A New Gene Coding for a Differentiation Antigen Recognized by Autologous Cytolytic T Lymphocytes on HLA-A2 Melanomas

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Summary

It has been reported previously that antitumor cytolytic T lymphocyte (CTL) clones can be isolated from blood lymphocytes of HLA-A2 melanoma patients, after stimulation in vitro with autologous tumor cells, and that some of these CTL clones lyse most HLA-A2 melanomas. A first antigen recognized by such CTL clones was previously shown to be encoded by the tyrosinase gene. We report here the identification of another gene that also directs the expression of an antigen recognized on most melanomas by CTL clones that are restricted by HLA-A2. The gene, designated Melan-A, is unrelated to any known gene. It is 18 kb long and comprises five exons. Like the tyrosinase gene, it is expressed in most melanoma tumor samples and, among normal cells, only in melanocytes.

When blood lymphocytes of melanoma patients are stimulated in vitro with autologous tumor cells in the presence of IL-2, one often observes proliferation of responder cells that exert a cytolytic activity on the tumor cells. From these responder cells it has been possible to obtain clones of cytolytic T lymphocytes (CTL)[1], that lyse the autologous melanoma cells but do not recognize autologous fibroblasts, EBV-transformed autologous B cells, or targets cells that are lysed by NK-like effectors (1–5). Several groups have derived from HLA-A2 melanoma patients CTL that lyse not only the autologous tumor cells but also a large proportion of the melanoma cell lines derived from other HLA-A2 patients (6–9). Such CTL have been derived from both blood cells and from tumor-infiltrating lymphocytes.

By immunoselection with CTL clones derived from blood lymphocytes of patient SK29(AV), two distinct antigens were described that were shared by a high proportion of HLA-A2 melanomas. The antigens were named SK29-Aa and SK29-Ab (9). The latter is encoded by the tyrosinase gene, which is expressed only in melanocytes and melanomas (10).

We report here the identification of a second gene that codes for antigen SK29-Aa. The cloning approach involved the transfection of a cDNA library in COS cells, which are monkey kidney cells transfected with the gene coding for the SV40 large T antigen (11, 12). When these cells are transfected with plasmids containing the SV40 origin of replication, these plasmids replicate so as to produce 10^4–10^5 copies per cell (13), resulting in a high level of expression of the cloned cDNA. Our data suggest that transfecting cDNA libraries into COS cells will be widely applicable for the identification of antigens recognized by T lymphocytes.

Materials and Methods

Cell Lines. Tumor cell lines LB39-MEL and LB33-MEL were derived from the metastatic melanomas of patients LB39 and LB33, respectively. The melanoma cells were cultured in Iscove's medium supplemented with 10% FCS (both from GIBCO BRL, Gaithersburg, MD), L-arginine (116 mg/liter), L-asparagine (36 mg/liter), and L-glutamine (216 mg/liter). Melanoma cell lines SK29-MEL and SK23-MEL, and melanocyte samples, were gifts from Dr. L. Old (Memorial Sloan-Kettering Cancer Center, New York). SK29-MEL was cloned by limiting dilution and clone SK29-MEL.1 was selected for further experiments (9, 14). The obtention of the antigen-loss variants of SK29-MEL has been described (9, 15). Melanoma cell line NA8-MEL was a gift from Dr. F. Jotereau (INSERM Unité 211, Nantes, France). SK29-MEL, SK23-MEL and NA8-MEL cells were cultured in DMEM (GIBCO BRL) containing 10% FCS,

[1] *Abbreviation used in this paper:* CTL, cytolytic T lymphocyte.

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10 mM Hepes and 4.5 g/liter glucose. COS-7 cells (American Type Culture Collection CRL 1651; Rockville, MD) were cultured in DMEM containing 10% FCS. WEHI-164 clone 13 (W13), a gift of Dr. T. Espevik (University of Trondheim, Trondheim, Norway; 16), was cultured in RPMI-1640 (GIBCO BRL) supplemented with 5% FCS.

Derivation and Culture of CTL Clones. Mixed lymphocyte-tumor cultures of PBL and tumor cells isolated from patient LB39 were performed as previously described (4) except that the medium was supplemented with 5 U/ml of human recombinant IL-4 (a gift from R. Devos, Roche Research, Gent, Belgium). Derivation, long-term culture, and specificity analysis of CTL clones from PBL of patient SK29(AV) were previously described (9, 15).

Assay for Cytolytic Activity. The protocol was previously described (4). Target cells were treated for 48 h with 50 U/ml of human recombinant IFN-γ (Boehringer Mannheim, Mannheim, Germany). CTL and 1,000 ^{51}Cr-labeled targets were incubated at various ratios in V-bottom microwells in a final volume of 200 μl. Chromium release in the supernatant was measured after 4 h of incubation.

Construction of the cDNA Library. Total RNA was isolated from clone SK29-MEL.1 and poly(A)$^+$ RNA was prepared by oligo-dT binding (mRNA purification kit; Pharmacia Fine Chemicals, Piscataway, NJ). mRNA was converted to cDNA, ligated to EcoRI adaptors, and inserted into the EcoRI site of expression vector pcDNAI/Amp (Invitrogen Corporation, Oxon, UK) as described in the SuperScript plasmid system kit (GIBCO BRL). Recombinant plasmids were electroporated into JM101 *Escherichia coli* bacteria with a Genepulser (Bio-rad Laboratories, Richmond, CA) at 1 pulse at 25 μF and 2,500 V, that were selected with ampicillin (50 μg/ml). Plasmid DNA from pools of bacteria was prepared as follows. Titrated suspensions of bacteria were seeded at 50, 100, or 150 bacteria per well in U-bottom microwells in 0.3 ml of tryptose, yeast extract, glycerol, phosphate, nitrate (TYGPN) medium (17). 10 microplates of each dilution were prepared. Bacteria were cultured for 48 h at 37°C. Control aliquots were titrated on agar, and only the set of microcultures containing close to 100 colonies per well were kept for the DNA extraction. Plasmid DNA was prepared in the microplates by the alkaline lysis method as described (17). The isopropanol-precipitated DNA were resuspended in 50 μl of Tris 10 mM, EDTA1 mM, pH 7.4, containing 20 ng/ml of RNase.

Transfection of COS-7 Cells and Screening of Transfectants. DNA from pools of bacteria was transfected by the DEAE-dextran-chloroquine method into COS-7 cells basically as described by Aruffo (17). 1 d before transfection, COS cells were seeded in flat-bottom tissue culture microwells at 15,000 cells per well in 100 μl of DMEM containing 10% FCS. For transfection, medium was discarded and replaced by 30 μl of DEAE-Dextran/DNA mixtures. These mixtures were prepared for duplicate transfections in V-bottom microwells by adding sequentially: 25 μl of plasmid DNA from the cDNA library, 5 μl of Tris 10 mM, EDTA 1 mM, pH 7.4, containing 200 ng of plasmid pcDNAI/Amp-A2 (plasmid pcDNAI/Amp containing the HLA-A2.1 gene isolated from a CTL clone of patient SK29 [9]), 5 μl of DMEM-NS (DMEM medium supplemented with 10% decomplemented NuSerum IV [Collaborative Biomedical Products, Bedford, MA]), and 35 μl of DMEM-NS supplemented with 0.8 mg/ml DEAE-Dextran (Pharmacia) and 200 μM chloroquine. The COS cells were incubated for 4 h at 37°C. DEAE-Dextran/DNA was discarded and replaced by 50 μl of PBS containing 10% DMSO. After 2 min at room temperature, PBS-DMSO was replaced by 200 μl of DMEM supplemented with 10% FCS. Transfected COS cells were incubated for 48 h at 37°C. The medium was then discarded and 1,000–2,000 CTL were added in 100 μl of Iscove's medium containing 10% human serum and 25 U/ml IL-2 (a gift of Biogen, Geneva, Switzerland). After 24 h, the supernatant was collected and its TNF content was determined by its cytolytic effect on WEHI-164 clone 13 cells (W13) (16) as previously described (18).

Analysis of the Structure of Gene Melan-A. A genomic library of 700,000 independent cosmids was constructed in cosmid c2RB with DNA from melanoma cell line LB33-MEL as described (19). Southern blots were prepared with DNA isolated from 22 groups of 70,000 cosmids. ^{32}P-labeled cDNA clone AaG1 hybridized to nine groups. The group that produced the strongest hybridization band was subcloned, and a cosmid that contained the Melan-A sequence was identified by colony hybridization with the labeled cDNA. The cosmid was sequenced using primers deduced from the sequence of cDNA AaG1, using the Cycle-Sequencing Kit (United States Biochemical Corp., Cleveland, OH). Complete sequences were obtained for introns 1, 2, and 4, and for the 3′ and 5′ ends of intron 3. The size of intron 3 was estimated as follows. Southern blots were prepared with cosmid DNA digested with EcoRI and Bgl II. A 7-kb EcoRI fragment hybridized with ^{32}P-labeled oligonucleotides deduced from the sequences of both ends of intron 3. The computer search for sequence homology was done with program FASTA on GenBank database release 79.0.

Transfection of a Melanoma Cell Line. Melanoma line NA8-MEL was transfected by the calcium phosphate precipitation method, as described (18). Briefly, 7.5 × 10^5 cells were seeded in 4.5 ml of medium. 24 h later, they were transfected with 2 μg of plasmid pSVtkneoβ and 20 μg of plasmid pcDNAI/Amp containing cDNA AaG1. After 48 h, the cells were seeded in microcultures at 2,000 cells/well in 200 μl of medium containing 2 mg/ml of neomycin analog G418 (GIBCO BRL). NeoR colonies were selected for further experiments.

PCR Assays for Melan-A Expression. Isolation of total RNA from tumor samples was performed as described (20). RNA from melanocyte culture was a gift from Dr. Old. Reverse transcription was performed on 2 μg of total RNA with an oligo(dT) primer. cDNA corresponding to 100 ng of total RNA (10^4 cell equivalents) was amplified for 33 cycles by PCR with the following primers: sense 5′-ACTGCTCATCGGCTGTTG-3′, antisense 5′-TCAGCCATGTCTCAGGTG-3′. These primers are located in exons 2 and 5 to exclude the amplification of genomic DNA. An aliquot of the PCR reaction was run on a 1% agarose gel stained with ethidium bromide. To ensure that the RNA were not degraded, the cDNA products were tested for the presence of human β-actin.

Results

Blood lymphocytes of HLA-A2 melanoma patient LB39 were stimulated in vitro with irradiated cells of autologous tumor cell line LB39-MEL. From the responder T cell population, we isolated cytolytic T cell clone LB39-CTL-1/95, which lysed the autologous melanoma cell line (Fig. 1). This CTL clone also lysed melanoma cells from several other HLA-A2 melanoma patients, suggesting that its target antigen was presented by HLA-A2 (Fig. 1). This was confirmed by the observation that a previously described cell variant of melanoma line SK29-MEL that had lost HLA-A2 was not lysed by CTL 1/95 (Fig. 1).

The antigen recognized by CTL 1/95 appeared to be different from the previously described melanoma antigen SK29-Ab, which is encoded by tyrosinase, because an antigen-loss variant of SK29-MEL, which was resistant to antityros-

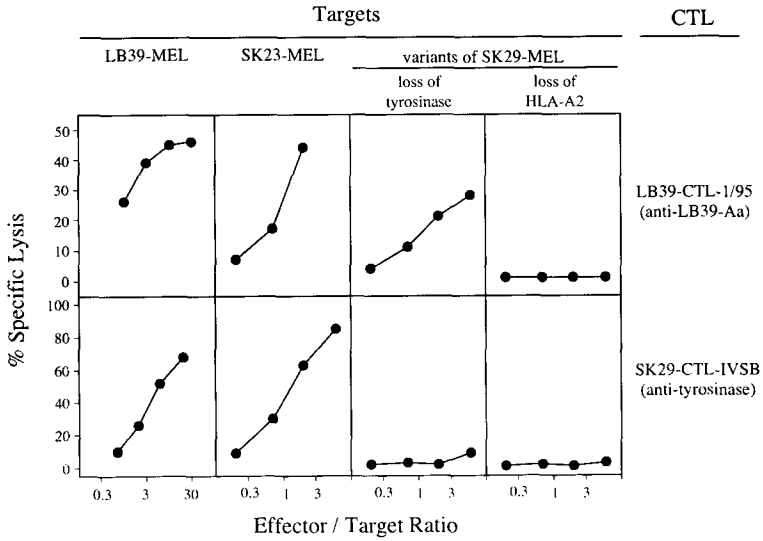

Figure 1. Lysis by CTL clone LB39-CTL-1/95 of autologous melanoma LB39-MEL, HLA-A2 allogeneic melanoma SK23-MEL, and variants of allogeneic melanoma SK29-MEL. One variant, named 29.1.5.86, had been selected for resistance to an antityrosinase CTL, whereas the other, named 29.1.22, had lost expression of HLA-A2. Antityrosinase CTL SK29-CTL-IVSB was tested on the same targets.

inase CTL clone SK29-CTL-IVSB, was still lysed by CTL 1/95 (Fig. 1). The target antigen of CTL 1/95 was named LB39-Aa. When CTL 1/95 was tested on other antigen-loss variants obtained from melanoma SK29-MEL, it showed the same lytic pattern as CTL clones obtained from patient SK29(AV) that recognize an antigen described as SK29-Aa (9). This suggested that antigen LB39-Aa was the same antigen as SK29-Aa.

Identification of a cDNA Coding for Antigen LB39-Aa. Because melanoma line SK29-MEL was lysed by CTL 1/95, we used a cDNA library that had been prepared with RNA from this cell line. This library had been prepared with expression vector pcDNAI/Amp. This vector carries the SV40 origin of replication which enables it to replicate autonomously in COS cells to large copy numbers (13). This library was divided into 800 pools of 100 bacteria. Each pool was expected to contain approximately 25 different cDNAs because only 50% of the plasmids contained an insert and the cloning was not directional. The HLA-A2.1 gene of patient SK29(AV) was also cloned into plasmid pcDNAI/Amp.

Each cDNA pool was cotransfected with the HLA-A2 construct into duplicate microcultures of COS-7 cells. After 48 h, the transfected COS cells were tested for expression of antigen LB39-Aa by their ability to stimulate the production of TNF by CTL 1/95. The CTL were added to the transfectants and after 24 h the concentration of TNF in the culture supernatant was measured by its cytotoxic effect on W13 cells. The amounts of TNF found in the supernatant showed a wide variation ranging from 2 to 7 pg/ml in most microcultures (Fig. 2). Six values stood out above 8 pg/ml. Among those we found two pairs of high duplicates. From each of the two pools of bacteria corresponding to these duplicate positives, we subcloned 800 bacteria. Plasmid DNA was extracted from each of the 1,600 bacteria and cotransfected into COS cells with the HLA-A2 construct. One clone, named AaG1, was found to confer recognition by CTL 1/95: COS cells transfected with this cDNA clone stimulated TNF release by the CTL (Fig. 3). They also stimulated CTL clone SK29-CTL-10/196, an HLA-A2–restricted CTL known to recognize on SK29-MEL the SK29-Aa antigen (9) (Fig. 3). The antityrosinase CTL IVSB was not stimulated by COS cells transfected with cDNA AaG1 (Fig. 3).

Stable transfectants were made in order to confirm the results obtained with COS cells. We used HLA-A2 melanoma cell line NA8-MEL which was not recognized by anti-Aa CTL 1/95. The cells were cotransfected with the pcDNAI/Amp plasmid containing cDNA AaG1 and with a plasmid conferring resistance to geneticin. Clones were isolated from the geneticin-resistant transfectants. They proved sensitive to lysis by CTL 1/95 (Fig. 4 *A*) and they also stimulated the production of TNF by this CTL (Fig. 4 *B*).

Structure of the Gene Coding for Antigen LB39-Aa. The sequence of cDNA clone AaG1 proved to be 675-bp long. When a Northern blot prepared with RNA of melanoma cell line SK29-MEL was hybridized with this cDNA, a band of ~0.75 kb was observed, suggesting that clone AaG1 was incomplete. It was used to screen again the cDNA library derived from SK29-MEL and a cDNA clone of 760 bp, named Aa84/3, was obtained. This cDNA clone was strictly identical to AaG1 except for the addition of 85 bp at the 5′ end.

The sequence of cDNA Aa84/3 is shown on Fig. 5. No significant homology with any gene recorded in data banks was observed. The putative protein comprises 118 amino acids. It has no signal sequence. It is rich in proline residues (9%) and contains a hydrophobic region spanning residues 27–48 (Fig. 5).

To isolate the gene corresponding to cDNA AaG1, DNA was prepared from 22 groups of 70,000 cosmids of a human genomic library. The cDNA hybridized to nine cosmid groups. We subcloned the group that produced the strongest hybridization band and we identified one cosmid that hybridizes with cDNA clone AaG1. Genomic sequences corresponding to exons and adjacent intron regions were obtained by sequencing the cosmid with primers deduced from the cDNA

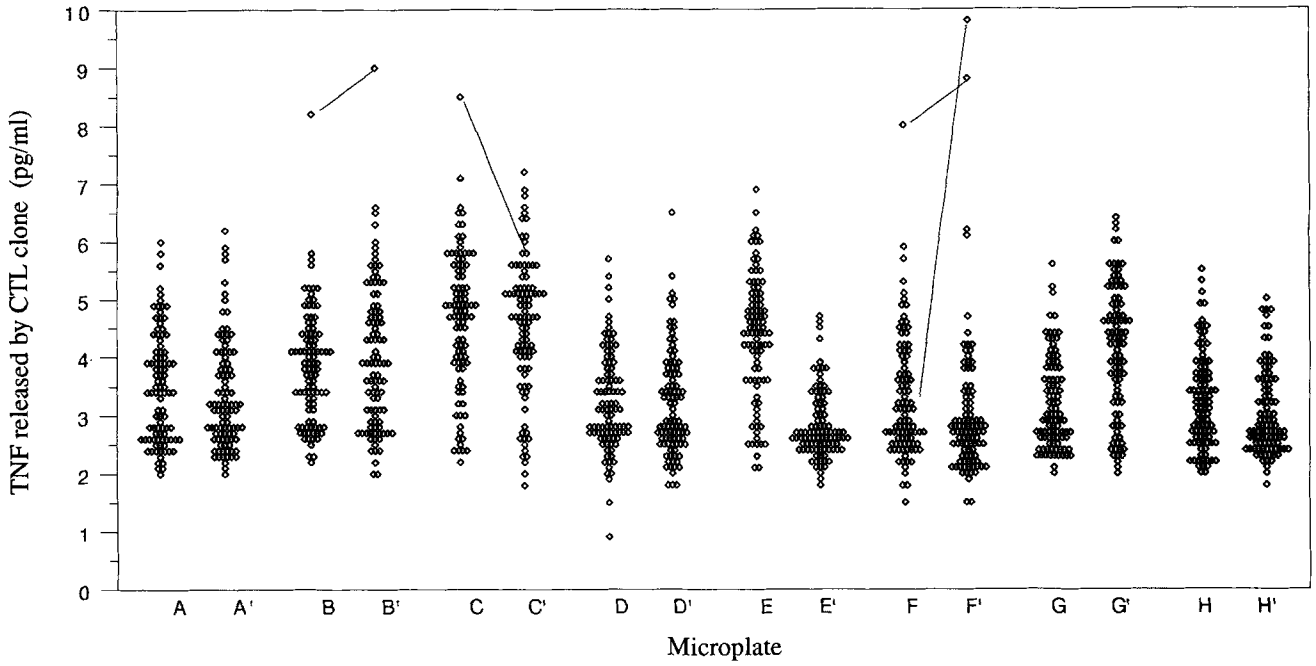

Figure 2. Stimulation of CTL 1/95 by COS cells cotransfected with HLA-A2 and pools of approximately 25 different cDNAs obtained from melanoma cell SK29-MEL. Each symbol represents the TNF content of one individual microculture, and the duplicate microplates are named A and A', B and B', etc. Duplicates of seemingly positive microcultures are connected with a line. Each pool of cDNAs was transfected into duplicate microcultures. Both the HLA-A2 gene and the cDNAs were cloned into expression vector pcDNAI/Amp. They were cotransfected with DEAE-Dextran into subconfluent COS cells. 48 h after transfection, CTL 1/95 (2,000 cells per well) was added. The culture supernatants were harvested 1 d later and tested on W13 cells for their TNF content.

sequence. The comparison of the cosmid and cDNA sequences indicated that gene Melan-A comprises five exons (Figs. 5 and 6). Further intron sequences were obtained using primers deduced from the first sequences. This led to the complete sequence of introns 1 (1,512 bp), 2 (5 kb), 4 (1,462 bp), and a partial sequence of intron 3 (Fig. 6). By hybridizing restriction fragments of the cosmid with oligonucleotides cor-

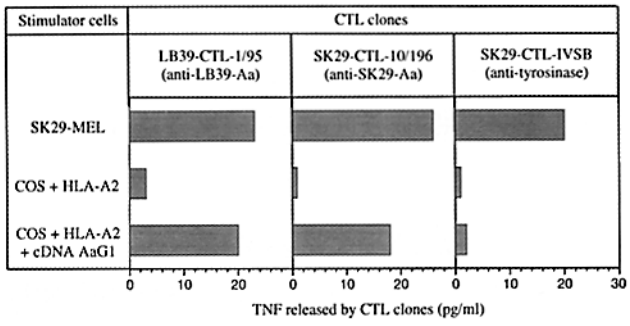

Figure 3. Stimulation of CTL 1/95 by COS cells transfected with pcDNAI/Amp constructs containing HLA-A2 and cDNA AaG1 as described in Materials and Methods. Control stimulator cells included HLA-A2 melanoma SK29-MEL and COS cells transfected with HLA-A2 alone. The transfectants also stimulated HLA-A2–restricted CTL clone 10/196 of patient SK29, which has been previously found to be directed against antigen LB39-Aa, and did not stimulate antityrosinase CTL clone SK29-CTL-IVSB.

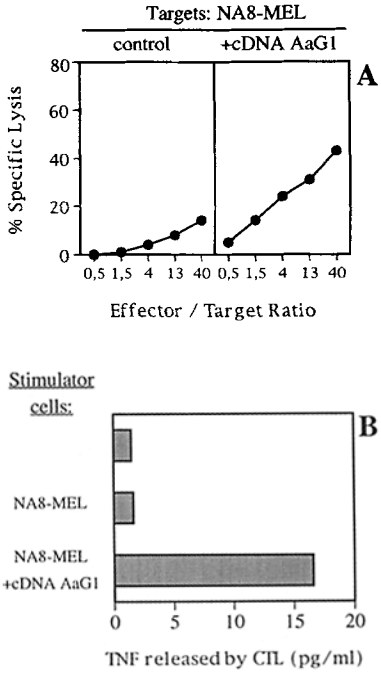

Figure 4. Recognition of antigen LB39-Aa expressed in a transfectant obtained by cotransfecting HLA-A2 melanoma cell line NA8-MEL with the pcDNAI/Amp-cDNA AaG1 construct and pSVtkneoβ. (*A*) Lysis of geneticin-resistant transfectant clone NA8-MEL.c1.1 by anti-LB39-Aa CTL 1/95. (*B*) Production of TNF by CTL 1/95 stimulated with clone NA8-MEL.c1.1.

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                                             (1)..CCG      3

TCAGAAATCTAAACCCGTGACTATCATGGGACTCAAAACCAGCCCAAAAAATAAG   58

TCAAAACGATTAAGAGCCAGAGAAGCAGTCTTCATACACGCGGCCAGCCAGCAGA  113

CAGAGGACTCTCATTAAGGAAG.. (2)..GTGTCCTGTGCCCTGACCCTACAAG  160


ATG CCA AGA GAA GAT GCT CAC TTC ATC TAT GGT TAC CCC AAG  202
Met Pro Arg Glu Asp Ala His Phe Ile Tyr Gly Tyr Pro Lys

AAG GGG CAC GGC CAC TCT TAC ACC ACG GCT GAA GA.. (3)..G  238
Lys Gly His Gly His Ser Tyr Thr Thr Ala Glu Glu

GCC GCT GGG ATC GGC ATC CTG ACA GTG ATC CTG GGA GTC TTA  280
Ala Ala Gly Ile Gly Ile Leu Thr Val Ile Leu Gly Val Leu

CTG CTC ATC GGC TGT TGG TAT TGT AGA AGA CGA AAT GGA TAC  322
Leu Leu Ile Gly Cys Trp Tyr Cys Arg Arg Arg Asn Gly Tyr

AGA GCC TTG ATG.. (4).. GAT AAA AGT CTT CAT GTT GGC ACT  358
Arg Ala Leu Met         Asp Lys Ser Leu His Val Gly Thr

CAA TGT GCC TTA ACA AGA AGA TGC CCA CAA GAA GGG TTT GAT  400
Gln Cys Ala Leu Thr Arg Arg Cys Pro Gln Glu Gly Phe Asp

CAT CGG GAC AGC AAA GTG TCT CTT CAA GAG AAA AAC TGT GAA  442
His Arg Asp Ser Lys Val Ser Leu Gln Glu Lys Asn Cys Glu

CCT GTG.. (5).. GTT CCC AAT GCT CCA CCT GCT TAT GAG AAA  478
Pro Val         Val Pro Asn Ala Pro Pro Ala Tyr Glu Lys

CTC TCT GCA GAA CAG TCA CCA CCA CCT TAT TCA CCT TAA      517
Leu Ser Ala Glu Gln Ser Pro Pro Pro Tyr Ser Pro OCH

GAGCCAGCGAGACACCTGAGACATGCTGAAATTATTTCTCTCACACTTTTGCTTG  572
AATTTAATACAGACATCTAATGTTCTCCTTTGGAATGGTGTAGGAAAAATGCAAG  627
CCATCTCTAATAATAAGTCAGTGTTAAAATTTTAGTAGGTCCGCTAGCAGTACTA  682
ATCATGTGAGGAAATGATGAGAAATATTAAATTGGGAAAACTCCATC[AATAAA]TG  737
TTGCAATGCATGATAAAAAAAAA                                  760
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Figure 5. Sequence of the five exons of gene Melan-A and sequence of the protein encoded by the longest open reading frame. The nucleotide indicated as the first in exon 1 corresponds to the 5′ end of cDNA clone Aa84/3. Box shows a polyadenylation site. A hydrophobic stretch of the protein is underlined. The cDNA sequence data are available from EMBL/GenBank/DDBJ under accession number HSU06654.

responding to each intron, a restriction map of the gene was obtained. An EcoRI fragment of 7 kb hybridized with oligonucleotides at the 5′ and 3′ sides of the partially sequenced intron 3 (Fig. 6). This allowed us to estimate the size of intron 3 at 9.5 kb. We concluded from these data that gene Melan-A has a length of ∼18.5 kb.

Expression of Gene Melan-A. The pattern of expression of gene Melan-A was analyzed by reverse transcription and PCR amplification of RNA prepared from various cells (Table 1). All of 26 melanoma tumor samples were positive. Among 21 melanoma cell lines we found 12 positives. No expression was found in tumor samples of other histological types. Among normal tissues, gene Melan-A was expressed only by melanocytes. Among skin biopsies some scored positive, presumably because they contained a higher proportion of melanocytes.

Table 1. *Expression of the Melan-A gene*

	Proportion of positive samples
Normal tissues	
Melanocytes	2/2
Skin	2/3
Liver	0/1
Kidney	0/1
Heart	0/1
Prostate	0/1
Breast	0/4
Ovary	0/1
Testis	0/2
Adrenals	0/3
Lung	0/2
Fetal brain	0/1
Cerebellum	0/1
Substantia nigra	0/1
Tumors	
Melanoma samples	26/26
Melanoma cell lines	12/21
Breast tumor samples	0/5
Sarcoma samples	0/5
Non small cell lung tumor samples	0/5
Renal carcinoma samples	0/4
Colon carcinoma samples	0/4

The expression of gene Melan-A was tested by reverse transcription and specific PCR amplification. The melanoma samples and cell lines were derived from a random sampling of HLA-A2 and non HLA-A2 patients. The amount of PCR product did not vary significantly among the positive samples. All RNAs presented here showed similar amplification of the β-actin cDNA.

Discussion

Both the tyrosinase gene (10) and the Melan-A gene (this report) have been identified as genes coding for antigens recognized by autologous CTL on melanoma cells, using a procedure that involves high level transient expression of HLA genes and cDNAs in COS cells. This method differs

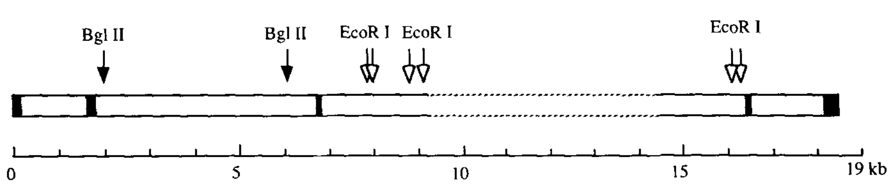

Figure 6. Structure of gene Melan-A with the exons represented as black boxes. (*Stippled line*) Part of intron 3 that was not sequenced. (*Unfilled arrows*) EcoRI restriction sites.

from the cosmid transfection approach that led to the identification of gene MAGE-1, which codes for tumor antigen MZ2-E (21). The cDNA-COS cell approach is definitely faster and more convenient than the cosmid approach. Recently, it has led to the identification of two additional genes that code for human tumor antigens recognized by autologous CTL (van der Bruggen, P., and B. Van den Eynde, personal communication). Moreover, we have carried out cloning experiments with a cDNA library of tumor MZ2-MEL, which expresses antigen MZ2-E. The cotransfection of the gene of HLA-A1, which is the presenting molecule, and of pools of the cDNA library led to the identification of several MAGE-1 cDNA clones. We therefore believe that this method will prove widely applicable for the identification of genes coding for antigens recognized by T lymphocytes. Besides its convenience, the COS-cDNA approach does not require the obtention of antigen-loss variants, whereas for the cosmid approach, these variants must be obtained to serve as transfection recipients. On the other hand, the COS-cDNA approach requires the prior cloning of the relevant HLA gene, which must be cotransfected into the COS cells.

The considerable episomal multiplication of the transfected plasmids generates a large amount of both the HLA molecules and the proteins encoded by the transfected cDNA clones. This probably has positive and negative consequences. On the positive side, the expression at the cell surface of a high copy number of a given HLA–peptide complex may be the only way to identify genes coding for antigens recognized by CTL of relatively low affinity. Differentiation antigens expressed by few cells throughout the body, such as those encoded by tyrosinase and Melan-A, may fall in this category because high affinity T cells may have been eliminated by tolerance, whereas low affinity T cells may have been spared.

On the negative side, we find that a number of antitumor CTL clones produce a significant level of TNF upon incubation with COS cells transfected with the HLA gene only. For some CTL clones this background TNF production is very low. But CTL clone LB39-CTL-1/95, which was used to identify gene Melan-A, produced a few picograms per milliliter of TNF when incubated with COS cells transfected with a plasmid containing the HLA-A2 gene. This accounts for the high background displayed in Fig. 2. These crossreactions are probably a consequence of the high expression of the HLA molecules, some of which presumably present peptides derived from proteins of the COS cells that resemble the original antigenic peptide recognized by the CTL.

Another drawback of the COS-cDNA cloning procedure seems to be the identification of inappropriate cDNA clones. We have described a CTL clone that recognizes an antigen restricted by HLA-A2 on the autologous lung tumor cell line. Using the COS-cDNA method, we isolated a cDNA clone (2C6) that transferred the expression of the antigen into COS cells (22). We have now identified another cDNA clone, whose sequence is unrelated to that of cDNA clone 2C6, and which also transfers the expression of the antigen into COS cells. Clearly, at least one of the two cDNA does not represent the appropriate gene, but codes for a peptide that shows enough similarity to the appropriate antigenic peptide, so that its presentation in high number by the COS cells stimulates the CTL clone. It will therefore be important to verify that cDNA clones that putatively code for an antigen can also confer recognition by the appropriate CTL when incorporated into stable transfectants that express a standard amount of the message. This has been observed both with tyrosinase and the Melan-A gene reported here. But in our view, the definitive evidence that the pertinent gene has been identified would be the observation that Southern blots prepared with the DNA of an antigen-loss variant show the loss of a band that is present in the original tumor cell when hybridized with the putative cDNA.

The Melan-A gene is the third gene that we have found to code for an antigen recognized on human tumors by autologous CTL. The first was gene MAGE-1 (21). It is silent in normal tissues except in testis and it is expressed by a significant proportion of tumors of different types: 40% of melanoma samples, 30% of lung tumors (23), and 20% of breast tumors (24). The second was the tyrosinase gene (10). Like the Melan-A gene, it is expressed only in melanocytes and in melanocytic tumors. Thus both antigen SK29-Ab, which is encoded by the tyrosinase gene, and antigen SK29/LB39-Aa, which is encoded by the Melan-A gene, are melanocytic differentiation antigens. A third differentiation antigen recognized by CTL on HLA-A2 melanomas was recently shown to be encoded by a cDNA coding for melanocyte lineage-specific antigen gp100 (25). The identification of these melanocytic differentiation antigens provides a basis for the observations of several groups that described antitumor CTL that recognized the autologous HLA-A2 melanoma cells as well as most melanomas derived from other HLA-A2 patients (5–9). Not surprisingly, CTL clones of this type have been shown to recognize A2 melanocytes as well (26). We have obtained HLA-A2–restricted antimelanoma CTL clones that recognize allogeneic melanoma lines that express neither the tyrosinase gene, nor the Melan-A and the gp100 genes. It is therefore very likely that other genes whose expression is restricted to the melanocytic cell lineage also direct the expression of antigens recognized by T lymphocytes.

The observation that proteins are specific for the melanocytic differentiation can provide target antigens for antitumor CTL responses in melanoma patients is in line with previous observations that sera of some melanoma patients contain antibodies against melanocyte differentiation antigens such as melanoma antigen gp75 (27, 28).

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