The detection and characterization of interferon-gamma from the Hawaiian green sea turtle (*Chelonia mydas*)

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ABSTRACT

Interferon-γ is of interest in the study of the immune system of the Hawaiian green sea turtle, *Chelonia mydas*. The close phylogenetic relationship between birds and reptiles led to the development of a molecular strategy, based on the amino acid and genetic sequence of chicken IFN-γ, to identify sea turtle IFN-γ. Oligonucleotide primers were constructed based on chicken DNA sequences corresponding to highly conserved amino acid regions of IFN-γ, and these primers were used to amplify potential, similar segments in sea turtle genomic DNA by PCR. No homologous sea turtle DNA fragments were obtained using this approach. Using an alternative technique designed to detect regions of homology over a longer IFN-γ DNA sequence, digoxigenin-labeled primers were synthesized to produce a larger chicken IFN-γ DNA probe. Sea turtle DNA was examined for homologous regions using the Southern blot technique. Again, no regions of homology were detected. Although the sequences of the turtle and chicken IFN-γ genes do not appear to be sufficiently related to detect homology by PCR amplification or DNA probe hybridization, further investigation is needed using other approaches to determine whether or not a IFN-γ homologous present in the *Chelonia mydas* genome.

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LIST OF ABBREVIATIONS

bp	base pairs
cDNA	complimentary DNA
ConA	Concanavalin A
DIG	digoxigenin
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
FP	Fibropapillomatosis
IFN-γ	Interferon-γ
IPTG	isopropylthio-β-D-galactoside
LB	Luria-Bertani
mRNA	messenger RNA
NIH	National Institutes of Health
Oligo	Oligonucleotide
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	Reverse transcriptase-PCR

CHAPTER 1
INTRODUCTION

1.1 Background

Proteins are biopolymers, made up of long chains of amino acids linked by peptide bonds, which show various levels of structural organization. Some proteins are combinations of different subunits that form multimeric proteins (on the quaternary structural level) that is based on the interactions of their tertiary structure, which gives it its overall three-dimensional shape, upon which its functions and biological activity depends. Its three-dimensional shape in turn is based the folding of its peptide backbone into secondary structures (including helices, loops, and coils), which in turn is based on the primary structure of its amino acid sequence.

Some proteins that have similar functions share similar three-dimensional shapes due to similar arrangements of secondary structures as a consequence of sharing similar amino acid sequences, with conserved amino acids occurring in identical positions throughout large stretches of the peptide structure. The sequences of the same protein isolated from different organisms shows this pattern as well, and allows the proteins to be classified based on similarities and differences in their amino acid sequences. This molecular taxonomy also provides information about protein function and evolutionary relationships. If the similarity among proteins of different organisms is significant over their entire sequence, then the proteins are considered homologous, and carry out similar functions. Sequence similarity also indicates an evolutionary relationship among proteins, suggesting that they evolved from a common ancestor. These homologous proteins can be grouped together in families - closely related proteins have the most similar sequences, while distantly related proteins have fewer similar sequences.

The ancestral protein of a given family slowly changes over time, as does the organism within which it evolves. However, the amino acids that are in positions critical to the maintenance of the three-dimensional structure of the protein, and hence critical to the protein's function, are usually found in the same identical position in different species. This is true of the protein studied here, interferon-γ, a protein that is critical for the immune system of various organisms, the structure of which has been evolutionarily conserved over time.

1.2 Interferons

Cytokines are regulatory peptides secreted by white blood cells and a variety of other cell types. They have pleiotropic regulatory effects on hematopoietic and other cells that participate in host defense and repair processes. Interferons are a class of cytokines that interfere with viral replication, blocking the spread of virus to uninfected cells. They are important modulators of the immune system.

There are two types of interferons, Type I and Type II. Type I interferons (which include IFN-α, IFN-ω, IFN-β, and IFN-τ), are all derived from the same ancestral gene, and have retained sufficient homology to act via the same cell surface receptor. IFN-α and β are considered antiviral interferons because they induce a state of resistance to viral replication in all cells. They bind to a common interferon receptor that is coupled to tyrosine kinase, which in turn phosphorylates STATs (signal transducing activators of transcription). The activated STATs bind to promoters of genes that induce the synthesis of host-cell proteins that contribute to the inhibition of viral replication, including oligo-adenylate synthetase and P1 kinase. The enzyme oligo-adenylate synthetase polymerizes

ATP into 2'-5' linked oligomers, which in turn activates an endoribonuclease that then degrades viral RNA. P1 kinase is a serine/threonine kinase that phosphorylates the eukaryotic protein synthesis initiation factor eIF-2, thereby inhibiting translation and consequently inhibiting viral replication.

Interferon-gamma (IFN-γ), the focus of this thesis, is a Type II interferon. It is a lymphokine that displays no molecular homology with Type I interferons, but it shares some important biological activities.

1.3 Induction of IFN-γ synthesis

IFN-γ inducers activate T cells either in a polyclonal (via mitogens or antibodies) or in a clonally restricted, antigen-specific manner. IL-1 and IL-12 released by phagocytes can activate γ:δ T cells to produce IFN-γ. Cells of the cytotoxic/suppressor phenotype (CD8+, Ly2) and cells of the T helper phenotype (CD4+, L3T4) are also involved in the production of IFN-γ. CD8+ T cells from individuals after infection with influenza or after immunization against rabies virus are stimulated to release IFN-γ when exposed to the corresponding viral antigens. Cytotoxic T cells derived from BALB/c mice that were immunized with influenza virus react specifically against influenza virus infected target cells in a MHC-restricted way and release IFN-γ. IFN-γ is produced during infection and antigen-specific IFN-γ-producing circulating CD4 + T helper cells are found in patients with recurring herpes labialis. Rabies virus-specific, MHC II-restricted, IFN-γ-producing T-helper cells are present in rabies vaccine recipients.

1.4 The IFN-γ Receptor

IFN-γ binds to a receptor (IFN-γR) that is expressed on the cell surface of most cells. IFN-γ first binds to two α-receptor chains that do not interact with each other, and which requires the β-chain in order to initiate cell signaling (Walter et al., 1995). The α-chain is identical in different cell types, however the β-chain differs and may confer different properties on the receptor. IFN-γRα and IFN-γRβ1 chains are members of the class-2 cytokine receptor family, which also include the receptors for IFN-α and -β.

The important role of the receptor was shown by Huang et al. (1993) in a study with mice that had a disrupted IFN-γ receptor gene (IFN-$\gamma R^{0/0}$ mice), and therefore no longer had a functional IFN-γ receptor. They showed no overt anomalies, and their immune system appears to develop normally, however, these mutant mice had a defective natural resistance. Compared to wild type normal mice, the IFN-$\gamma R^{0/0}$ mice had increased susceptibility to infection by *Listeria monocytogenes* & vaccinia virus despite normal cytotoxic T cell and T helper responses.

1.5 IFN-γ Signaling Pathway

The binding of IFN-γ to its receptor activates transcription of many quiescent genes. Transcriptional activation takes place via the phosphorylation of specific signal-transducing proteins, present in cytoplasm in inactive form (Lodish et al., 1995). Receptor binding of IFN-γ activates tyrosine kinases (JAK1 and JAK2). The tyrosine residues in the cytosolic domain of the receptor, as well as the cytosolic protein kinases JAK1 and JAK2 are phosphorylated. Once the phosphorylated receptor/JAK1/JAK2 complex is formed, STAT1α monomers bind to phosphotyrosine residues on the

activated complex. STAT1α molecules are then phosphorylated and released into the cytosol where they dimerize into GAFs (γ activation factor). The STAT1α dimer translocates to the nucleus and activates the transcription of genes controlled by the GAF-RE (response element) (Decker et al., 1991). Only the dimer is capable of binding to the target DNA sequence GAS (γ-IFN activation site). GAS is a consensus immediate response element (TTNCNNNAA) present in genes that are activated immediately after IFN-γ receptor-ligand binding. This binding activates the transcription of specific sets of genes. It was demonstrated experimentally by Shuai et al. (1993) that IFN-γ-dependent translocation of STAT1α to the nucleus depends on a critical tyrosine residue. Cells expressing wild type-STAT1α were treated with IFN-γ and the protein became localized to nucleus. Translocation did not occur in cells expressing mutant STAT1α (in which tyrosine-701 was replaced by phenylalanine).

1.6 IFN-γ Effects

IFN-γ has various effects on the immune system, including the modulation of expression of MHC antigens, the stimulation of macrophage activity, and the modulation of B, T, and NK cell activity.

1.6.1 Modulation of expression of MHC antigens

IFN-γ increases the expression of MHC class I molecules, TAP transporter genes, and the Lmp2 and Lmp7 components of the proteasome (Janeway et al., 1997). This enhances the ability of host cells to present viral peptides to CD8 T cells should infection occur. The increase in MHC I expression also protects uninfected host cells against attack by NK cells. IFN-γ also induces/enhances the expression of MHC class II antigens

on macrophages and T cells. The genes encoding MHC (HLA) class II need several hours of activation by IFN-γ (Amaldi et al., 1989).

1.6.2 Stimulation of macrophage activity

IFN-γ plays a role in the stimulation of macrophage activity, including tumoricidal activity and the destruction of parasites by IFN-activated macrophages. Macrophages are primed for tumoricidal activity (macrophage activating factor) by IFN-γ, which activates macrophages to kill tumor cells by releasing reactive oxygen intermediates, and by producing other cytotoxic molecules such as TNF-α, which synergizes with IFN-γ in macrophage activation. Tumor cell lysis by activated macrophages is an important mechanism of natural resistance to cancer. Th1 cells activate macrophages thru cell contact and focal secretion of IFN-γ. Activated macrophages have the ability to fuse their lysosomes more efficiently to phagosomes, make oxygen radicals and nitric oxide (NO) (which both have potent antimicrobicidal properties), and synthesize antimicrobicidal peptides (Janeway et al., 1997).

Intracellular parasite killing is activated significantly when infected macrophages are exposed to IFN-γ. Production of reactive oxygen intermediates and secretion of H_2O_2 are correlated w/capacity of macrophages to kill intracellular parasites. Munoz-Fernandez et al. (1992) showed that T cell-derived IFN-γ and TNF-α synergistically activate mouse macrophages for the killing of intracellular *Trypanosoma cruzi*, through a nitric oxide-dependent mechanism. The effect can be either preventative or curative, as shown in a study by Murray et al. (1985), who showed that in cells infected w/*Leishmania donovani,* parasite killing is also enhanced when already infected monocytes are treated with IFN-γ. In another experiment they showed that exposure to

recombinant IFN-γ leads to substantial activation of H_2O_2–releasing capacity accompanied by a stimulation of intracellular killing of *Toxoplasma gondii.*

IFN-γ induces indoleamine 2,3-dioxygenase (IDO), an enzyme of tryptophan catabolism. IDO activity has been implicated in the killing of intracellular parasites such as *T.gondii, Chlamydia trachomatis, & Chlamydia psitacci*. The inhibition of growth results from tryptophan starvation of the parasites. In mutant cell lines lacking the capacity to synthesize IDO, inhibition by IFN-γ of intracellular parasites is reduced (Daubener et al., 1999).

1.6.3 Modulation of B, T, and NK cell activity:

T-cells: IL-12 and IFN-γ are produced by macrophages and natural killer cells in the early phases of responses to viruses and intracellular bacteria. The CD4 T cells initially stimulated in presence of IL-12 and IFN-γ tend to develop into Th1 cells, in part because IFN-γ inhibits the proliferation of Th2 cells (Gajewski et al., 1990).

Cytotoxic T cells react with target cells via their MHC class I antigens, whose expression is stimulated by IFN-γ, and in this way their cytotoxic activity is boosted by IFN-γ. IFN-γ also restricts infection by inhibiting viral replication (inducing an antiviral state), and by conditioning infected cells for destruction by cytotoxic T cells, which increases the chances that infected cells will be recognized as target cells for cytotoxic attack by CD8+ T cells (Janeway et al., 1997).

IFN-γ is also capable of stimulating accessory cells to induce T suppressor cells. Stimulation of MHC class II expression on macrophages is followed by an increase in ability of these macrophages to induce the generation of T suppressor cells (Janeway et al., 1997).

Effector T cell recruitment: TNF-α and IFN-γ released by activated T cells act synergistically to change the shape of endothelial cells, allowing an increased blood flow, increased vascular permeability, and increased emigration of leukocytes, fluid, & protein into the site of infection (Janeway et al., 1997). .

Ig Isotype switching: Th1 cells (which are poor initiators of antibody responses) participate in isotype switching by releasing IFN-γ. IFN-γ induces the expression of IgG3 and IgG2a, while inhibiting IgM, IgG1, and IgE (Janeway, et al., 1997)

Effects on NK Cells: The activity of NK cells increases when exposed to IFN-α and IFN-β, and IL-12. IL-12 (with IFN-α) elicits production of large amounts of IFN-γ by NK cells. This secreted IFN-γ is crucial in controlling some infections before T cells have been activated to produce IFN-γ. In a study by Wallach (1983), mice that lack T and B cells are initially quite resistant to the pathogen *Listeria monocytogenes.* However, depletion of NK cells or mutations in the genes encoding TNF-α or IFN-γ or their receptors, renders them highly susceptible - they die a few days after infection, before adaptive immunity can be induced. He also demonstrated that NK cell activity is boosted by IFN-α/β and IFN-γ, and that interferon can protect target cells against NK cell activity.

1.7 Structure of IFN-γ

The human IFN-γ protein consists of 166 amino acids, 23 of which constitute the signal peptide (Figure 1.1). It contains two Asn-X-Ser/Thr glycosylation sites on its exposed surface (Rinderknecht et al., 1984). There are two molecular forms of human IFN-γ: one is 20kDa and the other 25 kDa; both are glycosylated at position 25, and the

25 kDa form is also glycosylated at position 97. It was demonstrated by Rinderknecht et al. (1984), that the residues at both the N- and C-termini are critical in the interaction with receptors. Recombinant IFN-γ with a truncated C-terminus showed substantially reduced antiviral activity, and truncation of N-terminus results in loss of secondary structure. Antibodies directed toward both the N- & C-terminus neutralize *in vitro* bioactivity of the molecule.

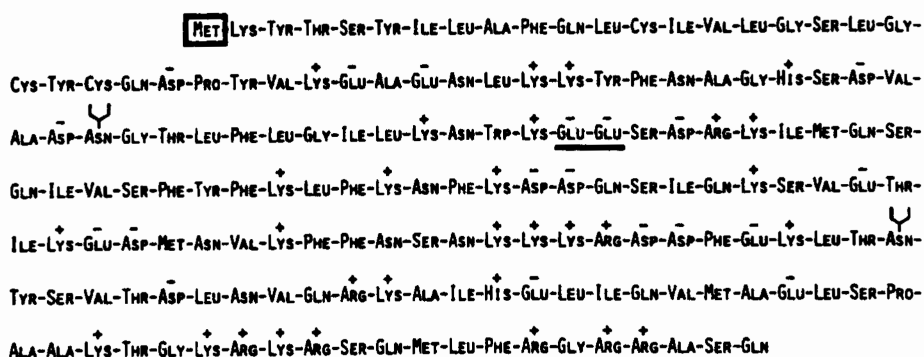


Figure 1.1: Amino acid sequence of human IFN-γ (Devos et al., 1982).

IFN-γ is found in its dimeric form in solution (Figure 1.2). Each subunit contains six α-helices (labeled A-F) that comprise approximately 62% of the structure, and the dimeric structure is stabilized by the intertwining of helices across the subunit interface with multiple intersubunit interactions (Ealick et al., 1991).

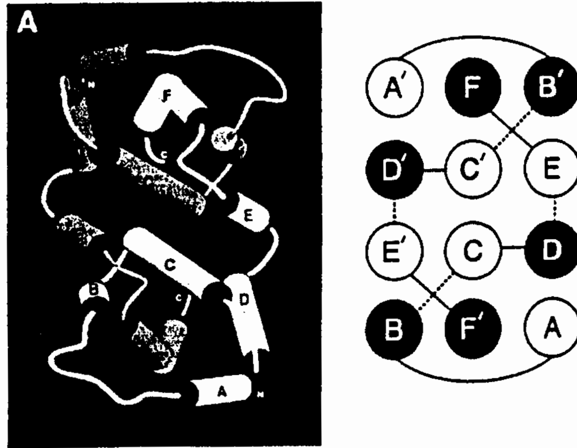


Figure 1.2: Schematic drawings of the recombinant human IFN-γ dimer, showing intertwining of the α-helices and intersubunit interactions (Ealick et al., 1991).

The human IFN-γ gene is located on chromosome 12. It is approximately 6kb in length, and contains 3 introns and 4 exons (Figure 1.3).

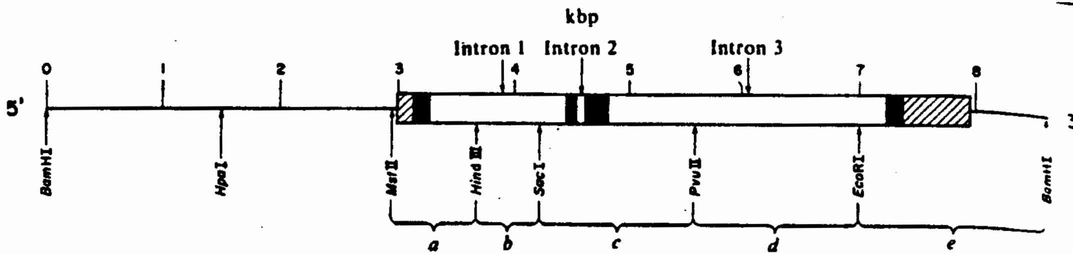


Figure 1. 3: The IFN-γ gene, showing intron and exon positions (Gray & Goeddel, 1982).

1.8 IFN-γ in Different Species

1.8.1 Homology in mammals

Figure 1.4 shows the amino acid sequences of IFN-γ from various mammals, showing the positions of the six α-helices A-F. The boxed areas show regions that are completely conserved. Sequence identities between the mammals range from 87% homology between rats and mice to 39% homology for human & rat sequences (Ealick et

al., 1991); among non-human primates (macaques & mangabey) there was 96-99% homology (Villinger, et al., 1995) (see Figure 1.5).

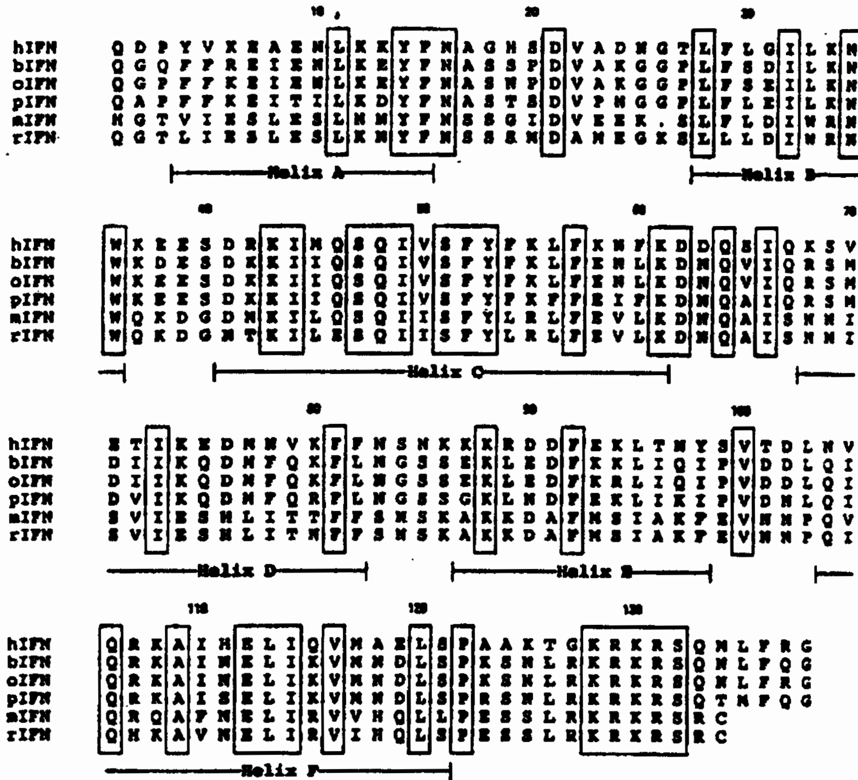


Figure 1.4: Amino acid sequences of IFN-γ from human, bovine, sheep, pig, mouse, and rat (Ealick et al., 1991).

The most highly conserved regions occur in helices C & F (the most buried helices in the dimer), and a short basic stretch at beginning of C-terminus (the Lys-Arg-Lys-Arg-Ser motif), while the most variable region is the loop between helices A & B. This loop may account for the high species specificity of IFN-γ; IFN-γ from one species generally displays poor affinity for the receptor from another species.

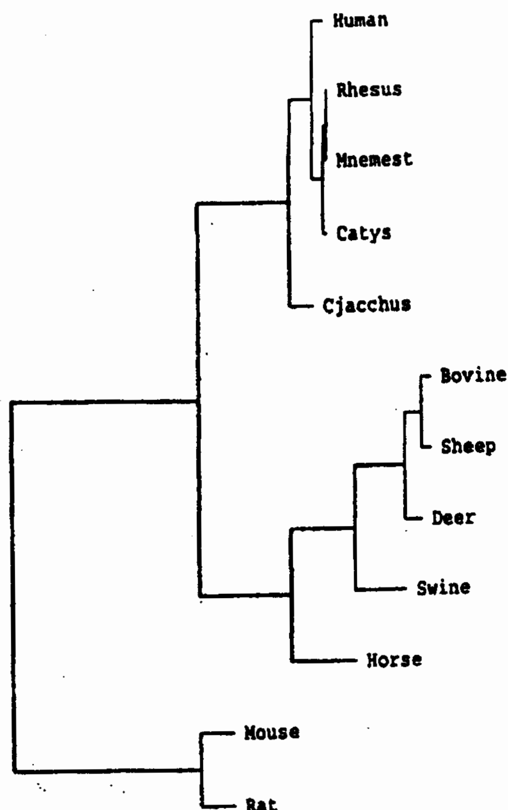


Figure 1.5: Evolutionary distance analysis of IFN-γ from various mammals (Villinger et al., 1995).

1.8.2 IFN-γ in Different Species: Avian

IFN-γ isolated from the chicken has been cloned. It produces IFN-γ activity that shares physiochemical properties with mammalian IFN-γ (Digby & Lowenthal, 1995). Chicken IFN-γ protein shares significant amino acid homology with mammalian IFN-γ; in particular it also contains the highly conserved motifs that are present in all mammalian IFN-γ (Figure 1.6). There is 35% homology between chicken & equine IFN-γ, and 32% homology between the chicken & human proteins, with two regions that are

highly conserved, including one in the C-terminal region: the Lys-Arg-Lys-Arg-Ser motif which is conserved in all IFN-γ proteins.

```
Human   MKYTSYILAFQLCIVLGSLG----CYCQDPYVKEAENLKKYFNAGHSDVADNGTLFLGIL
Avian   MTCQTYNL-FVLSVIMIYYGHTASSLNLVQLQDDIDKLKADFNSSHSDVADGGPIIVEKL
        *      * * *       *                 **  **  ****** *      *

Human   KNWKEESDRKIMQSQIVSFYFKLFKNFKDDQSIQKSVETIKEDMNV--KFFNSNKKKRDD
Avian   KNWTERNEKRIILSQIVSMYLEMLEN---TDKSKPHIKHISEELYTLKNNLPDGVKKVKD
        *****     *  ***** *     *             *               **  *

Human   FEKLTNYSVTDLNVQRKAIHELIQVMAELSPAAKTGKRKRSQMLFRGRRASQ
Avian   IMDLAKLPMNDLRIQRKAANELFSILQKLVDPPSF-KRKRSQSQ---RRCNC
           *      **  ****  **      *       ******     **
```

Figure 1.6: Amino acid homology comparison between chicken (avian) and human IFN-γ (Digby & Lowenthal, 1995).

1.8.3 Phylogenetic systematics: Relationship between birds and reptiles

Animals that are members of the phylum Chordata Vertebrata all possess a vertebral column and are a diverse group, containing members adapted to life in the sea, on land, and in the air. The principal classes of vertebrates are fishes that live in the water, and land-dwelling Tetrapods, which include Amphibia, Reptilia and Mammalia (see Figure 1.7). The relationship between birds and reptiles can be better understood by considering two vertebrate clades; the amniotes and the diapsids.

Amniota (amniotes) is a major vertebrate clade, which includes reptiles, birds and mammals. They all share in common an extraembryonic membrane called an amnion, formed from embryonic cells but located outside the body of the embryo, an important adaptation to life on land. Evidence for amniote phylogeny has been obtained from studies of amino acid sequences in proteins. For a particular protein, the rate of substitution is used to determine patterns of relationship among different taxa; the more distantly related two taxa are, the more differences there are between homologous proteins (see Figure 1.8).

An example of a protein that is conserved among amniotes is myoglobin. The amino acid sequence of the main component myoglobin from skeletal muscle of Pacific green sea turtle (Chelonia mydas caranigra) has been determined. The globin is 153 residues in length, and about 38, 52, 47 and 86 substitutions are noted in comparison with the myoglobins of other reptiles, mammals, birds and fish, respectively. The inferred pattern of structural stabilization and conservation of two loci are typical of tetrapod myoglobin (Watts et al., 1983).

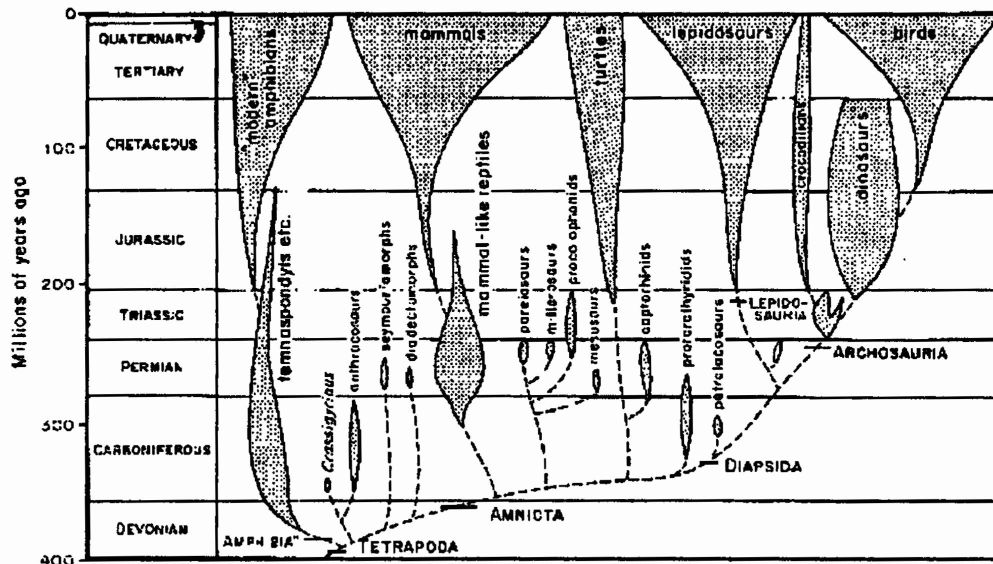


Figure 1.7: The phylogeny of the early amniotes during the Carboniferous and Permian times.

Myoglobin:	[[[[Turtle] Lizard] Crocodilian] [Bird [Mammal]]]
β-Hemoglobin:	[Snake [Crocodilian [Bird [Mammal]]]]
Lens α-crystallin A:	[Mammal [Crocodilian [Lizard [Bird]]]]
Cytochrome c:	[Bird [Snake [Mammal]]]
"Standard Morphological":	[[Turtle [[Lizard [Snake]] [Crocodilian [Bird]]]] Mammal]

Figure1.8: Protein homology of Amniote taxa based on the rate of amino acid substitution.

Members of the group Reptilia include the ectothermic snakes, lizards, crocodiles, turtles, and the endothermic birds (see www.ucmp.berkeley.edu/diapsids/diapsids.html). All members of Reptilia are diapsids, except for the anapsids (turtles). The main diagnostic physical character for a diapsid is the presence of two openings on each side of the skull (the upper and lower temporal openings). Birds are considered diapsids (as are reptiles) because they are descended from certain dinosaurs (diapsids) that ancestrally have the paired skull openings along with other physical characteristics in common w/diapsids. The close phylogenetic relationship between birds and reptiles allows us to hypothesize that this will be reflected in close homology of the effector proteins of their immune systems. Therefore, the knowledge of chicken cytokines should enable us to learn more about the same molecules in the green sea turtle.

1.9 The Need for Current Study: The Hawaiian Green Sea Turtle

The Hawaiian green turtles (*Chelonia mydas*) are sea turtles that form a genetically discrete population and are geographically isolated to the Hawaiian Islands. Sea turtles are designated worldwide as threatened and endangered species, and population declines have been prominent as the result of nesting habitat loss, human-associated factors, and diseases such as fibropapillomatosis.

Fibropapillomatosis (FP) is a debilitating, tumor-forming, transmissible disease of relatively recent significance that affects mainly green sea turtles. Several infectious agents (herpesvirus, retrovirus, and papillomavirus) have been associated with the disease, however the etiological agent has not been isolated or characterized (Landsberg et al., 1999). FP is characterized by multiple cutaneous papillomas, fibromas, and

fibropapillomas, as well as visceral fibromas. The most common sites of infection are on the flippers, eyes, oral cavity, and less frequently, internal organs (Herbst et al., 1999). The size, site, and locally invasive nature of these tumor growths cause normal breathing and feeding ability to be impaired, thereby contributing to stranding and death (Eckert et al., 1997). Identifying the cause, understanding the pathogenesis of the disease, and evaluating the immune system of the green turtle is necessary in order to minimize the impact of the disease on populations of the endangered species.

Green turtles with FP have been found to be immunosuppressed, based on lymphopenia in tumored turtles, and it is thought to be a sequela to the disease, with poor body condition (with hepatic atrophy and splenic lymphoid depletion), as a likely contributing factor (Aquirre et al., 1995). In order to properly evaluate the immune response of green turtles, it is necessary to investigate the humoral and cell-mediated aspects of their immune system. There is considerable information on measuring humoral immunity of green turtle (Benedict & Pollard, 1972; Herbst & Klein, 1995), however information on the cell-mediated immune response of green turtles is limited (Work, et al., 2000). Of primary interest is the evaluation of T cell-like responses, since T cells play a major role in tumor immunology.

1.10 Investigation of Interferon-γ in the Hawaiian Green Sea Turtle

Since interferon-γ is an important modulator of T-cell activity, it is hence of interest in the study of the turtle immune system. It is not known whether this lymphokine exists in turtles and until now, it has not been previously investigated in these animals. Because it is known to exist in other members of the tetrapod and reptilian

groups, and because of the important role it plays in these organisms, one can assume that it does exist in some form in turtles. As previously stated above, the close phylogenetic relationship between birds and reptiles allows us to assume that this will be reflected in close homology of the effector proteins of their immune systems. We can therefore take advantage of what is known about chicken IFN-γ, including its amino acid and genetic sequence, and apply it to the study of green sea turtle IFN-γ.

The strategy of this research project is based on the premise that the conserved amino acid segments of IFN-γ that exist between the chicken and mammals (such as humans) are important to the structure and function of the molecule, and therefore should also be conserved in the amino acid sequence of the hypothetical IFN-γ molecule in turtles. Figure 1.6 shows amino acid sequence homology of human and chicken IFN-γ in which the asterisks signify amino acids that are identical in the two species. Of particular interest are segments that contain many adjacent identical amino acids such as SHSDVAD, LSQIVSMY, QRKAANELF, and KRKRSQS. These segments are assumed to be conserved between the chicken and the green sea turtle sequences as well. In order to elucidate the genetic sequence of the hypothetical turtle IFN-γ, the chicken DNA codon sequences that correspond to these amino acid segments were used as primer sequences to be used in the PCR amplification of the IFN-γ gene, and to generate chicken IFN-γ probes for use in screening for homologous turtle RNA/cDNA and genomic DNA sequences.

CHAPTER 2
MATERIALS AND METHODS

2.1 Field Samples

Whole blood and organ tissue samples of the Hawaiian green sea turtle (*Chelonia mydas*) were obtained from normal and tumored animals from Kaneohe Bay, O'ahu, by the National Marine Fisheries Service in Honolulu. Stranded turtles showing terminal stages of fibropapillomatosis were euthanized and samples were then collected. A qualified veterinarian collected all samples under strict NIH guidelines. Chicken blood and organ tissue samples were collected from animals at a poultry slaughterhouse, and from the Department of Animal Sciences at the University of Hawai'i at Mānoa.

2.2 Processing of Blood and Tissue Samples

2.2.1 Isolation of Mononuclear Lymphocytes from Whole Blood

2.2.1.1 Chicken and Human Blood samples: The blood sample was transferred to sterile 5ml snap-cap tube (Fisher Scientific, Springfield, NJ) and spun down in a centrifuge on the high setting (2000 rpm) for 10 minutes. The buffy coat (containing white blood cells) was carefully removed using a Pasteur pipette, and transferred to a 5ml tube, and then brought up to the desired final volume using RPMI 1640 (RPMI medium 1640 [1x] w/25mM HEPES buffer w/ L-glutamine, GibcoBRL, Rockville, MD). The cell suspension was layered carefully over an equal volume of Histopaque (Ficoll) solution (1:1 ratio) (Sigma, St. Louis, MO) and the gradient was centrifuged for 15 minutes on the low setting (1300 rpm). The interface layer of white cells was transferred to a new 5ml tube, filled to the top with RPMI, and was then centrifuged on the low setting for 10

minutes. The supernatant was removed, and the pellet was resuspended in RPMI, and spun down again. The pellet was then resuspended in 2ml RPMI w/5%FBS.

2.2.1.2 Turtle Blood Samples: The blood sample was mixed with PBS in a 1:2 ratio, and was then layered over Histopaque (Ficoll) solution (1:1 ratio), and the gradient was centrifuged for 20 minutes at 300g (1300 rpm). The bottom layer was resuspended in PBS, mixed and re-spun. This wash process was repeated twice and the pellet was then brought up to the desired final volume with RPMI w/5%FBS.

2.2.2 Organ Tissue Samples

The chicken spleens and the green sea turtle liver were processed for RNA and DNA extraction - RNA and DNA were extracted from fresh chicken splenic tissue. However only DNA was extracted from the turtle liver that was stored frozen at -20°C, a temperature that is inadequate to prevent RNA degradation. In both cases, 6-9g of the tissues was cut into small portions and homogenized through a strainer containing a small amount of PBS (GibcoBRL). The homogenate was pooled in a 50ml centrifuge tube, centrifuged at 3000g for 10 minutes, and the tissue pellet was used for subsequent RNA and DNA extraction.

2.3 Lymphocyte Proliferation

The mononuclear cells were counted using a haemocytometer, and the concentration was then adjusted to ~5.0×10^6 cell/ml using RPMI w/10%FBS. The cells were added to a 96-well microtiter plate (NUNC, Naperville, IL) at 100µl/well. Concanavalin A (a polyclonal mitogen, used to stimulate T-cells to produce IFN-γ) was added to the wells (containing the cells to be stimulated) at a final concentration of 5µg/ml, with the final

total volume of 200µl/well. For the control cells, 100µl of RPMI w/10%FBS was added for a final total volume of 200µl/well. The cells were then incubated in a humid 8.0% CO_2 incubator at 37°C, (for turtle mononuclear cells, a humid 8.0% CO_2 incubator at 32°C is used). Cells were harvested 48 hours after mitogen stimulation (A kinetics study showed that after 36-48 hours, maximum stimulation was elicited in humans).

2.4 Total RNA Isolation

2.4.1 From Mononuclear cells

After mitogen stimulation was complete, the mononuclear cells were removed aseptically and pooled. The harvested cells were spun by centrifugation at 12,000g for 5 minutes and pelleted (Cells can be stored at -70°C, until ready for use). Cells were then lysed in TRIzol reagent (GibcoBRL) (a mono-phasic solution of phenol and guanidine isothiocyanate), which maintains the integrity of RNA, while disrupting cells and dissolving cell components. The solution was mixed with 100% chloroform (0.2ml chloroform per 1ml TRIzol reagent), incubated for 2-3 minutes, and then followed by centrifugation. This separates the mixture into a lower phenol-chloroform (organic) phase and an upper aqueous phase, which contains the RNA. The RNA was precipitated by mixing the aqueous solution with 100% isopropanol (0.5ml isopropanol per 1ml TRIzol reagent) followed by incubation at room temperature for 10 minutes. The solution was centrifuged at 12,000g for 10 minutes, the supernatant was removed, and the pellet was then washed with 75% ethanol (1ml 75% ethanol per 1ml TRIzol reagent). The sample was mixed, and then centrifuged at 9,500g for 5 minutes. The pellet was dried (air-dry or vacuum-dry) and was then resuspended in nuclease-free water to the desired volume.

2.4.2 From Tissue Samples

Tissue (chicken spleen) samples were processed (see Section 2.2.2), separated into 500mg portions, and placed in 5ml of TRIzol Reagent and incubated at 25°C for 5 minutes. 1ml of chloroform was added to each 5ml sample (0.2ml chloroform per 1ml TRIzol reagent), and the tubes were capped and the tubes were shaken vigorously for 30 seconds, than incubated for 3 minutes at 25°C. The samples were centrifuged for 15 minutes, which separates the mixture into a lower red, phenol-chloroform phase and a colorless upper aqueous phase. The aqueous phase was transferred to a fresh tube, and RNA was precipitated from the aqueous phase by adding 2.5ml isopropanol (0.5ml isopropanol per 1ml TRIzol reagent) to the solution. The samples were incubated for 10 minutes at 25°C and centrifuged for 10 minutes to pellet the RNA. The RNA pellet was washed once by adding 5ml of 75% ethanol (1ml 75% ethanol per 1ml TRIzol reagent) and mixed by vortexing, followed by centrifugation for 5 minutes. The RNA was briefly dried (for 5 minutes) and was then redissolved in 2ml of DEPC treated water. The sample was stored at -70°C.

2.5 Complimentary DNA (cDNA) Preparation by Reverse Transcription

Single-stranded cDNA was synthesized from the isolated RNA using the Reverse Transcription System (Promega Corporation, Madison, WI). The reverse transcriptase reaction was primed using Oligo $(dT)_{15}$ Primer (0.5μg per 1μg RNA) in a solution containing 5mM $MgCl_2$, 1x Reverse Transcriptase Buffer (10mM Tris-HCl [pH 9.0 at 25°C], 50mM KCl, 0.1% Triton X-100), 1mM each dNTP, 1U/μl Recombinant RNasin Ribonuclease Inhibitor, 15u/μg AMV Reverse Transcriptase (High Concentration), and

nuclease-free water. Using a thermocycler, the solution was heated at 42°C for 20 minutes, then at 100°C for 5 minutes, and then cooled down to 0°C.

2.6 Genomic DNA Purification

Genomic DNA was purified from samples using the Wizard Genomic DNA Purification Kit (Promega Corporation).

2.6.1 Isolation of Genomic DNA from Whole Blood Samples

The mixed whole blood sample was transferred to a sterile tube containing Cell Lysis Solution (9ml/3ml of whole blood sample), and then incubated at room temperature for 10 minutes with gentle mixing 2-3 times during the incubation to lyse the red blood cells. The sample was centrifuged at 2000g for 10 minutes, then the supernatant was removed and discarded without disturbing the cell pellet, then the tube was vortexed vigorously until the white blood cells were resuspended (10-15 seconds).

Nuclei Lysis Solution (3ml/3ml blood sample volume) was added to the tube containing the resuspended white cells, then the solution was pipetted 5-6 times to lyse the white blood cells. RNase Solution (15µl/3ml sample volume) was added to the nuclear lysate, the sample was incubated in a 37°C water bath for 15 minutes, and then cooled to room temperature. Protein Precipitate Solution (1ml/3ml sample volume) was added to the nuclear lysate and the mixture was vortexed vigorously for 20 seconds, followed by centrifugation at 200g for 10 minutes at room temperature, after which a dark brown pellet was visible.

The supernatant was transferred to a 15ml centrifuge tube containing 3ml of room temperature isopropanol, and mixed gently by inversion until white thread-like strands of

DNA formed a visible mass. The tube was centrifuged at 2000g for 1minute at room temperature, after which the DNA was visible as a small white pellet. The supernatant was removed and one sample volume of 70% ethanol was added to the DNA, followed by gentle inversion of the tube to wash the DNA pellet and the sides of the tube. The solution was then centrifuged again, after which the ethanol was removed and the pellet was air-dried. DNA Rehydration Solution (250µl/3ml sample volume) was added to the tube and incubated at 4°C overnight. The DNA was stored at 4°C.

2.6.2 Isolation of Genomic DNA from Animal Tissue

Tissue (chicken spleen and turtle liver) samples were processed (see Section 2.2.2) and separated into 500mg portions, then added to 6ml Nuclei Lysis Solution and incubated at 65°C for 30 minutes. RNase Solution (30µl) was added to the nuclear lysate and the solution was mixed and then incubated for 30 minutes at 37°C. The solution was then cooled to room temperature for 5 minutes. Protein Precipitation Solution (2ml) was added, the tube was vortexed vigorously for 20 seconds and the solution was chilled on ice for 5 minutes.

The solution was centrifuged at 2000g for 5 minutes at room temperature (the precipitated protein forms a white pellet) and the supernatant, containing DNA, was removed and transferred to a fresh tube containing 6ml of room temperature isopropanol. The tube was gently mixed by inversion until white thread-like strands of DNA formed a visible mass, and was then centrifuged for 5 minutes at 2000g (the DNA forms a white pellet). The supernatant was discarded, then 6ml of room temperature 70% ethanol was added to wash the pellet, followed by centrifugation for 5 minutes. The ethanol was removed and the DNA pellet was air-dried for 10-15 minutes. DNA Rehydration

Solution (1ml) was added to the tube and incubated at 4°C overnight. The DNA was stored at 4°C.

2.7 PCR Amplification

2.7.1 Primer Selection

Human IFN-γ gene amplification was performed using existing human IFN-γ gene forward and reverse primers. For chicken/turtle IFN-γ gene amplification, we used highly conserved sequences in the IFN-γ gene, and then selected primers that are specific to the chicken IFN-γ gene that were then constructed (from Integrated DNA Technologies, Inc., Coralville, IA, and GibcoBRL) and used for PCR.

SET 1:

Forward Primer: **5'**-TAC TGA GCC AGA TTG TTT CGA TGT AC-**3'**

Reverse Primer: **5'**-AAG AGT TCA TTC GCG GCT TTG CGC TG-**3'**

SET 2:

Forward Primer: **5'**-AGT CAT TCA GAT GTA GCT GAC-**3'**

Reverse Primer: **5'**-AGA CTG GCT CCT TTT CCT TTT-**3'**

The Primer Set 1 Forward sequence corresponds to the amino acid sequence LSQIVSMY, and the Primer Set 1 Reverse sequence corresponds to the amino acid sequence QRKAANELF. The Primer Set 2 Forward sequence corresponds to the amino acid sequence SHSDVAD, and the Primer Set 2 Reverse sequence corresponds to the

amino acid sequence KRKRSQS. Set 1.1 and 2.1 were Desalted-purified, while Set 1.2 and 2.2 were Cartridge purified.

2.7.2 PCR Amplification

The cDNA samples were mixed with 5μM of both Forward and Reverse primers, as well as PCR SuperMIX (GibcoBRL). Initially, PCR SuperMIX was used, containing 22U/ml *Taq* DNA Polymerase, 22mM Tris-HCl (pH8.4), 55mM KCl, 1.65mM $MgCl_2$, 220μM each dNTP, and stabilizers. Later, to improve PCR specificity and yield, PLATINUM PCR SuperMIX (GibcoBRL) containing PLATINUM *Taq* Antibody, was used. The samples are then placed in a thermocycler & processed for the optimized conditions:

1. 94°C for 5 min.
2. 94°C for 1 min., 55°C for 1min., 72°C for 1min. 30 sec (35 cycles)
3. 72°C for 7 min.

2% agaraose gels are prepared using 1x TAE buffer, HGT Agarose, and ethidium bromide (5mg/ml). The 10μl of sample was mixed with loading dye and placed in a 2% agarose gel (5μl of 50bp or 100bp ladder is used for reference) for gel electrophoresis (ran at 70mV, 45mA).

In order to produce blunt fragments for subsequent plasmid incorporation, the PCR was performed on the turtle cDNA samples using *Pfx* DNA Polymerase (*Taq* DNA Polymerase does not produce blunt fragments). The cDNA samples were mixed with 10mM of both forward and reverse chicken IFN-γ gene primers (Set 1), along with a solution containing 1x *Pfx* Amplification Buffer (50mM Tris-HCl (pH 8.0), 50mM KCl, 1mM DTT, 0.1 mM EDTA, stabilizers, and 50% glycerol), 0.3mM each dNTP, 1mM $MgSO_4$, 2.5 units of PLATINUM *Pfx* DNA Polymerase (GibcoBRL), and distilled water.

The samples were then placed in a thermocycler & processed for optimized conditions (see above). The samples were then analyzed using 2% agarose gel.

2.8 pCR Blunt Vector Amplification

2.8.1 Transformation using pCR Blunt vector

A blunt-ended PCR cloning system (Zero Blunt PCR Cloning Kit, Invitrogen Corporation, Carlsbad, CA) was used in order to incorporate the desired gene segment into a plasmid vector. The pCR-Blunt vector is a 3.5kb plasmid that allows the incorporation of blunt PCR products, and carries a Kanamycin-resistant segment for selecting transformants (see Figure 4.1). The blunt PCR product was first ligated into the pCR-Blunt plasmid by mixture with 25ng of linearized, blunt pCR-Blunt, 10x Ligation Buffer (with ATP), T4 DNA Ligase (4U/μl), and sterile water, and incubated at 16°C for 1 hour. The solution is centrifuged briefly and then placed on ice in preparation for transformation (or may be stored at -20°C until ready for use).

The next step was transformation using One Shot Competent Top10 *E. coli* cells. The ligation reaction product was added to one vial of Top10 cells, which was used for transformation, and 0.5M β-mercaptoethanol was also added to each vial and mixed. The solution was incubated at 0°C for 30 minutes, then for exactly 45 seconds at 42°C, and finally placed on ice for 2 minutes. Pre-warmed (37°C) SOC medium was added to each vial, and the vial was mixed at 37°C for 1 hour at 225rpm. The vial with the transformed cells was placed on ice, and 200μl was then spread on separate, labeled LB agar plates containing 50μg/ml kanamycin (pre-treated with 4μl IPTG and 40μl X-gal), then incubated at 37°C overnight. Blue-colored colonies were then isolated (ideally ten

colonies should be used). In order to analyze the transformants, antibiotic-resistant transformants were inoculated into 4ml LB medium containing 50µg/ml kanamycin and grown overnight at 37°C.

2.8.2 Miniprep Isolation

The plasmid was then isolated using a miniprep protocol for subsequent sequencing using M13 Forward and Reverse primers. Each LB medium bacterial culture was aliquoted into Eppendorf tubes (~1ml/tube) and pelleted by centrifugation, with subsequent removal of all medium. Cell Suspension Buffer (210µl) was added to the pellet and the cells were resuspended, followed by addition of Cell Lysis Solution (210µl) with gentle mixing and incubation at room temperature for 5 minutes. Neutralization Buffer (280µl) was then added and the solution is mixed, followed by centrifugation at 12,000g for 10 minutes. The supernatant was loaded into a spin-cartridge/wash tube and centrifuged at 12,000g for 1 minute, and the flow-thru was discarded. Wash Buffer (700µl) was added to the spin-cartridge/wash tube and centrifuged at 12,000g for 1 minute, and then centrifuged again to remove residual wash buffer. The plasmid was then eluted by adding warm water (75µl) to the center of the spin-cartridge, incubating it for 1 minute at room temperature, followed by centrifugation at 12,000g for 2 minutes. An *Eco*RI digest was performed in order to release the insert from the plasmid, and the products were visualized by gel electrophoresis.

2.9 Complimentary DNA sequencing

Samples were sent to the Biotechnology/Molecular Biology Instrumentation and Training Facility at the University of Hawai'i at Manoa for DNA sequencing. The

samples were prepared with 3.2-pmole primers; M13 primers were used for plasmid insert sequencing, while the Primer Set 1 and 2 Forward and Reverse primers were used for the genomic sequencing.

2.10 PCR DIG-Probe Synthesis

Digoxigenin-11dUTP (DIG-dUTP) can be incorporated by *Taq* DNA polymerase during PCR and the resulting DNA fragment can be used as a probe for use in dot blots and Southern Blots. DIG-probes were synthesized using the PCR DIG-Probe Synthesis Kit (Roche Biochemicals, Indianapolis, IN). A PCR reaction volume of 50μl was made up of the following: 5μl of 10x PCR Expand High Fidelity buffer with $MgCl_2$, 5μl of 10x PCR DIG dNTP mix containing dATP, dCTP, dGTP (2mM each), 1.3mM dTTP, and 0.7mM DIG-11-dUTP, 2.5μl each of forward and reverse primer pair, 5μl template DNA, 1μl Enzyme mix containing 20mM Tris-HCl (pH 7.5), 100mM KCl, 1mM dithiothreitol, 0.1mM EDTA, 0.5% Tween 20, 0.5% Nonidet P40, and 50% glycerol, and 29.2μl dH_2O. Each probe was synthesized under the same PCR conditions as above. In order to obtain probes with more efficient DIG incorporation, the DIG dNTP mix was diluted 1:1 with dNTP Stock solution containing 2mM each of dATP, dCTP, dGTP, and dTTP. The PCR cycling parameters that were used are listed in Section 2.7.2.

2.11 Restriction Digests of Genomic DNA

Restriction digests were performed by two methods: Digest A consisted of 5μl of genomic DNA, 2μl of restriction enzyme, 2μl of buffer, and 1μl of H_2O; Digest B consisted of 8μl of genomic DNA, 1μl of restriction enzyme, and 1μl of buffer. In both

methods, the digests were mixed by vortex and spun down, and then incubated in at 37°C for 24-36 hours.

The following enzymes and their corresponding buffers were used:

Enzyme	Buffer	Source
1. *Acc*I	NEBuffer 4	New England Biolabs, Beverly, MA
2. *Bam*HI	Buffer B	Promega
3. *Bsa*BI	NEBuffer 2	New England Biolabs
4. *Eco*RI	Buffer H	Promega
5. *Eco*RV	MultiCore Buffer	Promega
6. *Hind*III	Buffer B	Promega
7. *Kpn*I	Buffer J	Promega
8. *Nar*I	NEBuffer 1	New England Biolabs
9. *Nco*I	NEBuffer 4	New England Biolabs
10. *Pst*I	Buffer H	Promega
11. *Sac*I	Buffer J	Promega
12. *Sal*I	*Sal*I Buffer	New England Biolabs
13. *Sma*I	Buffer J	Promega

2.12 Southern Blot and Dot Blotting

Genomic DNA was prepared for blotting by restriction digestion using various restriction enzymes and their appropriate buffer (see Section 2.11). The DNA fragments were then separated on a 0.8% TAE agarose gel containing ethidium bromide alongside a 1kb DNA ladder marker (GibcoBRL). The gel was depurinated in 0.25M HCl for 10 minutes, rinsed in dH_2O, then treated with Denaturation solution (0.5N NaOH, 1.5M NaCl) for 1 hour, followed by neutralization in 0.5M Tris-HCl (pH 7.5), 3M NaCl. The DNA was blotted overnight on to a positively charged Nylon Membrane (Roche Biochemicals) by downward capillary transfer using 20x SSC. (For the dot blots, a dilution series of the DNA target was prepared and denatured for 10 minutes at 95°C, then spotted on positively charged Nylon membranes (Roche Biochemicals) at 1µl-5µl volumes). The DNA was immobilized by baking the membrane in a vacuum oven at

80°C for 30 minutes, followed by UV cross-linking for 2-3 minutes on a UV transilluminator, and a 5x SSC rinse.

The blot was treated with Prehybridization solution (High SDS Buffer: 7% SDS, 50% formamide, 5x SSC, 2% Blocking Reagent (Roche Biochemicals), 50mM sodium phosphate pH 7.0, 0.1% N-lauroylsarcosine) for 2 hours at 55°C. The blot was then placed in Hybridization solution (containing DIG-probe in High SDS buffer at the optimized concentration) and allowed to hybridize overnight. The next day, the hybridization solution was poured off (and was reused several times by storage at -20°C, thawing and denaturing at 68°C for 10 minutes). The membrane was washed twice in 2x Wash solution (2x SSC, 0.1% SDS) for 5 minutes, followed by washing twice in 0.5% Wash solution (0.5x SSC, 0.1% SDS) at 65°C for 15 minutes.

After hybridization and post-hybridization washes, the membrane was equilibrated for 1 minute in Wash Buffer (100mM maleic acid, 150mM NaCl pH 7.5, 0.3% Tween). The membrane was blocked using 1x Blocking Reagent for 1 hour, followed by incubation with anti-DIG-alkaline phosphatase (1:5000 or 1:2500 dilution) for 1 hour. The membrane was washed twice with Wash Buffer with Tween, followed by equilibration in Detection buffer (100mM Tris-HCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$) for 2 minutes. Finally, after pouring off the Detection buffer, the membrane was incubated in BCIP/NBT Phosphatase Substrate solution (Kirkegard & Perry Laboratories, Gaithersburg, MD) until the desired spots or bands were detected (15-30 minutes), and rinsed in dH_2O to prevent over-development.

CHAPTER 3
RESULTS

3.1 Human Samples

Preliminary studies were carried out with human samples in order to test and practice the methodology and ensure that the strategy was functional. Lymphocytes were obtained from whole human blood using the Ficoll/Histopaque isolation technique. The cells were then cultured on 96-well microtiter plates and mitogenically stimulated using Concanavalin A (Con A). After 48 hours of stimulation at 37°C, the cells were harvested, and total RNA was isolated by the TRIzol Reagent method. Complementary DNA (cDNA) was synthesized from mRNA contained in the total RNA using the Reverse Transcriptase System and Oligo(dT) primers, and underwent subsequent PCR amplification using human IFN-γ primers. As shown in Figure 3.1, the PCR product corresponds to the expected fragment size (427 bp) of the human IFN-γ gene.

A kinetic study was performed in order to ascertain the time period necessary to obtain the optimal amount of IFN-γ mRNA production. The procedure above was repeated, with cells undergoing Con A stimulation for 12, 24, 36, and 48 hours. As shown in Figure 3.2, the optimal time period corresponded to approximately 36-48 hours.

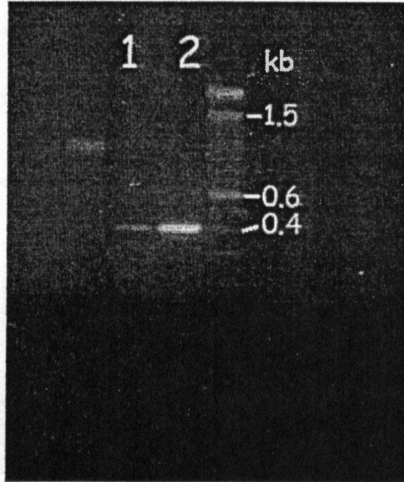


Figure 3.1: 2% TAE agarose gel showing RT-PCR products, using human IFN-γ primers, from mRNA extracted from human lymphocytes cultured in RPMI alone (1) or in the presence of Concanavalin A (2) for 48 h.

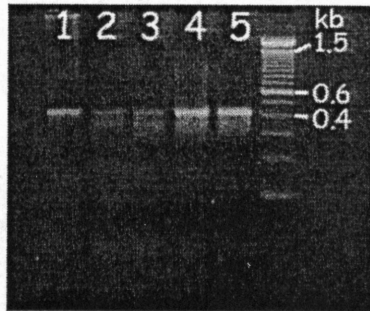


Figure 3.2: 2% TAE agarose gel showing RT-PCR products from mRNA extracted from human lymphocytes cultured in RPMI alone (1) or with Concanavalin A for 12 h (2), 24 h (3), 36 h (4), and 48 h (5).

3.2 Chicken & Turtle Samples

3.2.1 PCR Products Using Primer Set 1

Blood samples were obtained from two turtles during their necropsies. The samples were processed according to the previously described protocols, including lymphocyte isolation and stimulation, TRIzol total RNA isolation, cDNA synthesis, and PCR/*Taq* Polymerase amplification using chicken IFN-γ primers Set 1.1. Figure 3.3

shows three bands corresponding to fragment sizes of approximately 165 bp, 60 bp, and >25 bp. (The >25 bp fragments were shown to correspond to unused primers, and were eliminated by decreasing primer concentration from 100μM to 10μM).

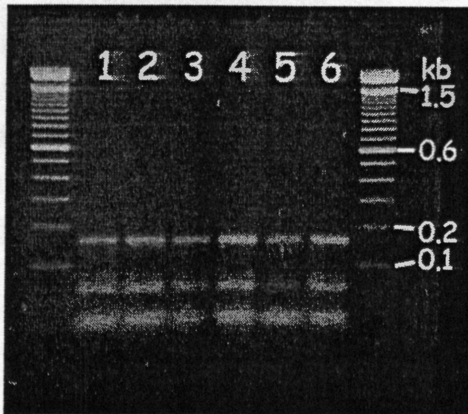


Figure 3.3: 2% TAE agarose gel showing RT-PCR (using *Taq* Polymerase) products, using chicken IFN-γ primers Set 1.1 (100μM), from mRNA extracted from turtle lymphocytes cultured in RPMI (1, 4) and with Concanavalin A for 24 h (2, 5), and 48 h (3, 6).

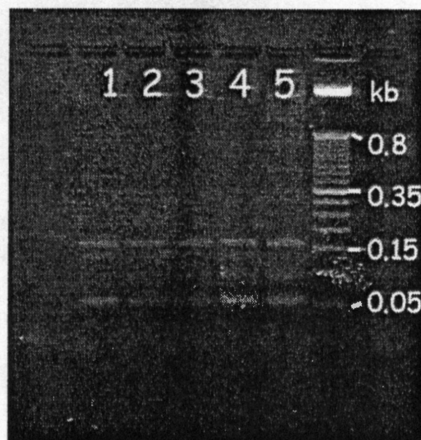


Figure 3.4: 2% TAE agarose gel showing RT-PCR (*Pfx* Polymerase) products, using chicken IFN-γ primers Set 1.1 (10μM), from mRNA (of different dilutions) extracted from turtle lymphocytes cultured with Concanavalin A for 24 h.

The cDNA was then amplified using *Pfx* Polymerase in order to obtain blunt PCR products to be used with the pCR-Blunt Vector for isolation and sequencing of the gene

fragments. Figure 3.4 shows the two bands corresponding to fragment sizes of approximately 165 and 60 bp.

The Zero Blunt PCR Cloning Kit was used for transformation and cloning of the two DNA fragments. Five distinct transformed *E. coli* colonies (kanamycin-resistant) were isolated and propagated in LB medium containing kanamycin. The plasmids were then isolated by the miniprep protocol, and the inserts were released by digestion with *Eco*RI. Figure 3.5 shows the released inserts from the plasmids of the five colonies. The inserts from Colonies 1 and 3 contain an internal *Eco*RI cutting site as indicated by the two resulting bands.

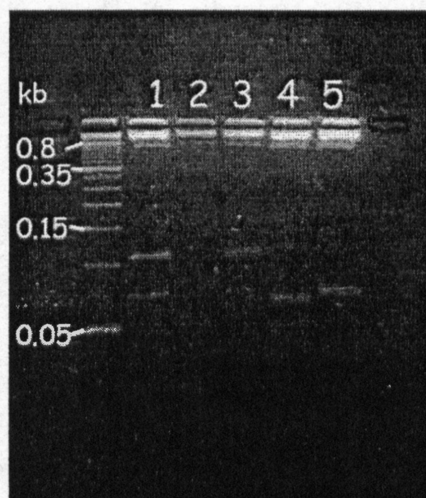


Figure 3.5: 6% TAE agarose gel showing released inserts from the plasmids of transformed cells from Colony 1 (1), Colony 2 (2), Colony 3 (3), Colony 4 (4), and Colony 5 (5). (Note: The inserts from Colonies 1 and 3 contain an internal *Eco*RI cutting site as indicated by the two resulting bands).

Subsequent PCR amplification of the inserts verifies that the band sizes of the inserts correspond to the fragment sizes of the two bands that were of interest (165 bp/60 bp), as shown in Figure 3.6. Amplification of the inserts from Colonies 1 and 3 resulted in single bands, as expected.

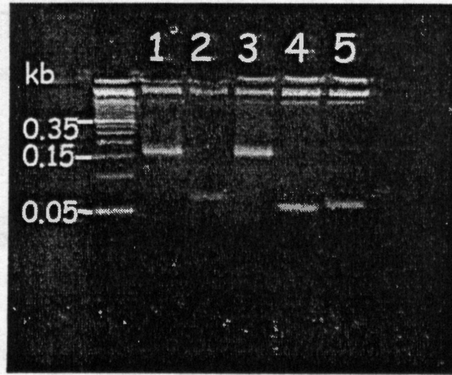


Figure 3.6: 5% TAE agarose gel showing released inserts from the plasmids of transformed cells from Colony 1 (1), Colony 2 (2), Colony 3 (3), Colony 4 (4), and Colony 5 (5). (Note: The amplification of the inserts from Colonies 1 and 3 resulted in a single band as expected).

Scaled-up production of the five pCR-Blunt plasmids was performed, the inserts were isolated and amplified, and subsequently sequenced using M13 primers (For complete sequences of plasmids, see Appendix 1). The sequences were analyzed, and the insert sequences were isolated (using the *Eco*RI recognition site GAATTC) and are shown in Table 3.1. The sequences were compared with each other using the BLAST 2 Sequence Program. The sequences from Colonies 1 and 3 were found to be nearly identical (the 88% identity was due to mismatching of the "N" bases), and Colonies 2, 4 and 5 were nearly identical to each other.

The turtle sequences were compared with sequences contained in the GenBank database using the BLAST program, and alignment matches were found with the primer sequences derived from the chicken IFN-γ sequence, however no other significant matches were found. In addition, based on the chicken IFN-γ sequence, the 164 bp turtle sequence was found to be 40 bp shorter than the 204 bp size expected from the corresponding chicken DNA segment.

Colony 1 Insert:
AAGAGTTCAT TCGCGGCTTT GCGCTGGGAA GCTTCAGACC
TCAATTGCGA GCTTTCGAGA **GAATTC**AGAT TGCCGCTGTT
GCTTTGGAAG CAGGCCACTC CCAGTTGTGG CATCGATGAA
CTGGTGTTGG CACTCGG**GTA CATCGAAACA ATCTGGCTCA GTA**

Colony 2 Insert (*inverse*):
AAGAGTTCAT TCGCGGCTTT GCGCTGGGGT TACCTAATC
GTACATCGAA ACAATCTGGC TCAGTA

Colony 3 Insert:
AAGAGTTCAT TCGCGGCTTT GCNCTGGGAA GCTTCAAACC
TCAATNGCNA GCTTTCNANA **NAATTC**AAAT NGCCGNTGTT
GCTTTGGAAG CAGGCCACTC CCAGNTGNGG CATNGANGAA
CTGGTGNTGG CACTCGG**CTA CATNGAAACA ATCTGGCTCA NNA**

Colony 4 Insert:
AAGAGTTCAT TCGCGNCTTT GCGCTGGGTA CATCGAAACA
ATCTGGCTCA GTA

Colony 5 Insert:
AAGAGTTCAT TCGCGGCTTT GCGCTGCACT GG**GTACATCG**
AAACAATCTG GCTCAGTA

Table 3.1: Sequences of inserts obtained from pCR-Blunt plasmids from colonies. The chicken IFN-γ Set 1.1 Primers and internal *Eco*RI recognition sites are indicated in bold.

The sequences were then run through the Open Reading Frame (ORF) Finder program, which searches for sequences that can be translated into amino acid sequences. Open reading frames in the inserts from Colony 1 and 3 were found, but only the primer regions (shown in Bold) matched the chicken sequence:

[**L S Q I V S M Y** P S A N T S S S M P Q L G V A C F Q S N S G N L N S L E S S Q L R S E A S **Q R K A A N E L F**]

The sequence is 17 amino acids shorter than of that the known chicken IFN-γ sequence. Although this may be due to a deletion event that occurred in the course of evolution, it is unlikely, since there are no other regions of significant alignment in the sequence.

To further investigate this discrepancy, PCR amplification was performed using chicken cDNA and Primer Set 1.1, to verify that they work with the DNA sequence from which they were derived. The same two fragments were produced at approximately 165 bp and 60 bp, identical in size to the turtle fragments, and approximately 34 bp short of the 204 bp fragment expected. The experiment was repeated with human cDNA, with identical resulting bands.

Because identical results were obtained with all the cDNA preparations (from turtle, chicken, and human), a contamination was suspected, and various possible sources were investigated including the PCR SuperMIX, the template DNA, and both forward and reverse Set 1.1 primers. Different PCR SuperMIX lots were used in combination with different templates and primers, with no significant differences. New chicken mRNA was obtained from the processing of new blood samples (as before), but the same bands resulted from PCR amplification. A new set of primers (identical sequence, but Cartridge-purified) was obtained (designated: Set 1.2) and tested with different templates and PCR SuperMIX lots (including Platinum PCR SuperMIX), yielding the same results. Various PCR parameters were tested, including different annealing temperatures and time variables, with little or no differences detected. In addition, PCR was performed using the same template using Primer Set 2.1, yielding no bands (see Figure 3.7). Finally, a PCR run was performed with Primer Set 1.2 but with no template present, and the same 165 bp/60 bp bands were still present.

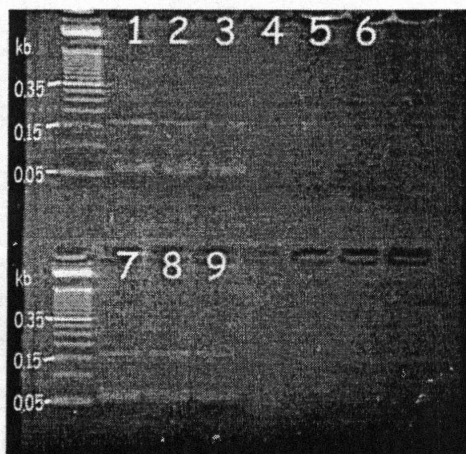


Figure 3.7: 4% TAE agarose gel showing RT-PCR products from mRNA extracted from turtle lymphocytes cultured in RPMI (1, 4, 7); in Concanavalin A for 24 h (2, 5, 8), and 48 h (3, 6, 9); using PCR SuperMIX in (7, 8, 9) and Platinum PCR SuperMIX in (1-6), and Primer Set 1 in (1-3, and 7-9) and Set 2 in (4-6);

PCR amplification was performed using different concentrations of Primer Set 1.2: 10μM, 5μM, 2μM, 1μM, 0.1μM, and 0.01μM, with no template. As shown in Figure 3.8, the bands became fainter as the concentration of primer decreased, disappearing altogether at a concentration of 2μM. In addition, when PCR was performed with no template and only one of the primer pair (forward only or reverse only), or no primers, the bands were absent (see Figure 3.9). These results indicated that, for reasons that remain unclear, artifactual PCR products of 60 bp and 165 bp are produced by gene amplification of Primer Set 1.

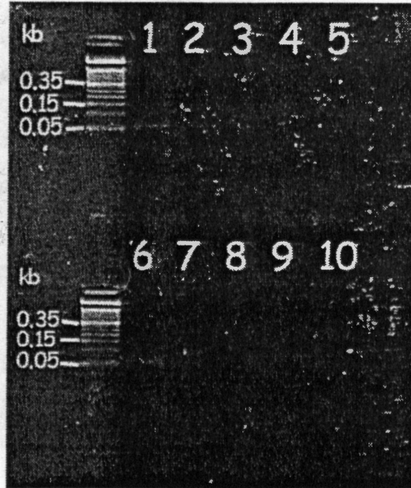


Figure 3.8: 4% TAE agarose gel showing the concentration-dependent effect of Primer Set 1 [10μM (1, 6), 5μM (2, 7), 2μM (3, 8), 1μM (4, 9), 0.1μM (5, 10)] on the presence of the fragments produced with PCR in the presence (1-5) and absence (6-10) of template.

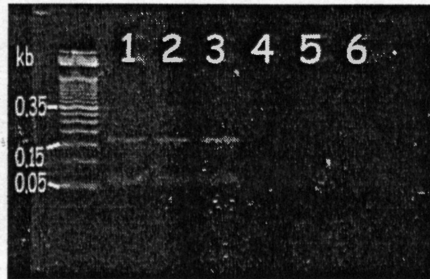


Figure 3.9: 4% TAE agarose gel showing the dependence of both primers being present for the production of the two fragments produced with PCR in the absence of template; RT-PCR products from mRNA, using Primer Set 1, extracted from chicken lymphocytes cultured in RPMI (1), and Concanavalin A for 48 h (2), and PCR with no template (3); PCR with no template and Forward Primer Set 1 only (4), Reverse Primer Set 1 only (5), and PCR with no template and no primers (6).

On occasion, when performing PCR on different chicken cDNA templates using Primer Set 1.2, a larger band (approximately 850 bp) appeared along with the 165 bp and 60 bp fragments, and sometimes the approximately 850 bp band appeared as the exclusive product (see Figure 3.10 and 3.11). This high molecular weight product was not obtained without the chicken cDNA template. The primer pair selected for the study did, indeed, hybridize to something in the chicken RNA/cDNA preparation. The

size of this band corresponded to that of IFN-γ genomic DNA (expected size of 845 bp) (Kaiser et al., 1998). However, this primer pair did not appear to produce the expected IFN-γ RNA/cDNA fragment (of 204 bp in length) for either the chicken or turtle peripheral blood cells.

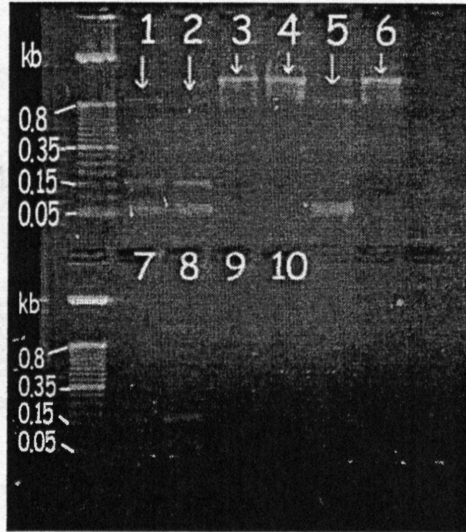


Figure 3.10: 2% TAE agarose gel showing RT-PCR products from mRNA extracted from chicken lymphocytes (1-6) and human lymphocytes (7-10); using Primer Set 1 (1, 2, 5, 7, 8) and Set 2 (3, 4, 6, 9, 10); using PCR SuperMIX (5, 6), and Platinum PCR SuperMIX (1-4, 7-10). (Note: When chicken template was used, note the appearance (see arrows) of 845 bp band along with the 160 bp and 60bp bands when Primer Set 1 was used (1, 2, 5); and the 1340 bp band with Primer Set 2 (3, 4, 6)).

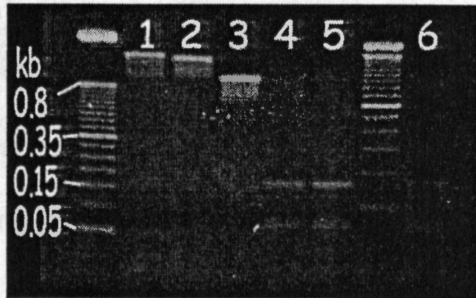


Figure 3.11: 2% TAE agarose gel showing RT-PCR products from mRNA extracted from chicken lymphocytes cultured with Concanavalin A for 48 h using Primer Set 2 (1), Primer Set 2 Forward and Set 1 Reverse (2), Set 1 Forward and Set 2 Reverse (3), Set 1 Forward and Reverse (4, 5, 6).

A genomic DNA preparation was performed from a new chicken blood sample using the Wizard Genomic DNA Purification Kit followed by PCR amplification using Primer Set1.2. As shown in Figure 3.12, the expected 845 bp band is visible along with the 165 bp and 60 bp bands. PCR was then performed using 5µM of each primer. As shown in Figure 3.13, the 845 bp appeared exclusively, without the presence of the primer artifacts.

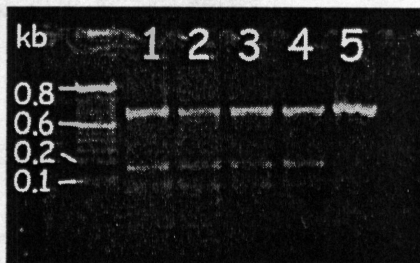


Figure 3.12: 2% TAE agarose gel showing PCR products from genomic DNA extracted from chicken blood cells, using Primer Set 1 at 10µM (1-4) and 5µM (5).

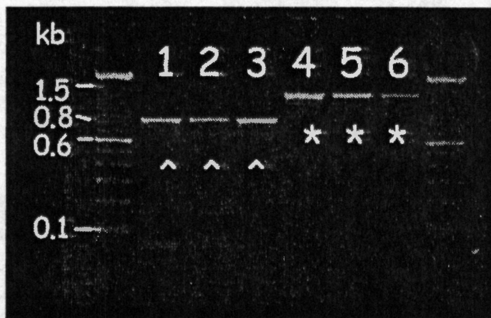


Figure 3.13: 2% TAE agarose gel showing PCR products from genomic DNA extracted from chicken blood cells, using Primer Set 1 at 10µM (1) and 5µM (2, 3); and Set 2 at 10µM (4) and 5µM (5, 6). Note: (^) indicates the 845bp fragment and (*) indicates the 1340bp fragment.

3.2.2 PCR Products Using Primer Set 2

A second set of primers (designated Primer Set 2) was selected which corresponded to conserved sequences outside the fragment flanked by the original primer pair. PCR amplification using this Primer Set 2 did not produce any primer artifacts, as obtained with Primer Set 1. As shown in Figure 3.10 and 3.11, the large band that was produced did not correspond to the 344 bp fragment expected from PCR amplification of cDNA synthesized from chicken IFN-γ mRNA. However, it did match the fragment size expected from PCR amplification of the IFN-γ gene (expected size: 1340 bp), most likely was due to the genomic DNA contamination of the original mRNA prep (see above). PCR amplification was performed using the chicken genomic DNA prep and Primer Set 2.2 (Cartridge-purified), and, as shown in Figure 3.13, the expected 1340 bp fragment was produced.

3.2.3 Processing of Tissue Samples from Chicken and Turtle

Isolating RNA from chicken and turtle blood samples using the TRIzol reagent method has proven to be inefficient, as evident from the above experiments that have resulted in little or no amplification products of the appropriate size cDNA. Alternatively, cDNA was prepared from whole organ fragments in order to increase the yield of the RNA/cDNA extraction step.

Whole spleens were obtained from chickens and were homogenized and processed according to the TRIzol Reagent protocol. Large amounts of RNA were extracted, from which cDNA was synthesized. As seen in Figure 3.14, cDNA PCR products of the correct size were obtained, indicating successful RNA extraction, cDNA

preparation and PCR amplification. Band sizes shown correspond to products made using Primer Set 1 and Primer Set 2 and are of the expected mRNA sizes (204 bp and 344 bp, respectively). Some genomic DNA contamination was also present in these RNA preps, and their PCR products can be seen as larger band sizes (845 bp and 1340 bp, respectively).

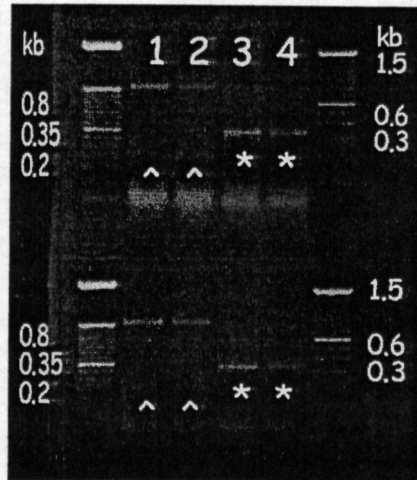


Figure 3.14: 2% TAE agarose gel showing PCR products from cDNA synthesized from RNA extracted from chicken spleens, using Primer Set 1 at 10μM (1) and 5μM (2); and Set 2 at 10μM (3) and 5μM (4). Some genomic DNA amplified products are present, however the expected cDNA (mRNA) band sizes are indicated. Note: (^) indicates the 204bp fragment and (*) indicates the 344bp fragment.

In addition to extracting total RNA, genomic DNA was also extracted from chicken spleens using the Wizard Genomic DNA Purification Kit, resulting in a higher yield than that obtained from whole blood (data not shown).

An archived frozen liver specimen from a healthy sea turtle was obtained from the Department of Wildlife and Fisheries, and was processed only for genomic DNA extraction using the Wizard Genomic DNA Purification Kit. In order to obtain RNA that has not undergone extensive degradation, fresh turtle tissue samples are necessary,

and therefore this liver tissue was not used for RNA extraction. Since spleens were not available due to lack of accessible sick animals, it was not possible to prepare splenic RNA/cDNA for analysis. In order to confirm that the DNA extraction was successful, PCR of the turtle genomic DNA was performed using highly conserved β-actin primers (Clontech), using human and chicken genomic DNA as β-actin positive controls. As seen in Figure 3.15, the turtle genomic DNA extraction was successful since β-actin amplification products of identical size were obtained for the turtle, chicken, and human genomic DNA templates.

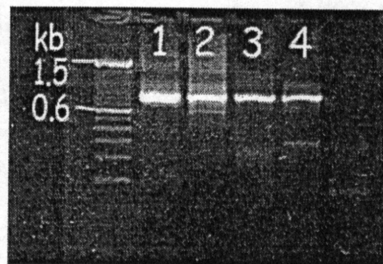


Figure 3.15: 2% TAE agarose gel showing PCR products of β-actin primers using a β-actin Control (1), human genomic DNA (2), chicken genomic DNA (3), and turtle genomic DNA (4).

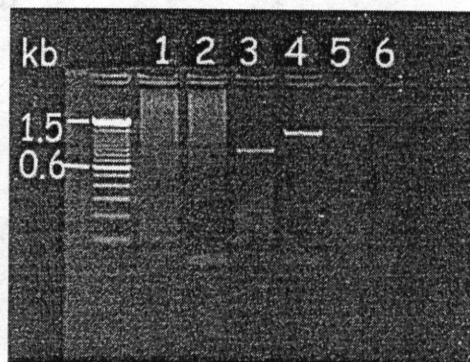


Figure 3.16: 2% TAE agarose gel showing PCR products of human genomic DNA and Primer Set 1 and Set 2 (1 and 2), chicken genomic DNA and Primer Set 1 and Set 2 (3 and 4), and turtle genomic DNA and Primer Set 1 and Set 2 (5 and 6).

As seen in Figure 3.16, PCR amplification of genomic human and turtle DNA using the chicken Primer Sets 1 and 2 was unsuccessful, yielding no PCR products, while PCR with genomic chicken DNA produced the expected 845 bp and 1345 bp bands.

3.2.4 PCR DIG-Probes and Southern Blot

While it was not possible to detect IFN-γ-related sequences by PCR using the two sets of primer pairs, it was possible that homologous sequences could be detected by hybridization of turtle genomic DNA to the fragments generated using these primer pairs. Digoxigenin-labeled probes were synthesized using the DIG-PCR Probe Synthesis Kit (Boehringer Mannheim). DIG-Probe was synthesized using Primer Set 1.2 (see Figure 3.17A), and was tested using a positive control (non-labeled PCR product produced using Primer Set 1.2 and chicken genomic DNA template) dot blot on a nylon membrane (positively-charged) (see Figure 3.17B).

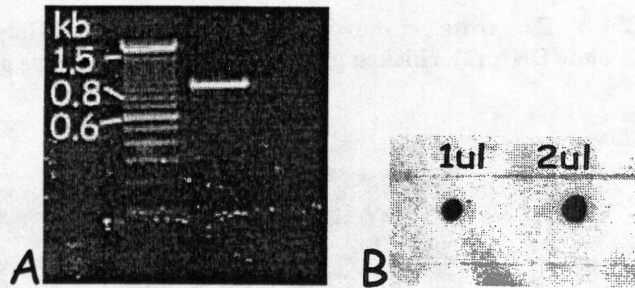


Figure 3.17: (A) 2% TAE agarose gel showing DIG-1.2 Probe synthesized by PCR (expected size = 845 bp) Note: DIG-dUTP is incorporated into the PCR product, which increases the molecular weight of the fragment. (B) Dot Blot showing DIG-1.2 Probe with positive control 1.2 fragment (1μl & 2μl spots).

In addition, a DIG-Probe was synthesized using Primer Set 2.2 (see Figure 3.18A), and was tested using a positive control (non-labeled PCR product produced using Primer Set 2.2 and chicken genomic DNA template) dot blot on a nylon

membrane (positively-charged) (see Figure 3.18B). These results indicated that the DIG-probes were appropriately labeled and were capable of hybridizing to the corresponding DNA sequences.

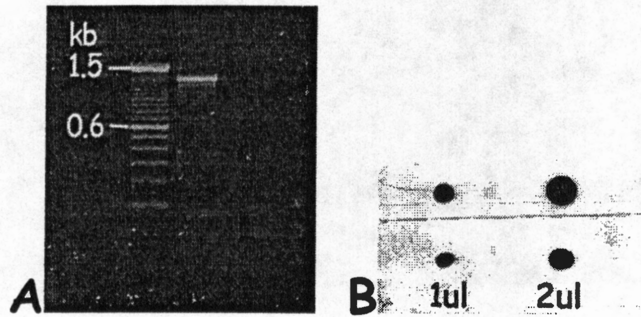


Figure 3.18: (A) 2% TAE agarose gel showing DIG-2.2 Probe synthesized by PCR (expected size = 1340 bp) Note: DIG-dUTP is incorporated into the PCR product, which increases the molecular weight of the fragment. (B) Dot Blot showing DIG-2.2 Probe with positive control 2.2 fragment (1μl & 2μl spots).

Southern Blots were performed (Boehringer Mannheim) as detailed in the Materials and Methods section. Genomic chicken DNA was digested using *Eco*RI, and run on a 0.8% agarose gel, along with the non-labeled positive controls. DNA was blotted from the gel by downward capillary transfer to a positively charged nylon membrane. Although the transfer was complete, as evidenced from strong bands with the positive control fragment, genomic bands corresponding to the IFN-γ were not readily visible in initial experiments (data not shown).

Genomic chicken DNA was digested using *Hind*III, and run on a 0.8% agarose gel, along with the non-labeled positive controls. The DIG-(2.2) Probe, synthesized using chicken IFN-γ Primer Set 2.2, was used to probe the Southern blot (see Figure

3.19) and a faint band corresponding to the *Hind*III restriction digest of chicken γ-IFN was visible in the Southern Blot (expected size = 1879 bp).

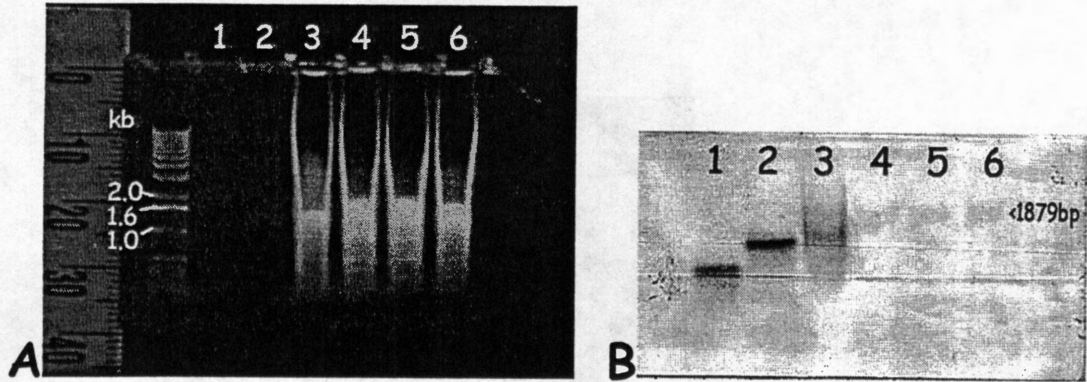


Figure 3.19: (A) 0.8% TAE agarose gel showing (1) 1.2-positive control, (2) 2.2-positive control, (3) 20μl of chicken *Hind*III digest (w/1μl 2.2-positive control spike), (4) 30μl of chicken *Hind*III digest, (5) 50μl of chicken *Hind*III digest, and (6) 25μl of chicken *Hind*III digest. (B) Southern Blot of the same gel.

With the expectation that the turtle γ-IFN gene would contain similar sequences and restriction digest splice sites, genomic turtle DNA was digested using *Hind*III and run on a 0.8% agarose gel, along with the non-labeled positive controls was hybridized using the DIG-(2.2) Probe (see Figure 3.20), however no bands were present.

In order to confirm that the turtle genomic restriction fragments were being properly transferred to the nylon membrane during the Southern Blot, the DIG-labeled turtle β-actin PCR product (~900bp) was used as a positive control. Using the PCR DIG Probe Synthesis Kit, a DIG-probe was produced from genomic turtle DNA and β-actin primers (see Figure 3.21).

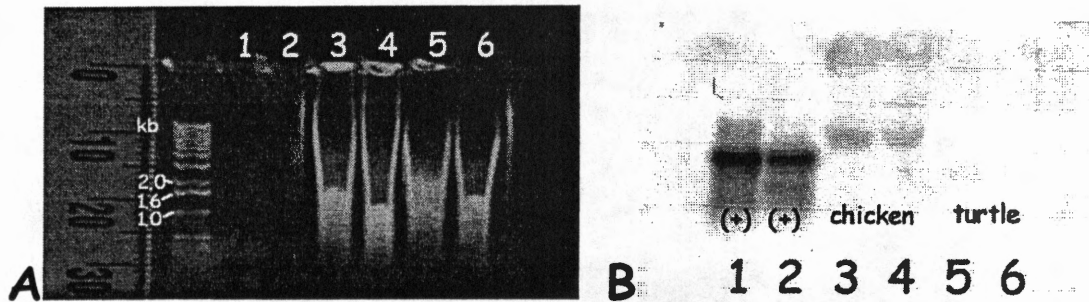


Figure 3.20: (A) 0.8% TAE agarose gel showing (1) 2.2-positive control (1:200), (2) 2.2-positive control (1:1000), (3 and 4) 50µl of chicken *Hind*III digest, and (5 and 6) 50µl of turtle *Hind*III digest (B) Southern Blot of the same gel.

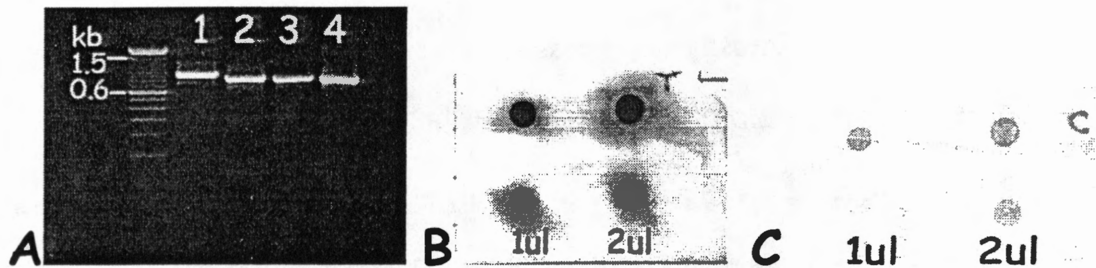


Figure 3.21: (A) 2% TAE agarose gel showing (1) β-actin Probe synthesized from turtle genomic DNA by PCR (Note: DIG-dUTP is incorporated into the PCR product, which increases the molecular weight of the fragment), (2) β-actin from turtle genomic DNA, (3) β-actin from chicken genomic DNA, and (4) β-actin positive control. (B) Dot Blot showing β-actin Probe with positive control turtle β-actin fragment (1µl & 2µl spots), and (C) Dot Blot showing β-actin Probe with positive control chicken β-actin fragment (1µl & 2µl spots).

Genomic chicken and turtle DNA was digested using *Kpn*I, and run on a 0.8% agarose gel, along with the non-labeled positive controls. The Southern blot shown in Figure 3.22 shows the turtle β-actin probe hybridizing to the positive control fragment as well as to the digested turtle genomic DNA. This shows that the turtle genomic DNA is being transferred to the nylon membrane, and is capable of hybridizing to a homologous DIG-labeled probe.

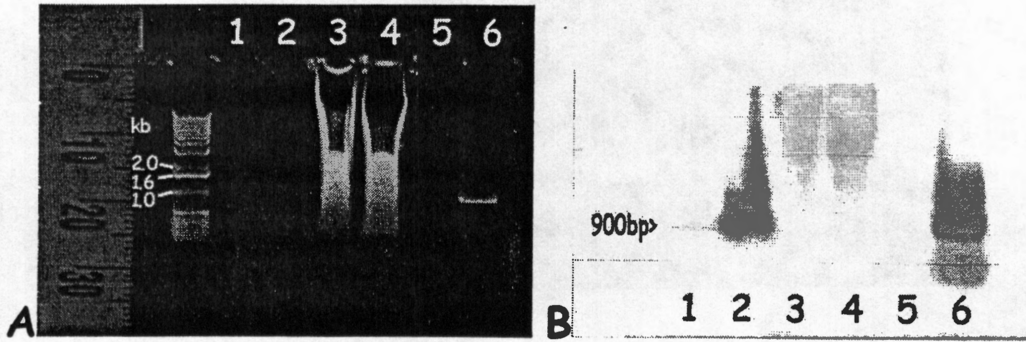


Figure 3.22: (A) 0.8% TAE agarose gel showing (1) chicken β-actin positive control, (2) turtle β-actin positive control, (3) chicken genomic digest using *Kpn*I, (4) turtle genomic digest using *Kpn*I, (5) (blank), (6) β-actin positive control. (B) Southern Blot of the same gel with turtle β-actin Probe.

In order to investigate the possibility that the fragments produced using the previous restriction enzymes were not small enough to be visualized, genomic turtle DNA was digested with a panel of commonly used restriction enzymes to generate smaller restriction fragments (Figure 3.23A). The DIG-(2.2) Probe, synthesized using chicken IFN-γ Primer Set 2.2, was used to probe the Southern blot and Figure 3.23B shows no visible bands, except for the positive control. However there did seem to be some non-specific binding of the probe to elements in the digests. To test if this was due to non-specific binding of the anti-DIG antibody, a Southern blot was performed in which no probe was used for hybridization, and no reaction occurred.

Turtle genomic DNA was then digested with two restriction enzymes at the same time in order to produce even smaller fragments, and the DIG-labeled β-actin probe was used. As shown in Figure 3.24, the combination of *Kpn*I and *Sac*I (seen in Lane 2) and the combination of *Hind*III and *Bam*HI (seen in Lane 3) yielded two bands (~2.0 kb and ~1.2kb) each when probed with the DIG-labeled β-actin probe, but yielded no bands when hybridized to the DIG-(2.2) probe (data not shown).

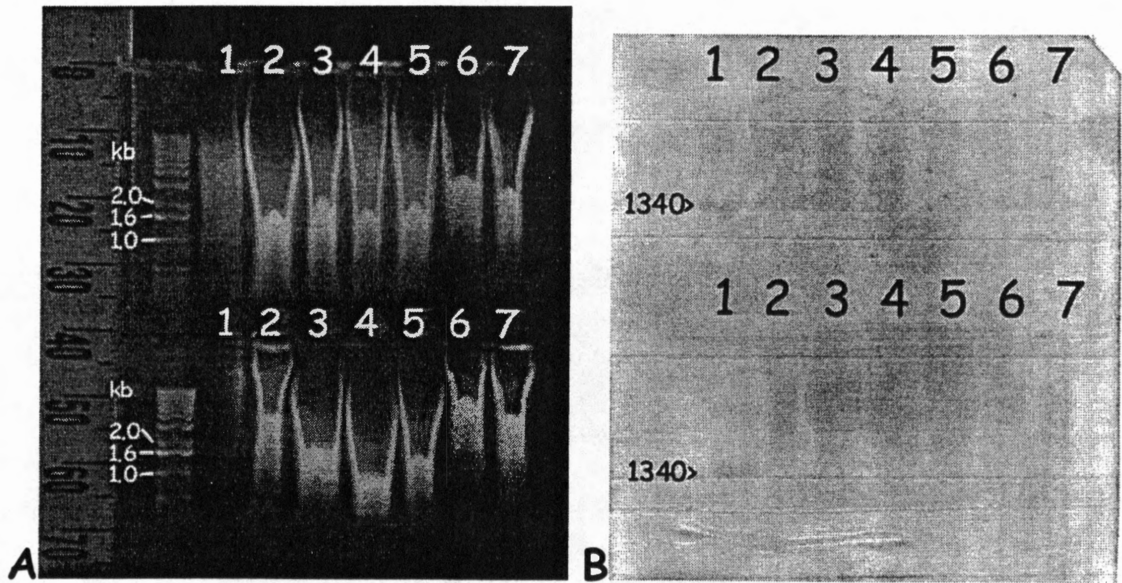


Figure 3.23: (A) 0.8%TAE gel showing genomic turtle DNA digests. Top Panel: (1) 2.2 positive control, (2) *Acc*I digest, (3) *Bam*HI digest, (4) *Bsa*BI digest, (5) *Eco*RI digest, (6) *Eco*RV digest, (7) *Kpn*I digest; Bottom Panel: (1) 2.2 positive control, (2) *Nar*I digest, (3) *Nco*I digest, (4) *Pst*I digest, (5) *Sac*I digest (6) *Sal*I digest, (7) *Sma*I digest. (B) Southern Blot of the same gel with DIG-(2.2) Probe.

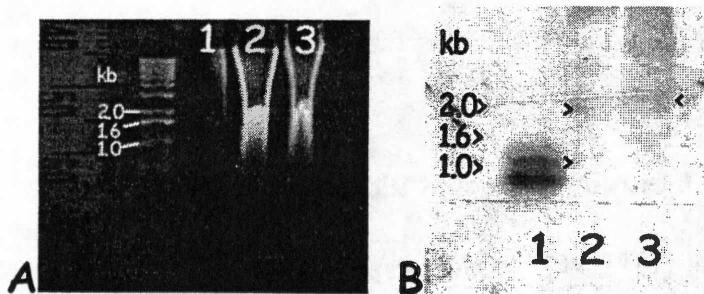


Figure 3.24: (A) 0.8% TAE showing genomic turtle DNA digests (1) turtle β-actin positive control, (2) turtle DNA digest using *Kpn*I and *Sac*I and (3) *Hind*III and *Bam*HI. (B) Southern Blot of the same gel with turtle β-actin Probe (arrows indicate the position of bands). Numbers on the blot indicate molecular weight markers.

CHAPTER 4
DISCUSSION

4.1 An Overview of The Strategy

In order to investigate the interferon-γ (IFN-γ) molecule in the Hawaiian green sea turtle *(Chelonia mydas)*, the original strategy of this research project involved the isolation of turtle lymphocytes and mitogen stimulation in culture. The cells are then harvested and an RNA extraction is performed, followed by cDNA synthesis from mRNA by RT-PCR. Using primers derived from the chicken IFN-γ gene, the turtle cDNA is then amplified by PCR and examined for a product that corresponds to the turtle IFN-γ gene. Southern blots would then be performed with the amplified product in order to confirm that the turtle IFN-γ PCR products contain homologous sequences to chicken IFN-γ. This would be accomplished by creating Digoxigenin-containing PCR probes synthesized using the primers, and hybridization of these probes to restriction enzyme digests of turtle DNA.

4.1.2 Lymphocyte Proliferation Assay and RT-PCR

By isolating lymphocytes present in blood, a population of cells of the immune system is obtained, including macrophages (mononuclears) and polymorphonuclear cells. Using a lymphocyte proliferation assay, the mononuclears can be grown in culture in the presence of Concanavalin A (a mitogen), which causes the macrophages to secrete various lymphokines, including IFN-γ. Therefore, mononuclears stimulated by Con A would produce an abundance of mRNA, some of which should encode for the IFN-γ polypeptide homologue.

After a period of time (36-48 hours), the cells are harvested (after accumulating vast amounts of IFN-γ mRNA), and the total RNA is extracted by the TRIzol technique. The TRIzol Reagent is a mono-phasic solution of phenol and guanidine isothiocyanate that maintains the integrity of RNA during cell homogenization or lysis, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation, separates the solution into an organic phase and an aqueous phase, containing the RNA. After the transfer of the aqueous phase, the RNA is recovered by precipitation with isopropanol.

DNA that is complimentary to the RNA (cDNA) is synthesized by the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) method. In the reaction, cDNA is produced and amplified using reverse transcriptase (an enzyme that uses RNA as a template to make DNA) and Oligo(dT) primers. The Oligo(dT) primers are a string of 15 dT bases that hybridize to the polyA tail present only on the mRNA species, ensuring that cDNA is synthesized exclusively from this population of RNA. The cDNA is then amplified by PCR using the primers constructed from the chicken IFN-γ gene, resulting in a product that corresponds to the IFN-γ gene in the green sea turtle.

4.1.3 Primer Selection

The primers used (Primer Set 1 and Set 2) were chosen specifically because they corresponded to segments in the chicken IFN-γ gene that encoded highly conserved, specific amino acid sequences. As discussed earlier, investigation of the known chicken IFN-γ amino acid sequence has revealed that there are regions of homology that are conserved in the various species in which this molecule has been studied. This is understandable, considering the importance of the role of IFN-γ in the immune system.

Figure 1.6 shows the alignment of the amino acid sequence of the chicken and human IFN-γ polypeptides, with the asterisks signifying exact matches. Primer Set 1 Forward and Reverse sequences corresponds to two regions of the peptide in which a series of identical amino acids occur: the segments LSQIVSMY and QRKAANELF, respectively. By using the ORF Finder database, the exact DNA codon sequences that corresponded to these amino acid chains were obtained; Set 1Forward primer (26bp) was chosen in the 5' to 3' direction, and Set 1 Reverse primer (26bp) is in the 3' to 5' direction. After PCR amplification of cDNA (derived from chicken mRNA) using the Set 1 primers, a gene product of 204bp should result, since this is the expected length of the DNA segment between and including the primers.

Primer Set 2 Forward and Reverse sequences correspond to two other regions of amino acid sequence homology, the segments SHSDVAD and KRKRSQS, located outside of the Set 1 sequences. Again, Set 2 Forward primer (21bp) is in the 5' to 3' direction, while Set 2 Reverse is in the 3' to 5' direction. After PCR amplification of the cDNA (derived from chicken mRNA), a gene product of 344 bp should result.

PCR amplification of green sea turtle cDNA derived from mRNA using Primer Set 1 and Set 2 should result in gene segments of sizes similar to those of the chicken, assuming that the gene is conserved between it and the turtle. If there are any insertion or deletion events, which normally occur in the evolution of any gene, the size of the resulting gene segment would vary in length, being longer or shorter than the original segment.

4.2 The Initial Studies

4.2.1 RNA Extraction and cDNA Amplification

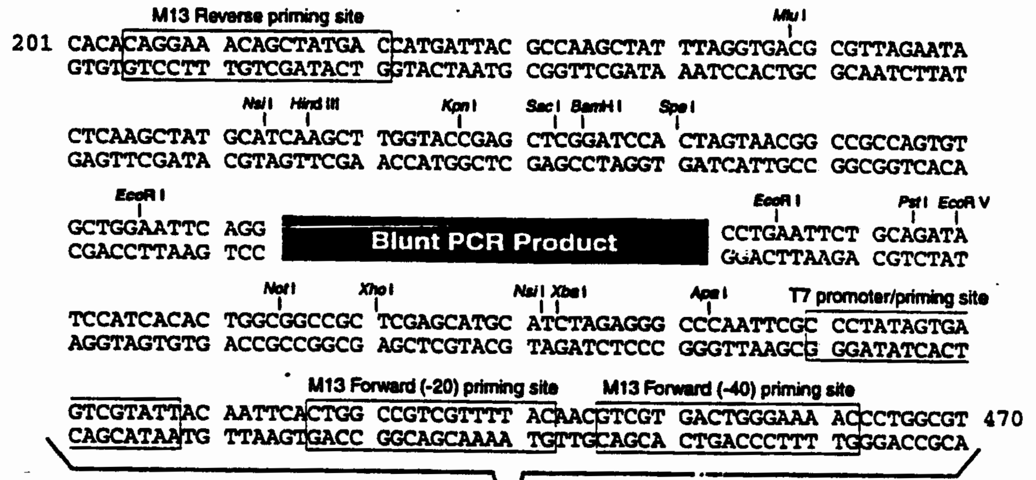
Initially it was necessary to test and practice the methodology on human samples to ensure that the process was sound. Lymphocytes were isolated from human blood, and grown in culture with and without Con A, a known mitogen, in accordance with a standard lymphocyte proliferation assay. The cells were harvested at defined time points, RNA was extracted, and cDNA was synthesized and subsequently amplified by PCR using known human IFN-γ primers. The resulting gene product of the expected length (427 bp) was seen (see Figure 3.1). The kinetic study revealed an increase in the amount of mRNA/cDNA from 12 hours to 48 hours (as would be expected with the accumulation of mRNA over time), which can be seen with the increasing intensity of the gel bands (see Figure 3.2). Note that even in cells grown in the absence of Con A, the PCR product can still be seen, revealing a basal level of IFN-γ mRNA being produced in these cells.

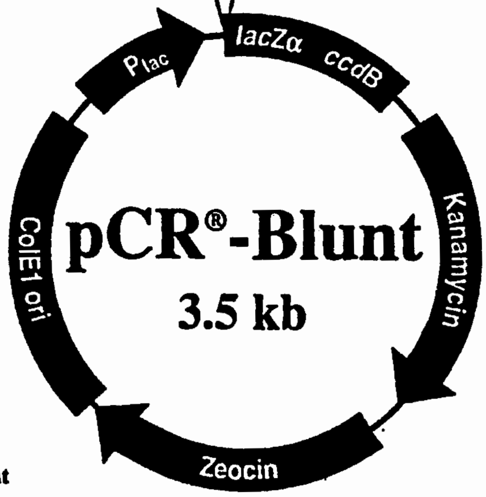
Chicken blood samples were obtained and processed in the same manner as the human samples. When the cDNA derived from chicken RNA was amplified by using the constructed chicken IFN-γ Primer Set 1, a PCR product was seen. Unfortunately, this product was not further investigated at the time to verify if it was the segment of correct size and make-up. In retrospect, the product was not of the expected size, 204 bp, but was closer to 800 bp in length. However, at that time, that the primers worked in the PCR amplification at all was the only concern. The arrival of the turtle samples soon thereafter resulted in movement of the project to the next phase.

Turtle blood samples were obtained and processed according to established protocols similar to the human Lymphocyte Proliferation Assay, and cDNA derived from turtle RNA was synthesized as usual. PCR amplification using Primer Set 1 resulted in three products of different lengths (165 bp, 60 bp and ~25 bp), of which the smallest one was quickly identified as excess primer and eliminated by reducing the primer concentration (see Figure 3.3 and 3.4). The next step was to isolate these DNA segments separately, and produce a sufficient quantity to allow for subsequent sequencing of the segments. This is usually done through a plasmid vector.

4.2.2 The pCR Blunt Vector

In order to obtain enough quantity of DNA to sequence the desired segments, the pCR-Blunt plasmid vector was used. A blunt-ended PCR gene product can be inserted into the plasmid in a polylinker region of the plasmid by using a T4 DNA ligase. The polylinker region contains restriction sites for various restriction enzymes, including *Eco*RI, as well as M13 Forward and Reverse priming sites. The process of transformation, in which the bacteria are treated to make them temporarily permeable to small DNA molecules, is used to introduce the plasmid into the host cells. The pCR-Blunt vector carries ColE1 Ori (origin of replication), which allows the plasmid DNA to be produced in quantities sufficient for sequencing. The incorporation of the DNA segment into the plasmid can be verified by release of the insert by digestion with *Eco*RI. After isolating the plasmid, the region within the M13 priming sites that contains the inserted gene segment of interest is sequenced.





Comments for pCR®-Blunt
3513 nucleotides

Lac promoter/operator region: bases 95-216
M13 Reverse priming site: bases 205-221
LacZ-alpha ORF: bases 217-570
Multiple Cloning Site: bases 248-393
T7 promoter priming site: bases 400-419
M13 Forward (-20) priming site: bases 427-442
M13 Forward (-40) priming site: bases 446-462
Fusion joint: bases 571-579
*ccd*B lethal gene ORF: bases 580-882
Kanamycin resistance ORF: bases 1231-2025
Zeocin resistance ORF: bases 2232-2606
ColE1 origin (pUC-derived): bases 2674-3387

Figure 4.1: Map of pCR-Blunt Vector (Invitrogen Corporation).

The plasmid contains a gene that confers resistance to kanamycin, which is used as a marker to select the desired cell colonies that are grown on LB agar/medium containing the antibiotic. The vector also contains a segment of DNA derived from the *lac* operon

of *E. coli* that codes for β-galactosidase, and whose synthesis can be induced by isopropylthio-β-D-galactoside (IPTG). This gene fragment is capable of intra-allelic complementation with a defective form of β-galactosidase encoded by the host that is transformed by the plasmid. *E. coli* that is exposed to IPTG synthesizes both fragments of the enzyme and form blue colonies when plated on media containing the chromogenic substrate 5-bromo-4-chloro-3-indoylyl-β-D-galactoside (X-gal) (Sambrook et al.1989). Insertion of foreign DNA into the polycloning site of the plasmid abolishes complementation by inactivating the β-galactosidase coding fragment, and hence bacteria carrying the recombinant plasmids give rise to white colonies.

The gene segments were amplified by PCR using Platinum *Pfx* DNA polymerase produces blunt-ended DNA products that were then ligated into the linearized, blunt pCR-Blunt plasmid using T4 DNA ligase. The ligation reactions are added to solutions containing Top 10 Competent *E. coli* cells, some of which pick up the plasmids and become "transformed". The cells are then incubated overnight on LB agar plates that contain kanamycin, as well as IPTG and X-gal. Five antibiotic-resistant transformants (white colonies) were selected, the plasmid DNA from these colonies was isolated, digested with *Eco*RI, and visualized by gel electrophoresis. Colonies 1 and 3 showed two bands of ~105bp and 60 bp, while Colonies 2, 4 and 5 showed bands 50-60 bp in size (see Figure 3.5). The presence of two fragments in from Colonies 1 and 3 indicated that a probable internal *Eco*RI splice site was present in the inserted DNA fragment. Subsequent PCR amplification of these plasmids produced a single band as expected (see Figure 3.6).

4.2.3 Analysis of pCR-Blunt Inserts

Further propagation of the cells from the five colonies on fresh media produced a large quantity of plasmids, and the isolated plasmids were sent to the Biotechnology/Molecular Biology Instrumentation Lab at UH Manoa to be sequenced using the M13 primers. The sequences (see Table 3.1) were analyzed by first identifying the *Eco*RI splice sites (GAATTC), thereby revealing the location of the inserted PCR products. The sequences were compared with each other using the BLAST 2 Sequence Program. The sequences from Colonies 1 and 3 were found to be nearly identical (the 88% identity was due to mismatching of the "N" bases), and Colonies 2, 4 and 5 were nearly identical. The 164/165bp length of Colonies 1 and 3 plasmids correlate to the 165bp fragment seen in the previous gels, while the plasmids of Colonies 2, 4 and 5 roughly correlate to the 60bp fragment. An internal *Eco*RI splice site was found within the inserts of Colonies 1 and 3 as expected, resulting in the two bands seen after digestion, indicated in bold in Table 3.1.

According to the chicken IFN-γ sequence from which the primers were chosen, the 164 bp turtle sequence was found to be 40 bp shorter than the 204 bp size expected from the corresponding chicken DNA segment. Initially it was thought that this discrepancy in size was due to a deletion in the turtle IFN-γ gene, reflecting the evolutionary distance of the two species. The BLAST/GenBank Program was used to find sequence homology of the turtle insert sequences with the chicken IFN-γ gene to confirm a deletion event, but they matched only in the primer sequences (which was expected since the primer sequences were derived from the known sequence of chicken IFN-γ gene), with no other similarities apparent. The insert sequences were analyzed

using the Open Reading Frame (ORF) Program, which searches for a start codon in the DNA segment, then translates the sequence to the corresponding amino acid chain. The insert from Colonies 1 and 3 was translated as a single open reading frame flanked by the amino acid segments LSQIVSMY and QRKAANELF, corresponding to the primer sequences of Primer Set 1 Forward and Reverse (see Table 4.1). The ORF Finder Program was not able to an open reading frame for insert sequences of Colonies 2, 4, and 5.

Turtle sequence:
LSQIVSMYPSANTSSSMPQLGVACFQSNSGNLNSLESSQLRSEAS**QRKAANELF**

Chicken sequence:
LSQIVSMYLEMLENTDKSKPHIKHISEELYTLKNNLPDGVKKVKDIMDLAKLP
MNDLRI**QRKAANELF**

Table 4.1: Amino acid sequences derived from the 165bp insert fragment, and the corresponding chicken IFN-γ sequence (Note: the sequences that corresponds to the primer DNA sequences derived from the chicken IFN-γ gene are indicated in bold).

When compared to the amino acid sequences of chicken IFN-γ segment, the turtle sequence was found to be ~14 amino acids short. In addition, the two sequences were analyzed for any alignment of key conserved amino acids known. However, there were no significant matches between the turtle fragment-derived amino acid sequence and the chicken IFN-γ amino acid sequence, except for the segments corresponding to the primer sequences, as expected. The two sequences did not seem to show any significant matching, but the amplified turtle gene segment did encode a complete open reading frame.

In order to investigate the above discrepancies, it was necessary to ensure that each step in the method of the original strategy was functioning correctly, starting with the PCR amplification procedure.

4.2.4 PCR Optimization

Different PCR cycle parameters were tested, including different annealing temperatures and durations. The PCR parameters used are listed in Section 2.7.2. The PCR cycle consists of three main steps that occur at different temperatures: (1) the denaturation step, in which the double-strands of the DNA molecule are separated into single strands, occurs at 94-95°C; (2) the annealing step, in which the primers bind to there complementary sequences on the DNA molecule, usually occurs between 50-70°C; and (3) the elongation step, in which the DNA polymerase adds nucleotides to the primer complementary to the DNA template and elongating the strand, occurs at 72°C (68°C for *Pfx* Polymerase). These steps are repeated for 25-35 cycles.

In order to optimize these conditions, it is necessary to adjust the parameters such as temperature and duration. The denaturation and elongation temperatures are standard and were kept the same (95°C and 72°C, respectively), but the duration of each step was extended from 45 seconds each, to 1 minute and 1 minute 30 seconds for the denaturation step and the elongation step, respectively, ensuring that the samples reached those temperatures. In addition, a "hot start" step (95°C for 5 minutes) was added before the PCR run to ensure that the sample was properly denatured at the starting point, and an extended elongation step (72°C for 7 minutes) was added at the end of the completed PCR run.

The optimal temperature for the annealing step is dependent on the melting temperature of the primers (T_M), based on its length and nucleotide composition. The optimal temperature for annealing is T_M-5°C, where $T_M(°C) = 2(N_A + N_T) + 4(N_G + N_C)$

and N is the number of each nucleotide (A, T, G, C) in the primer. The results are shown in Table 4.2.

Primer	T_M	T_M-5°C
Set 1 Forward	74°C	69°C
Set 1 Reverse	80°C	75°C
Set 2 Forward	60°C	55°C
Set 2 Reverse	60°C	55°C

Table 4.2: Melting Temperatures of Primer Set 1 and 2, and their corresponding optimal annealing temperatures.

For Primer Set 1, the calculated optimal annealing temperature was very close to the standard elongation temperatures. Temperatures ranging from 45°C to 65°C were tested in different PCR runs, with no significant differences among the results. Therefore an annealing temperature of 55°C was used for all PCR runs for both primers.

4.2.5 Initial PCR Amplifications using Primer Set 1 and Primer Set 2

In order to further investigate the discrepancies, it was necessary to verify that Primer Set 1 was functioning properly in the first place. The first step was to ensure the primers gave the correct results when used with a positive control, namely a chicken genetic template, as was initially performed. Because the primers were constructed from the known genetic sequence of the chicken IFN-γ gene, PCR amplification using chicken cDNA and Primer Set 1 should give the expected results of a 204 bp fragment (see Section 4.2.2). However the PCR amplification resulted in the identical bands obtained when using the turtle template: 165 bp and 60 bp bands, approximately 34 bp

short of the 204 bp fragment expected. In addition, when PCR was performed using human genomic DNA the identical bands were seen again.

Initially the problem was thought to lie with possible poor quality of the Primer Set 1 batch itself. A second batch of higher purity (Cartridge-purified instead of only Desalted-purified) was ordered and designated Set 1.2 (to differentiate it from the first set, "1.1"). Unfortunately, there was no difference seen in the results of PCR runs, the same two bands were seen with Primer Set 1.2. A different set of primers, Primer Set 2 (see 4.1.2) was then constructed to eliminate the possibility that the nucleotide composition of Primer Set 1 may somehow be affecting the performance of PCR. PCR amplification using the turtle template and Primer Set 2 resulted in no band, and hence no amplification products at all (see Figure 3.7).

The fact that PCR amplification using Primer Set 1 showed the same two bands (165 bp and 60 bp), with chicken, turtle, human templates, as well as with no template at all, pointed towards some sort of contamination present in the procedure.

An attempt was made to isolate the 165 bp and 60bp bands by running the samples on 2% GTG agarose, a low melting point agarose, and excising the individual band fragments, thereby removing and separating them from the rest of the gel. The low melting point of the gels allowed the pieces to be placed in Eppendorf tubes and melted in a heating block at 65°C. The separate samples containing the individual separated bands were then re-amplified by PCR, and again, both bands reappeared together.

The different components that made up the PCR reaction volume were the first place to investigate for contamination. Different PCR DNA polymerases (as well as

different manufacturer lots), including PCR SuperMIX (GibcoBRL), Platinum PCR SuperMIX (GibcoBRL) (both of which use a *Taq* DNA polymerase), Platinum *Pfx* DNA polymerase, and *Pfu* Turbo DNA polymerase, all yielded the same results. Nuclease-free water, used in the reaction volume, was tested from different bottles, again showing no differences.

Two experiments were then performed that helped to elucidate the reasons for the presence of the 165 bp and 60 bp bands. The first experiment involved PCR amplification using no template and different concentrations of Primer Set 1.2. As shown in Figure 3.8, the bands became fainter as the concentration of primer decreased, disappearing altogether at a concentration of 2μM. This indicated that the presence of the bands was primer concentration-dependent. In addition, when PCR was performed with no template and only one of the primer pair (forward only or reverse only), or no primers, the bands were absent (see Figure 3.9).

Other experiments provided important clues to the cause of the discrepancies. On occasion, when performing PCR on different chicken cDNA templates using Primer Set 1.2, a larger band (approximately 850 bp) appeared along with the 165 bp and 60 bp fragments, and sometimes the approximately 850 bp band appeared as the exclusive product (see Figure 3.10 and 3.11). This band was isolated and sequenced, and the resulting sequence was inputted into the BLAST Program. It was found to match with a segment of the whole chicken IFN-γ gene (the 84% identity was due to mismatching of the "N" bases). This indicated that the large band was produced by the PCR amplification of genomic chicken DNA that contaminated the original RNA prep. The expected size of 845 bp, corresponding to the size that would be produced by Primer Set

1.2 with genomic DNA, confirmed this hypothesis. This finding also confirmed that the primer pair selected for the study did, indeed, hybridize to the chicken IFN-γ gene. However, this primer pair did not appear to produce the expected IFN-γ RNA fragment for either the chicken or turtle peripheral blood cells.

To confirm the hypothesis that the 845 bp PCR product was due to the amplification of the chicken IFN-γ gene, a genomic DNA preparation was performed from a new chicken blood sample using the Wizard Genomic DNA Purification Kit, followed by PCR amplification using Primer Set1.2. As shown in Figure 3.12, the 845 bp band is visible along with the 165 bp and 60 bp bands. In order to test the hypothesis that the 165 bp and 60 bp were PCR product artifacts caused by the primers, and that the effect was primer concentration-dependent, PCR was performed using 5μM of each primer. As shown in Figure 3.13, at this primer concentration the 845 bp appeared exclusively, without the presence of the primer artifacts.

The forward and reverse primers of Set 1 may somehow have spontaneously produced artifacts of length 165 bp and 60 bp. This phenomenon was primer concentration-dependent as well as independent of the presence of the DNA template. (Also note in Figure 3.10, 3.11, and 3.12 that the intensity of the 845 bp band and that of the 165/60 bp artifacts are inversely related; intensity of the 845 bp band is higher when the artifacts are absent, and lower (or absent) when the 165/60 bp artifacts are present. This maybe related to the availability of the primers for PCR amplification, and their sequestering in the production of the artifacts).

PCR amplification using this Primer Set 2 did not produce any primer artifacts, as did Primer Set 1. But, as shown in Figure 3.10 and 3.11, the large band that was

produced did not correspond to the 344 bp fragment expected from PCR amplification of cDNA synthesized from chicken mRNA. However, it did match the fragment size expected from PCR amplification of the IFN-γ gene (expected size: 1340 bp), which was due to the speculated genomic DNA contamination of the original mRNA prep (see above). PCR amplification was performed using the chicken genomic DNA prep and Primer Set 2.2 (Cartridge-purified), and, as shown in Figure 3.13, the expected 1340 bp fragment was produced.

4.2.7 RNA Extraction from Organ Tissue

The lack of PCR products from the amplification of cDNA pointed to the inefficiency of cDNA synthesis of the original RNA extractions from the chicken and turtle blood samples. Upon reviewing the previous studies on cloning chicken IFN-γ gene, it was noted that the experiments were performed on chicken splenocytes, and not on blood samples. It was thought that the inefficiency of the RNA extractions was due to a low concentration of RNA in the starting blood samples, and that perhaps the use of organ (spleen) tissue would offer the greater amount of sample needed. Therefore, spleens were obtained from freshly killed chicken samples, and processed according to the TRIzol protocol (see Section 2.4.2), and cDNA was synthesized and amplified by PCR. As seen in Figure 3.14, PCR of the samples using Primer Set 1 and Set 2 yielded the band fragments of sizes 204 bp and 344 bp, as expected from the amplification of cDNA synthesized from mRNA. (Note that larger band fragments of sizes 845 bp and 1340 bp were present as well, signifying a slight amount of genomic DNA contamination in the samples).

While it would have been ideal to carry out similar experiments with turtle splenic RNA, fresh turtle tissue was not available at the time these studies were carried out.

4.3 The Later Studies

The initial lack of success of the RNA extractions prompted a new strategy in which genomic DNA would be the focus. Genomic DNA is more stable than RNA and can be extracted from tissue samples that are stored for long periods of time at -20°C (Tissues used for RNA work have to be immediately frozen at -70°C, or processed for extraction immediately after the sample is removed from the organism, since the RNA starts to degrade immediately after the tissue is removed).

4.3.1 Genomic DNA Extraction from Organ Tissue

Extraction of genomic DNA from organ tissue using the Genomic DNA Purification Kit (see Section 2.6.2) is a fast and easy method, and yields an abundance of genomic DNA material.

Homogenization of the tissue (chicken spleens and turtle liver) sample through a strainer removes the cytoplasmic membranes and the connective tissue, extruding a pulp of nuclei and cytoplasmic material, while the Nuclei Lysis Solution then removes the nuclear membranes, leaving only proteins and genetic material. Adding RNase destroys any RNA present, while the Protein Precipitation Solution precipitates out the protein. The genomic DNA that is left in the supernatant solution is then precipitated out using isopropanol, washed using ethanol, and air-dried. The genomic DNA is then rehydrated in TE buffer. This material was then used to perform PCR using the Primer Set 1 and

Set 2 to synthesize probes for use in Southern Blots, and to make genomic DNA restriction digests.

4.3.2 PCR DIG Probes

The PCR fragments synthesized using chicken template DNA and the chicken IFN-γ Primer Set 1 and Set 2 contain sequences that are specific segments of the IFN-γ gene and can be used as probes to detect the presence of complementary nucleic acids in complex mixtures of as a total cellular DNA. This is possible because of the high specificity of nucleic acid hybridization.

A label attached to the probe allows for subsequent detection. The PCR DIG Probe Synthesis Kit (see Section 2.10) allows for direct digoxigenin (DIG)-labeling of DNA fragments generated by PCR. DIG-dUTP (see Figure 4.2), included in the dNTP nucleotide Mix, is incorporated into the PCR product. A sheep anti-DIG antibody-alkaline phosphatase conjugate is allowed to bind to the DIG probe. The signal is then detected with the colorimetric 5-bromo-4-chloro-indoyl-phosphatase/nitroblue tetrazolium (BCIP/NBT) phosphatase substrate, which turns dark purple on contact with the antibody-conjugate.

The DIG-dUTP nucleotide is complementary to dATP and is incorporated in place of dTTP, and the DIG-dUTP:dTTP ratio is an important consideration in probe preparation. Since the PCR product was of a lower yield than usual, the recommendation from the manufacturer was to dilute the stock mix (which contains a ratio of 1:2 of DIG-dUTP:dTTP) 1:1 with a dNTP mix, which increased the yield considerably. However, this may have caused some decrease in the signal sensitivity.

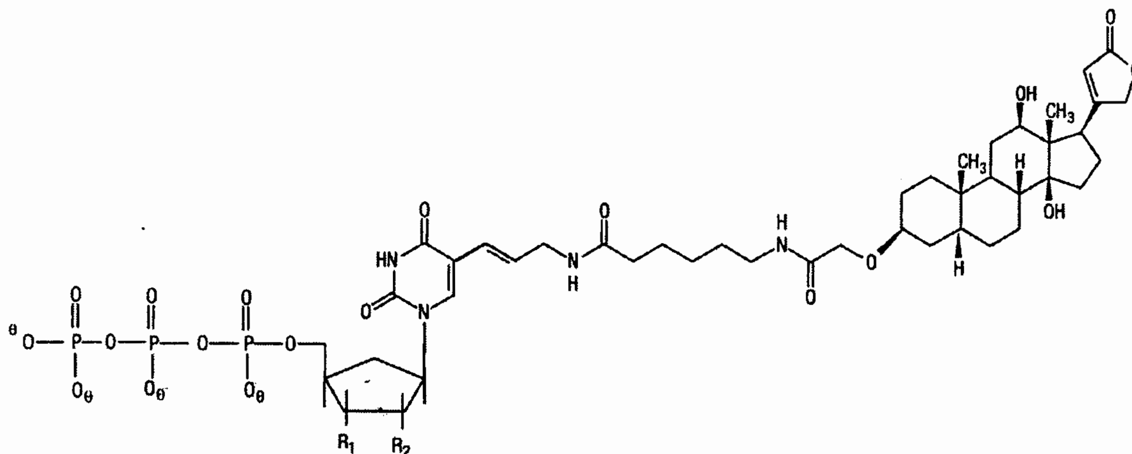


Figure 4.2: Digoxigenin (DIG)-labeled dUTP (R_1=OH, R_2=H).

DIG PCR probes were made using Primer Set 1 and Set 2 from chicken genomic DNA and were designated DIG-(1.2) and DIG-(2.2), respectively. In order to test the efficiency of the hybridization of the probe and the signal sensitivity, it was necessary to perform dot blots using prepared positive controls, which were PCR fragments made using Primer Set 1 and Set 2 from chicken genomic DNA. Various amounts of these positive controls were spotted onto nylon membranes, which are microporous, positively charged ultra-pure nylon cast on a polyester support. The DNA is secured on to the membranes by baking in a vacuum oven at 80°C for 30 minutes, followed by UV-cross linking for 3 minutes using a transilluminator. The initial dot blots had very weak signals, but after many adjustments of the DIG probe concentration and positive control concentrations, the dot blots seen in Figures 3.17 and 3.18, show that hybridization was successful and signal was clearly visible. The probes were then ready for use in the Southern blotting technique.

4.3.3 Southern Blot

The Southern blot technique is used for the detection of specific DNA sequences in a complex mixture of fragments obtained from the restriction digest of genomic DNA. The genomic DNA is digested using restriction enzymes and then subjected to gel electrophoresis to separate the fragments according to size. The restriction fragments are denatured with an alkaline solution (NaOH), and then transferred onto a nylon membrane by blotting, immobilizing the fragments onto the membrane as a replica of the gel. The membrane is then hybridized with a probe of a specific DNA sequence. The DNA restriction fragment that contains sequences complementary to the probe hybridizes, and its location on the membrane is revealed by a detection signal.

It was first necessary to make sure that all the conditions for the Southern blot were functioning properly, including using the proper buffers for the procedure, using the proper blotting apparatus, as well as optimizing the DIG Probe concentrations. A High SDS buffer containing formamide was chosen for the Pre-hybridization and Hybridization solution since it is optimal for use with DNA. The DIG PCR probes DIG-(1.2) and DIG-(2.2), were used as probes in the Southern blots, and initially the 1.2 and 2.2 positive controls were used to test the efficiency of transfer during the blot. The positive controls transferred successfully, and the DIG Probe concentration was optimized to insure adequate signal strength, while eliminating background signal.

4.3.4 Southern Blot of Genomic Chicken DNA

The next step was to blot digested chicken DNA, and *Eco*RI was used to digest genomic chicken DNA. After many attempts, an adequate signal from the probes could not be generated with the genomic digests, even though a strong signal was seen from very dilute concentrations of the positive controls.

Different approaches were taken to attempt to elucidate the source of the problems. To ensure that the genomic DNA was being blotted during the procedure, an optional depurination step was taken to improve the transfer of the DNA onto the gel. Also, the duration of blotting was increased from overnight to 24hours to 36 hours. Examination of the gel remnants after the blotting procedure showed no traces of ethidium bromide stained nucleic acid residue, indicating that the DNA was being transferred.

Increasing the DIG Probe concentrations to try to boost the signal was also tried (the positive control was diluted from 1:10 to 1:10000 to compensate), but no signal was detected from the genomic transfer. Increasing the gel load volume was then tried, from 10μl to 20μl, which made it necessary to pour thicker gels as well as to increase the blotting times to 36+ hours.

To ensure that the genomic DNA was being properly digested by the restriction enzyme, different ratios of DNA:enzyme:buffer were tried (including 8μl:1μl:1μl and 16μl:2μl:2μl and 5μl:2μl:2μl + 1μl water), etc., all yielding no results. Different lots of *Eco*RI were used as well as different concentrations of the enzyme, with still no results. Then different restriction enzymes, in addition to *Eco*RI, were used for the genomic

digests, including *Bam*HI and *Hind*III. Finally an extremely faint signal was seen with the *Hind*III genomic digest.

In order to boost the signal to make sure that band seen was not background or a ghost signal, different approaches were used. This included increasing the gel load of the genomic digest from 20μl to 50μl, pouring thicker gels to accommodate the increase in volume, as well as increasing the blot transfer time from 24hours to 72+ hours, and increasing the proportion of DNA in the digest from 50% to 80% of the total digest volume. These approaches resulted in significant enhancement of hybridization signal intensity.

To boost the signal even more, the DIG probe concentration was increased tenfold; the anti-DIG antibody concentration was increased twofold. Finally, the duration of the antibody incubation step was also increased from 30 minutes to 60 minutes. These final changes markedly increased the chicken IFN-γ hybridization and provided adequate sensitivity to initiate turtle genomic DNA hybridization studies.

Sequence analysis was performed to try to explain why a band was obtained by Southern blot of the *Hind*III genomic digest and not from the *Eco*RI or *Bam*HI digest. Using the Webcutter 2.0 program, which finds restriction enzyme splice sites in a given nucleotide sequence, the full chicken IFN-γ gene was analyzed. The gene is 6765 bp in size, and the PCR fragment which would be produced using Primer Set 2 (i.e. the DIG-(2.2) Probe) runs from position 4126 and 5466 (1340 bp). *Hind*III cuts twice within the IFN-γ gene at positions 3373 and 5252, creating a fragment of 1879 bp in length which would hybridize to the DIG-(2.2) Probe. *Bam*HI cuts in only one position (5430), and therefore does not create a genomic DNA fragment small enough to beresolved by gel

electrophoresis and visualized by hybridization with the DIG-(2.2) probe. *Eco*RI cuts in positions 1535 and 2629, however these do not overlap with the segment contained within the DIG-(2,.2) Probe would not be visualized either.

4.3.5 Southern Blot of Genomic Turtle DNA

The final part of this project was to investigate whether or not the hypothetical turtle IFN-γ gene contains sequences that would hybridize to sequences derived from the chicken IFN-γ gene. This would be accomplished by performing a Southern blot using genomic turtle DNA and the DIG-(2.2) Probe derived from the chicken IFN-γ gene. Figure 3.20 shows an attempt at this using genomic turtle *Hind*III digest, but no strong signal was observed. A very slight signal was seen in the higher molecular weight region, so another blot was attempted, but no signal was seen in this blot. Because the gene sequence of turtle IFNγ has not been elucidated, it is not known whether it would contain similar *Hind*III recognition sites to the chicken IFN-γ gene. There was a possibility that other restriction enzymes would reveal more information. However, before pursuing other digests, preliminary studies had to be done to show that the genomic turtle DNA was in fact being transferred properly during the blotting procedure. This was done by examining turtle genomic DNA hybridization to an appropriate positive control could probe.

β-actin is a cytoskeletal protein that is ubiquitously expressed and is one of the most abundant proteins in a cell. This protein is encoded by a large, highly conserved gene family, and is one of the most conserved proteins in the cell. It was therefore not surprising to find that β-actin primers produce PCR products in genomic DNA from human, chicken and turtle and that the product from all three organisms was nearly

identical in length. This housekeeping gene is therefore a good standard for quantitative expression studies, as well as a good positive control for cDNA synthesis and PCR amplification.

A turtle DIG-β-actin probe was constructed using genomic turtle DNA template (see Figure 3.21) and was shown to hybridize to β-actin positive controls constructed by using both chicken and turtle DNA. The intensity of the signal was weaker with the chicken PCR fragment, perhaps indicating slight differences in the gene sequences of the two species (causing inefficient hybridization). A blot of genomic *Kpn*I digests of both the chicken and the turtle genomic DNA confirmed that both DNA preparations are transferred on to the membrane properly. The weaker signal was again apparent with the chicken genomic DNA samples when compared to that of the turtles, seen in both the positive controls, and the genomic DNA.

A panel of twelve different restriction enzymes was used to digest separate samples of genomic turtle DNA, with the thought that they may have splice sites in regions that would hybridize to the chicken IFN-γ DIG-(2.2) Probe. No bands were seen, except for the positive control. There was some signal visible however; so further experiments were carried out to elucidate the source.

To rule out that the signal visible was caused by non-specific binding of the antibody to the DNA digest, a Southern Blot was run according to protocol, with the omission of the probe hybridization step. There was no detectable signal. It was hypothesized that this may have been caused by probe hybridization to incomplete DNA digests, however further investigation is needed.

Genomic turtle DNA was digested with pairs of restriction enzymes in order to create smaller fragments that would perhaps be better suited for hybridization to the DIG probes. When digested with a combination of *Kpn*I and *Sac*I, and the combination of *Hind*III and *Bam*HI, and probed with the turtle β-actin DIG-labeled probe, two sets of bands were visible. The same digests showed no visible signal, and hence no hybridization to the chicken IFN-γ DIG-labeled probe.

4.4 Conclusions

It has been shown that the interferon system is phylogenetically very old and is ubiquitous, being found in all classes of vertebrates, as well as evidence of interferon-like substances present in the cells of insects and higher plants and in bacteria. Evidence exists that IFN production occurs in fishes and reptiles, and specifically, IFN production has been induced in the tortoise (*Testudo graeca, Testudo hermani*). *Testudo graeca* kidney & peritoneal cells (TGK & TGP) cells were challenged by a variety of animal viruses, including the Semiliki virus and the West Nile virus, which caused the stimulation of IFN induction, measured by the antiviral effect of IFN in CPE & Plaque Inhibition assays (Galabov 1981). These studies demonstrated the functioning of a IFN system in turtles, analogous to that found in mammals and birds. However the biological activity studied in those experiments may have been due to the presence of IFN-α or IFN-β in the cell extract, and not necessarily an IFN-γ molecule.

It therefore seems very likely that IFN-like molecules would be found in the species studied in this research project, the Hawaiian green sea turtle. In particular, IFN-γ, which has been shown to be conserved among vertebrates and to play a very

important role in the immune systems of these animals, must also exist in the green sea turtle. Although no concrete evidence has been demonstrated here for the existence of this molecule, further investigation is necessary and should reveal more information.

The β-actin gene sequence is conserved among humans, chickens, and sea turtles, as demonstrated in this study by the ability of human-derived β-actin gene primers to produce amplification products in all three species. This is possible because the DNA sequence has remained virtually unchanged over the course of the evolutionary history of these three species, being encoded by a highly conserved gene family. This is reflected in the fact that it is one of the most conserved proteins in eukaryotic cells, with amino acid residues identical in 80% of the position among amoebas and animals.

The IFN-γ gene sequence, although highly conserved in amino acid sequences among vertebrates, is not necessarily identical in DNA sequences. This is due to different codon preferences between species coding for the same amino acid. As demonstrated in this study, although human and chicken IFN-γ share 39% homology in their amino acid sequence, the primers derived from the chicken IFN-γ gene were not able to produce a PCR product in from human genomic DNA. Therefore, the DNA sequence of the turtle IFN-γ gene, if it exists, is probably not closely related to that of the chicken gene, however the amino acid sequences may share homology. Because PCR primer hybridization is very specific, the sequence needs to be nearly identical to that of the chicken gene-derived primers for PCR amplification to occur. Southern blot was used since hybridization of probes during blotting may detect other regions of homology, and also may detect short stretches of sequence homology. But the

sensitivity of the genomic blotting technique may not have been adequate to detect low levels of homology.

In order to address the issues discussed, the following steps can be taken in the future. (1) Since fresh turtle organ samples were not available at the time of this study, samples must be obtained for examination of turtle splenic RNA/cDNA for IFN-γ homologous sequences, as originally planned. (2) Switching to a chemiluminescence detection system, which may increase the signal strength, may increase the sensitivity of the Southern Blot using the DIG-labeled probes. (3) The use of degenerate primers for amplification during PCR would help to resolve the codon preference discrepancies among the different species. In addition, studying the DNA sequences of proteins that have already been elucidated from the green sea turtle, and determining the codon preferences of the sea turtle, would allow the synthesis of primers based on conserved amino acid sequences translated using the turtle codon preferences. (4) Finally, the construction of a turtle cDNA library enriched for cytokine transcripts would be a prudent and worthwhile endeavor.

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