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PARTIAL MOLECULAR CHARACTERIZATION OF THE GAMMA CONSTANT HEAVY CHAIN GENE IN THE DOG

By

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ABSTRACT

PARTIAL MOLECULAR CHARACTERIZATION OF THE GAMMA CONSTANT HEAVY CHAIN GENE IN THE DOG

By

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The ability to isolate and sequence Immunoglobulin G (IgG) heavy and light chain genes in a variety of species has facilitated genetic engineering of antibody molecules. This has allowed the production of antibody molecules with desired specificities and effector function. This thesis initiated the molecular characterization of the gamma constant heavy (CH) chain gene encoding IgG constant heavy chains in the canine. A rabbit constant heavy gamma complementary deoxyribonucleic acid (cDNA) probe was used to screen a canine genomic DNA bacteriophage library in order to identify the canine gamma CH chain gene. A 16.0 kilobase (Kb) fragment was isolated containing the canine CH gamma gene. Polymerase chain reaction was utilized to amplify this isolated fragment using heavy chain primers. Successful amplification confirms the 16.0 Kb fragment contains a sequence which is representative of an immunoglobulin gamma CH gene.

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INTRODUCTION

Immunoglobulins are the mediators of humoral immune responses within the body and are responsible both for specific protection against bacteria and viruses as well as for normal and disease-related immune reactions (1). Since immunoglobulins are involved in many diseases, they have proven to be invaluable research tools in assay systems for evaluating conditions involving inflammation, autoimmunity, graft rejection and idiotype-mediated network regulation.

Immunoglobulins, also known as antibodies, are categorized according to their protein structures. There are five classes of immunoglobulins: immunoglobulins M (IgM), G (IgG), D (IgD), A (IgA) and E (IgE). Immunoglobulins are present in varying concentrations within the body and exhibit differing biological properties (2). Immunoglobulin G is the major antibody synthesized during secondary immune responses. It exists in vivo as either surface immunoglobulin of B lymphocytes or as free monomers in plasma (2). IgG crosses the placenta and diffuses readily into extravascular body spaces neutralizing bacterial toxins and binding microorganisms to enhance phagocytosis (2).

At the protein level, the IgG molecule consists of two heavy and two light polypeptide chains held together by disulfide bonds. These chains are comprised of constant heavy (CH), variable heavy (VH), constant light (CL) and variable light (VL) regions (3). At the molecular level, these regions are encoded by three separate gene loci - kappa light chain, lambda light chain and heavy chain genes. Each of these loci contains constant and variable genes (4). The immunoglobulin heavy chains are encoded by four linked families of genes - variable heavy (VH), diverse heavy (D), joining heavy (JH) and constant heavy (CH) - with one gene from each set aligning to form a functional heavy chain gene (4). The family of constant heavy gamma genes encode for IgG heavy chains and are the basis for the division of IgG molecules into its subclasses - For example in humans: IgG1, IgG2a, IgG2b, IgG2c, IgG3 and IgG4 (4,5,6).

IgG heavy chains are well characterized in humans, mice and rabbits (4). In humans, there are eleven CH genes which are denoted in the following order - mu, delta, gamma1, gamma2a, gamma2b, gamma2c, gamma3, gamma4, epsilon, alpha1, and alpha2 (8). Three pseudogenes have been identified (pseudogenes do not produce functional proteins) (5). In mice, there are eight CH genes which are arranged as follows - mu, delta, gamma3, gamma1, gamma2b, gamma2a, epsilon and alpha. There are no pseudogenes present (9). In rabbits, there are seven CH genes identified for IgM, IgG, IgE, IgA1,

there are seven CH genes identified for IgM, IgG, IgE, IgA1, IgA2, IgA3 and IgA4 (10). However, CH genes are not as extensively characterized in other species such as canine, bovine, ovine, equine and porcine (11).

Comparison of IgG gene nucleotide sequences has shown that 70-80% homology exists between many mammalian species (5,12). This observation has permitted the successful use of human, mouse and rabbit CH gamma complementary deoxyribonucleic acid (cDNA) probes in the identification of IgG genes (12). These cDNA probes were synthesized by using reverse transcriptase to copy an IgG messenger ribonucleic acid (mRNA) template and were then used to screen both cDNA and genomic DNA libraries of other species (13).

Isolation and sequencing of IgG heavy and light chain genes has facilitated genetic engineering of antibody molecules. Transfection of these genes into the appropriate bacterial or mammalian cell lines, has allowed the production of chimeric antibodies with improved biological and antigen-binding properties (14). Classical hybridoma technology involves the fusion of an efficient myeloma cell line with a source of antibody-producing cells (15). The ability to genetically engineer antibodies has two main advantages over hybridoma technology. The first advantage has been to produce antibody molecules with desired specificities or with the particular combinations of specificity and effector function (3). The second advantage has been in the development of chimeric immunoglobulin molecules (3,15). These advantages have provided solutions to previous failures in human hybridoma development because efficient fusion myeloma cell lines are not available (15). Currently, there are sources of chimeric antibodies comprised of murine and human IgG gene components (15). These antibody molecules normally consist of a murine variable region which possesses a selected antigen specificity in conjunction with a human CH gamma region (15,16,17,18). Chimeric antibodies of this kind have proven to be less immunogenic than murine monoclonal antibodies previously used in human immunotherapy (14).

In the canine, the development of hybridomas secreting canine monoclonal antibodies has not been successful; a good fusion myeloma cell culture line has not been identified. This research project has initiated the molecular characterization of the CH gamma gene encoding canine IgG heavy chains. The intent was to provide a foundation for the future development of canine chimeric IgG molecules. There have been four subclasses of IgG identified in the dog: IgG1, IgG2a, IgG2b and IgG2c (19). These isotypes were determined by immunochemical and serological techniques. Amino acid sequence data is available for the variable regions of canine IgM, IgG and IgA heavy chains (20). However, there has not been any nucleotide sequence data generated on a canine IgG gene. In order to begin the investigation of canine CH gamma genes, three objectives were established for this thesis project: 1) isolation of canine genomic DNA from peripheral blood lymphocytes and construction of a canine genomic DNA bacteriophage library; 2) screening of the canine genomic DNA bacteriophage library to identify the canine immunoglobulin gene; and 3) construction of a restriction map of the isolate.

There were two main approaches used in this project. The first involved the selection of a rabbit CH gamma cDNA probe for screening of the canine genomic DNA library. This probe was obtained from Dr. Katherine Knight of Loyola University. Dr. Knight has conducted extensive research on the rabbit's immunoglobulin variants or allotypes and the immunoglobulin genes which encode them. Her efforts have provided a model for the study of immunoglobulin gene regulation in other species (21,22,23). This particular probe was used successfully in screening a bovine recombinant bacteriophage library. The bovine IgG CH genes isolated were utilized in the construction and characterization of hapten-binding bovine/murine chimeric IgG1, IgG2 and IgG3 molecules (11).

The second approach involved the use of the polymerase chain reaction on canine genomic DNA. This was incorporated in the hope of amplifying regions of immunoglobulin VH and VL DNA sequences which could be subsequently used as canine-

specific probes and ultimately be sequenced. This would allow screening of the canine genomic DNA library and canine genomic DNA, by hybridization analysis, with a probe of greater homology. The outcome of these approaches will be discussed later.

The materials and methods sections of this thesis will provide a detailed guide for the continuation of this research project. Results from this thesis project will be used to determine the nucleotide sequence of canine Ig heavy chain. In addition, the isolated gene may be transfected within a suitable mammalian cell line (14). Further application of these results could be made in the construction of canine chimeric antibodies and ultimately in establishing a genetically engineered source of canine IgG molecules.

MATERIALS AND METHODS

Many of the methods described throughout this section were modified from procedures outlined in 1) Molecular Cloning- A Laboratory Manual, 2) Basic Methods in Molecular Biology and 3) Guide to Molecular Cloning Techniques - Methods in Enzymology, Volume 152.

In this section, all chemicals unless otherwise stated were purchased from generic vendors through the Michigan State University General Stores.

All DNA molecular weight markers and restriction enzymes used throughout this project were obtained from Boehringer Mannheim Biochemicals. Restriction endonuclease digests of DNA were prepared according to the instructions provided by Boehringer Mannheim Biochemicals and using the buffers supplied.

Preparation of the rabbit CH gamma cDNA probe

The rabbit CH gamma cDNA probe was kindly supplied by Dr. Katherine Knight, Department of Microbiology at Loyola University. The probe consists of a 3.4 kilobase (Kb) Eco

RI/Hind III restriction fragment which has been cloned into plasmid pBR322.

Preparation of the rabbit gamma cDNA probe involved:

1) transformation of Escherichia coli (E. coli) HB101 bacteria with the plasmid construct
2) amplification of the plasmid/probe construct
3) isolation of plasmid/probe DNA
4) restriction endonuclease digestion of plasmid/probe DNA
5) random primed labeling of the probe

Transformation of Escherichia coli (E. coli) HB101 bacteria with plasmid construct

E. coli HB101 bacteria (Bethesda Research Laboratories) was first transformed with a rabbit CH gamma cDNA plasmid clone. The bacteria were first made competent for the uptake of plasmid pBR322. E. coli HB101 bacteria were removed from storage at -70°C and partially thawed. An inoculating loop was flame-sterilized and cooled to room temperature. A loopful of bacteria was introduced into 200 milliliters (ml) of Luria-Bertani culture medium (LB), consisting of 1% bacto-tryptone (Difco), 0.5% bacto-yeast extract (Difco) and 1% sodium chloride. This culture was grown at 37°C with moderate shaking to an optical density (O.D.) of 0.2 to 0.3 measured at 600 nanometers (nm). The culture was shaken without creating frothy bubbles on the surface. The bacterial cells were centrifuged at 3,000 g for 5 minutes at 4°C. The pellet was washed in 40 ml of a "salt" solution (40 millimolar (mM) potassium acetate, 60 mM calcium chloride, 15% sucrose, 45 mM manganese chloride and 10 mM rubidium chloride) and then centrifuged as previously described. The pellet was resuspended in 4 ml of "salt" solution and dispensed into 200 microliter (ul) aliquots. These aliquots were iced for 5 minutes, frozen in a CO_2/ethanol bath and stored at -70°C.

One aliquot of competent bacteria was thawed on ice and incubated with 7 ul of dimethylsulfoxide (DMSO) for 5 to 10 minutes at room temperature on a rotator. Four microliters of the plasmid preparation were added to the mixture and incubated on ice for 15 minutes. The cells were frozen in a CO_2/ethanol bath for 30 seconds and thawed at 37°C for 30 seconds. After a subsequent incubation on ice of 30 minutes, the cells were heat shocked at 42°C for 1 to 2 minutes and further incubated with 1 ml of LB medium for 30 minutes at 37°C in a shaker bath with mild rotation. The cells were microfuged at 150 g for 2 minutes and resuspended in 200 ul of the supernatant.

Transformed and untransformed bacteria (negative control) were plated on LB/ampicillin agar plates (50 milligrams (mg)/liter LB) which were inverted and incubated at 37°C for 12 to 16 hours. Ampicillin (Sigma Chemical Company) was

used at a stock concentration of 50 mg/ml in double-distilled water. Transformation was confirmed by the appearance of colonies on these plates.

Amplification of plasmid/probe clone

One of the transformed colonies was lifted from the plate using a sterile glass pipet and placed into 100 ml of LB medium with ampicillin (100 mg/liter LB). The culture was grown overnight with continuous shaking at 37°C. The culture was added to a 2000 ml erlenmeyer flask containing 500 ml of LB/ampicillin medium and grown to an $O.D._{600}$ of 0.4 to 0.5. Chloramphenicol (Sigma Chemical Company) was added (0.17 mg/ml culture medium) and incubation was continued for 12 to 16 hours. Chloramphenicol was added to the culture in order to inhibit bacterial protein synthesis and prevent replication of the bacterial chromosome (24). However, replication of the plasmid does not cease and the copy number of the plasmid increases.

Isolation of plasmid/probe DNA

The amplified culture was centrifuged at 1,500 g for 15 minutes at 4°C. The bacterial pellet was thoroughly resuspended in 12 ml of a freshly prepared solution of 25 mM Tris-HCl, 10mM ethylenediamine tetraacetic acid-trisodium salt (EDTA), 15% sucrose and 24 mg lysozyme (Sigma Chemical

Company) and incubated in ice water for 20 minutes. The suspension was mixed with 24 ml of 0.2 molar (M) sodium hydroxide and 1% sodium dodecyl sulfate (SDS) by careful inversion. The mixture was incubated further for 10 minutes, mixed with 15 ml of 3 M sodium acetate (pH 4.6) and returned to ice water for 20 minutes. This preparation was then ultracentrifuged at 52,000 g for 20 minutes at $4^{O}C$. The supernatant was recovered, avoiding the white precipitate at the surface, and incubated with 50 ul RNAase (1mg/ml, Boehringer Mannheim Biochemicals) for 20 minutes at $37^{O}C$. The supernatant was then extracted with phenol:chloroform/isoamyl alcohol (Sigma Chemical Company) prepared 1:1. The DNA was precipitated using 0.6 volume of isopropyl alcohol. The precipitated DNA was collected by centrifugation at 9,600 g for 30 minutes at room temperature. The DNA pellet was washed in 70% ethanol at room temperature, allowed to dry after decanting most of the ethanol and then resuspended in 1.8 ml of a solution containing 10 mM Tris-HCl, 1 mM EDTA and 1 M sodium chloride (pH 8.0).

The plasmid DNA was purified by centrifugation over pZ523 columns (5 Prime - 3 Prime, Inc) and then resuspended in double-distilled water. The quantity of DNA collected was determined by spectrophotometry. The O.D. of resuspended DNA was measured at 280, 260, 250 and 230 nm. The following ratios were calculated and the ranges indicated represent normal values for quality and quantitation of DNA:

Ratio	Acceptable Range	Indication
260/280	1.8-2.0	DNA purity
260/250	1.0-1.5	carbohydrate contamination
230/260	0.4-0.5	protein contamination

$O.D._{260}$ = 1 is equivalent to 50 ug/ml of double-stranded DNA.

Restriction endonuclease digestion of plasmid/probe DNA

Purified plasmid/probe DNA was digested overnight with Eco RI and Hind III restriction endonucleases (Boehringer Mannheim Biochemicals) in Buffer B at 37°C in order to recover the 3.4 kb fragment from the remaining plasmid DNA. The digested preparation was electrophoresed in 1 X Tris-acetate EDTA (TAE) buffer (0.08 M Tris-HCl, 4 mM EDTA and 0.23% glacial acetic acid) on a 1.5% agarose (Sea Plaque low melting temperature agarose, FMC) gel (15 cm x 10 cm) at 50 V for 4 hours. The gel was stained in an ethidium bromide solution (0.3 mg/500 ml doubl-distilled water) for 1 hour. The gel was rinsed in double-distilled water and then placed on an ultraviolet illuminator box (254 nm, VWR Scientific) with a ruler as a distance marker. The gel was photographed using a polaroid camera (Type 667 black/white polaroid film) with a threaded glass filter to eliminate any extraneous light surrounding the gel. The camera was set at f/5.6 and an exposure time of 1 second was used.

The 2 resulting fragments on the gel were measured on the photograph using a pair of dividers to span the distance from the bottom of a well to the bottom of the fragment, and then compared to the ruler in the photograph. The lengths of the fragments were estimated by performing a least-squares analysis in relation to the migration of fragment lengths of a Hind III digest of bacteriophage lambda DNA (Boehringer Mannheim Biochemicals). This analysis was performed by computer algorithm (25). The lower band corresponding to the 3.4 kb Eco RI/Hind III fragment was excised from the gel with a scalpel blade and stored in a sterile polypropylene tube at 4°C. This fragment was used as the probe throughout this project.

Random primed labeling of the probe

The 3.4 kb insert DNA was labeled using a random primed labeling kit (Boehringer Mannheim Biochemicals). The insert DNA was melted at 65°C for 15 to 20 minutes and then 18 ul of the probe (0.54 ug/ul) was placed in a screw-capped microfuge tube. The probe was boiled for 10 minutes in order to separate the DNA strands of the probe and then immediately transferred to ice for 5 to 10 minutes to keep the strands separated. In a separate tube, 2 ul of dATP, dGTP, dTTP and 4 ul of random hexamers were combined. This reaction mixture was added to the probe along with 2 ul

Klenow enzyme and 10 ul alpha-^{32}P-dCTP (10 uCi/ul, New England Nuclear research products). The preparation was incubated in a 37°C waterbath for 30 minutes, vortexed and then returned to the waterbath for 90 minutes.

The labeling reaction was terminated by addition of 4 ul of 0.2 M EDTA and subsequent incubation at 65°C for 10 minutes. Approximately 1 ml of water (heated to 65°C) was added to dilute the agarose. At this point, the labeled probe could be stored at -70°C or added to a hybridization mixture. Prior to addition of the radiolabeled probe to a hybridization mixture, the probe was boiled for 5-10 minutes and then quick cooled on ice for 5-10 minutes.

Isolation of canine genomic DNA

Fifteen milliliters of whole blood was collected in EDTA and centrifuged at 750 g for 20 minutes. The buffy coat was transferred to a 15 ml conical polypropylene tube, mixed in phosphate-buffered saline (0.14 M sodium chloride, 3 mM potassium chloride, 8 mM sodium phosphate monobasic and 1 mM potassium phosphate monobasic) and centrifuged as above. The supernatant was aspirated and erythrocytes were lysed by mixing with 7 ml double-distilled water for 15 seconds and immediately adding an equal volume of 2 X EDTA phosphate-buffered saline (0.3 M sodium chloride, 20 mM EDTA-trisodium salt and 16 mM potassium phosphate monobasic). The suspension was centrifuged at 194.5 g for 20 minutes and the supernatant was discarded. The final leukocyte pellet was mixed with 5 ml of lysis buffer (10 mM Tris-HCl (pH 7.4), 0.1 M sodium chloride, 0.1 M EDTA and 2% SDS) and incubated at 55°C for 30 minutes. Proteinase K (Boehringer Mannheim Biochemicals) was added (400 ug/ ml lysis buffer) and the incubation continued overnight.

DNase-free RNase (Boehringer Mannheim Biochemicals) was added (100 ug/ ml lysis buffer) and the tube was incubated at 37°C for 1 hour. The suspension was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol mixture (50/48/2) by incubating at 37°C for 30 minutes with constant inversion and centrifuging at 292 g for 2 minutes. The upper aqueous phase was collected with a wide bore plastic pipet, and then extracted with a chloroform/isoamyl alcohol mixture (24/1) twice as described above. The final aqueous supernatant was mixed with 500 ul 3 M sodium acetate and allowed to stand at room temperature for 15 minutes. One volume of -20°C absolute ethanol was added and mixed by inversion. The white stringy precipitate was transferred with a wide bore pipet to a 1.5 ml screw cap tube and washed twice in 70% ethanol. The pellet was air dried for 1 hour and 70 ul 0.1 M Tris-HCl (pH 7.4) added. The DNA was allowed to go into solution overnight. The DNA was quantified by spectrophotometry as described previously.

Construction of canine genomic library

Sixty micrograms of canine genomic DNA was partially digested with 3 units of Sau3A restriction endonuclease (Boehringer Mannheim Biochemicals) in Buffer A in a 1 ml reaction volume. After 2.5, 5, 10 and 15 minutes of digestion, 150 ul aliquots were removed from each timed reaction tube. Approximately 30 ul from each of the timed digests were electrophoresed in 1 X TAE buffer on a 0.5% SeaKem GTG agarose (FMC) minigel (15 cm x 10 cm) in addition to an undigested control, at 50 V for 2 hours. The gel was stained in ethidium bromide (described previously) and the digest achieving the best 9-20 Kb distribution of fragments was selected for preparation of insert DNA for the lambda bacteriophage library. This distribution of fragments is optimal for ligation in the EMBL3 vector arms used in making this library. The insert was ligated into the EMBL3 vector by combining 6 ul (0.36 ug) of the 10 minute Sau 3A digest, 1 ul EMBL3 BamHI arms, 1 ul ligase (Bethesda Research Laboratories) and 2 ul 5 X ligation buffer (Promega EMBL3 BamHI Arms Cloning System). This reaction mixture was incubated overnight at 16^{O}C and in vitro packaged using Promega's Packagene Lambda DNA Packaging System. The library was titered by preparing 10-fold serial dilutions of packaged phage in 1 ml SM buffer (0.1 m sodium chloride, 8 mM magnesium sulfate, 0.5 M Tris-HCl, pH 7.5 and 0.01%

gelatin). Ten microliters of each dilution was incubated with 200 ul of an overnight culture of E. coli K803 bacteria (K803) (Promega) for 30 minutes at 37°C. The overnight culture of K803 was prepared by inoculating 100 ml of sterile LB medium with a loopful of K803 and then shaking the culture overnight at 37°C. The culture could then be stored at 4°C for up to 2 weeks. Four milliliters of melted top agar (1% bacto-tryptone, 0.085 M sodium chloride and 0.8% bacto-agar) was pipetted into each tube, vortexed, poured over 90 mm TB agar plates (1% bacto-tryptone, 0.085 M sodium chloride and 1.5% bacto-agar) and swirled to achieve a uniform thickness over the surface of the plates. After hardening of the top agar the plates were inverted and incubated overnight at 37°C. The appearance of clear plaques indicated the bacteria which have been infected with a bacteriophage/insert construct. Clear plaques were counted and the phage titer was calculated using the following equation:

Original titer =(# plaques x 1 ml/"x" ml added x 1/dilution)
Titer units are plaque forming units/ml (pfu/ml)
For example: 10^{-1} dilution = 136 plaques = 1.36×10^5 pfu/ml
10^{-2} dilution = 36 plaques = 3.6×10^5 pfu/ml
Average titer = 2.48×10^5 pfu/ml

This titered bacteriophage preparation was then amplified in order to have a stock of bacteriophage particles. Five microliters of the above titered packaging mix was mixed with 200 ul of an overnight culture of K803 and allowed to adsorb for 20 minutes at 37°C. Ten milliliters of melted top agar was added to the bacteriophage and K803 preparation and then poured over a 150 mm TB agar plate. Six plates were prepared in this manner and were then incubated at 37°C without inversion for 6-10 hours. The plates were removed to storage at 4°C for a few hours. The soft agar was scraped into sterile 50 ml polypropylene tubes and mixed with chloroform (2 ml/100 ml agar). The suspension was centrifuged at 3,000 g for 10 minutes and the supernatant, containing the amplified library, was decanted into a sterile tube and stored at 4°C. The amplified library was titered as above. This amplified library was titered at 2.1 x 10^9 pfu/ml.

Screening of amplified canine genomic DNA library

Primary screening plates were prepared by incubating 9.5 ul of the canine amplified library (2 x 10^4 pfu/plate) with 200 ul of K803 and plating on 150 mm TB plates with 10 ml of melted top agar. It was observed that plating with 2 x 10^4 pfu gave clear plaques which were evenly spread out and not overcrowded on the plate. The plates were inverted and incubated overnight at 37°C along with negative control plates which had only K803.

The plate covers were removed and plates were air dried at room temperature for 30 minutes. Each plate was labeled with a number and a corresponding nitrocellulose filter was placed on the agar with forceps. After allowing 20 minutes for adsorption of plaques, the filters were punched with 6-10 holes through the filter and agarose using a clean 18-gauge needle. The filters were removed and washed sequentially (60 seconds/ wash) by immersing into 100 ml of each of the following solutions: 1) 0.2 M sodium hydroxide, 1.5 M sodium chloride, 2) 2 x SSC, 0.4 M Tris-HCl (pH 7.4) and 3) 2 x SSC (0.3 M sodium chloride, 0.03 M sodium citrate). While immersed, the filters were washed in the buffers by gentle rotation. The filters were dried with the plaque sides up for 1 hour at room temperature and then baked in a vacuum dryer for 2 hours at 80°C.

These filters were prehybridized for 12-16 hours at 42°C in 40% deionized formamide (Boehringer Mannheim Biochemicals), 10% dextran sulfate (Sigma Chemical Company), 0.6 M sodium chloride and 0.06 M sodium citrate and then hybridized at 42°C with the radiolabeled rabbit CH gamma cDNA probe for 24 hours. The filters were stringency washed in 0.015 M sodium chloride, 0.015 M sodium citrate and 0.05% SDS. These washes accommodated for approximately 20% mismatch (26). The first wash was performed at room temperature for 15 min and was followed by 3 successive washes at 60°C for 15-30 minutes each. The filters were air dried for 30 minutes and then exposed to X-ray film with 1 intensifying screen for 2-3 days at -70°C. After development of the film, positive plaques were identified by the presence of dark signals on the autoradiograph. The positive plaques were matched to the corresponding filters and plates. These plaques were then picked from the plates using a sterile glass pipet tip. They were placed in 1 ml of SM buffer for 20 minutes. Dilutions were made from this supernatant (usually from the undiluted concentration to a dilution of 10^{-8}) in order to determine which plates gave the best distribution of plaques. The dilutions were plated with K803 on 90 mm TB plates for secondary and tertiary screenings.

Purification of positive bacteriophage clone

A single plaque of the positive bacteriophage clone was used to prepare a plate lysate stock. The plaque was mixed with 100 ul of K803, incubated at 37°C for 20 minutes and then mixed with 3 ml of melted top agar and poured onto a LB agar plate. The plate was incubated without inversion for 6-8 hours at 37°C. When confluent lysis had occurred, the soft top agar was gently scraped off into a sterile 50 ml polypropylene centrifuge tube. The plate was rinsed with 5 ml of SM buffer to remove any remaining top agar. Eight plates were prepared in this way. These suspensions were mixed with 100 ul of chloroform and then rotated for 15 min at 37°C. The tubes were centrifuged at 4000 g for 10 minutes at $4^{O}C$. The supernatants were stored with 1 drop of chloroform at $4^{O}C$.

Five hundred milliliters of LB medium was inoculated with 500 ul of an overnight culture of K803 bacteria. The culture was incubated at $37^{O}C$ with vigorous shaking until the O.D.$_{600}$ reached 0.5. It was then inoculated with 2 ml of plate lysate stock. The incubation was continued with shaking until lysis had occurred (12-16 hours). At this point, 10 ml of chloroform was added and the culture was shaken for 10 minutes.

The culture was cooled to room temperature. Pancreatic DNase I and RNase (Boehringer Mannheim Biochemicals) were added to a final concentration of 1 ug/ml and incubated for 30 minutes at room temperature. Solid sodium chloride was added (29.2 g/500 ml) and dissolved by swirling. The culture was put on ice for 1 hour. Cellular debris was removed by centrifugation at 11,000 g for 10 minutes at $4^{O}C$. Solid polyethylene glycol (molecular weight 8,000) was added to the supernatant (50 g/500ml) and dissolved by slow stirring at room temperature. The culture was then cooled in ice water for 1 hour. The precipitated bacteriophage particles were collected by centrifugation at 11,000 g for 10 minutes at $4^{O}C$. The supernatant was discarded and the pellet was resuspended in 8 ml of SM buffer. The suspension was extracted with an equal volume of chloroform/isoamyl alcohol, vortexed for 30 seconds and centrifuged at 3,000 g for 15 minutes at 4°C. Bacteriophage particles were removed from the aqueous phase by centrifugation at 44,100 g for 2 hours at 4°C. The supernatant was poured off and the pellet was allowed to dissolve in 2 ml of SM buffer overnight.

Proteinase K (Boehringer Mannheim Biochemicals) and SDS were added to the suspension at final concentrations of 50 micrograms (ug)/ml and 0.5% respectively. After mixing by inversion, the tube was incubated for 1 hour at 56°C. The digestion mixture was cooled to room temperature and extracted with an equal volume of phenol. The phases were separated by centrifugation at 3,000 g for 5 minutes at room temperature. The aqueous phase was extracted with a 50:48:2 mixture of phenol and chloroform/isoamyl alcohol, centrifuged and the recovered aqueous phase was extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The final aqueous phase was mixed with 1/10 volume 3 M sodium acetate (pH 7.0) and 2 volumes of 100% ethanol (stored at -20°C). The solution was incubated for 30 minutes at room temperature and then centrifuged at 12,000 g for 2 minutes at 4°C. The bacteriophage pellet was washed twice in 70% ethanol, air dried at room temperature for 2 hours and then dissolved in TE buffer (0.01 M Tris-HCl, pH 7.4, 0.1 mM EDTA, pH 8.0). The DNA was quantified as described previously.

Pulsed field gel electrophoresis of bacteriophage DNA

Intact bacteriophage DNA (4 ug) was electrophoresed using the Clamped Homogenous Electric Fields (CHEF)-DRII system (Bio-Rad). The DNA was loaded into a 1% SeaKem GTG (FMC) agarose gel (14.5 cm x 12.5 cm) and submerged into 0.5 X Tris-borate EDTA (TBE) buffer (0.089 M Tris-borate, 0.089 M boric acid and 2 mM EDTA). The gel was electrophoresed for 24 hours at pulse times of 50 seconds (initial) ramped to 90 seconds (final) at 200 V at 14°C with buffer recirculation.

After the run , the gel was stained in ethidium bromide and photographed as described previously. The length of the intact bacteriophage DNA fragment was measured and compared to the ruler in the photograph. The length was estimated by performing a least-squares analysis by computer algorithm in relation to migration of lambda ladder marker (Bio-Rad).

Isolation of positive insert DNA

Intact bacteriophage DNA was digested overnight with Sal I restriction endonuclease (Boehringer Mannheim Biochemicals) in Buffer H at 37°C. The digest was submerged in 1 X TAE buffer and electrophoresed overnight on a 0.5% SeaKem GTG (FMC) agarose gel (25 cm x 15 cm) at 40 V. After staining the gel with ethidium bromide, DNA fragments of approximately 8 Kb were excised from the gel. It was determined by hybridization analysis with the rabbit CH gamma cDNA probe, that the 8 Kb fragment of the Sal I digest contained the positive insert DNA (hybridization analysis technique will be discussed later). These gel slices were melted at 65°C and kept liquid at 37°C.

The DNA was recovered from the agarose by electroelution. A stock of 1% SeaKem GTG (FMC) agarose was prepared in 1 x TAE buffer and 1.5 ml was placed in a 2.0 ml polypropylene tube. After hardening for 5-10 minutes, 500 ul of liquified gel slices were added and allowed to harden for 10-15 minutes. A final layer of 1% agarose was applied and hardened. Approximately 200 ul of 1 X TAE was added to the top of the tube so that a convex meniscus extended above the tube. The closed portion of the cap was cut open with a scalpel blade. A small piece of dialysis membrane (8-15 kDa molecular weight cut-off) was placed over the top of the tube with forceps, taking care not to introduce any air bubbles into the buffer. The membrane was fixed in place by inserting the opened cap. The tip of the tube was cut off with a scalpel blade. This electroelution cell was placed in a plexiglass holder and immersed in a submarine electrophoresis chamber with the capped end facing the cathode. The electrophoresis chamber was filled with 1 X TAE buffer. After removing all air bubbles surrounding the electroelution cell, a current of 70 mA was passed through the apparatus for 2 hours.

After this period, the current was reversed for 1 minute to remove the electroeluted DNA from the membrane to the buffer. The cap and membrane were carefully removed with forceps and the buffer was transferred to a clean 2.0 ml tube. The eluted DNA was precipitated by adding 1/10 volume 3 M sodium acetate and 2.5 volumes of ice cold 100% ethanol. The tube was centrifuged at 12,000 g for 20 minutes. The pellet was washed twice in 70% ethanol and then dried in a speed-vac (Savant) on high temperature for 10 minutes. The DNA was resuspended in approximately 100 ul of TE buffer and quantified.

Restriction mapping of insert

Intact bacteriophage DNA and electroeluted insert DNA were digested with a series of restriction endonucleases and appropriate buffers. Initial single digests were prepared overnight with Eco RI, Bam HI, Hind III, Pvu II and Bgl II at 37°C. Double digest combinations of these enzymes were also prepared. Each digest contained 4 ug of the appropriate DNA. A second set of single digests were prepared overnight with Acc I, Hind III, Pst I, Hind II at 37°C and Sma I (25°C). Double digest combinations of these enzymes were also prepared. These digests were electrophoresed in 1 X TAE buffer on 1.0% SeaKem GTG (FMC) agarose gels (25 cm x 15 cm) overnight at 50 V. The gels were stained with ethidium bromide and then photographed as described earlier. These gels were then denatured in 1.5 M sodium chloride and 0.5 M sodium hydroxide for 45 minutes and then neutralized in 1 M Tris-HCl (pH 7.5) and 1.5 M sodium chloride for 15 minutes. DNA fragments in the gel were transferred overnight by capillary action in 10 X SSC (1.5 M sodium chloride, 0.15 M sodium citrate) to a piece of nylon membrane (Bio-Rad) by the method of Southern (27).

The nylon membranes were rinsed in 2 X SSC, air dried for 1 hour and then baked in a vacuum dryer for 2 hours at 80°C. The membranes were prehybridized, as described previously for library screening of filters, and then hybridized with the radiolabeled rabbit CH gamma cDNA probe for 24 hours.

After hybridization, stringency washes were performed, as described previously for filters. The membranes were air dried for 30 minutes and then exposed to Kodak XAR film with 1 intensifying screen each, for 1-3 days at -70°C.

The lengths of the DNA fragments generated on these gels were measured as decribed earlier. These lengths were estimated by performing a least-squares analysis in relation to the migration of fragment lengths of a Hind III digest of the bacteriophage lambda DNA. The lengths of the hybridizing fragments on the autoradiographs were measured using a ruler and estimated as described.

Hybridization analysis of dog, rabbit, horse, cattle, sheep, and human genomic DNA with rabbit CH gamma cDNA probe

High molecular weight DNA (10 ug) isolated from each species was digested overnight at 37°C with Eco RI restriction endonuclease (4 U/ ug) in buffer A. Canine genomic DNA was digested with a variety of restriction endonucleases and their appropriate buffers: Bam HI, Bgl II, Hae III, Hind III, Not I, Nru I, Kpn I, Pst I, Pvu II, Rsa I, Sal I, Xba I, Sma I (25°C) and Taq I (65°C). These digests were electrophoresed overnight in 1 X TAE buffer on a 0.8% SeaKem GTG agarose gels (25 cm x 10 cm) at 50 V.

These gels were treated as described for the restriction mapping gels. The lengths of the hybridizing fragments were measured and analyzed as described previously.

Polymerase chain reaction analysis of canine genomic and insert DNA

Immunoglobulin heavy and light chain primers were synthesized by the Michigan State University Macromolecular Structural Facility. The heavy chain (HC) 5' end primer sequence was obtained from a paper by Y.L. Chiang et al, 1989 (28). The HC 3'end primer sequence was derived by comparing amino acid sequences of CH1 regions (amino acids 142 to 154) in mouse, human, rabbit and guinea pig immunoglobulin G molecules (29). Both light chain (LC) 5' and 3' primer sequences were obtained from a paper by Y.L. Chiang et al, 1989 (28). These sequences are listed below:

HC 5': 5' CCCGAATTCGATGTGCATCTTCAGGAGTCGGGACCT 3'
HC 3': 5' CCCGAATTCCTCAGGGAAATAGCCCTTGACCAGGCA 3'
LC 5': 5' CCCGAATTCGACATTGTGCTGACCCAATCTCCAGCTTC 3'
LC 3': 5' CCGAATTCGATGGATACAGTTGGTGCAGCATCAGCCCG 3'

Primers were desalted by running over C18 Sep-Pak columns (Waters). They were then dried in a speed-vac and reconstituted in double-distilled water to a final concentration of 2 mM. Reaction mixes were prepared in sterile, siliconized microfuge tubes for human, cattle, sheep, horse and dog DNA samples. Each heavy chain reaction mix consisted of 32 ul sterile double-distilled water, 10 ul 10 X reaction buffer (Perkin Elmer), 16 ul dNTP mix (Perkin Elmer), 10 ul each primer, 10 ul DNA template (0.1 ug/ul), 10 ul 1 mM tetramethylammonium chloride (TMAC) and 2 ul of a 1:4 dilution (2.5 U/reaction) of Taq Polymerase enzyme (Perkin Elmer). TMAC was added to reaction mixes in order to reduce non-specific priming events. Each light chain reaction mix consisted of 52 ul double-distilled water, 10 ul 10X reaction buffer, 16 ul dNTP mix, 2.5 ul each primer, 10 ul DNA template, 5 ul TMAC and 2 ul of a 1:4 dilution (2.5 U/reaction) of Taq Polymerase enzyme. All the reagents were spun down, vortexed gently and spun down once more.

Mineral oil (100 ul) was layered over each sample to prevent evaporation.

The tubes were loaded into the DNA Thermal Cycler (Perkin Elmer)and incubated at 94°C (1 minute), 55°C (2 minutes) and 72°C (3 minutes) for heavy chain and light chain primers. After 30 cycles, 20 ul samples from each reaction were electrophoresed on a 10% polyacrylamide gel (8 cm x 7 cm) at 180 volts for 1 hour. The gel was then stained in ethidium bromide, rinsed and photographed as described previously. Lengths of amplified products were measured as described previously and then their sizes were estimated by performing a least-squares analysis in relation to migration of fragments of a Hae III digestion of pBR322 DNA (Boehringer Mannheim Biochemicals).

RESULTS

The rabbit CH gamma cDNA probe was isolated from plasmid pBR322 containing a 3.4 Kb Eco RI/Hind III restriction fragment. This fragment represents the gene encoding the CH gamma chain of rabbit IgG. The plasmid DNA was isolated as described in the material and methods, and digested with Eco RI and Hind III restriction endonucleases in order to separate the 3.4 Kb fragment from the remaining plasmid pBR322 DNA. The digested DNA was electrophoresed and the separated fragments observed after ethidium bromide staining (figure 1). The 3.4 Kb fragment was excised from the gel and radiolabeled for use as the rabbit CH gamma cDNA probe throughout this project.

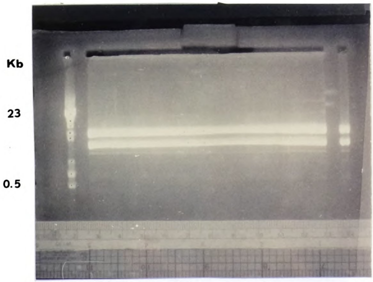

Figure 1 Ethidium bromide stained agarose gel (1.5%) of plasmid DNA with Eco RI and Hind III restriction endonucleases.

A canine genomic DNA bacteriophage library was constructed and screened using the radiolabeled rabbit CH gamma cDNA probe. The genomic library was constructed using the Promega EMBL3 Bam HI Arms Cloning System. Canine genomic DNA was digested with Sau 3A restriction endonuclease in order to prepare genomic insert fragments between 9 and 20 Kb which are required for insertion within the EMBL3 vector arms. Digests were prepared and samples removed after 2.5, 5, 10 and 15 minutes, in order to select the digest achieving the best 9-20 Kb distribution of fragments. The samples were electrophoresed, as described in the materials and methods, and after the gel was ethidium bromide stained, it was observed that the 10 minute Sau 3A digest gave the best distribution of fragments (figure 2). The bacteriophage library was then constructed, as described previously, with the canine DNA from this timed digest.

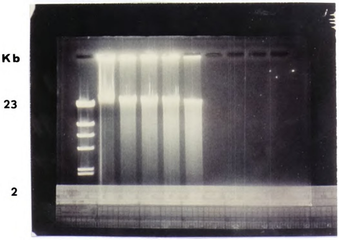

Figure 2 Ethidium bromide stained agarose gel (0.8%) of timed Sau 3A digests of canine genomic DNA for construction of genomic DNA library

The canine genomic DNA bacteriophage library was titered at 2.48 x 10^5 plaque forming units (pfu)/ml. The library was then amplified, as described previously, and titered at 2.1 x 10^9 pfu/ml. From this amplified library primary TB/agar plates were prepared and nylon filter lifts were made for screening by hybridization with the rabbit CH gamma cDNA probe. Approximately 8 x 10^5 primary plaques were screened (figure 3a). From these primary screenings, 15 positive plaques were chosen for further screening. After secondary and tertiary plaque purifications (figures 3b and 3c), 14 of the 15 plaques were eliminated as they no longer hybridized to the probe. Therefore, 1 positive bacteriophage clone was identified by hybridization to the rabbit CH gamma cNA probe. This clone contained a canine insert fragment which hybridized to the probe and its further characterization is described below.

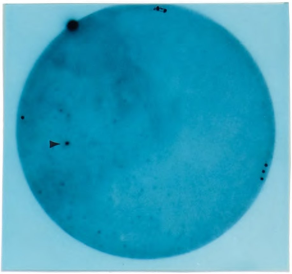

Figure 3 a) Autoradiograph of a nylon filter lift from a primary plate. This plate has 1 positive clone.

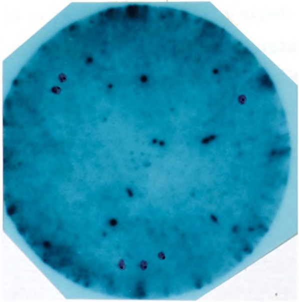

Figure 3 b) Autoradiograph of a lift from a secondary plate. This plate has 8 positive clones.

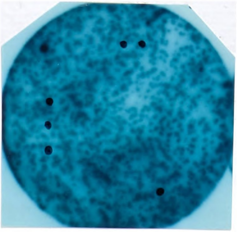

Figure 3 c) Autoradiograph of a lift from a tertiary plate. All plaques are positive after hybridization with the rabbit CH gamma cDNA probe.

This positive bacteriophage clone was grown in culture with K803 bacteria in order to accumulate a stock of the clone. The intact phage DNA was isolated as described previously, and the size estimated by electrophoresing the DNA on a Clamped Homogenous Electric Fields system (CHEF-DR II) (figure 4). The bacteriophage DNA was estimated to be approximatly 48 Kb.

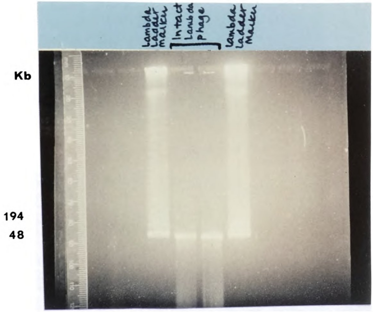

Figure 4 Clamped Homogenous Electric Fields agarose gel (1%) of intact bacteriophage DNA stained with ethidium bromide.

In order to recover the canine insert DNA contained within the bacteriophage clone, the phage DNA was digested with Sal I restriction endonuclease. This enzyme was used as it cuts at restriction sites which are exterior to the Bam HI restriction sites within which the insert DNA was cloned (figure 5). The Sal I digest preparation was

electrophoresed as described previously (figure 6), and sizes of the fragments were estimated (table 1). It was observed after staining the gel with ethidium bromide, that the 19.6 and 7.9 Kb fragments of this Sal I digest were of equal intensity. The 9.7 Kb fragment was less intense.

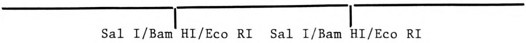

Figure 5 Diagram of restriction sites surrounding the cloning regions of the EMBL3 vector arms

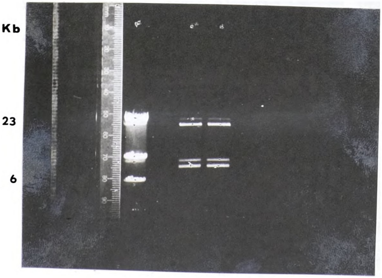

Figure 6 Ethidium bromide stained agarose gel (0.7%) of Sal I digest of intact bacteriophage DNA.

Table 1 Sizes of fragments generated from Sal I digestion of intact bacteriophage DNA

(Kb)
22.3
19.6
9.7
7.9

In order to determine which fragment of the Sal I digest of the bacteriophage DNA corresponded to the canine insert DNA, the digested DNA was transferred to a nylon membrane and hybridized with the rabbit CH gamma cDNA probe. After development of the autoradiograph, it was observed that the 7.9 Kb fragment of the Sal I digest hybridized to the probe (figure 7). The 19.6 and 9.7 Kb fragments represented the left and right arms of EMBL3 cloning vector respectively. The 22.3 Kb fragment was attributed to incomplete digestion of the bacteriophage construct DNA. The 7.9 Kb fragment corresponded to the canine insert which was contained within the isolated bacteriophage clone. This insert DNA contains a sequence which is at least 80% homologous to the 3.4 Kb rabbit CH gamma cDNA probe. This percentage of homology is based on the stringency conditions of the hybridization washes performed on the nylon membrane after hybridization with the rabbit CH gamma cDNA probe.

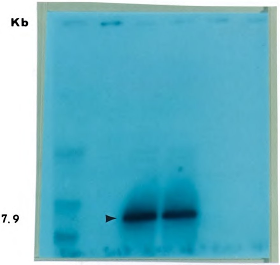

Figure 7 Autoradiograph of hybridized southern transfer of Sal I digest of intact bacteriophage DNA.

In order to further characterize the canine insert DNA, restriction mapping was initiated. Since the 3.4 Kb rabbit CH gamma cDNA probe contains the gamma gene sequence it was necessary to determine which portion of the 7.9 Kb fragment actually hybridized to the 3.4 Kb rabbit CH gamma cDNA sequence. The canine insert DNA was initially digested with Eco RI, Bam HI and Hind III restriction endonucleases. These digests were prepared individually and in double combinations. The digests were electrophoresed as described previously, and the gel stained with ethidium bromide (figure 8). The sizes of the fragments generated were estimated (table 2). Of these digests, only Hind III gave digestion of the insert DNA into smaller fragments. The insert DNA was not digested by Eco RI and Bam HI. It was observed that the length of the undigested fragments in the Eco RI and Bam HI digests were greater than 7.9 Kb. The sum of the resulting fragments of the Hind III digest was also greater than 7.9 Kb.

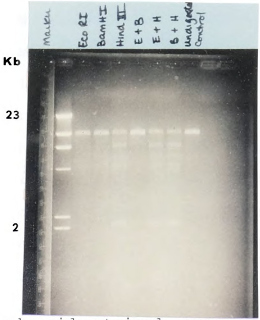

Figure 8 Ethidium bromide stained agarose gel (0.8%) of Eco RI, Bam HI and Hind III digests of canine insert DNA.

Table 2 Sizes (Kb) of fragments generated by Eco RI, Bam HI and Hind III single and double digests of the canine insert DNA

Eco RI	Bam HI	Hind III	(E+B)	E+H)	(B+H)	U
10.9	10.9	10.9	10.9	10.9	10.9	10.9
		7.4		7.4	7.4	
		5.8		5.8	5.8	
		4.7		4.7	4.7	
		2.0		2.0	2.0	
		30.8		30.8	30.8	

U= undigested canine insert DNA control

As restriction mapping is dependent upon cutting of the fragment of interest with more than one endonuclease, additional digest were prepared. These digests were also prepared in order to determine why the sum of the resulting Hind III digestion fragments was greater than 7.9 Kb. Both the intact bacteriophage construct DNA and the canine insert DNA were digested with the following endonucleases: Acc I, Hind III, Pst I, Sma I and Hind II. Single digests (figures 9 and 10) and double digest combinations (figures 11, 12, 13 and 14) were prepared. The digests were electrophoresed on agarose gels, transferred to nylon membranes and the membranes were hybridized with the rabbit CH gamma cDNA probe. The sizes of the resulting fragments for the single digests were estimated and totalled (see table 3). The sizes of the resulting fragments for the double digest combinations were estimated and totalled (tables 4 and 5). The sums of the resulting fragments in all the single and double digests of the canine insert DNA were greater than

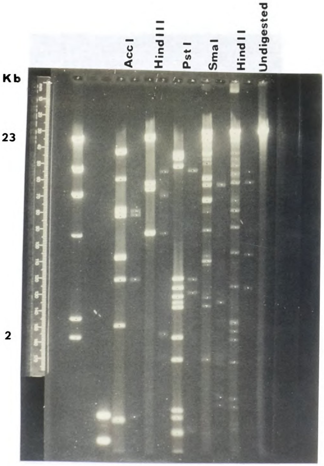

Figure 9 Ethidium bromide stained agarose gel (0.8%) of Acc I, Hind III, Pst I, Sma I and Hind II digests of intact bacteriophage construct DNA and canine insert DNA. For each digest, left lane = intact phage DNA and right lane = canine insert DNA

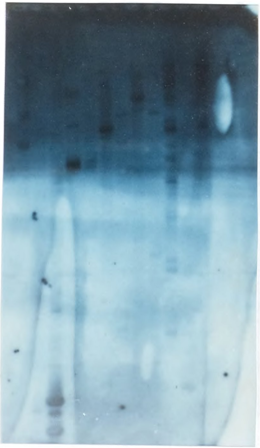

Figure 10 Autoradiograph of Southern transfer of gel pictured previously

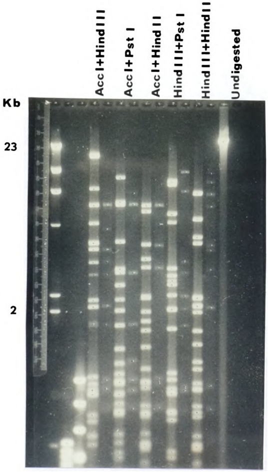

Figure 11 Ethidium bromide stained agarose gel (0.8%) of double digest combinations of Acc I, Hind III, Pst I and Hind II restriction enzymes of intact bacteriophage construct DNA and canine insert DNA. For each digest, left lane= intact phage DNA and right lane= canine insert DNA

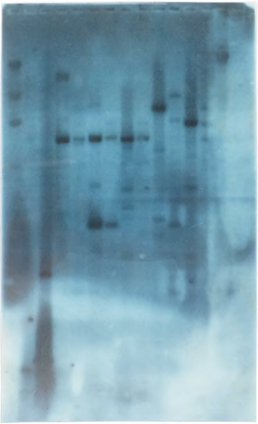

Figure 12 Autoradiograph of Southern transfer of gel pictured previously

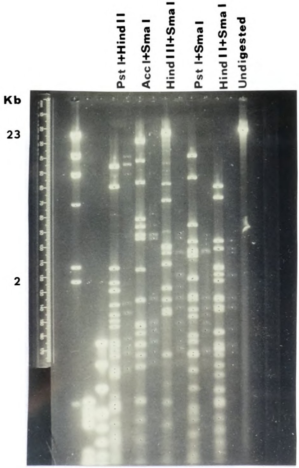

Figure 13 Ethidium bromide stained agarose gel (0.8%) of double digest combinations of Acc I, Hind III, Pst I, Hind II and Sma I of intact bacteriophage DNA and canine insert DNA. For each digest the left lane= intact bacteriophage DNA and right lane= canine insert DNA

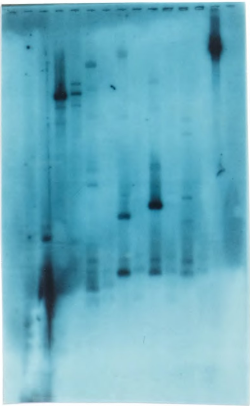

Figure 14 Autoradiograph of Southern transfer of gel pictured previously

Table 3 Sizes of fragments generated by single digests (Kb) (* indicates the fragments which hybridized with rabbit CH gamma cDNA probe)

	Intact	Insert
Acc I	*15.3	*5.4
	*8.5	*5.2
	5.6	3.0
	*5.3	1.4
	*5.1	15.0
	3.5	
	3.0	
	2.2	
	1.4	
	49.9	
Hind III	27.4	*9.7
	*7.8	*6.7
	7.3	4.3
	*4.4	2.1
	46.9	22.8
Pst I	13.3	*10.3
	10.9	3.0
	*3.0	*2.7
	*2.8	16.0
	2.6	
	2.5	
	2.1	
	1.9	
	1.5	
	1.4	
	1.3	
	43.3	
Sma I	15.6	7.9
	13.3	2.6
	12.1	*1.9
	8.8	1.6
	*7.9	1.5
	6.4	15.5
	*5.5	
	*4.5	
	3.6	
	3.5	
	3.3	
	*2.7	
	*2.5	
	*1.9	
	91.6	

Hind II	16.0	10.2
	13.9	* 8.3
	11.6	3.7
	9.6	2.9
	* 8.3	22.2
	6.4	
	5.6	
	4.7	
	3.7	
	2.9	
	2.3	
	2.2	
	2.1	
	1.9	
	1.6	
	1.5	
	94.3	
Undigested	52.2	

Table 4 Sizes of fragments generated by double digests (Kb) (* indicates the fragments which hybridized with rabbit CH gamma cDNA probe)

	Intact	Insert
Acc I + Hind III	6.8	* 5.4
	*5.3	4.0
	4.2	3.3
	*3.5	2.9
	*3.2	2.7
	2.9	2.2
	2.7	* 1.9
	2.2	1.5
	2.1	1.4
	1.9	25.3
	1.5	
	1.4	
	1.38	
	1.35	
	1.2	
	1.19	
	1.12	
	1.10	
	45.04	
Acc I + Pst I	8.6	* 6.3
	5.6	* 5.4
	*5.3	* 2.7
	*3.5	1.9
	2.8	1.6
	*2.7	1.4
	2.5	1.3
	2.2	1.2
	2.1	21.8
	1.9	
	1.7	
	1.6	
	1.5	
	1.4	
	1.35	
	1.3	
	1.2	
	1.14	
	1.1	
	1.06	
	1.03	
	51.58	

Acc I + Hind II	*5.3	*5.3
	4.9	4.3
	*3.5	3.4
	*3.0	2.8
	2.8	1.5
	2.3	1.4
	2.0	18.7
	1.9	
	1.8	
	1.7	
	1.5	
	1.43	
	1.4	
	1.28	
	1.24	
	1.14	
	1.10	
	1.00	
	39.29	

Hind III + Pst I	7.9	*9.8
	*7.4	*6.6
	*4.7	3.0
	4.3	*2.7
	2.8	2.3
	*2.7	2.1
	2.6	1.9
	2.5	1.4
	2.3	1.3
	2.1	31.1
	1.9	
	1.5	
	1.45	
	1.4	
	1.3	
	1.25	
	1.20	
	1.10	
	1.00	
	56.10	

Hind III + Hind II	*6.3	9.6
	4.5	*7.9
	3.7	*6.5
	*3.5	*5.3
	2.3	3.5
	*2.1	3.3
	2.08	2.8
	1.8	2.1
	1.7	1.4
	1.6	1.3
	1.5	43.7
	1.4	
	1.37	
	1.28	
	1.25	
	1.23	
	1.10	
	1.09	
	39.80	
Undigested	34.1	

Table 5 Sizes of fragments generated by double digests (Kb)
(* indicates fragments which hybridized with rabbit CH gamma cDNA probe)

	Intact	Insert
Pst I + Hind II	*5.3	*6.5
	3.7	1.8
	2.3	1.7
	2.0	1.45
	1.9	1.4
	1.78	1.36
	1.68	1.30
	1.6	15.51
	1.55	
	1.44	
	1.4	
	1.37	
	1.34	
	1.26	
	1.23	
	1.21	
	1.1	
	1.06	
	1.05	
	0.98	
	0.94	
	36.19	
Acc I + Sma I	5.7	3.0
	3.5	2.9
	*3.2	1.8
	3.0	*1.6
	*2.9	1.5
	2.3	1.44
	*1.8	1.40
	*1.6	1.30
	1.57	14.94
	1.5	
	1.4	
	1.34	
	1.30	
	1.10	
	1.00	
	0.90	
	34.11	

Hind III + Sma I	*5.6	2.6	
	4.3	2.1	
	3.1	2.0	
	2.75		1.87
	2.7	*1.7	
	2.6	*1.6	
	*2.4	1.5	
	2.0	1.44	
	1.8	1.40	
	*1.74	16.14	
	1.70		
	1.30		
	1.20		
	1.10		
	0.90		
	35.19		

Pst I + Sma I	2.8	2.8
	2.7	2.6
	*2.6	*1.9
	2.5	*1.7
	2.4	1.68
	2.1	1.60
	*1.89	1.50
	1.8	1.40
	*1.7	15.18
	1.66	
	1.6	
	1.5	
	1.44	
	1.36	
	1.34	
	1.24	
	1.2	
	1.05	
	1.03	
	1.0	
	0.94	
	0.90	
	0.87	
	45.72	

Hind II + Sma I	2.8	2.65
	*2.6	2.6
	*2.3	2.1
	2.1	*1.85
	2.0	*1.75
	1.9	1.5
	*1.8	1.4
	1.77	1.36
	*1.72	1.30
	1.5	16.51
	1.43	
	1.4	
	1.35	
	1.3	
	1.2	
	1.14	
	1.09	
	1.06	
	1.0	
	0.96	
	0.92	
	0.88	
	34.22	
Undigested	33.7	

7.9 Kb. It was observed that the sums of the resulting fragments of all the digests on the canine insert DNA were approximately 16.0 Kb. We then had to assume that the canine insert DNA was a 16.0 Kb fragment which had been inserted by chance within the bacteriophage clone. Upon Sal I digestion of the bacteriophage construct DNA, the canine insert was digested at an internal Sal I site and yielded two 7.9 Kb fragments. Since these two fragments were of the same size they could not be separated by resolution on the gel described previously (figure 6) and migrated as a "doublet". The intensity of the 7.9 Kb fragment after staining with ethidium bromide was due to the presence of the "doublet".

Given that the insert appeared to be 16.0 Kb and could not be easily used for restriction mapping, several alternative approaches were undertaken to isolate the canine IgG constant heavy gene. In order to confirm the use of the rabbit CH gamma cDNA probe for hybridization of mammalian Ig gamma genes, Eco RI restriction endonuclease digests were prepared on rabbit, dog, human, cow, sheep and horse genomic DNA. The digests were electrophoresed on agarose gels, transferred to a nylon membrane and the membrane hybridized with the rabbit CH gamma cDNA probe (figure 15). The probe hybridized strongly to digested fragments in the rabbit, cow and sheep. However, very weakly to the digested fragments in the dog, human and horse (table 6).

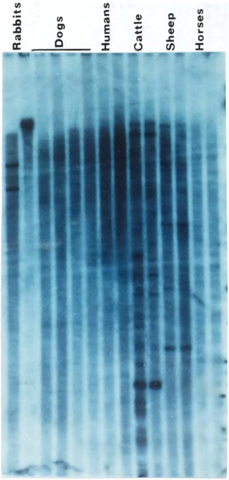

Figure 15 Autoradiograph of Southern transfer of Eco RI digest of rabbit, dog, human, cow, sheep and horse genomic DNA hybridized with radiolabeled rabbit CH gamma cDNA probe.

Table 6 Sizes of fragments (Kb) which hybridized to rabbit CH gamma cDNA probe after Eco RI digestion

Rabbit	Dog	Human	Cow	Sheep	Horse
16.0	9.4	33.2	4.5	16.6	18.9
10.6	8.9	25.6	4.0	6.9	14.5
	3.2		3.86	2.8	5.8
			2.3		5.2
					5.1
					2.5

Due to the weak hybridization signal observed above for the Eco RI digest dog genomic DNA, 14 other restriction endonuclease digests were prepared on the dog genomic DNA in order to establish if there could be other fragments generated which may hybridize to the rabbit probe. If a single fragment of a particular digest hybridized and could be isolated, this fragment could be further used as a canine-specific probe for the canine Ig gamma gene. Dog genomic DNA was digested with the following endonucleases: Bam HI, Bgl II, Hae III, Hind III, Kpn I, Not I, Nru I, Pst I, Pvu II, Rsa I, Sal I, Xba I, Sma I and Taq I. These digests were electrophoresed on an agarose gel, transferred to nylon membrane and the membrane hybridized with the rabbit CH gamma cDNA probe (figure 16) . The sizes of the hybridizing fragments were estimated (table 7).

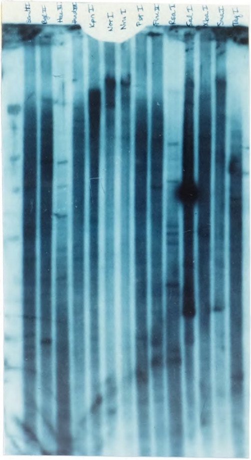

Figure 16 Autoradiograph of Southern transfer of dog genomic DNA digested with multiple restriction enzymes

Table 7 Sizes of hybridizing fragments (Kb) of dog genomic DNA digested with multiple restriction endonucleases

Bam HI	Bgl II	Hae III	Hind III	Kpn I	Not I	NruI
2.4	10.2	10.2	18.1	19.7	15.9	15.3
2.1	2.2	7.6		5.2		
2.0		5.5				

Pst I	Pvu II	Rsa I	Sal I	Xba I	Sma I	Taq I
5.7	7.1	12.9	6.7	14.8	2.7	6.4
2.2		7.8	2.6			1.7
		4.9				
		4.7				
		4.3				
		3.1				
		2.0				
		1.9				

A comparison was made between the hybridizing fragments generated from the Sma I and Pst I digests of the dog genomic DNA (table 7) and the hybridizing fragments of these same digests on the canine insert DNA (table 3). In both Pst I digests there were similar fragments of approximately 2.0 Kb. In the Sma I digest of the dog genomic DNA there was a 2.7 Kb fragment and in the same digest of the canine insert DNA, two fragments of 1.9 and 1.5 Kb. This could suggest that the dog CH gamma gene may be located on a stretch of DNA which approximately 2.0 Kb.

As hybridization analysis of repeated multiple digests of dog genomic DNA yielded inconsistent hybridizing fragments, another approach was undertaken. During the hybridization analyses described above, primers became available for amplification of variable heavy and light chain (VH and VL) regions of the Ig gamma gene, polymerase chain reaction (PCR) analysis was performed on dog, horse, cow, sheep and human genomic DNA. This approach was taken in an effort to amplify canine-specific regions of the Ig gamma gene. PCR was performed across species in order to establish if the primers would amplify sequences in these species. Initial heavy chain amplification products were electrophoresed on a polyacrylamide gel as described previously, and the gel was stained with ethidium bromide (figure 17). It was observed that there was more than one heavy chain amplification product in each of the species. The fragment sizes were estimated (table 8). However, it was noted that the canine amplified products were smaller than expected. These products were expected to be 500 bp or greater.

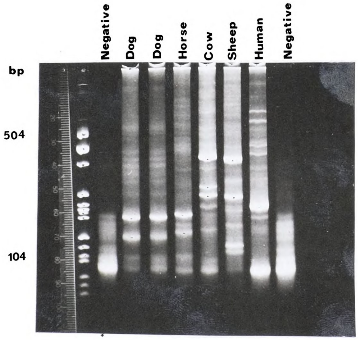

Figure 17 Heavy chain amplifications of dog, horse, cow, sheep and human genomic DNA electrophoresed on a 4% polyacrylamide gel. The gel was stained with ethidium bromide.

Table 8 Sizes (bp) of heavy chain amplified products

Dog	Horse	Cow	Sheep	Human
173	176	361	355	994
140	144	245	211	759
		218		449
				397
				188

In order to confirm that the heavy chain amplified products in the dog were representative of a Ig heavy chain sequence, the 173 and 140 bp fragments were excised from an agarose gel, radiolabeled as described for the rabbit CH gamma cDNA probe and used to hybridize Southern transfers of Sma I and Kpn I digests (figure 18) of each of the species mentioned above. It was hypothesized that if these products hybridized across species, it would be an indication that these heavy chain amplified products were representative of the Ig gamma heavy chain gene and shared homology with Ig gamma heavy chain sequences in the other species. The 173 bp radiolabeled fragment hybridized only to dog genomic DNA digested with the two restriction endonucleases. The sizes of the hybrdized fragments were estimated (table 9). The 140 bp radiolabeled product did not hybridize to digested DNA in any of the species. This experiment was repeated and yielded the same results.

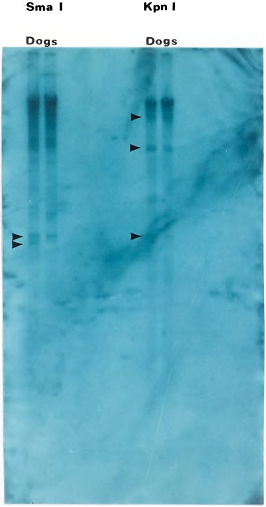

Figure 18 Autoradiograph of Southern transfer of Sma I and Kpn I digests of dog, horse, sheep, cow and human genomic DNA hybridized with radiolabeled 173 bp heavy chain amplified product of the dog.

Table 9 Sizes (Kb) of fragments which hybridized to radiolabeled 173 bp heavy chain amplified product of the dog.

	SmaI	Kpn I
Dog	4.2	25.6
	4.4	11.9
		4.2

Light chain amplifications were performed on each of the species as described for the heavy chain amplifications. The amplified products were electrophoresed as described for the heavy chain amplified products. The gel was stained with ethidium bromide (figure 19) and the sizes of the fragments estimated (table 10). The sizes of the variable light chain amplified products of dog, horse, cow and sheep were in the expected range of approximately 400 bp or greater.

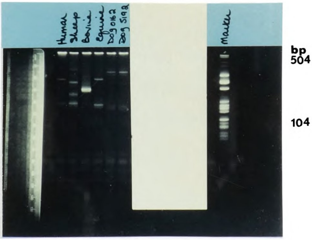

Figure 19 Light chain amplifications of human, sheep, cow, horse and dog genomic DNA electrophoresed on a 4% polyacrylamide gel and stained with ethidium bromide.

Table 10 Sizes (bp) of light chain amplified products

Human	Sheep	Cow	Horse	Dog
224	476	357	530	778
166	302			
130	232			

Variable heavy chain amplification was performed on the canine insert DNA recovered from the positive bacteriophage clone and was repeated on dog, cow, sheep, horse and human genomic DNA. These amplifications were electrophoresed as described previously and the gel stained with ethidium bromide (figure 20). The sizes of the amplified products were estimated (table 11). This time the heavy chain amplified products were closer to the expected range for the canine insert DNA as well as the genomic DNA of the species mentioned above.

This confirms that the canine insert DNA contains a sequence which is representative of the Ig gamma gene. However, due to time constraints, no further characterization was done on the amplified product of the canine insert DNA.

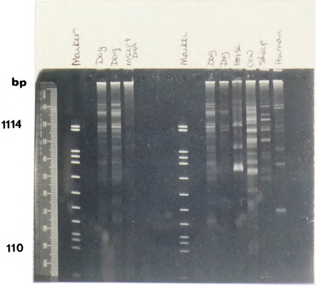

Figure 20 4% polyacrylamide gel of heavy chain amplified products from amplifications of canine insert DNA and genomic DNA of dog, horse, cow, sheep and human

Table 11 Sizes (bp) of heavy chain amplified products from amplification of canine insert DNA and genomic DNA of dog, horse, cow, sheep and human.

Dog	Insert	Horse	Cow	Sheep	Human
2385	2288	2035	1126	937	2198
1768		1511	657	763	1894
1426		1278	441	477	634
979		979		415	470
		763			219

DISCUSSION

The majority of the results presented in this paper were dependent on the molecular hybridization of the rabbit CH gamma cDNA probe to unknown nucleic acid sequences in dogs, horses, cattle, sheep and humans. The probe consisted of a 3.4 Kb cDNA fragment, encoding the rabbit CH gamma gene, which had been cloned into Eco RI and Hind III restriction sites within the plasmid pBR322. The plasmid/probe construct was transformed within E. coli HB101 bacteria, amplified and the DNA isolated. Digestion of this plasmid/probe construct DNA with Eco RI and Hind III restriction endonucleases separated the 3.4 Kb cDNA fragment from the remaining plasmid DNA (figure 1). This fragment was radiolabeled with alpha-^{32}P and subsequently used as the rabbit CH gamma cDNA probe.

The rabbit CH gamma cDNA probe was used to screen a canine genomic DNA bacteriophage library. In screening a genomic DNA bacteriophage library it was possible to search for the entire canine Ig gamma gene. The library was prepared using the fragments generated from a 10 minute Sau 3A digest of dog genomic DNA and insertng them into EMBL3 bacteriophage vector arms (figure 2). The Sau 3A restriction endonuclease was used in order to generate a 9-20 Kb distribution of fragments for insertion within the vector arms. The library was amplified and the stock titered at 2.1 x 10^9 pfu/ml. From this amplified library primary screening plates were prepared and nylon filter lifts made in order to screen plaque replicas of the plates by hybridization with the rabbit CH gamma cDNA probe. Approximately 8 x 10^5 primary plaques were screened and 15 primary plaques were chosen for further screening. Secondary and tertiary screening eliminates false positives which may have hybridized to the probe. Fourteen of the fifteen primary plaques were eliminated by these screenings. One positive bacteriophage clone remained after subsequent secondary and tertiary screenings. This bacteriophage clone contained a canine insert fragment which hybridized to the rabbit CH gamma cDNA probe.

In order to further characterize this bacteriophage clone it was necessary to accumulate a purified stock of the clone. From this stock, intact bacteriophage construct DNA was isolated and electrophoresed on a Clamped Homogenous Electric Fields system (CHEF-DR II). This system is used for electrophoresis of chromosome size DNA molecules which are usually larger than 50 Kb. Separation of DNA molecules of this magnitude is not possible by conventional agarose gel electrophoresis. Once the bacteriophage construct DNA had been resolved with this system, the size of the DNA was estimated by performing a least-squares analysis by computer algorithm in relation to migration of lambda ladder marker

(24). The bacteriophage construct DNA was approximately 48 Kb.

The canine insert DNA had to be separated from the bacteriophage DNA in order to characterize it separately from the bacteriophage DNA. The intact phage DNA was digested with Sal I restriction endonuclease. This enzyme was used as it cuts at restriction sites which are exterior to the Bam HI sites within which the insert DNA was cloned (figure 5). After digestion and separation of the digested phage DNA by electrophoresis, the transferred DNA was hybridized with the rabbit CH gamma cDNA probe. Of the 4 fragments generated after digestion (table 1) only the 7.9 Kb fragment hybridized. This implied that this fragment contained a sequence which was at least 80% homologous to the rabbit CH gamma cDNA sequence. The degree of homology was based on the stringency conditions of the hybridization washes performed on the nylon membrane after hybridization with the probe (26).

As the probe used was 3.4 Kb, it was necessary to determine which portion of the canine insert DNA had actually hybridized to the probe. Restriction mapping was initiated on the insert DNA. By digesting the insert DNA into smaller fragments and then hybridizing these fragments with the probe, it would be possible to eliminate portions of the 7.9 Kb fragment which would not possess homologous sequences to the probe. The canine insert DNA was digested with Eco RI, Bam HI and Hind III endonucleases in single and double digest combinations. It was observed that the insert was not digested by Eco RI and Bam HI. However, it was digested by Hind III. The sizes of the resulting fragments were estimated and the total sizes were greater than the expected 7.9 Kb (table 2). The Hind III digest was repeated along with 4 other restriction endonucleases: Acc I, Pst I, Sma I and Hind II (figures 9 an 10). Double digest combinations of these enzymes were also prepared (figures 11,12,13 and 14). Digests were prepared on both the intact bacteriophage construct DNA and the canine insert DNA. The sums of the resulting fragments in the digests of the canine insert DNA were still greater than the expected 7.9 Kb. The sums were approximately double the size, that is approximately 16.0 Kb. The assumption was then made that the insert DNA was 16.0 Kb and the 7.9 Kb fragment generated after Sal I digestion was two 7.9 Kb fragments being resolved simultaneously. This explained why the 7.9 Kb fragment was very intense after the agarose gel was stained with ethidium bromide (figure 6) The assumption that the canine insert DNA was 16.0 Kb may also be supported by the fact that the EMBL3 bacteriophage vector used in the construction of the canine genomic DNA library can only accommodate insert fragments which are between 9 and 20 Kb. A fragment of 7.9 Kb should have never been cloned into the vector arms. Due to the magnitude of the insert fragment and the appearance of partial digestion products, it was not possible to continue with the restriction mapping of this canine insert DNA at this time.

In order to isolate the canine CH gamma gene, two other approaches were undertaken. The first approach was to confirm the use of the rabbit CH gamma cDNA probe for hybridization of mammalian Ig gamma genes. Eco RI digests of rabbit, dog, human, cow, sheep and horse genomic DNA were electrophoresed, transferred to a nylon membrane and hybridized with the rabbit probe. The probe hybridized in all the species, however, the strength of the hybridization signals varied. The probe appeared to strongly hybridize with rabbit, cow and sheep genomic DNA but weakly with dog, human and horse genomic DNA (figure 15). Further digests were prepared on dog genomic DNA to observe whether stronger hybridization signals could be demonstrated with other fragments of genomic DNA and if a single fragment of a particular digest hybridized and could be isolated, the fragment could be used as a canine-specific probe for the canine Ig gamma gene. Digests included Bam HI, Bgl II, Hae III, Hind III, Kpn I, Not I, Nru I, Pst I, Pvu II, Rsa I, Sal I, Xba I, Sma I and Taq I (figure 16). Numerous fragments hybridized with the rabbit CH gamma cDNA probe. Hind III, Not I, Nru I and Xba I digests generated single fragments (table 7), however, these fragments were too large to radiolabel and use as probes. Pvu II and Sma I digests yielded 7.1 Kb and 2.7 Kb fragments respectively, however, when these digests were repeated these fragments did not reappear after hybridization. This may have been due to incomplete digestions and inadequate technical ability at the time of these experiments.

The comparisons made between the hybridizing fragments generated from the Sma I and Pst I digests of dog genomic DNA (table 7) and the hybridizing fragments of these same digests on the canine insert DNA (table 3)suggest that the canine Ig gamma gene may be located on a stretch of DNA approximately 2.0 Kb. However, without confirmed restriction mapping this cannot be concluded.

The second approach under taken was polymerase chain reaction (PCR) analysis of genomic DNA in a variety of species and the canine insert DNA. This approach was undertaken in an effort to amplify canine-specific regions of the Ig gamma gene. Primer sequences became available for amplification of variable heavy and light chain regions of murine Ig gamma gene. The primer sequences, described in the materials and methods section, were obtained from a paper by Y.L. Chiang et al, 1989 (28). Initial heavy chain amplifications of dog, horse, cow, sheep and human genomic DNA yielded products which were much smaller than expected. Since primer sequences amplified sequences which encoded Ig gamma chains from amino acid positions 0-154 and had two Eco

RI flanking regions (approximately 18 bp), it was expected that the amplified products should be approximately 516 bp or greater. The initial 173 bp and 140 bp canine heavy chain amplified products, despite their small sizes and in an effort to continue research with this approach, were radiolabeled and used to hybridize Southern transfers of Sma I and Kpn I digests of each of the species mentioned above (figure 18). It was hypothesized that if these products hybridized across species, they may be representative of the Ig gamma heavy chain gene. However, the 173 bp fragment only hybridized to fragments in the canine digests. The 140 bp fragment did not hybridize to any fragments. This result implies that the products were canine-specific, however , more than likely not representative of Ig gamma gene as there was no cross-hybridization with the digests of the other species.

Light chain amplification products were expected to be approximately 405 bp or greater. This was based on the primer sequences which amplified sequences which encoded variable light gamma chains from amino acid positions 0-117 and had two Eco RI flanking regions (approximately 18 bp). Light chain amplifications on the dog and the horse yielded products which were 778 and 530 bp, respectively, and in the expected range. These results were not used further in this project, however, future applications could be made if the 778 bp canine product could be combined with a canine heavy chain variable region amplified product produce a canine antigen-binding site.

Heavy chain amplification was also performed on the canine insert DNA and repeated on the genomic DNA of the mentioned species. The sizes of the amplified products were much greater than previously recorded (table 9). The amplification of the canine insert implies that the insert contains a sequence which is representative of the Ig gamma gene. A single band was generated by the amplification of the insert DNA as compared to 4 bands in the amplification of the dog genomic DNA. This may be due to the amplification of other homologous sequences in the genomic DNA. The insert DNA yielded a specific sequence when amplified by the heavy chain primers. Due to time constraints, the 2288 bp amplified product from the canine insert DNA was not further characterized. It is recommended that this fragment could be electrophoresed on an agarose gel, transferred to nylon membrane and then hybridized with the rabbit CH gamma cDNA probe in order to confirm that this fragment possesses the intact canine Ig gamma gene. The amplified fragment could be excised from an agarose gel and electroeluted, as described for the canine insert DNA in the materials and methods section. The DNA could then be radiolabeled and used to hybridize Southern transfers of digests of dog, cow, sheep, horse and human genomic DNA as were the initial dog heavy chain products.

The results discussed in this thesis have shown that the rabbit CH gamma cDNA probe has been successfully used to isolate a canine CH gamma gene. Even though restriction mapping was not completed at the end of this project, the results of the digestions performed on the canine insert DNA have established that a 16.0 Kb fragment contains the gene. A section of recommended procedures will be included in order to assist in the continuation of the restriction mapping of this fragment. The ability to amplify the canine insert DNA with variable heavy primers proves that the insert isolated from the canine genomic DNA bacteriophage library contains the variable heavy region in addition to the canine constant heavy region of the gamma gene. These results will be used in further characterization of the canine CH gamma gene. This will lead to the transfection of this gene, along with the amplified canine variable light chain gene, into a cell line for the establishment of a genetically engineered source of canine IgG.

CONCLUSIONS

This Master's thesis project has initiated the molecular characterization of the canine constant heavy gamma gene. The rabbit CH gamma cDNA probe was used to isolate a clone from a canine genomic DNA library. This clone contains a 16 Kb insert which possesses the canine constant heavy gamma gene. Restriction mapping of this isolated insert was not completed in this project, however, the results and futher research recommendations provided in this thesis will assist in continuing the molecular characterization of this gene.

This canine insert DNA was sucessfully amplified using variable heavy chain gamma gene primers. This proves that the insert DNA contains the variable heavy gamma gene in addition to the constant heavy gamma gene. With further characterization of both of these genes and both variable and heavy light chain genes, transfection of a suitable cell line with these genes will be possible. This will ultimately create a genetically engineered source of canine IgG.

RECOMMENDATIONS

This section has been included in order to provide a guideline for continued work using the results of this project. The following experiments are recommended to overcome some of the restriction mapping and in vitro amplification problems which were encountered during this project.

1. In order to eliminate partial restriction endonuclease digestion products, it is recommended that the digestion incubation times and the amount of enzyme be increased. This could ensure complete digestion of the intact phage DNA and recovered insert DNA.

2. Individual fragments resulting from digest preparations could be excised from gels, electroeluted and then digested with a second set of restriction enzymes. This process would aid in tracking fragments during progressive digestions.

3. Bal 31 is a restriction nuclease with double stranded DNA exonuclease activity (13). This enzyme progressively and bidirectionally removes mononucleotides from both strands of linear DNA. However, if one end of the insert DNA were to be labeled, the other end could be digested with

of linear DNA. However, if one end of the insert DNA were to be labeled, the other end could be digested with Bal 31. DNA could be removed at certain time points and then electrophoresed on gels. The resulting fragments could be digested with other enzymes.

4. The rabbit CH gamma cDNA probe could be broken into two or three smaller fragments which could be radiolabeled and used to hybridize Southern blots of the restriction digests. This process could aid in orientating the restriction fragments generated. The enzymes used would be dependent on the sequence of the probe (30).

5. Recently, primers have been made for the specific amplification of heavy-chain variable regions genes from mouse hybridoms cells (31). These primers may react more specifically with the heavy-chain variable sequences than the primers used previously in this project. There is a direct cloning kit - Ig-Prime System (Novagen) which has been developed for polymerase chain reaction amplification and cloning of immunoglobulin gene variable regions. These approaches may produce a more specific amplified product from dog genomic DNA which could be used as a species-specific probe.

6. The variable heavy gamma chain amplified product from the canine insert DNA should be electroeluted and

radiolabeled for use as a canine-specific hybridization probe. This probe could be used to hybridize genomic DNA digests for fragments containing the variable heavy gamma chain gene. These amplification products could be subcloned, in addition to any hybridizing fragments of the genomic DNA digests, and sequenced. Polymerase chain reaction products could also be sequenced using the asynchronous polymerase chain reaction to prepare the template (32). These sequences could then be compared to published sequences for variable heavy and constant heavy gamma genes in other species.

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