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**DEFINITION OF A CYTOTOXIC LYMPHOCYTE
EPITOPE OF THE SIN NOMBRE HANTAVIRUS G2
GLYCOPROTEIN**

by

Cindy M. Vollaro

A Thesis

Submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

In Partial Fulfillment of the Requirement for the

Degree of Master of Science

in

Biology

May 5, 1999

Approved by:

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Abstract

Sin Nombre virus is a hantavirus first recognized in New Mexico in 1993. This virus is responsible for causing Hantavirus Pulmonary Syndrome, an acute, life threatening illness characterized by pulmonary edema, capillary leaking, and extreme respiratory distress. CD8⁺ cytotoxic T-cell lines specific for Sin Nombre virus were isolated from the peripheral blood mononuclear cells (PBMC) of a donor (NM3) who was naturally infected with the Sin Nombre virus, and has survived hantavirus pulmonary syndrome (HPS). Cytotoxic T lymphocyte (CTL) assays showed that one of these cell lines, 10K, specifically recognizes a nine amino acid epitope, TAHGVGIIP (amino acids 664-672 of the precursor GPC protein), which is located in the G2 protein after cleavage. Another cell line, 10c27, specifically recognized an eight amino acid epitope, AHGVGIIP (amino acids 665-672 of the precursor GPC protein), located in the G2 protein after cleavage. Using polymerase chain reaction (PCR) and CTL assays, the recognition of these epitopes was shown to be restricted by the B35.01 Class 1 human leukocyte associated antigen (HLA) allele. This information will be useful in creating a vaccine for use in immunizing people against the Sin Nombre hantavirus, as well as elucidating the pathogenesis of this disease.

Acknowledgements

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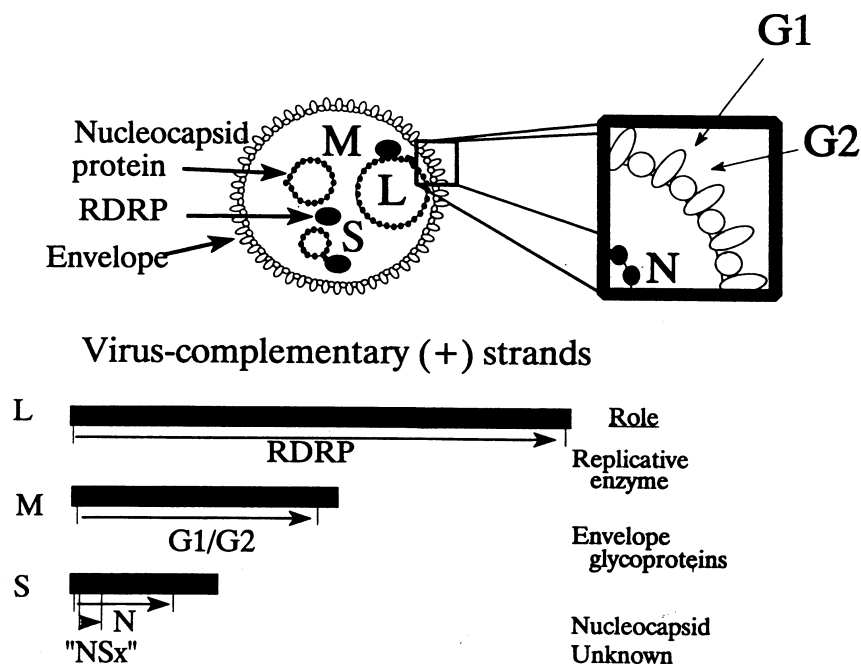
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Introduction

Hantaviruses

Hantaviruses, which are members of the family *Bunyaviridae*, are enveloped negative-sense single stranded RNA viruses whose genome is divided into three genome segments termed, S, M, and L, representing Small, Medium and Large, respectively (Hart and Bennett, 1994). The small segment (approximately 2000 nucleotides) codes for the nucleocapsid protein, N, the medium segment (approximately 3700 nucleotides) codes for the two envelope glycoproteins, G1 and G2, and the large segment (approximately 6500 nucleotides) codes for the viral transcriptase, L. The general structure and genome segments of a hantavirus are shown in Figure 1 (Hjelle *et al.*, 1995).

Figure 1. General structure of Hantavirus Showing Genome and Basic Virion Structure and Coding Assignments (Hjelle *et al.*, 1995)



Hantaviruses spread through direct contact with or inhalation of aerosolized rodent excreta or secretions, such as feces, urine and saliva (Zaki *et al.*, 1995). These viruses constitute the only genus in the *bunyaviridae* family that is not transmitted by an insect vector (Hart and Bennett, 1994). The rodents that carry hantaviruses are persistently infected with the virus and do not show any signs of disease (Hart and Bennett, 1994).

Hantaviruses cause two distinct types of disease in humans: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS).

Hemorrhagic Fever with Renal Syndrome (HFRS)

The hantaviruses that cause HFRS include Hantaan virus, which is the prototype hantavirus, Seoul virus, and Dobrava virus. The rodent hosts for the hantaviruses which cause HFRS are usually indigenous to the Old World (Hjelle, 1995).

The main signs of HFRS are hematologic abnormalities, prominent (often severe) renal involvement and increased vascular permeability (Duchin *et al.*, 1994). There are five distinct phases of HFRS: febrile, hypotensive, oliguric, diuretic, and convalescent (Butler and Peters, 1994). Approximately thirty to forty percent of infected patients have only a minimal illness, and only twenty to thirty percent of patients have a moderate or severe illness (Duchin *et al.*, 1994).

Hantavirus Pulmonary Syndrome (HPS)

Hantavirus Pulmonary syndrome was first recognized in 1993. This disease is caused by hantaviruses carried by rodent hosts that are indigenous to the New World

(Hjelle, 1995). Two strains of hantaviruses that cause HPS are the Sin Nombre virus and the Black Creek Canal virus (Morzunov *et al.*, 1998).

This disease is characterized by extreme respiratory distress, including pulmonary edema and capillary leaking in the lung (Ennis *et al.*, 1997). This disease passes through three phases: prodromal, cardiopulmonary, and convalescent (Butler and Peters, 1994). In the U.S., as of April 7, 1999, 211 cases of HPS have been identified spread across 30 states. Two of these cases occurred in 1999, and of the reported cases the overall mortality rate is 43%, or 90 deaths (Young, 1999).

Sin Nombre Virus

Sin Nombre virus, shown in Figure 2, was the first isolated hantavirus shown to cause HPS. This virus was discovered in New Mexico in May of 1993, and was originally termed the Muerto Canyon Virus and the Four Corners Virus. The rodent vector of Sin Nombre virus is *Peromyscus maniculatus*, commonly known as the deer mouse (Chapman and Khabbaz, 1994).

Figure 2. Electron Micrograph of Sin Nombre Virus (Hjelle, 1995)



The structure of Sin Nombre virus is consistent with the structure of other hantaviruses. The S segment of the genome is 2059 nucleotides in length and codes for the nucleocapsid protein, which is 428 amino acids long. The M genome segment is 3696 nucleotides in length and codes for the GPC precursor protein which is cleaved into the G1 protein (652 amino acids) and the G2 protein (488 amino acids) after translation (Spiropoulou *et al.*, 1994) (Figure 3). The L segment of the genome is 6,562 nucleotides coding for a putative 2,153 amino acid long L polymerase (Chizhikov *et al.*, 1995).

Figure 3. Amino Acid Sequence of Sin Nombre Precursor G1 and G2 Protein, Genbank Accession Number AAA75530 (Spiropoulou *et al.*, 1994). Boldface indicates G2 protein. Highlighted area indicates protein expressed by recombinant vac/G2Afl2 virus.

```

1  mvgwvciflv vlttataglt rnlyelkiec phtvlgqgy vtgsveitpi lltqvadlki
61  esscnfdlhv ptttqkynq vdwtkksstt estnagattf eaktkeinlk gtcnippttf
121 eaayksrktv icydlacnqt hclptvhlia pvqtcmsvrs cmigllsri qviyektycv
181 tgqlieglcf ipthtialtq pghtydtmtl pvtcflvakk lgtqlklave lekltgvs
241 tensfqgyyi cfigkhsepl fvptmedyrs aelftrmvln prgedhdpdq ngqglmriag
301 pvtakvpste ttetmqgiaf agapmyssfs tlvrkadpey vfspgiaes nhsvcdkktv
361 pltwtgflav sgeiekitgc tvfctlagpg asceaysetg ifnissptcl vnkqvkrfgs
421 egrinfmcqr vdqdvvyen gqkkviltkt lvigqciytf tslfslipgv ahslavelcv
481 pglhwatta llitfcfgwl lipavtliil kilrlltfsc shystesfkf vilervkvey
541 qktmgsmvcd ichhecetak elethkscp egqcpymti testesalqa hfaickltnr
601 fgenlkkslk rpevrkgcyr tlgvfryksr cyvglvwgil ltteliiwaa sadtplmesg
661 wsdtahvgvi ipmktdleld falasssys yrrklvnpa qeetlpfhfq ldkqvvhai
721 qnlghwmdgt fniktafhcy geckkyaypw qtakcfekd yqyetswgcn ppdcpgvgtg
781 ctacgvylkd lrsvgkayki vslkytrkvc iqlgteqtck hidvndclvt psvkvcmitg
841 isklqpgdtl lflgpleqgg iilkqwctts cvfgdpgdim sttsgmrcpe htgsfrkicg
901 fattptceyq gntvsgfqrn matrdsfqsf nvtephitsn rlewidpdss ikdhinmvln
961 rdvsfqdlsd npckvdlhtq sidgawgsgv gftlvctvgl tecanfitsi kacdsamcyg
1021 atvtnllrgs ntvkvvgkkg hsgslfkccch dtdcteegla asppldrvt gynqidskv
1081 yddgappcti kcwfksgew llgilngnwv vvavlivili lsillfsffc pvrskrknkan

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Cytotoxic T Lymphocytes

Cytotoxic T lymphocytes are T cells which recognize antigens presented on an infected cell and lyse the infected cell. These cytotoxic cells are usually CD8⁺, however, there are some which have been shown to be CD4⁺. Cytotoxic T lymphocytes have been shown to play a major role in recovery from viral infections. Some cytotoxic T lymphocytes can produce cytokines which can help to modify the immune response by stimulating other immune cells (Coleman, Lombard and Sicard, 1992).

Pathogenesis of HPS

Current research on the pathogenesis of HPS suggests that elevated levels of cytokine production in the lungs contribute to the respiratory distress and pulmonary edema associated with HPS (Mori *et al.*, 1999). These cytokine-producing cells have been shown to be localized specifically in the lungs of patients who have died from HPS. It is believed, but still needs to be determined, that the cytokine producing cells, which may cause the capillary-leak syndrome, include activated T lymphocytes that have infiltrated the lungs. Elevated levels of cytokine producing cells were not observed in patients who did not have acute lung disease (Mori *et al.*, 1999).

The Major Histocompatibility Complex

The major histocompatibility complex (MHC) is the set of genes that determine what a cell recognizes as “self” and “non-self”. This complex presents the antigenic peptide or epitope which is recognized by the T cell receptor on lymphocytes. In humans, this gene complex is referred to as the human leukocyte-associated (HLA)

complex (Coleman, Lombard and Sicard, 1992). These genes, located on chromosome 6, encode the three classes of HLA antigens designated Class I, II and III. These genes are highly polymorphic, resulting in many different alleles for each of the classes and, therefore, many different HLA molecules. Each class of HLA antigens has a different role in the immune response.

Class I Antigens

There are three HLA Class I genes designated HLA-A, HLA-B, and HLA-C. Each of the different alleles of these genes are distinguished by numbering the distinct alleles, such as HLA-A2, HLA-B35 or HLA-B7. In some cases, very similar alleles are subtyped, such as HLA-B35.01 and HLA-B35.02 (which differ by only a few nucleotides).

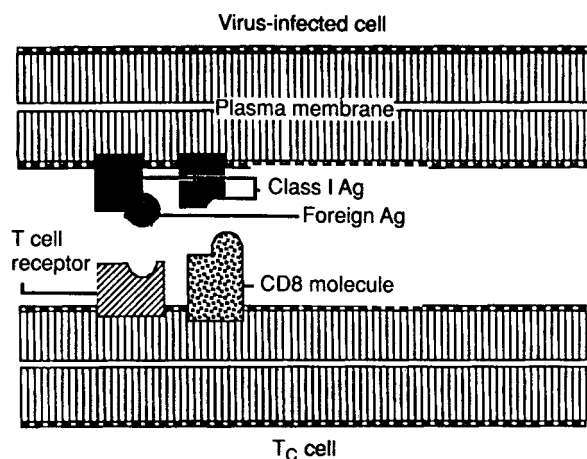
Each of the HLA genes codes for a peptide binding protein that is expressed on the surface of the cell. Since each person carries two alleles for each class I antigen, there is a wide variety of Class I HLAs which can be expressed on the cell surface.

Class I HLA Restriction of an Epitope

The peptide binding region of class I antigens restrict the foreign peptide antigens that will be presented for recognition to CD8⁺ cytotoxic T-lymphocytes. For a specific antigenic determinant, or epitope, to be recognized by a cytotoxic T cell, the larger antigen, such as a virus, must be processed by an infected cell, and presented as small peptides on the cell surface by the appropriate Class I molecule (Figure 4). CD8⁺ cytotoxic T lymphocytes will only recognize an epitope, usually nine amino acids in

length, in the context of a specific class I molecule, a phenomenon called HLA restriction (such as an HLA-A7 restricted epitope).

Figure 4. Recognition of Antigen By Human Cytotoxic CD8⁺ Cells. Foreign Antigen is recognized by CD8⁺ cytotoxic T cells only when it is associated with “self” class I HLA molecules. Binding of the complex to the T cell receptor is apparently enhanced by accessory association of other self class I molecules with CD8 molecules adjacent to the T cell receptor (Coleman, Lombard and Sicard, 1992).



Usually, the HLA Class I molecule's motif, or format for a recognizable epitope, consists of two anchoring amino acids, which are usually conserved, and other non-anchor residues, which can be more diverse (Reammensee Friede and Stevanovic, 1995). When defining an epitope, it is extremely important to realize that this epitope can only be recognized by CD8⁺ cytotoxic T lymphocytes in the context of the appropriate HLA antigen.

Previous Studies on Human Hantavirus-Specific T Lymphocytes

Research on cytotoxic T lymphocyte cell lines specific for the Sin Nombre virus has identified an HLA-C7 restricted epitope, ERIDDFLAA (amino acids 234-242), on

the nucleocapsid protein as well as a HLA-B35 restricted epitope, LPIILKALY (amino acids 131-139), also on the nucleocapsid protein. Both of these epitopes were recognized by CD8⁺ T cell lines. Another epitope, GIQLDQKII (amino acids 372-380), on the nucleocapsid protein, was recognized by a CD4⁺ T cell line (Ennis *et al.*, 1997).

Epitopes for Hantaan virus have also been defined. One CD8⁺ T cell line was specific for a HLA-B51 restricted epitope on amino acids 12-20 of the nucleocapsid protein. Another CD8⁺ T cell line was specific for an HLA-A1 restricted epitope on amino acids 421-429 of the nucleocapsid protein. Also a CD4⁺ T cell line has been shown to be specific for the G1 protein (VanEpps, Schmaljohn and Ennis., 1999).

Goals of Thesis

The goal of this project was to define and characterize the epitope of previously created CD8⁺ T lymphocyte lines specific for the Sin Nombre virus. The cell lines, isolated from donor NM3, who was infected with the Sin Nombre virus naturally, and had survived HPS, had been selected for G2 specificity, but the specific epitope causing the cytotoxic response was still unknown. The work presented here verified the G2 specificity of several clones by use of recombinant vaccinia virus constructs, and identified the minimum epitope recognized by two cell lines using peptide-based CTL assays. The human leukocyte-associated antigen (HLA) restriction of the epitope was also determined by use of PCR and CTL assays.

Methods

Sin Nombre Virus Specific T Cell Lines

Cell lines used were previously isolated as described (Ennis *et. al.*, 1997). Briefly, a bulk culture was established from the peripheral blood mononuclear cells (PBMC) of a patient (NM3) previously diagnosed with HPS. This bulk culture was stimulated with anti-CD3 monoclonal antibody (12F6 at 0.1µg/ml) and gamma-irradiated allogeneic PBMC feeders, and screened against autologous Epstein Barr Virus transformed B-lymphoblastoid cell lines (BLCLs) infected with recombinant vaccinia constructs which expressed various Sin Nombre viral proteins. This screen indicated T cell specificity for the Sin Nombre G2 protein (Scott, unpublished data).

The bulk culture was cloned by limiting dilution. Resulting cell lines were determined to be CD8⁺ by flow cytometric analysis and screened against the target cells infected with a G2 vaccinia construct and/or a vaccinia construct expressing the first half of the G2 protein (designated vac/G2 Afl2) (data not shown). Clonal cell lines showing G2 specificity were chosen for further analysis. Cell lines were courteously provided by Zachary Scott and John Cruz.

T-Cell Line Maintenance

T-cell lines were maintained at 37° C, 6.0% CO₂ in AIM-V medium (Life Technologies) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Life Technologies) and recombinant human interleukin-2 (IL-2), 30 units/ml (Collaborative Biomedical Products). Every two weeks cell lines were stimulated with an anti-CD3

monoclonal antibody, 12F6 at 0.1 µg/ml and gamma-irradiated allogeneic PBMC feeder cells as previously described (Walker *et al.*, 1989; Littaua *et al.*, 1991; Takahashi *et al.*, 1991).

Sin Nombre Vac/G2Afl2 Recombinant Vaccinia Virus

A vaccinia viral construct containing the 15 preceding amino acids and the first 211 amino acids of the Sin Nombre G2 protein (amino acids 638-863 of the precursor GPC protein, 226 amino acids total) was created as previously described (Earl and Moss, 1991) and generously provided by Masanori Terajima. This construct is designated vac/G2Afl2 (Refer to Figure 3).

Peptide Synthesis

All peptides based on the G2 protein (Figure 5) were synthesized at the Protein Chemistry Core Facility at the University of Massachusetts Medical Center, under the direction of Robert Carraway, Ph.D., using an automated Rainin Symphony peptide synthesizer.

Selection of Target Cells

Two different types of target cells were used, Epstein Barr Virus transformed B-lymphoblastoid cell lines (BLCLs) or Hmy2-C1R cell lines.

BLCLs can process an antigen (such as the recombinant vaccinia virus), and present it on their surface in conjunction with a class I HLA antigen. Also, soluble

Figure 5. Synthesized Peptide Sequences. Panel A shows original peptides made. Panel B shows truncations of Peptide 3 made following screening of peptides of Panel A.

A. Overlapping Peptide Truncations of vac/G2Afl2 Protein (20 amino acids)

Peptide	Sequence		Amino Acids
01	MGILLTTELI	IWAASADTPL	637-657
02	IWAASADTPL	MESGWSDTAH	647-667
03	MESGWSDTAH	GVGIIIPMKTD	657-677
04	GVGIIIPMKTD	LELDFALASS	667-687
05	LELDFALASS	SSYSYRRKLV	677-697
06	SSYSYRRKLV	NPANQEETLP	687-707
07	NPANQEETLP	FHFQLDKQVV	697-717
08	FHFQLDKQVV	HAEIQNLGHW	707-727
09	HAEIQNLGHW	MDGTFNIKTA	717-737
10	MDGTFNIKTA	FHCYGECKKY	727-747
11	FHCYGECKKY	AYPWQTAKCF	737-757
12	AYPWQTAKCF	FEKDYQYETS	747-767
13	FEKDYQYETS	WGCNPPDCPG	757-777
14	WGCNPPDCPG	VGTGCTACGV	767-787
15	VGTGCTACGV	YLDKLR SVGK	777-797
16	YLDKLR SVGK	AYKIVSLKYT	787-807
17	AYKIVSLKYT	RKVCIQLGTE	797-817
18	RKVCIQLGTE	QTCKHIDVND	807-827
19	QTCKHIDVND	CLVTPSVKVC	817-837
20	CLVTPSVKVC	MIGTISKLQP	827-847
21	MIGTISKLQP	GDTLLFLGPL	837-857
22	GDTLLFLGPL	EQGGIILN	847-865

B. Truncations of Peptide 03. Aligned according to consensus sequence. (9-14 amino acids)

Peptide	Sequence	Amino Acids
03	MESGWSDTAHGVGIIIPMKTD	657-676
3A	ESGWSDTAHGVGII	658-671
3B	WSDTAHGVGIIIPMK	661-674
3C	TAHGVGIIIPMKTD	664-676
3D	SDTAHGVGI	662-670
3E	TAHGVGIIP	664-672
3F	AHGVGIIPM	665-673
3G	HGVGIIPMK	666-674

antigen (such as the peptides) can be bound to the cell surface in conjunction with the HLA antigen (Coleman, Lombard and Sicard, 1992). Autologous or allogeneic BLCL lines were selected as target cells depending on the experiment performed. To define the epitope, autologous BLCL lines were used to ensure matching class I HLAs. To determine HLA restriction, allogeneic BLCL lines were used which were partially HLA matched in order to determine which HLA allele was restricting the recognition of the epitope (VanEpps, Schmaljohn, and Ennis, 1999)

Hmy2-C1R is a human plasma cell line lacking endogenous HLA-A and HLA-B antigens (Storkus *et al*, 1987). This cell line was transfected with either B35.01 or B35.02 as described (Hayashi, *et al.*, 1989; Ooba, *et al.*, 1989). These cell lines were kindly provided by Dr. Masafumi Takaguchi.

Preparation of Target Cells

Virus infected target cells were prepared by infecting either BLCLs or Hmy2-C1R cell lines with the vac/G2 Afl2 virus at a multiplicity of infection (M.O.I) of 15 for 1 hour at 37° C. Infected cells were then diluted in 1 ml of RPMI 1640 supplemented with 10% FBS and incubated at 37° C for approximately 14 hours before being ⁵¹Cr labeled.

⁵¹Cr labeling of cells was performed by incubating target cells with 0.25 mCi of ⁵¹Cr (in the form of Na₂⁵¹CrO₄) in 100 µl of RPMI 1640/10% FBS for 1 hour at 37° C. After the target cells were ⁵¹Cr labeled, they were washed three times and resuspended at 1.5 X 10⁴/ml in RPMI 1640 supplemented with 10% heat-inactivated FBS.

Peptide pulsed targets were prepared by incubating uninfected ^{51}Cr labeled cells (either BLCLs or Hmy2-C1R) with the indicated concentration of peptides for 30 minutes before effectors were added.

Cytotoxic T-Cell (CTL) Assay (^{51}Cr Release Assay)

Cytotoxic T-cell (CTL) ^{51}Cr release assays were performed in 96-well round bottom plates as previously described using either BLCLs or Hmy2-C1R cell lines as target cells (Ennis, *et al.*, 1997; Littaua *et al.*, 1991; Walker *et al.*, 1989). Target cells were prepared as described above, and 1.5×10^3 cells were added to each well of a 96 well round bottom plates in 100 μl . 100 μl of effector cells (T cell lines) were added at Effector:Target (E:T) ratios as indicated. Each sample was assayed in triplicate.

Plates were incubated at 37°C for 4 hours. The media was then harvested from individual wells using the Skatron supernatant collection system (Skatron Instruments) and counts per minute (cpm) for each sample were measured using a gamma counter.

Specific lysis was calculated as (experimental release-spontaneous release)/(maximum release-spontaneous release) X 100 (VanEpps, Schmaljohn and Ennis, 1999). Negative controls were uninfected/unpulsed targets. Spontaneous lysis (determined by spontaneous release/maximum release X 100) was <30% for most assays.

RNA Isolation

RNA isolation was performed from 1×10^7 BLCLs from Donor NM3 as specified by the UltraSpec RNA Isolation kit (Biotecx Laboratories, Inc.). Briefly, cell pellets were lysed with UltraSpec solution, chloroform was added, and the sample was

incubated on ice for 5 minutes. After centrifugation for 15 minutes at 12,000g (at 4° C), the aqueous phase was collected and an equal volume of isopropanol was added. The sample was placed on ice for 10 minutes then centrifuged for 10 minutes at 12,000g (at 4° C). The sample was then washed twice with 1 ml of 75% ethanol by vortexing and then centrifuging for 5 minutes at 7,500g (at 4° C). The RNA pellet was then air dried and resuspended in diethyl pyrocarbonate (DEPC) treated water.

cDNA Synthesis

Complementary DNA was synthesized from total RNA. 2 µg of RNA was added to 45 µl of DEPC treated water and 0.5 µg of oligo dT and incubated at 72° C for 2 minutes, then placed at 37° C for 5 minutes. The sample, 100 Units of RNase Inhibitor (Pharmacia Biotech), 1X reaction buffer, 2 mM dNTP, and 500 Units of Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies) were placed in a 1.5 ml Eppendorf tube in a final volume of 100 µl. The reaction mixture was incubated at 37° C for 60 minutes then 95° C for 10 minutes.

Polymerase Chain Reaction (PCR)

PCR was performed on the cDNA to isolate the HLA-B cDNA as previously described (Ennis *et al.*, 1990, Little and Parham, 1991) by use of the GeneAmp kit and a DNA thermal cycler (Perkin-Elmer). The reaction mixture included 1 µl of cDNA, 25 pmol of primer 5P2T (specific for class I HLA molecules), 25 pmol of primer 3B (specific for HLA-B), 1X XLBII buffer, 2 units of r *Tth* DNA polymerase, 0.8 mM dNTP, and 0.8 mM Mg(OAc)₂ in 100 µl. Primer sequences are shown in Figure 6. 60

μ l of mineral oil was added before the tube was placed in the thermal cycler. The thermal cycler's program was as follows: 60 seconds at 94° C, 30X (30 seconds at 94° C then 5 minutes at 65° C), 72° C for 10 minutes. PCR product was detected by electrophoresis.

Gel Electrophoresis

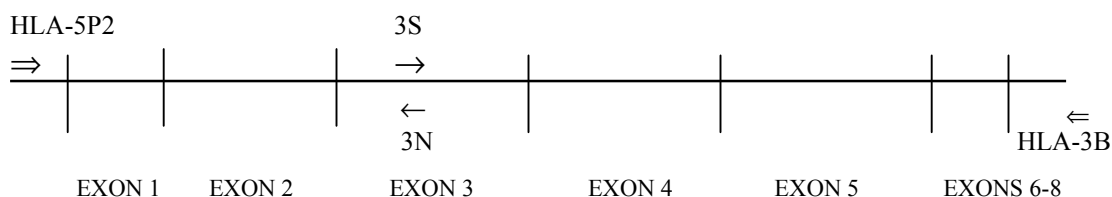
A 1% agarose gel was made in TAE buffer with ethidium bromide (final concentration 0.1 mg/ml) . The gel was placed in TAE buffer with ethidium bromide (final concentration approximately 0.05 mg/ml). A ϕ X174/Hae III digest was used as DNA size markers. 5 μ l of PCR product was run with an equal amount of 2X loading buffer (0.083% bromophenol blue, 0.83% xylene cyanol FF, 5% Ficoll). A PCR product previously determined to be a B35.02 sequence was loaded as a positive control. The gel was run at 100 V for approximately 30 minutes.

Purification of PCR Product

The PCR product was purified with the Qiagen QIAquick Purification Kit using the microcentrifuge method. Briefly, 5 volumes of Buffer PB (250 μ l) was added to 1 volume (50 μ l) of PCR reaction. The sample was placed in a QIAquick column and centrifuged for 60 seconds at 10,000 g. The flow through was discarded, and the column was washed with 750 μ l of Buffer PE and centrifuged for 60 seconds at 10,000 g. DNA was eluted into a clean 1.5 ml centrifuge tube with 30 μ l of 10mM Tris-HCl pH 8.5 by letting stand for 1 minute and then centrifuging at 10,000 g. Purification was confirmed by electrophoresis.

Figure 6. DNA Sequence of Priming and Sequencing Oligonucleotides. A. Schematic of HLA-B PCR product showing PCR primers (large arrows), sequencing primers (small arrows), and exon boundaries (vertical lines). Arrowheads are at the 3' hydroxyl end of each oligonucleotide primer and point in the direction of polymerase extension. B. Sequences of priming and sequencing oligonucleotides. Underlined regions indicate enzyme cutting sites listed above the region. Sequencing oligonucleotides S are derived from the sense strand, and N from the antisense strand. (modified from Ennis *et al.*, 1990; Little and Parham, 1991)

A.



B.

Priming oligonucleotides

Primer	Encompasses	5'	3'
HLA-5P2	5' untranslated region	gggc <u>gtcgac</u> ggactcagaatctccccagacgcgag	
HLA-3B	3' untranslated region	ccgca <u>agctt</u> ctggggaggaaacacaggtcagcatgggaac	

Sequencing oligonucleotides

Primer	Encompasses	5'	3'
3S	exon 3, nucleotides 429-450	cggcaaggattacatcgcctg	
3N	exon 3, nucleotides 429-450	cagggc gatgtaatccttgccg	

Sequencing of DNA

DNA sequencing was done by the Nucleic Acid Facility at the University of Massachusetts Medical Center, using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). Primers used were 3S and 3N (Figure 6). Products were electrophoresed on a 4.75% poly-acrylamide gel, and sequences were analyzed using ABI 373 stretch Automated Sequencer, and ABI Prism Sequencing 2.1.1 (ABI)

Results

G2Afl2 Peptide Screening

Overlapping peptides of 20 amino acids in length were made spanning the entire G2 Afl2 region. Each of these peptides overlapped by ten amino acid regions at the amino and carboxy ends (Figure 5A).

These peptides were screened in two separate CTL assays, at 25 µg/ml, with a CD8⁺ T cell line, 3c12, isolated from donor NM3 at an Effector:Target (E:T) ratio of 10:1. The viral construct vac/G2Afl2 was used as a positive control to show G2 protein cytotoxic specificity. It is important to note that a negative lysis is equivalent to 0.0%, and any lysis of greater than 100% is equivalent to 100%.

The 3c12 cell line was shown to specifically lyse autologous BLCLs infected with the vac/G2Afl2 virus at up to 66.5%, and peptide 03 pulsed autologous BLCLs at 133.5%. However uninfected/unpulsed autologous BLCLs were lysed at only up to 8.9%, and autologous BLCLs pulsed with peptides 01, 02, and 04-22 ranged from 0-12.8%. This indicates cell line 3c12 has cytotoxic specificity for Peptide 03 (Table 1).

Since neither peptide 02 nor peptide 04 was recognized, it was determined that the area containing the G2 epitope would be a sequence that was only completely contained in peptide 03.

Cell lines 10K and 10c27 were also tested in a CTL assay against autologous BLCLs pulsed with peptide 02, 03, or 04 with vac/G2Afl2 infected autologous BLCLs as a positive control to determine if other NM3 cell lines would also recognize this peptide

Table 1. CD8⁺ Cell line 3c12 (Donor NM3) Shows Cytotoxic Specificity for the Recognition of Peptide 03. E:T = 10:1, Peptide concentrations were 25 µg/ml.

Experiment #	Target- NM3 BLCLs	% Lysis
1	Vac/G2Afl2	<u>66.5</u>
1	Uninfected/Unpulsed	6.8
2	vac/G2Afl2	<u>57.0</u>
2	Uninfected/Unpulsed	8.9
2	01 (aa 637-657)	2.3
2	02 (aa 647-667)	2.3
2	03 (aa 657-677)	<u>133.5</u>
2	04 (aa 667-687)	6.8
2	05 (aa 677-697)	3.7
1	06 (aa 687-707)	7.3
1	07 (aa 697-717)	12.4
2	08 (aa 707-727)	-0.6
2	09 (aa 717-737)	4.9
2	10 (aa 727-747)	7.4
2	11 (aa 737-757)	5.1
2	12 (aa 747-767)	4.1
2	13 (aa 757-777)	-0.6
2	14 (aa 767-787)	2.3
1	15 (aa 777-797)	12.8
1	16 (aa 787-807)	4.8
2	17 (aa 797-817)	8.3
2	18 (aa 807-827)	0.7
2	19 (aa 817-837)	3.7
1	20 (aa 827-847)	1.9
1	21 (aa 837-857)	3.2
1	22 (aa 847-867)	0.6

(Table 2). Both 10K and 10c27 were shown to specifically lyse peptide 03-pulsed BLCLs (specific lysis 96-99%).

Screening of Peptide 03 Truncations

Once it was determined that the epitope being recognized was contained exclusively in peptide 03, three 14 amino acids peptides were made based on the sequence of peptide 03 (designated 3A, 3B, and 3C) (Figure 5B). These truncations were screened in a CTL assay at 25 µg/ml, with cell lines 10K and 10c27 at an E:T of 10:1. Autologous BLCLs pulsed with each peptide at 25 µg/ml were used as the targets. Autologous BLCLs pulsed with peptide 03 at 25 µg/ml was used as the positive control, and unpulsed autologous BLCLs were the negative control (Table 3).

This assay showed that both 10K and 10c27 recognize peptide 3B and peptide 3C. That indicates that the epitope that the cell lines are recognizing is located in the overlapping region of peptides 3B and 3C. This region corresponds to amino acids 664-674 of the precursor GPC protein.

Screening of Overlapping Areas of Peptides 3B and 3C

Three nine amino acid long peptides were then made of the region which overlaps in peptides 3B and 3C. These peptides were designated 3E, 3F and 3G based on their starting amino acids (sequences are shown in Figure 5B). Another nine amino acid long peptide was made extending to the left of the overlapping portions of 3B and 3C. This peptide was designated 3D (sequence shown in Figure 5B).

Table 2. CD8⁺ Cell Lines 10K and 10c27 (Donor NM3) Show Cytotoxic Specificity for the Recognition of Peptide 03. E:T = 10:1, Peptide concentrations were 25 µg/ml.

Target- NM3 BLCLs	% Lysis	
	10K	10c27
vac/G2Afl2	84.4	93.8
Uninfected/Unpulsed	1.0	2.6
02 (aa 647-667)	-2.8	-0.1
03 (aa 657-677)	96.0	98.9
04 (aa 667-687)	-0.4	1.0

Table 3. CD8⁺ Cell Lines 10K and 10c27 (Donor NM3) Show Cytotoxic Specificity for the Overlapping Portion of Peptides 3B and 3C. E:T = 10:1, Peptide concentrations were 25 µg/ml.

Target-NM3 BLCLs	Amino Acid Sequence	% Lysis	
		10K	10c27
Uninfected/Unpulsed		1.0	2.6
03 (aa 657-677)	MESGWSDTAHGVGIIIPMKTD	96.0	98.9
3A (aa 658-671)	ESGWSDTAHGVGII	-0.2	-1.1
3B (aa 661-674)	WSDTAHGVGIIIPMK	88.3	92.3
3C (aa 664-676)	TAHGVGIIIPMKTD	83.3	81.7

A CTL assay with cell lines 10K and 10c27 was done with these peptides using autologous BLCLs pulsed with the peptides at a concentration of 25 µg/ml. Autologous BLCLs pulsed with peptide 03 at a concentration of 25 µg/ml were used as a positive control, and unpulsed autologous BLCLs were used as a negative control (Table 4).

This assay showed that cell line 10K recognized peptide 3E exclusively. However, cell line 10c27 recognized both 3E and 3F at a concentration of 25 µg/ml. Cell line 10c27 also seems to have greater avidity for the peptide than cell line 10K, since the percent specific lysis is higher.

Neither peptide 3D (data not shown) nor peptide 3F were recognized by either cell line 10K or 10c27 at a concentration of 25 µg/ml.

Overview of Recognition of Peptide 03 Truncations

An overview of the results of experiments using truncations of peptide 03 indicates which amino acids are essential for epitope recognition (Table 5). Cell line 10K only recognizes peptides containing the entire nine amino acid sequence of peptide 3E (aa 664-672). In contrast, cell line 10c27 recognizes peptide truncations which contain only amino acids 665-672. The lack of response of both cell lines to peptide 3A indicates that the proline at position 672 is an essential part of the epitope recognized by both cell lines. The lack of response of cell line 10K to peptide 3F indicates that the threonine at position 664 is an essential part of the epitope recognized by this cell line. However, since 10c27 does recognize this peptide, this indicated that the threonine at position 664 is not an essential part of the epitope for this cell line. The lack of

Table 4. CD8⁺ Cell Line 10K shows Cytotoxic Specificity for peptide 3E, but CD8⁺ Cell Line 10c27 Shows Cytotoxic Specificity for Both Peptides 3E and 3F. E:T = 10:1, Peptide concentrations were 25 µg/ml.

Target-NM3 BLCLs	Amino Acid Sequence	% Lysis	
		10K	10c27
Uninfected/Unpulsed		0.9	-1.6
03 (aa 657-677)	MESGWSD TAHGVGI IPMKTD	74.5	102.7
3E (aa 664-672)	TAHGVGI IP	51.4	93.5
3F (aa 665-673)	AHGVGI IPM	5.4	99.8
3G (aa 666-674)	HGVGIIPMK	-0.8	2.5

Table 5. Peptides to Which Cell Lines 10K and 10c27 Show a Positive Response. Amino acids which are shown to be essential for recognition in at least one cell line are indicated by bold face in the peptide sequence which is recognized.

Peptide	Sequence	Recognition by Cell Line at 25 µg/ml	
		10K	10c27
03 (aa 657-677)	MESGWSD TAHGVGI IPMKTD	+	+
3A (aa 658-671)	ESGWSDTAHGVGI I	-	-
3B (aa 661-674)	WSD TAHGVGI IPMK	+	+
3C (aa 664-676)	TAHGVGI IPMKTD	+	+
3D (aa 662-670)	SDTAHGVGI	-	-
3E (aa 664-672)	TAHGVGI IP	+	+
3F (aa 665-673)	AHGVGI IPM	-	+
3G (aa 666-674)	HGVGIIPMK	-	-

recognition of peptide 3G of both cell line 10K and 10c27 indicates that the alanine at position 665 is essential for recognition for both cell lines.

Dose Dependent Recognition of Peptides 3E and 3F

A dose dependent response for peptide 3E (aa 664-672) is shown for both cell lines 10K and 10c27 at an E:T of 10:1 (Figure 7 and Table 6). Peptide 3E is maximally recognized by these cell lines at concentrations greater than or equal to 0.25 μ g/ml. The cells lines also show partial recognition down to 2.5 ng/ml, at which point the peptide is then at a concentration too low to be recognized.

However, only cell line 10c27 exhibits a dose dependent response for peptide 3F (aa 665-673), whereas cell line 10K does not recognize peptide 3F at any concentration (Figure 8 and Table 7). The recognition of peptide 3F by cell line 10c27 is strong at concentrations as low as 25 ng/ml, with partial recognition at 2.5 ng/ml.

Figure 7. Both Cell Lines 10K and 10c27 Exhibit a Dose Response for Peptide 3E

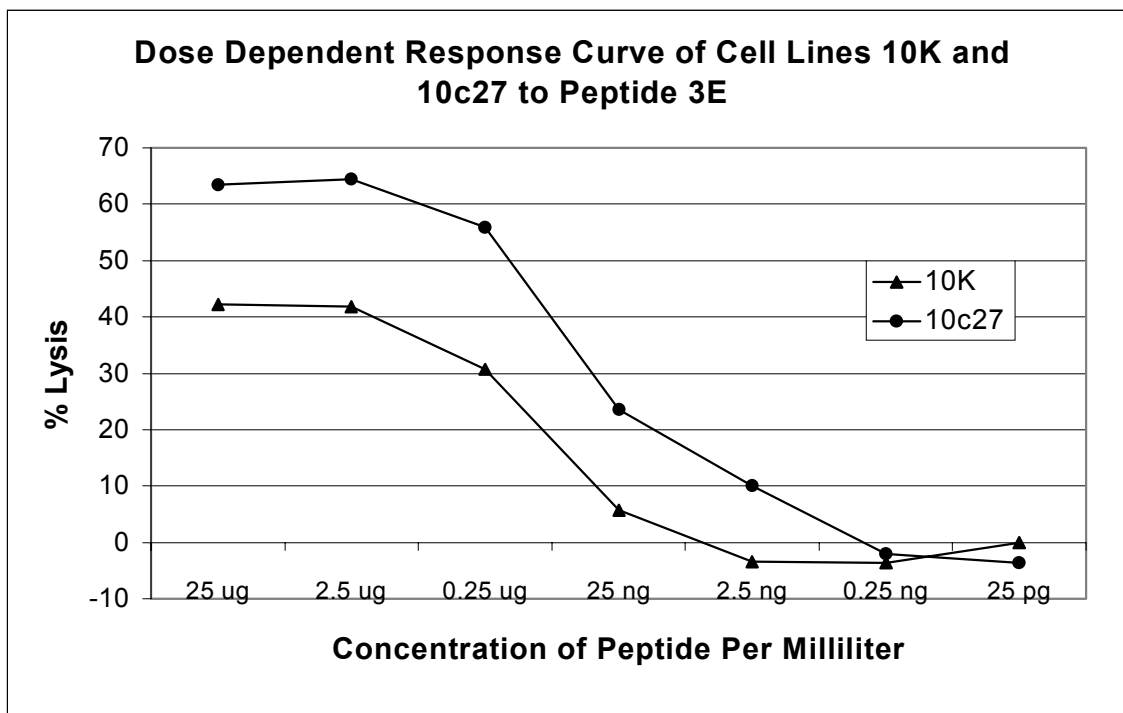


Table 6. Dose Dependent Response of Cell Lines 10K and 10c27 to Peptide 3E. Target cells are NM3 BLCLs pulsed with peptide 3E at the concentrations indicated. The E:T ratio was 10:1. Peptide 03 pulsed targets were included as a positive control, and unpulsed targets were included as a negative control. Bold face indicates strong recognition, and underlining indicates slight recognition.

Peptide	% Lysis of Target NM3 BLCLs								
	03	Unpulsed	3E						
	25 µg/ml		25 µg/ml	2.5 µg/ml	0.25 µg/ml	25 ng/ml	2.5 ng/ml	0.25 ng/ml	25 pg/ml
10K	45.5	-2.7	42.2	41.8	30.6	5.6	-3.4	-3.7	-0.9
10c27	76.4	-2.2	63.4	64.5	56.0	23.5	10.0	-2.1	-3.6

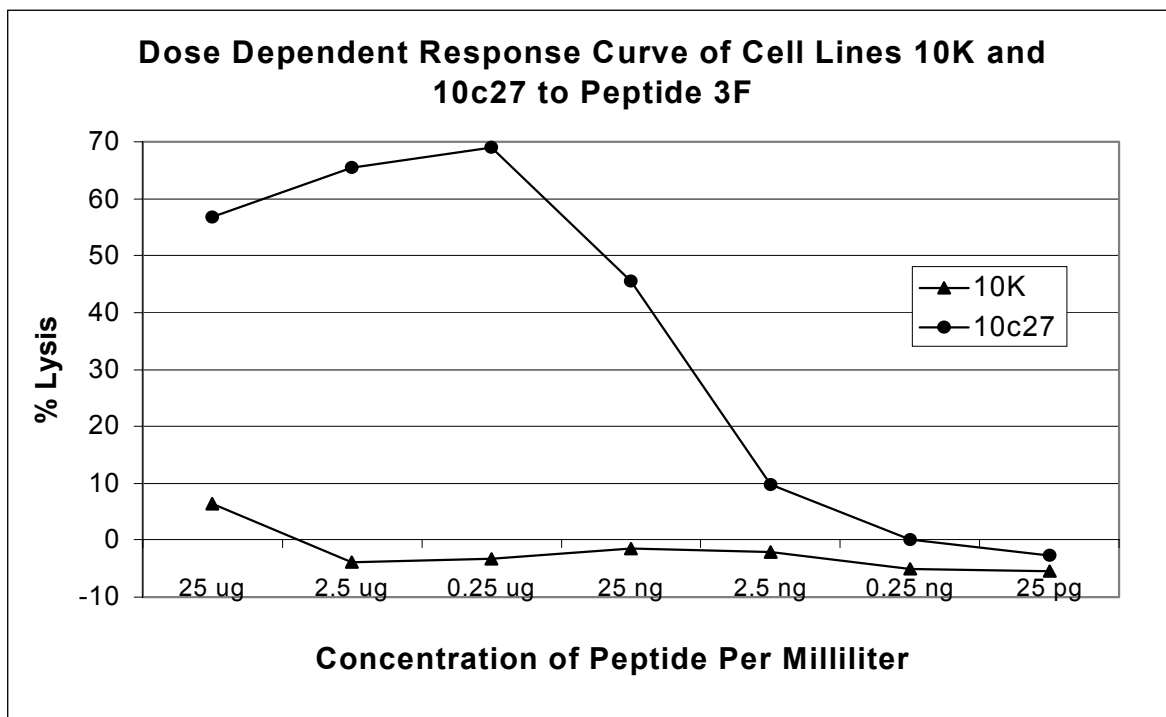
Figure 8. Cell Line 10c27 Exhibits a Dose Response for Peptide 3F

Table 7. Dose Dependent Response of Cell Lines 10K and 10c27 to Peptide 3F. Target cells are NM3 BLCLs pulsed with peptide 3F at the concentrations indicated. The E:T ratio was 10:1. Peptide 03 pulsed targets were included as a positive control, and unpulsed targets were included as a negative control. Bold face indicates strong recognition and underlining indicates slight recognition.

Peptide	% Lysis of Target NM3 BLCLs								
	03	Unpulsed	3F						
	25 µg/ml		25 µg/ml	2.5 µg/ml	0.25 µg/ml	25 ng/ml	2.5 ng/ml	0.25 ng/ml	25 pg/ml
10K	<u>45.5</u>	-2.7	6.4	-3.9	-3.2	-1.5	-2.1	-5.1	-5.5
10c27	<u>76.4</u>	-2.2	56.8	65.5	69.1	45.6	<u>9.7</u>	0.1	-2.7

HLA Restriction of Vac/G2Afl2 Recognition

To determine which HLA antigen was restricting the recognition of the epitope contained in the vac/G2Afl2 construct, NM3 cell line 10F was screened in CTL assays against vac/G2Afl2 infected BLCLs from various donors sharing at least one HLA Class 1 allele (Table 8). Cell line 10F recognizes Donor 9 (CCL011) who shares only HLA-B35, indicating this is the restricting HLA antigen. Cell line 10F also recognizes Donor 3 (3099), Donor 5 (MB) and Donor 8 (CCL002) who share HLA-A2, HLA-B35 and HLA-C4. Donor 4 (KS), who shares HLA-B35 and HLA-C4, is also recognized. Although all allogeneic target cells cell line 10F recognizes express the HLA-B35 allele, cell line 10F does not recognize every target expressing HLA-B35. Therefore, the recognized epitope is restricted by a specific HLA-B35 subtype. It is also shown that the restricting subtype for recognition is not HLA-B35.02, since Donor 1 (JC) is not recognized and is known to be HLA-B35.02.

Determination of HLA-B35 Subtype of NM3

To define the HLA-B35 subtype of NM3, RNA was isolated from the NM3 BLCLs, cDNA was made, and PCR was performed using HLA-B specific primers. The presence of a PCR product was confirmed by electrophoresis. After purification, the PCR product was sequenced. Comparison of the sequence to known B35 subtypes show that donor NM3 has HLA-B35.01 (Appendix I). It is also clear that NM3 is homozygous for B35.01, since direct sequencing of the PCR product showed that there was only one HLA-B sequence present.

Table 8. Recognition of Allogeneic BLCLs Expressing the Sin Nombre Virus G2 Protein. HLA alleles shared by both target cells and NM3 are shown. Subtypes, if known, are designated by listing after the decimal point. All target cells (with the exception of the uninfected NM3 negative control cells) were infected with vac/G2Afl2. Alleles shared are shown. Alleles which are shared are bold if there was a cytotoxic response from at least one cell line. E:T = 10.

	Target	HLA Alleles Expressed			% Lysis 10F
		HLA-A	HLA-B	HLA-C	
Experiment 1	NM3	2	35	4	58.7
	NM3/uninf.				-0.8
	Donor 1 (JC)		35.02	4	-4.7
	Donor 2 (Va12)		35	4	0.2
	Donor 3 (3099)	2	35	4	59.3
	Donor 4 (KS)		35	4	58.7
	Donor 5 (MB)	2.1	35	4	44.7
	Donor 6 (GJ)	2			-1.7
Experiment 2	NM3	2	35	4	78.4
	NM3/uninf.				2.8
	Donor 7 (CCL051)	2		4	10.2
	Donor 8 (CCL002)	2	35	4	88.8
	Donor 9 (CCL011)		35		108.5
	Donor 10 (CCL031)		35	4	9.1
	Donor 11 (CB)	2	35	4	4.5

Recognition of Peptide 03 by CTL Lines 10K and 10c27 is HLA-B35.01 Restricted

Once the donor, NM3, was determined to have the HLA-B35.01 gene, it was possible to confirm that this was, in fact, the HLA allele restricting recognition of the G2 epitope recognized by cell line 10K, using target cells only expressing various forms of HLA-B35. The cell lines used were HMY-C1R cells which either expressed only HLA-B35.01, only HLA-B35.02 or no HLA-A or HLA-B (HMY-C1R control). NM3 BLCLs were used as a positive control. Unpulsed target cells were included for each type of target as a negative control. The E:T was 10:1. This CTL assay shows that cell line 10K is clearly restricted by HLA-B35.01. Cell line 10c27 was also apparently restricted by HLA-B35.01. However, at the peptide concentration of 25 μ g/ml used in this assay, there was also significant lysis of the HMY-C1R B35.02 and control cells (Table 9).

HLA-B35.01 restriction of cell line 10c27 was further confirmed in a CTL assay using a lower E:T ratio (5:1) and lower peptide concentrations (2.5 μ g/ml-2.5 ng/ml). Peptides 3E and 3F were used, since they had been shown to contain the optimal epitope (Table 10). This assay did show that cell line 10c27 is B35.01 restricted. It is important to note that the lower levels of lysis are due to the lowered E:T ratio and lowered peptide concentrations and do not indicate that the cell lines are not lysing the targets.

Table 9. Cell Line 10K Recognizes a HLA-B35.01 Restricted Epitope on Peptide 03. Recognition by cell line 10c27 also seems to be HLA-B35.01 restricted, but high background lysis require confirmation. E:T = 10:1. Peptide concentrations were 25 $\mu\text{g/ml}$.

Target	% Lysis	
	10K	10c27
NM3/peptide 03	<u>67.3</u>	<u>85.3</u>
NM3/unpulsed	-2.6	-0.2
HMY-C1R B35.01/peptide 03	<u>87.0</u>	<u>105.7</u>
HMY-C1R B35.01/unpulsed	-1.1	0.4
HMY-C1R B35.02/peptide 03	18.2	<u>56.7</u>
HMY-C1R B35.02/unpulsed	1.2	1.4
HMY-C1R control/peptide 03	15.3	<u>48.0</u>
HMY-C1R control/unpulsed	-4.1	-2.4

Table 10. Recognition of Peptides 3E and 3F by Cell Line 10c27 is HLA-B35.01 Restricted. E:T = 5:1

Targets	% Lysis by Cell Line 10c27								
	Unpulsed	Peptide 3E				Peptide 3F			
		2.5 $\mu\text{g/ml}$	0.25 $\mu\text{g/ml}$	25 ng/ml	2.5 ng/ml	2.5 $\mu\text{g/ml}$	0.25 $\mu\text{g/ml}$	25 ng/ml	2.5 ng/ml
HMY-C1R B35.01	0.8	<u>16.2</u>	<u>24.6</u>	<u>23.1</u>	0.5	<u>18.0</u>	<u>30.9</u>	<u>23.5</u>	<u>12.3</u>
HMY-C1R B35.02	-3.2	5.2	1.2	-0.6	-0.1	6.6	1.3	0.3	0.4
HMY-C1R control	0.0	8.0	-1.6	-3.6	-4.9	2.6	-2.6	-4.6	-6.7

Discussion

Definition of B35.01 Restricted Epitopes in the Sin Nombre Virus G2 Protein

Studies presented here have defined the first CD8⁺ cytotoxic T cell epitopes to be located in the Sin Nombre virus G2 protein. CTL assays with peptide pulsed targets have shown that NM3 T cell line 10K recognizes the nine amino acid sequence TAHGVGIIP (aa 664-672) of the G2 protein of the Sin Nombre hantavirus. Another NM3 T cell line, 10c27, recognizes the eight amino acid sequence AHGVGIIP (aa 665-672) of the Sin Nombre virus G2 protein. Both of these epitopes have been shown to be restricted by HLA B35.01 based on PCR analysis of NM3 HLA genes and CTL assays with HMY-C1R cells expressing HLA-B35 alleles.

New Motif for B35.01

The epitopes that were defined for these Sin Nombre virus specific cell lines do not fit the current predictions for HLA-B35 motif. The motifs that are predicted are epitopes which contain the amino acid Proline at position 2 of the epitope, and amino acids Tyrosine, Phenylalanine, Methionine, Leucine, or Isoleucine at position 9 of the epitope (Rammensee, Friede and Stevanovic, 1995). The nine amino acid epitope that was described here contains amino acid Alanine at position 2 and amino acid Proline at position 9. Using the search strategies available at the BIMAS HLA Peptide Binding Predictions website (http://bimas.dcrf.nih.gov/molbio/hla_bind/), the epitope that was defined was not the best match in the entire M segment protein. In fact, this program, which ranks possible epitopes by their predicted half-time of dissociation, indicates that

this sequence is one of the least likely to be recognized (Parker, Bednarek and Coligan, 1994). This indicates that the predicted motifs may need to be revised.

Future Work

The eight amino acid peptide that is believed to be the epitope recognized by cell line 10c27 needs to be synthesized. Once this peptide is tested, the exact minimum epitope recognized by cell line 10c27 will be conclusively defined.

Both cell lines should also be tested against G2 proteins of other hantaviruses to ascertain if there is any cross reactivity. A preliminary search on the Entrez database (<http://www.ncbi.nlm.nih.gov/Entrez/>), showed that other hantavirus G2 proteins do not contain the exact epitope recognized by the cell lines, however there may be similar amino acid sequences located in the G2 proteins of other hantaviruses which could be recognized. The recognized peptides should likewise be screened with T cell lines isolated from other patients infected with hantavirus types to determine whether they can be recognized in a cross reactive fashion.

Also, the background lysis that was shown in screening of cell line 10c27 against HMY-C1R targets at high E:T ratios should be further analyzed to determine if 10c27 might be slightly cross-reactive with other subtypes of B35.

Cytotoxic T cell lines should be isolated and characterized from other patients who have had Sin Nombre virus infection. This would make it possible to define other epitopes which may be restricted by different HLA alleles.

This information can be used to help determine the exact pathogenesis of Hantavirus Pulmonary Syndrome. Since patients with different HLA alleles seem to

react to the virus differently, it would be interesting to compare the patients' disease progress to the response that is obtained *in vitro*. Specifically, current research indicates that patients who express the HLA-B35 allele tend to have more severe HPS (Koster *et al.*, 1998). It would be interesting to determine what exactly is causing an increase in the severity of HPS in patients expressing HLA-B35, and whether this specific epitope may have a part in this reaction.

The cytokine production of these cell lines should also be determined. Previous studies have indicated that T cells producing high amounts of cytokines could cause capillary leaking in the lungs, a symptom characteristic of HPS (Mori *et al.*, 1999).

Finally, after defining other T cell epitopes of Sin Nombre virus, a vaccine could conceivably be made by combining different viral peptides in a harmless construct. This vaccine should be able to induce a cytotoxic T cell response in any person having the restricting HLA molecules of the epitopes used. In fact, if there are several epitopes which can cause a cross reactive response for different viruses and also have different HLA restrictions, it could be possible to create one vaccine for protection against more than one type of hantavirus.

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Appendix I: Comparison of NM3 HLA-B35 Sequence to Known B35 Sequences

Sequences were obtained from (Mason and Parham, 1998). Alignment done with Align Plus 3.0 (Scientific and Educational Software). Molecules were shortened to contain only the region spanned by the NM3 HLA-B35 sequenced product. The large region, which is not present in NM3, corresponds to the region in which the primers overlapped.

- . indicates base is same as reference molecule (NM3)
- indicates base not present
- n indicates base was undetermined

Reference molecule:	NM3	1 - 640	(640 bps)	Homology
Sequence 2:	B3501.SEQ	1 - 1087	(1087 bps)	98%
Sequence 3:	B3502.SEQ	1 - 1087	(1087 bps)	98%
Sequence 4:	B3503.SEQ	1 - 1090	(1090 bps)	98%
Sequence 5:	B3504.SEQ	1 - 1089	(1089 bps)	98%
Sequence 6:	B3505.SEQ	1 - 1089	(1089 bps)	98%
Sequence 7:	B3506.SEQ	1 - 1089	(1089 bps)	98%
Sequence 8:	B3507.SEQ	1 - 1089	(1089 bps)	98%
Sequence 9:	B3508.SEQ	1 - 1089	(1089 bps)	98%
Sequence 10:	B35091.SEQ	1 - 1089	(1089 bps)	98%
Sequence 11:	B35092.SEQ	1 - 547	(547 bps)	74%
Sequence 12:	B3510.SEQ	1 - 526	(526 bps)	72%
Sequence 13:	B3511.SEQ	1 - 526	(526 bps)	72%
Sequence 14:	B3512.SEQ	1 - 526	(526 bps)	72%
Sequence 15:	B3513.SEQ	1 - 546	(546 bps)	74%
Sequence 16:	B3514.SEQ	1 - 546	(546 bps)	74%
Sequence 17:	B3516.SEQ	1 - 1089	(1089 bps)	98%
Sequence 18:	B3517.SEQ	1 - 546	(546 bps)	74%
Sequence 19:	B3518.SEQ	1 - 546	(546 bps)	74%
Sequence 20:	B3519.SEQ	1 - 546	(546 bps)	75%
Sequence 21:	B3520.SEQ	1 - 546	(546 bps)	75%
Sequence 22:	B3521.SEQ	1 - 546	(546 bps)	75%
Sequence 23:	B3522.SEQ	1 - 546	(546 bps)	74%
Sequence 24:	B3523.SEQ	1 - 546	(546 bps)	75%
Sequence 25:	B3524.SEQ	1 - 546	(546 bps)	75%
Sequence 26:	B3525.SEQ	1 - 546	(546 bps)	75%

Alignment type: Global DNA
Parameters: Mismatch 2; Open Gap 4; Extend Gap 1

```

NM3          (      1) -ccgccatgtcccggcccggccgnggggagccccgnttcacgcagtgagg
B3501.SEQ    (    101) -.....C.....C.....
B3502.SEQ    (    101) -.....C.....C.....
B3503.SEQ    (    101) a.....C.....
B3504.SEQ    (    101) -.....C.....C.....
B3505.SEQ    (    101) -.....C.....C.....

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B3506.SEQ ( 101) -.....c.....c.....
B3507.SEQ ( 101) -.....t...c.....c.....
B3508.SEQ ( 101) -.....c.....c.....
B35091.SEQ ( 101) -.....c.....c.....
B35092.SEQ ( 28) -.....c.....c.....
B3510.SEQ ( 28) -.....c.....c.....
B3511.SEQ ( 28) -.....c.....c.....
B3512.SEQ ( 28) -.....c.....c.....
B3513.SEQ ( 28) -.....c.....c.....
B3514.SEQ ( 28) -.....c.....c.....
B3516.SEQ ( 101) -.....c.....c.....
B3517.SEQ ( 28) -.....c.....c.....
B3518.SEQ ( 28) -.....c.....c.....
B3519.SEQ ( 28) -.....c.....c.....
B3520.SEQ ( 28) -.....c.....c.....
B3521.SEQ ( 28) -.....c.....c.....
B3522.SEQ ( 28) -.....c.....c.....
B3523.SEQ ( 28) -.....c.....c.....
B3524.SEQ ( 28) -.....c.....c.....
B3525.SEQ ( 28) -.....c.....c.....

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NM3 ( 50) cnacgtggacgacaccagttcgtgaggttngacagcgacgccgagtc
B3501.SEQ ( 150) .t.....c.....
B3502.SEQ ( 150) .t.....c.....
B3503.SEQ ( 151) .t.....c.....
B3504.SEQ ( 150) .t.....c.....
B3505.SEQ ( 150) .t.....c.....
B3506.SEQ ( 150) .t.....c.....
B3507.SEQ ( 150) .t.....c.....
B3508.SEQ ( 150) .t.....c.....
B35091.SEQ ( 150) .t.....c.....
B35092.SEQ ( 77) .t.....c.....
B3510.SEQ ( 77) .t.....c.....
B3511.SEQ ( 77) .t.....c.....
B3512.SEQ ( 77) .t.....c.....
B3513.SEQ ( 77) .t.....c.....
B3514.SEQ ( 77) .t.....c.....
B3516.SEQ ( 150) .t.....c.....
B3517.SEQ ( 77) .t.....c.....
B3518.SEQ ( 77) .t.....c.....
B3519.SEQ ( 77) .t.....c.....
B3520.SEQ ( 77) .t.....c.....
B3521.SEQ ( 77) .t.....c.....
B3522.SEQ ( 77) .t.....c.....
B3523.SEQ ( 77) .t.....c.....
B3524.SEQ ( 77) .t.....c.....
B3525.SEQ ( 77) .t.....c.....

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NM3 ( 100) cgaggacggagccccgggcgccatggatagagcaggaggggcccggagtat
B3501.SEQ ( 200) .....
B3502.SEQ ( 200) .....
B3503.SEQ ( 201) .....
B3504.SEQ ( 200) .....
B3505.SEQ ( 200) .....
B3506.SEQ ( 200) .....
B3507.SEQ ( 200) .....
B3508.SEQ ( 200) .....
B35091.SEQ ( 200) .....

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B35092.SEQ (127)
 B3510.SEQ (127)
 B3511.SEQ (127)
 B3512.SEQ (127)
 B3513.SEQ (127)
 B3514.SEQ (127)
 B3516.SEQ (200)
 B3517.SEQ (127)
 B3518.SEQ (127)
 B3519.SEQ (127)a.....g.....
 B3520.SEQ (127)
 B3521.SEQ (127)
 B3522.SEQ (127)
 B3523.SEQ (127)
 B3524.SEQ (127)
 B3525.SEQ (127)

NM3 (150) tgggaccggaacacacagatcttcaagaccaacacacagactnaccgaga
 B3501.SEQ (250)t.....
 B3502.SEQ (250)t.....
 B3503.SEQ (251)t.....
 B3504.SEQ (250)t.....
 B3505.SEQ (250)t.....
 B3506.SEQ (250)t.....
 B3507.SEQ (250)t.....
 B3508.SEQ (250)t.....
 B35091.SEQ (250)t.....
 B35092.SEQ (177)t.....
 B3510.SEQ (177)g.g.....t.....
 B3511.SEQ (177)g.g.....t.....
 B3512.SEQ (177)g.g.....t.....
 B3513.SEQ (177)g.g.....t.....
 B3514.SEQ (177)t.....
 B3516.SEQ (250)t.....
 B3517.SEQ (177)t.....
 B3518.SEQ (177)t.....
 B3519.SEQ (177)t.....
 B3520.SEQ (177)c.....t.....
 B3521.SEQ (177)t.....
 B3522.SEQ (177)t.....
 B3523.SEQ (177)t.....
 B3524.SEQ (177)t.....
 B3525.SEQ (177)t.....

NM3 (200) gagcctgcggaacctgcgcggctactacaaccagagcgaggccgggtctc
 B3501.SEQ (300)
 B3502.SEQ (300)
 B3503.SEQ (301)
 B3504.SEQ (300)
 B3505.SEQ (300)
 B3506.SEQ (300)
 B3507.SEQ (300)
 B3508.SEQ (300)
 B35091.SEQ (300)
 B35092.SEQ (227)
 B3510.SEQ (227)
 B3511.SEQ (227)
 B3512.SEQ (227)

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B3513.SEQ ( 227) .....
B3514.SEQ ( 227) .....
B3516.SEQ ( 300) .....
B3517.SEQ ( 227) .....
B3518.SEQ ( 227) .....
B3519.SEQ ( 227) .....
B3520.SEQ ( 227) .....
B3521.SEQ ( 227) .....
B3522.SEQ ( 227) .....
B3523.SEQ ( 227) .....
B3524.SEQ ( 227) .....
B3525.SEQ ( 227) .....

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NM3 ( 250) acatcatccagaggatgtatggctgcgacctggggcccgcgggcgc-t-
B3501.SEQ ( 350) .....c.-
B3502.SEQ ( 350) .....-t
B3503.SEQ ( 351) .....c.-
B3504.SEQ ( 350) .....c.-
B3505.SEQ ( 350) ...c.c.....c.....c.....c.-
B3506.SEQ ( 350) .....c.-
B3507.SEQ ( 350) .....c.-
B3508.SEQ ( 350) .....c.-
B35091.SEQ ( 350) .....c.-
B35092.SEQ ( 277) .....c.-
B3510.SEQ ( 277) .....c.-
B3511.SEQ ( 277) .....c.-
B3512.SEQ ( 277) .....c.-
B3513.SEQ ( 277) .....c.-
B3514.SEQ ( 277) .....c.-
B3516.SEQ ( 350) .....c.-
B3517.SEQ ( 277) .....c.-
B3518.SEQ ( 277) .....c.-
B3519.SEQ ( 277) .....c.-
B3520.SEQ ( 277) .....c.-
B3521.SEQ ( 277) .....c.-
B3522.SEQ ( 277) ...c.c.....c.....c.....g.....g.....c.-
B3523.SEQ ( 277) .....t.....c.-
B3524.SEQ ( 277) .....c.-
B3525.SEQ ( 277) .....c.-

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NM3 ( 298) cctccgcgggcatgaccagtccgc-----
B3501.SEQ ( 399) .....ctacgacggcaaggattacatcgccc
B3502.SEQ ( 399) .....a.....a...ctacgacggcaaggattacatcgccc
B3503.SEQ ( 400) .....t...ctacgacggcaaggattacatcgccc
B3504.SEQ ( 399) .....t...ctacgacggcaaggattacatcgccc
B3505.SEQ ( 399) .....ctacgacggcaaggattacatcgccc
B3506.SEQ ( 399) .....a.....t...ctacgacggcaaggattacatcgccc
B3507.SEQ ( 399) .....ctacgacggcaaggattacatcgccc
B3508.SEQ ( 399) .....ctacgacggcaaggattacatcgccc
B35091.SEQ ( 399) .....a.....a...ctacgacggcaaggattacatcgccc
B35092.SEQ ( 326) .....a.....a...ctacgacggcaaggattacatcgccc
B3510.SEQ ( 326) .....ctacgacggcaaggattacatcgccc
B3511.SEQ ( 326) .....ctacgacggcaaggattacatcgccc
B3512.SEQ ( 326) .....ctacgacggcaaggattacatcgccc
B3513.SEQ ( 326) .....t...ctacgacggcaaggattacatcgccc
B3514.SEQ ( 326) .....ctacgacggcaaggattacatcgccc
B3516.SEQ ( 399) .....ctacgacggcaaggattacatcgccc
B3517.SEQ ( 326) .....ctacgacggcaaggattacatcgccc

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B3518.SEQ ( 326) .....a.....a...ctacgacggcaaggattacatcgccc
B3519.SEQ ( 326) .....ctacgacggcaaggattacatcgccc
B3520.SEQ ( 326) .....ctacgacggcaaggattacatcgccc
B3521.SEQ ( 326) .....ctacgacggcaaggattacatcgccc
B3522.SEQ ( 326) .....a.....a...ctacgacggcaaggattacatcgccc
B3523.SEQ ( 326) .....ctacgacggcaaggattacatcgccc
B3524.SEQ ( 326) .....ctacgacggcaaggattacatcgccc
B3525.SEQ ( 326) .....ctacgacggcaaggattacatcgccc

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NM3 ( 322) ---acgaggac-tgagctcctggaccgcgcgacaccgcggtcagatc
B3501.SEQ ( 449) tga.....c.....
B3502.SEQ ( 449) tga.....c.....
B3503.SEQ ( 450) tga.....c.....
B3504.SEQ ( 449) tga.....c.....
B3505.SEQ ( 449) tga.....c.....
B3506.SEQ ( 449) tga.....c.....
B3507.SEQ ( 449) tga.....c.....
B3508.SEQ ( 449) tga.....c.....
B35091.SEQ ( 449) tga.....c..c.....
B35092.SEQ ( 376) tga.....c..c.....c.....g.....
B3510.SEQ ( 376) tga.....c.....
B3511.SEQ ( 376) tga.....c.....
B3512.SEQ ( 376) tga.....c.....
B3513.SEQ ( 376) tga.....c.....
B3514.SEQ ( 376) tga.....c.....
B3516.SEQ ( 449) tga.....c.....
B3517.SEQ ( 376) tga.....c.....
B3518.SEQ ( 376) tga.....c..c.....c.....g.....
B3519.SEQ ( 376) tga.....c.....
B3520.SEQ ( 376) tga.....c.....
B3521.SEQ ( 376) tga.....c.....
B3522.SEQ ( 376) tga.....c.....
B3523.SEQ ( 376) tga.....c.....
B3524.SEQ ( 376) tga.....c.....
B3525.SEQ ( 376) tga.....c.....

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NM3 ( 368) acccagcgcaagtgggaggcgcccgtgtggcggagcanctgaganccta
B3501.SEQ ( 499) .....g.....g....
B3502.SEQ ( 499) .....g.....g....
B3503.SEQ ( 500) .....g.....g....
B3504.SEQ ( 499) .....g.....g....
B3505.SEQ ( 499) .....g.....g....
B3506.SEQ ( 499) .....g.....g....
B3507.SEQ ( 499) .....g.....g....
B3508.SEQ ( 499) .....g.g....g....
B35091.SEQ ( 499) .....g.....g....
B35092.SEQ ( 426) .....g.....g....
B3510.SEQ ( 426) .....g.....g....
B3511.SEQ ( 426) .....g.....g....
B3512.SEQ ( 426) .....g.....g....
B3513.SEQ ( 426) .....g.....g....
B3514.SEQ ( 426) .....a.....gtg....g....
B3516.SEQ ( 499) .....g.....g....
B3517.SEQ ( 426) .....g.....g....
B3518.SEQ ( 426) .....g.g....g....
B3519.SEQ ( 426) .....g.....g....
B3520.SEQ ( 426) .....g.....g....
B3521.SEQ ( 426) .....a.....g.....g....

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B3522.SEQ ( 426) .....g.....g....
B3523.SEQ ( 426) .....g.....g....
B3524.SEQ ( 426) .....g.....g....
B3525.SEQ ( 426) .....g.....g....

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```

NM3 ( 418) cctggagggcctgtgcgtggagtggctccgcagatacctgganaacggga
B3501.SEQ ( 549) .....g.....
B3502.SEQ ( 549) .....g.....
B3503.SEQ ( 550) .....g.....
B3504.SEQ ( 549) .....g.....
B3505.SEQ ( 549) .....g.....
B3506.SEQ ( 549) .....g.....
B3507.SEQ ( 549) .....g.....
B3508.SEQ ( 549) .....g.....
B35091.SEQ ( 549) .....g.....
B35092.SEQ ( 476) .....g.....
B3510.SEQ ( 476) .....g.....
B3511.SEQ ( 476) .....g.....
B3512.SEQ ( 476) .....g.....
B3513.SEQ ( 476) .....g.....
B3514.SEQ ( 476) .....g.....
B3516.SEQ ( 549) .....ga.....g.....
B3517.SEQ ( 476) .....g.....
B3518.SEQ ( 476) .....g.....
B3519.SEQ ( 476) .....g.....
B3520.SEQ ( 476) .....g.....
B3521.SEQ ( 476) .....c.....g.....
B3522.SEQ ( 476) .....g.....
B3523.SEQ ( 476) .....g.....
B3524.SEQ ( 476) .....c.....g.....
B3525.SEQ ( 476) .....c.....g.....

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```

NM3 ( 468) aggagacgctgcancgcgcggaccccccaagacacacgtgaccaccac
B3501.SEQ ( 599) .....g.....
B3502.SEQ ( 599) .....g.....
B3503.SEQ ( 600) .....g.....
B3504.SEQ ( 599) .....g.....
B3505.SEQ ( 599) .....g.....
B3506.SEQ ( 599) .....g.....
B3507.SEQ ( 599) .....g.....
B3508.SEQ ( 599) .....g.....
B35091.SEQ ( 599) .....g.....
B35092.SEQ ( 526) .....g.....-----
B3510.SEQ ( 526) -----
B3511.SEQ ( 526) -----
B3512.SEQ ( 526) -----
B3513.SEQ ( 526) .....g.....-----
B3514.SEQ ( 526) .....g.....-----
B3516.SEQ ( 599) .....g.....
B3517.SEQ ( 526) .....g.....-----
B3518.SEQ ( 526) .....g.....-----
B3519.SEQ ( 526) .....g.....-----
B3520.SEQ ( 526) .....g.....-----
B3521.SEQ ( 526) .....g.....-----
B3522.SEQ ( 526) .....g.....-----
B3523.SEQ ( 526) .....g.....-----
B3524.SEQ ( 526) .....g.....-----
B3525.SEQ ( 526) .....g.....-----

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NM3      ( 518) cccgtctctgaccatgaggccaccctgaggtgctgggcctgggcttcta
B3501.SEQ ( 649) .....
B3502.SEQ ( 649) .....
B3503.SEQ ( 650) .....
B3504.SEQ ( 649) .....
B3505.SEQ ( 649) .....
B3506.SEQ ( 649) .....
B3507.SEQ ( 649) .....
B3508.SEQ ( 649) .....
B35091.SEQ ( 649) .....
B35092.SEQ ( 547) -----
B3510.SEQ ( 526) -----
B3511.SEQ ( 526) -----
B3512.SEQ ( 526) -----
B3513.SEQ ( 547) -----
B3514.SEQ ( 547) -----
B3516.SEQ ( 649) .....
B3517.SEQ ( 547) -----
B3518.SEQ ( 547) -----
B3519.SEQ ( 547) -----
B3520.SEQ ( 547) -----
B3521.SEQ ( 547) -----
B3522.SEQ ( 547) -----
B3523.SEQ ( 547) -----
B3524.SEQ ( 547) -----
B3525.SEQ ( 547) -----

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```

NM3      ( 568) ccctgaggagatcacnctgacctggcancgggatggcgaggaccaaactc
B3501.SEQ ( 699) .....a.....g.....
B3502.SEQ ( 699) .....a.....g.....
B3503.SEQ ( 700) .....a.....g.....
B3504.SEQ ( 699) .....a.....g.....
B3505.SEQ ( 699) .....a.....g.....
B3506.SEQ ( 699) .....a.....g.....
B3507.SEQ ( 699) .....a.....g.....
B3508.SEQ ( 699) .....a.....g.....
B35091.SEQ ( 699) .....a.....g.....
B35092.SEQ ( 547) -----
B3510.SEQ ( 526) -----
B3511.SEQ ( 526) -----
B3512.SEQ ( 526) -----
B3513.SEQ ( 547) -----
B3514.SEQ ( 547) -----
B3516.SEQ ( 699) .....a.....g.....
B3517.SEQ ( 547) -----
B3518.SEQ ( 547) -----
B3519.SEQ ( 547) -----
B3520.SEQ ( 547) -----
B3521.SEQ ( 547) -----
B3522.SEQ ( 547) -----
B3523.SEQ ( 547) -----
B3524.SEQ ( 547) -----
B3525.SEQ ( 547) -----

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NM3      ( 618) aggacactgancttgtggagacc-----

```

```
B3501.SEQ ( 749) .....g.....agaccagcaggagatagaaccttcag
B3502.SEQ ( 749) .....g.....agaccagcaggagatagaaccttcag
B3503.SEQ ( 750) .....g.....agaccagcaggagatagaaccttcag
B3504.SEQ ( 749) .....g.....agaccagcaggagatagaaccttcag
B3505.SEQ ( 749) .....g.....agaccagcaggagatagaaccttcag
B3506.SEQ ( 749) .....g.....agaccagcaggagatagaaccttcag
B3507.SEQ ( 749) .....g.....agaccagcaggagatagaaccttcag
B3508.SEQ ( 749) .....g.....agaccagcaggagatagaaccttcag
B35091.SEQ ( 749) .....g.....agaccagcaggagatagaaccttcag
B35092.SEQ ( 547) -----
B3510.SEQ ( 526) -----
B3511.SEQ ( 526) -----
B3512.SEQ ( 526) -----
B3513.SEQ ( 547) -----
B3514.SEQ ( 547) -----
B3516.SEQ ( 749) .....g.....agaccagcaggagatagaaccttcag
B3517.SEQ ( 547) -----
B3518.SEQ ( 547) -----
B3519.SEQ ( 547) -----
B3520.SEQ ( 547) -----
B3521.SEQ ( 547) -----
B3522.SEQ ( 547) -----
B3523.SEQ ( 547) -----
B3524.SEQ ( 547) -----
B3525.SEQ ( 547) -----
```