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

By Raffaella Lupetti,* Patrizia Pissara,* Alessandro Verrecchia,* Cinthia Farina,* Gabriella Nicolini,* Andrea Anichini,* Claudio Bordignon,† Mariafulisa Sensi,* Giorgio Parmiani,* and Catia Traversari‡

From the *Division of Experimental Oncology D, Istituto Nazionale Tumori, 20133 Milan, Italy; and the ‡Gene Therapy Program, Istituto Scientifico San Raffaele Hospital, 20132 Milan, Italy

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 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)\(^1\) 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 a 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, Me-2559/1, Me12657, Me17088/1, Me4023) and primary (Me20842) melanoma cell lines, established in our laboratory, the carcinoma cell 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, M22-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 Dermopatico dell’Immacolata, Rome, Italy), were kept in melanocyte growth medium (MGM-2; Clonetics). 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.

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

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 LN GFR) driven by the SV40 promoter was constructed as described previously (19). An amphotropic packaging cell line was established by infection of the GP4 env Am 12 line and immunoselection for \Delta LN GFR expression by the use of magnetic beads (Dynabeads M-450; Dynal A/S, Norway). 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 the target transcript.

Separation of Nuclear and Cytoplasmic RNA. Cells (2–3 × 10^6) were harvested, washed with PBS, and then carefully resuspended in 400 μl 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 vigorously vortexing for 10 s and then centrifuged for 30 s at 15,000 rev/min. The supernatant containing the cytoplasmic RNA was washed with buffer A to eliminate residual cytoplasmic or nuclear material (i.e., DNA and nuclear mRNA) in the cytoplasmic compartment. Cytoplasmic and nuclear RNAs were purified from the cell fractions using the RNaseasy 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 μl of water containing 200 μM of each dNTP, 0.6 μM of each primer, 1× 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'-TTCCGGCAGAAACATCCATCC-3') and the TRP-2-INT2 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'-ACCTCACAACACTCATCCTT-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 sequenced to 10 pg/μl, a concentration equivalent to 1,026,119,1,193,338, and 1,251,907 molecules/μl, 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 SK BR 3, negative for expression of TRP-2, TRP-2-INT2, and gp100, was added. TRP-2 was amplified with primers PR 3 and TRP-2-L. For TRP-2-INT2, in addition to PRIT-1b and INT2-1260, we also tested the sense primer PR 15 (5'-AATTGGGAGTCCAGTGGCC-3'), located in intron 2, and the antisense primer PR 16 (5'-ATGAGCATCCAGGCACT-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, 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 M e18732 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, TRP-2-INT2, and gp100 in4 was determined experimentally for the reference M e18732 cDNAs, starting from 2 μl 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, 9 plus n cycles. TRP-2 was amplified by primer pair PR 3 plus TRP-2-L; TRP-2-INT2 by PR 15 plus PR 16; 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 M e18732. 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 M e18732 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 PR 3...
plus TRP-2L (TRP-2) the threshold was 0.01%, and for PR 15 plus PR 16 (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 M. e18732 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 amplified 2–INT2 in the reference melanoma. 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.dcmr.nih.gov:80/molbio/hla_bind/) for the presence of potential HLA-A*68011–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 °C. Various concentrations of the peptides were added to 3HCr-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*6801 as Restriction Element for CTL 128. Melanoma cell line M. e18732 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*6801 (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 melanoma 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, together 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 DNA Encoding the Melanoma Antigen Recognized by CTL 128. A cDNA library was constructed in expression vector pcDNA3.1 with poly(A) + RNA extracted from M. e18732 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 melanoma cell line that was not recog-
nized by CTL 128 acquired, when transfected with cDNA 131, the ability to induce TNF release, and became sensitive to lysis by CTL 128 (data not shown). The sequence of the cDNA 131 proved to be 3535 bp long (Fig. 3), with nucleotides (nt) 1–994 and 3081–3347 identical to two noncontiguous regions of TRP-2 cDNA (17), which has been recently identified as a melanoma antigen of the melanocyte lineage (12). The first region contained exon 1, whose 5′-UTR lacked the first 15 bases, and exon 2, whereas the second region encompassed exons 3 and 4, of the TRP-2 gene, respectively. The sequences between nt 995 and 3080 and that downstream of nt 3347 showed no significant homology with any sequence recorded in the data-banks, and were likely to be retained intron sequences. Insignificant homology with any sequence recorded in the database was observed and 3080 and that downstream of nt 3347 showed no significant homology with any sequence recorded in the data-banks, and were likely to be retained intron sequences. Insignificant homology with any sequence recorded in the database was observed.

Figure 3. Sequence of cDNA 131. The ATG start codon of the TRP-2 cDNA, and the first stop codon (TAG) in the same ORF, are doubly underlined. Exonic and intronic sequences are uppercase and lowercase, respectively. Positions of the primers used in the study for amplification of TRP-2–INT2 were indicated by horizontal arrows. The nucleotide sequence downstream of nt 3347 of cDNA 131 proved to be 3535 bp long (Fig. 3), with nucleotides (nt) 995–3080 (2086 nt) was comparable with intron 2 of TRP-2, truncated at nt 176. Sequencing of intron 4, amplified by PCR from genomic DNA of Me18732, revealed the presence of a polyadenine stretch of 10 nt, starting from nt 177 (data not shown). This finding supports the hypothesis of a fortuitous priming of this sequence by the oligo-dT used for construction of the cDNA library. Thus, cDNA 131 appeared to be composed of a partially spliced form of the melanocyte differentiation antigen TRP-2, containing exons 1–4 with retention of intron 2 and the initial portion of intron 4.

Identification of the Sequence Encoding the Peptide Recognized by CTL 128. Three subfragments of ~1.5, 0.2, and 2 kb, respectively (5′-end 1500, 200, and 3′ end; Fig. 4), were obtained by digesting cDNA 131 with HindIII, then cloned into pcDNAI vector and transfected into COS-7 cells along with pcDNA3/A*6801. At this step, the presence in the 200 and 3′-end fragments of start codons regulating their translation was not investigated. The level of TNF released by CTL 128 in the presence of COS-7 cells transfected with the 5′-end 1500 fragment was comparable to that stimulated by cDNA 131 (Fig. 4), indicating that the antigenic peptide was encoded within this region. Since CTL 128 was not stimulated by COS-7 cells cotransfected with both the fully spliced TRP-2 cDNA and HLA-A*6801 (Fig. 4), we hypothesized that the sequence coding for the antigen recognized by CTL 128 could be entirely or partially located in the intronic portion of the TRP-2 gene present in cDNA 131. This notion received support by the lack of recognition of COS-7 cells transfected with the 5′-end 800 cDNA fragment (Fig. 4), derived by truncation of the 5′-end 1500 fragment at a BstUI site and comprising exon 1 and half of exon 2. The intronic localization of the sequence encoding the antigenic peptide was confirmed by the ability of a PCR-amplified fragment encompassing the first 434 bp of intron 2 (INT2-434; see Materials and Methods) to convey expression of the antigen (Fig. 4). A sequence that coded for a decapeptide (EVISCKLIK; Fig. 3) that possessed anchor residues (positions 2, 9, and 10) corresponding to the HLA-A*6801 peptide-binding motif (27) was present in the same ORF of the previous exons.

The sense primer KS-INT2 (which generates an ATG sequence that coded for a decapeptide (EVISCKLIK; Fig. 3) that possessed anchor residues (positions 2, 9, and 10) corresponding to the HLA-A*6801 peptide-binding motif (27) was present in the same ORF of the previous exons.) was used to generate fragments including (INT2-166) or not (INT2-107) the entire putative peptide-encoding sequence. Since CTL 128 was not stimulated by COS-7 cells cotransfected with both the fully spliced TRP-2 cDNA and HLA-A*6801 (Fig. 4), we hypothesized that the sequence coding for the antigen recognized by CTL 128 could be entirely or partially located in the intronic portion of the TRP-2 gene present in cDNA 131. This notion received support by the lack of recognition of COS-7 cells transfected with the 5′-end 800 cDNA fragment (Fig. 4), derived by truncation of the 5′-end 1500 fragment at a BstUI site and comprising exon 1 and half of exon 2. The intronic localization of the sequence encoding the antigenic peptide was confirmed by the ability of a PCR-amplified fragment encompassing the first 434 bp of intron 2 (INT2-434; see Materials and Methods) to convey expression of the antigen (Fig. 4). A sequence that coded for a decapeptide (EVISCKLIK; Fig. 3) that possessed anchor residues (positions 2, 9, and 10) corresponding to the HLA-A*6801 peptide-binding motif (27) was present in the same ORF of the previous exons. The sense primer KS-INT2 (which generates an ATG start site with the appropriate Kozak consensus sequence) was used to generate fragments including (INT2-166) or not (INT2-107) the entire putative peptide-encoding sequence. Only fragment INT2-166 was able to stimulate TNF release by CTL 128 (Fig. 4). Both 10- and 9-mer peptides, EVISCKLIK and EVISCKL, were then synthesized and incubated with the HLA-A*6801 homozygous LCL line LB. The decapeptide was able to sensitize LB cells to lysis by CTL 128, with half-maximum lysis ob-
tained at a concentration of ~100 pM, whereas the nonapeptide had a very low efficiency (Fig. 5). A control peptide, WTGPNCERKK, 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–INT2222-231.

To exclude the possibility that TRP-2–INT2222-231 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–INT2222-231 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–INT2222-231 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).
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%) | 16/19 (84%) |
| Melanocyte cell lines                                   | 0/4        | 4/4   |
| Fresh melanoma samples                                 | 7/13 (54%) | 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.

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

| Melanoma cell lines | R recognition by CTL 128 | TRP-2-INT2* | TRP-2* |
|---------------------|--------------------------|-------------|--------|
| M e18732            | +                        | 100         | 100    |
| M e12657            | +                        | 187         | 34     |
| M e20842            | +                        | 28          | 17     |
| M e2559/1           | +                        | 6           | 5      |
| M e4023             | +                        | 55          | 22     |
| SK23-MEL-A *6801†   | +                        | 15          | 35     |
| MZ2-MEL-A *6801†    | −                        | 0.4         | 0.5    |
| M e17088/1          | −                        | <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. M e18732 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.

†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 cytomtery with specific mAbs (data not shown).
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+ 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–INT2222-231 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

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

| Cytoplasmic mRNA | TRP-2–INT2 | TRP-2 | gp100-in4 | gp100 |
|------------------|-----------|-------|-----------|-------|
| 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%.
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).

**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.

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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 sensitive quantitative RT-PCR, could not be evidenced in Northern blots (data not shown) and probably accounts for the percentage of TRP-2+ fresh melanomas that fail to express TRP-2-INT2. In all normal tissues containing TRP-2+ 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 expression at levels allowing CTL 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 transformed 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
The TRP-2–INT2222-231 peptide is unknown, but the very high-affinity effector. The HLA-binding affinity of other melanoma antigens (37, 38), suggesting that CTLs may be expressed by melanoma cells in the context of more frequent HLA 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.

As a result of the minimum level of TRP-2–INT2 mRNA expression, allowing quantification 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 O R F 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 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 in its cytoplasmic compartment 0.2% of the level 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–INT2222-231 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–INT2222-231 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).

A 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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