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Induction of murine erythroleukemia cell differentiation is associated with methylation and differential stability of poly(A)$^+$ RNA transcripts

Ioannis S. Vizirianakis, Asterios S. Tsiftsoglou *

Laboratory of Pharmacology, Department of Pharmaceutical Sciences, Aristotle University of Thessaloniki, Thessaloniki 540 06, Greece

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Abstract

Murine erythroleukemia (MEL) cells exposed to DMSO were assessed for their ability to methylate poly(A)$^+$ RNA and accumulate RNA transcripts of globin and nonglobin genes (c-myc, β-actin and MER5). Cells were pulse-labeled with L-[methyl-^{3}H]methionine, cytoplasmic RNA was isolated, selected for poly(A)$^+$ RNA and analyzed by HPLC chromatography for methylated nucleosides. When MEL cells were exposed to inhibitors of RNA methylation (neplanocin A, 3-deazaneplanocin A and cycloleucine) and assessed for their ability to differentiate by DMSO, accumulate RNA transcripts, produce hemoglobin, methylate poly(A)$^+$ and poly(A)$^-$ RNA and synthesize *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH), we observed the following: (a) MEL cells treated with DMSO underwent hypermethylation in poly(A)$^+$ RNA that preferentially occurred at the 5′-cap structures (7-methylguanosine and 2′-*O*-methylcytidine and 2′-*O*-methyluridine); (b) inducer-treated MEL cells exhibited a decrease in the intracellular level of SAH that led to a lower ratio of SAH/SAM, an event that favors methylation; and (c) treatment of MEL cells with inhibitors of RNA methylation suppressed methylation of poly(A)$^-$ and poly(A)$^+$ RNA, reversed the ratio SAH/SAM seen in differentiated MEL cells and prevented differentiation to occur. Moreover, we observed that treatment of MEL cells with selective inhibitors of RNA methylation caused fragmentation of β^{major} globin and c-myc mRNAs, two RNA transcripts coded by developmentally regulated genes, while had no detectable effect on the structural integrity of poly(A)$^+$ RNA transcripts transcribed by two housekeeping genes (β-actin and MER5). These data indicate that induction of erythroid cell differentiation of MEL cells is associated with changes in methylation of poly(A)$^+$ RNA and selective differential stability of RNA transcripts, two events that might be related to each other.

Keywords: Cell differentiation; RNA transcript; Poly(A)$^+$; (Murine erythroleukemia cell)

1. Introduction

Differentiation of murine erythroleukemia (MEL or Friend) cells into mature hemoglobin producing cells is associated with a series of morphological and biochemical events reviewed elsewhere [1–3]. Among these, hypomethylation of DNA was observed in terminally differentiated MEL cells [4], an event that has attracted considerable attention in recent years in developmentally regulated cellular systems [5,6]. Biochemical studies with cordycepin (3′-deoxyadenosine) (an inhibitor of polyadenylation and methylation of RNA) and more recently with N^6-methyladenosine have suggested that initiation of commitment of inducer-treated MEL cells to terminal erythroid maturation may depends on both the synthesis and posttranscriptional modifications of RNA, such as methylation [7–11].

During posttranscriptional methylation of RNA, methyl-groups are transferred from SAM into specific base residues of RNA via RNA methyltransferases. Mammalian cells contain several RNA species (snRNAs, tRNAs, rRNAs, mRNAs) most of which are methylated at base residues located at unique structures like the 5′-cap in mRNA and the 5′-end of snRNAs [12–15]. Unfortunately, little is known about the biological role of RNA methylation in cell growth and differentiation [16–19].

In the present study, we extended our preliminary studies [9,20] and explored the potential role of RNA methylation in growth and differentiation of MEL cells. In particu-

Abbreviations: DMSO, dimethyl-sulfoxide; HMBA, hexamethylene-bis-acetamide; UDP-4, 2-(3-ethylureido)-6-methylpyridine; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; MEL, murine erythroleukemia.

* Corresponding author. Fax: +30 31 997631; e-mail: tsiftsoglou@olymp.ccf.auth.gr.

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lar, we investigated the following questions: (a) Do differentiating MEL cells undergo alterations in the synthesis and methylation of RNA? (b) If yes, do these changes occur simultaneously or independently during growth and differentiation? (c) Do poly(A)$^+$ RNA transcripts undergo changes in methylation, to what extend and at which base residues? (d) Do the changes in RNA methylation occur during differentiation as a result of alterations in the intracellular level of SAM and SAH? (e) Are the changes in RNA methylation causally related to induction of MEL cell differentiation and production of hemoglobin? and finally, (f) Does inhibition of RNA methylation affect stability of poly(A)$^+$ RNA transcripts?

To approach these questions, MEL cells were treated in culture with and without DMSO and pulse-labeled with L-[methyl-^{3}H]methionine. The ability of control and differentiating cells to methylate their total cytoplasmic RNA as well as poly(A)$^+$ RNAs was assessed at different times with the use of a modified reversed-phase HPLC chromatographic method based on standard procedures [21–23]. We analyzed [methyl-^{3}H]-labeled poly(A)$^+$ RNA fractions at the level of nucleosides and assessed the intracellular levels of SAM and SAH, two active intermediates in the methylation cycle. In a complementary series of experiments, MEL cells were exposed to both inducers of differentiation and inhibitors of RNA methylation and examined whether such MEL-treated cells mature, produce globin mRNA and hemoglobin and accumulate certain RNA transcripts and in particular those that play a critical role in growth and differentiation (e.g., c-myc). Here, we wish to present evidence indicating that induction of hemoglobin synthesis and terminal erythroid maturation in MEL cells are associated with changes in methylation of cytoplasmic RNA as well as of poly(A)$^+$ RNA. Moreover, we observed that suppression of RNA methylation led to blockade of MEL cell differentiation and selective degradation of globin and c-myc RNA transcripts.

2. Materials and methods

2.1. Chemicals and biochemicals

Dimethylsulfoxide (DMSO) was purchased by Mallinckrodt, (St. Louis, MO, USA). SAM, SAH, cycloleucine, HMBA, hypoxanthine, methylated and unmethylated base and nucleoside standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and were of the highest grade available. Neplanocin A and 3-deazaneplanocin A were kindly donated by Dr. Victor E. Marquez (National Cancer Institute, USA). UDP-4, a potent inducer of hemoglobin synthesis in MEL cells, was synthesized in our laboratory [24]. Analytical-grade potassium dihydrogen phosphate was purchased from Merck (Darmstadt, Germany). L-[methyl-^{3}H]Methionine (80.0 Ci/mmol) and [5,6-^{3}H]uridine (39.6 Ci/mmol) were obtained from NEN Research Products (Boston, MA, USA) and [^{35}S]methionine (1000 Ci/mmol) from Amersham (UK). P1 nuclease was purchased from Boehringer Mannheim (Mannheim, Germany), calf intestine alkaline phosphatase from BDH (Poole, UK) and tobacco acid pyrophosphatase from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions (1 mM) of nucleoside standards were prepared by dissolving appropriate amounts in 0.02 M KH_2PO_4 solution (pH 5.6) and stored at -20°C. Standards of various concentrations were prepared by appropriate dilutions of the stock solutions.

2.2. Cell cultures

Cells employed throughout this study were MEL-745PC-4A a clone of MEL-745 cells obtained after subcloning and subsequent testing of clones derived for high degree of inducibility. All cultures were maintained in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) fetal calf serum (Gibco, Long Island, NY) and antibiotics (penicillin and streptomycin 100 μg/ml). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2 and maintained at densities that permitted logarithmic growth ($1 \cdot 10^5$ to $1 \cdot 10^6$ cells/ml). Cell viability was assessed as reported elsewhere [10].

2.3. Induction and assessment of differentiation

Cells were incubated with no drug and the inducing agent in the absence or presence of an inhibitor as indicated in the text. At certain timed-intervals during incubation, the proportion of differentiated (hemoglobin-producing cells) was assessed cytochemically with benzidine-H_2O_2 solution [25]. The number of committed cells was assessed by the plasma clot clonal assay as described by Gusella et al. [26].

2.4. Pulse labeling with L-[methyl-^{3}H]methionine and isolation of [methyl-^{3}H]poly(A)$^+$ RNA

MEL cells treated in culture under various conditions were removed from cultures (samples of $1.0–1.5 \cdot 10^8$ cells) at different times and pulse-labeled in fresh methionine-free DMEM containing 10% v/v FCS for 3 h with L-[methyl-^{3}H]methionine (44 μCi/ml, 80.0 Ci/mmol) in the presence of 20 μM adenosine, 20 μM guanosine and 20 mM sodium formate. These agents were added in order to diminish the incorporation of [methyl-^{3}H] groups into purine ring via the de novo biosynthesis pathway and increased exclusive transmethylation of RNA [27]. This procedure however, was not used in early experiments shown in Fig. 2A, where pulse-labeling of cells was carried out in normal DMEM without the addition of adenosine, guanosine and sodium formate. The latter conditions were applied in order to record kinetic changes in the overall methylation of cytoplasmic RNA. Cells were

then collected, washed with PBS solution (3 ×) and processed for isolation of total cytoplasmic RNA [28]. Duplicate aliquots of 10 μl of isolated cytoplasmic RNA were counted for radioactivity and the extent of RNA methylation (cpm/100 μg RNA per3 h) was assessed. Isolated [methyl-^{3}H]-labeled cytoplasmic RNA was then separated into poly(A)$^+$ RNA fraction by passing twice throughout an oligo(dT)-cellulose column according to Kingston [29]. The purity of poly(A)$^+$ RNA fraction was checked by agarose gel electrophoresis.

2.5. Alkaline hydrolysis of [methyl-^{3}H]poly(A)$^+$ RNA

Samples of [methyl-^{3}H]-labeled poly(A)$^+$ RNA (5 μg or as otherwise is indicated) were dissolved in 50 μl of 0.3 M KOH and the mixture was incubated at 37°C for 19 h [30]. At the end of this period, 50 μl of 0.3 M $HClO_4$ solution were added and the resulting insoluble $KClO_4$ was removed by centrifugation (12000 × g). The supernatant was collected, mixed with 50 μl of 0.2 M glycine buffer pH 9.2 containing 1.4 U of calf intestine alkaline phosphatase and incubated at 37°C for 1 h [31,32]. The products derived were lyophilized, dissolved in water and injected (20 μl) into the HPLC column. This method yields nucleosides, 2′-*O*-methylated dinucleotides and intact 5′-end cap structures.

2.6. Enzymatic hydrolysis of [methyl-^{3}H]poly(A)$^+$ RNA

[Methyl-^{3}H]-labeled poly(A)$^+$ RNA (3 μg) dissolved in 50 μl solution containing 30 mM CH_3COONa pH 5.3, 2 mM $ZnCl_2$ was first digested with 2.1 U of P1 nuclease at 37°C for 5 h and subsequently with acid pyrophosphatase (0.8 U/ml) at 37°C for 1 h in a buffer solution containing 20 mM Tris · HCl, pH 7.5, and 2 mM $MgCl_2$ [32]. After addition of 50 μl buffer solution (0.2 M glycine, pH 9.2), incubation continued for an additional hour in the presence of 1.4 U of alkaline phosphatase. Finally, the reaction mixture was lyophilized, the residue was dissolved in 20 μl of water and analyzed by HPLC. This method yields methylated nucleosides originating from the 5′-cap structure as well as from other parts of the mRNA molecules.

2.7. HPLC separation of [methyl-^{3}H]-labeled poly(A)$^+$ RNA digestion products

A Varian (Sugar Land, Texas, USA) HPLC gradient system consisted of two Model 2510 dual-piston pumps, a Model 2584 high pressure solvent mixer, a Rheodyne Model 7125 sample injector and a Model 2550 UV-visible spectrophotometric detector was used. The reversed-phase (C18, 10 μm) Techsil 10 C_{18} column (25 cm × 4.6 mm i.d.) was purchased from HPLC Technology (Macclesfield, UK). Integrations and retention times were obtained using a Varian Model 4290 electronic integrator. Fractions of 0.5 ml/30 s of the effluent were collected with a LKB (Bromma, Sweden) 2112 Redirac fraction collector and the radioactivity was measured with a Packard liquid scintillation counter. Samples (20 μl) of hydrolytic products of [methyl-^{3}H]poly(A)$^+$ RNA were loaded on the reversed-phase column equilibrated at 1 ml/min with 0.02 M KH_2PO_4, pH 5.6. Chromatographic runs were performed at ambient temperature using a 3-step linear gradient system of 0.02 M KH_2PO_4, pH 5.6 and methanol as follows: (a) 0–2% methanol, t = 10 min; (b) 2–5% methanol, t = 10 min; (c) 5–35% methanol, t = 20 min. The effluent was monitored at 254 nm and fractions of 0.5 ml/30 s were collected. Each fraction was then assessed for radioactivity. The column was re-equilibrated for at least 15 min with the low-strength eluent between two different runs. Using this procedure, the retention times were reproducible.

2.8. Detection and quantitation of SAM and SAH levels in cellular extracts by HPLC analysis

Exponentially growing MEL cells ($1 \cdot 10^5$ cells/ml) were treated with one or more than one agents as indicated in the text. At different times, cells ($2 \cdot 10^6$) were removed from cultures and labeled for 3 h at 37°C with 10 μCi/ml [^{35}S]methionine (1000 Ci/mmol at reference date) in DMEM supplemented with 5% (v/v) FCS. By the end of this period, cells were resuspended in 1 M $HClO_4$ solution, sonicated for 10 s and centrifuged at 15000 × g for 15 min at 4°C. The supernatant was collected and analyzed by HPLC. Samples of 20 μl of acid-soluble cell extracts were loaded on the reversed-phase column (RP-18, 5 μm, 25 cm × 4.6 mm I.D.) and analyzed using a linear gradient system consisting of 0.02 M KH_2PO_4, pH 3.8 and methanol. Concentrations of methanol ranging from 0 to 35% were used for elution within 30 min. The flow rate was 1 ml/min and detection of SAM and SAH was carried out at 254 nm. Fractions collected were counted for radioactivity in a liquid scintillation counter.

2.9. Assessment of the steady-state level of RNA transcripts by Northern blot hybridization analysis

Cytoplasmic RNA prepared from control and MEL cells treated with agents at various timed-intervals, as indicated in the text, was assessed for the steady-state level of RNA transcripts coded by β^{major} globin, c-myc, β-actin and MER5 genes, with the use of [^{32}P]-labeled probes, as previously described [10,33]. The former two genes are regulated and expressed developmentally, while the latter two are considered house-keeping genes. The probes used were 7.0 kb for β^{major} globin mRNA [10], 1.7 kb for c-myc mRNA [34], 350 bp for β-actin mRNA [35] and 1.4 kb for MER5 mRNA [36]. The MER5 probe was kindly

donated by Dr. M. Obinata of Dept. of Cell Biology, Tohoku University, Sendai, Japan.

3. Results

3.1. Growth and differentiation of MEL cells are associated with alterations in synthesis and methylation of total cytoplasmic RNA as well as poly(A)$^+$ RNA

As shown in Fig. 1 continuous exposure of cells to DMSO (1.5% v/v) led to a gradual accumulation of committed cells, an early reduction in the synthesis of cytoplasmic RNA and a slightly lower rate of growth. MEL cells grown in culture from a relatively low ($5 \cdot 10^4$ cells/ml) to a high density of cells ($1-2 \cdot 10^6$ cells/ml) exhibited growth phase dependent alterations in the rate of RNA synthesis (Fig. 1C). The rate of RNA synthesis in DMSO-treated cells was about 25–30% of that of control cells after 24 h incubation (Fig. 1C). Both control and DMSO-treated MEL cells exhibited changes in the state of methylation of total cytoplasmic RNA, as we observed from the results shown in Fig. 2A. The overall methylation increased between 12 to 36 h incubation and decreased at later times as cells entered the plateau phase of growth. In particular, DMSO-treated cells underwent hypermethylation of RNA as compared to control untreated cells (Fig. 2A). However, the magnitude of methylation of total cytoplasmic RNA (incorporated [methyl-^{3}H]-groups/100 μg per 3 h) decreased at later times but remained at levels substantially above those of control cells. Since control and DMSO-treated cells continue to cycle for 72 h, these data indicate that growth and differentiation of MEL cells are associated with alterations both in synthesis and methylation of cytoplasmic RNA. The fact that DMSO-treated cells exhibited decreased RNA synthesis but higher level of methylation indicates that RNA is methylated at a greater extent in differentiating cells.

To increase the transmethylation of poly(A)$^+$ RNA and to suppress any non-specific incorporation of [methyl-^{3}H]-groups into purine ring due to de novo purine biosynthesis, we employed medium containing adenosine, guanosine and sodiun formate as routinely used by others [22,27,37]. Labeling of MEL cells with L-[methyl-^{3}H]methionine in methionine-free DMEM increased the incorporation of [methyl-^{3}H]-groups into RNA fractions (transmethylation) by 4-fold as compared to RNA fractions prepared from MEL cells and labeled with L-[methyl-^{3}H]-methionine in regular DMEM. This allowed us to isolate more radioactive poly(A)$^+$ RNAs and to study their pattern of methylation in both control and DMSO-treated MEL cells (see Fig. 2B). Induction of differentiation of cells by DMSO led to increased transmethylation (incorporated [methyl-^{3}H]-groups/100 μg per 3 h) of poly(A)$^+$ RNAs after 36 h incubation as compared to untreated cells, as seen in Fig. 2B.

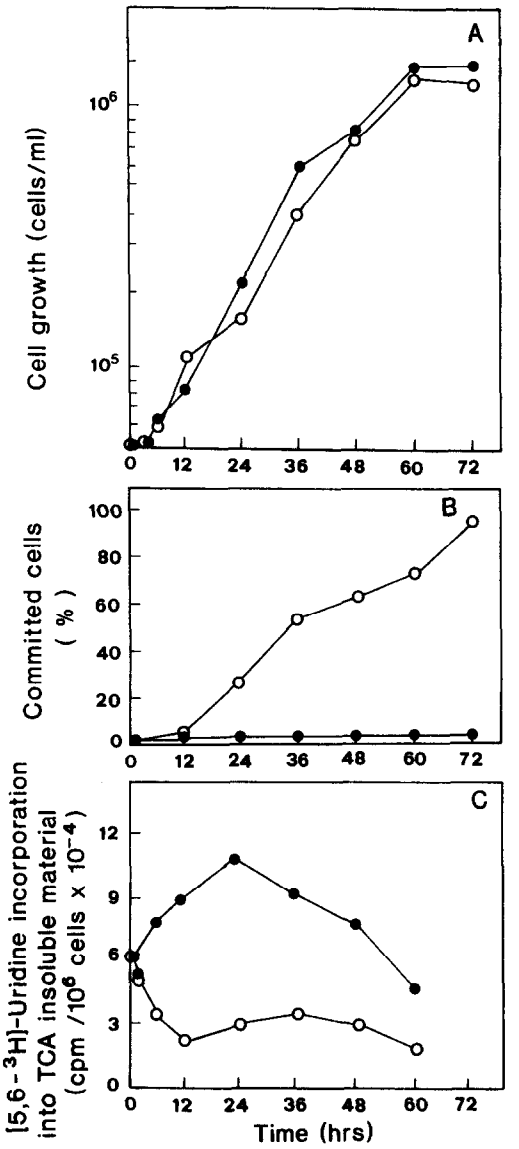

Fig. 1. Cell growth, commitment and rate of RNA synthesis in control and DMSO-treated cells. MEL-745PC-4A cells were incubated in DMEM supplemented with 10% FCS in the presence (-○-) and absence (-●-) of DMSO (1.5% v/v). At times indicated, cell growth was determined (panel A). In parallel, cells were removed from cultures and assessed for commitment by the plasma clot clonal assay (panel B) according to Gusella et al. [26]. The rate of RNA synthesis (panel C) was also measured as follows: Cells ($1 \cdot 10^6$) were removed from cultures and pulse-labeled with 2 μCi/ml [5,6-^{3}H]uridine (spec. act. 39.6 Ci/mmol) at 37°C for 90 min. By the end of this period, cells were precipitated with an ice-cold solution of TCA (10% w/v), the insoluble material was collected on filters (Whatman GF/B) and washed with 5% (w/v) TCA solution. The filters were washed thereafter with ethanol-ether (1:1), air-dried and counted for radioactivity in a scintillation counter. The values indicated are the mean value of two separate experiments.

3.2. Hypermethylation of poly(A)$^+$ RNA occurs at specific base residues

The results described above imply that changes in transmethylation of RNA species occur during MEL cell erythroid maturation. An important question then to be asked

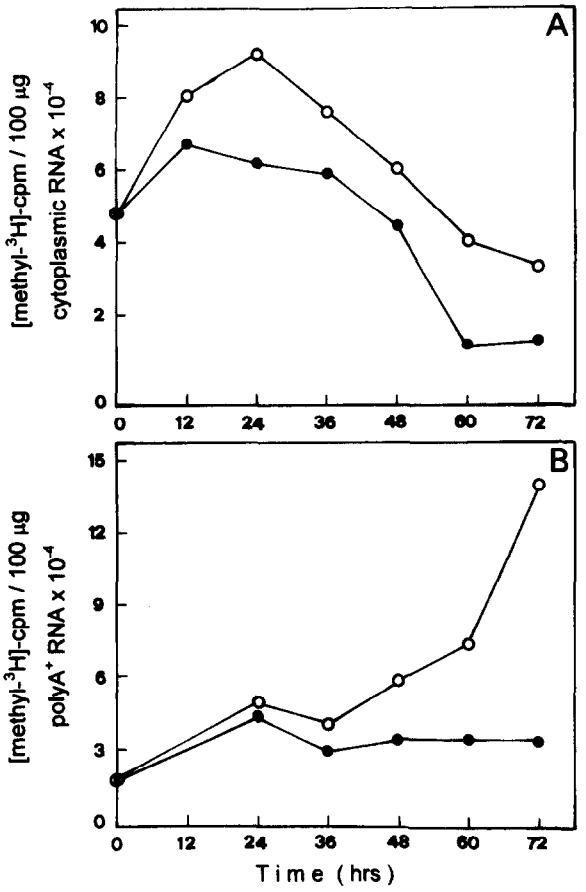

Fig. 2. Kinetics of methylation of total cytoplasmic as well as poly(A)$^+$ RNA in control and DMSO-treated MEL cells by pulse-labeling of cells with L-[methyl-^{3}H]methionine. Panel A: MEL-745PC-4A cells were incubated as indicated under Fig. 1. At time intervals indicated, samples of cells ($3–7 \cdot 10^7$) were removed from cultures and assayed for cytoplasmic RNA methylation as follows: Cells were washed out of any drug, resuspended in fresh normal DMEM supplemented with 10% (v/v) FCS (at density $8 \cdot 10^6$ to $1 \cdot 10^7$ cells/ml) and pulse-labeled with 30 μCi/ml of L-[methyl-^{3}H]methionine (spec. act. 80.0 Ci/mmol) at 37°C for 3 h. By the end of this time, total [methyl-^{3}H]-cytoplasmic RNA was purified and counted for radioactivity in a liquid scintillation counter. Cytoplasmic RNA methylation was estimated by the incorporation of [methyl-^{3}H]-groups per 100 μg RNA/3 h. The results presented are the average obtained from two separate experiments. Panel B: MEL-745PC-4A cells were incubated as indicated under Fig. 1. At time intervals indicated, cells were removed from culture and pulse-labeled in methionine-free DMEM with L-[methyl-^{3}H]-methionine (44 μCi/ml, 80.0Ci/mmol) for 3 h in the presence of adenosine, guanosine and sodium formate. In order to select purified [methyl-^{3}H]poly(A)$^+$ RNA from isolated [methyl-^{3}H]cytoplasmic RNA we carried out two rounds of oligo(dT)-cellulose chromatography, as described under Section 2. Poly(A)$^+$ RNA methylation was estimated by the incorporation of [methyl-^{3}H]-groups per 100 μg of poly(A)$^+$ RNA/3 h. The results presented are the average obtained from two separate experiments. Control (-●-) and DMSO-treated (-○-) MEL cells.

was where does this methylation occur in poly(A)$^+$ RNA? Two different but complementary approaches were applied to detect qualitative and quantitative changes in highly purified methylated poly(A)$^+$ RNA derived from control and differentiating MEL cells. Briefly, the first approach involved alkaline hydrolysis of [methyl-^{3}H]poly(A)$^+$ RNA, dephosphorylation of the products and separation of the nucleosides derived by HPLC (Fig. 3). This approach yielded nucleosides, 2′-*O*-methylated dinucleotides and intact 5′-cap structures of mRNA (Fig. 4A) [30]. The second, involved enzymatic digestion of [methyl-^{3}H]poly(A)$^+$ RNA that also breaks down the 2′-*O*-methylated dinucleotides and the intact 5′-cap structures of mRNA to the level of nucleosides (Fig. 4B) [32]. So, the alkaline hydrolysis of [methyl-^{3}H]-labeled poly(A)$^+$ RNA allowed us to detect methylated nucleosides having 2′-OH free, while the enzymatic digestion methylated nucleosides derived from the 5′-cap structures and 2′-*O*-methylated dinucleotides as well as other methylated derivatives from elsewhere of the poly(A)$^+$ RNA. Furthermore, the enzymatic hydrolysis of RNA allowed detection of methylated nucleosides that otherwise were unstable in alkaline conditions (e.g. 7-

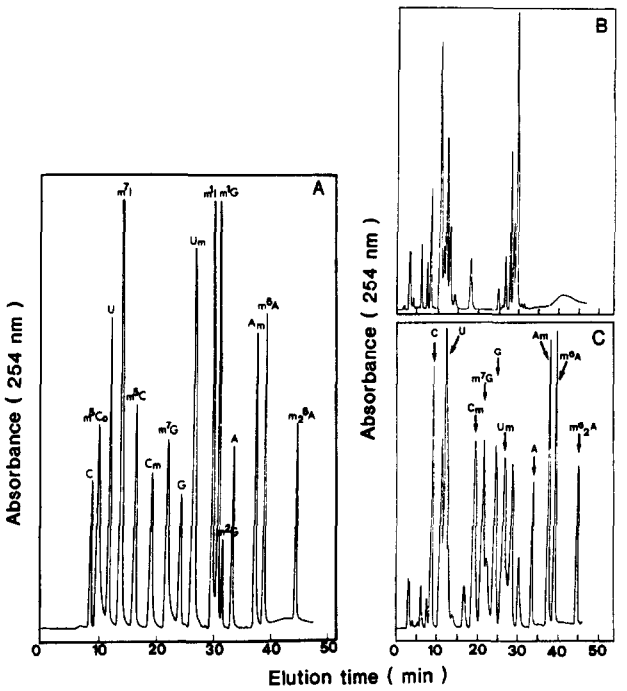

Fig. 3. HPLC elution pattern of known methylated and non-methylated nucleosides and bases. Panel A: A mixture of 1–2 nmol of each substance indicated in a total volume of 20 μl was injected on a reversed-phase column (Techsil 10 C_{18}, 25 cm×4.6 mm) and chromatographed using a gradient system of two solvents as described under Section 2. Panels B and C: Cytoplasmic [methyl-^{3}H]poly(A)$^-$ RNA was isolated from control MEL cells and hydrolyzed by alkali treatment as described under Section 2 for poly(A)$^+$ RNA. The RNA hydrolysis products mixed (panel C) or not (panel B) with known internal standards and analyzed by the HPLC method (shown in panel A). The peak of absorbance of added known methylated and unmethylated nucleosides is indicated by the arrows. 5 μg of RNA was hydrolyzed for each run. Abbreviations: C, cytidine; m^5C_o, 5-methylcytosine; U, uridine; m^7I, 7-methylinosine; m^5C, 5-methylcytidine; C_m, 2′-*O*-methylcytidine; m^7G, 7-methylguanosine; G, guanosine; U_m, 2′-*O*-methyluridine; m^1I, 1-methylinosine; m^1G, 1-methylguanosine; m^2G, N_2-methylguanosine; A, adenosine; A_m, 2′-*O*-methyladenosine; m^6A, N^6-methyladenosine and m^6_2A, N^6,N^6-dimethyladenosine.

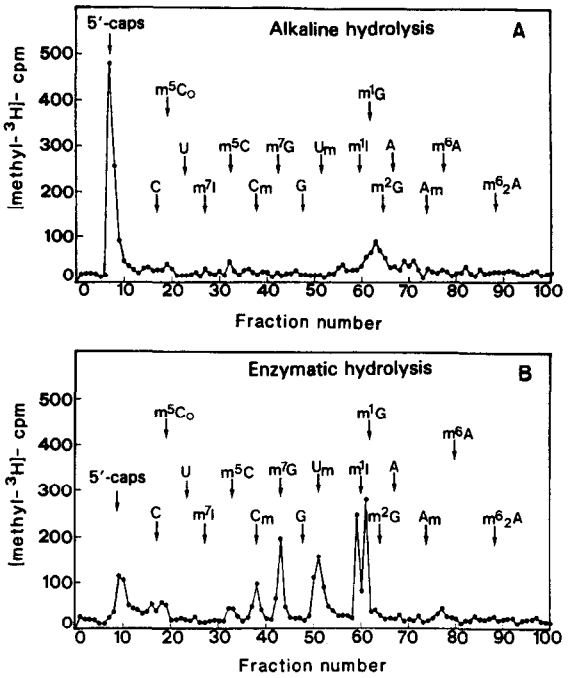

Fig. 4. HPLC analysis products derived from alkaline hydrolysis and/or enzymatic digestion of [methyl-^{3}H]poly(A)$^+$ RNA. Samples of 3 μg of [methyl-^{3}H]poly(A)$^+$ RNA prepared from DMSO-induced MEL cells (60 h of incubation) were hydrolyzed by alkali (panel A) or digested with P1 nuclease first and acid pyrophosphatase thereafter (panel B) to yield nucleotides. The nucleotides were then converted to nucleosides by alkaline phosphatase and analyzed by HPLC as described in Fig. 3. The arrows indicate the retention time of known nucleosides and bases at 254 nm absorbance length.

methylguanosine, 1-methylinosine, 1-methyladenosine, 3-methylcytidine) [30].

Detailed HPLC analysis of [methyl-^{3}H]poly(A)$^+$ RNA prepared from control and DMSO-treated MEL cells by using the HPLC system described in Fig. 3, is shown in Fig. 5. The left panels indicate the alkaline hydrolysis profile of methylated nucleosides derived from [methyl-^{3}H]poly(A)$^+$ RNA partially contaminated with poly(A)$^-$ RNA (one round purification via oligo(dT)-cellulose column). The right panels indicate the time dependent accumulation of alkaline hydrolysis products derived from highly purified [methyl-^{3}H]poly(A)$^+$ RNA from control and differentiating MEL cells. It is interesting to note that treatment of cells with DMSO led to gradual accumulation of 5'-cap structures (fractions 7–10), as confirmed by the enzymatic hydrolysis with acid pyrophosphatase, P1 nuclease and alkaline phosphatase (Fig. 4B). m^{7}G, C$_m$, U$_m$, and another as yet unidentified methylated nucleoside (fraction 59) were detected in 5'-cap-structures (Fig. 4). These changes were pronounced after 60 h treatment with DMSO. The only internal methylated nucleoside detected in poly(A)$^+$ RNAs from DMSO-treated cells was m^{1}G (fraction 61) as confirmed by the alkaline hydrolysis and the parallel enzymatic digestion of poly(A)$^+$ RNA (Fig. 4). These results are in agreement with the hypermethylation of poly(A)$^+$ RNA seen in DMSO-treated MEL cells (Fig. 2B).

3.3. Alterations in the level of SAM and SAH during growth and differentiation of MEL cells

Changes in the extent and pattern of methylation of cytoplasmic as well as of poly(A)$^+$ RNA seen during MEL cell differentiation can result from analogous alterations in the level of SAM and SAH, since the ratio SAH/SAM regulates the rate of methylation of macromolecules within the cells [38]. By using an HPLC system similar to that described before (Fig. 6D), we observed that control and DMSO-treated MEL cells pulse-labeled with [^{35}S]methionine underwent changes in the intracellular level of SAM and SAH (Fig. 6A and B). After an early increase, the level of SAM in control cells gradually dropped to a lower level. The level of SAH increased after 12 h of incubation and remained constant thereafter in these cells. Overall the ratio SAH/SAM increased in control cells after an early transient reduction (Fig. 6C). In contrast, DMSO-treated MEL cells exhibited a gradual reduction in the level of SAM just after an early increase. Similarly, the level of SAH markedly decreased in such inducer-treated cells (Fig. 6A and B). These changes in the level of both SAM and SAH kept the ratio SAH/SAM at a level significantly lower than that of control cells (Fig. 6C).

3.4. Inhibitors of RNA methylation affected cell growth and prevented erythroid differentiation of MEL cells induced by various agents

To demonstrate whether the changes seen in RNA methylation in differentiating MEL cells are causally related to induction of erythroid maturation, we employed an additional experimental approach. We exposed cells separately to neplanocin A, 3-deazaneplanocin A and cycloleucine, three selective inhibitors of RNA methylation [39–41] and asked if such treated MEL cells continue to differentiate by inducers, produce globin and non-globin mRNAs and synthesize hemoglobin. These agents are claimed to have selective action on methylation reactions as judged from the work published elsewhere [42–50]. Treatment of cells with each of RNA methylation inhibitor at concentrations known to block RNA methylation (neplanocin A $1 \cdot 10^{-6}$ M, 3-deazaneplanocin A $1 \cdot 10^{-5}$ M and cycloleucine $4 \cdot 10^{-2}$ M) [39–41,51], in the presence of DMSO decreased cell growth (Fig. 7A) whereas the viability of MEL cells was affected to a lesser degree, as expected (Fig. 7B) [51]. However, co-treatment of MEL cells with each inhibitor and inducer prevented erythroid differentiation to occur. No substantial amount of hemoglobin was produced (Fig. 7C). The fact that the inhibitors of RNA methylation prevented MEL cell differentiation promoted by HMBA, UDP-4 and hypoxanthine,

three other inducers in addition to DMSO (Table 1), suggests that RNA methylation may be part of the central process leading to differentiation and not an isolated event specific to an inducer. Finally, all the inhibitors of RNA methylation prevented erythroid differentiation of MEL cells by sodium butyrate, another potent inducing agent (data not shown).

3.5. *Treatment of MEL cells with RNA methylation inhibitors reduced methylation of poly(A)⁻ and poly(A)⁺ RNA and increased the ratio SAH / SAM*

To verify that treatment of MEL cells with each inhibitor of RNA methylation in the absence and presence of inducer DMSO prevents RNA methylation at concentrations used, we measured methylated RNA fractions in control and DMSO-treated cells. While neplanocin A caused a pronounced inhibition of methylation of poly(A)⁻ and poly(A)⁺ RNA, both 3-deazaneplanocin A and cycloleucine exerted a more selective inhibitory effect on methylation of poly(A)⁺ RNA rather than of poly(A)⁻ RNA (Table 2). This suggests that the inhibition effect of latter agents on methylation of poly(A)⁺ RNA is more or less specific.

As illustrated in Table 2, exposure of MEL cells to either neplanocin A or 3-deazaneplanocin A for even one hour led to rapid accumulation of SAH, whose level was hardly detectable prior treatment in both control and DMSO-treated cells (data not shown). Longer exposure (24 h) of DMSO-treated cells to neplanocin A and 3-deazaneplanocin A led to accumulation of SAH (Table 2) prior to commitment. In contrast to neplanocin A and 3-deazaneplanocin A treatment, exposure of cells to cycloleucine led to rapid depletion of SAM, without affecting

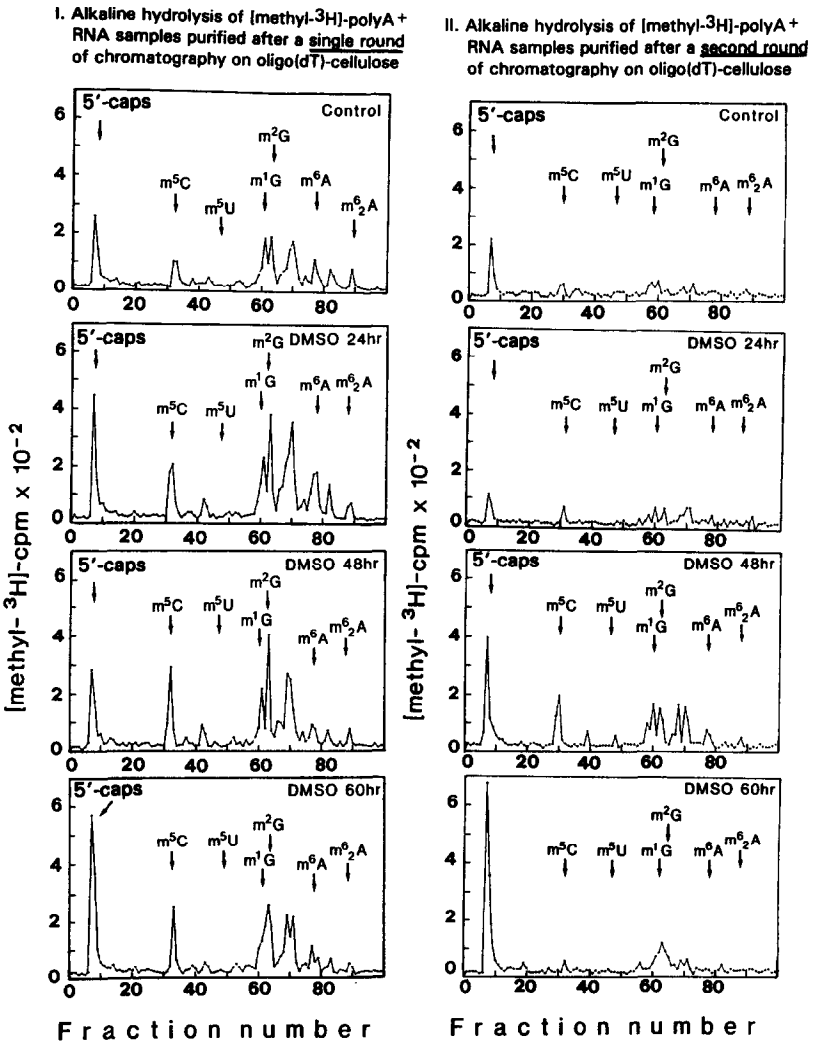

Fig. 5. HPLC analysis products derived from alkaline hydrolysis of [methyl-^{3}H]poly(A)$^+$ RNA prepared from control and DMSO-treated MEL cells. MEL-745PC-4A cells were incubated in the absence or presence of DMSO (1.5% v/v). At times indicated, [methyl-^{3}H]poly(A)$^+$ RNA was purified after one (left panels) or two (right panels) rounds of chromatographic separation of identical [methyl-^{3}H]cytoplasmic RNA samples (see Section 2) and hydrolyzed (5 μg) by alkali treatment. The products were co-chromatographed with known methylated nucleosides on the HPLC system as shown in Fig. 3. Fractions of 0.5 ml/30 s were collected and counted for radioactivity.

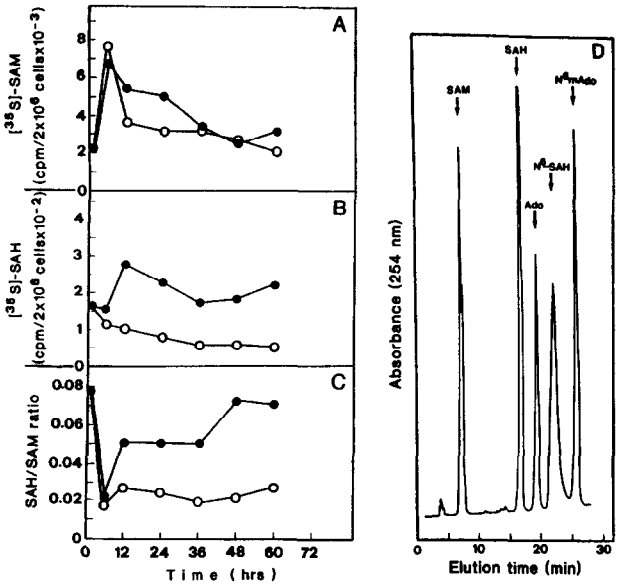

Fig. 6. Alterations in the intracellular levels of SAM and SAH in control and DMSO-treated MEL cells. MEL-745PC-4A cells were incubated in the presence (-○-) or absence (-●-) of DMSO (1.5% v/v). At time intervals indicated, cells ($2 \cdot 10^6$) were removed from cultures, pulse-labeled with 10 μCi/ml [^{35}S]methionine (spec. act. 1000 Ci/mmol) at 37°C for 3 h. Acid soluble cellular extracts were prepared and analyzed by HPLC, as shown in the right panel (D). (Panel D: A mixture consisted of 1–3 nmol of each of the compounds indicated above (20 μl total volume) was injected on a reversed-phase column (RP-18, 5 μm, 25 cm×4.6 mm) and chromatographed as described under Section 2). The level of SAM and SAH at each time point was estimated by the amount of radioactivity corresponding to SAM (panel A) or SAH (panel B) derived from each sample. The values of ratio SAH/SAM were also estimated and are shown in panel C. Abbreviations: SAM, *S*-adenosylmethionine, SAH, *S*-adenosylhomocysteine, Ado, adenosine, N^6-SAH, *S*-N^6-methyladenosylhomocysteine and N^6-mAdo, N^6-methyladenosine. N^6-SAH was prepared using known methods [11,75,76].

the level of SAH as expected. Overall, the reversion in the ratio SAH/SAM was less dramatic in DMSO-treated cells exposed to cycloleucine for 24 h than to neplanocin A and 3-deazaneplanocin A (Table 2). These data indicate that the blockade of MEL cell differentiation caused by these agents is associated with alterations in RNA methylation and changes in the intracellular level of SAM and SAH.

3.6. The inhibitors of RNA methylation caused differential stability of house- (β-actin, MER5) and non-house-keeping genes (β^{major} globin, c-myc)

To investigate whether inhibition of RNA methylation that leads to blockade of MEL cell differentiation also affects accumulation of RNA transcripts of various genes, some of which are house-keeping (β-actin, MER5) and others are developmentally regulated (β^{major} globin, c-myc), we carried out the following study: we exposed cells to each inhibitor in the presence and absence of the inducer DMSO for various times and then assessed the steady-state level of intact and degraded poly(A)$^+$ RNA transcripts. This study has shown the following: (a) treatment of MEL cells with DMSO alone led to cytoplasmic accumulation of β^{major} globin mRNA (Fig. 8A)., while caused marginal effect on RNA transcripts coded by two housekeeping genes (β-actin, MER5) (Fig. 8C and D). However, exposure of cells to each inhibitor of RNA methylation in the presence or absence of inducer altered the pattern of accumulation of c-myc RNA transcripts and caused fragmentation of β^{major} mRNA, an event previously observed with N^6-methyladenosine, another inhibitor of MEL cell differentiation [10]. In addition, exposure of MEL cells separately with each methylation inhibitor alone led to relatively high levels of β^{major} mRNA although fragmented, as compared to control untreated cells (Fig. 8A). Fragmentation was also observed in RNA transcripts coded by c-myc (Fig. 8B), a growth-gene involved in the regulation of commitment to maturation in MEL cells [52,53]; (b) Northern blot hybridization analysis for β-actin and MER5 transcripts, indicated that the

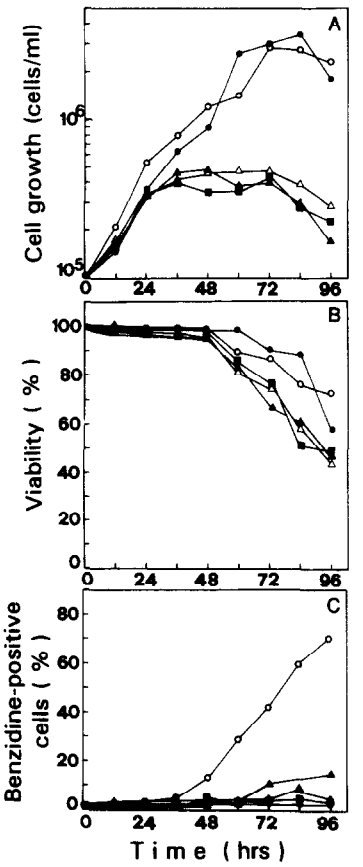

Fig. 7. Effects of neplanocin A, 3-deazaneplanocin A and cycloleucine on cell growth, viability and DMSO-induced differentiation of MEL cells. Exponentially growing MEL-745PC-4A cells were treated in culture with the following additions: none (-●-), DMSO (1.5% v/v) (-○-), DMSO (1.5% v/v)+neplanocin A ($1 \cdot 10^{-6}$ M) (-△-), DMSO (1.5% v/v)+3-deazaneplanocin A ($1 \cdot 10^{-5}$ M) (-■-) and DMSO (1.5% v/v)+cycloleucine ($4 \cdot 10^{-2}$ M) (-▲-). Cell growth (panel A) and viability (panel B) were determined 48 h later, while the proportion of differentiated cells (panel C) was scored after 96 h as described elsewhere [10]. Each time point represents the mean value of two separate experiments.

Table 1
Inhibition of MEL cell differentiation by neplanocin A, 3-deazaneplanocin A and cycloleucine in the presence of different inducing agents

Treatment	Concn. (M)	Cell growth (% of control)	Viability (%)	Benzidine-positive cells (%)
none	–	100	98.8	< 1
DMSO	0.210	103	99.2	69.6
DMSO + neplanocin A	$0.210 + 1 \cdot 10^{-6}$	50.6	97.0	1.9
DMSO + 3-deazaneplanocin A	$0.210 + 1 \cdot 10^{-5}$	39.3	97.0	1.8
DMSO + cycloleucine	$0.210 + 4 \cdot 10^{-2}$	52.8	95.4	15.9
HMBA	$5 \cdot 10^{-3}$	34.8	96.8	76.4
HMBA + neplanocin A	$5 \cdot 10^{-3} + 1 \cdot 10^{-6}$	29.2	89.2	9.5
HMBA + 3-deazaneplanocin A	$5 \cdot 10^{-3} + 1 \cdot 10^{-5}$	24.7	87.6	10.2
HMBA + cycloleucine	$5 \cdot 10^{-3} + 4 \cdot 10^{-2}$	23.6	86.7	25.0
UDP-4	$2.5 \cdot 10^{-4}$	53.9	98.1	74.0
UDP-4 + neplanocin A	$2.5 \cdot 10^{-4} + 1 \cdot 10^{-6}$	47.2	97.6	6.0
UDP-4 + 3-deazaneplanocin A	$2.5 \cdot 10^{-4} + 1 \cdot 10^{-5}$	39.3	97.0	4.1
UDP-4 + cycloleucine	$2.5 \cdot 10^{-4} + 4 \cdot 10^{-2}$	32.5	97.5	14.0
Hypoxanthine	$5.5 \cdot 10^{-3}$	74.1	98.4	40.7
Hypoxanthine + neplanocin A	$5.5 \cdot 10^{-3} + 1 \cdot 10^{-6}$	38.2	98.0	2.9
Hypoxanthine + 3-deazaneplanocin A	$5.5 \cdot 10^{-3} + 1 \cdot 10^{-5}$	37.1	98.3	2.8
Hypoxanthine + cycloleucine	$5.5 \cdot 10^{-3} + 4 \cdot 10^{-2}$	31.5	97.4	12.1

Exponentially growing MEL-745PC-4A cells were incubated with and without an inducing agent (DMSO, HMBA, UDP-4, hypoxanthine) at its optimum inducing concentration as well as with the inducer in the presence of neplanocin A, or 3-deazaneplanocin A and/or cycloleucine at concentrations indicated. Cell growth and viability were determined after 48 h whereas the proportion of differentiating cells after 96 h of incubation. Each value indicated is the mean value of two separate experiments.

steady-state level of both these RNA transcripts remained constant since no substantial degradation occurred (Fig. 8C and D). In addition, considering that the cytoplasmic RNA we prepared was intact as shown by the ethidium bromide staining pattern of the RNA samples (Fig. 8E), we conclude that inhibition of RNA methylation in MEL cells selectively affects stability of RNA transcripts coded by two developmentally regulated genes (β^{major} globin and c-myc) that play a critical role in MEL cell differentiation.

4. Discussion

The central mechanism(s) which govern the initiation of commitment of MEL cells to terminal erythroid maturation

Table 2
Effect of neplanocin A, 3-deazaneplanocin A and cycloleucine on RNA methylation of MEL cells grown in the presence and absence of DMSO

Treatment	Concn. (M)	Methylation of RNA [a] (spec. act. of [methyl-^{3}H]RNA)		Ratio SAH/SAM
		poly(A)$^-$ RNA (cpm/100 μg RNA)	poly(A)$^+$ RNA (cpm/100 μg RNA)	
A. Without DMSO				
None	–	354761 (100)	127783 (100)	0.023 [b]
Neplanocin A	$1 \cdot 10^{-6}$	124834 (35.2)	18099 (14.2)	0.854 [b]
3-deazaneplanocin A	$1 \cdot 10^{-5}$	302340 (85.2)	27931 (21.9)	1.185 [b]
Cycloleucine	$4 \cdot 10^{-2}$	438080 (123.5)	70956 (55.5)	0.230 [b]
B. With DMSO				
DMSO	0.210	503551 (141.9)	133071 (104.1)	0.045 [c]
DMSO + Neplanocin A	$0.210 + 1 \cdot 10^{-6}$	210042 (59.2)	18750 (14.7)	0.940 [c]
DMSO + 3-deazaneplanocin A	$0.210 + 1 \cdot 10^{-5}$	524732 (147.9)	16823 (13.2)	1.023 [c]
DMSO + Cycloleucine	$0.210 + 4 \cdot 10^{-2}$	649592 (183.1)	8482 (6.6)	0.097 [c]

For the measurement of RNA methylation MEL cells were incubated in culture with and without DMSO (1.5% v/v) as well as separately with neplanocin A, 3-deazaneplanocin A and/or cycloleucine at concentrations indicated in the presence and absence of DMSO. After 24 h incubation, cells were pulse-labeled with L-[methyl-^{3}H]methionine as described in Section 2. By the end of this time, total cytoplasmic [methyl-^{3}H]RNA was isolated and separated into poly(A)$^-$ and poly(A)$^+$ fractions by oligo(dT)-cellulose chromatography. The specific activity of each RNA species was measured with the use of a liquid scintillation counter. The ratio SAH/SAM was calculated as reported elsewhere [74].

[a] Numbers in parentheses indicate the level of methylation as compared to that of control cells.

[b,c] These numbers indicate the level of SAH/SAM ratio after: [b] 1 h and [c] after 24 h incubation of each methylation inhibitor indicated, as reported under Section 2.

are not as yet fully understood. Earlier studies suggested that initiation of commitment of MEL cells depends on the synthesis of new RNA and proteins [1–3,54]. It has also been shown that MEL cell differentiation is associated with hypomethylation of DNA [4], an event that may not be considered as a crucial initiative event, but still quite important for gene activation [5,6]. Earlier studies with cordycepin that synchronizes commitment of MEL cells [7] and blocks polyadenylation and methylation of RNA [8] indicated that induction of commitment depends on a methionine-sensitive event [10]. In this study, we extended earlier studies from this laboratory [9,20] and explored the role of RNA methylation in induction of MEL cell differentiation.

That poly(A)$^+$ RNA methylation occurs in MEL cells like in other eukaryotic cells is not surprising since methylated bases have been detected in different mRNAs [12], as well as in other RNA species (tRNAs, rRNAs, snRNAs) [13–15]. What is interesting in this case, however, is that the extent of methylation of cytoplasmic RNA increased in differentiating MEL cells while the overall RNA synthesis markedly decreased. Kinetically, cells entering the plateau phase of growth after 60–72 h incubation exhibited a reduction in the rate of RNA methylation, while DMSO-treated cells continued to methylate their RNA at a higher extent. This finding suggests that hypermethylation of cytoplasmic RNA may be a part of the differentiation process rather than a cell-cycle related event. The increase

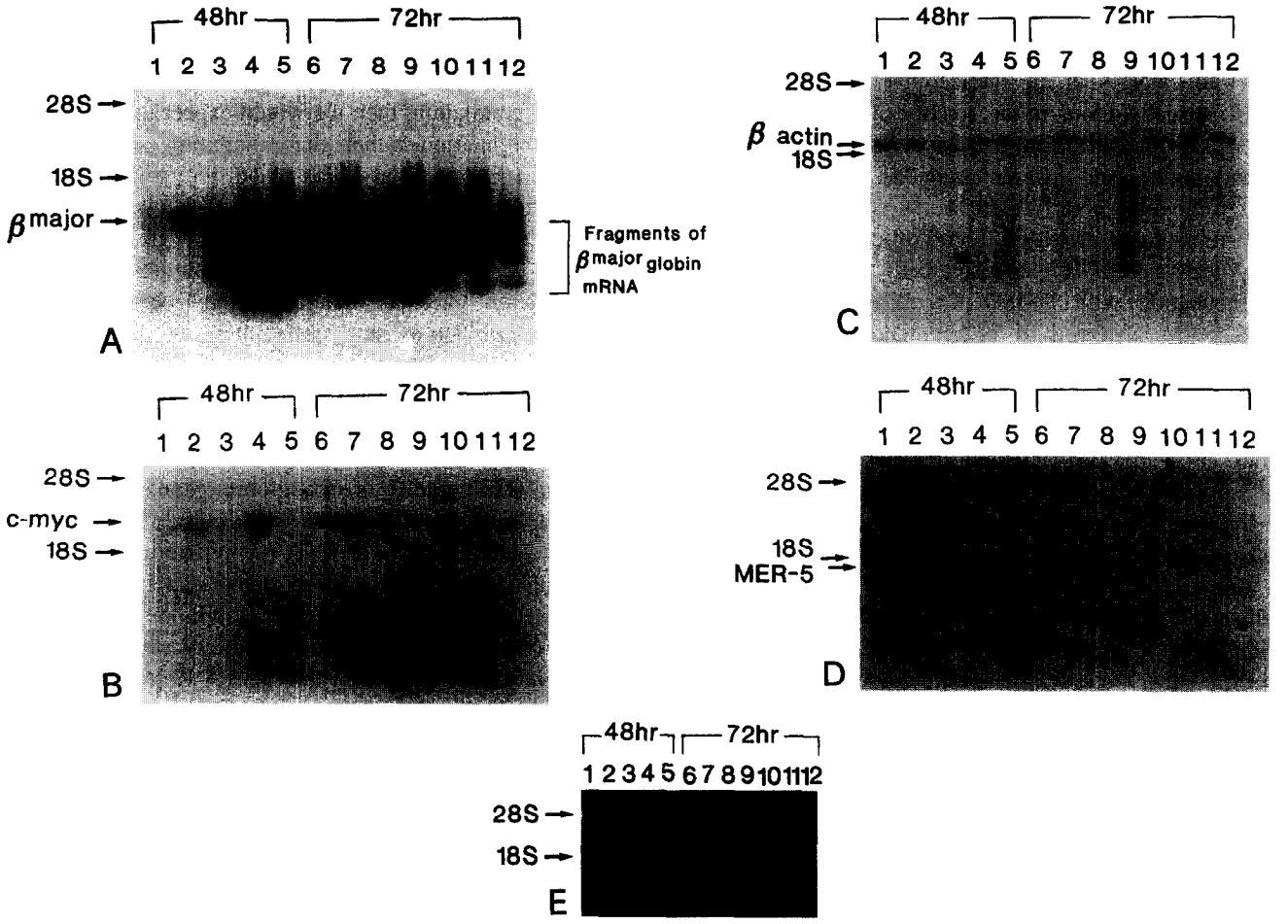

Fig. 8. Assessment of the effect of neplanocin A, 3-deazaneplanocin A and cycloleucine on cytoplasmic accumulation of β^{major} globin, MER5, c-myc and mouse β-actin RNA transcripts in control and DMSO-treated cells (Northern blot analysis). Samples of 10 μg total cytoplasmic RNA prepared from MEL-745PC-4A cells exposed to either each inhibitor of RNA methylation only or to both DMSO and an inhibitor as indicated below and for different times shown in the panels were electrophoretically separated on 1% agarose gel, transferred onto a nylon (Hybond-N, Amersham) membrane and hybridized at 65°C with ^{32}P-labeled DNA fragments coding either for β^{major} globin mRNA (7.0 kb) [10], or for mouse cytoplasmic β-actin mRNA (350 bp) [35], or for c-myc mRNA (1.7 kb) [34], or for MER5 mRNA (1.4 kb) [36], according to a method described by Church and Gilbert [33]. The filter was then washed at 65°C and autoradiographed using Kodak XAR-5 film. The autoradiograms obtained are shown above. Panel E shows the corresponding ethidium bromide staining patterns of electrophoresed RNA samples (the positions of 28S and 18S rRNAs are indicated). MEL cells were treated with the following additions: Lane 1, none; Lanes 2 and 6, DMSO (1.5% v/v); Lanes 3 and 7, DMSO (1.5% v/v) + neplanocin A ($1 \cdot 10^{-6}$ M); Lanes 4 and 8, DMSO (1.5% v/v) + 3-deazaneplanocin A ($1 \cdot 10^{-5}$ M); Lanes 5 and 9, DMSO (1.5% v/v) + cycloleucine ($4 \cdot 10^{-2}$ M); Lane 10, neplanocin A ($1 \cdot 10^{-6}$ M); Lane 11, 3-deazaneplanocin A ($1 \cdot 10^{-5}$ M) and Lane 12, cycloleucine ($4 \cdot 10^{-2}$ M).

in methylation of RNA was observed several hours after the reduction of RNA synthesis, but not simultaneously.

HPLC analysis of [methyl-^{3}H]poly(A)$^+$ RNA revealed methylated constituents at 5′-cap structure (m^7G, C_m, U_m) and hardly detectable levels of m^6A (Figs. 4 and 5). It must be noted that 1-methylguanosine (m^1G) was detected in poly(A)$^+$ RNA from DMSO-treated cells for the first time. The gradual accumulation of [methyl-^{3}H]-labeled 5′-cap structure constituents in DMSO-treated cells (Fig. 5) indicates that mRNAs are relatively hypermethylated at the 5′-cap structure during differentiation as well as at other base residues. At some sites, methylation occurs on the base residue while in others at 2′-OH position of ribose. Unfortunately, the analytical methods employed here did not permit us to demonstrate whether transmethylation of RNA at internal base residues occurs randomly or at discrete regions of RNA molecules.

Alterations in RNA methylation observed during DMSO-induced MEL cell differentiation can be attributed either to changes in the intracellular level of SAM and SAH or to alterations in the activity of RNA methyltransferases. As shown in Fig. 6A, the level of SAM decreased in both control and differentiating MEL cells by different rates following an early increase. In contrast, the level of SAH remarkably increased in control cells, while dramatically decreased in differentiating cells from the first hours of incubation with DMSO. Overall, a lower ratio of SAH/SAM observed in differentiated cells (Fig. 6C). These changes in the ratio SAH/SAM appear to affect the degree of RNA methylation, since RNA methyltransferases may no longer be inhibited by SAH [38]. Technical difficulties in achieving detectable level of RNA methylation in vitro did not allow us to assess endogenous RNA methyltransferase activity in control and DMSO-treated cells (data not shown).

Although the data presented thus far indicate that MEL cell differentiation is associated with both qualitative and quantitative changes in poly(A)$^+$ RNA methylation at specific sites, the precise role of these changes in maturation of MEL cells remains elusive. Hypermethylation of course could increase hydrophobicity of RNA and may facilitate RNA transport from nucleus into cytoplasm as reported by Camper et al. [37]. Alternatively, hypermethylation may affect the tertiary structure and conformation of RNA in a way that renders it capable to interact with trans-acting proteins like in other cases [55–62]. Furthermore, changes in methylation of RNA at several sites may affect its stability, an event that occurs during MEL cell differentiation [63–65] and is developmentally regulated [65]. A study showing an increase in methylation of 5.8 S rRNA in normal tissues versus neoplastic cells (HeLa and Novikoff hepatoma) has been reported [18]. Similarly, the role of RNA methylation was also examined in L5 myoblast cell differentiation [19].

To further support the notion that there may be a causal relationship between methylation of RNA and specific events of MEL cell differentiation, we employed a reverse but complementary approach mentioned above. Knowing that RNA methylation can be inhibited by agents like neplanocin A, 3-deazaneplanocin A and cycloleucine which have been already used in many cellular systems as specific inhibitors of methylation reactions [39–51], it was reasonable to investigate whether selective inhibition of methylation of nuclear RNA and most importantly of cytoplasmic poly(A)$^+$ RNA leads to: (a) alterations in SAH/SAM ratio, (b) changes in the steady-state accumulation and stability of RNA transcripts coded by various genes (MER5, β-actin, c-myc and β^{major} globin), and (c) blockade of maturation. We are aware that although the methylation inhibitors employed in this study are specific inhibitors of active methylation cycle, one can argue that they can affect methylation of other macromolecules in addition to RNA. We reason that their effect on DNA methylation must be negligible, since this process is already suppressed in differentiating MEL cells [4]. Furthermore, if DNA methylation was a rate-limiting step in MEL cell differentiation, then we would expect to see induction of differentiation rather than inhibition since evidence exists to indicate that MEL cell differentiation is associated with DNA hypomethylation [4]. However, the relatively high steady-state level of β^{major} globin mRNA in cells exposed exclusively to methylation inhibitors and fail to undergo terminal maturation may be due to DNA hypomethylation as seen by treatments of MEL cells with 5-aza-cytidine and 5-aza-2′-deoxy-cytidine [66]. DNA hypomethylation can occur at 5′-end upstream sequences adjacent or distant from the globin genes that contain transcription cis-control elements in a way seen in LCR region (HS region) of the globin genes [67–69]. The findings that co-treatment of MEL cells with DMSO and each of the inhibitors of RNA methylation blocked induction of erythroid maturation, suppressed the steady-state level accumulation of β^{major} globin and c-myc RNA transcripts and decreased their stability while had no detectable effect on β-actin and MER5 RNAs, suggest that RNA methylation and RNA stability may be related to each other in MEL cell differentiation.

The findings presented here are also in agreement with previous studies showing that N^6-methyladenosine [10,11], 3-deazaadenosine [70], cordycepin [7], 5′-methylthioadenosine [71,72] and 5′-*S*-isobutylthioadenosine [71], which modulate the methylation cycle [8,11,73] inhibit initiation of MEL cell differentiation. Methylation of RNA may be a critical event in induction of erythroid differentiation of MEL cells. Moreover, knowing that commitment of MEL cells depends on the synthesis of new RNA and proteins as well as on methylation and differential stability of poly(A)$^+$ RNAs, it is reasonable to assume that inhibition of RNA methylation may block initiation of differentiation by affecting stability of critical RNA transcripts. Of course, this is an interesting working hypothesis pending further investigation and approval.

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