-adenosine methyltransferase, and suggests a functional conservation of this region in genes from widely divergent species. The second homologous gene identified ($p = 1.4e^{-88}$) is from *Saccharomyces cerevisiae*. It encodes SPO8, which was shown previously to be an activator of genes involved in the sporulation pathway (Smith et al., 1988; Esposito & Klapholz, 1991). MT-A70 and SPO8 share 72% amino acid similarity and 54% identity over a region of 270 amino acids near their carboxyl ends (Fig. 6B). The consensus methylation motifs both fall within the region of MT-A70 that is most highly conserved with SPO8, suggesting that SPO8 may also function as a methyltransferase, although no evidence for this has been reported because the molecular function of SPO8 is unknown (Esposito & Klapholz, 1991).

A BLAST search of the database of ESTs identified 36 human cDNA entries with long regions of sequence identical to pMT-A70. These EST clones are derived from libraries prepared from human, fetal brain, heart, kidney, liver, spleen, and parathyroid tumor, confirming that this gene is expressed in a wide variety of tissues. The sequences of many of these cDNA clones indicate that the pMT-A70 pre-mRNA undergoes alternative splicing. A subset of 13 of the EST clones that

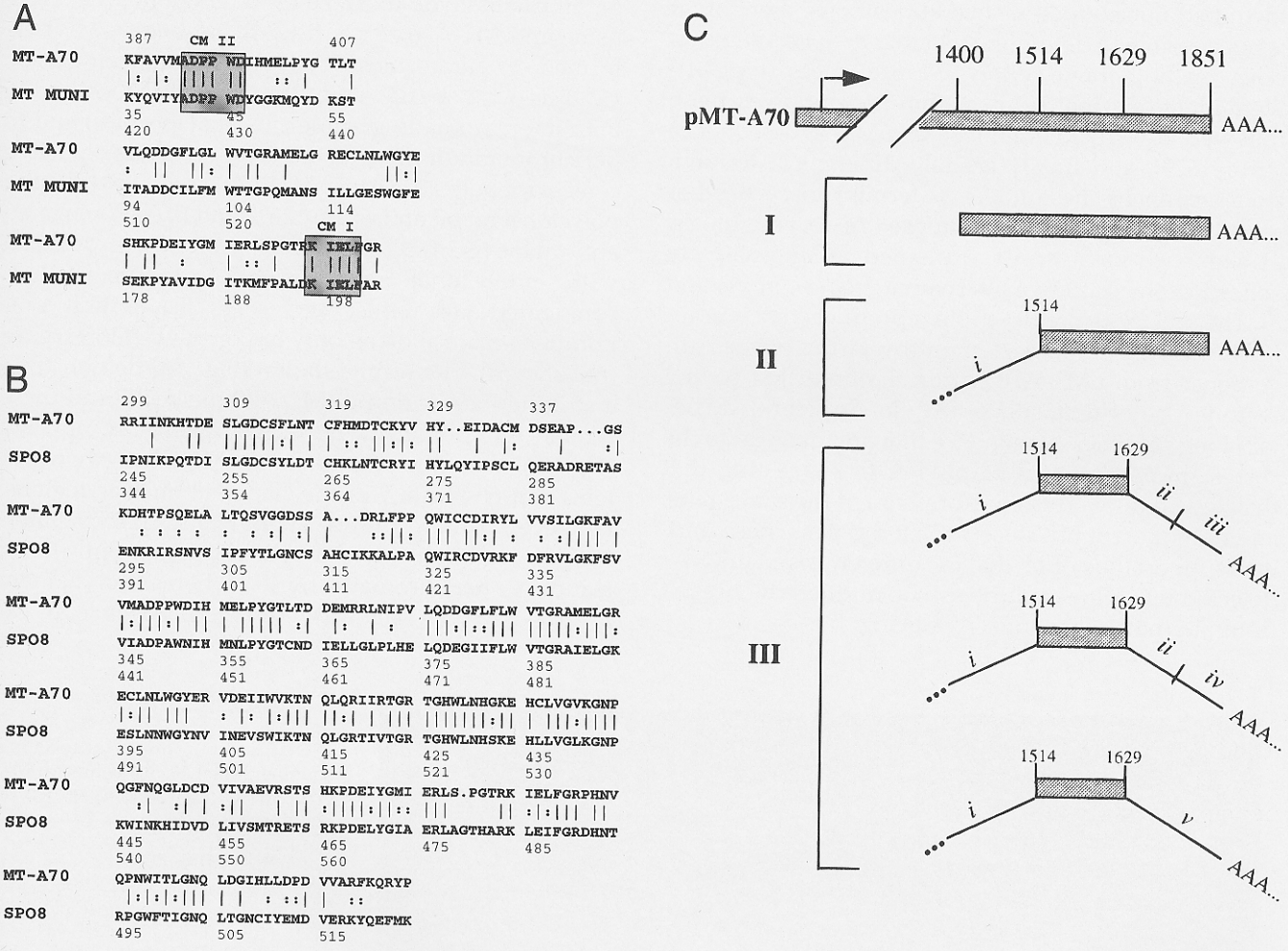

FIGURE 6. Results of BLAST search for homologous proteins. **A:** Alignment of similar regions of MT-A70 and M. Mun1 methyltransferase. "|" denotes identical amino acids and ":" denotes similar amino acids. Regions that correspond to consensus DNA methyltransferase motifs are highlighted. **B:** Alignment of similar regions of MT-A70 and SPO8. **C:** Schematic representation of three goups of EST sequences that contain regions of identity to the 3′-portion pMT-A70. Stippled box represents pMT-A70 sequence. Thin lines represent EST sequence not homologous to pMT-A70. Blocks of sequence that appear to be alternatively included exons that can be deduced from alignment of the EST sequences are denoted by lowercase Roman numerals. GenBank accession numbers for the EST sequences in each group are listed in Materials and Methods.

contain regions of sequence identity to pMT-A70 at the 3′ end were aligned with the pMT-A70 sequence (Fig. 6C). Three classes of EST sequences are represented schematically, with each type appearing to result from different patterns of alternative exon inclusion. Two EST sequences (Fig. 6C, type I) were identified that are identical to pMT-A70 from positions 1337 and 1388 to the 3′ terminus at position 1851 (for GenBank accession numbers, see Materials and Methods). At least four EST sequences (type II) were identified that are identical to pMT-A70 sequence from position 1514 to the 3′ terminus at 1851, but contain varying lengths of sequence upstream of postion 1514. This upstream sequence is similar or identical among these ESTs, but bears no similarity to any region of pMT-A70. This finding is consistent with the inclusion of an exon that is not present in pMT-A70 (denoted *exon i*). The full sequence of this putative exon (~174 nt, not shown) is present in only a single EST entry and it includes unidentified nucleotides at several positions, therefore it is not possible to determine whether *exon i* contains an open reading frame. Nine EST sequences (type III) were identified that begin within the putative *exon i*, followed by 116 nt identical to pMT-A70 sequence (1514–1629), and finally entirely different 3′ termini. Comparison of these novel 3′ termini to each other reveals that they are also composed of varying blocks of sequence most easily explained as alternatively spliced exons (putative alternative *exons ii*, *iii*, *iv*, and *v*). These cDNAs predict protein products that are different at their carboxyl termini by virtue of their divergence from pMT-A70 within the predicted coding region. Also of note, all 36 EST clones identified contain long stretches of sequence that are identical to the corresponding regions of pMT-A70, suggesting that they are all derived from expression of the same gene, and not different but closely related genes. These findings support the notion that the different-sized mRNAs detected using the multiple-tissue northern blot result from alternative pre-mRNA splicing.

Subnuclear localization of MT-A70 in HeLa cells

With the use of antibodies and specific nucleic acid probes, it has become increasingly apparent that many nucleoplasmic components are nonrandomly located throughout the nucleus (Fakan, 1994; Misteli & Spector, 1996). Of recent interest is the observation that apparently disparate components of the mRNA transcription and processing machinery can be co-localized and specifically interact. For example, the large subunit of RNA polymerase II has been shown to co-localize with the splicing protein SC35 in nuclear "speckles" (interchromatin granule clusters) and the physical interaction of these components has been documented by immunoprecipitation (Kim et al., 1997; McCracken et al., 1997).

In an experiment to explore the possible distribution of MT-A70 among discrete structural domains of HeLa cell nuclei, the anti-MT-A70 antisera was used to probe paraformaldehyde-fixed HeLa cells. Double labeling using anti-MT-A70 and anti-U2 B″ antibodies was performed on paraformaldehyde-fixed HeLa cell nuclei. U2 B″ is a U2-specific snRNP protein that localizes to both interchromatin granule clusters (speckles) and to perichromatin fibrils, and therefore serves as a positve control for both structures. MT-A70 was detected using polyclonal rabbit antiserum 12622 and FITC-conjugated anti-rabbit IgG (Fig. 7A). U2 B″ protein was detected with monoclonal antibody 4G3 and Texas Red-conjugated anti-mouse IgG (Fig. 7B). Rabbit pre-immune serum showed only background fluorescence (not shown). The results shown (Fig. 7A) demonstrate a striking localization of MT-A70 to speckles in these human interphase nuclei. U2B″ protein also appears (Fig. 7B) in speckles, demonstrating that these two proteins can co-localize in the same subnuclear regions. However, a more diffuse staining for U2B″ in interspeckle regions was observed, consistent with localization to perichromatin fibrils, whereas no staining for MT-A70 was seen in these regions. This is an ex-

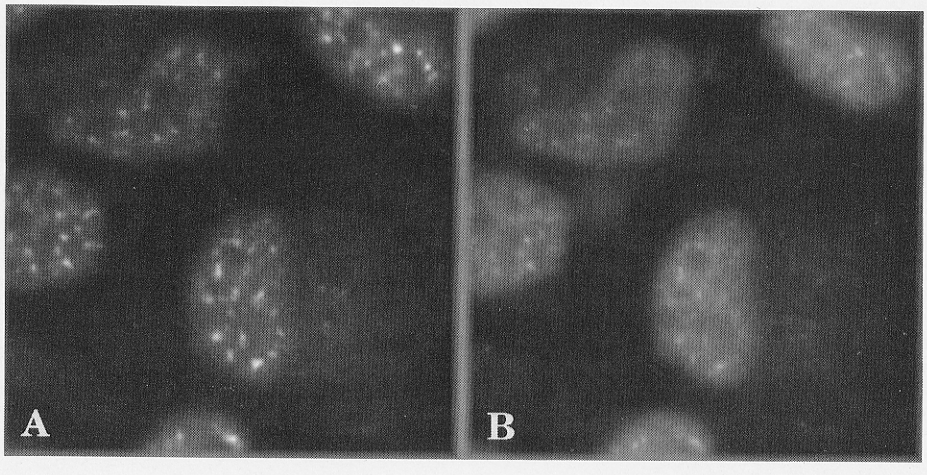

FIGURE 7. Immunofluorescence staining of HeLa cells using anti-MT-A70 antibody. HeLa cells grown on chambered slides were fixed with paraformaldehyde and incubated with rabbit antiserum 12622 (anti-MT-A70) followed by FITC-conjugated anti-rabbit IgG or monoclonal antibody 4G3 (anti-U2 B″) and Texas Red-conjugated anti-mouse IgG. Cells were visualized with an epifluorescence microscope to detect the FITC-conjugate (**A**, MT-A70) and the Texas red conjugate (**B**, U2 B″). Rabbit pre-immune serum showed only background fluorescence (data not shown).

citing result that suggests that mRNA m^{6}A-MT could indeed be associated with nuclear pre-mRNA splicing components.

DISCUSSION

Here we report the purification of a critical component of the mRNA m^{6}A-MT complex and its cDNA. HeLa cell nuclear extracts contain an activity that catalyzes the formation of m^{6}A in mRNA with the same sequence specificity observed in vivo (Narayan & Rottman, 1988). This activity requires a 200-kDa protein, MT-A, and a second protein factor, MT-B, with an apparent M_r = 875 kDa that has not been further characterized (Bokar et al., 1994). A 70-kDa subunit of MT-A binds to and can be UV crosslinked to [^{3}H-*methyl*]-AdoMet. This protein, MT-A70, has been purified, partially sequenced, and its cDNA has been isolated and characterized. The protein product of this cDNA was expressed as a recombinant fusion protein in *Escherichia coli*, and this recombinant protein was used to immunize rabbits. The resulting antiserum specifically recognizes a 70-kDa protein in HeLa nuclear extracts by western blot, and this 70-kDa protein co-elutes exactly with MT-A activity when fractionated on both Mono Q and Mono S columns. The anti-6H-MTA-70 antiserum also is capable of depleting nuclear extract of m^{6}A-MT activity, and the activity can be restored with partially purified MT-A. These findings are highly suggestive that this cDNA does in fact encode a critical subunit of the mRNA m^{6}A-MT. An alternative possibility is that the cDNA encodes a homologous enzyme that shares epitopes with the actual enzyme, thereby leading to the production of cross-reactive antibodies that can deplete m^{6}A-MT activity from the nuclear extract. The finding that both the AdoMet-binding protein and the immunoreactive 70-kDa protein co-elute precisely with m^{6}A-MT activity on several different chromatography columns makes it most likely that the cDNA does in fact encode a subunit of the authentic mRNA m^{6}A-MT. One means of proving this directly will be to reconstitute m^{6}A-MT activity in vitro using recombinant protein. Initial attempts at demonstrating methyltransferase or AdoMet-binding activity with the recombinant protein have been unsuccessful. There are many reasons why this may have been the case, including nonnative protein conformation, interference of the polyhistidine extension, absence of posttranslational modifications, and the likely necessity of other subunits of MT-A.

The predicted amino acid sequence of MT-A70 contains several interesting features identified by online computer analysis using PSORT—Prediction of Protein Localization Sites. The Robbins and Dingwall consensus motif (Robbins et al., 1991) for nuclear targeting is present beginning at amino acid 4 (KK QLDSLRE RLQ RRRKQ) and another near match at postion 162 (KK GPGEVAGTVT GQKRR). Using a second method (Klein et al., 1985), there is a predicted transmembrane domain from amino acids 377 to 393. We do not have any experimental evidence regarding whether m^{6}A-MT can localize to the nuclear membrane. An RNA binding motif was not found.

A BLAST search using the MT-A70 protein sequence resulted in the identification of only two proteins with significant homology. A DNA N^6-adenosine methyltransferase from mycoplasma, M. Mun1, contains three 23–30-amino acid regions with 47–61% similarity and about 40% identity to MT-A70. The similar regions contain sequences homologous to consensus methylation motifs I and II, identified by comparison of numerous prokaryotic DNA methyltransferases (Timinskas et al., 1995). Both the relative order of the consensus motifs (i.e., II, I), and the relative spacing (134 amino acids in MT-A70 and 155 amino acids in M. Mun1) are conserved. This finding is consistent with functionally conserved methyltransferase motifs in widely divergent organisms. Also of note, double-stranded adenosine deaminase (dsRAD) has also been reported to contain domains that are homologous to these same consensus methylation motifs (Hough & Bass, 1997). Although the significance of this finding is not yet known, it is interesting that two separate mRNA nucleoside modification enzymes, dsRAD and MT-A70, contain similar functionally conserved motifs.

The other gene found to have similarity to MT-A70 is SPO8, a gene that is involved in induction of sporulation in *S. cerevisiae*. The similarity between MT-A70 and SPO8 is striking, with 54% amino acid identity over roughly half of the proteins. The region of MT-A70 that is homologous to SPO8 includes the putative methyltransferase domains, making it tempting to speculate that SPO8 also functions as a nucleic acid methyltransferase. SPO8 expression results in activation of IME2, a gene critical for sporulation. However, the molecular mechanism by which SPO8 affects IME2 expression has not been reported (Smith et al., 1988). It is important to note that m^{6}A has not been detected in *S. cerevisiae* mRNA (unpubl. obs.).

The presence of m^{6}A in mRNA isolated from a variety of mammalian cell lines suggests that m^{6}A-MT is widely expressed in mammalian tissues. Northern blot analysis of polyadenylated RNA from a panel of human tissues confirms this expectation. Surprisingly, in addition to the 2-kb transcript seen in HeLa cell mRNA, all of the tissues tested also contain a 3-kb transcript that hybridizes strongly with the pMT-A70 cDNA-derived probe, even though the blot was washed using high stringency conditions. Two possible explanations for this finding are the presence of a closely related gene that is also expressed in the same tissues, and alternative splicing of a pre-mRNA transcribed from the gene encoding MT-A70. Although we do not have experimental evidence directly addressing these two

alternatives, the more likely explanation is alternative processing, based upon the results of EST database analysis. Because the 2-kb and 3-kb transcripts are present in each tissue in roughly similar amounts, it would be expected that cDNAs encoding the two forms would be present in the EST database with roughly similar frequency. All 36 EST clones identified as similar to MT-A70 contained long stretches (roughly 100–500 nt) of sequence identity or near identity (>98%). Many of the EST sequences also contain similar or identical sequence insertions at discreet positions relative to MT-A70. Most of these examples (some of which are represented schematically in Fig. 6C) occur in more than one EST clone isolated from different EST libraries by different research groups. Together, these findings are best explained by alternative exon inclusion during processing of a pre-mRNA that is transcribed from a single gene, although they do not rule out the presence of another similar gene that gives rise to the 3-kb transcript.

Whether expression of the enzyme is regulated either tissue specifically or developmentally has not been addressed directly to date. Several lines of evidence suggest that its expression is regulated. The multiple-tissue northern blot reveals that levels of both the 2- and 3-kb mRNA vary relative to each other within the individual tissues, and relative to β-actin mRNA when compared between tissues. In addition, Tuck has shown that m^{6}A-MT activity is elevated in nuclear extracts prepared from a number of transformed cell lines relative to nontransformed cells in culture or to normal tissues, and these differences are not simply due to a difference in mitotic rate (Tuck, 1992; Tuck et al., 1996). Adenovirus transformation of rat embryo cells (REC) in culture resulted in a 7.5-fold increase in m^{6}A-MT activity in nuclear extracts. The effect of v-*ski* expression on m^{6}A-MT activity was also studied. V-*ski* is a virally encoded oncogene that has been shown to be involved in both transformation and myogenesis (Colmenares & Stavnezer, 1989; Sutrave & Hughes, 1991). Expression of the v-*ski* protein in a mouse sarcoma cell line (mouse L cells) results in a paradoxical suppression of the tumorigenic potential of the cells. Interestingly, v-*ski*-producing cells had a 47% decrease in isolatable methyltransferase activity as compared to mock-transfected cells, supporting the hypothesis that a transformed phenotype correlates with increased m^{6}A-MT activity. The availability of the MT-A70 cDNA will now make it possible to determine whether similar patterns of expression occur at the mRNA level in these systems.

In summary, a critical protein subunit of the enzyme complex that catalyzes the formation of m^{6}A in mRNA has been purified, and its cDNA has been characterized. This cDNA, along with the anti-MT-A70 antisera, will finally allow us to develop the requisite tools to specifically modulate m^{6}A-MT activity in cultured cells, and thereby determine the effects of m^{6}A on mRNA metabolism and function. These tools will also provide the means to better understand the composition of the enzyme complex, and to begin to explore the tissue specificity and developmental regulation of this interesting posttranscriptional modification of eukaryotic mRNA.

MATERIALS AND METHODS

Methyltransferase assay

The assay that was employed for detecting m^{6}A-MT activity has been reported in detail (Bokar et al., 1994), and is described briefly. A T7 transcript containing 60 nt of the 3′ terminus of bovine prolactin mRNA containing the naturally occurring methylation site, linked to a 30-nt poly-A tail was synthesized with [α-^{35}S]UTP. This substrate RNA is incubated with [^{3}H-*methyl*]AdoMet along with an aliquot of HeLa nuclear extract or fractions thereof. After incubation, the substrate RNA is recovered and separated from the [^{3}H-*methyl*]AdoMet by binding to oligo-dT cellulose and filtration on a 96-well filter plate with vacuum. The RNA is then eluted with dH_2O and the incorporated [^{3}H-*methyl*]group quantified by scintillation counting. Differences in recovery due to varying salt concentrations and nuclease degradation are corrected by monitoring the ^{35}S recovery. Results are reported as fmol of [^{3}H-*methyl*]group incorporated per pmol of RNA.

Purification of MT-A70

HeLa cell m^{6}A-MT was purified using a modification of the procedure described previously (Bokar et al., 1994). Chromatography media, columns, and FPLC system were from Pharmacia Biotech. Nuclear extract was prepared from pellets of HeLa cells from 10–20 L of suspension culture (National Cell Culture Facility), as described. This typically yielded 20 mL of nuclear extract containing approximately 150 mg of protein per 10 L of suspension culture (approximately 6×10^9 cells). Extracts were dialyzed overnight and loaded onto a DEAE-Sepharose column (1.5 cm × 10 cm) in buffer A (50 mM Tris, pH 8.0, 10% v/v glycerol, 1.5 mM $MgCl_2$, 0.5 mM EDTA) containing 0.1 M $(NH_4)_2SO_4$. The flow-through fraction (DS-A) contained MT-A activity, and the bound fraction (DS-B), which eluted with buffer A containing 0.5 M $(NH_4)_2SO_4$, contained MT-B activity. Reconstitution of methyltransferase activity requires the presence of both MT-A and MT-B. Fraction DS-B was dialyzed against buffer A with 0.2 M KCl and was further purified on a HiTrap-Q column, using a step gradient; MT-B activity eluted between 0.35 and 0.55 M KCl. This fraction, QS-B, was utilized throughout the remainder of this study in all supplementation assays as the source of MT-B. MT-A was further purified as follows. Fraction DS-A was dialyzed against buffer A with 0.15 M KCl, and was loaded onto a HiTrap-Q column (5 mL) equilibrated with the same buffer, and eluted using a step gradient. The fraction that eluted between 0.15 and 0.3 M KCl (QS-A) was then dialyzed against buffer B (20 mM HEPES, pH 7.6, 10% v/v glycerol, 1.5 mM $MgCl_2$, 0.5 mM EDTA) with 0.1 M NaCl and loaded onto a HiTrap-SP

column (5 mL). The column was developed with a linear gradient of 0.1 M–0.6 M NaCl over 100 mL. Fractions containing MT-A activity were pooled (fraction SP-A). Fraction SP-A was concentrated by diluting 1:2 with buffer B, binding to a Mono S (HR5/5) column, and eluting in 1 mL using buffer B with 1 M NaCl. This concentrated fraction was then loaded onto a Superose 6 Prep column (HR 10/30) equilibrated in buffer A with 0.2 M KCl. Fractions containing MT-A activity (Sup6-A) were next diluted 1:2 with buffer A, loaded onto a HiTrap-Heparin column (1 mL), and eluted with a linear gradient of 0.1–0.6 M KCl. Fractions were used directly for [^{3}H-*methyl*]SAM UV crosslinking experiments and methyltransferase assays, or active fractions were pooled (HS-A). For peptide sequencing, proteins in fraction HS-A were bound to Strataclean resin (Stratagene, 10 μL resin/100 μg protein), which was then loaded directly onto a preparative 8% SDS-polyacrylamide gel. An aliquot of the sample was also UV crosslinked to [^{3}H-*methyl*]AdoMet (see below) and run in a parallel lane. After electrophoresis, the proteins were transferred to PVDF membrane; the crosslinked lane was excised and treated as described below, and the preparative lanes were stained with Coomassie blue dye. A visible 70-kDa band was excised and direct amino acid microsequencing was performed on tryptic peptides eluted from the membrane (W.S. Lane, Harvard Microchemistry Facility, Cambridge, Massachusetts).

[^{3}H-*methyl*]AdoMet UV crosslinking

Aliquots of the heparin Sepharose column fractions (50 μL) were mixed with 15 μL of 5 μM [^{3}H-*methyl*]AdoMet (85 Ci/mmol, Dupont-NEN). Reactions were incubated under short-wave UV light in a Stratalinker (Stratagene) for 10 min, and the proteins were then extracted and concentrated using Strataclean resin (10 μL of slurry/reaction). The recovered protein was separated on an 8% SDS-polyacrylamide gel, followed by transfer to PVDF membrane, treatment with En^{3}Hance spray (Dupont-NEN), and fluorography for 24–72 h.

cDNA cloning

A HeLa cell cDNA library constructed using the plasmid pCMV SPORT (Life Technologies) was screened using the GeneTrapper system (Life Technologies) and a degenerate oligodeoxynucleotide with the seqence GARATHTAYGGNA TGATHGA. Approximately 2,000 transformants were obtained, and approximately 10% of these were positive upon rescreening by hybridization to the same oligodeoxynucleotide. Ten of these were further analyzed by partial nucleotide sequencing. One (pMT-A70) was sequenced through the entire insert.

Northern blot and 5′-RACE PCR

Total cellular RNA was prepared from HeLa cells using Trizol reagent (Life Technologies) as recommended by the manufacturer. Polyadenylated RNA was prepared by selection with oligo-dT cellulose using standard techniques. For northern blot analysis, 1–3 μg of HeLa cell polyadenylated RNA was separated on a 1.5% agarose, 8 M urea gel and transferred to GeneScreen (BioRad) membrane. Alternatively, a multiple-tissue northern blot (MTN II, Clontech) was used. The membranes were hybridized to a probe generated using a random primed DNA labeling kit (Boehringer Mannheim) with the entire pMT-A70 cDNA insert as template. The membranes were subsequently stripped and rehybridized with probe generated by random primed labeling of β-actin cDNA.

The 5′-end of the MT-A70 cDNA was PCR amplified using the 5′-RACE system (Life Technologies) as recommended by the manufacturer. The reverse transcription reaction was primed with the gene-specific oligodeoxynucleotide CCA TGCAAGCATCAATTTC, which is complementary to the mRNA at positions 991–1009 of the cDNA sequence. The PCR amplification was performed using the upstream primer supplied with the 5′-RACE kit and the downstream oligodeoxynucleotide primer TCGTCTAGAGTGCATCATCTTG TAGGAGACC, which is complementary to the mRNA at positions 400–419 of the cDNA and linked to a *Sal* I restriction site. The PCR product was subcloned into the *Sal* I site of the BlueScript SK$^-$ plasmid (Stratagene) and was sequenced using a combination of internal and external sequencing primers.

Expression and purification of recombinant MT-A70

The coding region of pMT-A70 was subcloned into the plasmid pProEX HTb (Life Technologies) as follows. A unique *Sst* I site lies 17-nt downstream from the initiation codon in pMT-A70. pMT-A70 was digested with *Sst* I and *Not* I (which cuts in the vector downstream of the cDNA insert). The oligodeoxynucleotides ATGTCGGACACGTGGAGCT and CCACGTGTCCGACAT were annealed and ligated with the gel-purified *Sst* I–*Not* I fragment and with pProEX HTb that had been digested with the enzymes *Ehe* I and *Not* I. The resulting construct, p6HisMT-A70, contains a recombinant gene that contains the coding sequence for six histidine residues followed by a spacer region and the entire coding region of pMT-A70 linked in frame. DH10B (Life Technologies) cells transformed with this construct were grown in 200-mL cultures overnight, and were induced with IPTG at a final concentration of 0.6 mM for 3 h. The cells were harvested by centrifugation at 4,000 $\times$ *g* for 20 min, and pellets were solubilized in 6 M GuHCl, 0.1 M $NaHPO_4$, 0.01 M Tris, pH 8.0 (solubilization buffer) for 1 h with stirring. The solubilized preparation was centrifuged at 10,000 $\times$ *g* for 20 min, and the supernatant was loaded onto a HiTrap chelating column (1 mL) pre-equilibrated with 10 mM Ni_2SO_4, and then solubilization buffer. The GuHCl was removed by exchanging the solubilization buffer with 50 mM Tris, pH 8.0, 100 mM KCl, 20 mM imidazole (buffer C) over 20 column volumes, and the recombinant protein was then eluted with buffer C containing 400 mM imidazole. Fractions were analyzed by SDS-PAGE and fractions containing recombinant 6H-MT-A70 were desalted using buffer C without imidazole on BioRad 10DG desalting columns. Aliquots of this fraction were analyzed by electrophoresis and >90% of the protein was contained within a single band with an apparent M_r = 75 kDa (not shown). This fraction was used to immunize rabbits (Pocono Rabbit Farms and Laboratories, Inc., Canandensis, Pennsylvania). Three antisera (12621, −2, −3)

were obtained. All three were capable of detecting recombinant 6H-MT-A70 by both ELISA and western blot (see below).

Western blots and Mono Q and S chromatography

HeLa nuclear extract was diluted with buffer A (without glycerol) to an $(NH_4)_2SO_4$ concentration of 50 mM and was fractionated on a Mono Q (HR5/5) column using a linear gradient of 50–500 mM $(NH_4)_2SO_4$ over 20 column volumes; 1-mL fractions were collected. The peak fraction of MT-A activity was diluted 1:1 with buffer B (without glycerol), and loaded onto a Mono S column equilibrated with buffer B containing 0.1 M NaCl. The column was developed with a linear gradient of 0.1–1.0 M NaCl over 20 column volumes; 1-mL fractions were collected.

Aliquots of nuclear extract and Mono Q and Mono S column fractions were separated by electrophoresis using SDS-PAGE on 8% gels. The proteins were transferred to PVDF membrane using a semi-dry electroblotting apparatus at 200 mA for 1 h. The transfer buffer was 10 mM Tris, 4 mM glycine, 0.004% SDS, 20% methanol. The membranes were incubated sequentially with 5% nonfat dry milk in 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20 (TBST) for 30 min, primary antibody for 18 h (12622, 1:10,000 in blocking buffer), secondary antibody for 1 h (horseradish peroxidase conjugated goat anti-rabbit IgG, 1:10,000 in blocking buffer, Jackson Laboratories), and were then developed with LumiGlo reagent (KPL Laboratories) and fluorography for 1–10 min. Washes were performed with TBST for 45 min between each incubation.

Sequence information and computer assisted homology searches

The nucleotide and protein sequences reported in this work are available in the GenBank database (accession number AF014837). The GenBank nonredundant (nr) and EST databases were searched online with the BLAST search engine using the default parameters at http://www.ncbi.nlm.nih.gov:80. Nucleic acid sequence alignments and amino acid sequence alignments were performed with CLUSTALW using the default parameters at http://alfredo.wustl.edu/msa. Identification of protein domains was performed using PSORT at http://psort.nibb.ac.jp/cgi.bin/okumura. Accession numbers for the EST sequences described in Figure 6C are: type I, N39589, W93679; type II, N55548, W04670, R42072, W95745; type III, N94880, N95688, N66219, W58127, F09834, N66255, C00061.

Immunofluorescent staining of HeLa cell nuclei

HeLa monolayer cells were grown on chambered slides as described previously (Matera & Ward, 1993). Cells were fixed in 4% paraformaldehyde, washed, and pre-incubated with normal goat serum as described (Matera et al., 1995). Primary antibody 12622 (anti-MT-A70) was used at a dilution of 1:50. Control antibody 4G3 (anti-U2B″) was used at a dilution of 1:200. Fluorochrome-conjugated secondary antibodies were diluted in normal goat serum and incubated for 30 min. Images were acquired using a Zeiss Axioplan epifluorescence microscope and a cooled CCD camera as described (Matera et al., 1995).

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