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Limited Gene Activation in Tumor and Normal Epithelial Cells Treated with the DNA Methyltransferase Inhibitor 5-Aza-2′-deoxycytidine

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ABSTRACT

It remains unclear to what extent drugs targeting transcriptional repressor complexes affect global gene expression in cells derived from target and nontarget human tissues. To address this question, we used genome-wide expression analysis using microarrays to analyze the response of three tumor and one normal epithelial cell line to treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-CdR). Notably, we found that 5-aza-CdR treatment induced a limited number of genes (mean, 0.67%; range, 0.17–1.8% of 25,940 genes screened) in each cell line tested. The majority of the gene expression changes that followed 5-aza-CdR treatment were conserved in tumor and normal cells, including genes that function in cell proliferation, differentiation, immune presentation, and cytokine signaling. In contrast, 5-aza-CdR treatment induced the expression of cancer-testis class tumor antigens only in tumor cell lines. To explain this tissue-specific response, we analyzed the mechanism of transcriptional regulation of the prototype member of this tumor antigen gene family, *MAGE-1*. Taken from our analysis of *MAGE-1* gene regulation, we propose that 5-aza-CdR–mediated gene activation has two distinct requirements: 1) the reversal of promoter hypermethylation, and 2) the presence of transcriptional activators competent for the activation of hypomethylated target promoters. This latter requirement for gene activation by 5-aza-CdR is probably mediated by sequence-specific transcription factors and may account for the limited number of human genes induced by 5-aza-CdR treatment. This revised model for gene activation by 5-aza-CdR has important implications for the use of DNA methyltransferase inhibitors in clinical settings.

Aberrant DNA hypermethylation at gene promoters represses transcription and plays a key role in human carcinogenesis (Jones and Baylin, 2002). Recognition of the importance of epigenetic alterations in human cancer has spurred the investigation of DNA methyltransferase inhibitors as cancer therapeutics (Bender et al., 1998b; Karpf and Jones, 2002). One such agent, 5-aza-2′-deoxycytidine (5-aza-CdR), is a potent inhibitor of genomic and promoter-specific DNA methylation (Momparler, 1985; Bender et al., 1998b). Despite the increased interest in DNA methylation as a human cancer drug target, there is controversy in this area derived from mouse genetic studies. Whereas several studies have revealed a strong antitumorigenic effect from crossing DNA methyltransferase I (DNMT1)-deficient mice with mice genetically predisposed to intestinal cancer, other studies have demonstrated that *DNMT1*-targeted mice have an increased incidence of lymphomas (Laird et al., 1995; Eads et al., 2002; Trinh et al., 2002; Eden et al., 2003; Gaudet et al., 2003). It is unclear to what extent these data, which use genetic disruption of the *DNMT1* gene in mice, are relevant for understanding the pharmacological targeting of DNA methylation in humans. However, an important issue raised by these investigations is that there is a need for a more complete understanding of the potential benefits and limitations of DNA methylation as a human cancer drug target. An important component of this understanding will come from a precise evaluation of the molecular effects of 5-aza-CdR (and other DNA methylation inhibitors) in both target (cancerous) and nontarget (normal) human tissues.

As a first step toward this goal, we used DNA microarrays to conduct a genomic analysis of gene expression changes

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ABBREVIATIONS: 5-aza-CdR, 5-aza-2′-deoxycytidine; DNMT1, DNA methyltransferase I; HMEC, human mammary epithelial cells; IFN, interferon; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcriptase-polymerase chain reaction; PCR, polymerase chain reaction; IRF7, interferon regulatory factor 7A; MHC, major histocompatibility complex; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

elicited by 5-aza-CdR treatment in tumor and normal epithelial cell lines. DNA microarrays are particularly well-suited for evaluating the molecular consequences of drug treatment, especially in instances in which the compound under study is believed to act by regulating gene transcription, as is the case for 5-aza-CdR (Karpf et al., 1999). We found that 5-aza-CdR treatment induced a more limited scope of gene expression changes than might be expected for an agent that perturbs a fundamental transcriptional regulatory mechanism. Moreover, many of the genes responsive to 5-aza-CdR treatment were regulated similarly in tumor and normal cells. A number of these genes fell into distinct functional classes, including ones involved in cell proliferation, cell differentiation, antigen presentation, and interferon signaling. In contrast, cancer-testis tumor antigen genes were remarkable in that they were induced only in tumor cell lines. To further dissect this result, we analyzed the mechanisms underlying the activation of prototype tumor antigen gene *MAGE-1* by 5-aza-CdR. Although genomic DNA and the *MAGE-1* promoter were demethylated in both tumor and normal cell lines after treatment with 5-aza-CdR, only tumor cell lines displayed competency for activating an unmethylated MAGE-1 promoter construct. These results suggest a mechanism accounting for the limited extent of gene activation in 5-aza-CdR treated cells. We propose that 5-aza-CdR–mediated gene activation requires both the inhibition of DNA methylation as well as the presence of *trans*-factors that mediate the activation of hypomethylated target genes.

Materials and Methods

Cell Culture and 5-aza-CdR Treatments. Colon cancer cell lines were cultured as described previously (Karpf et al., 2001), and human mammary epithelial cells (HMECs) were cultured as recommended by the manufacturer (Cambrex Bio Science Walkersville, Walkersville, MD). 5-aza-CdR (Sigma, St. Louis, MO) was solubilized in phosphate-buffered saline. Table 1 describes the cell lines and 5-aza-CdR treatments in the experiments used for cDNA microarray analyses. Each cell line was subcultured 2 days after the initial 5-aza-CdR treatment. Because the RKO cell line was less sensitive to 5-aza-CdR treatment (Fig. 1), we added a second microarray experiment using this cell line in which cells were treated with a higher concentration of 5-aza-CdR (Table 1). To control for the ~50% reduced growth rate of HMECs compared with the cancer cell lines (data not shown), we treated these cells with 5-aza-CdR on 2 consecutive days and measured changes in gene expression both at early and later time points (Table 1). To control for gene expression changes coincident with cellular exposure to exogenous nucleosides (not related to the specific activity of 5-aza-CdR), we conducted microarray analyses of HT29 cells and HMECs treated with 2′-deoxycytidine (Sigma) at equimolar concentrations and identical treatment strategies as were used for 5-aza-2′-deoxycytidine. Human metastatic melanoma lines YUSAC 2, YUSIT 1, YUGEN 8, and LOX were maintained as described previously (Grossman et al., 1999). Normal human melanocytes were isolated from discarded foreskins and propagated as described elsewhere (Bowen et al., 2003). Melanoma cell lines were treated once with 5 μM 5-aza-CdR and harvested 72 h later. Because melanocytes displayed a reduced growth rate compared with melanoma cell lines (data not shown), these cells were treated with 5 μM 5-aza-CdR twice and harvested 6 days after the initial treatment.

Western Blotting. Protein extracts from colon cancer cell lines and HMECs were isolated as described previously (Karpf et al., 2001). Protein extracts were quantified using the D_c protein assay (Bio-Rad, Hercules, CA) to ensure equivalent protein loading. Melanoma and melanocyte cell lysates were recovered as described previously (Grossman et al., 1999). Western blotting was conducted as described previously (Karpf et al., 2001). DNMT1 protein was detected using an antibody (TB) kindly provided by Dr. A. Robert MacLeod (MethylGene, Inc. Montreal, Quebec, Canada). α-Tubulin protein was detected using antibody N356 from Amersham Biosciences Inc. (Piscataway, NJ). MAGE-1 protein was detected using antibody MA454 from NeoMarkers (Fremont, CA). Ponceau S total protein stain was obtained from Sigma.

Alu Microsatellite Southern Blot. Genomic DNA was harvested using the PureGene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). DNA samples were digested for 3 h at 37°C with 2 units of HpaII or MspI restriction enzymes (New England Biolabs, Beverly, MA) per 1 μg DNA. After digestion, DNAs were reisolated using Qiaspin columns (QIAGEN, Valencia, CA) and requantified by spectrophotometry. Next, 250 ng of each digested DNA sample was separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Southern blotting was performed as described previously (Sambrook and Russell, 2001), except ULTRAhyb Oligo Hybridization Buffer (Ambion, Austin, TX) was used for hybridization. The Alu microsatellite repeat probe used was the “Alu 3” probe described previously (Kochanek et al., 1993). Oligonucleotide probes were end-labeled using T4 polynucleotide kinase (Invitrogen, Carlsbad, CA) in the presence of [^{32}P]dATP (ICN Biomedicals Inc., Costa Mesa, CA) and were purified over Micro Bio-Spin 6 columns (Bio-Rad) before hybridization.

RNA Extractions and RT-PCR. RNA and mRNAs extractions were performed as described previously (Karpf et al., 1999). cDNAs were synthesized from equivalent amounts of total RNAs using Superscript II Reverse Transcriptase (Invitrogen). The primers used for amplification of MAGE-1 cDNA were: forward, 5′-ACTGCAAGCCTGAGGAAGCC-3′; reverse, 5′-TGGGTTGCCTCTGTCGAGTG-3′. The primers used for amplification of GAPDH cDNA were: forward, 5′ TGAAGGTCGGAGTCAACGGA 3′; reverse, 5′ CCATTGATGACAAGCTTCCCG 3′. p16INK4a RT-PCR was performed as described previously (Xing et al., 1999).

Microarray Construction, Control Elements, and Hybridizations. We used five human cDNA microarrays for gene expression profiling: Human Drug Target (formerly known as Human Genome GEM 1) and Human Foundation 1–4 (formerly known as Human Genome GEM 2–5) (Incyte, Palo Alto, CA). The identity of each cDNA clone was verified by resequencing. We screened a total of 47,650 clones representing 25,940 unique gene/expressed se-

TABLE 1

Cell lines and drug treatments used for DNA microarray experiments

25,940 genes were analyzed using Incyte GEM 1–5 DNA microarrays.

Cell Type	Genotype[a]	Treatment(s)[b]
HT29 colon cancer	p53 −, APC −, MMR +	0.5 μM 5-aza-CdR, day 5
HCT116 colon cancer	p53 +, APC +,[c] MMR −	0.5 μM 5-aza-CdR, day 5
RKO colon cancer	p53 +, APC +, MMR −	0.5 and 2.0 μM 5-aza-CdR, day 5
Human mammary epithelial cells	p53 +, APC +, MMR +	1.0 μM 5-aza-CdR (2×), days 4 and 9

[a] p53, adenomatous polyposis coli, and mismatch repair genotype are indicated (+ indicates wild type).
[b] 5-aza-CdR–treated cells were compared to vehicle (phosphate-buffered saline)-treated cells.
[c] Activating β-catenin mutation.

quence tag clusters, according to an analysis of the LifeSeq Foundation database, release 9, performed on January 28, 2003 (Incyte). Microarrays contained yeast DNA fragments as controls for reverse transcription and to determine hybridization efficiency and detection sensitivity. Human housekeeping genes were arrayed to assess the quality of the mRNA samples. Reverse-transcription reactions and hybridizations were performed as described previously (Yue et al., 2001). All hybridizations were conducted in duplicate for each set of Cy3-dCTP (control) and Cy5-dCTP (5-aza-CdR–treated)-labeled samples.

Microarray Data Acquisition and Analysis. Duplicate microarray experiments were scanned in the Cy3 and Cy5 channels with GenePix scanners (Axon Instruments Inc., Union City, CA) at 10-μm resolution. The signal was converted at a resolution of 16 bits per pixel, yielding a dynamic range of 65,536. Background-subtracted element signals were used to calculate Cy3:Cy5 ratios. Spots were analyzed only if they met the following criteria: 1) at least one reading (Cy3 or Cy5) had a signal to background ratio $\geq$2.5; 2) at least one reading had a signal intensity >250 units; and 3) the spot size was $\geq$40% of the spotted area. Differential gene expression values for each element were calculated as $\log_2$ (Cy5 signal/Cy3 signal). To meet the criteria for differential expression, we required that a given clone showed at minimum 1.8-fold (0.85 on a $\log_2$ scale) expression change (mean of the duplicate experiments) in at least one of the six experiments analyzed. The 1.8-fold limit corresponds to a tolerance interval of 99.5% for 99% of the elements (Yue et al., 2001). In addition, any clone showing greater than 60% coefficient of variation was removed from further analysis. Both induced and repressed genes that met these criteria for differential expression were included in our analysis. In control experiments using 2′-deoxycytidine, only 11 genes showed significant changes in gene expression, and these were excluded from further analysis (data not shown). Clones meeting each of the criteria for differential expression were clustered using a hierarchical clustering algorithm (Ward's method, data standardized) with JMP statistical discovery software (SAS Institute, Inc., Cary, NC).

Sodium Bisulfite DNA Sequencing of *MAGE-1* Promoter. Genomic DNAs were isolated as described above and were chemically converted with sodium bisulfite using the CpGenome DNA Modification Kit (Serologicals Corp., Norcross, GA). We amplified the 5′-CpG island of the MAGE-1 gene (−105 to +227 relative to the transcription start site) using primers similar to those described previously (De Smet et al., 1999). PCR products were directly cloned into the PCR 2.1-TOPO vector (Invitrogen) and individual clones were sequenced in both directions using the ABI Prism (DNA sequencing facility, University of Utah School of Medicine, Salt Lake City, UT). Between 10 and 15 independent clones were sequenced for each sample type.

Cell Transfections and Promoter-Luciferase Assays. The MAGE-1 5′-CpG island (−96 to +223 relative to the transcriptional start site) was cloned into the pGL3 basic vector (Promega, Madison, WI). The MAGE-1 promoter construct was methylated in vitro with HpaII methyltransferase (New England Biolabs) according to the manufacturer's instructions. Mock methylation was accomplished by incubating plasmids in the reaction mix in the absence of enzyme. All data accumulated using the methylated MAGE-1 promoter construct were normalized to the effect of HpaII methylation on the pGL3-promoter construct (Promega). This construct consists of the Firefly luciferase gene driven by the simian virus 40 promoter, which does not contain HpaII methyltransferase acceptor sites (Maier et al., 2003). Luciferase assays were conducted using the dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. In each transfection, Firefly luciferase activity was normalized to the activity of an RSV-Renilla luciferase expression control (Promega). All luciferase assay transfections were performed in triplicate in 24-well plates using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay. Nuclear protein extracts were isolated as described previously (Wang et al., 1995) and were quantified as described above for Western blotting. A 20-μg sample of each nuclear extract was incubated for 20 min at room temperature with radiolabeled probe in the presence of binding buffer [1 μg of poly(dI-dC), 15 mM HEPES, 0.5 mM EDTA, 1 mM dithiothreitol, 30 mM KCl, and 5% glycerol], with or without 25× unlabeled competitor oligonucleotides. Probes were labeled with [^{32}P]dATP as described above for Southern blotting. For each sample, 40,000 cpm of purified labeled oligonucleotides was used. Protein/DNA complexes were resolved through 5% polyacrylamide gels

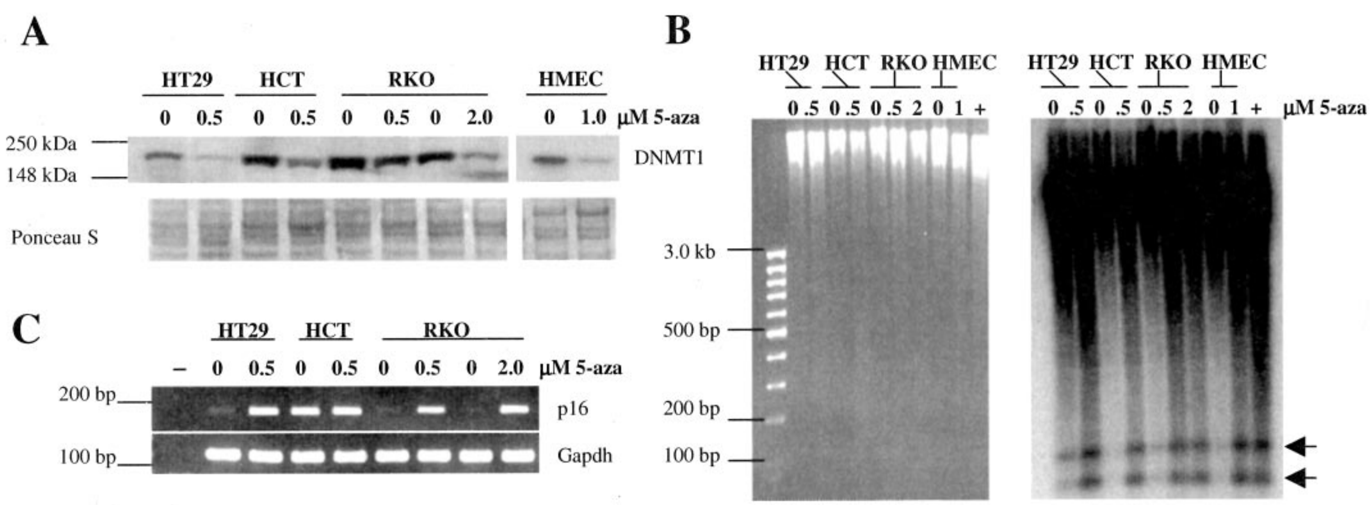

Fig. 1. Inhibition of DNA methylation in 5-aza-CdR–treated tumor and normal epithelial cells. A, DNMT1 levels in soluble nuclear protein extracts. Cells were treated with 5-aza-CdR once (HT29, HCT, and RKO) or twice (HMEC) at the indicated concentrations, and soluble nuclear protein extracts were isolated either 5 (HT29, HCT, and RKO) or 7 (HMEC) days later and used for Western blot analysis. Ponceau S staining of the blot confirmed equivalent protein loading. B, Alu microsatellite DNA methylation. Cells were treated with 5-aza-CdR as in A, and Southern blot analysis was conducted as described under *Materials and Methods*. Ethidium bromide staining (left) and Alu Southern blotting (right) of HpaII-digested genomic DNAs are shown. Two low molecular mass bands (arrows) corresponding to HpaII-digestion products of unmethylated Alu microsatellite repetitive sequences are indicated. +, a control digestion using MspI, a methylation-insensitive isoschizomer of Hpa II. C, p16INK4a gene expression. Tumor cell lines were treated with 5-aza-CdR as in A, and RNAs were isolated and used to determine the expression of p16INK4a by RT-PCR. GAPDH amplification confirmed equivalent cDNA input. −, no template cDNA.

for at least 4 h at 250 V. The probe corresponded to the −59 to −35 region of the *MAGE-1* promoter relative to the transcription start site (the "B" probe) (De Smet et al., 1995). The sequence of this probe is as follows: 5′-GGGTGCCCGGATGTGACGCCACTGA-3′ MAGE-1 promoter (top strand), and 5′-TCAGTGGCGTCACATCCGGGCA-CCC-3′ MAGE-1 promoter (bottom strand), where the underlined C indicates the residue that was synthesized either with dC or d5mC. A nonspecific nuclear factor-κB competitor oligonucleotide was obtained from Promega.

Results

Global Gene Expression Analysis of 5-aza-CdR–Treated Tumor and Normal Cells. We used cDNA microarrays to analyze gene expression changes in four human cell types after treatment with 5-aza-CdR. We used three colon adenocarcinoma cell lines (HT29, HCT116, and RKO) and one normal cell type (HMECs) (Table 1). HMECs are a well-established normal epithelial cell model and are, therefore, appropriate for measuring 5-aza-CdR–induced gene expression changes in nontarget (normal) epithelial cells. Any gene expression changes observed after 5-aza-CdR treatment of HMECs should be unrelated to the reactivation of genes or pathways silenced by DNA methylation during tumorigenesis. Cells were treated with 5-aza-CdR as described in Table 1 and mRNA was harvested for microarray analyses. To assess the efficacy of 5-aza-CdR treatment for inhibiting DNA methyltransferase in the cell lines, we analyzed three parameters: 1) the level of DNMT1 protein in soluble nuclear extracts (Fig. 1A); 2) the extent of methylation of Alu microsatellite repeats in genomic DNA (Fig. 1B); and 3) the reactivation of the known methylation-silenced gene *p16INK4a* (Fig. 1C). Each of these assays verified that DNA methylation was strongly inhibited in the experiments used for microarray analyses. Note that monoallelic expression of *p16INK4a* in HCT116 cells before5-aza-CdR treatment was expected (Myohanen et al., 1998).

A total of 1390 genes (5.3%) were differentially expressed after 5-aza-CdR treatment in one or more of the six microarray experiments delineated in Table 1. Figure 2A presents a dendrogram grouping the patterns of differential gene expression in the six microarray experiments taken from hierarchical clustering of the 1390 differentially expressed genes. As expected, the two RKO comparisons (differing in drug concentration) and the two HMEC comparisons (differing in time of treatment) each clustered close together (Fig. 2A). The overall proportion of differentially expressed genes in individual experiments ranged from 0.49 to 2.5% (Fig. 2B), whereas the proportion of genes that were induced ranged from 0.17 to 1.8%. Increasing treatment length (in HMECs) or drug concentration (in RKO cells) resulted in an approximate 3-fold increase in the number of differentially expressed genes (Fig. 2B).

Functionally Related Gene Clusters Differentially Expressed in Tumor and Normal Cells after Treatment with 5-aza-CdR. Our data indicated that a number of functionally related gene clusters were differentially expressed in cell lines after 5-aza-CdR treatment. Consistent with our previous observations (Karpf et al., 1999), we found that interferon (IFN) response genes were activated (Fig. 3A). This response occurred in the HT29, HCT116, and HMEC cell lines but was absent in RKO cells (Fig. 3A). The IFN responsive genes activated by 5-aza-CdR treatment can be separated into three general categories: 1) those whose classic function is to respond to virus infection (*2′-5′oligoadenylate synthetase*, *MX-1*, and *PKR*); 2) transcription factors that control the activation status of the IFN pathway [*STAT1/ISGF-3* and *interferon regulatory factor 7A (IRF7)*]; and 3) downstream IFN-pathway target genes (e.g., *interferon-inducible proteins 9–27* and *35*). We previously suggested that the activation of the IFN pathway after 5-aza-CdR treatment might result from the reactivation of methylation-silenced transcription factors mediating IFN responsiveness (Karpf et al., 1999). Our microarray data (Fig. 3A) suggested *STAT1* and *IRF7* as candidates for this type of mechanism. However, whereas Northern and Western blot analyses confirmed the induction of STAT1 and IRF7 by 5-aza-CdR treatment, methylation-specific PCR analysis of the promoter of these two genes failed to detect promoter methylation either before or after 5-aza-CdR treatment in tumor cell lines (data not shown). Moreover, IFN-pathway activation in HMECs after 5-aza-CdR treatment argues against this model. Alternatively, the activation of the IFN pathway after 5-aza-CdR treatment may be related to the induction of endogenous retroviruses and retroviral-like elements. Consistent with this idea, we found evidence for the activation of these elements in 5-aza-CdR–treated cell lines (Fig. 3B).

Many of the genes differentially expressed after 5-aza-CdR treatment in both tumor and normal cell lines were down-regulated (Fig. 2B). Among these were a number of genes associated with cell proliferation, including *Cdc2*, *PCNA*, *DNA topoisomerase II*, *c-myc*, *Ki-67*, *tubulin*, and *eukaryotic translation factors* (Fig. 3C). A second large group of down-regulated genes encoded ribosomal proteins (Fig. 3D). Both of these responses were conserved across the four cell types tested. In contrast, a number of gene clusters showed more

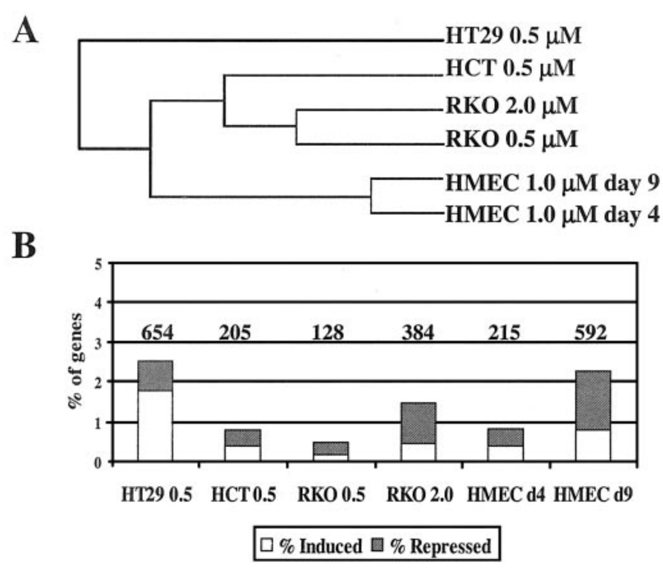

Fig. 2. Global gene expression response of 5-aza-CdR–treated tumor and normal epithelial cells. A, dendrogram outlining the hierarchical relationship between differentially expressed gene patterns obtained in the six microarray experiments described in Table 1. Clustering was performed as described under *Materials and Methods*. B, the number of induced and repressed genes in the microarray experiments listed in Table 1 was quantified and is graphed as a proportion of the number of genes screened (25,940). Differential gene expression was determined as described under *Materials and Methods*. The total number of differentially expressed genes in each experiment is indicated above the bars.

cell-specific responses to 5-aza-CdR. For example, 5-aza-CdR treatment induced classic markers of epithelial cell differentiation, including *keratin* and *cytokeratin* genes and *vimentin* (Fig. 3E). The activation patterns of differentiation markers showed a high degree of cell-type specificity; especially notable was the distinction between the pattern in HMECs versus the pattern in the colon cancer lines (Fig. 3E). In addition, 5-aza-CdR treatment elicited the activation of *MHC class I* genes and the class I light-chain molecule *β-2 microglobulin* (Fig. 3F). This particular response was absent in the HCT116 cell line (Fig. 3F).

Functionally Related Gene Clusters Uniquely Expressed in Tumor Cells after Treatment with 5-aza-CdR. We identified one large cluster of functionally related genes activated specifically in the tumor cell lines after 5-aza-CdR treatment. This cluster consisted of genes encoding tumor antigens, including *MAGE*-, *GAGE*-, *PAGE*-, and *XAGE-type cancer-testis antigens* (Fig. 4A). This response was remarkable for three reasons: 1) the level of gene induction was robust, 2) it occurred in all three tumor cell lines tested, and 3) it was absent in HMECs. These data led us to examine 5-aza-CdR–mediated activation of tumor antigen genes in more detail. Initially, we examined the regulation of *MAGE-1* as a representative member of this gene cluster because *MAGE-1* is the prototype and founding member of the cancer-testis tumor antigen gene family (van der Bruggen et al., 1991), and it has previously been shown to be regulated by DNA methylation (Weber et al., 1994; De Smet et al., 1996; Serrano et al., 1996). At the outset, we confirmed that *MAGE-1* expression was induced by 5-aza-CdR treatment in tumor cell lines but not in HMECs, at both the mRNA (Fig. 4B) and the protein levels (Fig. 4C).

Mechanism of *MAGE-1* Gene Regulation by 5-aza-CdR. An important earlier study found a link between genomic DNA hypomethylation and *MAGE-1* gene expression (De Smet et al., 1996). Thus, it was surprising that

A

Genbank ID	Gene Name	HT29 0.5 μM	HCT 0.5 μM	RKO 0.5 μM	RKO 2.0 μM	HMEC day 4	HMEC day 9
2281070	STAT1/ISGF-3	1.76	0.61				0.84
2098580	interferon regulatory factor 7A	1.62	0.64				
338651	2'5' oligoadenylate synthetase	2.9				1.39	1.29
5410450	MX1	1.28				0.75	0.92
189505	PKR	1.48	0.7				
186559	ISG-54K	2.38	0.89			0.61	0.78
33917	gamma-interferon inducible	1.62				0.57	
35183	ISG12	4.07	2.74			3.62	4.31
184570	interferon-induced 17-kDa/15-kDa	1.72	0.83				
177801	interferon-inducible protein 9-27	4.02	1.17			0.51	0.83
12655016	interferon-induced protein 35	1.08				0.78	0.62
11065720	IFRG28	0.96					

B

Genbank ID	Gene Name	HT29 0.5 μM	HCT 0.5 μM	RKO 0.5 μM	RKO 2.0 μM	HMEC day 4	HMEC day 9
257086	endogenous retroviral RTVL-H2					0.95	0.92
2290551	endogenous retrovirus-H LTR					0.9	0.92
2104909	endogenous retrovirus H	0.65				1.06	1.09
332612	pol polyprotein	1.14	0.82				
2226003	Tigger1 transposable element	1.19	0.79				
35995	pTR5 repetitive sequence	1.6	1.09		0.94		
35996	pTR7 repetitive sequence	2.09	1.5		0.87		
3808035	trinucleotide repeats region	1.85	1.16		1		

C

Genbank ID	Gene Name	HT29 0.5 μM	HCT 0.5 μM	RKO 0.5 μM	RKO 2.0 μM	HMEC day 4	HMEC day 9
29840	CDC2				-0.98	-1.41	-1.49
12653440	PCNA	-0.51			-0.7	-0.91	-0.74
292829	DNA topoisomerase II	-1.01	-0.6	-0.54	-0.96	-2.11	-2.41
34815	c-myc	-0.86	-0.78		-0.74	-0.56	-1.29
415818	Ki-67	-0.99	-0.65	-0.84	-1.61	-1.9	-3.03
14424571	tubulin alpha 1	-0.87	-0.69	-0.78	-0.54	-0.61	-0.6
31099	elongation factor-1-beta				-0.55	-0.52	-1.13
31103	elongation factor-1-gamma	-1.26	-0.86		-0.93	-0.53	-1.22
12653438	translation initiation factor 3	-1.38	-0.79	-0.59	-0.68	-0.66	-1.16
12006048	EF1a-like protein	-1.28	-0.7		-0.55		-1.17
8980659	SNF2-like protein		-0.62	-0.59	-0.99	-1.18	-1.25

D

Genbank ID	Gene Name	HT29 0.5 μM	HCT 0.5 μM	RKO 0.5 μM	RKO 2.0 μM	HMEC day 4	HMEC day 9
292434	ribosomal protein L26	-0.79	-0.57		-0.86		-0.94
36147	ribosomal protein S6	-1.1			-0.94		-0.98
1754632	ribosomal protein S13	-0.96			-0.82		-0.98
337507	ribosomal protein S25	-0.9	-0.57		-0.88		-0.86
607794	ribosomal protein L9	-1.1	-0.71	-0.57	-0.9	-0.54	-0.89
3077778	ribosomal protein L37	-1.02	-0.56		-0.68		-0.74
35569	polyA binding protein	-1.09	-0.67		-0.78		-0.67
14043525	ribosomal protein L4	-1.25	-0.65				-1.02
12803646	ribosomal protein S27	-0.7					-0.87
495125	ribosomal protein L11	-0.89			-0.81		-0.63

E

Genbank ID	Gene Name	HT29 0.5 μM	HCT 0.5 μM	RKO 0.5 μM	RKO 2.0 μM	HMEC day 4	HMEC day 9
12653736	keratin 8		0.93	0.59	0.73	0.62	
7339829	keratin 18		1.37		0.78		
402644	cytokeratin 20	1.35	0.52				
4321794	keratin 16					1.24	1.25
3901029	cytokeratin type II					1.07	0.86
34070	cytokeratin 15		0.52			-0.7	-1.04
13111800	vimentin		1.72	3.21	3.67		-0.63

F

Genbank ID	Gene Name	HT29 0.5 μM	HCT 0.5 μM	RKO 0.5 μM	RKO 2.0 μM	HMEC day 4	HMEC day 9
699597	HLA-A26	1.07			0.52	0.64	1.02
187600	MHC class I	1.22			0.73		0.85
32234	HLA class I locus C	1.75		0.56	0.9		1.21
342845	MHC class I protein	1.26		0.55	0.84		0.82
784923	HLA-B*1301	1.57		0.81	1.1	0.58	1.27
5926695	HLA Class I section 7/20	1.55			0.77		0.99
5926708	HLA Class I section 18/20	1.37			0.6		0.92
13186098	HLA-B	1.63			0.84		1.06
187783	HLA-C-alpha-2	1.48		0.53	0.83		1.03
188538	HLA G	1.36			0.72		0.95
342847	beta-2-microglobulin	1.28					1.25

Fig. 3. Functionally related gene clusters differentially expressed in 5-aza-CdR–treated tumor and normal epithelial cells. Common gene names and GenBank identification numbers are indicated. The six columns indicate the six microarray comparisons described in Table 1. Imbedded in the graph are the $\log_2$ expression values of differentially expressed genes. Negative and positive numbers indicate gene repression and gene activation after 5-aza-CdR treatment, respectively. Black squares indicate that gene expression was unchanged in that experiment. The degree of intensity of green or red indicates the degree of gene repression and gene induction, respectively. A, interferon response gene cluster. B, endogenous retrovirus and repetitive DNA element gene cluster. C, cell proliferation-related gene cluster. D, ribosomal protein gene cluster. E, epithelial cell differentiation marker gene cluster. F, MHC I gene cluster.

5-aza-CdR treatment, which inactivated DNMT1 and elicited genomic DNA hypomethylation equally well in tumor and normal cell lines (Fig. 1B), only led to the induction of *MAGE-1* and other tumor antigen genes in tumor cell lines (Fig. 4A). To investigate this phenomenon, we first analyzed the methylation status of the *MAGE-1* promoter CpG island in representative tumor and normal cell lines. As expected, we found that in both HT29 cells and HMECs, the *MAGE-1* promoter is heavily methylated before 5-aza-CdR treatment (Fig. 5A). In both HT29 cells and HMECs, 5-aza-CdR treatment elicited varying degrees of hypomethylation of the majority of the CpG residues within this region (Fig. 5A). These results did not explain why *MAGE-1* was differentially activated by 5-aza-CdR treatment in the two cell types. However, we noted that within the *MAGE-1* CpG island are two CpGs contained within the context of two putative Ets transcription factor-binding sites; previously these sites were shown to be important regulators of *MAGE-1* transcription (De Smet et al., 1995). Critically, a recent study showed that methylation of a CpG in the context of an Ets site prevents binding and activation by Ets family transcription factors (Maier et al., 2003). Thus, we interrogated the role of methylation of these putative Ets sites on *MAGE-1* gene expression. In HT29 cells, these sites were fully methylated before treatment and became significantly less methylated after treatment (Fig. 5A, right two stars), thus closely correlating with the activation status of the *MAGE-1* gene (Fig. 4). In contrast, in HMECs, these sites were only partially methylated either before or after 5-aza-CdR treatment; surprisingly, in HMECs, the Ets sites also showed a slightly increased level of methylation after treatment (Fig. 5A). Thus, in HT29 cells, but not in HMECs, the activation status of the *MAGE-1* gene closely paralleled the methylation level of the Ets sites. The reduced level of methylation of the Ets sites in HMECs before treatment suggests that HMECs may be incapable of activating the *MAGE-1* promoter irrespective of the methylation status of these sites. To test this hypothesis, we constructed a MAGE-1 promoter luciferase reporter vector to assess the competency of these cell lines for activating an unmethylated MAGE-1 promoter. As predicted, we found that the unmethylated MAGE-1 promoter construct was significantly more active (~20-fold) in the colon cancer cell lines than in HMECs (Fig. 5B). This result indicates that when the MAGE-1 promoter is unmethylated, tumor cell lines present a more favorable status for promoter activation than do HMECs.

We next analyzed the effect of methylation of the putative Ets sites on MAGE-1 promoter activity. To accomplish specific methylation of the Ets sites, we took advantage of the fact that these two sites are two of only three HpaII methyltransferase acceptor sites within the MAGE-1 promoter CpG island. Notably, the third HpaII methyltransferase acceptor site is also a putative Ets site containing a centrally located CpG and is located shortly upstream of the other two putative Ets sites (Fig. 5A, left-most star). Thus, HpaII methylation specifically tests the contribution of methylation of these three putative Ets sites to the regulation of the MAGE-1 promoter. To normalize our data, we used HpaII methyltransferase to modify a model mammalian cell promoter (SV40) that does not contain HpaII methyltransferase acceptor sites but is cloned into the same vector background as that used for MAGE-1 (pGL3) (Maier et al., 2003). As predicted, we found that methylation of the MAGE-1 Ets sites dramatically repressed MAGE-1 promoter activity in the colon cancer lines (Fig. 5B). Surprisingly, HpaII methylation also restricted the low level of MAGE-1 promoter activity present in HMECs. These data suggest that these cell lines contain transcriptional activators that are competent to bind and activate the MAGE-1 promoter only when the CpG residues residing within the putative Ets sites are unmethylated. To test this hypothesis, we conducted electrophoretic mobility shift assay (EMSA) analysis using HT29 cell nuclear extracts and radiolabeled probes corresponding to either the unmethylated or methylated MAGE-1 promoter-derived Ets site (Fig. 5C). We observed that HT29 cell nuclear extracts

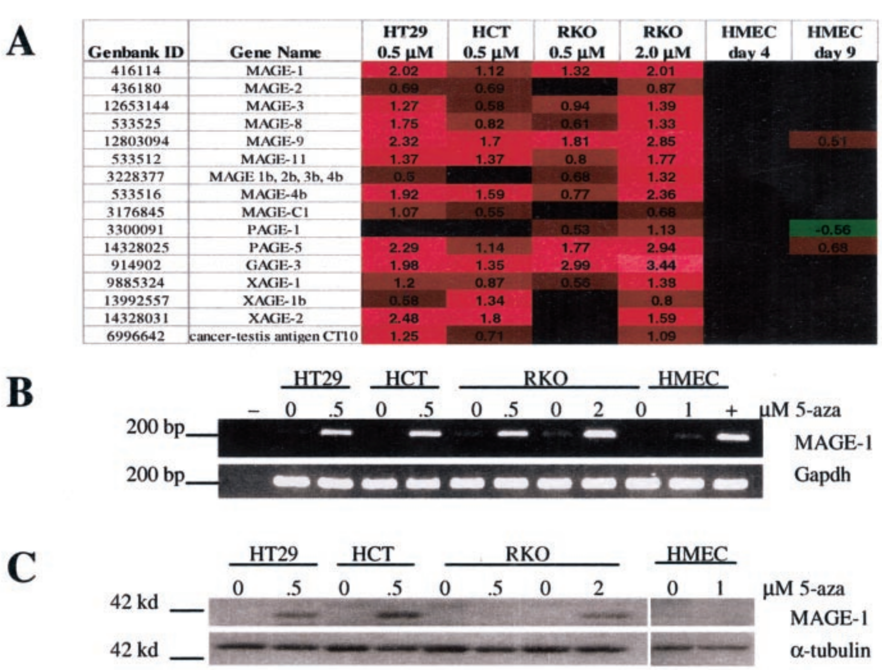

Fig. 4. Cancer-testis–type antigens and *MAGE-1* are activated by 5-aza-CdR treatment in tumor cell lines but not in HMECs. A, cancer-testis antigen gene cluster. Microarray data are presented as described in Fig. 3. B, RT-PCR analysis of *MAGE-1* expression. Cells were treated as described in Fig. 1, and RNA extracts were isolated either 5 (HT29, HCT, and RKO) or 7 (HMEC) days after treatment and used for RT-PCR analysis. −, negative PCR control (no cDNA); +, positive PCR control (testis cDNA). GAPDH amplification confirmed equivalent cDNA input. C, induction of MAGE-1 protein expression in colon cancer cell lines treated with 5-aza-CdR. Cells were treated as described in B, and proteins were harvested for Western blot analysis of *MAGE-1* expression. The blot was reprobed with an antibody directed against α-tubulin to confirm equivalent protein loading.

contain specific binding activities that are sensitive to Ets site methylation (Fig. 5C). Note that the two complexes indicated by arrows in Fig. 5C are not formed in the presence of the singly methylated probe (right-hand gel). HCT116 and RKO nuclear extracts gave results similar to those seen with HT29 (data not shown). In addition, 5-aza-CdR treatment did not affect the level of the specific binding activities contained in HT29 cell nuclear extracts (Fig. 5C). Finally, we found that HMEC nuclear extracts have a much reduced level of this binding activity compared with HT29 cells (Fig. 5D). This result offers a model for explaining the low level of MAGE-1 promoter activity in HMECs (Fig. 5B), as well as the absence of *MAGE-1* gene induction after 5-aza-CdR treatment in this cell type (Fig. 4).

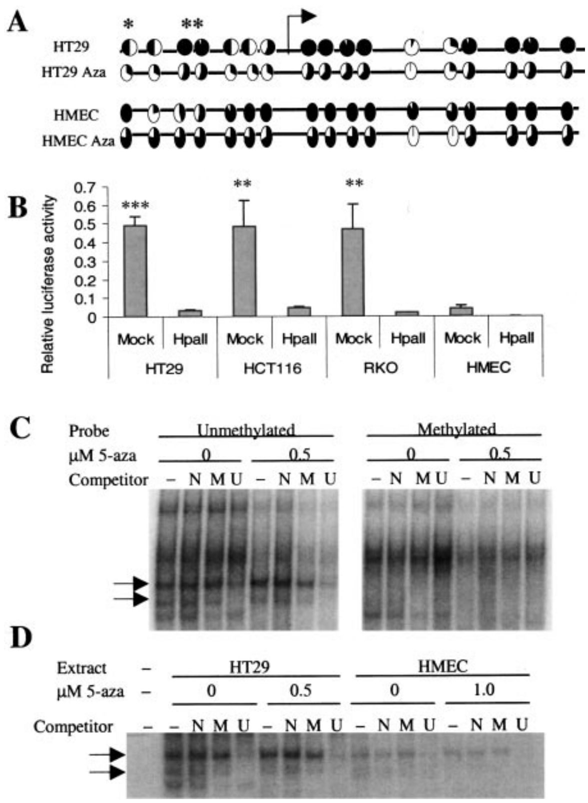

Fig. 5. Mechanism of *MAGE-1* gene activation by 5-aza-CdR. A, methylation status of the *MAGE-1* promoter CpG island in HT29 cells and HMECs. Cells were treated as described in Fig. 1, and genomic DNAs were isolated 5 (HT29) or 7 (HMEC) days later and used for sodium bisulfite DNA sequence analysis as described under *Materials and Methods*. Each circle represents a CpG site, and the extent of methylation of the site is directly related to the degree to which the circle is filled in. The horizontal arrow indicates the transcriptional start site, and stars indicate the position of three CpG sites lying within putative Ets transcription factor binding sites. B, MAGE-1 promoter activity in tumor and normal cell lines. MAGE-1 promoter activity was assessed by promoter luciferase assay as described under *Materials and Methods*. HpaII methylation was accomplished as described under *Materials and Methods*. Analysis of variance testing revealed a significant difference between the activities of the mock-methylated MAGE-1 promoter construct in each of the three colon cancer cell lines compared with HMECs. **, $p < 0.01$; ***, $p < 0.001$. C, EMSA analysis of HT29 nuclear extracts binding to a MAGE-1 promoter derived Ets site. HT29 cells were treated as described in Fig. 1, and nuclear protein extracts were harvested 5 days later and used for EMSA analysis as described under *Materials and Methods*. Binding to probes either fully unmethylated or singly methylated at the Ets site CpG was assessed. Equivalent counts were loaded in each lane; running conditions required that the free probe exit the gel. Competition experiments used either a nonspecific nuclear factor-κB consensus site competitor (N), a specific MAGE-1 Ets site competitor singly methylated at the Ets site CpG (M), or a specific MAGE-1 Ets site competitor that was fully unmethylated (U). Arrows indicate the position of two protein/DNA complexes with specific affinity for the unmethylated but not the methylated MAGE-1 Ets site probe. D, HMEC nuclear extracts have reduced levels of MAGE-1 promoter binding activities compared with HT29 cells. HT29 cells and HMECs were treated as described in Fig. 1, and 5 (HT29) or 7 (HMEC) days later, nuclear extracts were harvested and used for EMSA as described in C. Arrows indicate the position of two protein/DNA complexes with high affinity for the unmethylated, but not methylated, probe.

***MAGE-1* Gene Regulation in Melanoma Cell Lines and Melanocytes.** Our observations indicate that 5-aza-CdR treatment leads to *MAGE-1* gene induction in colon cancer cell lines but not in HMECs. Because the MAGE-1 tumor antigen was originally discovered in melanomas (van der Bruggen et al., 1991) and has been best characterized in this system, we wished to test the effect of 5-aza-CdR treatment on *MAGE-1* gene expression in melanoma cell lines and in normal human melanocytes. Four distinct human melanoma cell lines and four different human primary melanocyte cell cultures were treated with 5-aza-CdR as described under *Materials and Methods,* and MAGE-1 expression was analyzed by Western blot (Fig. 6A). Because many melanomas express MAGE-1, it was not surprising that MAGE-1 was expressed before 5-aza-CdR treatment in three of the four melanoma cell lines examined (LOX, YUSIT 1, and YUGEN 8). However, we also observed that 5-aza-CdR treatment further induced MAGE-1 expression in two of three melanoma cell lines that were already MAGE-1–positive (LOX and YUSIT 1) and in the one cell line that was previously MAGE-1–negative (YUSAC 2) (Fig. 6A). In contrast, MAGE-1 protein expression was undetectable both before and after 5-aza-CdR treatment in all four primary melanocyte cell cultures tested (two representative examples are shown in Fig. 6A). Because these data were reminiscent of our results using colon cancer cell lines and HMECs, we next examined the activity level of the MAGE-1 promoter in melanoma lines and melanocytes using the unmethylated MAGE-1 promoter luciferase construct. The activity of the unmethylated MAGE-1 promoter was significantly reduced in melanocytes compared with each of the melanoma cell lines (Fig. 6B). Furthermore, methylation of the Ets sites reduced MAGE-1 promoter activity in both melanoma cell lines and melanocytes, although the effect was more dramatic in the melanoma cell lines (Fig. 6B).

Discussion

DNA methylation-mediated gene silencing is a common epigenetic alteration in human cancer and has increased interest in drugs, such as 5-aza-CdR, that target fundamental elements of transcriptional control. The potential importance of these essential processes in normal tissues, however, requires a complete understanding of the molecular pharmacology of compounds under investigation as therapeutic agents. For this reason, we conducted a study using gene expression microarrays to probe molecular responses of tumor and normal epithelial cells to 5-aza-CdR treatment. Our results indicate that 1) 5-aza-CdR treatment elicits a limited set of gene expression changes; 2) 5-aza-CdR stimulates gene expression changes that are conserved between normal and cancer cells; 3) 5-aza-CdR stimulates tumor-specific gene expression changes; and 4) changes in the expression of specific genes in response to 5-aza-CdR requires the presence of transcriptional activators in addition to promoter demethylation.

DNA methylation has a postulated role in a variety of important cellular processes, including the regulation of tissue-specific gene expression, genomic imprinting, X chromosome inactivation, tumor-specific gene silencing, and the suppression of transposable elements and endogenous retroviruses (Baylin and Bestor, 2002). Thus, it was somewhat surprising that we found the cellular transcriptional response to 5-aza-CdR treatment to be limited both in human tumor and normal cells, with an average of 0.67% of genes becoming activated in our microarray analyses. In contrast to our results, genetic disruption of *DNMT1* in mouse fibroblasts resulted in the dysregulation of up to 10% of genes analyzed (Jackson-Grusby et al., 2001). These differences suggest that genetic knockouts of DNA methylation pathways in mice may not accurately predict cellular responses to the pharmacological targeting of DNA methylation in humans. If true in vivo, the lack of global dysregulation of gene expression in 5-aza-CdR–treated human cells could prove advantageous for methyltransferase inhibitors as cancer therapeutics.

Many of the transcriptional responses to 5-aza-CdR treatment were conserved in tumor and normal cells. Included in this list are proliferation-related and ribosomal protein genes, which were repressed in each of the four cell types examined. It is possible that the repression of these genes is an indirect effect related to the restriction in cell proliferation that accompanies 5-aza-CdR treatment (Bender et al., 1998a; Karpf et al., 1999), although a more direct effect cannot be excluded. A second response conserved in tumor and normal epithelial cells was the induction of classic markers of epithelial cell differentiation, best represented by keratin genes. This response indicates that colon tumor cells and HMECs show similar differentiation responses to 5-aza-CdR treatment as that seen in important early studies using mouse embryonic cells (Constantinides et al., 1977; Jones and Taylor, 1980).

The activation of *MHC I* genes after 5-aza-CdR treatment was noteworthy because *MHC I* gene repression is a commonly observed event in human tumorigenesis; recent studies have demonstrated that the *MHC I* gene is regulated by DNA methylation in human tumor cells (Gilboa, 1999; Nie et al., 2001; Serrano et al., 2001). However, we observed that 5-aza-CdR treatment also led to the activation of *MHC I* in HMECs, indicating either that DNA methylation regulates *MHC I* expression more generally or that 5-aza-CdR–mediated activation of *MHC I* is not dependent on methylation changes. Regardless of the mechanism of *MHC I* activation, the induction of *MHC I* by 5-aza-CdR could have positive clinical implications related to the fact that tumor cells, unlike normal cells, often express antigens perceived by the immune system as foreign.

Previously, we reported that the IFN signal transduction pathway is activated by 5-aza-CdR treatment in HT29 cells (Karpf et al., 1999). In the current study, we extended this observation to other cell lines (HCT116 cells and HMECs). In a situation analogous to that mentioned above for *MHC I*, the activation of IFN target genes after 5-aza-CdR treatment in HMECs suggests that tumor-specific methylation events do not lead to the silencing of the IFN pathway. Also consistent with this idea is our observation that the STAT1 and IRF7 transcription factors are induced by 5-aza-CdR treatment in colon cancer cell lines in the absence of detectable promoter methylation changes (data not shown). However, accumulating data in multiple systems suggest that inhibition of DNA methylation and the activation of IFN signaling are intimately linked. For example, disruption of the *DNMT1* gene in mouse embryonic fibroblasts coincides with the activation of IFN target genes (Jackson-Grusby et al., 2001). In addi-

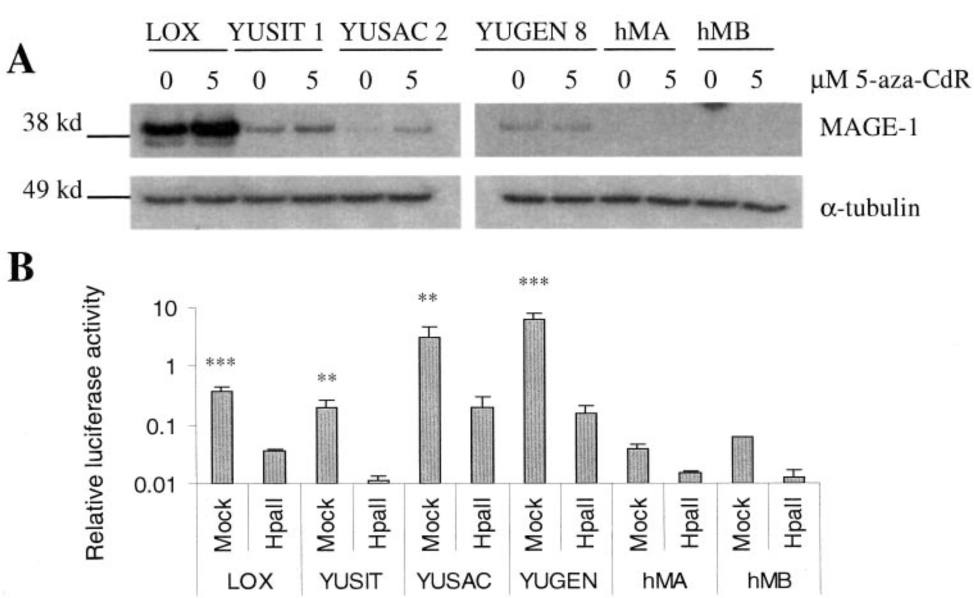

Fig. 6. Regulation of *MAGE-1* expression in melanoma cell lines and melanocytes. A, melanoma cell lines and primary human melanocyte cell cultures were treated with 5-aza-CdR as described under *Materials and Methods,* and the expression of MAGE-1 was assessed by Western blot analysis. Two representative examples of four human melanocyte cell cultures tested are shown. The blot was reprobed with an antibody directed against α-tubulin to confirm equivalent protein loading. B, MAGE-1 promoter activity. MAGE-1 promoter activity was assessed by promoter luciferase assays as described under *Materials and Methods*. HpaII methylation was accomplished as described under *Materials and Methods*. Analysis of variance testing revealed a significant difference between the activity level of the unmethylated MAGE-1 promoter construct in each of the four melanoma cell lines compared with the activity of this same construct in the melanocyte cell cultures (hMA or hMB). **, $p < 0.01$; ***, $p < 0.001$.

tion, 5-aza-CdR treatment activates IFN-responsive genes both in bladder cancer cells and in normal human fibroblasts (Liang et al., 2002). One mechanism suggested to explain the activation of IFN signaling in DNA methyltransferase-targeted cells is that it constitutes a cellular response to the induction of endogenous retroviral elements (Liang et al., 2002). In the current study, we make correlative observations consistent with this hypothesis. For instance, we noted that in HMECs (a cell line showing a robust IFN response), the expression of human endogenous retrovirus was induced after 5-aza-CdR treatment. In addition, we observed that the pattern of induction of retroviral and repetitive DNA elements in the six microarray comparisons roughly correlated with the magnitude of IFN target gene induction in these experiments. Lending further support for this model is the finding that endogenous retroviral and repetitive DNA elements are activated in *DNMT1* −/− mouse fibroblasts, in parallel with the induction of IFN target genes (Jackson-Grusby et al., 2001).

We observed that 5-aza-CdR treatment leads to the activation of a large number and variety of tumor antigen genes, including *MAGE-1*, in three genetically distinct colon cancer cell lines. This response was absent in HMECs. Similarly, we found that 5-aza-CdR treatment activated the expression of *MAGE-1* in melanoma cell lines but not in normal human melanocytes. These results indicate that tumor and normal cells have different abilities to activate *MAGE-1* after DNA hypomethylation. These data are intriguing because they suggest that prior or concurrent treatment with DNA methyltransferase inhibitors could increase the therapeutic usefulness of tumor immunotherapy strategies (e.g., vaccines) targeting MAGE antigens.

To begin to gain a mechanistic understanding of the phenomenon of tumor cell-specific activation of MAGE antigens after 5-aza-CdR treatment, we examined the mechanism of regulation of expression of *MAGE-1*, the prototype member of this gene family. In agreement with previous reports, we found that the *MAGE-1* promoter CpG island is methylated in nonexpressing cells and that 5-aza-CdR–induced reactivation correlated with promoter hypomethylation. Also illustrating the important role of DNA methylation in the regulation of *MAGE-1* gene expression was our observation that methylation of three consensus Ets sites in the MAGE-1 promoter blocks MAGE-1 promoter activity. However, these data alone could not explain the absence of MAGE-1 induction in normal epithelial cells (HMECs or melanocytes) treated with 5-aza-CdR. In this regard, experiments using an unmethylated MAGE-1 promoter luciferase construct proved informative. An unmethylated MAGE-1 promoter construct was highly active in tumor cell lines but showed significantly reduced activity in normal cell types, indicating that tumor cells present a more favorable state for MAGE-1 promoter activity. Notably, these data are consistent with the expression profile of MAGE-1 (and other tumor antigens), which is almost entirely restricted to tumor tissues. One potential mechanism accounting for the favorable transcriptional status of the MAGE-1 promoter in tumor cells, which is also consistent with the lack of expression before 5-aza-CdR treatment, invokes the presence of *trans*-activating factors specifically targeting the unmethylated *MAGE-1* gene promoter. A second, nonexclusive model is that specific transcriptional repressors act only on the methylated MAGE-1 promoter and that these are displaced after 5-aza-CdR treatment. In support of the first model, we observed that tumor cell nuclear extracts contain factors competent for specific binding to the unmethylated *MAGE-1* promoter and that normal cells have reduced levels of this binding activity. Future studies will focus on the identification of the specific transcriptional activators and repressors that regulate activity at the endogenous *MAGE-1* promoter.

More generally, the results of this study suggest a model wherein 5-aza-CdR–induced gene reactivation has two distinct requirements: 1) the reversal of promoter DNA hypermethylation, and 2) the presence of transcriptional activators competent for activation of the target promoter (Fig. 7). This model predicts three distinct states of gene activation: silenced, permissive, and activated. For many genes, 5-aza-CdR treatment may be sufficient for the induction of the transcriptionally permissive state. However, for specific gene activation to occur, sequence-specific activators must be present that are competent for the activation of target promoter. This second and distinct requirement for gene activa-

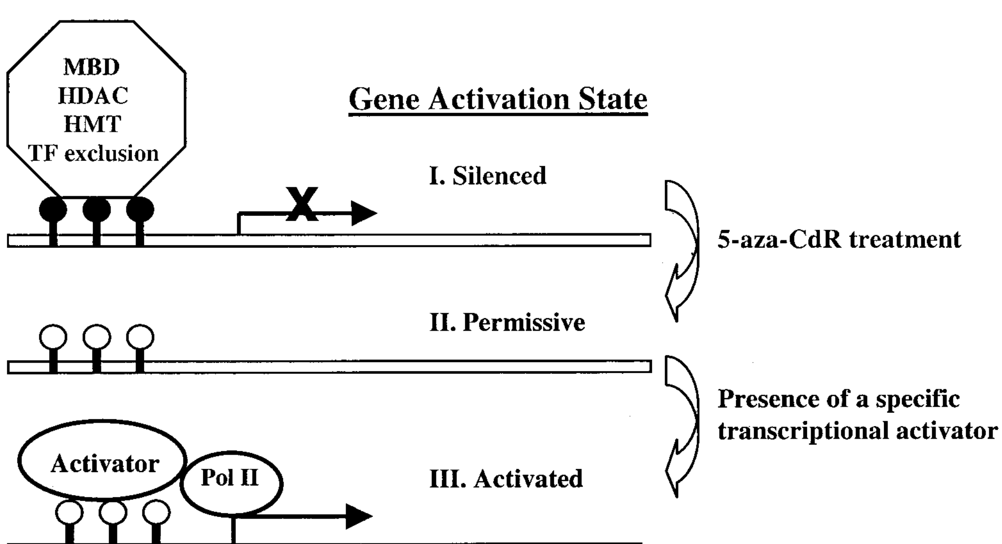

Fig. 7. A model for gene activation by 5-aza-CdR. A methylated promoter can be directly repressed by methylated DNA binding proteins (MBD), histone deacetylases (HDAC), or histone methyltransferases (HMT) and can be repressed indirectly by the exclusion of sequence-specific transcriptional activators (TF). 5-aza-CdR treatment reverses these effects and converts a silenced promoter into a permissive promoter. Sequence-specific transcriptional activators are required to convert a permissive promoter into an activated one. ●, methylated CpG sites; ○, unmethylated CpG sites; →, transcriptional start sites.

tion could account for the limited scope of gene activation in 5-aza-CdR–treated cells, as is seen in both tumor and normal cell types.

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