MOLECULAR AND CELLULAR BIOLOGY, Dec. 2009, p. 6182–6191 Vol. 29, No. 23
0270-7306/09/$12.00 doi:10.1128/MCB.00973-09

S-Adenosyl Homocysteine Hydrolase Is Required for Myc-Induced mRNA Cap Methylation, Protein Synthesis, and Cell Proliferation▽

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Received 23 July 2009/Returned for modification 1 September 2009/Accepted 23 September 2009

The c-Myc proto-oncogene promotes mRNA cap methylation, which is essential for almost all mRNA translation. The mRNA cap methylation reaction produces an inhibitory byproduct, *S*-adenosyl homocysteine. Here we report that Myc promotes upregulation of *S*-adenosyl homocysteine hydrolase (SAHH), an enzyme which hydrolyzes *S*-adenosyl homocysteine, thus neutralizing its inhibitory effects, and this is required for c-Myc-induced mRNA cap methylation. c-Myc-induced mRNA cap methylation was repressed by inhibiting the expression or activity of SAHH, whereas the same treatments did not have a significant effect on c-Myc-induced transcription or other c-Myc-dependent methylation events. The selective inhibition of mRNA cap methylation afforded by SAHH repression revealed that c-Myc-induced cap methylation could be correlated with the core c-Myc functions of protein synthesis, cell proliferation, and cell transformation.

The Myc proteins c-Myc, N-Myc and L-Myc promote cell growth and proliferation and are essential for development (12, 25). Deregulated expression of the Myc proteins results in unrestrained cell proliferation and promotes tumor formation. The c-Myc gene, in particular, is recognized to be a potent oncogene in many organs and tissues and is deregulated in a significant proportion of human tumors.

The Myc proteins are basic helix-loop-helix leucine zipper (bHLH-LZ) proteins which form heterodimers with another such protein, Max. Myc-Max heterodimers form transcription factors which both activate and repress transcription (8, 10, 18, 27). The Myc proteins have a major impact on cellular transcription, activating or repressing approximately 10% of RNA polymerase II (RNA pol II)-transcribed genes (mRNA and microRNA) and activating RNA pol I- and III-transcribed genes. Many cofactors which mediate Myc-regulated transcription have been isolated, with the majority of these regulating chromatin structure and the recruitment and activity of the RNA polymerases (8, 12, 38).

Myc proteins have recently been found to regulate gene expression by promoting mRNA cap methylation (5, 9). The mRNA cap is an inverted 7-methylguanosine group added to the 5′ end of RNA pol II-transcribed genes, which stabilizes the RNA and promotes splicing, nuclear export, and translation (1, 14, 35, 36). The 7-methylguanosine cap is added to nascent RNA by the action of three enzymes. A triphosphatase removes the terminal phosphate of the first transcribed nucleotide, a guanylyltransferase adds GMP to create the guanosine cap, and an RNA guanine-7 methyltransferase methylates the guanosine cap at the N-7 position to create the 7-methylguanosine cap (36). Methylation of the guanosine cap is critical for mRNA binding to eukaryotic translation initiation factor 4E (eIF4E), the subsequent formation of the eIF4F complex, and the initiation of translation (14).

mRNA cap methylation occurs predominantly during the early stages of transcription as the 5′ end of the nascent RNA emerges from the RNA pol II complex (26, 28). At this stage, the enzyme which catalyzes the addition of the 5′ guanosine cap, RNA guanylyltransferase and 5′ phosphatase/capping enzyme (RNGTT/CE), and the enzyme which catalyzes the methylation of the guanosine cap, RNA guanine-7 methyltransferase (RNMT; also called hMet), are recruited to TFIIH-phosphorylated RNA pol II (4, 24, 39). c-Myc and N-Myc were found to bind to TFIIH, enhance its recruitment to transcription initiation sites, and promote RNA pol II phosphorylation, correlating with enhanced mRNA cap methylation (6, 9).

Experimental results have suggested that Myc-induced mRNA cap methylation has a significant effect on gene expression and may be a critical effector of Myc. First, Myc induces mRNA cap methylation of most of its RNA pol II-transcribed target genes. Second, Myc induces a larger increase in mRNA cap methylation than transcription for most of its targets (5). Myc-induced cap methylation is not simply a result of transcriptional regulation of the mRNA cap methylation apparatus, and Myc can promote mRNA cap methylation of a subset of mRNAs independently of transcription. Specifically, Myc mutants which are defective for transcription were found to promote mRNA cap methylation of some mRNAs and promote cell proliferation, albeit at a reduced rate compared to that induced by the wild-type Myc protein (9). Although use of these synthetic mutants correlated Myc-induced cap methylation with increased cell proliferation, the contribution of mRNA cap methylation to mediating the biological effects of wild-type Myc remained to be established.

The cap methylation reaction requires the methyl donor *S*-adenosyl methionine and produces a byproduct, *S*-adenosyl homocysteine, which inhibits subsequent methylation reactions

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▽ Published ahead of print on 5 October 2009.

(3). Following the finding that Myc upregulates mRNA cap methylation of many genes, an intriguing question was how efficient mRNA cap methylation was possible while *S*-adenosyl homocysteine was being generated. In this paper, Myc is demonstrated to upregulate *S*-adenosyl homocysteine hydrolase (SAHH), an enzyme which hydrolyzes and thus neutralizes *S*-adenosyl homocysteine. Myc-induced mRNA cap methylation but not transcription requires upregulation of SAHH. Myc-induced protein synthesis, cell proliferation, and cell transformation also require upregulation of SAHH; thus, Myc-induced mRNA cap methylation could be correlated with mediating the core Myc functions.

MATERIALS AND METHODS

Cell culture. P493-6 B cells were cultured in 10% fetal calf serum-RPMI medium. Rat1A cells, TGR cells (rat fibroblasts), Phoenix packaging cells, and mouse embryonic fibroblasts (MEFs) were cultured in 10% fetal calf serum-Dulbecco's modified Eagle medium. Immortalized mammary epithelial cells (IMECs) were cultured according to reference 11. T lymphoblasts were generated from murine spleen and maintained in medium containing interleukin-2 (IL-2) according to reference 37. Cells were washed out of IL-2 medium on day 5 and then incubated in media for 24 h with IL-2 (20 ng/ml), IL-7 (5 ng/ml), or IL-15 (20 ng/ml). All cell lines were made by retroviral infection using Phoenix packaging cells according to standard protocols, followed by drug selection. Rat1A and IMEC lines were infected with retroviruses carrying the plasmids LXSH, LXSH c-MycWT, and c-Myc ΔMBII and were selected with 150 μg/ml hygromycin (Calbiochem). MEFs were infected with pBabe and pBabe c-Myc, and TGR cells were infected with pBabe and pBabe c-MycER and selected with 2 μg/ml puromycin (Sigma).

Cell treatments. P493-6 cells were incubated with 0.2 μg/ml doxycycline (Sigma) for 2 days to repress c-Myc expression. For each cell line, the two independent SAHH small interfering RNAs (siRNAs) and the cyclophilin B control were purchased from the siGenome collection (Dharmacon). Rat 1A cells (1×10^5) and IMECs (4×10^5) were plated in six-well plates. Twenty-four hours later, cells were transfected with 2 or 4 μl, respectively, 50 μM siRNA, according to the manufacturer's instructions. For c-Myc siRNA the procedure was as described above except 6 μl was used. Transfection with cyclophilin B and no transfection gave equivalent results in all assays (not shown). For activation of the fusion protein consisting of Myc and the mutated estrogen receptor (MycER), cells were incubated in 100 nM 4-hydroxytamoxifen (Sigma) for 2 h and, when used, 20 μg/ml cycloheximide 30 min earlier. For tubercidin experiments, cells were incubated with 100 nM tubercidin or vehicle control.

Cell proliferation. Cells were transfected with siRNA, and 24 h later 5×10^4 Rat1A cells and/or 2×10^5 IMECs were plated and cell counts began. Rat1A cells (5×10^4) were plated, 24 h later 100 nM tubercidin was added, and 24 h later cell counts began. At each time point cells were trypsinized and counted using a hemocytometer.

Transformation assay. The transformation assay was performed as in reference 7. Cells were treated with siRNA, and 24 h later 2×10^4 Rat1A cells and 4×10^4 IMECs were plated in duplicate. Seven days after plating the sizes of the colonies originating from 100 plated cells were measured and representative micrographs were taken.

Western blots. Cells were washed in phosphate-buffered saline and lysed in F buffer (10 mM Tris, pH 7.05, 50 mM NaCl, 50 mM NaF, 10% glycerol, 0.5% Triton X-100) plus protease inhibitors. Extracts were normalized by Bradford assay (Bio-Rad). Western blotting was carried out according to standard procedures, and primary antibodies directed against the following proteins were used: c-Myc (N-262), β-tubulin (ac-18), and poly(ADP-ribose) polymerase (Santa Cruz Biotechnology) and SAHH (M07A; Abnova).

RT-PCR. RNA was extracted with TRIzol reagent (Invitrogen), and reverse transcription-PCR (RT-PCR) was performed using a One-Step RT-PCR kit (Invitrogen) according to the manufacturer's instructions. ^{32}P-labeled primers were used to allow visualization and quantitation of the product by phosphorimager. PCRs were determined to be in the linear range by titration of input. Primer sequences are available on request.

Homocysteine assay. The homocysteine assay was performed using the Axis homocysteine enzyme immunoassay kit (Axis-Shield) according to the manufacturer's instructions. Two days following siRNA treatment, 25 μl from 3 ml medium in the well was used in the assay. At this point cells were also counted to allow the amount of homocysteine per cell to be calculated.

Chromatin IP. The chromatin immunoprecipitation (IP) assay was performed using the Upstate Biotechnology chromatin IP kit according to the manufacturer's instructions. For each IP, 4×10^6 cells and 1 μg antibody were used. Chromatin IP primers are as follows: SAHH gene transcriptional start site (TSS), 5′TTCGCCCGTTTCCATCACGAGTGC3′) and 5′GCATGCTGGG ACTTGTAGTCCACG3′; SAHH gene CACGTG (position +7650 with respect to the TSS), 5′GTGTCCCTGTGTGATTAAATGTGTTCTCC3′ and 5′CATCTCGTTCTCAGCAATGTCCAGG3′; hemoglobin-coding region (Hbb), 5′GTTCACTAGCAACCTCAAACAGACACC3′ and 5′ATAACAG CATCAGGAGTGGACAGATCC3′.

Cell labeling. For [^{35}S]cysteine and [^{35}S]methionine labeling, 2 days following siRNA treatment or 2.5 h following tubercidin treatment, cells were incubated without cysteine and methionine for 30 min and then with 22 μCi [^{35}S]cysteine and [^{35}S]methionine for 15 min. Cells were lysed, protein was extracted, and trichloroacetic acid (TCA) was precipitated. For [*methyl*-^{3}H]methionine labeling, 2 days following siRNA treatment, cells were incubated with 3 μCi [*methyl*-^{3}H]methionine per well for 3 h and then RNA, DNA, and protein were extracted according to standard protocols and TCA was precipitated. For the protein methylation experiment, cells were incubated for 30 min with 20 μg/ml cycloheximide prior to labeling. Counts were measured using a scintillation counter.

Cap methylation. Relative cap-methylated mRNA levels were determined by IP followed by RT-PCR according to reference 5. In summary, cellular RNA was TRIzol extracted and further purified by phenol-chloroform extraction. For each IP, 10 μl purified anti-7-methylguanosine or control antibody was prebound to 25 μl protein A/G-Sepharose and then incubated with 2 to 4 μg cellular RNA for 30 min at room temperature. Immunoprecipitates were washed rapidly three times, and immunoprecipitated RNA was purified by phenol-chloroform extraction and used as a substrate for RT-PCR. The whole procedure was performed in phosphate-buffered saline–0.01% Triton–0.1 mg/ml bovine serum albumin–1 mM dithiothreitol. In addition IPs were performed with 2 μl RNasin and 0.01 mg/ml poly(U). The assay was performed after cells were treated for 2 days with siRNA or 3 h with tubercidin.

RESULTS

The SAHH gene is a Myc target gene. Our working hypothesis was that the *S*-adenosyl homocysteine generated in the cell by Myc induction of mRNA cap methylation must be metabolized in order to maintain the reaction, and we therefore investigated the role of the SAHH enzyme in this process.

The expression level of SAHH enzyme in response to elevated Myc expression was investigated in a range of cell lines. Western blotting was performed on cell extracts from P493-6 B cells in which constitutively high levels of exogenous Myc were expressed or repressed by incubation with doxycycline and in Rat-1A fibroblasts, primary MEFs, and IMECs which were transduced with exogenous Myc by retroviral infection. In all cell lines, elevated Myc protein expression was found to correlate with increased SAHH protein expression (Fig. 1A). In the Rat1A cells and IMECs, the increase in SAHH expression was found to be partially dependent on the Myc Box II domain (MBII), a conserved region of the transactivation domain. SAHH protein was also upregulated in response to N-Myc (data not shown).

The level of expression of SAHH in response to expression of endogenous Myc was also investigated. Murine primary T lymphocytes were incubated with the cytokines IL-2, IL-7, and IL-15. The Myc level was highest in lymphocytes incubated with IL-2 and lowest in lymphocytes incubated with IL-7, and SAHH levels correlated with Myc levels (Fig. 1A). Endogenous Myc expression was repressed in Rat1A fibroblasts using siRNA, and SAHH level also correlated with Myc level (Fig. 1A).

The mechanism of SAHH regulation was further investigated by examining the expression level of SAHH mRNA in

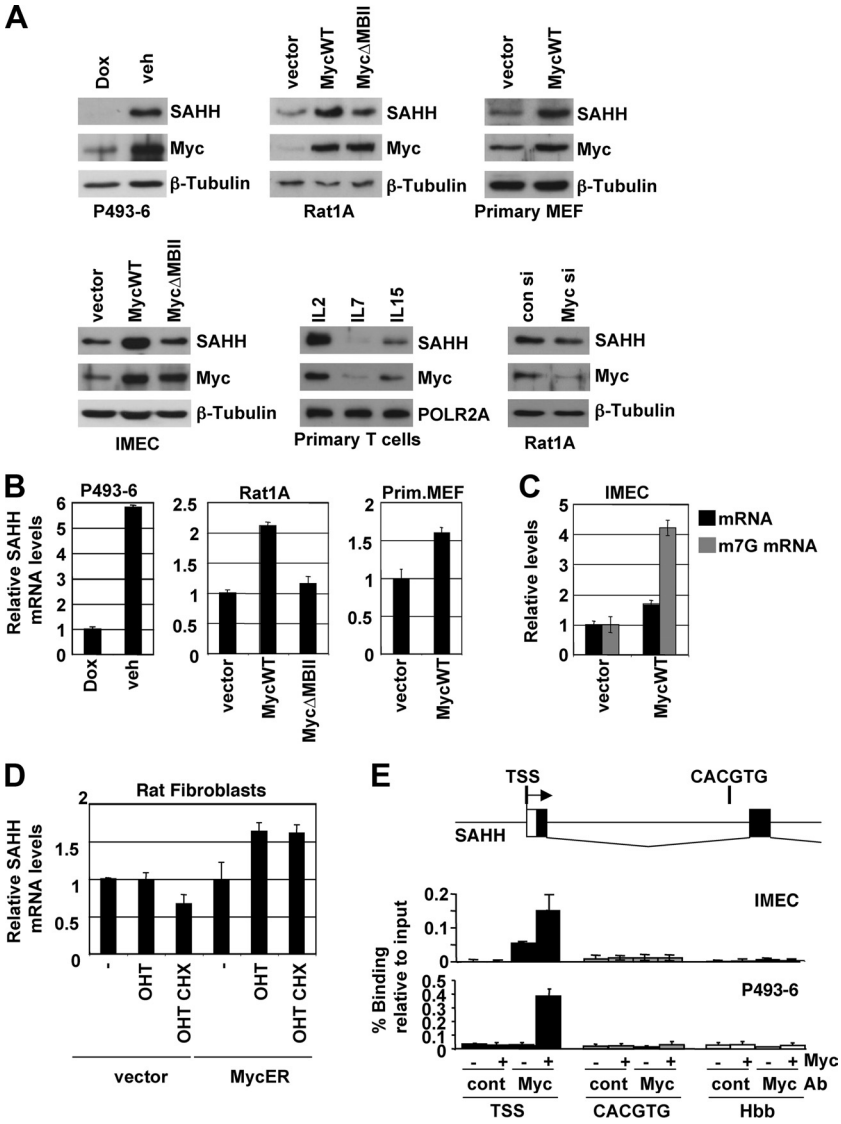

FIG. 1. The SAHH gene is a Myc target gene. (A) P493-6 cells were incubated with doxycycline (Dox) to inhibit exogenous Myc expression or vehicle control (veh). Rat1A fibroblasts, primary MEFs, and IMECs were infected with retroviruses to express vector control, MycWT, or MycΔMBII, and pools of cells were drug selected. Primary murine T cells were incubated with IL-2, IL-7, and IL-15 for 24 h. Rat1A cells were incubated with a control siRNA (con si; cyclophilin B siRNA) or an siRNA directed against Myc (Myc si) for 24 h. Western blotting was performed to detect SAHH, Myc, β-tubulin, or POLR2A expression, as loading controls. (B) RNA was extracted from the cell lines indicated, and RT-PCR was performed to detect SAHH mRNA levels relative to GAPDH (or 18S rRNA for P493-6). Prim., primary. (C) In the IMEC lines indicated, SAHH mRNA was detected by RT-PCR (black bars) and m7G (cap-methylated) SAHH mRNA was detected by anti-m7G IP followed by RT-PCR (gray bars). (D) Rat fibroblasts expressing the vector control or MycER were incubated with 4-hydroxytamoxifen (OHT) for 2 h to activate MycER and with cycloheximide (CHX) 30 min prior to OHT to inhibit protein synthesis. RT-PCR was used to detect SAHH mRNA levels relative to GAPDH levels. (E) Chromatin IP was performed in P493-6 cells incubated with Dox (low Myc; −) or the vehicle control (high Myc; +) and IMECs expressing the vector control (−) or c-Myc (+). Polyclonal control antibodies (cont) or anti-c-Myc antibodies (Myc) were used for immunoprecipitation, as indicated. Myc was found to bind significantly to the SAHH TSS but not to the E-box (CACGTG) found in intron 1 at +7645 or to Hbb.

response to changes in Myc expression level. RNA was extracted from P493-6 B cells, Rat1A fibroblasts, primary MEFs, and IMECs, and SAHH mRNA levels were determined by RT-PCR, relative to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA (or relative to 18S rRNA levels for P493-6 cells) (Fig. 1B and C). In all cell lines, elevated Myc expression correlated with increased SAHH mRNA expression levels. The relative cap methylation of SAHH mRNA in IMECs was also measured by anti-7-methylguanosine antibody immunoprecipitation performed on total cellular RNA, followed by RT-PCR to detect the immunoprecipitated (cap-methylated, m7G) SAHH mRNA (Fig. 1C). As has been observed for other Myc transcriptional target genes (5), the Myc-induced increase in cap methylated SAHH mRNA was significantly higher than the increase in total SAHH mRNA.

In order to determine that the SAHH gene is a direct Myc target gene, MycER was used (13, 20). MycER is inactive when expressed in cells and is rapidly activated by addition of the

estrogen receptor ligand, 4-hydroxytamoxifen. MycER can also be activated in the presence of the protein synthesis inhibitor cycloheximide, allowing the direct effects of Myc on transcription to be observed. Rat fibroblasts expressing MycER or the vector control were incubated in 4-hydroxytamoxifen for 2 h, with or without prior incubation in cycloheximide (Fig. 1D). SAHH mRNA levels were found to be induced rapidly in response to Myc activation and independently of protein synthesis.

In order to investigate whether Myc bound directly to the SAHH gene promoter, the chromatin IP assay was performed on P493-6 cells in which exogenous Myc was either constitutively expressed or repressed by incubation with doxycycline and on IMECs expressing exogenous Myc or the vector control (Fig. 1E). Myc binding to the SAHH gene TSS and a conserved E-box in intron 1 (CACGTG) was investigated. Hbb was used as a negative control. As has been observed with many Myc target genes, Myc was found to be enriched at the TSS of the SAHH gene but not at the conserved E-box in intron 1 (Fig. 1E) (19). There are numerous E-boxes with the loose consensus sequence (CANNTG) in the vicinity of the TSS, and it is likely that Myc binds to these. Additionally, the Myc transactivation domain can be recruited to chromatin, presumably via protein-protein interactions (9).

SAHH gene transcription and mRNA cap methylation were demonstrated to be upregulated by Myc (Fig. 1B). An alternative means of demonstrating that a gene can be upregulated in a transcription-independent manner is to demonstrate that it can be upregulated by mutants of Myc which are defective for transactivation (9). An obvious question was whether SAHH can be upregulated by these transactivation-defective mutants, and this was found to be the case. Expression of N-MycBM, which contains four point mutations in the basic region, and N-MycΔC, a truncation mutant which has the DNA-binding domain deleted, induced expression of SAHH, albeit at a reduced extent compared to N-MycWT (data not shown). As expected, upregulation of SAHH induced by the transactivation-defective mutants was independent of upregulation of the SAHH mRNA and correlated with an increase in cap-methylated SAHH mRNA (not shown).

In summary, Myc binds to the SAHH gene promoter and SAHH expression correlates with endogenous and exogenous Myc protein expression in cells from a range of lineages, including primary cells. SAHH mRNA expression increases rapidly and independently of protein synthesis in response to Myc. Myc also induces increased cap methylation of SAHH. Therefore, the SAHH gene is a Myc target gene.

Myc-induced SAHH expression is necessary for Myc-induced mRNA cap methylation but not transcription. In order to investigate the biological significance of Myc-induced SAHH expression, transfection of two independent siRNAs was used to reduce SAHH protein levels in Rat1A fibroblasts and IMECs. SAHH protein expression was reduced in cells expressing exogenous Myc to levels equivalent to those observed in the corresponding vector control cells (Fig. 2A and B). It is important to emphasize that this level of reduction of SAHH protein allowed the role of Myc-induced SAHH expression, rather than the role of SAHH in general, to be investigated. The same cells and vector control cells were also transfected with control siRNA. Figure 2A and B are Western blots of cell extracts 2 days after siRNA transfection, and SAHH levels in SAHH siRNA-transfected cells did not fall below this level on subsequent days (not shown).

In order to determine whether the elevated SAHH expression found in Myc-expressing cells had a detectable biochemical effect, levels of one product of the SAHH catalyzed reaction, homocysteine (Hcy), were measured. In Rat1A fibroblasts, Myc expression resulted in an increase in Hcy from 4.7 to 7.9 μmol/10^6 cells, consistent with increased SAHH expression (Fig. 2C). The increase in Hcy was dependent on Myc-induced SAHH expression, since transfection with SAHH siRNAs reduced the Hcy levels to 5.4 and 4.3 μmol/10^6 cells.

The hypothesis that Myc-induced mRNA cap methylation requires upregulation of SAHH was tested. In Rat1A fibroblasts, Myc induction of total mRNA and cap-methylated (m7G) mRNA was investigated for three Myc target genes, the Nol5a, NPM, and eIF4E genes (Fig. 2D). In cells expressing exogenous Myc, upregulation of SAHH was left intact or inhibited by transfection of SAHH-directed siRNAs (Fig. 2A). Myc induced a 1.6-fold increase in Nol5a and eIF4E mRNA and a 1.5-fold increase of NPM mRNA, and inhibition of Myc induction of SAHH expression did not result in a significant change in induction of these transcripts (Fig. 2D). Myc induced mRNA cap methylation by 3.1-fold for Nol5a, 2.7-fold for NPM, and 2-fold for eIF4E. Inhibition of SAHH upregulation resulted in a significant reduction in mRNA cap methylation for all three Myc target genes.

The same experiment was carried out with IMECs, and the expression and cap methylation of the three Myc target genes, the Nol5a, NUCL, and NPM genes, was investigated (Fig. 2E). Myc induced a 1.6-fold increase in Nol5a mRNA, a 1.4-fold increase in NUCL mRNA, and a 2-fold increase in NPM mRNA, and inhibition of Myc-induced SAHH upregulation did not result in a significant change in induction of these transcripts (Fig. 2E). Myc induced mRNA cap methylation by 4.6-fold for Nol5a, 4.2-fold for NUCL, and 3.5-fold for NPM, and inhibition of SAHH upregulation resulted in a significant reduction in mRNA cap methylation for all three Myc target genes.

Therefore the Myc-dependent increase in SAHH expression is required for Myc-induced cap methylation but not transcription.

Myc-induced SAHH expression does not alter total Myc-dependent RNA, DNA, and protein methylation. *S*-Adenosyl homocysteine has the potential to inhibit other methylation reactions in addition to mRNA cap methylation (3). Therefore, the effect of Myc-induced SAHH expression on other methylation reactions was investigated. Rat1A fibroblasts were incubated with [*methyl*-^{3}H]methionine, which is readily converted into the methyl donor *S*-[*methyl*-^{3}H]adenosyl methionine in the cell, resulting in labeled methylated substrates. Following the incubation, RNA, DNA, and protein were isolated and methyl incorporation was determined. When protein methylation was studied, cells were also incubated with the protein synthesis inhibitor cycloheximide to prevent [*methyl*-^{3}H]methionine incorporation as a result of protein synthesis. Inhibition of protein synthesis by cycloheximide was confirmed by measuring [^{35}S]methionine incorporation into protein (not shown).

Over 95% cellular RNA is rRNA and tRNA, both of which

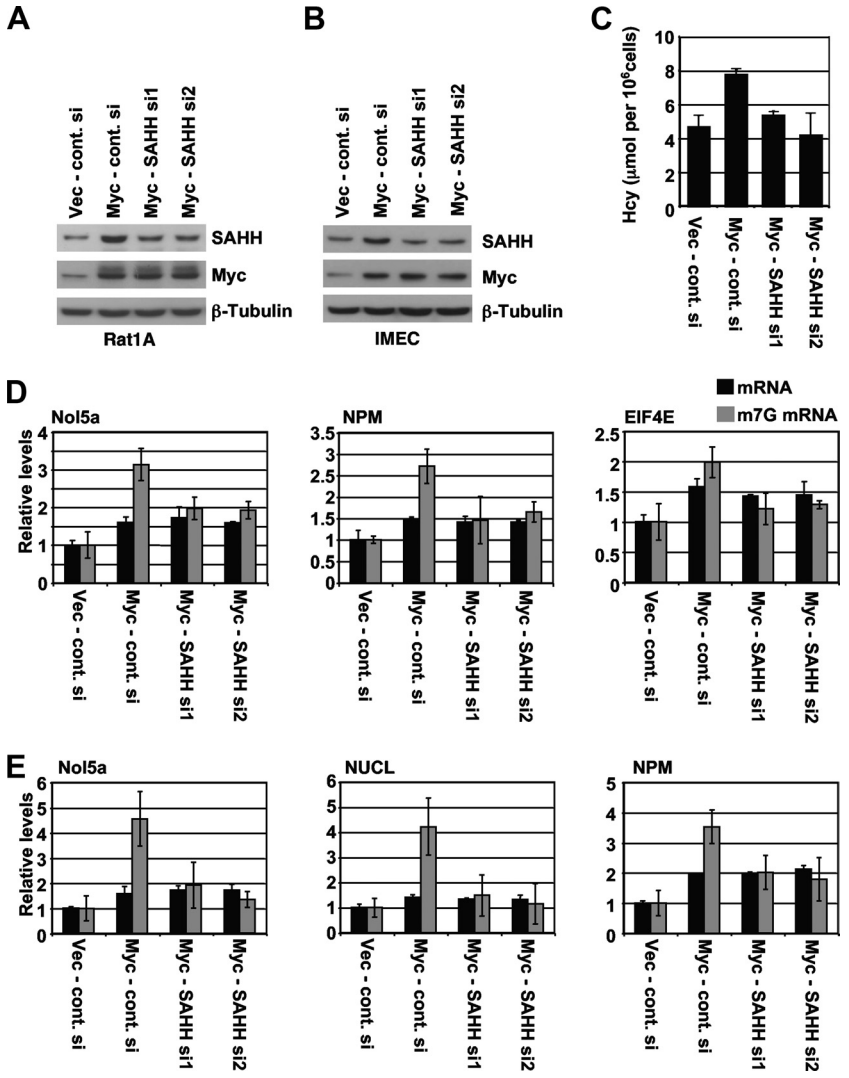

FIG. 2. Myc-induced SAHH expression is necessary for Myc-induced mRNA cap methylation. In Rat1A cells (A) and IMECs (B), SAHH expression was reduced in cells expressing exogenous Myc to a level equivalent to that found in vector control cells by transfection of two independent siRNAs directed against SAHH (SAHH si1 and SAHH si2). Vector control (vec) and exogenous c-Myc-expressing cells (Myc) were incubated with control siRNA (cont. si). Western blotting was performed on cell extracts to detect SAHH, Myc, and β-tubulin. (C) In the Rat1A cells, homocysteine levels in the extracellular medium were measured ($n = 3$). In Rat1A cells (D) and IMECs (E), mRNA and m7G (cap-methylated) mRNA levels were measured as described for Fig. 1C, for the Myc target genes encoding Nol5a, NPM, NUCL, and eIF4E ($n = 4$). For each gene examined, the values for m7G mRNA in exogenous c-Myc-expressing cells were significantly higher (t test; $P < 0.01$) in cells transfected with control siRNA than in cells transfected with SAHH siRNA.

are methylated on many residues. Myc target genes include those encoding a collection of rRNA-processing enzymes (40), and elevated Myc expression was found to increase total RNA methylation in Rat1A cells (Fig. 3A). However, inhibition of SAHH upregulation did not significantly inhibit RNA methylation.

The Myc cofactor DNMT3a is a DNA methyltransferase which mediates Myc-dependent transcriptional repression (2), and elevated Myc expression was also found to increase DNA methylation in our experimental systems (Fig. 3B). Inhibition of Myc-dependent SAHH expression also did not inhibit DNA methylation.

Cellular proteins are also the target of protein methylation. Increased H3K27 dimethylation was found at a subset of Myc target gene promoters (23), and Myc has been found to bind to ASH2, a component of complexes containing histone methyltransferase activity (21). Elevated Myc expression increased total protein methylation, and again this was not significantly SAHH dependent (Fig. 3C). Therefore, although Myc-dependent SAHH upregulation may be critical for other methylation events in addition to mRNA cap methylation, evidence for this was not found under these experimental conditions (see Discussion). The primary effect of Myc-induced SAHH upregulation appeared to be to permit Myc-induced mRNA cap methylation.

Myc-induced SAHH expression is necessary for Myc-induced protein synthesis. Myc promotes increased protein synthesis, probably via the collaboration of a number of

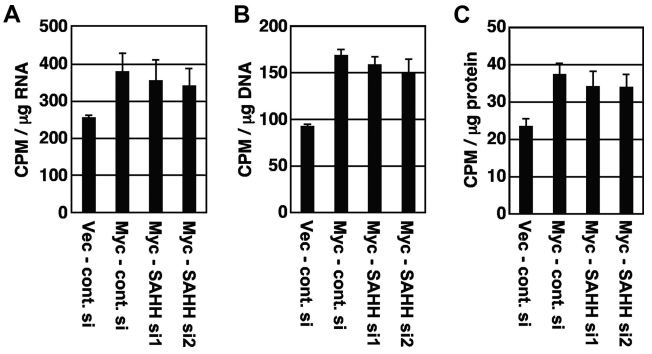

FIG. 3. Myc-induced SAHH expression does not regulate total RNA, DNA, and protein methylation. SAHH expression was reduced in Rat1A cells expressing exogenous Myc to a level equivalent to that found in vector control cells by transfection of two independent siRNAs directed against SAHH (SAHH si1 and SAHH si2). Vector control (vec) and exogenous c-Myc-expressing cells (Myc) were incubated with control siRNA (cont. si). Methylation of (A) RNA, (B) DNA, and (C) protein was determined by measuring [*methyl*-^{3}H]methionine incorporation ($n = 3$). For all three panels, the values for cells transfected with vector control siRNA were significantly lower than the values for cells expressing c-Myc and transfected with control siRNA (*t* test; $P < 0.0001$). The values for c-Myc/control siRNA and c-Myc/SAHH siRNAs were not significantly different (*t* test; $P > 0.01$).

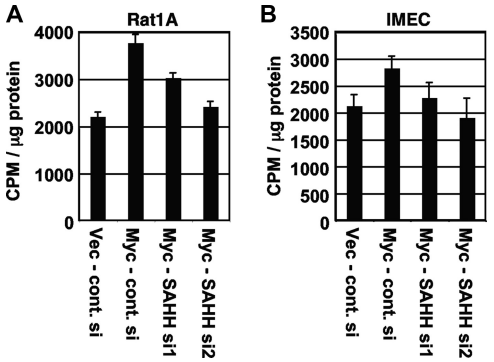

FIG. 4. Myc-induced SAHH expression is necessary for Myc-induced protein synthesis. SAHH expression was reduced in (A) Rat1A cells and (B) IMECs expressing exogenous Myc to a level equivalent to that found in vector control cells by transfection of two independent siRNAs directed against SAHH (SAHH si1 and SAHH si2). Vector control (vec) and exogenous c-Myc-expressing (Myc) cells were incubated with control siRNA (cont. si). The protein synthesis rate was analyzed by measuring incorporation of [^{35}S]methionine and [35S]cysteine into TCA-precipitable material ($n = 5$). For both panels, the values for cells expressing exogenous c-Myc and transfected with siRNA were significantly higher than the values for cells expressing vector control and transfected with control siRNA and for cells expressing exogenous c-Myc and transfected with SAHH siRNAs (*t* test; $P < 0.01$).

mechanisms including increased mRNA production, mRNA cap methylation, increased rRNA and tRNA production, and increased translation factor and ribosomal protein production (6, 18, 32). To determine the contribution of SAHH upregulation and mRNA cap methylation to Myc-induced protein synthesis, radiolabeled amino acid incorporation into proteins in response to elevated Myc expression was measured, with upregulation of SAHH left intact or inhibited by transfection of siRNAs (Fig. 4). In Rat1A cells and IMECs (Fig. 4A and B, respectively), Myc induced an increase in protein synthesis. Inhibition of SAHH upregulation resulted in a significant reduction in protein synthesis, equivalent to that found in vector control cells. The rate of protein degradation in the same cell lines was also measured using standard pulse-chase protocols and was not found to be significantly Myc dependent or altered by repression of SAHH expression (not shown). Therefore, Myc-induced SAHH expression is required for mRNA cap methylation and protein synthesis.

Myc-induced SAHH expression is necessary for Myc-induced cell proliferation and cell transformation. Myc promotes cell proliferation and, under certain conditions, anchorage-independent cell proliferation, i.e., cell transformation. In order to determine whether Myc-induced SAHH expression and mRNA cap methylation are rate limiting for cell proliferation, Rat1A fibroblasts and IMECs expressing exogenous Myc or vector control were counted on consecutive days. In cells expressing exogenous Myc, SAHH expression was left intact or inhibited using siRNAs (Fig. 5A and B). Elevated Myc expression induced an increase in cell proliferation compared to the vector control in both cell lines, and this increase was partially dependent on SAHH upregulation.

Anchorage-independent cell growth was also inhibited by reducing SAHH expression in the same cell lines, as measured by plating cells in suspension in soft agar (Fig. 5C and D). Vector control cells did not grow in an anchorage-independent manner for either cell line, whereas approximately 90% of Rat1A cells and 60% of IMECs expressing exogenous Myc that were plated in suspension grew into colonies (not shown). Inhibition of Myc-induced SAHH upregulation in both cell lines expressing exogenous Myc did not inhibit the percentage of plated cells that grew into colonies (not shown) but did reduce the colony size (Fig. 5C and D). One week after plating, over 10% of Rat1A fibroblasts expressing exogenous Myc had grown into colonies larger than 100 μm in diameter, whereas when SAHH expression was reduced to vector control levels, only approximately 2% of plated cells reached this size (Fig. 5C). Similarly, 23% of IMECs expressing exogenous Myc plated in suspension grew into colonies larger than 50 μm, whereas when SAHH expression was reduced to vector control levels, only 5 to 10% of plated cells reached this size (Fig. 5D).

In summary, Myc-induced SAHH expression is required to permit Myc-induced mRNA cap methylation, cell proliferation, and cell transformation.

Inhibition of mRNA cap methylation correlates with reduced Myc-induced protein synthesis and cell proliferation. For independent evidence that mRNA cap methylation is required for Myc-induced protein synthesis and cell proliferation, mRNA cap methylation was inhibited by culturing Rat1A fibroblasts with the drug tubercidin. Tubercidin is an adenosine analogue that has been reported to inhibit both mRNA cap methylation and transcription (31). However, in our experimental systems, by titrating the concentration of tubercidin used, it was found that 100 nM tubercidin does not inhibit transcription, as determined by measuring the rate of [^{3}H]uridine incorporation into RNA and oligo(dT)-purified RNA (predominantly mRNA) (not shown). In order to investigate Myc-dependent transcription and mRNA cap methylation, Rat1A fibroblasts expressing Myc or the vector control were incubated in 100 nM tubercidin for 3 hours and the relative

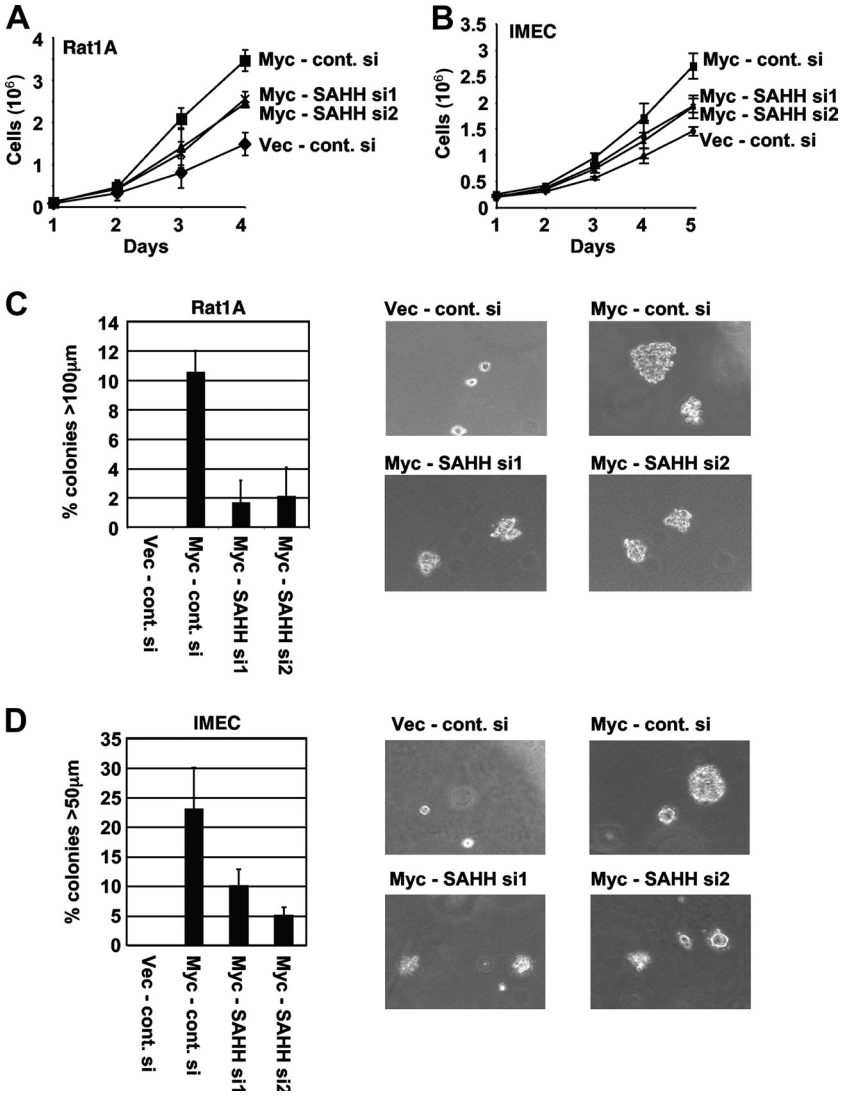

FIG. 5. Myc-induced SAHH expression is required for Myc-induced cell proliferation and cell transformation. SAHH expression was reduced in (A and C) Rat1A cells and (B and D) IMECs expressing exogenous Myc to a level equivalent to that found in vector control cells by transfection of two independent siRNAs directed against SAHH (SAHH si1 and SAHH si2). Vector control (vec) and exogenous c-Myc-expressing cells (Myc) were incubated with control siRNA (cont. si). (A and B) Cell proliferation was analyzed by counting cells each day ($n = 4$). (C and D) Cell transformation was investigated using the soft agar transformation assay. At day 7, representative micrographs were taken and number of plated cells which form colonies over 50 or 100 μm was determined ($n = 2$).

transcript level and mRNA cap methylation were determined for three Myc target genes, the Nol5a, NPM, and eIF4E genes (Fig. 6A). For all three Myc target genes, the tubercidin treatment did not inhibit either the transcript level or mRNA cap methylation in vector control cells. However, in cells expressing exogenous Myc, tubercidin treatment significantly inhibited mRNA cap methylation but not transcription.

Protein synthesis in response to tubercidin treatment was determined by measuring incorporation of labeled amino acids into acid-precipitable material (Fig. 6B). Tubercidin treatment did not inhibit protein synthesis in vector control cells but did inhibit protein synthesis in cells expressing exogenous Myc. Similarly, vector control cell proliferation was unchanged by tubercidin treatment, whereas the proliferation rate of exogenous Myc-expressing cells was inhibited (Fig. 6C). The effect of the same tubercidin treatment on total RNA, DNA, and protein methylation was measured as described for Fig. 3 (Fig. 6E and F). Tubercidin did not have a significant effect on total RNA, DNA, or protein methylation in either Rat1A fibroblasts expressing Myc or the vector control.

In summary, tubercidin treatment inhibited Myc-induced mRNA cap methylation but not transcription or the other Myc-dependent methylation events investigated, and this correlated with a reduction in Myc-induced protein synthesis and cell proliferation.

DISCUSSION

In this paper, the SAHH gene was demonstrated to be a novel Myc target gene whose upregulation is required to per-

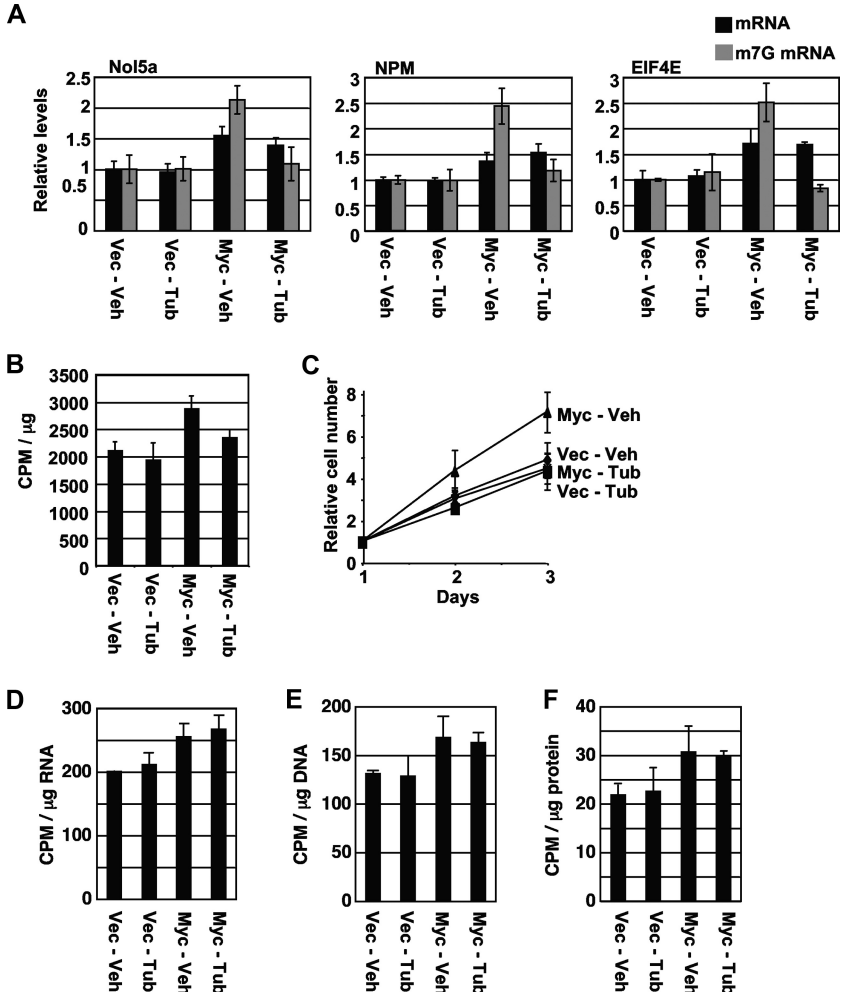

FIG. 6. Myc-induced cap methylation correlates with Myc-induced protein synthesis and cell proliferation. (A) Rat1A cells were incubated with 100 nM tubercidin (Tub) or vehicle control (Veh) for 3 h, and RNA was extracted. mRNA and m7G mRNA levels for three Myc target genes were measured as described for Fig. 1 ($n = 3$). (B) The protein synthesis rate was analyzed by measuring incorporation of [^{35}S]methionine and [^{35}S]cysteine into TCA-precipitable material ($n = 3$). (C) Cell proliferation was analyzed by counting cells each day ($n = 3$). Methylation of (D) RNA, (E) DNA, and (F) protein was determined by measuring [*methyl*-^{3}H]methionine incorporation ($n = 3$).

mit Myc-induced mRNA cap methylation. As described in the introduction, Myc induces mRNA cap methylation of a relatively large number of genes, and we have demonstrated here that the inhibitory byproduct of this reaction, *S*-adenosyl homocysteine, accumulates to such an extent that it needs to be hydrolyzed by SAHH in order for the cap methylation reaction to be maintained. Myc regulation of mRNA cap methylation was previously correlated with enhanced recruitment of TFIIH to promoters and increased RNA pol II phosphorylation (9). TFIIH-phosphorylated RNA pol II recruits the cap methyltransferase RNMT into the proximity of its substrate, the 5′ mRNA cap (29). Data presented here demonstrate that, although increased TFIIH recruitment and RNA pol II phosphorylation may be necessary to promote Myc-induced mRNA cap methylation, they are not sufficient to do so without the accompanying upregulation of SAHH.

Following the finding that Myc regulates cap methylation as well as transcription, a key question was whether Myc-induced mRNA cap methylation is a mechanism that has a significant impact on Myc-regulated cellular events (6). By inhibiting the upregulation or activity of SAHH, mRNA cap methylation could be selectively repressed, leaving Myc-induced transcription and other methylation events unaffected. Inhibition of SAHH also inhibited the core Myc functions of protein synthesis, cell proliferation, and cell transformation, thus correlating mRNA cap methylation with Myc biological function and providing the first evidence that Myc-induced mRNA cap methylation is an essential effector of Myc.

SAHH has also been implicated in regulating mRNA cap methylation during *Xenopus laevis* development (31). SAHH is translocated from the cytoplasm to the nucleus during gastrulation, and inhibitors of SAHH inhibited both mRNA methylation and transcription. This study complements a large body of work which demonstrates that the processes of transcription and mRNA cap methylation are mechanistically codependent (1, 26). Shortly after transcription has initiated, DSIF (DRB [5,6-dichloro-1-β-D-ribofuranosylbenzimidazole] sensitivity-inducing factor) is recruited to RNA pol II, and the subsequent

recruitment of NELF (negative elongation factor) mediates pausing of the polymerase. Recruitment of RNA guanylyltransferase and RNGTT/CE to the complex at this stage relieves repression of transcription, although this does not require the enzymatic activity of the enzyme (22). Transcription elongation can be further enhanced by the cap methyltransferase. The *Schizosaccharomyces pombe* cap methyltransferase, Pcm1p, has been shown to recruit P-TEFb (positive elongation factor) to chromatin, and the *Saccharomyces cerevisiae* cap methyltransferase, Abd1p, promotes RNA pol II progression through a subset of genes, although again this is independent of the enzymatic activity of the cap methyltransferase (15, 33). Furthermore *S*-adenosyl homocysteine, a cap methyltransferase inhibitor, was found to inhibit transcription initiation but not elongation (17). However, inactivation of Abd1p in vivo was not found to dramatically reduce the levels of transcripts of the genes investigated, and therefore the cap methyltransferase is more likely to be required for or to enhance transcription of certain genes rather than be an integral part of the transcription cycle for all genes (34). Our study also indicates that cap methylation is not required for transcription elongation for the genes investigated in this study, that is, inhibition of cap methylation of Myc target genes does not inhibit transcription.

SAHH has the potential to regulate other methylation events if the methylation reaction has become inhibited by elevated *S*-adenosyl homocysteine levels. However, in this study, Myc-induced SAHH upregulation was not found to regulate total cellular RNA, DNA, or protein methylation. RNMT may be inhibited by a lower concentration of *S*-adenosyl homocysteine than other methyltransferases, or the local concentration of the *S*-adenosyl homocysteine generated by mRNA cap methylation may rise to inhibitory levels. It is worth noting that in *Xenopus* SAHH was found to bind to the cap methyltransferase and localize to transcriptionally active lampbrush chromosomes, an interaction which, one may speculate, evolved because cap methylation is acutely sensitive to *S*-adenosyl homocysteine levels (30). It also remains a possibility that Myc-induced methylation of specific, as yet unmapped, residues within RNA molecules, DNA regions, or proteins is regulated by *S*-adenosyl homocysteine abundance and therefore levels of SAHH enzyme. Until such sites of Myc-induced methylation have been mapped, it will not be possible to test this hypothesis.

An obvious question is whether upregulation of SAHH in the absence of Myc upregulation can promote mRNA cap methylation. In order to address this question, we transduced cells with a variety of mammalian expression constructs designed to express exogenous SAHH. Unfortunately all our cell systems were exquisitely sensitive to elevated SAHH levels, and expression of even limiting levels of exogenous SAHH resulted in a loss of cell viability (not shown). Presumably, elevation of SAHH expression alone above endogenous levels is toxic. This finding is consistent with previous publications which have documented a loss of cell viability in response to elevated SAHH levels (16). Since SAHH levels can be elevated in response to Myc (Fig. 1), elevated Myc expression must provide other signals which prevent SAHH-mediated toxicity.

In summary, we now understand that Myc-induced mRNA cap methylation requires at least two events, that is, increased TFIIH recruitment and upregulation of SAHH. Furthermore, Myc-induced SAHH upregulation plays a key role in mediating critical biological effects of Myc, correlating mRNA cap methylation with Myc function.

ACKNOWLEDGMENTS

This work was funded by an MRC Career Development Award and a Tenovus Scotland grant to V.H.C.

We thank Michael Cole for the gift of the anti-7-methylguanosine antibody and Oliver Vugrek for SAHH cDNA. We thank Michael Johnston, Jeff Brady, Doreen Cantrell, and members of the Cowling lab for useful discussions.

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