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Differential Gene Expression in Human Glioma Cells: Correlation With Presence or Absence of DNA-Dependent Protein Kinase

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The human glioma cell line M059J is deficient in DNA-dependent protein kinase (DNA-PK) due to a frameshift mutation in *PRKDC*, the gene for its catalytic subunit, while cell line M059K, isolated from the same malignant tumor, has normal DNA-PK activity. DNA-PK is required for double-strand DNA break repair, and its absence is responsible for increased radiosensitivity of M059J. We show that transcripts of several melanoma antigen subfamily A (*MAGE-A*) genes, the expression of which is restricted to tumor and germ-line cells, are present in M059K, but that their expression is strongly downregulated in M059J. Normal levels of *MAGE-A* expression are restored in the *PRKDC*-complemented cell line M059J/Fus1, suggesting that the presence of DNA-PK is required for *MAGE-A* gene transcription. We also show that the *MAGE-A1* promoter is methylated in M059J, while the promoter is demethylated in M059K and M059J/Fus1. Other genes, including all three major histocompatibility class I (*HLA*) genes, *BENE*, and an unnamed gene related to *CNIL* (CORNICHON-like), display an opposite expression profile (i.e., they are upregulated in the DNA-PK-deficient cell line, but show low levels of expression in both M059K and in the *PRKDC*-complemented cell line). For these genes, differential expression does not correlate with DNA methylation in upstream promoter sequences. Our results suggest that the presence of DNA-PK can exert effects on gene expression by various mechanisms and pathways, thus affecting overall cell physiology even in the absence of DNA damage.

Malignant glioma cell lines DNA-dependent protein kinase Melanoma-associated antigen DNA methylation RNA fingerprinting

MANY protein kinases are components of signal transduction pathways that regulate gene expression, and much research effort is directed toward understanding which protein kinases and signals affect which genes. Signals are typically transduced through cascades of protein kinases, and the end targets often are nuclear proteins involved in transcription and transcriptional regulation (7,29). DNA-dependent protein kinase (DNA-PK) is a nuclear, heterotrimeric serine/threonine kinase that is activated by DNA, particularly by double-stranded DNA ends. Its components are a 470-kDa catalytic subunit (PRKDC) and the 70-/86-kDa Ku proteins, which form a DNA binding heterodimer. Results of both genetic and biochemical experiments established that DNA-PK plays a central role in the repair of double-strand DNA breaks (2,12,26,33). Nevertheless, its precise function during this process as well as its physiological substrates remain largely unknown. Its DNA binding properties and its abundance suggest that it might fulfill a structural role by aligning the broken DNA ends. Furthermore, evidence has been presented that the kinase function (i.e., protein phosphorylation) is directly required for the repair process (23,32). However, it is generally assumed that the kinase also plays a role in signaling to the cell the presence of DNA breaks. Candidate

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downstream targets for such a signaling function include the tumor suppressor protein p53 and the proto-oncogene c-*abl* (31,46).

Early studies with cell-free transcription systems identified DNA-PK as a template-dependent kinase activity (5,27). DNA-PK was found to phosphorylate many components of the transcription machinery, including the C-terminal domain of RNA polymerase II, general transcription factors, as well as transcriptional activators (17). Therefore, the notion that DNA-PK may have a role in transcriptional regulation under normal physiological conditions has attracted considerable attention, and several studies supporting such a role have been published. To explain how DNA-PK could be activated in the absence of DNA ends, it has been proposed that activation of PRKDC could be achieved by its association with DNA binding proteins other than Ku70/86 (39,48). Furthermore, the PRKDC/Ku complex was found to bind in a sequence-specific manner to a promoter element in the long terminal repeat (LTR) of mouse mammary tumor virus (MMTV) and to downregulate transcription, possibly by phosphorylation of the glucocorticoid receptor (21). The presence of DNA-PK in the cell was also reported to lead to reduced DNA binding and activity of transcription factor NF-κB due to phosphorylation of its inhibitor IκBα (35). A study showing decreased RNA polymerase II transcription in extracts from DNA-PK-deficient cells also suggests a transcriptional role for DNA-PK, albeit an indirect one (47).

Here we used a human glioma cell system to identify candidate target genes for DNA-PK. M059K contains wild-type DNA-PK activity, while M059J is DNA-PK deficient (1). M059J is still the only human DNA-PK-deficient cell line available, and because M059K was isolated from the same tumor, this cell pair has provided a useful model system for cellular and molecular studies addressing the function of DNA-PK. In the present screen for differentially expressed genes, we also included a M059J cell line that had been complemented with a fragment of chromosome 8 containing *PRKDC* (24). The hypothesis underlying the present study is that DNA-PK is an effector regulating gene expression even without being specifically activated by DNA damage-induced DNA ends. We identified several genes that are differentially expressed between M059K and M059J, and each of these genes shows a similar level of expression in both M059K and the *PRKDC*-complemented cell line, suggesting that DNA-PK is the cause for their differential expression. Among the genes identified are several members of the *MAGE* subfamily A genes, which encode tumor-specific antigens (14).

MATERIALS AND METHODS

Cell Culture

M059K and M059J were purchased from ATCC and maintained in DMEM/Ham's F-12 (Gibco-BRL) supplemented with 10% fetal calf serum. M059J/Fus1 (24) was a generous gift from Dr. C. Kirchgessner (Stanford, CA). M059J/Fus1 was grown in the same medium in the presence of 250 μg/ml geneticin (G418) to ensure the maintenance of the chromosome 8 fragment.

RNA Arbitrarily Primed PCR (RAP-PCR)

RNA was isolated from growing cells using the RNeasy midi kit from Qiagen. For RAP-PCR, purified RNA was additionally treated with RQ DNAseI (Promega). RAP-PCR reactions using various pairs of arbitrary primers were performed as described previously (37), except that no radioactively labeled dNTP was included. Unlabeled PCR products were electrophoresed on nondenaturing polyacrylamide gels and detected by silver staining (8). Differentially expressed products were eluted from the gel, amplified with the same two arbitrary primers, and directly sequenced.

Gene Expression Analysis

For semiquantitative reverse transcriptase PCR (RT-PCR), random-primed or oligo-dT-primed cDNA was made using Invitrogen Life Technologies' Superscript first-strand synthesis system. cDNA was diluted fourfold in TE (10 mM Tris, pH 7.6, 0.2 mM EDTA) and various amounts were used in PCR reactions using the gene-specific primers shown in Table 1. Primers were selected using the Primer3 software of the Whitehead Institute/MIT Center for Genome Research. Amplification was for 20 cycles in a 25-μl reaction containing 1 μCi [α-^{32}P]dCTP (3000 Ci/mmol), 50 μM dNTPs, and 0.8 μM primers. PCR products were analyzed on 5% nondenaturing polyacrylamide gels and visualized by autoradiography. Appropriate exposures were scanned and bands were quantitated using the NIH image 1.62 software. For Northern blot analysis, RNA was electrophoresed on formaldehyde-agarose gels and transferred to Nylon membranes following standard protocols. To generate hybridization probes, PCR products amplified from cDNA with *BENE*- and *MAGE-A10*-specific primers (see Table 1) were gel purified and labeled by random hexamer-primed DNA synthesis with DNA polymerase Klenow fragment.

DNA Methylation Analysis

PCR methylation assays were performed similar to those described previously (15). Total DNA from

TABLE 1
OLIGONUCLEOTIDE PRIMERS FOR RT-PCR AND DNA METHYLATION ASSAYS

	Forward	Reverse	PCR Product Size (bp)		
			cDNA	Genomic	Reference
RT-PCR assays					
PRKDC	CCAGTCCCTGAGTTGATGCCTT	CCTTCTCATGACCCAGGAGTAG	352	3187	
Ku86	TGGCCCCCAAAGACAAACCAAG	TTTTGGCTCCCCTTGAATGGCC	184	975	
BENE	CTGCAAGGATGGGTGATGTA	GCTGAAGGCATGGAGAATGT	285	6003	
XM086	TCATGAATGCCGACACTTTG	TACTCCCCCACACCACTGAT	392	5014	
MAGE-A1	CGGCCGAAGGAACCTGACCCAG	GCTGGAACCCTCACTGGGTTGCC	421	3307	14
MAGE-A2	AAGTAGGACCCGAGGCACTG	GAAGAGGAAGAAGCGGTCTG	236	317	14
MAGE-A3	TGGAGGACCAGAGGCCCCC	GGACGATTATCAGGAGGCCTGC	725	805	14
MAGE-A6	TGGAGGACCAGAGGCCCCC	CAGGATGATTATCAGGAAGCCTGT	727	807	14
MAGE-A10	GGTCCTGCCAGACAGTGAGT	GGGTGCAGTAGCCCTCTATG	362	362	
MAGE-A11	TGTGGGCACTCTAGAGGAGTT	GGGAAAAGGACTCAGGGTCT	188	188	
MAGE-A12	CCTCCCCACTACCATCAACTAT	CATCAGATGCCTCCAACACA	488	488	
HLA-C	ACCGGGAGACACAGAAGTACAA	TCTCCTTCCCATTCTTCAGGTA	351	597	
HLA-A	GCTTCTACCCTGCGGAGATCA	CCTGGGCACTGTCACTGCTT	390	1058	28 (reverse)
HLA-B	GCTTCTACCCTGCGGAGATCA	GACAGCCAGGCCAGCAACA	260	353	28 (reverse)
HLA-C(2)	GCTTCTACCCTGCGGAGATCA	TCAGAGCCCTGGGCACTGTT	382	1053	28 (reverse)
Methylation assays					
MAGE-A1 (a)	GTTCCCGCCAGGAAACAT	GGGGCTCTCTATTTGGAG	N/A	196	15
MAGE-A1 (b)	GCCACTGACTTGCGCATT	GGGGCTCTCTATTTGGAG	N/A	159	15
BENE (c)	CTTGGAAAAGGGCAAAACAG	CGGGAGTCTCCTCCTAGACC	N/A	222	
XM086 (d)	CACTCCGATAGGCGAAACTG	TCTCGGAGGAAACTTGAAGC	N/A	198	
HLA-C (e)	CTCAGGGTCTCAGGCTCCAA	AGCAGCAGGATGAGGGTTC	N/A	287	

Primers for *MAGE-A1, -A2, -A3*, and *-A6*, the locus-specific *HLA* primers (reverse only), and the primers for the methylation assay of the *MAGE-A1* promoter were as described in the references given. All other primers were designed with the Primer3 software program of the Whitehead Institute. The predicted lengths (in bp) of the cDNA or genomic amplification products are given. The size of all PCR products was consistent with their predicted size as judged by their electrophoretic migration relative to end-labeled *Hpa*II-digested plasmid DNA (data not shown). Selected PCR products were also confirmed by sequencing.

M059K, M059J, and M059J/Fus1 was prepared by lysis and digestion of cells in proteinase K and 0.5% SDS, organic extraction, and ethanol precipitation. After treatment with RNAseA, the sample was reextracted and reprecipitated. Each DNA was digested separately by *Hpa*II (methylation sensitive), *Msp*I (methylation insensitive), and *Xho*I (does not cut in the amplified segments). Digested DNA (100 ng) was used in PCR reactions containing [α-^{32}P]dCTP as described above for RT-PCR, except that amplification was for 25–30 cycles. The primers used are shown in Table 1. To induce DNA demethylation, cells were treated with 2 μM 5′-aza-2′-deoxycytidine (aza-C; Sigma) for 4 days. Control cells were treated with the corresponding amount of solvent (DMSO). RNA was isolated and analyzed by RT-PCR as described above.

RESULTS

Three closely matched human glioma cell lines were used in this study: M059J, which is DNA-PK deficient due to a frame-shift mutation in exon 32 in *PRKDC*, and M059K and M059J/Fus1, which express active DNA-PK (1,3,24). M059K contains a wild-type *PRKDC*, and M059J/Fus1 had been generated by complementation of M059J with a fragment of chromosome 8 containing *PRKDC*. We first wanted to confirm the status of DNA-PK in these three cell lines. PCR amplification of exon 32 from total DNA showed the wild-type sequence in M059K, while both M059J and M059J/Fus1 revealed the mutated sequence (data not shown), consistent with the expectation that M059J/Fus1 contained a single copy of the wild-type gene alongside four to five copies of the mutated gene (3,24). In agreement with a previous report (19), RT-PCR analysis showed about 10 times reduced levels of *PRKDC* mRNA in M059J compared with M059K, and in M059J/Fus1 the mRNA levels were restored to about 50% of the levels seen in M059K (Fig. 1A). Western blot analysis and kinase assays showed that PRKDC protein and DNA-PK kinase activity were absent in M059J, while in M059J/Fus1 both protein amount and kinase activity were restored to about 50% of the level in M059K (data not shown). The levels of both Ku86 mRNA (Fig. 1A, B) and Ku70 protein (Western blot analysis not shown) were similar in all three cell lines. These

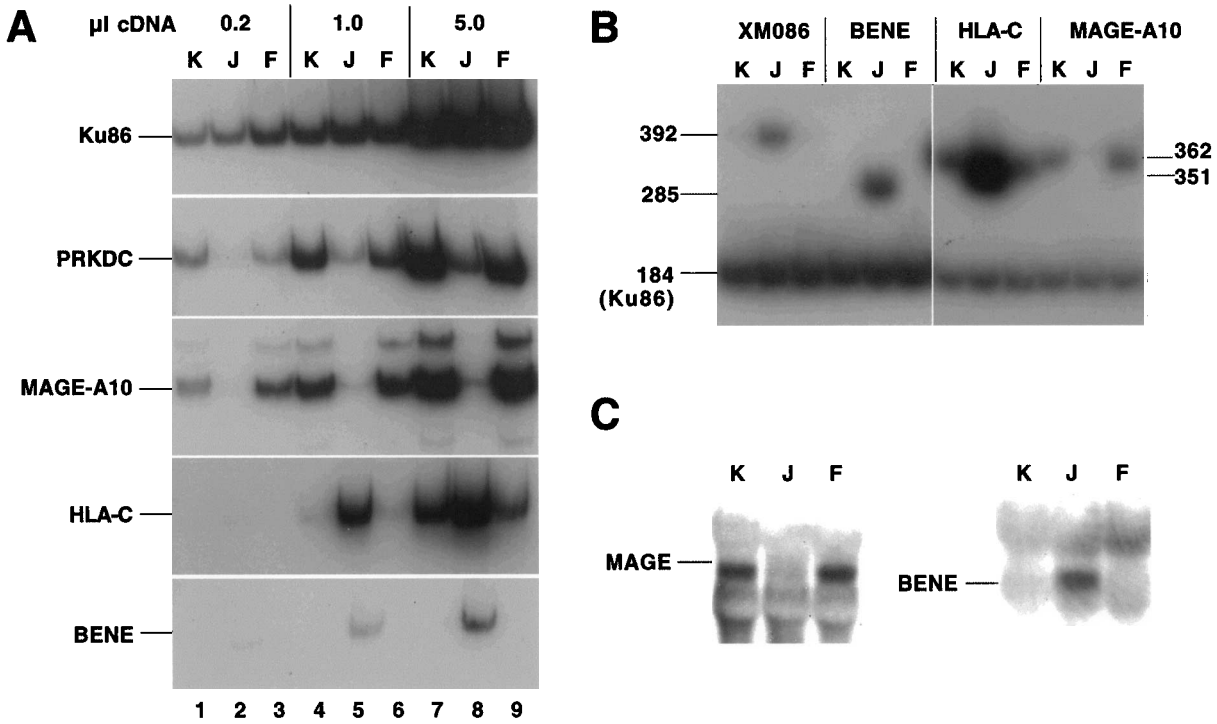

Figure 1. Differentially expressed genes in human glioma cell lines M059K, M059J, and M059J/Fus1. (A) Semiquantitative RT-PCR assays. cDNA (0.2, 1, and 5 µl) was used in amplification reactions with five sets of gene-specific primers, as indicated to the left. In all figures, the cell lines are abbreviated as follows: K, M059K; J, M059J; F, M059J/Fus1. Based on the dilution series, one can estimate that *MAGE-A10* expression is downregulated at least 25 times in M059J (band in lane 8 is weaker than bands in lanes 1 and 3) and *BENE* is upregulated at least 25 times in M059J (weak band in lane 2, but no bands visible in lanes 7 and 9). A similar comparison of band intensities indicates that *PRKDC* mRNA levels are reduced about 10 times from M059K to M059J, but in M059J/Fus1 they are restored to about 50% of the levels in M059K. (B) RT-PCR assays with Ku86 as internal standard. Amplification reactions as in (A), except that a 184-bp segment of the constitutively expressed Ku86 mRNA was coamplified with the sequence of the differentially expressed gene, as indicated on top. The PCR products are labeled by their predicted size in bp. (C) Northern blot analysis. RNA isolated from M059K (K), M059J (J), and M059J/Fus1 (F) was analyzed by Northern blot hybridization using a probe specific for *MAGE-A10* and *BENE*, respectively. The MAGE probe is likely to hybridize to various members of the subfamily.

results confirmed that the DNA-PK-related properties of the cell lines that we were using were as previously described.

To identify potential downstream target genes of DNA-PK signaling, we searched for differentially expressed transcripts between M059K, M059J, and M059J/Fus1 cells. RNA was isolated from untreated, growing cells and analyzed by RNA arbitrarily primed PCR (37). RAP-PCR products that either amplified from both M059K and M059J/Fus1 RNA but not from M059J RNA, or that were only amplified from M059J RNA were isolated and sequenced. The following four differentially expressed genes were identified: *MAGE-A10, BENE, HLA-C* (Fig. 1A–C), and an unnamed gene encoding a protein with similarity to *CNIL*, a "CORNICHON-like" protein (GenBank XM_086429; herein referred to as "*XM086*") (Fig. 1B, see also Fig. 5).

MAGE-A10 mRNA was expressed in the DNA-PK-containing cell lines M059K and M059J/Fus1, but was barely detectable in M059J. Semiquantitative PCR of serially diluted cDNA showed that its mRNA was downregulated at least 25 times in the DNA-PK-deficient cell line (Fig. 1A). The differential expression was confirmed by coamplification of a segment of the constitutively expressed Ku86 mRNA and by Northern blot analysis (Fig. 1B, C). *MAGE-A10* is a member of a family of genes that show tumor- and germ-line-specific expression (14). We therefore examined whether other *MAGE* genes were also differentially expressed in the present cell lines. Figure 2 shows that most of the *MAGE* genes that had detectable levels of mRNA in M059K cells were strongly downregulated in M059J cells and their expression was rescued in the *PRKDC*-complemented cell line M059J/Fus1. *MAGE* genes showing this expression pattern include *MAGE-A1, -A2, -A3, -A6, -A10*, and *-A11*. *MAGE-A4* was weakly expressed in M059K, was not detectable in M059J, and was only partially rescued in M059J/Fus1 (not shown). *MAGE-A12* expression differed from the typical *MAGE-A* profile in that the downregulation in M059J was less (Fig. 2).

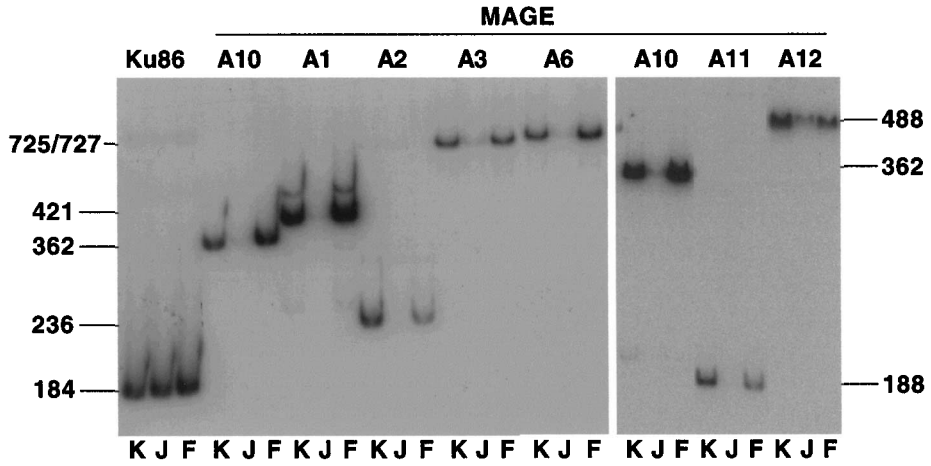

Figure 2. Expression of *MAGE-A* genes in cell lines M059K, M059J, and M059J/Fus1. Seven different members of the *MAGE-A* subfamily (as indicated on top) and the constitutively expressed Ku86 gene were analyzed by RT-PCR as in Figure 1. The predicted sizes (in bp) of the PCR products are indicated. All *MAGE-A* genes that show detectable expression in M059K (lanes K) are downregulated in M059J (lanes J) and their expression is fully or partially restored in M059J/Fus1 (lanes F).

No significant expression of *MAGE-A5, -A8*, and *-A9* was detected (data not shown). The specificity of all PCR primers was confirmed by sequencing the amplified DNA products.

The other three genes displayed a converse expression profile (i.e., their mRNA levels were increased in the M059J-deficient cell line, but in the *PRKDC*-complemented cell line M059J/Fus1 their mRNA levels were reduced to the same low level present in M059K). Semiquantitative PCR of serially diluted cDNA indicated that *HLA-C* mRNA expression was increased about 5 times in M059J over the expression in the other two cell lines (Fig. 1A, B). The sequence of the amplified product was 100% identical to GenBank entry M99389 [human MHC class I *HLA* heavy chain (HLA-Cw-0304) mRNA]. *HLA-C* is one of three genes encoding major histocompatibility class I molecules. We therefore examined the expression of the other two genes, *HLA-A* and *HLA-B*, using recently described locus-specific RT-PCR primers (28). Figure 3 shows that all three "classical" *HLA* mRNAs showed the same M059J-upregulated expression pattern. Similar to the *HLA* genes, the expression of *BENE* and *XM086* was increased in the DNA-PK-deficient cell line M059J. However, the differential expression was more pronounced: both of these transcripts were almost nondetectable in M059K and M059J/Fus1 and their levels were increased at least 25 times in M059J (Fig. 1A, B). The M059J-specific expression of *BENE* mRNA was also demonstrated by Northern blot analysis (Fig. 1C).

DNA methylation was found to be a major mechanism by which *MAGE* genes are repressed in normal cells (15). We therefore examined whether DNA methylation in the *MAGE-A1* promoter was different between M059K and M059J cells. The *MAGE* promoters are CG rich, but they are not classified as CpG islands by the criteria used in an annotated human genome assembly [(30); http://genome.ucsc.edu/]. Using a previously described PCR-based DNA methylation assay (15) we found that two *Hpa*II sites in the

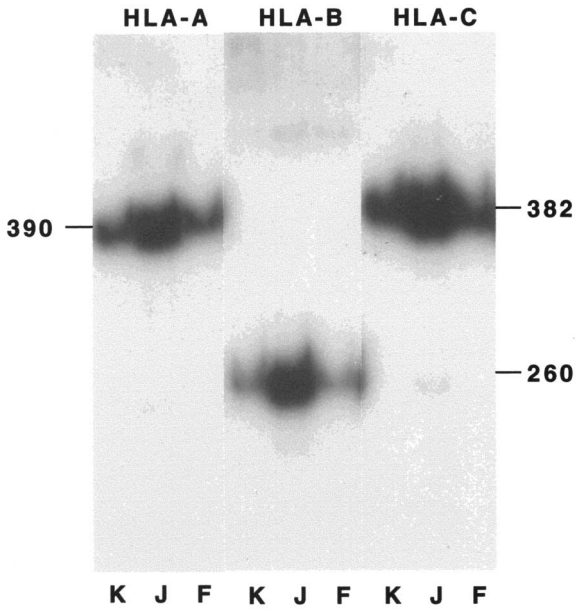

Figure 3. Expression of classical *HLA* genes in cell lines M059K, M059J, and M059J/Fus1. Locus-specific primer sets (see Table 1) were used to analyze by RT-PCR the expression of *HLA-A, HLA-B*, and *HLA-C*. The predicted sizes (in bp) of the PCR products are indicated. Note that all three HLA genes are upregulated (about fivefold) in M059J (lanes J) compared with M059K and M059J/Fus1 (lanes K and F).

MAGE-A1 promoter were completely resistant to digestion in DNA from M059J cells, but sensitive in both M059K and M059J/Fus1 cells (Fig. 4A, lanes 1–3, product a). The same sites were cleaved by the methylation-insensitive isoschizomer *Msp*I in all three cell lines (Fig. 4, lanes 4–6), while the expected PCR product was formed from genomic DNA digested with *Xho*I (which does not cut in the amplified segment) (Fig. 4, lanes 7–9). This result indicated that these sites were indeed methylated in M059J cells, but at least one of these sites was demethylated in M059K and M059J/Fus1 cells. Thus, promoter methylation correlated with the low expression of *MAGE-A1* in M059J but complementation with a chromosomal fragment containing *PRKDC* led to both demethylation and reactivation of the *MAGE-A1* promoter. Even though all *MAGE-A* genes are located on the X chromosome, our results were not affected by the presence of an inactive X chromosome, because the present glioma cell lines were derived from a male patient (1).

The promoters for *BENE* and *XM086* have not been characterized yet, but the fact that both of these genes have a CpG island encompassing the 5′ ends of the longest identified mRNAs (30) makes it very likely that the promoter is located upstream from these 5′ ends. To investigate the methylation status of these promoters, we designed various primer pairs that would amplify a genomic DNA segment containing two *Hpa*II sites (Fig. 4B) and used them in PCR reactions with *Hpa*II-, *Msp*I-, and *Xho*I-digested DNA from the three cell lines. The result showed that in

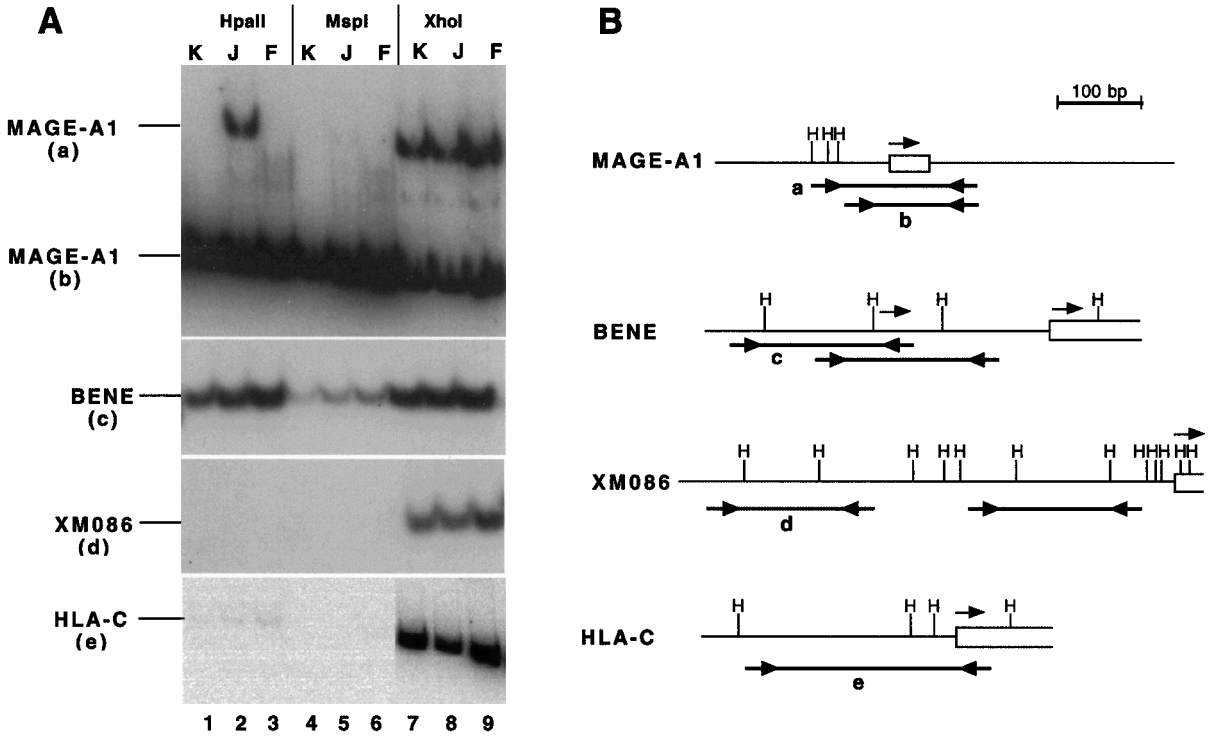

Figure 4. Methylation analysis of differentially expressed promoters in cell lines M059K, M059J, and M059J/Fus1. The methylation status of selected *Hpa*II sites in the *MAGE-A1* and in putative *BENE* and *XM086* promoters was analyzed with a PCR assay. Genomic segments containing two *Hpa*II sites were amplified from genomic DNA digested with *Hpa*II, *Msp*I, and *Xho*I. (A) Amplification products of *Hpa*II-, *Msp*I-, and *Xho*I-digested DNA (as indicated on top) from M059K (lanes 1, 4, 7), M059J (lanes 2, 5, 8), and M059J/Fus1 (lanes 3, 6, 9). *MAGE-A1* promoter segments a and b (see B) were amplified separately and combined before electrophoresis. Segment b is devoid of *Hpa*II sites and serves as an additional control. For both *BENE* and *XM086* only the result of one primer pair (c and d, respectively) is shown; the second primer pair (see B; not labeled) gave a similar result. Note that the sites in the *MAGE-A1* promoter are fully methylated in M059J (lane 2, a), but at least one site is demethylated in M059K and M059J/Fus1 (lanes 1 and 3, a). On the hand, the other three promoters analyzed do not show a difference in methylation between the three cell lines, but *BENE* is largely methylated (lanes 1–3, c), while *XM086* and *HLA-C* are largely demethylated (lanes 1–3, d and e). It is unclear why *Msp*I did not completely digest the genomic target sequence of the *BENE* primers (lanes 4–6, c). (B) Maps of the promoters for *MAGE-A1* and *HLA-C*, and of putative promoters for *BENE* and *XM086*. The segments amplified are indicated by thick lines with inward-facing arrows, the first exon by an open box, the transcription start site by a short arrow. The second arrow in the *BENE* map indicates a possible alternate start site. Two different primer sets were used for the putative *BENE* and *XM086* promoters, assaying a total of three and four *Hpa*II sites, respectively. H, *Hpa*II site. The maps shown correspond to the following coordinates on the human genome assembly (Dec. 22, 2001 draft assembly; http://genome.ucsc.edu/): *MAGE-A1*: chromosome X, 148680052-0591; *BENE*: chromosome 2, 109176316-6855; *XM086*: chromosome 1, 215006556-7155. The map for the *HLA-C* promoter is based on GenBank entry AJ318865.

the putative *BENE* promoter the targeted *Hpa*II sites were resistant to digestion, suggesting that they were largely methylated (Fig. 4A, lanes 1–3, c). On the other hand, the targeted *Hpa*II sites in the putative *XM086* promoter were sensitive to *Hpa*II digestion, indicating that they were mostly demethylated (Fig. 4A, lanes 1–3, d). Finally, we analyzed the *HLA-C* promoter, which also overlaps with a CpG island. Two *Hpa*II sites located 29 and 56 bp upstream of the transcription start site showed only minimal resistance to digestion with *Hpa*II, indicating a minimal level of methylation (Fig. 4A, lanes 1–3, e). A similar result was also obtained with primers specific for the *HLA-B* promoter (data not shown). Most importantly, and in contrast to the result seen with the *MAGE-A1* promoter, there was no difference in the methylation status of these three promoters between the three cell lines. We conclude that DNA methylation does not play a major role in the observed differential expression of *BENE, XM086*, and *HLA* genes in M059K, M059J, and M059J/Fus1.

The demethylating agent 5′-aza-2′-deoxycytidine (aza-C) has been widely used to induce the expression of genes that are repressed by DNA methylation. Indeed, aza-C was found to activate *MAGE* gene expression in cell lines that do not normally express *MAGE* (15,16). In agreement with these findings, we found that aza-C treatment of M059J induced *MAGE-A10* gene expression (Fig. 5, lane 4). *MAGE-A1* expression was also analyzed and was found to be induced as well (data not shown). aza-C treatment did not affect the high expression of these two *MAGE* genes in M059K and M059J/Fus1 (Fig. 5, lanes 1, 2, 5, 6). However, aza-C induced the repressed *BENE* gene in M059K and M059J/Fus1 (Fig. 5, lanes 2 and 6), consistent with methylation in its upstream promoter sequence. On the other hand, *XM086* and *HLA* expression was only minimally induced (less than twofold) in any of the cell lines, the slight induction possibly reflecting a low level of methylation. aza-C had no significant effect on the highly expressed Ku86 mRNA in all three cell lines (Fig. 5). These findings suggest that DNA methylation may have some repressive effect on several of the genes analyzed, but that other regulatory mechanisms normally dominate over the effect of DNA methylation.

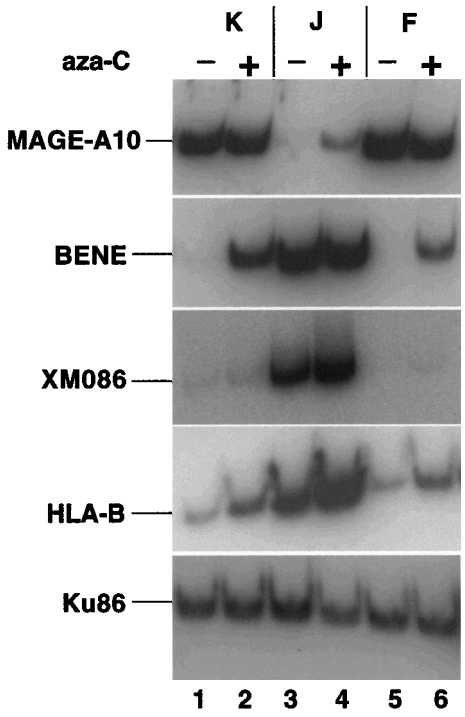

Figure 5. Effect of the demethylating agent aza-C on gene expression in cell lines M059K, M059J, and M059J/Fus1. Semiquantitative RT-PCR assays as in Figure 1A. The genes analyzed are indicated to the left. RNA was either from untreated cells (– lanes) or aza-C-treated cells (+ lanes). aza-C induces expression of the repressed *MAGE-A10* mRNA in M059J (lane 4) and of the repressed *BENE* mRNA in both M059K and M059J/Fus1 (lanes 2, 6). aza-C only slightly stimulates expression of *XM086* and *HLA-B* in all three cell lines. aza-C has no effect on the constitutively expressed Ku86 mRNA, but also does not further stimulate the expression of *MAGE-A10* in M059K and M059J/Fus1 (lanes 2, 6) and *BENE* in M059J (lanes 4). All the aza-C induction results were confirmed with additional RT-PCR reactions using a different amount of input cDNA (data not shown).

DISCUSSION

M059K and M059J are a pair of glioma cell lines that were derived from the same tumor, but differ in their sensitivity toward ionizing radiation and chemotherapeutic agents (1). M059K is resistant and M059J is sensitive. While the two cell lines are near pentaploid and have different karyotypes (3,24), the only known difference between their genomic DNA sequences is a frame-shift mutation in *PRKDC* in M059J (3). This mutation results in about 10- to 20-fold lower steady-state levels of *PRKDC* mRNA (presumably due to reduced stability of the mRNA) and in a complete absence of DNA-PK activity (19,34). We have been interested in whether DNA-PK may have a role in regulating gene expression, either by its presence or by its kinase activity. We therefore began to search for transcripts that are differentially expressed between M059K and M059J. Because the mutation in *PRKDC* may not be the only genetic difference between M059K and M059J, we also included in the screen a previously described *PRKDC*-complemented cell line (24). This cell line, M059J/Fus1, was generated by introducing into M059J a fragment of chromosome 8 containing *PRKDC*. This fragment is estimated to be about 10 Mb in size and thus clearly

contains many other genes in addition to *PRKDC*. While we cannot fully rule out that any complementation effects are due to genes other than *PRKDC*, such a scenario is considered unlikely because it would require that such a gene be mutated in M059J and wild-type in M059K and at the same time regulates gene expression [see also discussion in (24)]. In the present study we identified several genes that show strong differential expression between M059K and M059J. Most importantly, in the *PRKDC*-complemented M059J cell line the expression level of most of these genes is fully reverted to the one seen in M059K, suggesting that these genes are candidate downstream targets of DNA-PK signaling. It is important to note that this "reversal" of the gene expression profile rules out the possibility that the increased expression of *BENE, XM086*, and *HLA* genes in M059J is due to gene amplification.

MAGE-A genes were the only putative DNA-PK targets identified in this study with an expression that is positively correlated with the presence of DNA-PK. MAGE proteins belong to a family of tumor-specific antigens that were originally identified in melanoma cells and have attracted much attention because of their potential use as targets for cancer immunotherapy (6,14,18,22). The family comprises at least 25 human genes, 12 of which belong to the A subfamily. The cellular function of the encoded proteins is not known. There is no *MAGE* gene expression in healthy tissues, except for in testis and placenta. The expression of the various members of *MAGE* genes varies in different tumor types and tumor-derived cell lines and generally correlates with more progressed, metastatic tumor stages (9). Current research is testing the idea that the *MAGE* expression profile could be used to diagnose the type of tumor or its progression status (38,44). The present result is an example of very different *MAGE* gene expression profiles in two closely related tumor cell lines. Even though we did not attempt to quantify absolute expression levels of the various *MAGE* genes in M059K and M059J, *MAGE-A1, -A2, -A3, -A6, -A10, -A11*, and *-A12* were readily detected in M059K, but the expression of all *MAGE-A* genes was reduced in M059J. While the degree of downregulation may vary, our more detailed analysis of *MAGE-A10* (Fig. 1A) and *MAGE-A1* (data not shown) indicates that for these genes the difference in mRNA levels is at least 25 times. Weak or absent expression of *MAGE-A5, -A8*, and *-A9* is consistent with findings in other tumor cell lines (14). Different *MAGE* gene expression in closely related cell lines is not without precedent. Independently cultured cell lines of the erythroleukemia cell line K562 have been found to differ with respect to their *MAGE-A1* gene expression, and the differential gene expression was correlated with differential DNA methylation in the *MAGE* promoters (16,43). The cause for the differential *MAGE* gene expression in the erythroleukemia cells is not known. Here we show that a nonexpressing tumor cell line with methylated *MAGE* promoters can be converted to a *MAGE-A*-expressing cell line with demethylated *MAGE* promoters by a fragment of chromosome 8 containing *PRKDC*. It has been proposed that the demethylation of the *MAGE* promoters, as in M059K, is a "consequence of a genome-wide demethylation process that occurs in many cancers and is correlated with tumor progression" (15).

All other putative DNA-PK target genes identified in this study are negatively correlated with DNA-PK expression. The upstream regions of *BENE* and *XM086* and the *HLA* promoters, all constituting typical CpG islands, were investigated for differences in DNA methylation between the three cell lines. No differences were found, suggesting that DNA methylation is not involved in the differential expression of these genes. However, our studies discovered clear differences in promoter methylation and in the response toward aza-C between *BENE* and *XM086*. These findings suggest that *BENE* and *XM086* are regulated by a different mechanism, even though both genes show the same "on–off" type of expression that is inversely correlated with DNA-PK.

Intriguing is the inverse relationship between *HLA* and *MAGE* expression. HLA proteins are cell surface glycoproteins that present antigen peptides to cytotoxic T cells and serve an important function in self-recognition. HLA expression is often downregulated or lost in cancer cells or during tumor progression, possibly helping these cells to escape immune recognition (11,42). Thus, the higher *MAGE* expression, the demethylated *MAGE* promoters, as well as the lower *HLA* expression in M059K and M059J/Fus1 cells all support the notion that these DNA-PK-proficient cell lines represent a more progressed cancer cell than M059J.

It is not possible at this time to assess the significance of the M059J-specific expression of *BENE* and the *CNIL*-like gene *XM086*, because little is known about both of these genes and their encoded proteins. *BENE* is a proteolipid that shows tissue- and cell line-specific expression (36). Its function is unknown, but *BENE* was recently found to be associated with cholesterol-enriched membrane rafts (13). *XM086* mRNA represents an unnamed gene distantly related to CORNICHON (*CNIH*). This gene was originally identified in Drosophila, where it is involved in epidermal growth factor (EGF) signaling during embryogenesis (41). In mammals, *CNIH* and *CNIL* belong to a multigene family (25), and human *CNIH*

was reported to be upregulated in alloactivated T cells (45). These genes appear to encode integral membrane proteins.

Differential gene expression between DNA-PK-containing and DNA-PK-deficient cells has previously been reported. In an attempt to identify genes that are induced by DNA-PK in response to DNA damage, a recent study used RNA from untreated and irradiated M059K and M059J cells to probe Atlas human cancer cDNA expression arrays (20). While the arrays suggested the presence of a number of differentially expressed genes, the regulation of only one gene, *RFC37*, could be confirmed by Northern blot analysis, but its increased expression in M059J was independent of radiation. Subtractive hybridization was used in a related study to identify genes that were differentially expressed between various irradiated mouse and hamster DNA-PK-proficient and DNA-PK-deficient cell lines (10). Only the expression of a laminin gene correlated consistently with the presence of DNA-PK (independent of irradiation), and reintroduction of murine *Prkdc* into the deficient cell line partially rescued laminin expression. Differential expression of all other isolated genes was only seen in specific cell types, indicating that the genetic background contributed to the DNA-PK dependence of expression. Whether the differentially expressed genes identified in this study also show DNA-PK-correlated expression in other (e.g., mouse or hamster) DNA-PK plus and minus cells remains to be seen. It is important to note that the present M059K and M059J glioma cell lines carry a mutated p53 (4), and it is very likely that the observed gene expression profile is specific for tumor cells.

In summary, we show that three related malignant glioma cell lines have distinct gene expression profiles that correlate with the presence or absence of DNA-PK. For the following three reasons we believe that the observed gene regulation is an indirect consequence of low constitutive DNA-PK activity, and not a rapid and direct effect of the activation of DNA-PK. First, the *MAGE-A1* promoter is demethylated in M059K and methylated in M059J, but complementation of M059J with a chromosome fragment containing *PRKDC* leads to complete conversion to the demethylated state. Such drastic changes in methylation usually reflect major cell differentiation processes or "reprogramming" (40). Second, differential gene expression was seen in untreated cells (i.e., DNA-PK in M059K and M059J/Fus1 was not specifically activated by irradiation or treatment with radiomimetic drugs), and attempts to modulate the expression of the identified genes by activating or inhibiting DNA-PK were unsuccessful (R. Ai and P. Labhart, unpublished results). Finally, the differential expression appears to be the result of at least three distinct regulatory mechanisms: a) *MAGE* expression is positively correlated with DNA-PK, b) *BENE* expression is negatively correlated with DNA-PK and its promoter is methylated and aza-C inducible, and c) *XM086* and *HLA* are negatively correlated with DNA-PK and their promoters are demethylated. Thus, it is unlikely that DNA-PK directly regulates a common transcription factor binding to the promoters of these genes.

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