A Peptide Recognized by Human Cytolytic T Lymphocytes on HLA-A2 Melanomas Is Encoded by an Intron Sequence of the *N*-Acetylglucosaminyltransferase V Gene

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Summary

A cytolytic T lymphocyte (CTL) clone that lyses many HLA-A2 melanomas was derived from a population of tumor-infiltrating lymphocytes of an HLA-A2 melanoma patient. The gene coding for the antigen recognized by this CTL was identified by transfection of a cDNA library. It is the gene which has been reported to code for *N*-acetylglucosaminyltransferase V (GnT-V). Remarkably, the antigenic peptide recognized by the CTL is encoded by a sequence located in an intron. In contrast to the fully spliced GnT-V mRNA, which was found in a wide range of normal and tumoral tissues, the mRNA containing the intron region coding for the antigen was not found at a significant level in normal tissues. This mRNA was observed to be present in about 50% of melanomas. Our results suggest that a promoter located near the end of the relevant intron is activated in melanoma cells, resulting in the production of an mRNA coding for the antigen.

Cytolytic T lymphocytes (CTL) directed against human tumor cells have been obtained by culture of irradiated tumor cells with autologous blood lymphocytes, or with tumor infiltrating lymphocytes (reviewed in reference 1). From these responder T cell populations, it has been possible to derive stable anti-tumor CTL clones that do not lyse autologous fibroblasts, EBV-transformed autologous B cells, or target cells that are lysed by NK-like effectors (2–6).

Two main categories of antigens recognized on human melanomas by autologous CTL have been described so far. Some antigens are encoded by genes that are expressed in a wide range of tumors of different histological types but not in normal tissues except in testis and placenta. Examples are the MAGE gene family (7, 8), the BAGE gene (9), and the GAGE gene family (10). Others are differentiation antigens encoded by genes that are expressed only in melanocytes and in melanomas, such as those encoded by tyrosinase (11–14) and those encoded by Melan-A^{MART-1} (15, 16) $gp100^{Pmel17}$ (17, 18), and $gp75^{TRP1}$ (19).

We report here the identification of another gene coding for a melanoma antigen, which is recognized by a CTL clone on a majority of HLA-A2 melanomas. The antigenic peptide which binds to HLA-A2.1 is encoded by an intron sequence of the gene that codes for *N*-acetylglucosaminyltransferase V.

Material and Methods

Cell Lines. Tumor cell lines NA8-MEL, NA17-MEL, and NA74-MEL were derived from the metastatic melanomas of patients NA8, NA17, and NA74. Cell line MZ2-MEL is a subclone of the original MZ2-MEL melanoma line, and was described previously as MZ2-MEL.43 (5, 20). This subclone expresses tyrosinase. Subline MZ2-MEL.2.2.5, used for construction of the genomic library, was derived as described (10). Melanoma cell lines SK29-MEL and SK23-MEL, and RNA extracted from cultivated melanocytes were gifts from Dr. L. Old (Memorial Sloan-Kettering Cancer Center, New York). Tumor cell lines LB373-MEL and LB168-MEL were derived from melanoma patients LB373 and LB168 respectively. Tumor cell lines MI10221 and 526-mel were kindly provided by Dr. G. Parmiani (Istituto Nazionale Tumori, Milan, Italy) and Dr. S.A. Rosenberg (National Cancer Institute, Bethesda, MD), respectively. CTL clone NA17-CTL-213, referred to as CTL 213, was derived from TIL of patient NA17 (6). Lymphoblastoid cells NA17-EBV were derived from patient NA17 by standard techniques.

Assay for Cytolytic Activity. The chromium release assay was performed as described elsewhere (5). Briefly, 1,000 ^{51}Cr-labeled

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target cells were incubated with CTL 213 at different effector-to-target ratios. Chromium release in the supernatant was measured after 4 h of incubation.

Construction of the cDNA Library. Poly-A$^+$(RNA) was obtained from MZ2-MEL cells using the Fastrack mRNA extraction kit (Invitrogen Corporation, Oxon, UK). mRNA was converted to cDNA using an oligo-dT(NotI, EcoRI) primer. The cDNA was ligated to BstXI adaptors, digested with NotI and inserted into the BstXI and NotI sites of expression vector pcDNAI/Amp (Invitrogen Corporation, Oxon, UK). Recombinant plasmids were electroporated into DH5α *Escherichia coli* bacteria and selected with ampicillin (50 μg/ml). Bacteria were amplified to saturation and plasmid DNA was extracted by the alkaline lysis method (21).

Transfection of COS-7 Cells. Transfection experiments were performed by the DEAE-dextran-chloroquine method (11, 15, 22). Briefly 1.5×10^4 COS-7 cells were treated with 100 ng of plasmid pcDNAI/Amp-A2, a recombinant plasmid containing the HLA-A2.1 gene isolated from a CTL clone of patient SK29 (23), and 100 ng of DNA of a pool of the cDNA library. Transfected COS-7 cells were tested in a CTL stimulation assay after 48 h.

CTL Stimulation Assay. Transfectants were tested for their ability to stimulate the production of Tumor Necrosis Factor (TNF) by CTL (24). Briefly, in microwells containing target cells, 2,500 CTL were added in 100 μl of RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) containing 10% human serum and 25 U/ml r-hu-IL2. After 48 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on WEHI-164 clone 13 cells (25) in a MTT colorimetric assay (24, 26).

DNA Sequencing and Homology Search. DNA sequencing analysis was performed by specific priming with synthetic oligonucleotides. The sequencing reactions were performed by the dideoxy-chain termination method (T7 Sequencing Kit; Pharmacia, Uppsala, Sweden, ΔTaq™ Cycle-Sequencing Kit; USB, Cleveland, OH). The computer search for sequence homology was done with the blast program.

Production of Fragments of cDNA 560

Progressive Deletions in cDNA 560. The plasmid was opened with NotI and SphI before digestion with exonuclease III. This treatment was performed with the Erase-a-base System (Promega, Madison, WI). After ligation, the plasmids were electroporated in TOP 10F′ *E. coli* bacteria and selected with ampicillin (50 μg/ml). Clones were isolated, plasmid DNA was extracted from each clone and transfected into COS-7 cells together with the HLA-A2.1 gene.

PCR Fragments of cDNA 560. Fragments were generated from cDNA clone 560 by PCR amplification. They were blunted, phosphorylated and subcloned in vector pcDNAI/Amp digested by EcoRV. To generate PCR fragment 47-185, we used VB45 (5′-GATGTGTTCATACGCTGTGTGGT-3′) as sense primer, and VB56 (5′-TCAGCTTTTGGGTGGGTTGAACTTGG-3′) as anti-sense primer. To generate PCR fragment 35-82, we used as sense primer VB72 (5′-GCCGCCATGGTCCTGCCTGATGTG-3′) which generates an ATG (*underlined*) that has the appropriate Kozak consensus sequence, and YG15 (5′-CTAGTGTAAGACAGAAAACCACACAGCGTATGAA-3′) as anti-sense primer.

Screening of the Genomic Library. A genomic library of 1.1×10^6 independent λ phages was constructed with DNA from MZ2-MEL2.2.5 by standard techniques (21). A labeled cDNA 560 fragment (nt 47 to 185) was used as a probe (probe B) for colony hybridization. A positive phage was isolated and digested with SacI. The fragments were size-fractionated in a 0.7% agarose gel and blotted on a nitrocellulose filter. The filter was hybridized with probe A, a ^{33}P-labeled oligonucleotide (5′-GGTTTCTCGAAGAAGGAACTGC-3′) specific of exon "A" of GnT-V cDNA (positions 1651 to 1672). 5.5- and 8.5-kb fragments, hybridizing with probe A and probe B, respectively, were then subcloned in plasmid pTZ19R (Pharmacia Fine Chemicals, Piscataway, NJ) and partially sequenced.

mRNA Analysis. Anchored PCR amplifications of 5′ and 3′ ends of the cDNA coding for NA17-A antigen were performed. cDNA was synthesized using total RNA from HLA-A2 melanoma cell line LB24-MEL as template.

5′ End Amplification. 5′ end amplification was performed using the 5′-Amplifinder™ RACE Kit (Clontech, Palo Alto, CA). Positions of primers are indicated in figure 7. Primers used for cDNA synthesis were YG104 (5′-CAGCGTATGAACACATCAGGC-3′) or VB56 (5′-CTTTTGGGTGGGTTGAACTTGG-3′). cDNA was ligated to Amplifinder™ anchor as described in the kit. A first round of PCR amplification was done with anti-sense primers YG104 or YG20 (5′-AGGACCATCAGGCAGGAC-3′) and sense Amplifinder anchor primer. A second round of amplification was realized with anti-sense primers YG20 or YG31 (5′-CACTATGCTCTCCTCCACCAAG-3′) and the sense Amplifinder anchor primer. Amplified products were cloned in vector pCR-Script™ SK(+) (pCR-Script™ SK(+) Cloning Kit; Stratagene, La Jolla, CA) and sequenced.

3′ End Amplification. The primer used for cDNA synthesis was EDP1260 (5′-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTT-3′, described by Frohman [27]). A first PCR amplification was done with sense primer VB72 (5′-ATGGTCCTGCCTGATGTG-3′) and anti-sense primer EDP1260. A second PCR amplification was done with sense primer VB45 (5′-GATGTGTTCATACGCTGTGTGGT-3′) and anti-sense primer EDP1260. The amplified product was cloned as above.

In Vitro Transcription Assay. The 1389-bp HindIII-SacI restriction fragment of the 8.5-kb genomic subclone was inserted in transcription vector pGEM3Zf(−) (Promega, Madison, WI). 1 μg SacI-digested plasmid was diluted to a final volume of 50 μl with 10 μl 5× SP6 buffer (GIBCO BRL), 5 μl each NTP at 10 mM, 2.5 μl dithiotreitol at 0.1 M, 2 μl (20U) RNase Inhibitor (GIBCO BRL) and 3 μl (45U) SP6 RNA polymerase (GIBCO BRL). This mixture was incubated at 37°C for 2 h. 1 μl (1U) RQ1 DNase (Promega) was added and the mixture was incubated again for 45 min at 37°C. RNA was ethanol precipitated, resuspended at a final concentration of 2 μg/μl and serially diluted in 2 μg/μl yeast tRNA (Boehringer Mannheim Corp., Indianapolis, IN). 1 μl of 10^{-2} and 10^{-4} RNA dilutions was reverse transcribed as described in the RT-PCR assays section, except that YG104 or VB56 primers (4 μl at 10 μM) were used instead of an oligo-(dT-15) primer. To exclude contamination with plasmid DNA, control reactions were included where no MoMLV reverse transcriptase was added. 1/40 of the completed reactions were engaged in 30 PCR cycles with YG118 (5′-AGGGGAAAATCAACACCAAGATG-3′) as sense primer and YG20 as anti-sense primer. PCR products were fractionated by agarose gel electrophoresis. Control reactions yielded no detectable product.

Antigenic Peptides and CTL Assay. Peptides were synthesized on solid phase using F-moc for transient NH_2-terminal protection as described (28) and characterized by mass spectrometry. All peptides were >90% pure as indicated by analytical HPLC. Lyophilized peptides were dissolved in DMSO and stored at −80°C. They were tested by chromium release assay as previously de-

scribed (29). In this peptide sensitization assay, target cells were ^{51}Cr-labeled for one hour at 37°C and washed extensively. 1,000 target cells were then incubated in 96-well microplates in the presence of various concentrations of peptides for 30 min at 37°C and CTL 213 (10,000 cells) were added. Chromium release was measured after 4 h at 37°C.

RT-PCR Assays. Total RNA was extracted by the guanidine-isothiocyanate procedure as described (30). Reverse transcription was performed on 2 μg of total RNA in a reaction volume of 20 μl with 4 μl of 5× reverse transcriptase buffer (GIBCO BRL), 2 μl of a 20 mM solution of oligo(dT-15) primer, 4 μl of dNTPs at 2.5mM each, 20 U of RNasin (Promega), 2 μl of 0.1 M dithiotreitol and 200 U of MoMLV reverse transcriptase (GIBCO BRL). The reaction was incubated at 42°C for 60 min.

For PCR, $1/40$ of the cDNA reaction was supplemented with 2.5 μl of 10× thermostable DNA polymerase buffer (Finnzymes OY, Expoo, Finland), 0.5 μl each of 10 mM solution of dNTP, 0.625 μl each of 20 μM solution of primers, 0.5 U of DynaZyme™ (Finnzymes OY) and water to a final volume of 25 μl. For amplification of NA17-A cDNA (PCR "I-C"), VB45 was used as sense primer (primer i) and (5′-CTCTACTTCCTCCTGATTGTTGAG-3′) as anti-sense primer (primer c). For amplification of GnT-V cDNA (PCR "A-B"), primer a (5′-GATCTACTTGGACATTATTCACAC-3′) was used as sense primer and primer b (5′-CATCCATTTGCGATAGCTTCCAG-3′) as anti-sense primer. For amplification of cDNA 560, VB45 was used as sense primer, and SL6 (5′-CAATGTCGGTCTTTTCTGTC-3′) as anti-sense primer. The ability of oligonucleotide SL6 to prime DNA synthesis in presence of template cDNA was tested with sense primer SL5 (5′-GAAGAAGAGCAGGTCAGCAGTC-3′), located in positions 345 to 366 of cDNA 560. This control reaction yielded specific amplification products with several tumoral RNAs.

PCR were performed for 30 cycles (1 min at 94°C, 2 min at 62°C and 2 min at 72°C). 10 μl of the PCR product were fractionated on a 1.5% agarose gel. The quality of RNA preparations was checked by PCR amplification of β-actin cDNA with primers (5′-GGCATCGTGATGGACTCCG-3′) and (5′-GTCGGAAGGTGGACAGCGA-3′) for 21 cycles of 1 min at 94°C, 2 min at 65° and 2 min at 72°C. PCR assays for tyrosinase expression were performed as described (11), except that the number of cycles varied between 30 and 38 cycles.

For quantitative expression measurements, cDNA was synthesized as above. RNA obtained from MZ2-MEL cells was used undiluted or serially diluted in *E. coli* tRNA for each series of quantitative PCR. 0.2 μCi of labeled dCTP (3,000 Ci/mmol) were added to the PCR mix. The number of cycles was reduced to 24 for PCR I-C, to 25 for PCR A-B and to 18 for β-actin PCR so that a linear dilution curve of the standard was obtained. Accurate quantitation was obtained using the phosphor-imager technology (Phosphor-Imager; Molecular Dynamics, Sunnyvale, CA).

Results

An Anti-melanoma CTL clone Restricted by HLA-A2.1. Melanoma cell line NA17-MEL was established from a cutaneous metastasis of patient NA17. Anti-tumor CTL were obtained from tumor-infiltrating lymphocytes by culturing fragments of a metastasis in presence of IL-2 (6). This CTL population was restimulated with irradiated NA17-MEL cells under limiting dilution conditions, and stable anti-tumor CTL clones were obtained (6).

CTL clone NA17-CTL-213, further referred to as CTL 213, lysed the autologous melanoma cell line but lysed neither K562 nor autologous EBV-transformed B cells (Fig. 1). The target antigen of this CTL was named NA17-A. CTL clone 213 lysed 12 other melanoma cell lines carrying the HLA-A2 haplotype among the 15 that were tested, suggesting that antigen NA17-A is presented by HLA-A2 (31). It also produced TNF in the presence of NA17-MEL and other HLA-A2 melanoma cell lines (Fig. 2).

The lysis inhibition observed with two distinct anti-HLA-A2 monoclonal antibodies confirmed that HLA-A2 was the presenting molecule for the antigen recognized by CTL 213 (31). This was confirmed further by transfection of the HLA-A2.1 gene into melanoma line MZ2-MEL, which does not carry the HLA-A2 gene. The acquisition of the HLA-A2.1 sequence rendered this cell line capable of stimulating TNF release by CTL 213 (Fig. 2).

A cDNA Coding for Antigen NA17-A. A cDNA library was constructed in expression vector pcDNAI/Amp with poly A^+ (RNA) extracted from MZ2-MEL cells. The library was divided into 640 pools containing each ~90 bacteria carrying an insert. Plasmid DNA of each pool was cotransfected into COS-7 cells with plasmid pcDNAI/Amp-A2, containing the HLA-A2.1 gene. Duplicate microcultures were transfected and screened two days later for their ability to stimulate TNF release by CTL 213. The DNA of one of the 640 pools produced in both duplicates a level of TNF that was significantly above the average and this was confirmed in a second transfection experiment. Bacteria of the positive pool were cloned and their plasmid

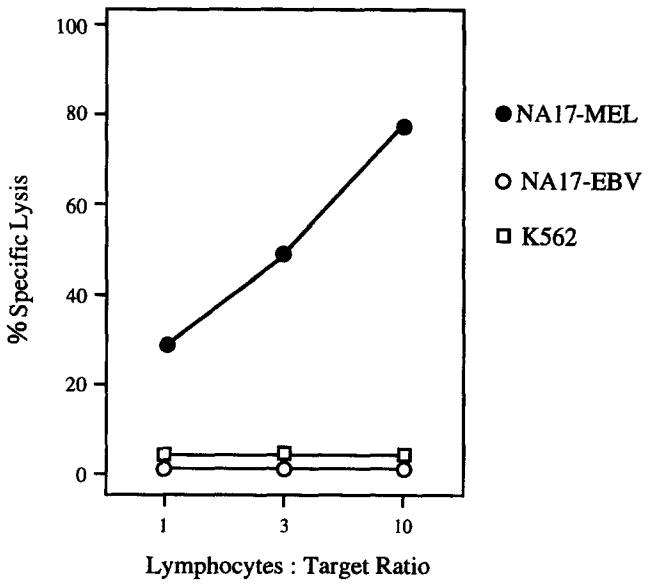

Figure 1. Cytolytic activity of CTL clone 213 on cells of the autologous melanoma line (*NA17-MEL*), on autologous EBV-transformed B cells (*NA17-EBV*), and on K562 cells. Target cells were chromium labeled and incubated with the CTL at different effector-to-target (E/T) ratios. Chromium release was measured after 4 h.

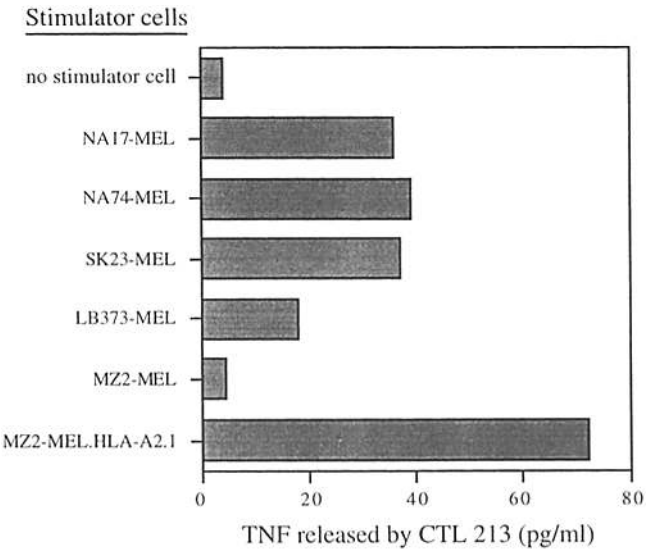

Figure 2. Recognition by CTL 213 of autologous melanoma NA17-MEL and of other HLA-A2 melanoma cell lines NA74-MEL, SK23-MEL, and LB373-MEL. Melanoma cell line MZ2-MEL does not carry HLA-A2. MZ2-MEL.HLA-A2.1 is a MZ2-MEL subclone transfected with a HLA-A2.1 gene. 2,500 CTL were added to 10,000 melanoma cells, and the TNF content of the supernatant was tested after 24 h on WEHI-13 cells.

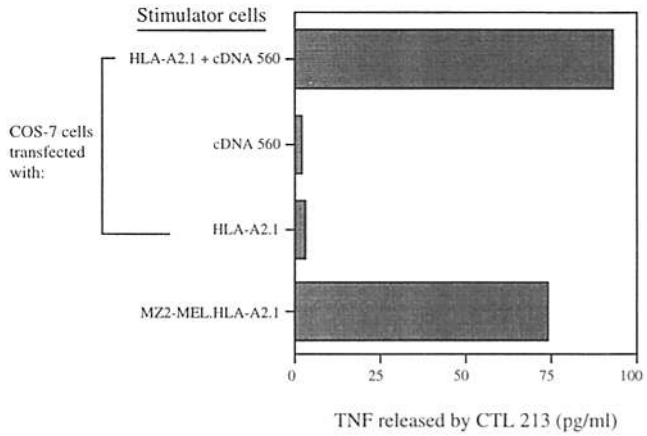

Figure 3. Stimulation of CTL 213 by COS-7 cells cotransfected with HLA-A2.1 and cDNA 560 cloned in pcDNAI/Amp. CTL were added 1 d after the transfection and the TNF content of the supernatant was estimated 1 d later by testing its toxicity of WEHI-13 cells.

DNA was cotransfected with the HLA-A2.1 construct as before. Two out of 1,150 clones stimulated TNF release by CTL 213. The result obtained with one of these, namely cDNA clone 560, is shown in Fig. 3.

The sequence of cDNA 560 proved to be 2,237 bp long. By searching GenBank, we found that nucleotides (nt) 84 to 230 were identical to a region of a cDNA reported by Saito and coworkers to code for *N*-acetylglucosaminyltransferase V (GnT-V)[1] (32). The sequence upstream of nt 84 and that downstream of 230 showed no significant homology with any sequence recorded in databanks.

The cDNA library derived from the MZ2-MEL cell line was searched for sequences related to cDNA 560 by colony hybridization. Using as a probe a XbaI restriction fragment of cDNA 560 extending from position 1 to 973 (Fig. 4), we identified several clones (not shown in Fig. 4) which differed completely from cDNA 560 up to position 230 but were completely identical after this position. The composition of cDNA 560 appeared therefore to be particularly complex with a central fragment (84-230) identical to the GnT-V cDNA (Fig. 4, region B) and two completely unrelated terminal fragments. This raised the possibility that cDNA 560 was an artefactual ligation product of unrelated cDNAs. RT-PCR experiments carried out with MZ2-MEL RNA and involving a sense primer in positions 47 to 69 (VB45) and an anti-sense primer in positions 608 to 628 (SL6) failed to yield an amplified product, consistent with the notion that, in cDNA 560, at least two fragments were artefactually combined.

[1]*Abbreviations used in this paper:* GnT-V, *N*-acetylglucosaminyltransferase V; RT, reverse transcription.

Identification of the Antigenic Peptide. To identify the sequence coding for the antigenic peptide recognized by CTL 213, we produced progressive deletions of the 3′ end of cDNA clone 560 with exonuclease III. By cotransfecting the truncated clones into COS-7 cells together with the HLA-A2.1 construct, we found that the region ranging from nt 1 to 291 transferred the expression of antigen NA17-A (Fig. 5). Using PCR amplified fragments cloned in expression vector pcDNAI/Amp, we identified a 48-bp fragment (nt 35 to 82) that transferred the expression of the antigen (Fig. 5). Another amplified sequence extending from nt 47 to 185 proved unable to transfer the expression of NA17-A. We concluded that the starting point of the sequence coding for the antigenic peptide was located between nt 35 and 47. We observed that nt 38-67 coded for a decapeptide corresponding to the consensus sequence of the peptides binding to HLA-A2.1 (33). This peptide, VLPDVFIRCV, was synthesized and incubated with T2 cells, which are HLA-A2 cells with an antigen processing defect resulting in an increased capacity to present exogenous peptides (34, 35). T2 cells incubated with the peptide were lysed by CTL 213. Nonapeptide VLPDVFIRC sensitized T2 cells equally well, with half-maximum lysis obtained at peptide concentrations of 1 nM, whereas nonapeptide LPDVFIRCV and octapeptide VLPDVFIR failed to confer recognition by the CTL (Fig. 6).

Antigen NA17-A Is Encoded by an Intron. The coding sequence of the antigenic peptide was located on cDNA 560 in front of the region corresponding to the published GnT-V cDNA (Fig. 4). Accordingly, it seemed that either this sequence belonged to an unrelated cDNA which had been ligated in front of the GnT-V sequence, or it belonged to the GnT-V gene, as part of an alternative exon or an unspliced intron. To explore the second possibility, we set out to isolate the GnT-V gene.

A λ gene library prepared with DNA of the MZ2-MEL cell line was screened with probe B, corresponding to nt

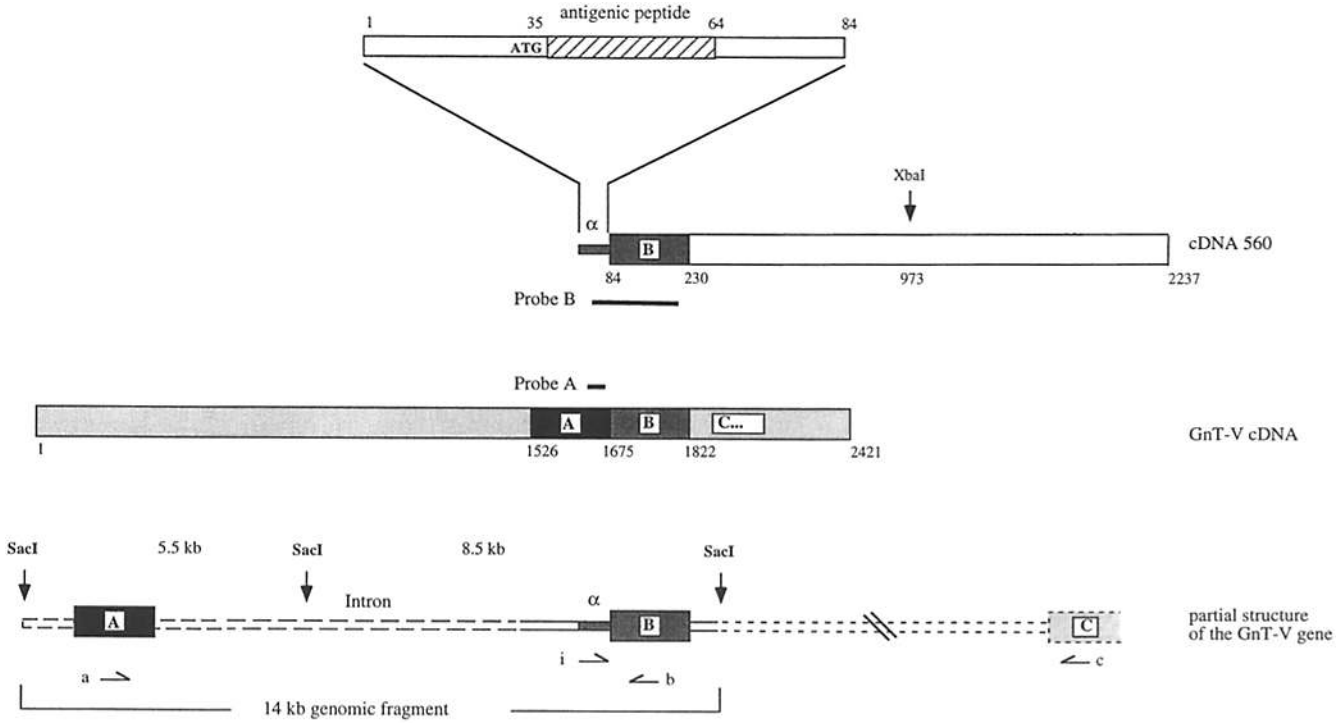

Figure 4. Comparison of cDNA 560, of the GnT-V cDNA reported by Saito et al. (32) and of a genomic clone containing part of the GnT-V gene. The limits of the 14-kb genomic fragment are indicated. The exons of the GnT-V gene fragment are represented as full boxes. Most of the intron located between exons A and B (*large stippled line*) was not sequenced but its approximate length could be evaluated from the length of restriction fragments. The right part (*small stippled lines*) of the representation of the GnT-V gene is purely hypothetical. Probes A and B used in hybridization experiments are indicated by bold lines. The arrows noted a, b, i, and c represent the locations of the primers used for RT-PCR as described in the text.

47-185 of cDNA 560 (Fig. 4). A positive clone with a 14-kb insert was identified. The digestion of this insert with SacI produced two fragments of 8.5 and 5.5 kb, respectively. The first hybridized with probe B. The 5.5-kb fragment hybridized with an oligonucleotide (Fig. 4, probe A) corresponding to a sequence of the reported GnT-V cDNA (32) located a few nucleotides upstream of the region (B) shared with cDNA clone 560. Thus it appeared that the

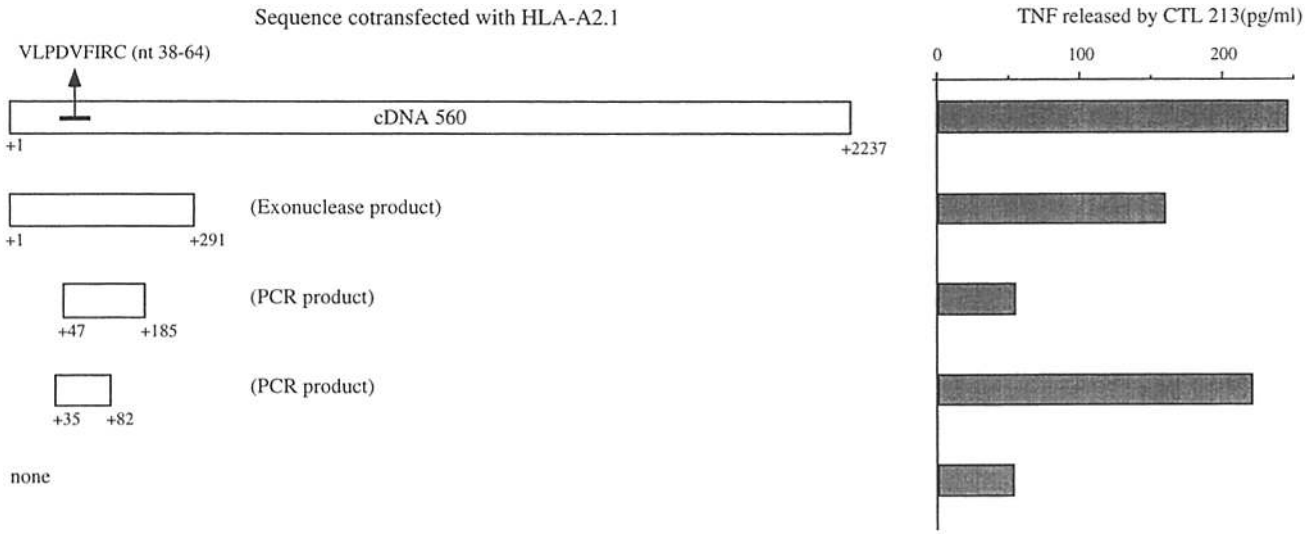

Figure 5. Identification of the region coding for the antigenic peptide recognized by CTL 213. The limits of the exonuclease products and PCR fragments that were cloned into pcDNAI/Amp and transfected into COS-7 cells together with the HLA-A2.1 gene are indicated. Transfected cells (30,000 were incubated for 24 h with 2,500 CTL and the TNF in the supernatant was measured by its toxicity on WEHI-13 cells.

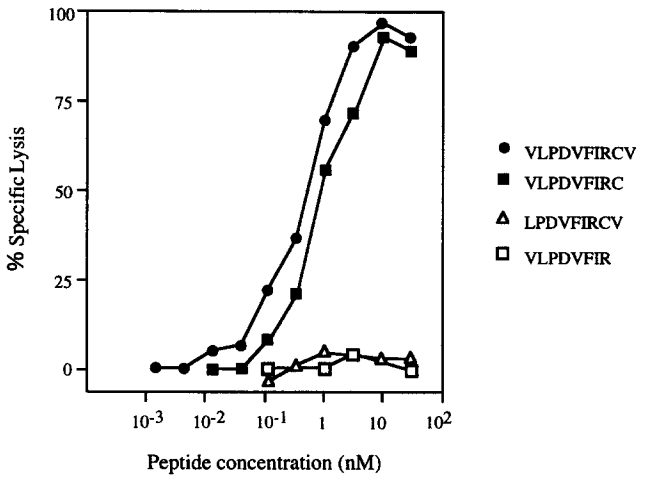

Figure 6. Lysis by CTL 213 of T2 cells, which express HLA-A2, after incubation with synthetic peptides corresponding to sequences of cDNA 560. Target cells were chromium labeled and incubated for 30 min with the peptides at the concentrations indicated. CTL 213 was then added at an E/T ratio of 10. Chromium release was measured after 4 h.

8.5-kb genomic fragment contained the GnT-V exon (B) shared with cDNA 560, whereas the 5.5-kb fragment contained the preceding GnT-V exon (A).

Partial sequencing of the 8.5-kb fragment (Fig. 7) showed that it contained a sequence identical to exon B and, immediately in front of it, a sequence identical to the initial 84 bp of cDNA 560, which contain the sequence coding for the antigenic peptide. This region ends with a consensus splice acceptor sequence. Downstream of region B, the sequence of the 8.5-kb genomic fragment corresponded neither to that of cDNA 560, nor to that of the GnT-V cDNA. The two first nucleotides of this sequence are GT, as expected for the beginning of an intron.

In the 5.5-kb SacI fragment of the genomic clone (Fig. 7) we found a sequence identical to the 150 bp sequence of the GnT-V cDNA (positions 1526 to 1675) that immediately precedes region B. This exon (A) is preceded and followed by consensus splice sites.

We conclude that the genomic clone contains two exons (A and B) which are present in the GnT-V cDNA. They are separated by an intron of at least 8 kb (Fig. 4). The sequence coding for the antigenic peptide is located near the very end of this intron. Its phase is different from that of the large open reading frame which runs through all the GnT-V cDNA.

Characterization of the mRNA Coding for Antigen NA17-A. The intron region coding for the antigenic peptide could be available for translation as a result of the presence of incompletely spliced Gnt-V messenger in the cytosol. Alternatively, a normally silent promoter region located in the intron between exons A and B (Fig. 4) could be activated in some melanoma cells to produce an additional messenger species. In the first hypothesis, 5′ anchored PCR would be expected to yield long and diverse products corresponding to incomplete cDNA molecules, since the intron is at least 8 kb long. In the second hypothesis, and in so far as the relevant promoter would be located near the end of the intron, complete 5′ anchored PCR products could be obtained indicating the new transcription starting point.

Three independent anchored PCR experiments were performed on RNA of melanoma cell line LB24-MEL, which is highly recognized by CTL 213 (Table 1). The primers are shown in Fig. 7. In experiment I, reverse transcription (RT) was primed with oligonucleotide YG104, located in the intron. The cDNA was ligated to an anchor oligonucleotide and submitted to two consecutive series of 30 amplification cycles with the anchor complementary primer and as anti-sense primers the reverse transcription primer in the first series of amplification and primer YG20 (Fig. 7) in the second. The three cloned PCR products that were sequenced started at positions -247, -198 and -91 relative to exon B (Fig. 7, vertical arrows). In experiments II (RT primer YG104, second anti-sense YG31) and III (RT primer VB56, second anti-sense YG20), PCR products with 5′ ends scattered between -288 and -96 were identified. These results suggest the existence of an imprecise transcription origin located in the distal part of the intron.

To exclude the possibility of artefactual anchored PCR results due to a secondary structure in the RNA molecule blocking reverse transcription, we cloned in a transcription vector a HindIII-SacI restriction fragment containing exon B and the last 1087 bases of the preceding intron (Fig. 7). Sense RNA was synthesized in vitro and reverse transcribed with primer YG104. PCR was performed on the cDNA product with anti-sense primer YG20 and sense primer YG118 which starts at position -588, i.e., 300 nt upstream of the earliest putative transcription starting point identified by anchored PCR. A specific 531-bp band was obtained, indicating that the reverse transcriptase can run across the putative transcription start region.

To identify the 3′ end of the messenger containing the peptide coding region, we used an oligo-dT primer for reverse transcription of LB24-MEL RNA. The cDNA was then submitted to two series of PCR amplification cycles with oligo-dT primer and two overlapping sense primers located in the peptide coding region. The sequence of the PCR product was identical to the end of the intron, followed by the complete 3′ end (746 bp) of the published GnT-V cDNA. It seems therefore that the antigen coding messenger starts with an unusual transcription site located in an intron but shows no further transcription or splicing difference with the normal messenger. In the antigen-coding messenger, the open reading frame starts 49 bp before exon B and it is interrupted 222 nt later, in the initial part of the putative exon "C" of the GnT-V gene.

Expression of the Normal GnT-V Enzyme. We designed RT-PCR assays that distinguish the expression of the fully spliced GnT-V transcript (32) from that of the transcript coding for antigen NA17-A. An RT-PCR performed with primers located in exons A and B (Fig. 4; primers a and b) amplifies a 206-bp fragment only from the fully spliced messenger. One sample of each of the large variety of normal adult tissues appearing in Table 3 was tested. All were positive. So were all of 29 melanoma samples.

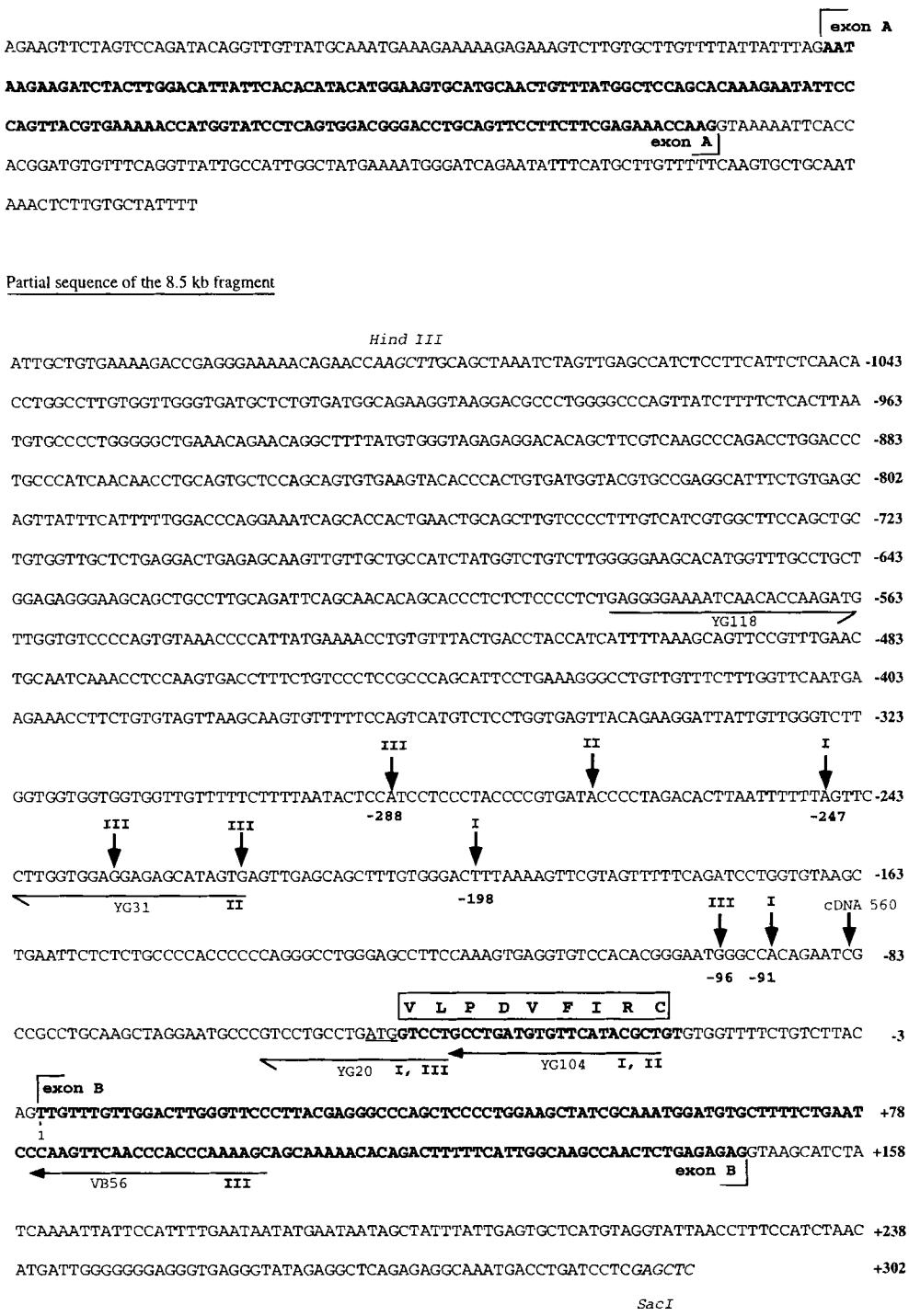

Figure 7. Sequences surrounding the exons present in the two SacI restriction fragments of the 14-kb genomic insert. Exons appear in bold, introns in plain text. The peptide-encoding sequence also appears in bold. It is immediately preceded by a start codon (*underlined*). Nucleotides of the 8.5-kb fragment are numbered from the first nt of exon B. Positions of primers used in 5′ anchored PCR experiments are indicated by horizontal arrows: bold arrows indicate primers used for reverse transcription, thin arrows those used for the final cycles of PCR amplification. Roman numbers below each primer indicate in which experiment it was used. Vertical arrows represent 5′ extremities of cloned anchored PCR products, and are noted I, II, or III to indicate the experiment where they were obtained. Each arrow represents one sequenced clone, except for arrow noted II which represents the extremity of eight different clones. The vertical arrow noted cDNA 560 shows the 5′ start of cDNA 560. SacI and HindIII restriction sites appear in italics. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers X91652 and X91653.

Expression of the Antigen-coding Sequence in Tumors. An RT-PCR assay named I-C, performed with a sense primer located near the end of the intron that precedes exon B and an anti-sense primer located downstream exon B in the GnT-V transcript (Fig. 4, primers i and c), amplifies a 271-bp fragment only from the GnT-V transcript that codes for antigen NA17-A. This assay yielded no amplification product with genomic DNA, indicating that the intron located between exons B and C is of considerable length.

13 HLA-A2 tumor cell lines were tested with a quantitative version of RT-PCR I-C and also for their ability to stimulate TNF release by CTL 213 (Table 1). The 8 HLA-A2 tumor cell lines that stimulated TNF release by CTL

Table 1. *TNF Assay and Quantitative RT-PCR Results on HLA-A2 Tumor Cell Lines*

HLA-A2 tumor cell lines	Recognition by CTL-213 in TNF assay*	Expression of the messenger coding for antigen NA17-A‡
LB24-MEL	+++	298%
NA17-MEL	++	175%
MZ2-MEL.HLA-A2	+++	100% (reference)
NA74-MEL	++	50%
SK23-MEL	++	32%
LB373-MEL	+	30%
526-MEL	+	19%
MI15-MEL	+	14%
LB168-MEL	+	8%
LB96SARC	−	3%
SK29-MEL	−	1%
NA8-MEL	−	1%
LB23-SARC	−	1%
LB37-NSCLC	−	1%

*The TNF assay was performed by adding 2,500 CTL 213 to 10,000 tumor cells and testing the supernatants 24 h later on WEHI-13 cells.
‡cDNA synthesis and 25 cycles of PCR I-C amplification were performed with trace amounts of [^{32}P]dCTP as described in Materials and Methods. Results were quantitated on Phosphor-Imager and normalized according to the expression of β-actin. Results are expressed as a percentage of the level of expression in melanoma line MZ2-MEL.

Table 2. *Expression of NA17-A in Tumor Samples*

Type of tumor	Number of samples tested*	Number of samples with a Na17-A Expression‡ >12.5%	2–12.5%	<2%§
Adrenal gland	1	0	0	1
Bladder	15	0	0	15
Brain	**10**	**1**	2	7
Breast	25	0	2	23
Colon-rectum	10	0	4	6
Head and neck	15	0	0	15
Kidney	16	0	0	16
Leukemia	8	0	3	5
Lung	31	0	0	30
Lymphoma	2	0	0	2
Melanoma	**42**	**20**	9	13
Neuroblastome	3	0	0	3
Ovary	1	0	0	1
Pancreas	4	0	1	3
Prostate	14	0	0	14
Sarcoma	**22**	**1**	2	19
Skin	3	0	0	3
Stomach	2	0	0	2
Testis	11	0	0	11
Thymus	1	0	0	1
Thyroid	1	0	0	1
Uterus	3	0	0	3

*cDNA synthesis and 30 cycles of PCR I-C were performed as described in Materials and Methods.
‡Semi-quantitative measurement was obtained by comparing ethidium bromide fluorescence of PCR products in samples to that in eightfold diluted MZ2-MEL.43 RNA (12.5%).
§The limit of detection was evaluated to 2%.

213 had a level of expression of NA17-A messenger between 300 and 8% of that observed in the MZ2-MEL cell line. There was a good correlation between the level of the NA17-A messenger and the amount of TNF released by the CTL. The five HLA-A2 tumor cell lines that were negative in the TNF assay expressed less than 3% of the amount of NA17-A messenger found in MZ2-MEL cells.

42 melanoma samples and 198 samples of tumors other than melanoma were tested by RT-PCR I-C (Table 2). About half the melanoma samples expressed levels of NA17-A messenger that should lead to antigen expression (More than 12% of that of MZ2-MEL), while tumors of other types did not, with the exception of one brain tumor and one sarcoma.

Expression of the Antigen-coding Sequence in Normal Tissues. A variety of normal tissue samples were tested by RT-PCR I-C for the expression of the RNA coding for antigen NA17-A (Table 3). All the 54 samples were negative except for one breast sample (out of six tested) and one brain sample (out of five), which contained substantia nigra. These samples gave signals evaluated to 3 and 4%, respectively, of the level observed in the MZ2-MEL cell line.

Whereas two skin samples were negative in the RT-PCR assay, a melanocyte culture was clearly positive. We felt that the expression of the NA17-A messenger might be related to in vitro culture conditions and we reexamined the presence of the messenger in the skin. We evaluated the expression of the NA17-A coding sequence relative to that of the tyrosinase gene which is known to be expressed specifically in melanocytes. We considered that if the NA17-A messenger is produced by normal melanocytes, which constitute a few percent of the cells of the basal layer of the epidermis, the expression of this messenger in the skin sample ought to be decreased relative to that found in the MZ2-MEL melanoma line proportionately to that of the tyrosinase messenger. In the two skin samples that were tested we observed a level of tyrosinase mRNA averaging 7.5% of that found in MZ2-MEL cells. For the NA17-A coding sequence the average was only 0.45%, suggesting that skin melanocytes do not express a significant level of the sequence coding for antigen NA17-A.

Table 3. *Expression of NA17-A in Normal Tissues*

Type of tissue	Number of positive results/number of samples tested*
Adrenal gland	0/3
Bladder	0/4
Brain	1/5 (4%)‡
Breast	1/6 (3%)§
Cerebellum	0/1
Colon	0/3
Epididyme	0/2
Heart	0/1
Kidney	0/3
Liver	0/3
Lung	0/3
Muscle	0/1
Ovary	0/2
Prostate	0/1
Scar	0/2
Skin	0/2
Stomach	0/1
Testis	0/3
Thymocytes	0/1
Uterus	0/1
Placenta	0/1
Foetal testis	0/1
Foetal brain	0/3
Umbilical cord	0/1

*cDNA synthesis and 30 cycles of PCR I-C were performed as described in Materials and Methods. The percentage of NA17-A expression compared to NA17-A expression in MZ2-MEL cell line was evaluated in the two positive samples.

‡The brain sample was assayed by quantitative radioactive PCR as described.

§The breast sample was evaluated by performing 38 PCR I-C cycles and comparing ethidium bromide fluorescence of products to those obtained on serially diluted MZ2-MEL RNA.

Discussion

Tumor antigen NA17-A is expressed by approximately 50% of HLA-A2 melanomas. It is a product of the gene that codes for *N*-acetylglucosaminyltransferase V (GnT-V). This enzyme is located in the Golgi apparatus. It catalyzes the transfer of *N*-acetylglucosamine in β(1,6)-linkage to the α(1,6)-linked mannose of biantennary *N*-linked oligosaccharides (35a). This enzyme is active in many normal tissues. Its activity in many cell types has been shown to increase significantly after infection by various transforming viruses or transfection with oncogenes, such as *ras* (36–40). For several cell lines, increase in the activity of GnT-V appears to correlate with increase in the metastatic potential (41, 42).

Remarkably, the region coding for the peptide that binds to HLA-A2.1 to form antigen NA17-A is located in an intron. The results of anchored-PCR amplification suggest that messengers coding for the antigenic peptide are generated by the activation in melanoma cells of a promoter located in this intron. No consensus TATA box can be found in the rather diffuse region where starting points of 5′ anchored-PCR clones were found, and this may account for the absence of a precise transcription starting point. The examination of the putative promoter region did not reveal any transcription factor binding sequence that seemed likely to be responsible for the transcriptional activation in melanoma. We intend to clone this region into a reporter vector, as a first step in analyzing the activation of the putative intron promoter in the melanoma cells.

The AUG codon, which immediately precedes the antigen coding sequence, does not benefit from a favorable upstream sequence for a translation start: instead of the consensus purine, there is a C in position −3. But there is a G in position +4, which ought to compensate for this.

The decapeptide VLPDVFIRCV, which is recognized in association with the HLA-A2.1 molecule by CTL directed against NA17-A is a consensus HLA-A2.1 binding peptide (33) with anchor residues L and V in positions 2 and 10, respectively. Interestingly, nonapeptide VLPDVFIRC induces lysis by CTL 213 equally well, even though it lacks the COOH-terminal consensus anchor residue.

The mRNA that codes for the NA17-A peptide could also code for several other HLA-binding peptides that are not encoded by the normal GnT-V mRNA. This is because the AUG located in front of the NA17-A peptide opens a reading frame which is out of phase with the normal GnT-V coding sequence and which codes for a polypeptide of 74 amino acids. Thus, the abnormal GnT-V messenger could code for several tumor antigens recognized by T lymphocytes.

We examined whether antigen NA17-A is a melanocytic differentiation antigen like those encoded by tyrosinase, Melan-A^{MART-1}, gp 100^{Pmel17} and gp75, or whether its expression is restricted to tumors. The messenger coding for antigen NA17-A seemed not to be expressed at a significant level in the skin melanocytes, indicating that antigen NA17-A is not a differentiation antigen. The expression of the NA17-A messenger appears to be tumor-specific, even though most normal tissues produced weak signals after 38 PCR cycles. But these weak signals may result from the amplification of incompletely spliced but otherwise normal GnT-V messenger, which is very unlikely to produce the antigenic peptide since the peptide-coding sequence is located near the end of a very long intron containing multiple stop codons in all reading frames. Even if these weak signals represented the appropriate message, the level of expression would most probably be too low to produce enough peptide for CTL recognition. We conclude that it is very likely that the expression of antigen NA17-A is sufficiently tumor-specific to be a useful target for cancer immunotherapy.

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