[CANCER RESEARCH 62, 961–966, February 15, 2002]

Advances in Brief

Analysis of Gene Induction in Human Fibroblasts and Bladder Cancer Cells Exposed to the Methylation Inhibitor 5-Aza-2′-deoxycytidine[1]

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Abstract

Hypermethylation of the promoters of cancer-related genes is often associated with their inactivation during tumorigenesis. Several preclinical and clinical trials have been developed to use DNA methylation inhibitors, such as 5-aza-2′-deoxycytidine (5-Aza-CdR) in attempts to reactivate silenced genes in human cancers. We used high-density oligonucleotide gene expression microarrays to examine the effects of 5-Aza-CdR treatment on human fibroblast cells (LD419) and a human bladder tumor cell line (T24). Data obtained 8 days after recovery from 5-Aza-CdR treatment showed that more genes were induced in tumorigenic cells (61 genes induced; ≥ 4-fold) than nontumorigenic cells (34 genes induced; ≥ 4-fold). Approximately 60% of induced genes did not have CpG islands within their 5′ regions, suggesting that some genes activated by 5-Aza-CdR may not result from the direct inhibition of promoter methylation. Interestingly, a high percentage of genes activated in both cell types belonged to the IFN signaling pathway, confirming data from other tumor cell types.

Introduction

The abnormal methylation of CpG islands in the promoters of tumor-associated genes is increasingly being recognized as a mechanism for their transcriptional inactivation during human cancer development (1, 2). The fact that a significant proportion of tumor suppressor genes have been demonstrated to be subject to methylation silencing in cancer has led to the realization that abnormal methylation could be a useful therapeutic target in cancer (3). Several clinical trials have been developed recently to explore the use of DNA methylation inhibitors in cancer treatment (4–7). The nucleoside analogues 5-aza-cytidine and 5-Aza-CdR[4] are the most potent known inhibitors of DNA methylation (8) and are currently being tested as treatments for myeloid dysplastic syndromes and other diseases (7).[5]

The aza nucleotides are incorporated into the DNA of replicating cells, where they serve as mechanism-based inhibitors of DNA methyltransferases because of their abilities to form covalent complexes with the enzymes (9, 10). Early experiments showed that drugs that inhibit DNA methylation are powerful inducers of cellular differentiation (8), and these drugs are routinely used to reactivate silenced genes (11, 12). Most studies investigating the induction of gene expression by aza nucleoside analogues have focused on the reactivation of known genes, and few studies have been done to conduct a more global analysis of the patterns of gene expression induced by these compounds (13). In this study, we used a gene expression chip containing 6600 human genes to investigate global changes in gene expression induced by transient treatment of human fibroblasts or human bladder cancer cells with 5-Aza-CdR. Nontumorigenic fibroblast cells were included in this analysis because few studies have examined the results of methylation inhibition on normal cells. These data may be important, because normal cells are potential targets for inappropriate gene activation in patients undergoing therapy with methylation inhibitors. Our results show that 8 of the 33 genes induced by 5-Aza-CdR treatment of normal fibroblasts were also induced in the human bladder cancer cell line T24. Approximately twice as many genes were induced by treatment of the tumor cell line than in fibroblasts, and IFN-induced genes were frequently activated in both cell types treated with 5-aza-CdR. About 40% of the genes induced had CpG islands within their promoters, suggesting that a considerable portion of genes activated by aza nucleosides may be induced by mechanisms not directly involving promoter CpG island demethylation.

Materials and Methods

Tissue Culture/5-Aza-CdR Treatment

The human fibroblast cell strain LD419 was established in our laboratory, and the T24 human bladder transitional carcinoma-derived cell line was obtained from the American Type Culture Collection (Rockville, MD). All cell lines were maintained in McCoy's medium supplemented with 10% heat-inactivated FCS, 25 units/ml of penicillin, and 25 μg/ml of streptomycin. Cells were plated at 3×10^5 cells/100-mm dish and treated the next day with 10^{-6} M 5-Aza-CdR (Sigma Chemical Co., St. Louis, MO). The medium was changed 24 h after treatment, and RNA was extracted at days 3 and 8 from exponentially growing cultures. Two independent 5-Aza-CdR treatment experiments were performed, and the corresponding total RNAs from the two experiments were pooled.

RNA Extraction

Total RNA was isolated from 2×10^6 cells using a standard protocol. First, cells were lysed with a buffer containing 4 M guanidine isothiocyanate (Life Technologies, Inc., Palo Alto, CA), 0.5% *N*-lauryl sarcosine (Sigma Chemical Co.), 25 mM sodium citrate (Fisher Scientific, Fair Lawn, NJ), and 0.1 M 2-mercaptoethanol (Sigma Chemical Co.). A standard phenol/chloroform extraction was then performed, followed by precipitation for 1 h at −20°C with 50% isopropanol/50% lysis buffer. This was centrifuged for 10 min at $10{,}000 \times g$, and the RNA pellet washed twice with 70% ethanol prepared with and dissolved in diethylpyrocarbonate-treated water.

Oligonucleotide Array Analysis

cRNA Preparation. Total RNA (10 μg) was used as starting material for the cDNA preparation. The first- and second-strand cDNA synthesis was

Received 9/17/01; accepted 12/28/01.

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[1] This work was supported by NIH Grant 1RO1 CA 83876-01, the Danish Cancer Society, and the University of Aarhus.

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[4] The abbreviations used are: 5-Aza-CdR, 5-aza-2′-deoxycytidine; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

[5] M. Daskalakis, T. T. Nguyen, P. Guldberg, C. Nguyen, G. Kohler, P. Wijermans, P. A. Jones, and M. Lubbert, Demethylation of a target hypermethylation *P15/INK4B* gene in patients with myelodysplastic syndrome by 5-aza-2′-deoxycytidine treatment, submitted for publication.

performed using the SuperScript Choice System (Life Technologies, Inc.) according to the manufacturer's instructions, except using an oligo-dT primer containing a T7 RNA polymerase promoter site. Labeled cRNA was prepared using the BioArray High Yield RNA Transcript Labeling kit (ENZO). Biotin-labeled CTP and UTP (Enzo) were used in the reaction, together with unlabeled nucleotide triphosphates. After the *in vitro* transcription reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen).

Array Hybridization and Scanning. Fifteen μg of cRNA were fragmented at 94°C for 35 min in a fragmentation buffer containing 40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, 30 mM magnesium acetate. Before hybridization, the fragmented cRNA in a 6xSSPE-T hybridization buffer [1 M NaCl, 10 mM Tris (pH 7.6), and 0.005% Triton] was heated to 95°C for 5 min and subsequently to 40°C for 5 min before loading onto the Affymetrix probe array cartridge. The probe array was then incubated for 16 h at 45°C at constant rotation (60 rpm). The washing and staining procedure was performed in the Affymetrix Fluidics Station. The probe array was exposed to 10 washes in 6xSSPE-T at 25°C, followed by four washes in 0.5xSSPE-T at 50°C. The biotinylated cRNA was stained with a streptavidin-phycoerythrin conjugate (final concentration, 2 μg/μl; Molecular Probes, Eugene, OR) in 6xSSPE-T for 30 min at 25°C, followed by 10 washes in 6xSSPE-T at 25°C. An antibody amplification step was added using normal goat IgG (final concentration, 0.1 mg/ml; Sigma Chemical Co.) and anti-streptavidin antibody (goat) biotinylated (final concentration, 3 μg/ml; Vector Laboratories). This was followed by a staining step with a streptavidin-phycoerythrin conjugate (final concentration, 2 μg/μl; Molecular Probes) in 6xSSPE-T for 30 min at 25°C and 10 washes in 6xSSPE-T at 25°C.

The probe arrays were scanned at 560 nm using a confocal laser-scanning microscope with an argon ion laser as the excitation source (Hewlett Packard GeneArray Scanner G2500A). The readings from the quantitative scanning were analyzed by the Affymetrix Gene Expression Analysis Software. All six microarray analyses (untreated, 3 days, and 8 days after 5-Aza-CdR treatment for T24 and LD419) were done in replicates, starting from the same total RNA sample.

Calculation of Expression Changes. The readings were scaled to a global intensity of 150, as published previously (14), and subjected to pairwise comparisons using the Software GeneChip Analysis Suite 3.3.

Hierarchical Clustering Analysis. The dendrogram was generated using the programs Cluster and Treeview (15), using the following filtering criteria: absolute call, present (*P*) or marginally present (*M*) in minimum one sample; maximum − minimum average difference, ≥300. The values were log transformed, median centered, and normalized before the hierarchical clustering analysis.

RT-PCR Assay

Total RNA was reverse transcribed using 2 mg of RNA and random hexamers, deoxynucleotide triphosphates (Boehringer Mannheim, Mannheim, Germany), and Superscript II reverse transcriptase (Life Technologies, Inc.) in a 50-ml reaction. The mixture was placed at room temperature for 10 min, 42°C for 45 min, and 90°C for 3 min and then rapidly cooled to 0°C. cDNA (100 ng) was PCR amplified using the following condition: *KRT8* expression; one cycle of 95°C for 2 min, followed by 95°C for 1 min, 52°C for 45 s, and 72°C for 1 min 30 s, for 26 cycles and one cycle of 72°C for 4 min. The conditions for *KRT17*, *H19*, *TIMP3*, and *STAT1* were similar to *KRT8*, except the cycle number for *KRT17* was 24, and annealing temperature for *H19* was 55°C; for *TIMP3*, it was 60°C; for *STAT1*, it was 48°C. The PCR conditions for *GAPDH* were 1 cycle of 94°C for 2 min, 20 cycles of 94°C for 1 min, 58°C for 30 s, and 72°C for 45 s, and finally 1 cycle of 72°C for 2 min. The PCR primers used were: KRT8, 5′-AAC AAC AAG TTT GCC TCC TTC ATA G-3′ (sense) and 5′-GAG GAC AAA TTC GTT CTC CAT C-3′ antisense; KRT17, 5′-AAT GCC AAC ATC CTG CTA CAG-3′ (sense) and 5′-CAT CCT TGC GGT TCT TCT CT-3′ (antisense); H19, 5′-GACTCAGGAATCG-GCTCTGG-3′ (sense) and 5′-GCC AAG GTG GCT CAC ACT CAA-3′ (antisense); TIMP3, 5′-GGA ATT CAT GAC CCC TTG GCT CGG G-3′ (sense) and 5′-GGA ATT CAG GGT CTG GCG CTC AGG-3′ (antisense); STAT1, 5′-ACA GTC TTG GCA CCT AAC GTG-3′ (sense) and 5′-GAC ATC TGG ATT GGG TCT TCC T-3′ (antisense); and GAPDH, 5′-CAG CCG AGC CAC ATC G-3′ (sense) and 5′-TGA GGC TGT TGT CAT ACT TCT C-3′ (antisense).

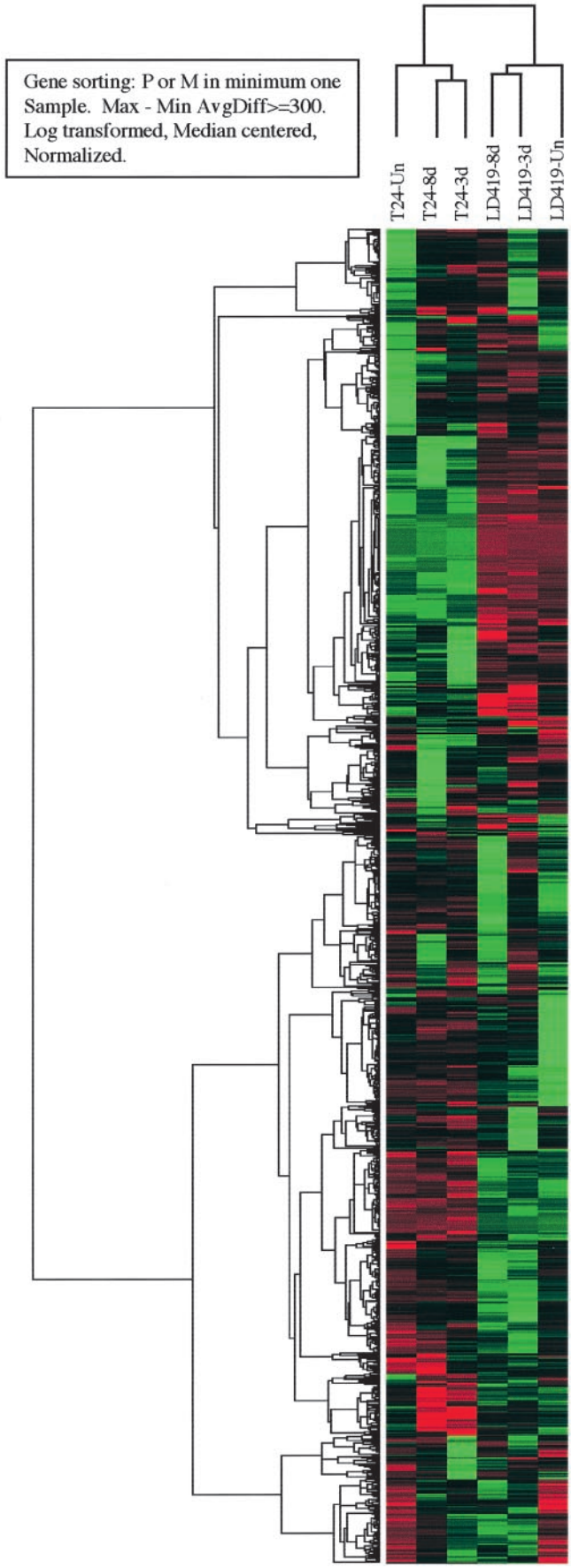
Results

Global Changes in Gene Expression Induced by 5-Aza-CdR. We first assessed the global changes in gene expression induced by 5-Aza-CdR in the bladder tumor cell line (T24) and human fibroblast cell strain (LD419) exposed to 1 μM 5-Aza-CdR for 24 h. Hierarchical clustering of gene expression in untreated and treated cells is shown in Fig. 1. As expected, the greatest differences in gene expression were seen between untreated cell types, and there were fewer differences between the two treated samples (days 3 and 8) than the untreated samples. This result was not surprising because the differences between the cell types were not only attributable to their tumorigenic potentials but also because T24 cells have an epithelial origin whereas the fibroblasts are mesenchymally derived. The oligonucleotide arrays were therefore clearly able to distinguish between the cell types, and ~1000 of the 6600 genes analyzed had observable expression differences. T24 cells analyzed 3 days after drug treatment had measurable changes in gene expression, and many of these changes persisted 8 days after treatment. This was an important observation because this drug is known to have immediate cytotoxic effects on cells, and some of the genes induced immediately after drug treatment (*i.e.*, 3 days) may be more related to response to stress than to prolonged changes in gene expression. Changes in expression of genes in fibroblasts were also found, and some of these also persisted 8 days after treatment (Fig. 1).

5-Aza-CdR Induces More Genes in Bladder Cancer Cells Than Human Fibroblasts. We next focused our attention on those genes that were up- or down-regulated by >4-fold 8 days after drug treatment and categorized the genes as to whether or not they are known to be in IFN pathways (Table 1). A total of 34 genes were found to be 4-fold overexpressed in human fibroblasts 8 days after 5-Aza-CdR treatment, and 10 of these (29%) are known to be inducible by IFN (Table 1). The level of induced expression varied from 4.3-fold for the *ANXA 3* to a maximum of 42-fold for an IFN-induced gene, *IF127*. Interestingly, 8 of the genes (indicated in bold type) were also up-regulated in T24 cells treated with the demethylating agent (Table 2). A total of 11 genes were found to be down-regulated in the fibroblasts by drug treatment. The 5′ ends of the genes up-regulated and down-regulated in this manner were analyzed in the BLAST database[6] to determine whether they might have promoter CpG islands. The data showed that only 10 up-regulated and 7 down-regulated genes in the fibroblasts definitely contained 5′ CpG islands. However, some of the associated mRNAs in the BLAST database may not include full-length mRNA sequences and hence may be missing the actual transcription start sites. Therefore, determining the exact number of up-regulated genes with 5′ CpG islands will require further analysis in the future. There is only sparse information indicating that methylation of non-CpG island promoter down-regulates expression (16); therefore, the mechanism by which the drug activates genes without CpG islands remains unclear. It is also possible that these genes were up-regulated in response to other genes that did contain CpG islands within their promoters.

The drug was more effective in inducing prolonged changes in gene expression in the T24 bladder cancer cell line than in fibroblasts (Table 2). The level of induced expression varied from 4.1-fold for the *SP100* to almost 60-fold for the *SAA1*. It was striking that 33 of these genes (54%) are known to be inducible by IFN, again underlining the fact that IFN pathways are highly inducible by 5-Aza-CdR treatment. Interestingly, 24 of the 61 genes induced by drug treatment contained CpG islands within their 5′ regions. This result is consistent with the observation that tumor cells often contain abnormal hypermethylation

[6] Internet address: www.ncbi.nlm.nih.gov/BLAST/.



of CpG islands within their promoters (1, 2). In the T24 cell line, the 5-Aza-CdR treatment induced expression of several tumor antigens, such as members of the *MAGE*, *GAGE*, and *SSX* gene families. These genes were shown previously to be moderately to highly expressed in several types of solid tumors but silent in normal tissue except male germinal cells and constitute promising targets for immunotherapy (17–19). Only two genes were down-regulated 8 days after drug treatment.

To ensure that the microarray data represented real changes in expression, we next used RT-PCR on a subset of induced genes to validate the changes. We examined five representative genes for their expression after 5-Aza-CdR treatment (*KRT8*, *KRT17*, *H19*, *TIMP3*, and *STAT1*) by RT-PCR (Fig. 2). *KRT17*, *H19*, and *STAT1* were induced by 5-Aza-CdR in both cell lines. *KRT8* and *TIMP3* were only induced in fibroblasts by 5-Aza-CdR (Fig. 2). Four of these 5 genes have CpG island in their 5′ end regions. All 5 genes tested showed up-regulation by RT-PCR analysis, as had been observed on the microarrays showing that the two independent methods supported each other.

Discussion

The important role of DNA methylation during tumorigenesis and the efficiency of 5-Aza-CdR in reversing DNA methylation changes has led to the suggestion that drug-induced demethylation may be an effective therapeutic intervention in cancer (7). The relationship between demethylation by 5-Aza-CdR and gene expression has been well studied (11, 12, 20). In this study, we wanted to assess the global effects of 5-Aza-CdR on gene expression in both tumorigenic and nontumorigenic cells. 5-Aza-CdR is a cytotoxic agent in addition to being a demethylating agent, and little is known as to how normal cells respond to drug exposure. Previous experiments have shown that 5-Aza-CdR can suppress the growth rates of several types of human tumor cell lines but not fibroblast cells after recovery from the immediate cytotoxic effects of treatment (11).

This study showed clearly the effectiveness of the microarray expression analysis in distinguishing between tumor cells of epithelial origin and normal human fibroblasts. Both cell types responded with prolonged changes in the expression levels of genes that could be observed 8 days after treatment. The number of genes up-regulated (at least 4-fold in the different cell types) represented only a minority of those analyzed, 34 of 6600 (0.5%) in fibroblasts and 61 of 6600 (0.9%) in T24 cells, respectively. This seems, at first, to be a relatively low number of genes induced by drug treatment in view of the fact that profound changes in methylation occur during tumorigenesis and in the formation of cell lines (21–23). One possible explanation for the low number of genes observed to be up-regulated could be that 5-Aza-CdR induces clonal expression of genes with the percentage of treated clones responding to the drug varying from 1 to 50% (8, 24). Indeed, we have found clonal variations in the levels of p16 expression induced by 5-Aza-CdR in T24 cells (20). Because genes such as *p16* could alter cell growth rates and alter the clonal composition of the surviving cell populations, there is a need for caution in interpreting the expression data. The genes identified here therefore represent those genes that were most susceptible to activation by drug treatment. It should also be noted that promoter methylation is likely to occur in many other genes in addition to those we found to be up-regulated after 5-Aza-CdR treatment.

Fig. 1. Hierarchical clustering based on gene expression data from T24 and LD419 5-Aza-CdR untreated and treated cells (3 and 8 days after treatment). The dendrogram is based on 1065 genes that passed the filtering criteria (see “Materials and Methods”). The hierarchical clustering analysis was generated using the programs Cluster and Treeview (15).

Table 1 *Genes altered ≥4-fold after 5-Aza-CdR treatment of the LD419 fibroblast cell line*

Accession	Symbol	Function	Fold change[a] 3 days after treatment			Fold change[a] 8 days after treatment			5′ CpG island (CGI)
			Average	Exp 1	Exp 2	Average	Exp 1	Exp 2	
Up-regulated genes[b]									
Genes inducible by interferon[c]									
K02765	***C3***	**Complement component**	**21.2**	**~17.7**	**~24.6**	**29.0**	**~28.6**	**29.3**	**N**
Z19574	***KRT17***	**Cytokeratin**	**13.6**	**~12.8**	**~14.4**	**19**	**~16.6**	**~21.4**	**N**
Y00503	*KRT19*	Cytokeratin	2.5	2.2	2.7	7.1	8.7	5.4	CGI
M13955	*KRT7*	Cytokeratin	2.8	1.4	4.1	11.9	5.3	18.5	CGI
X74929	*KRT8*	Cytokeratin	18.5	~25.3	11.6	29	~40.3	17.7	N
M26167	*PF4V1*	Cytokine family	2.3	~2.6	~1.9	5.4	~4.8	~6.0	N
J03474	***SAA1***	**Cytokine stimulated**	**3.3**	**~4.1**	**~2.4**	**18.2**	**~18.7**	**~17.7**	**N**
X67325	*IFI27*	IFN induced	18.8	~16.9	~20.7	42.1	~32.5	~51.7	N
D89050	*OLR1*	LDL receptor	4.2	3.9	4.5	7.7	9.6	5.7	N
M97936	***STAT1***	**Transcription factor**	**11.3**	**~16.8**	**~5.8**	**14.8**	**~23.8**	**5.8**	**CGI**
Other genes									
U07919	*ALDH6*	Aldehyde dehydrogenase	3.3	3.4	3.1	4.6	4.9	4.3	CGI
L20591	*ANXA3*	Annexin	2.9	2.5	~3.3	4.3	4.1	~4.4	N
X16662	*ANXA8*	Annexin	4.1	~3.0	~5.2	10.6	~6.5	~14.6	N
U70063	*ASAH*	Ceramidase	0.5	~−1.0	1.9	6.0	~5.1	6.8	CGI
D13639	*CCND2*	Cyclin	4.6	3.8	5.4	40.0	49.4	30.6	CGI
M83186	*COX7A1*	Cytochrome *c* oxidase	3.4	1.1	5.6	12.0	5.3	~18.7	CGI
D55696	*PRSC1*	Endopeptidase	4.9	~10.8	−1	15.4	~22.8	8	N
Y08374	*CHI3L1*	Environment change Resp.	16.2	13.8	18.5	18.5	17.1	19.9	N
U28727	*PAPPA*	Growth factor activator	3.7	4.7	2.7	11.1	~17.5	4.6	N
U90551	***H2AFL***	**Histone**	**3.3**	**2.5**	**~4.0**	**12.0**	**7.4**	**~16.6**	**N**
Z80780	***H2BFH***	**Histone**	**2.8**	**~1.6**	**~3.9**	**6.1**	**~7.4**	**4.8**	**N**
X57985	*H2BFQ*	Histone	4.5	~4.8	~4.1	6.7	~6.0	~7.4	CGI
M32053	***H19***	**Imprinted gene**	**4.3**	**4**	**~4.6**	**9.5**	**5.5**	**~13.5**	**CGI**
U31201	*LAMC2*	Laminin	−1.6	~−1.8	~−1.4	6.5	~4.3	~8.6	N
U41518	*AQP1*	Membrane protein	4.4	5.2	3.6	23.0	19.2	26.7	N
D90224	*TNFSF4*	Membrane protein (type ii)	4.5	~4.1	~4.8	8.6	~6.6	10.6	N
X05232	*MMP3*	Metalloproteinase 3	3.5	~4.0	~2.9	9.6	~8.8	~10.3	N
D45917	*TIMP3*	Metalloproteinase 3 inhibitor	2.8	2.9	2.7	4.8	5.1	4.5	CGI
U37283	*MAGP2*	Microfibril-associated	2.6	~2.9	~2.3	11.5	~9.5	~13.5	N
X13100	*MYH3*	Myosin	2.7	~1.6	~3.7	8.6	~8.2	~9.0	N
Y00757	*SGNE1*	Neuroendocrine protein	4.7	~1.8	~7.5	6.6	~6.4	~6.7	N
X17042	*PRG1*	Proteoglycan	2.6	2.1	3	4.7	4.3	5.1	N
U10687	***MAGEA4***	**Tumor transformation**	**4.1**	**2.1**	**~6.1**	**10.5**	**~8.8**	**~12.2**	**N**
Down-regulated genes[b]									
D21063_at	*MCM2*	Cell cycle	−14.4	~−19.2	−9.5	−22.4	~−24.4	~−20.4	CGI
X51688_at	*CCNA2*	Cell cycle	−11.3	~−11.2	~−11.3	−11.15	~−12.7	−9.6	CGI
X65550_at	*MKI67*	Cell cycle	−5.5	~−4.1	~−6.8	−6.6	~−4.9	~−8.3	CGI
X85137_s_at	*KNSL1*	Cell cycle	−3.1	−3.9	−2.2	−4.7	−4.4	−5	N
L25876_at	*CDKN3*	Cell cycle	−4.4	−4.8	−3.9	−4.05	−4	−4.1	N
L36033_at	*CDF1*	Cytokine	−1.8	−1.4	−2.1	−8.6	~−8.5	−8.7	CGI
X62534_s_at	*HMG2*	Nuclear protein	−3.9	−4	−3.7	−5.3	−5.2	−5.4	CGI
D82345_at	*TMSNB*	Thymosin β	−4.2	−3.5	−4.9	−7	~−8.0	−6	N
U74612_at	*FOXM1*	Transcription factor	−7.9	−9.2	−6.6	−9	−11.6	−6.4	CGI
U61145_at	*EZH2*	Transcription factor	−4.3	~−5.4	−3.1	−6.65	~−6.1	~−7.2	CGI
D14657_at	*KIAA0101*	Unknown function	−3	−3.6	−2.4	−4.85	−5.1	−4.6	N

[a] A "~" preceding the fold change value indicates that the expression level for the untreated sample was under the calculated noise level; therefore, the fold change is an approximation.
[b] Bold represents genes induced in both LD419 and T24 cells.
[c] Based on a PubMed search; CGI, 5′ CpG island; Exp, experiment; N, no known 5′ CpG island.

This study also shows that fewer genes were down-regulated than up-regulated by treatment, 11 of 6600 (0.16%) in fibroblasts and 2 of 6600 (0.03%) in T24 cells, respectively. These genes could potentially be directly down-regulated by 5-Aza-CdR or by the induction of negative regulatory factors. Our previous experiments have shown that 5-Aza-CdR heritably inhibits cell growth in tumor but not in fibroblast cell lines (11). Therefore, the roles of the 5 down-regulated cell cycle genes in fibroblast cells will require further study.

It was interesting that a substantial proportion of genes (~64%) induced in both fibroblasts and tumor cells did not contain CpG islands within their 5′ regions. However, as mentioned earlier, the exact locations of the transcriptional sites are not always known; therefore, this percentage may be an overestimate. The human genome project has shown that ~60% of genes do not have CpG islands in their 5′ regions (25). Our results suggest that 5-Aza-CdR did not specifically target genes with methylated 5′ CpG islands. This is interesting because most investigators seeking to link methylation to silencing have focused on the methylation of 5′ CpG islands. Methylation of CpG-poor promoters may also affect gene expression in ways that have not yet been investigated in detail. Alternatively, induction of genes acting upstream of the set that we observed may have been responsible for the global changes in expression. Several other recent studies have also indicated that silenced genes with unmethylated CpG islands or without CpG island promoters can be induced by 5-Aza-CdR (26–28). Distinguishing between these two possibilities will be important in understanding the mechanisms underlying the epigenetic changes in gene control. It was also interesting that the drug induced the expression of genes such as cytokeratins that are not normally expressed in fibroblasts.

The induced genes in both human LD419 fibroblasts and T24 bladder cancer cell lines were frequently in the IFN pathways. Recent work with colon cancer cell lines also showed that 5-Aza-CdR activates the IFN signaling pathway, and this may be a mechanism to inhibit tumor cell growth (13). Our results also show a strong induction of genes in the IFN signaling pathways such as *STAT1, SAA1, KRT17,* and other downstream genes. The mechanisms by which 5-Aza-CdR activates the IFN signaling

Table 2 *Genes altered ≥4 fold after 5-Aza-CdR treatment of the T24 tumor cell line*

Accession	Symbol	Function	Fold change[a] 3 days after treatment			Fold change[a] 8 days after treatment			5′ CpG island (CGI)
			Average	Exp 1	Exp 2	Average	Exp 1	Exp 2	
Up-regulated genes[b]									
Genes inducible by interferon[c]									
X89986	*BIK*	Apoptosis	6.2	~5.4	~7.0	5.5	~4.4	~6.5	N
U37518	*TNFSF10*	Apoptosis	12.1	~13.7	10.5	9.4	~10.2	8.6	N
J04080	*C1S*	Complement component	3.4	~2.7	~4.0	14.6	~9.2	~20.0	N
K02765	***C3***	**Complement component**	**10.7**	**~9.0**	**~12.3**	**37.7**	**~36.5**	**~38.8**	**N**
Z19574	***KRT17***	**Cytokeratin**	**29.5**	**~36.2**	**22.7**	**33.4**	**~38.5**	**28.2**	**N**
M13207	*CSF2*	Cytokine family	4.7	3.5	5.9	5.7	5.5	5.9	N
M57731	*GRO2*	Cytokine family	6.6	8.1	5	4.8	5.4	4.2	CGI
U64197	*SCYA20*	Cytokine family	22.0	~15.9	~28.1	21.7	~12.8	~30.5	N
M21121	*SCYA5*	Cytokine family	4.5	3.5	5.4	21.7	~22.2	21.1	N
L37036	*SCYB5*	Cytokine family	3.5	3.9	3.1	4.6	4.3	4.8	CGI
U83303	*SCYB6*	Cytokine family	8.4	11.1	5.6	10.0	13.9	6	N
J03474	***SAA1***	**Cytokine stimulated**	**12.2**	**~8.0**	**~16.3**	**59.5**	**~46.8**	**~72.2**	**N**
U04636	*PTGS2(COX2)*	Inflammation	22.9	24.2	21.6	9.9	9.2	10.6	CGI
U22970	*G1P3*	IFN induced	9.9	~10.4	~9.3	77.6	~71.7	~83.4	N
M24594	*IFIT1*	IFN induced	4.6	4.6	4.5	16.1	15.5	16.7	N
M14660	*IFIT2*	IFN induced	4.7	2.2	~7.2	10.3	4.2	~16.4	N
U52513	*IFIT4*	IFN induced	2.7	3	2.4	8.8	9.9	7.7	N
D28915	*MTAP44*	IFN induced	2.7	3	2.4	5.6	5.9	5.3	N
M33882	*MX1*	IFN induced	8.4	8.8	8	27.7	33.3	22	CGI
M30818	*MX2*	IFN induced	3.3	4.7	1.9	8.8	12.1	5.5	N
X02874	*OAS1*	IFN induced	5.6	5	6.1	12.6	10.6	14.6	N
M87434	*OAS2*	IFN induced	4.2	3.8	4.6	8.5	9.3	7.7	N
U53830	*IRF7*	IFN regulatory factor	3.7	4.1	3.3	6.0	5.8	6.2	N
Y00081	*IL6*	Interleukin	21.1	~27.2	15	29.9	~31.9	27.9	N
M24283	*ICAM1*	Membrane protein (type i)	14.4	~18.0	10.8	12.4	~13.6	11.2	CGI
U70981	*IL13RA2*	Membrane protein (type i)	4.8	~6.4	3.1	11.6	~13.4	9.7	N
U36501	*SP100*	Nuclear autoantigen	0.3	1.6	−1	4.1	4.1	4.1	N
L10343	*PI3*	Protease inhibitor	3.1	1.7	4.5	15.5	12.2	18.8	N
X04470	*SLPI*	Proteinase inhibitor	9.2	~13.1	5.2	12.2	~18.9	5.5	N
X65965	*SOD2*	Superoxide dismutase	5.3	5.2	5.3	4.7	4.4	4.9	N
X57579	*INHBA*	TGF-β family	3.4	3.3	3.5	5.2	5	5.3	CGI
M59465	*TNFAIP3*	TNF-α induced	6.3	7.4	5.1	6.1	7	5.2	CGI
M97935	***STAT1***	**Transcription factor**	**1.9**	**2**	**1.7**	**4.4**	**4.5**	**4.3**	**CGI**
Other genes									
U87459	*CTAG1*	Cancer antigen	7.9	7.1	8.6	10.9	7.5	14.3	CGI
X54925	*MMP1*	Collagenase	7.4	~5.4	~9.3	27.9	~11.7	~44.1	N
M30703	*AREG*	Growth factor	8.9	~6.9	~10.9	5.8	~4.8	6.7	CGI
U90551	***H2AFL***	**Histone**	**4.1**	**~3.5**	**~4.6**	**9.5**	**~5.5**	**~13.4**	**CGI**
L19779	*H2AFM*	Histone	3.2	3	3.3	5.4	5.3	5.4	CGI
Z80780	***H2BFH***	**Histone**	**2.5**	**~2.6**	**2.3**	**6.9**	**~7.0**	**6.8**	**CGI**
AB000115	*GS3686*	Hypothetical protein	6.4	6.6	6.1	12.3	10.8	13.8	N
M32053	***H19***	**Imprinted gene**	**10.7**	**10**	**11.4**	**6.6**	**7.1**	**6.1**	**CGI**
M80563	*S100A4*	Malignant suppression	18.5	~15.3	~21.7	12.8	~13.0	12.5	N
X55740	*NT5*	Membrane protein	1.9	2.4	1.3	5.7	7.3	4.1	CGI
M60278	*DTR*	Membrane protein (type I)	2.9	2.8	3	6.6	5.3	7.8	CGI
M29277	*MCAM*	Membrane protein (type I)	3.3	1.4	5.2	18.5	10.3	26.7	CGI
J02973	*THBD*	Membrane receptor	4.7	~5.8	3.5	6.0	~5.7	6.2	CGI
U31875	*HEP27*	Metabolism	0.3	−1.1	1.7	7.9	5.3	10.4	N
U66726	*DAZL*	RNA binding protein	13.9	~7.1	~20.7	13.6	~7.1	~20.0	?
U25997	*STC1*	Secreted protein	8.1	~6.5	~9.6	17.0	~11.0	~23.0	N
U32315	*STX3A*	Syntaxin family	1.9	~2.1	1.6	4.5	~4.8	4.2	CGI
X04741	*UCHL1*	Thiol protease	5.1	~2.5	~7.6	17.2	~14.8	~19.6	CGI
X79200	*SSX2*	Transcription factor	4.7	~3.0	~6.3	11.0	~4.9	~17.0	N
S82471	*SSX3*	Transcription factor	6.1	4	~8.2	12.8	6.3	~19.2	N
X99133	*LCN2*	Transport	4.4	~2.2	~6.5	5.6	~4.3	~6.9	CGI
U19147	*GAGE6*	Tumor-specific expression	6.1	~3.1	~9.0	21.7	~8.4	~34.9	N
L18877	*MAGEA12*	Tumor transformation	5.6	4.5	6.6	16.9	14.4	19.4	N
L18920	*MAGEA2*	Tumor transformation	6.4	~3.2	~9.5	22.6	~14.9	~30.2	CGI
U03735	*MAGEA3*	Tumor transformation	8.2	~4.3	12	18.5	~7.1	29.8	CGI
U10687	***MAGEA4***	**Tumor transformation**	**8.1**	**5.4**	**~10.8**	**15.5**	**5.9**	**~25.1**	**N**
M31551	*PAI2*	Urokinase inhibitor	2.6	~1.4	~3.8	43.7	~20.5	~66.8	N
X99699	*HSXIAPAF1*[c]	XIAP associated factor	4.9	~6.7	3	9.7	~13.7	5.7	N
Down-regulated genes[b]									
M35252_at	*TM4SF3*	Membrane protein	1.1	1.1	1	−4.7	−5.3	−4	N
X07696_at	*KRT15*	Cytokeratin	−1.6	−1.5	−1.7	−14.9	−9.8	~−19.9	N

[a] A "~" preceding the fold change value indicates that the expression level for the untreated sample was under the calculated noise level; therefore, the fold change is an approximation.

[b] Bold represents genes induced in both LD419 and T24 cells.

[c] Based on a PubMed search; "CGI" means 5′ CpG island; "N" means no known 5′ CpG island.

pathways are not clear; however, major functions induced by IFN induce antiviral activities in addition to inhibition cell growth, control of apoptosis, and activity of immune system (29). The activation of the IFN signaling pathway may have therapeutic implications, because pretreatment with 5-Aza-CdR sensitizes human colon cancer cell lines to subsequent IFN treatment (13). Demethylation of endogenous retrotransposons by 5-Aza-CdR may lead to their transcription (30, 31) and could also possibly activate the IFN pathway.

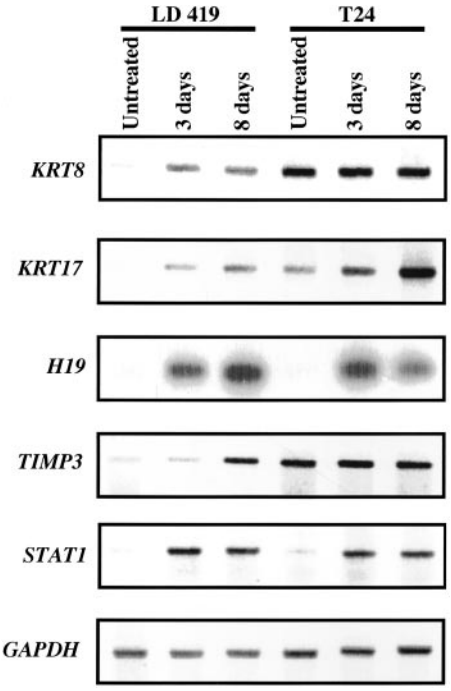


Fig. 2. RT-PCR analysis of gene expression data from LD419 and T24 5-Aza-CdR untreated and treated cells (3 and 8 days after treatment). *GAPDH* expression level is used as a control.

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Cancer Res 2002;62:961-966.