Characterization of the GAGE Genes That Are Expressed in Various Human Cancers and in Normal Testis

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ABSTRACT

The GAGE-1 gene was identified previously as a gene that codes for an antigenic peptide, YRPPRPRY, which was presented on a human melanoma by HLA-Cw6 molecules and recognized by a clone of CTLs derived from the patient bearing the tumor. By screening a cDNA library from this melanoma, we identified five additional, closely related genes named GAGE-2–6. We report here that further screening of this library led to the identification of two more genes, GAGE-7B and -8. GAGE-1, -2, and -8 code for peptide YYWPRPRY. Using another antitumor CTL clone isolated from the same melanoma patient, we identified antigenic peptide, YYWPRRRY, which is encoded by GAGE-3, -4, -5, -6, and -7B, and which is presented by HLA-A29 molecules. Genomic cloning of GAGE-7B showed that it is composed of five exons. Sequence alignment showed that an additional exon, which is present only in the mRNA of GAGE-1, has been disrupted in gene GAGE-7B by the insertion of a long interspersed repeated element retropon. These GAGE genes are located in the p11.2–p11.4 region of chromosome X. They are not expressed in normal tissues, except in testis, but a large proportion of tumors of various histological origins express at least one of these genes. Treatment of normal and tumor cultured cells with a demethylating agent, azadeoxycytidine, resulted in the transcriptional activation of GAGE genes, suggesting that their expression in tumors results from a demethylation process.

INTRODUCTION

Human cancer cells bear antigens that are recognized by CTLs derived from the patient bearing the tumor. These antigens consist of membrane-bound class I MHC molecules with short peptides derived from cellular proteins. A number of these antigenic peptides are encoded by genes that are expressed at high frequency in cancers of various histological origins but not in normal tissues, except in male germ cells. The expression of these genes in germ cells does not result in the formation of antigens because these cells does not produce class I MHC molecules (1–4). The genes coding for these tumor-specific shared antigens include the members of several gene families, such as the MAGE, GAGE, BAGE, SXX, and LAGE-1/ny-eso-1 genes (5–9).

GAGE-1 was identified as a gene coding for a tumor antigen of melanoma cell line MZ2-MEL. Five cDNAs sharing 80–98% nt identity with the GAGE-1 sequence were identified by screening a MZ2-MEL cDNA library with a GAGE-1 probe (6), and two additional homologous cDNAs were identified by screening for genes expressed in cell lines from the LNCaP prostate cancer model (10). One of these cDNAs corresponds to an additional GAGE gene, GAGE-7; the other, called PAGE-1, differs from the GAGE sequences, essentially by the presence of insertions and deletions (10). In addition, more distant genes were recently identified by searching expressed sequence tag sequences (11). Contrary to the GAGE genes, these genes, also named PAGE, are expressed in normal male and female reproductive tissues, prostate, testis, fallopian tube, uterus, and placenta as well as in prostate cancer, testicular cancer, and uterine cancer (11). The GAGE and PAGE putative proteins do not show significant homology with any polypeptide recorded in data banks, and nothing is known about their function. Here, we report the identification of two additional GAGE genes, GAGE-7B and -8, the definition of the exon-intron structure of the GAGE genes, and their chromosomal localization. We also report that GAGE-3, -4, -5, -6, and -7B code for a new antigenic peptide presented by melanoma cells.

MATERIALS AND METHODS

Cell Lines. Melanoma cell line MZ2-MEL was derived from an abdominal metastasis of patient MZ2, and a number of subclones were obtained. Subclone MZ2-MEL.3.0 was obtained by limiting dilution. Subline MZ2-MEL.43 was derived by limiting dilution from MZ2-MEL.3.0 cells that had survived mutagen treatment (12, 13). Subline MZ2-MEL.3.1 was obtained by extending the culture of subclone MZ2-MEL.3.0 for >150 generations. Subline MZ2-MEL.F+, which does not express antigen F, was selected from subclone MZ2-MEL.3.1 with autologous CTL clone 76/6 (13) Subline MZ2-MEL.3.1+HLA-A29 was obtained by transfecting the HLA-A29 gene into subline MZ2-MEL.3.1. Melanoma cell lines were grown as described previously (13, 14). Autologous CTL clones 76/6 and 22/23 were derived from blood mononuclear cells of patient MZ2 and grown in conditions similar to those described previously (15). PBLs were isolated using Lymphoprep (Nycomed Pharma, Oslo, Norway) and grown in Iscove’s medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Life Technologies, Inc.), 100 units/ml interleukin 2 (Biogen, Geneva, Switzerland), and 0.3% (v/v) phytohemagglutinin-P (Difco Laboratories, Detroit, MI). The fibroblast culture was derived from a human lung sample and maintained in Iscove’s medium supplemented with 10% FCS, 1-arginine (116 mg/ml), 1-asparagine (36 mg/ml), and L-glutamine (216 mg/ml).

Transfection of COS-7 Cells. Transfection experiments were performed by the DEAE-dextran-chloroquine method (16). Briefly, 1.5 × 10⁶ COS-7 cells were transfected with 50 ng of plasmid pcDNA1/Amp (Invitrogen, Carlsbad, CA) containing cDNA of HLA-A29 or Cw6 and 50 ng of each of the GAGE cDNAs cloned in pcDNA1/Amp. The HLA cDNAs were isolated from a cDNA library prepared with RNA extracted from subline MZ2-MEL.43. Transfected COS-7 cells were tested in a CTL stimulation assay after 24 h.

CTL Stimulation Assay. Transfectants were tested for their ability to stimulate the production of TNF by CTL (15). Briefly, in microcultures containing target cells, 1500 22/23 CTLs or 1000 76/6 CTLs were added to 100 μl of Iscove’s medium (Life Technologies, Inc.) containing 10% human serum and 20 units/ml recombinant human interleukin 2. After 24 h, the supernatant was collected, and its TNF content was determined by testing its cytotoxic effect on
cells of WEHI-164 clone 13 (17) in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (15, 18).

**Antigenic Peptides and CTL Assay.** Peptides were synthesized on solid phase using N-(9-fluorenyl)methoxycarbonyl for transient NH₂-terminal protection, as described previously (19), and characterized by mass spectrometry. All peptides were >90% pure, as indicated by analytical high-performance liquid chromatography. Lyophilized peptides were dissolved at 20 mg/ml in DMSO, diluted to 2 mg/ml in 10 mM acetic acid, and stored at −20°C. Peptides were tested in a chromium release assay, as described previously (20). In this peptide sensitization assay, target cells were Cr-labeled for 1 h at 37°C and washed extensively. One thousand target cells were then incubated in 96-well microplates in the presence of various concentrations of peptide for 15 min at 37°C before CTLs were added as an effector:target ratio of 5:1. Chromium release was measured after 4 h at 37°C.

**Construction and Screening of cDNA Libraries.** The cDNA library was derived from MZ2-MEL-43, as described previously (6).

**Construction and Screening of Human Genomic Libraries.** Genomic DNA isolated from PBLs of patient MZ2 was partially digested with Sau3AI, size-fractionated by NaCl density gradient centrifugation, and ligated with BamHI-EcoRI-digested LambdaGEM-11 cloning vector (Promega, Madison, WI). The cosmid library was constructed with DNA from the renal cell carcinoma cell line LE9211-RCC, as described previously (21). Screening of the libraries was performed using standard techniques (22) and the PCR-generated GAGE-1 probe.

**DNA Sequencing.** Fragments of λ clones containing GAGE genes were subcloned in plasmids and sequenced using a λ Taq Cycle-sequencing kit (United States Biochemical, Cleveland, OH), and primers were 5’-labeled with [γ-<sup>32</sup>P]ATP. Sequences were also obtained directly from λ clones and cosmids.

**Genomic Southern Blot Analysis.** Transfer and hybridization of genomic DNA were performed using standard techniques (22). The GAGE-specific probe was a PCR-amplified fragment corresponding to nt 18–309 of the GAGE-1 cDNA. This fragment was purified from low melting point agarose and labeled by incorporation of [α-<sup>32</sup>P]dCTP (3000 Ci/ml) upon primer extension (23). This probe hybridizes to all known GAGE genes because of their high homology. Washing conditions were: 0.2× SSC-0.1% SDS at 65°C for the human blot and 2× SSC-0.1% SDS at 60°C for the zoo blot, which was obtained from Clontech Laboratories (Palo Alto, CA).

**Analysis of Somatic Cell Hybrids.** DNA isolated from the human × rodent hybrids was digested with EcoRI and used to prepare Southern blots, as described previously (24). The hybrids designated with the GM prefix were derived from human cell lines containing translocations between chromosome X and an autosome, resulting in the presence of only a portion of the X chromosome. They were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The PCR analysis was performed with primers VDE23 (sense, 5’-AGGAAACCTTAGGAGTAC-3’, nt 453–470 of the GAGE-1 cDNA sequence) and VDE25 (antisense, 5’-CAAGGACGCTGTA-AAGCT-3’, nt 613–630 of the GAGE-1 cDNA sequence), which amplify a GAGE genomic fragment of 0.7 kb. Thirty-five cycles of PCR were performed, each cycle consisting of a denaturation at 94°C for 60 s, an annealing at 50°C for 60 s and an extension at 72°C for 60 s. PCR was preceded by a 3-min incubation at 94°C and followed by a 72°C final soak for 10 min. Amplified products were electrophoresed through 2% agarose gels and visualized by ethidium bromide staining.

**FISH.** Chromosome preparations were obtained from phytohemagglutinin-stimulated normal PBLs cultured for 72 h. To obtain bands chromosomes, we inoculated some harvests with 5-bromodeoxyuridine, as described previously (25). Cytogenetic harvests and slide preparations were performed using standard methods. The slides were stored at −80°C before use. FISH with metaphase chromosomes was performed as described previously (26). Briefly, the slides were denatured for 2 min in 70% formamide-2× SSC (pH 7.0) at 70°C and dehydrated in ice-cold ethanol. A cosmid that contained gene GAGE-7B was used as a probe. The digoxigenin-labeled cosmid DNA (100 ng) was preannealed with 10 mg of COT-1 DNA (Life Technologies, Inc.) and dissolved in Hybriol VII (Oncor; 50% formamide-2× SSC), denatured at 75°C for 5 min, and applied to the slides. Hybridization was allowed to proceed overnight in a humid chamber at 37°C. The slides were then washed using the formamide wash procedure and revealed with the FITC-digoxigenin detection kit and amplification protocol for dual-color FISH (Oncor). Slides were counterstained by mounting in an antifade solution containing 1 mg/ml phenylenediamine and 0.3 mg/ml propidium iodide. Metaphase spreads were examined and photographed using a Zeiss Axioshot microscope and the appropriate UV-filter combinations. We considered signals to be specific only if they were detected on each chromatid of a single chromosome. Chromosome identification was performed by simultaneous hybridization with the a-satellite repeat probe or by R-banding using the 5-bromodeoxyuridine incorporation, as described previously (25).

**RT-PCR Assay for the Expression of GAGE Genes.** Total RNA was extracted by the guanidine-isothiocyanate procedure, as described (27). Reverse transcription was performed on 2 μg of total RNA in the presence of 2 μl oligo(DT)₄ primer in a reaction volume of 20 μl. One-twentieth of the cDNA product was used for PCR amplification. For specific amplification of the GAGE-1, -2, and -8 cDNAs, primers were VDE44 (sense, 5’-GCACAA-GACCGTCAGTAG-3’) and VDE24 (antisense, 5’-CCATCAGGACCCTCTCA-3’). For specific amplification of the GAGE-3, -4, -5, -6, and -7B cDNAs, primers were VSI1 (sense, 5’-GACCGGCTAGTGACT-3’) and VDE24 (antisense, 5’-CCATCAGGACCCTCTCA-3’). Both amplifications were performed as follows: after a first denaturation step at 94°C for 5 min, 30 cycles of amplification were performed (1 min at 94°C, 2 min at 58°C, and 2 min at 72°C), and then a final extension step terminated the reaction. PCR products were analyzed by agarose gel electrophoresis. RNA integrity was checked by reverse transcription and amplification of the β-actin mRNA.

**Aza-2’-deoxycytidine Treatment and mRNA Expression Analysis.** Cells were incubated for 72 h in culture medium containing 1 μM 5-aza-2’-deoxycytidine (Sigma, Deisenhofen, Germany). Fibroblasts were treated at passage 4. Total RNA purification and cDNA synthesis were performed as described (28). The primers used for PCR amplification of the GAGE cDNAs were VDE24 and VDE44 (6).

**RESULTS**

The GAGE Gene Family Contains at Least Eight Members. We previously identified six closely related cDNAs, named GAGE-1–6, by probing a cDNA library made with RNA from melanoma cell line MZ2-MEL-43 (6). A second screening of the MZ2-MEL-43 cDNA library yielded two additional cDNAs. One of these differed only by a single base from the sequence of the GAGE-7 described by Chen et al. (10) and encoded the same putative protein. Because we did not know whether this new cDNA corresponded to a new gene or to another allele of GAGE-7, we named it GAGE-7B. The additional cDNA was named GAGE-8. Fig. 1 shows that the sequences of GAGE-2, -3, -4, -5, -6, -7B, and -8 differ from each other mainly by single nt substitutions. The GAGE-1 cDNA differs from the seven others by the presence of a 143-bp insertion (Fig. 1).

Because most GAGE cDNAs present only a few single-nucleotide differences, there was a possibility that some of them were allelic products. However, the MZ2-MEL-43 cells must contain a single allele of each GAGE gene because they contain a single X chromosome, where the GAGE genes are located (see below). Indeed, both karyotyping and absence of Xist expression indicated that, despite their female origin, MZ2-MEL-43 cells contain a single X chromosome. We conclude that a minimum of eight GAGE genes are expressed in MZ2-MEL-43 cells.

**Genomic Structure of the GAGE Genes.** A GAGE genomic clone was isolated from a phage library constructed with DNA from patient MZ2. The sequence of the insert revealed that it contained DNA GAGE-7B and that this gene comprises five exons (Fig. 2; see also Fig. 1). An open reading frame coding for a product of 117 amino acids spans exons 2–5 (Fig. 1).

The fourth intron contained two regions (Fig. 2, 4A and 4B) that

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* The sequence data reported in this paper have been deposited with the GenBank database (accession nos. AF055473, AF055474, and AF055475).

* C. De Smet, unpublished results.
Fig. 1. A, alignment of the nt sequences of GAGE-1–8 cDNAs. The sequences are ordered according to their degree of homology to the GAGE-1 sequence. •, nt that are identical to the GAGE-1 sequence; □, nt that are identical to the GAGE-1 sequence; ▲, limits of the exons. Initiation and termination codons are in boldface type. Horizontal arrows, locations of the primers VDE44, VDE24, and VV1 used for the analysis of GAGE expression by RT-PCR. •, comparison between GAGE putative proteins; ▲, amino acid residues that are identical to the GAGE-1 sequence; □, antigenic peptides presented by HLA-Cw6 and -A29 molecules.

B

HLA-Cw6 peptide

HLA-A29 peptide
showed strong sequence homology with region 4', which is present only in the GAGE-1 cDNA (Figs. 1 and 2). A genome database search revealed that the region of 561 bp located between 4'A and 4'B corresponds to a truncated L1 retroposon belonging to the LINE family (29). This LINE element is flanked by a perfect 13-bp target site duplication and contains part of the reverse transcriptase coding region, the 3' untranslated region, and the poly(A) tail of the original retroposon.

We concluded that GAGE-1 should contain an additional exon corresponding to the region disrupted by the LINE insertion in the GAGE-7B intron. This hypothesis was supported by the presence of an acceptor splicing site at the 5' end of 4'A and a donor splicing site at the 3' end of 4'B. To confirm the existence of an undisrupted exon 4' in gene GAGE-1, we performed a PCR with MZ2 genomic DNA using a sense primer in 4'A and an antisense primer in 4'B. As expected, in addition to the fragment of 0.7 kb corresponding to the genes with a LINE insertion, the PCR produced a 140-bp fragment, the sequence of which corresponded exactly to the insertion of GAGE-1 (data not shown). This confirmed that GAGE-1 contains an additional exon that is skipped in the other GAGE genes because it has been disrupted by a LINE insertion.

By screening a cosmid library with a GAGE probe (corresponding to nt 18–309 of the GAGE-1 cDNA), we obtained a cosmid clone containing gene GAGE-8. This gene had the same exon-intron structure as the GAGE-7B gene, including the LINE insertion.

The same probe was used to hybridize a Southern blot, made with PstI-digested human genomic DNA from 11 men. The probe revealed four bands (of 0.9, 1.4, 2.8, and 3.7 kb) in all of the DNAs, whereas two other bands (of 4.3 and 5.2 kb) were present in only some subjects, indicating the existence of polymorphism (Fig. 3). The bands of 0.9 and of 5.2 kb were expected from the sequence of gene GAGE-7B. The other bands should correspond to other members of the family. In addition, the strong intensity of the 0.9- and 2.8-kb bands suggests the stacking of several DNA fragments corresponding to different genes. Thus, this hybridization pattern confirmed the existence of several GAGE genes.

Southern hybridization with the GAGE probe revealed the presence of homologous genes in the monkey but not in the other mammals analyzed (mouse, rat, dog, cow, and rabbit). This indicated either that these species do not contain sequences that are homologous to GAGE genes or that these sequences are not sufficiently homologous to be detected by hybridization, even in low-stringency conditions.

**PAGE-1 Belongs to the GAGE Gene Family.** When compared to the GAGE-2 sequence, PAGE-1 shows two insertions (35 and 126 bp) and two short deletions (24 and 12 bp; Fig. 4). The insertion of 35 bp is located precisely between the first and the second exon and its sequence is homologous to the 5' end of the first intron in the GAGE-8 gene. Thus, it seems that, in PAGE-1, the use of a splicing donor site located downstream from the site used in the GAGE genes lengthens the first exon of 35 bp. The last 59 bp of the second insertion are a repetition of a sequence located just upstream from this insertion (Fig. 4). GAGE-2 shares 78% of identical residues with PAGE-1, whereas an identity of >56% was reported by Chen et al. (10). This difference is probably due to the addition of the gaps in the calculations of Chen et al. (10). The resemblance between PAGE-1 and the GAGE genes indicates clearly that it belongs the GAGE family. Therefore, we suggest renaming PAGE-1 as GAGE-9.

**Chromosomal Localization of the GAGE Genes.** The chromosomal localization of the GAGE genes was initially determined by Southern blot analysis of a panel of hamster or mouse × human somatic cell hybrids. Hybridization of human DNA with the GAGE-1 probe (nt 18–309) detected a single EcoRI band of 4.3 kb, indicating that the EcoRI sites defining this fragment are conserved in all GAGE genes (Fig. 5). No hybridization signal was observed with hamster or mouse DNA or with the 23 hybrids that covered the 22 human autosomes. A hybridization signal was detected only with the DNA from hybrid GM06318B that contained the human X chromosome (Fig. 5). The absence of a signal with the DNA from GM06317 that contained the human Y chromosome suggested that the GAGE genes do not reside in a pseudoautosomal region.
To refine the localization of the GAGE locus, hybrid cell lines containing only fragments of the human X chromosome were analyzed by hybridization and by PCR with GAGE primers. No signal was observed in hybrid GM09412 containing the Xpter–p21 region and in hybrid GM10095 containing the Xq13–qter region, indicating that the GAGE genes are located in Xp21–Xq13.

The position of the GAGE locus was also determined by FISH. Several cosmids containing GAGE genes were used as probes. All of them hybridized in the p11.2–p11.4 region of chromosome X (Fig. 6).

Antigenic Peptides Encoded by the GAGE Genes. A panel of stable autologous CTL clones was obtained previously against human melanoma cell line MZ2-MEL by stimulating blood mononuclear cells from patient MZ2 with irradiated autologous tumor cells (12). Tumor cells, selected in vitro for resistance to some of these CTL clones, remained sensitive to others, indicating that several different antigens were present on the MZ2-MEL cells (13). One of these antigens, named MZ2-F, was the antigenic peptide YRPRPRRY, presented by HLA-Cw6 (Fig. 1). This peptide is encoded by GAGE-1, GAGE-2, and also GAGE-8. We showed previously that cells expressing GAGE-1 or GAGE-2 are recognized by the CTLs directed against this peptide (6). We found that the antigenic peptide can also be processed from the GAGE-8 protein because COS-7 cells transfected with HLA-Cw6 and GAGE-8 stimulated the release of TNF by 76/6 CTLs (Fig. 7).

A number of other CTL clones, including CTL 22/23, were shown to lyse several MZ2-MEL subclones but not MZ2-MEL.3.1, a line that had been passed in vitro for >150 generations and had lost one HLA haplotype coding for HLA-Cw*1601, -A29, and -B44 (Fig. 8). The sequences encoding these HLA molecules were isolated from a cDNA library of patient MZ2 and stably transfected into MZ2-MEL.3.1. The cells transfected with HLA-A29 were lysed by the 22/23 CTLs, demonstrating that the presenting HLA molecule is A29 (Fig. 8). We then transfected COS-7 cells with HLA-A29 cloned in pcDNA1/Amp and a panel of expression plasmids containing cDNA coding for various tumor specific shared antigens, including members of the MAGE, BAGE, and GAGE families (6, 7, 30). Cells cotransfected with the cDNA of GAGE-3, -4, -5, -6, and -7B stimulated the release of TNF by 22/23 CTLs (Fig. 7). We searched the sequences of
the corresponding proteins for a peptide that was not encoded by GAGE-1, 2, or 8 and that contained consensus anchor residues for HLA-A29, namely E in position 2 and Y in position 9 (31). Only the nonapeptide YYWPRPRRY (Fig. 1) fulfilled these conditions. It was tested in a cytotoxicity assay with CTL 22/23 and produced half-maximal lysis of autologous EBV-B target cells at $10^6$ nM (Fig. 9).

Expression of the GAGE Genes. RT-PCR with specific primers allowed us to analyze the expression of the genes coding for one or the other antigenic peptide. GAGE transcripts were detected in a significant proportion of malignant tumors of various histological types. The highest fraction of positive tumors were found in melanoma as well as in esophageal and lung carcinomas (Table 1). In addition, GAGE-1, 2, and 8 mRNAs were also detected in 15% of prostate adenocarcinomas ($n = 20$), 10% of breast carcinomas ($n = 153$), and 17% of sarcomas ($n = 12$). The expression of GAGE-3, -4, -5, -6, and -7B was not determined in these tumors. No expression of GAGE was found in colorectal carcinomas and renal carcinomas (data not shown).

Expression of GAGE Genes Can Be Induced by DAC Treatment. To assess whether expression of GAGE genes can be induced by demethylation, we treated several cultured tumor and normal cells with DAC. RT-PCR analysis revealed the induction of GAGE-1, -2, and -8 in treated cells from the sarcoma cell line LB23 and from the melanoma cell lines Mi665 and SK23-MEL. GAGE-1, -2, and -8 and GAGE-3, -4, -5, -6, and -7B mRNAs were detected after treatment of phytohemagglutinin-stimulated PBLs (data not shown).

DISCUSSION

The GAGE genes belong to the category of genes exemplified by the MAGE genes. These genes are silent in most normal adult tissues but are expressed in cancers of various histological origin (5). Because the expression of these genes is shared by many different tumors, the antigens they code are promising targets for cancer immunotherapy. The only normal tissues that have been found to express these MAGE-type genes are testis and, in some instances, placenta, two tissues regarded as immunologically privileged sites (32). In testis, expression of the MAGE-type genes in the male germ cells should not result in antigen expression because these cells do not express class I MHC molecules (33). This is supported by the observation that in mice, a CTL response against a tumor antigen encoded by P1A, which is also expressed in testis, did not generate antitestis autoimmune effects (4).

What could explain the expression pattern of the MAGE-type genes? The activation of MAGE-A1 in cancer cells is due to the demethylation of its promoter, a process that appears to be the consequence of a genome-wide demethylation occurring in certain tumors (34). It is tempting to speculate that demethylation could also explain the activation of the other MAGE-type genes in tumors. The activation of genes MAGE-A1, -A2, -A3, -A4, -B1, and -B2, BAGE, GAGE, and P1A in cells treated with the demethylating agent DAC.
...show regulation by androgens in the prostate cancer cell lines. These analyses of expression were done essentially by Northern blot, using the PAGE-1 and GAGE-7 cDNAs as probes. However, given its high homology with the other GAGE genes, the GAGE-7 probe was probably not specific for GAGE-7 but hybridized with mRNA from all of the GAGE genes. Thus, we believe that the “GAGE-7 expression” reported by these authors probably corresponds to the expression of one or several undetermined members of the GAGE family. The same may also be true for the PAGE-1 probe, which contained blocks of sequence with >85% identity with GAGE sequences.

Besides their expression patterns, most of the MAGE-type genes share a common location on the X chromosome. The MAGE genes are clustered in three loci: Xq28 for the MAGE-A family (30), Xp21.3 for the MAGE-B family (41), and Xq27 for MAGE-C1 (42); the GAGE genes are located in Xp11.2–p11.4, the SSX genes map to Xp11.2 (43); NY-ESO-1 maps to Xq28 (8); and P1A has also been localized on the X chromosome (P. Chomez, personal communication). It is unclear at this stage whether there is a functional reason for the location of all these genes on the X chromosome.

Hybridization of Southern blots made with DNA from different eukaryotic species (including six mammalian species) did not reveal sequences homologous with GAGE genes, except in the monkey. This indicates that the GAGE genes are poorly conserved, even within mammals. This feature is shared with gene P1A, which appears to be devoid of a human homologue, and with the MAGE genes, which share only ~60% identity with their mouse homologues with respect to the coding region (28, 37, 44). This rapid evolution suggests that these genes have undergone species-specific adaptive selection. Such a selection has been demonstrated for genes involved in intergenomic conflicts as occur between host and pathogen. Another group of genes exhibiting a high level of sequence divergence includes those transcribed in reproductive tissues. For example, the Y chromosome-encoded SRY sex determination gene encodes a protein that contains domains that evolve rapidly (45, 46). The X chromosome-encoded Pem homeobox gene, which is selectively expressed in placenta, testis, epididymis, and ovary, has also been shown to undergo high rates of sequence divergence (47).

GAGE-1 has an additional exon (exon 4') with respect to the other GAGE genes. In the other genes, exon 4' is skipped because it has been disrupted by the insertion of a LINE retroposon. This suggests a scenario in which a GAGE ancestor gene has been duplicated before the insertion of a LINE element in one of the two resulting copies. GAGE-2–8 would then be generated through duplication of the disrupted copy. Because exon 4' encodes the 29 COOH-terminal resi-

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<th>No. of samples</th>
<th>Expression of GAGE-1, -2, and -8</th>
<th>Expression of GAGE-3, -4, -5, -6, and -7B</th>
<th>% of samples expressing GAGE-1, -2, and -8 and/or GAGE-3, -4, -5, -6, and -7B</th>
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* See Fig. 1 for the sequence and the location of the primers used for RT-PCR analysis. A more sensitive RT-PCR protocol explains the higher percentage of tumors expressing GAGE genes with respect to the data reported previously (6).

Fig. 9. Lysis by CTL 22/23 of autologous EBV-B cells incubated with peptide YYWPRPRRY. MZ2-EBV cells were Cr-labeled and incubated with 22/23 CTLs at an E:T ratio of 5:1 in the presence of the peptide at the indicated concentrations. Chromium release was measured after 4 h.

Table 1. Expression of the GAGE genes in tumors

<table>
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<tr>
<th>Tumor</th>
<th>No. of samples</th>
<th>Expression of GAGE-1, -2, and -8</th>
<th>Expression of GAGE-3, -4, -5, -6, and -7B</th>
<th>% of samples expressing GAGE-1, -2, and -8 and/or GAGE-3, -4, -5, -6, and -7B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutaneous melanoma (primaries)</td>
<td>79</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cutaneous melanoma (metastases)</td>
<td>211</td>
<td>7</td>
<td>1</td>
<td>54</td>
</tr>
<tr>
<td>Esophageal squamous cell carcinoma</td>
<td>18</td>
<td>7</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Esophageal adenocarcinoma</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Lung squamous cell carcinoma</td>
<td>83</td>
<td>28</td>
<td>4</td>
<td>44</td>
</tr>
<tr>
<td>Lung adenocarcinoma</td>
<td>42</td>
<td>13</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Head and neck carcinoma</td>
<td>92</td>
<td>21</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Bladder carcinoma (superficial)</td>
<td>35</td>
<td>1</td>
<td>0</td>
<td>34</td>
</tr>
<tr>
<td>Bladder carcinoma (infiltrating)</td>
<td>40</td>
<td>8</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Leukemia</td>
<td>76</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

* See Fig. 1 for the sequence and the location of the primers used for RT-PCR analysis. A more sensitive RT-PCR protocol explains the higher percentage of tumors expressing GAGE genes with respect to the data reported previously (6).

... supports this hypothesis (34–36).6 Genes such as MAGE-B3 and MAGE-B4, which are poorly induced or not induced at all by DAC, are not expressed in tumor cells (37). The demethylation hypothesis predicts that demethylated tumors ought to express frequently several MAGE-type genes. This was observed within the MAGE and GAGE families, whose members are frequently expressed together by the same tumors.7 We also frequently observed that the same tumors expresses genes belonging to different families of MAGE-type genes.7 However, the coexpression is not absolute and certain tumors express only one or a few MAGE-type genes. Demethylation is also likely to account for expression in testis, as a decrease in the level of DNA methylation was observed during spermatogenesis (38, 39). Undermethylation was also observed in placenta (40). In testis, it is not known whether the different MAGE-type genes are activated at the same stage of spermatogenesis. This question could be addressed by in situ hybridization or by replacing these genes by reporters in transgenic mice.

Chen et al. (10) reported that GAGE-7 was expressed in all of the analyzed the tumorigenic and nontumorigenic LNCaP sublines, whereas expression of PAGE-1 was increased 5-fold in the metastatic cell sublines with respect to the nontumorigenic LNCaP. In addition, these authors reported that neither GAGE-7 nor PAGE-1...
dyes of the putative protein, its skipping leads to their substitution by 7 unrelated amino acids encoded by exon 5. Because nothing is presently known about the function of the GAGE proteins, the effects of this substitution are obscure. Exon skipping associated with LINE insertions has been observed in the dystrophin gene (48, 49) and in the sodium channel gene Scn8a (50). Two possible causes have been proposed to explain this skipping. The two reported insertions in the dystrophin gene were 0.6 and 2 kb long, increasing exon length beyond the usual limit of 400 bp, which is thought to interfere with the exon definition step of splicing (51). Such a size effect could explain exon skipping in the GAGE genes, in which the LINE insertion resulted in an exon length of 707 bp. The insertion in Scn8a resulted in an exon of 300 bp, which is within the normal range. Exon skipping was in this case ascribed to the poly(U) sequence of the LINE element because insertion of polypyrrimidine tracts is known to inhibit splicing (52).

Two antigenic peptides derived from GAGE proteins have been identified. One derives from proteins GAGE-1, -2, and -8 and is presented by HLA-Cw6. The other derives from GAGE-3, -4, -5, -6, and -7B and is presented by HLA-A29, an allele that is expressed by only 6% of Caucasians. Patients eligible for anti-GAGE immunotherapy can be identified by HLA typing and by testing by RT-PCR the expression of the two groups of GAGE genes in their tumor samples. In addition to the two identified antigenic peptides, the GAGE proteins are likely to comprise other peptides combined with various class I molecules to form antigens. Their identification ought to increase the fraction of patients who are eligible for anti-GAGE immunotherapy.

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REFERENCES

Characterization of the GAGE Genes That Are Expressed in Various Human Cancers and in Normal Testis

Olivier De Backer, Karen C. Arden, Mauro Boretti, et al.


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