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The Bovine Lactoferrin Gene;
Defining the Minimal Promoter Region

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requirements for the degree of Master of Science in Biochemistry

Dedication

This thesis is dedicated to the memory of my grandfather,
Ted Bonney.

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Abstract

Lactoferrin is an 80 kDa glycoprotein with two lobes, each of which bind a single iron atom. Originally isolated from milk, lactoferrin has since been identified in a variety of exocrine secretions and in the secretory granules of neutrophils. A number of functions have been proposed for lactoferrin, some of which are related to the capacity of the protein to bind iron tightly but reversibly. The proposed functions include iron transport in the gut, antimicrobial activity and modulation of the activity of the immune system.

The synthesis of lactoferrin in the mammary gland is developmentally regulated with changes in protein concentration in milk being correlated with changes in lactoferrin mRNA in mammary tissue. Most species studies have identified high levels of lactoferrin during involution and late pregnancy into early lactation. The amounts of lactoferrin during the lactational phase are much lower, especially in bovine milk. The regulation of bovine lactoferrin expression was studied in the belief that knowledge of the factors influencing expression will provide insight into the function of lactoferrin in the mammary gland.

A 2.5 kb fragment of bovine genomic DNA, including the region immediately upstream of the transcription start point, has been subcloned into luciferase reporter gene vectors. The 2.5 kb fragment has been sequenced and a number of putative response elements identified. Promoter activity was tested by transient expression in the human endometrial carcinoma cell line RL 95-2. 5'- and 3'- deletion analysis of the promoter was used to establish regions which confer transcriptional regulation and the minimal promoter region.

A recent report on the sequence of the cDNA for caprine (goat) lactoferrin suggests that the transcription start point for the mRNA for this protein may be further upstream than that reported for the mRNA of bovine lactoferrin. In view of the high level of sequence identity between the two cDNA's in this region an attempt was made to reinvestigate the transcriptional start point for bovine lactoferrin using DNA footprinting.

Abbreviations

AP-1	activator protein 1
bp	base pair
β -Gal	beta galactosidase
BRL	Bethesda research laboratories
BSA	bovine serum albumin
CAP	calf alkaline phosphatase
CAT	chloramphenicol acetyl transferase
COUP-TF	chicken ovalbumin upstream promoter-transcription factor
Da	Dalton
DES	diethylstilbestrol
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribose nucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetate
EGF	epidermal Growth Factor
ERE	estrogen response element
ERM	estrogen response module
FCS	foetal Calf Serum
IL-6	interleukin 6
IL-8	interleukin 8
kb	kilobase
kDa	kilo Dalton
MGF	mammary gland factor
mRNA	messenger RNA
NF-IL 6	nuclear factor interleukin 6
NF-IL 8	nuclear factor interleukin 8

NF- κ B	nuclear factor kappa B
nt	nucleotide
Oct-1	octamer transcription factor-1
OD	optical density
ONPG	<i>o</i> -nitrophenyl- β -galactoside
PBS	phosphate buffered saline
PCR	polymerase Chain Reaction
RNA	ribose nucleic acid
RNase	ribonuclease
SDS	sodium dodecylsulphate
STAT	signal transducer and activator of transcription
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBP	TATA-box binding protein
TE	Tris EDTA
tRNA	transfer ribose nucleic acid
UTR	untranslated region
UV	ultraviolet

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Chapter One: Introduction

1.1 Lactoferrin

Bovine lactoferrin, like other members of the transferrin family, is a glycoprotein with a single polypeptide chain of approximately 700 amino acids and a molecular mass of close to 80kDa. The structure of lactoferrin has been well studied by X-ray crystallography which has demonstrated that the protein is bilobal in nature, having N-terminal and C-terminal lobes (Anderson *et al.*, 1987). Each lobe consists of two domains between which a ferric ion (Fe^{3+}) and a bicarbonate ion are bound. There is high internal homology between lobes which evoked the hypothesis that the 80kDa lactoferrin arose through the duplication of an ancestral gene.

The sequence and structure of lactoferrin places it in the class of transferrin iron binding proteins which include serum transferrin, ovotransferrin and melanotransferrin. Lactoferrin was originally isolated from human milk and bovine whey (Johanson, 1960) and has subsequently been found in external fluids such as tears, saliva and other exocrine secretions (Lönnerdal *et al.*, 1976; Masson *et al.*, 1966). Lactoferrin has also been shown to be present in the leukocytes (Baggiolini *et al.*, 1970; Hurley *et al.*, 1993). Investigation of milk from a variety of mammals, including humans, goats, pigs, horses, mice and cattle, has identified lactoferrin as a significant component. Due to the similarities to the structure and properties of serum transferrin, the biological properties of lactoferrin are thought to be related to its tight but reversible binding of iron. Although lactoferrin is distributed in a wide range of mammalian tissues, no clear role has been demonstrated for its physiological function. Researchers have proposed several functions for lactoferrin including bacteriostatic and bacteriocidal activity, intestinal iron absorption and modulation of the inflammatory response (reviewed in Sánchez *et al.*, 1992 and Jones *et al.*, 1994).

The promoter regions of the mouse and human lactoferrin genes have been isolated and studied extensively, demonstrating that the expression of lactoferrin is

hormonally regulated in the reproductive organs of these species. Of the two, mouse lactoferrin expression has been studied in greater depth.

1.2 Lactoferrin Expression in the Mouse

Mouse lactoferrin was originally isolated from uterine luminal fluid (Teng *et al.*, 1986). Experiments on immature mice demonstrated that estrogen dramatically increased lactoferrin mRNA expression and protein secretion in the uterus. Progesterone and testosterone, other candidate hormones known to regulate the reproductive tract, had no effect (Pentecost & Teng, 1987). Evidence which supported the regulation of lactoferrin by estrogen *in vivo*, was obtained by measuring lactoferrin mRNA and protein levels in the reproductive tract of adult mice at different stages in the estrous cycle. Again lactoferrin expression increased concurrently with high estrogen levels (Newbold *et al.*, 1992, Walmer *et al.*, 1992).

Further study of lactoferrin expression from mouse uterine endothelium compared the effect of other hormones involved in the estrous cycle (McMaster *et al.*, 1992) and demonstrated that estrogen stimulation was blocked by progesterone, but the action of progesterone alone did not reduce lactoferrin mRNA levels. This observation led to the hypothesis that the level of lactoferrin expression in the uterus is controlled by a balance of estrogen and progesterone (McMasters *et al.*, 1992). During the estrus cycle the highest levels of estrogen occur at the time of mating and implantation of the fertilised egg. It has been suggested that the elevated levels of lactoferrin may protect the mucosal surfaces from infection and from inflammatory agents which may have been introduced into the uterus during mating (Dalton *et al.*, 1994).

The expression of lactoferrin in the mouse is tissue specific. *In vivo* studies of adult mouse mammary glands demonstrated that the expression of lactoferrin mRNA and protein was not under the control of estrogen as occurs in the mouse uterus (Teng *et al.*, 1989). This supports the evidence of Green and Pastewaka, (1978) who demonstrated that there was an upregulation of lactoferrin in response to prolactin but not estrogen in mammary explants in pregnant mice. In liver cells the estrogen

receptor is present but lactoferrin is not expressed indicating that other factors must also be involved in the regulation of lactoferrin gene expression.

The promoter region of mouse lactoferrin has been isolated and characterised, and sequence analysis has identified several putative *cis*-acting elements (Liu and Teng, 1992). A deletion analysis of the mouse lactoferrin promoter using CAT assays of cell extracts from transiently transfected human endometrial cells (RL 95-2), demonstrated that the minimal promoter region was between nucleotides -234 to +1 relative to the transcription start point. A CAT reporter gene containing 600 bp of the 5' region of the mouse lactoferrin promoter demonstrated estrogen responsiveness when co-transfected with the estrogen receptor and the estrogen hormone. This length of sequence was found to contain an imperfect estrogen response element (ERE) overlapping a chicken ovalbumin upstream promoter (COUP) element beyond the boundary of the minimal promoter region at position -349 to -329 relative to the transcription initiation site (Liu and Teng, 1991). Electrophoretic mobility shift assays and DNase I footprinting experiments using this region of mouse lactoferrin promoter sequence demonstrated that the COUP transcription factor (COUP-TF) and the estrogen receptor compete directly for this element (Liu and Teng, 1992, Liu *et al.*, 1993). The identification of the estrogen response element and the binding of the estrogen receptor to this element support the *in vitro* and *in vivo* evidence of lactoferrin expression in the uterus but is insufficient to explain the tissue specific regulation of lactoferrin in the mouse.

Many other factors have been identified as having a role in controlling lactoferrin expression in the mouse. These include epidermal growth factor (EGF), which mimics estrogen in its stimulation of lactoferrin mRNA and protein levels (Nelson *et al.*, 1991). EGF is present in the mouse uterus and its production and that of its receptor is stimulated by estrogen. Nelson *et al.*, (1991) proposed that EGF acts as a local effector to amplify the expression of lactoferrin in response to estrogen *in vivo*. An EGF response element has been identified within the mouse lactoferrin promoter and reporter gene analysis indicates that the action of the response element is promoter specific (Shi and Teng, 1996). *Cis*-elements responsive to cyclic AMP

(cAMP) have been identified in the mouse lactoferrin promoter (Shi and Teng, 1994). One of these lies within the minimal promoter region and acts with a noncanonical TATA box to give the basal activity of reporter genes observed in RL 95-2 cells (Shi and Teng, 1996). Further characterisation of this promoter will be required to identify the molecular mechanisms responsible for the tissue specific regulation of lactoferrin in the mouse.

1.3 Lactoferrin Expression in Humans

Lactoferrin has been identified in the secondary granules of neutrophils (Rado *et al.*, 1984), in milk (Johanson, 1960) and breast carcinomas (Campbell *et al.*, 1992). Promoter sequences for human lactoferrin have been isolated from the DNA of placenta and human promyelocytic leukaemic cells (HL 60 cells) which do not normally express lactoferrin (Johnston *et al.*, 1992 and Teng *et al.*, 1992).

On comparison of the human and mouse promoter sequences several *cis*-acting elements were found to be conserved. Of particular interest was the conservation of the ERE overlapping the COUP element. Mobility shift assays and DNase I footprinting experiments similar to those used for the mouse lactoferrin promoter were performed on the human lactoferrin promoter. In contrast, there was no competition between the estrogen receptor and the COUP-TF for these sequences in the human promoter (Teng *et al.*, 1992). This was thought to be due to a one base-pair difference between the sequences of mouse and human lactoferrin promoter in this region. The ERE response element is imperfect and recent evidence suggests that the interaction of an estrogen related receptor hERR1 stabilises the binding of the estrogen receptor which then modulates lactoferrin expression (Yang *et al.*, 1996). The major conclusion from these experiments is that lactoferrin is under the control of both species-specific and tissue-specific mechanisms.

Human lactoferrin gene sequences have been used to create transgenic mice which secrete recombinant human lactoferrin in milk which differs from natural lactoferrin only in the glycosylation pattern (Nuijens *et al.*, 1997).

An alternative RNA molecule for human lactoferrin has been isolated in breast, placenta, lung, uterus, and a wide variety of other tissues, as opposed to RNA isolated from carcinoma cell lines (Siebert and Huang, 1997). The authors of this work suggest a truncated message, which does not contain exon I, may be derived from alternative splicing of the original transcript or through transcription from an alternative promoter within exon I. This may provide different protein trafficking signals for secretion and cellular localisation or a tissue specific function for the lactoferrin protein.

1.4 Regulation of Lactoferrin in the Mammary Gland

The Lactation Cycle

The supply of nutrients to the young of mammals through the mammary gland is regulated by complex interactions among pituitary, adrenal, ovarian and placental hormones to control mammary growth and differentiation (lactogenesis). Through these mechanisms, milk content and supply are co-ordinated with the development of the neonate. The changes that occur during the pregnancy, suckling and drying off period are described as the lactation cycle (Figure 1). Mammary glands undergo several physiological transitions within a lactation cycle. The cycle begins with a period of mammary development (mammogenesis), during which time the cells of the gland differentiate such that the mammary epithelia develop the correct biochemical machinery necessary for lactation. This is followed by lactogenesis which is the transition to active milk synthesis. Colostrum, the initial mammary secretion at lactogenesis, contains high concentrations of immunoglobulins which are important in conferring resistance to the new born and the mammary gland. The transition to an active state of milk synthesis marks the beginning of lactation.

Milk synthesis and secretion increases to a production peak, then declines until milk removal is stopped either at weaning or, in the case of the dairy animal due to management practices. Following weaning the mammary gland undergoes involution, a process whereby the gland undergoes major morphological, ultrastructural and

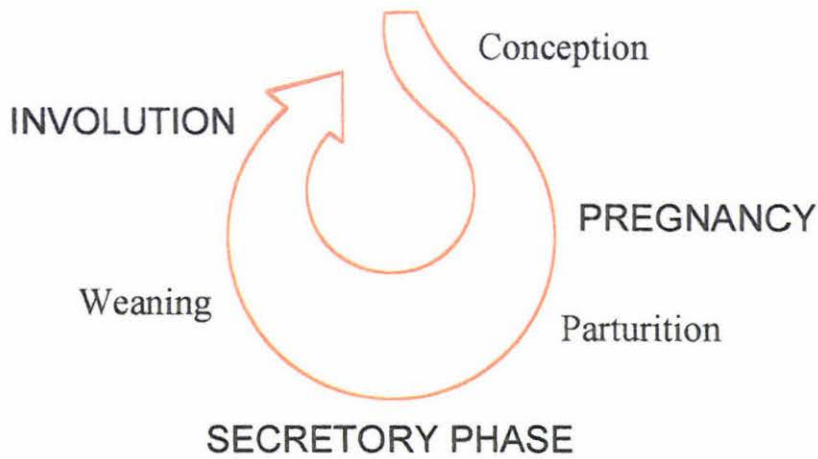


Figure 1: The Lactation Cycle

functional changes. The epithelial cells rapidly form large vacuoles filled with unsecreted milk components and there is rapid decline in synthetic and secretory functions. (Holst *et al.*, 1987).

These functional changes are reflected in the changing composition of lacteal secretions. For example, milk specific proteins in the cow, such as casein, α -lactalbumin and β -lactoglobulin concentrations decrease while serum derived proteins such as albumin and immunoglobulins increase along with lactoferrin synthesised in mammary epithelia. Involution results in a mammary gland structure with the potential to redevelop ductules and alveoli for the next lactation cycle (Hurley, 1988, 1989). The transition to active milk synthesis and to involution is associated with an increase in susceptibility to intramammary infection (Hurley., 1988).

Regulation of Lactoferrin in the Mammary Gland

Bovine lactoferrin was initially identified in the whey component of bovine milk (Sørensen and Sørensen, 1939). Since then the main focus has been on the expression of lactoferrin at the different stages in the lactation cycle; development, lactation and involution (Schanbacher *et al.*, 1993; Hurley and Rejman, 1993). Schanbacher *et al.*, (1993) compared the secretion of lactoferrin with that of α s1-casein in the mammary gland (Figure 2). Lactoferrin levels were high during pregnancy and development of the mammary gland, decreased at birth and were very low during lactation. At involution the level of lactoferrin secretion increased dramatically to a peak. The secretion of α s1-casein appeared to be the opposite of this, with highest levels being observed during lactation. The level of bovine lactoferrin expression has been compared to the level of human lactoferrin expression (Table 1). Both show a similar pattern of secretion throughout the lactation cycle but there are marked species differences in the absolute level of lactoferrin secretion especially during lactation where bovine lactoferrin levels are 1/10 of human lactoferrin levels. The level of lactoferrin synthesis has been shown to increase during the lactation phase only if intramammary infection occurs (Harmon *et al.*, 1976; Hurley and Rejman, 1993). Bovine lactoferrin inhibits the growth of coliform bacteria associated with bovine mastitis. This effect has been attributed the ability of lactoferrin to sequester iron, rendering it unavailable for utilisation in bacterial growth (Bishop *et al.*, 1976). Bovine lactoferrin can also damage the outer cell membrane of some gram-negative bacteria by releasing lipopolysaccharide molecules and changing cell permeability (Ellison & Giehl, 1991).

	Human (mg/mL)	Bovine (mg/mL)
Development	6-15	2-10
Lactation	1-2	0.02-0.2
Involution	>40	>20-100

Table 1: Comparison of the Lactoferrin Levels in the Mammary Gland at Different Stages in the Lactation Cycle in Humans and Cattle (Schanbacher *et al.*, 1993)

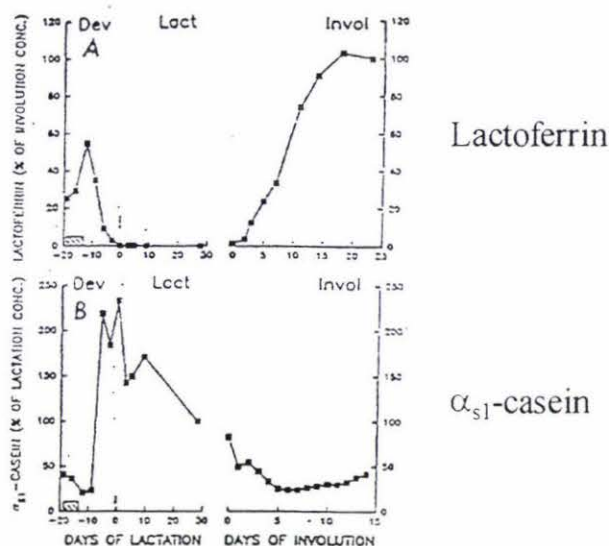


Figure 2: Lactoferrin and α_{S1} -Casein Expression Throughout the Lactation Cycle (Schanbacher *et al.*, 1993)

Northern hybridisation analysis was used to identify the mechanism of lactoferrin regulation (Figure 3; Schanbacher *et al.*, 1993). This was also used to determine whether the high level of lactoferrin expression seen at involution was not just an accumulation effect. Mammary glands were sampled at different stages of the lactation cycle and analysed for mRNA expression of the major milk proteins including lactoferrin. The expression of lactoferrin mRNA appears to parallel the secretory pattern of the protein and is inversely proportional to the expression of the other milk specific protein mRNAs assayed. Expression of lactoferrin mRNA was high at mammogenesis and parturition, low during lactation and peaked during involution. The mRNAs for most of the milk proteins persisted well into involution, long after milk synthesis had ceased, except for α -lactalbumin which was down-regulated early in involution. α -lactalbumin is not only a major milk protein but is also the rate-limiting protein component of the lactose synthetase, the enzyme responsible for lactose synthesis (Akers, 1985) (Lactose is the major carbohydrate component of milk.). Therefore the expression of α -lactalbumin mRNA is an excellent indicator of lactogenesis. The expression of the other milk proteins

including the caseins and β -lactoglobulin, also appear to parallel their secretion profiles. Schanbacher *et al.*, (1993) suggest that the persistence into involution may indicate long term stability of these mRNAs. Taken together, the evidence presented in the secretion profile and northern hybridisation analysis indicate that lactoferrin is developmentally regulated and the data in Table 1 also suggests that the regulation of lactoferrin expression is species-specific.

Lactoferrin in the bovine mammary gland can be derived from mammary epithelial cells or from neutrophils. Therefore, the changes in the amount of lactoferrin protein may stem either from the hormones controlling the lactation cycle or factors controlling the immune response, or a combination of the two. Candidate hormones for regulation of expression of lactoferrin include prolactin, estrogen and progesterone.

Prolactin is a protein hormone normally secreted in a pulsatile fashion from the pituitary gland, but at parturition a surge of secretion occurs in conjunction with the onset of lactation. The prolactin receptor has a tyrosine kinase activity and is similar to the insulin and growth hormone receptors. Prolactin has been shown to stimulate the expression of lactoferrin in the mouse mammary gland but *in vitro* tissue culture experiments by Schanbacher *et al.*, (1993) demonstrated that prolactin treatment could not induce the expression of lactoferrin in primary bovine mammary cells. Prolactin does have positive influences on the expression of milk proteins, in particular α -lactalbumin and the caseins (Akers, 1985). As previously described, estrogen has a positive effect on the expression of lactoferrin in the mouse uterus. There is no documented evidence for the expression of bovine lactoferrin in the uterus. The absence of the ERE/COUP element (Seyfert *et al.*, 1994), suggests that it is unlikely that the estrogen receptor will bind to the bovine lactoferrin promoter and regulate transcription.

It has been postulated that progesterone may play a role in regulating lactoferrin expression in the bovine mammary gland (H. Bain, 1995).

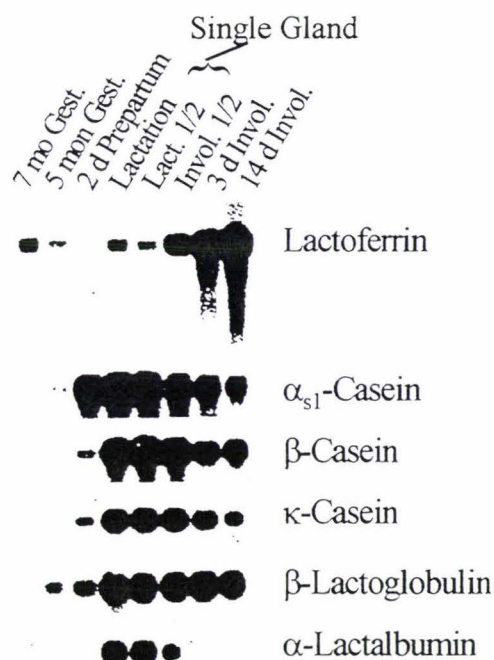


Figure 3: Northern Hybridisation of Major Milk Proteins and Lactoferrin (Schanbacher *et al.*, 1993) The first three lanes in this figure are the result of samples taken during pregnancy until two days before birth. The fourth lane is the result from fully lactating mammary gland tissue. The lanes with the parenthesis above are a special case where one half of the gland has been milked (Lact. 1/2) and the other unmilked and left to involute (Involu. 1/2). The last two lanes are the results of samples taken at different stages from a naturally involuting gland.

1.5 Bovine Lactoferrin

The coding sequence for the bovine lactoferrin gene is dispersed over 17 exons spanning 34.5 kb of genomic DNA (Seyfert *et al.*, 1994). The sequence displays evolutionary conservation of exon sizes and their contribution to the various domains of the protein molecule in all species. A genomic clone of bovine lactoferrin containing ~2.5 kb of DNA 5' to the lactoferrin coding sequence has been isolated and sequenced (H. Bain, 1995). The sequence was analogous to that of Seyfert *et al.*, (1994) but also includes additional 1000 bp upstream of the 5' end as reported by Seyfert *et al.*, (1994) and constitutes the promoter region of the bovine lactoferrin

gene. A comparison with the promoter sequences of mouse and human lactoferrin genes shows sequence elements longer than 10 bp with more than 80% homology at ten locations. Overall the bovine lactoferrin sequence shows a higher degree of homology to the human than the mouse promoter sequence (Seyfert *et al.*, 1994) but does lack some of the sequence motifs for transcriptional enhancers found in the promoters of both human and mouse genes. In particular the COUP/ERE sequence responsible for hormonal regulation in the uterus of the mouse is absent from the bovine lactoferrin promoter.

Two reporter genes were prepared from the genomic clone, containing approximately 2500 bp and 565 bp of the promoter region of bovine lactoferrin. The shorter construct was used in transient transfection experiments in COS cells. These experiments demonstrated that the bovine lactoferrin promoter sequence was active in the physiological system provided by these cells (H. Bain, 1995). Computer sequence homology searches identified putative transcriptional regulatory elements in the bovine lactoferrin promoter. These included nuclear factor interleukin-6 and progesterone receptor consensus sequences. These may be of particular relevance to the regulation of the lactoferrin gene in mammary tissue throughout the lactation cycle, and during infection e.g. mastitis (Harmon *et al.*, 1976).

Lactoferrin in mammary gland secretions can be derived from two sources, mammary gland epithelial cells in the regular lactation cycle and from neutrophil secondary granules at times of infection. These are distinguishable due to tissue specific glycosylation patterns of the protein depending on its source (Hurley *et al.*, 1989).

Progesterone

Homology searches of the bovine lactoferrin promoter against the transcription factor database (H. Bain, 1995) have identified two potential progesterone receptor consensus sequences approximately 1000 bp upstream of the transcription initiation site. Progesterone is one of the major hormones controlling the lactation cycle. The level of progesterone increases steadily throughout pregnancy but there is a rapid

decline just prior to parturition. Circulating progesterone acts to stimulate mammary alveolar growth and suppress milk synthesis (Kuhn, 1985). *In vitro* experiments have shown that the addition of progesterone to mammary tissue prevents the hormonal induction of the mRNAs for α -lactalbumin, galactosyltransferase (lactose synthetase) and casein mRNA (Khun, 1989; Turkington and Hill., 1969). The sudden decrease in progesterone is the primary lactogenic signal which is followed by a dramatic increase in circulating prolactin. Prolactin has a positive effect on the expression of milk specific protein genes (see later).

Circulating progesterone levels appear to correspond to the changes in lactoferrin protein and mRNA expression. Both factors are at high levels during pregnancy when mammaryogenesis occurs and low during lactation. After pregnancy the estrous cycle may begin again even if the cow is still lactating. The cyclic changes in progesterone and estrogen do not appear to have any effect on the expression of milk proteins in the bovine mammary gland (Akers, 1985). Experiments in rats have shown that the progesterone receptor appears to diminish from the mammary gland, being undetectable after day one of lactation but reappearing at weaning (day 28; Khun, 1989). This may be why progesterone from the estrous cycle does not inhibit established lactation. The northern hybridisation performed by Schanbacher *et al.*, (1993) demonstrated that milking only one half of the gland induced the rapid expression of lactoferrin and the decline of other milk proteins in the non-milked half (Figure 2).

The rapid induction of involution suggests that in addition to systemic signals, other signals originating within the mammary gland co-operate in the *in vivo* regulation of lactoferrin expression. A change in phosphorylation state of the various regulatory components of the cell provides a means of cross-talk between signal transduction pathways, which may act to signal the change in milk demand and the rapid induction of lactoferrin expression at the beginning of involution. The progesterone receptors are also excellent candidates for involvement in these processes (Moudgil, 1990). A change in the phosphorylation state of the progesterone receptor by proteins such as cAMP-PK may change the ability to activate transcription (Bailey *et al.*, 1986). A

change in phosphorylation state of the progesterone receptor could explain the rapid induction of lactoferrin mRNA expression at involution. Overall progesterone and its receptor are excellent candidates for involvement in the molecular mechanism controlling changes in mammary lactoferrin expression.

Recently the molecular mechanism controlling the activation of the β -casein gene in the mouse mammary cell line HC11 has been determined (Groner *et al.*, 1994). Prolactin stimulation of milk protein gene expression is mediated by a STAT protein (signal transducer and activator of transcription, STAT5) also known as the mammary gland factor (MGF). Upon prolactin binding to its receptor, Jak kinase, a tyrosine kinase, is able to phosphorylate MGF/STAT5. Phosphorylation promotes the dimerisation which is necessary for consensus sequence binding and also serves as a nuclear localisation signal. Once in the nucleus MGF/STAT5 can bind to its response element in the β -casein gene, modulating transcription. The MGF/STAT5 response element is also found in the bovine β -casein gene at position -87 to -99 and is thought to modulate transcription of this gene (Groner *et al.*, 1994). The absence of the MGF response element from the bovine lactoferrin promoter sequence is not surprising considering that the expression of bovine lactoferrin appears to be opposite to the casein genes.

Interleukin-6

Lactoferrin may have a function in the immune response to infection in the mammary gland. When an infection, such as mastitis occurs, leukocytes and lactoferrin increase in the bovine mammary gland (Harmon *et al.*, 1976). Computer sequence homology searches have identified several putative nuclear factor interleukin-6 (NF-IL6) recognition sequences within the bovine lactoferrin promoter sequence. Interleukin-6 (IL-6) is involved in the acute phase response which occurs after injury or infection (Gauldie *et al.*, 1987). The presence of the NF-IL6 recognition sequences in the lactoferrin promoter suggests that the molecular mechanism employed in activating lactoferrin at times of injury or intramammary infection may involve IL-6. Recent evidence from the involuting human endometrium describes the interaction of progesterone and interleukin-8 (IL-8). Interleukin-8 acts as a chemo-attractant

activating factor for neutrophils and T cells. The mRNA for IL-8 is present in endometrial cells and progesterone has been shown to stabilise the mRNA allowing greater translation of the interleukin-8 receptor (Arici *et al.*, 1996). Similar events may occur in bovine mammary tissue between interleukin-6 and progesterone. Progesterone may enhance transcription of lactoferrin and also stabilise the mRNA for the interleukin-6 receptor. The increase in interleukin-6 receptor may in turn enhance the expression of lactoferrin through the action of interleukin-6 and its receptor on the putative response elements in the bovine lactoferrin promoter sequence.

The Function of Bovine Lactoferrin in the Mammary Gland

The question of lactoferrin function in the mammary gland during development, at involution and at times of injury or infection remains unanswered but the identification of putative *cis*-acting elements provides starting points for an investigation. Studies by Hurley *et al.*, (1993) demonstrated the inhibitory action of lactoferrin on bovine mammary cell growth *in vitro*. This effect was species specific and independent of iron status. These authors suggest that lactoferrin may contribute to a pool of growth inhibitory factors, preventing mammary cell proliferation during periods of inflammation or at involution. Immunostaining for lactoferrin in involuting mammary tissue located lactoferrin at the basal region of alveolar epithelia and suggests that it is associated with basal membrane components (Hurley *et al.*, 1992). This observation is supported by the demonstration of selective binding of lactoferrin to heparin sulphate (Zou *et al.*, 1992), which suggests that lactoferrin may bind *in vivo* to proteoglycans in the basement membrane and extracellular matrix. The function of lactoferrin at this site may involve binding extracellular iron, thus protecting alveolar epithelia from extracellular free radicals generated during stromal remodelling after infection, during involution and preparation of the gland for lactation through out pregnancy. Lactoferrin may also act as a general antibacterial agent at times of development and involution as the mammary gland is most susceptible to infection at these times (Hurley, 1988).

1.6 Aims and Justification of Project

The isolation of the promoter region of bovine lactoferrin (H Bain, 1995) has allowed a preliminary investigation of the molecular mechanisms controlling the expression of the gene. Bovine lactoferrin expression is species-specific and developmentally regulated in the bovine mammary gland and the identification of the factors controlling gene expression should reflect these characteristics. Promoter regions of protein coding genes contain *cis*-elements which serve to bind *trans*-acting protein factors. The binding of these factors may act positively or negatively on the initiation of transcription. Many putative *cis*-acting elements have been identified in the bovine lactoferrin promoter; in particular the progesterone response elements and the NF-IL6 elements are favourable candidates for the regulation of lactoferrin in the mammary gland.

Before a study aimed at the identification of *cis*-elements and *trans* acting factors involved in the transcription of bovine lactoferrin can begin, the minimal promoter sequence necessary for transcription initiation must be defined. The primary goal of the experiments presented in this thesis was the identification of the minimal promoter region and regions of sequence that might confer positive and negative regulation of lactoferrin expression. The first step was to establish whether the expression of the reporter genes constructed by H. Bain (1995) could be supported in the human endometrial carcinoma RL 95-2 cell line. These cells represent a better physiological system for the study of the bovine lactoferrin promoter than the COS cells used by H. Bain (1995). Confirmation of expression allowed the beginning of a deletion analysis from the 5' and 3' ends of the promoter sequence and the transient transfection of the deleted constructs into RL 95-2 cells.

The minimal promoter contains the site of RNA polymerase II assembly and transcription initiation. The position of the transcription initiation site has recently come into question with the publication of the goat lactoferrin cDNA sequence (Le Provost *et al.*, 1994). This sequence closely matches the sequence of bovine lactoferrin but also overlaps the putative transcription initiation site described by

Seyfert *et al.*, (1994) and Schanbacher *et al.*, (1993), plus the putative TATA box where RNA polymerase is thought to bind. To address this question, DNase I footprinting of this region with TBP was attempted. With the definition of the minimal promoter region and areas that confer regions of positive and negative regulation it will be possible to begin unravelling the molecular mechanism controlling bovine lactoferrin expression.

Chapter Two: Materials and Methods

2.1 Materials and Reagents

All chemicals used in this study were of analytical quality. The sources of these chemicals are listed below.

T4 DNA ligase, 1 kb DNA ladder, penicillin-streptomycin, trypsin, Dulbecco's Modified Eagles Medium (DMEM), Ham's F 12 Medium, and Fetal Calf Serum (FCS) were purchased from Gibco Bethesda Research Laboratories (BRL), Life Technologies Ltd, USA.

Restriction endonucleases were purchased from New England Biolabs, Beverly, MA, USA or from Gibco Bethesda Research Laboratories (BRL), Life Technologies Ltd, MD, USA.

Proteinase K and Calf Alkaline Phosphatase (CAP) with appropriate buffers, were obtained from Boehringer Mannheim, Germany.

Taq polymerase was obtained from Promega, Madison, USA, Boehringer Mannheim, Germany and Gibco Bethesda Research Laboratories (BRL), Life Technologies Ltd, MD, USA.

The luciferase assay system, Wizard maxiprep resin and Maxicolumns were purchased from Promega, Madison, USA.

Supplies of dNTPs were from Pharmacia Biotech, Uppsala, Sweden.

Agarose, ethidium bromide, lysozyme, ampicillin, tetracycline, ammonium persulphate, TEMED, DMSO, DMEM, O-nitrophenyl- β -galactoside (ONPG) used in the β -galactosidase assays, HEPES buffer used in tissue culture and the enzyme RNase A were obtained from the Sigma Chemical Company, Saint Louis, MO, USA.

Sequenase kits (version 2.0) were purchased from the United States Biochemical Corporation (USB), Cleveland, Ohio.

Pfu Polymerase was obtained from Stratagene, La Jolla, CA, USA.

Tissue culture flasks and transfection plates were from Falcon, NJ, USA and the Corning Costar Corporation Cambridge, MA, USA.

Custom oligonucleotides were obtained from Oligos Etc., Wilsonville, OR, USA or Gibco Bethesda Research Laboratories (BRL), Life Technologies Ltd, MD, USA.

Polaroid film, type 667, was from Fabriqueau Royaume Uni par Polaroid (UK) Ltd, Hertfordshire, England.

The BRESA-CLEAN™ DNA Purification Kit was supplied by Bresatec Ltd, Adelaide, SA.

Dynabeads M-280 were from Dynal, Oslo, Norway.

Cryotubes were obtained from Nunc Inc., Naperville, IL, USA.

Radioisotopes were purchased from Dupont, NEN Research Products, Boston, MA, USA

The acrylamide premix came from Bio-Rad Laboratories, CA, USA.

X-ray film was purchased from the Fuji Photo Film Company, Japan.

X-ray film developer and fixer were from Kodak Ltd, Victoria, Australia.

Bacterial Cell Line:

E. coli XL-1 blue Genotype: *recA1, endA1, GyrA96, thi, hsdR17, supE44, relA1, λ⁻, lac⁻, [F⁺, proAB, lacI^q, ZΔM15, Tn10]* was from Stratagene, La Jolla, CA, USA.

Tissue Culture Cell Line:

The human endometrial carcinoma cell line known as RL 95-2 was purchased from the American Type Culture Collection, Rockville, Maryland, USA.

Plasmid DNA:

The group of pGL2 vectors was obtained from Promega, Madison, USA. This included a basic vector (pGL-2B) a control vector (pGL-2C) and an enhancer vector (pGL-2E).

The long (2449 bp) and short (565 bp) constructs of the bovine lactoferrin promoter in the pGL-2E vector were initially prepared by Dr H. Bain (1995) as well as four 5' deletion mutants of the long (2499 bp) construct.

The β-galactosidase plasmids pCH110 and pSVβ-gal were from Pharmacia Biotech, Uppsala Sweden. and Promega, Madison, USA respectively.

2.2 Methods**2.2.1 Bacterial Cell Culture*****Maintenance of Cells***

E. coli XL-1 was used as a host for all plasmids used in this work. All the plasmids used carry the β-lactamase gene, therefore bacteria were streaked onto agar plates containing ampicillin (100 μg/mL) to select only those bacteria containing the appropriate plasmid. All work with bacteria was carried out using aseptic technique and standard protocols according to Sambrook *et al.*, (1989).

Preparation of Competent Cells

Competent cells were prepared by one of two methods, both of which involve the use of CaCl₂ treatment performed on ice. The *E.coli* XL-1 strain was maintained on LB with tetracycline (10 µg/mL). In each case the cells were harvested at the middle of the logarithmic phase (O.D. of ~0.5) to maximise transformation efficiency. The standard method of CaCl₂ treatment is documented in Sambrook *et al.*, (1989). The “Supercompetent” method can be found in Inoue *et al.*, (1990). The success of this protocol is thought to be due to the slow growth of the culture at lower temperatures compared to the standard protocol.

Transformation Methods

Initially transformation of *E.coli* was carried out by heat shock followed by recovery at 37°C using the standard method of Sambrook *et al.*, (1989). Later in this work a second, more simple protocol was used. It relies on the pre-warming of the agar plates at 37°C. The competent cells were thawed on ice then approximately 50 ng of DNA was added to 100 µL of cells. This mixture was incubated on ice for 5 minutes then 80 µL and 20 µL were transferred to pre-warmed plates. These were left to incubate overnight at 37°C (Pope, B. & Kent, H.M., 1996).

2.2.2 Preparation of DNA

Small Scale Preparations

The rapid boil technique was utilised to isolate DNA from 5 mL bacterial cultures for screening purposes (Holmes & Quigley., 1981).

Large Scale Preparations

Large scale preparations of plasmid DNA was carried out using the Wizard™ Maxiprep DNA purification system (Promega), or by alkaline lysis followed by caesium chloride gradient centrifugation (Sambrook *et al.*, 1989).

Analysis of DNA Preparations

Diagnostic restriction digests were performed on all DNA preparations. The quantity and purity of large scale preparations of DNA was determined by spectrophotometric analysis (Sambrook *et al.*, 1989).

2.2.3 Manipulation of DNA

Restriction Digest of DNA

Restriction digests of plasmid DNA were carried out according to the manufacturers instructions. The amount of DNA digested ranged from 0.5 μg up to 3 μg in a reaction of 30 μL . When a restriction digest was being performed on DNA prepared by the rapid boil technique, an additional incubation at 37°C with RNase A (0.7 $\mu\text{g}/\mu\text{L}$) was performed. This digested large RNA molecules and aided in the visualisation of the DNA fragments on agarose gels.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to visualise the results of a restriction digest or PCR reaction (Sambrook *et al.*, 1989). The gels were prepared from low electroendosmosis grade agarose in 1 \times TAE buffer (40 mM Tris, 2 mM Na_2EDTA pH 8.0) containing ethidium bromide ($1.0 \mu\text{g mL}^{-1}$) and 1 \times TAE buffer (40 mM Tris, 2 mM Na_2EDTA pH 8.0). The resolution of DNA fragments depends upon the percentage of agarose and the size of the fragments. 0.7% agarose gels were used for the routine separation of DNA fragments up to 3 kb in size. Samples for electrophoresis were mixed with loading dye (0.25% bromophenol blue, 40% sucrose), at a ratio of 1:5, then loaded onto the agarose gels. Electrophoresis was performed in 1 \times TAE at voltages of 30-80 V. At the end of a run, DNA fragments were visualised under short wavelength UV light (305 nm) and photographed using polaroid film 667. DNA fragment sizes were estimated by comparison with the 1kb molecular size ladder.

Cloning of DNA Fragments

Isolation of DNA fragments from agarose gels was performed using the freeze squeeze method (Thuring *et al.*, 1975) or using glass milk resins, such as BRESA-CLEAN™, according to the manufacturers instructions.

Phosphatase and ligation reactions were all performed according standard protocols as documented in Sambrook *et al.*, (1989) and Ausubel *et al.*, (1990).

2.2.4 Polymerisation Chain Reaction (PCR)

Polymerisation chain reactions were performed according to standard methods. The reaction volume was normally 50 μL , but volumes of 100 μL were used in DNA footprinting experiment. Table 2 describes the reaction components used in the amplification of a template using PCR. The enzyme *Pfu* polymerase was used instead of *Taq* polymerase when the PCR products were to be cloned. A profile of the PCR cycle used in these experiments is shown in Figure 4. The 10 \times PCR buffers for *Taq* and contains *Pfu* polymerases were supplied by the manufactures of these enzymes.

Component	Volume
sH ₂ O	23.5 μL
10X PCR Buffer	5 μL
MgCl ₂ 4 mM	5 μL
dNTPs 3 mM	5 μL
Primer #1 50 ng/ μL	5 μL
Primer #2 50 ng/ μL	5 μL
Template 1ng/ μL	1 μL
Taq Polymerase 5 U/ μL	0.5 μL
Total Volume	50 μL

Table 2: PCR Reaction Components

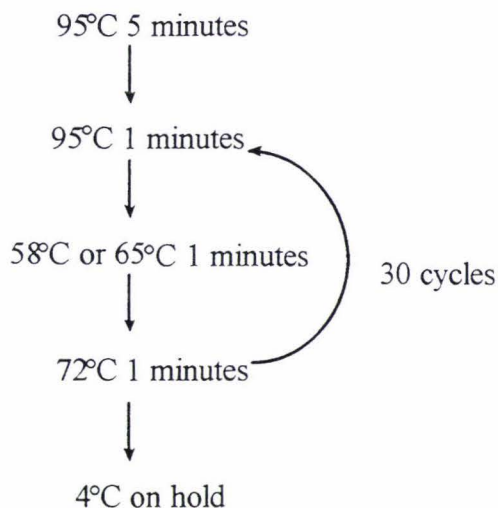


Figure 4: PCR Reaction Profile

2.2.5 Sequencing of DNA

All sequencing reactions were carried out in accordance with the protocol described in the Sequenase version 2.0 kit which is developed from the method described by Sanger *et al.*, (1977). Double-stranded sequencing was carried out essentially as described by Chen & Seeburg., (1985). Templates for single-stranded sequencing were prepared by PCR amplification using a set of primers with one that had been biotinylated at the 5' end. M-280 dynabeads were then used according to the manufacturers instructions to generate a single-strand template.

2.2.6 Maintenance of Tissue Culture Cells

Tissue culture was carried out using a human endometrial carcinoma cell line, RL 95-2. Cells were maintained in DMEM and Ham's F-12 medium in a 1:1 ratio which was supplemented with 10 mM HEPES buffer and 2 g of NaHCO₃ in a volume of 2 L (pH 7.2). This mixture was filter sterilised in the laminar flow tissue culture hood and stored in 180 mL aliquots at 4°C. Before the use in tissue culture the medium was supplemented with 20 mL of foetal calf serum (FCS) (~10% of the total volume), also

2 mL of a penicillin/streptomycin solution and 250 μ L of bovine insulin giving a final concentration of 2 μ g/ μ L. The cells were grown in 75 cm² tissue culture flasks containing approximately 15 mLs of media and maintained at 37°C in an incubator with a 5% CO₂ humidified atmosphere. Media was exchanged every 2-3 days.

Cells stored under liquid nitrogen in cryotubes were rapidly thawed in a water bath at 37°C for 40 seconds. The tubes were then transferred to a 70% ethanol solution before opening. Upon opening the cells were transferred to tissue culture flasks containing medium and incubated for 24 hours. After this period, the medium was changed to remove the DMSO used in the freezing procedure. The cells in the flasks were grown to 80% confluence prior to passage.

Passage of Cells

Cells were passaged at 80 % confluence. The cells were rinsed with 3 mL of PBSE (0.14 M NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄.7H₂O, 1.5 mM KH₂PO₄ pH 7.2 and 0.5 mM EDTA) to remove any fetal calf serum which may inhibit the trypsin used in the next step. A volume of 3 mL of a PBSE solution containing 0.25% trypsin was added to the flasks and then incubated for 10-15 minutes at room temperature. This solution was then transferred to a plastic 15 mL conical flask containing 3 mLs of media and centrifuged at ~250×g for 5 minutes. The supernatant was then discarded and the cell pellet resuspended in 10 mLs of medium ready to seed another tissue culture flask or the wells on a tissue culture plate.

Preparation of Frozen Stocks

At the end of the cell passage process the cells were resuspended in FCS containing 10% DMSO. This was then dispensed to cryotubes in 1 mL aliquots. To begin the freezing process the tubes were rolled in tissue paper and placed at -70°C. The cryotubes were transferred to liquid nitrogen for storage after 24 hours at -70°C.

2.2.7 Transient Transfection Procedure

Transfection plates were seeded at 20% confluence to maximise transfection efficiency according to Ausubel *et al.*, (1990). After a period of 24 hours the medium was exchanged and two hours later the transfection procedure begun. The calcium phosphate precipitation method of transfecting was utilised for all experiments (Ausubel *et al.*, 1990). Transfections in large wells (60 mm) contained 20 μg of luciferase reporter gene and 5 μg of β -galactosidase plasmid (pCH110 or pSV β -gal). In small wells (22 mm) 4 μg of reporter luciferase reporter gene and 1 μg of β -galactosidase plasmid was used. The DNA was added to 25 μL buffer A (0.5 M CaCl_2 , 0.1 M HEPES, pH 7.05-7.12) and the total volume made up to 50 μL with sterile water. Fifteen minutes prior to transfection 50 μL of buffer B (0.28 M NaCl, 0.05 M HEPES, 0.75 mM NaH_2PO_4 , 0.75 mM Na_2HPO_4 , pH 7.05-7.12, also filter sterilised) was added to each tube and mixed (Table 3).

Component	60mm wells: Volume	22mm wells: Volume
sH ₂ O	75 μL	15 μL
Buffer A	125 μL	25 μL
β -Gal plasmid (0.5 $\mu\text{g}/\mu\text{L}$)	10 μL	2 μL
Luciferase reporter plasmid (0.5 $\mu\text{g}/\mu\text{L}$)	40 μL	8 μL
Buffer B	250 μL	50 μL
Total Volume	500 μL	100 μL

Table 3: Transfection Reaction Components

The solutions were added to the cells and incubated for 6 hours. This allows the precipitate to sediment over the cells. At the end of this period the media was removed, the cells washed with medium then replaced with fresh complete medium. This removes the remnants of the calcium phosphate precipitate which is toxic to the cells. Cells were incubated for a further 18 hours before harvesting. This allows for the production of luciferase and β -galactosidase. The cells were washed twice with a PBS solution (0.14 M NaCl, 2.7 mM KCl, 6.5 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 mM KH_2PO_4

pH 7.2) then lysed with an alkali lysis reagent according to the instructions supplied in the Luciferase Assay SystemTM kit (Promega). See Figure 5 for the overall strategy of the transfection procedure.

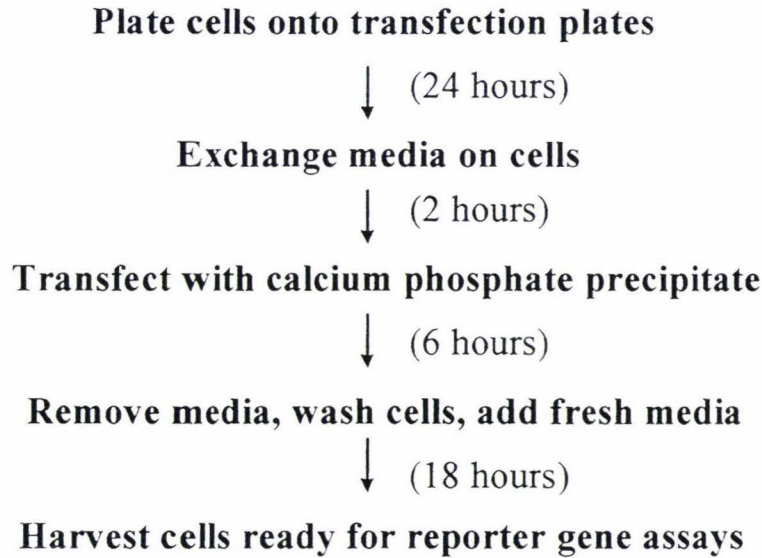


Figure 5: Outline of Transfection Procedure

2.2.8 Reporter Gene Assays

Assays for luciferase activity were carried out using 20 μ L of the cell lysate according to the instructions in the Luciferase Assay SystemTM kit (Promega).

β -galactosidase assays were carried out using 20 μ L of cell lysate according to the method described by Herbomel *et al.*, (1984).

2.2.9 DNA Footprinting Experiments

Preparation of a Radiolabelled Template for Footprinting Experiments

The primers BP-1 and BP-2 (Appendix, Figure 34) were labelled with ³²P using T4 polynucleotide kinase according to standard protocol (Sambrook *et al.*, 1989). Templates for the footprinting reactions were then prepared with one labelled and one

unlabelled primer in a PCR reaction using the 2.8 kb genomic clone of the bovine lactoferrin promoter as the template. The success of the PCR reaction was checked by sampling 1/10 of the reaction by agarose gel electrophoresis. Double-stranded footprinting templates, with one strand labelled at the 5' end were then purified from the PCR reaction mixture using non-denaturing polyacrylamide gel electrophoresis (6% acrylamide, 1× TBE at 30W). The radiolabelled templates were identified by exposing the gel to X-ray film for 30 seconds. The bands were excised and the templates eluted from the acrylamide matrix overnight in 400 µL of 2 M ammonium acetate at 37°C.

DNase I Titration Reactions

The radiolabelled template was then used in titration reactions with DNase I (10 U/µL) to determine the optimal time for DNA digestion and optimal concentration of enzyme to give a suitable digestion pattern. Enough template was used in the reaction to provide ~20000 cpm. This was incubated for 15 minutes at room temperature with 5 µL of a 2× footprinting buffer which would support TBP binding to the template (40 mM HEPES-pH 7.9, 50 mM KCl, 4 mM spermidine, 0.2 mM EDTA, 0.05% NP-40, 20% glycerol 1 mM DTT, 200 µg/mL BSA, 4 mM MgCl₂), and 1 µL of dI.dC. Enzyme was then added (1 µL) to give a total volume of 20 µL. The incubation times ranged from 15 seconds to 2 minutes. The reaction was stopped by adding DNase I stop solution (250 mM Tris pH 8.0, 250 mM EDTA, 4% SDS). Then, 1 µg tRNA carrier and 2 µL of proteinase K were added and the mixture heated at 65°C for 15 minutes. The reactions were then ethanol precipitated overnight, centrifuged and resuspended in 10 µL of loading dye (95% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 10 mM EDTA). These samples were loaded and electrophoresed on a denaturing acrylamide gel (6% acrylamide, 4 M urea, 1× TBE at 30W for 1.5 hours) then fixed, dried, and autoradiographed according to Sambrook *et al.*, (1989).

Chapter Three: Results

3.1 Preparation and Analysis of Reporter Gene Constructs

3.1.1 Introduction

Promoters consist of *cis*-acting DNA sequences, situated distal and proximal to the transcription start point. *Trans*-acting protein factors can bind to these DNA motifs and protein-protein interactions between bound transcription factors can occur. Other soluble factors can also associate forming the transcription initiation complex which drives gene expression. The isolation of the promoter region for bovine lactoferrin (H. Bain, 1995) provided a starting point for this investigation into the control of gene expression of bovine lactoferrin. A computer sequence homology search has identified many putative *cis*-acting DNA sequences in the bovine lactoferrin promoter which bind known transcription factors (Figure 22 and H Bain, 1995). Before the transcription factors which act on the bovine lactoferrin promoter can be identified it is necessary to define the minimal promoter region which can support transcription.

The strategy used to define the minimal promoter region was to reduce the length of the promoter sequence at the 5' and 3' ends until the ability to drive transcription ceased. Promoter activity was assayed by the introduction of promoter mutants, constructed in a reporter gene plasmid, into the RL 95-2 human endometrial cells in tissue culture followed by testing for reporter gene expression. The RL 95-2 cell line was selected because it was also used in the characterisation of both human and mouse lactoferrin promoters (Teng *et al.*, 1992). By using the same physiological system it may be possible to compare the factors controlling lactoferrin expression across species. A deletion analysis was used to define the minimal promoter region for mouse lactoferrin with reporter constructs used in RL 95-2 cells (Liu and Teng, 1991). The mouse lactoferrin promoter was contained within nucleotides -234 to -21 relative to the transcription initiation site. Further deletions into the promoter sequence completely abolished transcriptional activity. The strategy employed to identify the boundaries of sequence which constitutes the minimal functional bovine lactoferrin promoter, a series of 5' deletion mutants was created using nested

restriction endonuclease sites. A series of 3' deletion mutants using the polymerisation chain reaction (PCR). The bovine lactoferrin promoter fragments used in this study are described in Table 4 and Figure 6.

Construct	Promoter Size (bp)	Deletion Type	Vector
2.8kbBlue	2800	Genomic clone	Blue script
Long/2499	2499	3'	pGL-2E
1855	1855	5'	pGL-2E
1444	1444	5'	pGL-2E
1285	1285	5'	pGL-2E
Short/565	565	5'	pGL-2E
321	321	5'	pGL-2E
BP2464	2464	3'	pGL-2E
BL2444	2444	3'	pGL-2E
BP530	530	3'	pGL-2E
BL510	510	3'	pGL-2E

Table 4: Promoter Fragments Used in Deletion Analysis

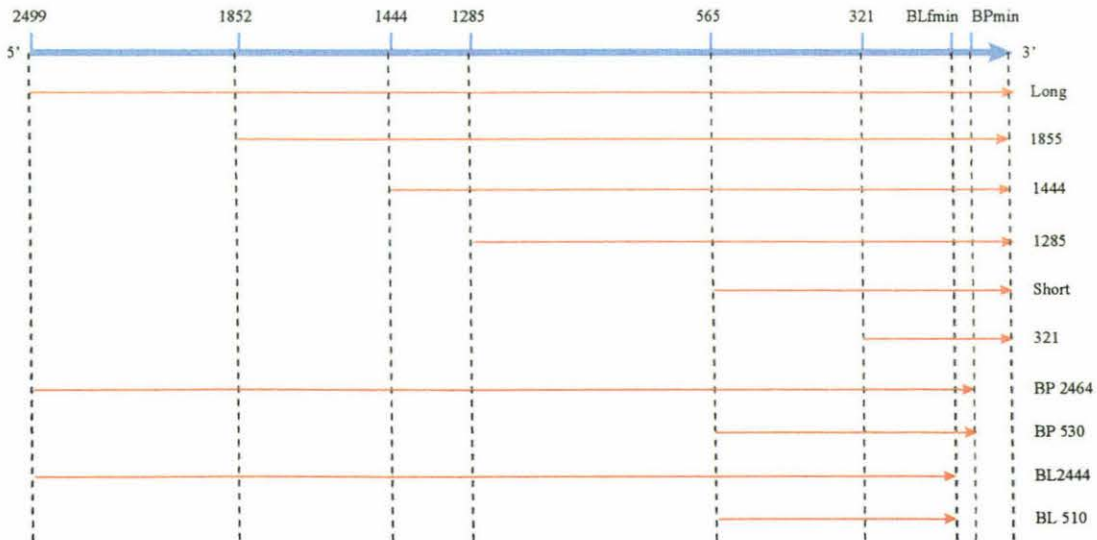


Figure 6: Promoter Fragments Used to Prepare Reporter Gene Constructs

3.1.2 Preparation of Reporter Gene Constructs

Introduction

The 2.8 kb genomic clone containing approximately 2.5 kb of promoter sequence and exon I of the lactoferrin gene had previously been used to create a long (2499 bp) and short construct (565 bp). A 2499 bp fragment, the result of removing exon I, and a 565 bp restriction fragment, prepared using a convenient restriction site, were cloned into the vector pGL-2E (H. Bain, 1995 and Figure 7). This vector was used in the Promega luciferase reporter gene system and contains the coding region for firefly (*Photinus pyralis*) luciferase. The expression of this gene product was used to assay for transcription directed by the bovine lactoferrin promoter in transfected eukaryotic cells. The pGL-2E vector (Appendix, Figure 35) contains an enhancer element downstream of the luciferase gene. This element increases the rate of gene transcription in order to detect low levels of promoter activity. The 565 bp construct had previously been used to transiently transfect COS cells and successfully show that the promoter construct was active (H. Bain, 1995).

Sequencing the Short, 565 bp, Construct

Both the 2499 bp and 565 bp promoter fragments were produced by PCR using an oligonucleotide primer to incorporate a *Bgl* II restriction site at the 3' end to allow cloning into pGL-2E. The 2499 bp construct had been sequenced previously and shown to be free of errors. The 565 bp construct was sequenced to check for errors, to validate the promoter activity seen in the COS cell line and also allow the development of 5' and 3' deletion mutants. Single stranded templates were generated to sequence the 565 bp construct. The 565 bp template was PCR amplified (Figure 8) using sets of primers in which one was biotinylated (Figure 9) to give a product of approximately 615 bp. A single strand of template was generated using DYNA™ beads and the MPC™ according to manufacture's instructions. The single stranded templates were then used in sequencing reactions with the pGL-2E specific primers 2825 and 2826, and also bovine lactoferrin promoter primers 3G, 4A, 5B, and 6Q (Figure 9 for strategy). The entire 565 bp was completely sequenced on both strands and found to contain no errors.

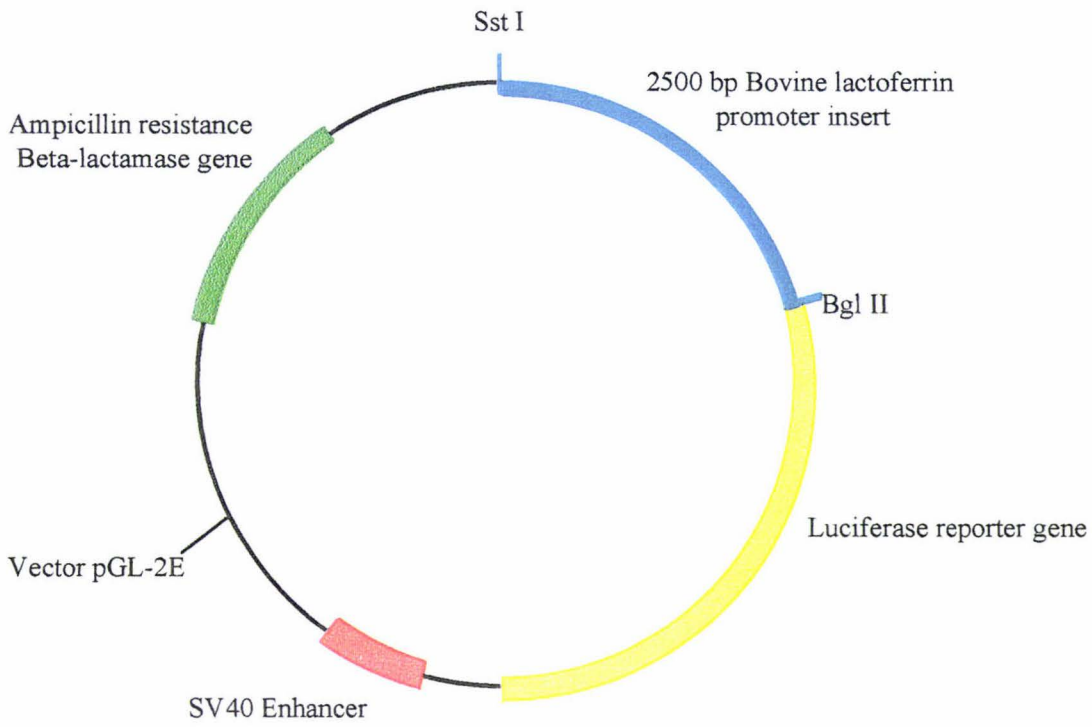
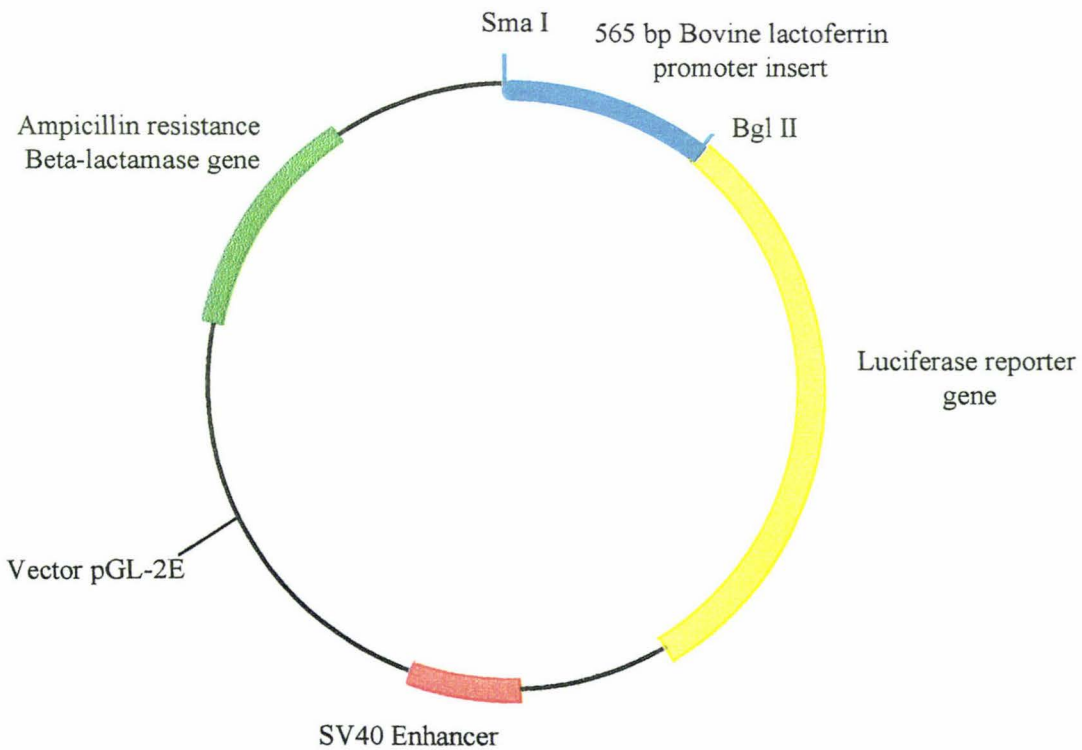
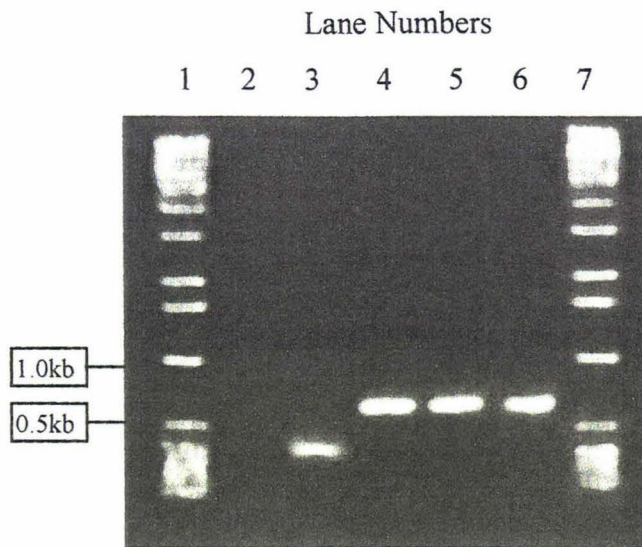
A: Long Bovine Lactoferrin Reporter Gene Construct**B: Short Bovine Lactoferrin Reporter Gene Construct****Figure 7: Schematic Diagram of the Long and Short Reporter Gene Constructs**

Figure 8: PCR Product Analysis

Analysis of PCR products; A 5 μ L aliquot of each PCR reaction was analysed by electrophoresis on a 1% agarose gel containing 1 \times TAE. The DNA was stained using ethidium bromide (0.25 μ g/mL) and visualised on an UV transilluminator.

1: BRL 1 kb ladder

2: Negative control (no DNA template)

3: Positive control, Factor XI promoter fragment

4: & 5: A 615 bp fragment amplified from 565 bp template using the primers 2825 & 2859 (biotinylated) (565 bp plus 2 \times 25 bp primers equals 615 bp)

6: A 615 bp fragment amplified from 565 bp template using the primers 2826 & 2858 (biotinylated) (565 bp plus 2 \times 25 bp primers equals 615 bp)

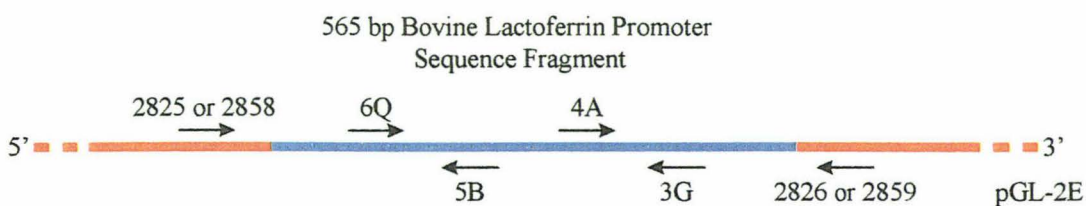


Figure 9: Schematic Diagram of the Sequencing Strategy Utilised in the Single Stranded Sequencing of the 565 bp Short Construct

3.1.3 Construction of the BL510 3' Deletion Mutant

Introduction

BL510 was the first 3' deletion mutant to be made. The initial construction of the long and short reporter genes involved creating a restriction site just 5' to the ATG in the bovine lactoferrin promoter sequence. Promoter sequences were cloned into pGL-2E using this restriction site at the 3' end (Figure 14). The distance between the putative transcription start point and the ATG is 40 bp (Seyfert *et al.*, 1994). On cloning the long and short constructs, the genomic ATG was lost leaving the ATG of the luciferase gene 39 bp downstream from the 3' multiple cloning site and 79 bp between the putative transcription start point and the ATG of the luciferase gene (Figure 10). It was thought that this extra sequence may be sub-optimal for transcription efficiency and/or translation of the reporter gene. To reduce this distance a 3' deletion mutant of the bovine lactoferrin sequence was created. A suitable restriction site to do so was not available therefore, a primer, BLfmin, was designed with an extension to introduce a *Bgl* II restriction endonuclease site for cloning (see figure 10). The primer annealed 5' to the transcription start site and removed 55 bp upstream of the 3' multiple cloning site. An overall strategy for the preparation of BL510 3' deletion mutant preparation appears in Figure 14.

Vector DNA Preparation for the BL510 Construct

The multiple cloning site of the plasmid pGL-2E was digested with *Bgl* II and *Sma* I to generate complementary ends for the 3' deleted insert. This gave a fragment of ~5800 bp. Phosphate groups were removed with calf intestinal phosphatase and the completeness of digestion determined by agarose gel electrophoresis (Figure 11).

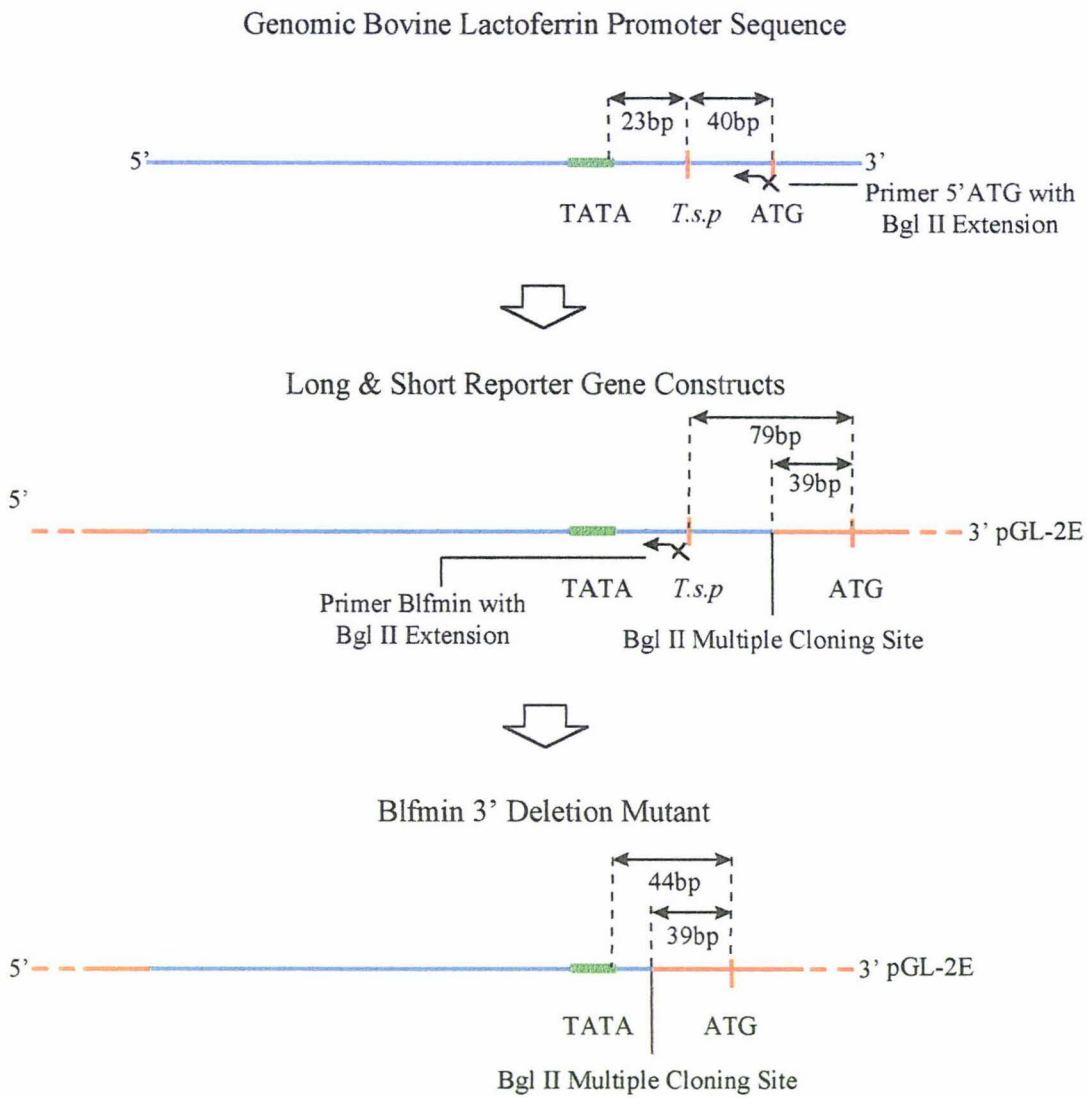
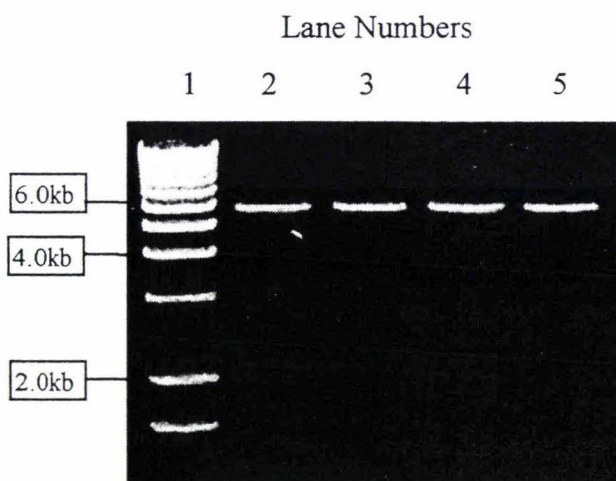


Figure 10: Diagram Demonstrating Distances Between Bovine Lactoferrin Promoter Elements and Reporter Gene Elements

Figure 11: Analysis of Vector Preparation

Analysis of restriction digest; A 5 μ L aliquot of each digest reaction was analysed by electrophoresis on a 1% agarose gel containing 1 \times TAE. The DNA was stained using ethidium bromide (0.25 μ g/mL) and visualised on an UV transilluminator.

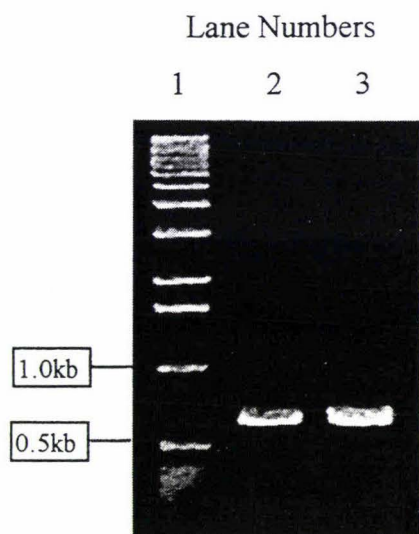
1: BRL 1 kb ladder

2-5: The vector pGL-2E linearised with *Bgl* II and *Sma* I restriction endonucleases to produce a fragment of approximately 5850 bp.

The vector DNA was purified by phenol/chloroform extraction, ethanol precipitated and quantitated by agarose gel electrophoresis (Figure 13).

Insert Preparation for the BL510 Construct

The preparation of the BL510 insert involved a PCR reaction using the primer BLfmin with the complementary primer 2825, the short construct (565) as a template and *Pfu* polymerase. The reason for using the short construct instead of the long (2499) was its size for sequencing and that a suitable cloning strategy could be devised (Figure 14).

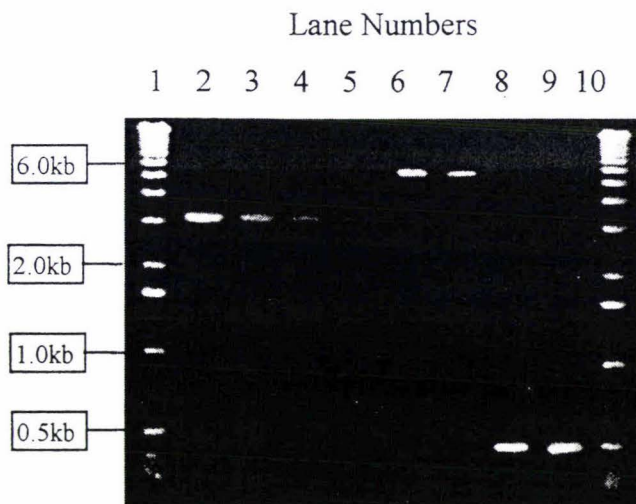
Figure 12: PCR Product Analysis

Analysis of PCR products; A 5 μ L aliquot of each PCR reaction was analysed by electrophoresis on a 1% agarose gel containing 1 \times TAE. The DNA was stained using ethidium bromide (0.25 μ g/mL) and visualised on an UV transilluminator.

1: BRL 1 kb ladder

2 and 3: Fragments of ~540 bp, the result of amplifying with the primers 2825 and BLfmin.

The resulting 540 bp fragment contains a new *Bgl* II site and the original *Sma* I site (Figure 12). Successful PCR reactions were pooled, gel purified then digested with *Sma* I and *Bgl* II. The resulting 510 bp fragment had a blunt end due to the *Sma* I cut and a staggered end from *Bgl* II allowing directional cloning. The insert DNA was purified by phenol/chloroform extraction, ethanol precipitated and quantitated by agarose electrophoresis (Figure 13).

Figure 13: Quantitation Gel

Analysis of restriction digest and gel purification; A 5 μ L aliquot of diluted samples was analysed by electrophoresis on a 1% agarose gel containing 1 \times TAE. The DNA was stained using ethidium bromide (0.25 μ g/mL) and visualised on an UV transilluminator.

1: BRL 1 kb ladder

2-5: Quantitation standards, 100 ng, 50 ng, 20 ng, 10 ng / 5 μ L

6 & 7: Vector DNA preparation 1/5 and 1/10 dilution

8 & 9: Insert. Digested PCR products giving fragment sizes of 510 bp.

10: BRL 1 kb ladder

Ligation reactions were carried out varying the estimated concentration of DNA to obtain the optimum 3:1 ratio for insert to vector as described by Sambrook *et al.*, (1989). Ligated plasmid DNA was introduced into *E.coli* XL-1 blue cells by the heat shock recovery method (Sambrook *et al.*, 1989) and transformants selected from colonies grown on LB amp plates. Plasmid DNA was prepared from transformants with the rapid boil method (Holmes & Quigley, 1981) and analysed by restriction digestion with *Sma* I and *Bgl* II. Positive clones released the insert from the vector giving two fragments, insert of 510 bp and vector size of \sim 5800 bp (Figure 15).

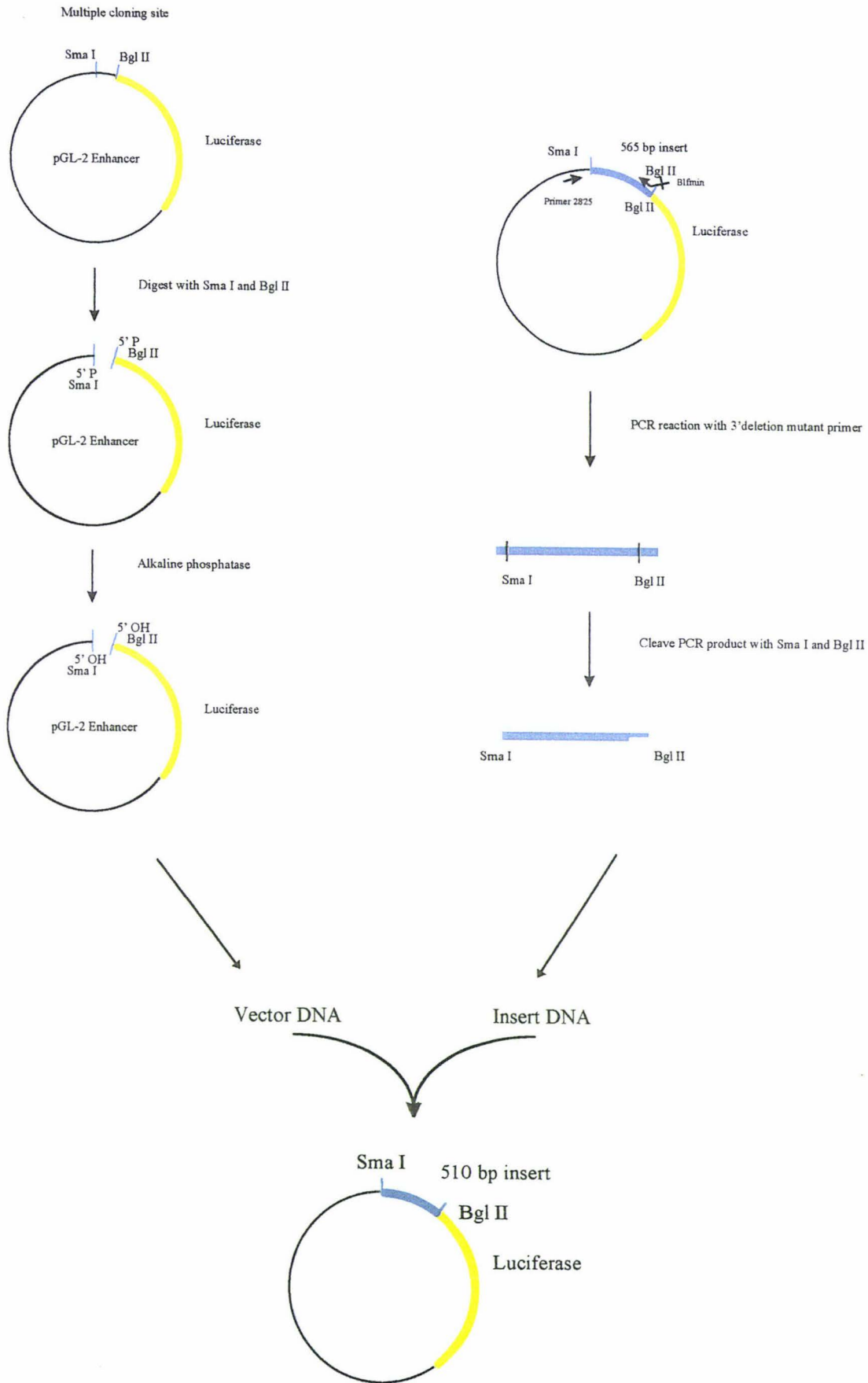
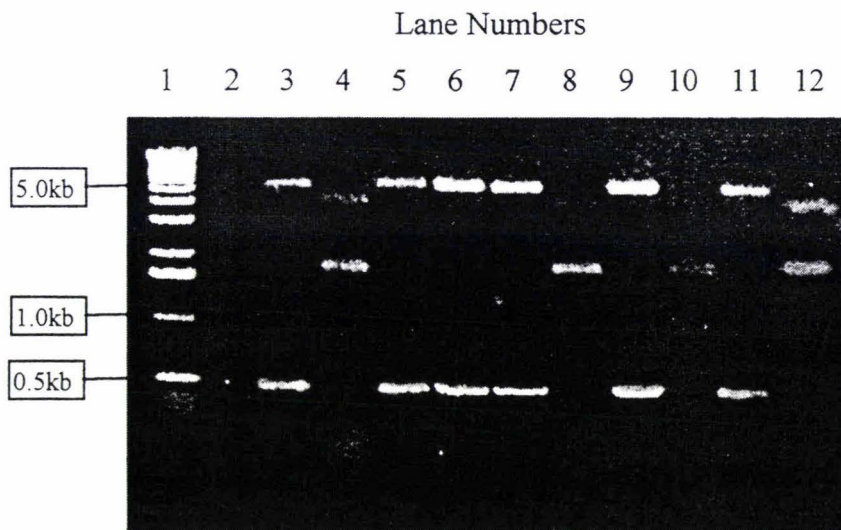


Figure 14: Outline of the Strategy Used to Create the BL510 3' Deletion Mutant

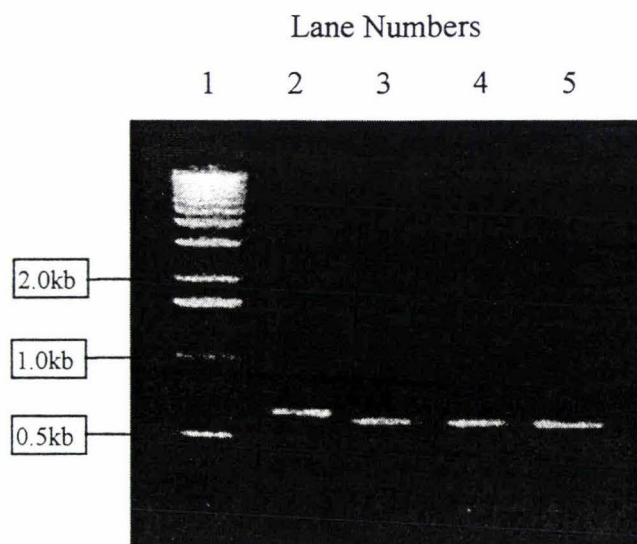
Figure 15: Diagnostic Digest Results for BL510 Transformants

Diagnostic digest of transformations; A 5 μ L aliquot of rapid boil plasmid DNA was digested with the appropriate enzymes then the total reaction (30 μ L) were analysed by electrophoresis on a 1% agarose gel containing 1 \times TAE. The DNA was stained using ethidium bromide (0.25 μ g/mL) and visualised on an UV transilluminator.

1: BRL 1 kb ladder

2 - 12: Rapid boil plasmid preparations digested with *Bgl* II and *Sma* I. Positive clones are identified as containing a fragment at 5850 bp and at 510 bp as occurs in lanes 3, 5, 6, 7, 9 and 11.

A single clone was then selected and prepared on a large scale. Because the 3' deletion mutant was produced by using PCR, it was checked by single-stranded sequencing for random mutations introduced by *Pfu* polymerase. The same strategy which was used for the short construct was also used for BL510. The template was amplified using the same biotinylated primer sets and internal sequencing primers (Figures 16 and 10). The sequence was free of errors and the construct was produced on the large scale.

Figure 16: PCR Product Analysis

Analysis of PCR products; A 5 μ L aliquot of each PCR reaction was analysed by electrophoresis on a 1% agarose gel containing 1 \times TAE. The DNA was stained using ethidium bromide (0.25 μ g/mL) and visualised on an UV transilluminator.

1: BRL 1 kb ladder

2: Positive control. Short construct amplified with primers 2825 and 2826 resulting in a 615 bp fragment.

3 - 4: The 3' deletion mutant amplified with 2825 and 2859 (the biotinylated primer) giving a 560 bp fragment.

5: The 3' deletion mutant amplified with 2826 and 2858 (the biotinylated primer) giving a 560 bp fragment.

3.1.4 Construction of BL2444 and BP2464 3' Deletion Mutants

Introduction

The BL510 construct used in transfection experiments gave low levels of promoter activity (Table 6 and Figure 23). One possible reason for the low level of activity may be that the 3' deletion was too close to the putative TATA box. Therefore a further set of 3' deletion mutants was made removing 35 bp 5' to the multiple cloning site. The primer used in the mutagenesis reaction was called BPmin and contained an extension tail with a *Bgl* II restriction site similar to the primer BLfmin (Appendix, Figure 34). Deletion mutants constructed using BLfmin have the prefix BL in their title and those that were constructed using BPmin have BP in their title. An elaborate strategy for the construction of the BL2444 and BP2464 reporter constructs was devised and is described in Figure 17.

Preparation of Inserts for BL2444 and BP2464 Constructs

The primers BLfmin and BPmin were each used in PCR reactions with the primer 8D, originally designed for sequencing (Appendix, Figure 33). The long construct was used as a template with *Pfu* polymerase. The reactions were analysed by agarose gel electrophoresis. The reaction containing BLfmin and 8D produced a product of 776 bp, and the BPmin/8D reaction, a product of 796 bp. Successful PCR reactions were purified by phenol/chloroform extraction, precipitated with ethanol, then digested with *Pvu* II and *Bgl* II creating fragments with blunt and sticky ends respectively. The BL2444 fragment has a size of 528 bp and BP2464 a size of 546 bp. The fragments were gel purified using BRESA-CLEAN™ (Bresatec) and quantitated by agarose gel electrophoresis.

Preparation of Vector for BL2444 and BP2464 Constructs

The same vector was used in the preparation of both BL2444 and BP2464. This involved cutting the long construct with *Pvu* II and *Bgl* II to create complementary ends for the insert and then phosphatasing with CAP. The digest results in a fragments of ~6970 bp and 583 bp. The 6970 bp fragment was gel purified using BRESA-

CLEAN™ (Bresatec) then run on a quantitation gel to determine the mass of purified DNA.

Ligations using vector DNA and insert DNA were prepared at the optimal ratio and these were then transformed using the simple transformation method (Pope & Kent., 1996). Positive clones were detected by digestion of DNA with *Pvu* II and *Bgl* II to give fragments of 528 bp for BL2444 and 546 bp for BP2464 with a vector fragment of ~6970 bp for both clones (Figure 18). Plasmid DNA was prepared by the rapid boil method from 3 possible positive clones for each construct; a total of six preparations. The region which had been produced by PCR mutagenesis was checked for errors using single stranded sequencing of the rapid boil DNA with internal primers 8D, 7Z, 6Q, 5B, 4A, and 3B (Appendix, Figure 33). The 3' deletion mutant clones BL2444 and BP2464, which were shown to be free of PCR-induced errors were produced on the large scale using Wizard Maxipreps™ (Promega).

3.1.5 Construction of the BP530 3' Deletion Mutant

The BP530 3' deletion mutant was made to complement BL510 and to test the effect on transcriptional activity of removing less of the 3' end of the bovine promoter sequence. Once BP2464 was constructed BP530 was prepared by digestion of the bovine sequence and the 5' multiple cloning site using *Sma* I. This resulted in two fragments, one of ~ 6400 bp and the other ~ 2000 bp. The 6400 bp fragment was gel purified with BRESA-CLEAN™ (Bresatec) and the size verified by agarose gel electrophoresis. This was then used in ligation reactions to join the blunt ends together. Transformation was performed by the simple method described by Pope & Kent, (1996) and positive clones were selected using a *Sma* I restriction digest of rapid boil DNA to give a linear fragment of ~ 6400 bp. A positive clone was selected and prepared on a large scale using a Wizard Maxiprep™. The maxiprep was then checked to ensure the correct clone had been selected (Figure 19). The overall strategy for the construction of BP530 is shown in Figure 17. The construct was sequenced at the 5' end of the promoter sequence to ensure the correct mutation had been produced.

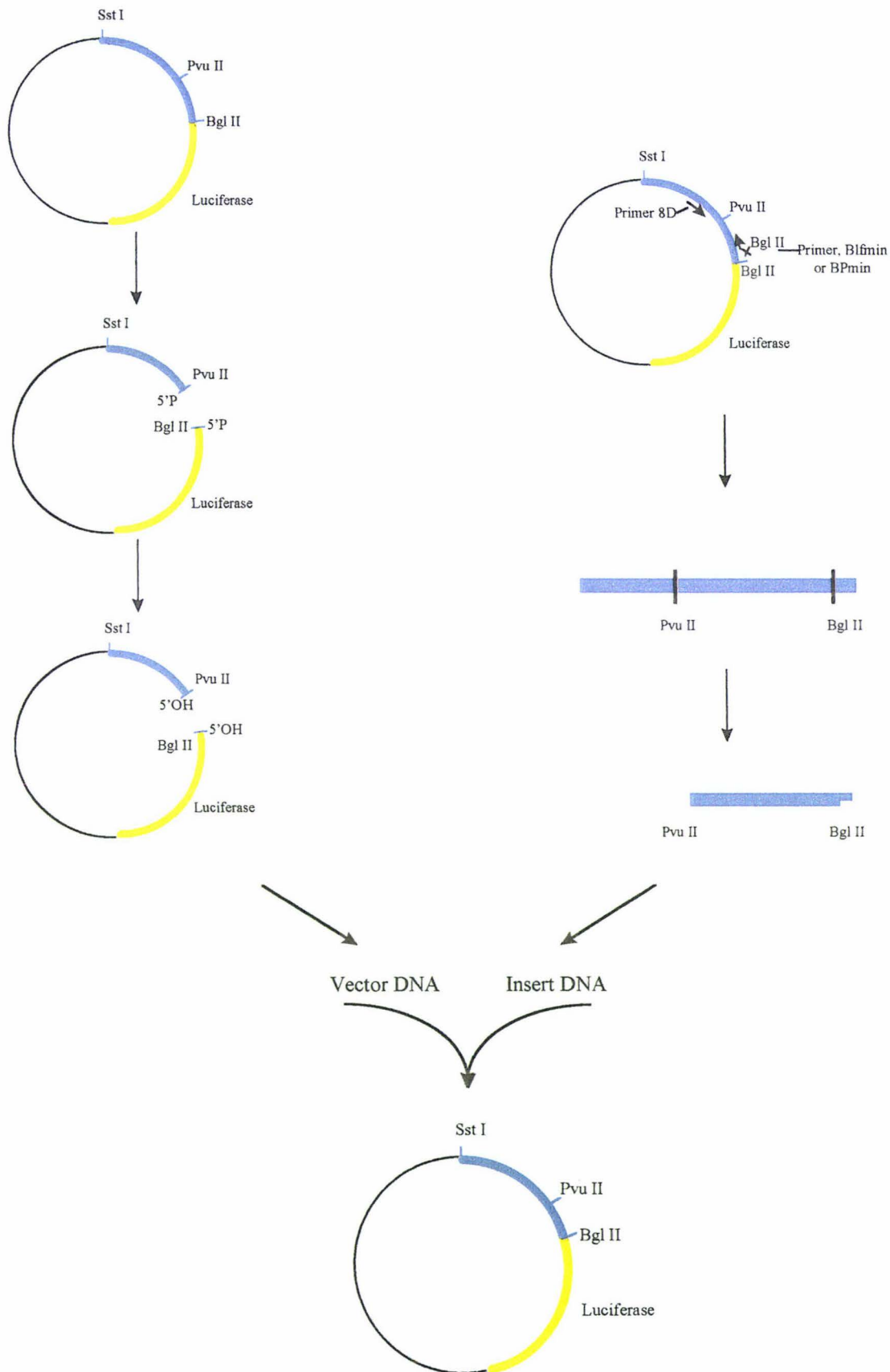
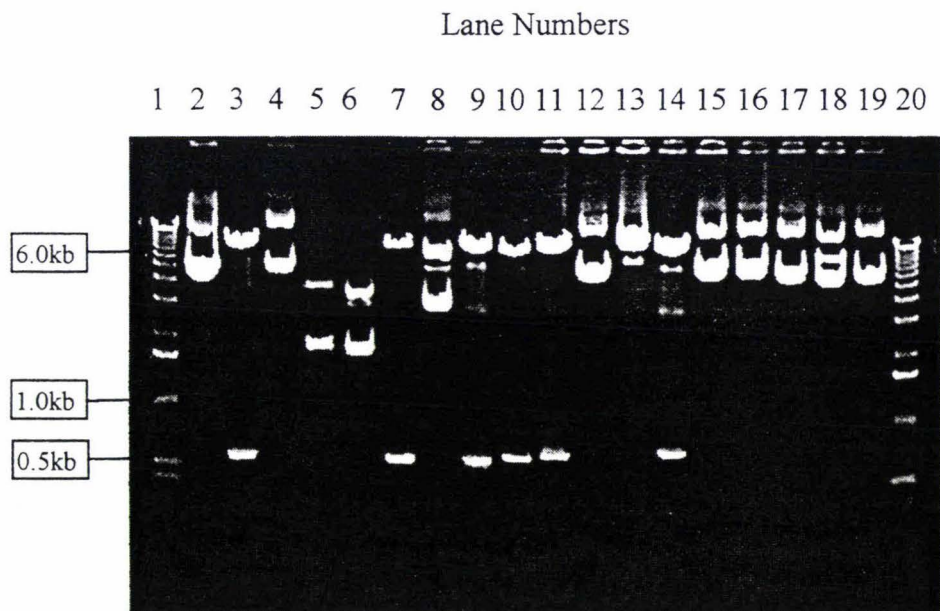


Figure 17: Outline of the Strategy Used to Create the BLfmin and BPmin 3' Deletion Mutants of the 2499 bp Promoter Fragment

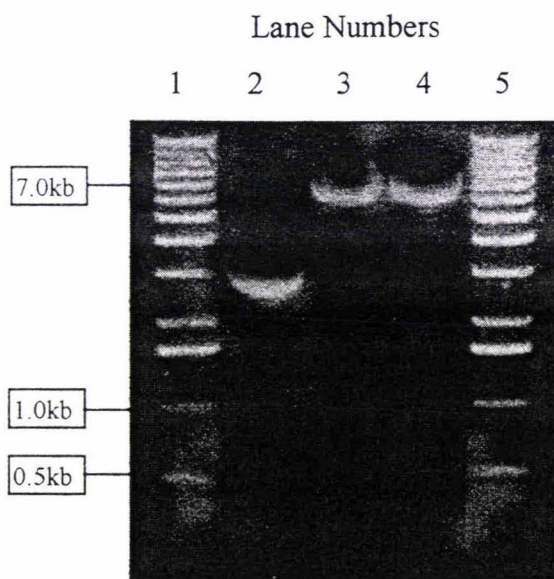
Figure 18: Identification of Positive Clones for BL2444 and BP2464 Mutants

Diagnostic digest of plasmids isolated from transformants; A 5 μ L aliquot of rapid boil plasmid DNA was digested with the appropriate enzymes then the total reaction (30 μ L) was analysed by electrophoresis on a 1% agarose gel containing 1 \times TAE. The DNA was stained using ethidium bromide (0.25 μ g/mL) and visualised on an UV transilluminator.

1: BRL 1 kb ladder

2 - 9: Rapid boil plasmid preparations of the 2444 bp 3' deletion mutant digested with Bgl II and Pvu II. Positive clones are identified as containing a fragment at 6970 bp and at 528 bp as occurs in lanes 3, 7 and 9.

10 - 19: Rapid boil plasmid preparations of the 2464 bp 3' deletion mutant digested with Bgl II and Pvu II. Positive clones are identified as containing a fragment at 6970 bp and at 546 bp as occurs in lanes 10, 11 and 14.

Figure 19: Maxiprep DNA analysis of BP530 3' Deletion Construct

Restriction digest of large scale plasmid preparation; 0.5 μg aliquots of 530 bp 3' deletion mutant DNA were digested with the appropriate enzyme then the total reactions (30 μL) were analysed by electrophoresis on a 1% agarose gel containing 1 \times TAE. The DNA was stained using ethidium bromide (0.25 $\mu\text{g}/\text{mL}$) and visualised on an UV transilluminator.

1: BRL 1 kb ladder

2 : Uncut supercoiled plasmid DNA of BP530 3' deletion mutant construct.

3 - 4: BP530 3' deletion mutant plasmid DNA cut with *Sma* I resulting in a linear fragment of 6400 bp

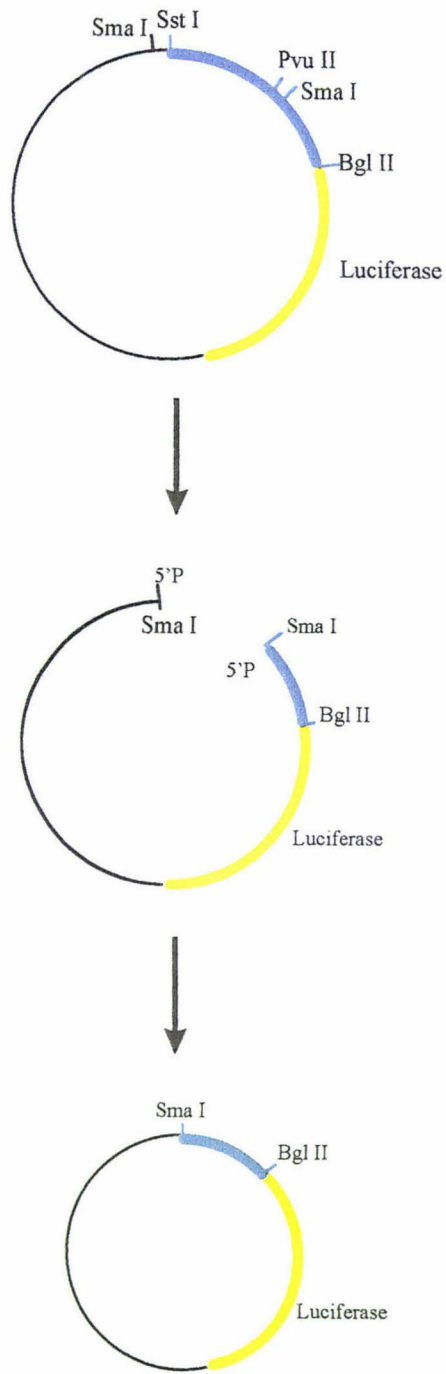


Figure 20: Strategy for the Construction of BP530 3' Deletion Mutant

3.1.6 Preparation of 5' Deletion Mutants

The preparation of 5' deletion mutants used to define the minimal promoter, and which had been constructed by H. Bain and K. Allen is documented below.

The 1855 bp 5' Deletion Mutant

The 1855 bp 5' deletion mutant was constructed using the 2499 bp construct. The total size of the plasmid is 7790 bp. A *Pst* I site is located 647 bp downstream of the 5' multiple cloning site and is not found elsewhere in the vector providing a convenient deletion site. The 2499 bp construct has a total size of 8353 bp and was cut with *Pst* I and *Sst* I which is located in the 5' multiple cloning site, creating a 647 bp fragment and one of ~7700 bp. The 7700 bp fragment had two sticky ends which were end-filled using T4 DNA polymerase allowing ligation of this fragment to form the 1855 bp 5' deletion mutant. This was prepared on a large scale and the deletion site verified by sequence analysis.

The 1444 bp 5' Deletion Mutant

A similar strategy to that above was used to prepare the 1444 bp 5' deletion mutant. A *Xho* I restriction site in the promoter sequence that occurs 1055 bp downstream of the 5' multiple cloning site was used. The 2499 bp construct was used in a double digest with *Xho* I and *Sst* I. This generates two fragments, one of 1055 bp and the other ~7300 bp. The 7300 bp fragment was used to create the mutant. It had a sticky ends at the *Xho* I site which was end filled with T4 DNA polymerase before ligation and transformation were performed. After large scale preparation the deletion site was verified by sequence analysis.

The 1285 bp and 321 bp 5' Deletion Mutants

The 1285 bp and 321 bp 5' deletion mutants were constructed using *Bal* 31 nuclease. *Bal* 31 is predominantly a 3' exonuclease that removes mononucleotides from both 3' termini of the two strands of linear DNA. *Bal* 31 also has a weaker single-strand-specific endonucleolytic activity. Therefore, the products of digestion of double

stranded DNA with *Bal* 31 are truncated molecules carrying blunt ends or short protruding 5' termini. *Bal* 31 was used on linearised long and short construct DNA to produce 5' deletion mutants. The constructs were first linearised at the 5' multiple cloning site then digested with *Bal* 31. These termini of the deletion mutants were then repaired with Klenow DNA polymerase. At the end of this step the restriction enzyme *Hind* III was added to cut the linear mutated DNA at the 3' multiple cloning site. This allowed the insertion of the deleted fragment into an appropriately prepared vector. Vector preparation involved cleaving pGL-2E with *Sma* I and *Hind* III followed by the removal of phosphate groups to prevent vector self ligation. The blunt end and staggered end produced by *Sma* I and *Hind* III, respectively, allowed for directional cloning. The *Bal* 31 prepared inserts and the cut pGL-2E were used in ligation reactions followed by transformation of *E.coli* XL-1 blue bacterial cells. After selection for positive recombinant colonies on LB amp plates single colonies were selected and used in small scale plasmid preparations. Positive clones of deletion mutants were identified by comparison of fragment size after analysis using PCR. These positive clones were then sequenced at the 5' end to determine the size of the deletion mutants created and verify the sequence.

3.2 The *In Vitro* Functional Analysis of Lactoferrin Promoter Deletion Mutants

3.2.1 Introduction

All 5' and 3' deletion mutants listed in table 4 were analysed *in vitro* functional assays in RL 95-2 human endometrial cells. The cell line provided the *trans*-acting protein factors which act on the *cis*-elements in the promoter sequence. The ability of the *cis*-elements to recruit these transcription factors within the nucleus, form the preinitiation complex and drive transcription, was determined by the amount of luciferase reporter gene expressed. Luciferase levels were assayed to determine the efficiency of a given construct to initiate transcription. The assay relies on the production of a photon of light with the breakdown of a luciferin substrate, therefore the amount of light produced is a measure of the luciferase concentration. Luciferase activity determined by this method is a sum of the positive and negative *cis*-acting sequences present in the length of bovine lactoferrin promoter sequence. The level of transcription is also determined and may be limited by the transcription factors available in the tissue culture system used. All *in vitro* functional assays included a β -Galactosidase vector to account for differences in cell numbers and transfection efficiencies between experiments.

3.2.2 Optimisation of Transfection Procedure

The calcium phosphate precipitate method of transfecting cells was used in all experiments. Initially a glycerol shock at the end of the calcium phosphate precipitate incubation was performed in an attempt to improve transfection efficiency. This was found to have no effect and was not continued. Estimation of cell numbers suitable for transfection proved to be the most difficult factor in performing these experiments. All promoter reporter gene constructs were prepared in the vector pGL-2E which contains an SV40 enhancer element to increase transcription efficiency. Initially cell numbers used in transfection experiments were difficult to optimise. The luciferase assay is sensitive and linear over an enormous range but the assay for β -galactosidase used to normalise the luciferase assays is limited by the

spectrophotometric analysis. Any value higher than 1.0 was discarded and hence the luciferase result for that replicate is also lost. Therefore suitable cell numbers for transfection were determined empirically to find β -galactosidase within the appropriate range. The time schedule of experiments was carried out as described in Chapter Two: Materials and Methods.

3.2.3 Examination of the 5' Deletion Mutants of the Bovine Lactoferrin Promoter

The RL 95-2 cells were transfected with 5' deletion mutants 1855, 1444, 1285, 321 and the long, and short construct. The amount of each construct used was 20 μ g in 60 mm plates and 4 μ g in 22 mm plates. 5 μ g of β -Galactosidase reporter gene was added to the 60 mm plates, 1 μ g on the 22 mm plates. After transfection and incubation (Chapter Two: Materials and Methods), the endometrial cells were harvested and cell extracts assayed for luciferase and β -Galactosidase activity. The experiments were performed several times with replicates ranging from 4-8 per construct in each experiment. To account for different cell numbers and transfection efficiencies between experiments the luciferase value for a given replicate was divided by its β -Galactosidase value. The normalised values for a reporter construct were then averaged and this value expressed as a percentage of the activity of the long construct reporter gene (Table 5). Results are expressed as the average +/- the standard error of the mean. These results appear graphically in Figure 21 and are compared to a sequence alignment of putative *cis*-acting elements in the promoter sequence for each reporter gene construct (Figure 22).

Reporter Gene Construct	Activity as % of the Long Construct Activity	Standard Error of the Mean
Long Construct 2499	100	2.29
1855	111.08	3.55
1444	82.04	3.46
1285	70.74	4.23
Short Construct 565	121.90	3.22
321	77.21	1.65

Table 5: 5' Deletion Mutant Activity

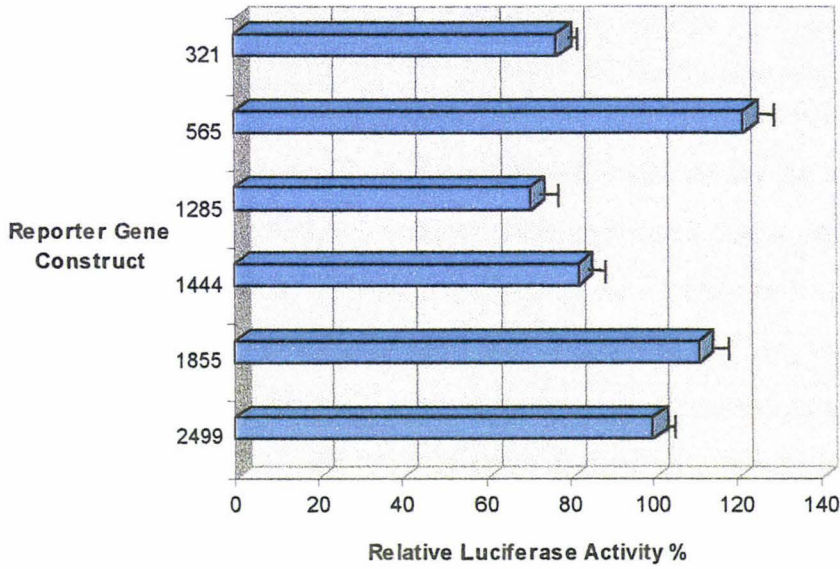


Figure 21: Relative Activity of 5' Deletion Mutants

RL 95-2 cells were transiently transfected with constructs containing different lengths of promoter sequence at the 5' end. The relative Luciferase activity was calculated by setting the luciferase assay result of the long construct to 100%. The other constructs results were then expressed as a percentage of this value.

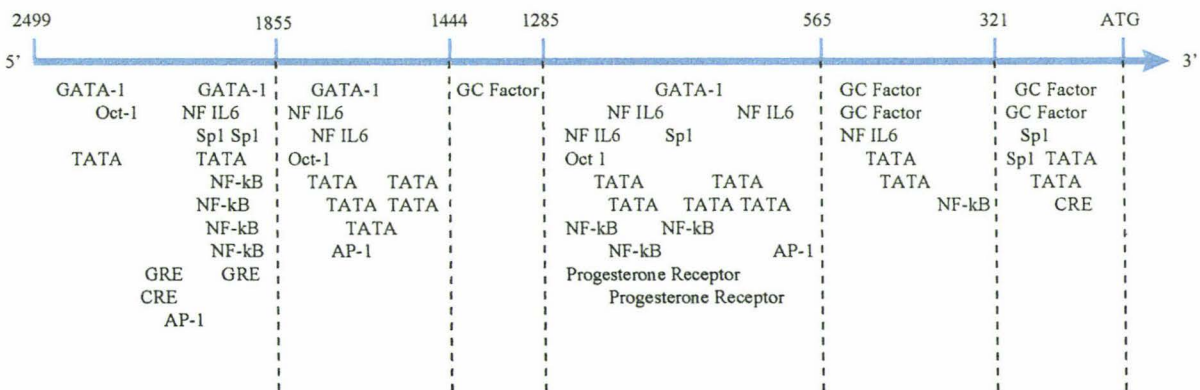


Figure 22: Putative *cis*-acting Factors in the Deletion Mutants

The blue line represents the 2499 bp bovine lactoferrin promoter sequence. The numbers represent the different 5' deletion mutants. The dashed lines represent the boundaries of the deletion mutants and the putative *cis*-acting elements are listed within these.

3.2.4 Examination of the 3' Deletion Mutants of the Bovine Lactoferrin Promoter

Short Construct 3' Deletion Mutants

The BP530, BL510 3' deletion mutants and the short construct were used to transiently transfect the endometrial cell line RL 95-2. As for the 5' deletion mutants, luciferase and β -Galactosidase activity were measured and a normalised value for each replicate calculated. Several experiments were performed using each construct with 4-8 replicates. The short construct was used as an isogenic control and the normalised values for the 3' deletion mutants were expressed as a percentage of the short construct activity. The level of error in these results was calculated using the standard deviation of the result. The level of error in the 3' deletion mutants is less than the values obtained in the 5' deletion analysis due to the lower levels of expression seen for these constructs (Table 6). The 3' deletion mutants were used to analyse the putative *cis*-elements clustered at the putative transcription initiation site. The results in the table are also represented graphically (Figure 23).

Reporter Gene Construct	Activity as % of the Short Construct Activity	Standard Error of the Mean
Short Construct 565 bp	100	3.21
530 bp	22.40	0.79
510 bp	30.70	0.90

Table 6: Short Construct 3' Deletion Mutant Activity

The results of the short construct 3' deletion mutant activity are expressed as a percentage of short construct activity.

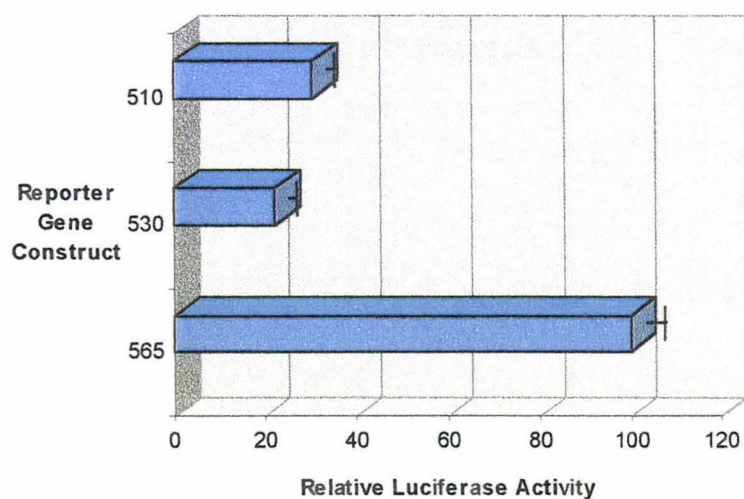


Figure 23: Relative Activity of Short Construct 3' Deletion Mutants

RL 95-2 cells were transiently transfected with constructs containing different lengths of promoter sequence at the 3' end. The relative Luciferase activity was calculated by setting the luciferase assay result of the short construct to 100%. The results from the other constructs were then expressed as a percentage of this value.

Long Construct 3' Deletion Mutants

The BL2444 and BP2464 3' deletion mutants of the large construct were transiently transfected and their normalised values expressed as a percentage of long construct expression. The average results of several experiments with these mutants is demonstrated in Table 7(a) and graphically in Figure 24.

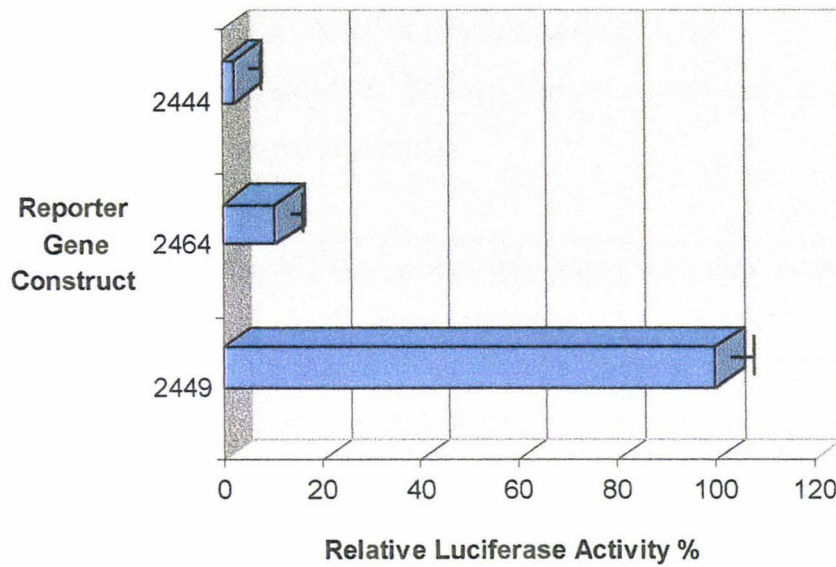


Figure 24: Relative Activity of Long Construct 3' Deletion Mutants

RL 95-2 cells were transiently transfected with constructs containing different lengths of promoter sequence at the 3' end. The relative Luciferase activity was calculated by setting the luciferase assay result of the long construct to 100%. The results from the other constructs were then expressed as a percentage of this value.

Reporter Gene Construct	Activity as % of the Long Construct Activity	Standard Error of the Mean
Long Construct 2499 bp	100	2.29
2464 bp	10.00	1.14
2444 bp	2.52	0.61

Table 7(a): Long Construct 3' Deletion Mutant Activity

The results of the long construct 3' deletion mutant activity are expressed as a percentage of the 2499 bp construct activity.

Reporter Gene Construct	Activity as % of the Long Construct Activity	Standard Error of the Mean
Short Construct 565 bp	100	3.22
530 bp	26.41	0.54
510 bp	36.39	1.20

Table 7(b): Short Construct 3' Deletion Mutant Activity

The results of short construct 3' deletion mutant activity expressed as a percentage of long 2499 bp construct activity.

Comparison of 3' Deletion Mutants

Of the 3' Deletion mutants the BP2464 and BP530 constructs carry the same 3' deletion as do the BL2444 and BL510 constructs, therefore it is useful to compare the activity of all these mutants. This was done by expressing the activity of the short construct 3' deletion mutants as a percentage of long construct activity (Table 7 (b)) and comparing these to long construct 3' deletion mutants (Figure 25).

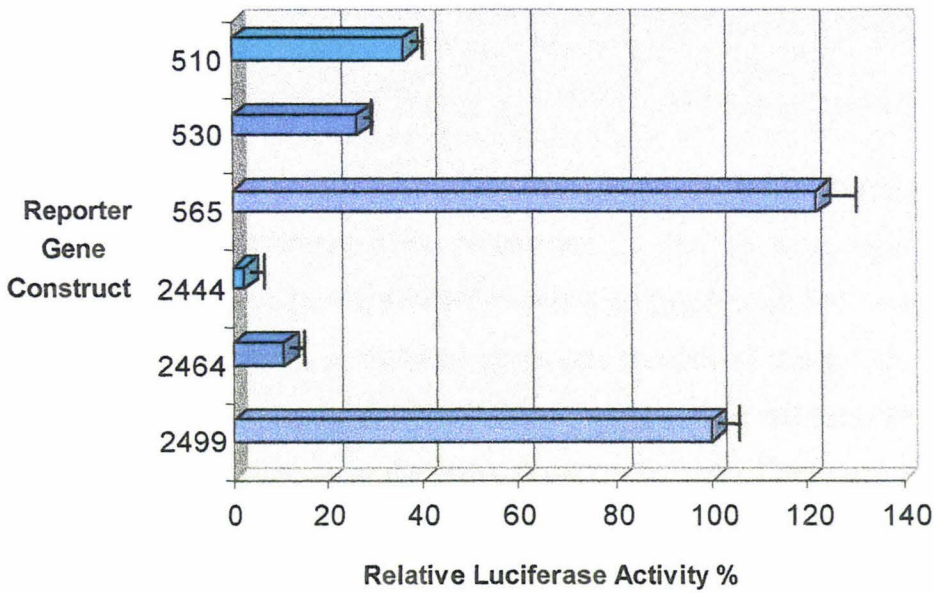


Figure 25: Relative Activity of 3' Deletion Mutants

RL 95-2 cells were transiently transfected with constructs containing different lengths of promoter sequence at the 3' end. The relative Luciferase activity was calculated by setting the luciferase assay result of the long construct to 100%. The other constructs results were then expressed as a percentage of this value. Bars of the same colour carry the same 3' deletion.

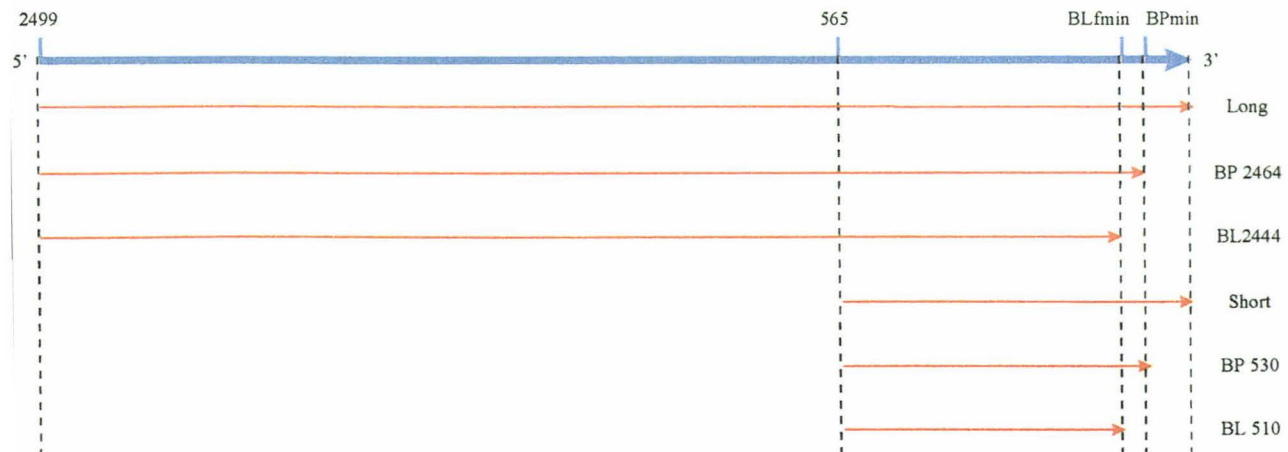


Figure 26: Schematic Diagram of the 2499 bp Long and 565 bp Constructs with BLfmin and BPmin 3' Deletion Mutants

3.3 Identification of TBP Binding Site

3.3.1 Introduction

Transcription initiation on protein encoding genes represents a major control point for gene expression in eukaryotes. RNA polymerase II (Pol II) transcription is an endpoint for a multitude of signal transduction pathways involved in cell development and differentiation. The core, or minimal promoter, consists of the TATA box and transcription start site. The gene specific DNA elements can be enhancer or silencer regions either close to or far from the transcription start site. These are bound by gene specific regulatory proteins that interact with transcriptional cofactors to either activate or inhibit transcription. The principle stages of transcription involve assembly of the preinitiation complex, initiation of transcription with the formation of the first phosphodiester bond, promoter clearance when Pol II leaves the initiation complex and starts elongation and finally, transcript elongation.

The general initiation factors are the basal transcription machinery, a universal set of proteins that recognises the core promoter and initiates transcription. This group of proteins is comprised of Pol II, TFIIA, TFIIB, TFIID (TBP-TBP associated factors (TAFs)), TFIIIE, TFIIF and TFIIH. Assembly of the preinitiation complex is thought to involve step wise process *in vitro* (Roeder, 1996). The first step is the binding of TFIID to the TATA box, an interaction which is then stabilised by TFIIA. The binding of TBP component of TFIID to the TATA element through minor groove contacts, forms a stable complex with an unprecedented DNA distortion and a resulting bend that brings sequences upstream and downstream of the TATA-box element into closer apposition (Buratowski *et al.*, 1989). TFIIB interacts directly with TBP-TFIIA and with DNA sequences downstream of the TATA and recruits a RNA Pol II-TFIIF complex to the promoter. TFIIB has been implicated in transcription start site selection by RNA Pol II. Crystallographic analysis of a TFIIB-RNA Pol II complex indicates that the minimal distance between TFIIB and the active site of the enzyme corresponds roughly to that between the TBP-TATA complex and the initiation site on the DNA (Roeder, 1996 and references within). TFIIIE binds to

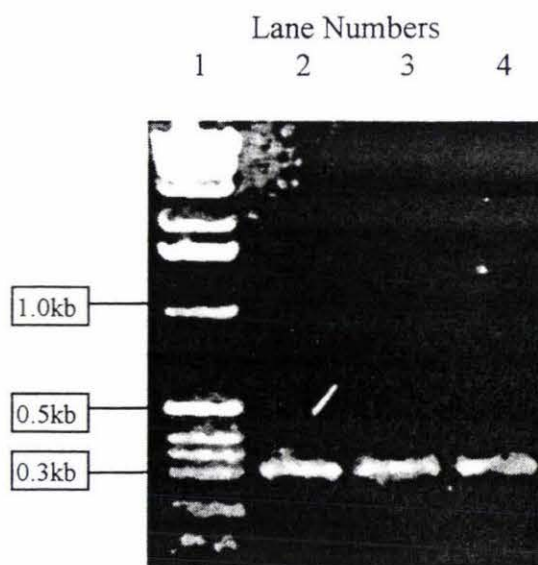
the preinitiation complex, recruits TFIID and modulates the helicase and kinase activities of TFIID and is thought to be involved in promoter melting (Kaiser and Meisterernst, 1996). At this stage the protein-DNA contacts within the preinitiation complex are thought to extend to position +30. The preinitiation complex that is formed with the minimal basal factors results from sequence-specific DNA-protein (TBP) interactions, position specific but sequence independent DNA-protein interactions (e.g. TFIIB), and a large number of protein-protein interactions. The TATA-box element is usually located ~ 25 bp upstream of the transcription initiation site (Lewin, 1994). Computer sequence analysis has identified several putative TATA-box elements in the bovine lactoferrin promoter sequence (H. Bain, 1995). Also the primer extension experiments by Seyfert *et al.*, (1994) suggest that transcription initiation occurs ~ 40 bp upstream of the ATG. A TATA-box element can be identified ~ 25 bp upstream of this putative transcription initiation site as occurs in the generic promoter. Seyfert *et al.*, (1994) also performed computer homology at the transcription initiation site for human, bovine and mouse lactoferrin (Figure 27). The homology at this region appears to be high, especially between the human and bovine lactoferrin promoter sequences. This is good evidence for the above described position of the transcription initiation site and hence the TATA-box element position. However, the recent publication of the goat lactoferrin cDNA (Le Provost *et al.*, 1994) has brought into question the transcription initiation site and TATA-box element position.

Figure 28 shows the sequence homology between the bovine genomic DNA and goat cDNA. The sequence in black is the bovine genomic DNA. Note the putative transcription initiation site described by Seyfert *et al.*, (1994) and the putative TATA-box which is thought to drive transcription from this point. Both are within the mRNA sequence described for goat lactoferrin (blue sequence, Figure 28). The initiation of transcription from within or 5' to a TATA box element would be an unprecedented occurrence for a RNA polymerase II promoter. The homology between the bovine lactoferrin promoter sequence and this portion of goat lactoferrin cDNA is much greater than is seen when comparisons are made between bovine promoter sequence and the promoter sequences of human and mouse lactoferrin. This may be due to the bovine and caprine species both being ruminants.

The goat cDNA is derived from mRNA. There is close match of this sequence to the bovine lactoferrin promoter sequence and another putative TATA box element can be identified in the bovine lactoferrin promoter the appropriate distance upstream of where the goat lactoferrin cDNA terminates. Hence, it is necessary to question the evidence reported by Seyfert *et al.*, (1994) as to the position of the transcription initiation site and therefore the TATA box element position.

3.3.2 DNA Footprinting

DNase I footprinting was used in an attempt to identify which TATA box element has the stronger affinity for TBP. These preliminary experiments were performed by amplifying a 300 bp region of the bovine lactoferrin promoter using the 2.8 kb genomic clone which contains the ATG and some of exon I. Two oligonucleotide primers, BP-1 and BP-2 (Appendix, Figures 33 and 34), were designed to locate both putative TATA box elements approximately in the centre of the 300 bp template (Figure 30). One of each of the primers in the oligonucleotide set was labelled with ³²P and used in separate PCR reactions with a complementary unlabelled primer (see Figure 29). The result was two DNA templates labelled on either the sense or antisense strand. Aliquots of each template were used in titration reactions with DNase I to optimise for digestion time and enzyme concentration. Unfortunately, these preliminary experiments were unsuccessful because a suitable titration reaction template could not be obtained after several attempts. Additional experiments are required to obtain a suitable titration result before identification of the TBP binding site can be determined. The identification of which TATA box element is being employed will help in defining the minimal promoter region and the possible molecular mechanisms involved in the expression of bovine lactoferrin.

Figure 29: PCR Product for DNA Footprinting

Analysis of footprinting templates; A 5 μ L aliquot of each PCR reaction was analysed by electrophoresis on a 1% agarose gel containing 1 \times TAE. The DNA was stained using ethidium bromide (0.25 μ g/mL) and visualised on an UV transilluminator.

1: BRL 1 kb ladder

2: A 300 bp fragment produced from the 2.8 kb genomic construct as a template using the primers P³²BP-1 and BP-2.

3: A 300 bp fragment produced from the 2.8 kb genomic construct as a template using the primers BP-1 and P³²BP-2.

4: A 300 bp fragment produced in a control from the 2.8 kb genomic construct as a template using the primers BP-1 and BP-2 both unlabelled.

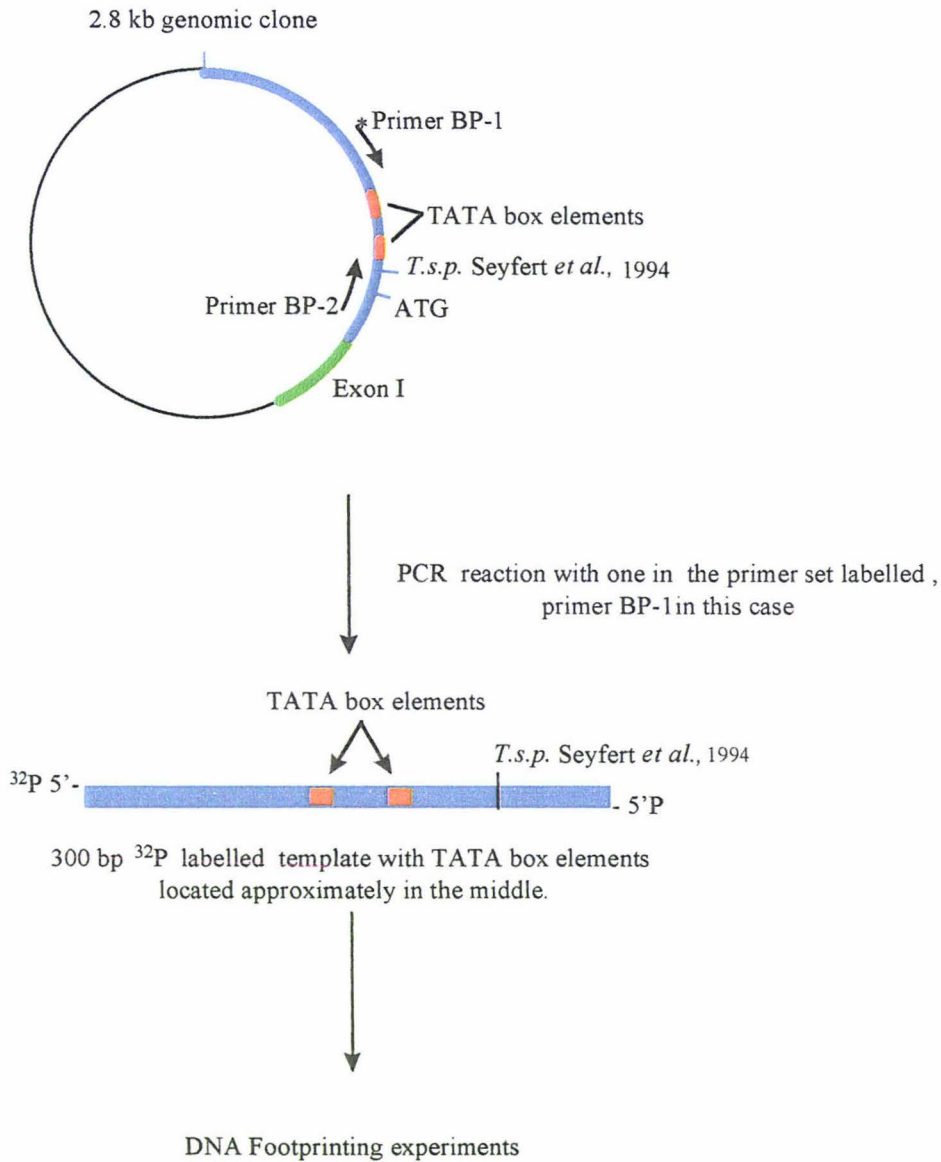


Figure 30: Strategy for Template Preparation for use in Footprinting Experiments

Chapter Four: Discussion & Conclusions

4.1 Introduction

The isolation of the bovine lactoferrin promoter by H. Bain (1995) has provided the basis for further investigation into the molecular mechanisms controlling the expression of the bovine lactoferrin gene. The regulation of bovine lactoferrin gene expression in the mammary gland appears to be developmentally regulated and is species specific. The lactoferrin protein levels vary dramatically throughout the lactation cycle and analysis of RNA levels suggests that the regulation of bovine lactoferrin gene expression occurs at the level of transcription (Schanbacher *et al.*, 1993). The goal of the work presented in this thesis was to identify the minimal promoter region and other regions of sequence that confer positive and negative regulation on the transcription of bovine lactoferrin.

Primary mammary cells, which offer the best physiological system to investigate the activity of the bovine lactoferrin promoter, were used by H. Bain (1995) with bovine lactoferrin promoter fragments in vectors containing the human growth hormone reporter gene. Unfortunately these constructs had very low activity within primary bovine mammary cells even though the cells were actively synthesising lactoferrin protein. Expression of the lactoferrin human growth hormone constructs was also undetectable in the mouse mammary epithelial cell line COMMA-1D. This result may have been a consequence of low sensitivity of the human growth hormone assay as the mouse lactoferrin promoter in the vector pSV-CAT did have activity in this cell line (Liu and Teng, 1991). Therefore an alternative assay system was developed.

Lactoferrin promoter reporter gene constructs were prepared in the pGL-2E vector which contains the luciferase reporter gene and SV40 enhancer element to elevate the promoter activity. The vector pGL-2E is a component of the Promega luciferase assay system, a system which is very sensitive for transcriptional activation in eukaryotic cells.

Two constructs had been prepared previously by H. Bain (1995) containing different lengths of the bovine lactoferrin promoter. These were designated the long and short constructs (Figure 7). Bovine lactoferrin promoter activity was demonstrated for the short construct in COS cells. COS cells are a fibroblast cell line initially derived from African green monkey kidney cells. As these cells do not represent a physiological system for the expression of bovine lactoferrin, the human endometrial carcinoma cell line RL 95-2 was obtained for use in this study. The RL 95-2 cell line has been used to study both the mouse and human lactoferrin promoters (Teng *et al.*, 1992) and was considered a step closer to representing the physiological environment of the bovine lactoferrin promoter. The ideal situation would be to transfect involuting primary bovine mammary cells with reporter gene constructs since the highest level of lactoferrin expression is observed at this stage of mammary development (Schanbacher *et al.*, 1993). The technology required to develop such a system is not currently available in the Department of Biochemistry at Massey University.

4.2 Promoter Construct Sequence Analysis

Both the long and short bovine lactoferrin promoter reporter gene constructs had been prepared by PCR from the original genomic clones (H. Bain, 1995). The long construct had been sequenced previously to check for errors introduced through PCR. Sequence analysis of the short construct confirmed that this promoter sequence was also free of PCR-induced errors. This work validated the previous results of the transient transfection of COS cells with this construct (H. Bain, 1995).

4.3 Deletion Analysis

The primary goal of this work was to identify the minimal promoter region necessary for basal transcription. Therefore a series of deletion mutants was constructed in the vector pGL-2E and analysed for transcriptional activity using transient transfection of RL 95-2 cells. Nested restriction sites within the promoter sequence and PCR mutagenesis were used to create a series of 5' and 3' deletion mutants respectively. The preparation of the 3' deletion mutants represents a major component of this thesis.

4.4 Preparation of 3' Deletion Mutants

The long and short constructs were used to prepare 3' deletion mutants. One reason for the construction of these mutants was to reduce the distance between the putative transcription start point of the bovine lactoferrin promoter and the start codon of the luciferase gene to the distance of the putative transcription start point and the start codon in the reported lactoferrin sequence. The primer BLfmin was used to introduce a *Bgl* II restriction endonuclease site 5' to the putative transcription initiation site described by Seyfert *et al.*, (1994). This primer was used initially to create a 3' deletion mutant of the short construct called BL510. DNA sequence analysis confirmed that the construct was free of PCR-induced errors. This primer was also used in the construction of a 3' deletion mutant of the long construct using a different cloning strategy (section 3.1.5). This long construct deletion mutant, called BL2444, was also sequenced and found to be free of PCR-induced errors.

The 3' deletion mutant BL510 demonstrated very low transcriptional activity in initial transient transfections of RL 95-2 cells which may have been due to removal of the putative transcription initiation site. Therefore a primer was designed to create 3' deletion mutants which had 35 bp 5' to the multiple cloning site removed. This region included the putative transcription initiation site so this too was removed. The primer, BPmin, was used initially to prepare a 3' deletion mutant, BP2464, of the long construct. The cloning strategy was the same as that used in the construction of BL2444 and sequencing confirmed the absence of errors introduced by PCR. Simple restriction endonuclease deletion of a 5' region in the BP2464 construct was used to prepare the short construct 3' deletion mutant, BP530. This construct was sequenced at the 5' end of the promoter to check that the correct mutant had been prepared.

4.5 Transient Transfection of Reporter Gene Constructs

Promoter activity of deletion mutants was evaluated using transient transfection of RL 95-2 cells followed by assay of cell lysates for luciferase activity. Relative luciferase activity was normalised for transfection efficiency and cell number by measuring β -galactosidase activity produced by co-transfection with the control vector pCH110 or pSV β -gal. In this process it was assumed that both the β -galactosidase and the luciferase plasmids were introduced into the RL 95-2 cells with the same efficiency and that the reporter genes were expressed independently of each other.

4.6 Analysis of Deletion Mutants

5' Deletion Mutants

The results for the 5' deletion analysis are presented in Chapter 3: Results, in Table 5 and Figure 21. Discussion of these results will start from the 5' end of the largest bovine lactoferrin promoter fragment.

There was no significant difference between the 2499 and 1852 constructs when mean expression of each construct and the respective standard error of the mean values were compared (Table 5). This implies that removing approximately 650 bp from the 5' end of the largest construct had no effect on the transcriptional activity of the bovine lactoferrin promoter. A large number of putative *cis*-acting elements lie in this 650 bp region but the removal of these appears to have no effect on transcription when the constructs are analysed in the environment of the RL 95-2 cells (compare Figures 21 and 22). The above trend was similar when the activity of the mouse lactoferrin promoter was analysed in RL 95-2 cells in that only 1739 bp upstream of the transcription initiation site was necessary for the maximum activity of transfected CAT reporter genes (Liu and Teng, 1991).

In contrast to the 2499 and 1855 constructs, a significant decrease in luciferase expression was observed for the next 5' deletion mutant construct, 1444. This suggests that elements within the 400 bp between 1852 and 1444 confer positive effects on transcription. The protein factors that bind in this region may promote

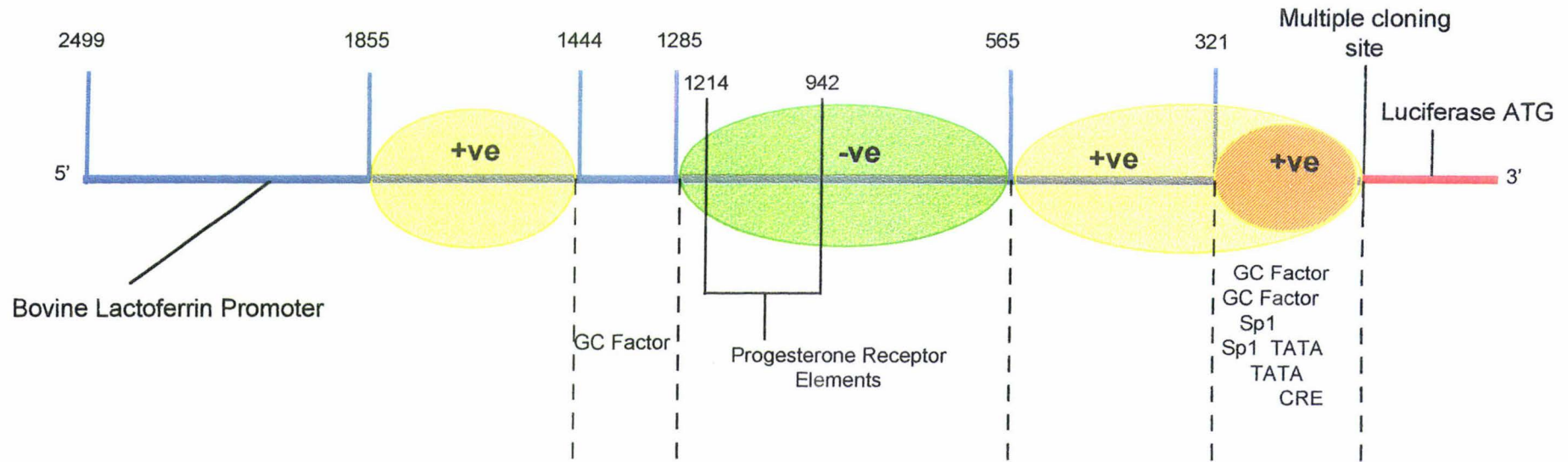


Figure 31: This is a summary of the transient transfection results of the 5' deletion mutants. Regions of sequence that are highlighted are those thought to modulate gene expression. Sequence elements that are described in the discussion are also represented

formation of the initiation complex or stimulate RNA polymerase II promoter clearance.

There was no significant difference in luciferase expression between the 1444 and 1285 constructs (Table 5), which suggests that 160 bp between 1444 and 1285 do not carry any critical *cis*-acting sequences. Indeed, by computer homology, only a GC factor binding sequence was identified in this region (Figure 22).

An increase in promoter activity was observed for the 565 construct which had the highest level of expression of all the constructs tested. The increase in expression observed when 720 bp are removed from the 1285 construct to create the 565 construct suggests that some of sequence elements in the 720 bp upstream of the 565 construct, may have a negative effect on bovine lactoferrin expression in RL 95-2 cells. This 720 bp region contains the progesterone receptor response elements (Figures 22 and 31). Comparing this deletion analysis to that of the mouse lactoferrin promoter carried out by Teng *et al.*, (1992), a similar effect was seen for the constructs carrying the estrogen response module. The shortest construct containing this module was 600 bp and had a lower activity than both the minimal promoter construct of 250 bp and constructs of lengths greater than 600 bp long. A reason for the lower activity of this construct may be that the estrogen receptor was bound to the estrogen response module but was unable to activate transcription because the appropriate effector was not bound, the result being inhibition of transcription. When RL 95-2 cells were co-transfected with the 600 bp construct and the estrogen receptor expression plasmid in the presence of diethylstilbestrol (DES), an estrogen agonist, the expression increased above that of the minimal promoter construct. A similar situation may occur in the bovine lactoferrin mediated by the progesterone receptor response elements.

The removal of approximately 240 bp from the 565 bp construct creating the 321 construct produced a decrease in promoter activity (Table 5 and Figure 21). The level of expression was similar to that seen for both the 1285 and 1444 constructs. The sequence elements responsible for the high level of transcriptional activity seen for

the 565 bp construct are most likely to be located in this 240 bp region. Again comparing this promoter deletion analysis to that of the mouse lactoferrin gene, 321 bp is similar in size to the minimal promoter region for the mouse gene. The mouse lactoferrin minimal promoter resides within the -234 to -21 region relative to the transcription initiation site and contains the noncanonical TATA-box, two CAAT elements (these elements bind members of the CAAT enhancer binding protein (C/EBP) family including NF- κ B) and a GC rich region. Analysis of Figure 22 shows that there are several putative *cis*-elements in the 312 construct, many similar to those identified in the mouse lactoferrin promoter. These include GC elements and TATA-box elements but their relevance to transcription is not known at this stage and so the 5' boundary of the minimal promoter of bovine lactoferrin is still to be defined (Figure 31).

3' Deletion Mutants

Table 6 and Figure 23 present the results of the 3' deletion mutants of the short 565 bp construct and are expressed as a percentage of this construct.

Removal of the 3' region of the 565 construct to produce the BL510 and BP530 mutants has reduced the level of promoter activity by approximately 70 %. This is a dramatic decrease considering the 565 construct demonstrated the highest level of expression in the 5' deletion analysis and indicates these regions of sequence are important for transcription or some other step in the formation of the final protein product.

Table 7(a) presents the results for BL2444 and BP2464, 3' deletion mutants of the long 2499 bp construct. Figure 24 is a graphical comparison of the 3' deletion mutant construct activity when compared to the long construct. Again there is a large decrease in promoter activity, 90 % for the BP2464 construct and 97% for the BL2444 construct, indicating that sequences important for reporter gene expression may reside within the deleted regions.

Table 7(b) is a summary of the promoter activity of the short construct and the 3' deletion mutants of the short construct, expressed as a percentage of the long construct promoter activity. This allows the comparison of the long construct 3' deletion mutants and the short construct 3' deletion mutants.

The comparison is shown in Figure 25. As already stated in the 5' deletion analysis, the short construct demonstrated a higher level of promoter activity than the long construct. This was also seen for the corresponding BLfmin and BPmin 3' deletion mutants of the long and short constructs. Both the 3' deletion mutants of the short construct (BL510 and BP530) demonstrated approximately two times the level of promoter activity seen for the corresponding long construct 3' deletion mutants (BL2444 and BP2464).

This evidence supports the suggestion that the 565 bp construct contains sequences that are important for, and act positively in, modulating transcription and conversely that the 2499 bp long construct contains some sequences which act to decrease the level of transcription. These sequences may be located in the region between the 1285 and 565 constructs (see 5' Deletion Mutants and Figure 31).

The reason for the large decrease in the BLfmin and BPmin 3' deletion mutant activities may be associated with transcription efficiency but could also involve some post-transcriptional event. The drop in the luciferase expression of the BLfmin mutants is probably not associated with the removal of the transcription start point as the level of promoter activity in the BPmin mutants is similar even though the putative transcription start point is present. This conclusion supports the current idealised model for the assembly of RNA polymerase II in that the TATA-box position normally determines the physical location of the transcription start point, not the sequence at the transcription start point (Roeder, 1996).

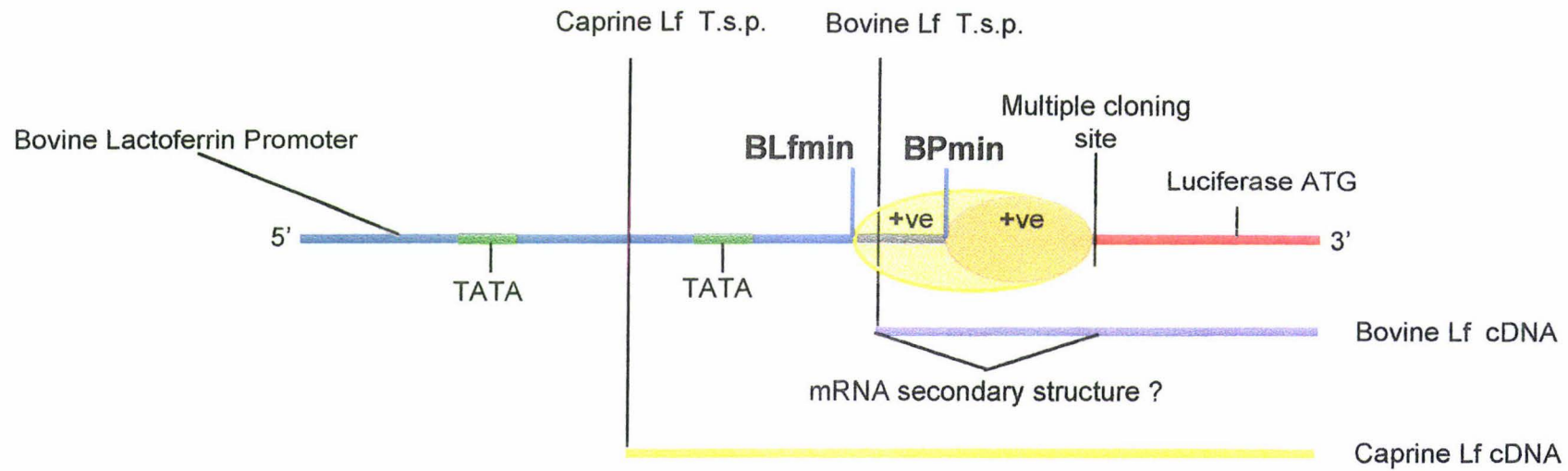


Figure 32: This is a summary of the effects that regions of bovine lactoferrin sequence, removed to make the Blfmin and BPmin reporter gene constructs, have on transcriptional activity. Included are the factors that may have some importance in this region; the BLf t.s.p. position (Seyfert *et al.*, 1994), the Caprine Lf t.s.p. described by Le Provost *et al.*, (1994) and the relevant TATA-box elements.

The reporter gene assays used in this study to detect the effects of the changes made in the construction of the 5' and 3' deletion mutants, cannot distinguish if the change was made at the level of transcription or a post-transcriptional event. What we currently know about promoter organisation allows the presumption that changes made to the 5' end of the promoter (5' deletion mutants) effect reporter gene expression at the level of transcription. This cannot be presumed in the case of 3' deletion mutants because the mRNA transcript may be directly affected by changes made at the 3' end of the promoter. The change to the mRNA transcript may affect subsequent events in gene expression such as translation control and mRNA stability.

All of the sequence removed from the 3' end in the BPmin constructs and the majority of the sequence, except two base pairs, in the BLfmin constructs are known to be transcribed into mRNA (Schanbacher *et al.*, 1993 and Seyfert *et al.*, 1994). Sequence elements in this region may affect the efficiency of transcription but, more probably, a post-transcriptional event is occurring. This could include mRNA stability or a translation control mechanism. mRNA stability has previously been implicated as a control mechanism in bovine lactoferrin gene expression by Schanbacher *et al.* (1993 and personal communication).

When Schanbacher *et al.*, 1993 cloned the bovine lactoferrin gene they had difficulty in synthesising the cDNA 5' from the mRNA molecule. They proposed that this was due to an unusual mRNA secondary structure that occurred around base +237 where cDNA synthesis halted. Direct sequencing of the mRNA itself was necessary to obtain the putative 5' untranslated region (UTR). The 5' terminus of the UTR corresponded to that reported by Seyfert *et al.* (1994). Schanbacher and co-workers (1993) used the RNA-FOLD program to investigate the possible secondary structures that may occur in bases +5 to +205, a region which includes the AUG translation start codon of the gene. A strong secondary structure was predicted for this molecule, a stem-loop complex with a stability sufficient to inhibit translation. When this prediction was contrasted to that for the secondary structure of the human lactoferrin gene in the same region, the human gene was shown to be less stable and hence not

capable of inhibiting translation. The difficulty in cDNA cloning and the predicted stem-loop secondary structures for bovine lactoferrin both suggest that secondary structure at the 5' UTR may contribute to the regulation of the bovine lactoferrin gene. Furthermore the translation initiation sequence differs greatly from the optimal Kozak consensus sequence, which is in contrast to the other milk protein genes (Schanbacher *et al.*, 1993).

Replacement of the bovine lactoferrin 5' UTR with random sequences to disrupt the predicted complex stem-loop secondary structure did not affect the expression of the bovine lactoferrin gene (Schanbacher *et al.* personal communication). The exact strategy for these experiments is unknown but in light of the cDNA sequence for goat lactoferrin (Le Provost *et al.*, 1994) it is possible be that Schanbacher *et al.*, (personal communication) did not include enough sequence at the 5' end. Until the exact position of the TATA-box element and the transcription initiation site have been identified the role of mRNA secondary structure in affecting the expression from the 3' deletion mutants in this study must remain speculative.

Although it can be said that the 3' end of the minimal promoter region for bovine lactoferrin is in the proximity of the 3' deletion constructs presented here (i.e. -1 to +34 relative to the transcription initiation site described by Seyfert *et al.*, 1994) it will be necessary to define this region more clearly using additional deletions 5' to those presented here. The first step may be to remove the putative TATA-box to see if this has any effect on transcription. This would also complement DNase I footprinting of this region as described in section 3.3.

4.7 DNase I Footprinting

An attempt was made to investigate the conflicting evidence on the location of the transcription initiation site using DNase I footprinting, but was unsuccessful due to both technical difficulties and time constraints. These experiments need to be carried out in conjunction with a further 3' deletion analysis to remove the most 3' TATA-

box element which is thought to be the putative TBP recognition site. The effect of removing this TATA-box element will be drastic if it is essential for transcription.

4.8 Final Discussion and Conclusion

The initial goal of the work for this thesis was to sequence the short construct of the bovine lactoferrin promoter prepared by H Bain, (1995) and validate the promoter activity seen in COS cells. The sequence was found to free of any PCR introduced errors. The next step was to establish if the bovine lactoferrin promoter had activity in the human endometrial cell line RL 95-2. The RL 95-2 cells represent a better physiological system than COS cells and have been used to study both the mouse and human lactoferrin promoters (Liu and Teng, 1992). Both the long and short constructs of bovine lactoferrin had transcriptional activity in these cells. Having established that this was a viable system to study the bovine lactoferrin promoter, a deletion analysis of the 5' and 3' ends was begun. A series of both 3' and 5' deletion constructs were used in transient transfection experiments using RL 95-2 cells.

The primary goals of this work were to identify the minimal promoter region necessary for basal transcription and also regions that confer positive and negative regulation upon the basal activity of the promoter. A region of positive regulation was found upstream of the 1444 5' deletion mutant and the region between 1444 and 565 appeared to contain negative elements. The 565 construct contained strong positive regulatory elements but the effects of these elements were not seen until the 565-1855 region was removed. The 321 5' deletion mutant demonstrated lower activity than the 565 construct confirming that positive elements important for activation of transcription are located between 565 and 321 (summarised in Figure 31). The 5' boundary of the minimal promoter is close to the 321 mutation point but further analysis is needed to define the 5' end of the minimal promoter.

The 3' deletion mutants of the long and short constructs confirm the results obtained for the 5' deletion mutants. Both sets show dramatic decrease in transcription although the short construct 3' deletion mutants have a higher activity than the long

construct 3' deletion mutants. This may be due to the positive region in the 565 construct identified in the 5' deletion analysis (321-565). Analysis of the 3' deletion mutants also demonstrated that there are negative elements active in the long construct. The high degree of secondary structure predicted for the 5' UTR (Schanbacher *et al.*, 1993) and the results of the 3' deletion analysis suggest that a post-transcriptional mechanism may play a role in regulating the activity of the bovine lactoferrin gene. Changes in this transcript introduced through the 3' deletion analysis may have altered transcript stability and/or translation control, affecting the level of transcription observed in the reporter gene assays (Figure 32). The action of activators is dependent upon the basal transcription apparatus, therefore further delineation of the minimal promoter boundaries must be performed to provide a reference point to investigate activator function. Once this is accomplished the bovine lactoferrin promoter will provide a useful model in the study of tissue specific and induced eukaryotic gene expression.

There must be some caution in interpreting the results of the transfection experiments as the RL 95-2 cells were transformed using an SV40 virus and it is not known if they express T-antigen. T-antigen is a viral protein which is thought to act on the SV40 enhancer element and the basal transcription machinery giving high levels of expression (Banerji *et al.*, 1981; McKnight and Tjian., 1986). The mechanism of its action is not known. It is not normally expressed in endometrial cells and it is not known if it was present in these experiments.

4.9 Future Directions

Clarification of Minimal Promoter Region

It will be necessary to define the minimal promoter more clearly at its 5' and 3' ends. Further 5' and 3' deletion constructs should be made and assayed for functionality in transiently transfected RL 95-2 cells until the ability of the promoter to support transcription ceases. The deletion of the putative TATA-box at the 3' end of the promoter would help clarify the uncertainty regarding the position of the transcription initiation site. Once the minimal promoter has been established the regions which act to regulate the basal transcription complex can be confirmed. This study has identified regions of promoter sequence that modulate expression in either a positive or negative way. These regions can then be used in further deletion analyses to identify the specific *cis*-elements that are acting within enhancers and repressors. For example, a positive region of sequence has been identified between the 1852 and 1444 constructs, a range of approximately 400 bp. Strategic deletion analysis of this region would aid in identifying the *cis*-sequences present which are acting to modulate transcription.

It may be necessary to investigate the role of the SV40 enhancer found in the pGL-2E vector. Deletion mutants need to be placed in reporter vectors that do not contain the SV40 enhancer element, such as pGL-2B. The use of such constructs will determine if the enhancer is overriding the endogenous effects of the bovine lactoferrin promoter.

Transcription factor Binding Sites

When specific *cis*-sequences are implicated in modulating promoter expression electromobility shift assays (EMSA) may be used to determine if proteins will interact with this sequence. Using a short radiolabelled template that contains the sequence to be investigated, incubation with either a cell nuclear extract or a specific protein will determine whether there is a significant protein-DNA interaction. The specificity of a DNA protein interaction can be investigated using a competitor of unlabelled template, where excess of unlabelled template abolishes the mobility shift seen on a polyacrylamide gel. A non-specific template could also be tested and used as a

control. Supershift experiments can also be used where an antibody to a specific protein thought to be involved in the protein-DNA interaction is added. A further shift in band mobility implies that the protein antigen is involved in the interaction.

DNase I footprinting could be used to identify specific DNA protein interactions over a wider region. As was attempted for the identification of the TATA-box element, a region of sequence may be amplified from the promoter sequence using PCR. This can then be incubated with a nuclear extract from RL 95-2 cells and protein binding sites identified after unbound sequence has been digested with DNase I and the resulting fragments electrophoresed on a denaturing polyacrylamide gel. Nuclear extracts from bovine mammary gland at different stages in the lactation cycle are available for use in the above type of experiments. Such experiments may provide some of the answers as to the physiological mechanisms acting in the bovine mammary gland to regulate lactoferrin *in vivo*.

Specific Transcription Factors

Progesterone: There is evidence that progesterone may modulate the expression of the bovine lactoferrin gene (Chapter 1: Introduction). Initially it will be important to demonstrate that the promoter is responsive to progesterone. This could be accomplished by supplementation of the media in which the RL 95-2 cells are grown with the progesterone hormone as well as the co-transfection of the RL 95-2 cells with reporter gene constructs and an expression plasmid for the progesterone receptor. If the cells are responsive, EMSA and DNase I footprinting experiments may be used with enriched nuclear extracts or purified progesterone receptor itself. This work could be complemented by reblotting the northern hybridisation of Schanbacher *et al.*, (1993) with a probe for the progesterone receptor. This would determine if the progesterone receptor is present in bovine mammary cells and if it cycles during the lactation cycle.

Interleukin-6: There are seven putative NF-IL 6 binding sites in the bovine lactoferrin sequence. As stated in Chapter 1: Introduction, the only time lactoferrin levels increase during lactation is when infection occurs. Interleukin 6 is an inflammatory

cytokine and may act to modulate the expression of bovine lactoferrin during times of infection. It will be necessary to establish if the promoter is responsive to interleukin-6 in the RL 95-2 cells. Experiments such as the EMSA and DNase I footprinting experiments described above for progesterone responsiveness, will need to be performed.

Once a specific transcription factor binding site or module has been identified it may be further characterised through the construction of heterologous reporter genes. Such experiments were performed for the estrogen response module (ERM) of the mouse lactoferrin promoter (Teng *et al.*, 1992). The ERM was placed either in front of the mouse lactoferrin minimal promoter or simian virus 40 (SV40) promoter sequences. The estrogen responsiveness of these reporter gene constructs was then tested in RL 95-2 cells co-transfected with an estrogen receptor expression plasmid and the tissue culture medium supplemented with estrogen. When estrogen responsiveness was established, specific point mutations were used to identify key DNA-binding sites necessary for protein interaction (Teng *et al.*, 1992). These type of experiments may be used to determine the functional significance of a modules transcriptional activity and its context within a promoter.

Transcription Initiation Site

Continuation of the DNase I footprinting experiments to identify the major TATA-box element will allow further clarification of the 3' end of the minimal promoter. It is possible that both putative TATA-box elements are utilised providing a method for differential expression of the lactoferrin gene. The transcription initiation site could be determined by primer extension analysis experiments similar to those performed by Seyfert *et al.*, (1994) on the bovine lactoferrin gene and Le Provost *et al.*, (1994) on the goat lactoferrin gene. The strategy used to do so should include primers that on annealing, disrupt the mRNA secondary structure predicted in the 5' UTR and so prevent the inhibition of cDNA synthesis. To complement these experiments a 3' deletion construct in which the putative TATA-box described by Seyfert *et al.*, (1994) is removed should be constructed and analysed.

mRNA Stability

The role of changes in mRNA stability may become clear with a stricter definition of the minimal promoter region and the identification of the transcription start point. Once these are established it will be possible to substitute the 5' UTR with random sequences and observe the effect, on the level of reporter gene expression. It may also be useful to perform a northern hybridisation of reporter genes mRNAs expressed in and isolated from RL 95-2 cells. This would determine if the 5' UTR is having a post-transcriptional effect or if some other transcriptional mechanism is acting.

Physiological System

Primary bovine mammary cells represent the best physiological system for the study of the bovine lactoferrin promoter constructs *in vitro*. This cell line is not currently available in the Department of Biochemistry at Massey University. A better physiological system than that provided by the RL 95-2 cells would be a bovine epithelial or neutrophilic cell line which is known to secrete lactoferrin. Both of these cell types would provide at least the basal transcription components for lactoferrin gene expression.

4.10 Summary

In summary, the expression of the short bovine lactoferrin promoter construct in COS cells has been validated through the sequencing of this construct. Expression of the long and short bovine lactoferrin reporter gene constructs has been established in the human endometrial carcinoma cell line RL 95-2. This is important because both the human and mouse lactoferrin genes have been shown to be active within these cells and this cell line represents the best physiological system available at the present time. A strategic 3' deletion analysis has been performed on both the long and short constructs. These constructs were then tested in a transient transfection procedure in RL 95-2 cells and these assayed for reporter gene activity. These type of experiments were also performed on a series of previously constructed bovine lactoferrin promoter 5' deletion mutant reporter genes. Using reporter gene assays, regions that confer positive and negative regulation upon the bovine lactoferrin promoter have been

defined but the precise boundaries of the minimal promoter require further investigation.

Analysis of the literature and other lactoferrin gene sequences has cast doubt on the putative transcription initiation site described by Seyfert *et al.*, (1994). Evidence for goat lactoferrin cDNA suggest that the putative transcription initiation point may occur at position -36 (Le Provost *et al.*, 1994) relative to that described by Seyfert *et al.*, (1994). The possibility exists that there may be multiple active TATA-box elements and transcription initiation sites.

These investigations provide the beginning to defining the minimal functional promoter region of the bovine lactoferrin gene, and will enable further studies into the molecular mechanisms involved in the tissue specific and developmental regulation of this gene. With these insights, the elucidation of the biological function of bovine lactoferrin and the lactoferrins of other species may also be possible.

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Appendix

Figure 33: Nucleotide Sequence of the Bovine Lactoferrin Promoter 2.8 kb Genomic Clone Showing Primer Annealing Sites Used for Cloning and Sequencing

1 GAGCTCAGGATGGAGGACATGACTTTGTGAATCCTCCTTCAATGTATTCC 50
 51 AGGTCGGAAAGCCTAGGGCAACTTTTGGTTGTCCGTTGAGGCCACTGAAT 100
 101 CCATGTTCTCAAGCCTTTACCTGGCTATTTCTCTACCTGAAGTTCTCTT 150
 151 GGTAGATAGGTAACCTTTCTTTCAGGTACCTAGGTCAAATGCTCTTCTTC 200
 201 AAAGCCTTATGGGCTCCTGATGATGCAAACCGGTCAAATGGCCCTCCTTC 250
 251 GGCTCCCTCGCTCACTCTATTTATGCTGCCTTGCCTCTTCCGTGGCCTGT 300
 301 GCCCACCCCACCCTCGGTGTGCCGTGAGCTCCTTAGGGCCAGGACGGGAC 350
 351 CCCCTTCTCTGTGTCCCTCTTGGTACCCACCAGTTCTGGCTTGGATGAGT 400
 401 GAATGAATGAATGTTGATAACAGATCCATGGAACATTGTCTTCTGGGCAG 450
 451 CCCCACCTCCTTTGT CAGCTTCAGATGGCCTTGGGGGCTGTTCTGTTGGC 500
 501 CTGAGGCTGGGACATTCCCTTGGAGACACAGCATGAAAACAGTCTGCTTAC 550
 551 TCCAATCCTGCCTCCAGGGCAATCCCTCAGCTCAGCCTCTCAGTTGTGGC 600
 601 CCCAGGTTCTCTATGTTCCCTGCCAACTCTGTATCAGACATGAGAGAATCT 650
 651 GCAGGCATCTTACCTCCCAACCCATCCTTTTTCTAATTTGCACTTGGAGA 700
 701 TACAGACCTGGGTTGTGACATGTCTTAATTCTTTTATAATCACGGAAGGG 750
 751 CAAAGCAAGTTGCTAATTTAGATACAAAGATGCTTCAGCACTCCTGAGAA 800

801 TTAGTCAATTTTGTGTTACTTCATTATTTTTGTAATGGCTTATTGCAGTT 850
851 ATTGATGAAAGCAACTTTTAATGGTGCAACACTGTGTTTCCAAACCATGA 900
901 GAGACCCTGGATCCGTCACCCCAAAGCTGACTGGTGATTCTCCCACTGA 950
951 ACCTTGGATCCCCTCCTGTGCCAGCAAGCAGGATCCCTAGTCAGACTC 1000
1001 CACTCATGTGCCTTGGCAAGATCCACGCCAGGATAGAGGGGCCCCACAG 1050
1051 CCTCCTCGAGGGCCTCCAAGACTTGGGCTGGCTCTTCTTCGCCATGGTGC 1100
1101 CAAGTGCCGCCCACTGCATATCCACCCCAACAGGGCCGCCTCCTGAGGT 1150
1151 GTTGCCCCTCTGCTCCTGGAAACCCTTTGTGTACTCAGTAGTCTAAGCAA 1200
1201 AGAATCAAGGCCAGCTTTTCAGGACAGACAGATTTCAGAATAACATACTG 1250
1251 TCTAGACTAACCACAGAGGGAATTTCTCTCACTGTTAGTACCTGACTTC 1300
1301 TTCACTTAGTATCTCCTGGAGCTAAGTGCTCATCAGTACTTGCATGGTGG 1350
1351 CCCTTTCTCTCTGGGCCCCCCAGTATGTTCCAGAGCACCATGCTCTATGT 1400
1401 GACAGCCCCCGCCTTTTTTTTTAAACGTTTTTGGCCACACCTCACAGCATA 1450
1451 TGGGATCTTAGTTCCCCCAAATGGGTCTGAACCTACACATGCTGCAATGG 1500
1501 AAGCGCCAGGGAAGTCCTCCCCACCCCTTGGGGGACACTTAGTTTGCTT 1550
1551 GCAATCAGTGAACGATAAGCAGGGCTGCACTGGAGACCCCTGCGTGGGAG 1600
1601 TTGTTGTGCTTCAAGGGAGTGTCTTCAAGGATGCAGAGCAGAGTTCTAG 1650
1651 CTTTAGAACTGAAAACCAGCCTCCTGAAACAGGGTCAGCCTGTGTACTGA 1700
1701 GGACAAAATAGGACATTTATCAAATGAGGTTCTGTCTCCACCTCATA 1750

8D
→

1751 TTGCCACAAAACAACACAAGGGGTAGGATATCCTTTTCATTGGCAAATGA 1800
 1801 GGGACCAGGAGACAGCCTTTGGGCACTTAGGCCTCTGGTTCTGTTTTCTG 1850
 1851 GGAGCTGTATTGCGGTCTCAGGAGGACCCCAGGGGCAGTCTGGGTCAGAC 1900
 1901 TCTGGGCAGCCTCTGCCAGCTGGACCAGGCTGCCGTGGACCCCGGGCCAG 1950
 1951 GCAGCGGGCCCTCTTTCAAACACTCCAGGCTGGCTCTGCGTGCAGATGCAA 2000
 2001 GGGTCTCCGTCTGTCTTAACTGGTTCCCAAGCACTTTAGATACCTTCTCT 2050
 2051 ATAGTCAAGCTGATCCGCAAAGATTCACCCTAGGACCCCTGCTCTGGATC 2100
 2101 CCGCTCTCTAGGAGGCACTGAGACCGGAGCGGGGACAAAACCCAGGGACT 2150
 2151 GCCACTCCCGAAGGGCTGCGGACAAGTGGGAAAGAAAGAGCATCCCCCAA 2200
 2201 CTAGGCAGCGCTGGGGAAGTGTGAGAGGTGGGTGTGGGTGGGTATCCTCT 2250
 2251 CCCCGAGCGCCAAGCCCCGCCAGGCACCTTTCTCGCTCCCTCGGTCTCC 2300
 2301 ACCCCCACTCTTCCCCCTTCCCCCGGTTTTTTCCCCTCTAGGAACCAGCA 2350
 2351 GACCTCGGGAGAGGGGAGGAGGGAGGCTGGGGCGCTTATAGGACCACAGG 2400
 2401 GCGGGGCAAACCTCGTGAGGTCACCGAGCACTGGATAAAGGGACGCAGAA 2450
 2451 CGAGCGCAGGTGGCAGAGCCTTCGTTCCGGAGTCGCCCCAGGACGCCAGC 2500
 2501 CATGAAGCTCTTCGTCCCCGCCCTGCTGTCCCTTGGAGCCCTTGGTGAGT 2550
 2551 GCAGGTATGAGTGGGGGCGGGGCATGCCCTCCAGGTAGGTGTCCCGGT 2600
 2601 CCCACGCCCCGCTGTGGGAGCATCCAGTCCCCTCACCCCTCAGTGTAGG 2650
 2651 CGTCTGGGTCCTCGCTCCCCGCGTGTGGGCCTCCTTGTCCCGCAGCAG 2700

Diagrammatic annotations:

- 7Z: Boxed label with a left-pointing arrow above the sequence at positions 1851-1880.
- 6Q: Boxed label with a right-pointing arrow above the sequence at positions 2001-2050.
- 5B: Boxed label with a left-pointing arrow above the sequence at positions 2051-2100.
- 4A: Boxed label with a right-pointing arrow above the sequence at positions 2201-2250.
- BP-1: Boxed label with a right-pointing arrow above the sequence at positions 2251-2300.
- 3G: Boxed label with a left-pointing arrow below the sequence at positions 2351-2400.
- BLf-min: Boxed label with a left-pointing arrow below the sequence at positions 2451-2500.
- BP-min: Boxed label with a left-pointing arrow below the sequence at positions 2451-2500.
- 5'ATG: Boxed label with a left-pointing arrow below the sequence at positions 2501-2550.
- BP-2: Boxed label with a left-pointing arrow below the sequence at positions 2501-2550.

2701 GCAGTTTGACACGGGCACCTGCCGCAGAGGAGGCGCCGGGAGCGAGCGGA 2750

2751 GCGGATCCCGCCCCTCGCCTGGGGAGGAGCCCGGAGCTGGGTTCCGAGA 2800

2801 AGCCGGCGGGTCCGGTGCCCAGAGCTGCCGAGCTC 2835

BLf-min 5'-GGGGAAGATCTGCGTCCCTTTA-3'

BP-min 5'-GGGGAAGATCTGCCACCTGCGC-3'

BP-1 5'-CCAAGCCCCGCCAGGCACCTTTCT-3'

BP-2 5'-TCATACCTGCACTCACCAAGGGCTC-3'

Figure 34: Primer Sequences Synthesised for Use in this Study

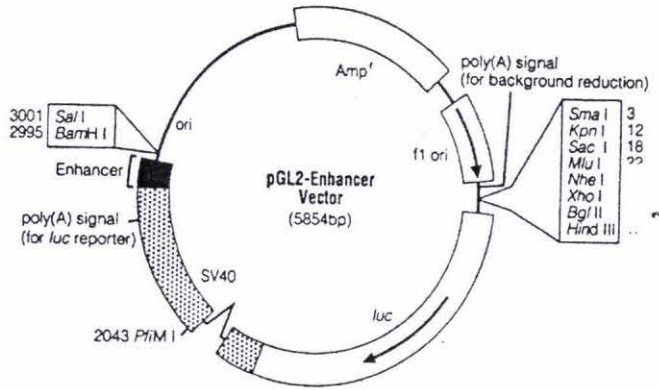


Figure 35: pGL2-Enhancer Vector Map

The pGL2-Enhancer Vector contains an SV40 enhancer inserted downstream of the luciferase gene. This allows testing of putative promoter sequences with the potential increase in transcription provided by the enhancer activity.

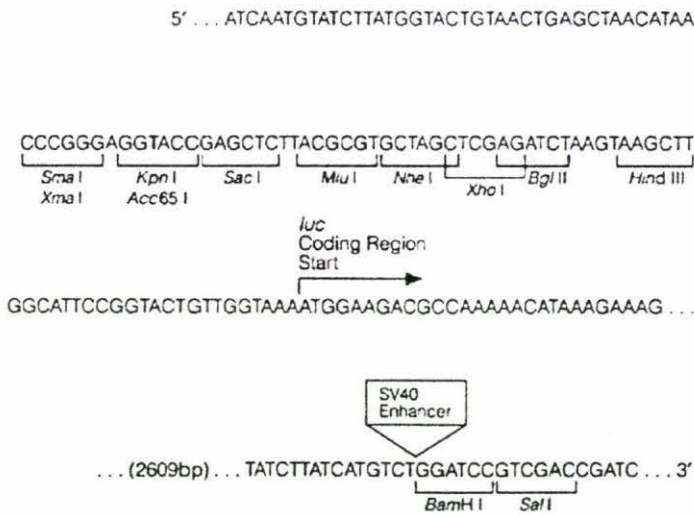


Figure 36: pGL2 Vector Multiple Cloning Region

Shown are the upstream and downstream cloning sites. The position of the enhancer is shown as an insertion into the vector sequence.

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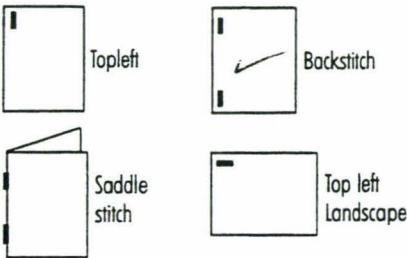
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