

# Translation of a Retained Intron in Tyrosinase-related Protein (TRP) 2 mRNA Generates a New Cytotoxic T Lymphocyte (CTL)-defined and Shared Human Melanoma Antigen Not Expressed in Normal Cells of the Melanocytic Lineage

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## Summary

We report here the identification of a new shared human melanoma antigen recognized by a human leukocyte antigen (HLA)-A\*68011-restricted cytotoxic T lymphocyte clone (CTL 128). The cDNA encoding this antigen is composed of a partially spliced form of the melanocyte differentiation antigen tyrosinase-related protein (*TRP*)-2, containing exons 1–4 with retention of intron 2 and part of intron 4 (*TRP-2-INT2*). The sequence coding for the antigenic epitope is located at the 5' end of intron 2 and is available for translation in the same open reading frame of the fully spliced *TRP-2* mRNA. This peptide is also recognized by CTL 128 when presented by the HLA-A\*3301, a member of the HLA-A3-like supertype that includes the HLA-A\*68011. Quantitative reverse transcription PCR analysis carried out on total and/or cytoplasmic mRNA demonstrated that, in contrast to the fully spliced *TRP-2* mRNA expressed in melanomas, normal skin melanocytes, and retina, the *TRP-2-INT2* mRNA could be detected at significant levels in melanomas but not in normal cells of the melanocytic lineage. Instead, in these normal samples, both the spliced and the unspliced transcript of *gp100* were expressed at high levels. Absence of endogenous *TRP-2-INT2* expression in melanocytes was also confirmed by lack of recognition of HLA-A\*68011-transduced, TRP-2<sup>+</sup> melanocyte lines by CTL 128. These results indicate that a partially spliced form of a differentiation antigen mRNA, present in the cytoplasmic compartment of neoplastic but not normal cells of the melanocytic lineage, can be the source of a melanoma-restricted T cell epitope.

Key words: melanoma • antigen • T cell • specificity • intron-encoded epitope

Several studies have demonstrated the existence of an MHC class I-restricted CTL response specifically directed against human tumors (1). The role of such tumor-specific effectors in mediating the regression of metastatic melanomas has been suggested by the results of several clinical trials of both adoptive immunotherapy (2, 3) and vaccination (4–6). The determinants recognized by such CTLs have been studied extensively on melanoma cells, and several antigens have been characterized at the molecular level (1). According to the pattern of gene expression in normal and neoplastic cells, these antigens can be divided into three classes which may have different clinical relevance.

The first class includes antigens that are shared between melanomas and tumors of other histological type, but not by normal tissues with the exception of testis and placenta

(1, 7, 8). However, these tissues, since they are negative for expression of MHC class I molecules (9) needed for antigen presentation, cannot be targeted by T cells. Clinical trials based on the use of these antigens are in progress in patients affected by melanoma and other neoplastic diseases, and no relevant side-effects have been detected in the treated patients (1, 4).

The second class contains tissue-specific antigens expressed in normal and neoplastic cells of the melanocytic lineage (1, 10–12). Recently, the treatment of HLA-A2 melanoma patients with a gp100 peptide, modified to increase its binding to this HLA allele, in IFA plus IL-2, was shown to be associated with a significant clinical response rate (5). Even though no significant side-effects were observed in that or other similar clinical studies, the develop-

ment of cross-reacting responses against normal tissues (i.e., skin melanocytes and pigmented retinal cells) must be carefully considered when self-differentiation antigens are used as vaccines (13).

The third class includes antigens expressed only by the tumor cells from which they have been isolated (1), since these antigenic epitopes are usually generated by a point mutation occurring in otherwise ubiquitously expressed proteins. The lack of natural tolerance against these antigens may allow the induction of a strong immune response, while avoiding the development of potential autoimmune reactions. However, until a panel of widely shared mutations has been discovered, the clinical application of such antigens is limited to the treatment of those few patients whose tumor carries the given mutation.

The existence of a fourth set of tumor antigens has been recently suggested based on the pattern of reactivity of a panel of CTL clones able to recognize the autologous and HLA-matched allogeneic melanomas, but not melanocytes or other targets of different histological origin (14). Here we report the characterization of a melanoma antigen belonging to this group and found to be encoded by a retained intron in a partially spliced transcript of the tyrosine-related protein 2 (TRP-2)<sup>1</sup> differentiation antigen.

The features of this new shared melanoma-specific antigen may allow the development of safer and more efficacious immunotherapy trials.

## Materials and Methods

**Cell Lines.** The melanoma cell line Me18732 was established from a metastatic lesion obtained from a surgical specimen of patient 18732, admitted for surgery to the Istituto Nazionale Tumori (Milan, Italy). PBLs of this patient were typed serologically as HLA-A2/28, -B44/51, -C2/5. The human metastatic (Me17697, Me2559/1, Me12657, Me17088/1, Me4023) and primary (Me20842) melanoma cell lines, established in our laboratory, the carcinoma lines CALU3, SKBR3, and SKOV3 (American Type Culture Collection, Rockville, MD), and the WEHI-164.13 cells (15) were cultured in 10% FCS/RPMI 1640. The melanoma lines LB33, MZ2-MEL, and SK23-MEL, and the COS-7 cell line were provided by Prof. T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium) and maintained in 10% FCS/RPMI 1640 (melanomas) or 10% FCS/DMEM (COS-7). Normal human epidermal melanocyte lines (NHEM)-2489, -2311, and -4528 (Clonetics, San Diego, CA), and DL-1 melanocytes, the gift of Dr. M. De Luca (Istituto Dermatologico dell'Immacolata, Rome, Italy), were kept in melanocyte growth medium (MGM-2; Clonetics). The C1R-A\*03301 transfectant, the homozygous EBV-transformed lymphoblastoid cell line (LCL) SCHU (HLA-A\*0301, B\*0702, C7), AMA-1 (HLA-A\*6802, -B\*5301, -C4), and WT-100-bis (HLA-A11, B35, C4) were provided by Dr. Soo Young Yang (Memorial Sloan-Kettering Cancer Center, New York); the EBV-LCL JHAF (HLA-A\*31011, -B51, -C8) and LB (HLA-A\*68011, -B\*40011, -C2/3) were obtained from

American Type Culture Collection. EBV-LCL cells were maintained in 10% FCS/RPMI 1640. CTL 128 (CD3<sup>+</sup>, CD4<sup>-</sup>, CD8<sup>+</sup>, TCR  $\alpha\beta$ <sup>+</sup>) was derived and grown as described previously (14). Its lytic activity was tested in a standard chromium-release assay (14) in the presence or absence of HLA-specific mAbs.

**Subcloning of the HLA-A\*68011 Allele.** cDNA obtained from Me18732 total RNA was amplified by PCR using a primer pair suitable for amplification and directional cloning of the HLA-A full-length coding region (gift of Dr. Soo Young Yang). The 1.1-kb PCR product was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen Corp., Abingdon, Oxon, UK). A plasmid clone (pcDNA3/A\*6801) encoding the HLA-A\*68011 allele was identified by diagnostic restriction mapping and sequencing.

**Construction and Screening of the cDNA Library.** Poly(A)<sup>+</sup> RNA was isolated from Me18732 cells using the FastTrack mRNA extraction kit (Invitrogen Corp.). The library was constructed converting 5  $\mu$ g of poly(A)<sup>+</sup> RNA to cDNA with the SuperScript Choice System kit (GIBCO BRL, Gaithersburg, MD) using an oligo-dT primer containing a NotI site at its 5' end. cDNAs were then ligated to BstXI adapters (Invitrogen Corp.) and digested with NotI. After size fractionation, cDNAs were unidirectionally cloned into the BstXI/NotI site of the mammalian expression vector pcDNA3.1 (Invitrogen Corp.). Recombinant plasmids were electroporated into DH5- $\alpha$  *Escherichia coli* and selected with ampicillin (50  $\mu$ g/ml). The library was divided into 1,300 pools of  $\sim$ 100 cDNA clones. Each pool of bacteria was amplified to saturation, and plasmid DNA was extracted and transfected (100 ng) together with pcDNA3/A\*6801 (100 ng) into COS-7 cells by the DEAE-dextran-chloroquine method (16). Using the same technique, in other experiments COS-7 cells were cotransfected with 100 ng of pcDNA3/A\*6801 vector and 100 ng of expression vectors encoding one of the following melanoma antigens: Melan-A/MART-1, tyrosinase, gp100, MAGE-1, -2, -3, -4, -12, BAGE-1, -2, GAGE-1, -2, -3, -4, -5, -6 (provided by Dr. P. van der Bruggen, Ludwig Institute for Cancer Research), TRP-1 (provided by Dr. R.S. Wang, National Cancer Institute, National Institutes of Health, Bethesda, MD). Full-length TRP-2 cDNA was amplified by reverse transcription (RT)-PCR using specific primers located in the 5'-untranslated region (UTR; PRIT-1b, 5'-GGAGAAAAGTACGACAG-3') and at the end of exon 8 (TRP-2L, 5'-ACCCTAGGCTTCTTCTGTGTATCTCTT-3'), cloned into pcDNA3, and sequenced to verify correspondence to the published cDNA sequence (17). Transfected COS-7 cells or stimulating cell lines were tested for their ability to induce TNF release by CTL 128, as described previously (18).

**DNA Sequencing.** Sequencing was performed with an automated DNA sequencer (ABI Prism 377; Perkin-Elmer Corp., Norwalk, CT) using the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Corp.). The computer search for the sequence homology was done with the program FASTA EMBL-Heidelberg.

**Production of Subfragments of cDNA 131.** Subfragments of cDNA 131 (5'-end 1500, 5'-end 800, 200, and 3' end) were obtained by digestion of the plasmid with HindIII and BstUI and cloned into the pcDNA1 plasmid (Invitrogen Corp.). From the 5'-end 1500, the smaller fragments INT2-434, -166, and -107 were generated by PCR amplification. The sense primer KS-INT2 (5'-GCCGCCATGTATTCTGTTAGAGATAACA-3') was used for amplification of all fragments. This primer generates an ATG start codon (underlined), with the appropriate Kozak consensus sequence, in the same open reading frame (ORF) as TRP-2. The antisense primers used for amplification of the INT2-434, -166,

<sup>1</sup>Abbreviations used in this paper: LCL, lymphoblastoid cell line; LNGFR, low-affinity nerve growth factor receptor; nt, nucleotide(s); ORF, open reading frame; RT, reverse transcription; TRP, tyrosinase-related protein; UTR, untranslated region.

and -107 fragments, respectively, were SP6 (5'-ATTTAGGT-GACTATAG-3') located in the pcDNA1 plasmid, INT2-as1 (5'-ATCCGCTCGAGCATGAAATTACTTCCC-3'), and INT2-as2 (5'-ATCCGCTCGAGGATAATTCTACGAC-3'), located in intron 2 of *TRP-2*. The antisense primers INT2-as1 and -as2 contained an XhoI restriction site (underlined) for directional cloning.

**Retroviral HLA-A\*68011 Gene Transfer into Melanocytes.** A retroviral vector encoding the full-length *HLA-A\*68011* cDNA, under the control of the viral LTR, and the truncated form of the human low-affinity nerve growth factor receptor ( $\Delta$ LNGFR) driven by the SV40 promoter was constructed as described previously (19). An amphotropic packaging cell line was established by infection of the GP<sup>+</sup>env Am 12 line and immunoselection for  $\Delta$ LNGFR expression by the use of magnetic beads (Dynabeads M-450; Dynal A.S., Oslo, Norway) coated with the LNGFR-specific mAb 20.4 (American Type Culture Collection). Transduction of melanocytes and SK23-MEL was performed by cultivation with retrovirus-containing supernatant in the presence of polybrene (0.8 mg/ml). Three rounds of infection of at least 4 h were performed.

**Separation of Nuclear and Cytoplasmic RNA.** Cells ( $2-3 \times 10^6$ ) were harvested, washed with PBS, and then carefully resuspended in 400  $\mu$ l of buffer A (10 mM Hepes, pH 8.0, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol). After 10 min of incubation on ice, cytoplasmic membranes were disrupted by the addition of 20  $\mu$ l NP-40 (10%), and the suspension was vigorously vortexed for 10 s and then centrifuged for 30 s at 15,000 rev/min. The supernatant containing the cytoplasmic RNA was transferred to a new tube. The pellet containing the intact nuclei was washed with buffer A to eliminate residual cytoplasmic RNA. Cytoplasmic and nuclear RNAs were purified from the cell fractions using the RNeasy mini kit (QIAGEN Inc., Chatsworth, CA). The absence of contaminating nuclear material (i.e., DNA and nuclear mRNA) in the cytoplasmic compartment was checked with specific primers flanking at least one intron of *MAGE-3* (20) or complementary to intron-exon junctions flanking exon 8 of *p53* (21).

**Expression of *TRP-2* and Its Partially Spliced Form *TRP-2-INT2*.** cDNA corresponding to 150 ng of total RNA from melanoma lines and fresh samples (tumors, skin, and retina) was amplified by PCR in 25  $\mu$ l of water containing 200  $\mu$ M of each dNTP, 0.6  $\mu$ M of each primer,  $1 \times$  PCR buffer, 1 U of Taq-Gold (all reagents from Perkin-Elmer Corp.). *TRP-2* cDNA was amplified using the sense primer PR3 located in exon 2 (5'-TTC-GGCAGAACATCCATTCC-3') and the TRP-2L antisense primer, originating an amplicon of 1186 bp. *TRP-2-INT2* was amplified using the sense primer PRIT-1b and the antisense primer INT2-1260 (5'-ACCTCACCAACTCACATCTT-3'), located in the 5'-UTR and in intron 2, respectively, and giving an amplification product of 977 bp. PCR amplification was performed for 30 cycles: 1 min at 94°C, 1 min at 55°C (58°C for *TRP-2*), and 1 min at 72°C. Primer positions were chosen in order to avoid contamination from genomic DNA. *Glyceraldehyde-3-phosphate-dehydrogenase* (*GAPDH*) cDNA was amplified with specific primers (22).

**Evaluation of Primer Pair Sensitivity.** Plasmids containing *TRP-2-INT2*, *TRP-2*, and *gp100* cDNAs were suspended to 10 pg/ $\mu$ l, a concentration equivalent to 1,026,119, 1,193,338, and 1,251,907 molecules/ $\mu$ l, respectively, serially twofold diluted, and amplified by PCR under the conditions described above. To mimic the target complexity of "true" cDNA sample, cDNA corresponding to 50 ng of total RNA from the carcinoma line SKBR3, negative

for expression of *TRP-2*, *TRP-2-INT2*, and *gp100*, was added. *TRP-2* was amplified with primers PR3 and TRP-2L. For *TRP-2-INT2*, in addition to PRIT-1b and INT2-1260, we also tested the sense primer PR15 (5'-AATTGGCAGTTCAGTGTCC-3'), located in intron 2, and the antisense primer PR16 (5'-AT-TGCAGGTACAGGAGCCAT-3'), located in intron 4, giving an amplicon of 906 bp. To amplify *gp100*, a previously described primer pair giving an amplicon of 361 bp was used (23). After an initial incubation at 94°C for 10 min and a final incubation at 72°C for 7 min, temperature cycling was conducted, with minor modifications, according to the original stepdown PCR parameters (24). Cycling was repeated 37 times, divided into 6 groups, with a denaturation of 20 s at 94°C and an elongation of 1 min 15 s at 72°C. The six groups consisted of three, six, six, six, nine, and seven cycles. Annealing temperatures lasted 40 s and decreased by 3°C from one group of cycles to the next, starting at 64°C in the first three cycles, 61°C in the following six, etc., and ending with 50°C in the last seven cycles. For *gp100*, extension time at 72°C lasted for 1 min in all cycles. Amplification products were size fractionated on agarose gels in the presence of ethidium bromide, digitally acquired under UV transillumination (Eagle Eye II, Still Video System; Stratagene Inc., La Jolla, CA), and analyzed densitometrically (ImageQuant version 3.3 software; Molecular Dynamics, Sunnyvale, CA). For each primer pair, the OD of gel bands (expressed in arbitrary units on a linear scale) was plotted against the initial concentration (expressed in picograms per microliter on a logarithmic scale) of target plasmid present in the sample. The minimum concentration of target plasmid detectable by each primer pair was indicated by the lowest point in the linear amplification range of the curve. The mean of this value, evaluated in three experiments and expressed as number of molecules, was considered as the sensitivity of each primer pair.

**Quantitative RT-PCR Analysis.** cDNA samples prepared from total or cytoplasmic RNA of Me18732 were used as a reference, i.e., a sample whose level of expression of a given transcript is arbitrarily defined as 100%. *GAPDH* expression was used to normalize experimental results. The linear amplification range of *GAPDH*, *TRP-2*, *gp100*, *TRP-2-INT2*, and *gp100-in4* was determined experimentally for the reference Me18732 cDNAs, starting from 2  $\mu$ l cDNA, a volume corresponding to 100 ng total RNA. For each primer pair, the appropriate number of cycles to attain exponential amplification was found for a certain range of dilutions, varying the number of the final cycles of the stepdown PCR, performed as described above. The total number of cycles for *GAPDH* was 22 (groups of 3, 6, 6, and 7 cycles), and for *gp100*, *TRP-2-INT2*, *TRP-2*, and *gp100-in4* was 30, 33, 35, and 40, respectively, according to groups of 3, 6, 6, 6, 9 plus  $n$  cycles. *TRP-2* was amplified by primer pair PR3 plus TRP-2L; *TRP-2-INT2* by PR15 plus PR16; and *gp100*, *gp100-in4*, and *GAPDH* by a pair of published primers (10, 22, 23). Linear regression analysis of experimental OD values allowed construction of standard curves for Me18732. Serial dilutions of cDNAs from all samples to be quantified were analyzed in these cycling conditions. Their OD values were compared with the appropriate standard curve always included in each PCR run, and normalized to the expression level of the *GAPDH* gene.

The lowest point in the linear amplification range of the calibration curve of a given primer pair, corresponding to a dilution expressed as a percentage of the first dilution of Me18732 cDNA, arbitrarily taken as 100%, was considered the minimum sensitivity threshold for that primer pair. The absence of a detectable amplification product, or OD values falling below this threshold, were reported as less than this threshold value. For primer pair PR3

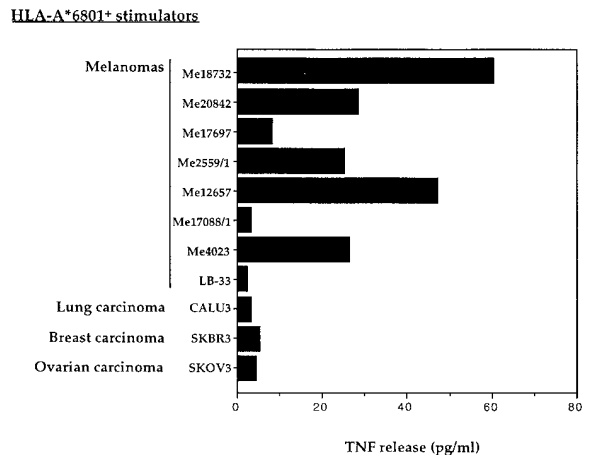
plus TRP-2L (*TRP-2*) the threshold was 0.01%, and for PR15 plus PR16 (*TRP-2-INT2*) 0.1%. To quantify the level of *TRP-2-INT2* expression in fresh specimens (skin and retina samples) in which the proportion of melanocytic cells is unknown and variable, the method described was modified as follows. The same cDNA dilutions from Me18732 total RNA were amplified using 31 (for *TRP-2*) and 33 (for *TRP-2-INT2*) cycles of stepdown PCR; after densitometric analysis, the OD results were expressed in arbitrary units. cDNA dilutions of skin and retina samples were amplified with *TRP-2* primers for *n* number of cycles (ranging from 40 to 45) in order to obtain, after amplification, an OD of the gel band within the range covered by the Me18732 cDNA. For *TRP-2-INT2*, the same quantities of starting material were amplified for *n* plus at least 2 cycles to reflect the difference in the number of cycles (31–33) needed to detect *TRP-2* and *TRP-2-INT2*, respectively, in the reference melanoma.

**Peptides and CTL Assays.** The *TRP-2-INT2* sequence was analyzed at a Web site of the National Institutes of Health ([http://bimas.dcrn.nih.gov:80/molbio/hla\\_bind/](http://bimas.dcrn.nih.gov:80/molbio/hla_bind/)) for the presence of potential HLA-A\*6801-binding peptides. Lyophilized peptides, 90% pure as indicated by analytical HPLC, were purchased from Primm Srl (Milan, Italy), dissolved at 10 mM concentration in DMSO, and stored at  $-80^{\circ}\text{C}$ . Various concentrations of the peptides were added to  $^{51}\text{Cr}$ -labeled target cells. After 1 h of incubation at room temperature, CTL 128 was added at an E/T ratio of 20:1. Lysis was measured 4 h later. Alternatively, stimulator cells were incubated for 1 h at room temperature with a fixed concentration of peptides; after extensive washing, CTL 128 was added, and the TNF release was evaluated 18–20 h later on WEHI-164.13 cells.

## Results

**Identification of HLA-A\*68011 as Restriction Element for CTL 128.** Melanoma cell line Me18732 was established from a metastatic lesion of patient 18732. Lymphocytes of the patient were typed as HLA-A2 and HLA-A28 by serological methods and then as HLA-A\*0201 and HLA-A\*68011 (hereafter referred to as HLA-A\*6801) by sequence-specific oligonucleotide probe (SSOP) subtyping (25). HLA-restricted antitumor CTL clones were obtained from mixed lymphocyte tumor culture, as described (14). In particular, CTL clone 128 recognized the autologous tumor, and its lytic activity was inhibited by an mAb recognizing HLA-A2 and -A28, but not by an anti-HLA-A2 and -A69 mAb, indicating that HLA-A28 (A\*6801) was the restricting molecule for CTL 128 (data not shown).

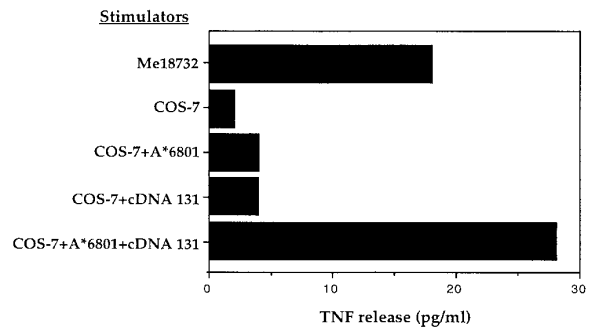
**Antigen Specificity of CTL 128.** To evaluate the frequency of expression of the antigen recognized by CTL 128, a panel of HLA-A\*6801 tumor lines was tested in a CTL stimulation assay. Five out of eight melanoma cell lines induced TNF release by CTL 128 (Fig. 1). However, no reactivity was observed with three HLA-A\*6801 carcinoma lines of different histological origin (Fig. 1). The pattern of reactivity displayed by CTL 128 towards the melanoma lines tested did not correlate with expression of previously described melanoma antigens in these cell lines, as assessed by RT-PCR (data not shown). This was confirmed by the lack of TNF release by CTL 128 in the presence of COS-7 cells cotransfected with plasmid pcDNA3/A\*6801, to-



**Figure 1.** Recognition by CTL 128 of HLA-A\*6801<sup>+</sup> melanoma and carcinoma cell lines.  $1.5 \times 10^3$  CTLs were added to  $2.5 \times 10^4$  stimulator cells, and the TNF content of the supernatant was tested 20 h later on WEHI-164.13 cells.

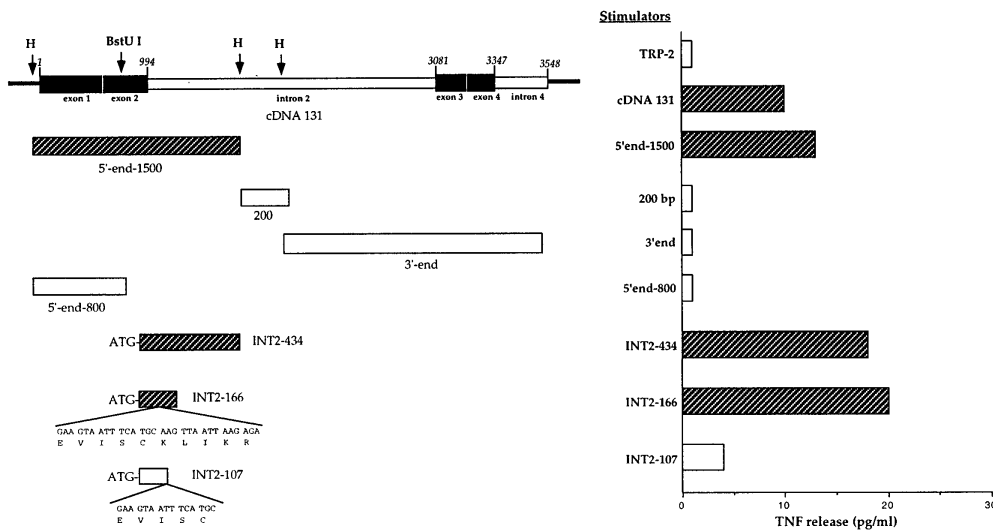
gether with most of the cDNAs known to encode shared melanoma antigens (see Materials and Methods; data not shown). These results suggested that CTL 128 recognized a new antigen shared by a number of melanomas.

**Cloning of a cDNA Encoding the Melanoma Antigen Recognized by CTL 128.** A cDNA library was constructed in expression vector pcDNA3.1 with poly(A)<sup>+</sup> RNA extracted from Me18732 cells. Plasmid DNA from pools of 100 recombinant clones was cotransfected into COS-7 cells together with plasmid pcDNA3/A\*6801. The DNA of one pool induced the production of a high level of TNF by CTL 128 (data not shown). By subcloning the bacteria of this pool, we obtained several clones that were able to transfer expression of the antigen. The results obtained with one of these clones, cDNA 131, are shown in Fig. 2. Recognition of the antigen was independent of the high, artificial expression level achieved in COS-7 cells. Indeed, an HLA-A\*6801<sup>+</sup> melanoma cell line that was not recog-



**Figure 2.** Stimulation of CTL 128 by COS-7 cells transfected with cDNA 131 and HLA-A\*6801 cDNA. COS-7 cells were transfected with HLA-A\*6801 and with cDNA 131. The production of TNF by CTL 128 was measured after 24 h of coculture with the transfected cells, using the TNF-sensitive cell line WEHI-164.13. As control, COS-7 cells were transfected with cDNA 131 or HLA-A\*6801 alone.





**Figure 4.** Identification of the sequence coding for the antigenic peptide recognized by CTL 128. *Top left*, Exon-intron organization of cDNA 131. Exons (black boxes) and introns (white boxes) are indicated; the horizontal line at the extremities represents pcDNA3.1 vector; the numbering of the sequence is relative to the 5' end of cDNA 131. Subfragments derived from cDNA 131 and PCR products, shown below cDNA 131, were cloned into expression vectors and transfected into COS-7 cells with the HLA-A\*6801 cDNA, and those recognized by CTL 128 (striped boxes) are indicated. The peptide-encoding sequences present in the PCR fragments INT2-166 and INT2-107 are shown (bottom left). The spliced

full-length form of *TRP-2* cDNA was obtained by PCR as described (see Materials and Methods). TNF release by CTL 128 was evaluated on WEHI-164.13 cells (right). Striped bars, Recognition of cDNA 131 and its subfragments.

tained at a concentration of  $\sim 100$  pM, whereas the nonapeptide had a very low efficiency (Fig. 5). A control peptide, WTGPNCKERKK, derived from exon 2 of the fully spliced *TRP-2* gene (17) able to bind to HLA-A\*6801, was not recognized (data not shown). Given the localization of the antigenic epitope within intron 2 of *TRP-2*, the antigen was named TRP-2-INT2 and the epitope TRP-2-INT2<sub>222-231</sub>.

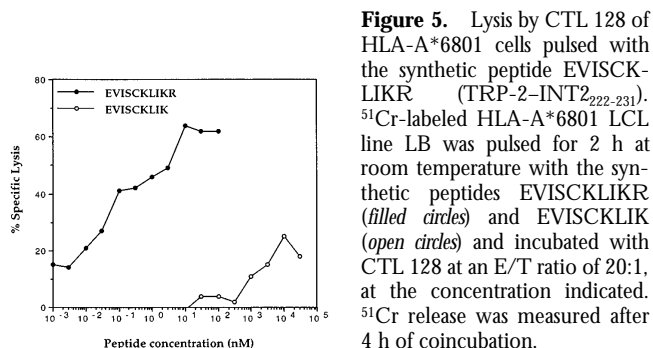
To exclude the possibility that TRP-2-INT2<sub>222-231</sub> may have been generated by a mutation that occurred in the tumor, a 2152-bp fragment spanning the entire intron 2 (nt 995-3080 in cDNA 131) was amplified by PCR from genomic DNA of CTL 128 and from a different melanoma, MZ2-MEL. The PCR products were cloned, sequenced, and transfected together with pcDNA3/A\*6801 into COS-7 cells. All clones had the expected sequences and were able to transfer expression of the antigen (data not shown).

**Presentation of the Antigenic Peptide by Alleles of the HLA-A3-like Supertype.** HLA-A\*6801, along with A3, A11, A31, and A\*3301, belongs to an A3-like supertype of HLA-A alleles with similar peptide-binding characteristics (28). To investigate whether the TRP-2-INT2<sub>222-231</sub> peptide can be

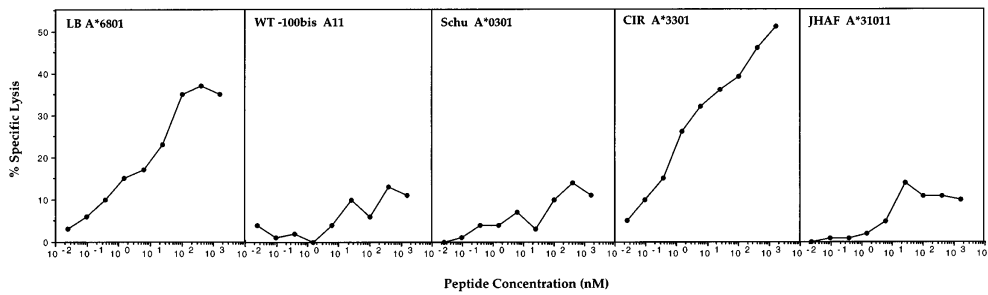
presented to CTL 128 by HLA alleles of the A3-like supertype, EBV LCL cells expressing such alleles were used as targets for CTL 128 after pulsing with the peptide. Among the A3-like supertype alleles, only A\*3301 was able to present TRP-2-INT2<sub>222-231</sub> peptide with the same efficiency as A\*6801 (Fig. 6).

**Expression of the Sequence Encoding TRP-2-INT2 in Tumors and Normal Tissues.** Expression of transcripts retaining intron 2 was analyzed by RT-PCR with the PRIT-1b plus INT2-1260 primer pair. The completely spliced *TRP-2* messenger was detected with the PR3 plus TRP-2L primer pair. Among the tumors tested, only melanomas proved positive for *TRP-2* and *TRP-2-INT2* expression (Table 1), whereas 17 carcinomas (4 ovary, 4 breast, 4 lung, 2 pancreas, and 3 bladder) were negative (data not shown). 84% (16 out of 19) and 68% (13 out of 19) of melanoma cell lines analyzed expressed *TRP-2* and *TRP-2-INT2*, respectively. In fresh melanoma samples, the normal form of *TRP-2* was expressed in 69% (9 out of 13) of the analyzed samples and *TRP-2-INT2* in 54% (7 out of 13; Table 1). Expression of *TRP-2-INT2* in the absence of *TRP-2* was never observed. This result is in agreement with the notion that *TRP-2* is a melanocytic differentiation antigen (12) and that the same promoter drives the synthesis of a common messenger from which both antigens arise. Normal tissues in which *TRP-2* is known to be present were analyzed for *TRP-2-INT2* expression. 4 melanocyte cell lines, 13 skin samples, and 1 retina tested were all negative in the RT-PCR assay (Table 1).

**Quantitative RT-PCR Analysis for TRP-2-INT2 Expression.** To quantitate levels of expression of *TRP-2* and *TRP-2-INT2* in both neoplastic and normal cells, primer pair sensitivity (i.e., the minimum number of target molecules detectable) as well as amplification efficiency had to be comparable. As detailed in Materials and Methods, seri-



**Figure 5.** Lysis by CTL 128 of HLA-A\*6801 cells pulsed with the synthetic peptide EVISCKLIKR (TRP-2-INT2<sub>222-231</sub>). <sup>51</sup>Cr-labeled HLA-A\*6801 LCL line LB was pulsed for 2 h at room temperature with the synthetic peptides EVISCKLIKR (filled circles) and EVISCKLIK (open circles) and incubated with CTL 128 at an E/T ratio of 20:1, at the concentration indicated. <sup>51</sup>Cr release was measured after 4 h of coincubation.



**Figure 6.** Recognition by CTL 128 of the TRP-2-INT2<sub>221-231</sub> peptide when presented by HLA alleles of the A3-like supertype. <sup>51</sup>Cr-labeled LCL cells were incubated with CTL 128 at an E/T ratio of 20:1, in the presence of peptide TRP-2-INT2<sub>222-231</sub> at different concentrations. <sup>51</sup>Cr release was measured after 4 h of coincubation.

ally diluted plasmids containing *TRP-2*, *TRP-2-INT2*, and *gp100* cDNA were amplified for 37 cycles with specific primers. After densitometric analysis, the sensitivity of the PR3 plus TRP-2L primer pair was determined in the order of 34 molecules for *TRP-2*, whereas 19 was the lowest number of molecules of *gp100* detectable with the previously described primers (23). For *TRP-2-INT2*, the primer pair PR15 plus PR16, which displays an even higher sensitivity (four molecules), was chosen for this analysis. All primer pairs showed a nearly identical amplification efficiency, as indicated by parallelism of regression lines in the linear amplification range of the curves (data not shown).

Therefore, levels of expression of *TRP-2* and *TRP-2-INT2* in nine melanoma cell lines were determined by quantitative RT-PCR on cDNA derived from total RNA. Me18732 was arbitrarily considered to have 100% expression of these genes and was used as the reference standard (Table 2). All measurements were normalized on *GAPDH* expression to take into account differences in template integrity. Expression of *TRP-2-INT2* in the absence of *TRP-2* was never observed. The six tumor lines that stimulated TNF release by CTL 128 expressed both *TRP-2* and *TRP-2-INT2*. The level of expression of *TRP-2-INT2* mRNA ranged from 6 to 187% of that present in

Me18732, and that of *TRP-2* from 5 to 35%. Among the three melanoma lines not recognized by CTL 128, LB33 was below the threshold of sensitivity of the primer pair used for expression of both transcripts, whereas Me17088/1 expressed low levels (0.1%) of *TRP-2* mRNA only; MZ2-MEL\*6801, a melanoma cell line stably transfected with *HLA-A\*6801*, expressed low levels of both *TRP-2* (0.5%) and *TRP-2-INT2* (0.4%). Since RT-PCR analysis performed on total RNA does not distinguish the cytoplasmic, partially spliced *TRP-2-INT2*, from which the antigenic peptide is generated, from pre-*TRP-2* mRNA of nuclear origin, RNA was extracted from both the nucleus and the cytosol of MZ2-MEL-A\*6801. A strong PCR signal was obtained from nuclear RNA, whereas the *TRP-2-INT2* message in the cytoplasm accounted for 0.2% of that present in the same compartment of Me18732. The absence of TNF release by CTL 128 in the presence of MZ2-MEL-A\*6801 demonstrates that cytoplasmic *TRP-*

**Table 1.** Expression of *TRP-2-INT2* and *TRP-2* mRNA in Tumors and Normal Tissues

	No. of samples with antigen expression/no. samples tested*	
	TRP-2-INT2	TRP-2
Melanoma cell lines	13/19 (68%) <sup>‡</sup>	16/19 (84%)
Melanocyte cell lines	0/4	4/4
Fresh melanoma samples	7/13 (54%) <sup>‡</sup>	9/13 (69%)
Skin	0/13	13/13
Retina	0/1	1/1
Carcinomas	0/17	ND

\*cDNA synthesis and PCR were performed as described in Materials and Methods.

<sup>‡</sup>TRP-2-INT2 expression was detected only in samples positive for TRP-2.

**Table 2.** Quantitative RT-PCR of *TRP-2* and *TRP-2-INT2* mRNA Expression and Recognition by CTL Clone 128

Melanoma cell lines	Recognition by CTL 128		
	TRP-2-INT2*	TRP-2*	
Me18732	+	100	100
Me12657	+	187	34
Me20842	+	28	17
Me2559/1	+	6	5
Me4023	+	55	22
SK23-MEL-A*6801 <sup>‡</sup>	+	15	35
MZ2-MEL-A*6801 <sup>‡</sup>	-	0.4	0.5
Me17088/1	-	<0.1	0.1
LB33	-	<0.1	<0.01

\*Expression of TRP-2-INT2 and TRP-2 mRNA in the different melanoma samples was assessed by quantitative RT-PCR. Me18732 was arbitrarily considered to have 100% expression of these genes, and the limit of detection for TRP-2-INT2 and TRP-2 was 0.1 and 0.01%, respectively.

<sup>‡</sup>Cell line obtained by calcium-phosphate transfection of pcDNA3/A\*6801 and selection in G418. Expression of the transfected *HLA-A\*6801* allele was verified by flow cytometry with specific mAbs (data not shown).

**Table 3.** Quantitative RT-PCR of *TRP-2-INT2*, *TRP-2*, *gp100*, and *gp100-in4* mRNAs in the Cytoplasm of Human Melanocytes

Cytoplasmic mRNA	<i>TRP-2-INT2</i>	<i>TRP-2</i>	<i>gp100-in4</i>	<i>gp100</i>
		%		
Me18732	100	100	100	100
NHEM-2489	<0.1	21	776	79
NHEM-2311	<0.1	133	1039	360
NHEM-4528	0.1	614	2363	155
DL-1	<0.1	15	5000	1613

Expression of the different transcripts was measured by quantitative RT-PCR. Me18732 was arbitrarily considered to have 100% expression of these genes. The limit of detection for *TRP-2-INT2* was 0.1%.

*2-INT2* transcript levels of at least >0.2% of that found in Me18732 are needed for functional recognition of target cells.

To exclude the possibility of even low contamination of pre-*TRP-2* nuclear message, quantitative analysis of *TRP-2* and *TRP-2-INT2* expression in four melanocyte cell lines at their first in vitro passages was performed with cDNA derived from cytoplasmic RNA. Expression levels of *gp100* and its partially spliced form *gp100-in4* mRNAs, containing sequences coding for CTL-defined antigens expressed by melanomas and melanocytes (1, 10), were also included in the analysis. As reported in Table 3, *TRP-2-INT2* mRNA of the cultured skin melanocytes was either undetectable (3/4) or at the lower limit of detection (1/4), whereas *TRP-2* mRNA ranged from 15 to 614% of that expressed by Me18732. However, all melanocyte lines expressed *gp100* and particularly *gp100-in4* at high levels, from 79 to 1,613% and from 776 to 5,000%, respectively, of that found in Me18732. Fig. 7 shows the gel bands used for densitometric analysis.

We then examined samples of three *TRP-2*<sup>+</sup> normal skin tissue and one retina. Since in fresh specimens the proportion of melanocytic cells is variable and generally very low, we used a slightly modified protocol, detailed in Materials and Methods. Total RNA was analyzed, since attempts to separate nuclei from cytoplasmic compartments

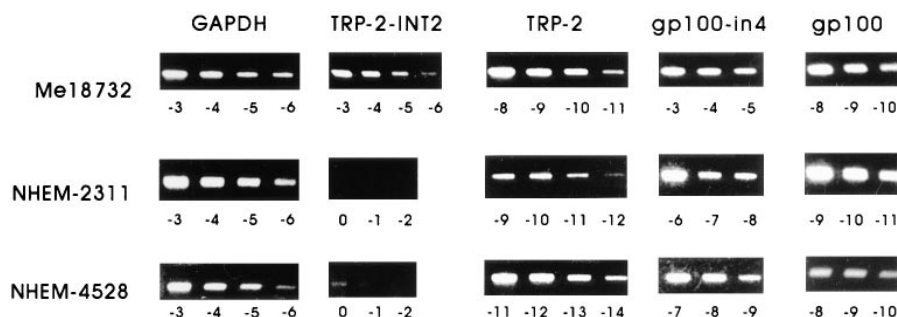
were unsuccessful, as demonstrated by a consistent level of nuclear contamination in cytoplasm (data not shown), due to the high level of cellular damage and/or deaths resulting from the enzymatic treatment used to prepare single cell suspensions. Results are reported in the original OD values and are shown in Fig. 8. An amplification product for *TRP-2* was obtained in each of the four samples (three skin tissue and one retina) analyzed, whereas *TRP-2-INT2* was undetectable. In this case too, both *gp100* and *gp100-in4* were expressed at high levels (data not shown).

The absence of expression of endogenous *TRP-2-INT2* in normal melanocytic cells was also confirmed by the lack of recognition by CTL 128 of melanocyte lines transduced with *HLA-A\*6801* (data for the DL-1 line are shown in Fig. 9). The transduced *HLA-A\*6801* molecule was able to present the endogenous *TRP-2-INT2* product as shown by recognition of the *HLA-A\*6801*-transduced SK23-MEL. Moreover, expression of *HLA-A\*6801* by the transduced melanocytes was confirmed by their ability to functionally present the exogenously provided *TRP-2-INT2*<sub>222-231</sub> peptide to CTL 128.

Taken together, these results indicate that the presence of a transcript corresponding to the unspliced *TRP-2* gene accompanies *TRP-2* mRNA expression in the cytoplasmic compartment of the majority of melanoma cells. This phenomenon does not occur in normal cells, despite the presence of comparable or even higher levels of *TRP-2*, allowing a partially spliced form of a differentiation antigen encoding mRNA to become the source of a melanoma-restricted T cell epitope.

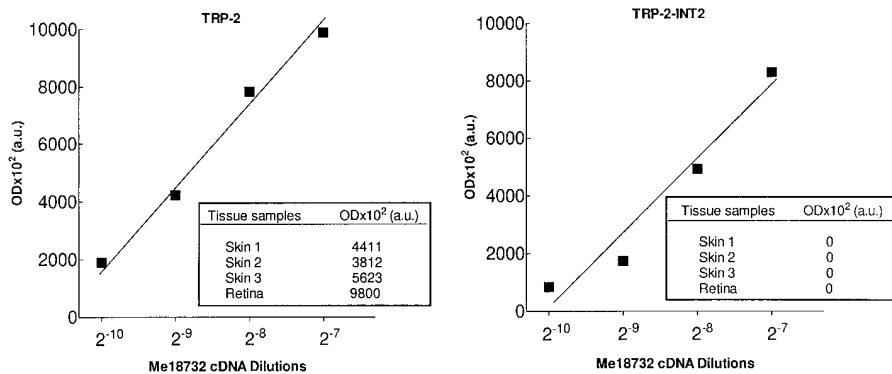
## Discussion

This study reports the molecular identification of an antigen (*TRP-2-INT2*) belonging to a new class of shared melanoma antigens absent from melanocytes. The epitope recognized by *HLA-A\*6801*-restricted CTL 128 is encoded within intron 2 of a partially spliced mRNA of the *TRP-2* gene. In its completely spliced form, *TRP-2* is a member of the tyrosinase-related gene family encoding an enzyme acting in the melanogenic pathway and containing an epitope recognized in an HLA-restricted fashion by T cells on melanomas and melanocytes (12). The sequence coding for the peptide recognized by CTL 128 is located at



**Figure 7.** Quantitative RT-PCR analysis. Serial twofold dilutions of cDNA derived from cytoplasmic RNA of DL-1 and NHEM-4528 melanocytes were amplified and simultaneously compared with the reference Me18732, as described in Materials and Methods. Depending on the sample and on the gene tested, starting dilutions ranged from 0 to -11 (i.e., 2<sup>0</sup> = undiluted, 2<sup>-11</sup> = 1/2,048). Results obtained after densitometric scanning of the amplified products are shown in Table 3.





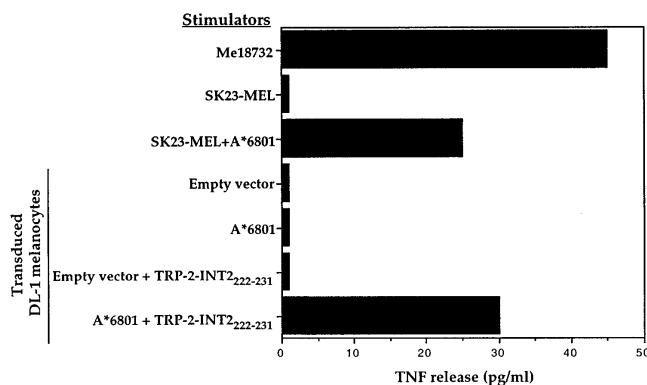
**Figure 8.** Expression of *TRP-2-INT2* and *TRP-2* on normal tissues. Expression was tested by quantitative RT-PCR on cDNA derived from total RNA. Results are expressed in arbitrary OD units (a.u.) after densitometric analysis of the amplified products, as described in Materials and Methods. For Me18732, the plotted OD points for *TRP-2* and *TRP-2-INT2* correspond to four identical and consecutive serial dilutions, chosen in order to obtain, with the lowest one, a still detectable amplification signal for *TRP-2-INT2*. Amplification of *TRP-2* from normal samples was performed in conditions (i.e., cDNA amount and cycle number) allowing the attainment of an OD value included in the range covered by Me18732. *TRP-2-INT2* was consequently amplified using comparable parameters (see Materials and Methods). (Insets) OD arbitrary units (a.u.) are reported for normal samples.

the 5' end of intron 2, which is available for translation because of a failure in the splicing pathway. An increasing body of evidence suggests the immunological significance of unconventionally produced epitopes. Indeed, epitopes encoded in alternative ORFs (29), within introns (10, 16), under the putative control of a cryptic promoter (30), or governed by a variant of conventional translation mechanisms (31) have been described. It has also been proposed that the increased expression in splice variants of pigment genes that occurs in melanoma compared with normal melanocytes may contribute to the pool of melanoma-specific antigenic peptides (32).

*TRP-2-INT2* expression was not observed in melanomas lacking *TRP-2*. Moreover, no *TRP-2-INT2* was found when *TRP-2* expression was 0.1% of that found in Me18732. Such a low level of *TRP-2* transcription, detected by the very sensitive quantitative RT-PCR, could

not be evidenced in Northern blots (data not shown) and probably accounts for the percentage of *TRP-2*<sup>+</sup> fresh melanomas that fail to express *TRP-2-INT2*. In all normal tissues containing *TRP-2*<sup>+</sup> cells of melanocytic origin (i.e., skin and retina) and in three out of four melanocyte samples that had levels of *TRP-2* ranging from 21 to 133% of that expressed by Me18732, *TRP-2-INT2* was undetectable (Table 3, and Figs. 7 and 8). Furthermore, *TRP-2-INT2* was still at the lower threshold of detectability (i.e., 0.1%) in a melanocyte line that displayed more than a sixfold increase (614%) of *TRP-2* compared with Me18732 (Table 3, and Fig. 7). Transduction of melanocyte lines with the *HLA-A\*6801* allele confirmed the absence of endogenous *TRP-2-INT2*<sub>222-231</sub> expression at levels allowing CTL 128 recognition (Fig. 9).

Northern blot analysis has indicated that *TRP-2* and, in general, pigment gene expression are low in human melanoma compared with the average expression in melanocytes (33). This finding was confirmed by our quantitative PCR analysis in which two out of four melanocyte lines had increased levels of *TRP-2* over that of Me18732, one of the tumors that displayed the highest level of expression for this gene (Tables 2 and 3). We had similar findings with *gp100*, whose levels of expression in three out of four melanocyte lines were 1.3–16 times higher than those of Me18732. Given the lower expression of *TRP-2* in transfected compared with normal cells, these results rule out the possibility that the presence of the unspliced form of the *TRP-2* gene in melanoma is simply related to high levels of gene transcription. Rather, our findings suggest that the splicing pathway that involves this gene is operating in normal melanocytes but is altered in melanoma cells, allowing the transport of certain intron-containing transcripts into the cytoplasm. As also observed for *gp100-in4* by Robbins et al., the intron-encoded epitope is expressed after transfection into COS-7 cells (10). Lack of adjacent regulatory sequences might be responsible for the splicing failure in COS-7 cells (34), since our cDNA clone is probably



**Figure 9.** Lack of recognition by CTL 128 of HLA-A\*6801<sup>+</sup>-transduced melanocytes. DL-1 melanocytes and the SK23-MEL lines were transduced by a retroviral vector encoding *HLA-A\*6801*.  $1.5 \times 10^3$  CTLs were added to  $10^4$  transduced and untransduced stimulator cells, and the TNF content of the supernatant was tested 20 h later on WEHI-164.13 cells. Efficiency of transduction was evaluated by the ability of the transduced cells to present the *TRP-2-INT2*<sub>222-231</sub> peptide.

truncated at its 3' end by a fortuitous priming of a polyadenine stretch in intron 4. However, the presence of unspliced mRNAs in the cytoplasm cannot be considered a distinguishing feature of transformed cells, but appears to be gene specific. Indeed, weak consensus sequences at intron-exon boundaries may determine a very low splicing efficiency and ubiquitous retention of intervening sequences in mRNA even in normal cells (35). Moreover, although the mechanism has not been investigated, expression of the unspliced form of the differentiation antigen *gp100* (*gp100-in4*) has been found in normal melanocytes at levels comparable to or even higher than that found in melanomas (10), a finding confirmed by our quantitative PCR analysis (Table 3).

*TRP-2-INT2* codes for a putative protein of 237 amino acids that runs, using the same ORF of *TRP-2*, from the start codon in position 400 to the terminator site (nt 1111) located in intron 2, just 18 nt downstream of the peptide-coding region. Wild-type *TRP-2* protein (519 amino acids) was identified as the DOPAchrome tautomerase involved in melanin biosynthesis (17, 36). The *TRP-2-INT2* translation product, devoid of the transmembrane domain, is expected to have a different localization from *TRP-2*, which exists as a melanosome membrane-bound form. Therefore, the functional role, if any, of *TRP-2-INT2* in the tumor cells needs to be addressed.

Although the minimum level of *TRP-2-INT2* mRNA expression allowing CTL-mediated recognition has not been established, an HLA-A\*6801 melanoma line expressing in its cytoplasmic compartment 0.2% of the level found in Me18732 failed to induce TNF production by CTL 128. However, a melanoma line (Me 2559/1) expressing *TRP-2-INT2* at levels equal to 6% of that found in Me18732 (Fig. 1, and Table 2) was recognized. On the basis of the frequency of hybridizing cDNA colonies, Me18732 appears to express ~20 *TRP-2-INT2* mRNA molecules per cell (data not shown). Accordingly, 6% expression corresponds to one molecule per cell and could be considered the minimum level of *TRP-2-INT2* that allows CTL 128-mediated recognition. This level of expression is lower than that required for CTL recognition of most of the other melanoma antigens (37, 38), suggesting that CTL 128 is a high-affinity effector. The HLA-binding affinity of the *TRP-2-INT2*<sub>222-231</sub> peptide is unknown, but the very low concentration (100 pM) required to sensitize cells to

lysis (see Fig. 5) supports this hypothesis. Detection of high-affinity effectors specific for epitopes encoded by intron 2 suggests that this sequence may be a source of "non-self" antigenic epitopes. Indeed, CTLs recognizing autoantigens have been shown to escape intrathymic clonal deletion only when expressing low-affinity receptors, while high-affinity CTLs against autoantigens are deleted (39). Furthermore, in peptide sensitization assays, many differentiation antigen-derived peptides are active only at concentrations higher than those of the tumor-specific peptides (40). Taken together, these observations strongly indicate that *TRP-2-INT2* codes for a new type of tumor antigen shared among melanomas, but absent in normal tissues.

Analysis of the structural similarities in the antigen-binding groove of HLA molecules and the identification of cross-reactive peptides have allowed grouping of HLA alleles into supertypes (41). Based on its peptide-binding characteristics, HLA-A\*6801 belongs to the HLA-A3-like supertype that also comprises A3, A11, A31, and A\*3301 (28). Among the alleles of the A3-like supertype, recognition of *TRP-2-INT2*<sub>222-231</sub> peptide was also observed when it was presented by A\*3301, which, along with A\*6801, is expressed by 13% of the world's population (28). It has been previously reported that identical peptides derived from viral proteins (HBcAg from hepatitis B) or from tumor antigens (*TRP-2*) can be recognized in the context of different HLA alleles, HLA-A31 and A68 or HLA-A31 and A33, respectively, by specific and differentially HLA-restricted CTL clones (42, 43). Moreover, in agreement with our findings, single T cells have been shown to recognize a *TRP-2* melanoma-associated peptide when presented by both HLA-A31 and A\*3301 (43).

Apart from the *TRP-2-INT2*<sub>222-231</sub> peptide, screening introns and intron-exon junctions of *TRP-2-INT2* for the presence of known motifs revealed peptide sequences that are predicted to bind with high efficiency several HLA-A and -B alleles, including HLA-A2, frequently present in all major ethnic groups. This suggests that other tumor antigens with the same degree of lineage and tumor specificity may be expressed by melanoma cells in the context of more frequent HLA-A and -B alleles. Furthermore, it is possible that other specific antigens can be generated in melanoma and other tumors by mechanism(s) of altered splicing of lineage-related genes.

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We thank Dr. K. Fleischhauer (Istituto Scientifico San Raffaele) for DNA typing of the *HLA-A* locus, and Dr. S. Battocchio (Division of Pathology, Istituto Nazionale Tumori) for providing mammary skin tissue samples. We are also indebted to Ms. M.T. Radice for oligonucleotide synthesis, Ms. D. Penso for help with the automated DNA sequencer, Ms. A. Molla for technical assistance, and Mr. M. Azzini and Ms. G. Barp for editing.

R. Lupetti is the recipient of a fellowship from the Italian Association for Cancer Research (AIRC), Milan. This work was supported in part by grants from the AIRC and from the European Community Biomed2 Program (BMH4-CT95-1627).

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Received 11 July 1997 and in revised form 8 July 1998.

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