To the Graduate Council:

I am submitting herewith a thesis written by Glenn P. Niemeyer entitled "Applications Of The Polymerase Chain Reaction In Retroviral-Mediated Gene Transfer Of A Foreign Gene Into Hematopoietic Cells Of Dogs And Cats". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master Of Science, with a major in Comparative and Experimental Medicine.

Dr. Clinton D. Lothrop, Jr., Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of The Graduate School

APPLICATIONS OF THE POLYMERASE CHAIN REACTION IN RETROVIRAL-MEDIATED GENE TRANSFER OF A FOREIGN GENE INTO HEMATOPOIETIC CELLS OF DOGS AND CATS

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ABSTRACT

Gene transfer using retroviral vectors has become a widely used approach for introducing foreign genes into mammalian cells. Because of the efficiency of retroviral-mediated gene transfer, this system can be utilized to deliver therapeutically important genes into deficient cells as a treatment modality for certain genetic diseases of both man and animals. This study was undertaken to develop methods to analyze the many operational steps involved in retroviral-mediated transfer of foreign genes into hematopoietic progenitor cells of cats and dogs. Polymerase chain reaction protocols were developed which permitted us to detect, with a high degree of sensitivity and accuracy, retroviral transduced genes in experimental animals. In addition, the use of a retroviral vector expressing the cDNA for the human multidrug resistant gene (MDR1) as a dominant selectable marker was investigated.

A Moloney murine leukemia virus based retroviral vector was used to transfer the bacterial neomycin resistance gene (neo^R) into canine and feline hematopoietic cells. Four cats and five dogs were lethally irradiated and reconstituted with autologous bone marrow that had been transduced with either the N2 or SAX retroviral vector. Bone marrow was obtained from gene transfer recipients at various times post-transplantation and tested for the expression of neo^R by the in vitro drug resistant colony forming unit granulocyte-macrophage assay. These results were corroborated utilizing the PCR method on gene transfer recipient bone marrow DNA. Experimental results showed expression and presence of retroviral vector sequences in all four cats and two dogs 30 to 48 day post-transplantation. Further sequential evaluation of feline bone marrow cells showed a low level of drug-resistant colony formation as well as the presence of vector sequences for greater than 200 days. Sequential expression and PCR analysis of canine bone marrow samples proved negative after approximately 80 days.

A retroviral vector containing a full-length human MDR1 cDNA was used to evaluate the potential of in vivo selection in gene transfer protocols. Bone marrow cells from two dogs were transduced with the MDR1 retroviral vector. These animals were then administered myelosuppressive drugs at various time points post-transplantation. The number of drug resistant colony forming units increased five-fold

in one animal during in vivo selection but decreased substantially once drug administration was stopped. PCR primers specific for human MDR1 cDNA were developed. These primers do not amplify canine or feline MDR genes. PCR analysis of bone marrow DNA from the two human MDR1 cDNA recipient dogs failed to detect the desired MDR1 sequences at multiple time points post-transplantation. Technical difficulties associated with the MDR1 retroviral vector used in this study, namely inefficient packaging and the generation of replication-competent virus, precluded additional studies with this vector. A new MDR1 expressing vector was constructed, and this vector (LMDR1) along with the PCR methodologies developed in this study should prove valuable in future experiments.

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CHAPTER I

INTRODUCTION

The molecular approach to the study of disease examines the intricate interaction of individual genes and their products. In the early 1900's, Archibold Garrod perceived the direct relationship between genes and gene products in his "one gene-one enzyme hypothesis". It was Garrod who first recognized that specific gene products have unique functions, and that the deficiency of a certain gene product may cause a specific pathological phenotype to appear. Since that time, advances in technology have allowed researchers to elucidate the causes of many genetic diseases. More specifically, scientists have characterized many of these diseases as exhibiting a pattern whereby the absence of a gene product results in the failure of specific molecular events.[1] As disease processes are increasingly understood, it may be possible to intervene in these processes by restoring essential genes by gene transfer. This therapeutic introduction of a normal gene for a deficient gene by gene transfer is known as gene therapy.

Gene transfer using retroviral vectors has become a widely used approach for introducing foreign genes into mammalian cells.[2,3] The usefulness of a retroviral based gene delivery system becomes apparent when one examines the unique molecular make up and life cycle of the retrovirus. Retroviruses have efficient mechanisms to enter host cells, as well as mechanisms to integrate their genomes stably into cellular DNA (Fig. 1).* In addition, retroviral infection of a susceptible cell type does not usually cause lysis or death of the target cell. Replication-competent retroviruses have at least three protein coding genes: gag, pol, and env. These proteins are involved in viral encapsidation and assembly, integration and synthesis of viral DNA by reverse transcription, and viral budding and entrance, respectively. Each of these genes is trans-acting, meaning that all of the proteins required for infection and integration are carried within the viral capsule. There are in addition to trans-acting regions within the viral genome, cis acting regulatory sequences that are required for proviral integration and expression. These sequences do not appear to encode any protein and are limited

*All tables and figures may be found in the appendices.

to two types: (1) sequences that form the long terminal repeat (LTR) or are necessary for its synthesis, and (2) sequences necessary for the packaging of genomes into virions. Because of these characteristics, it is possible to construct a recombinant retroviral vector capable of efficiently infecting and integrating its genome into target cells with only cis acting regulatory sequences.[4] In this type of gene delivery system, trans-acting proteins are provided by a packaging line. Packaging cell lines can be generated by introducing the retroviral structural genes gag, pol, and env encoded within a plasmid into a tissue cell line (NIH-3T3) by conventional calcium-phosphate-mediated DNA transfection.[5] When the cloned proviral form of a recombinant retroviral vector is introduced into this cell line, the genomic RNA generated by the vector combines with the constitutively expressed retroviral structural proteins of the packaging line resulting in the release of the retroviral vector into the culture medium. This cell line is now commonly referred to as a "producer cell line".[6] (Fig. 2) Transduction of the recombinant retroviral construct into target cells can be accomplished by culturing the target cells in the presence of supernatant collected from the producer cell line, or one can simply cocultivate producer and target cells.

The hematopoietic system is an excellent target for gene transfer because of well-developed procedures for bone marrow transplantation, the wide distribution and increased number of hematopoietic cells, and the existence of many diseases that affect hematopoietic cells. Most genetically defined hematologic diseases are the result of expression of the genetic defect by the progeny of the hematopoietic stem cell.[7,8] Correction of a defect in the pluripotent stem cell could possibly cure the disease in the mature daughter cells.

Hematopoietic stem-cells (HSCs) have two major distinguishing characteristics: the capacity for self-renewal and the capability to differentiate into all lineages of hematopoietic cells.[9] The mechanisms which govern the growth and differentiation of this cell type are not yet well understood and remain a central unanswered question of stem cell physiology. Totipotent or pluripotent stem-cells make up a very small minority of the total hematopoietic cell population (0.05 to 0.1% of cells).[10] Of these, the majority are in the G0 or G1 phase of the cell cycle. Studies by A. Dusty Miller suggest that cellular replication is required for the integration of retroviral vectors into

the host genome.[11] Lack of stem-cell replication along with the infrequency of this cell type make retroviral transduction a relatively improbable event.

A large amount of research has focused on the utilization of murine blood and bone marrow cells in the development of retroviral based transduction protocols.[12-17] In murine models exogenous genes, such as human β-globin gene, have been shown to be stably integrated and expressed in a lineage specific manner in bone marrow transplant recipients reconstituted with retroviral transduced stem cells.[18-22] While this pioneering work with mice has led to considerable advances in retroviral mediated gene transfer, there are many theoretical risks that must be considered if human gene therapy is to be realized. These potential risk factors can only be studied in animals, and at best they provide only an approximation of what might happen in humans. Large animal models such as primates, dogs and cats have allowed us to examine the efficacy of murine based protocols in species which may approach 1000 times the size of a mouse. The dog and cat are excellent models in this regard, because they like humans are randomly bred. Studies in experimental marrow transplantation in animals such as dogs have formed the basis for clinical bone marrow transplantation programs used in many centers worldwide.[23] In addition, canine models exist for human genetic disorders such as pyruvate kinase deficiency, cyclic hematopoiesis, α-iduronidase deficiency, and hemophilia A and B.[24-28]

Our laboratory and that of A. Dusty Miller at the Fred Hutchinson Cancer Research Center in Seattle have shown that retroviral vectors can be used to transfer exogenous genes into canine and feline hematopoietic cells cultured in vitro.[29-31] Subsequent studies in our lab extended these in vitro results to show that an exogenous gene could be expressed in vivo, with existing methodologies, by canine and feline gene transfer recipients.[32-33] In these experiments each of the transplanted cats expressed the exogenous gene 30 days post-transplantation, and evidence was presented to indicate the presence of this gene after more than 200 days. Analysis of canine gene transfer recipients also detected the exogenous gene in bone marrow cells following autologous transplantation, but the fraction of transduced cells declined quickly over a period of weeks.

Although these results are encouraging, they are not optimal in terms of expression and stability exhibited by the transferred gene. In general, the expression of exogenous genes introduced into hematopoietic cells of large animals has been short lived, or at very low levels with periods of positive colony formation interspersed with negative results. These observations may reflect the cell type in which transduction is actually occurring. Gene transfer into a differentiated cell type will result in the expression of the gene in a restricted lineage. Because differentiated cells have a limited life span, expression of the transferred gene will be short lived. In contrast, transfer into a pluripotent stem cell should result in the continued presence of the gene in all hematopoietic lineages for the life of the animal. However, transfer into only a small number of these stem cells may account for the observed intermittent expression pattern due to differential activation of different stem-cell clones.[34] The information gained from these important animal studies suggests that existing transduction methodologies must be improved if we are to achieve stable transfer and expression of exogenous genes in the majority of bone marrow cells.

This study was undertaken to develop methods to analyze the many operational steps involved in the transfer of foreign genes into hematopoietic progenitor cells of cats and dogs. With current methodologies, the transduction efficiency of HSCs of large animals is very low, therefore, polymerase chain reaction (PCR) protocols were developed to permit sensitive detection of retroviral transduced genes in these animals.[35] With PCR it is possible to detect small numbers of transduced cells in the presence of hundreds of thousands of untransduced cells.[36-37] The PCR protocols developed in this study are essential components in ascertaining the biological safety aspects of the procedures, as well as in the analysis of bone marrow transplant recipients. The augmentation of <u>in vitro</u> colony data with PCR analysis has led to important findings in the area of retroviral-mediated gene transfer in large animals. In addition, a current area of research focus in our laboratory is the utilization of a retroviral vector expressing the human multidrug resistant (MDR1) cDNA as a dominant selectable marker.[38] The MDR1 cDNA encodes a plasma membrane glycoprotein known as P-glycoprotein. The P-glycoprotein serves as an energy-dependent drug efflux pump, resulting in decreased cellular accumulation of cytotoxic drugs. Retroviral transfer of the full-length MDR1 cDNA confers the complete multidrug resistance phenotype to a wide vareity of drug-sensitive cell lines including

hematopoietic cells. The use of a retroviral vector containing the MDR1 cDNA in gene transfer protocols should allow for the in vivo selection of a transduced cell population. In vivo selection in gene transfer recipients consists of the administration of myelosuppressive concentrations of substrate for the multi-drug transporter. Preliminary results presented in this study suggest that retroviral mediated gene transfer followed by in vivo selection should ensure proper and stable expression of the exogenous gene. However, technical difficulties associated with the MDR1 retroviral vector used in this study, namely inefficient packaging and the generation of replication-competent virus, precluded additional studies with this vector. Therefore, a new MDR1 expression vector was constructed. This vector (LMDR1) along with the PCR methodologies developed in this study should prove valuable in future experiments.

CHAPTER 2

MATERIALS AND METHODS

Animals:

Dogs and cats of both sexes were used in this study. All animals had been previously vaccinated and dewormed and were disease free for at least two months before experimental study. Animals were housed in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care. This study was approved by the institutional Animal Care and Concerns Committee.

Transplantation Protocol:

Autologous bone marrow transplants were performed in this study. The pretransplant conditioning regime routinely consisted of decontaminating the gastrointestinal tract with neomycin and/or metronidazole prior to irradiation. Bone marrow was aspirated and treated in accordance to the procedures outlined below until infused into the donor animals that had received 7.5 Gy (0.125 Gy/min) total body irradiation from a Cobalt 60 source. Animals were kept in a clean isolated room, treated with antibiotics and fluids as indicated until bone marrow reconstitution was evident based on complete blood counts (CBCs).

Cell Collection:

Bone marrow was obtained by precutaneous aspiration of long bones using xylazine and oxymorphone or pentobarbital sedation. Bone marrow was collected in 12 cc syringes containing 4 cc of heparinized (160 units/ml, Gibco, Grand Island, NY) Iscoves modified Dulbecco's medium (IMDM, Gibco) and placed on wet ice. Mononuclear marrow cells were prepared by diluting the whole marrow, by addition of one-third its total volume, with heparinized IMDM (20 units/ml) and separating by Ficoll-Hypaque gradient sedimentation (Pharmacia, Piscataway, NJ). The low-density (< 1.077 g/cm^3) mononuclear cells were aspirated and washed three times with IMDM supplemented with 100 u/ml penicillin, 100 μg/ml streptomycin (Gibco) and 10% fetal

calf serum (Whittaker Walkersville, MD). The cells were then counted using a hemocytometer. Viability was consistently >90% using a trypan blue exclusion test.

Retroviral Vectors:

The recombinant retroviral vectors N2, SAX and HaMDR 1/A were used in this study.[39-41] N2 and SAX are each derived from the Moloney Murine Leukemia Virus and contain the bacterial neo^R gene. In addition to the neo^R gene, SAX contains a human adenosine deaminase (ADA) gene expressed from an internal SV40 promoter. The expression of the neo^R gene confers resistance to the neomycin analogue G418 in eukaryotic cells. HaMDR1/A is a recombinant retrovirus derived from the Harvey Murine Sarcoma Virus (Ha-MSV). In this construct, the human MDR1 cDNA, isolated from a human multidrug-resistant cell line, was placed in a Ha-MSV genome from which the viral *ras* oncogene was deleted. This vector efficiently transfers a multidrug resistance phenotype to a variety of cell types.

Retroviral Packaging Lines:

The retroviral vectors N2, SAX and HaMDR1/A were used to characterize the infectability of canine and feline hematopoietic progenitor cells. These vectors were transfected into the amphotropic packaging cell line PA12 by calcium phosphate precipitation.[42] The high titer clones V-6, S3A and HaMDR1/A-1 were used throughout these studies as a source of viral supernatants. Conditioned medium from V6 and S3A routeinly had a titer of 3 to 5 x 10^6 drug resistant colony-forming units/ml (CFU/ml). HaMDR1/A-1 had a titer of approximately 8 x 10^4 CFU/ml. Helper virus contamination was present in all three cell-lines.

Transduction Procedures:

Bone marrow cells were exposed to conditioned medium from the cell lines V-6, S3A and HaMDR1/A-1. In this supernatant protocol, 3 x 10^6 nucleated bone marrow cells were mixed with 7.5 ml conditioned medium. Polybrene (Sigma, St. Louis, MO), a polyanion used to increase viral adhesion, was added at a concentration of 12 μg/ml, and the suspension incubated for four hours with gentle mixing every 15

minutes. After transduction, cells were washed three times with complete IMDM and plated in the colony-forming unit granulocyte-macrophage (CFU-GM) assay or infused into donor animals.

CFU-GM Assay:

Canine or feline CFU-GM were determined by plating 7.5 x 10^5 nucleated marrow cells in 1 ml IMDM containing 15% pre-selected fetal calf serum (Whittaker, Walkersville, MD) 10% neutropenic dog serum in 0.8% methylcellulose (Fisher, Fair Lawn, NJ). Neutropenic serum obtained from day three or day four cyclic hematopoietic dogs served as a source of colony-stimulating activity (CSA).[43] Each patch of neutropenic serum was tested for CSA before use. For analysis of neo^R gene expression, the antibiotic G418 (Gibco), was present at the concentration of 750 to 2000 μg/ml. A G418 dose response curve was generated for each experiment, and assays where done in quadruplicate. Expression of the MDR1 cDNA was tested for by the addition of vinblastine or adriamycin to the assay at the concentrations of 20 and 100 ug/ml, respectively. The cultures were maintained at 37°C in humidified air and 5% CO_2. Colonies (>50 cells) and clusters (20-50 cells) were counted 6-8 days after plating.

DNA Isolation:

Bone marrow was collected from experimental animals at periodic time points following transplantation and stored frozen at -70°C until time of DNA preparation. In instances where peripheral blood served as a source of genomic DNA, 10 ml blood was collected in EDTA tubes (Becton Dickinson, Rutherford, NJ). The blood samples were stored on ice until low-density mononuclear cells could be separated by Ficoll-Hypaque gradient sedimentation. DNA was isolated by sodium dodecyl sulfate (Gibco) - Proteinase K (Sigma) digestion, followed by three rounds of phenol/chloroform (Amresco Inc., Solon, OH) extraction.[44] After the third extraction with phenol/chloroform, the aqueous phase was transferred to a new centrifuge tube and 0.2 volumes of 10 M ammonium acetate (Sigma) was added. Two volumes of room temperature ethanol (IBI, New Haven, CT) were added and the solution was swirled until thoroughly mixed. The DNA immediately formed a precipitate that was removed

from the ethanolic solution with a heat sealed capillary tube. The DNA precipitate was then washed twice with 70% ethanol and allowed to air dry at room temperature. The precipitate was dissolved in sterile Milli-Q water (Millipore, Bedford, MA.). DNA was quantitated by spectrophotometric analysis at wavelengths of 260 nm and 280 nm using a Shimadzu UV-160 spectrophotomer (Shimadzu, Kyoto, Japan).

Neo^R PCR Analysis:

Bone marrow DNA (1 μg) was specifically amplified for neo^R, LTR and env sequences by the PCR method.[35] The PCR reaction conditions were as follows: neo^R - 30 cycles of 94°C for 2 minutes and 45 seconds, 60°C for 2 minutes, 72°C for 3 minutes and 30 seconds followed by one cycle of 94°C for 2 minutes and 45 seconds, 60°C for 2 minutes, 72°C for 10 minutes; LTR - 30 cycles of 94°C for 2 minutes and 30 seconds, 64°C for 2 minutes, 72°C for 3 minutes and 30 seconds followed by one cycle of 94°C for 2 minutes and 30 seconds, 64°C for 2 minutes, 72°C for 10 minutes; env - 30 cycles of 94°C for 2 minutes and 45 seconds, 58°C for 2 minutes, 72°C for 3 minutes and 30 seconds followed by one cycle of 94°C for 2 minutes and 45 seconds, 58°C for 2 minutes, 72°C for 10 minutes. Reagents were obtained from Perkin-Elmer Cetus (Norwalk, CT.) in the form of a Gene-Amp® kit. Oligonucleotides were synthesized by the Midland Certified Reagent Co. (Midland, TX). The sequences of these primers are as follows: Neo 1, CAAGATGGATTGCACGCAGG; Neo 5, CCCG CTCAGAAGAACTCGTC; LTR 1, GTTTCCAGGGTGCCCCAAGG; LTR 2, CACT CAGAGGAGACCCTCCC; ENV 1, ACCTGGAGAGTCACCAACC; ENV 3, TACTT TGGAGAGGTCGTAGC. Primer concentrations were optimized at between 0.1 and 1 μM per 100 μl reaction. A master mix of all reagents (water, buffer, dNTP's and primers) was prepared and purified of all extraneous DNA by centrifugation through an ultrafiltration membrane with a molecular weight cut off of 10,000 daltons. (Millipore, Bedford, MA). The master mix was then aliquoted into individual siliconized tubes with a pipet fitted with an aerosol resistant pipet tip (Continental Laboratory Products, San Diego, CA). To reduce evaporation and refluxing, each reaction mixture was overlayed with 50 μl of mineral oil (Sigma). To enhance amplification, Taq DNA polymerase (2.5 units) was added after the samples were heated to 95°C during the first denaturation step.[45] An automated thermal cycler was used for all reactions (Twin Block* System Temperature Cycler, Ericomp, San Diego, CA).

MDR1 PCR Analysis:

DNA was prepared from either peripheral blood of normal dogs and cats or bone marrow from canine gene transfer recipients as described above. One microgram of this DNA was utilized in each reaction. The sequences of the primers are as follows: T-1, ACTTCCTCTCATGATGCTGGTGTT; T-2, ACATCTCATACAGTCAGAGTTCAC; T-3, TCACCAATAACTTCATTAATCTTA; T-4, TCATATTTTGCTTTTGGATGAAGC. Thermal cycler parameters for each given primer pair were: T-1 and T-2 - 30 cycles of 94°C for 1 minute and 45 seconds; 65°C for 2 minutes; 72°C for 4 minutes and 30 seconds followed by one cycle of 94°C for 1 minute and 45 seconds, 65°C for 2 minutes, and 72°C for 10 minutes; T-4 and T-2 - 30 cycles of 94°C for 1 minute; 60°C for 2 minutes; 72°C for 2 minutes and 30 seconds followed by one cycle of 94°C for 1 minute; 60°C for 2 minutes and 72°C for 10 minutes; T-1 and T-3 - 30 cycles of 94°C for 1 minute, 57°C for 2 minutes, 72°C for 2 minutes and 30 seconds followed by one cycle of 94°C for 1 minute, 57°C for 2 minutes and 72°C for 10 minutes. The optimal concentration for primers was between 0.1 and 0.5 μM per 100 μl reaction.

Southern Blot Analysis:

Sample analysis was conducted after removing the mineral oil by chloroform and then concentrating each sample by vacuum centrifugation (Jovan, Inc. Winchester, VA). One-half of each sample was loaded onto an agarose gel (3% Nusieve Agarose plus 1% GTG Seakem Agarose, FMC Bioproducts, Rockland, ME) and electrophoresed in 40 mM tris-acetate buffer. Ethidium bromide was present at a concentration of 5 μg/ml. Reaction products were visualized by UV (200 nM) illumination. Further analysis was carried out by the method of Southern.[46] The gel was soaked in 0.2 N NaOH, 0.6 M NaCl for 30 minutes at room temperature to denature the DNA. This was followed by neutralization of the gel by replacing the denaturing solution with 0.5 M Tris pH 7.5, 1.5 M NaCl and shaking gently for 30 minutes on an orbital shaker. Transfer to nylon membranes (Cuno, Inc,. Meridian, CT) was carried out overnight by capillary action using 20 x SSC (175.3 g of NaCl and 88.2 g of sodium citrate in one liter of water, pH 7.0). After transfer the membrane

was washed in 2 x SSC two times for 15 minutes each with agitation on an orbital shaker and then allowed to air dry at room temperature. The membrane was then washed for 1 hour in 0.1 x SSC, 0.5% SDS at 65°C. Prehybridization solution was then added to the membrane in a plastic bag at a volume of 5 ml per 100 cm^2 of membrane. Prehybridization buffer was 5 x SSC, 10 x Denhardt's (0.2% Ficoll 400, 0.2% polyvinylpyrrolidone - 360 and 0.2% bovine serum albumin), 0.05 M $NaPO_4$ (pH 6.7), 500 μg/ml salmon sperm DNA, 5% dextran sulfate, and 50% formamide. The sealed bag was then incubated overnight at 42°C. Following prehybridization, the prehybridization buffer was replaced with hybridization buffer which consisted of: 5 x SSC, 1 x Denhardt's, 0.02 M $NaPO_4$ (pH 6.7), 100 ug/ml salmon sperm DNA, 10% dextran sulfate and 50% formamide. For standard analysis, the hybridization probe was produced by PCR amplification of positive controls (V-6 cellular DNA for neo^R, LTR and Env; pHaMDR1/A-1 for MDR1), and then radiolabeled by random primer extension utilizing α-^{32}P-dCTP (New England Nuclear, Dupont, Wilmington, DE).[47] DNA probe hybridization was at 42°C for 20 hours. After this period, each membrane was washed (one time for 15 minutes with 2 x SCC, 0.1% SDS at room temperature; one time for 15 minutes with 0.1 x SSC, 0.1% SDS at room temperature and two times for 30 minutes each with 0.1 x SSC, 0.1% SDS at 60°C) to remove nonspecifically bound probe and exposed to X-ray film (XAR-5, Eastman Kodak Company, Rochester, NY).

MDR1 Vector Construction:

The construction of pHaMDR1/A and pLNCX have been described previously.[41,48,49] pLMDR1 containing the human MDR1 gene inserted between the two long terminal repeats of the recombinant retroviral vector LNCX was constructed as follows. A 4.1 Kb SacII-XhoI fragment encoding the entire MDR1 cDNA was isolated from plasmid pHaMDR1/A (Fig. 3). Digestion of this fragment with BstUI followed by a filling-in reaction using the Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA) generated a 3.9-Kb blunt ended fragment (Fig. 4). Plasmid MDR1-SP72 was obtained by cutting plasmid pSP72 (Promega, Madison, WI) with SmaI followed by ligation with the 3.9-Kb MDR1 fragment. This construct left a unique BamHI site at the 5-prime terminal and a unique ClaI at the 3-prime terminal of the MDR1 cDNA (Figs. 5 and 6). In a second step, DM1 competent cells

(Gibco, BRL, Gaithersburg, MD) were transformed with pLNCX. The DM1 strain of competent cells lacks both the dam and dcm methylases; therefore, GATC and CC(A/T)GG sites can be cut by many restriction endonucleases which otherwise are inhibited by dam or dcm methylation. Following transformation individual ampicillin resistant colonies were isolated and grown for 8 hours at 33°C in Luria Broth. LNCX plasmid DNA was isolated by standard alkaline lysis preparation and digested with HindIII. The linearized molecule was then filled in with the Klenow fragment of DNA polymerase I and digested with BclI removing a 1.9-Kb fragment encoding the neo^R gene and the immediate early promoter. Phosphorylated Bam HI linkers (New England Biolabs, Beverly, MA) were ligated to the remaining 4.6 Kb portion of pLNCX. This was followed by Bam HI digestion to generte cohesive ends. The fragment was then purified by electrophoresis and gel-digestion utilizing GELase (Epicentre Technologies, Madison, Wisconsin). Re-ligation with T4 DNA ligase (New England Biolabs, Beverly, MA) yielded pL with unique Bam HI, XhoI and ClaI cloning sites (Fig. 7). Finally, the 3.9-Kb MDR1 cDNA was excised from MDR1-SP72 as a BamHI - ClaI fragment and ligated to previously BamHI-ClaI digested pL to yield the 8.6-Kb pLMDR1 (Fig. 8).

CHAPTER 3

RESULTS

Retroviral Transduction and Bone Marrow Transplantation in Cats and Dogs:

Four cats and five dogs that had been lethally irradiated (7.5 Gy) were reconstituted with autologous bone marrow that had been transduced with either the N2 or SAX retroviral vector. The multiplicity of infection (MOI), which is the ratio of viral particles to bone marrow mononuclear cells, ranged from less than 0.008 to greater than 2. Bone marrow reconstitution was evident in all four cats by 20 days post-transplantation based on evaluation of polymorphonuclear leukocyte (PMN) and platelet counts. The PMN and platelet counts had returned to at least 5,000 PMN/μl and 50,000 platelets/μl in the four cats by 18.5 ± 5.4 (mean ± SD) and 16.0 ± 4.7 days, respectively. Bone marrow reconstitution was evident in each of the five dogs by 30 days. The PMN and platelet counts had returned to at least 5,000 PMN/μl and 50,000 platelets/μl in the five dogs by 26.8 ± 0.8 and 26.6 ± 1.7 days, respectively. Because reconstitution was evident in each animal, this indicates that retroviral transduction did not preclude bone marrow reconstitution in an autologous transplantation protocol. Hematologic complications and some gastrointestinal toxicity were noted to various degrees in most animals; however, the severity was not greater than that observed previously from lethal irradiation and autologous bone marrow transplantation alone. The cell doses for transplantation, and MOI are summarized in tables 1 and 2.

CFU-GM Analysis:

Bone marrow was obtained from the transplanted animals at various times post-transplantation and was tested for expression of the neo^R gene with the in vitro colony forming unit (CFU) assay. This assay is based on CFU-GM formation in the presence or absence of the antibiotic G418. Expression of the neo^R gene was evident in two of five dogs (table 2) 48 days post-transplantation but had declined by 85 days post-transplant. In the three dogs with low multiplicity of infection, G418-resistant CFU were not detectable post-transplantation. In contrast, bone marrow obtained from each of the four cats (table 1) indicated the expression of the neo^R gene in all four cats 30

days post-transplantation based on the CFU assay. In each of these four animals the MOI was greater than two. Sequential evaluation of bone marrow cells for G418-resistant CFU showed that the fraction of G418 resistant CFU declined over a period of several months, but in three cats there was still a low level of drug-resistant CFU-GM after 200 days.

DNA Analysis in Feline Gene Transfer Recipients:

DNA from transplanted animals was prepared from bone marrow mononuclear cells both before and after retroviral transduction to test for the presence of neo^R, LTR and env sequences by the PCR method. (Fig. 9). Bone marrow DNA from transplanted animals had previously tested negative for neo^R sequences by standard Southern hybridization techniques. This suggests that the neo^R gene was present in fewer than 5% of the bone marrow cells. Bone marrow DNA reexamined by PCR and Southern blotting proved positive for neo^R sequences in three of four animals. The neo^R gene was detected in two animals 26 days post-transplantation, and sequential evaluation of bone marrow mononuclear cell DNA showed that in Martina the neo^R sequences were detectable 214 and 609 days following transplantation. (Fig. 10) The neo^R gene was also detected in Yankee 26, 67, 214, 609 and 978 days following transplantation (Fig. 11). Andy was neo^R positive by PCR at only one evaluation point 154 days post-transplantation.

Bone marrow DNA was also analyzed for the presence of the amphotrophic retrovirus 4070A envelop (env) gene. Envelope sequences were detected in Martina at 26 and 210 days post-transduction. Andy was also positive for env 154 days post-transplant, this coincides with his only positive detection for neo^R sequences. Yankee was negative for env sequences at 26, 96 and 209 days post-transplant. The presence of the helper virus genome in two of the three transplanted cats means that a localized helper virus infection may have contributed to the persistent expression of the transferred neo^R gene.

DNA Analysis in Canine Gene Transfer Recipients:

DNA was prepared from bone marrow mononuclear cells prior to and after retroviral transduction and analyzed for neo^R, LTR and env sequences by PCR and Southern blotting. In two of the five animals, ZB and Scamp II, the neo^R gene was present in bone marrow DNA 48 days following retroviral transduction and bone marrow transplantation. Neo^R sequences were not detected in any animal after 48 days. Bone marrow DNA was also analyzed for LTR sequences to confirm the presence of recombinant proviral sequences in the gene transfer recipients. ZB and Scamp II were each positive for LTR sequences at 48 days post transplantation. In agreement with Neo^R analysis, LTR sequences were not detected at evaluation points after 40 days. Amphotrophic env sequences were not detected in any sample post-transduction. (Fig. 12, 13 and 14).

In vivo Selection Following Retroviral Transduction and Bone Marrow Transplantation in Dogs:

The HaMDR1/A vector used in this study contains a cDNA for the human multidrug resistance gene under the transcriptional control of Harvey murine sarcoma virus (Ha-MSV) long terminal repeat sequences. Two dogs (Barbara, Moloney) were irradiated (7.5 Gy) and transplanted with autologous bone marrow that had been transduced with HaMDR1/A. Two additional dogs (George, Dolby) served as nontransduced controls. The multiplicity of infection was not noted; however, given that supernatant from the producer cell line HaMDR1/A-1 in general has a titer in the 10^4 range one should expect a MOI far less than those observed in the feline transduction procedures. All four dogs were treated with 0.5 $\mu g/m^2$, 1.0 $\mu g/m^2$, 2.0 $\mu g/m^2$ and 2.0 $\mu g/m^2$ vinblastine 6, 14, 28 and 48 days following transplantation to select for cells expressing the MDR1 cDNA. The four animals tolerated the treatments with minor gastrointestinal and hematologic complications.

CFU-GM Analysis of in vivo selected animals:

Bone marrow was obtained from the transplanted animals at various times post-transplantation and was tested for the expression of the MDR1 cDNA with the CFU-

GM assay. Both dogs transduced with the MDR1 retroviral vector had a low level of drug resistant bone marrow cells 26 days following transplantation (Table 3). The number of drug resistant CFU-GM increased five-fold in one animal (Barbara) during in vivo selection but decreased substantially once vinblastine treatment was stopped. Control animals had no drug resistant CFU-GM throughout the experimental period. These results indicate that a retroviral vector containing the MDR1 cDNA may be useful as an in vivo selectable marker but continuous selection with vinblastine may be necessary to obtain long term expression of an exogenous gene in hematopoietic cells.

DNA Analysis of MDR1 Gene Transfer Recipients:

Initially, DNA was prepared from peripheral blood of normal dogs and cats and amplified with a series of PCR primers to examine the specificity of each primer pair. The primer pairs shown in figure 15 are specific for the human MDR1 cDNA and do not amplify the endogenous canine or feline multidrug resistant genes (Figs. 16, 17 and 18). To evaluate for the presence of the human MDR1 cDNA in the two dogs transduced with HaMDR1/A, bone marrow DNA was prepared from time periods before and after the transduction procedure. Utilizing the primer pairs A and B the PCR method was used to analyze each sample. The MDR1 cDNA was not detected in any bone marrow sample by PCR followed by Southern blotting.

Vector LMDR1:

A retroviral vector containing the MDR1 cDNA may prove useful in future studies. Efficient expression of the exogenous gene would allow for in vivo selection and, therefore, enrichment of transduced cells. However, the vector, HaMDR1/A, used in the current study transfers the desired multidrug resistance phenotype inefficiently and is susceptible to helper virus generation. Therefore, a plan was devised to improve upon HaMDR1/A. The basis for the LMDR1 vector is the LNCX vector as shown in figure 7. Many of the features that make LNCX useful, such as the inability to yield helper virus by homologous recombination with the retroviral genome present in the packaging cells and the included mutations to block viral protein synthesis, are retained in the LMDR1 construct. In essence, the only features removed from LNCX were the neo^R sequences and the CMV immediate early promoter.

However, in order to ensure the DNA sequences necessary for efficient retroviral mediated gene transfer are present and are in the appropriate conformation, restriction enzyme mapping and PCR analysis were performed on the newly derived vector (Figs. 19 and 20). The following restriction enzymes were utilized in the mapping procedure: Bam HI, Hind III, Nhe I, and Eco RI. Bam HI and Hind III each cut the construct once at the 5-prime and 3-prime end of the inserted MDR1 cDNA, respectively. Nhe I has two sites in the vector cutting once in each of the LTR's. Eco RI has four recognition sequences in the plasmid construct. Gel-electrophoresis of the plasmid following digestion with the above enzymes generated fragments of the expected sizes. Bam HI and Hind III each produced one fragment of 8634 bp, Nhe I generated two fragments of 5538 and 3096 bp and Eco RI generated four restriction fragments of 3006, 2811, 1602 and 1215 bp. To confirm these results further, PCR analysis was performed using the MDR1 cDNA specific primer pair A (fig. 15). This primer pair faithfully produces an amplicon that spans virtually the entire MDR1 cDNA. PCR results were also positive verifying the presence of an intact MDR1 cDNA within the vector. The structure of LMDR1 is shown in figure 8. This vector contains a copy of the human MDR1 full-length cDNA bound by long-terminal repeats (LTR) and the extended packaging sequence of LNCX. The complete nucleotide sequence and restriction analysis of pLMDR1 as well as the intermediate pL are given in appendices C and D, respectively.

CHAPTER 4

DISCUSSION

Gene transfer into repopulating stem cells of the mouse has been demonstrated by many research groups over the past few years. However, to study the feasibility of gene therapy for humans, it is necessary to evaluate gene transfer efficiencies into hematopoietic stem cells of large outbred animals. Unfortunately, in contrast to the relatively efficient transduction of murine HSCs, gene transfer into hematopoietic progenitors of large animals such as dogs, cats or nonhuman primates has not been as successful. In general, bone marrow cells from large animals transduced in vitro have not been maintained at high levels in vivo after autologous transplantation.[50-55] The purpose of this study was to develop methods to analyze, with a large degree of accuracy and sensitivity, the many operational steps involved in gene transfer procedures in large animals such as dogs and cats.

Gene transfer using retroviral vectors has become a widely used approach owing to the highly-efficient stable integration of foreign gene sequences in a wide variety of cell types. The actual transduction procedure requires three phases: (1) construction of retroviral vector clones and packaging cell lines, (2) transduction of target cells by the retroviral vector and (3) analysis of the transduced target cells. In this study we have applied polymerase chain reaction methodologies to several aspects of our gene transfer program. First, we have utilized PCR to analyze bone marrow DNA from gene transfer recipients for the presence of the exogenous neo^R and MDR1 genes. Secondly, we have employed PCR in ascertaining critical safety concerns such as the generation of contaminating replication-competent retrovirus, or helper virus. And lastly, in hopes of improving upon what we have accomplished in these studies, PCR was applied with other molecular biology techniques in the construction of a new retroviral vector LMDR1.

The results of the current study demonstrate that an exogenous gene can be expressed in retroviral transduced canine and feline hematopoietic cells. In this study, the bone marrow of five dogs and four cats was transduced with either the N2 or SAX retroviral vector. Transduction efficiencies at the time of infusion ranged from zero to

12.5% G418 resistant CFU-GM. The multiplicity of infection, the ratio of viral particles to bone marrow mononuclear cells, in these procedures varied greatly. It is of importance to note that in animals with a low MOI transduction efficiencies were poor. In general, a high MOI is required for efficient transduction, because repopulating stem cells are quite rare in bone marrow.

The size of the animal as well as the viral titer plays a critical role in determining the MOI. The titer of a given producer cell line is largely determined by the retroviral vector construct, with the maximum obtainable titer dependent upon the vector size, the length of the gag region present, and whether the inserted gene contains sequences that reduce the stability of the vector genome.[56-58] The size of the animal is also important, because the larger the animal the more cells required for reconstitution. The more bone marrow cells needed for reconstitution, the more viral supernatant needed to transduce them. Gene transfer procedures in larger animals thus become both cost and equipment prohibitive. For example, a 20 lb dog would require approximately 9×10^8 cells to reconstitute the bone marrow after lethal irradiation. In order to achieve a MOI of two, it would require 1.8 liters of viral supernatant previously titered at 1×10^6 cfu/ml. Reduction of the viral titer to 1×10^4 cfu/ml and still achieving the desired MOI of two would require 180 liters of viral supernatant. Increasing the body weight of the experimental animal to 30 lbs using viral titers of 1×10^6 or 1×10^4 cfu/ml would require 2.7 and 270 liters of viral-superantnat, respectfully. This example highlights the importance of high titer vectors in retroviral-mediated gene transfer.

Bone marrow was obtained from gene transfer recipients at times from approximately 30 days to greater than 300 days post-transplantation and tested for the expression of neo^R by the <u>in vitro</u> drug resistant CFU assay. These experiments showed expression of the retroviral vector in all four cats and one dog 30 to 48 days post-transplantation. Sequential evaluation of bone marrow from these animals showed a distinct species difference in the expression of the neo^R gene between dogs and cats transduced with similar MOIs. The two dogs that had a MOI greater than two had a low but detectable number of G418 resistant CFU-GM for approximately 80 days. Within several weeks, even this low level of expression was lost. The cats with MOIs greater than two however, showed that although the fraction of G418-resistant CFU

declined over a period of several months there still was a low level of drug-resistant CFU-GM after 200 days. Expression of the neo^R gene after 200 days in the cats suggest that transduction of the HSC pool may have occurred. The basis for the difference seen in these studies between dogs and cats is not understood, but perhaps there is a species variation in the number of receptors for the amphotropic envelope protein on hematopoietic cells of dogs and cats.

Besides the MOI, another important parameter that determines the efficiency of retroviral gene transfer into hematopoietic stem cells is the cycling status of the target cell. Retroviral integration is blocked in nonreplicating cells, and HSCs in bone marrow tend to be in G_0 of the cell cycle.[59] In cyclic hematopoietic (CH) dogs, hematopoietic progenitor cells undergo periods of proliferation at predictable intervals, and one would think that transduction of bone marrow cells with a retroviral vector at the time of maximal hematopoiesis would increase transduction efficiencies. This however, was not the case and bone marrow from CH dogs at the time of maximal hematopoiesis was no more transducable with the N2 retroviral vector than marrow from normal dogs.[30] This suggests that transduction of canine bone marrow cells, with existing retroviral vectors, may be limited by some other factor(s) other than target cell cycling status. Transduction of feline bone marrow cells may not be limited by such factor(s). The variance in transduction efficiencies between species is worthy of future investigation if a large animal model for gene therapy is to be realized.

Bone marrow DNA from transplant recipients was analyzed at various time points post-transplantation for neo^R, LTR and env sequences by PCR and Southern blotting. The sensitivity of PCR is such that one has to exercise meticulous laboratory technique, as well as to utilize well characterized negative controls.[60] In the initial phase of sample analysis, it became apparent that sample contamination, by either previously generated amplicon or producer cell genomic DNA, was going to hinder rapid and accurate processing of results. To eliminate this problem, a master mixture of all reagents used in the PCR reaction except Taq polyermase and experimental DNA were purified of any extraneous DNA by centrifugation through an ultrafiltration membrane. In this process during centrifugation, the DNA is brought into pressurized contact with the membrane. The passage or retention of the DNA in the solution is determined by the size of the DNA fragments and the effective pore size of the

membrane. The pore size utilized for these procedures had a 30,000 nominal molecular weight limit. Given that the primers used in the PCR are short single stranded DNA (approximately 6600 Daltons) these effectively passed through the membrane while any contaminating DNA greater than 46 base pairs is retained by the membrane.

PCR analysis of gene transfer recipient bone marrow DNA corroborated the in vitro colony data. At times post-transduction when percent G418 resistant colonies were elevated, the neo^R gene was clearly detectable by PCR. However, in two felines that were transduced with the N2 retroviral vector, helper virus genome was also detected. Helper virus is generally produced following homologous recombination between the vector and the packaging system and has been a consistent problem with PA12-N2 based producer lines (Fig. 21)[61] The presence of the helper virus genome in the transplanted cats means a localized bone marrow infection may have contributed to the persistent expression of the neo^R gene in the transplanted animals. In addition, two of the four transplanted cats unexpectedly developed diabetes mellitus. In one of these cats (Andy), the viral envelope sequence was clearly detected by PCR before the development of the disease. The exact cause of the diabetes mellitus in these two cats was not ascertained; however, viruses are thought to be a common environmental factor inducing diabetes in susceptible humans.[62] Another important consideration is the duration in which the helper genome persisted. Earlier studies examining the fate of amphotropic leukemia retrovirus in rhesus monkey concluded that murine amphotrophic viruses are not acute pathogens in primates. Rhesus monkeys that were transplanted with autologous skin fibroblasts shown to be producing amphotropic virus successfully cleared the virus within 125 days.[3] Since the helper viral genome was still detectable in some cats 210 days post-transplantation, differences may exist in the ability of species to eliminate the murine amphotrophic virus from their circulation. These results underscore the importance of the use of large animal models to test the several novel aspects of retroviral technology needed for human gene therapy.

We have shown by both expression and DNA sequence analysis that an exogenous gene can be successfully transferred via a recombinant retroviral vector into canine and feline hematopoietic cells. However, improvements in current retroviral vector transduction protocols are necessary to achieve stable transfer and expression of

exogenous genes in the majority of bone marrow cells. One way of improving the apparent rate of transfer and expression of exogenous genes may be by applying selective pressure in vivo following transduction and transplantation. This can be accomplished by utilizing retroviral vectors that contain a dominant selectable marker. A dominant selectable marker would confer an advantageous phenotype to those cells expressing the marker. One such marker is the human multidrug resistance (MDR1) gene. The human MDR1 gene encodes a 170-Kd molecular weight plasma membrane protein termed P-glycoprotein.[63,64] P-glycoprotein functions as an energy-dependent transport protein that extrudes certain hydrophobic cytotoxic agents out of cells.

Retroviral vectors containing full-length human and mouse MDR cDNAs have been constructed and introduced into various packaging cell lines.[65,66] Virions from these producer cells can efficiently confer the multidrug resistance phenotype to a wide variety of cell types. We have utilized one such vector and producer cell line, HaMDR1/A and HaMDR1/A-1, respectively, to transduce canine hematopoietic cells. Of the two dogs transplanted with bone marrow transduced by the MDR1 retroviral vector, each had a low level of drug resistant colonies after 26 days. The transduced population of cells developed resistance to two unrelated cytotoxic drugs vinblastine and adriamycin. In vivo selection dramatically increased the number of drug resistant CFU-GM in one animal during vinblastine treatment, but once the treatment was stopped drug resistant colony numbers decreased to near control levels. Throughout the selection period, control marrow never developed significant numbers of drug resistant colonies indicating that the drug resistance seen in experimental animals marrow cells was due to the expression of the exogenous human MDR1 cDNA and not an endogenous canine multidrug resistance gene.

Three sets of PCR primers specific for the human MDR1 cDNA were designed so that experimental animals could be analyzed for the presence of the human MDR1 cDNA following transduction. These primers were chosen because the sequences are in regions of P-glycoprotein that are not well conserved between mouse and man.[67] Preliminary data indeed showed that these primer pairs were specific for human MDR1 cDNA and did not cross-react with either the canine or feline MDR species. PCR analysis of bone marrow DNA from the two HaMDR1/A recipient dogs at multiple time points post-transplantation failed to detect the desired MDR1 cDNA sequences

even though in vitro colony data suggested that MDR1 was being expressed. Although a discrepancy between DNA and drug resistance colony data exists, this information is suggestive that MDR1 may be useful as a dominant in vivo selectable marker.

A recombinant retroviral vector based on the replication-defective Harvey murine sarcoma virus, from which the viral *ras* gene was removed and replaced with the human MDR1 cDNA, was used in the above study. We encounter several problems associated with this (HaMDR1/A) vector. The viral construct is quite large (8.6 Kb) and is packaged inefficiently by the PA12 cells. In addition, the HaMDR1/A-1 cell line produces contaminating helper virus that hinders ones ability to accurately determine transduction efficiencies and poses a serious health threat to gene transfer recipients. For these reasons, a new retroviral vector was constructed based on the LN series of vectors that has recently been used in the first gene therapy protocol in humans.[68] This new vector (LMDR1) has design features that prevent the generation of helper virus by homologous recombination with the retroviral genome present in the packaging cells, and includes mutations to block the production of viral proteins that may have antigenic or other unwanted properties. There are, in addition to the above features, unique cloning sites on either end of the MDR1 cDNA. This facilitates the addition of another gene; thereby, allowing the use of the dominant drug resistance phenotype to introduce passenger genes into bone marrow as an alternative approach to gene therapy. LMDR1 should also prove useful in chemotherapy research. The ability to generate multidrug-resistant canine or feline bone marrow cells with high efficiency by retroviral vector may make it possible to alleviate the myelosuppression associated with the administration of chemotherapeutic agents in the treatment of tumors; thus, allowing dose intensification that might achieve a cure.

The results of the present study show that recombinant retroviral vectors can successfully transfer an exogenous gene into canine and feline bone marrow cells. However, many aspects of the retroviral transduction procedure must be optimized in order to achieve both long-term stability and expression of the exogenous gene. If these problems can be solved, then the methodologies applied in this study will be the heart of a medical revolution in the treatment of genetic diseases of both man and animal.

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APPENDICES

APPENDIX A

Table 1. Gene transfer in feline hematopoietic cells

Animal	Vector	Cell dose/ kg x 10^{-8}	Days Post Transplantation	CFU-GM -G418 Infected	CFU-GM -G418 Control	CFU-GM +G418 Infected	CFU-GM +G418 Control	%G418 Resistant Infected	%G418 Resistant Control	MOI
Sam	N2	2.2	0	ND		ND		ND		>2
			30	378	ND	28	ND	7.4	ND	
			66	490	199	0	0	<0.2	<0.5	
			94	979	ND	0	ND	<0.1	ND	
Andy	N2	2.0	0	932	781	55	0	5.9	<0.1	>2
			30	627	ND	35	ND	5.6	ND	
			66	404	199	0	0	<0.2	<0.2	
			94	1336	ND	5	ND	0.4	ND	
			154	551	ND	19	ND	3.4	ND	
			194	563	779	15	8	2.7	1.0	
			223	480	766	16	3	3.3	0.4	
			348	210	621	1	6	0.5	1.0	
			368	335	296	3	0	0.9	<0.3	
			729	327	365	3	0	0.9	<0.2	
Martina	N2	5.5	0	1211	638	40	3	3.3	0.4	>2
			26	540	ND	30	ND	5.5	ND	
			67	349	779	4	8	1.1	1.0	
			96	240	755	0	3	<0.4	0.4	
			214	190	621	6	6	3.1	1.0	
			248	390	296	4	0	1.0	<0.3	
			609	600	365	21	0	3.5	<0.2	
			978	287	198	0	0	<0.3	<0.5	
Yenkee	SAX	3.7	0	468	540	0	0	<0.2	<0.2	>2
			26	420	ND	13	ND	3.1	ND	
			67	386	779	18	8	4.7	1.0	
			96	414	766	9	3	2.2	0.4	
			214	419	621	15	6	3.6	1.0	
			248	975	296	35	0	3.6	<0.3	
			609	257	365	9	0	3.5	<0.2	
			978	209	198	12	0	5.7	<0.5	

Table 1. Gene transfer in feline hematopoietic cells. The data are presented as total CFU-GM of five individual culture dishes (7.5 x 10^5 cells/dish). Control marrow (uninfected marrow from the same animal [day 0 only] or marrow from an uninfected cat) was assayed in parallel to check for "background" G418-resistant CFU-GM. The multiplicity of infection (MOI) is the ratio of viral particles, as estimated by the titer of drug-resistant 3T3 CFUs, to BM mononuclear cells. Abbreviation: ND, not done.

Table 2. Gene transfer into canine hematopoietic cells following autologous bone marrow transplantation.

Animal	Vector	Cell dose/ kg x 10^{-8}	Days Post Transplan-tation	CFU-GM -G418 Infected	CFU-GM -G418 Control	CFU-GM +G418 Infected	CFU-GM +G418 Control	% G418 Resistant Infected	% G418 Resistant Control	MOI
Mighty Dog	SAX	2.7	0	ND		ND		ND		<0.008
			20	258		0		0.0		
Ringling	N2	3.2	0	ND		ND		ND		0.025
			20	252		0		0.0		
Powerhouse	SAX	4.6	0	427		0		0.0		0.1
			28	162		0		0.0		
			60	35		0		0.0		
Scamp II	N2	0.93	0	167	835	8	0	4.8	0.0	>2
			48	1263	ND	56	ND	4.4	ND	
			85	739	ND	4	ND	0.5	ND	
			108	1282	1753	14	0	1.1	0.1	
ZB	N2	1.3	0	160	800	20	0	12.5	0.0	>2
			48	530	ND	3	ND	0.6	ND	
			85	625	ND	11	ND	1.8	ND	
			108	1888	1753	1	0	0.05	0.0	

Table 2. Gene transfer in canine hematopoietic cells. The data are presented as total CFU-GM of 5 individual culture dishes (7.5 x 10^5 cells/dish) or as the %G418 resistant CFU-GM (CFU-GM + G418/CFU-GM-G418. Control marrow (uninfected marrow from the same animals [day 0 only] or marrow from an uninfected dog) was assayed in parallel to check for "background" G418-resistant CFU-GM. The multiplicity of infection (MOI) is the ratio of viral particles, as estimated by the titer of drug-resistant 3T3 CFUs, to bone marrow mononuclear cells. ND - not done.

Table 3. Vinblastine and adriamycin resistant CFU-GM in control and MDR infected canine bone marrow cells following autologous bone marrow transplantation.

Animal	Days Post Transplant	Control	Vinblastine†	Adriamycin†	% Drug Resistant CFU-GM Vinblastine	Adriamycin
*Barbara	26	24	2	1	8.1	4.2
	41	73	31	ND	42.4	ND
	71	548	8	7	1.4	1.3
*Moloney	26	95	10	3	10.5	3.2
	41	104	6	ND	5.8	ND
	71	344	3	3	0.9	0.9
George	26	613	0	0	<0.2	<0.2
	41	250	0	nd	<0.4	ND
	71	125	0	0	<0.8	<0.8
Dolby	26	122	0	0	<0.8	<0.8
	41	157	0	ND	<0.6	ND
	71	ND	ND	ND	ND	ND

*Barbara and Moloney were transplanted with autologous bone marrow that had been infected with the MDR1 retroviral vector. George and Dolby were transplanted with autologous bone marrow without infection with the MDR retroviral vector. The dogs were treated with 0.5 mg/m^2, 1.0 mg/m^2, 2.0 mg/m^2 and 2.0 mg/m^2 vinblastine 6, 14, 26 and 48 days following transplantation. †Vinblastine and adriamycin were added to the in vitro CFU-GM assay at 20 and 100 $\mu g/ml$, respectively. The data are summarized as the number of CFU-GM/2.25 x 10^6 cells or the % drug resistant CFU-GM (experiment of CFU-GM/control CFU-GM).

APPENDIX B

Figure 1. The retroviral life cycle. 1. Adsorption and uptake (infection), 2. The single-stranded (SS) RNA is converted to double-stranded (DS) DNA by reverse transcriptase, 3. DNA integrates randomly into a chromosome to form a provirus, 4. The provirus serves as a template for mRNA to yield both viral proteins and new full length genomic RNA, 5. Viral RNA and newly formed viral proteins combine and by budding release an infectious virus.

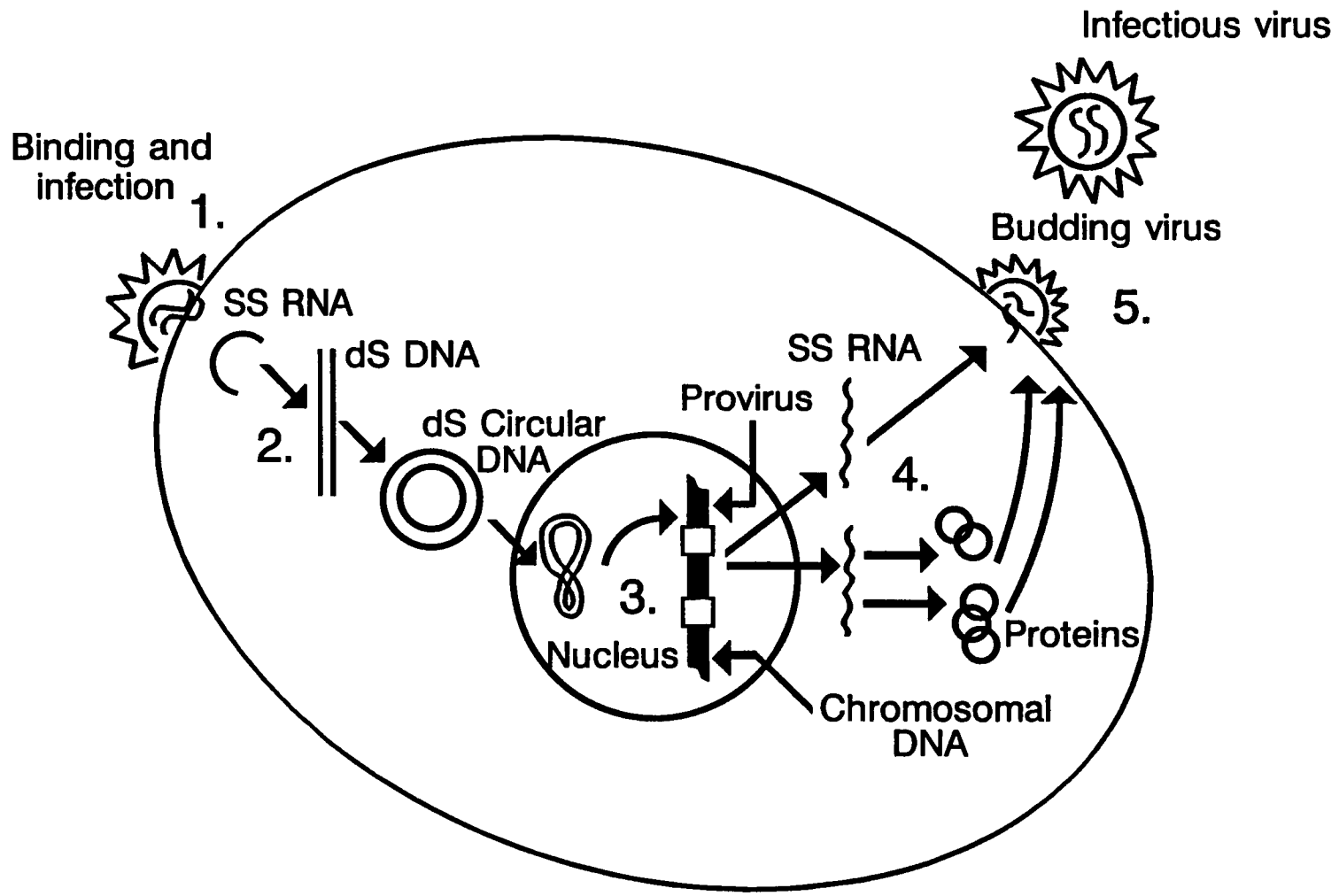

Infectious virus
Binding and infection
1.
Budding virus
5.
SS RNA
dS DNA
SS RNA
2.
dS Circular DNA
Provirus
4.
3.
Proteins
Nucleus
Chromosomal DNA

Figure 2. Procedures for the generation of packaging and producer-cell lines.

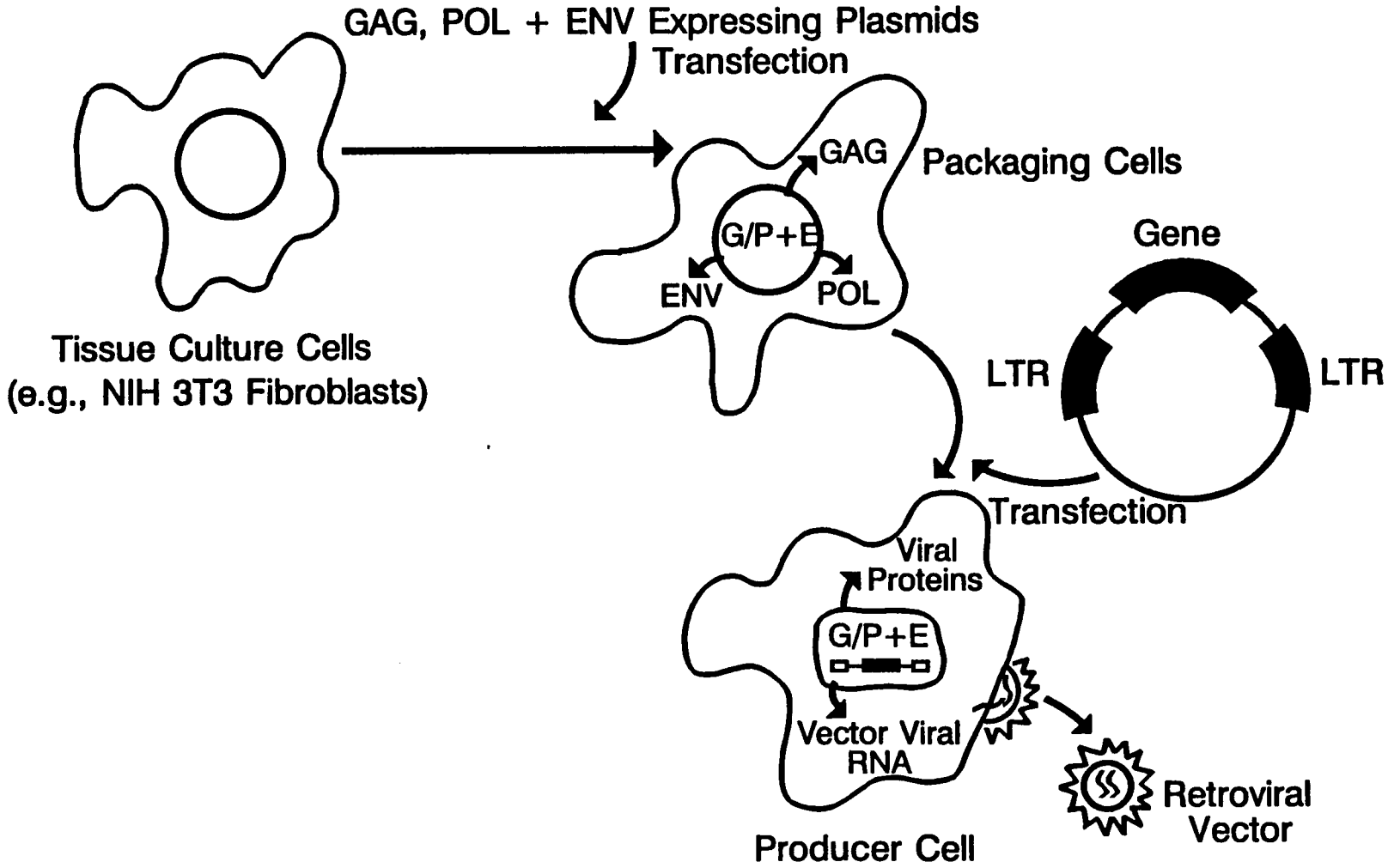

GAG, POL + ENV Expressing Plasmids
Transfection
Tissue Culture Cells
(e.g., NIH 3T3 Fibroblasts)
GAG
Packaging Cells
G/P+E
ENV
POL
Gene
LTR
LTR
Transfection
Viral
Proteins
G/P+E
Vector Viral
RNA
Retroviral
Vector
Producer Cell

Figure 3. Sequential digestion of pHaMDR1/A with Sac II and XhoI yields a 4.1 Kb fragment encoding the entire MDR1 cDNA.

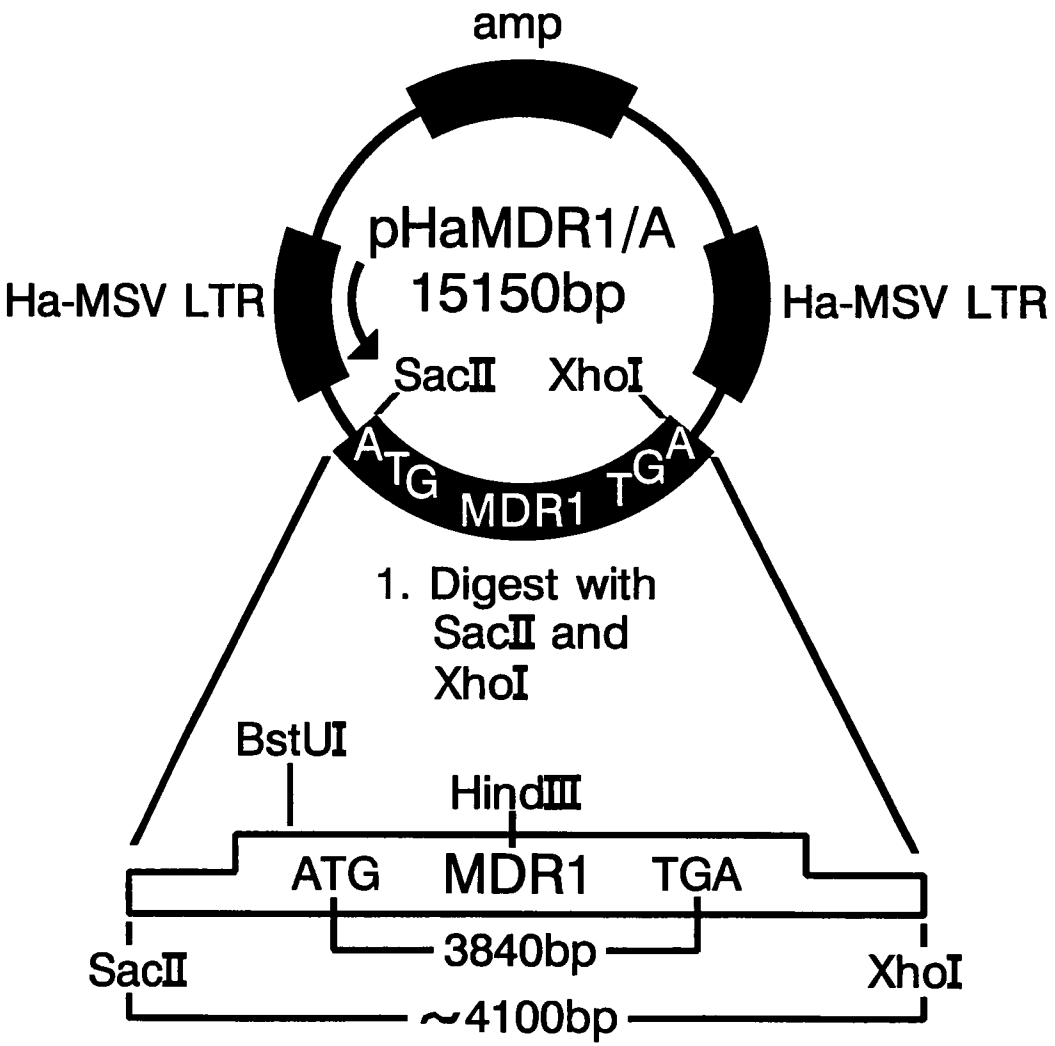

amp
pHaMDR1/A
15150bp
Ha-MSV LTR
Ha-MSV LTR
SacII
XhoI
ATG
MDR1
TGA
1. Digest with
SacII and
XhoI
BstUI
HindIII
ATG
MDR1
TGA
3840bp
SacII
XhoI
~4100bp

Figure 4. Refinement of the MDR1 cDNA sequence isolated from pHaMDR1/A by the removal of 5-prime extraneous sequences. Digestion of the 4.1 - Kb Sac II - XhoI fragment obtained in figure 3 with BstUI followed by a filling-in reaction using the large fragment of DNA polymerase I (Klenow) generates a 3.9 - Kb blunt ended MDR1 cDNA. The coding region of MDR1 is approximately 3.8 - Kb.

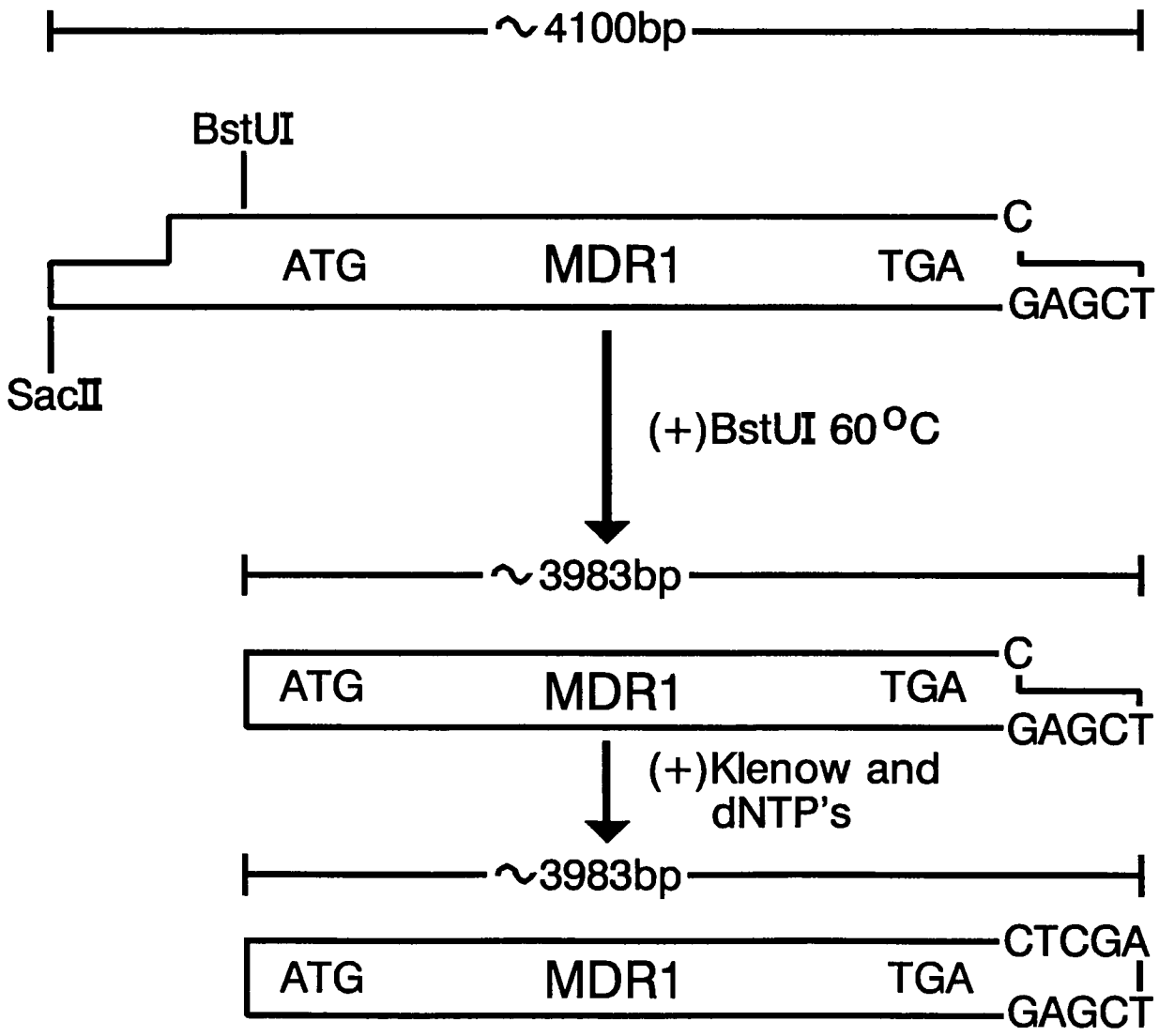

~4100bp
BstUI
C
ATG
MDR1
TGA
GAGCT
SacII
(+)BstUI 60°C
~3983bp
C
ATG
MDR1
TGA
GAGCT
(+)Klenow and
dNTP's
~3983bp
CTCGA
ATG
MDR1
TGA
GAGCT

Figure 5. Cloning of the MDR1 cDNA into the multi-cloning site of pSP72. pSP72 is first digested with SmaI and treated with calf intestine alkaline phosphatase. Ligation of the linear pSP72 fragment and the MDR1 cDNA is achieved utilizing T4 DNA ligase.

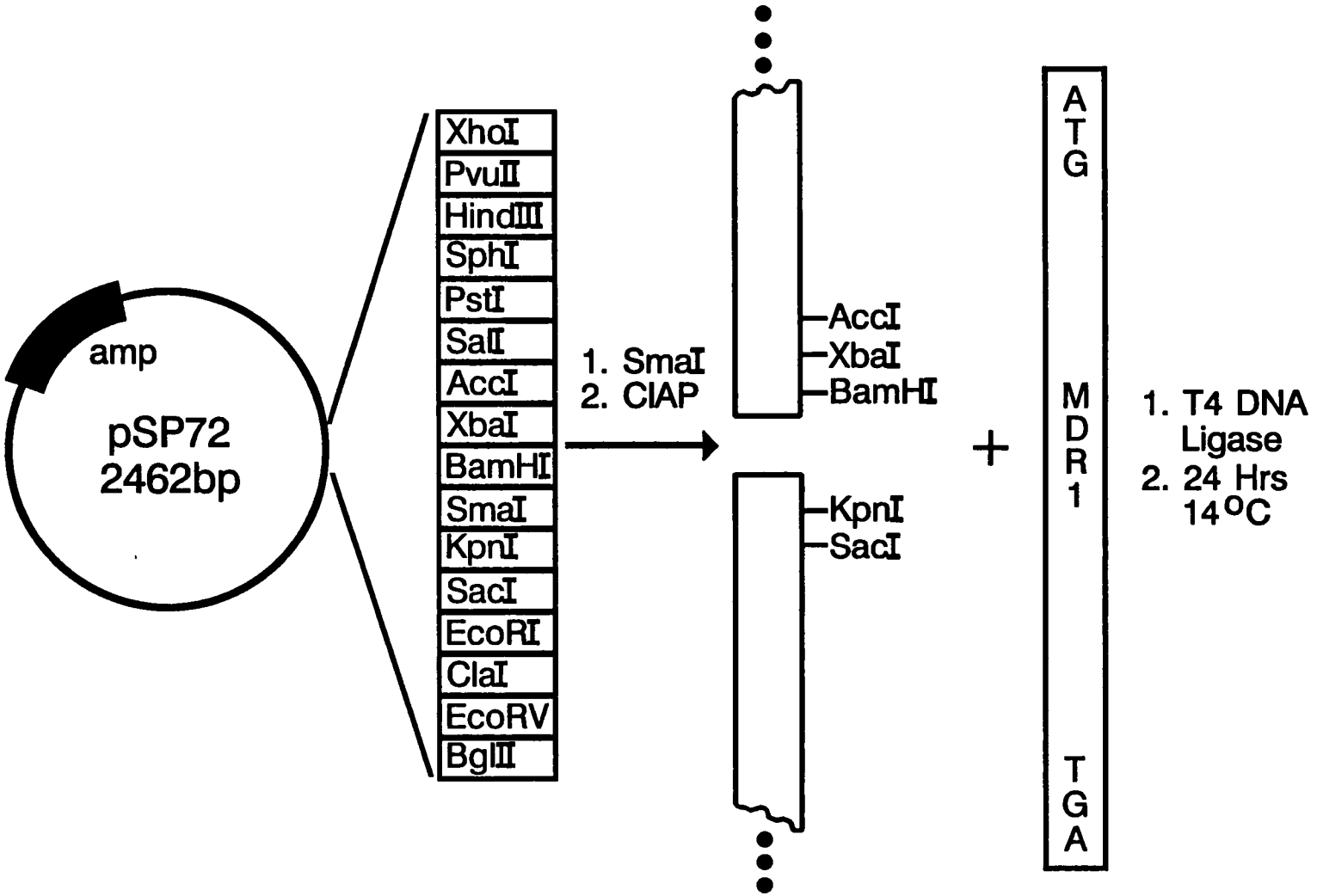
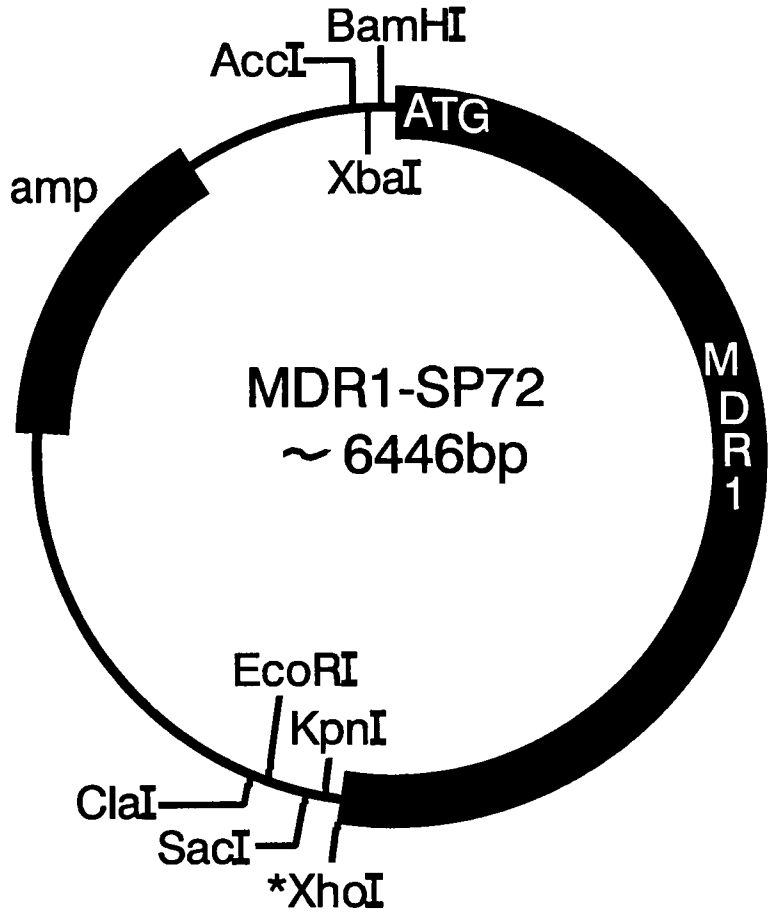

amp
pSP72
2462bp
XhoI
PvuII
HindIII
SphI
PstI
SalI
AccI
XbaI
BamHI
SmaI
KpnI
SacI
EcoRI
ClaI
EcoRV
BglII
1. SmaI
2. CIAP
AccI
XbaI
BamHI
KpnI
SacI
+
ATG
MDR1
TGA
1. T4 DNA Ligase
2. 24 Hrs 14°C

Figure 6. MDR1-SP72 is a useful cloning intermediate due to unique restriction sites on either end of the MDR1 cDNA.



*XhoI is recreated from XhoI fill-in ligated to SmaI

Figure 7. Formation of pL from pLNCX. The neo^R gene and the immediate early promoter from human cytomegalovirus were removed from pLNCX as a BclI - Hind III fragment. BamHI linkers were ligated to the remaining retroviral vector backbone. Digestion of this backbone with BamHI followed by re-ligation with T4 DNA ligase generated pL. pL contains unique BamHI, HpaI and ClaI cloning sites.

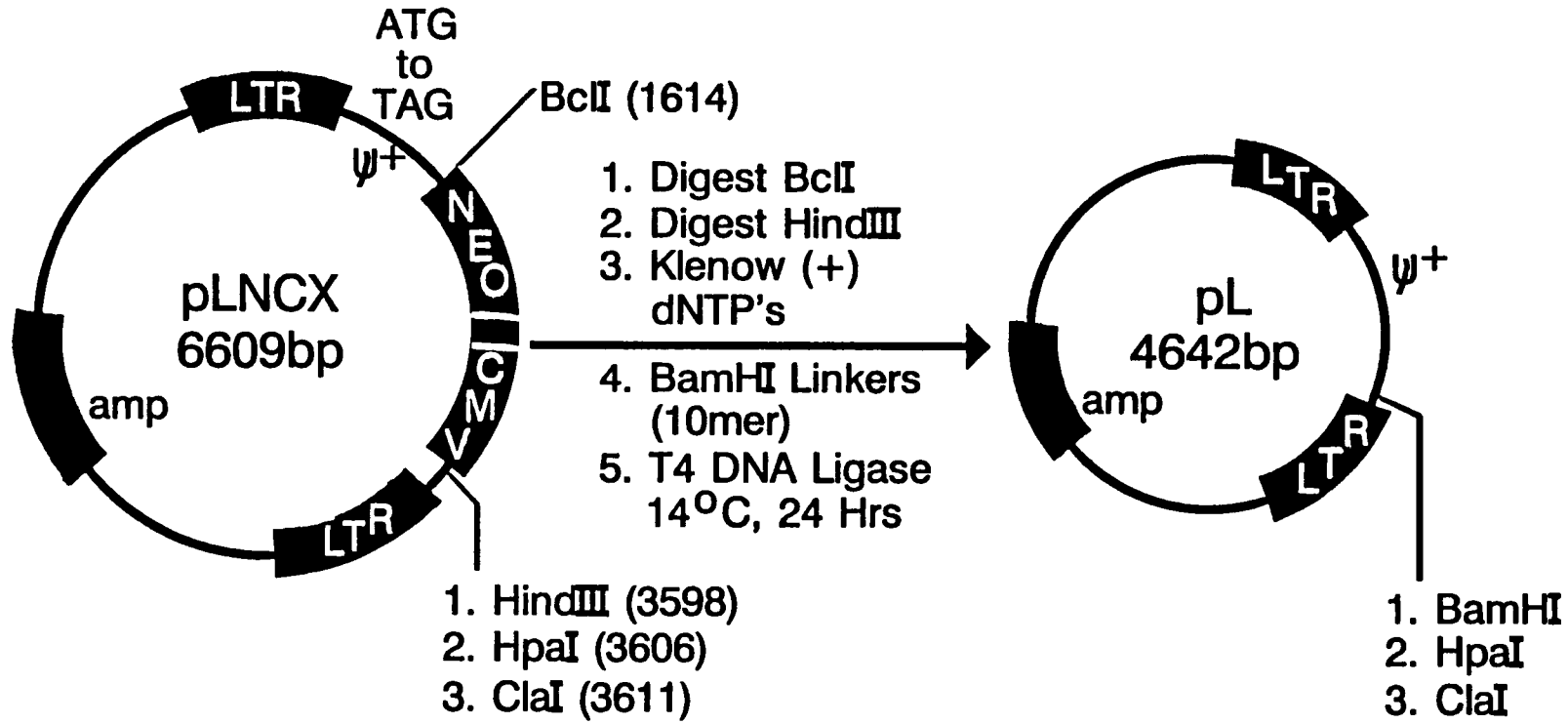

ATG
to
TAG
BclI (1614)
LTR
ψ+
NEO
pLNCX
6609bp
amp
CMV
LTR
1. Digest BclI
2. Digest HindIII
3. Klenow (+)
dNTP's
4. BamHI Linkers
(10mer)
5. T4 DNA Ligase
14°C, 24 Hrs
1. HindIII (3598)
2. HpaI (3606)
3. ClaI (3611)
LTR
ψ+
pL
4642bp
amp
LTR
1. BamHI
2. HpaI
3. ClaI

Figure 8. Formation of pLMDR1. The 3.9 - Kb MDR1 cDNA was excised from MDR1-SP72 as a BamHI-ClaI fragment and ligated to previously BamHI-ClaI digested pL to yield pLMDR1. pLMDR1 retains many of the desirable features of pLNCX, in addition pLMDR1 has unique cloning sites on either end of the MDR1 cDNA to facilitate the addition of another gene.

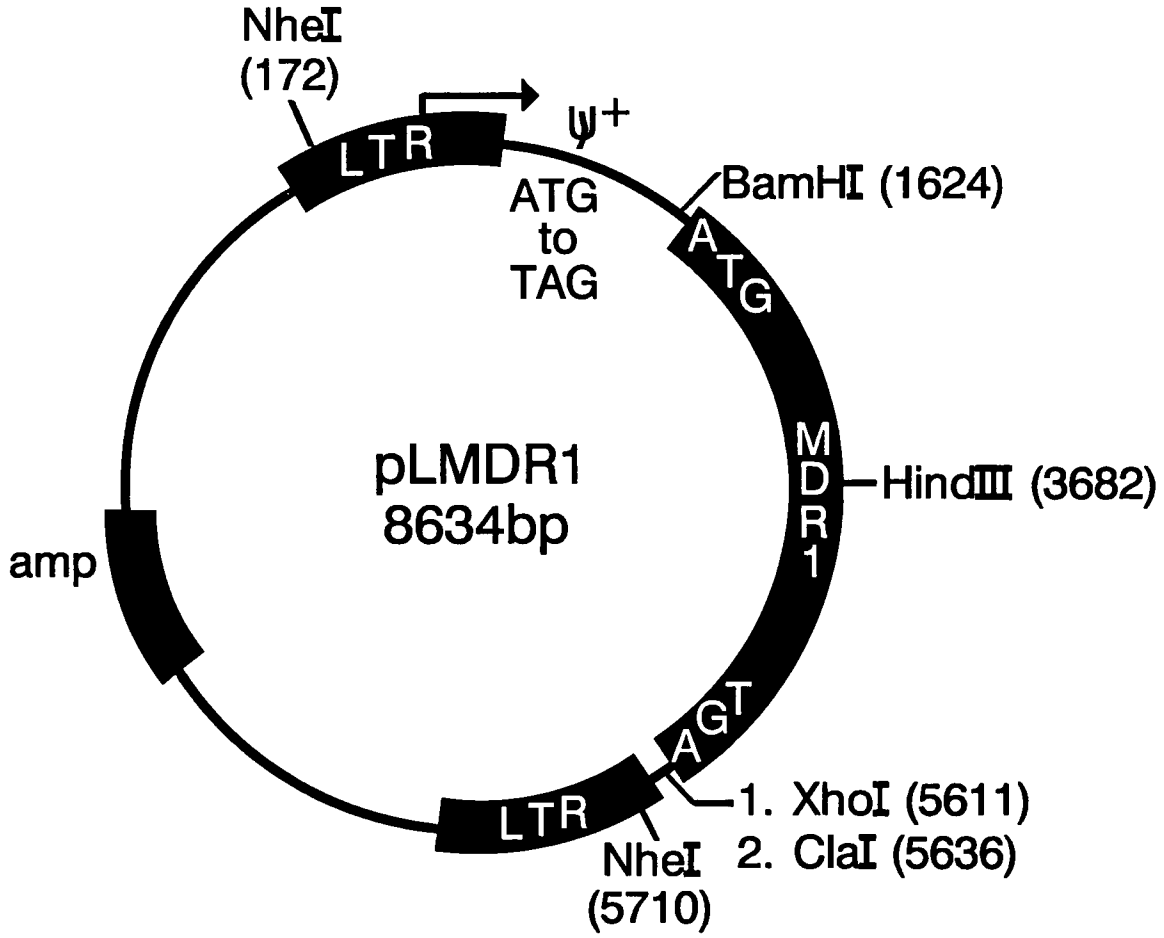

NheI
(172)
LTR
ψ+
ATG
to
TAG
BamHI (1624)
ATG
MDR1
HindIII (3682)
pLMDR1
8634bp
amp
AGT
LTR
1. XhoI (5611)
2. ClaI (5636)
NheI
(5710)

Figure 9. PCR analysis of the N2 vector and PA12 packaging construct. (A) Oligonucleotide primers were chosen to amplify two distinct regions of the N2 retroviral vector: the transcriptional control region (LTR; primers LTR 1 and LTR 2), and the neomycin phosphotransferase (NEO) gene (NEO; primers Neo 1 and Neo 5). The SAX retroviral vector is similar to N2 and these primer pairs would generate the same results. (B) Diagrammed is a representation of the packaging construct present in PA12 cells. Oligonucleotide primers ENV1 and ENV3 amplify a 375 bp region of the amphotropic (4070A) envelope gene. This primer pair is used in safety studies to detect helper virus.

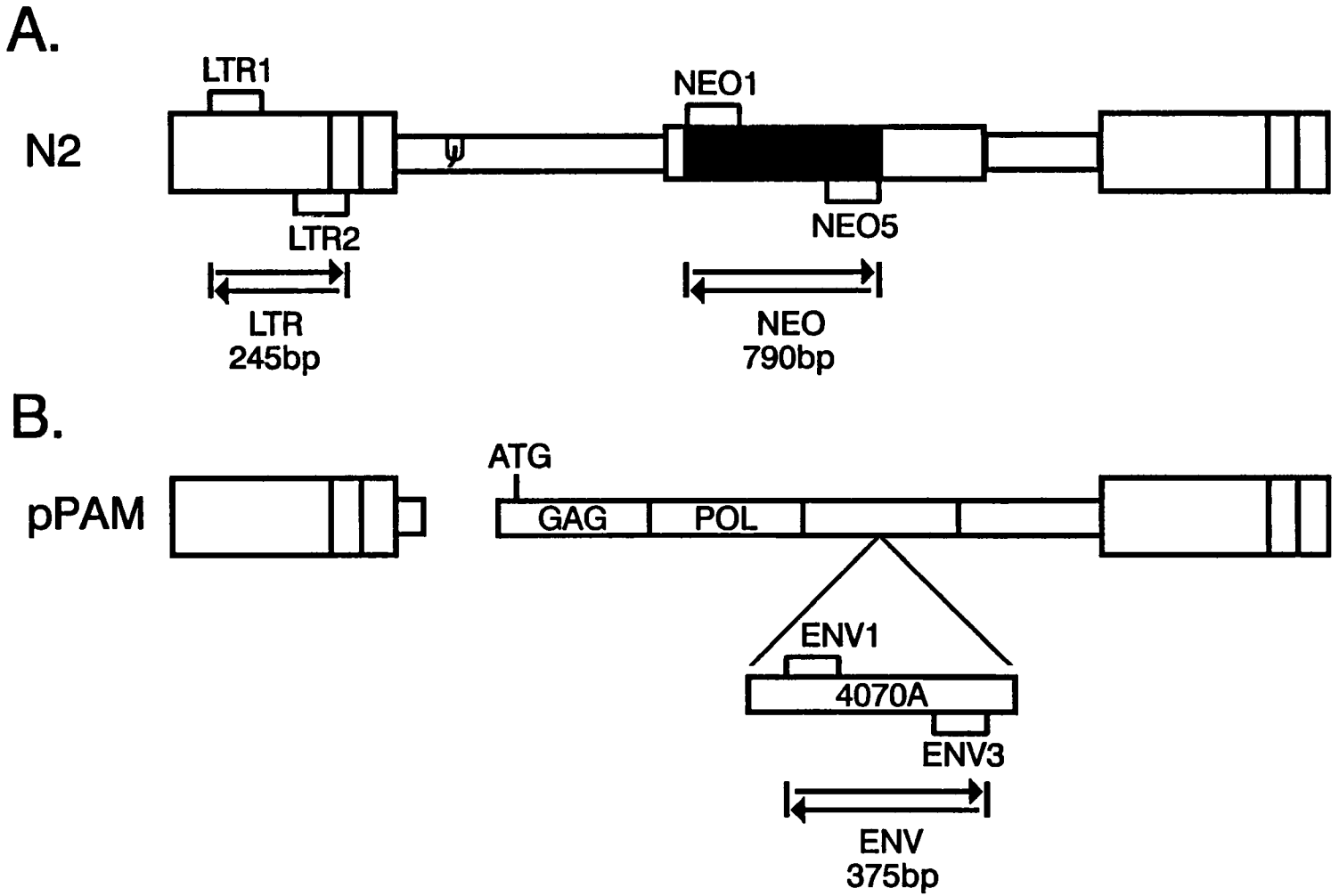

A.
LTR1
NEO1
N2
ψ
LTR2
NEO5
LTR
245bp
NEO
790bp
B.
ATG
pPAM
GAG
POL
ENV1
4070A
ENV3
ENV
375bp

Figure 10. Sequential evaluation of feline (Martina) bone marrow DNA for the neo^R gene. The lanes are: 1. negative control, 2. pre-transplant, 3. 214 days post-transplant, 4. 609 days post-transplant, 5. 978 days post-transplant, 6. positive control. Lanes 7 and 8 are empty.


1 2 3 4 5 6 7 8

Figure 11. Sequential evaluation of feline (Yenkee) bone marrow DNA for the neo^R gene. The lanes are: 1. positive control, 2. pre-transplant, 3. 26 days post-transplant, 4. 67 days post-transplant, 5. 96 days post-transplant, 6. 214 days post-transplant, 7. 609 days post-transplant, 8. 978 days post-transplant.

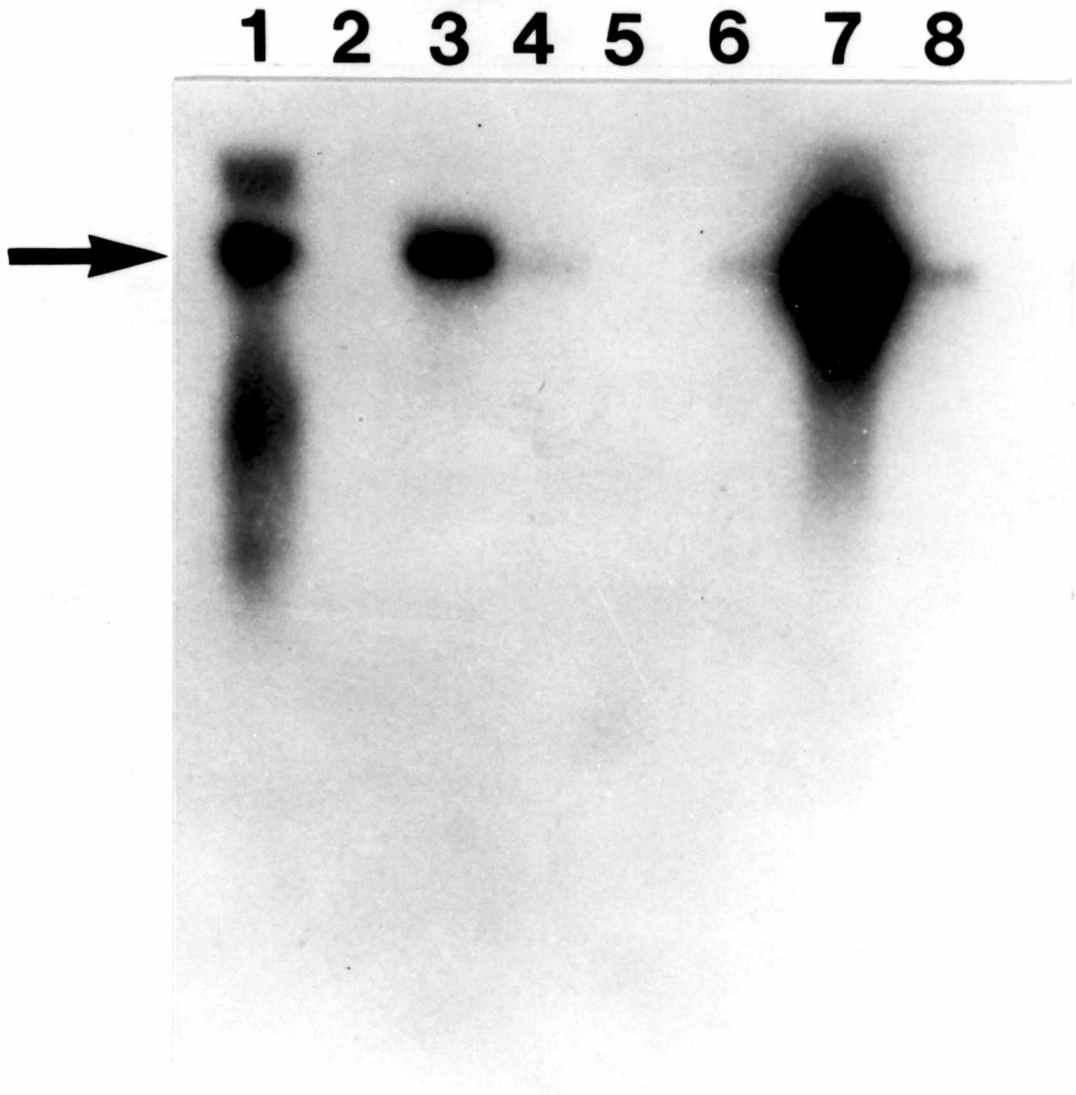

1 2 3 4 5 6 7 8

Figure 12. Sequential evaluation of canine bone marrow DNA for the neo^R gene. The lanes are: 1. Empty, 2. ZB pre-transplant, 3. ZB 48 days post-transplant, 4. Empty, 5. Scamp II pre-transplant, 6. Scamp II 48 days post-transplant.


neo^R
1 2 3 4 5 6

Figure 13. Sequential evaluation of canine bone marrow DNA for viral long terminal repeat sequences. The lanes are: 1. Empty, 2. ZB pre-transplant, 3. ZB 48 days post-transplant, 4. Empty, 5. Scamp II pre-transplant, 6. Scamp II 48 days post-transplant.

LTR

1 2 3 4 5 6

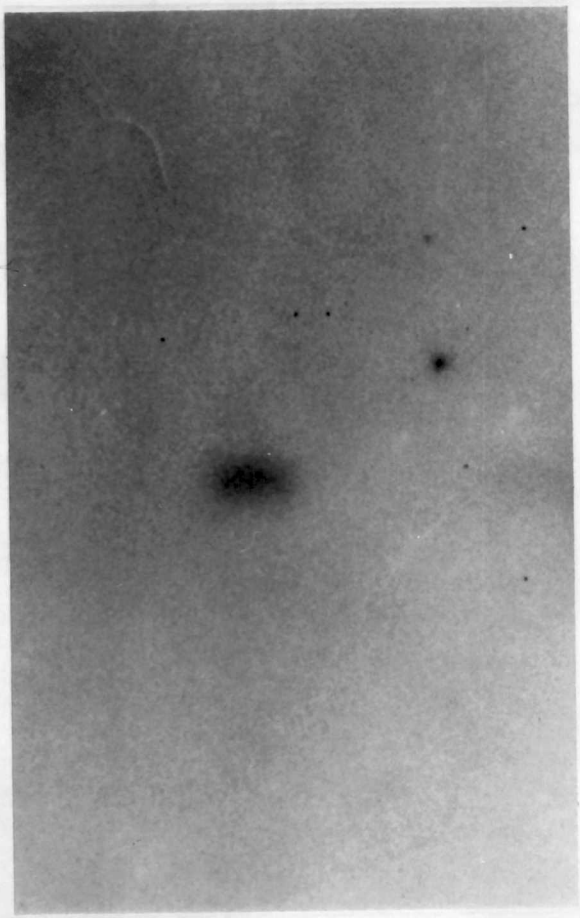


Figure 14. Sequential evlauation of canine bone marrow DNA for the amphotropic envelope gene of the 4070 A retrovirus. The lanes are: 1. Empty, 2. ZB pre-transpant, 3. ZB 48 days post-transplant, 4. Empty, 5. Scamp II pre-transplant, 6. Scamp II 48 days post-transplant, 7. Scamp II 85 days post-transplant, 8. Positive control.

env

1 2 3 4 5 6 7 8

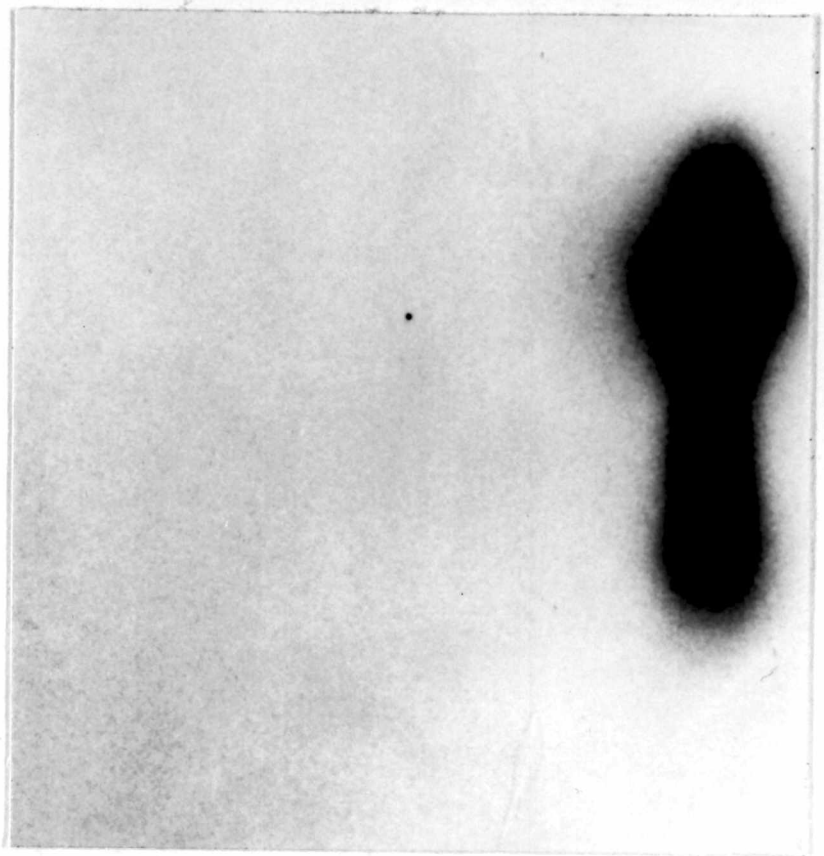


Figure 15. PCR analysis of the MDR1 cDNA. Oligonucleotide primers were chosen to amplify three distinct regions of the MDR1 cDNA. These primers were chosen because their sequences are in regions of P-glycoprotein that are not well conserved between mouse and man.

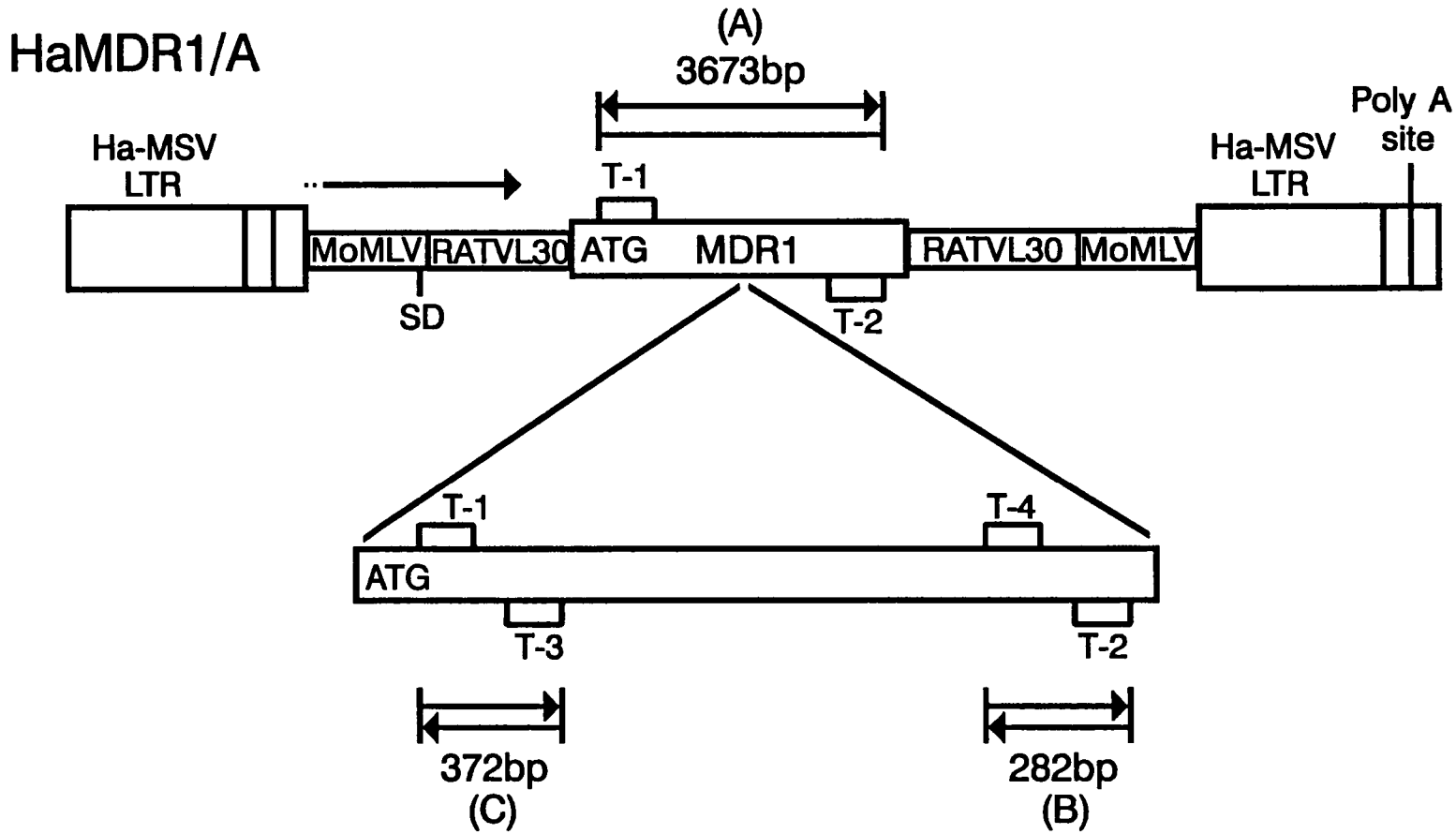

HaMDR1/A
(A)
3673bp
Poly A
site
Ha-MSV
LTR
Ha-MSV
LTR
T-1
MoMLV
RATVL30
ATG
MDR1
RATVL30
MoMLV
SD
T-2
T-1
T-4
ATG
T-3
T-2
372bp
(C)
282bp
(B)

Figure 16. Specificity of the MDR1 cDNA primer pair A. This primer pair generates a 3673 base pair amplicon. The lanes are: 1. Empty, 2. Canine (Tyson), 3. Canine (Spike), 4. Positive control, 5. Feline (Andy), 6. Feline (Yenkee), 7. Negative control, 8. Empty.

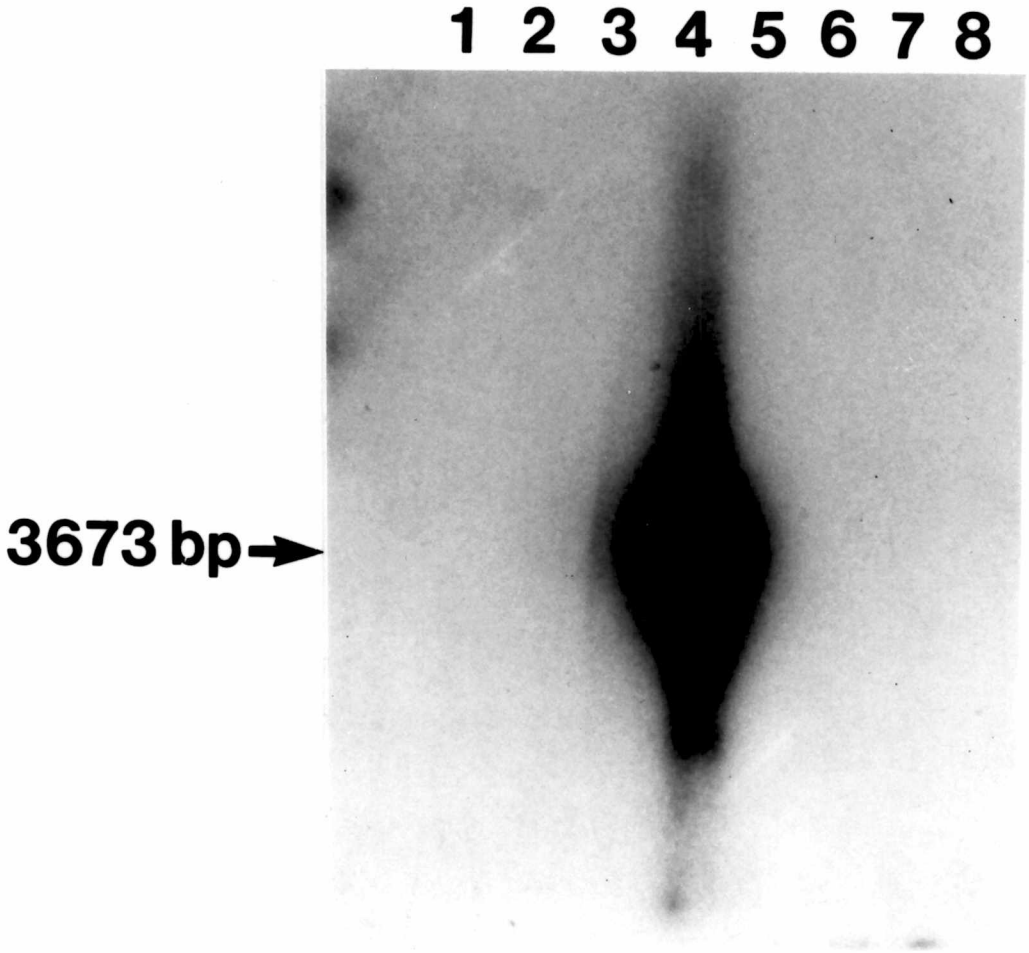

1 2 3 4 5 6 7 8
3673 bp

Figure 17. Specificity of the MDR1 cDNA primer pair B. This primer pair generates a 282 base pair amplicon. The lanes are: 1. Empty, 2. Empty, 3. Canine (Tyson), 4. Canine (Spike), 5. Positive control, 6. Feline (Andy), 7. Feline (Yenkee), 8. Negative control.

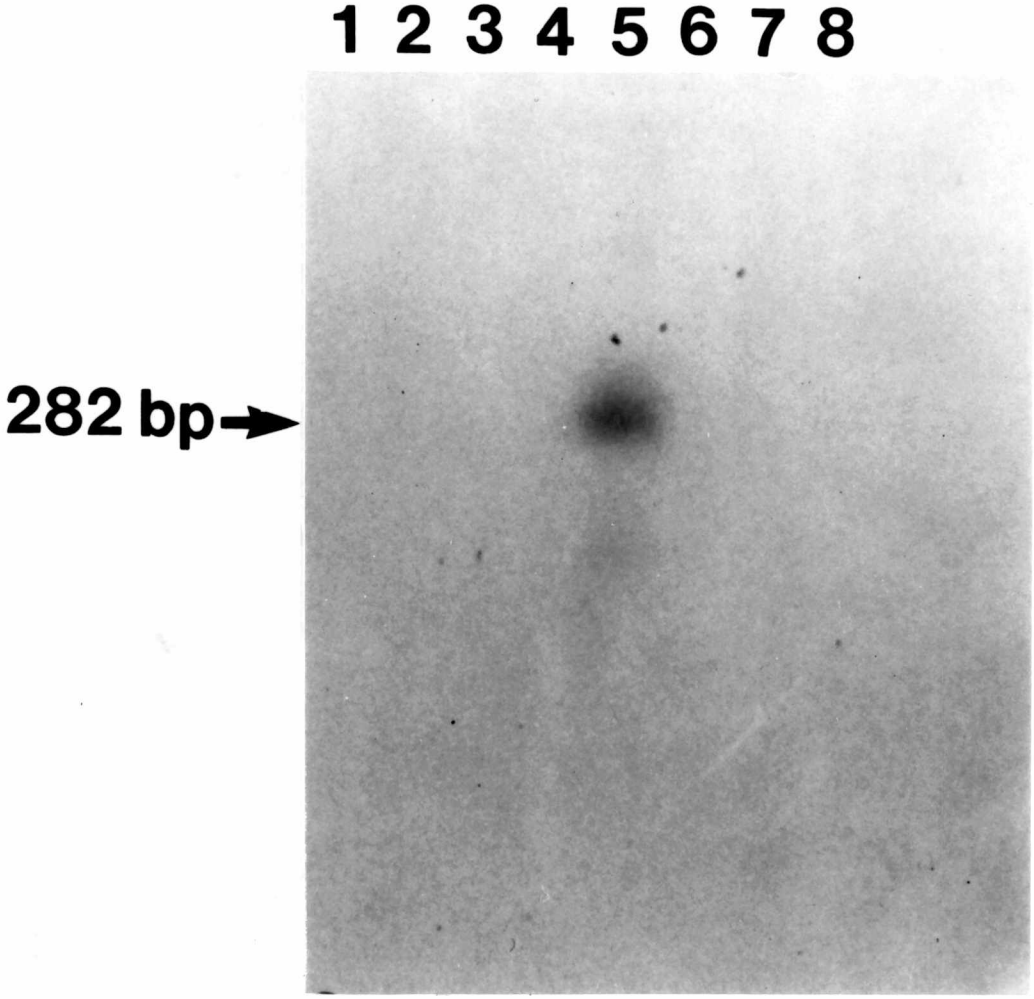

1 2 3 4 5 6 7 8
282 bp

Figure 18. Specificity of the MDR1 cDNA primer pair C. This primer pair generates a 372 base pair amplicon. The lanes are: 1. Canine (Tyson), 2. Canine (Spike), 3. Positive control, 4. Feline (Andy), 5. Feline (Yenkee), 6. Negative control, 7. Empty, 8. Empty.

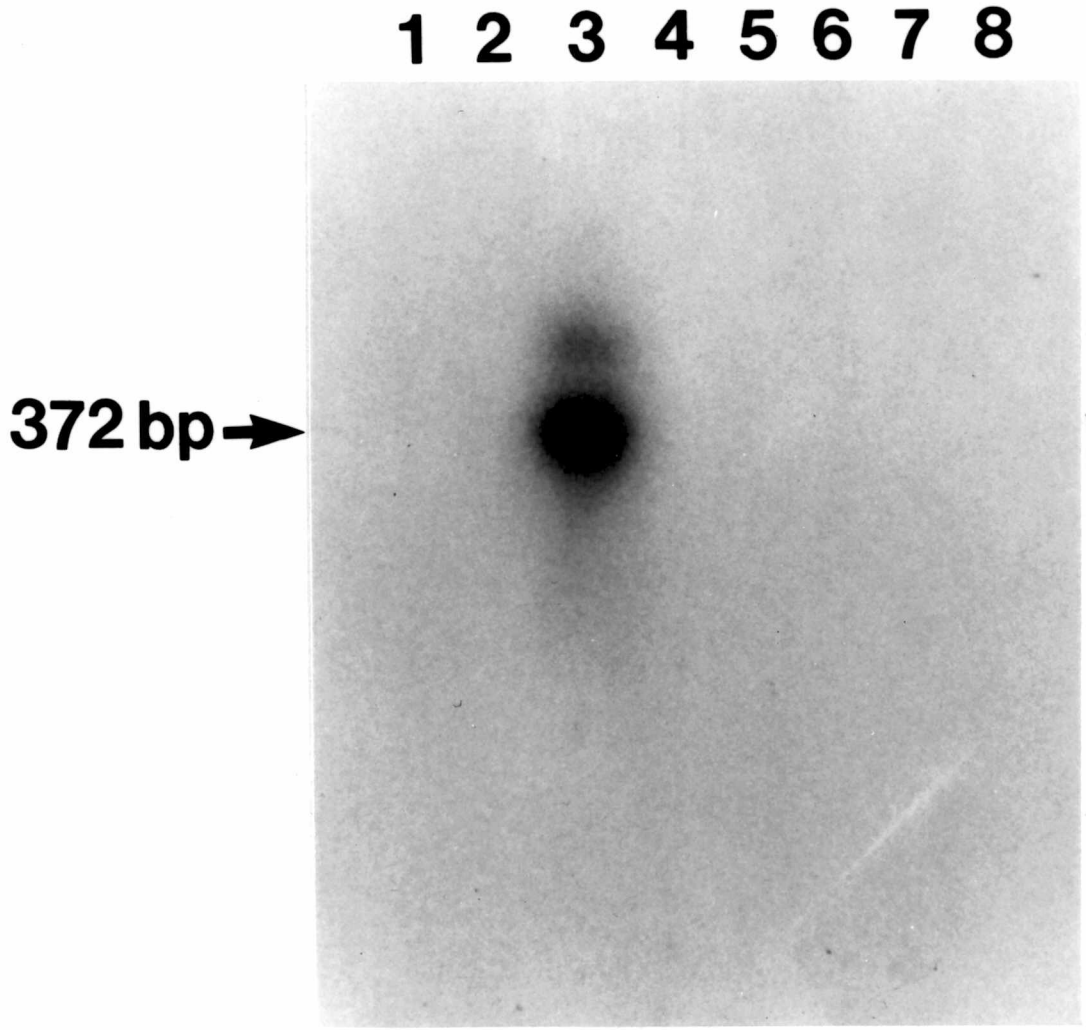

1 2 3 4 5 6 7 8
372 bp

Figure 19. Restriction enzyme analysis of pL and pLMDR1. The lanes are: 1. pL uncut, 2. pL Bam HI, 3. pL Nhe I, 4. pLMDR1 uncut, 5: pLMDR1 Bam HI, 6. pLMDR1 Hind III, 7. pLMDR1 Nhe I, 8. pLMDR1 Eco RI, 9. Lamda Hind III standards.

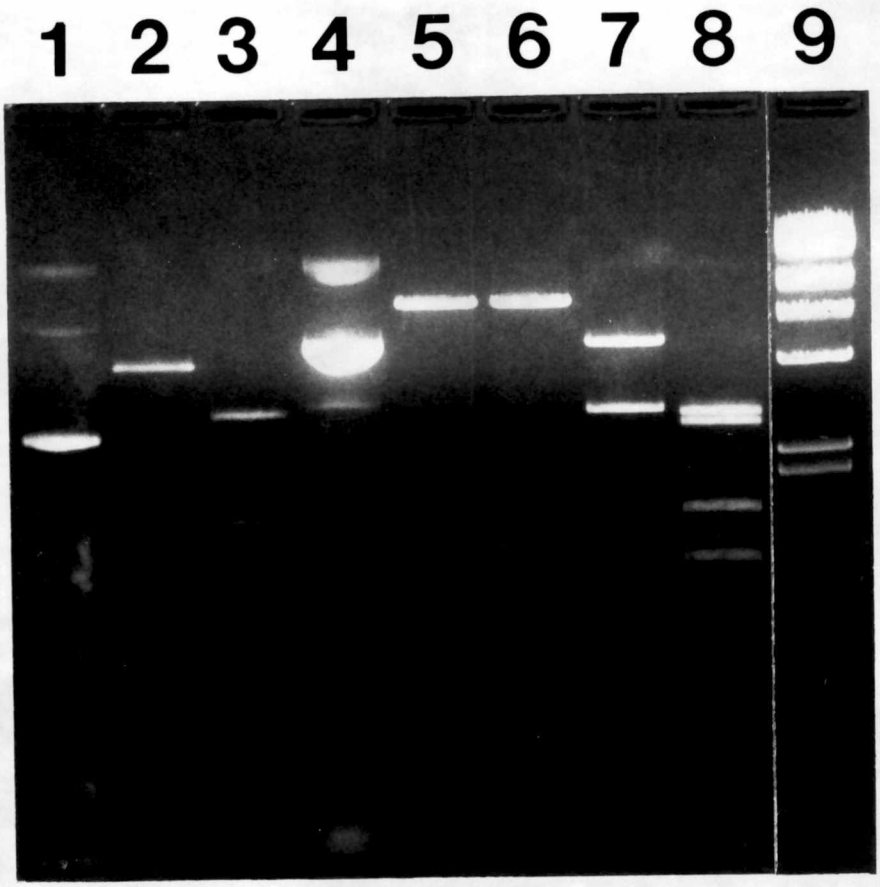

1 2 3 4 5 6 7 8 9

Figure 20. PCR analysis of pLMDR1. Analysis was performed utilizing MDR1 cDNA specific primer pair A. This primer pair amplifies a 3673 bp. region of the MDR1 cDNA. The lanes are: 1, Lamda Hind III standards, 2. pLMDR1, 3. MDR1-SP72, 4. (+) control pHaMDR1/A, 5. (-) control no DNA.

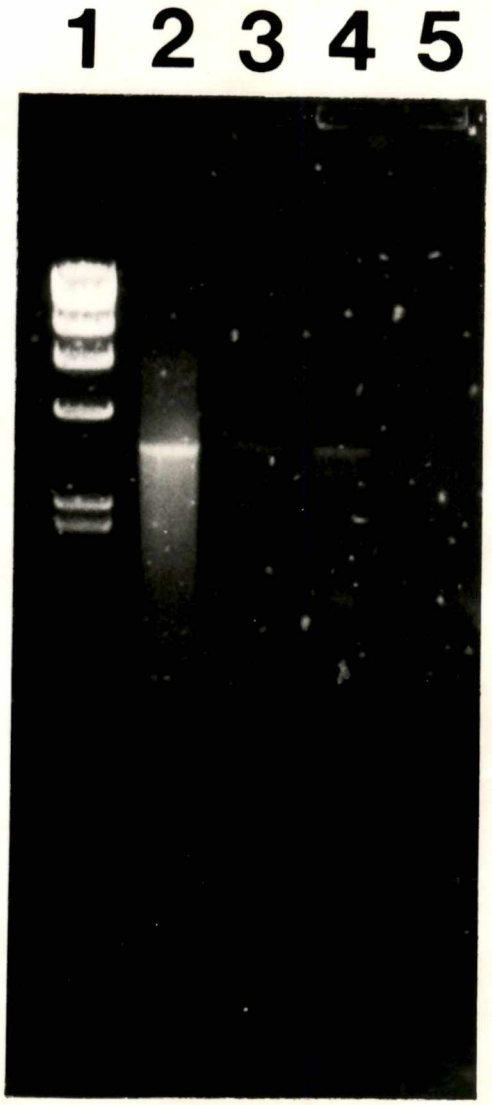
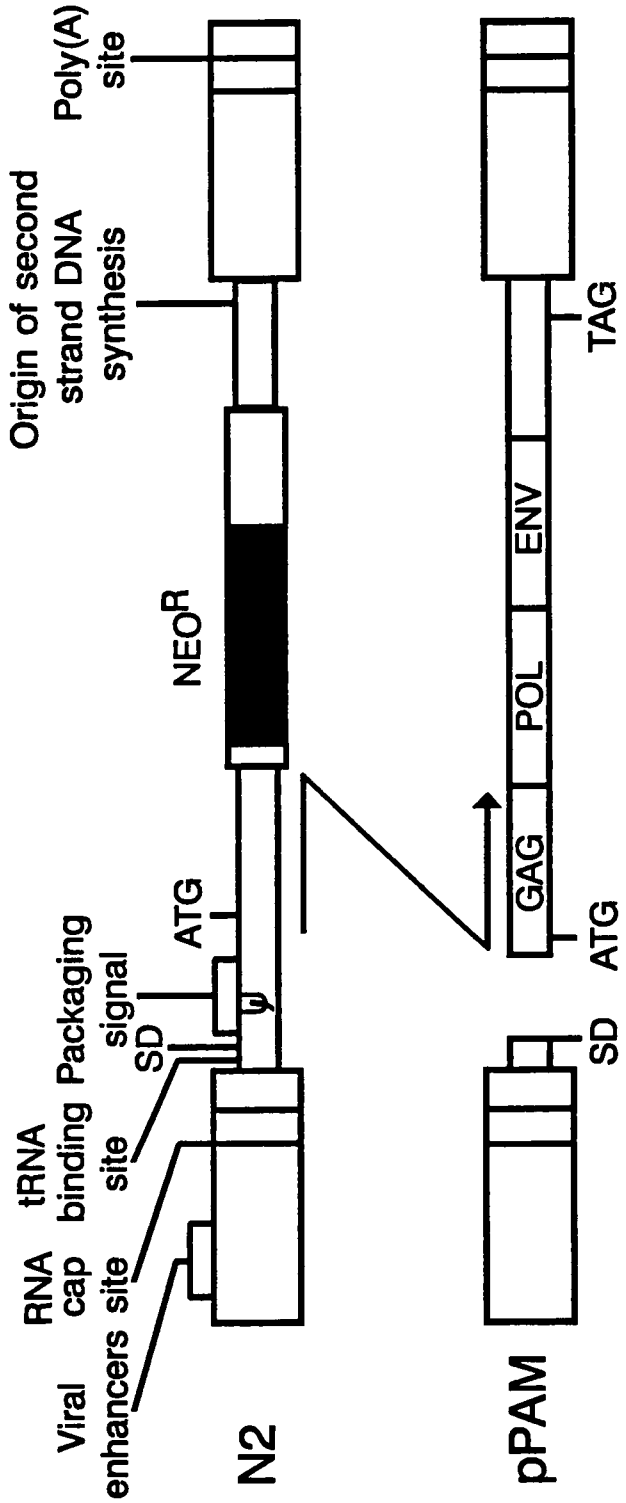

1 2 3 4 5

Figure 21. Generation of wild-type virus (Helper Virus). Recombination events between homologous regions of the retroviral vector N2 and the packaging construct pPAM present in PA12 cells leads to formation of helper virus.


Poly(A) site
Origin of second strand DNA synthesis
NEO^R
ATG
Packaging signal
SD
tRNA binding site
RNA cap site
Viral enhancers
N2
GAG
POL
ENV
TAG
ATG
SD
pPAM

APPENDIX C

===03-MAR-1992==========PC/GENE===

```
*******************************
* RESTRICTION-SITE ANALYSIS *
*******************************

Done on DNA sequence PLMDR1
Total number of bases is: 8634.
Analysis done on the complete sequence.

                    ------------------------------------

List of cuts by enzyme
======================

AatII     :   954  3431  8564
AccI      :  3409  3714  6521
AciI      :    68   117   127   297   419   540   902   933  1066  1107  1248
              1331  1397  1496  1515  1553  1604  1616  1626  1656  4004  4898
              5310  5334  5961  6263  6427  6466  6476  6518  6543  6581  6586
              6612  6629  6680  6687  6708  6791  6819  6946  6965  7086  7204
              7331  7348  7702  7793  7992  8030  8159  8195  8272  8381  8488
              8527
AcyI      :   951  3428  6129  8179  8561
Afa16RI   :  8011
AflII     :   177  1207  5715
AflIII    :  2266  6749
AhaIII    :  1692  7508  7527  8219
AluI      :   172   176   262   333   554   797   877   885  2059  2151  2280
              2326  2389  2401  2491  2515  2725  2771  2955  3094  3148  3397
              3562  3684  3747  3979  4550  4579  4700  4884  5166  5200  5254
              5418  5595  5625  5710  5714  5801  5876  6095  6338  6391  6410
              6691  6917  7007  7053  7310  7831  7931  7994
AlwNI     :   374  2329  2401  2930  4806  4836  4859  4887  5418  5421  5841
              5916  7165
ApaI      :  3228
ApaLI     :  2134  6565  7063  8309
Asp718I   :   619  4416  5617  6160
AsuI      :   124   312   343   388   403   416   471   769   899   969   982
              1186  1397  1420  1656  2591  2601  2874  2910  3017  3224  3225
              3380  4827  4878  5810  5855  5885  5930  5945  5958  6013  6310
              7684  7763  7780  8002  8618
AsuII     :  2776
AvaI      :   384   615  1630  5611  6090  6123  6156
AvaII     :   403   416   471   769   899  1420  1656  2591  2601  3017  3380
              4878  5945  5958  6013  6310  7780  8002
BalI      :   801  1312  2299  5177
BamHI     :  1624
BbeI      :  6132
BbvI      :  1112  1140  1797  2010  2068  2313  2428  2529  2734  3571  4092
              4666  4862  4910  5027  5095  5405  5427  6329  6400  6497  6641
              6659  7078  7168  7171  7377  7680  7869  8071
BbvII     :   587  1973  2070  3593  8620
BcefI     :   957  1357  3099  4586  7251
BcgI      :   677  8147
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BclI	:	4670										
BetI	:	107	4511	4782	6955	7102	7933					
BglI	:	7762										
BglII	:	1899	2864									
BinI	:	1253	1557	1609	1619	1632	1652	3262	3285	3611	3669	3865
		3981	4016	6296	7310	7396	7398	7494	7495	7958	8275	8279
Bme142I	:	5476	6130	6625	6995							
Bpu10I	:	315	418	1484	5858	5933	5960					
BsaAI	:	2269	3268	6503								
BsgI	:	2911										
BsiI	:	5195	6916	8300	8619							
BsiYI	:	161	164	309	310	384	385	386	413	582	664	685
		717	718	750	765	766	785	786	789	991	995	1340
		1417	1467	1468	1595	1636	1637	2314	2610	2982	2983	2984
		3300	3734	4011	4836	4887	4912	4913	5671	5697	5852	5853
		5927	5928	5955	6123	6124	6204	6225	6257	6258	6291	6306
		6307	6326	6327	6330	6400	6771	6789	6955	7234		
BsmI	:	5361										
BsmAI	:	321	673	694	748	942	1040	1151	1273	1332	1350	1530
		2181	5036	5144	5666	6213	6234	6289	6392	7703	8479	
Bsp120I	:	3224										
BspHI	:	1841	1952	3179	3474	3485	4544	4667	7469	8477	8582	
BspMI	:	4404	5326									
BsrI	:	589	1232	1256	2250	3226	3618	3719	4077	4790	5476	6130
		6494	6529	7157	7170	7280	7686	7804	7847	8118	8286	
BssHII	:	535	586	6076								
BstEII	:	1288										
BstNI	:	458	766	944	957	1370	1395	1418	1438	1468	1977	2053
		2914	3208	3274	4089	4134	4477	4813	4888	4978	4993	5293
		5461	6000	6307	6777	6898	6911					
BstXI	:	1828	3235	4602								
Bsu90I	:	1625										
CauII	:	304	380	616	617	745	787	932	5053	5847	5922	6157
		6158	6286	6328	6395	6430	7129	7825	8176			
Cfr10I	:	7722										
CfrI	:	799	933	1310	1331	2297	3111	4598	5175	8030		
ClaI	:	5636										
CviJI	:	67	113	119	125	172	176	262	307	314	333	345
		383	390	422	554	567	640	738	797	801	877	885
		935	971	983	1109	1188	1312	1333	1398	1445	1451	1477
		1495	1580	1776	1810	1828	2059	2123	2151	2280	2299	2326
		2389	2401	2491	2515	2524	2578	2665	2725	2757	2771	2876
		2911	2930	2955	2967	3094	3113	3148	3160	3226	3310	3319
		3355	3367	3397	3562	3670	3684	3700	3747	3765	3809	3922
		3979	4002	4092	4123	4137	4522	4550	4579	4600	4642	4657
		4700	4715	4811	4829	4840	4859	4884	4965	4998	5051	5095
		5166	5177	5200	5220	5245	5254	5290	5302	5312	5418	5464
		5595	5609	5625	5710	5714	5801	5812	5850	5857	5876	5887
		5925	5932	5964	6095	6108	6279	6338	6342	6391	6410	6443
		6488	6528	6672	6691	6764	6775	6793	6819	6917	7007	7053
		7058	7083	7162	7227	7238	7281	7310	7673	7685	7726	7752
		7756	7765	7831	7921	7931	7994	8032	8619			
CviQI	:	489	620	867	1484	2469	2632	3689	4234	4417	5618	6161
		6556	8121									
CviRI	:	195	654	1128	1304	1879	1885	2136	2383	2437	2563	2707

	2713	2892	2961	3265	3525	3559	3813	3826	4246	4413	5015
	5321	5359	5733	6188	6194	6388	6567	7065	7361	7696	7786
	7885	7979	8067	8311							
DdeI :	76	218	308	334	425	696	990	1219	1491	2180	2373
	2607	3685	4030	4224	4373	4403	4807	5024	5162	5756	5851
	5926	5967	6148	6236	6559	7024	7433	7599	8139	8565	
DpnI :	14	238	1247	1379	1564	1611	1616	1626	1646	1901	2557
	2866	3256	3292	3618	3639	3663	3872	3988	4023	4327	4672
	5074	5776	6290	7317	7392	7403	7411	7489	7501	7606	7947
	7965	8011	8269	8286	8322						
DraII :	471	1420	2874	3224	6013	8618					
DrdI :	6444	6857									
DsaI :	120	903	1619	1821	3128	3657	4595	4601			
Eam1105I :	1221	6205	6251	7642							
Ecl136II :	554	5625	6095								
Eco31I :	673	694	748	1350	6213	6234	6289	7703			
Eco57I :	2423	2820	2905	3940	4355	4783	7297	8309			
Eco78I :	6130										
EcoHI :	306	382	618	619	747	789	934	5055	5849	5924	6159
	6160	6288	6330	6397	6432	7131	7827	8178			
EcoNI :	993	1593	3732								
EcoRI :	1	1603	2818	5629							
EcoRII :	456	764	942	955	1368	1393	1416	1436	1466	1975	2051
	2912	3206	3272	4087	4132	4475	4811	4886	4976	4991	5291
	5459	5998	6305	6775	6896	6909					
EcoRV :	280	356	1872	1932	5823	5898					
Esp3I :	954	1052	1285	1344	1518	6404					
FauI :	56	1076	1407	1563	1592	1614	1626	6251	6454	6464	
Fnu4HI :	120	123	936	1110	1126	1129	1334	1811	2024	2057	2327
	2417	2543	2723	3560	4000	4106	4655	4851	4924	5016	5084
	5313	5416	5419	6343	6389	6486	6539	6655	6673	6676	6794
	6949	7092	7157	7160	7366	7694	7883	8033	8060	8155	8384
FnuDII :	63	122	535	537	588	1112	1114	1336	1621	6076	6078
	6350	6352	6455	6796	7377	7707	8200	8532			
FokI :	641	1341	1501	1653	1804	3000	3145	3316	3718	4051	4517
	4570	4774	4987	5251	5335	6181	6437	7608	7789	8076	
FsiI :	1	57	1081	1603	1890	2478	2811	2818	3196	3750	5629
GdiII :	927	939	1325	3105	4592	8024					
GsuI :	1981	2411	3704	4001	4094	4834	4909	5648	7712		
HaeI :	801	1312	2299	2578	3310	3367	3765	3809	4715	5095	5177
	6764	6775	7227								
HaeII :	5478	6132	6627	6997							
HaeIII :	125	314	345	390	801	935	971	983	1188	1312	1333
	1398	2299	2578	2876	2911	3113	3226	3310	3367	3765	3809
	4600	4715	4829	5095	5177	5312	5812	5857	5887	5932	6764
	6775	6793	7227	7685	7765	8032	8619				
HgaI :	598	850	1103	4842	6444	6860	7438	8168			
HgiAI :	556	2138	2944	4873	4974	5627	6097	6569	7067	8228	8313
HgiCI :	461	619	1343	2048	4416	4591	5617	6003	6128	6160	7590
HgiJII :	556	569	740	3228	5000	5627	6097	6110	6281		
HhaI :	537	539	588	590	857	1114	5477	6078	6080	6131	6352
	6455	6485	6626	6659	6929	6996	7096	7270	7379	7772	7865
	8202	8534									
HinP1I :	535	537	586	588	855	1112	5475	6076	6078	6129	6350
	6453	6483	6624	6657	6927	6994	7094	7268	7377	7770	7863

		8200	8532									
HindII	:	8183										
HindIII	:	3682										
HinfI	:	441	660	1000	1011	1956	3060	3452	3568	3610	4371	5116
		5390	6150	6649	6724	7120	7637					
HpaII	:	108	304	380	616	744	786	932	4512	4783	5052	5847
		5922	6157	6285	6327	6395	6429	6956	7103	7129	7319	7723
		7757	7824	7934	8176							
HphI	:	7	97	1357	1383	2212	3069	3117	3984	4464	4765	4830
		5326	6371	6380	7485	7712	8128	8334	8369			
KasI	:	6128										
KpnI	:	623	4420	5621	6164							
Ksp632I	:	1271	1512	2399	2773	2932	4861	5063	5570	6633	8437	
MaeI	:	173	436	832	870	882	1595	3919	4309	5711	5978	7244
		7497	7832									
MaeII	:	951	1282	1323	2268	3267	3313	3428	5248	6502	7452	7868
		8241	8561									
MaeIII	:	181	1195	1223	1288	1432	2200	2839	3123	3516	3895	4217
		4743	5060	5117	5719	6403	6498	7105	7168	7284	7567	7898
		7956	8109	8297								
MboI	:	12	236	1245	1377	1562	1609	1614	1624	1644	1899	2555
		2864	3254	3290	3616	3637	3661	3870	3986	4021	4325	4670
		5072	5774	6288	7315	7390	7401	7409	7487	7499	7604	7945
		7963	8009	8267	8284	8320						
MboII	:	587	1288	1499	1689	1692	1909	1941	1978	2075	2342	2416
		2497	2727	2790	2949	3593	4335	4833	4878	5080	5587	6620
		7411	7482	8237	8315	8424	8620					
McrI	:	936	4783	6665	7089	8012	8161					
MfeI	:	4229										
MlaAI	:	5612										
MlyI	:	994	3054	3446	4380	5110	5384	6144	6658	7114	7646	
MmeI	:	1039	1306	2254	2655	2898	3192	3358	6222	6964	7148	
MnlI	:	39	55	434	587	651	677	703	782	984	1082	1370
		1473	1506	1512	1515	1521	1551	1554	1569	1576	1592	1625
		1659	1847	1955	2399	2436	2614	2709	2956	2975	3200	3213
		3244	3299	3304	3689	3727	3728	3798	4000	4429	4547	4808
		5071	5084	5231	5570	5606	5620	5976	6128	6147	6192	6217
		6243	6323	6356	6386	6648	6856	6931	7180	7580	7661	7809
		8015	8608									
MseI	:	178	1208	1320	1350	1374	1691	2153	2186	2656	2804	2861
		3754	4115	5365	5504	5515	5549	5589	5597	5601	5605	5716
		6530	7455	7507	7512	7526	7579	7814	7853	8218	8590	
MstI	:	7864										
MwoI	:	119	298	374	551	2335	2521	2569	2719	2898	3316	4102
		4336	4712	4856	4890	5092	5251	5318	5406	5415	5841	5916
		6092	6573	6623	6688	6802	7374	7762	7882			
NarI	:	6129										
NcoI	:	1821	3128	4595								
NdeI	:	4394	6572									
NheI	:	172	5710									
NlaIII	:	204	1406	1825	1845	1956	1974	2101	3132	3183	3478	3489
		4014	4029	4548	4599	4671	5107	5404	5742	6388	6493	6753
		7473	7964	7974	8052	8088	8481	8586				
NlaIV	:	112	296	389	405	463	621	770	901	970	1102	1345
		1422	1626	1657	2050	2592	3068	3225	3226	4003	4131	4418

		4521	4593	4966	4997	5619	5947	6005	6130	6162	6311	6781
		6820	7592	7686	7727	7938	8528					
NsiI	:	2565										
NspBII	:	122	262	333	1621	2059	2326	2955	3562	4579	5418	5801
		5876	6258	6461	7091	7336	8277					
NspI	:	5107	6388	6753								
PacI	:	5601	5605									
PflKI	:	109	305	381	617	745	787	933	4513	4784	5053	5848
		5923	6158	6286	6328	6396	6430	6957	7104	7130	7320	7724
		7758	7825	7935	8177							
PflMI	:	4836	4887									
PleI	:	1008	3068	3460	4365	5124	5398	6158	6643	7128	7631	
PmaCI	:	2269	3268									
PpuMI	:	471	1420	6013								
PshAI	:	720										
PssI	:	474	1423	2877	3227	6016	8621					
PstI	:	1130	1306	5017	7887							
PvuI	:	8012										
PvuII	:	262	333	2059	2326	2955	3562	4579	5418	5801	5876	
RleAI	:	115	585	2922	2994	4675	4851	6126				
RsaI	:	490	621	868	1485	2470	2633	3690	4235	4418	5619	6162
		6557	8122									
SacI	:	556	5627	6097								
SacII	:	123	1622									
SapI	:	2932	4861	6633								
SauI	:	990	1219									
ScaI	:	2633	8122									
SciI	:	5613										
ScrFI	:	304	380	458	616	617	745	766	787	932	944	957
		1370	1395	1418	1438	1468	1977	2053	2914	3208	3274	4089
		4134	4477	4813	4888	4978	4993	5053	5293	5461	5847	5922
		6000	6157	6158	6286	6307	6328	6395	6430	6777	6898	6911
		7129	7825	8176								
SduI	:	466	556	569	740	2138	2789	2944	3228	3273	4873	4974
		5000	5208	5627	6008	6097	6110	6281	6569	7067	8228	8313
SecI	:	120	302	378	457	467	615	679	764	765	903	942
		943	955	956	1368	1437	1446	1466	1467	1619	1631	1821
		2597	2912	3128	3206	3272	3320	3590	3657	3924	4475	4595
		4601	4907	4977	4991	5291	5845	5920	5999	6009	6156	6219
		6305	6306	6909								
SfaNI	:	401	663	1140	1835	2572	2692	2948	3576	4092	4991	5418
		5444	5943	6203	6415	6550	6586	6626	6846	7898	8089	8338
SfeI	:	1126	1302	2534	3410	4124	4701	5013	5695	7014	7205	7883
SmaI	:	617	6158									
SpeI	:	869										
SsoII	:	302	378	456	614	615	743	764	785	930	942	955
		1368	1393	1416	1436	1466	1975	2051	2912	3206	3272	4087
		4132	4475	4811	4886	4976	4991	5051	5291	5459	5845	5920
		5998	6155	6156	6284	6305	6326	6393	6428	6775	6896	6909
		7127	7823	8174								
SspI	:	2529	2617	2802	3833	5519	8446					
StuI	:	3809	4715									
StyI	:	467	679	1446	1821	2597	3128	3320	3590	3924	4595	4907
		6009	6219									
TaqI	:	1003	1239	1550	1561	2776	2850	3443	5114	5612	5627	5636

	6849	8293									
TaqIIA :	1382	1425	4489	8328	8345						
TaqIIB :	6651	7990	8175								
Tth111I :	608	1020	1418	6149	6496						
Tth111II :	287	363	639	832	1843	1926	2706	3067	4301	4915	4996
	5572	5830	5905	7339	7346	7378					
VspI :	2186	2656	5601	7814							
XbaI :	5977										
XcaI :	3715	6522									
XcmI :	2985	3235	3318	3699	3728	5253					
XhoI :	5611										
XhoII :	1624	1644	1899	2864	3290	3616	3986	7390	7401	7487	7499
	8267	8284									
XmaI :	615	6156									
XmaIII :	933										
XmnI :	3101	3933	8241								

Total number of cuts is : 2000.

Sorted list of enzymes by number of cuts

CviJI : 162	Tth111II : 17	PssI : 6	XcaI : 2
MnlI : 68	HinfI : 17	BbvII : 5	StuI : 2
BsiYI : 64	HaeI : 14	AcyI : 5	SauI : 2
AciI : 56	MaeII : 13	BcefI : 5	BcgI : 2
AluI : 52	XhoII : 13	TaqIIA : 5	XmaI : 2
ScrFI : 47	AlwNI : 13	Tth111I : 5	PacI : 2
SsoII : 47	TaqI : 13	Asp718I : 4	DrdI : 2
SecI : 47	RsaI : 13	HaeII : 4	AflIII : 2
Fnu4HI : 44	StyI : 13	PstI : 4	PflMI : 2
HaeIII : 40	CviQI : 13	Eam1105I : 4	NheI : 2
NlaIV : 39	MaeI : 13	AhaIII : 4	BclI : 1
AsuI : 38	FsiI : 11	Bme142I : 4	HindIII : 1
DpnI : 38	SfeI : 11	ApaLI : 4	BglI : 1
MboI : 38	HgiCI : 11	VspI : 4	AsuII : 1
CviRI : 37	HgiAI : 11	EcoRI : 4	HindII : 1
DdeI : 32	PvuII : 10	BalI : 4	BsgI : 1
MseI : 32	MlyI : 10	BsiI : 4	NarI : 1
BbvI : 30	MmeI : 10	KpnI : 4	BamHI : 1
MwoI : 30	PleI : 10	EcoNI : 3	MstI : 1
NlaIII : 29	BspHI : 10	BstXI : 3	PvuI : 1
EcoRII : 28	Ksp632I : 10	Ecl136II : 3	Bsu90I : 1
BstNI : 28	FauI : 10	XmnI : 3	Eco78I : 1
MboII : 28	HgiJII : 9	TaqIIB : 3	MfeI : 1
PflKI : 26	GsuI : 9	AccI : 3	Bsp120I : 1
HpaII : 26	CfrI : 9	BsaAI : 3	ClaI : 1
MaeIII : 25	DsaI : 8	PpuMI : 3	BbeI : 1
HinP1I : 24	HgaI : 8	NcoI : 3	XhoI : 1
HhaI : 24	Eco57I : 8	SacI : 3	ApaI : 1
BinI : 22	Eco31I : 8	SapI : 3	Afa16RI : 1
SfaNI : 22	AvaI : 7	NspI : 3	NsiI : 1

SduI : 22	RleAI : 7	AflII : 3	SciI : 1
BsrI : 21	XcmI : 6	AatII : 3	BstEII : 1
FokI : 21	BetI : 6	BssHII : 3	Cfr10I : 1
BsmAI : 21	EcoRV : 6	BspMI : 2	XbaI : 1
CauII : 19	GdiII : 6	SmaI : 2	XmaIII : 1
EcoHI : 19	McrI : 6	ScaI : 2	MlaAI : 1
HphI : 19	Bpu10I : 6	PmaCI : 2	PshAI : 1
FnuDII : 19	Esp3I : 6	NdeI : 2	KasI : 1
AvaII : 18	DraII : 6	BglII : 2	SpeI : 1
NspBII : 17	SspI : 6	SacII : 2	BsmI : 1

List of non cutting selected enzymes
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AgeI , AvrII , BsaBI , BspMII , DraIII , Eco47III, Eco56I ,
EspI , FseI , HpaI , MluI , NaeI , NotI , NruI ,
RsrII , SalI , SfiI , SgrAI , SnaBI , SphI , SplI ,
Sse8387I, SwaI

Total number of selected enzymes which do not cut: 23

===03-MAR-1992==========PC/GENE===

===03-MAR-1992==========PC/GENE===

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      3        9       15       21       27       33       39       45
      |        |        |        |        |        |        |        |
  1 GAA TTC ATA CCA GAT CAC CGA AAA CTG TCC TCC AAA TGT GTC CCC
    CTT AAG TAT GGT CTA GTG GCT TTT GAC AGG AGG TTT ACA CAG GGG

 46 CTC ACA CTC CCA AAT TCG CGG GCT TCT GCT CTT AGA CCA CTC TAC
    GAG TGT GAG GGT TTA AGC GCC CGA AGA CGA GAA TCT GGT GAG ATG

 91 CCT ATT CCC CAC ACT CAC CGG AGC CAA AGC CGC GGC CCT TCC GTT
    GGA TAA GGG GTG TGA GTG GCC TCG GTT TCG GCG CCG GGA AGG CAA

136 TCT TTG CTT TTG AAA GAC CCC ACC CGT AGG TGG CAA GCT AGC TTA
    AGA AAC GAA AAC TTT CTG GGG TGG GCA TCC ACC GTT CGA TCG AAT

181 AGT AAC GCC ACT TTG CAA GGC ATG GAA AAA TAC ATA ACT GAG AAT
    TCA TTG CGG TGA AAC GTT CCG TAC CTT TTT ATG TAT TGA CTC TTA

226 AGG AAA GTT CAG ATC AAG GTC AGG AAC AAA GAA ACA GCT GAA TAC
    TCC TTT CAA GTC TAG TTC CAG TCC TTG TTT CTT TGT CGA CTT ATG

271 CAA ACA GGA TAT CTG TGG TAA GCG GTT CCT GCC CCG GCT CAG GGC
    GTT TGT CCT ATA GAC ACC ATT CGC CAA GGA CGG GGC CGA GTC CCG

316 CAA GAA CAG ATG AGA CAG CTG AGT GAT GGG CCA AAC AGG ATA TCT
    GTT CTT GTC TAC TCT GTC GAC TCA CTA CCC GGT TTG TCC TAT AGA

361 GTG GTA AGC AGT TCC TGC CCC GGC TCG GGG CCA AGA ACA GAT GGT
    CAC CAT TCG TCA AGG ACG GGG CCG AGC CCC GGT TCT TGT CTA CCA

406 CCC CAG ATG CGG TCC AGC CCT CAG CAG TTT CTA GTG AAT CAT CAG
    GGG GTC TAC GCC AGG TCG GGA GTC GTC AAA GAT CAC TTA GTA GTC

451 ATG TTT CCA GGG TGC CCC AAG GAC CTG AAA ATG ACC CTG TAC CTT
    TAC AAA GGT CCC ACG GGG TTC CTG GAC TTT TAC TGG GAC ATG GAA

496 ATT TGA ACT AAC CAA TCA GTT CGC TTC TCG CTT CTG TTC GCG CGC
    TAA ACT TGA TTG GTT AGT CAA GCG AAG AGC GAA GAC AAG CGC GCG

541 TTC CGC TCT CCG AGC TCA ATA AAA GAG CCC ACA ACC CCT CAC TCG
    AAG GCG AGA GGC TCG AGT TAT TTT CTC GGG TGT TGG GGA GTG AGC

586 GCG CGC CAG TCT TCC GAT AGA CTG CGT CGC CCG GGT ACC CGT ATT
    CGC GCG GTC AGA AGG CTA TCT GAC GCA GCG GGC CCA TGG GCA TAA

631 CCC AAT AAA GCC TCT TGC TGT TTG CAT CCG AAT CGT GGT CTC GCT
    GGG TTA TTT CGG AGA ACG ACA AAC GTA GGC TTA GCA CCA GAG CGA

676 GTT CCT TGG GAG GGT CTC CTC TGA GTG ATT GAC TAC CCA CGA CGG
    CAA GGA ACC CTC CCA GAG GAG ACT CAC TAA CTG ATG GGT GCT GCC

721 GGG TCT TTC ATT TGG GGG CTC GTC CGG GAT TTG GAG ACC CCT GCC
    CCC AGA AAG TAA ACC CCC GAG CAG GCC CTA AAC CTC TGG GGA CGG
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 766 CAG GGA CCA CCG ACC CAC CAC CGG GAG GTA AGC TGG CCA GCA ACT
     GTC CCT GGT GGC TGG GTG GTG GCC CTC CAT TCG ACC GGT CGT TGA

 811 TAT CTG TGT CTG TCC GAT TGT CTA GTG TCT ATG TTT GAT GTT ATG
     ATA GAC ACA GAC AGG CTA ACA GAT CAC AGA TAC AAA CTA CAA TAC

 856 CGC CTG CGT CTG TAC TAG TTA GCT AAC TAG CTC TGT ATC TGG CGG
     GCG GAC GCA GAC ATG ATC AAT CGA TTG ATC GAG ACA TAG ACC GCC

 901 ACC CGT GGT GGA ACT GAC GAG TTC TGA ACA CCC GGC CGC AAC CCT
     TGG GCA CCA CCT TGA CTG CTC AAG ACT TGT GGG CCG GCG TTG GGA

 946 GGG AGA CGT CCC AGG GAC TTT GGG GGC CGT TTT TGT GGC CCG ACC
     CCC TCT GCA GGG TCC CTG AAA CCC CCG GCA AAA ACA CCG GGC TGG

 991 TGA GGA AGG GAG TCG ATG TGG AAT CCG ACC CCG TCA GGA TAT GTG
     ACT CCT TCC CTC AGC TAC ACC TTA GGC TGG GGC AGT CCT ATA CAC

1036 GTT CTG GTA GGA GAC GAG AAC CTA AAA CAG TTC CCG CCT CCG TCT
     CAA GAC CAT CCT CTG CTC TTG GAT TTT GTC AAG GGC GGA GGC AGA

1081 GAA TTT TTG CTT TCG GTT TGG AAC CGA AGC CGC GCG TCT TGT CTG
     CTT AAA AAC GAA AGC CAA ACC TTG GCT TCG GCG CGC AGA ACA GAC

1126 CTG CAG CAT CGT TCT GTG TTG TCT CTG TCT GAC TGT GTT TCT GTA
     GAC GTC GTA GCA AGA CAC AAC AGA GAC AGA CTG ACA CAA AGA CAT

1171 TTT GTC TGA AAA TTA GGG CCA GAC TGT TAC CAC TCC CTT AAG TTT
     AAA CAG ACT TTT AAT CCC GGT CTG ACA ATG GTG AGG GAA TTC AAA

1216 GAC CTT AGG TCA CTG GAA AGA TGT CGA GCG GAT CGC TCA CAA CCA
     CTG GAA TCC AGT GAC CTT TCT ACA GCT CGC CTA GCG AGT GTT GGT

1261 GTC GGT AGA TGT CAA GAA GAG ACG TTG GGT TAC CTT CTG CTC TGC
     CAG CCA TCT ACA GTT CTT CTC TGC AAC CCA ATG GAA GAC GAG ACG

1306 AGA ATG GCC AAC CTT TAA CGT CGG ATG GCC GCG AGA CGG CAC CTT
     TCT TAC CGG TTG GAA ATT GCA GCC TAC CGG CGC TCT GCC GTG GAA

1351 TAA CCG AGA CCT CAT CAC CCA GGT TAA GAT CAA GGT CTT TTC ACC
     ATT GGC TCT GGA GTA GTG GGT CCA ATT CTA GTT CCA GAA AAG TGG

1396 TGG CCC GCA TGG ACA CCC AGA CCA GGT CCC CTA CAT CGT GAC CTG
     ACC GGG CGT ACC TGT GGG TCT GGT CCA GGG GAT GTA GCA CTG GAC

1441 GGA AGC CTT GGC TTT TGA CCC CCC TCC CTG GGT CAA GCC CTT TGT
     CCT TCG GAA CCG AAA ACT GGG GGG AGG GAC CCA GTT CGG GAA ACA

1486 ACA CCC TAA GCC TCC GCC TCC TCT TCC TCC ATC CGC CCC GTC TCT
     TGT GGG ATT CGG AGG CGG AGG AGA AGG AGG TAG GCG GGG CAG AGA

1531 CCC CCT TGA ACC TCC TCG TTC GAC CCC GCC TCG ATC CTC CCT TTA
     GGG GGA ACT TGG AGG AGC AAG CTG GGG CGG AGC TAG GAG GGA AAT
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1576 TCC AGC CCT CAC TCC TTC TCT AGG CGG GAA TTC CGA TCT GAT CCC
     AGG TCG GGA GTG AGG AAG AGA TCC GCC CTT AAG GCT AGA CTA GGG

1621 GCG GGA TCC CCC GAG GTC GGG ATG GAT CTT GAA GGG GAC CGC AAT
     CGC CCT AGG GGG CTC CAG CCC TAC CTA GAA CTT CCC CTG GCG TTA

1666 GGA GGA GCA AAG AAG AAG AAC TTT TTT AAA CTG AAC AAT AAA AGT
     CCT CCT CGT TTC TTC TTC TTG AAA AAA TTT GAC TTG TTA TTT TCA

1711 GAA AAA GAT AAG AAG GAA AAG AAA CCA ACT GTC AGT GTA TTT TCA
     CTT TTT CTA TTC TTC CTT TTC TTT GGT TGA CAG TCA CAT AAA AGT

1756 ATG TTT CGC TAT TCA AAT TGG CTT GAC AAG TTG TAT ATG GTG GTG
     TAC AAA GCG ATA AGT TTA ACC GAA CTG TTC AAC ATA TAC CAC CAC

1801 GGA ACT TTG GCT GCC ATC ATC CAT GGG GCT GGA CTT CCT CTC ATG
     CCT TGA AAC CGA CGG TAG TAG GTA CCC CGA CCT GAA GGA GAG TAC

1846 ATG CTG GTG TTT GGA GAA ATG ACA GAT ATC TTT GCA AAT GCA GGA
     TAC GAC CAC AAA CCT CTT TAC TGT CTA TAG AAA CGT TTA CGT CCT

1891 AAT TTA GAA GAT CTG ATG TCA AAC ATC ACT AAT AGA AGT GAT ATC
     TTA AAT CTT CTA GAC TAC AGT TTG TAG TGA TTA TCT TCA CTA TAG

1936 AAT GAT ACA GGG TTC TTC ATG AAT CTG GAG GAA GAC ATG ACC AGG
     TTA CTA TGT CCC AAG AAG TAC TTA GAC CTC CTT CTG TAC TGG TCC

1981 TAT GCC TAT TAT TAC AGT GGA ATT GGT GCT GGG GTG CTG GTT GCT
     ATA CGG ATA ATA ATG TCA CCT TAA CCA CGA CCC CAC GAC CAA CGA

2026 GCT TAC ATT CAG GTT TCA TTT TGG TGC CTG GCA GCT GGA AGA CAA
     CGA ATG TAA GTC CAA AGT AAA ACC ACG GAC CGT CGA CCT TCT GTT

2071 ATA CAC AAA ATT AGA AAA CAG TTT TTT CAT GCT ATA ATG CGA CAG
     TAT GTG TTT TAA TCT TTT GTC AAA AAA GTA CGA TAT TAC GCT GTC

2116 GAG ATA GGC TGG TTT GAT GTG CAC GAT GTT GGG GAG CTT AAC ACC
     CTC TAT CCG ACC AAA CTA CAC GTG CTA CAA CCC CTC GAA TTG TGG

2161 CGA CTT ACA GAT GAT GTC TCT AAG ATT AAT GAA GTT ATT GGT GAC
     GCT GAA TGT CTA CTA CAG AGA TTC TAA TTA CTT CAA TAA CCA CTG

2206 AAA ATT GGA ATG TTC TTT CAG TCA ATG GCA ACA TTT TTC ACT GGG
     TTT TAA CCT TAC AAG AAA GTC AGT TAC CGT TGT AAA AAG TGA CCC

2251 TTT ATA GTA GGA TTT ACA CGT GGT TGG AAG CTA ACC CTT GTG ATT
     AAA TAT CAT CCT AAA TGT GCA CCA ACC TTC GAT TGG GAA CAC TAA

2296 TTG GCC ATC AGT CCT GTT CTT GGA CTG TCA GCT GCT GTC TGG GCA
     AAC CGG TAG TCA GGA CAA GAA CCT GAC AGT CGA CGA CAG ACC CGT

2341 AAG ATA CTA TCT TCA TTT ACT GAT AAA GAA CTC TTA GCG TAT GCA
     TTC TAT GAT AGA AGT AAA TGA CTA TTT CTT GAG AAT CGC ATA CGT

2386 AAA GCT GGA GCA GTA GCT GAA GAG GTC TTG GCA GCA ATT AGA ACT
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     TTT CGA CCT CGT CAT CGA CTT CTC CAG AAC CGT CGT TAA TCT TGA

2431 GTG ATT GCA TTT GGA GGA CAA AAG AAA GAA CTT GAA AGG TAC AAC
     CAC TAA CGT AAA CCT CCT GTT TTC TTT CTT GAA CTT TCC ATG TTG

2476 AAA AAT TTA GAA GAA GCT AAA AGA ATT GGG ATA AAG AAA GCT ATT
     TTT TTA AAT CTT CTT CGA TTT TCT TAA CCC TAT TTC TTT CGA TAA

2521 ACA GCC AAT ATT TCT ATA GGT GCT GCT TTC CTG CTG ATC TAT GCA
     TGT CGG TTA TAA AGA TAT CCA CGA CGA AAG GAC GAC TAG ATA CGT

2566 TCT TAT GCT CTG GCC TTC TGG TAT GGG ACC ACC TTG GTC CTC TCA
     AGA ATA CGA GAC CGG AAG ACC ATA CCC TGG TGG AAC CAG GAG AGT

2611 GGG GAA TAT TCT ATT GGA CAA GTA CTC ACT GTA TTC TTT TCT GTA
     CCC CTT ATA AGA TAA CCT GTT CAT GAG TGA CAT AAG AAA AGA CAT

2656 TTA ATT GGG GCT TTT AGT GTT GGA CAG GCA TCT CCA AGC ATT GAA
     AAT TAA CCC CGA AAA TCA CAA CCT GTC CGT AGA GGT TCG TAA CTT

2701 GCA TTT GCA AAT GCA AGA GGA GCA GCT TAT GAA ATC TTC AAG ATA
     CGT AAA CGT TTA CGT TCT CCT CGT CGA ATA CTT TAG AAG TTC TAT

2746 ATT GAT AAT AAG CCA AGT ATT GAC AGC TAT TCG AAG AGT GGG CAC
     TAA CTA TTA TTC GGT TCA TAA CTG TCG ATA AGC TTC TCA CCC GTG

2791 AAA CCA GAT AAT ATT AAG GGA AAT TTG GAA TTC AGA AAT GTT CAC
     TTT GGT CTA TTA TAA TTC CCT TTA AAC CTT AAG TCT TTA CAA GTG

2836 TTC AGT TAC CCA TCT CGA AAA GAA GTT AAG ATC TTG AAG GGC CTG
     AAG TCA ATG GGT AGA GCT TTT CTT CAA TTC TAG AAC TTC CCG GAC

2881 AAC CTG AAG GTG CAG AGT GGG CAG ACG GTG GCC CTG GTT GGA AAC
     TTG GAC TTC CAC GTC TCA CCC GTC TGC CAC CGG GAC CAA CCT TTG

2926 AGT GGC TGT GGG AAG AGC ACA ACA GTC CAG CTG ATG CAG AGG CTC
     TCA CCG ACA CCC TTC TCG TGT TGT CAG GTC GAC TAC GTC TCC GAG

2971 TAT GAC CCC ACA GAG GGG ATG GTC AGT GTT GAT GGA CAG GAT ATT
     ATA CTG GGG TGT CTC CCC TAC CAG TCA CAA CTA CCT GTC CTA TAA

3016 AGG ACC ATA AAT GTA AGG TTT CTA CGG GAA ATC ATT GGT GTG GTG
     TCC TGG TAT TTA CAT TCC AAA GAT GCC CTT TAG TAA CCA CAC CAC

3061 AGT CAG GAA CCT GTA TTG TTT GCC ACC ACG ATA GCT GAA AAC ATT
     TCA GTC CTT GGA CAT AAC AAA CGG TGG TGC TAT CGA CTT TTG TAA

3106 CGC TAT GGC CGT GAA AAT GTC ACC ATG GAT GAG ATT GAG AAA GCT
     GCG ATA CCG GCA CTT TTA CAG TGG TAC CTA CTC TAA CTC TTT CGA

3151 GTC AAG GAA GCC AAT GCC TAT GAC TTT ATC ATG AAA CTG CCT CAT
     CAG TTC CTT CGG TTA CGG ATA CTG AAA TAG TAC TTT GAC GGA GTA

3196 AAA TTT GAC ACC CTG GTT GGA GAG AGA GGG GCC CAG TTG AGT GGT
     TTT AAA CTG TGG GAC CAA CCT CTC TCT CCC CGG GTC AAC TCA CCA
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3241 GGG CAG AAG CAG AGG ATC GCC ATT GCA CGT GCC CTG GTT CGC AAC
     CCC GTC TTC GTC TCC TAG CGG TAA CGT GCA CGG GAC CAA GCG TTG

3286 CCC AAG ATC CTC CTG CTG GAT GAG GCC ACG TCA GCC TTG GAC ACA
     GGG TTC TAG GAG GAC GAC CTA CTC CGG TGC AGT CGG AAC CTG TGT

3331 GAA AGC GAA GCA GTG GTT CAG GTG GCT CTG GAT AAG GCC AGA AAA
     CTT TCG CTT CGT CAC CAA GTC CAC CGA GAC CTA TTC CGG TCT TTT

3376 GGT CGG ACC ACC ATT GTG ATA GCT CAT CGT TTG TCT ACA GTT CGT
     CCA GCC TGG TGG TAA CAC TAT CGA GTA GCA AAC AGA TGT CAA GCA

3421 AAT GCT GAC GTC ATC GCT GGT TTC GAT GAT GGA GTC ATT GTG GAG
     TTA CGA CTG CAG TAG CGA CCA AAG CTA CTA CCT CAG TAA CAC CTC

3466 AAA GGA AAT CAT GAT GAA CTC ATG AAA GAG AAA GGC ATT TAC TTC
     TTT CCT TTA GTA CTA CTT GAG TAC TTT CTC TTT CCG TAA ATG AAG

3511 AAA CTT GTC ACA ATG CAG ACA GCA GGA AAT GAA GTT GAA TTA GAA
     TTT GAA CAG TGT TAC GTC TGT CGT CCT TTA CTT CAA CTT AAT CTT

3556 AAT GCA GCT GAT GAA TCC AAA AGT GAA ATT GAT GCC TTG GAA ATG
     TTA CGT CGA CTA CTT AGG TTT TCA CTT TAA CTA CGG AAC CTT TAC

3601 TCT TCA AAT GAT TCA AGA TCC AGT CTA ATA AGA AAA AGA TCA ACT
     AGA AGT TTA CTA AGT TCT AGG TCA GAT TAT TCT TTT TCT AGT TGA

3646 CGT AGG AGT GTC CGT GGA TCA CAA GCC CAA GAC AGA AAG CTT AGT
     GCA TCC TCA CAG GCA CCT AGT GTT CGG GTT CTG TCT TTC GAA TCA

3691 ACC AAA GAG GCT CTG GAT GAA AGT ATA CCT CCA GTT TCC TTT TGG
     TGG TTT CTC CGA GAC CTA CTT TCA TAT GGA GGT CAA AGG AAA ACC

3736 AGG ATT ATG AAG CTA AAT TTA ACT GAA TGG CCT TAT TTT GTT GTT
     TCC TAA TAC TTC GAT TTA AAT TGA CTT ACC GGA ATA AAA CAA CAA

3781 GGT GTA TTT TGT GCC ATT ATA AAT GGA GGC CTG CAA CCA GCA TTT
     CCA CAT AAA ACA CGG TAA TAT TTA CCT CCG GAC GTT GGT CGT AAA

3826 GCA ATA ATA TTT TCA AAG ATT ATA GGG GTT TTT ACA AGA ATT GAT
     CGT TAT TAT AAA AGT TTC TAA TAT CCC CAA AAA TGT TCT TAA CTA

3871 GAT CCT GAA ACA AAA CGA CAG AAT AGT AAC TTG TTT TCA CTA TTG
     CTA GGA CTT TGT TTT GCT GTC TTA TCA TTG AAC AAA AGT GAT AAC

3916 TTT CTA GCC CTT GGA ATT ATT TCT TTT ATT ACA TTT TTC CTT CAG
     AAA GAT CGG GAA CCT TAA TAA AGA AAA TAA TGT AAA AAG GAA GTC

3961 GGT TTC ACA TTT GGC AAA GCT GGA GAG ATC CTC ACC AAG CGG CTC
     CCA AAG TGT AAA CCG TTT CGA CCT CTC TAG GAG TGG TTC GCC GAG

4006 CGA TAC ATG GTT TTC CGA TCC ATG CTC AGA CAG GAT GTG AGT TGG
     GCT ATG TAC CAA AAG GCT AGG TAC GAG TCT GTC CTA CAC TCA ACC
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4051 TTT GAT GAC CCT AAA AAC ACC ACT GGA GCA TTG ACT ACC AGG CTC
     AAA CTA CTG GGA TTT TTG TGG TGA CCT CGT AAC TGA TGG TCC GAG

4096 GCC AAT GAT GCT GCT CAA GTT AAA GGG GCT ATA GGT TCC AGG CTT
     CGG TTA CTA CGA CGA GTT CAA TTT CCC CGA TAT CCA AGG TCC GAA

4141 GCT GTA ATT ACC CAG AAT ATA GCA AAT CTT GGG ACA GGA ATA ATT
     CGA CAT TAA TGG GTC TTA TAT CGT TTA GAA CCC TGT CCT TAT TAA

4186 ATA TCC TTC ATC TAT GGT TGG CAA CTA ACA CTG TTA CTC TTA GCA
     TAT AGG AAG TAG ATA CCA ACC GTT GAT TGT GAC AAT GAG AAT CGT

4231 ATT GTA CCC ATC ATT GCA ATA GCA GGA GTT GTT GAA ATG AAA ATG
     TAA CAT GGG TAG TAA CGT TAT CGT CCT CAA CAA CTT TAC TTT TAC

4276 TTG TCT GGA CAA GCA CTG AAA GAT AAG AAA GAA CTA GAA GGT GCT
     AAC AGA CCT GTT CGT GAC TTT CTA TTC TTT CTT GAT CTT CCA CGA

4321 GGG AAG ATC GCT ACT GAA GCA ATA GAA AAC TTC CGA ACC GTT GTT
     CCC TTC TAG CGA TGA CTT CGT TAT CTT TTG AAG GCT TGG CAA CAA

4366 TCT TTG ACT CAG GAG CAG AAG TTT GAA CAT ATG TAT GCT CAG AGT
     AGA AAC TGA GTC CTC GTC TTC AAA CTT GTA TAC ATA CGA GTC TCA

4411 TTG CAG GTA CCA TAC AGA AAC TCT TTG AGG AAA GCA CAC ATC TTT
     AAC GTC CAT GGT ATG TCT TTG AGA AAC TCC TTT CGT GTG TAG AAA

4456 GGA ATT ACA TTT TCC TTC ACC CAG GCA ATG ATG TAT TTT TCC TAT
     CCT TAA TGT AAA AGG AAG TGG GTC CGT TAC TAC ATA AAA AGG ATA

4501 GCT GGA TGT TTC CGG TTT GGA GCC TAC TTG GTG GCA CAT AAA CTC
     CGA CCT ACA AAG GCC AAA CCT CGG ATG AAC CAC CGT GTA TTT GAG

4546 ATG AGC TTT GAG GAT GTT CTG TTA GTA TTT TCA GCT GTT GTC TTT
     TAC TCG AAA CTC CTA CAA GAC AAT CAT AAA AGT CGA CAA CAG AAA

4591 GGT GCC ATG GCC GTG GGG CAA GTC AGT TCA TTT GCT CCT GAC TAT
     CCA CGG TAC CGG CAC CCC GTT CAG TCA AGT AAA CGA GGA CTG ATA

4636 GCC AAA GCC AAA ATA TCA GCA GCC CAC ATC ATC ATG ATC ATT GAA
     CGG TTT CGG TTT TAT AGT CGT CGG GTG TAG TAG TAC TAG TAA CTT

4681 AAA ACC CCT TTG ATT GAC AGC TAC AGC ACG GAA GGC CTA ATG CCG
     TTT TGG GGA AAC TAA CTG TCG ATG TCG TGC CTT CCG GAT TAC GGC

4726 AAC ACA TTG GAA GGA AAT GTC ACA TTT GGT GAA GTT GTA TTC AAC
     TTG TGT AAC CTT CCT TTA CAG TGT AAA CCA CTT CAA CAT AAG TTG

4771 TAT CCC ACC CGA CCG GAC ATC CCA GTG CTT CAG GGA CTG AGC CTG
     ATA GGG TGG GCT GGC CTG TAG GGT CAC GAA GTC CCT GAC TCG GAC

4816 GAG GTG AAG AAG GGC CAG ACG CTG GCT CTG GTG GGC AGC AGT GGC
     CTC CAC TTC TTC CCG GTC TGC GAC CGA GAC CAC CCG TCG TCA CCG

4861 TGT GGG AAG AGC ACA GTG GTC CAG CTC CTG GAG CGG TTC TAC GAC
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     ACA CCC TTC TCG TGT CAC CAG GTC GAG GAC CTC GCC AAG ATG CTG

4906 CCC TTG GCA GGG AAA GTG CTG CTT GAT GGC AAA GAA ATA AAG CGA
     GGG AAC CGT CCC TTT CAC GAC GAA CTA CCG TTT CTT TAT TTC GCT

4951 CTG AAT GTT CAG TGG CTC CGA GCA CAC CTG GGC ATC GTG TCC CAG
     GAC TTA CAA GTC ACC GAG GCT CGT GTG GAC CCG TAG CAC AGG GTC

4996 GAG CCC ATC CTG TTT GAC TGC AGC ATT GCT GAG AAC ATT GCC TAT
     CTC GGG TAG GAC AAA CTG ACG TCG TAA CGA CTC TTG TAA CGG ATA

5041 GGA GAC AAC AGC CGG GTG GTG TCA CAG GAA GAG ATC GTG AGG GCA
     CCT CTG TTG TCG GCC CAC CAC AGT GTC CTT CTC TAG CAC TCC CGT

5086 GCA AAG GAG GCC AAC ATA CAT GCC TTC ATC GAG TCA CTG CCT AAT
     CGT TTC CTC CGG TTG TAT GTA CGG AAG TAG CTC AGT GAC GGA TTA

5131 AAA TAT AGC ACT AAA GTA GGA GAC AAA GGA ACT CAG CTC TCT GGT
     TTT ATA TCG TGA TTT CAT CCT CTG TTT CCT TGA GTC GAG AGA CCA

5176 GGC CAG AAA CAA CGC ATT GCC ATA GCT CGT GCC CTT GTT AGA CAG
     CCG GTC TTT GTT GCG TAA CGG TAT CGA GCA CGG GAA CAA TCT GTC

5221 CCT CAT ATT TTG CTT TTG GAT GAA GCC ACG TCA GCT CTG GAT ACA
     GGA GTA TAA AAC GAA AAC CTA CTT CGG TGC AGT CGA GAC CTA TGT

5266 GAA AGT GAA AAG GTT GTC CAA GAA GCC CTG GAC AAA GCC AGA GAA
     CTT TCA CTT TTC CAA CAG GTT CTT CGG GAC CTG TTT CGG TCT CTT

5311 GGC CGC ACC TGC ATT GTG ATT GCT CAC CGC CTG TCC ACC ATC CAG
     CCG GCG TGG ACG TAA CAC TAA CGA GTG GCG GAC AGG TGG TAG GTC

5356 AAT GCA GAC TTA ATA GTG GTG TTT CAG AAT GGC AGA GTC AAG GAG
     TTA CGT CTG AAT TAT CAC CAC AAA GTC TTA CCG TCT CAG TTC CTC

5401 CAT GGC ACG CAT CAG CAG CTG CTG GCA CAG AAA GGC ATC TAT TTT
     GTA CCG TGC GTA GTC GTC GAC GAC CGT GTC TTT CCG TAG ATA AAA

5446 TCA ATG GTC AGT GTC CAG GCT GGA ACA AAG CGC CAG TGA ACT CTG
     AGT TAC CAG TCA CAG GTC CGA CCT TGT TTC GCG GTC ACT TGA GAC

5491 ACT GTA TGA GAT GTT AAA TAC TTT TTA ATA TTT GTT TAG ATA TGA
     TGA CAT ACT CTA CAA TTT ATG AAA AAT TAT AAA CAA ATC TAT ACT

5536 CAT TTA TTC AAA GTT AAA AGC AAA CAC TTA CAG AAT TAT GAA GAG
     GTA AAT AAG TTT CAA TTT TCG TTT GTG AAT GTC TTA ATA CTT CTC

5581 GTA TCT GTT TAA CAG CTT AAT TAA TTA AGC CTC GAG GGT ACC GAG
     CAT AGA CAA ATT GTC GAA TTA ATT AAT TCG GAG CTC CCA TGG CTC

5626 CTC GAA TTC ATC GAT AAA ATA AAA GAT TTT ATT TAG TCT CCA GAA
     GAG CTT AAG TAG CTA TTT TAT TTT CTA AAA TAA ATC AGA GGT CTT

5671 AAA GGG GGG AAT GAA AGA CCC CAC CTG TAG GTT TGG CAA GCT AGC
     TTT CCC CCC TTA CTT TCT GGG GTG GAC ATC CAA ACC GTT CGA TCG
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5716 TTA AGT AAC GCC ATT TTG CAA GGC ATG GAA AAA TAC ATA ACT GAG
     AAT TCA TTG CGG TAA AAC GTT CCG TAC CTT TTT ATG TAT TGA CTC

5761 AAT AGA GAA GTT CAG ATC AAG GTC AGG AAC AGA TGG AAC AGC TGA
     TTA TCT CTT CAA GTC TAG TTC CAG TCC TTG TCT ACC TTG TCG ACT

5806 ATA TGG GCC AAA CAG GAT ATC TGT GGT AAG CAG TTC CTG CCC CGG
     TAT ACC CGG TTT GTC CTA TAG ACA CCA TTC GTC AAG GAC GGG GCC

5851 CTC AGG GCC AAG AAC AGA TGG AAC AGC TGA ATA TGG GCC AAA CAG
     GAG TCC CGG TTC TTG TCT ACC TTG TCG ACT TAT ACC CGG TTT GTC

5896 GAT ATC TGT GGT AAG CAG TTC CTG CCC CGG CTC AGG GCC AAG AAC
     CTA TAG ACA CCA TTC GTC AAG GAC GGG GCC GAG TCC CGG TTC TTG

5941 AGA TGG TCC CCA GAT GCG GTC CAG CCC TCA GCA GTT TCT AGA GAA
     TCT ACC AGG GGT CTA CGC CAG GTC GGG AGT CGT CAA AGA TCT CTT

5986 CCA TCA GAT GTT TCC AGG GTG CCC CAA GGA CCT GAA ATG ACC CTG
     GGT AGT CTA CAA AGG TCC CAC GGG GTT CCT GGA CTT TAC TGG GAC

6031 TGC CTT ATT TGA ACT AAC CAA TCA GTT CGC TTC TCG CTT CTG TTC
     ACG GAA TAA ACT TGA TTG GTT AGT CAA GCG AAG AGC GAA GAC AAG

6076 GCG CGC TTC TGC TCC CCG AGC TCA ATA AAA GAG CCC ACA ACC CCT
     CGC GCG AAG ACG AGG GGC TCG AGT TAT TTT CTC GGG TGT TGG GGA

6121 CAC TCG GGG CGC CAG TCC TCC GAT TGA CTG AGT CGC CCG GGT ACC
     GTG AGC CCC GCG GTC AGG AGG CTA ACT GAC TCA GCG GGC CCA TGG

6166 CGT GTA TCC AAT AAA CCC TCT TGC AGT TGC ATC CGA CTT GTG GTC
     GCA CAT AGG TTA TTT GGG AGA ACG TCA ACG TAG GCT GAA CAC CAG

6211 TCG CTG TTC CTT GGG AGG GTC TCC TCT GAG TGA TTG ACT ACC CGT
     AGC GAC AAG GAA CCC TCC CAG AGG AGA CTC ACT AAC TGA TGG GCA

6256 CAG CGG GGG TCT TTC ATT TGG GGG CTC GTC CGG GAT CGG GAG ACC
     GTC GCC CCC AGA AAG TAA ACC CCC GAG CAG GCC CTA GCC CTC TGG

6301 CCT GCC CAG GGA CCA CCG ACC CAC CAC CGG GAG GTA AGC TGG CTG
     GGA CGG GTC CCT GGT GGC TGG GTG GTG GCC CTC CAT TCG ACC GAC

6346 CCT CGC GCG TTT CGG TGA TGA CGG TGA AAA CCT CTG ACA CAT GCA
     GGA GCG CGC AAA GCC ACT ACT GCC ACT TTT GGA GAC TGT GTA CGT

6391 GCT CCC GGA GAC GGT CAC AGC TTG TCT GTA AGC GGA TGC CGG GAG
     CGA GGG CCT CTG CCA GTG TCG AAC AGA CAT TCG CCT ACG GCC CTC

6436 CAG ACA AGC CCG TCA GGG CGC GTC AGC GGG TGT TGG CGG GTG TCG
     GTC TGT TCG GGC AGT CCC GCG CAG TCG CCC ACA ACC GCC CAC AGC

6481 GGG CGC AGC CAT GAC CCA GTC ACG TAG CGA TAG CGG AGT GTA TAC
     CCC GCG TCG GTA CTG GGT CAG TGC ATC GCT ATC GCC TCA CAT ATG
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6526 TGG CTT AAC TAT GCG GCA TCA GAG CAG ATT GTA CTG AGA GTG CAC
     ACC GAA TTG ATA CGC CGT AGT CTC GTC TAA CAT GAC TCT CAC GTG

6571 CAT ATG CGG TGT GAA ATA CCG CAC AGA TGC GTA AGG AGA AAA TAC
     GTA TAC GCC ACA CTT TAT GGC GTG TCT ACG CAT TCC TCT TTT ATG

6616 CGC ATC AGG CGC TCT TCC GCT TCC TCG CTC ACT GAC TCG CTG CGC
     GCG TAG TCC GCG AGA AGG CGA AGG AGC GAG TGA CTG AGC GAC GCG

6661 TCG GTC GTT CGG CTG CGG CGA GCG GTA TCA GCT CAC TCA AAG GCG
     AGC CAG CAA GCC GAC GCC GCT CGC CAT AGT CGA GTG AGT TTC CGC

6706 GTA ATA CGG TTA TCC ACA GAA TCA GGG GAT AAC GCA GGA AAG AAC
     CAT TAT GCC AAT AGG TGT CTT AGT CCC CTA TTG CGT CCT TTC TTG

6751 ATG TGA GCA AAA GGC CAG CAA AAG GCC AGG AAC CGT AAA AAG GCC
     TAC ACT CGT TTT CCG GTC GTT TTC CGG TCC TTG GCA TTT TTC CGG

6796 GCG TTG CTG GCG TTT TTC CAT AGG CTC CGC CCC CCT GAC GAG CAT
     CGC AAC GAC CGC AAA AAG GTA TCC GAG GCG GGG GGA CTG CTC GTA

6841 CAC AAA AAT CGA CGC TCA AGT CAG AGG TGG CGA AAC CCG ACA GGA
     GTG TTT TTA GCT GCG AGT TCA GTC TCC ACC GCT TTG GGC TGT CCT

6886 CTA TAA AGA TAC CAG GCG TTT CCC CCT GGA AGC TCC CTC GTG CGC
     GAT ATT TCT ATG GTC CGC AAA GGG GGA CCT TCG AGG GAG CAC GCG

6931 TCT CCT GTT CCG ACC CTG CCG CTT ACC GGA TAC CTG TCC GCC TTT
     AGA GGA CAA GGC TGG GAC GGC GAA TGG CCT ATG GAC AGG CGG AAA

6976 CTC CCT TCG GGA AGC GTG GCG CTT TCT CAT AGC TCA CGC TGT AGG
     GAG GGA AGC CCT TCG CAC CGC GAA AGA GTA TCG AGT GCG ACA TCC

7021 TAT CTC AGT TCG GTG TAG GTC GTT CGC TCC AAG CTG GGC TGT GTG
     ATA GAG TCA AGC CAC ATC CAG CAA GCG AGG TTC GAC CCG ACA CAC

7066 CAC GAA CCC CCC GTT CAG CCC GAC CGC TGC GCC TTA TCC GGT AAC
     GTG CTT GGG GGG CAA GTC GGG CTG GCG ACG CGG AAT AGG CCA TTG

7111 TAT CGT CTT GAG TCC AAC CCG GTA AGA CAC GAC TTA TCG CCA CTG
     ATA GCA GAA CTC AGG TTG GGC CAT TCT GTG CTG AAT AGC GGT GAC

7156 GCA GCA GCC ACT GGT AAC AGG ATT AGC AGA GCG AGG TAT GTA GGC
     CGT CGT CGG TGA CCA TTG TCC TAA TCG TCT CGC TCC ATA CAT CCG

7201 GGT GCT ACA GAG TTC TTG AAG TGG TGG CCT AAC TAC GGC TAC ACT
     CCA CGA TGT CTC AAG AAC TTC ACC ACC GGA TTG ATG CCG ATG TGA

7246 AGA AGG ACA GTA TTT GGT ATC TGC GCT CTG CTG AAG CCA GTT ACC
     TCT TCC TGT CAT AAA CCA TAG ACG CGA GAC GAC TTC GGT CAA TGG

7291 TTC GGA AAA AGA GTT GGT AGC TCT TGA TCC GGC AAA CAA ACC ACC
     AAG CCT TTT TCT CAA CCA TCG AGA ACT AGG CCG TTT GTT TGG TGG

7336 GCT GGT AGC GGT GGT TTT TTT GTT TGC AAG CAG CAG ATT ACG CGC
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     CGA CCA TCG CCA CCA AAA AAA CAA ACG TTC GTC GTC TAA TGC GCG

7381 AGA AAA AAA GGA TCT CAA GAA GAT CCT TTG ATC TTT TCT ACG GGG
     TCT TTT TTT CCT AGA GTT CTT CTA GGA AAC TAG AAA AGA TGC CCC

7426 TCT GAC GCT CAG TGG AAC GAA AAC TCA CGT TAA GGG ATT TTG GTC
     AGA CTG CGA GTC ACC TTG CTT TTG AGT GCA ATT CCC TAA AAC CAG

7471 ATG AGA TTA TCA AAA AGG ATC TTC ACC TAG ATC CTT TTA AAT TAA
     TAC TCT AAT AGT TTT TCC TAG AAG TGG ATC TAG GAA AAT TTA ATT

7516 AAA TGA AGT TTT AAA TCA ATC TAA AGT ATA TAT GAG TAA ACT TGG
     TTT ACT TCA AAA TTT AGT TAG ATT TCA TAT ATA CTC ATT TGA ACC

7561 TCT GAC AGT TAC CAA TGC TTA ATC AGT GAG GCA CCT ATC TCA GCG
     AGA CTG TCA ATG GTT ACG AAT TAG TCA CTC CGT GGA TAG AGT CGC

7606 ATC TGT CTA TTT CGT TCA TCC ATA GTT GCC TGA CTC CCC GTC GTG
     TAG ACA GAT AAA GCA AGT AGG TAT CAA CGG ACT GAG GGG CAG CAC

7651 TAG ATA ACT ACG ATA CGG GAG GGC TTA CCA TCT GGC CCC AGT GCT
     ATC TAT TGA TGC TAT GCC CTC CCG AAT GGT AGA CCG GGG TCA CGA

7696 GCA ATG ATA CCG CGA GAC CCA CGC TCA CCG GCT CCA GAT TTA TCA
     CGT TAC TAT GGC GCT CTG GGT GCG AGT GGC CGA GGT CTA AAT AGT

7741 GCA ATA AAC CAG CCA GCC GGA AGG GCC GAG CGC AGA AGT GGT CCT
     CGT TAT TTG GTC GGT CGG CCT TCC CGG CTC GCG TCT TCA CCA GGA

7786 GCA ACT TTA TCC GCC TCC ATC CAG TCT ATT AAT TGT TGC CGG GAA
     CGT TGA AAT AGG CGG AGG TAG GTC AGA TAA TTA ACA ACG GCC CTT

7831 GCT AGA GTA AGT AGT TCG CCA GTT AAT AGT TTG CGC AAC GTT GTT
     CGA TCT CAT TCA TCA AGC GGT CAA TTA TCA AAC GCG TTG CAA CAA

7876 GCC ATT GCT GCA GGC ATC GTG GTG TCA CGC TCG TCG TTT GGT ATG
     CGG TAA CGA CGT CCG TAG CAC CAC AGT GCG AGC AGC AAA CCA TAC

7921 GCT TCA TTC AGC TCC GGT TCC CAA CGA TCA AGG CGA GTT ACA TGA
     CGA AGT AAG TCG AGG CCA AGG GTT GCT AGT TCC GCT CAA TGT ACT

7966 TCC CCC ATG TTG TGC AAA AAA GCG GTT AGC TCC TTC GGT CCT CCG
     AGG GGG TAC AAC ACG TTT TTT CGC CAA TCG AGG AAG CCA GGA GGC

8011 ATC GTT GTC AGA AGT AAG TTG GCC GCA GTG TTA TCA CTC ATG GTT
     TAG CAA CAG TCT TCA TTC AAC CGG CGT CAC AAT AGT GAG TAC CAA

8056 ATG GCA GCA CTG CAT AAT TCT CTT ACT GTC ATG CCA TCC GTA AGA
     TAC CGT CGT GAC GTA TTA AGA GAA TGA CAG TAC GGT AGG CAT TCT

8101 TGC TTT TCT GTG ACT GGT GAG TAC TCA ACC AAG TCA TTC TGA GAA
     ACG AAA AGA CAC TGA CCA CTC ATG AGT TGG TTC AGT AAG ACT CTT

8146 TAG TGT ATG CGG CGA CCG AGT TGC TCT TGC CCG GCG TCA ACA CGG
     ATC ACA TAC GCC GCT GGC TCA ACG AGA ACG GGC CGC AGT TGT GCC
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8191 GAT AAT ACC GCG CCA CAT AGC AGA ACT TTA AAA GTG CTC ATC ATT
     CTA TTA TGG CGC GGT GTA TCG TCT TGA AAT TTT CAC GAG TAG TAA

8236 GGA AAA CGT TCT TCG GGG CGA AAA CTC TCA AGG ATC TTA CCG CTG
     CCT TTT GCA AGA AGC CCC GCT TTT GAG AGT TCC TAG AAT GGC GAC

8281 TTG AGA TCC AGT TCG ATG TAA CCC ACT CGT GCA CCC AAC TGA TCT
     AAC TCT AGG TCA AGC TAC ATT GGG TGA GCA CGT GGG TTG ACT AGA

8326 TCA GCA TCT TTT ACT TTC ACC AGC GTT TCT GGG TGA GCA AAA ACA
     AGT CGT AGA AAA TGA AAG TGG TCG CAA AGA CCC ACT CGT TTT TGT

8371 GGA AGG CAA AAT GCC GCA AAA AAG GGA ATA AGG GCG ACA CGG AAA
     CCT TCC GTT TTA CGG CGT TTT TTC CCT TAT TCC CGC TGT GCC TTT

8416 TGT TGA ATA CTC ATA CTC TTC CTT TTT CAA TAT TAT TGA AGC ATT
     ACA ACT TAT GAG TAT GAG AAG GAA AAA GTT ATA ATA ACT TCG TAA

8461 TAT CAG GGT TAT TGT CTC ATG AGC GGA TAC ATA TTT GAA TGT ATT
     ATA GTC CCA ATA ACA GAG TAC TCG CCT ATG TAT AAA CTT ACA TAA

8506 TAG AAA AAT AAA CAA ATA GGG GTT CCG CGC ACA TTT CCC CGA AAA
     ATC TTT TTA TTT GTT TAT CCC CAA GGC GCG TGT AAA GGG GCT TTT

8551 GTG CCA CCT GAC GTC TAA GAA ACC ATT ATT ATC ATG ACA TTA ACC
     CAC GGT GGA CTG CAG ATT CTT TGG TAA TAA TAG TAC TGT AAT TGG

8596 TAT AAA AAT AGG CGT ATC ACG AGG CCC TTT CGT CTT CAA
     ATA TTT TTA TCC GCA TAG TGC TCC GGG AAA GCA GAA GTT
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Total number of bases is: 8634.

Sequence name: PLMDR1

===03-MAR-1992==========PC/GENE===

APPENDIX D

===04-MAR-1992==========PC/GENE===

****
* RESTRICTION-SITE ANALYSIS *
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Done on DNA sequence PL
Total number of bases is: 4642.
Analysis done on the complete sequence.

List of cuts by enzyme
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AatII	:	954	4572									
AccI	:	2529										
AciI	:	68	117	127	297	419	540	902	933	1066	1107	1248
		1331	1397	1496	1515	1553	1604	1616	1626	1969	2271	2435
		2474	2484	2526	2551	2589	2594	2620	2637	2688	2695	2716
		2799	2827	2954	2973	3094	3212	3339	3356	3710	3801	4000
		4038	4167	4203	4280	4389	4496	4535				
AcyI	:	951	2137	4187	4569							
Afa16RI	:	4019										
AflII	:	177	1207	1723								
AflIII	:	2757										
AhaIII	:	3516	3535	4227								
AluI	:	172	176	262	333	554	797	877	885	1633	1718	1722
		1809	1884	2103	2346	2399	2418	2699	2925	3015	3061	3318
		3839	3939	4002								
AlwNI	:	374	1849	1924	3173							
ApaLI	:	2573	3071	4317								
Asp718I	:	619	2168									
AsuI	:	124	312	343	388	403	416	471	769	899	969	982
		1186	1397	1420	1818	1863	1893	1938	1953	1966	2021	2318
		3692	3771	3788	4010	4626						
AvaI	:	384	615	1628	2098	2131	2164					
AvaII	:	403	416	471	769	899	1420	1953	1966	2021	2318	3788
		4010										
BalI	:	801	1312									
BamHI	:	1624										
BbeI	:	2140										
BbvI	:	1112	1140	2337	2408	2505	2649	2667	3086	3176	3179	3385
		3688	3877	4079								
BbvII	:	587	4628									
BcefI	:	957	1357	3259								
BcgI	:	677	4155									
BetI	:	107	2963	3110	3941							
BglI	:	3770										
BinI	:	1253	1557	1609	1619	1632	2304	3318	3404	3406	3502	3503
		3966	4283	4287								
Bme142I	:	2138	2633	3003								
Bpu10I	:	315	418	1484	1866	1941	1968					
BsaAI	:	2511										
BsiI	:	2924	4308	4627								

BsiYI	:	161	164	309	310	384	385	386	413	582	664	685
		717	718	750	765	766	785	786	789	991	995	1340
		1417	1467	1468	1595	1679	1705	1860	1861	1935	1936	1963
		2131	2132	2212	2233	2265	2266	2299	2314	2315	2334	2335
		2338	2408	2779	2797	2963	3242					
BsmAI	:	321	673	694	748	942	1040	1151	1273	1332	1350	1530
		1674	2221	2242	2297	2400	3711	4487				
BspHI	:	3477	4485	4590								
BsrI	:	589	1232	1256	2138	2502	2537	3165	3178	3288	3694	3812
		3855	4126	4294								
BssHII	:	535	586	2084								
BstEII	:	1288										
BstNI	:	458	766	944	957	1370	1395	1418	1438	1468	2008	2315
		2785	2906	2919								
Bsu90I	:	1625										
CauII	:	304	380	616	617	745	787	932	1855	1930	2165	2166
		2294	2336	2403	2438	3137	3833	4184				
Cfr10I	:	3730										
CfrI	:	799	933	1310	1331	4038						
ClaI	:	1644										
CviJI	:	67	113	119	125	172	176	262	307	314	333	345
		383	390	422	554	567	640	738	797	801	877	885
		935	971	983	1109	1188	1312	1333	1398	1445	1451	1477
		1495	1580	1633	1718	1722	1809	1820	1858	1865	1884	1895
		1933	1940	1972	2103	2116	2287	2346	2350	2399	2418	2451
		2496	2536	2680	2699	2772	2783	2801	2827	2925	3015	3061
		3066	3091	3170	3235	3246	3289	3318	3681	3693	3734	3760
		3764	3773	3839	3929	3939	4002	4040	4627			
CviQI	:	489	620	867	1484	2169	2564	4129				
CviRI	:	195	654	1128	1304	1741	2196	2202	2396	2575	3073	3369
		3704	3794	3893	3987	4075	4319					
DdeI	:	76	218	308	334	425	696	990	1219	1491	1764	1859
		1934	1975	2156	2244	2567	3032	3441	3607	4147	4573	
DpnI	:	14	238	1247	1379	1564	1611	1616	1626	1784	2298	3325
		3400	3411	3419	3497	3509	3614	3955	3973	4019	4277	4294
		4330										
DraII	:	471	1420	2021	4626							
DrdI	:	2452	2865									
DsaI	:	120	903	1619								
Eam1105I	:	1221	2213	2259	3650							
Ecl136II	:	554	2103									
Eco31I	:	673	694	748	1350	2221	2242	2297	3711			
Eco57I	:	3305	4317									
Eco78I	:	2138										
EcoHI	:	306	382	618	619	747	789	934	1857	1932	2167	2168
		2296	2338	2405	2440	3139	3835	4186				
EcoNI	:	993	1593									
EcoRI	:	1	1603									
EcoRII	:	456	764	942	955	1368	1393	1416	1436	1466	2006	2313
		2783	2904	2917								
EcoRV	:	280	356	1831	1906							
Esp3I	:	954	1052	1285	1344	1518	2412					
FauI	:	56	1076	1407	1563	1592	1614	1626	2259	2462	2472	
Fnu4HI	:	120	123	936	1110	1126	1129	1334	2351	2397	2494	2547
		2663	2681	2684	2802	2957	3100	3165	3168	3374	3702	3891

		4041	4068	4163	4392							
FnuDII	:	63	122	535	537	588	1112	1114	1336	1621	2084	2086
		2358	2360	2463	2804	3385	3715	4208	4540			
FokI	:	641	1341	1501	2189	2445	3616	3797	4084			
FsiI	:	1	57	1081	1603							
GdiII	:	927	939	1325	4032							
GsuI	:	1656	3720									
HaeI	:	801	1312	2772	2783	3235						
HaeII	:	2140	2635	3005								
HaeIII	:	125	314	345	390	801	935	971	983	1188	1312	1333
		1398	1820	1865	1895	1940	2772	2783	2801	3235	3693	3773
		4040	4627									
HgaI	:	598	850	1103	2452	2868	3446	4176				
HgiAI	:	556	2105	2577	3075	4236	4321					
HgiCI	:	461	619	1343	2011	2136	2168	3598				
HgiJII	:	556	569	740	2105	2118	2289					
HhaI	:	537	539	588	590	857	1114	2086	2088	2139	2360	2463
		2493	2634	2667	2937	3004	3104	3278	3387	3780	3873	4210
		4542										
HinP1I	:	535	537	586	588	855	1112	2084	2086	2137	2358	2461
		2491	2632	2665	2935	3002	3102	3276	3385	3778	3871	4208
		4540										
HindII	:	1639	4191									
HinfI	:	441	660	1000	1011	2158	2657	2732	3128	3645		
HpaI	:	1639										
HpaII	:	108	304	380	616	744	786	932	1855	1930	2165	2293
		2335	2403	2437	2964	3111	3137	3327	3731	3765	3832	3942
		4184										
HphI	:	7	97	1357	1383	2379	2388	3493	3720	4136	4342	4377
KasI	:	2136										
KpnI	:	623	2172									
Ksp632I	:	1271	1512	2641	4445							
MaeI	:	173	436	832	870	882	1595	1719	1986	3252	3505	3840
MaeII	:	951	1282	1323	2510	3460	3876	4249	4569			
MaeIII	:	181	1195	1223	1288	1432	1727	2411	2506	3113	3176	3292
		3575	3906	3964	4117	4305						
MboI	:	12	236	1245	1377	1562	1609	1614	1624	1782	2296	3323
		3398	3409	3417	3495	3507	3612	3953	3971	4017	4275	4292
		4328										
MboII	:	587	1288	1499	2628	3419	3490	4245	4323	4432	4628	
McrI	:	936	2673	3097	4020	4169						
MlyI	:	994	2152	2666	3122	3654						
MmeI	:	1039	1306	2230	2972	3156						
MnlI	:	39	55	434	587	651	677	703	782	984	1082	1370
		1473	1506	1512	1515	1521	1551	1554	1569	1576	1592	1984
		2136	2155	2200	2225	2251	2331	2364	2394	2656	2864	2939
		3188	3588	3669	3817	4023	4616					
MseI	:	178	1208	1320	1350	1374	1638	1724	2538	3463	3515	3520
		3534	3587	3822	3861	4226	4598					
MstI	:	3872										
MwoI	:	119	298	374	551	1849	1924	2100	2581	2631	2696	2810
		3382	3770	3890								
NarI	:	2137										
NdeI	:	2580										
NheI	:	172	1718									

NlaIII	:	204	1406	1750	2396	2501	2761	3481	3972	3982	4060	4096
		4489	4594									
NlaIV	:	112	296	389	405	463	621	770	901	970	1102	1345
		1422	1626	1955	2013	2138	2170	2319	2789	2828	3600	3694
		3735	3946	4536								
NspBII	:	122	262	333	1621	1809	1884	2266	2469	3099	3344	4285
NspI	:	2396	2761									
PflKI	:	109	305	381	617	745	787	933	1856	1931	2166	2294
		2336	2404	2438	2965	3112	3138	3328	3732	3766	3833	3943
		4185										
PleI	:	1008	2166	2651	3136	3639						
PpuMI	:	471	1420	2021								
PshAI	:	720										
PssI	:	474	1423	2024	4629							
PstI	:	1130	1306	3895								
PvuI	:	4020										
PvuII	:	262	333	1809	1884							
RleAI	:	115	585	2134								
RsaI	:	490	621	868	1485	2170	2565	4130				
SacI	:	556	2105									
SacII	:	123	1622									
SapI	:	2641										
SauI	:	990	1219									
ScaI	:	4130										
ScrFI	:	304	380	458	616	617	745	766	787	932	944	957
		1370	1395	1418	1438	1468	1855	1930	2008	2165	2166	2294
		2315	2336	2403	2438	2785	2906	2919	3137	3833	4184	
SduI	:	466	556	569	740	2016	2105	2118	2289	2577	3075	4236
		4321										
SecI	:	120	302	378	457	467	615	679	764	765	903	942
		943	955	956	1368	1437	1446	1466	1467	1619	1853	1928
		2007	2017	2164	2227	2313	2314	2917				
SfaNI	:	401	663	1140	1951	2211	2423	2558	2594	2634	2854	3906
		4097	4346									
SfeI	:	1126	1302	1703	3022	3213	3891					
SmaI	:	617	2166									
SpeI	:	869										
SsoII	:	302	378	456	614	615	743	764	785	930	942	955
		1368	1393	1416	1436	1466	1853	1928	2006	2163	2164	2292
		2313	2334	2401	2436	2783	2904	2917	3135	3831	4182	
SspI	:	4454										
StyI	:	467	679	1446	2017	2227						
TaqI	:	1003	1239	1550	1561	1644	2857	4301				
TaqIIA	:	1382	1425	4336	4353							
TaqIIB	:	2659	3998	4183								
Tth111I	:	608	1020	1418	2157	2504						
Tth111II	:	287	363	639	832	1838	1913	3347	3354	3386		
VspI	:	3822										
XbaI	:	1985										
XcaI	:	2530										
XhoII	:	1624	3398	3409	3495	3507	4275	4292				
XmaI	:	615	2164									
XmaIII	:	933										
XmnI	:	4249										

Total number of cuts is : 1232.

Sorted list of enzymes by number of cuts
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CviJI	: 85	SduI	: 12	BetI	: 4	DrdI	: 2
AciI	: 51	NspBII	: 11	PssI	: 4	SacII	: 2
BsiYI	: 50	HphI	: 11	FsiI	: 4	Asp718I	: 2
MnlI	: 39	MaeI	: 11	Eam1105I	: 4	NspI	: 2
ScrFI	: 32	MboII	: 10	TaqIIA	: 4	KpnI	: 2
SsoII	: 32	FauI	: 10	EcoRV	: 4	AatII	: 2
SecI	: 29	HinfI	: 9	GdiII	: 4	SauI	: 2
AsuI	: 27	Tth111II	: 9	Ksp632I	: 4	Afa16RI	: 1
Fnu4HI	: 26	MaeII	: 8	PstI	: 3	BglI	: 1
AluI	: 25	Eco31I	: 8	AhaIII	: 3	Cfr10I	: 1
NlaIV	: 25	FokI	: 8	BcefI	: 3	NdeI	: 1
HaeIII	: 24	TaqI	: 7	Bme142I	: 3	Bsu90I	: 1
DpnI	: 23	HgaI	: 7	TaqIIB	: 3	XcaI	: 1
PflKI	: 23	HgiCI	: 7	AflII	: 3	BamHI	: 1
HinP1I	: 23	CviQI	: 7	HaeII	: 3	AccI	: 1
HpaII	: 23	XhoII	: 7	DsaI	: 3	Eco78I	: 1
HhaI	: 23	RsaI	: 7	BsiI	: 3	MstI	: 1
MboI	: 23	HgiAI	: 6	ApaLI	: 3	ClaI	: 1
DdeI	: 21	SfeI	: 6	PpuMI	: 3	SspI	: 1
FnuDII	: 19	Esp3I	: 6	RleAI	: 3	HpaI	: 1
EcoHI	: 18	HgiJII	: 6	BspHI	: 3	NarI	: 1
CauII	: 18	Bpu10I	: 6	BssHII	: 3	PshAI	: 1
BsmAI	: 18	AvaI	: 6	HindII	: 2	ScaI	: 1
CviRI	: 17	McrI	: 5	GsuI	: 2	PvuI	: 1
MseI	: 17	Tth111I	: 5	EcoRI	: 2	SapI	: 1
MaeIII	: 16	PleI	: 5	BalI	: 2	BsaAI	: 1
BbvI	: 14	MlyI	: 5	SacI	: 2	AflIII	: 1
MwoI	: 14	CfrI	: 5	SmaI	: 2	XbaI	: 1
BstNI	: 14	StyI	: 5	XmaI	: 2	BstEII	: 1
BsrI	: 14	HaeI	: 5	EcoNI	: 2	VspI	: 1
BinI	: 14	MmeI	: 5	BcgI	: 2	BbeI	: 1
EcoRII	: 14	AlwNI	: 4	BbvII	: 2	KasI	: 1
NlaIII	: 13	PvuII	: 4	Ecl136II	: 2	SpeI	: 1
SfaNI	: 13	AcyI	: 4	Eco57I	: 2	XmnI	: 1
AvaII	: 12	DraII	: 4	NheI	: 2	XmaIII	: 1

List of non cutting selected enzymes
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AgeI , ApaI , AsuII , AvrII , BclI , BglII , BsaBI ,
BsgI , BsmI , Bsp120I , BspMI , BspMII , BstXI , DraIII ,
Eco47III, Eco56I , EspI , FseI , HindIII , MfeI , MlaAI ,
MluI , NaeI , NcoI , NotI , NruI , NsiI , PacI ,
PflMI , PmaCI , RsrII , SalI , SciI , SfiI , SgrAI ,

SnaBI , SphI , SplI , Sse8387I, StuI , SwaI , XcmI , XhoI

Total number of selected enzymes which do not cut: 43

===04-MAR-1992==========PC/GENE===

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      3        9       15       21       27       33       39       45
      |        |        |        |        |        |        |        |
  1 GAA TTC ATA CCA GAT CAC CGA AAA CTG TCC TCC AAA TGT GTC CCC
    CTT AAG TAT GGT CTA GTG GCT TTT GAC AGG AGG TTT ACA CAG GGG

 46 CTC ACA CTC CCA AAT TCG CGG GCT TCT GCT CTT AGA CCA CTC TAC
    GAG TGT GAG GGT TTA AGC GCC CGA AGA CGA GAA TCT GGT GAG ATG

 91 CCT ATT CCC CAC ACT CAC CGG AGC CAA AGC CGC GGC CCT TCC GTT
    GGA TAA GGG GTG TGA GTG GCC TCG GTT TCG GCG CCG GGA AGG CAA

136 TCT TTG CTT TTG AAA GAC CCC ACC CGT AGG TGG CAA GCT AGC TTA
    AGA AAC GAA AAC TTT CTG GGG TGG GCA TCC ACC GTT CGA TCG AAT

181 AGT AAC GCC ACT TTG CAA GGC ATG GAA AAA TAC ATA ACT GAG AAT
    TCA TTG CGG TGA AAC GTT CCG TAC CTT TTT ATG TAT TGA CTC TTA

226 AGG AAA GTT CAG ATC AAG GTC AGG AAC AAA GAA ACA GCT GAA TAC
    TCC TTT CAA GTC TAG TTC CAG TCC TTG TTT CTT TGT CGA CTT ATG

271 CAA ACA GGA TAT CTG TGG TAA GCG GTT CCT GCC CCG GCT CAG GGC
    GTT TGT CCT ATA GAC ACC ATT CGC CAA GGA CGG GGC CGA GTC CCG

316 CAA GAA CAG ATG AGA CAG CTG AGT GAT GGG CCA AAC AGG ATA TCT
    GTT CTT GTC TAC TCT GTC GAC TCA CTA CCC GGT TTG TCC TAT AGA

361 GTG GTA AGC AGT TCC TGC CCC GGC TCG GGG CCA AGA ACA GAT GGT
    CAC CAT TCG TCA AGG ACG GGG CCG AGC CCC GGT TCT TGT CTA CCA

406 CCC CAG ATG CGG TCC AGC CCT CAG CAG TTT CTA GTG AAT CAT CAG
    GGG GTC TAC GCC AGG TCG GGA GTC GTC AAA GAT CAC TTA GTA GTC

451 ATG TTT CCA GGG TGC CCC AAG GAC CTG AAA ATG ACC CTG TAC CTT
    TAC AAA GGT CCC ACG GGG TTC CTG GAC TTT TAC TGG GAC ATG GAA

496 ATT TGA ACT AAC CAA TCA GTT CGC TTC TCG CTT CTG TTC GCG CGC
    TAA ACT TGA TTG GTT AGT CAA GCG AAG AGC GAA GAC AAG CGC GCG

541 TTC CGC TCT CCG AGC TCA ATA AAA GAG CCC ACA ACC CCT CAC TCG
    AAG GCG AGA GGC TCG AGT TAT TTT CTC GGG TGT TGG GGA GTG AGC

586 GCG CGC CAG TCT TCC GAT AGA CTG CGT CGC CCG GGT ACC CGT ATT
    CGC GCG GTC AGA AGG CTA TCT GAC GCA GCG GGC CCA TGG GCA TAA

631 CCC AAT AAA GCC TCT TGC TGT TTG CAT CCG AAT CGT GGT CTC GCT
    GGG TTA TTT CGG AGA ACG ACA AAC GTA GGC TTA GCA CCA GAG CGA

676 GTT CCT TGG GAG GGT CTC CTC TGA GTG ATT GAC TAC CCA CGA CGG
    CAA GGA ACC CTC CCA GAG GAG ACT CAC TAA CTG ATG GGT GCT GCC

721 GGG TCT TTC ATT TGG GGG CTC GTC CGG GAT TTG GAG ACC CCT GCC
    CCC AGA AAG TAA ACC CCC GAG CAG GCC CTA AAC CTC TGG GGA CGG
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 766 CAG GGA CCA CCG ACC CAC CAC CGG GAG GTA AGC TGG CCA GCA ACT
     GTC CCT GGT GGC TGG GTG GTG GCC CTC CAT TCG ACC GGT CGT TGA

 811 TAT CTG TGT CTG TCC GAT TGT CTA GTG TCT ATG TTT GAT GTT ATG
     ATA GAC ACA GAC AGG CTA ACA GAT CAC AGA TAC AAA CTA CAA TAC

 856 CGC CTG CGT CTG TAC TAG TTA GCT AAC TAG CTC TGT ATC TGG CGG
     GCG GAC GCA GAC ATG ATC AAT CGA TTG ATC GAG ACA TAG ACC GCC

 901 ACC CGT GGT GGA ACT GAC GAG TTC TGA ACA CCC GGC CGC AAC CCT
     TGG GCA CCA CCT TGA CTG CTC AAG ACT TGT GGG CCG GCG TTG GGA

 946 CGG AGA CGT CCC AGG GAC TTT GGG GGC CGT TTT TGT GGC CCG ACC
     CCC TCT GCA GGG TCC CTG AAA CCC CCG GCA AAA ACA CCG GGC TGG

 991 TGA GGA AGG GAG TCG ATG TGG AAT CCG ACC CCG TCA GGA TAT GTG
     ACT CCT TCC CTC AGC TAC ACC TTA GGC TGG GGC AGT CCT ATA CAC

1036 GTT CTG GTA GGA GAC GAG AAC CTA AAA CAG TTC CCG CCT CCG TCT
     CAA GAC CAT CCT CTG CTC TTG GAT TTT GTC AAG GGC GGA GGC AGA

1081 GAA TTT TTG CTT TCG GTT TGG AAC CGA AGC CGC GCG TCT TGT CTG
     CTT AAA AAC GAA AGC CAA ACC TTG GCT TCG GCG CGC AGA ACA GAC

1126 CTG CAG CAT CGT TCT GTG TTG TCT CTG TCT GAC TGT GTT TCT GTA
     GAC GTC GTA GCA AGA CAC AAC AGA GAC AGA CTG ACA CAA AGA CAT

1171 TTT GTC TGA AAA TTA GGG CCA GAC TGT TAC CAC TCC CTT AAG TTT
     AAA CAG ACT TTT AAT CCC GGT CTG ACA ATG GTG AGG GAA TTC AAA

1216 GAC CTT AGG TCA CTG GAA AGA TGT CGA GCG GAT CGC TCA CAA CCA
     CTG GAA TCC AGT GAC CTT TCT ACA GCT CGC CTA GCG AGT GTT GGT

1261 GTC GGT AGA TGT CAA GAA GAG ACG TTG GGT TAC CTT CTG CTC TGC
     CAG CCA TCT ACA GTT CTT CTC TGC AAC CCA ATG GAA GAC GAG ACG

1306 AGA ATG GCC AAC CTT TAA CGT CGG ATG GCC GCG AGA CGG CAC CTT
     TCT TAC CGG TTG GAA ATT GCA GCC TAC CGG CGC TCT GCC GTG GAA

1351 TAA CCG AGA CCT CAT CAC CCA GGT TAA GAT CAA GGT CTT TTC ACC
     ATT GGC TCT GGA GTA GTG GGT CCA ATT CTA GTT CCA GAA AAG TGG

1396 TGG CCC GCA TGG ACA CCC AGA CCA GGT CCC CTA CAT CGT GAC CTG
     ACC GGG CGT ACC TGT GGG TCT GGT CCA GGG GAT GTA GCA CTG GAC

1441 GGA AGC CTT GGC TTT TGA CCC CCC TCC CTG GGT CAA GCC CTT TGT
     CCT TCG GAA CCG AAA ACT GGG GGG AGG GAC CCA GTT CGG GAA ACA

1486 ACA CCC TAA GCC TCC GCC TCC TCT TCC TCC ATC CGC CCC GTC TCT
     TGT GGG ATT CGG AGG CGG AGG AGA AGG AGG TAG GCG GGG CAG AGA

1531 CCC CCT TGA ACC TCC TCG TTC GAC CCC GCC TCG ATC CTC CCT TTA
     GGG GGA ACT TGG AGG AGC AAG CTG GGG CGG AGC TAG GAG GGA AAT
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1576 TCC AGC CCT CAC TCC TTC TCT AGG CGG GAA TTC CGA TCT GAT CCC
     AGG TCG GGA GTG AGG AAG AGA TCC GCC CTT AAG GCT AGA CTA GGG

1621 GCG GGA TCC CGA GCT TGT TAA CAT CGA TAA AAT AAA AGA TTT TAT
     CGC CCT AGG GCT CGA ACA ATT GTA GCT ATT TTA TTT TCT AAA ATA

1666 TTA GTC TCC AGA AAA AGG GGG GAA TGA AAG ACC CCA CCT GTA GGT
     AAT CAG AGG TCT TTT TCC CCC CTT ACT TTC TGG GGT GGA CAT CCA

1711 TTG GCA AGC TAG CTT AAG TAA CGC CAT TTT GCA AGG CAT GGA AAA
     AAC CGT TCG ATC GAA TTC ATT GCG GTA AAA CGT TCC GTA CCT TTT

1756 ATA CAT AAC TGA GAA TAG AGA AGT TCA GAT CAA GGT CAG GAA CAG
     TAT GTA TTG ACT CTT ATC TCT TCA AGT CTA GTT CCA GTC CTT GTC

1801 ATG GAA CAG CTG AAT ATG GGC CAA ACA GGA TAT CTG TGG TAA GCA
     TAC CTT GTC GAC TTA TAC CCG GTT TGT CCT ATA GAC ACC ATT CGT

1846 GTT CCT GCC CCG GCT CAG GGC CAA GAA CAG ATG GAA CAG CTG AAT
     CAA GGA CGG GGC CGA GTC CCG GTT CTT GTC TAC CTT GTC GAC TTA

1891 ATG GGC CAA ACA GGA TAT CTG TGG TAA GCA GTT CCT GCC CCG GCT
     TAC CCG GTT TGT CCT ATA GAC ACC ATT CGT CAA GGA CGG GGC CGA

1936 CAG GGC CAA GAA CAG ATG GTC CCC AGA TGC GGT CCA GCC CTC AGC
     GTC CCG GTT CTT GTC TAC CAG GGG TCT ACG CCA GGT CGG GAG TCG

1981 AGT TTC TAG AGA ACC ATC AGA TGT TTC CAG GGT GCC CCA AGG ACC
     TCA AAG ATC TCT TGG TAG TCT ACA AAG GTC CCA CGG GGT TCC TGG

2026 TGA AAT GAC CCT GTG CCT TAT TTG AAC TAA CCA ATC AGT TCG CTT
     ACT TTA CTG GGA CAC GGA ATA AAC TTG ATT GGT TAG TCA AGC GAA

2071 CTC GCT TCT GTT CGC GCG CTT CTG CTC CCC GAG CTC AAT AAA AGA
     GAG CGA AGA CAA GCG CGC GAA GAC GAG GGG CTC GAG TTA TTT TCT

2116 GCC CAC AAC CCC TCA CTC GGG GCG CCA GTC CTC CGA TTG ACT GAG
     CGG GTG TTG GGG AGT GAG CCC CGC GGT CAG GAG GCT AAC TGA CTC

2161 TCG CCC GGG TAC CCG TGT ATC CAA TAA ACC CTC TTG CAG TTG CAT
     AGC GGG CCC ATG GGC ACA TAG GTT ATT TGG GAG AAC GTC AAC GTA

2206 CCG ACT TGT GGT CTC GCT GTT CCT TGG GAG GGT CTC CTC TGA GTG
     GGC TGA ACA CCA GAG CGA CAA GGA ACC CTC CCA GAG GAG ACT CAC

2251 ATT GAC TAC CCG TCA GCG GGG GTC TTT CAT TTG GGG GCT CGT CCG
     TAA CTG ATG GGC AGT CGC CCC CAG AAA GTA AAC CCC CGA GCA GGC

2296 GGA TCG GGA GAC CCC TGC CCA GGG ACC ACC GAC CCA CCA CCG GGA
     CCT AGC CCT CTG GGG ACG GGT CCC TGG TGG CTG GGT GGT GGC CCT

2341 GGT AAG CTG GCT GCC TCG CGC GTT TCG GTG ATG ACG GTG AAA ACC
     CCA TTC GAC CGA CGG AGC GCG CAA AGC CAC TAC TGC CAC TTT TGG

2386 TCT GAC ACA TGC AGC TCC CGG AGA CGG TCA CAG CTT GTC TGT AAG
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     AGA CTG TGT ACG TCG AGG GCC TCT GCC AGT GTC GAA CAG ACA TTC

2431 CGG ATG CCG GGA GCA GAC AAG CCC GTC AGG GCG CGT CAG CGG GTG
     GCC TAC GGC CCT CGT CTG TTC GGG CAG TCC CGC GCA GTC GCC CAC

2476 TTG GCG GGT GTC GGG GCG CAG CCA TGA CCC AGT CAC GTA GCG ATA
     AAC CGC CCA CAG CCC CGC GTC GGT ACT GGG TCA GTG CAT CGC TAT

2521 GCG GAG TGT ATA CTG GCT TAA CTA TGC GGC ATC AGA GCA GAT TGT
     CGC CTC ACA TAT GAC CGA ATT GAT ACG CCG TAG TCT CGT CTA ACA

2566 ACT GAG AGT GCA CCA TAT GCG GTG TGA AAT ACC GCA CAG ATG CGT
     TGA CTC TCA CGT GGT ATA CGC CAC ACT TTA TGG CGT GTC TAC GCA

2611 AAG GAG AAA ATA CCG CAT CAG GCG CTC TTC CGC TTC CTC GCT CAC
     TTC CTC TTT TAT GGC GTA GTC CGC GAG AAG GCG AAG GAG CGA GTG

2656 TGA CTC GCT GCG CTC GGT CGT TCG GCT GCG GCG AGC GGT ATC AGC
     ACT GAG CGA CGC GAG CCA GCA AGC CGA CGC CGC TCG CCA TAG TCG

2701 TCA CTC AAA GGC GGT AAT ACG GTT ATC CAC AGA ATC AGG GGA TAA
     AGT GAG TTT CCG CCA TTA TGC CAA TAG GTG TCT TAG TCC CCT ATT

2746 CGC AGG AAA GAA CAT GTG AGC AAA AGG CCA GCA AAA GGC CAG GAA
     GCG TCC TTT CTT GTA CAC TCG TTT TCC GGT CGT TTT CCG GTC CTT

2791 CCG TAA AAA GGC CGC GTT GCT GGC GTT TTT CCA TAG GCT CCG CCC
     GGC ATT TTT CCG GCG CAA CGA CCG CAA AAA GGT ATC CGA GGC GGG

2836 CCC TGA CGA GCA TCA CAA AAA TCG ACG CTC AAG TCA GAG GTG GCG
     GGG ACT GCT CGT AGT GTT TTT AGC TGC GAG TTC AGT CTC CAC CGC

2881 AAA CCC GAC AGG ACT ATA AAG ATA CCA GGC GTT TCC CCC TGG AAG
     TTT GGG CTG TCC TGA TAT TTC TAT GGT CCG CAA AGG GGG ACC TTC

2926 CTC CCT CGT GCG CTC TCC TGT TCC GAC CCT GCC GCT TAC CGG ATA
     GAG GGA GCA CGC GAG AGG ACA AGG CTG GGA CGG CGA ATG GCC TAT

2971 CCT GTC CGC CTT TCT CCC TTC GGG AAG CGT GGC GCT TTC TCA TAG
     GGA CAG GCG GAA AGA GGG AAG CCC TTC GCA CCG CGA AAG AGT ATC

3016 CTC ACG CTG TAG GTA TCT CAG TTC GGT GTA GGT CGT TCG CTC CAA
     GAG TGC GAC ATC CAT AGA GTC AAG CCA CAT CCA GCA AGC GAG GTT

3061 GCT GGG CTG TGT GCA CGA ACC CCC CGT TCA GCC CGA CCG CTG CGC
     CGA CCC GAC ACA CGT GCT TGG GGG GCA AGT CGG GCT GGC GAC GCG

3106 CTT ATC CGG TAA CTA TCG TCT TGA GTC CAA CCC GGT AAG ACA CGA
     GAA TAG GCC ATT GAT AGC AGA ACT CAG GTT GGG CCA TTC TGT GCT

3151 CTT ATC GCC ACT GGC AGC AGC CAC TGG TAA CAG GAT TAG CAG AGC
     GAA TAG CGG TGA CCG TCG TCG GTG ACC ATT GTC CTA ATC GTC TCG

3196 GAG GTA TGT AGG CGG TGC TAC AGA GTT CTT GAA GTG GTG GCC TAA
     CTC CAT ACA TCC GCC ACG ATG TCT CAA GAA CTT CAC CAC CGG ATT
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3241 CTA CGG CTA CAC TAG AAG GAC AGT ATT TGG TAT CTG CGC TCT GCT
     GAT GCC GAT GTG ATC TTC CTG TCA TAA ACC ATA GAC GCG AGA CGA

3286 GAA GCC AGT TAC CTT CGG AAA AAG AGT TGG TAG CTC TTG ATC CGG
     CTT CGG TCA ATG GAA GCC TTT TTC TCA ACC ATC GAG AAC TAG GCC

3331 CAA ACA AAC CAC CGC TGG TAG CGG TGG TTT TTT TGT TTG CAA GCA
     GTT TGT TTG GTG GCG ACC ATC GCC ACC AAA AAA ACA AAC GTT CGT

3376 GCA GAT TAC GCG CAG AAA AAA AGG ATC TCA AGA AGA TCC TTT GAT
     CGT CTA ATG CGC GTC TTT TTT TCC TAG AGT TCT TCT AGG AAA CTA

3421 CTT TTC TAC GGG GTC TGA CGC TCA GTG GAA CGA AAA CTC ACG TTA
     GAA AAG ATG CCC CAG ACT GCG AGT CAC CTT GCT TTT GAG TGC AAT

3466 AGG GAT TTT GGT CAT GAG ATT ATC AAA AAG GAT CTT CAC CTA GAT
     TCC CTA AAA CCA GTA CTC TAA TAG TTT TTC CTA GAA GTG GAT CTA

3511 CCT TTT AAA TTA AAA ATG AAG TTT TAA ATC AAT CTA AAG TAT ATA
     GGA AAA TTT AAT TTT TAC TTC AAA ATT TAG TTA GAT TTC ATA TAT

3556 TGA GTA AAC TTG GTC TGA CAG TTA CCA ATG CTT AAT CAG TGA GGC
     ACT CAT TTG AAC CAG ACT GTC AAT GGT TAC GAA TTA GTC ACT CCG

3601 ACC TAT CTC AGC GAT CTG TCT ATT TCG TTC ATC CAT AGT TGC CTG
     TGG ATA GAG TCG CTA GAC AGA TAA AGC AAG TAG GTA TCA ACG GAC

3646 ACT CCC CGT CGT GTA GAT AAC TAC GAT ACG GGA GGG CTT ACC ATC
     TGA GGG GCA GCA CAT CTA TTG ATG CTA TGC CCT CCC GAA TGG TAG

3691 TGG CCC CAG TGC TGC AAT GAT ACC GCG AGA CCC ACG CTC ACC GGC
     ACC GGG GTC ACG ACG TTA CTA TGG CGC TCT GGG TGC GAG TGG CCG

3736 TCC AGA TTT ATC AGC AAT AAA CCA GCC AGC CGG AAG GGC CGA GCG
     AGG TCT AAA TAG TCG TTA TTT GGT CGG TCG GCC TTC CCG GCT CGC

3781 CAG AAG TGG TCC TGC AAC TTT ATC CGC CTC CAT CCA GTC TAT TAA
     GTC TTC ACC AGG ACG TTG AAA TAG GCG GAG GTA GGT CAG ATA ATT

3826 TTG TTG CCG GGA AGC TAG AGT AAG TAG TTC GCC AGT TAA TAG TTT
     AAC AAC GGC CCT TCG ATC TCA TTC ATC AAG CGG TCA ATT ATC AAA

3871 GCG CAA CGT TGT TGC CAT TGC TGC AGG CAT CGT GGT GTC ACG CTC
     CGC GTT GCA ACA ACG GTA ACG ACG TCC GTA GCA CCA CAG TGC GAG

3916 GTC GTT TGG TAT GGC TTC ATT CAG CTC CGG TTC CCA ACG ATC AAG
     CAG CAA ACC ATA CCG AAG TAA GTC GAG GCC AAG GGT TGC TAG TTC

3961 GCG AGT TAC ATG ATC CCC CAT GTT GTG CAA AAA AGC GGT TAG CTC
     CGC TCA ATG TAC TAG GGG GTA CAA CAC GTT TTT TCG CCA ATC GAG

4006 CTT CGG TCC TCC GAT CGT TGT CAG AAG TAA GTT GGC CGC AGT GTT
     GAA GCC AGG AGG CTA GCA ACA GTC TTC ATT CAA CCG GCG TCA CAA
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4051 ATC ACT CAT GGT TAT GGC AGC ACT GCA TAA TTC TCT TAC TGT CAT
     TAG TGA GTA CCA ATA CCG TCG TGA CGT ATT AAG AGA ATG ACA GTA

4096 GCC ATC CGT AAG ATG CTT TTC TGT GAC TGG TGA GTA CTC AAC CAA
     CGG TAG GCA TTC TAC GAA AAG ACA CTG ACC ACT CAT GAG TTG GTT

4141 GTC ATT CTG AGA ATA GTG TAT GCG GCG ACC GAG TTG CTC TTG CCC
     CAG TAA GAC TCT TAT CAC ATA CGC CGC TGG CTC AAC GAG AAC GGG

4186 GGC GTC AAC ACG GGA TAA TAC CGC GCC ACA TAG CAG AAC TTT AAA
     CCG CAG TTG TGC CCT ATT ATG GCG CGG TGT ATC GTC TTG AAA TTT

4231 AGT GCT CAT CAT TGG AAA ACG TTC TTC GGG GCG AAA ACT CTC AAG
     TCA CGA GTA GTA ACC TTT TGC AAG AAG CCC CGC TTT TGA GAG TTC

4276 GAT CTT ACC GCT GTT GAG ATC CAG TTC GAT GTA ACC CAC TCG TGC
     CTA GAA TGG CGA CAA CTC TAG GTC AAG CTA CAT TGG GTG AGC ACG

4321 ACC CAA CTG ATC TTC AGC ATC TTT TAC TTT CAC CAG CGT TTC TGG
     TGG GTT GAC TAG AAG TCG TAG AAA ATG AAA GTG GTC GCA AAG ACC

4366 GTG AGC AAA AAC AGG AAG GCA AAA TGC CGC AAA AAA GGG AAT AAG
     CAC TCG TTT TTG TCC TTC CGT TTT ACG GCG TTT TTT CCC TTA TTC

4411 GGC GAC ACG GAA ATG TTG AAT ACT CAT ACT CTT CCT TTT TCA ATA
     CCG CTG TGC CTT TAC AAC TTA TGA GTA TGA GAA GGA AAA AGT TAT

4456 TTA TTG AAG CAT TTA TCA GGG TTA TTG TCT CAT GAG CGG ATA CAT
     AAT AAC TTC GTA AAT AGT CCC AAT AAC AGA GTA CTC GCC TAT GTA

4501 ATT TGA ATG TAT TTA GAA AAA TAA ACA AAT AGG GGT TCC GCG CAC
     TAA ACT TAC ATA AAT CTT TTT ATT TGT TTA TCC CCA AGG CGC GTG

4546 ATT TCC CCG AAA AGT GCC ACC TGA CGT CTA AGA AAC CAT TAT TAT
     TAA AGG GGC TTT TCA CGG TGG ACT GCA GAT TCT TTG GTA ATA ATA

4591 CAT GAC ATT AAC CTA TAA AAA TAG GCG TAT CAC GAG GCC CTT TCG
     GTA CTG TAA TTG GAT ATT TTT ATC CGC ATA GTG CTC CGG GAA AGC

4636 TCT TCA A
     AGA AGT T
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Total number of bases is: 4642.

Sequence name: PL

===04-MAR-1992==========PC/GENE===

VITA

Glenn Paul Niemeyer was born in Hialeah, Florida on October 2, 1960. He attended high school in that city until his senior year when his family moved to Maryville, Tennessee. He graduated from high school in Maryville and then entered the University of Tennessee. He received a Bachelor of Arts degree in Biology in March of 1986. The following month he began work as a supervisor for an air freight company. In the Fall of 1989 he entered the graduate program in comparative and experimental medicine at The University of Tennessee at Knoxville. He received a Master of Science degree in August, 1992.