Controlled gene expression using acute phase response elements

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by

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Declaration

This thesis is the original work of the author except where otherwise stated.

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Previous work in our laboratory involved the creation of an inducible gene expression system based on the promoter of the major acute phase protein in humans, C-reactive protein (CRP). The aims of this project were to further characterise the CRP based expression vector and to modify it for use in pigs. The green fluorescent protein (GFP) was also evaluated as a reporter of transient inducible gene expression.

The 30kb fragment containing the human CRP gene was sequenced and analysed for the presence of elements that may be responsible for the low basal levels of expression of the gene and for sequences that are responsible for the sexually dimorphic pattern of expression of the human CRP gene in transgenic mice.

It was planned to use the information from these analyses to modify the expression vector for use in pigs. However, because it is not currently known how the CRP-based acute phase expression vector would behave in other species, the promoter of the major acute phase protein in pigs, ITIH4, was isolated. Due to the absence of homology between the pig ITIH4 promoter and the human CRP promoter, the pig ITIH4 promoter was further characterised.

The investigations focused on the inducibility of the promoter in response to cytokine stimulation and the effect modification of the promoter would have on promoter activity and inducibility. It was found that the promoter was induced by IL-6 but not IL-1 (except at low concentrations). A combination of IL-1 and IL-6 was shown to result in a decrease in the inducibility of the construct by IL-6. Mutation of the promoter in order to decrease the basal level of expression and enhance inducibility was unsuccessful.

In order to develop a system that will facilitate studies of inducible gene expression *in vitro* a destabilised variant of GFP was evaluated as a reporter gene. Although small increases in fluorescence intensity could be detected following stimulation, the analyses suggest that GFP is not as sensitive as other reporter genes for studying inducible gene expression.

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Finally, a special thanks to my parents, sisters, family and friends for being so understanding and supportive over the last three years.

Abbreviations

A	Adenosine
ANOVA	Analysis of variance
APC	Antigen presenting cell
ASP	Antisense promoter
BAC	Bacterial artificial chromosome
BBC	British Broadcasting Cooperation
BCG	Bacille Calmette-Guerin
bp	Base pairs
BSA	Bovine serum albumin
°C	Degrees Celsius
C	Cytosine
CAT	Chloramphenicol acetyl transferase
cDNA	complementary DNA
C/EBP	CCAAT/enhancer binding protein
cm^2	Centimetre squared
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CRP	C-reactive protein
CSF	Classical swine fever
Da	Dalton
dATP	2'-deoxyadenosine 5'- triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dNTP	2'-deoxynucleoside 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dH_2O	Distilled water
DIG	Digoxigenin
DIG-POD	Digoxigenin peroxidase
DMF	Dimethylformaldehyde
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid

ECACC	European Collection of Animal Cell Cultures
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme linked immunoabsorbant assay
EMSA	Electromobility shift assay
ES	Embryonic stem
EU	European Union
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
G	Guanine
x g	Acceleration in the earth's gravity field
GCG	Genetics Computer Group
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
h	Hours
HCl	Hydrochloric acid
hCRP	human C-reactive protein
HNF	Hepatocyte nuclear factor
IgA	Immunoglobulin A
IFNγ	Interferon-γ
IHRP	Inter-α-trypsin inhibitor related protein
IL	Interleukin
IPTG	Isopropylthiogalactoside
ITIH4	Inter-α-trypsin inhibitor heavy chain 4
ITN	Independent Television News
KAc	Potassium acetate
kb	kilobase
KCl	Potassium chloride
kDa	KiloDalton
kg	Kilogram
LF-A1	Liver factor A1
LINE	Long interspersed nuclear element
LPS	Lipopolysaccharide
M	Molar

MAP	Major acute phase protein
MCS	Multiple cloning site
mg	Milligram
MHC	Major histocompatibility complex
min(s)	Minute(s)
ml	Millilitre
mM	Millimolar
mm	Millimetre
mRNA	messenger RNA
nm	Nanometer
NF-κB	Nuclear factor-κB
NF-IL-6	Nuclear factor-interleukin-6
NS	No stimulation
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	A measure of the acidity of a solution
pmol	Picomoles
PolyA	Polyadenylation
PRRSV	Porcine reproductive and respiratory syndrome virus
QTL	Quantitative trait loci
RE	Responsive element
RNA	Ribonucleic acid
Rnase	Ribonuclease
ROI	Region of interest
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
sec	Seconds
SLY	Suilysin
spi	Serine protease inhibitor
STAT	Signal transducer and activator of transcription
SV40	Simian virus 40

T	Thymidine
TNF-α	Tumour necrosis factor - α
Tris	2-amino-2-(hydroxymethyl)1,3-propandiol
U	Units
μg	Microgram
UK	United Kingdom
μl	Microlitre
US	United States
UV	Ultraviolet
V	Volts
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Introduction

Chapter 1: Introduction

In the last 50 years there has been a large increase in animal production and animal densities (Knap and Bishop, 2000). This means that in areas where farming is intense infections can spread rapidly from one animal to another, and it is often necessary to slaughter large numbers of healthy animals just to prevent the spread of disease. Recent examples of this scenario include the outbreak of foot and mouth disease in the UK in 2001, which rapidly spread throughout the country and necessitated the culling of 6 million animals (ITN News Report). Another example is the outbreak of classical swine fever in the Netherlands in 1997, which lasted for over a year and during which, an estimated 10 million healthy pigs were killed to prevent the spread of infection (Stegeman *et al.*, 2000). The outbreak of foot and mouth disease in the UK in 2001 is estimated to have cost tax payers more than £2.3 billion (BBC News Report) and it is estimated that recent outbreaks of classical swine fever in several EU countries have amounted to several billion Euro (Moennig, 2000; Saatkamp *et al.*, 2000; Stegeman *et al.*, 2000).

In developing countries the economic impact of parasitic infections is reflected in a loss in the production potential of livestock. Climatic conditions in developing countries provide ideal conditions for parasite development and as a result parasitic infections in livestock in these countries are often endemic. Many livestock production systems in these countries are characterised by animals with decreased susceptibility to many endemic parasitic infections but with poor productivity performance (for example, lower outputs of meat, milk, hides, skins) (reviewed by Perry and Randolph, 1999). Ngategize *et al.* (1993) calculated that ovine fascioliasis in the highlands of Ethiopia resulted in a financial loss of US$23 million annually.

Antibiotics are used extensively in the livestock industry, not just as a means of controlling infection but also as growth enhancers. However, there is concern that strains of bacteria resistant to antibiotics used in animals (particularly those antibiotics that are also used in human medicine) could transfer this resistance to

human pathogens. Moreover, antibiotics are not effective against viral infections. The other method of controlling infection currently in use is vaccination. The aim of vaccination is to provide the animal with protection against infectious disease before it encounters the infectious organism. Vaccination, if successful, can also be used as a means of controlling the spread of infectious disease and eventual elimination of the pathogen from the population. However, in spite of the fact that there have been some very successful vaccination programs, it remains that for many diseases no vaccine is available, the available vaccine does not provide complete protection (e.g. Meng, 2000) or when a vaccine is available it is not desirable to use it for economic reasons (for example, foot and mouth vaccine, classical swine fever vaccine). Infectious disease remains an important threat to livestock production and as a result there is a need to continue to develop and investigate new control strategies.

The introduction to this thesis will first of all describe how pathogens cause disease. Understanding the stages of pathogenesis is important as each stage offers the opportunity to block the development of disease. I will then describe the current means for controlling infection, with a particular emphasis on vaccination. Finally, I will discuss novel approaches to increasing animal resistance to infectious disease using transgenic technology.

1.1 The stages of pathogenesis

Pathogenesis is defined as the mechanism of disease development. Pathogenesis can be caused in a number of different ways (discussed by Singleton, 1999) as it is dependent on the causative agent – some pathogens produce toxins or other substances that destroy tissue or disrupt physiological processes, others invade particular cells or tissues. The interactions between the host and the pathogen are also believed to play an important role in pathogenesis. In some diseases, the immune system itself can play a part in cellular and tissue damage (Bergeron *et al.*, 1998; Thanawongnuwech *et al.*, 2001). Pathogenesis is a multi-stage process and requires the pathogen to breach a number of host defences. Understanding the

stages of pathogenesis is important as it not only informs us of how pathogens cause disease, it also provides us with the opportunity to develop methods to halt the development of disease. The various stages of pathogenesis will be discussed in the following sections. This discussion will be limited to infectious diseases of humans and swine.

1.1.1 Route of infection

The skin is usually an effective barrier to infection. However, if the skin is broken, this provides a way for pathogens to enter the body. The mucous membranes of the respiratory, intestinal and genitourinary tracts are also susceptible to infection and infectious disease often begins at these sites. To cause disease, the pathogen must first enter the body. Many infectious diseases, particularly those of the respiratory tract, can be spread in aerosol droplets. This allows infection to spread rapidly from one animal or person to another (Jernigan *et al.*, 1996; Jobert *et al.*, 2000; Lever *et al.*, 2000; Elbers *et al.*, 2001). Another means of infection is the ingestion of infected food. For example, swill feeding to pigs is believed to be a major source of infection (Horst *et al.*, 1996; Fritzemeier *et al.*, 2000). Therefore, one area of current research is the conditions necessary for survival of pathogens. The classical swine fever virus, for example, has been shown to be capable of surviving in frozen pork for more than four years and that only pasteurisation or cooking is capable of killing the virus (Edwards, 2000). On the other hand *Mycobacterium tuberculosis* has been shown to have a poor aerosol survival rate over a period of one hour (Lever *et al.*, 2000). Understanding the route of infection and the conditions for the survival of the pathogen provide the first opportunity to block the development of disease.

1.1.2 Adhesion

The cells of the respiratory, intestinal and urogenital tract are covered with mucous secretions. These secretions are moved through their respective tracts by peristalsis. Therefore it is important that the pathogen is able to adhere to the cells of these

tracts in order to prevent it from being swept along and eventually being expelled from the body. Bacterial adherence is dependent on two factors: appendages on the bacterial cell surface and host cell receptors.

Appendages on the bacterial cell surface that allow binding to host cells are called adhesins. Bacterial adhesins are often fimbriae. Fimbriae are long protrusions built from protein subunits that mediate binding to mammalian cells and non-living surfaces. The fimbriae of *Escherichia coli* were reviewed recently by van den Broeck *et al.* (2000). It is believed that fimbriae are responsible for the fact that some strains of *E. coli* can only cause infections in some species or colonise a particular part of the intestine. Strains of *E. coli* possessing the K88 antigen have been implicated in porcine neonatal and postweaning intestinal infections (Jones and Rutter, 1972). The K88 antigen allows the organisms to attach and colonise the lower small intestine (Blomberg *et al.*, 1993; reviewed by Jin and Zhao, 2000). The infection then spreads to the proximal part of the intestine (reviewed by van den Broeck *et al.*, 2000). A marked variation in the susceptibility of pigs to infection by K88+ *E. coli* strains has been observed (Rutter *et al.*, 1975). For example, the Chinese Meishan breed of pig has increased resistance compared to the Minzhu breed (Michaels *et al.*, 1994). This difference may be due to the inability of the bacteria to adhere to the appropriate receptor (three antigenic variants of the K88 fimbriae have been described and they bind to different receptors on the piglet intestinal epithelium) or the absence of the receptor in the porcine intestine. The presence of receptors for the K88 fimbriae in the porcine intestinal epithelium has been reported to be age-dependent (Conway *et al.*, 1990; Willemsen and deGraf, 1992) and explains why neonatal and post-weaning pigs are most susceptible to infection and disease by these strains of *E. coli*.

Preventing adhesion of the pathogens to the mucosal surface is one way of preventing the pathogenic process. Oral immunisation of piglets containing a receptor for K88 has shown that they were protected against a challenge with virulent K88+ *E. coli* (van den Broeck *et al.*, 1999a) although this protection is

dependent on the presence of a receptor for K88 (van den Broeck *et al.*, 1999b). In addition, egg-yolk antibodies obtained from hens immunized with fimbrial antigens from enterotoxigenic K88+ *E. coli* were capable of protecting neonatal and early weaned pigs against enterotoxigenic *E. coli* infection (Marquardt *et al.*, 1999).

It should be noted that fimbriae are not the only adhesins bacteria possess. *Bordetella* species use a filamentous haemagglutinin and pertactin for adhesion (reviewed by Brennan and Shahin, 1996). While fimbriae could be demonstrated on some isolates of *Actinobacillus pleuropneumoniae*, lipopolysaccharide (LPS) is believed to be the major adhesin involved in adherence to porcine respiratory tract cells (Bélanger *et al.*, 1990; 1994). The capsular hyaluronic acid of *Pasteurella multocida* has also been implicated as an important adhesin (Esslinger *et al.*, 1994; Pruimboom *et al.*, 1996). In summary, it is believed that there are multiple adhesins capable of interacting with different receptors and that these may be used in a stepwise fashion during colonisation (Jacques and Paradis, 1998).

1.1.3 Invasion of mammalian cells

The pathogenesis of some infectious disease is caused by the ability of organisms to invade mammalian cells. The ability to invade and survive in mammalian cells protects the pathogen from the host's immune response and may allow it to disseminate. Some authors have shown that virulent *Streptococcus suis* (a causative agent of meningitis in pigs) are capable of invading epithelial cells of human origin (Norton *et al.*, 1999). In addition, intracellular bacteria have been found in circulating monocytes of pigs (Williams and Blakemore, 1990; Busque *et al.*, 1998) and it has been suggested that this is how *S. suis* disseminates and reaches the blood-brain barrier. However, it is still not clear how *S. suis* breaches the blood-brain barrier. While high levels of bacterial adhesion to phagocytic cells have been observed, other authors reported that the bacteria were not ingested (Segura and Gottschalk, 1999; reviewed in Gottschalk and Segura, 2000). Moreover, no invasion of brain microvascular epithelial cells has been observed although it has been

suggested that the secretion of toxic factors could affect the endothelial cells resulting in an increase in the permeability of the blood-brain barrier (Charland *et al.*, 2000).

One type of cell that is particularly prone to invasion and has been extensively investigated is the M cell (membranous epithelial cell). M cells transport antigens across mucosal epithelia to the underlying lymphoid tissues where protective immune responses are generated (reviewed by Jepson and Clark, 1998). In pigs, the follicle-associated epithelium of the terminal ileum is composed entirely of M cells (Jepson and Clark, 1998). M cells are targeted by bacterial species capable of intracellular survival, such as *Salmonella*. Although the major function of M cells is to activate an immune response at mucosal surfaces, in a non-immune host it may take up to a week for sufficient IgA to accumulate. By this time the invading organism may have already spread to other organs or invaded macrophages (Patrick and Larkin, 1995).

1.1.4 Evasion of the immune system

The immune system is the body's defence against infectious disease. The immune system can be divided into two groups. The innate immune response is an early non-specific defence mechanism which acts by changing the environment of the host and attacking the invading organism while the slower reacting but more specific acquired immune response develops (reviewed by Fearon and Locksley, 1996). The acquired immune response is responsible for life-long immunity to infectious disease. These two branches of the immune system will be discussed in the following sections.

1.1.4.1 Innate immunity

The aim of the innate immune response is to destroy the pathogen, determine the location and extent of the challenge and facilitate the acquired immune response.

The innate immune response employs a number of different strategies to deal with invading organisms. This non-specific immune response is triggered when receptors expressed primarily on the surface of monocytes and macrophages (but also found on dendritic cells and B cells among others). These receptors, known as Toll-like receptors (reviewed by Akira, 2001), are responsible for the expression of acute phase reaction products including inflammatory cytokines (Akira *et al.*, 2001). One of the effects of the release of the inflammatory cytokines, which include interleukin-1 and interleukin-6, is induction of the acute phase response (discussed in section 1.1.4.1.1), which is one of the early stages of the inflammatory response. A summary of the non-specific defence mechanisms is as follows:

- Phagocytosis – the body contains specialised cells that are involved in the ingestion and destruction of "foreign" material, such as microorganisms. These cells are known as phagocytes and include macrophages.
- Opsonisation – opsonisation of bacteria, e.g. by complement or antibodies, can make them more susceptible to ingestion by macrophages (e.g. Rozenberg-Arska *et al.*, 1986; McKinlay *et al.*, 1993). The importance of opsonisation as a defence mechanism has been shown by studies in which defective opsonisation function has resulted in recurrent reinfection (Baker *et al.*, 1993).
- Complement – complement consists of a number of different proteins that become activated in a sequential manner. The complement 'cascade' can be activated in a number of ways. Gram negative bacteria can trigger the alternative complement pathway (as their outer membrane contains lipopolysaccharide) and antibody-antigen complexes can activate the classical pathway. C-reactive protein, a constituent of the acute phase response has also been reported to be capable of activating complement (Mold *et al.*, 1999) and linkage between complement and development of the acquired immune response (reviewed by Carroll and Prodeus, 1998). A number of the components of the complement cascade have physiological functions, e.g. C3b increases the binding of a bacterium to a phagocyte (enhances phagocytosis); C5a is a chemoattractant and attracts cells of the immune system to an infected site; the C5b6789 complex can cause lysis of Gram-negative bacteria. The complement

system is believed to play an important role in protection against infection from *Neisseria meningitidis*. Individuals with defects in the terminal complement components have been shown to be at increased risk of invasive disease (Ross and Densen, 1984; Sjohölm, 1990; Figueroa and Densen, 1991; Mayatepek *et al.*, 1993; Fijen *et al.*, 1999).

- Inflammatory response – the inflammatory response involves the release of a number of signalling molecules, including the release of histamine which increases the permeability of small blood vessels. Cytokines are also released during the inflammatory response. These cytokines have pleiotrophic effects, including activation of macrophages, increasing the binding of leukocytes to endothelial cells, allowing them to pass between endothelial cells to the affected tissue and activation of the acute phase response which is discussed in the next section.

1.1.4.1.1 The acute phase response

The acute phase response is a systemic reaction induced following events such as infection, inflammation or tissue injury. It is characterised by changes in the serum levels of several plasma proteins, termed acute phase proteins. A large number of behavioural, physiological, biochemical and nutritional changes also occur (reviewed by Gabay and Kushner, 1999). These changes include fever, anorexia, somnolence and lethargy, anaemia in chronic disease, hypoferremia, cachexia and alteration of intracellular hepatic constituents. All of these changes are a result of and regulated by inflammation-associated cytokines. A simplified diagram of the acute phase response is illustrated in Figure 1.1.

The acute phase response changes the normal homeostatic environment of the organism and it is presumed that these changes contribute to defensive or adaptive capabilities (Gabay and Kushner, 1999). Indeed, several studies have reported the ability of acute phase proteins to provide protection against bacterial infection in mice (Mold *et al.*, 1981; Yother *et al.*, 1982; Vogels *et al.*, 1993; Szalai *et al.*, 1995;

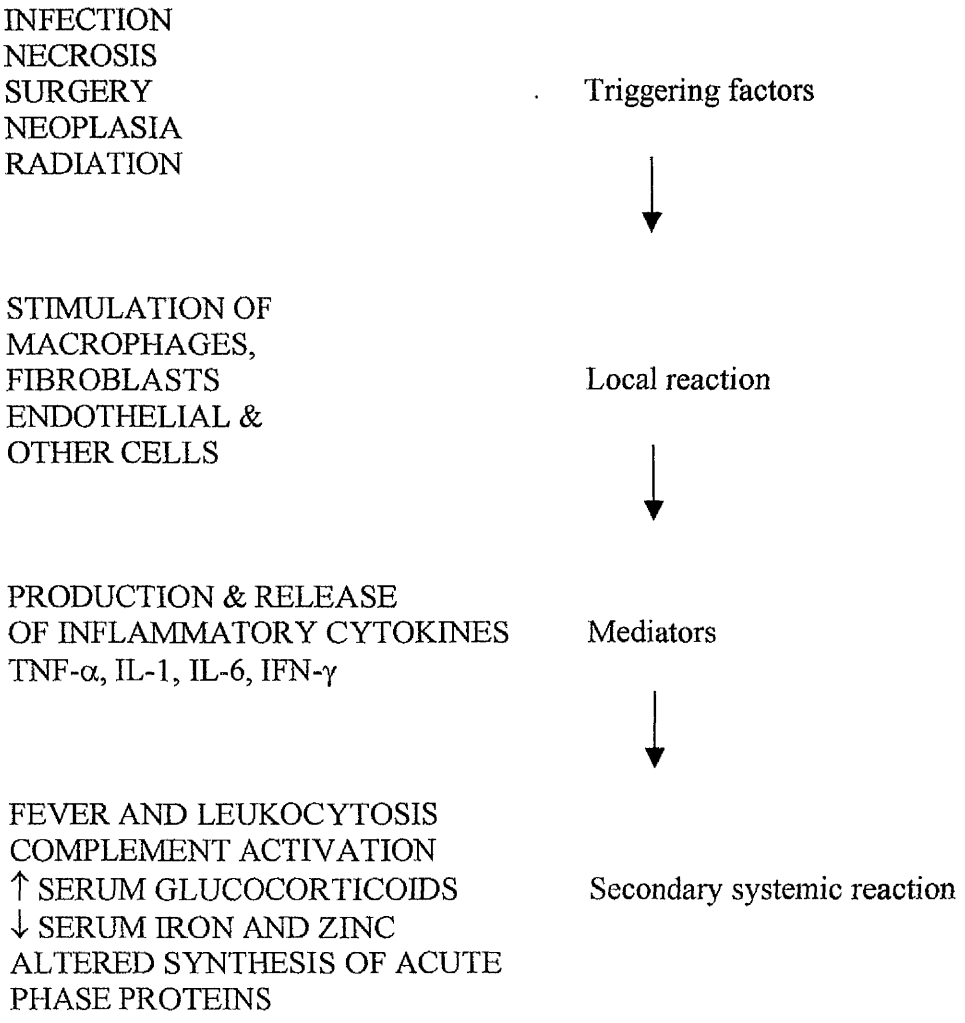


Figure 1.1: Simplifed cascade of the acute phase response (Koj, 1996)

Hochepied *et al.*, 2000). The changes in the synthesis of the acute phase proteins reflect the presence and intensity of inflammation and they have long been used as a clinical guide to diagnosis and management (Gabay and Kushner, 1999). In this regard, there is also immense interest in veterinary medicine in using the acute phase response as a diagnostic tool and indicator of infection.

1.1.4.1.2 Acute phase proteins

The main characteristic of the acute phase response is the change in the plasma concentration of a number of proteins synthesised by hepatocytes can be either increased or decreased during the acute phase response. Clinically, the term acute phase protein is reserved for proteins whose plasma concentration is at least 50% higher than normal values (Engler, 1988).

Figure 1.2 shows the kinetics of the acute phase response in humans. Normally, acute phase proteins are synthesised at a very low basal level. However, upon induction there is an increase in the serum concentration of these proteins. This is followed by a decrease to pre-induction levels. The level of induction and the length of time taken to return to pre-induction levels vary from protein to protein. At this stage more than 30 proteins have been identified as acute phase proteins (see Gabay and Kushner, 1999). However, the exact physiological function of many of these proteins and their role in the acute phase response is unknown. Some of the major acute phase proteins in humans and their putative functions are described in Table 1.1.

C-reactive protein is the major acute phase protein in humans. Levels of CRP can rise from a basal level of < 1μg/ml to more than 300μg/ml during the acute phase response. Administration of CRP prior to challenge with *Streptococcus pneumoniae* has been shown to provide protection against a lethal challenge (Mold *et al.*, 1981; Yother *et al.*, 1982). CRP is thought to act by binding to phosphorylcholine residues on the pneumococcal surface and thereby function as an opsonin (Mold *et al.*, 1981).

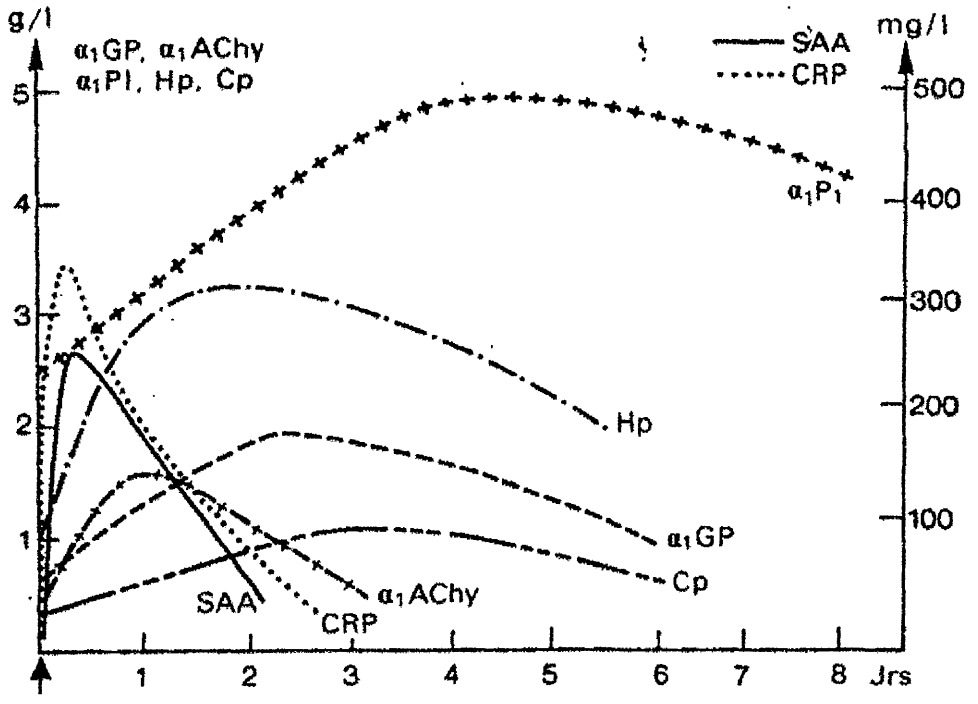


Figure 1.2: Kinetics of the acute phase response (adapted from Engler (1988))

Key: Jrs (French): Days

SAA: Serum amyloid A

CRP: C-reactive protein

α_1 Achy: α_1-antichymotrypsin

Cp: Ceruloplasmin

α_1GP: α_1-acid glycoprotein

Hp: Haptoglobin

α_1P_1: α_1-antitrypsin

Table 1.1: Some of the major acute phase proteins in humans and their functions

Protein	Fold increase/ decrease	Function	References
C-reactive protein	200 – 1000 X increase	Protection against pneumococcal and other bacterial infections. Other functions are described in section 1.4.4.1	Mold *et al.* (1981); Yother *et al.* (1982); Szalai *et al.* (1995,1997,2001)
Serum amyloid A	1000 X increase	Influencing cholesterol metabolism during inflammatory states and causing adhesion and chemotaxis of phagocytic cells and lymphocytes. Has also been shown to have an anti-inflammatory role.	Reviewed by Uhlar & Whitehead (1999)
Haptoglobin	2-5 X increase	Facilitates the removal of haemoglobin by the reticuloendothelial system Host defence response to infection and inflammation	Singh-Majkic (1998) Dobryszycka (1997)
α_1-acid glycoprotein	2-5 X increase	Immunoregulatory functions Inhibition of the transport of molecules through the endothelial layer Protection against Gram negative infections	Costello *et al.* (1979); Bennett & Schmid (1980); Lainé *et al.* (1990); Vasson *et al.* (1996) Haraldsson & Rippe (1987); Muchitsch *et al.* (1996) Hochepied *et al.* (2000)
α_1-antitrypsin	2-5 X increase	Protects the lower respiratory tract from damage by neutrophil elastase	Crystal (1990)

Fibrinogen	2-5X increase	Role in inflammation – generation of a fibrin matrix to prevent blood loss and trap inflammatory cells	Dunn & Willoughby (1981)
		May prevent dissemination of inflammatory agent	Dunn & Simmons (1982) Dvorak *et al.* (1992)
		Endothelial cell retraction	Senior *et al.* (1986) Skogen *et al.* (1988) Francis *et al.* (1993)
		Induction of signalling pathways in endothelial cells	Kowalski (1968); Plow & Edgington (1986); Lorenzet *et al.* (1992)
Albumin		Maintenance of normal oncotic pressure Regulation of tissue fluid distribution Binding of substances e.g. bile acids, heavy metals Metabolism of endogenous substances, e.g.lipids Maintenance of microvascular integrity Anticoagulant	Reviewed by Nicholson *et al.* (2000)
		Inactivation of a small group of compounds	Peters (1996)
		Plasma buffer	Doweiko & Nompleggi (1991)
		Antioxidant	Holt *et al.* (1984)

Although CRP was initially thought to be specific for *S. pneumoniae* it has since been shown that there are phosphorylcholine epitopes on pili of *N. meningitidis* and *Neisseria gonorrhoeae* as well as on a protein of *Pseudomonas aeruginosa* (Weiser *et al.* 1998a). CRP has been shown to bind to the phosphorylcholine residues of *Haemophilus influenzae* (Lysenko *et al.* 2000) and to provide protection when challenged with *H. influenzae* (Weiser *et al.*, 1998b) and *Salmonella typhimurium* (Szalai *et al.*, 2000).

The innate immune response is a short-lived response that attacks the invading organism while the acquired immune response, which is responsible for the life-long protection against an infectious agent that follows infection, develops. The acquired immune response will be discussed in the next section.

1.1.4.2 Acquired immunity

Lymphocytes are the most important cells of the acquired immune response. There are two types, which develop in the bone marrow but mature in different sites. T cells mature in the thymus and B cells in bone marrow. T cells can be divided into a number of different subsets (see Figure 1.3) and have a wide variety of roles, including cytotoxicity and defence against intracellular and extracellular pathogens (reviewed by Chtanova and Mackay, 2001). CD4+ T cells can be further subdivided into Th1 and Th2 cells. These cells differ in the cytokines produced and consequently bias the immune response. For example, cytokines secreted by Th1 cells are involved in defence against intracellular pathogens whereas those secreted by Th2 cells are involved in humoral immunity and the defence against extracellular parasites (reviewed by Chtanova and Mackay, 2001). The reduced ability of neonatal T cells to produce both Th1 and Th2 cytokines has been proposed as the reason for increased susceptibility of neonates to infections such as *Toxoplasma* and herpes simplex virus (Sullender *et al.*, 1987; Wilson, 1991). In addition, Pollard *et al.* (1999b) found that production of IL-10 and IFN-γ by peripheral blood mononuclear cells (PBMCs) from children after a meningococcal infection was age-

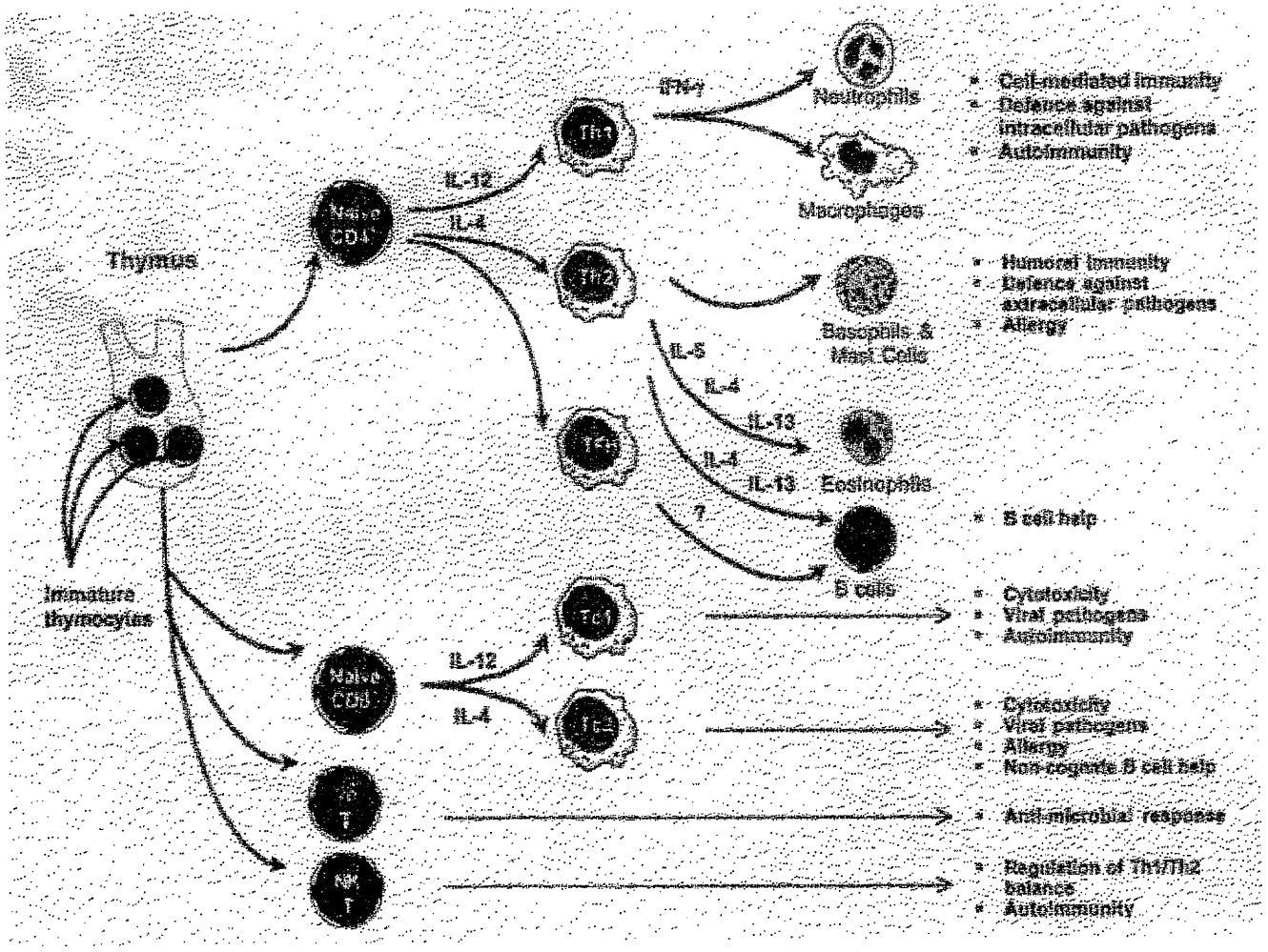


Figure 1.3: Subsets of effector T cells and their functions (Chtanova and Mackay, 2001)

dependent. The balance of cytokine production in youngest children was skewed towards a Th1 response whereas that of older children was skewed towards a Th2 response. PBMCs from adults were also shown to produce more cytokine in response to meningococcal antigens compared to PBMCs from children. Pollard *et al.* (1999b) proposed that the poor efficacy of the serogroup B meningococcal vaccine in young children and the poor bactericidal response to infection in this age group (Pollard *et al.*, 1999a) may be related to impaired function of the cellular components of the immune system.

The acquired immune response is specific for the invading organism and results in the production of antibodies and life-long immunity. To produce antibodies, an antigen (normally a small foreign protein molecule, which is capable of inducing an immune response) is taken up and processed by antigen presenting cells (APCs). APCs include dendritic cells and macrophages. However, antigens from intracellular pathogens can also be processed and displayed on the surface of the infected cell. Antigens derived from extracellular pathogens are displayed to T cells via MHC Class II molecules whereas those derived from intracellular pathogens are displayed via MHC Class I molecules. The T cell binds to the processed antigen and MHC molecule via the T cell receptor. This activates the T cell and it begins to secrete cytokines. Some of these cytokines stimulate B cells that have bound the same antigen and some of these B cells differentiate into plasma cells that secrete antibodies. Some of the activated B cells form memory cells – they no longer secrete antibodies but they are primed so that they will be able to respond rapidly should the organism be exposed to the same antigen again. This is the idea behind vaccination, which will be discussed in section 1.2.3.

In spite of all the sophisticated defence mechanisms the body has available microbes have still managed to devise means of evading the immune response as discussed in the following section.

1.1.4.3 Microbial strategies for evading the immune response

Microbes activate the immune response because some of their surface structures are recognised as being "foreign". The presence of these structures tends to activate the innate immune response making the bacterium susceptible to phagocytosis and complement-mediated cell lysis. Therefore, in order to evade the immune response microbes have developed a number of strategies that prevent their host from recognising them as foreign as well as physical means to make them resistant to the immune response. Physically, Gram-positive bacteria have a thick cell wall and are therefore resistant to complement, as the C5b6789 complex is unable to gain access to the outer membrane to cause lysis. Gram-negative bacteria are more susceptible to complement as their cell wall is much thinner. However, their LPS is believed to prevent access of complement to the outer membrane of the cell by steric hindrance (e.g. Harvill *et al.*, 2000). Bacteria can also avoid complement-induced lysis by binding protectin (CD59), a glycolipid-tailed molecule that prevents lysis of host cells by complement (Rautemaa *et al.*, 1998; Rautemaa *et al.* 2001). In addition, bacteria may encode genes that are associated with increased resistance to complement-mediated killing (Heffernan *et al.*, 1992; Levin and Wessels 1998). Bacteria are also capable of inhibiting the complement cascade, e.g. by inactivating C3b (Alitalo *et al.*, 2001). Although bacteria appear to have developed means of avoiding cell lysis by complement, the deposition of complement on their surface may have opsonic properties. Moreover, some of the products of complement act as chemoattractants.

As discussed in section 1.1.4.1 opsonisation of bacteria is an important defence mechanism. One mechanism for evasion of opsonisation is the expression of a host-cell receptor-like protein by bacteria called Mac (Lei *et al.*, 2001). Mac bound to CD16 on the surface of human polymorphonuclear leukocytes and inhibited opsonophagocytosis and production of reactive oxygen species, which resulted in significantly decreased pathogen killing. While resistance of bacteria to complement-dependent opsonisation is a problem, there have been reports of

complement-independent pathways for pathogens that are resistant to complement-dependent opsonisation and phagocytosis by neutrophils (Grunwald *et al.*, 1996).

Many virulent pathogens are encapsulated and the capsule is often targeted in an immune response. One of the strategies pathogens have developed is to incorporate sialic acid residues into the polysaccharide of their capsule. Sialic acid residues are found on the surface of eukaryotic cells and consequently an immune response is not activated against such structures. For example, the polysialic acids of the Group B meningococcal capsule are chemically identical to those found on some human tissues (Finne *et al.*, 1983; 1987; Finne, 1985; Bitter-Suermann and Roth, 1987) and are therefore poorly immunogenic. Bacteria are also capable of changing the structure of some of their cell surface molecules and this may affect antigenic structure (e.g. McNeil and Virji, 1997). If this occurs this means that even if the body has already been exposed to this pathogen, it may not recognise it. Another means bacteria employ for evading the immune response is the shedding of surface components. For example, *N. meningitidis* are known for shedding endotoxin (LPS) in the form of membrane blebs (DeVoe and Gilchrist 1973; Troelstra, 1998). The release of endotoxin into the bloodstream may induce a strong pro-inflammatory response which may be responsible for the pathophysiology of severe meningococcal disease (Brandtzaeg *et al.*, 1989; reviewed by van Deuren *et al.*, 2000). Shedding surface components may also direct the immune response away from the bacterial cell.

If the microorganism evades the immune response then it is capable of causing disease as discussed in the next section.

1.1.5 Pathogenesis

The ability of the invading organism to cause disease appears to be dependent on a number of factors. These include the presence of virulence factors in the pathogen, the immune status of the host and the presence of other infectious agents. For

example, pigs may harbour a variety of strains or serotypes of *S. suis* in nasal cavities and virulent and avirulent forms appear to exist (reviewed by Gottschalk and Seguna, 2000). Moreover, virulent strains can be isolated from healthy animals, suggesting that clinical disease may be due to the disturbance of the immune balance due to other causes, e.g. other infectious diseases or stress (Gottschalk and Seguna 2000). This idea appears to be supported by a study by Wills *et al.* (2000) who proposed that the absence of pneumonia in pigs infected experimentally with porcine reproductive and respiratory syndrome virus (PRRSV) may be due to the absence of bacterial pathogens. In their study they investigated the effect of a concurrent infection with PRRSV and *Salmonella choleraesuis*. They found that animals infected with both organisms showed more severe signs of disease compared to those infected individually. PRRSV replicates preferentially in macrophages leading to destruction and it has been proposed that this affects the ability of the immune system to function effectively and leaves the animal more susceptible to infection and clinical disease. *Bordetella bronchiseptica* has also been implicated in increasing susceptibility to infection with *P. multocida* and to be capable of acting in synergy with PRRSV to predispose pigs to infections of the upper respiratory tract (Brockmeier *et al.*, 2001). There are a number of ways that pathogens can cause disease and these are described in the following sections.

1.1.5.1 Toxin-mediated pathogenesis

Different bacteria secrete different types of toxins. Some act by altering the cellular activities of the host cell, others are cytotoxic. *S. suis* produces a haemolytic toxin known as suilysin (SLY) (Gottschalk *et al.*, 1995). This is a thiol-activated toxin that forms transmembrane pores and has relatively high similarity to the well-characterised thiol-activated toxin of *S. pneumoniae*, pneumolysin (Jacobs *et al.*, 1994; Segers *et al.*, 1998). Antibodies against SLY appear to protect against infection (Jacobs *et al.*, 1994), suggesting this toxin is an important virulence factor. However, while most European strains are SLY+ there is variability in virulent North American strains (Gottschalk *et al.*, 1998; Staats *et al.*, 1999; reviewed in

Gottschalk and Segura, 2000). In addition, it has been shown that a *S. suis* mutant lacking the *Sly* gene was non-haemolytic and non-cytotoxic for cultured macrophage-like cells, avirulent in a mouse infection model but only slightly attenuated in a porcine model of systemic infection (Allen *et al.*, 2001). This suggests that other virulence factors may be involved in disease and the importance of SLY remains to be clarified. SLY+ strains have been shown to be cytotoxic (Norton *et al.*, 1999) but the mode of action remains to be determined. SLY may contribute to the development of meningitis in pigs by damaging the endothelial cells of the blood-brain barrier and thereby increasing the permeability of the blood-brain barrier (reviewed by Gottschalk and Seguna, 2000).

The adenylate cyclase toxin of *Bordetella pertussis* has been shown to cause apoptosis in macrophages infected with *B. pertussis* (Khelef *et al.*, 1993; Khelef and Guiso, 1995). *B. bronchispetica* has also been shown to be cytotoxic for murine macrophages and it has been proposed that this may be due to the adenylate cyclase produced by this organism (Brockmeier and Register, 2000). Brockmeier and Register (2000) propose that destruction or cytotoxicity to alveolar macrophages could lead to increased survival of the virulent phase of *B. bronchiseptica* by avoiding innate immune clearance.

Cholera toxin acts on mucosal cells in the small intestine. It stimulates the enzyme adenylate cyclase and this results in an increase in the intracellular levels of cAMP. The increase in cAMP appears to stimulate secretion of chloride ions in the gut lumen. This is followed by an outflow of water to the gut and the characteristic rice-water stools (Patrick and Larkin, 1995). Other toxins, such as botulism and tetanus toxins act on muscle cells causing either paralysis (botulism) or uncontrollable contractions (tetanus).

1.1.5.2 Release of other bacterial products that contribute to disease

Bacteria may secrete a range of other products that contribute to their invasiveness or lead to tissue destruction. For example, *S. pneumoniae* secretes neuraminidase and hyaluronidase. Neuraminidase has been shown to cleave the *N*-acetylneuraminic acid from glycolipids, lipoproteins and oligosaccharides on cell surfaces and in body fluids (Camara *et al.*, 1994). Neuraminidase has been shown to play a role in the colonisation of the nasopharynx and development of otitis media in the chinchilla model (Tong *et al.*, 2000). Hyaluronidase breaks down the hyaluronic acid component of mammalian connective tissue and extracellular matrix. This may aid the spread of bacteria. Moreover, breakdown products of hyaluronic acid have been shown to stimulate chemokine production by macrophages *in vitro* (McKee *et al.*, 1996).

1.1.5.3 Adverse effects as a result of induction of the immune response

One of the key questions in the study of the pathogenesis of meningitis caused by *S. suis* is how the organism breaches the blood-brain barrier. Gottschalk and Seguna (2000) suggest that *S. suis* may have two mechanisms for breaching the blood-brain barrier. In SLY+ strains, the toxin may destroy the endothelial cells as mutants lacking SLY have been shown to be non-cytotoxic (King *et al.*, 2001; section 1.1.5.1). As adhesins have also been shown to be associated with the virulence of this organism Gottschalk and Seguna (2000) suggest that adherence of SLY- strains to the endothelial cells results in the stimulation of cytokine production from these cells (possibly cyto-necrotic cytokines, such as TNF-α) and alterations of the blood-brain barrier allowing the passage of leukocytes to the brain. These alterations have been proposed as being responsible for entry of *S. suis* into the brain (discussed by Gottschalk and Seguna, 2000).

The ability of microorganisms to stimulate the production and over-production of pro-inflammatory or tissue-destroying cytokines is often associated with

pathogenesis (reviewed by Svanborg *et al.*, 1999). For example, release of endotoxins in the blood stream, which sometimes follows chemotherapy of Gram negative infections with antibiotics, is associated with septic shock e.g. in severe meningococcal disease (reviewed by van Deuren *et al.*, 2000). Septic shock is a result of endotoxins acting on macrophages and other cells and inducing the secretion of cytokines such as IL-1β and TNF-α. These cytokines can recruit others and a fall in blood pressure and blockage of blood vessels by white blood cells leads to organ failure.

This section has described how microorganisms cause disease. The following section will describe methods currently in use for controlling and preventing infectious disease.

1.2 Controlling infectious disease

1.2.1 Culling

Culling remains the method of choice when there is an outbreak of a highly infectious disease that is capable of infecting large numbers of animals and spreading over large distances. This was the method of choice for controlling the outbreaks of foot and mouth disease in the UK in 2001 and classical swine fever in the UK in 2000 and The Netherlands in 1997. When an infected animal is detected all animals on the farm are killed and destroyed. In addition the premises and equipment are disinfected. Around infected herds a protection zone with a radius of around 3km and a surveillance zone are established. In these zones no movement of animals is allowed. Herds in a protected zone are checked weekly (reviewed by Stegeman *et al.*, 2000). The aim is to create a ring that allows rapid detection of infected herds and prevention of the spread of the disease. However, it appears this method is only effective if the outbreak is detected before any movement of animals occurs. The rapid spread of foot and mouth disease in the UK and classical swine fever in The Netherlands is believed to be due to a lack of awareness and the

presence of the virus for a number of weeks before the first infected herd was detected and the length of time taken to cull infected animals, thereby allowing the virus to spread (Stegeman *et al.*, 2000; Ferguson *et al.*, 2001). While this method may be suitable for controlling contained outbreaks of highly infectious diseases, if there are a large number of infected herds, this method proves to be a costly and challenging way of eliminating the infection.

1.2.2 Antibiotics

Antibiotics are microbial or synthetic products that selectively kill certain microorganisms. Some antibiotics are effective against a broad range of microorganisms whereas others have a more limited range. Antibiotics function by disrupting some normal bacterial physiological process. For example, the β-lactam antibiotics, which include penicillin, act by binding to enzymes involved in peptidoglycan synthesis and this results in a weakening of the bacterial cell envelope in growing cells and eventually lysis of the bacterium. However, many bacteria have acquired genes encoding enzymes known as β-lactamases which inactive the β-lactam antibiotics (reviewed by Bush, 1999).

Although some bacteria are naturally resistant to some antibiotics, e.g. *Mycoplasma* species are resistant to penicillins because they lack a cell wall; *Mycobacterium leprae* is resistant to isoniazid because the *katG* gene, whose gene product is responsible for the toxicity of isoniazid, is a pseudogene (Eiglmeier *et al.*, 1997), resistance to antibiotics can also be acquired. Bacteria can become resistant to drugs as a result of a mutation that alters the target site of the antibiotic. For example, missense mutations in the *katG* gene of *Mycobacterium tuberculosis* are associated with isoniazid resistance (Heym *et al.*, 1995; Heym and Cole, 1996). These type of mutations normally only lead to resistance to a single antibiotic. However, antibiotic resistance can also be transferred on plasmids and this may result in a bacterium acquiring a number of antibiotic resistance genes and thus becoming resistant to a wide-range of antibiotics. For example, many β-lactamase-producing Gram-

negative bacteria have become multidrug resistant due to the acquisition of additional resistance determinants (reviewed by Bush, 1999). Acquisition of resistance determinants has also been the means by which methicillin-resistant *Staphylococcus aureus* have emerged (Ayliffe, 1997; Chambers, 1997; Smith and Jarvis, 1999). This is why there is concern about the use of antibiotics used both in human and veterinary medicine as growth enhancers in animals. For example, vancomycin and erythromycin are two antibiotics used as growth enhancers in animals and in human medicine for treatment of methicillin-resistant *S. aureus* (vancomycin) and Gram-positive infections (erythromycin). The use of glycopeptide antibiotics as growth enhancers has been shown to be associated with increased occurrence of resistance. Avoparcin was used extensively in the EU as a growth enhancer in animals and it is noteworthy that when use of this antibiotic was banned in the late 1990s due to fear of the spread of vancomycin-resistant enterococci to humans, the number of vancomycin-resistant enterococci decreased (Aarestrup *et al.*, 2001). However, the exact risk of transmission of vancomycin resistant strains from animals to humans remains to be clarified (Stobberingh *et al.*, 1999).

The emergence of multi-drug resistant strains of bacteria has lead to concern that a super-bug will emerge that is resistant to all available drugs. Moreover, antibiotics are not effective against viruses and viruses have the ability to mutate rapidly and quickly acquire resistance to anti-viral drugs. The other means of protection against infectious disease is vaccination, which is discussed in the following section.

1.2.3 Vaccination

As discussed in section 1.1.1.4 the end result of exposure to an infectious organism is the production of memory B cells which are primed to activate the immune response rapidly should the individual be exposed to the same pathogen again. The aim of vaccination is to induce the same response but without requiring the individual to suffer the disease.

Protection against infectious disease by vaccination can be done in two ways: (i) exposing the individual to antigens from the infectious agent and (ii) administering preformed antibody from an immune animal. Vaccination, if successful, can also be used as a means of controlling the spread of infectious disease and eventual elimination of the pathogen from the population. However, before deciding to use vaccination to control a specific disease it must be established that the vaccine will induce an immune response that will give protection against the disease in question and also that the risks of vaccination are lower than those of contracting the disease itself. There are a number of different types of vaccine available (reviewed by Ellis, 1999) and these will be discussed in the following sections.

1.2.3.1 Live vaccines

Live vaccines are the ideal vaccine as they induce lifelong immunity with minimal reactogenicity and usually only one or two doses are required. Live vaccines are normally viral vaccines (polio, measles, PRRSV, swine influenza) although there are a few bacterial ones (BCG and Ty21a). The vaccines are made by attenuating the organism by technical or biological means so that while it can replicate similarly to the wild virus or bacterium it will at most cause a mild disease. The organism can be attenuated by passaging it in cell culture until it is attenuated (e.g. Weill-Halle, 1957). Other means of obtaining an attenuated virus for use in a vaccine are selecting for temperature sensitive strains that do not replicate at 37°C (e.g. Maasab and DeBorde, 1985). In the case of live bacterial vaccines, the bacterial strain used in the BCG vaccine is *Mycobacterium bovis* bacille Calmette-Guerin which was attenuated by 231 successive *in vitro* subculturings over 13 years. The Ty21a *Salmonella typhi* vaccine strain was created by chemical mutagenesis followed by *in vitro* selection (Germanier and Furer, 1975). One of the concerns regarding live attenuated vaccine strains made by passaging or chemical mutagenesis is that the mutation responsible for attenuation is unknown and there is a possibility that the vaccine strain could revert to the wild type strain. These concerns have been relieved due to recombinant DNA technology, which allows the creation of defined

mutations. In addition at least two mutations located in different parts of the genome are carried out in order to reduce the possibility of the strain reverting. Recombinant DNA technology can also be used to create recombinant bacteria where the genes responsible for virulence are eliminated or are not expressed *in vivo*. An example is the live attenuated cholera vaccine currently in use (Levine *et al.*, 1988; Tacket *et al.*, 1999; reviewed in Ryan and Calderwood, 2000).

1.2.3.2 Dead vaccines

The advantage of dead or inactivated vaccines is that the organism cannot replicate in the host. They are generally well tolerated although if the vaccine components are not purified they made cause reactogenicity (e.g. whole cell pertussis vaccine) if given by the parenteral route. Cultivated bacterial cells are inactivated by heat or chemical agents; virus particles are inactivated chemically, usually with formalin and then adjuvanted by an aluminium salt. Inactivated viral vaccines are particularly attractive, as viral epitopes conferring immunity are usually conformational and therefore difficult to reproduce by, for example, recombinant technology. Both live and inactivated polio vaccines have been produced and these have been shown to be equally effective in providing protection against polio (reviewed by Murdin *et al.*, 1996). One of the major concerns regarding dead vaccines is that it is absolutely essential that all viral particles or bacterial cells are inactivated.

1.2.3.3 Acellular vaccines

Acellular vaccines consist of purified components from bacterial cultures that are capable of inducing an immune response. In addition, advances in recombinant DNA technology and the identification of immunogenic virulence factors means that these components can now be expressed and purified from cultures of *E. coli*, for example. These vaccines are attractive because it is not necessary to work with large amounts of hazardous material. An acellular vaccine currently in use is the pertussis vaccine. This vaccine consists of various combinations of purified proteins from *B.*

pertussis, including pertussis toxin and various combinations of filamentous haemagglutinin, pertactin and adenylate cyclase toxin (e.g. Sato *et al.*, 1984; Podda *et al.*, 1991; Edwards, 1993; Edwards *et al.*, 1995; Decker *et al.*, 1995). Other subunit vaccines are tetanus and diphtheria. These vaccines are produced by detoxifying the tetanus and diphtheria toxins with formalin or glutaraldehyde (e.g. Ramon, 1923). Toxins can now also be inactivated using recombinant DNA technology so that they lose their toxicity but maintain their immunogenicity (e.g. pertussis and cholera toxins; Rappuoli *et al.*, 1996). Other bacterial components used in vaccines are the capsular polysaccharide. The vaccine currently in use against *S. pneumoniae* contains polysaccharide against 23 serotypes of pneumococci although this vaccine has low efficacy in young children due to their immature immune system (White, 1988; Alonsodevelasco *et al.*, 1995). Conjugation of capsular polysaccharide to a protein carrier as in the case of the *Haemophilus influenzae* type b vaccine, has resulted in improved immune responses in young children (Goldblatt, 1998) and similar vaccines for use against *S. pneumoniae* have been licensed for use in the United States (Black *et al.*, 2001).

1.2.3.4 New vaccines –genetically modified *Salmonella* and DNA vaccines

One of the problems with vaccines against mucosal pathogens administered via the parenteral or oral route is that a mucosal immune response is not induced. As discussed in section 1.2.3.1 one of the advantages of the live vaccine is that it mimics natural infection and induces a good immune response. With recombinant DNA technology it has become possible to engineer viral and bacterial vectors to express foreign antigens. The most commonly used vectors are vaccinia and adenoviral (viral vectors) (e.g. Lubeck *et al.*, 1994) and *Salmonella* (bacterial) (e.g. Roberts *et al.*, 1994). However, an improved understanding of the pathogenesis and physiology of other bacteria mean that there is now a wider range of vectors in use (e.g. Gentschev *et al.*, 2000). These viral and bacterial vectors are designed to only survive long enough in the host to induce protective immunity. They are particularly useful because different species can be used to infect different parts of the

mammalian system (respiratory, intestinal or genitourinary tract) resulting in a potent immune response against the expressed antigen.

The possibility of introducing naked DNA, i.e. plasmid DNA containing the bacterial antigen under the control of a eukaryotic promoter is also being investigated (reviewed by Donnelly *et al.*, 2000; Gurunathan *et al.*, 2000). DNA vaccination has been particularly successful against viral diseases.

This section has discussed the various types of vaccines currently available and those under development. While there are some effective vaccines available, some of the existing ones are not so effective, e.g. the efficacy of the BCG vaccine ranges from 0-80% (discussed by Behr, 2001) and there have been outbreaks of PRRS despite vaccination leading to concern about the efficacy of this vaccine (Meng, 2000). Moreover, vaccination against some viral infections, e.g. influenza, is difficult due to the ability of the influenza virus to undergo antigenic drift and shift. In the case of the foot and mouth and classical swine fever vaccines, it is not desirable to use them in EU countries for economic reasons. Therefore, attention is turning to other methods of improving disease resistance, particularly in animals, using transgenic technology.

1.3 Increasing disease resistance in animals using transgenic technology

Selective breeding has been the traditional way for improving desirable traits in livestock such as production potential and disease resistance. Until recently selective breeding has focused on improving production potential. However, there is concern that selecting traits which improve animal production could be having a negative effect on animal health by selecting against traits that could be involved in immunocompetence (reviewed by Knap and Bishop, 2000). Attention is now being drawn to using genetic variation in immunocompetence to select for animals with increased resistance to infectious disease. Resistance to infectious disease is

controlled by additive quantitative trait loci (QTL). Genetic variation in resistance to infectious disease is therefore a result of additive effects of QTLs that regulate innate and acquired immunity (Wilkie and Mallard, 1999). This means that not only will there be genetic variation in susceptibility to disease between breeds but also between individuals. For example, as discussed in section 1.1.2, the ability of K88+ strains of *E. coli* to cause disease in pigs is dependent on the presence of a receptor in the porcine intestine. Although selective breeding can produce animals with increased resistance to infectious disease (Pryzytulski and Porzeczkowska, 1980) this is generally not successful or advisable because some pathogens (e.g. intracellular pathogens) induce the cell-mediated immune response whereas others (i.e. extracellular pathogens) induce the acquired immune response. Consequently, selection for resistance to one pathogen may result in susceptibility to another. This is discussed by Wilkie and Mallard (1999) and they propose that simultaneously selecting for cell-mediated immunity and acquired immunity may have more advantages than selecting for each individually. This was supported by the finding that pigs with a high immune response responded better to vaccination and also had the best weight gain. Müller and Brem (1991) also discuss the advantages and disadvantages of selective breeding and conclude that improving disease resistance in livestock by genetic means is a difficult and time-consuming task requiring long-term strategies.

Transgenic technology offers a new approach to increasing the disease resistance of farm animals. The strategies aim at the stable or transient expression of components known to influence non-specific or specific host defence mechanisms against infectious pathogens, such as those genes inducing or conferring innate and acquired immunity as well as specific disease resistance genes (Müller and Brem, 1996). The following sections will describe first of all the various methods currently being investigated and used in the generation of transgenic animals and secondly, some of the strategies being used to increase the resistance of animals to infectious disease.

1.3.1 Methods used for generation of transgenic animals

The methods currently in use and those under investigation for generating transgenic animas were reviewed recently by Wheeler and Walters (2001) and Wolf *et al.* (2000). Transgenic technology is used widely to create transgenic mice in order to investigate things such as complex signalling pathways, gene expression and the effect of gene mutations on for example, physiology or progression of a disease. Transgenic mice are relatively easy to produce and have a short generation time and a large number of mice can be quickly obtained. The creation of transgenic farm animals has been hampered to some extent by technical difficulties, the expense and the long generation time. However, in recent years there have been advances in transgenic technology and new approaches have been proposed to facilitate the generation of transgenic livestock. The various methods used to create transgenic animals will be discussed in the following sections.

1.3.1.1 Pronuclear DNA microinjection

Pronuclear microinjection is the classic method of gene transfer in farm animals and was developed by Brem *et al.* (1985) and Hammer *et al.* (1985). In this method DNA fragments are injected into the pronuclei of a zygote. As a result the DNA fragments can integrate randomly, which is a drawback of this method. In addition, species-specific modifications of this technique are necessary. For example, the cytoplasm of zygotes from cattle and pigs is opaque and these zygotes have to be centrifuged prior to microinjection so that the pronuclei become visible (Wolf *et al.*, 2000). The efficiency of this technique in farm animals is lower than in mice. One of the drawbacks of this technique as mentioned above has been the random integration of the introduced DNA fragment into the genome, which can have major effects on expression of the introduced gene or alter the expression of endogenous genes if the introduced DNA inserts itself within a gene. However, new techniques, such as Cre recombinase system and matrix attachment regions (discussed in section 1.3.2) may overcome this limitation.

1.3.1.2 Viral vectors

Retroviral vectors function by transferring the genetic information as an RNA molecule. This is then reverse transcribed and integrated into the host genome. Integration of the DNA into the host genome occurs mainly in dividing cells. Therefore, there were problems with infection of early embryo cells which resulted in delayed integration and genetic mosaics characterised by different insertion sites in different tissues (reviewed in Chan *et al.*, 1998). To overcome these problems the Moloney murine leukaemia virus has been used. Although Chan *et al.* (1998) reported a high efficiency of gene transfer using this virus, there were questions regarding the proper expression of the transgene. A major drawback of retroviral vectors is that they can only accommodate less than 10kb of sequence and this limits the type of DNA that can be transferred. In addition, the long terminal repeats flanking retroviral genomes have been reported to interfere with mammalian promoters (Wells *et al.*, 1999).

1.3.1.3 Sperm-mediated transfer

This approach is based on the ability of mammalian spermatozoa to bind exogenous DNA. The ability of spermatozoa to bind endogenous DNA is believed to be due to the presence of DNA-binding proteins on the sperm surface. Internalisation of exogenous plasmid DNA appears to result in extensive rearrangement of the DNA before it undergoes recombination with genomic DNA (Zoraqi and Spadafora, 1997). This method has not fulfilled expectations and has low efficiency and repeatability. Alternatives to this method are intracytoplasmic sperm injection (Perry *et al.*, 1999) and intratesticular transfection of germ cells (Kim *et al.*, 1997).

1.3.1.4 Pluripotent stem cells

Embryonic stem (ES) cells are important tools in experimental genetics. They form the basis for the generation of knockout mice, site-directed insertions and

chromosomal rearrangements. Attempts have therefore been made to isolate ES cells from livestock species. However, so far germ-line competent pluripotent cell lines have not been established for any mammalian species other than the mouse (reviewed in Wolf *et al.*, 2000).

1.3.1.5 Nuclear transfer using transfected cells

Nuclear transfer experiments were initially described by Willadsen (1986) and Prather *et al.*, (1987; 1989). Modifications of the method were described by Campbell *et al.* (1996) and Wilmut *et al.*, (1997). This method involves the transfection of the DNA into cultured cells. A nucleus from these cells is then transferred into a metaphase II oocyte from which the genetic material has been removed. The embryo is then transferred to the animal. This method results in the production of 100% transgenic offspring. However, the method is also associated with a high incidence of abortions and premature death. This method appears to offer the best prospects in the future development of transgenic technology.

1.3.2 Improving transgenic technology

One of the problems until recently with transgenic technology was the inability to determine the site of integration of the transgene. This was a problem because insertion of the transgene in a region under strong negative control could mean that the transgene would not be expressed. Moreover, there was the possibility that the transgene could insert itself within a coding gene and disrupt the function of this gene. In order to prevent the effects of long range controls on expression of the transgene it has been attempted to create transgenic animals using yeast artificial chromosomes (Brem *et al.*, 1996; Fujiwara *et al.*, 1999). Another approach has been the use of matrix attachment regions or scaffold attachment regions which can bind the nuclear matrix and/or chromosome scaffold and act as boundary elements, protecting the transferred DNA constructs from regulatory effects of the neighbouring chromatin (McKnight *et al.*, 1996). More recently, there have been

reports of the use of the Cre-recombinase system to allow site-specific introduction of the transgene. This means that the inserted gene has the correct chromatin structure and regulatory elements required for gene expression and avoids the problems associated with randomly integrated transgenes (Kolb *et al.*, 1999; Kolb, 2001).

1.3.3 Embryonic transfer

There are two methods in use for obtaining embryos and embryonic transfer – surgical and non-surgical. These methods have been discussed by Hazeleger and Kemp (2001). The simplest method for embryo collection is flushing of the uterus after the donor has been slaughtered. This method results in high recovery rates but it means that the donor can only be used once. Hygienic collection of the embryo is important and those that have been collected immediately after slaughter have higher developmental rates *in vitro* (Hazeleger and Kemp, 1999). In surgical collections the genital tract is exposed and the oviducts and/or upper parts of the uterine horns are flushed retrograde. More recently, an endoscope has been used. This allows the collection and transfer of embryos through a small incision in the abdominal wall. Surgical embryo transfers carried out by exposing the reproductive system result in a mean pregnancy rate of 60%. Endoscopic procedures result in a mean pregnancy rate of 14 - 40% for transfers into the uterus and 33% for tubal transfers (reviewed by Hazeleger and Kemp, 2001). A disadvantage of surgical collections and transfers is that skilled personnel, anaesthesia and surgical facilities are required. Non-surgical transfer of embryos involve transfer of embryos into the uterus transcervically. Factors affecting the success rate of non-surgical transfers have not yet been investigated fully. However, Yonemura *et al.* (1996) found that their technique, which involves putting the catheter deeply into the cervical canal gave good results because it prevents embryos sticking to the cervical mucus. Other factors that are thought to be important are the developmental stages of the embryo and it appears to be important that the recipients have ovulated approximately 24h before to 12h after the donors (Hazeleger and Kemp, 1999). In addition, hygiene

has also been reported to be important with most successful transfers obtained by groups that use antibiotics in the transfer medium and prevent contamination of the uterus with vaginal material (Hazeleger and Kemp, 1999).

1.3.4 Transgenic approaches for increasing the disease resistance of animals

A wide variety of methods have been proposed for increasing the disease resistance of animals using transgenic technology as shown in Figure 1.4. This section will discuss some of these methods including, the identification of disease resistant genes or genes that are responsible for susceptibility to infection, congenital immunisation, and intracellular immunisation.

1.3.4.1 Identification of disease resistance genes

Resistance or susceptibility to infectious disease is thought to be not only due to genetic differences resulting in variation of the immune response (discussed in section 1.3) but is also due to the presence or absence of specific "disease resistance" genes. Probably the best characterised protein giving protection against infectious disease is the Mx1 protein, which confers resistance to influenza virus. This is a highly conserved protein that is present in all eukaryotes examined including humans, cattle, pigs, rats, horses, hamsters, fish and yeast (Horisberger & Gunst, 1991). However, it remains unclear why, despite the presence of Mx proteins, that some species, for example, humans and pigs, remain susceptible to influenza virus infection.

A variation on this theme is the identification of genes that predispose the animal to infection. Improvements in transgenic technology, as discussed in section 1.3.1.4 and 1.3.1.5 mean it may now be possible to create animals where the disease susceptibility gene has been knocked out, e.g. pigs lacking the gene that encodes the receptor for the K88 antigen of *E. coli*.

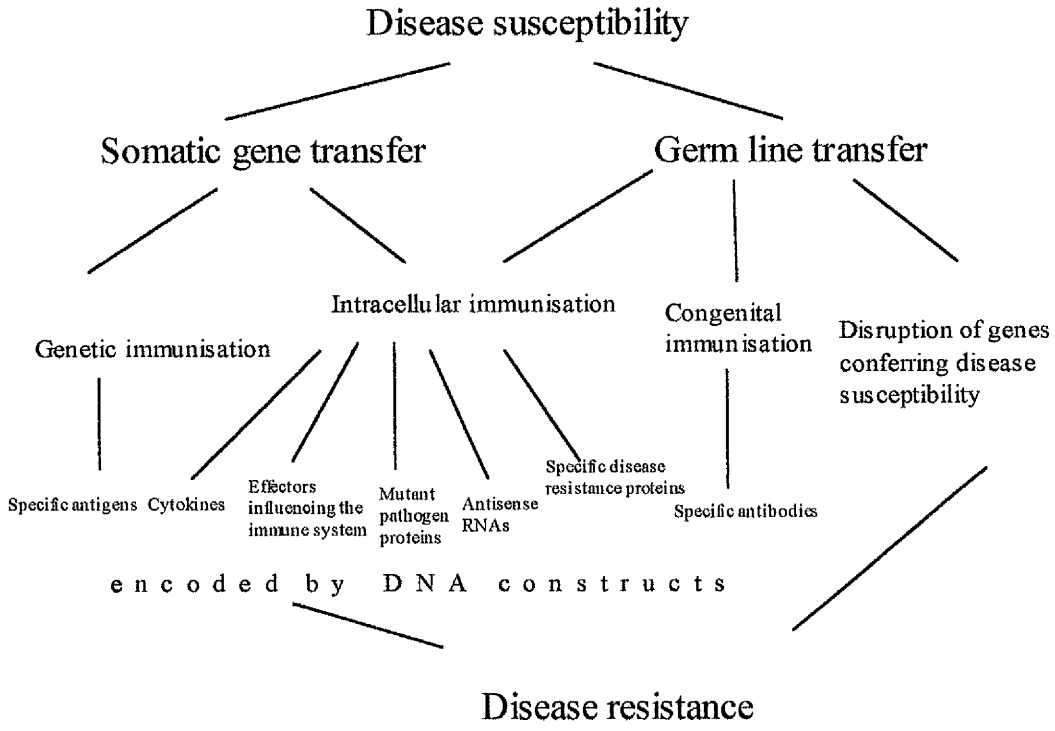


Figure 1.4: Transgenic approaches for increasing disease resistance in animals (Müller & Brem, 1996).

Identification of disease resistance genes is not an easy task (discussed by Malo and Skamene, 1994). The difficulty in identifying disease resistance genes is reflected by the limited number of such genes that have been identified to date. However, the availability of animal genetic maps and the generation of genetic markers may facilitate the discovery of new disease resistant genes.

1.3.4.2 Congenital immunisation

Congenital immunisation is defined as transgenic expression and germ line transmission of a gene encoding an immunoglobulin specific for a pathogen. Although early studies showed that expression of the transgene could result in significant inhibition of gene rearrangement and expression of endogenous immunoglobulin genes (Ritchie *et al.* 1984; Rusconi & Köhler, 1985; Nussenzweig *et al.* 1987; Stall *et al.* 1988), work by Lo *et al.* (1991) showed that it was possible to express murine IgA in pigs without affecting expression of the endogenous porcine immunoglobulin genes. Weidle *et al.* (1991) also reported the successful expression of a mouse monoclonal antibody in pigs. The ability to produce transgenic animals that constitutively express recombinant antibodies or antibody fragments against pathogens of the species in question may improve its resistance to infectious disease (e.g. Castilla *et al.* 1998; Lorenzen *et al.* 2000). More recent variations on this theme are the creation of transgenic commensal bacteria expressing antibodies against mucosal pathogens and these transgenic bacteria may provide protection against mucosal infections as long as they remain present (Whaley & Zeitlin, 2000). One of the main limitations of congenital immunisation is that the antibody expressed is pathogen-specific. This means that the pathogen can evade the transgenic animal's immunity simply by changing its antigenic determinants, e.g. the ability of influenza virus to undergo antigenic shift and drift.

Other approaches to improving animal resistance to infectious disease include the expression of protein-based antimicrobials that are specific for a particular pathogen. An example is the peptidoglycan hydrolase lysostaphin. This protein is secreted by

Staphylococcus simulans and has bactericidal activity against *Staphylococcus aureus*, a major cause of mastitis in cattle. Investigations have shown that transgenic mice expressing this protein under the control of the ovine β-lactoglobulin gene, not only express and secrete this protein in milk but also they have enhanced protection when challenged with *S. aureus* (Kerr *et al.* 2001). Therefore, transgenic animals expressing this protein may be protected from mastitis caused by *S. aureus*.

1.3.4.3 Intracellular immunisation

Intracellular immunisation is defined as the intracellular expression of transgenic products that inhibit the replication of pathogens in host organisms (Müller and Brem, 1996). This approach can include the transfer of cytokine encoding gene constructs or the introduction of specific disease resistance genes into the germ line, for example, transfer of the *Mx1* gene, a murine gene that confers resistance to influenza virus infection, into pigs (Müller *et al.* 1992).

Although there are several transgenic approaches for producing animals with increased resistance to infectious disease the limiting factor in all of these approaches is that the animals will only be protected against a specific pathogen. In an attempt to overcome the limitations of these approaches we have designed and created an inducible expression system that will allow the controlled expression of protective cytokine genes in response to infection. The generation of the expression system is described in the next section.

1.4 Generation of the CRP-based acute phase expression vector

Cytokines are the signalling molecules of the immune response and have a wide variety of roles, including the activation of phagocytes and other immune cells and controlling the immune response – pro-inflammatory and anti-inflammatory cytokines have been reported. Because cytokines can stimulate the innate immune

response it is reasoned that administration of cytokines prior to infectious challenge will activate the innate immune response and result in protection against a wide range of pathogens. Indeed, it has been shown that the cytokine GM-CSF, when administered prior to an infectious challenge is capable of providing protection against a variety of pathogens (Tanaka *et al.*, 1989; Frenck *et al.*, 1990; Fontt *et al.*, 1996; Hebert and O'Reilly, 1996). For example, Hebert and O'Reilly (1996) showed that administration of GM-CSF enhanced bactericidal activity of alveolar macrophages and that pre-treatment with GM-CSF significantly improved the survival of mice challenged with pneumococci compared to mice pre-treated with saline (Figure 1.5).

Although administration of cytokines has been shown to provide protection against an infectious challenge in small animals, it is not feasible to administer cytokines to farm animals routinely because of the high cost. Therefore, transgenic technology offers a new approach for "administering" cytokines to animals. Because cytokines play a critical role in the regulation of the immune response, it is not surprising that constitutive expression of cytokines in transgenic mice has resulted in a number of undesirable side-effects (Taverne, 1993; Lang *et al.*, 1987). The study by Lang *et al.* (1987) which investigated the constitutive expression of GM-CSF in transgenic mice revealed that this resulted in a number of pathologies including opacity of the eye, wasting muscles and eventually death around six weeks of age. These studies indicated that controlled expression of the cytokine gene in transgenic animals is essential. Our work has focused on the cytokine GM-CSF and exploited the kinetics of the acute phase response to control expression of the gene.

The acute phase response was chosen as the basis for our inducible expression system for two reasons. Firstly, the acute phase response is induced by infection. This means that the cytokine gene will be "switched on" naturally in response to infection and therefore, does not require the administration of exogenous substances to induce the expression of the gene. The second reason was because of the kinetics of the major acute phase protein in humans, C-reactive protein in transgenic mice

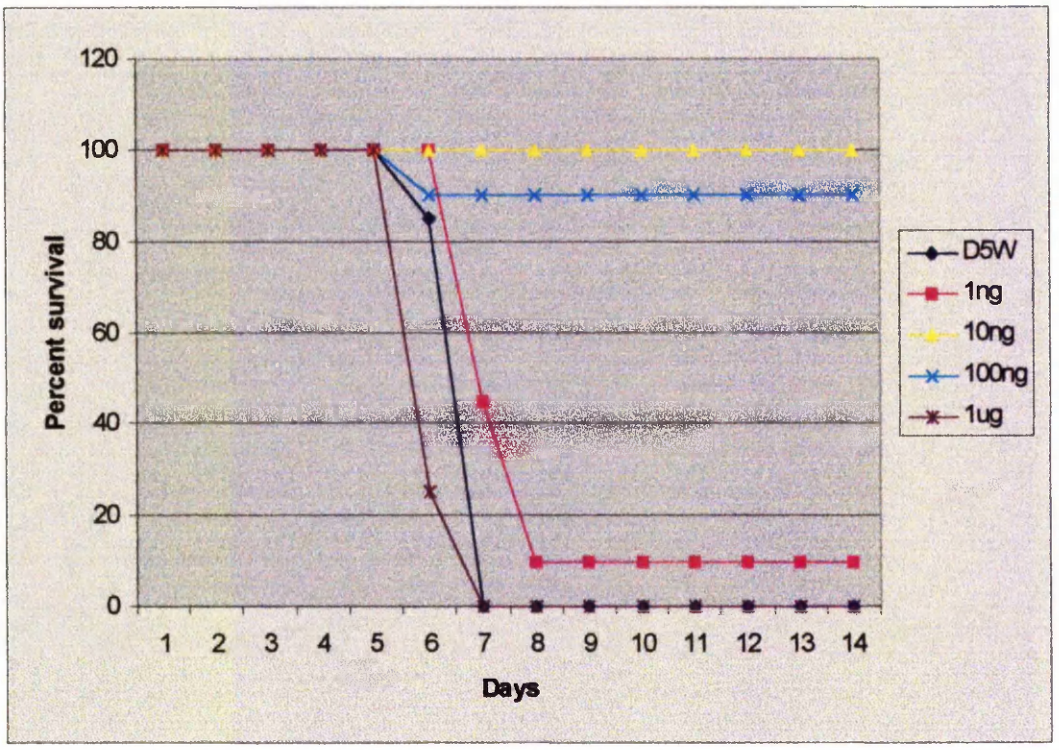


Figure 1.5: Ability of GM-CSF to provide protection against a pneumococcal infection (adapted from Hebert & O'Reilly, 1996). Mice were administered GM-CSF at various concentrations (1ng to 1μg) twice daily for 14 days following exposure to an aerosol of type III pneumococci. Treatment with 10ng and 100ng of GM-CSF resulted in a significant increase in survival of the mice. However, treatment with lower or higher doses of GM-CSF did not result in a significantly higher survival rate compared to controls.

(Figure 1.6). In the absence of a stimulus CRP is expressed at very low levels. However, following stimulation (infection, inflammation, and tissue injury) levels of CRP can rise 1000-fold within 24 hours and return to normal levels within 48 hours. Therefore, by using the promoter of the CRP gene to control the expression of the GM-CSF gene it was expected that the GM-CSF gene would be regulated with CRP kinetics. Expression of the GM-CSF gene under the control of the CRP promoter should stimulate the animal's immunity during the early stages of infection because GM-CSF will be induced earlier than normal during an infection and may therefore, enhance protection against the infectious agent. Controlled expression of the GM-CSF gene should prevent the deleterious side effects observed following long-term expression of GM-CSF (Lang *et al.*, 1987). Although GM-CSF may not seem to be an ideal choice for use in an acute phase expression system, the fact that the biological effects of constitutive expression of this cytokine are known means that it will give an early indication of how controlled the expression system is.

The creation and analysis of the CRP-GM-CSF construct is described in detail by Williams (2001) and Burke *et al.* (manuscript in preparation) and is based on constructs made and investigated by Murphy *et al.* (1995 and Figure 1.7). The 5.6kb *Bam*HI-*Bam*HI fragment containing the human CRP gene was used for cloning the CRP-GM-CSF construct into the background of the 30kb fragment. The CRP gene was removed by inverse PCR with primers that had engineered *Not*I sites at each end to facilitate cloning. The promoter and signal peptide of the CRP gene were fused to the murine GM-CSF gene using primers that also had engineered *Not*I sites at each end. The CRP-GM-CSF fusion was then cloned into the *Not*I sites of the shuttle vector and it was then attempted to clone this into the background of the 30kb fragment. However, since attempts to do this were unsuccessful, the CRP-GM-CSF fusion was instead cloned into the smaller construct C79 (Figure 1.7). C79 exhibited low basal levels of expression and high levels of induction *in vivo* and it was the construct whose kinetics most resembled the 30kb fragment. The C79 construct containing the CRP/GM-CSF fusion was termed GM-C79 and was used to generate a line of transgenic mice.

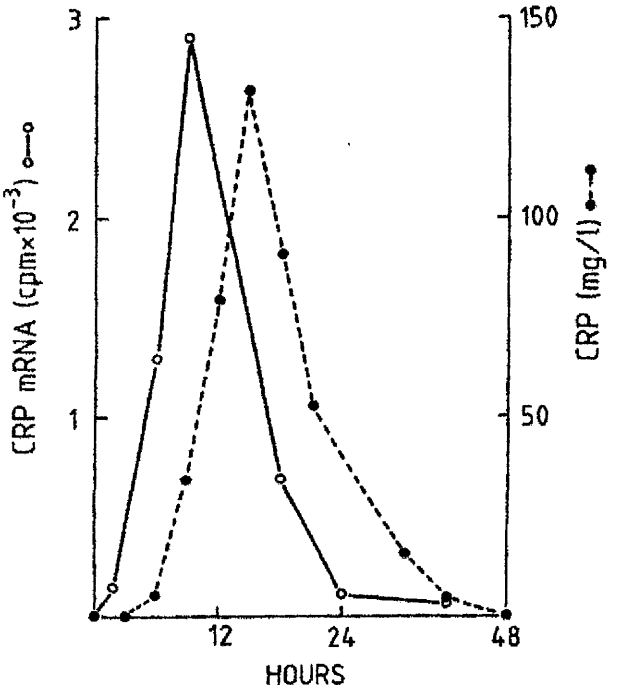


Figure 1.6: Regulation of the human CRP gene in transgenic mice (Ciliberto *et al.* 1987b). This group analysed expression of the human CRP gene in transgenic mice by measuring mRNA and protein synthesis following stimulation with LPS. The results show that protein synthesis (right-hand side of graph) parallels mRNA synthesis with a time-lag of approximately 5 hours.

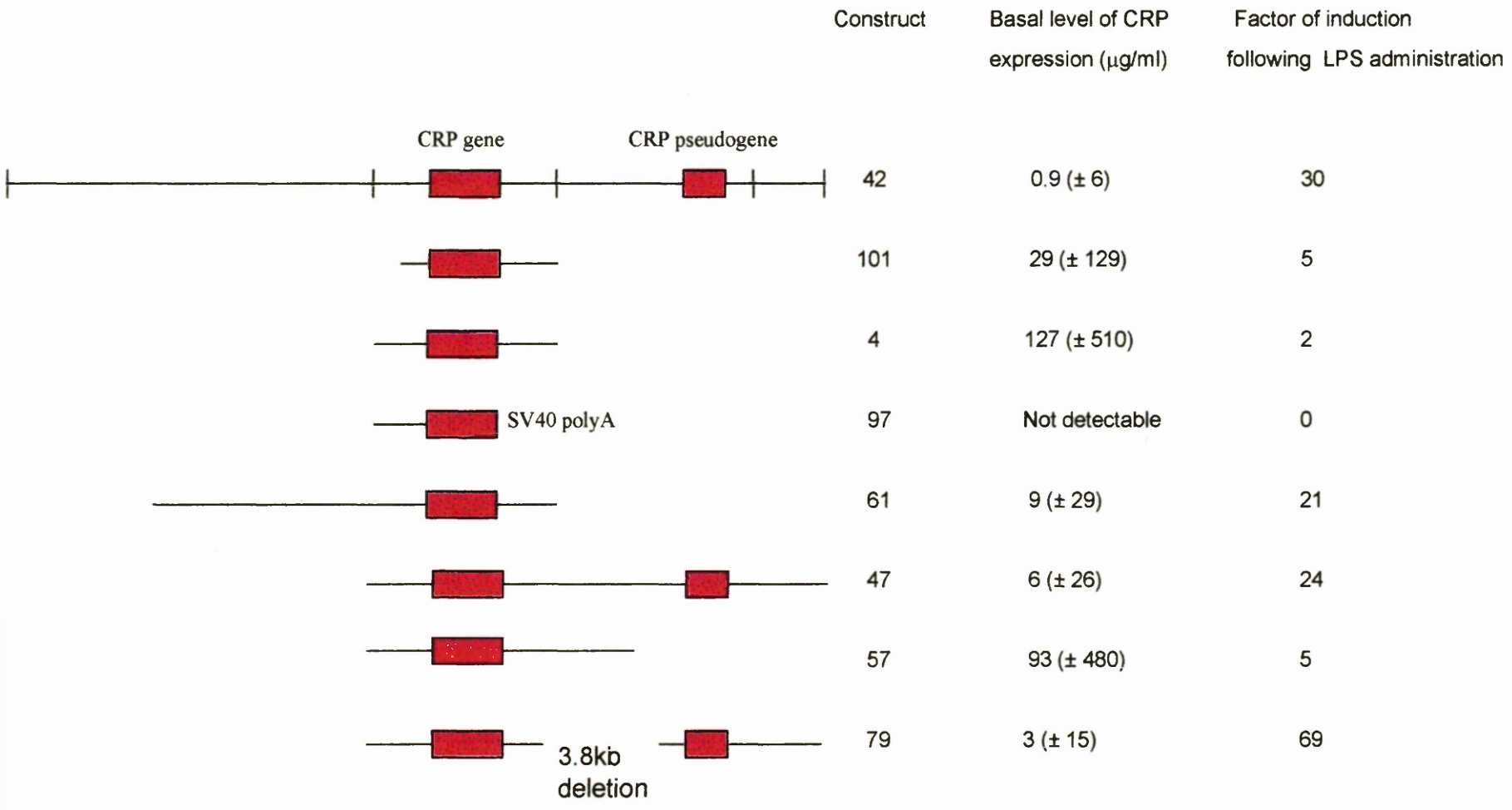


Figure 1.7: Schematic diagram of the constructs made by Murphy *et al.* (1995) and the levels of CRP expression prior to and following stimulation with LPS

Transgenic mice carrying the GM-C79 construct were identified by PCR and Southern blotting (Williams, 2001) and these founder mice were used to establish our current lines. To determine if the GM-CSF gene was regulated with acute phase kinetics in transgenic mice the mice were administered LPS to induce the acute phase response and the amount of GM-CSF produced was measured over a 48 hour time period (Figure 1.8)

From the graph it can be seen that following administration of LPS the levels of GM-CSF rise with peak expression occurring 12 hours post-stimulation. By 24 hours the level of GM-CSF is starting to fall and by 48 hours it is back to pre-induction levels (in the case of female transgenics). This indicated that the CRP promoter was driving expression of the GM-CSF gene with acute phase kinetics. However, it is also seen that the male transgenics exhibit higher basal levels of GM-CSF compared to female transgenics prior to stimulation (1146 ± 571 pg/ml (male) compared with 266 ± 53 pg/ml (female)). This phenomenon, whereby male transgenics have higher basal levels of expression of the transgene, has already been reported by Szalai *et al.* (1995) in mice harbouring the human CRP transgene.

In summary, previous work in our laboratory has resulted in the creation of an inducible expression system that results in expression of GM-CSF with acute phase kinetics following an inflammatory stimulus. Although the male transgenics have higher basal levels of expression (possibly a consequence of using the CRP promoter as the backbone to the vector), the system is controlled enough to avoid the deleterious effects reported by Lang *et al.* (1987). The majority of our GM-CSF transgenics survive for at least 12 months and they do not have the pronounced bilateral opacity of the eye that was also reported by this group. We now wish to develop the system further, to see if we can eliminate the sexually dimorphic pattern of expression and secondly, to develop the system for use in pigs.

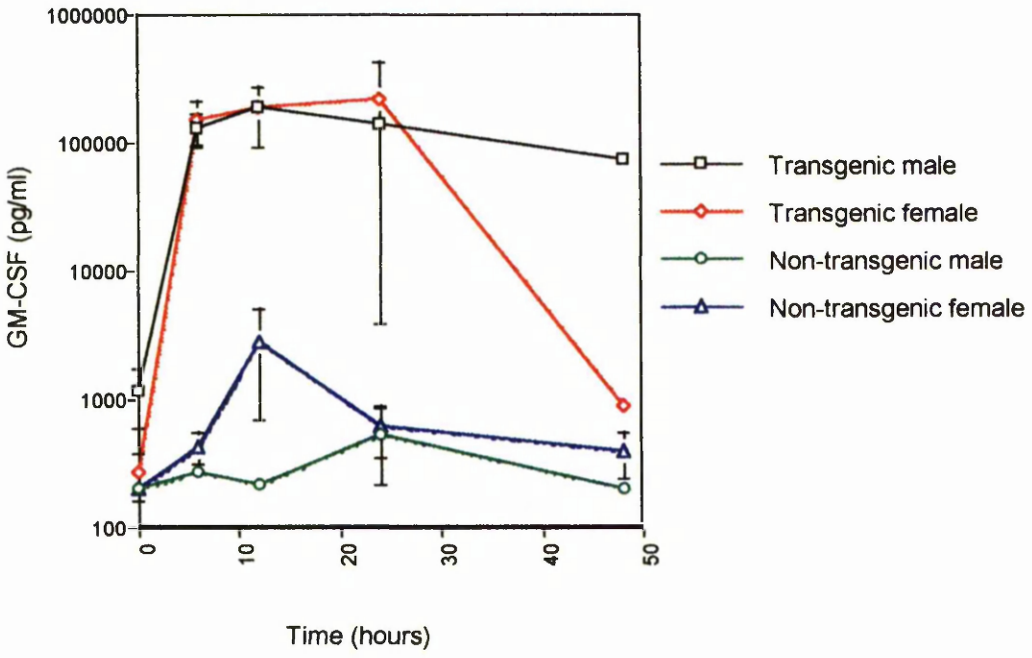


Figure 1.8: Levels of GM-CSF in the serum of GM-C79 transgenic and non-transgenic mice (log scale) following stimulation with LPS (Burke *et al.* in preparation). The levels of GM-CSF in the serum of non-transgenics is much lower than that of GM-C79 transgenics following stimulation with LPS. In addition, while the basal level of expression of GM-CSF in the serum of female transgenics is similar to that of non-transgenics, the basal level of expression of GM-CSF in male transgenics is about 5-fold higher. It should be noted that although the increased basal level of expression in male transgenics is only 0.6% of the maximal amount of GM-CSF produced this is still sufficient to cause pathology.

1.5 Aims of thesis

The aims of this thesis are illustrated in Figure 1.9. We have shown that the acute phase expression system is functional in transgenic mice and we now wish to see if it is possible to modify the expression vector for use in animals of economic importance. This project investigates the modification of the expression vector for use in pigs. Modification of the expression vector may be necessary for use in pigs for a number of reasons. First of all, we do not know how this construct will behave in pigs – will it be regulated the same way as in mice (i.e. as a human acute phase reactant) or will it be regulated as the porcine CRP gene is (CRP is not regulated in the same manner in pigs and humans)? Secondly, due to the current controversy surrounding genetically modified foods, transgenic pigs containing human DNA sequences may be unacceptable to consumers. The acute phase expression vector will therefore be developed in two ways:

- Analysis of the sequences involved in regulating the CRP gene with a view to identifying elements that are critical for the low basal levels of expression of the gene and those responsible for the sexually dimorphic pattern of expression. Due to the expense and difficulty in making transgenic animals it is desirable that the acute phase expression vector is as perfect as possible before attempting to make transgenic pigs.
- In parallel, it was decided to isolate the promoter from the major acute phase protein in pigs and examine its potential to drive expression of heterologous genes with acute phase kinetics. If it turns out that the acute phase expression vector based on the human CRP gene is not functional in pigs, then there is the possibility of creating one based on the porcine major acute phase protein. Alternatively, our knowledge of the elements involved in regulating the CRP gene could be used to modify the expression vector for use in pigs.

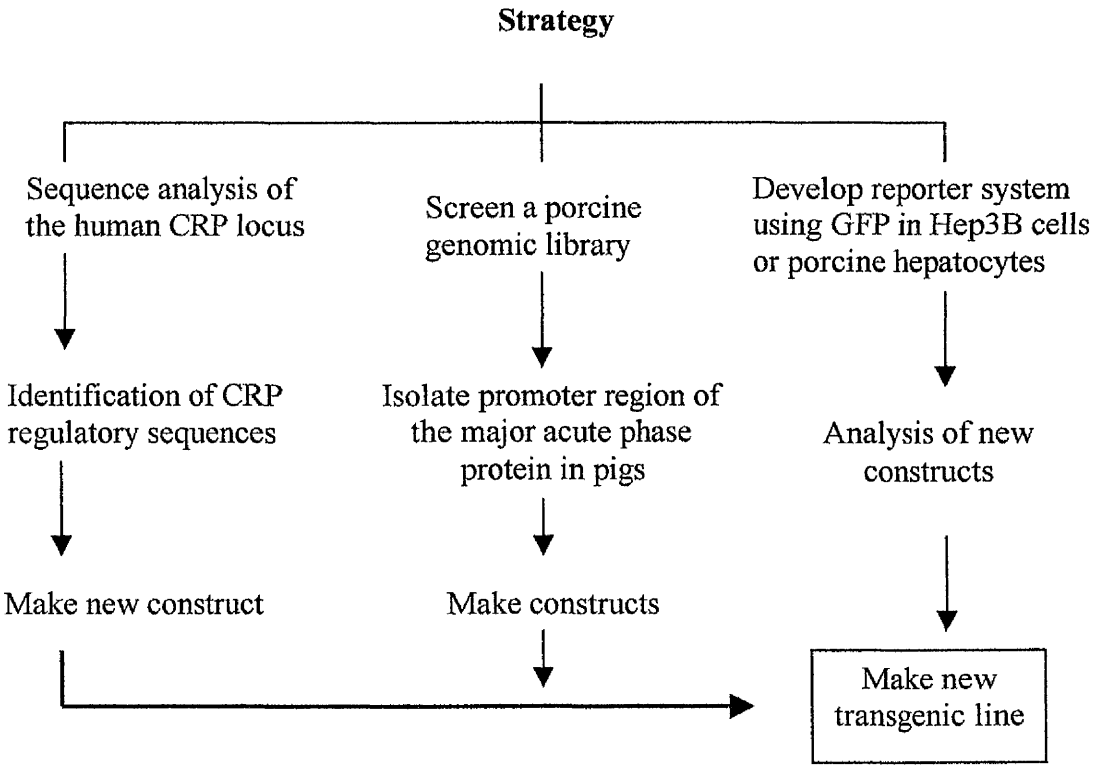


Figure 1.9: Proposed strategy for the project "Controlled gene expression using acute phase response elements"

To modify the expression vector for use in pigs, an understanding of the regulation of pig-MAP or ITIH4, the major acute phase protein in pigs, as well as identification of the sequences involved in its regulation needs to be carried out.

The work described above will necessitate a large number of gene expression studies to be carried out. Therefore, a system that is cheap to use with high throughput is desirable. The green fluorescent protein (GFP) has been marketed as an ideal reporter gene for studying gene expression as it does not require the use of exogenous substrates for detection and allows real-time analysis. The final aim of the project is therefore, to evaluate GFP as a reporter gene for studying inducible gene expression.

Materials & Methods

Chapter 2: Materials and Methods

2.1 Materials

All chemicals were supplied from Sigma and Fisher Scientific unless otherwise stated. Restriction enzymes were supplied by Promega and Boehringer Mannheim (Roche).

2.2 Bacterial strains

The bacterial strains used in this project were for transformation purposes.
Epicurian Coli® XL-1 blue competent cells (Stratagene): Genotype *recA1 endoA1 gyrA96 thi-1 supE44 relA1 lac*[F' *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})
Epicurian Coli® SoloPack® Gold competent cells (Stratagene): Genotype Tet^{r} Δ(*mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte[F' *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r}) Amy Cam^{r})
Novablue Singles (Novagen): Genotype *endA1 hsdR17*($r_{k12}^{-}m_{k12}^{+}$) *supE44 thi-1 recA1 gyrA96 relA1 lac*[F' *proA*$^{+}$*B*$^{+}$ *lacI*^{q}*ZΔM15 ::*Tn*10*]

2.3 Cell lines and primary porcine hepatocytes

Hep 3B, a human hepatoma cell line that has been used extensively for studies of the acute phase response was obtained from the European Collection of Animal Cell Cultures (ECACC 86062703), Cambridge, UK.
COS-7 (ECACC 87021302), a monkey kidney cell line, was supplied by Dr C Rush.
Primary porcine hepatocytes were supplied by Mr L Nelson and Mr S Keach, Liver Research Unit, Department of Medicine, University of Edinburgh, UK.

2.4 List of vectors used

Vector	Description	Manufacturer
pPCRScript	Cloning vector for PCR products	Stratagene
pBluescript (S/K-)	General purpose cloning vector	Stratagene (supplied by Dr C Gray)
pZero	General purpose cloning vector	Invitrogen (supplied by Dr C Gray)
pCAT-Control	Chloramphenicol acetyltransferase (CAT) mammalian expression vector where CAT gene is under the control of the SV40 promoter	Promega (supplied by Dr A Pridmore)
pCAT3-Basic (pCAT3B)	Promoterless CAT mammalian expression vector	Promega
C3:EGFP	Enhanced GFP mammalian expression vector where GFP is under the control of CMV promoter	Clontech (supplied by Dr C Rush)
pd2EGFP-Control (GFP-C)	Destabilised GFP mammalian expression vector where GFP is under the control of the SV40 promoter	Clontech
pd2EGFP-Basic (GFP-B)	Promoterless GFP mammalian expression vector	Clontech

2.5 Methods

2.5.1 Molecular Biology Techniques

2.5.1.1 Small scale preparation of plasmid DNA

A single colony was used to inoculate 3-5ml of Luria broth supplemented with the appropriate antibiotic and grown overnight at 37°C with shaking (approximately 230 rpm). The plasmid DNA was extracted from the cells using the alkaline lysis method described by Sambrook *et al.* (1989). The overnight culture was transferred to a 1.5ml eppendorf and the cells were harvested by centrifugation at 13 000 rpm for 3 min in a microfuge. The supernatant was removed from the cells and the cells resuspended in 100µl GTE solution (25mM Tris, pH8; 50mM glucose; 10mM EDTA) containing 100mg/ml Rnase A by vortexing. Then 200µl 1% SDS/0.2M NaOH was added and the tubes mixed by inversion before being placed on ice for 5 mins. To neutralise the solution 150µl KAc was added and the solution was mixed and placed on ice for a further 5 mins. After this incubation the tubes were centrifuged at 12 000 x g for 10 mins. The supernatant (0.4ml) was removed and transferred to a fresh microfuge tube. To this, 0.8ml of 100% ethanol was added and the solution was incubated at room temperature for 2 mins before being centrifuged at 12 000 x g for 3 mins. The supernatant was removed, taking care not to disturb the pellet, and 1ml of 70% ethanol was added. The tubes were centrifuged at 12 000 x g for 3 mins and the supernatant removed and the pellet left to air-dry. The pellet was the resuspended in 30µl of TE. The resuspended DNA was then stored at –20°C.

DNA prepared by this method was suitable for restriction enzyme digest and PCR. However, for DNA to be sequenced DNA was prepared using the Qiagen Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.5.1.2 Large scale preparation of plasmid DNA

Colonies from a freshly streaked agar plate were used to inoculate 100ml Luria broth supplemented with the appropriate antibiotic and grown at 37°C overnight with shaking (230 rpm). The cells were then harvested by transferring the cell suspension to 50ml Falcon tubes and centrifuging at 1 500 x g for 10 mins. Plasmid DNA was obtained using the Stratagene Midiprep Kit according to the manufacturer's instructions. DNA was eluted in either 1ml or 0.5ml elution buffer. If the DNA was to be used for transfection the eluate was divided between a number of tubes (each containing 200-250µl eluate) and 0.1 volume of NaAc pH 4.8 was added followed by 0.2 volumes of ice-cold 100% ethanol. The solutions were mixed and incubated on ice for 30 mins. The tubes were then centrifuged at 12 000 x g for 10 mins at 4°C. The supernatant was removed and the pellets were washed twice with 0.5ml 70% ethanol (12 000 x g for 2 mins at 4°C). The supernatant was removed and the pellet left to air-dry. Once dry, the pellet was resuspended in 30µl sterile dH_2O.

2.5.1.3 Small scale preparation of bacterial artificial chromosome (BAC) DNA

BAC DNA, obtained from the clones isolated from the library screen (sections 2.5.1.7 and 4.2.3), was prepared using a modified Qiagen protocol and the reagents supplied with the Qiagen DNA prep kits. A single colony from a freshly streaked agar plate was used to inoculate 3ml Luria Broth supplemented with 12.5µg/ml chloramphenicol and grown overnight with shaking at 37°C in a plastic universal. The following day the universal was centrifuged at 3 000 rpm for 10 mins. The supernatant was removed and the pellet was resuspended in 0.3ml Buffer P1 and the resuspended pellet was transferred to a 1.5ml eppendorf. To lyse the cells, 0.3ml Buffer P2 was added and the tubes were mixed by inversion. The tubes were left at room temperature for 5 mins. After this time, 0.3ml Buffer P3 was added and the tubes mixed by inversion before being placed on ice for 5 mins. The tubes were then centrifuged at 10 000 rpm for 10 mins at 4°C. The supernatant was added to an equal volume of ice-cold isopropanol and mixed by inversion. The tubes were

placed on ice for 5 mins and then centrifuged at 10 000 rpm for 15 mins at 4°C. The supernatant was removed and the pellets washed with 0.5ml 70% ethanol and centrifuged at 10 000 rpm for 5 mins at 4°C. The pellet was left to air-dry and resuspended in 30-40µl elution buffer.

2.5.1.4 Cloning DNA

2.5.1.4.1 Digestion of DNA with restriction enzymes

Digestions were carried out in 0.5ml eppendorf tubes. A typical 20µl reaction consisted of:

1-3µl DNA
2µl 10X enzyme buffer (supplied with the enzyme)
0.5µl enzyme (normally supplied at a concentration of 10 units/µl)
sterile distilled water to 20µl

The reaction mixture was centrifuged briefly and placed at 37°C for 1-3 hours. Reactions were typically carried out in a volume of 20µl. If larger amounts of DNA were to be digested then the volumes of reagents used were scaled up accordingly. If a double digest was carried out 1µl of each restriction enzyme was used in multi-core buffer (Promega) if the enzyme buffer was not compatible with both enzymes being used.

2.5.1.4.2 Separation of DNA bands through agarose gels

DNA fragments were separated by agarose gel electrophoresis using 1.5 % (w/v) agarose for smaller fragments (<1kb) and 0.5% (w/v) agarose for larger fragments (>1kb). Agarose was made up in 1X TAE (Amresco) with ethidium bromide added to a final concentration of 0.5µg/ml. Electrophoresis was carried out in a tank of TAE. Before loading the DNA samples onto the gel 0.2 volumes of loading buffer

(0.25% bromophenol blue; 0.25% xylene cyanol FF; 40% (w/v) sucrose in water) was added. The agarose gels were run at 100V for an appropriate time until the bands were clearly separated. The DNA bands were located using UV light.

To identify bands of the correct size a 1kb ladder (Gibco) was used. This has a range of 0.075-12.216kb.

2.5.1.4.3 Purification of DNA from agarose gels

DNA samples were run out through the agarose gel and the band to be purified was located under UV light and cut out using a scalpel. The DNA was then extracted from the gel using the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.5.1.4.4 Ligation

DNA that had been purified from agarose gels was used to set up the ligation reaction with an insert to vector ratio of approximately 3:1. The insert to vector ratio was determined by running various volumes of the purified DNA on an agarose gel and comparing the intensity of the bands by eye. The ligation reaction was set up as follows:

x μl insert
x μl vector
1μl ligase buffer
1μl ligase
x μl water (to bring final volume to 10μl)

The reactants were mixed gently together and then incubated at room temperature for 3-4 hours. The reaction was stopped by incubating the mixture at 70°C for 10 mins. The mixture was then used for transformation or stored at -20°C.

2.5.1.4.5 Bacterial transformation

Epicurian Coli XL1-blue competent cells or Epicurian Coli XL-10 competent cells (Stratagene) or Novablue Singles Competent cells (Novagen) were used for transformation. The DNA (1µl from a ligation reaction) was transformed into the cells according to the manufacturer's instructions. The transformation mixtures (200µl for XL1-blue and XL-10 competent cells; 50µl for Novablue cells) were plated on Luria agar containing the appropriate antibiotic. The plates were then incubated at 37°C overnight. For blue/white colour screening 100µl 2% X-gal (supplied by Melford Laboratories and made up in DMF) and 100µl 1mM IPTG (Melford Laboratories) were spread on the plates 1 hour before plating the bacteria.

2.5.1.4.6 Screening colonies for recombinants

If no colour screening was used random colonies were picked and if blue/white screening was used white colonies were picked from the plate and grown overnight in 3ml Luria broth with the appropriate antibiotic. DNA was prepared as described in section 2.5.1.1 and digested with enzymes that would release the insert. Positive clones were identified as those that gave bands of the expected size for insert and vector when analysed on an agarose gel and compared with the known molecular weights of a DNA marker. Additional confirmatory experiments included PCR on the putative positive clones and sequencing of the inserts.

Occasionally recombinants were screened by PCR using the method of Kim (1996). Colonies were picked from an agar plate using a sterile toothpick and streaked on a fresh agar plate containing the appropriate antibiotic before being inoculated in 20µl DNA Extraction buffer (pH 8.0) [1M KCl; 100mM Tris-HCl; 10mM EDTA] in 0.5ml eppendorf tubes and heated to 99°C in a PCR machine for 10 mins. The tubes were then placed on ice for 5 mins before being centrifuged for 5 mins to pellet the denatured cell debris. The supernatant was then transferred into a 1.5ml eppendorf and diluted 10-fold with sterile dH_2O. For PCR, 3µl of this was used in a 50µl PCR

reaction volume as described in section 2.5.1.5. The results of the PCR were confirmed by taking a colony from the streaked agar plate and preparing the DNA as described for screening recombinants by DNA digestion.

2.5.1.5 Polymerase Chain Reaction (PCR)

PCR reactions were carried out in 0.5ml eppendorfs and cycled in a thermocycler. Reactions were typically carried out in 100µl volumes but if a large number of reactions were to be carried out the volume was reduced to 50µl.

PCR reaction with Taq DNA polymerase (Gibco)

10µl 10X reaction buffer (supplied with the enzyme)

3µl 100mM $MgCl_2$

4µl dNTP (5mM dATP, dCTP, dGTP, dTTP)

2µl Primer 1 (60pmol/µl)

2µl Primer 2 (60pmol/µl)

2µl DNA

0.5µl Taq

76.5µl sterile distilled water

PCR reaction with Vent DNA polymerase (New England Biolabs)

10µl 10X reaction buffer (supplied with the enzyme)

2µl 50mM $MgSO_4$

4µl dNTP (5mM dATP, dCTP, dGTP, dTTP)

2µl Primer 1 (60pmol/µl)

2µl Primer 2 (60pmol/µl)

2µl DNA

0.5µl Vent

77.5µl sterile distilled water

The reactions were centrifuged briefly and were then overlaid with 30µl of mineral oil to prevent evaporation of the reaction during cycling. The reactions were heated to 94°C to denature the DNA; then cooled to allow annealing of the primers (this temperature was dependent on the annealing temperature of the primers and varied from 50-60°C); then heated to 72°C to allow primer extension. The length of time at 72°C was dependent on the size of the sequence to be amplified (as a rough guide 1 min. was allowed for every 1kb of DNA to be amplified). A typical reaction was cycled at:

95°C for 3 mins
94°C for 45 sec)
55°C for 30 sec) 30 cycles
72°C for 1 min)
72°C for 5 mins

For analysis, 10µl of the PCR product were run through an agarose gel by electrophoresis.

2.5.1.5.1 Cloning PCR products in PCR Script

For analysis and sequencing the PCR products were cloned in PCR Script according to the manufacturer's instructions.

2.5.1.6 DNA sequencing and analysis

DNA sequencing was carried out by BaseClear (Leiden, the Netherlands), at the PNACL (Leicester University, UK) or at the MBSU at Glasgow University, UK. The reactions were carried out on either LiCor or ABI Sequencers with a combination of Dye-primer and Dye terminator chemistry. DNA sequences were assembled using SeqMan (DNAStar software package) and sequences were analysed using other programs from the DNAStar package, GCG (Devereux *et al.*, 1984),

NIX (HGMP), BLAST (Altschul *et al.*, 1997) and various transcription factor search programs found on the TRANSFAC home page. The website addresses of these search engines are given in Table 2.1.

2.5.1.7 Screening the porcine PigE BAC genomic library

The PigE BAC library (Anderson *et al.*, 2000) was screened with the assistance of Dr S Anderson, Roslin Institute, Edinburgh, UK using the porcine ITIH4 cDNA clone described by Buchman *et al.* (1990) as a probe. Details of the library can be found at: http://www.hgmp.mrc.ac.uk/Biology/descriptions/pig_BAC.html

2.5.1.7.1 Preparation of the filters

The genomic library has been gridded on Hybond N nylon membranes, which are stored at -20°C. The filters were allowed to thaw and were placed in hybridisation solution (7% SDS; 0.5M $NaHPO_4$ (pH7.2); 1mM EDTA). The filters were separated from each other with nylon mesh. This prevents a lot of background if the filters overlap and also allows the probe to bind more efficiently. The filters were rolled up and placed in the hybridisation tube (3 filters per tube) with 15ml of hybridisation solution and the tubes were placed at 65°C.

2.5.1.7.2 Hybridisation and identification of positive clones

While the procedure described in section 2.5.1.7.1 was carried out, the probe was labelled using the High Prime System (Boehringer/Roche) ^{32}P-labelled dCTP according to the manufacturer's protocol. 500µl of the hybridisation solution was added to the labelled probe and 250µl of this solution was added to each hybridisation tube and incubated overnight at 65°C with gentle rotation.

The following day the filters were washed twice with Wash 1 (2x SSC; 0.1% SDS) and then washed twice for 15 mins with Wash1 at 65°C. The filters were then

Table 2.1: Website addresses for sequence search engines

Search engine	Web address
BLAST	http://www.ncbi.nlm.nih.gov/BLAST
NIX	http://www.hgmp.mrc.ac.uk
TRANSFAC	http://transfac.gbf.de
MatInspector	Accessed via the TRANSFAC home page
Alibaba 2	http://wwwiti.cs.uni-magdeburg.de/~grabe/alibaba2

washed twice with Wash 2 (1x SSC; 0.1% SDS) at 68°C for overnight. The filters were then exposed at -70°C for approximately 5 hours. The films were developed and positive clones were identified by aligning the films with the original filters and working out the position of the clone using the guide supplied by the HGMP. The putative positive clones were then picked from the glycerol stock at -70°C and streaked onto Luria agar plates containing chloramphenicol (12.5μg/ml) and incubated at 37°C overnight.

2.5.1.8 List of primers used

A list of primers used during this project is given in Appendix 1.

2.5.1.9 List of constructs made

A list of constructs made during this project is given in Appendix 2.

2.5.2 Tissue Culture Methods

2.5.2.1 Standard passage of Hep 3B cell line

Hep 3B cells were obtained from the ECACC and grown in Minimal Essential Medium Eagle M2279 (Sigma) supplemented with 10% foetal bovine serum (Gibco), 1% non-essential amino acids (Gibco), 1% L-glutamine (Gibco) (referred to here as the growth medium). Once the cells were confluent the growth medium was removed and the cell monolayer washed twice with sterile PBS (PBS tablets (Oxoid) dissolved in dH_2O and autoclaved). To split the cells, 5ml trypsin/EDTA (Gibco) was added to the flask and any excess was removed. The flasks were then incubated at 37°C for 5 mins. The cells were resuspended in 10ml growth medium. The resuspended cells were divided between a number of $75cm^2$ flasks and 25ml growth medium added. The caps were loosely closed and the flasks were incubated at 37°C with 5% CO_2.

2.5.2.1.1 Standard passage of COS-7 cell line

COS-7 cells were grown in Dulbecco's Modified Eagles Medium (Gibco) supplemented with 10% FBS and 1% L-glutamine (Gibco) (referred to as the growth medium). Once the cells were confluent they were split as described for Hep 3B cells in section 2.5.2.1. When the cells had detached from the flask the cells were resuspended in 10ml growth medium. The resuspended cells were divided between a number of 75cm^2 flasks and 25ml growth medium added. The caps were loosely closed and the flasks were incubated at 37°C with 5% CO_2.

2.5.2.1.2 Isolation and growth of primary porcine hepatocytes

Primary porcine hepatocytes were isolated by Mr L Nelson and Mr S Keach from a non-fasting pig weighing approximately 13kg using the method of Nelson *et al.* (2000). The isolated hepatocytes were transported from Edinburgh to Glasgow on ice and then seeded in 6 well or 60mm plates immediately at a density of 120 000 viable cells /cm^2. The cells were grown in Williams E medium with supplements as described by Nelson *et al.* (2000). The following day the cells were washed using Hanks Balanced Salt Solution and fresh medium was added. The cells were incubated for a further 24 hours before transfections were carried out.

2.5.2.2 Cryopreservation of mammalian cells

The cells were grown until confluent in 75 cm^2 flasks and were then trypsinised (as described in section 2.5.2.1.1) and resuspended in 10ml growth medium. The cells were pelleted by centrifugation (3 000 rpm for 4 mins) and the supernatant was discarded. The cells were then resuspended in 1-3ml (depending on the size of the pellet) growth medium. The cells were aliquoted into 900µl quantities in cryotubes and 100µl DMSO was added. The cells were then frozen to -70°C by placing them in a box surrounded by isopropanol in a -70°C freezer overnight. The cryotubes were then transferred to a liquid nitrogen store.

2.5.2.3 Recovery of cells from liquid nitrogen storage

A vial of cells was removed form liquid nitrogen storage and quickly defrosted at 37°C. The cells were resuspended in 10ml growth medium and then pelleted by centrifugation at 200 x g for 10 min. The supernatant was discarded and the pellet resuspended in 5ml of growth medium. The cell suspension was transferred to a 25cm^2 flask and incubated at 37°C with 5% CO_2.

2.5.2.4 Counting cells using a haemocytometer

Following resuspension of the cells in growth medium as described in section 2.5.2.1 and 2.5.2.1.1, 200µl were transferred to a 1.5ml eppendorf containing 300µl growth medium. In order to visualise the cells 500µl Trypan Blue was added and the contents of the tube were mixed. With the coverslip of the haemocytometer in place a small volume of the suspension was transferred to the counting chambers by capillary action. The number of cells in 10 squares was counted. Each square represents a total volume of 0.1mm^3 (10^{-4} cm^3). The cell concentration per ml was determined as follows:

Cells per ml = average number of cells per square x dilution factor x 10^4

2.5.2.5 Transfection of DNA into mammalian cells

2.5.2.5.1 Seeding cells for transfection

Once the cells became confluent, the cells were split as described in section 2.5.2.1. Following resuspension in 10ml growth medium the cells were centrifuged at 1 500 rpm for 3 mins. The supernatant was removed and the cells were resuspended in 5ml PBS and centrifuged again. The cells were then resuspended in 5ml growth medium and the number of cells per ml was determined using a haemocytometer. In 6 well plates cells (both Hep 3B and COS-7) were seeded at a density of 2x10^5 cells

in a final volume of 2ml and in 24 well plates the cells were seeded at a density of $4x10^4$ cells in a final volume of 1ml. The cells were then incubated at 37°C with 5% CO_2 overnight. The next day the cells were normally 60-80% confluent and ready for transfection.

2.5.2.5.2 Transfection of mammalian cells

Transfections were carried out using GenePorter–2 transfection reagent (Gene Therapy Systems) according to the manufacturer's instructions. Experiments were carried out to determine the optimal amount of DNA to use for transfection of Hep 3B cells. In the experiments described in this thesis, 2μg DNA was used for transfections in 6 well plates (experiments using the CAT reporter gene) and 0.75μg DNA for transfections in 24 well plates (experiments using the GFP reporter gene).

2.5.2.6 Interleukin stimulation of transfected cells

Stocks of human recombinant interleukin-6 (Sigma) and human recombinant interleukin-1β (R&D Systems) were made up in PBS/0.1% BSA and frozen in single use aliquots at -70°C. Approximately 24 hours post-transfection the growth medium was removed from the cells and the cells were washed with PBS. Sufficient medium for the experiments was pre-warmed to 37°C and supplemented with 1% non-essential amino acids, 1% L-glutamine (no FBS was used in these experiments in control or test samples) and with or without (controls) the appropriate concentration of cytokines. For cells growing in 6 well plates 2ml stimulation medium was added. For cells growing in 24 well plates 1ml stimulation medium was added. The cells were then incubated at 37°C with 5% CO_2 for a further 24-48 hours.

2.5.2.7 Harvesting of transfected cells

Cells were harvested using the lysis buffer (solution 8) supplied with the CAT ELISA kit (Roche Molecular Biochemicals) diluted 1:5 prior to use. The cells were

washed 3 times with 2ml of cold PBS (4°C) and 0.5ml lysis buffer was added to each well of a 6 well plate. The cells were left at room temperature for 30 mins and the supernatant was then transferred to a 1.5ml eppendorf. The cell extract was centrifuged at 13 000 x g for 10 mins at 4°C. The supernatant was transferred to a fresh eppendorf and frozen in an ice/ethanol bath before being stored at -70°C until analysis.

2.5.2.8 Analysis of cell extracts using CAT ELISA

For measuring promoter activity using the CAT reporter gene a CAT ELISA (Roche Molecular Biochemicals) was used according to the manufacturers instructions. Briefly, the microtiter plates are supplied with pre-bound antibody to CAT. The cell extracts containing the CAT enzyme are added to the wells and the enzyme binds to the anti-CAT antibodies on the surface of the microtiter plate. A digoxigenin-labelled antibody to CAT (anti-CAT-DIG) is then added and binds to CAT. Then an antibody to digoxigenin conjugated to peroxidase (anti-DIG-POD) is added and binds to digoxigenin. The peroxidase substrate ABTS is added and the peroxidase enzyme catalyses the cleavage of the substrate giving a coloured reaction product. The absorbance of the sample was determined using a Dynatech Microplate Reader at 450nm (reference wavelength 490nm).

2.5.2.9 Total protein determination using Bradford Reagent

The results from the CAT ELISA were normalised with respect to protein concentration using the method of Bradford (1976). Standard curves were prepared by diluting a 3mg/ml stock solution of BSA in PBS 1:2 giving a range from 3000μg/ml to 46.875μg/ml. 5μl of sample, standard or PBS (blank) were added to the wells of a flat bottomed 96 well plate. To correct the calibration curve (due to interference with the assay by the cell lysis buffer) 5μl of the diluted lysis buffer was added to the wells containing the standards and the blank and 5μl PBS was added to the wells containing the test samples. 200μl of Bradford Reagent was then added to

each well and the samples were incubated at room temperature for approximately 10 mins. The absorbance was then read at 600nm on a Dynatech microplate reader. Total protein concentrations of the samples was determined using BioLinx software.

2.5.2.10 Quantitating GFP fluorescence

GFP fluorescence was detected using a Zeiss Axioskop 20 microscope equipped with a HB50 microscope illuminator. Filter set 09 (Zeiss) was used. The filters used were as follows:

- Excitation: BP450-490nm
- Beamsplitter: FT510
- Emission: LP520

Fluorescent cells were visualised using a PC-CAM video camera and the fluorescence quantitated using Openlab software (Improvision). To quantitate GFP fluorescence, an image from a field of view of cells transfected with the GFP-B vector was taken. This image served as the background and it was "subtracted" from images containing fluorescent cells using the 'subtract background' function in the Openlab program. This meant that the fluorescence being measured was actual GFP fluorescence rather than GFP fluorescence plus background fluorescence. The fluorescence from a number of cells (at least 100 if possible) was measured using the 'Measurement' function in Openlab. To carry out this procedure individual cells were outlined to create a 'region of interest' (ROI). The software calculated the fluorescence present by calculating the mean number of pixels present in the ROI. To calculate the total fluorescence intensity per cell, the mean number of pixels of the ROI was multiplied by the area of the ROI. The results presented in this thesis are the mean values of the total fluorescence intensity of the selected cells.

2.5.2.11 Statistical analysis

Statistical analysis was carried out using StatView software. The results in this thesis are reported as the mean ± standard error of the mean and are the results from

one set of experiments performed in triplicate. For statistical analysis, samples receiving treatment were compared individually with the sample containing no treatment using the Mann Whitney U Test. The ANOVA Test (with Bonferroni correction) was used to compare samples with each other. P values <0.05 were deemed significant.

Results

Chapter 3: Further characterisation of the CRP-based acute phase expression vector

3.1 Introduction

Ciliberto *et al.* (1987b) screened a human genomic library and isolated a clone containing a 30kb insert containing the CRP gene. They subsequently showed that this 30kb fragment contained all the sequences required for correct regulation of the human CRP gene as an acute phase protein in transgenic mice (Ciliberto *et al.*, 1987a). Further work by Murphy *et al.* (1995) to delineate the regions necessary for controlled expression in transgenic mice revealed that there were sequences both upstream and downstream of the CRP gene involved in regulating its expression. As discussed in section 1.4 we have developed an expression system based on the constructs analysed by Murphy *et al.* (1995). Although the system is functional in transgenic mice, it requires some modification in order to obtain more tightly regulated expression and to eliminate the sexually dimorphic pattern of expression seen in the transgenic mice.

This chapter will describe the sequencing and analysis of the 30kb fragment containing the human CRP gene in order to identify regions of the fragment that are important for the controlled expression of the CRP gene and the sexually dimorphic pattern of expression that is seen in transgenic mice.

3.2 Sequencing of the region approximately 15kb upstream of the human CRP gene

3.2.1 Background and strategy

The 30kb fragment can be subdivided into four smaller fragments as illustrated in Figure 3.1 – a 15kb *Cla*I – *Bam*HI fragment, a 5.6kb *Bam*HI – *Bam*HI fragment, a 6.6kb *Bam*HI – *Bam*HI fragment and a 3kb *Bam*HI – *Cla*I fragment. Previous work in our laboratory by Dr B Burke involved the subcloning of the 5.6kb, 6.6kb and 3kb fragments into the pUC vector, which were then sequenced by primer walking. In this method of sequencing, sequencing commences from each end of the fragment to be sequenced. Primers are then designed from the newly generated sequence to continue sequencing and so on until the sequences overlap. The sequences are then assembled into contigs using sequence assembly programs.

This section of the project continued the work started by Dr B Burke to complete the sequencing of the 30kb fragment. Before commencing to sequence the 15kb upstream region of the 30kb fragment the sequence obtained by Dr B Burke was compared with those in the nucleotide database. This analysis was carried out to see if it was possible to identify a clone being sequenced as part of the human genome sequencing project containing the human CRP gene. The sequences were analysed using the NIX program in late 1998 but no evidence was obtained that such a clone existed at that time. Therefore, it was decided to proceed with sequencing the 15kb upstream region of the 30kb fragment.

For sequencing the upstream region of the 30kb fragment, the clone 15kb3D was used (see Figure 3.1). This clone had been made previously by Mr D Hayward and it lacks the 5.6kb and 6.6kb *Bam*HI - *Bam*HI fragments. For sequencing the 15kb3D clone there were two possibilities:

1. Sequence the entire clone by primer walking and
2. Subclone the 15kb3D clone and sequence the subclones.

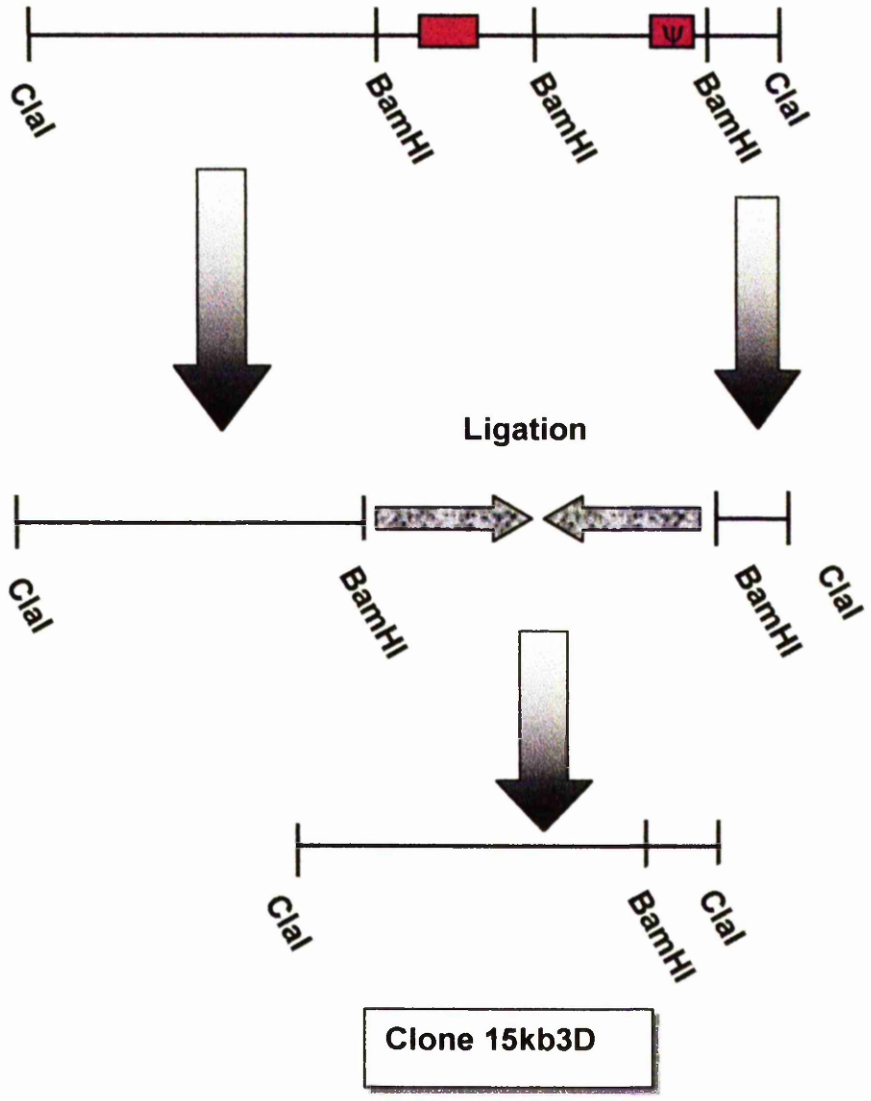


Figure 3.1: Illustration of the 30kb CRP fragment and the creation of clone 15kb3D. The CRP gene and the CRP pseudogene (ψ) are indicated by pink boxes. Clone 15kb3D was created by digesting the 30kb fragment with *Bam*HI to release the 5.5kb and 6.5kb *Bam*HI-*Bam*HI fragments. The 15kb *Cla*I-*Bam*HI fragment was then ligated to the 3kb *Bam*HI-*Cla*I fragment.

It was decided to take the second approach. This involved making a restriction map of Clone 15kb3D, cloning fragments of a suitable size and then sequencing the clones by primer walking. This work is described in the following sections.

3.2.2 Restriction map of Clone 15kb3D

Clone 15kb3D was first of all digested with *Cla*I and *Bam*HI to verify the DNA was indeed from Clone 15kb3D. The plasmid DNA was subsequently digested with enzymes not located in the vector because this would allow mapping of the insert and identification of fragments for subcloning. Initially the clone was digested with a single enzyme. The enzymes chosen were: *Acc*I, *Apa*I, *Kpn*I, *Nde*I and *Spe*I. Figure 3.2 shows the results of these digests.

As seen in Figure 3.2, most of the fragments formed were greater than 12kb. This suggested that combinations of these enzymes could be suitable for creating a restriction map. To begin mapping, a double digest with one of the chosen enzymes and *Cla*I was carried out because the position of the restriction sites for this enzyme in the clone was already known. In addition, a double digest with *Kpn*I and *Spe*I was also carried out. An example of these digests is illustrated in Figure 3.3. Table 3.1 summarises the results from a number of digests and from these results a putative restriction map was drawn (Figure 3.4).

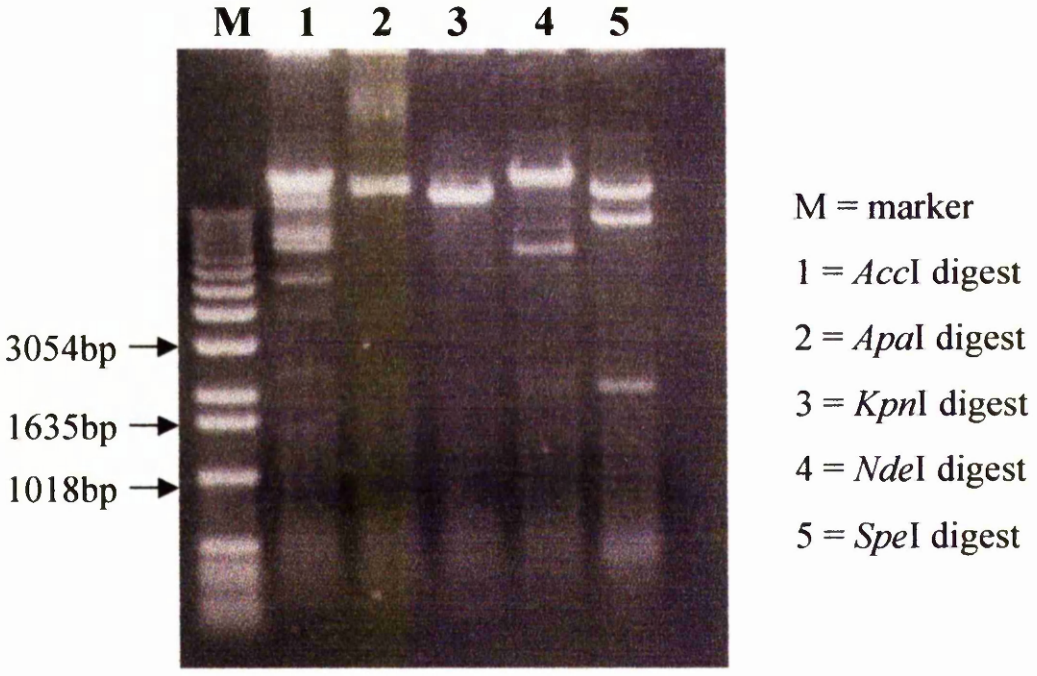


Figure 3.2: Digest of 15kb3D with *Acc*I, *Apa*I, *Kpn*I, *Nde*I and *Spe*I. Relative bands on the DNA ladder are indicated with arrows.

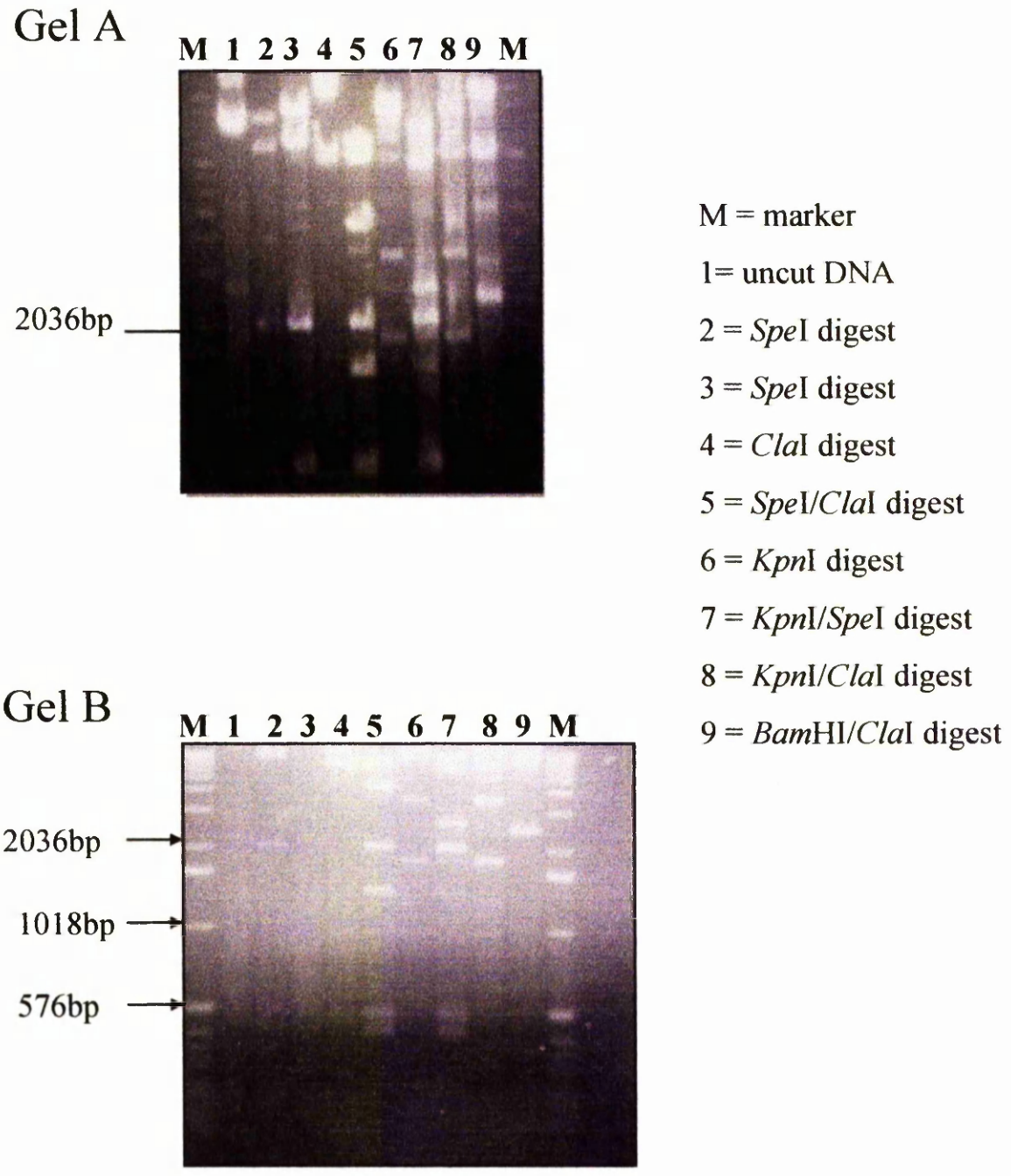


Figure 3.3: Digest of 15kb3D with *Kpn*I, *Spe*I and *Cla*I to make a restriction map. Gel A shows the digested fragments on a 0.5% agarose gel and Gel B shows the digested fragments on a 1.5% agarose gel. Relative bands on the DNA ladder are indicated by arrows.

Table 3.1: List of enzymes and size of fragments obtained following digestion of the 15kb3D clone.

Enzyme(s)	Size of fragments formed after digest (bp)
*Spe*I	15,000; 10,000; 2,000
*Kpn*I	13,000; 13,000
*Cla*I	18,000; 8,000
*Kpn*I/*Cla*I	13,000; 8,000; 3,500; 1,900
*Kpn*I/*Spe*I	10,000; 8,000; 2,500; 2,000; 570; 394

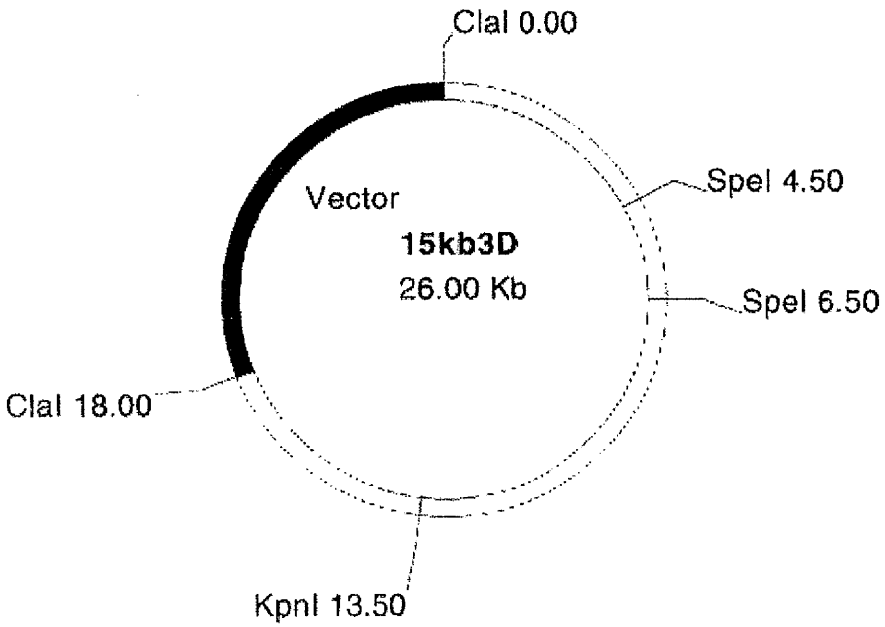


Figure 3.4: Putative restriction map of 15kb3D

3.2.3 Subcloning 15kb3D and identification of positive clones

Analysis of the restriction map showed that there were a number of fragments that were of suitable size for subcloning. It was decided to subclone the following fragments: *Spe*I/ *Cla*I (4.5kb) named CSI; *Spe*I/ *Spe*I (2kb) named SI; *Kpn*I/ *Spe*I (7kb) named SK7; *Cla*I/ *Kpn*I (3.5kb) named CKI as illustrated in Figure 3.5 (Gel A).

Following digestion of the 15kb3D clone and the standard cloning vector, pBluescript SK- (Stratagene), with the appropriate enzymes the CSI, CKI, SK7 and SI fragments and the digested vector were cut from the agarose gel and the DNA extracted using the Qiagen Gel Extraction Kit. The digested vector was then ligated to the appropriate 15kb3D digest and transformed in XL1-Blue competent cells. Following transformation a number of colonies were picked and plasmid DNA was isolated from each. Positive CKI and CSI clones were identified following digestion of the DNA with *Cla*I to linearize the plasmid, which gave a fragment of 7.5kb (CSI) and 6.5kb (CKI). The result was confirmed by digesting the clone with the appropriate enzymes to release the insert (Figure 3.5, Gels B, C and D).

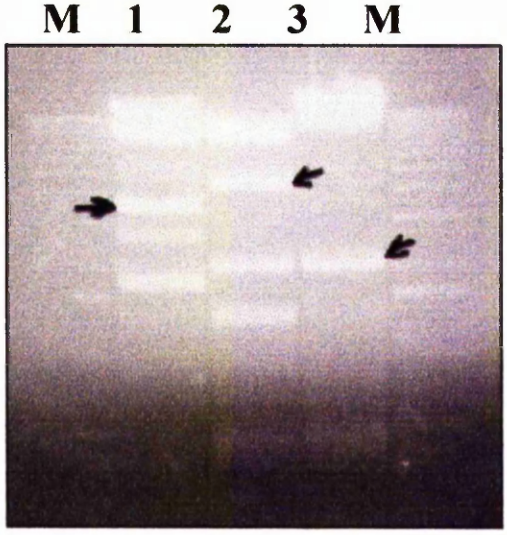
Figure 3.5: Isolation of fragments for cloning and identification of positive CKI and CSI clones. A small arrow indicates the 1.6kb band (the brightest band) of the DNA ladder in each picture.

Gel A: Digestion of 15kb3D with the appropriate enzymes to release fragments of a size suitable for subcloning. The fragments chosen for cloning are indicated with arrows.

Gel B: Identification of positive CKI clones. M = marker; Lanes 1 and 2 contain undigested pBluescript. Lanes 3-6 contain DNA digested with *Cla*I from putative positive clones containing the 3.5kb CKI fragment. The expected size of a fragment from a positive clone is 6.5kb. Therefore, lanes 3, 4 and 6 appear to be positive.

Gel C: Identification of positive CSI clones. M = marker; Lanes 1-12 contain DNA digested with *Cla*I from putative positive clones containing the 4.5kb CSI fragment. The expected size of a fragment from a positive clone is 7.5kb. Therefore, lane 5 appears to be positive.

Gel D: Confirmation of positive clones. The sample in Gel B lane 6 was digested with *Cla*I and *Kpn*I to release the insert (lane 1). This reaction gives two fragments of the expected size (3kb vector and 3.5kb insert). Sample 5 in Gel C was digested with *Cla*I and *Spe*I to release the insert (lane 2). This reaction also gives two fragments of the expected size (3kb vector and 4.5kb insert).



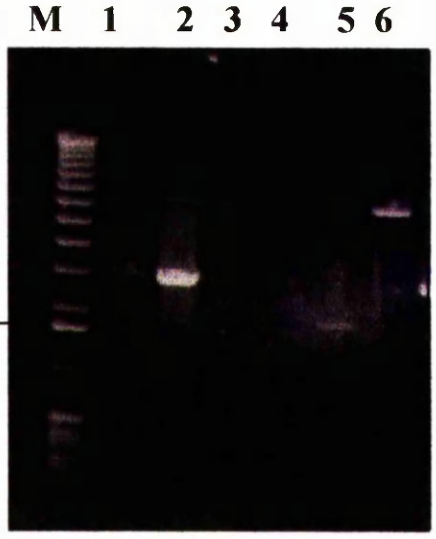
Gel A

M = marker

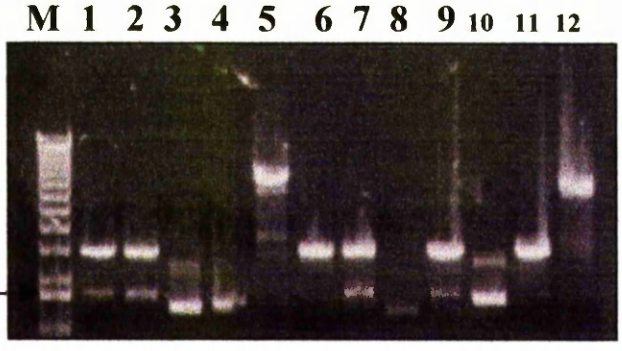
1 = *Cla*I/*Kpn*I digest

2 = *Cla*I/*Spe*I digest

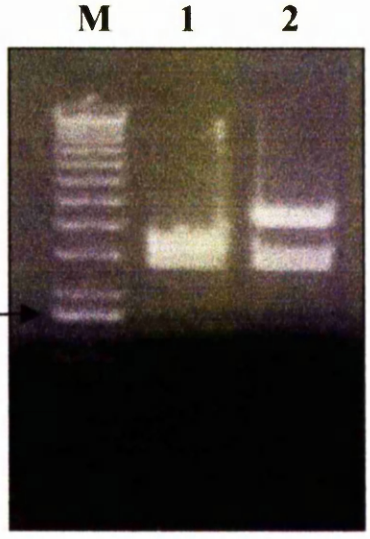
3 = *Spe*I digest



Gel B



Gel C



Gel D

Attempts to clone the 2kb *Spe*I/*Spe*I fragment into pBluescript SK- were not successful. This was thought to be because *Spe*I was the only enzyme cutting the vector thus increasing the probability of the vector self-ligating. Therefore, it was decided to clone the SI fragment into a different vector, pZErO-2 (Invitrogen). This vector encodes a protein that is lethal to the cell unless the gene is disrupted by the insertion of foreign DNA. Theoretically any surviving cells should contain the insert. Cloning the SI fragment in pZErO-2 was successful and positive clones are shown in Figure 3.6. Cloning the SK7 fragment into pBluescript was also difficult, possibly due to the large size of this fragment. Although subcloning this fragment was considered, it was first of all decided to try cloning this fragment into pZErO-2. This strategy was also successful and positive clones are shown in Figure 3.6.

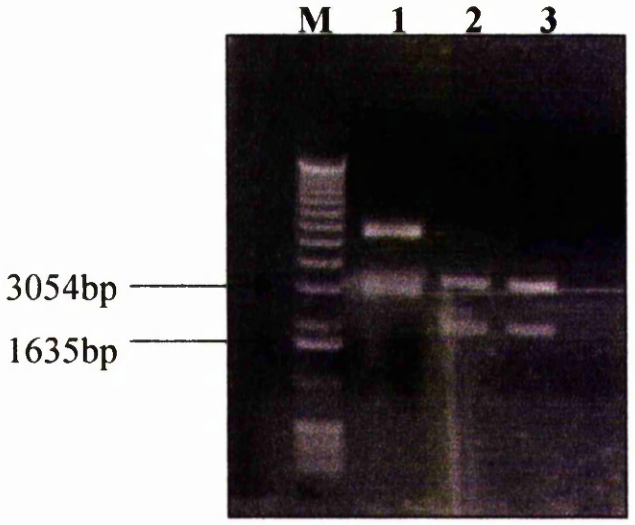
3.2.4 Sequencing the subclones

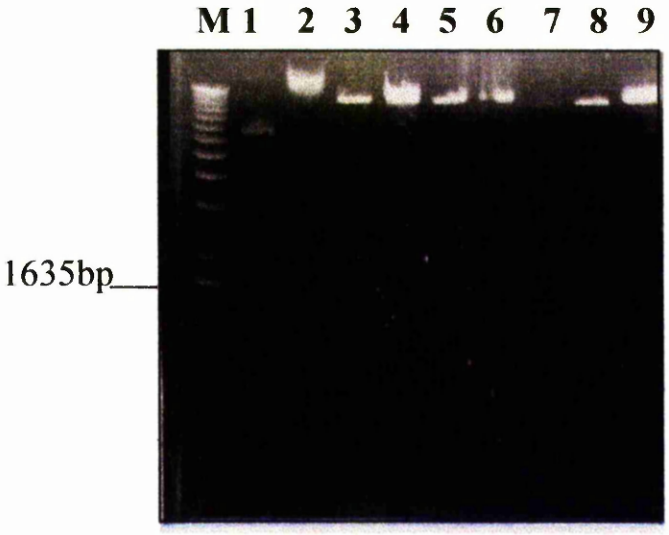
The subclones were sequenced by BaseClear (Leiden, the Netherlands) by primer walking on one strand only. The first set of reactions was carried out using standard primers located in the vectors (T7 and M13 reverse). For the next round of sequencing primers were designed from near the end of the newly generated sequence and used to amplify the middle sections of the subclones. These PCR products were then cloned in PCRScript and sequenced using standard primers. The reason for this additional cloning was that longer sequencing runs could be obtained using standard primers. The only exception to this strategy was the SK7 fragment, which was sequenced entirely by primer walking by BaseClear. The sequences were assembled into contigs using the sequence assembly program SeqMan. When the subclones had been sequenced primers were designed to link the fragments together and then to link the 15kb upstream region to the 15kb downstream region that had been sequenced by Dr B Burke. The assembly of the 30kb fragment is illustrated in Figure 3.7.

Figure 3.6: Identification of positive SI and SK7 clones for sequencing

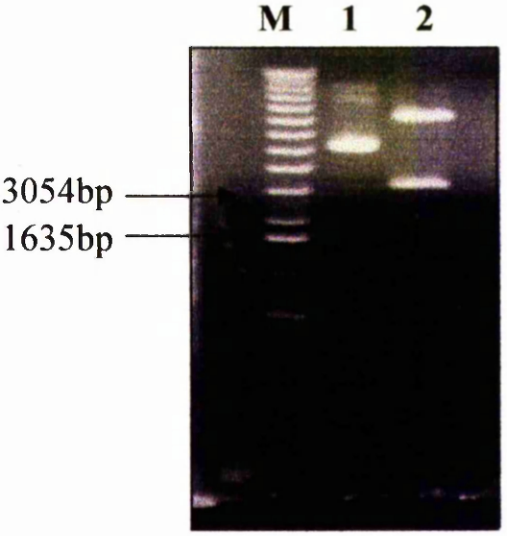
Gel A: Identification of positive SI clones by digesting DNA from putative positive clones with *Spe*I. Lanes 1-3 show plasmid DNA from different clones digested with *Spe*I. Positive clones are those which give two fragments of 3.3kb (vector) and 2kb (insert). Therefore, samples in lanes 2 and 3 are positive. The 1.6kb band of the ladder is indicated with an arrow.

Gels B and C: Identification of positive SK7 clones by digesting DNA from putative positive clones with *Kpn*I. Lanes 1-9 of Gel B show plasmid DNA from different clones digested with *Kpn*I. Positive clones are those which give a fragment of 10.3kb. Therefore, samples in lanes 2-9 are positive. This result was confirmed by digesting one of the clones with *Kpn*I and *Spe*I (Gel C). Lane 1 of Gel C shows undigested DNA. Lane 2 shows a positive clone digested with *Kpn*I and *Spe*I, which gives two bands of the expected size of 3.3kb (vector) and 7kb (insert). The 1.6kb and 3kb bands of the ladder are indicated with arrows.



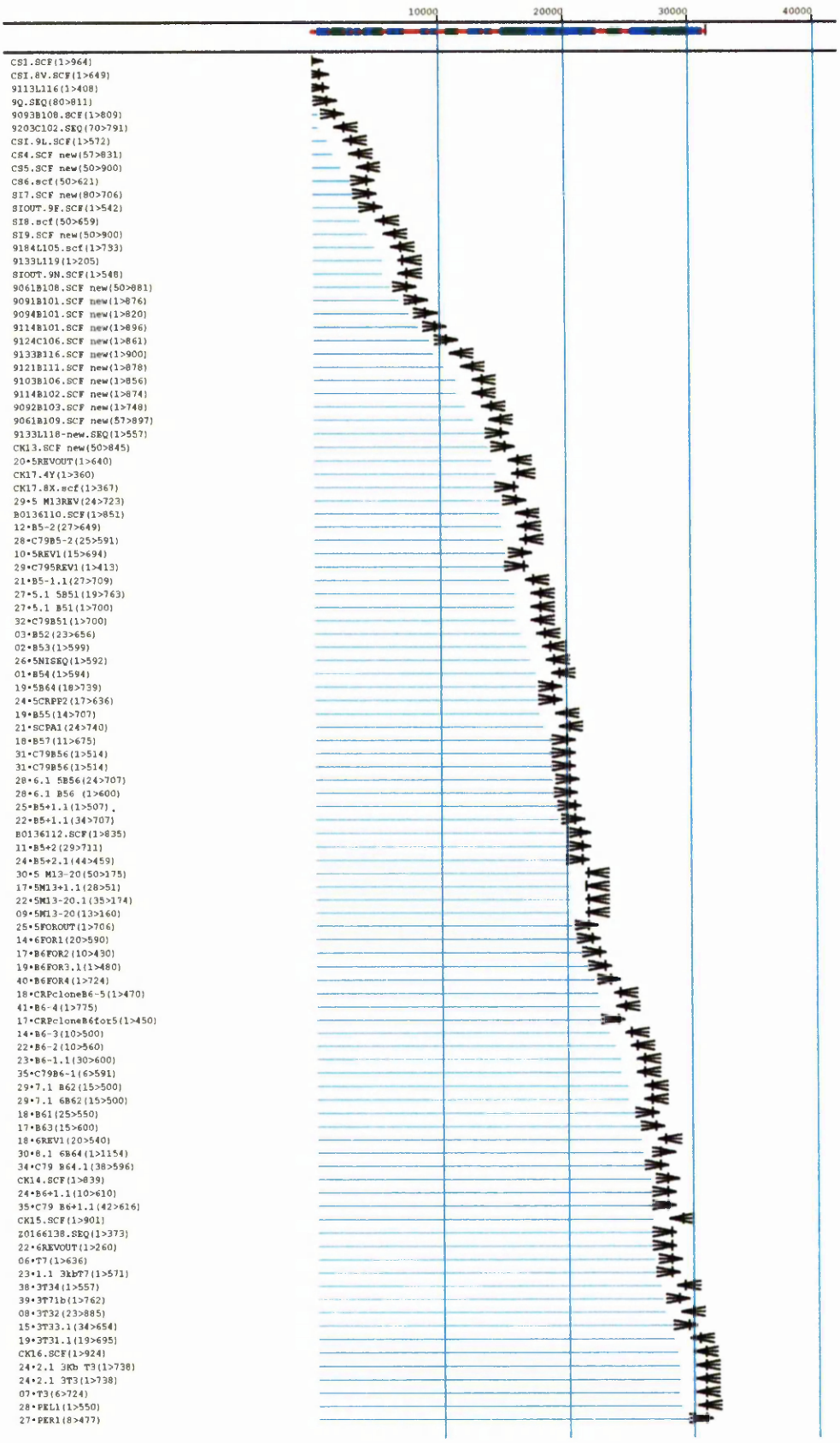


Gel B



Gel C

Figure 3.7: Assembly of the sequences of the 15kb *Cla*I/*Bam*HI CRP fragment and linkage to the downstream *Bam*HI/*Cla*I fragment sequenced by Dr B Burke. The sequences were assembled using the sequence assembly program SeqMan. The arrows refer to the DNA strand on which the sequencing reactions were carried out (forward facing arrows refer to sequencing on the positive strand and backward facing arrows refer to sequencing on the negative strand).


10000
20000
30000
40000
CS1.SCF(1>964)
CSI.8V.SCF(1>649)
9113L116(1>408)
9Q.SEQ(80>811)
9093B108.SCF(1>809)
9203C102.SEQ(70>791)
CSI.9L.SCF(1>572)
CS4.SCF new(57>831)
CS5.SCF new(50>900)
CS6.scf(50>621)
SI7.SCF new(80>706)
SIOUT.9E.SCF(1>542)
SI8.scf(50>659)
SI9.SCF new(50>900)
9184L105.scf(1>733)
9133L119(1>205)
SIOOT.9N.SCF(1>548)
9061B108.SCF new(50>881)
9091B101.SCF new(1>876)
9094B101.SCF new(1>820)
9114B101.SCF new(1>896)
9124C106.SCF new(1>861)
9133B116.SCF new(1>900)
9121B111.SCF new(1>878)
9103B106.SCF new(1>856)
9114B102.SCF new(1>874)
9092B103.SCF new(1>748)
9061B109.SCF new(57>897)
9133L118-new.SEQ(1>557)
CK13.SCF new(50>845)
20•5REVOUT(1>640)
CK17.4Y(1>360)
CK17.8X.scf(1>367)
29•5 M13REV(24>723)
B0136110.SCF(1>851)
12•B5-2(27>649)
28•C79B5-2(25>591)
10•5REV1(15>694)
29•C795REV1(1>413)
21•B5-1.1(27>709)
27•5.1 5B51(19>763)
27•5.1 B51(1>700)
32•C79B51(1>700)
03•B52(23>656)
02•B53(1>599)
26•5NISEQ(1>592)
01•B54(1>594)
19•5B64(18>739)
24•5CRPP2(17>636)
19•B55(14>707)
21•SCPA1(24>740)
18•B57(11>675)
31•C79B56(1>514)
31•C79B56(1>514)
28•6.1 5B56(24>707)
28•6.1 B56 (1>600)
25•B5+1.1(1>507)
22•B5+1.1(34>707)
B0136112.SCF(1>835)
11•B5+2(29>711)
24•B5+2.1(44>459)
30•5 M13-20(50>175)
17•5M13+1.1(28>51)
22•5M13-20.1(35>174)
09•5M13-20(13>160)
25•5FOROUT(1>706)
14•6FOR1(20>590)
17•B6FOR2(10>430)
19•B6FOR3.1(1>480)
40•B6FOR4(1>724)
18•CRPcloneB6-5(1>470)
41•B6-4(1>775)
17•CRPcloneB6for5(1>450)
14•B6-3(10>500)
22•B6-2(10>560)
23•B6-1.1(30>600)
35•C79B6-1(6>591)
29•7.1 B62(15>500)
29•7.1 6B62(15>500)
18•B61(25>550)
17•B63(15>600)
18•6REV1(20>540)
30•8.1 6B64(1>1154)
34•C79 B64.1(38>596)
CK14.SCF(1>839)
24•B6+1.1(10>610)
35•C79 B6+1.1(42>616)
CK15.SCF(1>901)
Z0166138.SEQ(1>373)
22•6REVOUT(1>260)
06•T7(1>636)
23•1.1 3kbT7(1>571)
38•3T34(1>557)
39•3T71b(1>762)
08•3T32(23>885)
15•3T33.1(34>654)
19•3T31.1(19>695)
CK16.SCF(1>924)
24•2.1 3Kb T3(1>738)
24•2.1 3T3(1>738)
07•T3(6>724)
28•PEL1(1>550)
27•PER1(8>477)

3.2.5 Sequence details

The sequence has been lodged in the GenBank database (accession number AF442818) and the length of the sequence is 30234 bases. The CRP gene in this sequence is located from 18026-18980, the bone fide cap site (Arcone *et al.*, 1988) at position 17922, PolyA at position 20195 and the CRP pseudogene is from position 26533 to 27357. A restriction map of the 30kb fragment using the enzymes used to map the 15kb upstream region shows that the rough restriction map created in section 3.2.2 was quite accurate (Figure 3.8) although this region appears to be slightly longer in length than first predicted (16kb rather than 15kb). One difference between this sequence and that reported by Murphy *et al.* (1995) is the absence of the *Bam*HI site in the 15kb upstream region at position –12.7kb. The sequencing result confirms the experimental findings of Dr B Burke who noticed that this site appeared to be absent in the 30kb CRP fragment that we have.

3.2.6 A restriction map of the 30kb CRP fragment

In order for the heterologous gene to be controlled with acute phase kinetics it is desirable to clone the fusion construct (acute phase promoter-heterologous gene) in the background of the 30kb fragment. Initial attempts at using the 5.6kb *Bam*HI – *Bam*HI fragment as a shuttle vector have been unsuccessful as the fragment was incorporated into the 30kb fragment in the wrong orientation (Williams, 2001). Therefore, the ability to identify unique restriction sites that may facilitate the incorporation of a shuttle vector in the correct orientation may facilitate cloning the fusion construct in the background of the 30kb fragment. Since we now have the entire sequence of the 30kb CRP fragment it is possible to create a detailed restriction map.

The 30kb fragment was searched for unique restriction enzyme sites using the MapDraw program. A list of the unique restriction sites found is given in Table 3.2. The results show that there are two unique sites flanking the CRP gene, *Bco* 63I at

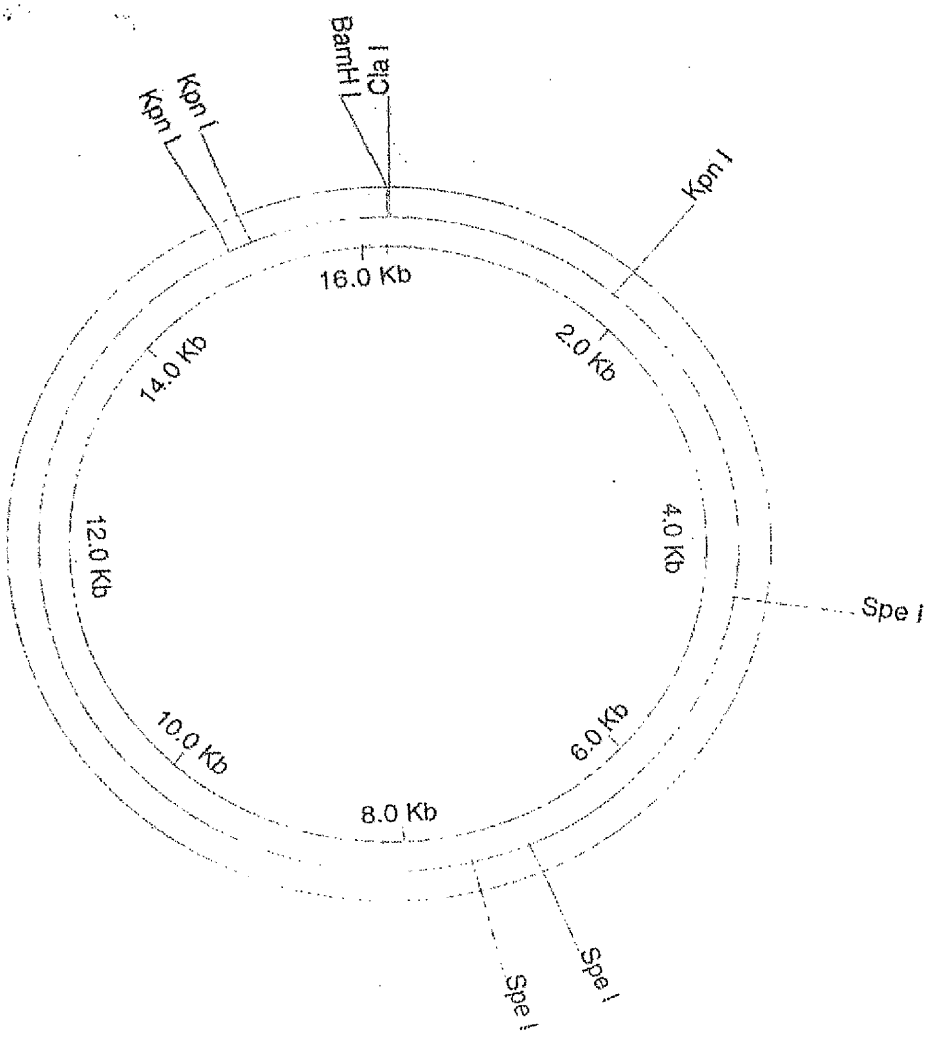


Figure 3.8: A restriction map of the *Cla*I – *Bam*HI upstream region of the 30kb CRP fragment with the restriction enzymes *Kpn*I and *Spe*I.

Table 3.2: Location of unique restriction enzyme sites in the CRP 30kb fragment

Position	Restriction enzyme site
414	*Ahy* AI
414	*Bsu* MI
415	*Xho* I
417	*Sci* I
2614	*Sty* SKI
3581	*Eco* KI
8902	*Pme* I
9248	*Age* I
9248	*Pin* AI
9742	*Aci* I
15283	*Bsr* BI
15283	*Mbi* I
17907	*Bco* 63I
17912	*Bsr* BRI
18312	*Eci* AI
18315	*Sna* BI
20725	*Sty* SQ
22183	*Sty* SJ
22997	*Uba* 1220 I
22998	*Cfr* 9I
22998	*Psp* A1I
22998	*Xma* I
23000	*Cfr* J4I
23000	*Sma* I
23050	*Bbe* AI
23051	*Kas* I
23052	*Nar* I
23053	*Ehe* I
23055	*Bbe* I

position 17907 and *Sty* SQ at position 20725. However, a search of the REBASE database (Roberts & Macelis, 1999) showed that neither of these restriction enzymes is currently available commercially. Commercially available isochizomers for *Bco* 63I that only cut the 30kb fragment once were sourced (*Bsa*BI and *Mam*I are two examples) but isochizomers of *Sty* SQ that only cut the 30kb fragment once were not found.

The absence of usable restriction enzyme sites in the 30kb fragment suggests that if the CRP/GM-CSF construct cannot be cloned in the background of the 30kb fragment, the 30kb fragment or the existing acute phase expression vector will have to be modified to some extent. One approach would be the introduction of restriction enzyme sites not currently present in the 30kb fragment using long range PCR to facilitate cloning of the CRP-GM-CSF fusion. An alternative approach could involve either the removal of sequences that are responsible for the high basal levels of expression in the existing expression vector or the addition of sequences that are necessary for low basal levels of expression. The following sections describe the analysis of the 30kb fragment for such elements.

3.3 Comparison of the 30kb sequence with the first working draft of the human genome

The sequencing of the 30kb CRP fragment was completed in June 1999. Although it was anticipated that sequencing of the human genome would be complete at some stage during this project, we did not know when. As it turns out, the first working draft of the human genome was not released until 20 months after we had completed sequencing the 30kb fragment (International Human Genome Sequencing Consortium, 2001). Sequencing the 30kb CRP fragment was deemed necessary in order to identify the elements involved in regulating the human CRP gene. I also wanted this information for the parallel studies to modify the expression vector for use in pigs (as discussed in section 1.5).

When the working draft became available the 30kb fragment was compared with the human genome using the BLAST search engine. As expected, the 30kb sequence mapped to a region on chromosome 1 containing the CRP gene (Figure 3.9). There was good agreement between the CRP sequence reported here and those of the human genome sequence (99% homology). An interesting observation was that the GT repeat found in the CRP intron (Lei *et al.*, 1985) is shorter in the 30kb CRP fragment compared with that of the human genome sequence (Figure 3.10). This fits in with the recently published findings of Szalai *et al.* (2001) that polymorphisms in the GT region of the intron of the human CRP gene occur.

3.3.1 Comparison of the 30kb CRP sequence with other sequences in the nucleotide databases

The 30kb sequence was compared with other sequences in the nucleotide databases to see if there were any other genes or potential regulatory elements present in the fragment. These analyses were done using BLAST and NIX (section 2.5.1.6).

The sequences were first analysed using the NIX program which is a combination of a number of different nucleotide search programs and gives a good overview of the types of sequence present (Figure 3.11). The sequence was analysed a number of times throughout this project as the nucleotide databases are updated on a regular basis. The last analysis on the 30kb fragment was carried out in June 2001. Most of the non-coding regions of the 30kb fragment appear to be composed of the repeated elements that are found throughout the human genome. The only regions in the 30kb fragment with homology to genes or pseudogenes are the regions containing the human CRP gene and the human CRP pseudogene. This result confirms the finding in Figure 3.9. A BLASTx search, however, revealed that there was a region of approximately 2kb (from nucleotides 1 to 2056) with homology to p40, one of the open reading frames of a LINE-1 (long interspersed nuclear element). Although L1 elements were initially believed to be non-functional, in the past few years there have been reports of these elements affecting the regulation of downstream genes.

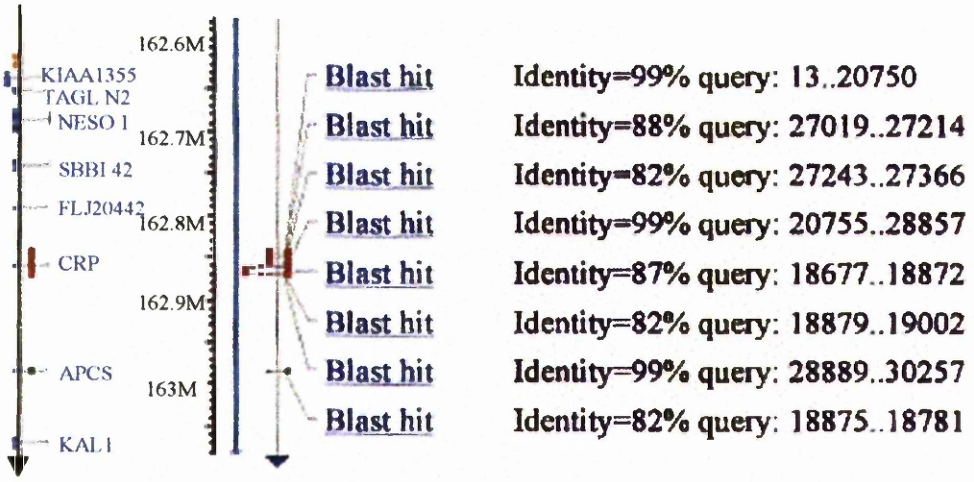


Figure 3.9: Mapping the 30kb fragment containing the human CRP gene to a region on chromosome 1 of the human genome containing the CRP gene using BLAST. It is evident from this figure that there do not appear to be any genes in the vicinity of the CRP gene.

```
Query: 18197 acagtggagcatccttcatgg--------tgtgtgtgtgtgtgtgtgtgtgtgt 18244
              ||||||||||||||||||||        ||||||||||||||||||||||||||
Sbjct: 62713 acagtggagcatccttcatggtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgt 62768


Query: 18245 gtggtgtgtaactggagaaggggtcagtctgtttctcaatcttaaattctatacgt 18300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 62769  gtggtgtgtaactggagaaggggtcagtctgtttctcaatcttaaattctatacgt 62824
```

Figure 3.10: Comparison of the GT repeat of the CRP intron in the 30kb CRP fragment (Query sequence) and in the working draft of the human genome (Sbjct).

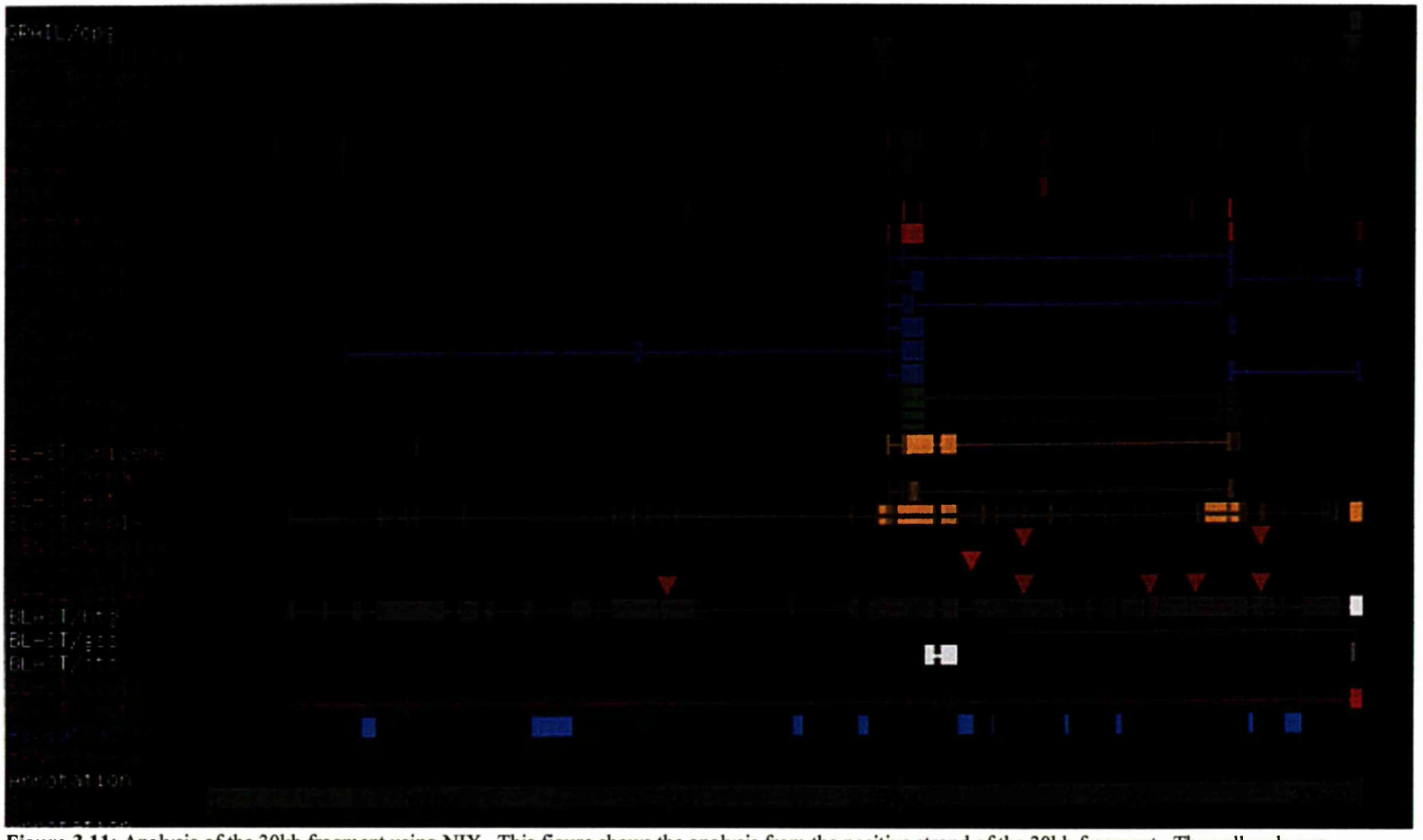


Figure 3.11: Analysis of the 30kb fragment using NIX. This figure shows the analysis from the positive strand of the 30kb fragment. The yellow boxes correspond to regions with high levels of homology to sequences present in the EMBL database. The green boxes correspond to regions with high levels of homology to protein sequences in the TREMBL and SWISSPROT databases. As reported in the text the only regions that exhibited homology to sequences in the database were the regions containing the CRP gene and the CRP pseudogene. Although GRAIL suggests a number of promoter sites these are only marginal predictions according to the NIX classification.

Yang *et al.* (1998) published the first report of a LINE element affecting the regulation of a downstream gene. This was found to be due to the presence of an enhancer region 20kb 5' to the apolipoprotein(a) transcription start site and was located within the LINE element. This element responded to members of the Ets transcription factor family and is important for enhancing the expression of the apolipoprotein(a) gene. The 30kb fragment was searched for the sequence CCCGGAAG, which was shown by Yang *et al.* (1998) to be functionally active. However, this sequence was not found in the 15kb upstream region of the 30kb CRP fragment.

The second report of a L1 element affecting regulation of a downstream gene investigated was the one by Speek (2001). Speek reported that an antisense promoter (ASP) of human L1 retrotransposons could drive the transcription of adjacent cellular genes and also possibly modulate their expression. The presence of this promoter upstream of the CRP gene could therefore theoretically influence its expression. Therefore, the region of the 30kb CRP fragment containing ORF1 of the L1 element was compared with the consensus sequence of the antisense promoter region described by Speek (2001) using the GCG Gap program. It was found that there was 90% homology between the sequence found in the 30kb CRP fragment and the consensus sequence given by Speek (2001) (Figure 3.12).

Analysis of the 30kb fragment using the BLAST search engine allowed the identification of one factor that may have an effect on the regulation of the CRP gene. However, BLAST does not allow the identification of transcription factor binding sites and other regulatory regions because the program was designed for similarity not motif searches. Searching for transcription factor binding sites must be done manually or by using specialised transcription factor and regulatory element search engines that are widely available on the Internet. Analysis of the 30kb fragment for transcription factor binding sites and regulatory sequences is described in the following sections.

Figure 3.12: Comparison of the 30kb CRP fragment (top) with the consensus antisense promoter found in human L1 elements as described by Speek (2001)

3.3.2 Analysis of the sequence for regulatory elements

Gene expression can be controlled in a number of ways and at a number of different levels – transcription and translation and the steps in between and following these processes. CRP is believed to be regulated at the transcriptional level (Ciliberto *et al.*, 1987a) and transcription factors affecting regulation of the CRP gene have already been described (e.g. Morrone *et al.*, 1988; Majello *et al.*, 1990; Li and Goldman, 1996). For transcription to occur DNA must be dissociated from nucleosomes, DNA must be unwound to enable transcription factors to bind and negative regulators of transcription must be removed while positive regulators must bind to the DNA. Factors governing all these processes have been identified. CRP has been reported to be under negative control (Arcone *et al.*, 1988) and that elements involved in repressing transcription in the absence of stimulation are located both upstream and downstream of the CRP promoter (Murphy *et al.*, 1995). This work focused on analysis of the 30kb sequence for elements that may be involved in processes governing the negative regulation of the CRP gene.

The 30kb sequence was analysed by searching the sequence manually for the consensus binding sequences of transcription factors and regulatory elements that have been reported in the literature to be involved in regulation of eukaryotic genes. This approach was favoured over using the available transcription factor databases and search engines mainly due to the large volume of sequence to be analysed. The 30kb fragment was initially searched for the transcription factor binding sites that have been reported by Arcone *et al.* (1988); Li *et al.* (1990) and Li and Goldman (1996). Following this the 30kb fragment was searched for the consensus sequences of a number of transcription factor binding sites and regulatory elements. The consensus sequences used in these analyses are shown in Table 3.3 and the locations of these sites in the 30kb fragment (coding strand) are given in Appendix 3.

As illustrated in Figure 3.13, which only takes into account a few of the potential transcription factors that could bind in this region, the distribution of the

Table 3.3: Consensus binding sequences for transcription factors and other regulatory elements known to be involved in regulating gene expression and used for analysis of the 30kb CRP fragment and porcine ITIH4 promoter region (see Chapters 4 and 5).

Factor/ binding site	Recognition Sequence	Function of binding factor	Reference
ARE	TGTTCT	Androgen response element. This is only half of the element GGTACAnnnTGTTCT. However, half of the sequence can function in co-operation with other enhancer elements nearby.	(Zhou *et al.* 1997) (Zilliacus *et al.* 1995)
CACCC box	ACACCC	Important in transcriptional regulation	(Horie & Takeishi, 1997)
GAGA box	GAGGAGGGAGG AG	Growth hormone sensitivity	(Le Cam *et al.* 1994)
HIP1	ATTCN$_{(1-30)}$GCCA	Acts on TATA-less promoters of housekeeping genes to specify the site of transcription initiation	(Faisst & Meyer, 1992)
HNF-5	T(G/A)TTTG(C/T)	This factor is liver-specific. Binding sites for this factor are located close to other liver-specific factors.	(Faisst & Meyer, 1992)
IL-6 RE	CTGGGA	Implicated in the regulation of Class 2 acute phase genes; sequence recognised by factors such as IL-6 RE binding protein and STAT3	(Wegenka *et al.* 1993) Simar-Blanchet *et al.* 1996) (Weinhold *et al.* 1997)
LF-A1	TGAACC TGGCCC	Binding factor that has been shown to interact with the promoter regions of several liver-specific genes - haptoglobin and α_1-antitrypsin	(Hardon *et al.* 1988)

	TGAGAGA TGAGAA T/AGGAAAA	Motifs found in rabbit CRP promoter	(Li *et al.* 1990)
	TGAAGCA TGGAAAA TGGAGGA	Motifs found in the three IL-6 responsive elements of human haptoglobin	(Oliviero & Cortese, 1989)
	TGGGAAA	Distal regulatory element for rat a1-acid glycoprotein	(Prowse & Baumann, 1988)
NF-IL-6	TT/GNNGNAAT/G	This factor is involved in acute phase reactions and a homologue of this factor may play a role in the glucocorticoid induction of the a1-acid glycoprotein gene	(Faisst & Meyer, 1992)
STAT3 (acute phase response factor)	TTCC(G/C/A/T)GGAA (palindromic sequence)	Originally defined as a protein that binds to an IL-6 RE in the promoter of several acute phase genes and can confer IL-6 responsiveness to a heterologous promoter. Growth hormone can also induce binding of STAT3 containing complexes to APRE. Therefore, possibility that GH may also regulate the expression of acute phase response genes in the liver.	(Wegenka *et al.* 1993) (Campbell *et al.*1995) (Subramanian *et al.* 1995)
Sp1	GGGCG	Transcription factor that has a loose binding specificity to G-rich sequences upstream of TATA boxes	(Faisst & Meyer, 1992)
Universal silencer	TCTCTCCNA 5'ANCCTCTCT 3'	negative regulation of eukaryotic genes	(Haecker *et al.* 1995) (Ogbourne & Antalis, 1998)

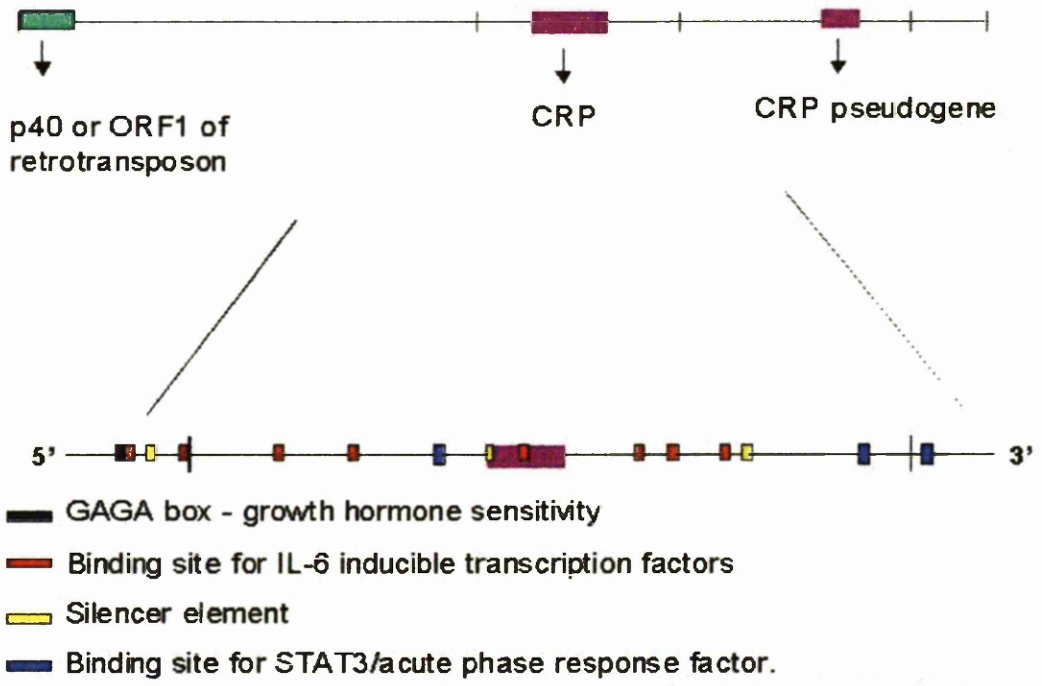


Figure 3.13: Putative transcription factor binding sites and regulatory elements surrounding the human CRP gene.

transcription factor binding sites appears to be numerous and complex. This makes it difficult to assign a role for these elements. Nonetheless, regions where there is a cluster of consensus binding sites are potentially important because interactions of transcription factors with each other are important. As well as acting to positively regulate gene transcription, transcription factors can modulate gene transcription by preventing other transcription factors binding (steric-hindrance) and by competing for overlapping binding sites. One such region is located approximately 2kb upstream of the CRP gene and contains a number of putative regulatory elements that will be discussed in the following sections. While understanding the mechanisms involved in regulating the CRP gene is desirable, this knowledge is not essential in order to make an improved expression vector. However, knowledge of the regions that are involved in regulating expression (particularly, the negative regulation) of the gene is important if we cannot clone the CRP-GM-CSF fusion into the background of the 30kb fragment and need to use a smaller construct. This information may also be useful for modifying the expression vector for use in pigs.

Using information from the literature on the influence of various regulatory elements on gene expression a picture can be drawn of regions in the 30kb fragment that may be involved in its regulation. Comparing the 30kb fragment with for example, the GM-C79 construct, for the presence and absence of these elements can further enhance this analysis. A more detailed analysis of the 30kb fragment was therefore confined to a number of transcription factor binding sites and regulatory elements that had the potential to be important and relevant based on information from the literature. The sequence analysis, therefore, focused on the following potential regulatory elements: polypurine/polypyrimidine rich regions due to the fact that they have the potential to adopt secondary structures that may affect gene expression; a silencer element identified by Haecker *et al.* (1995); STAT binding sites and a growth hormone responsive element identified by Le Cam *et al.* (1994).

3.3.2.1 Polypurine/polypyrimidine repeats

Polypurine/polypyrimidine repeats may affect eukaryotic gene transcription by forming unusual structures that may affect the formation or dissociation of nucleosomes and/or the binding of transcription factors to the DNA (reviewed by Wells *et al.*, 1988; Brahmachari *et al.*, 1997). Since these structures have been shown to be formed only under certain conditions *in vitro* this may determine when gene transcription occurs. The only published polypurine/polypyrimidine repeat in the CRP 30kb fragment is the GT repeat that is found in the CRP intron. However, analysis of the 30kb fragment showed that it also contained a number of polypurine/polypyrimidine rich regions as shown in Table 3.4.

CA repeats are very common in human DNA and account for 0.5% of the genome and are often highly polymorphic. CT repeats are also common (0.2% of genome) and occur approximately every 50kb. As a result, this makes interpretation of the results shown in Table 3.4 difficult. For example, there are five homopyrimidine rich regions in the 30kb fragment, but the significance of this is not clear. Therefore, analysis of these regions has taken into account the location of the polypurine/polypyrimidine rich region in the 30kb fragment and the transcription factor binding sites located nearby. As a result, the analysis has focused on the 65bp CT rich region located at positions 15982-16046, approximately 2kb upstream of the CRP gene. This region is absent from all the constructs made by Murphy *et al.* (1995) apart from construct C61. C61 had extended upstream sequences which were believed to contain a negative regulatory element that functioned independently of the negative element located downstream of the CRP gene. The CT rich region is also located in a segment of the 30kb fragment containing a number of consensus sequences for transcription factors and regulatory elements as illustrated in Figure 3.14.

Table 3.4: Polypurine/polypyrimidine rich regions in the CRP 30kb fragment

Position	Sequence
13164-13177	CAGGAGAGAGACAG
15982-16046	TTTTCCTTCCTTCCTCCCTCTCTCCCTCCCATCCTTC CTTTTTTCTCTTTTCTTTCTTCTTTCTCT
16528-16558	AAAGGGAAAAAGGGGGAAGTGGGAGGGGAAA
17283-17309	TTGTGGGGTGGGGGGAGGGGGGAGGGA
24602-24620	AGGAGGAAGAGAGAAGAGG
24840-24882	CTCTCTCCATCTCTATTTCTCTCTGCCTCTCCCTCTC TTTTTC
25743-25696	ACACATGCACACACACACACACTCACGTAAACACACA CACTTCA
28746-28779	TCTCTCTCTTCCCTCTCTGGTCCCTGCTCTCGCC

5′ TAGATTTAATGAGAGAGGTGTTTGTTTATGTTGGCTTCACTAGAGTGGTGAA

GGCCAATGGCTGATTTCCATGGATTGAGGAGGGAACAGGAGATGGAAAATAGA

GGTTGTCAACCCACGTAAACTTTTCCTTCCTTCCTCCCTCTCTCCCTCCCATCC

TTCCTTTTTTCTCTTTTCTTTCTTCTTTCTC 3′

Figure 3.14: Putative transcription factor binding sites in a region approximately 2kb upstream of the CRP gene (Positions –2065 to –1876 relative to cap site are shown). Putative binding sites include a motif found in the promoter of the rabbit CRP gene, STAT binding site, the GAGA box, one of the motifs found in the IL-6 responsive element of the human haptoglobin gene and a silencer element. The CT rich region is underlined.

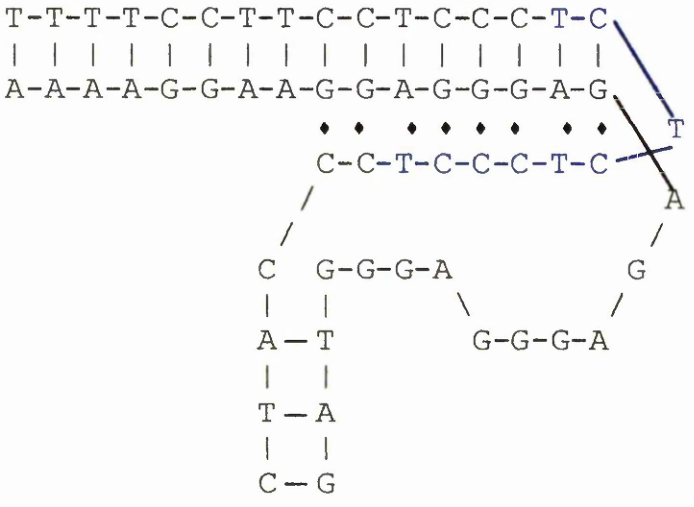


Figure 3.15: Putative H-DNA structure that could be formed from the homopyrimidine mirror repeat located in the 65bp CT rich region. Watson-Crick base pairs are indicated with straight lines and Hoogsteen base pairs are indicated by ♦. The silencer element reported by Haecker *et al.* (1995) is shown in blue.

The ability to form H-DNA (i.e. triple stranded DNA) is dependent on the presence of a mirror repeat in the polypurine/polypyrimidine rich region. Such a repeat is present in the 65bp CT rich region and the H-DNA structure that could be formed is shown in Figure 3.15. It should also be noted that the consensus sequence reported by Haecker *et al.* (1995) (section 3.3.2.2) as a silencer element forms part of the H-DNA structure. Therefore, if this DNA structure was formed *in vivo* there is the possibility that transcription factors binding to this site or in this region would be affected. As mentioned above and shown in Figure 3.14, the region 5' of the CT repeat contains putative binding sites for a number of transcription factors. Therefore, binding of these transcription factors could be affected if this structure was formed. Although the mirror repeat is only 8 bases in length, the mirror repeat reported by Glaser *et al.* (1990) was 11 bases in length and Bhaumik *et al.* (1998) reported polypurine repeats as short as 6 bases being capable of adopting a triplex conformation *in vitro*.

3.3.2.2 Silencer elements

As well as being controlled positively by enhancer elements, gene transcription can also be controlled negatively by silencer elements. Haecker *et al.* (1995) reported a silencer element that was believed to act with other regulatory elements in strongly repressing synthesis of the ovalbumin gene in hens when eggs are not being laid. Regulation of the ovalbumin gene is therefore somewhat similar to that of CRP in that it is under strong negative control while also being capable of being strongly induced.

The 30kb fragment was searched for the consensus sequence of the universal silencer element defined by Haecker *et al.* (1995). Two of these elements were located upstream of the CRP gene, one on the coding strand and one on the non-coding strand. Another three elements are found in the coding sequence of the CRP gene (one on the coding strand and two on the non-coding strand). The sixth element is found in the CRP pseudogene. The results reported here are those that

matched the consensus sequence 100%. However, there are several more putative silencer elements in the 30kb fragment as elements that differ from the consensus sequence by a single base have been found also.

3.3.2.3 Comparison of the GM-C79 construct with the 30kb CRP fragment

It is known that the GM-C79 construct contains most but not all of the sequences required for tightly controlled expression. Therefore, this construct was compared with the 30kb fragment for the presence of the previously described regulatory elements.

There are two putative silencer elements on the coding strand of the GM-C79 construct – at position + 139 and + 4843 and none on the non-coding strand. The GM-C79 construct obviously lacks two of the silencer elements located in the CRP gene as well as one located 5' of the *Bam*HI site. The transcription factor-rich region (Figure 3.13), including the CT rich-region present in the 30kb CRP fragment, is also absent from the GM-C79 construct.

3.3.2.5 Growth hormone responsive elements

The second aim of the sequence analysis was to identify regions that may be responsible for the sexually dimorphic pattern of expression seen in the CRP and GM-C79 transgenic mice. Since this pattern of expression is seen in both the CRP and GM-C79 transgenics this analysis focused on the GM-C79 construct.

3.3.2.5.1 STAT5

The STAT family is an important family of transcription factors with the ability to affect transcription in a number of ways. Their potential role in mediating the growth hormone signal was investigated because several of the STAT family members have been reported to be themselves regulated by growth hormone

(STAT3, STAT5). STAT5 itself has also been implicated as being the "major transducer of the pulsatile pattern of growth hormone secretion seen in male rodents into sex-specific patterns of gene expression" (Herrington *et al.*.2000).

The GM-C79 construct was searched for binding sites for STAT5. This analysis was done using the transcription factor search programs MatInspector (Quandt *et al.*, 1995) and Alibaba 2. These analyses have shown that there are a number of putative binding sites for STAT5.

Three putative STAT5 binding sites were found in the GM-C79 construct and are shown in Table 3.5. Two of the STAT5 binding sites are located upstream of the CRP cap site (approximately 1.2 and 0.9kb respectively) and one of them is approximately 1.3kb downstream. The two STAT5 binding sites upstream of the CRP cap site flank a GAGA-like box (section 3.3.2.5.2), being located approximately 100bp upstream and downstream, respectively. The putative STAT5 binding sites in the GM-C79 construct exhibit considerable homology to those reported by Varin-Blank *et al.* (1998) as being involved in the growth hormone mediated expression of the *C4-Slp* gene (Table 3.5).

3.3.2.5.2 GAGA box

The GAGA box was shown by Le Cam *et al.* (1994) to be involved in both growth hormone stimulation and activation of the rat serine protease inhibitor 2.1 gene since mutations that destroy the symmetry of the GAGA box or partial deletion of the 5' end of the GAGA box motif abolish growth hormone stimulation and reduce promoter activity by at least 90%.

Two putative GAGA boxes have been identified in the 30kb CRP fragment. One GAGA box is located approximately 2.1kb upstream of the CRP gene and the other is located about 1.2kb upstream. This is a lot further upstream that that of the

Table 3.5: Putative growth hormone responsive elements in GM-C79. + or – refers to coding (+) or non-coding (-) strand.

Reported binding sites for STAT5 or growth hormone responsive elements (5' to 3')	Sequence present in GM-C79 (5' to 3')	Position relative to cap site (+1)	Reference
TTC TCA GAA (STAT5)	TTC TCA TAA	-1100 to –1092 (-)	Varin-Blank *et al.* (1998)
TTC TCA GAA (STAT5)	TTC TCT GAA	-794 to –786 (-)	Varin-Blank *et al.* (1998)
TTC TGA GAA (STAT5)	TTC AGA GAA	-794 to –786 (+)	Meyer *et al.* (1997); Feltus *et al.* (1999); Rusterholz *et al.* (1999)
TTC CCA GAA (STAT5)	TTC CCA GAA	+1281 to +1296 (-)	Varin-Blank *et al.* (1998)
GAGGAG GGA GGAG (GAGA box)	GAGGAG CAA GGAG	-1053 to –1041 (+)	Le Cam *et al.* (1994)

GAGA box of the rat serine protease inhibitor 2.1 gene which is located about 50 bases upstream of the gene.

The GAGA boxes in the 30kb fragment differ slightly from that reported by Le Cam *et al.* (1994) (Table 3.5) although the 5' and 3' regions are conserved. The GAGA boxes may be involved in controlling promoter activity or possibly proteins bound to this box could interact with the STAT5 proteins as a STAT5 binding site has been located on either side of one of the GAGA boxes.

Summary of results

1. The 15kb 5' region of the 30kb CRP fragment was sequenced and linked to the downstream region, thereby completing the sequence of the 30kb CRP fragment.
2. Analysis of the 30kb fragment has identified a number of putative regulatory elements:
   - An ORF from a retrotransposon that contains an antisense promoter
   - A number of polypurine/polypyrimidine rich regions
   - A region containing a number of consensus sequences for a number of transcription factors involved in inducing acute phase gene expression
   - A number of putative silencer elements have been identified that may have a negative effect on CRP expression
3. Analysis of the GM-C79 construct for elements that may be involved in the sexually dimorphic pattern of expression in the transgenic mice identified two elements that may be responsible for this:
   - STAT5 binding sites with considerable homology to those already reported in the literature to mediate sexually dimorphic patterns of expression
   - GAGA box which has been reported as a growth hormone responsive element

As mentioned in section 1.5, one of the aims of the sequence analysis was to identify regulatory elements that may facilitate modification of the expression vector for use in pigs. The next chapter describes the first step to modify the expression vector, the isolation of the promoter of the major acute phase protein in pigs.

Chapter 4: Isolation and characterisation of the porcine ITIH4 promoter

4.1 Introduction

Characterisation of the porcine acute phase response has only begun in the last 10 years and it remains to be investigated at the molecular level. The major acute phase protein in pigs is known as pig-MAP or ITIH4. A number of studies have reported the isolation of cDNA clones containing the porcine ITIH4 gene (Buchman *et al.*, 1990; Hashimoto *et al.*, 1996). However, no work was done to isolate the promoter. The aim of this work was to isolate the promoter region of the pig ITIH4 gene with the view to using this creating an acute phase expression vector. By analysing the promoter and comparing it with the human CRP promoter, we also hoped to answer the following question:

Are the regulatory elements that control expression of the human CRP gene also controlling expression of the pig ITIH4 gene?

If the regulatory elements were the same as the human CRP gene then an expression vector could be more easily created since the regulation of the human CRP gene is partially understood. The sequence analyses described in the previous chapter would also facilitate the modification of the acute phase expression vector. On the other hand, if the regulatory elements were different to the human CRP gene then the pig ITIH4 promoter would have to be further characterised before an acute phase expression vector could be made.

This chapter will describe the isolation and analysis of the promoter of the porcine ITIH4 (pig-MAP) gene. To isolate the promoter two strategies were employed. First of all I attempted to make a probe that could be used to screen a porcine genomic library constructed in the lambda vector. Because problems were

experienced making a probe, it was instead decided to screen a pig BAC genomic library using the ITIH4 cDNA clone described by Buchman *et al.* (1990) as a probe. An advantage of this approach was that the BAC clones contained inserts of approximately 150kb. Therefore there was a good chance that an isolated clone would contain all the elements required for regulation of the porcine ITIH4 gene.

4.2 Isolation of a clone containing the pig-MAP/ITIH4 gene

4.2.1 Amplification of a section of the pig ITIH4 gene to make a probe for screening a porcine genomic library

When work began on this section of the project it was believed that the major acute phase protein in pigs was called pig-MAP and that the only sequence available was the N-terminal amino acid sequence reported by González-Ramón *et al.* (1995). Therefore, to make a probe degenerate oligonucleotides were designed from this sequence. Due to the extensive homology reported between the N-terminal of pig-MAP and the human protein PK-120 (Nishimura *et al.*, 1995; González-Ramón *et al.*, 1995), oligonucleotides were also designed from the cDNA sequence of PK-120 that corresponded to the region of pig-MAP the degenerate oligonucleotides had been designed from. The porcine genomic DNA used in these experiments was supplied by Dr R Wales (PIC).

Based on the cDNA sequence of PK-120 the expected size of the PCR product was approximately 500bp. However, successive PCRs failed to give a product of the expected size. Changing the reaction conditions and reagents used also failed to give a positive result. It was suspected that one or more introns could be located between the primer pairs and that the PCR product to be formed was much larger than expected. This was confirmed when it was realised that pig-MAP and the IHRP protein reported by Hashimoto *et al.* (1996) were the same protein. This realisation gave us access to a wealth of DNA sequence data, including the genomic organisation of the homologous human ITIH4 gene (Saguchi *et al.*, 1995). The

oligonucleotides designed from the PK-120 (human ITIH4) cDNA sequence actually amplify a region of approximately 3kb (this calculation is based on the length of the introns reported by Saguchi *et al.*, 1995) rather than 500bp. This meant that the extension time being used in the PCR was too short to amplify the region that the oligonucleotides were binding to.

Resolving the nomenclature of the pig-MAP/ITIH4 gene opened up a lot of possibilities for obtaining a clone containing the ITIH4 gene. First of all, knowledge of the genomic structure of the human ITIH4 gene would facilitate the designing of oligonucleotides to ensure they bound in the same exon (assuming the structure of the porcine and human ITIH4 genes was the same). In addition, knowledge of the cDNA sequence of the pig ITIH4 gene meant that the oligonucleotides could be porcine rather than human specific. However, although information was available regarding the DNA sequence of the pig ITIH4 gene, none of the reported sequences contained any information on the sequence of the promoter region.

At this stage of the project two slightly different strategies were employed in parallel. The first was the amplification of a polymorphic microsatellite from an ITIH4 clone and secondly, screening a porcine genomic library using an ITIH4 cDNA clone.

4.2.2 PCR using oligonucleotides from a polymorphic microsatellite

The pig genome is currently being mapped and a large database is maintained at the Roslin Institute (Hu *et al.*, 2001). From this database it was possible to source a set of primers that had been used to amplify a polymorphic microsatellite that has been identified in a clone containing ITIH4. The primers were supplied by Professor Merete Fredholm (The Royal Veterinary & Agricultural University, Fredriksberg, Denmark) and give a PCR product of 160-174bp. Using these primers with the pig genomic DNA gave a band of the expected size as shown in Figure 4.1. This band

was then cloned and sequenced. Sequencing of this clone revealed that it came from the ITIH4 gene.

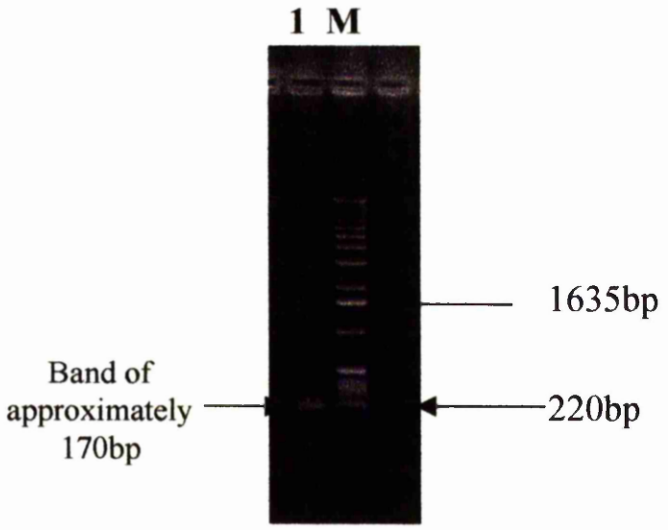


Figure 4.1: PCR using oligonucleotides from a polymorphic satellite. The bands of 1.6kb and 220bp of the ladder are indicated with arrows. A PCR product of approximately 170bp can be seen in Lane 1.

From this PCR product a probe could have been made to screen a porcine genomic library. However, while sourcing these primers we became aware of the existence of a porcine genomic BAC library that had been constructed by the PiGMAP research group at the Roslin Institute, UK. Screening this library had the advantage that due to the large size of the inserts (approximately 150kb) there was a good chance that any isolated clone would contain the entire porcine ITIH4 gene (useful for future analysis) and also possibly all the elements required for regulation of the gene itself. The following sections describe the library screen and isolation of positive clones.

4.2.3 Screening the PigE BAC library

The library screen was carried out at the Roslin Institute, UK, using the ITIH4 cDNA clone described by Buchman *et al.* (1990) (EMBL accession number M29507) as a probe. The filters and the probe were prepared as described in section 2.5.1.7.1 and 2.5.1.7.2 and details of the library screen are given in section 2.5.1.7. The PigE BAC library has been gridded on filters (for example, see Figure 4.2) and positive clones are identified by first of all identifying which filter the positive clone is on. The filter is then orientated so that the panel in which the positive clones are located in can be identified. The microtitre co-ordinates of the 4 x 4 array are then worked out to identify the putative positive clones. The library screen resulted in the identification of eight putative positive clones, which are shown in Table 4.1

Although, eight putative positive clones were identified it was noted that some signals were weaker than others. A possible reason for this was the ITIH4 clone binding to sequences that are similar to ITIH4 such as other members of the ITI family. Therefore, before proceeding to isolate the ITIH4 promoter it was necessary to confirm the clones actually contained the ITIH4 gene.

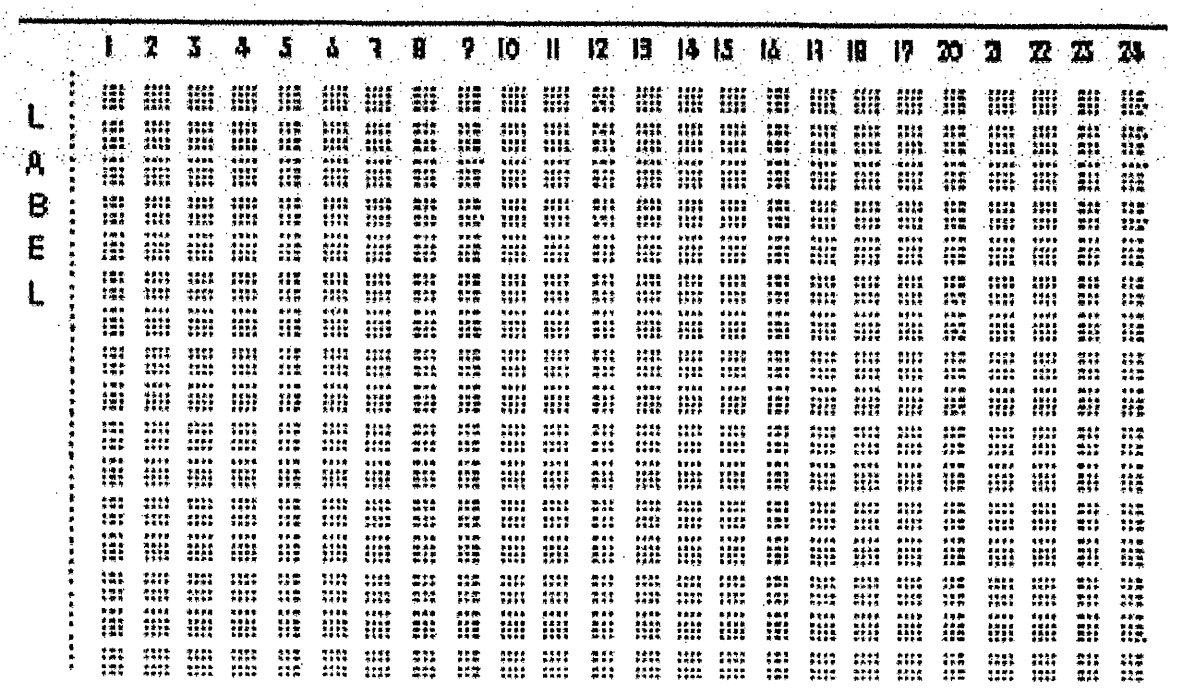


Figure 4.2: Example of one of the panels on a filter, illustrating the 4 x 4 array

Table 4.1: Putative positive BAC clones

PigE BAC library clone identification	Clone renamed as:
PigE BAC 037a02	BAC 1
PigE BAC 044h21	BAC 2
PigE BAC 049g22	BAC 3
PigE BAC 096n06	BAC 4
PigE BAC 203b11	BAC 5
PigE BAC 231l07	BAC 6 (did not grow and was excluded from further analysis)
PigE BAC 246l06	BAC 7
PigE BAC 258c15	BAC 8

4.2.4 Confirmation of positive clones

To confirm the clones were positive two PCRs were carried out. For the first PCR, primers were designed from near the beginning of the cDNA sequence of the probe (ITIH4 F1 and ITIH4 R1). Because it was suspected that several introns were located in the porcine ITIH4 gene (based on information from the genomic organisation of the human ITIH4 gene) the primers were designed relatively close together to increase their chances of binding to sites within the same exon. The expected size of the PCR product based on the cDNA sequence of the ITIH4 gene was 105bp. The results suggested that BAC1, BAC2 and BAC7 were positive as bands of this size were obtained but the small size of the PCR product meant that it was difficult to distinguish the PCR product from the primers (Figure 4.3).

However, the sequence reported by Buchman *et al.* (1990) is actually much shorter than the cDNA sequence of the pig ITIH4 gene reported by Hashimoto *et al.* (1996). Consequently, the primers used in the first PCR are located approximately 700bp from the start of the cDNA sequence reported by Hashimoto *et al.* (1996). For the second PCR primers PK-120 F1 and ITIH4 R1 were used. Their locations are indicated in Figure 4.4. The size of the PCR product expected to be formed based on the cDNA sequence of the pig ITIH4 gene was approximately 764bp whereas the expected size of the PCR product based on the genomic structure of the human ITIH4 gene was 4.3kb. PCR on the BAC clones using these primers confirmed that BAC1, BAC2 and BAC7 were positive because they gave a PCR product of 4.3kb (Figure 4.4).

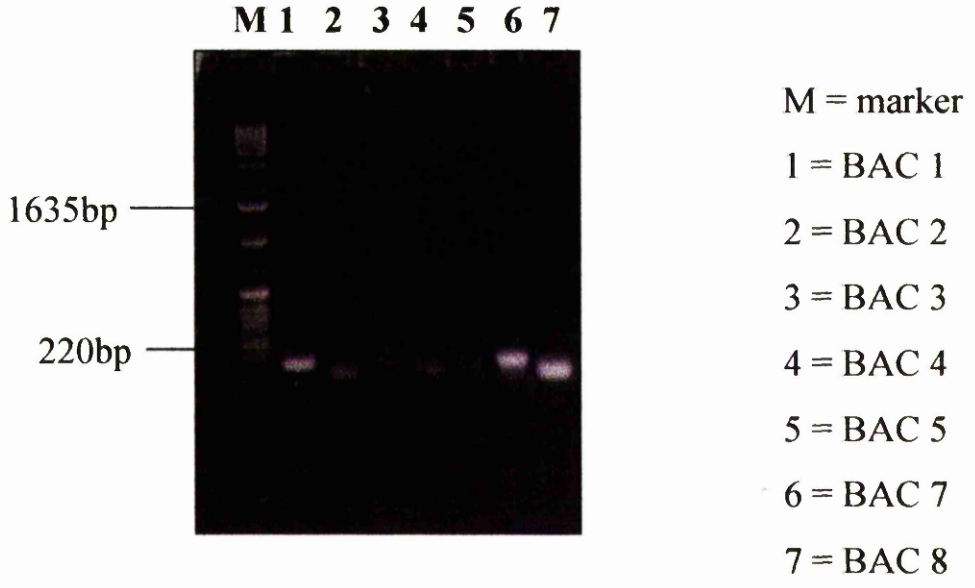


Figure 4.3: PCR to confirm isolated BAC clones contain the ITIH4 gene. The expected size of the PCR product was approximately 100 bp. Therefore, BAC1, BAC2, BAC7 and BAC8 appear to be positive. The reason for the difference in size of the PCR products is not known.

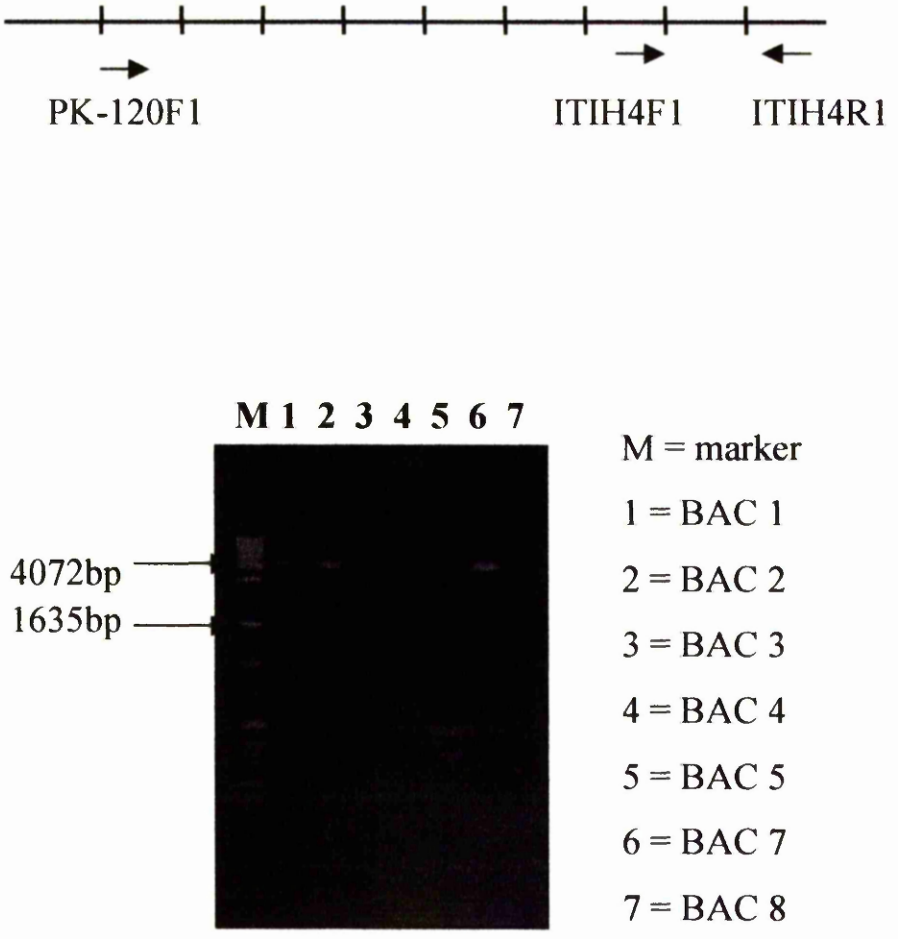


Figure 4.4: Location of primers in the cDNA sequence of the porcine ITIH4 gene used to confirm the isolated BAC clones contain the porcine ITIH4 gene (each line represents 100bp) and the PCR carried out to confirm the isolated BAC clones contain the ITIH4 gene. The expected size of the PCR product was 4.3kb. Therefore, BAC1, BAC2 and BAC7 are positive.

Before proceeding to try to isolate the promoter region of the porcine ITIH4 gene it was decided to confirm that the 5' end of the ITIH4 gene was present in the BAC clones (BAC1 was chosen for this analysis). This was done using a primer set (MAP.IN and MAP.OUT) aimed at amplifying a region of 100bp based on the cDNA sequence of the pig ITIH4 gene. However, the expected size of the PCR product based on the structural organisation of the human ITIH4 gene was actually 1.4kb due to the presence of an intron. The presence of the intron in the pig ITIH4 gene and the 5' end of the pig ITIH4 gene in BAC1 were confirmed by this PCR as a PCR product of the expected size of 1.4kb was formed (see Figure 4.5) with this primer pair. This region was also shown to be present in BAC 7 but not BAC 2. The clone BAC 1 was therefore chosen as the template for all further experiments.

This section has described the isolation of a clone containing the porcine ITIH4 gene and confirmation that the 5' end of the gene is present in BAC1, the clone selected for further characterisation. The following section will describe the strategies employed to isolate the promoter and upstream sequences of the porcine ITIH4 gene.

4.3 Isolation of the promoter of the pig ITIH4 gene

To isolate the promoter of the ITIH4 gene a number of strategies were considered. The most obvious one was to digest the BAC clone and probe for fragments that contained the 5' end of the pig ITIH4 gene. The main problem with this approach was that it was difficult to obtain sufficient quantities of BAC DNA for this work. At the time this work was carried out commercially available kits for the large scale preparation of BAC DNA were not readily available. I also considered sequencing out from the 5' end of the pig ITIH4 gene but there was concern that DNA prepared using our methods or the modified alkaline lysis method described by Brosch *et al.* (1998) would not be suitable for DNA sequencing. Therefore, our first strategy for isolating the promoter of the pig ITIH4 gene was based on the available sequence for the human ITIH4 promoter.

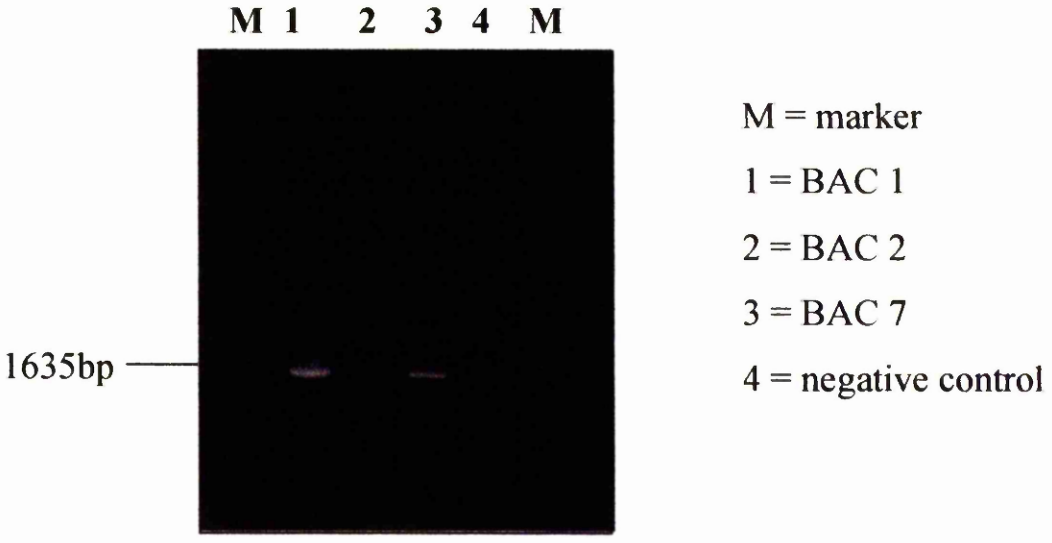


Figure 4.5: Confirmation by PCR that the proposed intron of 1.4kb and the 5' region of the pig ITIH4 gene are present in BAC 1. This region is also present in BAC 7 but absent in BAC 2.

4.3.1 Using the sequence from the promoter of the human ITIH4 gene to isolate the corresponding sequences from the pig gene

A primer, 5' IHRP, was designed from the promoter of the human ITIH4 gene (EMBL U42015) [Saguchi *et al.* (1995)] and a primer ITIH4.OUT was designed from the cDNA sequence of the pig ITIH4 gene. PCR was carried out using BAC1 DNA as template and this gave a product of the expected size of 1.7kb (Figure 4.6). The purified PCR product was then cloned in PCRScript and sequenced.

4.3.2 Sequence results and analysis using BLAST

The sequence was first of all analysed by comparing it with other nucleotide sequences in the database using the BLAST program. For this analysis a 7 word search was used with an expect value of 10. The results from the BLAST search were surprising due to the apparent limited homology to the promoter of the human ITIH4 gene (Figure 4.7). As expected the 3' end of the sequence has homology to the 5' end of the pig ITIH4 gene. After that, there are only a few regions of the promoter with homology to the human ITIH4 promoter and upstream sequences. Therefore, it appears that I was fortunate to isolate the porcine ITIH4 promoter as the oligonucleotide designed to amplify the 5' end of the promoter is located in a region with little homology to the human ITIH4 promoter. In spite of these findings the fact that there is some homology to the human ITIH4 promoter and particularly the homology in the 3' end to the published cDNA sequence of the pig ITIH4 gene suggested that the promoter of the porcine ITIH4 gene had been isolated. The porcine ITIH4 promoter was also compared with the working draft of the human genome when it became available. Parts of the porcine ITIH4 promoter isolated here mapped to a region on chromosome 3 containing the human ITIH4 gene (Figure 4.8). However, it should be noted that only a small section of the porcine ITIH4 promoter is mapping in this region. This is to be expected as a comparison between DNA sequences from two different species is being made.

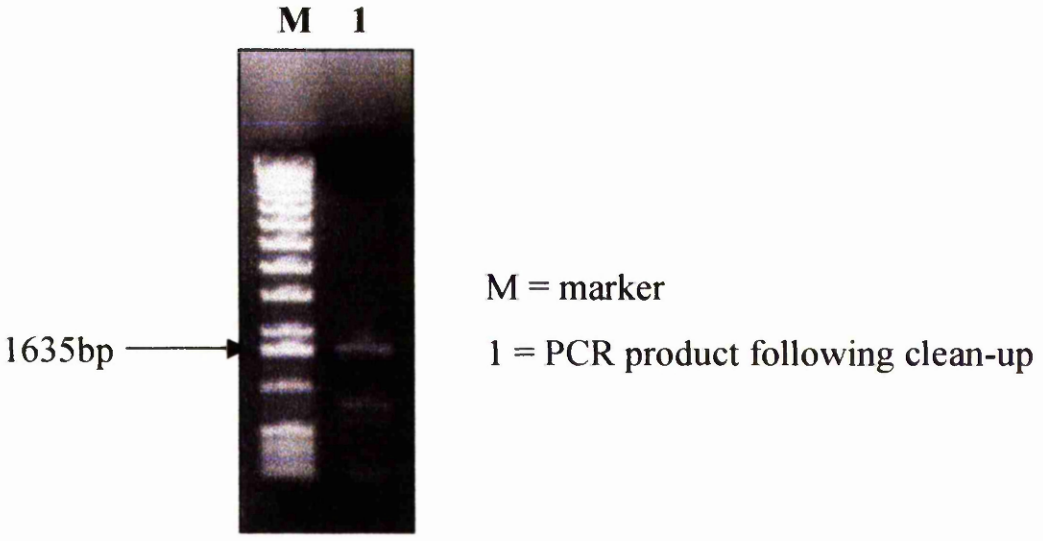


Figure 4.6: Isolation of the promoter region of the pig ITIH4 gene. The PCR product formed (Lane 1) was of the expected size of approximately 1.7kb. Other bands may be non-specific PCR products. The 1.6kb band of the DNA ladder is indicated with an arrow.

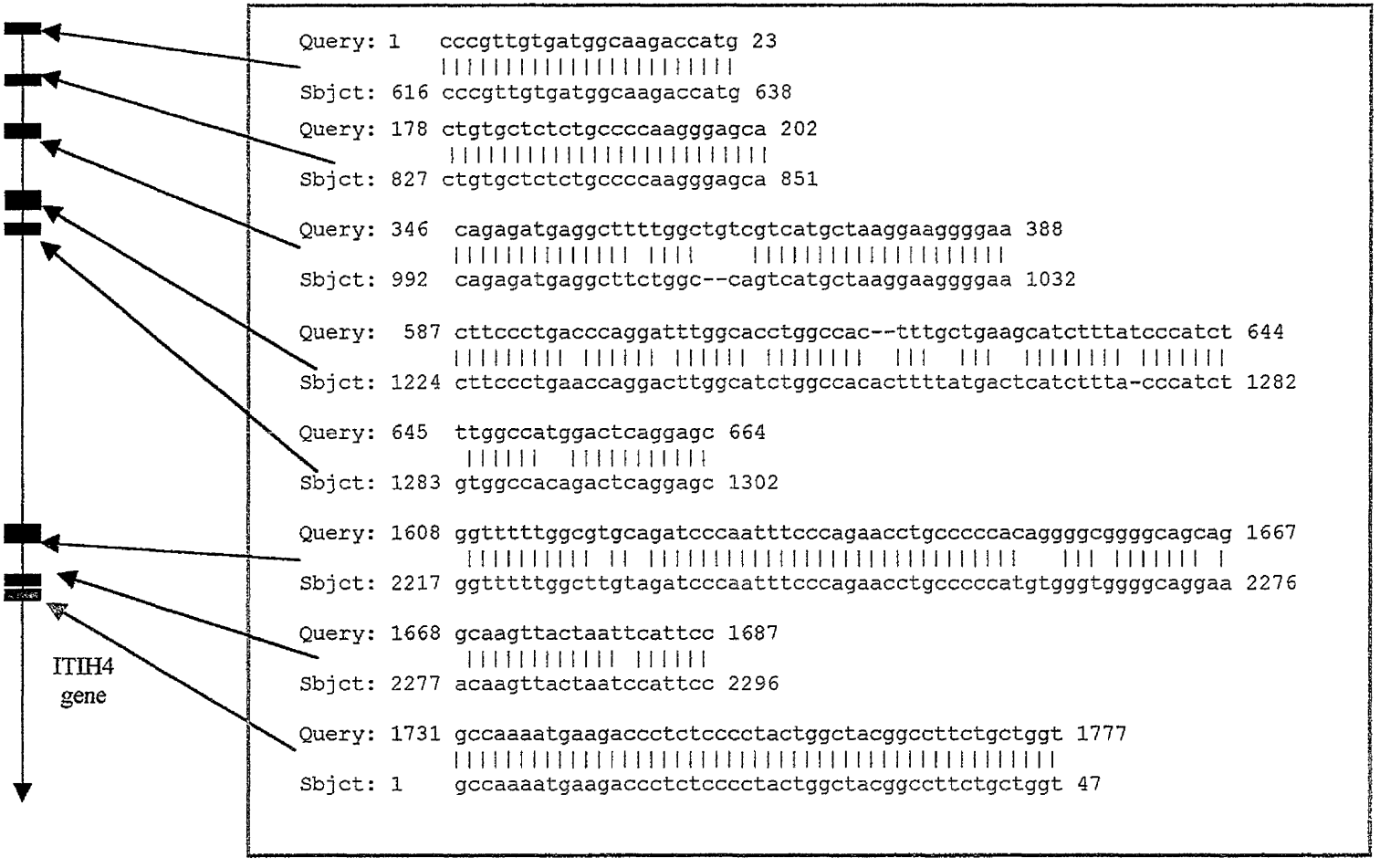

ITIH4
gene
Query: 1 cccgttgtgatggcaagaccatg 23
Sbjct: 616 cccgttgtgatggcaagaccatg 638
Query: 178 ctgtgctctctgccccaagggagca 202
Sbjct: 827 ctgtgctctctgccccaagggagca 851
Query: 346 cagagatgaggcttttggctgtcgtcatgctaaggaaggggaa 388
Sbjct: 992 cagagatgaggcttctggc--cagtcatgctaaggaaggggaa 1032
Query: 587 cttccctgacccaggatttggcacctggccac--tttgctgaagcatctttatcccatct 644
Sbjct: 1224 cttccctgaaccaggacttggcatctggccacacttttatgactcatcttta-cccatct 1282
Query: 645 ttggccatggactcaggagc 664
Sbjct: 1283 gtggccacagactcaggagc 1302
Query: 1608 ggttttggcgtgcagatcccaatttcccagaacctgcccccacaggggcggggcagcag 1667
Sbjct: 2217 ggttttggcttgtagatcccaatttcccagaacctgcccccatgtgggtggggcaggaa 2276
Query: 1668 gcaagttactaattcattcc 1687
Sbjct: 2277 acaagttactaatccattcc 2296
Query: 1731 gccaaaatgaagaccctctcccctactggctacggccttctgctggt 1777
Sbjct: 1 gccaaaatgaagaccctctcccctactggctacggccttctgctggt 47

Figure 4.7: Schematic diagram illustrating regions of the isolated pig ITIH4 promoter with homology to the human ITIH4 promoter (blue boxes) and the cDNA sequence of the pig ITIH4 gene (red box) as determined by a BLASTn search.

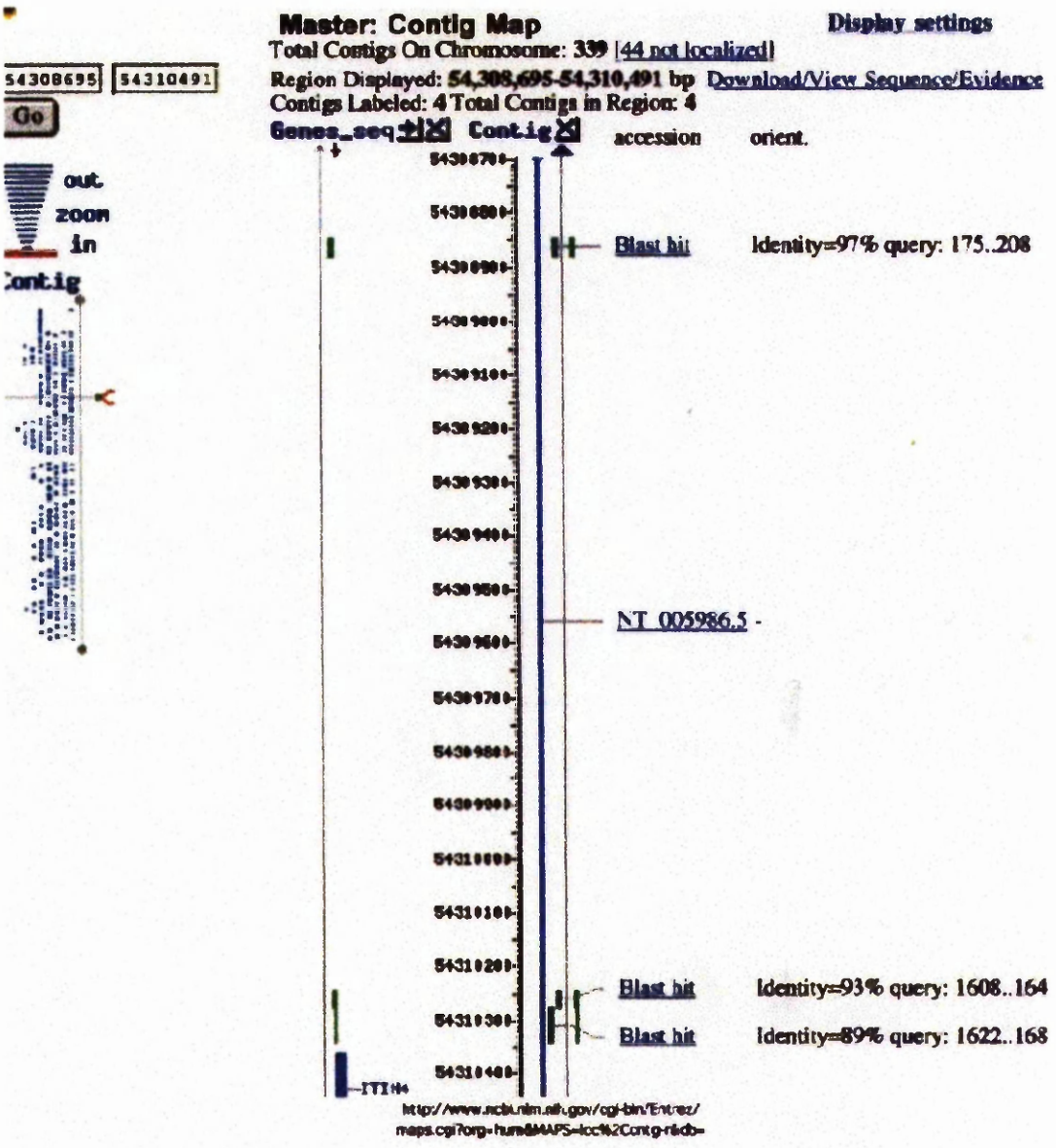


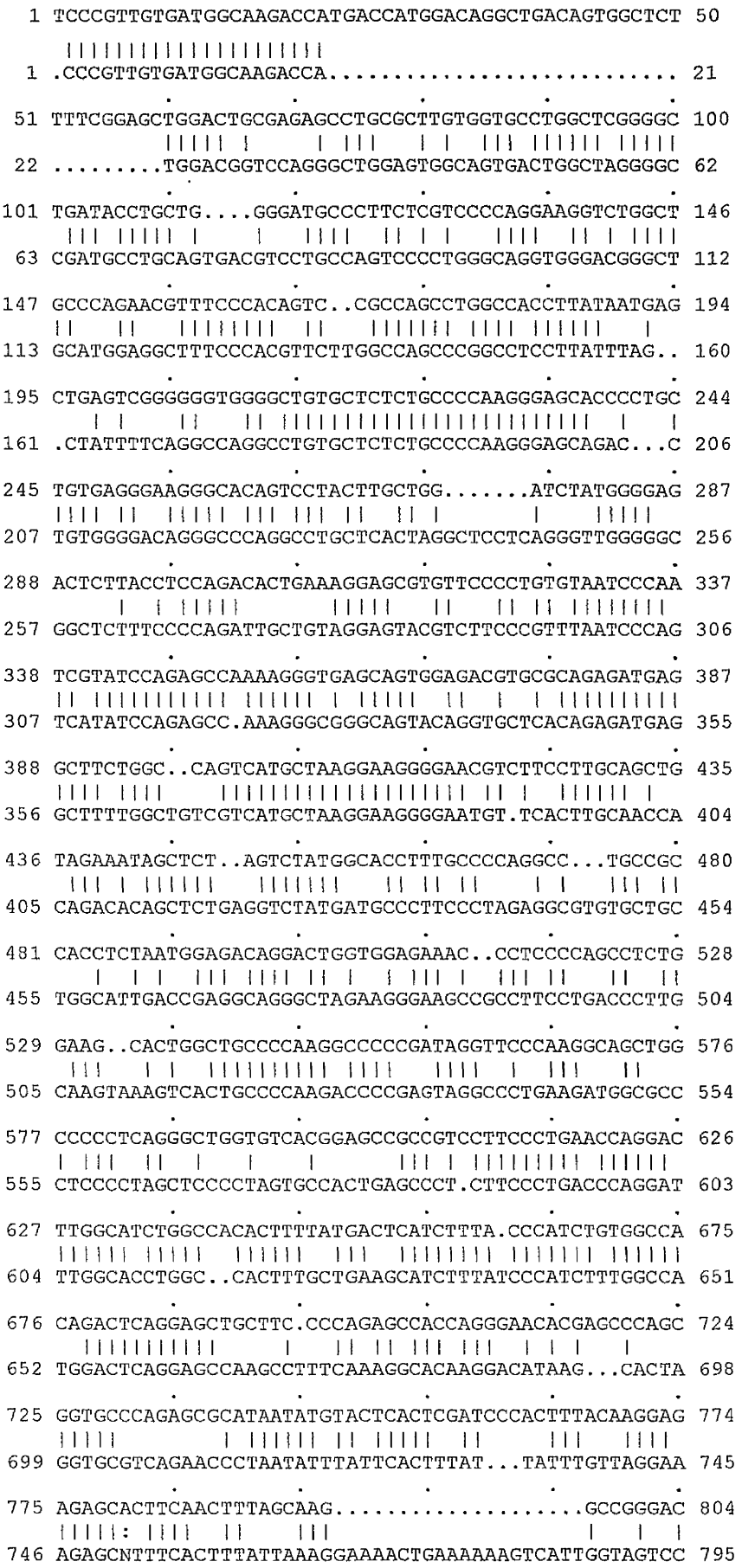
Figure 4.8: Comparison of the pig ITIH4 promoter with the working draft of the human genome using the BLAST program. This figure shows a segment of human chromosome 3 (region 54,308,695 to 54,310,491bp). The location of the human ITIH4 gene is indicated. Regions of the pig ITIH4 promoter exhibiting homology in this region are referred to in the diagram as Blast hit. It is evident from this figure that parts of the isolated pig ITIH4 promoter are mapping to the promoter of the human ITIH4 gene.

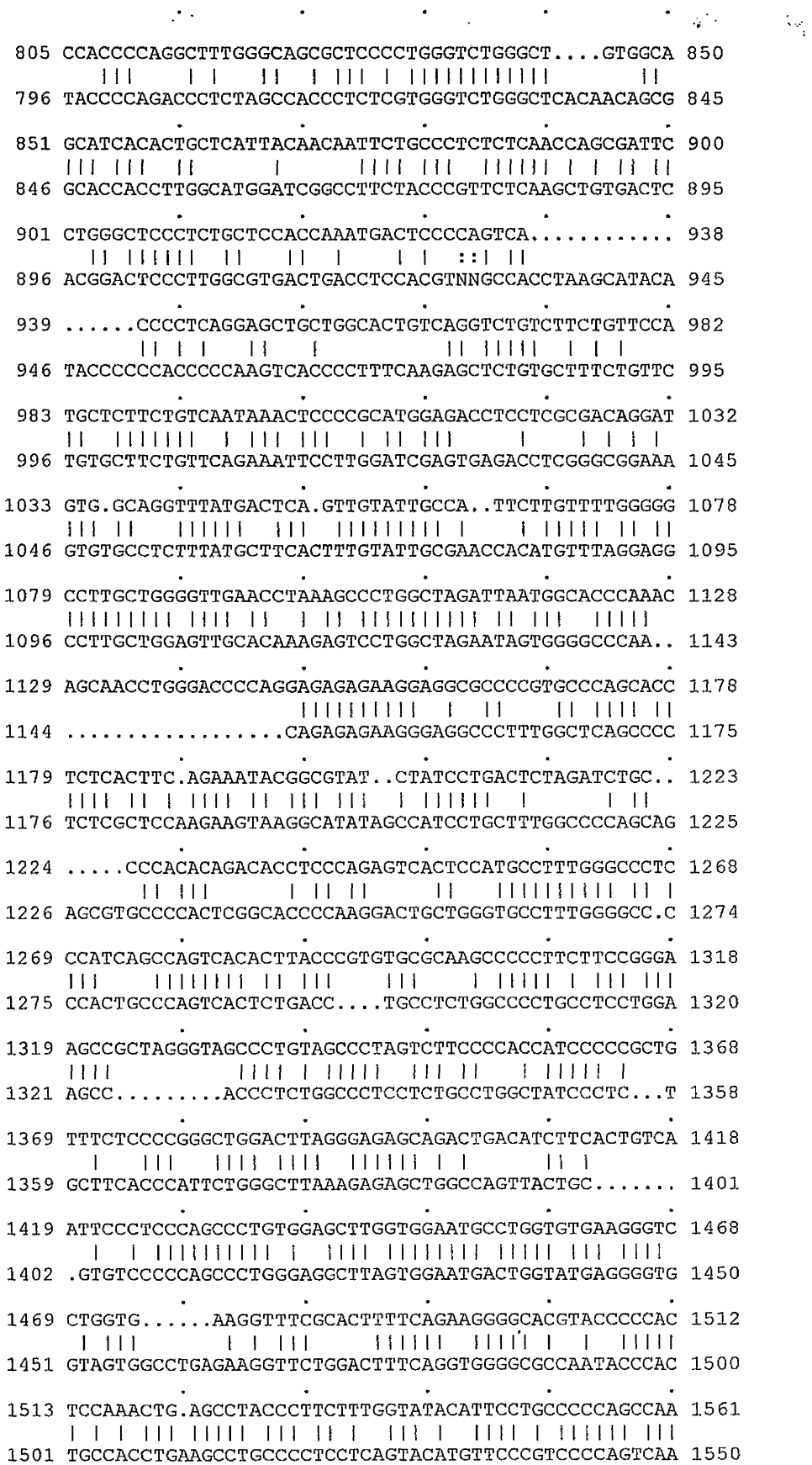
4.3.3 Sequence analysis using GCG Gap program

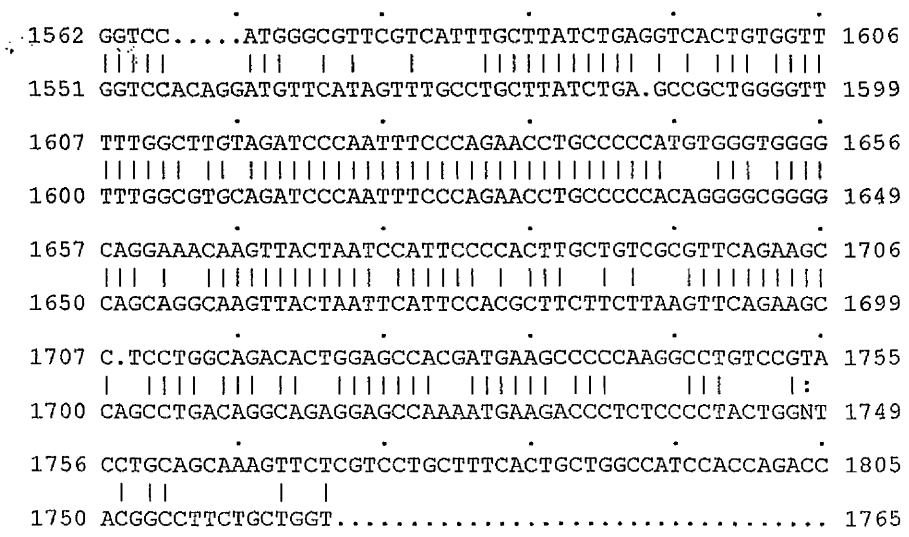
The sequence was analysed using BLAST to determine if the isolated sequence had any homology to other sequences in the nucleotide database. In order to determine exactly where the human and pig ITIH4 promoters differed, the human and the pig ITIH4 promoters were compared using the GCG Gap program (Figure 4.9). This analysis suggested that the promoters were more homologous. Homology between the two promoters is calculated to be 65%. The reason for the different results obtained with the BLAST and Gap programs is probably due to how the data is analysed. Gap creates a global alignment that maximises the number of matched residues and minimises the number and size of gaps. BLAST on the other hand is a local alignment tool that searches for regions of similarity and only reports sequences where the alignment score meets or exceeds a threshold or cut off score (details of this can be found on the BLAST website (Table 2.1)). The smallest search sequence used by BLAST is 7 letters. Therefore, it appears likely, as shown in Figure 4.9, that because many of the regions exhibiting homology are only a few nucleotides in length BLAST calculated that they were below the cut off score and so they were not reported. Moreover, it is clear from Figure 4.9 that without the introduction of gaps to maximise the number of matched residues the homology between the promoters would be much less. Therefore, these results indicate that caution must be taken when interpreting the results as the results depend on the method used for analysis.

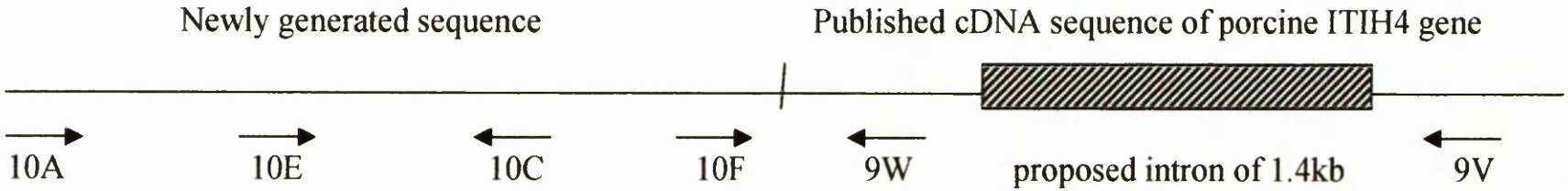
The final experimental procedure carried out to confirm that the sequence isolated was the pig ITIH4 promoter was a series of PCRs. For these reactions primers were designed against the newly generated sequence (some with homology to the human ITIH4 promoter, others without) and primers designed from the published cDNA sequence of the pig ITIH4 gene. All of the PCRs give products of the expected size as shown in Figure 4.10.

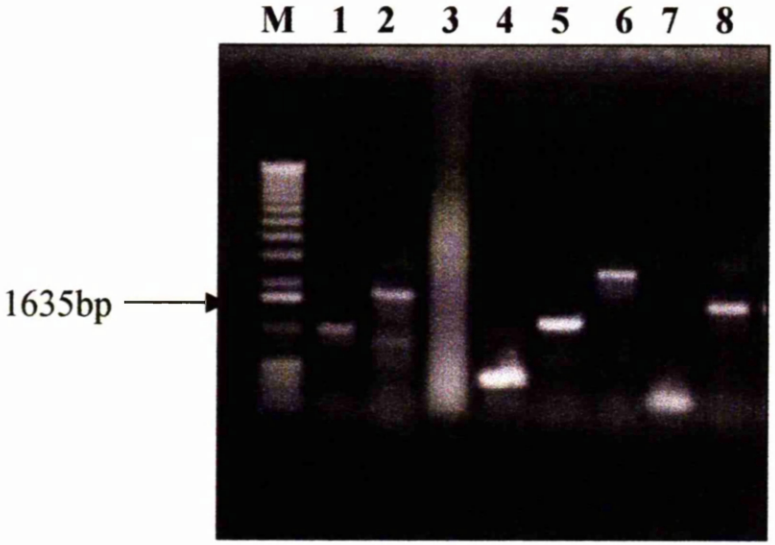
Figure 4.9: Comparison of the pig ITIH4 and human ITIH4 promoter regions using the GCG Gap program. The human ITIH4 promoter sequence is on the top row and the porcine ITIH4 promoter sequence is on the bottom row.











Lane	Reaction	Expected size	Actual size
1	10A+10C	~1kb	1kb
2	10A+9W	~1.7kb	1.7kb
3	10A+9V	2.8 to 3kb	Smear
4	10E+10C	~500bp	~400bp
5	10E+9W	~1kb	1.1kb
6	10E+9V	~2.8kb	2.5kb
7	10F+9W	~170bp	~170bp
8	10F+9V	~1.6kb	1.6kb

Figure 4.10: PCR to confirm that the promoter isolated is that of the pig ITIH4 gene. As shown in the table all PCR reactions gave a PCR product of the expected size. The exception was the PCR product in Lane 3 which gave a smear. This was because the extension time used on the PCR was not long enough to allow a specific PCR product to be formed.

4.4 Sequence analysis of the promoter region of the pig and human ITIH4 genes

Comparison of the porcine ITIH4 promoter with other sequences in the nucleotide database did not reveal any homology to other acute phase promoters (apart from the human ITIH4 promoter). The lack of homology to the human CRP promoter was confirmed when the pig ITIH4 promoter was compared with the 30kb CRP fragment using the GCG gap program.

The results of the sequence analysis of the porcine ITIH4 promoter can be summarised as follows:

- Some but limited homology to the human ITIH4 promoter
- No homology to the human CRP promoter

These findings were somewhat unexpected because the ITIH4 gene in pigs and the CRP gene in humans are the major acute phase proteins in these species. Therefore, it was expected that they would have some sequences in common and that this would allow us to perhaps identify an "acute phase regulatory element" that was common to all acute phase genes and responsible or at least involved in their induction and regulation. The limited homology of the porcine ITIH4 promoter with the human ITIH4 promoter was also surprising and it seems I was actually fortuitous in isolating the porcine ITIH4 promoter using an oligonucleotide from the human ITIH4 promoter, as homology in the region the oligonucleotide was designed from is very low. However, the differences in the promoters of the human and pig ITIH4 genes is in part to be expected because in pigs ITIH4 is a major acute phase proteins whereas in humans ITIH4 is only a minor one.

These observations suggested that analysis of the promoters of the human CRP gene and the porcine and human ITIH4 genes could give some insights into the different regulation of these genes. This analysis was carried out by searching the region 1kb upstream of the translational start site of each gene for binding sites for

inflammation-induced transcription factors. In addition, binding sites for reported regulatory elements found in the promoters of acute phase genes and for binding sites for ubiquitous transcription factors that have been reported to interact with inflammation-induced transcription factors were also searched for. The pig ITIH4 promoter was also searched for binding sites that had been reported by Saguchi *et al.* (1995) in the human ITIH4 promoter. Most of these transcription factor binding sites have already been described in Table 3.3.

The results of these analyses are illustrated in Figures 4.11, 4.12, 4.13. The most striking observation is the completely different organisation of the promoter region of each gene. Although all of the promoters contain binding sites for IL-1 and IL-6 inducible transcription factors, such as NFIL-6 and IL-6RE, the location of these factors varies in each promoter. All of the promoters differ in the number and/or location of other ubiquitous transcription factor binding sites, such as Sp1 and liver-enriched transcription factors such as HNF-5 and LF-A1. An interesting observation is that the acute phase response elements identified by Arcone *et al.* (1988) as being essential for induction of the CRP gene are absent from the promoter of both the ITIH4 genes. A motif found in the rabbit CRP promoter is found in both the human CRP promoter and the pig ITIH4 promoter but not the human ITIH4 promoter.

A big difference between the CRP and ITIH4 genes is that the ITIH4 promoters lack a TATA box. Although this is not unusual, TATA-less promoters are normally associated with "housekeeping" genes. The exact function of the ITIH4 genes is unknown but it is not expected that a major acute phase protein would fall into this category. Both the human and pig ITIH4 promoters contain binding sites for a transcription factor that binds to TATA-less promoters. It is interesting that there are two putative binding sites for this transcription factor in the porcine ITIH4 promoter whereas there is only one in the human ITIH4 promoter. This could result in greater activation of the pig ITIH4 promoter compared to the human ITIH4 promoter.

Figure 4.11: Human CRP promoter illustrating various transcription binding sites and regulatory elements

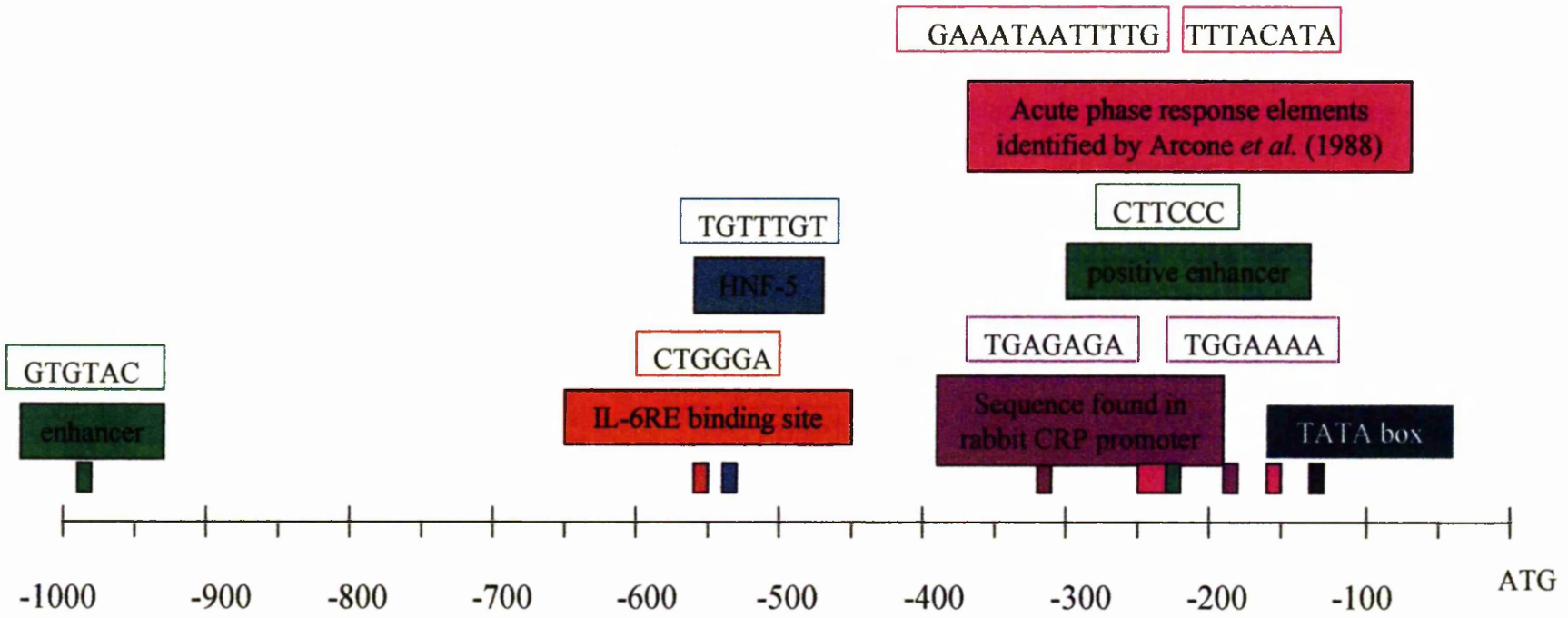


Figure 4.12: Pig ITIH4 promoter region illustrating the binding sites for various transcription factors

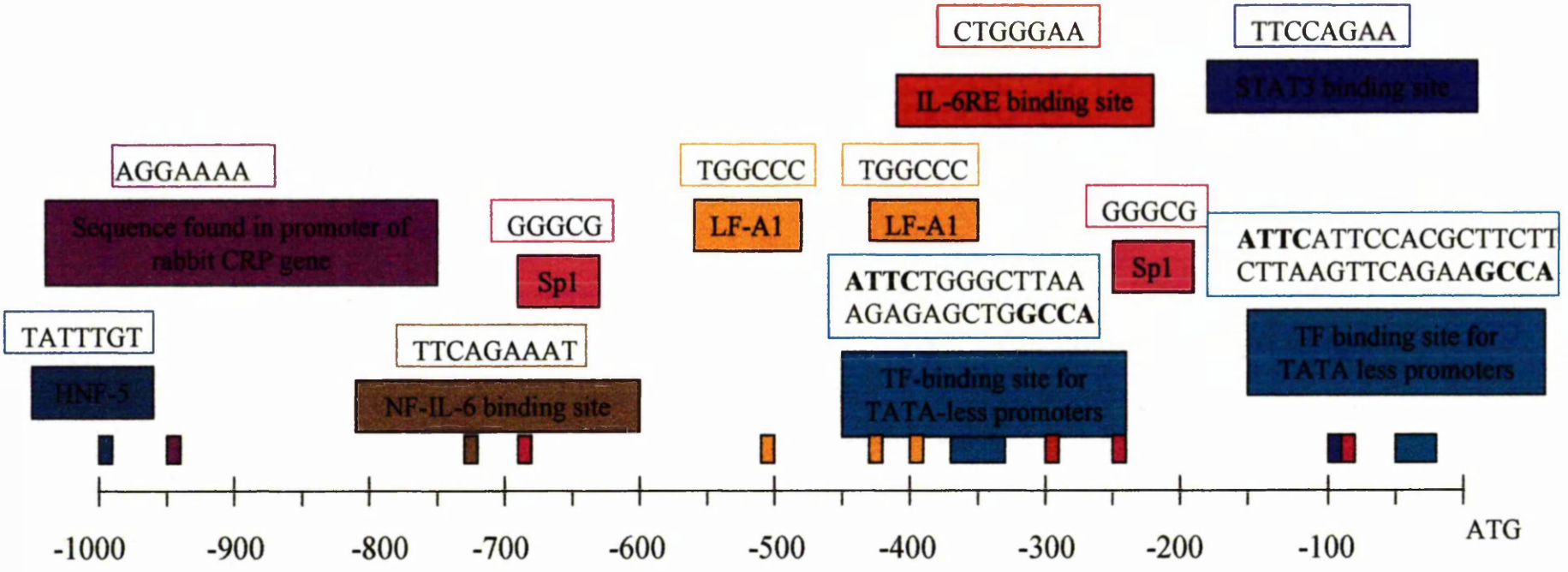
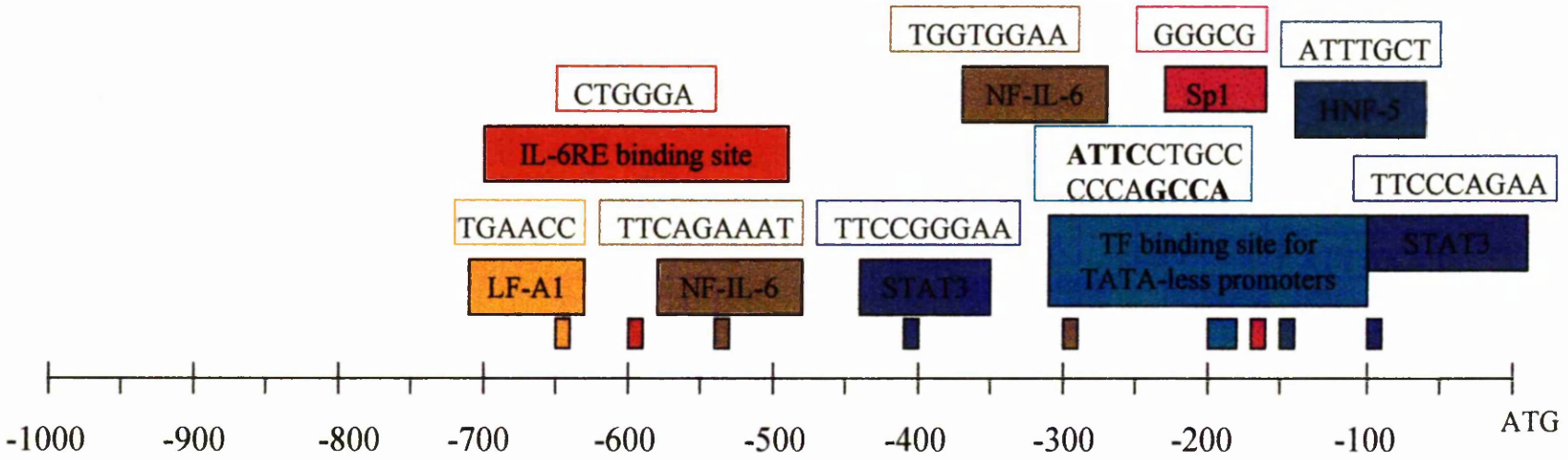


Figure 4.13: Human ITIH4 promoter illustrating the binding sites for various transcription factors



Summary of results

1. A clone containing the porcine ITIH4 gene was isolated by screening a porcine BAC genomic library.
2. The promoter of the porcine ITIH4 gene was isolated
3. Comparison of the porcine ITIH4 promoter with the human ITIH4 promoter revealed that there was limited homology between the two promoters
4. No homology was observed between the pig ITIH4 promoter and the human CRP promoter
5. Comparison of the transcription factor binding sites in the promoter regions of the human CRP gene and the porcine and human ITIH4 genes reveals a very different structural organisation

The main aim of this analysis was to investigate if the porcine ITIH4 promoter contains the same regulatory elements as the human CRP promoter. Since this is not the case it is now necessary to further characterise the porcine ITIH4 promoter to understand its regulation and to establish a system that will facilitate these studies *in vitro*.

Chapter 5: Biological analysis of the porcine ITIH4 promoter in tissue culture

In Chapter 4 the isolation and sequence analysis of the promoter of the porcine ITIH4 gene was described. In this chapter, a functional analysis of the promoter will be described as well as the establishment of an *in vitro* system for studying the porcine acute phase response. Although an immortalised porcine hepatocyte cell line has been made (Liu *et al.* 1999), this cell line is not available commercially at the present time. Therefore, we have examined the potential of the human hepatoma cell line, Hep 3B, as an *in vitro* system for studying expression of the porcine ITIH4 gene.

5.1 Creation of the expression vector

To confirm the ITIH4 promoter was functional and responded to an inflammatory stimulus a number of expression studies were carried out using the chloramphenicol acetyltransferase (CAT) reporter gene. The vector used in these studies, pCAT3-Basic (pCAT3B) vector (Promega), is a promoterless vector containing the CAT gene and has been designed for studying mammalian gene expression. This vector has a MCS that facilitates the cloning of the desired promoter.

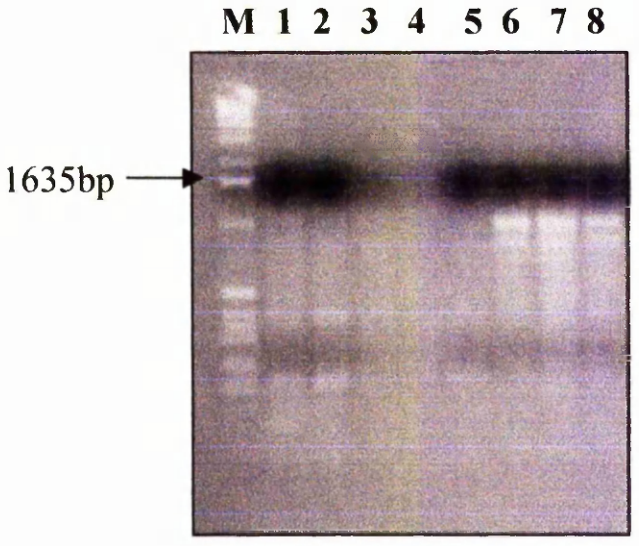
The pig ITIH4 promoter isolated in section 4.3.1 was amplified with primers 5'PPR and PPR 3', which were designed to introduce a *Kpn*I site at the 5' end of the promoter and a *Xho*I site at the 3' end to allow cloning in the MCS of the pCAT3B vector. It is important to note that the region amplified for expression studies did not contain any of the signal peptide sequence of the pig ITIH4 gene. This was to ensure that the reporter gene product was retained in the cell and not secreted into the medium.

The amplified PCR product gave a band of the expected size of 1.8kb. This was cloned into PCRScript and transformed into XL-10 competent cells. Following selection on agar containing X-gal/IPTG, a number of white colonies were screened for clones containing the insert. Once a positive clone had been isolated DNA was prepared and digested with *Kpn*I and *Xho*I to release the insert. The purified DNA was then cloned into the pCAT3B vector, which had also been digested with *Kpn*I and *Xho*I. Following ligation of the insert to the vector and transformation in XL-10 competent cells, the resulting colonies were screened by PCR (Figure 5.1, Gel A). Positive clones were confirmed by digesting the DNA with *Kpn*I and *Xho*I (Figure 5.1, Gel B). To confirm the clone contained the promoter of the pig ITIH4 gene it was sequenced. This clone was termed PPR-CAT3B. This clone was then tested in Hep 3B to see if it was functional and how it responded to an inflammatory stimulus.

5.2 Establishing Hep 3B as a model system for studying gene expression

Because there are no immortalised porcine cell lines currently available, the expression studies were carried out in the immortalised human hepatoma cell line Hep 3B. This cell line has been used extensively in studies of the acute phase response *in vitro*. The first sets of experiments looked at the expression of the pCAT-Control vector, pCAT3B vector (with no insert) and the CRP-CAT construct. The pCAT-Control served as a positive control. In this vector the CAT gene is under the control of the SV40 promoter and this results in constitutive expression of the CAT gene. The pCAT3B vector is a promoterless vector into which the pig ITIH4 promoter was cloned. This vector also served as a negative control and the background expression from pCAT3B was subtracted from the results obtained in the expression studies with the pig ITIH4 promoter. The CRP-CAT construct was made by Shirley Hanley and contains the minimal sequences necessary for acute phase expression of the human CRP gene in tissue culture systems (Arcone *et al.*, 1988; Li *et al.*, 1990). The construct has been tested previously in this system

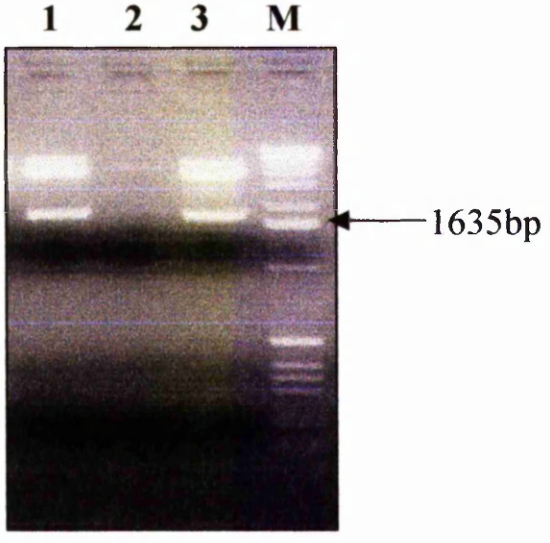
Figure 5.1: Identification of putative positive clones containing the pig ITIH4 promoter in the pCAT3B vector by PCR (Gel A). The expected size of the PCR product from positive clones was 1kb. Other bands present may have been due to non-specific binding of the primers to the template DNA. Clones 6,7 and 8 appeared to be positive. This result was confirmed by digesting DNA from these clones with *Kpn*I and *Xho*I to release the insert of 1.8kb as shown in Gel B.



Gel A

M = marker

1-8 = putative positive clones



Gel B

1 = Clone 6 from Gel A digested with *Kpn*I and *Xho*I

2 = Clone 7 from Gel A digested with *Kpn*I and *Xho*I

3 = Clone 8 from Gel A digested with *Kpn*I and *Xho*I

(Williams, 2001) is known to be expressed and induced in Hep 3B following stimulation with cytokines. Figure 5.2 illustrates the results of these experiments.

These results show that:

- CAT is expressed in Hep3B and can be detected using a CAT ELISA
- The pCAT-3B vector with no insert has very low background levels of expression. This means that in future experiments where a promoter is cloned in this vector any CAT detected above this level is due to promoter activity rather than background expression
- The cell line Hep 3B is responsive to stimulation with IL-1 and IL-6

It should be noted that although the induction of the CRP-CAT construct is only 2-fold, which is much lower than seen *in vivo*, this finding is in agreement with the results of Williams (2001) and is related to the length of the promoter used to make the construct. Other constructs investigated by Williams (2001) with extended 5' sequences exhibited higher levels of induction. In addition, several authors have shown both *in vivo* and *in vitro* that the levels of induction of the CRP or reporter gene are dependent on the sequences present in the construct examined (Arcone *et al.*, 1988; Ganter *et al.*, 1989; Murphy *et al.*, 1995).

In summary, the results from these control experiments meant that the Hep 3B cell line could be tested as an *in vitro* system for studying promoter activity of the porcine ITIH4 gene.

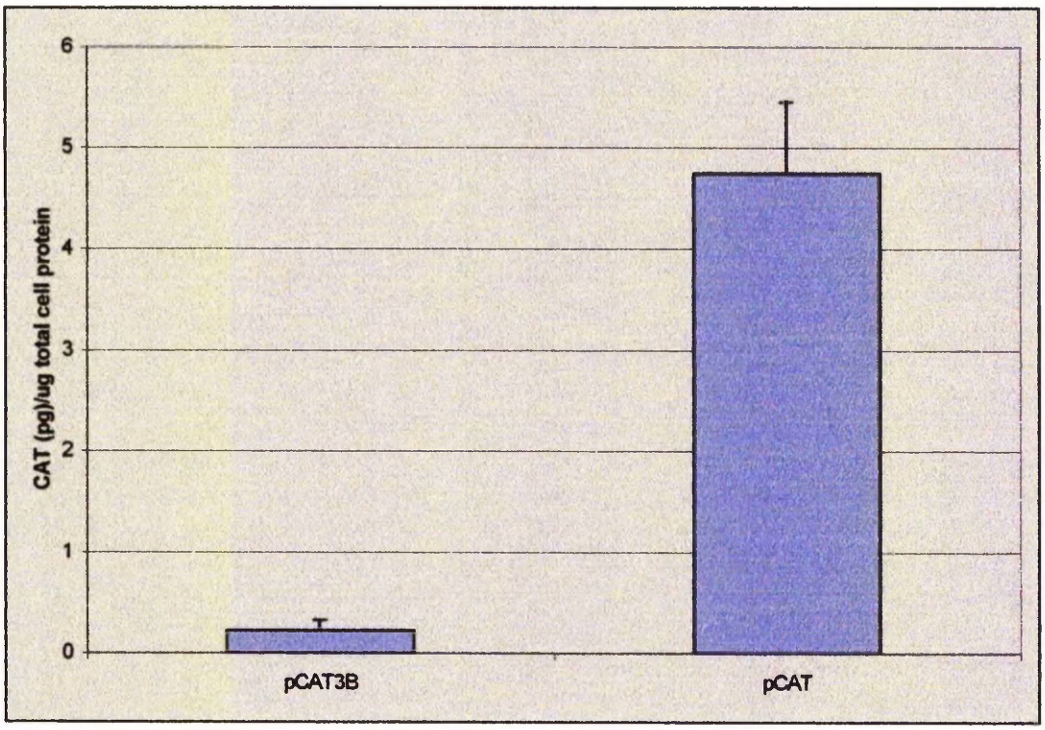


Figure 5.2 (a): Expression of pCAT3B and pCAT (negative and positive control vectors) in Hep3B

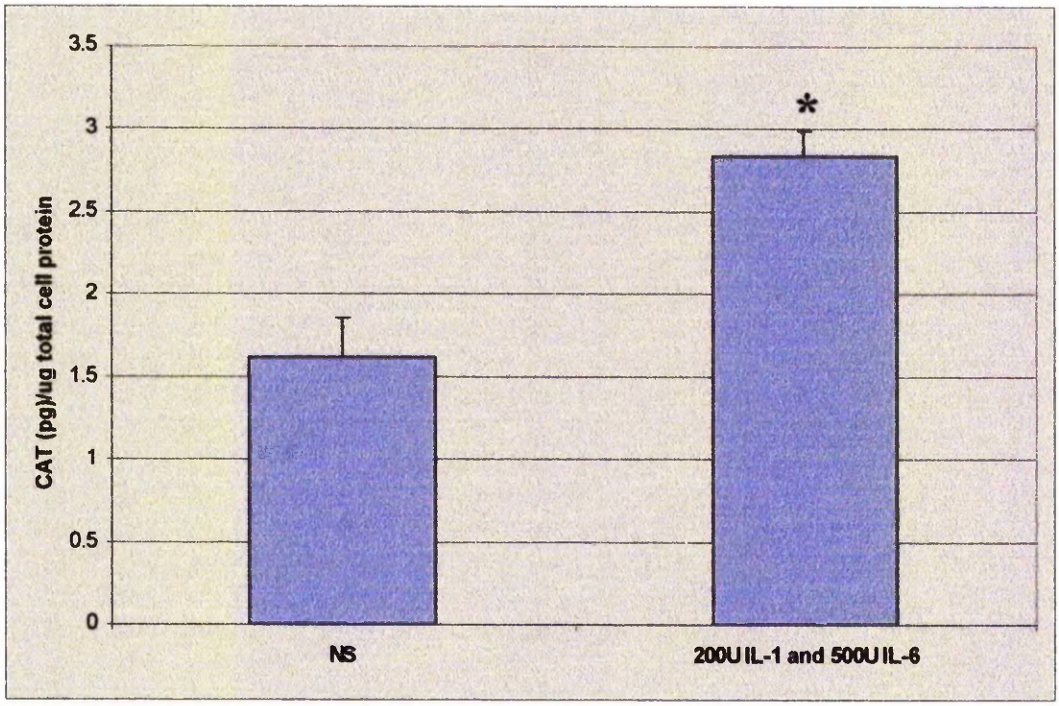


Figure 5.2(b): Expression of CRP-CAT in Hep3B 24 hours post-stimulation with IL-1β and IL-6. * refers to a significant increase in expression ($p<0.05$ Mann Whitney U Test)

5.3 Stimulation of the PPR-CAT3B construct with various concentrations of cytokines

The first set of experiments in Hep 3B examined the expression of the PPR-CAT3B construct in the presence and absence of cytokines. Examination of the construct in the absence of stimulation was carried out to determine the background levels of expression of the construct. Attempts to induce expression of the CAT gene were carried out using recombinant human IL-1β and IL-6. These two pro-inflammatory cytokines are known to play a role in the induction of acute phase proteins and also act in synergy to induce and enhance expression of the human CRP gene. The effect of each of these cytokines and a combination of both on induction of the porcine ITIH4 gene was investigated in Hep 3B as described in the following sections.

5.3.1 Stimulation with IL-6

In section 1.4.2.1 interleukin-6 was described as being the major mediator of the acute phase response. IL-6 has been reported to induce the expression of both the human and pig ITIH4 genes *in vitro* (Piñeiro *et al.*, 1999; González-Ramón *et al.*, 2000). In the experiments described here, the effect of various concentrations of human recombinant IL-6 on the pig ITIH4 promoter was examined. The cells were stimulated with 5U, 50U or 500U IL-6 in serum-free medium 24 hours post-transfection and the cells were harvested 48 hours later. The amount of CAT produced was measured by ELISA (section 2.5.2.8).

Analysis of the samples following stimulation shows that even in the absence of stimulation the pig ITIH4 promoter is driving expression of the CAT gene. This cannot be accounted for by background transcription of the CAT gene because it is much higher than that of the pCAT3B vector alone. Following stimulation with 5 and 50 units of IL-6 only a small increase in expression was seen. This increase in expression was less than two fold and was not significant (Mann Whitney U Test).

However, following stimulation with 500U IL-6 there was a 6-fold increase in CAT expression which was significant ($p < 0.05$ Mann Whitney U Test).

5.3.2 Stimulation with IL-1β

Piñeiro *et al.* (1999) and González-Ramón *et al.* (2000) reported that IL-1β did not have any effect on expression of the endogenous ITIH4 gene in humans and pigs. Therefore, the effect of IL-1β on the pig ITIH4 promoter *in vitro* was examined. Hep 3B cells were transfected with the PPR-CAT3B construct and stimulated with various concentrations (5-500U) of human recombinant IL-1β 24 hours post-transfection. After a 48 hour incubation period the cells were harvested and analysed for CAT expression.

Figure 5.4 shows that stimulation with 5U IL-1β resulted in a small but significant increase in expression ($p < 0.05$ Mann Whitney U Test). However, at higher concentrations (50 and 500U), there was no significant increase in expression. These results suggest that the porcine ITIH4 gene is a Class II acute phase protein as it is induced most strongly by IL-6.

5.3.3 Stimulation with a combination of IL-6 and IL-1β

The results from the above experiments showed that the promoter of the ITIH4 gene was inducible in Hep 3B when stimulated with IL-6. However, the level of induction was much lower than expected (induction of up to 30-fold has been reported *in vivo* (Lampreave *et al.*, 1994)). Since it is unlikely that one cytokine is responsible for induction of any acute phase gene the combination of different concentrations of cytokines on promoter activity was examined. Induction of the human CRP gene has been reported to be the result of IL-1β and IL-6 acting in synergy (Ganter *et al.*, 1989; Zhang *et al.*, 1995). Therefore, the effect of a combination of both IL-1β and IL-6 on induction of the porcine ITIH4 gene was investigated. 500U of IL-6 had been shown to result in a 6-fold increase in

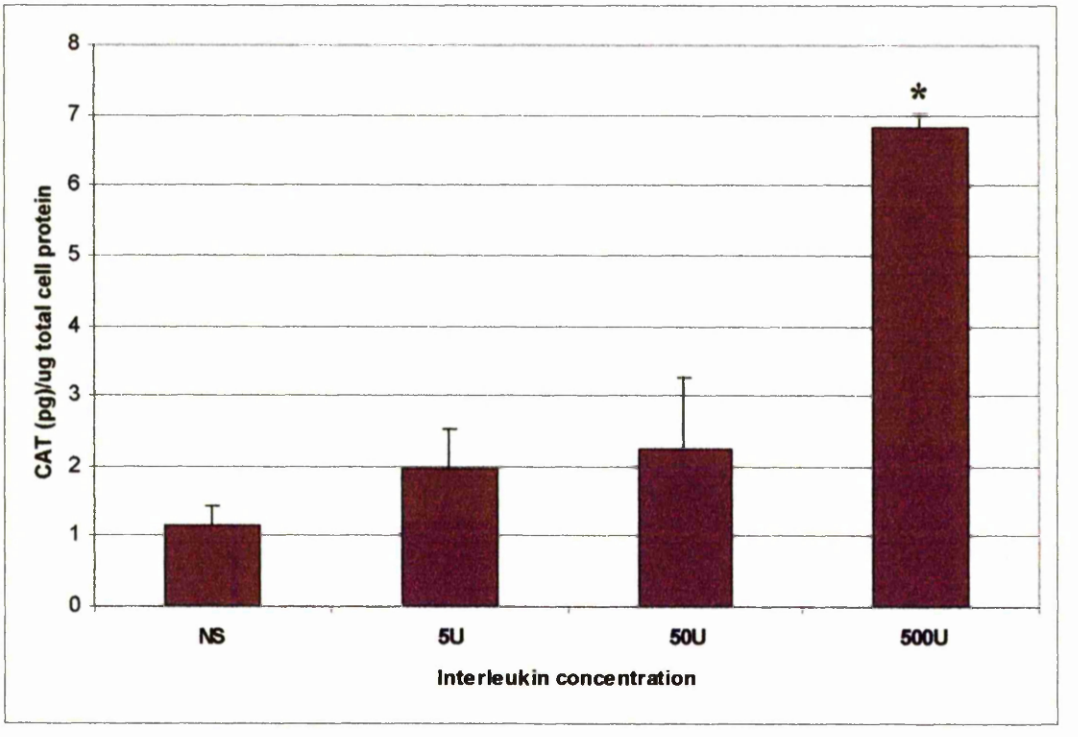


Figure 5.3: Expression of PPR-CAT3B in Hep 3B 48 hours post-stimulation with various concentrations of IL-6. * refers to a significant increase in expression ($p<0.05$ Mann Whitney U Test)

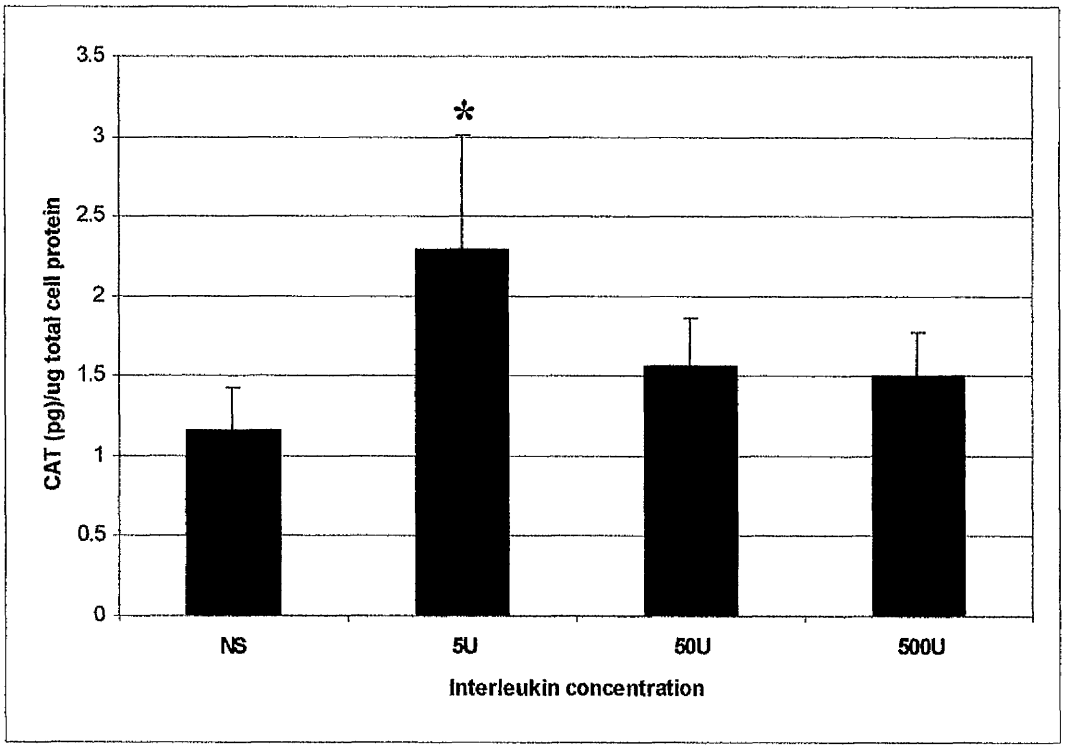


Figure 5.4: Expression of PPR-CAT3B in Hep 3B 48 hours post-stimulation with various concentrations of IL-1β. * refers to a significant increase in expression ($p<0.05$ Mann Whitney U Test)

expression, therefore the effect of 500U IL-6 plus various combinations of IL-1β (5-500U) was examined. The results from these experiments are shown in Figure 5.5.

A combination of 500U IL-6 and 5U IL-1β resulted in a 6-fold increase in expression, which was similar to that seen in cells stimulated with 500U IL-6 alone. Therefore, the addition of 5U IL-1β did not enhance the inducibility of the construct. A combination of 500U IL-6 and 50 or 500U of IL-1β resulted in a decrease in expression of the CAT gene, which was dose dependent. A combination of 500U IL-6 and 50U IL-1β only results in a 2.5 fold increase in expression, although this is still significantly higher than in cells receiving no stimulation ($p < 0.05$ Mann Whitney U Test). The combination of 500U IL-6 and 500U IL-1β abolishes the inducibility of the construct by 500U IL-6 alone. The level of expression of the CAT gene is more or less the same as that of cells receiving no stimulation (Figure 5.5).

The results obtained when the PPR-CAT3B construct was stimulated with IL-1β and IL-6 separately are in good agreement with those of González-Ramón *et al.* (2000) who investigated the inducibility of the endogenous porcine ITIH4 gene *in vitro* following stimulation with IL-1 and IL-6. Although González-Ramón *et al.* (2000) did not examine the effect of a combination of IL-1 and IL-6 on expression of the endogenous porcine ITIH4 gene, the inhibition of the IL-6 induced activity of the ITIH4 promoter by IL-1 is consistent with ITIH4 being classed as a Class II acute phase protein. These findings suggest that Hep 3B may be suitable for a functional analysis of the porcine ITIH4 promoter *in vitro*. To verify this I wished to analyse the constructs in porcine hepatocytes. As mentioned previously, an immortalised porcine hepatocyte cell line is not currently available. However, I was able to source some primary porcine hepatocytes and I planned to test the ITIH4 constructs in these cells before carrying out any further work.

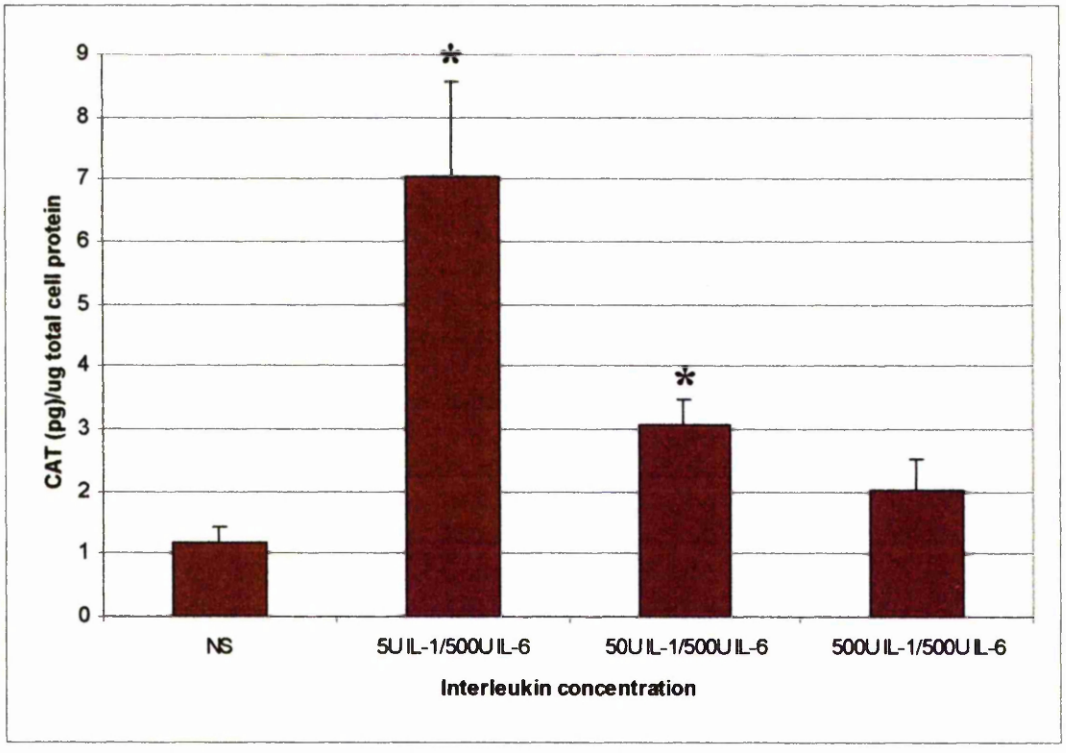


Figure 5.5: Expression of PPR-CAT3B in Hep 3B 48 hours post-stimulation with 500U IL-6 and various concentrations of IL-1β. * refers to a significant increase in expression ($p<0.05$ Mann Whitney U Test)

5.4 Analysis of the constructs in primary porcine hepatocytes

Before carrying out any further work on the pig ITIH4 promoter I wanted to confirm the findings from the above experiments in primary porcine hepatocytes. Primary porcine hepatocytes were kindly supplied by Mr L Nelson and Mr S Keach (Liver Research Group, Department of Medicine, University of Edinburgh, UK). The hepatocytes were isolated using the method described by Nelson *et al.* (2000). The cells were seeded in 6 well plates and transfected as described in section 2.5.2.5.2 with 2μg of C3:EGFP. C3:EGFP is a vector containing the enhanced variant of GFP under the control of the CMV promoter. Preliminary experiments suggested that transfection was possible and successful because fluorescent cells could be seen when the cells were examined using a fluorescent microscope. Further experiments to confirm these findings, to optimise conditions and to test the porcine ITIH4 constructs were not possible due to the outbreak of foot and mouth disease in the UK which prevented access to farms and the movement of samples.

5.5 Generation of mutants to decrease basal level of expression and increase inducibility

Although I was not able to confirm the findings reported in section 5.3 in primary porcine hepatocytes, I believed that Hep 3B was a usable system for biological studies of the porcine ITIH4 promoter. In Hep 3B, the ITIH4 promoter was behaving as a typical Class II acute phase promoter. Although the levels of induction of the PPR-CAT3B construct were different to those of the endogenous ITIH4 gene *in vitro* and the ITIH4 gene *in vivo*, a similar phenomenon has already been reported for the human CRP gene (Arcone *et al.*, 1988). Transiently transfected constructs containing the human CRP promoter were induced stronger than the endogenous gene *in vitro* and the levels of induction never match those seen *in vivo*. Therefore, it was decided to continue our studies on the ITIH4 promoter using Hep 3B cells for analysis.

5.5.1 Comparison of the human and pig ITIH4 promoters

Although the promoter region of the ITIH4 gene was driving expression of the CAT gene and was inducible, the fact that basal level expression could be detected was not acceptable for reasons already discussed in section 1.4. Therefore, it was attempted to create a construct whereby the basal level of expression would be reduced while maintaining or increasing the inducibility of the construct. As the human ITIH4 gene is not induced to the same extent as the porcine ITIH4 gene *in vivo*, the promoter regions of these two genes were compared to see if differences in binding sites for transcription factors could account for the differences in expression. As reported in section 4.3.3 although there is 65% homology between the human and the pig ITIH4 promoters the distribution and organisation of putative transcription factor binding sites is very different. The literature was searched to see if any of the putative transcription factor binding sites could influence or account for the different levels of expression of the human and porcine ITIH4 genes.

The work focused on the putative binding sites for the LF-A1 transcription factor that had been identified by searching the pig ITIH4 promoter for transcription factor binding sites that had been reported by Saguchi *et al.* (1995) in the human ITIH4 promoter. Hardon *et al.* (1988) suggested that the affinity of the LF-A1 transcription factor for its binding site in the promoter region of the haptoglobin and haptoglobin-related genes as well as the number of binding sites to which LF-A1 could bind strongly influenced the basal levels of expression of the gene. They proposed that decreased affinity of LF-A1 for its binding site in the promoter of the haptoglobin gene due to a base substitution in its binding site was correlated with lower basal levels of expression. It was interesting to note that in the region 1kb upstream of the human and porcine ITIH4 translation start codons there are three putative binding sites for LF-A1 in the porcine ITIH4 promoter whereas there is only one binding site in the corresponding region of the human ITIH4 promoter (Figures 4.12 and 4.13). It was therefore decided to delete these binding sites from the porcine ITIH4 promoter to see what effect it would have on the basal levels of transcription. Figure

5.6 shows the location of the LF-A1 binding sites in the pig ITIH4 promoter relative to each other. They LF-A1 sites are found in a region approximately 300-550 bases upstream of the pig ITIH4 translation start codon. From 5' to 3' the LF-A1 sites were termed the alpha, beta and gamma.

5′ AGCCCCTCTCGCTCCAGAAGTAAGGCATATAGCCATCCTGCTTTGGCCC

CAGCAGAGCGTGCCCCACTCGGGCACCCCAAGGACTGCTGGGTGCCTT

TGGGGCCCCACTGCCCAGTCACTCTGACCTGGCCTCTGGCCCCTGCCTCC

TGGAAGCCACCCTCTGGCCCTCCTCTGCCTGGCTATCCCTCTGCTTCACC

CATTCTGGGCTTAAAGAGAGCTGGCCAGTTCACTGCGTGTCCCCCAGC 3′

Figure 5.6: Location of the putative LF-A1 binding sites with respect to each other. The region shown in this figure is from positions –556 to –312 relative to the first nucleotide of the translational start codon (+1). The binding sites are shown in green.

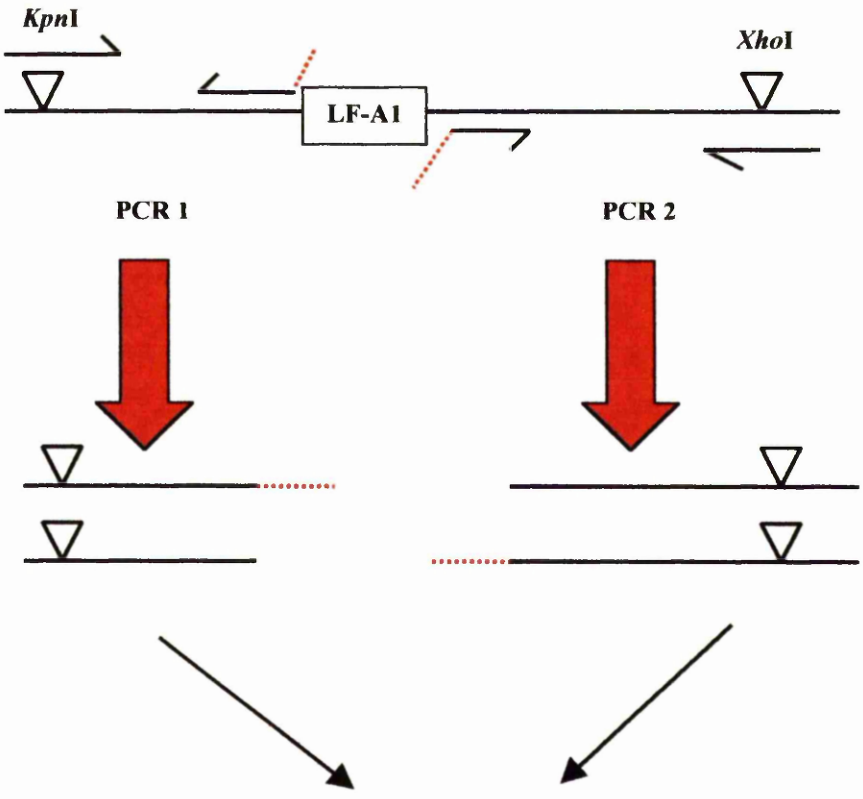
5.5.2 Deleting the LF-A1 binding sites from the pig ITIH4 promoter

The mutant constructs were created in a series of PCRs designed to knock out each binding site and to delete the entire region containing these binding sites (region underlined with dotted line in Figure 5.6). The method of creating the mutant constructs is illustrated in Figure 5.7.

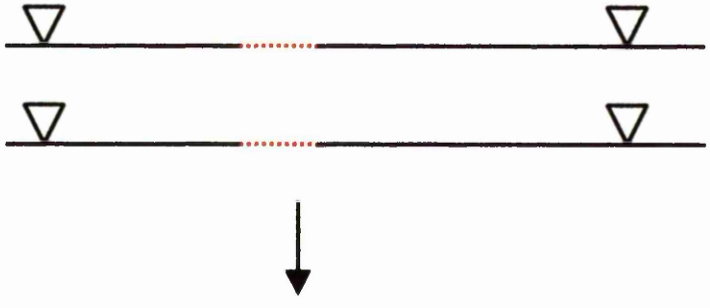
Although the initial strategy was to use standard primers located in the PCRScript vector so that the restriction enzyme sites that had been introduced previously for cloning (section 5.1) could be used this was unsuccessful. Therefore, the primers that had been used to introduce the restriction enzyme sites at each end were used instead. The oligonucleotides to delete the LF-A1 binding sites were designed so that one half of the oligonucleotide was from one side of the LF-A1 binding site and the tail of the oligonucleotide was from the other. This resulted in an oligonucleotide with the LF-A1 binding site missing. In the first set of PCRs the two halves of the fusion product were created. Figure 5.8 shows the amplification products from each half of the PCR. The expected size of the PCR products was approximately 1.3kb for the left-hand PCR and approximately 500bp for the right-hand PCR.

The third PCR was carried out to fuse the products of each half of the fusion together. The PCR products from the fusion PCR were then cloned in PCRScript and sequenced to ensure that the LF-A1 binding sites had been deleted. These clones were then digested with *Kpn*I and *Xho*I to release the insert (Figure 5.9). The insert was then cloned in the pCAT3-Basic vector these constructs were subsequently analysed in Hep 3B.

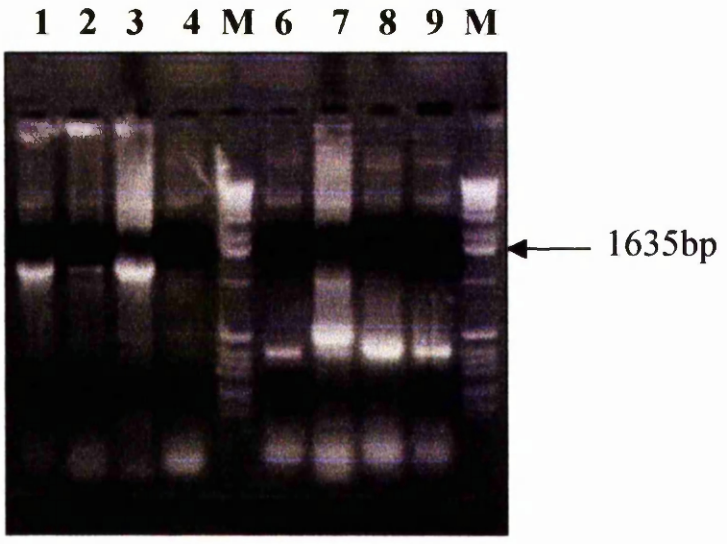
Figure 5.7: Generation of LF-A1 mutants. The mutants were made by amplifying each half of the promoter by PCR with one primer from each set (the central primers in this figure) designed so that they lacked the LF-A1 site and that their 5' ends (shown with a red broken line) were complementary to each other. The other primers in each set contained engineered restriction enzyme sites to facilitate later cloning. PCR 1 and PCR 2 resulted in the creation of two PCR products with overhanging ends (red broken line) that were complementary to each other. The third PCR was carried out with a low annealing temperature for the first 3 cycles to allow the complementary ends to bind to each other. The PCR product created from PCR3 was, therefore, the pig ITIH4 promoter lacking a LF-A1 site or the entire region containing the LF-A1 sites.



PCR 3 with low annealing temperature for the first 3 cycles



Cloning and sequencing of the constructs and analysis of expression in Hep 3B



1 = ΔALL (PCR 1)

2 = Δα (PCR 1)

3 = Δβ (PCR 1)

4 = Δγ (PCR 1)

M = marker

6 = ΔALL (PCR 2)

7 = Δα (PCR2)

8 = Δβ (PCR 2)

9 = Δγ (PCR 2)

Figure 5.8: Amplification of each half of the fusion. Amplification of the left side of the fusion (PCR 1) is shown to the left of the marker and amplification of the right side of the fusion (PCR 2) is shown to the right of the marker. The 1.6kb band of the DNA ladder is indicated with an arrow.

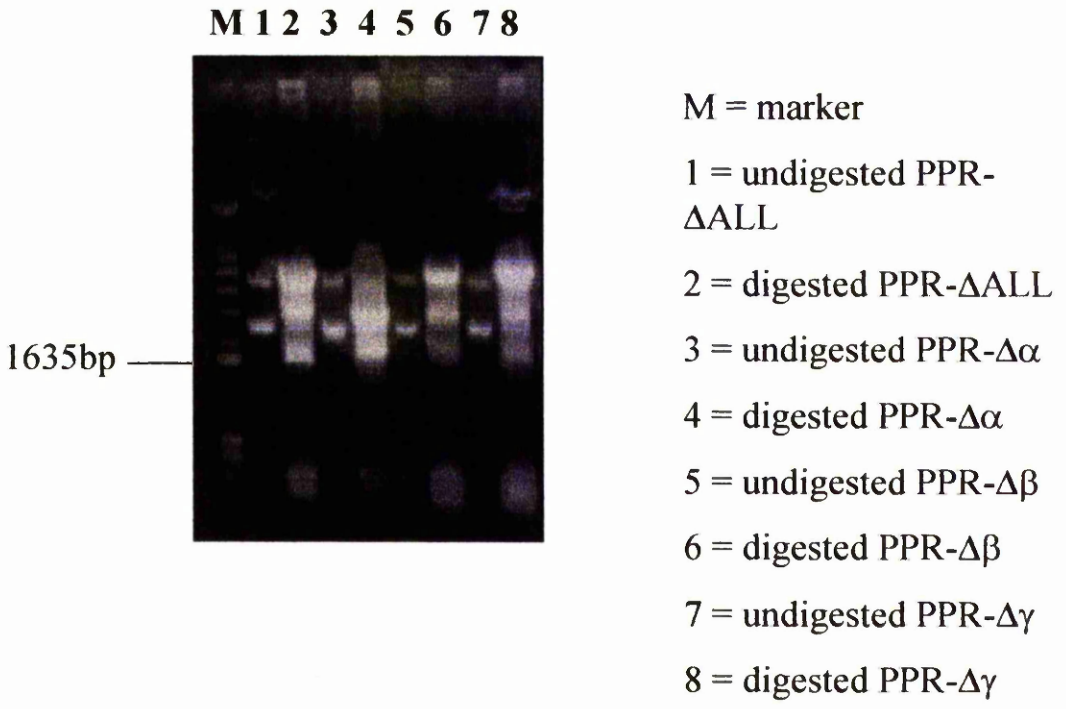


Figure 5.9: Clones containing the mutated pig ITIH4 promoter following digestion with *Kpn*I and *Xho*I to release the insert. All digests result in the release of a band of approximately 1.7kb. The 1.6kb band of the DNA ladder is indicated with an arrow.

5.5.3 Analysis of the mutated constructs in Hep 3B

Hep3B cells were transfected with each of the constructs and the cells were stimulated with 500U IL-6. The cells were harvested 48 hours later and the amount of CAT produced measured. The results from these experiments are given in Table 5.1.

The data from these experiments can be analysed in two ways: first of all the effect of the mutation on the inducibility of the constructs and secondly the effect on the basal levels of expression.

As shown in Table 5.1, the level of induction of the mutated constructs following stimulation with 500U IL-6 varies from 2.3 fold to 4.2 fold. Therefore, it appears the mutation has affected the inducibility of the construct to some extent as the wild-type construct was induced 6-fold following stimulation with 500U IL-6.

When compared with the wild-type construct it is seen that all the constructs exhibit increased basal levels of expression. This is particularly true for the PPR-Δγ construct whose basal levels of expression are 6-fold higher than the wild-type construct. The level of expression of the constructs following stimulation with 500U IL-6 does not vary as much when compared to the wild-type in the absence of stimulation. In the presence of IL-6 the mutated constructs at best only exhibit a 2.4 fold increase in expression over the wild-type construct under the same conditions. Both the PPR-ΔALL and PPR-Δγ construct have higher levels of CAT compared to the other two constructs when stimulated with IL-6. This suggests that the Δγ -LF-A1 site may be responsible for most of the effects seen with the PPR-ΔALL construct.

A summary of the effect of the mutation on the amount of CAT produced when compared with the wild-type construct is given in Table 5.2.

Table 5.1: Expression of LF-A1 mutants in Hep3B. Results are given as mean ± SEM. * refers to significant increases in expression ($p<0.05$ Mann Whitney U Test) a) following stimulation with 500U IL-6 and b) when compared with the wild-type construct.

				Fold increase in expression over wild-type construct	
Construct	No stimulation (CAT (pg/μg total cell protein)	500U IL-6 CAT (pg/μg total cell protein)	Fold increase in expression	No stimulation	500U IL-6
PPR-CAT3B	1.16 ± 0.265	6.83 ± 0.183	5.9X*		
PPR-ΔALL	4.38 ± 1.8	15.35 ± 2.13	3.5X *	3.77X *	2.24X *
PPR-Δα	3.67 ± 1.1	8.8 ± 4.01	2.4X	3.16X *	1.29X
PPR-Δβ	2.39 ± 0.59	10.1 ± 1.01	4.18X *	2.06X	1.46X *
PPR-Δγ	7.25 ± 0.56	16.47 ± 0.91	2.27X *	6.25X *	2.41X *

Table 5.2: Effect of deleting putative LF-A1 binding sites on the background expression and stimulation of the PPR-CAT3B construct in Hep 3B following stimulation with IL-6. + refers to enhanced expression compared to the wild-type construct. – refers to no significant change in expression compared to the wild-type construct

	Effect on background	**Effect on stimulation**
PPR-ΔALL	+	+++
PPR-Δα	+	-
PPR-Δβ	-	+
PPR-Δγ	++	+++

Mutation of the putative LF-A1 sites was carried out in an attempt to reduce the basal level of expression of the construct while maintaining or increasing the inducibility of the construct. While I was successful in increasing the amount of CAT produced, the mutations have in fact increased the basal levels of expression and decreased the inducibility of the constructs. The γ-LF-A1 site appears to be responsible for these changes. In order to draw some conclusions about how the γ-LF-A1 site could be affecting the expression of the pig ITIH4 gene, the region absent from the ΔALL construct was analysed using the transcription factor search program Alibaba 2. The results from this analysis are shown in Figure 5.10. The results show that there are a number of putative binding sites for the ubiquitous transcription factor Sp1. Sp1 has already been reported to associate with other inflammation induced transcription factors to modulate gene expression and it can affect gene expression in both a positive and negative manner (Look *et al.*, 1995; Li *et al.*, 1998b; Ripe *et al.*, 1999; Uhlar & Whitehead, 1999; Kato *et al.*, 2000; Ping *et al.*, 2000). In Figure 5.10 it can be seen that a number of the putative Sp1 sites overlap with the putative LF-A1 sites including the γ-site. Therefore, it could be hypothesised that the removal of the γ-LF-A1 site could disrupt the basal transcription machinery in some manner by affecting the binding of other transcription factors in this region.

```
seq(    0..   59)     tggcccagcagagcgtgccccactcgggcaccccaaggactgctgggtgcctttggggc
Segments:
2.3.1.0    14   27                    ------Sp1-----
2.3.1.0    20   34                          -------Sp1-----
1.6.1.0    27   36                                 -AP-2alph-
2.1.1.2    34   43                                        -----PR---
2.3.1.0    55   68                                                  -----
seq(   60..  119)     cccactgcccagtcactctgacctggcctctggcccctgcctcctggaagccaccctctg
Segments:
2.3.1.0    55   68    -Sp1-----
2.3.1.0    63   72       ----Sp1---
9.9.29     65   74         ----AP-1--
2.1.1.4    73   86                 -------ER-----
2.1.2.1    75   84                   -RAR-alph-
2.1.2.2    75   84                   --RXR-beta
2.1.2.3    75   84                   -T3R-alph-
2.3.3.0    75   84                   -CPE_bind-
2.3.1.0    81   93                         ------Sp1----
1.6.1.0    84   93                            ----AP-2--
9.9.539    85   94                             ----NF-1--
2.3.2.3    87   96                               ----BRF1--
2.3.1.0    87   101                              -------Sp1-----
2.2.1.1    89   98                                 ---GATA-1-
2.3.1.0    94   108                                      -------Sp1-----
9.9.539    103  112                                             ----NF-1--
9.9.539    113  122                                                     ----NF-
2.3.1.0    117  126                                                       ---
seq(  120..  179)     gccc
Segments:
9.9.539    113  122   1--
2.3.1.0    117  126   -Sp1---

22 segments in this sequence identified as potential binding sites
```

Figure 5.10: Analysis of the region absent from the PPR-ΔALL construct. This region was searched for putative transcription factor binding sites using the program Alibaba 2. From this figure it can be seen that a number of putative Sp1 transcription factor binding sites overlap with the putative LF-A1 binding sites (putative LF-A1 binding sites are shown in red and underlined), including the Δγ-LF-A1 site.

Summary of results

1. Porcine ITIH4 promoter is induced by 500U IL-6. Induction by IL-6 is dose-dependent.
2. Porcine ITIH4 promoter is not induced by IL-1 at concentrations greater than 50U. This result suggests the porcine ITIH4 gene is a Class II acute phase protein.
3. A combination of IL-1 and IL-6 inhibits the IL-6 induced activation of the porcine ITIH4 promoter. This result is further confirmation that pig ITIH4 is a Class II acute phase protein.
4. Hep 3B appears to be a usable system for studying the porcine ITIH4 promoter.
5. Primary porcine hepatocytes can be transfected with GFP.
6. A number of mutated constructs were made in an attempt to reduce the basal level of expression seen with the PPR-CAT3B while maintaining or increasing the inducibility of the construct. However, analysis of these constructs in Hep 3B revealed that the basal level of expression was increased while the inducibility of the constructs was decreased.

The ability to transfect the primary porcine hepatocytes with GFP suggested that in the future this reporter gene could be used for studying gene expression *in vitro*. The main advantage of GFP reporter gene over the CAT reporter gene is that it would allow real-time analysis. In addition, if a suitable method for quantitating GFP fluorescence could be established then another advantage of the GFP reporter gene would be that quantitating changes in gene expression would be less labour intensive.

In this chapter a number of constructs have been described with varying levels of expression in Hep 3B. It was thought that these constructs could also be used to evaluate GFP as a reporter gene in Hep 3B. The following chapter describes the evaluation of GFP as a reporter gene of transient inducible gene expression.

Chapter 6: Evaluation of GFP as a reporter gene of inducible gene expression

6.1 Introduction

In Chapter 5 use of the CAT reporter gene for analysing gene expression of the porcine ITIH4 gene was reported. However, this method for studying gene expression is laborious and expensive. In particular, it is not useful for studying inducible gene expression, as the cells have to be harvested at pre-determined time points and so although changes in gene expression can be seen, the time these changes occur cannot be accurately determined. More importantly for us, because CAT is a stable protein which accumulates in the cell over time, the decrease in gene transcription and subsequently protein synthesis seen during resolution of the acute phase response *in vivo* will not be seen.

The green fluorescent protein (GFP) allows real-time analysis of gene expression *in vitro* as it is synthesised by the cells themselves and no exogenous substrates or co-factors are required for its synthesis or detection. In addition, there have also been reports of GFP being used as a reporter gene *in vivo* (Chiocchetti *et al.* 1997). One of the main attractions of GFP is that it can be easily detected using a fluorescence microscope or UV light. There are several GFP mutants now available that have been created for optimised expression in mammalian cells as well as destabilised variants that can be used for studies of inducible gene expression since GFP itself is a very stable protein. In order to evaluate GFP as a reporter of gene expression in Hep 3B we used the promoter of the pig ITIH4 gene and the γ-LF-A1 mutant, as well as two forms of GFP – a stabilised and destabilised variant.

6.2 Transfection of COS-7 and Hep 3B cells with C3:EGFP and GFP-C

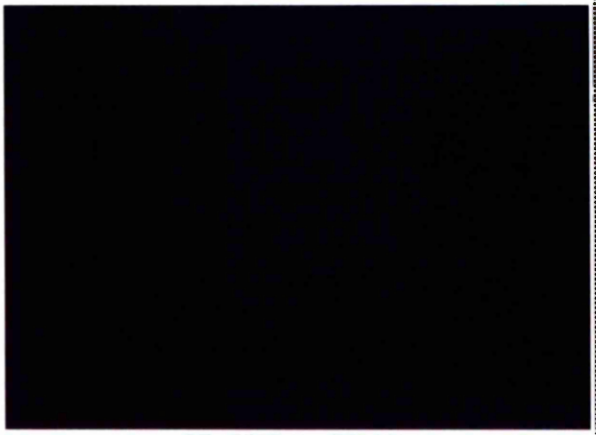
6.2.1 Optimising the transfection conditions

In order to establish the system, COS-7 cells, a monkey kidney derived cell line, which has previously been reported to express GFP (Sakai *et al.*, 1997; Yano *et al.*, 1997), was used. To determine the optimum amount of DNA to use in the transfection experiments the cells were seeded in 24 well plates and transfected with various amounts of the control vectors C3:EGFP and GFP-C ranging from 0.5μg to 2μg. Similarly, Hep 3B cells were also transfected with the same amount of the control GFP vectors. Following analysis of the cells over a 72 hour period it was deemed that 0.75μg DNA was suitable for the transfections as larger amounts of DNA were inducing cell death. Figure 6.1 shows some images from these experiments. It is seen that Hep 3B cells were transfected as well as COS-7 cells but the kinetics of expression of the two GFP vectors is different. The destabilised variant of GFP appeared much brighter compared to the stabilised variant. In addition, transfection with the C3:EGFP vector resulted in higher numbers of fluorescent cells compared with the GFP-C construct. These observations were observed in both COS-7 and Hep 3B cells at all time points examined. These observations were examined further in Hep 3B.

6.2.2 Comparison of the kinetics of expression of C3:EGFP and GFP-C in Hep 3B

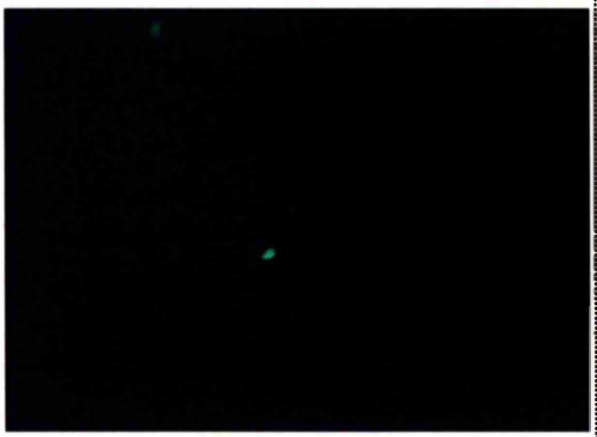
The C3:EGFP vector contains a stabilised variant of the enhanced green fluorescent protein. It is a vector that has been designed to allow cloning of heterogeneous proteins to the C-terminal of GFP. In this vector the Cytomegalovirus (CMV) promoter drives expression of the GFP gene. The GFP-C vector contains a destabilised variant of enhanced GFP with a half-life of 2 hours. This variant of GFP was purchased to enable studies of transient inducible gene expression to be

Figure 6.1: GFP expression in Hep3B and COS-7 cells. The images on the left side of the figure show Hep 3B cells transfected with either the C3:EGFP construct (contains a stabilised variant of GFP) or GFP-C (contains a destabilised variant of GFP). The images on the right show COS-7 cells transfected with the same two vectors. In both cell lines it is clear that there are more fluorescent cells in the images from cells transfected with the C3:EGFP vector. Cells transfected with the GFP-C vector do appear to be brighter than those transfected with the C3:EGFP vector.



C3 : EGFP Hep3B 48h

C3 :EGFP COS-7 48h



GFP-C Hep3B 48h

GFP-C COS-7 48h

carried out. This vector has been designed as a control vector to optimise transfection conditions. The GFP gene in this vector is under the control of the SV40 promoter.

Due to the fact that the two vectors under examination contained different variants of GFP with GFP expression being driven by a different promoter, the expression of GFP from each vector was examined every 24h over a 120 hour period using a fluorescence microscope. Analysis of the cells was carried out in two ways:

1. The number of fluorescent cells per well was counted. However, if this number was high, then an estimate of the number of fluorescent cells in a field of view was taken.
2. The fluorescence from a selection of cells was quantitated.

Following transfection of Hep 3B with C3:EGFP and GFP-C a cell count of fluorescent cells was taken as illustrated in Figure 6.2. Over time the number of fluorescent cells in wells transfected with both the C3:EGFP and GFP-C construct increases until 72h when the number stabilises. However, the number of fluorescent cells in wells transfected with the GFP-C construct is much lower than wells transfected with the C3:EGFP construct. At 72h there are only 5-6 fluorescent cells in a field of view compared to 30+ cells in wells transfected with the C3:EGFP vector at the same time point. These differences are evident in images taken from the cells over a 72h period. (Figure 6.3).

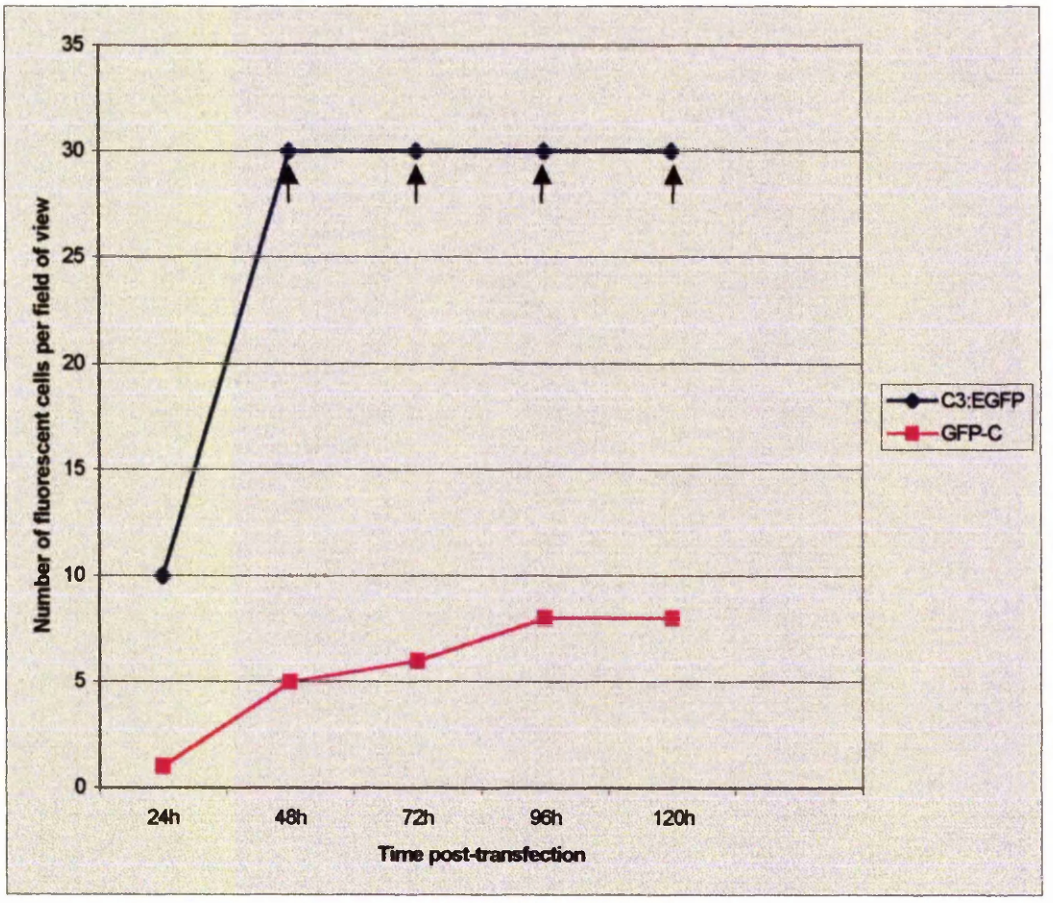
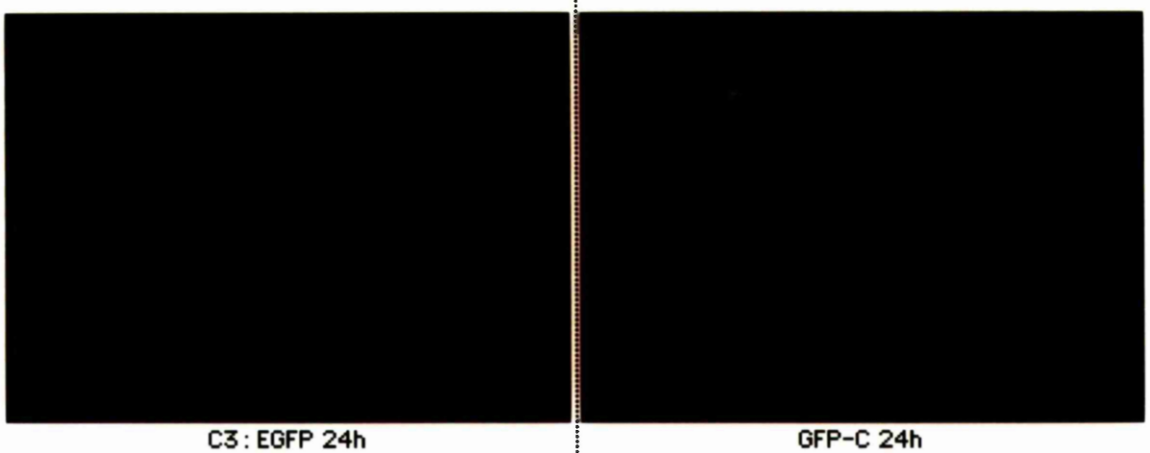


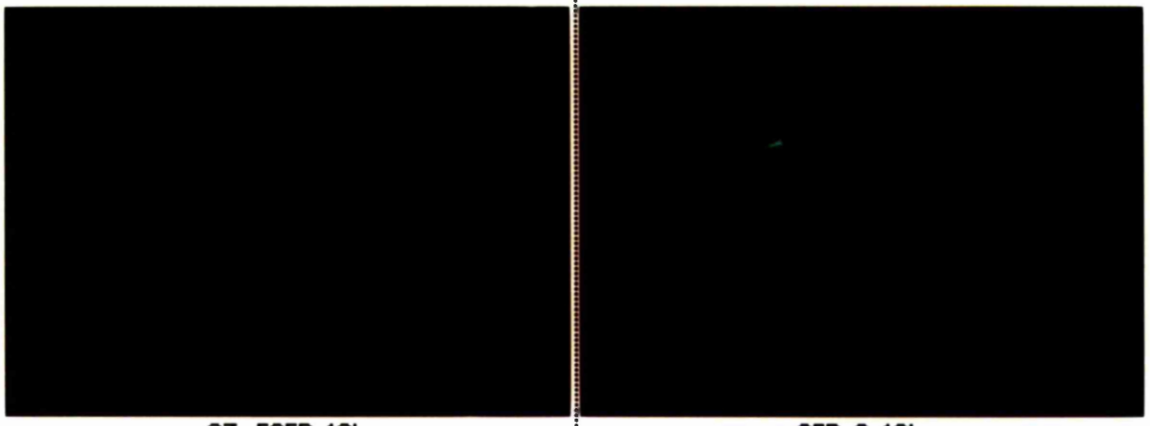
Figure 6.2: Number of fluorescent cells per field of view in Hep 3B cells transfected with either C3: EGFP or GFP-C. Arrows indicate cell counts of more than 30 cells. This graph shows that at all time points there are more fluorescent cells in wells transfected with the C3:EGFP vector compared to the GFP-C vector. It also appears that the number of fluorescent cells appears to increase with time although this can only be inferred from analysis of cells transfected with the GFP-C vector as fluorescent cells transfected with the C3:EGFP vector were too numerous to count.

Figure 6.3: Transfection of Hep 3B cells with C3:EGFP and GFP-C. This figure shows representative images from different fields of view taken over a 72h period. Images on the left show cells transfected with the C3:EGFP vector and images on the right show cells transfected with the GFP-C vector. At 24h post-transfection not many fluorescent cells can be seen from cells transfected with either of the two vectors. However, cells transfected with the GFP-C vector do appear brighter than those transfected with the C3:EGFP vector. By 48h the differences are more pronounced and it can be seen that there are more fluorescent cells from cells that have been transfected with the C3:EGFP vector compared to those transfected with the GFP-C vector. However, cells transfected with the GFP-C vector appear much brighter compared to those transfected with the C3:EGFP vector. These differences are still apparent 72h post-transfection.



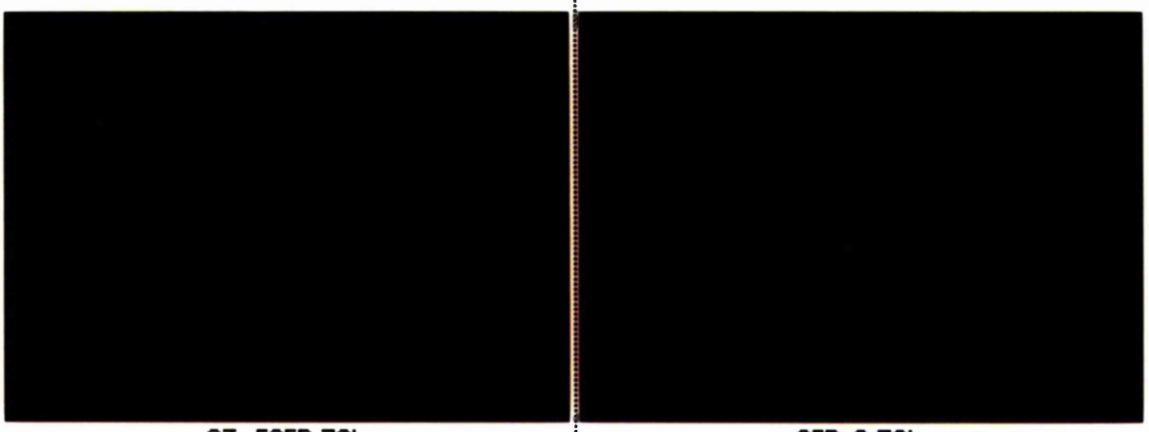
C3 : EGFP 24h

GFP-C 24h



C3 : EGFP 48h

GFP-C 48h



C3 : EGFP 72h

GFP-C 72h

The images taken over the 72h period also suggest that the fluorescence from the cells transfected with the GFP-C vector is much higher than that of cells transfected with the C3:EGFP construct. To quantitate the levels of fluorescence images from at least four different fields of view were taken from the wells transfected with the GFP-C and C3:EGFP constructs and the fluorescence intensity from a selection of cells was taken.

These results showed that the expression and the kinetics of expression appeared to be different for the two vectors. In cells transfected with the C3:EGFP vector a large number of fluorescent cells are visible at all time points but the fluorescence intensity at 24 hours is quite low (Table 6.1 and Figure 6.3). By 48 hours however, the fluorescence intensity appears stronger and the level of fluorescence intensity appears to peak around 72h post-transfection after which it stabilises. A statistical analysis by ANOVA (with Bonferroni correction) revealed that the only significant increases in expression were seen between 48h and 72h and between 24h and 120h and 48h and 120h.

In contrast, cells transfected with GFP-C always seem to fluoresce more strongly than those transfected with the C3:EGFP construct (Table 6.1 and Figure 6.3) although the number of cells fluorescing is much lower compared to that of the C3:EGFP construct (Figure 6.2 and 6.3). Cells transfected with GFP-C are almost 4 times more fluorescent than those transfected with C3:EGFP. The fluorescence intensity of cells transfected with GFP-C also increases with time and also peaks at 72h post-transfection after which time the fluorescence intensity stabilises (Table 6.1). However, a statistical analysis by ANOVA (with Bonferroni correction) showed that there was no significant difference between the fluorescence intensity at any time point.

Table 6.1: Comparison of the levels of fluorescence exhibited by the stabilised GFP (C3:EGFP) and destabilised GFP (GFP-C) control vectors in Hep 3B 24-120h post-transfection

	Mean fluorescence per cell (pixels) post-transfection				
Construct	24h	48h	72h	96h	120h
C3:EGFP	6942 ± 772.5	7349.9 ± 606.3	11533.6 ± 970.3	9380.1 ± 831.5	13592.8 ± 2392.5
GFP-C	23494.8 ± 5968.7	26507.5 ± 4590.5	49384 ± 8462.8	40089.3 ± 8018.3	47006.3 ±10853.8

The results of these experiments did lead to some concern that the destabilised variant of GFP may not be suitable for studying transient inducible gene expression due to the low number of fluorescent cells present in cells that were transfected with the destabilised variant. It has been reported that 10 000 molecules of GFP are necessary for accurate detection (Patterson *et al.*, 1997). Therefore, there was concern that the acute phase promoters, with their typically low basal levels of expression, would not be strong enough to allow enough GFP molecules to accumulate in the cell for detection and that this would be exacerbated by the destabilised nature of the GFP molecule. While this "problem" could be advantageous in the sense that basal levels of expression too low for detection may allow induction of the gene to be observed, it would cause problems if using GFP to quantitate levels of gene expression as it would not be possible to evaluate how well the construct is being regulated.

However, I could not rule out the possibility that the differences between the two control vectors were due to the promoters driving expression of the GFP gene since there have been reports of the CMV promoter functioning better than the SV40 promoter *in vitro* in different cell lines (Sutherland & Williams, 1997; Zarrin *et al.*, 1999). The acute phase promoters, being liver-specific, could result in higher levels of GFP expression compared to the SV40 promoter and hence, a larger number of fluorescent cells. Therefore, it was decided to use the promoters of the pig ITIH4 gene and the Δγ-LF-A1 mutant to drive expression of the GFP gene and to evaluate the system as a reporter of inducible gene expression.

6.3 Preparation of the GFP constructs

To determine if the acute phase promoters described in this thesis could drive expression of GFP with acute phase kinetics the ability of the promoters to drive the expression of the stabilised variant of GFP as well as the destabilised variant was examined. Therefore, for these studies I examined the ability of the porcine ITIH4 promoter as well as the Δγ-LF-A1 mutant to drive expression of the GFP mutant.

The Δγ-LF-A1 mutant was chosen for these analyses because this promoter had given the highest levels of expression of the CAT gene. Due to the fact that the C3:EGFP vector was not designed for promoter studies it had to be slightly modified to allow the EGFP gene expression to be driven by the acute phase promoters rather than CMV.

6.3.1 Modification of the C3:EGFP vector

The C3:EGFP vector was modified by removing the CMV promoter and replacing it with the acute phase promoters. The C3:EGFP vector was digested with *Vsp*I and *Nhe*I to remove the promoter region as illustrated in Figure 6.4. This was then replaced with one of the acute phase promoters.

6.3.2 Amplification of the acute phase promoters for cloning in the modified C3:EGFP vector

For these analyses the following acute phase promoter sequences were used: the pig ITIH4 promoter (Chapter 4) and the pig ITIH4 promoter without the γ-LF-A1 site (section 5.5.2). For cloning in the modified C3:EGFP vector, the promoters were amplified again by PCR using primers that had engineered *Vsp*I and *Nhe*I sites at each end. Figure 6.5 shows the results of this PCR and all of the reactions give products of the expected size of approximately 1.8kb.

The PCR products were cloned into PCRScript and transformed into XL-10 competent cells. Following transformation and selection on X-gal/IPTG, colonies were screened for the presence of the insert by PCR using primers NH1 and NH2, which amplify the middle section of the pig ITIH4 promoter.

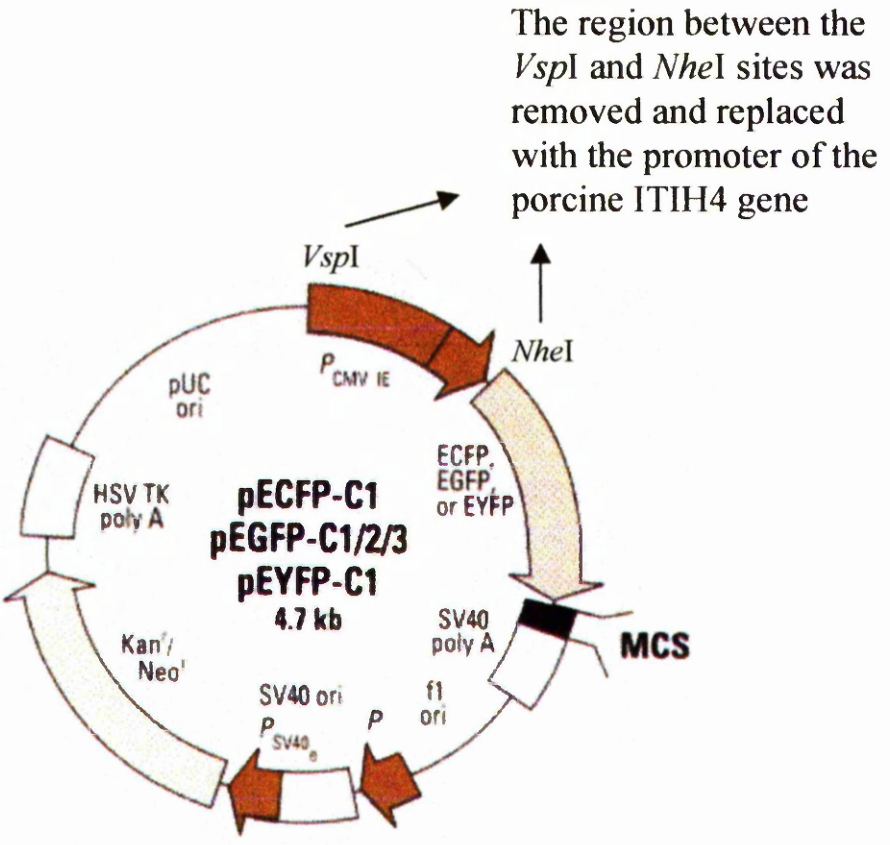


Figure 6.4: Modification of the C3:EGFP expression vector

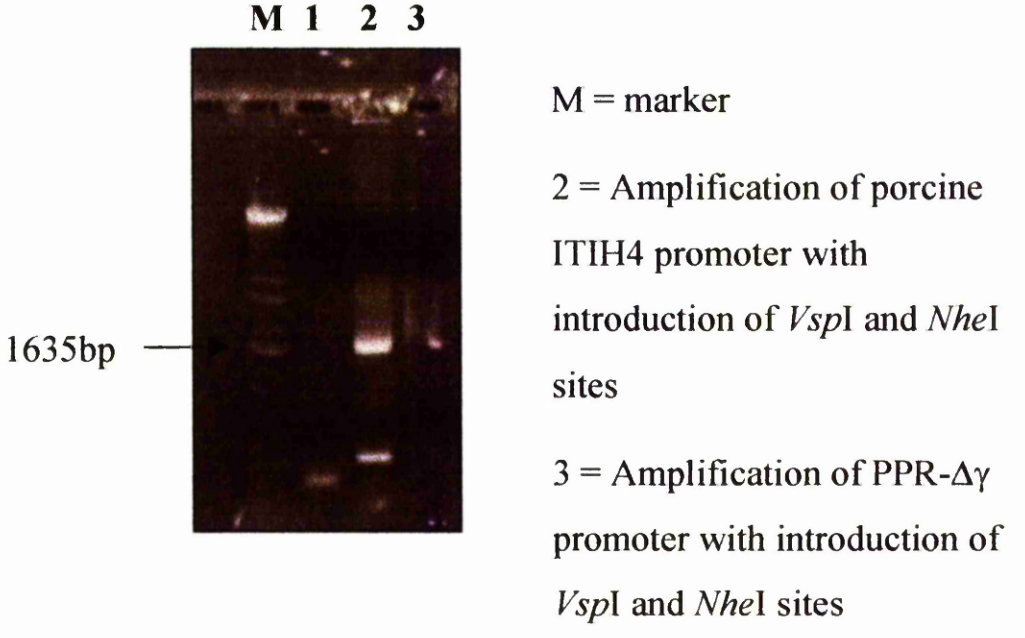


Figure 6.5: Amplification of the acute phase promoters for cloning in the modified C3:EGFP vector. The expected size of the PCR products is 1.7kb. Other bands seen may be due to non-specific binding of the primers to the template DNA.

6.3.3 Cloning the acute phase promoters into C3:EGFP

Once positive clones had been identified the insert was cut from the PCRScript vector using *Vsp*I and *Nhe*I. The bands of the correct size were cut from the gel and the purified fragments were then ligated to the previously digested and purified C3:EGFP vector and transformed into XL-10 competent cells. Following incubation overnight at 37°C, random colonies were picked from each plate and plasmid DNA was obtained. To check if the colonies contained the correct insert the DNA was digested with *Sac*I. This enzyme were known to have only one restriction site in the C3:EGFP vector as well as only cutting the ITIH4 promoter once. Figure 6.6 shows the results of some of these digests and the identification of positive clones. These results were confirmed by PCR using internal primers.

The constructs were named as follows:

Construct	
PPR-EGFP	Pig ITIH4 promoter as described in Chapter 4 and Chapter 5
Δγ-EGFP	Pig ITIH4 promoter lacking the γ-LF-A1 site (section 5.5.2)

The promoters from these constructs were also cloned in the GFP-B vector as described in the following section.

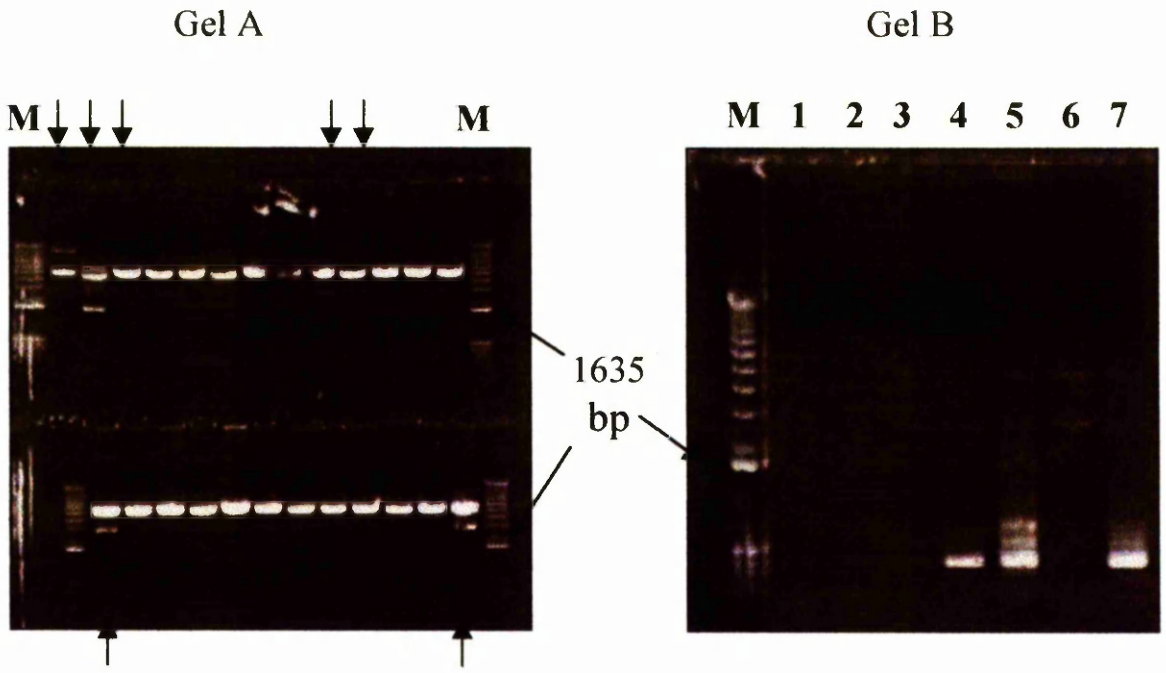


Figure 6.6: Identification of clones containing the pig ITIH4 promoter or the PPR-Δγ promoter in the modified C3:EGFP vector.

Gel A: Top row: Screening for positive clones containing the pig ITIH4 promoter by digesting plasmid DNA with *Sac*I. Expected size of fragments are 2-3kb and 4-5kb. Putative positives are (indicated with arrows) give a bright band of approximately 5kb and a fainter band of approximately 3kb.

Bottom row: Screening for positive clones containing the PPR- Δγ promoter. Putative positives are expected to give fragments of the same size as those of the pig ITIH4 promoter and are shown in lanes 1 and 12 (indicated by arrows).

Gel B: PCR to confirm that the putative positive clones are positive. Expected size of PCR product is approximately 500bp. * = positive clones

Lane 1 = Clone 1 (pig ITIH4 promoter)

Lane 2 = Clone 2 (pig ITIH4 promoter)

Lane 3 = Clone 3 (pig ITIH4 promoter)

Lane 4 = Clone 8 (pig ITIH4 promoter)*

Lane 5 = Clone 9 (pig ITIH4 promoter)*

Lane 6 = Clone 1 (PPR- Δγ promoter)*

Lane 7 = Clone 12 (PPR- Δγ promoter)*

6.3.4 Cloning the acute phase promoters in GFP-B

The pig ITIH4 promoter and the LF-A1-Δγ mutant cloned in the C3:EGFP vector were also cloned into the *Kpn*I and *Xho*I sites in the MCS of the GFP-B vector. The promoters had already been amplified with a *Kpn*I site at the 5' end and a *Xho*I site at the 3' end for cloning in the pCAT3B vector (section 5.1). Therefore, plasmid DNA from the PCRScript vectors containing the acute phase promoters with the engineered *Kpn*I and *Xho*I sites was prepared and digested with *Kpn*I and *Xho*I. Similarly, the GFP-B vector was also digested with *Kpn*I and *Xho*I. The promoters were then ligated to the digested GFP-B vector and transformed into XL-10 competent cells. Following transformation, random colonies were screened for the presence of the insert. Figure 6.7 shows the results of these analyses and the identification of positive clones.

The constructs were named as follows:

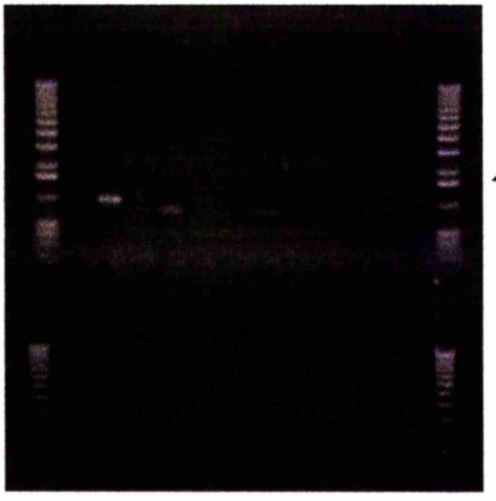
Construct	
PPR-dEGFP	Pig ITIH4 promoter as described in Chapter 4 and 5
Δγ-dEGFP	Pig ITIH4 promoter lacking the γ-LF-A1 site (section 5.5.2)

DNA from all the constructs was prepared as described in section 2.5.1.2 in preparation for transfection in Hep 3B.

Figure 6.7: Identification of clones containing the porcine ITIH4 promoter or the PPR-Δγ promoter in the GFP-B vector

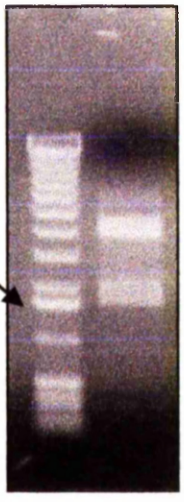
Clones containing the porcine ITIH4 promoter were identified by PCR (Gel A). Only Clone 2 (indicated) appears positive. This result was confirmed by digesting DNA from this clone with *Kpn*I and *Xho*I to release the insert of 1.8kb (Gel B). Clones containing the PPR-Δγ promoter were identified by digesting a number of clones with *Kpn*I to linearise the plasmid. This result was confirmed by PCR as shown in Gel C.

Gel A

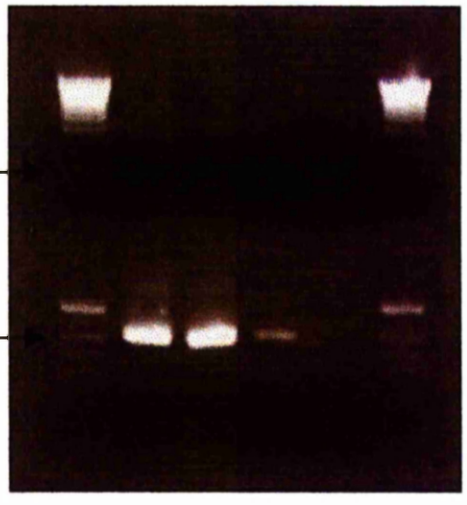


1635bp

Gel B



Gel C



1635bp

394bp

M = marker

1-3 = PCR on putative positive clones

4 = negative control

6.4 Transfecting Hep 3B with the stabilised and destabilised variants of GFP under the control of an acute phase promoter

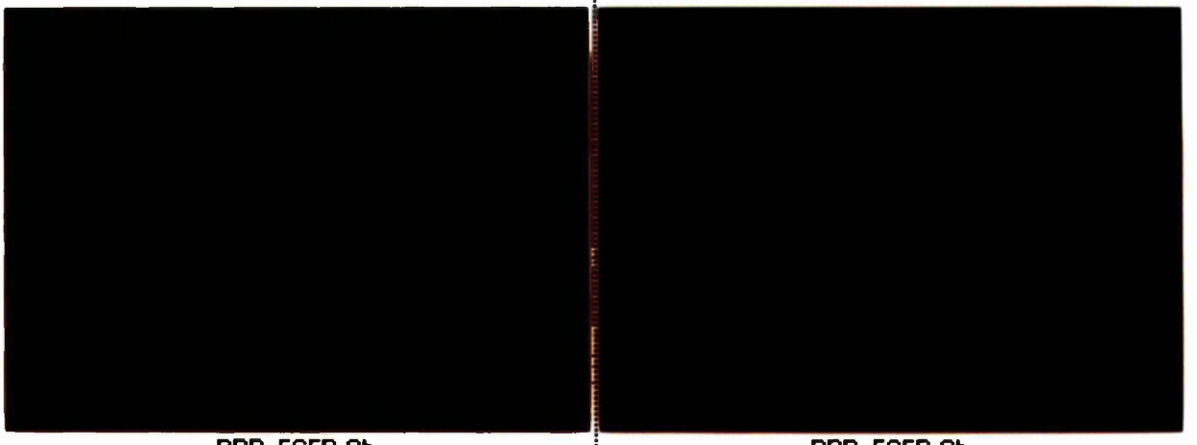
For analysis, Hep 3B cells were transfected with 0.75μg of each construct as described in sections 2.5.2.5.2. Following a 24 hour incubation period the cells were analysed using a fluorescence microscope and the number of fluorescent cells counted. Images were also taken at this time point and the fluorescence intensity of the cells measured. The cells were then stimulated with cytokines and examined 24h and 48h later. At each time point an estimate of the number of fluorescent cells in a field of view or per well if the number of fluorescent cells was low was taken and the fluorescence intensity from a number of cells was also measured.

6.5 Expression of EGFP and d2EGFP under the control of an acute phase promoter in Hep 3B

The expression of GFP under the control of the acute phase promoters resembled that of the control constructs (section 6.2.2) with a high number of fluorescent cells in wells transfected with the stabilised variant and a lower number of fluorescent cells in wells transfected with the destabilised variant. The cells transfected with the destabilised variant also appeared to have higher levels of fluorescence compared to the stabilised variant (Figures 6.8 and 6.9 compared with Figures 6.13 and 6.14). The fluorescence intensity of the cells was measured arbitrarily (as described in section 2.5.2.10), and some variation is seen when experiments are repeated on different days. Therefore, for analysis of these results I examined the changes in expression rather than examining the exact levels of fluorescence.

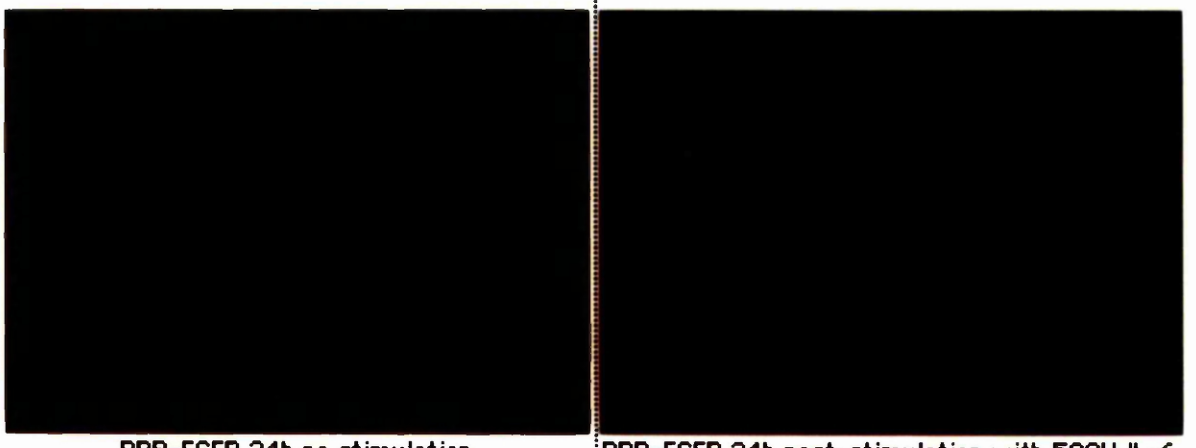
Figure 6.8: Representative image of Hep 3B cells transfected with the PPR-EGFP construct before and after stimulation with IL-6. 24h post-transfection (0h post-stimulation) only a few fluorescent cells can be seen. The fluorescence from these cells is not very strong. 24h later, the number of fluorescent cells has increased and the cells appear much brighter. There appear to be more fluorescent cells from cells transfected with C3:EGFP and stimulated with IL-6 compared to cells receiving no stimulation, both 24h and 48h post-stimulation. In addition cells that have been stimulated with IL-6 appear brighter than those receiving no stimulation.

Figure 6.9: Representative image of Hep 3B cells transfected with the Δγ-EGFP construct before and after stimulation with IL-6. 24h post-transfection (0h post-stimulation) only a few fluorescent cells can be seen. The fluorescence from these cells is not very strong, although they do appear much brighter compared to cells transfected with PPR-EGFP at the same time period. 24h later, the number of fluorescent cells has increased and the cells appear much brighter. There appear to be more fluorescent cells from cells transfected with C3:EGFP and stimulated with IL-6 compared to cells receiving no stimulation, both 24h and 48h post-stimulation. In addition cells that have been stimulated with IL-6 appear brighter than those receiving no stimulation.



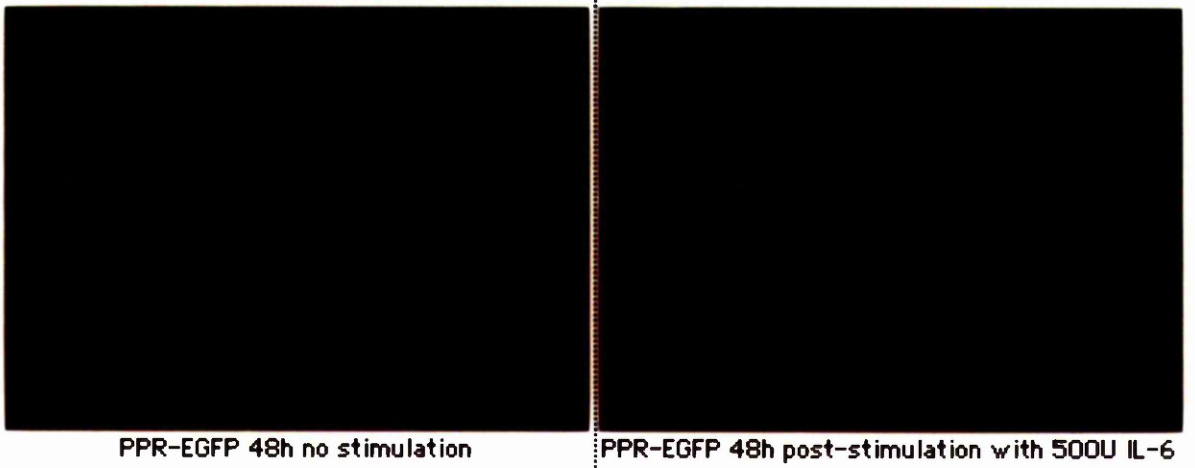
PPR-EGFP 0h

PPR-EGFP 0h



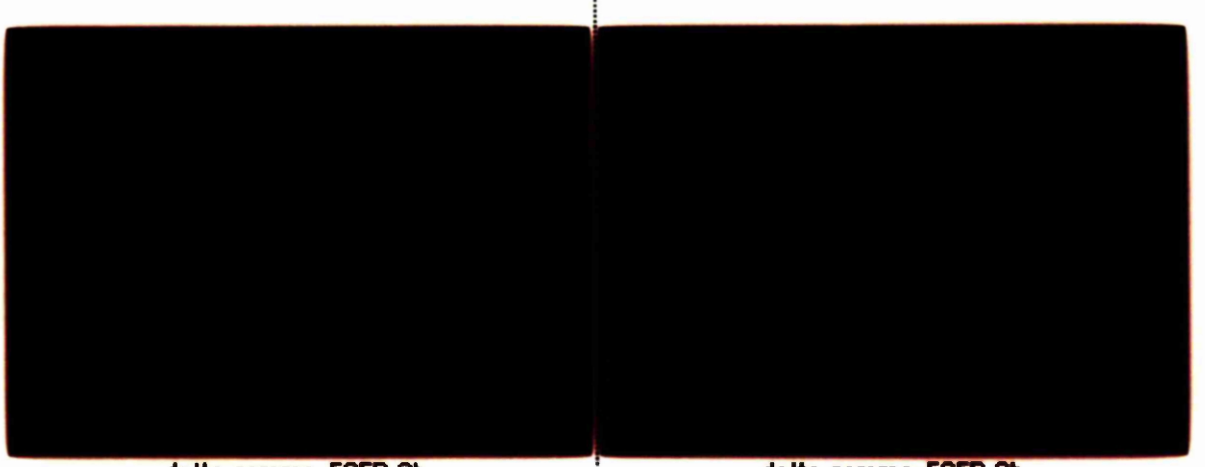
PPR-EGFP 24h no stimulation

PPR-EGFP 24h post-stimulation with 500U IL-6



PPR-EGFP 48h no stimulation

PPR-EGFP 48h post-stimulation with 500U IL-6



delta gamma-EGFP 0h

delta gamma-EGFP 0h

delta gamma-EGFP 24h no stimulation

delta gamma-EGFP 24h post-stimulation with 500U IL-6

delta gamma-EGFP 48h no stimulation

delta gamma-EGFP 48h post-stimulation with 500U IL-6

6.5.1 Expression of PPR-EGFP and Δγ-EGFP in Hep 3B

Cells in wells transfected with the stabilised variant of GFP under the control of the pig ITIH4 promoter or the pig ITIH4 promoter without the γ-LF-A1 site were already fluorescent 24h post-transfection (0h post-stimulation in Figure 6.10). This result confirms the earlier experiments with the CAT reporter gene where it appeared the pig ITIH4 promoter was capable of driving constitutive expression of the reporter gene. Following stimulation with 500U IL-6 the fluorescence intensity increases with time. There is a significant increase in fluorescence intensity in cells transfected with PPR-EGFP and stimulated with IL-6 at both 24 hours and 48 hours post-stimulation ($p<0.05$ Mann Whitney U Test). Cells transfected with the Δγ-EGFP construct also showed a small increase in fluorescence following stimulation with IL-6. However, these increases were not significant (Figure 6.10 a and b).

There were, however, some differences between the results in these experiments and those obtained with the CAT reporter system. The level of induction following stimulation with IL-6 was much lower compared to that seen with the CAT constructs. While stimulation of the PPR-CAT3B construct with 500U IL-6 resulted in a 6-fold increase in CAT expression, stimulation of the PPR-EGFP construct only resulted in a 1.3-fold increase in expression. Similarly, there was only a 1.5-fold (24h) and 1.6 fold (48h) increase in fluorescence in cells transfected with the Δγ-EGFP construct following stimulation. A 2.4-fold increase in CAT was seen in cells transfected with PPR-Δγ-CAT3B following stimulation compared to cells that received no stimulation. This suggests that quantitating GFP fluorescence with an imaging system is not as sensitive for detecting changes in gene expression as the CAT ELISA.

The number of fluorescent cells was also counted at each time point. It was thought that an increase in gene expression would be associated with a corresponding increase in cell number. This was because, as stated previously, a certain number of molecules of GFP need to be present in the cell to allow accurate detection of

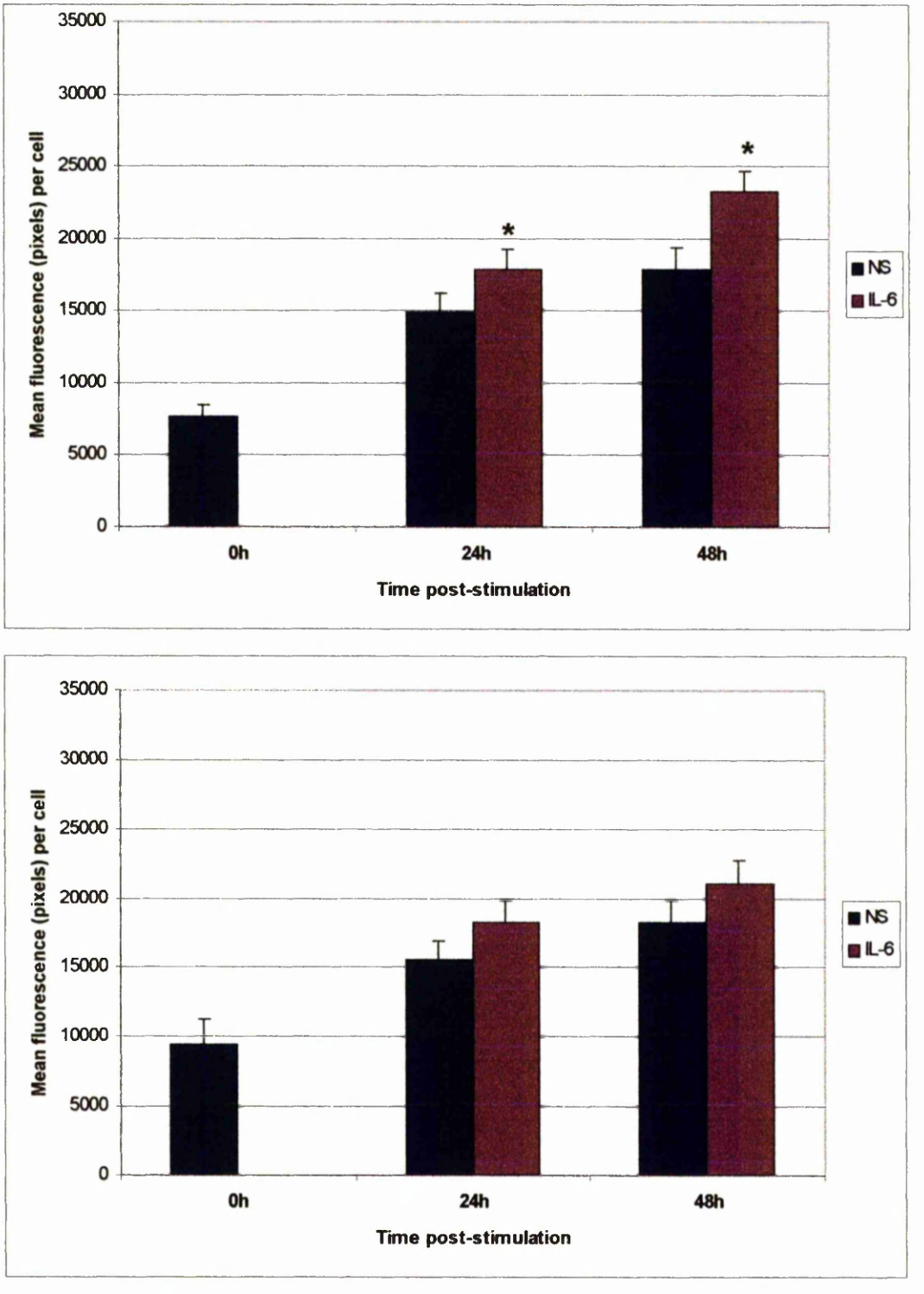


Figure 6.10 a and b: Expression of PPR-EGFP (top (a)) and Δγ-EGFP (bottom (b)) in Hep 3B following stimulation with 500U IL-6. * refers to a significant increase in expression.

fluorescence. Therefore, the increased expression of GFP in cells following stimulation should allow the detection of fluorescence from cells that were previously expressing GFP at levels too low for detection. The results shown in Figure 6.11 not only agree with this but also agree with the results obtained with the CAT expression system. The lowest number of fluorescent cells was seen in wells transfected with the PPR-EGFP construct. Following stimulation the number of fluorescent cells increased. At 24 and 48 hours the number of fluorescent cells was roughly equivalent to those of cells transfected with the Δγ-EGFP construct. Stimulation of cells transfected with the Δγ-EGFP construct resulted in an increase in the number of cells to an extent that the numbers appeared to be similar to those seen in cells transfected with the C3:EGFP construct. Therefore, this method of analysis resembles more closely what was seen with the CAT reporter system than quantitating the fluorescence intensity of the cells.

6.5.2 Expression of PPR-dEGFP and Δγ-dEGFP in Hep 3B

The results from the experiments with the stabilised variant of GFP showed that the acute phase promoters were capable of driving expression of the stabilised variant of GFP. Although the results suggested that there could be problems in quantitating the changes in gene expression, this problem could be due to the high levels of GFP present initially and the accumulation of GFP in the cell. Subsequent increases in GFP expression may therefore be difficult to visualise and detect. This problem would not have been expected with the destabilised variant due to the fact that the protein is not accumulating in the cell.

As before, the cells were examined over a 72h period post-transfection. Examination of cells transfected with both of the destabilised constructs revealed no fluorescent cells 24h post-transfection. In cells transfected with the PPR-dEGFP construct, fluorescent cells could be detected 24h post-stimulation and an increase in fluorescence was seen following stimulation with 500U IL-6 at both 24 and 48h post-stimulation. However, this increase was only significant at 48h ($p<0.05$ Mann

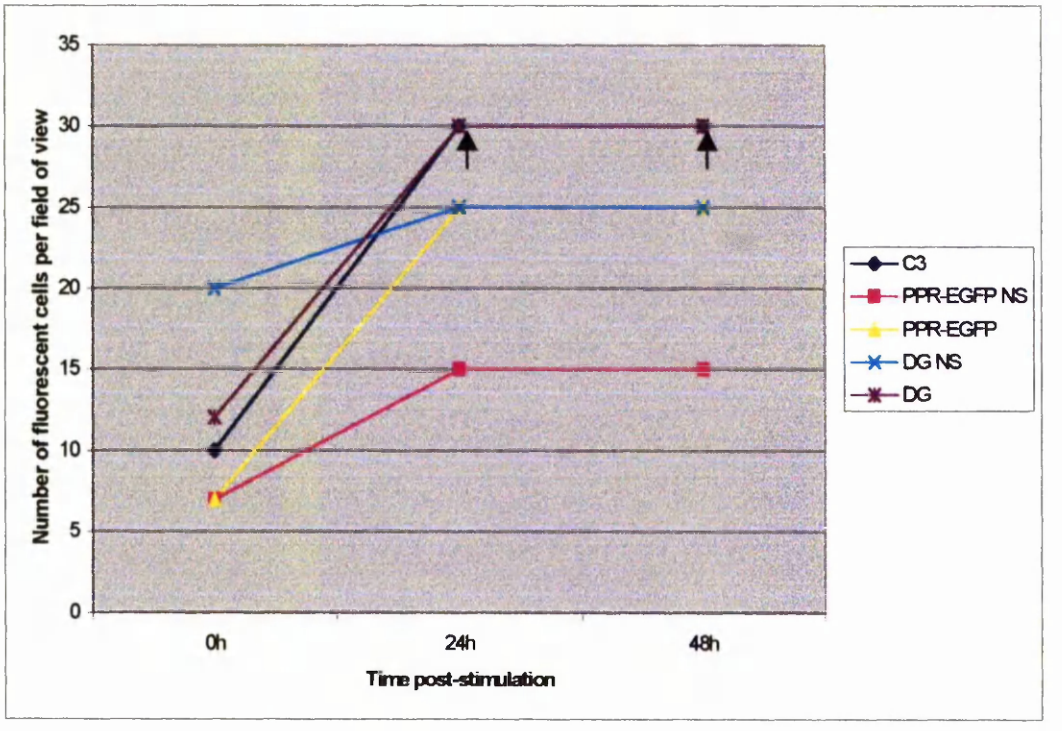


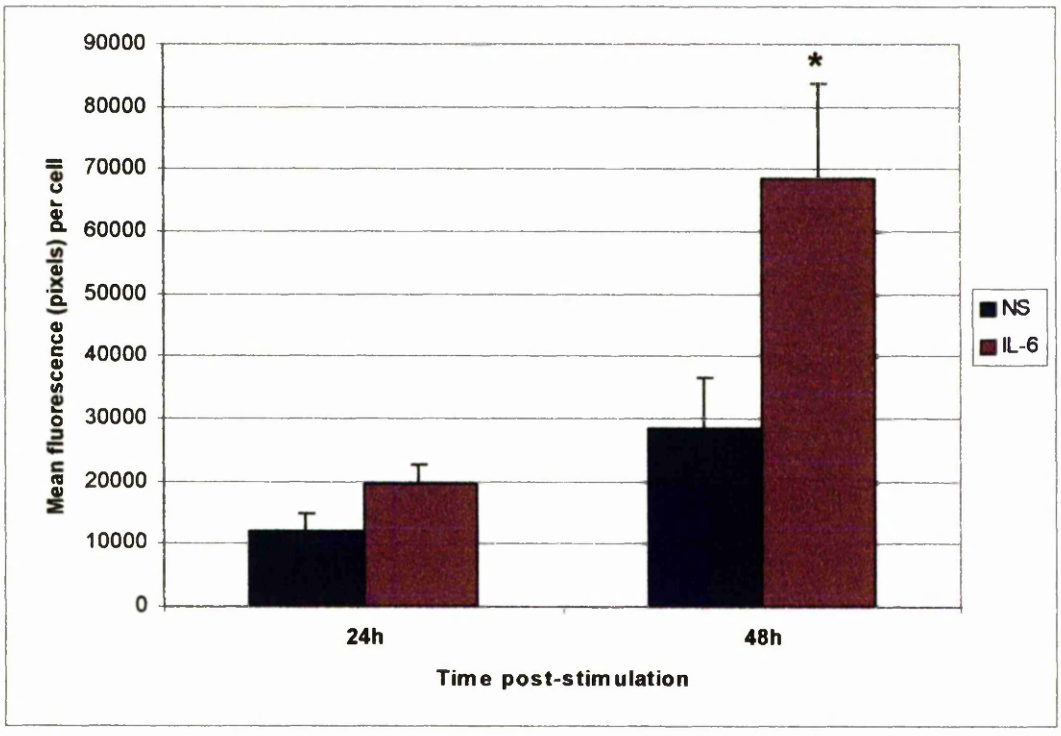
Figure 6.11: Number of fluorescent cells from Hep 3B cells transfected with the stabilised variant of GFP under the control of the acute phase promoters. DG = Δγ-EGFP construct and NS = no stimulation. Arrows indicate cell counts of more than 30 cells. This graph shows that over time the number of fluorescent cells increases. In addition, the number of fluorescent cells increases following stimulation with IL-6.

Whitney U Test). The increase in fluorescence was 2.4-fold which was more than seen with the PPR-EGFP construct at this time point (Figure 6.12)

Cells expressing dEGFP under the control of the Δγ-LF-A1 mutant were also visible 24h post-stimulation. However, no significant increases in fluorescence were seen in cells when stimulated with 500U IL-6 at any time point (Mann Whitney U Test) (Figure 6.12).

Although, as mentioned earlier, I did not analyse the actual values obtained in these experiments due to the arbitrary nature of the measurements, it was clear from the images of the cells (and also suggested by the measurements) that the fluorescence intensity of the dEGFP constructs was higher than that of the stabilised GFP constructs. This finding is in agreement with the earlier observation that the GFP-C vector had higher levels of fluorescence compared to the C3:EGFP vector (Figures 6.13 and 6.14 compared with Figure 6.3).

The number of fluorescent cells in wells transfected with the dEGFP constructs was also examined. As with the control vector, the number of fluorescent cells was much lower compared to those transfected with the stabilised GFP under the control of the acute phase promoters. However, in agreement with the observations reported for the stabilised acute phase GFP constructs and the CAT reporter system, the wells with the lowest number of fluorescent cells were those transfected with the PPR-dEGFP construct (no stimulation) and the wells with the highest number of fluorescent cells were those transfected with the Δγ-dEGFP construct and stimulated with IL-6 (Figure 6.15).



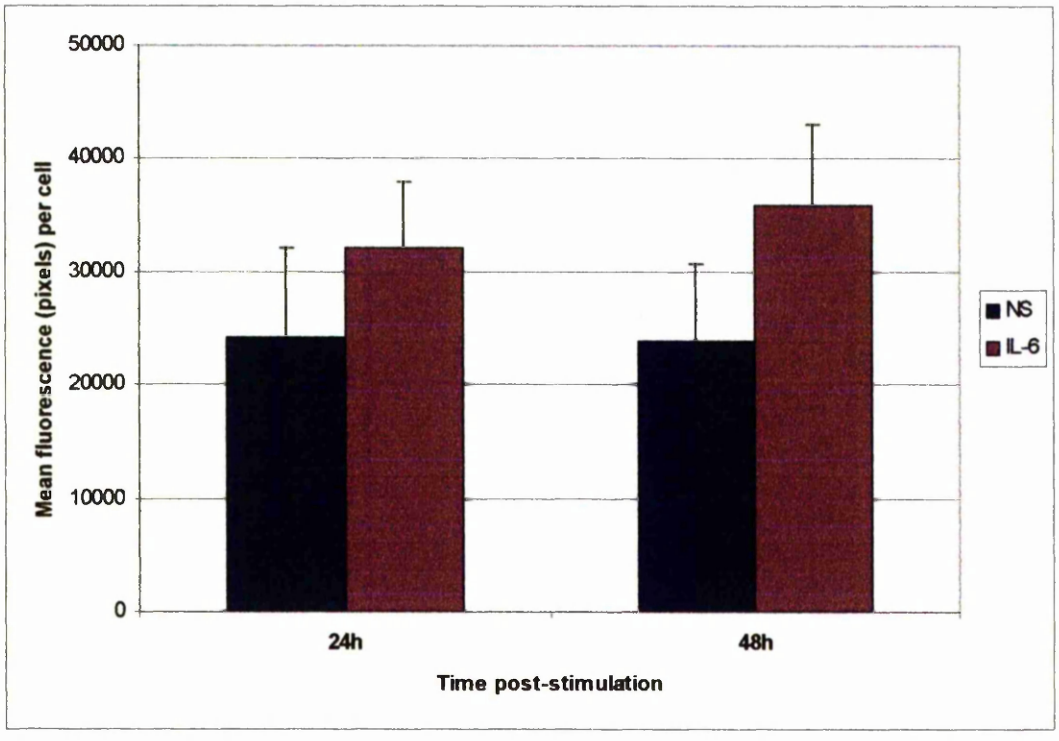
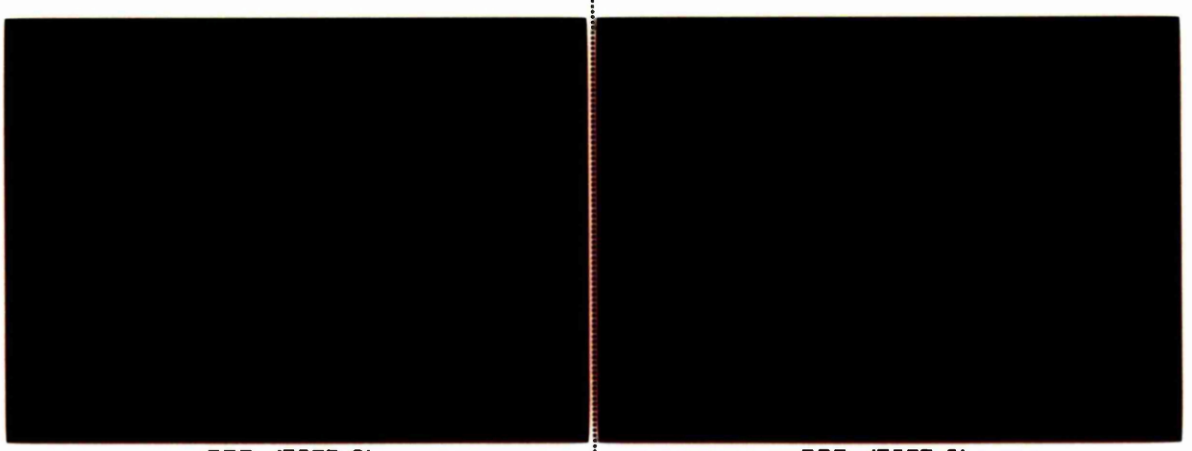


Figure 6.12: Expression of PPR-dEGFP (top) and Δγ-dEGFP (bottom) in Hep 3B following stimulation with IL-6. * refers to a significant increase in expression ($p < 0.05$ Mann Whitney U Test).

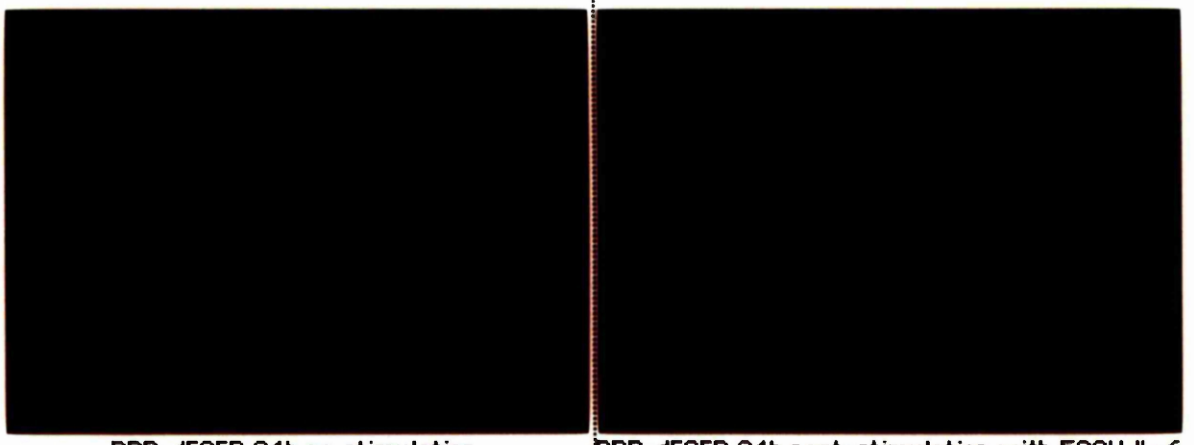
Figure 6.13: Representative image of Hep 3B cells transfected with the PPR-dEGFP construct before and after stimulation with IL-6. In the absence of stimulation few cells can be seen at any time point. Stimulation with IL-6 results in the appearance of a few fluorescent cells. The number of fluorescent cells is much lower compared to that seen in cells transfected with the stabilised variant of GFP. However, the fluorescence from cells transfected with the destabilised variant appears much stronger compared to that from cells transfected with the stabilised variant (c.f. Figure 6.9).

Figure 6.14: Representative image of Hep 3B cells transfected with the Δγ-dEGFP construct before and after stimulation with IL-6. In the absence of stimulation few cells can be seen at any time point. Stimulation with IL-6 results in the appearance of a few fluorescent cells. The number of fluorescent cells is much lower compared to that seen in cells transfected with the stabilised variant of GFP. However, the fluorescence from cells transfected with the destabilised variant of GFP appears much stronger compared to that from cells transfected with the stabilised variant (c.f. Figure 6.10).



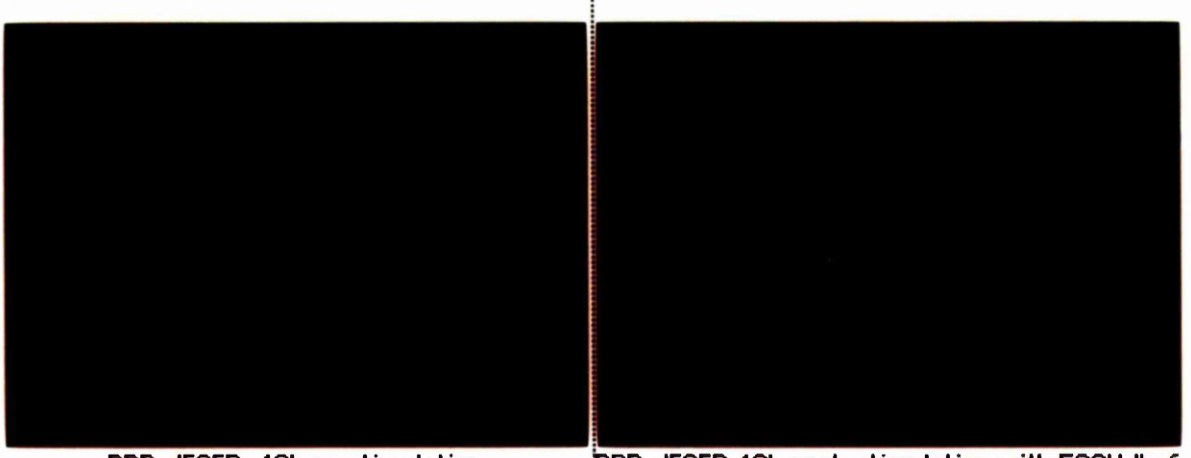
PPR-dEGFP 0h

PPR-dEGFP 0h



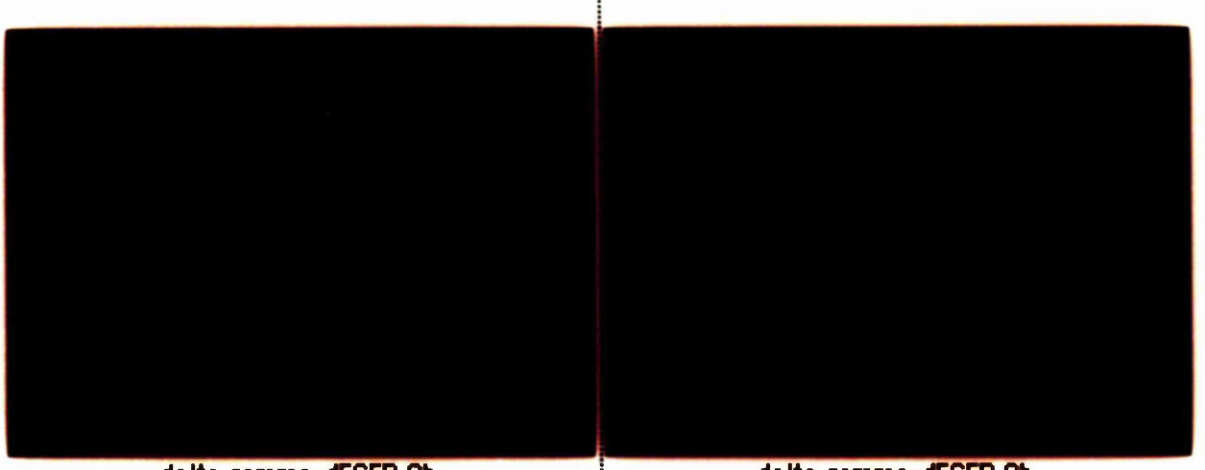
PPR-dEGFP 24h no stimulation

PPR-dEGFP 24h post-stimulation with 500U IL-6



PPR-dEGFP 48h no stimulation

PPR-dEGFP 48h post-stimulation with 500U IL-6



delta gamma-dEGFP 0h

delta gamma-dEGFP 0h

delta gamma-dEGFP 24h no stimulation

delta gamma-dEGFP 24h post-stimulation with 500U IL-6

delta gamma-dEGFP 48h no stimulation

delta gamma-dEGFP 48h post-stimulation with 500U IL-6

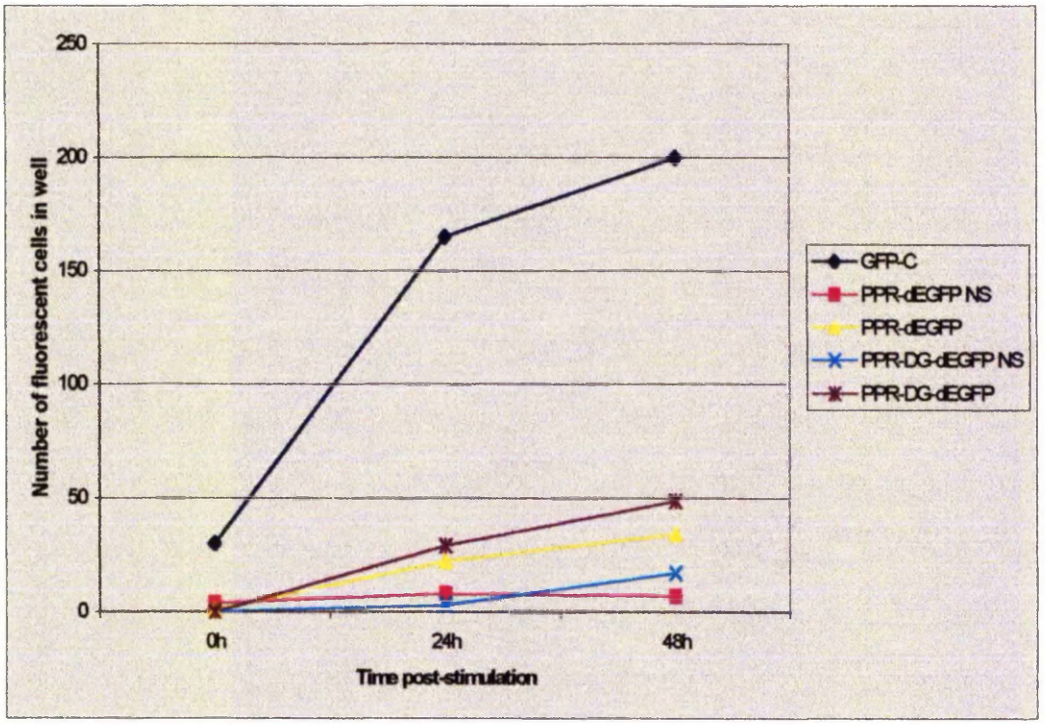


Figure 6.15: Number of fluorescent cells from Hep 3B cells transfected with the destabilised variant of GFP under the control of the acute phase promoters. DG = $\Delta\gamma$ and NS = no stimulation. Over time, the number of fluorescent cells increases both in the presence and absence of IL-6. However, stimulation with IL-6 does result in an increase in the number of fluorescent cells when compared with cells receiving no stimulation.

Summary of results

1. Both stabilised and destabilised variants of GFP under control of a viral promoter are expressed in Hep 3B.
2. Both stabilised and destabilised variants of GFP under the control of the pig ITIH4 promoter and a mutated version (PPR-Δγ) are also expressed in Hep 3B.
3. Although changes in the levels of fluorescence following stimulation of the transfected cells with IL-6 could be detected these changes were much less than seen with the CAT reporter system.
4. Counting the number of fluorescent cells in the presence and absence of stimulation agreed with the hypothesis that following stimulation there would be an increase in the number of fluorescent cells due to the ability to detect fluorescence in cells where the levels had previously been too low for detection. The results from this analysis were also in agreement with those of the CAT reporter system, in terms of the relative strength and inducibility of the two promoters examined.

In conclusion, while GFP could be used as a means of visualising inducible gene expression, quantitating its fluorescence is more difficult. A combination of the arbitrary nature of the measurements, the small changes in fluorescence that are detected, the heterogeneous nature of fluorescence between individual cells, the need to analyse individual cells and the variation in day to day experiments complicates quantification of inducible gene expression using GFP as a reporter gene. Another method of quantitation, such as FACS, may be more suitable for quantitating the changes in fluorescence.

Discussion

Chapter 7: Discussion

This project had three main aims:

1. Sequencing and analysis of the 30kb fragment containing the human CRP gene
2. Isolation and characterisation of the promoter of the major acute phase protein in pigs
3. Evaluation of GFP as a reporter of inducible gene expression.

From these analyses it was hoped to develop an improved acute phase expression vector based on the CRP gene for use in transgenic mice as well as a modified expression vector based on either the human CRP gene or the porcine ITIH4 gene for use in pigs. The development of GFP as a reporter system would have facilitated these studies.

Chapter 3 described the sequencing and analysis of the 30kb CRP fragment and the identification of a number of sequences that may be involved in the negative regulation of this gene and the sexually dimorphic pattern of the human CRP gene that is seen in transgenic mice. In Chapter 4, the isolation of the promoter of the porcine ITIH4 gene was described. Once this sequence had been isolated it had been planned to develop the acute phase expression vector for use in pigs using the information we had gained from analysis of the 30kb CRP fragment. However, due to the fact that these promoters were completely different, we instead concentrated on characterising the promoter of the porcine ITIH4 gene and establishing an *in vitro* system for its analysis (Chapter 5). Rather than comparing the porcine ITIH4 promoter with the human CRP promoter, the porcine ITIH4 promoter was compared with its human homologue in an attempt to identify sequences that would allow modification of either the porcine ITIH4 promoter or the human CRP promoter. Sequences of interest were those for responsible for increased inducibility and/or low basal levels of expression of the ITIH4 gene. Finally, to analyse these sequences we evaluated GFP as a reporter gene of inducible gene expression using the acute phase promoters to drive expression of the gene (Chapter 6).

The results from these experiments give new insight into the factors regulating expression of acute phase proteins as well as an evaluation of a number of *in vitro* systems for studying the acute phase response. In this chapter, these findings and their contribution to the further understanding of the acute phase response will be discussed.

7.1 Sequencing and analysis of the 30kb fragment containing the human CRP gene

Sequencing the 30kb fragment containing the human CRP gene was deemed necessary in order to identify the sequences necessary for tightly controlled expression of the CRP gene and for parallel studies to modify the expression vector for use in pigs. The aim of the sequence analysis was to identify elements that are responsible for the low basal levels of expression of the human CRP gene, as well as those responsible for the increased basal levels of expression of the CRP/GM-CSF transgene in male transgenic mice. The results from this analysis could be used to design a more tightly controlled expression vector based on either the promoter of the human CRP gene or the porcine ITIH4 gene.

Genomic strategies to identify mammalian regulatory sequences were reviewed recently by Pennacchio & Rubin (2001). Some of the points raised in this review will be discussed here in relation to the analysis of the 30kb fragment containing the human CRP gene.

The first point raised by Pennacchio & Rubin (2001) is that analysis of non-coding DNA with a view to identifying regulatory elements and predicting their function is extremely difficult compared to the analysis of coding DNA where function can sometimes be predicted from sequence analysis alone. Analysis of the 30kb fragment using NIX showed that at least 25kb of the fragment is composed of non-coding DNA. Therefore, sequence analysis alone, as it does not take into account other factors such as DNA bending, conformation changes, chromatin remodelling,

will not be sufficient to identify regulatory elements and needs to be carried out in parallel with functional studies.

Early studies to understand the regulation of the human CRP gene have been experimental based (Arcone *et al.*, 1988; Toniatti *et al.*, 1990; Murphy *et al.*, 1995). The study by Murphy *et al.* (1995), however, revealed that functional studies need to be carried out in transgenic mice as constructs sufficient for controlled expression *in vitro* were not sufficient for controlled expression *in vivo*. As transgenic mice are expensive to produce and maintain, as well as requiring individuals with expertise to generate and analyse the mice, this may in part explain why, since the mid-1990s, very few papers have been published on the molecular aspects of the regulation of the human CRP gene.

In the last few years, the completion of the sequencing of mammalian genomes and the availability of transcription factor search programs and databases (for example, the TRANSFAC (Wingender *et al.*, 2001) and COMPEL (Kel-Margoulis *et al.*, 2000) databases) means that computational analysis is another means for analysing non-coding DNA for regulatory elements (reviewed by Pennacchio & Rubin, 2001). This was the approach that was employed for analysing the 30kb fragment. Pennacchio & Rubin (2001) describe three strategies for computational sequence analysis of non-coding regions. The main strategy employed by us was using information from sequence analyses of other acute phase genes or genes regulated in a similar manner to acute phase genes to predict regions of the 30kb fragment that may be important for its regulation. It was evident from the initial analysis of the 30kb fragment, as described in section 3.3.2, that it would not be possible to predict regions that could be important for regulation of the CRP gene by this approach alone. Therefore, the results described in Chapter 3 are at the moment speculative and a functional analysis needs to be carried out to confirm the predictions.

As mentioned earlier this functional analysis will need to be carried out *in vivo*. As it was not possible for us to make new lines of transgenic mice we began to

investigate an alternative means for analysing the 30kb fragment *in vivo*. During the course of this project it was reported that CRP is expressed in the human respiratory tract (Gould & Weiser, 2001). We have now begun investigations (in collaboration with Dr Weiser) to see if we can detect human CRP in the respiratory tract of our CRP transgenic mice. Preliminary data show that the CRP transgene is being expressed in cells of the respiratory tract of the transgenic mice. Therefore, in the future it may be possible to deliver the constructs to these cells using a genetically engineered respiratory virus. This would be an alternative to making transgenics and would facilitate the *in vivo* study of a larger number of constructs.

Analysis of the 30kb fragment resulted in the identification of a number of putative regulatory elements that may responsible for the low basal levels of expression of the CRP gene, as well as those that may be responsible for the increased expression of the CRP transgene in male transgenics. The CT rich region found about 2kb upstream of the human CRP gene was particularly interesting because a similar type of element, found in the promoter of a heat-shock gene, was shown to have an important role in the regulation of this gene (Glaser *et al.*, 1990). One of the ways this region was thought to affect gene expression was by the formation of a conformational structure known as H-DNA (reviewed by Wells *et al.*, 1988). The CRP promoter has already been described as having elements similar to those found in heat shock promoters (Arcone *et al.*, 1988). Therefore, it was interesting that the CT rich region present in the 30kb fragment also has the potential to adopt the H-DNA structure due to the presence of a mirror repeat (Figure 3.15). If this structure was formed *in vivo* there is a strong possibility that transcription factors binding in its vicinity would be affected. It is particularly noteworthy that a putative silencer element would be affected by the formation of this structure. Since formation of H-DNA is dependent on the pH and/or high helicity of the DNA, this may affect when, for example, transcription factors can bind to the silencer element and consequently would determine when transcription of the CRP gene will occur.

If these elements were shown to be functional then they could be used to modify expression of the pig ITIH4 gene. In Chapter 5 it was described how a comparative genomic analysis was carried out to identify regulatory elements responsible for the lower levels of expression and inducibility of the human ITIH4 gene compared with the pig ITIH4 gene. This analysis was carried out in order to decrease the basal level expression seen with the section of the pig ITIH4 promoter that I had isolated. An alternative approach would be to make a series of deletion constructs of the pig ITIH4 promoter, as done for the human CRP promoter by Arcone *et al.* (1988) to identify the minimal sequences required for promoter activity and inducibility of the gene. The promoter of the pig ITIH4 gene could then be modified by introducing the CT rich element or the silencer element. Haecker *et al.* (1995) reported that the silencer element found in the promoter of the hen ovalbumin gene was position independent, could function upstream or downstream of a reporter gene and was able to affect the promoter activity of a heterologous gene. Analysis of the promoter region of the pig ITIH4 gene did not reveal the presence of a sequence that matched the consensus for the silencer element. Therefore, it would be interesting to investigate what effects these elements would have on the basal levels of expression of the pig ITIH4 promoter.

The significance of the ORF of the L1 element found in the 5' end of the 30kb fragment is presently not clear. This element contained the antisense promoter (ASP) described by Speek (2001) (Figure 3.12). One of the ways Speek suggests that the ASP could affect the regulation of downstream genes is based on the observation by Greger *et al.* (1998) that activation at an upstream promoter could affect activation of a downstream promoter if transcription of the upstream gene continued past the PolyA site and into the promoter of a downstream gene. Although I did not find any evidence that there was another gene in the 30kb fragment 5' to the CRP gene, Woo *et al.* (1985) reported the presence of a PolyA signal 5' of the CRP TATA box.

Another possible role for the L1 element was the creation of the CRP pseudogene. Esnault *et al.* (2000) have shown that LINE elements which transpose through the reverse transcription of their own transcript can also mobilise transcribed DNA not associated with a LINE sequence. This results in the retroposition of the transcribed gene and the formation of new copies that have features characteristic of naturally occurring processed pseudogenes (Esnault *et al.*, 2000). The LINE ORF1 has been shown to be essential for pseudogene formation.

The other aim of the sequence analysis was to identify putative growth hormone regulated elements with a view to removing them in order to eliminate the sexually dimorphic pattern of expression seen in the CRP and GM-C79 transgenic mice. The analysis has focused on the GM-C79 construct and on binding sites for the transcription factor STAT5. STAT5 has been implicated in the mediation of the sexually dimorphic pattern of expression of a number of murine liver-specific genes. Analysis of the GM-C79 construct showed that it contained a number of STAT5 binding sites that exhibited considerable homology to those that were identified experimentally by Varin-Blank *et al.* (1998) to be involved in mediating the sexually dimorphic pattern of expression of the *C4-Slp* gene. The pulsatile pattern of expression of growth hormone in male rodents has been suggested to mediate the translocation of activated STAT5 into the nucleus (Subramanian *et al.*, 1995). The continuous secretion of growth hormone in female rodents has been shown to dephosphorylate STAT5 (Gebert *et al.*, 1999) and so it is not expected that it would be translocated to the nucleus. Consequently, it can be speculated that the activation of STAT5 in male rodents and its binding to the DNA could enhance transcription from the CRP promoter. It is interesting to note that Szalai *et al.* (1998) proposed that the mediator of the sexually dimorphic pattern of expression in the CRP transgenic mice would be comparable to growth hormone and display a sexually dimorphic pattern of expression. They also suggested that in humans, this mediator did not appear to be under androgen control. STAT5 thus appears as a strong candidate for the proposed mediator of the sexually dimorphic pattern of expression of the human CRP gene in transgenic mice - it has a sexually dimorphic pattern of

expression in mice due to the different patterns of growth hormone secretion in male and female mice. Because humans do not exhibit the same patterns of growth hormone secretion as mice, STAT5 would not be expected to be regulated the same way in humans, and consequently no sexually dimorphic pattern of expression of CRP would be expected to be seen in humans (and this is the case).

Although the role of STAT5 in regulation of acute phase genes is unknown it has been shown that signal transduction does not occur via the gp130 pathway (the pathway used by IL-6 to induce expression of acute phase genes) (Heinrich *et al.*, 1998). Binding sites for the various STAT factors exhibit considerable homology to each other with binding sites for STAT1 also being reported as binding sites for STAT5 and STAT6 on one strand and STAT3 on the other. While deleting or mutating elements that are binding sites for STAT3 as well as STAT5 may affect the inducibility of the construct, removing or mutating binding sites specific for STAT5 may be one way of eliminating the sexually dimorphic pattern of expression of the human CRP gene in transgenic mice.

It should be born in mind that while removing the STAT5 binding sites may make a more tightly controlled construct in mice, the effect this modification of the construct would have if the construct was used in other species is unknown. It is not clear if the sexually dimorphic pattern of expression of acute phase and other transgenes (e.g.GM-CSF (Metcalf, 1988)) seen in transgenic mice is rodent-specific and a result of how these genes are regulated normally in rodents or the consequence of introducing a transgene.

In summary, the sequence analysis has identified a number of elements that may be involved in regulating CRP expression in transgenic mice. A functional analysis of these sites can now be carried out.

7.2 Characterisation of the pig ITIH4 promoter in vitro

One of the reasons for the analysis of the 30kb CRP fragment was to facilitate the modification of the acute phase expression vector for use in pigs. However, isolation and sequence analysis of the pig ITIH4 promoter revealed that it did not have any homology to the human CRP promoter. As mentioned several times in this thesis, one of the advantages of using CRP as the backbone for our acute phase expression vector is that regulation of this gene is partially characterised. Therefore, the presence of the same regulatory sequences in the porcine ITIH4 promoter would have greatly facilitated the design of an acute phase expression vector based on the porcine ITIH4 gene. Now, before this can be seriously attempted some understanding of the regulation and the elements affecting the regulation of the porcine ITIH4 gene needs to be carried out. The experiments described in Chapter 5 describe two things: firstly, they are functional studies on the porcine ITIH4 promoter. Secondly, they examine the possibility of using a human hepatoma cell line as a means of studying the porcine acute phase response at the molecular level in the absence, at present, of any better *in vitro* system.

The experiments carried out investigated the basal expression and inducibility of the 1.8kb region upstream of the porcine ITIH4 gene. The analyses were carried out 48 hours post-stimulation, as this had been shown to be the peak time of expression of the pig ITIH4 gene both *in vivo* and *in vitro* (Lampreave *et al.*, 1994; González-Ramón *et al.*, 2000). CAT was always detected, even in the absence of stimulation. Control experiments confirmed that this expression of CAT was due to the ITIH4 promoter rather than background expression.

Stimulation with the cytokines IL-6 and IL-1β confirmed the *in vitro* findings from studies on the endogenous ITIH4 gene in primary porcine hepatocytes (González-Ramón *et al.*, 2000). As reported in the literature, stimulation with IL-1β does not affect expression of the porcine or human ITIH4 gene (González-Ramón *et al.*, 2000). Therefore, our results were in good agreement with this although a small

increase in expression was seen following stimulation with 5U IL-1 β (section 5.3.2). These findings support the classification of the pig ITIH4 gene as a Class II acute phase protein.

Stimulation with IL-6 induced expression of the CAT gene and the level of induction was dose dependent. The increase in expression seen in cells transfected with the PPR-CAT3B following stimulation with IL-6 was twice as high as that seen when the endogenous porcine ITIH4 gene was stimulated (González-Ramón *et al.*, 2000). This is in spite of the fact that in our experiments a lower concentration of IL-6 was used (500U compared to 1000U) and I did not use dexamethasone. Although dexamethasone, a synthetic glucocorticoid, is frequently used along with cytokines to enhance the stimulation of acute phase genes *in vitro*, there have been some reports of dexamethasone having a negative effect on gene expression (Steel & Whitehead, 1991). Therefore, dexamethasone was omitted from our experiments since it was unknown what effect it would have on expression of the ITIH4 gene.

In both our experiments, and those of González-Ramón *et al.* (2000), the level of induction seen following stimulation with cytokines was much lower (6-fold and 3-fold respectively) than that seen *in vivo*. Lampreave *et al.* (1994) reported that *in vivo* synthesis of the pig ITIH4 gene increased 30-fold following a turpentine injection. González-Ramón *et al.* (2000) offer several possibilities for the lower levels of expression seen with the endogenous ITIH4 gene *in vitro* compared to *in vivo* including the use of human cytokines rather than porcine cytokines for stimulation. In their experiments human cytokines may not have bound effectively to their receptors on porcine hepatocytes and the signal transduced may have been affected. For our experiments, the use of human cytokines was deemed to be justified as the analyses were being carried out in a human cell line. Using porcine cytokines with a human cell line may have resulted in the same problem as using human cytokines with a porcine cell line.

Hep 3B cells were chosen for our analysis because they have been used extensively in studies of the acute phase response and at present an immortalised porcine hepatocyte cell line is not commercially available. Therefore, another possibility for the difference in results between González-Ramón *et al.* (2000) and the results reported here is the fact that different cells were used for the analyses. There have been several reports of different results being obtained when different cell lines and primary cells are used (Weinhold *et al.*, 1997) as well as differences between *in vivo* and *in vitro* results (Simar-Blanchet *et al.*, 1998). For this reason I wanted to test the constructs in primary porcine hepatocytes. Transfection of the primary porcine hepatocytes with the C3:EGFP construct suggested that transfection of these cells would be possible. However, due to the outbreak of foot and mouth disease in the UK in February 2001 we were unable to get any primary porcine hepatocytes to carry out these analyses.

An interesting observation in the literature is that almost all studies on the acute phase response *in vitro* are carried out at 37°C. *In vivo*, an acute phase response is likely to be accompanied by fever and a study by Karlsson *et al.* (1998) reported that induction of acute phase proteins by IL-6 *in vitro* had a different effect at 40°C compared to 37°C. For example, they found that expression of the human haptoglobin gene increased 4-7 fold following stimulation with IL-6 at 37°C. However, at 40°C there was a 23-fold increase in expression compared to controls following stimulation with IL-6. It may be speculated that carrying out the experiments with the PPR-CAT3B construct at a higher temperature could affect the inducibility of the construct and possibly result in higher levels of induction.

A simple explanation for the differences between the results of González-Ramón *et al.* (2000) and those reported in this thesis may be that the promoter region I isolated does not contain all of the elements necessary for controlled expression. It may also be due to the nature of the construct since Arcone *et al.* (1988), in their study on expression of the human CRP gene *in vitro*, found that it was more difficult to induce expression of the endogenous gene compared to transiently transfected

constructs. Overall though, our findings support those of González-Ramón *et al.* (2000) in the classification of the porcine ITIH4 gene as a Class II acute phase protein and the use of Hep 3B as an *in vitro* system for studying the regulation of the porcine ITIH4 gene.

The most unexpected finding from our experiments was that stimulation with a combination of IL-6 and IL-1β affected the inducibility of the PPR-CAT3B construct. This result was inititally unexpected, as IL-1β had not been shown to have any effect on the inducibility of the construct. However, this phenomenon, whereby IL-1β inhibits the activity of IL-6 and inducibility of the gene in question has already been reported for another two acute phase genes, fibrinogen and C1 inhibitor. A study by Zuraw & Lotz (1990) showed that stimulation of Hep G2 cells with IL-6 or IFN-γ results in an increase in C1 inhibitor secretion which is dose dependent. A combination of IL-6 and IFN-γ has an additive effect on C1 inhibitor secretion whereas a combination of IL-1 and IFN-γ synergistically increases C1 inhibitor secretion. However, in contrast they showed that IL-1 antagonises the effect of IL-6 on C1 inhibitor. This effect was maximal when IL-1 was added prior to or at the same time as IL-6.

In the case of the fibrinogen gene, a Class II acute phase gene, IL-1 is believed to act by inhibiting the activation of STAT1 by IL-6. This activity has been shown to be specific for STAT1 as another IL-6 inducible STAT, STAT3, is not affected (Shen *et al.*, 2000). The ability of STAT1 to form heterodimers is known and it has also been proposed that the IL-1 inducible transcription factor NF-κB can reduce the binding affinity of STAT1/STAT3 to acute phase gene promoters (Zhang & Fuller, 1997). Further experiments, such as EMSA, could be carried out to determine if there are members of the STAT family binding to the ITIH4 promoter and if so, which ones.

In summary, the conclusion from these experiments was that the porcine ITIH4 gene encodes a Class II acute phase protein and that the human hepatoma cell line, Hep

3B, is a usable system for studying the regulation of the porcine ITIH4 gene *in vitro*. The results of these experiments, in particular the downregulation of IL-6 induced activation of the porcine ITIH4 gene, may be of interest to those interested in understanding the signal transduction pathways involved in the regulation of the acute phase genes.

7.3 What makes an acute phase protein an acute phase protein?

The results from the sequence analysis of the fragment containing the regulatory elements for the human CRP gene and the promoter of the pig ITIH4 gene gave us the opportunity to study the elements involved in regulating acute phase protein gene expression. The species-specificity of the acute phase response and the exact role of the acute phase proteins during the acute phase response have puzzled researchers for some time. Acute phase proteins in one species are not necessarily acute phase proteins in another. For example, the major acute phase protein in pigs is only a minor acute phase protein in humans. Our finding that the promoter of major acute phase protein in pigs had more homology to its human homologue, a minor acute phase protein in humans, than to the major acute phase protein in humans allowed some sequence analysis to be carried out to investigate the species-specificity of the acute phase response.

Ciliberto *et al.* (1987a) proposed that the species specificity of the acute phase response was due to the absence of specific transcription factors or changes in the DNA sequence of the promoter which affects binding of the transcription factors and consequently gene expression. In mice, CRP is only a minor acute phase protein, yet transgenic mice for the human CRP gene express human CRP as it is in humans (Ciliberto *et al.*, 1987a). This suggests that it is changes in the promoter that affects the binding of transcription factors rather than the absence of the necessary transcription factors. The work in this project involved the analysis of the promoters of the major acute phase proteins in humans and pigs as well as comparison of the promoter of the major acute phase protein in pig and its human homologue. The

results of these analyses, discussed in the following sections, may give some insight into the species-specificity of the acute phase response.

7.3.1 Comparison of the pig ITIH4 promoter with the CRP promoter

The pig ITIH4 promoter did not have any homology to the CRP promoter as revealed by a BLAST search of the nucleotide database. Comparison of the pig ITIH4 promoter and the human CRP promoter by searching them for the binding sites of elements known to affect the regulation of acute phase genes showed that the two genes had a very different promoter organisation (Figures 4.11 and 4.12). In fact, elements that had been identified by Arcone *et al.* (1988) to be involved in the acute phase induction of the human CRP gene were absent in the pig ITIH4 promoter. This result seems to suggest that acute phase genes are regulated independently, in the sense that just because a protein is a major acute phase protein does not mean that its promoter will have homology to the promoters of other major acute phase proteins.

7.3.2 Comparison of the pig ITIH4 promoter with the human ITIH4 promoter

Further insights into how the promoter can affect the regulation of the acute phase genes can be gained by comparing the promoter of the pig ITIH4 gene with that of the human ITIH4 gene. The promoters of pig and human ITIH4 genes have homology of 65%. This is surprising on the one hand because ITIH4 in pigs is a major acute phase protein whereas ITIH4 in humans is only a minor acute phase protein so one would not expect so much homology. It is surprising on the other hand because the ITIH4 genes in pigs and human are quite homologous (over 75%), therefore the homology between the promoters is maybe lower than expected. The most surprising aspect is that when the promoters are compared the promoter organisation is completely different. Even though there are binding sites for the transcription factors analysed in both the promoters of the ITIH4 genes, their

position and number varies and it can be postulated that this is the key to the differences in gene expression of the human and pig ITIH4 genes.

I attempted to use this information to manipulate expression of the pig ITIH4 gene. Hardon *et al.* (1988) had suggested a correlation between the binding of the transcription factor LF-A1 to its binding site and the levels of gene expression. As mentioned in section 5.5.1 the presence of three putative binding sites for LF-A1 in the promoter of the pig ITIH4 gene and only one in the human ITIH4 gene suggested that this may account for the lower inducibility of the human ITIH4 gene. It was hoped that mutating these sites in the pig ITIH4 gene would reduce the basal levels of expression while maintaining or increasing the inducibility of the constructs. The results showed though that the mutation did affect not only the inducibility of the construct but also the basal transcription machinery. This may be because the LF-A1 transcription factor is a bi-functional transcription factor involved in both induction and regulation of the basal transcription machinery. Alternatively, deleting the binding site for this transcription factor affected the structural organisation of the promoter. For example, the LF-A1 transcription factor may function by steric hindrance – allowing or preventing the access of other transcription factors to the promoter. In addition, two of the LF-A1 sites were shown to overlap with putative Sp1 binding sites (Figure 5.10). Sp1, a ubiquitous transcription factor has been reported as being capable of both inducing and repressing gene expression (see section 5.5.3). It is therefore possible that making a base substitution in the binding site for LF-A1 would only have decreased its affinity for the binding site and consequently may have had a different effect on gene expression.

7.4 Developing GFP as a reporter of transient gene expression

Sequence analysis allows the identification of a whole range of putative regulatory elements and regions. However, without a functional analysis the significance of these findings can only be speculated. It is clear from the literature that regulation of

the CRP gene and eukaryotic genes in general is complex. Therefore, attempting to identify the regions important in regulating the CRP and pig ITIH4 genes will involve numerous *in vitro* expression studies. This was one of the reasons for wanting to develop GFP as a reporter system. GFP also allows real time analysis and would be interesting to see the switching on and off of gene expression in response to various stimuli. In the future, there is also the possibility of creating transgenic mice where an acute phase promoter drives expression of GFP. This would allow the expression of the acute phase gene to be monitored *in vivo*.

Initial experiments comparing transfection of the two GFP control vectors in COS-7 (control cell line) and Hep 3B (experimental cell line) showed that both cell lines could be transfected with both the GFP vectors. The main difference was the expression of the two vectors, both the levels of fluorescence and the number of fluorescent cells. This was investigated further in Hep 3B over a 120h period.

One of the differences between the two constructs is the number of fluorescent cells present following transfection. Transfections with the C3:EGFP vector result in a large number of fluorescent cells whereas the GFP-C vector results in a few fluorescent cells although the number increases with time but never to the extent of that seen with the C3:EGFP vector. The reason for these differences was originally thought to be due to the expression levels and half-life of the GFP-C vector. Viral promoters have been reported to have varying levels of efficiency depending on the cell line (Sutherland & Williams, 1997; Zarrin *et al.*, 1999) with the CMV promoter often being reported to be stronger than the SV40. Therefore, since GFP in the C3:EGFP vector is under the control of the CMV promoter and the GFP in the GFP-C vector under the control of the SV40 promoter it was thought that this may be contributing to the differences seen. Lower levels of expression of GFP in cells transfected with the GFP-C construct would be exacerbated by the short half-life of the GFP molecule. Consequently there was a possibility that a sufficient number of GFP molecules were not accumulating in the cell to allow detection.

However, this theory did not make sense when the fluorescence intensity of the cells was measured because cells transfected with the destabilised variant of GFP were much brighter compared to cells transfected with the C3:EGFP vector. It was suggested that mutations may have been introduced to the destabilised GFP variant to enhance its fluorescence due to the low number of GFP molecules expected to be produced (Dr G Palmer, Clontech, personal communication). Further evidence that the differences in expression of the two vectors are connected to the GFP molecule rather than the promoters was obtained when the acute phase promoters were used to drive expression of the stabilised and destabilised variants of GFP. As seen with the control vectors, transfections with the stabilised variant resulted in a higher number of cells but with lower levels of fluorescence compared to cells whereas the opposite was the case with cells transfected with the destabilised variant. This suggests that intrinsic properties of GFP may be responsible for the differences in the levels of expression seen.

The intrinsic properties of GFP may limit its use as a reporter system for inducible gene expression, particularly when using the destabilised variant for the following reasons:

1. Fluorescence is dependent on the formation of a chromophore. This process is oxygen-dependent and a delay of more than one hour can occur between chromophore formation and detection of fluorescence (Piston *et al.*, 1999). Therefore, the protein may be degraded before a sufficient number of molecules can accumulate in the cells.
2. Only ~ 20% of GFP molecules are expected to fold correctly (Tsien, 1998). This limitation in combination with a slow rate of chromophore formation and a short-half life of the protein would be particularly problematic due to the fact that GFP cannot be detected if a sufficient number of molecules folded correctly are not present in the cell.
3. Mutations introduced to GFP and creation of fusion proteins can affect the transcription and translation of the molecule (Sacchetti *et al.*, 2001). The

destabilised GFP vector used in our experiments was designed by fusing the degradation domain of the mouse ornithine decarboxylase to the C-terminal of EGFP (Li *et al.*, 1998a). The effect of this modification on transcription or translation of the GFP gene has not been investigated.

4. Transfection efficiency of the plasmids. Analysis of the cells appears to suggest that the C3:EGFP vector has a higher transfection efficiency compared to the GFP-C vector. However, this is misleading because the apparent lower transfection efficiency of the GFP-C vector may be due to the fact that a sufficient number of GFP molecules are not present in the cells to allow detection.

Although studies have been carried out to correlate transcription and translation of the GFP gene (Sacchetti *et al.*, 2001), no work has been done to determine the relationship between transcription and translation and fluorescence of the destabilised variant. This is an important relationship to establish as it has implications for studying inducible gene expression as discussed in the next section. It may also help determine if the low number of fluorescent cells is due to the intrinsic properties of GFP or the absence or low levels of fluorescence or the transfection efficiency of the plasmid. Some of these questions could be answered by analysing the RNA content of the cells to see if the GFP gene is being transcribed and to what extent.

7.4.1 Quantitating GFP fluorescence

Although GFP would possibly allow the visualisation of switching on and off of gene expression, quantitating the fluorescence over time is difficult. Niswender *et al.* (1995); Subramanian *et al.* (1996); Patterson *et al.* (1997) and Piston *et al.* (1999) have discussed the problems encountered with quantitating fluorescence in studies of transient gene expression. The problems not only include those mentioned in section 7.4 but also the fact that the fluorescence is not homogeneous between the cells. When the cells are examined using a fluorescence microscope considerable

variation in the fluorescence intensity between one cell and another is seen. As stated in section 2.5.2.10, the mean fluorescence per cell was calculated by summing the total fluorescence intensity values from, if possible, at least 100 cells. The variation in fluorescence from cell to cell has the effect of decreasing the mean value from these analyses and consequently the differences between cells receiving stimulation and those receiving none is reduced. Analysing cells in this manner (i.e. measuring the fluorescence from individual cells) also makes quantitating fluorescence over time difficult because it is difficult to choose the same cells for analysis each time. In addition, over time, as the cells divide, the fluorescence will also appear to decrease but this may be due to plasmid loss from the cell during cell division rather than a decrease in gene expression. Therefore, an alternative method, such as FACS, may be better.

Although some changes in fluorescence intensity were detected following stimulation with IL-6, these changes were usually small. It is not yet clear how changes in the number of protein molecules are related to fluorescence and gene expression. For example, if 10 000 molecules of GFP are necessary for accurate detection, how many molecules are necessary to detect a change in expression? If a large number of molecules are required then a big increase in gene expression may only be correlated with small but significant changes in fluorescence intensity. Detecting changes in fluorescence intensity may also be complicated, at least in the case of the destabilised GFP molecule if it has been mutated to enhance fluorescence, by the fact that the levels of fluorescence present in the cell initially may be so high as to prevent any further changes being detectable. This would explain why no significant changes in expression following stimulation with IL-6 were seen when the Δγ-dEGFP and Δγ-dEGFP constructs were examined.

The idea that levels of fluorescence need to be low initially in order to detect changes in fluorescence appears to be supported by the observation that in cells transfected with dEGFP there was an increase in the number of fluorescent cells following stimulation with IL-6. This was presumably related to an increase in gene

expression allowing detection of fluorescence in cells where previously the fluorescence levels were too low for detection. The results from these analyses (i.e. comparing the number of fluorescent cells) were in agreement with what was seen with the CAT reporter system in terms of the relative strength of the promoters to each other.

GFP and CAT as reporter systems have been compared (Albano *et al.*, 1998) and this study found that GFP was as good as CAT for quantitating heterologous protein production. GFP has also been reported to be more sensitive than other reporter systems *in vivo* (Chiochetti *et al.*, 1997). However, the results from the experiments described in this thesis suggest that GFP is not as sensitive as other reporter genes for quantitating small changes in gene expression.

7.5 Modification of the acute phase expression vectors – is it possible?

7.5.1 Modification of the CRP acute phase expression vector

The identification of regulatory sequences is difficult because it may be the organisation and interactions of transcription factors that is important rather than specific sequences. Ogbourne & Antalis (1998) have also suggested a similar idea in that it is DNA sequences and their context within a given promoter rather than the DNA binding proteins that determine the repressor mechanisms at a given promoter. These hypotheses are supported by the work done in this project on the pig ITIH4 promoter. Although the promoters of the pig and human ITIH4 gene exhibit a reasonable amount of homology, the promoter organisation is very different.

Ideally, we want to clone the CRP/GM-CSF fusion in the background of the 30kb fragment. Although sequencing of the 30kb fragment meant it was possible to generate a restriction map of unique restriction sites, none of the sites would have been suitable for cloning. Nonetheless, this project has identified at least two regions that may have an influence on regulation of the CRP gene and are worth

exploring further. In particular, if the CT repeat located approximately 2kb upstream of the CRP gene is important in regulating its expression, this would still allow a small but more tightly controlled acute phase expression vector to be made.

The second aim of the analysis was to identify putative growth hormone responsive elements. The sexually dimorphic pattern of expression of human CRP in transgenic mice is seen both in mice with the full length 30kb fragment and the smaller C79 construct. Therefore, identification of these elements is important since even if we are successful in cloning the CRP/GM-CSF fusion into the background of the 30kb fragment this pattern of expression will remain. A number of binding sites for growth hormone responsive elements were identified – two putative GAGA boxes and several binding sites for STAT5. In future constructs these sites could be mutated and analysed in transgenic mice to see if they have any effect on the sexually dimorphic pattern of expression. Alternatively, analysis could be carried out to see if STAT5 actually binds to the putative STAT5 binding sites in the GM-C79 construct.

7.5.2 Modification of the acute phase expression vector for use in pigs

For the acute phase expression system to function effectively, tightly controlled expression of the heterologous gene is essential. At this stage the elements necessary for controlled expression of the porcine ITIH4 gene are unknown and there is no model system available for its analysis *in vitro*. The work in this project is the first step to understanding the regulation of the porcine ITIH4 gene and the establishment of a model system for molecular analysis of the porcine acute phase response. The results of the gene expression studies show that the section of the promoter I have isolated is both functional and inducible in Hep 3B and that it behaves as a Class II acute phase protein.

Although we believe Hep 3B is a usable system for studying the porcine acute phase response at the molecular level there is obviously some concern about analysing the

porcine constructs in a human cell line. For this reason, I wanted to analyse the constructs in primary porcine hepatocytes in order to verify the findings in Hep 3B. Initial experiments to see if it was possible to transfect the cells with GFP appeared to be successful. This was an important breakthrough because it means that a) the constructs made during the course of this project can be tested in primary porcine hepatocytes and b) it can be attempted to make an immortalised porcine liver cell line, for example using the vector and method described by Park *et al.* (1996).

It is important that a model system for studying ITIH4 gene expression *in vitro* that reflects the *in vivo* situation is established to facilitate studies of the porcine acute phase response. Studies with the human CRP gene *in vitro* have shown that analyses in different cell lines will give different results. They have also shown that the minimal promoter for controlled expression varies from cell line to cell line and that the minimal promoter for controlled expression *in vitro* is not sufficient for controlled expression *in vivo*. Studies on the endogenous porcine ITIH4 gene (González-Ramón *et al.*, 2000) and the porcine ITIH4 promoter (this thesis) show that they are not induced to the same extent as *in vivo*. It should be stressed though that while it is important to be aware of the limitations of the *in vitro* systems and to exercise caution when interpreting the results, these systems have contributed greatly to our understanding of the acute phase response. At present, the lack of knowledge on the porcine acute phase response, particularly at the molecular level and the absence of established systems for studying the porcine acute phase response both *in vivo* and *in vitro* hinders further development of the acute phase expression vector for use in pigs.

7.6 Conclusion

The ultimate goal of this project was to develop a modified expression vector for use in pigs. Although we did not achieve this, the work carried out during the course of this project has opened up numerous areas for further development, both for us to develop the acute phase expression system and for those that are interested in understanding the acute phase response at the molecular level.

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Appendices

Appendix 1: List of primers

Name	Sequence	Function
CSI.T7F1	CTG ATA CCC TTT CTT CCA C	Sequencing CSI clone
CSI.MRF1	CCT TCC TAA TCT GTC ATA CC	Sequencing CSI clone
CS4	CAT AAT AGT CTT GGA AGT GC	Completing CSI sequence
8V1	AGG TGG GAT GCC AGT CTG CC	Completing CSI sequence
9L1	ACC TCA GAA TGT GAG CTT ATC	Completing CSI sequence
CSI-MR.OUT	CCC AGC CTT GTG GAA CTA TG	Linking CSI clone to SI clone
SI.T7F1	GCA GCC GTG TTT CTA TGA TG	Sequencing middle section of SI clone and linkage to CSI clone
SI.MRF1	TAA GTA CCA TGC TGT ACC C	Sequencing middle section of SI clone and linkage to CSI clone
9N1	TTG AGA AGT CTT CAT AAT GC	Linking SI and SK7 clones
SK7.C2	GTT TGG ATG CAA ATT AGT CC	Linking SK7 clone to SI clone
SK7	CTG CCC ATA GTT GGT TCC	Linking SK7 clone to CKI clone
CKI.T7F1	TAG AGT GGT GAA GGC CAA TG	Sequencing CKI clone
38.3T34	AGG GCC TCA GAA AGC TTA C	Sequencing CKI clone
PK-120 F	ATC TAC AGC CTC ACC GTG GAC	Forward primer designed for human PK-120 cDNA sequence (Nishimura *et al.* 1995). Used to make probe for screening pig genomic library for pig-MAP/ITIH4 clone.

PK-120 R	GTT GCC GTC CAG GAC TGT TTC	Reverse primer designed form human PK-120 cDNA sequence (Nishimura *et al.* 1995). Used to make probe for screening pig genomic library for pig-MAP/ITIH4 clone.
MAP-DF	CAY AAT AAY GAY ATH AAY ATH	Degenerate forward primer designed from MAP N-terminal protein sequence (Gonzalez-Ramon *et al.* 1995).
MAP-DR	NAC HAT RAA RTT NCC RTC	Degenerate reverse primer designed from MAP N-terminal protein sequence (Gonzalez-Ramon *et al.* 1995).
MAP F1	CAC AAG AAC GAC ATC AAC ATC	New degenerate primers MAP clone designed with the help of a pig codon usage table. Forward primer.
MAP R1	GGT CAC CAC CTT RSW GGC	New degenerate primers for isolating MAP-clone designed with the help of a pig codon usage table. Reverse primer.
ITIH4 F1	CTT TGT GCA TTA CTT TGC CC	Primer designed from ITIH4 sequence of Buchman *et al.* (1990). EMBL: M29507. Forward primer
ITIH4 R1	GGT CTG CTG GAT TTT CCT G	Primer designed from ITIH4 sequence of Buchman *et al.* (1990). EMBL: M29507. Reverse primer
MAP.OUT	AAA TCG GGA CGA GAC CTT GGA GTC	Primer designed from cDNA sequence of pig ITIH4 gene (Hashimoto *et al.* 1996). Obtain upstream sequences of pig ITIH4 gene

ITIH4.OUT	ACC AGC AGA AGG CCG TAG CC	Primer designed from cDNA sequence of pig ITIH4 gene (Hashimoto *et al.* 1996). Obtain upstream sequences of pig ITIH4 gene
MAP.IN	TAC TGG CTA CGG CCT TCT GC	Primer designed from cDNA sequence of pig ITIH4 gene (Hashimoto *et al.* 1996). Used with primer MAP.OUT to confirm the upstream sequences of the pig ITIH4 gene are present in the BAC clones.
5' IHRP	TCC CGT TGT GAT GGC AAG ACC	Primer designed from promoter of human ITIH4 gene (EMBL U42015; Saguchi *et al.* 1995). Used to isolate corresponding sequences of the pig ITIH4 gene.
NH1	AAG GAA TTT CTG AAC AGA AGC	Sequencing pig ITIH4 promoter
NH2	GGC CAC TTT GCT GAA GCA TC	Sequencing pig ITIH4 promoter
NH3	TTT GCC TGC TTA TCT GAG CC	Sequencing pig ITIH4 promoter
5' PPR	TCG ATA GGT ACC TCC CGT TGT GAT GGC AAG ACC	Amplifying 5' end of pig ITIH4 promoter with introduction of *Kpn*I site (underlined)
PPR 3'	GCA GAT CTC GAG TTT GGC TCC TCT GCC TGT CAG GC	Amplifying 3' end of pig ITIH4 promoter with introduction of *Xho*I site (underlined)

PPR-ALL R	GAT AGC CAG GCA GAG GAA AGC AGG ATG GC	Deleting all LF-A1 sites in pig ITIH4 promoter. Reverse primer.
PPR-ALLF	GCC ATC CTG CTT TCC CTG CCG GCT ATC	Deleting all LF-A1 sites in pig ITIH4 promoter. Forward primer.
PPR-α R	GGG CAC GCT CTG CTG AAG CAG GAT GGC	Deleting LF-A1 α-binding site in pig ITIH4 promoter. Reverse primer.
PPR-α F	GCC ATC CTG CTT CAG CAG AGC GTG CCC	Deleting LF-A1 α-binding site in pig ITIH4 promoter. Forward primer.
PPR-β R	GTC TCC AGG AGG CAG GAGGCA GGT CAG AGT G	Deleting LF-A1 β-binding site in pig ITIH4 promoter. Reverse primer.
PPR-β F	CAC TCT GAC CTG CCT CCT GCC TCC TGG AAG C	Deleting LF-A1 β-binding site in pig ITIH4 promoter. Forward primer.
PPR-γ R	GAT AGC CAG GCA GAG GAG AGG GTG GCT TCC AGG	Deleting LF-A1 γ-binding site in pig ITIH4 promoter. Reverse primer.
PPR-γ F	CCT GGA AGC CAC CCT CTC CTC TGC CTG GCT ATC	Deleting LF-A1 γ-binding site in pig ITIH4 promoter. Forward primer.
5' PPR1	TTA GTT ATT AAT TCC CGT TGT GAT GGC AAG ACC	Amplification of pig ITIH4 promoter with introduction of *Vsp*I site. Forward primer.

3 PPR1	GGT AGC GCT AGC TTT GGC TCC TCT GCC TGT CAG GC	Amplification of pig ITIH4 promoter with introduction of *Nhe*I site. Reverse primer.

Appendix 2: List of constructs

Name of construct	Description
pCSI	ClaI/SpeI subclone for sequencing 30kb CRP fragment
pCKI	ClaI/KpnI subclone for sequencing 30kbCRP fragment
pSI	SpeI/SpeI subclone for sequencing 30kb CRP fragment
pSK7	SpeI/KpnI subclone for sequencing 3kb CRP fragment
pCRP-CAT	~260bp of the CRP promoter in promoterless pCAT enhancer (Shirley Hanley)
pPPR	Pig ITIH4 promoter and some coding sequence in PCR Script
pPPR (K/X)	Pig ITIH4 promoter with engineered *Kpn*I and *Xho*I sites and without coding sequence in PCRScript
pPPR-CAT3B	Pig ITIH4 promoter in pCAT3-Basic vector
pPPR-EGFP	Pig ITIH4 promoter in C3:EGFP
pPPR-dEGFP	Pig ITIH4 promoter in dEGFP-Basic
pPPR-ΔALL	Pig ITIH4 promoter with all LF-A1 sites deleted in PCRScript
pPPR-ΔALL-CAT3B	Pig ITIH4 promoter with all LF-A1 sites deleted in pCAT3-Basic
pPPR-Δα	Pig ITIH4 promoter with α LF-A1 site deleted in PCRScript
pPPR-Δα-CAT3B	Pig ITIH4 promoter with α LF-A1 site deleted in pCAT3-Basic
pPPR-Δβ	Pig ITIH4 promoter with β LF-A1 site deleted in PCRScript
pPPR-Δβ-CAT3B	Pig ITIH4 promoter with β LF-A1 site deleted in pCAT3-Basic
pPPR-Δγ	Pig ITIH4 promoter with γ LF-A1 site deleted in PCRScript
pPPR-Δγ-CAT3B	Pig ITIH4 promoter with γ LF-A1 site deleted in pCAT3-Basic

pPPR-Δγ-EGFP	Pig ITIH4 promoter with γ LF-A1 site deleted in C3:EGFP
pPPR-Δγ-GFP	Pig ITIH4 promoter with γ LF-A1 site deleted in dEGFP-Basic

Appendix 3: Location of putative transcription factor binding sites in the 30kb CRP fragment

KB	Sil	Sil	IL-6	Tran	Rb1	Rb2	Rb3	Hp1	Hp2	Hp3	Dis	GAG A box	ARE	NF-IL-6	GC	GRE	STAT 1	STAT 3	STAT	NFkB
0													417	464					428	840
1			324 125					762						871 968	323					783 839
2			001 824 959			526							483	750		474				
3						550								255 646			944 950	944	270 386 547 590 950	852
4						003 709				643			176	045						
5						977		959	640		490			246 623 640			361	361	367 723	
6																			834	
7			961			374								413 703 870 911			720		726	
8							461							320 331 573					662 937	
9				401 511		069			377				965		090					
10				473		204								940					592	
11	056												154							
12														238 836		751				

13						226 494				889			394	120 731						
14									166					062 166 953	325				755	
15					866	224 244		951				934			177 182	795				
16			171 663			726					664	870		077 746 927	545					
17					695	169 236		847					206 783	041 785	285 292 299 462					
18		059	656			327								797	132 587					
19			176 196 735	306		151 295 355			643	890	197 736								733	
20			017							087										
21							291	413						369 557					671	
22			199			122				164									053	
23			158	680 944		554							824	521						
24													427 486	640 716 782	759				695	
25					152		735	051					547	170 491						
26							903	246							295					
27	691		374										633 941			932				
28				793										485	609				174	

													586					545	
29			159 288						315				315 378	586				236 513	289
30					043								412						

Key: Sil (silencer element); IL-6 (binding site for IL-6 inducible factor); Tran (sequence that has been shown to be involved in transcriptional regulation); Rb1 (motif found in the promoter of the rabbit CRP gene); Rb2 (motif found in the promoter of the rabbit CRP gene); Hp1 (binding site for IL-6 inducible factor in human haptoglobin gene); Hp2 (binding site for IL-6 inducible factor in human haptoglobin gene); Hp3 (binding site for IL-6 inducible factor in human haptoglobin gene); Dis (distal regulatory element in the rat α_2-macroglobulin gene); ARE (androgen response element); GC (GC box); GRE (glucocorticoid responsive element)