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Cloning and Analysis of MAGE-1-Related Genes+

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The spectrum of MAGE gene expression in the human melanoma cell line DM150 was examined using reverse transcription polymerase chain reaction and cDNA cloning. We have isolated five full-length cDNAs from DM150 which were identified as MAGE-1, MAGE-3, MAGE-12 and two previously undescribed MAGE genes, MAGE-3b and MAGE-X2. DNA sequence analysis of the coding regions of the MAGE-3b and MAGE-X2 genes revealed 83% and 88% identity with MAGE-1, while MAGE-3b was 98% homologous with the full length MAGE-3 clone. The predicted amino acid sequences of MAGE-X2 and MAGE-3b contain consensus HLA-A1 peptide binding motifs, suggesting that, like MAGE-1, they may code for tumor-associated antigens. In addition, a nonamer peptide encoded by both the MAGE-3 and MAGE-12 genes was shown by direct binding studies to contain an aggretope for HLA-A2.

The MAGE-1 gene, which codes for the tumor-associated antigen, MZ2-E, was isolated from a human melanoma cell line that was recognized by autologous CTL (1). MAGE-1 is expressed in 40% of melanomas and belongs to a family of 14 genes that are 80-99% identical (2, 3). MAGE genes are also expressed in other tumor types, including lung and breast carcinomas, and squamous cell carcinoma of head and neck (1, 4, 5), but not expressed in normal tissues with the exception of the testes (1, 4). The lack of expression of MAGE genes in normal adult tissues makes them attractive potential targets for CTL in the antigen-specific immunotherapy of cancer. We have studied expression of MAGE-

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Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; CTL, cytotoxic T lymphocyte; REN, restriction endonuclease; $\beta 2\mu$, beta-2 microglobulin; bp, base pairs.

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family genes in the human melanoma cell line DM150. We present the full-length sequence of 5 expressed genes including 2 previously unreported MAGE genes and discuss their potential to encode novel tumor antigens.

Materials and Methods

RT-PCR. Total RNA was prepared from the melanoma cell line DM150 using RNAzol (TEL-TEST, Inc.). cDNA was synthesized from 4 μg of total RNA using random primers in a 40 μl reaction mixture according to the manufacture's instructions (Perkin-Elmer Cetus, Inc.). The mixture was incubated at room temperature for 10 min, 42°C for 15 min, heated to 95°C for 5 min, and then chilled on ice. Twenty μl of the cDNA mixture was used for PCR amplification in a 100 μl reaction containing 0.2 μM of each primer. Primers were: (A) 5'-CCGCTCGAGGCCTGCTGCCCTGACGAGAG (MAGE-1 position -25 to -6 sense); (B) 5'CCGCTCGAGTCCCCCTCCCACTGGCCTTGGCTGC (MAGE-1 position 939-965 anti-sense). Primers were designed to contain terminal XhoI restriction sites. PCR was performed using the following parameters: denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 10 min extension at 72°C after the last cycle. PCR products were digested with XhoI and cloned into plasmid pBluescript II SK(-). Clones were analyzed by DNA sequencing using Sequenase Version 2.0 as per manufacturer's instructions (United States Biochemical, Inc.). The sequence was compiled and analyzed with the Genetics Computer Group Sequence Analysis Software Package Version 7.0.

cDNA synthesis. Total RNA from 1 x 10^8 DM150 melanoma cells was poly(A) selected by two rounds of oligo(dT)-cellulose spin column purification (Pharmacia). cDNA was synthesized utilizing a ZAP-cDNA synthesis kit (Stratagene) and 5 μg of oligo(dT)primed poly(A)+ mRNA. The unamplified library consisted of 1.2 x 10^6 independent clones. The cDNA library was amplified by the plate lysate method and 500,000 plaques were screened at 30,000 plaques per 15 cm plate using standard methods (6).

Library screening. A 500 bp MAGE-3 DNA probe was obtained by RT-PCR of DM150 RNA with CHO8 and CHO9 oligonucleotide primers as described elsewhere(1). Twenty-five nanograms of MAGE-3 probe fragment were ^{32}P-labeled by the RadPrime DNA labeling system (BRL) and hybridized to nitrocellulose filters containing recombinant plaques in 50% formamide, 6x SSC, 10% dextran sulfate, 5 mM EDTA, 0.1% SDS, and 10 mM Tris-HCL (pH 7.4) at 42°C. Filters were washed twice in 2 x SSC, 0.1% SDS at room temperature followed by 2 x SSC, 0.1% SDS at 68°C. Positive plaques were purified and inserts were excised from the Lambda ZapII vector as pBluescript phagemid by in vivo excision using the ExAssist/SOLR System (Stratagene).

Acid elution and peptide loading. Sixty percent confluent cultures of DM150 cells in 60 mm dishes were washed twice with cold PBS, and 2 ml of ice cold citrate-phosphate buffer [0.131 M citric acid/0.066 M Na_2HPO_4, 290 mOsmol/kg H_2O (isosmotic), pH 3.3] were added to plates on ice (7). After 2 min, cells were washed 3 times with cold culture medium/10% FCS and then incubated in 1 ml of peptide binding solution containing 100 μg/ml peptide and 10 μg/ml β2μ in medium/10% FCS. Cells were incubated 30 min on ice, followed by 30 min at 37°C. Cells were removed by trypsinization and incubated with antibody W6/32 [pan-class I (framework position) of ternary but not acid eluted complexes], or mA2.1 which is specific for HLA-A2 ternary complexes. Secondary staining was performed with FITC goat anti-mouse $F(ab)'_2$ and fixed cells were subjected to FACS analysis. Peptides (Macromolecular Resources, 90% pure) were: MAGE-1, EADPTGHSY; tyrosinase, YMNGTMSQV; and the putative MAGE-A2 binding peptide, FLWGPRALV.

Results and Discussion

The DM150 cell line is an HLA-A_1^+, -A_2^+ melanoma cell line which has recently been engineered to express human B7 (CD28 ligand) on its surface in

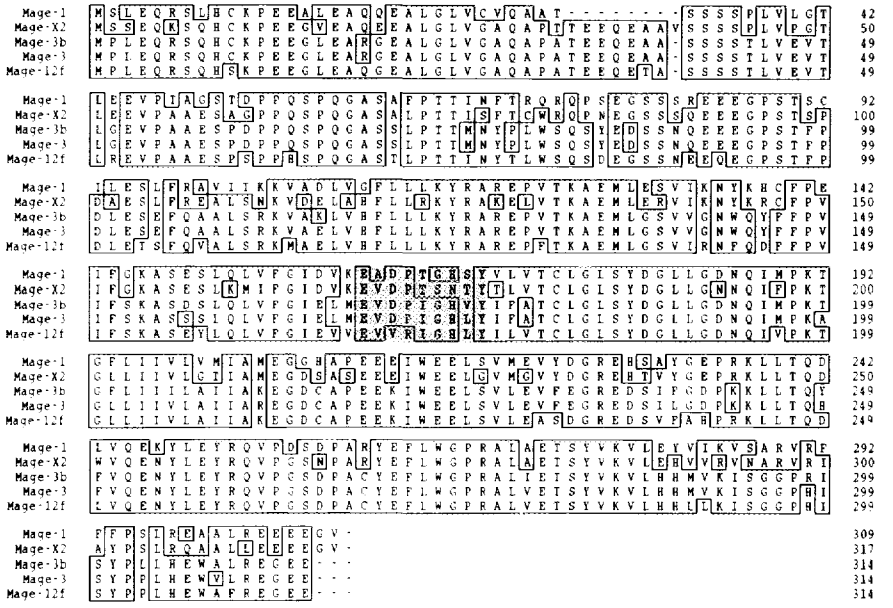

Figure 1. Alignment of amino acid sequence of MAGE-1, X2, 3b, 3, and 12f. Numbering is relative to the initiating methionine residue. Spaces (-) have been introduced for maximum alignment. Conserved regions are boxed. The amino acid residues corresponding to potential HLA-A1-binding nonamers are shaded.

preparation for a human vaccine trial(8). To determine if DM150 expressed the shared tumor antigens encoded by the MAGE-family genes, RNA was isolated from DM150 and screened for MAGE-specific sequences by two methods. Using previously reported DNA sequence data from the MAGE-1 gene, PCR primers were designed such that full length MAGE-1 related cDNAs were generated. As a second approach, a cDNA library generated from DM150 was screened using a MAGE-3 probe under low stringency hybridization conditions. Five distinct MAGE family genes, MAGE-1, 3, 3b, X2, and 12, were isolated and their amino acid sequences are shown in Figure 1.

A gene highly homologous to MAGE-1 was isolated which differed from the published DNA sequence at positions 94 and 813. The position 94 A to G transition results in a threonine to alanine substitution, while the position 813 mutation is silent. The sequence of cloned products from 2 independent RT-PCR reactions demonstrated these differences. In contrast, the DNA sequence of RT-PCR clones isolated from the human melanoma cell line DM13 were identical to the previously reported DNA sequence of MAGE-1. Thus, the differences noted in the DM150 cell line likely represent polymorphisms of the MAGE-1 gene. A cDNA clone encoding the complete MAGE-3 gene was identified; the nucleic acid sequence of this gene is identical to MAGE-3 sequence reported recently (3).

We also isolated a full length cDNA clone (designated MAGE-12f) which is highly homologous to the GenBank sequence for MAGE-12 (4), but differs at

seven positions. At nucleotides 43, 146, and 317, single base pair deletions are noted in the GenBank sequence that alter the reading frame of the MAGE-12 gene and probably represent sequencing errors. Of the 4 remaining discrepancies with MAGE-12f, 3 would result in amino acid changes (assuming realignment of the Genbank sequence with our sequence to maintain the open reading frame homologous to MAGE family genes). These differences may due to polymorphisms of the MAGE-12 gene.

Two novel MAGE genes were isolated. MAGE-3b is 98% and 96% homologous to MAGE-3 at the nucleic acid and amino acid level, respectively, and 83% and 69% homologous to MAGE-1. MAGE-X2 shares greatest homology with MAGE-1 (88% at nucleotide and 77% at amino acid level). However, beginning at base pair 95, the MAGE-1 gene contains a 24 base pair deletion relative to the MAGE-X2 sequence, and 21 base pairs compared with MAGE-3, 3b and 12. This deletion, which consists of 7 or 8 codons, preserves the reading frame alignment among these genes (Figure 1). Thus, although MAGE-X2 is most homologous to MAGE-1, it shares with the other MAGE genes the extra coding information in this region. Interestingly, this region from amino acid 33 to 39 is highly conserved in MAGE genes 3, 3b, 12, and X2. It will be of interest to determine if this conserved region encodes an important functional domain, and whether MAGE-1 protein activity is significantly different from other MAGE family genes that retain this coding segment. Taken together, these data indicate that expression of at least 5 MAGE-family genes is coordinately upregulated in the DM150 line.

The HLA-A1-restricted MAGE-1 encoded nonamer recognized by clone MZ2-E has been mapped to amino acids 168-176 (9). The corresponding positions in the MAGE-3, 3b, 12 and X2 genes are highlighted in Figure 1. The HLA-A1 consensus anchor motif consists of either a Thr/Ser/Met residue in position 2 or Asp/Glu/Ala/Ser/Thr in position 3, a proline in position 4 and a tyrosine in position 9 (10,11). As compared with the MAGE-1 sequence (EADPTGHSY), MAGE-3, 3b and X2 maintain the HLA-A1 anchor binding motif, although amino acids at minor sites are not identical; this would presumably alter the T cell receptor recognition of these peptides and could change the affinity of the peptides for the HLA-A1 binding pocket. Thus, the MAGE-3b and -X2 genes, like MAGE-1 and -3, encode proteins that are candidate tumor antigens restricted by HLA-A1. MAGE-12 has a valine residue in anchor position 3 (like MAGE-2) and an arginine in position 4 and is therefore unlikely to bind to HLA-A1 with high affinity.

The amino acid motif which defines the binding of peptides to HLA-A2 has been described in great detail (12). Using these data, the amino acid sequences of the MAGE-family genes were analyzed for favorable HLA-A2 binding motifs. The peptide FLWGPRALV (amino acid residues 271-279), shared by MAGE-3 and MAGE-12, was identified and was noted to contain appropriate anchor residues at positions 2 and 9, and favorable minor residues at positions 1, 4, and 8. To directly test the ability of this peptide to bind HLA-A2 molecules, the technique of mild acid elution and peptide loading was performed with

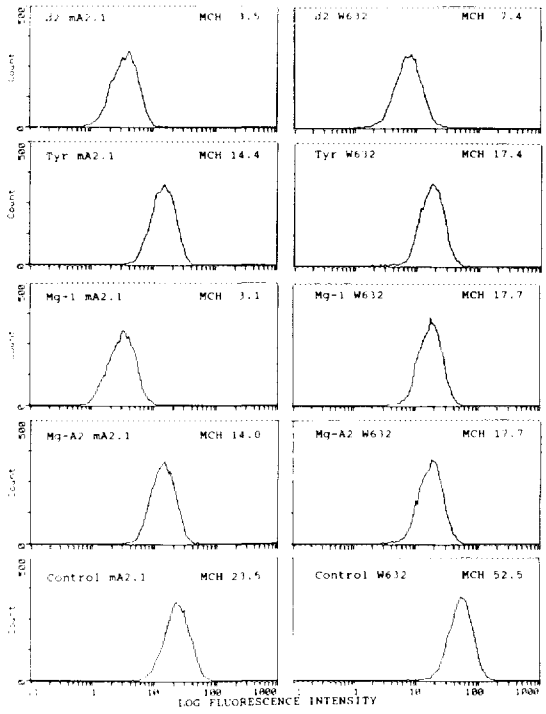

Figure 2. Mage-3 and -12 genes encode an HLA-A_2 binding peptide. Ternary complexes of class I molecules, $\beta 2\mu$, and peptide on DM150 cells were disrupted by acid elution, and reconstitution of HLA-A2 alleles by specific nonamer peptides was assayed. Control DM150 cells (bottom panel) were not acid treated, while top panels show the results of acid elution, followed by incubation with $\beta 2\mu$ in the absence of peptide. Central panels demonstrate reconstitution of specific ternary complexes after addition of the indicated peptides. Staining was with antibody mA2.1 (HLA-A2-specific) or W6/32 (pan class I). Mean chanel fluorescence values (MCH) are shown.

DM150 cells (Fig. 2). Untreated control cells stained with W6/32 (pan-class-I antigen reactive) or mA2.1 (HLA-A2 specific) demonstrate a clonal pattern with the mean channel fluorescence shown in the panels. Acid elution without peptide addition (top panels) resulted in an approximate 85% decrease in staining with each antibody as compared with controls. Upon addition of the tyrosinase peptide [a positive control with known high affinity for HLA-A2 (13)] significant staining was regained with both antibodies, as expected if ternary complexes with HLA-A2 and $\beta 2\mu$ are induced by this peptide. The MAGE-1 HLA-A1 binding peptide was used as a negative control for HLA-A2 binding. This peptide did not induce reassembly HLA-A2 ternary complexes, while W6/32 staining was restored (which represents binding of the MAGE-1 peptide to HLA-A1 molecules on DM150). The experimental peptide (referred to as Mg-A2) showed a pattern of binding indistinguishable from the tyrosinase peptide. Therefore, these data confirm that the nonamer FLWGPRALV encoded by MAGE-3 and -12 does indeed bind to HLA-A2 molecules. Experiments are underway to

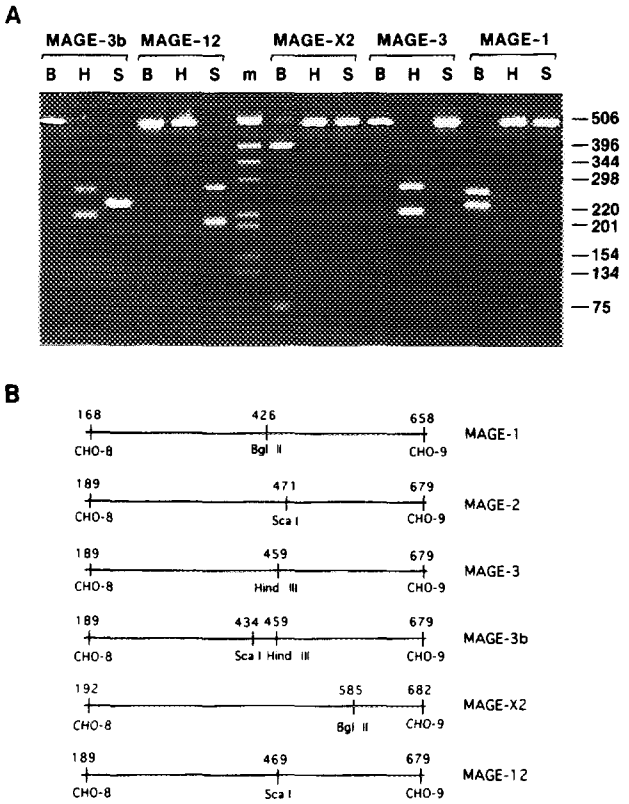

Figure 3. PCR-REN analysis of CHO8/CHO9 PCR products from MAGE cDNA clones. A. Agarose gel analysis of PCR products cleaved with BglII (B), HindIII (H), or ScaI (S). Size of marker fragments (m) is indicated. B, Restriction map of MAGE gene CHO8/CHO9 PCR-REN products shown in panel A. Numbering corresponds to nucleic acid sequence with position 1 corresponding to the "A" in the initiating ATG.

determine whether this peptide is presented by HLA-A2$^+$ human melanomas in a manner that can be targeted by antigen-specific CTL.

A rapid screening method for distinguishing MAGE-1, -2, and -3 expression has been developed using PCR amplification of cDNA from the region corresponding to exon 3, followed by restriction enzyme digestion (14). This method was used to analyze the pattern of each MAGE cDNA clone isolated from DM150. The cDNA clones were amplified with CHO8 and CHO9 primers, and the resultant PCR products were digested with either BglII, HindIII, or ScaI and size fractionated on agarose gels. The PCR-REN pattern of each clone is shown in Fig. 3A and schematically in Fig. 3B. As previously described (14), amplified DNA from MAGE-1 yields two fragments (258 and 232 bp) when digested with BglII, while amplified DNA from MAGE-3 digested with HindIII yield two fragments (270 and 220 bp). PCR-REN analysis of amplified DNA from MAGE-3b yielded a unique pattern. Digestion with HindIII yielded fragments of 270 and 220 bp, while ScaI generated a doublet at 245 bp. The HindIII pattern of MAGE-3b is identical to that of MAGE-3, but MAGE-3b contains a ScaI site while

MAGE-3 does not, thus permitting distinction between these genes. Digestion of the MAGE-X2 CHO8/CHO9 fragment with BglII generates two fragments of 393 and 97 bp, while no HindIII or ScaI sites are noted. Digestion of the MAGE-12 PCR product with ScaI yields 281 and 209 bp fragments [which is indistinguishable from the MAGE-2 gel pattern (14)].

In summary, accurate analysis of MAGE-gene expression is possible using the PCR-REN conditions described here. The two newly described MAGE genes (X2 and 3b) can be distinguished from other family members by the method described. These data will aid in the analysis of MAGE gene expression in tumor samples from patients during MAGE antigen-specific immunotherapy trials.

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