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**The responsiveness of the bovine lactoferrin promoter to
cytokines and glucocorticoids**

**A thesis presented to Massey University in partial fulfilment of the requirement for
the degree of Master of Science in Biochemistry**

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Dedication

This thesis is dedicated to my parents, Sandra and Gary Allen, and to my twin sister, Kim Allen, for all their support and encouragement during the past two years.

Acknowledgments

I would like to thank my supervisors Kathryn Stowell and John Tweedie for all their time, expertise, enthusiasm and support that made this study possible. I would also like to thank all the occupants of the Twilight Zone and Carole Flyger, our laboratory manager, for their patience and tolerance. I acknowledge the financial support of the Palmerston North Medical Research Foundation and Massey University Research Fund, which made many of the experiments possible.

Abstract

Lactoferrin is an iron-binding protein found in many bodily secretions and in the secondary granules of polymorphonuclear leukocytes. While there are many proposed functions for the lactoferrin protein - e.g. for iron storage, antibacterial properties, or a role in inflammation, the specific function(s) of lactoferrin have yet to be elucidated.

Evidence that lactoferrin may be involved in inflammation was observed by Harmon *et al.* (1976) where after the induction of bovine mammary infections, a significant increase in secreted lactoferrin protein was seen during the early phase of the infection. As this increase was during the period of the acute phase response, this suggested that lactoferrin, as was the case with other proteins induced during this time, may have a role in the inflammatory response. The bovine lactoferrin (bLf) promoter contains many putative binding sites for inflammatory modulators, which suggests that the increases in lactoferrin seen during inflammation may be due to activation of lactoferrin gene transcription by these specifically-induced transcription factors. Substantiation of this suggestion would provide further evidence for a specific role for lactoferrin during inflammation.

To investigate the cytokine-responsiveness of the bLf promoter, constructs corresponding to various lengths of the putative bLf promoter were linked to the luciferase reporter gene and introduced, by transient transfection, into RL95-2 human endometrial carcinoma cells. Cytokines, glucocorticoids or expression vectors for transcription factors were added to the cells, or potential 'masking' factors in the media such as phenol red or insulin were removed. The luciferase activity of the transfected cells was monitored for significant variation from the basal levels.

The addition of cytokines with or without phenol red or insulin did not cause any significant changes in bLF promoter activity. In phenol red-free media, increases in luciferase reporter gene activity were observed after the co-transfection of an expression vector for NF-IL6, the addition of dexamethasone and also the addition of dexamethasone together with the co-transfection of a glucocorticoid receptor

expression vector. These data provided evidence that lactoferrin transcription may be induced by inflammatory factors which support the suggestion that lactoferrin has a role in the inflammation process.

Abbreviations

| | |
|-----------------|------------------------------------------------------------|
| A | adenine |
| AP-1 | activator protein-1 |
| APR | Acute Phase Response |
| ATP | adenosine triphosphate |
| bLf | bovine Lactoferrin |
| bp | base pair |
| BSA | bovine serum albumin |
| BRL | Bethesda Research Laboratories |
| C | cytosine |
| C-terminal | carboxyl terminal |
| cAMP | cyclic adenosine monophosphate |
| CAT | Chloroamphenicol acetyl transferase |
| cDNA | complementary DNA |
| CDP | CCAAT displacement protein |
| C/EBP | CCAAT enhancer binding protein |
| COUP-TF | chicken ovalbumin upstream promoter - transcription factor |
| CO ₂ | carbon dioxide |
| cpm | counts per minute |
| CRE | cyclic AMP response element |
| CREB | CRE binding protein |
| DBD | DNA-binding domain |
| dex | dexamethasone |
| DMEM | Dulbecco's modified Eagle's media |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DNaseI | deoxyribonuclease I |
| DTT | dithiothreitol |
| <i>E. coli</i> | <i>Escherichia coli</i> |
| EDTA | ethylene diamine tetra-acetate |
| EGF | epidermal growth factor |

| | |
|---------------------------|-------------------------------------------------------|
| EGFRE | epidermal growth factor response element |
| EMSA | Electrophoretic Mobility Shift Assay |
| ER | Estrogen Receptor |
| ERE | Estrogen Response Element |
| FCS | Fetal calf serum |
| G | guanine |
| GCG | Genetics Computing Group |
| GM-CSF | granulocyte-monocyte-colony stimulating factor |
| GR | Glucocorticoid Receptor |
| GRB-2 | Growth factor bound-2 |
| GRE | Glucocorticoid Response Element |
| GTP | Guanosine triphosphate |
| HCMV | human cytomegalovirus |
| HEPES | N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid |
| hGH | human growth hormone |
| HIV | human immunodeficiency virus |
| hLf | human lactoferrin |
| HNF4 | hepatocyte nuclear factor 4 |
| HRP | horse radish peroxidase |
| HSF-1 | hepatocyte stimulating factor-1 |
| hsp90 | heat shock protein 90 |
| IFN- β 2 | Interferon-beta2 |
| IFN- γ | Interferon-gamma |
| IFN- γ R- α | Interferon-gamma receptor-alpha |
| IGF-I | Insulin Growth Factor-I |
| IGF-II | Insulin Growth Factor-II |
| IL-1 α or β | Interleukin one-alpha or beta |
| IL-1R1 | Interleukin-one receptor type I |
| IL-1RII | Interleukin-one receptor type II |
| IL-6 | Interleukin-six |
| IL-6RE | Interleukin-six response element |
| IL-6R α | Interleukin-six receptor α chain |

| | |
|------------|---------------------------------------------|
| IL-8 | Interleukin-eight |
| IL-11 | Interleukin-eleven |
| IRE | Insulin Response Element |
| IRS-1 | Insulin Receptor Substrate-1 |
| JAK | <i>Janus</i> kinase |
| JNK | jun N-terminal kinase |
| kb | kilobase |
| kDa | kiloDalton |
| LAK | lymphokine killer cell |
| LBD | Ligand Binding Domain |
| Lf | lactoferrin |
| LPS | lipopolysaccharide |
| MAPK | mitogen activated protein kinase |
| MCF-7 | mammary gland carcinoma cell line |
| MEK | Map Kinase Kinase |
| mERM | mouse Estrogen Response Module |
| mLf | mouse lactoferrin |
| mRNA | messenger ribonucleic acid |
| mSOS | mouse Son of Sevenless |
| MW | molecular weight |
| NADH | nicotinamide adenine dinucleotide |
| NFIL-6 | nuclear factor interleukin-six |
| N-terminal | amino terminal |
| oligo | oligonucleotide |
| OPNG | o-Nitrophenol β -D-Galacto-pyranoside |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PBSE | phosphate buffered saline EDTA |
| PC12 | rat pheochromocytoma cells |
| PCR | polymerase chain reaction |
| PDGF | platelet-derived growth factor |
| pGL2E | pGL2-Enhancer |

| | |
|-------------------|----------------------------------------------------|
| pGL3B | pGL3-Basic |
| pGL2C | pGL2-Control |
| PI-3 | phosphatidyl inositol-3 |
| PMN | polymorphonuclear leukocytes |
| PMSF | phenyl methane sulfonyl fluoride |
| pSV- β -gal | pSV- β -galactosidase expression vector |
| RA | retinoic acid |
| RAR | retinoic acid receptor |
| RARE | retinoic acid response element |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| RT-PCR | reverse transcriptase polymerase chain reaction |
| S-S | disulphide bond |
| SDS | sodium dodecylsulphate |
| SH2 | src homology domain 2 |
| SH3 | src homology domain 3 |
| Stat | Signal Transducers and Activators of Transcription |
| SV40 | Simian virus 40 |
| T | thymine |
| T75 | 75 cm ² tissue culture vented flasks |
| TAE | Tris acetate EDTA |
| TAT | tyrosine aminotransferase |
| TBE | Tris Boric acid EDTA |
| TBST | Tris buffered saline triton X-100 |
| TBP | TATA-box binding protein |
| TEMED | N,N,N',N'-Tetramethylethylenediamine |
| TNF | Tumour Necrosis Factor |
| TGF- β | transforming growth factor- β |
| Tris | Tris-(hydromethyl) aminomethane |
| tsp | transcription start point |
| UV | ultra violet light |

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Chapter One - Introduction

1.0 Lactoferrin

Lactoferrin is an 80 kDa glycoprotein which is found in mucosal secretions including milk, urine, gastric fluid, and male and female reproductive tract secretions (Masson *et al.*, 1966 ; Masson *et al.*, 1969 ; Nagasawa *et al.*, 1972 ; Tauber *et al.*, 1975 ; Segars and Kinkade, 1977 ; Mason and Taylor, 1978 ; Dalton *et al.*, 1994). Lactoferrin is also stored in the secondary granules of polymorphonuclear leukocytes (Masson *et al.*, 1969 ; Baggiolini *et al.*, 1970 ; Pryzwansky *et al.*, 1979 ; Rado *et al.*, 1984 ; Cramer *et al.*, 1985 ; Fouret *et al.*, 1989). Lactoferrin is found in high levels in human milk (>2 mg/ml), lower levels in horse, guinea pig and mouse milk (0.2-2 mg/ml) and lower levels still in the milk of cow, sheep and pig (20-200 µg/ml). The milk of rat, rabbit and dog have low to insignificant lactoferrin concentrations (reviewed in Schanbacher *et al.*, 1993 ; Masson and Heremans, 1971). There have been many roles proposed for lactoferrin including bacteriostasis, iron absorption, gene regulation and growth stimulation. A significant amount of experimental evidence also suggests that lactoferrin has a role in inflammation.

1.1 The Inflammation Process

Inflammation is a cumulative homeostatic process where after infection, trauma or injury, the body sets in motion processes where the pathogen is isolated and destroyed. In addition, systems are activated which prevent ongoing tissue damage and enhance the repair processes which return the body's systems to normal function. The initial reactions in the inflammation process are collectively called the acute phase response (APR). The initiation of APR occurs by release of mediators such as the alarm cytokines, interleukin-1 (IL-1) and tumour necrosis factor (TNF), from platelets and mononuclear phagocytes at the site of tissue damage. These cytokines cause the release of a secondary wave of cytokines, such as interleukin-6 (IL-6) and interleukin-8 (IL-8), from adjacent stroma. The overall effect of these initial events is to activate

macrophages and platelets, release cytokines and cause secretion of chemotactic mediators, such as transforming growth factor- β (TGF- β), to recruit leukocytes to and retain them in the affected tissue. The mediators of the APR can be separated into 4 groups - the IL-1-type cytokines (e.g. IL-1 α and IL-1 β), the IL-6-type cytokines (e.g. IL-6, interleukin-11 (IL-11)), growth factors (e.g. insulin) and glucocorticoids. While the cytokines are the primary activators of APR gene expression, the growth factors and glucocorticoids modulate the action of the cytokines (reviewed by Baumann and Gauldie, 1994). As IL-6, IL-1 and IFN- γ are among the major cytokines implicated in the inflammatory response, the details and modes of their action are described below.

1.1.1 Interleukin-1

Interleukin-1 is a major pleiotropic cytokine whose biological effects include inducing fever, sleep, hypotension, neutrophilia and T- and B-cell growth and differentiation (reviewed in Lennard, 1995). Interleukin-1 α and -1 β are both produced as precursors by activated monocytes and macrophages (reviewed by Saklatvala *et al.*, 1996) and are cleaved to yield the mature forms. Both have similar tertiary structures, bind to the same receptors and have comparable biological activities (reviewed in Bankers-Fullbright *et al.*, 1996 ; Dinarello, 1996), although there is evidence that IL-1 α and IL-1 β can induce different signal transduction pathways (Vassiliadis *et al.*, 1997).

There are two IL-1 receptors - IL-1RI and IL-1RII. IL-1RI is found in T cells, fibroblasts and endothelial cells, IL-1RII is found in B cells, monocytes and neutrophils (reviewed in O'Neill, 1996). Although both receptors can bind the IL-1 α or - β ligand, only IL-1RI is thought to be involved with signal transduction, with IL-1RII proposed to act as a 'decoy'. IL-1RII, either membrane-bound or released in soluble form, binds the IL-1 ligand and so prevents ligand binding to the true IL-1 receptor. This inhibits IL-1 signal transduction (reviewed in Bankers-Fullbright *et al.*, 1996 ; Colotta *et al.*, 1994). IL-1 has been postulated to activate the sphingomyelin pathway, lipid metabolism, G proteins, cAMP-dependent kinase and protein kinase C. Also mitogen activated protein kinase (MAPK) and Jun N-terminal kinase (JNK) signal transduction pathways are also implicated (reviewed in Bankers-Fullbright *et al.*, 1996). The transcription factors that IL-1 is known to regulate include AP-1 (by inducing *jun* and

fos gene expression and post-translationally regulating the jun and fos gene products), NF- κ B (by stimulating the degradation of the I κ B α - the NF- κ B inhibitor, and by post-translational modification) and nuclear factor-IL-6 (NF-IL6) (by increasing gene expression and post-translational modification) (reviewed in Bankers-Fullbright *et al.*, 1996). IL-1 causes an increase in gene expression of factors such as cytokines, cytokine receptors, growth factors, clotting factors and proinflammatory factors, while reduced gene expression of oncogenes, neuropeptides, specific cell surface receptors (eg platelet derived growth factor (PDGF) receptor) and extracellular matrix proteins (eg fibronectin) is observed (reviewed by Dinarello, 1996). IL-1 can act synergistically with other cytokines such as IL-6 to increase specific gene transcription e.g. acid glycoprotein and C3 in hepatocytes (reviewed by Dinarello, 1996 ; Baumann and Gauldie, 1994).

1.1.2 Interleukin-6

Interleukin-6 (IL-6) (also called IFN- β 2, HSF-1, 26 kDa protein, myeloma/plasmacytoma growth factor) is one of the major cytokines involved in the inflammatory response. IL-6 is produced by cells such as fibroblasts, endothelial cells and endometrial stromal cells (reviewed by Sehgal, 1990). IL-6 has a vast number of cellular actions including stimulating antibody production in B-cells, neural development in PC12 cells, acute phase protein synthesis in hepatocytes, proliferation of T-cells and growth stimulation of keratinocytes (reviewed by Kishimoto *et al.*, 1992). The gene targets for IL-6 stimulation in hepatocytes include acute phase proteins hemopexin, haptoglobin and serum amyloid A. IL-6 has an inhibitory effect on the expression of genes encoding albumin, transferrin and fibronectin. Targets outside hepatocytes include immunoglobulins (B-cells), complement factor B/ C3 (fibroblasts) and α_1 -antitrypsin (monocytes) (reviewed by Sehgal, 1990 ; Baumann and Gauldie, 1994). The transcription factors activated by IL-6 include NF-IL6 (through an as yet undefined pathway) and Stat 1 α and Stat3 (through the Jak-Stat pathway).

The interleukin-6 family of receptors all contain a common gp130 receptor component (β -chain) which is involved in signal transduction and the gp80 component which is

involved in ligand binding. The gp130 component only associates with the receptor after IL-6 stimulation (reviewed in Taga, 1997 ; Schindler and Darnell, 1995). Both gp80 and gp130 are required for a high affinity ligand binding site. Dimerisation of gp130 is required to obtain an active hexameric receptor complex. Jak proteins are thought to associate with the dimerised gp130 subunits in the membrane-proximal regions in a ligand-dependent manner (reviewed by Schindler and Darnell, 1995).

1.1.3 Interferon-gamma

Interferon- γ (IFN- γ) is produced in T cells and large granular lymphocytes. The cellular effects of interferon- γ include suppression of B cell immunoglobulin production and B cell proliferation, modulation of gene expression in monocytes and macrophages, and clonal deletion by T cells (reviewed by Young and Hardy, 1995).

Two components of the IFN- γ receptor have been identified. It has been proposed that Jak1 associates with the cytoplasmic domain of IFN γ R- α , which is then phosphorylated on specific tyrosine residue(s) (reviewed in Schindler and Darnell, 1995). The transcription factors activated by IFN- γ using the Jak-Stat pathway include Stat1 α (reviewed by Ihle, 1996).

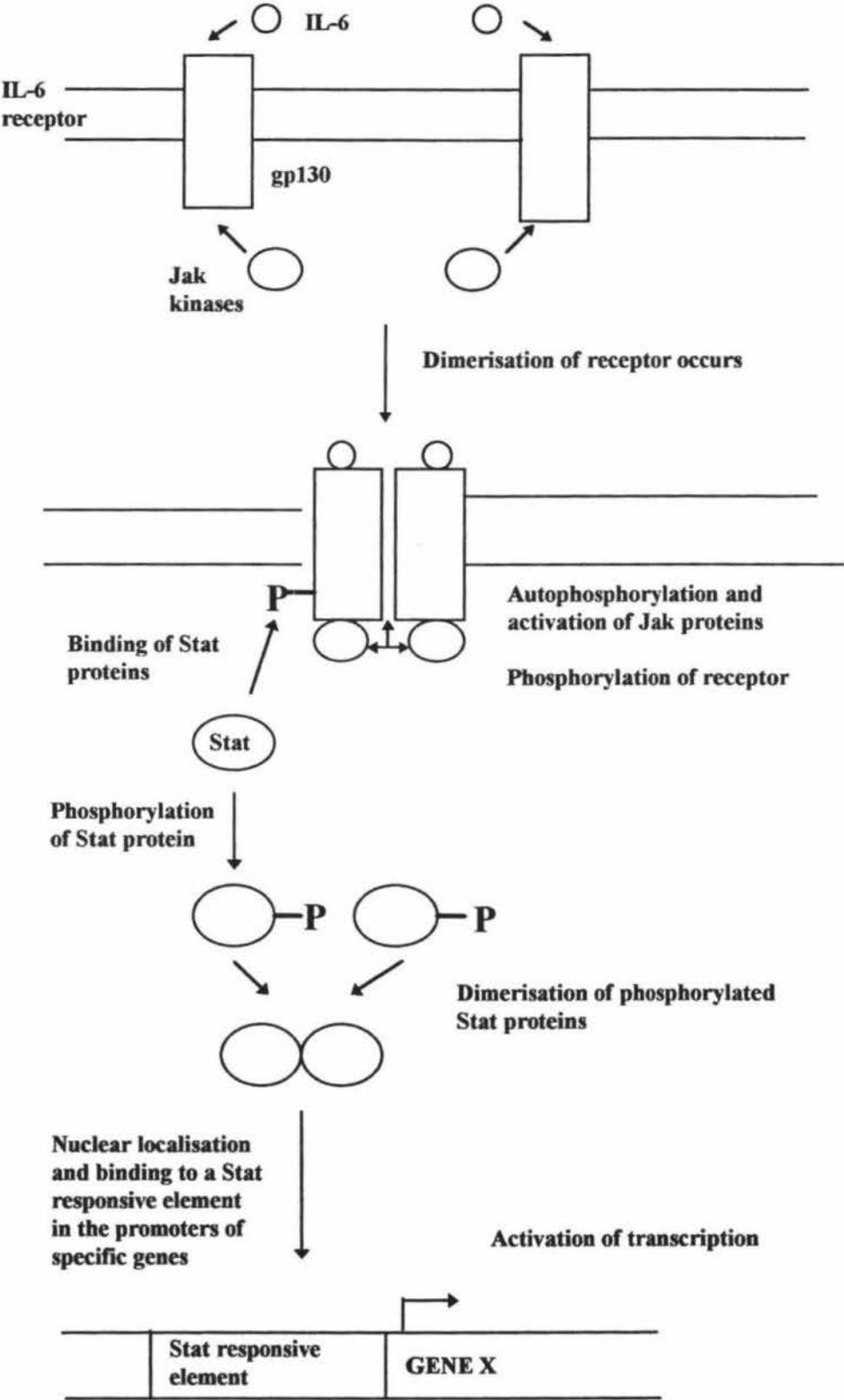
1.1.4 The Jak-Stat pathway

The Jak-Stat signal transduction pathway has been implicated in the action of IL-6 and IFN- γ . *Janus* protein tyrosine kinases (Jaks) are composed of a carboxyl kinase domain and a nonfunctional kinase domain at the amino terminus of the protein. There are 4 members of the Jak family which include Jak1, Jak2, Jak3 and Tyk2, which are all widely expressed except Jak3 (reviewed by Ihle, 1996 ; Ihle and Kerr, 1995 ; Schindler and Darnell, 1995 ; Briscoe *et al.*, 1996). Jak proteins associate with the intracellular domains of the cytokine cell surface receptors.

Upon ligand binding to the α -chain of the cytokine receptor, the receptor and attached Jak proteins dimerise to form receptor-Jak complexes. This places the Jaks in position to cross-phosphorylate each other and activate their kinase activity. The receptor is then phosphorylated on specific tyrosine residue(s) which allows the recruitment and binding of specific substrates via their SH2 protein domains. These bound proteins can subsequently be tyrosine phosphorylated (figure 1). With IL-6 signal transduction, it is thought that the gp130 protein aggregates to IL-6 receptor (IL-6R), after binding of ligand to IL-6 receptor, and then interacts with the Jak proteins (reviewed by Ihle, 1996 ; reviewed by Ihle and Kerr, 1995 ; Schindler and Darnell, 1995 ; Taga, 1997). IL-6 activates Jak1, Jak2 and Tyk2 and IFN- γ activates Jak1 and Jak2 - but it seems that only Jak1 is involved in signal transduction (reviewed by Ihle and Kerr, 1995).

Stat (signal transducers and activators of transcription) proteins are proposed substrates that are recruited to the activated cytokine receptor. Stat proteins contain a carboxyl SH2 domain, an SH3-like domain with a DNA-binding region in the middle of the protein. Tyrosine phosphorylation in the SH2 domain is required for Stat protein homo- or hetero-dimerisation, which is required for nuclear translocation and DNA binding (figure 1). A proposed serine or threonine phosphorylation is required for Stat protein activation (reviewed by Ihle, 1996 ; Ihle and Kerr, 1995 ; Schindler and Darnell, 1995 ; Briscoe *et al.*, 1996). IL-6 activates Stat1 α and Stat3 while IFN- γ activates Stat1 α (reviewed in Schindler and Darnell, 1995).

Figure 1. The proposed Jak-Stat signal transduction pathway



1.2 Potential roles for lactoferrin in inflammation

A variety of roles have been proposed for lactoferrin in the inflammation process. Lactoferrin helps to control monocyte and macrophage activity (McCormick *et al.*, 1991) and other cells involved in the inflammatory response. These activities include the negative feedback regulation of myelopoiesis (granulocyte and macrophage production) (Broxmeyer *et al.*, 1978) by decreasing the production of granulocyte-macrophage stimulating activity from monocytes (Broxmeyer *et al.*, 1980 ; Bagby *et al.*, 1983). Lactoferrin interacts with polymorphonuclear leukocytes and reduces the surface charge. This enhances the 'stickiness' of the leukocyte and allows a variety of cell-cell interactions (Boxer *et al.*, 1982). Lactoferrin enhances the accumulation and adherence of neutrophils to tissues at the site of injury, possibly due to the reduction of neutrophil surface charge (Birgens, 1984). Nishiya and Horwitz (1982) showed that lactoferrin could enhance natural killer activity by monocytes. Enhancement of lymphokine killer cell (LAK) cytotoxicity by lactoferrin has been observed (Shau *et al.*, 1992). These activities may be a primary defence against tumorigenesis (Bezault *et al.*, 1994). Djeha and Brock (1992) found that apolactoferrin could overcome the inhibitory effect of non-transferrin-bound-iron on T-lymphocytes and so allow lymphocyte function during inflammation. Lactoferrin is also thought to inhibit the synthesis of antibodies (Winton *et al.*, 1981).

Crouch *et al.* (1992) suggested that lactoferrin could act to inhibit the release, but not the biological activity, of various inflammatory mediators (such as IL-1 β , IL-2 and TNF), and so act in a negative feedback mechanism which would prevent the recruitment and activation of leukocytes at sites of inflammation. Oseas *et al.* (1981) stated that neutrophilic lactoferrin acts to retain neutrophils at the sites of inflammation to amplify the response. Machnicki *et al.* (1993) also describe a preventative action of lactoferrin during infection where lactoferrin could block TNF- α release and induce IL-6 release. While Paul Eugene *et al.* (1993) observed that lactoferrin together with LPS augmented the production of IL-6, IL-1 β , TNF- α and prostaglandins, Shinoda *et al.* (1996) showed that lactoferrin and lactoferricin (an active peptide derived from a pepsin hydrolysate of lactoferrin) could stimulate the release of IL-8 from

polymorphonuclear leukocytes (PMN). While the lactoferrin derived from neutrophils is not a direct gene regulation event, there is evidence that TNF- α can partly mediate the release of lactoferrin from PMNs (Koivuranta-Vaara *et al.* 1987). Apo-lactoferrin may mediate the hyposideremia (drop in plasma iron concentration) during inflammation (Van Snick *et al.*, 1974). Evidence that lactoferrin was essential for the antibacterial power of polymorphonuclear leukocytes was provided by Bullen and Armstrong (1979).

The complement system is a family of ~20 proteins in the blood. They function through a cascade of proteolytic reactions which lyse target cells by inducing large pores in the cell membrane and attract macrophages to take up and destroy antigen-antibody complexes. The classical complement pathway is normally activated by antibody-antigen complexes (Becker and Deamer, 1991 ; Stryer, 1988 ; Lewin, 1994). Rainard (1993) observed that lactoferrin could also activate the classical complement pathway. This implicates lactoferrin in the activation of one of the primary responses to infection and inflammation. The variety of inflammatory roles attributed to lactoferrin provides evidence that a primary role for lactoferrin may be in the inflammatory process.

Further evidence for a role for lactoferrin in inflammation was noted by Harmon *et al.* (1975) and Harmon *et al.* (1976) where upon induction of infections in bovine mammary tissue, an increase in lactoferrin concentration was observed in the mammary secretions. A 30-fold increase in lactoferrin concentration was seen 90 hours after inoculation. This effect was not due to a concentrating effect resulting from decreased milk production, nor was likely to be due to release by polymorphonuclear leukocytes (Harmon *et al.*, 1976). Harmon *et al.* (1975) showed that the actual increases in lactoferrin observed upon inflammation were related to the severity of the infection. This induction of lactoferrin occurred in the acute phase of the inflammation response and so may implicate cytokines in the regulation of lactoferrin gene expression. Rejman *et al.* (1995) found that after the intramammary infusion of recombinant IL-1 β into cows, higher concentrations of lactoferrin were seen in mammary secretions from day 7 and 14 of involution. In clinically-induced mastitis, high concentrations of IL-6, IL-1 β and TNF were seen (Shuster *et al.*, 1993 ; Sordillo and Peel, 1992 ; Shuster *et al.*,

1995). Sone *et al.* (1997) observed that milk macrophages increased the production of IL-1 β , IL-6, TNF- α and IFN- γ following viral infection. Therefore IL-6, IL-1, IFN- γ or other cytokines present in the mammary gland following infection could be involved in the increase of lactoferrin observed during inflammation.

1.3 Structure of Lactoferrin

Human lactoferrin is composed of N- and C-terminal lobes joined by a short α -helix. Each lobe is formed from two interconnected globular domains connected by β -strands, has the same folding pattern and one ferric ion can bind at the interface between the two domains. The iron atoms are coordinated by four protein ligands - two tyrosines, one histidine and one aspartate residue. Binding of a carbonate or bicarbonate ion along with the iron is required (Anderson *et al.*, 1987 ; Anderson *et al.*, 1989). Nagasako *et al.* (1993) suggest that lactoferrin may be able to bind iron at sites additional to the stated iron-binding sites, although no further observations of this type have been reported. Some flexibility is seen in the orientation of the two lobes and within the domains (Baker *et al.*, 1990). Metz-Boutigue *et al.* (1984) predicted a MW of ~82,000, with internal homology between the N- and C-domains. Lactoferrin shares similarities with transferrin, including a similar molecular weight, similar tertiary structures and similar metal iron-binding sites. However lactoferrin binds iron with a higher affinity than transferrin particularly at low pH (Aisen and Leibman, 1972).

Predictions from the structure of the bovine lactoferrin gene, indicated that the bovine lactoferrin protein, like other members of the transferrin family, had two lobes. The similarity of the structure of bovine Lf to other lactoferrins has been confirmed by structural studies (Baker *et al.*, 1998). The ligands involved in iron binding were conserved (Seyfert *et al.*, 1994 ; Baker *et al.*, 1998). Goodman and Schanbacher (1991) and Pierce *et al.* (1991) found that the amino acids involved with and surrounding the iron binding site were conserved, but 17 disulphide (S-S bonds) were predicted compared to 16 S-S in human lactoferrin. Five potential glycosylation sites have been detected (Pierce *et al.*, 1991). Le Provost *et al.* (1994) found that like the

bovine lactoferrin protein, the goat lactoferrin has high homology to other members of the transferrin family with conserved iron binding sites and disulphide bonds. The structure of lactoferrin which enables the protein to bind iron links lactoferrin to the inflammatory process as a modulator of free radical production (Section 1.4.2).

Comparisons between lactoferrin from mucosal secretions and neutrophils show that the two proteins are similar in molecular weight, iron release under acidic conditions, isoelectric point and in uptake by the liver (Moguilevsky *et al.*, 1985) but differ in N-glycosylation (Derisbourg *et al.*, 1990).

1.4 Other roles for lactoferrin

1.4.1 Antibacterial properties of lactoferrin

Much evidence has shown that lactoferrin has bacteriostatic or bacteriocidal activity against a variety of microorganisms. Rainard (1986) and Arnold *et al.* (1980) found that apo-lactoferrin could inhibit or kill gram positive and gram negative bacteria, rods and cocci, aerotolerant anaerobes and facultative anaerobes. The addition of iron removed this bacteriocidal or bacteriostatic activity. Bacteriocidal or bacteriostatic effects of apo-lactoferrin - but not iron-loaded lactoferrin have also been reported by Dionysius *et al.* (1993), Arnold *et al.* (1982), Bishop *et al.* (1976) and Oram and Reiter (1968). Citrate can also block the bovine lactoferrin bacteriostatic effect (Law and Reiter, 1977 ; Bishop *et al.*, 1976).

One proposed mechanism of bacteriostatic action is the sequestering of environmental iron from the microbe with subsequent inhibition of microbial growth. Another mechanism has been proposed to account for the bacteriocidal activity. Ellison *et al.* (1988) showed that lactoferrin could damage the outer membrane of gram negative bacteria and cause changes in the cellular permeability. Ellison *et al.* (1990) provided further evidence that lactoferrin acts at the outer membrane, to cause the release of LPS and sensitize the bacteria to various antibiotics and detergents. This effect is modulated by calcium and magnesium. Ellison and Giehl (1991) observed that

lysozyme and lactoferrin together can have bacteriocidal effects, potentially through osmotic damage of the bacterial outer membrane. This effect was blocked by iron-saturation and increased calcium concentrations.

Tomita *et al.* (1991) found that antibacterial potency of pepsin-digested lactoferrin was >8 fold greater than that of undigested lactoferrin. But unlike native lactoferrin, the activity was retained in the presence of iron. Bellamy *et al.* (1992) identified the lactoferrin antibacterial domain, distinct from the iron-binding region, in a loop between disulphide bonds. This active peptide (named lactoferricin) was more potent than the native lactoferrin and had bacteriocidal effects. Yamauchi *et al.* (1993) noted that the bacteriostatic activity was modulated by calcium and magnesium and enhanced by lysozyme. This activity damaged the bacterial outer membrane. Lactoferricin was found to bind directly to the cell membrane surface, which may disrupt the cell permeability (Bellamy *et al.*, 1983). The antibacterial loop may act as an amphipathic α -helix to cause the effects seen (Odell *et al.*, 1996). Lactoferricin also had inhibitory effects on the growth of fungi and yeast, again possibly due to a disruption of cell membrane function (Bellamy *et al.*, 1994). Another possible mechanism of bacteriostasis could be the agglutination of bacteria via interaction of the glycans of lactoferrin with the bacterial fimbriae (Teraguchi *et al.*, 1996). Lactoferrin also has proposed antiviral effects against HCMV infection and HIV-induced cytopathic effect (Harmsen *et al.*, 1995).

This proposed antibacterial role could have possible effects in the protection of the neonatal digestive tract. For example, Teraguchi *et al.* (1995) found that lactoferrin inhibited the bacterial translocation from the intestine to the mesenteric lymph nodes possibly due to the suppression of bacterial overgrowth in the gut. This effect is feasible as lactoferrin was not completely digested by the neonatal digestive system (Britton and Koldovsky, 1989) and could still bind iron (Spik *et al.*, 1982a ; Spik *et al.*, 1982b). But holo-lactoferrin was more resistant to trypsin digestion than apo-lactoferrin, and trypsin-digested apo-lactoferrin upon iron addition had reduced iron binding compared to undigested apo-lactoferrin (Brines and Brock, 1983). The experimental observations that lactoferrin can cause bacteriostasis or bacteriocidal

effects on a wide range of microorganisms directly implicates lactoferrin in the inflammatory response.

1.4.2 Lactoferrin as an antioxidant

Lactoferrin has also been proposed to act as an antioxidant to protect tissues from reactive hydroxyl radicals. Britigan *et al.* (1989) and Aruoma and Halliwell (1987) showed that apo-lactoferrin could inhibit the formation of the hydroxyl radicals possibly by binding the iron and so preventing an iron-catalysed reaction. This was confirmed by Shinmoto *et al.* (1992). But this process seemed dependent on the iron status of the lactoferrin and the environmental conditions, which determine whether lactoferrin is an iron scavenger or supplier. Bannister *et al.* (1982) observed that iron-saturated lactoferrin increased the production of hydroxyl radicals. Under acidic conditions, lactoferrin (by supplying iron) catalysed the production of hydroxyl radicals by neutrophils (Bennett *et al.*, 1981).

1.4.3 Role for lactoferrin in iron absorption

The role of lactoferrin in the absorption of iron from the dietary tract is still under much debate. Chierici *et al.* (1992) found that lactoferrin may be involved in neonatal iron absorption after experiments with lactoferrin supplementation of infant formulae. Cox *et al.* (1979) provided evidence that lactoferrin could donate iron to the small intestine via interaction with brush border cell surface receptors. Davidson and Lonnerdal (1989) found that this receptor had affinity for iron-saturated lactoferrin, but partially digested lactoferrin could still donate iron. Davidsson *et al.* (1994) and De Laey *et al.* (1968) found that lactoferrin did not enhance iron absorption but actually inhibited it. Fairweather-Tait *et al.* (1987) and Schulz-Lell *et al.* (1991) found that lactoferrin did not influence the iron absorption in the digestive tract.

Hepatocytes have been reported to take up holo- or apo-lactoferrin via clathrin-dependent endocytosis, release the iron, degrade the now apo-lactoferrin protein and

recycle the cell surface lactoferrin binding sites (McAbee *et al.*, 1993 ; McAbee *et al.*, 1995).

1.4.4 Interaction of lactoferrin with DNA

There has been some evidence of a lactoferrin interaction with DNA. Garre *et al.* (1992) found that lactoferrin was rapidly internalized and localised to the nucleus. He and Furmanski (1995) described a potential role for lactoferrin as a transcription factor when they identified lactoferrin DNA binding sites, some which could activate gene transcription upon lactoferrin addition. Zhao and Hutchens (1994) found evidence for lactoferrin-mediated hydrolysis of DNA or RNA. There is some evidence for DNA bound on the cell membrane surface of B cells and monocytes acting as a binding site for lactoferrin (Bennett *et al.*, 1983). But the functional significance of these observations is unclear as lactoferrin can bind to double-stranded DNA, via an interaction with arginine residues found in the lactoferrin N-terminal domain, that is independent of nucleotide sequence (van Berkel *et al.*, 1997).

1.4.5 Lactoferrin as a Growth factor

There is evidence that lactoferrin can act as a growth factor for a number of different cell types. Hashizume *et al.* (1983) indicated that bovine lactoferrin and human lactoferrin could stimulate the growth of human B- and T-lymphocytic cell lines. Human lactoferrin can stimulate the growth of phytohemagglutinin-stimulated lymphocytes (Mazurier *et al.*, 1989) and can act as a maturation factor for B cells (Zimecki *et al.*, 1995). A proposed mechanism for lactoferrin-induced growth stimulation involves lactoferrin participating in oxoreduction reactions that activate the NADH oxidase at the cell outer membrane (Sun *et al.*, 1991). There is also evidence that lactoferrin, regardless of its iron state, can act as an activator of thymidine incorporation into DNA in rat crypt enterocytes (Nichols *et al.*, 1987 ; Nichols *et al.*, 1990). Ismail and Brock (1993) observed that lactoferrin does not act as an iron donor to a promonocytic cell line. This suggested that transfer of iron is not involved in the mechanism for growth stimulation. The action of lactoferrin to stimulate the growth or

maturation of lymphocyte cells involved in the inflammation response directly implicate lactoferrin in the acute phase response.

1.4.6 Receptors for lactoferrin

Receptors for lactoferrin have been reported in hepatocytes (Bennatt and McAbee, 1997), in the intestinal brush border (Mazurier *et al.*, 1985 ; Hu *et al.*, 1988 ; Gislason *et al.*, 1993), peripheral blood lymphocytes (Mazurier *et al.*, 1985), mouse peritoneal cells (Van Snick and Masson, 1976), monocytes (Birgens *et al.*, 1983) and various microorganisms (Bonnah *et al.*, 1995 ; Naidu *et al.*, 1991). Evidence from Rochard *et al.* (1989) indicated that the N-terminal domain of human lactoferrin binds to a specific blood human lymphocyte lactoferrin receptor while Yu and Schryvers (1993) implicated both an N- and C-lobe interaction with bacterial receptors. The fucosylated glycans on lactoferrin are proposed to be required for receptor recognition (Davidson and Lonnerdal, 1988). There is also evidence that lactoferrin can bind non-specifically to bacterial cell surfaces via an interaction between N-terminal arginine residues and bacterial cell surface lipopolysaccharides (van Berkel *et al.*, 1997). The exact biological significance of the interaction of lactoferrin and these receptors has yet to be fully elucidated.

1.5 Regulation of lactoferrin expression in the body

1.5.1 Regulation of lactoferrin expression in reproductive tissues

Teng *et al.* (1986) identified an estrogen-induced secretory protein from mouse uterine luminal fluid. The protein was 70 kDa, a single polypeptide, strongly basic, glycosylated and secreted by uterine epithelial cells. This protein was identified by Pentecost and Teng (1987) as lactoferrin. A 300-fold increase in lactoferrin was seen after estrogen addition. Teng *et al.* (1989) found that the regulation of lactoferrin expression was tissue-specific. The lactoferrin in immature mouse uterus was induced

by estrogen in a time- and dose-dependent manner while mammary lactoferrin was not. This regulation of mouse lactoferrin by estrogen has also been reported by McMaster *et al.* (1991), Walmer *et al.* (1992), Shigeta *et al.* (1996) and Newbold *et al.* (1992). Shigeta *et al.* (1996) found that there was no increase in lactoferrin mRNA in the non-reproductive tissues (liver or spleen) after addition of estrogen. Walmer *et al.* (1995) found that unlike in the mouse where the lactoferrin gene is expressed throughout the endometrium, lactoferrin gene expression in humans is restricted to a number of glandular epithelial cells located deep in the zona basalis.

1.5.2 Regulation of lactoferrin expression in the mammary gland

Lactoferrin levels in milk vary quite dramatically between species with humans having high levels, cows and sheep lower levels, and rats and dogs none (reviewed in Levay and Viljoen, 1995). The reason for the differences in lactoferrin concentration between the human and bovine have been proposed to be due to strong secondary structure and destabilising elements in the bovine mRNA (Schanbacher *et al.*, 1993). Also, a lack of specific transcription factor binding sites (Seyfert *et al.*, 1994) or a non-optimal translation initiation consensus sequence in the bLf promoter (Schanbacher *et al.*, 1993) may cause the low levels of bovine lactoferrin observed. Sanchez *et al.* (1988) found that in the bovine, lactoferrin was the highest (0.8 mg/ml) in the colostrum and, after decreasing rapidly, lowest in the mature milk (0.09 mg/ml). Smith *et al.* (1991) found that lactoferrin was 30-50% of the total protein from the dry secretion from the involuted bovine mammary gland.

This expression is opposite to that of other milk proteins which are highest in the milk and lowest in the colostrum. There is an active upregulation of lactoferrin mRNA and translation during involution (Schanbacher *et al.*, 1993), indicating that a transcriptional effect, and not a mRNA stability effect, causes the changes in lactoferrin levels seen. Welty *et al.* (1975) and Sanchez *et al.* (1992) also saw this pattern of lactoferrin expression. Molenaar *et al.* (1996) found that lactoferrin was expressed at different stages of mammary development and at different sites from other milk proteins. Lactoferrin was synthesized in higher levels in involuting mammary tissue

compared with lower levels in lactating tissue, and synthesis of lactoferrin occurred despite the decline of synthesis of milk-specific proteins (Hurley *et al.*, 1993). Hurley *et al.* (1993) found that lactoferrin was associated with mammary epithelial cells - in the basal regions of alveolar epithelial cells.

Green and Pastewka (1978) indicated that lactoferrin production in the mammary gland was regulated by prolactin. But Hurley *et al.* (1994) using explants of bovine mammary gland, found that under the conditions used, prolactin had no effect on lactoferrin expression. Schanbacher *et al.* (1993) observed that lactoferrin secretion *in vitro* from bovine mammary cells seemed independent of prolactin. Therefore, although there is a definite pattern of lactoferrin expression during the development of the mammary gland, the molecular mechanisms behind these changes remains unclear.

1.5.3 Regulation of neutrophil lactoferrin

Masson *et al.* (1969) found that lactoferrin was first produced in myeloid cells at the stage of the promyelocyte. Lactoferrin was located in the specific or secondary granules in polymorphonuclear leukocytes (PMN) (Baggiolini *et al.*, 1970 ; Pryzwansky *et al.*, 1979 ; Rado *et al.*, 1984 ; Cramer *et al.*, 1985). Fouret *et al.* (1989) found that lactoferrin was not produced until the late stage of neutrophil differentiation. A limited number of studies have been performed on the regulation of lactoferrin in PMN leukocytes. TNF- α was found to decrease lactoferrin gene transcription by 70% in normal bone marrow cells (Srivastava *et al.*, 1991). Shirsat *et al.* (1992) when comparing the lactoferrin DNA sequence to the sequence of another leukocyte-expressed protein, found a region of shared nucleotides which could be involved in regulation of granulocyte differentiation.

1.6 The Lactoferrin Gene

The human lactoferrin gene is found on chromosome 3 q21→ q23 (McCombs *et al.*, 1988), the mouse lactoferrin gene is found on chromosome 9 (Teng *et al.*, 1987) and the bovine lactoferrin gene is located on chromosome 22q24 (Schwerin *et al.*, 1994).

1.6.1 The Lactoferrin Promoter

The lactoferrin promoter has been isolated from a variety of species. Lui and Teng (1991) characterised the 5' flanking region of the mouse lactoferrin promoter. Elements corresponding to putative TATA box, CAAT boxes, GC boxes, PU boxes and a COUP-ERE were identified.

Teng *et al.* (1992) isolated the human lactoferrin promoter. The elements conserved between the mouse and human lactoferrin promoter include the noncanonical TATA box, the CAAT-like sequence, putative Sp1 binding site in a G-C rich region, PU box, COUP-ERE and $\frac{1}{2}$ palindromic EREs. The TATA box, CAAT-like sequence and Sp1 are elements typical of housekeeping genes while the other elements are typical of inducible genes. Johnston *et al.* (1992) isolated human lactoferrin cDNA from a chronic myelogenous leukemia library. The sequence differed from the cDNA isolated from mammary gland by only 2 bp - but the same protein sequence was predicted. This indicated that neutrophilic and glandular lactoferrin were the products of the same gene. Putative sequence elements identified in the human lactoferrin promoter included TATA (-30), CCAAT (-59), CACCC (-68), Sp1 (-24), GATA-1 (-367) and GRE (-350). The presence of a hexamer CTGGA (-280) that is found in some acute phase response genes was also identified.

Seyfert *et al.* (1994) investigated the bovine lactoferrin promoter. Putative binding sites for CCAAT-enhancer binding protein and Sp1 were identified. Putative consensus sequences identified in another study by Bain, (1995) include binding sites for TBP, AP1, Stat1, Stat3, NF-IL6, GRE and NF- κ B (figure 3).

The functionality of various putative elements have been investigated in the human and mouse lactoferrin promoters. Liu and Teng (1992) identified the mouse estrogen response module (mERM) - a putative ERE overlapping a COUP (chicken ovalbumin upstream promoter) element (-349 to -329) which conferred estrogen responsiveness. The evidence presented indicated that ER and COUP-TF may compete for the same binding site. The level of ER determines the estrogen responsiveness while the COUP-TF modulates the estrogen-stimulated transcriptional activation. Lui *et al.* (1993) confirmed that COUP-TF represses the stimulation of mERM by estrogen possibly due to competition between ER and COUP-TF for an identical contact site in mERM. However this mechanism is thought to be more complex as Shigeta *et al.* (1996) found that upon the addition of estrogen to developing neonatal reproductive tract tissue, increased lactoferrin mRNA or protein occurred, but the expression or levels of ER and COUP-TF remained unchanged.

As with the mERM, the COUP-ERE element in the human lactoferrin promoter was found to act as an enhancer in response to estrogen with the responsiveness being both hormone- and receptor-dependent. However, unlike the mERM, only the ER not COUP-TF bound to the COUP-ERE, indicating a different mechanism for estrogen activation between human and mouse (Teng *et al.*, 1992). Yang *et al.* (1996) found evidence that hERR1 - an orphan receptor may interact with the human ER through protein-protein contacts and may participate in the estrogen stimulation of the human lactoferrin gene.

Shi and Teng (1994) characterized mitogen response elements in the mouse lactoferrin promoter. A CRE (cAMP response element) which binds CREB and AP1 and an element which is stimulated by EGF and TGF α were also identified. Shi and Teng (1996) defined the elements required for basal activity as a CRE (cAMP response element) and the TATA box. A mitogen response element (EGFRE/CRE) was also identified. Nelson *et al.* (1991) identified EGF as an estrogen-inducible mediator of uterine mouse lactoferrin transcription and translation.

Experiments by Lee *et al.* (1995) indicated that an overlapping retinoic acid response (RARE)-ERE element may regulate mouse lactoferrin expression by acting as a signalling switch modulator that mediates multihormonal responsiveness. Retinoic Acid Receptor (RAR) and ER could act as positive regulators of the element while COUP-TF is a negative regulator. It was proposed that in breast and reproductive tissues, this element would mainly confer the ER activity, with retinoic acid (RA) regulating this activity, but in hemopoietic cells this element mediates RA activity. These observations suggested that during the terminal differentiation process in myeloid cells, retinoic acid and its receptors may be involved in lactoferrin gene regulation.

A comparison of the ~800 bp of the promoter and exon I sequence of the human, mouse and bovine lactoferrin genes by Bain, (1995) showed a 63% (hLf) and 54% (mLf) homology. There was no homology seen with the regions of the mouse and human promoter that contain the COUP-ERE element. Although both the human and mouse lactoferrin promoters contain the COUP-ERE element, the action of gene regulation by this element seems species-specific. In regions of the Lf promoter which are conserved across species, differences in the regulation of promoter activity occur. This may indicate that a role for bovine lactoferrin in inflammation may be specific only for the bovine and not for human or mouse. Alternatively, different molecular mechanisms may be involved in mediating a species-specific lactoferrin inflammatory response.

1.7 Aim of the research

There are many proposed functions for the lactoferrin protein but no clear role has yet been determined. The aim of this study was to further implicate lactoferrin as an inflammatory protein by providing evidence that the lactoferrin gene is regulated by cytokines or glucocorticoids.

In Harmon *et al.* (1976) a significant increase in lactoferrin secreted protein was seen during the early phase of a bovine mammary tissue infection. The bovine lactoferrin promoter contained many putative binding sites for inflammatory modulators e.g. cytokine or glucocorticoid-induced transcription factors (Bain, 1995). These observations indicated that the increases in lactoferrin seen upon inflammation may be due to activation of lactoferrin gene transcription by these mediators.

The cytokine-responsiveness of the bLf promoter was investigated by transient transfection of RL95-2 cells with reporter gene constructs containing various lengths of the bLf promoter linked to the luciferase reporter gene. Transcriptional activity was measured in the presence and absence of cytokines and dexamethasone as well as expression vectors for NF-IL6 and GR, transcription factors known to be involved in the inflammatory response.

Chapter Two - Materials and Methods

2.1 Materials

The pGL2E and pGL3B luciferase reporter vectors and multicore buffer were from Promega Corporation, WI, USA. The majority of restriction enzymes, T4 ligase, ligase buffer and LB base media were from Life Technologies Inc., MD, USA. *Nco* I was from New England Biolabs Inc. MA, USA. Sephaglas™ Band Prep Kit was from Pharmacia LKB Biotechnology, Uppsala, Sweden. Proteinase K and calf alkaline phosphatase were from Boehringer Mannheim, West Germany. Ampillicin, RNase and chicken egg white lysozyme were from Sigma Chemical Company, St Louis, MO, USA. The QIAGEN plasmid preparation kit was from Quiagen Pty Ltd, Victoria, Australia.

The RL95-2 human endometrial carcinoma cells were purchased from the American Type Culture Collection. The African green monkey kidney COS cells were a gift from Kerry Loomes, University of Auckland.

DMEM media, F12 media, DMEM-F12 base medium and media additives - L-leucine, L-lysine-HCl, L-methonine, MgCl₂·6H₂O, MgSO₄(an.), CaCl₂·2H₂O, NaHCO₃, charcoal stripped and delipidated bovine calf serum and DMSO were all tissue culture grade from Sigma Chemical Company, St Louis, MO, USA. Crystalline zinc bovine insulin (4 mg/ml), glutamine (200 mM), penicillin (5000 U/ml)- streptomycin (5000 µg/ml), 2.5% trypsin, HEPES buffer and bovine calf serum were from Life Technologies Inc., MD, USA. Cryotubes were from Nunc Inc., Naperville, IL, USA.

Interleukin-6 (human recombinant) and interferon- γ (human recombinant) were obtained from Life Technologies Inc., MD, USA and both were tissue culture grade. Dexamethasone (crystalline) and Interleukin-1 α (human, recombinant) were from Sigma Chemical Company, St Louis, MO, USA.

The 0.2 μ M SartoLab P media filters were from Sartorius AG, Germany. The filters for sterilisation of transfection buffers were Supor Acrodisc 32 0.2 μ M filters from Gelman Sciences, MI, USA.

The 75 cm² canted neck 0.2 μ M vented flasks were from Sarstedt. The 60 mm plates (60 x 15 mm γ -irradiated) were from Falcon, NJ, USA. The 12-well plates (22.1 x 17.6 mm) with lids were from Corning Costar Corporation, MA, USA. The 150 mm plates were from Nunc Inc., Naperville, IL, USA.

The Luciferase Assay Kit was from Promega Corporation, WI, USA. This contained the cell lysis buffer (x5), luciferase assay buffer and the luciferase assay substrate. The pSV- β -galactosidase control plasmid was from Promega Corporation, WI, USA. The MSV-C/EBP β expression vector was a gift from Gareth Morgan, Dunn School, School of Pathology, University of Oxford, UK. The RSV-GR expression vector was a gift from Dr Tsai, Baylor College of Medicine, Houston, USA. The cell scraper (23 cm) was from Nunc Inc., Naperville, IL, USA. The polystyrene luminometer cuvettes were from Crellin B.V., Rotterdam, The Netherlands. The Bradford protein determination reagent was from Biorad Laboratories, CA, USA.

Synthetic oligonucleotides, γ -³²P-ATP (3000 Cimmol⁻¹) and T4 kinase (10 U/ μ l) were from Life Technologies Inc, MD, USA. Acrylamide (40% 29:1) and SDS-PAGE low range molecular weight standards were from Bio Rad Laboratories, CA, USA. The DE-81 ion exchange membrane and 3 mm Whatman paper was from Whatman,

England. The x-ray film was from Fuji Photo Film Company, Japan. Developer and fixer were from Eastman Kodak, NY, USA.

The 0.45 μ M nitrocellulose blotting membrane was from Sartorius AG, Germany. The 3 mm paper was from Whatman, England. The ECL western blotting analysis kit which contains the HRP-labelled secondary antibodies and chemiluminescence reagents were from Amersham Life Sciences, UK. The G410 antibodies were kindly supplied by Dr M. Grimes (Massey University, New Zealand).

All other chemicals were analytical grade or better.

2.2 Methods

2.2.1 Preparation of plasmid constructs

pGL2E2464 (2.5 kbp) (-2464 to +36) and pGL2E520 (556 bp) (-520 to +36) were previously prepared by H. Bain (PhD thesis, 1995) by the following methods.

pGL2E2464

A 2.8 kb fragment isolated from a bovine genomic library was sequenced to confirm that it contained the bovine lactoferrin promoter region. The polymerase chain reaction (PCR) was used to remove the start codon and to insert a 3' *Bgl* II restriction site at the 3' end of the product. Enzyme digests with *Sst* I and *Bgl* II produced a 2.5 kb fragment which was inserted directly into the multiple cloning site of the pGL2-Enhancer (pGL2E) vector after digestion with the same enzymes. This construct was called pGL2E2464.

pGL2E520

pGL2E520 was prepared by digesting the 2.5 kb PCR product with *Sma* I and *Bgl* II. To produce a 556 bp fragment. This fragment was inserted directly into a pGL2E vector which had been digested with *Sma* I and *Bgl* II. Both clones were sequenced to ensure that they were free of PCR-induced errors.

Both the 2.5 kb and 556bp fragments were subcloned into pGL3-Basic (pGL3B) vector by digesting pGL2E2464 and pGL3B with *Sst* I and *Bgl* II and pGL2E520 and pGL3B with *Sma* I and *Bgl* II respectively.

pGL2E2087

The -2087 to +36 clone was prepared previously in pGL2E by the following method. pGL2E2464 was digested with *Kpn* I to remove the 5' ~ 380 bp region and the ~ 8 kb fragment gel purified. The fragment was then religated to produce the pGL2E2087 construct.

pGL2E1816

The -1816 - +36 clone had been prepared previously by H.B. Bain as follows. pGL2E2464 was digested with *Pst* I and *Sst* I to remove the 5' ~ 650 bp region and the ~ 7.7 bp size fragment gel purified. Cohesive ends were filled with T4 DNA polymerase before religation to produce pGL2E1816.

Subcloning of the -2087 to +36 and -1816 to +36 fragments

2.2.2 Isolation of inserts

The -2087 to +36 and -1816 to +36 fragments were subcloned into pGL3B by the following method ; both pGL2E1816 and pGL2E2087 were initially digested with *Kpn* I in React 4 at 37° for 1 hr (as there is a *Kpn* I restriction site in the multiple cloning site of pGL2E) and then for a further hour after addition of *Bgl* II and 100 mM NaCl. The inserts were gel purified using the Sephaglas™ Band Prep Kit according to manufacturers instructions.

2.2.3 Preparation of vectors

pGL3B was digested with *Kpn* I and *Bgl* II as described above. 1 µg calf alkaline phosphatase (1 U/µl) was then added and the tubes incubated at 37° for 15 minutes to remove 5' phosphate groups. 2 µl of proteinase K (10 mg/ml) and 4 µl of SDS (10%)

were then added and the tubes incubated for 1 hr at 37° to remove the phosphatase. The vector DNA was then purified by phenol/chloroform extraction followed by ethanol precipitation.

2.2.4 Ligation of vector and insert

50 ng of insert and 48 ng vector (vector : insert ratio 1 : 3) were added to 2 µl T4 Ligase Buffer (x5), 1 µl T4 ligase and 7 µl H₂O (total of 10 µl) and incubated overnight at 16° to allow ligation to occur.

2.2.5 Transformation

(i) Ultra-competent XL-1 blue cells were produced by C. Flyger, Department of Biochemistry, Massey University by the following method. XL-1 cells were streaked onto LB-tetracycline plates and incubated overnight. 10-12 large colonies were inoculated into 300 ml SOB broth (20 g/l tryptone, 5g/l yeast extract, 0.5 g/l NaCl, 2.5 ml 1 M KCl, with 5 ml of 2 M MgCl₂ added prior to use) and grown at 22° or 18° until an A₆₀₀ of 0.6 was reached. The cells were placed on ice for 10 minutes and centrifuged at 4000 rpm for 10 minutes. The cells were resuspended in 12 ml ice cold TB (10 mM PIPES, 15 mM CaCl₂, 250 mM KCl pH 6.7, 55 mM MnCl₂.4H₂O) and centrifuged at 4000 rpm (GSA rotor) for 10 minutes. After repeating this washing, 1-2 ml of cold DMSO was then added to the cells which were then dispensed into 500 µl aliquots and snap-frozen using liquid N₂ before storage at -70°.

(ii) Transformation of XL-1 cells was carried out according to Pope and Kent (1996) as follows. Half of the ligation reaction and 100 µl of competent XL-1 cells were mixed and placed on ice for 5 minutes. 400 µl of LB broth was added and varying amounts of the ligation-bacteria mixture were spread over prewarmed LB-amp plates, which were incubated overnight at 37°.

2.2.6 Isolation of plasmid DNA

Mini-prep isolation of plasmid DNA was by the rapid-boil procedure of Holmes and Quigley, (1981). Single colonies of ampicillin-resistant bacteria were grown overnight in 5 ml LB Broth. 1.5 ml of the culture was pelleted for 1 minute at 12000g. The pellet was resuspended in 350 µl STET (8% sucrose, 50 mM EDTA, 50 mM Tris pH 8.0, 0.5% triton X-100) and 25 µl freshly prepared lysozyme (10 mg/ml) was added. The resulting mixture was placed in a boiling water bath for 40 seconds and centrifuged immediately for 10 minutes at 12000 rpm. The gelatinous pellet was removed and an equal volume of isopropanol added. The mixture was placed at -20° or -70° for 30 minutes. The DNA pellet was collected by centrifugation at 12000g for 15 minutes and was recentrifuged after washing with cold 95% ethanol. The dried pellet was resuspended in 50 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

2.2.7 Large scale preparation of plasmid DNA

A single colony from a correct clone was inoculated into a 5 ml LB Broth containing ampicillin and grown overnight at 37°. All of this culture was used to inoculate a flask containing 500 ml of LB Broth containing ampicillin which was then incubated overnight at 37°. The QIAGEN Plasmid Kit was used to produce large scale plasmid DNA for transfections as described in the QIAGEN Plasmid Handbook. This method is based on alkaline lysis of bacteria, followed by neutralization to precipitate chromosomal DNA and protein. Plasmid DNA is purified by anion exchange chromatography using a modified DEAE-silica gel.

2.2.8 Quantitation of DNA

The plasmid DNA was quantitated using the nucleic acid (200 - 350 nm) scan programme on the Pharmacia Biotech Ultrospec 300 UV/Visible Spectrophotometer. The purity of the DNA was assessed by the A260/A280 absorbance ratio. A ratio of

1.8 is expected for pure DNA, ratios greater than 1.8 indicated RNA, ratios less than 1.8 indicated protein contamination.

Functional assays

2.2.9 Routine maintenance and passage of RL95-2 endometrial carcinoma cells

All tissue culture manipulations prior to harvesting were performed in a laminar flow workstation.

Preparation of tissue culture media

(i) Cells were grown in T75 tissue culture vented flasks using DMEM-Ham's F12 (1:1) media. The media was prepared according to the manufacturers instructions with 2 g/L sodium bicarbonate and 10 mM HEPES added before filter sterilization. The pH was adjusted to 6.8 so that the final pH would be approximately 7.1 as the pH rises 2-3 units upon filter sterilization. Prior to use, 20 ml bovine calf serum, 2 ml penicillin (5000 U/ml) -streptomycin (5000 µg/ml) and 250 µL insulin (4 mg/ml) were added to 180 ml of media.

(ii) Phenol red-free media was made from a DMEM-F12 base medium without phenol red. To 1 litre of media, 1.2 g NaHCO₃, 0.05905 g L-leucine, 0.09125 g L-lysine-HCl, 0.01724 g L-methionine, 0.0612 g MgCl₂, 0.0488 g MgSO₄, and 0.1545 g CaCl₂ were added prior to sterilisation. Two ml glutamine (200 mM) was added immediately prior to use.

Establishment of RL95-2 cells from frozen stocks

RL95-2 (human endometrial carcinoma) cells were grown from stocks stored in liquid nitrogen. The frozen RL95-2 cells were quickly thawed, centrifuged and the pellet resuspended in DMEM-F12 media. The cell suspension was added to 12 ml of DMEM-F12 media in T75 tissue culture flasks and placed in a 37°, 5% CO₂ incubator in a humidified atmosphere.

Passage of RL95-2 cells for cell maintenance, transfections and cell extract preparation

The cells were grown to 80-100% confluence in T75 flasks before passage into new flasks to maintain stocks, or onto plates for transfections and cell extract preparation. Cells were passed by washing with 4 ml of 0.25% trypsin in PBSE (0.14 M NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·2H₂O, 0.5 mM EDTA). The flasks were then left lying flat for ~ 10 minutes. Flasks were knocked to help dislodge the cells and the cells were resuspended by aspiration with 10 ml media. One ml of this suspension was added to T75 tissue culture flasks containing 12 ml media for the maintenance of stocks. Two drops (with a plastic disposable pipette) were added to each well of a 12-well tissue culture plate containing 800 µL media for transfections or 0.5 ml of cell suspension was added to 60 mm plates containing 4.5ml media for cell extract preparation. Cells were placed in a 37°, 5% CO₂ incubator in a humidified atmosphere.

Freezing of cells for liquid nitrogen storage

Cells for liquid nitrogen storage were prepared by passing minimally-passed cells and resuspending in bovine calf serum containing 10% DMSO. These cells were then dispensed into cryotubes in 1 ml aliquots and slowly frozen at -70° before transferring to liquid nitrogen.

2.2.10 COS cell maintenance

COS cells were maintained on DMEM media containing 10% bovine calf serum and 2 ml penicillin (5000 U/ml) -streptomycin (5000 µg/ml) using the methods described for RL95-2 cells. For preparation of cell extracts 14 ml of media and 1-1.5 ml of cell suspension were added to 150 mm plates.

2.2.11 Transfections

Two drops (using a plastic disposable pipette) from a 10 ml suspension of RL95-2 cells were added to each well (of a 12-well tissue culture plate) containing 800 µL of media and left in the incubator overnight. Fresh media was added to the cells two hours prior to transfection. Cells were transfected using calcium phosphate co-precipitation as described in Ausubel *et al.* (1991). 50 µL of Buffer A (0.5 M CaCl₂, 0.1 M HEPES, pH 7.05-7.12) was added to 50 µL of DNA and water. 100 µL of Buffer B (0.28 M NaCl, 0.05 M HEPES, 0.75 mM sodium dihydrogen phosphate, 0.75 mM disodium hydrogen phosphate) was added to the DNA and the mixture was immediately vortexed. After a 15 minute incubation at room temperature, the DNA suspension was dispersed into the medium on the prepared cells. 2 µg of pSV-β-gal control vector and 4 µg of reporter vector were used unless otherwise stated. Each treatment was carried out in triplicate within each transfection experiment. Cells were incubated in the presence of the DNA precipitate for 6 hours and then washed twice with media without additions. The cells were then incubated with fresh complete media for 18 hours in the 37°, 5% CO₂ incubator. Cytokine was added after washing or at indicated times before harvesting. All cytokines were reconstituted according to the suppliers instructions and dispensed into aliquots to avoid excessive freeze-thaw cycles. Dexamethasone was reconstituted in absolute ethanol to a concentration of 25 mM. This was added to the cells in the concentrations and times indicated for each experiment.

2.2.12 Harvesting

Cells were harvested as described in the Luciferase Assay System Technical Bulletin. Media was removed from the cells, which were then washed twice with PBS (0.14 M NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·2H₂O). 80 µL of 1x cell lysis solution (25 mM Tris-phosphate pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) was added to each well. After 15 minutes, the cells in lysis solution were scraped off the well base and centrifuged at 12000g for 15 seconds to remove cellular debris. Cell extract was then transferred to a new microfuge tube and stored at -20°.

2.2.13 Luciferase assay

Assays for reporter gene activity were carried out as described in the Luciferase Kit Technical Bulletin by mixing 100 µL of luciferase assay reagent (20 mM tricine, 1.07 mM (MgCO₃)₄Mg(OH)₂·5H₂O, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 µM DTT, 270 µM coenzyme A, 470 µM Luciferin, 530 µM ATP, final pH 7.8) at room temperature with 20 µL of cell extract in a luminometer cuvette. Readings were taken in a luminometer at 5-10 second intervals until a maximum value was reached.

2.2.14 β-galactosidase assay

β-galactosidase assays were carried out as described in Herbomel *et al.* (1984). 400 µL β-galactosidase assay buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgCl₂) and 200 µL ONPG (2 mg/ml ONPG in 60 mM Na₂HPO₄, 40 mM NaH₂PO₄) were mixed with 20 µL of cell extract and incubated at 37° for 2-3 hours until an adequate colour was produced. 500 µL of 1 M sodium carbonate was then added to stop the reaction. Absorbances were measured at 420 nm against a blank containing 20 µL cell lysis buffer. The β-galactosidase values, expressed as total absorbance units, were used to normalise the luciferase values.

2.2.15 Determination of protein concentration.

Bradford reagent (1 ml, 1:4 dilution) was added to 20 μ l of BSA standard protein (in the range of 100-1600 μ g/ml) or cell extract and mixed. The absorbance at 595 nm was read after 15 minutes. The BSA standards were used to prepare a standard curve from which the protein concentrations of the cell extracts were determined.

Electrophoretic mobility shift assays

2.2.16 Cell extract preparation for electrophoretic mobility shift assay (EMSA)

COS cells were grown overnight to a confluence of 50% on 150 mm plates and fresh media was added two hours prior to transfection. Transfections were carried out as described in Section 2.2.11 as follows : 45 μ g of expression vector was added to 240 μ l of Buffer A and sterile H₂O (total volume of 480 μ l). To this mixture, 480 μ l of Buffer B was added and after a 15 minutes at room temperature, all the mixture was incubated with the cells for 18 hours. The cells were then washed with media without additions and incubated for a further 30-36 hours in fresh complete media. Cell extracts were prepared in a manner similar to that described in Ladas *et al.* (1992). Cells were washed with PBS and 1.5 mL of TEN buffer (40 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.15 M NaCl) was used to harvest the cells. After centrifugation at 12000g for 1 minute, the pellet was resuspended in 300 μ L extraction buffer (40 mM Hepes pH 7.9, 0.4 M KCl, 1 mM DTT, 10 % glycerol, 0.1 mM PMSF and 0.1% aprotinin). The cell suspension was freeze-thawed three times using liquid nitrogen to disrupt the cells. The cell extract was centrifuged at 4° at 14000g for 5 minutes and the supernatant was dispensed into 30 μ L aliquots, snap-frozen in liquid nitrogen and stored at -70°. 10 μ l of cell extract was analysed by 8% SDS-PAGE to determine the effectiveness of the cell extract preparation.

2.2.17 Labeling of oligonucleotides

Oligonucleotides were labeled as follows. 2 μl water, 1 μl 10x kinase buffer (0.5 M Tris.HCl pH 7.5, 0.1 M MgCl_2 , 50 mM DTT, 0.5 mg/ml BSA), 1 μl oligonucleotide (100 ng/ μl), 5 μl γ - ^{32}P -ATP (10 $\mu\text{Ci}/\mu\text{l}$) and 1 μl T4 polynucleotide kinase (10 U/ μl) were incubated at 37° for 1 hour. A 6x excess of complementary oligonucleotide (100 ng/ μl), 2.5 μl 1 M KCl and 31.5 μl H_2O was then added and the mixture was heated at 95° and cooled slowly to allow annealing to occur. An equal volume of 2x gel shift buffer (40 mM Tris pH 7.6, 16% Ficoll, 100 mM KCl, 0.4 mM EDTA, 1 mM DTT) was added and the labeled probe was gel purified by electrophoresis on a 10% polyacrylamide gel (37 cm gel fitted with 0.4 mm spacers) in 1x TBE at 30W. The gel was exposed to X-ray film and the labeled annealed oligonucleotide was excised from the gel and eluted by incubation overnight at 37° in 50 mM KCl. The gel suspension was mixed by vortexing, centrifuged and the supernatant transferred to a new microcentrifuge tube. 1 μl of the supernatant was analysed for incorporation of radioactivity using Cerenkov counting.

2.2.18 Electrophoretic mobility shift assays (EMSA)

(i) Varying amounts of cell extract, 10 μl 2x gel shift buffer, 1 μl poly(dI-dC) and water to a total volume of 20 μl was incubated on ice for 10 minutes. 1 μl of labelled oligonucleotide (~10,000 cpm/ μl) was added and the mixture incubated at room temperature for a further 15 minutes. Half of each mixture was then analysed by electrophoresis on a 4% acrylamide gel (BRL V15.17 apparatus fitted with 0.75 mm spacers) in 0.25x TBE for 1.5-2 hours at 200V. The gels were dried on to DE-81 membrane and exposed to X-ray film overnight at -70° using intensifying screens.

(ii) Double-stranded unlabeled oligonucleotides for competition assays were prepared by mixing equal amounts (10 μl of 1 $\mu\text{g}/\mu\text{l}$ solutions) of complementary single-stranded oligonucleotides. The mixture was heated at 90° for 10 minutes and then cooled slowly to room temperature. Dilutions corresponding to 5 ng/ μl , 50 ng/ μl and

100 ng/ μ l were prepared in H₂O and stored at -20°. For competition gel shifts, 5, 50 or 100 ng of competitor DNA was added to the reaction mix prior to the 10 minute incubation on ice.

Protein gels and western blots

2.2.19 Cell extract preparation for western blots

Cell extracts were prepared as described above in a manner similar to that in Ladas *et al.* (1992). RL95-2 cells were grown on 60 mm plates overnight. Cells were washed, harvested and stored as described previously in Section 2.2.16 using 500 μ L of TEN buffer and 50 μ L extraction buffer.

2.2.20 Protein gel and western blotting

Proteins in the cell extract (5-10 μ L) were separated by polyacrylamide (8%) gel electrophoresis in the presence of SDS (Laemli, 1970) using the buffer system described by Ornstein (1964) and Davis (1964). Proteins were transferred to nitrocellulose membrane by electroblotting with transfer buffer (25 mM Tris, 192 mM Glycine pH 8.3) for 45 minutes at 0.5A. The gel was stained using Coomassie Blue (0.1% R-250 40% methanol, 10% acetic acid) and destained with Destain I (50% methanol, 10% acetic acid) and Destain II (5% methanol, 7% acetic acid) to determine if efficient transfer had occurred. The membrane was blocked for 30 minutes using 5% low fat milk in TBST (130 mM Tris base, 25 mM NaCl, pH 7.6, 1% Triton X-100) or 3% BSA in TBST. The primary antibodies used were G410 (monoclonal antibodies against phosphotyrosine used at a 1:1000 dilution in the blocking solution). Membranes were incubated for 45 minutes with the primary antibodies, and after washing with TBST, were incubated with horse radish peroxidase (HRP)-conjugate mouse secondary antibodies (from the ECL detection kit)(1:10,000 dilution). Bands were detected using the ECL reagents (Amersham) as described in the manufacturers

instructions. Chemiluminescent detection is more sensitive and safer than other current detection methods. Detection occurs by the addition of substrates to the membrane after washing off excess HRP-conjugate peracid to the primary and secondary affixed conjugate. This resulted in the production of the oxidised form of the HRP enzyme. Luminol is oxidised under alkaline conditions by HRP and hydrogen peroxide resulting in an excited luminol state. This excited state decayed to the ground state by emitting light (428 nm) which is detected by x-ray film. The presence of a chemical enhancer e.g. phenols, increases light output and emission time (ECL Western blotting protocol).

Chapter Three - Functional Assays of the bovine lactoferrin promoter in pGL2E

3.1 Introduction

The work described in this chapter is an initial attempt at investigating the putative role for lactoferrin in the inflammatory response (see Chapter One).

To investigate an inflammation-induced transcriptional activation of the bLf promoter, constructs corresponding to various lengths of the putative bLf promoter were linked to the luciferase reporter gene and transfected into RL95-2 human endometrial carcinoma cells.

RL95-2 (human endometrial carcinoma) cells were used in this study as they have been used previously to examine the regulation of the human and mouse lactoferrin promoter (Shi and Teng, 1996 ; Lui *et al.*, 1993 ; Liu and Teng, 1992 ; Teng *et al.*, 1992 ; Lui and Teng, 1991 ; Shi and Teng, 1994 ; Yang *et al.*, 1996). The effect of the addition of glucocorticoids and cytokines to RL95-2 cells transfected with various promoter-reporter constructs, was studied. As the complement of transcription factors in these cells has not yet been established, some experiments were carried out with co-transfection of expression vectors for specific transcription factors. The potential confounding effect of phenol red in the medium on regulation by cytokines and glucocorticoids was investigated by the use of phenol red-free medium.

Initially the promoter-reporter constructs were made in the vector pGL2-Enhancer (pGL2E) which contains the powerful SV-40 enhancer element. This vector was used because previous work (Bain, 1995) indicated low levels of expression from the bLf promoter. Studies in the pGL2E vector, described in this chapter suggested that the enhancer element in this construct may interfere with the normal activity of the bLf promoter. For this reason, subsequent experiments were carried out using pGL3-Basic

(pGL3B) vector, which lacks the SV40 enhancer. These experiments are described in Chapter Four.

3.1.1 Transfection methods

Transfections are commonly used to introduce the DNA of interest into mammalian cells in order to study mammalian gene regulation within the environment of the intact cell. Transient transfections occur when there is no integration of the transfected DNA into the cell chromosomal DNA. Assays to detect activity are performed 12 - 72 hours after transfection (Alam and Cook, 1990). There are currently four well-known methods of transfection :- liposome-, calcium phosphate precipitate, DEAE-dextran-mediated-transfections and electroporation (Ausubel *et al.*, 1991). The first three methods involve increasing the endocytic DNA uptake by enhancing DNA-cell surface interactions. Electroporation involves the passive movement of DNA through artificially-induced pores (Alam and Cook, 1990). Electroporation can be used on all cell types and involves a high voltage pulse to induce pores in the plasma membrane, through which DNA can enter the cell. This method has a high transfection efficiency but large amounts of DNA and cells are required. Liposome-mediated transfection involves liposomes (containing cation and neutral lipids) which can fuse with the cell membrane to facilitate the movement of the DNA into the cell. This method has higher efficiency and greater reproducibility than the other methods and only a small amount of DNA is required to obtain a high transfection frequency. With both the calcium phosphate and DEAE-dextran-induced transfections, the DNA is in a chemical environment which is endocytosed into the cells by unknown mechanisms. The DEAE-dextran is more reproducible as a number of factors such as reagent or media pH and DNA purity can affect the calcium phosphate method. But importantly, the cells transfected by the calcium phosphate method tend to contain a representative sampling of the different plasmids in the precipitate (Ausubel *et al.*, 1991). This method is also cost-effective and within the budget allocated for this research project.

Moreover, the calcium phosphate precipitate method had been used previously with RL95-2 cells (Shi and Teng, 1996 ; Liu and Teng, 1992 ; Lui *et al.*, 1993 ; Liu and Teng, 1991 ; Shi and Teng, 1994 ; Teng *et al.* ; 1992; Yang *et al.*, 1996 ; Curtis *et al.*,

1997), and as this method was successful during the initial stages of this research, it was used for all subsequent transfection experiments.

3.1.2 Reporter genes

An assay system using a reporter gene is the most commonly used experimental tool to study promoter function.

Reporter genes are used to investigate the activity of promoters, under a variety of controlled conditions, in order to elucidate the mechanisms of gene regulation. Reporter gene constructs are fusions containing the promoter under investigation linked to a reporter gene. The ability of the promoter to direct transcription is thought to be reflected by the amount of reporter protein produced. The reporter protein must be detectable in a sensitive assay, have a minimal effect on the physiology of the transfected cells (Ausubel *et al.*, 1991), and thus be sensitive to changes in transcription due to interactions between the bLf promoter sequence and transcriptional modulators.

There are a variety of well known reporter genes used in transfections - these include the bacterial CAT (chloroamphenicol acetyl transferase) gene, the firefly luciferase (*Photinus pyralis*) gene and the hGH (human growth hormone) gene (reviewed in Bronstein *et al.*, 1994 ; Alam and Cook, 1990). The CAT reporter gene is the most frequently used reporter and is a widely accepted measure of promoter activity. The CAT enzyme assays are reliable and relatively fast. The hGH reporter system relies on an immunological assay of protein secreted into the media. The luciferase reporter gene is also frequently used because its assay is quicker and more sensitive than the CAT reporter assay and requires neither radioactivity nor specific antibodies. Due to the sensitivity, weak promoters or cells which transfect poorly can be investigated (Ausubel *et al.*, 1991) and smaller amounts of plasmids can be used (Alam and Cook, 1990). This avoids the anomalous effect that may occur with large amounts of plasmid i.e. the competition for limited amounts of transcription factors between the control

plasmid elements and the promoter construct under investigation (Alam and Cook, 1990).

3.1.3 Luciferase assay

Because of the advantages of the luciferase reporter assay system over other assay systems, the Promega luciferase assay system was used in this research. Luciferase assays are rapid and sensitive and are based on the reaction by which the firefly luciferase protein supplied with substrate can emit light (562 nm) in the presence of ATP (Luciferin + ATP + O₂ $\xrightarrow{\text{Mg}^{2+}}$ oxyluciferin + AMP + PPi + CO₂ + light) (reviewed in Bronstein *et al.*, 1994 and Gould & Subramani, 1988). The Promega luciferase assay system produces a greater enzymatic turnover of the luciferase and so increases the light intensity. This system has a 100-fold greater sensitivity than the CAT (chloroamphenicol acetyl transferase) assay and <10⁻²⁰ mole of luciferase can theoretically be detected (Promega Technical Manual). Another advantage is that the light emission is proportional to the luciferase activity which directly reflects the activity of the promoter region inserted in front of the luciferase reporter gene. The pGL2C vector, which has the luciferase gene controlled by the strong SV40 promoter, was used as a control to check the luciferase assay and reagents.

3.1.4 β -galactosidase assay

A bacterial β -galactosidase expression vector was co-transfected with the luciferase reporter vectors as β -galactosidase is a useful internal standard to normalize for variations in cell number, extract preparation and transfection efficiency (Alam and Cook, 1990). Bacterial β -galactosidase catalyses the hydrolysis of various β -galactosides such as lactose. The usefulness of β -galactosidase was seen in Edlund *et al.* (1985) where normalizing CAT assay results with β -galactosidase levels reduced the standard deviation of the CAT measurements from 22% to 8%. The spectrophotometric assay to detect β -galactosidase levels involves monitoring the

hydrolysis of ONPG (o-nitrophenyl- β -D-galactopyranoside) at 420 nm (Alam and Cook, 1990). As cell lines can contain endogenous β -galactosidase, performing the assays at pH 8 will limit this effect as this pH is suboptimal for the endogenous β -galactosidase, and has only a limited effect on the bacterial β -galactosidase produced from the transfected plasmid (Bronstein *et al.*, 1994).

3.2 RL95-2 human endometrial carcinoma cells

Previous studies have shown that lactoferrin is normally expressed and secreted by uterine cells (Pentecost and Teng, 1987 ; Teng *et al.*, 1989), and so the lactoferrin promoter is functional in these cells *in vivo*. Therefore, the RL95-2 cell line represents an authentic system within which to study the transcription of bovine lactoferrin. While there was limited evidence that RL95-2 cells are responsive to the cytokines used in this study, the cell type and source of the cells make it likely that the cells are responsive. Previous experiments have shown that RL95-2 cells contain EGF receptors (Lelle *et al.*, 1993) and type I,II and III TGF- β receptors (Dumont *et al.*, 1995). These cells can mediate EGF-induced gene activity (Sallot *et al.*, 1996), TGF- β can inhibit cell growth (Korc *et al.*, 1987) and retinoic acid causes a reversion of the RL95-2 cells towards a normal differentiated phenotype (Carter *et al.*, 1996).

RL95-2 cells are derived from an adenosquamous endometrial carcinoma. The cells are non-stromal epitheloid (Way *et al.*, 1983). RL95-2 cells have some of the features of typical glandular epithelial and secretory cells, but vary from normal endometrial cells in size, nuclear membrane invagination, nuclear-to-cytoplasm ratio, having ≥ 3 prominent nuclei per cell, loss of contact inhibition and have the ability to undergo unlimited cell division (Sundareshan and Hendrix, 1992).

3.3 The bovine lactoferrin promoter constructs

Two constructs, one 2.5 kb construct representing the entire putative promoter (-2464 to +36) (named 2464) and the other, a 556 bp construct (named 520) representing a truncated promoter (-520 to +36) (figure 2) were used to investigate the cytokine responsiveness of the bovine lactoferrin promoter. A number of putative cytokine-responsive transcription factor binding sites corresponding to IL-6RE, Stat1, Stat3, GRE, NF- κ B and AP-1 had been previously detected (Bain, 1995) (figure 3). There were significantly more of these putative elements in the longer than in the shorter construct. Therefore it was likely that differences in promoter activity between the two constructs would be seen in the event of cytokine-induced transcriptional modulation. The first set of experiments were carried out using constructs which had been previously prepared in the pGL2-Enhancer vector while the second set of experiments, were with constructs in the pGL3-Basic vector.

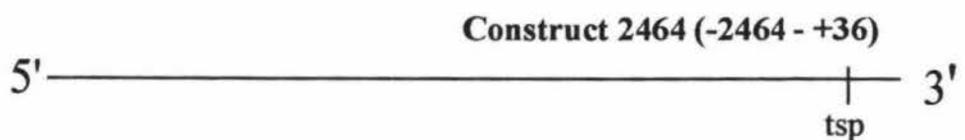


Figure 2. The bLf promoter constructs

Construct 520 (-520 - +36)

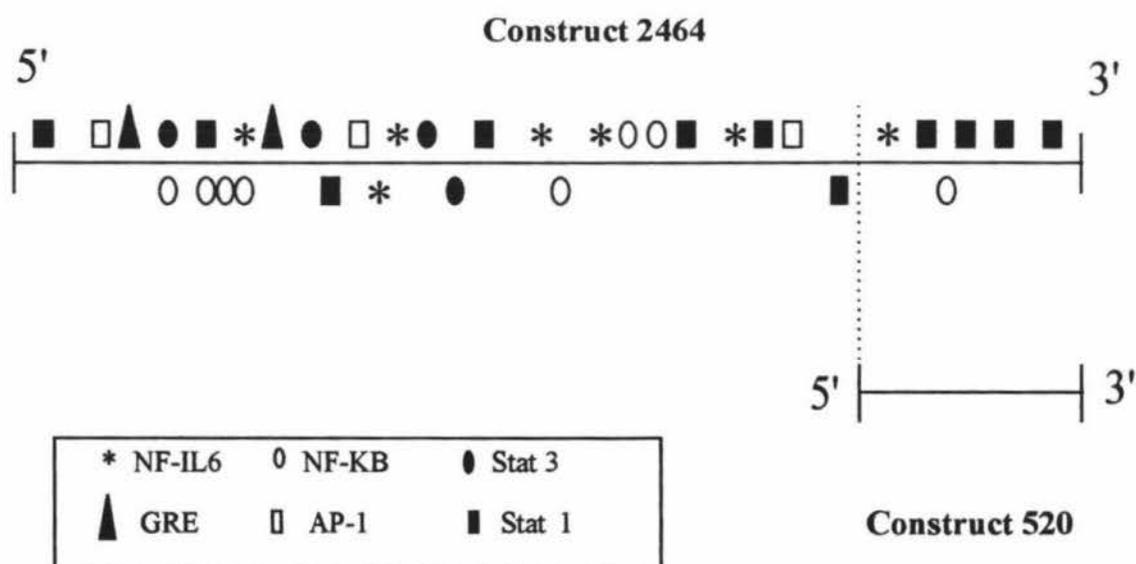


Figure 3. Putative inflammation-induced transcription factor binding sites in the bLf promoter constructs (from Bain, 1995)

3.4 The pGL2-Enhancer (pGL2E) vector

The pGL2E reporter vector (containing the SV40 enhancer element) (Appendix 1) was used as the bLf promoter was expected to support only low levels of transcriptional activity. The SV40 enhancer element present in the pGL2E vector will "often result in transcription of *luc*⁺ at high levels" (Promega pGL3B Luciferase Reporter Vector Technical Manual, 1994), therefore the SV40 enhancer element may also contribute to the luciferase levels. As a result, the luciferase levels produced will depend in part on the promoter construct and in part on the enhancer element. However since the enhancer element is common in all pGL2E constructs, any change in expression should be due to interactions involving the bLf promoter sequence.

3.5 Optimization of pGL2E constructs in RL95-2 cells

Optimization of transient transfection of RL95-2 cells with the pGL2E constructs was carried out to define the conditions needed to produce optimal luciferase and β -galactosidase levels. The luciferase levels reflect the activity of the promoter region inserted in front of the luciferase reporter gene. Control vectors were transfected to monitor the background levels of transcription from the reporter vector (pGL2E) and to verify the activity of the assay reagents (pGL2-Control).

Cells were incubated in the presence of DNA-calcium phosphate precipitate for 6 and 18 hours. Incubation for 6 hours produced the highest luciferase and β -galactosidase values. Once this was determined, cells (incubated for 6 hours with the calcium phosphate precipitate) were washed and then incubated for 18, 36 and 48 hours prior to harvesting. Incubation for 18 hours produced the optimal results (table 1) (figure 4). Cells were transfected with various amounts of both pGL2E construct and pSV- β -galactosidase vectors together to test for optimal results. 4 μ g of pGL2E or pGL3B constructs and 2 μ g of pSV- β -gal vector were found to produce optimal levels. Insulin was added to the media in all experiments unless otherwise stated.

| Time (hr) | Luciferase values (relative light units) | β -galactosidase values (A ₄₂₀) |
|-----------|---------------------------------------------|------------------------------------------------------|
| 18 | 0.21 \pm 0.07 | 0.72 \pm 0.12 |
| 24 | 0.13 \pm 0.05 | 0.25 \pm 0.14 |
| 36 | 0.03 \pm 0.01 | 0.03 \pm 0.01 |

Table 1. Optimisation results of three experiments with pGL2E520 in RL95-2 cells after 18, 24 or 36 hours incubation after the 6 hour incubation of the calcium phosphate precipitate.

Figure 4. Optimization protocol for transient Transfections of pGL2E constructs in RL95-2 human endometrial carcinoma cells

Plate RL95-2 cells Human endometrial carcinoma cells

24 hours



Change media

2 hours



Add DNA in Calcium Phosphate precipitate

5-6 hours



Wash cells

18 hours



Harvest cells

**Luciferase assay
β-galactosidase assay**

3.6 Protein concentrations vs β -galactosidase value

Protein concentration of cell extracts were initially determined to see if these values instead of β -galactosidase absorbance values could be used to standardise the luciferase values. Cell number can be normalised by the protein content, therefore it can be assumed that luciferase levels are also proportional to the cell number which is proportional to protein concentration. Therefore the protein levels could indicate the number of cells producing the luciferase protein. Cells were transfected with 2 μ g of pSV- β -galactosidase and 4 μ g luciferase reporter vector and after 18 hours, the β -galactosidase activity, luciferase activity and protein concentrations were determined. Subsequent analysis showed that protein concentrations were not proportional to β -galactosidase values (figure 5b) and so could not be used for normalisation of luciferase activity. The β -galactosidase values showed a good correlation with the luciferase activity (figure 5a). This could be due to the transfection efficiency not being influenced by cell numbers. This result agreed with Alam and Cook (1990) who stated that standardizing the data to a second enzyme activity is a suitable control for variability in cell extract collection and transfection efficiency, but standardizing by the amount of total protein only acts as a control for the collection of the extract.

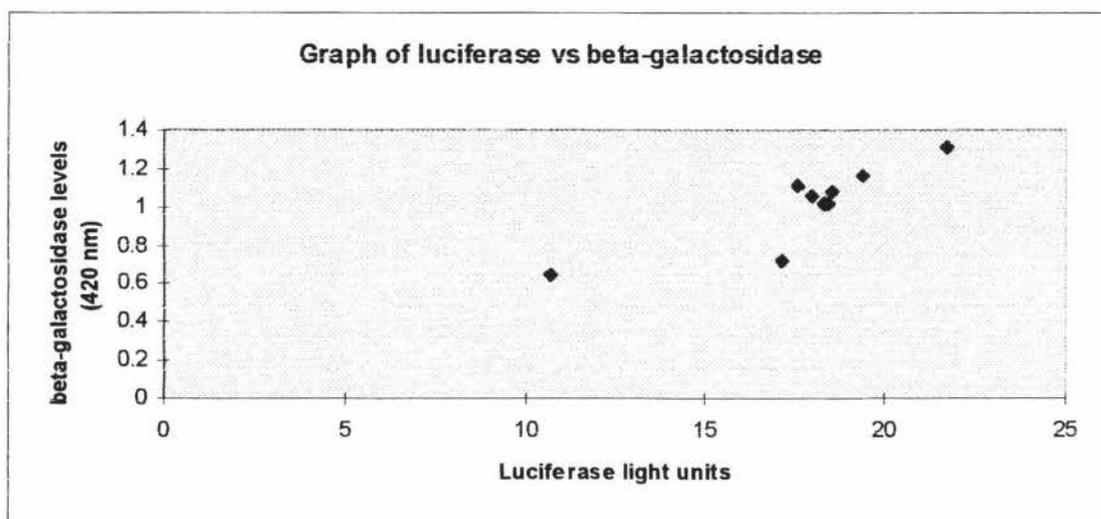


Figure 5a. Comparison of luciferase activity with β -galactosidase activity. RL95-2 cells were transiently transfected with 4 μ g of pGL2E luciferase reporter construct and 2 μ g pSV- β -gal. Luciferase activity is measured in light units and β -galactosidase activity is represented as absorbance at 420 nm.

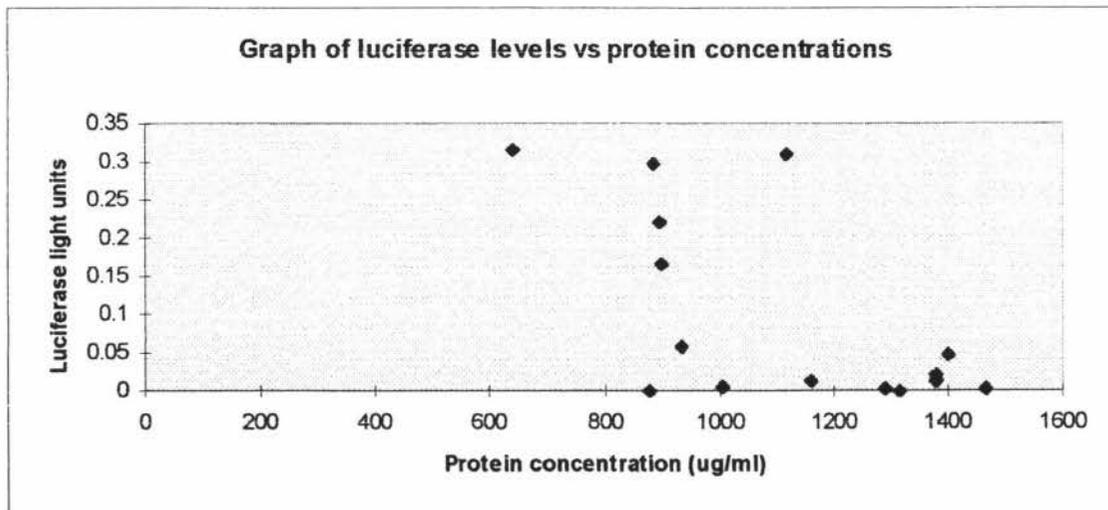


Figure 5b. Comparison of protein concentration with luciferase activity. RL95-2 cells were transiently transfected with 4 μ g of luciferase reporter vector and 2 μ g pSV- β -galactosidase. Luciferase activity is measured in light units and β -galactosidase activity is represented as absorbance at 420 nm.

3.7 Transcriptional activity of pGL2E constructs

RL95-2 cells were transfected with pGL2E2464 and pGL2E520 together with appropriate controls as described previously. All experiments were performed in triplicate within each experiment and an average was calculated. Where the experiment was repeated, an average was calculated from the individual experiments and the standard deviation was determined. The basal transcription levels for pGL2E2464 (-2464 to +36) was set arbitrarily at 100% and pGL2E520 (-520 to +36) was calculated to be $107 \pm 18\%$. Therefore both constructs had the same activity within experimental error. Both constructs had $>100x$ the activity of the pGL2E vector alone ($0.4 \pm 0.2\%$) (figure 6) while pGL2-Control had half the activity ($49 \pm 7\%$) of pGL2E2464. This result was not unexpected as fully functional promoters have been reported at lengths shorter than the 556 bp length of the shorter construct. For example, the minimal functional human Factor IX promoter is ~ 300 bp (Anson *et al.*, 1984) while the minimal human topoisomerase II α promoter is 249 bp (Isaacs *et al.*, 1996).

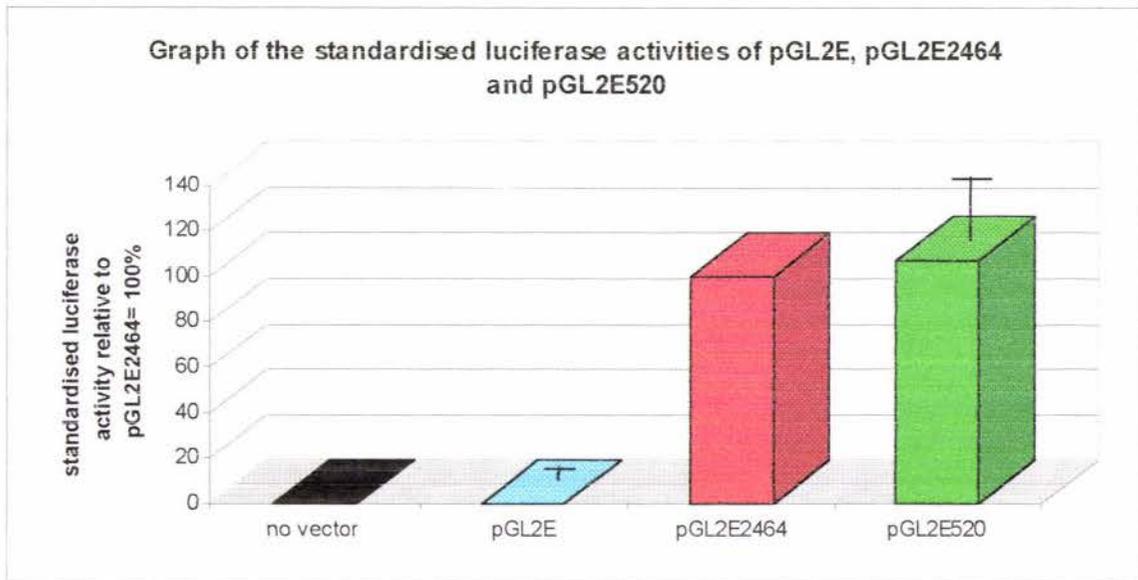


Figure 6. Bovine lactoferrin promoter activity. RL95-2 cells were transiently transfected with 4 μg of pGL2E2464 or pGL2E520 and 2 μg of pSV- β -gal. Cells transfected with 2 μg of pGL2E and 2 μg of pGL2C acted as controls. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the average of >10 separate experiments where each experiment contained triplicates of the reporter constructs. The standard deviation (S.D.) of the mean is shown. The luciferase activity of pGL2E2464 was arbitrarily set at 100% and all other values adjusted accordingly.

3.7.1 Addition of cytokines to RL95-2 cells containing the pGL2E constructs

The number of experiments that address the cytokine-responsiveness of RL95-2 cells has been limited. Okadome *et al.* (1996) incubated IFN- γ and IL-1 with RL95-2 cells for 10 days. Gene expression was not investigated in these experiments but the cytokines did not show anti-proliferative activity at the levels used. Other cell types have used cytokines in a variety of experiments investigating cytokine-induced gene activation. The cytokines were used in amounts ranging from <0.1 to 100 ng with incubation times from 1 to >24 hours (table 2). Therefore, the RL95-2 cells containing the pGL2E constructs were incubated with 0.5 - 100 ng of cytokines (after the removal of the calcium phosphate precipitate) for 18 hours. The response of the bLf promoter to IL-6, IL-1 α and IFN- γ was investigated. There was no significant difference in

promoter activity in the presence or absence of IL-6 (figure 7). Similar results were obtained for the addition of 0.5 - 100 ng IL-1 α and IFN- γ (data not shown). Table 3 shows a sample of the raw data and of the calculations involved.

| Cell type | Amount used | Incubation time | Reference |
|---------------------------------------------------------|--------------|-----------------|--------------------------------|
| IL-6 | | | |
| Primary cultured hepatocytes | 2-100 ng/ml | 24 hours | Huang <i>et al.</i> (1995) |
| " | 10 ng/ml | 6 - 72 hours | " |
| HepG2 cells | 100 ng/ml | 1 - 10 hours | Nakajima <i>et al.</i> (1993b) |
| " | 10 ng/ml | 5 hours | Lamb <i>et al.</i> (1995) |
| Hep3B cells | 7.5 ng/ml | 48 hours | Morrone <i>et al.</i> (1988) |
| IL-1 (α or β) | | | |
| murine fibroblasts | 4 ng/ml | 24 hours | Altmeyer <i>et al.</i> (1995) |
| bovine endometrial cells | 1-100 ng | 24 hours | Davidson <i>et al.</i> (1995) |
| HepG2 cells | 1 ng/ml | 12-16 hours | Rogers (1996) |
| rat small intestinal epithelial cells | 0.01-5 ng/ml | 12-24 hours | Andoh <i>et al.</i> (1996) |
| IFN-γ | | | |
| HepG2 cells | 5 ng/ml | 5 hours | Lamb <i>et al.</i> (1995) |

Table 2. Examples of the cell types, amounts of cytokines and time of incubations used in tissue culture experiments investigating cytokine-induced transcriptional effects.

| Construct | Luciferase units | β -gal. values (420 nm) (total absorbance) | Ratio | Average | Relative % |
|------------------------------|------------------|-----------------------------------------------------|-------|---------|----------------------------|
| pGL2E2464 | 3.326 | 0.347 | 9.6 | 9.5 | 100% |
| | 3.591 | 0.368 | 9.8 | | |
| | 2.864 | 0.311 | 9.2 | | |
| pGL2E2464 (+) 100 ng IL-6 | 3.883 | 0.359 | 10.8 | 9.7 | 102% (9.7/9.2 x 100) |
| | 4.140 | 0.460 | 9.0 | | |
| | 3.450 | 0.374 | 9.2 | | |

Table 3. Example of the raw data and the subsequent calculations to obtain the relative standardised luciferase values.

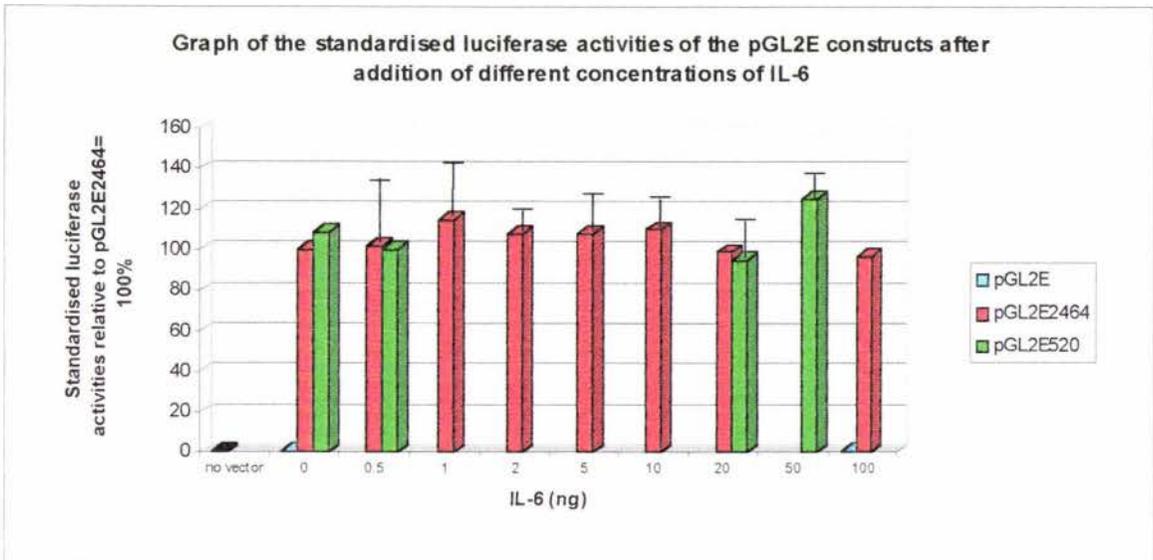


Figure 7. The effect of IL-6 on bLf promoter activity. RL95-2 cells were transfected with 4 μ g of pGL2E2464 or pGL2E520 and 2 μ g of pSV- β -gal. Cells transfected with 2 μ g of pGL2E and 2 μ g of pGL2C acted as controls. Interleukin-6 (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the average of >5 separate experiments where each experiment contained triplicates of the reporter constructs. The standard deviation (S.D.) of the mean is shown. The luciferase activity of pGL2E2464 (0 ng IL-6) was arbitrarily set at 100% and all other values adjusted accordingly.

Modifications of the transient transfection conditions

The addition of the cytokines to RL95-2 cells containing the bLf promoter constructs did not produce any changes in transcription. In order to determine if this was due to the incubation conditions or components in the medium, a number of modifications were made. These included the removal of insulin, the time of incubation with cytokine and co-transfection with an expression vector for a transcription factor which might influence the bLf promoter.

3.7.2 Change of cytokine incubation time

Various groups have reported using shorter cytokine incubation times for a variety of different cell types. Nakajima *et al.* (1993b) used a 3-5 hour incubation with IL-6 in HepG2 cells. Gerhartz *et al.* (1996) used a 7 minute incubation with HepG2 cells and a 15 minute incubation with COS-7 cells. As cytokine induction of cellular activities is a rapid process, the short length of these incubation times is reasonable. Luciferase is moderately unstable in mammalian cells as it is susceptible to proteolysis (Bronstein *et al.*, 1994). It is not known if luciferase produced during the 18 hour cytokine incubation period is significantly degraded by cellular proteolytic enzymes. This degradation could obscure the detection of transcriptional activation by cytokines. An experiment with a cytokine incubation time of 2 hours was performed to investigate this possibility.

The addition of IL-6 to cells containing pGL2E constructs, 2 hours prior to harvesting (with harvesting 18 hours after removal of the calcium phosphate precipitate) did not significantly affect promoter activity of the pGL2E constructs (figure 8). Similar results were seen for a 4 hour IL-6 incubation (results not shown). This result suggested that the luciferase protein was relatively stable 2, 4 or 18 hours after production and that proteolysis of the luciferase protein was not 'masking' cytokine-induced transcriptional activation.

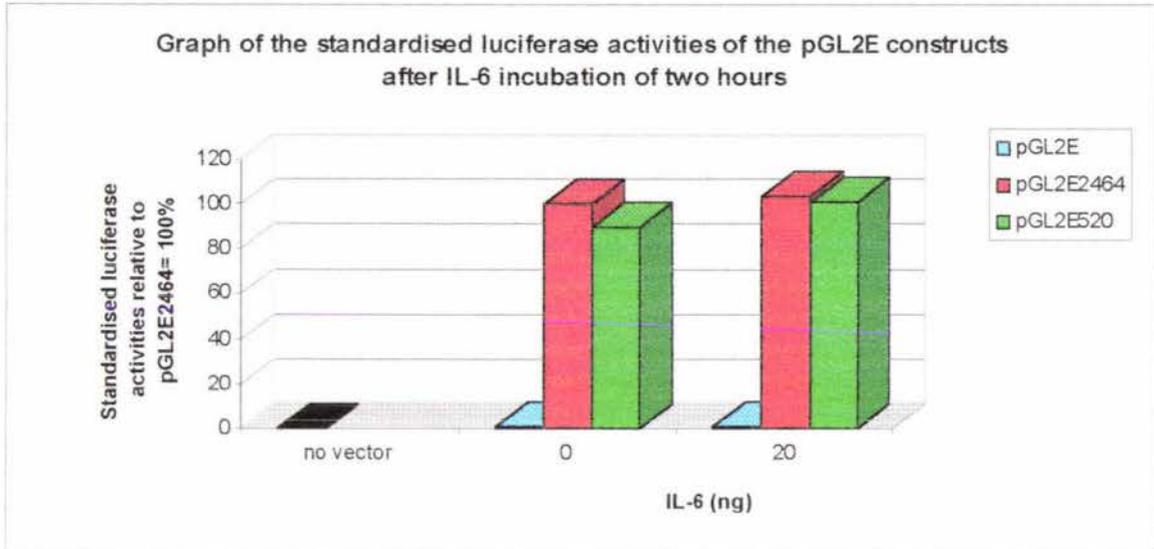


Figure 8. The effect of a 2 hour incubation with IL-6 on *bLf* promoter activity. RL95-2 cells were transfected with 4 μg of pGL2E2464 or pGL2E5202 and 2 μg of pSV- β -galactosidase. Cells transfected with 2 μg of pGL2E and 2 μg of pGL2C acted as controls. Interleukin-6 (2 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the results of 1 experiment where the experiment contained triplicates of the reporter construct, therefore error bars have not been included. The luciferase activity of pGL2E2464 (0 ng IL-6) was arbitrarily set at 100% and all other values adjusted accordingly.

3.7.3 Removal of insulin from the tissue culture media

Insulin is a peptide hormone that is required for the growth of RL95-2 human endometrial carcinoma cells (Way *et al.*, 1983). Insulin has the potential to affect many steps in the process of gene expression from transcription through to post-translational protein modification. The primary targets for insulin are gene transcription, translation and the stability of mRNA. The exact mechanisms for increasing or decreasing mRNA stability are as not yet known. More information is available concerning the mechanism of insulin regulation of protein synthesis, which includes the regulation of ribosomal or initiation factors (reviewed by O'Brien and Granner, 1996 ; Myers and White, 1996).

There are two possible mechanisms for the transcriptional effects of insulin. The first mechanism involves plasma membrane-initiated signal transduction cascades which result in the phosphorylation or dephosphorylation of nuclear factors affecting nuclear translocation, DNA binding and/or transactivation. The second mechanism involves the cellular internalization of insulin by intracellular receptors which transport the insulin to the nucleus (reviewed by O'Brien and Granner, 1996).

Much research has been directed at the potential signal transduction cascades that mediate insulin-induced transcriptional effects. Insulin binds to three related receptors - the insulin receptor (with the highest affinity), the IGF-I (Insulin Growth Factor-I) receptor and the IGF-II (Insulin Growth Factor-II) receptor (with lower affinity). The insulin receptor is thought to be primarily involved in metabolic signaling while the IGF receptor is thought to be primarily involved in mitogenic signaling. The receptors belong to the receptor protein tyrosine kinase superfamily, where extracellular binding of the ligand induces a conformational change that activates the intracellular kinase domain. Receptor dimerization and autophosphorylation of the tyrosine kinase domain occurs producing signaling molecule recognition sites. Tyrosine phosphorylation of the receptor-bound signaling molecules begins a cascade of reactions with end effects which include altering specific enzymatic activities and gene activation (reviewed by De Meyts *et al.*, 1995 ; Myers and White, 1996 ; White and Kahn, 1994).

Insulin binds to the extracellular domain of an insulin receptor. This binding activates the intracellular tyrosine kinase domain which can then recognise and phosphorylate specific substrates. The IRS-1 (Insulin Receptor Substrate-1) is a major physiological substrate for the insulin receptor tyrosine kinase in the mitogen-activated protein kinase pathway. While this pathway seems to be more complicated, basically IRS-1 binds to the adaptor protein GRB-2 (Growth factor Receptor Bound-2) which then binds mSOS - a p21^{ras}-guanine nucleotide exchange factor. mSOS (mouse Son Of Sevenless) causes the production of active p21^{ras}-GTP (a small GTP binding protein), which then activates Raf-1 (Protein kinase), which consequently activates MEK (Map Kinase Kinase). MEK activates MAPK (Mitogen Activated Protein Kinase) isoforms, from which direct or indirect activation of transcription factors can occur. Potential targets for this cascade may involve the serum response element and the AP-1 motif (through regulation of *c-jun* or *c-fos* homo- or heterodimers). Insulin can also act through other IRS-1-associated proteins like phosphatidylinositol-3-kinase (PI-3-kinase). PI-3-kinase produces phosphatidyl inositol 3,4,5 trisphosphate - a proposed 'secondary messenger' with potential targets including protein kinase B and certain Protein Kinase-C isoforms (reviewed in Sutherland *et al.*, 1996 ; O'Brien and Granner, 1996 ; Myers and White, 1996).

There is also evidence that insulin can regulate the JAK (*Janus* Kinase)-Stat (Signal Transducers and Activators of Transcription) pathway- a pathway involved in certain cytokine-induced effects. Ceresa and Pessin (1996) found that with Chinese hamster ovary cells, the addition of insulin (1-3 nM) activated the phosphorylation of serine residues on Stat3 but not the Stat1 α protein. In hepatoma cells, O'Riordain *et al.* (1995) found that insulin accentuated the IL-6 activation or inhibition of various acute phase response genes. Campos and Baumann (1992) found that insulin reduced the IL-1 and IL-6-type cytokine-induced activation of various acute phase response genes. Campos *et al.* (1996) demonstrated that the inhibition observed by insulin correlated with a reduction in Stat3 and IL-6R α transcription and protein levels. The exact connection between these results and insulin signaling pathways has yet to be elucidated.

Genes which are regulated by insulin include those encoding the expected metabolic enzymes, as well as oncogenes and transcription factors. For example, in different tissues, insulin either stimulates, inhibits, or has no effect on NF-IL6 gene expression. *Cis*-acting elements that mediate the effects of insulin (insulin response elements (IREs)) have been identified although a consensus has yet to be determined (reviewed by O'Brien and Granner, 1996). The exact mechanisms of insulin/IRE action seem to be diverse and have yet to be elucidated for most genes.

Tissues not primarily involved in the metabolic effects of insulin have also shown insulin-regulated gene expression (O'Brien and Granner, 1996). Therefore it was possible that endometrial cells could also show this type of regulation. As RL95-2 cells require insulin in the media, this could indicate that insulin-induced gene regulation is important for these cells, or that, like some other tissue culture cells, RL95-2 cells require insulin only for cell proliferation. The effects of insulin on the basal reporter gene expression could involve the direct alteration of the promoter activity or through alteration of the cellular levels or activities of endogenous transcription factors. The effects of insulin on potential cytokine induction in RL95-2 cells is unknown, but as insulin inhibits IL-6 action in hepatoma cells, the potential effect of insulin in the experiments described in this thesis cannot be discounted.

Insulin was removed from the media two hours prior to transfection. RL95-2 cells were transfected with pGL2E2464 and pGL2E520 as described previously. Upon removal of insulin from the media, the RL95-2 cells survived although they appeared to be physically smaller. This result could indicate a role for insulin in cell growth or proliferation. The basal transcription rates of the pGL2E constructs appeared unchanged (figure 9). Addition of IL-6 did not change the transcriptional activity of the bLf promoter constructs in the absence of insulin (figure 10).

These results show that insulin did not affect the basal levels of transcription of the pGL2E constructs, which suggests that there are no IREs in the bLf promoter. As a consensus IRE has not yet been identified, this result could not be checked by sequence analysis. Another possibility is that while RL95-2 cells are responsive to insulin (seen by the morphological changes in the absence of insulin) the pathways e.g

MAPK and JAK/Stat potentially involved in insulin-induced transcriptional effects, are nonfunctional in these cells. The removal of insulin did not change IL-6-induced effects, so the insulin did not inhibit a possible cytokine-induced transcriptional activation.

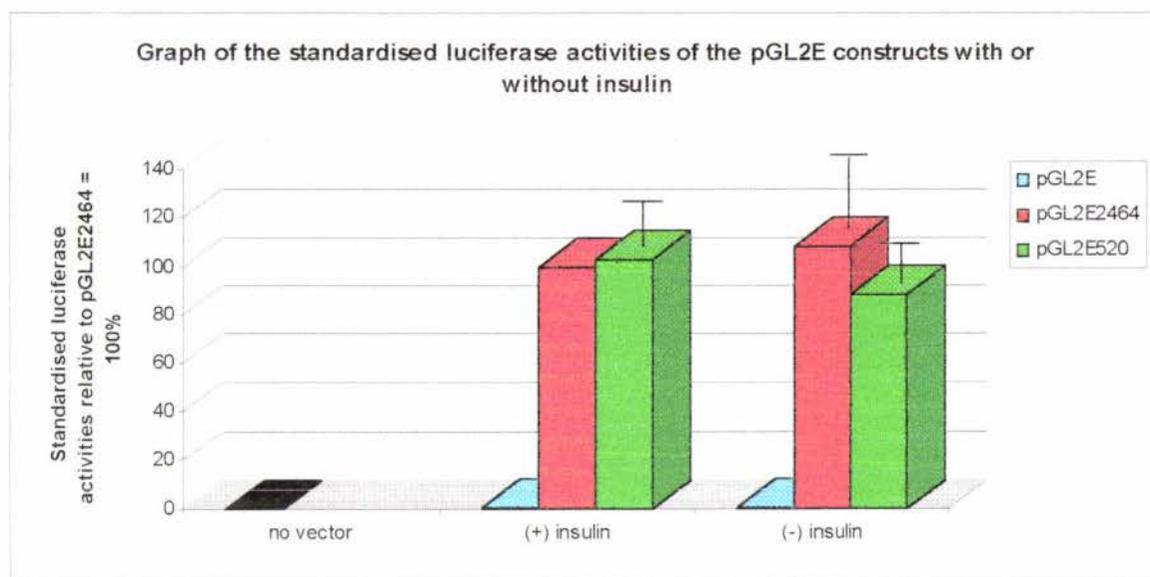
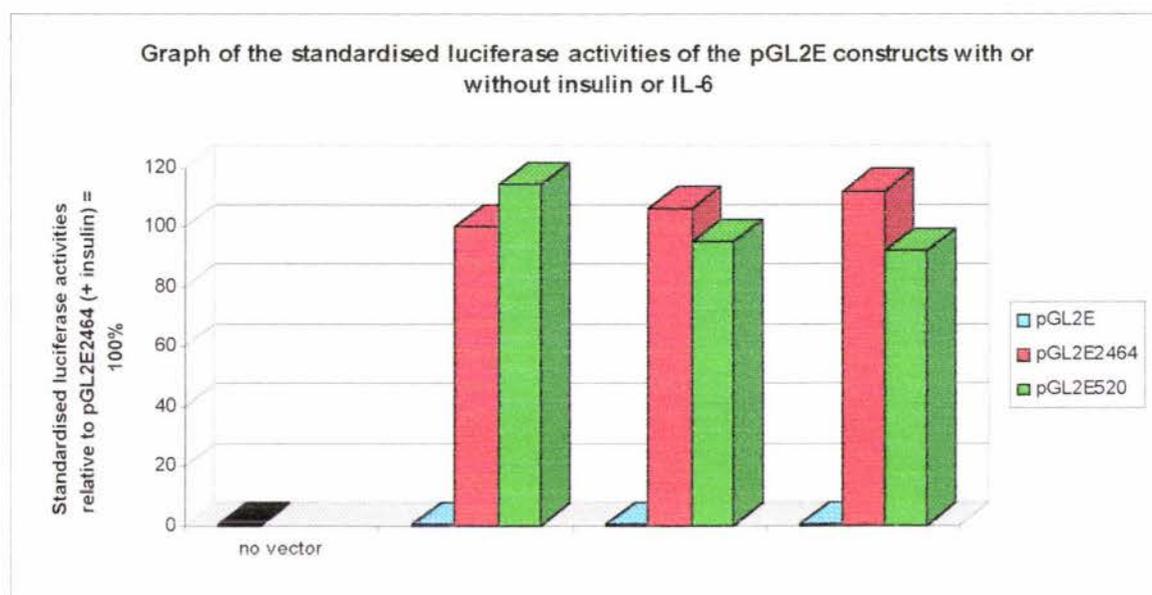


Figure 9 The effect of insulin on the transcriptional activity of the *bLf* promoter. RL95-2 cells grown in media with or without insulin (5 $\mu\text{g/ml}$) were transfected with 4 μg of pGL2E2464 or pGL2E520 and 2 μg of pSV- β -gal. Cells transfected with 2 μg of pGL2E and 2 μg of pGL2C acted as controls. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the results of 2 separate experiments where each contained triplicate transfected using the reporter constructs. The standard deviation (S.D.) of the mean is shown. The luciferase activity of pGL2E2464 (+ insulin) was arbitrarily set at 100% and all other values adjusted accordingly.



| | | | |
|----------------------------|---|---|---|
| Insulin) (5 μ g/ml) | + | + | - |
| IL-6 (20 ng) | - | + | + |

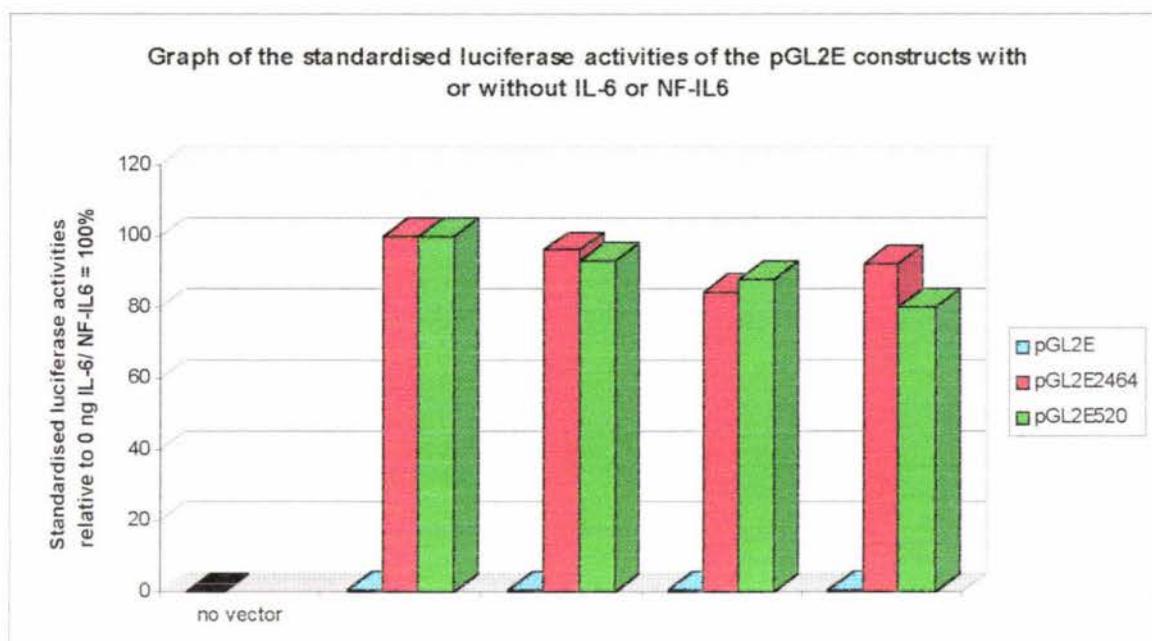
Figure 10. The effect of insulin and IL-6 on the transcriptional activity of the *bLf* promoter. RI.95-2 cells, grown in media with or without insulin (5 μ g/ml) were transfected with 4 μ g of pGL2E2464 or pGL2E520 and 2 μ g of pSV- β -gal. Cells transfected with 2 μ g of pGL2E and 2 μ g of pGL2C acted as controls. Interleukin-6 (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the results of 1 experiment where the experiment contained triplicates of the reporter constructs, therefore error bars have not been included. The luciferase activity of pGL2E2464 (0 ng IL-6, + insulin) was arbitrarily set at 100% and all other values adjusted accordingly.

3.7.4 Addition of NF-IL6 expression vector

As previously stated, RL95-2 cells are different from normal endometrial cells in several morphological parameters (Sundareshan and Hendrix, 1992). The consequence of these differences on the amount, availabilities, and activities of specific protein factors necessary for basal transcription or cytokine-induced signal transduction is not known. NF-IL6 (C/EBP- β) is a leucine zipper-containing activating transcription factor which has high homology to C/EBP (a liver nuclear factor) (Akira *et al.*, 1990 ; Poli *et al.*, 1990). NF-IL6 can homodimerize or heterodimerize with other leucine zipper-containing proteins (Poli *et al.*, 1990). For example Kinoshita *et al.* (1992) have identified a protein (NF-IL6 β) which has high homology to NF-IL6. NF-IL6 β is inducible by IL-6, IL-1 and glucocorticoids, is a stronger transactivator than NF-IL6 and can dimerize with NF-IL6. This heterodimer can bind to the same binding site as the NF-IL6 homodimer. NF-IL6 can also physically interact with other transcription factors, such as NF- κ B through its leucine zipper domain and the Rel domain of NF- κ B (Stein *et al.*, 1993 ; Matsusaka *et al.*, 1993). This interaction causes a synergistic enhancement of transcription (Matsusaka *et al.*, 1993).

NF-IL6 is induced by IL-6, IL-1, tumour necrosis factor and lipopolysaccharides (reviewed in Bankers-Fullbright *et al.*, 1996). Poli and Cortese (1989) and Akira *et al.* (1990) found that NF-IL6 bound to the promoters of various acute phase and cytokine promoters - indicating a potential role in inflammation. Activation of the lactoferrin promoter by NF-IL6 could indicate a role for lactoferrin in inflammation. NF-IL6 has been implicated in IL-6 and IL-1 α signal transduction (reviewed in Bankers-Fullbright *et al.*, 1996) and putative NF-IL6 DNA binding sites have been identified in the bLf promoter (Bain, 1995). Limiting levels of NF-IL6 in RL95-2 cells could potentially reduce or inhibit any transcriptional regulation mediated by IL-1 and IL-6. For this reason, RL95-2 cells were co-transfected with the NF-IL6 expression vector (MSV-C/EBP β) (Friedman *et al.*, 1989) and various bovine lactoferrin promoter reporter constructs. The transfected cells were cultured in the presence or absence of IL-6.

Addition of NF-IL6 did not affect basal transcription levels of the reporter constructs. The addition of IL-6 in the presence of NF-IL6 also had no effect (figure 11). The negative result seen could have been due to the MSV-C/EBP β expression vector not being correctly expressed in RL95-2 cells, but experiments presented in Chapter Five will show that this vector can produce sufficient NF-IL6 protein to bind to a consensus DNA binding site.



| | | | | |
|--------------|---|---|---|---|
| IL-6 (20ng) | - | + | - | + |
| NF-IL6 (1µg) | - | - | + | + |

Figure 11. The effect of NF-IL6 and IL-6 on the *b1.f* promoter. RL95-2 cells were transiently transfected with 4 µg of pGL2E2464 or pGL2E520 and 2 µg of pSV-β-gal. 1 µg MSV-C/EBPβ was co-transfected with the reporter constructs as indicated. Cells transfected with 2 µg of pGL2E and 2 µg of pGL2C acted as controls. Interleukin-6 (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β-galactosidase absorbance values at 420 nm. The values presented are the results of 2 individual experiments where each experiment contained triplicates of one of the reporter constructs, therefore error bars have not been included. The pGL2E2464 and pGL2E520 data was analysed separately with the (0 ng IL-6 and NF-IL6) luciferase activities arbitrarily set at 100% and all other values adjusted accordingly.

3.8 Removal Of Phenol Red From The Tissue Culture Media

Phenol red has been reported to act as an estrogenic analogue. Berthosis *et al.* (1986) found that phenol red (phenol sulfonphthalein) structurally resembles some non-steroidal estrogens and that it had significant estrogenic activity at the concentrations present in tissue culture media. Welshon *et al.* (1988) investigated the effects of phenol red on MCF-7 and other cells. These authors found there was an indication of a sparingly water-soluble contaminant of the phenol red which caused the predominant estrogenic activity. Other researchers have also found that phenol red and the estrogenic activity could be separated indicating estrogenic contaminants. Bindall *et al.* (1988) found that lipophilic impurities in the phenol red preparation were responsible for estrogen receptor binding and the growth promoting activity.

The phenol red preparation was also found to contain other contaminants which could cause significant cellular effects. Grady *et al.* (1991) found that a contaminant of the phenol red preparation had pH-dependent cytotoxic effects on MCF-7 cells and that this activity was distinct from the contaminants that caused estrogenic activity. This could have substantial effects on experiments using susceptible cells, as even a small or transient rise in pH could cause cell death.

Although there is evidence that phenol red itself may not be causing the predominant estrogenic effect, there is evidence that phenol red may have other cellular effects. Gruffat *et al.* (1991) found that phenol red acted as a substrate for thyroperoxidase and so inhibited thyroglobulin iodination, and so must be removed for specific studies of thyroid cell function. Driscoll *et al.* (1982) found that phenol red was partially metabolised by hepatocytes.

The estrogen receptor belongs to the steroid and thyroid hormone receptor superfamily (Evans, 1988). When the ligand binds to the intracellular receptor, activation of the receptor occurs. The receptor can then translocate into the nucleus and bind to estrogen responsive elements (EREs) in the promoter of specific genes. This binding may inhibit or assist the binding of specific transcription factors or cause the remodelling of the chromatin DNA. Inhibition of specific transcription factors by

protein-protein interactions has also been indicated. Therefore the estrogenic analogues may be activating or inhibiting transcription of specific genes, potentially affecting basal or cytokine-induced transcription. The human and mouse lactoferrin promoters both contain an estrogen response module (Lui and Teng, 1992 ; Teng *et al.*, 1992), although an ERE has yet to be demonstrated in the bovine lactoferrin promoter.

There is evidence that RL95-2 cells may or may not be sensitive to estrogen. Way *et al.* (1983) found that RL95-2 cells have low levels of cytoplasmic estrogen receptors. But Sundareshan and Hendrix (1992) compared early and late passage RL95-2 cells for any differences. They found that the early passage cells (<30 times subcultured) had the characteristics of the primary tumour while the later passage cells (>200 times subcultured) had a faster rate of growth (shorter doubling time), were less serum dependent and had altered morphology. The early passage cells were previously found to be estrogen-positive (Way *et al.*, 1983) while the later passage cells estrogen-negative - although this was noted by a personal communication and the data was not shown. Lui and Teng (1991) and Lui and Teng (1992) found that RL95-2 cells had little estrogen responsiveness without the addition of exogenous estrogen receptors.

The DMEM-F12 media used to maintain the RL95-2 cells contained phenol red as an indicator of media pH changes. Therefore due to the potential activating, cytotoxic, metabolic or other as-yet-unknown effects of the phenol red preparation, the phenol red was removed from the media. Therefore a base media deficient in phenol red was used to maintain the RL95-2 cells in an attempt to minimise any effect phenol red or contaminants may have.

The fetal calf serum used in the complete media may also contain hormones or other activating molecules which may affect cell growth or promoter activity. For example components in fetal calf serum have been found to enhance gonococcal invasion into HeLa cells (Gomez-Duarte *et al.*, 1997), diminish the transfer of phospholipids to hepatocytes (Hoeskstra and Scherphof, 1979) and induce alkaline phosphatase activity (Watanbe *et al.*, 1994). Of potential consequence to this work using the RL95-2 endometrial carcinoma cell line is research by Fleming (1995), where a protein factor in

fetal calf serum was found to stimulate epithelial cells, of the Ishikawa endometrial carcinoma cell line, to differentiate and form multicellular structures. As fetal calf serum could potentially affect the RL95-2 cells, attempts to maintain the cells on serum which had been charcoal-stripped (to remove potentially active hormones and other proteins) were performed. These experiments were not successful indicating necessary growth factors were absent from the stripped media. Therefore normal fetal calf serum was used. This result is contrary to research by Lui and Teng (1992), Curtis *et al.* (1997), Lui *et al.* (1993) and Yang *et al.* (1996) where RL95-2 cells were grown on charcoal-stripped bovine serum. Whether this disparity is due to differences in the source or method of charcoal stripping of the fetal calf serum or to some other effect has yet to be determined.

3.8.1 Basal Transcription Rates of the pGL2E bLf constructs

The RL95-2 cells grown on phenol red-free media were similar in appearance to cells grown on media containing phenol red. The removal of phenol red did not alter the luciferase/ β -galactosidase ratios or transcriptional activities previously obtained for the pGL2E constructs in media containing phenol red (table 4) (figure 12). Therefore the phenol red or contaminants did not appear to affect the basal transcription rates of the pGL2E bLf promoter constructs in RL95-2 cells.

| | Phenol red-containing media | Phenol red-free media |
|-----------|-----------------------------|-----------------------|
| pGL2E | 0.4 \pm 0.21% | 0.4 \pm 0.14% |
| pGL2E2464 | 100% | 100% |
| pGL2E520 | 107 \pm 18% | 123 \pm 19% |

Table 4. Comparison of the transcriptional levels of the pGL2E constructs in media with or without phenol red. No significant difference is seen between phenol red or phenol red-free media. The large error seen in the pGL2E520 phenol red-free media result is due to the small sample number.

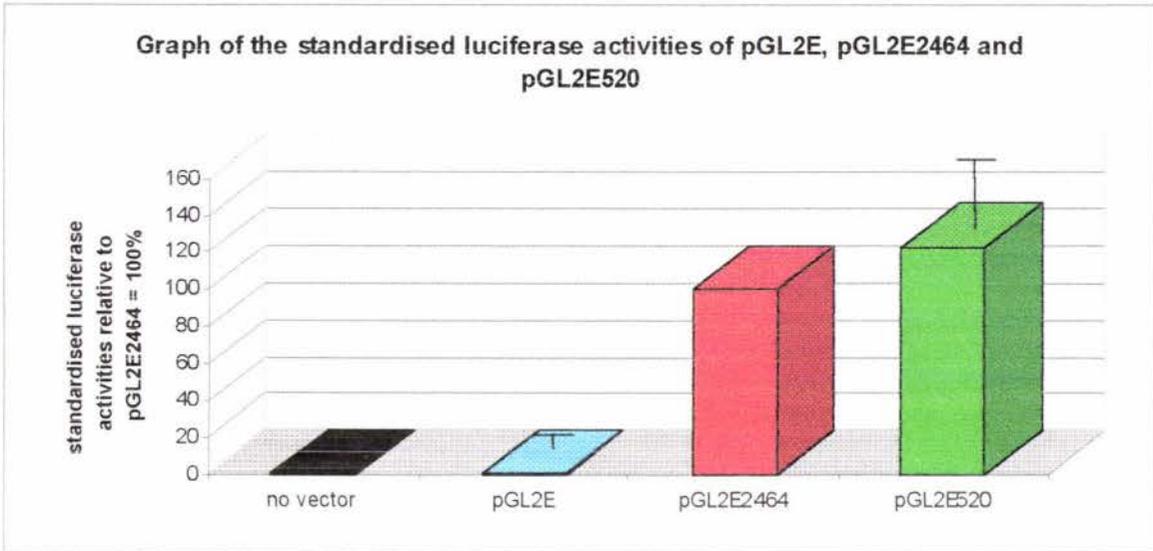


Figure 12. The *bLf* promoter activity in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μg of pGL2E2464, pGL2E520 and 2 μg of pSV- β -gal. Cells transfected with 2 μg of pGL2E and 2 μg of pGL2C acted as controls. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the average of >2 separate experiments where each experiment contained triplicates of the reporter constructs. The standard deviation (S.D.) of the mean is shown. The luciferase activity of pGL2E2464 was arbitrarily set at 100% and all other values adjusted accordingly.

3.8.2 Addition of cytokines to cells containing pGL2E *bLf* constructs

Cytokines have been used in a variety of experiments with other cell types, in amounts ranging from <0.1 to 100 ng with incubation times from 1 to >24 hours (table 2). Therefore as with the experiments with phenol red-containing media, the RL95-2 cells containing the pGL2E constructs were incubated with 0.5 - 100 ng of cytokines for 18 hours. The addition of IL-6 and IFN- γ to RL95-2 cells containing the pGL2E constructs did not cause changes in the transcriptional activity (figure 13). These results were the same to what was seen in experiments with phenol red-containing

media. This indicated that the phenol red was not 'masking' any potential activation or inhibitory transcriptional effects of these cytokines.

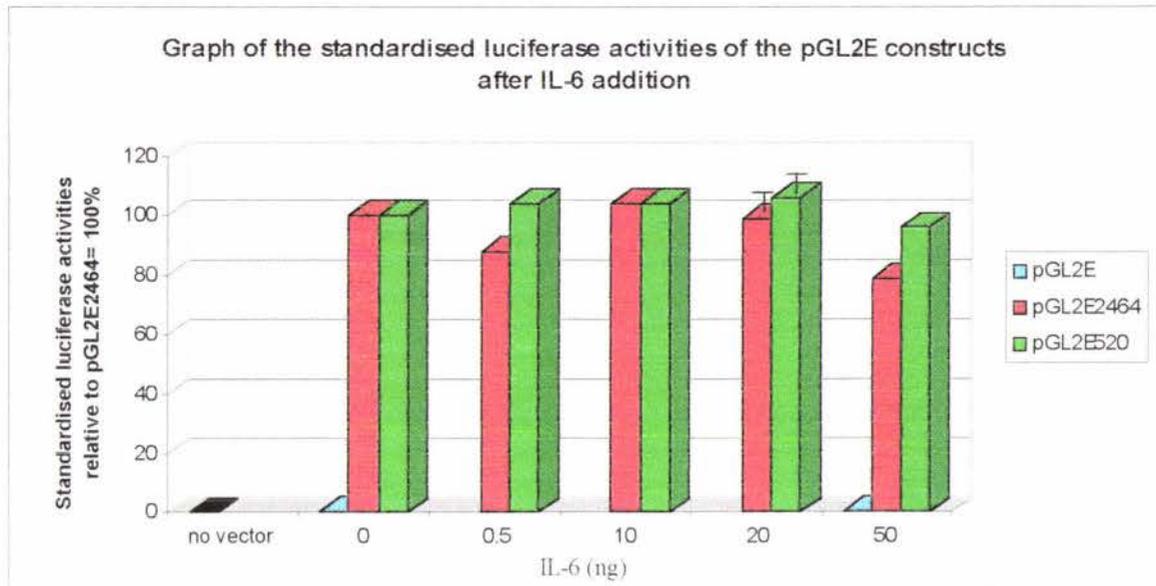


Figure 13. The effect of IL-6 on bLf promoter activity in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μ g of pGL2E2464, pGL2E520 and 2 μ g of pSV- β -gal. Cells transfected with 2 μ g of pGL2E and 2 μ g of pGL2C acted as controls. Interleukin-6 (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the average of >2 individual experiments where each experiment contained triplicates of the reporter constructs, therefore certain error bars have not been included. The standard deviation (S.D.) of the mean is shown. The luciferase activity of pGL2E2464 (0 ng IL-6) was arbitrarily set at 100% and all other values adjusted accordingly.

Modifications of transient transfection in phenol red-free media

As the addition of IL-6, to RL95-2 cells grown in phenol red-free media and containing the pGL2E bLf constructs, did not elicit transcriptional changes, modifications in the transfection conditions were used. As with the experiments in phenol red-containing media, manipulation of the cytokine incubation time and the presence or absence of insulin in the media was performed.

3.8.3 Change of cytokine incubation time

Researchers have used short incubation times to obtain cytokine-induced modulation of transcription (Nakajima *et al.*, 1993b ; Gerhartz *et al.*, 1996). Therefore, a 2 hour, instead of an 18 hour incubation was performed. Addition of cytokines 2 hours prior to harvesting, did not cause transcriptional increases of the pGL2E constructs in RL95-2 cells grown on phenol red-free media (figure 14). These results show that changing the length of cytokine incubation does not cause a cytokine-induced effect - with or without phenol red.

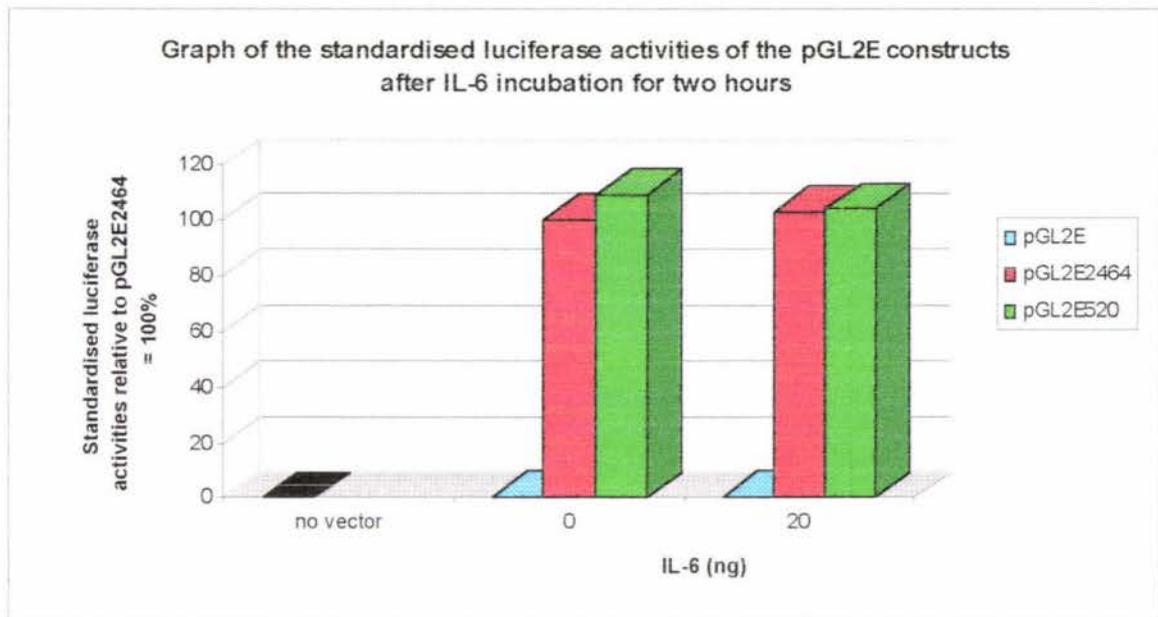
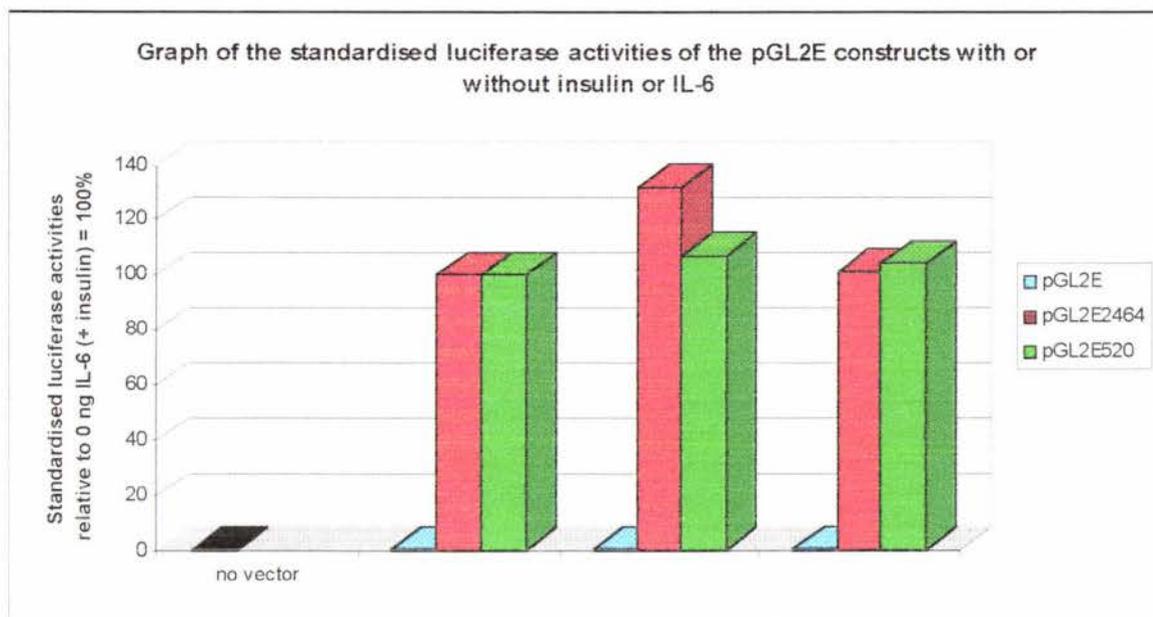


Figure 14. The effect of a two hour incubation with IL-6 on bLf promoter activity in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μg of pGL2E2464, pGL2E520 and 2 μg of pSV- β -gal. Cells transfected with 2 μg of pGL2E and 2 μg of pGL2C acted as controls. Interleukin-6 (2 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the results of 1 experiment where the experiment contained triplicates of the reporter constructs, therefore error bars have not been included. The luciferase activity of pGL2E2464 (0 ng IL-6) was arbitrarily set at 100% and all other values adjusted accordingly.

3.8.4 Removal of insulin from the tissue culture media

As mentioned previously (section 3.7.3), insulin could affect gene transcription by interacting directly with the bLf promoter or by modulating the activity or levels of other transcription factors. The removal of insulin from the media did not affect the basal transcriptional rates of the pGL2E constructs in RL95-2 cells grown in phenol red-free media (data not shown). Addition of IL-6 after removal of the insulin did not cause changes in the transcriptional activity in transfections using the pGL2E

constructs (figure 15). This result is the same to what is seen after the removal of insulin in media containing phenol red. Therefore the phenol red does not 'mask' an insulin-induced modulation of the bLf promoter.



| | | | |
|-------------------|---|---|---|
| Insulin (5 µg/ml) | + | + | - |
| IL-6 (20 ng) | - | + | + |

Figure 15. The effect of insulin and IL-6 on the transcriptional activity of the bLf promoter in phenol red-free media. RL95-2 cells grown in phenol red-free media with or without insulin (5 µg/ml) were transfected with 4 µg of pGL2E2464 or pGL2E520 and 2 µg of pSV-β-gal. Cells transfected with 2 µg of pGL2E and 2 µg of pGL2C acted as controls. Interleukin-6 (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β-galactosidase absorbance values at 420 nm. The values presented are the results of 1 experiment where the experiment contained triplicates of the reporter constructs, therefore error bars have not been included. pGL2E2464 and pGL2E520 data was analysed separately with 0 ng IL-6 (+ insulin) luciferase activities arbitrarily set at 100% and all other values adjusted accordingly.

3.9 Chapter Summary

The basal transcription rates for pGL2E520 and pGL2E2464 were similar under all conditions tested indicating that the minimal bLf promoter may be less than 556 bp. The addition of cytokines to RL95-2 cells containing the pGL2E constructs did not elicit any changes in transcription from the bLf promoter. Reduction in cytokine incubation time, removal of phenol red (containing a potential estrogenic analogue), removal of insulin (a potential transcription modifier), and addition of NF-IL6 (a transcription factor involved in specific cytokine signal transduction) did not elicit a cytokine-induced transcriptional effect. Another factor that may be masking a cytokine-induced transcriptional effect is the SV40 enhancer element present in the pGL2E vector. To investigate this possibility, the bLf promoter regions were subcloned into the pGL3-Basic vector and the transfection experiments were repeated.

Chapter Four - Functional Assays of the bovine lactoferrin promoter in pGL3-Basic.

4.1 Introduction

The pGL2E reporter construct (containing the SV40 enhancer element) was initially used to investigate the bLf promoter. As the SV40 enhancer element may also contribute to the transcription of the reporter constructs, the luciferase levels produced will depend in part on the promoter and in part on the enhancer element. The effect of this enhancer on masking the true basal transcription levels of the constructs, or any cytokine-induced transcriptional changes is not known. This 'masking' could be due to the inability of the transcriptional system to increase an already maximal transcription rate due to the presence of the SV40 enhancer. All of the functional assays using the bLf promoter in pGL2E gave essentially identical results, regardless of the promoter length or addition of cytokines. Therefore these experiments were repeated using an alternate vector.

The pGL3-Basic (pGL3B) vector does not contain an SV40 enhancer element (Appendix 1) and so the “expression of luciferase activity.... depends in insertion and proper orientation of a functional promoter upstream of *luc+*” (Promega Technical Manual). To test the theory of an enhancer-induced 'masking' effect, the -2464 to +36 and -520 to +36 constructs were subcloned into pGL3B vector as described in the Methods section 2.2.1.

The pGL3B vector has been modified (when compared to the pGL2E series vectors) to increase luciferase expression and improve the *in vivo* vector stability. The changes to the Firefly (*Photinus pyralis*) luciferase cDNA include removal of the signal for peroxisome targeting, the improvement of codon usage for mammalian cells and the changing of DNA sequences to remove transcription factor consensus binding sequences and prevent spurious transcription. Changes to the vector sequence included

the insertion of the Kozak consensus sequence (Kozak, 1986) to increase the efficiency of translation initiation and the replacement of the SV40 early poly(A) signal with the late signal, to increase the efficiency of transcription termination and polyadenylation of the luciferase transcript (Promega Technical Bulletin).

4.2 Optimisation of the transfection of pGL3B constructs in RL95-2 cells

Optimisation of the transfection of pGL3B constructs in RL95-2 cells was performed in the same way as for the pGL2E constructs. The optimal conditions for the pGL3B constructs in RL95-2 cells were the same as for the pGL2E constructs (figure 4) i.e. a time of six hours for calcium phosphate precipitate incubation followed by an 18 hour incubation after the precipitate removal. Table 5 shows the ratio of luciferase/ β -galactosidase levels in lysates from transfected cell with incubation times of 18, 24 and 30 hours after the removal of the calcium phosphate precipitate.

| Time of incubation | Construct | Luciferase/ β -gal values |
|--------------------|-----------|---------------------------------|
| 18 hours | pGL3B2464 | 1.27 |
| | pGL3B520 | 3.12 |
| 24 hours | pGL3B2464 | 0.96 |
| | pGL3B520 | 2.66 |
| 30 hours | pGL3B2464 | 0.93 |
| | pGL3B520 | 2.36 |

Table 5. The luciferase/ β -galactosidase values produced by incubation times after calcium phosphate precipitate removal of 18, 24, and 36 hours in RL95-2 cells for the optimization experiment.

4.3 Transcriptional rates of the pGL3B constructs

In contrast to the constructs in pGL2E, pGL3B2464 (2.5 kb) and pGL3B520 (556 bp) did not have the same transcription rates. When compared to pGL3B520 which was arbitrarily set at 100%, pGL3B2464 had $31.1 \pm 7.5\%$ the activity (figure 16). The fact that the pGL3B2464 construct had $\approx 3x$ less activity than pGL3B520 suggested the presence of a repressive element in the region between -2464 and -520 of the bLf promoter. A potential silencer element has been identified in the 5' end of the hLf promoter between the -800 to -916 region when Lf promoter was expressed in neutrophils (Khann-Gupta *et al.*, 1997). Further investigation is needed to more accurately define the position of the repressive region and to determine if the mechanism in endometrial cells is similar to the regulation of lactoferrin in neutrophils.

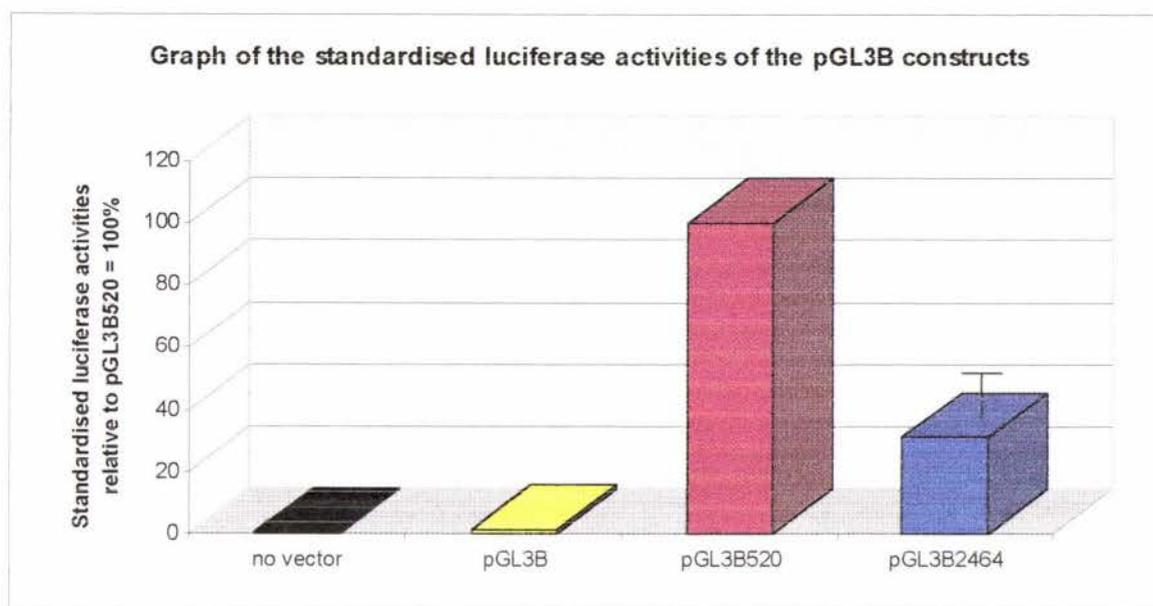


Figure 16. Basal transcription of the *bLf* promoter using the pGL3-Basic vector. RL95-2 cells were transiently transfected with 4 μg of pGL3B2464 or pGL3B520 and 2 μg of pSV- β -gal. Cells transfected with 2 μg pGL3B and 2 μg of pGL2C acted as controls. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the average of >7 separate experiments where each experiment contained triplicates of the reporter constructs. The standard deviation (S.D.) of the mean is shown. The luciferase activity of pGL3B520 was arbitrarily set at 100% and all other values adjusted accordingly.

For comparison, the levels of reporter gene activity of the pGL3B and pGL2E constructs is shown in figure 17 and table 6. Because of the vector differences, the results of experiments using pGL2E and pGL3B constructs cannot be meaningfully compared. However these data suggest that the SV40 enhancer element present in pGL2E 'masks' the differences between the two constructs (pGL2E2464 and pGL2E520) by elevating the transcription levels of both the constructs to a maximal level which results in both constructs having similar activities.

| Construct | Relative. luciferase activities | % increase compared to the 2464 construct vector |
|-----------|---------------------------------|--------------------------------------------------|
| pGL2E | 0.4 ±0.2% | - |
| pGL2E2464 | 100% | 0% |
| pGL2E520 | 107 ±18% | 7% |
| pGL3B | 0.59 ±0.45% | - |
| pGL3B2464 | 9.6 ±2.5% | 0% |
| pGL3B520 | 31.9 ±11.1% | 332% |

Table 6. Basal transcriptional activities of the pGL2E and pGL3B constructs in RL95-2 cells expressed relative to pGL2E2464 = 100%.

Both the pGL2E and pGL3B vectors had similar basal transcription rates. This was somewhat unexpected as the enhancer element in pGL2E was expected to increase the transcription when compared to pGL3B. However the pGL3B vector (due to its altered luciferase and more stable vector structure) may produce luciferase which is more stable, which may explain the similar basal levels seen. In both cases, transcriptional activity due to vector alone is extremely low.

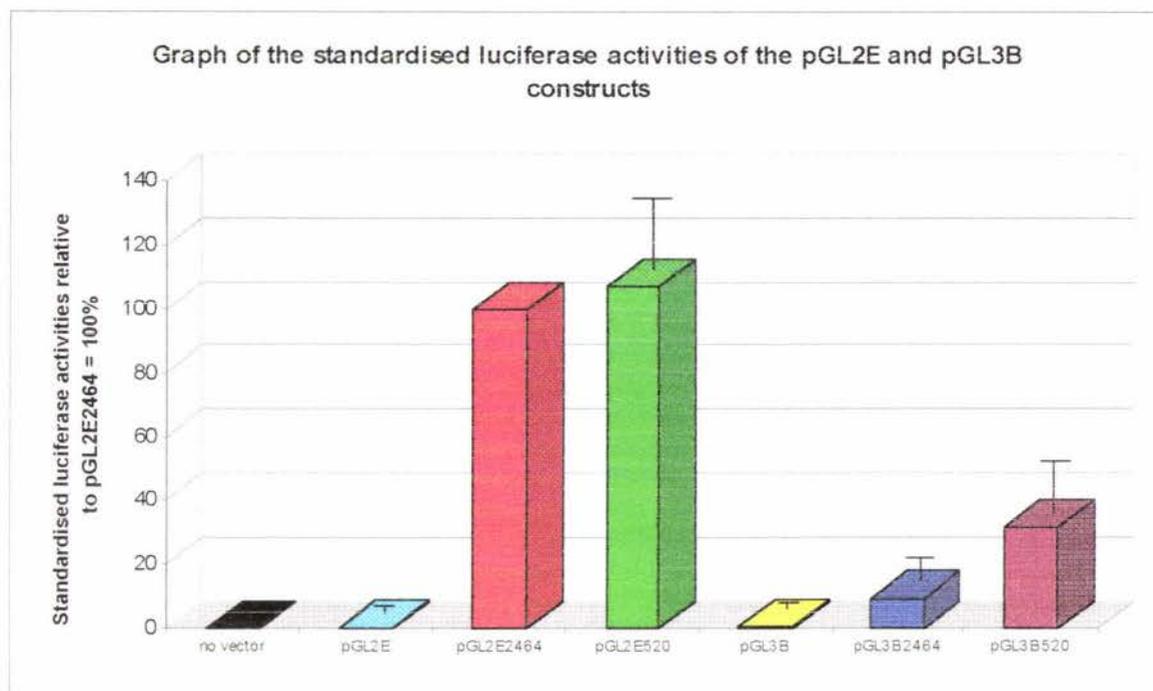


Figure 17. Comparison of transcriptional activity of the bLf promoter in pGL2E and pGL3B vectors. RL95-2 cells were transfected with 4 μg of pGL2E2464, pGL2E520, pGL3B2464 or pGL3B520 and 2 μg of pSV- β -gal. Cells transfected with 2 μg of pGL2E, 2 μg pGL3B and 2 μg of pGL2C acted as controls. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the average of >7 separate experiments where each experiment contained triplicates of the reporter constructs. The standard deviation (S.D.) of the mean is shown. The luciferase activity of pGL2E2464 was arbitrarily set at 100% and all other values adjusted accordingly.

4.3.1 Addition of cytokines

The cytokine responsiveness of RL95-2 cells transfected with pGL3B bLf constructs was tested over a range of IL-6, IL-1 and IFN- γ from 0.5 - 100 ng. IL-6, IL-1 α and IFN- γ were added as described for the pGL2E constructs (section 3.7.1). The addition

of IL-6 (figure 18), IL-1 α or IFN- γ (data not shown) for 18 hours after transfection did not cause any significant changes in promoter activity. Therefore the SV40 enhancer element in the pGL2E vector was not masking a cytokine-induced transcriptional activation.

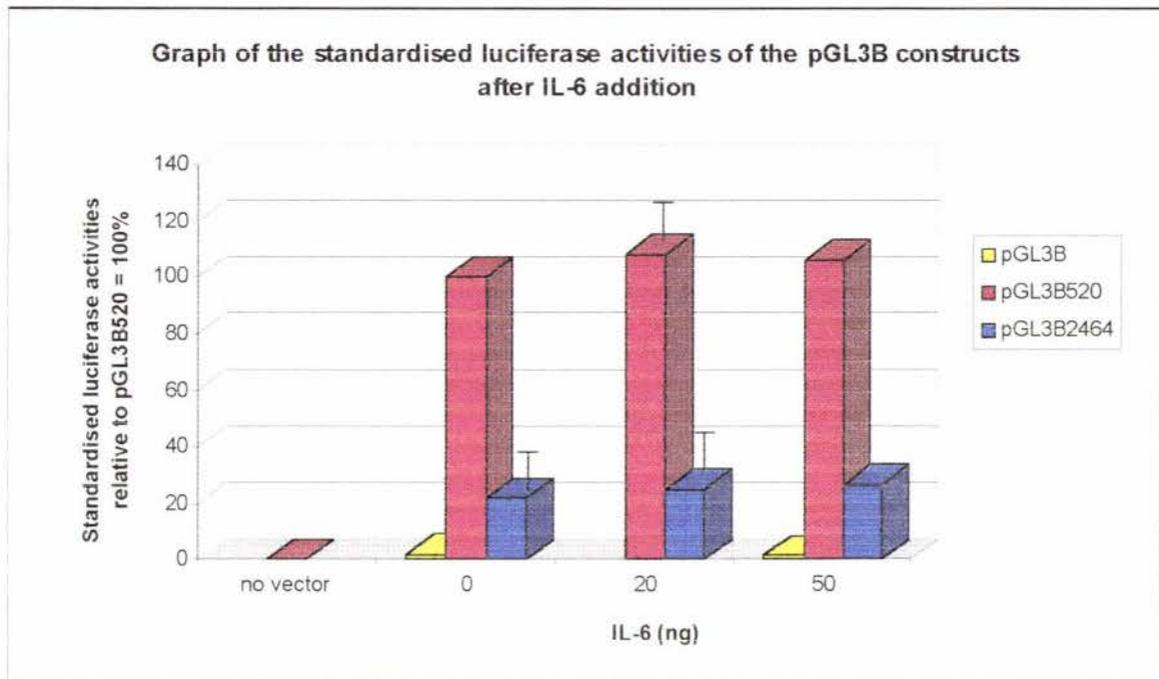


Figure 18. The effect of IL-6 on the transcriptional activity of the hLf promoter in pGL3B. RL95-2 cells were transfected with 4 μ g of pGL3B2464 or pGL3B520 and 2 μ g of pSV- β -gal. Cells transfected with 2 μ g of pGL3B and 2 μ g of pGL2C acted as controls. Interleukin-6 (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The standard deviation (S.D.) of the mean is shown. The values presented are the results of 2 separate experiments where each experiment contained triplicates of the reporter constructs. As the transfections with 50 ng IL-6 were performed only once, no error bars have been included. The luciferase activity of pGL3B520 (0 ng IL-6) was arbitrarily set at 100% and all other values adjusted accordingly.

Modifications of the transient transfection conditions

Since the addition of IL-6, IL-1 α and IFN- γ to RL95-2 cells transfected with the pGL3B bLf constructs, for 18 hours, did not elicit transcriptional changes, the transfection conditions were modified. The manipulation of the cytokine incubation time, the presence of insulin in the media, and the effects of co-transfection with the expression vectors for relevant transcription factor(s) were examined.

4.3.2 Change in IL-6 incubation time

As mentioned previously, various authors have used shorter cytokine incubation times for a variety of experiments e.g. 3 - 5 hours (Nakajima *et al.*, 1993b) and 7 - 15 minutes (Gerhartz *et al.* 1996). Luciferase is moderately unstable in mammalian cells as it is susceptible to proteolysis (Bronstein *et al.*, 1994) and it is not known whether proteolysis, during the 18 hour incubation time, could 'mask' cytokine-induced transcriptional activation by removing the excess luciferase produced. As this was a possibility, reporter gene activities after a 2 hour exposure to cytokines were examined.

As with pGL2E constructs, the addition of IL-6 to cells transfected with the pGL3B constructs 2 hours prior to harvesting, did not affect the levels of promoter activity seen (figure 19). Therefore proteolysis or other cellular events did not appear to be responsible for 'masking' a cytokine-induced transcriptional activation.

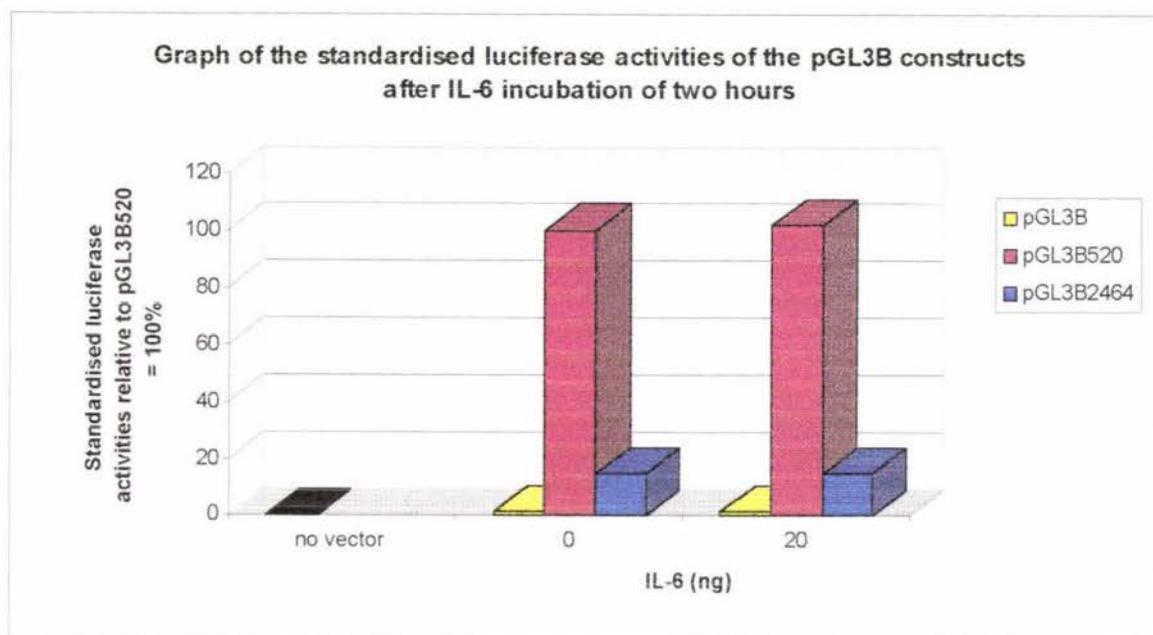
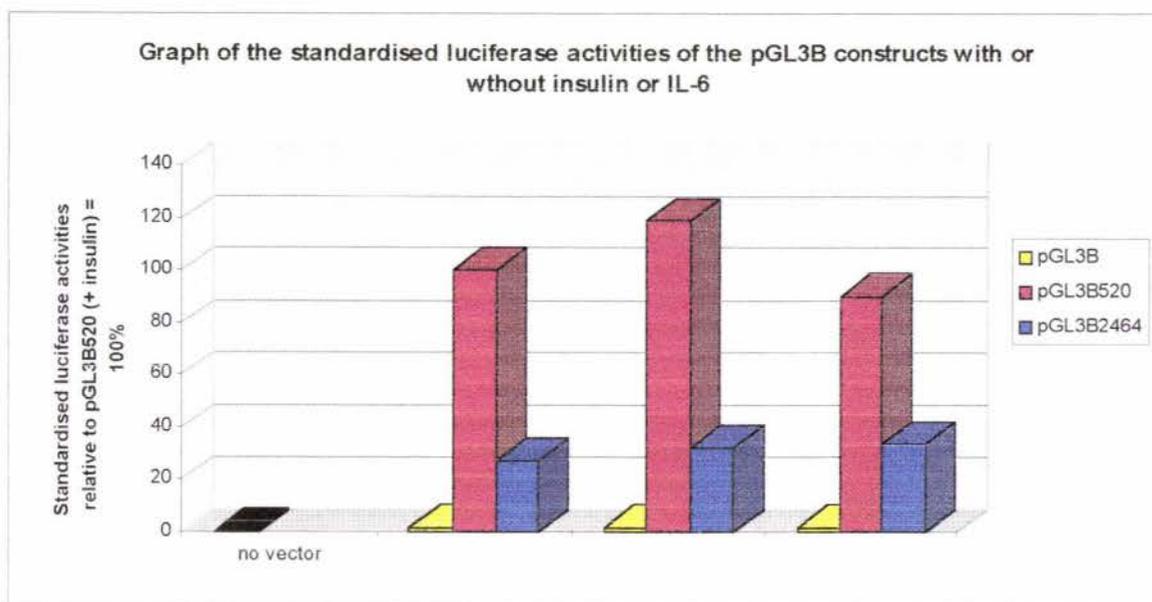


Figure 19. The effect of a 2 hour incubation with IL-6 on the transcriptional activity of the *bLf* promoter in pGL3B. RL95-2 cells were transfected with 4 μg of pGL3B2464 or pGL3B520 and 2 μg of pSV- β -gal. Cells transfected with 2 μg of pGL3B and 2 μg of pGL2C acted as controls. Interleukin-6 (2 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the results of 2 separate experiments where each experiment contained triplicates of the reporter constructs, therefore error bars have not been included. The luciferase activity of pGL3B520 was arbitrarily set at 100% and all other values adjusted accordingly.

4.3.3 Removal of insulin

As discussed previously, insulin has many potential effects on gene expression (section 3.7.3). The presence or absence of insulin did not affect transcription from the bLf promoter in constructs prepared in pGL2E. There were significant differences between basal transcriptional activation of the bLf promoter constructs prepared in pGL2E and pGL3B. Therefore, it was important to test the effect of insulin on transcription of the 2464 and 520 constructs in pGL3B.

Removal of insulin from the media did not alter the basal transcription rates of the pGL3B constructs (data not shown). Addition of IL-6, either with or without insulin, did not significantly affect the pGL3B promoter construct transcription rates (figure 20). Therefore, insulin did not appear to be 'masking' a cytokine-induced transcriptional effect in the pGL3B constructs.



| | | | |
|----------------------------------|---|---|---|
| Insulin (5 $\mu\text{g/ml}$) | + | + | - |
| IL-6 (20 ng) | - | + | + |

Figure 20. The effect of insulin and IL-6 on the transcriptional activity of the *bLf* promoter in pGL3B. RL95-2 cells, grown in media with or without insulin (5 $\mu\text{g/ml}$) were transfected with 4 μg of pGL3B2464 or pGL3B520 and 2 μg of pSV- β -gal. Cells transfected with 2 μg of pGL3B and 2 μg of pGL2C acted as controls. Interleukin-6 (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the results of 2 separate experiments where each experiment contained triplicates of the reporter constructs, therefore error bars have not been included. The luciferase activity of pGL3B520 (0 ng IL-6, + insulin) was arbitrarily set at 100% and all other values adjusted accordingly.

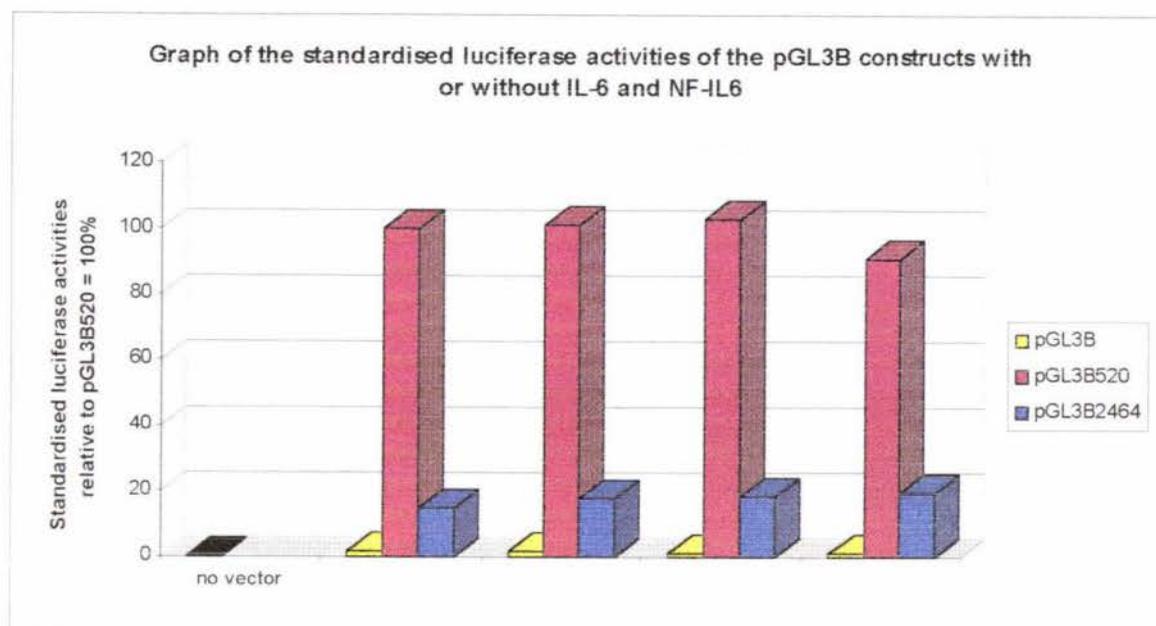
These results show that insulin did not affect the basal levels of transcription of either the pGL2E or the pGL3B constructs. The reasons for this have been discussed previously (section 3.7.3). The removal of insulin did not appear to cause any IL-6-induced effects, so the presence of insulin was not responsible for the lack of cytokine-induced transcriptional activation.

4.3.4 Addition of NF-IL6

IL-6 did not cause any changes in transcriptional activation of the bLf promoter constructs using pGL3B. Therefore, an expression vector for NF-IL6 was added to transfections of RL95-2 cells using the pGL3B bLf constructs.

As with pGL2E constructs, the addition of NF-IL6 did not affect basal transcription levels of the pGL3B constructs. Cytokine-induction of pGL3B constructs in the presence of NF-IL6 did not vary significantly from basal levels (figure 21).

These results indicated that the lack of IL-6 and IL-1 α -induced activation or inhibition of the long and short bLf promoter constructs was not due to a lack of NF-IL6. This result however, does not rule out a disruption in the NF-IL6 signal transduction pathway.



| | | | | |
|--------------------|---|---|---|---|
| IL-6 (20ng) | - | + | - | + |
| NF-IL6 (1 μ g) | - | - | + | + |

Figure 21. The effect of NFIL-6 and IL-6 on the transcriptional activity of the bLf promoter in pGL3B. RL95-2 cells were transfected with 4 μ g of pGL3B2464 or pGL3B520 and 2 μ g of pSV- β -gal. 1 μ g MSV-C EBP β was co-transfected with the reporter constructs as indicated. Cells transfected with 2 μ g of pGL3B and 2 μ g of pGL2C acted as controls. Interleukin-6 (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the results of 2 separate experiments where each experiment contained triplicates of the reporter constructs, therefore error bars have not been included. The luciferase activity of pGL3B520 (0 ng IL-6 or NF-IL6) was arbitrarily set at 100% and all other values adjusted accordingly.

4.4 Removal of Phenol Red

The DMEM-F12 media used to maintain the RL95-2 cells contained phenol red as an indicator of media pH changes. Because of the potential of phenol red to confound experiments involving cytokines or steroid hormones, a series of experiments were carried out using phenol red-free media.

4.4.1 Basal transcriptional activities of the pGL3B constructs

The removal of phenol red did not alter the transcriptional activities previously obtained for transfections using the pGL3B constructs in phenol red media (table 7) (figure 22). Therefore phenol red or contaminants did not appear to affect the basal transcription rates. For comparison, table 8 and figure 23 show that the basal transcription of the pGL2E and pGL3B constructs is similar in media with or without phenol red.

| | Phenol red-containing media | Phenol red-free media |
|-----------|-----------------------------|-----------------------|
| pGL3B | 1.6% | 1.2 ±0.6% |
| pGL3B520 | 100% | 100% |
| pGL3B2464 | 31.1 ±7.5% | 31.1 ±8.4% |

Table 7. Comparisons of the transcriptional rates of the pGL3B constructs in media with or without phenol red.

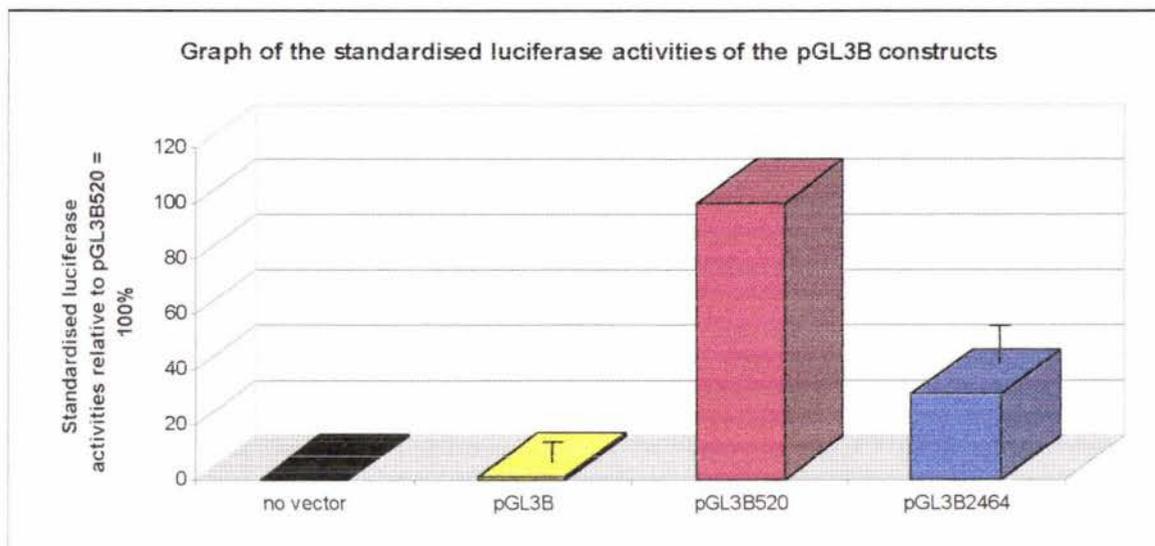


Figure 22. The transcriptional activity of the *bLf* promoter in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μg of pGL3B2464 or pGL3B520 and 2 μg of pSV- β -gal. Cells transfected with 2 μg pGL3B and 2 μg of pGL2C acted as controls. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the average of >18 separate experiments where each experiment contained triplicates of the reporter constructs. The standard deviation (S.D.) of the mean is shown. The luciferase activities of pGL3B520 was arbitrarily set at 100% and all other values adjusted accordingly.

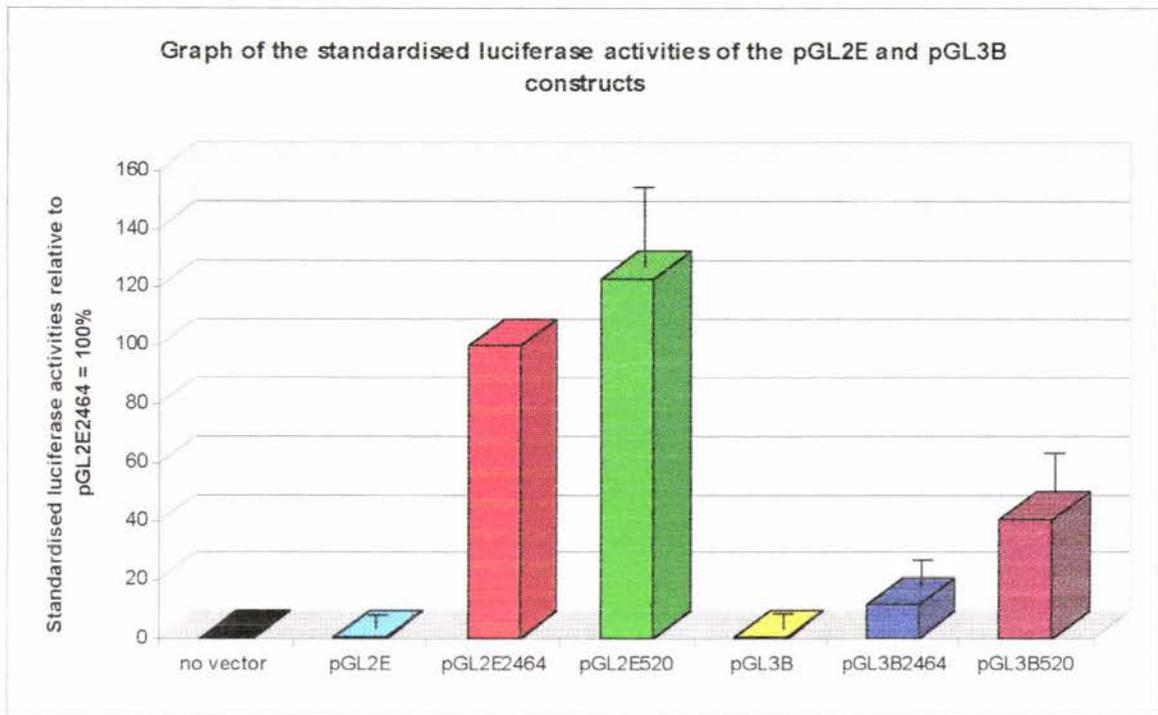


Figure 23. Comparison of the activity of the *bLf* promoter in pGL2E and pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μg of pGL2E2464, pGL2E520, pGL3B2464 or pGL3B520 and 2 μg of pSV- β -gal. Cells transfected with 2 μg of pGL2E, 2 μg pGL3B and 2 μg of pGL2C acted as controls. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the average of >18 separate experiments where each experiment contained triplicates of the reporter constructs. The standard deviation (S.D.) of the mean is shown. The luciferase activity pGL2E2464 was arbitrarily set at 100% and all other values adjusted accordingly.

| | Phenol red-containing media | Phenol red-free media |
|-----------|-----------------------------|-----------------------|
| pGL2E2464 | 100% | 100% |
| pGL2E520 | 107 ±18% | 123 ±19% |
| pGL3B2464 | 9.6 ±2.5% | 11.5 ±4% |
| pGL3B520 | 31.9 ±11% | 41.0 ±11.2% |
| pGL2E | 0.4 ±0.21% | 0.4 ±0.14% |
| pGL3B | 0.59 ± 0.45% | 0.44 ± 0.20% |

Table 8. Comparisons of the pGL2E and pGL3B constructs in media with or without phenol red relative to pGL2E2464 = 100%.

4.4.2 Addition of cytokines

The response of the bLf promoter constructs in pGL3B to IL-6 was investigated in RL95-2 cells grown on phenol red-free media. Cells, transfected with bLf pGL3B constructs, were incubated with 0.5 - 100 ng of cytokines for 18 hours. The addition of IL-6 (figure 24), for a 18 hour incubation, did not cause bLf promoter activities to significantly differ from basal levels. This result indicated that the phenol red was not masking a cytokine-induced transcriptional activation or inhibition.

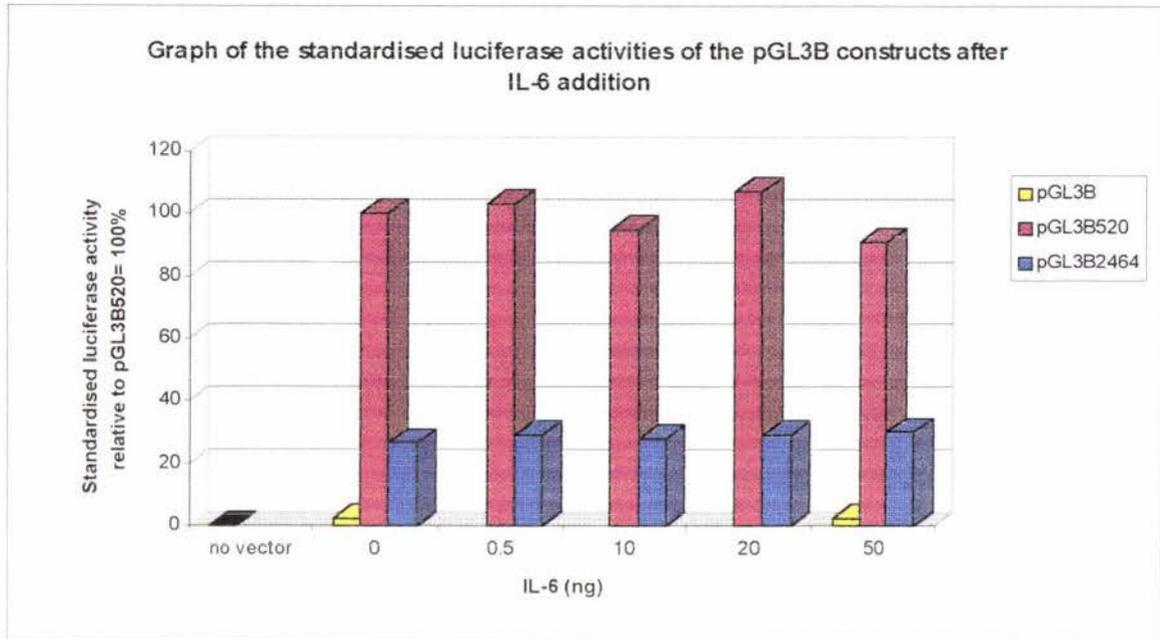


Figure 24. The effect of IL-6 on *bLf* promoter activity in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μ g of pGL3B2464 or pGL3B520 and 2 μ g of pSV- β -gal. Cells transfected with 2 μ g pGL3B and 2 μ g of pGL2C acted as controls. Interleukin-6 (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the results of 2 separate experiments where each experiment contained triplicates of the reporter constructs, therefore error bars have not been included. The luciferase activity of pGL3B520 (0 ng IL-6) was arbitrarily set at 100% and all other values adjusted accordingly.

Modifications of transient transfection

IL-6 in phenol red-free media had no effect on bLf promoter activity in pGL3B. Therefore, the transfection conditions were modified. These included manipulation of the cytokine incubation time, the presence of a relevant transcription factor and the presence or absence of insulin in the media.

4.4.3 Change in cytokine incubation time.

As with pGL2E constructs, the addition of IL-6 2 hours prior to harvesting, did not affect the levels of promoter activity seen for the pGL3B constructs, in transfections of RL95-2 cells in phenol red-free media (figure 25).

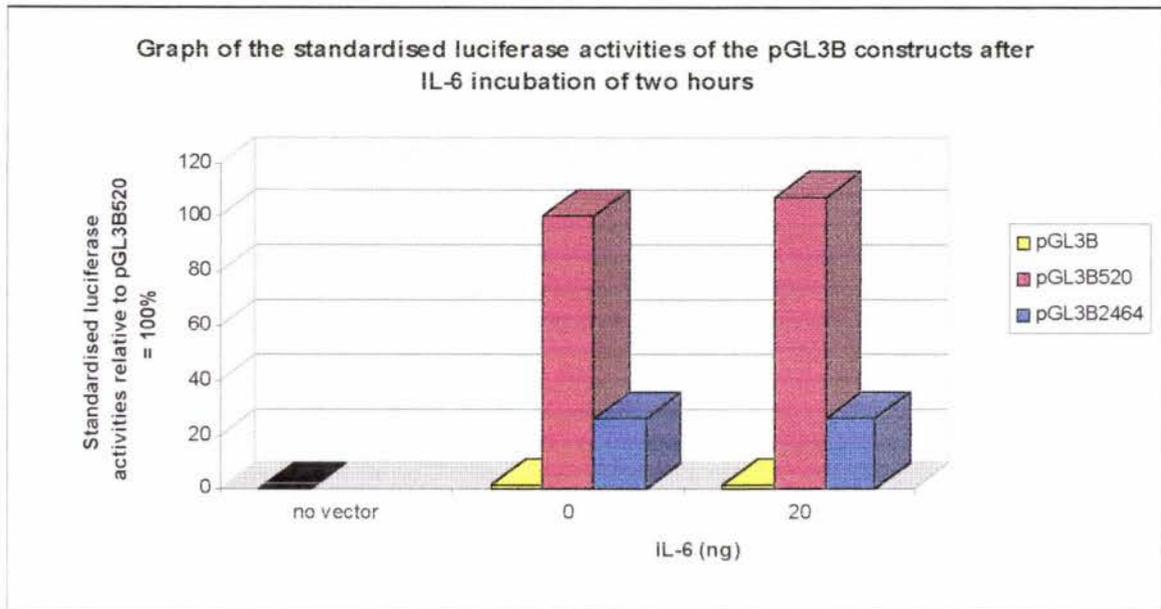
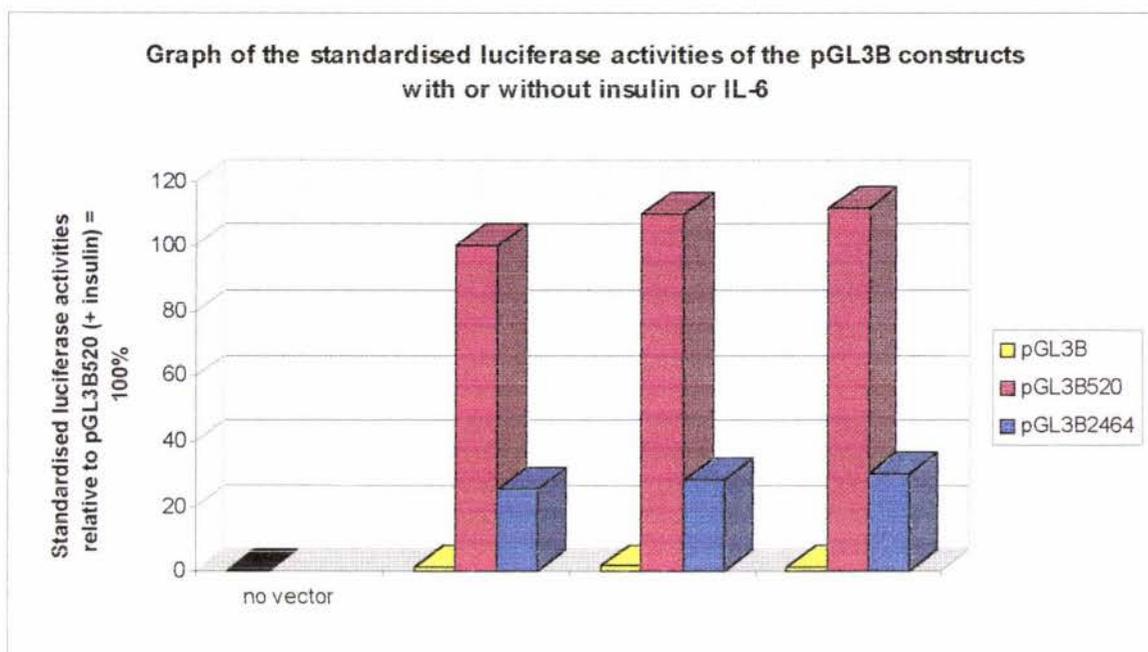


Figure 25. The effect of a 2 hour incubation with IL-6 on the *bLf* promoter activity in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μg of pGL3B2464 or pGL3B520 and 2 μg of pSV- β -gal. Cells transfected with 2 μg pGL3B and 2 μg of pGL2C acted as controls. Interleukin-6 (2 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the results of 1 experiment where the experiment contained triplicates of the reporter constructs, therefore error bars have not been included. The luciferase activity of pGL3B520 (0 ng IL-6) was arbitrarily set at 100% and all other values adjusted accordingly.

4.4.4 Removal of insulin from transfections using pGL3B constructs

The removal of insulin from the media did not alter the basal transcriptional activities of the pGL3B constructs in the RL95-2 cells (data not shown). Addition of IL-6 did not significantly affect the transcriptional activity of the promoter constructs (figure 26). This result indicated that the phenol red did not 'mask' insulin-induced transcriptional effects.



| | | | |
|-------------------------------|---|---|---|
| Insulin (5 $\mu\text{g/ml}$) | + | + | - |
| IL-6 (20 ng) | - | + | + |

Figure 26. The effect of insulin and IL-6 on the transcriptional activity of the *bLf* promoter in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media with or without insulin (5 $\mu\text{g/ml}$) were transfected with 4 μg of pGL3B2464 or pGL3B520 and 2 μg of pSV- β -gal. Cells transfected with 2 μg pGL3B and 2 μg of pGL2C acted as controls. Interleukin-6 (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the results of 1 experiment where the experiment contained triplicates of the reporter constructs, therefore error bars have not be included. The luciferase activity of pGL3B520 (+ insulin) was arbitrarily set at 100% and all other values adjusted accordingly.

4.4.5 Addition of NF-IL6 to transfections using pGL3B constructs

RL95-2 cells were co-transfected with an expression vector MSV-C/EBP β (NF-IL6) and the luciferase bLf pGL3B reporter constructs to test the effect of additional intracellular NF-IL6.

For pGL3B constructs, increases in MSV-C/EBP β (NF-IL6) produced increasing amounts of luciferase activity (figure 27) (table 9). Each value was normalised with the basal transcriptional activity (no MSV-C/EBP β) arbitrarily set at 100%. This was performed in order to more easily observe the activation induced by NF-IL6 and to compare the results between the constructs.

| NF-IL6 expression vector (μ g) | 0 | 0.25 | 0.5 | 1 | 2 |
|-------------------------------------|------|---------------|---------------|---------------|----------------|
| pGL3B | 100% | - | - | 264 \pm 35% | 416 \pm 90 |
| pGL3B520 | 100% | 168 \pm 5% | 324 \pm 56% | 507 \pm 80% | 833 \pm 134% |
| pGL3B2464 | 100% | 167 \pm 33% | 338 \pm 25% | 515 \pm 95% | 817 \pm 97% |

Table 9. The transcriptional activation seen after the co-transfection of MSV-C/EBP β . Each constructs activation was determined separately by arbitrarily setting the luciferase activity of 0 μ g MSV-C/EBP β to 100%.

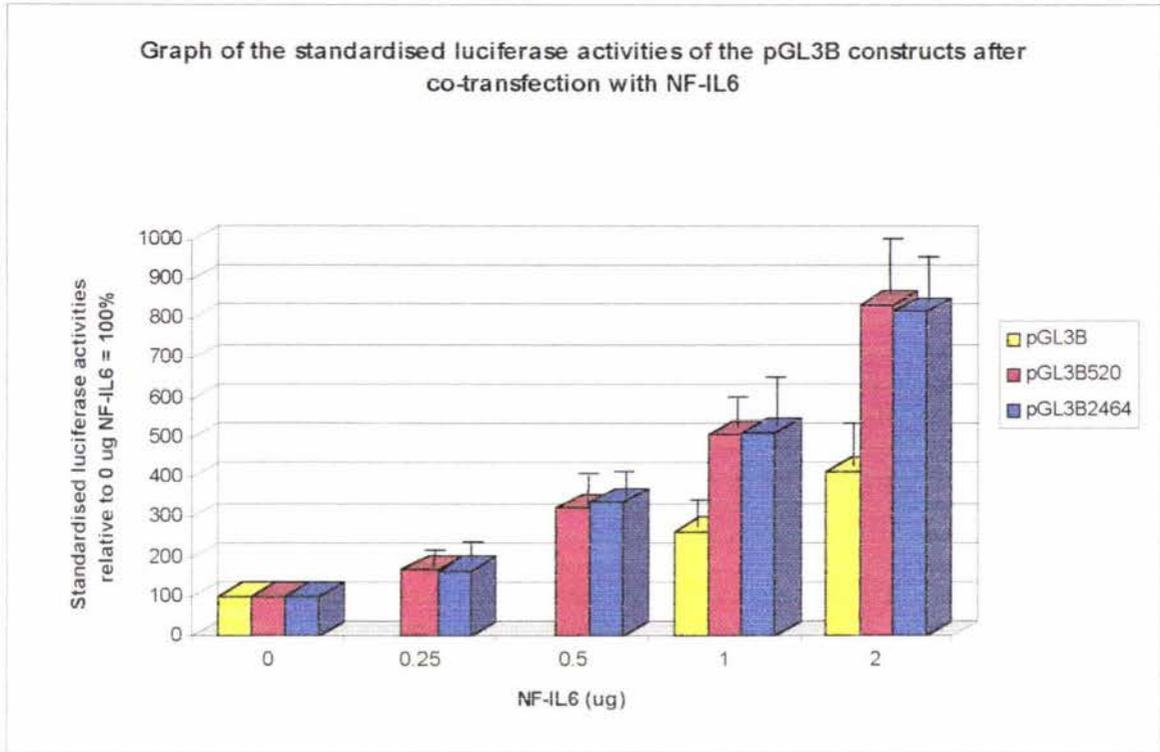


Figure 27. The effect of NF-IL6 on the transcription activity of the *bLf* promoter in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μg of pGL3B2464 or pGL3B520 and 2 μg of pSV- β -gal. MSV-C EBP β was co-transfected with the reporter constructs in the amounts indicated. Cells transfected with 2 μg pGL3B and 2 μg of pGL2C acted as controls. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the average of 4 separate experiments where each experiment contained triplicates of the reporter constructs. The standard deviation (S.D.) of the mean is shown. Each value was determined separately with the luciferase activity in the absence of MSV-C/EBP β arbitrarily set at 100% and all other values adjusted accordingly.

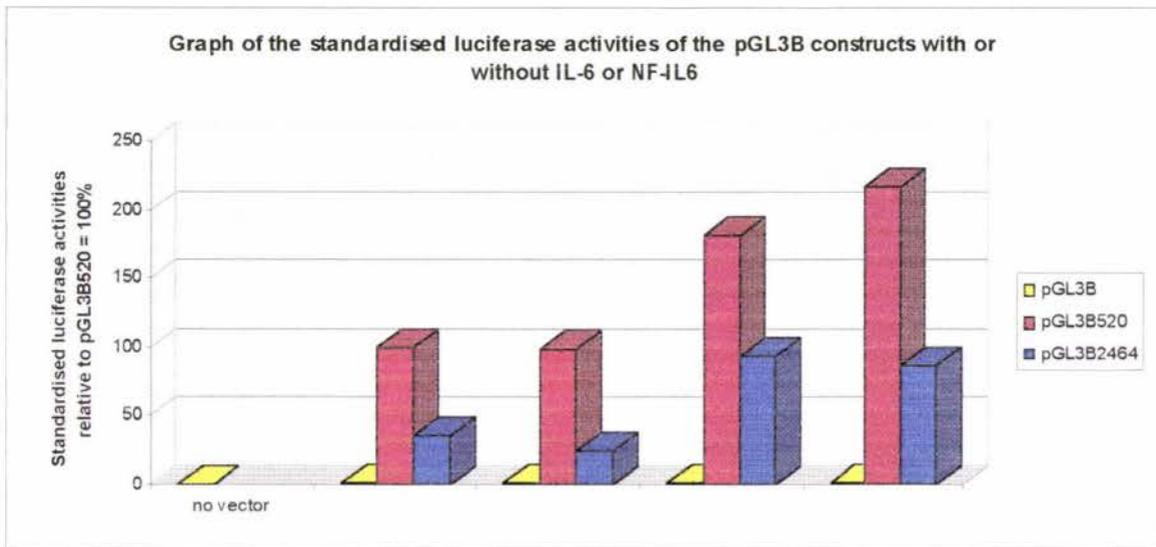
These results suggest that the activation due to increased amounts of expression vector for NF-IL6 in the cell is the same for both pGL3B2464 and pGL3B520. This activation is approximately twice than observed for the pGL3B vector alone. Therefore although some activation may have resulted from increases in the background vector

transcription possibly due to vector sequences, additional activation resulted from interactions with the bLf promoter sequences within the constructs. As pGL3B2464 and pGL3B520 had the same levels of NF-IL6-induced transcriptional activation, this suggested that a NF-IL6 binding site is present in the -520 to +36 region of the bLf promoter.

This activation was not seen in media containing phenol red. The observation that NF-IL6-induced activation occurred only in the absence of phenol red may be explained by the work by Ray *et al.* (1997). These authors used the endometrial adenocarcinoma cell line Ishikawa and found that the estrogen receptor (ER) could directly interact with NF-IL6 and NF- κ B and inhibit the ability of these proteins to bind to DNA and affect gene transcription. Stein and Yang (1995) also provided evidence of direct interaction between ER, NF- κ B and NF-IL6 through the DNA binding domain / region D of ER, the Rel domain of NF- κ B and bZip region of NF-IL6. NF- κ B is a protein induced under a wide range of conditions including exposure to LPS or inflammatory cytokines. Its binding motif is found in a wide range of inducible genes including those encoding acute phase response proteins and immunoregulatory molecules. A synergistic interaction with NF-IL6 affecting transcription has been proposed (reviewed in May and Ghosh, 1997 ; Baldwin, 1996).

If phenol red (or contaminants) has estrogenic activity, then it is possible that phenol red may activate the estrogen receptor, which can then bind and inhibit NF-IL6. In phenol red-free media, the NF-IL6 is not inhibited and can therefore bind to the IL-6REs in the promoter of specific genes and hence activate transcription.

The addition of IL-6 to cells transfected with MSV-C/EBP β did not cause any additional activation of the bLf promoter (figure 28). There are two possible explanations for this observation.



| | | | | |
|---------------|---|---|---|---|
| IL-6 (20 ng) | - | + | - | + |
| NF-IL6 (1 µg) | - | - | + | + |

Figure 28. The effect of NF-IL6 and IL-6 on the transcriptional activity of the *bLf* promoter in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 µg of pGL3B2464 or pGL3B520 and 2 µg of pSV-β-gal. 1 µg of MSV-C EBPβ was co-transfected with the reporter constructs as indicated. Cells transfected with 2 µg pGL3B and 2 µg of pGL2C acted as controls. Interleukin-6 (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β-galactosidase absorbance values at 420 nm. The values presented are the results of 2 separate experiments where each experiment contained triplicates of the reporter constructs, therefore error bars have not been included. The luciferase activity of pGL3B520 (0 ng IL-6, 0 µg NF-IL6) was arbitrarily set at 100% and all other values adjusted accordingly.

The first explanation is that NF-IL6-induced gene activation occurs by increasing the cellular levels of the NF-IL6 protein, which then bind to the DNA. Evidence of this mechanism was seen in Akira *et al.* (1990) where it was found that NF-IL6 (in tissues where it is not normally expressed) was induced after stimulation by IL-1, IL-6 TNF or lipopolysaccharides (Akira and Kishimoto, 1992 ; Baumann *et al.*, 1989 and reviewed

in Bankers-Fullbright *et al.*, 1996). Therefore the final result of the co-transfection of the NF-IL6 expression vector (i.e increased NF-IL6) would mimic the result of an active IL-6 signal transduction pathway. If this pathway was active in the RL95-2 cells, then addition of IL-6 to the cells may cause an increase in transcription from the bLf promoter sequence. The addition of IL-6 did not cause transcriptional activation under a variety of conditions. This suggests that the NF-IL6-signal transduction pathway in RL95-2 cells is non-functional or impaired under these conditions. Non-functionality could occur at any stage in the signal transduction pathway (receptor levels, absence of or low signal transduction components) or the endogenous NF-IL6 gene may malfunction.

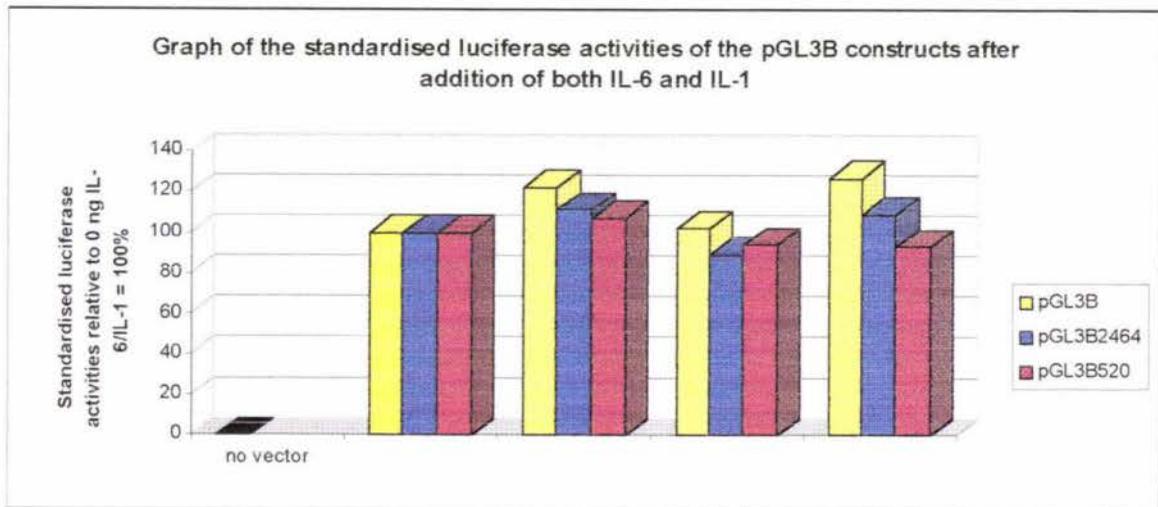
Another possible mechanism for NF-IL6-induced gene activation occurs by the post-translational modification and consequent activation of pre-existing NF-IL6. Phosphorylation of NF-IL6 is thought to cause nuclear translocation, increased transcriptional efficiency of the NF-IL6 protein (reviewed in Bankers-Fullbright *et al.*, 1996) or increased DNA binding (Akira *et al.*, 1992). Trautwein *et al.* (1993) found that phosphorylation within the activation domain of NF-IL6 enhanced the potential of NF-IL6 to activate transcription. Nakajima *et al.* (1993a) observed that NF-IL6 was phosphorylated on specific threonine residues by MAPK. This phosphorylation is required for DNA binding. The Ras/MAPK signaling pathway is activated after stimulation of gp130 (reviewed in Taga, 1997). This observation may provide a potential connection between the activation of NF-IL6 and the activation of the IL-6 receptor.

As NF-IL6 has been reported to be phosphorylated, the activation seen upon NF-IL6 co-transfection suggests that the RL95-2 cells lacked NF-IL6 and that the NF-IL6 expressed after the co-transfection of the expression vector was activated without an applied external stimulus. The stimulus that activates NF-IL6 may come from substances in the media, fetal calf serum, or substances released by neighbouring cells. The potential lack of endogenous NF-IL6 in the RL95-2 cells may explain why no transcriptional activation of the bLf constructs was seen after IL-6 or IL-1 addition. The addition of IL-6 to the RL95-2 cells co-transfected with NF-IL6 had little effect. This may be because the NF-IL6 proteins are already activated to a maximal level.

It is possible that both mechanisms (transcriptional activation and post-translational modification of NF-IL6) act together to achieve the maximum activation of the NF-IL6 protein, and hence the maximum activation of responsive genes. This would complicate the elucidation of the mechanism involved in NF-IL6-induced activation of the bLf promoter in RL95-2 cells grown on phenol red-free media.

4.4.6 Addition of IL-1 α and IL-6

Baumann *et al.* (1989) reviewed various acute phase promoters which require the synergistic action of IL-6 and IL-1 α to obtain transcriptional activation. To test whether this is the case for the bLf promoter, 20 ng of IL-6 and IL-1 α were added to the RL95-2 cells containing the pGL3B bLf constructs was tested. These multiple cytokine additions did not result in any changes to transcriptional activity of cells transfected with the pGL3B constructs (figure 29). This result indicated that the synergistic action of IL-1 α and IL-6 is not required for the transcriptional activation of the pGL3B bLf constructs in RL95-2 cells.



| | | | | |
|---------------|---|---|---|---|
| IL-6 (20 ng) | - | + | - | + |
| IL-1α (20 ng) | - | - | + | + |

Figure 29. The effect of IL-6 and IL-1α on the transcriptional activity of the bLf promoter in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media with or without insulin were transfected with 4 μg of pGL3B2464 or pGL3B520 and 2 μg of pSV-β-gal. Cells transfected with 2 μg pGL3B and 2 μg of pGL2C acted as controls. Interleukin-6 and interleukin-1α (18 hours prior to harvesting) were added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β-galactosidase absorbance values at 420 nm. The values presented are the results of 2 separate experiments where each experiment contained triplicates of the reporter constructs, therefore error bars have not been included. Each construct's data was determined separately with the luciferase activity of 0 ng IL-6 and IL-1α arbitrarily set at 100% and all other values adjusted accordingly.

4.5 Dexamethasone- a synthetic glucocorticoid

The bLf promoter contains two putative GREs are at positions -2029 and -1854. The construct pGL3B2464 contains both putative GREs, while pGL3B520 does not contain either (figure 3). These constructs were used in transfection experiments in an attempt to determine whether these putative GRE(s) are functional in the bovine lactoferrin promoter.

4.5.1 Background

Glucocorticoids are steroid hormones with immunosuppressive and anti-inflammatory properties. At physiological levels, glucocorticoids appear to both enhance and suppress immune function (reviewed in Wilckens and De Rijk, 1997). Glucocorticoids are proposed to have roles during sepsis, intrathymic T-cell selection, peripheral T-cell selection and B cell antibody production (reviewed in Wilckens and De Rijk, 1997).

Glucocorticoids are hydrophobic molecules which diffuse through the plasma membrane into the cytoplasm and bind to the intracellular glucocorticoid receptor (GR). GRs belong to the thyroid/steroid hormone superfamily (reviewed by Evans, 1988). The glucocorticoid receptor contains a C-terminal ligand binding domain (LBD), a central DNA binding domain (DBD) and transactivation domains at the N-terminal ($\tau 1$) and on both sides of the LDB ($\tau 2/\tau C$). The $\tau 1$ transactivation domain is thought to bind with co-activators such as CREB binding protein or the chromatin remodeller Swi3p (reviewed in McEwan *et al.*, 1997).

The response to glucocorticoids is initiated by the binding of the glucocorticoid ligand to the cytoplasmic GR which is complexed to hsp90 protein. Following the steroid binding, the hsp90 dissociates and the now activated GR is translocated to the nucleus and forms a dimer active in transcriptional regulation (reviewed in McEwan *et al.*, 1997 ; Burnstein and Cidlowski, 1989 ; Almawi *et al.*, 1996).

Several mechanisms have been proposed for glucocorticoid action on gene transcription. Glucocorticoids are able to activate the synthesis of acute phase proteins directly but are mainly thought to synergistically enhance the effects of IL-1 and IL-6 (Baumann and Gauldie, 1994). The first mechanism involves the activated GR binding to glucocorticoid responsive elements (GREs) in the promoter of specific genes. The GRE is a conserved palindromic sequence with each GRE half site binding one GR molecule. This GR/GRE binding may physically prevent adjacent transcription factors from binding or may facilitate the binding of other transcription factors, transcription initiation factors or chromatin remodelling factors (reviewed in Almawi *et al.*, 1996, McEwan *et al.*, 1997). Dexamethasone (a synthetic glucocorticoid) may also have other effects which could affect transcription or protein levels such as the modulating the stabilization of mRNA (reviewed in Rousseau, 1984).

Protein-protein interactions have also been proposed to occur. Interactions between specific transcription factors may facilitate or stabilize DNA binding. There is also evidence that activated GR may act as an antagonist and bind to some transcription factors e.g NF- κ B or the *c-fos* and *c-jun* components of AP-1, and prevent nuclear translocation (reviewed in Almawi *et al.*, 1996 ; McEwan *et al.*, 1997 ; Pfahl, 1993 ; Scheinman *et al.*, 1995).

4.5.2 Addition of dexamethasone to transient transfection experiments

Dexamethasone is a potent synthetic glucocorticoid which has been used in tissue culture experiments to elicit gene activation and/or inhibition and cytokine responsiveness in a variety of cells (reviewed by Baumann *et al.*, 1989). Since the 2.5 kb bLf construct contains two putative GREs, the effect of the addition of dexamethasone was tested in transient transfection experiments.

The effect of dexamethasone (or other steroids and hormones) in tissue culture experiments is sometimes obscured by high background transcription due to potential

activating molecules contained in the bovine calf serum and in the media itself. Therefore phenol red-free media and charcoal-stripped bovine calf serum should be used where possible. Whilst the RL95-2 cells survived in media without phenol red, the cells died in media prepared with charcoal-stripped serum. This indicated that elements stripped by the charcoal are required for RL95-2 cell survival, therefore normal serum was used in these experiments.

4.5.3 Addition of dexamethasone to RL95-2 cells transfected with pGL3B constructs

At the time these experiments were carried out, there was no information available about the amounts, or incubation times, of dexamethasone addition to RL95-2 cells. Therefore, the initial concentration of dexamethasone added to the cells, after removal of the calcium phosphate precipitate, was 1 mM. Cells were then incubated for 18 hours prior to harvesting. At this concentration, the RL95-2 cells clumped together and increased numbers of dead cells were seen. It was necessary to dissolve dexamethasone in absolute ethanol, therefore the effect of absolute ethanol alone was investigated. The addition of absolute ethanol alone resulted in increased cell death but no clumping, therefore, at the 1 mM dexamethasone level, both the ethanol and dexamethasone had an effect on the survival. The ethanol-induced cell death could be due to the presence of impurities e.g. benzene used in the production of absolute ethanol or to a general effect of ethanol on the cells.

Lower levels of dexamethasone, from 0.05 - 0.25 mM, were then added to the cells without the cell death and clumping observed at the higher concentrations. These levels of dexamethasone were subsequently used in transfections using the pGL3B constructs. Addition of dexamethasone to transfections using the pGL3B bovine lactoferrin promoter constructs resulted in transcriptional activation (table 10) (figure 30). No transcriptional activation was observed with the constructs when absolute

ethanol alone was added to the cells (data not shown), suggesting that the response observed was due to the dexamethasone itself.

| Dexamethasone (mM) concentration | 0 | 0.05 | 0.1 | 0.25 |
|----------------------------------|------|----------|----------|----------|
| pGL3B | 100% | 151 % | 178 ±28% | 287 ±65% |
| pGL3B520 | 100% | 156 ±22% | 168 ±17% | 265 ±66% |
| pGL3B2464 | 100% | 292 ±63% | 306 ±71% | 356 ±47% |

Table 10. The transcriptional activations of the pGL3B, pGL3B520 and pGL3B2464 after dexamethasone addition. Each value was normalised with the basal transcriptional activity (0 mM dexamethasone) arbitrarily set at 100%. This was performed in order to observe more easily the activation induced by dexamethasone and to compare the results between the constructs.

At the concentrations of dexamethasone used, the pGL3B vector alone and the 556 bp (-520 to +36) construct showed similar responses. This could have been due to vector sequences responding to dexamethasone or to a general transcription-activating effect of dexamethasone on the cells. These activations were approximately 2x lower than the activation observed with the 2.5 kb (-2464 to +36) construct. This effect was particularly significant with 0.05 mM and 0.1 mM dexamethasone but not at 0.25 mM. This may have been due to a non-specific effect of the higher dexamethasone levels on cell growth. The results suggested that elements present only in 2.5 kb fragment were responsible for the additional dexamethasone-induced transcriptional activation. As the 2.5 kb construct contained two putative GREs, these elements were prime candidates for the dexamethasone activation seen.

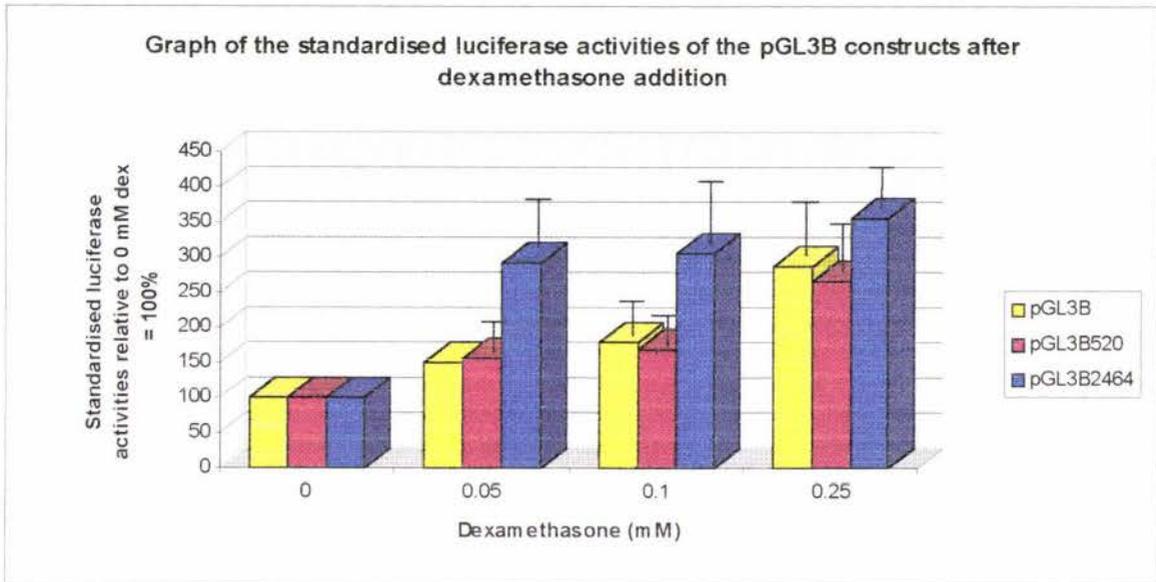


Figure 30. The effect of dexamethasone on the transcriptional activity of the bLf promoter in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μ g of pGL3B2464 or pGL3B520 and 2 μ g of pSV- β -gal. Cells transfected with 2 μ g pGL3B and 2 μ g of pGL2C acted as controls. Dexamethasone (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the average of >3 separate experiments where each experiment contained triplicates of the reporter constructs. The standard deviation (S.D.) of the mean is shown. Each value was determined separately with the luciferase activity in the absence of dexamethasone arbitrarily set at 100% and all other values adjusted accordingly.

In retrospect, the levels of dexamethasone used with the RL95-2 cells were greater than that used with other cell types (table 11). The significance of the higher dexamethasone levels required by the endometrial carcinoma cell line used in these experiments is not known. Lower concentrations of dexamethasone may have resulted in transcriptional activation as there was no difference in luciferase activity in the presence of both 0.05 mM and 0.1 mM dexamethasone. However, there was insufficient time to repeat these experiments using lower concentrations of dexamethasone.

| Cell type | Amounts/time of dexamethasone incubation | Reference |
|------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|
| Airway smooth muscle cells HepG2 liver hepatoma cells mouse fibroblast L929 cells C6 glioma cells | 1 nM to 1 μ M, 22 hrs 1 μ M, 24 hrs 1 μ M 24 hrs 10 nM, 18 hrs | Hardy <i>et al.</i> (1996) Baumann <i>et al.</i> (1989) Kralli and Yamamoto, (1996) Tonner <i>et al.</i> (1997) |

Table 11. Examples of the amounts and incubation times of dexamethasone used in experiments with other cell types.

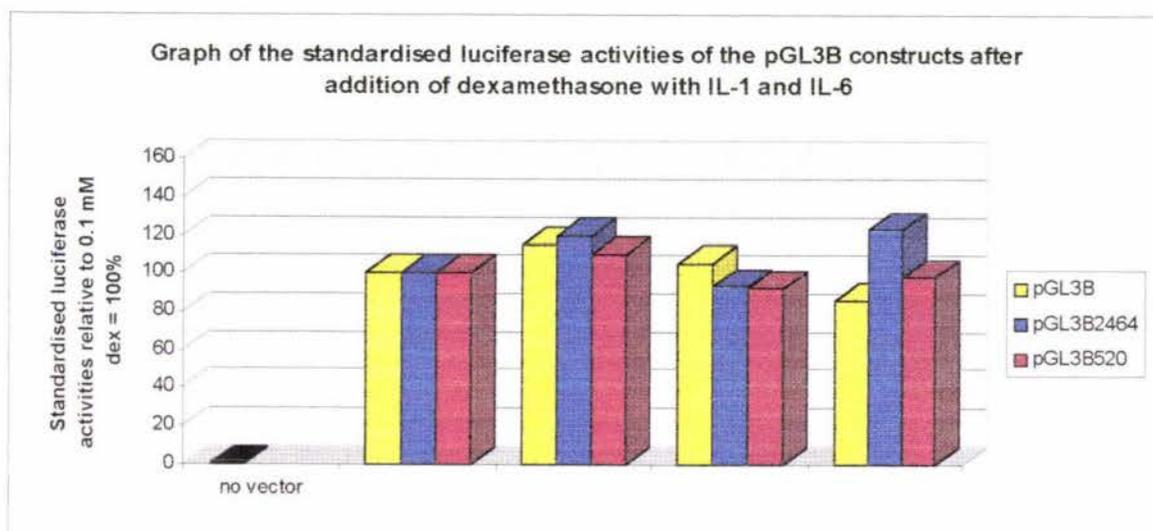
4.6 The action of cytokines and glucocorticoids.

Glucocorticoids have been found to up-regulate cytokine receptor expression for IL-1R, IL-6R and IFN- γ R (reviewed in Almawi *et al.*, 1996). For example, dexamethasone can induce the expression of the 80 kDa subunit of the IL-6 receptor (Geisterfer *et al.*, 1995 ; Baumann *et al.*, 1993) The upregulation of IL-6 signal transduction pathway components by glucocorticoids has also been reported (reviewed in Wilckens and De Rijk, 1997).

It has been reported that certain cell lines (such as hepatoma cell lines) require the addition of dexamethasone together with IL-6 and/or IL-1 in order for the promoters of certain acute phase protein genes to be responsive to cytokines. Also the synergistic action of IL-6 and IL-1 α may also be required for a cytokine response (reviewed in Baumann *et al.*, 1989).

4.6.1 Addition of dexamethasone with IL-1 α and IL-6

The addition of dexamethasone with IL-6, IL-1 α or IL-6 and IL-1 α together did not result in any changes in the transcriptional activity of pGL3B, pGL3B2464 or pGL3B520 in transfection experiments containing these constructs (figure 31). This is contrary to what is seen with a variety of acute phase promoters (reviewed in Baumann *et al.*, 1989).



| | | | | |
|------------------------|---|---|---|---|
| IL-6 (20 ng) | - | + | - | + |
| IL-1 α (20 ng) | - | - | + | + |
| Dexamethasone (0.1 mM) | + | + | + | + |

Figure 31. The effect of dexamethasone, IL-6 and IL-1 α on the transcriptional activity of the bLf promoter constructs in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μ g of pGL3B2464 or pGL3B520 and 2 μ g of pSV- β -gal. Cells transfected with 2 μ g pGL3B and 2 μ g of pGL2C acted as controls. Dexamethasone, interleukin-6 and interleukin-1 α (18 hours prior to harvesting) were added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the results of 2 separate experiments where each experiment contained triplicates of the reporter constructs, therefore error bars have not been included. Each reporter construct's data was analysed separately with the luciferase activity of (0.1 mM dexamethasone) arbitrarily set at 100% and all other values adjusted accordingly.

4.7 Experiments to investigate the putative GREs present in the bLf promoter

A number of transfection experiments were carried out in an attempt to localise the functional GRE(s) in the bovine lactoferrin promoter. The putative GREs are at positions -2029 and -1854. The construct pGL3B2464 contains both putative GREs and shows transcriptional activation upon addition of dexamethasone, while pGL3B520 does not contain the putative GREs and shows no activation above the background levels. In order to further investigate the role of these putative GREs, the addition of dexamethasone to two other constructs was analysed.

pGL2E2087 is a construct containing a DNA fragment from -2087 to +36 in the bLf promoter and consequently the construct contains both putative GREs but lacks the 5'~380 bp present in pGL2E2464. pGL2E1816 is a construct containing sequences from -1816 to +36 and does not contain the putative GREs, but contains the bLf region between -520 and -1816 which is absent in pGL3B520 (figures 32 and 33). Both of these constructs were subcloned into the pGL3B vector and tested for activation by dexamethasone. To confirm the identity of the clones, 5 µl of plasmid DNA was digested with *Bgl* II and *Kpn* I as described in section 2.2.2. This confirmed the correct size of the insert. Diagnostic digests were carried out to confirm the identity of the clones. 5 µl of plasmid DNA were digested with *Xba* I and *Nco* I in multicore buffer. pGL2E, pGL3B, pGL2E2087 and pGL2E1816 were used as controls.

4.7.1 Basal transcription of pGL3B2087 and pGL3B1816

Both pGL3B2087 and pGL3B1816 had similar transcription rates, and had 3x greater activity than pGL3B2464 (table 12) (figure 34). This supports the theory that the 2.5 kb construct contained a putative repressive element. These results suggest that the repressive element lies somewhere within the ~380 bp at the 5' end of the 2.5 kb fragment i.e. between the -2464 to -2087 region relative to the transcription start site of +1.

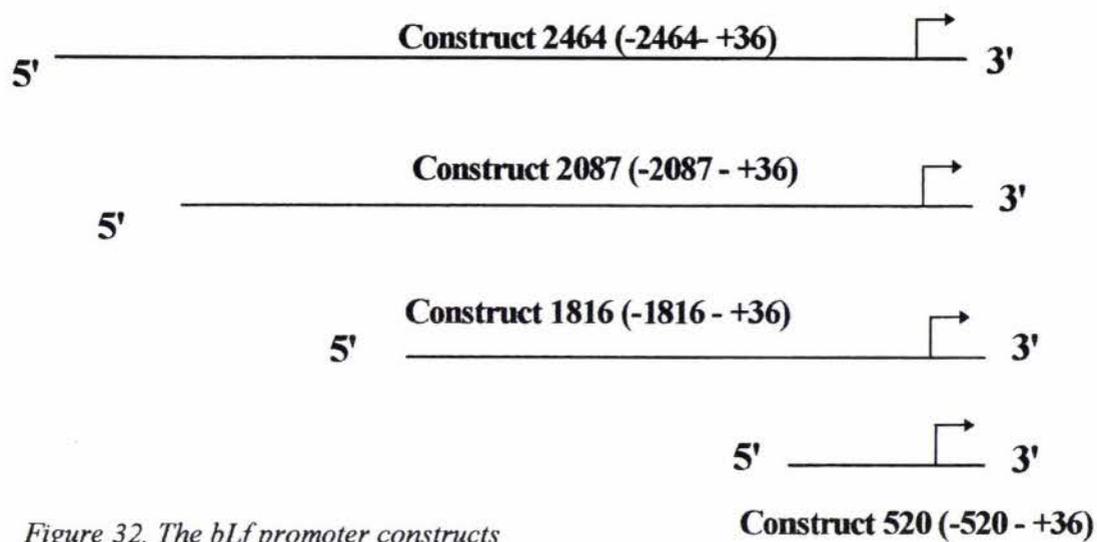


Figure 32. The bLf promoter constructs

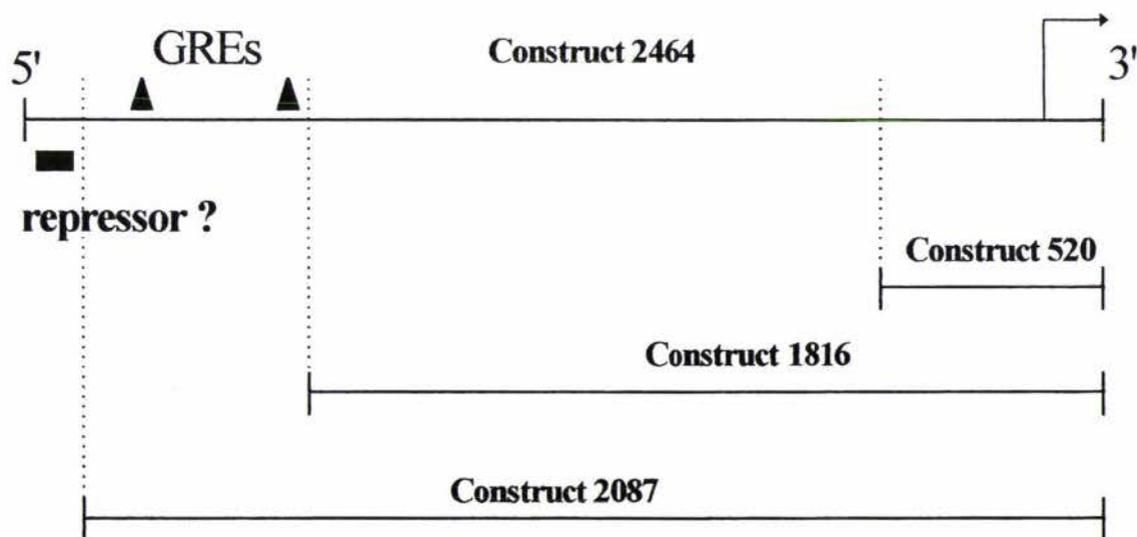


Figure 33. Putative GREs in the bLf promoter constructs
(Bain, 1995)

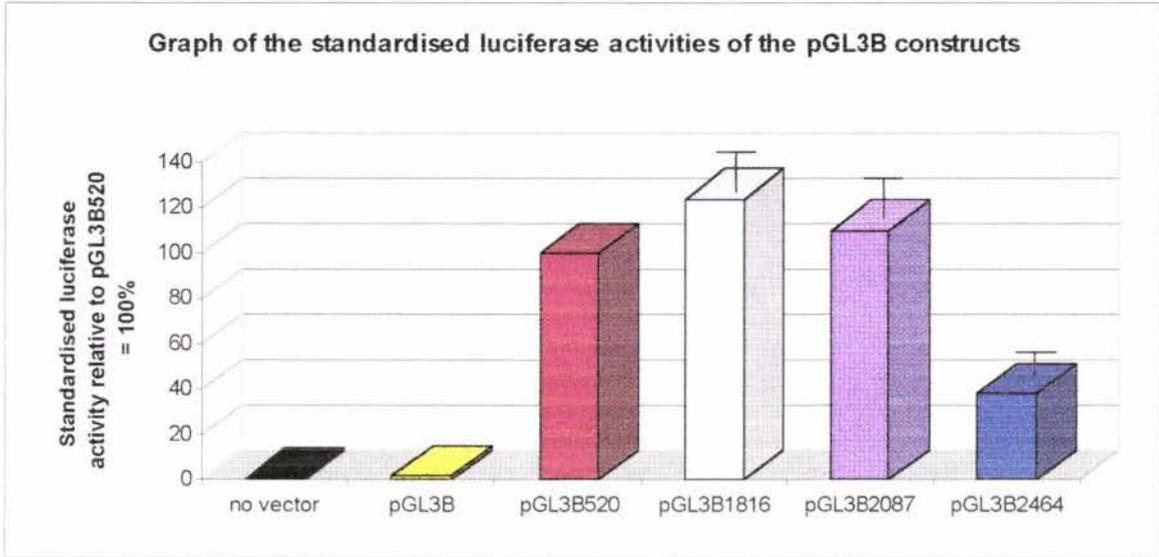


Figure 34. Comparison of the transcriptional activity of the *bLf* promoter in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μg of pGL3B2464, pGL3B520, pGL3B2087 or pGL3B1816 and 2 μg of pSV- β -gal. Cells transfected with 2 μg pGL3B and 2 μg of pGL2C acted as controls. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the average of 5 separate experiments where each experiment contained triplicates of the reporter constructs. The standard deviation (S.D.) of the mean is shown. The luciferase activity of pGL3B520 was arbitrarily set at 100% and all other values adjusted accordingly.

| Construct | Relative luciferase activities |
|-----------|--------------------------------|
| pGL3B | 1.5 ±0.2% |
| pGL3B520 | 100% |
| pGL3B1816 | 124 ±8% |
| pGL3B2087 | 110 ±13% |
| pGL3B2464 | 37.5 ±6% |

Table 12. Transcriptional rates of the pGL3B constructs in RL95-2 cells relative to pGL3B520 – 100%.

A potential silencer element has been identified in the 5' end of the hLf promoter between the -800 to -916 region (Khann-Gupta *et al.*, 1997). Three 8 bp repetitive elements which bind the CCAAT displacement protein CDP/cut were identified as a silencer element. CDP/cut binding occurred only in cells that do not express lactoferrin (Khann-Gupta *et al.*, 1997). Since uterine cells do express lactoferrin (Pentecost and Teng, 1987 ; Teng *et al.*, 1989) it would seem unlikely that the repression seen in the -2464 to -2087 bLf promoter region is due to CDP/cut-induced repression. Nevertheless, the ~380 bp bLf region was scanned for the repetitive elements identified in the hLf promoter (ATGTATTT / ATGTATTC / AGTATTCT). While some similarities were seen with the bLf sequence and the hLf repetitive regions (figure 35), the distance between the bLf putative repetitive elements was 14-56 bp and not the 6-7 bp seen in the hLf repressive region. The significance of these results need to be investigated further.

4.7.2 Addition of dexamethasone to cells containing the pGL3B bLf constructs

The difference in transcriptional activity of the 520 and 2464 constructs were greatest at 0.1 mM dexamethasone, therefore, RL95-2 cells were transfected with pGL3B2464, pGL3B520, pGL3B2087 and pGL3B1816 and cultured in the presence of 0.1 mM dexamethasone. The results showed that the cells transfected with pGL3B, pGL3B520 and pGL3B1816 had similar transcription levels, which were ~2x less than that of cells transfected with pGL3B2464 and pGL3B2087 (table 13) (figure 36). Therefore the constructs with the putative GREs were activated compared to those constructs which lacked the putative GREs. This result provided evidence that the putative GREs may be involved in the activation by dexamethasone.

| Construct | 0 mM | 0.1mM dexamethasone |
|-----------|------|---------------------|
| pGL3B | 100% | 161 ±16% |
| pGL3B520 | 100% | 163 ±17% |
| pGL3B1816 | 100% | 165 ±16% |
| pGL3B2087 | 100% | 270 ±14% |
| pGL3B2464 | 100% | 283 ± 61% |

Table 13. Degree of the activation seen after the addition of 0.1 mM dexamethasone to RL95-2 cells containing pGL3B, pGL3B520, pGL3B1816, pGL3B2087 or pGL3B2464. Each value was normalised with the basal transcriptional activity (0 mM dexamethasone) arbitrarily set at 100%. This was performed in order to observe more easily the activation induced by dexamethasone and to compare the results between the constructs.

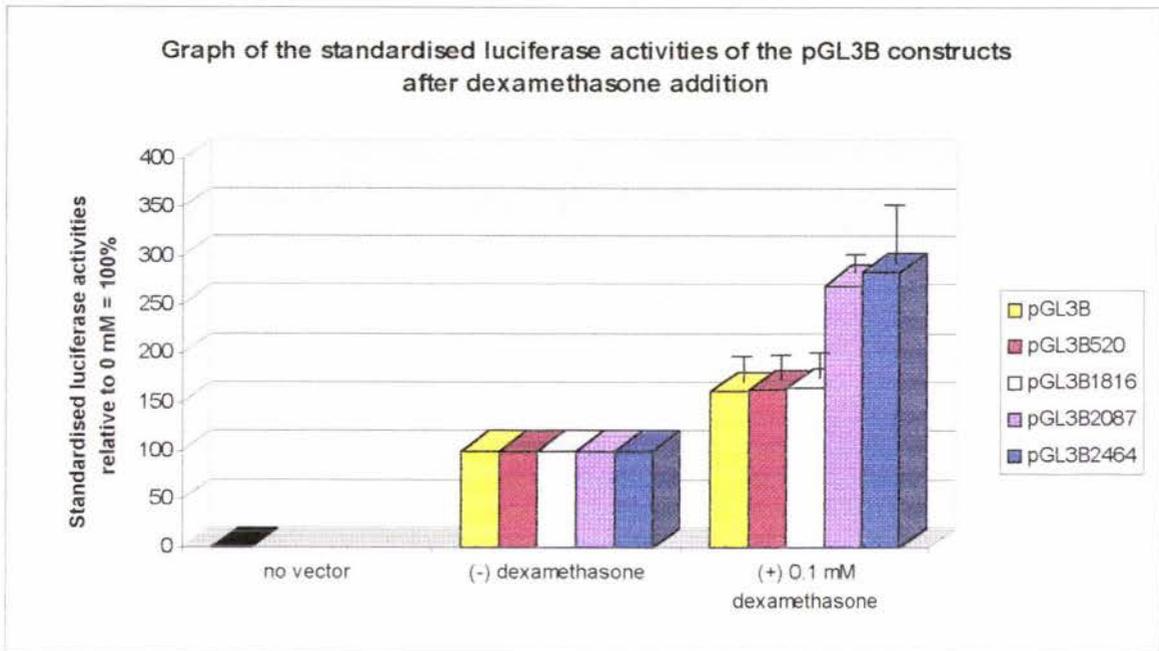


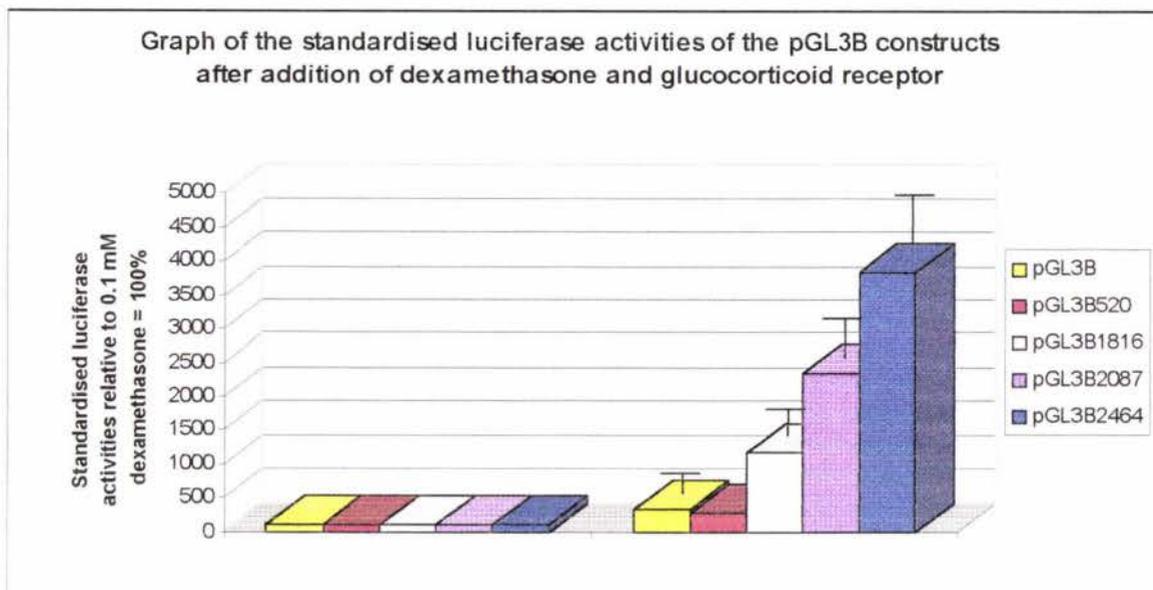
Figure 36. The effect of dexamethasone on transcriptional activity of the bLf promoter in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μ g of pGL3B2464, pGL3B520, pGL3B2087 or pGL3B1816 and 2 μ g of pSV- β -gal. Cells transfected with 2 μ g pGL3B and 2 μ g of pGL2C acted as controls. Dexamethasone (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the average of 5 separate experiments where each experiment contained triplicates of the reporter constructs. The standard deviation (S.D.) of the mean is shown. Each value was determined separately with the luciferase activity in the absence of dexamethasone arbitrarily set at 100% and all other values adjusted accordingly.

The cellular levels of GR within the RL95-2 cells is not known and any effect of dexamethasone may be affected by limiting amounts of GR. Therefore, co-transfections of RL95-2 cells with bLf pGL3B promoter constructs and the human glucocorticoid receptor (GR) expression vector (RSV-GR) were carried out to determine whether additional glucocorticoid receptor in the cells would increase the activation observed upon the addition of dexamethasone. This would only have an effect if the cellular glucocorticoid receptor levels were not already at saturation levels.

4.7.3 Co-transfection of glucocorticoid receptor to RL95-2 cells

Co-transfection of RL95-2 cells with the human RSV-GR expression vector (Hollenberg *et al.*, 1985) and pGL3B, pGL3B520, pGL3B1816, pGL3B2087 and pGL3B2464 constructs was carried out. No change in transcription was seen with glucocorticoid receptor (GR) co-transfection (data not shown). With other nuclear receptors of the same receptor superfamily, it has been found that binding to DNA occurs in a ligand-independent manner and the receptors may act as transcriptional repressors in the absence of the ligand (reviewed in Pfahl, 1993). As the co-transfection of GR alone did not cause inhibition, this could indicate that this type of regulation is not occurring. The effect on transcriptional activation of both dexamethasone and GR was then tested.

Co-transfection of RL95-2 cells with bLf promoter constructs and RSV-GR, in the presence of dexamethasone, produced a substantial increase in levels of transcription from the bLf promoter constructs, compared to that seen with dexamethasone alone. pGL3B and pGL3B520 showed ~300% activation compared to the level seen with dexamethasone alone (100%). This result strengthens the conclusion that an active GRE-like element exists in the pGL3B vector and may allow GR to bind weakly. Addition of dexamethasone to cells containing pGL3B1816 and RSV-GR produced an activation which is significantly greater than what is seen with pGL3B and pGL3B520. Addition of dexamethasone to cells containing GR and pGL3B2087 or pGL3B2464 again produced significant activations above that of dexamethasone alone (table 14) (figure 36). Therefore with dexamethasone alone, the shorter (pGL3B520 and pGL3B1816) constructs and the pGL3B vector had a similar degree of activation which was also lower than that of the longer (pGL3B2087 and pGL3B2464) constructs (figure 36). But upon the addition of GR and dexamethasone, the longer the bLf construct, the greater the transcriptional activation seen. In order to simplify the results seen, figure 37 shows the activation of each construct, after addition of dexamethasone and GR, normalised to the transcriptional levels seen for dexamethasone alone.



| | | |
|-------------|---|---|
| 0.1 mM dex | + | + |
| 2 µg RSV-GR | - | + |

Figure 37. The effect of dexamethasone and GR on the transcriptional activity of the bLf promoter in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 µg of pGL3B2464, pGL3B520, pGL3B1816 or pGL3B2087 and 2 µg of pSV-β-gal. 2 µg of RSV-GR was co-transfected with the reporter constructs as indicated. Cells transfected with 2 µg pGL3B and 2 µg of pGL2C acted as controls. 0.1 mM dexamethasone (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β-galactosidase absorbance at 420 nm. The values presented are the average of 4 separate experiments where each experiment contained triplicates of the reporter constructs. The standard deviation (S.D.) of the mean is shown. Each value was determined separately with the luciferase activity of (0.1 mM dexamethasone with 0 µg GR) arbitrarily set at 100% and all other values adjusted accordingly.

| Construct | 0.1 mM dexamethasone | 0.1 mM Dex + 2 μ g GR |
|-----------|----------------------|---------------------------|
| pGL3B | 100% | 344 \pm 73% |
| pGL3B520 | 100% | 274 \pm 0% |
| pGL3B1816 | 100% | 1178 \pm 146% |
| pGL3B2087 | 100% | 2353 \pm 262% |
| pGL3B2464 | 100% | 3832 \pm 725% |

Table 14. The transcriptional activations of bLf constructs in RL95-2 cells seen after the addition of GR and dexamethasone relative to the transcriptional levels seen with 0.1 mM dexamethasone alone arbitrarily valued at 100%. This was performed in order to more easily observe the activation induced by GR and to compare the results between the constructs.

There are several possible explanations of these results. One reason could be that RL95-2 cells have only a minimal level of glucocorticoid receptor so that upon activation by dexamethasone, there would be competition between Glucocorticoid Response Elements (GREs) for GR binding. The dexamethasone-induced transcriptional activation seen with pGL3B, pGL3B520 and pGL3B1816 could indicate the presence of a strong GRE(s) in the pGL3B vector, but not in the -1816 to +36 bp region of the bLf promoter. The higher transcriptional activation seen with pGL3B2087 and pGL3B2464 could indicate the presence of strong or multiple GREs present in both constructs (-2087 to -1816 region) (figure 38). The addition of GR to the cells would result in excess cellular GR and may allow weaker GREs to compete for GR binding. The presence of such element(s) in pGL3B1816 may explain the \sim x4 fold increase in activity compared to pGL3B and pGL3B520 (in the -520 to -1816 region). Additional elements in the -1816 to -2087 region could explain the additional \sim 2 fold activation of pGL3B2087 when compared to pGL3B1816. This could be either due to a cumulative effect of weaker GREs and/or due to the pair of putative strong GREs between -2029 and -1854. pGL3B2464 showed a further \sim 2 fold increase in transcription over that observed for pGL3B2087. This represents a \sim 10x activation compared to pGL3B. This again could be due to a weak GRE in the -2087 to -2464

region or due to a dexamethasone-GR-activated enhancer element or transcription factor binding site present upstream of the putative GRE pair in the same region. These possible scenarios are presented in figure 39.

Examples of the cooperative effects of GREs have been seen in the TAT (tyrosine aminotransferase) and tryptophan oxidase genes. In the TAT gene, two GREs act cooperatively. The distal GRE can independently induce transcription, the proximal GRE cannot, but the cooperative effect of both is greater than the single GRE effect (Jantzen *et al.*, 1987). Two GREs in the tryptophan oxidase promoter enhance transcription in an additive manner. Each GRE can act independently, with one also requiring additional upstream sequences (Danesch *et al.*, 1987). Also weaker GREs may require the binding of additional transcription factors to obtain maximal glucocorticoid induction.

Although the binding of GR to GREs seems a probable explanation, researchers have also discovered that activated GR can interact directly with certain transcription factors and thus affect their activity. Protein-protein interactions can occur between GR, AP-1 and NF- κ B (reviewed by McEwan *et al.* ; 1997, Pfahl, 1993). Ray and Prefontaine (1994) found that transcriptional activation by NF- κ B was inhibited by dexamethasone-activated GR and that a physical association was detected between the GR and the p65 subunit of NF- κ B. Mukaida *et al.* (1994) and Scheiman *et al.* (1995b) have also discovered that dexamethasone-activated GR can physically interact with NF- κ B and prevent binding to its cognate *cis*-element. A second independent mechanism has been proposed for the inhibition of NF- κ B activity. In its inactive state, NF- κ B binds to I κ B α (its cytoplasmic inhibitor), but upon activation of NF- κ B, the degradation of the inhibitor allows nuclear translocation of NF- κ B (reviewed in Vanderburg, 1997 ; Baldwin, 1996 ; May and Gosh, 1997). The second proposed mechanism involves dexamethasone inducing the production of I κ B mRNA and protein. Therefore, upon dexamethasone addition, the NF- κ B binds to the newly produced I κ B α , thus reducing nuclear translocation and inhibiting NF- κ B action (Scheinman *et al.*, 1995a ; Scheinman *et al.*, 1995b ; Auphan *et al.*, 1995).

Figure 38. Proposed model to explain bLf constructs transcriptional activation after addition of dexamethasone

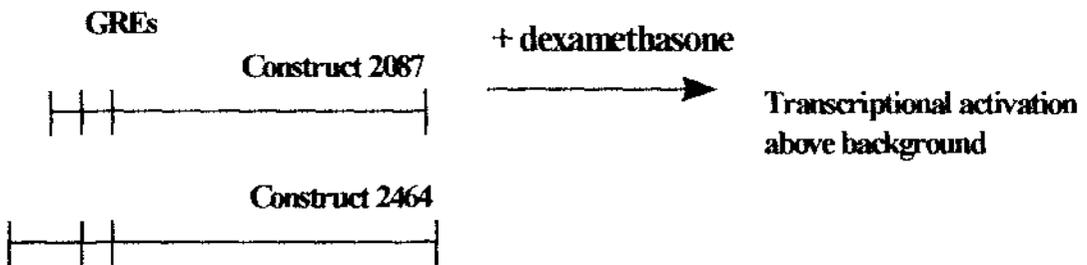
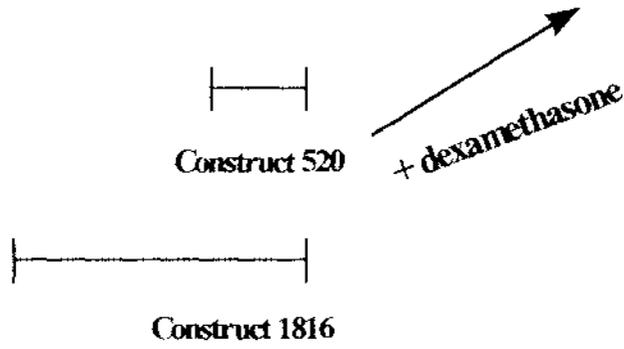
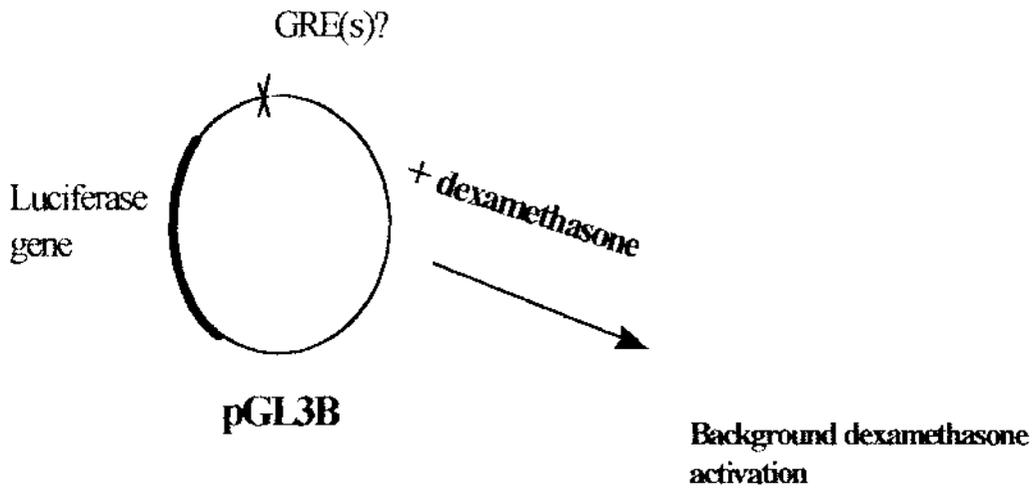
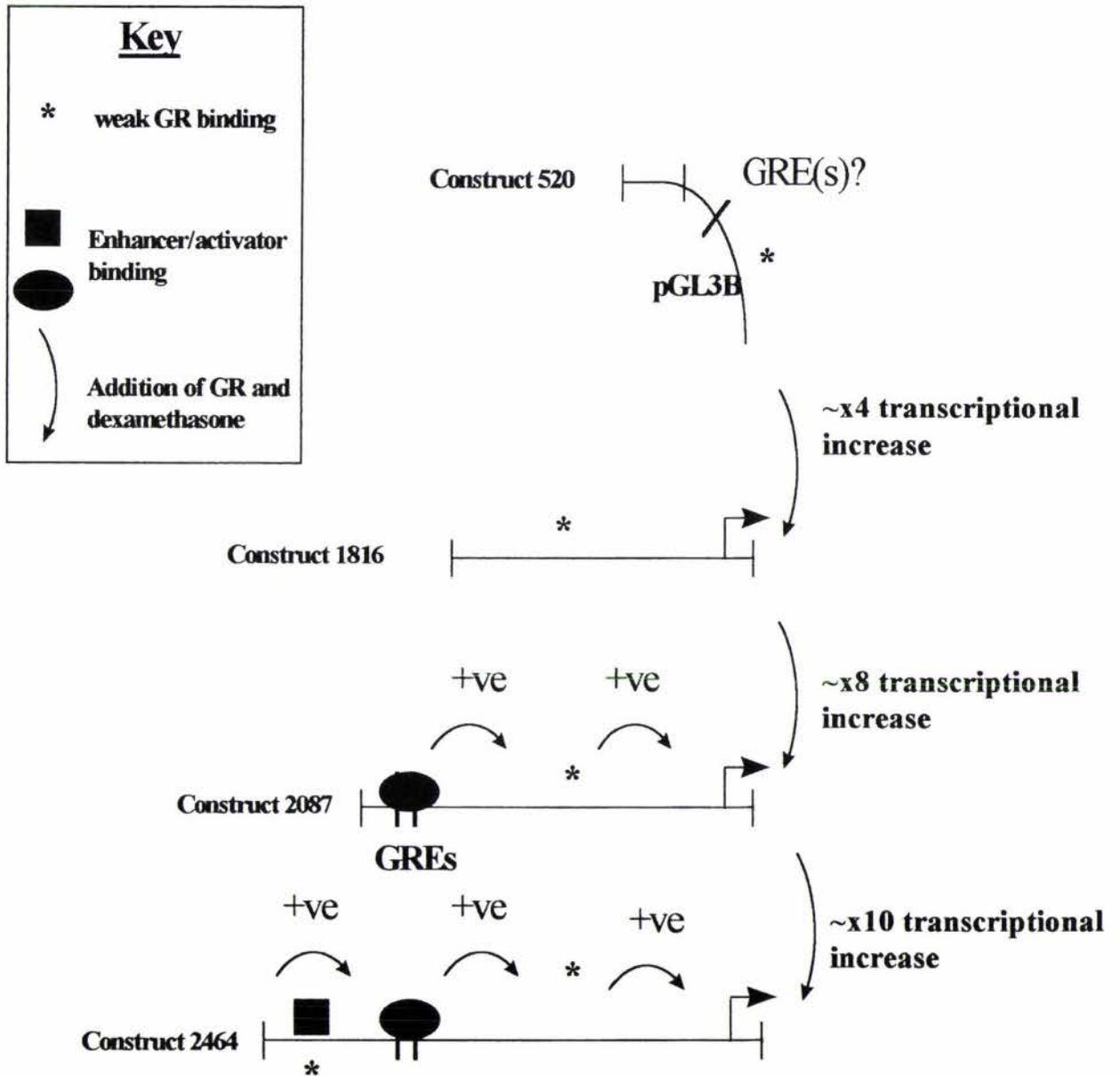


Figure 39. Proposed model of the transcriptional activation by addition of excess glucocorticoid receptor in the presence of dexamethasone



The activity of AP-1 (activator protein-1), which exists as a protein complex of c-Fos and c-Jun (Chiu *et al.*, 1988 ; Rausher *et al.*, 1988), is modulated by factors including cytokines and oncoproteins. The AP-1 binding site is recognized by c-Jun homodimers or c-Jun/c-Fos heterodimers (reviewed by Angel and Karin, 1991 ; Pfahl, 1993). Yang-Yen *et al.* (1990) and Jonat *et al.* (1990) found evidence of a direct protein-protein interaction between c-Jun or c-Fos and the dexamethasone-activated GR. This interaction inhibited their mutual DNA binding abilities and transcriptional activities. This was also observed by Schule *et al.* (1990).

Both of AP-1 and NK- κ B have putative binding sites in the bLf promoter. If these factors play an inhibitory role for the bLf promoter, then the GR and dexamethasone-induced activation seen, may be a lifting of repression as these factors are prevented from binding. From the results seen, binding sites of varying strengths could range through the promoter constructs. Those in the pGL3B vector and in both pGL3B2464 and pGL3B2087 may be especially susceptible to a dexamethasone-induced inhibition of transcription factor binding, and so show activation upon dexamethasone addition. Therefore, the addition of GR could accentuate the 'repression release' as more GR-transcription factor interactions could occur, and allow release of inhibitory factors from strong binding sites within pGL3B1816 and pGL3B2087. It is also possible that the activated GR could activate GREs in the endogenous promoters of transcription factor genes, which in turn increase the intracellular levels of specific transcription factors, which could then activate the promoter constructs.

Another possibility that could account for the results observed is that the RL95-2 cells produce negligible amounts of GR. The activation observed upon the addition of dexamethasone may not be due to GR/GRE interactions but to an 'indirect' effect. For example, dexamethasone inducing activating transcription factors or repressing inhibitory factors that can bind in the pGL3B vector or within pGL3B2087 and pGL3B2464 constructs. The addition of GR, could then allow GR/GRE interactions to occur.

While the results observed seem to be significant, several factors should be taken into account when considering experiments using glucocorticoids. The levels of dexamethasone used in the experiments were at pharmacological ranges (1×10^{-4} M) and well above 'physiological' levels. The levels of cortisol within the plasma are between 1.6 and 5.7×10^{-7} M with an equivalent dexamethasone concentration of $0.5 - 2 \times 10^{-8}$ M (O'Riordain *et al*, 1995). The fact that the RL95-2 cells used are not the same as 'physiological endometrial cells' and may be more glucocorticoid-insensitive (i.e reduced receptor levels, less effective signal transduction pathways) may explain the discrepancies. The minimal amounts of dexamethasone required to elicit a transcriptional response in RL95-2 cells were not tested in the presence of GR, so it was likely that with excess intracellular GR, lower amounts of dexamethasone will be required.

Synthetic glucocorticoids may differ from endogenous glucocorticoids both in receptor affinity and transcription factor interactions (reviewed in Wilckens and De Rijk, 1997), which may suggest that dexamethasone has limited value in mimicking physiological actions.

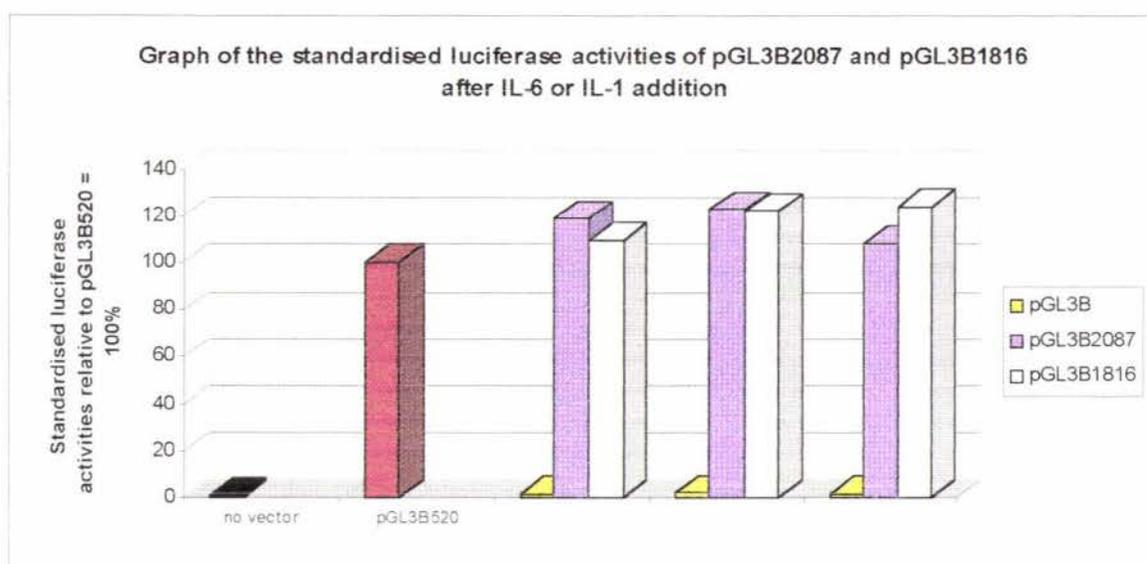
Oshima and Simans (1992) found that with hepatoma cells, there was a direct effect with growth conditions e.g. cell density (cell-to cell contact) and amount of media on the glucocorticoid-induced gene regulation. Also glucocorticoid sensitivity can vary during the cell cycle (reviewed in Burnstein and Cidlowski, 1989).

All of these potential influences should be taken into account when using synthetic chemicals to mimic biological systems.

4.7.4 Addition of IL-6 and IL-1 α

Experiments were carried out to confirm that the lack of cytokine responsiveness seen with the 2.5 kb construct was not due to the presence of a repressive element near the 5' end of the bLf promoter. Transfections in RL95-2 cells with pGL3B2087 and

pGL3B1816 were carried out in the presence of IL-6 and IL-1 α . No significant activation was seen with either cytokine with either construct (figure 40). Therefore in the context of RL95-2 cells, the bLf promoter was not responsive to cytokines even after the removal of a putative repressive region or putative GREs.



| | | | | |
|-----------------------|---|---|---|---|
| IL-6 (20 ng) | - | - | + | - |
| IL-1 α (20 ng) | - | - | - | + |

Figure 40. The effect of IL-6 and IL-1 α on the transcriptional activity of the bLf promoter in pGL3B in phenol red-free media. RL95-2 cells grown in phenol red-free media were transfected with 4 μ g of pGL3B2087 or pGL3B1816 and 2 μ g of pSV- β -gal. Cells transfected with 2 μ g pGL3B and 2 μ g of pGL2C acted as controls. Interleukin-6 or interleukin-1 α (18 hours prior to harvesting) was added in the amounts indicated. The standardised luciferase levels represent the ratio of luciferase light units to β -galactosidase absorbance values at 420 nm. The values presented are the results of 1 experiment where the experiment contained triplicates of the reporter constructs, therefore error bars have not been included. The luciferase activity of pGL3B520 was arbitrarily set at 100% and all other values adjusted accordingly.

4.8 Chapter summary

The reduction of the basal transcription rate of the pGL3B2464 construct compared to the other pGL3B constructs indicated the presence of a repressive element in the -2464 to -2087 bp region of the bLf promoter. This result also showed that the SV40 enhancer element in pGL2E was masking the transcriptional activation differences observed between the -2464 to +36 and -520 to +36 constructs in pGL2E.

The addition of cytokines to RL95-2 cells, grown on phenol red-containing media, and containing the pGL3B constructs, did not elicit any changes in promoter activity. A reduction in cytokine incubation time, the removal of insulin and the addition of NF-IL6 did not elicit transcriptional activation. When cells were grown on phenol red-free media, similar results were seen under the same conditions.

The co-transfection of an expression vector for NF-IL6 to cells grown on phenol red-free media, and transfected with pGL3B constructs, elicited transcriptional activation of the pGL3B2464 and pGL3B520 reporter constructs. As both pGL3B520 and pGL3B2464 had the same levels of activation, a NF-IL6 binding site common to both constructs (in the -520 to +36 region) was indicated.

The addition of dexamethasone to cells grown on phenol red-free media, and transfected with the pGL3B constructs, elicited an increased transcription of the pGL3B2087 and pGL3B2464 bLf promoter constructs. This indicated the importance of two putative GREs in the -1816 to -2087 bp region. The co-transfection of glucocorticoid receptor resulted in transcriptional activation, where the longer the bLf promoter construct, the greater the activation seen. Proposed theories to account for these results include weak GR binding due to excess intracellular GR, and physical interaction and inhibition of repressive factors by the GR.

Chapter 5 - Investigation of the protein factors implicated in the bLf promoter transcriptional activation.

5.1 *In vitro* binding assays with NF-IL6 and consensus sequence for NF-IL6

Co-transfection of pGL3B520 and pGL3B2464 with an NF-IL6 expression vector resulted in an increase in reporter gene expression. These results indicated that an element responsive to NF-IL6 existed in the bLf promoter in the region -520 to +36. Several putative NF-IL6 binding sites had been identified in the bLf promoter. Bain (1995), using the GCG (University of Wisconsin Genetic Computer Group) programme "Findpatterns", identified a NF-IL6 binding site at -491 (relative to transcription start point (tsp) +1). In this study, the GCG programme "Gap" identified a putative NF-IL6 binding site at position +12, while "BestFit" identified a putative NF-IL6 binding site at -386 (relative to tsp = +1)(Figure 41). The most homologous putative NF-IL6 binding site (+12) was used in an Electrophoretic Mobility Shift Assay (EMSA) to test its ability to bind to NF-IL6. COS-7 cells were transfected with an expression vector for NF-IL6 and cell extracts prepared as described in section 2.2.16.



Figure 41. Diagram of the putative NF-IL6 binding sites (*) (from Bain (1995) and this study) in the -520 to +36 region of the bLf promoter relative to tsp = +1.

NF-IL6 or HNF-4 were not available so the presence of NF-IL6 or HNF-4 could not be verified by western blotting.

Electrophoretic Mobility Shift Assays (EMSAs) were performed using an HNF-4 control oligonucleotide (see Appendix 2) which had been used previously in other studies (Crossley *et al.*, 1992) ; a control oligonucleotide for NF-IL6 (Akira *et al.*, 1990) and an oligonucleotide representing the putative NF-IL6 binding site from the bLf promoter together with the appropriate cell extract. The cell extract containing HNF-4 produced a retarded band with the HNF-4 oligonucleotide, indicating that under the transfection conditions used, sufficient detectable quantities of protein were produced to cause an electrophoretic mobility band shift (figure 42). Retarded band(s) were observed with the control cell extract, but at different position(s) from those observed with the HNF-4 cell extract. Whether this result is significant is unknown, but any further research was beyond the scope of this study.

The NF-IL6-enriched cell extract and the NF-IL6 control oligonucleotide produced a dark smear containing possibly three retarded bands (figure 42) corresponding to a complex between protein and oligonucleotide. Therefore cell extracts from COS cells transfected with the two expression vectors were suitable for EMSA. The control cell extract produced a faint retarded band(s) at a similar position to that seen with the NF-IL6 enriched cells extract (figure 42). This suggests that the COS cells contain sufficient endogenous NF-IL6 or C/EBP to be detectable under the conditions used. These retarded bands could correspond to monomers or homodimers of NF-IL6 or heterodimers of NF-IL6 with another bZIP protein binding to the oligonucleotide. NF-IL6 can homodimerize or heterodimerize with other leucine zipper-containing proteins. For example Kinoshita *et al.* (1992) have identified a protein which has high homology to NF-IL6 and can dimerize with NF-IL6. This heterodimer can bind to the same binding site as the NF-IL6 homodimer. These results have also shown that the 'experimental system' and gel shift buffer (previously used for HNF-4 gel shifts) were suitable to detect NF-IL6 binding to the appropriate sites.

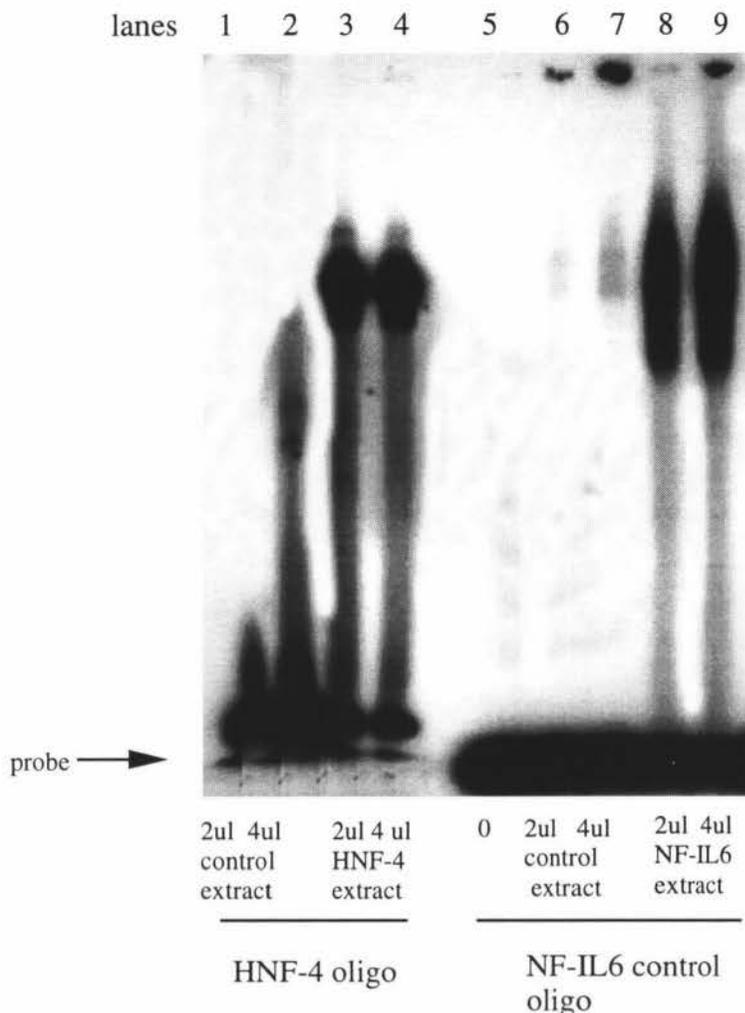


Figure 42. EMSA of COS cell extracts with control consensus sequence for HNF-4 and NF-IL6. 2-4 ug of NF-IL6-enriched HNF-4-enriched, or control cell extracts were analysed by EMSA using 1 ug of poly(dI-dC) as a nonspecific competitor. 1 ul of labelled NF-IL6- control oligo (10,000 cpm) or HNF-4 oligo (10,000 cpm) were used as probes. Electrophoresis was carried out on a 4% polyacrylamide gel 0.25 x TBE 1.5-2 hours at 200V. The gels were dried onto DE-81 membrane and exposed to x-ray film overnight at -70 C. Lanes 5 contain probe only. Lanes 1 and 2 contain 2 and 4 ul of control cell extract with HNF-4 oligo, lanes 3 and 4 contain 2 and 4 ul of HNF-4 enriched cell extract with HNF-4 oligo. Lanes 6 and 7 contain control cell extract with NF-IL6 control oligo, lanes 8 and 9 contain 2 and 4 ul of NF-IL6-enriched cell extract with the NF-IL6 control oligo.

The oligonucleotide representing a putative NF-IL6 site in bLf did not produce any retarded bands with either the control or NF-IL6-enriched extracts (figures 43). This result suggested that NF-IL6 (from either control or NF-IL6-enriched cell extracts) was not binding with sufficient strength to the putative NF-IL6 binding site. The results were confirmed by further EMSA experiments.

To ensure that the retarded bands observed with the NF-IL6-enriched extract and NF-IL6 control oligonucleotide were specific protein-DNA complexes, competition assays were performed. The addition of 10x excess (5 ng) unlabelled NF-IL6 control competitor produced a faint retarded band, which was much reduced in intensity from the retarded band produced with the NF-IL6-enriched cell extract alone. The addition of 100x excess (50 ng) or 200x excess (100 ng) unlabelled competitor totally removed the retarded bands. This indicated that the binding of NF-IL6 was specific for the control oligonucleotide. The addition of the oligonucleotide representing the putative NF-IL6 site from the bLf promoter did not result in a significant decrease of the retarded band, indicating that NF-IL6 does not bind, even weakly, to the putative oligonucleotide. Similar results were observed with the HNF-4 competitor DNA (included as a non-specific competitor) (figure 44).

These experiments showed that the putative NF-IL6 binding site at +12 of the bLf promoter could not bind NF-IL6 at detectable levels. However, there are other putative NF-IL6 binding sites present in the bLf 531 construct for example in positions -491 and -386 (figure 41), so either one of these could bind NF-IL6 and cause the activation observed.

While a functional NF-IL6 binding site was not identified in the bLf promoter using EMSA, this assay system provides a basis for future experiments such as EMSA and DNaseI footprinting.

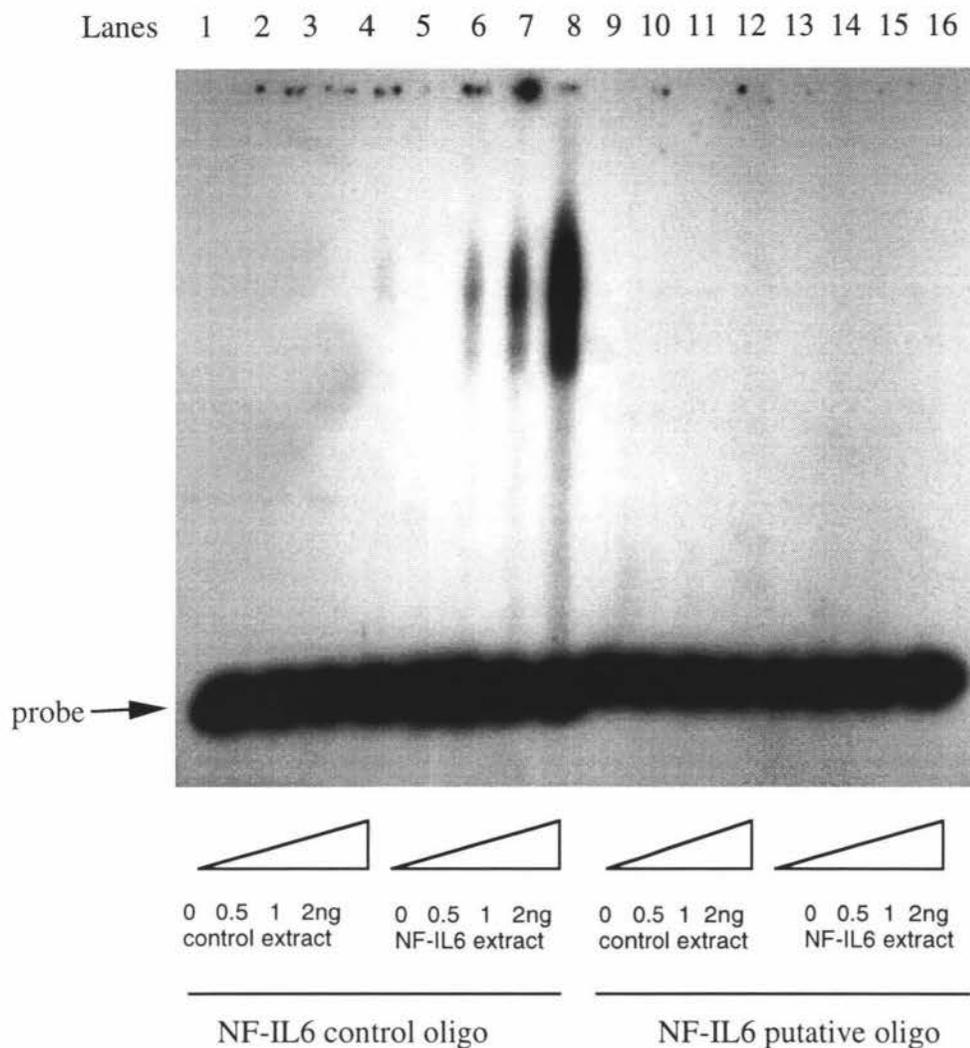


Figure 43. EMSA of NF-IL6-enriched COS cell extracts and NF-IL6 consensus sequence. 0.5 - 2 ug of NF-IL6 - enriched or control cell extract were analysed by EMSA using 1 ug of poly(dI-dC) as a nonspecific competitor. 1 ul of labelled NF-IL6 control oligo (10,000 cpm) or labelled NF-IL6 putative oligo (10,000 cpm) were used as probes. Electrophoresis was carried out on a 4% acrylamide gel with 0.25x TBE for 1.5 - 2 hours at 200 V. The gel was dried onto DE-81 membrane and exposed to x-ray film overnight at -70 C. Lanes 1, 5, 9 and 13 contain probe only. Lanes 2-4 contain control cell extract with the NF-IL6 control oligo. Lanes 6-8 contain NF-IL6-enriched cell extract with the NF-IL6 control oligo. Lanes 10-12 contain the control cell extract with the NF-IL6 putative oligo. Lanes 14-16 contain the NF-IL6-enriched cell extract with the NF-IL6 putative oligo.

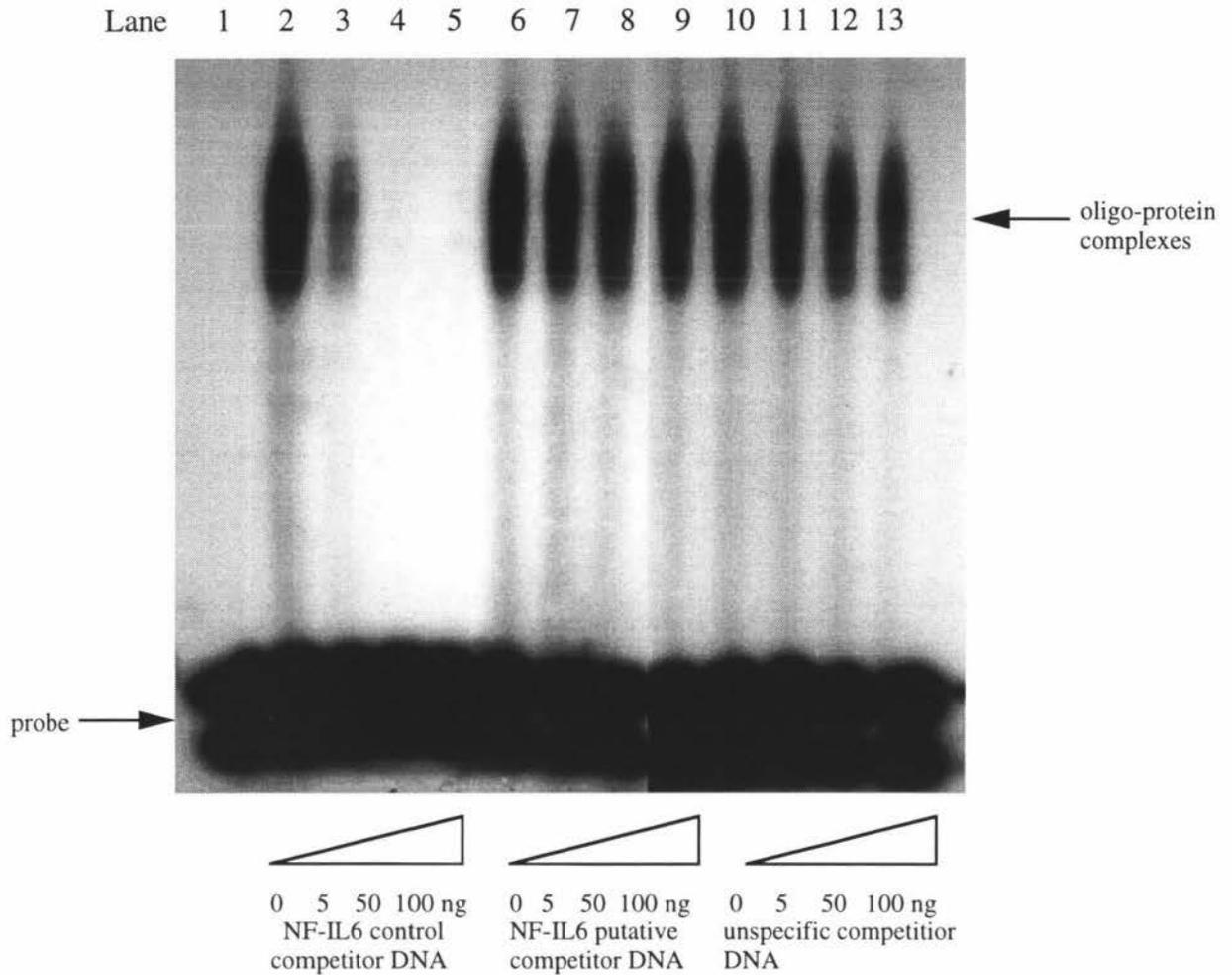


Figure 44. Competition EMSA of NF-IL6 control oligo and NF-IL6-enriched extract. 2 μ l of NFIL-6-enriched cell extract was analysed by EMSA using 1 μ g of poly(dI-dC) as a nonspecific competitor and the indicated amounts of unlabelled competitive DNA (control, putative or unspecific (1589/1590)). 1 μ l of labelled NF-IL6 control oligo (10,000 cpm) was used as a probe. Electrophoresis was carried out on a 4% acrylamide gel 0.25x TBE for 1.5-2 hours at 200V. The gel was dried onto DE-81 membrane and exposed to x-ray film overnight at -70 C. Lane 1 contains probe only. Lanes 2, 6 and 10 contain cell extract with no competitor DNA. Lanes 3, 4 and 5 contain 5, 50 and 100 ng of unlabelled NF-IL6 control DNA. Lanes 7, 8 and 9 contain 5, 50 or 100 ng of unlabelled NF-IL6 putative DNA while Lanes 11, 12 and 13 contain 5, 50 or 100 ng of unlabelled unspecific DNA (1589/1590).

5.2 Attempt to determine the cytokine-responsiveness of RL95-2 cells

The potential of the RL95-2 cells to respond to various cytokines used in this study is not known. The addition of IL-6, IL-1 α or IFN- γ to RL95-2 cells transfected with bLf promoter constructs in pGL2E and pGL3B did not cause any changes in the transcription of the reporter constructs. But the co-transfection of an NF-IL6 expression vector, a transcription factor implicated with both IL-6 and IL-1 signal transduction, produced transcriptional activation of both the pGL3B520 and pGL3B2464 constructs. This observation that NF-IL6 can activate the bLf promoter, but that the addition of IL-6 and IL-1 to the RL95-2 cells does not, may indicate that the RL95-2 cells are dysfunctional in some part of the IL-6 or IL-1 signal transduction pathways that activate NF-IL6. It would be informative to determine whether the RL95-2 cells used in this study are in fact responsive to cytokines. This knowledge would help to determine whether the negative results seen upon cytokine addition were due to the non-responsiveness of the bLf promoter constructs or because the RL95-2 cells themselves cannot respond to the cytokine. Possible reasons for non-responsiveness could include low levels of membrane-bound cytokine receptors or disruption of signal transduction pathways due to an incorrect balance of the proteins involved in signaling.

A possible method for testing cytokine responsiveness involves transfecting RL95-2 cells with a reporter construct containing a promoter known to be strongly responsive to cytokines. An appropriate change in reporter gene activity upon cytokine-addition would signal cytokine responsiveness. This method, at present, was not viable as there is still much dispute about the responsiveness of many potential cytokine-responsive promoters.

A second method involves the detection of the activation events proposed to occur during the cytokine-activated signal transduction pathway. Upon the binding of cytokines to cytokine receptors, a variety of events occur including the activation of JAK kinases. Stat proteins, which are recruited to the activated receptor, are phosphorylated on specific tyrosine residues by the activated JAK kinases. This

phosphorylation allows the dimerization and translocation of Stat proteins into the nucleus (reviewed by Ihle, 1996 ; Ihle and Kerr, 1995 ; Schindler and Darnell, 1995 ; Briscoe *et al.*, 1996). The presence of phosphotyrosine residues in the Stat proteins is an indication of the correct receptor action and a functional signal transduction pathway. But the presence of phosphorylation does not necessarily mean the correct nuclear translocation and DNA binding of the activated transcription factors.

Extracts were prepared from RL95-2 cells that had been incubated with and without cytokine. Initially an 18 hour incubation was performed but previous studies have indicated that cytokine activation is a transient and rapid event occurring within minutes of cytokine addition (Gerhartz *et al.*, 1996), therefore a time course of cytokine incubation from 1 - 20 minutes was carried out. The cell extracts were analysed by SDS-PAGE. There were no visible differences in Coomassie-stained protein bands after cytokine addition (figure 45). The proteins were electroblotted onto nitrocellulose and incubated with G410 anti-phosphotyrosine antibody and horse radish peroxidase (HRP)-labelled secondary antibody. The bound antibody was detected by chemiluminescence with ECL reagents (Amersham).

Initial results showed that some but not all of the visible protein bands were detected. With increasing incubation time, an increase in the presence of high molecular weight bands were seen. This result was obtained only once. The most reproducible result obtained is seen in figure 46 where the majority of the higher molecular weight protein bands have bound the antibodies. A protein (ovalbumin) in the ladder reacted with the ECL reagents (figure 46(a))- indicating that a non-specific interaction may have occurred with the secondary antibody.

A control experiment carried out using secondary antibody in the absence of the primary antibody, produced a reaction with the ECL reagents (figure 47). This result indicated that the secondary antibody could bind non-specifically to some of the cellular proteins. Arrows (b) and (c) on figure 46 indicate the general positions of the protein bands which bound the secondary antibody. Blocking of the membrane with 5% non-fat milk instead of 3% BSA did not promote more specific binding. Therefore, it appeared that the secondary antibody could bind to cellular proteins in the absence of

any primary antibody. As there appeared to be no significant difference between any of the cell extracts, as well as the non-specificity of the secondary antibody, these experiments could not be used to determine whether RL95-2 cells are responsive to cytokines.

More conclusive results may have been obtained with a partial purification scheme where Stat proteins were immunoprecipitated with anti-Stat antibodies. The immunoprecipitate could then be analysed by SDS-PAGE followed by western blotting and immunodetection using the G410 antiphosphotyrosine antibodies. Experiments of this type should show whether the phosphate status of Stat proteins could be influenced by treatment of RL95-2 cells with cytokines. Unfortunately, these experiments could not be carried out due to monetary constraints.

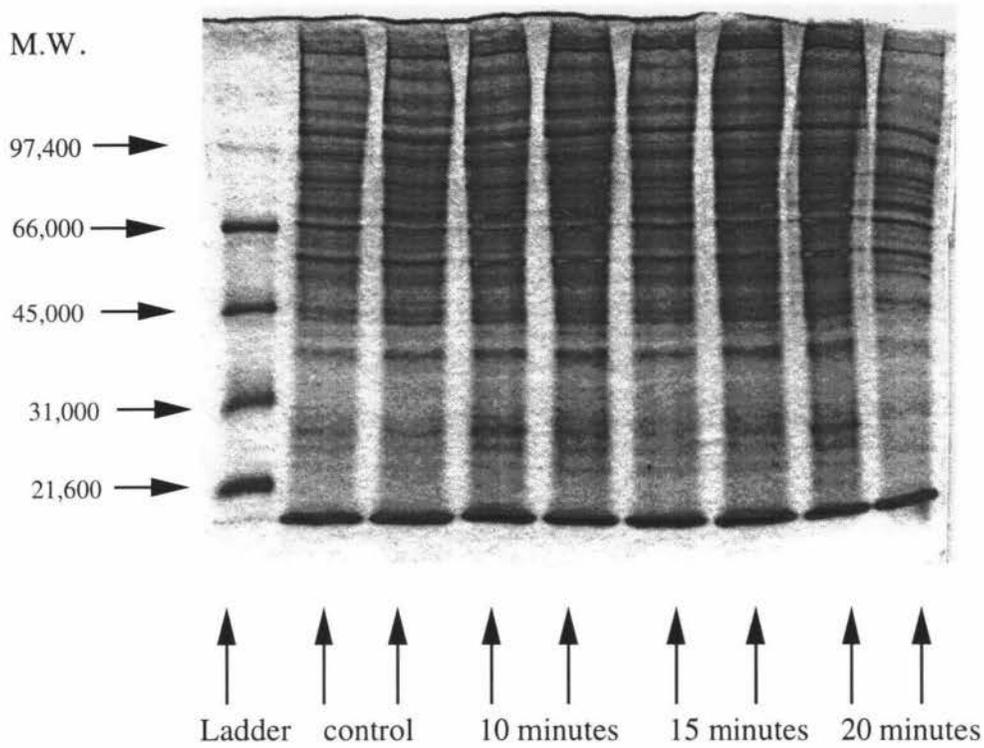


Figure 45. Electrophoresis of cellular proteins. 20 ng of IL-6 was added to RL95-2 cells for the times indicated and the cell extracts prepared as described in section 2.2.16. 10 ul of cell extract was analysed by 8% SDS-PAGE 200V for 1 hour then stained by Coomassie Blue. The control extracts did not have IL-6 addition.

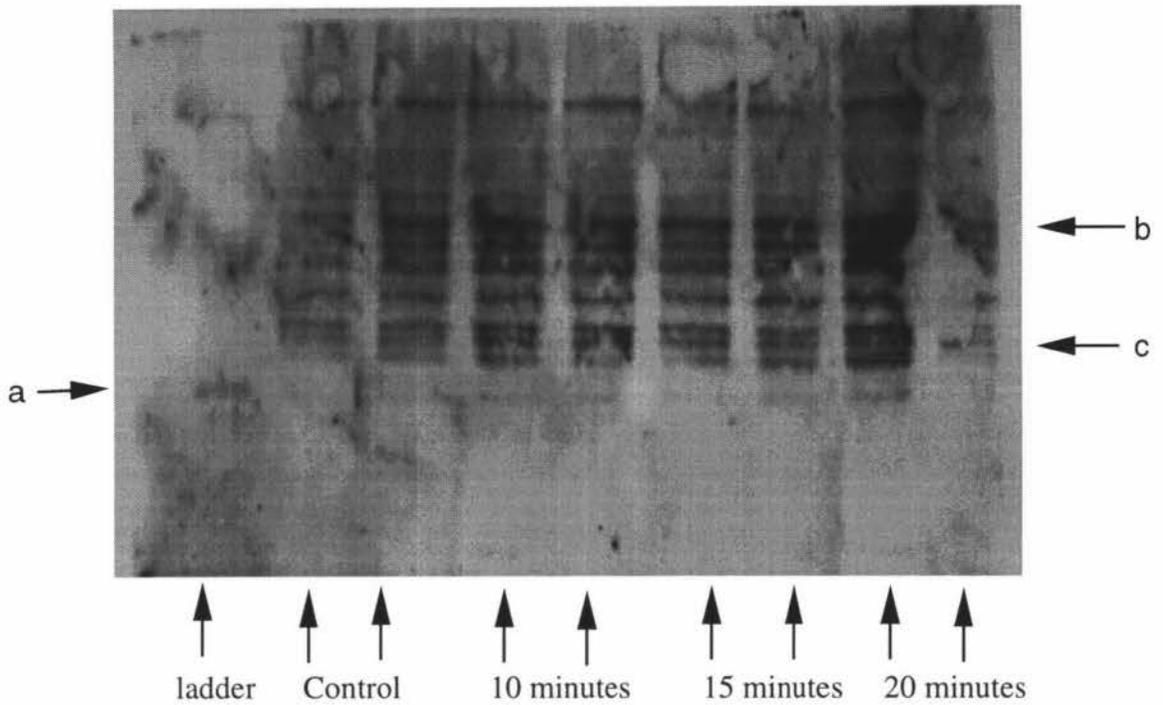


Figure 46. Western blot of RL95-2 cell extracts after IL-6 addition. 20 ng of IL-6 was added to RL95-2 cells for the times indicated and the cell extracts prepared as in section 2.2.16. 10 μ l of cell extract was separated by 8% SDS-PAGE and the proteins transferred to nitrocellulose by electroblotting for 45 minutes at 0.5 A. The membrane was blocked with 3% BSA for 30 minutes at room temperature. Primary antibody at a 1:1000 dilution were incubated with the membrane for 45 minutes. Then the membrane was incubated for a further 45 minutes with the secondary antibody. The bands were detected using ECL reagents. Variations in the density of the samples was reflected by variations in cell extract protein concentration. Arrow (a) represents a band labelled in the ladder. This protein could be ovalbumin. Arrows (b) and (c) indicate the main group of bands that the secondary antibody can bind to without the presence of the primary antibody. The control cell extract did not have IL-6 addition.

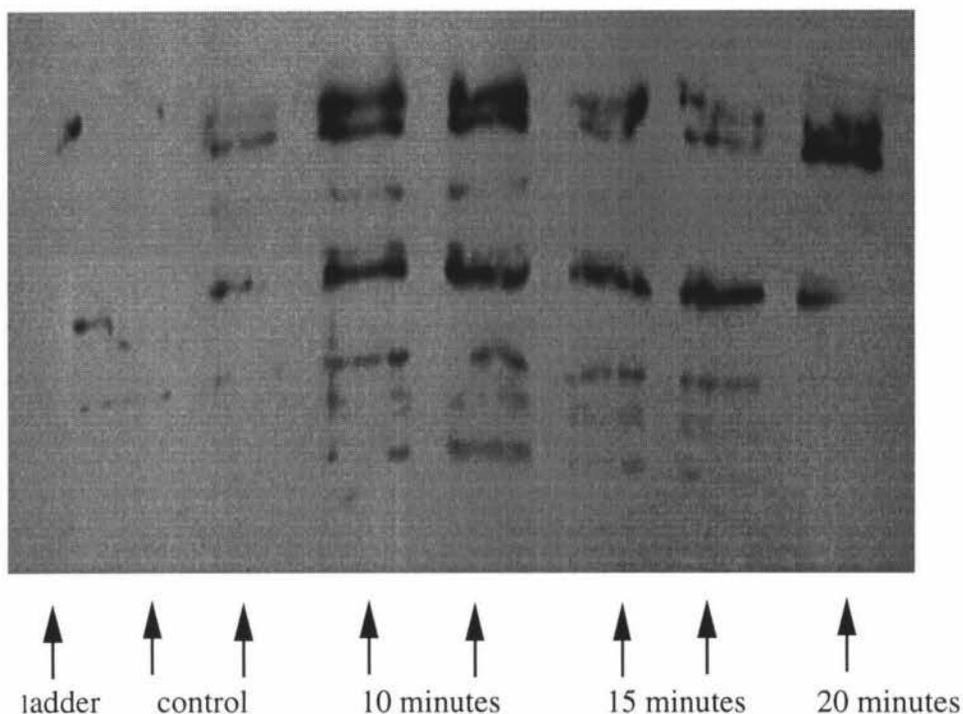


Figure 47. Non-specificity of the secondary antibody. Western blot of RL95-2 cell extract after IL-6 addition with secondary antibody only. 20 ng of IL-6 was added to the RL95-2 cells for the times indicated and the cell extracts prepared as indicated in section 2.2.16. 10 ul of cell extract was separated on 8% SDS-PAGE. The protein bands were transferred to nitrocellulose by electroblotting for 45 minutes at 0.5 A. The membrane was blocked with 3% BSA for 30 minutes at room temperature. The secondary antibody at a 1:10,000 dilution were incubated with the membrane for 45 minutes. The bands were then detected by ECL reagents. The control cell extract did not have IL-6 addition.

Chapter 6 - Summary and Future Plans

6.1 Overall Summary

The investigation into the basal transcription of the bLf promoter showed that in the pGL2E reporter vector, the 2464 (-2464 to +36 region) construct and the 520 (-520 to +36 region) construct had similar transcription rates. But in the pGL3B reporter vector, the -2464 to +36 construct had ~3x lower activity than the -520 to +36 construct, indicating a potential repressive region in the 5' part of the 2.5 kb construct. This result suggests that the SV40 enhancer element present in pGL2E appeared to 'mask' the differences in transcriptional levels between the 2.5 kb and 556 bp constructs. pGL3B2087 and pGL3B1816 had the same transcription rates as the pGL3B520 construct. This suggested that the 5' repressive element is contained within ~380 bp at the 5' end of the 2.5 kb construct (-2464 to -2087 bp region).

The addition of IL-6, IL-1 α and IFN- γ to the -2464 to +36 and -520 to +36 bLf constructs in both the pGL2E and pGL3B vectors produced no significant changes in the transcription rate. IL-6 and IL-1 α added to pGL3B2087 and pGL3B1816 also showed no significant differences in the transcriptional activity. IL-6 and IL-1 α were added together (to investigate a potential synergistic action) to all of the bLf constructs but no significant differences were observed. Phenol red (and accompanying impurities) present in the media may have potential estrogenic or cytotoxic activities. The use of phenol red-free media had no effect on the relative transcriptional activity from the bLf promoter. The removal of the insulin (a potential modulator of signal transduction cascades from the media) did not affect basal levels of the bLf constructs or cause a cytokine-induced transcriptional effect.

The co-transfection of an expression vector for NF-IL6, a transcription factor which may be involved in IL-6- and IL-1-induced signal transduction pathways, had no effect on basal transcription. In addition, NF-IL6 had no interleukin 6-induced transcriptional effect on cells grown on media containing phenol red. But in media deficient of phenol

red, a transcriptional activation was observed (relative to pGL3B) with both the 2.5 kb and 556 bp constructs in pGL3B after the addition of NF-IL6. No further activation was seen after IL-6 addition. This results indicates a common NF-IL6 binding site in the -520 to +36 bp region. A possible explanation for this result is that the phenol red (or impurities) activated estrogen receptors, which then bound to the NF-IL6 protein and prevented DNA binding. This theory could explain why NF-IL6 could activate transcription only in the absence of phenol red in the media. Electrophoretic Mobility Shift Assays (EMSA) were performed using a putative NF-IL6 binding site (+12) common to both 2.5 kb and 556 bp constructs. NF-IL6 binding was not detected with this putative binding site but other putative sites have yet to be investigated.

The addition of dexamethasone to the pGL3B2464 and pGL3B2087 constructs resulted in transcription levels twice that of the pGL3B520, pGL3B1816 and the pGL3B vector alone. The activation seen was significantly above the basal levels and confirmed the importance of two putative GREs (in the -2087 to -1816 bp region). The co-transfection of the glucocorticoid receptor expression vector into cells stimulated by dexamethasone, caused significant activation above that seen with dexamethasone alone. The results showed that the longer the construct, the greater the transcriptional activation. Various theories were proposed to account for this observation, including the additive effects of weak as-yet-unidentified GREs and the protein-protein interaction of GR with potential modulating transcription factors.

There are a number of factors that must be taken into account when using transfections and reporter gene assays to investigate mammalian gene regulation.

6.2 Factors related to transfections

There are a variety of factors which need to be considered when using transient transfections of mammalian cell lines to monitor reporter gene activity. There is an assumption that equivalent numbers of the different plasmids are taken up by a cell during transfection, and that one plasmid (e.g. the reporter plasmid) is not

preferentially adsorbed in favour of another (e.g. the β -galactosidase expression vector). Evidence that equivalent uptake may occur was noted in Ausubel *et al.* (1991) who reported that cells transfected by the calcium phosphate method tended to contain a representative sampling of the different plasmids in the precipitate. However, the effects of plasmid size, structure or sequence on the transfection process is not known. It is also assumed that transcription and translation from the genes of the different plasmids occurs independently, and that the genes of one plasmid are not preferentially expressed over the genes of the other.

The potential problems of using adherent cell culture include that non-homogeneous cell growth causing the production of gradients for the supply of certain substrates such as nutrients and added factors such as cytokines, dexamethasone and insulin. Also optimal pH and oxygen levels may also form gradients (Noe and Werner, 1993). Because of these assumptions it is necessary to carry out many replicates of transient transfection experiments in order to increase the significance of the results obtained.

6.3 Factors related to the use of reporter genes to measure transcription rates

There are also a number of potential concerns with using reporter genes to measure transcriptional activity. Firstly, the reporter system may measure enzyme activity which is only an indirect measure of transcriptional activation (Latchman, 1993). There is an assumption that enzyme activity correlates with the enzyme protein levels, and that the levels of reporter protein are related to the levels of reporter gene mRNA, and that these results correlate to activity of the promoter construct. As it is possible for regulation to occur at both the RNA processing and stability stages as well as the translation, processing or activity steps, this assumption, in some cases, may not be correct. To provide a more reliable measure of transcriptional activity that would be unaffected by translational regulation, the use of PCR has been described (Morales and Gottlieb, 1993). In this very sensitive method, PCR is used to quantitate reporter gene mRNA. This is still an indirect method to determine mRNA concentration but a linear relationship was seen between mRNA and cDNA under the experimental conditions

used. Other options include RNase protection assays, which is a commonly used method to quantitate RNA.

A second concern is that there may be potential elements within a gene that may regulate the activity of its own promoter. In the case of a reporter construct, a promoter is separated from these potential regulatory elements, and this may affect the normal activity of the promoter (Ausubel *et al.*, 1991). The effect of the removal of promoter elements from their 'natural chromatin environment' into the 'unnatural environment' of the vector DNA is not known (Alam and Cook, 1990). Another concern is that the reporter gene itself may contain elements that affect the activity of the promoters linked to it. Also, other factors which should be considered include spurious transcription of the reporter gene induced by vector sequences and specific cellular activators. The potential result of this effect is that the reporter gene activity levels seen may not truly reflect the actual activity of the promoter construct (Alam and Cook, 1990). In addition, functional assays need to be supported with *in vitro* binding assays such as EMSA and DNaseI footprinting. These potential effects indicate the importance of controls such as : the reporter gene vector without the promoter, which could indicate vector-induced reporter gene activation and the use of a reference constant to all transient transfection experiments which can reduce any variation seen between individual transfections.

All of these factors should be considered when analysing the results of transfection and reporter gene experiments. Nevertheless, transient transfections of reporter gene constructs remain a very useful and simple assay system with which to analyse promoter activity, and provide a starting point for additional studies.

6.4 Future plans

While the results of this research are significant, they are preliminary, and further research is needed to confirm these results and relate their biological relevance to the actual situation in inflamed mammary tissue. The following sections outline proposed experiments which may help to elucidate the mechanisms involved in the transcriptional activation of the bLf promoter.

6.4.1 Further investigation into the proposed repressive area in the 5' ~380 bp region of the putative bLf promoter

The repressive element which is present in the -2464 to -2087 bp region of the bLf promoter should be investigated further. The exact identity and position of this repressor could be determined by the production and subsequent reporter gene expression of a narrow range of 5' deletion mutants. Once the specific sequence has been identified then computer searches of lactoferrin promoters from other species may indicate whether the repression is likely to be common to all lactoferrin genes or unique to bovine lactoferrin. This would lead into a subsequent study aimed at characterising the mechanism of repression. The isolation of the 380 bp fragment to use in experiments to see if this region can also act as a repressor with a heterologous promoter, may also help to elucidate the repression mechanism. Types of repression could include the removal of an activator (either by transcriptional or translational repression or a posttranslational modification or protein-protein interaction) or the induction of an inhibitor. Also chromatin structure may have an effect.

To investigate whether the mechanism of transcriptional repression identified with the hLf promoter (Khanna-Gupta *et al.*, 1997) is similar to the mechanism of bLf promoter repression, *in vivo* functional assays and *in vitro* binding assays could be carried out to see if the CDP/cut protein can interact in the -2464 to -2087 bp region of the bLf promoter. The results of these experiments may help to confirm whether the

mechanism for bLf repression is similar to that for hLf repression, which involves CDP/cut protein binding to the hLf repressive region.

6.4.2 Further investigation into RL95-2 cells responsiveness to specific cytokines

One important result that needs to be clarified is whether the RL95-2 cells are responsive to IL-6, IL-1 α and IFN- γ . If the cells are not responsive, then the lack of promoter activation or inhibition seen after cytokine addition could be attributed to the absence of the correct signal transduction pathways. Once the cells are known to have sufficient receptors and active cytokine-induced signal transduction pathways, it could be concluded that the bovine lactoferrin promoter is unaffected by cytokines at least in the reporter gene assay system used in this study.

Attempts, using antibodies, directed at identifying the activated state of Stat proteins proved inconclusive as the negative control using only secondary antibody also produced a positive result. A modification of this protocol where immunoprecipitation with a Stat antibody is carried out prior to addition of the anti-phosphotyrosine antibody may overcome this difficulty. In the future, after more extensive research into cytokine action has been performed, the use of a reporter gene attached to a known and verified cytokine-responsive promoter may also be an option. The use of immunofluorescence and confocal microscopy to detect cell surface cytokine receptors on the RL95-2 cells would indicate the presence although not the functionality of the cytokine receptors.

6.4.3 Further investigation into NF-IL6-induced transcriptional activation of the bLf promoter

Further work is needed to confirm and extend the result where transcriptional activation of bLf constructs in pGL3B was observed after the co-transfection of MSV-C/EBP β (NF-IL6) into cells grown in phenol red-free media. Further EMSAs could be performed with the other putative NF-IL6 binding sites identified in the bLf promoter or DNase I footprinting could be used as a more global detection system for NF-IL6 binding sites on the bLf promoter. The absence of transcriptional activation in the presence of phenol red-containing media could be studied further by determining the effect of the addition of the estrogen receptor expression plasmid in *in vivo* functional assays. EMSA or DNase I footprinting with NF-IL-6 could be carried out in the presence and absence of ER protein. EMSAs with cell extracts prepared from RL95-2 cells grown on phenol red-containing media may indicate if sufficient estrogen receptors (potentially induced by the phenol red preparation) are present to inhibit NF-IL6 binding to a common consensus sequence. The detection of ER levels (using antibodies to detect ER protein or RT-PCR to detect ER mRNA levels) in cells grown with or without phenol red, may show variations in the amounts of ER which correspond to the presence of phenol red.

6.4.4 Further investigation into the dexamethasone and glucocorticoid-induced transcriptional activation of the bLf promoter

EMSAs and DNaseI footprinting could be carried out to determine if the glucocorticoid receptor can bind to the putative GREs identified by the *in vivo* functional assays. Variation of the conditions used may determine whether multiple GR/GRE binding occurs at different strengths, and at as-yet-unidentified sites. The use of cell extracts from dexamethasone-activated RL95-2 cells may indicate if sufficient GR was normally present in the RL95-2 cells to cause GR/GRE binding. This information could indicate whether the activation seen with dexamethasone alone and with dexamethasone together with the expression vector RSV-GR is by the same

mechanism. Also placing the putative GREs before a heterologous promoter may determine whether the GREs can directly act as enhancers.

6.4.5 Further experiments under more physiological conditions

To determine whether the above experiments with RL95-2 cells are physiologically valid, investigation of the bovine lactoferrin promoter in a more physiological environment should be considered. The RL95-2 cells are endometrial cells and not bovine mammary cells where the increase in lactoferrin gene expression is observed. It needs to be established that the transcriptional activation seen with NF-IL6 and dexamethasone can also be reproduced in mammary cells. This should be possible as mammary cells have been shown to be responsive to glucocorticoids, IL-6 and IL-1 β (Warburton *et al.*, 1993 ; Jahn *et al.*, 1987). It will then need to be determined how the transcriptional activation observed can be related to the NF-IL6 and dexamethasone-induced activation. The activation of the endogenous bovine lactoferrin gene by the glucocorticoids or cytokines would also provide more definite evidence of a significant physiological effect. Experiments such as time course northern blots could be used to investigate the expression of the glucocorticoid receptor in comparison with the expression of lactoferrin during the normal mammary gland cycle and in response to infection. If glucocorticoids are important in lactoferrin gene regulation, a correlation should be seen between GR and lactoferrin gene expression.

The production of bovine mammary tissue culture cells has many problems including the lack of tissue availability, the influence of the cell preparation on the cells, the difficulty of the preparation process and the lack of definition of the cell culture conditions (described in Talhouk *et al.*, 1993). But some experimental systems using primary bovine mammary cells have been described which could be used to observe the effects of cytokines, glucocorticoids or NF-IL6 on the regulation of the lactoferrin gene under a more physiological environment. Talhouk *et al.* (1993) described a system where bovine mammary epithelial cells were cultured that could synthesize and secrete lactoferrin. Hurley *et al.* (1994) used explants of bovine mammary tissues to

investigate the production of lactoferrin. The addition of endotoxin and TNF- α to these cells inhibited the synthesis of lactoferrin under the conditions used.

The most 'physiological' system to investigate lactoferrin regulation was used by Rejman *et al.* (1995) where the mammary glands of cows were infused with IL-1 β and the levels of lactoferrin in the mammary secretions were observed. A similar system could be used to investigate cytokine and glucocorticoid action on the bovine lactoferrin promoter.

In conclusion, there is much further research that needs to be performed to elucidate the mechanisms of transcriptional activation of the bLf gene. This proposed research will help to confirm the relevance of the results seen in this research. The observations from this study and the proposed future work may be important to elucidate mechanisms of eukaryotic transcriptional regulation and mechanisms involved in the inflammatory response. But more specifically, the experimental observations from this research may help to determine the mechanism of regulation of bovine lactoferrin and to confirm the role of lactoferrin in inflammation.

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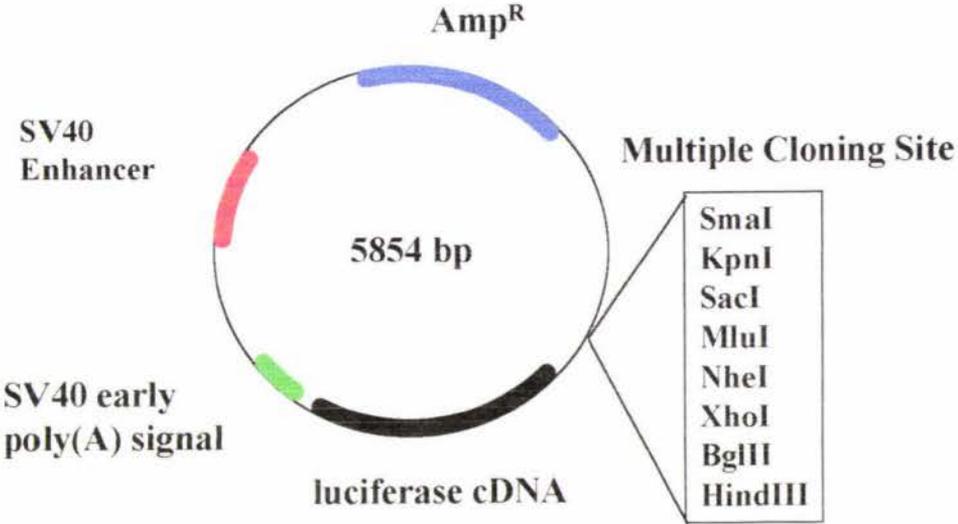
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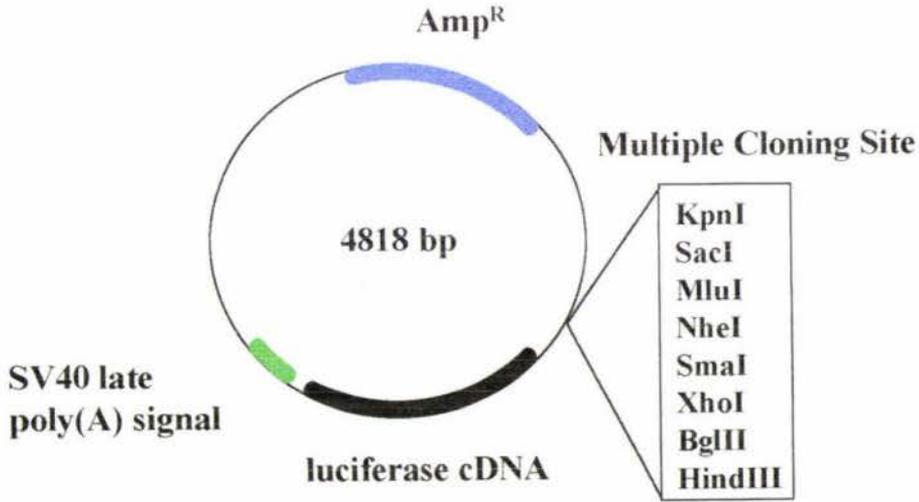
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Appendix 1.

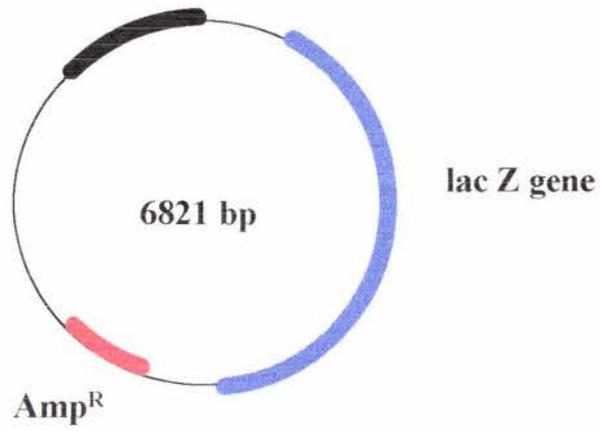


pGL2-Enhancer Vector



pGL3-Basic Vector

SV40 promoter and enhancer



β -galactosidase vector

Appendix 2.

HNF-4 oligonucleotide

LF-A1/HNF4 site in the α_1 antitrypsin gene promoter

1589 GAC TCA GAT CCC AGC CAG TGG ACT TAG CCC CTG TTT

1590 AAA CAG GGG CTA AGT CCA CTG GCT GGG ATC TGA GTC