Molecular Studies of TNF-α and IL-6 Gene Expression upon FcγRI Activation

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Contents

List of Figures

List of Tables

Summary

The high affinity Fc receptor for IgG, FcγRI, is expressed constitutively on the surface of macrophages and monocytes and plays a crucial role in the co-ordination of the humoral and cellular arms of the human immune system. The activation of the receptor by aggregation at the cell surface leads to recruitment of accessory signalling molecules the activation of a wide range of responses including antibody-directed cell cytotoxicity. However, the nature of the response is dependent on the differentiation state of the cell. It has been previously reported that in the monocyte-like U937 cell line when the cell is differentiated to a more macrophage-like phenotype a molecular switch causes an absolute change in the signal cascade that FcγRI initiates. However the effects of this switch on levels of gene expression in the nucleus have not been investigated. The work presented here details the development of two complementary assay systems to measure the levels of expression of the cytokines TNF-α and IL-6 in response to the activation of FcγRI in monocytes and macrophages. A competitive RT-PCR assay was developed to measure levels of mRNA in the cell and a reporter gene assay was developed to study in more detail the promoter sequences of the two genes. Both assays were shown to work in principle using the TNF-α mRNA and gene promoter, and transfection experiments utilising the TNF-α promoter coupled to a reporter gene showed a change in expression in response to induced cell differentiation. Although the work proceeded no further than this stage, it provided two valuable tools for further examination of the novel mechanism FcγRI employs to control gene expression.

Chapter 1: Introduction

1.1 The Human Immune System

The human body is a rich source of organic materials in a relatively poor environment and as such it is an ideal environment for microorganisms to grow in. In some cases these colonising microorganisms have no detrimental effect on the body's normal function and in a few cases, such as that of the bacteria found in the digestive tract, their presence is beneficial. However, most microorganisms would severely compromise the human body if there were no mechanisms to fight infection. To combat these infectious agents, the body has evolved an array of very effective defence systems, which can identify and neutralise foreign intruders which get past the physical barriers of the skin and mucosal membranes. These defensive adaptations are collectively called the immune system.

1.1.1 The Cells of the Immune System

The basis of the human immune system is a highly evolved family of cells, collectively called leucocytes, which arise from haemopoietic stem cells and provide the means to identify and destroy foreign antigens. The differentiation of the stem cells occurs along two distinct lineages: lymphoid and myeloid.

1.1.1.1 Lymphoid Cell Lines

The lymphoid cell line produces cells called lymphocytes. These cells are responsible for the specific recognition of antigens and thus are the cells that initiate a specific immune response against invading microorganisms or other pathogens. Lymphocytes derive from bone marrow stem cells and then mature in several locations in the body, producing functionally different, terminally

differentiated cells. Cells that mature in the thymus are called T cells, while cells that mature in the bone marrow are called B cells.

T cells identify pathogens and direct a particular response from the immune system to destroy them. Their ability to identify foreign molecules comes from their possession of a cell surface receptor called the T-cell receptor. This is a highly specific but also highly heterogeneous molecule and it allows the T cell population as a whole to specifically identify a large range of foreign pathogens. T cells can be identified as belonging to two main groups: T-helper or T_H cells and T-cytotoxic or T_C cells. T_H cells assist other leucocytes in performing their functions, while T_C cells function to destroy cells that have been infected by intracellular pathogens such as viruses.

B cells are involved in the production of antigen-binding molecules called antibodies. Like T cells the B cell population carries a cell-surface antigen-binding receptor. This B cell receptor is a membrane-bound version of the soluble antibody molecule. Like the T cell receptor it is highly heterogeneous and each individual B cell will bind one specific antigen. When this binding occurs the B cell is activated and will divide and differentiate into plasma cells which will then secrete large quantities of soluble antibody specific to the detected antigen.

1.1.1.2 Myeloid Cell Lines

The myeloid cell line generates cells called phagocytes. These cells are primarily involved in the destruction of antigenic particles by engulfing them, internalising in vacuoles and degrading them. Phagocytes can be subdivided into two groups: mononuclear phagocytes and polymorphonuclear granulocytes.

Mononuclear phagocytes are derived from bone-marrow stem cells and are relatively long-lived, persisting in the body for months or years. Upon exiting the bone marrow, newly formed cells join a pool of cells called monocytes, which circulate in the bloodstream. These circulating cells will then migrate from the circulation to specific tissues of the body where they differentiate into cells called macrophages. Macrophages throughout the body do not exist as a single population of uniform cells, but rather as a number of tissue-specific sub-populations. They are concentrated in the tissues, such as the liver (where they are called Kupffer cells, the kidneys (mesangial phagocytes) and the lungs (alveolar macrophages), where they are most likely to encounter the pathogenic particles that they are designed to eliminate. The work presented in this thesis is focused on gene function in macrophages and therefore their diversity, activation and function are covered in greater detail in **Section 1.3**.

Polymorphonuclear granulocytes are derived from bone marrow stem cells like mononuclear phagocytes but have a much shorter life span, living in the circulation for only 2 to 3 days. Several types have been identified on the basis of differential staining with cell dyes, the most common of which are neutrophils, basophils and eosinophils. All granulocytes possess a highly convoluted nucleus and numerous cytoplasmic granules. These granules are compact stores of enzymes and other molecules used to digest antigenic particles and elicit further immune responses. The granules can either be used internally where they fuse with vacuoles containing phagocytosed particles, or their contents can be released into the extracellular environment when the granules fuse with the plasma membrane; a process called degranulation.

1.1.2 Soluble Molecules of the Immune System

In addition to the diverse family of cells described above, a large array of soluble, extracellular

molecules are involved in the mediation of the immune response. Typically, these molecules are generated in lymphocytes and then secreted into the serum, normally in a controlled manner, in response to a specific signal. Two types of molecules particularly relevant to the work described in this thesis are immunoglobulins, or antibodies, and cytokines.

1.1.2.1 Immunoglobulins

Antibodies or Immunoglobulins (Ig) are a family of glycoproteins that specifically bind to antigen molecules and particles. They are soluble and found in the serum as well as most tissue fluids. There are five structurally and functionally distinct classes: IgG (gamma), IgA (alpha), IgE (epsilon), IgD (delta) and IgM (mu).

The basic immunoglobulin molecule is formed from 2 pairs of identical polypeptides called the heavy and light chains. Each chain consists of several similar domains called immunoglobulin folds. The typical immunoglobulin fold is formed from two layers of extended β-sheets, one of three β-strands the other of four β-strands. These two sheets are roughly parallel to each other and surround an inner portion packed with hydrophobic amino acid side chains.

The light chain consists of two Ig domains. The N-terminal domain exhibits great variation in its amino acid sequence and is classified as a variable-type domain and specifically referred to as the V_L or variable-light chain domain. The C-terminal domain sequence is more conserved and is thus classified as a constant-type domain and specifically called the C_L or constant-light chain domain. Humans, and indeed all vertebrates, possess two types of light chain, called kappa (κ) and lambda (λ). An individual immunoglobulin molecule can possess either two κ-chains or two λ-chains but not one of each type.

The heavy chain consists of between four and five Ig domains. The variable/constant system of classification employed for the light chain is also used to identify the domains of the heavy chain. As with the light chain, the N-terminal domain of the heavy chain is a variable type, specifically the V_H domain. The remaining domains are all constant-type and simply numbered one to three (or four) from N- to C-terminal (C_H1, C_H2, C_H3, C_H4). There are five types of heavy chain in humans, γ, α, δ, ε, and μ, each immunoglobulin containing an identical pair of one of these types. The five types of heavy chain define the five classes of immunoglobulin described above.

The heavy and light chains are joined together by covalent disulphide bridges and form a Y-shaped molecule with two antigen-binding arms, the Fab region (from antigen-binding Fragment), and a tail, the Fc region (constant Fragment) (see **Figure 1.1.1.1**). The variable domains at the N-termini of a heavy/light chain pairing align to form a hyper-variable binding pocket specific for one particular antigen molecule. The five classes of immunoglobulin all share this basic structure although two of the five are typically found in multimeric forms. IgA typically exists as a dimer, with the two Ig molecules facing in opposite directions and linked through a protein assembly called the J-chain. IgM monomers form a pentameric ring structure, again held together by a J-chain assembly. The structures of all five classes of immunoglobulin are shown in detail in **Figure 1.1.2.1**.

The antigen binding capacity of immunoglobulins allows them to both neutralise antigen particles and identify them for disposal by the immune system. The five classes are found in differing concentrations at different locations within the body and serve specific functions.

IgG is the most common immunoglobulin class, making up 70-75% of the circulating pool under normal conditions. It is also found in the interstitial fluids of the tissues and is the only

immunoglobulin class that can cross the placenta, via receptor-mediated endocytosis, to provide the gestating fetus with an immune capability.

IgM is usually confined to the circulation and, as a pentamer, is most effective at cross-linking antigenic particles. It is the first immunoglobulin produced after a primary immune challenge, typically being produced 2 to 3 days after an antigen is first detected by the body. After this primary phase, it is replaced by IgG as the principal immunoglobulin to respond to subsequent challenges by the same antigen (a change known as isotype switching).

IgA is the primary immunoglobulin found in the secretions of mucosal membranes such as those in the gastro-intestinal and respiratory systems. Here it defends against microbial invaders which have entered the body through the air or in ingested food. IgA is also the main immunoglobulin present in mammalian milk and provides the neonate with much needed immunity against gastro-intestinal infections.

IgE is the class of immunoglobulin associated with the allergic response, also known as type I hypersensitivity. This response, against harmless environmental antigens such as pollen or animal hair, is characterised by the release of molecules which generate an acute inflammatory response. IgE plays a central role in the sensitization and activation of the mast cells that are the primary source of these inflammatory agents in an allergic reaction. This immunoglobulin class is also central in the defence against parasitic infestation.

IgD is the least understood of the five immunoglobulin classes and its functions are not clear. It is also the least abundant in serum, normally occurring at trace levels of less than 1% of the total amount of immunoglobulin, but it is found in abundance in the membranes of B-cells, leading to the suggestion that it plays a role in antigen-triggered lymphocyte differentiation.

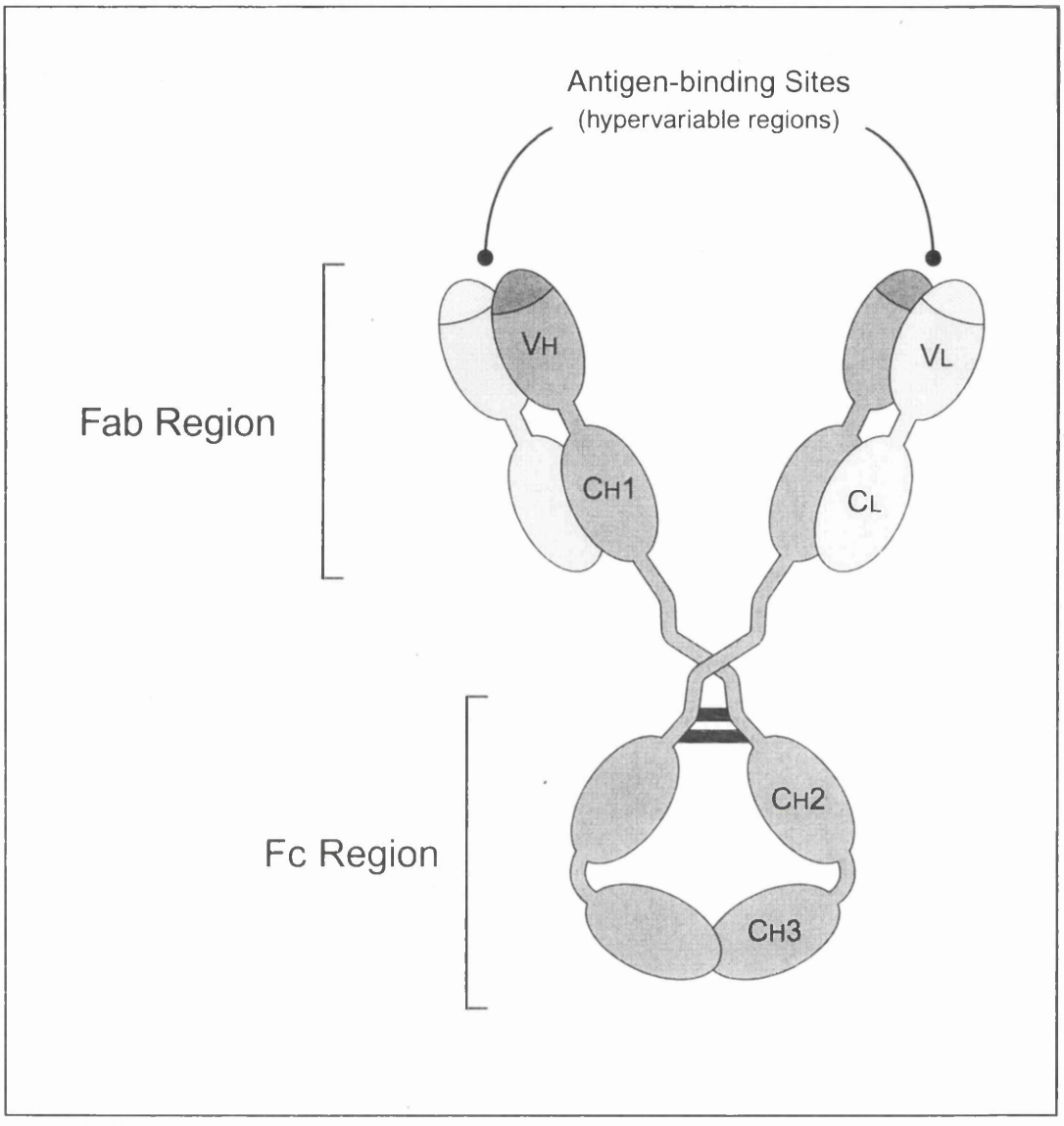


Figure 1.1.1.1 Basic Structure of Immunoglobulin Molecule

The basic structure of an immunoglobulin monomer is illustrated. The heavy and light chains each consist of a number of Ig-family domains, the heavy chain consisting of 4 such chains and the light chain consisiting of two. The variable-type domains at the end of each arm of the structure contain the hypervariable regions which form the specific, antigen-binding pockets. The heavy chains are linked by several covalent bonds formed by disulphide links.

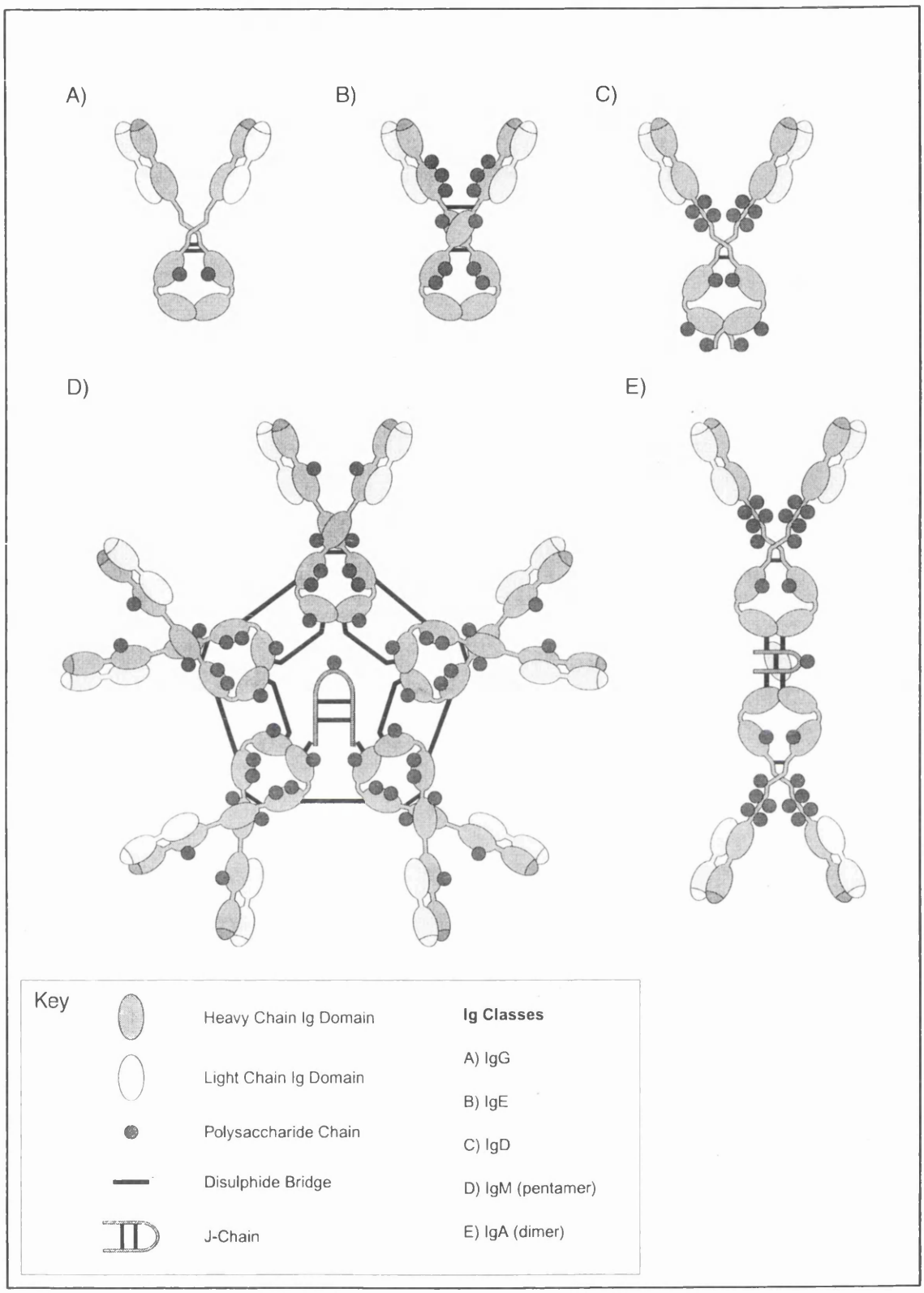


Figure 1.1.2.1 Structures of the Five Immunoglobulin Classes

The five classes of immunoglobulin share the same basic, monomeric structure but feature unique characteristics that allow them to carry out their specific functions within the immune system.

1.1.2.2 Cytokines

The term cytokine refers to a wide range of hormone-like peptides and glycopeptides that act as messengers and elicit cellular functions in every area of the immune system. They are classified as belonging to one of a number of a number of cytokine families that contain functionally or structurally similar members. They are functionally active at very low concentrations (several pg/ml) due to the high affinity with which specific cell-surface receptors bind them.

Interferons are primarily characterised as antiviral agents but they are also function as regulators of cell growth and differentiation. There are three types: IFN-α, IFN-β and IFN-γ. IFN-α, of which there are at least 20 variants, is produced by leukocytes in response to activation by viral particles or nucleic acids and has a substantial antiviral effect. IFN-β is a single protein produced by fibroblasts, again in response to viruses or nucleic acids, and has similar antiviral capability to IFN-α. IFN-γ is a much less potent antiviral agent than IFNs -α or -β, and is produced in a single form by lymphocytes. It is of particular relevance to the work presented in this thesis as an experimental regulator of the differentiation state of the U937 cell line (see **Section 2**).

Interleukins are a large family and were originally defined as molecules that are made by and act on leukocytes, although it has subsequently been shown that there are exceptions to both these rules. They are mainly produced by T-cells and display a wide variety of functions, with indivdual examples often displaying multiple functions dependent on their location in the body and the cells they interact with. IL-6, one of the two cytokines being examined in the work in this thesis is described in greater detail below.

IL-6 is produced by many different cell types. The main sources *in vivo* are monocytes, fibroblasts

and endothelial cells, although macrophages, T-cells, B-cells, smooth muscle cells and mast cells amongst others, will produce IL-6 upon stimulation The IL-6 molecule is a chain of 185 amino acids generated from a precursor protein of 212 amino acids. It is glycosylated at several residues and variation in the size of these post-translational modifications leads to several different forms being secreted, with molecular masses ranging from 21.5 to 28 kDa. The multi-functionality of IL6 is evident from the long list of alternative names it possesses (over 30). It plays roles in the differentiation of stem cells into neutrophils and monocytes, the differentiation of B-cells into immunoglobulin-producing plasma cells and the secretion of Ig from these cells to the extent that serum levels of IgG can rise 120-400 fold. IL-6 also effects non-immune system cells, promoting proliferation of kidney mesangial cells and keratinocytes. It is also one of the major growth regulation factors in many human myelomas. The IL-6 gene is approximately 5kb in length and contains five exons. Its promoter sequence contains a number of regulatory sequences which allow induction by a number of stimuli, including glucocorticoids, cAMP and NF-κB (see **Section 1.4**).

A third family of cytokines of particular relevance to the work in this thesis are the tumour necrosis factors (TNFs). This family consists of two molecules (α and β). TNF-α is the second cytokine examined in the work presented here. It is secreted by macrophages, monocytes and other lymphocytes, and is a primary mediator of the inflammatory response together with IL-1 and IL-6. Human TNF-α is a non-glycosylated 17 kDA polypeptide of 157 amino acids. The secreted form is produced from a 233 amino acid precursor protein by the post-translational modification of an N-terminal signal peptide. It shares approximately 30% sequence identity with TNF-β and forms dimers and trimers *in vivo*. The TNF-α mRNA is approximately 3.6kb in length and comprises four exons.

1.2 Fc Receptors

1.2.1 Immunoglobulin Classes and Fc Receptor Families

The antibody or immunoglobulin (Ig) molecules of the mammalian immune system play a vital role in the specific detection and removal of antigenic particles from the body. While they may appear superficially similar in structure, there are five distinct classes, found in different locations within the body and which have very different roles in the immune system.

The five classes of immunoglobulin are: IgG, IgA, IgM, IgE and IgD (see **Section 1.1.3**). The functionality of each class of immunoglobulin molecule is mediated by cell surface receptors and a different family of receptors has evolved for each class of immunoglobulin. These receptors all bind the immunoglobulin molecule on the Fc portion and are therefore known as Fc receptors with their nomenclature being derived from the particular class of immunoglobulin they bind. This is summarised in **Table 1.2.1** below:

Table 1.2.1 Immunoglobulin Classes and Fc Receptors

Immunoglobulin Class	Fc Receptor
IgG	FcγR
IgA	FcαR
IgM	FcμR
IgE	FcεR
IgD	FcδR

There are several different receptors in each Fc receptor family and these are not necessarily structurally related but are nevertheless are given the simple nomenclature FcRI, FcRII, FcRIII and so on. As with the immunoglobulins they bind, the Fc receptor families have distinct cellular distributions and this defines the nature of the immune response to antigen recognised by antibodies. For example, the high affinity receptor for IgE, FcεRI, is found on the surface of mast cells and its activation through the binding of IgE immune complexes results in the classic features of the allergic response; the activation of FcεRI leads to mast cell degranulation and release of inflammatory factors such as histamines and leukotrienes.

The work presented here focuses on the IgG activation of macrophages through Fcγ receptors.

1.2.2 Fcγ Receptors

1.2.2.1 Fcγ Receptor Classes

There are 3 classes of Fcγ receptor. These have distinct characteristics but are closely related in terms of structure, and their genes are located in close proximity to each other on human chromosome 1, suggesting a common evolutionary origin. The basic structures of the Fcγ receptors are illustrated in **Figure 1.2.1.1** and their characteristics are summarised in **Table 1.2.2** below.

All 3 classes of Fcγ receptor are type 1 integral membrane proteins with a single extracellular region and (with the exception of the GPI-anchored FcγRIIIb subclass) single membrane-spanning and intracellular regions. The extracellular region in all three classes is composed of several domains belonging to the Ig superfamily. These are globular domains comprised of seven β-strands arranged in two β-sheets of three and four strands. This type of domain is also found repeated in

Table 1.2.2 Fc Gamma Receptors

Receptor Class	CD Number	Molecular Weight	Affinity	Expression
FcγRI	CD64	72kDa	High	Macrophages, Monocytes, Neutrophils^{+}, Eosinophils^{+}
FcγRII	CD32	40kDa	Low*	Monocytes, Macrophages, Neutrophils, Basophils, Eosinophils
FcγRIII	CD16	50-80kDa	Low-Medium*	Monocytes^{+}, Eosinophils^{+}, Macrophages, Natural Killer Cells, T Cells

* only binds polymeric IgG

$^{+}$ expression is inducible

immunoglobulin molecules, from where it derives its name.

1.2.2.2 FcγRI: Structure and Function

FcγRI is the high affinity receptor for IgG and can bind monomeric IgG with an association constant (Ka) of 10^{8}-10^{9} M^{-1} under physiological conditions. Its extracellular region comprises 292 amino acids and consists of 3 Ig superfamily domains. The Ig domain is the repeated tertiary structure in immunoglobulin molecules (hence its name) and is also found in a wide range of other proteins. It consists of seven β-strands of amino acids arranged in two β-sheets of 3 and 4 strands. The outer two domains of FcγRI share sequence identity with the two extracellular domains found in FcγRII and FcγRIII. The third domain, closest to the cell membrane, differs significantly from these and is essential for binding IgG at high affinity. However, experiments involving the generation of chimeric receptors, where domain 3 of FcγRI is added to the two domains of FcγRII, have indicated that the addition of domain 3 is not sufficient to confer high affinity binding characteristics to FcγRII. This

suggests that small variations in the amino acid compositions of domains 1 and 2 between FcγRI and FcγRII, which allow monoclonal antibodies to distinguish FcγRI from FcγRII, are also vital for conferring the high affinity of ligand binding characteristic to FcγRI .

As with the other high affinity Fc receptors such as FcαRI and FcεRI, FcγRI does not possess any identified signalling motifs within its intracellular tail. These motifs would normally be used to recruit and activate intracellular signalling molecules such as soluble tyrosine kinases and monomeric G-proteins as the first steps in a signal cascade. In order to activate signal transduction pathways within the cell FcγRI must recruit molecules that possess these sequences.

FcγRI activates a large array of cellular functions. It is the mediator of endocytosis and phagocytosis of IgG. Because FcγRI can bind monomeric IgG, these functions are only activated when IgG is complexed with antigen and can cross-link multiple receptors. FcγRI also activates intracellular signalling pathways. Because it has no intrinsic signal activation motif within its intracellular tail, the receptor does this by recruiting accessory molecules embedded in the cell membrane which do possess such sequences in their cytosolic regions. The two molecules particularly relevant to the work presented in this thesis are the gamma (γ-) chain and FcγRII.

1.2.2.3 The Gamma Chain

The gamma (γ-) chain was first identified as a component of the multi-subunit high affinity receptor for IgE, FcεRI, on mast cells (Perez-Montfort *et al*, 1983). The γ-chain was found to associate with the α-chain which binds IgE and was necessary for the cell surface expression of FcεRI. Later the same γ-chain was shown to associate with the α-chain of FcγRI in the membrane of macrophages (Ernst *et al*, 1993). The γ-chain is a 7 kDa, type I membrane-spanning polypeptide and is normally

found as a homodimer in the membrane; the two chains being linked by a single disulphide bridge (see **Figure 1.2.1**). The γ-chain belongs to a family of membrane-associated signal transduction molecules that also includes the ζ-chain component of the T-cell receptor (Orloff *et al*, 1990), and it shares the distinct structural characteristics shown by these molecules. The extracellular portion of the chain is minimal, the only notable feature being the presence of a cysteine residue that forms a covalent disulphide bridge, to form the homodimer. The membrane-spanning region is a single α-helix and the intracellular domain features a classic ITAM signalling motif (Samuelson *et al*, 1992). The γ-chain associates with FcγRI through non-covalent interactions between their transmembrane α-helices (Harrison *et al*, 1995).

1.2.2.4 FcγRII

FcγRII is structurally related to FcγRI (see **Figure 1.2.1**) with two important exceptions. It has only two extracellular Ig domains and binds IgG at lower affinities than FcγRI. In fact, it is unable to bind monomeric IgG at detectable levels and will only bind IgG when aggregated as part of an immune complex. It also has a more extensive cytoplasmic region than FcγRI, and includes an intracellular signal-mediating motif. The two subclasses of FcγRII, a and b, possess motifs which elicit functionally opposite responses. FcγRIIa has a non-classical ITAM (Immuno-Tyrosine Activation Motif) sequence, while FcγRIIb has an ITIM (Immuno-Tyrosine Inhibition Motif).

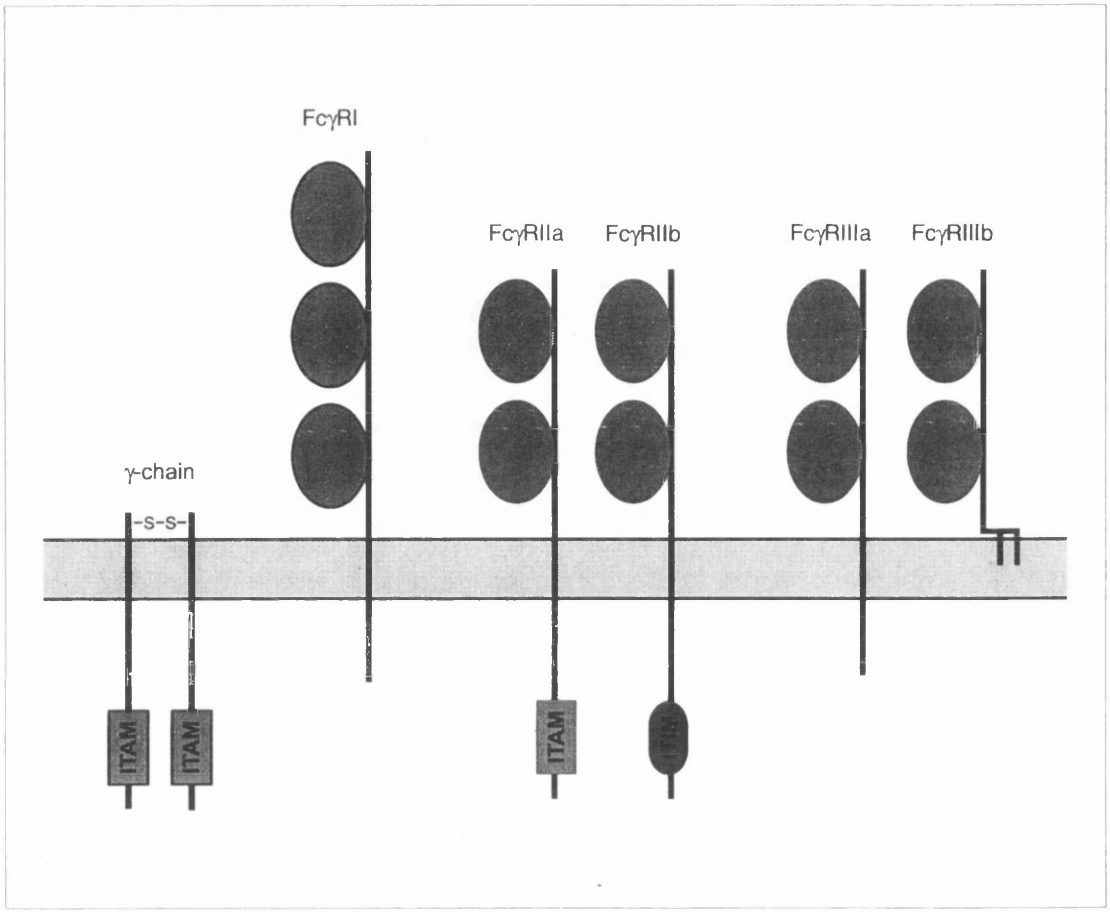


Figure 1.2.1 Fc gamma Receptors

Schematic representation of the three classes of Fc gamma receptor as well as the γ-chain accessory signalling molecule embedded in the cell plasma membrane (Top of picture shows the extracellular environment while the bottom shows the cytsol of the cell). Both the γ-chain and FcγRIIa feature ITAM (Immuno-Tyrosine Activation Motif) sequences in their cytosolic tails while FcγRIIb feature an ITIM (Immuno-Tyrosine Inhibition Motif) sequence.

1.2.3 FcγRI-Mediated Cell Signalling in U937 Cells

The U937 cell line provides an experimental system whereby the functionality of FcγRI can be examined in the context of a controlled differentiated cell type. Treatment with dbcAMP results in the U937 cell differentiating from its inactive monoblast-like phenotype to a more macrophage-like state (Sheth *et al*, 1988), while treatment with IFN-γ results in cell activation and a functional monocyte-like phenotype (Ishizuka *et al*, 1995). This model system allows controlled differentiation and thereby overcomes the major problem of heterogeneity normally encountered when using macrophages harvested from tissues.

While FcγRI will bind and internalise monomeric IgG, aggregation through binding immune complexes is required for internalisation and activation of the full range of cellular responses. These responses differ between in monocytes and macrophages. U937 cells were used to explore the underlying nature of the difference in signalling pathways.

Briefly, in IFN-γ primed U937 cells, activation of FcγRI results in a single calcium spike lasting 1-2 minutes and activation of phospholipase D. In cells differentiated with dbcAMP, activation of FcγRI results in a prolonged calcium signal caused by the activation of I_{CRAC} and in these cells phospholipase C and not phospholipase D is activated by the receptor (Melendez *et al*, 1998). The mechanism underlying this switch in signalling cascades activated by FcγRI was shown to result from a switch in the accessory molecule recruited by FcγRI. Thus, in IFN-γ primed cells, FcγRI recruits the γ-chain whereas in dbcAMP differentiated cells, FcγRI recruits FcγRII for signal transduction.

FcγRI, FcγRIIa and γ-chain are all constitutively expressed on the surface of U937 cells. However,

treatment with IFN-γ or dbcAMP does alter the levels of expression. Treatment with IFN-γ generates a transient 15-fold increase in the levels of expression of FcγRI mRNA (Allen and Seed, 1989) and a subsequent up regulation in surface-expressed protein. IFN-γ also results in an increase in expression of both FcγRIIa (Davis *et al*, 1994) and γ-chain (Ernst *et al*, 1993) although mRNA levels of both of these molecules increased by only twofold. Upon treatment with dbcAMP, the level of FcγRI mRNA falls by about 50% although this change is not reflected in the amount of surface-expressed receptor, which is maintained at a steady level (Davis *et al*, 1994). Levels of FcγRIIa and γ-chain mRNA are increased and result in increased surface expression of both accessory molecules (Davis *et al*, 1994).

It is this change in the relative amounts of FcγRI, FcγRIIa and γ-chain on the cell surface which is at the heart of the switch in cell signalling differentiation causes. Treatment with IFN-γ causes a differentiation state where FcγRI recruits γ-chain exclusively as its accessory signalling molecule, whereas treatment with dbcAMP causes FcγRI to recruit and signal through FcγRIIa when it is aggregated (Melendez *et al*, 1998).

Although both FcγRIIa and γ-chain both have ITAM motifs, these initiate different signalling cascades. With FcγRIIa, a first step in this cascade is the activation of the phospholipase PIP_2-PLC, which generates IP_3 by cleaving membrane-bound PIP_2. The secondary messenger, IP_3 then activates calcium channels in intracellular stores (IP_3 receptor) and the plasma membrane (I_{CRAC}) resulting in a sustained increase in cytosolic calcium levels (Melendez *et al*, 1998; Davis *et al*, 1994). A γ-chain-mediated activation results, in the first instance, in the recruitment and activation of the phospholipase, PtdCho-PLD. This causes the downstream activation of sphingosine kinase and the generation of a transient calcium signal (Melendez *et al*, 1998 (2), Davis *et al*, 1994) observed as a single calcium spike lasting 1-2 minutes. The absolute nature of the switch in FcγRI signalling

which occurs with differentiation is dramatic and this tight control observed in U937 cells points to the potential for very different roles that FcγRI can elicit from a tissue macrophage compared to its circulating monocyte precursor.

1.3 Activation of Macrophages

Macrophages can be generally described as a population of ubiquitously distributed mononuclear phagocytes responsible for numerous homeostatic, immunological, and inflammatory processes. Their wide tissue distribution makes these cells ideally suited to provide an immediate defence against pathogens prior to a more organised mobilisation of immune system cells and signals. Because macrophages participate in both specific immunity via antigen presentation and cytokine production, as well as nonspecific immunity against bacterial, viral, fungal, and cancerous pathogens, it is not surprising that macrophages display a wide range of functional and morphological phenotypes.

1.3.1 Macrophage Heterogeneity

Examination of the body's macrophages reveals a highly heterogeneous population of cells which can be classified into two broad groups: those found in the bloodstream, which exhibit a more monotype-like, undifferentiated nature, which are termed *circulating macrophages* resident in tissues, and those which are generally differentiated and specialised to function in their location in tissues, called *residential macrophages*.

1.3.1.2 Circulatory Macrophages

Circulatory macrophages are young cells but already possess migratory, chemotactic and phagocytic activities, as well as Fc gamma receptors expressed in their plasma membranes. Under migration into tissues, circulatory macrophages undergo further differentiation (usually lasting at least one day) to become multifunctional tissue macrophages. It can be argued that monocytes can be included in

the circulating macrophage population and should be considered fully functional for their location, changing phenotype in response to factors encountered in specific tissue after migration.

1.3.1.2 Residential Macrophages

It has also long been recognized that macrophages isolated from different sites in the body display a diversity of phenotypes and capabilities. Because macrophage function is dependent in part on signals received from the immediate tissue microenvironment, it is suggested that macrophage heterogeneity may arise from unique conditions within specific tissues. For example, the sterile, anaerobic environment of the spleen or peritoneum will impart different constraints on macrophages found there than does the aerobic environment of the alveolar macrophage, which contains numerous external factors. These different constraints produce different phenotypes, illustrated in experiments using antibodies directed against specific membrane antigens. For instance, human breast milk macrophages express an antigen not observed on monocytes, alveolar macrophages, or peritoneal cells. Furthermore, human alveolar macrophage express high levels of MHC class II antigen, whereas the opposite is found for peritoneal macrophages.

1.3.1.3 Generation of Macrophage Heterogeneity

The macrophage population in a particular tissue may be maintained by three mechanisms: influx of monocytes from the circulating blood, local proliferation and biological turnover. Under normal steady-state conditions, the renewal of tissue macrophages occurs through local proliferation of progenitor cells and not via monocyte influx. Originally, it was thought that tissue macrophages were long-living cells. More recently, however, it has been shown that depending on the type of tissue, their viability ranges between 6 and 16 days. Given this relatively rapid turnover of cells there

exists, in a particular locus, a population of macrophages displaying a spectrum of maturational states and an attendant diversity of function.

Because macrophages are responsible for numerous inflammatory processes, it becomes important to distinguish between normal or steady state cell turnover and differentiation and induced haematopoiesis associated with immunological challenge. Production of the macrophage lineage from bone marrow-derived monocytes is normally controlled by M-CSF, which is constitutively produced by many cell types. In response to invasive stimuli and inflammation, monocyte numbers increase dramatically, as do serum levels of M-CSF. In addition, GM-CSF appears in the serum. Although there appear to be a large overlap of macrophage progenitors able to respond to M-CSF or GM-CSF, the very different structures and signal transduction mechanisms of the receptors for M-CSF and GM-CSF suggest that the differentiation pathways they initiate, would be dissimilar.

M-CSF-derived macrophages are larger, have a higher phagocytic capacity, and are highly resistant to infection by virus compared to GM-CSF-derived macrophages. Conversely, GM-CSF-derived macrophages are more cytotoxic against TNF-α-resistant tumour targets and express more MHC class II antigen.

The production of functionally distinct macrophage populations gives the non-specific immune system added flexibility to respond to immunological or inflammatory stimuli. It is probable that the nature of an immune response is dictated in large part by the functional phenotypes of the macrophages present within the area of the immune challenge.

1.3.2 Macrophage Activation

1.3.2.1 Macrophage Activation is a Two-Stage Process

The term "activated macrophage" is reserved for macrophages possessing specifically increased functional activity. The process of activation is not to be confused with differentiation, which is a process through which differentiated macrophages acquire only an increase *ability* to perform specific functions. Characteristically, residential tissue macrophages are relatively quiescent immunologically, having low oxygen consumption, low levels of major histocompatibility complex (MHC) class II gene expression, and little or no cytokine secretion. Resident macrophages are, however, phagocytic and chemotaxic and retain some proliferative capacity.

There are two stages of macrophage activation, the first being a primed stage in which macrophages exhibit enhanced MHC class II expression, antigen presentation, and oxygen consumption, but reduced proliferative capacity. The agent that primes macrophages for activation is IFN-γ, a product of stimulated T cells. But many other factors, including IFN-α, IFN-β, IL-3, M-CSF, GM-CSF and TNF-α can also prime macrophages for selected functions.

Primed macrophages respond to secondary stimuli to become fully activated, a stage defined by their inability to proliferate, high oxygen consumption (through NADPH oxidase), killing of extracellular and intracellular parasites, tumour cell lysis, and maximal secretion of mediators of inflammation, including TNF-α, IL-1, IL-6, reactive oxygen species, and nitric oxide produced by iNOS. Agents capable of providing secondary signals are diverse and include LPS and antibody-associated antigen.

1.3.3 Cell Surface Receptors Mediate Macrophage Activation

The cell-surface receptors that bind macrophage-activation signals, and as a result initiate intracellular signalling cascades, can be divided into two broad groups. Those that mediate activation of the *innate* immune response include the LPS receptor and the Toll family of receptors. Receptors that initiate and coordinate the *specific* immune response include Fc receptors (see **Section 1.2**).

1.3.3.1 The LPS Receptor

Lipopolysaccharide (LPS) is a component of the cell walls of Gram-negative bacteria. It is recognised by the human immune system as an adjuvant antigen, that is, one that elicits a non-specific response, boosting the activity and effectiveness of the whole immune system. LPS is primarily detected by a specific, cell-surface receptor called the LPS receptor (Wright *et al.* 1990).

The LPS receptor (LPS-R), also known as CD14 using the CD system of nomenclature, is a 53 kDa protein composed of 356 amino acids (Bazil *et al.* 1986). Its mature form requires cleavage of a 19-amino acid peptide and N-linked glycosylation at 4 sites on the polypeptide chain. The receptor is anchored to the cell surface by linkage to GPI molecule that embeds in the outer of the two lipid layers that make up the plasma membrane.

LPS-R is strongly expressed on the surface of monocytes and most tissue macrophages, and to a lesser extent on the surface of granulocytes. It is not expressed on the myeloid progenitor cells that mature to these cell types. Expression can also be induced in non-myeloid cell types, including hepatocytes and epithelial cell types, upon exposure to LPS.

As well as the cell-surface expressed form of the receptor, there are at least two soluble forms of LPS-R. Cells shedding the surface-bound receptor, resulting a 48 kDa molecule, produce one form (Bazil *et al.* 1991). The second form of soluble receptor is secreted from cells before the addition of the GPI anchor. The soluble LPS receptor is present in whole blood in amounts 100-1000 times greater than the membrane-bound receptor.

In binding LPS, the LPS receptor associates with serum protein called LBP (LPS Binding Protein) that acts as a lipid transfer enzyme, moving monomers of LPS to the LPS receptor binding site (Hailman *et al.* 1994). The activation of the LPS receptor by binding the LPS/LPB complex results in the activation of cells and release of pro-inflammatory cytokines such as TNF-α. Also up-regulated is the expression of cell adhesion molecules. This allows phagocytic cells such as macrophages to directly engage and destroy the bacterial source of the LPS.

1.3.3.3 The Toll Receptor Family

The Toll receptors (TLRs – Toll-Like Receptors) are a family of structurally similar cell surface receptors that bind a wide range of ligands and initiate key features of the innate immune response. The function of Toll receptors in the immune response was first discovered in *Drosophila* (Lemaitre *et al.* 1996) and subsequently an analogue were found and characterised in the mammalian immune system (Medzhitov *et al.* 1997). After this initial discovery came the rapid identification of several additional TLRs within the human genome. Ten TLRs have been found so far, classified as TLR1 to TLR10.

While individual TLRs bind specifically to one or two ligands, the TLR family as a whole will bind a wide range of microbe-derived ligands. These include LPS and LTA, bound by TLR4, PGN and

lipoproteins, bound by TLR2, and unmethylated bacterial DNA, bound by TLR9.

The TLR family all share a similar structure. Embedded in the plasma membrane the typical TLR possesses a large extracellular portion made up of between 12 and 23 leucine-rich repeat domains with a cysteine-rich domain flanking the transmembrane region. The intracellular portion consists of a Toll/IL-1 receptor homology domain (TIR).

TLR activation leads to an immune response mainly through gene regulation via the NF-κB transcription factor system. This TLR-mediated immune response is available to a wide range of cell types that form the first line of defence against an immune challenge. Macrophages, neutrophils and dermal, gut and lung epithelial cells all express various TLRs on their surfaces. Upon exposure to microbes they are then recruited from the plasma membrane to phagosomes where they help facilitate the destruction of invading cells.

1.3.4 Functions of Activated Macrophages

The central regulatory role that macrophages play in the immune response is reflected in the vast array of substances they can produce. These substances allow such diverse functions as inflammatory response initiation and regulation, phagocytosis (see below) and destruction of microbes and reorganisation of tissues (see **Figure 1.3.4.1**).

1.3.4.1 Phagocytosis and Microbicidal Functions

The engulfment of invading bacterial cells and other microbes, a process known as phagocytosis, is an essential part of the immune response as this provides the main mechanism of clearing microbes

from the body. Macrophages will not only engulf microbial cells, but also possess mechanisms to degrade them and process the antigenic markers they carry for presentation to other cells as an initiatory step in the specific immune response. The process begins with the macrophage attaching to the microbe and then engulfing the cell in an area of macrophage plasma membrane. This phagosome then detaches from the plasma membrane and is held in the cytosol. Organelles called lysosomes then fuse with the phagosome. Lysosomes are small endosomes that contain the components of a variety of cell-killing mechanisms. These include reactive oxygen species (including hydrogen peroxide, the superoxide anion and the hydroxyl radical), reactive nitrogen species (including nitric oxide) and also oxygen-independent and enzymic mechanisms (including proteases, lysozyme and hydrolases). These components are released into the phagosome and proceed to degrade the cell. Selected antigen molecules are then taken from the phagosome and displayed in the surface of the macrophage in association with the MHC class II protein. This whole process is summarised in **Figure 1.3.4.2**.

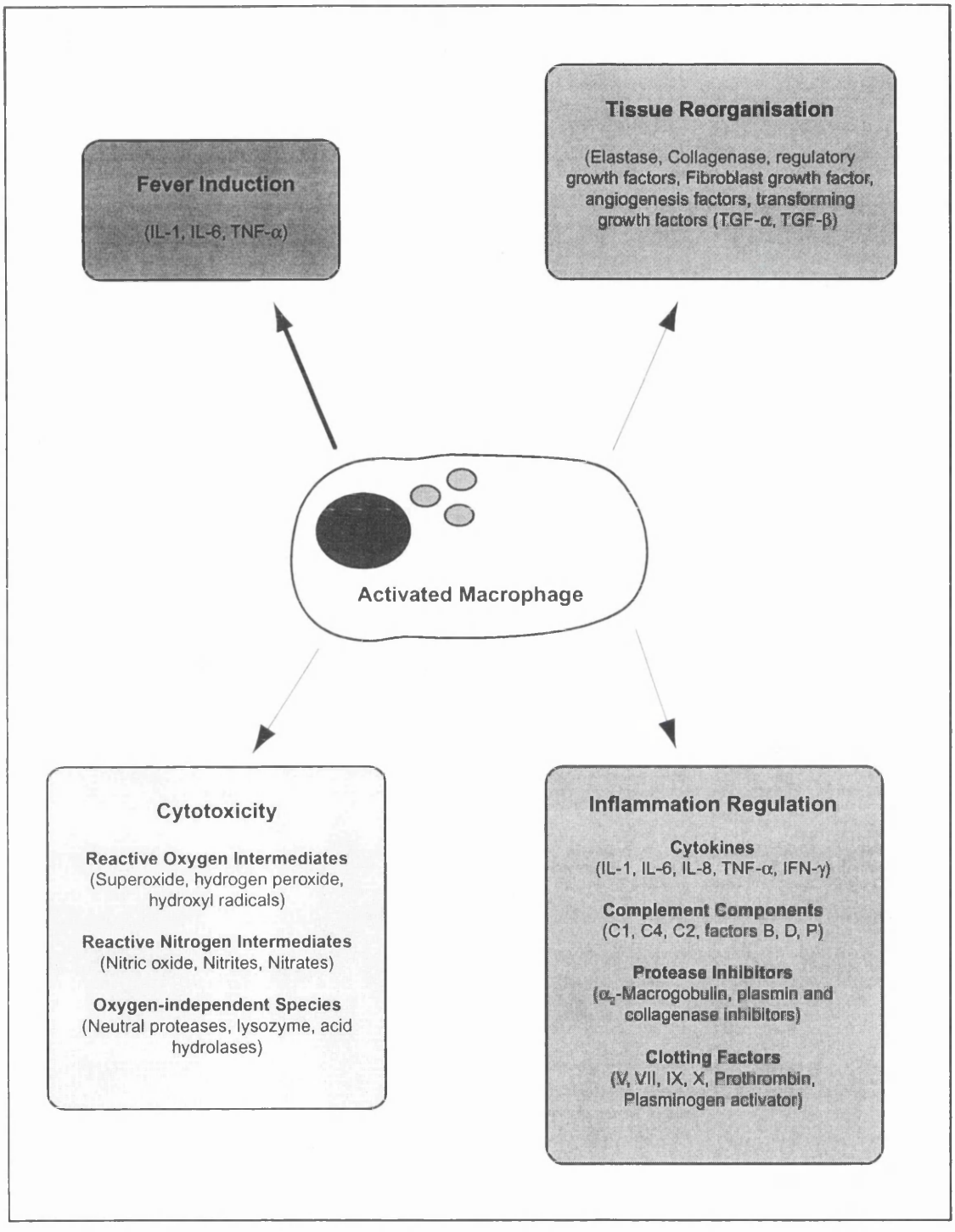


Figure 1.3.4.1 Macrophage Function and Molecule Secretion

The vast array of substances an active macrophage can secrete allows it to perform a wide range of regulatory and direct functions in the immune response.

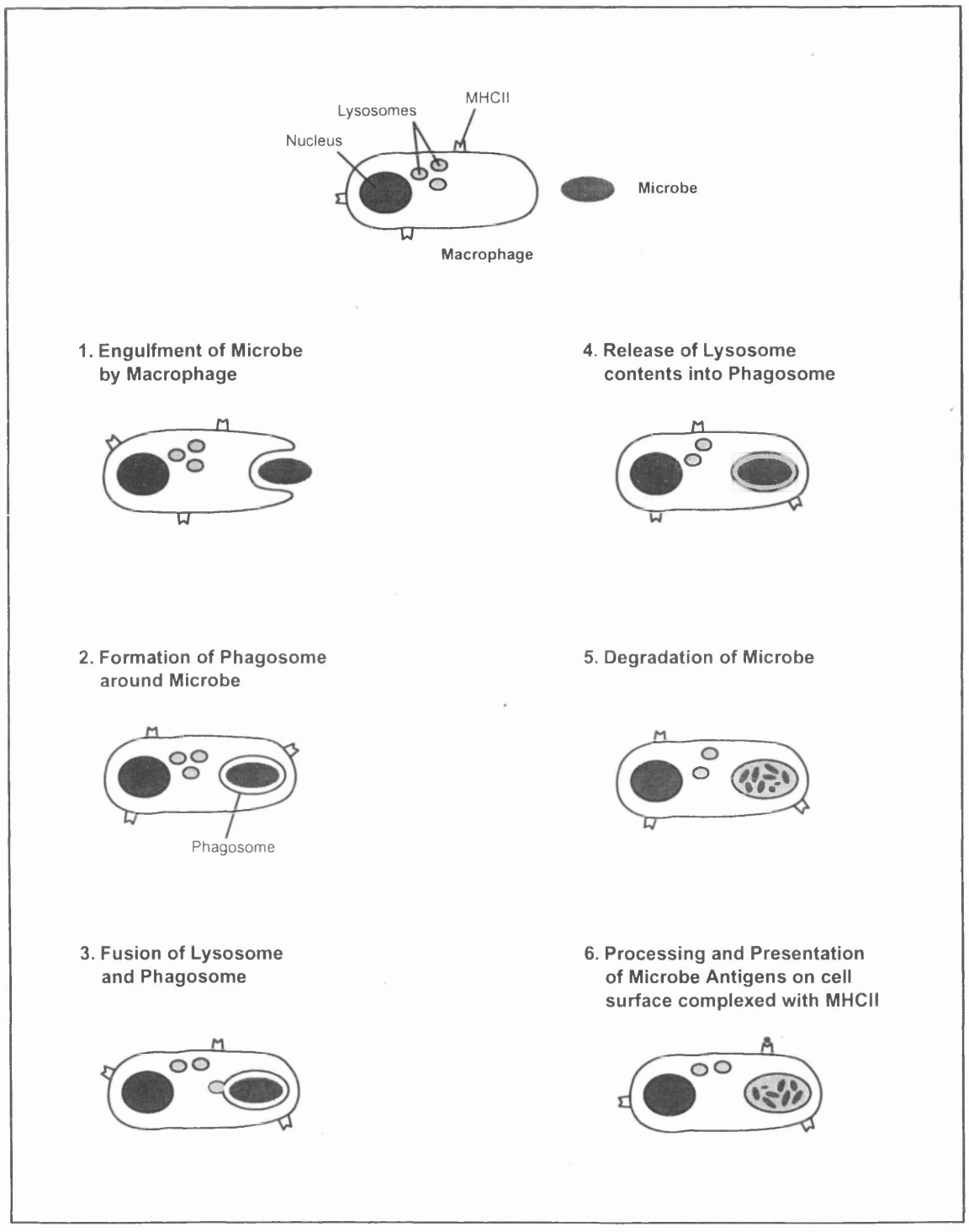


Figure 1.3.4.2 Phagocytosis and Degradation of Microbial Cells

The phagocytic killing of microbial cells by macrophages is important as a way of clearing invading cells from the host and also in initiating the specific immune response by the processing and presentation of antigen.

1.4 Regulation of Gene Expression

The central mechanism in the response of macrophages (and indeed all cells) to stimulus is an alteration of the levels of gene expression in the nucleus. Gene expression controls the cell at the most fundamental level, dictating which functions the cell can perform and how it responds to the environment around it.

1.4.1 Strucuture of the Eukaryotic Gene

The control of expression of genes in eukaryotic cells is inherently linked to the structures which make up the genes themselves. Although a gene, in the form of chromosomal DNA held in the nucleus, is a relatively homogenous polymer when compared to the panaromic range of protein structures, the information contained in the nucleotide sequence allows regulation of its expression with a high degree of control and finesse.

At the most basic level, a gene can be divided into two parts: the part that encodes a protein and the part that does not. However, the protein-coding part of the gene is not a single, unbroken length of DNA. The coding region of the gene consists of one or more lengths of sequence called exons, which are interspersed with non-coding sections called introns. The number of exons can vary greatly. The multiple genes encoding the histone proteins that form the chromosomal DNA-protein complex contain a single, uninterrupted coding region, as do genes encoding the interferon family of extracellular signalling molecules. In contrast, the coding transcript for an immunoglobulin light or heavy chain is made from a large number of exons, chosen from an even larger selection at the locus on the chromosome. Indeed, given the fact that a huge variety of different proteins (immunoglobulins) can be produced from this alternative splicing of many exons it is perhaps

inaccurate to describe the exons as belonging to a *single* gene at all.

The transcription of an RNA molecule from genomic DNA results in a sequence that incorporates both exons and the introns interspersed between them. It is only after this initial transcription step that processing occurs to remove the introns and produce a mRNA molecule that can be translated into a polypeptide chain by the ribosomal machinery in the cytosol.

Upstream of the first exon in a gene lies a region of variable length called the promoter. Within this region lie specific lengths of sequence that can bind to and associate with proteins that control the expression of the gene.

1.4.2 The Promoter Regulates Transcription of the Gene

The gene promoter begins immediately upstream of the transcription initiation trinucleotide codon (usually ATG) and can stretch upstream for many kilobases of sequence. This large length allows multiple protein complexes, including the RNA polymerase complex, to bind and affect expression, often in very subtle and complex ways.

One promoter element that is essential for many genes is the so-called TATA box. This is a short consensus sequence found approximately 25 nucleotides upstream of the transcription initiation site. It is found in many genes and across many species and is crucial for the correct positioning of the transcriptional apparatus.

1.4.3 Multiple Systems Control Gene Expression

The vast number of extracellular signals a cell can react to are translated, through cell surface receptors, to a smaller number of regulatory systems within the cytosol and nucleus. Through these systems, a signal is transduced from the cell surface to the nucleus by a cascade of molecules, each transferring the message to the next. An example of a system of great importance in the regulation of immune system genes in general and the TNF-α and IL-6 genes in particular is the NF-κB.

1.4.4 The NF-κB Gene Regulation System

1.4.4.1 NF-κB Regulates Many Immune System Genes

NF-κB (Nuclear Factor Kappa B) is a eukaryotic transcription factor that has been implicated in the regulation of a wide variety of genes producing cytokines, growth factors and other soluble molecules of the immune system. It was first described as a transcription factor necessary of the transcription of the gene for the immunoglobulin kappa light chain in B cells, from where it derives its name (Sen *et al*, 1986). NF-κB has since been described in virtually all cell types studied.

1.4.4.2 Structure of NF-κB

The name NF-κB is used to describe a number of different proteins. The NF-κB transcription factor is a dimer made up of members of the rel family of proteins (See **Figure 1.4.4.1**). Both hetero- and homodimers can be formed. Each of the rel proteins contains a large, highly conserved domain of 300 amino acids, called the rel homology domain or RHD. This domain is responsible for the DNA-binding and dimerisation of the proteins and is also the binding site for an inhibitory protein, IκB,

which keeps NF-κB in an inactive form in the cytosol (see below). The NF-κB initially described as a transcription factor in kappa chain expression in B cells is a dimer of the p50 and p65 subunits. Other combinations produce slight variations in the DNA-binding motif within the RHD and thus results in genes having a preference for one combination of subunits over another (Kunsch *et al*, 1992). Most of the combinations of rel proteins produce NF-κB factors which will activate transcription, although several combinations result in transcription repression (Brown *et al*, 1994).

1.4.4.3 Activation of NF-κB

In it's resting state, NF-κB sits in the cytosol, bound to IκB. This inhibitory protein masks the nuclear localisation signal of NF-κB and keeps it in the cytoplasm. Like NF-κB, IκB is, in reality, a family of proteins that share structural features. The most obvious of these are a series of domain repeats, called ankyrin repeats, which vary in number from individual between the IκB proteins. The number of ankyrin repeats affects the specificity of binding to NF-κB that the inhibitors exhibit.

NF-κB is activated by a wide variety of signals. These include stress-related species such as reactive oxygen intermediates, molecules suggestive of bacterial infection such as LPS and cytokines such as IL-1 and TNF-α. Signals such as these all indicate that tissue damage of one kind or another has occurred and an increase in immune system activity is required. The signal, whatever its source, ultimately leads to the activation of an IκB kinase. This enzyme phophorylates the IκB leading its degradation and dissociation from the NF-κB. This then allows the NF-κB to localise to the nucleus where it associates with NF-κB binding sites on cell promoters and facilitates upregulation of gene expression.

The genes which NF-κB can bind to and activate are numerous but all many are involved in subsequent immune functions, including cell migration and repair (cell adhesion molecules such as

VCAM-1 and ELAM-1), further activation of immune cells (cytokines such as IL-1, IL-2, G-CSF and TNF-α), inflammation (acute phase response proteins such as angiotensinogen and serum amyloid protein) and the regulation of the NF-κB response itself (IκB proteins). The process of activation is summarised in **Figure 1.4.4.2.**

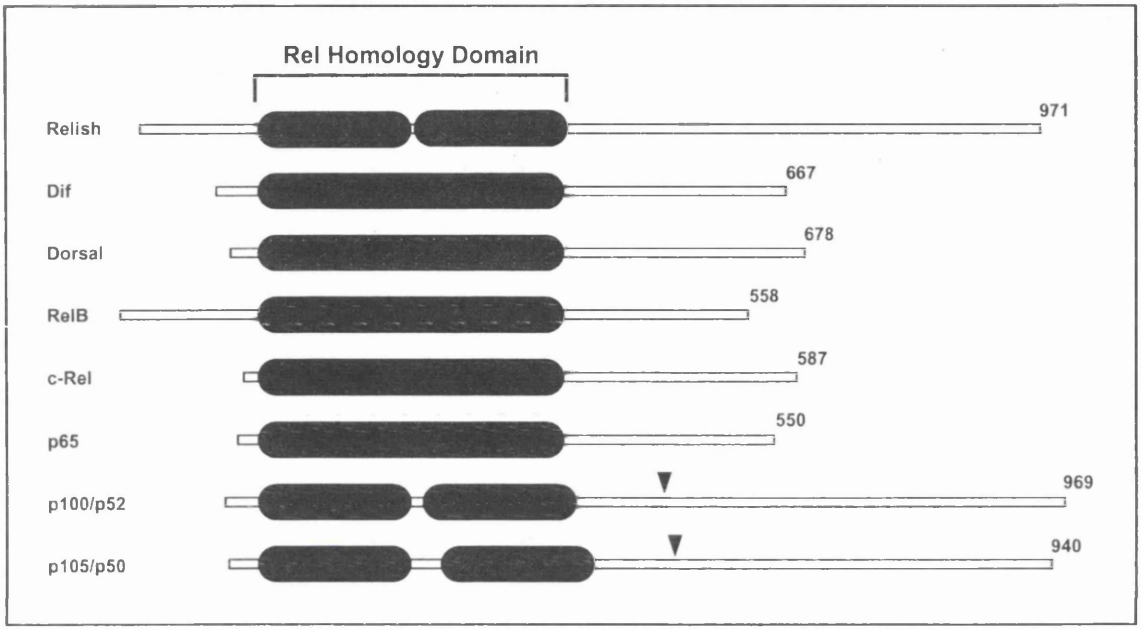


Figure 1.4.4.1 The Rel Family of Proteins

The Rel family of proteins form the dimer NF-κB transcription factors. The amino acid length of each protein is displayed at the right hand side and the two black arrows show the positions where endoproteolytic cleavage occurs to form the p52 and p50 subunits.

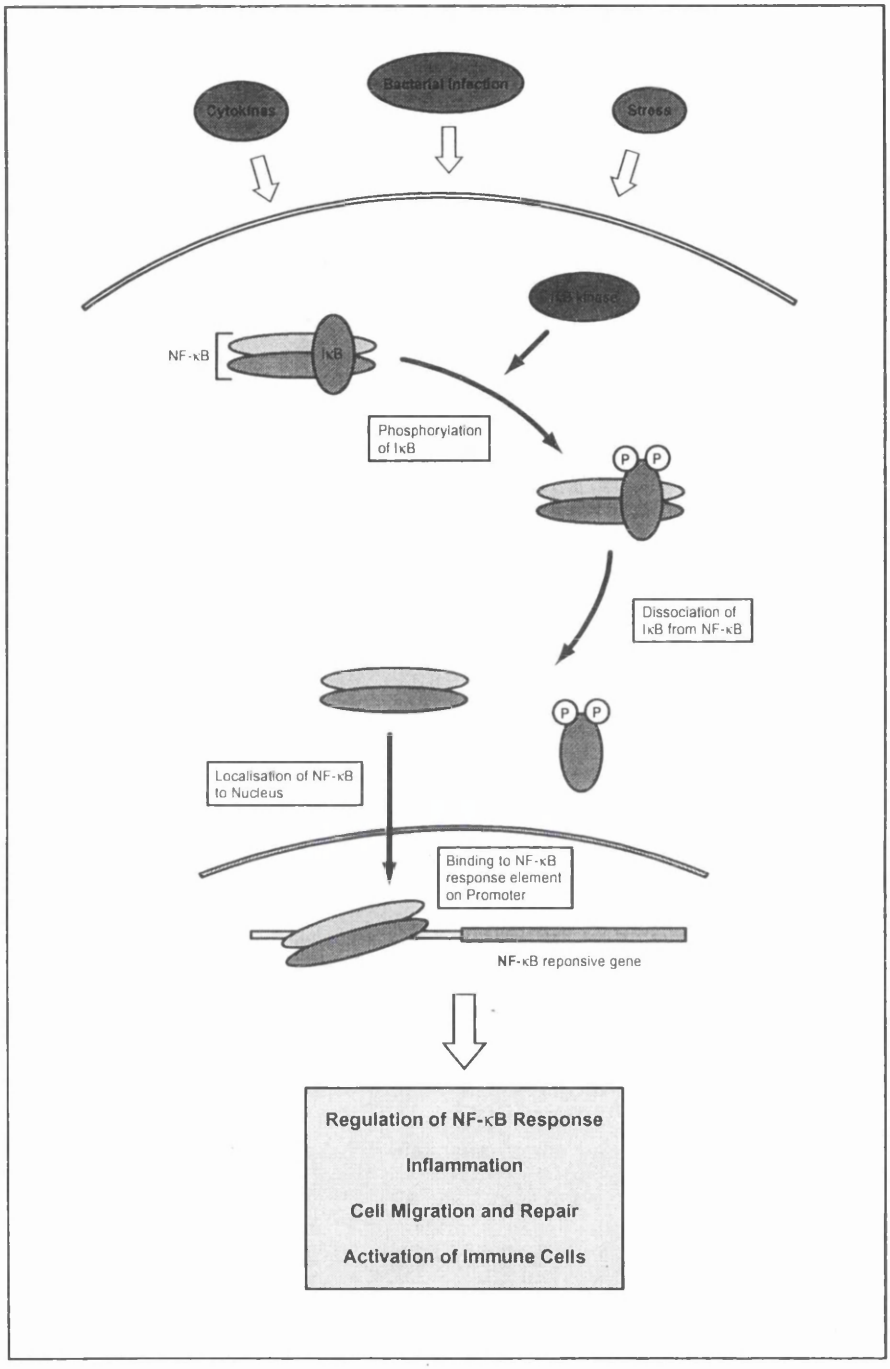


Figure 1.4.4.2 Activation of NF-κB

The activiation of NFκB by multiple signals results in an increase in activity of many parts of the immune system

Chapter 2: Materials and Methods

2.1 Human Cell Culture

2.1.1 Cell Culture Conditions

The cell type utilised for the work described in this thesis was the U937 cell line, an immortalised, human monoblastic cell line which grows in suspension. The cells were grown at 37°C in a water-saturated atmosphere containing 6% CO_2 in air. All cell manipulations were carried out using standard aseptic technique in a sterile Class II laminar flow hood. Cells were typically grown in cultures of 10ml in sterile, plastic 50ml flasks, and were spilt 1:2 every fourth day. The medium used for cell culture was RPMI 1640 supplemented with 10% fetal calf serum.

2.1.2 Treatment of U937 cells

2.1.2.1 Treatment with IFN-γ

Cells were treated with IFN-γ to produce an activated monocyte phenotype. A 10ml culture of cells was resuspended in 5ml of RPMI medium and allowed to recover for 15 minutes. 50µl of 100mM IFN-γ was then added to the culture by pipette and mixed by gentle swirling of the culture medium to give a final concentration of 1mM. The cells were incubated for 3 hours before a further 5ml of RPMI medium was added to the culture and the cells were then left overnight before use.

2.1.2.2 Treatment with dbcAMP

Cells were treated with dibutyryl cyclic AMP (dbcAMP) to differentiate them into a macrophage-like phenotype. The dbcAMP was stored in a stock solution of 30mM at –70°C and the required

final concentration in the culture of cells was 1mM. Therefore, for a 10ml culture 333µl was added to give a 1 in 30 dilution. This was done by simply pipetting the dbcAMP into the flask and mixing with the medium by gentle swirling. The cells were then incubated for 48 hours before use to allow full differentiation to occur.

2.1.3 Transfection and Harvest of U937 Cells

2.1.3.1 Transfection of Cells

Cell cultures were split approximately 18 hours before transfection to ensure a suitable dilution (see section 2.1.1). Four hours before transfection the cell density was measured using a haemocytometer. For each transfection 3 x 10^6 cells were transferred from the 100ml culture flask to a 60mm plastic Petri dish and RPMI medium was added to a final volume of 4ml. These plated cultures were then placed back in the 37°C incubator. The plasmid DNA to be transfected was removed from storage at -20°C and allowed to thaw on ice. The desired amount of DNA was then transferred by Gilson pipette to a sterile Eppendorf tube and Effectene buffer was added to a total volume of 150ml. 8ml of Enhancer reagent was then added by Gilson pipette and the solution was mixed by vortex for 1 second before being incubated at room temperature on the bench top for 3 minutes. 25ml of Effectene transfection reagent was added by pipette and the Eppendorf tube was vortex mixed for 10 seconds and then incubated at room temperature on the bench top for 7 minutes. The plate of cells was removed from the 37°C incubator and a suitable volume of RPMI 1640 medium was warmed to 37°C. 1ml of RPMI medium was added to the Eppendorf containing the tranfection reagent/DNA solution and was mixed with it by pipetting up and down rapidly but smoothly 5 times. 1ml of this liquid was then taken from the Eppendorf and added drop wise to the plate of cells, which were gently agitated throughout the addition to ensure mixing, thus giving a total volume of 5mls. The

plates of cells were then placed back in the 37°C incubator for the required incubation period

2.1.3.2 Cell Harvest and Lysis (Protocol 1)

Materials: 5x Passive Lysis Buffer (Promega)

A suitable volume of 1x PLB was prepared by adding volumes of distilled, autoclaved water to one volume of 5x PLB in a 13ml plastic tube. The resulting 1x PLB was mixed by vigorous shaking for several seconds and was then allowed to equilibrate to room temperature on the bench top. The previously transfected 5ml cell culture was removed from incubation and spun down to a pellet by centrifuge. The supernatant was removed by pouring and inverting the tube on a layer of tissue paper for approximately 15 seconds. 400ml of 1x PLB was then added to the tube and the pellet was disturbed and mixed by pipetting the volume of lysis buffer up and down several times. The lysate was then transferred to a sterilised Eppendorf tube and Luciferase activity was measured immediately.

2.1.3.3 Cell Harvest and Lysis (Protocol 2)

This modified protocol was identical to protocol 1, described above, with the exception of the following steps which were performed before the luciferase measurements were taken.

The lysate was transferred to a sterile Eppendorf tube and was incubated at room temperature for 15 minutes. It was then spun in a bench top microfuge for 30 seconds at 13,000rpm and 4°C to pellet cell debris. The cleared supernatant was then transferred to a clean Eppendorf tube and Luciferase activity was measured immediately.

2.2 Bacterial Cell Culture

2.2.1 Cell Culture Conditions

Bacterial cell cultures were used to grow plasmid DNA to provide templates for PCR reactions and for transfection into mammalian cells. The strain used in this work was DH5α. All bacterial cell cultures were grown in 5ml (miniprep) or 100ml (maxiprep) volumes of LB Broth medium, when growing in suspension, or on 100mm plates of LB with Agar when growing colonies on a solid medium. Cells on LB Agar plates were grown at 37°C in a normal air atmosphere.

2.2.2 Preparation of Cell Culture Media

2.2.2.1 Preparation of LB Broth Medium

To prepare medium for a 100ml culture of cells, 100ml of distilled water was placed in a 500ml conical flask. To this was added 2g of powdered LB. The flask was then stoppered with a piece of cotton wool and a aluminium foil cover and autoclaved to sterilise. The medium was then allowed to cool before use.

To prepare medium for 5ml miniprep cultures, 100ml of LB broth was prepared in a Stott-type screw-top bottle was used instead of a conical flask. The medium was then pipetted into sterile miniprep tubes under aseptic conditions.

2.2.2.2 Preparation of LB Agar Plates

400ml of distilled water was placed in a 500ml Stott bottle. 12.8g of powdered LB with Agar was added and the mixture was autoclaved. The medium was allowed to cool to below 40°C before the required antibiotics were added. After gentle mixing by inversion, the medium was poured into clean 100mm plastic cell culture plates using standard aseptic techniques. The medium was allowed to set before the plates were sealed with Sarin Wrap and stored, inverted, at 4°C.

2.2.2.3 Addition of Antibiotics

Antibiotics were added by pipette before addition of bacterial cells to liquid broth or pouring of LB Agar plates. The two types used were Ampicillin and Kanamycin. Both were stored in stock solutions of 100mg/ml at –20°C and were added to a final solution in the medium of 50µg/ml.

2.2.3 Transformation of Bacterial Cells

The required number of 50µl aliquots of bacterial cells, each in a 1.5ml Eppendorf tube, were taken from storage at –70°C and allowed to thaw gently on ice. 2µg of DNA was added to each aliquot by pipette and mixed with the cells by gentle agitation of the cells with the pipette tip. The tubes were placed on ice for 15 minutes, then heat shocked in a 37°C heat block for 30 seconds and placed back on ice for 2 minutes. 500µl of LB Broth medium (without antibiotic) was then added to each Eppendorf tube and the tubes were incubated at 37°C for 1 hour. 25µl and 100µl from each tube were plated out on to LB Agar containing the appropriate antibiotic and when the liquid had been absorbed into the medium the plates were inverted and incubated overnight at 37°C.

2.2.4 Cell Culture Preparations

2.2.4.1 Miniprep Cell Cultures

Miniprep cell cultures were set up to grow plate-grown bacterial colonies in a larger volume of liquid medium for extraction of plasmid DNA and for seeding Maxiprep cultures. 5ml of LB Broth medium, containing the appropriate antibiotic, was added to a sterile glass test tube (with lid) by pipette under aseptic conditions. Previously grown colonies were picked from an agar plate with sterile plastic pipette tips and one tip was placed in each test tube. The tubes were then incubated at 37°C overnight in an air incubator while being constantly being agitated in a rotator. Plasmid DNA was then harvested from the Miniprep cultures using a Qiagen miniprep kit.

2.2.4.2 Maxiprep Cell Cultures

2ml of a Miniprep culture was added to 100ml of LB Broth medium with antibiotics in a conical flask. This was then stoppered with a cotton wool plug and incubated overnight at 37°C in a shaking incubator. Plasmid DNA was then harvested using a Qiagen Maxiprep kit.

2.3 DNA Manipulation

2.3.1 Acid Phenol Extraction of Total Cell RNA

Materials: Guanidinium thiocyanate (GTC) buffer:

4M GTC

25mM Sodium citrate

0.5% Sarcosylic acid

100mM β-Mercaptoethanol

2M Sodium acetate, pH 4.0

Phenol (water saturated), pH 4.0

Chloroform/Isoamyl alcohol (24:1)

Isopropanol

80% Ethanol

Nuclease-Free Water

A 10ml culture of U937 cells was resuspended in 500µl of GTC buffer to lyse the cells. The lysate was then transferred to a sterile Eppendorf tube. 50µl of 4M Sodium Acetate was added, followed by 500µl of Phenol and 100µl of Chloroform/Isoamyl alcohol. The tube was securely shut and vortexed for several seconds, then placed on ice for 15 minutes. The aqueous and phenol components of the mixture were then separated by centrifuging the tube at 13,000 rpm for 20 minutes. This was done at 4°C. The upper aqueous layer containing the RNA was carefully removed by pipette and transferred to a new Eppendorf tube. The RNA was precipitated out by the addition of 500µl of Isopropanol and collected in a pellet by again centrifuging the tube for 20 minutes (13,000rpm, 4°C). The supernatant was poured off and the pellet of RNA was washed with 500µl of 80% ethanol. The

tube was centrifuged again for 5 minutes and the ethanol was carefully removed by pipette. The pellet was finally resuspended in 50µl of nuclease free water and stored at –70°C.

2.3.2 Restriction Enzyme Digests

Materials: Restriction Enzyme Buffer (Bohringer Mannheim)

Nuclease-free Water (Promega)

Restriction enzymes were used to cleave plasmid DNA and PCR products at specific sites. 10µl of DNA was placed in a sterile 0.5ml Eppendorf tube. To this was added 2µl of restriction enzyme buffer and 6µl nuclease-free water to a total volume of 18µl. 2µl of restriction enzyme was then added and the reagents were mixed by gently flicking the tube. If any of the liquid had been displaced to the sides of the tube they were returned to the bottom by pulse spinning in a desktop centrifuge for several seconds. The tube was then incubated at 37°C for 4 hours.

2.3.3 Preparation of cDNA from Total Cell RNA

Materials: Total cell RNA (see 2.3.1)

Sterile Water (Invitrogen)

Oligo dT primer (Invitrogen)

Rnase Inhibitor (Invitrogen)

5x RT Buffer (Invitrogen)

100mM dNTPs (Invitrogen)

80mM Sodium pyrophosphate (Invitrogen)

AMV Reverse Trancriptase (Invitrogen)

0.5mM EDTA (Invitrogen)

Phenol-Chloroform (Invitrogen)

4M Ammonium acetate (Invitrogen)

100% Ethanol

Nuclease-free water (Promega)

5-10µl of RNA was added to a sterile 0.5ml Eppendorf tube. Sterile water was then added to a final volume of 11.5µl. 1µl of Oligo dT primer was then added and the tube was incubated at 65^{0}C for 10 minutes to removed any secondary structure in the RNA. The following were then added in order:

1µl Rnase Inhibitor

4µl 5x RT Buffer

1µl 100mM dNTPs

1µl 80mM Sodium pyrophospate

0.5µl AMV Reverse Transcriptase

The tube was then incubated at 42^{0}C for 60 minutes followed by 2 minutes at 95^{0}C to denature the RNA-cDNA hybrids. The cDNA was then purified by doing an acid phenol extraction. 1µl of EDTA and 20µl of Phenol-Chloroform were added to the tube which was then vortexed for several seconds to mix the contents and then spun in a desktop centrifuge at 13,000rpm for 5 minutes. This separated the mixture into an upper, aqueous layer and a lower phenol layer. The aqueous layer was removed carefully by pipette and transferred into a new sterile Eppendorf tube. 22µl of Ammonium Phospate and 88 µl of Ethanol were then added and the tube was vortexed for several seconds before being stored at –70^{0}C overnight to precipitate the cDNA. After thawing the tube was spun at 13,000rpm and 4^{0}C in a desktop centrifuge for 15 minutes to the pellet the cDNA. The supernatant

was then removed and the cDNA was resuspended in 50µl of Nuclease-free water (Promega) and stored at –20°C.

2.3.4 Polymerase Chain Reaction

Materials: Sterile Water

10x *Taq* Polymerase Buffer (Promega)

25mM Magnesium Chloride (Promega)

100mM dNTPs (Promega)

Oligonucleotide Primers

Template DNA

Taq thermostable DNA polymerase (Promega)

The *Taq* Polymerase Buffer, Magnesium Chloride, dNTPs, Primers and template DNA were all removed from storage at –20°C and allowed to thaw on ice. When this was complete each was mixed gently by flicking and placed back on ice with the exception of the magnesium chloride which was vortexed for 20 seconds to ensure it was completely dissolved in solution and then placed on ice. The substrates were then added to a sterile, 1.5ml Eppendorf tube in the shown above and mixed by gentle tapping before the *Taq* polymerase was removed from storage at –20°C and added to the tube. The tube was then placed in the PCR thermocycler and the required program was run. After the program was completed the PCR reaction was stored at –20°C until required.

2.3.5 Visualisation on Agarose Gels

Materials: Powdered Agarose

50x TAE

Distilled Water

Ethidium Bromide

2.3.5.1 Preparation of 1-2% Agarose gel (with Ethidium Bromide)

Agarose gel was prepared by first adding 100ml of distillled water to a 500ml conical flask. 2ml of 50x TAE was added to this and mixed by gentle swirling. 1-2g of Agarose was then added depending on the percentage gel required. This mixture was gently heating in a microwave oven until the agarose had completely melted. After allowing the liquid to cool slightly, 5µl of Ethidium bromide was added and mixed by gently swirling. The mouth of the flask was then covered with saran wrap, allowed to solidify, and kept at room temperature until required.

2.3.5.2 Pouring of Agarose gel

The flask of agarose gel was heated gently in a microwave oven until liquid and 10ml was removed by pipette and placed on a glass slide (6cm by 4cm) which had been previously cleaned with distilled water and 70% ethanol. A comb was placed at one end to create wells and the gel was allowed to solidfy. The slide and gel were then placed in a gel tank, covered with 1x TAE and allowed to equilibrate with the buffer for several minutes.

2.3.5.3 Separation of Nucleic Acids on Agarose Gel

The DNA/RNA samples were then mixed with 3µl of Orange G running dye and added to the lanes by dropwise pipetting and the gel was run at a constant voltage of 75 volts until the dye-front had

reached the bottom of the gel. The gel was then removed from the tank and placed on an ultraviolet transilluminator and a polaroid of the fluorescing gel was taken.

2.4 Luciferase Assays

2.4.1 Single Luciferase Assay

Materials: Lypholised Single Luciferase Assay Reagent (Promega)

Luciferase Assay Reagent Buffer I (Promega)

2.4.1.1 Preparation of Reagents

1x Luciferase Assay Reagent I (LAR I) was prepared by reconstituting the Single Luciferase Assay substrate in 10ml of buffer. The reagent was mixed thoroughly by inversion and aliquoted into 1ml volumes in sterile Eppendorf tubes, which were stored at –70°C until used. Approximately 45 minutes before the assay was performed, the required number of aliquots of LAR were removed from storage at –70°C and allowed to equilibrate to room temperature on the benchtop. The luminometer was also prepared by setting a delay phase of 2 seconds followed by a measurement phase of 10 seconds.

2.4.1.2 Measurement of Firefly Luciferase Activity

100µl of LAR I was pipetted into a clean luminometer tube. 20µl of cell lysate was added to this and mixed by pipetting up and down five times. The tube was then placed in the luminometer and the luciferase activity recorded.

2.4.2 Dual Luciferase Assay

Materials: Luciferase Assay Buffer II (Promega)

Lypholised Luciferase Assay Substrate (Promega)

Stop and Glo™ Buffer (Promega)

Stop and Glo™ Substrate (Promega)

2.4.2.1 Preparation of Reagents

1x Luciferase Assay Reagent II (LAR II) was prepared by reconstituting the Luciferase Assay Substrate in 10ml of buffer. The reagent was mixed thoroughly by inversion and aliquoted into 1ml volumes in sterile Eppendorf tubes, which were stored at –70°C until used. 50x Stop and Glo™ reagent was prepared by adding 200µl of buffer to the lypholised substrate. After the substrate had been reconstituted by gentle inversion the solution was stored at –70°C until required.

Stop and Glo™ buffer was aliquoted into 1ml volumes in sterile Eppendorf tubes and stored at –70°C until required.

Approximately 45 minutes before the assay was performed the required number of aliquots of LAR and Stop and Glo™ buffer were removed from storage and allowed to equilibrate to room temperature on the benchtop. When this has occurred 20µl of 50x Stop and Glo™ substrate was added to each 1ml volume of Stop and Glo™ buffer to give 1x Stop and Glo™ reagent. The luminometer was also prepared by setting a delay phase of 2 seconds followed by a measurement phase of 10 seconds.

2.4.2.2 Measurement of Firefly and Renilla Luciferase Activities

100µl of LAR was pipetted into a clean luminometer tube. 20µl of cell lysate was added to this and mixed by pipetting up and down five times. The tube was then placed in the luminometer and the Firefly Luciferase activity was recorded. The tube then removed from the luminometer and 100µl of 1x Stop and Glo™ reagent was added and mixed by pipetting up and down 5 times. The tube was then placed back in the luminometer and the measurement of Renilla Luciferase activity was taken.

Chapter 3: Competitive RT-PCR

3.1 Introduction

Messenger RNA (mRNA) is transcribed from genomic DNA and is the template for protein translation. It is a relatively short-lived molecule, only made by the cell when it is required and quickly degraded after the appropriate number of protein molecules has been translated from it. Therefore the abundance of a mRNA molecule, transcribed from a particular gene within a cell, is often directly related to the activation state of the gene and provides a quantifiable way of measuring this activation.

3.1.1 RT-PCR

RT-PCR consists of two separate reactions. The first, the reverse transcription step, uses an enzyme derived from the AMV virus called reverse transcriptase. This enzyme synthesises DNA from a single-strand RNA template and in the context of the RT reaction synthesises DNA complementary to mRNA isolated from cells. This complementary DNA (cDNA) is more stable than RNA as it is not susceptible to degradation by the ubiquitous RNase enzymes found on most surfaces and in most reagents in a laboratory. The cDNA can also be used as a template for the PCR reaction, which forms the second part of the RT-PCR process. Again, this reaction relies on a non-mammalian enzyme, a bacteria-derived thermostable DNA polymerase called *Taq*, which can resist denaturation at the high temperatures required to melt DNA strands apart. The *Taq* enzyme catalyses the extension of DNA in a 5' to 3' direction, using a complementary strand as a template. In the case of the PCR reaction the DNA which is extended is an oligonucleotide primer which anneals to a specific sequence within a piece of DNA. The use of two of these primers, one forward and one reverse, allows a short sequence within this piece of DNA to be amplified many times, producing millions of copies from one original.

3.1.2 Competitive RT-PCR

While RT-PCR is an effective technique for establishing a certain mRNA is expressed in a cell it is less useful, in its basic form, for quantifying the amount of mRNA. The efficiency of both the RT and PCR reactions can vary significantly, so for a given RT-PCR reaction it is not possible to deduce the amount of mRNA initially present from the amount of DNA that results at the end of the reaction. An internal control is required as a baseline against which the amount of DNA produced in the PCR reaction can be measured. If this internal control is amplified by the same primers as the fragment of interest then it is possible to quantify the amount of original mRNA. This the basis for competitive PCR, where a fragment is amplified and measured against an internal control competing with it for primers.

The work presented in this chapter focuses on attempting to quantify the levels of TNF-α and IL-6 mRNA in stimulated U937 cells using a Competitive RT-PCR assay.

3.2 Results

3.2.1 Preparation of cDNA

A 10ml culture of U937 cells was treated overnight with IFN-γ and then RNA was extracted from the cells using the acid-phenol method. 10μl of this RNA was immediately used to generate cDNA. 10μl of this cDNA and 10μl of the RNA used to produce it were run on a 2% Agarose gel stained with Ethidium Bromide (**Figure 3.2.1.1**). This showed the RT reaction had generated cDNA molecules of a wide of sizes, showing up as a continual smear down the gel. The RNA had not been degraded substantially by RNase action as the ribosomal 28S and 18S RNA molecules are visible as the two significant bands on the gel.

3.2.2 Initial Reactions and Optimisation

3.2.2.1 Positive Control – FcγRI

To evaluate the PCR reaction at the early stages a positive control, amplifying a portion of the cDNA for FcγRI was used. The primer pair for this produced a fragment of approximately 300bp in length. These primers were then used on two separate templates. The first was the cDNA prepared from IFN-γ-treated U937 cells and the second was the FcγRI cDNA in a CDM vector backbone. As a further control a reaction was carried out which amplified the entire FcγRI cDNA using primers specific for sequences in the CDM vector which bordered the cloning site of the plasmid.

10ml samples of each reaction were run on a 2% agarose gel and the DNA bands were visualised with Ethidium bromide under UV light (**Figure 3.2.2.1**). The gel showed that the PCR reaction had

produced a clean band of the correct size from two different preparations of cDNA (Lanes B and C) and also from the FcγRI-CDM vector DNA (Lane D). The full length FcγRI cDNA was also synthesised successfully (Lane E). There were no clearly visible non-specific products of different sizes to the predicted products.

3.2.2.2 Sequence Analysis and Primer Design – TNF-α and IL-6

The sequences for the TNF-α and IL-6 cDNAs were obtained by searching the GENBank database (www.ncbi.nih.org). The 3'-untranslated regions of the mRNA were identified and complementary oligonucleotides were designed for fragments within this region (see **Figure 3.2.2.2**). Primers were designed to incorporate the following characteristics:

- 20-25 nucleotides in length
- Approximately 50% G/C content
- Terminal nucleotides either G or C
- Lack of repeated nucleotides (e.g. AAAA)

The desired size of the PCR product to be produced was between 300 and 500 base pairs. This would produce a fragment which could be easily visualised on an agarose gel while remaining small enough to give a relatively straightforward PCR reaction. The pair of primers for each cDNA consisted of a forward primer which would anneal to the anti-sense strand of cDNA and a be extended in the same direction as the reading frame to produce the coding strand, and a reverse primer which would anneal to the sense strand and be extended to produce the antisense strand of the fragment. The primer characteristics are summarised in **table 3.2.2.2** below.

Table 3.2.2.2

cDNA	Forward Primer	Reverse Primer	Fragment Size
TNF-α	CTT AGG CCT TCC TCT CTC CAG ATG	CCG ATT ACA GAC ACA ACT CCC	395bp
IL-6	GTT AAT GGG CAT TCC TTC TTC TGG	GAG GTA AAG CCT ACA CTT TCC AAG	298bp

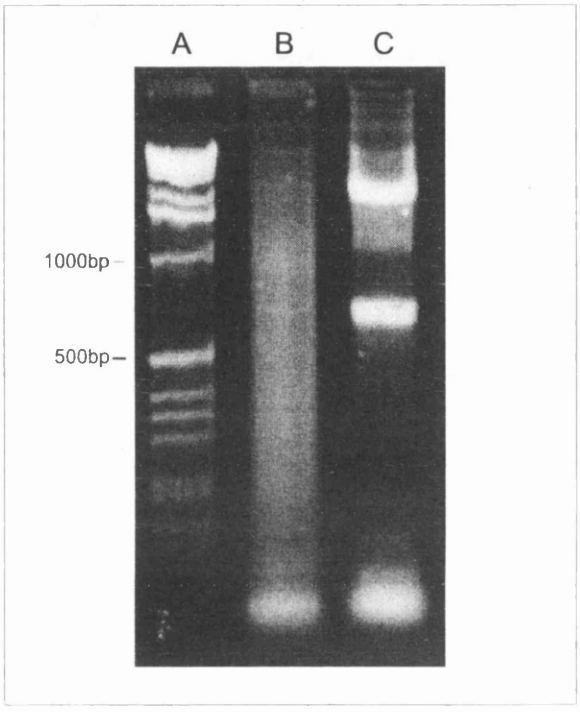


Figure 3.2.1.1 Preparation of cDNA

Lane B shows cDNA prepared by reverse transcription from the total cell RNA shown in Lane C. Lane A contains 1kb DNA ladder markers.

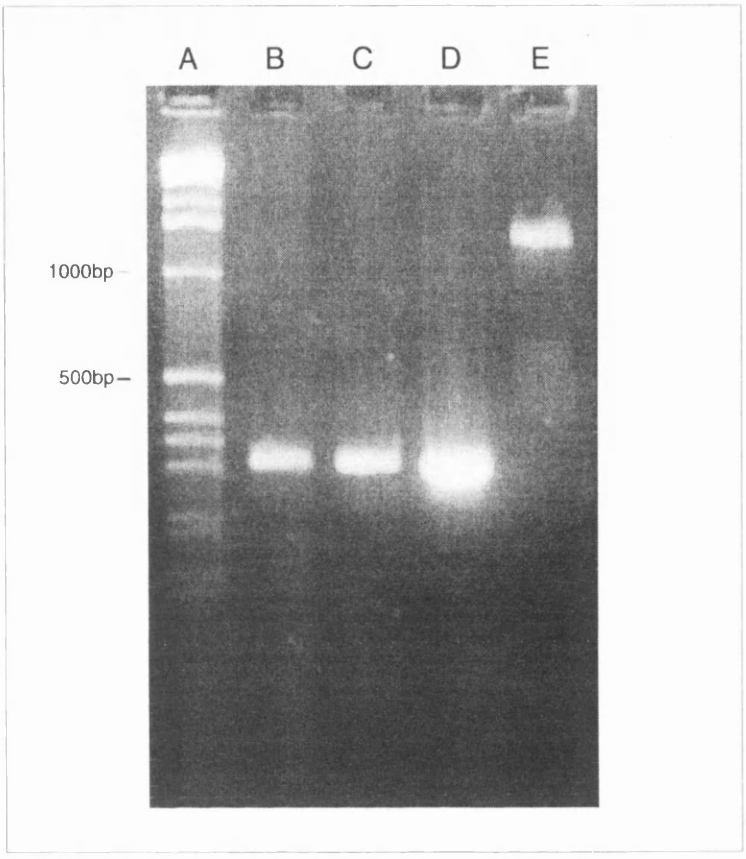


Figure 3.2.2.1 PCR Postive Control - FcγRI

PCR reaction positive control using internal to primers to FcγRI with cDNA prepared from IFN-γ-treated U937 cells (Lanes B,C), FcγRI cDNA in CDM vector (Lane D) and external primers with FcγRI cDNA in CDM vector (Lane E). Markers (Lane A) are 1kb DNA ladder.

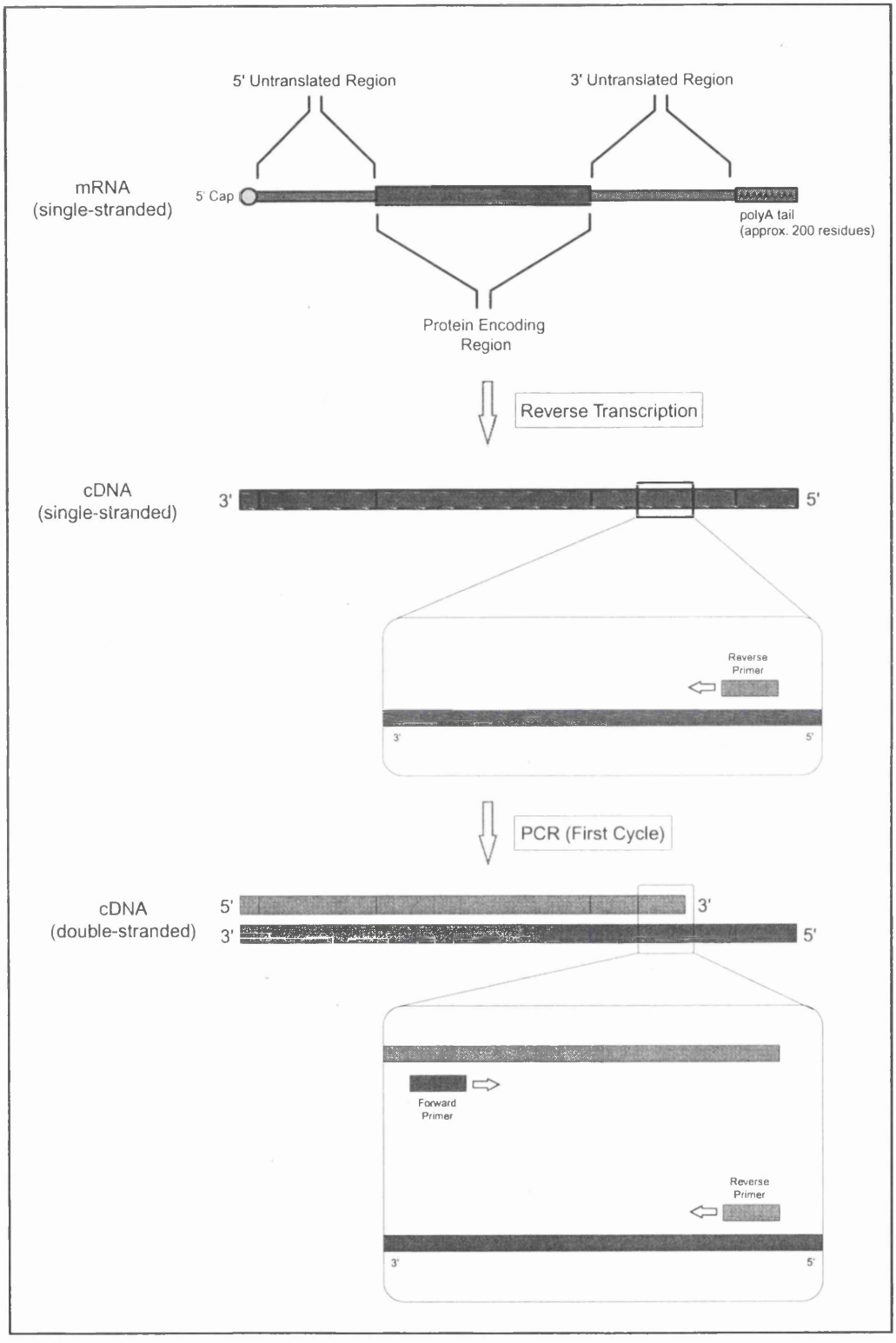


Figure 3.2.2.2 Structures of mRNA and cDNA molecules

3.2.2.3 Initial PCR reaction - TNF-α and IL-6

A 35 cycle PCR reaction was run using the primer pairs for TNFα and IL-6 against two preparations of cDNA from IFN-γ-treated U937 cells. A reaction using the forward and reverse external CDM primers against FcγRI-CDM was used as a positive control. 10ml samples were run on a 2% agarose gel and visualised with ethidium bromide under UV light (**Figure 3.2.2.3**). The reactions using the TNF-α primers produced a product of the predicted size of just under 400bp, but also a number of non-specific products at sizes smaller than this. The reactions using the IL-6 primer pair also produced the predicted fragment as well as several non-specific products. It was likely that this was due to the relatively low annealing temperature of 50°C and that the reaction had to be optimised for annealing temperature to produce a specific amplification.

3.2.2.4 Optimisation of Annealing Temperature – TNF-α and IL-6

A series of 35 cycle PCR reactions were carried out where the annealing temperature was set at 52.5°C, 55°C, 57.5°C and 60°C. This series was done using both sets of primer pairs with the cDNA described previously. 10ml samples were then run on a 1% agarose gel (**Figure 3.2.2.4**). Both the TNF-α and IL-6 primers produced correct-sized fragments. The number of other non-specific products diminished and the amount of the specific product increased as the annealing temperature rose. From these results, an annealing temperature of 57.5°C was chosen as the preferred annealing temperature for all future reactions using these primers.

3.2.2.5 Optimisation of Cycle Number – TNF-α and IL-6

PCR reactions were carried out at a range of cycle numbers (15, 20, 25, 30, 35, 40) using either

the TNF-α or IL-6 forward/reverse primer pairs. Melting, annealing and extension temperatures during the cycle phase of the reaction were 93°C, 57.5°C and 72°C respectively. 10µl samples of each reaction were run and visualised on 2% agarose gels (**Figure 3.2.2.5 (TNF-α)** and **Figure 3.2.2.6 (IL-6)**). The results were similar for both the TNF-α and IL-6 reactions. In each case there was no visible product at 15 and 20 reaction cycles. A product band appeared at 25 cycles and this increased in intensity as the number of cycles was increased to 40.

3.2.2.6 Optimisation of Magnesium Concentration – TNF-α and IL-6

Magnesium is an essential cofactor of the Taq polymerase enzyme and therefore variations in the concentration of Mg^{2+} ions in the PCR reaction may have a profound effect on the efficiency of the reaction. Previous reactions had magnesium chloride added to a final concentration of 1.5mM. This produced a successful reaction but it was necessary to investigate whether this was producing the best possible results or if a slight change in the amount of magnesium added would result in a vast change in reaction efficiency; a possibility given the small volumes being pipetted. 35 cycle PCR reactions were run using either the TNF-α or IL-6 primer pairs and with magnesium added at final concentrations of 1mM, 1.5mM and 2mM. 10µl samples from each reaction were run and visualised on 2% agarose gels (**Figure 3.2.2.7**). In both reaction series all 3 concentrations of magnesium gave a positive result although the amount of specific product was reduced when the magnesium concentration was higher.

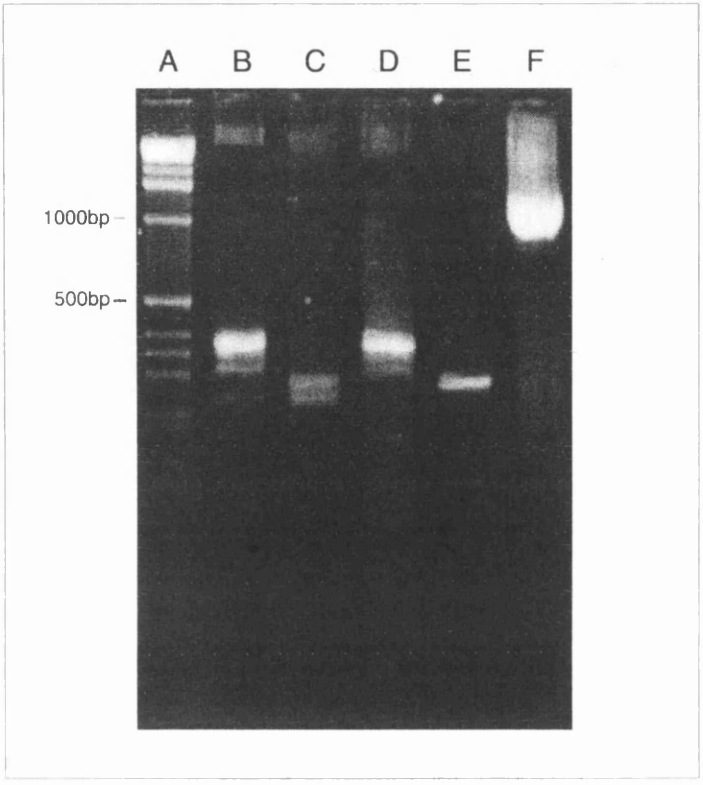


Figure 3.2.2.3 PCR Reaction - TNF-α, IL-6

PCR reaction using INF-γ-treated U937 with TNF-α forward/reverse primer pair (Lanes B and C) and IL-6 forward/reverse primer pair (Lanes D and E). Positive control (Lane F) was CDM external forward/reverse primers with FcγRI-CDM plasmid DNA. Markers (Lane A) were 1kb DNA ladder.

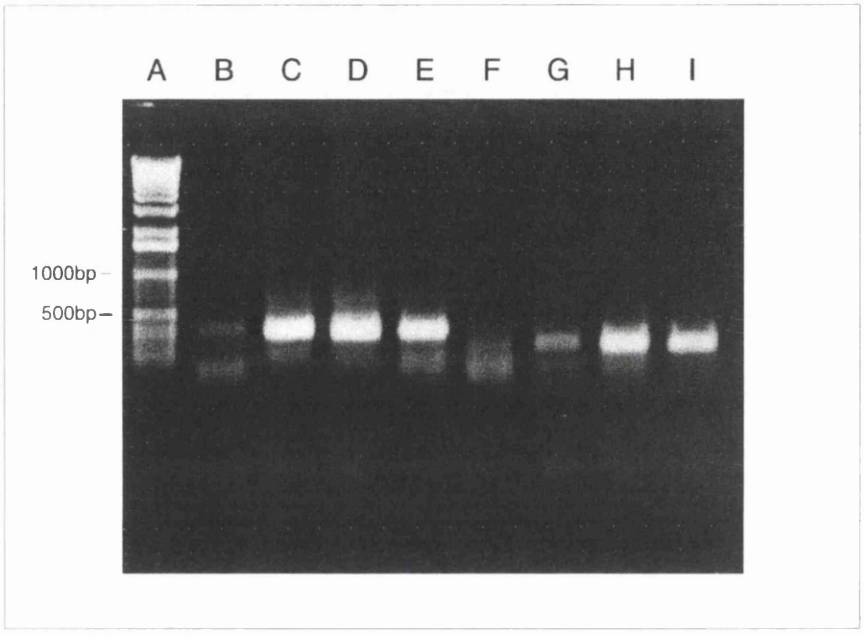


Figure 3.2.2.4 Optimisation of Annealing Temperature

PCR reactions were performed with temperatures for the annealing phase of the cycle set at 52.5^0C, 55^0C, 57.5^0C and 60^0C using the TNF-α primer pair (Lanes B-E) and the IL-6 primer pair (Lanes F-I). Lane A contains 1kb DNA ladder markers.

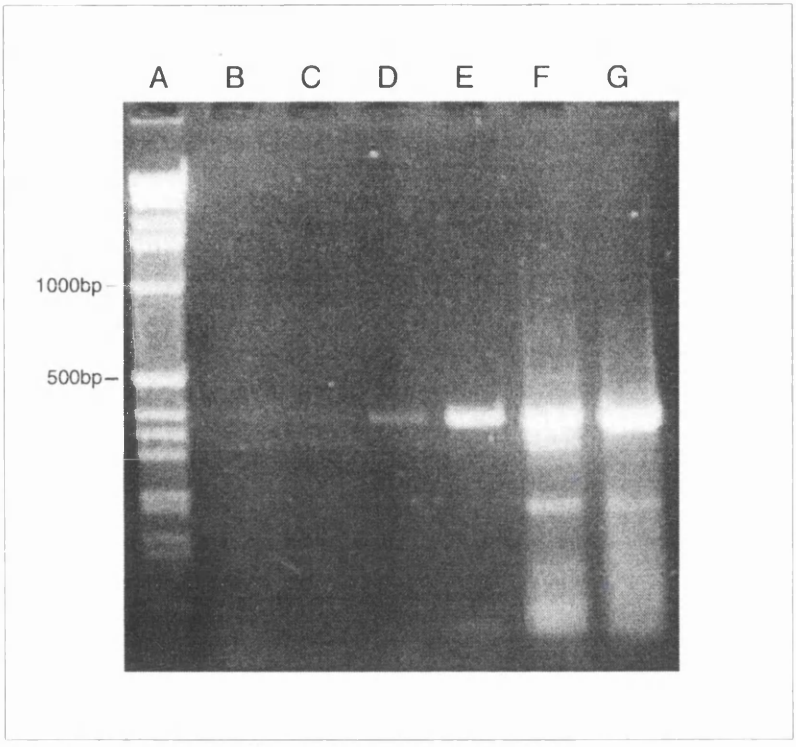


Figure 3.2.2.5 Optimisation of Cycle Number - TNF- α

PCR reactions were run at 15 cycles (Lane B), 20 cycles (Lane C), 25 cycles (Lane D), 30 cycles (Lane E), 35 cycles (Lane F) and 40 cycles (Lane G) using IFN-γ-treated U937 cell cDNA and TNF-α primers. Lane A contains 1kb DNA ladder markers.

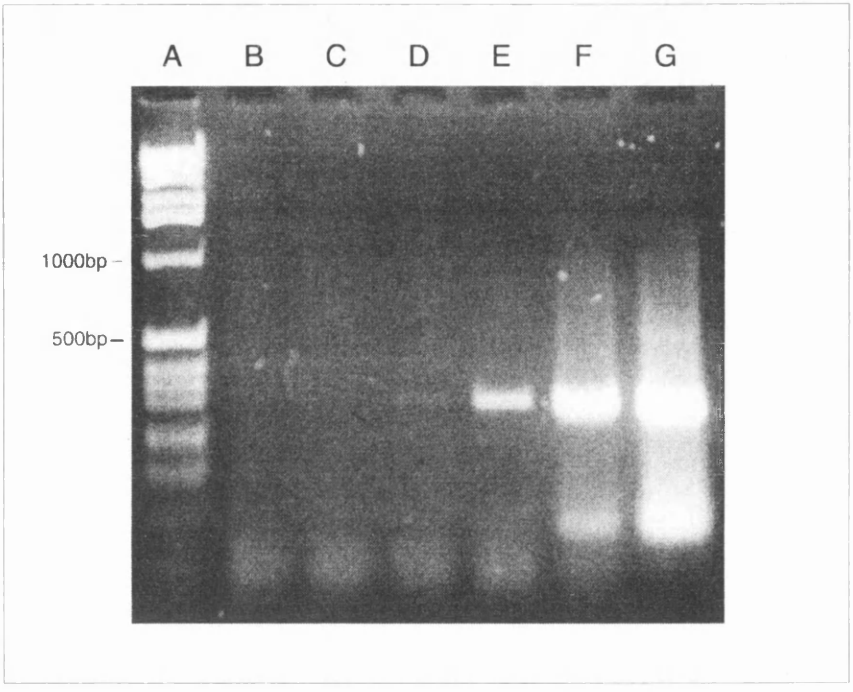


Figure 3.2.2.6 Optimisation of Cycle Number - IL-6

PCR reactions were run at 15 cycles (Lane B), 20 cycles (Lane C), 25 cycles (Lane D), 30 cycles (Lane E), 35 cycles (Lane F) and 40 cycles (Lane G) using IFN-γ-treated U937 cell cDNA and IL-6 primers. Lane A contains 1kb DNA ladder markers.

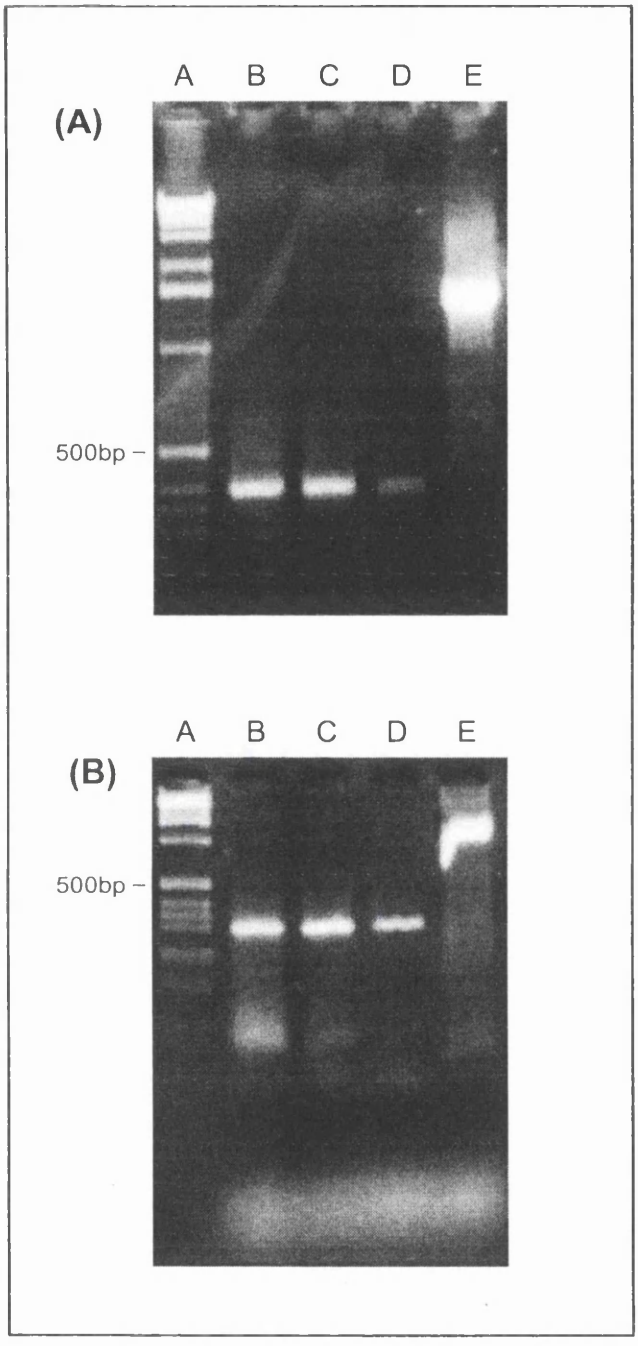


Figure 3.2.2.7 Optimisation of Magnesium Concentration

PCR reactions were performed using IFN-γ-treated U937 cDNA using TNF-α (A) and IL-6 (B) primers. Magnesium concentration was 2mM (Lane B), 1.5mM (Lane C) and 1mM (Lane D). Lane A contains the 1kb DNA ladder markers and Lane E contains the FcγRI positive control.

3.2.3 Restriction Enzyme Digests of PCR products

To confirm that the PCR products were the predicted TNF-α and IL-6 fragments multiple restriction enzyme digests were carried out on each fragment.

3.2.3.1 Selection of Restriction Enzymes

Restriction maps showing the restriction enzyme cut sites in the sequence of the TNF-α and IL-6 fragments were generated using the GCG computer software package. This was used to select the enzymes with which to digest each fragment. The enzyme picked and the predicted fragments they would produce are summarised in **Figure 3.2.3.1.**

3.2.3.2 Restriction Digests of PCR Fragments

The putative TNF-α and IL-6 fragments were digested by the enzymes described above according to the protocol described in section 2.3.2. 10μl aliquots of the reactions were run and visualised on 2% agarose gels (**Figures 3.2.3.2 and 3.2.3.3**). The fragments generated in each case were as predicted in the restriction maps and therefore it was concluded that the two fragments observed were amplified from the 3' untranslated regions of the TNF-α and IL-6 mRNAs.

3.2.4 Subcloning of PCR Fragments in pCR2.1 Plasmid Vector

PCR-generated fragments of DNA were subcloned into the pCR2.1 vector for use in generating the deleted versions of the fragments to be used in the competitive PCR reactions. The subcloning reaction was performed using DNA taken directly from the PCR reaction tube after a fraction of this

had been analysed on an agarose gel to ensure there were no non-specific products. The protocol was as described in **section 2.2.5**. Bacterial colonies were selected to inoculate miniprep cultures and plasmid DNA was prepared from these. A restriction digest was performed using the enzyme EcoRI, which excises any DNA inserted into the pCR2.1 plasmid. A fraction of the digest was run on an agarose gel to visualise the plasmid DNA and any inserts. **Figures 3.2.4.1** and **3.2.4.2** show visualisation of digests of plasmid DNA which did contain the TNF-α and IL-6 PCR fragments inserted at the multiple cloning site. The gel for the TNF-α minipreps (**Figure 3.2.4.1**) shows inserts of approximately 400bp in lanes I and K while the gel for the IL-6 minipreps (**Figure 3.2.4.2**) shows an insert of approximately 300bp in lane K. The plasmids were amplified by Maxiprep and the plasmid DNA was stored at –20°C until required.

3.2.5 Generation of Deletion Constructs

3.2.5.1 Overview of the Protocol

A PCR protocol was devised to generate a forms of the TNF-α and IL-6 PCR fragments which had a central portion of their sequence deleted. The undeleted form of the fragment was used as a template and two separate reactions were carried out with this. The first utilised the original Forward primer and a reverse primer which would anneal to sequence within the fragment, rather than at the end. This primer also featured a sequence of 10 nucleotides at its 5'end which did not anneal to the template. The second reaction used the original Reverse primer and an internal forward primer with series of 10 nucleotides at its 5' end complementary to the overhanging 10 nucleotides on the internal reverse primer.

These two reactions generated two PCR products, which were only part of the original template. The

first was analogous to the beginning of the original template with the addition of the 10-nucleotide overhang at the end. The second was analogous the end of the original template, this time with the 10 nucleotide overhang at the beginning of the fragment. The middle portion of the original template sequence was not found in either of the two daughter products.

To join these two fragments together, thus generating a form of the original fragment with a length of its sequence deleted, a third reaction was performed using both daughter fragments as template and the original Forward and Reverse primers. The 10-nucleotide complementary sequences allowed single strands from each fragment to anneal togther and be extended, creating a single, complete fragment. The three reactions described above are summarised in **Figure 3.2.5.1.**

3.2.5.2 Design of Internal primers

The sequence of the TNF-α and IL-6 PCR fragments were analysed and internal primers were designed following the criteria previously described (see **Section 3.2.1**). The primers and the fragments they will generate are shown in **Figures 3.2.5.2 A** and **B.**

3.2.5.3 PCR Reactions and Subcloning of Fragments

Following the 3-reaction protocol described above, fragments were generated using the combination of original terminal and internal primer pairs for both the TNF-α and IL-6 PCR fragments. The separate fragment were generated first (**Figure 3.2.5.3**) and then combined to produce the deleted versions of the TNF-α and IL-6 PCR (hereafter called ΔTNF-α and ΔIL-6) fragments (**Figure 3.2.5.4**). The ΔIL-6 fragment was present in similar quantities to both the undeleted fragments which were generated as positive controls in parallel. The ΔTNF-α fragment was present in much

lower quantities but was visible. It was therefore purified from the gel before both fragments were subcloned into the vector pCR2.1 and plasmid DNA minipreps were prepared for each putative construct. The EcoRI digests of these minipreps are shown in **Figure 3.2.5.5**. Inserts of the correct size were present in one of the ΔTNF-α constructs (Lane D) and in two of the ΔIL-6 (Lanes H and K). These plasmids were used to make The plasmids were then amplified by Maxiprep and the plasmid DNA was stored at -20^0C until required.

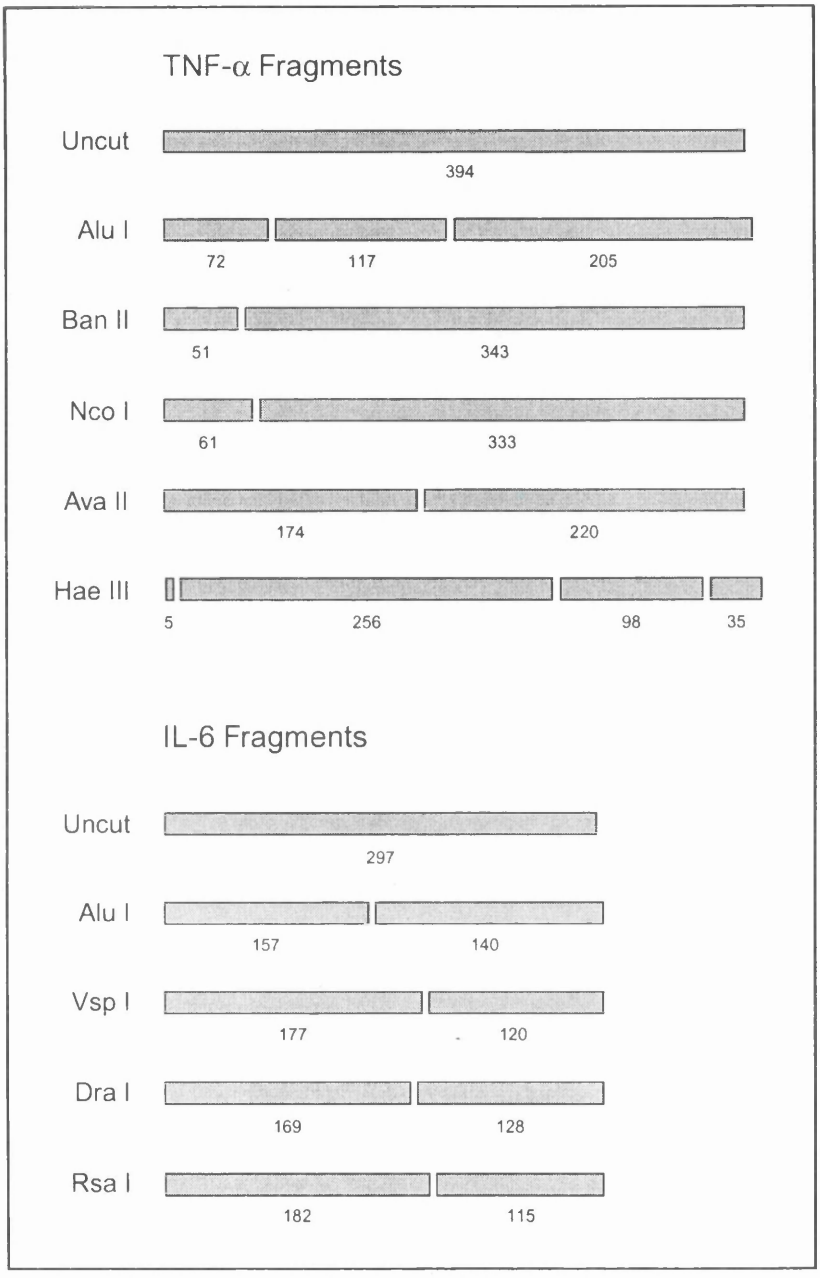


Figure 3.2.3.1 Restriction Enzyme Digest Maps

Sequence analysis predicted the following restriction enzyme digest products when the TNF-α and IL-6 PCR fragments were digested with the enzymes listed. The digest products are shown in the order in which they are found moving along the sense strand of the fragment in a 5' to 3' direction.The units of length shown are base pairs.

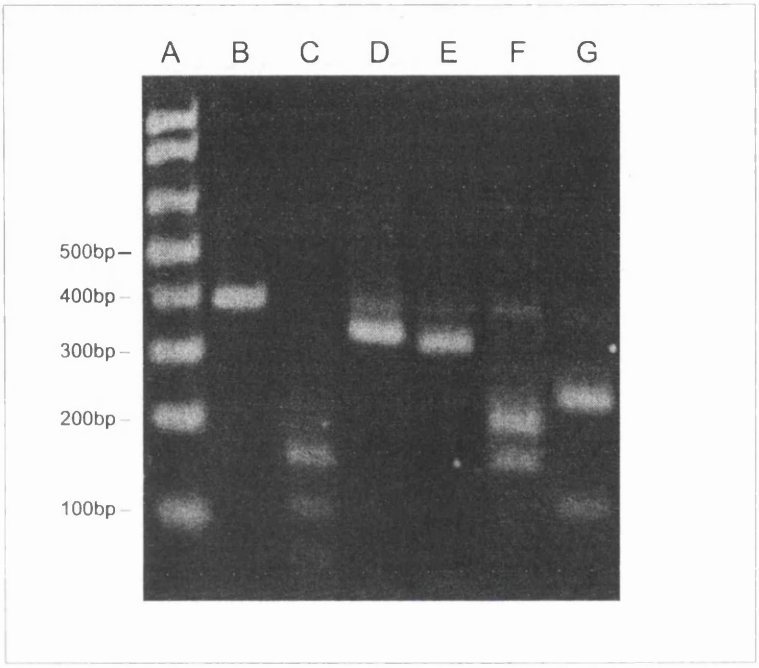


Figure 3.2.3.2 Restriction Digests of TNF-α PCR Fragment

Lanes C to G show restriction digest products using the following restriction enzymes: Alu I (Lane C), Ban II (Lane D, Nco I (Lane E), Ava II (Lane F) and Hae III (Lane G). Lane B contains undigested TNF-α fragment and Lane A contains 100bp DNA ladder markers.

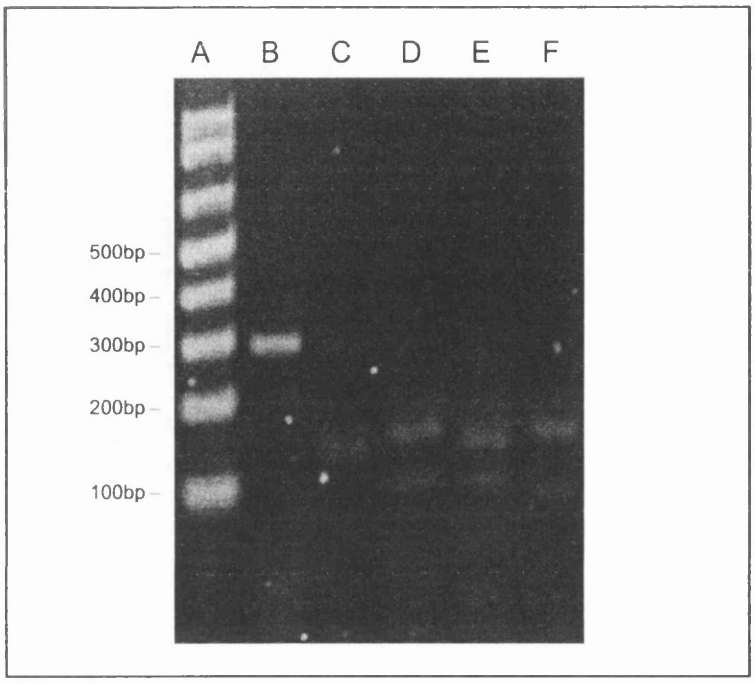


Figure 3.2.3.3 Restriction Digests of IL-6 PCR Fragment

Lanes C to F show restriction digests of the IL-6 PCR fragment with the following restriction enzymes: Alu I (Lane C), Vsp I (Lane D), Dra I (Lane E) and Rsa I (Lane F). Lane B contains uncut IL-6 PCR fragment and Lane A contains 100bp DNA ladder markers.

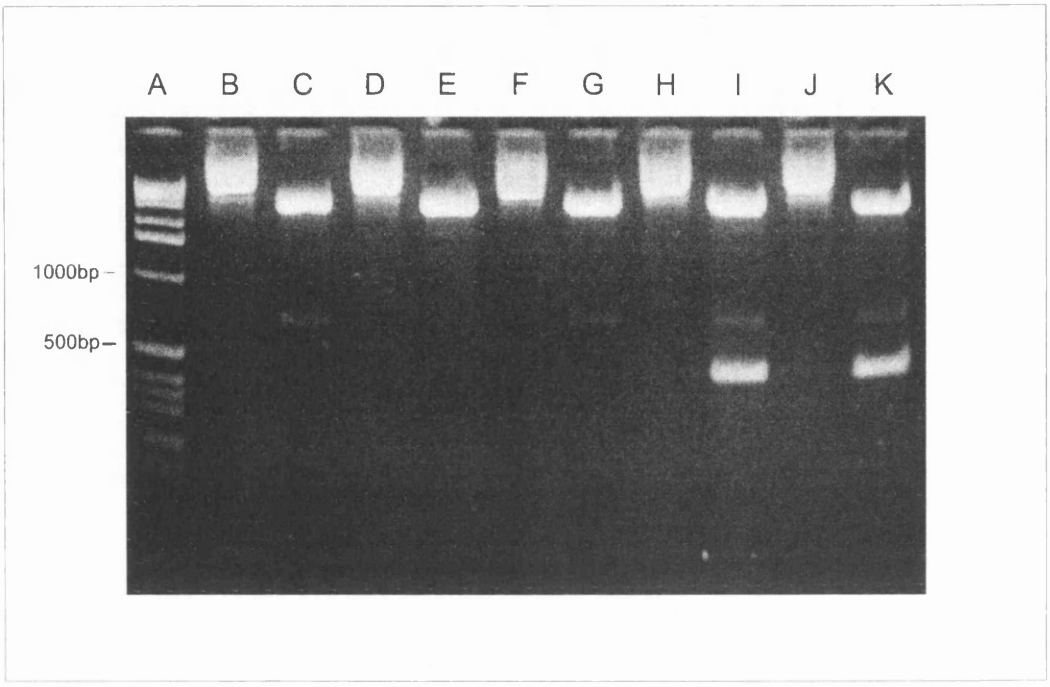


Figure 3.2.4.1 TNF-α Miniprep Digests

Lanes B-K show five plasmid DNA minipreps uncut and cut with EcoRI (Miniprep 1 is shown uncut in Lane B and cut in Lane C, etc.). Lane A contains 1kb DNA ladder markers.

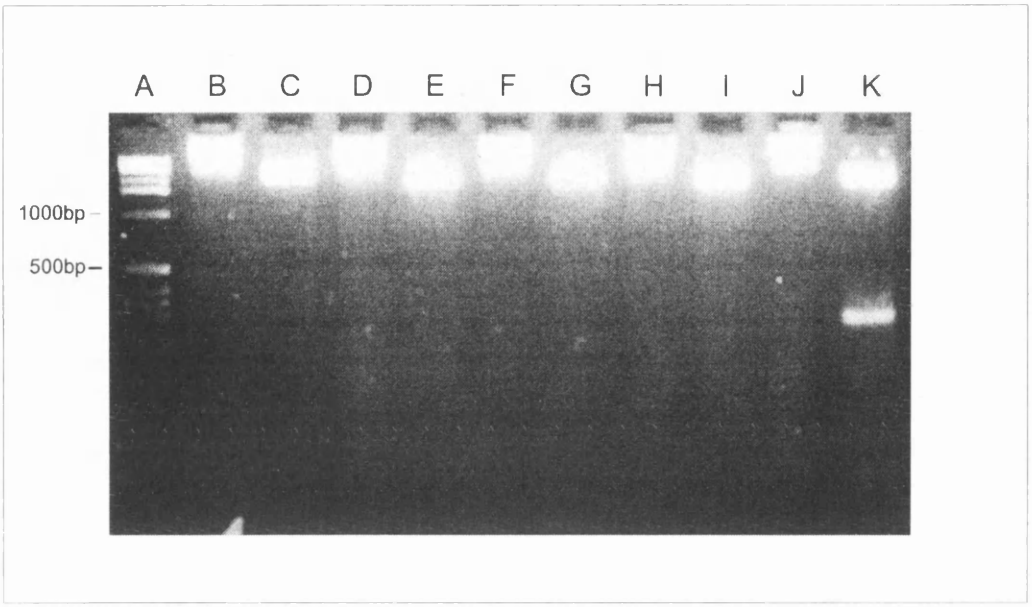


Figure 3.2.4.2 IL-6 Miniprep Digests

Lanes B-K show five plasmid DNA minipreps uncut and cut with EcoRI (Miniprep 1 is shown uncut in Lane B and cut in Lane C, etc.). Lane A contains 1kb DNA ladder markers.

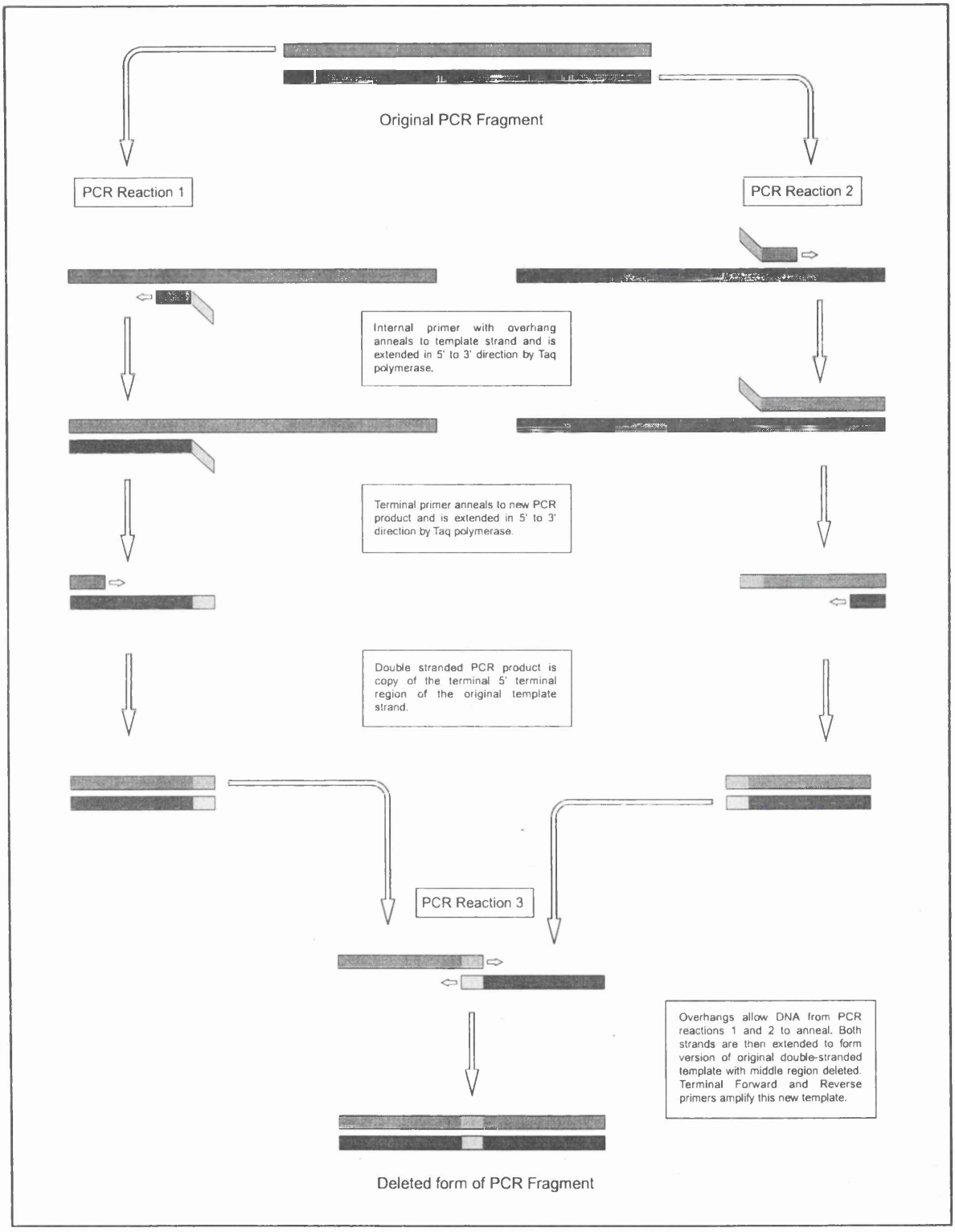
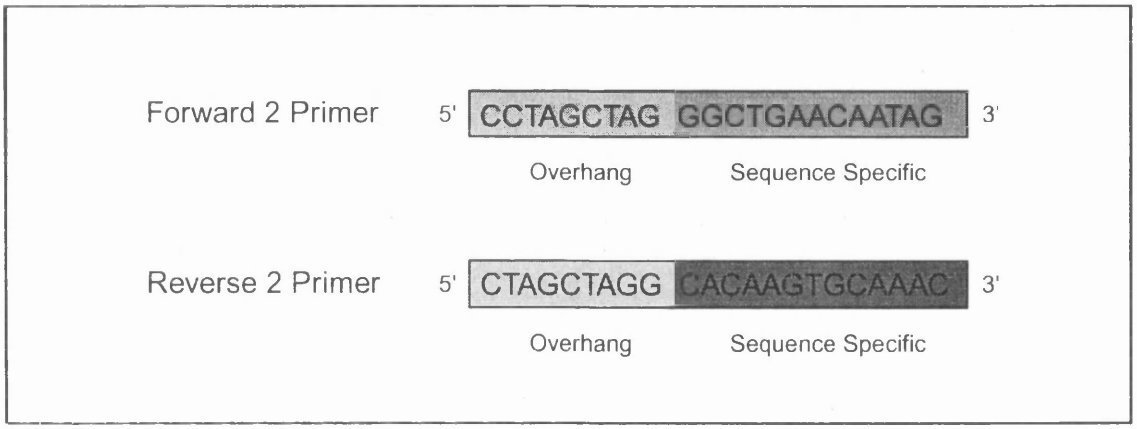


Figure 3.2.5.1 Generation of PCR Fragment with Internal Deletions



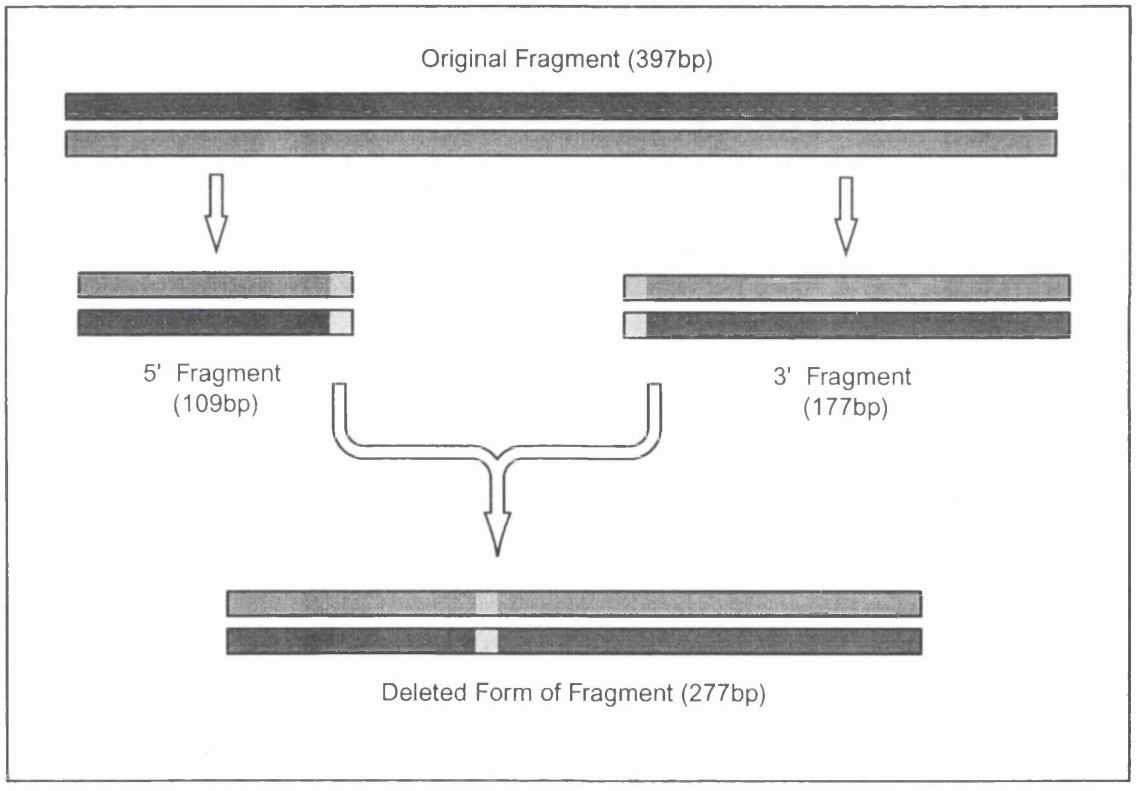
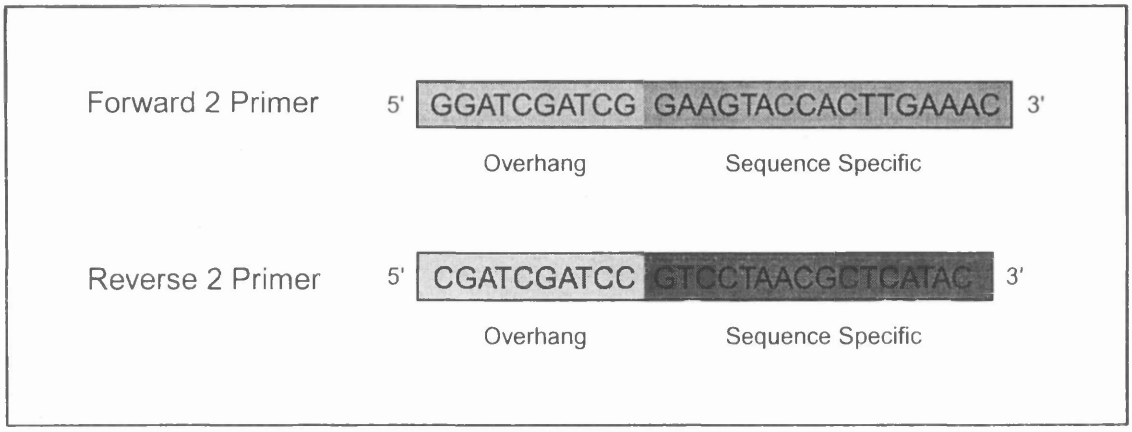


Figure 3.2.5.2 (A) TNF-α Internal Primers and Deletion Fragment



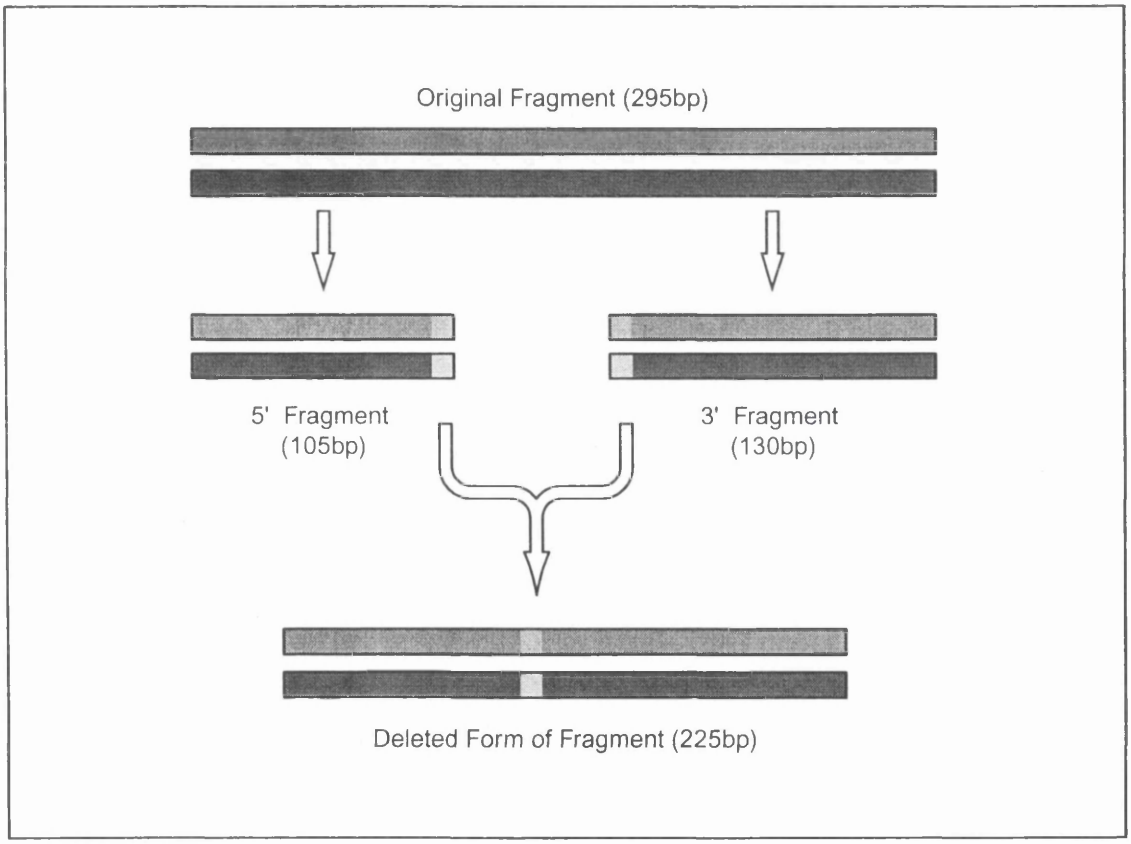


Figure 3.2.5.2 (B) IL-6 Internal Primers and Deletion Fragment

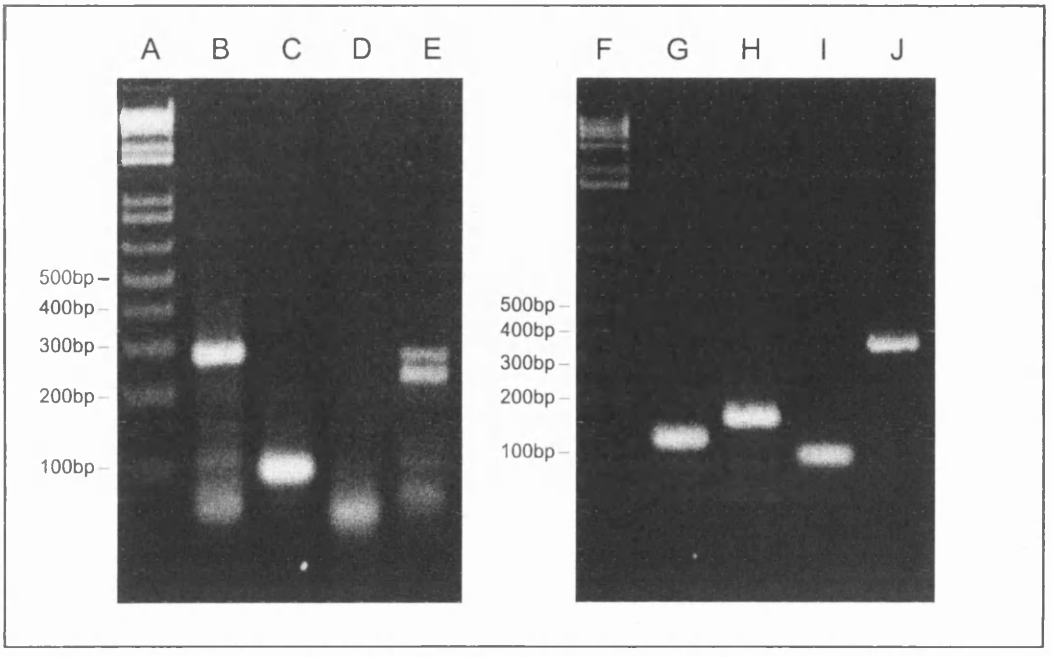


Figure 3.2.5.3 Internal PCR Reactions

Four fragments were generated using terminal and internal primers and purified TNF-α and IL-6 PCR fragments as the template molecules. The reaction products are visualised above, together with positives controls.

Lanes A and F contain 100bp DNA ladder markers. Lanes B and E contain original IL-6 fragment postives controls, B using purified IL-6 fragment as a template and E using IFN-γ-treated U937 cell cDNA as a template. Lane C contains the 5' IL-6 fragment (105bp). Lane G contains the 3' IL-6 fragment (130bp). Lane H contains the 3' TNF-α fragment (177bp). Lane I contains the 5' TNF-α fragment (109bp). Lane J contains an original TNF-α fragment postitive control using IFN-γ-treated U937 cell cDNA as a template.

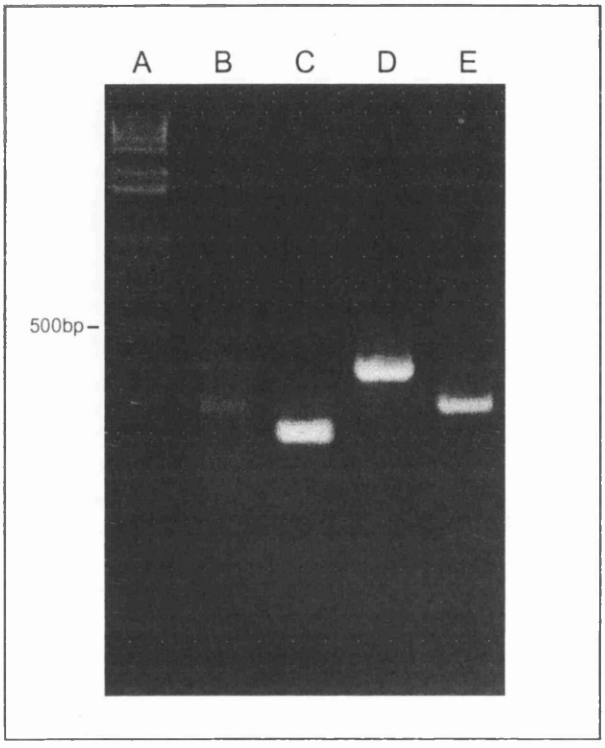


Figure 3.2.5.4 Deletion PCR Fragments

Deleted PCR fragments were generated by annealation and extension of previously generated terminal fragments. Lane B shows the deleted form of the TNF-α fragment (277bp), compared with the original length fragment (397bp) in Lane D. Lane C shows the deleted form of the IL-6 fragment (225bp) compared with the original length fragment (295bp) in Lane E.

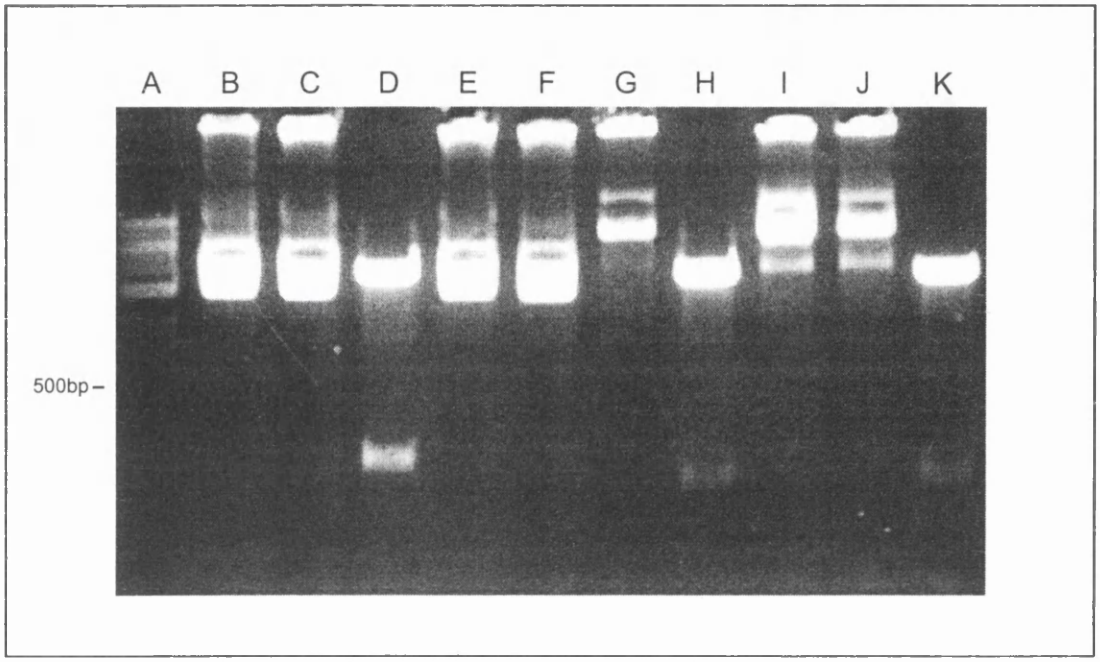


Figure 3.2.5.5 ΔTNF-α/ΔIL-6 Fragment-pCR2.1 Minipreps

Plasmid miniprep DNA was digested with EcoRI. Lanes B-F show five ΔTNF-α minipreps and Lanes G-K show five ΔIL-6 minipreps.

3.2.6 Competitive PCR Reactions

To test whether accurate measurements of mRNA were possible using a PCR system with a competitive template, reactions were performed using known amounts of the original and deleted PCR fragments in the form of the plasmids previously constructed. These reactions would show if a relative amount of each construct would generate a relative difference in band intensities.

3.2.6.1 Single Template Reactions

Each plasmid construct was used as the single template in four separate PCR reactions to evaluate each one individually. The reactions were 35 cycles in length and the annealing temperature used was 55^{0}C. Purified TNF-α and IL-6 PCR fragments were used as positive controls. The results are shown in **Figure 3.2.6.1**. Both the TNF-α plasmids produced very strong, clean bands of the correct size. The plasmid carrying the undeleted form of the IL-6 fragment also produced a strong, clean band of the correct size, but the plasmid carrying the deleted form did not produce any visible product at all. Subsequent attempts to generate a product with this plasmid, under a number of different conditions, all failed and so only the pair of TNF-α plasmid were used in further competitive PCR reactions.

3.2.6.2 Competitive Reaction 1

A series of four reactions were performed in which the amounts of two plasmids were varied by a thousand fold in opposite directions. This was to establish a general idea of the amount of PCR product the plasmids would generate relative to each other. The amount of DNA used was 1-1000pg. The results are shown in **Figure 3.2.6.2.** With an initial amount of 1000pg, the TNF-α construct

generated a large amount of product, but when the initial amount was dropped to 100pg the amount of product decreased substantially. At an initial amount of 100pg the product was no longer visible on the gel. The ΔTNF-α construct did not produce any visible product when 1pg was used as the initial template, but when this amount was increased to 10pg the amount of product the reaction gave was comparable to that produced by 1000pg of the TNF-α construct. The amount of product remained at this level as the amount of ΔTNF-α plasmid was increased to 100 and 1000pg.

3.2.6.3 Competitive Reaction 2

To establish whether the rapid decrease in product from theTNF-α was due to competition from the ΔTNF-α construct the TNF-α construct was kept constant at 100pg while the ΔTNF-α construct was increased from 1pg to 50pg. The results of the reaction are shown in **Figure 3.2.6.3**. The gel showed a smooth increase the amount of ΔTNF-α product as the initial amount of plasmid increased and a corresponding decrease in the amount of product from the TNF-α plasmid. This showed that the reaction off the TNF-α construct was being limited in some way, as a constant amount of template should have given a constant amount of product.

3.2.6.4 Mathematical Model of PCR Reaction – Primer Concentration

The most likely candidate for a limiting reagent was the primer pair. To establish whether this was the case, a mathematical model of the primer concentration over time was constructed. The details of the calculations and the resultant data are shown below in **Figure 3.2.6.4**, and analysed in greater detail in the discussion section of this chapter (**Section 3.3**). The data suggested that the lack of primers would limit the amount of one of the products (in this case the TNF-α product) if the initial amount of was significantly less than the initial amount of competing template (the ΔTNF-α

plasmid). The plasmid preps were analysed by spectrophotometry and it was found that the TNF-α concentration was nearly a thousand-fold less than previously thought. Therefore, new preparations of each plasmid were prepared and subject to multiple spectrophotometric measurements to ensure that the concentrations of each were comparable.

3.2.6.5 Competitive Reaction 3

A series of reactions were performed where the initial amount of TNF-α plasmid was decreased from 50-1pg and the initial amount of the ΔTNF-α plasmid was increased from 1-50pg. The results are shown in **Figure 3.2.6.5**. The TNF-α product showed a smooth decrease as the initial concentration was reduced and the ΔTNF-α product showed a smooth increase as the initial concentration was increased. The crossover point, where the amount of product from each template were equal occurred at between 20pg and 30pg for both plasmids, suggesting that the plasmids would produce equal amounts of product under equal reaction conditions and therefore one could be used to quantitate the amount of the other present in a sample.

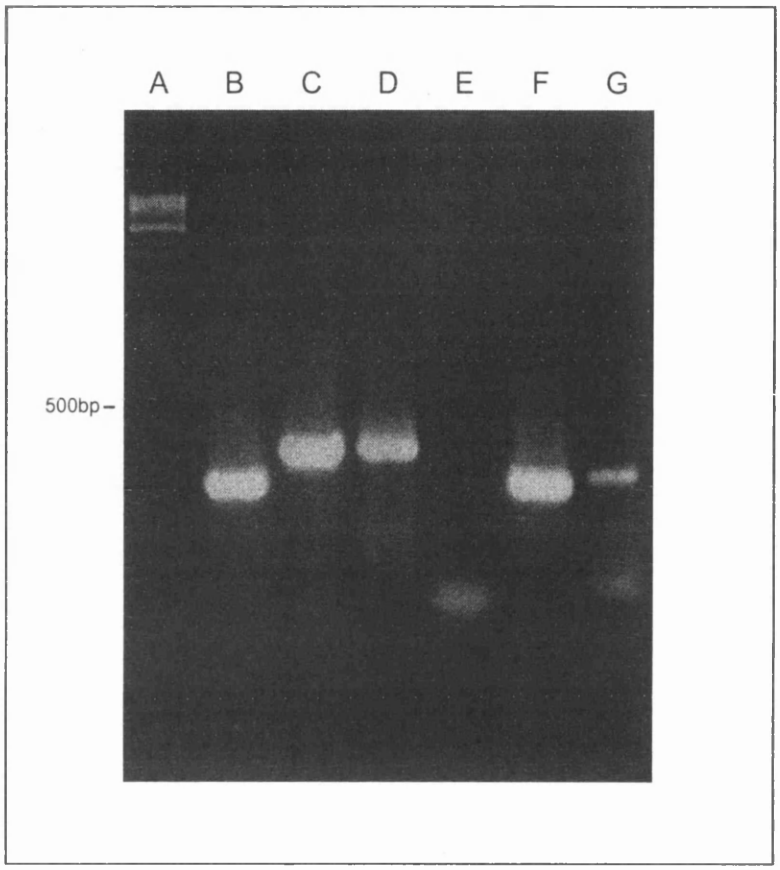


Figure 3.2.6.1 Plasmid Contruct Test Reactions

Lanes B and C show 35 cycle PCR reactions using the deleted and undeleted TNF-α plasmid constructs respectively. Lane D hows an identical control reaction using purified TNF-α fragment as template. Lanes E and F show reactions using the IL-6 deleted and undeleted plasmid constructs with an IL-6 postitive control, using purified IL-6 fragment DNA as the template. Lane A contains a 100bp DNA marker ladder.

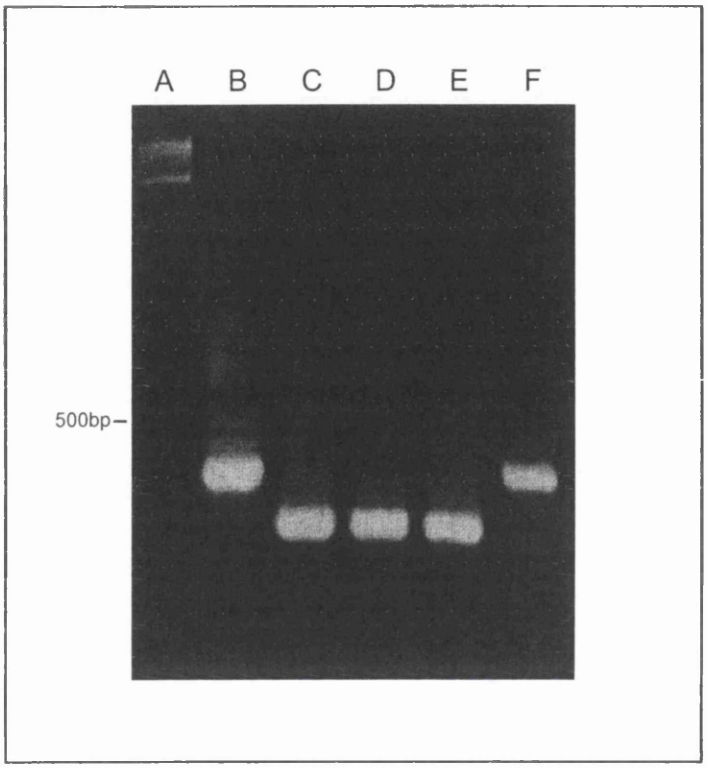


Figure 3.2.6.2 Competitive PCR Reaction 1

Lanes B to E show the results of a series of four competitive PCR reactions using the deleted and undeleted TNF-α fragment plasmid constructs as templates. Lane B shows 1000pg of undeleted plasmid vs. 1pg of deleted plasmid. Lane C shows 100pg undeleted vs. 10pg deleted plasmid. Lane D shows 10pg undeleted vs. 100pg deleted plasmid. Lane E shows 1pg undeleted vs. 1000pg deleted plasmid. Lane F shows the purified, 400bp TNF-α positive control and Lane A contains 100bp DNA ladder markers.

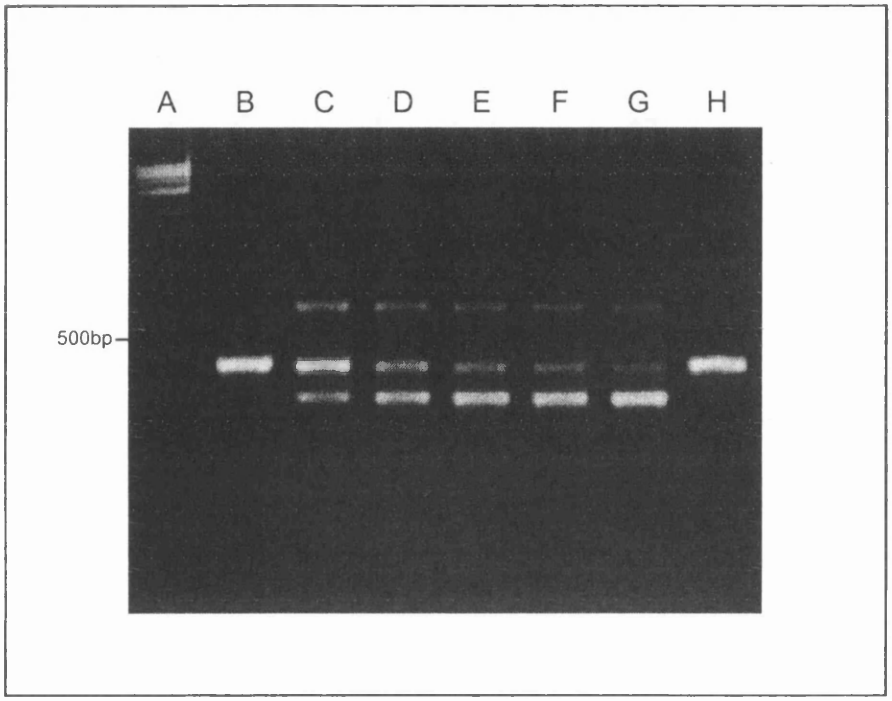


Figure 3.2.6.3 Competitive PCR Reaction 2

Lanes B to G show a competitive PCR reaction series where the undeleted plasmid template (upper band) was kept constant at 100pg and the deleted plasmid (lower band) was varied from 1pg to 50 pg (1, 10, 20, 30, 40, 50pg) from lane B to lane G. Lane H contains the TNF-α postiive control and Lane A contains 100bp DNA ladder markers.

To determine whether the amount of primer was a limiting factor in the PCR reaction, the amounts of PCR product generated and primers consumed throughout the reaction were calculated:

The reaction modelled was one where no pCR2.1-ΔTNF-αF plasmid was present and the initial amount of pCR2.1-TNF-αF was 100pg. The reaction was 25 cycles long.

pCR2.1-TNF-αF Plasmid = 4300 base pairs

Molecular Weight of pCR2.1-TNF-αF Plasmid = 4300 bases x 650Da per base

= 2795kDa

So, 1 mole of pCR2.1-TNF-αF Plasmid = 2,795,000g

There are 100pg of plasmid in the reaction

Therefore, the number of moles of plasmid = $\frac{100 \times 10^{-12}g}{2795000g} = 3.58 \times 10^{-17}$ moles

This is effectively the amount of PCR product at cycles = 0. The first cycle of the reaction will double this amount as each plasmid acts as a template for the generation of one double-stranded product molecule. With each subsequent cycle the amount of PCR product will double again. This is shown in Table 4.2.6.4.

This shows that after 25 cycles this PCR reaction generates 1.2 nanomoles of TNF-αF.

Each individual PCR reaction requires 1 molecule of template DNA and 1 pair of primers (1 forward and 1 reverse)

So, 1 mole of template needs 1 mole of primers

Therefore the number of moles of primer used up during a reaction cycle is equal to the number of *new* moles of product generated in that cycle (see Table 3.2.6.4, Column 2)

The concentration of the stock mix of Primers was 27.86μM. The volume in each reaction was 1μl, therefore the initial amount of Primers in each reaction was:

$27.86\mu M \times 1 \times 10^{-6} L = 27.86$ pmoles

Figure 3.2.6.4 Mathematical Modelling of Primer Consumption in PCR Reactions

The number of Primer pairs used in each cycle of the reaction will equal the number of new PCR products generated in that cycle, and, assuming a reaction in which all available templates are used, that number will be equal the total amount of PCR product in the previous reaction.

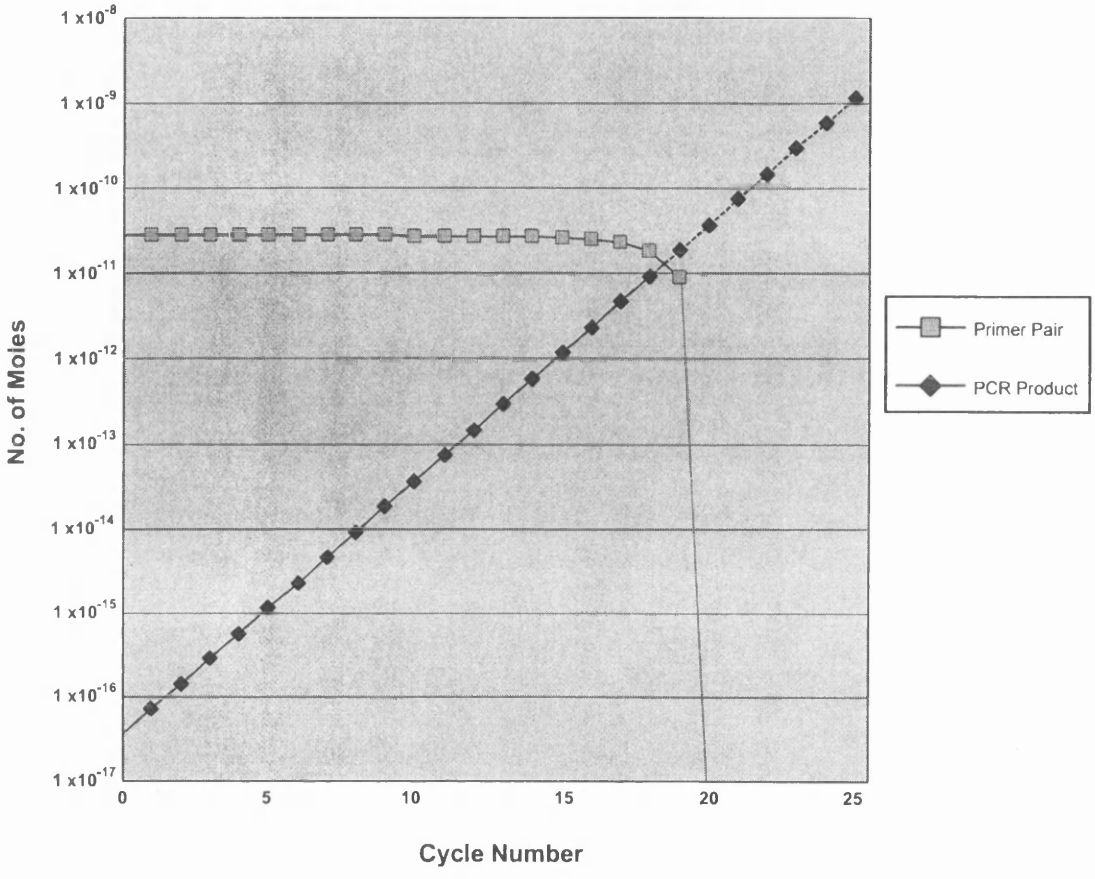
Therefore the consumption of Primers during the course of reaction can be calculated and is shown in Column 3 of Table 4.2.6.4, below.

Table 3.2.6.4

Cycle Number	Amount of PCR Product (moles)	Amount of Primer Pair (moles)
0	3.58×10^{-17}	2.786×10^{-11}
1	7.16×10^{-17}	2.786×10^{-11}
2	1.43×10^{-16}	2.786×10^{-11}
3	2.86×10^{-16}	2.786×10^{-11}
4	5.73×10^{-16}	2.786×10^{-11}
5	1.15×10^{-15}	2.786×10^{-11}
6	2.3×10^{-15}	2.786×10^{-11}
7	4.6×10^{-15}	2.7855×10^{-11}
8	9.2×10^{-15}	2.8651×10^{-11}
9	1.84×10^{-14}	2.7842×10^{-11}
10	3.68×10^{-14}	2.7822×10^{-11}
11	7.33×10^{-14}	2.7786×10^{-11}
12	1.47×10^{-13}	2.7713×10^{-11}
13	2.93×10^{-13}	2.7566×10^{-11}
14	5.86×10^{-13}	2.7273×10^{-11}
15	1.17×10^{-12}	2.6687×10^{-11}
16	2.34×10^{-12}	2.5517×10^{-11}
17	4.69×10^{-12}	2.3177×10^{-11}
18	9.38×10^{-12}	1.8487×10^{-11}
19	1.88×10^{-11}	9.1071×10^{-12}
20	3.75×10^{-11}	0
21	7.51×10^{-11}	0
22	1.5×10^{-10}	0
23	3.0×10^{-10}	0
24	6.0×10^{-10}	0
25	1.2×10^{-9}	0

Figure 3.2.6.4 (continued)

The results are shown below in graphical form



These results show that this PCR reaction will run out of primers before the 20th cycle has been reached.

Figure 3.2.6.4 (continued)

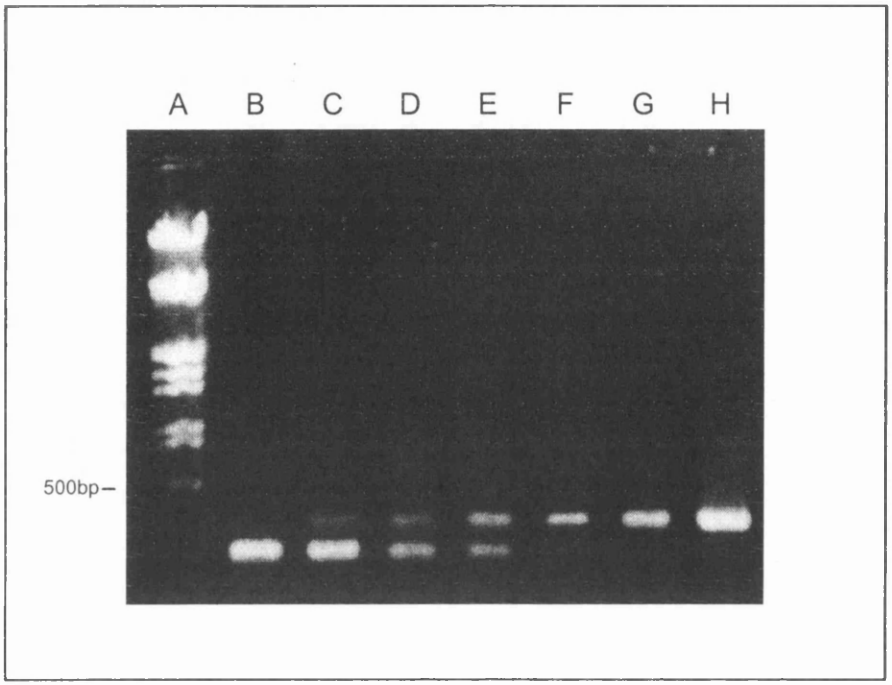


Figure 3.2.6.5 Competitive PCR Reaction 3

A series of competitive reactions were performed in which both the undeleted and deleted plasmids were varied. The undeleted plasmid was decreased from lane B to lane G (50, 40, 30, 20, 10, 1pg) and the deleted plasmid was increased from lane B to lane G (1, 10, 20, 30, 40, 50pg). Lane H contains the TNF-α positive control and lane A contains 100bp DNA ladder markers.

3.3 Discussion

The purpose of the work described in this chapter was to develop an assay which could be used to investigate the activation states of a number of genes under a number of conditions. While this investigation was not reached the work which preceded it did give valuable insights into the process of assay development and the specific methodologies involved.

The process involved the establishment of the PCR reaction as a reproducible and consistent assay method, and the construction of plasmids to act as internal controls against which cell-derived genetic signals could be quantified.

3.3.1 Primer Design and PCR Reaction Optimisation

It was decided to design the primer pairs required for the PCR reaction on paper and not to use a sequence analysing computer program. The expression of TNF-α, IL-6 and IL-10 has been widely reported in U937 cells and similar cell types, so it was thought likely that finding a signal, under the right conditions, would be relatively straight forward given the levels of expression previously described and the central role these cytokines play in the operation of macrophages and monocytes. There were five criteria used to limit the choice of primer sites. The first four are listed in **section 3.2.2.2**. The length of the primer, the first of these criteria was important in striking a balance between an oligonucleotide which would possess a high degree of specificity for a particular sequence (necessary when seeking to amplify one mRNA signal out many thousands) and one which would rapidly anneal to the template molecule during the limited time allowed for this in the annealation phase of the PCR cycle. In practice the primers designed and used varied between 24 and 30 nucleotides. This variation was to comply as much as possible with the other three criteria.

The fifth criterion was the length of the fragment of DNA the primer pair would generate. A length of several hundred bases would ensure that the fragment was large enough to be visible when run on an ethidium-stained agarose gel, but would also avoid the problems associated with amplifying lengthier pieces of DNA (e.g. secondary structures in the template molecule).

The PCR reaction shown in **Figure 3.2.2.3** was the first reaction performed using the primers and using two separate preparations of U937 cDNA. Its success, and the success of the reactions which followed, showed that this simple method of primer design was robust enough for the types of PCR used in this work.

The optimisation of the reaction involved analysing the effect of factors such as cycle number, annealing temperature and reagent concentration on the reaction, both in terms of product quantity and specificity.

3.3.2 Development of the TNF-α Competitive PCR Assay

The generation of the internal control for the TNF-α PCR reaction was achieved using internal PCR primers, again designed on paper, in a series of reactions to create the ends of the PCR product, excluding the middle region, and anneal these together. These deleted fragments were then subcloned into the pCR2.1 vector to create the undeleted and deleted template constructs.

These plasmids were then used to determine whether two templates which used the same primers but generated different products could be used in one reaction to generate products the amounts of which accurately reflected the initial amounts of template.

The first reaction (**Figure 3.2.6.2**) showed that the plasmid templates were not behaving as had been predicted. The rapid decrease in the amount of TNF-αF being produced from the pCR2.1-TNF-α plasmid when a comparatively small amount of the pCR2.1-ΔTNF-α plasmid was added suggested that a reagent was not available in excess and thereby limited the production of the TNF-αF to the extent that it was not visible when run on the agarose gel stained with ethidium bromide. To understand why this was, it is first important to understand the factors which dictate the amount of product a PCR reaction will be able to generate. Two theoretical scenarios are described below:

(1) In a theoretical reaction vessel with two templates, which utilise the same primers as well as other reagents, and which contains an excess of all these reagents, the amount of each product generated will again only be limited by the amount if template initially present and the number of cycles the PCR reaction proceeds through. This is a model of the "competitive" PCR reaction, perhaps more accurately termed a parallel PCR reaction, in which the two templates effectively exist independently of each other.

(2) The second scenario, where the independence of the two templates is destroyed, occurs when a reagent (for example, the primer pair) is not in excess. In this case, the amount of product will be limited by the reagent and will not continue to increase at an exponential rate will each successive reaction. For example, if there are initially 100pmoles of primer pairs and 1 mole of primer pairs is required to generate 1 mole of PCR product, then the maximum amount of PCR product that can be generated is 100pmoles, no matter how many cycles the reaction proceeds for. This is the total amount of product for all templates in the reaction vessel. In a two-template reaction the maximum amount of product from one template becomes dependent on that template's initial amount *relative* to the initial amount of the other template. A plasmid which represents 50% of the total amount of initial template will only generate 50% of the total amount of PCR product allowed by the limited

reagent. To continue the example above, this plasmid will only generate a maximum of 50pmoles (50% of the 100pmoles allowed) of PCR product. On its own, this relationship between the two different templates does not cause a problem when trying to quantify initial amounts of template DNA. If the reaction were allowed to run without limit for a longer time, the final relative amounts of each product would be the same.

However, the practical consideration of measuring the final amounts of DNA brings to light a potential problem. The method of separating DNA of different lengths on an agarose gel and visualising it by staining with ethidium bromide has practical limitations. As demonstrated in the cycle number optimisation experiments (**Section 3.2.2.5**), the agarose gel system will not allow the detection of DNA below a certain level. Below approximately 25 reaction cycles the amount of DNA produced was not sufficient to show up against the background levels of fluorescing ethidium bromide. Also, above approximately 35 reaction cycles the band intensity reaches a saturation point and does not increase in the linear fashion seen with smaller amounts of DNA (lower cycle numbers). These two factors mean the agarose-ethidium detection method for DNA has a limited range within which quantification can be made accurately.

In **Figure 3.2.6.3** the evidence of a reagent-limited reaction was seen again, this time over a narrower range of DNA concentrations. The band of undeleted fragment (TNF-αF) should have remained at a constant intensity from Lanes B-G. The fall in intensity suggested that the amount of PCR product was not being dictated solely by the initial amount of the pCR2.1-TNF-α plasmid and the cycle number, but also by the amount of limiting reagent and the relative amounts of pCR2.1-ΔTNF-α plasmid also present. The mathematical analysis of the reaction, described in detail in **Figure 3.2.6.4**, showed that the primer concentration used may have been the factor limiting the 25-cycle reaction.

In **Figure 3.2.6.3**, the intensity of the ΔTNF-αF band was greater than the TNF-αF band in lane C, where the initial amount of pCR2.1-ΔTNF-α plasmid was supposed to be 10-fold less than that of pCR2.1-TNF-α. This suggested that the amounts of DNA added to the reaction were not accurate, that the amount of pCR2.1-ΔTNF-α plasmid was greater than that of the pCR2.1-TNF-α plasmid, and hence the TNF-αF was decreasing as its template's relative abundance decreased. A spectrophotometric analysis of the plasmid preparations used in the reaction confirmed that the preparation of pCR2.1-ΔTNF-α DNA was more concentrated than previously thought (probably through experimental error when the preparation was originally made and measured).

With new preparations of plasmid DNA, measured several times to ensure the estimated concentration of each was accurate, the reaction was performed again, this time successfully. The relative amounts of the ΔTNF-αF and TNF-αF PCR products seen in **Figure 3.2.6.5** accurately mirror the known initial amounts of template DNA. The reaction may have been limited by the primer concentration (unchanged from previous reactions) this did not matter when considering the relative amounts of each product. This reaction showed that this system could be used to measure an unknown message against an internal control of known concentration if the factors which affect the amplification and measurement of the PCR products are known and controlled.

Chapter 4: Reporter Gene Assay

4.1 Introduction

4.1.1 Reporter Assay Systems

When examining the regulation of expression of a gene *in vivo*, measurement of encoded protein is the most physiologically relevant way of assessing the regulatory factors involved. However it may be difficult to assay the protein of interest as it may be difficult to isolate and quantify. In addition, other factors may regulate protein levels, such as the stability of the encoded protein. Measurement of gene transcription can performed by using a reporter gene.

The basic principle of a reporter gene system is to couple the regulatory sequences of the gene of interest to the coding sequence for a more easily assayed gene product, the reporter gene. This is typically an enzyme not naturally present in the cell type being studied: common reporter gene includes alkaline phosphatase, chloramphenicol acetyl transferase, and luciferase. The experimental enzyme can then be harvested and used in a quantifiable *in vitro* reaction.

4.1.2 Luciferase Reporter Assay

The term Luciferase refers to a number of unrelated proteins, which catalyse similar chemical reactions. These reactions typically produce light as their primary product and form the enzymatic basis of the phenomenon of bioluminescence, found in several species of insect and plant. These enzymes form the basis for a reporter gene system where light is generated and the amount detected is related to the level of expression of the luciferase gene. Using this principle and considered experimental design, the action of the promoter sequence can be determined.

The two major luciferase enzymes used in reporter systems of this type are found naturally in the Firefly, *Photinus pyralis*, and the sea pansy, *Renilla reniformis*. While their uncommon and specific function suggests a common ancestry and mechanism, the luciferase enzymes in these two species are distinct from each other in evolutionary terms and differ in their substrate.

4.1.2.1 *Photinus pyralis* Firefly Luciferase

This enzyme is a 61kDa monomeric protein that is active immediately upon primary translation and does not require any post-translational modifications such the cleavage of a signal sequence or glycosylation. The light-generating reaction catalysed is a single step oxidation step. The substrate Beetle Luciferin is converted to Oxyluciferin. ATP and molecular oxygen are required as cosubstrates and ionic Magnesium (Mg^{2+}) is required as an enzymic cofactor. (See **Figure 4.1.2.1 A**)

4.1.2.2 *Renilla reniformis* Sea Pansy Luciferase

This is a 36kDa protein that is 3% glycosylated in its natural form, although like Firefly Luciferase it is fully active immediately upon translation. The reaction catalysed is similar but distinct from the Firefly reaction described above. Again, it is a one-step oxidation reaction utilizing the substrate Coelenterazine and molecular oxygen. (See **Figure 4.1.2.2 B**)

4.1.3 Luciferase Reporter Plasmids

The luciferase gene used in the reporter gene system is encoded on a plasmid containing the cDNA for the natural luciferase mRNA. Several different types of luciferase plasmid were used in the work reported here and they are described in detail below.

4.1.3.1 pGL3-Basic

This plasmid (shown in **Figure 4.1.3.1**), of just under 5000bp in length, contains the coding sequence of the cytosolic form of Firefly luciferase (*luc*+) with a multiple cloning site upstream of the start codon to allow insertion of regulatory DNA sequences. A synthetic poly(A) sequence upstream of this prevents opportunistic translation of the gene by the random binding of transcription factors upstream of the coding region. It also contains the ampicillin resistance gene (Amp^r) to allow for selection when the plasmid is transformed into bacterial strains.

This plasmid allows background levels of expression of the *luc*+ gene to be evaluated. It also provides the vector into which the promoter sequence of interest can be inserted.

4.1.3.2 pGL3-Control

This plasmid (shown in **Figure 4.1.3.2**) is similar to pGL3-Basic but with the addition of an SV40 promoter between the multiple cloning site and the luciferase gene and an SV40 enhancer sequence immediately downstream of the SV40 late poly(A) signal found at the end of the coding sequence. The SV40 promoter and enhancer are derived from the SV40 virus and genes coupled to them are constitutively activated, to varying degrees, in a wide range of eukaryotic cells. Therefore the pGL3-Control plasmid provides a positive control plasmid for expression of Firefly luciferase *in vivo*.

4.1.3.3 pRL-SV40

This plasmid (shown in **Figure 4.1.3.3**) contains the SV40 Early Enhancer/Promoter sequence driving expression of R*luc*, an engineered form of the luciferase found in *Renilla reniformis*. This

provides constitutive expression of the *Renilla* luciferase. The plasmid also contains the ampicillin resistance gene, Amp^r, to allow for selection when growing the plasmid in bacterial culture.

4.1.4 Single and Dual Luciferase Assays

While transfecting a cell culture with one luciferase reporter plasmid can show that the cells will express active luciferase enzyme, it cannot be used to measure promoter activation without an internal control for transfection efficiency. Without this control it would be unclear whether any variation in luciferase activity seen between experimental conditions was due a differential activation of the gene by the promoter or different levels of plasmid DNA in the cells. An internal control provides a baseline of activity, which can be directly related to transfection efficiency, and thus experimental data on promoter activation can be normalised against this baseline giving a more accurate result. To provide this internal control in the luciferase system, two luciferase reporter plasmids are co-transfected into the cell. The first is the experimental plasmid containing the promoter sequence of interest coupled to the Firefly luciferase gene. The second plasmid consists of a viral promoter sequence coupled to the Renilla luciferase gene, which will give constitutive expression of the Renilla luciferase. As the Firefly and Renilla luciferases use different substrates, their activities can be measured independently of each other without having to separate them from each other when the cells are harvested and cell lysates are prepared.

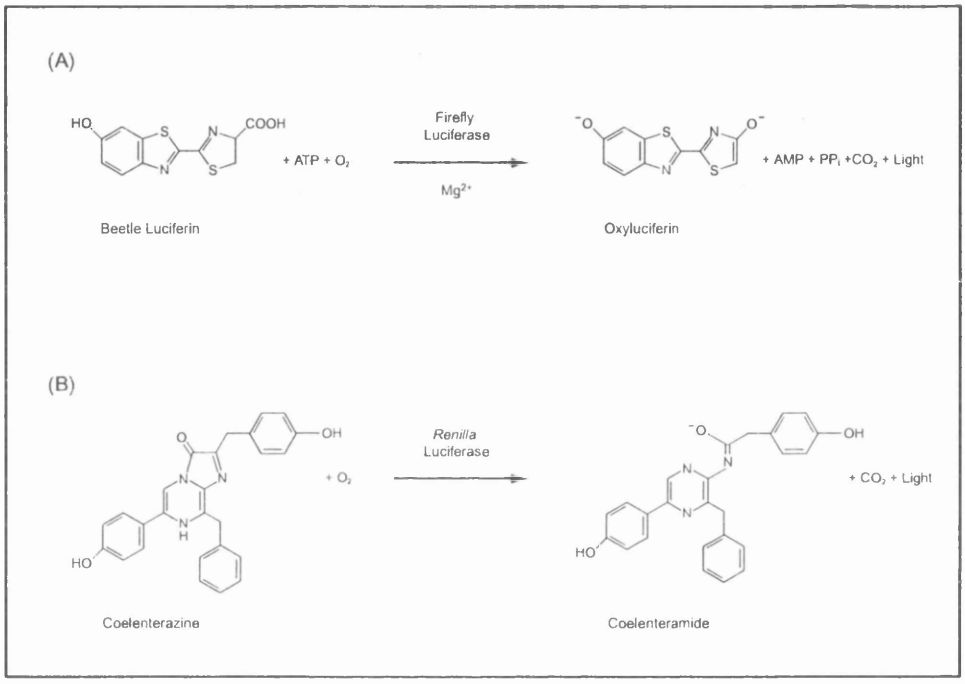


Figure 4.1.2.1 Reactions catalysed by Firefly and *Renilla* Luciferases

The light-generating reactions catalysed by the Firefly and *Renilla* luciferases are both one-step processes and utilise different substrate molecules, an indication of the distinct and separate evolutionary origins of the two enzymes.

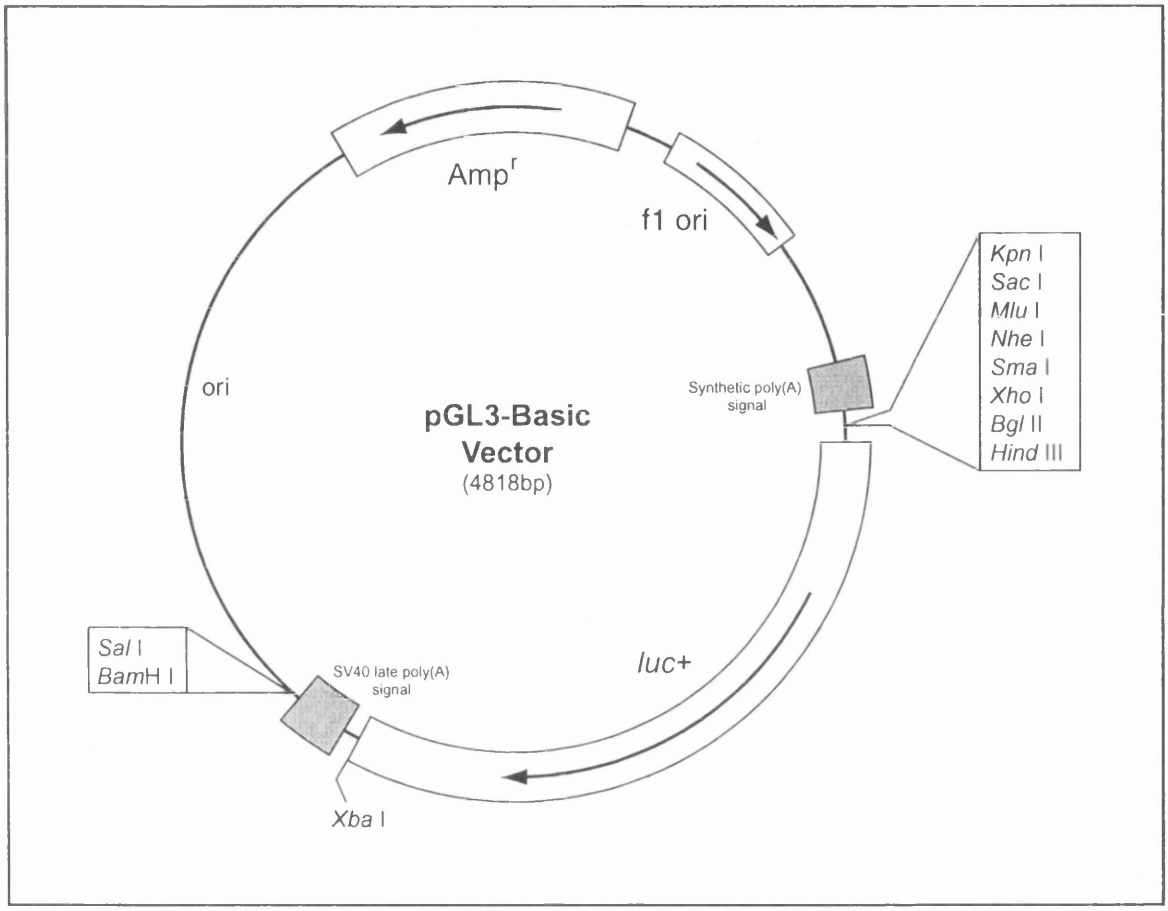


Figure 4.1.3.1 Schematic of pGL3-Basic Luciferase Expression Plasmid

The pGL3-Basic plasmid features the Firefly luciferase enzmye coding sequence with a multiple cloning site immediately upstream.

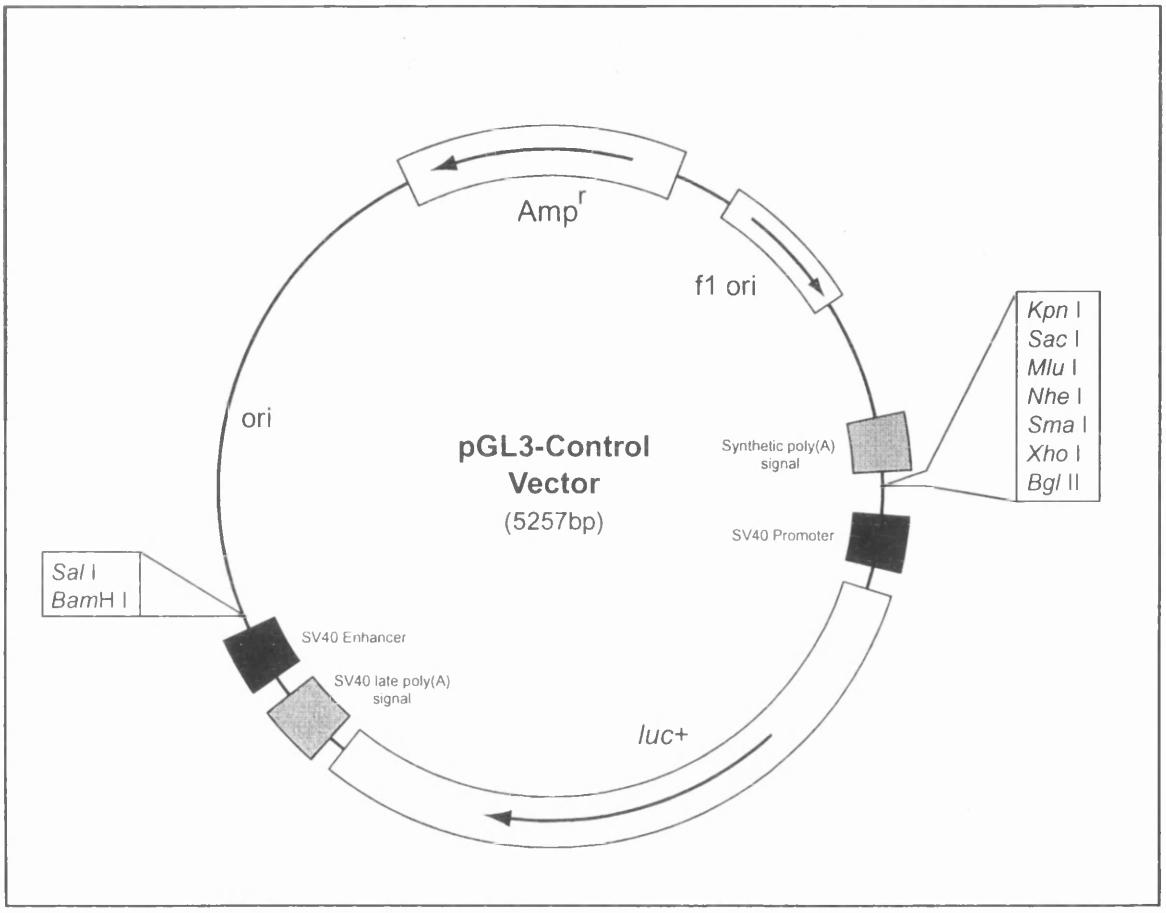


Figure 4.1.3.2 Schematic of pGL3-Control Luciferase Expression Plasmid

The pGL3-Control plasmid features the Firefly luciferase coding sequence coupled to the SV40 upstream promoter and downstream enhancer elements. This ensures constitutive expression across a wide range of eukaryotic cell types.

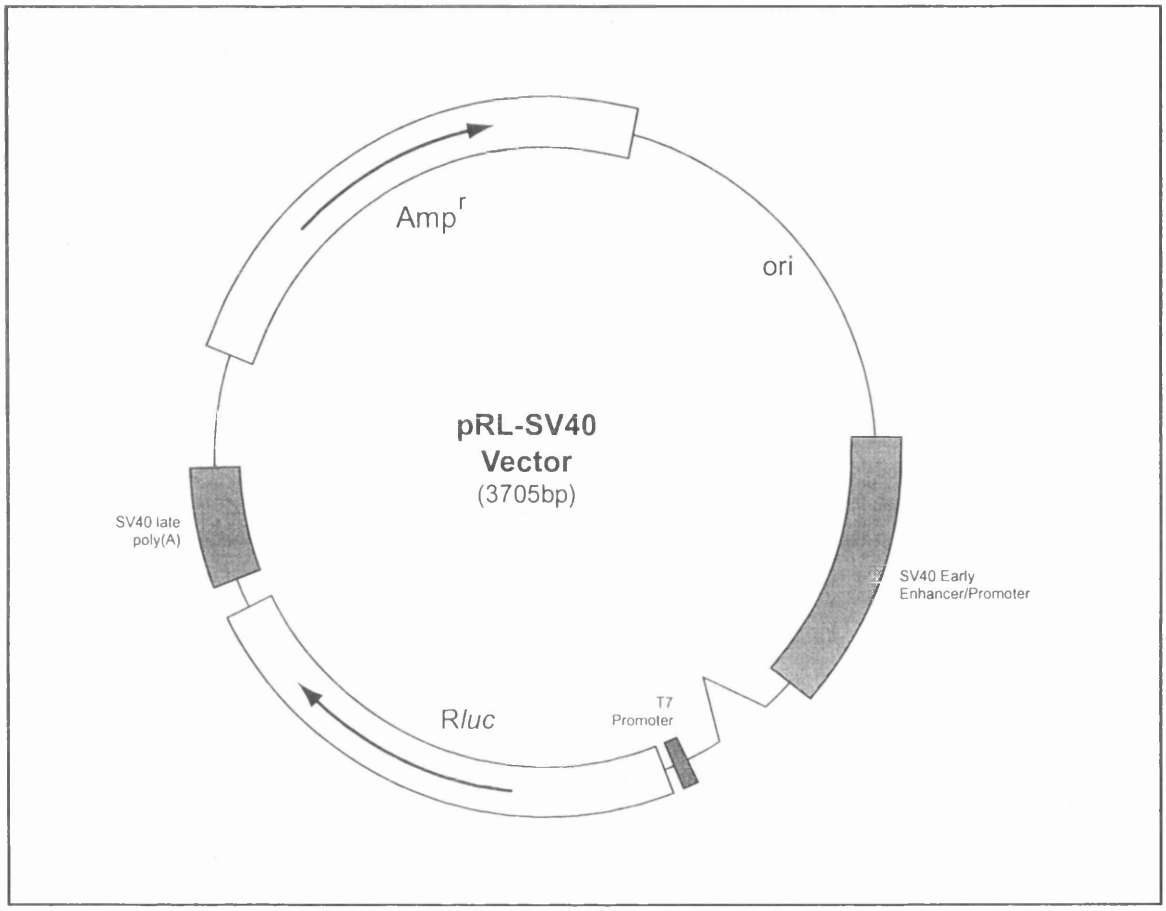


Figure 4.1.3.3 Schematic of pRL-SV40 Luciferase Expression Plasmid

The pRL-SV40 plasmid couple the *Renilla* luciferase coding sequence to a combined SV40 upstream promoter/enhancer. This provides constitutive expression across a wide range of eukaryotic cell types.

4.2 Results

4.2.1 Preparation of Plasmid DNA

Preparations of PGL3-Control, pGL3-Basic and pRL-SV40 plasmid DNA were made by transforming bacteria with plasmid DNA and growing colonies on agar plates, selecting for ampicillin resistance. Suitable colonies were picked and used to grow cultures in LB medium. Plasmid DNA was purified from these cultures by maxiprep. The concentration of DNA was measured spectrophotometrically.

4.2.2 Single Luciferase Transfections

A successful Luciferase assay system is one in which sensitivity and accuracy is balanced against an efficient use of resources. It was therefore necessary to investigate the effect of several experimental variables on measurable Luciferase activity to arrive an optimal set of conditions for further work. The variables tested were the amount of DNA transfected, the time of incubation after transfection, and the concentration of Enhancer, a reagent used in the transfection process.

4.2.2.1 Amount of DNA Transfected

1μg, 2μg, 5μg and 10μg of either pGL3-Control or pGL3-Basic plasmid DNA were transfected into 3 x 10^6 cells in a final volume of 5ml of RPMI medium. These cultures were then incubated for 24 hours and the cells were harvested using lysis protocol 1 and cell lysates were assayed immediately for Luciferase activity using the single luciferase assay system. The results are plotted in **Figure 4.2.2.1**.

Luciferase activity initially increased with the amount of pGL3-Control DNA transfected and reached a maximum with 5μg. The activity then decreased approximately 100-fold when 10μg of DNA was transfected.

Luciferase activity when cells were transfected with pGL3-Basic did not vary significantly with the amount of DNA between 1 and 5μg, although there was a dramatic decrease in activity when 10μg was used. Activity was negligible when compared to the pGL3-Control-transfected cells, being between and 500 and 4000-fold less for corresponding amounts of DNA.

While 5μg of DNA gave the highest activity it was decided to use 2μg of DNA in subsequent transfections. This would give an activity which was typically only 10% less than when using 5μg but which would consume only 40% of the DNA. This would allow more transfections to be carried out using the same preparations of DNA.

4.2.2.2 Period of Incubation

2μg of either pGL3-Control or pGL3-Basic DNA was transfected into 3 x 10^6 cells in a final volume of 5ml of RPMI medium and the cells were then incubated for 24, 48 or 72 hours before being harvested using lysis protocol 1 and immediately assayed using the single luciferase assay system. The results are plotted in **Figure 4.2.2.2**.

Luciferase activity was highest at the 24-hour time point and decreased rapidly after this, although activity was still large enough to be measured with a high degree of accuracy. This was true for both the pGL3-Control-transfected cells and the pGL3-Basic-transfected cells, although the difference in magnitude between the two plasmids was 1000-1500 fold throughout the time course.

4.2.2.3 Concentration of Enhancer Reagent

2μg of pGL3-Control DNA was transfected into 3 x 10^6 cells in a final volume of 5ml RPMI medium with either 4μl, 6μl, 8μl, 10μl or 12μl of Enhancer reagent. The volume of buffer was altered in each case to give each DNA/Enhancer/Effectene mixture a consistent volume. The cell cultures were incubated for 24 hours and harvested using lysis protocol 2, before being assayed immediately using the single luciferase assay system. The results are plotted in **Figure 4.2.2.3**. There was no significant variation in the measured luciferase activity for the five experimental conditions.

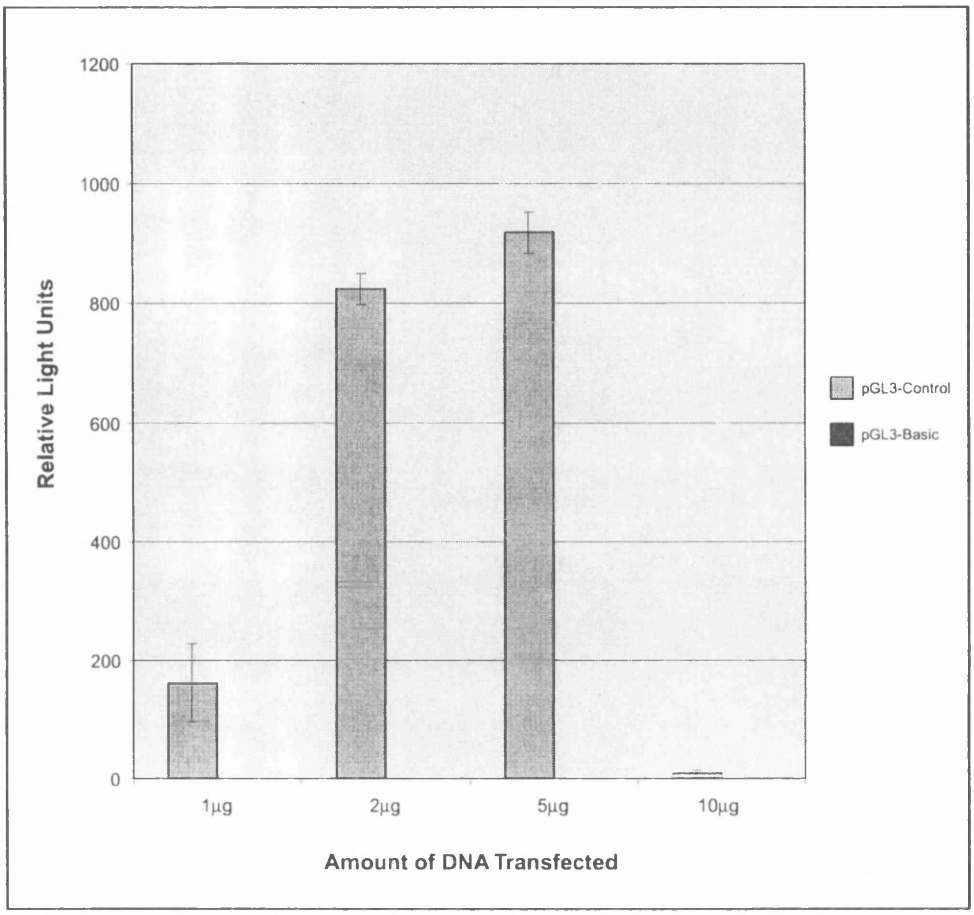


Figure 4.2.2.1 Firefly Luciferase Activity vs. Amount of Plasmid DNA Transfected

Cells were transfected with 1-10µg of either pGL3-Control or pGL3-Basic plasmid DNA, incubated for 24 hours and harvested. Firefly luciferase activity was then assayed. The graph shows the mean of three separate transfections, each measured in triplicate, and the standard deviation of the mean.

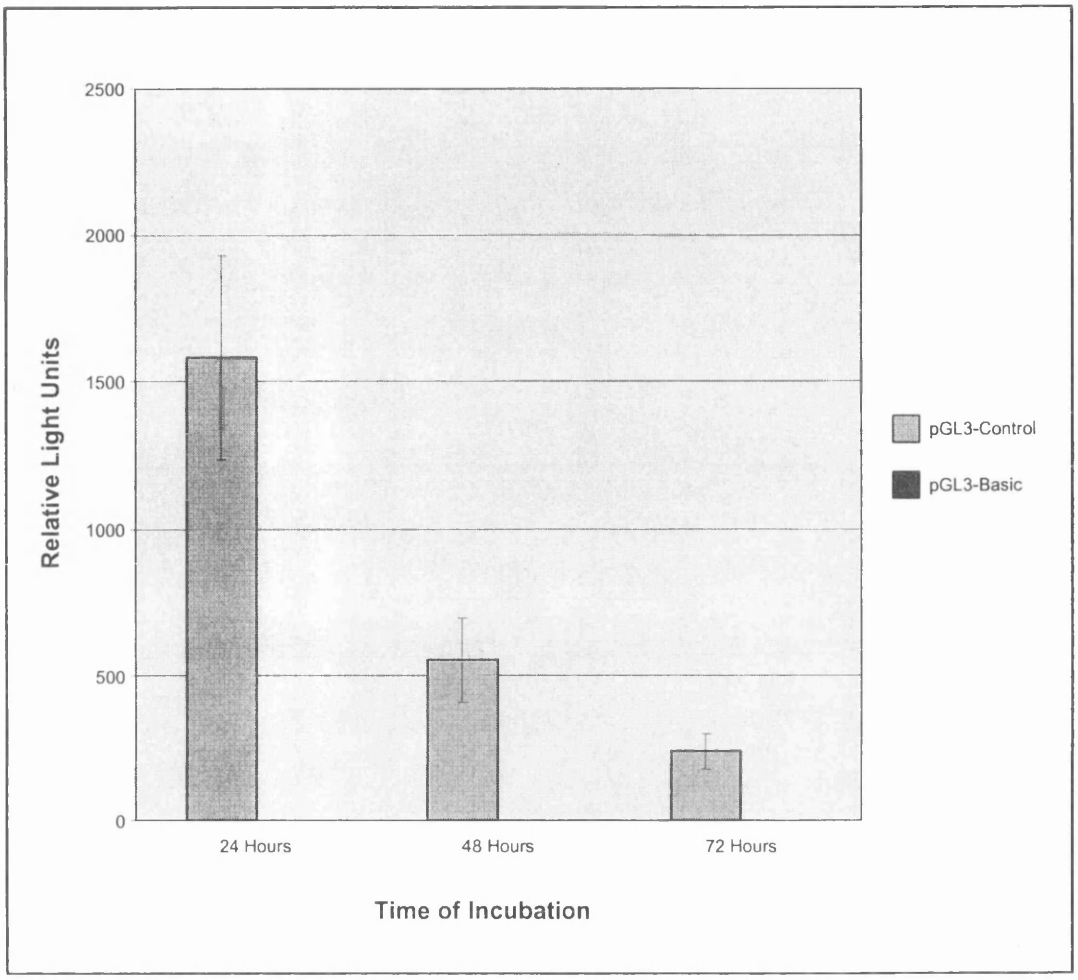


Figure 4.2.2.2 Firefly Luciferase Activity vs. Time of Incubation

Cells were transfected with 2μg of pGL3-Control or pGL3-Basic plasmid DNA and incubated for 24, 48 and 72 hours. They were then harvested and Firefly luciferase activity was assayed. The graph shows the mean of three separate transfections, each measured in triplicate, and the standard deviation of the means.

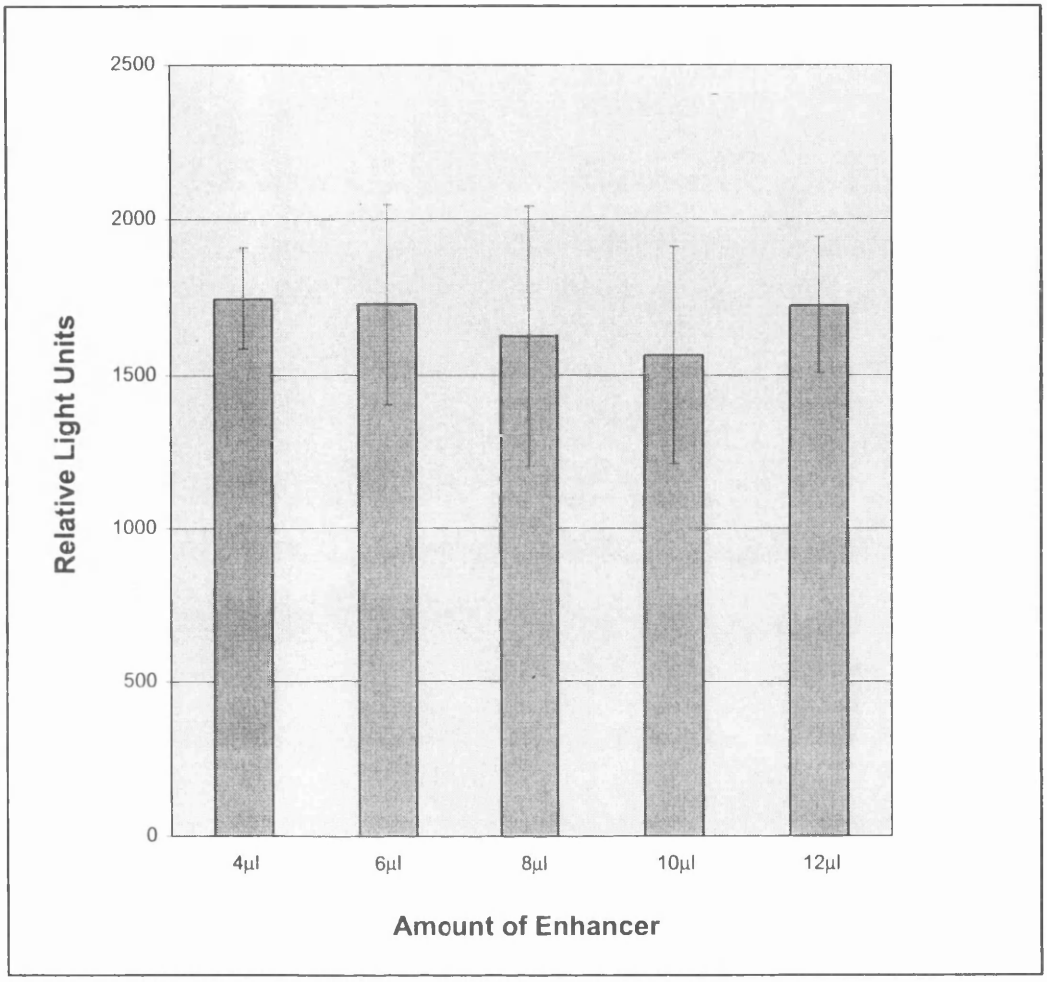


Figure 4.2.2.3 Firefly Luciferase Activity vs. Amount of Enhancer

Cells were transfected with 2µg of pGL3-Control plasmid DNA. The amount of Enhancer regeant used in the transfectionwas varied from 4 to 12µl. The cells were incubated for 24 hours, harvested and Firefly luciferase activity was measured. The graph shows the means of three separate transfection series, each of which was measured in triplicate, and the standard deviation of the means.

4.2.3 Dual Luciferase Transfections

4.2.3.1 Initial Transfection

2μg of pRL-SV40 DNA was cotransfected with 2μg of either pGL3-Control or pGL3-Basic DNA into 3 x10^6 cells in a final volume of 5ml of RPMI medium. The cultures were incubated for 24 hours and the cells harvested using lysis protocol 2. Firefly and Renilla Luciferase activity in the lysates was then measured, using the Dual Luciferase Assay protocol. The results are plotted in **Figure 4.2.3.1.**

The results showed a large difference in measured activity between the two types of Luciferase and also a difference in Renilla luciferase activity depending on which Firefly luciferase plasmid it was co-transfected with. In the co-transfection of pRL-SV40 and pGL3-Control, where the two luciferase genes were under the control of similar SV40 promoter elements, the Renilla luciferase's activity was typically more than twice that of the Firefly luciferase. When co-transfected with pGL3-Basic, the activity of the Renilla Luciferase was much reduced, falling to below that of Firefly luciferase as expressed by the pGL3-Control plasmid.

4.2.3.2 Variation in Amount of pRL-SV40 DNA Transfected

2μg of pGL3-Control DNA was co-transfected with either 2μg, 1μg, 0.5μg, 0.25μg or 0.1μg of pRL-SV40 DNA in cell cultures of 3 x 10^6 cells in a final volume of 5ml RPMI medium. The cultures were incubated for 24 hours, harvested and assayed using the Dual Luciferase Assay protocol. The results are plotted in **Figure 4.2.3.2.**

The results showed a steady decrease in Renilla luciferase activity followed a decrease in the amount of pRL-SV40 DNA transfected. Initially, with the amounts of pRL-SV40 and pGL3-Control DNA the same (2μg each) the activity of Renilla luciferase was approximately 8 fold that of the Firefly luciferase. As the activity of Renilla luciferase fell there was a steady increase in Firefly luciferase activity, and when the amount of pGL3-Control DNA transfected was 8 fold that of pRL-SV40, the activities of the two luciferases were comparable. When the Firefly luciferase activity was used as a control for transfection efficiency and the two activities were expressed as a ratio, the Renilla luciferase activity being normalied by the Firefly luciferase, the result was a linear relationship between the amount of pRL-SV40 DNA transfected into the cells and the activity of the Renilla luciferase expressed by the cells.

4.2.4 Generation of Promoter-Luciferase Plasmid Constructs

The strategy for making pGL3 luciferase expression plasmids containing the TNF-α and IL-6 gene promoters consisted of two main stages. In the first stage the promoters would be amplified from cell DNA by PCR and in the second stage the promoter fragments would be subcloned into the pGL3-Basic plasmid at the multiple cloning site.

4.2.4.1 Sequence Analysis and PCR Primer Design

The promoter sequence of each gene was retrieved from the Genbank database and primers were designed to amplify a region at least 1kb in length immediately upstream of the transcription initiation start codon. The primers were designed following the criteria set out in section **3.2.2.2.** Two sets of primers were designed to make available four possible combinations of forward and reverse primers. The primer sequences are shown in **Table 4.2.4** below.

Table 4.2.4 Primers for PCR of Gene Promoters

Primer	Sequence (5'-3')
TNF-α Forward 1	GAT GTG ACC ACA GCA ATG GGT AGG
TNF-α Reverse 1	CAT GCT TTC AGT GCT CAT GGT GTC C
TNF-α Forward 2	GAA TGT CCA GGG CTA TGG AAG TCG AG
TNF-α Reverse 2	CAG TGC TCA TGG TGT CCT TTC CAG
IL-6 Forward 1	GAG ACA CCA TCC TGA GGG GAA GAG GG
IL-6 Reverse 1	CTT GTG GAG AAG GGA GTT CAT AGC
IL-6 Forward 2	GGA ATC CTC CTG CAA GAG ACA CCA TC
IL-6 Reverse 2	CTA AGG ATT TCC TGC ACT TAG TTG TG

4.2.4.2 PCR Amplification of Promoter Sequences

PCR amplification was performed on genomic DNA prepared from untreated U937 cells. Initially, each primer pair was used separately giving two primer combinations for each promoter. This produced the result seen in **Figure 4.2.4.1**. The primer pairs for the IL-6 promoter sequence did not produce a fragment of the correct size but both primer pairs for the TNF-α promoter did produce a fragment of the correct size. Further PCR reactions using all four combinations of the IL-6 primers, over a range of conditions failed to generate the IL-6 promoter fragment.

4.2.4.3 Construction of pGL3-TNFP Plasmid

Instead of trying to subclone the TNF-α promoter fragment directly into the pGL3-Basic plasmid, it was decided first to subclone the fragment into the pcDNA3.1/V5-His-TOPO vector. The fragment could then be excised using two restriction enzymes and inserted into the multiple cloning site of the pGL3-Basic plasmid in the correct orientation.

The first subcloning step used the TA cloning method, where the fragment has a single deoxyadenosine (A) overhang at the 3' end of each strand and the vector has a single deoxythymidine (T) overhang at the end of each strand, which will anneal together. However, the PCR fragment had been generated with the polymerase Pfu, which does not leave an overhang on the fragment. Therefore the fragment was given an overhang by incubation with Taq polymerase at 72^0C for 30 minutes. The Taq was added directly to the PCR reaction so no further dÁTP needed to be added. The PCR fragment was then gel purified and subcloned into the pcDNA3.1/V5-His-TOPO vector. This plasmid was used to transfect bacterial cells, from which miniprep cultures were prepared. The plasmid DNA prepared from these cultures was purified and digested to excise the promoter insert. The results of the digest are shown in **Figure 4.2.4.2.** Lane F showed a positive result with an insert of the correct size.

This plasmid and the pGL3-Basic plasmid were then both digested with the restriction enzymes *Asp*718 (a Neoschizomer of *Kpn* I) and *Xho* I, and the products were run on a gel (**Figure 4.2.4.3**) and purified from this. The excised TNF-α promoter fragment and the linearised pGL3-Basic were ligated together and the resulting plasmid was amplified by bacterial transformation and miniprep culture. The plasmid DNA isolated from these minipreps was then digested with *Asp*718 and *Xho* I to excise the insert. The digests are shown in **Figure 4.2.4.4.** All ten plasmid clones contained the TNF-α promoter insert. The plasmid in clone 1 was used generate larger amounts of DNA by

maxiprep, which was stored for use in transfections.

4.2.5 Dual Luciferase Transfection with pGL3-TNFP Plasmid

4.2.5.1 Treatment with IFN-γ and dbcAMP

To establish a baseline of expression for the pGL3-TNFP plasmid, cells were transfected with both it and pRL-SV40 and then treated with IFN-γ and/or dbcAMP for periods of 18 and 36 hours.
A culture of cells large enough to provide 3 million cell aliquots for each of the conditions was grown and transfected with 2μg of pGL3-TNFP and 2μg of pRL-SV40 DNA per 3 million cells. After 6 hours the cells were split into individual aliquots and treated. They were incubated, harvested and assayed as before. The results are plotted in **Figure 4.2.5.1**.

After 18 hours there is small increase in activity over the control (no treatment) when the cells were treated with IFN-γ. This is true for both the individual treatment and the dual treatment with dbcAMP. Treatment with dbcAMP alone did not significantly alter the expression from the control.

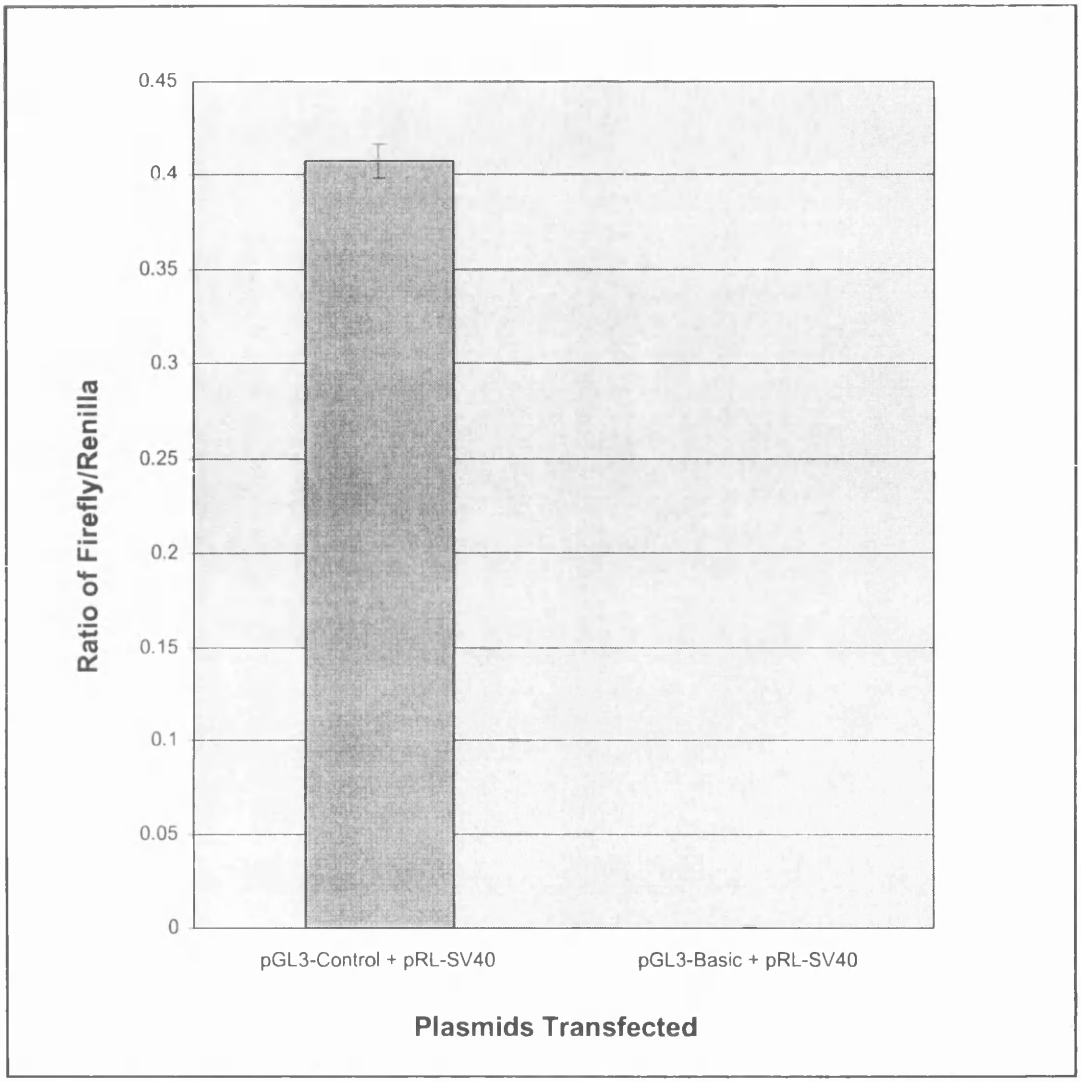


Figure 4.2.3.1 Dual Luciferase Assay

Cells were transfected with 2µg of pGL3-Control or pGL3-Basic and 2µg of pRL-SV40 plasmid DNA and incubated for 24 hours before harvesting and assay of Firefly and Renilla luciferase activities. The graph shows the means of three separate transfections, each of which measured in triplicate, and the standard deviation of these means.

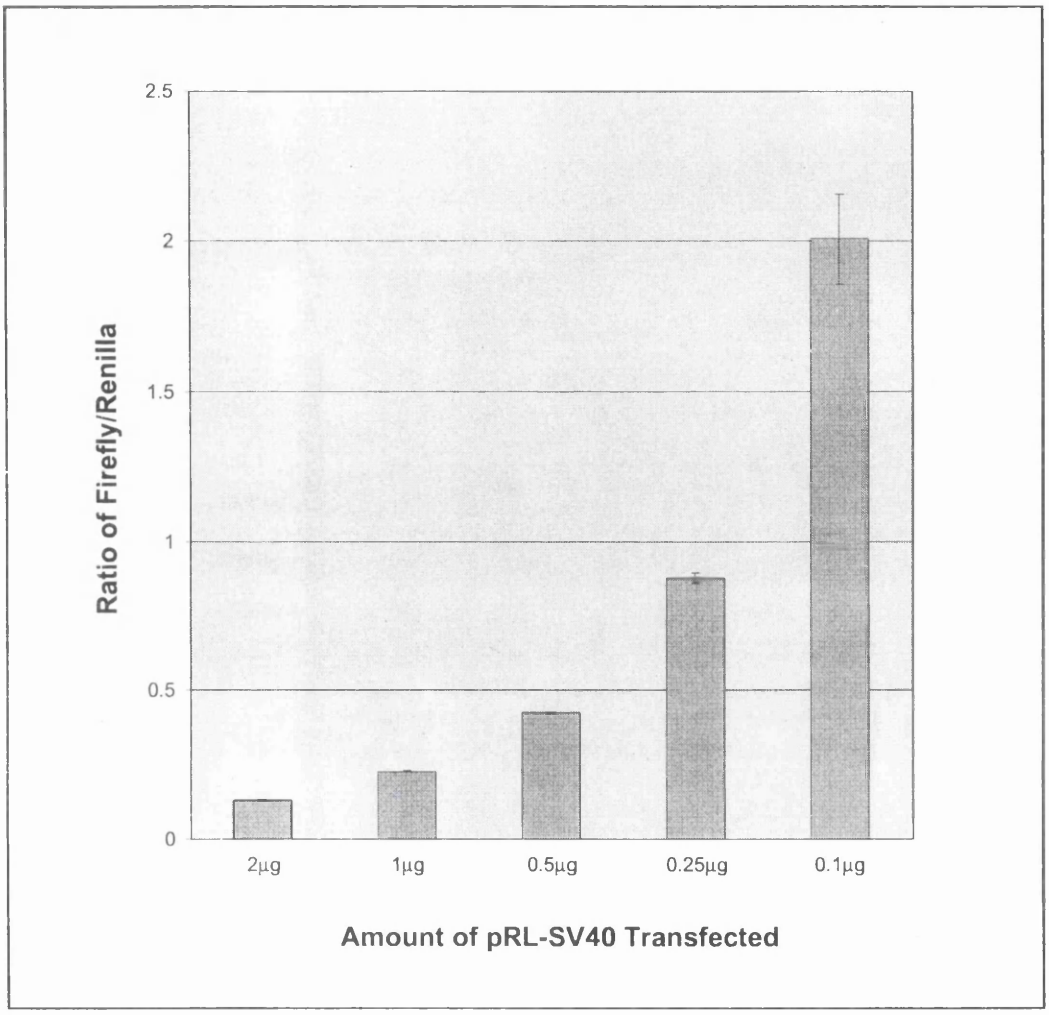


Figure 4.2.3.2 *Renilla* Luciferase activity vs. Amount of pRL-SV40 transfected

Cells were transfected with 2µg of pGL3-Control and varying amount of pRL-SV40 plasmid DNA, incubated for 24 hours and harvested before Firefly and Renilla luciferase activities were assasyed. The graph shows the means of three separate transfection, each of which was measured in triplicate, and the standard deviations of those means.

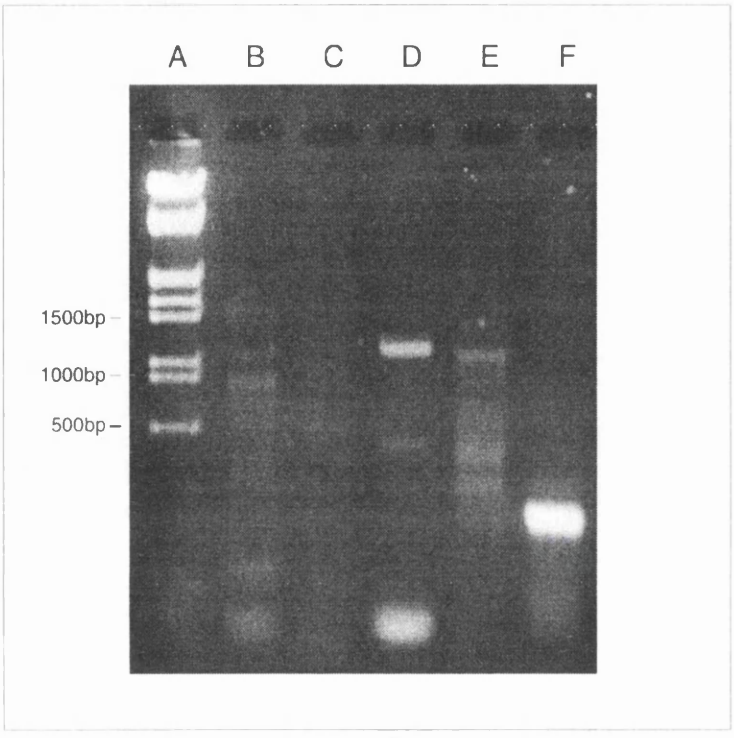


Figure 4.2.4.1 PCR of IL-6/TNF-α Promoter Sequences

A PCR reaction was performed using a template of genomic DNA isolated from untreated U937 cells. Primer pairs for the promoter sequences of IL-6 (Lanes B and C) and TNF-α (Lanes D and F) were used against this. Lane F shows the IL-6F positive control.

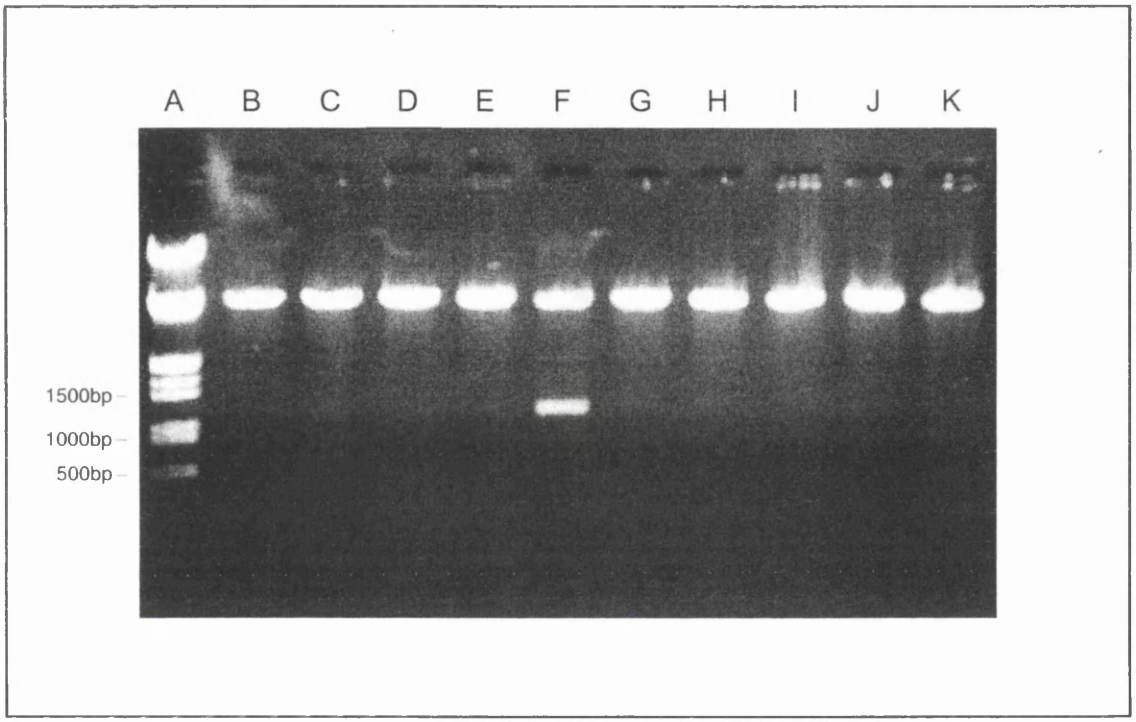


Figure 4.2.4.2 Miniprep Digest of pcDNA3.1-TNFP Plasmid Construct

Gel-purified TNFP PCR product was subcloned into pcDNA3.1/V5-His-TOPO vector and miniprep cultures were generated from this construct. Lanes B-K show 10 of these plasmid DNA preps digested with *Eco*RI.

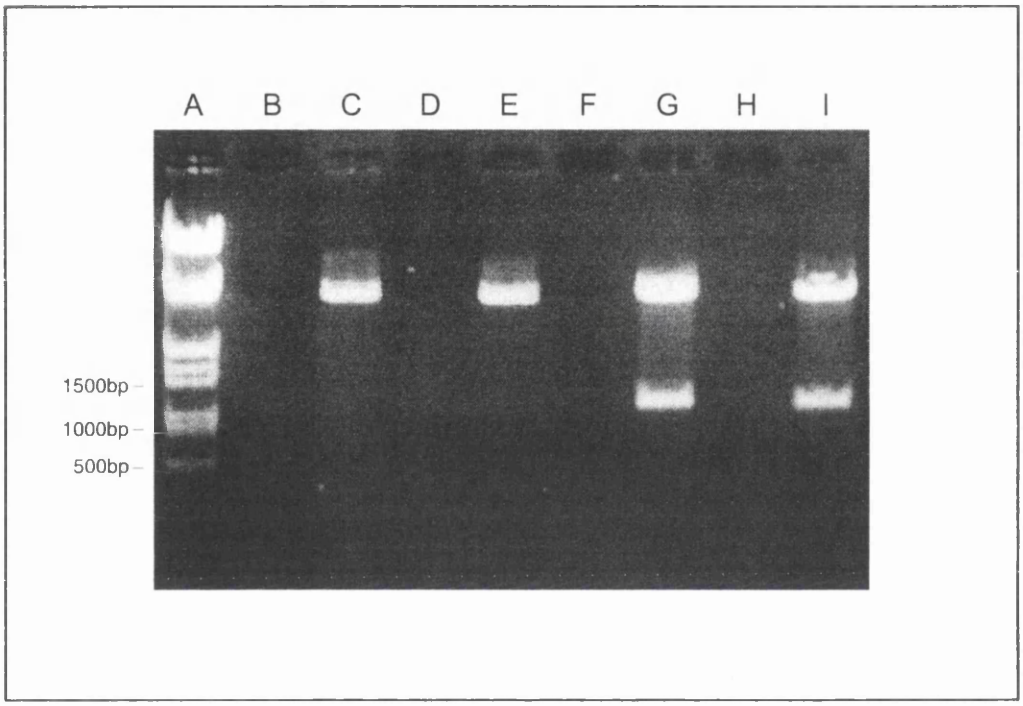


Figure 4.2.4.3 Digest of pGL3-Basic and pcDNA3.1-TNFP Plasmids

The plasmids pGL3-Basic and pcDNA3.1-TNFP were digested with the restriction enzymes *Asp*718 and *Xho* I.

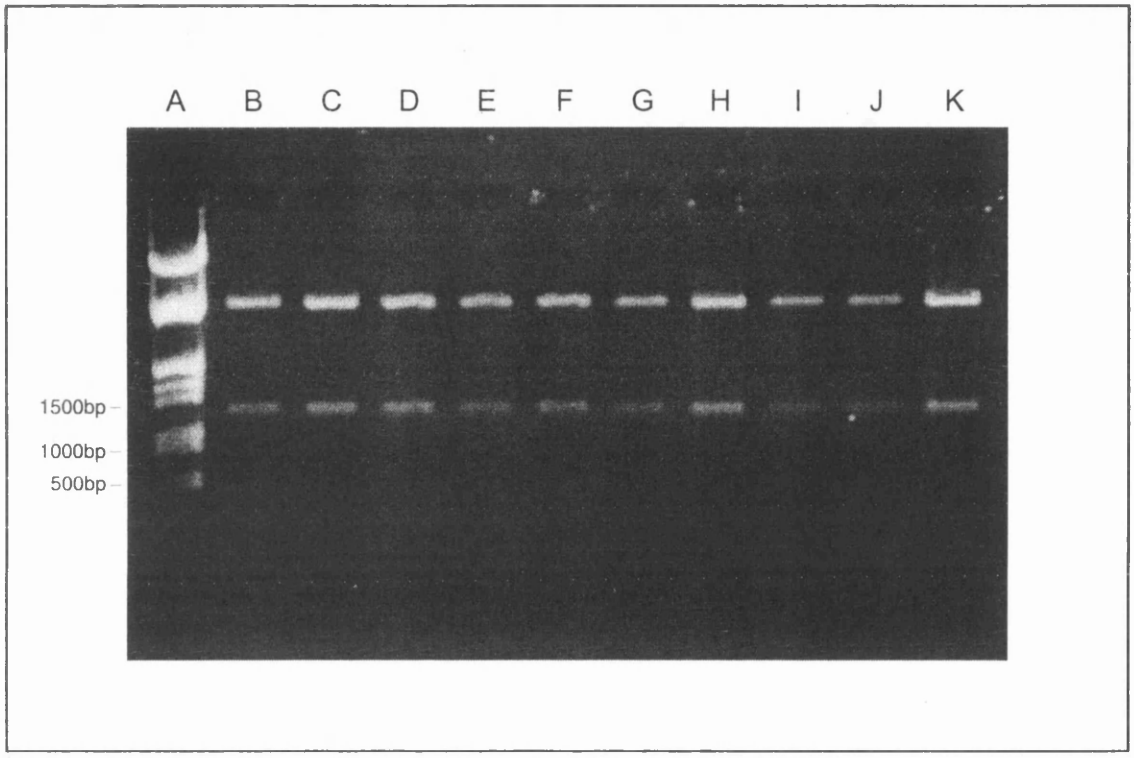


Figure 4.2.4.4 Digest of pGL3-TNFP Plasmid DNA

The TNFP fragment was ligated into the multiple cloning site of the pGL3-Basic luciferase expression plasmid. This construct was used to transform bacteria and miniprep cultures were grown from these cells before the plasmid was purified from them and digested with *Asp*718 and *Xho* I to excise the promoter fragment. Lanes B-K show ten of these digests.

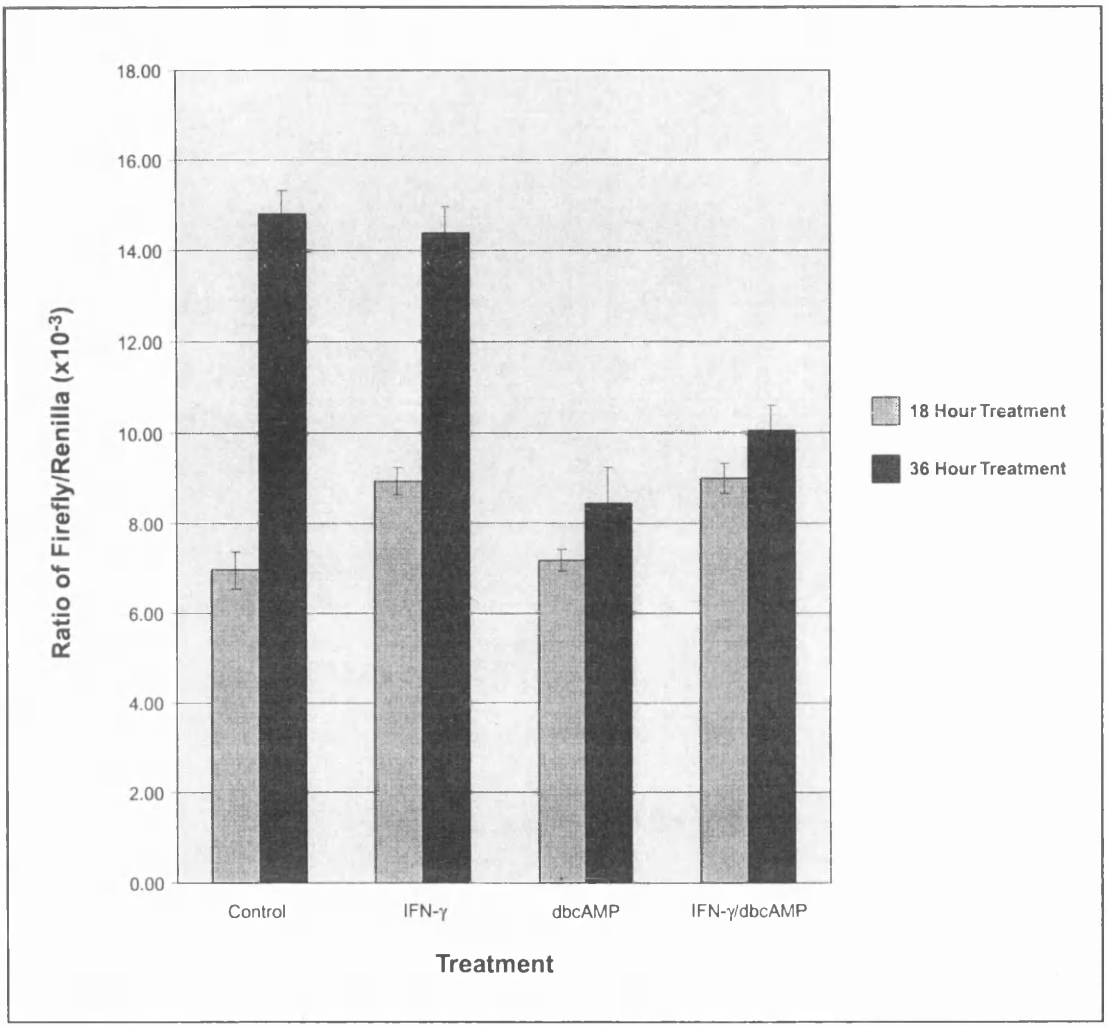


Figure 4.2.5.1 pGL3-TNFP Response to IFN-γ/dbcAMP Treatment

Cells were transfected with 2μg of pGL3-TNFP and 2μg of pRL-SV40 plasmid DNA then treated with IFN-γ and/or dbcAMP before incubation for 18 and 36 hours. The cells were then harvested and Firefly and Renilla luciferase activities were assayed. The graph shows the mean values of three separate transfections, each measured in triplicate, and the standard deviations of the means.

4.3 Discussion

This chapter describes work carried out to create a reporter gene assay for the study of the gene regulation of cytokine promoters. The work was successful in generating a reporter plasmid construct consisting of the TNF-α promoter coupled to the firefly luciferase cDNA.

4.3.1 Single Luciferase Assays

The U937 cell line had been used extensively in the lab prior to this work, but had never been successfully transfected with DNA. The initial transfections, which were carried out using the pGL3-Control plasmid was the positive control and the pGL3-Basic plasmid was the baseline for no promotion of gene expression (although the gene itself was present), showed that the transfection reagent used was successful in transfecting the cells. The technique involved a one-step addition of the DNA-reagent mix and observation of the culture of cells after this did not reveal a large toxic effect in terms of the number of viable cells left after the incubation period. The expression of the luciferase protein was easily detected with the activity of the positive control generally being 3 to 4 orders of magnitude greater than that of the negative.

While it was encouraging to find the reporter system working well from the outset, it was important to more closely characterise the expression of the enzyme to provide the data needed to plan effective experiments in the future. It was unlikely that the cytokine promoters would generate the same kind of expression as the SV40 promoter/enhancer combination of the pGL3-Control plasmid, so it may have been necessary to increase (or decrease) the amount of DNA transfected to produce enough active enzyme to be measured accurately and easily. The measurement of activity over a range of amounts of DNA (**Figure 4.2.2.1**) showed that the amount of DNA chosen (2μg)

was adequate to give a high activity, that half this amount still gave acceptable values and that there was a limit as DNA transfected increased beyond which the activity declined sharply. This decline in activity was not accompanied by a decline in cell vigour as observed through the microscope, so the reason may have been a more subtle toxic effect of the DNA or transfection reagent.

The incubation time was also a crucial factor when considering future experiments which would involve treatment with IFN-γ and dbcAMP followed by incubation periods to allow activation or differentiation to occur and cross-linking of Fc receptors, which would again require an incubation period to allow cellular activation to transpire. The activity dropped off steadily from 24 hours of incubation so it would be useful for subsequent work to allow this time period of incubation between transfection and harvest. However, the activity after 72 hours, although just over 6-fold less than after 24 hours, was still orders of magnitude higher than the baseline and so it would be possible for incubation periods of this length to generate useful data.

4.3.2 Dual Luciferase Assays

To control for transfection efficiency when measuring and comparing the levels of firefly luciferase activity in separate transfections, an internal control, the *Renilla* luciferase pRL-SV40 plasmid, was used. This was co-transfected with the firefly luciferase plasmid and measured using the Stop and Glo dual luciferase system.

Initial experiments showed the Firefly and *Renilla* plasmids could be co-transfected and expressed successfully in the U937 cell line. The fact that there was also a linear relationship between the amount of *Renilla* plasmid DNA transfected and the light intensity measured (see **Section 4.2.3.2**) suggested the pRL-SV40 plasmid as a good choice for an internal control versus the

pGL3-Promoter plasmid.

4.3.3 Construction of the Promoter-Luciferase Plasmid Constructs

The promoter of a gene is only present at the level of nuclear genomic DNA, with only one copy available for amplification in each cell. However, unlike the amplification of cDNA derived from cell mRNA the activation state of the cell in this case should have little bearing on the subsequent PCR amplification if the genomic DNA is correctly purified from a culture of cells. Therefore, the major variable in the success of such an amplification is the primer pair used. To provide a greater chance of amplifying the desired length of promoter sequence, two sets of forward and reverse primers were designed for each of the promoters. This provided four combinations of forward and reverse primers to use in each promoter sequence amplification. The result of numerous reactions, with variations in all the parameters, was a promoter sequence for TNF-α successfully amplified but no sequence from the IL-6 promoter. Given that genomic DNA *in vivo* is closely associated with histone proteins in a complex, 3-dimensional arrangement, it is possible that new secondary and tertiary structures could be created when the DNA is separated from the protein during purification. This new conformation may even persist to some extent when the genomic DNA is denatured during the PCR cycle. This could explain the lack of any significant amplification product in any of the IL-6 promoter PCR reactions.

The design of the primer sets for the PCR reactions also had a bearing on the subsequent subcloning protocol used to create the complete Luciferase-Promoter construct. Two approaches were considered. The first, which was eventually utilised, involved the two-step, two-vector protocol described in **Section 4.3.4**. The second approach involved the modification of sequence at the termini of the promoter fragment. The insertion of the promoter sequence into the multiple

cloning of the pGL3-Basic plasmid required that the ends of the promoter featured complementary overhangs, in the correct orientation, to the restriction sites available on the plasmid. Analysis of the promoter sequence revealed, as expected, that the termini of the hypothetical fragment did not contain these restriction sites. Therefore, primers were designed which, although mostly complementary to the genomic DNA, contained nucleotide changes that would introduce the required restriction sites into a subsequently amplified PCR fragment.

However, several preliminary PCR reactions showed no evidence that the primers could be mutated in this way and still retain the desired level of affinity for the specific sequences in the promoters. Therefore the two-step process was chosen even though this would introduce additional sequence from the pcDNA3.1 vector at either end of the promoter. It was considered that this would not inhibit the function of the promoter in regulating expression of the luciferase gene to an extent that would limit the plasmid's experimental uses, given that the multiple cloning site of the pGL3-Basic plasmid would itself introduce intervening nucleotides.

4.3.4 Experiments with the pGL3-TNFP Plasmid

The pGL3-TNFP plasmid was used in only one preliminary experiment during the work covered by this thesis. The U937 monocyte/macrophage differentiation system requires treatment of the cells with either IFN-γ or dbcAMP to give the desired phenotypes. Therefore, before the effects of cross-linking and activating FcγRI on the expression of luciferase through the TNF-α promoter could be measured, the baseline changes in expression due to the treatments had to be assessed.

The result of this experiment (**Figure 4.2.5.1**) suggested that the TNF-α promoter was down-regulating the expression of luciferase as the U937 cell was differentiated to a more macrophage-

like phenotype, while IFN-γ had no effect on the promoter individually and did not alter the effect of the dbcAMP when used to co-treat the cells with it. What this suggested for future experiments where FcγRI was activated was that in the IFN-γ-treated cells the process of activation itself would not contribute significantly to any up- or down-regulation in expression of the luciferase gene and therefore any results would not be complicated by the effect of this variable. The same would not be true in the dbcAMP-differentiated cells but if parallel controls were performed to measure the contribution of differentiation on expression this variable would not obscure any effects the FcγRI was having on the expression of the gene.

Chapter 5: Conclusion

5.1 Conclusion

This thesis describes the work undertaken in the design and development of two assay systems intended for use in measuring gene expression downstream of activation of the high affinity Fc receptor for IgG, a key component of the immune system. The main objectives that were achieved are summarised below:

- Lengths of sequence from the 3'-untranslated regions of the human TNF-α and IL-6 mRNAs were amplified by RT-PCR to generate DNA fragments, which were subcloned into plasmid vectors
- An internal PCR methodology was used to delete regions from these fragments and subsequent subcloning of the fragments created plasmid constructs for use in quantifying mRNA by competitive PCR
- The accurate quantification of DNA was demonstrated in primciple using the pair of TNF-α fragment plasmids
- U937 cells were transfected with two types of luciferase reporter gene and expression of these genes was demonstrated through measurement of light given off by their enzymic action
- The promoter sequence of the TNF-α gene was amplified by PCR and inserted as the regulatory sequence in a luciferase expression vector
- The promoter-reporter construct was used to investigate the effect induced differentiation of the U937 cell line had on TNF-α expression

In essence, this work is the first stage in investigating the relationship between FcγRI and the TNF-α and IL-6 genes. The process of developing an assay by applying a number of basic

molecular tools (the enzymes used to perform RT-PCR, the luciferase expression vectors) and information (nucleic acid sequences for the genes and mRNAs of TNF-α and IL-6) to an *in vitro* cell model such as the U937 monocyte/macrophage system was one which demonstrated the unexpected complexities one has to contend with when approaching biological research. However, the nature of the problems encountered meant that those complexities were investigated and understood to a high degree, an important benefit when proceeding forward using these tools.

In terms of future work, I see these two assays as ways in which to investigate the final stage in translation of information from extracellular signal to the ultimate cellular response: the expression of signal molecules which would provide the next step in the wider co-ordinated immune response. Previous work cited in this thesis (Melendez *et al*, Davis *et al*, Harrison *et al*) has described in detail the complex and novel way in which FcγRI operates at the centre of the immune system, linking the humoral and cellular arms together. Utilisation of these assays would allow the nuclear events triggered by the FcγRI-activated signalling cascades to be elucidated and thus provide an unbroken description of the signal through the macrophage and monocyte cell types.

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