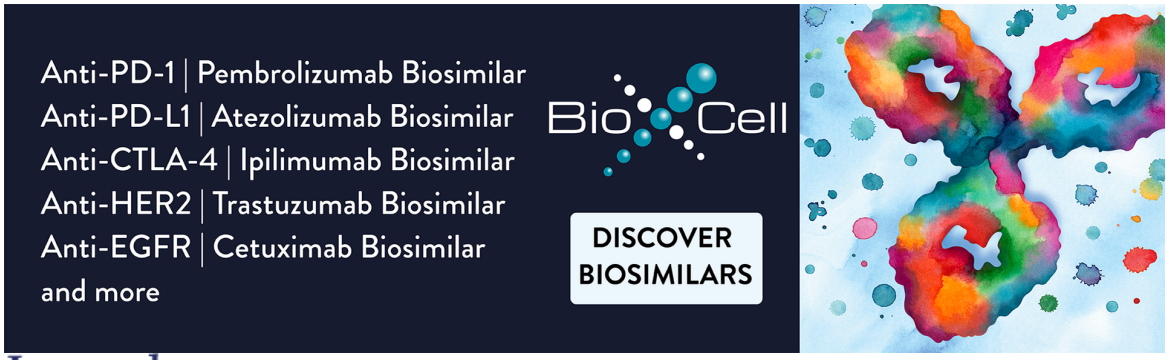

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The Intronic Region of an Incompletely Spliced *gp100* Gene Transcript Encodes an Epitope Recognized by Melanoma-Reactive Tumor-Infiltrating Lymphocytes

Paul F. Robbins,[1] Mona El-Gamil, Yong F. Li, Ellen B. Fitzgerald, Yutaka Kawakami, and Steven A. Rosenberg

Recent studies have characterized a number of the Ags that are recognized by melanoma-reactive T cells. Although the majority of tumor Ags appear to represent nonmutated gene products, a variety of epitopes have been shown to arise from either mutated or alternatively processed transcripts. Here, we report that the screening of a cDNA library with a HLA-A24-restricted melanoma-reactive T cell cloid derived from tumor infiltrating lymphocytes resulted in the isolation of a variant of the *gp100* gene that had retained the entire fourth intron of this gene, termed *gp100*-in4. The *gp100*-in4 transcript could be detected by reverse transcriptase-PCR but could not be detected in Northern blots conducted with melanoma RNA, indicating that it represents a relatively rare transcript. Read-through of this transcript into the region corresponding to the fourth intron gave rise to an additional 35 amino acids not found in the normal gp100 glycoprotein, and a peptide within this region conforming to the HLA-A24 consensus motif (VYFFLPDHL) was shown to be recognized by the T cell cloid. The sequence of the intron was identical with that of a previously isolated genomic *gp100* clone, and T cells that recognized the *gp100*-in4 gene product were found to recognize HLA-A24-matched allogeneic melanoma cell lines and melanocytes, demonstrating that this represents a nonmutated epitope. These results further extend the types of Ags that can be recognized by melanoma-reactive T cells to aberrant transcripts of melanosomal genes. *The Journal of Immunology,* **1997, 159: 303–308.**

Studies conducted over the past several years have indicated that tumor-reactive T cells isolated from melanoma patients recognize a variety of products. Some of the Ags recognized by melanoma-reactive T cells represent nonmutated gene products the expression of which in tissues of normal adults appears to be restricted to the testis. These include MAGE-1, MAGE-3, BAGE, and GAGE, which are expressed in 30 to 70% of melanomas as well as in additional tumor types (1–4). A number of nonmutated genes that are expressed in normal melanocytes but not in other normal tissues also appear to encode Ags recognized by melanoma-reactive T cells. These include the *MART-1/MelanA*, *gp100*, tyrosinase, and *gp75* melanocyte lineage gene products (5–10).

Melanoma-reactive T cells have also been shown to recognize mutated products of the *CDK-4* (11) and *β-catenin* (12) genes, as well as the product of a previously undescribed gene, termed *MUM-1* (13). T cell clones isolated from a renal cancer patient reacted with a point-mutated HLA-A2 gene product that was expressed in the autologous tumor (14).

Additional mechanisms have also been found to be involved in generating Ags recognized by tumor-reactive T cells. The T cell epitope identified in *MUM-1* appeared to span an intron-exon boundary (13). A novel product of the *N*-acetylglucosaminyltransferase V (*GnT-V*)[2] gene, transcribed from a cryptic promoter located near the 3′ end of one of the GnT-V introns, encoded a T cell epitope recognized by HLA-A2-restricted melanoma-reactive T cells (15).

This study demonstrates that HLA-A24-restricted, melanoma-reactive T cells recognize a nonmutated peptide encoded by an aberrant transcript of the *gp100* gene. This transcript contained the fourth intron of the *gp100* gene, and the T cell epitope was encoded within this region. Peptides derived from additional introns present in *gp100*, as well as the introns present in other genes that have been shown to encode tumor Ags, thus represent potential targets for tumor-reactive T cells.

Materials and Methods

Cell lines

The isolation of the 293-A24 cell line, which stably expressed HLA-A24, has been previously described (10). The 293-A24 line was grown in DMEM containing 7.5% fetal bovine serum, and melanoma cell lines were isolated in this laboratory and grown in RPMI 1640 media containing 5% fetal bovine serum. Melanoma-reactive CTL were derived from tumor-infiltrating lymphocyte (TIL) cultures grown in AIM V medium (Life Technologies, Gaithersburg, MD) containing 1000 IU/ml of IL-2 as previously described (16). T cell cloids were derived by culturing cells under limiting dilution conditions by stimulation with anti-CD3 Ab (OKT-3, Ortho Pharmaceuticals, Raritan, NJ) as previously described (17). Briefly, 5×10^4 irradiated (30 Gy) allogeneic PBMC and 10^4 irradiated (120 Gy) autologous EBV B cells were plated in round-bottom 96-well plates with 2 to 100 T cells per well. Cells were cultured in RPMI 1640 medium containing 10% human AB serum and 30 ng/ml OKT3 Ab and 120 IU/ml of IL-2. The medium was changed on days 5 and 8, and clones were assayed 12 to 16 days after stimulation. T cells were then expanded by plating 5×10^4 to 10^5 cells in a T25 flask in 25 ml of medium with

Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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[1] Address correspondence and reprint requests to Paul F. Robbins, Surgery Branch, National Cancer Institute, National Institutes of Health, Building 10, Room 2B42, Bethesda, MD 20892.

[2] Abbreviations used in this paper: Gnt-V, *N*-acetylglucosaminyltransferase V; TIL, tumor-infiltrating lymphocyte; RT, reverse transcriptase; GM, granulocyte-macrophage.

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Table I. *Recognition of gene transfectants and melanoma cell lines by T cell cloid 5A*

Stimulator Cell Line[a]	Transfected Gene	HLA-A24 Expression	GM-CSF Release (pg/ml)
293-A24	*MART-1*	+	<8
	gp100	+	<8
	Tyrosinase	+	<8
	gp75	+	<8
	β-catenin	+	17
	Clone *D12-3*	+	80
888 mel	None	+	17
1290 mel	None	+	18
501 mel	None	+	25
1300 mel	None	+	<8
397-A24 mel	None	+	45
397 mel	None	−	<8
SK23 mel	None	−	<8
None		−	<8

[a] 5×10^3 T cells were incubated with 5×10^4 target cells for 18 h, and the release of cytokine was measured using a GM-CSF ELISA.

2.5×10^7 irradiated PBMC and 5×10^6 irradiated EBV B cells in medium containing 30 ng/ml OKT3 Ab. The T cell cloid 5A was isolated from a plate initially containing 100 responding T cells per well, at which density essentially all wells were positive for growth. The T cell cloids 15, 16, and 18 were isolated from wells initially plated with 10 T cells per well; at this density 27% of the wells were positive for growth.

The normal melanocyte cell line 2488 (HLA-A2, A24) was obtained from Clonetics (San Diego, CA), and the melanocyte cell lines 1070 (HLA-A24, A29) and 1085 (HLA-A2, A3) were kindly provided by Dr. Meynard Herlyn (The Wistar Institute, Philadelphia, PA).

cDNA library construction and screening

A cDNA library was constructed from 5 mg of poly(A)$^+$ mRNA that had been twice purified using the poly(A) tract isolation system (Promega, Madison, WI). A directional cDNA library was produced using random primers according to the manufacturer's instructions (Novagen, Madison, WI). Briefly, random primers containing two T residues at the 5′ end were used for reverse transcription of oligo(dT)-purified mRNA. Following ligation of the oligomer 5′-GCTTGAATTCAAGC-3′ to the cDNA, DNA was digested with *Hin*dIII, resulting in clones containing an *Eco*RI site at the 5′ end and a *Hin*dIII site at the 3′ end. The pCDNA3 eukaryotic expression vector was modified by digesting with *Hin*dIII and filling in the overhang with Klenow, then ligating the resultant product to destroy this *Hin*dIII site. This plasmid was then digested with *Xba*I, and the oligomer CTAGAAGCTT containing a *Hin*dIII site was ligated into the *Xba*I site. The modified pCDNA3 vector was then digested with *Eco*RI and *Hin*dIII, and used for ligation with the cDNA library. Pools containing ~200 cDNAs were generated by estimating the titer following plating of transformed DH510B *Escherichia coli* on plates containing 100 mg/ml of ampicillin, and 200 bacteria were grown for 48 h in a 1-ml block (Advanced Genetics Technologies Corporation, Gaithersburg, MD). The plasmid DNA was purified using the Wizard 9600 purification system (Promega), and transfection and screening assays were conducted as previously described (12). Granulocyte-macrophage (GM)-CSF release was detected using an ELISA assay conducted with an Ab pair obtained from Endogen (Cambridge, MA).

Sequencing and PCR analysis

Sequences of the isolated cDNA clones were determined using an ABI Prism 310 automated capillary electrophoresis instrument (Perkin-Elmer, Foster City, CA). Cycle sequencing reactions were conducted using AmpliTaq DNA polymerase FS enzyme reaction kits (Perkin-Elmer). Sequences were compared with the GenBank database using the BLAST algorithm. Reverse transcriptase (RT)-PCR reactions were conducted using 200 ng of poly(A)$^+$ RNA and using oligo(dT) for reverse transcription. Semiquantitative PCR reactions were conducted using 0.1 volume of the above RT reaction and at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min for either 15, 20, or 25 cycles. The PCRs were conducted using a primer located within the normal *gp100* open reading frame upstream of the region containing the fourth intron (5′-AACTGACGATGC CTGCATCT-3′) and a second primer complementary to a sequence within the fourth intron of *gp100* 5′-TCAAAGGGGACTGAGGGACA-3′).

```
          Exon 4 | Intron 4
              W  G  E  G  L  P  S  Q  P  I  I  H  T  C  V  Y  F  F  L  P
gp100-in4 TGGGGTGAGGGACTCCCTTCTCAGCCTATCATCCACACTTGTGTTTACTTCTTTCTACCT

              D  H  L  S  F  G  R  P  F  H  L  N  F  C  D  F  L  *
gp100-in4 GATCACCTTTCTTTTGGCCGCCCCTTCCACCTTAACTTCTGTGATTTTCTCTAATCTTCA

gp100-in4 TTTTCCTCTTAGATCTTTTCTCTTTCTTAGCACCTAGCCCCCTTCAACTCTATCATAATT

gp100-in4 CTTTCTGGCAACTCTTCCTCAATTGTAGTCCTACCCCATGGAATGCCTCATTAGGACCCC

gp100-in4 TTCCCTGTCCCCCCATATCACAGCCTTCCAAACACCCTCAGAAGTAATCATACTTCCTGA

gp100-in4 CCTCCCATCTCCAGTGCCGTTTCGAAGCCTGTCCCTCAGTCCCCTTTGACCAGTAATCTC

                                                  Intron 4 | Exon 5
gp100-in4 TTCTTCCTTGCTTTTCATTCCAAAAACTTCAGGCC
```

FIGURE 1. Partial sequence of the *gp100*-in4 cDNA including the entire sequence of intron 4 and translation of the intronic sequence. The T cell peptide epitope is underlined.

Peptide synthesis

Peptides were synthesized using an AMS 222 multiple peptide synthesizer (Gilson Co., Inc., Worthington, OH) and standard F-moc chemistry. Peptide purification was conducted using an R2 reversed-phase HPLC column (Perseptive Biosystems, Framingham, MA) with an acetonitrile gradient in water containing 0.05% trifluoroacetic acid. Peptides were estimated to be >90% pure, and the mass of identified peptides verified by mass spectrometry.

Results

Isolation of cDNA clones

Previous studies conducted with TIL 1290 demonstrated that this cell line recognized a mutated product of the *β-catenin* gene in the context of HLA-A24 (12), as well as *p15*, a gene that appeared to be expressed in a number of normal tissues (18). In an attempt to identify additional Ags recognized by TIL 1290, a number of T cell cloids were generated from this cell line by limiting dilution. Several of the T cell cloids that were isolated from this patient recognized *β-catenin*, including T cell cloid 5A, but this T cell cloid also recognized several melanoma cell lines that naturally expressed HLA-A24 but did not contain the *β-catenin* mutation (Table I). The 5A T cell cloid recognized two melanomas derived from the autologous patient, 888 mel and 1290 mel, as well as an HLA-A24-positive allogeneic melanoma, 501 mel, but failed to recognize the 1300 melanoma, which also expressed HLA-A24. The T cell cloid 5A did, however, recognize a transfectant of the 397 mel cell line, which expressed HLA-A24 but not the parental 397 cell line, providing strong evidence that this T cell line recognized a widely shared Ag in the context of HLA-A24. This cloid did not recognize transfectants of the 293-A24 cell line expressing *MART-1*, *gp100*, or tyrosinase, but recognized transfectants expressing the mutated *β-catenin* gene product. Previous observations indicated that the mutated *β-catenin* gene was expressed in melanomas derived from patient 888 and was not present any of the 12 allogeneic melanomas tested including the 397 melanoma, indicating that this was a relatively rare mutation (12). Thus, it appeared that the T cell cloid 5A contained at least two populations of T cells, one of which recognized the unique *β-catenin* gene product and a second population that recognized a shared product in the context of HLA-A24. The T cell cloid 5A was then used to screen an autologous cDNA library by transfecting pools that contained ~200 cDNAs into a stable transfectant of the 293 cell line expressing high levels of HLA-A24 (293-A24). Three positive pools were isolated following the screening of a total of 576 pools

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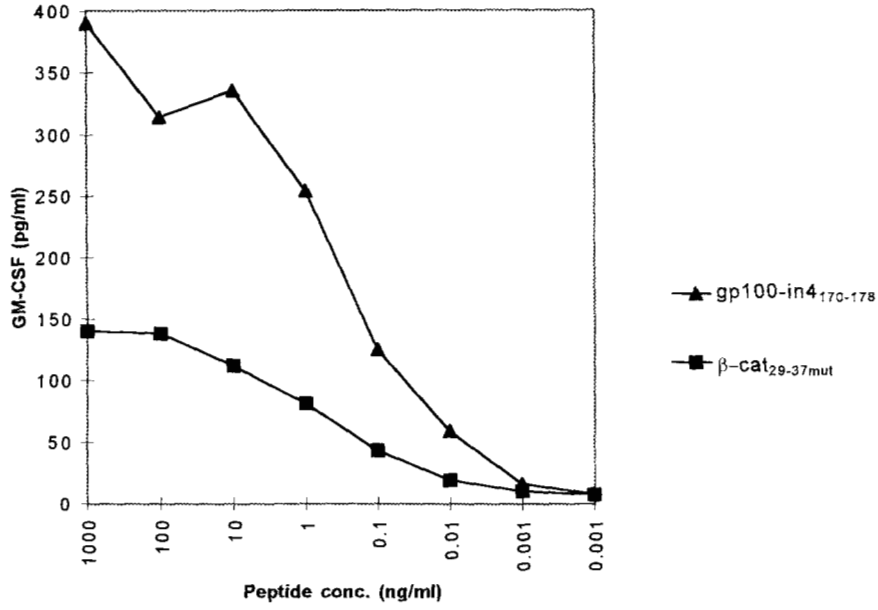

FIGURE 2. Autologous 888 EBV B cells were incubated with the indicated concentrations of either peptide for 2 h, followed by incubation of 5×10^4 B cells with 5×10^3 cloid 5A T cells for 18 h. The release of cytokine from stimulated T cells was then measured in a GM-CSF ELISA assay.

containing ~100,000 cDNA clones, but PCR analysis indicated that two of the three pools contained *β-catenin* (data not shown). Individual colonies were then isolated from the single positive pool that failed to express *β-catenin*. A single cDNA clone that was isolated from this pool, designated *D12-3*, was found to stimulate strong cytokine release following transfection into 293-A24 cells (Table I).

Sequence analysis of positive cDNA clone

A comparison of the sequence of the *D12-3* clone with the GenBank database revealed that the 5′ end of this clone was identical to a previously isolated *gp100* cDNA clone and contained 11 bp of the 5′ untranslated region. Additional sequencing conducted with *gp100*-specific primers revealed that, in addition to containing the entire *gp100* coding region plus 67 bp of 3′ untranslated sequence, the *D12*-3 clone contained an insertion of 388 bp following nucleotide 469 of the normal *gp100* coding region. Alignment of the D12-3 sequence with that of a genomic *gp100* sequence demonstrated that the additional sequence corresponded to the fourth intron of *gp100*, leading to the designation of the *D12*-3 cDNA clone as *gp100*-in4 (Fig. 1). The entire fourth intron of *gp100* was present in the *gp100*-in4 clone, and this sequence as well as the residues from the adjoining exons were identical to a previously isolated genomic *gp100* sequence (R. A. Spritz, unpublished observation). The only other difference noted between *gp100*-in4 and a previously published *gp100* cDNA sequence (19) was the presence of an insertion of the dinucleotide GA following nucleotide 1174 of the *gp100* coding region.

Identification of the T cell epitope in gp100-*in4*

The failure of T cell cloid 5A to recognize cells transfected with the normal *gp100* cDNA (Table I) suggested that the epitope may be encoded by the intronic sequence present in the *gp100*-in4 cDNA clone. The intronic sequence present in the *gp100*-in4 clone contained an in-frame stop codon that would prematurely terminate translation of *gp100*, but read-through from the normal *gp100* coding region into the intronic sequence would result in the addition of 35 amino acids that are not present in the normal gp100 glycoprotein (Fig. 1). Seven peptides encoded within the intronic region that conformed to the HLA-A24 binding motif (20) were

Table II. *Recognition of gp100-in4 by T cell cloids*

Stimulator Cell Line[a]	Peptide	GM-CSF Release of T Cell Cloid (pg/ml) 15	16	18
888 EBV B	None	<8	<8	<8
888 EBV B	gp 100-in4$_{170-178}$	380	910	270
888 EBV B	β-cat$_{29-37mut}$	<8	<8	<8
293-A24/gp100-in4	None	160	680	41
293-A24/pCDNA	None	<8	<8	<8
888 mel	None	83	160	13
397 mel	None	<8	<8	<8
397-A24 mel	None	295	680	56
None	None	<8	<8	<8

[a] Responding T cells were cultured at 5×10^3/well with the 293-A24 cell lines that had veen stably transfected with either *gp100-in4* or *pCDNA3*, 888 EBV B cells pulsed with the indicated peptides at 1 μg/ml for 2 h prior to the addition of T cells or the indicated melanomas, all added at 10^5/well. The release of GM-CSF was measured following 18 h of incubation.

then synthesized and tested for recognition by T cell cloid 5A following incubation with autologous 888 EBV B cells: VWKT WGEGL, VYFFLPDHL, YFFLPDHLSF, FFLPDHLSF, SF GRPFHLNF, PFHLNFCDF, and PFHLNFCDFL. The peptide VYFFLPDHL, designated gp100-in4$_{170-178}$, was the only peptide of the seven tested that stimulated significant cytokine release from T cell cloid 5A when pulsed on HLA-A24-expressing target cells (Fig. 2). The gp100-in4$_{170-178}$ peptide stimulated significant cytokine release from cloid 5A when used to pulse targets at concentrations as low as 10 pg/ml. The T cell cloid 5A also recognized *β-catenin*, and these T cells recognized target cells pulsed with as little as 10 pg/ml of the peptide epitope that had previously been identified in this Ag, β-cat$_{29-37mut}$.

Characterization of gp100-*in4 expression and recognition by TIL*

Further analysis of the *gp100*-in4 gene product was conducted using three T cell cloids that were isolated from TIL 1290. The three T cell cloids 15, 16, and 18 recognized the gp100-in4$_{170-178}$ peptide but not the β-cat$_{29-37mut}$ peptide (Table II). In addition, the

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Table III. *Recognition of* gp100-in4 *by HLA-A24-restricted T cell lines and cloids*

Stimulator Cell Line[a]	HLA-A24 Expression	Peptide	Transfected Gene	GM-CSF Release in Responding T Cells (pg/ml)			
				T cell cloid 15	TIL 1290	TIL 888	TIL 1541
293-A24	+	None	*β-catenin*	<8	91	<8	83
293-A24	+	None	*gp100-in4*	772	188	<8	<8
293-A24	+	None	*gp100*	<8	8	<8	<8
293-A24	+	None	Tyrosinase	<8	<8	30	<8
293-A24	+	None	*pCDNA3*	<8	<8	<8	<8
888 mel	+	None	None	255	450	147	130
1290 mel	+	None	None	108	104	32	52
928 mel	+	None	None	32	23	30	<8
1102 mel	+	None	None	42	21	49	<8
1300 mel	+	None	None	20	18	101	<8
397-A24 mel	+	None	None	1229	504	71	<8
397 mel	−	None	None	<8	14	<8	<8
2488 melanocyte	+	None	None	361	125	186	<8
1070 melanocyte	+	None	None	433	145	150	<8
1085 melanocyte	−	None	None	<8	<8	<8	<8
888 EBV B	+	None	None	<8	10	<8	37
888 EBV B	+	gp100-in4$_{170-178}$	None	497	399	<8	44
888 EBV B	+	β-cat$_{29-37mut}$	None	<8	115	<8	124
None	−	None	None	<8	<8	<8	<8

[a] Either 5 × 10^3 cells (cloid 15) or 5 × 10^4 cells (TIL) were incubated with 10^5 of the indicated stimulator cells, and GM-CSF release was measured after 18 h.

Table IV. *Recognition of* gp100 *isolates by HLA-A24- and HLA-A2-restricted T cells*

T Cells	Stimulator Cell Line	Transfected Gene	GM-CSF (pg/ml)
1290 cloid 15	293-A24	*gp100-in4*	>1000
	293-A24	*gp100*	<8
	293-A24	*pCDNA3*	<8
	888 mel	None	124
	624 mel	None	<8
TIL 620	293-A2	*gp100-in4*	10
	293-A2	*gp100*	208
	293-A2	*pCDNA3*	9
	888 mel	None	35
	624 mel	None	354
TIL 1200	293-A2	*gp100-in4*	<8
	293-A2	*gp100*	218
	293-A2	*pCDNA3*	8
	888 mel	None	41
	624 mel	None	>1600

[a] The cytokine release following coincubation of TIL with 293-A24 or 293-A2 transfectants or melanomas for 18 h was measured in a GM-CSF ELISA assay.

cytokine release of the three T cell cloids in response to the stable *gp100*-in4 transfectants was comparable to that stimulated by HLA-A24$^+$ melanomas. These results indicate that high levels of expression, as seen in the transient transfection system, were not required for stimulation of T cell responses.

Three independently derived TIL lines derived from patient 888 were then analyzed for their ability to recognize the *gp100*-in4 gene product. One TIL, designated TIL 888, was derived from a melanoma metastasis resected in 1989, and a second, TIL 1290, was isolated from a recurrent pelvic tumor mass in 1992. In 1995, a small s.c. tumor was resected, and a third T cell line, TIL 1541, was derived from this lesion. TIL 1290 was found to recognize 293-A24 transfectants expressing *gp100*-in4 as well as *β-catenin*, and T cell cloid 15, derived from TIL 1290, also recognized *gp100*-in4 transfectants (Table III). TIL 1541 recognized *β-catenin*, and TIL 888 recognized tyrosinase, but neither TIL responded to transfectants expressing *gp100*-in4. In agreement with these results, TIL 1290 recognized targets pulsed with the gp100-in4$_{170-178}$ and the β-cat$_{29-37mut}$ peptides. Two melanocytes that expressed HLA-A24, 2488 and 1070, were recognized by T cell cloid 15 cells as well as by TIL 1290, indicating that normal melanocytes express *gp100*-in4 at sufficient levels to allow recognition by T cells. TIL 888, which had previously been shown to recognize tyrosinase, was also stimulated by HLA-A24-expressing melanocytes. In agreement with previous findings (12), TIL 1541 recognized targets pulsed with the β-cat$_{29-37mut}$ peptide, but did not recognize targets pulsed with the gp100-in4$_{170-178}$ peptide, and failed to recognize either the HLA-A24$^+$ melanocytes 2488 and 1070 or allogeneic HLA-A24$^+$ melanomas.

The ability of TIL that respond to the normal *gp100* gene product to recognize the *gp100*-in4 gene product was then examined. Two previously characterized HLA-A2-restricted TIL responded to the normal *gp100* gene product but failed to recognize the *gp100*-in4 gene product following transfection into the HLA-A2-expressing target cell line 293-A2 (Table IV). Previous results indicated that TIL 620 predominantly recognized the epitope G9$_{209}$, which is encoded by the region downstream of the intronic sequence in this clone, and G9$_{154}$, which is encoded by sequences both upstream and downstream of the intron in the *gp100*-in4 clone (21). Previous results also demonstrated that TIL 1200 predominantly recognized the G9$_{154}$ peptide epitope derived from *gp100* (21). Thus, these polyclonal HLA-A2-restricted TIL did not appear to recognize additional T cell epitopes encoded by the intronic sequence in the *gp100*-in4 clone. In addition, these results indicate that little if any splicing of the *gp100*-in4 transcript takes place following transient transfection, since splicing of this intron from the *gp100*-in4 cDNA should regenerate the normal *gp100* reading frame encoding the G9$_{154}$ and G9$_{209}$ epitopes.

The level of expression of the aberrantly spliced message containing the fourth intron was then examined by carrying out RT-PCR with poly(A)$^+$ mRNA from melanoma and melanocytes cell lines. The RT reactions were primed with oligo(dT) primers, and PCR was then conducted with a 5′ primer corresponding to a sequence upstream of the fourth intron and a 3′ primer complementary to a region within the fourth intron, giving rise to a 442-bp product. The relative levels of expression in these cells of products

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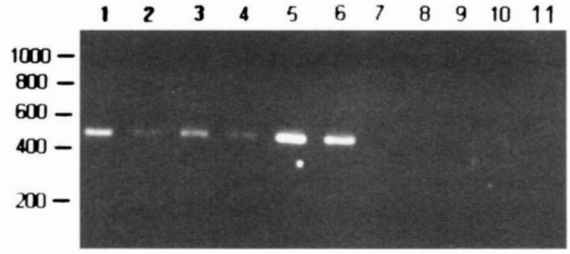

FIGURE 3. The RT-PCR reaction was conducted as described in *Materials and Methods*, run out on a 1% agarose gel with a 100-bp ladder. *Lane 1*, 1300 melanoma (mel); *lane 2*, 397 mel; *lane 3*, 928 mel; *lane 4*, 1102 mel; *lane 5*, 888 mel; *lane 6*, 2488 melanocyte; *lane 7*, 2488 melanocyte RT negative control (no RT added to reaction); *lane 8*, 888 mel RT negative control; *lane 9*, TIL; *lane 10*, PBL; *lane 11*, no RNA added to RT reaction.

containing the fourth *gp100* intron were estimated by carrying out PCR amplification for 15, 20, and 25 cycles. While amplification for 15 cycles revealed little or no signal from any of the samples, after 20 cycles it appeared that the intronic product was expressed at relatively comparable levels in a melanocyte cell line and in five different melanoma cell lines but not in TIL or PBL (Fig. 3).

Discussion

The recent isolation of a number of the Ags recognized by melanoma-reactive T cells has revealed that a variety of mechanisms may be involved in generating T cell epitopes. While a number of the genes that encode tumor Ags appear to represent normal transcripts of nonmutated genes, mutated genes (11–14) as well as the intronic regions of certain genes (13, 15) have been shown to encode tumor Ags. In addition, the product of an alternative open reading frame of the *TRP-1* gene has been shown to encode a T cell epitope recognized by HLA-A31-restricted, melanoma-reactive T cells (22).

The cDNA clone characterized in this report appears to have resulted from the failure to utilize a 5′ splice donor site in the fourth intron of *gp100*, resulting in the retention of this intron in the final cDNA transcript. This product encoded a T cell epitope that was recognized in the context of the HLA-A24 class I restriction element. The *gp100*-in4 product, although expressed at lower levels than the normal *gp100* gene product, appeared to be expressed at similar levels in melanomas and normal cultured melanocytes, and no mutations were found in the intronic region of this clone.

Previously, use of an alternative splice acceptor in the ninth intron of *gp100* was found to generate a product containing seven additional amino acids not present in other *gp100* cDNA clones that have been described (23). This intron is only 102 bp long but does not contain the long polypyrimidine tract generally found at the 3′ end of most introns, which may contribute to the usage of an alternative splice acceptor site. The 3′ end of the fourth intron of the *gp100* gene also does not contain a long polypyrimidine tract, which might lead to the use of an alternative splice acceptor either within this intron or downstream of the intron but should not give rise to a product that has retained an entire intron. Although the 5′ splice donor appears to conform to the general consensus sequence, it appears that this site may not uniformly be recognized as a splice donor site. Other examples of cDNAs that contain intronic sequences have been described in addition to the examples cited above (24, 25). A number of tyrosinase cDNA variants have been isolated, including products that that appear to result from the use of alternative 5′ and 3′ splice sites, as well as a product that has retained a portion of one of the introns of tyrosinase (26).

It is not clear what is responsible for the lack of splicing of the intron present in the *gp100*-in4 gene product after transfection into target cells. Experiments examining the splicing of two introns of the β-globin gene, however, indicate that removal of one of the introns can significantly impede the rate of splicing of the second intron (27). Thus, the lack of adjacent introns in the *gp100*-in4 product may be responsible for the failure of this transcript to be spliced.

The *gp100*-in4 gene product appears to be expressed in melanomas at relatively low levels, since preliminary experiments have indicated that it is difficult to detect these transcripts on Northern blots (data not shown). Although this would be expected to result in the expression of only low levels of the gp100-in4 peptide epitope, T cells have been shown to recognize very small numbers of cell surface peptide-MHC complexes (28). In addition, the gp100-in4 peptide epitope may bind to HLA-A24 class I molecules with high affinity and may be very efficiently processed and presented on the melanoma and melanocyte cell surface.

These findings raise the possibility that additional introns from *gp100*, as well as the introns present in other genes, may encode T cell epitopes. The majority of introns encode only short open reading frames, however, and thus may not encode any peptides capable of binding to a particular class I MHC allele. In addition, differences in splicing efficiency may result in the generation of only certain gene transcripts at detectable levels. The low levels of expression of transcripts containing introns may also result in a failure to induce T cell tolerance to these products, however, which might facilitate the generation of tumor-reactive T cells using immune manipulations such as vaccination and cytokine administration.

References

1. Van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. DePlaen, B. Van Den Eynde, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science 254:1643.*
2. Gaugler, B., B. Van Den Eynde, P. Van der Bruggen, P. Romero, J. J. Gaforio, E. De Plaen, B. Lethe, F. Brasseur, and T. Boon. 1994. Human gene *MAGE-3* codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J. Exp. Med. 179:921.*
3. Van Den Eynde, B., O. Peeters, O. De Backer, B. Gaugler, S. Lucas, and T. Boon. 1995. A new family of genes coding for an antigen recognized by autologous cytolytic T lymphocytes on a human melanoma. *J. Exp. Med. 182:689.*
4. Boel, P., C. Wildmann, M. L. Sensi, R. Brasseur, J-C. Renauld, P. Coulie, T. Boon, and P. Van der Bruggen. 1995. BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. *Immunity 2:167.*
5. Brichard, V., A. Van Pel, T. Wolfel, C. Wolfel, E. De Plaen, B. Lethe, P. Coulie, and T. Boon. 1993. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med. 178: 489.*
6. Coulie, P. G., V. Brichard, A. Van Pel, T. Wolfel, J. Schneider, C. Traversari, S. Mattei, E. D. De Plaen, C. Lurquin, J-P. Szikora, J-C. Reauld, and T. Boon. 1994. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med. 180:35.*
7. Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, K. Sakaguchi, E. Appella, J. R. Yannelli, G. J. Adema, T. Miki, and S. A. Rosenberg. 1994. Identification of a human melanoma antigen recognized by tumor infiltrating lymphocytes associated with in vivo tumor rejection. *Proc. Natl. Acad. Sci. USA 91:6458.*
8. Kawakami, Y., S. Eliyahu, C. H. Delgaldo, P. F. Robbins, L. Rivoltini, S. L. Topalian, T. Miki, and S. A. Rosenberg. 1994. Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc. Natl. Acad. Sci. USA 91:3515.*
9. Wang, R. F., P. F. Robbins, Y. Kawakami, X. Q. Kang, and S. A. Rosenberg. 1995. Identification of a gene encoding a melanoma tumor antigen recognized by HLA-A31-restricted tumor-infiltrating lymphocytes. *J. Exp. Med. 181:799.*
10. Robbins, P. F., M. El-Gamil, Y. Kawakami, E. Stevens, J. Yannelli, and S. A. Rosenberg. 1994. Recognition of tyrosinase by tumor infiltrating lymphocytes from a patient responding to immunotherapy. *Cancer Res. 54:3124.*
11. Wolfel, T., M. Hauer, J. Schneider, M. Serrano, C. Wolfel, E. Klehmann-Hieb, E. De Plaen, T. Hankeln, K.-H. Meyer Zum Buschenfelde, and D. Beach. 1995. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science 269:1281.*

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12. Robbins, P. F., M. El-Gamil, Y. F. Li, Y. Kawakami, D. Loftus, E. Appella, and S. A. Rosenberg. 1996. A mutated *β-catenin* gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. *J. Exp. Med. 183:1185.*
13. Coulie, P. G., F. Lehmann, B. Lethe, J. Herman, C. Lurquin, M. Andrawiss, and T. Boon. 1995. A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. *Proc. Natl. Acad. Sci. USA 92:7976.*
14. Brandle, D., F. Brasseur, P. Weynants, T. Boon, and B. J. Van den Eynde. 1996. A mutated HLA-A2 molecule recognized by autologous cytotoxic T lymphocytes on a human renal cell carcinoma. *J. Exp. Med. 183:2501.*
15. Guilloux, Y., S. Lucas, V. G. Brichard, A. VanPel, C. Viret, E. De Plaen, F. Brasseur, B. Lethe, F. Jotereau, and T. Boon. 1996. A peptide recognized by human cytolytic T lymphocytes on HLA-A2 melanoma is encoded by an intron sequence of the *N*-acetylglucosaminyltransferase V gene. *J. Exp. Med. 183:1173.*
16. Rosenberg, S. A., B. S. Packard, P. M. Aebersold, D. Solomon, S. L. Topalian, S. T. Toy, P. Simon, M. T. Lotze, J. C. Yang, C. A. Seipp, C. Simpson, C. Carter, S. Bock, D. Schwartzentruber, J. P. Wei, and D. E. White. 1988. Use of tumor infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma: preliminary report. *N. Engl. J. Med. 319:1676.*
17. Walter, E. A., P. D. Greenberg, M. J. Gilbert, R. J. Finch, K. S. Watanabe, E. D. Thomas, and S. R. Riddell. 1995. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N. Engl. J. Med. 333:1038.*
18. Robbins, P. F., M. El-Gamil, Y. F. Li, S. L. Topalian, L. Rivoltini, K. Sakaguchi, E. Appella, Y. Kawakami, and S. A. Rosenberg. 1995. Cloning of a new gene encoding an antigen recognized by melanoma-specific HLA-A24 restricted tumor-infiltrating lymphocytes. *J. Immunol. 154:5944.*
19. Adema, G. J., A. J. de Boer, A. M. Vogel, W. A. M. Loenen, and C. G. Figdor. 1994. Molecular characterization of the melanocyte lineage-specific antigen gp100. *J. Biol. Chem. 269:20126.*
20. Kondo, A., J. Sidney, S. Southwood, M-F. del Guercio, E. Appella, H. Sakamoto, E. Celis, H. M. Grey, R. W. Chestnut, and R. T. Kubo. 1995. Prominent roles of secondary anchor residues in peptide binding to HLA-A24 human class I molecules. *J. Immunol. 155:4307.*
21. Kawakami, Y., S. Eliyahu, C. Jennings, K. Sakaguchi, X-Q. Kang, S. Southwood, P. F. Robbins, A. Sette, E. Appella, and S. A. Rosenberg. 1995. Recognition of multiple epitopes in the human melanoma antigen gp100 associated with in vivo tumor regression. *J. Immunol. 154:3961.*
22. Wang, R-F., M. Parkhurst, Y. Kawakami, P. F. Robbins, and S. A. Rosenberg. 1996. Utilization of an alternative open reading frame of a normal gene in generating a novel human cancer antigen. *J. Exp. Med. 183:1131.*
23. Kwon, B. S., C. Chintamaneni, C. A. Kozak, N. G. Copeland, D. J. Gilbert, N. Jenkins, D. Barton, U. Francke, Y. Kobayashi, and K. K. Kim. 1991. A melanocyte-specific gene, *Pmel* 17, maps near the silver coat color locus on mouse chromosome 10 and is in a syntenic region on human chromosome 12. *Proc. Natl. Acad. Sci. USA 88:9228.*
24. Matsumura, Y., M. Sugiyama, S. Matsumura, A. J. Hayle, P. Robinson, J. C. Smith, and D. Tarin. 1996. *Unusual retention of introns in CD44 gene transcripts* in bladder cancer provides new diagnostic and clinical oncological opportunities. *J. Pathol. 177:11.*
25. Svoboda, M., E. Ciccarelli, M. Tastenoy, A. Cauvin, M. Stievenart, and J. Christophe. 1993. Small introns in a hepatic cDNA encoding a new glucagon-like peptide 1-type receptor. *Biochem. Biophys. Res. Commun. 191:479.*
26. Porter, S., and B. Mintz. 1991. Multiple alternatively spliced transcripts of the mouse tyrosinase-encoding gene. *Gene 97:277.*
27. Lang, K. M., V. L. van Santen, and R. A. Spritz. 1985. The two intervening sequences of human β- and γ-globin pre-mRNAs are excised in a preferred temporal order in vitro. *EMBO J. 4:1991.*
28. Christinck, E. R., M. A. Luscher, B. H. Barber, and D. B. Williams. 1991. Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis. *Nature 352:67.*

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