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# IS THERE AN ASSOCIATION BETWEEN AN INCREASE IN c-*myc* RNA STEADY STATE LEVELS AND c-*myc* METHYLATION IN HL-60 CELLS TREATED WITH 3-DEAZA-(±)-ARISTEROMYCIN, AN INDIRECT INHIBITOR OF METHYLATION?

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**Abstract**—Alteration in gene expression of the proto-oncogene c-*myc* in HL-60 cells is associated with differentiation of these cells. We have studied the steady state levels of c-*myc* transcripts, the levels of transmethylation metabolites *S*-adenosylmethionine and *S*-adenosyl-homocysteine and the methylation pattern of the c-*myc* gene after treatment of HL-60 cells with the transmethylation inhibitor and granulocytic inducer, 3-deaza-(±)-aristeromycin. A transient increase in c-*myc* RNA levels after 45 min of drug exposure was observed which was accompanied by changes in the ratio of transmethylation metabolites in both whole cells and nuclei. The changes in transmethylation metabolites in whole cells, although compatible with levels frequently associated with hypomethylation of cellular components, caused no changes in methylation of c-*myc* DNA sequences of the HL-60 cells as detected by *Hpa*II or *Msp*I digestion and Southern blotting.

We have used the human promyelocytic leukemia cell line HL-60 [1], which has been extensively employed in studies of molecular mechanisms controlling proliferation and differentiation [2, 3], to investigate molecular targets for the adenosine analogue 3-deaza-(±)-aristeromycin (c$^3$Ari‖). c$^3$Ari induces HL-60 cells to differentiate in the granulocytic direction, and perturbs the levels of transmethylation metabolites in cells by inhibition of *S*-adenosyl-homocysteine (AdoHcy) hydrolase (EC 3.3.1.1) [4–6]. AdoHcy hydrolase has been proposed to play a key role in the regulation of biological methylation reactions by modulating the AdoMet/AdoHcy ratio [7], conceivably also *in vivo* DNA methylation. The biological significance of DNA methylation emerges from a great number of studies showing that alteration in methylation of DNA cytosine plays a role in the regulatory mechanisms of expression of certain genes [8–14] and in cell differentiation [15–20].

Numerous variables are known to be involved during HL-60 cell differentiation, among them the expression of the oncogene c-*myc* [2, 3, 21–24]. Although a down-regulation of c-*myc* mRNA is the most frequent finding in growth-arrested HL-60 cells at the onset of differentiation [25], we postulated that an early increase in c-*myc* RNA should be observed after c$^3$Ari induction if DNA hypomethylation of c-*myc* were a dominant molecular mechanism. The rationale for this hypothesis is the fact that despite numerous attempts to demonstrate alternative mechanisms, AdoHcy hydrolase is, so far, the only known cellular target for c$^3$Ari [4–6]. We show here that c$^3$Ari induces an early increase in c-*myc* RNA and an alteration in the ratio of transmethylation metabolites in whole cells formerly associated with cellular hypomethylation. We further demonstrate no relation between changes in the ratio of cellular transmethylation metabolites and c-*myc* DNA methylation.

## MATERIALS AND METHODS

*Chemicals.* c$^3$Ari was kindly supplied by the Dr Peter K. Chiang. AdoHcy and AdoMet were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Acetonitrile was HPLC grade, whereas all other reagents were of analytical grade.

*Equipment.* HPLC was carried out with a Rheodyne (Cotati, CA, U.S.A.) Model 7125 injector fitted with a 200-μL loop, a Perkin–Elmer Serie 4 pump (Perkin–Elmer), and an absorbance detector (Spectroflow Model 773 UV-VIS; Kratos, Ramsey, NJ, U.S.A.) operated at 254 nm and 0.001 a.u.f.s. Quantification was performed with an SP 4270 integrator (Spectra-Physics, San Jose, CA, U.S.A.).

The reversed-phase columns (analytical column, 250 mm × 4.6 mm i.d.; guard, 20 mm × 4.6 mm i.d.) used were Supelcosil $C_{18}$-DB, 5 μm particle size (Supelco, Bellefonte, PA, U.S.A.), and Supelguard $LC_{18}$-DB, 5 μm particle size (Supelco).

*Cells.* HL-60 cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% horse serum and 100 U/mL of streptomycin and penicillin,

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‖ Abbreviations: c$^3$Ari, 3-deaza-(±)-aristeromycin; AdoHcy, *S*-adenosyl-homocysteine; AdoMet, *S*-adenosyl-methionine; $^{m5}$C, 5-methylcytosine.

respectively. Cells were kept in a humidified 5% $CO_2$ atmosphere at 37°. Cell counts were determined by the use of a hemocytometer chamber.

*Drug treatment.* $c^3$Ari was dissolved in RPMI 1640 medium and used at a final concentration of 25 μM. The drug was present continuously before cells were harvested.

*Preparation of perchloric acid extract of cells.* About $10^7$ cells were homogenized in 100 μL 0.4 N perchloric acid and treated as described by Ueland *et al.* [26].

*Preparation of nuclear extracts.* Nuclei were extracted from $10^8$ cells as described previously [27]. AdoHcy and AdoMet were extracted from nuclei as described for whole cells.

*HPLC analyses of AdoHcy and AdoMet extracts.* The supernatants obtained by extraction of cells or nuclei, were mixed with equal volumes of 20 mM heptansulfonic acid. Two hundred microlitres of cell mixtures and 100 μL of nuclear mixtures, respectively, were subjected to HPLC.

AdoMet and AdoHcy were analysed by ion-pair chromatography by a modification of the method of Wagner *et al.* [28] in a single run eluting the column with a linear gradient of acetonitrile (7–12%) in 120 mM sodium dehydrogen phosphate pH 2.8, and 8 mM heptan sulfonic acid over 15 min. The flow rate was 1.5 mL/min.

Concentrations of AdoHcy and AdoMet in unknown samples were calculated by linear regression, using AdoHcy and AdoMet peak height standard curves of the compounds dissolved in the mobile phase.

*DNA isolation.* Logarithmically growing HL-60 cells were harvested after 45 min, 1.5, 3 and 36 hr. The doubling time for control cells was 18 hr. DNA was isolated from pellets of $10^7$ cells as described previously [29]. The purities and concentrations of the isolated DNAs were determined spectrophotometrically.

*Enzyme digestion.* Aliquots containing 4–8 μg of DNA were incubated with 30 and 45 U of *Hpa*II or *Msp*I, respectively (USB, United States Biochemical Corporation, Cleveland, OH, U.S.A.) as recommended by the manufacturer.

*Southern blots.* After digestion with the restriction endonucleases, DNA fragments were separated by electrophoresis in 0.8% agarose gel using a Tris–borate–EDTA buffer. Molecular mass markers, 1.0 kb ladder (Gibco, BRL) or *Hinc* II digested ΦX 174 phage DNA (USB) were included in every gel and were visualized by photographing the ethidium bromide-stained gels under UV light. Alkaline transfer of DNA to Nytran membranes (Schleicher & Schuell) was performed by vacuum blotting (30 cm $H_2O$ for 30 min; 2016 Vacugene Vacuum blotting Unit, LKB, Bromma), followed by neutralization in 50 mM Tris–HCl, pH 7.2. Filters were prehybridized overnight, and hybridization of DNA blots were performed at 65% for 36 hr according to the method by Church and Gilbert [30].

*Northern blots.* Cells were treated with the drug as described above, and total RNA was isolated from $10^7$ cells by the guanidinum/cesium chloride method [31]. Samples (20 μg) were electrophoresed in 0.8% agarose gels containing 2.2 M formaldehyde,

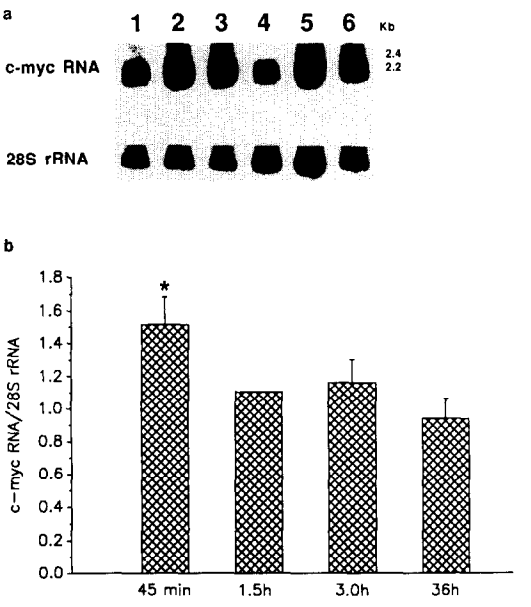


Fig. 1. (a) One typical northern blot showing the effect of $c^3$Ari on c-*myc* RNA steady state levels in HL-60 cells. Lanes 1 and 2, control cells; lanes 3–6, cells exposed to 25 μM $c^3$Ari for 45 min, 1.5, 3 and 36 hr, respectively. The probe was a 1.5 kbp *Cla*I/*Eco*RI fragment of the human c-*myc* gene as indicated in Fig. 3. The c-*myc* hybridization signals were validated by rehybridizing the membrane with a probe against 28S rRNA. (b) Relative c-*myc* RNA levels as determined by densitometric scanning of the autoradiograms as shown in (a). The relative levels of c-*myc* (±SD, N = 3) are presented with the amount of c-*myc* RNA in control cells chosen as 1. *$P < 0.025$ by Student's *t*-test.

followed by transfer to nylon membranes (Nytran-N, Schleicher and Schuell) by vacuum blotting (50 cm $H_2O$ for 4 hr) in 20 × SSC (1 × SSC is 0.015 M $Na_3$citrate, 0.15 M NaCl, pH 7.0). After blotting, the membranes were rinsed briefly with 2 × SSC and air dried. The RNA was fixed by baking at 80° for 2 hr. Prehybridization was carried out for 1 hr at 65° in 6 × SSC, 5 × Denhardt's solution (50 × Denhardt was obtained from the Sigma Chemical Co., St Louis, MO, U.S.A), 0.5% SDS and 10 μg/mL salmon sperm DNA (Sigma Chemical Co.). Hybridization was performed at 65° for more than 16 hr in the same buffer with the addition of the labelled probe. The hybridized membrane was washed in 2 × SSC (2 × 15 min), 2 × SSC/0.1% SDS (1 × 30 min) and 0.1 × SSC (1 × 10 min). All washes were performed at 65°.

Northern blot signals were renormalized by rehybridizing the filter with a probe containing 28S rRNA sequences.

*Probes.* The plasmid pE-H (a kind gift of Dominique Stehelin, Pasteur Institute, France) containing an 8 kb fragment of the c-*myc* gene was digested with restriction endonucleases and the fragments of interest were isolated from 0.8% agarose gel and purified by Gene Clean (BIO 101 Inc, La Jolla, CA, U.S.A.). A 2.45 kb fragment

Table 1. Effect (after 36 hr) of $c^3$Ari on the intracellular AdoMet and AdoHcy levels in HL-60 cells

| | N | pmol/$10^6$ cells* AdoMet | AdoHcy | AdoMet/AdoHcy* |
|---|---|---|---|---|
| Whole cells | | | | |
| Control | 5 | 104.2 ± 3.9 | 3.5 ± 0.1 | 30.1 ± 2.0 |
| 25 μM $c^3$Ari | 3 | 154.5 ± 2.6 | 47.8 ± 8.8 | 3.3 ± 0.2 |
| Nuclei | | | | |
| Control | 11 | 3.5 ± 1.0 | 0.5 ± 0.1 | 6.4 ± 0.6 |
| 25 μM $c^3$Ari | 4 | 2.2 ± 0.2 | 0.6 ± 0.1 | 3.5 ± 0.2 |

* Mean of N replicates ± SD.

obtained by digestion with *Cla*I/*Xba*I and a 1.5 kb fragment obtained by digestion with *Cla*I/*Eco*RI, were used as probes for Southern and northern blots, respectively (Fig. 3).

As a 28S rRNA probe, a 1.4 kbp *Bam*HI fragment of the plasmid pA (kindly provided by Dr R Aasland) was used.

Probes were labelled with [$^{32}$P]α-dCTP (3000 Ci/mmol; NEN Research Products, Boston, MA, U.S.A.) using a multiprime labelling kit (Amersham International, Amersham, U.K.).

## RESULTS

### *Effect of $c^3$Ari on the steady state levels of* c-myc *RNA in HL-60 cells*

Changes in the high levels of c-*myc* RNA have been observed in HL-60 cells induced to differentiate [1, 3, 21–24]. This prompted us to look at the level of c-*myc* transcripts in $c^3$Ari-treated cells. The addition of $c^3$Ari for 45 min resulted in a 40% increase of the 2.2 kb c-*myc* mRNA (Fig. 1) as compared to control cells. At longer exposures of HL-60 cells to this drug (1.5, 3 and 36 hr, respectively), the c-*myc* RNA levels gradually decreased to those found in untreated cells. To correct for variations in the RNA loading, the membrane was rehybridized with a probe against 28S rRNA. Relative c-*myc* mRNA levels as determined by densitometric scanning of the autoradiogram shown in Fig. 1a and corrected for variation in RNA loading, are presented in Fig. 1b.

Control cells were handled in the same way as drug-treated cells, except for the omission of $c^3$Ari. There were no significant variabilities in c-*myc*/28S rRNA ratios of control cells at different time points (data not shown), and the ratios in drug-treated cells were therefore related to the mean value of control cells measured at different time points.

### *Effect of $c^3$Ari on the intracellular AdoMet and AdoHcy levels in HL-60 cells and nuclei*

Treatment of cells with 25 μM $c^3$Ari for 36 hr, resulted in a 9–10-fold decrease in the AdoMet/AdoHcy ratio as a result of a profound increase in the cellular AdoHcy level, and only a minor increase in the AdoMet level (Table 1). The time course study with the drug revealed that the AdoMet/AdoHcy ratio rapidly decreased to about 50% compared to untreated cells during the first 1.5 hr of treatment and stayed constant until 36 hr (Fig. 2).

Since intracellular compartmentalization of AdoHcy has been illustrated [32], we also examined the AdoMet/AdoHcy ratio in nuclei from treated cells. A similar trend in the change in the AdoMet/AdoHcy ratio was observed in nuclei of HL-60 cells treated with $c^3$Ari as in whole cells, the ratio in nuclei of drug-treated cells decreased 40% compared to control cells (Table 1). However, the concentrations of AdoMet and AdoHcy were much lower in the nuclei of cells treated with $c^3$Ari than in whole cells, and the change in the AdoMet/AdoHcy ratio in nuclei was due to a decrease in the AdoMet level rather than an increase in the AdoHcy level (Table 2).

### *Effect of $c^3$Ari on the methylation pattern of* c-myc *DNA sequences*

Total DNA of logarithmically growing HL-60 cells was harvested after 45 min, 1.5, 3 and 36 hr in $c^3$Ari-stimulated and control cells. The DNA was then examined for its c-*myc* methylation pattern. For that purpose, the DNA was digested with the restriction endonucleases *Hpa*II and/or *Msp*I. *Hpa*II is methylation-sensitive and will not cleave C$^{m5}$CGG, whereas MspI cleaves both the unmethylated and methylated sequence. None of these enzymes, however, cleave the sequence $^{m5}$CCGG. Digested DNA was separated on an agarose gel and c-*myc* fragments were identified with a specific c-*myc* probe. This probe spans from nucleotide 1072 to 3507 in the c-*myc* DNA (numbering according to Spencer and Grouidine [25]; see Fig. 3). Figure 3 also presents the *Hpa*II (*Msp*I) motifs. The fragments that theoretically can be detected with the probe used here are listed in Table 2, i.e. if no methylation has occurred. Southern blot analyses of *Msp*I-, *Hpa*II- and *Hpa*II/*Msp*I-digested DNA showed no differences in *Hpa*II restriction pattern between control cells and the cells treated with $c^3$Ari (25 μM) for 45 min, 1.5, 3 and 36 hr, respectively (Fig. 4). Both *Msp*I and *Hpa*II produced the three distinct fragments of 1066, 820 and 560 bp, respectively, all of which were expected if c-*myc* was unmethylated. Cleavage by *Hpa*II, however, also gave a 700 bp

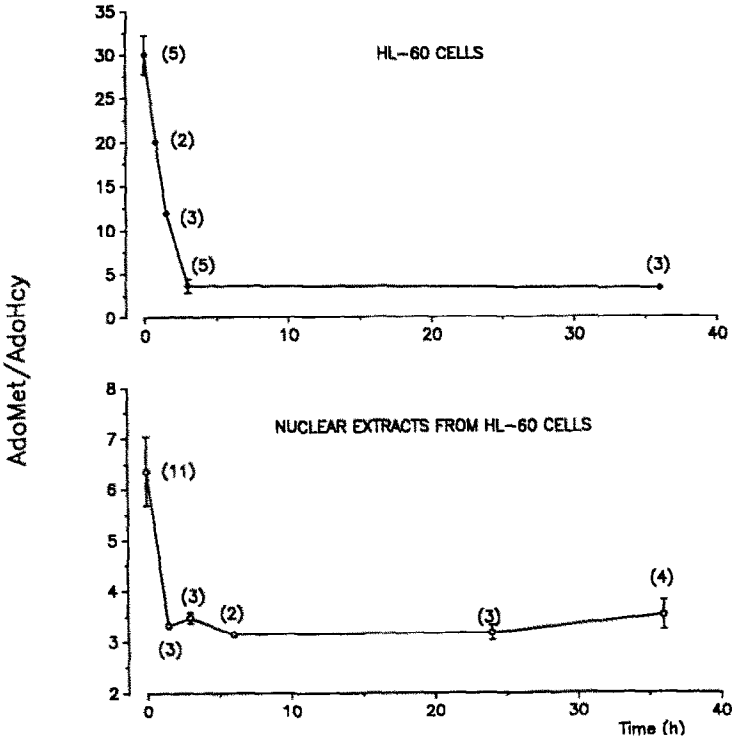


Fig. 2. Time course study of the AdoMet/AdoHcy ratio in whole HL-60 cells (upper), and nuclei of HL-60 cells (lower), after incubation of cells with 25 $\mu$M c$^3$Ari for 45 min, 1.5, 3 and 36 hr. The number of experiments is given in parenthesis at each time point.

Table 2. DNA fragments that will arise by restriction with *Msp*I or *Hpa*II if c-*myc* is unmethylated and the *Cla*I/*Xba*I probe is used for hybridization of Southern blots (see Fig. 3): the fragments found are given in bold

| Fragment size (bp) |
|---|
| **1066**, 11, 52, 43, 72, 24, **820**, 59, 66, 71, 4, 100, 69, 18, 44, **566**, 4, 170, 42, 363, 142, 106, 238, 395, 165, 2165 |

fragment which could be due to one or several methylation site(s). To exclude partial digestion, a large excess of enzyme was used and different DNA preparations were tested. We consistently reproduced the results. Due only partly to overlap of the probe with the 1066 bp fragment, the hybridization signal obtained is weaker than the other bands, where the probe completely spans the fragments (see Fig. 3). As also seen from the restriction map in Fig. 3 and Table 2, a number of small *Msp*I/*Hpa*II fragments (below 100 bp) will arise in our experiments that are not detectable by

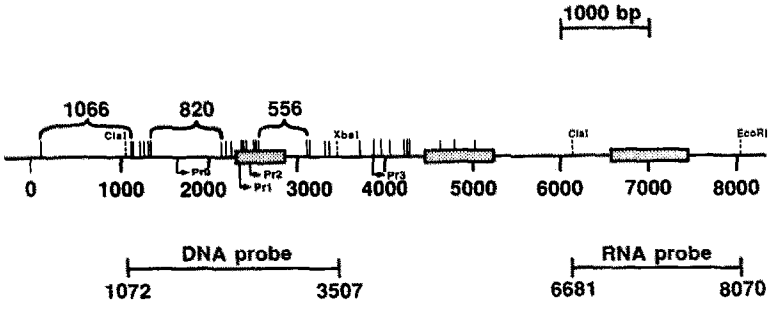


Fig. 3. Restriction map of the human 8 kb c-*myc* gene [25] with locations of the *Msp*I, restriction sites indicated ( | ). The figure also illustrates the *Cla*I, *Xba*I and *Eco*RI sites of importance to obtain the probes, used for Southern blots (DNA probe) and northern blots (RNA probe), respectively. ▒, c-*myc* exons; Pr, possible promoters.

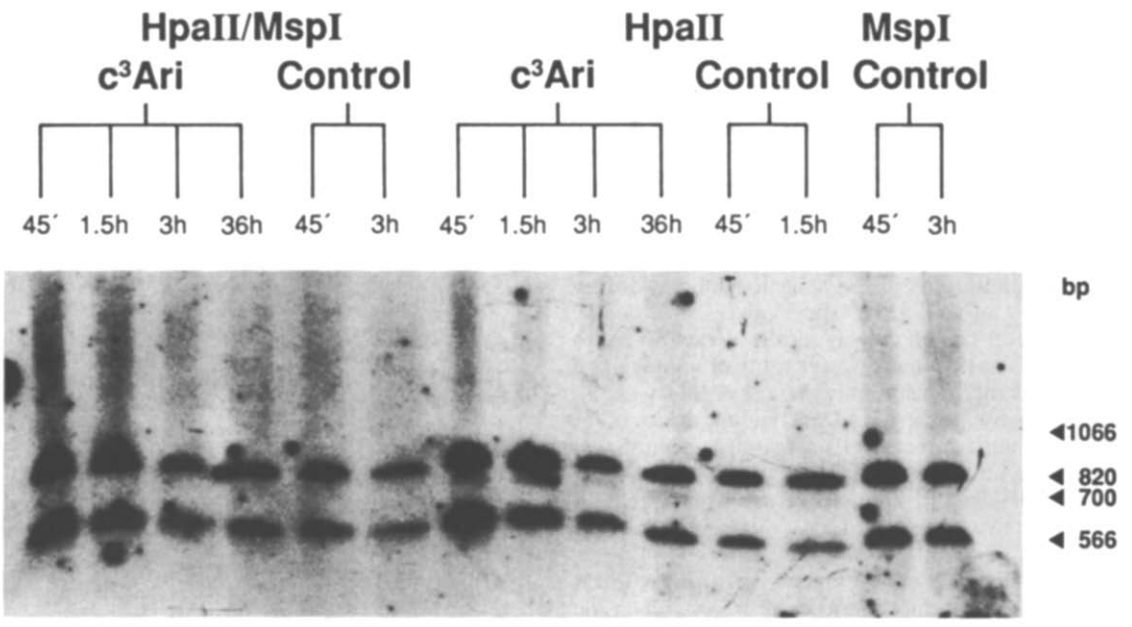


Fig. 4. Southern gel analysis determination of the state of methylation of c-*myc* DNA in control and $c^3$Ari-treated cells. Total DNA was extracted from control and $c^3$Ari-treated cells, digested with the restriction enzymes *Msp*I, *Hpa*II or *Hpa*II/*Msp*I and hybridized with the *Cla*I/*Xba*I c-*myc* probe (see Fig. 3). The arrows locate the size of the indicated main signals, 566 and 820 bp seen in all digests; and the weak signals 1066 bp seen in all digests and about 700 bp in all *Hpa*II digests seen in over-exposed films.

the method used. These fragments are either run off from the 0.8% agarose gel, not transferred sufficiently or washed out of the filters because the stringency was too great for a small number of coupled base pairs [33].

A lot of the small *Hpa*II/*Msp*I sites are clustered around the Pr1 and Pr2 promoters. Methylation in this region might have important biological implications, but presently we are not able to detect this by Southern blotting. Moreover, if the DNA methylation pattern in only a certain fraction of the cells becomes affected by the drug, the present method may not be sufficiently sensitive to detect this.

## DISCUSSION

The transient increase of c-*myc* RNA steady state levels 45 min after addition of $c^3$Ari, is the first report of oncogene expression observed shortly after the addition of this inducer. Yen and Guernsey [34] also reported increased c-*myc* RNA levels associated with the precommitment state during HL-60 myeloid differentiation, followed by a final decrease in advanced cultures where differentiation was essentially complete. However, decrease of c-*myc* transcripts and c-*myc* proteins are the most frequent early events observed in HL-60 cells induced to differentiate by several inducers [1, 3]. The c-*myc* RNA levels as measured in our work, also contrast to the early decrease reported for 1,25-dihydroxyvitamin $D_3$-induced monocytic differentiation [1]. However, both our data and those of Yen and Guernsey [34], show that HL-60 c-*myc* RNA levels can be either up- or down-regulated early after drug exposure, depending on the inducer. HL-60 c-*myc* RNA levels measured after time-intervals corresponding to several cycles of control cells, show, on the other hand, a consistent decrease as reported with granulocytic induction by retinoic acid [35, 36] and monocytic differentiation by 12-*O*-tetradecanoylphorbol-13-acetate [22]. Our data on c-*myc* RNA levels after exposure of HL-60 cells with 25 $\mu$M $c^3$Ari for 36 hr (two cell cycles in control cells), a dosing schedule causing HL-60 cell differentiation [5] and accumulation in a $G_2$ restriction point [37], also show that c-*myc* is subsequently down-regulated with this compound, albeit only to levels of control cells. Post-transcriptional stabilization of c-*myc* RNA has been reported with increases of RNA half-life from about 35 to 70 min [25], and should therefore be considered as a possible mechanism for the increase in c-*myc* RNA. However, the fact that we observed a very transient increase at only 45 min, with normalization at 90 min, makes this explanation rather unlikely.

The cellular mechanisms/targets for $c^3$Ari-induced differentiation remains speculative. We here pursued experiments based on the hypothesis that $c^3$Ari effects are closely related to hypomethylation. We observed a decrease in the AdoMet/AdoHcy ratio (Table 1) as expected for an AdoHcy hydrolase inhibitor by treatment of HL-60 cells with 25 $\mu$M

$c^3$Ari for 36 hr, consistent with our previous report [5]. About 90% reduction in the AdoMet/AdoHcy ratio was observed for whole cells after treatment with 25 $\mu$M $c^3$Ari (Table 1) as a result of a profound increase in the cellular AdoHcy level, and only a minor increase in the AdoMet level. Table 1 also shows that the AdoMet/AdoHcy ratio decreases with about 50% in nuclei by the same treatment due to a decrease in the AdoMet level, whereas the AdoHcy level is unchanged. The reasons for the relative insensitivity of the nuclear pool of AdoHcy to effects of $c^3$Ari, is speculative. If AdoHcy hydrolase in HL-60 cells is localized exclusively to cytosol, as has been shown for rat liver homogenate [38], some mechanisms may prevent AdoHcy excess in nuclei by moving and/or binding it to other cellular compartments. It has previously been shown that free and bound AdoHcy represent distinct intracellular kinetic compartments in rat liver [32], and these two pools might have different regulatory functions.

Our findings of $c^3$Ari-induced c-*myc* RNA levels and the change in the ratio of transmethylation metabolites after 45 min, fit to a hypothesis with a general hypomethylation status of cellular components, including regulatory sequences of DNA. On the other hand, the 10-fold reduction in AdoMet/AdoHcy ratio in whole cells, observed at other time-points which were not accompanied by a reduction in the steady state of c-*myc* RNA, points to additional mechanisms for c-*myc* RNA regulation [25].

We have revealed no change either in overall DNA methylation as measured by HPLC and UV detection of DNA bases [29] or in methylation of specific c-*myc* sequences as measured by restriction analysis and Southern blots, after treatment of HL-60 cells with a dose schedule of $c^3$Ari causing cell cycle perturbation, cytostasis and differentiation. Our results are consistent with the results of Giesler *et al.* [33] who found no alteration in the methylation pattern of c-*myc* during granulocytic differentiation of HL-60 cells induced by dimethyl sulphoxide.

In summary, our results indicate that cytosine methylation of DNA is relatively insensitive to changes in the intracellular ratio of AdoMet/AdoHcy [39], pointing to the existence of very complex relations between cellular transmethylation metabolites and DNA methylation.

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