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Two Antigens Recognized by Autologous Cytolytic T Lymphocytes on a Melanoma Result from a Single Point Mutation in an Essential Housekeeping Gene[1]

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ABSTRACT

We have pursued our analysis of antigens recognized by autologous cytolytic T lymphocytes (CTLs) on the melanoma cells of patient LB33. This patient enjoys an unusually favorable evolution, which is associated with a strong and sustained antitumor CTL response. We reported previously the analysis of two melanoma cell lines, MEL.A and MEL.B, which were derived from metastases removed from the patient at 5 years' distance. Autologous CTL clones derived from blood lymphocytes recognized several antigens presented by different HLA class I molecules on MEL.A. The MEL.B cells resisted lysis by these CTLs because they have lost expression of most HLA molecules, suggesting that they were selected *in vivo* by the anti-MEL.A CTL response. One of the MEL.A antigens was shown to result from a point mutation in the tumor. Here we report the cloning of a gene that encodes two other MEL.A antigens. This new gene, *MUM-2*, is expressed ubiquitously. In the melanoma cells of patient LB33, it contains a point mutation that changes one amino acid in the translated protein. Two different antigenic peptides, one presented to CTL by HLA-B44 molecules and another by HLA-C6 molecules, overlap and contain the mutated residue. Gene *MUM-2* is homologous to an essential yeast gene, *bet5*, that was recently shown to be implicated in the vesicular transport of proteins from the endoplasmic reticulum to the Golgi. In a mutant yeast with a disrupted *bet5* gene, both the wild-type and the mutated *MUM-2* genes could complement for *bet5* function. These results indicate that the antigenic mutation does not destroy the function of the protein, a function that is conserved in eukaryotic cells. The identification of these antigens suggests that point mutations could be the major cause of the strong immunogenicity of MEL.A cells.

INTRODUCTION

The list of antigens recognized on human tumors by autologous T lymphocytes increases regularly. A first group of these are the shared and tumor-specific antigens encoded by the *MAGE*-type genes. The latter, members of the *MAGE-A* (1), *MAGE-B* (2, 3), *MAGE-C* (4), *BAGE* (5), *GAGE* (6), and *LAGE/NY-ESO* (7, 8) gene families, are activated in many tumors by demethylation of their promoter region (9). They are not expressed in normal cells with the exception of male germ-line cells, which do not express HLA molecules. The antigens encoded by these genes are, therefore, truly tumor-specific, which makes them safe for use in immunotherapy. Clinical trials are proceeding with MAGE-encoded peptides: significant tumor regressions were detected in 7 of 25 metastatic melanoma patients vaccinated with a MAGE-3 peptide binding to HLA-A1 molecules (10, 11).

Another important category of tumor antigens are those generated by point mutations. They are strictly tumor-specific, but their individuality prevents them from being used in generic vaccines. One interesting aspect of their identification is that several of the mutations that generate tumor-specific antigens recognized by T cells also play a role in oncogenesis. An antigenic mutation in the CDK4 protein was shown to prevent binding of the inhibitor p16, thereby driving cells into cycle (12). A mutation in FLICE, a protein participating in the transmission of apoptotic signals through the Fas or TNF[3] receptors, rendered cells less sensitive to apoptosis (13). A mutation in the *β-catenin* gene was shown to be responsible for a decrease in turnover of the protein, leading to an increase in activity of the transcription factors bound to catenin (14, 15).

Much of our knowledge of tumor antigens recognized by T cells derives from the detailed analysis of the antitumor cytolytic T cell (CTL) responses of patients with an unusually favorable clinical evolution. Genes *MAGE*, *BAGE* and *GAGE* were identified because they encoded antigens recognized by autologous CTLs on the melanoma cells of patient MZ2. This patient remains without signs of cancer 13 years after abdominal metastases were treated with surgery, chemotherapy, and inoculations of irradiated autologous tumor cells. The oncogenic *CDK4* mutation mentioned above was found in the melanoma of patient SK29, who is disease-free more than 20 years after metastatic lymph nodes were resected. CTLs from this patient also led to the identification of the *tyrosinase* and *Melan-A/MART-1* genes as sources of melanoma antigens (16–18).

We are pursuing our analysis of the tumor-specific T-cell response of patient LB33, who is presently disease-free 10 years after the treatment of a metastatic melanoma (Fig. 1). Two tumor cell lines, MEL.A and MEL.B, were derived from metastases resected in 1988 and 1993, respectively. The patient developed a very strong CTL response against the MEL.A cells (19). A panel of anti-MEL.A CTL clones was derived from blood lymphocytes collected in 1990. These CTLs recognize at least five antigens presented by various HLA class I molecules. One of these antigens was found to result from a point mutation in an ubiquitously expressed gene that we called *MUM-1* (20). The mutation was absent from 300 tumor samples that were subsequently tested, making it unlikely that it participated in the development of the tumor. The function of the MUM-1 protein is unknown. The MEL.B cells were found to resist lysis by the anti-MEL.A CTLs because they have lost expression of HLA class I molecules, except HLA-A24, which did not present antigens to anti-MEL.A CTL. This strongly suggests that MEL.B cells were selected *in vivo* by the anti-MEL.A CTL response. From blood collected in 1993, new CTL clones were obtained that lysed the MEL.B cells. These CTLs recognize an antigen encoded by gene *PRAME*, which is overexpressed in many tumors, including acute leukemias (21, 22). MEL.A cells express *PRAME* but are not lysed by the anti-PRAME CTLs because they carry HLA-Cw7 molecules that bind to killer cell inhibitory receptors present on the CTLs, resulting in a complete inhibition of the activation of these lymphocytes.

Together, these results are suggestive of a role for antitumor T lymphocytes in the favorable clinical history of patient LB33. In this

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[3] The abbreviations used are: TNF, tumor necrosis factor; CTL, cytolytic T lymphocyte; ER, endoplasmic reticulum.

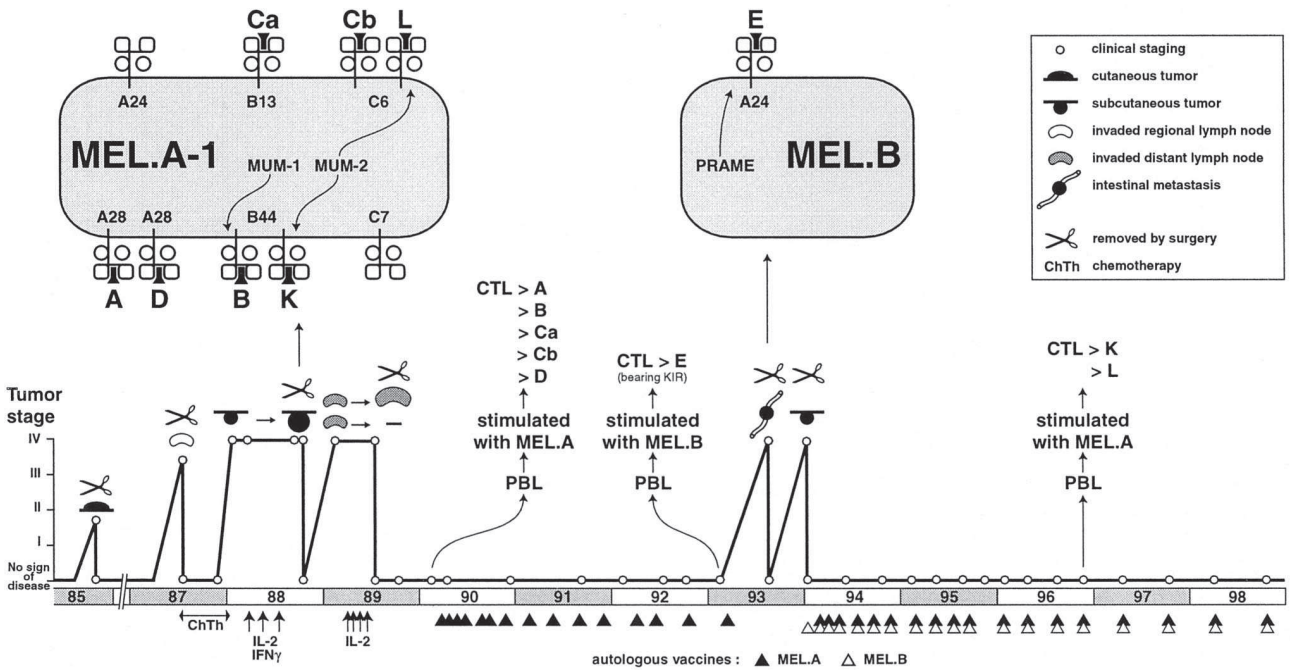

Fig. 1. Clinical evolution of melanoma patient LB33. The melanoma cell lines derived from metastases are represented with their respective antigens recognized by autologous CTLs. The obtention of the corresponding CTL clones is shown.

respect, the antitumor immune response of this patient represents what may be achievable with therapeutic anticancer vaccines. Therefore, we feel that additional information about this response is valuable, and we report on the identification of two new antigens recognized by autologous CTLs.

MATERIALS AND METHODS

Cell Lines. The clinical course of melanoma patient LB33 (HLA-A24, A28, B13, B44, Cw6, Cw7) and the obtention of the various LB33-MEL clonal cell lines and antigen-loss variants were described previously (19, 23). Melanoma cell line LB33-MEL.A was derived from a cutaneous metastasis resected from patient LB33 in 1988. Clonal cell line LB33-MEL.A-1 was derived from MEL.A by limiting dilution. The tumor cell lines were cultured with Iscove's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Life Technologies, Inc.), L-arginine (116 mg/L), L-asparagine (36 mg/L), and L-glutamine (216 mg/L). 293-EBNA cells (Invitrogen) were maintained in DMEM (Life Technologies, Inc.) with 10% FCS. WEHI-164c13 cells (24) were cultured in RPMI 1640 (Life Technologies, Inc.) with 5% FCS. LG2-EBV and K562 were grown in Iscove's medium containing 10% FCS.

Obtention and Use of Autologous CTL Clones. Blood mononuclear cells of patient LB33 were isolated by Lymphoprep (Nycomed, Oslo, Norway) density-gradient centrifugation and were cryopreserved. Autologous mixed lymphocyte-tumor cell cultures (MLTC), and derivation and long-term culture of CTL clones were carried out as described previously (19). Anti-LB33-K CTL clone LB33-CTL-407AB/1 and anti-LB33-L CTL clone LB33-CTL-407AB/12, referred to in this report as CTL 7/1 and 7/12, respectively, were derived from lymphocytes collected in 1996 and stimulated with MEL.A-1.1 cells (19). Sensitivity of target cells to lysis by the CTLs was evaluated by a standard ^{51}Cr-release assay over 4 h, with target tumor cells incubated for 48 h with IFN-γ (50 units/ml, Boehringer Mannheim, Mannheim, Germany).

Peptides were synthesized by conventional solid-phase peptide synthesis, using Fmoc for transient NH_2-terminal protection (25), and were characterized by mass spectrometry. They were solubilized at 10 mg/ml in DMSO, kept frozen at −20°C, and diluted in Iscove's medium immediately before use.

Construction and Screening of the cDNA Library. Total RNA was extracted from MEL.A-1 cells by the guanidine-isothiocyanate procedure. Poly(A+) RNA enriched with an oligo(dT)-cellulose column (Pharmacia Biotech) was converted to cDNA with the Superscript Choice System (Life Technologies, Inc., Gaithesburg, MD) using an oligo(dT) primer [5′-ATAAGAATGCGGCCGCTAAACTA(T)18VZ-3′; V = G, A, or C; Z = G, A, T, or C] containing a *Not*I site at its 5′ end. The cDNA was ligated to *Hin*dIII-*Eco*RI adaptors (Stratagene, Heidelberg, Germany), phosphorylated, digested with *Not*I, and inserted at the *Hin*dIII and *Not*I sites of expression vector pCEP4 (Invitrogen, San Diego, CA). *Escherichia coli* DH5α were transformed by electroporation with the recombinant plasmids and selected with ampicillin (50 μg/ml). The library was divided into 528 pools of about 100 cDNA clones. Each pool was amplified for 4 h and plasmid DNA was extracted using the QIAprep 8 plasmid Kit (Qiagen, Hilden, Germany). Duplicate microcultures of 293-EBNA cells (Invitrogen, San Diego CA), plated in flat-bottomed 96 microwells (3.5×10^4/well) 24 h before transfection, were cotransfected with 1.5 μl of lipofectAMINE reagent (Life Technologies, Inc.), 100 ng of plasmid DNA of each pool of the cDNA library, and 50 ng of plasmid pcDNA3 (Invitrogen) containing the HLA-B*4402 or HLA-Cw*0602 cDNA. After 24 h, CTLs (3000 cells/well) were added, and, after another 24 h, supernatant was collected, and its TNF content was measured by testing the cytotoxic effect on WEHI-164cl3 cells (24) in an MTT colorimetric assay (26).

Screening for MUM-2 Mutations. mRNA extracted from 270 tumor samples of various origins was converted to cDNA with M-MLV reverse transcriptase (Boehringer Mannheim) using an oligo(dT) primer. Because the mutation created a *Bsp*EI restriction site, PCR-amplified products (OPC527 5′-CCGATGACTGTCCACAACCTG and OPC522 5′-AGTTGGCACAGGTTCGGAAGG) were digested with this enzyme. In addition, approximately 100 amplified products (OPC538 5′-GTAACTCTTGACGGGCACTCG and OPC522) were purified using QIAquick PCR purification kit (Qiagen) and sequenced with primer OPC534 (5′-GGCCCCAGGCAGACATGAATT).

Structure of Gene *MUM-2*. The GeneBridge 4 Radiation Hybrid Panel (Research Genetics, Inc., Huntsville) was used to map the *MUM-2* gene. Genomic DNA (25 ng) from each of the 93 radiation hybrid clones were PCR-amplified with primers OPC 538 and OPC 524 (5′-GTGACCCACCAGTTACAGTA). PCR amplification was performed for 35 cycles of 1 min at 94°C, 2 min at 68°C, and 3 min at 72°C. PCR results were submitted for analysis to the web sites of the Whitehead Institute for Biomedical Research[4] and of the Genome Database.[5]

[4] Whitehead Institute for Biomedical Research web site: http://www-genome.wi.mit.edu/cgibin/contig/rhmapper.pl.

[5] Genome Database web site: http://www.ncbi.nlm.nih.gov/SCIENCE96.

To identify the gene, a labeled PCR product corresponding to nucleotides 184–493 of cDNA clone 7R was used to screen high-density filters spotted with the human genomic library RPCI6 constructed in vector pPAC4 by Dr. Pieter de Jong at the Roswell Park Cancer Institute (Buffalo, NY). The filters with this library, named 709, were made available through the Ressourcen-Zentrum/PrimärDatenBank (MaxPlanck-Institut für Moleculare Genetik, Berlin). A positive clone, named LLNLP709N1149Q2, was found. A 2750-bp portion of the DNA insert, around the *MUM-2* gene, was sequenced with the BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, Great Britain), using primers chosen in the cDNA sequence. The products of the sequencing reactions were purified on Centrisep CS-900 spin columns (Princeton Separations, Adelphia, NJ) and analyzed on an ABI 310 Sequencer (Perkin-Elmer).

To localize the 5′ end of the MUM-2 mRNA, we used the anchored PCR technique with the 5′RACE System for Rapid Amplification of cDNA Ends (Life Technologies, Inc.). cDNA produced with primer OPC534 was used as template for nested PCR amplifications using forward primers from the 5′RACE system and reverse primers OPC520 (5′-GAGAAGAAGGGCAGAGAGCGA) and OPC525 (5′-ACCAGCTCCACATACAGCGCA). The amplified product was cloned into vector pCR3.1 with the TA Cloning kit (Invitrogen). Ten clones were sequenced. The 5′ end of the two longest clones extended the sequence of cDNA clone 7R by 36 nucleotides. Comparison of this sequence with that of the pPAC4 clone indicated the presence of an additional exon, ending immediately upstream of the putative initiation codon (Fig. 6).

Disruption and Complementation in Yeast. Yeast cells were grown in YPD supplemented with adenine (40 mg/l) and 2% glucose as a carbon source. A chromosomal deletion of YML077 was created by replacing all but the first 39 and the last 36 nucleotides of the coding sequence with a kanamycin resistance gene. Plasmid pKANMX6 was used as template for PCR amplification with forward primer OPC607 5′-*ATGGGGATATATTCATTTTGGATCTTTGATAGGCATTGTA*GGTCGACGGATCCCCGGG and reverse primer OPC608 5′-*TCACTGATTAACCATTGGTGCTAGAAATGACTCGAGAACG*CATCGATGAATTCGAGCTCG. The italicized sequences correspond to parts of the YML077 coding sequence. A diploid strain with one disrupted copy of YML077 (ΔYML077CENPK2) was obtained by transforming the PCR product in the CENPK2 diploid yeast strain. After the induction of sporulation of the transformants, tetrads were dissected. After 3 days at 30°C, the spores examined for two independent disrupted mutants displayed a 2:2 segregation for viability. All of the viable spores were sensitive to geneticin.

For the complementation experiments, we cloned PCR products, consisting of the putative YML077 promoter region (450 bp upstream of the ATG), followed by the complete ORF and the following 200 bp, into the *Bam*HI and *Eco*RI sites of centromeric plasmid pRS416 (URA3), and into the *Bam*HI and *Sph*I sites of high-copy plasmid Yep24 (URA3). Forward primer was OPC662 5′-CGC*GGATCC*GGTGGATGGAAAACACGTTG and reverse primers were OPC663 5′-G*GAATTC*ATGACGAGTTTTGGACAGACC or OPC 668 5′-ACAT*GCATGC*ATGACGAGT TTGGACAGACC for pRS416 and Yep24, respectively. *Bam*HI, *Eco*RI, and *Sph*I sequences are italicized. The two constructs were transformed into ΔYML077CENPK2. After sporulation of the transformants, tetrads were dissected on YPD plates. Colonies that were URA^+ and resistant to geneticin were selected for analysis of growth.

Similarly, PCR products consisting of the putative YML077 promoter followed by the normal or mutated MUM-2 sequence were cloned into plasmids pRS416 or Yep24. Yeast DNA was used as a template for a PCR amplification with forward primer OPC662 and reverse primer OPC681 5′-CCC*AAGCTT*GCTCCACTGATTTATGATTTATATC, whereas plasmids pCEP4 containing the mutated or the wild-type 7R cDNA sequence were used as templates for an amplification with forward primer OPC682 5′-CCC*AAGCTT*ATGACTGTCCACAACCTGTAC and reverse primer OPC683 5′-ACGC*GTCGAC*GAGTGACAAAAAGTTTATTCTGTGC. PCR product OPC662-OPC681 digested with *Bam*HI and *Hin*dIII, and PCR product OPC682-OPC683, digested with *Hin*dIII and *Sal*I, were cloned into the *Bam*HI and *Sal*I sites of pRS416 or Yep24. ΔYML077CENPK2 was then transformed with these plamids. Sporulation and tetrad analysis were performed as described above.

RESULTS

Obtention of New Anti-MEL.A-1 CTL Clones. CD8 blood lymphocytes collected from patient LB33 in 1996 (Fig. 1) were stimulated with irradiated A^- cells, in the presence of interleukin 2 and interleukin 7. The A^- cells were derived from MEL.A-1 by selection *in vitro* for resistance to lysis by a CTL clone recognizing antigen LB33-A (19). These cells have lost expression of antigen A but carry the antigens recognized by other anti-MEL.A-1 CTL. Because antigen A is recognized by most of the CTLs that we obtained against the MEL.A-1 cells, we used the A^- variant to derive CTLs with other specificities. After two weekly restimulations with irradiated tumor cells, the lymphocytes were cloned by limiting dilution, and CTL clones 7/1 and 7/12 were obtained. They lysed the MEL.A-1 and A^- cells, which indicate that they did not recognize antigen LB33-A. These clones did not lyse autologous EBV-transformed B cells or NK-target cells K562 (Fig. 2).

To identify the HLA restriction of the CTLs, we tested their lytic activity on HLA haplotype-loss variants derived from MEL.A-1 cells (Fig. 2). CTL 7/1 did not lyse a variant that had lost expression of the A28-B44-C7 haplotype, but it did lyse a variant that had lost expression of the A24-B13-C6 haplotype. The opposite pattern of recognition was observed for CTL 7/12. The HLA-loss variants were transfected with HLA cDNA clones and tested for recognition by the CTLs. The results indicated that CTL clones 7/1 and 7/12 recognized antigens presented by HLA-B44 and HLA-C6 molecules, respectively (Fig. 2).

We previously identified an antigen of MEL.A-1 cells encoded by gene *MUM-1* and presented by HLA-B44 molecules (20). CTL 7/1 did not lyse autologous EBV-B cells incubated with the MUM-1.B44 peptide, which indicated that it recognized a new antigen, which we named LB33-K. The antigen recognized by CTL 7/12 was named LB33-L.

Identification of a cDNA Clone Encoding Antigens K and L. A cDNA library prepared with mRNA from MEL-A.1 cells was cloned into expression vector pCEP4. This plasmid contains the EBV origin of replication, resulting in episomal multiplication of the transfected plasmids in the human embryonic kidney cells 293 transfected with the EBV *EBNA-1* gene. The cDNA library was divided into 528 pools of about 100 bacteria. Plasmid DNA was extracted from each pool and cotransfected into duplicate microcultures of 293-EBNA cells with either an HLA-B44 or an HLA-C6 construct. After 48 h, the cells cotransfected with B44 were screened for the expression of the antigen by adding CTL clone 7/1 and measuring the production of TNF. Those cells cotransfected with C6 were screened with CTL clone 7/12. For each CTL, only one pool of cDNA resulted in stimulation, and, surprisingly, it was the same pool for both CTLs. The positive pool was subcloned, and cDNA clone 7R was found to transfer expression of antigen K or L into 293-EBNA cells cotransfected with HLA-B44 or HLA-C6 constructs, respectively (Fig. 3). Each CTL clone showed specificity for 293-EBNA cells cotransfected with cDNA 7R and only one HLA construct, indicating that the transfectants neither secreted TNF by themselves nor stimulated the CTLs without HLA restriction.

Similar results were obtained by transfecting into 293-EBNA cells a sequence containing the main open reading frame of cDNA 7R cloned into vector pCR3.1, which does not contain the EBV origin of replication. Thus, recognition by the two CTL clones of 293-EBNA cells transfected with cDNA 7R did not depend on a particularly high level of expression of the cDNA.

Presence of a Point Mutation in the cDNA. cDNA 7R was 712 bp long and contained a poly(A) tail and a polyadenylation signal (Fig. 4). When a Northern blot prepared with RNA extracted from

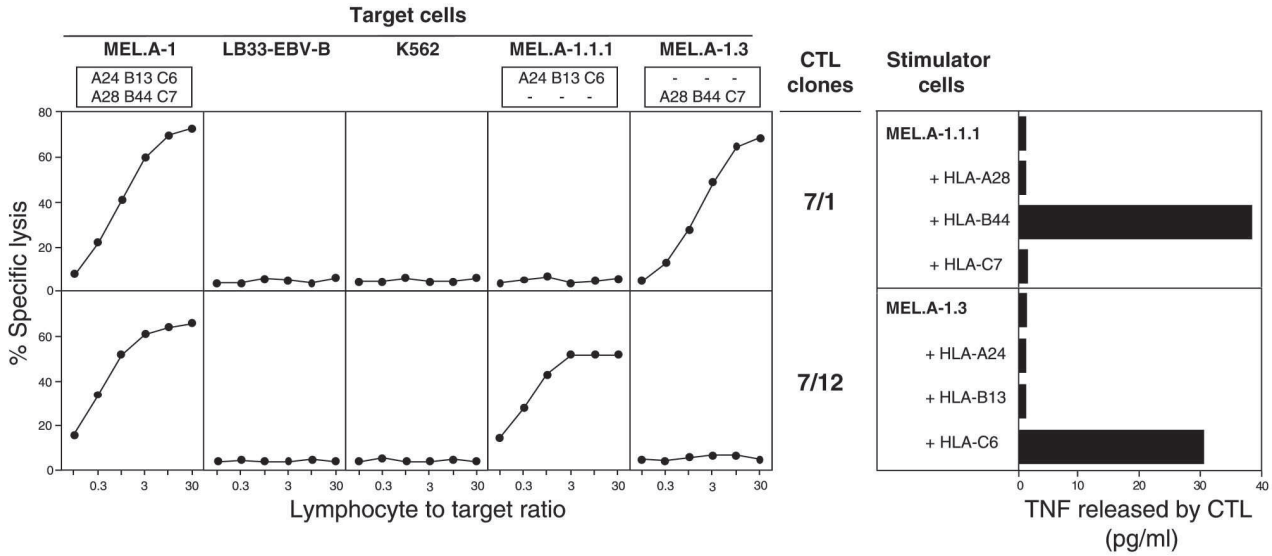

Fig. 2. Specificity of CTL clones 7/1 and 7/12. *Left*, sensitivity of ^{51}Cr-labeled target cells to lysis by CTL clones 7/1 and 7/12. MEL.A-1 is a clonal subline of the melanoma line MEL.A from patient LB33. MEL.A-1.1.1 cells, cloned from a population of MEL.A-1 cells after selections *in vitro* with autologous CTL clones (19), have lost expression of the HLA-A28-B44-C7 haplotype. MEL.A-1.3 is another immunoselected variant, which has lost expression of the A24-B13-C6 haplotype. *Boxed*, the profiles of HLA class I gene expression by the cell lines. *Right*, production of TNF by the CTL clones stimulated with the HLA-loss variants transfected with expression vector pcDNA3 containing cDNA encoding HLA class I molecules. TNF was detected by measuring the cytotoxicity of the culture medium on the TNF-sensitive WEHI-164c13 cells.

melanoma cells was hybridized with a PCR probe representative of cDNA 7R (nucleotides 7–513 on Fig. 4), a band of approximately 0.8 kb was observed, which suggested that cDNA 7R was complete. The corresponding gene, provisionally named *MUM-2* (Melanoma Ubiquitous Mutated), was found to be expressed ubiquitously, which suggested that mutations could be responsible for the expression of antigens K and L. RNA extracted from MEL.A-1 or from autologous EBV-B cells was converted into cDNA, and a fragment corresponding to nucleotides 7–513 of the MUM-2 cDNA was amplified by PCR and cloned. The sequences of 2 of 4 clones derived from the tumor cells were identical to that of cDNA 7R, whereas two other sequences differed by one nucleotide: C instead of G at position 394 (Fig. 4). The sequences of 5 clones derived from the EBV-B cells contained C at that position.

The C-to-G mutation was also found in cDNA prepared from melanoma metastases resected from patient LB33 in 1993 and 1994 (Fig. 1), indicating that the mutation occurred *in vivo*.

We screened approximately 270 tumor samples of different histological origins for the presence of this mutation, and sequenced the complete MUM-2 open reading frame in cDNA from 100 of these samples. No mutation was found, which indicated that *MUM-2* is unlikely to be a proto-oncogene.

Identification of the Antigenic Peptides. The C-to-G mutation changes arginine into glycine in the putative MUM-2 protein (Fig. 4). A MUM-2 peptide of 12 amino acids, containing the mutated residue at position 7, sensitized EBV-B cells to lysis by both CTL clones 7/1 and 7/12 (Fig. 5*A*). Shorter peptides, with various COOH- or NH_2 termini were then tested (Fig. 5*A*). The optimal peptide for recognition by CTL 7/1, SELFRSGLDSY, was 11 amino acids long, with the mutated residue at position 7. It contains glutamic acid at position 2 and tyrosine at the COOH terminus. This is the canonical binding motif for HLA-B44 molecules (27). This peptide was not recognized at all by CTL 7/12. When increasing concentrations of the peptide were incubated with EBV-B cells, one-half of maximal lysis was obtained at 200 nM (Fig. 5*B*). The corresponding normal peptide was not recognized, even at a concentration of 30 mM. In a competition

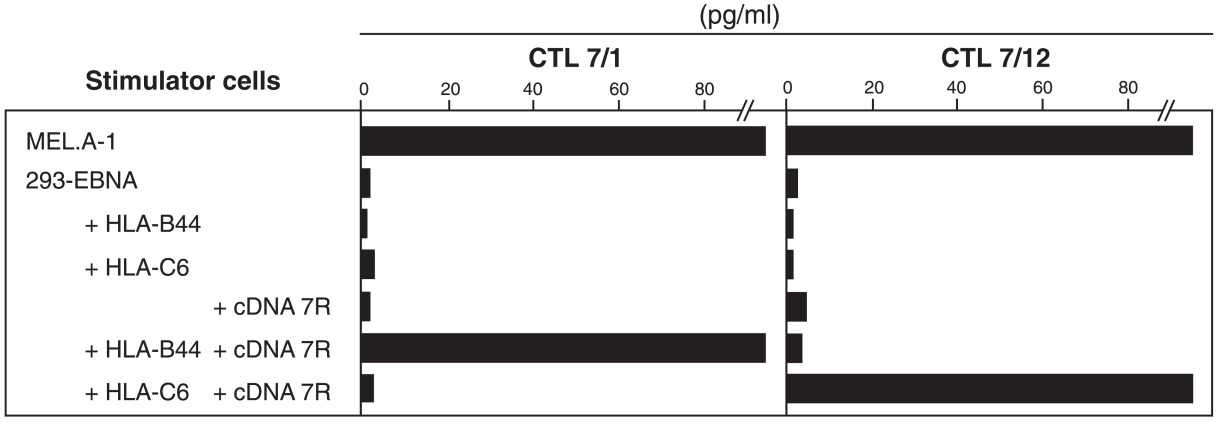

Fig. 3. Identification of a cDNA clone encoding antigens LB33-K and LB33-L. 293-EBNA cells (5.10^4/well) were cotransfected using lipofectamine with 50 ng of pcDNA3/HLA-B44 or pcDNA3/HLA-C6 and with 100 ng of pCEP4–7R. CTL clones (3.10^3/well) were added after 24 h, and medium was collected for TNF measurement after another 24 h.

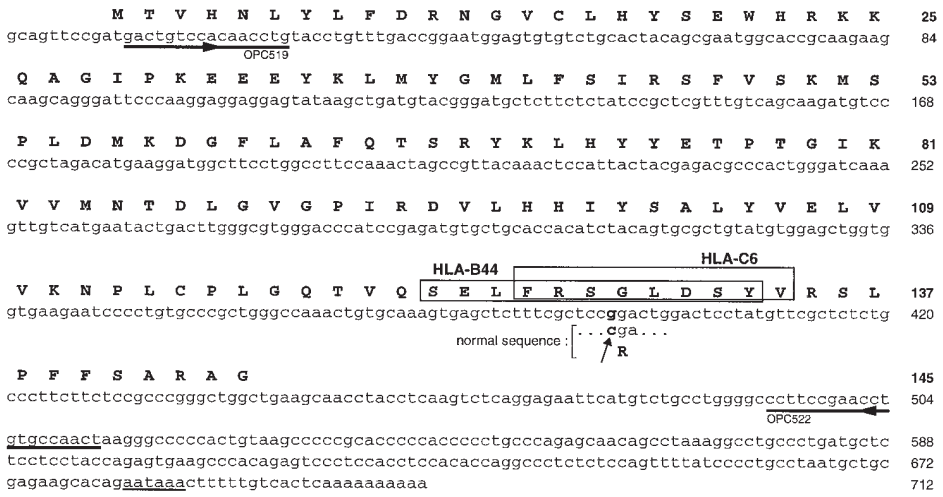
M T V H N L Y L F D R N G V C L H Y S E W H R K K 25
gcagttccgatgactgtccacaacctgtacctgtttgaccggaatggagtgtgtctgcactacagcgaatggcaccgcaagaag 84
OPC519
Q A G I P K E E E Y K L M Y G M L F S I R S F V S K M S 53
caagcagggattcccaaggaggaggagtataagctgatgtacgggatgctcttctctatccgctcgtttgtcagcaagatgtcc 168
P L D M K D G F L A F Q T S R Y K L H Y Y E T P T G I K 81
ccgctagacatgaaggatggcttcctggccttccaaactagccgttacaaactccattactacgagacgcccactgggatcaaa 252
V V M N T D L G V G P I R D V L H H I Y S A L Y V E L V 109
gttgtcatgaatactgacttgggcgtgggacccatccgagatgtgctgcaccacatctacagtgcgctgtatgtggagctggtg 336
HLA-B44
HLA-C6
V K N P L C P L G Q T V Q S E L F R S G L D S Y V R S L 137
gtgaagaatcccctgtgcccgctgggccaaactgtgcaaagtgagctctttcgctccggactggactcctatgttcgctctctg 420
normal sequence : ...cga...
R
P F F S A R A G 145
cccttcttctccgcccgggctggctgaagcaacctacctcaagtctcaggagaattcatgtctgcctggggcccttccgaacct 504
OPC522
gtgccaactaagggccccactgtaagcccccgcaccccaccccctgcccagagcaacagcctaaaggcctgccctgatgctc 588
tcctcctaccagagtgaagcccacagagtccctccacctccacaccaggccctctctccagttttatcccctgcctaatgctgc 672
gagaagcacagaataaacttttgtcactcaaaaaaaaaa 712

Fig. 4. Sequence of cDNA 7R and sequence of the protein encoded by the longest open reading frame. *Horizontal arrows*, primers OPC519 and OPC522, used to analyze the expression of gene MUM-2 by RT-PCR; underlined, the polyadenylation signal; *arrow*, nucleotide 394, which is mutated in the LB33 melanoma cells; *boxed*, the peptides corresponding to antigens LB33-K and -L, presented by HLA-B44 and -C6 molecules, respectively.

experiment, the normal peptide was shown to bind to HLA-B44 molecules, which indicated that the mutated amino acid is part of the epitope recognized by CTL clone 7/1.

The peptide that was optimally recognized by CTL 7/12 was 9 amino acids long, with the mutated residue at position 4. This peptide, FRSGLDSYV, was not recognized by CTL clone 7/1. Although the HLA-C6 binding motif is not defined precisely, the COOH-terminal valine residue is also present in other peptides reported to bind to HLA-C6 molecules (27). One-half maximal lysis was obtained with 3 nM of peptide FRSGLDSYV, whereas the corresponding normal peptide could not sensitize cells to lysis (Fig. 5*B*). Competition

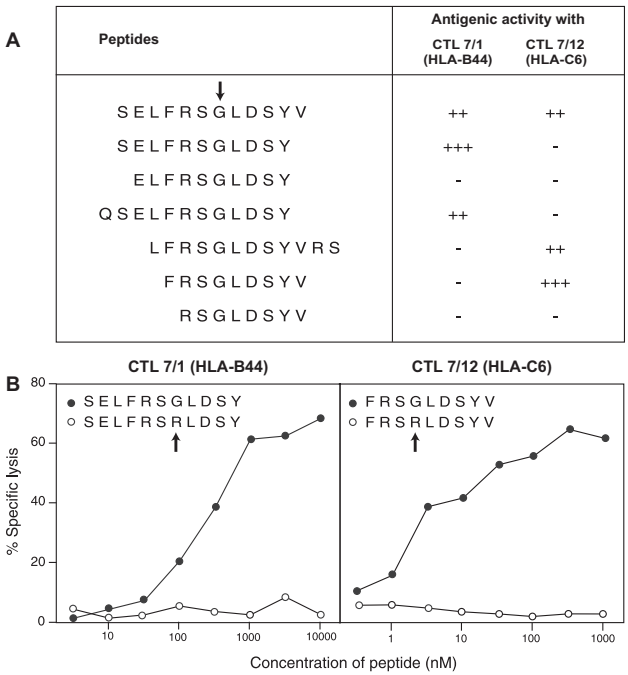

Fig. 5. Identification of the antigenic peptides. *A*, LB33-EBV-B cells were incubated for 15 min with increasing concentrations of the indicated peptides, and CTLs were added at an E:T ratio of 5. Lysis was measured after 4 h. The concentrations of peptide required to obtain one-half maximal lysis (L50) were calculated. The results are expressed as relative antigenic activities, defined as [L50 of the tested peptide ÷ L50 of the optimal peptide for this CTL]. The optimal peptides were SELFRSGLDSY (L50 = 200 nM) for CTL clone 7/1 and FRSGLDSYV (L50 = 3 nM) for CTL clone 7/12. Results are expressed semiquantitatively, with +++, ++, and − corresponding to relative antigenic activities of 1, 0.3, and <0.1, respectively. *B*, ^{51}Cr-labeled autologous EBV-B cells were incubated over 15 min at room temperature with the indicated concentrations of peptides, and CTL clones were added at an E:T ratio of 5. Chromium release was measured after 4 h.

experiments indicated that this normal peptide could also bind to HLA-C6 molecules.

Characterization of Gene *MUM-2*. Hybridization of a Southern blot suggested that gene *MUM-2* is a single copy gene, and the screening of DNA samples extracted from a panel of human-hamster radiation hybrids indicated that it is located on 17p13. The complete sequence of the *MUM-2* gene was obtained from a clone of a library of human DNA cloned into a PAC vector. The gene comprises 5 exons (Fig. 6).

The *MUM-2* cDNA contains an open reading frame encoding a putative protein of 145 amino acids, which had no significant homology with proteins of known functions. EST data banks contained murine sequences that could be aligned into a contig coding for a putative protein of 145 residues that, surprisingly, was identical to the human MUM-2 protein with the exception of two amino acids. We also found anonymous sequences from *C. elegans* and from plants that could code for protein fragments which were almost 50% identical to portions of MUM-2. Finally, a putative 159-amino-acid protein encoded by gene *YML077* (GenBank Z46373) on chromosome 13 of *Saccharomyces cerevisiae* showed 29% identity and 54% similarity with MUM-2. No significant homology was found with proteins encoded by bacterial sequences.

Because these results suggested that protein MUM-2 could be conserved, we surmised that this protein could exert an essential function in all eukaryotic cells. Considering the existence in yeast of the YML077 sequence, coding for a protein showing some homology with MUM-2, we decided to knock out the *YML077* gene. One copy of the gene was disrupted by homologous recombination in the diploid strain CENPK2. After sporulation, tetrad analysis showed a 2:2 segregation for viability, indicating that *YML077* did indeed control an obligatory function in yeast cells.

Because the YML077 and MUM-2 putative proteins are only 29% identical, this result did not prove that these proteins had similar functions. To examine this point, we complemented the disrupted ΔYML077 strain with the normal *MUM-2* allele, cloned in the high-copy vector YEP24 or in the low-copy centromeric plasmid pRS416 (Fig. 7). Somewhat unexpectedly, tetrad analysis showed a complementation of ΔYML077, even with the low-copy vector. These results demonstrated that *YML077* and *MUM-2* encode proteins that have a similar function, which is conserved in eukaryotic cells.

This provided us with an opportunity to investigate the effect of the antigenic mutation in the function of the MUM-2 protein. Complementation experiments were repeated in the ΔYML077 yeast strain with the wild-type and mutated *MUM-2* genes. The results indicated

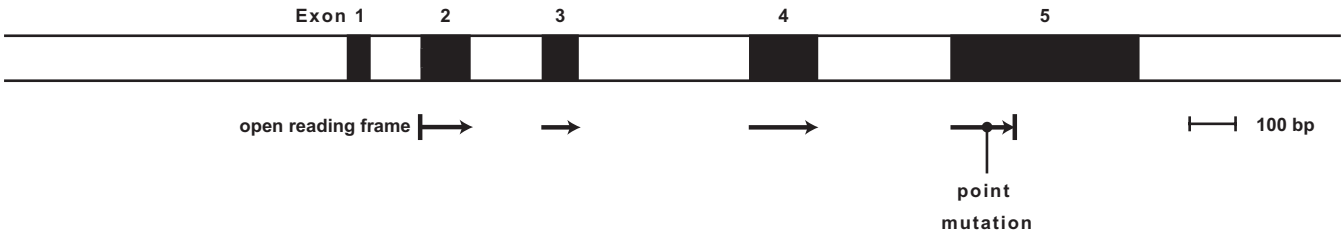

Fig. 6. Structure of gene *MUM-2*. A fragment (2750 bp) of a human genomic clone in vector pPAC4 is represented. *Black boxes*, the exons. This DNA sequence is available from EMBL/Genbak/DDBJ under accession number AF129332. The indicated 5′ end of exon 1 corresponds to the 5′ end of the longest cDNA clones obtained in an anchored PCR experiment, as described in "Materials and Methods."

that the mutated human allele complemented the disrupted yeast strain with the same efficiency as the wild-type allele. We concluded that the MUM-2 mutation present in the LB33 melanoma cells does not have an important effect on the function of the protein.

DISCUSSION

While we were completing the experiments described in this report, the group of S. Ferro-Novick identified a new essential gene in yeast, designated *bet5*, that plays a role in the vesicular protein transport between the ER and the Golgi apparatus (28). Gene *bet5* is identical to *YML077*, the yeast homologue of *MUM-2*. It was found in a screening for genes that suppressed the growth defect of a yeast strain with a mutated *bet3* gene. The latter encodes a small hydrophilic protein of the *cis*-Golgi that appears to mediate a late stage of ER-to-Golgi transport (29). The bet3 and bet5 proteins coimmunoprecipitate and are parts of a M_r 800,000 complex called TRAPP (transport protein particle) that participates in the targetting and/or fusion of ER-to-Golgi transport vesicles with their acceptor compartment (30). Because our complementation experiments indicated that the functions of the human MUM-2 and yeast YML077/bet5 proteins were conserved, we conclude that protein MUM-2 is involved in the vesicular transport of proteins.

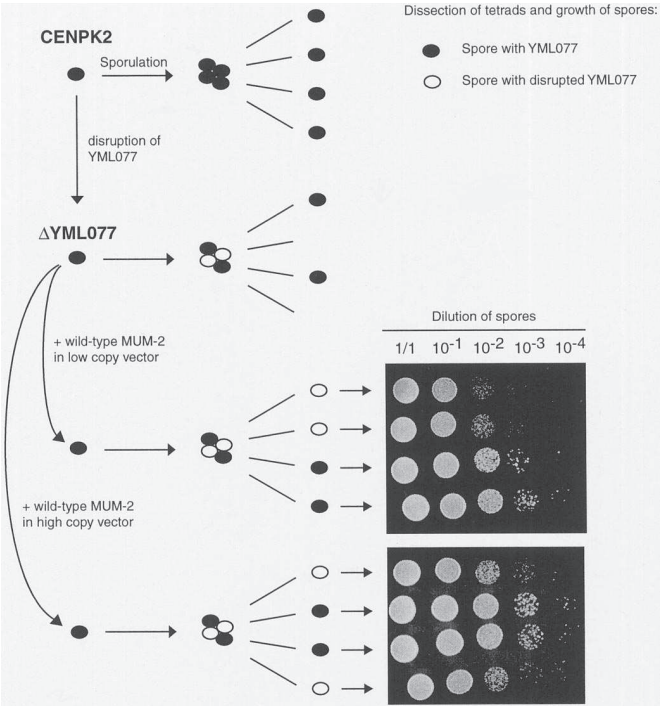

Fig. 7. Growth of spores of a tetrad of yeast CENPK2 disrupted for YML077 and complemented with a wild-type MUM-2 sequence. Diploid yeast strain CENPK2 was transfected with a construct consisting of a kanamycin resistance gene flanked with YML077 sequences. Integration of one disrupting construct by homologous recombination was selected by resistance to geneticin and confirmed by PCR. The disrupted strain was complemented with a wild-type MUM-2 sequence cloned downstream of the YML077 promotor in plasmids that were maintained at low- or high-copy numbers in the transformants. After sporulation, tetrads were dissected and dilutions of the amplified spores were plated to compare the growth of the wild-type or complemented yeasts.

Given that several of the mutations that were discovered in tumors because they encoded a CTL target antigen have been found to be involved in oncogenesis (12, 13, 15), we carefully examined this possibility for the *MUM-2* mutation. We did not detect mutations in the open reading frame of the *MUM-2* gene in about 100 tumors that were tested, suggesting that *MUM-2* is not a proto-oncogene. Considering the role of *MUM-2* in the transport of secreted proteins, we examined the possibility of a defect in protein secretion caused by the *MUM-2* mutation in MEL.A cells. Because a normal and a mutated *MUM-2* allele are coexpressed in MEL.A, such a defect would most probably be partial. We reasoned that the moderate level of HLA class I expression at the surface of MEL.A cells, which we systematically have to incubate with IFN-γ prior to CTL stimulation or lysis assays, could be a consequence of such a defect. MEL.A cells were transfected with expression vector pEF-BOS-Puro containing the wild-type or the mutated MUM-2 cDNA. We verified that clonal transfected lines overexpressed gene *MUM-2* and labeled them with the anti-HLA class I monoclonal antibody W6/32. We did not find any significant difference between the labeling intensities of the transfectants overexpressing the wild-type or the mutated *MUM-2* allele: expression of HLA class I molecules was comparable to that of the parental MEL.A cells. Altogether we could not link the *MUM-2* mutation to the tumoral phenotype of the LB33 melanoma cells. However, a role for the mutation cannot be ruled out, and more precise questions will be amenable to experimentation when the role of *MUM-2/bet5* in protein transport is defined.

The collection of autologous anti-MEL.A CTL clones is directed against at least nine antigenic specificities: five of them (A, B, Ca, Cb, and D) have been characterized previously (19); two (K and L) are analyzed here; and two others (F and H) have been described briefly (23). Four of these antigens have been identified thus far: antigen B is encoded by a mutated gene that we called *MUM-1* (20); antigens K and L are encoded by gene *MUM-2* (this report); and antigen A is encoded by a new, mutated, member of the RNA helicase gene family, which we called *MUM-3*.[6] The fact that most anti-MEL.A CTL seem to recognize mutated antigens does not result from the absence of other types of melanoma antigens on the tumor. For example, although the MEL.A cells do not express as many of the MAGE-type genes as MZ2-MEL cells, they express gene *MAGE-A3*. Anti-MAGE-A3.B44 CTLs were shown to lyse MEL.A cells, which indicates that there is no defect in the processing and presentation of MAGE antigens (31).

[6] J-F. Baurain, manuscript in preparation.

It is worth comparing the nature of the immunogenic antigens on MEL.A with that of the antigens recognized by autologous CTLs on the two other melanoma cell lines, MZ2-MEL and SK29-MEL, that were analyzed extensively. MZ2-MEL cells present at least eight antigens: seven of them are encoded by *MAGE*-type genes (5, 6, 31–35), and one is encoded by the *tyrosinase* gene (16). SK29-MEL cells carry at least five antigens: four melanocytic differentiation antigens and the mutated CDK4 peptide (12, 36–38).

Although conclusions cannot be drawn from only three cases, these observations suggest that different melanomas may display a highly immunogenic phenotype through very different mechanisms, possibly controlled by different genes. Activation of *MAGE*-type genes seems to be largely induced by DNA demethylation (9). Genome-wide demethylation may, thus, lead to a high level of expression of many antigens encoded by these genes, and this could be the major mechanism of antigenicity of MZ2-MEL cells. Another mechanism leading to immunogenicity of melanomas may be the up-regulation of genes encoding melanocyte differentiation antigens. There are indications that these genes have a common regulation of their trancription. The melanocyte-specific basic helix-loop-helix (bHLH) leucine zipper transcription factor encoded by the *mi* gene activates transcription of the *tyrosinase*, *TRP-1* and *Pmel17/gp100* genes through recognition of the same M-box element (39–42). In the *Melan-A/MART-1* gene, a tissue-specific promoter activity was identified in a 233-bp region that includes a bHLH consensus sequence found to be important in other melanoma-specific promoters (43).

Because of the preponderance of mutated antigens on MEL.A cells, it will be interesting to test whether or not there is a greater accumulation of mutations in the genome of the LB33 melanoma cells compared with other melanoma cell lines. Inactivation of genes involved in mismatch repair leads to a form of genetic instability observed in hereditary nonpolyposis colorectal cancer and in some sporadic cancers (39, 44). We did not find any microsatellite instability in the DNA of MEL.A cells, which suggests that the Mut's Mismatch Repair system, one of the three human DNA repair systems, is intact in these cells. But other DNA repair mechanisms, the integrity of which cannot be evaluated by microsatellite analysis, should be analyzed.

That two antigens presented to CTLs by different HLA molecules should result from the same point mutation was unexpected. The two antigenic peptides overlap but have different NH_2 and COOH termini. There are several other examples of overlapping peptides presented to T cells by different HLA class I molecules. Peptides EVDPIGHLY and MEVDPIGHLY, encoded by gene *MAGE-3*, are presented to CTLs by HLA-A1 and -B44 molecules, respectively (31, 35). Two peptides of the Melan-A/MART-1 protein, AAGIGILTV and AEEAAGIGILT, are recognized by CTLs restricted by HLA-A2 (45) and HLA-B45 (18), respectively. Similar observations were made with CTLs recognizing peptides derived from the HIV-1 nef (46) or envelope (47) proteins, from the influenza A nucleoprotein (48), or from the Epstein-Barr virus transactivator Zta (49). This clustering of epitopes, noticed and discussed by others (50), could result from some kind of specificity of the antigen-processing machinery for particular regions of a polypeptide. Likely candidates for such a bias in antigen presentation are the proteolytic enzymes of the proteasome. A practical consequence is that the search for new CTL epitopes within a given protein may be focused first on regions that are already known to encode an antigenic peptide.

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