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Immunology

A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma

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ABSTRACT **We have identified an antigen recognized on a human melanoma by autologous cytolytic T lymphocytes. It is encoded by a gene that is expressed in many normal tissues. Remarkably, the sequence coding for the antigenic peptide is located across an exon–intron junction. A point mutation is present in the intron that generates an amino acid change that is essential for the recognition of the peptide by the anti-tumor cytotoxic T lymphocytes. This observation suggests that the T-cell-mediated surveillance of the integrity of the genome may extend to some intronic regions.**

When blood lymphocytes or tumor-infiltrating lymphocytes of melanoma patients are stimulated *in vitro* with irradiated tumor cells of the same patient, they often proliferate to produce populations of cytolytic T lymphocytes (CTLs) that show specificity for the tumor cells. From such autologous responder cell populations, it is possible to derive stable anti-tumor CTL clones.

Several antigens recognized by such anti-tumor CTL clones have been identified by an approach based on gene transfection and recognition of the transfectants by the CTLs. So far, two main categories of antigens have been found on human melanomas. The first group contains antigens encoded by genes, such as *MAGE1, MAGE3, BAGE*, and *GAGE*, that are not expressed in normal tissues except for testis but are expressed in a significant proportion of tumors of various histological types (1–6). The second group contains differentiation antigens encoded by genes that are expressed only in melanocytes and melanomas, such as tyrosinase, Melan-A^{MART-1}, $gp100^{Pmel17}$, and gp75 (7–14). A third category of antigens could also be expected to be present on human tumors; namely, those caused by point mutations. We have observed in mouse tumor systems that point mutations can generate potent antigens recognized by syngeneic T lymphocytes (15–17). Recently, a mouse tumor antigen recognized on Lewis lung carcinoma cells has been shown to result from a mutation in the connexin gene (18).

We have carried out a systematic study of the antigens recognized by autologous CTLs on melanoma LB33-MEL. Two cell lines, LB33-MEL.A and LB33-MEL.B, were derived from metastases that were removed from patient LB33 in 1988 and 1993, respectively (19). A large number of CTL clones directed against LB33-MEL.A were obtained with blood lymphocytes collected from the patient in 1990. By selecting *in vitro* for resistance to lysis by some of these CTL clones, we obtained antigen-loss variants of LB33-MEL.A. These variants were resistant to various subsets of the panel of CTL clones, leading to the definition of five antigens on LB33-MEL.A. Remarkably, we observed that cell line LB33-MEL.B had lost the expression of the HLA class I molecules that presented these five antigens, suggesting that T-cell-mediated immune selection against the tumor cells bearing these antigens had occurred *in vivo*. We report here the identification of one of these antigens, named LB33-B. It is produced as a result of a point mutation.

MATERIALS AND METHODS

Cell Lines. Melanoma cell line LB33-MEL.A was derived from a cutaneous metastasis of patient LB33, and clone LB33-MEL.A-1 was obtained by limiting dilution (19). We have obtained and described (19) a large panel of autologous CTL clones that lysed LB33-MEL.A-1 cells and that were shown to recognize five distinct antigens on the melanoma cells. Clonal subline LB33-MEL.A-1.2, which does not express antigen LB33-B, was selected *in vitro* from LB33-MEL.A-1 cells with anti-B CTL clone LB33-CTL-159/5 (19). Clonal subline LB33-MEL.A-1.1.1, which does not express antigens LB33-A, -B, and -D, was selected from LB33-MEL.A-1 cells with anti-A CTL clone LB33-CTL-159/3 and anti-B CTL clone LB33-CTL-159/5. This antigen-loss variant proved to have lost the expression of a complete HLA haplotype, HLA-A28, B44, and Cw7 (19). All the LB33-MEL clonal cell lines were cultured in Iscove's medium (GIBCO) containing 10% (vol/vol) fetal calf serum (GIBCO), supplemented with L-arginine (116 mg/ml), L-asparagine (36 mg/ml), and L-glutamine (216 mg/ml). Human choriocarcinoma cell line JAR, which does not express major histocompatibility complex class I molecules (20), was obtained from the American Type Culture Collection. The class I-negative human B-cell line C1R (21), transfected with an *HLA-B*4402* cDNA (22), was provided by K. Fleischhauer (Istituto Scientifico H.S. Rafaele, Milan).

Lysis Test and CTL Stimulation Assay. Lytic activity of CTLs was tested in a 4-h chromium release assay. COS-7 transfectants were tested for their ability to stimulate the production of tumor necrosis factor by the CTL as described (23). Briefly, 3000 CTLs were added to microwells containing the transfected cells. After 24 h, supernatants were collected and the tumor necrosis factor content of these supernatants was determined by testing their cytotoxic effect on WEHI-164c13 cells (24) in a colorimetric assay (23).

Construction of the cDNA Library and Transfection of COS-7 Cells. The cDNA library was constructed basically as described (9). Briefly, poly(A)$^+$ RNA was isolated from LB33-MEL.A-1 cells with the mRNA extraction kit FastTrack (Invitrogen). It was converted to cDNA by using an oligo(dT) primer containing a *Not* I restriction site at its 5′ end. The cDNA was ligated to *Bst*XI adaptors, digested with *Not* I, and inserted between the *Bst*XI and *Not* I sites of expression vector pcDNAI/Amp (Invitrogen), as described in the SuperScript choice system kit (GIBCO/BRL). Recombinant plasmids

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Abbreviations: CTL, cytotoxic T lymphocyte; EBV, Epstein–Barr virus.

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were transfected by electroporation in *Escherichia coli* DH5α. The library was divided into 750 pools of ≈100 cDNA clones. Each pool was amplified to saturation and plasmid DNA was extracted. COS-7 cells (10^4 cells per well) were cotransfected by using the DEAE-dextran/chloroquine method (9, 25), with 100 ng of plasmid pcDNAI/Amp containing a *HLA-B*4402* cDNA (19) and ≈100 ng of plasmid DNA of a pool of the cDNA library. Transfected COS-7 cells were tested in a CTL stimulation assay after 48 h.

Transfection of JAR Cells. The cells were transfected by the calcium phosphate precipitation method, as described (23). Briefly, 10^6 cells were seeded in 20 ml of medium. Twenty-four hours later, they were cotransfected with 80 μg of plasmid pcDNAI/Amp containing cDNA 350/2 and with 8 μg of plasmid pcDNA3 (Invitrogen), which carries a neomycin-resistance gene and into which a cDNA encoding HLA-B*4402 was cloned. After 3 days, the transfectants were selected with the neomycin analog G418 (0.4 mg/ml) (GIBCO), and these neomyocin-resistant cells were used as targets in a lysis assay.

Production of Truncated Melanoma Ubiquitous Mutated 1 (MUM-1) cDNA. Plasmid pcDNAI/Amp containing cDNA clone 350/2 inserted at the *Bst*XI and *Not* I sites was digested with *Not* I and *Sph* I before treatment with exonuclease III, by using the Erase-a-Base system kit (Promega). After ligation the plasmids were transfected by electroporation in *E. coli* DH5α and selected with ampicillin. Plasmid DNA was extracted from the amplified colonies and cotransfected into COS-7 cells with the *HLA-B44* cDNA.

PCR Assay for the Expression of Gene MUM-1. Total RNA was extracted from samples of tumors and of normal tissues as described (26). To assess the presence of MUM-1 RNA spliced for introns 1 and 2, reverse-transcribed RNA was amplified by PCR using primers OPC181 and OPC204. OPC181 corresponds to nt 137–143 and 704–718 of cDNA clone 350/8 and, thus, straddles exons 1 and 2, and OPC204 corresponds to nt 2134–2157 of MUM-1 gene in exon 3. PCR conditions were 5 min at 94°C followed by 32 cycles of amplification (94°C for 1 min, 64°C for 2 min, and 72°C for 3 min). The presence of an amplified product of 155 bp, corresponding to RNA spliced for introns 1 and 2, was assessed visually on agarose gels stained with ethidium bromide. For incompletely spliced MUM-1 RNA containing intron 2, amplifications were carried out with primer OPC181 and with primer OPC172 that corresponds to nt 1271–1293 in intron 2. PCR conditions were similar but for an annealing temperature of 65°C. The amplified product was 592 bp long. Both spliced and unspliced MUM-1 RNAs were found to be expressed in all the cell lines and tissue samples tested, including LB33-MEL.A-1 cells, other melanoma cell lines such as SK29-MEL and MZ2-MEL.43, Epstein–Barr virus (EBV)-transformed B cells from patient LB33, and several normal tissues (liver, heart, muscle, colon, or bone marrow).

Sequence of the Normal and Mutated MUM-1 Alleles. DNA was extracted from LB33-MEL.A-1 cells and from blood mononuclear cells of patient LB33. The extracted DNA was amplified by PCR by using primers corresponding to nt 638–661 and 932–958 of cDNA clone 350/2, respectively. For each DNA sample, the products of six amplifications were pooled to minimize the representation of sequences containing PCR-induced errors, purified through a Quickspin column (Qiagen, Chatsworth, CA), and ligated into plasmid pCR-Script [PCR-Script SK(+) cloning kit; Stratagene]. Ligated products were transfected by electroporation into *E. coli* JM101. Plasmid DNA was extracted from eight colonies obtained from each ligation and sequenced.

DNA Sequence Analysis. DNA sequencing was performed with the ΔTAq cycle sequencing kit (United States Biochemical). Computer search for sequence homology was done with GenBank release 85 and the FASTA program (27). GenBank accession numbers of cDNA clones 350/2, 475/1, and 122/3 are U20908, U20897, and U20896, respectively.

PCR Assay for the Presence of the Mutated MUM-1 Allele. We used the amplification refractory mutation system PCR methodology that relies on the perfect nucleotide match needed at the 3′ end of primers to ensure specificity of DNA amplifications (28). DNA extracted from various cells as indicated in Fig. 4 was amplified with either of two forward primers corresponding to nt 773–795 of cDNA clone 350/2 with guanine at the 3′ end or to nt 772–795 with thymine at the 3′ end that ensured selective amplifications of either the normal or the mutated MUM-1 allele, respectively. The reverse primer corresponded to nt 1272–1293. PCR conditions included denaturation at 94°C for 5 min followed by 38 cycles of amplification under stringent conditions of annealing (94°C for 1 min, 62°C for 2 min, and 72°C for 3 min).

Isolation of Other MUM-1 cDNA Clones. cDNA clone 350/2 was used to screen the cDNA library derived from LB33-MEL.A-1 cells. Five cDNA clones were obtained, including 122/3 and 122/1, which extended the 3′ end of 350/2. cDNA 122/1 was used as a probe in another screening of the same library, and we obtained three other cDNA clones, including 14/9, which extended the 3′ end of 122/1. By using cDNA 14/9 as a probe, we obtained five other cDNA clones, including 17/12. cDNA 17/12 proved to lack intronic sequences that were present in cDNAs 350/2, 122/3, and 122/1. A 683-bp sequence at the 5′ end of 17/12 was amplified by PCR and used as a probe to screen a cDNA library prepared with mRNA extracted from MZ2-MEL.43 melanoma cells (5). One cDNA clone, 475/1, was found to hybridize with the 683-bp sequence.

Antigenic Peptides and CTL Assay. Peptides were synthesized by conventional solid-phase peptide synthesis, based on the Fmoc strategy. They were purified by reverse-phase HPLC and characterized by mass spectrometry. The binding affinities of the normal and the mutated MUM-1 peptides to HLA-B*4402 were compared by testing their ability to compete for binding with a tyrosinase peptide that was recognized on HLA-B44 by CTL clone MZ2-CTL-22/31 derived from melanoma patient MZ2 (V. Brichard, personal communication). Chromium-labeled C1R-B44 cells were incubated with various concentrations of the two competitor peptides for 15 min before addition of the tyrosinase peptide at 0.4 μM; 15 min later, the anti-tyrosinase CTLs were added at an effector-to-target ratio of 15:1. Lysis was measured 2 h later. Control competitor peptides included EBNA3C-derived peptide EENLLDFVRF presented to anti-EBV CTLs by HLA-B44 (29) and EBNA3A-derived peptide FLRGRAYGL presented by HLA-B8 (30).

RESULTS

A cDNA Clone Coding for Antigen LB33-B. Antigen LB33-B, which is recognized by autologous CTL clone LB33-CTL-159/5 on melanoma cell line LB33-MEL.A, appeared to be presented by class I molecule HLA-B44. A B^- antigen-loss variant that had lost the expression of B44 regained sensitivity to lysis by the anti-B CTLs after transfection with a cDNA coding for *HLA-B*4402*, the B44 allele of patient LB33 (Fig. 1).

To identify the gene coding for antigen LB33-B, a cDNA library prepared with RNA from LB33-MEL.A-1 cells was cloned into expression vector pcDNAI/Amp. This plasmid carries the simian virus 40 origin of replication, resulting in high copy numbers of the transfected plasmids in COS-7 cells, which express the simian virus 40 tumor antigen (25). The cDNA library was divided into 750 pools, each containing 100 bacteria, and DNA of each pool was cotransfected with that of a cloned HLA-B*4402 cDNA into duplicate microcultures of COS-7 cells. After 24 h, the transfected cells were tested for the expression of antigen LB33-B by adding CTL 159/5 to each

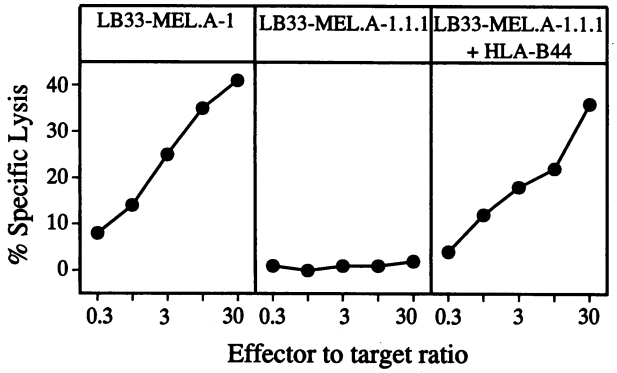

FIG. 1. Sensitivity of ^{51}Cr-labeled target cells to lysis by anti-B CTL clone 159/5. LB33-MEL.A-1 is a clonal subline of the melanoma cell line LB33-MEL.A from patient LB33. LB33-MEL.A-1.1.1 is a variant, selected *in vitro* for resistance to CTL 159/5, that proved to have lost the expression of a complete HLA haplotype, HLA-A28, B44, and Cw7 (19). These cells were transfected with vector pcDNA3 containing an *HLA-B*4402* cDNA obtained from the LB33 melanoma cells.

microculture. The production of tumor necrosis factor by the CTLs was measured after 24 h. One pool of cDNA proved positive. It was subcloned, and cDNA clone 350/2 was found to transfer the expression of antigen LB33-B (Fig. 2*A*). Stable transfectants obtained with a human cell line were also lysed by CTL 159/5, indicating that the expression of the antigen was not dependent on the very high gene copy number present in COS-7 cells (Fig. 2*B*).

The sequence of cDNA 350/2 showed no significant similarity to any sequence recorded in data banks (GenBank/EMBL, July 7, 1995). The corresponding gene was expressed in normal tissues such as liver, colon, muscle, and heart.

Identification of the Antigenic Peptide. To localize the region coding for the antigenic peptide, cDNA 350/2 was digested from the 3′ end with exonuclease III, and truncated cDNA clones and the HLA-B44 cDNA clone were cotransfected into COS-7 cells. The results indicated that the peptide-coding region was located between nt 430 and 860. We searched the three reading frames of this region for sequences

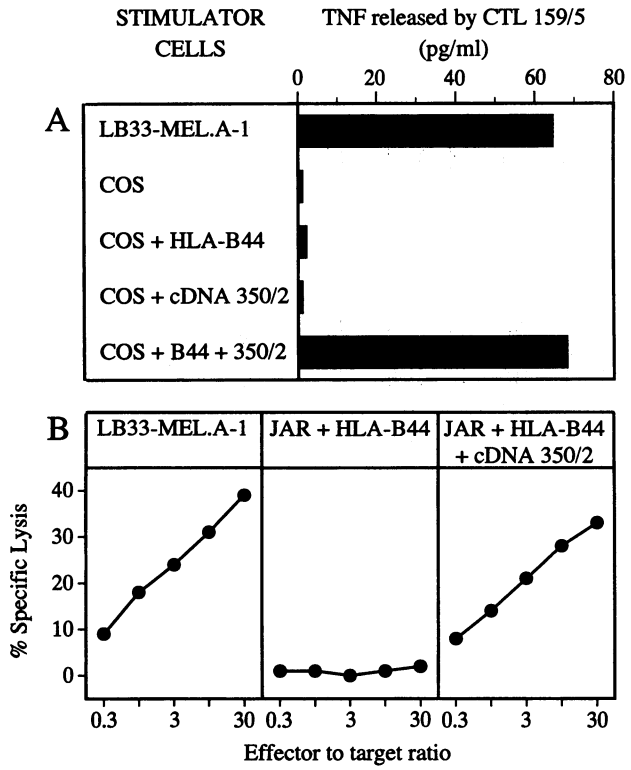

FIG. 2. (*A*) Stimulation of anti-B CTL clone 159/5 by COS-7 cells cotransfected with expression vector pcDNAI/Amp containing cDNA 350/2 and with pcDNA3 containing HLA-B44. Control stimulator cells included LB33-MEL.A-1 cells and COS-7 cells transfected with HLA-B44 or cDNA 350/2 alone. (*B*) Lysis by CTL clone 159/5 of a clone obtained by cotransfecting choriocarcinoma cell line JAR with the pcDNAI/Amp-350/2 and pcDNA3-HLA-B44 constructs. Control targets included LB33-MEL.A-1 and JAR transfected with the HLA-B44 construct alone.

coding for a peptide with Glu in position 2 and Phe in position 9 or 10, the recently identified HLA-B44 binding motif (22). Only one such sequence was found (namely, nonapeptide

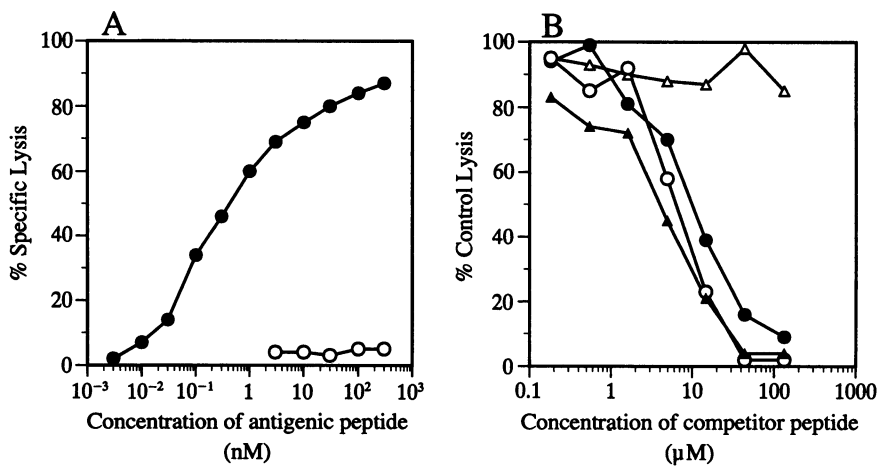

FIG. 3. (*A*) Lytic activity of anti-B CTL clone 159/5 on C1R-B44 cells incubated with synthetic peptides EEKLIVVLF (●) or EEKLSVVLF (○). The first peptide corresponds to the mutated form of gene MUM-1 that is found in the LB33 melanoma cells. The second peptide corresponds to the normal form of the gene. Target cells were incubated with the indicated concentrations of peptides, and CTL clone 159/5 was added at an effector-to-target ratio of 20:1. (*B*) Comparison of the HLA-B44 binding affinity of the normal and mutated MUM-1 peptides. The ability of these peptides to compete with a standard peptide for binding to HLA-B*4402 was measured with CTL clone MZ2-CTL-22/31, which recognizes a tyrosinase peptide bound to the HLA-B44 molecule. Results are presented as percentages of the specific lysis obtained with the tyrosinase–B44 peptide alone, which was 58%. In the absence of the tyrosinase–B44 peptide, the lysis of C1R-B44 cells was 1%. Competitor peptides included the mutated and normal MUM-1 peptides EEKLIVVLF (●) and EEKLSVVLF (○), respectively. Positive and negative controls were EBNA3C-derived peptide EENLLDFVRF (▲), which is recognized by anti-EBV CTL on HLA-B44, and EBNA3A-derived peptide FLRGRAYGL (△), which is recognized by anti-EBV CTL on HLA-B8.

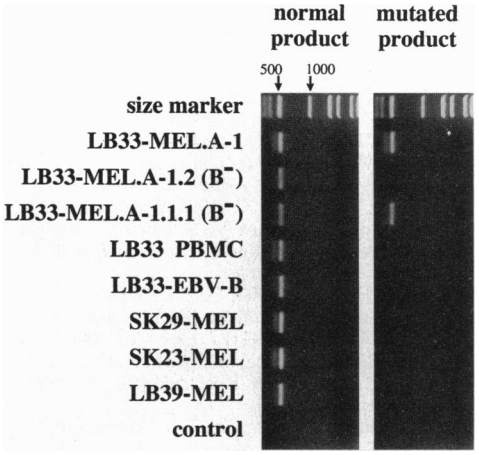

FIG. 4. Selective PCR amplification of the normal or the mutated form of gene MUM-1, with DNA extracted from the LB33 melanoma cell line, antigen-loss variants LB33-MEL.A-1.2 (HLA-B44$^+$) and -1.1.1 (HLA-B44$^-$) [which were selected for resistance to anti-B CTL 159/5 (19)], blood mononuclear cells and EBV-transformed B cells from patient LB33, and melanoma cell lines of patients SK29, SK23, and LB39. Similar results were obtained with the DNA from melanoma lines of 12 additional patients. Our interpretation is that, contrary to LB33-MEL.A-1.1.1, which has lost one HLA haplotype (19), the B$^-$ variant LB33-MEL.A-1.2 probably carries a deletion involving the mutated allele of gene MUM-1.

EEKLIVVLF), encoded by nt 782–808. This peptide was synthesized and it very efficiently sensitized HLA-B44$^+$ cells to lysis by the anti-LB33 CTLs, with a half-maximal effect at 0.4 nM (Fig. 3*A*).

A Mutation in the Sequence Coding for the Antigen. Because the gene coding for antigen LB33-B appeared to be expressed ubiquitously, we considered the possibility that the tumor might carry a point mutation in this gene. To compare the sequence of the gene in the tumor cells and in the normal cells of patient LB33, a 300-bp fragment containing the sequence coding for the antigenic peptide was amplified by PCR. The products of PCRs performed on DNA of LB33-MEL.A-1 cells and on DNA of mononuclear cells from blood were cloned and sequenced. The sequences of three out of eight clones derived from the DNA of LB33-MEL.A-1 cells were identical to that of cDNA 350/2, whereas the sequences of the five others differed by 1 nt: there was a guanine in place of the thymine corresponding to nt 795 of cDNA 350/2. The sequences of the eight clones derived from the DNA of the normal blood cells also had a guanine in that position. Thus one copy of the gene is mutated in the melanoma cells of patient LB33, and this point mutation replaces a serine with an isoleucine at position 5 of the antigenic peptide. A competition assay indicated that the normal and the mutated peptides bind with a similar affinity to the HLA-B44 molecule (Fig. 3*B*), but the peptide encoded by the normal sequence was not recognized by the anti-B CTLs (Fig. 3*A*). This suggests that the isoleucine generated by the point mutation is an essential part of the epitope recognized by anti-tumor CTL 159/5.

We propose the provisional name MUM-1 (melanoma ubiquitous mutated) for the gene encoding antigen LB33-B. To test cells for the presence of the normal or the mutated MUM-1 sequence, we used PCR primers with 3′-end nucleotides corresponding to either the normal or the mutated nucleotide. The normal sequence was found in the DNA of all the normal and tumor cells that were tested, including LB33 melanoma cells, 15 melanoma lines of other patients, blood mononuclear cells, and EBV-transformed B cells from patient LB33 (Fig. 4). The mutated sequence was found only in the two melanoma cell lines of patient LB33. It was also found in a tumor sample from this patient, indicating that the mutation did not occur *in vitro*.

Part of the Antigenic Peptide Is Encoded by an Intronic Sequence. Even though cDNA 350/2 was 1900 bp long, it contained only very small open reading frames. Several cDNA clones that hybridized with cDNA 350/2 were identified in cDNA libraries prepared with RNA of melanoma cell lines LB33-MEL.A-1 and MZ2-MEL.43. One of these cDNAs, named 475/1, consisted entirely of an open reading frame of 1300 nt. It was colinear with two separate regions of cDNA 350/2, suggesting that these two regions were exons and that cDNA 350/2 was incompletely spliced. Alignment with other cDNA clones that overlapped with the 3′ end of cDNA 350/2 led to the partial exon–intron structure shown in Fig. 5. It appears to represent the 5′ end of a gene that extends for several additional kilobases at the 3′ end. The two introns delineated in Fig. 5 have consensus acceptor and donor splice sites. The size of each intron was confirmed with PCR products obtained by amplifying DNA with primers located in the adjacent exons. These results leave little doubt regarding the intronic nature of these two sequences.

Remarkably, the sequence encoding the antigenic peptide straddles the junction between the end of exon 2 and the following intron and the point mutation that generates the antigen is located in position 6 of the intron. It is followed immediately by a stop codon, suggesting that the peptide derives from the C terminus of the translation product of an incompletely spliced mRNA.

Reverse transcription and PCR amplification revealed the presence of MUM-1 RNA spliced for introns 1 and 2 in all normal and tumor tissue samples tested. The MUM-1 RNA species containing intron 2 was also found to be ubiquitous. These results suggest that the lack of splicing of intron 2 is independent of the point mutation and that the specificity of the antigen for tumor LB33-MEL is caused by the mutation and not by a splicing difference.

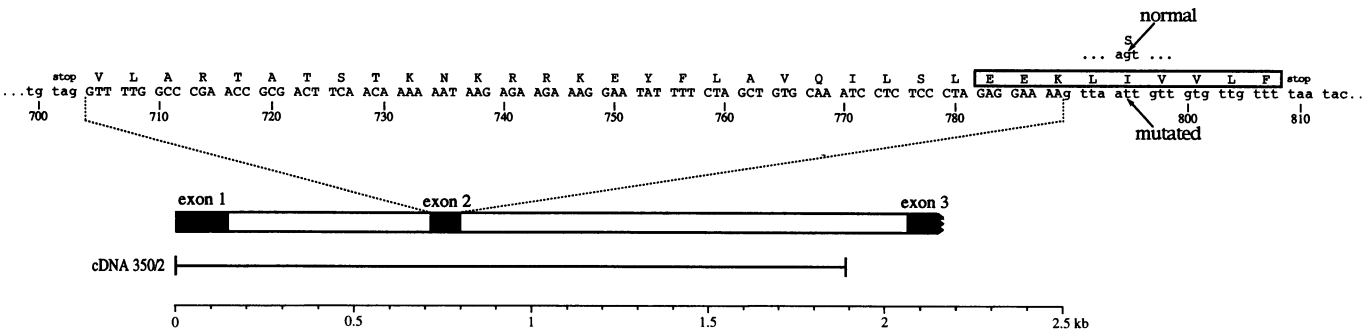

FIG. 5. Putative structure of the 5′ end of gene MUM-1. Exons and introns are represented as solid and open boxes, respectively. The sequence of exon 2, of the initial part of intron 2, and of the antigenic peptide are shown. The numbering of the sequence is relative to the 5′ end of cDNA clone 350/2. Exonic or intronic sequences are in uppercase and lowercase type, respectively. The mutated antigenic peptide recognized on HLA-B44 by CTL clone 159/5 is boxed. The nucleotides and amino acids corresponding to the MUM-1 sequence found in normal cells are indicated on top.

DISCUSSION

Previous experiments with mouse antigens revealed two mechanisms whereby point mutations can generate antigenic peptides (15–17). In some instances, an amino acid change transforms a peptide that is incapable of binding to a class I molecule into a peptide that binds well. In other instances, the peptide encoded by the normal sequence already binds, but because the gene is expressed in normal tissues, the T lymphocytes that recognize the antigen are presumably eliminated or anergized and this results in natural tolerance. Here, the amino acid change produces a peptide with a new epitope that can be recognized by a fresh set of T lymphocytes. The tumor antigen encoded by the mutated *MUM-1* gene belongs to the second category.

To our knowledge, this is the first instance where human CTLs obtained by stimulation with autologous tumor cells have been shown to recognize an antigen generated by a point mutation. The possibility that genes such as ras or p53, which are often mutated in human tumors, might produce antigens recognized by human T lymphocytes has been carefully examined (31, 32). Stimulation by cells incubated with mutated ras or p53 peptides produced human $CD8^+$ lymphocytes that responded specifically to the mutated peptides. But no evidence was obtained that these lymphocytes recognize cells expressing the mutated ras or p53 genes.

Some point mutations found in the genome of tumor cells may be irrelevant to the transformation of the tumor. They may arise because carcinogens that induce the mutation responsible for the transformation also produce irrelevant mutations. The loss of enzymes that correct replication errors is frequently observed in tumors, and it should also contribute to this phenomenon (33–35). Because the point mutation that generates an antigen on melanoma LB33-MEL is absent from any of the other tumors tested, it is possible that it represents one of these irrelevant mutations. Antigens such as LB33-B may accordingly be of limited use for cancer immunotherapy, since they can be used only to immunize the patient in whom they have been discovered. One the other hand, such antigens resulting from point mutations ought to be absolutely specific for the tumor, and technical progress may make the identification of such antigens so easy that treatment of patients bearing tumors with such individually specific tumor antigens will become feasible.

Our observation that the translation of some intronic regions can produce antigenic peptides extends the possible range of the surveillance of the integrity of the genome by T lymphocytes.

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