THE CLONING, SEQUENCING, EXPRESSION AND DEMONSTRATION OF BIOLOGICAL ACTIVITY OF FELINE RECOMBINANT INTERFERON-γ

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A Thesis submitted for the degree of Doctor of Philosophy

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To my wife Sally Anne and daughter Blythe,
and to my parents

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DECLARATION

The work described in this thesis was carried out by myself, with the following exceptions. The CPER assays were, for the most part, carried out by Dr. Catherine Lawrence in the laboratory of Professor O. Jarrett and the peptide synthesis was carried out by Miss. Kathryn McBride. In addition, Dr.Gill Webster and Mr. Ronnie Barron conducted the FACS analysis upon completion of cell labelling in the MHC class II assay.

David J. Argyle, July 1995

ABBREVIATIONS

A_{260} or A_{280}	absorbency at 260nm or 280nm
A	adenosine, adenine or Angstrom unit
Ab	antibody
AcMNPV	*Autographica californica* multiple-enveloped nu virus
ADCMC	Antibody Dependent Cell Mediated Cytotoxicity
Af-1	Accessory factor 1
APC	Antigen Presenting Cell
APS	ammonium persulphate
bp	base pair
BSA	bovine serum albumin
C	cytosine or cytodine
$^{o}C.$	degrees centigrade
Ca^{2+}	ionised calcium
CIITA	class II transactivator
cAMP	cyclic AMP
CD	cluster of differentiation (antigens)
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary (cells)
CO_2	carbon dioxide
CSA	cyclosporin A
CTL	cytotoxic T cell
d	(as a prefix) deoxy-

dd	(as a prefix) dideoxy-
Da	Dalton
DEA	diethylamine
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	one or more of the 4 deoxynucleoside triphospha
ds	double stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
FACS	fluorescent antibody cell sorting
FCS	foetal calf serum
FCV	feline calicivirus
FeLV	Feline Leukaemia Virus
FITC	fluoroesceine isothyocyanate
FIV	feline immunodeficiency virus
FPLC	fast protein liquid chromatography
g	gramme(s) or g-force
G	guanine or guanosine
ga	gauge
GAS	gamma activation sequence
gag	group-specific antigen encoding gene
H5	high five cells
HCL	hydrochloric acid
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphon
HIV	human immunodeficiency virus

HLA	human leukocyte antigen
HRP	horseradish peroxidase
IFN	interferon
IL	interleukin
Ig	immunoglobulin
IRF	interferon regulatory factor
ISGF3	interferon stimulated gene factor 3
Ka	dissociation constant
kDa	kilo-Dalton
LAK	lymphokine activated killer cell
l	litre(s)
LRU	laboratory reference unit
LT	leukotriene
LTR	long terminal repeat
MEM	minimal essential medium
Mg^{2+}	ionised magnesium
MHC	major histocompatibility complex
ml	millilitre(s)
mRNA	messenger RNA
MuLV	moloney murine leukaemia virus
NK	natural killer cell
No	nitric oxide
OD	optical density
o/n	overnight
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming units

PGE2	prostaglandin E2
PKC	protein kinase C
PLC	protein lipase C
pM	picomoles
poly-A	poly-adenosine
PTK	protein tyrosine kinase
RE	restriction endonuclease
RNA	ribonucleic acid
RNase	ribonuclease
r.p.m	revolutions per minute
RSV	Rous sarcoma virus
RT-PCR	reverse transcriptase PCR
Sarkosyl	*N*-lauroylsarcosine
SDS	sodium dodecyl sulphate
Sf	Spodoptera frugiperda
SPF	specific pathogen free
STAT	signal transducer and activator of transcription
T	thymine or thymidine
T cell	thymus derived lymphocyte
TAE	Tris/acetic buffer
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tris/borate buffer
TBS	Tris-buffered saline
TCID50	dilution of virus required to infect 50% of cultur
TCR	T cell receptor
TE	Tris/EDTA buffer
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TH	T helper cell
TK	thymidine kinase

Tm	melting temperature
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)aminomethane
TVT	transmissible venereal tumour
U	uracil
ul	microlitre(s)
ug	microgram(s)
UV	ultraviolet
v/v	volume/volume
VSV	Vesicular Stomatitis virus

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SUMMARY

Cytokines are intercellular messengers which orchestrate host defence, repair and haemopoiesis. The potential that cytokines have in the pharmacological manipulation of the immune and haemopoeitic systems has lead them to be at the forefront of biomedical research. In human medicine, cytokines now feature in many clinical trials ranging from those involving infectious diseases to those involving the treatment of cancer.

The potential benefits to therapeutics that cytokines offer is now being realised in veterinary medicine. The cat suffers from a number of neoplastic, infectious, immune mediated, and inflammatory diseases and, in addition, can provide a model for human diseases such as HIV. This thesis describes the cloning, sequencing and expression of feline recombinant interferon-γ with the ultimate aim of exploring the potential of this cytokine in the treatment and prevention of diseases in cats.

Initially feline specific cDNA, encoding interferon-γ, was amplified using the polymerase chain reaction (PCR). Messenger RNA was harvested from feline lymphocytes which had been incubated with a non-specific mitogen. This mRNA was then used to create a library of first strand cDNA molecules, from which the interferon specific cDNA was amplified using synthetic primers and the process of PCR. Subsequently, the products of PCR were sub-cloned into plasmid vector and sequenced. The feline interferon cDNA coding region was found to be 568bp, coding for a

peptide of 166 amino acids. In addition, the sequence showed a high degree of homology to the human interferon-γ molecule.

Expression of the interferon gene was achieved using the baculovirus system. This system produces a glycosylated product and can be scaled up to producing large quantities of recombinant protein. Since the ultimate aim was to use the interferon *in-vivo,* then both of these factors were important in the choice of expression system. Recombinant baculovirus, expressing interferon protein, was generated by homologous recombination of a transfer vector containing the interferon cDNA and linearized wild-type baculovirus. Expression and biological activity of the protein was determined by two biological assays. The first was a viral interference assay based upon the ability of interferon to protect cells against viral infection, and the second assay was based on the ability of interferon to up-regulate MHC Class II antigens on cells. Both assays confirmed that the baculovirus system was producing a biologically active protein. Immunological detection of the interferon protein was carried out using antisera raised against synthetic peptides. These peptides were selected by analysis of regions of the interferon protein sequence which were predicted to be immunogenic sites. Western blot analysis of the interferon protein, using polyclonal antisera, demonstrated the mature recombinant protein to be around 20kDa. Partial purification of the interferon protein was carried out using a combination of salt precipitation and ion exchange/FPLC chromatography.

The potential benefits and drawbacks that recombinant feline interferon-γ offers for the treatment of feline diseases is discussed in the final chapter. The advent of recombinant DNA technology, coupled with the ever increasing amount of experimental data demonstrating the biological

function of these cytokines, will undoubtedly lead to a rational approach to introducing these molecules into clinical trials. This thesis, in part, demonstrates an approach for taking a gene from the cloning stage towards clinical application.

PUBLICATIONS ARISING FROM THIS THESIS

Argyle, D.J., Smith, K., McBride, K., Fulton, R., and Onions, D.E. Nucleotide and predicted peptide sequence of feline Interferon-γ. *DNA Sequence*, 5:169-171, 1995.

Argyle, D.J., Smith, K., McBride, K., Fulton, R., and Onions, D.E. Nucleotide sequence of feline Interferon-γ cDNA. *EMBL Database submission*, 1994.

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Curran, J.A., Argyle, D.J., Cox, P., Onions, D.E. and Nicholson, L. Nucleotide sequence of the Equine interferon-gamma cDNA. *DNA sequence*, 4: 405-407, 1994.

Nicholson, L., Curren, J.A., Argyle, D.J., Cox, P. and Onions, D.E. Potential use of recombinant cytokines in the prevention and treatment of Equine infectious diseases. *Seventh International Conference on Equine Infectious Diseases (Conference proceedings)*, 1994.

CHAPTER 1

GENERAL INTRODUCTION

1.0 INTRODUCTION

1.1 GENERAL OVERVIEW

Cytokines are regulatory glycoproteins that can be produced by virtually every nucleated cell type in the body; they have pleiotropic regulatory effects on the haemopoeitic and many other cell types that participate in host defence and repair processes (Oppenheim, 1994). Cytokines therefore include lymphocyte-derived factors known as lymphokines, monocyte-derived factors known as monokines, haemopoeitic colony stimulating factors and connective tissue growth factors. It is difficult to define cytokines because of their pleiotropic actions but table 1.1 shows some of their characteristic features.

The field of cytokine research today has evolved from four originally independent sources. The first is the area of lymphokine research, carried out by immunologists; secondly, the area of interferon research, carried out initially by virologists; thirdly, the area of haemopoeitic research, studying the effects of the colony stimulating factors and fourthly, the area of growth factor research. Inasmuch as the cytokine field has emerged from different sources there is now, after much research, a unifying concept of the cytokine. However, the field of cytokine nomenclature is still confusing, with older names still being used and an ever increasing list of interleukins.

It has been difficult to assign unique biological activities to most cytokines because most cells express multiple receptor types on their surfaces and can thus respond to many different cytokines. Further, cytokines can have multiple biological functions which overlap, and so there exists

Cytokines are simple polypeptides or glycoproteins with a molecular weight usually below 30 kDa. Only one known cytokine, IL-12, is a heterodimer.
Constitutive production of cytokines is usually low or absent. Production is controlled by inducing stimuli at the level of transcription or translation.
Cytokines act in a paracrine or autocrine fashion and their action is usually transient.
Most cytokine actions can be attributed to altered patterns of gene expression in the target cell.

Table 1.1 The characteristic features of cytokines

considerable redundancy in their actions (reviewed by Oppenheim and Saklatvala 1994). These overlapping functions may provide alternative pathways or greater co-operation between cytokines in cellular signalling. Such interactions are further amplified by the capacity of certain cytokines to induce other cytokines, resulting in cascades and networks of interacting cell signals. While cytokines are intimately involved in orchestrating the non-specific and specific immune responses, they also influence the activities of connective tissue, neural, endothelial, epithelial and haemopoeitic cells involved in tissue repair and homeostasis. In addition, there is increasing evidence that cytokines may be involved in normal development (Oppenheim and Saklatvala, 1994).

Interferon was first described by Isaacs and Lindenmann (1957) as a factor produced by a variety of virus infected cells capable of inducing cellular resistance to homologous or heterologous viruses but its role in immune regulation was not suspected at this time. Several years later Wheelock (1965) described a functionally related virus-inhibitory protein produced by mitogen (PHA) activated T cells. Although in the virus inhibition assay the factor was indistinguishable from typical interferon, Wheelock noted that the PHA induced leukocyte-derived factor was inactivated at pH2. In 1973, Youngner and Salvin showed that interferon derived from lymphocyte cultures by mitogenic stimulation was antigenically distinct from "classical" interferon and proposed the use of the term type II interferon for the former. In 1980 the term type II interferon and immune interferon were replaced by the term interferon-gamma. At the same time, the names interferon-α and interferon-β were introduced for the two antigenically distinct varieties of type I interferons. It is now well established that interferon gamma is structurally distinct from the two

other interferon species known as alpha and beta interferons (Gray and Geodell 1982).

1.0.1 THE INTERFERON FAMILY

The Interferons are a family of glycoproteins related by their ability to protect cells from viral infection (Pestka and Langer *et al* 1987). The interferons can be divided into two very distinct families of proteins. The Interferon-α/β superfamily (also known as the type I interferons) include a group of structurally related genes and proteins, further subdivided into subfamilies IFN-α, IFN-β, IFN-ω, and IFN-τ. The second family consists of a single gene encoding the interferon-γ protein (type II interferon). Interferon-α/β and IFN-γ were initially distinguished from each other by their different stability's at pH 2 and distinct antigenic specificities. When the sequences of the genes and proteins of the major interferons were elucidated in the early 1980's it became clear that all members of the IFN-α/β family were structurally related to each other, but IFN-γ was completely distinct. In addition, it has been established that two types of Interferon receptor exist, one for IFN-α/β and one for IFN-γ (Vilcek and Oliveira 1994).

1.0.3 INTERFERON ALPHA AND BETA

The properties of interferon alpha and beta have been reviewed by Pestka and Langer *et al* (1987) and by De Maeyer and De Maeyer-Guignard (1994). Interferon-α is produced by peripheral blood mononuclear cells and is also known as leukocyte interferon or type I interferon. Twenty six IFN-α genes (including several pseudogenes) have been identified in man. These genes have common structures, i.e. they lack introns and appear to

derive from a common ancestral precursor. The reasons for the complexity of the IFN-α gene system remain unclear, but recent studies suggest different IFN-α species effect distinct arrays of biological responses in different cells (Farrar and Shreiber 1993).

Interferon-β is also known as type I interferon or fibroblast interferon because of its cell of origin. In man there is only one distinct type of IFN-β which is encoded by a single gene which is located next to the IFN-α locus in both man (chromosome 9) and the mouse. Based on the general organisation of their genes and proteins, IFN-α and IFN-β are thought to have evolved from a common ancestral precursor. However, IFN-β only shares limited antigenic relatedness to the IFN-α family, and the proteins display only 15-30% amino acid homology. Nevertheless, both forms of type I interferon bind the same receptor on the surface of target cells. Both forms of type I interferon are inducible by virus.

The human interferon beta gene codes for a protein of 166 amino acids with about 29% homology to human interferon-αD or IFN-α1. The bovine, equine and porcine interferon β gene families contain many genes, whereas the human and most other mammalian species contain only one. The lion and rabbit genomes contain two interferon β genes (Pestka and Langer *et al*, 1987).

Interferons other than α, β and γ have been reported such as Interferon-ω and τ which are also termed type I interferons (reviewed by De Maeyer and De Maeyer Guignard 1994). In humans, Interferon-ω shares 60% homology to the interferon-α gene. Interferon-τ has only been described in sheep and cattle and is also termed trophoblast interferon. These interferons are the major secretory products of the trophoblast of ruminant

ungulates during pregnancy, in the period immediately preceding attachment and implantation of the fertilised ovum. Interferon-τ shares most of the biological functions of the other interferons but is poorly responsive to viral induction.

A summary of the functions of interferon-α and β is shown in tables 1.2 and 1.3.

1.0.4 EVOLUTIONARY RELATIONSHIPS OF INTERFERON GENES

The homology between human interferon-α and β genes suggest that they originated from a common ancestral gene around 300 million years ago, about the time of the divergence of mammals, reptiles and birds from fish and amphibia. This result suggests that amphibia and fish contain only an interferon-α or β gene homologous to the mammalian counterparts, but not both, and is in agreement with weak hybridisation of human interferon beta, but not interferon alpha to amphibia and fish DNA. There appears to be no clear homology between the interferon alpha and beta genes with the interferon-gamma gene.

All three classes of Interferon genes appear to be evolving at a rate comparable to the immunoglobulin genes (Pestka and Langer *et al* 1987).

Antiviral effect	Broad spectrum antiviral activity
Effect on cell growth and division	Inhibition of replication of normal and tumour cells. IFN-α and β prolong G1, reduce the rate of entry into S phase, and lengthen S and G2, resulting in a slower replication rate. Cells show different sensitivities to these actions
Modulation of the expression of MHC class I and II	One of the mechanisms responsible for the immunomodulatory actions of IFN-α and β but modulation of MHC II is more specifically a function of IFN-γ.
Stimulation of macrophage activity	More a function of IFN-γ
Stimulation of NK cell activity	Contributes to the antitumour activity of IFN-α and β
Up or down-regulation of delayed type hypersensitivity	DTH can be up or down regulated by IFN-α or β, depending on the timing of action
Antitumoural activity	The mechanism of antitumour activity is only partially understood. Involves stimulation of immune effector cells and possibly direct antiproliferative activity

Table 1.2 A synopsis of the principal biological activities of IFN-α and β

PROPERTIES OF THE INTERFERONS

PROPERTY	IFN-α	IFN-β	IFN-γ
Nomenclature	Type I / leukocyte	Type I / Fibroblast	Type II / Immune
Major inducers	Virus	Virus / LPS ds-poly RNA	Antigens / Mitogens
M.W.(kDa)	20	20-25	17-34
amino-acids	165-166	166	143
subunit	Single Polypeptide	Single Polypeptide	Noncovalent Homodimer
pH stability	Stable	Stable	Labile
No. of genes	26	1	1
Chromosomal lcn.			
murine	4	4	10
human	9	9	12
presence of introns	none	none	3
Cellular source	T cells B cells Macrophages	Fibroblasts Epithelial cells	T cells NK cells

Table 1.3 The characteristic features of the different interferon species.

1.1 INTERFERON GAMMA

1.1.1 OVERVIEW

30 years of research into the actions of gamma-interferon has demonstrated the minor role that it plays as a direct anti-viral agent and shows that it has a more important role in the co-ordination of immune responses (reviewed by Farrar and Shreiber 1993)

Interferon-γ is unrelated to type I interferons both at the gene and protein levels Gray *et al* (1982). Moreover, IFN-γ is induced by a unique set of stimuli and is produced only by T lymphocytes and NK cells. Importantly, viral infection of these cells does not directly induce IFN production. Pace *et al* (1985) demonstrated that IFN-γ has a 10-100 fold lower specific anti-viral activity than the type I interferons but is 100-10,000 times more active as an immunomodulator than are the other classes of interferons. Thus the conclusion can be made that IFN-γ is primarily an immunomodulator with some anti-viral activity, while the type I interferons are primarily antiviral cytokines.

The human (Gray *et al,* 1982), murine (Gray and Goedell, 1983), bovine (Cerretti *et al,* 1986), canine (Zucker *et al*, 1992), ovine (Radford *et al,* 1991)
, equine (Curren *et al,* 1994), rabbit (Samudzi *et al,* 1991) and rat (Dijkema *et al,* 1986) interferon cDNA's have been cloned. Although there is a reasonable degree of homology between the DNA sequences of these genes, the different interferon-γ molecules are generally species specific.

1.1.2 MOLECULAR CHARACTERISTICS OF HUMAN AND MURINE INTERFERON-γ

Both humans and mice only contain one interferon-γ gene which is considerably more complex than the type I interferon genes. The human and murine genes are 6kb in size and contain four exons and three introns (Gray and Goedell 1982). Using *in situ* hybridisation techniques the gene has been localised to chromosome 12 in man and chromosome 10 in the mouse, Trent *et al* (1982), and Naylor *et al* (1984).

The molecular characteristics of human and murine interferon gamma species have been reviewed by Farrar and Schreiber (1993). Activation of the human gene leads to generation of a 1.2 KB mRNA that encodes a 166 amino acid polypeptide. Two polypeptides self-associate to form a homodimer with an apparent molecular weight of 34 kDa. Only the dimer can display biological activity, possibly because it is the only form of the molecule which can effect interferon-γ receptor dimerization. The mature human interferon-γ molecule has no cysteine residues and thus the homodimer is held together by non-covalent forces. The molecule is sensitive to extremes of heat and pH. The dimeric nature of the human interferon molecule is depicted in figure 1.1

The individual human and murine polypeptide chains contain two N-linked glycosylation sites (residues 25 and 97 in human interferon-γ and residues 16 and 69 in the murine form). These are independently and differentially glycosylated giving rise to subunits of differing molecular weights. Natural human and murine interferon-γ homodimeric molecules display molecular weights which range from 30-50KDa. Whereas glycosylation is

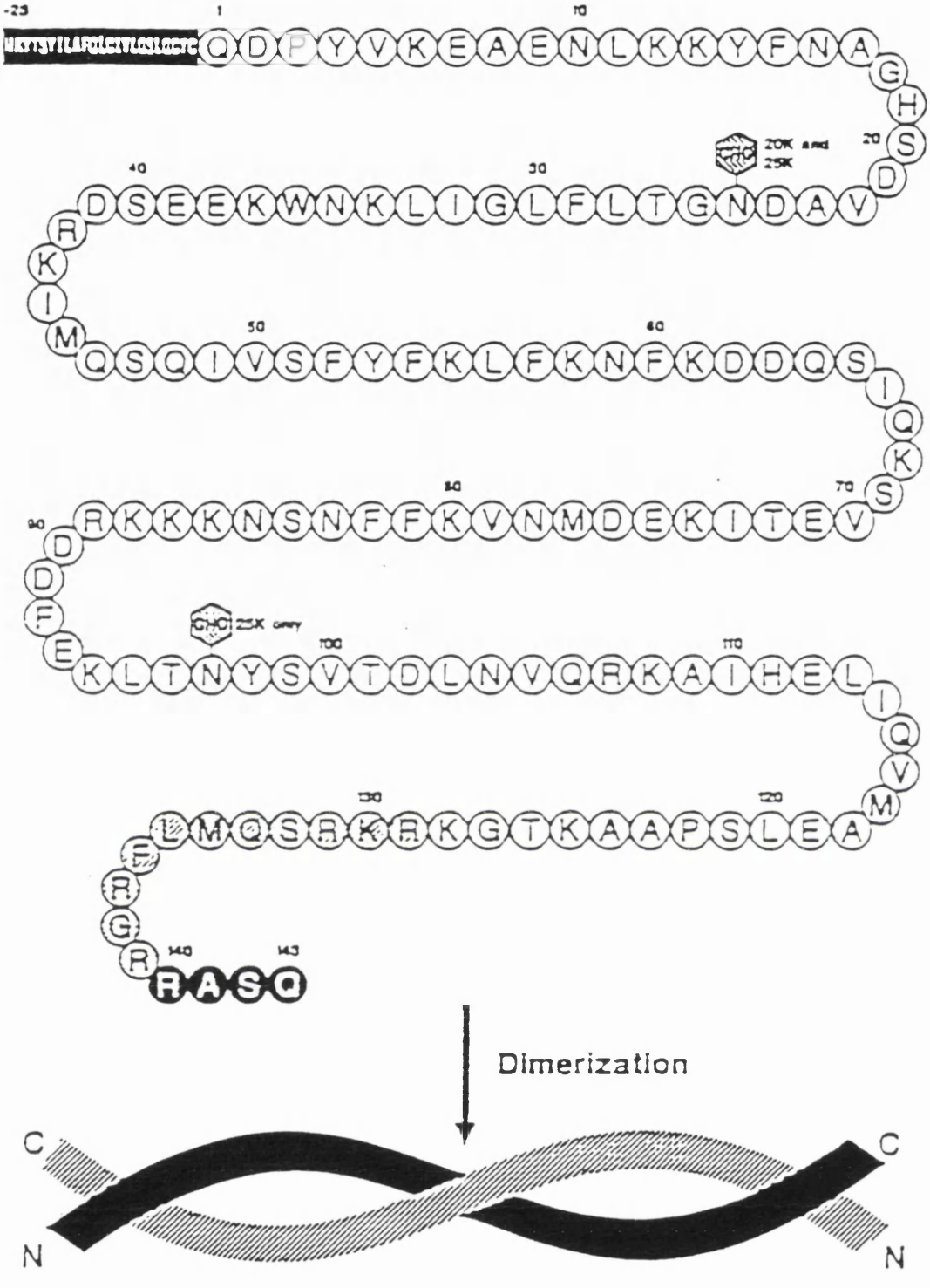


Figure 1.1 Schematic representation of the human IFN-γ molecule. IFN-γ is synthesised as a 166 amino acid polypeptide that contains a 23 amino acid signal sequence (shaded box). Two polypeptides self associate to form a noncovalent homodimer(from Farrar and Shreiber, 1993)

not important for full biological activity of the interferon molecule it appears to influence the biological half-life in the circulation.

Current data indicates that both the amino and carboxy terminal regions play critical roles in maintaining the active conformation of the protein. Recently, the X-ray crystallographic structure of human interferon-γ was resolved to 3.5A. (Ealick *et al* 1991). This study confirmed the dimeric nature of the mature protein. The individual subunits have a flattened prolate elliptical shape. However, the overall structure of the dimer is compact and globular. The molecule appears to be primarily helical (62%) and lacks beta sheet structure. Each subunit consists of 6 alpha helices held together by short non-helical regions. The dimer is formed by a unique intertwining of the helices across the subunit face which provides an opportunity for multiple interactions along each subunit. This type of subunit interaction is highly unusual and has only been seen in a few other proteins. The model predicts that the subunits associate in an antiparallel fashion, thereby leading to a juxtaposition of the amino and carboxy termini of the opposing polypeptide chains. Whereas the amino termini co-ordinates have been firmly established, the carboxy termini do not adopt a rigid conformation in solution, as determined using either X-ray crystallography or nuclear magnetic resonance approaches. Nevertheless, the model suggests that each interferon-gamma dimer may be able to bind two interferon-gamma receptors. The structure of the human interferon-γ is shown in figure 1.2.

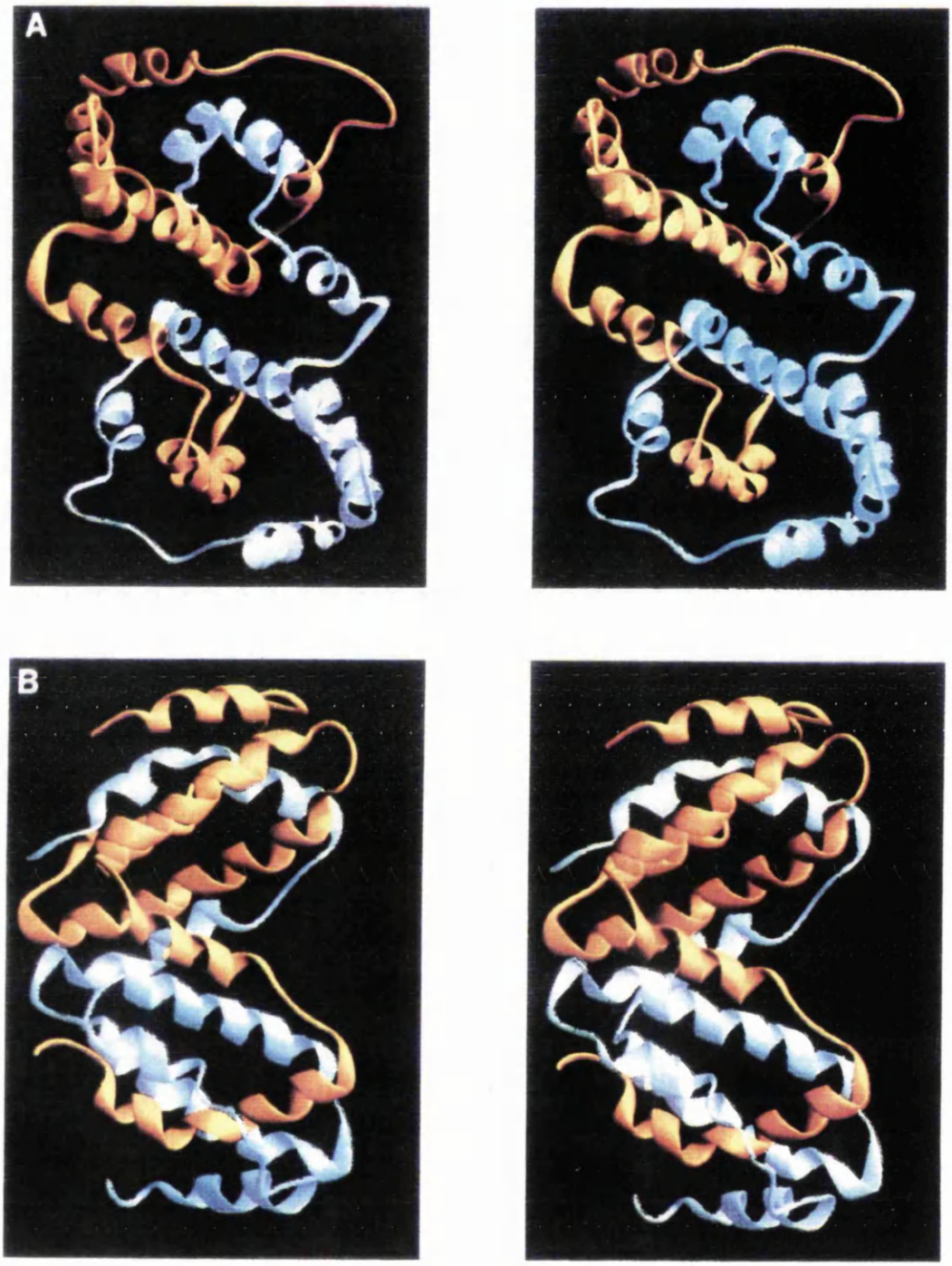


Figure 1.2 Stereo views of the human recombinant interferon-γ dimer (from Ealick *et al* 1991). (A) is a view approximately parallel to the dimer twofold axis and (B) is a view approximately perpendicular to the dimer two fold axis.

1.1.3 PRODUCTION OF INTERFERON-γ

The mechanisms involved in the production of human and murine interferon-γ have been reviewed by Farrar and Schreiber (1993), De Maeyer and De Maeyer Guignard (1994) and Vilcek and Le (1994). What follows is a synopsis of the current understanding.

The T lymphocyte represents the major cellular source of IFN-γ. All CD8+ T cell populations and certain subsets of CD4+ T cells can produce the protein. Interferon-γ biosynthesis has been demonstrated in the TH_1 helper T cell subset and also in TH_O cells which are thought to be a less well differentiated/activated CD4+ cell. The external stimuli which cause production of the protein are similar to those of other T cell derived cytokines. The major physiological stimulus is antigen presented in the context of MHC I or MHC II. In addition T cell-dependent production of IFN-γ is enhanced by products of activated T cells and macrophages such as IL-2, hydrogen peroxide, and leukotrienes LTB4, LTC4, and LTD4. The cellular sources of interferon-γ are represented diagramatically in figure 1.3.

The induction of interferon-γ appears to be regulated at both the level of gene transcription and mRNA stabilisation. T cell lymphokine gene expression has been reviewed by Fraser *et al* (1993). Interferon-γ is produced by T cells after stimulation of the T cell receptor (TCR). The TCR has no intrinsic tyrosine kinase function but cross-linking of the TCR complex leads to activation of cytoplasmic protein tyrosine kinases (PTKs) which interact with the TCR. Two *src*-family PTKs, *fyn* and *lck*, have been implicated in this signalling mechanism. Subsequently, there is

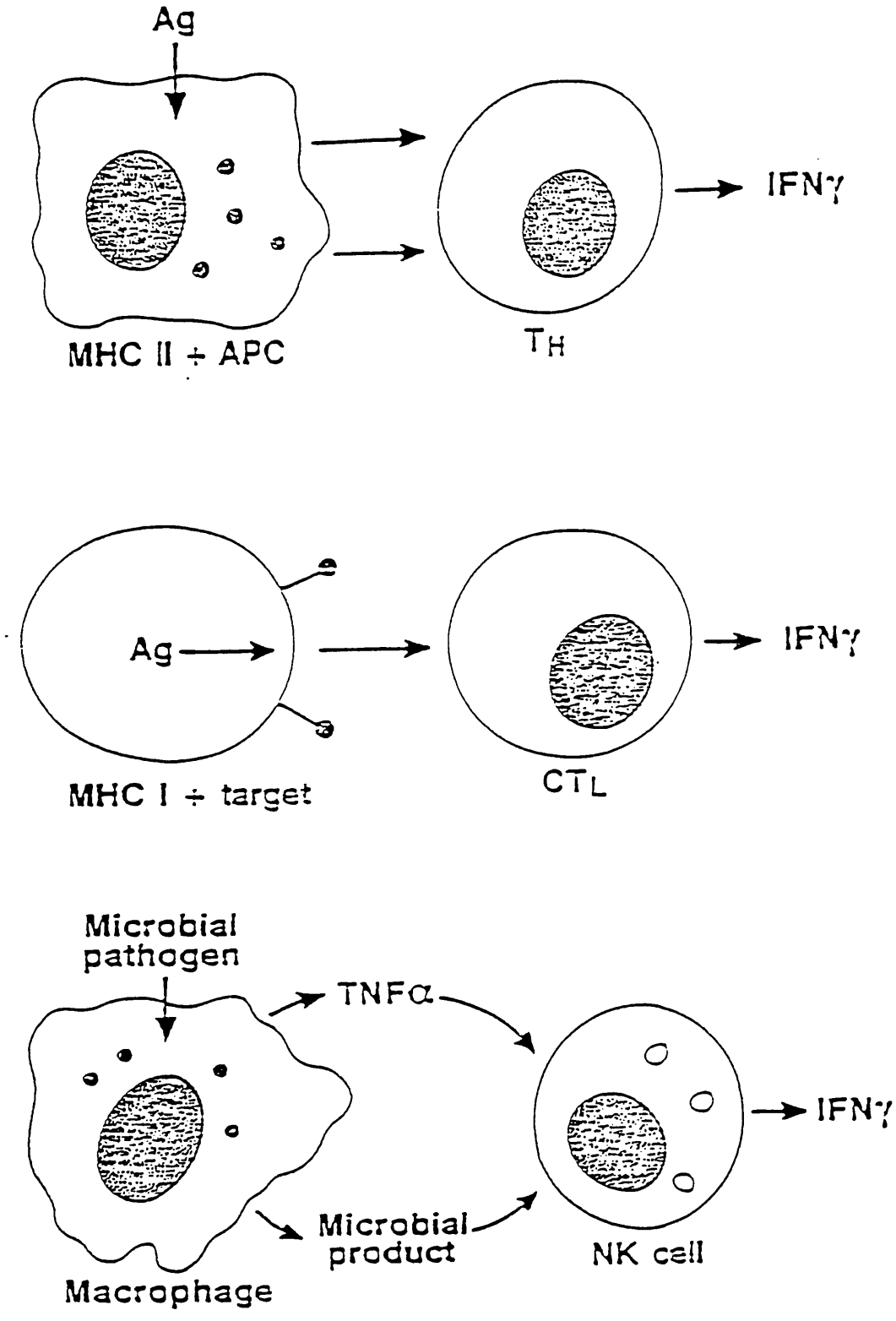


Figure 1.3 Sources and production of interferon-γ (from Farrar and Shreiber, 1993)

tyrosine phosphorylation of cellular proteins. Phospholipase C-γ1 (PLC) is one of the proteins whose tyrosine phosphorylation is induced following TCR stimulation and activation of PLC leads to the generation of inisotol triphosphate and diacylglycerol. The former leads to an increase in free calcium ions (Ca^{2+}), whereas the latter activates protein kinase C (PKC).

The generation of free calcium ions is considered to be an important event in mediating gene transcription and is inhibited by the immunomodulatory drug Cyclosporin A (CSA). The mechanism by which these early signalling events are transduced to the nucleus is unclear but the outcome is to activate transcription factors which regulate gene transcription. The nature of the transcription factors regulating interferon-γ gene transcription is largely unknown.

T cell activation involves the induction of lymphokine mRNA which belongs to a class of short-lived mRNA characterised by the presence of repeated $(AUUUA)_n$ sequences in their 3' non-coding region. The human interferon-γ mRNA bears these features of instability shared by most interleukin mRNA. It has been suggested that these AU motifs may be recognition sequences for inducible ribonuclease activity (Troutt and Kelso 1992). Interferon-γ mRNA stabilisation by activation of protein kinase C or increased cAMP in a cytolytic T cell clone has been recently demonstrated by Kaldy and Schmitt-Verhulst (1995). While an increase in calcium ions generates increased gene transcription, increased PKC activity does not. However, PKC and cAMP induced PKA activity have now been shown to stabilise interferon-γ mRNA by an unknown mechanism. cAMP levels can be increased by physiological doses of

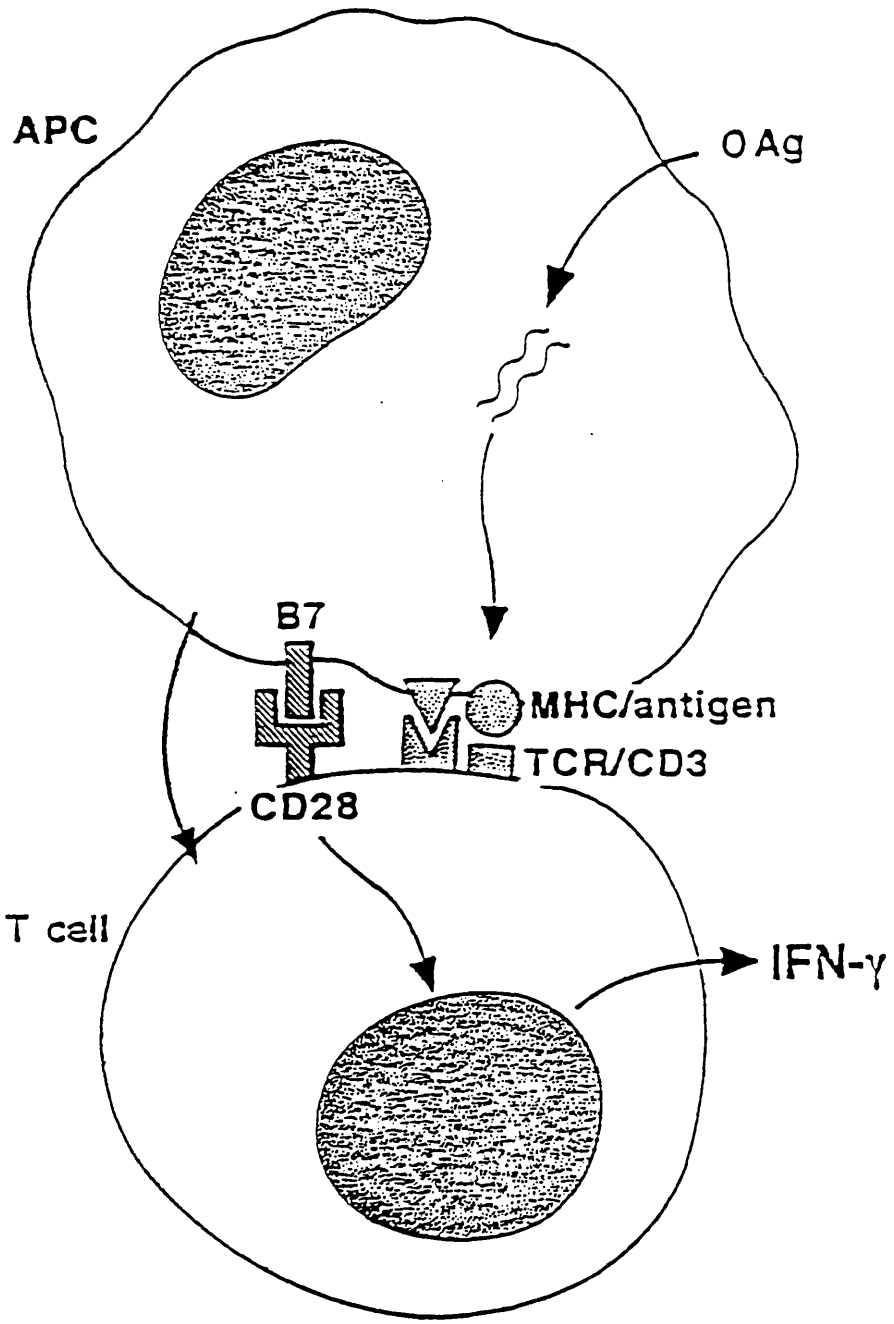


Figure 1.4 Shematic representation of the process of T cell activation and IFN-γ production

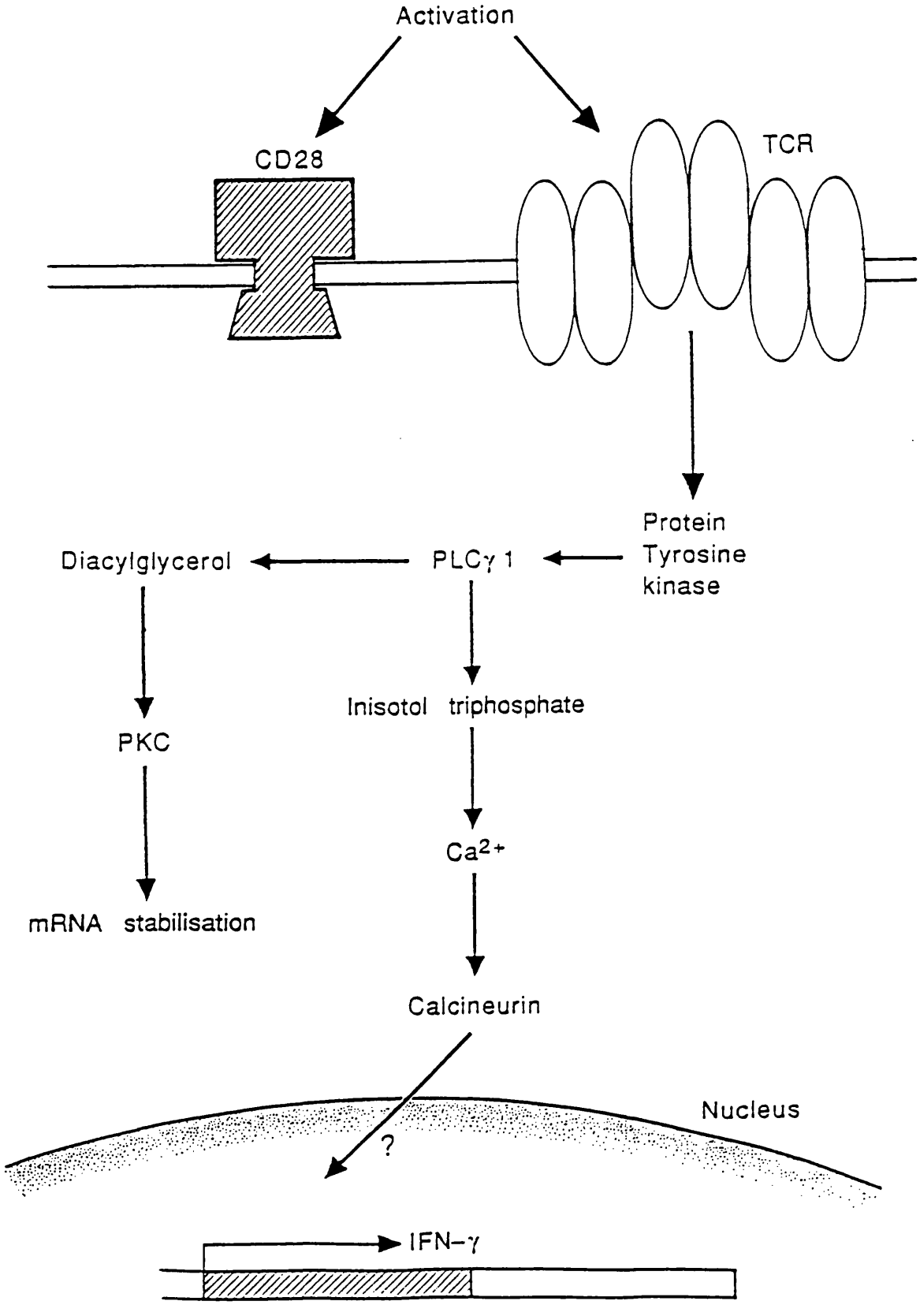
prostaglandin E2 (PGE2) or Histamine, both of which are potent inflammatory mediators.

While TCR-generated signals can contribute to the activation of cytokine gene expression, this alone is generally insufficient to induce gene transcription. Evidence suggests that accessory receptors are involved in full activation of the T cell (reviewed by Fraser *et al* 1993). One of the most studied "co-stimulatory" molecule is CD28, a 44kDa glycoprotein member of the Ig superfamily which is expressed by both CD4+ and CD8+ T cells. The ligand for CD28 is another member of the IG superfamily called B7, found on activated B cells and antigen presenting cells (APCs). CD28 signal transduction in the context of TCR-generated signals increases the mRNA level and secretion of a number of T cell derived cytokines such as IL-2, as well as Interferon-γ. The mechanisms of T cell activation are represented in figures 1.4 and 1.5.

Interferon-γ can also be produced by NK cells. Mitogens can induce interferon-γ production from human and murine NK cells expanded *in-vitro* with IL-2. in addition, production of IFN-γ can be rapidly induced from naive NK cells by the action of microbial products in conjunction with TNF-α.

Two recently described cytokines are noteworthy in terms of their respective abilities to regulate interferon-γ production in both a positive and negative fashion. IL-12, (formerly known as Natural Killer cell stimulatory factor (NKSF) produced from B cells and macrophages, has a positive effect on interferon-γ gene transcription and IL-10 , produced by TH2 cells, B cells and macrophages has a negative effect on interferon-γ production (reviewed by Farrar and Schreiber 1993).

Figure 1.5 Signalling events in Tcell activation and interferon-γ production



The exact mechanism of IL-12 induction is unclear but probably involves bacteria, bacterial products or parasitic infection. The mechanisms by which IL-12 stimulates production of interferon-γ is, as yet, undetermined. However, it is thought that IL-12 induces Interferon-γ production in T cells and NK cells in a manner that is at least partially distinct from the conventional pathway of T cell activation. IL-12 induction is not inhibited by Cyclosporin-A (CSA) which is known to antagonise Ca^{2+} induced gene transcription following activation of the TCR. It appears from studies on T cells that IL-12 induces interferon-γ production at the level of gene transcription and also, in conjunction with IL-2, stabilises the mRNA transcribed (Chan, S.H. *et al* 1992). The role of IL-12 in induction of interferon-γ has been reviewed by Trinchieri (1993). IL-12 is particularly effective in inducing interferon-γ production, either directly or with strong synergistic effect with several other inducers of interferon-γ production, including IL-2, mitogens, and ligands for TCR-CD3 and CD28 on T cells as well as IL-2 and CD16 ligands on NK cells. Anti-IL-12 antibodies inhibit interferon-γ production in response to various stimuli, both *in vivo* and *in vitro*, indicating that IL-12 is required for optimal production of Interferon-γ. IL-12 is thought to act both directly and indirectly, via the induction of interferon-γ, on T cells to generate TH_1 type responses. Interferon-γ has a positive feedback effect by enhancing the production of IL-12 by monocytes and macrophages. This may, in part, be due to the ability of Interferon-γ to block endogenous IL-10 production by macrophages. The existence of positive and negative feedback loops suggests that once either a TH_1 or TH_2 type response is determined early during the immune response, it remains stable, unless major changes take place in the balance of cytokine production during the response.

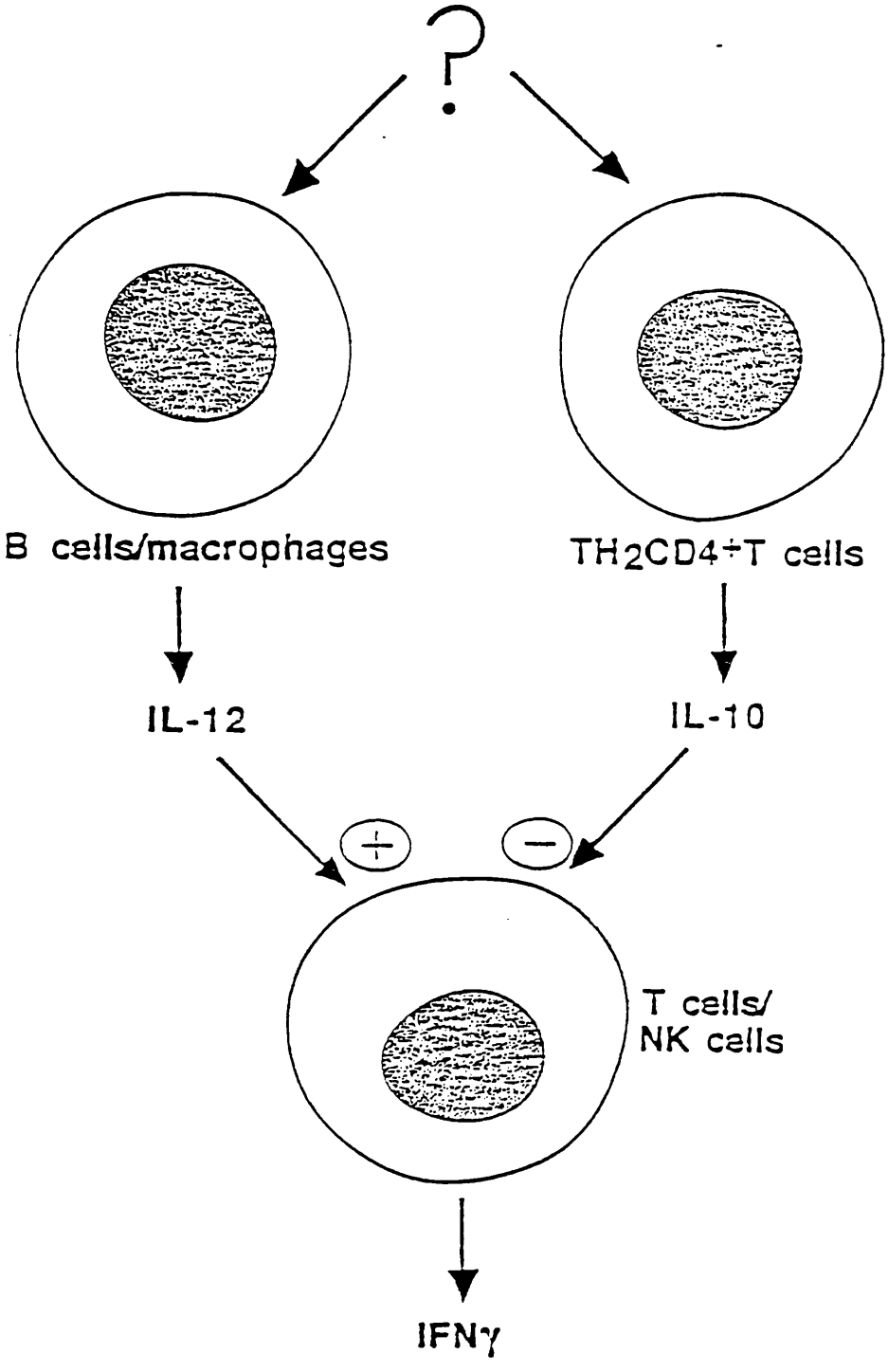


Figure 1.6 Schematic representation of the regulation of IFN-γ production by IL-10 and IL-12

The mechanisms by which IL-10 downregulates production of IFN-γ is currently being elucidated. The target for IL-10 appears to be the antigen presenting cell (APC) as well as the T cell. The current hypothesis is that IL-10 may inhibit the expression of accessory molecules required for full activation of the T cell for interferon-γ production. The regulation of interferon-γ production by IL-10 and IL-12 is shown diagramatically in figure 1.6.

It is worthy of note that Interferon-γ is rarely seen in the circulation of individuals undergoing immunological stimulation possibly due to the rapid removal of IFN-γ from the circulation by IFN-γ receptors which are ubiquitously expressed on all cells.

1.1.4 THE INTERFERON GAMMA RECEPTOR

Studies concerning the interferon-γ receptor has been reviewed by Pestka and Langer (1987) and, more recently, by Farrar and Schreiber (1993) and Vilcek and Oliveira (1994).

Interferon-gamma exerts its pleiotropic actions on cells through the interaction with a specific receptor which is ubiquitously expressed on all cells apart from the erythrocyte. Even platelets have a high level of receptor expression (around 300 receptors per cell) and it has been postulated that these cells may serve to transport interferon-γ through the circulation. The highest levels of receptor expression are actually seen in those tissues which are not normally associated with the immune system. In particular skin, nerve and syncitial trophoblast cells of the placenta express high levels of interferon-γ receptor. The receptor binds ligand with high affinity ($Ka=10^{9}$-$10^{10}M^{-1}$) and is expressed on most cells at

modest levels of around 200-25000 sites per cell. Human and murine interferon-γ receptors display strict species specificity in their ability to interact with human and murine interferon-γ.

The receptor is synthesised in the endoplasmic reticulum and is glycosylated as it moves from the endoplasmic reticulum into the golgi apparatus. Only fully glycosylated receptors are expressed at the cell surface. Fully mature receptors that are expressed at the plasma membrane have molecular masses that vary between 80 and 95 KD. These differences arise because of cell specific differences in glycosylation. Following ligand binding the intracellular domain of the receptor is phosphorylated on serine and threonine residues. The rate and extent of the phosphorylation correlates with the magnitude of the biological response. The phosphorylated receptor/ligand complex is internalised, enters an acidified endosomal compartment, and dissociates. Free interferon-γ is ultimately degraded in the lysosome. In many cells the receptor then enters a large intracellular pool of receptors that is usually 2-4 times larger than the cell surface pool. The ability of receptor recycling in macrophages remains controversial with some groups suggesting that this occurs while others suggest that internal receptors are degraded.

The complete characterisation of the human and murine interferon-γ receptor was made possible by the cloning of the respective cDNAs, Aguet *et al* (1988) and Gray *et al* (1989). The genes for human and murine receptors have been localised to chromosome 6 and 10 respectively. Both genes are around 30Kb in size and each consist of seven 7 exons. Upon activation the gene gives rise to a single 2.3kb mRNA transcript. The human and murine proteins are organised in a similar manner. The mature human and murine proteins consist of 472 and 451 amino acids,

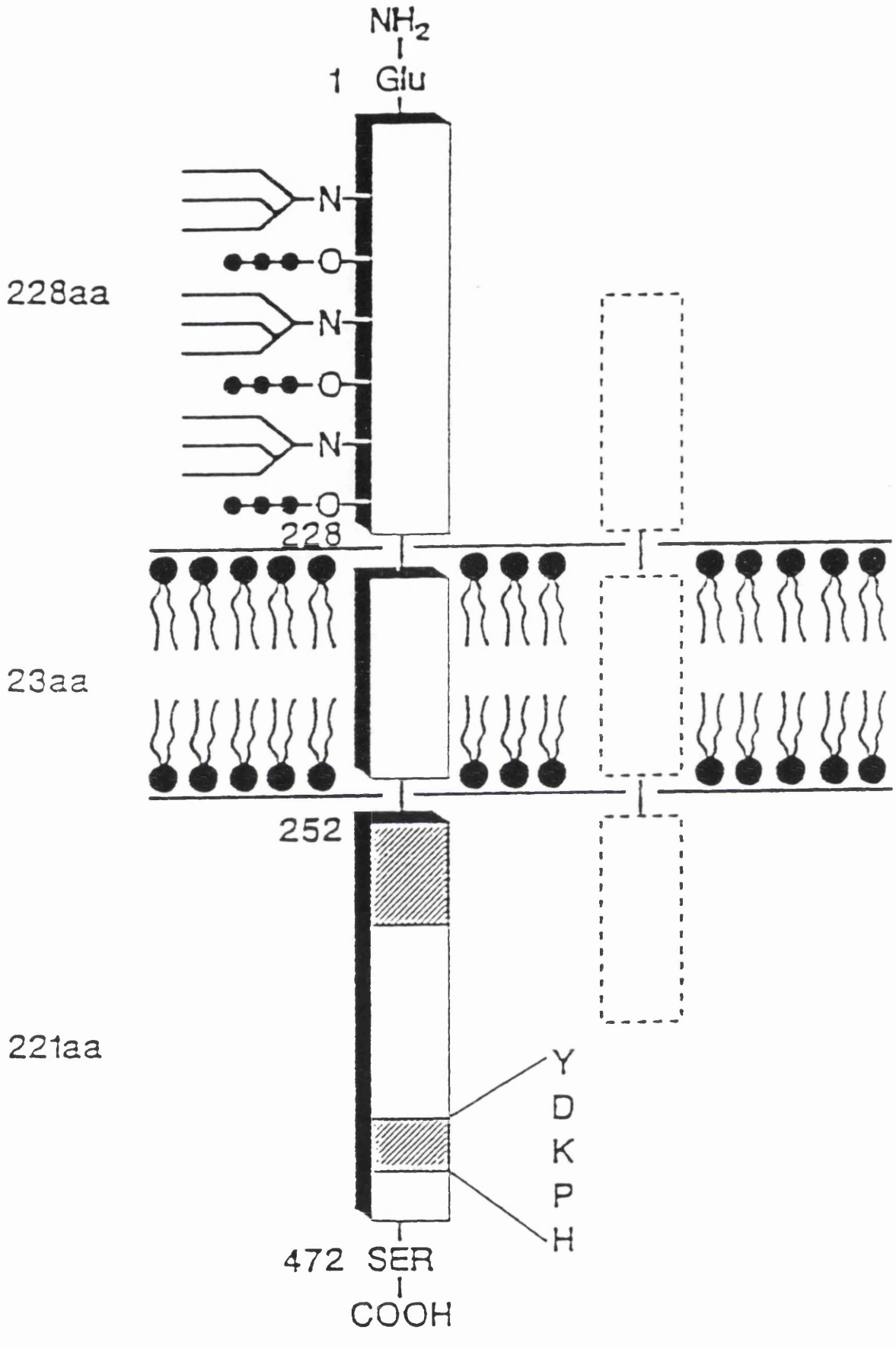


Figure 1.7 Diagram showing the structure of the human Interferon-γ receptor (from Farrar and Shreiber, 1993)

respectively, and have predicted molecular masses of 52.5 and 48.5 KDa. Both proteins are symmetrically orientated around single 23 amino acid transmembrane domains. Each possesses a 228 amino acid extracellular domain that contains 10 cysteine residues and 5 potential N-linked glycosylation sites which all appear to be occupied. The extracellular domain is sufficient to account for high affinity binding and receptor glycosylation is not critical for binding activity. The structure of the human interferon-γ receptor is shown in figure 1.7.

Based upon primary sequence comparisons, the interferon-γ receptor bears little identity to any other known proteins. More specifically, it is neither a member of the type one cytokine receptor family (haemopoeitic-characterised by the WSXWS motif) or the immunoglobulin superfamily. More refined sequence analysis suggests that the interferon-γ receptor belongs to a newly emerging class of receptor termed the type II receptor family whose members include the receptor for interferon alpha. The members of this family share a similarly organised 210 amino acid binding domain which contains conserved cysteine pairs at both amino and carboxy termini. The type two family seems to be only distantly related to the type I family.

The intracellular domains of the human and murine interferon-γ receptors are 221 and 200 residues, respectively. Both are rich in serine and threonine residues which together constitute 25% of the residues of this domain. In addition of the 6 tyrosine residues in the human receptor, 5 are conserved with the murine receptor, which is noteworthy considering the overall low sequence identity between the human and murine intracellular domains.

Expression of the human and murine cDNA across species boundaries confirmed that the receptor bound ligand in an appropriate species specific manner and that receptors expressed across species lines were unable to induce a functional response in transfected cells (Hemmi *et al*, 1989). This result suggested that additional species specific components were required for full activation of the interferon-γ receptor and supported conclusions reached from seminal experiments conducted by Jung *et al* (1987). This group used murine:human somatic cell hybrids to investigate the minimal requirements to form a functionally active human interferon-γ receptor in murine cells. Fusion of murine and human fibroblasts was known to generate stable cell hybrids that contained a full complement of murine chromosomes but only a random assortment of human chromosomes. All hybrids that contained human chromosome 6, bound human interferon-γ. However, responsiveness to the human ligand was observed only in cells that contained both human chromosome 6 and chromosome 21. This obligate requirement for two or more distinct species specific gene products led to the hypothesis that functionally active human receptors were composed of at least two distinct polypeptides: the receptor responsible for ligand binding and a species specific accessory protein(s) for full functionality.

Recently the identification and sequence of an accessory factor required for activation of the human and murine interferon-γ receptor has been reported, Soh and Donnely *et al* (1994), Hemmi and Bohni *et al* (1994). It is now well recognised that human chromosomes 6 and 21 and mouse chromosomes 10 and 16 are required for sensitivity to interferon-gamma as measured by the induction of class I major histocompatibility complex antigens. This indicates that the binding of interferon-gamma to the receptor is necessary but not sufficient for the induction of these antigens.

The newly cloned second component of the interferon-γ receptor has been termed accessory factor-1 (AF-1) by one group (Soh and Donnelly *et al* (1994)), and Interferon-γ β-chain by the other (Hemmi and Bohni *et al* (1994). Analysis of the sequence of this newly cloned component revealed that it shares some structural homology with the known components of the Interferon-α and Interferon-γ receptors, indicating that it is a new member of the Interferon-receptor family. Whether additional 'accessory factors' exist that are needed for a fully functional IFN-γ receptor is not yet clear. Soh *et al* (1994) found that AF-1 did not confer full antiviral protection against encephalomyocarditis virus in cells that express the binding portion (α-chain) of the human interferon-γ receptor, suggesting that yet another factor(s) encoded by human chromosome 21 is required. In contrast Hemmi *et al* (1994) found that the newly cloned β-chain of the murine interferon-γ receptor was sufficient to restore what appears to be full responsiveness to murine interferon-γ in cells that express the α-chain of the murine interferon-γ receptor.

1.1.5 INTERFERON-MEDIATED SIGNAL TRANSDUCTION

The interferons can induce an anti-viral state and modulate cell growth, differentiation and the immune response. Interferon α/β (type I) and IFN-γ (type II) induce distinct but overlapping sets of genes through independent cell surface receptors (Muller *et al,* 1993). The interactions of interferons with their specific cell-surface receptors is followed by the rapid activation of DNA binding factors which stimulate the transcription of a set of genes containing interferon response sequences (IRS's) homologous to the prototypic sequence GGGAAANNGAAACT (De Maeyer and De Maeyer Guignard 1994).

Until recently events in the signalling tranduction pathway after interferon binding to receptor have been poorly defined. Some reports had indicated that signalling involved protein kinases, receptor phosphorylation and/or ion fluxes. Other reports had suggested that internalisation of the receptor ligand complex was required for signal transduction (reviewed by Farrar and Schreiber, 1993). This, however, would be insufficient to mediate signalling events. This is based upon two observations. Firstly, whereas human interferon-γ receptors expressed in normal murine cells effect ligand internalisation and degradation with the same kinetics as functionally active receptors, no biological response is observed. Second, mutant human receptors lacking the carboxy terminal 39 amino acids when expressed in murine cells containing human chromosome 21, are also functionally inactive despite their ability to mediate ligand internalisation.

Recent work from several labs has lead to the elucidation of the backbone of the signal transduction pathways following interferon binding (Muller *et al* 1993; Watling *et al* 1993; Hunter, 1993). It has been demonstrated that interferons induce tyrosine phosphorylation and activation of transcription factor sub-units in the cytoplasm, which then translocate into the nucleus to initiate gene transcription. The initial insight into this pathway came from the observation that sub-units of an interferon-alpha transcription factor-Interferon Stimulated Gene Factor 3 (ISGF3), are localised in the cytoplasm. Following interferon alpha binding to the receptor, three of these cytoplasmic proteins p113, p84, p91 (Designated Signal Transducer and Activators of Transcription,or STAT, proteins) become phosphorylated on tyrosine, migrate to the nucleus and assemble into a complex together with p48, a sequence specific DNA-binding protein, to bind to the interferon-stimulable response elements and initiate gene transcription. The p91 and p84 subunits are derived from alternate

splicing of the same gene and differ only in a 38 amino acid extension at the carboxy terminal of p91. P113 is derived from a distinct gene but is related over its whole length to p91. p48 contains an amino terminal DNA-binding domain related in sequence to those of the transcription factors IRF-1, IRF-2 and cMyb, and a carboxy terminal domain required for the binding of the other ISGF3 subunits.

The next steps in elucidating signal transduction pathways came from the analysis of mutants of an HT1080 human fibrosarcoma cell clone, which expresses the bacterial gpt gene under the control of an interferon-α and interferon-γ inducible promoter, selected for non-responsiveness to interferon-α, Muller *et al* (1993). Mutant complementation group U3 cells lack a functional p91 gene and therefore express neither p91 or p84. Interferon-α responsiveness is restored to U3 cells by the expression of p91 or p84 complementary DNAs. Mutant group U2 cells lack a functional p48 gene and responsiveness is restored by expression of p48. Mutant group U1 cells, which contain functional p113, p91/p84 and p48 genes and bind interferon-α proved to be a mutant in the TYK2 protein tyrosine kinase (ptk) gene. Up to that time there was no known function for tyk2 and its related non receptor tyrosine kinases JAK1 and JAK2. However, this finding suggested that interferon-α binding to its receptor, which lacks intrinsic PTK activity, might lead to activation of TYK2, which in turn may lead to the phosphorylation of p91, p84 and p113.

The complementation of interferon resistant mutant cell lines has provided direct evidence for the involvement of the JAK family of non-receptor PTKs in the interferon signalling pathways. This family has three known members, JAK1, JAK2, and TYK2. Each is around 130kDa in mass and is characterised by the presence of a classical C-terminal protein tyrosine

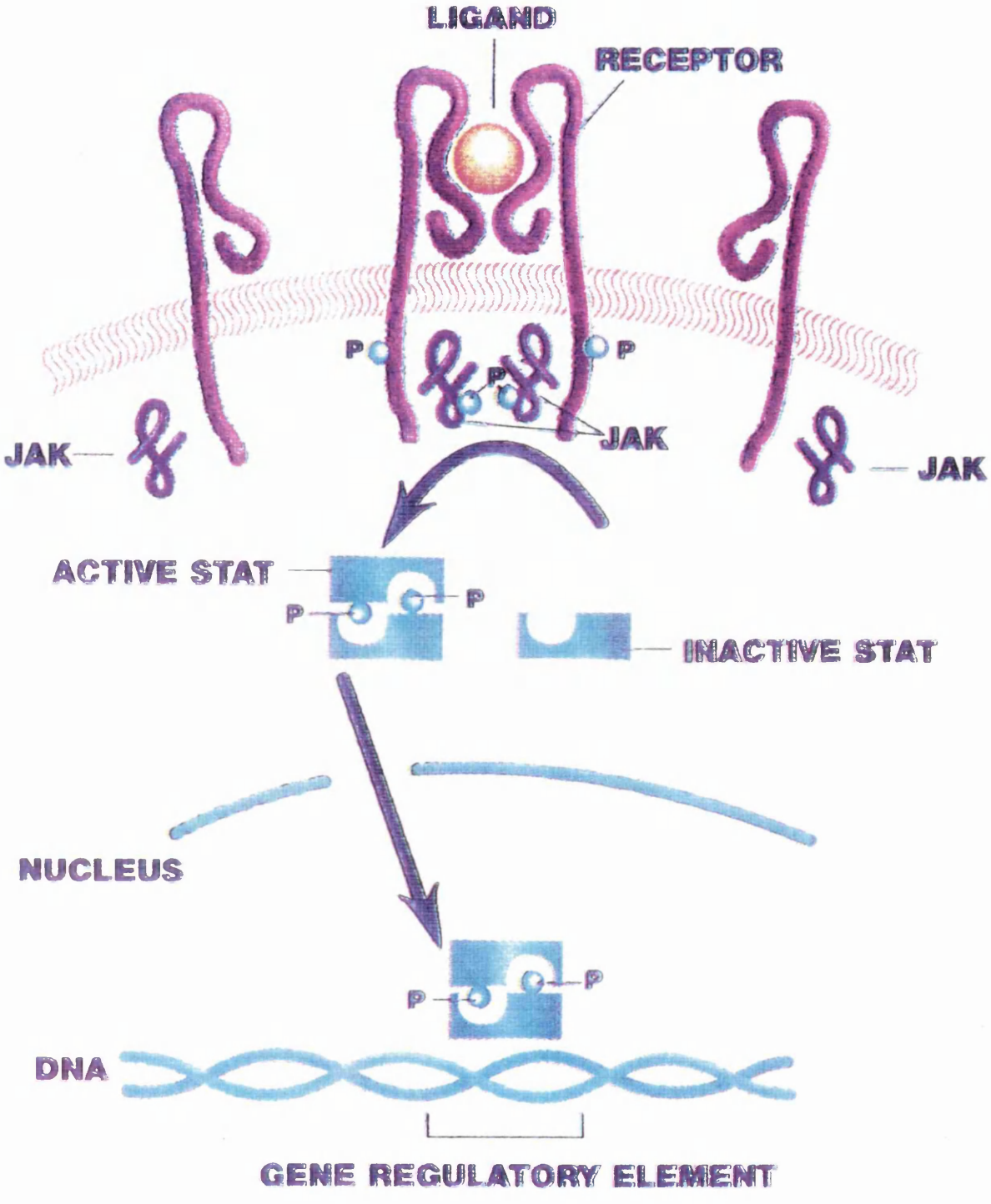


Figure 1.8 General scheme for the JAK-STAT mechanism of signal transduction. Ligand occupied receptors aggregate and form multimeric complexes with JAKs, which catalyse phosphorylation of the receptors, themselves and STAT proteins. The phosphorylated STATs migrate to the nucleus and bind to DNA regulatory elements

kinase domain, an adjacent kinase or kinase related domain and five further domains of substantial amino acid similarity with other members of the JAK family extending towards the amino terminus, Muller *et al* (1993).

The demonstration that TYK2 is required for interferon-α signalling then raised the question of how the interferon-α receptor would activate this tyrosine kinase and how the receptor functions. Interferon-γ has an unrelated receptor and induces expression of a different but overlapping set of genes through a distinct response element known as the Gamma Activation Sequence (GAS). The GAS is given by the sequence TTTCATATTACTCT. Interferon-γ stimulates tyrosine phosphorylation of cytoplasmic p91, but not p113 or p84. Phosphorylated p91 bind directly to the GAS element after translocation to the nucleus.

Interferon-γ induced phosphorylation of p91 also occurs at tyr701 and this modification is required for GAS binding. The failure of U3 cells to respond to interferon-γ suggests the essential role for p91. The selective phosphorylation of p91 after interferon-γ binding suggests that a PTK other than TYK2 is responsible.

Kerr and co-workers were able to demonstrate the possible nature of the PTK involved. They showed that the U4A mutant cell line, which responds to neither interferon-α or γ, expresses a truncated form of JAK1 mRNA and no JAK1 protein, can be complemented for both interferon-α and γ responses by expression of JAK1 cDNA.

In a second study the same group selected a new HT1080 cell line termed g1A, which was unable to respond to interferon-gamma but could respond to interferon-alpha and had functional p91, p84, p113 and p48 genes.

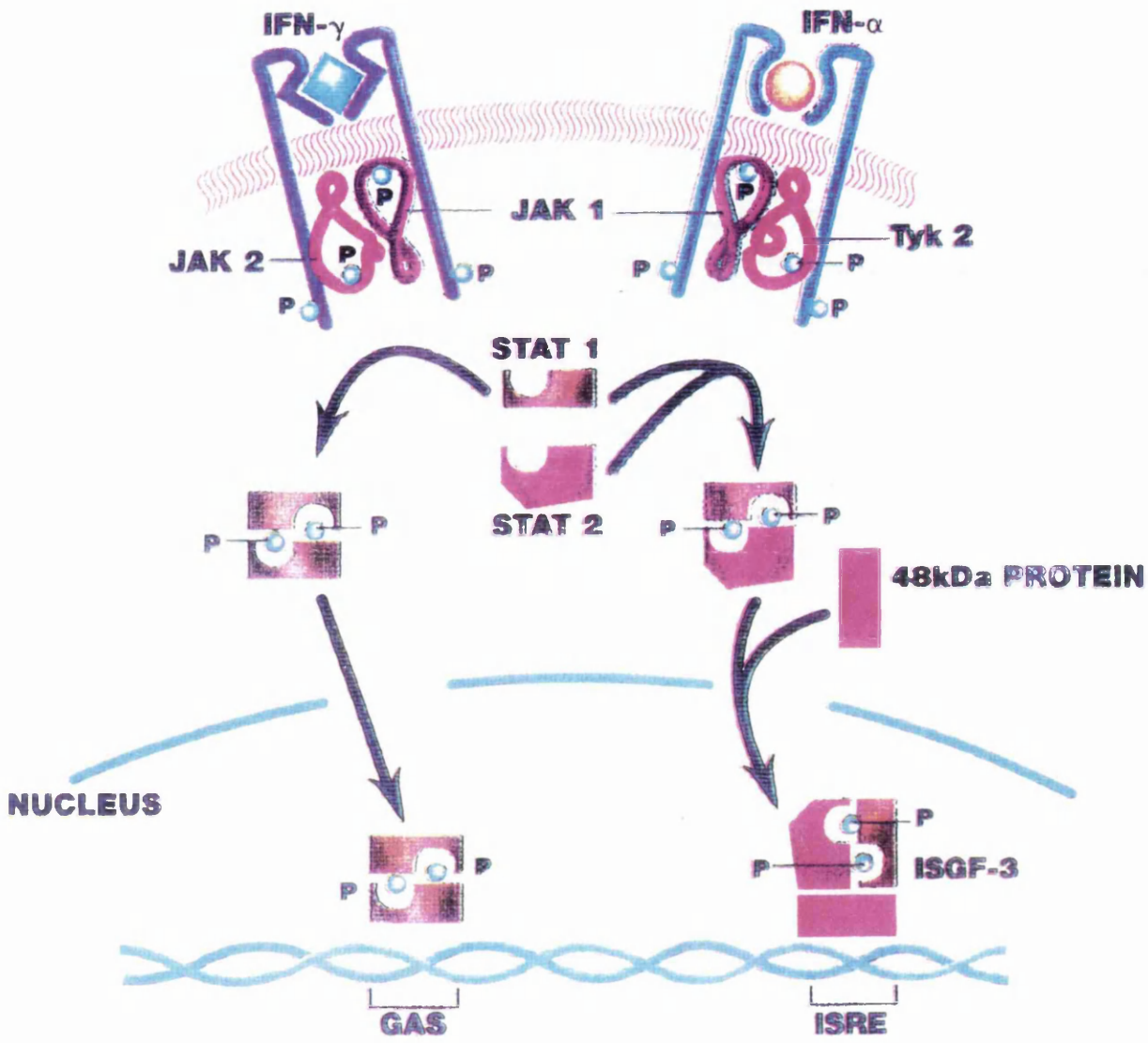


Figure 1.9 A model for IFN-α and IFN-γ activation of gene transcription. After IFN-γ binding to receptor, the receptor dimersises and binds JAK1 and JAK2. Both receptor and JAK1 and 2 become phosphorylated and catalyse the phosphorylation of STAT1. This then becomes an active gene regulatory factor.

Interferon-γ stimulates both JAK1 and JAK2 tyrosine phosphorylation in parental cells but neither JAK1 or JAK2 is phosphorylated in g1A cells. Expression of JAK2 cDNA in g1A cells restores interferon-γ responsiveness, and interferon-γ induced phosphorylation of JAK1 and JAK2. A full length JAK2 protein is detected in g1A cells which suggests that there is an inactivating point mutation in JAK2 in g1A cells (although the mutation could be in another protein which is required for the activation of JAK2).

Activation of the JAK family of PTKs can be summarised as follows. Interferon-γ elicits tyrosine phosphorylation of JAK1 and JAK2, but not TYK. The mechanism by which interferon binding with receptor causes this tyrosine phosphorylation of the PTKs is still uncertain. Johnson and Szente (1994) have suggested the following scenario. When interferon gamma binds with its specific receptor there is internalisation of the protein which is then degraded. Soon after internalisation the carboxyl terminal region of the fragmentary or intact interferon-γ molecule could bind to the receptor at a site that was initially inaccessible in the cytoplasm. Such binding could displace JAK2 from the same site and thereby free it to act upon other proteins. However, as stated above, ligand receptor internalisation is insufficient alone for signal transduction and a second co-factor is required. Activation of the janus kinases leads to phosphorylation of the receptor and the STAT protein P91 (GAF or Interferon-γ activation factor). STAT91 dimerises and migrates to the nucleus where it binds to the GAS element, initiating gene transcription. Figures 1.8 and 1.9 show the general sheme of the JAK-STAT mechanism of signal transduction.

Interferon-α induces phosphorylation of JAK1 and TYK2. Both U1 and U4A cells fail to respond to interferon-α and lack functional TYK2 and JAK1 respectively, which implies that both of these PTKs are required for the interferon alpha response. The failure of U4A and g1A cells to respond to interferon-γ indicates that both JAK1 and JAK2 are required for the interferon-γ response. The other conclusion that can be drawn from the analysis of the mutants is that the JAK PTKs cannot be placed in any linear order because if either is missing then the second is not phosphorylated. The exact mechanism of phosphorylation of these kinases following receptor binding has yet to be established.

There are still gaps in the understanding of signal transduction pathways mediated by the interferons. One of these is how different interferon alpha species induce transcription of different sets of genes. Another obvious gap is the mechanism by which receptor binding leads to phosphorylation of the PTKs.

1.2 INTERFERON-γ BIOLOGY

1.2.1 INTRODUCTION

Reported biological functions of interferon-γ have been reviewed by Farrar and Schreiber (1993), De Maeyer and De Maeyer Guignard (1994), Pestka and Langer (1987) and De Maeyer and De Maeyer Guignard (1992).

Despite the many documented functions of inteferons it is not easy to determine how essential interferons are in host defence and immune responses. The reason for this uncertainty is the extensive redundancy in cytokine actions. Some of the antiviral and immunomodulatory actions of

IFN-α/β of IFN-γ can be mimicked by other cytokines, notably by TNF or IL-1. To what extent these and other cytokines can substitute for the actions of the interferons is uncertain. Help for this problem is coming from the use of 'gene knockout' mice, that is mice which lack a functional interferon-γ gene or interferon-γ receptor gene. To generate mice lacking a specific functional gene, embryonic stem cells taken from the preimplantation mouse embryo are cultured and injected with a targeting vector in which DNA, homologous the the interferon-γ or interferon-γ receptor gene, is incorporated. Homologous recombination leads to removal of the essential portion of the gene. Embryonic stem cells carrying the mutation are selected and re-introduced into an early embryo.

Mice with a targeted deletion of the interferon-γ gene and mice with a targeted deletion in the interferon-γ receptor gene have recently been generated. Both types of mice develop normally and show no overt phenotypic abnormalities. In addition the immune system does not show any developmental abnormalities and receptor deficient mice are able to mount a normal cytotoxic T cell response against infection with lymphocytic choriomeningitis virus and develop undiminished antibody levels when immunised with various antigens. Despite this it may be that the deficiency is affecting the development of immune responses in a more subtle way. In addition, a complete deletion in the structural gene for interferon-γ or its receptor may be promoting the development of compensatory pathways that are not normally present when the interferon-γ system is intact. Thus it may be that studies using gene knockout mice may underestimate the importance of interferon-γ in the immune response.

Despite the ability of the receptor deficient mice to mount specific immune responses, these animals showed severe defects in the defence against

several infectious agents. Inoculation of a sublethal dose of *Listeria monocytogenes* into receptor deficient mice caused death in the majority of mice by the fifth day after infection. Increased mortality was accompanied by a more abundant multiplication of the organism in the organs of the mice. In addition, these mice were also more susceptible to infection with vaccinia virus than wild-type mice. This increased sensitivity was accompanied by a higher titre of virus in the organs of affected mice. Hence, it appears that interferon-γ may be restricting viral replication in the early stages of infection.

Infection of receptor deficient mice with the BCG strain of *Mycobacterium bovis* also demonstrated increased susceptibility compared to wild-type mice. In wild type mice there is development of granulomatous inflammatory lesions in their livers, spleens and lungs which reach a peak at 2-3 weeks following inoculation with *M.bovis.* Subsequently, the cellular immune response leads to the gradual elimination of mycobacteriam from the organs and complete recovery from infection. In contrast, in receptor deficient mice the elimination of the organism was impaired and all infected mice died within 7-9 weeks of infection. Extensive inflammatory lesions and large numbers of mycobacteriam were found in the livers, lungs and spleens. This increased susceptibility to infection with *M.bovis* has also been demonstrated in mice with a mutant interferon-γ structural gene.

The exact mechanism whereby interferon-γ promotes host resistance organisms requires further investigation. Interferon-γ is known to be the principal agent responsible for macrophage activation. The receptor deficient mice showed decreased formation of granulomatous lesions following infection with *M.bovis*. The presence of activated macrophages

in granulomas is thought to be essential for the elimination of mycobacteriam, Listeria and other infectious agents. Endogenous TNF-α is also thought to be important in granuloma formation during mycobacteriam infection, probably acting in concert with interferon-γ. In the infected, receptor deficient mice, production of endogenous TNF-α was also impaired. Another factor which may contribute to increased susceptibility to infectious agents is the important role that interferon-γ plays in the regulation of MHC class II expression. In the infected, receptor deficient mice MHC II expression was significantly lower than infected wild-type mice.

1.2.2 MHC CLASS ANTIGEN INDUCTION

Interferon-γ is an extremely pleiotropic cytokine that has a major physiological role in regulating immune and inflammatory processes. Clearly one of the major roles of IFN-γ is in the enhancement of MHC I expression and the induction of MHC II expression. IFN-γ acts at the level of MHC gene transcription and is important in promoting antigen presentation during the inductive phase of the immune response. Thus a prime target for interferon-γ action is the macrophage.

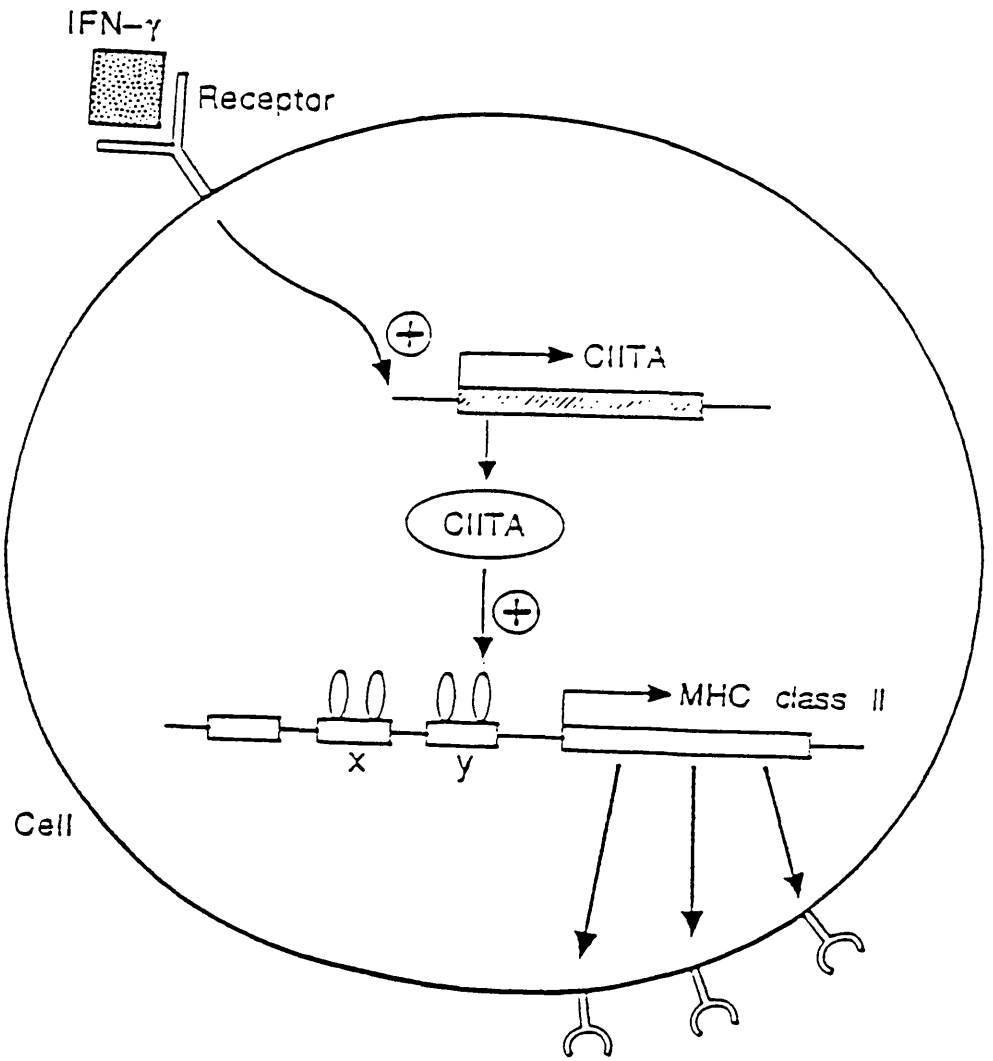
Work from several laboratories has shown that interferon gamma is one of the major cytokines responsible for activating or regulating the differentiation and function of mononuclear phagocytes. Interferon-γ has been shown to effect the differentiation of immature myeloid precursors into mature monocytes. It promotes antigen presenting function, not only through the induction of MHC II but also by increasing levels of intracellular enzymes which may be required for antigen processing. In addition, IFN-γ augments expression of macrophage cell surface proteins

such as ICAM 1 that enhance the functional consequences of the interaction between macrophages and T cells during antigen presentation.

MHC class II molecules present antigens to T lymphocytes and the tight regulation of their expression is of critical importance to the control of the immune response. However, the mechanism of interferon-γ induced MHC class II expression remains obscure. Induction of MHC class II genes is characterised by a long lag period and total dependence on *de novo* synthesis of protein. These genes thus exhibit a secondary response to interferon-γ which has led to the postulation of an intermediary activator which is itself induced by interferon-γ. It has not been possible to define interferon response elements in MHC II genes. In addition none of the now well documented interferon-γ inducible elements have been found to bind to class II promoter sequences. Furthermore, it has been suggested that the constitutive and inducible modes of class II expression are controlled by distinct regulatory mechanisms, Steimle *et al* (1994).

The MHC class II transactivator CIITA was recently cloned by genetic complementation of an MHC class II mutant B lymphocyte line (Steimle *et al* 1994). CIITA is expressed in a cell specific manner which correlates with MHC class II expression. CIITA expression has been studied in MHC class II negative cells. In these cells it was found that interferon gamma could induce CIITA expression and this precedes expression of MHC class II genes. It is now established that CIITA is required for both constitutive and inducible expression of MHC II molecules and that it is itself inducible by gamma interferon. It has also been found that expression of CIITA cDNA in the absence of interferon gamma is alone sufficient to switch on MHC class II expression. Thus the model for induced expression of MHC class II by interferon gamma is as follows.

Figure 1.10 A model for MHC Class II antigen induction by IFN-γ
(from Steimle et al, 1994)



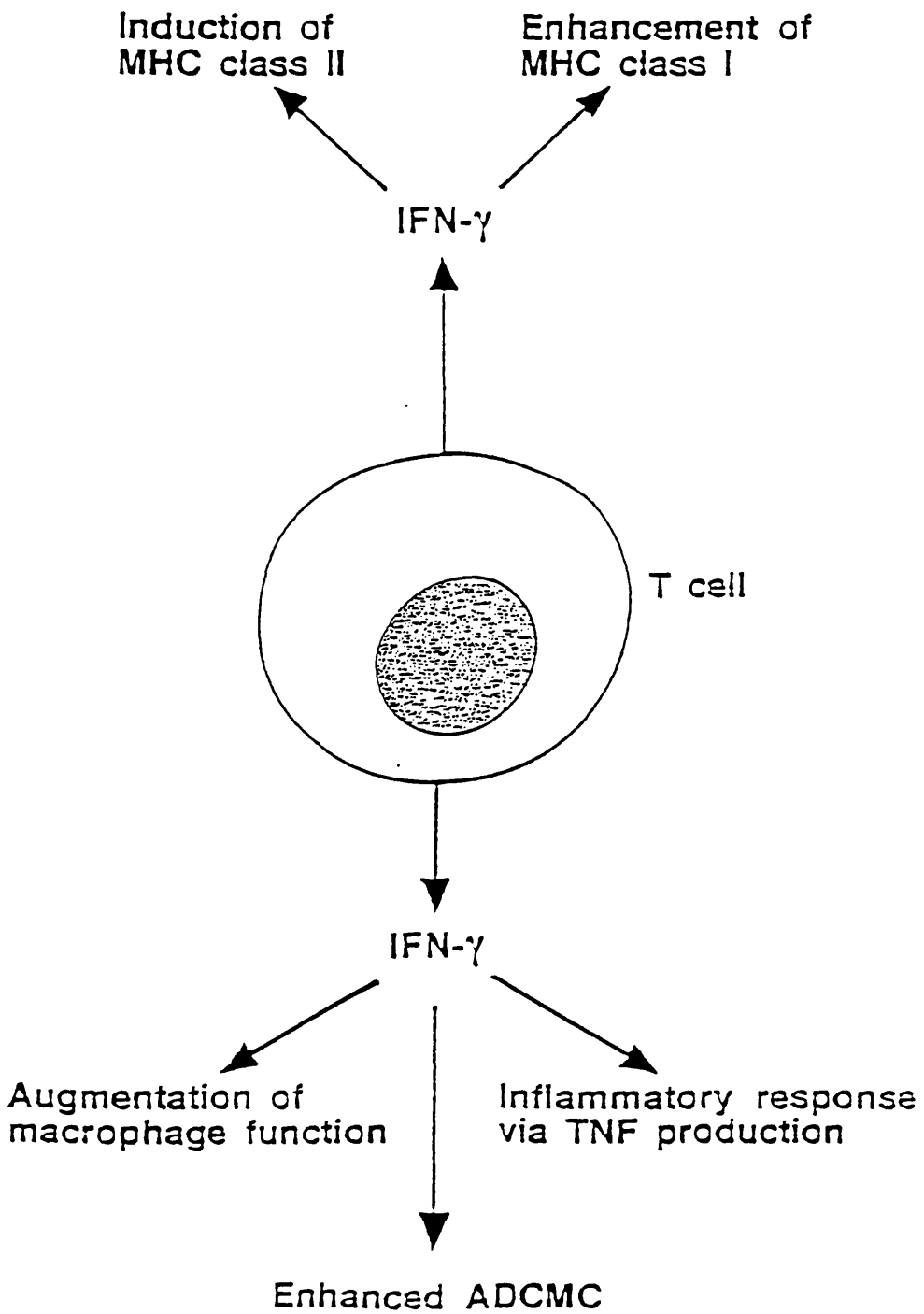
Interferon gamma induced intracellular signals activate expression of the CIITA gene. There is no direct evidence that there is binding of the CIITA to the MHC class II promoter and therefore CIITA may act as a gene and tissue specific co-activator. The model for MHC class II induction is represented in figure 1.10

The main target cell for interferon-γ is the macrophage. In experiments carried out in mice, the administration of recombinant murine interferon-gamma resulted in increased expression of MHC antigens on macrophages and on other cell types. In addition, interferon-gamma enhances MHC class II expression on T cells, B cells and tumour cells. Stimulation or expression of MHC class II antigens on certain tumours has been observed with both interferon-α and γ. Cells of established lines derived from human tumours such as melanoma, breast carcinoma can be induced to express class II antigens by interferon-γ. However, this is not a general property of all tumour cells and some are resistant to the effects of interferon-γ.

1.2.3 NON-SPECIFIC HOST DEFENCES

There is little doubt that IFN-γ is the major physiological macrophage activating factor and therefore is the primary cytokine responsible for inducing non-specific cell mediated mechanisms of host defence. Work from several laboratories has established interferon gamma's ability to activate non-specific cytocidal activity in macrophages towards a variety of intracellular and extracellular parasites and neoplastic cells. IFN-γ induces the expression of undefined structures on macrophages that recognise target cells, promote the elaboration of macrophage-derived cytocidal compounds such as reactive nitrogen and oxygen intermediates

Figure 1.12 The role of Interferon-γ in host defences



and TNF-α, and also reduces the susceptibility of macrophages to microbial infection. The importance of IFN-γ in the clearance of microbial pathogens has been demonstrated using animal models. Mice pre-treated with neutralising antibody to IFN-γ lose their capacity to resolve infection from microbial pathogens such as *Listeria monocytogenes*, *Toxoplasma gondii*, and *Leishmania majo*r. In addition the experiments carried out in gene 'knockout' mice, described above, have confirmed this.The induction of nitric oxide (NO) production is thought to be one of the principal mechanisms of macrophage cytotoxicity for tumour cells, bacteria, protazoa, helminths and fungi. NO is an unstable free radical gas that, in addition to its role in host defences, functions as an intercellular messenger in vasodilation and neurotransmission, At least three related genes encode NO synthases in different tissues. Endothelial and neural NO synthases are constitutive enzymes that are activated by Ca2+/calmodulin. In contrast, macrophages and many other cells contain a transcriptionally inducible NO synthase (iNOS) that remains undetectable until these cells are activated. In murine macrophages the most potent activators of gene transcription appear to be interferon-γ and bacterial lipopolysaccharide (LPS). The promoter region of the murine iNOS gene was recently shown to be responsive to interferon-γ and LPS.

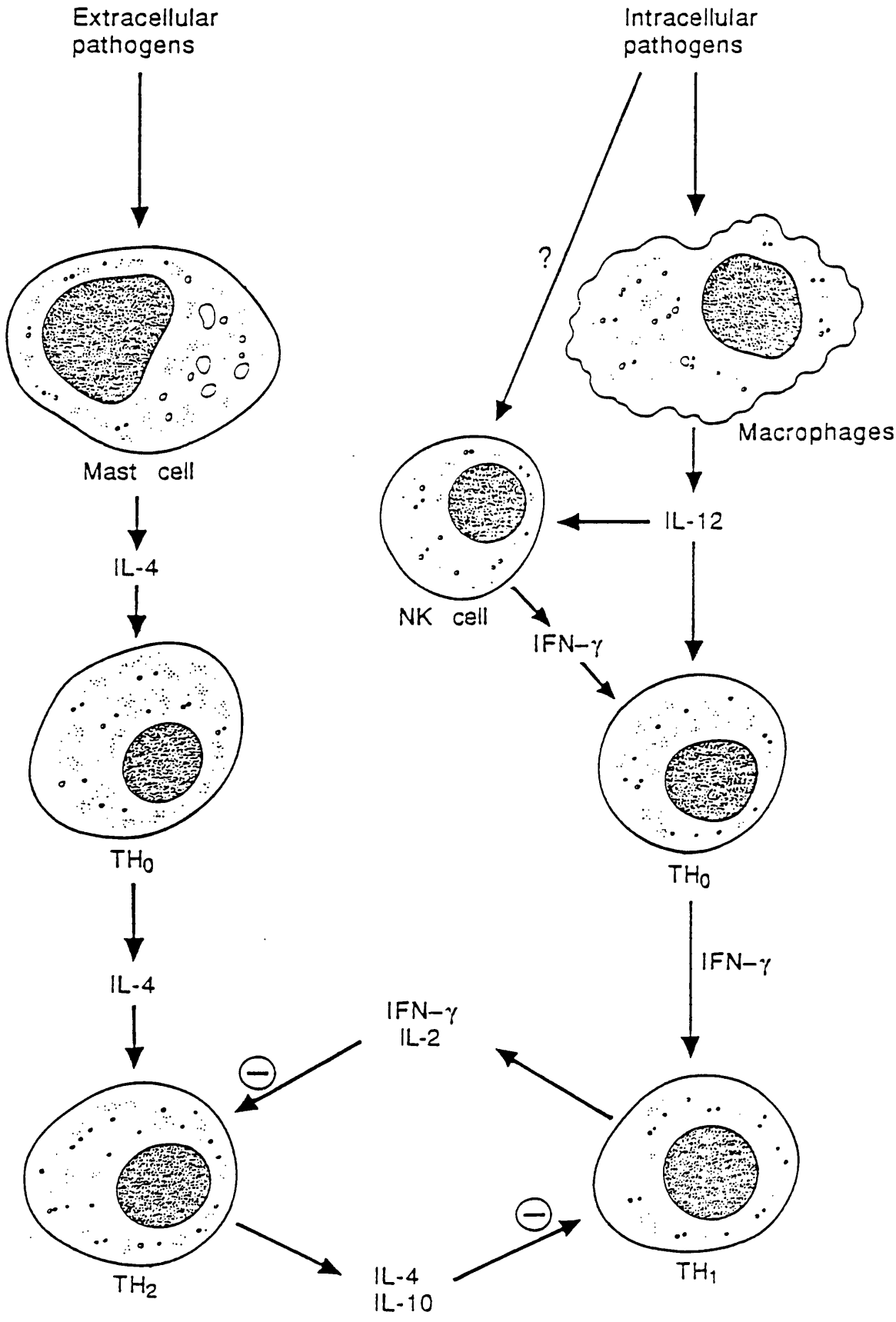
More recently, Interferon regulatory factor I (IRF-1) has been identified as a transcription factor critically important for the induced expression of the iNOS gene and NO synthesis in murine macrophages. IRF-1 and the related IRF-2 protein are transcription factors involved in the regulation of IFN-a/b genes and some IFN-inducible genes. IRF-1 functions as a transcription activator, whereas IRF-2 inhibits transcription. IRF-1 is strongly induced by the action of interferon-γ, (Vilcek *et al*, 1994).

1.2.4 IMMUNE EFFECTOR FUNCTIONS

In addition to enhancing non-specific cell mediated cytocidal activities, IFN also enhances the ability of macrophages to participate in other immune response effector functions. It enhances the expression of high affinity Fc receptors on macrophages and monocytes (FcγRI) and thereby enhances the capacity of these cells to take part in antibody dependent cellular cytotoxicity (ADCC). Interferon-γ also enhances the biosynthesis of a variety of complement proteins by macrophages and fibroblasts and regulates the expression of complement receptors on the mononuclear phagocyte plasma membrane thereby promoting humoral immunity through the enhancement of complement activity.

IFN-γ also exerts its effects on other cells of the immune system. It regulates immunoglobulin isotype switching on B cells and antagonises the ability of IL-4 to induce MHC II expression on murine B cells. These responses result from the direct effect of interferon-γ on the B cell. B cell responses are also affected indirectly by the ability of interferon-γ, in synergy with IL-12, to regulate the development of specific subsets of CD4+ T cells. Interferon-γ has a profound antiproliferative effect on TH2 cells but not TH_1. Thus interferon-γ plays a major role in determining the type of immune effector function that eventually develops during specific immune responses. The opposing effects of IL-10 and IFN-γ thereby serve to cross regulate the development of specific immune responses. IL-10 inhibits IFN-γ production by T cells and NK cells and thus diverts the response to the humoral pole. In contrast, IFN-γ inhibits the expansion of TH2 like cells which eliminates the source of IL-10 and diverts the response to that of cell mediated immunity. The involvement of

Figure 1.13 Schematic representation of the involvement of interferon-γ in the generation of Th1 and Th2 cells



interferon-γ in the generation of TH_1 and TH_2 cells is represented diagrammatically in figure 1.13.

1.2.5 INTERFERON AND TNF-α

Interferon-γ is intimately related to the regulation of TNF-α production. It is well established now that LPS induced macrophages produce increased amounts of TNF-α when pre-treated with interferon-γ. It was initially thought that interferon-γ treatment alone could induce TNF-α production but this was not substantiated in other laboratories and the initial findings may have been due to the increased sensitivity of cells to minute traces of LPS in the cell medium. Studies indicate that the enhancement of TNF production may be due to either increased gene transcription or increased stability of TNF mRNA. in addition, interferon-γ enhances TNF receptor expression on certain cell types. The role that interferon plays in TNF regulation demonstrates a further role in non-specific defence mechanisms by the enhancement of the inflammatory response (Farrar and Shreiber 1993).

During the inflammatory response, cells leave the circulation at the site of the response , but to do this, they must extravasate through the vascular endothelium. This process is intimately related to the up-regulation of cell surface adhesion molecules on the surface of endothelial cells. Interferon-γ has been found to up-regulate ICAM-1 and MHC class I molecules on these cells, while TNF-α has been found to up-regulate ICAM-1, ELAM-1 and MHC-class I (reviewed by Farrar and Schreiber 1993). Further, the two cytokines appear to act synergistically to amplify these effects. Thus, the co-operative ability of Interferon-γ and TNF-α to modulate cell migration serves to expand and amplify the overall inflammatory process.

1.2.6 INTERFERON AND NK CELLS

Natural killer cells probably function as an early defence line against viruses because of their ability to kill virus-infected cells as well as a variety of tumour cells. In both cases, the killing of the cells occurs in a non-MHC restricted fashion. NK cells are positively regulated by all three classes of interferons and can also be indirectly stimulated by inducers of interferon production such as viruses or mitogens. The mechanisms by which interferon enhances NK cell function have been reviewed by Reiter (1993) and Reiter and Rappaport (1993). In general, it is possible to define at least four mechanisms by which interferon may enhance or activate NK function. Firstly, IFN could induce NK cell proliferation; secondly, it could convert the NK precursor cell into an active NK cell; thirdly, it could enhance binding of NK cells to target cells, and fourthly, it could enhance the lytic efficiency of NK-target cell conjugates.

In contrast, interferon can also have a negative effect on both NK cell and lymphokine activated killer cell (LAK) mediated cytotoxicity. The negative modulatory effect of interferon on these cells has been reviewed by Reiter (1993). Pretreatment of target cells with interferon causes them to become refractory to the effects of NK cells and LAK cells but the cells still remain susceptible to killing by cytotoxic T cells. This suggests that interferon does not induce a general resistance to T cells but induces specific resistance to NK cells. Interferons have been found to have this protective effect on many cell types but the degree of protection may vary between cell phenotypes. Interestingly, tumour cells are found to be less protected than normal cells. Thus, interferon, by stimulating cytotoxic cells and by simultaneously protecting normal cells from lysis, might

thereby make the NK system and inducible selective defence system against tumour and virus-infected cells. However, because protection by interferon may extend to some transformed cells, there will be occasions when these cells will escape killing by NK and LAK cells

1.2.7 RESOLUTION FROM VIRAL INFECTION

There are essentially two ways in which the interferons are involved in the antiviral response and resolution of viral infections. The first, is the ability of interferon species to protect cells against viral infection by the activation of enzyme pathways which inhibit viral protein synthesis (described below). This is important in the early stages of infection and is a non-specific defence mechanism to limit viral replication and dissemination. As discussed earlier, interferon-α and β are far more potent antiviral agents as regards this process of viral limitation. Viruses differ in their capacity to induce interferon. Myxoviruses, paramyxoviruses and toga viruses are the best inducers; picorna viruses and poxviruses induce moderate amounts; herpesviruses are poor inducers, and adenoviruses are non-inductive. In general, viruses which dramatically inhibit cellular mRNA synthesis are poor inducers.

The ability of viruses to induce interferon and their susceptibility to the action of interferon are not necessarily related (Onions and Jarrett, 1994). For example, in dog cell cultures vaccinia virus does not induce interferon but its growth is inhibited when exogenous interferon is added to the culture. In contrast, in these cells influenza B induces interferon but is insensitive to its action.

Different strains of the same virus type may induce different quantities of interferon, and their virulance may be related to the amount of interferon induced. For example, certain strains of rubella virus which induce high levels of interferon are less teratogenic than strains which induce small amounts.

What follows is a description of the induction of the antiviral state by the interferon species. Several of the interferon-stimulated genes code for proteins responsible for the antiviral state, such as the dsRNA-dependent P1/eIF-2a kinase, the (2'-5') oligo adenylate synthetase, and the Mx proteins. These antiviral mechanisms have been reviewed by De Maeyer (1994).

The (2'-5') oligo adenylate synthetase, or (2'-5')An synthetase is constitutively expressed in many cells but its concentration can be increased by several orders of magnitude by the addition of interferon. Interferon-α and β are far more potent at enzyme induction than interferon-γ. IFN treatment alone does not significantly increase the intracellular concentration of the synthatase enzyme or increase the extent of the protein kinase phosphorylation. However, infection of IFN-treated cells with either EMC virus, reovirus, influenza virus, vaccinia virus, or simian 40 virus causes a significant increase in the intracellular concentration of the synthetase enzyme and/or the extent of eIF-2a phosphorylation. When activated by ds RNA, (2'-5')An synthetase polymerizes ATP into a series of 2'-5'-linked oligomers (ppp(A2'p)n) of which the trimer is the most abundant. These oligomers, collectively called 2-5A, then activate a latent endoribonuclease, designated RNase L, which is responsible for the antiviral activity. The third enzyme of this system, which is present in interferon untreated and treated cells, is a 2'-5'

phosphodiesterase that catalyses the degradation of 2-5A. The dsRNA required to activate the system are most likely to be intermediates or by-products of viral RNA replication. The system serves then to degrade viral RNA.

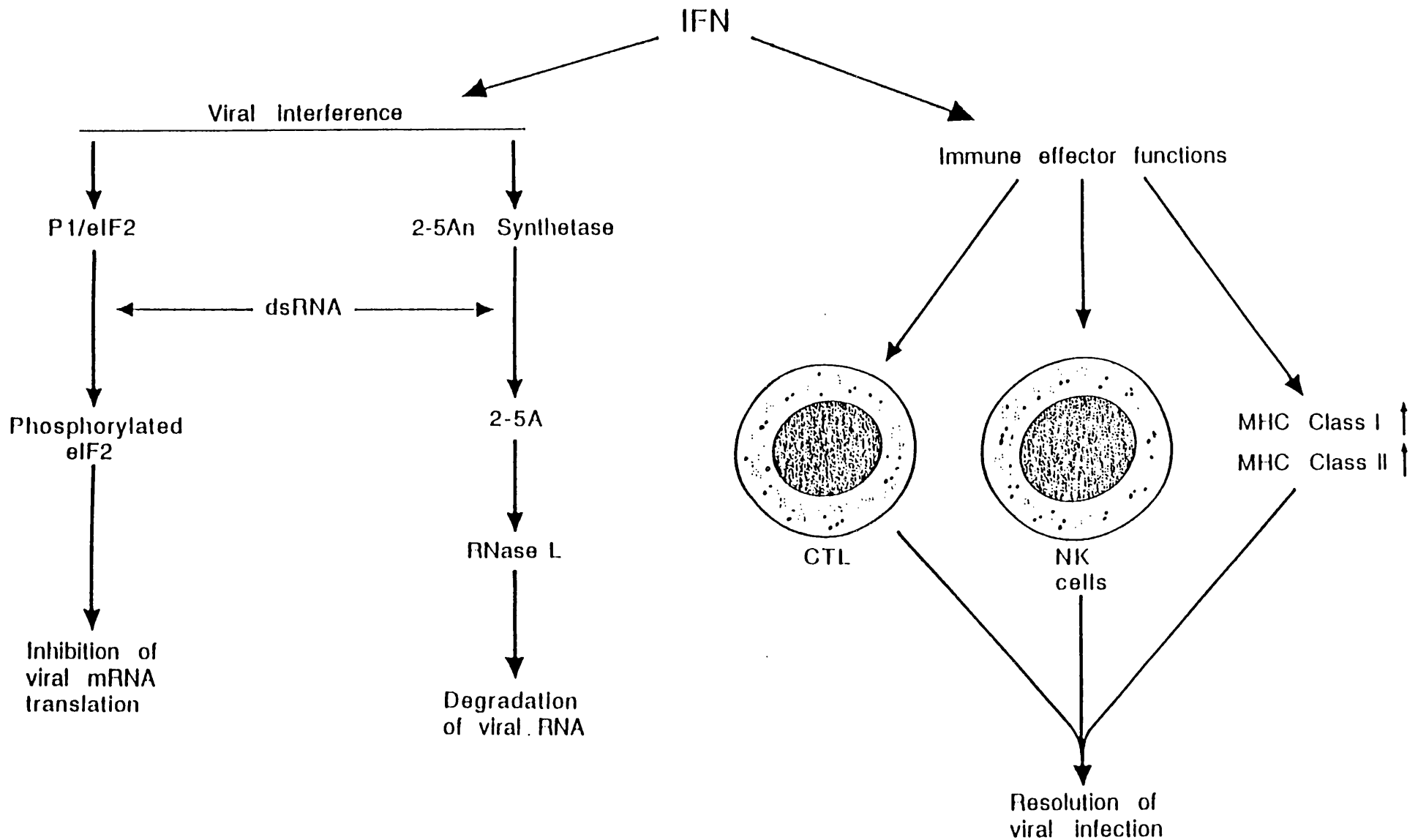
Activation of the protein kinase P1/eIF2 (a serine threonine kinase) is the second pathway of IFN-induced translational control that is dependent on the presence of dsRNA. P1/eIF2 is activated by interferon treatment and the presence of dsRNA. Following activation, it first autophosphorylates and then phosphorylates the a subunit of eIF2, the eukaryotic protein synthesis initiation factor. As a result, the recycling of the a subunit is inhibited and the initiation of translation cannot begin. This system then serves to prevent viral mRNA translation.

The Mx proteins are a family of interferon α/β inducible GTPases that display antiviral activity against specific viruses such as influenza virus.

The antiviral responses induced by interferon are depicted diagrammatically in figure 1.14. The individual importance of each system in the antiviral response has yet to be resolved.

The second mechanism by which interferon is involved in antiviral immunity is by its immunomodulatory actions, and this is really the domain of interferon-γ. As described, interferon-γ stimulates natural killer cells, cytotoxic T cells, and upregulates both MHC class I and II antigens. These effects produce a local stimulus for cell killing by these effector cells and, in addition, because interferon-γ is produced by NK cells and CTLs, there is a local positive feedback loop which amplifies the immune response.

Figure 1.14 Interferon-γ and the resolution from viral infection



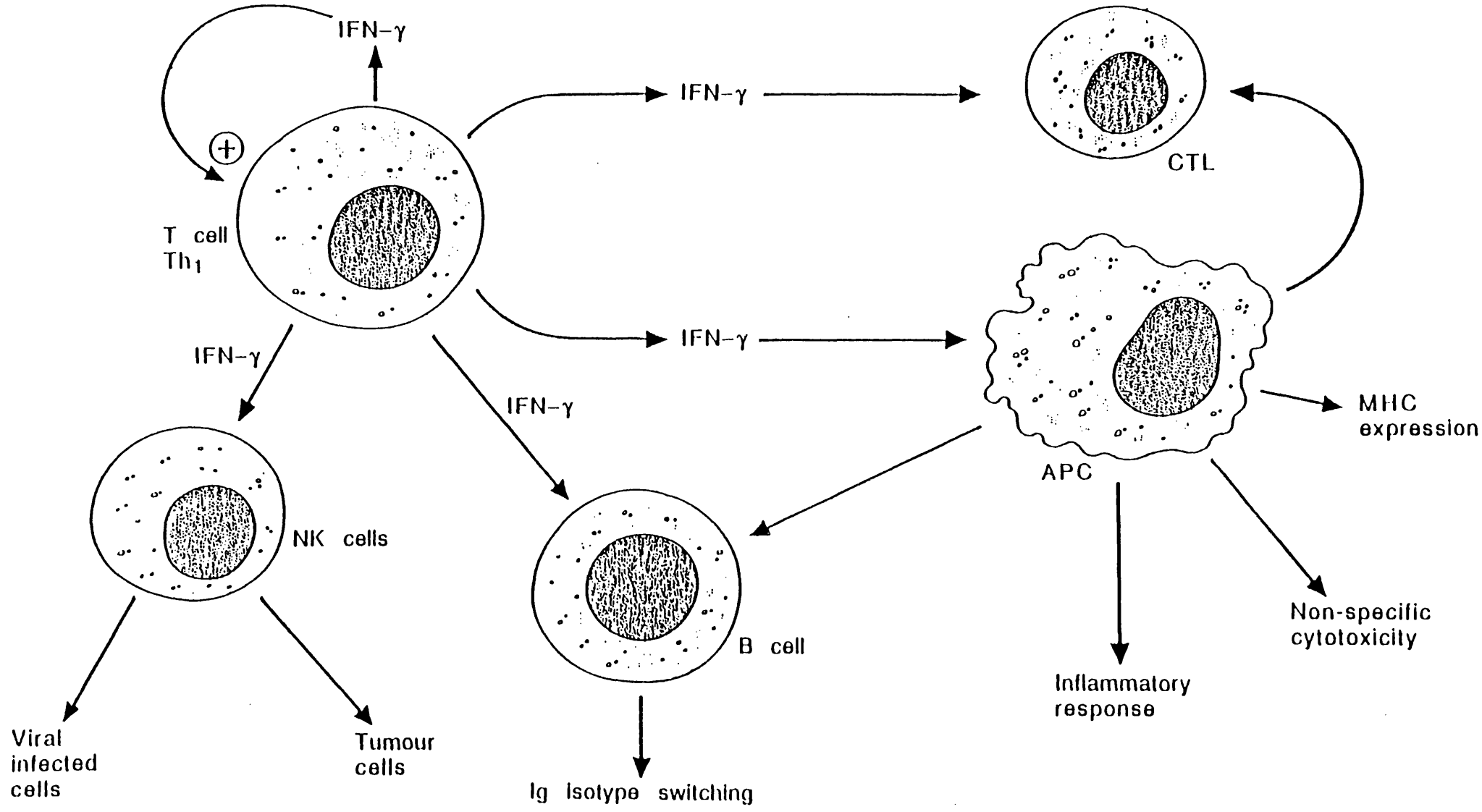
It has been suggested by one group (Ramsay *et al* 1993), that the major function of the effector cells (NK cells and CTLs) is to focus antiviral cytokines at the site of virus replication. This group used recombinant vaccinia viruses (rVV), constructed to encode murine cytokine genes, to examine the antiviral effects of these molecules. It was found, in the murine model, that in the absence of classical, anti-viral effector cells, animals were able to control infection with rVV infection when the rVV expressed interferon-γ. This group also suggest that TNF-α and IFN-γ synergise as antiviral agents. This is evidence that, while interferon-γ is important in directing the immune response against viruses, it may also be directly anti-viral.

As a final point on antiviral mechanisms, it is noteworthy that some viruses have evolved mechanisms which evade the immune response. For example, The human herpes virus EBV has "captured" the human IL-10 gene by recombination (Rhode *et al*, 1993). Expression of this in the host may direct the immune response away from the cell mediated pole and thus evade the antiviral CTL responses. In addition, a recent report (Alcami and Smith, 1995) has suggested that a variety of orthopoxviruses have captured the interferon-γ receptor gene which is able to bind and neutralise IFN-γ in a non-species specific manner. This interferes with the anti-viral action of this cytokine.

SUMMARY

The above review of the biological functions of interferon-γ underlines the pleiotropism of this cytokine and its intimate relationship with non-specific and specific effector arms of the body's defence mechanisms. One can appreciate now that the primary function of interferon-γ is that of a

Figure 1.15 Interferon-γ in specific and non-specific host defences



pivotal immunoregulatory cytokine rather that an anti-viral agent. The various roles that interferon plays in the host defence mechanisms therefore include a role in innate immunity (macrophages/neutrophils), lymphocyte migration, antigen recognition by T cells and effector T cell development. This is depicted in figure 1.15.

1.3 CLINICAL APPLICATION

1.3.1 OVERVIEW

Interferon-gamma is a pleiotropic cytokine which has numerous effects on the immune system and host defence. The pivotal role that interferon plays
in host defence and immunity has made it an attractive candidate in the treatment of infectious, immune related and neoplastic disorders. In addition, because of interferon-gamma's role in antigen presentation, the potential of immune interferon as a vaccine adjuvant is also being explored. The following paragraphs will briefly highlight some of the clinical applications being found for human recombinant interferon-gamma.

1.3.2 THE PHARMACOLOGY OF RECOMBINANT HUMAN INTERFERON-γ

The pharmacology of recombinant interferon-γ has been reviewed by Kruskal and Ezekowitz (1994). Recombinant human interferon-γ is an *E.coli* derived protein which is available by parenteral route only. The drug can be given by the intravenous and intramuscular routes but the usual route is subcutaneous, which provides the most sustained release.

Pharmacokinetic data, derived from healthy volunteers, demonstrate rapid clearance after intravenous administration. Mean half-times for a dose of 100ug/m2 by the i.v, i.m and s.c routes are, respectively, 0.6, 2.9, and 5.9 hours. In a large placebo-controlled clinical trial of interferon-γ (50ug/m^2 or 1.5ug/kg for patients less than 0.5 m^2) for chronic granulomatous disease, the drug was generally well tolerated. Adverse reactions reported significantly more frequently from treated patients included fever, headaches, chills, and erythema at the site of injection. The magnitude of these reactions was usually mild and they were effectively treated symptomatically (i.e. paracetemol). The drug gave fewer reactions when given at bedtime, and patients less than 10 years of age had fewer adverse reactions than older patients. Only four of the 128 patients on the interferon trial had to discontinue treatment because of toxicity. Patients treated with much higher doses (e.g.>100ug/m^2) rarely had more severe reactions, e.g. cardiovascular (hypotension, syncope, tachycardia, heart block, congestive heart failure, and myocardial infarction), pulmonary (bronchospasm, interstitial pneumonia), CNS (confusion, seizures, parkisonism, transient ischaemic attacks), haemostatic (deep vein thrombosis, pulmonary embolism), metabolic (hyperglycaemia and hyponatraemia), GI (bleeding, hepatic insufficiency, pancreatitis), and reversible renal insufficiency.

1.3.3 INTERFERON ADJUVANT PROPERTIES

The adjuvant properties of interferon have been reviewed by Heath and Playfair, (1992) and Heath and Playfair, (1993). Interferon-γ is a product of activated T cells, serving to activate macrophages to produce its array of pro-inflammatory cytokines, while also upregulating the MHC Class II molecule expression on these antigen presenting cells. These properties

make interferon-γ an ideal candidate for use as a vaccine adjuvant. Heath and Playfair (1991) have studied the effects of enhanced MHC expression and cytokine effects on lymphocyte trafficking, to adjuvanticity. It was found that both interferon-γ and the adjuvant saponin had effects on lymphocyte traffic, causing homing of labelled lymphocytes to an injection site in the skin. There was, however, an inverse relationship between the enhancement of lymphocyte traffic and adjuvant effects in different strains of mice. Balb/c mice, which showed a very poor adjuvant effect, showed strong lymphocyte homing to an interferon-γ injection site and the homing lymphocytes were predominantly CD8+ T cells. The relationship between CD8+ homing and lack of adjuvanticity in these mice is still unclear. In contrast there was a positive relationship between enhancement of MHC Class II expression in vivo and adjuvanticity in different mouse strains. There is therefore evidence that interferon-γ acts as an adjuvant by enhancing antigen presentation by up regulation of MHC class II antigens on antigen presenting cells. This is supported by the fact that interferon-γ must be administered at the same site as the antigen to produce an adjuvant effect and that passively transferred peritoneal cells from mice injected previously with interferon-γ and antigen prime the recipient mice more effectively than cells from donors that have received antigen alone, or mixtures of cells from antigen-injected and interferon-γ injected mice. In terms of measurable immune responses in the animal, interferon-γ is known to enhance T-cell help for antibody, secondary antibody responses, delayed type hypersensitivity and antibody affinity.

Playfair and DeSouza (1987) first demonstrated the adjuvant effect of interferon-γ when it was used with a *Plasmodium yoelli* murine malaria vaccine. The administration of 5000 units of interferon-γ intraperitoneally at the same time and site on two occasions, two weeks apart, conferred

over 90% immunity to mice challenged with a lethal dose of parasitized red blood cells. Moreover, they showed that antibody, delayed type hypersensitivity (DTH), and T-helper cell responses were all increased with the use of recombinant interferon-γ as an adjuvant. The increased survival was a result of the adjuvant effect of the interferon-γ rather than a direct result of the interferon on the parasitized cells, since no survival benefit was seen with interferon alone.

The administration of recombinant interferon-γ at the time of primary immunisation with glycoprotein G of vesicular stomatitis virus (Anderson 1988) enhanced the secondary antibody response to a booster dose of the viral protein. In each of these studies, interferon-γ was most effective when administered at the same time as the vaccine antigen. To ensure interferon-γ delivery with the antigen, Heath and Playfair (1990) conjugated biotinylated interferon with avidin antigen and compared the DTH and antibody response to avidin in mice immunised with the conjugate or avidin and interferon-γ alone. They noted a 50% increase in the DTH response and no significant increase in the antibody response in animal immunised with the conjugate. An explanation proposed to explain the increased adjuvanticity of the conjugate is that it allows specific activation of those antigen presenting cells that phagocytose the antigen, thus resulting in a more focused increase in the immune response.

In studies particularly relevant to the immunocompromised host, Heath, *et al*, (1989), Demonstrated that interferon-γ could increase the response rate to a malaria vaccine in different strains of immunocompromised mice. They inoculated several strains of immunodeficient mice with two, biweekly doses of *P. yoelli* vaccine mixed with interferon-γ as an adjuvant. Biozzi low responder mice, which normally produce low levels of

antibody, were 100% protected against lethal challenge by the addition of interferon-γ to the vaccine; mice administered only the vaccine were totally unprotected. Mice depleted of CD4+ cells by the use of anti-CD4 monoclonal antibody, showed 50-66% increased survival when interferon-γ was added to the vaccine.

Interferon-gamma has also been shown to be effective in other systems. Schijns, (1994), has demonstrated increased immunogenicity of an inactivated rabies vaccine in mice by administration of exogenous interferon-gamma.

While there is now a fairly large body of data on the use of cytokines as adjuvants in animals, data from human studies is more scarce. Two human clinical trials have been conducted with IL-2 and interferon-γ, both in haemodialysis patients as subjects and hepatitis B as the vaccine, as infection with this virus is a problem in dialysis patients.

Quiroga *et al* (1990) used a large group of haemodialysis patients, of which 41 received vaccine alone, and 40 vaccine plus $2x10^6$ units of interferon-γ/m^2 (a higher dose than needed in mice, which was approximately $2x10^5$ units/m^2). Similar proportions of patients seroconverted in both groups (81% and 89%, respectively), but the interferon-γ group responded earlier, and mean titres were higher, although not significantly so. In a small trial with healthy male students, interferon-γ added to hepatitis B vaccine, given intradermally, accelerated the development of protective levels of antibody.

1.3.4 INTERFERON AND CHRONIC GRANULOMATOUS DISEASE

Chronic granulomatous disease (CGD) is an immunodeficiency characterised by recurrent pyogenic infections beginning early in life. Phagocytes from CGD patients fail to produce superoxide and other reactive oxygen intermediates important in bacterial killing. This results from a failure of a membrane associated NADPH oxidase which contains a unique, phagocyte-specific cytochrome b. CGD is known to be genetically heterogenous, with a common X-linked form, and a less common variant X-linked and autosomal recessive form. Classical X-linked CGD results from deletions or mutations in the gp91*phox* gene, which encodes the phagocyte-specific cytochrome b.

The initial rationale for the use of interferon-γ in CGD came from in-vitro experiments demonstrating that it could increase the magnitude of the respiratory burst, and increase expression of gp91*phox* in phagocytes from normal patients. A large controlled trial of interferon-γ in X-linked CGD revealed a clear clinical benefit in reducing the number of serious infections with minimal toxicity, (reviewed by Kruskal and Ezeckowitz, 1994).

1.3.5 INTERFERON AND INFECTIOUS DISEASES

During the past decade , much work has been carried out to establish the role of interferon-γ in macrophage activation which is essential in the fight against intracellular pathogens. It has also been shown that interferon-γ also has an effect on granulocytes, including migration and adherence, phagoctosis and oxidative killing. Although most of the effects of

interferon-γ on the inflammatory response are beneficial to the host, interferon-γ has also been shown to be a pivotal mediator of LPS-induced Schwartzman reaction and lethality (reviewed by Kullberg and van t'Wout, 1994).

As a consequence of the importance of interferon-γ in killing of intracellular parasites, attention has been focused on the treatment of such conditions as *Toxpoplasma gondii* and *Leishmaniasis*. These, and other similar disorders are becoming increasingly important with the emergence of the AIDS epidemic. Leishmania has served as a model to study the regulation of killing mechanisms of macrophages. The profile of cytokines released in mice infected with *L. major* determines whether protective immunity or exacerbation occurs. Leishmania resistant strains of mice produce interferon-γ, but not IL-4, whereas susceptible strains produced IL-4 and IL-10 but not interferon-γ mRNA. The knowledge of the immunopathology of leishmania infection now opens the door to alternative therapy, as reviewed by Ho *et al* (1994). The treatment of leishmaniasis has not changed for over 70 years, pentavalent antimony being the drug of choice. However, the response rate is only 70-80%, and the toxicity is significant. A number of studies with interferon-γ in patients with visceral leishmaniasis has now been performed, and even those patients who had not responded to pentavalent antimony the clinical outcome has been encouraging.

Interferons were originally described as selective antiviral agents. As discussed above, it is now well established that while interferon-α and β are primarily antiviral cytokines, interferon-γ is primarily an immunomodulator with some antiviral activity. As a consequence interferons α and β have been more widely investigated as direct antiviral

agents and have been used in the treatment of acute and chronic virus infections with some success. Less success has been observed with interferon-γ when used as a direct antiviral cytokine. Where interferon-γ may be important in the treatment of viral diseases is in terms of immune enhancement, i.e. in the redirection of the immune response such that more efficient viral clearance occurs. Administration of bovine interferon-γ prior to experimental infection of cattle with bovine herpes virus I (BHV-1) reduces virus induced suppression of cell mediated cytotoxicity (Bielfeldt Ohmann and Babiuk, 1984).

In summary, Interferon-γ may become a useful therapeutic agent in the treatment of intracellular parasitism, especially in instances where the host may be immunocompromised. While the effectiveness of interferon-γ as an antiviral agent may be limited it may be beneficial in terms of immune enhancement of the host to aid viral clearance

1.3.6 INTERFERON IN AUTOIMMUNE DISEASE

There is conflicting evidence of the role that interferon-gamma plays in autoimmune disease, both in its possible protective role and also in its role in immunopathegenesis. Evidence that supports the idea that interferon-gamma produces immunopathology in autoimmune disease comes from experiments in transgenic mice which express interferon gamma under the control of a rat insulin promoter (Sarvetnick, 1988). These mice develop severe insulinitis and become hypoglycaemic thereby developing a syndrome similar to insulin dependent diabetes mellitus, which is commonly thought to be an autoimmune disease.

In contrast interferon-gamma appears to play a protective role in some murine models of experimental autoimmune encephalomyelitis (EAE) (Billiau, 1988), where administration of interferon-gamma seemed to enhance survival. However, when recombinant interferon-gamma is given to patients suffering from multiple sclerosis (the natural human disease which resembles murine EAE), there appears to be an exacerbation of clinical signs (Panitch, 1987). Together, these results suggest that interferon-gamma may play different roles in the development of autoimmune disease and may act through a variety of different mechanisms. Further experimental work needs to be carried out to determine the molecular mechanisms responsible for the apparent contradictory roles of interferon-gamma in autoimmune disease.

1.3.7 INTERFERON-GAMMA IN NEOPLASTIC DISEASE

The abnormal population of cells constituting a malignancy demonstrates temporal unrestricted growth preference over its normal counterparts, inhibition of differentiation, and the tendency to invade tissues and metastasise. The development of certain tumours is related to mutations in oncogenes that regulate cell growth and differentiation and/or to mutations or loss of tumour suppressor genes.

Interferons may be important in the treatment of cancer for two reasons. Firstly, they modulate the immune response and could therefore potentially augment the anti tumour response, and secondly, they can affect cell growth and differentiation. In terms of tumour treatment, interferon-α has warranted the greatest interest. A large-scale assessment of interferon-α as treatment for spontaneous malignancies began in the United States of America in 1979. By far, the most significant impact has been seen in

human patients with Hairy Cell leukaemia. Mechanisms of action of interferon-a probably differ depending on the tumour type. In hairy cell leukaemia, interferon-α's action may involve effects on differentiation, apoptosis and cell-cycle arrest, but in such tumours as gastro-intestinal malignancies the action appears to involve the potentiation of chemotherapeutic drugs such as 5-florouracil (Zwierzina, 1993).

The use of interferon-γ in malignancies has been disappointing, But has been used in such tumours as renal cell carcinoma (Farace *et al,* 1993), malignant melanoma (Charak *et al,* 1992) and ovarian and cervical cancer (Colombo *et al,* 1992 and Iwasaka *et al*, 1990, respectively) with varying results. The rationale behind treatment using interferon-γ is to augment the immune response, i.e. to enhance effector cell function and possibly to upregulate MHC expression on tumour cells. More success in the treatment of malignant disease with interferon-γ may come with the advent of gene therapy for cancer.

1.3.8 INTERFERON-GAMMA IN ALLERGIC DISEASE

Although the original definition of allergy by Von Pirquet in 1906 encompasses a whole spectrum of immune responses, the term allergy is now equivalent to type I hypersensitivity (reviewed by Banchereau and Miossec, 1994). The term atopy is currently used to describe the abnormal reaction and diseases which are associated with the production of IgE following low exposure to allergens. These IgE molecules can bind via their Fc portions to high affinity Fc receptors on basophils and mast cells. Exposure to allergen allows cross linking of Fc receptors on these cells and the production of inflammatory mediators which are responsible for the clinical signs associated with allergy. IL-4 is known to cause isotype

switching in B-cells to produce IgE, and IL-5 is a potent activator of eosinophils. Both IL-4 and IL-5 are cytokines produced by TH_2 cells, and, in addition, T cells isolated from inflammatory infiltrates from allergy patients have been found to be predominantly of the TH_2 type. Thus, imbalances in the TH_1/TH_2 system, biased toward the TH_2 type, may be involved in the manifestation of allergy.

An important feature of the TH_1/TH_2 system is the ability of one subset to regulate the other, i.e. th1 cytokines (IL-2, IFN-γ) downregulate the TH_2 response and TH_2 cytokines (IL-4, IL-5, IL-10) downregulate the TH_1 response. Thus, the TH_2 response may be reversed by boosting the TH_1 response and interferons, therefore, represent good candidates for such therapeutic application.

1.4 AIMS OF THE PROJECT

As described above, numerous clinical trials are in progress to assess the use of recombinant interferons in disease treatment and prevention. The aim of this project was to clone, sequence and express cDNA encoding feline interferon-gamma with the ultimate goal of examining the potential of this cytokine in diseases of felidae. This project forms part of a larger cytokine project being developed in the department of Veterinary Pathology at Glasgow University. The aims of this larger project are to clone and express a panel of feline cytokine genes in order to develop new strategies in disease control, prevention and in developing our understanding of the pathogenesis of various diseases.

The detailed aims of this project were as follows:

1) To amplify, using the polymerase chain reaction, cDNA encoding feline interferon-gamma from mRNA derived from mitogen stimulated, feline lymphocyte cultures.

2) To sub-clone the PCR product into plasmid vector and to sequence the recombinant insert.

3) To express the feline interferon-gamma cDNA as a glycosylated protein in a Baculovirus system.

4) To establish a biological assay for feline interferon-gamma based upon cytopathic effect reduction and MHC class II antigen induction.

5) To purify the resultant protein by FPLC and to assay for biological activity.

The proceeding chapters will describe the experimental approach to achieve these objectives and the results obtained.

CHAPTER 2

MATERIALS AND METHODS

2.0 MATERIALS

2.0.1 Chemicals
2.0.2 Radiochemicals
2.0.3 Complete Kits
2.0.4 Equipment
2.0.5 Experimental animals
2.0.6 Bacterial Strains
2.0.7 Cloning Vectors
2.0.8 Restriction Endonucleases
2.0.9 Other enzymes
2.0.10 Oligonucleotide Primers
2.0.11 Buffers, Solutions and Growth media
2.0.12 Bacterial Growth Media

2.1.0 GROWTH AND MANIPULATION OF MAMMALIAN CELLS

2.1.1 FEA Cell Line
2.1.2 F422 Cell Line
2.1.3 Primary T cell Cultures

2.2.0 GROWTH AND MANIPULATION OF INSECT CELLS

2.3.0 GROWTH AND MANIPULATION OF VIRUSES

2.3.1 Wild-type Baculovirus Strain

2.3.2 Titration of Baculovirus by Plaque Assay

2.3.3 Feline Calicivirus

2.3.5 Titration of Calici Virus by Plaque Assay

2.3.6 Vesicular Stomatitis Virus

2.3.7 Titration of VSV by Plaque Assay

2.4.0 GROWTH AND MANIPULATION OF BACTERIA

2.4.1 Transformation of Competent Bacteria

2.4.2 Screening and Selection

2.4.3 Isolation of Plasmid DNA.

2.4.4 Restriction Enzyme Digestion of Plasmid DNA.

2.5.0 MANIPULATION OF NUCLEIC ACIDS

2.5.1 mRNA Harvest and Purification

2.5.2 First Strand cDNA Synthesis

2.5.3 PCR Amplification

2.5.4 Agarose Gel Electrophoresis

2.5.5 Polyacrylamide Gel Electrophoresis

2.5.6 DNA Ligation

2.5.7 Extraction of DNA from Agarose Gels

2.5.8 Sequencing of DNA

2.6.0 PROTEIN ANALYSIS

2.6.1 SDS-Polyacrylamide Gel Electrophoresis

2.6.2 Western Blot Analysis of Protein Gels

2.0.1 MATERIALS

The following section lists only those materials used routinely. Less frequently used materials are detailed in the appropriate methods section.

2.0.1 CHEMICALS

All chemicals were of ultrapure or equivalent purity and were obtained from Gibco BRL, Pharmacia LKB, or The Sigma Chemical company, except:

Ampicillin (Penbritin™) Beecham Ltd.

Ethanol James Burroughs Ltd.

2.0.2 RADIOCHEMICALS

[Alpha ^{32}P] dCTP for labelling of DNA probes and [^{35}S] dATP alpha S for DNA sequencing were obtained from Amersham International plc.

2.0.3. COMPLETE KITS

Quick prep™ mRNA purification kit: Pharmacia

First strand cDNA synthesis kit : Pharmacia

TA cloning kit : Invitrogen

PCR kit : Cetus

Sequenase version 2.0 sequencing kit : Pharmacia

2.0.4. EQUIPMENT

Tissue culture flasks and other plasticware: Nucleon (Delta)

Collodian dialysis tubes : Sartorius

Autoradiography film: Amersham life science

Sequencing Apparatus: Pharmacia

2.0.5. EXPERIMENTAL ANIMALS

SPF cats: Specific pathogen free cats were obtained from a breeding unit and housed at Glasgow University. They were aged approximately 18 months at the time of lymphnode harvest. The study was carried out in accordance with home office regulations.

Rabbits: 4 SPF rabbits were used to raise antisera to synthetic peptides. The animals were maintained on a commercial diet in the University of Glasgow animal house and all procedures were carried out in accordance with Home Office Regulations.

2.0.6 BACTERIAL STRAINS

Escherichia coli (E.coli) DH5α™strain (Gibco BRL). This is a recombination negative strain which is provided competent. The strain can be used for blue-white screening as the ϕ80Δ*lac*ZΔM15 marker provides α-complementation of the β-galactosidase gene within vectors. The DH5α strain was used to amplify pCRscript SK(+) plasmids containing PCR products.

E.coli strain INVαF' (Invitrogen) is a recombination negative strain (*rec*A1) which enables stable replication of high copy number plasmids. The genotypes *end*A1 and *hsd*R17 improve the quality of minipreps. INVαF' confers blue-white screening of recombinant plasmids by alpha

complementation of β–galactosidase with the ϕ80Δ*lac*ZΔM15 genotype. This bacterial strain was used to amplify the Baculovirus transfer vector during the expression work. These bacterial strains are provided in the TA cloning vector kit (Invitrogen) and are competent.

2.0.7 CLONING VECTORS

pCR-script™SK(+) phagemid (Stratagene) is a 2961-bp phagemid designed by incorporating a *Srf* site into the pBluescript SK(+) phagemid. This cloning vector includes an ampicillin-resistance gene, a *lac* promotor for gene expression, T3 and T7 RNA polymerase binding sites, an f1 intergenic region for single stranded DNA rescue and the SK multiple cloning site, which is modified to include the *Srfl* restriction-endonuclease target sequence. The SK designation indicates the polylinker is oriented such that the *lac*-Z transcription proceeds from *Sac*1 to *Kpn*1.

pAcCL29-1 Baculovirus Transfer vector: The transfer vector used in the experiments described in this thesis was a polyhedrin promotor based expression vector termed pAcCL29-1, Livingstone and Jones (1989). The pAcCL29 series of transfer vectors were originally derived from the transfer vector pAcYM1. The major difference is that each vector has the M13 intergenic region necessary for single stranded DNA production, after superinfection of transformed bacteria with the M13 helper phage (e.g. M13 K07). This is particularly advantageous if modifications need to be made to the inserted foreign gene to study structure and function. The pAcCL29 vectors are also slightly smaller than pAcYM1 and should, therefore, be able to accept more foreign DNA before becoming

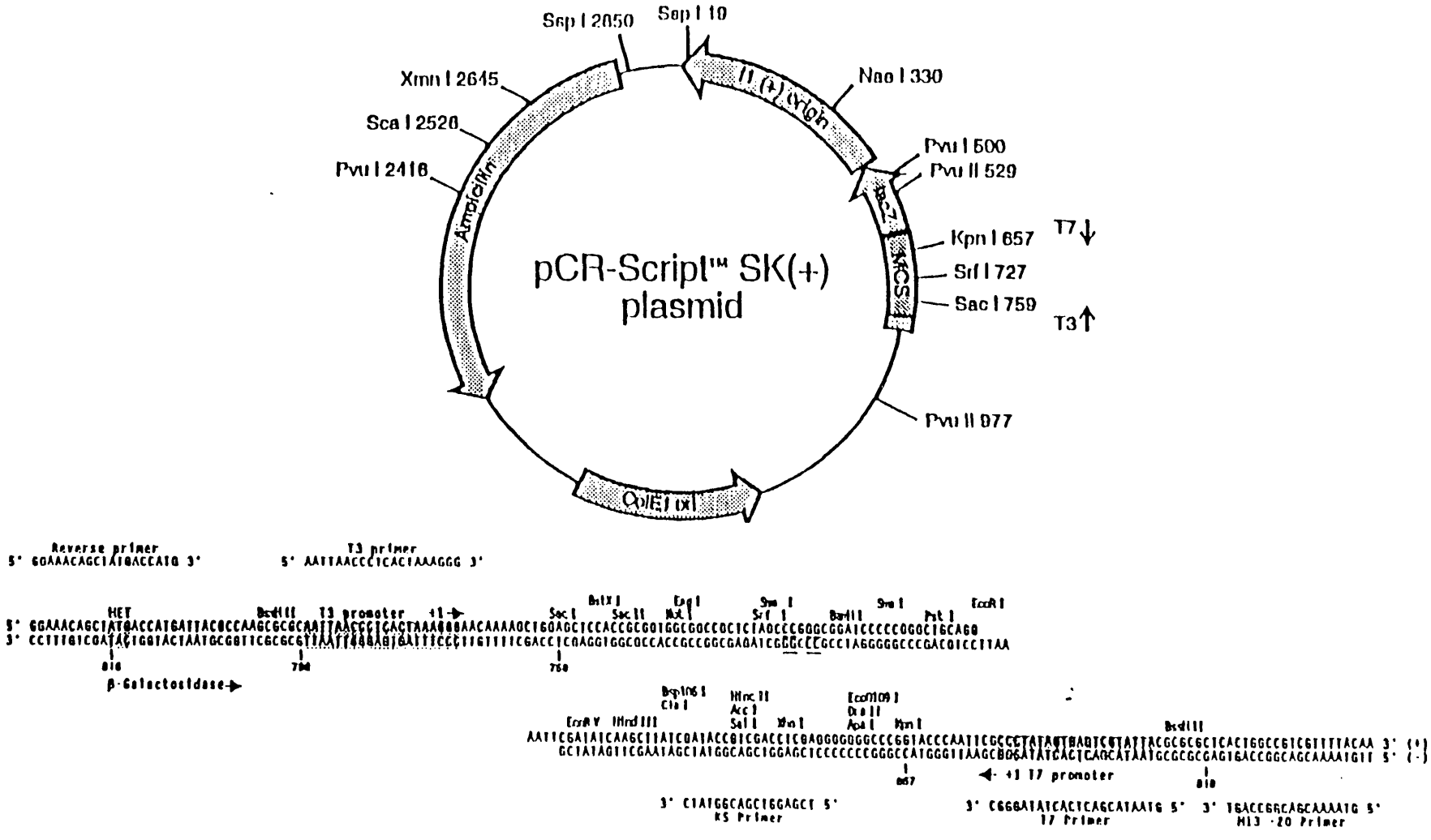
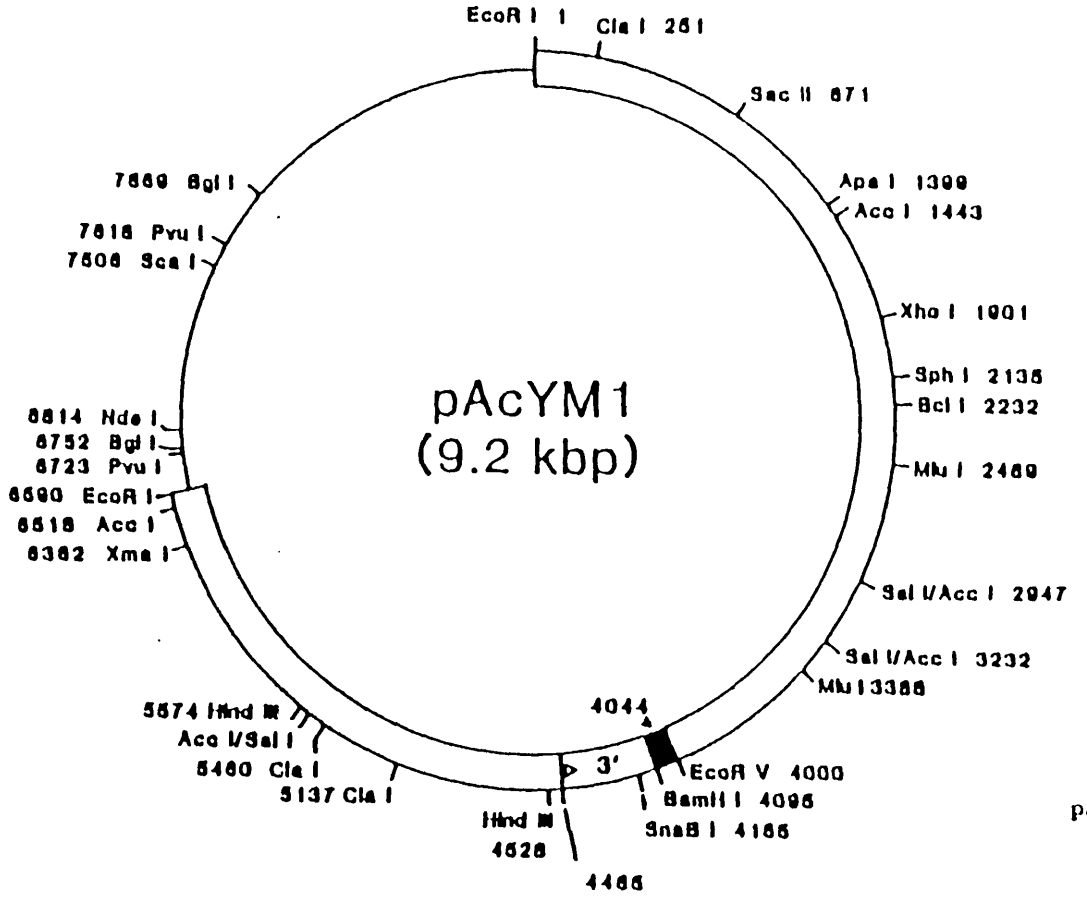
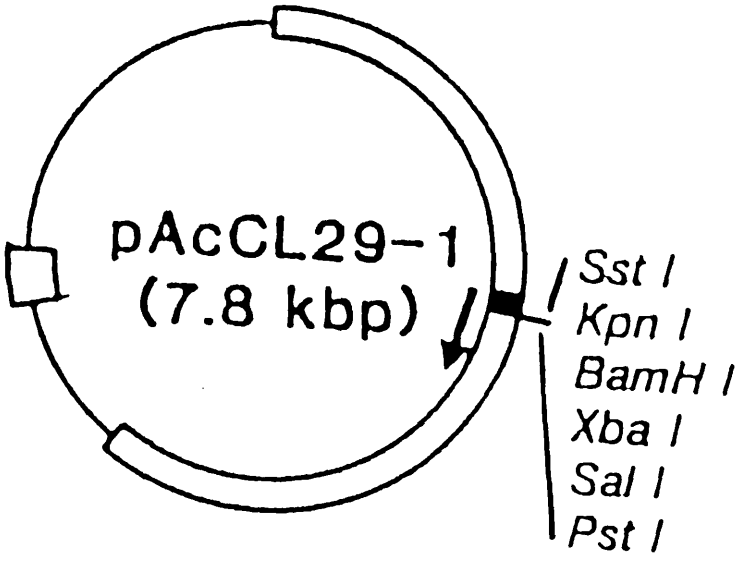


Figure 2.1 The pCR-Script SK(+) plasmid. The polylinker sequence is depicted beneath the circular map.






```
                                        +1                  Kpn I           Xba I
pAcCL29-1 AAAAAAACCTATAAATA CGGATCAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCG
                                                 Sst I    Sma I BamH I     Sal I

          CTGCAGGCATGCAAGCTGATCCGGTTATTAGTACATTTATTAAGCGCTAGATTCTGTGCC
          Pst I
```

Figure 2.2 The baculovirus transfer vector pAcCL29-1 is derived from pAcYM1. The sequence spanning the polylinker site is shown below the circular map of pAcCL29-1.

unstable. pAcCL29 has a single BamH1 cloning site whereas pAcCL29-1 has a multi-cloning site consisting *Sst* 1, *Kpn* 1, *Bam*H 1, *Xba* 1, *Sal* 1, *Pst* 1. The *Sal* 1 site is not unique (King and Possee 1992).

2.0.8 RESTRICTION ENDONUCLEASES

All were from Gibco BRL or from Northumbria Biologicals Ltd. All were supplied and used with appropriate reaction buffers.

2.0.9 OTHER ENZYMES

MuLV Reverse Transcriptase-Pharmacia (provided in cDNA synthesis kit)
Taq polymerase- Cetus (provided in their PCR kit)
Sequenase sequencing enzyme- Amersham (in the sequenase V.2.0 kit)
T4 DNA ligase- Stratagene (in their ligation kit)

2.0.10 OLIGONUCLEOTIDE PRIMERS

Primers for both PCR amplification and DNA sequencing were supplied by Alto Biosciences, University of Birmingham. They were supplied purified and lyophilised. After reconstitution with ultrapure, autoclaved water the concentration was determined by spectrophotometry.

2.0.11 BUFFERS, SOLUTIONS & GROWTH MEDIA

Where indicated, autoclaving was carried out at 121^{o}C for 20 minutes.

Antibiotics:

Ampicillin: sodium ampicillin at 100mg/ml in distilled water sterilised by filtration and stored in 1ml aliquots at -20°C. Used at a final concentration of 50ug/ml.

Penicillin/Streptomycin: purchased as a tissue culture grade solution from Gibco-BRL. Supplied at 1000IU/ml and stored at -20°C.

Buffers and solutions:

BHK21 medium: purchased as a 10x sterile liquid from Gibco BRL.

Concanavalin-A: (Sigma) stored dry at 4°c.

DEPC/DEPC Treated water: Diethyl pyrocarbonate (Sigma), DEPC water was prepared by the addition of two drops of DEPC to 500ml of water. The water was allowed to stand for 3 hours and then autoclaved. All procedures involving DEPC were carried out in a safety cabinet.

DMSO: dimethylsulphoxide (Fisons).

DNA Gel Loading Buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water. Stored at -20°C.

Dulbecco's MEM: purchased as a 1x sterile liquid from Gibco BRL.

EDTA (O.5M, pH 8.0): 186.1g. disodium ethylene diamine teraacetate.$2H_20$ added to 800ml. of distilled water. NaOH pellets (approximately 20g.) added to make the pH 8.0 (EDTA will then dissolve). Volume adjusted to 1 litre with distilled water and sterilised by autoclaving.

Ethidium bromide : ethidium bromide made to a working solution of 3mg/ml. with distilled water in a fume cupboard. Stored away from light.

Ficol-Hypaque: Purchased as a 100ml solution from Pharmacia Biotech. and stored at 4°C.

Foetal calf serum :Virus screened, mycoplasma screened, bacteriophage screened. Purchased from Gibco BRL and heat inactivated at 57°C for 30 minutes before storage at -20°C.

L-Glutamine: purchased as a 200mM (100X) aqueous solution from Gibco BRL.

Lysis solution: 25mM tris-HCL pH 8.0, 10mM EDTA, 50mM glucose.

Lysozyme: purchased as a sterile powder from Sigma chemicals and used at a concentration of 10mg/ml in STET solution.

Phosphate buffered saline: 137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4.7H_20$, 1.4mM KH_2PO_4 (pH 7.3).

Protein gel loading buffer: made as a 2 x stock. 100mM Tris-HCL (pH 6.8), 4% SDS, 20% glycerol, 0.1% Bromophenol Blue and 10% 2-β-mercaptoethanol.

RPMI 1640: purchased as a 1x sterile liquid from Gibco BRL

SDS (10%): 100g. sodium dodecyl sulphate made up to 1 litre with distilled water and sterilised by filtration.

SDS-PAGE running buffer: 25mM Tris base, 250mM glycine, 0.1%SDS pH 8.3.

Semi-dry transfer buffer: 48mM Tris, 39mM glycine, 0.1% SDS and 20% 2-β mercaptoethanol.

Sodium pyruvate: purchased as a 100mM solution from Gibco BRL and stored at 4°C.

STET Solution: 5% Triton X-100, 50mM EDTA, 50mM tris-HCL, 8% sucrose.

TAE (50X): 242g. Tris base, 57.1ml. glacial acetic acid, 100ml. 0.5M EDTA (pH 8.0) made up to 1 litre with distilled water. Used at a final concentration of 1x (1xTAE pH 8.15 is 40mM tris-acetate, 2mM EDTA)

TBE (10X/L): 89mM Tris base, 89mM boric acid, 2mM EDTA (pH 8.0).

TBS: 25mM Tris base, 137mM NaCl, pH to 7.6 with HCL.

TC100 Insect media: purchased from Gibco BRL as a sterile solution.

TE: 10mM Tris HCL, 1mM EDTA, pH adjusted as appropriate (made up as a 100x solution and sterilised by autoclaving).

Trypsin/EDTA: purchased as a 10x solution from Gibco-BRL and stored at -20°C.

X-gal: 5-bromo-4-chloro-3indolyl-β-D-galactosidase (Gibco BRL), stored dry at -20°C. and made to the required dilution using N,N-Dimethylformamide (Sigma)

2.0.12 BACTERIAL GROWTH MEDIA

L-agar: L broth containing 1.5% agar sterilised by autoclaving

LB medium (L-broth): 10g tryptone, 5g yeast extract and 10g NaCl made up to 1 litre with distilled water, and the pH adjusted to 7.5 with NaOH, followed by sterilisation by autoclaving.

SOC medium: L-broth, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose.

2.1.0 GROWTH AND MANIPULATION OF MAMMALIAN CELLS

2.1.1 FEA CELLS

A feline embryonic cell line was used in the assay procedure for feline recombinant interferon. This is a cell line designated FEA and derived from feline embryonic cells.

Maintenance: The cells were maintained in platicware at 37°C in 95% air, 5% CO2. The cells were grown as monolayer adherent cultures in 0.2ml/cm^2 surface area until they reached sub-confluence. Cells were then washed with trypsin/EDTA before replating in fresh medium at the correct density. The cells were split 1:3 approximately twice weekly.

Long term storage: Master and working cell banks were prepared of the FEA cells. Cells were trypsinised and washed in growth medium. After centrifugation, cells were resuspended at 5x10^6/ml in Dulbecco's MEM (supplemented with 20% FCS, 1% Glutamine, 1% Sodium pyruvate, penicillin/streptomycin and 10% w/v dimethyl sulphoxide (DMSO)). The cells were frozen in a cell freezer and stored under liquid nitrogen. After long term storage, frozen cells were rapidly thawed by immersion of their plastic container in sterile water at 37°C. Following thawing, the cells were washed in fresh medium. After centrifugation the cells were resuspended in growth medium and plated in plasticware at the appropriate density.

2.1.2 F422 CELL LINE

The F422 cells are an FeLV positive cell line derived from a naturally occurring lymphosarcoma (Rickard 1969). The cells were cultured in RPMI medium supplemented with 10% FCS and penicillin/streptomycin.

2.1.3 PRIMARY T CELL CULTURES.

A specific pathogen free (SPF) domestic cat was euthanased using intravenous pentabarbitone (euthatal). Using standard aseptic techniques the spleen and several mesenteric lymph nodes were removed and placed

in modified RPMI medium (RPMI 1640, penicillin/streptomycin, 10mM Hepes buffer, $5x10^{-5}$M β-mercaptoethanol, 2% FCS, and 2mM glutamine) and placed on ice. The spleen and lymph nodes were diced in medium on a sterile 9cm plate using a sterile scalpel. The medium containing cells was transferred to a separate sterile receptacle. The dead cells and contaminating red blood cells were removed by layering 15ml of cells on top of 15ml of ficoll hypaque (Pharmacia) and centrifuging at 3000 rpm for 15 minutes. The interface cells were removed and diluted with an equal volume of fresh medium. The cells were pelleted for 5 minutes at 1500 rpm, counted and used to make replicate 20ml suspension cultures at a concentration of $2.5x10^6$ cells/ml. The cultures contained 10% spleen derived lymphocytes and 90% lymph node derived lymphocytes. The cells were stimulated by the addition of the mitogen concanavalin-A at a concentration of 7.5ug/ml of culture medium and flushed with CO_2 and incubated at 37°C.

2.2.0 GROWTH AND MANIPULATION OF INSECT CELLS

Spodoptera frugiperda (Sf9) cells (Invitrogen) were cloned from the parent line IPLB-SF 21 AE. The parent line was derived from pupal ovarian tissue of the fall armyworm (*Spodoptera frugiperda*). These cells are the host for the propagation of wild-type or recombinant *Autographica californica* multiple nuclear polyhedrosis virus (AcMNPV). Sf9 cells can be propagated as both adherent monolayers and as non-adherent suspension cultures.

Maintenance: Cultures were maintained in plasticware at 28°C in TC100 medium (Gibco) supplemented with 10% FCS and penicillin/streptomycin. There was no requirement to flush the cells with CO_2 or maintain cells in a

CO_2 incubator. For the most part, cells were grown as adherent monolayer cultures until sub-confluence. The cells were then removed from the flask my mechanical disruption, counted by trypan blue exclusion (see below) and plated in fresh medium at the appropriate density.

For protein production the cells were grown as suspensions cultures in spinner culture flasks in TC100 complete medium. The cells were initially added to the spinner flasks at a concentration of 10^5 cells /ml of medium. The cells were allowed to go through two doublings (to about $5x10^5$ cells/ml) before the addition of virus to the cells. It was essential that the cells were in a logarithmic growth phase when virus was added. The spinner flasks were not overfilled (max. 100ml in a 250ml flask) so that an adequate surface area for gaseous exchange was maintained. For maximum protein production the virus was added at 10 pfu/cell.

Trypan blue exclusion: Cells were removed from the flasks by mechanical disruption and resuspension in 20ml of fresh growth medium. One hundred microlitres of this suspension was added to an equal volume of trypan blue (Gibco BRL) in an eppendorf tube. The cells were then counted using a haemocytometer, the dead cells appearing blue.

Long term storage: Both master and working banks of cells were established and stored under liquid nitrogen. For long term storage, cells were washed in growth medium and, after centrifugation, resuspended at a concentration of $5x10^6$ cells/ml in TC100 medium (supplemented with 10%FCS, penicillin/streptomycin and 10% w/v DMSO). The cells were then stored for 24 hours at -70°C and transferred to liquid nitrogen. To revive cells, a vial was removed from the liquid nitrogen and thawed by

rubbing the vial in the palm of the hand. The cells were washed, centrifuged and plated in plasticware at the appropriate density.

High Five™ CELLS (Invitrogen): this cell line was derived from *Trichoplusia ni* egg cell homogenates. Culturing was performed as for the Sf9 cells.

2.3.0 GROWTH AND MANIPULATION OF VIRUSES

2.3.1 WILD-TYPE BACULOVIRUS STRAIN

The baculovirus strain used throughout the project was generously provided by Dr. Jones at the NERC institute of Virology. The virus has been designated BacPAK6. BacPAK6 was generated by co-transfection of linearized AcRP6-SC with a plasmid designated PAK6. PAK6 was generated to have two *Bsu*36I sites which flank a β-galactosidase gene (Kitts and Possee 1993), thus recombinant BacPAK6 viral plaques stain blue with X-gal. The orf1629 sequence required to rescue restricted BacPAK6 viral DNA are present in most transfer vectors.

2.3.2 TITRATION OF BACULOVIRUS BY PLAQUE ASSAY

The method for titration of viruses by plaque assay was taken from King and Possee (1992). Plaque assays are carried out to determine virus titres in terms of plaque forming units per ml (pfu/ml) so that standard amounts of virus can be used to infect cells during experimental work. Plaque assays are also used to separate and titrate recombinant and wild type virus after co-transfections. The BacPAK6 virus is *lac*-z positive and therefore plaques generated by this virus will stain blue with X-gal.

Initially 35mm plates were seeded with 10^6 Sf9 cells per plate in 2ml of medium. The plates were then left at 28°C for 3 hours to allow the cells to settle and form a monolayer. Before commencing the assays, the cells were viewed microscopically to ensure that the cells had settled down and had formed a fairly sparse, even monolayer (50% confluence). If the cells become confluent too soon then no plaques or very tiny plaques will form.

Working in a safety hood, serial log dilutions of the virus to be titred were prepared, using growth medium as the diluent. The dilutions made depended on the source of the virus to be titred. Wild-type virus and recombinant virus replicate to a titre of about 1-2 x 10^8 pfu/ml and so the dilutions made for these ranged from 10^{-5} to 10^{-8}. Co-transfection supernatants contain virus between 10^2 and 10^4 pfu/ml and so dilutions ranging from undiluted to 10^{-4} were made. The aim was to obtain around 10 to 30 plaques per plate that could be easily counted. In addition dilutions were always duplicated.

Once the cells had been allowed to settle, the medium was removed using a pasteur pipette. The cells were then overlaid with 100ul of the appropriate dilution of virus, by adding dropwise to the centre of the dish. The dishes were then incubated at room temperature for one hour for the virus to adsorb to the cells.

During the 1 hour incubation period the agarose overlay was prepared. The agarose overlay consisted of 3% low gelling temperature agarose, which was autoclaved and maintained at 45°C in a water bath.

After 1 hour, the inoculum was removed from the cells. Equal volumes of agar and TC100 medium were mixed. 2ml of this was added to the sides of the 35mm dishes and allowed to set. All the inoculum was removed, as failure to do so may cause poor plaque formation or the agarose plug may fall out of the dish.

When the agarose overlay had set, this was then overlaid with 2ml of TC100/10% FCS. It was essential to add a liquid overlay to allow the development of well defined plaques. The reason for this is that the liquid overlay provides additional nutrients to supplement the half strength TC100 medium in the solid overlay (concentrated TC100 medium can not be used in the overlay as the salts precipitate from solution.)

The cells were incubated at 28°C for 3-4 days. Plaques were visualised by staining with neutral red. 0.5 ml of 0.1% neutral red in PBS was added to each plate. The neutral red was prepared fresh. After preparing the solution it was heated in the microwave for 10 seconds and then filtered through a 0.45u filter. After the solution had cooled, X-gal was added to a concentration of 0.8mg/ml. The cells were left to stain for around 3 hours at 28°C. Following this, the dye was removed and the plates inverted. The plates were left overnight at 4°C to allow clearing and better visualisation of plaques. Live cells take up the neutral red stain so that plaques appear as blue plaques (wild-type) or clear plaques (recombinant).

To determine the titre of virus, the plates were examined to find the dilution which gives a countable number of plaques (10 to 30). The titre was calculated as follows:

no. plaques x dilution factor x 10 (only 0.1 ml added) PFU/ml.

2.3.4 FELINE CALICIVIRUS

In the experiments described in this thesis a large plaque forming strain of calicivirus was used, designated FCV/Gl/PR.

2.3.5 TITRATION OF CALICIVIRUS BY PLAQUE ASSAY

Titration of calicivirus was performed in a 24 well plate system. FEA cells were grown to sub-confluency in tissue culture flasks at 37°C/5% CO_2. The cells were subsequently trypsinised, counted and resuspended in Dulbecco's complete MEM at a concentration of 2 x 10^5 cells/ml. One ml of the suspension was added to each well. The cells were allowed to incubate at 37°C/5%CO_2 for 24 hours before being examined microscopically to ensure a sub-confluent monolayer had been established. The medium was removed from the cells and wells were allocated cell and virus controls. Serial log dilutions of the calicivirus were made, and 100ul of each dilution was added drop wise to the centre of the cell monolayer in the appropriate well. The cells were allowed to incubate for 1.5 hours at 37°C/5%CO_2. Following incubation, the virus inoculum was removed using a plastic pipette and the cells were overlaid with agar/medium mix. The agar medium mix consisted of equal volumes of 2% sterile agarose and complete BHK21 medium (i.e. supplemented with penicillin/streptomycin, glutamine, and 10% FCS). Once the agar had set, the cells were incubated for 48 hours at 37°C/5% CO_2. Plaques were visualised after staining the monolayer with crystal violet/formalin. The titration of the virus was calculated by examination of the first well which

gave a countable number of plaques. The titration was calculated as follows, and was expressed as plaque forming units/ml (pfu/ml.).

Titre = no. plaques x dilution factor x 10 (only 100ul added to cells)

2.3.6 VESICULAR STOMATITIS VIRUS

Vesicular stomatitis virus is a group 2 pathogen as defined in the *Categorisation of Pathogens According to Hazard and Categories of Containment* 1990. In addition the organism is covered by the Specified Animals Pathogen Order 1993 which prohibits the holding or use of the organism unless a licence has been issued by the Scottish Office's Agriculture and Fisheries Department (SOAFD).

A licence was granted to use a mutant VSV and all assay procedures were carried out in a category II laboratory. The VSV mutant used in these assays was designated VSV*ts*E2 and is derived from the New Jersey strain of VSV. This mutant strain contains two independent mutations within the NS gene (Rae and Elliott, 1986) and is incapable of producing diseases in domestic livestock. In addition, because it is temperature sensitive, it is unable to sustain a transmission cycle between animals. The primary virus stock was kindly provided by Dr. Richard Elliott of the MRC Institute of Virology.

2.3.7 TITRATION OF VSV BY PLAQUE ASSAY

The titration procedure for VSV was identical to that used for calicivirus. However, the assay was performed at 31°C/5% CO_2 because the VSV used was a temperature sensitive mutant.

2.4.0 GROWTH AND MANIPULATION OF BACTERIA

2.4.1 TRANSFORMATION OF COMPETENT BACTERIA.

For transformation of the DH5α cells (Gibco BRL), 1-4ul of a ligation reaction was added to 0.1ml of competent cells and the mixture was held on ice for 30 minutes. The cells were then "heat-shocked" at 42°C for 45 seconds. The cells were placed on ice for two minutes. Following this, 80ul of SOC medium was added to the cells and the cells incubated at 37°C on an orbital shaker for 1 hour to allow for expression of plasmid-encoded antibiotic resistance. Following the incubation step the cells were plated out on appropriate selective culture plates at a volume of 100ul per plate.

For the transformation of the INVαF' cells (Invitrogen), much the same protocol was followed. However, the products of the ligation reaction were added to 50ul of cells, and, in addition, 2ul of 0.5M β-mercaptoethanol was added to improve transformation efficiency. After incubation and subsequent heat-shocking (described above), 450ul of SOC medium was added to the vials prior to incubation at 37°C on an orbital shaker.

2.4.2 SCREENING AND SELECTION

Transformed cells expressing antibiotic resistance were selected from non-transformed cells by growth on appropriate selective (antibiotic

containing) L agar plates. When a host/vector combination allowing blue/white selection of recombinant from non-recombinant plasmids on the basis of intact β–galactosidase activity was used, then X-gal (5-bromo, 4-chloro, 3-indolyl β-D galactoside) was incorporated into the L agar. The X-gal is a substrate of β-galactosidase which produces a blue colour on hydrolysis. Cells transformed by non-recombinant plasmids have intact β-galactosidase function and grow as blue colonies. In cells transformed by recombinant plasmids, the *lac*Z gene fragment in the plasmid is disrupted by DNA insertion and the β-galactosidase activity is absent. These cells grow as white colonies and can be selected on this basis. Colonies were picked and each colony placed into universals containing 10ml of L broth (+100ug/ml ampicillin). These colonies were grown overnight in L-broth by vigorous shaking at 37°C.

2.4.3 ISOLATION OF PLASMID DNA

Recombinant plasmid DNA was isolated according to the alkaline lysis method introduced by Birnboim & Doly, (1979) involving the denaturation of *E.coli* DNA and selective renaturation of covalently closed circle DNA.

i) Small scale isolation

A single colony was inoculated into 10ml of L broth and incubated at 37°C o/n. 1.5 ml of the o/n culture was pelleted, then resuspended in 300ul of STET (5% Triton X-100, 50mM EDTA, 50mM Tris-HCl, 8% sucrose) and 50ul of fresh lysozyme solution added (10mg/ml in STET). The solution was boiled for 45 seconds, then the lysate cleared in a micro centrifuge (13,000xg). The pellet was removed and the DNA precipitated from the solution with 0.5 volumes 7.5M ammonium acetate and 2-3

volumes ethanol in dry ice for 30 minutes. The precipitate was pelleted in a micro centrifuge, washed in 70% ethanol, dried and resuspended in 20ul dH_2O.

ii) Large scale purification.

High quality plasmid DNA was purified from a 500ml exponentially growing o/n culture. Cells were pelleted at 4000 rpm at 4°C for 10 minutes and resuspended in 20mls of lysis solution (25mM Tris-HCl (pH 8.0), 10mM EDTA, 50mM glucose). After 30mins on ice, 40mls of fresh 0.2M NaOH/1% SDS was added and the mixture was left on ice for a further 5 minutes. The lysates were neutralised with 30ml of potassium acetate (3M KoAc) solution for 15 minutes on ice. The lysates were clarified by centrifugation at 4000 rpm for 5 minutes at 4°C and the supernatant filtered through gauze swabs into a conical flask. DNA was precipitated by the addition of 0.6 volumes of isopropanol (54mls/500ml prep) for 30 minutes at room temperature and pelleted by centrifugation for 10 minutes at 4000 rpm and 4°C. The pellet was drained thoroughly and dried and then re suspended in 4ml of TE (10mM Tris, 1mM EDTA pH 8.0). Five g of caesium chloride were placed in a universal followed by the TE buffer containing the plasmid and 300ul of ethidium bromide. The refractive index was adjusted to 1.390 with CsCl or TE and the DNA separated on a density gradient by centrifugation at 55,000 rpm for 40 hours at 20°C. Closed circular DNA (the lower band) was removed by syringe and needle and the ethidium bromide removed by repeated extraction with isopropanol until no trace of colour was left. The CsCl was removed by extensive dialysis in collodion bags against TE pH 8.0 and the yield of DNA calculated on the basis that 50ug/ml solution of double stranded DNA would have an OD_{260} measurement of 1. The DNA was concentrated by extraction of the aqueous phase with butan-2-ol the

precipitated with 0.1 volumes of 3M sodium acetate, and 2-3 volumes of ethanol.

iii) Small scale preparation of sequencing grade plasmid.

1.5 ml of an o/n culture were decanted into an eppendorf tube and spun in a micro centrifuge at 14k for 2 minutes. The media was poured off and any residual media removed with a Gilson pipette. The pellet was re suspended in lysis solution and 300ul of fresh 1%SDS/0.2M NaOH added. The solution was thoroughly mixed by inverting several times and was placed on ice. After 5 minutes, 225ul of 3M KoAc was added to the tube, mixed and then placed on ice. After a further 5 minutes the solution was clarified by centrifugation at 14k for 5 minutes. 630ul of the supernatant was added to a fresh eppendorf tube and to this was added 630ul of 100% ethanol. The tube was shaken vigorously and the immediately centrifuged at 14k for 5 minutes. The ethanol was decanted and 1.5ml of 70% ethanol was added to the tube. The tube was vortexed and centrifuged at 14k for 2 minutes. The ethanol was removed and the remaining pellet dried in a vacuum dessicator for 1 minute, followed by resuspension in 16ul of distilled water.

2.4.4 RESTRICTION ENZYME DIGESTION OF PLASMID DNA.

Restriction enzyme digestion of plasmid DNA was carried out to confirm the presence of inserts of the correct size within small scale plasmid preps or the correct insertion of a cDNA into an expression vector. Typically, 1-2ug of plasmid DNA was digested in a reaction volume of 15ul. Restriction enzymes were used at 5-10 units per 15ul reaction in buffer provided by the manufacturer. Samples were digested in the presence of 100mM spermidine, which is reported to prevent endonuclease inhibition

by polyanions (Bouch, 1981). When samples were to be subjected to electrophoresis, the reaction was terminated by the addition of 0.2 volumes of DNA gel loading buffer.

2.5.0 MANIPULATION OF NUCLEIC ACIDS

2.5.1 mRNA HARVEST AND PURIFICATION.

mRNA harvesting and purification was carried out using the Quick Prep™ mRNA purification kit (Pharmacia). This kit employs the disruptive properties of guanidinium thiocyanate to lyse the T cells and allows the direct isolation of polyadenylated RNA by the use of oligo(dt) cellulose chromatography. Messenger RNA was purified in accordance with the manufacturers protocol (Pharmacia).

By far the most important factor in RNA purification is avoiding or inactivating RNAses which are ubiquitous and highly stable. RNA manipulations were carried out in disposable, sterile plasticware, which is essentially free of RNases, or in glassware which had been treated with diethylpyrocarbonate (0.1% DEPC, 12 hours, 37°C) and then autoclaved.

The method for mRNA purification using this kit was divided into two parts. The first procedure involved the lysis of the cells and separation of total RNA from other cellular components by centrifugation. Cells were

initially recovered from the flasks and subjected to centrifugation at 1500 rpm for 5 minutes. Following centrifugation, the supernatant was discarded and 1.5ml of extraction buffer (a buffered aqueous solution containing guanidinium thiocyanate and N-lauroyl sarcosine) was added to the cells. This ensures the rapid inactivation of endogenous RNase activity and the complete dissociation of cellular components from the mRNA. The cells were homogenised using a 2ml syringe and a 21ga needle by repeatedly aspirating the mixture through the needle. Following this, 3ml of elution buffer (10mM Tris-HCl (pH 4.4), 1mM EDTA) was added to the mixture and again the mixture was homogenised. The mixture is diluted 3 fold with elution buffer to lower the concentration of guanidinium thiocyanate. The concentration was then low enough to allow efficient hydrogen bonding between poly-A tracts on mRNA molecules and the oligo-(dt) attached to cellulose, but high enough to maintain complete inhibition of RNases. In addition, the three fold dilution causes a number of proteins to precipitate, allowing them to be easily removed by centrifugation. The elution buffer was placed in a water bath set at 65^{o}C and the mixture was subjected to centrifugation at 10,000 rpm for 10 minutes at room temperature in a Beckman JA20 rotor.

The second part involved isolation of mRNA from total RNA using oligo(dt) cellulose spun columns. Initially, the column was inverted several times to resuspend the matrix. The top and bottom closures were removed and the column placed in a 15ml falcon tube. This was subjected to centrifugation for 2 minutes at 350g (1300 rpm in our bench top omnifuge). Following centrifugation the bottom closure was replaced and the column placed upright in a test tube rack. 4ml of the supernatant obtained from centrifugation of the lysed cells was added to the surface of the cellulose spun column. The top closure was replaced and the tube was

inverted several times over a 10 minute period. This allows for the mRNA to bind to the column matrix. Prior to elution of the mRNA the column was washed 3 times in high salt buffer (10mM Tris-HCl (pH 7.4), 1mM EDTA, 0.5M NaCl) and twice in low salt buffer (10mM Tris-HCl (pH 7.4), 1mM EDTA, 0.1M NaCl). The mRNA was eluted from the column with three successive washes with elution buffer at 65°C, using 25ul of elution buffer for each wash. The entire procedure from cell lysis through to mRNA elution was carried out without pause.

Quantitation of mRNA was carried out using a spectrophotometer. The A_{260} value and the $A_{260/280}$ ratio were recorded. The concentration of mRNA was determined using the following calculation:

RNA (ug/ml) = A_{260} x D x 40 were D = dilution factor

Following quantitation, the mRNA was precipitated by the addition of 50ul of potassium acetate (2.5M potassium acetate (pH 5.0)) and 10ul of glycogen (10mg/ml glycogen in DEPC treated water) and 1ml of 95% ethanol. After incubation at -20°C for 1 hour the reaction was spun in a microcentrifuge at 13000 rpm for 15 minutes. The RNA was subsequently stored, precipitated, under ethanol at -70°C.

2.5.2 FIRST STRAND cDNA SYNTHESIS.

First strand cDNA synthesis was achieved using a First strand cDNA synthesis kit (Pharmacia) which employs Murine Leukaemia Virus (M-MuLV) reverse transcriptase to catalyse the reaction. For successful cDNA synthesis it was essential to use intact, undegraded RNA in the reaction. This was achieved by the use of the mRNA purification kit

(Pharmacia) as described above. The conditions for first strand synthesis were also optimised by the use of a first strand cDNA synthesis kit (Pharmacia) which permits full-length transcription of RNA's 7 kilobases or more in length. The protocol for synthesis of a 33ul first strand reaction was followed. One ug of mRNA was diluted in 20ul of DEPC-treated water and then incubated at 65°C. for 10 minutes to heat denature it, and placed on ice. The mRNA was added to 11ul of bulk first strand reaction mix (cloned, FPLC*pure* Murine Reverse Transcriptase, RNAguard, RNase/DNase-free BSA, dATP, dCTP, dGTP, and dTTP in aqueous buffer). In addition, 1ul of DTT (200mM aqueous solution) and 1ul of N*ot* I-d$(T)_{18}$ primer (0.2ug) was added to the mixture. The mixture was incubated for 1 hour at 37°C. The completed first strand reaction then contained a library of mRNA:cDNA heteroduplexes which were ready for PCR amplification.

2.5.3 PCR AMPLIFICATION.

Amplification of cDNA was achieved using the Polymerase Chain Reaction (PCR), described by Mullis, 1986. PCR amplification of cDNA involves the enzymatic synthesis of specific cDNA target sequences using two oligonucleotide primers which flank the cDNA region of interest and hybridise to opposite strands. PCR was carried out using the Gene Amp DNA Amplification kit (Perkin Elmer Cetus) using AmpiTaq recombinant Taq DNA polymerase. In most instances the reaction was performed in 0.5ml polypropylene microcentrifuge tubes and consisted of cDNA, 3' and 5' primers, 10x reaction buffer (100mM Tris-HCl (pH 8.3), 500mM KCl, 15mM $MgCl_2$, 0.01% (w/v) gelatin), dNTP mix (200uM final concentration of each dATP, dTTP, dGTP, dCTP), AmpliTaq DNA polymerase (final concentration of 2.5u/50ul), and autoclaved, ultra-

filtered water. The reaction was overlaid with mineral oil to prevent evaporation. The reactions were then subjected to thermal cycles of denaturation, annealing and polymerisation in an automated thermal cycler (Perkin Elmer). Upon completion of the PCR the products were visualised by either polyacrylamide gel electrophoresis or agarose gel electrophoresis before ligation into plasmid vector. A more detailed account of reaction conditions and PCR optimisation is given in the relevant chapter.

2.5.4 AGAROSE GEL ELECTROPHORESIS

DNA molecules of different sizes were separated by electrophoresis through 1% agarose in 1xTAE. More often, the minigel kits were used along with varying number of sample wells. The cast agarose gel was immersed in TAE buffer in the minigel apparatus. Each DNA sample was mixed 5:1 (v/v) with DNA gel loading buffer (*q.v.*) and carefully loaded into one of the wells. This was repeated for all of the samples. Electrophoresis was carried out at 70 volts. Following electrophoresis the separated DNA fragments were stained with ethidium bromide and identified by direct examination of the gel using an ultraviolet transilluminator. Fragment sizes were determined by comparison with DNA markers. The markers used were λ DNA/*Hind* III fragments (size range 125bp to 23 Kb) and DNA/*Hae* III fragments (size range 72bp to 1353bp). Both markers were obtained from Gibco BRL. Only the DNA/Hae III fragments were used in conjunction with PAGE. Gels were photographed using kodak film.

2.5.5 POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis was used to separate and accurately measure the size of DNA molecules less than 1kb in length. Acrylamide was used at concentrations between 3.5 and 8% w/v in TBE, depending on the size of DNA of interest. An acrylamide: *N,N'*-methlynbisacrylamide ratio of 19:1 was used. Polymerisation was achieved by addition of ammonium persulphate (0.08%, w/v) and TEMED (*N,N,N',N'*-tetramethylethylenediamine, 0.08% v/v). 15 x 17cm gels were cast at a thickness of 1.5mm between sealed, vertical, siliconised glass plates. Twenty well teflon combs were used. After removal of the comb and before the samples were loaded, the wells were thoroughly flushed with TBE to remove incompletely polymerised acrylamide. DNA samples were mixed 5:1 (v/v) with DNA gel loading buffer. A total volume of 10ul was loaded. Gels were run in TBE buffer at approximately 5V/cm until the marker dyes had run the desired distance. DNA bands were detected and photographed after staining with ethidium bromide for 15 minutes. Denaturing polyacrylamide gel electrophoresis using 6% acrylamide gels incorporating 7M (42% w/v) urea was used for DNA sequencing . This method is described below.

2.5.6 DNA LIGATION

All DNA ligations involved DNA molecules with either compatible cohesive or blunt-ended termini. Purified, precipitated insert DNA was resuspended in an appropriate volume of TE (pH 8.0) and mixed with vector DNA in TE. For plasmid vectors, the molar ratio of insert:vector was usually 10:1 and a reaction volume of 20ul was used. Included in the reaction was 10x reaction buffer, 1ul of T4 DNA ligase and water to make the volume to 20ul. The reaction was allowed to proceed overnight at

14°C. The protocol for insertion of PCR product into the pCR-script vector is slightly different and is described in the relevant chapter.

2.5.7 EXTRACTION OF DNA FROM AGAROSE GELS

This was achieved using the Geneclean II kit (BIO 101 inc.). DNA was subjected to electrophoresis using 1% agarose gels in TAE. After electrophoresis the gels were stained with ethidium bromide and the DNA visualised using an ultraviolet transilluminator as described. The appropriate DNA band was cut from the gel using a scalpel blade and transferred to an eppendorf tube.

The Geneclean kit II contains a specially formulated silica matrix called Glassmilk that binds single and double stranded DNA without binding DNA contaminants. To the gel slice in the eppendorf tube was added three volumes of Sodium iodide (NaI). The addition of three volumes keeps the final concentration of NaI above 4 molar. The tube was then incubated at 50°C for 10 to 15 minutes or until all of the agarose had melted. The tube was transferred to ice and the glassmilk added. The amount of glassmilk added depends on the mass of DNA contained in the NaI solution. Generally 5ul of glassmilk was added to solutions containing 5ug of DNA or less. 1ul of glassmilk was added for every 0.5ug of DNA above 5ug. Following the addition of glassmilk the tube was vortexed and incubated on ice for 15minutes to allow the DNA to bind to the silica matrix. After the incubation step the silica matrix was pelleted along with its bound DNA. The pellet was washed four times using the NEWWASH solution (NaCl, Ethanol and water), provided in the kit. After a final centrifugation the supernatant was discarded and the pellet dried for about 2 minutes in a vacuum dessicator. The bound DNA was eluted from the silica matrix by

the addition of 10ul of TE to the pellet, and after mixing, incubating for 15 minutes at 50°C. The solution was centrifuged for 1 minute and the supernatant containing the DNA removed. 1ul of the eluted DNA solution was subjected to agarose gel electrophoresis to estimate the concentration of DNA eluted.

2.5.8 SEQUENCING OF DNA

Sequencing of DNA was carried out according to the chain termination method of Sanger *et al* (1977) using the reagents and protocol of the "Sequenase" version 2.0 reagent kit (United States Biochemical). Plasmid DNA was first denatured by the addition of 4ul of 1N NaOH to 16ul of plasmid DNA solution. The reaction was incubated at 37°C for 15 minutes. The solution was neutralised with 8ul of 5M NH4OAc and precipitated under 100ul of ethanol at -70°C. The resulting precipitate was centrifuged at 14k for 15 minutes and then the ethanol removed and the precipitate dried in a vacuum dessicator for around 5 minutes. The pellet was resuspended in 7ul of distilled water. Following the denaturation step the protocol provided by the kit was followed.

The protocol for the sequencing reaction involves an annealing reaction, a labelling reaction and a termination step.

Annealing reaction:

For each of the four sequencing lanes, a single annealing reaction was used. The 7ul of DNA obtained in the denaturation step was combined with 2ul of reaction buffer (200mM Tris-HCL, pH 7.5; 100mM $MgCl_2$; 250mM NaCl) and 1ul of the appropriate sequencing primer, giving a total volume of 10ul. The reaction was incubated for 2 minutes at 65°C. The

reaction was allowed to cool to room temperature over a period of around 30 minutes. Slow cooling was achieved by placing a beaker of water at 65°C on the bench. Once the temperature was below 35°C, annealing was complete and the tubes were placed on ice. The annealed template was used within 4 hours.

Labelling reaction:

In the labelling reaction, the following was added to the annealed template/primer: 1ul of 0.1M DTT; 2.0ul of dilute labelling mix (dGTP); 0.5ul [α-^{35}S]dATP and 2.0ul of diluted sequenase enzyme. The reaction was prepared on ice and the enzyme was always added last. For standard reactions (reading sequences up to 500 or so bases from the primer), the labelling mix was diluted 5 fold (i.e. 4ul of mix combined with 16ul of water). The sequenase enzyme was diluted 1:8 in ice cold enzyme dilution buffer.

After the reaction had been set up the contents of the tube were mixed thoroughly and incubated for 2 to 5 minutes at room temperature.

Termination reactions:

Four tubes were labelled G, A, T and C. 2.5ul of the ddGTP termination mix was added to the tube labelled G. Similarly the A, T and C tubes were filled with 2.5 ul of the ddATP, ddTTP and ddCTP termination mixes, respectively. The tubes were then pre-warmed to 37°C for at least 1 minute. When the labelling incubation was complete, 3.5ul of the reaction was added to each of the tubes labelled G, A, T and C. The reactions were mixed and incubated at 37°C for 3-5 minutes. Following the incubation step the reaction was terminated by the addition of 4ul of termination solution to each tube. The tubes were stored on ice until ready to load on

to the gel. Samples labelled with ^{35}S can be stored at -20°C for up to 1 week. When the gels were ready for loading, the samples were heated to 75-80°C for 2 minutes and loaded onto the gel immediately using 2-3ul in each lane.

Six percent denaturing acrylamide gels were used to separate the labelled, terminated fragments and gels were run at 1400V. Aliquots of each reaction were run for 2 and 4 hours, usually allowing the reading of 250-300 bp of DNA sequence per primer.

After electrophoresis, the gel plate with the gel bonded to it, was prised from the gel apparatus. Urea was removed from the gel by immersion of the glass plate, with the gel attached, in 10% acetic acid and methanol for 15 minutes. The gel was transferred to paper and dried using a vacuum gel dryer. The dried gel was transferred to the dark room where it was overlaid with autoradiography film and placed in a light proof film cassette and exposed for 24 hours at room temperature. The following day the film was developed using a Kodak automatic processor. The sequencing film was read manually and the sequence data analysed.

Sequence analysis was carried out using the University of Wisconsin genetics computer group (G.C.G) sequence analysis software programmes. These programmes are run using the VAX computer and the VMS operating system. BESTFIT, which employs the "local homology" algorithm of Smith and Waterman (1981), was used to find the best homology between the sequences of PCR derived DNA and that of published interferon-γ sequences for other species.

2.6.0 PROTEIN ANALYSIS

2.6.1 SDS-POLYACRLAMIDE GEL ELECTROPHORESIS

One-dimensional gel electrophoresis under denaturing conditions (i.e. in the presence of 0.1% SDS), initially described by Laemmli (1970), separates proteins based on molecular size as they move through the polyacrylamide gel matrix towards the anode. For the most part, 12 % gels were used as they resolve proteins in the size range 10 to 55 KDa. The gels were cast in minigel vertical gel units (Bio-Rad Mini-Protean II) which incorporate rectangular glass plates separated by 0.75mm spacers. Initially, a resolving gel was prepared (3.3ml H_2O, 4.0ml acrylamide mix, 2.5ml 1.5M Tris (pH 8.0), and 0.1ml 10% SDS). Polymerisation was achieved by the addition of 0.1ml 10% ammonium persulphate (APS) and 4ul of Temed (*N,N,N',N'*-tetramethylethylenediamine) and the mixture was poured between the glass plates. The upper interface of the poured gel was covered with butanol to allow the development of an even surface. The resolving gel was allowed to set for 30 minutes while the stacking gel was prepared. The stacking gel consisted of 2.1 ml H_20, 0.5ml acrylamide mix, 0.38ml 1.0M Tris (pH 6.8) and 30ul of 10% SDS. Polymerisation was achieved by the addition of 30ul of 10% APS and 3ul of Temed. Prior to the addition of the stacking gel to the plates, the butanol was poured off and the interface thoroughly washed with distilled water. After the addition of the stacking gel, an 8 space teflon comb (Bio-Rad) was inserted into the plates to create the protein wells. The gel was allowed to set for around 20 minutes.

Protein loading buffer was added to each of the protein samples in a 1:2 v/v ratio and the samples boiled for 5 minutes prior to being loaded into the gel wells. The protein samples were subjected to electrophoresis at

200V in SDS-PAGE running buffer. Typically the electrophoresis was carried out for 30 to 45 minutes. Subsequently, the gels were subjected to electroblotting as described below.

2.6.2 WESTERN BLOT ANALYSIS OF PROTEIN GELS

Western blot analysis was carried out using the ECL™ method. This is a light emitting non-radioactive method for detection of immobilised specific antigens, conjugated directly or in-directly with horseradish peroxidase-labelled antibodies. Following SDS-PAGE, proteins were transferred from the gels to nitrocellulose membranes by the process of electroblotting. The gel was first removed from the electrophoresis glass plates and immersed in semi-dry transfer buffer. In addition, the nitrocellulose membrane (Hybond™-ECL) was soaked in transfer buffer for 5 minutes prior to the commencement of the blotting procedure. Two sheets of blotting paper were cut and soaked in transfer buffer. One sheet of the blotting paper was arranged on the blotting apparatus, over which was placed the nitrocellulose membrane. The gel was placed over this and the second sheet of blotting paper arranged over the gel to create a sandwich. Air bubbles were removed from the sandwich by rolling a glass tube over the upper sheet of blotting paper. The electoblotting was performed at 15V for 45 minutes.

Following the electroblotting, the nitrocellulose was removed and rinsed in 1X TBS. The non-specific binding sites were blocked by incubation of the membrane with non-fat milk powder (0.5% Marvel, 0.5% Tween in TBS) for 30 minutes on a shaker apparatus at room temperature. Subsequently, the primary antibody was added at a predetermined concentration, in 3% Marvel, 0.5% Tween in TBS. Following a two hour incubation at room

temperature, the membrane was subjected to 3 X 10 minute washes with 3% Marvel, 0.5% Tween in TBS. The secondary antibody (HRP labelled) was then added, again in 3% Marvel, 0.5% Tween in TBS, and incubated at room temperature for 1.5 hours. The membrane was washed 3 times for 10 minutes in TBS/0.5% Tween and subsequently developed.

Proteins were detected by chemiluminescence using ECL Western blotting reagents (Amersham life science). The entire detection procedure was carried out in a dark room. Equal volumes of ECL detection solution 1 and 2 were mixed to give sufficient volume to cover the membrane (1ml/ membrane). Excess buffer was drained from the membrane and the ECL solution added dropwise to the entire protein side of the membrane surface. The membrane was allowed to incubate for 1 minute without agitation. Excess solution was drained from the membrane by holding the corner next to some blotting paper. The membrane was sandwiched in cling film, any air bubbles removed and placed in a film cassette (protein side up). A sheet of autoradiography film (Hyperfilm-ECL, Amersham) was placed over the membrane and the cassette closed for, typically, 15 seconds. The film was developed using an automated processor

CHAPTER 3

CLONING AND SEQUENCING OF cDNA ENCODING FELINE INTERFERON-γ

3.1 INTRODUCTION

This chapter describes the cloning and sequencing of cDNA encoding feline interferon-γ. The ultimate aim of the project was to produce a biologically active protein that could eventually enter into clinical trials. Before the advent of recombinant DNA techniques the only way to obtain such biological agents was to purify them to homogeneity directly from animal tissue or from tissue culture supernatants. This is a time consuming procedure which gives low yields of product. This procedure is further disadvantaged by the potential contamination of the product with other agents. Many of the early clinical trials in the late 1970's and early 1980's involving interferon were conducted using a partially purified interferon-α species, which was later replaced with the recombinant product (reviewed by Gutterman 1994). Recombinant DNA techniques allow the actual coding sequence of the gene of interest to be cloned and subsequently expressed. The advantages of cloning the gene include increased yield of product and less problems with contamination. One of the major problems with proteins derived from laboratory based expression systems is that the product may not be identical to the native protein in terms of its post-translational modification. Differences in glycosylation and phosphorylation may alter the biological half-life of the protein and, in addition, it is now well recognised that animals can develop antibody responses to recombinant proteins, even if those proteins are species specific. However, the advantages of gene cloning far out weigh any disadvantages when compared to obtaining the protein directly from the animal.

There are several approaches one can take towards the cloning of genes. Several human cytokines were purified to homogeneity in sufficient

quantities to allow partial amino acid sequence to be determined. For human tumour necrosis factor-β, (Gray *et al* 1984), the complete amino acid sequence was derived and chemical synthesis of the entire gene from oligonucleotides was performed, prior to the natural gene being isolated. More often, however, determination of partial amino acid sequence has allowed the generation of degenerate oligonucleotides that could then be used to screen libraries of cDNA or genomic clones for the entire cytokine coding region. Many human cytokines were cloned by this approach, e.g., human interleukin 1β (March *et al*, 1985); human interleukin-6 (Hirano *et al* 1986); and human interleukin-12 (Gubler *et al* 1991; Wolf *et al* 1991).

Similar methodology has been used to detect cytokines from other species (e.g. mice, domestic animals and non-human primates), with the additional stage of cross-species screening of animal cDNA libraries with human DNA probes. Such approaches have been successful for Bovine Interferon-γ (Cerreti *et al* 1986), and Rat interferon-γ (Dijkema *et al* 1986).

Prior amino acid sequence may not be required if a specific and sensitive bioassay is available, or if the cytokine to be cloned is very highly conserved between species. Many of the first cytokine genes to be cloned were detected using a combination of hybrid selection of mRNA to cloned cDNA sequences followed by expression in Xenopus oocytes or rabbit reticulocyte lysates, e.g human interferon-γ (Devos *et al* 1982). The procedures were later modified so that mRNA was prepared from cloned cDNA using in-vitro RNA polymerase reactions prior to oocyte injection. Hybridisation selection and oocyte injection fell out of favour because of the inherent instability of the RNA preparations and the time taken for oocyte injection. As an alternative, expression of cDNA in monkey COS-7 cells became more widely used and many cytokines have been cloned by

expression cloning in COS-7 cells including human GM-CSF (Lee *et al* 1985); mouse interleukin-4 (Lee *et al* 1986), and Murine interferon-γ (Gray and Goeddel 1983)

A more powerful tool for cloning genes has now been provided by the introduction of the polymerase chain reaction (PCR) (Mullis, 1986) and this has been incorporated into the approach described here to clone cDNA encoding feline interferon-γ. This procedure initially involves the isolation of RNA from appropriate tissues and subsequent synthesis of a panel of RNA:cDNA heteroduplexes which represent all the genes expressed by the cells at the time of RNA harvest. The cDNA is synthesised using the enzyme reverse transcriptase which is an RNA dependent DNA polymerase. PCR is then used to amplify the cDNA encoding interferon-γ by using specific primers which flank the coding sequence. The use of PCR to amplify a specific cDNA sequence from RNA is termed Reverse Transcriptase PCR (RT-PCR). Because the actual feline interferon-γ sequence was unknown, primers had to be designed according to regions of homology between published sequences from other species. The basic experimental approach is shown schematically in figure 3.1.

Interferon-γ genes have now been cloned from a number of species including human (Gray *et al* 1982), murine (Gray and Goeddel 1983), equine (Curren *et al*, 1994), canine (Zucker *et al*, 1992), bovine (Ceretti *et al* 1986), porcine (Roger *et al* 1990) and ovine (Radford *et al* 1991), rabbit (Samudzi *et al*, 1991), and rat (Dijkema *et al*, 1986). The ovine and equine interferon-gamma genes were cloned following amplification by the polymerase chain reaction.

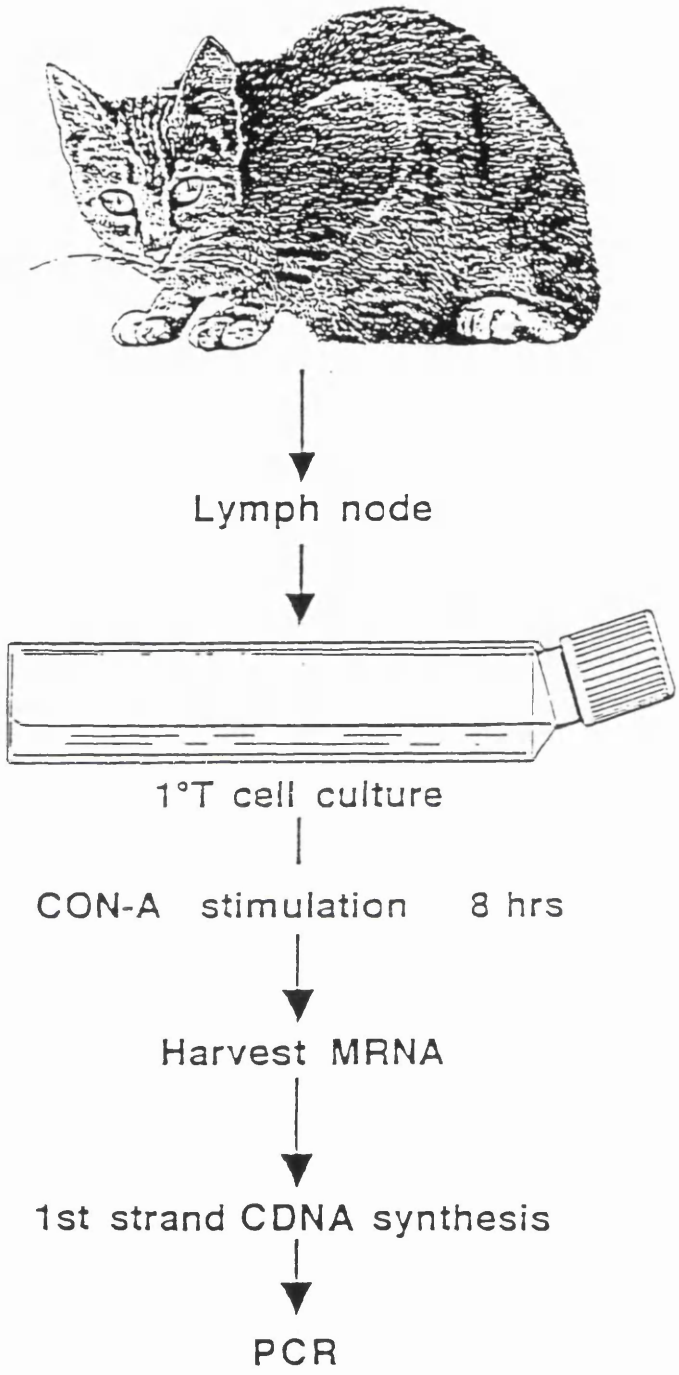


Figure 3.1: The sequence of events in the experimental procedure.

3.2 ISOLATION OF mRNA FROM STIMULATED LYMPHOCYTE CULTURES

The human interferon gamma gene contains 3 introns and is 6kb in size (Taya, 1982). Since the aim of the project was to determine the amino acid sequence and produce recombinant interferon it was technically easier to amplify the coding sequence from mRNA rather than chromosomal DNA.

For the whole experiment to proceed satisfactorily it was essential that the starting material, i.e. the mRNA, was full length and of high quality. The difficulty in isolating RNA is the ubiquity of ribonucleases which are very stable, active enzymes which require no co-factors to function. To eliminate ribonuclease contamination all the glassware used in the experimental procedure was DEPC treated and the mRNA isolation was carried out in a tissue culture fume hood and sterile surgical gloves were worn throughout the procedure.

Fresh lymph node tissue was used to establish T cell cultures as described in the materials and methods. The lymphocyte cultures were stimulated with the T cell mitogen concanavalin-A. Concanavalin-A is toxic to cells in high doses and the amount used in this experiment (7.5ug/ml) was taken from reported experimental data (Nagata *et al* 1987). Addition of the mitogen causes the enhancement of gene transcription and induces the cells to proliferate. Concanavalin-A is a plant derived non-specific polyclonal activator. Both concanavalin A and phytohaemaglutinin (PHA) are equally active in inducing interferon-gamma gene transcription (Kiener and Spitalny 1987). There is a time lag between addition of the mitogen and maximum RNA production and so the difficulty in these experiments is knowing when to harvest the mRNA as the time lag is unknown. To

optimise the yield, mRNA was harvested at 6, 12 and 18 hours. It was found that maximum mRNA harvest was achieved after 6 hours of stimulation. This correlated with the report of Nagata *et al* (1987)

mRNA was harvested using an mRNA purification kit from Pharmacia, as described in the materials and methods. The concentration and purity of the mRNA was determined from spectrophotometric readings. In the experiment described here, 5ug of mRNA was harvested from 10^7stimulated T cells. The mRNA sample had an OD $A_{260/280}$ reading of 1.95, indicating high purity mRNA.

3.3 FIRST STRAND cDNA SYNTHESIS

First strand cDNA synthesis was carried out as described in the Materials and Methods. The reaction was catalysed by Molony murine reverse transcriptase and the primer used was a N*ot*-I-d(T)18 primer provided in the kit. 1ug of mRNA was diluted to a volume of 20ul with DEPC treated water and incubated at 65°C. for 10 minutes. Following incubation, the 20ul of mRNA was added to 11ul of the bulk first strand reaction mix, along with 1ul of DTT and 1ul of the primer and the contents of the tube thoroughly mixed by pipetting. The reaction was allowed to proceed for 1 hour at 37°C., after which, 10ul of the sample was included in the PCR reaction, while the remainder was stored at -20°C. It was found that the best PCR results were obtained when the cDNA was used directly after preparation.

3.4 PCR AMPLIFICATION

Amplification of cDNA was achieved using the polymerase chain reaction (PCR) as described by Mullis, (1986). PCR amplification of cDNA involves the enzymatic synthesis of specific cDNA target sequences using two oligonucleotide primers which flank the cDNA region of interest and hybridise to opposite strands. In addition to the DNA to be amplified and the oligonucleotide primers, there are appropriate deoxyribonucleoside triphosphates (dNTP's), buffer and a thermostable DNA polymerase included in the reaction.

The primers are added in vast excess compared to the DNA to be amplified. They hybridise to opposite strands of the DNA and are oriented with their 3' ends facing each other so that synthesis of DNA by Taq polymerase (which catalyses the extension of DNA in the 5' to 3' direction) extends across the DNA between them. Repeated thermal cycles of denaturation, annealing and extension allow amplification of the DNA of interest.

Using the newly synthesised cDNA as template, PCR was carried out to amplify cDNA encoding feline Interferon-γ. The basic protocol is outlined in materials and methods and is shown schematically in figure 3.2.

3.5 DESIGN OF PCR PRIMERS

The most common cause of PCR failure is primer design. Primer design is the least predictable PCR parameter and it should be noted that some primers just do not work. For many PCR applications, primers are designed to be exactly complementary to the template. However, in this

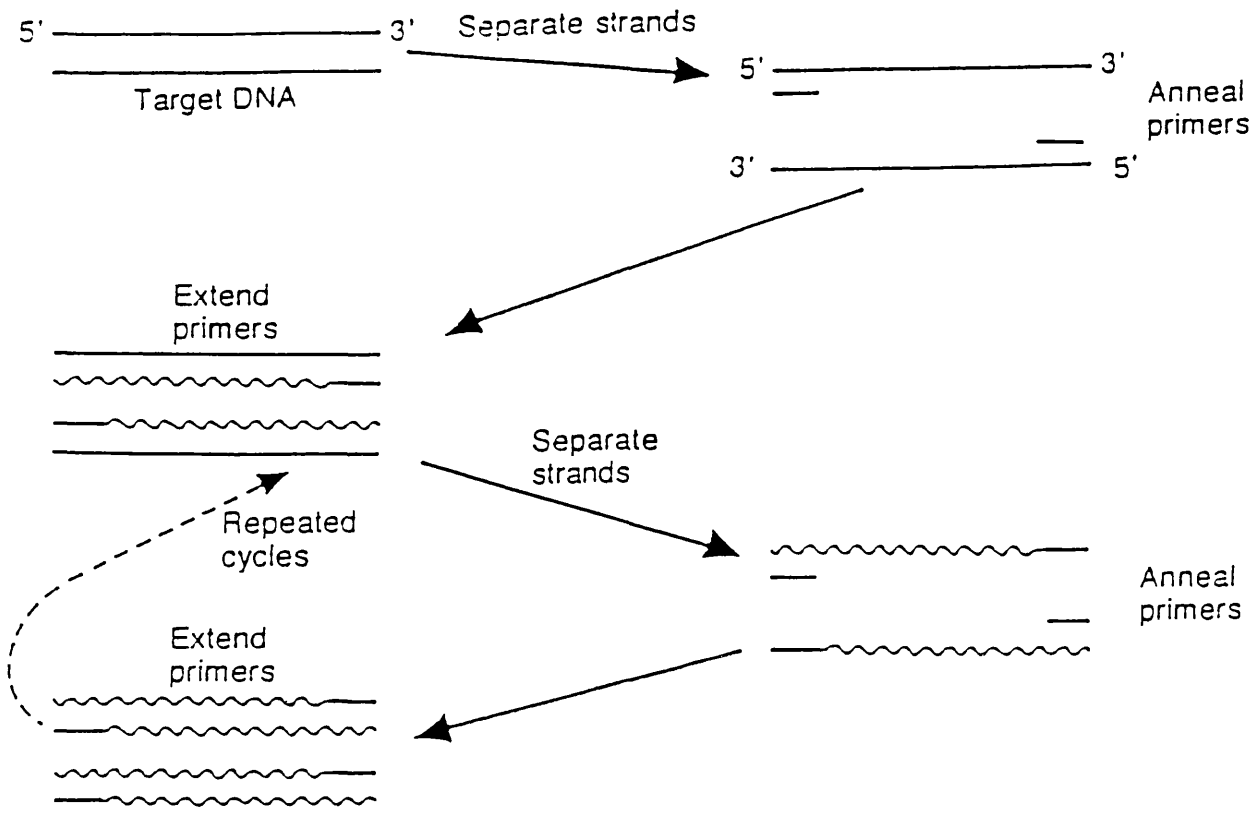


Figure 3.2: Diagrammatic representation of the Polymerase Chain Reaction

```
A 5'CTA CTG ATT TCA ACT TCT TTG GC 3' fe
  --- --- --- --- --- --- --- --     Hu
  --- -C- --- --- --- A-- CC- --     Bo
  --- -C- --- --- --- A-- CC- --     Ov
  T-C TG- GC- --T C-- C-- GC- --     Mu


B 5'CAA ATA TTG CAG GCA GGA CAA CC 3' Fe
  --- --- --- --- --- --- --- --     Hu
  --- --- --- --- --- --- GG- --     Bo
  --- --- --- --- --- --- G-- --     Ov
  --- --- G-- -T- --- -A- TT- TT     Mu
```

Figure 3.3 Line up of upstream (A) and down stream (B) feline primers with equivalent regions in the interferon sequence for other species.(-) indicates areas of homology.

instance the template sequence was unknown and so primers were selected from regions of homology between published sequences of other species.

To try and optimise the amplification the following general rules were followed in design of the PCR primers:

1) Primers were selected based upon regions of high degrees of homology between sequences published for other species.

2) Primers were designed to be 20-30 bases in length.

3) Primers were made which had a GC:AT ratio of around 1:1 and runs of polypurines or polypyrimidines were avoided where possible.

4) The ends of the primers consisted of a C or G to improve anchorage of the primer.

5) The calculated melting temperatures (Tms) for a given primer pair were balanced. In order to achieve this, the Tm was predicted from the general calculation of 2°C. for A or T and 4°C. for G and C (Thein and Wallace 1986).

The following 23 base long primers were used in the amplification process:

Upstream: 5' CTA CTG ATT TCA ACT TCT TTG GC 3' Tms 70°C

Downstream: 5' CAA ATA TTG CAG GCA GGA CAA CC 3' Tms 68°C

Other primers were used in an attempt to amplify the feline Interferon gamma cDNA but were all unsuccessful. The above primers were found to be the most efficient. . The most important primer is the 3' primer as mismatches in this primer are more likely to prevent extension. It is worth noting that computer programs are now available to aid in primer design. I had no access to such programs in these initial stages of the project and all primers used were designed manually.

3.6 PRODUCTS OF PCR AMPLIFICATION

PCR was carried out using the Gene Amp DNA Amplification kit (Perkin Elmer Cetus) using AmpiTaq recombinant Taq DNA polymerase. It was ensured that all reagents used were of a high purity. This was aided by the use of a PCR amplification kit (Cetus) in which all of the reagents were provided. The setting up of the PCR reaction was carried out in a separate laboratory away from the normal working space and under strict sterile conditions to avoid any contamination. A separate pipetteman was used in setting up the reactions which was set aside for PCR use only. In addition, aerosol resistant tips were used throughout the experiment.

The reaction was performed in 0.5ml polypropylene microcentrifuge tubes and consisted of 10ul of the cDNA reaction, 200pm of each primer, 5ul of 10x reaction buffer (100mM TRIS-HCl (pH 8.3), 500mM KCl, 15mM $MgCl_2$, 0.01% (w/v) gelatine), 5ul of dNTP mix (200uM final concentration of each dATP, dTTP, dGTP, dCTP), 5ul of AmpliTaq DNA polymerase (final concentration of 2.5u/50ul) and autoclaved, ultra-filtered water to bring the final volume to 50ul. The reaction was overlaid with mineral oil to prevent evaporation. The reactions were then subjected to

thermal cycles of denaturation (94°C, 1 min), annealing (52°C., 1min) and polymerisation (72°C., 1min) in an automated thermal cycler (Perkin Elmer). A positive and negative control was included in the reaction. The positive control was provided in the kit, and consisted of control template and appropriate control primers. The negative control consisted all the components of the PCR reaction without the addition of cDNA template. The positive control demonstrated that the reaction had proceeded satisfactorily and the negative control was used as an indicator of contamination.

After 30 PCR cycles, the products of the reaction were visualised on a 1% agarose gel by staining with ethidium bromide and using an ultraviolet transilluminator (figure 3.4). The reaction had generated a specific product of around 600kb which was then cloned into the *Srf* 1 site of the plasmid vector pCR script^{tm} SK+ (Stratagene). The pCR-Script SK(+) cloning system utilises blunt-ended ligation of PCR fragments without the need for post PCR treatment. The ligation was carried out using the protocol provided by the manufacturer. 4ul of the PCR product was added to the ligation reaction containing pre-digested vector DNA (1ul), T4 DNA ligase (1ul), 1ul of the rare 8-base recognising restriction enzyme *Srf* I (5' GCCCGGGC 3'), 1ul of pCR-script 10 x reaction buffer, 0.5ul of 10mM rATP and 1.5ul of ultra-pure water. Using the restriction enzyme in the ligation reaction maintains a high steady-state concentration of the digested vector DNA. The reaction was allowed to proceed at room temperature for 1 hour before heat treating at 65°C for 10 minutes. 2ul of the ligation reaction was used to transform DH5α competent bacteria as described in chapter 2. The resulting white colonies were screened for insert after small scale plasmid preparation and subsequent digestion with *Bam* HI and *Sst* I restriction endonucleases.

Sequencing plasmid preparations were made of the resulting positive clones from the PCR ligation and these clones were then sequenced. DNA sequencing was performed on both strands of DNA from three independent clones using a standard di-deoxy chain termination method employing the Sequenase version 2.0 kit (United States Biochemical) as described in the Materials and Methods. The PCR product was found to be 568 base pairs in length which included the PCR primers. In one of these clones there appeared to be a base change at nucleotide no. 143 (T Instead of A). However, two further clones were sequenced in this region and the consensus nucleotide was found to be A. In addition, one of the clones demonstrated third base wobble at nucleotide numbers 507 (T as compared to C in other clones) and 525 (A compared to C in other clones). Large scale caesium chloride preparations of recombinant plasmid were made for subsequent sequence analysis as described in materials and methods.

Sequence analysis was carried out using the university of Winconsin genetics computer group (G.C.G) sequence analysis software programmes. These programmes are run using the VAX computer and the VMS operating system. BESTFIT, which employs the "local homology" algorithm of Smith and Waterman (1981), was used to find the best homology between the sequences of PCR derived DNA and that of published interferon-gamma sequences for other species.

Figure 3.5 shows the primers used in the sequencing of feline interferon-γ. Figure 3.6 shows the nucleotide and predicted amino acid sequence for feline interferon-gamma. The sequence codes for a protein of 167 amino acids with a predicted molecular weight of 19.6 kd.

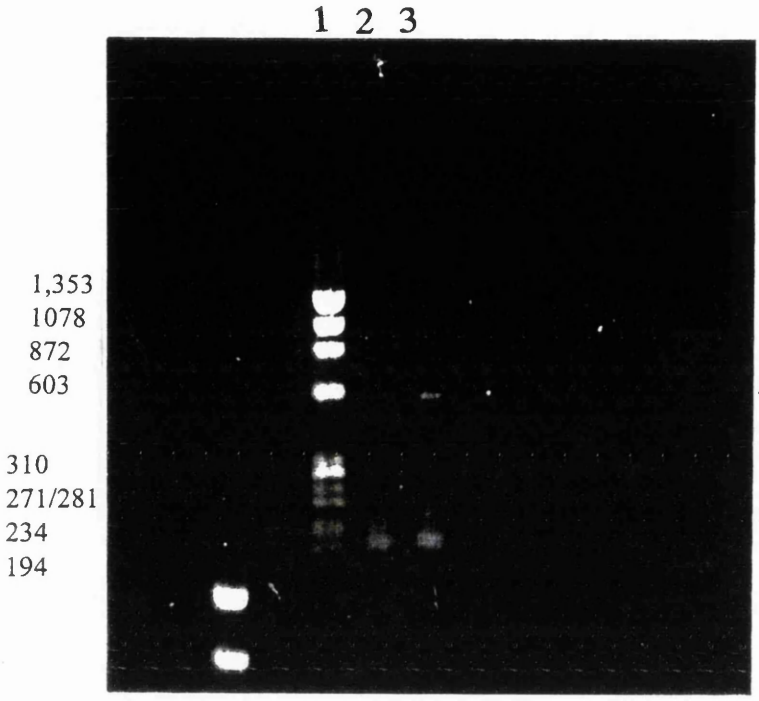


Figure 3.4: 1% Agarose gel showing lane 1, DNA/*Hae* III marker; lane 2, negative control, and lane 3, product of cDNA amplification reaction. The feint band corresponds to a 600bp molecule.

T3 PRIMER: 5' AATTAACCCTCAGTAAAGGG 3'

T7 PRIMER: 5' GTAATACGACTCACTATAGGGC 3'

PCR PRIMER 1: 5' CTACTGATTTCAACTTCTTTGGC 3'

PCR PRIMER 2: 5' CAAATATTGCAGGCAGGACAACC 3'

SEQUENCE PRIMER 1: 5' GTAATCCAGATGTAACAG 3'

SEQUENCE PRIMER 2: 5' CCATCAAGGAAGACATG 3'

SEQUENCE PRIMER 3: 5' GCAGATCATTCACAGGG 3'

SEQUENCE PRIMER 4: 5' CACTCTCCTCTTTCCAGTTC 3'

Figure 3.5 Primers used to sequence Feline interferon-gamma

```
                                                        M   N * Y   T   S
1       ctactgatttcaacttctttggcctaactctccgaaacg atg aat tac aca agt

         F   I   F   A   F   Q   L   C   I   I   L   C   S   S   G
55      ttt att ttc gct ttc cag ctt tgc ata att ttg tgt tct tct ggt

         Y   Y   C   Q   A   M   F   F   K   E   I   E   E   L   K
100     tat tac tgt cag gcc atg ttt ttt aaa gaa ata gaa gag cta aag

         G   Y   F   N * A   S   N   P   D   V   A   D   G   G   S
145     gga tat ttt aat gca agt aat cca gat gta gca gat ggt ggg tcg

         L   F   V   D   I   L   K   N   W   K   E   E   S   D   K
190     ctt ttc gta gac att ttg aag aac tgg aaa gag gag agt gat aaa

         T   I   I   Q   S   Q   I   V   S   F   Y   L   K   M   F
235     aca ata att caa agc caa att gtc tcc ttc tac ctg aaa atg ttt

         E   N   L   K   D   D   D   Q   R   I   Q   R   S   M   D
280     gaa aac ctg aaa gat gat gac cag cgc att caa agg agc atg gac

         T   I   K   E   D   M   L   D   K   L   L   N * T   S   S
325     acc atc aag gaa gac atg ctt gat aag ttg tta aat acc agc tcc

         S   K   R   D   D   F   L   K   L   I   Q   I   P   V   N
370     agt aaa cgg gat gac ttc ctc aag ctg att caa atc cct gtg aat

         D   L   Q   V   Q   R   K   A   I   N   E   L   F   K   V
415     gat ctg cag gtc cag cgc aaa gca ata aat gaa ctc ttc aaa gtg

         M   N   D   L   S   P   R   S   N   L   R   K   R   K   R
460     atg aat gat ctc tca cca aga tct aac ctg agg aag cgg aaa agg

         S   Q   N   L   F   R   G   R   R   A   S   K  stop
505     agc cag aat ctg ttt cga ggc cgt aga gca tcg aaa taa tgg ttg


550     tcc tgc ctg caa tat ttg
```

Figure 3.6, The above shows the nucleotide and predicted amino acid sequence for feline interferon-gamma. The nucleotides underlined represent the PCR primers. The amino acid sequence is shown to start at the first methionine. With the human equivalent the first 20 amino acids represent a leader sequence which is cleaved from the mature protein.

* Indicates possible sites for N-glycosylation.

3.8 DISCUSSION

PCR provides a rapid means to amplify cDNA's of interest and it has been employed to amplify several cytokine genes, including ovine (Radford *et al* 1991) and equine (Curran *et al*, 1994) interferon-gamma. This chapter has described the use of PCR to amplify cDNA encoding feline interferon-gamma and has outlined the importance of high quality template and PCR optimisation. Initial experiments using different primers and conditions were unrewarding and thus a great deal of time and effort went into optimising conditions. Each element of PCR can effect its outcome and thus all steps in the process were optimised.

The deciding factor in the success of PCR reactions for amplification of unknown sequences seems to hinge on primer design. The primers were selected according to high degrees of homology between published sequences of other species. In evolutionary terms, the feline sequence was likely to be more closely related to the human sequence than the murine sequence (This has been demonstrated at the karyotypic level where the feline and human organisation is more similar than that of the murine/feline of murine/human (O'Brien, 1986) and so the primers used were more closely related to the human sequence than the murine sequence. Radford, (1990), used bovine primers to amplify the ovine sequence with great success and found that the ovine and bovine sequences to be highly homologous (93%) and the protein is actually cross reactive in these species. Conservation of cytokine gene sequences across species improves the ability to amplify unknown sequences by the polymerase chain reaction.

Other conditions of the PCR reaction which were optimised included the concentration of Taq polymerase, dNTP's, Mg^{2+} and primers, and also the various parameters of the thermal cycling. The enzyme *Taq* DNA polymerase, from *Thermophilus aquaticus*, has the ability to withstand the repeated heating and cooling conditions which are inherent in PCR. However, it is not totally resistant to heat and should not be put through unnecessary denaturation steps. The enzyme's catalytic ability gradually diminishes when heated repeatedly and it has little activity following 30 cycles of template denaturation.

Increasing the amount of Taq polymerase above 2.5U per reaction can sometimes increase PCR efficiency, but only up to a point. Adding more enzyme can sometimes increase the yield of non-specific PCR products at the expense of the product of interest. In the experiment described here, Taq polymerase was used at 2.5U/reaction.

The differences in sequence in certain clones may have been a result of the lack of proof-reading capacity of the Taq polymerase used. More clones had to be sequenced in these anomalous regions to establish a consensus sequence. Proof-reading polymerases are now commercially available and should be considered in further amplification reactions. While the introduction of mutations by the Taq polymerase is possible (Taq polymerase has an error rate estimated at $2x10^{-4}$ nucleotides per cycle (Saiki, 1988), another explanation of differences between clones may be that, because the PCR was performed on cDNA which was presumably derived from more than one interferon-gamma mRNA molecule, it is possible that variations in mRNA transcripts or reverse transcription reaction products could have contributed to the differences between certain clones. Cerretti *et al,* (1986), found one base difference between the

sequence of two cDNA clones of bovine interferon-gamma, and two variations with the bovine interferon-gamma sequence of Dernyck (1983), which was also derived from a cDNA clone. Both of the latter variations altered the inferred amino acid sequence.

dNTP's were provided with the manufactures kit and, in this experiment, were used at the recommended concentration, i.e. in excess, and at a final concentration of 200uM for each dNTP. The concentration of dNTP's should not be increased in an attempt to increase the efficiency of the PCR reaction. When each dNTP is 200uM, there is enough to synthesise 12.5ug of DNA when half of the dNTP's are incorporated. dNTP's chelate magnesium and thereby change the effective optimal magnesium concentration. Moreover, dNTP concentrations > 200uM increase the error rate of the polymerase. Millimolar concentrations of dNTP's actually inhibit Taq polymerase (Gelfand 1989).

It is beneficial to have an optimum magnesium concentration because this can affect all of the following parameters: primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer dimer artefacts, the enzyme activity and fidelity. Taq polymerase requires free magnesium on top of that bound by template DNA, primers and dNTP's. Accordingly, PCR reactions should contain dNTP concentrations of 0.5 to 2.5 mM over the total dNTP concentration. The presence of EDTA or other chelators in the primer stocks or template DNA may disturb the apparent magnesium concentration.

In the experiment described here, PCR buffer (10x), supplied by the manufacturer, was used which contained free magnesium at a

concentration of 15mM, i.e. the final concentration in the PCR reaction was 1.5mM. While conducting preliminary experimental PCR reactions to optimise the process, it was found that varying the concentration of magnesium between 0.5 and 2.5 mM had little effect on improving PCR efficiency of feline interferon-γ amplification.

Primer concentrations of 10 to 50 pM per reaction are generally optimal for PCR reactions. Primers at higher concentration may promote mispriming and accumulation of non-specific products. However, for the amplification of the feline interferon-γ sequence, the optimal primer concentration was found to be 200pM per reaction, based upon standard spectrophotometric determination.

In thermal cycling, each step in the cycle requires a minimal amount of time to be effective while too much time at each step may be wasteful and deleterious to the Taq polymerase. The shortest possible times at each step are used. For templates less than 1Kb in length, 1 minute per cycle is adequate for synthesis of complete copies of the DNA stands.

It is critical in the denaturation stage that the double strands are completely separated. For PCR from genomic DNA it is generally required that the sample be heated to 95°C+ for approximately 5 to 10 minutes prior to the addition of the Taq polymerase due to the viscosity of the template. For the amplification of feline interferon cDNA, from first strand cDNA, the Taq polymerase was added to the reaction tube prior to the addition of the tubes to the thermal cycler as, in this type of reaction, it is less important to heat denature the DNA for long periods of time. In general the length of time for the denaturation process was taken to be 1 minute/kb. Thus, in this experiment, the denaturation step was 94°C for 1 minute.

It is critical that the primers anneal to the template in a stable fashion. Specificity is improved by raising the annealing temperature towards 60°C. However, where there may be primer/template mismatch the temperature needs to be lower to promote annealing of these degenerate primers. In the amplification of interferon-γ cDNA, the annealing temperature was varied between 45°C and 55°C, in preliminary experiments, to determine optimum annealing conditions. The optimum temperature for annealing was found to be 52°C and the annealing time was set at 1 minute.

The extension temperature was set at a standard 72°C and the time was set at 1 minute and 30 cycles of denaturation, annealing and extension were performed.

The PCR primers used in the reaction amplified a product of 568 base pairs (which included the PCR primers). The predicted coding sequence is 504 base pairs and codes for a protein of 167 amino acids. The mature protein may be smaller than this because, as in human interferon-gamma, the first twenty three amino acids may represent a leader or signal peptide sequence which is cleaved from the mature protein before secretion. The signal sequence is thought to develop a conformation that is recognised by channel proteins in the endoplasmic reticulum and thus serves to transport the protein through the ER to be ultimately secreted. The signal peptide is cleaved from the mature protein by a signal peptidase on the luminal side of the endoplasmic reticulum. It can be difficult to predict the site of signal peptide cleavage, but signal sequences do exhibit some common features (Stryer, 1987). They range in length, from 13 to 30 residues; the amino terminal part of the signal contains at least one positively charged

residue; a highly hydrophobic stretch forms the centre of the sequence and the cleavage site at the carboxyl-terminal end is preceded by a sequence of about 5 residues that is more polar than the hydrophobic core. However, the residues one before (-1 on the amino-terminal side) and three before (-3) the cleavage site have small neutral side chains. Alanine is most common but cysteine, threonine, serine and glycine can be found at the -1 and -3 positions. From these general rules one can predict that the cleavage of the signal sequence of the feline recombinant interferon-gamma would be at amino acid 23.

The feline nucleotide sequence shows significant homology of 78% to the human sequence and 63% homology to the murine sequence (Gray *et al* 1982 and Gray and Goeddel 1983). At the amino acid level the feline interferon-gamma shares 63% and 43% homology with the human and murine homologs respectively. A comparison of cDNA sequences and amino acid sequences is given in figure 3.7. There are three potential glycosylation sites in the feline sequence, between amino acids 2 and 4, 39 and 41 and between 107 and 109. These glycosylation sites are predicted from the amino acid sequence of the mature protein. Sugars can be linked onto the protein through the amide groups of asparagine in the short peptide sequence Asn-X-Ser/Thr. Whether these predicted glycosylation sites are a true reflection of the actual in-vivo glycosylation is a matter for debate. The glycosylation sites do appear to be highly conserved between species.

The three dimensional structure of human interferon-γ has been described by Ealick *et al*, (1991). The protein is primarily α helical, with 6 helices (designated A to F) in each subunit that comprise ~62% of the structure (figure 3.7). There is no β sheet structure. The mature protein is a dimer

and the dimeric structure of the human recombinant protein is stabilised by the intertwining of helices across the sub-unit interface with multiple intersubunit interactions

The amino acid sequence of interferon-γ derived from a number of species is given in figure 3.7. The diagram shows the helical assignment and the highly conserved regions between species. Based on this homology, one could predict that the feline interferon-γ molecule would have similar tertiary structure to the human recombinant protein (figure 3.8). Three of the most conserved regions in the sequence occur in helices C and F, which are the most buried helices in the dimer, and a short basic stretch at the beginning of the COOH terminal tail. The NH_2 and COOH termini of the mature molecule are thought to be critical for receptor binding or triggering of biological responses (Ealick *et al* 1991). One of the most variable regions between species is the loop between helices A and B. It has been suggested by Ealick *et al* (1991), that this region may account for receptor affinity i.e. IFN-γ from one species generally displays poor affinity for the receptor of another species.

In conclusion, PCR was used successfully to amplify cDNA encoding feline interferon-gamma and may provide a powerful tool to amplify other feline cytokine genes. The following chapters will discuss how this cDNA was then introduced into an expression system to produce recombinant feline interferon-γ protein.

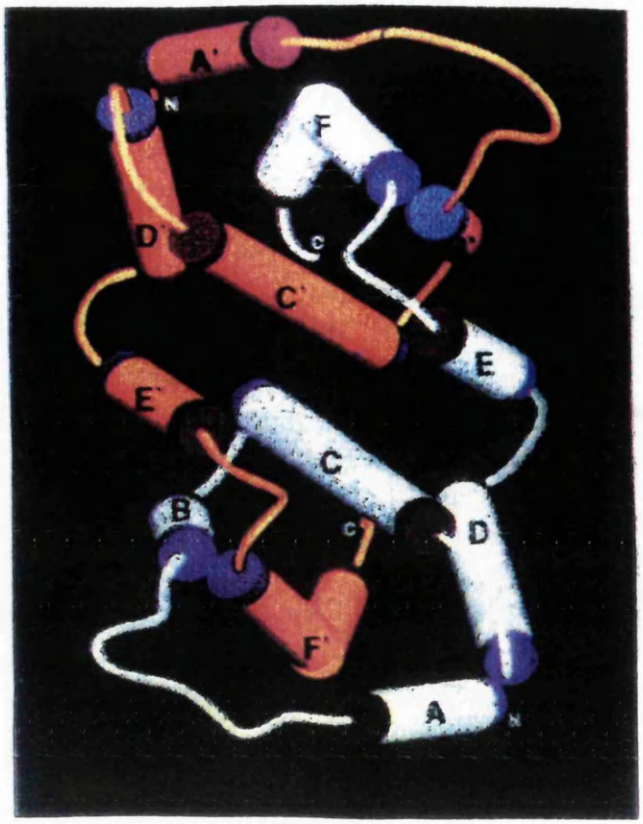
```
      1                                                  50
  Fe  MNYTSFIFAF QLCIILCSSG YYCQAMFFKE IEELKGYFNA SNPDVADGGS
 Hum  MKYTSYILAF QLCIVLGSLG CYCQDPYVKE AENLKKYFNA GHSDVADNGT
Hum2  MKYTSYILAF QLCIVLGSLG CYCQDPYVKE AENLKKYFNA GHSDVADNGT
 Bov  MKYTSYFLAL LLCGLLGFSG SYGQGQFFRE IENLKEYFNA SSPDVAKGGP
 She  MKYTSSFLAL LLCVLLGFSG SYGQGPFFKE IENLKEYFNA SNPDVAKGGP
 Cer  MKYTSYILAL QLCVLLGFSG SYGQGPFFKE IENLKEYFNA SNPDVAEGGP
  Eq  MKYTSFILAF QLCAILGSST YYCQAAFFKE IENLKEYFNA SNPDVGDGGP
 Pig  MSYTTYFLAF QLCVTLCFSG SYCQAPFFKE ITILKDYFNA STSDVPNGGP
RabbitMSYTSYILAF QLCLILGSYG CYCQDTLTRE TEHLKAYLKA NTSDVANGGP
 Rat  MSATRRVLVL QLC.LMALSG CYCQGTLIES LESLKNYFNS SSMDAMEGKS
 Mur  MNATHCILAL QL.FLMAVSG CYCHGTVIES LESLNNYFNS SGIDVEEKS.

      51                                                 100
  Fe  LFVDILKNWK EESDKTIIQS QIVSFYLKMF ENLKDDDQRI QRSMDTIKED
 Hum  LFLGILKNWK EESDRKIMQS QIVSFYFKLF KNFKDD.QSI QKSVETIKED
Hum2  LFLGILKNWK EESDRKIMQS QIVSFYFKLF KNFKDD.QSI QKSVETIKED
 Bov  LFSEILKNWK DESDKKIIQS QIVSFYFKLF ENLKDN.QVI QRSMDIIKQD
 She  LFSEILKNWK EESDKKIIQS QIVSFYFKLF ENLKDN.QVI QRSMDIIKQD
 Cer  LFIEILKNWK EESDRKIIQS QIVSFYFKLF ENFKDN.QVI QRSVDIIKQD
  Eq  LFLDILKNWK EDSDKKIIQS QIVSFYFKLF ENLKDN.QVI QKSMDTIKED
Pig   LFLEILKNWK EESDKKIIQS QIVSFYFKFF EIFKDN.QAI QRSMDVIKQD
RabbitLFLNILRNWK EESDKKIIQS QIVSFYFKLF DNLKDH.EVI KKSMESIKED
Rat   LLLDIWRNWQ KDGNTKILES QIISFYLRLF EVLKDN.QAI SNNISVIESH
Mur   LFLDIWRNWQ KDGDMKILQS QIISFYLRLF EVLKDN.QAI SNNISVIESH

      101                                                150
 Fe   MLDKLLNTSS SKRDDFLKLI QIPVNDLQVQ RKAINELFKV MNDLSPRSNL
 Hum  MNVKFFNSNK KKRDDFEKLT NYSVTDLNVQ RKAIHELIQV MAELSPAAKT
Hum2  MNVKFFNSNK KKRDDFEKLT NYSVTDLNVQ RKAIHELIQV MAELSPAAKT
 Bov  MFQKFLNGSS EKLEDFKKLI QIPVDDLQIQ RKAINELIKV MNDLSPKSNL
 She  MFQKFLNGSS EKLEDFKRLI QIPVDDLQIQ RKAINELIKV MNDLSPKSNL
 Cer  MFQKFLNGSS EKLEDFKKLI QISVDDMQIQ RKAINELIKV MNDLSPKSNL
  Eq  LFVKFFNSST SKLEDFQKLI QIPVNDLKVQ RKAISELIKV MNDLSPKANL
 Pig  MFQRFLNGSS GKLNDFEKLI KIPVDNLQIQ RKAISELIKV MNDLSPRSNL
RabbitIFVKFFNSNL TKMDDFQNLT RISVDDRLVQ RKAVSELSNV LNFLSPKSNL
Rat   LITNFFSNSK AKKDAFMSIA KFEVNNPQIQ HKAVNELIRV IHQLSPESSL
 Mur  LITTFFSNSK AKKDAFMSIA KFEVNNPQVQ RQAFNELIRV VHQLLPESSL

      151          168
 Fe   RKRKRSQNLF R.GRRASK*
 Hum  GKRKRSQMLF Q.GRRASQ*
Hum2  GKRKRSQMLF R.GRRASQ*
 Bov  RKRKRSQNLF R.GRRAST*
 She  RKRKRSQNLF R.GRRASM*
 Cer  IKRKRSQNLF R.GRRASM*
  Eq  RKRKRSQNPF R.GRRALQ*
 Pig  RKRKRSQTMF Q.GQRASK*
RabbitKKRKRSQTLF R.GRRASKY*
Rat   RKRKRSRC*
 Mur  RKRKRSRC*
```

Figure 3.7 Line up of known interferon gamma amino acid sequences. Bold text indicates areas of conservation.



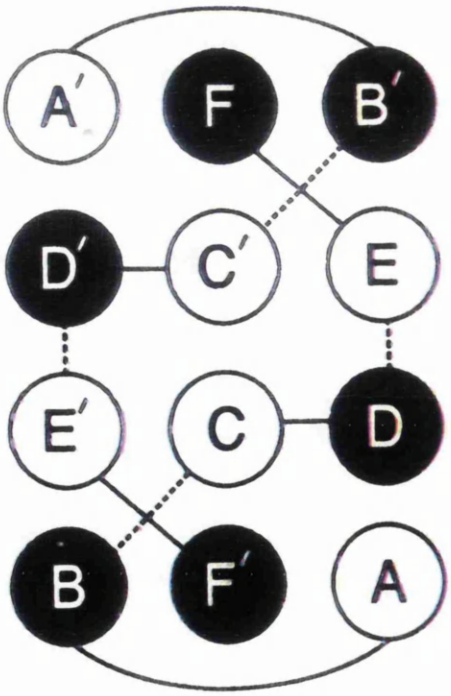


Figure 3.7 Schematic drawing of recombinant human interferon-γ dimer (from Ealick *et al*, 1991)

Chapter 4

EXPRESSION OF FELINE RECOMBINANT INTERFERON-γ IN BACULOVIRUS AND DEMONSTRATION OF BIOLOGICAL ACTIVITY

4.0 INTRODUCTION

4.0.1 GENERAL INTRODUCTION
4.0.2 BACULOVIRUS EXPRESSION AND REPLICATION
4.0.3 POST TRANSLATIONAL PROCESSING
4.0.4 GENERAL PRINCIPLES OF BACULOVIRUS EXPRESSION
4.0.5 ADVANTAGES AND DISADVANTAGES OF THE BACULOVIRUS SYSTEM
4.0.6 TRANSFER VECTORS
4.0.7 WILD-TYPE BACULOVIRUS

4.1 EXPERIMENT TO EXPRESS THE rFeIFN-γ PROTEIN IN A BACULOVIRUS SYSTEM

4.1.2 LIGATION OF INTERFERON cDNA INTO VECTOR pAcCL29-1
4.1.4 ISOLATION OF INFECTIOUS AcMNPV DNA
4.1.5 PURIFICATION OF INFECTIOUS VIRUS DNA
4.1.6 LINEARIZATION OF WILD-TYPE VIRUS DNA
4.1.7 CO-TRANSFECTION OF INSECT CELLS

4.0 INTRODUCTION

4.0.1 GENERAL INTRODUCTION

This chapter describes the expression of recombinant feline interferon-γ protein. The options for protein expression include those based on bacterial, yeast and mammalian cell systems, and systems utilising baculovirus to express genes in insect cells. The system which is chosen to express a gene of interest should reflect the desired end result. For this project a fully functional interferon-γ protein was required that had sufficient *in-vivo* half-life so that it could eventually be used as a therapeutic agent. Post-translational glycosylation of expressed genes occurs in both mammalian and baculovirus based systems. While glycosylation is unimportant for the biological function of interferon-γ, it may be important in extending biological half-life. In addition a system was required that would be highly efficient at protein expression, and a system that would not be difficult to scale up to producing large amounts of protein. For these reasons the Baculovirus system was chosen.

The lifecycle and applications of baculoviruses have been reviewed by Bishop (1992); King and Possee (1992), and Luckow and Summers (1988). *Autographica californica* nuclear polyhedrosis virus (AcMNPV), a member of the family Baculoviridae, has a wide host range and infects more than 30 species of Lepidopteran insects. The genome of AcMNPV consists of double stranded, circular, supercoiled DNA approximately 130 kb in length. During AcMNPV infection two forms of viral progeny are produced: extracellular viral particles (ECV) and occluded virus particles (OV). The latter are embedded in proteinaceous viral occlusions called

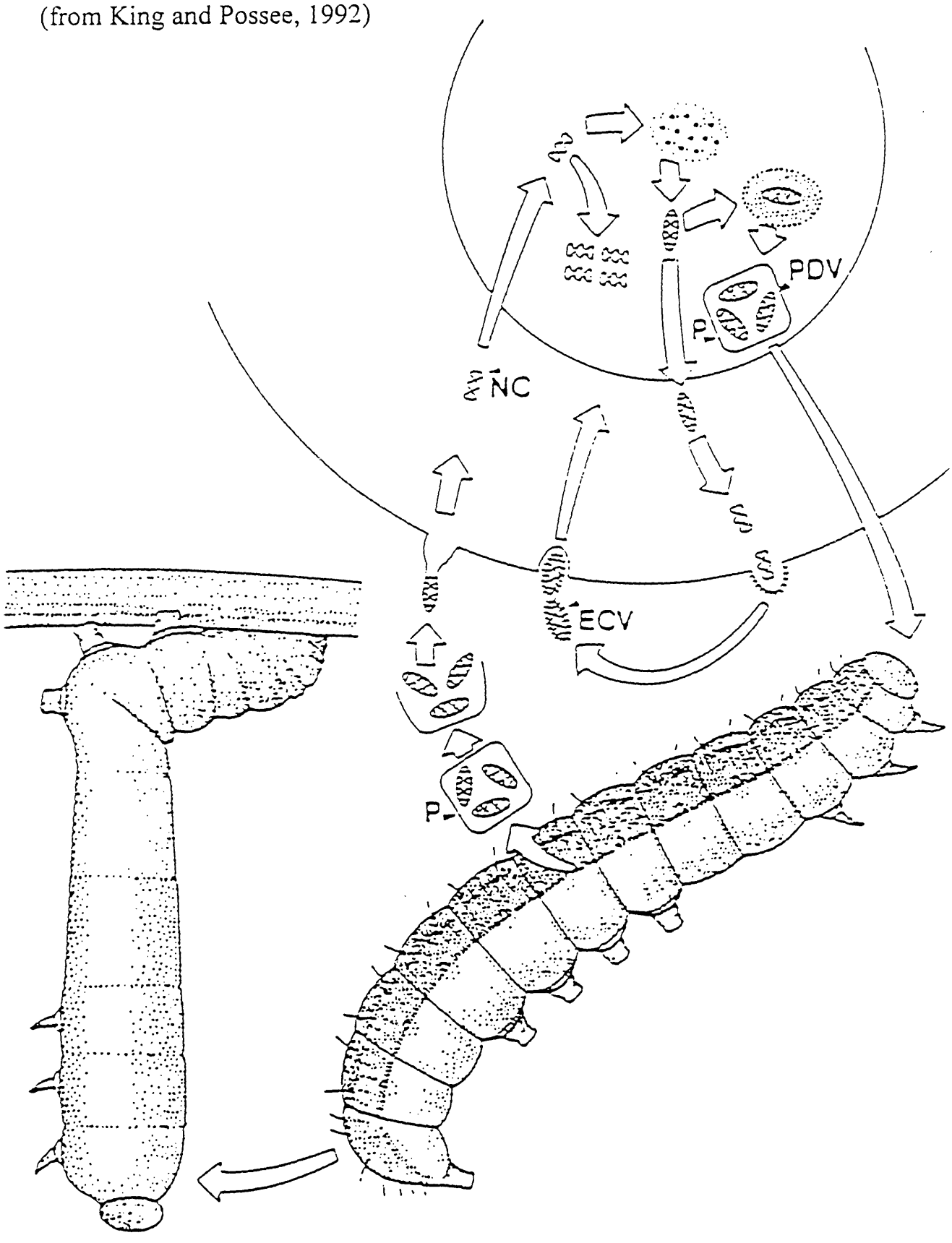
polyhedra. The polyhedrin protein is the major viral encoded structural protein of the virus and, in infected *Spodoptera frugiperda* cell cultures, accumulates to high levels.

The viral occlusions are an important part of the natural virus life cycle providing a means for horizontal transmission. When infected larvae die, the occlusion protein protects embedded viral particles from the environment. Ingestion of contaminated plant material by larvae then causes release of the virus particles in the gut to allow the life cycle to continue. Virus particles enter the cell by endocytosis or fusion and the viral DNA is uncoated at the nuclear pore or in the nucleus. DNA replication occurs around 6 hrs post infection but viral occlusions are not detected until 18 to 24 hpi. Extracellular virus particles reach a maximum between 36 and 48 hpi but the polyhedrin protein continues to accumulate for 4-5 days until the infected cells lyse. The natural baculovirus life cycle is represented diagrammatically in figure 4.1.

4.0.2 BACULOVIRUS GENE EXPRESSION AND REPLICATION

Baculovirus gene expression and replication has been reviewed by King and Possee 1992. The bi-phasic production of ECV and polyhedra observed in insect larvae is also found in cell culture and, in general, the processes are similar. The budded or ECV form of AcMNPV enters insect cells in culture by the process of adsorptive endocytosis. The nucleocapsids serve to translocate the DNA to the nucleus of the cell where virus replication is initiated. Baculovirus genes are expressed in a regulated fashion in infected cells. For convenience, virus gene expression has been divided into four phases. These are: immediate early (α), delayed early (β), late (γ) and very late (δ). In general, the expression levels

Figure 4.1. Diagrammatic representation of the life cycle of a baculovirus in insect larvae and the insect cell (P, polyhedra; ECV, budded extracellular virus; PDV, polyhedra-derived virus; NC, nucleocapsid).
(from King and Possee, 1992)



attained in each succeeding phase is higher than that of the preceding one. This is shown diagrammatically in figure 4.2.

A description of these genes has been reviewed by King and Possee (1992). Immediate, early genes are defined as those genes which can be transcribed in the presence of inhibitors of protein synthesis (e.g. cycloheximide). Two examples have been described, namely IE-1 and IE-N. Evidently, these virus genes are transactivated by host transcriptional factors and do not require *de novo* synthesis of other virus gene products.

The 39k delayed early gene is first detected in infected cells between 3 and 6 hours post infection (h.p.i.). It has been concluded that the early-immediate gene products are required to transactivate the delayed early genes.

The late genes are expressed in infected cells at around 6 h.p.i which coincides with the onset of virus DNA replication. The virus genes which are expressed during this period include, the basic protein, the capsid protein and the virus membrane glycoprotein.

The very late genes are transcribed at a time when the virus is assembling occlusion bodies within the nucleus of the infected cell (from about 15 h.p.i). The very late gene products include the polyhedrin gene, which forms the matrix of the occlusion body, and the p10 protein, which is most likely involved in polyhedra formation. Both polyhedra and p10 genes are not involved in the formation of infectious virus particles. These two very late genes have certainly been the major focus for development of baculovirus expression vectors, since their promotors are extremely

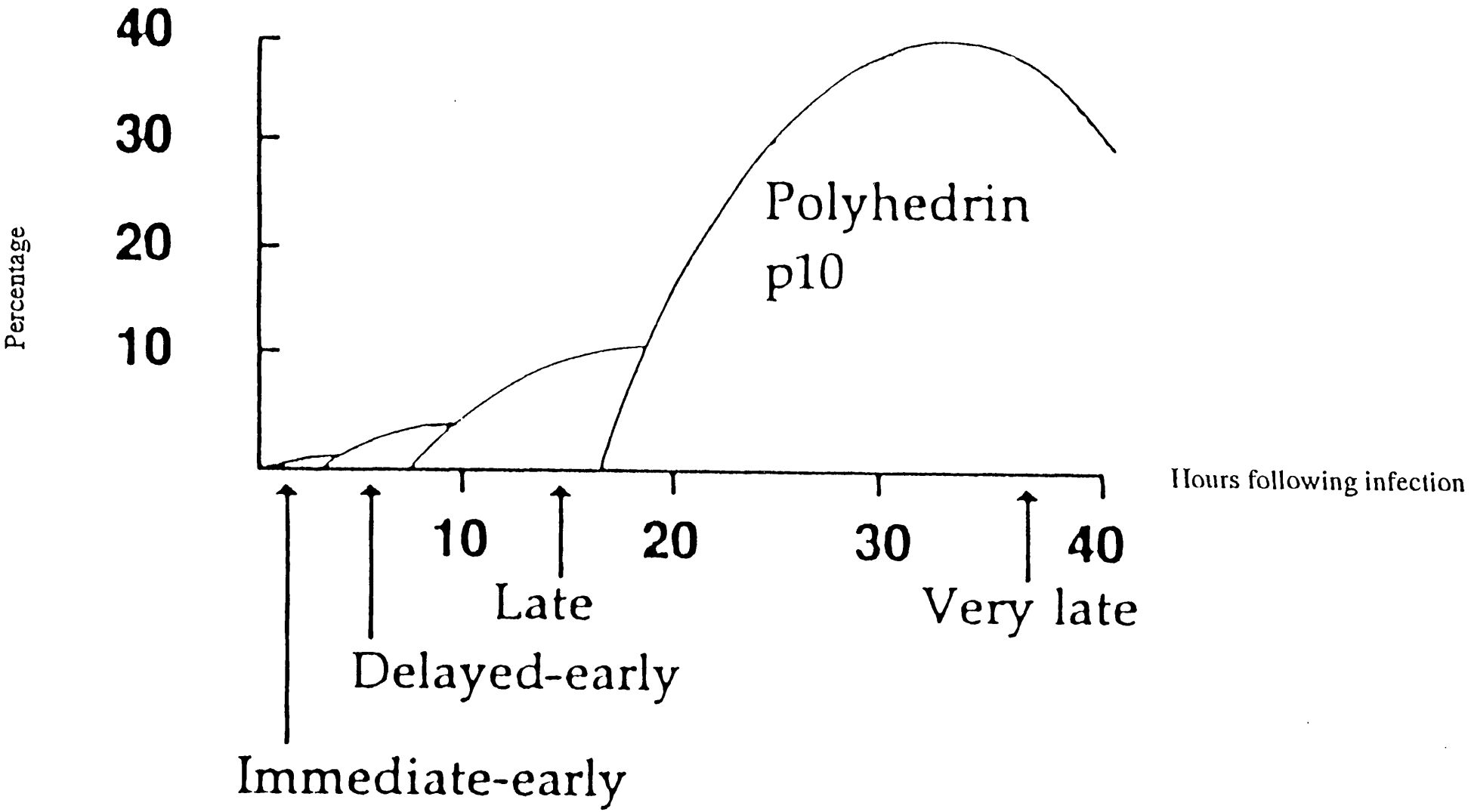


Figure 4.2 Schematic representation of the four phases of baculovirus gene expression in-vitro (from King and Possee 1992). The levels of gene expression are not drawn to scale.

efficient and can result in their combined proteins accounting for up to 50% of the total cell protein mass in the terminal stages of infection.

The polyhedrin gene of AcMNPV has been mapped and sequenced (cited by King and Possee 1992). This gene is non essential for replication or for the production of extracellular virus in cell culture. Deletion or insertional inactivation of the polyhedrin gene results in the production of occlusion negative viruses, which form plaques which are distinctively different from those of wild type, occlusion positive viruses. This provides a way of visually screening for recombinant viruses. The nonessential nature, the high levels of expression of the polyhedrin gene and the ease of visual screening make the promoter of this gene particularly suitable for engineering as an expression vector (Lucklow and Summers, 1988).

4.0.3 POST-TRANSLATIONAL PROCESSING OF FOREIGN PROTEINS

The insect cell appears to be capable of undertaking many of the processing events that are required for the formation of biologically active, heterologous proteins. The following processing events have been reported: glycosylation, phosphorylation, fatty acid acetylation, amidation, and proteolytic processing, including signal peptide cleavage (Reviewed by King and Possee, 1992).

While it is evident that recombinant proteins expressed in baculovirus systems undergo glycosylation, there are differences in the nature of the added oligosaccharides in insect cells as compared to mammalian cells. Mammalian cell extensively trim and modify the core oligosaccharide in terminal glycosylation events. These events usually result in the addition

of fucose, glucosamine-galactose and sialic acid residues to form complex, branched oligosaccharides. Insect cells lack, or only have very low levels, of the necessary fucose, galactose and sialic acid transferases. They also appear to lack the ability to process the core oligosaccharide which has been shown to contain a high proportion of mannose residues.

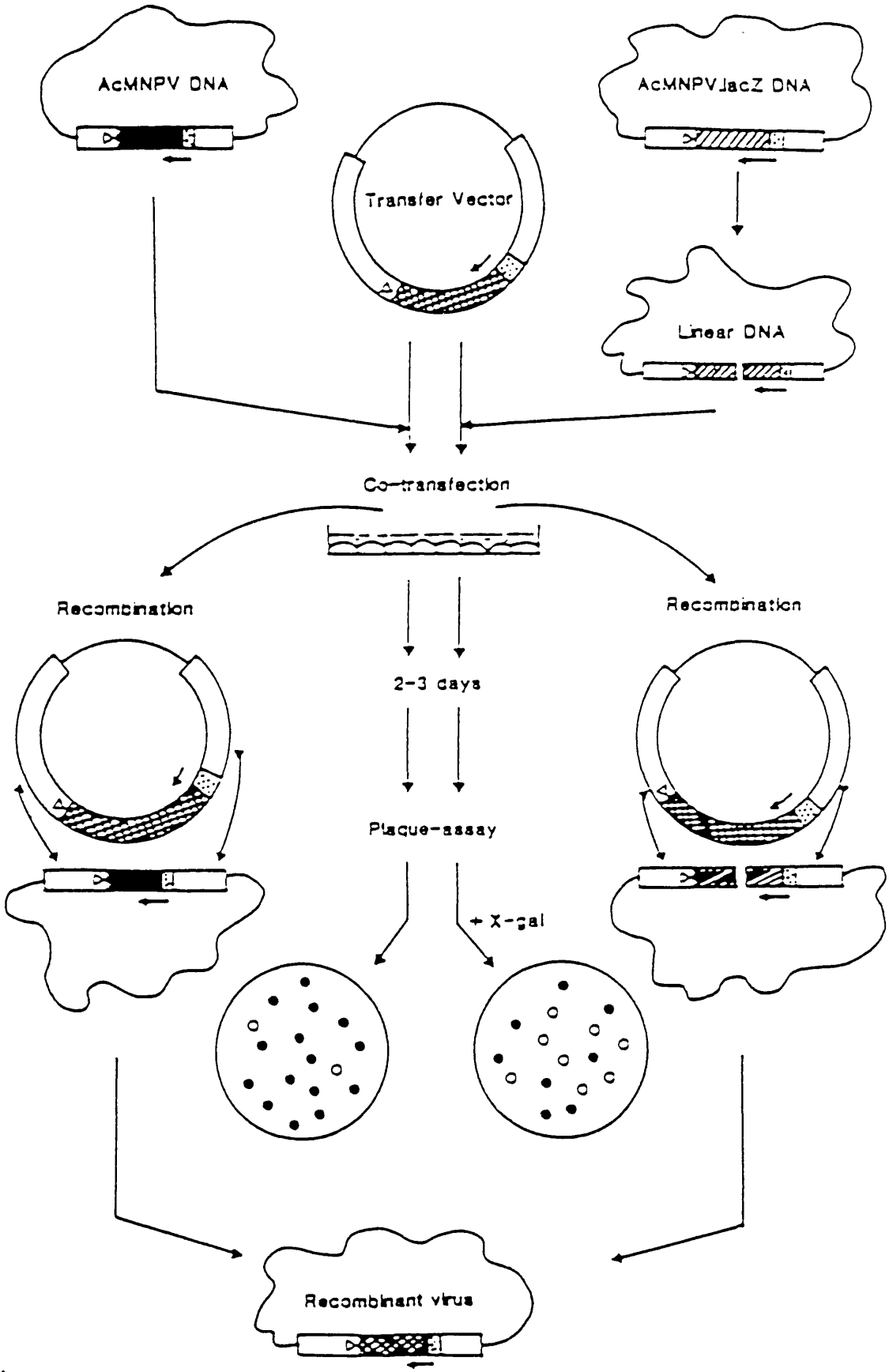
While post-translational modifications occur to proteins expressed from baculovirus vectors in insect cells, in some cases the efficiency of modification is not 100% (Bishop 1992). This is probably not unexpected for polyhedrin or p10 based expression vectors, since these are late promotors and at this stage of infection the cell is in a terminal state and the machineries involved in post-translational modification may be becoming increasingly inefficient.

Proteins synthesised by baculovirus vectors appear to be translocated in the insect cell in a manner corresponding to its own host cell. Most signal peptides are recognised and cleaved in the endoplasmic reticulum and the resulting protein is targeted to the membrane or is secreted, as appropriate. Amino-terminal sequence analysis has confirmed the correct signal peptide cleavage for, among others, human interferon-α (Maeda *et al,* 1985), and human interleukin-2 (Smith *et al,* 1985).

4.0.4 GENERAL PRINCIPLES IN BACULOVIRUS EXPRESSION

A consequence of the size of the baculovirus genome (around 150 kb) is that it cannot be directly manipulated to insert foreign DNA. To express a foreign gene in baculovirus, the gene of interest is cloned in place of the viral coat protein gene in a plasmid transfer vector carrying a small potion of the viral genome. The recombinant transfer vector is co-transfected into

Figure 4.3 Sequence of events in baculovirus gene expression
(from Watson et al, 1992)



insect cells with wild type baculovirus DNA. At low frequency, the transfer vector plasmid and the viral DNA's recombine through homologous sequences, resulting in the insertion of the foreign gene into the virus genome. Recombinant viral plaques develop which are visually different from wild-type viral plaques. The recombinant virus can then be put through several rounds of plaque purification and the expanded and used to infect fresh insect cells, resulting in high expression of the foreign protein, Watson *et al* (1992)

4.0.5 ADVANTAGES AND DISADVANTAGES OF THE BACULOVIRUS EXPRESSION SYSTEM

There is no ideal protein expression system and bacterial, yeast, mammalian and baculovirus systems all have advantages and disadvantages. The method chosen to express the gene of interest is based upon the nature of the recombinant protein and its final use. The advantages and disadvantages of the baculovirus system are given in table 4.1

4.0.6 TRANSFER VECTORS.

Most transfer vectors reported contain sequences from AcMNPV including the promoter of the polyhedrin gene and varying amounts of 5' and 3' viral DNA flanking the polyhedrin gene cloned into a high copy number bacterial plasmid (Lucklow and Summers 1988).

The transfer vector used in this experiment was a polyhedrin promotor based expression vector termed pAcCL29-1, Livingstone and Jones (1989). This is described in Materials and Methods.

ADVANTAGES OF BACULOVIRUS EXPRESSION	DISADVANTAGES OF BACULOVIRUS EXPRESSION
1) The products of the late expression of both the polyhedrin and p10 genes are not essential for replication of the virus and may, therefore, be replaced with foreign genes. 2) The polyhedrin and p10 genes have very strong promoters which allows expression of protein at very high levels. 3) The expression of very late gene promoters occurs in cells after the maturation of budded, infectious virus particles. Thus, if a cytotoxic protein is to be expressed it will not adversely affect virus replication. 4) The virus can accommodate large amounts of foreign DNA without affecting normal replication and DNA packaging; the nucleocapsid simply extends lengthways to accommodate the extra DNA. 5) The baculovirus replicates in eukaryotic cells which allows for post-translational modification. However, there may be differences in the patterns of glycosylation. 6) Baculoviruses replicate only in invertebrates and there is no risk to the user of the system. 7) Protein production is relatively easy to scale up.	1) Baculovirus infection of insect cells results in the death of the host and hence the necessity to reinfect fresh cultures of Sf9 cells. This may be inefficient on a commercial scale. 2) Post-translational processing is different in insect cells. Insect cells produce glycoproteins with simple, unbranched side-chains with a high mannose content.

Table 4.4

4.0.7 WILD TYPE BACULOVIRUS

Success with the baculovirus expression system is dependent on being able to infect cells efficiently with *Autographica californica* multiple nucleocapsid nuclear polyhedrosis virus (AcMNPV), thus obtaining maximum virus replication and optimum production of the desired protein King and Possee (1992). During co-transfection of plasmid transfer vector with circular wild-type baculovirus the recombination frequency is around 0.1 to 1%. Because of this, new ways of improving recombination frequency so as to make screening plaques more efficient have been developed, Kitts and Possee (1993), Bishop (1992).

In yeast and mammalian cells DNA molecules containing double-strand breaks are highly recombinogenic and in these systems linearization of the incoming DNA is routinely used to promote recombination with homologous systems. In insect cells, linear fragments of AcMNPV DNA, produced by restriction enzymes, have been used to map mutations in the virus genome by marker rescue, but linear baculovirus DNA is generally regarded as non-infectious. It was proposed that a linearized virus genome might be non-infectious but still be able to recombine with homologous sequences introduced into the host cell. If the homologous sequences spanned the two ends of the linearized baculovirus DNA, then the genome would be recircularized and restored to full infectivity.

This hypothesis was tested when it was discovered that a restriction enzyme (*Bsu*36I, *Mst*II, *Sau*I) did not digest AcMNPV DNA. This site could be inserted using synthetic oligonucleotides. Circular and linearized viral DNA's were compared in terms of their infectivity and recombinogenicity. It was confirmed that linear DNA co-transfected alone

reduced the yield of progeny virus to 93% and infectivity was reduced 15-150 times that of circular DNA. However, co-transfection of linearized virus with an appropriate transfer vector demonstrated that the linear DNA was proficient at recombination and that upto 30% of the progeny virus were recombinant. The use of linearized virus DNA in co-transfections represented a considerable improvement over previous methods for selecting recombinant virus. The system was refined further by the discovery that the *Bsu*36I RE site is also present in the bacterial *lac*-Z gene coding sequence. A virus AcMNPV*lac*-Z has been constructed with this coding sequence in place of the polyhedrin gene. After linearization any recombinant plaques that develop after homologous recombination should be colourless after staining with X-gal. King and Possee (1992) and Kitts, Ayres and Possee (1993).

The process described above can improve the proportion of recombinant viral progeny to around 30%. In the experiments which demonstrated this, the background of non-recombinant viruses came from two sources:1) viral DNA molecules that did not get cut by the restriction enzyme (a small proportion of uncut virus can make a large difference because uncut virus is 15 to 150 times more infective than linearized virus) and 2) linearized viral DNA that has recircularised inside the insect cell without recombination with the transfer vector. To remove background caused by the second factor, another approach was developed by Kitts and Possee (1993).

The new approach involved the construction of a virus such that restriction of the viral DNA would separate part of the essential gene downstream of the polyhedrin expression locus from the remainder of the viral genome. Even if the virus recircularised, the resulting virus genome would lack

essential sequences and would not produce viable progeny, whereas a double recombination between the linearized viral DNA and the transfer vector would generate a circular viral genome with an intact copy of the essential gene that would produce viable viruses. In addition, the incorporation of two or three restriction sites into the viral genome would reduce the background created by virus escaping restriction enzyme digestion. This is because the fraction of molecules that had not been cut at least once would be extremely small.

In the experiment described below (4.1), a wild type virus with two *Bsu*36I sites, designated BacPAK6, was used and was kindly provided by NERC institute of virology and environmental microbiology, Oxford. BacPAK6 had been generated by co-transfection of linearized AcRP6-SC with a plasmid designated PAK6. PAK6 was generated to have two *Bsu*36I sites which flank a β-galactosidase gene (Kitts and Possee 1993), thus recombinant BacPAK6 viral plaques would stain blue with X-gal. It was demonstrated by the group in Oxford, that the use of restricted BacPAK6 viral DNA to generate recombinant AcMNPV expression vectors yields a higher proportion of recombinant viruses than existing methods. This system has the additional advantage of being compatible with a wide range of existing vectors. The orf1629 sequence required to rescue restricted BacPAK6 viral DNA are present in most transfer vectors.

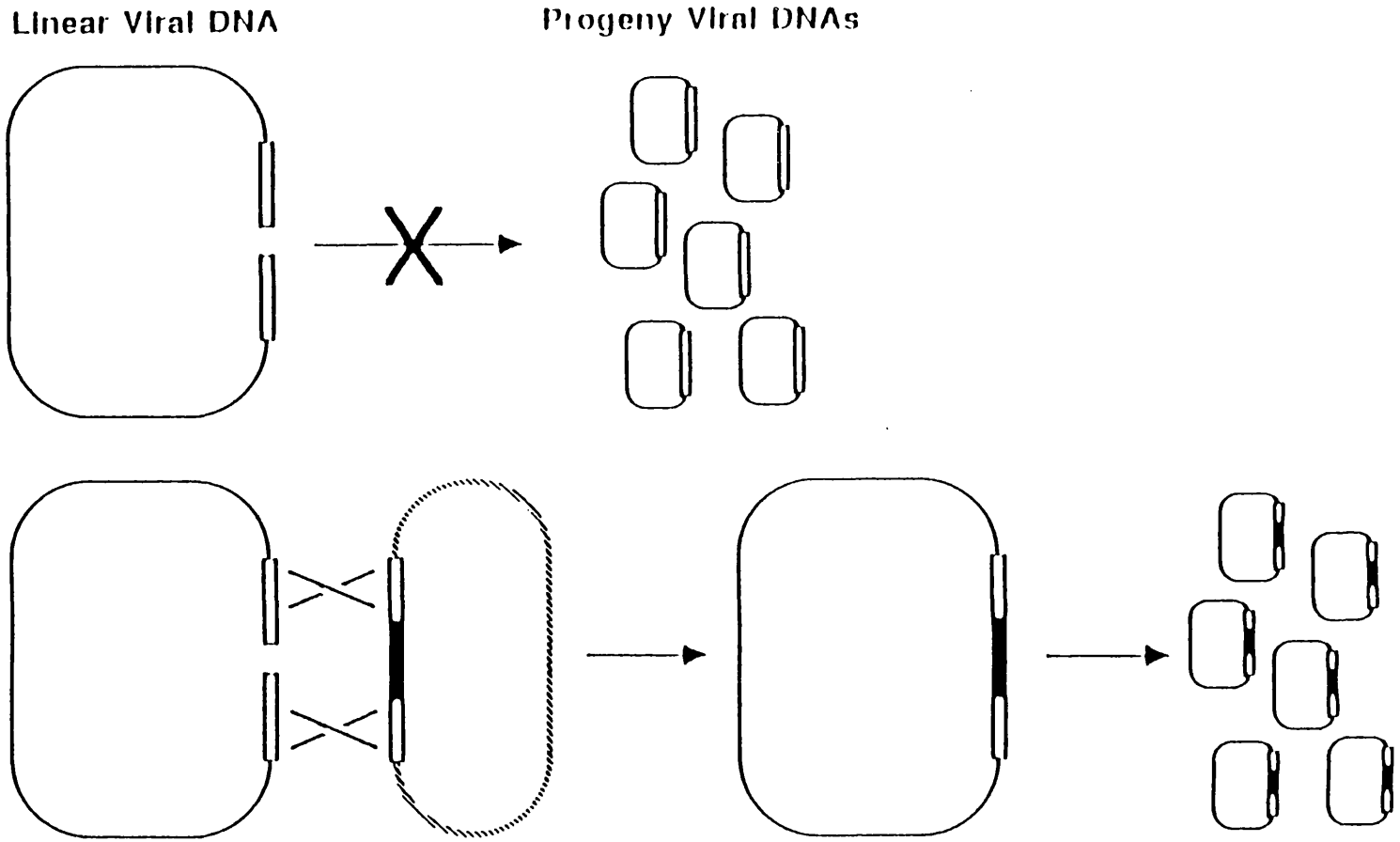


Figure 4.4 Rescue of linear virus DNA by recombination with a transfer vector (from King and Possee 1992). In the upper panel, linear DNA cannot replicate because the replication apparatus of the AcMNPV is, in part, removed. The lower panel shows that the ability to replicate is restored after homologous recombination with transfer vector.

4.1 EXPERIMENT TO EXPRESS THE rFeIFN-γ PROTEIN IN A BACULOVIRUS SYSTEM

4.1.1 LIGATION OF THE INTERFERON-GAMMA GENE INTO pAcCL29-1.

The amplified cDNA encoding feline interferon-gamma was originally cloned into the pCR script™ SK+ plasmid vector and sequenced (chapter 3). As previously described, five clones were sequenced, and one of these clones, designated clone 2, was found to have an interferon-γ cDNA insert in an orientation that would allow directional cloning into the *Sst*1 and *Bam*H1 sites of pAcCL29-1. Caesium chloride plasmid preparations were made of both interferon clone 2 and also pAcCL29-1. Both plasmids were then independently digested with *Sst*1 and *Bam*H1. The digested interferon clone 2 was subjected to electrophoresis using a 1% agarose gel. The band corresponding to the interferon insert was excised and purified as described in materials and methods. One microlitre of the product of gene cleaning was then subjected to agarose gel electrophoresis to check for the presence, purity and to estimate the concentration of insert. The insert was then included in a ligation reaction with the pre-digested pAcCL29-1 vector. The ligation was set up at a molar ratio of 1:10, vector:insert, i.e. 100ng of vector and 50ng of insert. The ligation reaction was incubated for 12 hours at 14°C and was then used to transform the *E.coli* bacterial strain INVαF' (Invitrogen) as described in Chapter 2. Twenty white bacterial colonies were screened for insert using the standard plasmid miniprep technique and subsequent digestion with *Sst*1 and *Bam*H1 restriction endonucleases. Six out of the 20 colonies had plamid clones which were positive for an insert of the correct size. One of these clones, designated clone 20, was grown in culture and purified on a caesium

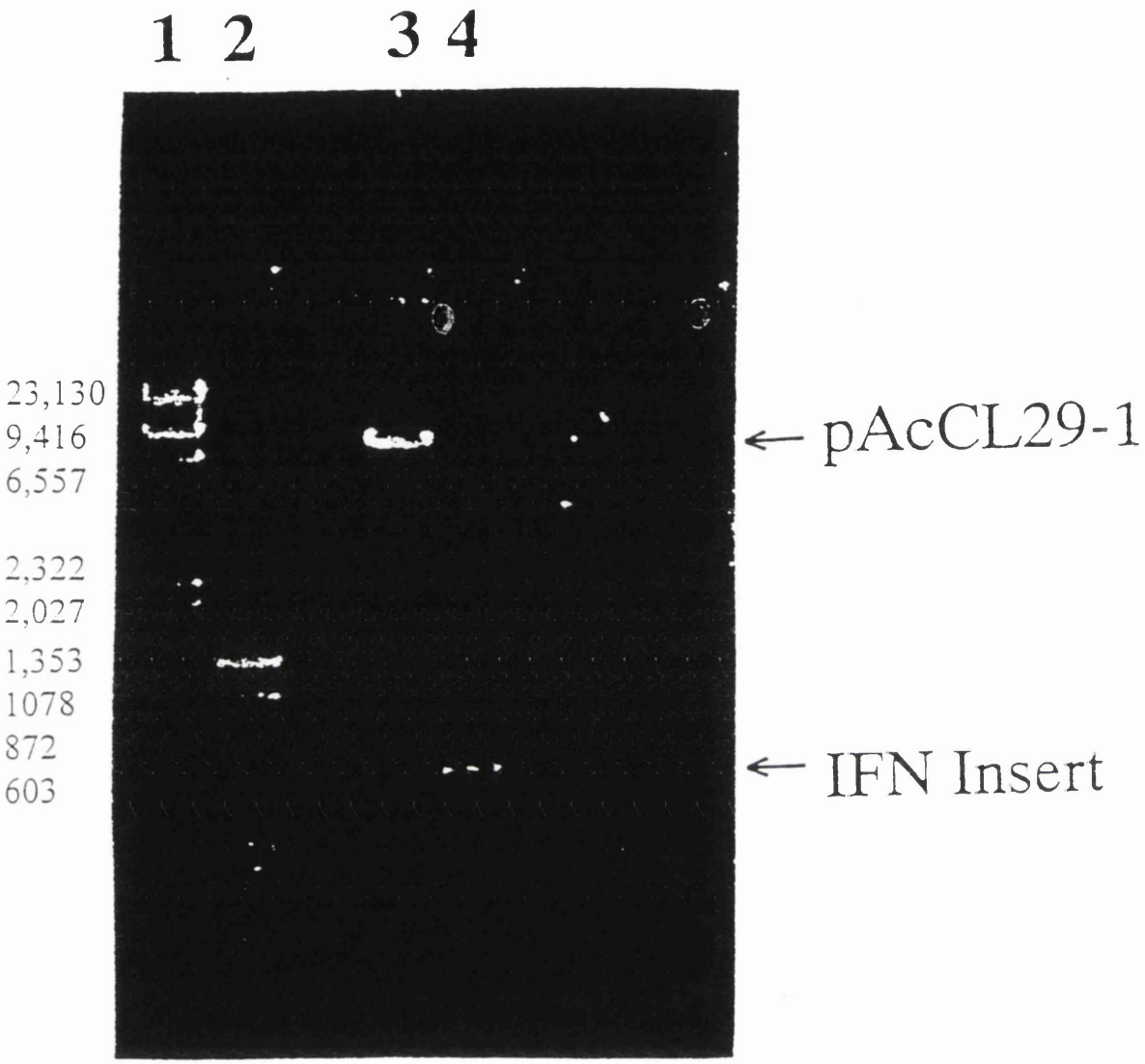


Figure 4.5 1% Agarose gel showing : lane1, λ DNA/*Hind* III marker; lane 2, DNA/*Hae* III marker; lane 3, 1ul of pAcCL29-1 digested vector, and lane 4, 1ul of digested, gene cleaned clone 2 interferon insert.

chloride gradient. The clone was then sequenced again using previously described primers and, in addition, primers which were designated 58 and 59 (see below), which confirmed that the insert was in the correct orientation. Sequencing of clone 20 demonstrated that there had been no alteration in the coding sequence. Primer 58 and 59 were synthesised according to published primer sequences of King and Possee (1992). Primer 58 is a 5' upstream primer which sequences through the promotor region of the polyhedrin gene. Primer 59 is a 3' downstream primer which sequences through the terminal 3' end of the polyhedrin gene.

Primer #58: 5' TGA TAA CCA TCT CGC AA 3'

Primer #59: 5' AGC GAG ATT GTA TGG TG 3'

These primers can be used to sequence across the cloning junction in any polyhedrin promotor based transfer vector. The 3' primer is only applicable to transfer vectors where the polyhedrin coding region has been deleted e.g. pAcYM1.

4.1.2 ISOLATION OF INFECTIOUS AcMNPV DNA

A large stock of AcMNPV (BacPAK 6) was grown up in Sf9 cells using 500ml spinner culture flasks. The cells were pelleted at low speed and the virus particles from the medium were then pelleted at 13k and 4°C overnight in a 6 x 250 MSE rotor. The pellets were soaked overnight in a small volume of TE. Following this the pellets were resuspended by vortexing and then spun at low speed (3k/10mins/chillspin) to remove large debris. The virus was layered on to a 10-50% (w/v) sucrose gradient (in TE). This gradient consisted of a 10% layer of sucrose on top of a 50%

cushion. Seven ml was used in each of two tubes (topped up with paraffin) and spun at 4°C/24k for 1 hour. The virus forms a tight band at the interface and was harvested in the usual way. The diluted virus was then pelleted at 30k for 1 hour and soaked overnight in TE (2ml). The virus was then stored at 4°C.

4.1.3 PURIFICATION OF INFECTIOUS VIRUS DNA

This method was used to prepare very pure, infectious virus DNA for co-transfections. The starting material was purified virus. Sterile solutions and aseptic technique was used throughout, as the DNA prepared would eventually be used to infect cells in culture. The method used was as follows: Four hundred ul of purified virus was taken, to which was added 100ul of 20% sarkosyl solution. This was incubated at 60°C for 30 minutes to lyse the virions. The lysed virions were then layered onto a cushion of caesium chloride (50% w/v in TE buffer) containing 12.5ul of ethidium bromide (10mg/ml). The sample was spun at 35000 rpm for 18 hours at 20°C. After centrifugation, the virus DNA appeared as two orange bands. The lower band corresponded to supercoiled DNA and the upper band was open circular DNA. Both bands were harvested as they are equally infectious.

The tube was placed in a retort stand and a beaker placed beneath the tube to catch any drips. A hole was bored into the base of the tube and both DNA bands were collected in a bijou. The ethidium bromide was removed by adding equal volumes of butanol and shaking gently. The phases were allowed to separate and the upper phase was discarded. This was repeated until all the ethidium had been removed. The sample was then dialysed against TE buffer at 4°C overnight to remove the caesium chloride. The

buffer was changed several times during this procedure. Following dialysis, the sample was transferred to a sterile bijou and stored at 4°C. Baculovirus should not be stored frozen and will remain infectious for in excess of one year at 4°C. In addition, viral DNA should never be ethanol precipitated as this destroys infectivity.

The concentration of viral DNA was determined by UV spectrophotometery. An OD reading was taken at 260nm and the concentration of DNA was calculated as follows:

DNA conc.= OD_{260} x 50 x dilution factor.

In this experiment, after TE dialysis, the concentration of DNA was found to be 8.5ug/ml with an $A_{260/280}$ of 2.0067.

4.1.4 LINEARIZATION OF WILD-TYPE VIRUS

The amount of linearized virus required for 1 calcium phosphate co-transfection is around 0.7-1ug (King and Possee 1992). Thus, enough virus was linearized for two reactions, namely the true co-transfection and a control. One hundred and fifty ul (1ug) of the above wild-type DNA was included in two restriction enzyme digestion reactions. Each reaction included 150ul of viral DNA, 18ul of buffer #3, 2ul of *Bsu*36I, 1.8ul of acetylated BSA, and 8.2ul of water to give a final volume of 180ul. The reaction was digested overnight at 37°C. One ul of each reaction was electrophoresed on a 1.5% agarose gel against uncut DNA and high molecular weight markers to ensure digestion had taken place. In both reactions there appeared to be full digestion (figure 4.7).

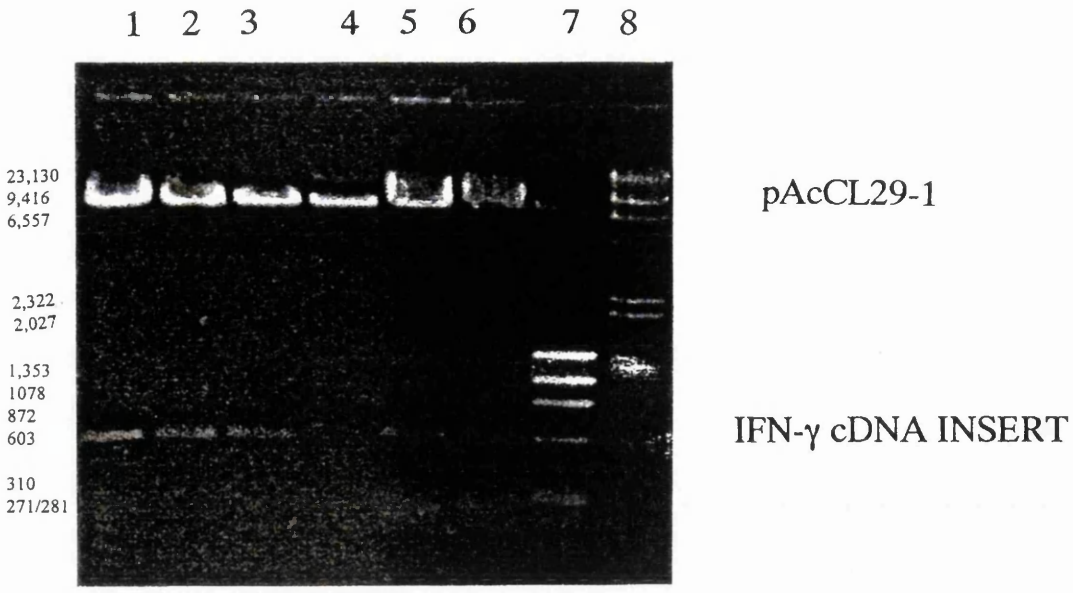


Figure 4.6 1% agarose gel: lanes 1-6 correspond to the 6 positive clones of pAcCL29-1 containing interferon cDNA insert after digestion with *Sst*1 and *Bam*H1; lane 7, DNA/*Hae* III marker, and lane 8, λDNA/*Hind* III marker.

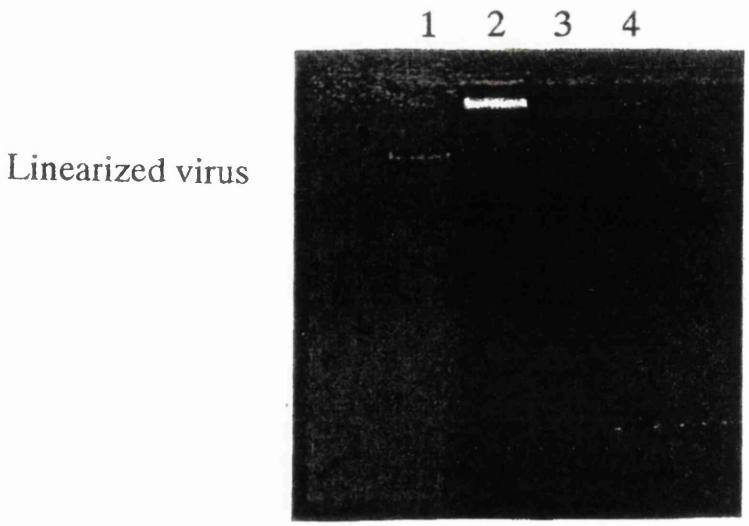


Figure 4.7 1.5% agarose gel showin- linearized wild-type virus (lane 1), uncut virus (lane 2), DNA/*Hae* III marker (lane 3) and λDNA/*Hind* III marker (lane 4)

4.1.5 CO-TRANSFECTION OF INSECT CELLS

Co-transfection of insect cells with linearized virus and transfer vector was achieved using the calcium phosphate co-precipitation method. The success of this method depends very much on forming the correct size of precipitate. To facilitate the success of this procedure, a standard calcium phosphate co-precipitation kit (Stratagene) was used. This kit employs two solutions, #1 and #2. #1 is 2.5M $CaCl_2$ and #2 is 2xBBS (pH 6.95) consisting of 50mM N,N-bis (2-hydroxyethyl)-2-aminoethanesulphonic acid and buffered saline, 280mM NaCl and 1.5mM Na_2HPO_4.

Enough 35mm dishes containing 10^6 Sf9 cells were prepared and allowed to settle for around 2-3 hours. Three co-transfection reactions were set up, namely, linearized viral DNA plus Transfer vector, linearized viral DNA alone and uncut wild-type virus alone, the latter two acting as controls. The reactions were set up as follows: 1ug of linearized viral DNA and 5ug of transfer vector were diluted with distilled, deionized water to 450ul to which was added 50ul of solution #1. Five hundred ul of solution #2 was added slowly and mixed gently by flicking the reaction tube with the index finger. The mixture was allowed to incubate at room temperature for 20 minutes. After incubation the medium was removed from the 35mm dishes and 1ml of each co-transfection mixture added to the appropriate plates. The cells were incubated for 1 hour at room temperature. Following this incubation, a further 1ml of TC 100 medium was added to the plates, which were then allowed to incubate overnight at 28°C. The following day, the medium was removed and the monolayer washed with calcium free phosphate buffered saline. Two ml of medium was then added to each plate and the plates allowed to incubate at 28°C for a further

48 hours. After 48 hours the co-transfection mixtures were harvested, filtered through a 0.45u filter and stored at 4°C. The next step was to separate recombinant and wild-type virus.

4.1.6 SEPARATION OF PARENTAL AND RECOMBINANT VIRUSES BY PLAQUE ASSAY

A detailed protocol for plaque assay has already been described (chapter 2) and this method follows much the same principles. The required number of 35mm dishes were prepared, as described, using 10^6 Sf9 cells /dish. The required dilutions of the co-transfection mixtures were then made in TC100 growth medium, viz: neat, 10^{-1}, 10^{-2} and 10^{-3}. The medium from the SF9 cells was removed and 100ul of each dilution was added to the appropriate dish. The dishes were allowed to incubate at room temperature for 1 hour. Following this incubation step, the inoculum was removed and replaced with a 2ml agarose/TC100 overlay. The dishes were then incubated at 28°C for 3 days and stained as described in Chapter 2.

Recombinant plaques are *lac-Z* negative and appear as white/clear plaques when dual stained with neutral red and X-gal. From the actual plaque assay performed, a total of 6 white plaques were visualised on the plates containing harvested co-transfection mixtures incorporating transfer vector and linearized virus. No blue plaques were observed on these plates. The linearized virus alone gave no plaques, either blue or white, and the wild-type virus alone gave a diffuse lawn of blue plaques alone on the stained plates. The 6 white plaques were picked into 1 ml of TC100 medium. 100ul of each of the plaque dilutions was re-plated onto Sf9 cells in 35mm dishes to begin plaque purification. In addition 400ul of each plaque pick solution (labelled #1 to #6) was added to 10^5 cells (in a 24 well plate). 0.5

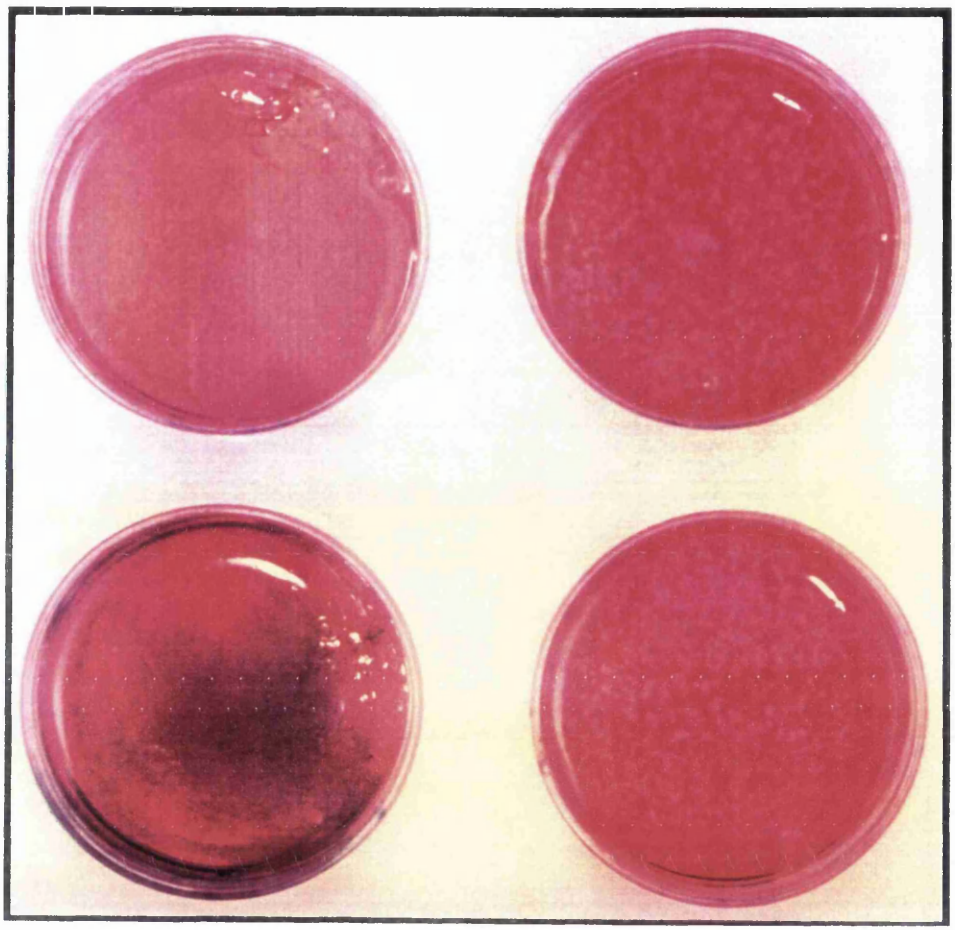
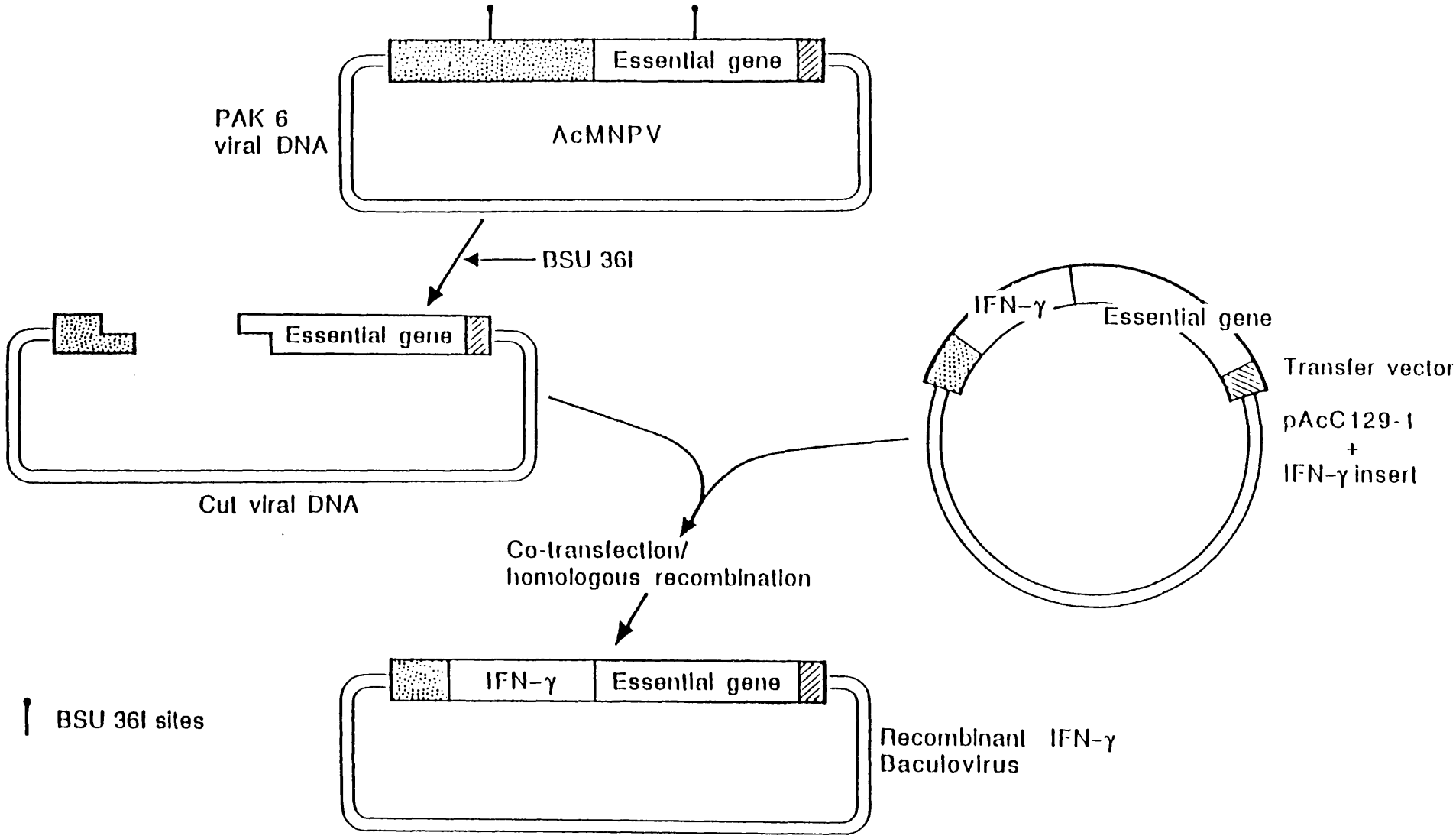


Figure 4.8 Separation of parental and recombinant viruses by plaque assay. upper plate (left) is the control plate; lower plate (left) contains wild-type virus showing diffuse blue colouration after X-gal staining; right upper and lower plates demonstrate clear staining recombinant viral plaques.

ml of TC100 medium was added to these wells and they were allowed to incubate for 3-4 days at 28°C.

After a four day incubation period the cells in the 24 well plates were microscopically examined. In each of the six wells that had been plated the cells had lysed and had floated off the base and into the medium. This indicated that viral replication was occurring. The supernatants from these cells were removed and clarified by centrifugation. The supernatants were then included in a standard cytopathic effect reduction assay to check for expression of interferon. In this crude assessment the individual supernatants (#1 to #6) were incubated with FEA cells, as described below, and subsequently challenged with feline calicivirus. $5x10^5$ FEA cells were used to seed each well in a 12 well assay plate. The cells were incubated overnight at 37°C/5%CO_2 and examined microscopically the following day to ensure that the cells had become just sub-confluent. Wells were assigned virus and cell controls. The medium was removed from the cells and 100ul of neat supernatant from samples #1 to #6 was added to the appropriate wells and allowed to incubate with the cells for 1 hour at 37°C/5%CO_2. Following this incubation, the virus innoculum was removed and replaced with an agarose overlay. The plates were then incubated for 48 hours at 37°C./5%CO_2 prior to staining with crystal violet/formalin (0.2% crystal violet in 10% formalin). The results of this assay are given in Table 4.2. All of the supernatants appeared to have some protective effect against calicivirus challenge in the assay, except for perhaps #4 and #6, but protection appeared to be stronger when supernatant #3 was used. As a consequence, the original plaque-pick #3 was used in subsequent plaque purifications.

Figure 4.9 Diagram showing the generation of recombinant baculovirus expressing the interferon gene



No. Recombinant virus	challenge with FCV
Plaque pick #1	~10 plaques
Plaque pick #2	~6 plaques
Plaque pick #3	~2 plaques
Plaque pick #4	>20 plaques
Plaque pick #5	~10 plaques
Plaque pick #6	>20 plaques
cell control	>20 plaques
wild-type viral control	>20 plaques

Table 4.2 Results of the crude CPER assay to assess whether recombinant virus was producing a biologically active protein.

4.1.7 PLAQUE PURIFICATION OF RECOMBINANT VIRUS STOCKS

As described above the original plaque-pick #3 was replaqued in a second assay and six plaques from this plate were picked into 1ml of TC100 growth medium. These plaques were put through a second round of plaque purification. Crude evidence of expression was then looked for using the same procedure, as described above. The virus from each plaque pick was expanded in 24 well plates over a 4 day period and the supernatants harvested and clarified by centrifugation. To test for expression, neat supernatant was used to protect feline fibroblasts in a standard cytopathic effect reduction assay (Maeger, 1987). However, before adding virus it was noted that the cells which had been incubated with neat supernatants had rounded up and floated into the medium. This effect was not seen in control cells which had been incubated with medium alone, medium + neat medium harvested from uninfected Sf9 cells and cells which had been incubated with medium + wild-type baculovirus PAK6. It was concluded that the baculovirus recombinants were indeed producing a product that was appearing to cause cytostasis. The greatest effect was observed in the assay wells containing final plaque-pick #6, and 100ul of this was used to infect $5x10^6$ cells in a medium sized flask in an attempt to amplify recombinant viral stocks.

4.1.8 AMPLIFICATION OF THE INTERFERON-γ CODING SEQUENCE FROM RECOMBINANT VIRUS USING PCR

PCR was used to establish that the recombinant viruses contained the interferon gene. 10ul of each of the 6 final plaque-picks were taken and

boiled for 10 minutes to lyse the viral particles. One ul of each of the boiled viruses was then included in a PCR reaction, using the original PCR primers described in chapter 3. Prior to adding the Taq polymerase the reactions were boiled again for a further 10 minutes to separate double stranded viral DNA. A total of 30 cycles were used of 94°C for 1 min; 55°C for 1 min and 72°C for 1 min. All six of the final plaques were positive for interferon-gamma cDNA. The results from the PCR amplification are given in Figure 4.10.

4.1.11 THE GENERATION OF RECOMBINANT VIRUS STOCKS

High titre working stocks of virus were generated to enable efficient, high multiplicity of infection during the production cycle. As described above 100ul of final plaque-pick #6 was used to amplify virus stocks. 5×10^6 Sf9 cells were plated in a T75 medium sized flask and allowed to settle for around 3 hours. The medium from the cells was then removed and 100ul of plaque pick #6 and 1ml of medium was added to the flask. Following a 1 hour incubation period, this medium was removed and replaced with 15ml of fresh medium. The cells were then incubated for 7 days at 28°C. Following the incubation, the supernatant was harvested and clarified by centrifugation. The supernatant was then included in a plaque assay to determine the viral titre (described in Chapter 2). This stock of virus had an apparent titre of 5×10^{10} PFU/ml and was subsequently designated the master stock of recombinant virus. This stock was then used to prepare a working stock of virus. Initially 10^5 Sf9 cells were seeded into a 500ml spinner culture flask containing 100ml of TC100 medium. The cells were allowed to go through two doublings (i.e. they were in logarithmic growth) and were subsequently infected with 0.1 pfu/cell of the master stock recombinant virus. The cells were incubated at 28°C for 1 week. The

medium was then clarified by centrifugation and filtered through a 0.2u filter and was then assayed for virus using the standard plaque assay technique as described. This virus stock had a titre of 3 x 10^8 PFU/ml and was used as the working stock of virus for subsequent protein expression. Figure 4.11 shows the sequence of events in generating virus stocks.

4.1.10 TIME COURSE STUDY OF INTERFERON PROTEIN PRODUCTION

For this experiment It was necessary to establish to optimum time (peak interferon concentration) to harvest supernatants from the baculovirus cultures. This would allow a set protocol for protein production to be established which would reduce variation between subsequent protein batches. Sf9 cells were grown to a sufficient density and used to seed a 100ml spinner flask at a density of 1 x 10^5 cells/ml. After seeding the cells in spinner culture, they were allowed to go through two doublings (as determined by cell counting) and then recombinant virus was added at a concentration of 10 pfu/cell when the cells were at 5 x 10^5 cells/ml and were still in logarithmic growth. The high pfu was used to ensure that the cells were all infected at the same time. 5ml aliquots were removed at 24 hour intervals and the protein production estimated by the standard CPER assay using calicivirus and feline fibroblasts (see below).

4.1.11 PRODUCTION OF RECOMBINANT PROTEIN

Following the time course study, a set protocol for protein production was established to minimise any variations between supernatant batches. The

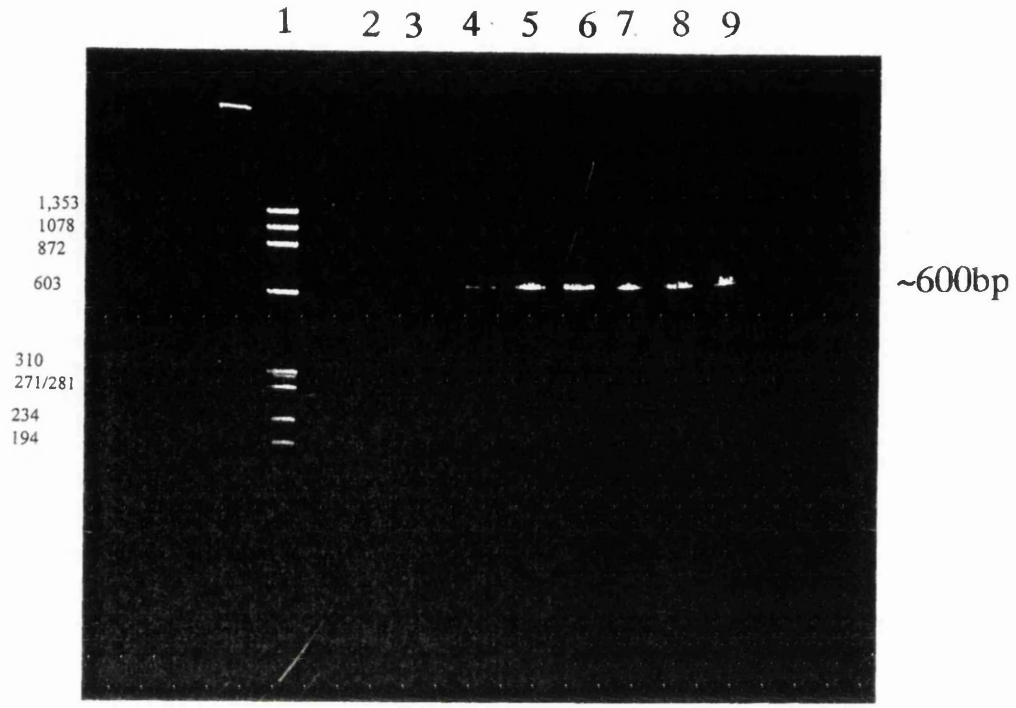


Figure 4.10 6% Polyacrylamide gel showing amplification of the interferon-γ coding sequence from recombinant virus using PCR. DNA/*Hae* III marker (lane 1), negative control (lane2), positive control (lane 3) and amplification from plaques 1 to 6 (lanes 4 to 9).

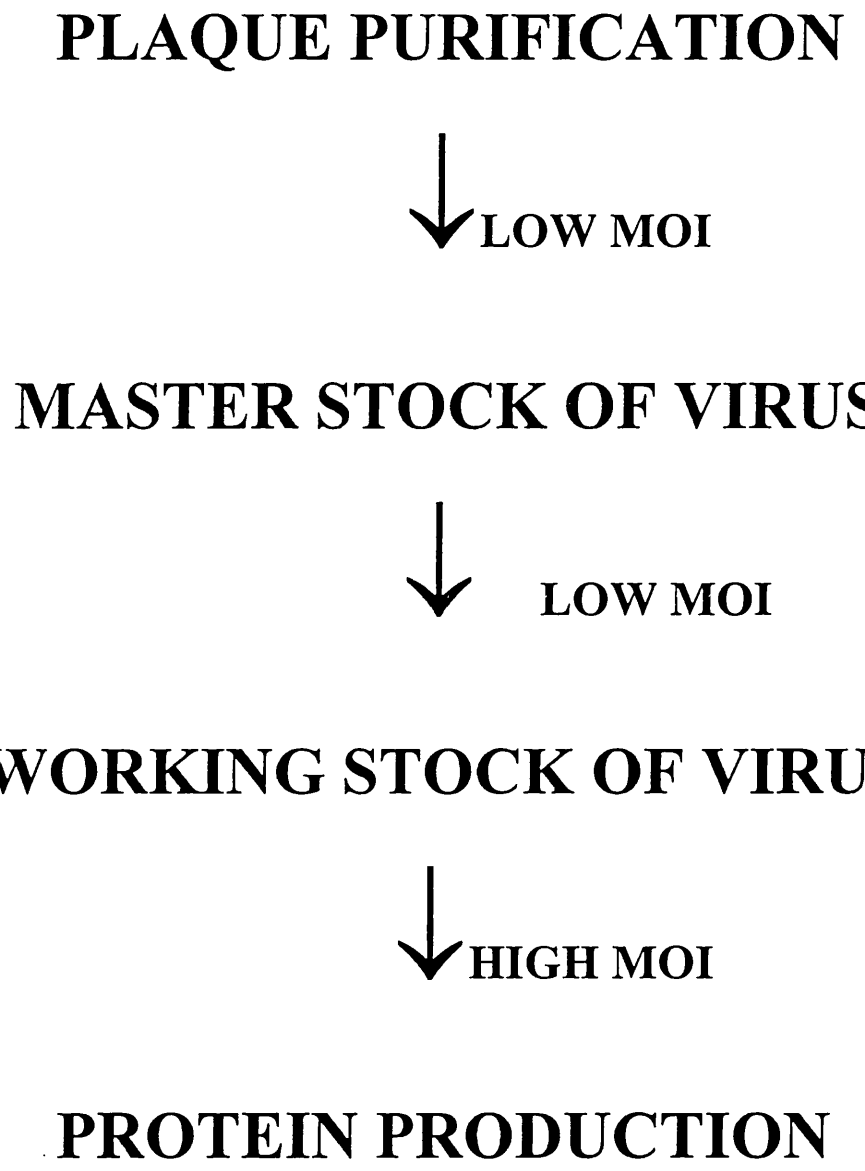


Figure 4.11 The generation of recombinant virus stocks

protocol consisted of infecting cells which were in logarithmic growth (cell viability>98%) and at a concentration of 5 x 10^5 cells per ml. For the production of the first batch of interferon protein, 1 x 10^7 Sf9 cells were used to seed 100ml of TC100 medium in a 500ml spinner culture flask. The cells were allowed to go through two doublings before being counted and subsequently infected with recombinant virus. The cells were infected when they had reached a concentration of 5 x 10^5 cells/ml (after 48 hours) and the infecting dose of virus used was 10 pfu/cell. The supernatant was harvested 5 days post infection and was subjected to centrifugation (1500 rpm/10mins) to remove cell debris. The product was then filtered through a 0.2um filter and stored at -20°C. This first batch of crude interferon protein was designated DA1 and was subsequently used in the bioassays described below.

In addition to the production of DA1, a second batch of crude interferon was produced, designated DA2. The conditions for production were as described above but the protein was produced by the infection of High Five (Invitrogen) insect cells (described in Chapter 2). These cells are reported to increase protein production 10 fold.

4.2 EXPERIMENT TO DEMONSTRATE BIOLOGICAL ACTIVITY OF RECOMBINANT INTERFERON-γ PROTEIN

4.2.1 INTRODUCTION

Interferons are related by their ability to induce the anti-viral state in cells, they are not themselves anti-viral agents. The cellular response to interferon and the subsequent inhibition of replication of an infecting virus, which can be measured, forms the basis of the cytopathic effect

reduction assay for interferon (CPER). The degree of inhibition of virus replication is related to the potency of the interferon preparation or sample; that is a measure of the degree to which an individual interferon preparation is able to protect cells of a particular type against productive infection by a virus of a certain type. Potency measurements are relative, not absolute, and depend on: the proportion of interferon molecules in a particular preparation which are biologically active in the antiviral assay; the sensitivity of the cell line to interferon; and the sensitivity of the virus to the anti-viral state established in the assay cells. Cell culture media and conditions add a further dimension to the complexity and variability of the antiviral assay. The potency of interferon preparations has therefore been quantified by the arbitrary assignment of anti-viral units defined in particular cell-virus systems, usually where some kind of end point can be observed, and expressed as the reciprocal of the dilution of an interferon preparation required to produce the end-point. The World Health Organisation (WHO) has established a number of human IFN international reference preparations (standards), the use of which allows individual laboratories to calibrate their assays in relation to the assigned potencies of these international reference preparations (IRP's) and thus to compare the results of their assays with other laboratories.

4.2.2 THE CYTOPATHIC EFFECT REDUCTION (CPER) ASSAY

This is by far the cheapest and most common form of assay to demonstrate biological activity of interferon preparations. In this type of assay cells are protected with serial dilutions of the interferon preparation and then challenged with a cytopathic virus. The cells are then cultured until maximum cytopathic effect is seen in virus control cells. At that point, the end point for the interferon sample, usually assigned to be the well in

which 50% of the cells are living, is either determined microscopically or following staining with a vital dye. Such assays produce sharply increased CPE as the interferon is diluted out and thus it is not always easy to construct a satisfactory dose-response curve. Often, only two or three points are on the rectilinear portion of the dose response curve. A modification of the CPER assay is the plaque reduction assay. This is a similar assay but is carried out using a reduced multiplicity of infection (moi) of the challenge virus and a suitable overlay material. The end-point chosen for this type of assay is usually 50% plaque reduction compared to the virus control. Other assays to measure the antiviral action of interferons rely on the measurement either of infectious virus or particular viral components. The plaque reduction assays were not used in our experimental work.

In this experiment to measure the antiviral action of the baculovirus supernatant derived from the time course study, a CPER assay was used. This CPER assay was used to demonstrate protection of feline embryonic fibroblasts (FEA cells) against challenge with Feline calicivirus after their incubation with supernatants.

Initially a stock of feline calicivirus was prepared in FEA cells and the titre determined as described in Materials and Methods. In parallel, a working bank and master bank of FEA cells were grown in tissue culture and subsequently stored under liquid nitrogen. A working stock vial of cells was thawed and revived, as described in Chapter 2, and amplified to be used in the assay. In the 24 well plate system, FEA cells were harvested and counted from tissue culture flasks. Wells were assigned cell controls and virus controls. The cells were used to seed 24 wells at a concentration of 2×10^5 cells/well in 1ml of medium (Dulbecos MEM/

10%FCS/glutamine/NaHCO3/penecillin/streptomycin and they were allowed to incubate for 24 hours at 37°C/5% CO_2. Following the incubation step the cells were examined microscopically to ensure that they had formed a sub-confluent monolayer. Subsequently, dilutions of the interferon preparations derived from days 2 through 5 in the time course study were then added to the appropriate wells. The cells were then allowed to incubate at 37°C for a further 24 hours. The control wells were incubated with medium alone or wild-type baculovirus. Following incubation, the challenge calicivirus was added to the medium overlaying the cells and the cells were incubated for a further 1 hour at 37°C/5% CO_2. The concentration of virus added was sufficient to cause 100% CPE, as determined when the virus was titrated, and, in this experiment, both 1 PFU and 4PFU per cell were used. Following the 1 hour incubation step, the medium was removed and replaced with fresh medium. The cells were examined daily until maximum cytopathic effect was seen in the virus controls (this was 48 hours). The medium was then removed and the cell layer stained using crystal violet/formalin (0.2% crystal violet in 10% formalin). The results of the time course study are given in Table 4.3. The assay was repeated for the day 5 preparation and, for this assay, the dye in the stained wells corresponding to dilutions of the day 5 harvest was eluted using 0.5% NaCl in 50% ethanol, and the optical density at 595nm was measured spectrophotometrically. The results of this are shown in table 4.4 and represented graphically in Figure 4.12.

An identical assay procedure was performed for the sample DA1 using the 24 well system. However, the assay was difficult to reproduce for this sample.

4.2.3 CPER ASSAY USING VESICULAR STOMATITIS VIRUS

Virtually all cells and cell lines will show some sensitivity to interferon-gamma of the appropriate species, but not all are suitable for use in an assay system (Meager, 1987). Interferon-gamma is highly species specific and thus, for this experiment, the feline FEA cell line was used with, initially, feline calicivirus as the challenge virus, in the CPER assay. The choice of viruses which are sensitive to interferon induced protection is wide but, for the anti-viral assays, only a few viruses have proved suitable. This has been established by interferon researchers who have developed the antiviral assay for the human and murine interferon systems. The three most common viruses used in the human and murine assays are: Encephalomyocarditis virus (EMCV), Vesicular stomatitis virus (VSV), and Semliki Forest virus (SFV). All three viruses are known to work well in the interferon assay system. Previously, Zack, 1986(cited in Khan, 1992) had reported the successful determination of interferon-γ titres using the CPER assay utilising VSV and feline embryonic fibroblasts.

Vesicular stomatitis virus is a rhabdovirus and causes a disease in ungulates and horses which is clinically indistinguishable from Foot and mouth disease. As a consequence, the use of vesicular stomatitis virus is restricted and is confined to laboratories which have special licence and category II facilities. Three serotypes of the virus are recognised: VSV-Indiana (VSV-1), VSV-New Jersey (VSV-NJ) and VSV-Algoas (VSV-A). VSV is capable of replicating in a wide range of cells derived from vertebrate and invertebrate hosts and serological surveys have shown that it infects man and arboreal and semi-arboreal hosts in addition to domestic animals (Reif *et al,* 1987). Vesicular stomatitis virus is a group 2 pathogen as defined in the *Categorisation of Pathogens According to Hazard and*

Categories of Containment 1990. In addition the organism is covered by the Specified Animals Pathogen Order 1993 which prohibits the holding or use of the organism unless a licence has been issued by the Scottish Office's Agriculture and Fisheries Department (SOAFD).

In the initial stages of the expression work there was no licence to use the VSV, and thus the calicivirus system was used. There was no evidence at this stage that any interferon preparation would protect FEA cells against challenge with calici virus. The calicivirus assay was used in a rather crude manor to initially assess whether the baculovirus system was producing interferon, and then used it to assess the profile of interferon production with time from Sf9 cells infected with recombinant virus. The advantage of the system was that it did not require category II facilities. While initial assays showed protection, the assay system became rather variable and it was difficult to reproduce a working assay for the sample DA1. As a consequence, it was decided to use the VSV assay system with FEA cells to try and standardise the assay procedure.

A licence was granted to use a mutant VSV and all assay procedures were carried out in a category II laboratory. The VSV mutant used in this assay was designated VSV*ts*E2 and is derived from the New Jersey strain of VSV. This mutant strain contains two independent mutations within the NS gene (Rae and Elliott, 1986) and is incapable of producing diseases in domestic livestock. In addition, because it is temperature sensitive, it is unable to sustain a transmission cycle between animals. The primary virus stock was kindly provided by Dr. Richard Elliott of the MRC Institute of Virology.

The assay procedure was essentially the same as that described for the calicivirus assay. The optimum temperature for virus replication is 31°C and, initially, FEA cells were cultured at this temperature to ensure that they would still grow. The cells appeared to double normally and were subsequently infected with the VSV mutant at a rate of 0.1 PFU/cell to amplify virus stocks. The stock of virus was subsequently titrated using a standard plaque assay procedure as described in materials and methods. The 24 well plate assays system was set up as described for the calici virus assay. The only differences being that, after incubation with interferon dilutions of DA1, the cells were challenged with VSVtsE2 at a rate of 4 PFU/cell and incubation was carried out at 31°C/5%CO_2 in a category II laboratory.

Subsequently, the assay procedure was repeated using a 96 well format for the sample DA1 and DA2. The assay procedure for the 96 well format was essentially identical to the 24 well system. The only difference being that the cells were added to the wells at a concentration of 2 x 10^4 cells/well in 100ul of medium. VSV virus was added at a concentration of 4pfu/cell and the assay was carried out in triplicate. Staining was carried out by the addition of 100ul of Amido Blue Black (0.05% solution in 9% acetic acid with 0.1M sodium acetate) to each well and leaving them for 1 hour. The cells were fixed for 1 hour by the removal of the well contents and the addition of 100ul of formalin acetate (10% formaldehyde solution in 9% acetic acid with 0.1M sodium acetate. After 1 hour, dye elution was carried out by the removal of the well contents and the addition of 100ul of 0.38% sodium hydroxide to each well. After a further hour of mixing, the optical density at 595nm was measured spectrophotimetrically. The O.D. measurements are shown in table 4.5 and are represented graphically in figure 4.13. The staining, fixing and elution protocols were taken from Balkwill, 1991.

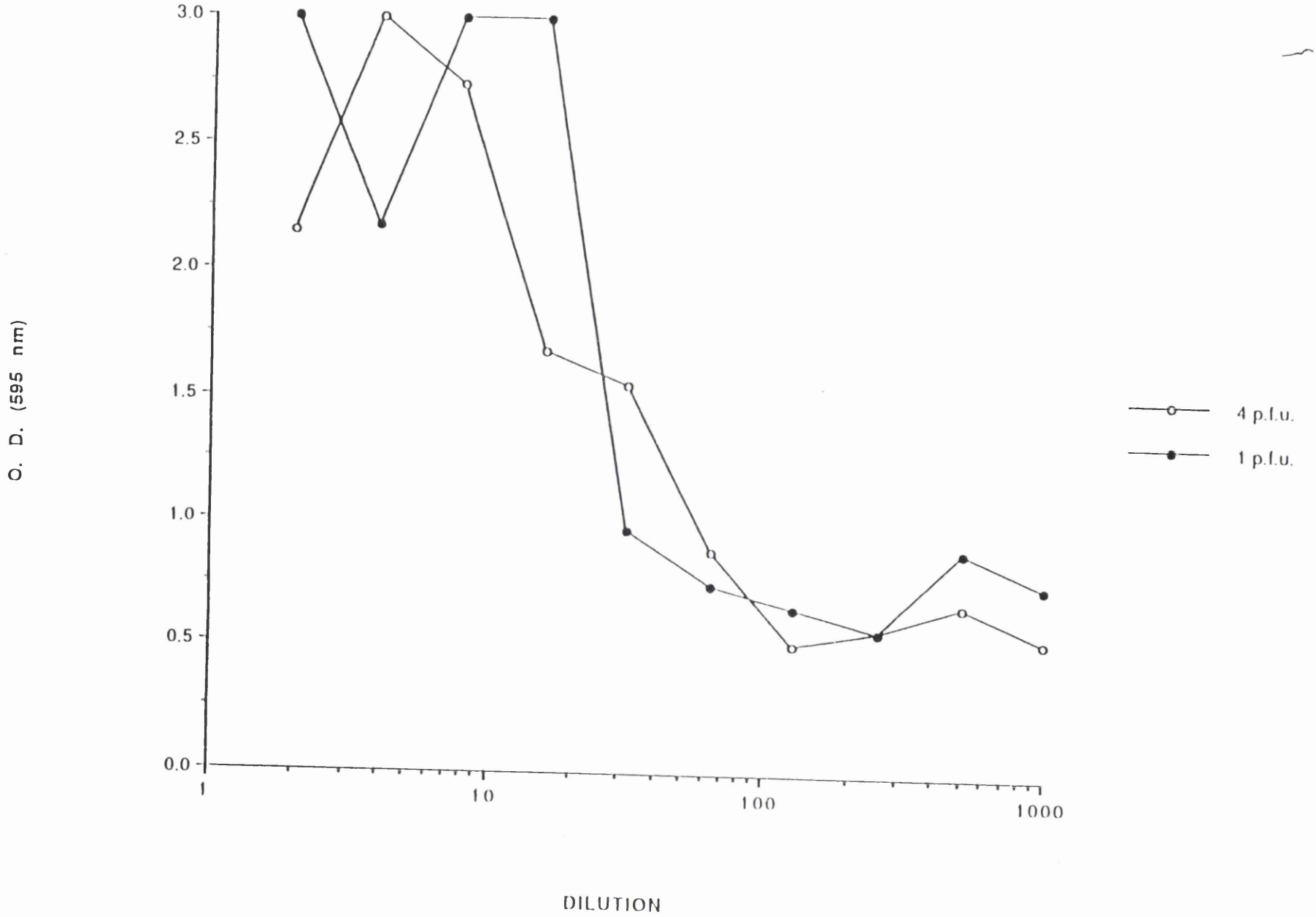
Dilution	day2	day3	day4	day5
1:4	+++	+++	+++	unreadable
1:16	++-	++-	+++	unreadable
1:64	- - -	- - -	++-	unreadable
1:256	- - -	- - -	- - -	unreadable
1:1024	- - -	- - -	- - -	unreadable

Table 4.3 Results of the time course study of recombinant protein production. (+) indicates protection against challenge with calicivirus where (+++) = 100% protection.

No.	Dilution	Day 5 Sample (O.D.)
1	1:2	3.0
2	1:4	2.179
3	1:8	3.0
4	1:16	3.0
5	1:32	0.968
6	1:64	0.745
7	1:128	0.659
8	1:256	0.568
9	1:512	0.893
10	1:1024	0.755

Table 4.4. O.D.(595) readings from the day 5 calicivirus assay in the time course study.

Fig. 4.12 Graph showing the antiviral effect of recombinant interferon in the calicivirus based CPER assay



4.2.4 RESULTS OF THE TIME COURSE STUDY

The results of the time course study are shown in Table 4.3. Protein production was evident from day 2 but maximum effect was seen on day five. From these results it is evident that the protein production begins early in infection, i.e. from 24-48 hours, and the protein is accumulating in a stable fashion through to day 5. The results from the day 5 wells were not included in Table 4.3 because, before addition of virus to these cells, the cells were rounded and had floated from the monolayer. As a consequence, this day 5 assay was repeated and the results plotted as O.D measurements. The O.D. measurements for the day 5 dilutions are shown graphically in Figure 4.12. The variability in O.D. measurements at high concentrations of interferon may be due to the toxic effects of interferon causing cytostasis. There is a sharp cut off point between protection and non-protection in the dilutions shown, with few points on the rectilinear portion of the graph. From the graph, the dilution which gave 50% protection was 1:32.

4.2.5 RESULTS OF THE VSV CPER ASSAY

The mean optical density measurements of the 96 well VSV assays performed for DA1 and DA2 are given in table 4.5 and are shown graphically in figure 4.13. The maximum O.D measurement shown was obtained from the cell controls and the minimum value was obtained from the virus controls. For both samples, DA1 and DA2, there is a gradual cut off between protection and non-protection with more points on the rectilinear portion of the graph than in the calici virus assay. For the sample DA1 the dilution which gave 50% protection was found to be 1:8583 and, for DA2, the value was 1:1968. Thus, for sample DA1 and

No	IFN Dilution	DA1 (O.D)	DA2 (O.D)
1	1:10	2.732	2.256
2	1:100	2.559	2.279
3	1:1000	2.405	2.059
4	1:10000	1.817	2.006
5	1:100000	1.563	1.250

CELL CONTROL: O.D 2.888

VIRUS CONTROL: O.D 0.994

Table 4.5 O.D (595) measurements for samples DA1 and DA2 in the 96 well VSV assay.

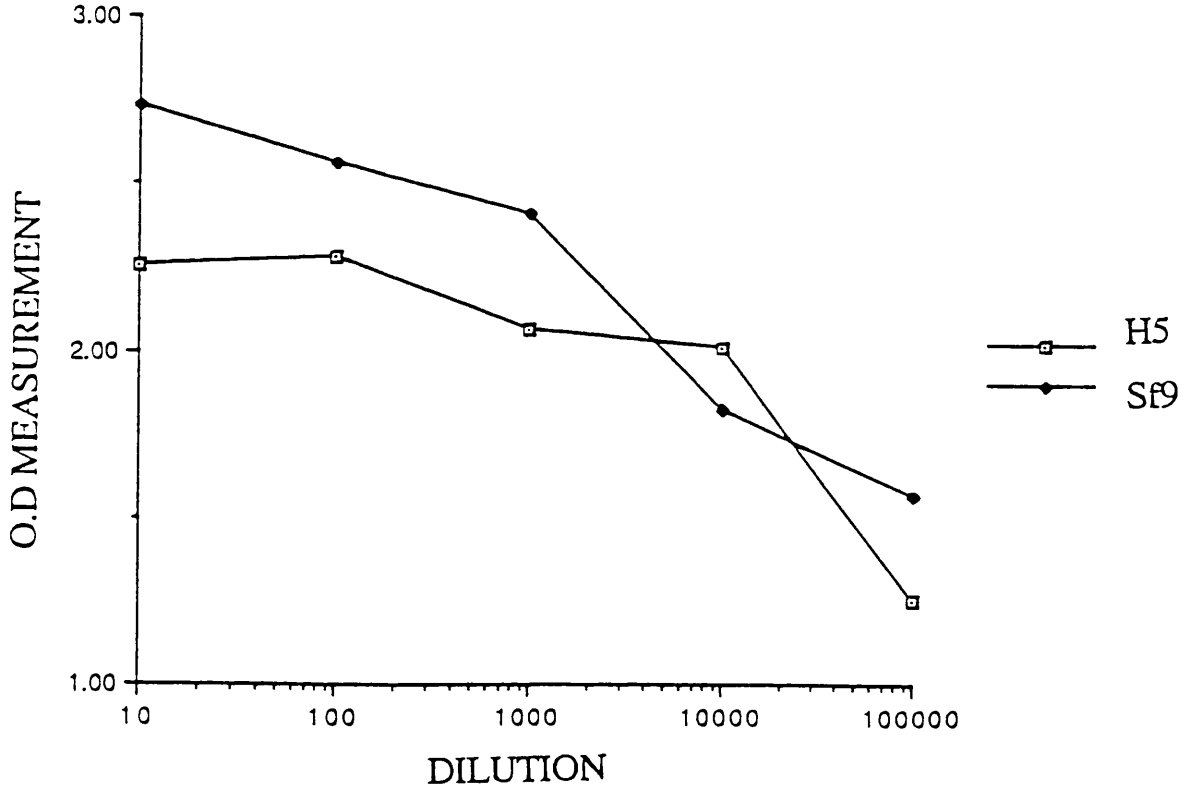


Fig. 4.13 Graph showing the results of the VSV based CPER assay for sample DA1

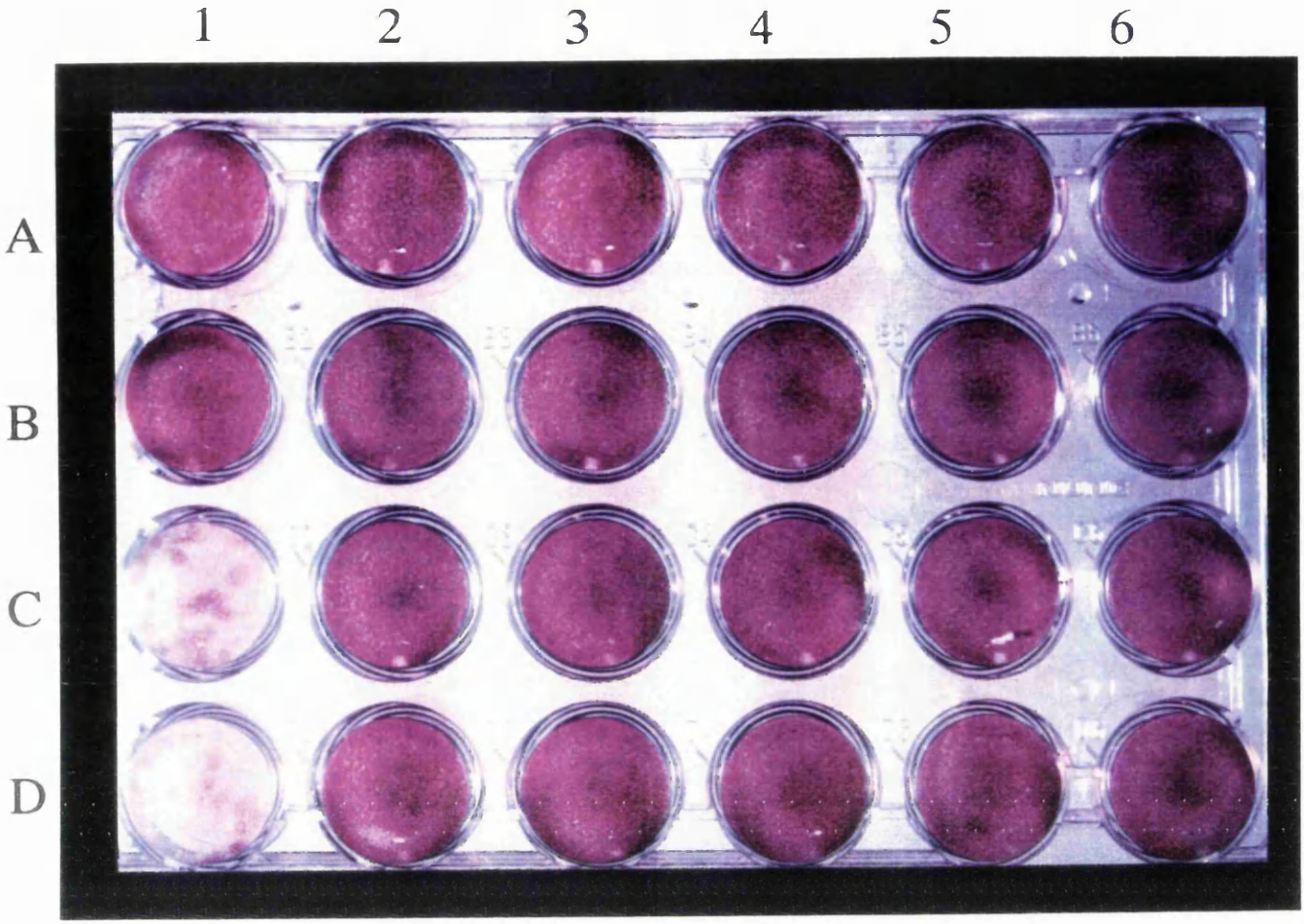


Figure 4.14: VSV/Interferon assay using 24 well format. Row A and B, Column 1 are cell controls. Row C and D, column 1 are virus controls. The remainder of row A is the assay using Interferon sample DA1 showing doubling dilutions from columns 2 to 6 (1:4 to 1:64). Row B is a duplicate of Row A. Rows C and D are as for A and B but using the sample DA2. It is clearly demonstrated that the interferon samples are protecting the cells from VSV challenge at these dilutions.

using this assay protocol, the concentration of interferon, in terms of laboratory reference units, was 8583/ml. Therefore, the Sf9/baculovirus system produced 8.583 x 10^6 LRU of feline recombinant interferon per litre. The High Five/ baculovirus system appears to have produced a lower value of 1.968 x 10^6 LRU/litre.

4.2.6 MHC CLASS II ANTIGEN INDUCTION ASSAY

As discussed in Chapter 1, one of the most important biological functions of interferon-gamma is the up-regulation of MHC class II antigens. This assay was used to demonstrate that the baculovirus derived recombinant feline interferon gamma, was able to up-regulate MHC class II antigens on the feline continuous T cell line, designated F422. The assay has been described in Current Protocols in Immunology (1994), and King and Jones (1983). The F422 cell line is discussed in Materials and Methods.

Initially F422 cells were grown in tissue culture and subsequently centrifuged, washed and resuspended in complete medium to a concentration of $3x10^5$ cells/ml. Two fold serial dilutions of the interferon preparation DA1 were made in 12 well tissue culture plates. Wells were also assigned to virus and cell controls. 1ml of the cell suspension was added to each well and the cells were allowed to incubate at 37°C/5%CO_2 for around 48 hours.

Following incubation, the cells in each well were divided in two to give a test (+) and negative control (-) for the subsequent flow cytometry analysis. The test cells were labelled with MHC class II anti-cat monoclonal antibodies (IgG_1) and the negative control cells were labelled with CD8 anti-cat monoclonal antibodies (IgG_1). The MHC class II

antigen is inducible on F422 cell lines (Willett-unpublished results) whereas the cells are CD8 negative.

Following incubation, the cells were transferred to labelled 5ml falcon tubes. To each tube was added 2ml of PBS/0.1%BSA/0.01% Sodium azide to wash the cells. The tubes were then centrifuged at 1500 rpm for 5 minutes at 4°C. and the supernatant discarded. 50ul of MHC class II monoclonal antibody was then added to the (+) cells and 50ul of CD8 monoclonal antibody was added to the (-) cells. Both the anti-cat MHC class II monoclonal antibody and the anti-cat CD8 monoclonal antibody were derived as supernatants from hybridoma cells and the amount used in this experiment was that advised by Willett, personal communication. The cells were incubated on ice for 15 minutes. Following incubation, The cells were washed again using 2ml PBS/BSA/azide, centrifuged and the supernatant discarded. 50ul of sheep anti-mouse IgG FITC conjugate (Sigma) was then added to each tube and the tubes allowed to incubate on ice for 15 minutes. The cells were then washed with 2ml of PBS/BSA/azide, centrifuged and the supernatant discarded. Subsequently, cell analysis was carried out using flow cytometry (EPICS cell sorter (Coulter Electronics, Luton), or the cells were fixed using 1% paraformaldehyde and analysed at a later stage. Isotype matched controls were used to set an analysis gate such that <1% were positive. Five thousand events were collected in LIST mode and percentage positive were calculated relative to isotype matched negative controls.

4.2.7 RESULTS OF MHC CLASS II INDUCTION ASSAY

The MHC Class II induction assay was performed on the baculovirus interferon preparation designated DA1. This is the same preparation that

had been used in the VSV assay described above and had titrated to give a value of 8583 laboratory units per ml in this assay. The results of the flow cytometry analysis is given in Figures 4.15 to 4.19. The gate labelled H in the figures represents background fluorescence, while the gate labelled L represents the percentage of cells which are demonstrating MHC class II up-regulation. The anti-cat MHC class II monoclonal antibody was used to demonstrate any changes in MHC class II expression on the cell line. The anti-cat CD8 monoclonal antibody was used to measure non-specific binding of mouse monoclonal antibody. The cell controls had been incubated in the absence of interferon-gamma. The virus controls were cells which had been incubated with Sf9 cell supernatants containing wild-type PAK6 baculovirus at a 1:32 dilution. Both of these controls were labelled with anti-cat MHC class II and anti-cat CD8 monoclonal antibodies in the same manner as the cells incubated with the interferon preparations. The results of the flow cytometry analysis are shown in Table 4.6. The assay procedure was carried out in duplicate and the figures shown in Table 4.6 are mean values.

All of the cells which were labelled with the anti-cat CD8 monoclonal antibody showed negligible fluorescence. Cell controls and wild-type virus controls showed base line fluorescence when labelled with the anti-cat MHC Class II monoclonal antibody indicating that there is some expression of MHC Class II antigen on these cells in the absence of interferon-gamma. However, incubation of the cells with interferon dilutions clearly enhances the expression of this antigen on these cells. This is shown by the marked shift in fluorescence as shown in Figure 4.17. Interestingly, at high concentrations of interferon (1:4 to 1:32), while MHC Class II was still up-regulated, the degree of up-regulation was less than for those cell incubated with lower dilutions. This may be because at

higher concentrations there is a higher concentration of TC100 medium which may be altering the pH or nutrient concentrations and thus the cells will not be growing under optimum conditions. The MHC class II induction assay seem to be very sensitive and up-regulation is still seen at dilutions of interferon DA1 of 1:4096.

In addition to measuring the degree of expression of MHC Class II antigens, the flow cytometry also allowed the analysis of cell populations. Figure 4.19 shows the forward and side scatter analysis of the cell populations in each sample which is a distribution analysis based upon cell size (forward scatter) and cell granularity (side scatter). With increasing dilution of the interferon sample the population of cells in gate L appears to alter. At higher concentrations of interferon (1:4, 1:8), the majority of the cells show low forward scatter and increased side scatter which may indicate that there may be a high proportion of non viable cells or cells undergoing apoptosis (cell granularity but small size) in these samples. As the interferon is increasingly diluted, the population of cells appears to shift to form a majority of cells showing increased forward scatter and decreased side scatter. This suggests that this is a more viable, activated population of T cells and correlates with the increased MHC Class II expression at lower interferon dilutions. In one study by Novelli *et al* (1994), the level of interferon-gamma receptor expression on the surface of a T cell population, was shown to influence whether interferon-gamma caused proliferation or apoptosis. High level expression of the receptor appears to be a marker for apoptosis. One can hypothesise that, in this experiment, a high concentration of interferon-gamma has increased receptor expression on the T cells and is promoting apoptosis. At higher dilutions, the level of receptor expression may be lower, and here the interferon-γ is promoting proliferation and activation. This system may be

Dilution	%fluorescence MHC II	%fluorescence CD8
1:4	31.1	negligible
1:8	38.9	negligible
1:16	44.9	negligible
1:32	63.55	negligible
1:64	69.9	negligible
1:128	68.85	negligible
1:256	69.3	negligible
1:512	66.95	negligible
1:1024	67.6	negligible
1:2048	70.55	negligible
1:4096	71.6	negligible
cell control	1.1	0.7
virus control	1.75	0.5

Table 4.6 Results of FACS analysis for the MHC Class II induction assay performed on the sample DA1.

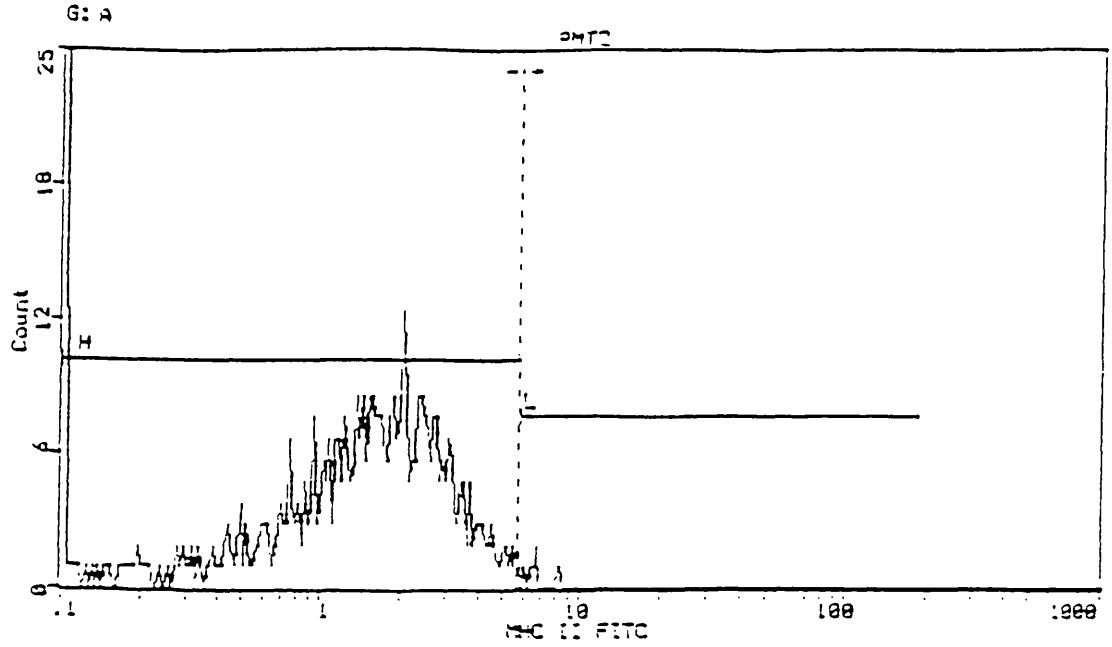


Fig. 4.15 FACS analysis of cell control: Cells incubated without interferon and subsequently labelled with anti-cat MHC Class II antibody. All of the cells are in gate H.

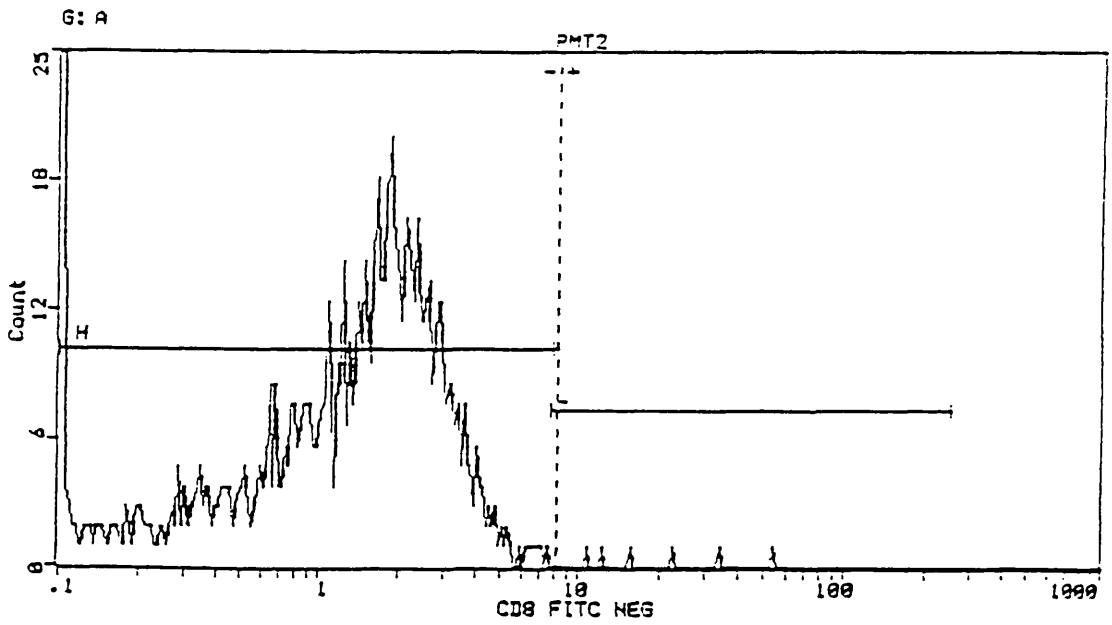


Fig. 4.16 FACS analysis of cells, incubated with interferon, but labelled with anti-cat CD8 antibody. The cells are FITC negative.

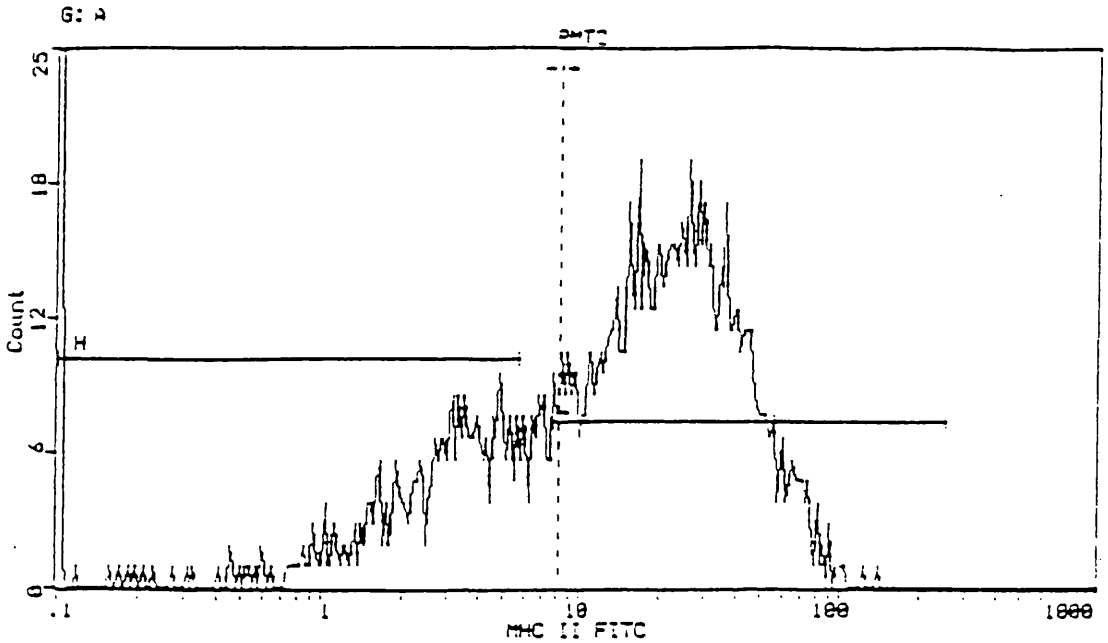


Fig. 4.17 FACS analysis of cells, incubated with 1:32 dilution of interferon, and subsequently labelled with anti-cat MHC Class II antibody. A clear shift of cells from gate H to gate L is demonstrated as compared to cells which have not been exposed to interferon.

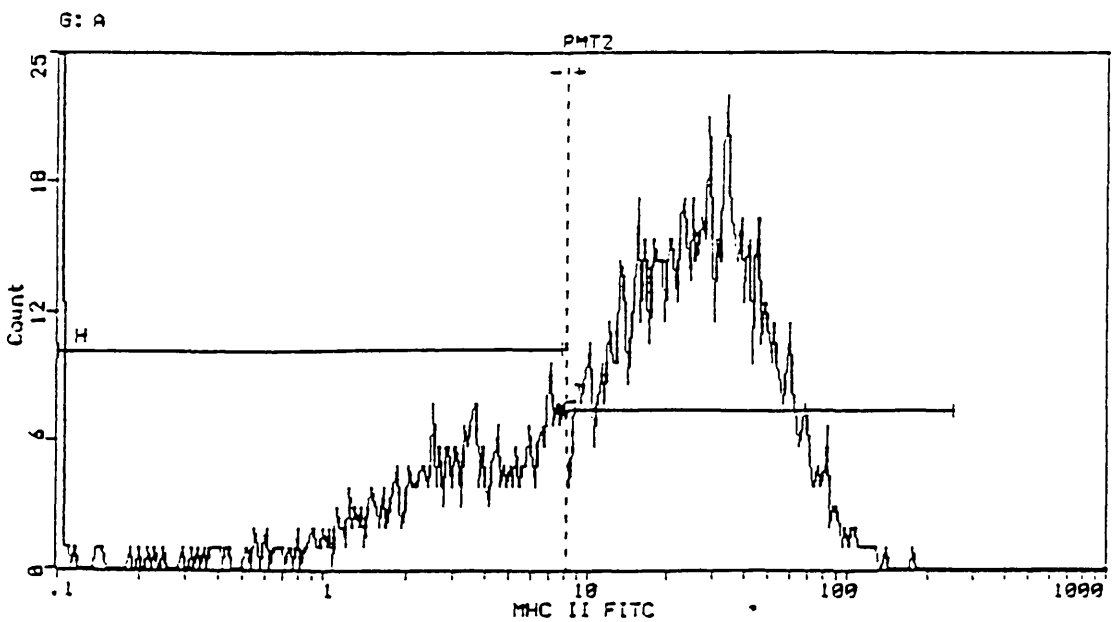


Fig. 4.18 FACS analysis of cells, incubated with interferon at 1:4096 dilution, and subsequently labelled with anti-cat MHC Class II antibody.

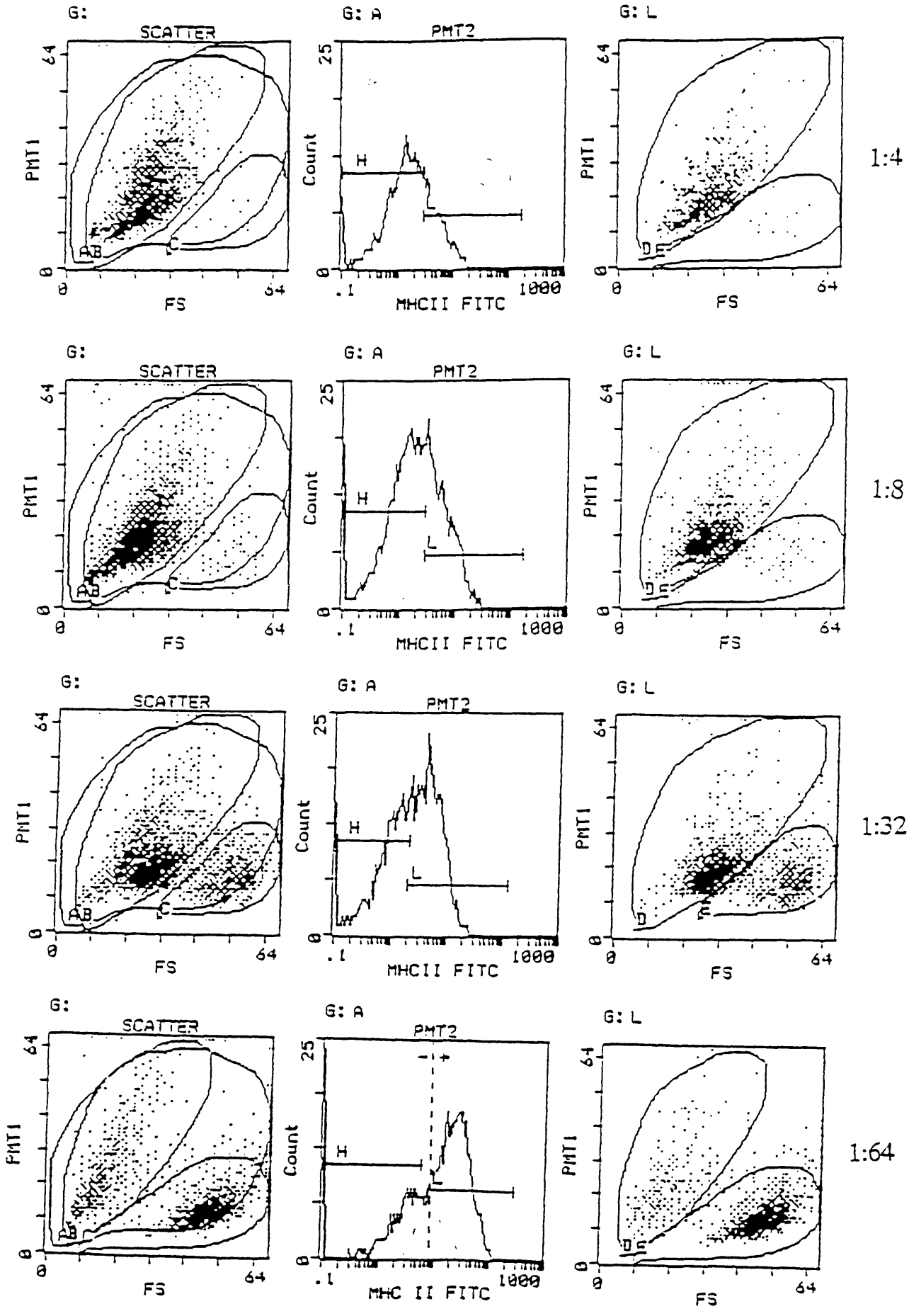


Figure 4.19 FACS analysis of cell poulations in the MHC Class II induction assay. The change in cell population in gate L, with increasing dilution of interferon is clearly demonstrated.

further complicated by environmental factors such as the higher concentration of TC100 medium in the wells which contained higher concentrations of interferon.

4.3 DISCUSSION

This chapter has described how cDNA encoding feline interferon-γ was cloned into the Baculovirus genome and stably expressed to produce a biologically active protein. Several expression systems have been used to express interferon-γ from other species. In man, interferon-γ was originally cloned in an expression system involving xenopus oocytes (Devos 1982) and, in the murine system, interferon-γ was originally cloned by the expression of the protein in COS cells and was subsequently expressed in *E.coli* (at a level of $5x10^4$ U/ml).

Since the original cloning experiments for human interferon-γ were performed, the protein has been expressed in a number of systems which include Yeast (Dernyk *et al* 1983), *E.coli* based systems (Simons *et al* 1984) giving levels of $0.5\text{-}2.5x10^4$ U/ml and $5x10^4$ U/ml, respectively, and CHO cells (Scahill *et al* 1983) and (Mory *et al*, 1986) which gave levels of expression in the order of $1\text{-}2 \times 10^8$ units/mg of protein.

The original interest in baculoviruses was based upon their potential as pesticides. However, studies involving gene replication by the virus and, in addition, the demonstration that removal or replacement of the very late polyhedrin gene did not affect replication, lead to an interest in baculoviruses as expression systems for cloned genes. The polyhedrin protein is produced at high levels and thus it was assumed that replacing the polyhedrin gene with another cloned gene would result in high levels

of transcription of the new gene and high levels of protein expression. These initial theories have been realised in recent expression experiments.

The first reports of the use of baculovirus as expression systems were published by Smith *et al* (1983) and Pennock *et al* (1984) who used AcMNPV to produce β-interferon and β-galactosidase, respectively, in SF9 cells. Maeda *et al* (1985) also expressed β-interferon using BmNPV system in the silkworm. In the succeeding years a wide variety of recombinant proteins have been expressed using this system (reviewed by King and Possee, 1992), including feline interferon-α (Sakurai *et al*, 1992) and human interleukin-2 (Smith *et al* 1985). Smiths original experiments, in which he expressed human interferon-β, were very encouraging. Interferon-β had already been expressed in an *E.coli*-based system and in mammalian cells. The highest levels of β-interferon production in these systems were $1x10^4$ U/ml and $5x10^4$ U/ml, respectively (cited in Smith *et al*, 1985). However, in Smith's baculovirus system the interferon was secreted at levels of $5x10^6$ U/ml. In addition the protein was glycosylated and the data indicated that the signal peptide at the amino terminal end of the primary translation product was removed. In addition, the interferon had no anti-viral effect on the Baculovirus.

The high levels of expression of foreign genes reported for baculovirus systems, the fact that foreign genes undergo post translational modification, and the ability to scale up protein production encouraged the use of this system for expression of feline recombinant interferon-gamma. The ultimate aim for the project was to take the recombinant protein towards clinical application. While glycosylation is unimportant for the biological activity of the interferon protein, it may be important in terms of extending the biological half-life of the protein. This is obviously an

important factor when considering therapeutic potential. Thus, baculovirus expression could potentially yield a product with high specific activity, potentially greater in-vivo half-life than yeast or *E.coli* derived proteins, and be easily scaled up to producing large amounts of protein for clinical use.

In this experiment, the cDNA encoding feline interferon-γ was successfully cloned into the transfer vector pAcCl29-1 and subsequently transferred into the baculovirus genome by the process of homologous recombination. The major problems in baculovirus expression systems have been concerning the yield of recombinant viruses and the screening process for recombinant viruses. The use of linearized PAK 6 wild-type virus and neutral red/x-gal in the screening process greatly facilitated the isolation of recombinant virus.

The subsequent establishment of master and working stocks of recombinant virus is a time consuming process and initially it was necessary to screen for expression and demonstrate that the protein product was biologically active before establishing these stocks. To do this, a fairly crude antiviral assay based around the protection of FEA cells against calicivirus infection was used. There was no evidence that even a biologically active interferon preparation would work in this system but, at this stage there was no licence to use VSV. These crude initial screening assays were encouraging, and, although the interferon preparation was not quantified, the baculovirus system appeared to be producing a biologically active protein. Subsequently, protein production was optimised in spinner cultures by infecting the cells while they were in logarithmic growth and using a high multiplicity of infection of the recombinant virus (10pfu/cell). The results from the time course study indicated, in the calicivirus system,

that protein could be detected 18 to 24 hours post infection (h.p.i) but stably accumulated to maximum protein production by day 5. Following five days incubation, the addition of the infected Sf9 supernatant to FEA cells seemed to cause the cells to lose adherence and shrink. It was concluded that this may be a toxic effect of high concentrations of interferon-gamma leading to cytostasis. Despite the fact that the calicivirus assay system seemed to be working, it required concentrated interferon preparations to cause protection, and it appeared that the assay was quite variable and difficult to reproduce. Initially, It was thought that this may have been due to poor protein yield or an inappropriate concentration of calicivirus in the assay. Attempts to further optimise the assay conditions (viz, altering the dose of challenge virus) were unrewarding and it was decided to carry out the same assay procedure but to use vesicular stomatitis virus as the challenge virus.

The vesicular stomatitis virus assay was performed on the sample DA1 and DA2, as described above, and gave more meaningful, reproducible results. In addition, the assay was performed in a 96 well plate format which could potentially facilitate assaying large numbers of interferon samples. The interferon preparation was quantified as the reciprocal of the dilution of interferon which gives 50% protection. This value was expressed in terms of laboratory reference units (LRU). Interferon-gamma is highly species specific and thus it is impossible to standardise these units in terms of international units (as described by WHO). At the beginning of this discussion the expression of interferon-gamma from other species is described. It is difficult to compare yields according to units of interferon between these systems for the reasons outlined. This is exemplified by the results gained by Mory *et al* (1986) compared to those gained by Zucker *et al* (1993). Mory expressed the human interferon-gamma molecule in CHO

cells and the resulting cell clones gave interferon levels which had a specific activity of 1-2 x 10^8 units/mg of protein using a VSV bases CPER assay. Zucker expressed the canine interferon-gamma molecule in *E.coli* and measured specific activity using the VSV-based CPER assay and using MDCK cells as the host cells. Zucker's results revealed a specific activity of canine recombinant interferon gamma of 640,000 laboratory reference units/mg of protein. No real comparisons can be drawn from these two, very different apparent specific activities because the assays were performed under different conditions and using different host cells. It is thus important to standardise a particular IFN-γ assay so that subsequent titrations can be referenced according to laboratory units. In this experiment, it is difficult to define the titration in terms of specific activity until an absolute value for interferon-γ production is gained in terms of mg of interferon/litre of baculovirus supernatant. However, it can be concluded from this experiment that the FEA/calicivirus assay system is a poor system for measuring biological activity of feline recombinant interferon-gamma and the FEA/VSV assay is a more reproducible assay.

The results of the VSV assay for sample DA2 demonstrated an apparent lower titre of interferon-γ production from the High Five cells. The High Five cells were cultured as described for the Sf9 cells but were more difficult to culture and appeared to have slower population doubling times. This may account for a sub-optimal protein production compared to that claimed by the supplier.

The expression of feline recombinant interferon-α(tau) in the baculovirus/silkworm system has recently been reported by Ueda, Sakurai, and Yanai (1992). Interestingly, this group demonstrated that their interferon preparation protected feline cells (Crandell feline kidney-CRFK)

against challenge with $10^{3.5}$ TCID50/ml/Feline herpesvirus (FHV) or $10^{5.5}$ TCID50/ml/Feline calicivirus (FCV). About 30 or 80 units/ml of rFeIFN-α decreased 50% of the number of plaques by FHV or the cytopathic effect (CPE) by FCV, respectively. Interferon-α is a far more potent antiviral cytokine than interferon-gamma (Farrar and Shreiber 1993). This may explain the poor reproducibility of the antiviral assay based upon FCV in our system compared to the assays carried out using feline recombinant interferon-alpha.

The MHC Class II induction assay appeared to be as sensitive as the anti-viral assays, especially at low concentrations, for the detection of biologically active recombinant protein. This is in accordance with reported assay performances being up to 10 times more sensitive than the antiviral assays (Current Protocols in Immunology, 1991). This is an easy assay to perform and may be more practical on a day to day basis considering the need for containment facilities for the use of VSV. The confirmation that the recombinant protein up-regulates MHC Class II is encouraging, considering that it is envisaged that the protein may be useful as a vaccine adjuvant.

Although it is difficult to quantify the baculovirus derived interferon, other than in terms of laboratory units, it can be concluded that recombinant interferon-gamma can be stably expressed in the baculovirus system and that the protein product appears to be biologically active. It is difficult to assess accurately the level of expression from the baculovirus system. During the plaque purification stages, recombinant plaques were chosen which appeared to give the highest level of expression in the crude anti-viral assays. These apparent differences in levels of expression of different phenotypes may not actually represent variations in the phenotype but may

be reflecting variations in experimental conditions. However, It has been advised that several alternative recombinant isolates are screened in case there are differences in the expression phenotype, although this is a rare occurrence (Bishop 1992). Usually the expression level of a foreign gene does not vary from experiment to experiment, provided the infection conditions are optimal (cell viability of >98%, cells in exponential growth, use of high titred virus stocks to establish one-step growth). Thus, for this experiment, infection conditions were set at: infection of cells in logarithmic growth at a rate of 10 pfu/cell of recombinant virus and subsequent harvest of the supernatant at 5 days post infection.

Many genes have now been expressed using the baculovirus system employing both the p10 and polyhedrin promotor system. Using either promotor system has resulted in high levels of expression in both tissue culture and larvae. The levels obtained often approach or exceed those of the polyhedrin or p10 proteins. By analysis of stained preparations of cell extracts, expression levels of 50% or more of the total cell protein have been frequently obtained (Bishop 1992). The reasons for low level expression of foreign genes may be many and varied and have been reviewed by King and Possee (1992) and by Bishop (1992). The gene product may directly or indirectly alter the rate of gene transcription by its effects on other functions of the host cell (although this is unlikely for species specific interferon-γ). In addition, there may be differences in mRNA translation efficiency for different genes. The latter may be due to codon usage, or due to the primary or secondary structure of the mRNA species in the vicinity of the translation initiation codon, or the secondary structures within the body of the mRNA that affect the speed of translation. No studies have been carried out to address these issues. Other, more obvious reasons for apparent low levels of protein production

include, infecting cells which are not in logarithmic growth or are of poor viability, using a low multiplicity of infectious virus or by mis-timing the harvest of baculovirus supernatants.

As described in Chapter 3, the first 23 amino acids represent a leader sequence which is cleaved from the mature protein. No protein sequence analysis is available for the feline recombinant protein, but the demonstration of biological function indicated that the protein had undergone post-translational processing, i.e. there was secretion of a functional protein which indicated that the signal sequence may be cleaved and homodimers formed.

The following chapter will discuss the immunological detection of the protein and subsequent partial purification.

CHAPTER 5

IMMUNOLOGICAL DETECTION OF FELINE RECOMBINANT INTERFERON-γ AND PARTIAL PURIFICATION USING ION EXCHANGE CHROMATOGRAPHY

5.0 INTRODUCTION

5.1 AMINO ACID SEQUENCE ANALYSIS

5.2 PEPTIDE SYNTHESIS

5.3 PREPARATION OF PEPTIDES FOR IMMUNISATION

5.4 IMMUNISATION PROTOCOL

5.5 COLLECTION AND PREPARATION OF SERUM

5.6 ELISA TO DETERMINE ANTIBODY TITRE

5.7 WESTERN BLOT ANALYSIS OF RECOMBINANT BACULOVIRUS PRODUCTS

5.8 PURIFICATION OF RECOMBINANT PROTEIN

5.9 BIOASSAY OF FPLC PRODUCTS

5.10 WESTERN BLOT ANALYSIS OF FPLC PRODUCTS

5.11 DISCUSSION

5.0 INTRODUCTION

While conducting the cloning and expression experiments, the only way of demonstrating the presence of recombinant interferon in the baculovirus supernatants was to conduct biological assays as described in Chapter 4. An alternative to measuring biological function is to detect the presence of recombinant protein by the use of labelled antibodies. The only commercially available antibodies were monoclonal antibodies for human and murine interferon-γ, and there was no evidence that they would be cross-reactive in the feline system. However, once the amino acid sequence could be predicted from the cDNA sequence, an analysis was performed to predict immunogenic peptides for synthesis and eventual use as immunogens. The immunogens were used to raise polyclonal antisera for eventual use in protein detection systems.

The production of antibodies that could recognise the feline recombinant protein, in addition to the biological assay, then aided in the purification of recombinant protein from the baculovirus supernatants. This chapter describes the processes of peptide design, synthesis and immunisation and finally discusses how immunological techniques and biological assays were used in the purification process.

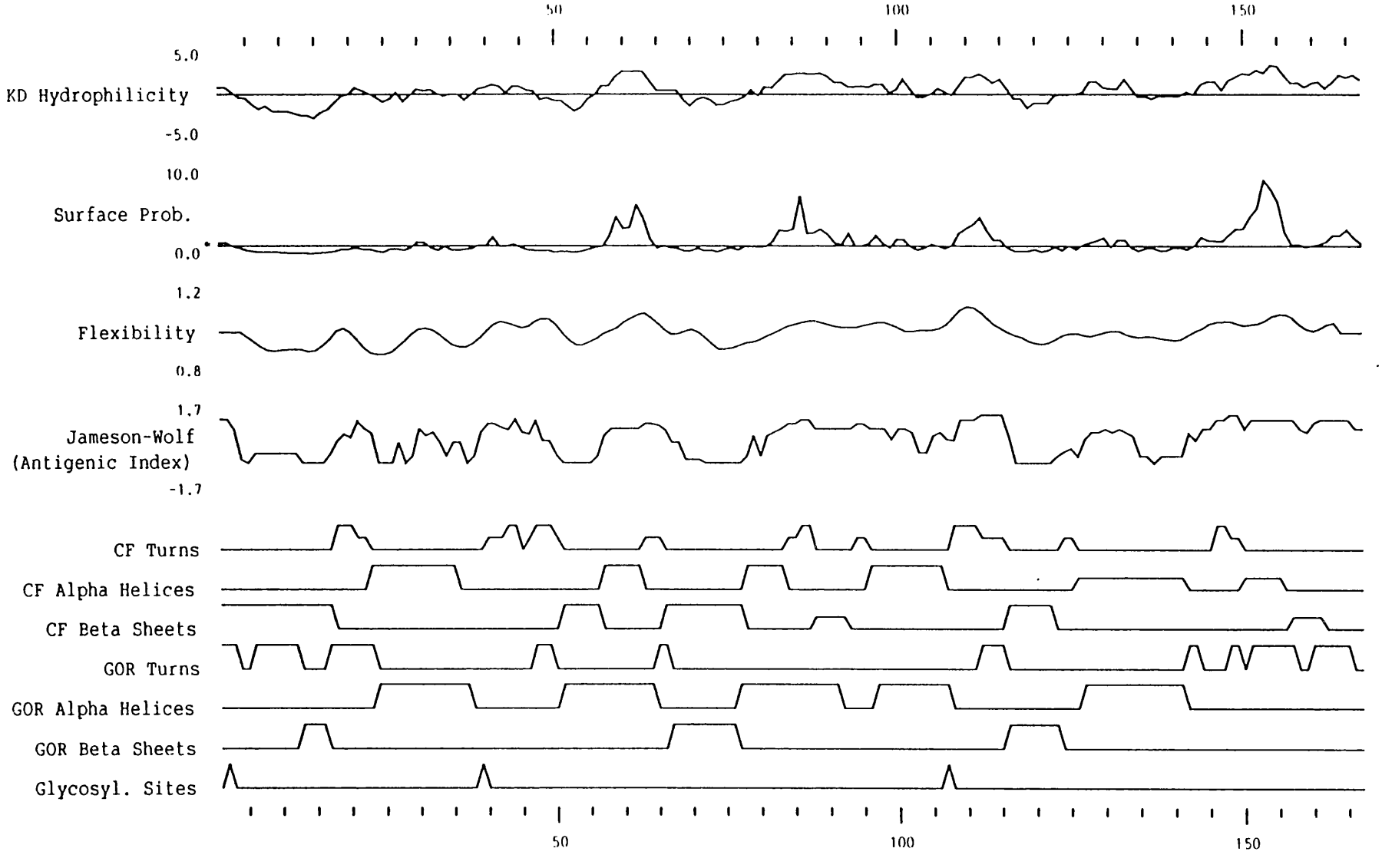
5.1 AMINO ACID SEQUENCE ANALYSIS

Choosing an appropriate peptide is critical in obtaining an antibody that will cross react with the recombinant protein. Initially, the amino acid sequence was predicted from the cDNA sequence using G.C.G sequence analysis software programmes. These software programmes also allow analysis of the predicted protein structure to determine critical regions

which are important in peptide design. The critical parameters in peptide design have been reviewed in Harlow and Lane (1988), Current Protocols in Molecular Biology (1994) and Current Protocols in Immunology (1994), and include regions of hydrophilicity, predicted antigenicity, secondary structure and the presence of glycosylation sites. The use of the *Peptide Structure* and *Peptide Plot* commands allow predictions of secondary structure (according to the Chou-Fasman method), hydrophobicity (according to the Kyte-Doolittle or Hopp-Woods method) and surface probability (according to the Emini method). Antigenicity, (expressed as an antigenic index according to the Jameson-Wolf method), is a cumulative index based upon all the variable antigenic parameters. *Plot Structure* plots these measurements in the output file from *Peptide Structure*. The measurements are then shown as parallel panels on a graph (Figure 5.1). The methods used for predicting these parameters were cited in the GCG sequence analysis software package programme manual (1991).

The results of the peptide structure analysis are shown in Figure 5.1. From these predictions peptides were selected which could be potentially immunogenic. Peptides were selected according to areas of high surface probability, hydrophophilicity and antigenic index. The premise is that an antibody raised against a peptide from a surface area or flexible area of the protein is more likely to represent a B cell epitope on the native protein. Areas of increased hydrophilicity indicate surface exposure and thus are more likely to be immunogenic. Potential glycosylation sites were avoided as glycosylated sites on the native protein may cause stearic hindrance of the antibody. An added parameter in peptide selection was the degree of homology between chosen peptide sequences for the recombinant feline interferon-γ and the peptide sequence of the rabbit interferon-γ protein.

Figure 5.1 Output from the Plotstructure program for peptide analysis



Peptides are more likely to be immunogenic if they differ from the native protein of the experimental animal which is raising the antisera. Taking these factors into consideration, two peptides were selected. The first was an internal peptide based on the protein region between amino acids 80 and 100, and the second was a peptide designed according to a region in the protein close to the carboxy terminus (amino acids 145 to 165). Several workers have noted that the carboxy-terminal sequences are often exposed and can be targeted for anti-peptide sequences. Both the amino termini and the carboxy termini are recognised as good immunogenic sites (reviewed in ab book). While analysis of the inferred amino acid sequence allows the prediction of the most suitable peptides for antibody production, it must be borne in mind that these are only predictions and the design of suitable peptides is still largely empirical. Figure 5.2 shows the sequence of the peptides chosen. The peptides were 20 amino acids in length. The size of peptide used can vary between about 6 and 20 amino acids. While short peptides are likely to be more soluble, they are usually less immunogenic than larger peptides.

Peptide 34 differs from the rabbit sequence by 8 amino acids and peptide 33 differs from the rabbit sequence by 3 amino acids. A line up of the two peptide sequences is given in figure 5.3.

5.2 PEPTIDE SYNTHESIS

Peptide synthesis was carried out using the 432A Protein Synthesiser (Applied Biosystems). In the conventional approach for preparing anti peptide antibodies, small peptides are conjugated to larger proteins to render them immunogenic. This method has several disadvantages which have

PRSNLRKRKRSQNLFRGRRA 33

ENLKDDDQRIQRSMDTIKED 34

Figure 5.2. Amino-acid sequence of peptide 33 and 34. Both peptides are 20 amino acids in length.

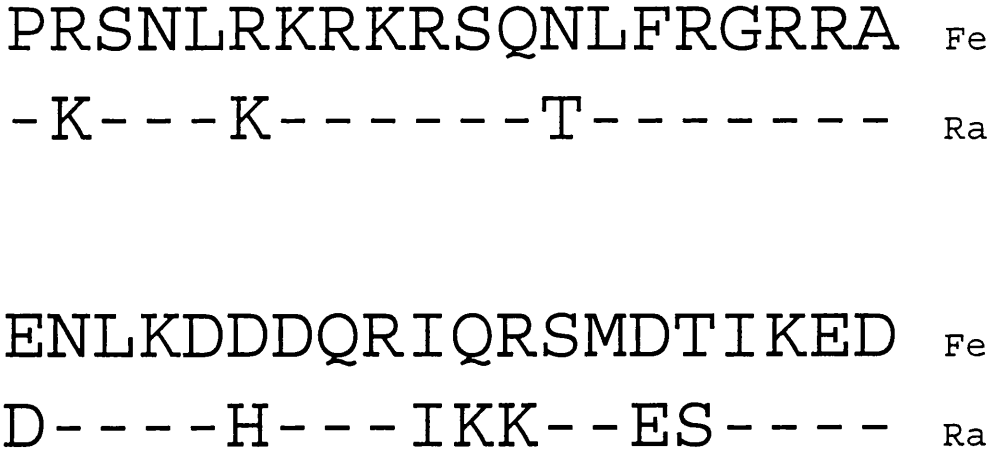


Figure 5.3. Amino acid line-up of peptide 33 and 34 with the rabbit equivalents. Peptide 33 shares 85% homology and peptide 34 shares 60% homology with the rabbit equivalents.

been reviewed in Current Protocols In Immunology, 1995. Firstly, the peptide represents only a fraction of the total molecular weight of the immunogen and thus antibodies to the peptide will only represent a fraction of the total antibody response. Secondly, the amount of peptide conjugated to the carrier is unknown and may be randomly distributed along the length of the carrier. Finally, the process of conjugation may alter immunological determinants on the peptide.

In this experiment, the multiple antigen peptide system (MAP) was used in the peptide synthesis in order to overcome the above difficulties and to try and optimise antibody production. This system was originally described by Tam (1988) and is reviewed in Current Protocols in Immunology (1995). The MAP system replaces the carrier protein by the arrangement of several copies of the peptide around an inner amino acid core. The core is quite small and the bulk of the complex is formed by up to 16 peptide antigens layered around the inner core matrix which is composed of lysine. The MAP core is oligimeric and contains non-cationic peptidyl and isopeptidyl lysine amide on both the N^{α} and N^{ε} termini of the lysine. As a result the core is not immunogenic. It is the dendritic peptide antigen chains on the MAP core that are likely to be mobile and contribute to the enhanced immunogenicity of the MAP system.

Following synthesis and purification, the peptides were lyophilised and stored at 4°C.

5.3 PREPARATION OF PEPTIDES FOR IMMUNISATION

To obtain a good immune response the peptide antibodies were introduced into the animals along with an adjuvant. Adjuvants are non-specific stimulators of the immune response and, in general, incorporate two components. The first component is a substance which allows the formation of a deposit and protects the antigen from rapid degradation. The second component is a non-specific stimulant of the immune response. These stimulators raise the local level of cytokines which stimulate antigen presenting cells and cause a local inflammatory response. In this experiment, a Non Ulcerative Freunds Adjuvant (NUFA) was used, which contains mineral oil for the formation of the antigenic deposit, and Bacillus Calmette Guerin (BCG) vaccine was used as a local immuno-stimulator. The NUFA was supplied in the incomplete form and was made complete for priming immunisations by the addition of *Mycobacterium tuberculosis* in the form of BCG vaccine. The BCG was added to the aqueous phase of the immunogen prior to emulsification with NUFA. The BCG preparation used was that of Evans Medical, formulated for intradermal rather than subcutaneous use (BCG vaccine BP). The subcutaneous formulation causes skin ulceration. The vial of BCG vaccine was reconstituted according to the manufacturer's instructions and 0.1ml was added to each 0.9ml of aqueous immunogen. The aqueous phase was then mixed with 2 volumes of adjuvant to produce a stable water-in-oil emulsion (see below). The BCG was only used for the primary vaccination and, for subsequent booster vaccinations, the aqueous immunogen was mixed with 2 volumes of adjuvant alone to form a stable emulsion.

Initially, the aqueous phase of the peptide was made up to the appropriate concentration using ultra pure, autoclaved water. The total volume

injected in to each rabbit was 1ml at each immunisation (0.5ml into each of the quadriceps muscle). The volume of immunogen prepared for each rabbit was slightly larger than this to account for losses during the mixing or immunisation process. The peptide/NUFA was made up in a ratio of 2:1 as described above. The mixing process was carried out using two syringes attached via twin luer-hubbed, syringe connector with a fine connecting channel, the connector allowing the contents to pass between the syringes to facilitate thorough emulsification. To check that the adjuvant and peptide were sufficiently mixed, a small amount was allowed to drip on to the surface of some water, if the drop dispersed then mixing was incomplete. However, if a discrete droplet was formed on the surface of the water then the mixture was ready for injection.

5.4 IMMUNISATION PROTOCOL

The animals chosen to raise the anti-sera were New Zealand rabbits. Two rabbits were used for each peptide and the intramuscular route was chosen for the immunisation. The dose of immunogen required to elicit the optimum immune response is empirically determined. Much of the immunogen injected may be degraded before it reaches the target cells and so the amount of immunogen injected does not necessarily reflect that which is going to be active. General guide lines on the dose used were taken from Harlow and Lane (1988) and Current Protocols in Immunology (1994). For the initial priming dose, 100ug of immunogen per rabbit was used. Subsequently, this was reduced to 10ug per rabbit for booster injections. While reducing the subsequent doses to 10ug may have the effect of reducing the yield of antibody, their tends to be an increase in antibody affinity.

Following primary immunisation, the rabbits were boosted at 3 weeks and subsequently every six weeks. 14 days following the third boost, the animals were bled to check for the presence of antibody by ELISA (see below).

5.5 COLLECTION AND PREPARATION OF SERUM

Blood was collected between 10 and 14 days after boosting with immunogen. The blood was collected from the marginal ear vein in to sterile glass bijou bottles. 10-15ml. of blood was collected, allowed to clot and the serum removed and aliquoted in to 1ml tubes and stored at -20°C.

5.6 ELISA TO DETERMINE ANTIBODY RESPONSE

To establish that the rabbits had developed an antibody response to the injected peptides, and to measure the level of antibody response, an indirect antibody Enzyme Linked Immunosorbent Assay (ELISA) was performed. For the ELISA experiment, an ELISA starter kit (Pierce) was used which provided all the reagents for the assay apart from antigen and primary antibody. The procedure was carried out using the protocol, provided by the manufacturer, for a two dimensional serial dilution assay. The primary antibody was serially diluted across the plate and the secondary antibody was diluted down the plate. Initially, each antigen (peptide 33 and 34) was prepared as a solution at 10ug/ml using coating buffer (BupH™ Carbonate in distilled water). Plates were assigned for either peptide 33 or peptide 34. One hundred ul of antigen solution was added to each well of the microtitre plate and allowed to incubate for 1 hour at room temperature. The plates were emptied and washed 3 times

with 100ul of wash buffer (prepared by adding 2.5ml of Surfact-Amps™ Tween 20, 5ml of Blocker™ BSA in PBS and 1 pack of BupH™ Dulbecco's PBS in 500ml of distilled water). The plates were incubated for 30 minutes with 100ul of blocking buffer (to block irrelevant antigenic sites), after which the plates were emptied. 100ul of wash buffer was added to each well and 100ul of the appropriate serum sample was added to all the wells in row A (see Figure 5.4). The primary antibody was serially diluted 2 fold from rows A through G (H was used as a control) and the plate was incubated for 1 hour followed by 3 washes with 100ul of wash buffer. The alkaline phosphatase labelled secondary antibody was diluted 1:3000 with blocking buffer. One hundred ul of wash buffer was added to each well and 100ul of the secondary antibody added to each well in column 1 (see Figure 5.4). This antibody was then serially 2 fold diluted across from column 1 through column 11 (12 was used as a control). The plates were incubated for 30 minutes and subsequently washed 3 times with 100ul of wash buffer. One hundred ul of wash buffer was then added to each well and allowed to incubate for a further 5 minutes. The wells were emptied and 100ul of the phophatase enzyme substrate added to each well (substrate was made by the addition of 1 5mg PNPP tablet to 10ml of DEA substrate buffer). The plates were incubated for 30 minutes and the optical density measured at 405nm using an automated ELISA reader.

The results of the ELISA for both peptide 33 and 34 are given in Tables 5.1 and 5.2. A minimal response was seen with peptide 33 (using serum designated T710) but a strong response was seen with peptide 34, using serum designated T711. A two fold serial dilution assay was performed in order to establish optimum concentrations for the primary and secondary antibodies when used in combination. The optimal combination of the

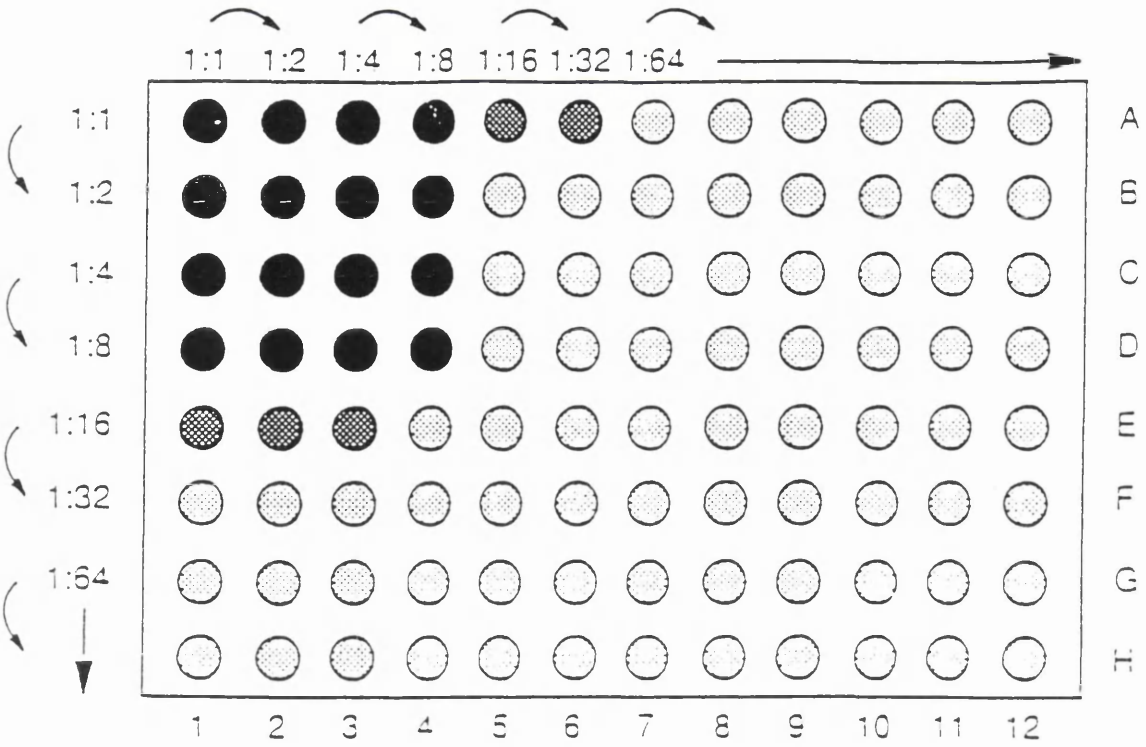


Figure 5.4.Schematic representation of ELISA protocol.

	1	2	3	4	5	6	7	8	9	10	11
A	.374	.150	.111	.119	.096	.088	.085	.082	.077	.075	.040
B	.512	.237	.147	.120	.101	.101	.088	.084	.082	.087	.038
C	.773	.323	.196	.136	.108	.100	.090	.083	.082	.080	.046
D	.786	.305	.194	.136	.116	.106	.102	.082	.081	.078	.040
E	.615	.267	.185	.125	.103	.094	.091	.078	.078	.082	.039
F	.568	.276	.182	.157	.115	.117	.091	.083	.086	.077	.040
G	.469	.245	.177	.130	.100	.094	.090	.084	.084	.078	.042
H	.085	.097	.096	.096	.085	.087	.089	.092	.084	.080	.035

Table 5.1 ELISA O.D.(405) readings for peptide 34 and serum sample T711. Column 11 is the control for the primary antibody and row H is the control for the secondary antibody.

	1	2	3	4	5	6	7	8	9	10	11	12
A	.094	.089	.085	.086	.118	.082	.079	.076	.074	.076	.042	.045
B	.099	.089	.088	.093	.086	.089	.091	.083	.082	.082	.041	.041
C	.094	.143	.107	.149	.095	.088	.086	.081	.080	.082	.045	.038
D	.090	.092	.093	.096	.087	.086	.084	.092	.094	.078	.040	.040
E	.081	.086	.093	.107	.099	.119	.103	.074	.073	.074	.044	.038
F	.084	.087	.124	.111	.095	.088	.084	.081	.081	.096	.041	.040
G	.083	.086	.087	.088	.081	.084	.083	.080	.079	.077	.039	.038
H	.088	0.93	.088	.085	.076	.085	.088	.082	.081	.078	.041	.037

Table 5.2 ELISA O.D (405) measurements for peptide 33 and serum sample T710. Column 12 is the control for the primary antibody and row H is the control for the secondary antibody.

primary and secondary antibodies was defined as the one which gives half the maximum reading on the microtitre plate reader. For the antigen 34, this was using the primary antibody at 1:4 and the secondary antibody at 1:12000.

5.7 WESTERN BLOT ANALYSIS OF RECOMBINANT BACULOVIRUS PRODUCTS

Western blot analysis was conducted, as described in Chapter 2, on both Sf9 supernatants containing recombinant protein and Sf9 cell lysates following reducing (SDS) polyacrylamide gel electrophoresis (SDS-PAGE). Ten ul of each of the cell lysate suspension, supernatant containing recombinant protein and supernatant containing wild-type baculovirus (BacPAK 6) were loaded on to the polyacrylamide gel as described in chapter 2. Electrophoresis was carried out at 200v for 45 minutes. The gel was then subjected to electroblotting (Materials and Methods), after which western blot analysis was performed. The primary antibody used in this procedure was rabbit anti-cat IFN-γ polyclonal antibody raised against peptide 34 and designated T711. The secondary antibody used was a horseradish peroxidase (HRP) labelled, sheep anti-rabbit antibody (Sigma). Primary antibody was used at a concentration of 1:3000 and the secondary antibody was also used at a concentration of 1:3000. Blots were visualised using the ECL process as described in materials and methods. The results of the Western blot analysis is give in Figure 5.5.

The autoradiographs demonstrate that the primary antibody is binding to a protein of around 20kDa in both the lanes containing Sf9 supernatant and the lanes containing cell lysates. This appears to be the only product

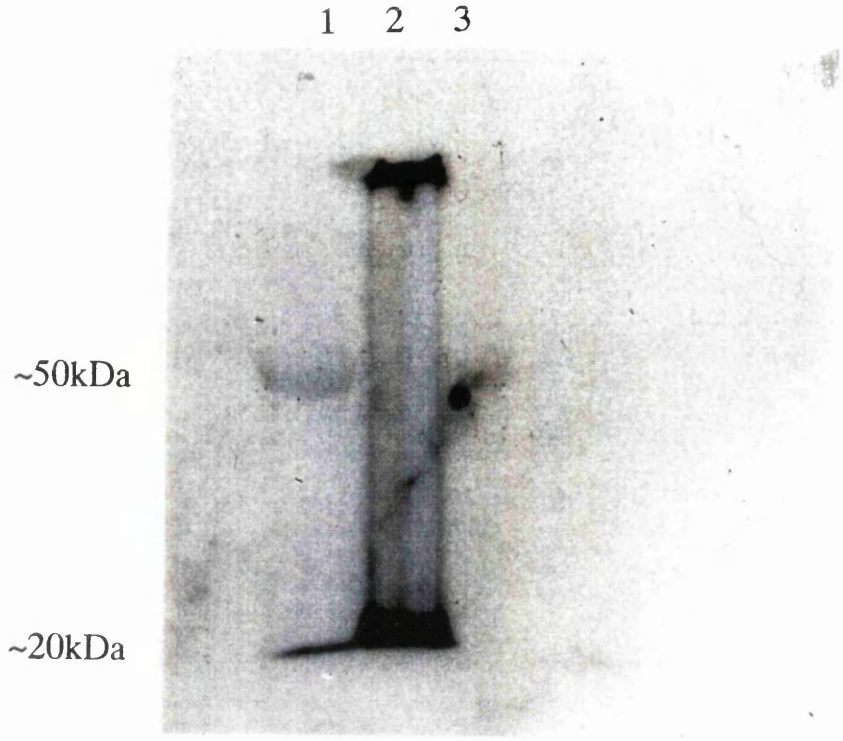


Figure 5.5 Western blot analysis of baculovirus derived IFN-γ. Supernatant (lane 1); cell lysate (lane 2), and wild-type virus supernatant (lane 3).

detected by this antibody, there is, however, a feint band corresponding to 55KDa which may represents non-specific binding to serum proteins. The negative control lane consists supernatant from Sf9 cells infected with wild-type PAK6 baculovirus. No protein bands were detected in this lane when blotted with antibody T711.

5.8 PARTIAL PURIFICATION OF RECOMBINANT PROTEIN

In order for the production of expressed protein to progress towards clinical application it was necessary to design a purification protocol for the protein. To achieve this, the purification method described for the murine interferon-g, Nagata *et al* (1987), was followed for the feline equivalent. This group used a combination of ion exchange chromatography and gel filtration following an ammonium sulphate precipitation step. Because of time constraints, only, ammonium sulphate precipitation and ion exchange chromatography have been conducted, but it is planned to complete the purification process using gel filtration. Ion exchange chromatography was carried out utilising FPLC.

Initially, 20ml of Sf9 supernatant, designated DA1 (as described in chapter 4), was subjected to filtration using a 0.2u filter. Solid ammonium sulphate was added to the supernatant to create a 30% solution (176g/l). This mixture was subjected to centrifugation at 10,000 r.p.m in a JA 10 rotor, and the precipitate discarded. Solid ammonium sulphate was added to the supernatant to make 85% saturation (400g/l), centrifugation carried out at 10,000 r.p.m, and the supernatant discarded. Ammonium sulphate precipitated all the protein in the mixture, including the serum proteins in the TC100 medium.

The resulting precipitate was dissolved using 5 volumes of buffer B (20mM Tris-HCL, 5mM benzamidine, 2mM dithiothreitol (DTT), 10% (v/v) glycerol, pH 8.0). The solution was then subjected to dialysis against buffer B to remove the salt. To do this in a small scale preparation, the solution was added to a collodian bag and dialysed using 8 volumes of buffer B. The dialysis was carried out at 4°C over a 24 hour period. Following dialysis, the solution was degassed by filtration using a 0.45u filter and stored at 4°C prior to addition to the chromatography column.

Ion exchange chromatography was carried out using a Mono Q HR 5/5 anion exchange chromatography column (Pharmacia). This type of column has been specifically designed for FPLC (Fast Protein Liquid Chromatography) of proteins and peptides. FPLC is a variant of High Pressure Liquid Chromatography (HPLC) and has been developed for the fast separation of biomolecules. Ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to an immobilised ion exchange group of opposite charge. Initially, the ion exchanger is equilibrated with buffer which allows the binding of the desired solute molecules. The solute molecules adsorb by displacing exchangeable counter ions. Unbound substances are washed out from the exchanger bed using starter buffer and then solute molecules are eluted from the column using an increasing salt concentration gradient. Molecules are released from the column in the order of their strengths of binding, the most weakly bound substances being eluted first. The column is then washed, re-equilibrated and is ready for the next sample.

The FPLC controller was first programmed to carry out the following tasks: to equilibrate the column with 3 volumes of buffer B, to load the sample (in a 2ml volume), to wash the column, then to elute molecules

using an increasing salt gradient over 30ml, and then to wash and re-equilibrate the column. The salt gradient was provided by buffer A (buffer B + 0.3M NaCl). Both buffer B and buffer A were degassed by filtration using a 0.45u filter.

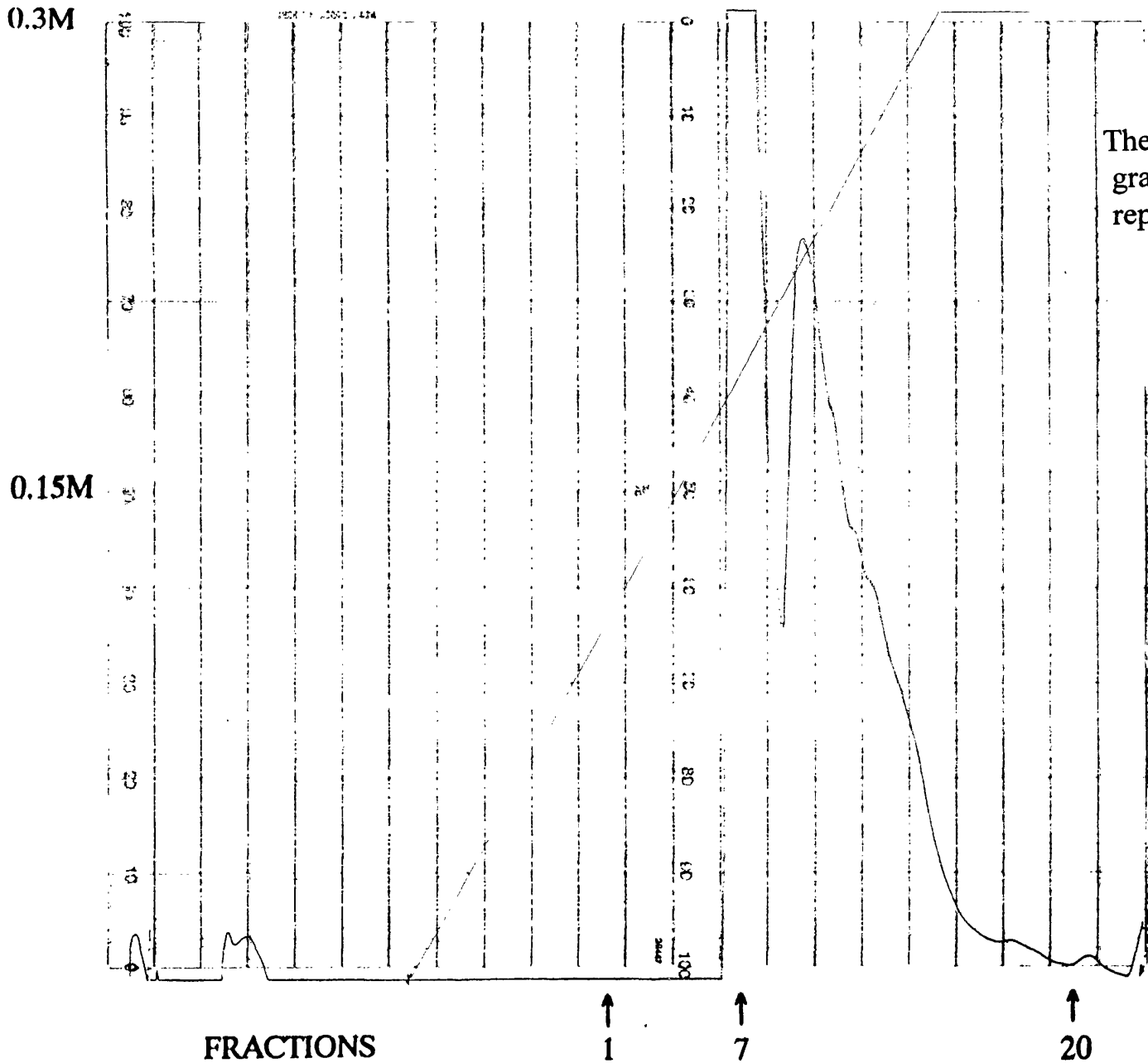
Fractions from the column were collected using a standard fraction collector (Pharmacia) and the elution profile was recorded graphically. The graphical representation of the column output is given in figure 5.6. From the profile we can see a major, narrow protein peak corresponding to 55% of the salt gradient. Subsequently, there is a larger, homogenous set of peaks. The protein mixture added to the column contained, not only interferon and serum proteins, but also viral proteins derived from the baculovirus.

Twenty fractions from the column were then taken and 200ul of each fraction was dialysed against PBS and glycerol (10% v/v) to remove any of the buffer components which could have interfered with subsequent bioassays. The samples were dialysed using micro collodian bags (Sartorius) and 8 volumes of the PBS/glycerol. These dialysed samples were then subjected to both a CPER assay (as described in chapter 4) and SDS-PAGE electrophoresis and subsequent western blotting using antibody T711.

5.9 BIOLOGICAL ASSAY OF FPLC PRODUCTS

The fractions collected from the column were numbered 1 to 20 and each were incorporated into a 96 well format CPER assay, as described in chapter 4, utilising VSV. The samples were incubated for 24 hours with the FEA cells at a dilution of 1:3 prior to the addition of virus. The virus

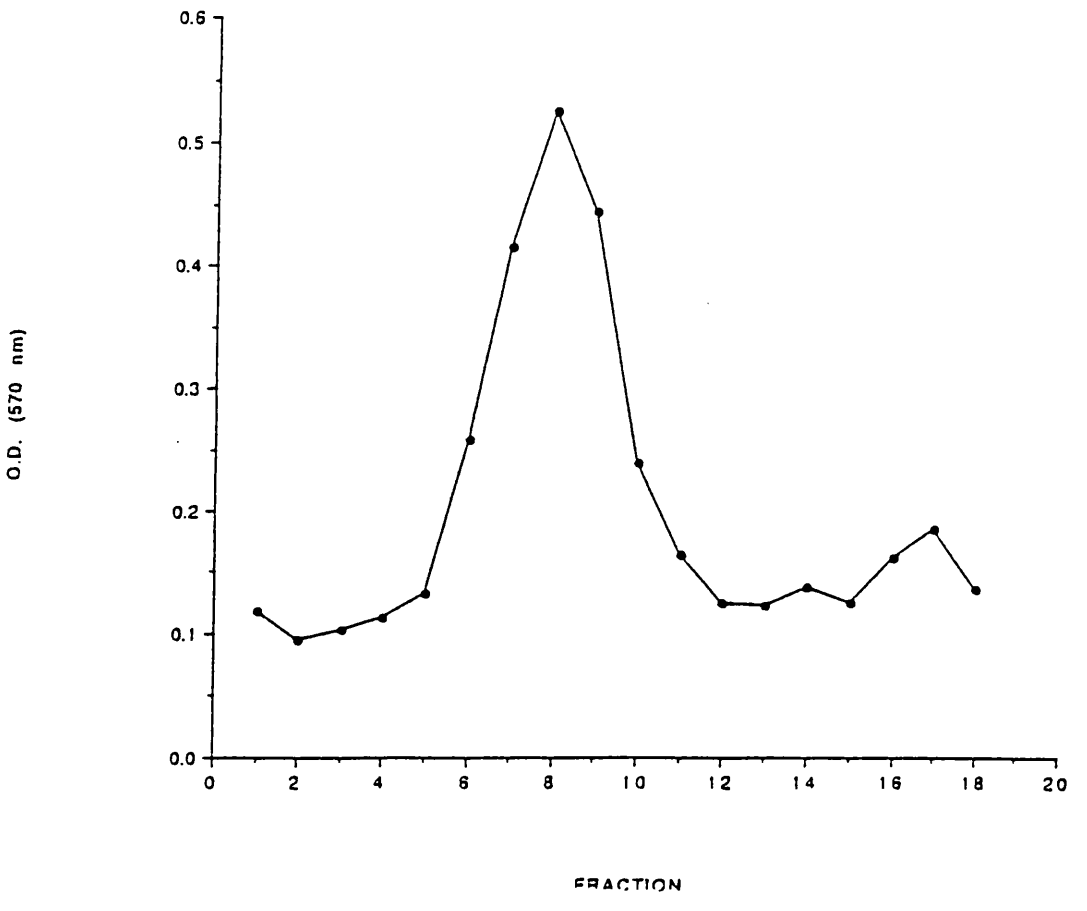
Figure 5.6 Graphical output from FPLC/ ion exchange chromatography of sample DA1



Fraction	O.D.(595nm)	Fraction	O.D.(595nm)
Cell Control	0.868	9	0.443
Virus control	0.095	10	0.238
1	0.118	11	0.164
2	0.095	12	0.125
3	0.103	13	0.123
4	0.112	14	0.137
5	0.132	15	0.124
6	0.259	16	0.162
7	0.416	17	0.184
8	0.525	18	0.136

Table 5.3 Results of CPER assay for FPLC fractions

Figure 5.7 INTERFERON ACTIVITY OF PURIFIED FRACTIONS



was added at 4 pfu/cell and incubated at 31°C/5%CO_2. Following a two day incubation, the cell layers were stained, as described in chapter 4, and a visual assessment of the monolayers performed. In addition, the dye in the assay wells was eluted an the O.D. measurements at 595nm recorded using an automated ELISA reader. The results are shown in Table 5.3 and depicted graphically in Figure 5.7. From the assay, it appeared that fractions 6, 7, 8, and possibly 9 were giving protection to the FEA cells against challenge with VSV. It would appear, therefore, that these are the fractions which contain the eluted interferon. This finding was further substantiated by western blotting.

5.10 WESTERN BLOT ANALYSIS OF FPLC PRODUCTS

10ul of each of Fractions 1 to 14 were subjected to SDS-PAGE electrophoresis, as described, and subsequent western blotting using antibody T711 as the primary antibody and HRP labelled sheep anti-rabbit antibody as the secondary antibody. The antibodies were used at the same concentrations as those described for previous western blots i.e. both were used at a concentration of 1:3000. Blots were visualised using the ECL method (Chapter 2). The results of the western blot are given in Figure 5.8.

It can be seen from the autoradiographs that the primary antibody is binding to a protein in fraction 7, and 8. The majority of the protein seems to be in fraction 7 as only feint bands can be seen in lanes corresponding to fractions 8 and 9. The size of the protein detected appears to be around 20KDa which is the same size as the protein detected in crude DA1 using the same antibodies and conditions.

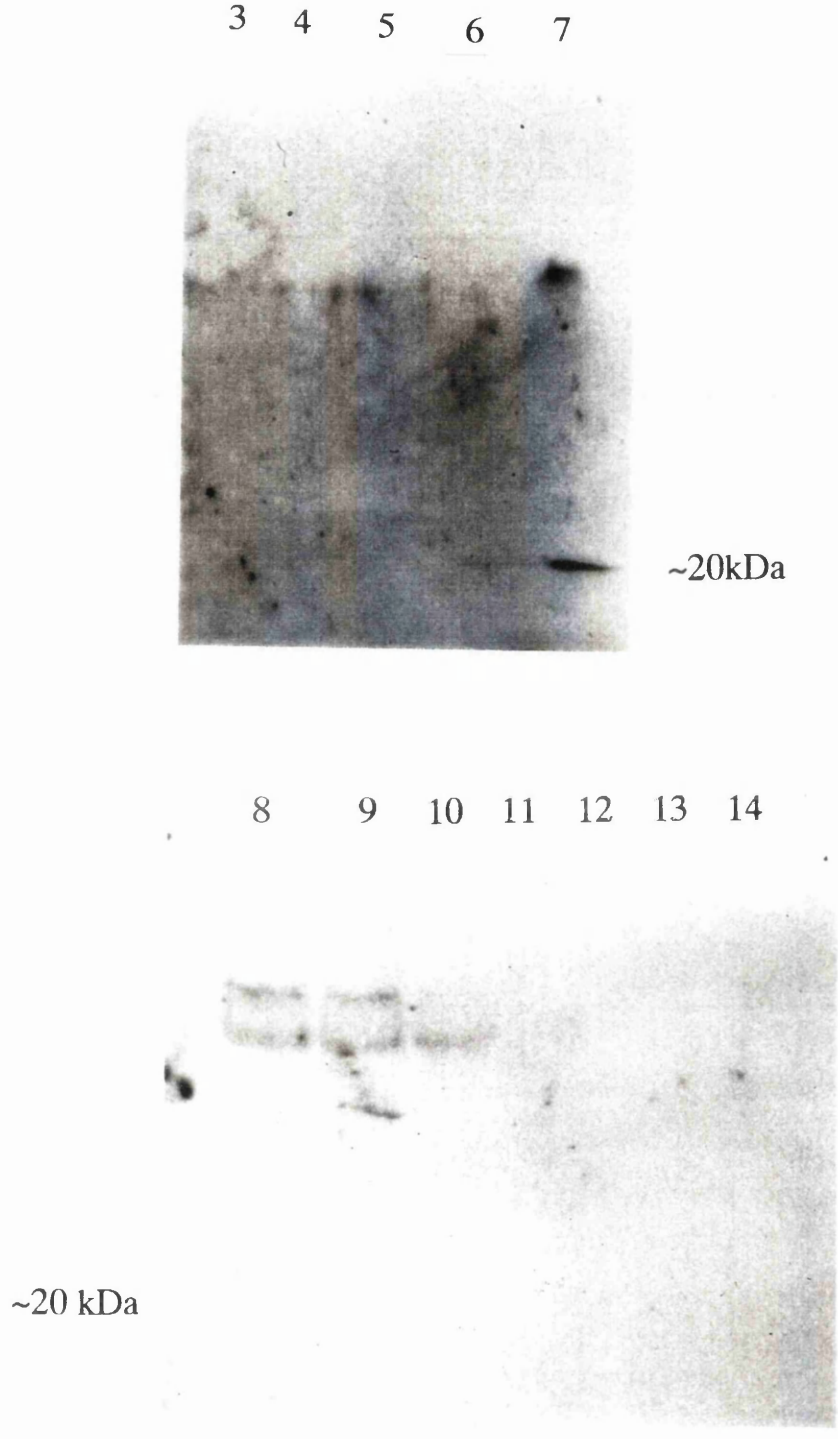


Figure 5.8 Western blot analysis of fractions 3 to 14, derived from FPLC.

Once the bioassay and western blots were complete, the graphical output from the FPLC was analysed to determine the peaks which corresponded to fractions 6 through 9. These fraction exactly corresponded to the first major protein peak (Figure 5.6). From this, it can concluded that interferon-γ protein is being eluted from the ion exchange column over a period of 3 to 4 fractions at a concentration of NaCl of around 0.15M.

5.11 DISCUSSION

This chapter describes how polyclonal antibodies were raised against synthetic peptides of interferon-γ and how these, along with the bioassay, were used to partially purify the interferon protein. Two peptides were synthesised for eventual immunisation of rabbits. However, it would appear that only one of the peptides, peptide 34, elicited a strong immune response in the rabbit. This may be because either the epitope chosen for peptide 33 was weakly immunogenic and/or that there was insufficient heterogeneity between the rabbit and feline equivalents to elicit an immune response (the feline peptide shared 85% homology to the rabbit equivalent). To determine the presence of antibody to the appropriate peptides, an ELISA was performed as described. The ELISA results for peptide 33 were very poor and indicated a poor response by the host. Despite this, a western blot of sample DA1 was performed, using polyclonal antisera to peptide 33. Unfortunately, the autoradiographs appeared blank (results not shown) and it was decided not to continue with antisera to peptide 33.

No available human or murine interferon-gamma antibodies, either mono or polyclonal, cross react in the feline system, and so the use of antibody T711 greatly facilitated detection of the recombinant protein. The

potential value of this antibody is that it would be possible to detect protein far faster than using the bioassay alone. Subjecting Sf9 supernatants to SDS-PAGE and comassie blue staining, without subsequent western blotting, is usually unrewarding. This is because after staining there are multiple bands corresponding, not only to the recombinant protein and serum protein in the TC100 medium, but also other viral proteins expressed by the recombinant virus. Even if a comparison is made against supernatants containing recombinant virus and supernatants containing wild-type virus it is often difficult to draw any conclusions about differences in the protein bands observed. This is because insertion of foreign genes in to the baculovirus genome will alter the pattern of expression of other viral, non recombinant proteins (King and Possee (1992).

Ion exchange chromatography was successfully performed on the sample DA1 to produce a partially purified interferon molecule. The conditions for chromatography were reproduced as described for the purification of murine recombinant interferon-γ (Nagata *et al*, 1987). The interferon appears to be eluted at a salt concentration of 0.15M which is in accordance with that observed for the murine equivalent which was eluted at a salt concentration of between 0.05M and 0.15M. The use of antibody T711 and western blot analysis, along with the sensitive bioassay, greatly facilitated the FPLC analysis. The interferon appears to be eluted in one single peak, but one could consider improving purification conditions by eluting over a greater volume (i.e. reducing the slope of the salt gradient) to improve separation of the interferon from irrelevant proteins.

A further extension to improving conditions for protein purification would be to produce the protein in serum free conditions. This would remove any

irrelevant serum proteins from the FPLC equation. The production of protein in serum free conditions can often be a difficult process. Initially the cells have to be adapted to serum free conditions by subsequent passage in reducing serum concentrations (King and Possee 1992). When the cells are growing under optimal conditions, they are then infected with recombinant virus as described in chapter 4. An alternative approach is to grow the cells in serum and subsequently infect the cells under the same conditions. Following a 24 hour incubation period the cells are harvested by centrifugation and resuspended in spinner culture using serum free medium (M. Harris personal communication). Both sets of serum free conditions were experimented with but the yield of protein fell using these procedures and it is possible that more work needs to be carried out on this approach to try and optimise conditions. One of the problems in using the latter method for serum free protein production is that, after a relatively long incubation period the non-adapted cells may lose viability and the capacity for post-translational modification of the recombinant protein.

Time has limited the experiment to only the partial purification of the recombinant protein, but it is planned to continue the process by using an additional gel filtration step, described by Nagata *et al* (1987) for the murine IFN-γ.

CHAPTER 6

GENERAL DISCUSSION

6.1 INTRODUCTION

6.2 THE TREATMENT OF RETROVIRUS INFECTIONS

6.3 INTERFERON AS A VACCINE ADJUVANT

6.4 INTERFERON IN THE TREATMENT OF NEOPLASIA

6.1 INTRODUCTION

At the outset, the aim of this project was to clone, sequence and express feline interferon-γ with to aim of examining the potential of this cytokine in the treatment and prevention of diseases in felidae. The amplification of feline specific cDNA encoding interferon-γ was achieved using the polymerase chain reaction, utilising primers selected according to highly conserved regions in sequences published for other species. This method had previously been adopted in the cloning of other cytokines genes including ovine and equine interferon-γ, and canine and feline IL-2. While this approach offers simplicity, care is required in the design of primers and PCR optimisation.

Expression in the baculovirus system was greatly facilitated by the use of the BacPAK 6 wild-type virus which allowed rapid isolation of recombinant virus. Subsequent expression studies were initially hampered by the lack of a reliable biological assay, but the use of the CPER assay, utilising vesicular stomatitis virus, eventually proved more reliable than the calicivirus based assay. The MHC class II induction assay was found to be as sensitive as the viral assays and may be more practical, considering the need for category II facilities when working with VSV.

The future aim of the project is to take feline recombinant interferon-γ into a clinical setting. Before this can happen, a phase I clinical trial needs to be conducted to assess the toxicity of interferon in experimental animals. It is intended to conduct this trial in experimental cats using three escalating doses of interferon-γ. Various clinical parameters, including temperature, presence of anorexia, weight changes, haematology, biochemistry and possibly cardiovascular signs, will be monitored

throughout this phase I trial. If the protein is then to be taken into the veterinary clinic as a therapeutic agent, an Animal Test Certificate (ATC) must be obtained. An ATC is used to assist in the development of a new veterinary product in the target species by providing data on efficacy. In addition, data is required on the safety and quality of the product. Non-clinical safety tests have to be conducted under the principles of Good Laboratory Practice (GLP) and quality depends on manufacture under good Manufacturing Practice (GMP).

Since their discovery, interferons have been extensively researched in the hope that they would prove beneficial in the treatment of human diseases, notably cancer and viral infections. The emergence of recombinant DNA techniques have allowed sufficient quantities of a variety cytokines to be produced and enter into clinical trials. At present, interferon-α has proved to be the most beneficial of the interferon species in terms of the treatment of neoplasia and chronic viral infections. Interferon-β has recently entered clinical trials for the treatment of multiple sclerosis, with encouraging results, while Interferon-γ has found a niche in the treatment of chronic granulomatous disease in humans (reviewed by Johnson *et al* (1994). Interferon-γ is also being examined for its potential as a vaccine adjuvant, especially in patients who are immunosupressed, and it may also have value in the treatment of neoplastic disease.

The use of recombinant cytokines in veterinary medicine has more often been limited to the use of human or murine proteins because the native protein has not been available. There are essentially two problems with this approach. Firstly, the long term use of recombinant human or murine cytokines in other species, despite the high degree of conservation between species, leads to the development of antibodies to the recombinant protein

by the host. While this may not be a problem in short term use of cytokines, say in the treatment of neoplasia, it would become a major factor in long term usage, for example, in the treatment of chronic haemopoietic disease. However, it is also note worthy that even native recombinant proteins can lead to the development of antibodies in the natural host. This may depend upon the type of system used to express the protein and may become important where there are differences in the glycosylation pattern such as that observed in the baculovirus system. The second problem associated with using a non-native recombinant protein is that the protein may not be cross reactive in that species. This is certainly true for interferon-γ, which is highly species specific, with only ovine and bovine interferon-γ species being cross reactive.

Recently, there has been an interest in cloning specific veterinary cytokines to examine their potential in veterinary diseases (reviewed by Campos *et al* 1991; Elmslie *et al*, 1991; MacEwen, 1990; MacEwen, 1993; Babuik, 1991; Tompkins and Tompkins, 1991; Lawman *et al* 1994). A number of veterinary cytokines have now been cloned from a variety of species. A limited number of clinical trials using veterinary cytokines are currently in progress, including the evaluation of canine rG-CSF in the management of chemotherapy induced myelosuppression; Feline rIFN-α(omega), in the treatment of feline acute respiratory infections; bovine interferon-α in the treatment of respiratory infections and bovine interferon-γ in the treatment of acute mastitis and the treatment and prevention of bovine viral diseases.

This thesis has described the sequence of events in taking a gene from the cloning stage towards purification and eventual clinical application. The work forms part of a larger project continuing at Glasgow concerned with

the expression of a panel of domestic animal cytokine genes in order to examine their therapeutic potential. The interest in the cloning and expression of feline cytokine genes stems from their potential in the treatment of neoplastic, haematological and infectious diseases. In addition, Interferon-γ is a good candidate as a vaccine adjuvant.

There are essentially three main areas where it is felt that recombinant interferon may have a role in treatment of diseases in cats. These areas are, the treatment of chronic retroviral infections; the treatment of neoplastic disease and the use of interferon-γ as a vaccine adjuvant. The use of cytokines in a clinical setting fall into two broad categories viz. the direct, parenteral administration of the recombinant protein, and the administration of the cytokines by gene transfer methods. In chapter 1, it was indicated that cytokines act in a paracrine or autocrine fashion. If the recombinant protein is given systemically then it must act in an endocrine fashion, but to do this the dose required often has to be high in order to obtain a clinical effect. This problem has precluded the use of many cytokines in the treatment of clinical cases because, in certain instances, the doses required for clinical benefit are toxic to the host. For example, while TNF-α has a direct anti-tumour effect, the systemic administration of the recombinant protein may lead to excessive morbidity. In contrast, gene transfer methods allow local production of cytokines in the area in which they are required and thus mimic the natural situation.

6.2 THE TREATMENT OF RETROVIRAL INFECTIONS

Exogenous interferon-γ may have a role in the treatment of retroviral infections in cats. The two major retroviral infections in cats are Feline leukaemia virus (FeLV) and Feline immunodeficiency virus (FIV). FeLV

is an oncovirus and is characterised by the ability to produce haemopoeitic tumours in cats. FIV is a lentivirus and is characterised by a long asymptomatic period and a progressive immunodeficiency similar to that seen with HIV infection in man. There have been recent reports of the use of combined interferon-α, Zidovudine and adoptive cell transfer in the treatment of established FeLV infection (Zeidner *et al,* 1993). The interferon was a human recombinant product and the study was limited by the production of neutralising antibodies to the interferon. This group reported that 50% of the cats cleared their viraemia and developed virus neutralising antibody which indicated both viral clearance and immune reconstitution. As discussed in Chapter 1, interferon-α is a more potent antiviral cytokine than interferon-γ and one could hypothesise that interferon-α would therefore be a better candidate for use as an antiviral agent than interferon-γ. However, one may consider a more appropriate role for interferon-gamma as an enhancer of immune effector functions. The exact role that cell mediated immunity plays in FeLV infection is, as yet, undefined, but as the immunological mechanisms which accompany infection are unravelled a clearer role for exogenous interferon-γ may appear.

Against this is a report by one group (Khan *et al* 1992) which suggests a deleterious role for interferon-γ in FeLV-C infection. FeLV-C infection is associated with erythroid aplasia causing severe aplastic anaemia in affected cats and is also associated with raised serum levels of interferon-gamma and TNF-α. These two cytokines may act synergistically to cause haemopoeitic suppression. Khan *et al* (1992) evaluated the effects of interferon-γ (purified feline interferon-γ from stimulated splenocytes) and TNF-α on feline bone marrow progenitors. They found that a combination of TNF-α and IFN-γ incubated with bone marrow cells caused marked

suppression of feline colony forming units-erythroid (CFU-E), burst forming units-erythroid (BFU-E), and colony forming units-fibroblasts (CFU-F), whereas colony forming units-granulocyte/macrophage (CFU-GM) were minimally affected. The effects of incubating the cells with these cytokines independently had minimal effects. These results not only suggest that these two cytokines may have a role in the pathogenesis of FeLV-C infection, but also they may have a role in regulating haemopoeisis in the cat.

FIV is a lentivirus infection which causes progressive immunosupression characterised by a decline in CD4+ T cell counts. The disease mimics that seen in HIV infected humans. Shortly after infection there is a brief febrile period involving a high level of viral replication. There then follows an asymptomatic phase of variable length and finally a terminal stage where the cat is susceptible to opportunistic infections and tumour development. Initially, many workers involved in HIV research concentrated on the terminal stages of the disease, but it is becoming apparent that important immunological processes may be occurring in the early stages of infection which may influence the subsequent disease course (Barcellini *et al*, 1994). The cat provides an ideal model for the human equivalent and much attention has focused on the immunological changes which occur during the course of this disease which, in experimental animals, can be followed from the point of infection.

The factors responsible for initiating a switch from asymptomatic to symptomatic disease in HIV and FIV infection has yet to be resolved. It has been proposed that changes in TH_1 and TH_2 T helper cell responses may mediate disease progression during HIV infection. Patients with TH_1 type responses, i.e. secretion of IL-2 and IFN-γ remain asymptomatic

whereas those that switch from TH_1 to TH_2, as indicated by a fall in IL-2 production and a rise in IL-4 production, go on to develop AIDS (Mosmann, 1994; Clerici, 1994). The mechanism by which retroviruses, including FeLV, cause immunosuppression is the subject of much research. Haraguchi *et al* (1995) have recently reported that a synthetic retroviral peptide, designated CKS-17, was able to regulate TH_1 and TH_2 cytokines in a negative and positive fashion, respectively. CKS-17 is a heptadecapeptide which represents the conserved domain of the transmembrane envelope protein, P15e, of feline and murine leukaemia virus. The regulation of T cell cytokines appears, from this study, to be due to regulation of IL-10 and IL-12.

A longitudinal study of cytokine production by cats infected with feline immunodeficiency virus has recently been reported (Lawrence *et al,* 1995). This group found that similar mechanisms of immune dysfunction, involving a switch from TH_1 to TH_2 T cell responses, may be occurring during FIV infection. Cats in the terminal stages of infection, produce low levels of IL-2 and high levels of IL-6, whereas those with high levels of IL-2 and low levels of IL-6 remain asymptomatic. As described in Chapter 1, interferon-γ plays an intimate role in the determination of whether a TH_1 or a TH_2 response occurs. Interferon down-regulates IL-10 production and the development of TH_2 responses. One might conclude that the long term administration of interferon-γ during the asymptomatic phase of FIV may maintain the TH_1 predominance and slow the progression of disease. However, further studies monitoring the changes in levels of IL-4, IFN-γ and antibody isotype during infection with FIV are required before a definitive role for T helper cell subsets in the immunopathology of FIV can be determined.

This kind of approach has, in part, been addressed by Clerici *et al* (1993). This group have used IL-12 in an attempt to restore HIV-specific cell-mediated immune responses in-vitro. As described in Chapter 1, IL-12 is not only is a potent inducer of interferon-γ but also promotes the establishment of TH_1 responses. In this study it is not clear, at present, whether IL-12 was directly acting on existing T cells to create a TH_1 predominance or whether it was acting through the generation of TH_1 T cell populations.

While retrovirus induced immune dysfunction may offer the possibility of immunological intervention with cytokines it is impossible to predict the outcome of such therapy. We do not fully understand the intricate cytokine network and it may be that combination cytokine therapy, as opposed to single agent therapy, may be more beneficial. Also the dose of cytokine to cause a beneficial clinical response may be too toxic or not tolerated by the host. In addition, the timing of therapy may be critical as administration of different cytokines may be more or less beneficial depending on the stage of disease. As the knowledge of cytokine interactions and the mechanisms of immune disruption in retroviral infections becomes clearer then a more rational approach to cytokine therapy may be adopted.

While one can speculate on the potential roles that cytokines may play in the resolution or the slowing of progression of retroviral infections, one also must consider the practicalities of treatment, especially in the veterinary clinical setting. The long term use of interferon-γ in cats with pre-clinical FIV may not be a practical one for several reasons. Firstly, the dose required to cause an immunomodulatory effect may be toxic to the host; secondly, the cost of treatment may be excessive despite recombinant

DNA technology aiming to reduce the costs of such therapies and thirdly, for treatment to be effective one would need to screen cats prior to the development of clinical FIV. In reality, veterinarians often see FIV positive cats when they have progressed to being clinically ill and, even if cats are tested positive prior to becoming ill, it may be difficult to convince owners that their cat requires treatment. However, the use of interferon-γ in FIV may be important in experimental FIV infection in order to help unravel the complexities of immunological dysfunction in this disease.

6.3 INTERFERON AS A VACCINE ADJUVANT

A clearer clinical role for recombinant interferon-γ is in its use as a vaccine adjuvant. The adjuvant properties of interferon-γ have been demonstrated in both human and murine systems and is discussed extensively in Chapter 1. Non-specific stimulation of the immune response by adjuvant formulations is often beneficial in cases of prophylactic or therapeutic vaccination. However, in certain instances, a more defined stimulation of the immune response using specific cytokines as adjuvants or co-adjuvants might be more effective. This can take the form of direct administration of the recombinant cytokine or expression of cytokines within live or disabled virus vectors. In addition, a more recent approach has been to administer the cytokine, again in the form of DNA, in a non-viral expression vector capable of expression in mammalian cells, i.e. so called DNA vaccination. Cytokines associated with adjuvanticity include IL-1, IL-2 and IFN-γ.

As discussed in Chapter 1, IFN-γ up-regulates MHC class II expression, enhancing antigen presentation, and suppresses the TH_2 lymphocyte response with resultant inhibition of humoral immunity. Thus, an

interferon-γ adjuvant might drive a vaccine induced immune response towards cell mediated mechanisms which correlate more effectively with protective immunity against viruses (Ramshaw *et al* 1992). Candidate live virus vectors for use in cats include feline herpesvirus, although the latent capacity of this virus may preclude its use. In latent virus infections there may be constitutive production of the inserted cytokine, the relevance of which is uncertain. However, if there was a scenario of superinfection after vaccination it may theoretically possible for the wild-type virus to recombine with the modified vector and have the cytokine gene transferred to the wild-type virus. Again the relevance of this is uncertain, but may allow the development of a new virus able to modify the host immune response.

Historically, the most effective vaccines against infectious agents have relied upon the use of attenuated or killed preparations of the wild-type infectious agent. Although these vaccines have been successful for a variety of diseases, some organisms are unable to be attenuated by traditional methods or are poorly immunogenic when administered as the killed preparation. In addition, attenuated vaccines have the added concern that the vaccine may be contaminated with virulent infectious agent or that attenuation is unsuitable for certain viruses which show latency or integrate in to the host genome. The use of recombinant DNA technology allows the production of vaccines using only those genes which are immunologically relevant so that there is only the minimal amount of information necessary to generate a protective immune response without the addition of irrelevant antigens that contribute to unwanted side-effects. The added advantage of recombinant vaccines is the ability to co-express cytokine genes in order to enhance the immune response.

Recombinant vaccines may be divided into several categories, but probably the two which have warranted the greatest attention are recombinant-vector vaccines and DNA vaccines. Recombinant vector vaccines use a viral vector to deliver relevant genes to generate an immune response. The most widely studied recombinant vaccine vehicle is Vaccinia Virus (VV). The gene encoding the protein to be expressed is inserted into the VV genome by the process of homologous recombination. Most genes are inserted into the Thymidine Kinase gene such that VV virulance is reduced. The advantage of these vector approaches are that multiple genes can be co-expressed so that fusion proteins can be produced. Cytokine genes can be conjugated to the relevant immunogen by this process. Vaccines have also been developed using other vectors. For example, an Avipox vaccine vector has been used to express gag and env genes of FeLV (Tartaglia *et al*, 1993). The disadvantage of using vectored vaccines is that, in addition to generating an immune response against the inserted gene, they also induce a response against the vector. This may in turn reduce the response against the insert or prevent the future use of the carrier for subsequent vaccinations (J.C Neil personal communication).

Nucleic acid or DNA vaccines have the advantage over vectored vaccines in that they can be administered repeatedly for booster inoculations without the generation of immunity to irrelevant antigens. In addition, the formulation of these vaccines are far easier in that they only require the simple purification of nucleic acid. In this approach, the genetic template is directly injected into the tissues resulting in the transfection of the host's cells and endogenous production of the antigen encoded by the genetic template. The plasmid DNA injected in to the host can be engineered to express both the relevant gene(s) and a cytokine gene(s) either as single or

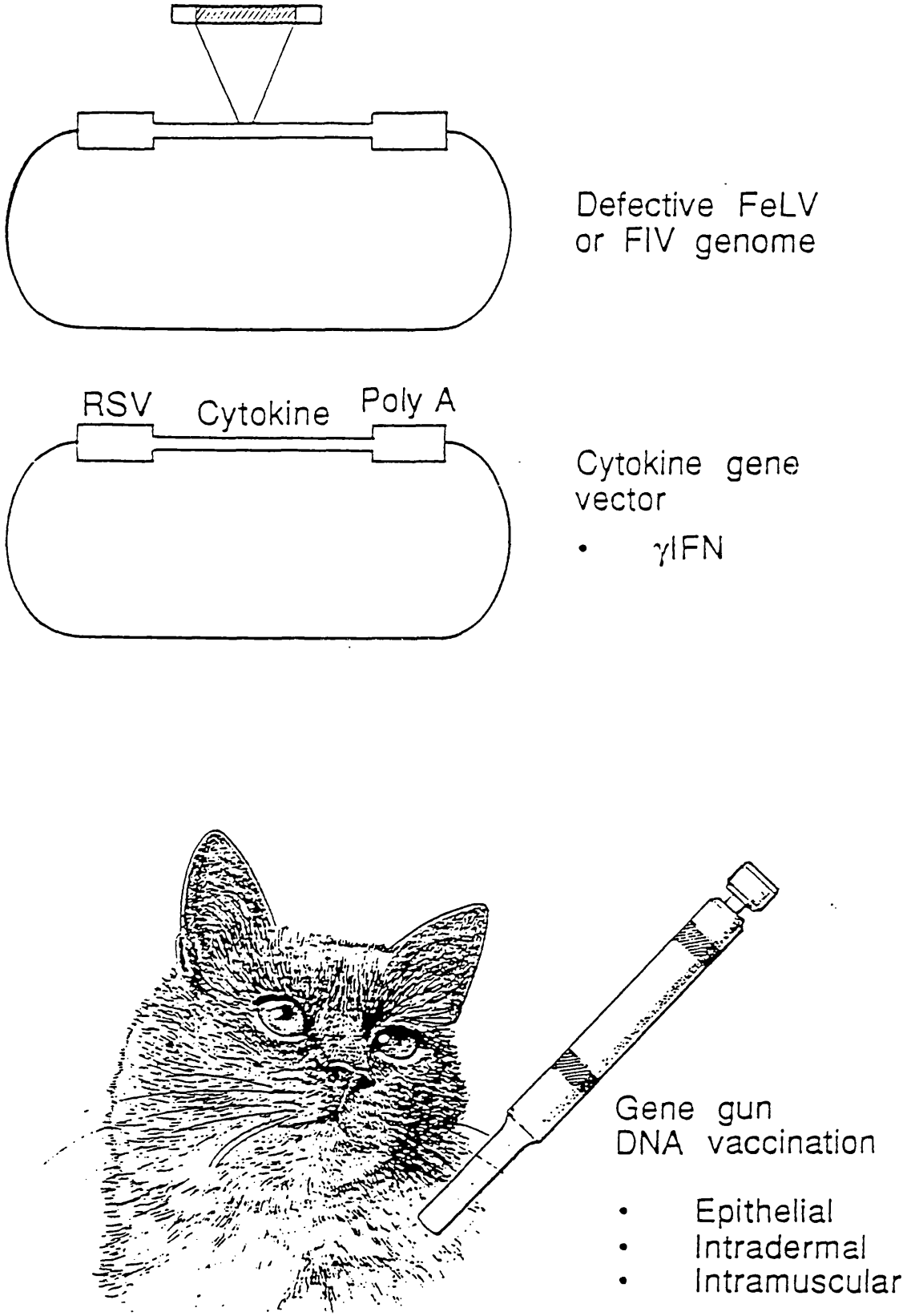


Fig. 6.1 DNA vaccination strategies for FIV and FeLV

fusion proteins. Alternatively, two plasmids can be injected, one expressing the required gene and one expressing single or multiple cytokine genes. This approach has been hindered by the method of injection of the vaccine. Direct injection into the muscle mass will lead to loss of some DNA along the fascial planes of the muscle and thus large amounts of DNA would have to be injected to ensure at least some entered the muscle cell. Ideally, it would be an advantage to be able to inject at multiple levels of the muscle in order to maximise the intracellular delivery of the DNA. To overcome these difficulties of delivery, a new approach has now been taken by some workers. This approach involves the adsorption of DNA onto gold particles which are then injected via a gene gun that delivers the DNA into the epidermal cells without the disruption of cell membranes. The motive force to accelerate the DNA particles is generated by release of a high pressure burst of helium gas within the gun.

DNA vaccination may become relevant in the cat in an attempt to protect against the retrovirus infections of FeLV and FIV. There are available plasmid constructs containing either replication defective FIV or FeLV DNA under the control of their own LTR promoters. It is planned to carry out a vaccination study using these constructs co-injected with a plasmid vector containing the feline recombinant Interferon-γ cDNA. The plasmid vector chosen to express interferon is a pRc/RSV plasmid (Invitrogen) which allows high levels of expression of the gene, in mammalian cells, under the control of an RSV LTR (Rous Sarcoma virus) promoter and enhancer.

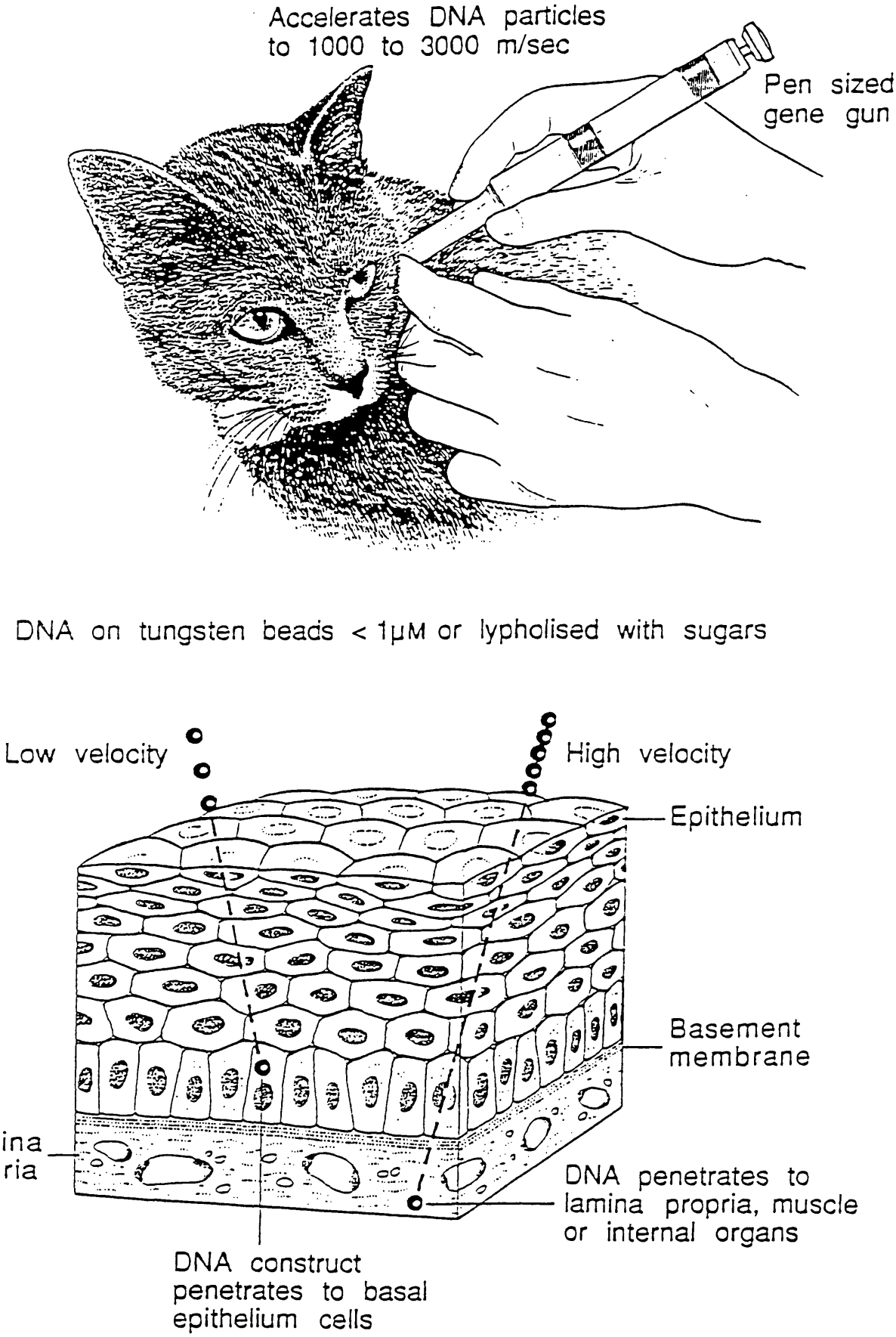


Fig. 6.2 Gene gun to vaccinate or introduce therapeutic genes

6.4 INTERFERON IN THE TREATMENT OF NEOPLASIA

The third main area where feline interferon-γ may be considered for clinical use is in the treatment of neoplasia. Interferons have both immunomodulatory actions and affect cell growth and differentiation. Interferon-γ, along with other immunomodulatory cytokines, may be important in the development or enhancement of the anti-tumour response. The area of tumour immunology is a controversial one and has been reviewed by Onions and Gorman (1989); Onions (1987), and Elmslie (1995).

The development of an anti-tumour immune response requires the existence of tumour specific antigens. The existence of tumour specific antigens in non-viral induced tumours in domestic animals has been poorly defined. A cancer cell represents altered self and tumour antigens can be considered under three broad categories: altered glycoproteins on the surface of the cancer cell as a result of transformation; viral epitopes, and the expression of differentiation antigens which are usually only present during development. The effector cells in the anti-tumour response include cytotoxic T cells (CTL's), B lymphocytes, macrophages, natural killer (NK) cells and lymphokine activated killer cells (LAK). Research over the past few years has demonstrated that cell mediated responses are more important than humoral immunity in the development of the anti-tumour immune response. Moreover, the relevance of each cell subset to the development of the response may vary with the stage of disease. In the early stages of neoplasia, NK cells may play a significant role, but as the disease progresses, CTL responses and macrophages may become more important. NK cells, LAK cells and macrophages lyse tumour cells in a non-MHC restricted fashion whereas the CTL response is MHC restricted.

Tumours may escape immune mechanisms for several reasons. While some tumours are antigenic, they may not necessarily be immunogenic. In addition some tumours have low levels of MHC Class I expression (e.g. Transmissible Venereal Tumour, TVT, in dogs), or produce, as yet undefined, soluble factors which down regulate the anti-tumour response. Another, more recent mechanism of immune escape has recently been defined. Activation of the T cell to lyse the tumour cell requires, not only recognition of antigen in the context of MHC, but also co-stimulation by accessory molecules. Some tumour cells do not possess these co-stimulatory molecules so that, after T cell binding, the T cell becomes anergic. The discovery of cytokines and their immunomodulatory properties lead to renewed interest in mechanisms of immune enhancement against tumours, the premise being that administration of exogenous cytokines will enhance immune effector cells.

Both IL-2 and IFN-γ are pivotal immunomodulatory cytokines and early clinical trials focused around these molecules. Greatest clinical success has been achieved using recombinant IL-2 in the treatment of renal cell carcinoma and malignant melanoma (reviewed by Foa 1992). IL-2 causes generalised immune enhancement by CTL activation, enhancement of NK activity, generation of LAK cells and in-vivo release of other cytokines such as IFN-γ. In addition, the administration of exogenous IL-2 can reverse T cell anergy. The use of recombinant interferon-γ has proved to be less beneficial with only moderate responses being observed.

The problem in using direct, systemic administration of cytokines is that the dose required to cause a clinical effect is often too high to be tolerated by the host. To minimise these side effects, studies have been carried out

to evaluate localised, peritumoural or paracrine cytokine therapy, either by intratumoural injection of recombinant proteins or by a gene transfer approach (reviewed by Yang and Sun (1995); Fujiwara *et al* (1994); Forni *et al* (1994); Pardoll (1992); Pardoll (1993); Rosenthal *et al* (1994); Sikora (1994) and Schmidt-Wolf and Schmidt-Wolf (1995).

The ultimate goal of cytokine gene therapy is to induce a sustained regional or even systemic antitumour response by transferring genes encoding specific cytokines into tumour bearing hosts, while avoiding systemic toxicity often associated with conventional cytokine immunotherapy. Gene delivery systems include both viral and non viral methods.

The cytokine gene therapy strategy may have applications for treatment of localised or regional tumours that are surgically unresectable or have disseminated. It has been suggested that local administration of *ex-vivo* cytokine transgene-modified tumour vaccine cells or transfection of tumour cells *in-vivo* to induce a tumour vaccine response at a tumour site or its draining lymphnode, may result in local as well as systemic anti-tumour effects against non-modified tumours. There have now begun several studies looking at this cytokine based gene therapy approach.

Some studies have focused on the transduction of interferon-γ genes into tumour cells. In mice, injection of IFN-γ transduced tumour cells gives rise to an increased cytotoxic T-lymphocyte response against tumour associated antigens. Restifo *et al* (1992) studied a murine sarcoma that had very low MHC class I expression and gave rise to no detectable immune response. Following retroviral gene transduction with interferon-γ, class I expression on the tumour was significantly increased and tumour

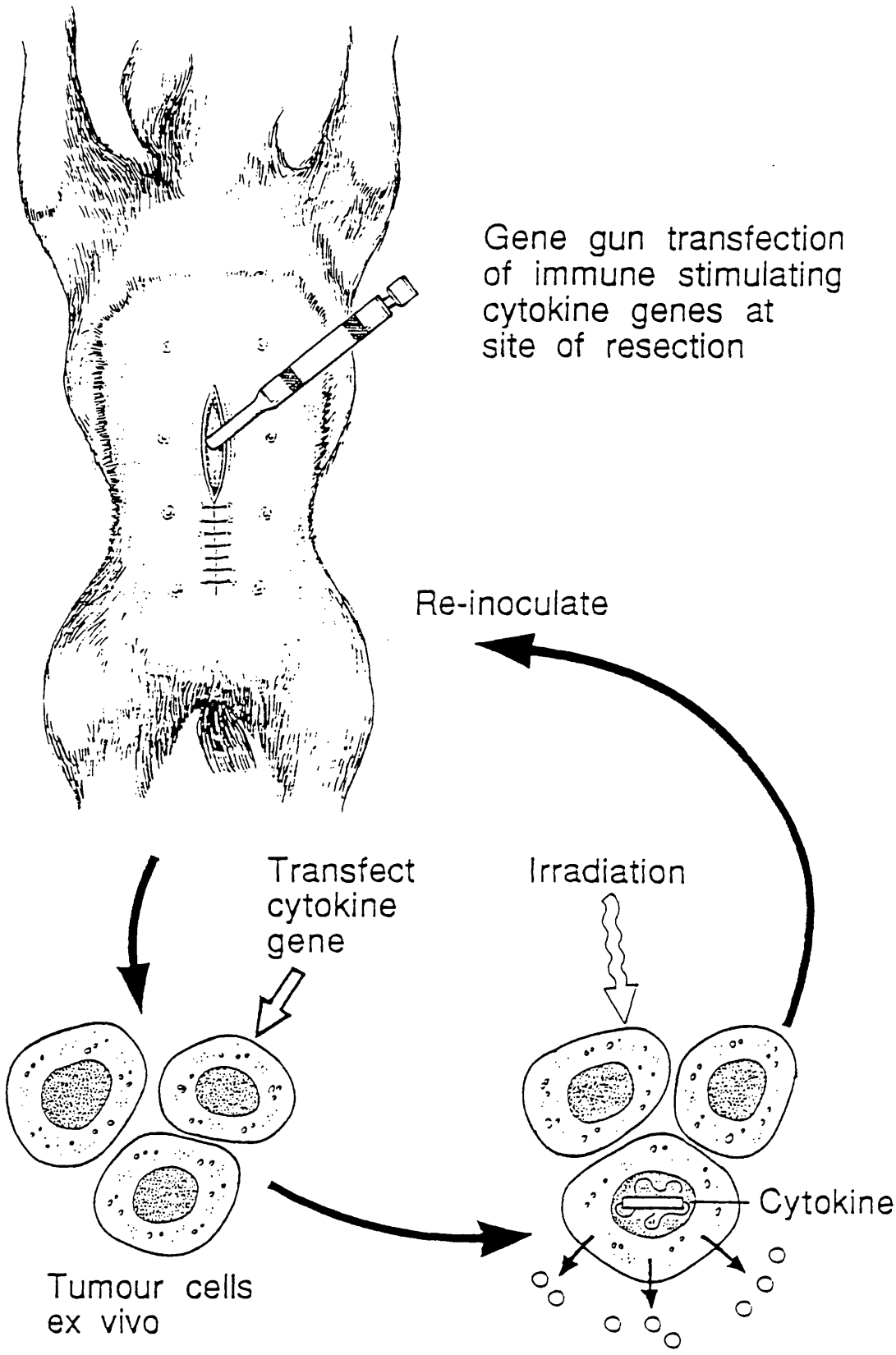


Fig. 6.3 Cytokine adjuvant therapy for cancer

infiltrating lymphocytes could be derived that successfully treated establish lung metastases from the parental unmodified tumour. More recently, Ogasawara and Rosenberg (1993) transduced a series of human tumours with retroviral vectors containing the interferon-γ gene. This group demonstrated a significant up-regulation of surface MHC antigens and, in addition, demonstrated that melanoma cells that have been transduced with the interferon-γ gene and express HLA.DR are able to stimulate cytokine release when co-cultured with autologous tumour infiltrating lymphocytes. At present a phase I clinical trial of human IFN-γ transduced autologous tumour cells in patients with disseminated malignant melanoma is being conducted (Seigler, 1994).

The mechanisms underlying the rejection of tumours after targeted gene transfer with various cytokines has been addressed by Hock *et al* (1992). This group demonstrated that all the cytokines tested required CD8+ T cell for complete long-term eradication of tumours but different cytokines activated heterogeneous transient tumour suppressive mechanisms. For interferon-γ this is mediated, in part by NK cells and macrophages.

While gene therapy in domestic animals would appear to be impracticable at the present time, there are important considerations. Domestic animals provide a population of naturally occurring tumours and are the closest model that we have to human equivalents. Thus the results of clinical trials in naturally occurring tumours in domestic animals can, in part be extrapolated to human medicine. It should, however, be borne in mind that cytokine gene therapy will probably never form the sole treatment for neoplasm but be an adjunctive treatment alongside more conventional therapies. Factors against the use of gene therapy, include the cost of treatment in domestic animals and the time delay involved in *ex-vivo*

tumour transduction. Both of these factors may be reduced considerably by the use of non-viral *in-vivo* methods for gene transduction (reviewed by Yang and Sun (1995). The most promising method may be the use of gene guns as described for DNA vaccination above. Another problem concerning gene therapy is that we are uncertain about the effects of long term constitutive expression of the transduced gene.

In summary, The cloning of domestic animal cytokine genes opens the door to explore their potential in therapy. While interferon-γ may eventually find a role in the treatment of retrovirus infections, at present the main clinical application would appear to be in the form of vaccine adjuvanticity. The use of interferon-γ in the treatment of neoplasia in humans has been, in part, disappointing but with the advent of gene therapy interferon-γ may eventually find a role in the fight against cancer.

Above, I have discussed the potential applications of these cytokines but practical considerations must also be borne in mind. These include practicalities of treatments, cost of treatments and efficacy and toxicity compared to conventional treatments. All of these points, along with pre-clinical data obtained from experimental animals, must be taken into consideration in the design of clinical trials.

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