A Human Endogenous Retroviral Sequence Encoding an Antigen Recognized on Melanoma by Cytolytic T Lymphocytes[1]

Francesca Schiavetti,[2] Joëlle Thonnard,[2] Didier Colau, Thierry Boon, and Pierre G. Coulie[3]

Cellular Genetics Unit, Christian de Duve Institute of Cellular Pathology, Université de Louvain, B-1200 Brussels [F. S., J. T., T. B., P. G. C.], and Ludwig Institute for Cancer Research, Brussels Branch, B-1200 Brussels [D.C., T.B.], Belgium

ABSTRACT

We have identified a gene encoding an antigen recognized by cytolytic T lymphocytes on the autologous tumor cells of a melanoma patient, AVL3. The gene shows homologies with members of the HERV-K family of human endogenous retroviruses, and it was provisionally named *HERV-K-MEL*. It contains many mutations that disrupt the open reading frames coding for all of the viral proteins. The *HERV-K-MEL* gene is not expressed in normal tissues with the exception of testis and some skin samples. It is expressed in most samples of cutaneous and ocular melanoma. It is also expressed in a majority of naevi and in a minority of carcinomas and sarcomas. The antigenic peptide, presented by HLA-A2 molecules, is encoded by a very short open reading frame present in the *env* region of a spliced *HERV-K-MEL* transcript. Anti-HERV.A2 CTLp could not be detected in the blood of three individuals without cancer but were present at a frequency of 3×10^{-5} among blood CD8 T cells in patient AVL3 and 6×10^{-7} in another HLA-A2 melanoma patient whose tumor expressed *HERV-K-MEL*. Anti-HERV.A2 CTL clones derived from each patient lysed melanoma cells. Analysis of T-cell receptor β chain sequences indicated that the anti-HERV.A2 CTL population was oligoclonal in patient AVL3 and probably monoclonal in the other patient. These results suggest that *HERV-K-MEL* is a source of antigens that are targeted by CTLs in melanoma patients and could therefore be used for vaccination.

INTRODUCTION

Over the last 10 years, many antigens were identified that are specifically recognized on human tumor cells by cytolytic T lymphocytes (1).[4] This work of antigen identification has been carried out mostly with melanoma, for which a comprehensive description of antigenicity becomes available. Melanoma antigens recognized by CTLs are either present on individual tumors such as the antigens resulting from point mutations or common to many tumors. Some of the common melanoma antigens, encoded by genes such as *tyrosinase*, *Melan-A/MART-1*, *gp100/Pmel17*, or *TRP-2*, are present also on normal melanocytes. Other common tumor antigens appear to be strictly tumor specific such as those encoded by the cancer-germ line genes. These genes are expressed in a variety of tumors but are silent in normal tissues with the exception of male germ-line cells, which do not express *HLA* genes and therefore cannot present antigens to T lymphocytes. About 10 cancer-germ line gene families have been identified, the first of which was the *MAGE* family, comprising >20 genes (2).

Clinical trials involving vaccination with antigenic peptides en-

coded by cancer-germ line genes of the *MAGE* family, notably *MAGE-3*, have been applied to metastatic melanoma patients (3–5). In one study involving a MAGE-3 peptide presented by HLA-A1, significant tumor regressions occurred in 7 of 25 patients who completed the trial, a frequency well above that reported for spontaneous regressions in metastatic melanoma patients (6). These tumor regressions that followed vaccination with a MAGE-3 peptide were mostly observed in patients with a not too advanced form of melanoma. However, in one case, five lung metastases regressed completely after vaccination, leaving the patient (AVL3) with no evidence of tumor over 4 months. It is worth noting that new metastases then appeared progressively at several visceral sites but not in the lungs.

The mechanisms underlying these clinical responses have not yet been clarified. As a first step toward this understanding, we could demonstrate that a CTL response against the MAGE-3.A1 peptide was induced by vaccination in a patient who responded clinically to the vaccine (7). The precise contribution of these CTLs to the tumor regression remains to be defined. They may reach the metastases and kill tumor cells or activate T cells that recognize other tumor-specific antigens and could participate in the tumor rejection process. We therefore tried to find, in patients who responded clinically to MAGE vaccination, CTLs directed against tumor antigens absent from the vaccine. We report here our first result of such an analysis.

MATERIALS AND METHODS

Derivation and Culture of CTL Clone 13. Blood mononuclear cells (3×10^6 cells) collected from patient AVL3 in March 1997, after vaccination with MAGE peptides (3), were stimulated with irradiated (100 Gy) AVL3-MEL cells (4×10^4 cells) in 2 ml of Iscove's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% HS[5], L-arginine (116 mg/L), L-asparagine (36 mg/L), L-glutamine (216 mg/L), and 5×10^{-5} M 2-mercaptoethanol (complete medium). On day 2, IL-6 (1000 units/ml) and IL-12 (5 ng/ml) were added. On day 8, the responding lymphocytes were restimulated with irradiated AVL3-MEL cells incubated for 2 days in medium containing IFN-γ (50 units/ml; Boehringer-Mannheim, Mannheim, Germany) in complete medium supplemented with IL-2 (5 units/ml), IL-6, and IL-12. On day 10, the concentration of IL-2 was raised to 25 units/ml. On day 14, the lymphocytes were restimulated as before but with IL-2 only. On day 23, they were cloned by limiting dilution in microwells containing irradiated IFN-γ-treated AVL3-MEL cells (5000/well) and irradiated LG2-EBV cells (10^5/well) in complete medium with IL-2 (50 units/ml). Several CTL clones were obtained. One of them was named AVL3-CTL-413/13, hereafter referred to as CTL 13.

Construction and Screening of the cDNA Library. Polyadenylated RNA from AVL3-MEL cells was converted to cDNA with the Superscript Choice System (Life Technologies, Inc.) using an oligodeoxythymidylic acid primer containing a *Not*I site at its 5′ end. The cDNA was ligated to *Hin*dIII-*Eco*RI adaptors (Stratagene), phosphorylated, digested with *Not*I, and ligated to plasmid pCEP4 (Invitrogen) digested with *Hin*dIII and *Not*I, and dephosphorylated. Recombinant plasmids were electroporated into *Escherichia coli* DH5α. The library was divided into pools of ~100 cDNA clones. 293-EBNA cells were transfected with 100 ng of plasmid DNA from a pool of the cDNA

---

library, 50 ng of expression vector pcDNA1/Amp containing a genomic HLA-A*0201 sequence, and 1.5 μl of LipofectAMINE (Life Technologies, Inc.). After 24 h, CTL 13 (3000 cells/well) was added. An additional 24 h later, half of the medium was collected, and its TNF content measured by testing the cytotoxic effect on WEHI-164c13 cells (8).

Localization of the Antigenic Peptide. Four fragments of cDNA were amplified by PCR, using forward primer OPC598 (5′-GTACCAGCTGCTAG-CAAG), located in plasmid pCEP4 immediately upstream of the cloned cDNA and reverse primers OPC599, OPC600, OPC601, and OPC591 shown on Fig. 2. The amplified products were purified on a Chroma Spin 100 column (Clontech) and cloned into plasmid pCR3 using the Eukaryotic TA Cloning Kit (Invitrogen). The recombinant plasmids were analyzed by PCR to determine the orientation of the inserted cDNA. The first three constructs were cotransfected into 293-EBNA cells with the HLA-A2 cDNA clone. The largest construct was used to obtain a stable transfectant from melanoma cells NA8-MEL.

Quantitative Measurement of *HERV-K-MEL* Expression. For each cDNA sample, *HERV-K-MEL* and *actin* copy numbers were measured with Real Time Quantitative PCR (TaqMan), using the qPCR Core-No ROX kit (Eurogentec, Liège, Belgium) on the ABI Prism 7700 Sequence Detection System (Applied Biosystems). *HERV-K-MEL* primers and fluorescent probe are shown on Fig. 2. PCR conditions were 95°C at 10 min followed by 45 cycles of 95°C at 15 s and 60°C at 2 min. *Actin* primers were 5′-ATTGC-CGACAGGATGCAGAA and 5′-TCAGCAAGCAGGAGTATGAC, and probe was 5′-(6-Fam)TCAAGATCATTGCTCCTCCTGAGCGC(Tamra). PCR conditions were 95°C at 10 min and 45 cycles of 95°C at 15 s and 60°C at 1 min. *HERV-K-MEL* copy numbers were normalized according to *actin* copy numbers and expressed as percentage of the copy number found in AVL3-MEL cells.

Detection, Cloning, and Analysis of Anti HERV-K-MEL CTLs. PBMC from three hemochromatosis patients and from melanoma patients AVL3 and EB81 were thawed and incubated at 10^7 cells/ml for 30 min at 4°C and 30 min at room temperature in Iscove's medium with 1% HS and peptide MLAVIS-CAV (20 μM). Cells were washed and distributed at $2–3 \times 10^5$ cells/microwell in complete medium with IL-2 (20 units/ml), IL-4 (10 ng/ml), and IL-7 (10 ng/ml). On day 7, half of the medium was replaced by fresh medium with peptide and cytokines as on day 0. On days 13 or 14, the presence of anti-HERV-K-MEL CD8$^+$ lymphocytes in the cultures was assessed with multimers of soluble HLA-A2 molecules loaded with the antigenic peptide (9). Recombinant HLA-A2 molecules were folded *in vitro* with β2-microglobulin and peptides MLAVISCAV (from HERV-K-MEL) or GLCTLVAML (from the BMLF1 protein of EBV), purified by gel filtration, biotinylated, and mixed with Extravidin-PE (Sigma, St. Louis, MO) or streptavidin-allophycocyanin (APC) (Molecular Probes, Eugene, OR). Lymphocytes were washed, incubated for 30 min at 37°C in PBS + 1% HS with titrated amounts of HERV-PE and BMLF1-APC multimers. Anti-CD8 antibodies coupled to FITC (SK1; Becton Dickinson) were added for 30 min at 37°C. The cells were washed, fixed with 0.5% paraformaldehyde in PBS, and analyzed by flow cytometry. Cells stained by multimer were sorted at 1 cell/well and stimulated with irradiated allogeneic PBMC (8×10^4/well), irradiated allogeneic HLA-A2 EBV-B cells (2×10^4/well) incubated with the MLAVISCAV peptide (20 μM) and washed, in medium with IL-2, IL-4, and IL-7 as above. The CTL clones were restimulated weekly by the addition of feeder cells and peptide-pulsed EBV-B cells.

RESULTS

Identification of a cDNA Encoding a New Tumor-specific Antigen Recognized by CTLs. Blood mononuclear cells collected from melanoma patient AVL3 were stimulated with irradiated autologous AVL3-MEL cells. Responder lymphocytes were restimulated on days 7 and 14 by the addition of tumor cells and IL-2 and cloned by limiting dilution on day 21. CTL clone 13 was obtained, which lysed AVL3-MEL cells but did not lyse autologous B cells transformed with EBV or the natural killer target cells K562 (Fig. 1A). Recognition of AVL3-MEL was inhibited by an anti-HLA-A2 monoclonal antibody. Cells of some allogeneic HLA-A2 melanoma lines were also lysed by
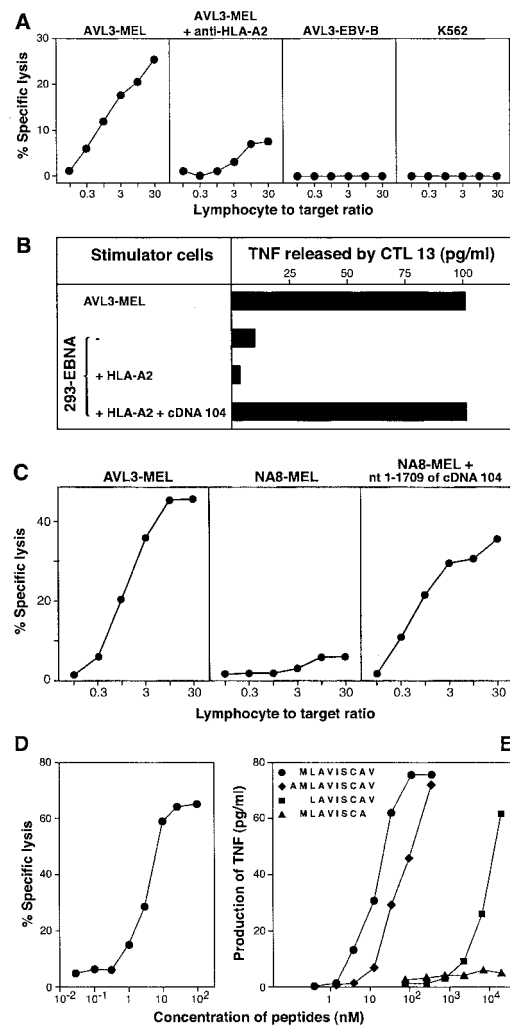

Fig. 1. *A*, sensitivity of ^{51}Cr-labeled target cells to lysis by CTL clone 13. AVL3-MEL is a melanoma cell line derived from a metastasis resected from patient AVL3. Anti-HLA-A2 monoclonal antibody BB7.2 (IgG2b) was used to inhibit lysis of AVL3-MEL cells by the CTL, by adding a 1:30 dilution of ascitic fluid from mice inoculated with the hybridoma cells. No inhibition was observed with an isotype-matched control antibody (anti-CD11b monoclonal antibody OKM1; data not shown). *B*, identification of a cDNA clone encoding the antigen recognized by CTL 13. CTL 13 was stimulated by AVL3-MEL cells or by 293-EBNA cells cotransfected with vectors pCEP4 containing cDNA clone 104 and pcDNA1/Amp containing an HLA-A*0201 sequence. The concentration of TNF released in the medium was measured using the TNF-sensitive WEHI-164c13 cells. *C*, lysis by CTL 13 of HLA-A2 melanoma cells NA8-MEL expressing a construct containing nucleotides 1-1709 of cDNA clone 104. Clonal transfectants were isolated from the G418-resistant population, and expression of the cloned cDNA was assessed by RT-PCR. *D*, lysis by CTL 13 of AVL3-EBV-B cells incubated with antigenic peptide MLAVIS-CAV. ^{51}Cr-labeled EBV-B cells from patient AVL3 were incubated for 30 min at 20°C with various concentrations of peptide before addition of CTL 13 at an E:T ratio of 5:1. Chromium release was measured after 4 h. *E*, production of TNF by CTL13 stimulated with peptides. LB37-NSCLC-5 is a clonal cell line derived from a non-small cell lung carcinoma. The cells carry HLA-A2 molecules and are not recognized by CTL clone 13. Tumor cells (20,000/well) were incubated with various concentrations of the indicated peptides and with CTL 13 (3000 cells/well) over 24 h. The concentration of TNF released in the medium was then measured.

CTL 13, suggesting that the CTL recognized an antigen that was shared between different tumors.

A cDNA library prepared with polyadenylated RNA from AVL3-MEL cells was cloned into expression plasmid pCEP4 and divided into 500 pools of ~100 recombinant clones. DNA prepared from each pool was cotransfected with an HLA-A2 construct into 293-EBNA cells. CTL 13 was added to the transfectants after 24 h. After another 24 h, the supernatant was collected and its TNF content was measured with the TNF-sensitive WEHI-164c13 cells. Four pools of cDNA
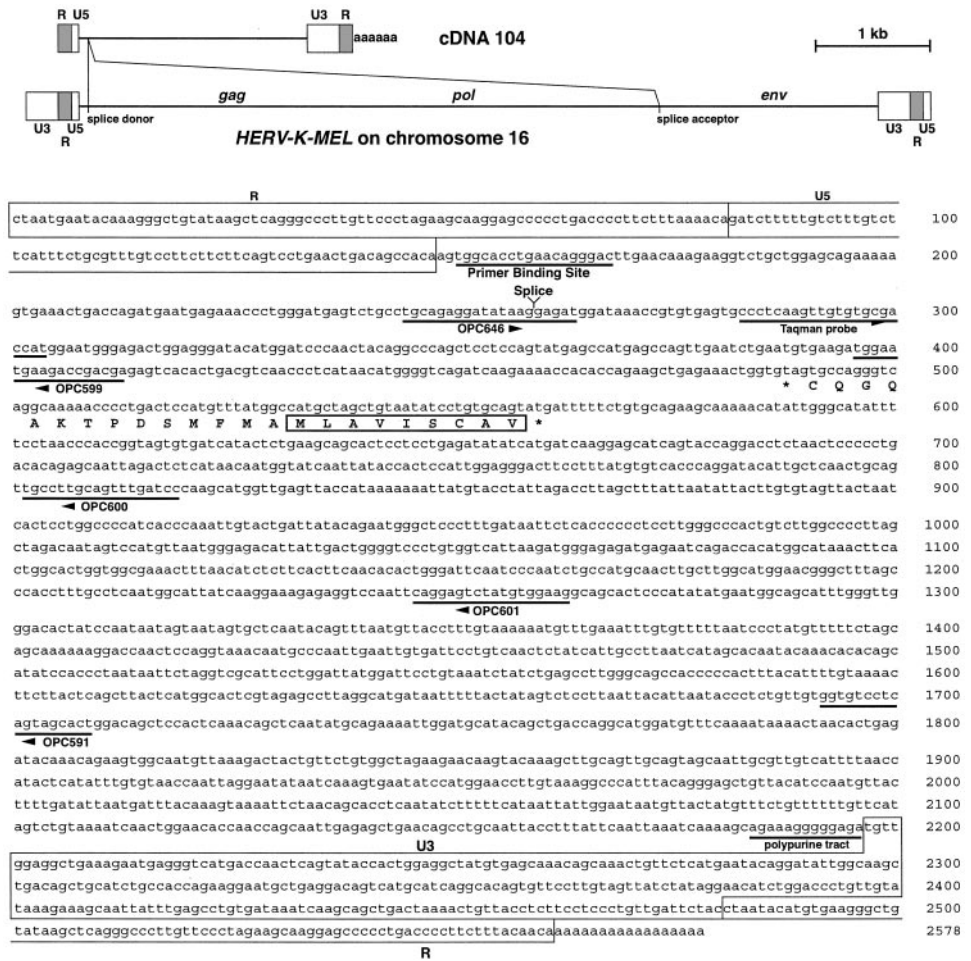
Fig. 2. Structure of the *HERV-K-MEL* DNA and spliced transcript and sequence of cDNA 104. Primers are indicated by *horizontal arrows*: OPC646, OPC600, and Taqman probe were used to analyze the expression of the HERV-K-MEL env transcript. OPC599, OPC600, OPC601, and OPC591 were used to localize the antigenic peptide, as detailed in "Materials and Methods." The LTR U3, R, and U5 regions are *boxed*. The primer binding site is complementary to a lysine tRNA. The antigenic peptide is *boxed*. The cDNA 104 sequence is present in databanks: AC018558.5, from 132664 to 132992 and from 137897 to 140200 (99% identical); and AC092357.2, from 82409 to 82667 and from 87643 to 89946 (100% identical).

proved positive. One of them was subcloned, and cDNA clone 104 was obtained (Fig. 1*B*).

The sequence of this cDNA clone was 2578 bp long. A PCR product corresponding to nucleotides 1–1709 of the cDNA was cloned into expression plasmid pCR3.1, and the construct was transfected into the HLA-A2 melanoma cells NA8-MEL, which were not recognized by CTL 13. A clonal transfectant that expressed the cDNA was lysed by the CTL (Fig. 1*C*), indicating that the antigen was encoded within the first 1700 nucleotides of the cDNA and that its expression did not depend on the high plasmid copy number obtained in 293-EBNA cells with plasmid pCEP4.

Characterization of cDNA Clone 104. The 5′ and 3′ ends of the cDNA contain sequences of 81 nucleotides that are almost identical and highly homologous to the R sequence of the LTRs of mouse mammary tumor retroviruses or HERV sequences (Fig. 2). cDNA 104 also contains other sequences that are typical of a retroviral transcript. A LTR U5 sequence follows the 5′ R sequence, and a LTR U3 sequence precedes the 3′ R sequence. An 18 nucleotide sequence that is perfectly complementary to the 3′ terminal nucleotides of the lysine (K) tRNA (UUU) follows the U5 sequence. Binding of the tRNA to this site primes for synthesis of the first strand of DNA by the reverse transcriptase, the first step of retroviral DNA synthesis. A polypurine tract, which can prime for the synthesis of the second strand of retroviral DNA, is found immediately upstream to the 3′ LTR. These features confirm that cDNA 104 is a retroviral transcript. The presence of a primer binding site for a lysine (K) tRNA suggests that the sequence belongs to the HERV-K family (10, 11). The cDNA does not contain an ORF that codes for a complete retroviral protein.

However, a reading frame spanning nucleotides 1372–2034, although it contains stop codons, corresponds to a protein sequence that is 78% identical to a part of the envelope protein of HML-6, a previously identified HERV-K sequence (Ref. 11; GenBank accession no. U60269).

Databanks contain two descriptions of a sequence in chromosome 16 that corresponds to the provirus of cDNA 104 (Fig. 2): one is 100% identical (AC092357.2), the other is 99% identical (AC018558.5). It is highly defective in the *gag*, *pol*, and *env* genes, because of the presence of many mutations. A comparison of this sequence with cDNA 104 confirms that the latter is a spliced *env* transcript of this proviral gene. We named the proviral sequence *HERV-K-MEL*.

Identification of the Antigenic Peptide. cDNA fragments corresponding to the first 413, 819, or 1260 nucleotides of cDNA 104 were amplified by PCR with primers shown on Fig. 2. The constructs were cotransfected into 293-EBNA cells with an HLA-A2 cDNA, and expression of the antigen by the transfectants was analyzed by adding CTL 13 and measuring the production of TNF. The results indicated that at least a part of the peptide-coding region corresponded to nucleotides 413–819 of the cDNA. This region contains small ORFs of 100 bp or less. The putative translation products were searched for a peptide containing the HLA-A2 binding motif. Five peptides of nine amino acids were predicted to bind with a significant affinity to HLA-A2 molecules. One of them, MLAVISCAV, sensitized AVL3-EBV-B cells to lysis by CTL 13, with half-maximal effect at 5 nM (Fig. 1*D*). It also stimulated the production of TNF by the CTL (Fig. 1*E*). Shorter peptides, without the M residue at position 1 or the V residue at position 9, were not recognized at all or recognized much
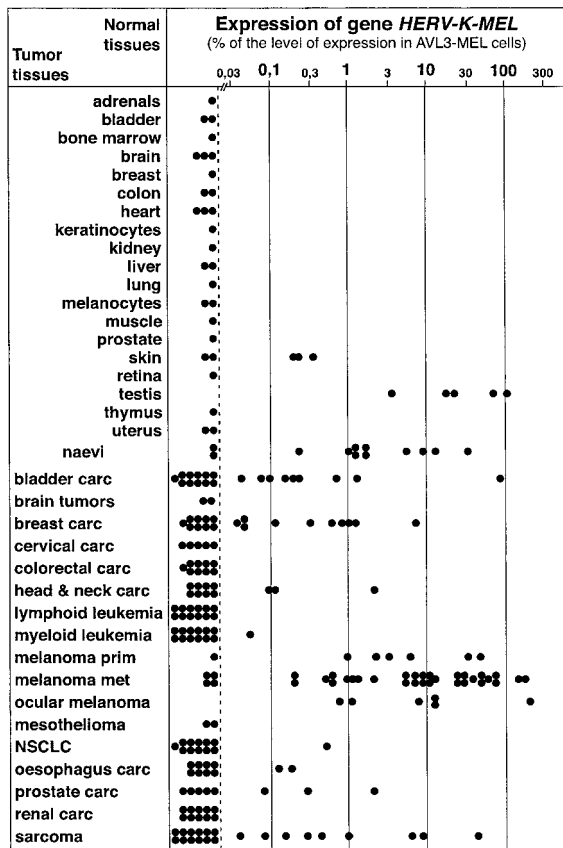
Expression of gene *HERV-K-MEL*
(% of the level of expression in AVL3-MEL cells)

Scale: 0.03 0.1 0.3 1 3 10 30 100 300

Normal tissues:
- adrenals
- bladder
- bone marrow
- brain
- breast
- colon
- heart
- keratinocytes
- kidney
- liver
- lung
- melanocytes
- muscle
- prostate
- skin
- retina
- testis
- thymus
- uterus
- naevi

Tumor tissues:
- bladder carc
- brain tumors
- breast carc
- cervical carc
- colorectal carc
- head & neck carc
- lymphoid leukemia
- myeloid leukemia
- melanoma prim
- melanoma met
- ocular melanoma
- mesothelioma
- NSCLC
- oesophagus carc
- prostate carc
- renal carc
- sarcoma

Fig. 3. Expression of the *HERV-K-MEL* gene analyzed on normal and tumor tissues. Expression of the *β-actin* gene and of the spliced env *HERV-K-MEL* transcript was assessed by reverse transcription of total RNA and quantitative real-time PCR amplification with primers and probe shown in Fig. 2. The level of expression of the *HERV-K-MEL* gene was corrected for the integrity of the RNA by taking into account the expression level of the *β-actin* gene. Each point represents the level of *HERV-K-MEL* expression measured in a sample of normal or tumor tissue. The results are expressed relative to the level of expression of the *HERV-K-MEL* gene measured in the AVL3-MEL cells. — — —, the limit of detection.

less efficiently (Fig. 1*E*). A longer peptide, with an additional alanine residue at the NH_2 terminus, was also recognized less efficiently. These results suggested that peptide MLAVISCAV is the antigenic peptide recognized by CTL clone 13.

This peptide results from the translation of a short ORF, the maximal length which (69 nucleotides) is shown in Fig. 2. It contains three AUG codons, and none of the six non-AUG codons that can serve as alternative initiators of translation (12), suggesting that a classical AUG codon is used. The first AUG does not fit the Kozak consensus motif observed for initiation of translation in vertebrates (13). The second AUG is followed by a G in position 4, the second most conserved position of the motif. The third AUG is preceded by a purine in position-3, the most conserved position of the motif and could therefore be the main initiator codon. Remarkably, this AUG codes for the NH_2-terminal residue of the antigenic peptide. In addition, the peptide coding sequence is immediately followed by a stop codon. These results suggest that the antigenic peptide corresponds to a very short translation product.

Expression of the *HERV-K-MEL* Gene. Expression of the spliced *HERV-K-MEL* transcript corresponding to cDNA 104 was tested on reverse-transcribed RNA with real-time quantitative PCR using primers shown in Fig. 2. The transcript was absent from most normal tissues (Fig. 3). It was expressed at a high level in testis and at a 300-fold lower level in 3 of 5 skin samples. EBV-transformed B cells and CTL clones did not express the *HERV-K-MEL* gene (data not shown). The gene was expressed in most melanoma samples, including primary and metastatic cutaneous melanomas, and primary ocular melanomas. Interestingly, it was also expressed in a majority of naevi. Most of the other types of tumors that were tested did not express this *HERV-K-MEL* transcript at a level that exceeded 1% of that found in the AVL3-MEL cells, with a few exceptions, including sarcoma and carcinoma samples.

Analysis of Anti-HERV-K-MEL CTL Responses. To study the frequency and diversity of anti-HERV.A2 (MLAVISCAV + HLA-A2) T lymphocytes in patient AVL3, we used A2/HERV multimers: recombinant HLA-A2 molecules folded with the antigenic peptide, biotinylated, and multimerized with avidin conjugated to phycoerythrin (9). Blood mononuclear cells (PBMC) collected from the patient in 1997, after vaccination with peptides MAGE-1.A1 and MAGE-3.A1 (Fig. 4*A*), were divided in 44 groups of 2×10^5 cells and stimulated on day 0 and day 7 by the addition of peptide MLAVISCAV and growth factors (IL-2, IL-4, and IL-7). On day 14, cells of all the cultures were incubated with the A2/HERV multimers and anti-CD8 antibodies and analyzed by flow cytometry. Cells labeled with the A2/HERV multimer were clearly identified in the $CD8^+$ fraction of 22 of 44 cultures (Fig. 4*B*). Considering that 20,000 $CD8^+$ lymphocytes were initially present in each group of PBMC, these results indicate a precursor frequency of anti-HERV.A2 T cells of 3×10^{-5} among blood $CD8^+$ T cells. Unfortunately, PBMC collected before MAGE vaccination could not be tested.

For each of 6 cultures randomly selected from the 22 that contained anti-HERV.A2 $CD8^+$ cells, cells stained by the A2/HERV multimer were sorted, seeded at 1 cell/well, and restimulated with peptide-pulsed irradiated HLA-A2 EBV-B cells, irradiated feeder cells, IL-2, and IL-4. Clones were obtained from each culture (Fig. 4*B*), and all of them were stained by the multimer. For one or two clones derived from each of the 6 cultures, cDNA was prepared and used to obtain the sequence coding for the TCRβ chain. Of 11 clones, 5 different TCRβ rearrangements were found (TCR 1–5 on Fig. 4*B*). TCR 2 was expressed by clones derived from 2 independent cultures. TCR 3 was expressed by clones derived from 3 independent cultures and was also expressed by CTL clone 13. Clones expressing TCR 1, 2, and 3 were used in lysis assays: they recognized autologous melanoma cells and HLA-A2 EBV-transformed B cells incubated with peptide MLAVISCAV (Fig. 4*C*).

We applied the same method of stimulation with peptide, detection with multimer, cloning, and TCR analysis to PBMC from another HLA-A2 patient (EB81) whose melanoma cells expressed the *HERV-K-MEL* gene at a high level. Of 192 cultures of 2×10^5 PBMC, 3 contained detectable anti-HERV.A2 lymphocytes. CTL clones derived from each of these 3 populations lysed autologous melanoma cells and expressed the same *TCRβ*-rearranged gene. These results suggested the presence of a monoclonal population of anti-HERV.A2 T cells at a frequency of 6×10^{-7} among CD8 cells in the blood of this patient. In contrast, anti-HERV.A2 lymphocytes could not be detected among 15×10^6 blood CD8 cells obtained from three HLA-A2 individuals without cancer.

DISCUSSION

Endogenous retroviral sequences, remnants of ancient germ-line infections with exogenous retroviruses, are estimated to comprise >1% of the entire human genome (10). Most of these sequences have accumulated point mutations and deletions and are unable to code for infectious virus particles. HERV sequences have been classified according to the tRNAs used to prime reverse transcription. Thus for the HERV-K family, lysine tRNAs can bind to the primer binding site. Homologs of HERV-K are found in Old World monkeys and other
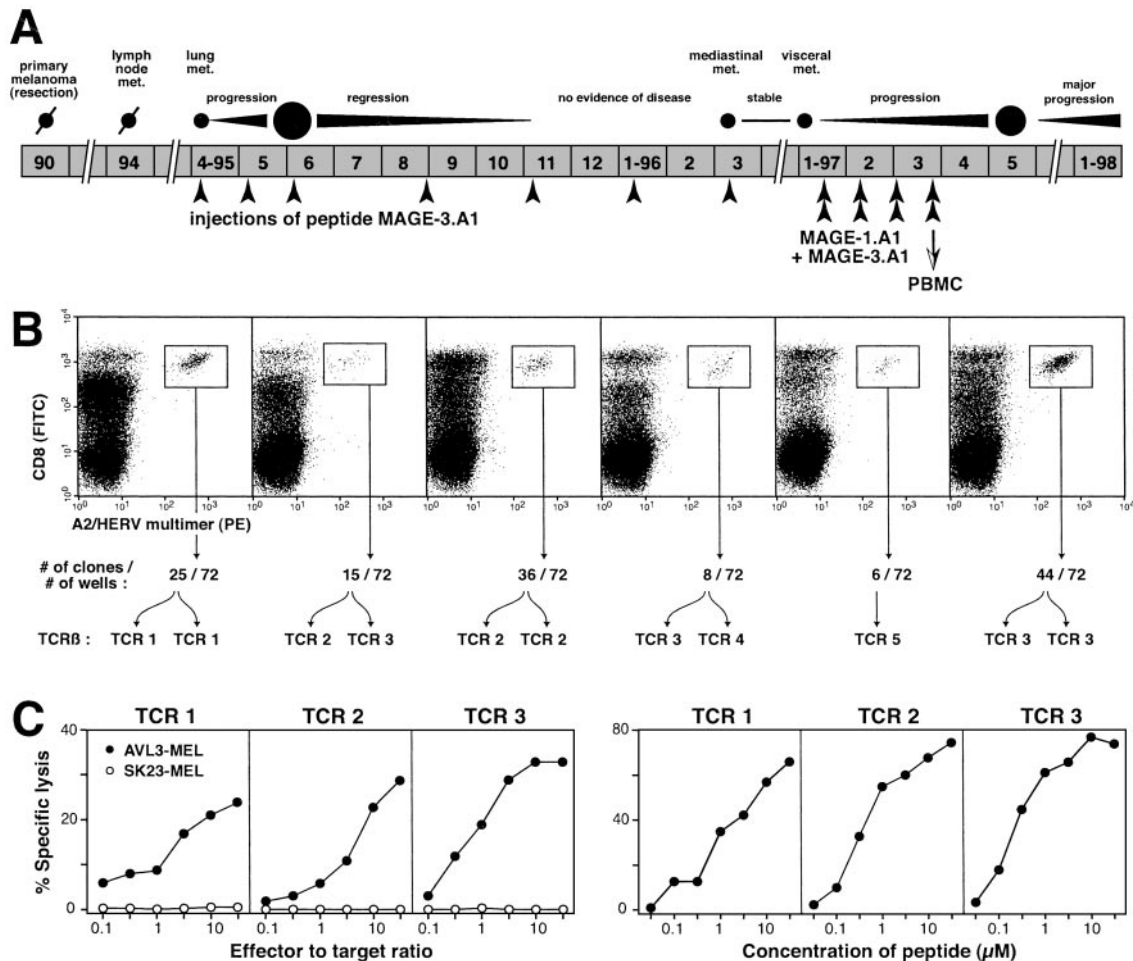
Fig. 4. Anti-HERV.A2 CTL clones derived from the blood of patient AVL3. *A*, clinical evolution of patient AVL3. *B*, derivation and diversity of CTL clones. PBMCs were thawed, incubated with peptide MLAVISCAV, washed, and distributed in microwells in the presence of IL-2, IL-4, and IL-7. On day 7, the cultures were restimulated with peptide and cytokines. On day 14, cells were incubated with the A2/HERV multimer and anti-CD8 antibodies, washed, fixed, and analyzed by flow cytometry. For six cultures, shown on the figure, that contained CD8 cells stained by multimer, positive cells were cloned and stimulated with irradiated peptide-pulsed EBV-B cells, feeder cells, and growth factors. The cloning efficiencies are indicated. Eleven clones that were stained by the A2/HERV multimer were kept for additional experiments. Total RNA was extracted and converted to cDNA. TCR-β sequences were obtained and indicated the presence of five different TCR-β rearrangements. *C*, lytic activity of three representative CTL clones, expressing three different TCR-β chain sequences. Target cells included the allogenic HLA-A2$^+$ melanoma cells SK23-MEL, which do not express the HERV-K-MEL transcript, and the autologous melanoma cells AVL3-MEL (*left panels*) or HLA-A2 EBV-transformed B cells incubated with the indicated concentrations of peptide MLAVISCAV (*right panels*). In the latter assays, the E:T ratio was 10:1.

primates but not in New World monkeys, suggesting that the primary germ-line infection occurred after the separation of these species 30–40 million years ago (14). The family is present in an estimated 30–50 copies/human genome and comprises sequences with large ORFs (11, 15). All these proviruses are defective. The prototype full-length sequence, described in 1986, is defective in the *gag* and *env* genes (16). However, some of the HERV-K sequences come very close to coding for infectious virus (15). One of them was shown to code for noninfectious virus-like particles that are produced constitutively by some human teratocarcinoma cell lines (17).

Several members of the HERV-K family are expressed in cancer tissues and cell lines, notably teratocarcinoma cells and the breast cancer cell line T47D (10), as well as in normal tissues. In a recent study, the expression of HERV-K envelope transcripts was analyzed in breast cancer. Expression was detected in 45% of the tumor samples and not detected in breast tissues that did not contain tumor cells (18). No other detailed analysis of the pattern of expression of HERV-K sequences in normal and tumoral tissues is available. For HERV-K-MEL, we focused on the spliced transcript that corresponds to the cDNA clone found in the AVL3-MEL tumor cells. Its pattern of expression differs from those found for other genes that encode

cancer-specific antigens. The only normal tissue that expresses *HERV-K-MEL* at a high level is testis. This is reminiscent of the expression of cancer-germ line genes such as the *MAGE-A* genes (19). For several of these genes, expression in tumors and in male germ-line cells results from DNA demethylation (20). Interestingly, HERV-K sequences are also expressed in male germ-line cells and in terato-carcinoma cells in which DNA is hypomethylated, and this expression increases after treatment with the demethylating agent 5-azacytidine (21). In urothelial tumors, HERV-K sequences were found to be hypomethylated compared with normal tissues but this did not result in transcription (22). Along the same line, we failed to detect *HERV-K-MEL* transcripts in normal cells that expressed gene *MAGE-1* as a result of a treatment with 5-azacytidine. It is therefore possible that hypomethylation of HERV-K sequences is necessary but not sufficient for their expression. Another result indicating a different regulation of expression of the cancer-germ line and *HERV-K-MEL* genes is that many melanoma samples that do not appear to express any of the cancer-germ line genes express *HERV-K-MEL* at a significant level. An interesting consequence of this observation is that vaccination with HERV-K-MEL antigens could be proposed to some of the patients that are not eligible for vaccination with MAGE antigens.

In contrast to cancer-germ line genes, *HERV-K-MEL* is expressed at a low level in some skin samples and at higher levels in most samples of naevi. Together with the expression in most samples of cutaneous or ocular melanoma, this suggests that *HERV-K-MEL* is expressed in cells of the melanocytic lineage. However, we found no expression in cultured melanocytes. If *HERV-K-MEL* is also silent in melanocytes *in vivo*, its expression could be an early marker of melanocyte transformation.

In mice, endogenous retroviral genes code for antigens recognized by tumor-specific CTLs. The *env* gene of a leukemia provirus is expressed in B16 melanoma cells (23). The corresponding gene product is recognized by syngeneic antibodies and CTLs. An antigen recognized by CTLs on the spontaneous leukemia LEC is encoded by a defective endogenous retroviral sequence of the murine Intracisternal A particle family (24). The antigenic peptide is derived from a 599-amino acid gag protein, and expression of the antigen by the leukemia results from transposition of the Intracisternal A particle sequence. An antigen present on a murine colorectal tumor is encoded by the *env* gene of an endogenous MuLV (25). This gene is silent in normal cells and expressed in several tumor cell lines. Finally, an antigenic peptide derived from the envelope protein of a retrovirus of the AKV (MuLV of the AKR strain) family is targeted by tumor-infiltrating lymphocytes (26). Thus far, human endogenous retroviruses have not been shown to code for antigenic peptides recognized by tumor-specific T lymphocytes.

The 2.5-kb *HERV-K-MEL* sequence described in this report is an expressed pseudogene that does not code for a retroviral envelope protein. Because of the presence of many mutations, one can even not define a main ORF in the *HERV-K-MEL* env transcript. That an antigenic peptide can be encoded by a small ORF is not new. On the basis of observations of antigen expression by cells transfected with promoterless gene fragments, we proposed in 1989 that antigenic peptides could be direct products of the transcription of short genetic regions or of the translation of a small part of a mRNA (27). The first proposal did not receive firm experimental support, whereas the second has been validated: many antigenic peptides are now known to be translated from small alternative ORF. The HERV.A2 peptide even appears to be translated from the smallest possible ORF, not longer than the peptide coding sequence itself. An interesting feature of an antigenic peptide encoded by such a minimal ORF is that it is expected to be presented by HLA class I molecules without any kind of processing by proteasomes. These results strengthen the concept that the fraction of our genome that is under immunosurveillance by T lymphocytes is larger than that encoding proteins.

Anti-HERV.A2 CTLs were detected at frequencies of the order of 10^{-5} and 10^{-6} among blood $CD8^+$ cells of two melanoma patients, whereas none could be found among 10^7 $CD8^+$ cells of normal donors. In addition, analysis of the anti-HERV.A2 CTL clones from the two patients showed that the same TCR-β chain was used by clones derived from different groups of PBMC, suggesting a previous clonal amplification. These results are compatible with an *in vivo* anti-HERV.A2 CTL activation in response to the tumor. They suggest that HERV-K-MEL is a useful source of tumor-specific antigens that could be used for vaccination of most patients with melanoma and a small proportion of patients with carcinoma.

For patient AVL3, our initial aim was to detect tumor-specific T cells that had been amplified after MAGE vaccination. Unfortunately, by the time the work of antigen identification was completed, blood cells collected before vaccination had been used to monitor anti-MAGE T cell responses, precluding us from analyzing the influence of vaccination on the frequency of anti-HERV.A2 CTLs. Our results indicate that tumor-specific CTLs directed against an antigen absent from the vaccine were present in patient AVL3 after vaccination with a MAGE peptide and rejection of lung metastases. Whether or not these CTLs played a role in the tumor regression remains to be analyzed.

REFERENCES

1. Parmiani, G., Anichini, A., and Castelli, C. New tumor restricted melanoma antigens as defined by cytotoxic T-cell responses. Melanoma Res., *7:* S95–S98, 1997.

2. Chomez, P., De Backer, O., Bertrand, M., De Plaen, E., Boon, T., and Lucas, S. An overview of the *MAGE* gene family with the identification of all human members of the family. Cancer Res., *61:* 5544–5551, 2001.

3. Marchand, M., van Baren, N., Weynants, P., Brichard, V., Dréno, B., Tessier, M-H., Rankin, E., Parmiani, G., Arienti, F., Humblet, Y., Bourlond, A., Vanwijck, R., Liénard, D., Beauduin, M., Dietrich, P-Y., Russo, V., Kerger, J., Masucci, G., Jäger, E., De Greve, J., Atzpodien, J., Brasseur, F., Coulie, P. G., van der Bruggen, P., and Boon, T. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene *MAGE-3* and presented by HLA-A1. Int. J. Cancer, *80:* 219–230, 1999.

4. Nestle, F. O., Alijagic, S., Gilliet, M., Sun, Y., Grabbe, S., Dummer, R., Burg, G., and Schadendorf, D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat. Med., *4:* 328–332, 1998.

5. Thurner, B., Haendle, I., Roder, C., Dieckmann, D., Keikavoussi, P., Jonuleit, H., Bender, A., Maczek, C., Schreiner, D., von den Driesch, P., Brocker, E. B., Steinman, R. M., Enk, A., Kampgen, E., and Schuler, G. Vaccination with MAGE-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. J. Exp. Med., *190:* 1669–1678, 1999.

6. Baldo, M., Schiavon, M., Cicogna, P. A., Boccato, P., and Mazzoleni, F. Spontaneous regression of subcutaneous metastasis of cutaneous melanoma. Plast. Reconstr. Surg., *90:* 1073–1076, 1992.

7. Coulie, P. G., Karanikas, V., Colau, D., Lurquin, C., Landry, C., Marchand, M., Dorval, T., Brichard, V., and Boon, T. A monoclonal cytolytic T-lymphocyte response observed in a melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene *MAGE-3*. Proc. Natl. Acad. Sci. USA, *98:* 10290–10295, 2001.

8. Espevik, T., and Nissen-Meyer, J. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J. Immunol. Methods, *95:* 99–105, 1986.

9. Altman, J. D., Moss, P. A. H., Goulder, P. J. R., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. I., McMichael, A. J., and Davis, M. M. Phenotypic analysis of antigen-specific T lymphocytes. Science (Wash. DC), *274:* 94–96, 1996.

10. Löwer, R., Löwer, J., and Kurth, R. The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequences. Proc. Natl. Acad. Sci. USA, *93:* 5177–5184, 1996.

11. Medstrand, P., Mager, D. L., Yin, H., Dietrich, U., and Blomberg, J. Structure and genomic organization of a novel human endogenous retrovirus family: HERV-K (HML-6). J. Gen. Virol., *78:* 1731–1744, 1997.

12. Shastri, N., Nguyen, V., and Gonzalez, F. Major histocompatibility class I molecules can present cryptic translation products to T cells. J. Biol. Chem., *270:* 1088–1091, 1995.

13. Kozak, M. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res., *15:* 8125–8132, 1987.

14. Johnson, W. E., and Coffin, J. M. Constructing primate phylogenies from ancient retrovirus sequences. Proc. Natl. Acad. Sci. USA, *96:* 10254–10260, 1999.

15. Tonjes, R. R., Czauderna, F., and Kurth, R. Genome-wide screening, cloning, chromosomal assignment, and expression of full-length human endogenous retrovirus type K. J. Virol., *73:* 9187–9195, 1999.

16. Ono, M., Yasunaga, T., Miyata, T., and Ushikubo, H. Nucleotide sequence of human endogenous retrovirus genome related to the mouse mammary tumor virus genome. J. Virol., *60:* 589–598, 1986.

17. Boller, K., Konig, H., Sauter, M., Mueller-Lantzsch, N., Lower, R., Lower, J., and Kurth, R. Evidence that HERV-K is the endogenous retrovirus sequence that codes for the human teratocarcinoma-derived retrovirus HTDV. Virology, *196:* 349–353, 1993.

18. Wang-Johanning, F., Frost, A. R., Johanning, G. L., Khazaeli, M. B., LoBuglio, A. F., Shaw, D. R., and Strong, T. V. Expression of human endogenous retrovirus K envelope transcripts in human breast cancer. Clin. Cancer Res., *7:* 1553–1560, 2001.

19. Boon, T., and Van den Eynde, B. Cancer vaccines: cancer antigens, shared tumor-specific antigens. *In:* S. A. Rosenberg (ed.), Principles and Practice of the Biologic

Therapy of Cancer, Ed. 3, pp. 493–504. Philadelphia: J. B. Lippincott Williams & Wilkins, 2000.

20. De Smet, C., Lurquin, C., Lethé, B., Martelange, V., and Boon, T. DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. Mol. Cell. Biol., *19:* 7327–7335, 1999.

21. Gotzinger, N., Sauter, M., Roemer, K., and Mueller-Lantzsch, N. Regulation of human endogenous retrovirus-K Gag expression in teratocarcinoma cell lines and human tumours. J. Gen. Virol., *77:* 2983–2990, 1996.

22. Florl, A. R., Lower, R., Schmitz-Drager, B. J., and Schulz, W. A. DNA methylation and expression of LINE-1 and HERV-K provirus sequences in urothelial and renal cell carcinomas. Br. J. Cancer, *80:* 1312–1321, 1999.

23. Hayashi, H., Matsubara, H., Yokota, T., Kuwabara, I., Kanno, M., Koseki, H., Isono, K., Asano, T., and Taniguchi, M. Molecular cloning and characterization of the gene encoding mouse melanoma antigen by cDNA library transfection. J. Immunol., *149:* 1223–1229, 1992.

24. de Bergeyck, V., De Plaen, E., Chomez, P., Boon, T., and Van Pel, A. An intracisternal A particle sequence codes for an antigen recognized by syngeneic cytolytic T lymphocytes on a mouse spontaneous leukemia. Eur. J. Immunol., *24:* 2203–2212, 1994.

25. Huang, A. Y. C., Gulden, P. H., Woods, A. S., Thomas, M. C., Tong, C. D., Wang, W., Engelhard, V. H., Pasternack, G., Cotter, R., Hunt, D., Pardoll, D. M., and Jaffee, E. M. The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product. Proc. Natl. Acad. Sci. USA, *93:* 9730–9735, 1996.

26. Yang, J. C., and Perry-Lalley, D. The envelope protein of an endogenous murine retrovirus is a tumor-associated T-cell antigen for multiple murine tumors. J. Immunother., *23:* 177–183, 2000.

27. Boon, T., and Van Pel, A. T cell recognized antigenic peptides derived from the cellular genome are not protein degradation products but can be generated directly by transcription and translation of short subgenic regions. A hypothesis. Immunogenetics, *29:* 75–79, 1989.