Abstract. Cytosine methylation, an epigenetic form of
regulating gene transcription, has gained importance upon
the discovery that genes involved in the carcinogenic process may
be regulated by this mechanism and, moreover, that certain cancers
respond to treatment with demethylation-promoting drugs.
Typically, the use of DNA methyltransferase inhibitor drugs
results in the up-regulation of important tumor suppressor genes,
previously down-regulated by the existence of abnormal cytosine
methylation within their promoters. Here, we show microarray
and RT-PCR results indicating that many genes are down-
regulated upon treatment of KAS 6/1 multiple myeloma cells
with Zebularine, a demethylating agent. Our findings suggest
that, in addition to the typical methylation inhibitor-induced up-
regulation of genes, removal of methylation in some genes may
have a profound down-regulating effect upon their expression.
The analysis of gene function showed that, of the down-
regulated genes, 38 are associated with cell proliferation and/or
cancer. Our analysis of the promoters of the subset of selected
genes containing CpG islands showed that the distribution of cis
elements differs between genes up- and down-regulated by
methylation. Finally, we propose a model which shows how
genes containing methylation sites within their basic promoters
and/or enhancer sequences are susceptible to down-regulation,
whereas genes methylated within silencer regions are up-
regulated, thus providing a model as to how DNA methylation
could induce such opposing effects on transcription.

Epigenetic Up-regulation of Gene Expression
in KAS 6/1 Human Multiple Myeloma Cells

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Landmark Genomic Scanning, epigenetic gene silencing,
microarray, cholesterol metabolism.

The epigenetic regulation of gene expression is controlled by
various developmental signaling pathways, that mediate the
execution of a pre-determined genetic program (1). The
developmental alteration of expression patterns occurs by
two major mechanisms, methylation of nucleosides in DNA
(usually cytosines), and post-translational modification of
histone proteins by specific protein kinases, methylases and
acyltransferases (2,3). These modifications control the activity of
various subsets of genes, and serve to determine the ultimate
tissue-specific expression pattern of a given cell (4,5). During
development, methylation of DNA is mediated primarily by
three DNA-5-cytosine-Methyltransferases, two de novo
methylating enzymes, DNMT-3A and DNMT-3B, which
work in concert with a third enzyme, DNMT-1, or
maintenance methyltransferase (6-8). Upon reaching a state
of developmental "maturity," the cell’s methylation patterns
are essentially set, and are maintained by DNMT-1 (9). The
substrate specificity for DNMT-1 differs from that of
DNMT-3A and DNMT-3B, in that it has a strict preference
for hemi-methylated sites, thus DNMT-1 utilizes the
methylation pattern present in the template strand to
maintain the cell’s epigenetic methylation pattern throughout
its lifetime (10,11). Methylation most often occurs in CpG
islands, which are defined as regions greater than 200 bp
with a G and C content greater than 50%, and an observed-
to-predicted ratio of CG dinucleotides greater than or equal
to 0.6 (12). Once methylation occurs, the methylated CpG
site is then bound by a family of specific proteins, which
induce the initiation of the epigenetic silencing process, by
starting a cascade of other types of chromatin-modifying
enzymes and proteins (13).

While the epigenetic control of gene expression is a
normal developmental event, aberrant DNA methylation
anomalies are known to occur in many types of cancer cells.
Hypomethylation of cellular DNA is known to lead to
genomic instability, probably due to the reactivation of
retrotransposons, previously silenced by methylation (14).
The activation of other silenced genes may also occur, some with developmental functions such as cell differentiation, and proliferation (15,16). Hypermethylation of DNA in tumor cells is known to occur primarily in the CpG islands, resulting in the epigenetic silencing and reduced expression of important tumor suppressor and pro-apoptotic genes (17). Much of the literature pertaining to the epigenetic silencing of important tumor suppressor genes examines the reactivation of these genes and how they affect cellular function. The classical model of epigenetic silencing typically describes the methylation of a tumor suppressor gene promoter (or CpG island) which results in the reduction of that gene’s expression level. The effects of methylation-induced silencing are often ameliorated by the use of various methyltransferase inhibitor drugs, mainly cytosine analogs, causing a reduction in promoter methylation and increased levels of gene expression (18). Previously, we have shown that, in multiple myeloma cells, certain genes can be reactivated by the use of the methyltransferase inhibitor Zebularine (19). Zebularine has greater bioavailability and decreased side-effects as compared to the 5-aza-cytidine family of demethylating drugs, thus, its use as a tool to study the discreet effects of gene methylation are enhanced relative to previously employed drugs (19).

Gene silencing by promoter methylation occurs due to changes in chromatin structure and the binding of methylcytosine (CpG) specific protein complexes, resulting in the inhibition of the binding of transcription factors and RNA polymerase II (20). In contrast, a gene whose expression is down-regulated by the use of methyltransferase inhibiting drugs may have methylation sites in regions of DNA that recruit inhibitors of gene transcription. In these situations, methylation can promote gene transcription instead of silencing genes by blocking the inhibitor protein from binding to its cognate sequence (21). Alternatively, reductions in expression levels could be due to the reactivation (by reducing methylation) of silenced inhibitor proteins, which are then able to bind to their respective targets sites (22,23). Transcription factors may act to inhibit gene expression, depending on other factors, such as neighboring CIS elements, and the presence of other DNA-binding proteins. Examples include the thyroid hormone receptor Beta-1 promoter and the inhibitor factor Oct-1 (24), and the glucocorticoid-responsive element (GRE) and glucocorticoid receptor (GR), which binds to it. Although not as well characterized as positive transcriptional elements, negative regulatory elements have been found in many genes (25-27). DNA sequences, CIS elements, associated with repression of transcription, can be found within promoters, coding and non-coding exons and in introns (28). The classical sequences are position-independent, silencer elements, mainly functioning by preventing the assembly of the general transcription factors (GTFs) necessary for RNA polymerase II to transcribe RNA. Other DNA sequences are position-dependent, negative regulatory elements (NREs) or repressors, and usually interfere with the binding of transcription factors to DNA elements upstream from the transcription initiation site (28).

Here, we show that, while the expression of many genes is up-regulated by the use of methyltransferase inhibiting drugs, there are others whose expression is down-regulated. Using the KAS 6/1 multiple myeloma cell line as a model system, we characterized the expression levels of many genes that are down-regulated in response to a reduction in DNA methylation levels induced by the methyltransferase inhibitor Zebularine. We also provide a bioinformatics based analysis of the promoter regions of these genes to propose a possible model to explain why these particular genes are responding in a negative fashion, while others are up-regulated in response to methylation inhibitor drugs.

Materials and Methods

Materials. KAS 6/1 cells were kindly provided by Dr. Dianne Jelinek, of the Mayo Clinic, Rochester, MN, USA. Zebularine (2-(1H)-pyrimidinone riboside, NSC 309132), a ribose nucleoside derivative compound, is available from the Developmental Therapeutics Program of the NCI (Laboratory of Medicinal Chemistry, National Cancer Institute, National Institutes of Health, Frederick, MD, USA) (29). Affymetrix DNA microarrays were obtained from Affymetrix (Santa Clara, CA, USA). Fetal bovine serum (FBS) was from Gemini Bio-Products (Woodland, CA, USA). RPMI 1640, ampicillin/streptomycin solution, and L-glutamine were from Cellgro (Mediatech, Herndon, VA, USA), IL-6 was obtained from PeproTech (Rock Hill, NJ, USA). Agarose, Taq polymerase and dNTPs were from Invitrogen (Carlsbad, CA, USA). 100 bp DNA molecular weight standard was from New England BioLabs (Beverly, MA, USA), other reagents were obtained from Sigma (Saint Louis, MO, USA), unless otherwise stated in the methods.

Cell treatment. The human multiple myeloma cell line KAS 6/1 was grown in RPMI 1640 medium supplemented with 10% FBS, 10 ng/ml IL-6, 50 Ìg/ml streptomycin, 2 mM L-glutamine, 10 ng/ml IL-6, in a humidified incubator, at an atmosphere of 5% CO2, at 37°C. Control cells received no treatment; Zebularine-treated cells received 200 ìM of the drug for a period of 48 hours; "remethylated" cells were treated with Zebularine as described, washed 3 times with fresh medium, diluted ten-fold in fresh medium containing 10 ng/ml IL-6, and allowed to grow for two weeks.

Restriction Landmark Genomic Scanning (RLGS). Restriction Landmark Genomic Scanning was performed as described in previous reports (30). Briefly, cell genomic DNA was extracted and sheared ends were blocked with nucleotide analogues (alpha-S-dGTP, alpha-S-dCTP, ddATP and ddTTP) in the presence of DNA polymerase I. Fragments were then cut with NotI, which is specific for non-methylated strands, and the cut ends were radiolabeled with [alpha-32P]-dGTP. This method ensured that highly methylated regions of DNA were not radiolabeled. The resulting fragments were
Table 1. PCR primers for each of the analyzed genes. Primers were designed using Primer3 program, with the exception of ANT3 and CD48.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANT3 or SLC25A61 solute carrier family 25, member A6; ADP/ATP translocase 3</td>
<td>5'- caccaagtccgacggcatccg -3' 5'- acggtgaggattctagttg -3'</td>
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<tr>
<td>CD48 antigen</td>
<td>5'-gaagcatgtgcctccagtggt-3' 5'-tgccattcttgctgctcacag -3'</td>
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<tr>
<td>CXCL12 chemokine (C-X-C motif ligand 12)</td>
<td>5'-tgctggaggtggatcgagtgga-3' 5'-gctttacagcggacagttaaag -3'</td>
</tr>
<tr>
<td>EGR1 early growth response 1</td>
<td>5'-ccaccaagtccgacggcatccg -3' 5'-gacaagggcaagagacatca -3'</td>
</tr>
<tr>
<td>GAML guanidinoacetoacetate N-methyltransferase</td>
<td>5'-tttgaatcagaggacggataca-3'</td>
</tr>
<tr>
<td>IGF1 insulin-like growth factor I</td>
<td>5'-aaggaggtgagatgattgac-3' 5'-ggtggtctagctcccacttca -3'</td>
</tr>
<tr>
<td>INSIG1 insulin induced gene 1</td>
<td>5'-ctgggatcactcactctccttc-3' 5'-aactataggagttcttaagga -3'</td>
</tr>
<tr>
<td>LSS lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)</td>
<td>5'-ccagctgcagccatcactaaca -3' 5'-ggagccagagctgtaagag -3'</td>
</tr>
<tr>
<td>SULF1 sulfatase 1</td>
<td>5'-gaggctcagggagcgcataaca -3' 5'-gtttgtagctacagaggatgga -3'</td>
</tr>
<tr>
<td>GAPDH glyceraldehyde 3-phosphate</td>
<td>5'-aggtgaaggtcggagtcaacgg-3' 5'-ccacgtctcctctcaggtgtg -3'</td>
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</tbody>
</table>

further cut with EcoR V and subjected to electrophoresis on a 0.8% agarose tube gel. The DNAs were cut in situ with Hind I and the tube gel was placed on the top of a 5% polyacrylamide gel for a second dimension electrophoresis of the DNA. The gels were dried and exposed to an X-ray film. Differences observed in the intensity of spots indicate changes in the degree of DNA methylation between samples. Restriction Landmark Genomic Scanning fragments were cloned using the strategy described by Smiraglia et al. (31). Sequences were aligned using BLAST to DNA databases such as GenBank and the UCSC human genome project to map their positions.

RNA extraction. RNA was extracted from cells using the TRIzol reagent (Invitrogen), as recommended by the manufacturer. RNA quantity and quality were assessed by agarose gel electrophoresis (1% gel) and spectrophotometry (absorbance ratio at wavelengths 260 and 280 nm). The RNAs used for microarray analysis were also evaluated by high-pressure liquid chromatography by Expression Analysis (Durham, NC, USA).

Affymetrix microarray analysis. Samples of RNA from control (untreated), Zebularine-treated and recovered cells were processed by Expression Analysis. The data obtained was then processed by several programs (Affymetrix Microarray Suite version 5.0) to normalize the data, statistically determine whether a given transcript was present or not, and whether there were significant differences in expression between the different duplicates (experiments 1 and 2) or groups (control, Zebularine-treated and remethylated).

The microarray assay was carried out with two sets of chips, U133A and U133B, with a total of 45,000 probe sets representing more than 39,000 transcripts, derived from approximately 33,000 well-substantiated human genes (based on the genome Build of April 2001). The set design uses sequences selected from GenBank, dbEST, and RefSeq. The U133A set was generated from cDNA data sequences previously represented on the Human Genome U95Av2 Array, whereas the U133B was generated primarily from ESTs clusters. Each array has approximately 22,000 "probe sets", each containing 11 DNA oligonucleotides of 25 bases corresponding to different regions of the 3' end of a given transcript. For some genes, several "probe sets" exist to allow assessment of different splice forms or transcripts with different polyadenylation sites. Three sets of chips were analyzed for each group of cells: two with probes generated from the same RNA (experiments 1 and 2) and one with probes generated from RNA obtained in a different experiment (experiment 3).

Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was used as a template for the synthesis of cDNA using the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV), according to the manufacturer’s protocol (Roche Applied Science, Indianapolis, IN, USA). The total RNA used per reverse transcription, using Oligo-p(dT)15 primers was 1 µg/50 µl. The PCR was carried out with 20 mM Tris (pH 8.4), 50 mM KCl, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, 1.5 mM MgCl2 (unless otherwise stated), 0.2 µM each primer, 1 U AmpliTag DNA polymerase (Invitrogen) and 60 µM/µl cDNA template. After an enzyme activation step (2 min, 96°C), cycles consisting of 30 sec denaturing (96°C), 30 sec annealing and 30 sec extension (72°C) were performed. Primers for each gene are indicated in Table 1. The PCR conditions were optimized in order to obtain data from the exponential phase of the reaction. Glyceraldehyde 3-phosphate expression (GAPDH) was used to normalize data. After PCR, the DNA products were run on a 2% Tris-acetate-EDTA (0.5 X TAE), etidium bromide-stained agarose gel. The size of the PCR products was estimated by comparison to the 100 bp Molecular Weight Standard. The fluorescence expression of each band was quantified using the UVP Digital Camera (GDS-800 System, UVP BioImaging Systems USA, Upland, CA, USA) and the LabWorks™ Analysis Software, Version 3.0.02.00, UVP. Fluorescence was also detected by the Typhoon 8600 Variable Mode Imager, from Molecular Dynamics (Amersham Pharmacia Biotech, Uppsala, Sweden) and analyzed by the ImageQuant 5.2 software (Molecular Dynamics).

Analysis of promoter sequences. The up- and down-regulated sequences were analyzed using the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat?hgvsid=363066957)(32) and CpGProD (CpG Island Promoter Detection) algorithm (http://phil.univ-lyon1.fr/software/cpgprod_query.html)(33), in order to identify the promoter with CpG islands within the first 1,500 bp upstream from the transcription initiation site. The regions corresponding to the identified CpG islands were then analyzed using the SIGSCAN Version 4.05 suite (http://bimas.dct.nih.gov/molbio/signal/) (34). In order to filter the results obtained, CIS elements with fewer than 4 bp were ignored. All the locations of the remaining set of identified CIS elements for each
Tables IIa and IIb.  
* There may be more than one probe set per gene and the same gene may be present on different chips. The total of genes present in the microarrays U133A plus U133B is 33,000. The presence of CpG islands was analyzed within the 1,500 bp upstream from the transcription initiation signal of the gene associated with each probe set, using the "CpGProD (CpG Island Promoter Detection)" program (33), open for public use at http://pbil.univ-lyon1.fr/software/cpgprod_query.html. Exp: experiment.

Table IIa. Genes down-regulated by Zebularine in the U133A microarray.

<table>
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<th>Rec</th>
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<td>0.12</td>
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<td>INSIG1</td>
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<td>1714.3</td>
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<td>SLC25A6</td>
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<td>277.3</td>
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<td>0.19</td>
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of the genes with CpG islands were then used to generate Figures 4a and 4b. The number of hits to each sequence of the CIS elements was computed and the expected random values, discounted. The total promoters analyzed for the genes down-regulated with Zebularine was 45 and that of genes up-regulated by the demethylating agent was 190. In order to compare both groups, the values for the up-regulated CIS elements were corrected by multiplying them by 45/190 (0.237). The consensi with over 25 hits for the down-regulated genes were selected to generate Table V, which shows the normalized number of hits within the up-regulated gene group and the ratio between the values of the up- and down-regulated number of hits to each consensus sequence.

Results

Restriction Landmark Genomic Scanning. In our previous report, changes in methylation status of multiple genes were identified upon treatment of cells with Zebularine, followed by Restriction Landmark Genomic Scanning (19). This assay uses methylation-sensitive restriction enzymes with a high CG and CpG island content to cleave genomic DNA at the CpG islands. Cleaved sites are radioactively labeled and, after further digestion with restriction enzymes, the fragments are

Table IIb. Genes down-regulated by Zebularine in the U133B microarray.

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<td>235278_at</td>
<td>16248.7</td>
<td>3291.8</td>
<td>16473.9</td>
<td>0.20</td>
<td>0.20</td>
<td>Weakly similar to cdcl2-like kinase associating cyclophilin na</td>
<td></td>
</tr>
<tr>
<td>234989_at</td>
<td>10142.1</td>
<td>2704.9</td>
<td>11609.9</td>
<td>0.27</td>
<td>0.23</td>
<td>Multiple endocrine neoplasia I TncRNA</td>
<td></td>
</tr>
<tr>
<td>227062_at</td>
<td>3367.4</td>
<td>906.0</td>
<td>3696.7</td>
<td>0.27</td>
<td>0.25</td>
<td>Multiple endocrine neoplasia I TncRNA</td>
<td></td>
</tr>
<tr>
<td>225239_at</td>
<td>15572.7</td>
<td>744.6</td>
<td>1662.5</td>
<td>0.48</td>
<td>0.45</td>
<td>Acyl-Coenzyme A dehydrogenase, very long chain ACADVL</td>
<td></td>
</tr>
<tr>
<td>221286_at</td>
<td>3600.2</td>
<td>1740.2</td>
<td>3690.6</td>
<td>0.48</td>
<td>0.47</td>
<td>NA ZFP36L2</td>
<td></td>
</tr>
<tr>
<td>200770_at</td>
<td>1214.9</td>
<td>581.4</td>
<td>1221.0</td>
<td>0.48</td>
<td>0.48</td>
<td>Serine hydroxymethyltransferase 2 SHMT2</td>
<td></td>
</tr>
<tr>
<td>222209_at</td>
<td>420.1</td>
<td>206.6</td>
<td>473.9</td>
<td>0.49</td>
<td>0.44</td>
<td>KIAA1040 protein KIAA1040</td>
<td></td>
</tr>
<tr>
<td>209737_at</td>
<td>1540.8</td>
<td>750.5</td>
<td>1713.7</td>
<td>0.49</td>
<td>0.44</td>
<td>Tetra tripeptide repeat domain 3 TTC3</td>
<td></td>
</tr>
</tbody>
</table>

Table V. Normalized number of hits within the up-regulated gene group and the ratio between the values of the up- and down-regulated number of hits to each consensus sequence.
Figure 1. Depiction of expression of probe-sets that do not comply with the 4 criteria established for main gene selection, but do fulfill two criteria. The relative expression in control cells and recovered cells is at least twice that of Zebularine-treated cells and this applies to both independent experiments. Figure 1A corresponds to the U133A microarray, and Figure 1B to the U133B microarray. The expression levels of each independent experiment for control, Zebularine and recovered cells are side by side. Boxes with greatest intensity of red correspond to higher expression.
Table IIIa. Gene function of down-regulated genes from U133A microarray.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITGB7, GPC4(54), KAI1(67)</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>IGF1(68), TGFBR2(69), SUFL1(70), SEPT10(71)</td>
<td>Cell growth</td>
</tr>
<tr>
<td>D2S448(72), CD48(73), TSC2(74), GPR37, RPIB9, AIP1(75), PIK3C2A(76), TM4SF2, CCL3(77), CAV1(78), INSG1(79)</td>
<td>Intracellular signaling</td>
</tr>
<tr>
<td>SCD(79), SHMT2(80), FASN, FADS2, SL25A6(81), ACADVL, QARS(82), DPYD, AD-017, FADS1, ID1*, FDF1*, SC4MOL*, LSS*, DHCR7*, SOLE*, HMGS1*, SOLE*, FDP5*, MVDR*, DFTF1*</td>
<td>Metabolism</td>
</tr>
<tr>
<td>D2S448(72), CD48(73), TSC2(74), GPR37, RPIB9, AIP1(75), PIK3C2A(76), TM4SF2, CCL3(77), CAV1(78), INSG1(79)</td>
<td>Other</td>
</tr>
<tr>
<td>SULF1(70), SEPT10(71), D2S448(72), CD48(73), TSC2(74), GPR37, RPIB9, AIP1(75), PIK3C2A(76), TM4SF2, CCL3(77), CAV1(78), INSG1(79)</td>
<td>Intracellular signaling</td>
</tr>
<tr>
<td>SCD(79), SHMT2(80), FASN, FADS2, SL25A6(81), ACADVL, QARS(82), DPYD, AD-017, FADS1, ID1*, FDF1*, SC4MOL*, LSS*, DHCR7*, SOLE*, HMGS1*, SOLE*, FDP5*, MVDR*, DFTF1*</td>
<td>Metabolism</td>
</tr>
<tr>
<td>D2S448(72), CD48(73), TSC2(74), GPR37, RPIB9, AIP1(75), PIK3C2A(76), TM4SF2, CCL3(77), CAV1(78), INSG1(79)</td>
<td>Other</td>
</tr>
<tr>
<td>SULF1(70), SEPT10(71), D2S448(72), CD48(73), TSC2(74), GPR37, RPIB9, AIP1(75), PIK3C2A(76), TM4SF2, CCL3(77), CAV1(78), INSG1(79)</td>
<td>Intracellular signaling</td>
</tr>
<tr>
<td>SCD(79), SHMT2(80), FASN, FADS2, SL25A6(81), ACADVL, QARS(82), DPYD, AD-017, FADS1, ID1*, FDF1*, SC4MOL*, LSS*, DHCR7*, SOLE*, HMGS1*, SOLE*, FDP5*, MVDR*, DFTF1*</td>
<td>Metabolism</td>
</tr>
<tr>
<td>D2S448(72), CD48(73), TSC2(74), GPR37, RPIB9, AIP1(75), PIK3C2A(76), TM4SF2, CCL3(77), CAV1(78), INSG1(79)</td>
<td>Other</td>
</tr>
<tr>
<td>SULF1(70), SEPT10(71), D2S448(72), CD48(73), TSC2(74), GPR37, RPIB9, AIP1(75), PIK3C2A(76), TM4SF2, CCL3(77), CAV1(78), INSG1(79)</td>
<td>Intracellular signaling</td>
</tr>
<tr>
<td>SCD(79), SHMT2(80), FASN, FADS2, SL25A6(81), ACADVL, QARS(82), DPYD, AD-017, FADS1, ID1*, FDF1*, SC4MOL*, LSS*, DHCR7*, SOLE*, HMGS1*, SOLE*, FDP5*, MVDR*, DFTF1*</td>
<td>Metabolism</td>
</tr>
<tr>
<td>D2S448(72), CD48(73), TSC2(74), GPR37, RPIB9, AIP1(75), PIK3C2A(76), TM4SF2, CCL3(77), CAV1(78), INSG1(79)</td>
<td>Other</td>
</tr>
</tbody>
</table>

Each probe set found to be down-regulated by Zebularine was assigned a main function based on Gene Ontology (GO) Consortium annotations (102), GeneBank and UniGene annotations, publications and bioinformatics prediction tools, such as SMART. Underlined genes are associated with the stimulation of cell proliferation, apoptosis inhibition, metastasis, angiogenesis or have otherwise been associated with the carcinogenic process; double-underlined genes are associated with anti-carcinogenic processes; genes underlined with dashed lines have been associated with the carcinogenic or with the inhibition of the carcinogenic process, depending on the experimental model or tumor type. Tables IIIa-b contain probe sets regulated according to 4 criteria: presence of signal in the control and recovered cells; relative expression in control cells and recovered cells is at least twice that of Zebularine-treated cells; differential expression of a given transcript from a Zebularine-treated and the control or recovered populations is statistically significant ($p< 0.05$); and all tree criteria apply to both independent experiments (RNA from cells treated at independent occasions). Table IIIa corresponds to probe-sets present in the U133A microarray and Table 3b to probe-sets present in the U133B. Table IIIc contains genes which are not regulated according to the 4 criteria above, but comply at least with two criteria: the relative expression in control cells and recovered cells is at least twice that of Zebularine-treated cells and this applies to both independent experiments.

Table IIIb. Gene function of down-regulated genes from U133B microarray.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPRY4, PDK1(88), INPP4A(89), HTPAP, TncRNA, PACAP(93), MGC39820, LOC540719, FLJ36769, EST, KIAA0141, FLJ34888, FLJ2747, FLJ10392, FLJ14564, FLJ20202, LAMC1, TUBB, BHLHB2(85), SOX2(86), DNAJC1(87)</td>
<td>Intracellular signaling</td>
</tr>
<tr>
<td></td>
<td>Other</td>
</tr>
<tr>
<td></td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Table IIIc. Gene function of down-regulated genes from the U133A and U133B microarray assays as assessed by less stringent criteria.

<table>
<thead>
<tr>
<th>U133A</th>
<th>U133B</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRCAM (90), SERPINE2(91), CCNG2(93), CDKN1A(94), AIP1(75), ARHQ, CXCL12(96), MAPK1, NIC, CHST12, GAMT, LSS*, MGC33202, UX51, ARFGAP3, MAGED2(98), PCM1(99), RNASE4, TBCD, TUBB, FOXD1(100), MAF(101)</td>
<td>ITGA4(92)</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGR1(95)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DELGEF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RASSF4(97)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MGC33202, UX51, FLJ00026 (DOCK8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAPK1, NIC, CHST12, GAMT, LSS*, MGC33202, UX51, ARFGAP3, MAGED2(98), PCM1(99), RNASE4, TBCD, TUBB, FOXD1(100), MAF(101)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGR1(95)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DELGEF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RASSF4(97)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MGC33202, UX51, FLJ00026 (DOCK8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAPK1, NIC, CHST12, GAMT, LSS*, MGC33202, UX51, ARFGAP3, MAGED2(98), PCM1(99), RNASE4, TBCD, TUBB, FOXD1(100), MAF(101)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGR1(95)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DELGEF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RASSF4(97)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MGC33202, UX51, FLJ00026 (DOCK8)</td>
</tr>
</tbody>
</table>

Figure 2. Semi-quantitative RT-PCR for microarray genes down-regulated by Zebularine. RNAs from control, Zebularine-treated and recovered cells were extracted and used as templates for the synthesis of cDNAs. PCR reactions were carried out with primers designed to hybridize with different exons of each gene. The number of cycles and PCR conditions were optimized to obtain semi-quantitative data (see Materials and Methods). The PCR reaction products for GAPDH are shown to assure equalization of total cDNA for each PCR reaction. The names of the genes are shown on the right.
sequentially resolved in agarose and polyacrylamide gels, yielding a two-dimensional image. Darker spots correspond to unmethylated DNA and are found more frequently in Zebularine-treated-derived DNA, as compared to control DNA (untreated) and DNA remethylated after Zebularine washout, followed by culture of cells for several days in the presence of IL-6. Of a total of 1360 spots, 23 DNA fragments were darker in DNA derived from cells treated with Zebularine. Of these, 7 were sequenced, all of which were found to contain CpG islands. Five sequences aligned with promoters of known genes, one with a predicted open reading frame and one with an EST (expressed sequence tag). Of these, one, identified as 2F55, which corresponds to the estrogen receptor 1 (ESR1), has a coding region that aligns with a microarray cluster (215552_s_at, from the U133A chip), and is shown elsewhere (19). Although present in very low levels, there was microarray detection, with the values for experiment 1+2 being: 124.4 control, 90.45 Zebularine and 136.55 recovered; for experiment 3, these values were, respectively, 111, 20 and 64.6. These data indicate that, although overall methylation of the CpG island of this gene is decreased by treatment with Zebularine, the gene expression decreases upon demethylation. This supports our initial hypothesis that methylation may be associated with increased gene expression. The down-regulation of estrogen receptor 1 (ESR1) by Zebularine was not expected, however, we, and others, have shown the negative effects of estrogen on the growth and survival of multiple myeloma cells (37).

**Microarray.** A systematic study of genes epigenetically regulated by Zebularine using total cell RNA as a probe in the expression cDNA microarray technique was described in a previous study aimed at the identification of genes silenced by methylation (19). This approach was chosen to complement data obtain by the restriction landmark genomic scanning, which indicated that promoter methylation was indeed associated with changes in gene expression. Here, we examined the dataset to search for genes up-regulated by methylation.

Three basic criteria were adopted to select the subset of probes of interest: the expression of genes from the control and remethylated cells had to be considered sufficiently high

<table>
<thead>
<tr>
<th>E.C. or gene symbol</th>
<th>Gene name</th>
<th>Down-regulated by Methylation?</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Zebul./ control</td>
<td>Zebul./ recovered</td>
</tr>
<tr>
<td>2.3.3.10</td>
<td>Hydroxymethylglutaryl-CoA synthase.</td>
<td></td>
<td>0.30</td>
<td>0.27</td>
</tr>
<tr>
<td>1.1.1.34</td>
<td>Hydroxymethylglutaryl-CoA reductase (NADPH).</td>
<td></td>
<td>0.60</td>
<td>0.63</td>
</tr>
<tr>
<td>2.7.1.36</td>
<td>Malonate kinase.</td>
<td></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2.7.4.2</td>
<td>Phosphomevalonate kinase.</td>
<td></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4.1.1.33</td>
<td>Diphosphomevalonate decarboxylase.</td>
<td></td>
<td>0.18</td>
<td>0.19</td>
</tr>
<tr>
<td>5.3.3.2</td>
<td>Isopentenyl-diphosphate delta-isomerase.</td>
<td></td>
<td>0.42</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.43</td>
<td>0.35</td>
</tr>
<tr>
<td>2.5.1.10</td>
<td>Geranyltransthase.</td>
<td></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2.5.1.29</td>
<td>Farnesyltransthase.</td>
<td></td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2.5.1.21</td>
<td>Farnesyl-diphosphate farnesyltransferase.</td>
<td></td>
<td>0.38</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Squalene monooxygenase.</td>
<td></td>
<td>0.44</td>
<td>0.42</td>
</tr>
<tr>
<td>1.14.99.7</td>
<td>Squalene monooxygenase.</td>
<td></td>
<td>0.45</td>
<td>0.46</td>
</tr>
<tr>
<td>5.4.99.7</td>
<td>Lanosterol synthase.</td>
<td></td>
<td>0.30</td>
<td>0.22</td>
</tr>
<tr>
<td>5.3.3.5</td>
<td>Cholestenol delta-isomerase.</td>
<td></td>
<td>0.71</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.47</td>
<td>0.61</td>
</tr>
<tr>
<td>1.3.3.2</td>
<td>Lathosterol oxidase.</td>
<td></td>
<td>0.63</td>
<td>0.66</td>
</tr>
<tr>
<td>1.3.1.21</td>
<td>7-dehydrocholesterol reductase.</td>
<td></td>
<td>0.43</td>
<td>0.42</td>
</tr>
<tr>
<td>1.14.13.72</td>
<td>Methylsterol monooxygenase.</td>
<td></td>
<td>0.36</td>
<td>0.39</td>
</tr>
<tr>
<td>SREBP1</td>
<td>Sterol regulated element binding protein 1.</td>
<td></td>
<td>0.29</td>
<td>0.26</td>
</tr>
<tr>
<td>SREBP2</td>
<td>Sterol regulated element binding protein 2.</td>
<td></td>
<td>0.42</td>
<td>0.45</td>
</tr>
</tbody>
</table>

The first column corresponds to the E.C. (Enzyme Commission) number for enzymes or gene symbol for transcription factors. The second column contains the gene names. The third and fourth columns correspond to the expression ratio between Zebularine (Zebul.) to control and Zebularine to recovered, respectively, in experiment 1. The last two columns correspond to the ratios, as in columns 3 and 4, but for experiment 2. Each gene may be associated with one or more probe sets, the results of each being present on a different line.
Semi-quantitative RT-PCR assays were carried out. RT-PCR indicates that these 13 genes may be added to the cancer-approach in the treatment of cancer (35,36,38). This in tumor cells and its inhibition may be a therapeutic search. Literature searches indicate that cholesterol biosynthesis is increased cholesterol metabolism, including key enzymes. Literature search was performed to determine how many genes usually found to inhibit cancer totaled 10. Genes associated with the carcinogenic process added up to 27, whereas genes already described as cancer-associated genes. Genes attributed to the carcinogenic process had to be down-regulated at least two-fold by the treatment with Zebularine, a strong indication that they are in fact targets of methylation. The application of less stringent selection criteria, by verifying that there was a down-regulation by Zebularine relative to the control and remethylated cells of at least 50% for both RNA experiments, added 20 down-regulated probe sets from chip U133A and 15 from chip U133B, as illustrated in Figures 1A and 1B. The regulated probe sets from chip U133A all correspond to genes with CpG islands, whereas 53% of the probe sets of chip U133B have predicted CpG islands in their promoters.

An interesting group of 13 genes are associated with cholesterol metabolism, including key enzymes. Literature searches indicate that cholesterol biosynthesis is increased in tumor cells and its inhibition may be a therapeutic approach in the treatment of cancer (35,36,38). This indicates that these 13 genes may be added to the cancer-associated group, adding up to 41 genes.

RT-PCR. Semi-quantitative RT-PCR assays were carried out with a subset of regulated genes to validate data obtained by the microarray studies (Figure 2 and Table IV). All chosen transcripts were decreased in the Zebularine-treated cells as compared to the normal and remethylated populations. Such results correlate well with the microarray data (Table IV, right side), and all showed a decrease in mRNA levels of a least two-fold by Zebularine, validating the use of the microarray technique in this study. The most regulated genes found were IGF1, LSS, INSIG1 and SULF1. IGF1 (Insulin growth factor 1) is a growth factor frequently found associated with the carcinogenic process (39). It binds to specific receptors and triggers cell proliferation, besides inhibiting apoptosis. In opposition to IGF1, SULF1 has been associated with the inhibition of the carcinogenic process (40). SULF1 encodes a heparin-degrading endosulfatase, a cell surface-associated enzyme that diminishes sulfation of heparan sulfate proteoglycans. Many peptide growth factors bind to glycosylated receptors and such binding is inhibited whenever SULF1 levels are high, decreasing the intracellular signaling that leads to cell proliferation (41).

LSS (Lanosterol synthase: 2,3-oxidosqualene-lanosterol cyclase) and INSIG (Insulin-induced gene 1) are genes associated with cholesterol metabolism (42,43). LSS and another 11 down-regulated genes participate in cholesterol biosynthesis, whereas INSIG1, SREBF1 and SREBF2 that were also down-regulated, in at least one of the experiments, are associated with the detection of intracellular cholesterol levels and the consequent genetic regulation of cholesterol synthesis enzyme transcription (42). The enzymes involved in cholesterol biosynthesis are shown in Table III (identified by an asterisk).

The cholesterol biosynthesis pathway is important not only for the generation of cholesterol and steroids, but also of isoprene polymers. Considering the 7 enzymes involved in the metabolism of acetyl-CoA to isoprene polymers, up to the synthesis of geranyl pyrophosphate, 4 are down-regulated at the mRNA level by Zebularine. From the conversion of geranyl pyrophosphate to cholesterol, yet another 8 enzymes are silenced. Among these enzymes are HMG-CoA reductase and squalene synthase, also known as Farnesyl-diphosphate farnesyltransferase or Squalene monooxygenase; both of these proteins are considered key enzymes in cholesterol biosynthesis (44). All of the 11 cholesterol biosynthesis enzyme genes regulated by Zebularine treatment contain CpG islands in their promoters, an indication that methylation may indeed be involved in mRNA-level regulation. Furthermore, one of the most down-regulated genes, INSIG-1, along with SREBP1 and SREBP2, all of which contain CpG islands, are important in the detection of cellular cholesterol levels and the transcriptional control of cholesterol-metabolism genes by steroids (42).

Promoter analysis of up- or down-regulated genes by methylation. The commonly accepted model of promoter methylation predicts that it causes inhibition of gene transcription. We compared the promoter sequences from sets of genes up- or down-regulated by Zebularine in order to identify CpG-rich CIS elements that might allow us to explain the opposite regulatory effects caused by DNA methylation on these gene groups. The CIS elements
Table V. Comparison of CIS element frequency of promoters from up- and down-regulated genes.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CAC-binding_protein</td>
<td>GGTGG</td>
<td>289</td>
<td>483</td>
<td>30.8</td>
<td>9.4</td>
<td>R04295</td>
<td>+</td>
</tr>
<tr>
<td>H1TF2</td>
<td>CCAAT</td>
<td>94</td>
<td>52</td>
<td>12.3</td>
<td>7.6</td>
<td>R00660</td>
<td>+</td>
</tr>
<tr>
<td>Sp1</td>
<td>GCCCGGCC</td>
<td>120</td>
<td>71</td>
<td>16.8</td>
<td>7.1</td>
<td>R00224</td>
<td>+</td>
</tr>
<tr>
<td>Pit-1</td>
<td>TAAAT</td>
<td>46</td>
<td>29</td>
<td>6.9</td>
<td>6.7</td>
<td>R00690</td>
<td>-</td>
</tr>
<tr>
<td>NF-E</td>
<td>CCAAT</td>
<td>63</td>
<td>43</td>
<td>10.2</td>
<td>6.2</td>
<td>R00561</td>
<td>+</td>
</tr>
<tr>
<td>NF-Y</td>
<td>ATGGG</td>
<td>62</td>
<td>43</td>
<td>10.2</td>
<td>6.1</td>
<td>R01081</td>
<td>+</td>
</tr>
<tr>
<td>SRF</td>
<td>CCAAT</td>
<td>63</td>
<td>49</td>
<td>11.6</td>
<td>5.4</td>
<td>R00039</td>
<td>?</td>
</tr>
<tr>
<td>CCAAT-binding_factor</td>
<td>ATGGG</td>
<td>63</td>
<td>49</td>
<td>11.6</td>
<td>5.4</td>
<td>R00231</td>
<td>+</td>
</tr>
<tr>
<td>CBF-B</td>
<td>ATGGG</td>
<td>63</td>
<td>49</td>
<td>11.6</td>
<td>5.4</td>
<td>R00232</td>
<td>?</td>
</tr>
<tr>
<td>CP1</td>
<td>CCAAT</td>
<td>63</td>
<td>49</td>
<td>11.6</td>
<td>5.4</td>
<td>R00564</td>
<td>+</td>
</tr>
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Down-regulated genes according to the 4 criteria mentioned in Table III and that contain CpG islands detected by the use of the USCG Genome Browser (http://genome.ucsc.edu/cgi-bin/hgBlat?hgsid=30306595) (32) and CpGProD (CpG Island Promoter Detection) algorithm (http://phil.univ-lyon1.fr/software/cpqporgd_query.html) (33), found within the first 1,500 bp upstream from the transcription initiation site were selected and the regions containing the CpG islands which were collectively screened for the presence of CIS elements using SIGSCAN Version 4.05 suite (http://bimas.dct.nih.gov/molbio/signal/) (34). The expected number of CIS elements for a given stretch of DNA, based on the random probability of their existence, considering an equal frequency of nucleotides, the length of the promoter region and the length of the consensus were subtracted from the actual number of CIS elements found and correspond to 'correct.' In other words, a 4 nucleotide consensus is expected to be found at every 44 or 136 bases; if the DNA stretch is of, say, 1360 bp, then the chance of randomly finding 10 consensi is high. If instead of 10, 4 0 consensi were found for that given CIS element, than the column labeled "correct." corresponds to 30 (40-10). This Table used data also from genes found to be up-regulated in the presence of Zebularine (further details in 18). As the number of genes picked from the up-regulated group (190) is greater than that of the down-regulated group (45), a correction factor of 45/190 was used for the up-regulated group in order to enable the comparison of the frequency between the up- and down-regulated gene groups (up-reg. normalized for # of genes); column 6. The first column contains the name of the CIS element, the second, the CIS element consensus sequence; column 6, to the ratio between the corrected frequency of a given CIS element in the down-regulated genes by the corrected and normalized value found in the up-regulated genes; column 7, to a reference number given by the CIGSCAN software; and column 8, to the histogram distribution of the position of the CIS elements (+ corresponds to CIS elements concentrated upstream of →
present in the sequences of regulated promoters containing CpG islands were identified using the SIGSCAN Version 4.05 suite (http://bimas.dctr.nih.gov/molbio/signal/) (34). As shown in Table V, many CIS elements differ in frequency when compared among the up- and down-regulated gene collections, reaching up to 9.4-fold higher frequency in down-regulated genes than in up-regulated genes. The positions of the CIS elements were also analyzed, as shown in Figures 3 and 4. Figure 3A corresponds to the overall distribution of the CIS element positions and indicates that, in general, genes that are up-regulated by methylation, CIS elements are most frequently found closer (up to 500 bp) to the transcription initiation site as compared to down-regulated genes. Figure 3B corresponds to a similar analysis performed exclusively with Sp1 CIS elements. In this case, the up-regulated genes are also found most frequently within the 500 bp immediately upstream from the transcription initiation site, whereas the Sp1 CIS-elements of the down-regulated genes show a clear bimodal distribution, concentrating themselves, nevertheless, upstream from the 500 bp closest to the transcription initiation site. Other histograms were generated for the distribution of the CIS elements shown in Table V. Two examples are found in Figures 4A and 4B, respectively. The latter CIS elements, corresponding to MAZ and one of the Sp1 consensi, show the same overall pattern as the one observed in Figures 3A and 3B, that is, there is a greater frequency of the CIS elements of up-regulated genes closer or within the basic promoter, whereas CIS elements from down-regulated

Figure 3. A. Histogram of overall CIS element distribution of up- and down-regulated genes. The distribution of the positions of CIS elements with consensi of at least 4 bp within promoter regions corresponding to CpG islands is shown. The value "0" corresponds to the transcription initiation site. Two histograms are show in parallel: one corresponding to down-regulated genes (white) and another, to up-regulated genes (black).

B. Histogram of overall Sp1 CIS element distribution of up- and down-regulated genes. The distribution of the positions of CIS elements recognized by the Sp1 transcription factor with consensi of at least 4 bp within promoter regions corresponding to CpG islands is shown. The value "0" corresponds to the transcription initiation site. Two histograms are show in parallel: one corresponding to down-regulated genes (white) and another, to up-regulated genes (black).
genes are found further upstream. Other CIS elements with similar patterns are identified with a "+" sign in Table V; opposite patterns are identified with a "-" sign, and irregular patterns, with a "?" sign.

Discussion

The overwhelming bulk of the literature concerning epigenetic changes in gene expression addresses genes that
are up-regulated upon demethylation, based on the concept that cytosine methylation attracts many proteins, including histone deacetylases, which cause changes in chromosome structure, impeding the access of the basic transcription factors, and of the RNA polymerase II machinery to the promoter. Here, we present data that characterize the genes that are down-regulated upon treatment with the methyltranferase inhibitor Zebularine, but which return to their previous expression levels after Zebularine washout in the presence of IL-6. Nevertheless, some reports have found that demethylation sometimes leads to the down-regulation, often to the same level, i.e., the number of regulated genes and gene-fold change, as well as up-regulation of gene expression, which is not compatible with the model described above (45). Alternatively, transcriptional inhibition could be attributed to indirect causes, such as the up-regulation of inhibitory transcription factors (previously silenced by methylation) or of any genes coding for inhibitory proteins involved in signaling pathways. We did observe, from our restriction landmark assay, data indicating that one down-regulated gene (ESR1) is in fact demethylated upon Zebularine treatment, and remethylated post-removal of the drug, in the presence of IL-6.

Computer analysis predicted the presence of CpG islands, frequent targets of methylation, in the majority of the genes down-regulated by Zebularine. The analysis of the distribution of CIS elements within the CpG islands of genes up-regulated by demethylation and those of down-regulated genes, indicates that the overall presence and localization of CIS elements from these two gene populations differ considerably. In order to explain this apparent contradiction, we propose a model (Shown in Figure 5) wherein methylation causes gene silencing when it occurs in regions associated with the basic promoter and with enhancer elements, whereas methylation may cause gene up-regulation when it occurs in regions containing inhibitory CIS elements upstream from the basic promoter. In the latter case, demethylation would cause the exposure of silencer regions that would then inhibit gene transcription. This two-way model is compatible with the preferential methylation patterns found in cancer cell genes, as methylation seems to preferentially silence tumor-suppressor and anti-apoptotic genes, while up-regulating the transcription of tumorigenic genes.

Upon the analysis of microarray results obtained with control, Zebularine-treated and remethylated cells, we previously found that 9 probe-sets (8 containing CpG islands) from the Affymetrix U133A chip and 2 probe-sets (neither containing a CpG island) from the Affymetrix U133B chip are up-regulated in the Zebularine-treated population (19). Such analysis was done based on 4 criteria: genes had to be significantly present in the Zebularine group; the difference between control versus Zebularine and Zebularine versus remethylated samples had to be significant (p<0.05); such difference had to be of at least 2-fold; and, finally, such criteria had to apply to two groups of RNA obtained from independently-treated cells. Using similar selection criteria, but altering the requirement of minimal gene expression in the control and remethylated populations instead, we showed that the probe sets associated with gene down-regulation upon Zebularine treatment are more consistent: 73 (79% containing CpG islands) and 18 (62.5% containing CpG islands) probe sets were significantly selected from microarray chips U133A and U133B, respectively (19). The comparison of these previously-obtained data, related to less stringent selection criteria with the results presented herein shows that, overall, there are similar numbers of probe-sets up- or down-regulated by Zebularine. Moreover, given that the percentage of genes with CpG islands is similar in both groups, there is an indication that methylation may indeed be the direct cause of most cases of gene-regulation, whether increasing or decreasing it. Some of the genes hereby identified have already been associated with methylation and cancer, such as GPC4 glypican 4, TGFB2, INSIG1 and EGR1.

Epigenetic silencing by methylation is considered an important step in the carcinogenic process, leading to the decreased expression of genes that inhibit tumor growth, such as tumor suppressor and apoptotic genes. Interestingly, our results indicate that methylation-induced gene up-regulation may also be important in the process of cell transformation and cancer, as 28 of the down-regulated genes have already been shown to be cancer-promoting, as opposed to 10 that are found to inhibit carcinogenesis (Tables IIIa-c). Another 13 genes are involved in cholesterol metabolism, which has been linked with tumorigenic states, indicating that multiple myeloma cells such as KAS 6/1 may have undergone, throughout their transformation process, changes in “housekeeping” gene methylation patterns in such a way as to favor pro-carcinogenic genes and repress tumor-suppressor genes (46,47).

Although little importance is usually given to the regulation of metabolism genes during carcinogenesis, the high frequency of genes associated with cholesterol metabolism that appear to be regulated by methylation in KAS 6/1 multiple myeloma cells cannot be disregarded (46). Eleven genes of the biosynthetic pathway of cholesterol synthesis were epigenetically regulated, including two key enzymes, HMG-CoA reductase and squalene synthase. All such enzymes have CpG islands in their promoters, which is not uncommon for housekeeping genes. Furthermore, 3 other genes, that were found to be down-regulated by Zebularine, are associated with intracellular levels of cholesterol and its metabolites. These are INSIG1 (48), SREBP1 (49) and SREBP2 (50). The current model for cell
<table>
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<td>A</td>
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<td>Up-regulation</td>
<td>Methylating (tumorigenic)</td>
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<td>Yes (tumorigenic)</td>
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detection and control of cholesterol levels involves these 3 proteins that, in conditions of high intracellular cholesterol levels, are in the form of complexed transmembrane proteins that are retained in the endoplasmatic reticulum. Upon decrease in cholesterol levels, SREBP1 and SREBP2, complexed to SCAP, a protease, dissociate from INSIG1 and move towards the budding portion of the endoplasmatic reticulum, whereupon SREBPs are sequentially cleaved by two proteases, releasing a soluble transcription factor moiety which migrates to the nucleus, binds to sterol-responding elements (SREs) and promotes transcription of the genes involved in cholesterol biosynthesis (51).

The question remains as to why KAS 6/1 cells over-express cholesterol-related genes. Many authors have shown that cholesterol biosynthesis and that of its intermediates is increased in tumor cells, including multiple myelomas (59). The key enzyme in cholesterol biosynthesis, HMG-CoA reductase, is frequently overexpressed in tumors and is not properly regulated by its inhibitors (51). A correlation has been found between the use of statins, cholesterol-lowering drugs that inhibit HMG-CoA reductase, and the decreased chance of development of cancer (47). Diets rich in fiber are known to decrease circulating cholesterol levels, and are coincidently associated with decreased incidence of cancer (52). The importance of cholesterol itself in cancer cells can be attributed to the need for this material in the construction of new membranes for ongoing cell proliferation. Cholesterol is particularly important in the formation of lipid rafts, where signaling proteins, frequently associated with the stimulation of proliferation and the inhibition of apoptosis, concentrate, along with proteins important in cellular adhesion (53). Moreover, some cancer cells shed large amounts of vesicles, some of which are particularly rich in cholesterol (54). Nevertheless, it is believed that cholesterol itself is not the key molecule important in the carcinogenic process, but some of the intermediates of its synthesis, particularly isoprenoids, essential for protein modification by prenylation and geranylgeranylation (55). Many authors have shown that cholesterol biosynthesis and that of its intermediates is increased in tumor cells, including multiple myelomas (56). Such post-translational modifications confer a hydrophobic moiety to proteins, which can then migrate and bind to the cell surface, interacting with other membrane proteins. An important protein family that depends upon such post-translational modifications to promote cancer are the small G-proteins, with prototypic members such as Ras (56). Therefore, by stimulating cholesterol biosynthesis, cancer cells can increase not only the levels of a main membrane component, but also promote proliferation.

Our data indicated that treatment of tumor cells with a demethylating drug is capable of altering gene expression, and that genomic demethylation manifests itself in both epigenetic up-, and down-regulation of gene expression. To explain these observations, we proposed a model in which we observed, by in silico sequence analysis, that the potential methylation target regions in the different promoters of the up-regulated and down-regulated genes differed. We noted that the constitutively up-regulated genes were probably inhibited due to methylation of silencer regions, while sequences involved in basic transcription were not methylated; whereas down-regulated genes were methylated around their basic promoter and/or in other transcription-promoting regions (Figure 5). Such a hypothesis can be sustained by CIS element mapping of the promoter regions of up- and down-regulated genes. In general, methylation-silenced genes have a greater frequency of CIS elements surrounding their basic promoter. This distribution is observed in the majority of CIS elements analyzed individually: 22 CIS elements are preferentially found upstream from the basic promoter in the genes up-regulated by methylation as compared to genes down-regulated by methylation, which have such CIS elements concentrated around the first 500 bp upstream of the transcription initiation site. On the other hand, only 6 CIS elements had the opposite distribution. The remainder of the genes had irregular patterns of CIS elements, and did not lend themselves to simple interpretation.

### Figure 5. Alternative Epigenetic Down-regulation of Gene Expression Model

The left portion of Box A corresponds to the traditional view of gene regulation by methylation. A gene region containing an enhancer sequence, the basic promoter (from 0 to –200 bp), the hnRNA coding region and the protein coding region are depicted. Our data suggests that whenever DNA methylation occurs close to the promoter region, the methylated DNA provokes the formation of a protein complex (trapezoid, hexagonal and triangular figures) that hinders the binding of the basic transcriptional machinery, including RNA polymerase. In this situation, gene transcription is decreased. The right portion of Box A shows what happens after methylation is inhibited by Zebularine; the basic transcriptional machinery (round or oblong figures) can bind and promote transcription, which may be increased by other transcription factors (round or oblong figures) bound to enhancer regions, but previously blocked due to methylation and methylated CpG binding factors. Box B (below) depicts a gene containing a silencer region, plus the hnRNA and the protein coding regions. Methylation, in this case, may occur upstream from the basic promoter, within the silencer region. In this situation, after the formation of protein complexes over the methylated DNA, silencing transcription factors cannot bind and are unable to inhibit DNA transcription, allowing the basic transcriptional machinery to work. After Zebularine treatment, demethylation of putative silencing regions allows the binding of inhibitory transcription factors that inhibit DNA transcription. The table below Boxes A and B summarizes the types of genes which could be regulated in these two different fashions, their relationship with the carcinogenic process, the effect of IL-6, and the potential therapeutic effect of Zebularine treatment.
In order to confirm our hypothesis, it would be necessary to show that the regions targeted by methylation inhibitors in the constitutively up-regulated genes correspond to silencer elements. Among the CIS elements that reside up-stream from the basic promoter in genes that are down-regulated upon Zebularine treatment are several known silencers, or elements that can act as gene silencers in some genes, by binding such factors as MAZ (57), Sp1 (58), CAC-binding protein (59), NF-E (60), LF-1 (61), and CIS elements bound by uncharacterized transcription factors (62,63). The phenomenon of methylation targeting to specific sequences (CpGs for example) is well understood. However, why only certain clusters or groups of CpG motifs are methylated, while others remain clear, is unknown, as the search for such methylation "signatures" has been fruitless thus far. It is possible that some of the silencer CIS elements identified here, and probably others still to be discovered, may aid in the recognition of methylation targeting sites. Tufarelli (64) showed an indirect mechanism of transcriptional silencing by methylation, while Debinski (65) showed that demethylation with 5-aza-2'-deoxycytidine led to the suppression of several cancer genes, including insulin receptor-related receptor (INSRR), ephrin-related receptor tyrosine kinase B3 (EphB3), latent transforming growth factor beta binding protein 4 (LTBP4) and the endoglycan PODXL. Further studies with the identified sequences, along with their positions and orientation within promoters, may aid in the understanding of the methylation process, and in the confirmation of whether gene silencer methylation can lead to gene activation.

In summary, demethylating KAS 6/1 multiple myeloma cells with the methyltransferase inhibitor Zebularine results in both up- and down-regulation of gene expression. Among the regulated genes, most of those associated with up-regulation by methylation are tumor suppressors, whereas genes down-regulated by methylation are tumor or proliferation promoters. However, in some genes, it is likely that the demethylation effects are the result of some indirect mechanism(s). Nevertheless, the array of regulated genes indicates that the treatment of cancer can be aided by the use of demethylating agents such as Zebularine, both by the re-activation of tumor-suppressor and pro-apoptotic genes, and by inhibition of genes that promote oncogenesis.

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