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Genetics

The activation of human gene *MAGE-1* in tumor cells is correlated with genome-wide demethylation

CHARLES DE SMET, OLIVIER DE BACKER, ISABELLA FARAONI*, CHRISTOPHE LURQUIN, FRANCIS BRASSEUR, AND THIERRY BOON†

Ludwig Institute for Cancer Research, Brussels Branch, and Cellular Genetics Unit, Université Catholique de Louvain, Brussels B-1200, Belgium

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ABSTRACT **Human gene *MAGE-1* encodes tumor-specific antigens that are recognized on melanoma cells by autologous cytolytic T lymphocytes. This gene is expressed in a significant proportion of tumors of various histological types, but not in normal tissues except male germ-line cells. We reported previously that reporter genes driven by the *MAGE-1* promoter are active not only in the tumor cell lines that express *MAGE-1* but also in those that do not. This suggests that the critical factor causing the activation of *MAGE-1* in certain tumors is not the presence of the appropriate transcription factors. The two major *MAGE-1* promoter elements have an Ets binding site, which contains a CpG dinucleotide. We report here that these CpG are demethylated in the tumor cell lines that express *MAGE-1*, and are methylated in those that do not express the gene. Methylation of these CpG inhibits the binding of transcription factors, as seen by mobility shift assay. Treatment with the demethylating agent 5-aza-2′-deoxycytidine activated gene *MAGE-1* not only in tumor cell lines but also in primary fibroblasts. Finally, the overall level of CpG methylation was evaluated in 20 different tumor cell lines. It was inversely correlated with the expression of *MAGE-1*. We conclude that the activation of *MAGE-1* in cancer cells is due to the demethylation of the promoter. This appears to be a consequence of a genome-wide demethylation process that occurs in many cancers and is correlated with tumor progression.**

Human melanoma cells express antigens that are recognized by cytolytic T lymphocytes derived from the blood of the tumor-bearing patient or from tumor infiltrating lymphocytes (1). A number of these antigens are encoded by genes of the *MAGE* family (2–4). Twelve *MAGE* genes are located in the q-terminal region of chromosome X (5, 6). Another cluster of several homologous genes, designated *MAGE*-Xp, has been identified in Xp21.3 (7). Presently, nothing is known about the function of these genes.

The *MAGE* genes are expressed not only in melanomas but also in tumors of other histological origins (2, 8, 9). These genes are silent in normal tissues except in testis and, for some, in placenta (6, 10). Recent data indicate that the expression of the *MAGE* genes in testis is restricted to the germ-line cells (11, 12). Because male germ-line cells lack expression of the major histocompatibility complex molecules (13), they are not expected to present *MAGE*-encoded antigens. With regard to cancer immunotherapy, the potential usefulness of the antigens encoded by the *MAGE* genes largely rests on the tumor-specific expression of these genes. For this reason, we have tried to understand the mechanisms governing this specificity.

The promoter region of *MAGE-1* contains several positive regulatory elements located between positions −792 and +47 (14). Two key elements, named B′ and B, drive 90% of the promoter activity (see Fig. 1*A*). They contain an identical but inverted Ets binding site and they bind to the same nuclear proteins, which appear to belong to the family of Ets transcription factors (14). The action of the B′ region was shown to be dependent on the presence of regulatory element A, which is located 40 bp upstream and binds to factors of the Sp1 family (14).

Transfection experiments with luciferase reporter plasmids showed that the *MAGE-1* promoter exerts transcriptional activity not only in tumor cell lines that express the gene but also in those that do not (14). Thus, cells that do not express *MAGE-1* nevertheless contain transcription factors capable of activating the *MAGE-1* promoter. This observation suggests that the tumor-specific expression of *MAGE-1* is not determined by the action of transcription factors present only in tumor cells, and that other mechanisms contribute to the transcriptional regulation of this gene. DNA methylation is known to be an important mechanism of gene regulation, as CpG methylation at specific promoter regions represses transcription (15, 16). We have investigated the role of DNA methylation in the control of *MAGE-1* transcription.

MATERIALS AND METHODS

Cell Cultures. The tumor cell lines were obtained and cultured as described (14). Peripheral blood lymphocytes (PBL) were isolated using Lymphoprep (Nycomed, Oslo) and grown in Iscove's medium (GIBCO) supplemented with 10% fetal calf serum (FCS) (GIBCO), 100 units/ml interleukin-2 (Biogen), and 0.3% (vol/vol) phytohemagglutinin (PHA)-P (Difco). The fibroblast culture was derived from a human lung sample and was maintained in Iscove's medium supplemented with 10% FCS, L-arginine (116 mg/ml), L-asparagine (36 mg/ml), and L-glutamine (216 mg/ml).

PCR Methylation Analysis. DNA was extracted from cell cultures as described by Blin and Stafford (17), except that DNA was finally purified by ethanol precipitation instead of dialysis. For the fibroblasts, the extraction was performed at passage 6. DNA was digested with *Hpa*II (10 units/μg) during 3 h at 37°C, extracted with phenol and chloroform-isoamyl alcohol (24:1), and recovered by ethanol precipitation. *Hpa*II (10 units/μg) was again added to the DNA and the digestion was performed at 37°C for an additional 3 h. After phenol extraction and ethanol precipitation, 100 ng of DNA was amplified in a volume of 25 μl PCR buffer (50 mM KCl/100 mM Tris, pH 8.3) containing 0.2 mM of each dNTP and 0.625 unit of *Taq* polymerase (Perkin–Elmer), and with a sense primer of either CDS21 (5′-GCCACTGACTTGCGCATT-3′:

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Abbreviations: PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin.

*Present address: Istituto di Medicine Sperimentale e Scienze Biochimiche, Università di Roma "Tor Vergata," 0013 Rome, Italy.
†To whom reprint requests should be addressed at: Ludwig Institute for Cancer Research, 74 Avenue Hippocrate–Université Catholique de Louvain 7479, B-1200 Brussels, Belgium.

MAGE-1 −42−−25) or CDS20 (5′-GTTCCCGCCAGGAAACAT-3′: *MAGE-1* −79−−62) and an antisense primer EDP4 (5′-GGGGCTCTCTATTTGGAG-3′: *MAGE-1* 100–117). Amplification was performed in a TRIO-Thermoblock (Biometra, Göttingen, Germany) for 30 cycles: 1 min at 94°C, 2 min at 64°C, 2 min at 72°C. PCR products were electrophoresed in agarose gels, transferred to nylon membranes and hybridized with the CDS22 probe (5′-AGAAGCGAGGTTTCCATT-3′: *MAGE-1* −13–5) as described (10), except that the CDS22 probe was labeled with [γ-^{33}P]ATP (1000–3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) using T4 polynucleotide kinase (BRL).

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from MZ2-MEL.2.2.5 cells by a method previously described (18). Mobility shift assays were performed as described (14). The synthetic oligonucleotides containing a 5-methyl-deoxycytidine were obtained from Pharmacia.

5-Aza-2′-Deoxycytidine Treatment and mRNA Expression Analysis. Treated cells were incubated for 72 h in culture medium containing 1 μM 5-aza-2′-deoxycytidine (Sigma). Fibroblasts were treated at passage 4. Total RNA purification and cDNA synthesis were performed as described (10). Primers and PCR conditions to amplify *MAGE-1* and the β-actin gene were described (10), except that 33 cycles were performed for *MAGE-1*. For the amplification of the tyrosinase gene the primers were VB17 (5′-GGATAGCGGATGCCTCTCAAAG-3′) and VB18 (5′-CCCAAGGAGCCATGACCAGAT-3′), and the cycle conditions were 94°C for 1 min, 65°C for 2 min, 72°C for 2 min, for 33 cycles. PCR products were electrophoresed in agarose gels and hybridized with oligonucleotidic probes recognizing a specific sequence of either *MAGE-1* (5′-TTGCCTCCTCACAGAGCC-3′), tyrosinase (5′-TATTTTTGAGCAGTGGCT-3′), or β-actin (5′-CCAACACAGTGCTGTCTGGC-3′). These probes were labeled with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) using T4 polynucleotide kinase (BRL).

Analysis of DNA for Overall Methyl-CpG Content. DNA from each sample was digested with *Xba*I (20 units/μg) for 2 h at 37°C. The DNA was then extracted with phenol and chloroform-isoamyl alcohol (24:1), recovered by ethanol precipitation, and resuspended in water at 50 μg/ml. The quality and the quantity of the DNA samples were checked on an agarose gel stained with ethidium bromide. For the methylation with M.*Sss*I, 5 μl of cut DNA were added to a mixture containing 1 μl Tris·HCl, 1 M (pH 8.0), 2 μl NEBuffer 2 10 × (New England Biolabs), 10 μl *S*-adenosyl-L-[*methyl*-^{3}H]methionine (14.4 Ci/mmol, 70 μM; Amersham), and 2 μl M.*Sss*I methylase (2 units/μl; New England Biolabs). For the methylation with *dam* methylase, the same amount of DNA was added to the following mixture: 1 μl Tris·HCl, 1 M (pH 7.5), 2 μl NEBuffer for *dam* methylase 10 × (New England Biolabs), 0.5 μl water, 10 μl *S*-adenosyl-L-[*methyl*-^{3}H]methionine, and 1.5 μl *dam* methylase (8 units/μl; New England Biolabs). Both reaction mixtures were incubated for 4 h at 37°C. The reactions were then stopped by 20 min of incubation at 80°C, and the incorporation of radioactivity was measured after precipitation with trichloroacetic acid and filtration through GF/C filters (Whatman). Under these conditions, the DNA substrate is fully methylated because ^{3}H incorporation was shown to be proportional to the amount of input DNA (between 16 ng and 500 ng). For each DNA sample, the incorporated radioactivity obtained with M.*Sss*I, which methylates the C in CpG dinucleotides, was reported to that obtained with *dam* methylase, which methylates the A in GATC sequences. Contrary to the C in CpG dinucleotides, the A in GATC sequences is never methylated in mammalian DNA. The percentage of methylated CpG was estimated from the ratio between M.*Sss*I and *dam* methylase ^{3}H incorporation (*Sss*I/*dam* ratio), as follows:

$$\%\ \text{of methylated CpG} = \left(1 - \frac{\textit{Sss}\text{I}/\textit{dam}\ \text{ratio}}{3.2}\right) \times 100$$

where 3.2 corresponds to the frequency of the *dam* recognition site, which is 1/256, reported to the frequency of the M.*Sss*I recognition site, which is 1/80 considering that the CpG doublet occurs at only ≈20% of the expected frequency (19). Each percentage of methylated CpG that is given corresponds to the mean of at least two values.

RESULTS

Essential Promoter Elements Have Demethylated CpG in the Cells Expressing *MAGE-1*. Both the B′ and B promoter elements of *MAGE-1*, which are essential for promoter activity (14), contain a CpG (Fig. 1*A*). The methylation status of these CpG was analyzed in cells with different *MAGE-1* expressions. Total genomic DNA was digested with the methylation-sensitive restriction enzyme *Hpa*II (CCGG), and two pairs of primers were used for PCR amplification of regions of the *MAGE-1* promoter (Fig. 1*A*). The sequence amplified with primers CDS21 and EDP4 includes no *Hpa*II sites and was used as positive control. Primers CDS20 and EDP4 bracket the two *Hpa*II sites that include the CpG of the B′ and B boxes (Fig. 1*A*). Therefore, amplification products can be obtained with the two last pairs of primers only when the CpG of the B′ and B elements are methylated, preventing *Hpa*II digestion (20).

We tested the DNA from eight tumor cell lines expressing *MAGE-1,* including six melanoma cell lines, a sarcoma cell line, and a head and neck carcinoma cell line. The PCR results

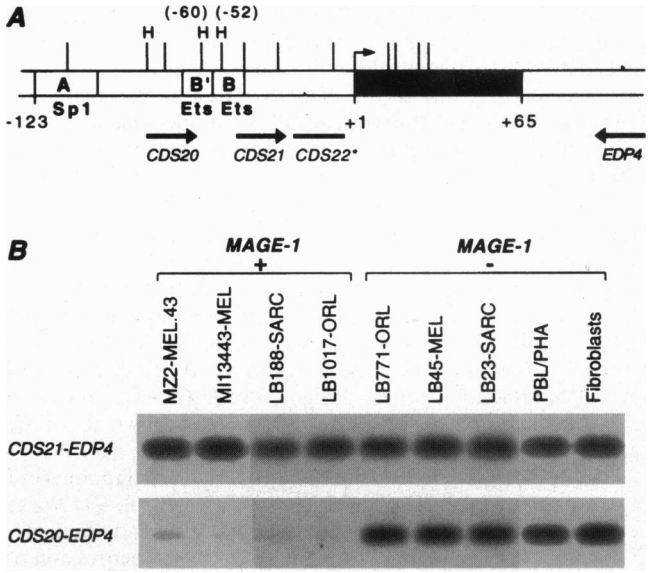

FIG. 1. (*A*) CpG content of the *MAGE-1* promoter sequence. The first exon in black. Positions of CpG dinucleotides are indicated by vertical bars and *Hpa*II sites are indicated (H). The A, B′, and B promoter elements and the binding site they contain are indicated. PCR primers are indicated by arrows, and oligonucleotide probe CDS22* by a solid bar. (*B*) Methylation status of the B′ and B promoter elements in cells that either do or do not express *MAGE-1*. DNA was extracted from tumor cell lines that express *MAGE-1* (*MAGE-1* +), two melanoma lines (-MEL), a sarcoma line (-SARC), and a head and neck carcinoma line (-ORL). DNA was also extracted from tumor cell lines that do not express *MAGE-1* (*MAGE-1* −), from PHA-activated PBL/PHA, and from primary fibroblasts. *Hpa*II-digested genomic DNA was submitted to PCR amplification using two pairs of primers: CDS21–EDP4 and CDS20–EDP4. PCR products were separated in agarose gels, blotted on nylon membranes, and hybridized with radiolabeled oligonucleotide CDS22*.

showed that at least one of the *Hpa*II sites of the B′ and B boxes was demethylated in all these tumor cell lines (Fig. 1*B*). In contrast, the CpG in boxes B′ and B were found to be methylated in all the tumor cell lines and normal cell cultures that do not express *MAGE-1*, including a head and neck carcinoma cell line, a melanoma cell line, a sarcoma cell line, peripheral blood lymphocytes (PBL) stimulated with PHA and normal fibroblasts (Fig. 1*B*).

With one exception, this analysis was performed on cells derived from male patients, because the DNA of female cells always yields amplification products copied from the constitutively methylated inactive X chromosome. The exception is melanoma cell line MZ2-MEL.43, which has lost the inactive X chromosome.

CpG Methylation Inhibits the Binding of Transcription Factors. The Ets binding sites in the B′ and B promoter elements were previously shown, by mobility shift assays, to bind to nuclear proteins of the MZ2-MEL melanoma cells that express *MAGE-1* (14). Competition experiments with consensus Ets-binding sequences indicated that the binding factors belong to the family of the Ets transcription factors (14). We assessed the effect of methylation of the CpG in boxes B′ and B on the binding of these nuclear proteins. An oligonucleotide probe corresponding to box B′ formed a retarded complex when it was incubated with a nuclear extract from MZ2-MEL cells (Fig. 2). The formation of this complex was efficiently inhibited by competitor oligonucleotides corresponding to either B or B′, confirming that both sequences bind to the same nuclear protein. No competition was observed with either the B′ or B sequence when they were methylated at the CpG located in the Ets motif (Fig. 2). In contrast, the B competitor carrying a methylated CpG outside the Ets binding site was still able to compete (Fig. 2). We conclude that boxes B′ and B lose their binding activity when the CpG in their Ets binding site is methylated (Fig. 2).

Azadeoxycytidine Induces *MAGE-1* Expression in Tumoral and Normal Cells. Weber and coworkers (21) have previously shown that the expression of *MAGE-1* is activated in melanoma cells treated with the demethylating agent 5-aza-2′-deoxycytidine. To assess this effect in other cell types, we treated with this agent tumor cells that do not express *MAGE-1*, such as sarcoma cell line LB23-SARC, melanoma cell lines MI.665.2-MEL, and SK23-MEL and choriocarcinoma cell line JAR. RT-PCR analysis revealed that the expression of *MAGE-1* was induced by azadeoxycytidine in all these cells, although only faintly in melanoma cell line MI.665.2-MEL (Fig. 3). Activation of *MAGE-1* by this treatment was also observed in PHA-stimulated PBL and normal fibroblasts, in contradiction with the observations of Weber and coworkers (21).

In contrast, none of these cell lines showed induction of the tyrosinase gene by azadeoxycytidine, consistent with the known requirement for melanocyte-specific transcription factors for the activation of the tyrosinase promoter (22).

***MAGE-1* Expression Correlates with Genome-Wide Hypomethylation.** We examined the overall degree of methylation of the DNA of 20 different tumor cell lines that do or do not express *MAGE-1*. The proportion of unmethylated CpG was estimated by measuring the incorporation of ^{3}H-*methyl* in total cellular DNA treated with methylase M.*Sss*I in the presence of *S*-adenosyl-[*methyl*-^{3}H]-methionine. M.*Sss*I methylates the C in CpG dinucleotides.

We evaluated the overall degree of DNA methylation of 8 tumor cell lines that express *MAGE-1* and 12 cell lines that do not. The results indicated an inverse correlation between the expression of *MAGE-1* and the overall level of DNA methylation (Fig. 4). An average of 29% methylated CpG was observed in the tumor cell lines that expressed *MAGE-1*, including one small cell lung carcinoma cell line, one head and neck carcinoma cell line, one leukemia cell line, one sarcoma

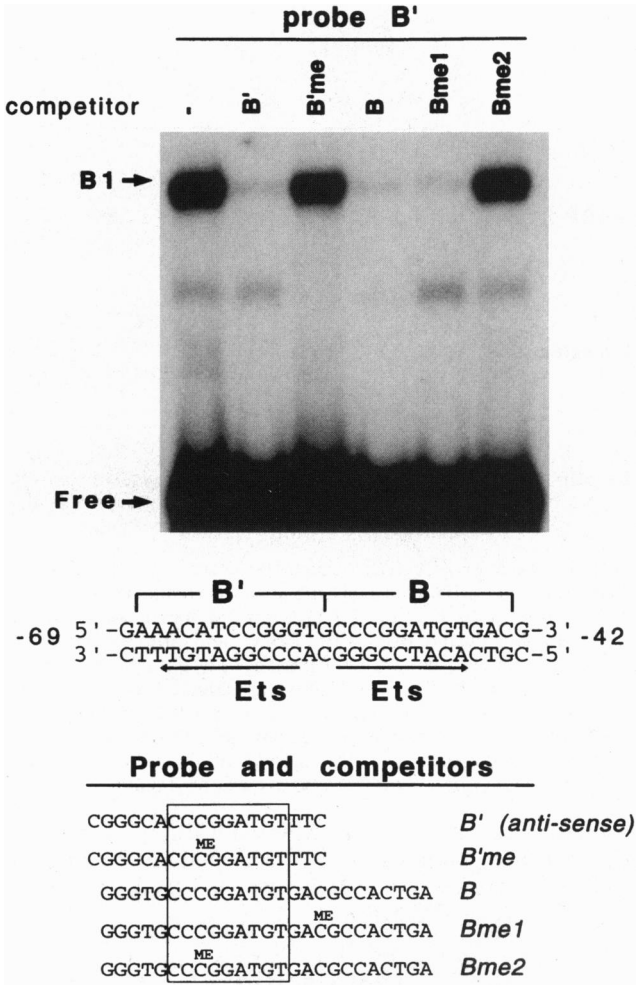

FIG. 2. Effect of CpG methylation on the binding activity of boxes B′ and B. A mobility shift analysis was performed by incubating a radioactively labeled probe corresponding to box B′ with a nuclear extract from MZ2-MEL cells. A 100-fold molar excess of cold competitor probe was added as indicated above each lane. B1 indicates the main retarded complex and Free corresponds to the unbound probe. The sequence of the B′ and B promoter elements of *MAGE-1* and the location of the Ets binding sites are given. The sequences of the oligonucleotides used as probe and competitors are given, and the Ets binding site is boxed. ME indicates the position of the methylated cytosine.

cell line, and four melanoma cell lines. By contrast, an average of 52% of the CpG were methylated in tumor cell lines that do not express *MAGE-1*, including a small cell lung carcinoma cell line, two head and neck carcinoma cell lines, a renal carcinoma cell line, a leukemia cell line, a choriocarcinoma cell line, and five melanoma cell lines (Fig. 4). However, two of these melanoma cell lines (MI.665.2-MEL and LB4-MEL) had only 28% and 31% of methylated CpG, indicating that a low level of DNA methylation does not necessarily lead to *MAGE-1* activation (Fig. 4). The methylation status of the *MAGE-1* promoter could not be analyzed in these two melanoma cell lines because they were derived from female patients. It is unlikely that the lack of *MAGE-1* expression in MI.665.2-MEL is due to the absence of some transcription factor, because transfection experiments with reporter plasmids previously showed that the *MAGE-1* promoter exerts transcriptional activity in this cell line (14). The important variation that we observed between the tumor cell lines is within the range of variation reported by others (23).

Normal human fibroblasts, which do not express *MAGE-1*, showed 60% methylated CpG, in agreement with previously

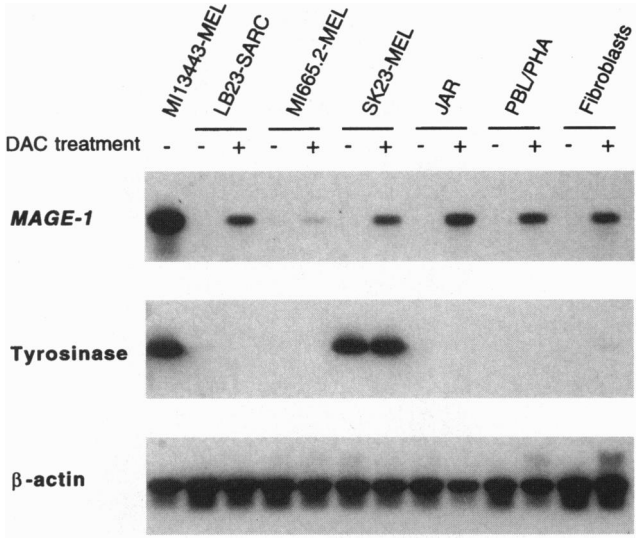

FIG. 3. Induction of *MAGE-1* expression after 5′-aza-2-deoxycytidine (DAC) treatment. Treated cells included two melanoma cell lines (-MEL), a sarcoma cell line (-SARC), a choriocarcinoma cell line (JAR), PHA-activated PBL (PBL/PHA), and primary fibroblasts. They were incubated for 72 h in the presence (+) or in the absence (−) of 1 μM azadeoxycytidine. Total RNA was extracted and submitted to RT-PCR using *MAGE-1*-, tyrosinase-, or β-actin-specific primers. PCR products were separated in agarose gels, transferred on nylon membranes, and hybridized with oligonucleotidic probes recognizing a sequence from either *MAGE-1*, tyrosinase, or β-actin.

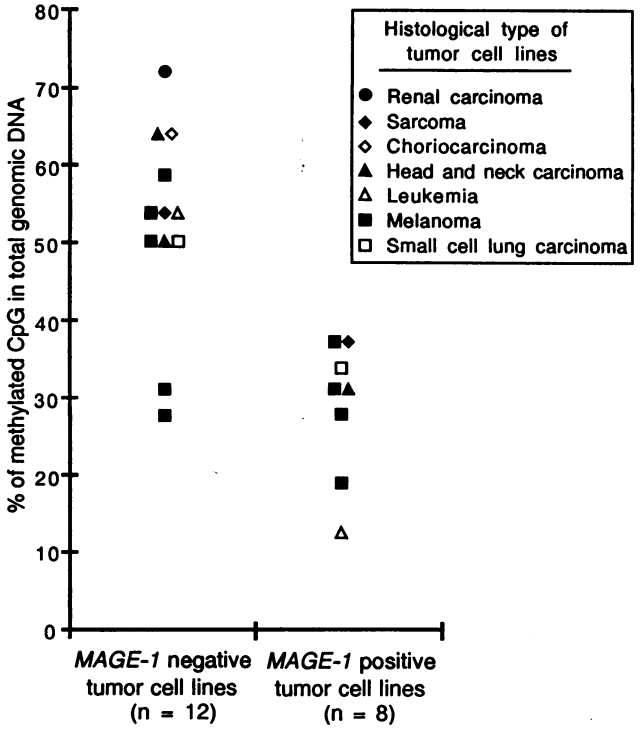

FIG. 4. Overall CpG methylation in 20 tumor cell lines of different histological types that either do or do not express *MAGE-1*. Cellular DNA was treated with either M.*Sss*I methylase or *dam* methylase in the presence of *S*-adenosyl-[*methyl*-^{3}H]-methionine. The incorporations of *methyl*-^{3}H were measured. The percentage of CpG methylation was deduced from the ratios of the *methyl*-^{3}H incorporation obtained with M.*Sss*I, which methylates the C in CpG dinucleotides, to that obtained with *dam* methylase, which methylates the A at GATC sites. Unlike the C in CpG, the A in GATC is never methylated in mammalian DNA. *MAGE-1* expression in the different cell lines was analyzed by RT-PCR.

reported values (24). In PHA-activated PBL, 69% of the CpG were methylated, whereas in PHA-activated PBL treated with azadeoxycytidine, where *MAGE-1* was activated, only 22% of the CpG were methylated.

DISCUSSION

We have investigated the role of DNA methylation in the control of expression of *MAGE-1*. Our results clearly indicate a striking correlation between expression and demethylation of CpG dinucleotides located in the Ets binding sites of the promoter. The methylation of these CpG dinucleotides inhibits the binding of transcription factors *in vitro*. A similar loss of binding activity was observed as a result of a double point mutation that reduced the *MAGE-1* promoter activity by 90% (14). Therefore, it appears very likely that DNA methylation of the essential B′ and B promoter elements is sufficient to repress the transcription of *MAGE-1* by inhibiting the binding of transcription factors.

Demethylation of the *MAGE-1* promoter appears to be sufficient to activate this gene in tumor cell lines. This results from the observation that the unmethylated *MAGE-1* promoter activates a reporter gene in cells where the gene *MAGE-1* is inactive (14). It is confirmed by the results obtained with azadeoxycytidine in tumor cell lines (ref. 21 and our results). Normal cells, like PHA-stimulated PBL and primary fibroblasts, also express *MAGE-1* after azadeoxycytidine treatment. These data indicate that the transcription factors necessary for the expression of *MAGE-1* are present in normal as well as in tumoral cells. We conclude that the tumoral specificity of the expression of *MAGE-1* is due to the demethylation of promoter regions, a process that occurs in some tumor cells but not in normal cells with the exception of germ cells.

The demethylation of the *MAGE-1* promoter seems to be a random consequence of a genome-wide demethylation. Global DNA demethylation is often observed in tumor cell lines and in tumor samples (23, 25, 26). Male germ-line cells also undergo a genome-wide demethylation (27, 28). The low level of overall DNA methylation that we observed in all the tumor cell lines expressing *MAGE-1*, an average of 29% methylated CpG versus 52% in the other tumor cells, shows that the activation of *MAGE-1* correlates with an important decrease in global DNA methylation. The correlation is not absolute because two melanoma cell lines that do not express *MAGE-1* were heavily demethylated. This suggests that global demethylation affects the genome randomly, and therefore does not always lead to the demethylation of the *MAGE-1* promoter region.

The expression of *MAGE-1* in melanoma is more frequent in metastases than in primary tumors (29). Consistent with the role that we propose for demethylation in *MAGE-1* activation, genome-wide hypomethylation is usually more pronounced in metastases than in primary tumors (26, 30, 31). Conversely, no expression of *MAGE-1* has been found in acute leukemias (32), a malignancy that retains a high level of methylation (33).

Experiments with reporter genes driven by the promoter of two other *MAGE* genes, *MAGE-3* and *MAGE-4*, showed that these promoters also are active in tumor cell lines where these genes are not expressed (E. De Plaen and J.-P. Szikora, unpublished data). The promoter of *MAGE-3* was shown to be hypomethylated in the tumor cell lines that express the gene. These observations suggest that, like the expression of *MAGE-1*, that of *MAGE-3* and *MAGE-4* is repressed by DNA methylation. Recently, other genes, namely *GAGE* and *BAGE*, were found to code for tumor-specific antigens (34, 35). Like *MAGE-1*, these genes are expressed in tumors of various histological origins but not in normal tissues, except in testis. We observed the induction of the expression of *GAGE* and *BAGE* in cells treated with azadeoxycytidine (data not shown), suggesting that the expression of these genes is controlled by

DNA methylation as well. This may explain why the expression of *MAGE-1* shows a correlation not only with that of the other *MAGE* genes, but also with that of the *BAGE* and *GAGE* genes. It seems probable that genome-wide demethylation in tumor cells leads to the activation of numerous genes. Among those, the genes that are not expressed in any normal tissue, except testis, may produce tumor-specific antigens because of the absence of immune tolerance. Interestingly, Frost and coworkers (36) have previously reported that strongly immunogenic variants can be selected from poorly immunogenic murine tumor cells treated with azadeoxycytidine.

Why DNA demethylation appears to occur frequently in the course of tumor progression remains an open question. An obvious possibility is that demethylation activates genes that confer a selective growth or invasion advantage. This has been shown for the *ras* gene in colonic carcinoma and small cell lung cancer (37).

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