Immunity, Vol. 6, 199–208, February, 1997, Copyright ©1997 by Cell Press

Characterization of an Antigen That Is Recognized on a Melanoma Showing Partial HLA Loss by CTL Expressing an NK Inhibitory Receptor

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Summary

Melanoma lines MEL.A and MEL.B were derived from metastases removed from patient LB33 in 1988 and 1993, respectively. The MEL.A cells express several antigens recognized by autologous cytolytic T lymphocytes (CTL) on HLA class I molecules. The MEL.B cells have lost expression of all class I molecules except for HLA-A24. By stimulating autologous lymphocytes with MEL.B, we obtained an HLA-A24-restricted CTL clone that lysed these cells. A novel gene, *PRAME*, encodes the antigen. It is expressed in a large proportion of tumors and also in some normal tissues, albeit at a lower level. Surprisingly, the CTL failed to lyse MEL.A, even though these cells expressed the gene *PRAME*. The CTL expresses an NK inhibitory receptor that inhibits its lytic activity upon interaction with HLA-Cw7 molecules, which are present on MEL.A cells and not on MEL.B. Such CTL, active against tumor cells showing partial HLA loss, may constitute an intermediate line of anti-tumor defense between the CTL, which recognize highly specific tumor antigens, and the NK cells, which recognize HLA loss variants.

Introduction

During the last 5 years, several human tumor antigens recognized by autologous cytolytic T lymphocytes (CTL) have been identified. So far three classes of antigen can be distinguished. The first class comprises antigens encoded by genes, such as *MAGE1*, *MAGE3*, *BAGE*, and *GAGE*, that are expressed in tumors of different histological origins, but not in normal tissues other than testis or placenta (van der Bruggen et al., 1991, 1994a, 1994b; Traversari et al., 1992a; Gaugler et al., 1994; Boël et al., 1995; Van den Eynde et al., 1995; Herman et al., 1996). The second class contains differentiation antigens encoded by genes that are only expressed in melanocytes and in melanoma cells: *tyrosinase* (Brichard et al., 1993, 1996; Wölfel et al., 1994; Kang et al., 1995), *Melan-A*$^{MART-1}$ (Coulie et al., 1994; Kawakami et al., 1994a; Castelli et al., 1995), *gp100*$^{Pmel\ 17}$ (Cox et al., 1994; Kawakami et al., 1994b, 1995), and *gp75*$^{TRP-1}$ (Wang et al., 1996). The third class includes antigens produced by point mutations in genes that are expressed ubiquitously, such as *MUM-1* (Coulie et al., 1995), CDK4 (Wölfel et al., 1995), β-catenin (Robbins et al., 1996), and HLA-A2 (Brändle et al., 1996).

We have studied the pattern of antigens recognized by autologous CTL on two melanoma cell lines derived from metastases that were removed from patient LB33 several years apart (Lehmann et al., 1995). Cell line LB33-MEL.A was obtained after surgery in 1988. A large number of CTL clones directed against MEL.A cells, a clonal line derived from LB33-MEL.A, were obtained with blood lymphocytes collected from the patient in 1990. Using a method to evaluate the frequency of precursors of anti-tumor CTL (CTLp) in the blood of melanoma patients, we found that patient LB33 clearly stood out among 15 patients with a frequency of anti-tumor CTLp of 1 out of 1000 blood mononuclear cells (Coulie et al., 1992; P. G. C., unpublished data). In vitro selection of melanoma cells that were resistant to these CTL clones indicated that at least five different antigens were recognized on the MEL.A cells by autologous CTL. These antigens are presented by HLA-A28, B13, B44, and Cw6. Antigen LB33-B, presented by HLA-B44 molecules, was found to be encoded by a gene that is expressed ubiquitously and that is mutated in the LB33 melanoma cells (Coulie et al., 1995). The patient remained disease-free from 1989 until 1993 when a metastasis was detected and was used to obtain the clonal cell line MEL.B, which proved resistant to lysis by all the CTL clones directed against the MEL.A cells. The MEL.B cells had lost expression of all HLA class I molecules except for HLA-A24, suggesting that in patient LB33 the melanoma cells have lost the expression of several HLA molecules under the selective pressure of an anti-tumor CTL response (Lehmann et al., 1995).

Using MEL.B cells to stimulate blood lymphocytes collected from the patient in 1994, we obtained CTL that lysed these cells. CTL clones were derived that recognized a new antigen, LB33-E, which is presented by HLA-A24 molecules. Surprisingly, the MEL.A cells were not lysed by these anti-LB33-E CTL (Lehmann et al., 1995). Here we report the identification of antigen LB33-E and provide an explanation for the specificity of the anti-LB33-E CTL for the MEL.B cells.

Results

A CTL Clone Recognizing MEL.B Cells

Blood lymphocytes collected from patient LB33 in 1994 were stimulated in vitro with irradiated MEL.B cells, a

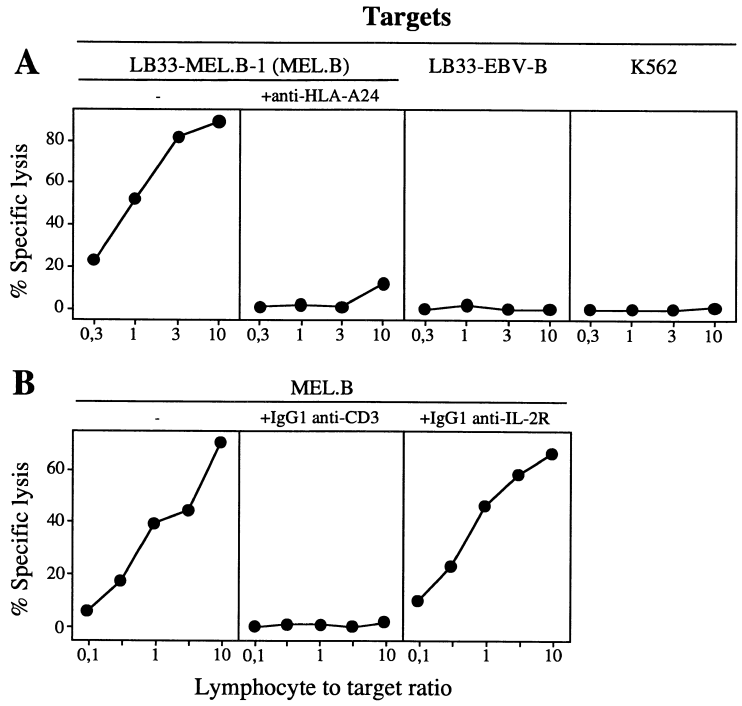

Figure 1. Lytic Activity of CTL Clone 17

(A) Sensitivity of ^{51}Cr-labeled target cells to lysis by anti-LB33-E CTL clone 17. LB33-MEL.B-1 (MEL.B) is a clonal subline of the melanoma cell line LB33-MEL.B derived from a metastasis resected from patient LB33 in 1993. These cells have lost expression of all HLA class I molecules except HLA-A24. LB33-EBV-B is an EBV-transformed B cell line from patient LB33. Anti-HLA-A24 monoclonal antibody C7709A2 was used to inhibit lysis of MEL.B cells by the CTL by adding a 1:45 dilution of ascitic fluid from mice inoculated with the hybridoma cells.

(B) Inhibition of the lytic activity of CTL 17 by an anti-CD3 monoclonal antibody. Target cells and CTL were incubated over 2.5 hr in the absence or presence of anti-CD3 monoclonal antibody UCHT-1 (20 μg/ml) or isotype-matched anti–interleukin-2 receptor β antibody CF1, and chromium release was measured.

clonal line derived from a melanoma metastasis resected from the patient in 1993. From the responder T cell population we isolated CTL clone 17, which lysed MEL.B cells but did not lyse autologous Epstein–Barr virus (EBV)-transformed B cells or natural killer (NK) target cells K562 (Figure 1A). The antigen recognized by CTL 17 was named LB33-E. By labeling MEL.B cells with anti–HLA class I monoclonal antibodies and measuring HLA class I gene expression, we observed that these cells had lost expression of all HLA class I alleles with the exception of HLA-A24 (Lehmann et al., 1995). As expected, an anti-HLA-A24 monoclonal antibody inhibited recognition by CTL 17, indicating that antigen LB33-E was presented by HLA-A24 molecules (Figure 1A). The CTL clone was typed TCR $\alpha\beta^+$, $CD3^+$, $CD4^-$, and $CD8^+$. An anti-CD3 monoclonal antibody abolished the lysis of MEL.B cells by CTL 17 (Figure 1B), indicating that the T cell receptor (TCR) of the CTL was involved in the recognition of antigen LB33-E.

Identification of a cDNA Coding for Antigen LB33-E

A cDNA library prepared with RNA from LB33 melanoma cells was cloned into expression vector pcDNAI/Amp. This plasmid contains the simian virus 40 (SV40) origin of replication resulting in a high copy number of the transfected plasmids in COS-7 cells, which express the SV40 T antigen (Seed and Aruffo, 1987). The cDNA library was divided into 900 pools of about 100 bacteria. Plasmid DNA was extracted from each pool and cotransfected into microcultures of COS-7 cells with an HLA-A*2402 cDNA clone isolated from the LB33 melanoma cells. After 24 hr the transfected cells were tested for the expression of antigen LB33-E by adding CTL 17 and measuring the production of tumor necrosis factor (TNF) after 24 hr. One pool of cDNA proved positive. It was subcloned, and cDNA clone 5E10 was found to transfer the expression of antigen LB33-E into COS-7 cells (Figure 2A).

When a Northern blot prepared with RNA of LB33 melanoma cells was hybridized with cDNA 5E10, a band of ±2.2 kb was observed, suggesting that the 1.5 kb 5E10 clone was incomplete. This cDNA clone was used to screen another cDNA library prepared with RNA from LB33 melanoma cells, and a novel cDNA clone, Hi2, was obtained. cDNA Hi2 was 2148 bp long and included the sequence of cDNA 5E10. Stable transfectants were obtained with cDNA Hi2 in a human HLA-A24 leukemia cell line. They were lysed by CTL 17, indicating that the expression of the antigen was not dependent on the high gene copy number present in COS-7 cells (Figure 2B).

Sequence and Expression of the Gene *PRAME*

cDNA Hi2 contains an open reading frame encoding a putative protein of 509 amino acids that has no signal sequence and shows no significant homology with known proteins (Figure 3). Two parts of cDNA Hi2 were identical to anonymous sequences present in data banks: nucleotides 1486–1589 corresponded to a sequence expressed by myeloid leukemia cells K562, and nucleotides 1983–2128 matched a cDNA fragment from promyelocytic leukemia cells HL-60 (Murakawa et al., 1994). In addition, nucleotides 1736–2067 of cDNA Hi2 were 97% homologous to a 332 bp cDNA from human testis (Pawlak et al., 1995). We propose to name *PRAME* (for preferentially expressed antigen of melanoma) the gene that corresponds to cDNAs 5E10 and Hi2.

Northern blots prepared with poly(A)$^+$ RNA of human tumor cell lines and normal tissues were hybridized with a probe corresponding to cDNA Hi2. Two bands of approximately 2.2 and 3 kb were observed with the RNA of several tumor cell lines, including leukemias K562 or HL-60, cervical and lung carcinomas, and melanoma

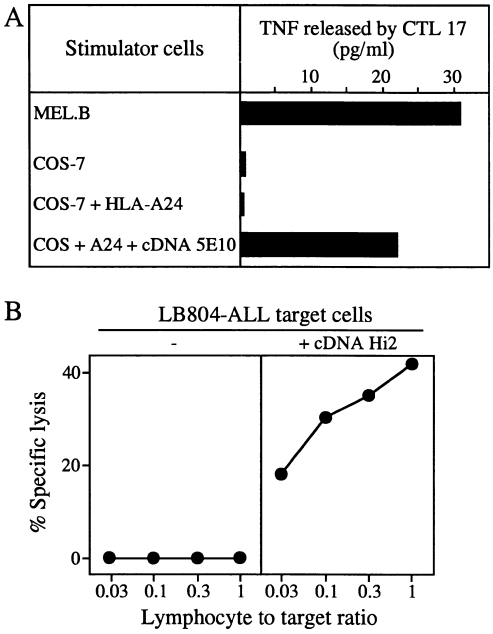

Figure 2. Identification of a cDNA Clone Encoding the Antigen Recognized by CTL 17

(A) Stimulation of CTL 17 by COS-7 cells cotransfected with vector pcDNAI/Amp containing cDNA clone 5E10 and vector pcDNA3 containing the autologous HLA-A*2402 cDNA. Control stimulator cells included the MEL.B cells and COS-7 cells transfected with HLA-A24 alone. The concentration of TNF released in the medium was measured using the TNF-sensitive WEHI-164 c13 cells.

(B) Lysis by CTL 17 of a clone obtained by transfecting HLA-A24 acute lymphocytic leukemia cell line LB804-ALL with expression vector pEF-PL3 containing the cDNA clone Hi2. Control target was the untransfected cell line LB804-ALL.

(Figure 4). No bands were obtained with the RNA of normal adult tissues except testis.

The expression of the *PRAME* gene in normal tissues was also studied by reverse transcription–polymerase chain reaction (RT–PCR) amplification (Figure 5). As expected based on the results obtained with the Northern blots, high levels of *PRAME* expression were found in testis. Endometrium samples expressed the *PRAME* gene at levels corresponding to 5%–30% of that found in the LB33 melanoma cells. Samples of ovary and adrenals expressed 2%–5% of that level. Lower levels of expression were detected in some samples of other tissues, including kidney, brain, or skin. We verified that the sequence of these PCR products amplified from normal tissues corresponded to that of *PRAME*.

A wide variety of tumor samples expressed the *PRAME* gene at a level exceeding 3% of that found in the LB33 melanoma cells (Table 1). The highest proportions of positive tumors were found among melanomas (91%), lung squamous cell carcinomas (78%) or adenocarcinomas (46%), renal carcinomas (41%), sarcomas (39%), head and neck squamous cell carcinomas (39%), and acute leukemias (33%). A high level of gene expression, comparable with that found in the LB33 melanoma cells, was found in most melanoma samples, 24% of lung squamous cell carcinomas, 35% of sarcomas, and 2% of renal carcinomas.

Absence of Lysis by CTL 17 of Several HLA-A24 Melanoma Lines Expressing *PRAME*

When we tested the lytic activity of CTL 17 on a panel of HLA-A24 melanoma cell lines expressing the *PRAME* gene at a high level, we observed that some of these cell lines were sensitive to lysis by the CTL, but several others were totally resistant (data not shown). Remarkably, CTL 17 also failed to lyse MEL.A cells, a clonal line that was derived from a metastasis resected from patient LB33 in 1988 (Figure 6A), even though these cells expressed *PRAME* and HLA-A24 at the same level as the MEL.B cells.

A major difference between the MEL.A and MEL.B cells is that the former also express all the other HLA class I alleles of patient LB33: A28, B13, B44, Cw6, and Cw7 (Lehmann et al., 1995). Our observation was therefore reminiscent of the inhibition of the cytolytic activity of NK cells, and a fraction of T cells, by major histocompatibility complex (MHC) class I molecules expressed on the target cells (Ljunggren and Karre, 1985; Moretta et al., 1992; Phillips et al., 1995). We therefore tested the lytic activity of CTL 17 against MEL.A cells in the presence of a monoclonal antibody recognizing HLA-B and HLA-C molecules. In these conditions, MEL.A cells were very efficiently lysed by the CTL (Figure 6A). We concluded that antigen LB33-E was expressed by both the MEL.A and MEL.B cells and that HLA-B or C molecules protected MEL.A from lysis by CTL 17.

To identify the inhibitory HLA-B or C molecules, we tested the lytic activity of CTL 17 on MEL.A-1.1.1, an HLA loss variant derived from MEL.A cells by several rounds of selection with autologous CTL clones restricted by HLA-A28 and HLA-B44 molecules. MEL.A-1.1.1 had lost an entire HLA class I haplotype: HLA-A28, B44, and Cw7 (Lehmann et al., 1995). These cells were efficiently lysed by CTL 17 (Figure 6B). No significant inhibition of lysis was observed after transfection of MEL.A-1.1.1 cells with an HLA-B44 construct, whereas transfection with a cDNA clone encoding HLA-Cw7 protected these cells from lysis by the CTL. These results suggested that an inhibitory receptor binding to HLA-Cw7 prevented CTL 17 from lysing MEL.A.

Involvement of NK Inhibitory Receptor p58.2

Several HLA class I–specific NK cell inhibitory receptors have been identified (Moretta et al., 1996). The inhibitory receptors specific for HLA-C molecules are represented by two members of the p58 molecular family, which belongs to the immunoglobulin superfamily (Colonna and Samaridis, 1995; Wagtmann et al., 1995; Moretta et al., 1996). The p58.1 receptors are recognized by the EB6 monoclonal antibody and interact with several HLA-C molecules, including Cw2, 4, and 6 (Moretta et al., 1990b). The p58.2 receptors are glycoproteins of 58 kDa recognized by the GL183 antibody. They show specificity for a subset of HLA-C molecules that contain serine and asparagine at positions 77 and 80, respectively, such as HLA-Cw1, 3, 7, and 8 (Colonna et al., 1993; Moretta et al., 1993; Biassoni et al., 1995; Colonna, 1996).

CTL 17 was labeled with monoclonal antibody GL183, indicating that it carried the p58.2 receptor (Figure 7).

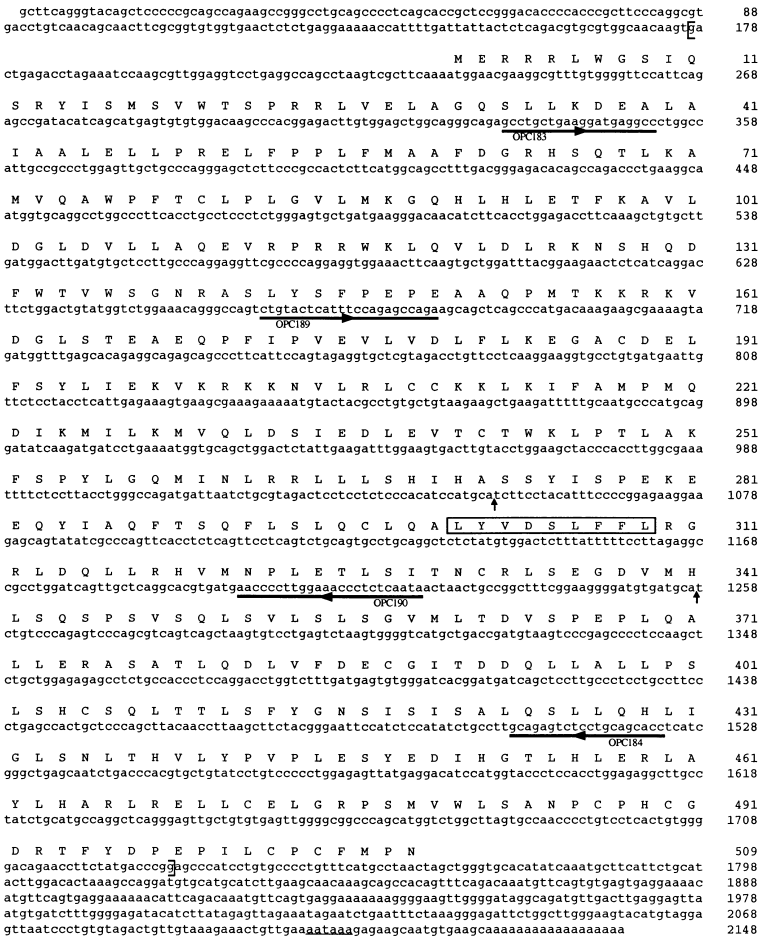

Figure 3. Sequence of cDNA Hi2 and Sequence of the Protein Encoded by the Longest Open Reading Frame

Primers OPC183, OPC184, OPC189, and OPC190 used for the analysis of *PRAME* expression by PCR are indicated by horizontal arrows. The polyadenylation signal is underlined. The LB33-E antigenic peptide is boxed. The sequence of cDNA 5E10 is delimited by the square brackets. It contains two NsiI sites, indicated with vertical arrows, which were used for the localization of the antigenic peptide.

The lytic activity of CTL 17 was tested against MEL.A cells in the presence of antibody GL183, aimed at blocking the interaction between p58.2 and HLA-Cw7 molecules (Figure 8). Addition of increasing concentrations of the antibody restored lysis of the MEL.A cells. No effect was observed with the isotype-matched monoclonal antibody EB6, which does not label CTL 17 (Figure 7), or with an anti–MHC class II monoclonal antibody that labels the CTL. These results indicated that blocking the p58.2 receptors on the CTL suppressed the inhibition of lysis of the MEL.A cells.

Taken together, the results indicate that the MEL.A cells express the *PRAME*-encoded antigen, but that they are not lysed by the anti-PRAME CTL 17 because they bear HLA-Cw7 molecules, which inhibit the lytic activity of the CTL upon engagement of the NK inhibitory receptor p58.2. As indicated above, CTL 17 recognized its target through a classical $\alpha\beta$ TCR. We also found that it did not express FcγRIII (CD16), a receptor expressed by NK cells (Figure 7).

Identification of the LB33-E Antigenic Peptide

To localize the region encoding the antigenic peptide, cDNA 5E10 was partially digested with NsiI (see Figure 3), and truncated cDNA clones were cotransfected into COS-7 cells with the HLA-A24 cDNA clone. The transfectants were tested for the expression of antigen LB33-E by adding CTL 17 and measuring the production of TNF by the CTL. The results indicated that the peptide-coding region corresponded to nucleotides 1051–1257 of cDNA Hi2.

This region contained four sequences coding for a peptide containing the HLA-A24 binding motif, namely tyrosine or phenylalanine at position 2 and phenylalanine, leucine, isoleucine, or tryptophan at position 9 or 10 (Kubo et al., 1994; Maier et al., 1994). These peptides were synthesized and incubated with EBV-transformed B cells derived from patient LB33. CTL 17 was added, together with the anti-HLA-B or C monoclonal antibody to block the inhibition by HLA-Cw7 molecules. Nonapeptide LYVDSLFFL sensitized the cells to lysis with a half-maximal effect at 100 nM (Figure 9). Ten amino acid peptides with an additional N-terminal or C-terminal residue, or an octamer without the C-terminal leucine residue, were recognized less efficiently (data not shown). In the absence of the anti-HLA-B or C antibody, EBV-transformed B cells from patient LB33 could not be sensitized at all to lysis by CTL 17, even when high concentrations of the antigenic peptide were used (Figure 9).

Discussion

We have identified a tumor antigen recognized by a CTL clone on melanoma cells that have lost the expression of several HLA molecules. The gene *PRAME*, which

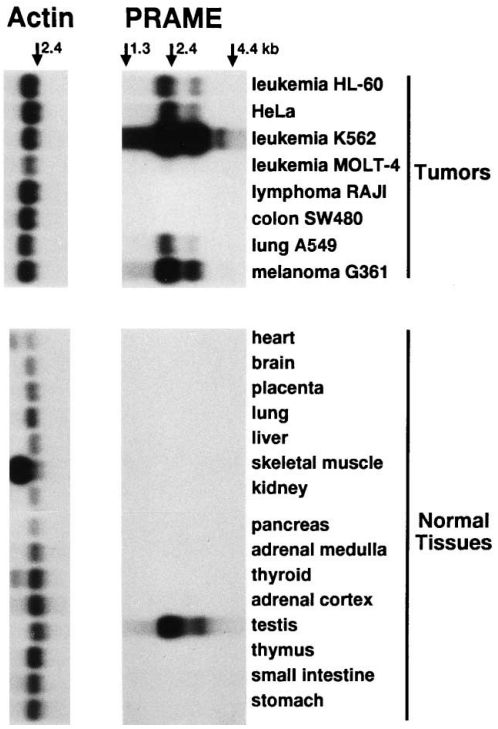

Figure 4. Northern Blot Analysis of the Expression of *PRAME* in Tumor Cell Lines and in Normal Tissues

Lanes contained 2 μg of poly(A)$^+$ RNA from tumor cell lines and from adult human tissues (Clontech). The three membranes were hybridized simultaneously with ^{32}P-labeled cDNA Hi2, washed under stringent conditions, and autoradiographed over 10 days to detect a low level of expression in the normal tissues. Control hybridization was performed on the same membranes with a β-actin probe.

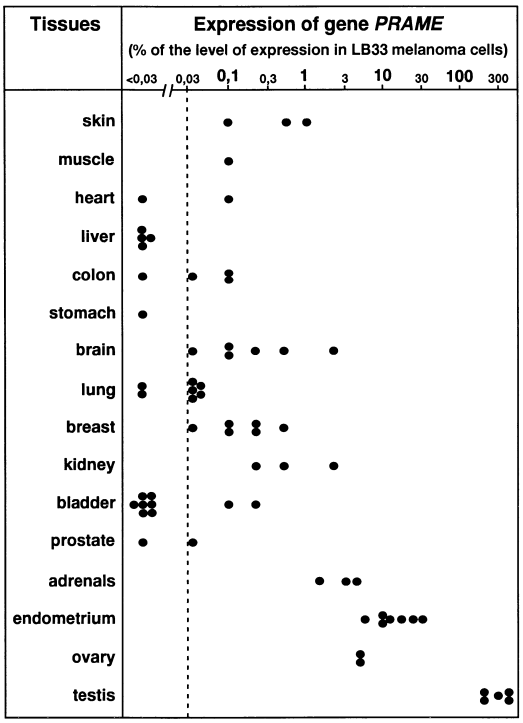

Figure 5. Expression of the *PRAME* Gene Analyzed with RT–PCR on Normal Tissues

Expression was tested by reverse transcription of total RNA and PCR amplification with primers OPC189 and OPC190 shown in Figure 3. The 561 bp product is not observed when genomic DNA is tested. A semi-quantitative measurement was obtained by combining a limiting number of PCR cycles, comparing the result with a standard curve of RNA from MEL.A cells, and making a correction for the integrity of the RNA by taking into account the expression level of the β-actin gene. Each point represents the level of *PRAME* expression found in a sample of normal tissue. The results are expressed relative to the level of expression of the *PRAME* gene measured in the MEL.A cells, which is equivalent to that measured in the MEL.B cells. The limit of detection is indicated with a broken line.

codes for this antigen, is expressed in most melanomas and lung squamous cell carcinomas and also in many sarcomas and acute leukemias. It shows a low level of expression in many normal tissues and an intermediate level in adrenals, ovary, and endometrium. The CTL clone that recognizes the *PRAME*-encoded antigen is remarkable in that, owing to the presence of a NK inhibitory receptor, it does not lyse cells that present the target antigen if they also bear the HLA-Cw7 molecule. Our observation that anti-PRAME CTL could be derived from LB33 lymphocytes collected in 1994 but not in 1990 (Lehmann et al., 1995) suggests that an anti-PRAME response occurred in vivo and provided CTL that could be restimulated in vitro. It will be interesting to find out whether or not anti-PRAME CTL always carry NK inhibitory receptors. Considering the expression of *PRAME* in some normal tissues, it may well be that anti-PRAME CTLp devoid of the inhibitory receptor undergo some form of tolerance. If this is the case, only anti-PRAME CTL with inhibitory receptors will be found. The alternative is that anti-PRAME CTLp with or without inhibitory receptor can be activated in vivo and that the response against the MEL.B cells, which have lost HLA-Cw7, happened to involve a CTL clone carrying the inhibitory receptor. In this case, anti-PRAME responses involving lymphocytes devoid of inhibitory receptor may be observed in other patients.

The expression of the *PRAME* gene shares several characteristics with that of genes of the *MAGE*, *BAGE*, and *GAGE* families. All of these genes are expressed in tumors of many different histological types. However, *PRAME* is expressed by a higher proportion of samples than any of the other genes, and it is also expressed by acute myeloid leukemias, which never express the *MAGE*, *BAGE*, or *GAGE* genes (Chambost et al., 1993; Shichijo et al., 1996). A second similarity among all these genes is their high level of expression in testis. The mouse gene family *SMAGE*, which is homologous to the human *MAGE* gene family, is expressed in testis by germinal cells (Chomez et al., 1995), and the MAGE1 and MAGE4 proteins were detected in human spermatogonia and spermatocytes (Takahashi et al., 1995). It is possible that the expression of *PRAME* in testis will also be restricted to germinal cells, which do not bear HLA molecules and should therefore not suffer from an anti-PRAME T cell response.

The activation of the *MAGE1* gene in tumor cells appears to be due to the demethylation of the promoter, and this was correlated with a genome-wide demethylation process (De Smet et al., 1996). We observed that

Table 1. Expression of PRAME by Tumor Tissues

Tumor Samples	n	Percent
Brain tumors	1 of 7	
Colorectal carcinomas	2 of 51	
Gastric carcinomas	1 of 2	
Melanomas		
Primary lesions	43 of 49	88
Metastases	144 of 152	95
Ocular	5 of 9	
Neuroblastomas	2 of 3	
Head and neck squamous carcinomas	17 of 44	39
Lung carcinomas		
Small cell lung carcinomas	1 of 4	
Non–small cell lung carcinomas		
Adenocarcinomas	12 of 26	46
Squamous cell carcinomas	51 of 65	78
Prostatic carcinomas	2 of 20	
Renal carcinomas	24 of 58	41
Bladder tumors		
Superficial	4 of 36	
Infiltrating	9 of 42	
Sarcomas	9 of 23	39
Mammary carcinomas	45 of 169	27
Thyroid carcinomas	3 of 5	
Acute leukemias	21 of 63	33
Tumor Cell Lines	**n**	**Percent**
Melanomas	72 of 74	97
Sarcomas	4 of 5	
Lung carcinomas:		
Small cell lung carcinomas	19 of 27	70
Non small cell lung carcinomas	2 of 2	
Mesotheliomas	2 of 18	
Head and neck tumors	2 of 7	
Bladder tumors	2 of 3	
Colorectal carcinomas	1 of 15	
Renal carcinomas	9 of 12	
EBV-transformed B cell lines	0 of 8	

Expression of the *PRAME* gene was tested by RT–PCR amplification of total RNA with primers OPC183 and OPC184 shown in Figure 3. The amplified product of 1191 bp was not observed when genomic DNA was tested. A semi-quantitative measurement was obtained as indicated in Figure 5, and samples were scored positive if their expression of the *PRAME* gene exceeded 3% of that found in MEL.A cells.

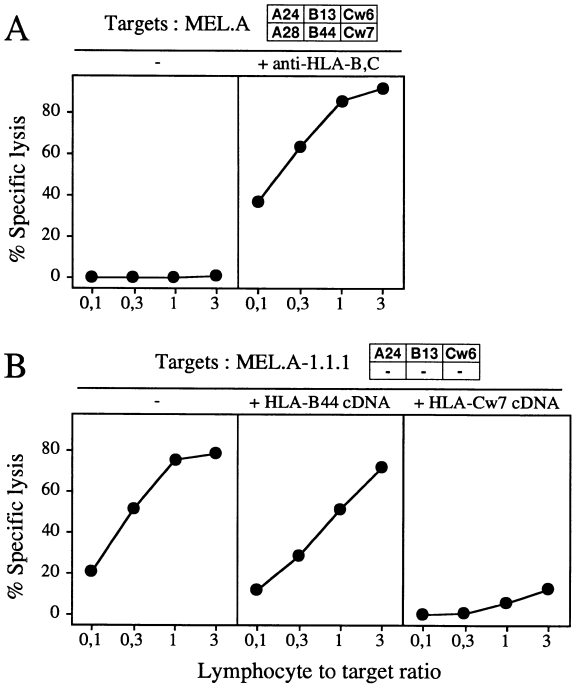

Figure 6. Inhibition of the Lytic Activity of CTL 17 by HLA Class I Molecules Expressed on the Target Cells

(A) Cytolytic activity of anti-PRAME CTL17 on autologous melanoma cells MEL.A, a clonal subline of the melanoma cell line LB33-MEL.A derived from patient LB33 in 1988. These cells express HLA-A24, A28, B13, B44, Cw6, and Cw7. Anti-HLA-B or C monoclonal antibody B1.23.2 was added in the lysis assay as a 1:60 dilution of ascitic fluid from mice inoculated with the hybridoma cells.

(B) Cytolytic activity of CTL 17 on MEL.A-1.1.1 cells. MEL.A-1.1.1 is a variant, derived from the MEL.A cells, selected in vitro for resistance to autologous CTL clones 159/3 and 159/5. It has lost the expression of a complete HLA haplotype: HLA-A28, B44, and Cw7. The MEL.A-1.1.1 cells were transfected with the autologous HLA-B*4402 or HLA-Cw*0704 alleles cloned into expression vectors, and clonal lines isolated from the transfected populations were tested for their sensitivity to lysis by CTL 17.

the treatment of primary fibroblasts or phytohemagglutinin A–stimulated blood mononuclear cells with demethylating agent 5-aza-2′-deoxycytidine activated the *MAGE1* gene and also the *PRAME* gene (data not shown). This effect of DNA demethylation may explain why *PRAME* is expressed in testis, since male germline cells undergo a genome-wide demethylation.

In contrast with the *MAGE* genes, *PRAME* is expressed in some normal tissues other than testis. Except for endometrium, which expresses up to 30% of the level found in the LB33 melanoma cells, the levels of expression in normal tissues corresponded to less than 3%–5% of that found in melanoma cells. We have observed that anti-MAGE1 CTL were unable to recognize tumor cell lines that expressed *MAGE1* at levels corresponding to less than 5% of that found in the melanoma cells that were efficiently lysed (B. L., unpublished data). It is therefore possible that the expression of *PRAME* in most normal tissues is insufficient for CTL recognition.

It is well established that lysis by NK cells is inhibited by the expression of MHC class I molecules on the target cells (Ljunggren and Karre, 1985; Moretta et al., 1992), and HLA-specific NK cell inhibitory receptors have been identified (Moretta et al., 1996). Inhibitory receptors were

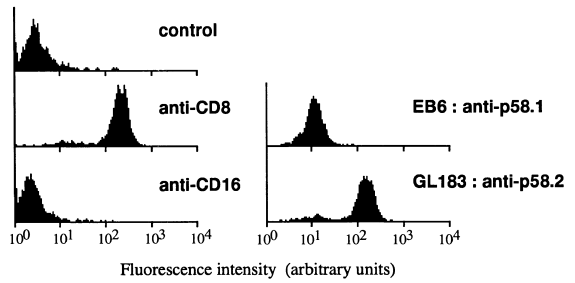

Figure 7. Expression of Surface Antigens by CTL 17

Cells were incubated with the indicated murine monoclonal antibodies or without antibody, washed, labeled with goat anti–mouse immunoglobulin antibodies coupled to fluorescein, and analyzed by flow cytometry.

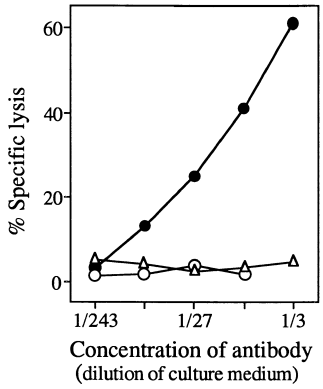

Figure 8. Lytic Activity of CTL 17 against MEL.A Cells in the Presence of an Anti-p58.2 Antibody

CTL 17 (10,000 cells per well) were incubated for 30 min at 4°C with the indicated concentrations of anti–p58.2 IgG1 monoclonal antibody GL183 (closed circles), anti–p58.1 IgG1 monoclonal antibody EB6 (open triangles), or anti–MHC class II IgG2a monoclonal antibody L243 (open circles). ^{51}Cr-labeled MEL.A cells were then added (1000 cells per well) and chromium release was measured after 4 hr.

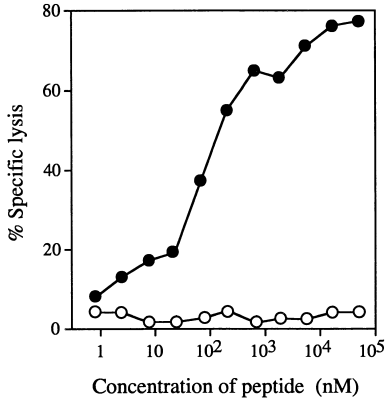

Figure 9. Lysis by CTL 17 of Autologous EBV-Transformed B Cells Incubated with the *PRAME*-Encoded Peptide LYVDSLFFL

^{51}Cr-labeled EBV-transformed B cells from patient LB33 were incubated first for 30 min at 4°C with anti-HLA-B or C monoclonal antibody B1.23.2 (closed circles; 1:50 dilution of ascitic fluid from mice inoculated with the hybridoma cells) or without antibody (open circles) and then for 30 min at 20°C with the indicated concentrations of peptide. CTL 17 were then added at an E:T ratio of 10:1, and chromium release was measured after 4 hr.

also shown to be present on a small fraction of T lymphocytes (Falk et al., 1995; Mingari et al., 1995, 1996; Phillips et al., 1995). The anti-PRAME CTL 17 bears the p58.2 inhibitory receptor, which recognizes HLA-Cw1, 3, 7, and 8 (Colonna et al., 1993; Biassoni et al., 1995). This explains why CTL 17 could not lyse some allogeneic HLA-A24 melanomas that expressed *PRAME*. These tumor cell lines were derived from patients expressing one of the HLA-C alleles that bind p58.2, and they could be lysed by CTL 17 in the presence of an anti-HLA-B or C antibody (data not shown). It is worth noting that CTL 17 does not lyse the prototype NK target K562 (Figure 1), indicating that although it bears an inhibitory receptor present on NK cells, it does not exert the MHC-nonrestricted lytic activity of these cells.

Two cDNA clones (clone 6 and clone 43) encoding p58.2 receptors have been reported (Wagtmann et al., 1995). The sequence of the p58.2 transcript present in CTL 17 encodes a mature protein of 327 residues that is identical to that coded by the p58 cDNA clone 43 (GenBank number U24075) with the exception of two amino acids. Residue 312 of the mature protein, located in the C-terminal ITIM (immunoreceptor tyrosine-based inhibition motif), is alanine in the published sequence and threonine in the cDNA of CTL 17. This threonine residue is the consensus found in the other reported p58.1 and p58.2 sequences. The second difference is residue 200 of the mature protein: the isoleucine of p58 clone 43 is replaced here by threonine, which is also the consensus found in the other p58 sequences. This amino acid is located in the C-terminal part of the second immunoglobulin domain, 25 residues apart from the transmembrane region.

CTL 17 may be representative of a novel category of anti-tumor T lymphocytes, situated between tumor-specific CTL and NK cells, that shows specificity for tumor cells that have lost expression of some, but not all, HLA class I molecules. Such partial HLA losses are frequently observed in tumors and may often result from selection by anti-tumor T cell responses (Smith et al., 1989; Garrido et al., 1995). The anti-tumor CTL with inhibitory receptors may therefore play a role after the classical CTL that recognize tumor-specific antigens presented by HLA class I molecules and before NK cells, which usually express multiple inhibitory receptors and may therefore preferentially target tumor cells that have completely lost the expression of MHC class I molecules. It is possible that some tumor-specific CTL bearing inhibitory receptors will prove to recognize, on tumor cells showing partial HLA loss, antigenic peptides that are expressed ubiquitously but are not recognized by these CTL on normal cells that express all HLA class I molecules.

Experimental Procedures

Cell Lines

The clinical course of melanoma patient LB33 (HLA-A24, A28, B13, B44, Cw6, Cw7) and the characterization of the various LB33-MEL clonal cell lines and antigen-loss variants have been described (Lehmann et al., 1995). Melanoma cell line LB33-MEL.A was derived from a cutaneous metastasis resected from patient LB33 in 1988. Clonal cell line LB33-MEL.A-1 (MEL.A) was derived from it by limiting dilution. Melanoma cell line LB33-MEL.B was derived from an intestinal metastasis resected in 1993 and used to derive clonal cell line LB33-MEL.B-1 (MEL.B) (Lehmann et al., 1995). One of the antigens recognized by autologous CTL on the MEL.A cells is encoded by a gene that is mutated in the MEL.A and MEL.B cells, but not in the normal cells of patient LB33 (Coulie et al., 1995). This demonstrates that MEL.A and MEL.B cells derive from the same tumor. Pre-B acute lymphocytic leukemia cell line LB804-ALL was derived from an HLA-24 patient. With the exception of LB804-ALL, all the tumor cell lines were cultured with Iscove's medium (GIBCO Laboratories, Grand Island, NY) containing 10% FCS (GIBCO), supplemented with L-arginine (116 mg/l), L-asparagine (36 mg/l), and L-glutamine (216 mg/l). LB804-ALL cells were cultured in Iscove's medium containing 10% human serum. COS-7 cells (American Type Culture Collection [ATCC], Rockville, MD) were maintained in DMEM (GIBCO) with 10% FCS. WEHI-164c13 cells (Espevik and Nissen-Meyer, 1986) were cultured in RPMI-1640 (GIBCO) with 5% FCS.

Anti-Tumor CTL Clones

Blood mononuclear cells of patient LB33 were isolated by Lymphoprep (Nycomed, Oslo, Norway) density-gradient centrifugation and cryopreserved. Autologous mixed lymphocyte tumor cell cultures (MLTC) and derivation and long-term culture of CTL clones were carried out as described previously (Lehmann et al., 1995). Anti-LB33-E CTL clone LB33-CTL-269/17, referred to in this report as CTL 17, was derived from lymphocytes collected in 1994 and stimulated with MEL.B cells. Anti-LB33-E CTL clone LB33-CTL-269/1, which we described previously (Lehmann et al., 1995), was derived from the same population of MLTC responder cells as CTL 17. CTL 17 was chosen for further experiments because it showed a better rate of proliferation than CTL clone 269/1.

Sensitivity of target cells to lysis by the CTL was evaluated by a standard ^{51}Cr-release assay over 4 hr. Target tumor cells were incubated for 48 hr in culture medium supplemented with IFNγ (50 U/ml; Boehringer Mannheim, Mannheim, Federal Republic of Germany). The TNF secretion assay was performed as described previously (Lehmann et al., 1995). Some lysis assays were performed in the presence of the following murine monoclonal antibodies: C7709A2, an anti–HLA-A24 immunoglobulin G2a (IgG2a) obtained in our laboratory; B1.23.2, an anti–HLA-B or C IgG2a (Lemonnier et al., 1982; Rebai and Malissen, 1983); L243, an anti–HLA-DR IgG2a obtained from ATCC; EB6 and GL183, anti–p58.1 and anti–p58.2 IgG1, respectively (Moretta et al., 1990a, 1990b); UCHT1 and CF1, anti–CD3 and anti–CD122 IgG1, respectively (Immunotech, Marseille, France). Peptides were synthesized by conventional solid-phase peptide synthesis, using Fmoc for transient N-terminal protection (Atherton et al., 1981), and characterized by mass spectrometry. They were solubilized at 20 mg/ml in DMSO, kept frozen at −20°C, and diluted in Iscove's medium immediately before use.

Construction and Screening of the cDNA Library

The cDNA library was constructed as described previously (Coulie et al., 1994). In brief, poly(A)$^+$ RNA was isolated from MEL.A cells with the mRNA extraction kit Fastrack (Invitrogen Corporation, Oxon, United Kingdom). It was converted to cDNA by using random primers. The cDNA was ligated to BstXI adaptors (Invitrogen) and inserted into the BstXI sites of expression vector pcDNAI/Amp (Invitrogen), as described in the SuperScript plasmid system kit (GIBCO BRL, Gaithersburg, MD). Recombinant plasmids were transfected by electroporation into E. coli DH5α and selected with ampicillin (50 μg/ml). The library was divided into 900 pools of about 100 cDNA clones. Each pool was amplified to saturation and plasmid DNA was extracted. COS-7 cells (2×10^4 per well) were cotransfected using the DEAE–dextran–chloroquine method (Seed and Aruffo, 1987), with about 100 ng of plasmid DNA of a pool of the cDNA library and with 100 ng of plasmid pcDNA3 (Invitrogen) containing an HLA-A*2402 cDNA isolated from the MEL.A cells (Lehmann et al., 1995). Transfected COS-7 cells were tested in a CTL stimulation assay after 24 hr.

Transfection of Tumor Cell Lines

Melanoma clonal line LB33-MEL.A-1.1.1 (MEL.A-1.1.1) (Lehmann et al., 1995) was transfected by the calcium phosphate precipitation method, as described previously (Traversari et al., 1992b). In brief, 1.2×10^6 cells were transfected with 30 μg of plasmid pcDNA3 containing an HLA-B*4402 cDNA or of vector pEF-PL3 containing an HLA-Cw*0704 cDNA. Plasmid pEF-PL3, provided by J.-C. Renauld (Ludwig Institute, Brussels), was derived from pEF-BOS (Mizushima and Nagata, 1990) by insertion of a puromycin resistance gene. The HLA cDNAs were isolated from the MEL.A cells (Lehmann et al., 1995). Clonal transfectants were isolated from the G418-resistant (1.5 mg/ml) or puromycin-resistant (0.7 μg/ml) populations. Acute leukemia cell line LB804-ALL, derived from patient LB804 (HLA-A2, A24, B35, B62, Cw3, Cw4) does not express the *PRAME* gene. These cells were transfected by electroporation with 50 μg of cDNA Hi2 cloned into expression vector pEF-PL3. A clone was isolated by limiting dilution from the puromycin-resistant (0.5 μg/ml) population.

Cloning the p58/p70 Sequences Expressed by CTL 17

RNA extracted from CTL 17 with the TRIzol reagent (GIBCO BRL) was converted to cDNA using oligo(dT) primers. Sequences corresponding to known p58.1, p58.2, or p70 products were amplified by PCR using oligonucleotides deduced from the leader sequences and 3′ ends of published sequences (Colonna and Samaridis, 1995; D'Andrea et al., 1995; Wagtmann et al., 1995): 5′-CACCATGTCGCTC ATGGTCGTCA-3′ (sense) and 5′-GACAGGGCTGTTGTCTCCCTAG-3′ (antisense). PCR conditions were 5 min at 94°C, followed by 32 cycles of amplification (94°C for 1 min, 64°C for 2 min, and 72°C for 3 min) using the Pfu DNA polymerase (Stratagene, La Jolla, CA), and followed by an incubation of 15 min at 72°C with the AmpliTaq DNA polymerase (Roche Molecular Systems, Branchburg, NJ). The amplified products were cloned into plasmid pCR3 using the Eukaryotic TA Cloning Kit (Invitrogen) and sequenced. In addition to a p58.2 cDNA clone, CTL 17 expressed sequences corresponding to other members of the immunoglobulin-related family of NK receptors such as the p50.3 or pKKA3 (GenBank number X94609; Bottino et al., 1996) and the p70 NKAT4 (GenBank number L41270; Colonna and Samaridis, 1995). The presence of these molecules on CTL 17 could not be detected using several of the available monoclonal antibodies.

DNA Sequencing and Homology Search

DNA sequencing was performed with the ΔTaq cycle sequencing kit (United States Biochemical, Cleveland, OH). Computer search for sequence homology was done with programs FASTA@EMBL–Heidelberg and blast@ncbi.nlm.nih.gov. The sequence of cDNA clone 5E10 (1554 bp) was totally included into that of cDNA clone Hi2 (2148 bp). The overlapping sequences were identical with the exception of one nucleotide, T at position 254 in cDNA Hi2 versus C in 5E10. The two sequences may correspond to two *PRAME* alleles expressed in MEL.A cells.

Northern Blot Analysis

Nylon membranes with UV-fixed poly(A)$^+$ RNA (2 μg per lane) extracted from normal adult tissues and from tumor cell lines (Clontech, Palo Alto, CA) were prehybridized for 4 hr at 65°C in 5× SSPE (20× SSPE is 3 M NaCl, 0.2 M NaH_2PO_4, 0.5 M EDTA [pH 7.4]), 10× Denhardt's solution, and 2% SDS and hybridized overnight at 65°C in the same solution containing 1.3×10^6 cpm per milliliter of a ^{32}P-labeled cDNA Hi2 probe and 100 μg/ml herring sperm DNA. The membranes were washed once at room temperature in 2× SSC, 0.05% SDS and two times for 20 min at 50°C in 0.1× SSC, 0.1% SDS and then autoradiographed.

PCR Assay for *PRAME* Expression

Total RNA extraction, reverse transcription of RNA, and PCR amplifications were performed as described previously (Van den Eynde et al., 1995). For the analysis of *PRAME* expression in tumor samples, PCR primers were OPC183 and OPC184, shown in Figure 3, and PCR conditions were 5 min at 94°C, followed by 30 cycles consisting of 1 min at 94°C, 2 min at 65°C, and 3 min at 72°C. We verified that with these conditions we were in the linear range of DNA amplification. The size of the product amplified from cDNA (1191 bp) could be distinguished from that amplified from contaminating DNA (>2.6 kb). The quality of RNA preparations was tested by PCR amplification of a human β-actin sequence. The quantities of the amplified DNA were visually assessed with agarose gels stained with ethidium bromide. They were compared with the products of RT–PCR amplifications of serial dilutions (1:1, 1:3, 1:9, and 1:27) of RNA from MEL.A cells. The level of expression of each sample was normalized for RNA integrity by taking into account the expression level of the β-actin gene. For the analysis of *PRAME* expression in normal tissues, we sought a more sensitive detection and performed PCR amplifications with more cycles. We observed that in these conditions nonspecific products were sometimes amplified with primers OPC183 and OPC184. We resorted to the use of another pair of primers, OPC189 and OPC190 (Figure 3), that allowed the specific amplification of a 561 bp fragment of *PRAME*. The size of the product amplified from contaminating DNA was different. PCR conditions were 5 min at 94°C followed by 34 cycles consisting of 30 s at 94°C, 2 min at 64°C, and 3 min at 72°C. The quantities of amplified DNA were compared with those obtained with a standard curve containing the products of RT–PCR amplifications of serial dilutions (1:128, 1:256, 1:512, and 1:1024) of RNA from MEL.A cells. The expression of samples was normalized for RNA integrity as above, and the results were expressed relative to the level of expression by MEL.A cells.

Acknowledgments

We thank Ms. T. Aerts, C. Muller, and C. Mondovits for expert technical assistance, Dr. J. Luchetti (Ludwig Institute, Brussels) for synthesis of peptides, Dr. J. Thonnard for HLA-C typing, F. Brasseur and M. Swinarska for the RNA preparations, and Ms. S. Depelchin and S. Khaoulali for secretarial help. We thank Drs. D. Olive and B. Van den Eynde for constructive suggestions during the initial phase of this work and Dr. J.-C. Renauld for providing us with plamid pEF.PL3. This work was supported by the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State (Prime Minister's Office, Science Policy Programming) and by grants from the Association contre le Cancer (Brussels, Belgium), the Fonds J. Maisin (Brussels, Belgium), the BIOMED1 and BIOMED 2 programs of the European Community, and the Fonds National de la Recherche Scientifique (FNRS) (grant TELEVIE) (Brussels, Belgium). H. I. was supported by research fellowships from the Uheara Memorial Foundation (Japan) and the Ciba–Geigy Foundation for the Promotion of Science (Japan). P. G. C. is a Research Associate with the FNRS.

Received July 26, 1996; revised November 13, 1996.

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GenBank Accession Number

The accession number of the cDNA clone Hi2 reported in this paper is U65011.