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Erythroid Differentiation and Regulatory Gene Expression are Modulated by Adenosine Derivatives Interfering With S-Adenosylmethionine Metabolic Pathway

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Abstract The differentiation of murine erythroleukemia cells and the expression of *SCL, Id1* and *c-myc* regulatory genes were studied. The first gene is a positive regulator of differentiation, while the other two are both negative regulators of differentiation and positive regulators of proliferation. Accordingly, our data show that when differentiation is stimulated *SCL* is upregulated while *Id1* and *c-myc* are, coordinately, downregulated. The cultures were treated with two adenosine derivatives, 3-deazaadenosine and 3-deazaaristeromycin, known to act on the metabolic pathway of the methyl donor S-adenosylmethionin, in order to assess the possibility of a coordinated modulation, by these drugs, of regulatory gene expression and erythroid cell differentiation. 3-Deazaaristeromycin caused the simultaneous down-regulation of *Id1* and *c-myc,* whereas 3-deazaadenosine caused their upregulation; both drugs produced a transient increase in *SCL* expression. The use of these drugs evidenced a predominant regulatory effect of negative regulators in the control of erythroid differentiation. The distinct effects of the two drugs on regulatory gene expression led to an increased differentiation induced by 3-deazaaristeromycin and to a reduced differentiation induced by 3-deaza-adenosine, if compared with controls. Southern analysis of DNA digested with methylation-specific restriction endo-nucleases showed that the administration of 3-deazaaristeromycin resulted in hypomethylation of *SCL* and *c-myc,* thus evidencing, in these cells, a clear correlation between DNA hypomethylation and differentiation but no straightforward correlation between DNA methylation and gene expression. J. Cell. Biochem. 81:401–412, 2001. © 2001 Wiley-Liss, Inc.

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Among the families of genes regulating differentiation, the lineage-restricted transcription factors that belong to the helix-loop-helix (HLH) class reportedly play a central role in growth, development, and in the oncogenic transformation of several cellular systems of the myogenic [Olson et al., 1991], neurogenic [Green et al., 1992], and haematopoietic [Visvader and Begley, 1991] lineages. HLH proteins have in common a conserved dimerization domain that mediates protein–protein interactions among various HLH members to form mostly heterodimers [Murre et al., 1989]; these dimeric proteins then bind DNA via a group of positively charged amino acids (the basic (b) region) directly adjacent to the HLH domain [bHLH proteins; Davis et al., 1990]. Subgroups of HLH proteins without a basic domain which antagonize the function of the above-mentioned bHLH proteins have also been described; these defective proteins still dimerize with other members of bHLH family but cannot bind DNA, and therefore act as negative regulators [Sun et al., 1991; Duncan et al., 1992; Kreider et al., 1992].

The haematopoietic system offers unique opportunities for the study of lineage commitment and differentiation driven by HLH lineage-restricted transcription factors. Among the

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genes coding for members of the HLH family, the most important regulators of erythroid differentiation appear to be *SCL* [Begley et al., 1989], a positive regulator, and *Id1* [Benezra et al., 1990], a negative regulator. The SCL protein, also known as TCL5 [Finger et al., 1989] or TAL [Chen et al., 1990], is normally expressed in erythroid, mast, and megakaryocyte cells [Visvader et al., 1991; Green et al., 1992], and is also activated by chromosomal rearrangements in up to 25% of human T-cell acute lymphoblastic leukemias [Brown et al., 1990]. Although it is known that the SCL protein plays a fundamental role in normal erythroid differentiation [Visvader et al., 1991], its physiologic role and the mechanism by which SCL deregulation contributes to leukemogenesis are still obscure. In addition to this positive regulation, erythroid differentiation is also negatively regulated by the expression of members of the Id-family, namely *Id1*, *Id2* [Sun et al., 1991], *Id3* [Christy et al., 1991], and *Id4* [Riechmann et al., 1994]. Besides SCL and the members of the Id-family, another gene involved in the control of the proliferation/differentiation switch in a great number of cell lineages is *c-myc*, whose product also possesses an HLH domain [Marcu et al., 1992]. C-myc protein levels appear, indeed, to be crucial for the initiation of the erythroid program [Kirsch et al., 1986], while the constitutive expression of *c-myc* has been shown to block inducer-mediated erythroid differentiation of murine erythroleukemia (MEL) cells [Coppola and Cole, 1986].

Among the possible mechanisms involved in the coordinated pattern of gene expression that finally results in the differentiated phenotype, methylation of cytosine moieties in the DNA of regulatory regions of eukaryotic genes [Szyf, 1996] appears to be an interesting candidate. In several eukaryotic genes involved in development and differentiation, initiation of gene transcription has been shown to correlate with hypomethylation of regulatory regions of these genes [Razin and Riggs, 1980]; by contrast, methylation in the promoter region has been shown to suppress gene transcription [Levine et al., 1992]. In this respect, the use of drugs able to modify the DNA methylation pattern has proven to be a valuable tool for the comprehension of molecular mechanisms of differentiation [Razin et al., 1986, 1988; Scarpa et al., 1996].

In early experiments, DNA hypomethylation and consequent gene activation were obtained with the cytidine analog 5-azacytidine (5-aza-C) [Taylor and Jones, 1979], which has been shown to undergo incorporation in DNA during replication and, as a consequence, to cause DNA methyltransferase (MeTase) inactivation [Taylor and Jones, 1982]. It has, however, now been shown that, owing to its incorporation into DNA, 5-aza-C produces a number of toxic effects that block DNA replication, RNA transcription, and protein synthesis. In addition, 5-aza-C is capable of inducing new developmental phenotypes also in organisms which do not contain 5-methylcytosine [Tamame et al., 1983], and the observed induction of gene expression cannot therefore be exclusively attributed to DNA demethylation. On the other hand, although the majority of experiments performed with 5-aza-C have shown the ability of this drug to reverse unnatural in vitro methylation of CpG islands, there is no evidence that 5-aza-C is really able to mimic some in vivo mechanism of gene derepression by direct "activation" of non-CpG island sites which would be otherwise naturally methylated [Bird, 1992]. The use of adenosine derivatives, such as 3-deazaadenosine (DZA), that interfere with the S-adenosylmethionine (SAM) metabolic pathway, has proven to be a better way, with fewer side effects, to investigate the effect of "naturally" induced hypomethylation on cell differentiation [Scarpa et al., 1984].

Gene targeting experiments have, by showing that a disrupting mutation into the DNA MeTase gene is lethal for the homozygous mutant mouse embryos [Li et al., 1992], demonstrated that DNA methylation is essential for mouse development. Moreover, the overexpression of bacterial Hha l MeTase in cultured murine fibroblasts also was lethal to such cells, and the surviving cell clones displayed increased tumorigenicity [Wu et al., 1996]. The constitutive overexpression of an exogenous eukaryotic DNA MeTase gene resulted in an increase in overall DNA methylation and in tumorigenic transformation of mouse fibroblasts [Wu et al., 1993], as well as in a de novo methylation of CpG island sequences in human fibroblasts [Vertino et al., 1996].

Even in the absence of severe effects, in vitro methylation of a number of gene promoters by bacterial MeTase has been shown to lead to

a decrement in promoter activity [Levine et al., 1991]. By contrast, a specific partial inhibition of DNA MeTase, such as that achieved by transfecting an antisense expression vector, resulted in the induction of the myogenic phenotype in the mesenchymal line 10T1/2, thus indicating that DNA demethylation is indeed an important event in the control of mammalian cell differentiation [Szyf et al., 1992]. Moreover, it is well known that many tumor cell lines overexpress DNA MeTase, whereas expression of antisense mRNA vs. DNA MeTase not only results in demethylation, but also inhibits tumorigenesis [Ramchandani et al., 1997]. Eukaryotic cells appear, therefore, to require, qualitatively and quantitatively, precise DNA methylation patterns. This positive correlation between demethylation and transcriptional activity has, however, been proven mainly on single genes and only occasionally on regulatory genes involved in differentiation. The aims of the present study were to clarify, in erythroid cells: (a) the occurrence of a coordinated modulation of regulatory gene expression and their relative importance in the induction of the differentiated phenotype; (b) the link between DNA methylation and differentiation; (c) the link between DNA methylation and regulatory gene activation. To reach these goals we used, in addition to the differentiative stimulus, two adenosine derivatives, DZA and 3-deazaaristeromycin (DZAri), which have been shown to act on the SAM metabolism through a partial block of the regenerating pathway mediated by the inhibitory effect on S-adenosylhomocysteine (AdoHCY) hydrolase and a consequent reduction in methyl transfer reactions [Cantoni, 1977]. Despite some similarities, these two drugs differ somewhat in their interference with the SAM cycle insofar as DZAri is a very poor substrate for AdoHCY hydrolase, while DZA is readily converted by this enzyme into 3-deaza-AdoHCY, with an efficiency similar or even higher than adenosine [Chiang, 1985]. Despite its ineffectiveness as a substrate, the carbocyclic analog DZAri has been shown to be one of the most potent competitive inhibitors of AdoHCY hydrolase, with K_i values ranging (depending on the source of the enzyme) from 10^{-6} to 10^{-9} M, i.e., at least two orders of magnitude below the K_i values of DZA.

The induction of differentiation of MEL cells in response to treatment with hexamethylenebisacetamide has been shown to correlate with a transient genome-wide hypomethylation of DNA, achieved by the replacement of 5-methylcytosine with cytosine residues [Razin et al., 1985, 1986]. Razin and coworkers [1988] have also demonstrated that exposure of MEL cells to DZA in combination with homocysteine (HCY) causes comparable inhibition of HMBA-induced hypomethylation and differentiation. In this work, we demonstrate that DZA and DZAri exert opposite effects on MEL cell terminal differentiation, a stimulatory effect being displayed by DZAri and an inhibitory one by DZA. These contrasting effects on differentiation are intriguing because of the common mechanism of action through DNA demethylation postulated for both drugs; they could, however, be partially explained by the different biochemical effects of DZA+HCY and DZAri on the SAM cycle. We could expect an expression pattern of genes that regulate is referred to genes differentiation and growth, assuming that their regulation is somehow affected by modifications in the DNA methylation status, as occurs in myoblasts [Scarpa et al., 1996]. In fact, the different levels of terminal differentiation and growth correlated well with the effects of modulation of expression exerted by either drug on regulatory genes, stressing, in particular, the greater importance of negative regulators (with respect to the positive one) in the control of differentiation. Exposure to DZA+HCY was unable to modify the DNA methylation pattern. By contrast, DZAri was found to cause hypomethylation. Although a correlation between hypomethylation and enhanced differentiation was observed, a straightforward correlation between hypomethylation and gene expression could not be inferred, because this hypomethylation occurred on the positive as well as on one of the negative regulatory genes.

METHODS

Chemicals

DZAri was produced by the Southern Research Institute, P.O. Box 55305, Birmingham, AL 35255-5305 USA, and kindly supplied by ViraChem Inc.

Cell Culture

MEL cells (GM/86 strain) were grown in suspension in F14 medium [Vogel et al., 1972] at 37°C in a humidified incubator equilibrated

with 5% CO_2 in air, and the cultures were seeded to keep cell density below 10^6/ml. In our experimental conditions, cells were counted with a Coulter Counter, mod. D_{n3}, harvested by centrifugation ($200 \times g$ for 7 min) and seeded at a density of 10^5 cells/ml in F14 with 10% FCS, which allowed cell growth without differentiation (growth medium, GM), and F14 with 10% FCS supplemented with 2% dimethylsulfoxide (DMSO), which induced differentiation (differentiation medium, DM). Experimental conditions included GM and DM with 6 μM DZA +50 μM HCY or with 4 μM DZAri. We also used 20 μM DZA together with HCY to stress the DZA effect on growth and differentiation. However, owing to the highly toxic effect of DZA at high dosages, the expression and methylation experiments were not carried out at this concentration and the abbreviation DZA+ HCY, unless otherwise specified, must be considered as 6 μM DZA together with 50 μM HCY. These drugs were added together to the appropriate culture medium and the experiments were stopped after a few hours and/or days, depending on the experimental design. For gene expression and methylation analysis, 4×10^7 cells were harvested by centrifugation and aliquots were concomitantly removed for benzidine reactivity determination and growth evaluation.

Growth and Differentiation

The experiments performed to assess the effect of the drugs on growth were carried out in multiwells in duplicate. Cells were seeded in GM at a density of 10^5 cells/ml and aliquots were counted 2 days after seeding. For each experimental condition two aliquots from each of the duplicate wells were counted with a Coulter Counter; the average ± standard error of the mean was calculated and the cell number was expressed as cells/ml.

To examine the effect of the drugs on differentiation, cells were seeded in DM at a density of 10^5 cells/ml in duplicate wells. MEL cells grew for several days in the presence of DMSO, undergoing changes similar to the normal maturation of red blood cells, including the production of hemoglobin as demonstrated by benzidine staining. Benzidine staining was performed by adding 10 μl of fresh benzidine, 0.2% in 0.5 M acetic acid solution containing 0.4% hydrogen peroxide (30%), to 100 μl of cell suspension. Cells containing hemoglobin turn blue in 5–10 min and the benzidine-positive cell percentages were determined by light microscopy. For each experimental condition, 4 and 5 days after induction, 400 cells/well were counted in two aliquots from each of the wells. The counts were expressed as a percentage of benzidine positive cells and the average ± standard error of the mean was calculated.

Northern Blots

For the extraction of total RNA, the cells were rinsed twice with cold PBS, pelletted and frozen at −80°C. Total RNA was extracted by RNAfast (Molecular System-Biotecx) and resuspended in water. The quantification and purity of preparations were assessed by reading 260/280 nm absorbances. Electrophoresis was performed in 1.1% agarose (BioRad) gels containing 2.2 M formaldehyde, with 20 μg samples of total RNA per lane, denatured for 15 min at 65°C in 2.2 M formaldehyde and 50% formamide. Blotting was performed by using a Trans-Vac-TE 80 (Hoefer) on Hybond-N membranes (Amersham) following the manufacturer's instructions. Prehybridization was performed at 42°C overnight in 6× SSPE, 5× Denhardt's, 0.1% SDS, 100 μg/ml denatured fragmented salmon sperm DNA and 50% formamide [Sambrook et al., 1989]. Hybridization was performed in the same buffer *plus* 5% dextran-sulfate, under high stringency conditions, with radiolabeled probes added for 20 h at 42°C. The following washes were performed: 3 × SSC, 0.1% SDS at room temperature, 3 × SSC, 0.1% SDS at 60°C; 0.2 × SSC, 0.1% SDS at 68°C [Sambrook et al., 1989]. The last high stringency wash for *c-myc* cDNA was omitted. Membranes were exposed at −80°C using Kodak X-OMAT XAR-5 films with a DuPont Lightning Plus intensifying screen. Plasmid Bluescript containing murine *SCL* cDNA probe (1612 bp) that encompasses the exons IV, V and a portion of VI [Begley et al., 1991], and plasmid Bluescript containing murine *Id1* cDNA probe [Benezra et al., 1990] were used to transform DH5α bacteria (GIBCO BRL) according to the manufacturer's instructions; plasmids were then propagated and the *SCL* probe was excised with *Not*I and *Sst*I (GIBCO BRL), while the *Id1* cDNA insert was excised with *Bam*HI and *Eco*RI (Boehringer Mannheim) using standard procedures. The human insert relative to *c-myc* cDNA third exon [Battey et al., 1983] is manufactured by Oncor. Blots were normalized with *r18S* RNA mouse

probe. Probes were labeled by random priming using the Amersham Megaprime DNA labeling system with [α-^{32}P]dATP (NEN 3000 Ci/mmol), the final specific activities being above 1.9×10^9 dpm/μg. All signals detected on the films were quantified by BIO IMAGE computerized densitometer (Millipore), referred to as integrated optical density (IOD) and the ratio with *r18S* signals expressed as a percentage of the highest signal in each blot (set at 100%). The membranes used were stripped twice by standard procedures and efficient stripping was checked by autoradiography.

Southern Blots

For Southern blot analysis, DNA was extracted according to Sambrook et al. [1989]. Digestions of genomic DNA were carried out for 1 h with two units of enzyme per μg of DNA with *Eco*RI and subsequently for 16 h with eight units of enzyme per μg of DNA either with restriction endonuclease *Msp*I specific to CCGG sequences (methylated or unmethylated) or with *Hpa*II specific to unmethylated CCGG sequences. Digests were electrophoresed under standard conditions in 0.6% agarose, using 20 μg/lane. Blotting was performed under standard conditions. Prehybridization was performed in 50% QuikHyb (Stratagene) and 50% formamide, with 100 μg/ml denatured fragmented salmon sperm DNA, for 4 h at 42°C. Hybridization was performed in the same buffer with the radiolabeled probe added for 20 h at 42°C. The following washes [Sambrook et al., 1989] were performed for *SCL*: 2 × SSC, 0.1% SDS at room temperature; 0.2 × SSC, 0.1% SDS at 60°C; 0.1 × SSC, 0.1% SDS at 68°C. For *Id1*: 2 × SSC, 0.1% SDS at room temperature; 2 × SSC, 0.1% SDS at 50°C; 0.2 × SSC, 0.1% SDS at 60°C. For *c-myc*: 2 × SSC, 0.1% SDS at room temperature; 2 × SSC, 0.1% SDS at 50°C; 0.2 × SSC, 0.1% SDS at 55°C.

The genomic *SCL* probe used for Southern blot is a 4.5 kb fragment encompassing exons Ia, lb, lIb, and approximately 1.7 kb of the 5′-flanking portion of the gene [Begley et al., 1994]. The probes used for *Id1* and *c-myc* were the same as in Northern blots.

RESULTS

Figure 1A shows the growth histograms of MEL cells, in both GM and DM, in the presence or absence of 4 μM DZAri or 6 μM DZA+50 μM HCY (low dosage) and 20 μM DZA+50 μM HCY (high dosage). After the induction of differentiation by DMSO, there was a reduction in cell proliferation (open bars). Cell proliferation also decreased upon addition of either DZAri or DZA to GM (black and gray bars, respectively). The cytostatic effect of DZAri and of DZA+HCY at a high dosage was also evident in DM, resulting in about the same growth reduction, while DZA+HCY at a low dosage (light gray bars) were practically ineffective. The independent administration of 6 μM DZA or 50 μM HCY caused no reduction in growth either in GM or in DM (data not shown). Figure 1B, C shows the variations in differentiation. In DM about 50%, at Day 4, and 75%, at Day 5, of the

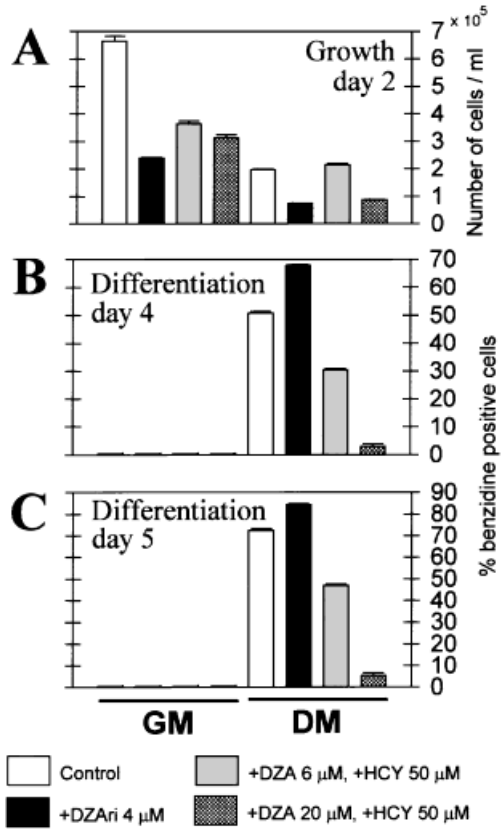

Fig. 1. Growth and differentiation patterns of treated and untreated MEL cells. (**A**) Growth at Day 2 of culture; (**B**) differentiation at day 4; (**C**) differentiation at Day 5. Columns 1–4 represent cultures grown in GM; columns 5–8 represent cultures grown in DM. Open bars indicate controls without drug addition; black bars indicate samples treated with DZAri 4 μM; light gray bars indicate samples treated with DZA 6 μM and HCY 50 μM; crosshatched gray bars indicate samples treated with DZA 20 μM and HCY 50 μM. For abbreviations, see Materials and Methods.

cells were benzidine positive (open bars). DZAri added to DM caused an increment in benzidine positive cells (black bars) which was greater at Day 4 than at Day 5. DZA+HCY added simultaneously to DM at a low dosage inhibited differentiation by approximately 40% (light gray bars), whereas the two drugs added independently showed a reduced inhibitory effect (data not shown). DZA+HCY added at a high dosage (crosshatched gray bars) produced a dramatic reduction in benzidine positive cells to about 5%. In GM we did not observe any differentiation with or without the drugs. Because of its extreme effect on growth and differentiation, DZA at a high dosage was not used in expression and methylation experiments which were performed with 6 μM DZA+50 μM HCY (hereafter, unless specified, the abbreviation DZA+HCY indicates 50 μM HCY plus DZA at a low concentration, i.e., 6 μM).

Figure 2 shows the expression of *SCL*, *c-myc*, and *Id*1, studied by Northern analysis, in DM with or without the drugs from 2 to 72 h after the induction of differentiation; the expression in GM, without the addition of DMSO, is also shown as a control.

Although *SCL* is reported as a positive regulator, its transcript was also detected in the absence of erythroid differentiation (Fig. 2A, lanes 1 and 2); the signal was, however, detected only after prolonged exposure (see also densitometries in Fig. 3). In GM alone we obtained clear evidence of two signals corresponding to 3.0 kb and 4.7 kb *SCL* transcripts, whereas in all the other conditions tested only the 4.7 kb transcript was detected [Begley et al., 1991]. In spite of prolonged exposures, only a very faint *SCL* signal was detected in DM at early times (up to 6 h, Fig. 2A, lanes from 3 to 8, and Fig. 2B, lanes 1, 4, 7), with or without the addition of either DZA+HCY or DZAri. The *SCL* signal, in DM without these drugs, remained low up to 24 h after the induction of differentiation (Fig. 2B, lane 2) and became evident at 72 h (Fig. 2B, lane 3). Both drugs caused an anticipation of expression (at 24 h, Fig. 2B lanes 5, 8), although at late times (72 h) DZA+HCY exerted a slight inhibition of *SCL* expression (Fig. 2B, lane 6); this reduction was not observed with DZAri (Fig. 2B, lane 9). *C-myc* expression was very high in GM (Fig. 2A, lanes 1, 2) but disappeared in MEL cells cultured in DM, with or without drugs, 2 h after the induction of differentiation (Fig. 2A,

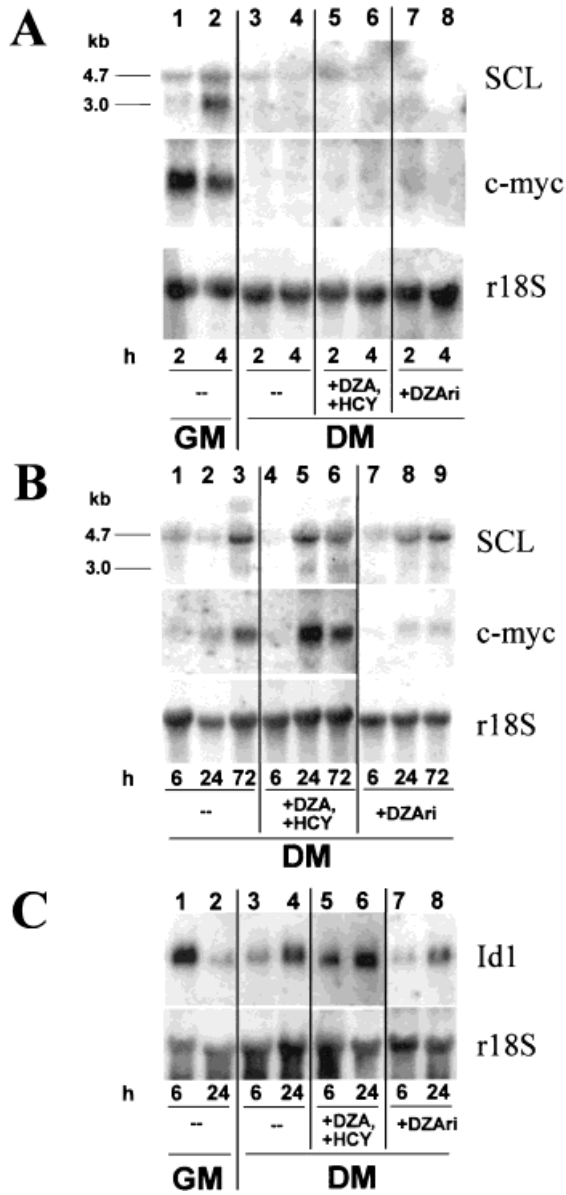

Fig. 2. Northern blot analysis of *SCL*, *c-myc*, and *Id1* transcripts in GM and DM from 2 to 72 h. The ribosomal RNA expression, *r18S*, is shown as blot normalization. The dimensions of *SCL* multiple transcripts are indicated. For abbreviations, see Materials and Methods. (**A**) *SCL* and *c-myc* expression at 2 and 4 h of growth or differentiation. **Lanes 1**, **2** display the controls in GM. **Lanes 3–8** display the samples grown in DM: without drugs (**lanes 3**, **4**); with addition of DZA 6 μM and HCY 50 μM (**lanes 5**, **6**); with addition of DZAri 4 μM (**lanes 7**, **8**). Exposure time of *SCL* blot was 12 days while that of *c-myc* was 4 days. (**B**) *SCL* and *c-myc* expression at 6, 24 and 72 h after differentiation. **Lanes 1–9** show the blots obtained in DM: without drugs (**lanes 1–3**); with addition of DZA 6 μM and HCY 50 μM (**lanes 4–6**); with addition of DZAri 4 μM (**lanes 7–9**). (**C**) *Id1* expression at 6 and 24 h of growth or differentiation. **Lanes 1**, **2** show the controls in GM, **lanes 3–8** the samples in DM. **Lanes 5**, **6** show the samples obtained with addition of DZA 6 μM and HCY 50 μM; **lanes 7**, **8** show the samples obtained with addition of DZAri 4 μM.

lanes from 3 to 8), and remained practically undetectable up to 6 h (Fig. 2B, lanes 1, 4, 7). In DM without drugs, *c-myc* expression increased from 24 to 72 h (Fig. 2B, lanes 2, 3) without, however, reaching the expression levels found in GM. The effects exerted by DZA+HCY and DZAri at late times (24 and 72 h), were quite different: DZA+HCY produced a marked increase in *c-myc* expression (Fig. 2B, lane 5) which dropped at 72 h (Fig. 2B, lane 6), whereas DZAri decreased the levels of *c-myc* transcripts (Fig. 2B, lanes 8, 9). *Id1* gene showed very high levels of expression in GM 6 h after seeding (Fig. 2C, lane 1), with a decrease after 24 h (Fig. 2C, lane 2), which was not, however, accompanied by MEL differentiation. Low levels of *Id1* transcripts were found after 6 h in DM (Fig. 2C, lane 3), followed by an increase at 24 h (Fig. 2C, lane 4). In DM, addition of DZA+HCY increased *Id1* transcriptional levels both at 6 and 24 h (Fig. 2C, lanes 5, 6). By contrast, DZAri addition to DM resulted in a decrease of *ld1* transcript both at 6 and 24 h (Fig. 2C, lanes 7, 8).

Figure 3 (left side) shows the expression patterns of the three genes considered, represented as densitometric values of the Northern blots previously shown, normalized to *r18S* RNA expression. The densitometric value of each gene in GM before shifting to DM is shown as an initial expression level (time 0). This value was also used, for each gene to normalize

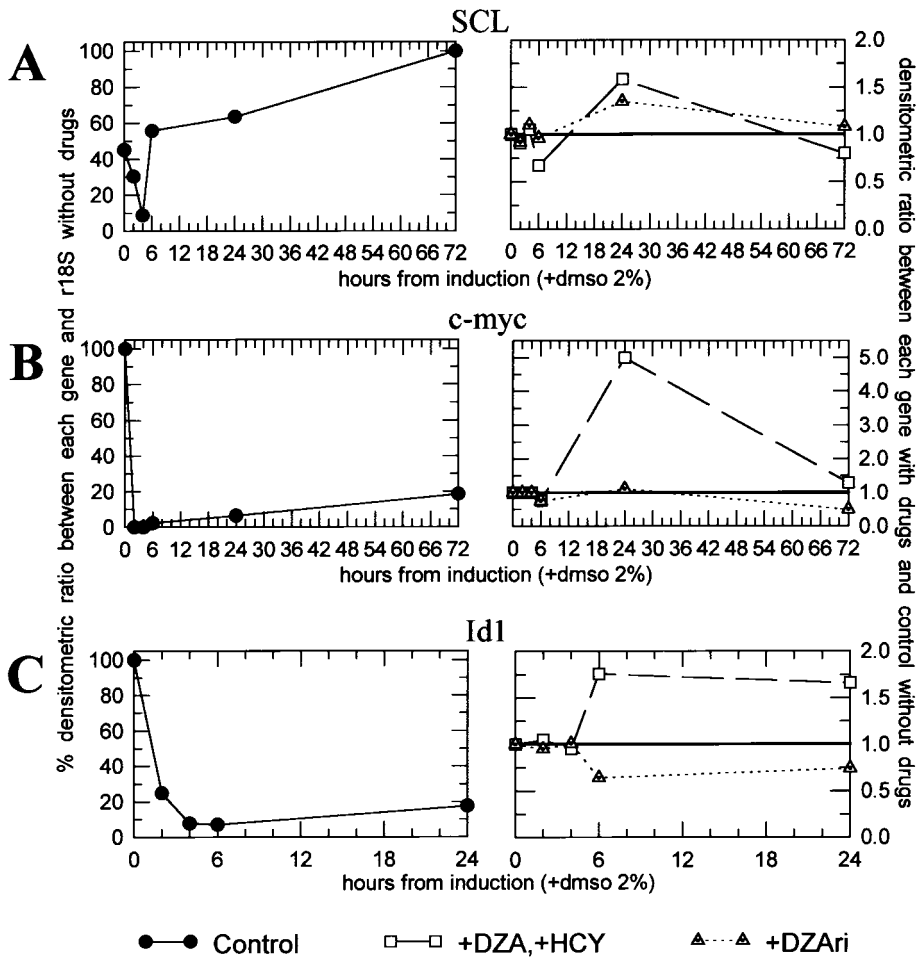

Fig. 3. Densitometric analysis of *SCL* (**A**), *c-myc* (**B**), and *Id1* (**C**) Northern blots from Fig. 2 in DM. On the left, the *Y*-axis shows the ratio between densitometric values (calculated as IOD) of each gene without drugs and *r18S* RNA expressed as a percentage of the relative highest signal set to 100% (filled circles). The times 0 indicate the relative initial levels of expression before DMSO addition. On the right, the *Y*-axis shows the ratio between the densitometric values of each gene in DM with the drugs (DZA+HCY: open boxes; DZAri: open triangles) and the relative controls without the drugs. The times in hours (*X*-axis) indicate the ages of the cultures which originated the samples. For abbreviations, see Materials and Methods.

the signals obtained in different blots. When showing the densitometric values of one gene without drug addition, the highest densitometric values in each panel were considered as 100%. Figure 3 (right side) shows the ratios between the *r18S* normalized signals of each of the three genes with the drugs and the relative control values (without drug addition), which are therefore shown as a straight line at 1.0. A direct comparison between culture conditions and the expression of each gene is therefore possible. At early times (up to 4 h after the induction of differentiation) we observed a sharp decrease in *SCL* signals (Fig. 3A, left side), regardless of drug addition; however, between 4 and 6 h after induction, *SCL* signals underwent a sharp increase which persisted up to 72 h. After the induction of differentiation *c-myc* and *Id1* decreased sharply and increased very slightly thereafter. In the differentiated control at 72 h, *c-myc* expression reached only 20% of time 0 (Fig. 3B left side); even *Id1* transcripts, in the same condition, increased slightly from 6 to 24 h to 20% of time 0 (Fig. 3C, left side). The addition of the two drugs (Fig. 3, right side) evidenced a coordination between these three genes. Their expression in the presence of DZA+HCY, which partially inhibits differentiation, showed a rapid increase in *Id1* at 6 h (1.7-fold compared with control) followed, at 24 h, by an increase in *c-myc* (5-fold) and *SCL* (only 1.5-fold). DZAri, which stimulates differentiation, by contrast produced a decrease in *Id1* expression at 6 h with no initial increase and a final (72 h) decrement (0.5-fold compared with control) of *c-myc* expression and a slight increase in *SCL* expression after 24 h.

Figure 4 shows the Southern blots of DNA extracted from MEL cells in DM, both treated and untreated with the drugs. The bands shown in Figure 4A were obtained through the hybridization with the *Id1* structural probe. *Msp*I (lanes 1, 2, 3) and *Hpa*II (lanes 4, 5, 6) restriction endonuclease treatments were very similar, with two main bands unchanged by the drugs. Since the *Id1* region studied includes three CCGG sites, we can deduce that they were not methylated and their methylation did not change. The bands shown in Figure 4B were obtained through hybridization with a *c-myc* third exon probe. In all the conditions tested, *Msp*I digestion yielded only a 6.2 kb band (lanes 1, 2, 3), while markedly larger fragments were obtained with *Hpa*II digestion (lanes 4, 5, 6). When digestion was performed with *Hpa*II, we observed a marked hypomethylation when the cells had been cultured with DZAri (lane 6): the resulting 7.7 kb band was much smaller than that of the control or of the DZA+HCY lanes, though not as small as that

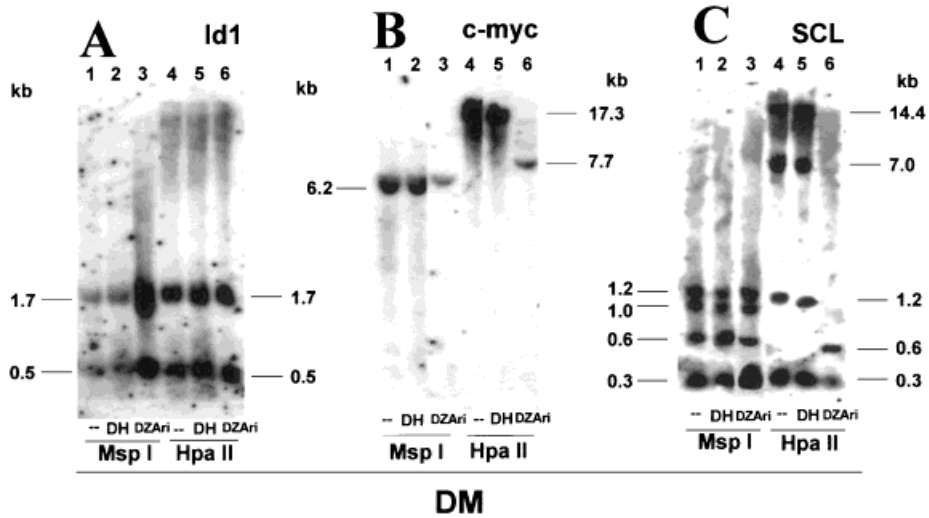

Fig. 4. Southern blot analysis of *Id1*, *c-myc*, and *SCL* in DM. Band patterns obtained by hybridization with (**A**) *Id1* structural probe, (**B**) *c-myc* structural probe, and (**C**) *SCL* 5′-flanking region probe. **Lanes 1–3** (A, B and C) show the band patterns after *Msp*I treatment in DM without drugs (**lanes 1**) and with addition of DZA 6 μM and HCY 50 μM (= DH, **lanes 2**) or with addition of DZAri 4 μM (**lanes 3**). **Lanes 4–6** (A, B, C) show the band patterns after *Hpa*II treatment in DM without drugs (**lanes 4**) and with addition of DZA 6 μM and HCY 50 μM (= DH, **lanes 5**) or with addition of DZAri 4 μM (**lanes 6**). For abbreviations, see Materials and Methods. The band dimensions are indicated.

obtained after *Msp*I digestion. Figure 4C shows the bands obtained through hybridization with the *SCL* 5′-flanking region probe. In all the conditions tested, we observed four bands following *Msp*I digestion (lanes 1, 2, 3). *Hpa*II digestion produced four bands both in the control and in the DZA+HCY treated samples (lanes 4, 5), two of which had the same molecular weight as the ones obtained with *Msp*I, while the other two had a much higher molecular weight. The DZAri sample (lane 6) evidenced, as in the case of *c-myc*, consistent hypomethylation, as demonstrated by the loss of the bands with the highest molecular weight and by the appearance of a low molecular weight band.

DISCUSSION

The necessary prerequisites for the activation of the differentiative cascade are both withdrawal from the cell cycle and activation of differentiation by positive regulatory genes. Our data are consistent with the expression of regulatory genes studied during MEL cell differentiation induced by DMSO described by other authors. It is well known [Benezra et al., 1990] that to activate the differentiative program *Id1* transcripts have to decrease transiently within few hours of induction. Our results show that in MEL cells *Id1* was clearly depressed by the addition of DMSO. This is in agreement with previous papers that have described the function of Id proteins in the progression from G_1-phase to S-phase in human fibroblasts [Hara et al., 1994], thus evidencing their role in the control of cell cycle progression also [Peverali et al., 1994]. The role of *Id1* as a negative regulator of differentiation and positive regulator of proliferation is not a unique case among regulators of differentiation. *MyoD*, a positive regulator of muscle differentiation [Davis et al., 1987], has the ability to arrest growth without inducing differentiation [Sorrentino et al., 1990], thereby also acting as a negative regulator of proliferation. *C-myc*, a positive regulator of proliferation known to be involved in cell cycle control [Marcu et al., 1992] was also studied. Coppola and Cole [1986] have demonstrated the inhibition of MEL cell differentiation by constitutive *c-myc* expression. Our results show that the induction of differentiation abruptly depresses the amount of *c-myc* transcripts in a very early phase of cell culture. *C-myc* downregulation, along with the induction by DMSO, may draw the cells out of the cycle. We also show a sharp (4–6 h) increase in *SCL* expression during the induction of differentiation, which increases up to 72 h and correlates strongly with its known role as a late positive regulator in the terminal differentiation of erythropoietic cells. These data indicate that only when *Id1* and *c-myc* are downregulated and *SCL* is upregulated may differentiation occur; this conclusion is in line with the negative and positive role of these genes in the regulation of differentiation.

In our experiments, the expression of regulatory genes involved with terminal differentiation and growth was modified in a coordinated manner by DZA+HCY and DZAri. It is noteworthy that DZA+HCY and DZAri exerted opposite effects on *Id1* expression (Fig. 3): from 4 to 6 h the first drug increased whereas the second decreased its expression. Likewise, DZA+HCY increased *c-myc* expression from 6 to 24 h, with only a minor increase in *SCL* expression in the same conditions. DZAri did not promote early *c-myc* expression but did promote a late signal decrease. DZAri also caused a minor increase in *SCL* expression. It can therefore be speculated that the coordinated expression of the genes studied allows terminal differentiation and that the drugs tested are able to induce the coordinated modulation of their expression which is, in turn, responsible for the observed effects on differentiation and growth. The use of the drugs evidenced a predominant regulatory effect on differentiation exerted by negative regulators compared to positive one.

The partially different mechanisms of action of DZA+HCY and DZAri on the SAM cycle described in the introduction may account for the difference in gene expression and MEL terminal differentiation induced by the drugs. Some differences in action have also been reported in a muscle cell system: DZA+HCY has been shown to stimulate differentiation in two different sets of clones [Scarpa et al., 1984, 1996], while DZAri did not show any effect in one case and had an effect which was very similar to that of DZA+HCY in the other; moreover, DZAri also displayed a cytostatic effect.

The drugs were therefore not only capable of modulating the expression of positive regulatory genes [Scarpa et al., 1996], but also of

acting on different classes of genes (negative/positive regulators of proliferation/differentiation). The methylation patterns of the three genes were subsequently studied. Unfortunately, *SCL* is the only one of these genes with a known 5′-flanking sequence in mouse, whereas for the other two we had to study the structural portion sequences. The comparison between *Msp*I and *Hpa*II bands showed some higher bands after *Hpa*II treatment which were absent after *Msp*I treatment for both *c-myc* and *SCL*. These data demonstrate that both genes have several methylated (at least in some of the experimental conditions) CCGG sites. As far as *Id1* is concerned, the digestion with *Msp*I and *Hpa*II showed the same bands, thus indicating a lack of a methylation pattern and a generalized hypomethylated status which was not modulated by any of the drugs used; it should, however, be kept in mind that only a structural portion of this gene was investigated.

In our experiments, *SCL* and *c-myc* genes in DM displayed a substantially similar modulation of the methylation patterns: methylated in the control (DMSO alone), unmodified by the addition of DZA+HCY, and hypomethylated after DZAri addition. These effects further suggest that, though the DZA+HCY mechanism of action is, at least partially, distinct from that of DZAri, each of these drugs exerts substantially the same effect on genes with quite distinct (if not opposite) functions. There is indeed a good correlation between the hypomethylating effect of DZAri and differentiation. By contrast, no straightforward correlation can be found in these cells between methylation and gene expression. In fact, the resulting *SCL* and *c-myc* expression shows a differentiated response to the same methylation stimulus depending on the kind of gene and, ultimately, on its function; only the SCL response is in line with current knowledge about the link between DNA hypomethylation and the activation of gene expression. On the other hand, the lack of a hypomethylating effect by DZA+HCY is in agreement with previous data according to which the HMBA-induced differentiation of MEL cells correlates with a transient genome-wide hypomethylation [Razin et al., 1985, 1986] and that exposure to DZA+HCY similarly inhibited both this hypomethylation and differentiation [Razin et al., 1988]. The lack of hypomethylation induction by DZA+HCY in MEL cells is, however, in contrast with our previous data in L5 myoblasts in which we demonstrated that the hypomethylating effect exerted by DZA+HCY increased the expression of the positive regulator *myogenin*. This suggests that the effect of the drugs may be different on different cell systems, but that whenever the hypomethylation of a positive regulator (*SCL* in MEL cells or *myogenin* in L5 cells) is obtained, regardless of the hypomethylating stimulus, the expression of this kind of genes is enhanced. However, a more complex way of modulation of negative regulators is likely to be involved.

From the data presented in this paper, we may conclude that the differentiation of MEL cells results from the coordinated expression of regulatory genes controlling the proliferation/differentiation switch, with a predominant effect of negative regulators. The drugs used were able to modulate, in a coordinated manner, the expression of these genes, thereby producing a definite differentiative switch. Although no straightforward correlation between the alteration of DNA methylation pattern and gene expression was found in this cellular system, the administration of DZAri resulted in hypomethylation (regardless of the gene considered) correlated with enhanced differentiation. These results highlight a difference in the mechanism of action of the two drugs, a general link between DNA hypomethylation and differentiation, and a distinct gene response of positive and negative regulators following DNA hypomethylation.

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REFERENCES

Battey J, Moulding C, Taub R, Murphy W, Stewart T, Huntington P, Lenoir G, Leder P. 1983. The human c-myc oncogene: structural consequences of translocation

into the IgH locus in Burkitt Lymphoma. Cell 34:779–787.

Begley CG, Aplan PD, Dovey MP, Nokahara K, Tchorz K, Kurlzberg J, Hershfield MS, Hoynes BT, Cohen DI, Waldmann TA, Kirsch IR. 1989. Chromosomal translocation in a human leukemic stem-cell line disrupts the T-cell antigen receptor chain diversity region and results in a previously unreported fusion transcript. Proc Natl Acad Sci USA 86:2031–2035.

Begley CG, Robb L, Rockman S, Visvader J, Bockamp EO, Chan YS, Green AR. 1994. Structure of the gene encoding the murine SCL protein. Gene 138:93–99.

Begley CG, Visvader J, Green AR, Aplan PD, Metcalf D, Kirsch IR, Gough NM. 1991. Molecular cloning and chromosomal localization of the murine homolog of the human helix-loop-helix gene SCL. Proc Natl Acad Sci USA 88:869–873.

Benezra R, Davis RL, Lockshon D, Turner DL, Weintraub H. 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell 61:49–59.

Bird A. 1992. The essential of DNA methylation. Cell 70:5–8.

Brown L, Cheng JT, Chen Q, Siciliano MJ, Crist W, Buchanon G, Baer R. 1990. Site-specific recombination of the tal-1 gene is a common occurrence in human T cell leukemia. EMBO J 9:3343–3351.

Cantoni GL. 1977. In: Salvatore F, Boker E, Zappia V, Williams-Ashman HG, Schlenk F, editors. The biochemistry of adenosylmethionine. New York: Columbia University Press. p 557–577.

Chen Q, Cheng JT, Tsai LH, Shneider N, Buchanon G, Carroll A, Crist W, Ozanne B, Siciliano MJ, Baer R. 1990. The TAL gene undergoes chromosome translocation in T cell leukemia and potentially encodes a helix-loop-helix protein. EMBO J 9:415–424.

Chiang PK. 1985. S-Adenosylhomocysteine hydrolase. Measurement of activity and use of inhibitors. In: Paton DM, editor: Methods in pharmacology, Vol. 6. Plenum Publishing Corporation. p 127–145.

Christy BA, Sanders LK, Lou LF, Copeland NG, Jenkins NA, Nathans D. 1991. An Id-related helix-loop-helix protein encoded by a growth factor-inducible gene. Proc Natl Acad Sci USA 88:1815–1819.

Coppola JA, Cole MD. 1986. Constitutive c-Myc oncogene expression blocks mouse erythroleukaemia cell differentiation but not commitment. Nature 320:760–763.

Davis L, Cheng P-F, Lassar AB, Weintraub H. 1990. The MyoD DNA binding domain contains a recognition code for muscle specific gene activation. Cell 60:733–746.

Davis RL, Weintraub H, Lassar AB. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51:987–1000.

Duncan M, Di Cicco-Bloom EM, Xiang X, Benezra R, Chada K. 1992. The gene for the helix-loop-helix protein, Id, is specifically expressed in neural precursors. Dev Biol 154:1–10.

Finger LR, Kogan G, Christopher G, Kurtzberg J, Hershfield MS, Nowell PC, Croce CM. 1989. Involvement of the TCL5 gene on human chromosome 1 in T cell leukemia and melanoma. Proc Natl Acad Sci USA 86:5039–5043.

Green AR, Lints T, Visvader J, Harvey R, Begley CG. 1992. SCL is coexpressed with GATA-1 in hemopoietic cells but is also expressed in developing brain. Oncogene 7:653–660.

Hara E, Yamaguchi T, Nojima H, Ide T, Campisi J, Okayama H, Oda K. 1994. Id-related genes encoding helix-loop-helix proteins are required for G1 progression and are repressed in senescent human fibroblasts. J Biol Chem 269:2139 –2145.

Kirsch IR, Bertness V, Silver J, Hallis GF. 1986. Regulated expression of the c-myb and c-myc oncogenes during erythroid differentiation. J Cell Biochem 32:11–21.

Kreider BL, Benezra R, Rovera G, Kadesch T. 1992. Inhibition of myeloid differentiation by the helix-loop-helix protein Id. Science 255:1700–1702.

Levine A, Cantoni GL, Razin A. 1991. Inhibition of promoter activity by methylation: possible involvement of protein mediators. Proc Natl Acad Sci USA 88:6515–6518.

Levine A, Cantoni G, Razin A. 1992. Methylation in the preinitiation domain suppresses gene transcription by an indirect mechanism. Proc Natl Acad Sci USA 89:10119–10123.

Li E, Bestor TH, Jaenisch R. 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 61:915–926.

Marcu KB, Bossone SA, Patel AJ. 1992. Myc function and regulation. Annu Rev Biochem 61:809–860.

Murre C, Schonleber McCaw P, Baltimore D. 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD and Myc proteins. Cell 56:777–783.

Olson EN, Brennan TJ, Chakraborty T, Cheng T, Cserjesi P, Edmonson D, James G, Li L. 1991. Molecular control of myogenesis: antagonism between growth and differentiation. Mol Cell Biochem 104:7–13.

Peverali FA, Ramqvist T, Saffrich R, Pepperkok R, Barone MV, Philipson L. 1994. Regulation of G1 progression by E2A and Id helix-loop-helix proteins. EMBO J 13:4291–4301.

Ramchandani S, MacLeod AR, Pinard M, von Hofe E, Szyf M. 1997. Inhibition of tumorigenesis by a cytosine-DNA methyltransferase antisense oligodeoxynucleotide. Proc Natl Acad Sci USA 94:684–689.

Razin A, Feldmesser E, Kafri T, Szyf M. 1985. In: Cantoni GL, Razin A, editors. Biochemistry and biology of DNA methylation. New York: Wiley–Liss. p 239–253.

Razin A, Levine A, Kafri T, Agostini S, Gomi T, Cantoni GL. 1988. Relationship between transient DNA hypomethylation and erythroid differentiation of murine erythroleukemia cells. Proc Natl Acad Sci USA 85:9003–9006.

Razin A, Riggs A. 1980. DNA methylation and gene function. Science 210:604–610.

Razin A, Szyf M, Kafri T, Roll M, Giloh H, Scarpa S, Carotti D, Cantoni GL. 1986. Replacement of 5-methylcytosine: a possible mechanism for transient DNA demethylation during differentiation. Proc Natl Acad Sci USA 83: 2827—2831.

Riechmann V, van Cruchten I, Sablitzky F. 1994. The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. Nucl Acid Res 22:749–755.

Sambrook J, Fritsch EF, Maniatis T. 1989. In: Ford N, Nolan C, Ferguson M, editors. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press.

Scarpa S, Lucarelli M, Palitti F, Carotti D, Strom R. 1996. Simultaneous myogenin expression and overall DNA

hypomethylation promote in vitro myoblast differentiation. Cell Growth Diff 7:1051–1058.
Scarpa S, Strom R, Bozzi A, Askamit RR, Backlund SP, Chen J, Cantoni GL. 1984. Differentiation of myoblast cell lines and biological methylation: 3-Deazaadenosine stimulates formation of multinucleated myofibers. Proc Natl Acad Sci USA 81:3064–3068.
Sorrentino V, Pepperkok R, Davis RL, Ansorge W, Philipson L. 1990. Cell proliferation inhibited by MyoD1 independently of myogenic differentiation. Nature 345: 813–815.
Sun X-H, Copeland NG, Jenkins NA, Baltimore D. 1991. Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. Mol Cell Biol 11: 5603–5611.
Szyf M. 1996. The DNA methylation machinery as a target for anticancer therapy. Pharmacol Ther 70:1–37.
Szyf M, Rouleau J, Theberge J, Bozovic V. 1992. Induction of myogenic differentiation by an expression vector encoding the DNA methyltransferase cDNA sequence in the antisense orientation. J Biol Chem 267:12831–12836.
Tamame M, Antequera F, Villanueva JR, Santos T. 1983. High-frequency conversion of a "fluffy" developmental phenotype in *aspergillus* spp. by 5-azacytidine treatment: evidence for involvement of a single nuclear gene. Mol Cell Biol 3:2287–2297.
Taylor SM, Jones PA. 1979. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 17:771–779.
Taylor SM, Jones PA. 1982. Mechanism of action of eukaryotic DNA methyltransferase. J Mol Biol 162:679–692.
Vertino PM, Yen RW, Gao J, Baylin SB. 1996. De novo methylation of CpG island sequences in human fibroblasts overexpressing DNA (cytosine-5-)-methylatransferase. Mol Cell Biol 16:4555–4565.
Visvader J, Begley CG. 1991. Helix-loop-helix genes translocated in lymphoid leukemia. TIBS 16:330–333.
Visvader J, Begley CG, Adams JM. 1991. Differential expression of the LYL, SCL and E2A helix-loop-helix genes within the hemopoietic system. Oncogene 6:187–194.
Vogel Z, Sytkowsky AJ, Niremberg MW. 1972. Acetylcholine receptors of muscle grown in vitro. Proc Natl Acad Sci USA 69:3180–3184.
Wu J, Herman JG, Wilson G, Lee RY, Yen RW, Mabry M, de Bustros A, Nelkin BD, Baylin SB. 1996. Expression of prokaryotic HhaI DNA methyltransferase is transforming and lethal to NIH 3T3 cells. Cancer Res 56: 616–622.
Wu J, Issa JP, Herman J, Bassett DE Jr, Nelkin BD, Baylin SB. 1993. Expression of an exogenous eukaryotic DNA methyltransferase gene induces transformation of NIH 3T3 cells. Proc Natl Acad Sci USA 90:8891–8895.