Promoter Demethylation and Histone Acetylation Mediate Gene Expression of *MAGE-A1, -A2, -A3*, and *-A12* in Human Cancer Cells

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Abstract

The broad range of expression of cancer-testis antigens in various tumor types makes the proteins encoded by human *MAGE* gene family promising targets for anticancer immunotherapy. However, a major drawback is their heterogeneous expression. In the current study, we have examined the influence of the DNA methylase inhibitor 5-aza-2′-deoxycytidine (5-aza-CdR) together with the histone deacetylase inhibitor trichostatin A on the expression of *MAGE-A1, -A2, -A3*, and *-A12* genes in different cell lines. Reverse transcription-PCR, Western blot analyses, and immunocytochemical staining show that trichostatin A was able to significantly up-regulate 5-aza-CdR-induced *MAGE* gene expression. Transient transfection assays with methylated reporter plasmids containing promoter fragments of the different *MAGE* genes show that trichostatin A was able to overcome gene silencing. In addition, the methylation status of the MAGE promoters was assessed by sodium bisulfite mapping in the various cell lines before and after stimulation with 5-aza-CdR and/or trichostatin A. In contrast to the methylation patterns, which clearly correlated with the basal MAGE RNA transcripts, up-regulation of the MAGE-A mediated by both agents only resulted in a reduction in promoter methylation ranging between 1% and 19%. In conclusion, our data show for the first time that not only hypermethylation but also histone deacetylation is responsible for the mechanism underlying *MAGE* gene silencing.

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Introduction

The human *MAGE* gene family encodes tumor-associated antigens, which are recognized by CTLs in conjunction with MHC class I molecules of various haplotypes on the tumor cell surface. The expression of *MAGE* genes is limited to the testicular germ cells (spermatogonia and primary spermatocytes), placenta (1), and different human tumor types (e.g., melanoma, lung, breast, bladder, and gastric carcinoma; refs. 2-7). The first characterization of the *MAGE-A1* gene was published in the year 1991 (8). Subsequent studies have identified new members of these cancer-testis antigens. The *MAGE-A* gene family comprises 12 members (*MAGE-A1* to *A12*) and is located in a cluster on the q-terminal region of the X chromosome (for review, see ref. 9). Contrary to the high homology of the *MAGE-A* genes, their promoters are less homologous. The promoter of MAGE-A1 contains binding sites for the transcription factors Ets and Sp1, whereby the Ets proteins are responsible for the high transcriptional activation. DNA hypermethylation of CpG dinucleotides at the 5′-end of the *MAGE-A1* gene may prevent the access of these activators to their motif and consequently induction of the promoter activity (10). These findings indicate a relationship between DNA hypermethylation and transcriptional repression of the MAGE-A1 promoter. Other *MAGE* genes and the unrelated *GAGE* and *LAGE-1* genes are presumed to be regulated in a similar manner (11-13).

The pattern of DNA methylation is generated during vertebrate development (14). Alterations in DNA methylation occur during the pathogenesis of human tumors and a global DNA hypomethylation has been observed in various carcinomas. Despite the global decrease in DNA methylation, hypermethylated regions leading to transcriptional silencing of many tumor suppressor and DNA repair genes have also been detected in malignant cells (15). This suggests that both DNA hypermethylation and hypomethylation play a crucial role during tumorigenesis, and transcriptional activation might be induced by promoter demethylation. However, several laboratories have shown that although tumor cells were treated by the DNA methylase inhibitor 5-aza-2′-deoxycytidine (5-aza-CdR) up-regulation of *MAGE-A* gene expression could not be always observed (2, 16-22).

Gene expression is determined not only by promoter methylation status but also by configuration of chromatin. Both histone acetyltransferases and histone deacetylases (HDAC) are involved in post-transcriptional modifications resulting in remodeling of chromatin. The strategy to overcome gene silencing by the use of HDAC inhibitors (HDACI) is not yet well established. HDACIs seem to possess antitumor activity and have successfully entered clinical trials, suggesting that they might contribute to a more tailored cancer treatment (23).

The failure of activating gene expression by 5-aza-CdR as well as the differential and heterogeneous expression patterns of *MAGE-A* genes in tumors have prompted us to examine in detail their inactivation. A successful induction of *MAGE* gene

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Table 1. MAGE-A1, -A2, -A3, and -A12 Gene Expression in Different Cell Lines before and after Stimulation

Cell line	MAGE-A1				MAGE-A2			
	Basal	5-Aza-CdR	Trichostatin A	5-Aza-CdR and trichostatin A	Basal	5-Aza-CdR	Trichostatin A	5-Aza-CdR and trichostatin A
Leukemic								
HL60	(+)	+++	(+)	+++	–	+++	–	+++
Hepatic								
HepG2	–	–	–	–	(+)	(+)	(+)	(+)
Prostate								
LNCaP	–	(+)	(+)	+++	(+)	+	+	+++
DU145	–	+++	(+)	++	–	+++	(+)	+++
Breast								
BT20	+++	+++	+++	+++	+++	+++	+++	+++
BT474	+++	+++	+++	+++	+++	+++	+++	+++
MDA-MB-468	+++	+++	+++	+++	+++	+++	+++	+++
T47D	+++	+++	+++	+++	+++	+++	+++	+++
MCF-7	(+)	++	+	+++	–	+	–	++
GI101	–	+	–	++	–	+	(+)	++
MDA-MB-231	–	+	–	++	+	++	+	++
MTSV1.7	(+)	+	+	++	+	++	+	++
Colon								
LoVo	(+)	++	+	+++	(+)	++	+	+++
Colo205	–	++	–	+++	–	+++	–	+++
WiDr	–	++	–	+++	–	+	–	++

NOTE: Cell lines were treated with 1 μmol/L 5-aza-CdR for 72 hours and/or with 0.5 μmol/L trichostatin A for the last 24 hours. –, none; (+), very weak; +, weak; ++, medium; +++, strong MAGE-A mRNA expression.

expression could prevent tumor cells from escaping an immune response by lymphocytes and possibly improve immunotherapy. In the current study, we have investigated the role of the HDAC in transcriptional repression of *MAGE-A1, -A2, -A3*, and *-A12* and show that both DNA methylation and histone deacetylation may be involved in gene silencing of *MAGE-A*. Treatment of the cell lines derived from a broad range of tumor types with inhibitors of DNA methylase (5-aza-CdR) and HDAC (trichostatin A) could synergistically induce and activate *MAGE-A1, -A2, -A3*, and *-A12* gene expression and partly lead to demethylation of their promoters. Furthermore, administration of trichostatin A enhanced the promoter-driven luciferase activity of transfected methylated reporter plasmids each containing one of the four MAGE-A promoters.

Results

Expression Patterns of MAGE-A1, -A2, -A3, *and* -A12 *in Different Cancer Cells before and after Treatment with 5-aza-CdR and Trichostatin A*

As summarized in Table 1, MAGE-A RNA expression was determined in a leukemic, a hepatic, two prostate, eight breast, and three colon cancer cell lines and analyzed by reverse transcription-PCR using primers specific for *MAGE-A1, -A2, -A3*, and *-A12* genes. Representative results derived from two breast cancer cell lines (MCF-7 and MDA-MB-231) and a colon cancer cell line (WiDr) are shown in Fig. 1. A heterogeneous basal expression pattern of these genes was detected in the cell lines tested. In nearly each cell line, either high or low basal expression levels of the four *MAGE-A* genes were simultaneously observed with exception of the breast cell lines MDA-MB-231 (Fig. 1B) and MTSV1.7 (Table 1) and the colorectal cell lines WiDr (Fig. 1C) and LoVo (Table 1). In contrast to the leukemic cells, hepatic, prostate, colon, and four of the eight breast cancer cell lines (MCF-7, MDA-MB-231, GI101, and MTSV1.7) showed negligible RNA transcripts, the four remaining breast cancer cell lines (BT20, BT474, MDA-MB-468, and T47D) had high basal RNA levels of MAGE-A1, -A2, -A3, and -A12 (Fig. 1; Table 1). Whereas the breast cancer cell line MDA-MB-231 expressed MAGE-A2 and -A3 but not MAGE-A1 and -A12 (Fig. 1B), the MCF-7 cell line expressed none of the *MAGE-A2, -A3*, and *-A12* genes and only showed a weak *MAGE-A1* gene expression (Fig. 1A).

To clarify whether both DNA methylation and histone deacetylation are involved in transcriptional repression of these genes, the various cell lines were incubated with 1 μmol/L of the DNA demethylating agent 5-aza-CdR for 72 hours and/or 500 nmol/L of the HDACI trichostatin A for the last 24 hours. Figure 1A to C shows representative examples of the MAGE-A1, -A2, -A3, and -A12 RNA expression in MCF-7, MDA-MB-231, and WiDr cells before and after stimulation with both agents done by reverse transcription-PCR. In Fig. 1D, the densitometric measurements of the unstimulated and 5-aza-CdR-up-regulated MAGE-A RNA levels found in MCF-7 cells are depicted as a bar graph. Stimulation of the MCF-7 cell line with 5-aza-CdR resulted in 10-, 20-, 22-, and 6-fold higher MAGE-A1, -A2, -A3, and -A12 transcript levels, respectively, than the unstimulated basal levels. As shown by the evaluations of the 5-aza-CdR-mediated stimulations in Table 1, similar results were obtained for the three breast cancer cell lines MDA-MB-231, GI101, and MTSV1.7. The constitutively high basal transcription of the *MAGE-A* genes in the other four breast cancer cell lines (MDA-MB-468, T47D, BT20, and BT474) could not further be up-regulated by 5-aza-CdR probably due to the saturated RNA levels in these cells. Treatment of the cell lines with trichostatin A alone had only small influence on *MAGE-A* gene expression. However, trichostatin A was able synergistically to enhance 5-aza-CdR-mediated MAGE-A transcription (Fig. 1; Table 1).

Table 1. MAGE-A1, -A2, -A3, and -A12 Gene Expression in Different Cell Lines before and after Stimulation (Cont'd)

MAGE-A3				MAGE-A12			
Basal	5-Aza-CdR	Trichostatin A	5-Aza-CdR and trichostatin A	Basal	5-Aza-CdR	Trichostatin A	5-Aza-CdR and trichostatin A
–	+++	–	+++	–	+	–	+
–	(+)	–	–	–	–	–	–
(+)	++	++	+++	–	(+)	(+)	+++
–	+++	(+)	+++	–	+	–	++
+++	+++	+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++	+++	+++
+++	+++	+++	+++	+++	+++	+++	+++
–	++	–	+++	–	+	–	+++
–	+++	+	+++	–	+	–	+
+	++	+	++	–	+	–	++
(+)	+	+	++	–	+	+	++
+	+++	+	+++	–	++	–	+++
–	+++	–	+++	–	+	–	++
+	++	+	+++	–	+	–	++

Administration of MCF-7 cells with both 5-aza-CdR and trichostatin A lead to 1.5-, 4-, 1.5-, and 7-fold higher *MAGE-A1, -A2, -A3*, and *-A12* gene expression, respectively, than the level observed in 5-aza-CdR-treated MCF-7 cells. Both agents together caused the strongest activation of the *MAGE-A2* gene in these cells, suggesting a variable effect on the different members of *MAGE-A* gene family by the substances (Fig. 1D). Whereas addition of 5-aza-CdR alone could hardly activate transcriptional gene expression in the prostate cancer cell line LNCaP, which was negative for MAGE-A1 and -A12 and disposed a minimally basal MAGE-A2 and -A3 RNA level, both components could synergistically up-regulate *MAGE-A1* (14-fold), *MAGE-A2* (3-fold), *MAGE-A3* (4-fold), and *MAGE-A12* (11-fold) gene expression in this cell line. The hepatic cell line HepG2 was the only one on which 5-aza-CdR and trichostatin A had no effect. In the other cell lines tested, trichostatin A could usually up-regulate twice the 5-aza-CdR-mediated *MAGE-A* gene expression (Table 1).

The synergistic effect of 5-aza-CdR and trichostatin A on MAGE-A RNA transcription also corresponded to the high protein level stimulated by these agents (Fig. 2). Figure 2A shows a representative example of a Western blot of the MAGE-A1 protein expression in MCF-7 cells before and after stimulation. In addition, Fig. 2B shows the fluorescence-immunostained MAGE-A1 proteins and antigens in the cell cytoplasm and on the MCF-7 cell membrane, respectively. The stimulation of the MCF-7 cells with 2-aza-CdR leads to a strong induction of the MAGE-A1 protein expression as indicated by the red staining of these antigens in the cytoplasm and on the cell surface (Fig. 2B).

Trichostatin A–Mediated Activation of the Transfected Reporter Plasmids Containing MAGE Promoter Fragments

To functionally test the influence of DNA demethylation and histone acetylation on promoter activity, we constructed a series of reporter plasmids in which we inserted sequences derived from MAGE-A1, -A2, -A3, and -A12 promoters immediately upstream of the luciferase gene (Fig. 3A). The DNA alignment of these promoter fragments showed an identity between 71% and 90%. *In vitro* DNA methylation of the cloned constructs was done by the DNA methylases *Hpa*II and *Sss*I, which methylate the second cytosine residue of the CCGG sequence and each cytosine in the CpG dinucleotides, respectively. We chose the breast cell line MCF-7 as a model system for our transient transfection studies (Fig. 3B and C) because this cell line is well characterized and has been shown to have a high DNA methylase activity (24). The unmethylated and methylated reporter plasmids were introduced together with a reference reporter plasmid into the cells and cell lysates were assayed for luciferase activity. The MAGE-A1 promoter had the highest basal activity in comparison with MAGE-A2, -A3, and -A12, which were 40%, 30%, and 30% of that measured for MAGE-A1, respectively (Fig. 3B). *Hpa*II-methylated MAGE-A2, -A3, and -A12 constructs decreased promoter activities to 13%, 29%, and 28%, respectively, compared with the unmethylated plasmids, which were arbitrarily set to 100% (Fig. 3C). DNA methylation of the MAGE-A1 promoter caused a complete loss of activity. *Sss*I-methylated promoter fragments showed qualitatively similar results (data not shown). Whereas trichostatin A was able to elevate the promoter-driven luciferase activity of the methylated MAGE-A2 and -A12 to their basal activities (Fig. 3C). Trichostatin A had small or no effect on the methylated MAGE-A1 and -A3 constructs. The similar response of MAGE-A2 and -A12 to trichostatin A could be explained by the identity of their promoters, which was ~90%, whereas the DNA alignment of MAGE-A1 and -A3 only showed a promoter identity of 72%. Trichostatin A was even able to up-regulate ~2-, 4-, 2.5-, and 4-fold the unmethylated MAGE-A1, -A2, -A3, and -A12 promoters, respectively (Fig. 3C).

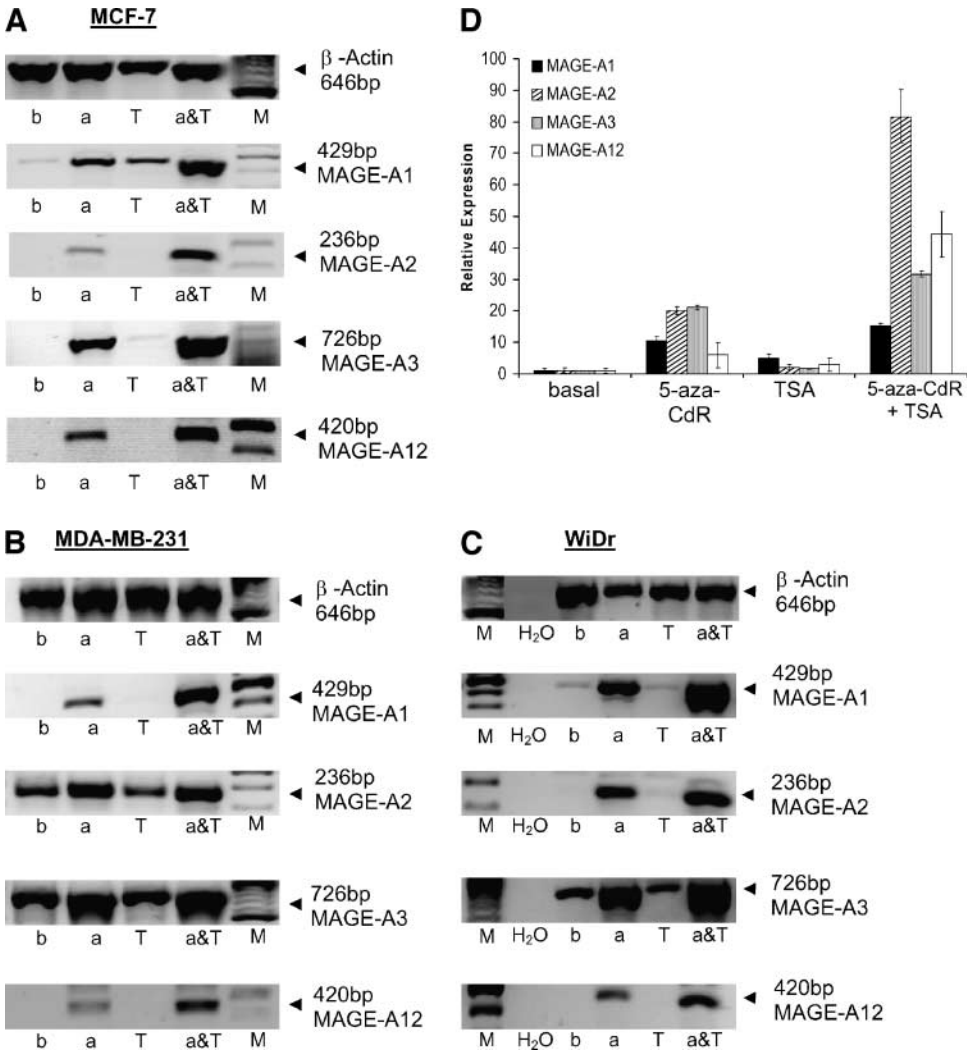

FIGURE 1. Analysis of MAGE-A1, -A2, -A3, and -A12 mRNA expression before and after stimulation of cell lines MCF-7 (**A**), MDA-MB-231 (**B**), and WiDr (**C**) with 5-aza-CdR and/or trichostatin A done by reverse transcription-PCR. The cells were treated with 1 μmol/L 5-aza-CdR for 72 hours and/or 0.5 mmol/L trichostatin A for the last 24 hours. **D.** Densitometric measurements of the PCR products representing the amplified MAGE-A mRNA transcripts in the MCF-7 cell line (**A**). The densitometric values of MAGE-A1, -A2, -A3, and -A12 RNA expression levels were normalized to those of β-actin. The normalized values are shown in relation to basal MAGE-A expression. b, basal level; a, 5-aza-CdR; T or TSA, trichostatin A; a&T, both agents together; M, marker.

DNA Methylation Analysis by Methylation-Sensitive PCR of MAGE-A2 Promoter

A preliminary analysis of the methylation status of MAGE-A promoters was carried out by methylation-sensitive PCR using genomic DNA derived from MCF-7 cells and digested by the restriction enzymes *Hpa*II (methylation-sensitive) or *Msp*I (methylation-insensitive) and promoter-specific MAGE-A2 primers (Fig. 4). The promoter sequence amplified by the primers 1 and 2 contains no *Hpa*II restriction sites and served as a positive control. The amplification of the downstream region of MAGE-A2 promoter containing four *Hpa*II sites with primers 1 and 3 identified the DNA methylation status (Fig. 4A). An inverse relationship between methylation status of MAGE-A2 promoter (Fig. 4B) and its gene expression (Table 1) was observed. The densitometric evaluation of the PCR products, which reflects promoter demethylation, shows that application of 5-aza-CdR, trichostatin A, and 5-aza-CdR plus trichostatin A reduced the amplified bands to 37%, 23%, and 37%, respectively, compared with the intensity of the basal band derived from untreated cells.

Comparison of MAGE-A *Gene Expression with the Promoter Methylation Status*

To define the promoter methylation patterns in more detail, we did bisulfite genomic sequencing. The MAGE-A1 promoter region contains two inverted motifs for binding of the Ets transcription factors at positions −64/−56 and −53/−45 relative to the transcription start site, which have been shown to drive 90% of the promoter activity in a melanoma cell line. DNA methylation of CpG sites located in the Ets elements inhibited the binding of these transcriptional activators *in vitro* (10). DNA

demethylation of the Ets binding sites seems to be sufficient to activate *MAGE-A1* gene expression in tumor cell lines (25). The transcription start site is localized in the region between −30 and +30 and responsible for basal activity of the MAGE-A1 promoter (26). In contrast to the investigated MAGE-A1 promoter, only little is known about MAGE-A2, -A3, and -A12 promoters. By DNA sequence analysis and alignments, we identified Ets consensus motifs at positions −99/−90, −143/−134, and −154/−145 of MAGE-A2, positions −113/−104 and −151/−141 of MAGE-A3, and position −16/−7 of MAGE-A12 promoter. The content of CpG dinucleotides is 11%, 9%, 10%, and 11% in the region extending from −100 to +222 of MAGE-A1, −443 to +120 of MAGE-A2, −363 to +119 of MAGE-A3, and −51 to +219 of MAGE-A12 promoter, respectively.

The DNA methylation status of MAGE-A1, -A2, -A3, and -A12 promoters was determined before and after incubation of the cell lines with 5-aza-CdR and/or trichostatin A. The breast cancer cell lines MDA-MB-468 and T47D with a high basal transcription of MAGE-A1, -A2, -A3, and -A12 and the MCF-7 and MDA-MB-231 cell lines almost negative for the four MAGE-A transcripts were chosen to identify the DNA methylation patterns using bisulfite-treated DNA amplified with methylation-specific primers. Representative examples of the methylation status of MAGE-A1 (Fig. 5A and B) and MAGE-A3 (Fig. 5C and D) promoters in the cell lines MCF-7 and MDA-MB-231 are illustrated in Fig. 5.

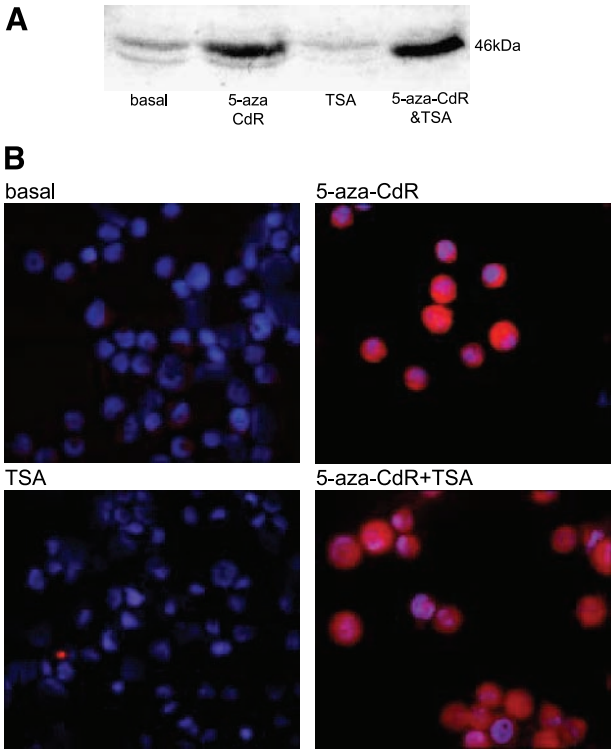

FIGURE 2. **A.** Protein expression of MAGE-A1 in MCF-7 cells before and after stimulation with 5-aza-CdR and/or trichostatin A. Western blot was done with the MAGE-A1 antibody monoclonal antibody clone MA454. **B.** Fluorescence microscopy of the MAGE-A1 antigen on MCF-7 cells. Magnification, ×40. The staining with the fluorochromes Alexa Fluor 546 (*red*) and 4′,6-diamidino-2-phenylindole (*blue*) visualize MAGE-A1 antibody binding and nuclear DNA, respectively.

As expected, the CpG sites of the MAGE-A promoters were nearly completely demethylated when the genes were active. In the breast cancer cell lines MDA-MB-468 and T47D, the constitutively high promoter activities corresponded to the rarely detected methylated CpG sites of the four MAGE promoters (data not shown). In both cell lines MCF-7 (Fig. 5A and C) and MDA-MB-231 (Fig. 5B and D), the low *MAGE-A* gene expression correlated to the highly methylated region flanking the start site and to the almost completely methylated Ets sites of the promoters. After treatment with 5-aza-CdR, both cell lines showed a heterogeneous methylation pattern of the four MAGE-A promoters (Fig. 5). The high promoter activities could not be explained by a specific 5-aza-CdR-demethylated pattern of the CpG dinucleotides of the investigated promoter fragments. However, in all cases, 5-aza-CdR caused a general decrease in overall DNA methylation of all four MAGE-A promoters, which ranged between 1% and 19% and is summarized in Table 2. A further DNA demethylation with both substances could not be observed (Fig. 5A and C).

Discussion

In the current study, we show that HDACI trichostatin A could synergistically up-regulate the 5-aza-CdR-mediated RNA expression. These findings show that both histone acetylation and DNA demethylation play an important role in the transcriptional activation of the *MAGE-A1, -A2, -A3*, and *-A12* genes. Whereas reverse transcription-PCR analysis showed a small effect of trichostatin A on transcription, transfection studies showed that trichostatin A was able to significantly up-regulate promoter-driven luciferase activity of the methylated and even unmethylated reporter plasmids containing each of the four promoter fragments with the exception of the methylated MAGE-A3 construct. In agreement with previous studies (25, 27), DNA methylation of transfected reporter constructs could strongly down-regulate promoter activity, which correlated to the methylation-dependent regulation of these genes. Whereas the methylation of the rare *Hpa*II CCGG motifs in the promoters was sufficient to down-regulate the activity, the methylation by the *Sss*I methylase had a negligible effect. Although the methylation status of the promoters correlated to the basal expression patterns of their genes, treatment with the methylation inhibitor 5-aza-CdR only resulted in a weak promoter demethylation, whose grade was not congruent to the highly activated RNA expression level.

For our investigations, we chose besides breast and prostate cancer cell lines different MAGE-negative cell lines HL60, Colo205, WiDr, and HepG2, whose *MAGE-A* gene expression has been shown not to be inducible despite extensive treatment with 5-aza-CdR (16-19). In all cell lines tested with the exception of HepG2 cells, we were able to stimulate *MAGE-A* expression with the demethylating agent. The additional treatment with trichostatin A resulted in a further increase in RNA transcripts. A possible explanation of the missing effect of 5-aza-CdR on *MAGE-A* expression in HepG2 cells could be a rapid remethylation by a high DNA methyltransferase activity or the inadequate levels of appropriate transcription factors necessary for the promoter activity.

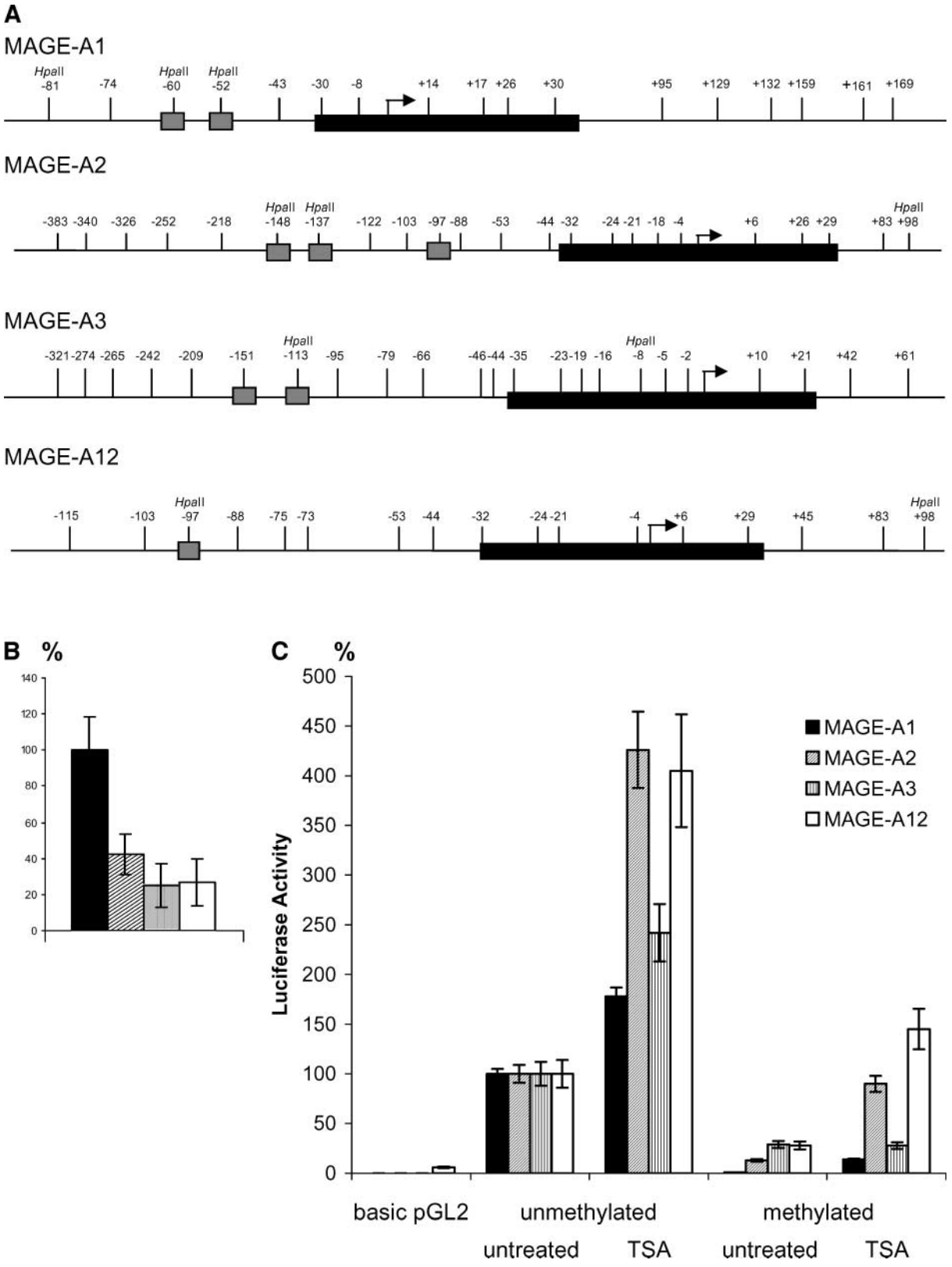

FIGURE 3. **A.** Schematic view of MAGE-A1, -A2, -A3, and -A12 promoter fragments. Black bars, region of the start site; gray squares, Ets motifs, including the *Hpa*II methylation sites; arrow, start site. **B.** Basal luciferase activity of the transiently transfected unmethylated MAGE-A promoter constructs in MCF-7 cells. The MAGE-A1 promoter activity was set to 100%. **C.** Luciferase activity of the transfected unmethylated and *Hpa*II-methylated plasmids before and after cell stimulation of MCF-7 cells with 100 ng/mL trichostatin A. The unmethylated MAGE-A promoters (**B**) were set to 100%.

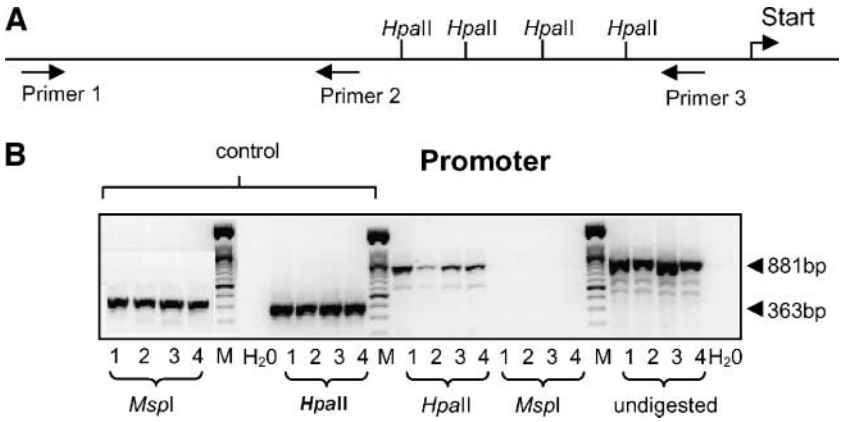

FIGURE 4. A. Schematic view of the MAGE-A2 promoter region. *Hpa*II restriction sites are marked. Arrows, primer binding sites. **B.** Methylation-sensitive PCR analysis of MAGE-A2 promoter derived from the MCF-7 cell line. After digestion with the methylation-sensitive restriction enzyme *Hpa*II (CCGG) and the insensitive isoschizomer *Msp*I, genomic DNA was amplified using control or methylation-sensitive primer pairs specific for the MAGE-A2 promoter. The sequence amplified with primers 1 and 2 contains no *Hpa*II site and represents the positive control. In contrast, the downstream region amplified with primers 1 and 3 contains four *Hpa*II sites. 1, basal; 2, 5-aza-CdR; 3, trichostatin A; 4, 5-aza-CdR/trichostatin A.

The basal MAGE-A1, -A2, -A3, and -A12 transcription was found to vary among the different cell lines. In four breast cancer cell lines (T47D, BT20, BT474, and MDA-MB-468), RNA levels were high, whereas in four other breast (MCF-7, GI101, MDA-MB-231, and MTSV1.7), three colorectal (Colo205, LoVo, and WiDr), a leukemic (HL60), a hepatic (HepG2), and two prostatic (LNCaP and DU145) cancer cell lines expression was low or absent. The low MAGE-A mRNA level observed in the slightly invasive MCF-7 cells is probably related to the high DNA methyltransferase activity in these cells (28). To gain further insight in the silencing of *MAGE-A* genes, we compared the RNA expression with their promoter methylation patterns and did sequencing of bisulfite-treated DNA. Although a relationship of basal transcription with the methylation status of the promoters could be found, there was no clear correlation between the methylation patterns and their 5-aza-CdR-stimulated and/or trichostatin A–stimulated gene expression. A significant 5-aza-CdR-mediated promoter demethylation leading to increased gene expression was missing. However, the 5-aza-CdR-stimulated overall promoter demethylation usually ranged from 1% to 19%, which could possibly explain the highly activated RNA transcription. The heterogeneous and random spreading of the demethylating agent in the cells and the insensitivity of some cells to these agents (18, 20) might likewise be the cause of the missing effect of 5-aza-CdR. Furthermore, the number of clones analyzed might be too small to obtain a statistically valid picture of the promoter methylation pattern. In agreement to our results, De Smet et al. (26) have also found a heterogeneous DNA methylation pattern of the MAGE-A1 promoter in a 5-aza-CdR-treated melanoma cell line. Whereas a smaller part of the clones was completely demethylated, a bigger part was nearly completely methylated, a finding that is obviously contradictory to a high activation by 5-aza-CdR. In addition, Janssen et al. (18) showed a strong 5-aza-CdR-induced *MAGE-A1* expression, which did not correlate with the number of demethylated CpG sites in the Ets motif. Moreover, our findings are comparable with the results published by Sigalotti et al. (27), who reported that the 5-aza-CdR-mediated overall DNA demethylation of CpG dinucleotides ranged from 6% to 12%. Similar to our data, Suyama et al. (21) failed to detect a clear relation between DNA demethylation of CpG dinucleotides in both Ets sites of the MAGE-A1 promoter and its transcriptional expression in different 5-aza-CdR-stimulated cell lines. In addition, they did not find any additional CpG sites whose demethylation could be responsible for the high *MAGE-A1* expression. Finally, Zhang et al. (29) showed in their transient transfection studies with liver cell lines that the drastically repressive effects on promoter activity might be caused by methylated CpG sites at positions −81 and −30 (29). Although we could not detect any hotspots in the 5-aza-CdR-mediated demethylation of CpG dinucleotides, which could contribute to the high promoter activities, we also observed a frequent demethylation at the positions −81 and −30 by 2-aza-CdR. To further explain the discrepancy, it is likely that the cell lines differ in their epigenetic composition and consequently activity of intracellular factors.

Several studies (16, 18, 19, 21, 30, 31) have already shown a stimulatory effect on *MAGE-A* gene expression by 5-aza-CdR. However, until now, the additional administration of trichostatin A has not been shown. HDACIs induce the histone hyperacetylation and usually affect expression of 8% to 10% of the genes. They are able to down-regulate as well as to up-regulate several genes. The genes up-regulated by trichostatin A inhibit tumor cell growth and are mainly involved in cell cycle arrest and apoptosis. The genes down-regulated by trichostatin A play a role in DNA synthesis. It is postulated that down-regulation of a gene could result from the direct effect of histone acetylation, which are usually deacetylated and therefore results in blocking the necessary transcription machinery. Alternatively, histone hyperacetylation could result in transcription of inhibitors that negatively regulate gene expression (32).

In addition to the *MAGE* gene family, the cancer-testis antigens, including the members of several other families, such as *GAGE/PAGE/XAGE, BAGE, SSX*, and *LAGE* (11-13, 31, 33, 34), seem all to be regulated by a similar mechanism. Besides the cluster of the *MAGE-A, -B*, and *-C* genes, the *GAGE/PAGE/XAGE, SSX*, and *LAGE* genes have been identified on the X chromosome (11, 12, 31). For the X-linked *GAGE* genes, a 5-aza-CdR-mediated activation in various cell lines could be described implying gene silencing by hypermethylation of their promoters (11-13, 31, 33). However, to our best acknowledgment, a regulation of these genes by HDACs and acetylases has until now not been investigated.

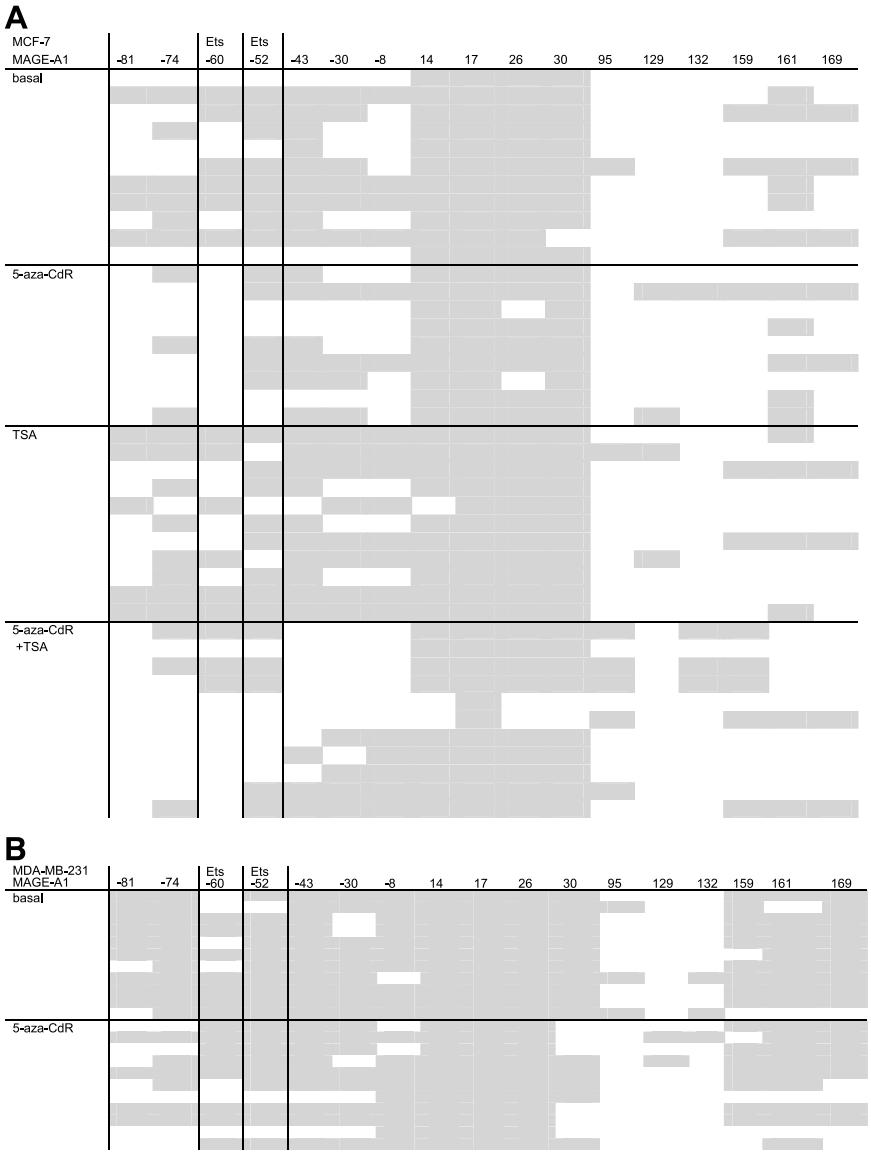

FIGURE 5. Methylation status of MAGE-A1 and MAGE-A3 promoters in the cell lines MCF-7 (**A** and **B**) and MDA-MB-231 (**C** and **D**) before and after stimulation of 5-aza-CdR and/or trichostatin A. Gray squares and empty squares, methylated and unmethylated cytosines in the CG sites, respectively.

Recent clinical studies could show that an immunotherapy with MAGE peptides had a minimal toxicity and caused induction of tumor-specific CTLs (35-38). A significant tumor regression could also be observed in several patients, pointing out that a vaccination with a MAGE-A peptide could be promising for the treatment of tumor patients. Nevertheless, heterogeneous intratumor expression of MAGE may hamper the effectiveness of immunotherapy. Moreover, in treatment of patients with myelodysplastic syndrome, chronic myeloid leukemia, and acute myeloid leukemia, 5-aza-CdR has exhibited significant activity (39). Phase II and III studies are ongoing, although clinical phase I and II studies with solid tumors have not yet been too promising (39, 40). Initial clinical trials indicate that HDACIs of several different structural classes exhibit clinical activity against a variety of human malignancies (41). In view of the promising results on the first clinical trials with HDACIs, our results on the synergistic effect of 5-aza-CdR and trichostatin A may have important implications for an improved MAGE-based cancer immunotherapy.

Materials and Methods

Cell Lines

The following cell lines were used for the gene expression studies: HL60 (acute promyelocytic leukemia cells); HepG2 (hepatocellular carcinoma cells); LNCaP and DU145 (metastatic prostate carcinoma cells); WiDr, LoVo, and Colo205 (colorectal adenocarcinoma cells); BT20, BT474, MDA-MB-468, T47D, MCF-7, GI101, and MDA-MB-231 (breast adenocarcinoma cells); and MTSV1.7 (breast epithelial cells).

FIGURE 5 *Continued.*

Cell Culture

The cell lines were maintained in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS and L-glutamine (Invitrogen). Cell viability was determined by trypan blue staining. Each cell line was stimulated by 5-aza-CdR (f.c. 1 μmol/L; Sigma-Aldrich, Taufkirchen, Germany) for 72 hours. 5-Aza-CdR-treated cells or a mock control were stimulated by trichostatin A (f.c. 500 nmol/L; Sigma-Aldrich) for 24 hours after the 48-hour 5-aza-CdR incubation.

Preparation of RNA, Genomic DNA, Protein, and Reverse Transcription

Cell lysates were divided into three portions for the preparation of genomic DNA, total RNA, and proteins. Genomic DNA was extracted from cell lines untreated or treated by 5-aza-CdR and/or trichostatin A using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) and quantified by spectrophotometry (BioPhotometer, Eppendorf, Hamburg, Germany). Total RNA was prepared using the RNeasy RNA Isolation kit (Qiagen) and was done according to the manufacturer's description. The cDNA synthesis was carried out using the SuperScript First-Strand System and priming with the oligo(dT) (Invitrogen). For the protein preparation, the unstimulated or stimulated cells were homogenized in a lysis buffer containing 25 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 10 mmol/L EDTA (Sigma-Aldrich), 1% NP40 (Roche, Mannheim, Germany) and the Protease Inhibitor Cocktail Set I (Calbiochem, San Diego, CA). Protein extracts were quantified with the Bio-Rad Protein Assay (Munchen, Germany) to ensure equivalent protein loading.

Western Blot

Total protein lysates (25 μg) were separated by SDS-PAGE and transferred onto the nitrocellulose membrane Hybond-C Extra (Amersham, Freiburg, Germany). After blocking, the membrane was probed with a 1:200 dilution of anti-MAGE-A1 monoclonal antibody clone MA454 (Zymed, San Francisco, CA), which is directed against the amino acid residues 57 to 219 of the human MAGE-A1 protein. Detection and staining of the protein were done using the peroxidase-conjugated secondary antibody (Sigma-Aldrich) and the enhanced chemiluminescence detection kit (Amersham).

Immunocytochemical Staining

The trypsinized cells were centrifuged at 110 × *g* for 3 minutes on adhesive slides (Superfrost Plus, Menzel

Table 2. Overall DNA Methylation of the Different MAGE-A Promoters in Breast Cancer Cell Lines

Cell line		MAGE-A1 (%)	MAGE-A2 (%)	MAGE-A3 (%)	MAGE-A12 (%)
MDA-MB-468	Basal	29	52	4	2
T-47D	Basal	8	48	13	24
MCF-7	Basal	49	88	79	98
	5-Aza-CdR	41	76	79	79
	Trichostatin A	62	—	74	—
	5-Aza-CdR and trichostatin A	38	—	77	—
MDA-MB-231	Basal	75	73	54	64
	5-Aza-CdR	69	66	51	63

Glassware, Braunschweig, Germany) at a concentration of 5×10^5 per area of 240 mm^2 in a cytocentrifuge (Hettich model 16 A, Tuttlingen, Germany). Cytospins were air-dried overnight and fixed with the Solution B containing formaldehyde of the Epimet kit (Micromet, Martinsried, Germany) according to the manufacturer's instructions. After blocking with a serum-free blocking reagent (Dako Cytomation, Glostrup, Denmark) for 20 minutes, slides were incubated for 30 minutes with a dilution of 1:25 of the anti-MAGE-A1 polyclonal antibody ab21472 (Abcam, Cambridge, United Kingdom), which recognizes the human epitope (amino acids 245-259). The application of the primary antibody was followed by incubation with Alexa Fluor 546 (red) goat anti-rabbit IgG for 45 minutes (Molecular Probes, Leiden, the Netherlands). The nuclear counterstaining was applied with Vectashield Mounting Medium with 4′,6-diamidino-2-phenylindole (blue; Vector Laboratories, Burlingame, CA).

DNA Methylation Analysis by Sodium Bisulfite Sequencing

For the sodium bisulfite conversion, 4 μg genomic DNA was denatured in 0.3 mol/L NaOH for 20 minutes at 42°C. The denatured DNA was incubated in a 600 μL solution of 3 mol/L sodium bisulfite, 0.5 mmol/L hydroquinone, and 1.6 mol/L NaOH (pH 5.0) for at least 16 hours at 50°C. The sodium bisulfite–treated DNA was desalted and concentrated by a column containing a silica matrix obtained from the PCR purification kit (Qiagen). Following precipitation and resuspension, 4 μL modified DNA was amplified with primers specific for MAGE-A1, -A2, -A3, and -A12 promoter fragments to investigate the methylation status.

PCR

PCR amplification of cDNA was done with primers specific for *MAGE-A1, -A2, -A3*, and *-A12* genes and PCR amplification of bisulfite-modified genomic DNA was done with methylation-specific primers. The reaction was done in a final volume of 40 μL containing PCR buffer, Q-Solution for analyses of CG-rich sequences (Qiagen), 200 μmol/L of each deoxynucleotide triphosphate (Roche, Mannheim, Germany), 0.5 μmol/L of each primer set, and 2.5 units Taq polymerase (Qiagen). Template DNA was amplified in 40 cycles.

For analysis of the promoter methylation patterns, the PCR products of bisulfite-treated genomic DNA were cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen) and at least 11 clones were picked for sequencing analysis. The constructs of the MAGE-A1, -A2, -A3, and -A12 promoter fragments were amplified with primers specific for the M13 sequences flanking the cloning site of the pCR4-TOPO vector. All clones were verified by digesting using restriction enzymes and DNA sequencing (Institute for Cellbiochemistry and Clinical Neurobiology, Hamburg, Germany).

PCR Methylation Analysis of MAGE-A2 Promoter

The genomic DNA isolated from cell lines untreated or treated by 5-aza-CdR and/or trichostatin A was twice digested for 10 hours with the methylation-sensitive and methylation-insensitive restriction enzymes *Hpa*II and *Msp*I (New England Biolabs, Frankfurt, Germany), respectively. Digested DNA (100 ng) was amplified in 35 cycles with three primers specific for the MAGE-A2 promoter and forming two primer sets. For the amplification of the positive control, a primer set binding at positions −174/−144 and +332/+304 was used, and for the amplification of the methylation-sensitive analysis, a set binding at positions −549/−524 and +332/+304 was used. After gel electrophoresis, the PCR product was densitometrically quantified using the software NIH Image 6.2 f.

Construction of Plasmids

Reporter plasmids were constructed by cloning the MAGE-A1 (−82/+116), MAGE-A2 (−549/+332), MAGE-A3 (−418/+12), and MAGE-A12 (−425/+115) promoter fragments into the *Bgl*II and *Hin*dIII sites of the pGL2-Luciferase reporter plasmid (Promega, Mannheim, Germany). All clones were verified by restriction digestion and DNA sequencing.

In vitro *Methylation of Plasmid DNA*

Reporter plasmid constructs (20 μg) containing the MAGE-A1, -A2, -A3, and -A12 promoters (pMAGE-A1Pro, -A2Pro, -A3Pro, and -A12Pro) were methylated by *Hpa*II or *Sss*I methylase (New England Biolabs) for 4 hours. Efficient methylation of the plasmid DNA was confirmed by its resistance to digestion with the methylation-sensitive restriction enzyme *Hpa*II. As a control a digestion with the isoschizomer *Msp*I was done.

Transfection and Luciferase Assay

The MCF-7 cell line was transfected with 0.5 μg reporter plasmids (pMAGE-A1Pro, -A2Pro, -A3Pro, and -A12Pro) using the FuGene 6 reagent (Roche Applied Science, Indianapolis, IN). For efficiency of the transfection assays, the CMV-β-galactosidase vector was additionally transfected as an internal control. Following 24-hour transfection, the cell line was incubated with trichostatin A (f.c. 0.5 mmol/L) for

24 hours and lysed using the Luciferase Reporter Gene Assay (Roche Applied Science). Promoter-driven luciferase activity was measured by a 20/20^n Luminometer (Turner Biosystems, Sunnyvale, CA) and normalized by the β-galactosidase activity (Galacto-Light, Applied Biosystems, Bedford, MA). Each transfection experiment was carried out in triplicate wells and repeated for at least thrice.

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References

1. Takahashi K, Shichijo S, Noguchi M, Hirohata M, Itoh K. Identification of MAGE-1 and MAGE-4 proteins in spermatogonia and primary spermatocytes of testis. Cancer Res 1995;55:3478–82.

2. De Smet C, Martelange V, Lucas S, Brasseur F, Lurquin C, Boon T. Identification of human testis-specific transcripts and analysis of their expression in tumor cells. Biochem Biophys Res Commun 1997;241:653–7.

3. Inoue H, Mori M, Honda M, et al. The expression of tumor-rejection antigen "MAGE" genes in human gastric carcinoma. Gastroenterology 1995;109:1522–5.

4. Liang G, Gonzales FA, Jones PA, Orntoft TF, Thykjaer T. Analysis of gene induction in human fibroblasts and bladder cancer cells exposed to the methylation inhibitor 5-aza-2′-deoxycytidine. Cancer Res 2002;62:961–6.

5. Miyashiro I, Kuo C, Huynh K, et al. Molecular strategy for detecting metastatic cancers with use of multiple tumor-specific MAGE-A genes. Clin Chem 2001;47:505–12.

6. Otte M, Zafrakas M, Riethdorf L, et al. MAGE-A gene expression pattern in primary breast cancer. Cancer Res 2001;61:6682–7.

7. Kufer P, Zippelius A, Lutterbuse R, et al. Heterogeneous expression of MAGE-A genes in occult disseminated tumor cells: a novel multimarker reverse transcription-polymerase chain reaction for diagnosis of micrometastatic disease. Cancer Res 2002;62:251–61.

8. van der Bruggen P, Traversari C, Chomez P, et al. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 1991;254:1643–7.

9. Chomez P, De Backer O, Bertrand M, De Plaen E, Boon T, Lucas S. An overview of the MAGE gene family with the identification of all human members of the family. Cancer Res 2001;61:5544–51.

10. De Smet C, Courtois SJ, Faraoni I, et al. Involvement of two Ets binding sites in the transcriptional activation of the MAGE1 gene. Immunogenetics 1995;42:282–90.

11. De Smet C, Lurquin C, Lethé B, Martelange V, Boon T. DNA Methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG rich promoter. Mol Cell Biol 1999;19:7327–35.

12. De Backer O, Arden KC, Boretti M, et al. Characterisation of the GAGE genes that are expressed in various human cancers and in normal testis. Cancer Res 1999;59:3157–65.

13. Lethé B, Lucas S, Michaux L, et al. LAGE-1, a new gene with tumor specificity. Int J Cancer 1998;76:903–8.

14. Otto SP, Walbot V. DNA methylation in eukaryotes: kinetics of demethylation and *de novo* methylation during the life cycle. Genetics 1990;124:429–37.

15. Esteller M, Herman JG. Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. J Pathol 2002;196:1–7.

16. Mori M, Inoue H, Mimori K, et al. Expression of MAGE genes in human colorectal carcinoma. Ann Surg 1996;224:183–8.

17. Serrano A, Garcia A, Abril E, Garrido F, Ruiz-Cabello F. Methylated CpG points identified within MAGE-1 promoter are involved in gene repression. Int J Cancer 1996;68:464–70.

18. Janssen BL, van de Locht LT, Fourkour A, et al. Transcription of the MAGE-1 gene and the methylation status of its Ets binding promoter elements: a quantitative analysis in melanoma cell lines using a real-time polymerase chain reaction technique. Melanoma Res 1999;9:213–22.

19. Tahara K, Mori M, Sadanaga N, Sakamoto Y, Kitano S, Makuuchi M. Expression of the MAGE gene family in human hepatocellular carcinoma. Cancer 1999;85:1234–40.

20. Bert T, Lubomierski N, Gangsauge S, et al. Expression spectrum and methylation-dependent regulation of melanoma antigen-encoding gene family members in pancreatic cancer cells. Pancreatology 2002;2:146–54.

21. Suyama T, Ohashi H, Nagai H, et al. The MAGE-A1 gene expression is not determined solely by methylation status of the promoter region in hematological malignancies. Leuk Res 2002;26:1113–8.

22. Karpf AR, Lasek AW, Ririe TO, Hanks AN, Grossman D, Jones DA. Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine. Mol Pharmacol 2004;65:18–27.

23. Caponigro F, Basile M, de Rosa V, Normanno N. New drugs in cancer therapy, National Tumor Institute, Naples, 17-18 June 2004. Anticancer Drugs 2005;16:211–21.

24. Pilat MJ, Schwab ED, Yao KL, Pienta KJ. Examination of the DNA methylation properties in nontumorigenic and tumorigenic breast epithelial cell lines. Anticancer Res 1998;18:2575–82.

25. De Smet C, De Backer O, Faraoni I, Lurquin C, Brasseur F, Boon T. The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation. Proc Natl Acad Sci U S A 1996;93:7149–53.

26. De Smet C, Loriot A, Boon T. Promoter-dependent mechanism leading to selective hypomethylation within the 5′ region of gene MAGE-A1 in tumor cells. Mol Cell Biol 2004;24:4781–90.

27. Sigalotti L, Coral S, Altomonte M, et al. Cancer testis antigens expression in mesothelioma: role of DNA methylation and bioimmunotherapeutic implications. Br J Cancer 2002;86:979–82.

28. Guo Y, Pakneshan P, Gladu J, Slack A, Szyf M, Rabbani SA. Regulation of DNA methylation in human breast cancer. Effect on the urokinase-type plasminogen activator gene production and tumor invasion. J Biol Chem 2002;277:41571–9.

29. Zhang J, Yu J, Gu J, et al. A novel protein-DNA interaction involved with the CpG dinucleotide at −30 upstream is linked to the DNA methylation mediated transcription silencing of the MAGE-A1 gene. Cell Res 2004;14:283–94.

30. Sigalotti L, Coral S, Nardi G, et al. Promoter methylation controls the expression of MAGE2, 3 and 4 genes in human cutaneous melanoma. J Immunother 2002;25:16–26.

31. Sigalotti L, Fratta E, Coral S, et al. Intratumor heterogeneity of cancer/testis antigens expression in human cutaneous melanoma is methylation-regulated and functionally reverted by 5-aza-2′-deoxycytidine. Cancer Res 2004;64:9167–71.

32. Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG, Davidsen SK. Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther 2003;2:151–63.

33. Lim JH, Kim SP, Gabrielson E, Park YB, Park JW, Kwon TK. Activation of human cancer/testis antigen gene, XAGE-1, in tumor cells is correlated with CpG island hypomethylation. Int J Cancer 2005;116:200–6.

34. Ruault M, van der Bruggen P, Brun ME, Boyle S, Roizes G, De Sario A. New BAGE (B melanoma antigen) genes mapping to the juxtacentromeric regions of human chromosomes 13 and 21 have a cancer/testis expression profile. Eur J Hum Genet 2002;10:833–40.

35. Thurner B, Haendle I, Roder C, et al. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J Exp Med 1999;190:1669–78.

36. Sadanaga N, Nagashima H, Mashino K, et al. Dendritic cell vaccination with MAGE peptide is a novel therapeutic approach for gastrointestinal carcinomas. Clin Cancer Res 2001;7:2277–84.

37. Marchand M, Punt CJ, Aamdal S, et al. Immunisation of metastatic cancer patients with MAGE-3 protein combined with adjuvant SBAS-2: a clinical report. Eur J Cancer 2003;39:70–7.

38. Karanikas V, Lurquin C, Colau D, et al. Monoclonal anti-MAGE-3 CTL responses in melanoma patients displaying tumor regression after vaccination with a recombinant canarypox virus. J Immunol 2003;171:4898–904.

39. Hennessy BT, Garcia-Manero G, Kantarjian HM, Giles FJ. DNA methylation in haematological malignancies: the role of decitabine. Expert Opin Investig Drugs 2003;12:1985–93.

40. Ruter B, Wijermans PW, Lubbert M. DNA methylation as a therapeutic target in hematologic disorders: recent results in older patients with myelodysplasia and acute myeloid leukemia. Int J Hematol 2004;80:128–35.

41. Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC. Clinical development of histone deacetylase inhibitors as anticancer agents. Annu Rev Pharmacol Toxicol 2005;45:495–528.