

# Sequence and expression pattern of the human MAGE2 gene

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Abstract. We reported previously identification of the human MAGE1 gene, which encodes an antigen recognized on human melanoma MZ2-MEL by autologous cytolytic T lymphocytes. In addition to MAGE1, melanoma MZ2-MEL expresses several closely related genes, one of which has been named MAGE2. The complete MAGE2 sequence was obtained and it comprises 3 exons homologous to those of MAGE1 and an additional exon homologous to a region of the first MAGE1 intron. Like the open reading frame of MAGE1, that of MAGE2 is entirely encoded by the last exon. The MAGE1 and MAGE2 sequences of this exon show 82% identity and the putative proteins show 67% identity. The MAGE2 gene is expressed in a higher proportion of melanoma tumors than MAGE1. It is also expressed in many small-cell lung carcinomas and other lung tumors, laryngeal tumors, and sarcomas. No MAGE1 and MAGE2 gene expression was found in a large panel of healthy adult tissues, with the exception of testis.

## Introduction

When the blood lymphocytes of a melanoma patient are stimulated in vitro with an autologous tumor cell line, it is often possible to obtain cytolytic T cells (CTL) that lyse the tumor cells and do not lyse the healthy cells of the same patient. From these responder T-cell populations it is possible to isolate anti-tumor CTL clones (Mukerii and McAlister 1983; De Vries and Spits 1984; Knuth et al. 1984). Such a panel of CTL clones has been obtained against the human melanoma cell line MZ2-MEL (Hérin et al. 1987). These CTL clones were used to select antigen-loss variants and this led to the definition of several different antigens on the MZ2-MEL cells, one of which was named MZ2-E (Van den Eynde et al. 1989). To identify the gene directing the expression of antigen MZ2-E, a cosmid library prepared with the DNA of the tumor cells was transfected into an E- antigen-loss variant. This led to the identification of the MAGE1 gene, which transfers the expression of this antigen (van der Bruggen et al. 1991; Traversari et al. 1992b). The gene is about 4.5 kilobases (kb) long and comprises three exons. The third exon contains an open reading frame coding for a protein of 309 amino acids. A nonapeptide encoded by this open reading frame combines to the class I major histocompatibility complex (MHC) molecule HLA-A1 to form antigen MZ2-E (Traversari et al. 1992a). The MAGE1 gene was found to be expressed in several melanoma tumors and in other types of tumors as well. No expression was found in a panel of healthy tissues (van der Bruggen et al. 1991; Brasseur et al. 1992), but we report here that the gene is expressed in testis.

When we analyzed *MAGE1* gene expression in the MZ2-MEL cell line, we found two other species of messenger RNA that hybridized with a *MAGE1* probe. The corresponding genes were named *MAGE2* and *MAGE3*. Because these genes may also code for tumor rejection antigens recognized by cytolytic T cells, it seemed worthwhile obtaining a complete description of their structure and expression. We report here the complete *MAGE2* gene sequence and its pattern of expression in a panel of healthy and tumor tissues.

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession number L18920.

## Materials and methods

*Cell lines.* The melanoma cell line MZ2-MEL was derived from an abdominal metastasis of patient MZ2 (Hérin et al. 1987). A number of subclones such as MZ2-MEL.43 and MZ2-MEL.3.1 were obtained. The subline MZ2-MEL.2.2, which does not express antigen MZ2-E, was obtained after repeated selection of MZ2-MEL.3.1 with an autologous anti-E CTL clone (Van den Eynde et al. 1989).

Hybridization with oligonucleotides. Oligonucleotides were labeled, using terminal deoxynucleotidyl transferase (BRL, Gaithersburg, MD) and  $[\alpha^{-32}P]$  dCTP (3000 Ci/mmole; Amersham, Arlington Heights, IL) to a specific activity of  $\sim 2.10^8$  cpm/µg. The probes were purified on Chroma Spin-10 columns (Clontech, Palo Alto, CA). Nitrocellulose filters were prehybridized for 2 h at 65°C in  $10 \times \text{Denhardt's solution}, 6 \times \text{standard sodium citrate (SSC) and}$ hybridized overnight to the labeled oligonucleotides (106-3.10<sup>6</sup> cpm/ml) in 50  $\mu$ l/cm<sup>2</sup> of 3.5  $\times$  SSC, 1  $\times$  Denhardt's solution, 25 mM sodium phosphate buffer (pH 7.0), 0.5% sodium dodecyl sulfate (SDS), 2 mM ethylenediaminotetraacetate, and 100 µg/ml denatured salmon sperm DNA at a temperature that was 5° C below the dissociation temperature of the oligonucleotide. After hybridization, the filters were washed twice at room temperature for 5 min each in 6  $\times$  SSC and then twice in 6  $\times$  SSC, 0.1% SDS at the dissociation temperature of the oligonucleotide probe for 5 min each.

Screening of the genomic library. The genomic library was derived from the MZ2-MEL.43 melanoma cell line as previously described (van der Bruggen et al. 1991). About 50 ng of DNA of each cosmid group was submitted to polymerase chain reaction (PCR) amplification in a 100  $\mu$ l reaction mixture, using the GeneAmp PCR reagent kit (Perkin Elmer, Norwalk, CT) and, as primers, the previously defined oligonucleotides CHO-8 and CHO-9 (van der Bruggen et al. 1991). Each PCR reaction (18  $\mu$ l) was then size-fractionated in agarose gels, blotted on nitrocellulose filters, and hybridized with oligonucleotide probe CHO-2 (van der Bruggen et al. 1991). A positive group of cosmids was then screened by colony hybridization with the CHO-2 probe (Sambrook et al. 1989).

Sequencing. Cosmid C6.3 was digested with Bam HI and Pst I, size-fractionated in agarose gels, and blotted on nitrocellulose filters. The blotted material was then hybridized with the oligonucleotides CHO-2, CDS-2, 5'-AGAACCACTGCATCTGTCGAC-3' (MAGE2, positions 1375–1355 anti-sense), or CDS-1, 5'-CCAATAATCCAGCGCTGCCT-3' (MAGE2 positions 71–90 sense). The hybridizing fragments were subcloned in pTZ18R and single-stranded DNA was produced. The nucleotide sequence was determined, using the T7 Sequencing kit (Pharmacia Piscataway, NJ) and synthetic primers. Sequence alignments with MAGE1 were performed with the GeneWorks computer program (Intelligenetics, Mountain View, CA). Sequence identity was estimated as the percentage of matching nucleotides contained in the aligned sequence.

Rapid amplification of the 5' cDNA end. The rapid amplification of the 5' cDNA end of MAGE2 mRNA was performed essentially as described by Frohman and co-workers (1988). The primer for the synthesis of the first cDNA strand was 5'-ACTTCCACTAGAG-TAG-3' (MAGE2 positions 2582-2567 anti-sense). For the amplification, we used 5'-GACCTGAGTCACCTTCTGAAAAC-3' (MAGE2 positions 1335-1313 anti-sense) as 3' primer and the primers described by Frohman and co-workers (1988) as 5' primers. The amplified products were cloned in pTZ18R, using the Sal I site of the 5' primer and a *Pst* I site located in the *MAGE2* sequence at position 280. The resulting clones were screened with the CDS-1 oligonucleotide probe, and five positive clones were sequenced.

Southern blot analysis. DNA extraction, electrophoresis, transfer to nitrocellulose filter, labeling of the *MAGE1* 2.4 kb *Bam* HI fragment, and hybridization were performed as described (Lurquin et al. 1989), except for the last step of washing, which was performed in  $0.5 \times SSC$  and 0.5% SDS at  $65^{\circ}C$ .

mRNA expression analysis. Total RNA was extracted by the guanidine-isothiocyanate procedure (Davis et al. 1986). For cDNA synthesis, total RNA (2  $\mu$ g) was diluted to a total volume of 20  $\mu$ l with 4  $\mu$ l 5  $\times$  HR-T buffer (BRL), 4  $\mu$ l dNTPs at 2.5 mM each, 2  $\mu$ l 0.1 M DTT (BRL), 1 µl of a 40 µM solution of oligo dT(15), 1 µl RNasin at 40 units/µl (Promega, Madison, WI), 1 µl MoMLV reverse transcriptase at 200 units/µl (BRL), and autoclaved water to 20 µl. This mixture was incubated at 42°C for 50 min and then diluted to 100 µl with water. Five microliters of this cDNA solution was used for PCR amplification performed in a volume of 50 µl containing 1 × PCR amplification buffer of the GeneAmp PCR reagent kit (Perkin Elmer), each primer at 0.5 µM, each dNTP at 200 µM (Perkin Elmer), and 1.25 units AmpliTaq DNA polymerase (Perkin Elmer). Primers were: 5'-CGGCCGAAGGAACCTGACC-CAG-3' (MAGE1 positions 26-47 sense) and 5'-GCTGG-AACCCTCACTGGGTTGCC-3' (MAGE1 positions 3331-3309 anti-sense) for MAGE1; 5'-AAGTAGGACCCGAGGCACTG-3' (MAGE2 positions 2251-2270 sense) and 5'-GAAGAGGAA-GAAGCGGTCTG-3' (MAGE2 positions 2567-2548 anti-sense) for MAGE2; 5'-GGCATCGTGATGGACTCCG-3' (human β-actin exon 3 sense), and 5'-GCTGGAAGGTGGACAGCGA-3' (human β-actin exon 6 anti-sense) for the human β-actin gene. The cDNA was first denatured at 94°C for 5 min, amplifications were then performed for 30 cycles (1 min 94°C, 3 min 72°C for MAGE1; 1 min 94°C, 2 min 68°C, 2 min 72°C for MAGE2 and the β-actin gene). Each reaction (18 µl) was size-fractionated in an agarose gel containing ethidium bromide and was visualized on an ultraviolet transilluminator.

### Results

A cDNA library prepared with RNA of the melanoma line MZ2-MEL was hybridized with a 2.4 kb probe covering exons 2 and 3 of the *MAGE1* gene (Fig. 1). This led to the identification of two cDNA species whose sequences were different from that of *MAGE1* but closely homologous. The corresponding genes were named *MAGE2* and *MAGE3* (van der Bruggen et al. 1991).

A cosmid carrying gene MAGE2. To isolate the MAGE2 gene, we screened a cosmid library prepared with the DNA of a subline of MZ2-MEL. DNA extracted from 14 groups of 50000 independent cosmids was submitted to PCR amplification with oligonucleotide primers corresponding to sequences of the third exon of MAGE1, which are common to the MAGE1, -2 and -3 cDNAs (van der Bruggen et al. 1991). The PCR products were then hybridized with a radioactive oligonucleotide corresponding to a region



Fig. 1. Comparison of MAGE1 and MAGE2 gene structure. The Bam HI (B) and Pst I (P) restriction sites are deduced from the sequences and are consistent with Southern blot results. White boxes show the exons of MAGE1 and MAGE2. Filled boxes indicate the open reading frames, and the little black box in MAGE1 corresponds to the region encoding the nonapeptide of antigen MZ2-E. Gaps (.....) were introduced for best alignment of related sequences. Arrows (>~) indicate the position of the oligonucleotides used for the RT-PCR analysis of MAGE1 and MAGE2 mRNA expression. Sequence identity is indicated in % of matching nucleotides for different portions of the genes.

of the MAGE2 cDNA that contained several mismatches with the MAGE1 and MAGE3 sequences. The PCR products of several cosmid groups were found to hybridize, and the group presenting the most intense signal was screened by colony hybridization with the same probe. A positive cosmid, C6.3, was identified. Restriction analysis and Southern blots revealed that this cosmid contained a 38 kb insert carrying a 6 kb and an 8 kb Bam HI fragment, which both hybridized to the MAGE2 oligonucleotide probe. These two fragments were subcloned in phagemid pTZ18R and their nucleotide sequence was determined. A sequence contained in the 6 kb fragment was found to contain regions that were strictly identical to the MAGE2 cDNA. The other fragment contained a highly similar sequence (90% identity), which was named MAGE12.

Sequence and structure of the MAGE2 gene. The MAGE2 gene is about 4 kb long, and the overall sequence identity with MAGE1 amounts to 77% (Figs. 1, 2). Remarkably, the comparison of the genomic sequence with that of the MAGE2 cDNA delineated four exons, which is one more than MAGE1 (Fig. 1).

To determine the transcription initiation point, we carried out reverse transcription on MZ2-MEL RNA with a *MAGE2*-specific primer located near the 5' end of the cDNA sequence. The product was tailed with a poly(A) sequence and amplified by anchored PCR (Frohman et al. 1988). Three PCR products were obtained. The largest added 55 nucleotides to the *MAGE2* cDNA, and we provisionally consider its starting point as the cap site. The two others had 5' ends located 8 and 15 nucleotides downstream, respectively.

The first exon of MAGE2 is longer than that of MAGE1. In MAGE2, the initiation point is located 45 nucleotides downstream of the putative initiation site of the MAGE1 gene, and the donor splice site of the first exon is located 176 nucleotides downstream of that of MAGE1 (Fig. 2). Except for a large deletion of 650 bp in the MAGE2 gene, the region corresponding to the first intron of MAGE1 is closely related to the MAGE2 sequence (76% identity). Remarkably, however, a new splice acceptor site and a new donor site have appeared in the MAGE2 sequence, creating a new exon that is homologous to an intron sequence of MAGE1. The presence of a C, instead of the G of the MAGE1 sequence, eight nucleotides upstream of the new acceptor site of MAGE2, probably contributes to the creation of this acceptor site (Fig. 2).

Like *MAGE1*, *MAGE2* contains a large open reading frame in the last exon, and the two genes have the same translation initiation point. Twenty-nine codons downstream of this ATG codon, *MAGE1* contains a small deletion when compared to the *MAGE2* sequence. This deletion, which corresponds to exactly 7 codons, preserves the reading frame alignment between the two genes. The MAGE2 protein, which is 314 amino acids long, presents 67% identity with that of MAGE1, which is 309 amino acids long.

Hydrophobicity pattern analysis of MAGE1 and MAGE2 putative proteins indicates that they have no signal peptide. It may be worth noting the presence of two stretches of 21 and 17 amino acids that are strictly identical in both MAGE1 and MAGE2 protein sequences (Fig. 2, residues 112-132 and residues 171-187 of the MAGE1 protein). These regions may constitute parts of the protein that are essential for its function.

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MAGE-1	tccgtgtgatcagggaagg	getgettaggagagggeage	gtccaggctctgccagacat	catgetcaggattetcaagg	agggetgaggg-teeetaag	-274
MAGE-2	ggtacacactggcaatgat	ctcaccccgagcacacccct	ccccccaatgccacttcggg	ccgactcagagtcagagact	tggtctgaggggggggagcagaca	-345
MAGE-1	accccactcccgtgacccaa	<u>ccc</u> ccactccaatgctcact	cccgtgacccaaccccctct	tcattgtcattccaaccccc	accccacatcccccacccca	-1.74
MAGE-2	caatcggcagaggatggcgg	tccaggctcagtctggcatc	caagtcaggaccttgaggga	tgaccaaaggcccctcccac	ccccaactcccccgacccca	-245
		-				
MAGE-1	<u>tc</u>	- <u>c</u> ctcaaccctgatg <u>cccat</u>	ccgcccagccattccaccct	cacecccaceccaceccca	cgcccactcccaccccacc	-93
MAGE-2	ccaggatetacageetcagg	atccccgtcccaatccctac	ccctacaccaacaccatctt	catgettacccccaccc	cccatccagatccccatc	-149
MAGE-1	caggcaggatecggttcccg	ccaggaaac	atccgggtgcccggatgtga	cgccactgacttgcgcattg	tggggcagagagaagcgagg	-4
MAGE-2	<u> </u>	ccttgccgtgaacccaggga	agtcacgggcccggatgtga	cgccactgacttgcacattg	gaggtcagaggacagcgaga	-49
	+1 exon 1 MAGE-1					
MAGE~1	LLECCATTCTGAGGGACGGC	GTAGAGTTCGGCCGAAGGAA	CCTGACCCAGGCTCTGTGAG	GAGGCAAGgtgagaggctga	gggaggactgaggaccccgc	97
MAGE-2	ttctcgccctgagcaacggc	ct-gacgtcggcggagggaa	gcaggcgcaGGCTCCGTGAG	GAGGCAAGGTAAGACGCCGA	GGGAGGACTGAGGCGGGCCT	51
			+1 exon 1 M2	AGE-2		
MAGE-1	cactccaaatagagagcccc	aaatattccagccccgccct	tgetgecagecetggeceae	ccgcgggaagacgtctcagc	ctgggctgcccccagacccc	197
MAGE-2	CACCCCAGACAGAGGGCCCC	CAATAATCCAGCGCTGCCTC	TGCTGCCGGGCCTGGACCAC	CCTGCAGGGGAAGACTTCTC	AGGCTCAGTCGCCACCACCT	151
MAGE-1	tgetecaaaageettgagag	acaccaggttcttctcccca	agetetggaateagaggttg	ctgtgaccagggcaggactg	gttaggagagggcagggcac	297
MAGE-2	CACCCCGCCACCCCC	-CGCCGCTTTAACCGCAGGG	AACTCTGGCgtaagagc			202
MAGE-1	aggetetgecaggeateaag	atcagcacccaagagggagg	gctgtgggcccccaagactg	cactecaatecccactecca	ccccattcgcattcccattc	397
	cccacccaacccccatctcc	tcagctacacctccaccccc	atccctactcctactccgtc	acctgaccaccacctccag	ccccagcaccagccccaacc	497
	cttctgccacctcaccctca	ctgcccccaaccccaccctc	atctctctcatgtgccccac	teccategectecccatte	tggcagaatccggtttgccc	597
	ctgctctcaacccagggaag	ccctggtaggcccgatgtga	aaccactgacttgaacctca	cagatetgagagaagecagg	ttcatttaatggttctgagg	697
	ggcggcttgagatccactga	ggggagtggttttaggctct	gtgaggaggcaaggtgagat	gctgagggaggactgaggag	gcacacaccccaggtagatg	797
	gecceaaaatgatecagtae	cacccctgctgccagccctg	gaccacccggccaggacaga	tgtctcagctggaccacccc	ccgtcccgtcccactgccac	897
	ttaacccacagggcaatetg	tagtcatagettatgtgace	ggggcagggttggtcaggag	aggcagggcccaggcatcaa	ggtccagcatccgcccggca	997
	ttagggtcaggaccctggga	gggaactgagggtteeccac	ccacacctgtctcctcatct	ccaccgccaccccactcaca	ttcccatacctaccccctac	1097
	ccccaacctcatcttgtcag	aatecetgetgteaaceeac	ggaagccacgggaatggcgg	ccaggcacteggatettgac	gtccccatccagggtctgat	1197
	ggagggaaggggcttgaaca	gggeetcaggggageagagg	gagggccctactgcgagatg	agggaggcctcagaggaccc	agcaccctaggacaccgcac	1297
	ccctgtctgagactgaggct	gccacttctggcctcaagaa	tcagaacgatgggggactcag	attgcatgggggtgggaccc	aggeetgeaaggettaegeg	1397
	gaggaagaggaggaggact	caggggaccttggaatccag	atcagtgtggacctcggccc	tgagaggtccagggcacggt	ggccacatatggcccatatt	1497
	tcctgcatctttgaggtgac	aggacagagetgtggtetga	gaagtggggcctcaggtcaa	cagagggaggagttccagga	tccatatggcccaagatgtg	1597
	ccccttcatgaggactggg	gatatccccggctcagaaag	aagggactccacacagtctg	gctgtccccttttagtagct	ctaggggggaccagatcaggg	1697
	atggcggtatgttccattct	cacttgtaccacaggcagga	agttggggggccctcaggga	gatggggtcttggggtaaag	gggggatgtctactcatgtc	1797
	agggaattgggggttgagga	agcacaggcgctggcaggaa	taaagatgagtgagacagac	aaggetattggaatecacac	cccagaaccaaaggggtcag	1897
MAGE-1	ccctggacacctcacccagg	atgtggcttctttttcactc	ctgtttccagatctggggca	ggtgaggacctcattctcag	agggtgactcaggtcaacgt	1997
MAGE-2		cctcctactt	gtctttccagATCTCAGGGA	GTTGATGACCTTGTTTTCAG	AAGGTGACTCAGGTCAACAC	1340
			exon 2 MAG	3E-2		
MAGE-1	agggacccccatctggtcta	aagacagagcggtcccagga	tctgccatgcgttcgggtga	ggaacatgagggaggactga	gggtaccccaggaccagaac	2097
MAGE-2	AGGGGCCCC-ATCTGGTCGA	CAGATGCAGTGGTTCTAGGA	TCTGCCAAGCATCCAGGTGG	AGAGCCTGAGgtaggattga	aāā•••	1422
MAGE-1	actgagggagactgcacaga	aatcagccctgcccctgctg	tcaccccagagagcatgggc	tgggccgtctgccgaggtcc	ttccgttatcctgggatcat	2197
	tgatgtcagggacggggagg	ccttggtctgagaaggctgc	gctcaggtcagtagagggag	cgtcccaggccctgccagga	gtcaaggtgaggaccaagcg	2297
	ggcacctcacccaggacaca	ttaattccaatgaattttga	tatetettgetgecettece	caaggacctaggcacgtgtg	gccagatgtttgtcccctcc	2397
	tgtccttccattccttatca	tggatgtgaactcttgattt	ggatttctcagaccagcaaa	agggcaggatccaggccctg	ccaggaaaaatataagggcc	2497
	ctgcgtgagaacagaggggg	tcatccactgcatgagagtg	gggatgtcacagagtccage	ccaecctcctggtagcactg	agaagccagggctgtgcttg	2597
	cggtctgcaccctgagggcc	cgtggattcctcttcctgga	gctccaggaaccaggcagtg	aggccttggtctgagacagt	atcctcaggtcacagagcag	2697
	aggatgcacagggtgtgcca	geagtgaatgtttgecetga	atgcacaccaagggccccac	ctgccacaggacacatagga	ctccacagagtctggcctca	2797
W00 1	est esst act at cast set a	tagaatcgacctctgctggg	conctataccet gagtacce	e teteacttectectteagGT	KOD 2 MAGE-1 TTTCAGGGG-ACAGGCCAAC	2896
MAGE-1	concorrections			ectecttear@	TCTGAGGGGGACAGGCTGAC	2250
MAGE-2				ອະເວດເປັນແຫຼ່ງຍາ ຄ	KOD 3 MAGE-2	
MACE 1	ന്ദ്രാദ്യാവാന്നന്നും	GAGGCCACAGAGGAGCACCA	AGCAGAAGATCTataaataa	gcetttgttagagtetecaa	ggttcagttctcagctgagg	2996
MAGE-1			ACCACA ACADOMICTIC Strangerdy			2299
MAGE-Z	AAGT'AGGACCCGAGGCACTG	GAGGAGCATTGA	AGGAGAAGATCTGEaag		v a -	
MAGE 1	ectet cacacactecet at a	exon 3 MAGE-1	CATTIGOCCAGOTOCTIGOCCA	CACTCCTGCCTGCTGCCCTG	M S L ACGAGAGTCATCATGTCTCT	3 3096
MAGE-1		tccccadGCCTGTGGGTCTT	CATTGCCCAGCTCCTGCCCG	CACTCCTGCCTGCTGCCCTG	ACCAGAGTCATCATGCCTCT	2447
MAGE-Z		axon 4 MAGE-2			MPL	3

Fig. 2. (For legend see p. 125)

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MAGE-1 MAGE-2	E O R S L H C TGAGCAGAGAGGAGTCTGCACT C TGAGCAGAGAGGAGTCAGCACT C E Q R S Q H C	K P E E A L BCAAGCCTGAGGAAGCCCTT BCAAGCCTGAAGAAGGCCTT C K P E E G L	E A O O E A L GAGGCCCAACAAGAGGCCCT GAGGCCCGAGGAGAGGCCCT E A R G E A L	G L V C V O GGGCCTGGTGTGTGTGCAGG GGGCCTGGTGGGTGCGCAGG G L V G A Q	A CTCTGCTACTGAGGAGCAG A P A T E E Q	30 3178 2547 36
MAGE-1 MAGE-2	A T S S S S GCCACCTCCTCCTCCTC T CAGACCGCTTCTTCCTCTC T Q T A S S S S	PLVLGT TCCTCTGGTCCTGGGCACCC TACTCTAGTGGAAGTTACCC TLVEVT	L E E V P T A TGGAGGAGGTGCCCACTGCT TGGGGGGAGGTGCCTGCTGCC L G E V P A A	G S T D P P Q GGGTCAACAGATCCTCCCCA GACTCACCGAGTCCTCCCCA D S P S P P H	S P Q G A S GAGTCCTCAGGGAGCCTCCG CAGTCCTCAGGGAGCCTCCA S P Q G A S	62 3275 2647 69
MAGE-1 MAGE-2	A F P T T I N CCTTTCCCACTACCATCAAC T GCTTCTCGACTACCATCAAC T S F S T T I N	F T R Q R Q P TTCACTCGACAGAGGCAACC FACACTCTTTGGAGACAATC Y T L W R Q S	S E G S S S CAGTGAGGGTTCCAGCAGCC CGATGAGGGCTCCAGCAACC D E G S S N	R E E E G P S GTGAAGAGGAGGGGCCAAGC AAGAAGAGGAGGGGCCCAAGA Q E E E G P R	T S C I L E S ACCTCTTGTATCCTGGAGTC ATGTTTCCCGAGAGCCTCCA M F P D L E S	96 3375 2747 103
MAGE-1 MAGE-2	L F R A V I CTTGTTCCGAGCAGTAATCA C CGAGTTCCCAAGCAGCAATCA C E F Q A A I S	FKKVADL TTAAGAAGGTGGCTGATTTG STAGGAAGATGGTTGAGTTG SRKMVEL	V G F L L L K GTTGGTTTTCTGCTCCTCAA GTTCATTTTCTGCTCCTCAA V H F L L L K	Y R A R E P ATATCGAGCCAGGGAGCCAG GTATCGAGCCAGGGAGCCGG Y R A R E P	V T K A E M L TCACAAAGGCAGAAATGCTG TCACAAAGGCAGAAATGCTG V T K A E M L	129 3475 2847 136
MAGE-1 MAGE-2	E S V I K N Y GAGAGTGTCATCAAAAATTA ( GAGAGTGTCCTCAGAAATTG ( E S V L R N C	K H C F P E CAAGCACTGTTTTCCTGAGA CCAGGACTTCTTTCCCGTGA Q D F F P V	I F G K A S E TCTTCGGCAAAGCCTCTGAG TCTTCAGCAAAGCCTCCGAG I F S K A S E	S L Q L V F G TCCTTGCAGCTGGTCTTTGG TACTTGCAGCTGGTCTTTGG Y L Q L V F G	I D V K E A CATTGACGTGAAGGAAGCAG CATCGAGGTGGTGGAAGTGG I E V V E V	162 3575 2947 169
MAGE-1 MAGE-2	D P T G H S Y ACCCCACCGGCCACTCCTAT ( TCCCCATCAGCCACTGTAC A V P I S H L Y	V L V T C L G STCCTTGTCACCTGCCTAGG ATCCTTGTCACCTGCCTGGG I L V T C L G	L S Y D G L TCTCTCCTATGATGGCCTGC CCTCTCCTACGATGGCCTGC L S Y D G L	L G D N Q I M TGGGTGATAATCAGATCATG TGGGCCACAATCAGGTCATG L G D N Q V M	P K T G F L I CCCAAGACAGGCTTCCTGAT CCCAAGACAGGCCTCCTGAT P K T G L L I	196 3675 3047 203
MAGE-1 MAGE-2	I V L V M I A AATTGTCCTGGTCATGATTG ( AATCGTCCTGGCCATAATCG ( I V L A I I )	AMEGGHA CAATGGAGGGGGGGCATGCT CAATAGAGGGGGGGCCTGTGCC AIEGDCA	P E E E I W E CCTGAGGAGGAAATCTGGGA CCTGAGGAGAAAATCTGGGA P E E K I W E	E L S V M E GEAGCTGAGTGTGATGGAGG GEAGCTGAGTATGTTGGAGG E L S M L E	V Y D G R E H TGTATGATGGAGGAGGAGCAC TGTTTGAGGGGAGGGAGGAC V F E G R E D	229 3775 3147 236
MAGE-1 MAGE-2	S A Y G E P R AGTGCCTATGGGGAGCCCAG ( AGTGTCTTCGCACATCCCAG ( S V F A H P R	K L L T Q D SAAGCTGCTCACCCAAGATT SAAGCTGCTCATGCAAGATC K L L M Q D	L V Q E K Y L TGGTGCAGGAAAAGTACCTG TGGTGCAGGAAAACTACCTG L V Q E N Y L	E Y R Q V P D GAGTACCGGCAGGTGCCCGA GAGTACCGGCAGGTGCCCGG E Y R Q V P G	S D P A R Y CAGTGATCCCGCACGCTATG CAGTGATCCTGCATGCTACG S D P A C Y	262 3875 3247 269
MAGE-1 MAGE-2	E F L W G P R AGTTCCTGTGGGGTCCAAGG ( AGTTCCTGTGGGGTCCAAGG ( E F L W G P R	A L A E T S Y GCCCTCGCTGAAACCAGCTA GCCCTCATTGAAACCAGCTA A L I E T S Y	V K V L E Y TGTGAAAGTCCTTGAGTATG TGTGAAAGTCCTGCACCATA V K V L H H	V I K V S A R TGATCAAGGTCAGTGCAAGA CACTAAAGATCGGTGGAGAA T L K I G G E	V R F F F P S GTTCGCTTTTTCTTCCCATC CCTCACATTTCCTACCCACC P H I S Y P P	296 3975 3347 303
MAGE-1 MAGE-2	L R E A A L H CCTGCGTGAAGCAGCTTTGA ( CCTGCATGAACGGGCTTTGA ( L H E R A L H	R E E E E G V SAGAGGAGGAGGAGGGGGGGGG GAGAGGGAGAAGAGTGAGT	OPA TGAGCATGAGTTGCAGCCAA TCAGCACATGTTGCAGCCAG	GGCCAGTGGGAGGGGGACTG GGCCAGTGGGAGGGGGGTCTG	GGCCAGTGCACCTTCCAGGG GGCCAGTGCACCTTCCAGGG	309 4075 3447 314
MAGE-1 MAGE-2	COGCGTCCAGCAGCTTCCCC 1 CCCCATCCATTAGCTTCCAC 1	FGCCTCGTGTGACATGAGGC FGCCTCGTGTGTGATATGAGGC	CCATTCTTCACTCTGAAGAG CCATTCCTGCCTCTTTGAAG	AGCGGTCAGTGTTCTCAG AGAGCAGTCAGCATTCTTAG	TAGTAGGTTTCTGTTCTATT CAGTGAGTTTCTGTTCTGT	4174 3547
MAGE-1 MAGE-2	GGGTGACTTGGAGATTTATC 7 GGATGACTTTGAGATTTATC 7	PTTGTTCTCTTTTGGAATTG PTTCTTTCCTGTTGGAATTG	ТТСААА?:GTTTTTTTTAAG ТТСАААТGTTCCTTTT-ААС	GGATGGTTGAATGAACTTCA AAATGGTTGGATGAACTTCA	GCATCCAAGTTTATGAATGA GCATCCAAGTTTATGAATGA	4273 3646
MAGE-1 MAGE-2	CAGCAGTCACACAGTTCT ( CAGTAGTCACACATAGTGCT (	STGTATATAGTTTAAGGGTA STTTATATAGTTTAGGGGTA	AGAGTCTTGTGTTTTATTCA AGAGTCCTGTTTTTATTCA	GATTGGGAAATCCATTCTAT GATTGGGAAATCCATTCCAT	TTTGTGAATTGGGATAAT TTTGTGAGTTGTCACATAAT	4369 3746
MAGE-1 MAGE-2	AACAGCAGTGGAATAAGTAC 1 AACAGCAGTGGAATATGTAT 1	ТТАСАААТСТСААААА ГТСССТАТАТТСТСААССАА	TGAGCAGTAAAATAGATGAG TTAGCAGTAAAATACATGAT	АТАААGААСТАААGАААТТА АСААGGAACТСА	AGAGATAGTCAATTCTTGCC AAAGATAGTTAATTCTTGCC	4465 3838
MAGE-1 MAGE-2	TTATACCTCAGTCTATTCTG 7 TTATACCTCAGTCTATTATG 7	ГААЛАТТТТТАЛДGATATAT ГАЛЛАТТАЛАА	GCATACCTGGATTTCCTTGG TATGTGTATGTTTTTG	CTTCTTTGAGAATGTAAGAG CTTCTTTGAGAATGCAAAAG	аааттааатстбаатаааба аааттааатстбаатааата	4565 3926
MAGE-1 MAGE-2	ATTCTTCCTGTtcactggct c	rtttcttctccatgcactg	agcatctgctttttggaagg	ccctgggttagtagtggaga	tgctaaggtaagccagactc	4665 3941
MAGE-1	atacccacccatagggtcgt a ggtgtggggctccgggtgag a atgatcttgggtggatcc	agagtetaggagetgeagte agtggtggagtgteaatgee	acgtaatcgaggtggcaaga ctgagctggggcattttggg	tgtcctctaaagatgtaggg ctttgggaaactgcagttcc	aaaagtgagagaggggtgag ttctggggggagctgattgta	4765 4865 4883

**Fig. 2.** Sequence alignment of *MAGE1* and *MAGE2* genes. *MAGE2* sequences corresponding to the exons with surrounding portions of intron and to the promoter region are aligned to the complete sequence of *MAGE1*. A correction to the previously described sequence of the *MAGE1* gene (van der Bruggen et al. 1991) was contributed by E. Paoletti and J. Tartaglia (Virogenetics, Troy, NY). Gaps, indicated by *dashes* (–), were introduced for optimal alignment. Exons are in *uppercase*. Untranscribed and intron sequences are in *lowercase*. *Arrows* (––––––), mark the 7 bp direct repeats in the promoter region. In this region we also indicated the potential binding sites for transcription factors AP2 (–––––), Ets (––––––), Myb (––––––), PuF (––––––) and ATF (–––––). The amino acid sequence of the putative protein encoded by both genes is represented. The amino acid sequence corresponding to the MZ2-E antigenic peptide is *boxed*.



Fig. 3. Reverse transcription and PCR amplification of MAGE2 mRNA. MAGE2-specific primers shown in Figure 1 were used to amplify reverse transcribed RNA (RT-RNA) from two non-smallcell lung carcinoma samples (LB175 and LB178) and from control melanoma cell line MZ2-MEL. MAGE2 amplification with the same primers was also carried out on genomic DNA extracted from melanoma cell line MZ2-MEL. PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide.



Fig. 4. Southern blot analysis of the *MAGE* genes. *Bam* HI-digested DNAs were from the melanoma cell lines of four patients (MZ2, LB34, LB33, and LB38), and from a sarcoma cell line of patient LB23. The *MAGE1* 2.4 kb *Bam* HI fragment was <sup>32</sup>P-labeled and used as a probe for hybridization. Final washings were in  $0.5 \times SSC$ , 0.1% SDS at 65° C.

The MAGE2 promoter. There are no CAAT or TATA motifs in the MAGE2 promoter. However, by screening its sequence from -445 to -1 for the presence of upstream promoter elements (UPE) as listed by Faisst and Meyer (1992), we identified consensus sequences for the binding of Myb, Ets-1, PuF, AP2, and ATF transcription factors (Fig. 2). Consensus binding sequences for the four latter transcription factors are also present in the corresponding region of MAGE1. In addition, it may be interesting to note the presence of a pair of identical 7 bp direct repeat sequences located in

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both genes at approximatively 160 bp upstream from the first transcription start point (Fig. 2). Unlike the promoters of the housekeeping genes, which also lack CAAT and TATA motifs and typically contain GC-rich regions carrying multiple Sp1 binding sites (Melton et al. 1986), the promoter regions of *MAGE1* and *MAGE2* show a high content of C (42% and 38%, respectively) but not of G (21% and 25%, respectively), and do not contain any Sp1 consensus binding sequence.

*MAGE2 gene expression.* To evaluate *MAGE2* gene expression in various cells, we performed reverse transcription and PCR amplification of their RNA. Pairs of specific oligonucleotide primers were chosen, which matched sequences located in different exons, so as to distinguish the PCR products originating from mRNA from those produced by contaminating DNA (Figs. 1, 3).

A large number of tissue samples from tumors of different histological types were tested (Table 1). In order to verify the quality of the RNA preparations, we also evaluated the  $\beta$ -actin mRNA. A very high proportion (85%) of melanoma samples expressed MAGE2. This is much higher than for MAGE1, which is expressed in about 40% of melanomas. No melanoma sample was found where MAGE1 was expressed and MAGE2 was not. Laryngeal tumors expressing MAGE2 were also more frequent (46%) than those expressing MAGE1 (23%). For lung tumors and sarcomas the distribution was different: those expressing MAGE1 were as numerous (35%) as those expressing MAGE2. Less than 10% of breast tumors were found to express the MAGE2 gene, whereas 20% express MAGE1. In these types of tumor, no correlation was observed between the expression of the two genes.

We also tested a panel of healthy adult and fetal tissues (Table 1). No *MAGE1* or *MAGE2* expression was observed, with the important exception of testis. The level of expression in testis was estimated to be approximatively one-third of that observed in the MZ2-MEL cell line.

Low restriction polymorphism of MAGE genes. DNA samples from four melanoma cell lines and one sarcoma cell line were digested with *Bam* HI. Southern blots were hybridized with the *MAGE1* 2.4 kb *Bam* HI probe (Fig. 1) and washed at medium stringency. Autoradiography revealed the same pattern of 13 bands for all cell lines (Fig. 4). The two bands corresponding to the *MAGE2* and *MAGE12* genes isolated from tumor MZ2-MEL were observed with all the tumor samples. These results suggest that the *MAGE* gene family contains 10–15 members and presents a low level of polymorphism. This latter observation is relevant to the possibility of setting up for *MAGE* genes a PCR expression test that is applicable to a wide variety of cancer patients.

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Tumor samples					Healthy tissues			
Histological	Expression of		Histological	Expressio	on of	Histological	Expression of	
type	MAGE1	MAGE2	type	MAGE1	MAGE2	type	MAGE1 M	MAGE2
Brain tumors			Melanomas			Adult tissues		
	0/2	0/2	LB 211	_	++++			
			MZ 2	++++	++++	Colon		-
Breast carcinomas			BB5	+++	++++			
LB 177	_	++	LB 40	_	+	Stomach	-	-
LB 222	++	-	LB 49	++++	++			
LB 171	++++	_	LB 124	++	++++	Liver	-	-
LB 231	++		LG 11	++	+++			
LB 232	+++	_	BB 1	-	+++	Medulla	-	-
LB 207	+	+	BB 2		++++			
LB 284	++	+++	BB 3		+++	Muscle	_	-
	6/34	3/34	BB 4	_	+++			
			LB 265	_	+++	Melanocytes	_	
Colon carcinomas			LB 271	+	+++	·		
LB 274	_	++++	LG 7	_	++	Skin	_	-
	0/11	1/11	LG 12	++	╉┿┿┿			
	0,11		LB 225	+++	<del>+++</del>	Lung	_	-
Larvngeal tumors				8/19	16/19			
LB 266	++++	_				Breast	_	_
LB 270	****	+++	Ovary tumors					
LB 575	++	++++	o tury turnoro	0/3	0/3	Uterus		_
LB 363	_			0.0	010			
LB 430	_	++++	Sarcomas			Thymocytes	_	
LB 512	_	***	LB 29		+	11191110491460		
LB 516	_	++++	LB 258	++	++	Testis		
LD 510	3/13	6/13	LB 230	++	-	CLO 64027	+++	++++
	5/15	0/15	LD 221	2/6	2/6	LB 451	+++	+++
Laukaamias				240	210			
Leukaennas	0/4	0/4	Thyroid tumors			Fetal tissues		
	0/4	0/4	Thyfold tulliors	0/3	0/3	1000003		
Lung corcinomos				0/5	015	Fibroblasts	_	_
Lung carchiomas						1 1010014313		
LB 175 NSCLC		TTT				Liver		
LD 170 NOCLU	$+\tau\tau$					(20  weaks)		
LD 162 NOCLU	_	+				(20  weeks)		
LD 195 NSCLC	++	++++				(22  weeks)	_	-
LB 200 NSCLC	+++	++				(35 weeks)	_	-
LB 212 NSCLU	++	+				(20 weeks)	—	-
LD 234 INSULU	++	-				Drain (26 montra)		
LB 259 NSCLU	-	+				Drain (20 weeks)	_	-
LD 204 INSCLU	+++	++++				Serloop (76 moder)		
LB 048 SCLU	+	++++				spieen (26 weeks)	-	-
LB 444 SCLC	-	+++						
	//19	9/19				1 nymus (26 weeks)	-	
						Lung (26 weeks)	_	_

	T	EL A MACE	ana hutimoral	hoolthy and	fotal ticcules
Table I.	Expression of MAC	ET and MAGE	genes by tumoral	, neariny, and	i ietai ussues

*MAGE1* and *MAGE2* gene expression was tested by specific RT-PCR amplification on total RNA. Only those tumor samples that express at least one of these genes are represented. Lung tumors noted NSCLC are non-small-cell lung carcinomas; SCLC are small-cell lung carcinomas. Absence of PCR product is indicated by –, and different levels of expression as deduced by band intensity of PCR products are represented by +, ++, +++, or ++++. All RNAs presented here showed similar amplification of the  $\beta$ -actin cDNA.

#### Discussion

Like *MAGE1*, the *MAGE2* gene is expressed in tumor cells and not in the healthy tissues that have been tested, with the exception of testis. Antigens encoded by

*MAGE2* should therefore qualify as tumor rejection antigens, since a T-lymphocyte response directed against these antigens should be capable of destroying tumors, with no unacceptable side effects on healthy tissues, except possibly on testis. In the absence of appropriate antibodies, we have not yet been able to determine which cell type expresses genes *MAGE1* and *MAGE2* in testis.

Even though no CTL recognizing a MAGE2derived peptide has yet been identified, MAGE2 constitutes a priori a more useful source of melanoma tumor rejection antigens than MAGE1 because it is expressed in almost 85% of melanoma samples, whereas MAGE1 is expressed in 40%. Like MAGE1, MAGE2 is also expressed in many tumors other than melanomas, notably lung tumors, sarcomas, and laryngeal tumors. The MAGE2 region, which is homologous to the MAGE1 region coding for the MZ2-E antigenic peptide, codes for a peptide that displays five differences with the MAGE1 peptide presented by HLA-A1 (Fig. 2). It is therefore not surprising that MAGE2 does not code for antigen MZ2-E. But we deem it likely that MAGE2 produces other antigenic peptides that bind to other HLA molecules.

It will be interesting to determine the promoter components that ensure tumor-specific MAGE gene expression. Whereas the multiplicity of 5' ends observed on MAGE2 cDNAs could be due to incomplete reverse transcription preceding anchored-PCR amplification, it could also reflect the nature of the MAGE2 promoter region, which contains no TATA or CAAT consensus sequence. Promoters lacking a TATA box have been divided into two classes (Smale and Baltimore 1989). The GC-rich promoters are found primarily in housekeeping genes (Melton et al. 1986), contain multiple Sp1 binding sequences, and present several, sometimes widely spread, transcription initiation sites. TATAlacking promoters which are not GC-rich constitute the second class. They usually regulate the transcription of genes involved in differentiation or development, like the mouse terminal deoxynucleotidyl transferase gene (Smale and Baltimore 1989), or the genes coding for the  $\delta$  chain of the T-cell receptor complex (Van den Elsen et al. 1986). They initiate transcription at one or a few tightly clustered start sites. The promoter of the MAGE2 gene appears to belong to this second class.

No *MAGE12* cDNA could be isolated from a cDNA library of the melanoma cell line MZ2-MEL. However, preliminary analysis of RNA samples of different origins by reverse transcription and PCR amplification with primers located in the longest open reading frame of the *MAGE12* gene, indicated that this gene is expressed in several tumor samples but not in healthy tissues, with the exception of testis. The *MAGE12* gene may therefore also produce tumor rejection antigens. It will be analyzed further for its structure and expression pattern.

The function of the MAGE proteins is still unknown, though they may play a role in embryonal development. Our failure to observe *MAGE1* and *MAGE2* gene expression in fetal tissues may be because the analyzed tissues came from fetuses of

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20–35 weeks. Since a number of genes have their expression restricted to an earlier phase of embryonal development (Pfeifer-Ohlsson et al. 1984), it will be important to examine earlier embryos. The absence of a signal peptide implies that the MAGE1 and MAGE2 proteins are neither surface nor secreted proteins. Precise intracellular localization of these MAGE proteins could help to understand their function. For this purpose, efforts are being made to obtain antibodies directed against these proteins.

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