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Cloning and sequencing of the cDNA for bovine lactoferrin

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This thesis is submitted to Massey University as partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

Dedication

This thesis is dedicated to my parents
Patricia and David Sidwell.

Thankyou for all your love and support.

Abstract

Bovine lactoferrin isolated from colostrum was partially sequenced by tryptic mapping and automated peptide sequencing. Homogeneous lactoferrin was used to raise polyclonal antibodies in rabbits. Specific anti-lactoferrin antibodies were isolated from the total rabbit gamma-globulin fraction by affinity chromatography on bovine lactoferrin Sepharose. These antibodies were used to quantify lactoferrin in various solutions (by electroimmuno-diffusion assay) and to demonstrate the *de novo* synthesis of lactoferrin in involuting bovine mammary tissue.

RNA was isolated from mammary tissue biopsies that were synthesizing lactoferrin. The presence of lactoferrin messenger RNA was verified by northern blot analysis. Complementary DNA (cDNA) was prepared from RNA samples and ligated into either the bacteriophage vector λ gt11 or the plasmid vector pGEM-2. Recombinant clones with cDNA inserts coding for bovine lactoferrin were identified by hybridisation to radiolabelled human lactoferrin cDNA. Several clones were isolated and characterised by restriction map analysis and DNA sequencing. The overlapping nucleotide sequence from these clones encoded most of the mature protein sequence for bovine lactoferrin.

Nucleotide sequence encoding the 5' end of the lactoferrin messenger RNA was isolated by enzymatic amplification of homopolymeric-tailed first strand cDNA. Specific oligonucleotide primers were used to direct the synthesis of lactoferrin-specific sequences by the polymerase chain reaction (PCR). Double-stranded products were produced by the inclusion of an oligonucleotide that would prime DNA synthesis from the homopolymeric tract on the 3' end of the first strand cDNA. The nucleotide sequence of the PCR products overlapped the 5'-most sequence of the cDNA clones and extended to encode the initiation codon for bovine lactoferrin.

The combined nucleotide sequence of the cDNA and PCR clones overlapped to encode the entire coding region for bovine lactoferrin and included 5' and 3' untranslated flanking sequences. The deduced amino acid sequence of the mature protein concurred with the amino acid sequence of the tryptic peptides prepared from bovine colostrum lactoferrin.

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Abbreviations

ADP	adenosine diphosphate
AHSG	α_2 -HS-glycoprotein
AMV	avian myeloblastosis virus
ATP	adenosine triphosphate
BAPNA	N α -benzoyl-DL-arginine-p-nitroanilide
Bas	basophil
BFU-E	blast forming unit-erythroid
bp	base pair
cDNA	complementary DNA
CDI	carbonyldiimidazole
CFU	colony forming unit
CFU-GEMM	multipotential colony forming unit
CMC	1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide
	metho- <i>p</i> -toluene sulphonate
CM-Sephadex	carboxymethyl-Sephadex
CP	ceruloplasmin
cpm	counts per minute
C-terminal	carboxyl terminal
CTP	cytosine triphosphate
dH ₂ O	deionised water
ddH ₂ O	deionised, distilled water
DEPC	diethylpyrocarbonate
DMF	dimethylformamide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOC	sodium deoxycholate
d(pC) ₂₀	deoxycytosine twenty-mer oligonucleotide
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
Fe-lactoferrin	iron-loaded lactoferrin
γ G	gamma-globulin
GM	granulocyte-macrophage
GM-CSF	GM colony stimulating factor
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
kb	kilobase pairs

K _d	dissociation constant
kDa	kilodalton
IEP	isoelectric point
IM	intramuscular (injection)
IPTG	β-D-isopropyl-thiogalactopyranoside
LB	Luria-Bertani broth
Lf	lactoferrin
Meg	megakaryocyte
M-MLV	Moloney murine leukemia virus
mRNA	messenger RNA
MW	molecular weight
NLS	n-lauryl sarcosine
N-terminal	amino terminal
NTA	nitrilotriacetate
O ₂ ⁻	superoxide anion
·OH	hydroxyl radical
oligo (dT)-cellulose	oligo (deoxythymidine)-cellulose
p97	melanotransferrin (oncofetal 97 kDa protein)
PB	phosphate buffer
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	phage dilution buffer
PHA	phytohemagglutinin
pO ₂	partial pressure of oxygen
Poly A ⁺ RNA	RNA having a polyadenylate tract at its 3' end
Poly A ⁻ RNA	RNA lacking a polyadenylate tract at its 3' end
POPOP	1,4 di-[2, -(5-phenyloxazolyl)] benzene
PPO	2,5-diphenyloxazole
RES	reticuloendothelial system
RME	receptor-mediated endocytosis
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RP-HPLC	reverse phase-HPLC
rpm	revolutions per minute
SAM	S-adenosyl-L-methionine
SC	subcutaneous (injection)

sdH ₂ O	sterile, distilled water
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SM	suspension medium
SSC	sodium chloride and sodium citrate solution
TAE	tris-acetate buffer containing EDTA
TBE	tris-borate buffer containing EDTA
TCA	trichloroacetic acid
TdT	terminal deoxynucleotidyl transferase
TE	tris-HCl buffer containing EDTA
Tf	transferrin
TFA	trifluoroacetic acid
TfR	transferrin receptor
T-lymphocyte	thymus derived lymphocyte
Tris	tris-(hydroxymethyl) aminomethane
TTP	thymidine triphosphate
TX-100	triton X100
UV	ultraviolet
(v/v)	volume:volume ratio
(w/v)	weight:volume ratio
X-Gal	5-bromo-4-chloro-3-indolyl- β -galactopyranoside

Chapter One

Introduction and literature review

Transition metals play an important role in the cellular metabolism of virtually all living organisms. In particular, the iron group, consisting of iron, copper and zinc, are involved in a variety of biological reactions. Some of the fundamental biological processes that these metals are associated with are listed in table 1.1. Undoubtedly the most biologically significant member of this group of transition metals is iron.

Iron (Fe) has two stable oxidation states in aqueous solution, the ferrous (Fe(II) or Fe^{2+}) and the ferric (Fe(III) or Fe^{3+}). This property allows iron to participate in a variety of redox reactions that are central to life as we know it (refer to table 1.1). Clearly iron is a very important, if not essential, ingredient for life. However, the same chemical properties that make iron so useful in biological reactions also make iron potentially toxic. Ferrous ions are capable of catalysing the production of highly reactive species such as the hydroxyl radical ($\cdot\text{OH}$) (Haber and Weiss, 1934) which is capable of rapidly destroying cellular function and viability. On the other hand, ferric ions are practically insoluble at physiological pH. Aquated ferric ions are hydrolytic and can not exceed approximately 10^{-17} M in neutral solution (Aisen and Listowsky, 1980). Consequently cells are faced with the problem of maintaining adequate amounts of iron in a non-toxic and bio-available form. The way many organisms, from bacteria to mammals, have overcome the dilemma of iron essentiality versus potential toxicity is by producing specialised iron-binding proteins. This discussion will deal almost exclusively with the iron-binding proteins found in the vertebrates.

1.1 The iron-binding proteins

The iron-binding proteins of vertebrates can be considered to fall into three functionally distinct groups: (i) the iron-containing proteins with catalytic or carrier functions, (ii) the proteins of iron storage and (iii) the iron transport proteins.

The first category of iron-binding proteins includes the heme-containing cytochromes (eg. cytochromes b, c₁, c and cytochrome oxidase) and oxygen

Table 1.1 Metabolically significant iron-group transition metal-binding proteins.

Metal	Metalloprotein	Catalytic activity and/or function
Zinc	Carboxypeptidase A	Proteolysis
	Phospholipase	Lipid metabolism
	Superoxide dismutase	O ₂ ⁻ scavenger
Copper	Cytochrome oxidase	Electron transport
	Plastocyanin and azurin	Plant electron transport
	Ascorbate oxidase	Oxidation of ascorbic acid
	Ceruloplasmin	Oxidation of Fe ²⁺ to Fe ³⁺
	Superoxide dismutase	O ₂ ⁻ scavenger
Iron	Myoglobin	Intracellular oxygen transport
	Haemoglobin	Extracellular oxygen transport
	Nitrogenase	Bacterial nitrogen fixation
	Succinate dehydrogenase	Intermediary metabolism
	Ferredoxin	Electron transport
	Transferrin	Iron transport
	Lactoferrin	Bacteriostasis
	Ferritin	Iron storage
	Cytochromes	Electron transport

carriers (eg. haemoglobin and myoglobin). Also in this first, broad group are the non-heme iron-containing enzymes and electron carriers. Table 1.1 contains a representative, but far from comprehensive list of the metabolically significant iron-containing proteins that fall into this first category. As indicated in table 1.2, this group of iron-binding (containing) proteins makes up the largest iron compartment in normal humans.

The second group, and the second largest iron compartment in humans (table 1.2), is comprised of the iron storage proteins, ferritin and haemosiderin. Ferritin is a large multimeric protein with a widespread distribution in animal cells. Twenty-four ferritin monomers combine to form an almost spherical protein capable of binding approximately 4,500 atoms of iron as an iron-oxide-phosphate complex (Drysdale, 1988). Although ferritin is apparently ubiquitous in animal cells, it is particularly abundant in mammalian liver,

spleen and bone marrow cells where it has a role in the recycling of iron for haemoglobin synthesis. In other tissues, ferritin may supply iron for the synthesis of iron-containing proteins and may also act as a sink for potentially poisonous free iron (Drysdale, 1976). Haemosiderin is an insoluble iron-binding protein found predominantly in the spleen, bone marrow and the Kupffer (macrophage) cells of the liver. It is believed to be a partially denatured form of ferritin (Smith *et al.*, 1983) and may function as a long term iron storage compartment (Harrison *et al.*, 1974). The first two groups of iron-binding proteins comprise in excess of 97 per cent of the iron store in the human body.

The third and smallest iron-binding protein compartment contains the iron transport proteins. These proteins play a pivotal role in vertebrate iron metabolism as they are involved with the distribution of iron from the storage compartments to the tissues of the body.

Table 1.2 Iron compartments in normal humans (from Smith et al., 1983).

Iron Compartment	Iron content (mg)	Total body iron (%)
Group I		
Haemoglobin	2500	67
Myoglobin	130	3.5
Other tissue iron (eg. enzymes)	8	0.2
Group II		
Iron storage		
Ferritin and haemosiderin	1000	27
Group III		
Iron transport (eg. Transferrin)	3	0.08
Labile Pool	80	2.2

The transferrin family of iron binding proteins is characterised by their ability to reversibly bind iron with great avidity. Serum transferrin is the most abundant and well studied member of the transferrin family. Figure 1.1 illustrates the role of transferrin in the delivery of iron to iron-requiring cells and the interaction of the other iron-binding proteins discussed in this section. The transferrin family, and in particular lactoferrin, will be discussed in greater detail in the main body of this chapter.

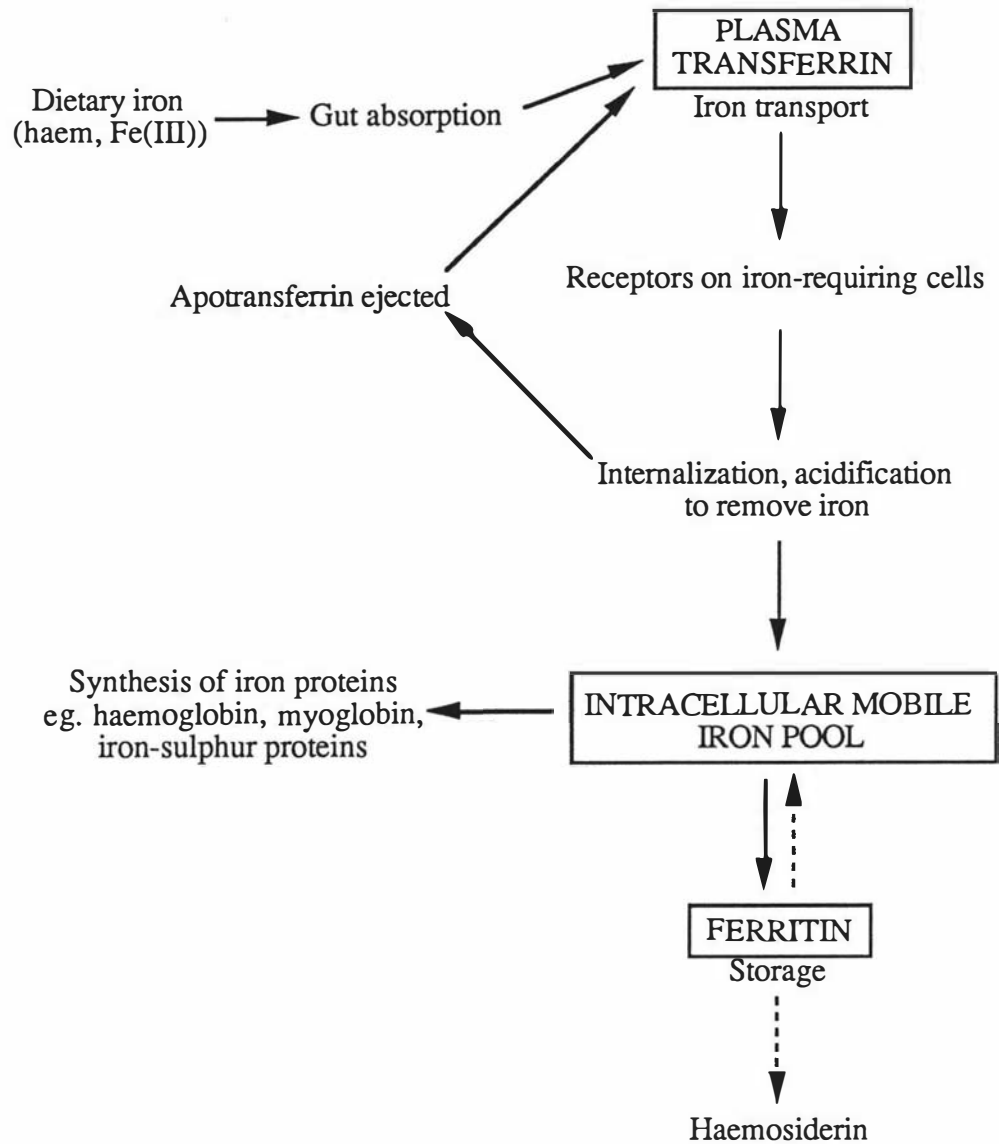


Figure 1.1 Mammalian iron metabolism. (Halliwell and Gutteridge, 1986).

The fourth iron compartment listed in table 1.2 is the labile iron pool. This pool is made up of an ill-defined compartment of intracellular and interstitial iron.

1.2 The transferrin family

The members of the transferrin family of iron-binding proteins are monomeric glycoproteins with the ability to bind metal ions tightly but reversibly. One of the most striking characteristics of the transferrins is the interdependence of metal and anion binding. Iron binding to the specific sites of transferrins requires the concomitant binding of anions (Aisen and Listowsky, 1980). Carbonate is the preferred anion but other anions, such as

oxalate and malonate, can replace carbonate to form stable metal-anion-protein complexes (Schlabach and Bates, 1975).

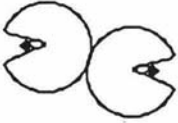
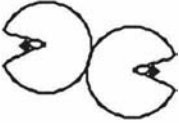
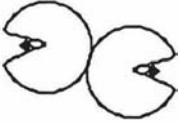
Several members of the transferrin family have been identified in vertebrates. Serum transferrin, lactoferrin, ovotransferrin and melanotransferrin are the best characterised members of the transferrin family. Some important characteristics of these four proteins are listed in table 1.3. In the early 1980's, amino acid sequence analysis identified two tumour-associated proteins with homology to the transferrins. These proteins are the transforming proteins from chicken B-cell lymphoma (Goubin *et al.*, 1983) and from human Burkitt lymphomas (Diamond *et al.*, 1983).

The following sections contain a brief description and some historical aspects of the four main members of the transferrin family. Many of the physical characteristics of these proteins are very similar. To avoid repetition, the common physical properties, such as metal binding, gross tertiary structure and molecular biology will be discussed in detail following a general description of each of the transferrins.

1.2.1 Serum transferrin

Iron is bound to transferrin for transport between storage compartments and iron-requiring tissues (Morgan, 1974). Transferrin (siderophilin, β 1-metal-combining globulin) was first isolated from serum in the 1940's (Laurell and Ingelman, 1947) and since then it has been the subject of intensive study. Over forty years of interdisciplinary research has yielded a wealth of information about the structure and function of this protein. Human serum transferrin consists of a single polypeptide chain with two covalently attached polysaccharide moieties. The molecular weight of the protein is 79570 (Thorstensen and Romslo, 1990), 6% of which is carbohydrate. The peptide component of human serum transferrin, consisting of 679 amino acid residues, has been completely sequenced (amino acid sequence, MacGillivray *et al.*, 1983; cDNA sequence, Yang *et al.*, 1984). The tertiary structure of rabbit serum transferrin has been determined (Bailey *et al.*, 1988) and will be discussed in greater detail later together with the structures of the other members of the transferrin family. The sequence of the two identical, N-linked, biantennary polysaccharide moieties was determined by Spik and co-workers (1975).

Table 1.3 Some properties of the members of the transferrin family of iron-binding proteins.

	Transferrin	Ovotransferrin	Lactoferrin	Melano-transferrin
Molecular weight	~80 kDa	~80 kDa	~80 kDa	~97 kDa
Distribution	Extracellular fluids (eg.serum)	Egg white	External secretions and neutrophils	Surface membranes of melanocytes
Proposed <i>in vivo</i> function	Delivery of iron to cells (especially reticulocytes)	Bacteriostasis (?)	Bacteriostasis (?)	Delivery of iron to melanocytes (?)
Predicted structure*	 Binds 2 Fe ³⁺ per molecule	 Binds 2 Fe ³⁺ per molecule	 Binds 2 Fe ³⁺ per molecule	 Binds 1 Fe ³⁺ per molecule

*Note: (?) indicates a putative function of these molecules. * see text for details on the predicted structure of these proteins. The characteristics and biological roles of the members of the transferrin family are also discussed in greater detail in the text.*

Transferrin has been isolated from the serum of all vertebrates studied to date (Morgan, 1974). It has also been found in the biological fluids of some invertebrates (Huebers *et al.*, 1984). The liver is the main, but not exclusive site of transferrin synthesis in humans (Morgan, 1974). Other sites of synthesis include the central nervous system, mammary gland, lymphocytes (T4⁺ subset), heart, lung, spleen, kidney, muscle, testes and the intestine (Bowman *et al.*, 1988). Serum transferrin, like serum albumin, is distributed throughout the extracellular fluids of the body (Brown, 1976).

As indicated in figure 1.1, transferrin plays a key role in the distribution of iron to cells. In addition to its role in iron transport, transferrin has antimicrobial activity and may be an integral part of the non-specific defence mechanisms of vertebrates (Morgan, 1974). The metabolic significance of

this protein is accentuated by the fact that mutations resulting in the absence of transferrin are very rare. The congenital absence of transferrin (atransferrinaemia) has been described in only a small number of children and results in severe anaemia, increased tissue iron stores and early death (Heilmeyer *et al.*, 1961; Goya *et al.*, 1972).

Iron-requiring cells initiate the uptake of iron from transferrin by receptor-mediated endocytosis (RME). Iron-saturated transferrin binds to specific receptors on the surface of the cell. The transferrin-transferrin receptor complexes are internalised and become localised in endosomes (Thorstensen and Romslo, 1990) where the iron is released from the transferrin by the rapid acidification of the endosome to a pH between 5 and 5.5. The released iron is then transported out of the vesicle and into the cytoplasm. Within the endosome, the acidic conditions ensure that apotransferrin remains bound to the transferrin receptor. The complex is then returned to the cell surface by exocytosis. At physiological pH, apotransferrin has a very low affinity for its receptor and is released. Apotransferrin and the membrane-bound receptor are available for another cycle of iron delivery.

The antimicrobial activity of transferrin has been attributed to its iron-binding properties. Micro-organisms require iron for growth and must compete for iron with the iron-binding proteins of the host. The virulence of many bacteria has been attributed, in part, to their ability to sequester iron from the environment (Bullen *et al.*, 1982).

Transferrin has been shown to be an essential growth factor for both normal and malignant proliferating cells (Thorstensen and Romslo, 1990). Proliferating cells need iron for cell growth and division, a requirement reflected by an increase in the number of cell-surface transferrin receptors (TfRs) during proliferation and periods of low iron status. Furthermore, Trowbridge and his co-workers (1984) have demonstrated that anti-TfR antibodies inhibit cell growth, providing that the antibody interferes with either transferrin binding to its receptor or with the endocytosis of the transferrin-TfR complex. Transferrin may also have a role in stimulating the growth of cells in a manner that is independent of the iron-transport function (Trowbridge and Lopez, 1982; James and Bradshaw, 1984).

1.2.2 Ovotransferrin

Ovotransferrin (conalbumin) is the major iron-binding protein found in hen egg white. Chicken ovotransferrin and chicken serum transferrin have identical polypeptide structure but they differ in their carbohydrate content (Williams, 1962). Ovo- and serum transferrin also differ in their sites of synthesis. Ovotransferrin is synthesized in the tubular gland cells of the oviduct magnum (Mandeles and Ducay, 1962; Williams, 1962; Palmiter, 1972a and 1972b) while serum transferrin is synthesized primarily in the liver. The complete amino acid sequence of chicken ovotransferrin was determined by Jeltsch and Chambon (1982).

The biological role of ovotransferrin remains obscure. A plausible function of this protein is bacteriostasis in the egg.

1.2.3 Melanotransferrin (p97 antigen)

p97 is a tumour-associated antigen found on the surface of most human melanoma cells. The distribution of this monomeric glycoprotein is virtually limited to transformed melanocytes and certain fetal tissues. However, p97 is also found in trace amounts in normal adult tissues (Brown *et al.*, 1981). The messenger RNA coding for p97 has been sequenced (Rose *et al.*, 1986). The deduced amino acid sequence shares extensive homology with other members of the transferrin superfamily. Like the members of the transferrin superfamily, p97 binds iron (Brown *et al.*, 1982). The gene coding for p97 is located on the same region of human chromosome 3 (3q21-3q29) as the genes coding for the other members of the transferrin superfamily (Plowman *et al.*, 1983; Yang *et al.*, 1984). On the basis of these similarities to the transferrin superfamily, Rose *et al.* (1986) proposed that p97 should be renamed melanotransferrin. They also went on to speculate that melanotransferrin has a role in iron transport. Melanoma cells, like other proliferating cells, have a high requirement for iron and melanotransferrin may represent an alternative method of iron delivery to that of the widely used transferrin–transferrin receptor system.

1.2.4 Lactoferrin

The presence of a red protein in bovine milk was first documented in the late 1930s (Sørensen and Sørensen, 1939). The isolation of this protein from bovine milk was first reported in 1960 (Groves, 1960). Groves called this protein the "red protein" due to its distinctive salmon pink colour when saturated with iron. In the same year, Johansson reported the isolation of the "red protein" from human milk (Johansson, 1960). The contemporary name for the "red protein" is lactoferrin and is derived from its initial identification in milk and from its similarity to serum transferrin.

As indicated above, lactoferrin (also called lactotransferrin and lactosiderophilin) was first identified in milk. In the years following its initial isolation from milk, it became apparent that lactoferrin was also present in a wide variety of other external secretions. Masson *et al.* (1966a; 1966b) used immunochemical techniques to demonstrate the presence of lactoferrin in saliva, nasal secretions, tears, bronchial mucus, hepatic bile, pancreatic juice, seminal fluid, cervical mucus and in urine. Lactoferrin is also found in neutrophils (Masson *et al.*, 1969). In these polymorphonuclear granulocytes (polymorphs), lactoferrin is localised in the secondary (specific) granules (Baggiolini *et al.*, 1970).

Lactoferrin shares many physical characteristics with serum transferrin (Aisen and Leibman, 1972). However, using column chromatography, amino acid analysis, electrophoretic and ultracentrifugal measurements, Gordon and co-workers (1963) demonstrated that lactoferrin (red protein) isolated from bovine milk was quite distinct from bovine serum transferrin. The physical characteristics of lactoferrin will be discussed in detail in subsequent sections.

Masson and Heremans (1971) demonstrated that lactoferrin could be isolated from the milk of a variety of mammals. These workers identified lactoferrin in the milk from guinea-pigs, cows, goats, mares, pigs, mice and humans. Milk from other mammalian species, such as dogs, rabbits and rats, was apparently devoid of lactoferrin.

Although lactoferrin can be identified in the milk from other mammals, only the human and bovine proteins will be discussed in detail in this review. The concentration of lactoferrin in the mammary secretion changes according to the functional status of the gland. Figure 1.2 depicts the functional cycle of the bovine mammary gland. The cycle begins as the immature gland becomes active in response to the cows first pregnancy. The first secretion produced by the gland after parturition is the colostrum. This is followed by normal milk production which continues until the calf is weaned. When milk is no longer required, the gland involutes and the 'dry' secretion is produced. The functional cycle is completed when the cow gives birth to another calf. Mammary gland infections, mastitis, can cause the disruption of the functional cycle. Acute mastitis causes tissue damage and reduced secretory capacity. This is illustrated in figure 1.2 by the arrows returning the cycle to the involution stage. In mild cases of mastitis, there is a cessation of milk secretion which is followed by a partial or complete recovery of mammary function (Schanbacher and Smith, 1975).

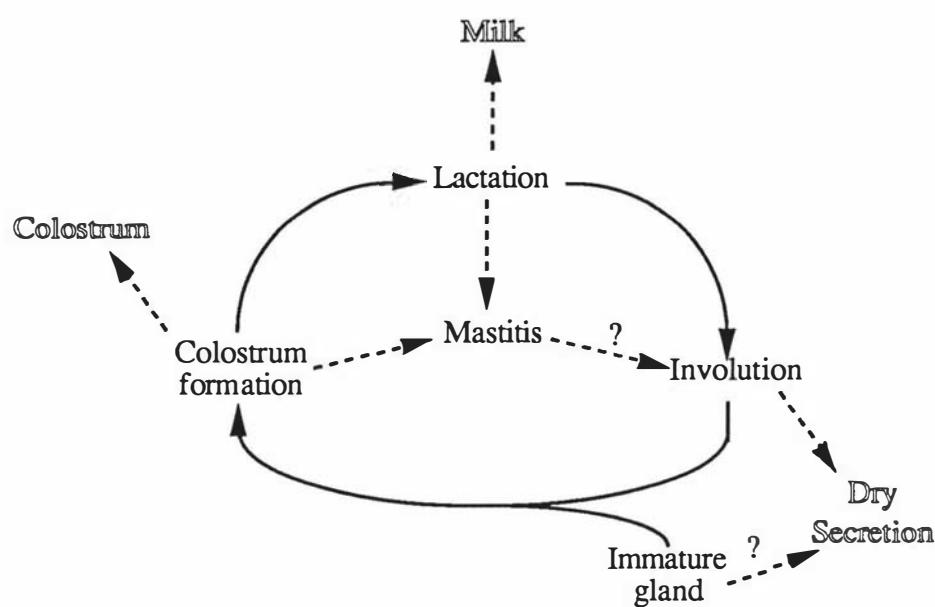


Figure 1.2 Diagrammatical representation of the functional cycle of the mammary gland and its secretions. (From Schanbacher and Smith, 1975).

The amount of lactoferrin in the gland varies during the cycle (Smith and Schanbacher, 1977; Sanchez *et al.*, 1988). Table 1.4 lists the concentration of lactoferrin in bovine mammary secretions.

Table 1.4 Concentration of lactoferrin in various secretions of the bovine mammary gland.

Type of mammary secretion	Lactoferrin concentration (mg/ml)
Colostrum	1 to 5
Normal milk	0.1 to 0.35
Early involution	1 to 8
Thirty days of involution	20 to 30
Clinical mastitis	1 to 8

From Smith and Schanbacher, 1977.

The lactoferrin content of mammary secretions also varies between species at similar stages in the functional cycle of the gland. In humans, for example, the concentration of lactoferrin in colostrum is approximately 6 mg/ml. The concentration decreases to around 1 to 2 mg/ml in normal milk (Lonnerdal *et al.*, 1976). That is, the concentration of lactoferrin in human milk is approximately 10-times greater than that in bovine milk. The high concentration of lactoferrin in human milk compared to bovine milk may have important implications with respect to the biological role of lactoferrin in these two species. The biological significance of lactoferrin in humans and cows will be discussed in detail later in this chapter.

The complete amino acid sequence of human lactoferrin has been determined (Metz-Boutigue *et al.*, 1984). Human lactoferrin contains two structurally heterogeneous N-linked glycans (Metz-Boutigue *et al.*, 1984). The sequence of the mRNA coding for human myeloid cell lactoferrin was partially determined by Rado and his colleagues (1987). The predicted amino acid sequence of the mRNA was almost identical to that reported for milk lactoferrin by Metz-Boutigue *et al.* (1984). A full length cDNA coding for human mammary lactoferrin has been isolated and sequenced by Powell and Ogden (1990).

At the outset of this study there was virtually no sequence information available for bovine lactoferrin. Recently, the complete amino acid sequence of bovine lactoferrin has been reported (This study; Mead and Tweedie, 1990).

1.3 Common physical properties of the transferrin family

The members of the transferrin superfamily share several physical characteristics. The best studied member of this family is serum transferrin and there is a wealth of information available on the physical characteristics of this protein. However, as lactoferrin, and in particular bovine lactoferrin, is the focus of this thesis, special reference will be made to this protein where possible.

1.3.1 Metal and anion binding properties of the transferrin family

The metal and anion binding characteristics of the transferrin family have been studied in detail for many years. The co-ordination of a metal cation requires the concomitant co-ordination of an anion (Schlabach and Bates, 1975). This mechanism of metal binding is apparently unique to this superfamily of proteins. Recent X-ray crystallographic studies have markedly improved our understanding of the mechanism of metal and anion binding by the transferrins. Consequently, this section will give only a brief description of the ion-binding features of the transferrin family. The details of metal- and anion-binding will be discussed in relation to the tertiary structure of the transferrins in the subsequent section.

Despite the advances in our understanding of metal and anion co-ordination by the transferrins, little is known about the mechanisms involved in the release of iron from these proteins. The release of iron from transferrins is triggered by low pH (below pH 6.0 for human transferrin and below pH 4.0 for human lactoferrin) (Mazurier and Spik, 1980). In addition, the binding of non-synergistic anions at secondary sites (Williams *et al.*, 1982) and the ionic strength of the media (Kretchmar and Raymond, 1988) also effect the release of iron from transferrins. Kretchmar and Raymond (1988) reported that as the ionic strength of the surrounding media decreases the release of iron from transferrin approaches zero implying that ions are required for the release of metal from the protein.

Metal-binding

The transferrins are capable of binding a wide variety of metal ions. Metal ions of varying size and oxidation states ranging from +2 to +5 can be co-ordinated by members of the transferrin family. These include Fe^{3+} , Cu^{2+} , Co^{3+} , Mn^{3+} , VO^{2+} , VO_2^+ , Al^{3+} , Th^{4+} and lanthanide cations (Ainscough *et al.*, 1979; Chasteen *et al.*, 1977; Harris and Carrano, 1984; Cochran *et al.*, 1984; Luk, 1971; Zak and Aisen, 1988; O'Hara and Bersohn, 1982; Harris *et al.*, 1981).

Synergistic anion-binding

In the absence of carbonate, transferrins are capable of co-ordinating a wide variety of anions (Schlabach and Bates, 1975). These authors compiled an extensive list of synergistic anions that could replace carbonate in forming stable Fe^{3+} -transferrin-anion complexes. These anions include oxalate, malonate, succinate, pyruvate, glycine, nitrilotriacetate and EDTA. Schlabach and Bates (1975) also identified two critical features of the synergistic anion; a carboxyl group and proximal ligand. Although these authors oriented the anion incorrectly in their model of iron binding, the two features that they suggested were crucial for iron binding have been verified by the determination of the molecular structure of the protein.

1.3.2 The tertiary structure of the members of the transferrin family

The tertiary structures of several members of the transferrin family have been solved by X-ray crystallography. Anderson *et al.* (1987) were the first to report the tertiary structure of a member of the transferrin family. They elucidated the structure of diferric human lactoferrin at a resolution of 3.2 Å. Following the initial characterisation of the molecular structure of human lactoferrin, Bailey and co-workers (1988) solved the structure of rabbit serum transferrin at a resolution of 3.3 Å. The overall secondary and tertiary structures of human lactoferrin and rabbit serum transferrin were found to be very similar and supported the theory of a strong phylogenetic link between the members of the transferrin family.

The structure of diferric human lactoferrin has been refined to 2.8 Å (Anderson *et al.*, 1989). The elucidation of the tertiary structure of lactoferrin has clarified many aspects of the unique chemistry of this family of proteins. Figure 1.3 illustrates an α -carbon ribbon diagram of the tertiary structure of diferric human lactoferrin at a resolution of 2.8 Å. This figure serves to illustrate several points about the molecular organisation of the transferrins.

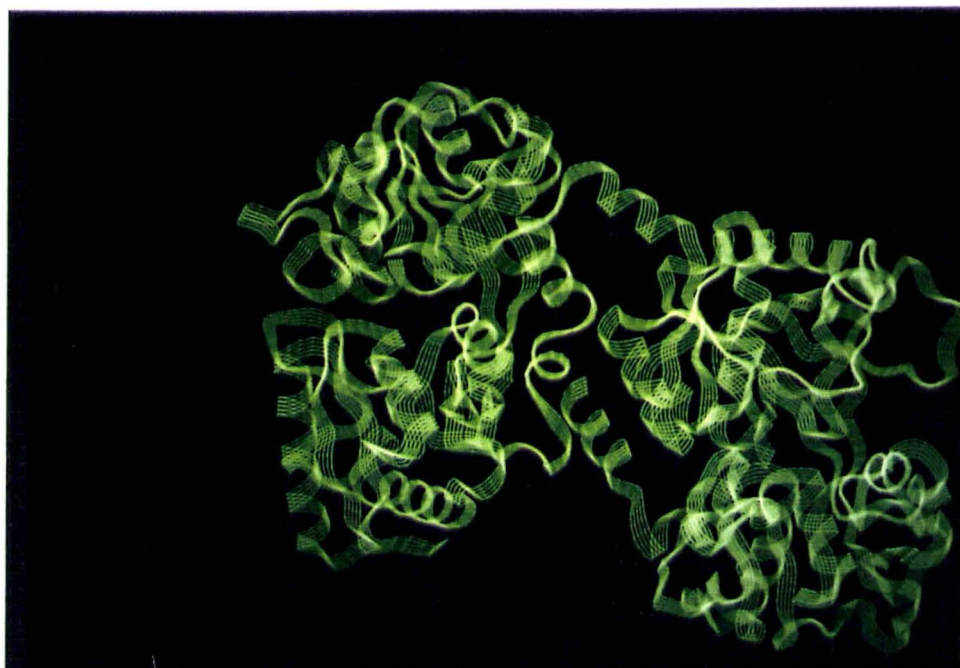


Figure 1.3 α -carbon ribbon diagram of the tertiary structure of human lactoferrin. The N-terminal lobe is on the left-hand side of the picture. The structural domains, clockwise from the lower left-hand corner, are termed N1, N2, C1 and C2 respectively. This illustration is courtesy of Dr B. F. Anderson (Massey University).

Human lactoferrin is folded into two globular lobes. Each lobe contains one iron binding site and the two lobes are connected by a short α -helix. The two-fold internal homology of the protein structure is quite striking. When the N- and C-terminal lobes of human diferric lactoferrin are superimposed approximately 85% of the structure aligns with a high degree of fidelity (Anderson *et al.*, 1989). Each globular lobe is divided into two domains, with an iron-binding site residing in the cleft between the domains. The four structural domains of human lactoferrin are labelled as follows; N1 (residues 1 to 90 and 252 to 320), N2 (91 to 251), C1 (345 to 433 and 596 to 663) and C2 (434 to 595). The structural homology between the domains is significantly higher than that of the complete lobes. That is, the structure of N1 and C1 domains superimpose with much higher fidelity than the complete

lobes. Likewise the N2 and C2 domains have significantly higher structural homology than the N- and C-terminal lobes (Anderson *et al.*, 1989). Thus it has been proposed that the transferrins have evolved from an ancient gene duplication event (Williams, 1982). Amino acid sequence analysis and the elucidation of the genomic organisation of the transferrin family have substantiated this assertion. Recent advances in determining the molecular structure of the transferrins have graphically highlighted the striking internal homology of these proteins.

Analysis of the tertiary structure of diferric lactoferrin has revealed a remarkable degree of structural homology with another group of binding proteins. The bacterial periplasmic binding proteins, which are involved in the active transport of certain sugars, amino acids and ions, bear strong structural and functional similarities to the transferrins. The domain size and structure, the substrate binding site and the ligand-induced structural changes are very similar in the two groups of proteins (Baker *et al.*, 1987). The molecular organisation of the sulphate-binding protein from *Salmonella typhimurium* (Pflugrath and Quioco, 1985) is remarkably similar to that of diferric lactoferrin. Interestingly, these proteins share little sequence homology (<10%; Baker *et al.*, 1987) but the topology of the binding domains are very similar. Baker and his colleagues (1987) proposed that these structural similarities may reflect divergent evolution from a common ancestral binding protein or, alternatively, convergent evolution to give a folding pattern that is dictated by physical and functional requirements.

Solving the molecular structure of diferric lactoferrin and serum transferrin has revealed several interesting aspects of metal and synergistic anion binding. As indicated above, the iron-binding sites are located in the clefts between the domains that make up each lobe. Structural studies have indicated that the co-ordination of the iron is essentially the same in both lobes.

The iron-binding sites are buried deeply within the folded protein. There are five ligands that bind the iron atom. Four of these ligands are provided by amino acid side chains and the fifth is supplied by a non-protein ligand. The four protein ligands are provided by one carboxylate oxygen (Asp60 and Asp 395; N- and C-terminal lobes respectively), two phenolate oxygens (Tyr92, Tyr192 and Tyr435, Tyr528) and one imidazole nitrogen (His253 and His597). The non-protein ligand is usually a (bi)carbonate ion. This

synergistic anion co-ordinates iron in a bidentate fashion and, in turn, is held in position by hydrogen bonding to side-chain donor-groups and to the N-terminal of an α -helix. This observation supports the earlier prediction by Schlabach and Bates (1975) that synergistic anions require two features: a carboxylate group and another electron donor ligand on a proximal carbon atom. Figure 1.4 depicts a schematic representation of the iron and anion-binding sites in the N-terminal lobe of human lactoferrin.

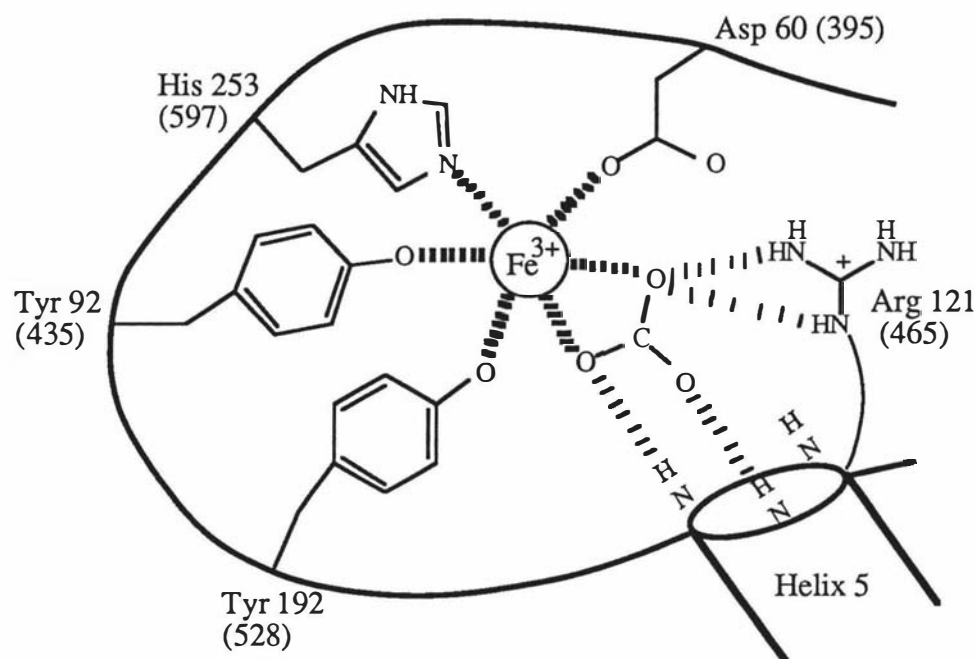
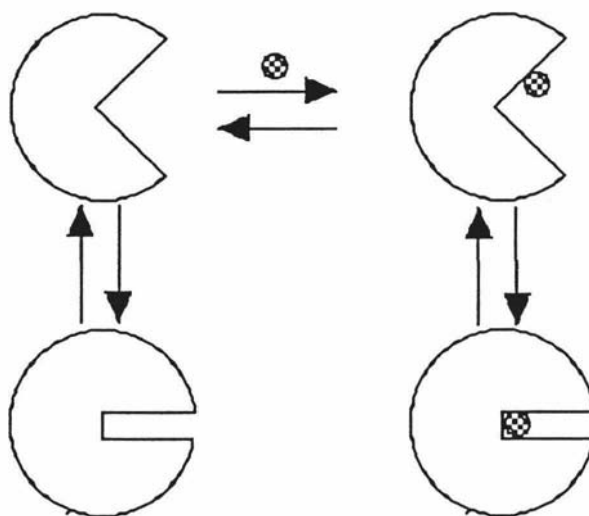


Figure 1.4 Schematic diagram of the metal and anion binding site in lactoferrin. The residue numbers are for the N-terminal lobe. The corresponding residues in the C-terminal lobe are given in parentheses. (Based on Baker et al., 1991).

The molecular structure of deglycosylated human apolactoferrin has been determined by Anderson *et al.* (1990). The crystallographic study of the iron-free form of lactoferrin revealed some surprising molecular movements apparently related to metal-binding. The N-terminal lobe of apolactoferrin assumes a wide 'open' structure with the N2 domain rotating 53° relative to the N1 domain to leave the iron-binding pocket wide open. The C-terminal lobe, however, remains in the 'closed' state with a structure very similar to that of the C-terminal lobe in diferric lactoferrin. Anderson and his colleagues (1990) proposed that the 'one open, one closed' structure represents the equilibrium between the two structures in aqueous solution.

The resulting crystal structure is probably due to the physical constraints imposed by the crystal packing of the apolactoferrin molecules and the chemical composition of the mother liquor. The large molecular movements observed in apolactoferrin relative to diferric lactoferrin resemble the 'hinge-bending' conformational changes seen in the bacterial periplasmic binding proteins. Quiocho and co-workers (1990; Sack *et al.*, 1989) have solved the tertiary structures of both liganded and unliganded periplasmic binding proteins. They determined that the periplasmic binding proteins are in equilibrium between open and closed forms. Figure 1.5 illustrates a possible scheme for conformational changes observed in liganded and unliganded lactoferrin. Each of the 'phases' in the scheme have been observed in crystal structures of the periplasmic binding proteins.



*Figure 1.5 Schematic diagram of the structural changes associated with metal binding to a single lobe of lactoferrin. The double arrows represent the equilibrium between the different molecular forms of the protein. (From Baker *et al.*, 1991).*

Interestingly, as indicated earlier, Kretchmar and Raymond (1988) determined that at very low ionic strength iron is no longer released from transferrin. Baker and his colleagues (1991) speculated that this may imply that opening of the liganded, closed form is inhibited at low ionic strength. In other words, the equilibrium is shifted towards the liganded, closed form at very low ionic strength effectively preventing the release of the co-ordinated ligand.

Anderson and his colleagues (1990) proposed the following scheme for ligands binding to apolactoferrin. The anion (carbonate) is attracted into the cleft by the presence of three basic side chains, which are buried in the closed structure. The anion then binds to the N2 domain and is held in place by forming hydrogen bonds with the main chain amino groups at the N-terminus of an α -helix and with side chain amino groups of Arg121 (see figure 1.4). The notion that the anion binds specifically to the apo-protein prior to metal binding is supported by anion competition experiments (Rogers *et al.*, 1977). With the anion in place, four of the six metal-binding ligands (Tyr92, Tyr192 from the N2 domain, and two CO_3^{2-} oxygens) are sufficiently close to allow the metal to bind. The metal (Fe^{3+}) binds to the N2 domain ligands and to the anion. Co-ordination of the metal is completed by rotation of the N2 domain and closure of the cleft which allows bonding between the two ligands on the N1 domain (Asp60 and His253) and the 'loosely' co-ordinated metal.

X-ray crystallographic studies of copper(II)- and oxalate-substituted human lactoferrin (Smith *et al.*, 1991) have illustrated that the ligand binding sites of lactoferrin can accommodate different sized ionic species. These studies highlighted the flexibility of the metal and anion binding sites. The large, solvent-filled cavity in the interdomain cleft can accommodate larger anions than carbonate. Consequently, few changes to the protein structure are required to accommodate these bulkier anions. This perhaps explains the ability of lactoferrin to bind a variety of metals and synergistic anions.

As expected by the high degree of amino acid sequence identity between human lactoferrin and rabbit serum transferrin, the overall folding pattern of these two proteins is very similar. The extensive sequence homology within the transferrin family (see Appendix III) implies that the tertiary structures of other transferrins will probably be very similar to that of human diferric lactoferrin. The notable exception to this prediction is melanotransferrin. Analysis of the amino acid sequence of melanotransferrin suggests that this protein contains only one metal-binding domain (Baker *et al.*, 1987) as only the N-terminal lobe of melanotransferrin contains all the protein ligands required for metal and anion binding. The C-terminal half of the protein lacks several amino acid side chains which have been shown to be involved in iron-binding in other transferrins. Furthermore, the disulphide bridge closest to the C-terminal is missing in melanotransferrin. This may allow the hydrophobic C-terminal sequence (714-738) to act as a transmembrane

anchor attaching melanotransferrin to the surface of the cell (Baker *et al.*, 1987). A diagrammatic prediction of the structure of melanotransferrin can be found in table 1.3.

The tertiary structure of diferric bovine lactoferrin has been determined by crystallographic studies to a resolution of 4.5 Å (Dr. M. Haridas and Prof. E.N. Baker, personal communication). At this resolution no detail can be drawn from the model but the overall folding pattern appears to be similar to that of diferric human lactoferrin.

1.3.3 The molecular biology of the transferrin family

Recent advances in the field of molecular biology have been applied to the study of the transferrin family and this analysis has revealed some interesting characteristics of this family of iron-binding proteins.

1.3.3.1 The genomic organisation of the transferrin family

The first member of the transferrin family to be characterised at the nucleotide level was chicken ovotransferrin. Jeltsch *et al.* (1987) reported the nucleotide sequence of the chicken ovotransferrin gene. The ovotransferrin gene is organised into 17 exons and 16 introns and spans 11.5 kb of genomic DNA. Schaeffer and colleagues documented the complete structure of the human transferrin gene (Schaeffer *et al.*, 1987). Like the chicken ovotransferrin gene, the human serum transferrin gene is organised into 17 exons and 16 introns. Although the two genes code for mRNA molecules of approximately the same length, the serum transferrin gene (33.5 kb) is approximately three-times the size of the ovotransferrin gene (Bowman *et al.*, 1988).

Nucleotide sequence analyses of both the avian and human transferrin genes have supported the hypothesis (Williams, 1982) that these present day genes evolved from an ancient gene duplication event (Park *et al.*, 1985). According to this hypothesis, the modern day bilobal transferrins are thought to have evolved from a primordial single lobe protein. Furthermore, the presence of internal sequence homology within each lobe is suggestive of an even more ancient duplication event. Gene duplication of a very ancient binding domain gave rise to the first lobe, subsequent duplication of the gene coding for this lobe produced the modern day transferrins (MacGillivray and Brew, 1975). Analysis of the mRNA sequence coding for chicken

ovotransferrin indicates that there is considerable sequence identity (~44%) between the four binding domains (Jeltsch and Chambon, 1982). Figure 1.6 depicts a simple model for the origin of the present day transferrin gene.

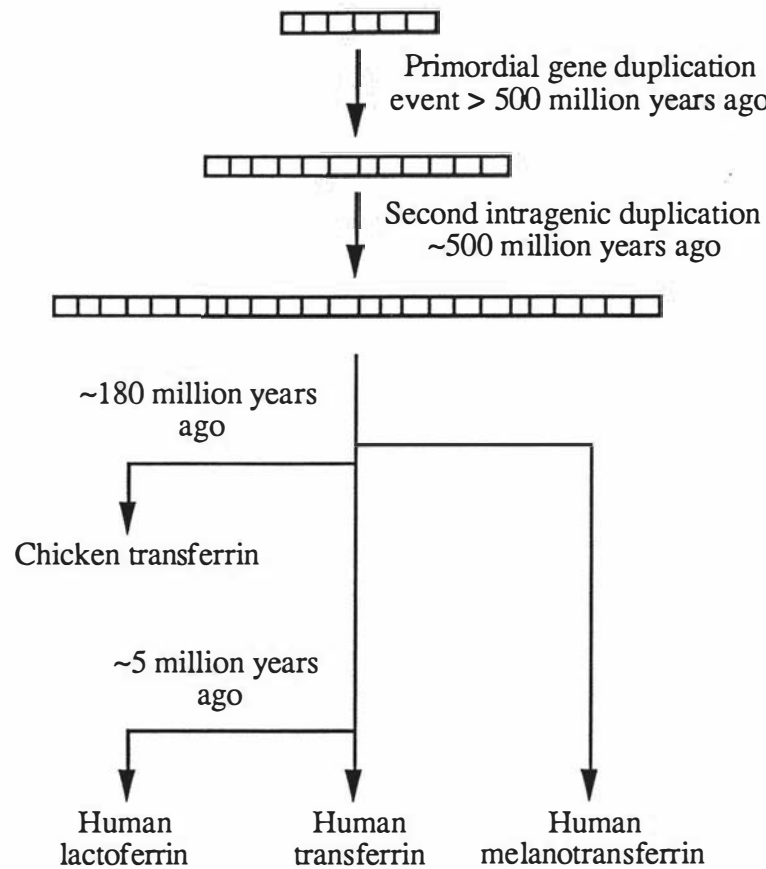


Figure 1.6 Schematic diagram of the evolution of the transferrin gene family. Based on Bowman et al., 1988.

To date, the gene coding for human lactoferrin has not been fully characterised.

1.3.3.2 The chromosomal organisation of the transferrin gene family

Chromosomal mapping studies in humans and mice have determined the chromosomal localisation of the genes coding for the members of the transferrin superfamily. The human serum transferrin gene was mapped to the long arm of chromosome 3 (3q) (Yang *et al.*, 1984). The presence of a human transferrin pseudogene on chromosome 3q has been reported by Schaeffer *et al.* (1987). The gene coding for the transferrin receptor has been located on the same chromosome ((3q22-3qter) Miller *et al.*, 1983; (3q26.2)

Rabin *et al.*, 1985) and is linked to the transferrin gene. Since the assignment of the transferrin gene to chromosome 3, several other members of the transferrin family have also been mapped to the same chromosome. Figure 1.7 illustrates the chromosomal assignments of the genes encoding the members of the transferrin superfamily. The chromosomal locations of some other functionally related proteins that are genetically linked to the transferrin family genes are also indicated in this figure.

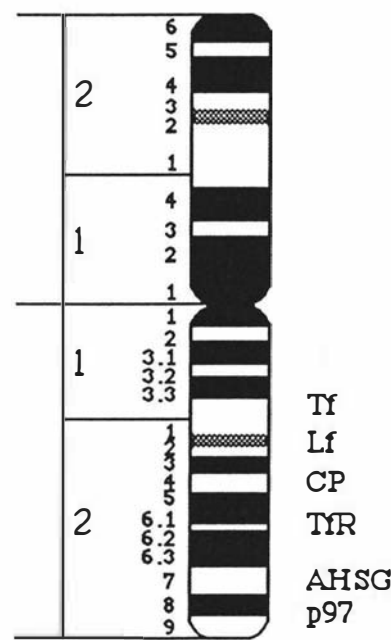


Figure 1.7 Regional assignment of transferrin family genes on human chromosome 3. The genes coding for the metal-binding proteins CP and AHSG are linked to the Tf gene on the long arm of this chromosome (3q). Tf, serum transferrin; Lf, lactoferrin; CP, ceruloplasmin; TfR, transferrin receptor; AHSG, α_2 -HS-glycoprotein and p97, oncofetal 97,000 Da protein (melanotransferrin). Reproduced from Bowman *et al.*, 1988.

The gene coding for the p97 antigen (melanotransferrin) was initially assigned to chromosome 3 by Plowman *et al.* (1983). Le Beau and co-workers (1985) refined the location of the melanotransferrin gene to within 3q28-29. Teng *et al.* (1987), using the cDNA encoding mouse lactoferrin, established the chromosomal location of human lactoferrin on chromosome 3q. *In situ* hybridisation studies refined the chromosomal assignment to 3q21-23 (McCombs *et al.*, 1987).

The transferrin gene is also linked to genes encoding other metal-binding proteins. Ceruloplasmin (CP) is a copper binding protein found in serum. Yang *et al.* (1986) mapped the ceruloplasmin gene to 3q25. Interestingly, although ceruloplasmin does not share sequence homology with transferrin, their biological functions appear to be related. The role of ceruloplasmin is thought to be the oxidation of ferrous iron to the ferric form. Transferrin can only bind iron specifically in the ferric form.

The gene encoding the calcium and barium-binding protein α_2 -HS-glycoprotein (AHSG) has also been mapped to chromosome 3q (Lee *et al.*, 1987). Magnuson and co-workers (1988) used *in situ* hybridisation to determine the precise location of the AHSG gene at 3q27-29.

The mouse homologue of human chromosome 3 is chromosome 9 (Naylor *et al.* 1982). The mouse lactoferrin gene locus has been mapped to mouse chromosome 9 (Teng *et al.*, 1987) and is linked to the transferrin gene (Bowman *et al.*, 1988).

1.3.3.3 Expression of the transferrin gene family

The characterisation of the transferrin family at the gene level has allowed some insight into the regulation of expression of these proteins. The tissue distribution of the members of the transferrin family has been discussed elsewhere in this chapter.

Regulation by iron

It is known that iron deficiency induces the expression of transferrin (Bowman *et al.*, 1988) and that increased mRNA synthesis from the transferrin gene appears to be tissue specific. McKnight and co-workers (1980a; 1980b) demonstrated that chickens fed on a low-iron diet showed increased transferrin mRNA synthesis in the liver but not in the oviduct. Similar tissue specific 'up-regulation' was noted in rats fed iron-deficient diets. Liver transferrin mRNA synthesis increased in response to low dietary iron, but synthesis of transferrin at other sites in the rat was unchanged (Idzerda *et al.*, 1986). When iron supplementation corrected the deficiency, the amount of transferrin mRNA returned to normal hepatic levels (McKnight *et al.*, 1980b).

Regulation by hormones

Transferrin synthesis is modulated by hormones. Table 1.5 lists hormones that induce the synthesis of transferrins.

Table 1.5 Hormonal modulation of transferrins.

Hormone	Species	Reference
Progesterone and estrogen	Rat	Horne and Ferguson, 1972. Lee <i>et al.</i> , 1978; McKnight and Palmiter, 1979; McKnight <i>et al.</i> , 1980b. Pentecost and Teng, 1987.
	Chicken	
	Mouse*	
Glucocorticoid	Rat	Jeejeebhoy <i>et al.</i> , 1972a.
Thyroxine	Rat	Jeejeebhoy <i>et al.</i> , 1972b.
Glucagon	Rat	Tavill <i>et al.</i> , 1972.

* Pentecost and Teng (1987) reported that lactoferrin is the major estrogen-inducible protein of mouse uterine tissue.

Molecular biology techniques have now been applied to the study of transferrin proteins. Stowell and colleagues (1991) at Massey University have reported the expression of cloned human lactoferrin in tissue culture. Site directed mutagenesis of the cloned gene, combined with crystal-structure analysis of the mutant proteins should give valuable insight to the unique characteristics of this protein family.

1.4 The biological role of lactoferrin

The biological significance of lactoferrin remains elusive but it is undoubtedly related to the ability of lactoferrin to bind iron tightly yet reversibly. Various functions have been ascribed to lactoferrin as a result of both *in vitro* and *in vivo* studies (Nemet and Simonovits, 1985). Many of these studies have been on the biological effects of human lactoferrin. This section will review the functions that have been described in the literature for lactoferrin from a variety of species. Care will be taken, however, to indicate the source of lactoferrin as it unclear whether it is possible to extrapolate the findings to lactoferrins from other species. Table 1.6 lists the principal biological functions attributed to lactoferrin.

Table 1.6 Biological functions of lactoferrin.

Biological activity of lactoferrin	Reference
Inhibits bacterial growth	Bullen <i>et al.</i> , 1972; Lonnerdal, 1985
Bactericidal activity	Arnold <i>et al.</i> , 1982; Bortner <i>et al.</i> , 1989
Enhances neonatal iron absorption	Birgens, 1984; Lonnerdal, 1985
Contributes to anaemia of chronic disease	Van Snick <i>et al.</i> , 1974; Birgens, 1984
Inhibits myelopoiesis	Broxmeyer <i>et al.</i> , 1987
Degrades mRNA	Furmanski <i>et al.</i> , 1989; Das <i>et al.</i> , 1976; Furmanski <i>et al.</i> , 1990

The functions of lactoferrin will be discussed in the context of their biological localisation. For the purposes of this discussion the distribution of lactoferrin will be categorised into three groups; mammary secretions, other external secretions, and polymorphonuclear leucocytes.

1.4.1 Lactoferrin in mammary secretions

The biological role for lactoferrin is probably best understood in the context of its presence in milk. Even so, the physiological function of lactoferrin in mammary secretions is not completely understood and is obviously complex. The biological significance of lactoferrin will be discussed in relation to its action in the mammary gland and in the neonate.

Lactoferrin and the neonate

(i) bacteriostasis

It has been recognised for several decades that breast-fed babies suffer less from enteritis than those fed with artificial milk formulas. In a review of infantile enteritis, Alexander (1948) reported that "Few breast-fed infants were admitted with enteritis. ... The majority of infants with enteritis, and almost all the severe cases, had been artificially fed before admission." Infantile enteritis is a serious disease which can result in vomiting and

diarrhoea. As infants are less able to conserve water than adults, they can become quickly, and sometimes irreversibly, dehydrated by continued fluid loss (Alexander, 1948).

One of the most significant micro-organisms causing infantile gastroenteritis is *Escherichia coli*. The disease is associated with particular serotypes of *E. coli*, but these serotypes can also be isolated from healthy infants (Bullen *et al.*, 1972). This indicates that the symptoms are due to a proliferation of the bacteria rather than an infection *per se*. Enteritis is also common among newly born calves and piglets, and particularly so in those deprived of colostrum. A feature of this condition is the rapid rise in the numbers of *E. coli* in the small intestine (Bullen *et al.*, 1972).

Milk contains several antimicrobial systems (Reiter and Oram, 1967; Reiter, 1978). These include specific factors, such as immunoglobulins, phagocytes and complement, and non-specific factors such as lysozyme, lactoperoxidase and lactoferrin. The bacteriostatic effects of lactoferrin have been known for many years (Reiter and Oram, 1967; Oram and Reiter, 1968). In particular, lactoferrin has been demonstrated to inhibit the growth of various strains of *E. coli* (Bullen *et al.*, 1972; Reiter *et al.*, 1975; Griffiths and Humphreys, 1977; Raptopoulou-gigi *et al.*, 1977; Spik *et al.*, 1978). Bullen and co-workers (1972) demonstrated that lactoferrin, in combination with specific *E. coli* antibody, was a more powerful inhibitor of bacterial growth than lactoferrin alone. The inhibition of growth was reversed by saturating the lactoferrin with iron. This indicates that lactoferrin may interfere with bacterial iron supply. *E. coli* sequesters iron from the environment by secreting the iron-chelating agent enterochelin. Antibody is thought to increase the efficacy of bacteriostasis by lactoferrin by interfering with the production of enterochelin (Griffiths and Humphreys, 1977). As indicated earlier, iron-binding to lactoferrin requires the concomitant binding of an anion, usually (bi)carbonate. Griffiths and Humphreys (1977) reported that bicarbonate was required for the bacteriostatic activity of lactoferrin.

Lactoferrin may play an important part in resistance to infantile enteritis caused by *E. coli*. In order to be effective in the gut, lactoferrin must survive passage from the mouth to the small intestine. The pH of the stomach contents of breast-fed babies remains relatively high (pH 6.0-6.5) about one hour after feeding (Mason, 1962). Even after ninety minutes Mason (1962) was unable to detect hydrolysed protein in aspirated gastric contents.

Therefore, it is conceivable that lactoferrin survives passage into the small intestine. Recent studies support this assumption. Lactoferrin has been identified in the faeces of breast-fed babies (Spik *et al.*, 1982; Davidson and Lonnerdal, 1987; Prentice *et al.*, 1987; Prentice *et al.*, 1989; Goldman *et al.*, 1990). This finding indicates that lactoferrin not only survives passage into the small intestine but also that a measurable amount reaches the faeces. Furthermore, Spik and co-workers (1982) demonstrated that lactoferrin isolated from the stools of breast-fed infants maintained its iron-binding capacity.

Lactoferrin may also play an important role in the preferential colonisation of the neonate gut with lactobacilli. Bullen and co-workers (1972) suggested that the antimicrobial systems in milk provide an environment that encourages the growth of *Lactobacillus bifidus*. Lactic acid bacteria have an extremely low requirement for iron (Reiter *et al.*, 1975). Consequently, the lactoferrin bacteriostatic system would not be as inhibitory to lactobacilli as to *E. coli*. The eventual colonisation of the gut contents with lactobacilli should, in turn, suppress the growth of coliform organisms (Bullen *et al.*, 1972).

(ii) Iron absorption and nutrition

In addition to a role in bacteriostasis, lactoferrin may be involved in iron absorption in the small intestine (Lonnerdal, 1985). Factors in human milk enhance the absorption of iron from the infants diet. The bioavailability of iron in human milk is much greater than in either cow's milk or infant milk formulas (Saarinen *et al.*, 1977; McMillan *et al.*, 1976). As lactoferrin is a major component of human milk, and is found in small quantities in the alternative infant diets, various researchers have speculated that lactoferrin aids in the absorption of iron. Fransson and co-workers have demonstrated that lactoferrin enhances the uptake of iron in suckling pigs (1983a) and weanling mice (1983b). Cox and colleagues (1979) working with adult human mucosal biopsies demonstrated that lactoferrin was capable of delivering iron to the cells lining the small intestine. On the basis of this finding, Cox *et al.* (1979) postulated the presence of lactoferrin-specific receptors on the surface of the mucosal cells. In 1988, Davidson and Lonnerdal reported the specific and saturable interaction of lactoferrin to rhesus monkey brush border membranes. The lactoferrin-receptors on these cells bind diferric monkey and human lactoferrin tightly ($K_d=9 \times 10^{-6}$), but did not bind either bovine lactoferrin or human serum transferrin. Specific

binding of lactoferrin to the small intestine mucosa was observed regardless of the age of the donor monkey. This finding may imply that lactoferrin is involved in iron uptake throughout the lifespan, and not only during infancy. However, deVet and van Gool (1974) found that apolactoferrin inhibits iron absorption in adult humans. De Laey *et al.* (1968) demonstrated similar inhibition of iron absorption in the presence of lactoferrin using everted duodenal sacs from rats and guinea pigs. These groups suggest that the function of lactoferrin is to bind excess iron in the gut and thus make it unavailable for absorption. Kawakami and co-workers (1988) suggested that the inhibition of iron absorption was possibly an experimental artefact produced by the level of the iron dose, the source of the lactoferrin used in the experiments and the species of experimental animal.

The mechanisms for iron absorption in man are poorly defined. Likewise, the significance of lactoferrin receptors in the small intestine remains obscure. The role of the lactoferrin receptors in the adult gut is even more puzzling. Lactoferrin is present in hepatic bile and pancreatic secretions (Masson *et al.*, 1966a). Hegnhøj and Schaffalitzky de Muckadell (1985) demonstrated that the concentration of lactoferrin in duodenal aspirates increased markedly following hormonal stimulation of the pancreas. In light of these findings it is tempting to speculate that lactoferrin, secreted into the duodenum in response to feeding, binds iron and then interacts with its receptor. Binding of lactoferrin to its specific receptor may then facilitate the mucosal uptake of iron by either receptor-mediated endocytosis or release of iron at the cell surface. As more data becomes available the enigma of a role for lactoferrin in iron absorption may be solved.

(iii) Growth factor

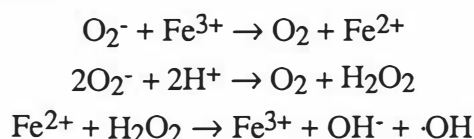
Animal studies have indicated that the gastrointestinal tract matures more rapidly if the newborn is allowed to suckle (Widdowson, 1985). The milk from a variety of mammals, including humans, cows, goats and sheep, have been shown to increase the proliferation of cells in tissue culture (Brown and Blakeley, 1983; Klagsbrun, 1978; Klagsbrun *et al.*, 1979). Nichols and co-workers (1987) demonstrated that human lactoferrin increased the incorporation of radiolabelled thymidine into DNA of rat crypt cells. The proliferative effect of lactoferrin appeared to be specific for crypt enterocytes as it had no effect on mouse 3T3 (fibroblast) cells. Nichols *et al.* (1987)

speculated that mucosal development may be another 'nutritional' function for lactoferrin.

Infant formulas are commonly used in refeeding young infants with diarrhoea. Nichols and co-workers (1989) demonstrated that refeeding formulas decreased the proliferation of rat crypt cells in an *in vitro* system. Addition of lactoferrin to infant formulas increased thymidine incorporation into DNA of rat crypt enterocytes. Consequently, lactoferrin supplementation of infant formulas may be particularly beneficial when refeeding neonates with mucosal atrophy.

(iv) Serum antioxidant

Iron is thought to have a role in the generation of potentially harmful oxygen radicals. In the Haber-Weiss reaction, iron catalyses the production of hydroxyl radical ($\cdot\text{OH}$):



The mechanisms for oxygen-derived radical damage will be discussed in detail later in this chapter. There are several antioxidant systems that protect tissues from oxygen radical injury. Over 90% of the preventive antioxidant activity in plasma is associated with apotransferrin and ceruloplasmin (Sullivan, 1988). Ceruloplasmin catalyses the oxidation of ferrous ions to the ferric form. Iron in the ferric form can be co-ordinated by transferrin. When associated with transferrin, iron is no longer able to promote the production of oxygen-derived free radicals (Sullivan, 1988).

In the adult human there is adequate apotransferrin in the serum to ensure that the plasma concentration of free iron is very low. In fact, most of the serum transferrin is in the iron-free form (apotransferrin). In the neonate, the iron status is quite different to that of the adult. The plasma concentration of transferrin and ceruloplasmin is lower in the 'term' neonate than in the adult. Concentrations of these 'antioxidant' proteins are even lower in the plasma of the preterm infant (Sullivan and Newton, 1988). The post-natal changes in iron metabolism result in near complete saturation of serum transferrin. Although the preterm infant has less total body iron than the term infant, lower gestational age also correlates with low serum transferrin concentrations (Scott *et al.*, 1975). The virtual cessation of erythropoiesis

after birth also adds to the iron burden in the newborn. Red cell senescence and lysis continue despite the temporary (6 to 8 weeks) pause in red cell synthesis. In the preterm this is exacerbated by the apparent unregulated absorption of iron from the diet (Shaw, 1982). Consequently, preterm infants may have virtually all their serum transferrin saturated with iron. Transferrin saturations of 100% have been noted in some preterm infants (Sullivan, 1988). Sullivan (1988) hypothesized that the predisposition of preterm neonates to oxygen radical-derived pathology was due to a developmentally low serum antioxidant activity combined with changes in iron metabolism following birth. Preterm infants are susceptible to a variety of oxygen radical-mediated disorders. Sullivan (1986; 1988) used retinopathy of prematurity as an example of these disease states. The retinopathy of prematurity (retrolental fibroplasia) results from oxidative damage to the developing retina. Retrolental fibroplasia was the leading cause of blindness in the United States between 1940 and the mid 1950s (Albert and Dryja, 1989). This disease is often a result of medical intervention when neonates suffering from respiratory distress syndrome are given increased oxygen supply to correct hypoxia. The increased oxygen tension leads to the over production of oxygen-derived radicals. Sullivan (1986) proposed that neonates are only susceptible to oxygen radical damage in the presence of increased free iron. Breast-fed premature infants have a lower incidence of retrolental fibroplasia than their artificially fed contemporaries (Cunningham, 1987). Sullivan (1988) proposed that the lactoferrin in human colostrum and milk can enter the blood stream of the neonate from the gastrointestinal tract. Lactoferrin, which is secreted from the mammary gland predominantly in the apo-form (3-5% iron-saturated; Fransson and Lonnerdal, 1980), is able to bind iron and thus decrease the catalysis of oxygen-derived radicals. In a rat model system, the injection of human apolactoferrin protected the animals from radical-mediated acute lung injury in a dose-dependent manner (Ward *et al.*, 1983).

In order to protect the neonate from oxidative stress, lactoferrin must enter the blood stream intact and in a biologically active form. Recent studies have indicated that lactoferrin can be isolated from the urine of breast-fed infants (Goldman *et al.*, 1989; Goldman *et al.*, 1990; Hutchens *et al.*, 1991a; Hutchens *et al.*, 1991b). Using stable isotopic enrichment studies, Hutchens and co-workers (1991b) illustrated that nearly all of the intact lactoferrin in the urine of breast-fed premature infants was of maternal origin. Although the significance of this finding is still under debate, it is interesting to

speculate that breast milk lactoferrin is available in the neonatal blood and may have a role in protecting the infant from oxidative stress. Hutchens and his colleagues (1991b) speculate that maternal lactoferrin in the blood of the neonate may have a role in the regulation of the development of the infant's immune system. The interaction of lactoferrin with the immune cells will be discussed in detail later in this chapter.

Lactoferrin and the mammary gland

Lactoferrin may have an important role in the protection of the bovine mammary gland against infection. As indicated earlier, the concentration of lactoferrin in bovine milk is substantially lower than that in human milk. Several workers have speculated that, due to the low concentration in bovine milk, lactoferrin may not have such a vital role in bacteriostasis in the gastrointestinal tract of the growing calf (Smith and Schanbacher, 1977; Reiter, 1978). However, lactoferrin does become a major component in the secretion from the involuting bovine mammary gland (see table 1.4; Smith *et al.*, 1971; Welty *et al.*, 1976; Smith and Schanbacher, 1977; Nonnecke and Smith, 1984a).

The onset of involution is a period of increased risk of mammary infection in the cow (Neave *et al.*, 1950). Neave and co-workers (1950) reported that cows are more susceptible to mammary infections at the beginning of the dry period than at any other stage in the functional cycle of the mammary gland. The infection rate is highest during the early stages of involution, being approximately seven times that observed during normal lactation. However, as involution progresses, the infection rate decreases to approximately one-fifth of that observed during normal lactation.

Mastitis is caused by several groups of pathogens, including staphylococci, streptococci and coliforms (Norcross and Stark, 1970). 'Coliform' is a general term used to describe members of the Enterobacteriaceae family which are fermentative gram-negative bacteria that inhabit the gastrointestinal tract of man and other animals without causing disease. It is well documented that bovine lactoferrin can inhibit the growth of mastitis-causing bacteria (Bishop *et al.*, 1976; Smith and Schanbacher, 1977; Nonnecke and Smith, 1984b; Rainard, 1986a; Rainard, 1986b). In addition, Bishop and co-workers (1976) demonstrated that lactoferrin was bactericidal for certain strains of *E. coli*. As indicated earlier, the concentration of lactoferrin in the

mammary secretion increases markedly after the onset of involution. Lactoferrin is a major whey protein in the secretions from fully involuted bovine mammary glands. In most cows the concentration of lactoferrin reaches 20 to 30 mg/ml approximately thirty days after the cessation of regular milking. However, lactoferrin concentrations in excess of 100 mg/ml have been reported in the dry secretion of some cows (Welty *et al.*, 1976). The high concentration of this natural bacteriostatic agent in the dry secretion has lead several investigators to suggest that lactoferrin may be a potent antimicrobial agent in the non-lactating bovine mammary gland (Smith *et al.*, 1971; Harmon *et al.*, 1975; Welty *et al.*, 1976; Smith and Schanbacher, 1977; Eberhart *et al.*, 1979; Smith and Oliver, 1981).

In addition to the direct increase in the concentration of lactoferrin in the lacteal secretion from the involuting mammary gland, the concentration of citrate decreases as involution progresses. Several investigators have reported that citrate can reverse the inhibition of bacterial growth by bovine lactoferrin (Bishop *et al.*, 1976; Smith and Oliver, 1981; Smith and Schanbacher, 1977). Coliform bacteria possess at least two iron-sequestering mechanisms (Smith and Schanbacher, 1977). The first iron-acquiring mechanism involves the production and secretion of the iron-binding catechol, enterochelin into the surrounding medium. The second mechanism, the citrate-mediated uptake of iron, utilises citrate in the growth media. Citrate is also thought to repress certain enzymes involved in the synthesis of enterochelin. Consequently, in the presence of citrate, coliform bacteria are thought to sequester iron from the medium by citrate- rather than enterochelin-mediated uptake.

Smith and Schanbacher (1977) suggested that the molar ratio of citrate to lactoferrin could be used to determine the degree of inhibition of coliform growth in mammary secretions. A molar ratio of citrate to apolactoferrin of 75 resulted in approximately 50% growth inhibition in an *in vitro* assay system, and a molar ratio of citrate to apolactoferrin of 300 or greater resulted in less than 10% growth inhibition (Bishop *et al.*, 1976). The molar ratios of citrate to lactoferrin in mammary secretions are listed in table 1.7.

Table 1.7 The molar ratio of citrate to lactoferrin in the secretions from the bovine mammary gland (from Smith and Schanbacher, 1977).

Bovine lacteal secretion	Molar ratio of citrate to lactoferrin
Colostrum	300
Normal milk	3,000
Fully involuted dry secretion	10

The *in vitro* growth inhibition data of Bishop and co-workers (1976) suggests that the molar ratios of citrate to lactoferrin in colostrum and normal milk indicate that these mammary secretions would have little or no effect on the growth of coliform bacteria. Conversely, the molar ratio of citrate to lactoferrin in the secretion from the fully involuted gland would indicate that this secretion should be a powerful inhibitor of coliform growth. Interestingly, the bovine mammary gland is more resistant to new coliform infection when fully involuted than at other stages of the functional cycle.

The presence of citrate in lacteal secretions may also have an effect on the inhibition of bacteria in the intestine of the new-born calf. Although the concentration of lactoferrin in normal milk is thought to be too low to be effective as an antimicrobial agent in the calf's gut, the concentration in colostrum is high enough to be effective (Reiter, 1978). However, the molar ratio of citrate to lactoferrin of 300 would indicate that colostrum would be a poor inhibitor of microbial growth. Reiter (1978) indicated that citrate is rapidly absorbed from the intestine of the calf and that bicarbonate, which is secreted into the gut, should allow colostral lactoferrin to be effective as an antimicrobial agent in this environment.

In order for citrate to be effective in reversing the bacteriostatic activity of bovine lactoferrin, it must first remove the iron bound to the protein so that the bacteria can then assimilate it. A model for the interaction of citrate with bovine lactoferrin resulting in the release of iron will be presented in the discussion of this thesis.

Another interesting point, in relation to the protective effect of lactoferrin in the bovine mammary gland, is that several workers have noted an increase in lactoferrin concentration following infection of the bovine mammary gland with mastitis-causing bacteria (Harmon *et al.*, 1975; Harmon *et al.*, 1976; Sanchez *et al.*, 1988). Harmon and co-workers (1976) demonstrated that

lactoferrin levels in milk increase by as much as 30 times normal levels in response to acute mastitis. Harmon and his colleagues (1976) suggested that although some of the lactoferrin is likely to have come from the degranulation of neutrophils, the number of these leucocytes at the point of infection was too low to account for the marked increase in the concentration of lactoferrin in the milk. These workers speculate that the increase in lactoferrin concentration was due to an increase in the synthesis of lactoferrin by the mammary epithelial tissue. The increase in the concentration of lactoferrin in the secretion from the infected gland further indicates that lactoferrin may be part of the natural defense mechanisms operating in the bovine mammary gland.

1.4.2 Lactoferrin in other external secretions

Masson and co-workers (1966) demonstrated the presence of lactoferrin in a variety of biological fluids. The function of lactoferrin in these extracellular fluids is thought to be bacteriostasis. As in mammary secretions, lactoferrin in other external secretions is likely to be an important component of the non-specific defense mechanisms protecting the epithelial surfaces against microbial invasion.

The concentration of lactoferrin in external secretions changes in response to certain stimuli. For example, the concentration of lactoferrin in nasal secretions increases in response to cholinergic challenge (Raphael *et al.*, 1989). Raphael and his colleagues (1989) demonstrated that lactoferrin was produced and secreted from the serous cells of submucosal glands of the nasopharynx.

1.4.3 Lactoferrin in polymorphonuclear leucocytes

Lactoferrin has been identified as a major component of the specific granules of neutrophils (Masson *et al.*, 1969). The biological significance of lactoferrin in the neutrophil has been a subject of intense study. It appears that lactoferrin plays an important role as a mediator of the acute inflammatory response. That is, lactoferrin is involved in adherence of neutrophils to the sites of tissue damage, the cytotoxic activity of the activated neutrophils and finally in the regulation of the inflammatory response. Table 1.8 lists some of the effects that lactoferrin exhibits on the white blood cells. A number of these putative functions will be discussed in detail below.

Neutrophils

Neutrophils are the most abundant type of leucocyte in the peripheral blood. The most distinguishing feature of these granulocytes (polymorphonuclear leucocytes) is the highly lobulated nucleus. Like other granulocytes, the neutrophils contain two types of granules in their cytoplasm; the primary or azurophilic granules (which are large lysosomes) and the secondary or specific granules. The neutrophils are phagocytic and their primary function is to engulf invading micro-organisms, particularly bacteria. These cells are highly mobile and can migrate out of small blood vessels in response to tissue damage. Mature, circulating neutrophils are virtually incapable of regenerating the contents of their granules once they have been released in response to phagocytosis. Consequently, neutrophils are capable of only a single 'burst' of activity, after which they degenerate (Wheater *et al.*, 1979). The neutrophils are involved primarily in the acute inflammatory response and are the first line of 'blood-borne' defense against foreign substances entering the body by routes other than the digestive tract.

As indicated above, lactoferrin is found in the specific granules of neutrophils. When stimulated by opsonized bacteria or immune complexes, the plasma membrane of the neutrophil invaginates to surround the foreign particle (phagocytosis). The azurophilic and specific granules fuse with the newly formed vacuole at the internal border. The granules then release their contents, such as proteases, lysozyme and myeloperoxidase, into the phagosome (Weissman *et al.*, 1980). The neutrophil has a variety of cytotoxic mechanisms which work in unison to destroy the foreign particle.

Perhaps the most potent of the killing systems available to the neutrophil is the production of highly reactive species from molecular oxygen. These species include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and, possibly, singlet oxygen (Weissman *et al.*, 1980).

The role of lactoferrin in the neutrophil cytotoxic systems is unclear but obviously complex. The probable functions of lactoferrin in these systems will be subdivided into four groups; bacteriostasis, regulation of radical formation, feedback inhibition of neutrophil function and development, and the involvement of other white blood cells which is mediated by lactoferrin.

Table 1.8 Interactions of lactoferrin with the white blood cells. Possible functionally relevant in vitro and in vivo findings.

Proposed functions	References
Regulation of myelopoiesis.	Broxmeyer <i>et al.</i> , 1978; Bagby <i>et al.</i> , 1981; Gentile and Broxmeyer, 1983.
Enhancement of hydroxyl radical production by neutrophils.*	Ambruso and Johnston, 1981.
Inhibition of hydroxyl radical production by neutrophils.*	Britigan <i>et al.</i> , 1989.
Promotion of neutrophil adhesiveness.	Oseas <i>et al.</i> , 1981; Boxer <i>et al.</i> , 1982.
Reduction of lysozyme regeneration.	Anderson and Tomasi, 1977.
Enhancement of monocyte-natural killer activity.	Nishiya and Horwitz, 1982.
Suppression of murine <i>in vitro</i> primary antibody response.	Duncan and McArthur, 1981.
Modulation of activation of the complement system.	Kijlstra and Jeurissen, 1982.
Protection against complement- and neutrophil-mediated lung injury.	Ward <i>et al.</i> , 1983.
Growth factor for human lymphocytes in serum-free medium.	Hashizume <i>et al.</i> , 1983.
Increases uptake and killing of an intracellular parasite by murine macrophages and human monocytes.	Lima and Kierszenbaum, 1985.
Gallium-67 binding activity.	Weiner <i>et al.</i> , 1981; Hoffer <i>et al.</i> , 1977.

*Notes: * indicates a contrast of opinion in the literature. Britigan et al., (1989) suggest that it is unlikely that the conditions required for potential ·OH catalysis by lactoferrin occur in vivo.*

Bacteriostasis

Lactoferrin deprives the invading bacteria of the iron which is essential for cell growth. In the hostile environment of the phagosome the bacteriostatic effect may be of little consequence as the other antimicrobial systems rapidly

destroy most micro-organisms. However, Bullen and Armstrong (1979) proposed that iron-free lactoferrin was essential for the bactericidal activity of polymorphonuclear leucocytes. Two lines of evidence support their findings. Firstly, patients with no neutrophil lactoferrin suffer from repeated infections (Spitznagel *et al.*, 1972). Secondly, when iron was delivered to neutrophils via phagocytosis of ferritin-antibody complexes, the bactericidal power of the cells was markedly reduced (Bullen and Armstrong, 1979). When treated with apoferritin-antibody complex, the neutrophils retained their bactericidal activity indicating that phagocytosis of the complex itself did not interfere with the killing mechanism of the cell.

Lactoferrin may play an important role in the inhibition of growth of micro-organisms in the extraphagosomal environment. As indicated earlier, lactoferrin has potent bacteriostatic activity when not fully saturated with iron. The retardation of bacterial growth is even more pronounced when lactoferrin is combined with specific antibody directed against bacterial epitopes (Bullen *et al.*, 1972).

A notable response to inflammation is a general decrease in the plasma iron concentration. The decrease in serum iron concentration, hyposideraemia, may be part of the non-specific defense mechanism of the host to retard the growth of invading micro-organisms. In addition, as we will see later, the hyposideraemia may also be a prophylactic measure to protect the host against oxidative stress. Van Snick and co-workers (1974) demonstrated that the hyposideraemia of inflammation is a three step process that involves lactoferrin:

- (i) Lactoferrin is released from the specific granules in response to inflammatory stimuli (eg. bacteria). Importantly, lactoferrin found in the specific granules is predominantly iron-free.

- (ii) In the extraphagosomal environment, apolactoferrin is able to bind iron with great avidity. *In vitro*, lactoferrin is able to remove iron from transferrin only when either the pH is below pH 7.0 or in the presence of high concentrations of citrate. Van Snick *et al.* (1974) showed that rats injected with human apolactoferrin showed a marked decrease in the plasma iron concentration, indicating removal of iron from transferrin.

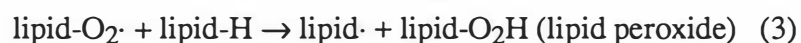
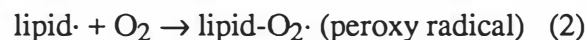
- (iii) Using immunofluorescence studies, van Snick and co-workers (1974) showed that iron-loaded lactoferrin was taken up by the reticulo-endothelial system (RES). In particular, these studies indicated that lactoferrin was bound and ingested by monocytes. Blockade of the RES

retarded the rate at which iron-loaded lactoferrin (Fe-lactoferrin) was cleared. These authors indicated that this implied the existence of Fe-lactoferrin receptors on the surface of monocytes and macrophages. The involvement of the monocyte-macrophage line will be discussed in detail later.

Regulation of oxygen-derived radical formation

Lactoferrin may also play an important role in the regulation of killing by reactive oxygen species. Ambruso and Johnston (1981) demonstrated that lactoferrin enhanced the production of the highly toxic hydroxyl radicals ($\cdot\text{OH}$). In sharp contrast to this finding, Britigan *et al.* (1989), demonstrated that lactoferrin actually inhibited the production of $\cdot\text{OH}$. Using sensitive spin-trapping techniques these authors showed that both lactoferrin and myeloperoxidase inhibited $\cdot\text{OH}$ production by activated neutrophils. Their explanation for this finding was that as neutrophils require iron for $\cdot\text{OH}$ formation, lactoferrin can suppress its production by removing the catalyst required for its production. The regulation of the production of oxygen derived radicals is clearly very important. The same reactive species that are used to kill invading organisms can also rapidly destroy host cells.

Oxygen-derived radicals arise during oxidative stress. The 'respiratory burst' of activated neutrophils (and excessive oxygen) cause the formation of hydrogen peroxide (H_2O_2) and superoxide radicals ($\cdot\text{O}_2^-$). Although these compounds may directly damage cell components, they more often combine to form hydroxyl radicals ($\cdot\text{OH}$). The hydroxyl radical can set off a radical chain reaction with devastating effects on cellular function and viability. For example, $\cdot\text{OH}$ attack on membrane lipids can initiate a chain reaction involving adjacent lipids as follows:



Not only lipids, but proteins and DNA are susceptible to $\cdot\text{OH}$ attack that can lead to radical chain reactions (Halliwell and Gutteridge, 1986). As indicated in figure 1.8, iron is involved in at least three reactions that lead to radical chain reactions. Clearly, there is a requirement for iron to be maintained in a form that will not promote these radical cascades. When bound to transferrin, iron is unable to promote $\cdot\text{OH}$ production (Halliwell and

Gutteridge, 1986; Sullivan, 1988). Britigan *et al.* (1989) demonstrated that lactoferrin decreases the production of $\cdot\text{OH}$ radicals. Lactoferrin retains iron under conditions that serum transferrin releases iron, indicating that lactoferrin may be a more potent anti-oxidation agent than transferrin. Consequently, lactoferrin may play an important role in providing protection against oxidative stress. As indicated earlier, the lactoferrin-mediated hyposideraemia of inflammation may be a physiological response aimed at reducing the damage caused by the over-production of oxygen-derived radicals.

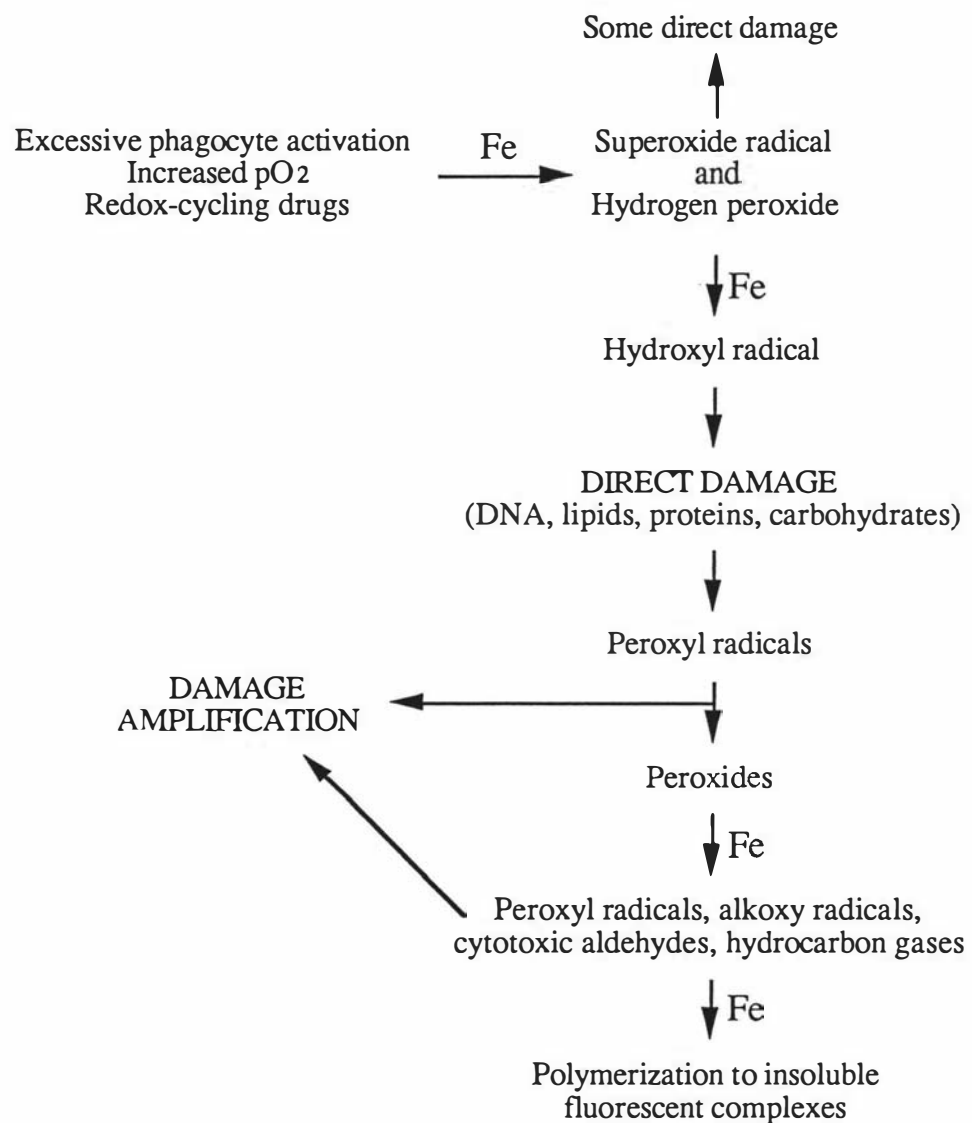


Figure 1.8 Mechanisms for cellular damage by oxygen-derived radicals and the involvement of iron. Based on Halliwell and Gutteridge, 1986.

Feedback inhibition of neutrophil function and development

Lactoferrin also has a role in the regulation of neutrophil development and function. Broxmeyer and co-workers (1978) were the first to demonstrate that lactoferrin inhibits the production of granulocyte-macrophage colony stimulating factor (GM-CSF).

GM-CSF is a cytokine which has a variety of effects on the myeloid lineage. It is a growth factor, stimulating the proliferation and maturation of the myeloid progenitor cells. Figure 1.9 illustrates the points of action of GM-CSF in the development of the granulocyte and monocyte lines. The suppression of GM-CSF production by lactoferrin may represent an important feedback mechanism for the regulation of granulocyte development in the bone marrow. In particular, regulating the number of circulating mature granulocytes which are kept within a narrow range in healthy adults.

The mechanism of regulation of GM-CSF production by lactoferrin has not been clearly defined. Broxmeyer and Platzer (1984) demonstrated that lactoferrin suppressed the release of GM-CSF from mouse peritoneal macrophages. Bagby and co-workers (1981; 1982; 1983a; 1983b) provided experimental evidence that lactoferrin suppressed the release of a monokine (or monokines) from mononuclear phagocytes that stimulate other cells (eg. T-lymphocytes) to release GM-CSF.

The degree of iron saturation appears to have a role in the functional activity of lactoferrin. Broxmeyer *et al.*, (1978; 1980) determined that fully saturated lactoferrin was more effective at suppressing GM-CSF release than partially or completely unsaturated forms of the protein.

In addition to the proliferative effect, GM-CSF has a vital role in the function of mature myeloid cells. Of particular interest, in relation to the effect of lactoferrin on the production of this cytokine, is the action of GM-CSF on macrophages and neutrophils. Table 1.9 shows some of the biological effects of GM-CSF on mature neutrophils and macrophages.

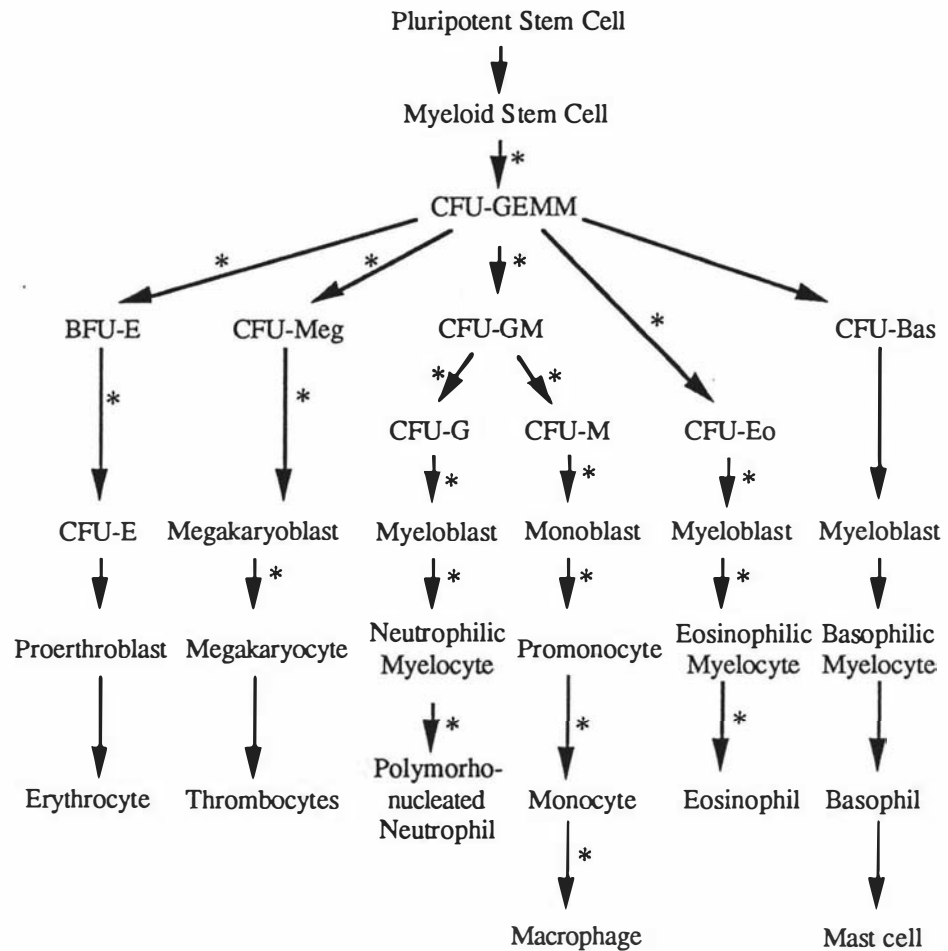


Figure 1.9 Haematopoiesis of the myeloid lineage. The points of action of GM-CSF on the development of the myeloid cells are indicated by the asterisks. Abbreviations: CFU, colony forming unit; GEMM, multipotential colony forming unit; BFU, Blast forming unit; E, erythroid; GM, granulocyte-macrophage; Bas, Basophil; Meg, Megakaryocyte; Eo, Eosinophil. (Adapted from a Schering-Plough and Sandoz Pharmaceuticals GM-CSF information bulletin).

Lactoferrin also interacts directly with neutrophils. Bennet *et al.* (1986) reported that lactoferrin binds to DNA on the surface of neutrophils. The significance of this apparently specific and saturable interaction is unknown.

Table 1.9 Some examples of the functional enhancement of GM-CSF on the activity of mature macrophages and neutrophils (from Gasson, 1991).

Neutrophils	Macrophages
Survival and protein synthesis	Cytokine release
Migration inhibition	Killing of parasites
Oxidative metabolism	Surface receptor antigen expression
Degranulation	Tumour cell killing
Cytokine secretion	Adherence
Recruitment	Oxidative metabolism
Platelet activating factor and leukotriene synthesis	
Antibody-dependent cell-mediated cytotoxicity	
Arachidonic acid release	
Changes in cell surface receptors	

The interaction of lactoferrin with other white blood cells

Lactoferrin, released from neutrophils during phagocytosis, interacts with a variety of white blood cells apart from neutrophils. Specific cell surface receptors for lactoferrin have been identified on leucocytes. Mazurier *et al.* (1989) identified lactoferrin receptors on phytohemagglutinin (PHA)-stimulated human peripheral blood lymphocytes (PBLs). These authors were unable to demonstrate the presence of lactoferrin receptors on the surface or within resting PBLs. This indicates that mitogen stimulation results in *de novo* synthesis of lactoferrin receptors rather than mobilisation of internal stores of the protein. Hashizume *et al.* (1983) identified lactoferrin as an essential growth factor for human B- and T-cell lines in serum-free tissue culture media. Mazurier and co-workers (1989) illustrated that lactoferrin promoted the proliferation of PHA-stimulated lymphocytes *in vitro*.

Lactoferrin associated with the lymphocyte receptors did not translocate iron into these cells, thus ruling out the proliferative effect of iron delivery into these cells. Sun *et al.*, (1991) proposed that diferric lactoferrin at the surface of the cell can participate in oxidoreduction reactions leading to the activation of Na^+/H^+ exchange. Plasma membrane oxidoreductase activity has been demonstrated to alter cellular functions involved in growth control (Sun *et al.*, 1991).

It is appealing to consider a physiological meaning for these *in vitro* findings. The question arises - does lactoferrin, released from neutrophils in response to an immunological stimulus, regulate the proliferation of stimulated lymphocytes in the blood? As *in vivo* data becomes available an answer to this intriguing question may be found.

Van Snick *et al.* (1974) determined that lactoferrin was taken up by mononuclear leucocytes. On the basis that iron-saturated lactoferrin was cleared from serum faster than the iron-free form, van Snick *et al.* (1974) proposed that these cells have surface receptors for lactoferrin. Birgens and his colleagues (1983) demonstrated the presence of saturable lactoferrin receptors on monocytes. The physiological significance of lactoferrin receptors on the monocyte/macrophage line is intriguing. Birgens *et al.* (1988) demonstrated that lactoferrin is capable of transferring iron to intracellular ferritin in monocytes. As van Snick and co-workers (1974) suggested, the delivery of iron to monocytes may be central in the hyposideraemia observed during inflammation. Lactoferrin, released from the monocyte after delivering iron to intracellular compartments, is no longer able to interact with the cell surface receptor (Birgens *et al.*, 1990). This is in sharp contrast to the 'recycling' scheme demonstrated for serum transferrin which is capable of repeated iron-delivery cycles.

What is the fate of lactoferrin after interaction with the monocyte/macrophage system? It appears that lactoferrin is rapidly cleared from the circulation by the liver (Regoeczi *et al.*, 1985). Whether specific lactoferrin receptors in the liver are responsible for the rapid clearance is still under debate. Liver macrophages (Kupffer cells), like other members of the monocyte line may have specific lactoferrin receptors. Presuming that these cells do have lactoferrin receptors, it may be unlikely that lactoferrin 'modified' by interaction with peripheral monocytes would bind to these receptors. Regoeczi *et al.* (1985) indicate that hepatocytes are responsible for the clearing of lactoferrin from serum. They also propose that the number of lactoferrin receptors required to account for clearance rates observed in rats would preclude the presence of specific receptors.

Lactoferrin may also interact with platelets (thrombocytes) and megakaryocytes. Raha *et al.* (1988) demonstrated that a peptide (KRDS, residues 39-42) from human lactoferrin inhibited the binding of monoclonal antibodies to glycoprotein IIb-IIIa on ADP-stimulated platelets and

megakaryocytes. Glycoprotein IIb-IIIa (GP IIb-IIIa) is the most abundant cell surface protein on platelets. This integrin is an adhesion receptor whose primary ligand is fibrinogen (Phillips *et al.*, 1988). KRDS inhibits both the binding of GP IIb-IIIa to fibrinogen and the aggregation platelets (Raha *et al.*, 1988; Fiat *et al.*, 1989). The significance of this finding is uncertain but it is interesting to speculate a function for lactoferrin (peptides) in platelet adhesion. Some of the lactoferrin released from neutrophils at the site of inflammation may be partially degraded by neutrophil proteases. The biologically active peptide KRDS may interact with GP IIb-IIIa and thus limit the adhesion of platelets to fibrinogen deposited at the site of tissue damage. This may represent yet another mechanism by which lactoferrin mediates the inflammatory response.

1.5 The aims of this study

Perspectives

Although lactoferrin was first identified in bovine milk, the human homologue has been the focus of more intensive study. Consequently, there is a wealth of information on the physical characteristics and biological activities of the human protein. Comparison of the physical characteristics of the members of the transferrin family has aided in the assignment of putative biological roles for these proteins.

Scientists in the department of Chemistry and Biochemistry at Massey University have had an interest in the structure and function of lactoferrin for over two decades. Lactoferrin research at Massey University is multidisciplinary, involving X-ray crystallographers, physical and inorganic chemists and biochemists. At the outset of this project the crystal structure of human lactoferrin had been solved at low resolution by Dr. E.N. Baker and his colleagues. Work had also begun on the elucidation of the molecular structure of bovine lactoferrin. Physico-chemical approaches, under the direction of Drs. A.M. Brodie and E. Ainscough, were being employed to define further the metal- and anion-binding characteristics of human and bovine lactoferrin.

In addition to the intrinsic interest in determining novel sequences, amino acid sequence data for bovine lactoferrin would clearly be of use in the related projects underway in the department.

Aim and outline of this thesis

The aim of the work outlined in this thesis was the elucidation of the entire amino acid sequence of bovine lactoferrin. Amino acid sequence data was to be determined by peptide mapping and sequencing and by molecular biology techniques.

Chapter two describes the isolation and partial characterisation of lactoferrin isolated from bovine colostrum. Rabbits were immunised with purified lactoferrin and the antibodies were used to determine the de novo synthesis of lactoferrin in involuting mammary tissue. Amino acid sequence data was determined by peptide mapping of homogeneous lactoferrin and sequencing of selected peptides. This data was used to verify that the amino acid sequence deduced from the nucleotide sequence of complementary DNA (cDNA) clones was that of bovine lactoferrin.

Chapter three details the synthesis, isolation and characterisation of cDNA clones coding for bovine lactoferrin. RNA isolated from involuting mammary tissue was used to direct the synthesis of cDNA. After cloning into the bacteriophage λ gt11, lactoferrin-specific sequences were identified by hybridisation to radiolabelled human lactoferrin cDNA. cDNA clones were characterised by restriction endonuclease mapping, Southern blot analysis and DNA sequencing.

The features of both the messenger RNA sequence and the deduced amino acid sequence for bovine lactoferrin are discussed in chapter four.

Chapter Two

Isolation and partial characterisation of bovine lactoferrin and studies on its biosynthesis in mammary tissue

2.1 Introduction

The aim of the work presented in this thesis was to synthesize and determine the sequence of the complementary DNA (cDNA) coding for bovine lactoferrin. Messenger RNA is required as the template for cDNA synthesis and can be isolated from a tissue which is actively synthesizing the protein of interest. The first step in this project was the identification of a source of messenger RNA (mRNA) coding for lactoferrin. In order to identify a tissue synthesizing lactoferrin, a means of identifying and quantitating the protein was required. To this end antibodies to lactoferrin were raised in rabbits and isolated from the sera. Purified antibody, specific for lactoferrin, was used to immunoprecipitate radiolabelled protein and also served as the basis of an assay system for lactoferrin.

As bovine lactoferrin can be isolated from the secretions of the mammary gland, mammary tissue was an obvious candidate as a source of mRNA coding for lactoferrin. Smith and Schanbacher (1977) reported that the concentration of lactoferrin in the mammary secretion of the cow changes according to the functional status of the gland. During normal lactation the concentration of lactoferrin in whey is 0.1 to 0.3 mg/ml but rises significantly after the onset of involution ("drying off"). Twenty to thirty days after the termination of regular milking the concentration of lactoferrin in the "dry" secretion was routinely 20-30 mg/ml. This dramatic increase may be due to a number of factors. The elevation of lactoferrin concentration may be due to an increased rate of synthesis of lactoferrin in the mammary tissue. Masson (1970) demonstrated that the secretory epithelial cells of the guinea pig mammary gland synthesize lactoferrin. Up-regulation of either transcription or translation of lactoferrin messenger RNA in this tissue may result in an elevation of lactoferrin in the lacteal secretion. The increase may also be due to exogenous synthesis of lactoferrin followed by transport to the mammary gland. For example, the degranulation of neutrophils in the regressing tissue may give rise to increased levels of lactoferrin in the dry secretion. However, Smith and Schanbacher (1977) suggested that the neutrophil content of

mammary tissue is too low to account for the marked elevation of lactoferrin concentration. Another possible explanation is that the rise in lactoferrin concentration may be a simple concentration effect. That is, as the gland regresses, the volume of the secretion decreases and the apparent concentration of lactoferrin increases correspondingly. This theory can be discounted in part by the observation that the volume of the 'dry' secretion from the mammary gland does not decrease significantly during the early stages of involution (A. Whetton, personal communication) when the concentration of lactoferrin has already increased significantly .

This chapter outlines the purification and partial characterization of bovine lactoferrin, and the isolation of antibodies specific for lactoferrin. In an attempt to elucidate the source of the increase in lactoferrin concentration of the dry secretion and to identify a source of mRNA for lactoferrin, newly synthesized mammary proteins were labelled *in vitro* and precipitated with lactoferrin specific antibodies. In one experiment, tissue biopsies were removed from a dairy cow at regular intervals over the first few weeks of involution. In a second study, the whey lactoferrin concentration from a dairy cow was monitored over the course of involution. A mammary biopsy was obtained from this animal twenty-five days after the cessation of regular milking. In both animal studies, tissue fragments from the mammary biopsies were incubated in the presence of a radiolabelled amino acid. The *de novo* synthesis of total protein was followed by the incorporation of radiolabel into trichloroacetic acid (TCA)-precipitable products. Lactoferrin synthesis was estimated by the immunoprecipitation of radiolabelled protein with lactoferrin specific antibodies, followed by liquid scintillation counting. Lactoferrin synthesis indicated the presence of mRNA coding for lactoferrin. Tissue biopsies which demonstrated the highest proportion of lactoferrin synthesis were selected for RNA extraction and the subsequent production of cDNA.

2.2 Materials

All chemicals were of reagent grade or higher and were supplied by Sigma Chemical Company, British Drug Houses (BDH), Serva (Sweden), or United States Biochemicals (USB). Agarose (Type I, low EEO) and trypsin was from Sigma Chemical Company, Mo. ^{125}I labelled bovine lactoferrin was kindly prepared by K.M. Stowell and M. Mock.

2.3 Methods

2.3.1 Protein Assays

(i) Direct spectrophotometric determination

The protein concentration of solutions of homogeneous proteins was determined by measurement of the absorption at 280 nm. The extinction coefficients listed in table 2.1 were used to calculate protein concentration.

Table 2.1 Extinction coefficients used to determine the concentration of proteins in solution.

Protein	E ²⁸⁰ for a 1% (w/v) solution	Reference
Bovine serum albumin	6.3	Stoscheck (1990).
Bovine lactoferrin	12.5	Morris, G. and Gartner, A. (Personal communication)
Rabbit gamma-globulin	15.4	Hudson and Hay (1976).

The absorbance of protein solutions at 280 nm was measured in quartz cuvettes with a variety of instruments including Cecil UV/Vis spectrophotometer, Aminco DW-2a UV/Vis spectrometer, Hitachi 101 spectrophotometer, or a Hewlett Packard Diode Array Spectrophotometer.

(ii) Chemical Protein Determination

The protein concentration of various solutions was measured by colorimetric methods, including the Biuret method and the method of Lowry *et al.* (1951).

(iii) Absorbance Spectra

The absorbance spectra of solutions containing lactoferrin were measured with either an Aminco SW2a UV/Vis spectrometer or a Hewlett Packard Diode Array Spectrophotometer.

2.3.2 Polyacrylamide gel electrophoresis

Polyacrylamide-SDS gel electrophoresis was performed according to the method of Laemmli (1970). Gels were cast and run in either homemade vertical electrophoresis stands or in a Pharmacia GE-4 gel electrophoresis apparatus. After electrophoresis gels were stained with Coomassie Brilliant Blue R250. When additional sensitivity was required gels were silver stained (Biorad) according to the manufacturers specifications.

Polyacrylamide gel electrophoresis was also performed using a Pharmacia Phast Gel Electrophoresis system. Gels were run, stained and destained according to the manufacturers specifications.

2.3.3 Immobilisation of Protein Ligands on Sepharose Beads

Various protein ligands were covalently bound to Sepharose beads via a ten atom spacer arm as described by Bethel *et al.* (1979) and outlined in figure 2.1.

2.3.3.1 Preparation of aminocaproic acid substituted Sepharose after activation of Sepharose CL-6B with 1,1-carbonyldiimidazole (CDI)

Sepharose CL-6B (50 g wet weight) was transferred to a scintered glass funnel in a fume hood and washed with several funnel volumes of deionised water (dH₂O) to remove preservatives. The resin was solvent exchanged from water to dimethylformamide (DMF) by sequential washing with 250 ml 70% ddH₂O/30% DMF (v/v), 250 ml 30% ddH₂O/70% DMF (v/v) and 500 ml of DMF. The resin was washed with 500 ml of distilled degassed DMF and transferred into a screw cap reaction vessel. 1,1-carbonyldiimidazole (CDI) (1.25 g) was dissolved in distilled degassed DMF, added to the resin, and mixed for 1-2 hours on a rotator. The resin was transferred to a scintered glass funnel and solvent exchanged back to water by reversing the order of the first solvent exchange. Distilled degassed DMF was used in each step of the solvent exchange back to water. The activated resin was washed with 500 ml distilled deionised water and transferred back to the reaction vessel.

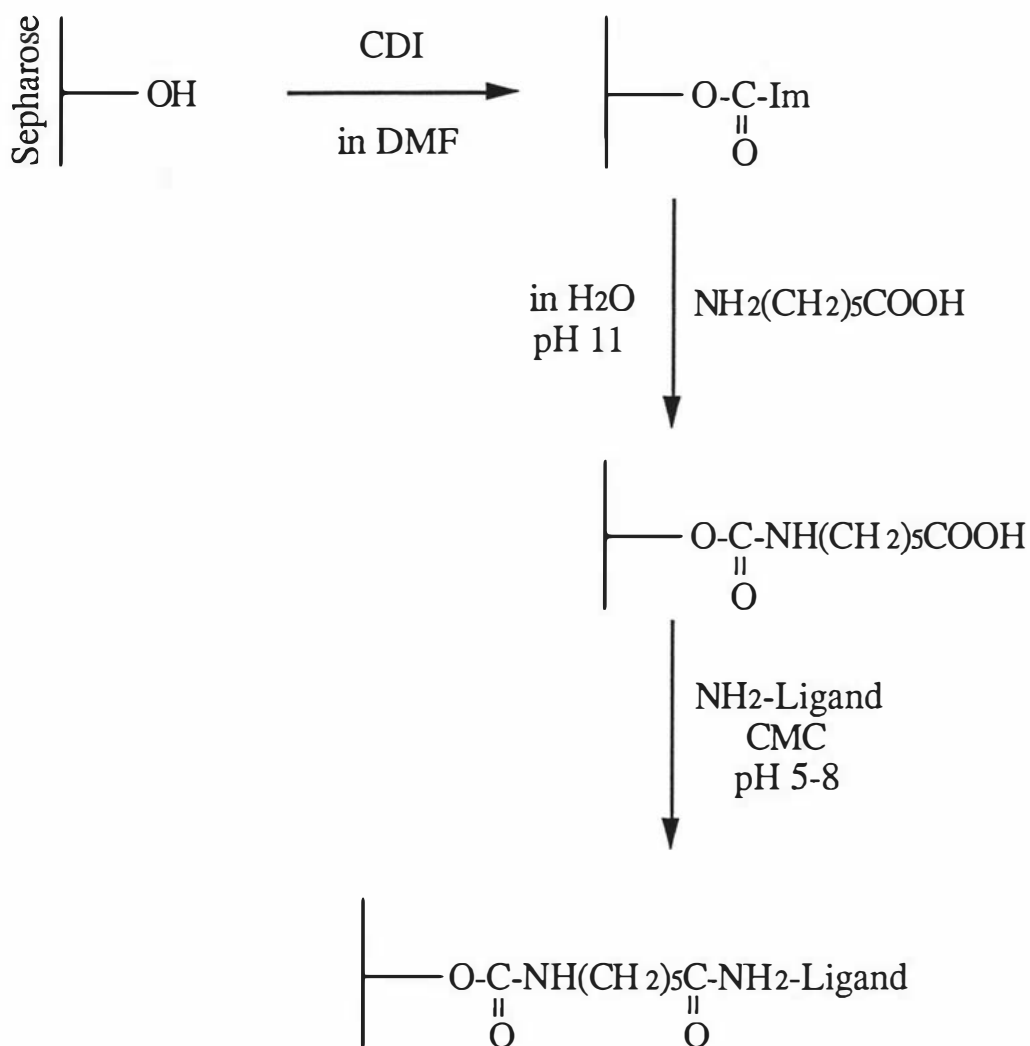


Figure 2.1 Schematic of the covalent attachment of ligands via free amino groups to CDI activated, aminocaproic acid substituted Sepharose CL-6B.

Aminocaproic acid (spacer arm) (5 g) was dissolved in 10 ml 1 M NaOH, titrated to pH 11 and added to the resin in the reaction vessel. The vessel was mixed on a rotator for 24 hours. The resin was transferred to the scintered glass funnel and washed with 500 ml ddH₂O. This was followed by sequential washes with 250 ml 0.1 M NaOH, 500 ml ddH₂O, 250 ml 0.1 M HCl, and finally with 500 ml ddH₂O. The washed resin was transferred to an automated titrator vessel (Radiometer) with water. After the addition of approximately 100 mg NaCl, the resin was titrated to pH 8.0 with 1.0 M NaOH. The volume of NaOH required to titrate the resin was recorded and used to calculate the degree of activation. The resin was washed with water on the scintered glass funnel and transferred to a measuring cylinder to record the volume of the substituted resin. The degree of activation, a measure of the substitution of aminocaproic acid on the resin, was estimated by dividing

the μ moles of carboxyl groups (which is equivalent to the mmoles of OH^- required to titrate the resin to pH 8.0), by the volume of the resin.

For example, the volume of 1 M NaOH required to titrate the resin to pH 8.0 was 1.288 ml, that is 1288 μ moles of OH^- . The volume of substituted resin was 58.0 ml. Therefore the degree of substitution was $1288/58.0 = 22$ μ moles COOH per ml of resin. This degree of substitution was routinely obtained.

2.3.3.2 Covalent attachment of lactoferrin and other ligands to aminocaproic acid substituted Sepharose

Activated resin (20 g) was titrated to pH 5.4 with 1 M HCl and stirred gently at room temperature. Electrophoretically pure bovine lactoferrin (50 mg) was added to the resin and the pH was adjusted to pH 5.4. 1-cyclohexyl-3-(2-morpholinyl)-(4-ethyl)carbodiimide metho-*p*-toluene sulphonate (CMC) (0.5 g) was dissolved in distilled water and titrated to pH 5.4. The solution of CMC was added to the resin slowly over a period of ten minutes. The resin was then transferred to a screw-cap reaction vessel and mixed on a rotator. After one hour at room temperature the pH was adjusted to pH 5.4. The resin was mixed gently by slow rotation for two or three days at 4°C. The resin was collected on a scintered glass funnel. The absorbance of the eluent was measured at 465 nm to determine the amount of lactoferrin that remained free in solution. The resin was washed with 500 ml 0.1 M NaCl to remove any protein which was not covalently bound to the resin. The absorbance of the washings was measured at 465 nm. The resin was washed thoroughly with phosphate buffer (PB, 10 mM NaH_2PO_4 pH 7.2) and was stored at 4°C in PB containing 0.1% (w/v) thiomersal as an antimicrobial agent.

Other protein ligands were covalently attached to activated Sepharose in an identical fashion. Amounts of resin and ligand were scaled according to the final volume of substituted resin required. The estimation of the degree of immobilisation was dependant on the ligand. For lactoferrin this was simply determined by measuring the absorbance of the washings at 465 nm, a wavelength characteristic of the iron binding sites of the protein. This method was inappropriate for other ligands as they do not absorb light in the visible region.

The attachment of antibody to Sepharose was followed by measuring the absorbance of the washings at 280 nm after removal of CMC by exhaustive dialysis of the washings against water. The water soluble carbodiimide (CMC) also absorbs strongly at 280 nm and its removal was necessary before the absorbance due to unreacted protein ligand could be measured.

The proteolytic enzyme trypsin was also covalently bound to activated Sepharose. The attachment of trypsin was followed by enzyme assay of the resin sample and washings after attachment.

2.3.4 Protein isolation and partial characterization

2.3.4.1 Isolation of lactoferrin from bovine colostrum

Bovine lactoferrin was isolated from colostrum essentially as described by Zagaloski and co-workers (1979). Bovine colostrum (12 l) was obtained from Massey University N^o 1 Dairy farm. The colostrum was defatted by centrifugation (Sorvall GS-3 rotor, 7500 rpm, 15 min, 15°C). The supernatant was strained through glass wool and pooled. The centrifugation step was repeated and the second supernatant was pooled and supplemented with iron. Iron supplementation ensured that all the lactoferrin in the sample was iron saturated. Brock *et al.* (1976) have reported that iron loaded lactoferrin is more resistant to proteolytic attack than the iron free form (apolactoferrin). Iron was added in 5-fold excess over that amount required to fully saturate the estimated amount of lactoferrin in the sample. 125 ml of 0.1 M FeNTA was added to the 10 l of defatted colostrum and mixed with an overhead stirrer at 4°C. Sephadex CM-50 resin (120 g wet weight) was washed with dH₂O on a scintered glass funnel and added slowly to the stirring colostrum. The sample was stirred slowly for 12 hours and then allowed to settle overnight at 4°C. The bright red resin was collected by centrifugation (GS-3, 7500 rpm, 10 minutes, 10°C) and transferred to a scintered glass funnel. The resin was washed with 35 l dH₂O at 4°C. To maintain a high flow rate, the resin was periodically removed and the funnel was washed with 500 ml 1 M HCl followed by 2 l dH₂O to remove protein deposits. The resin was washed at 4°C with 15 l of 0.025 M Tris-HCl pH 7.8 and then 15 l of 0.025 M Tris HCl pH 7.8, 0.25 M NaCl. Small aliquots (approximately 10 ml) of each wash were collected for protein determination by the Biuret method. The resin was then transferred to a Pharmacia column apparatus and washed overnight with 0.025 M Tris-HCl pH 7.8, 0.25 M

NaCl. Approximately 2.7 l of eluant, corresponding to a large A^{280} peak, was pooled and concentrated by ultrafiltration (Amincon, YM-50 membrane). The concentrated sample was dialysed overnight against two changes of 15 l 0.025 M Tris-HCl pH 7.8, 0.1 M NaCl at 4°C and finally against 5 l of the same buffer for 4 hours at 4°C.

The dialysed sample was loaded onto a CM-Sephadex (Sigma C-50-120) column equilibrated with 0.025 M Tris-HCl pH 7.8, 0.1 M NaCl at a flow rate of 1 ml/min at 4°C. The column was washed overnight with 0.025 M Tris-HCl pH 7.8, 0.1 M NaCl at the same flow rate. The column was eluted with 0.2 M NaCl in the equilibration buffer and then with 0.3 M NaCl in the same buffer. Lactoferrin was eluted from the column by a linear gradient (3.0 l) from 0.3 M NaCl to 0.7 M NaCl in 0.025 M Tris-HCl pH 7.8, at a flow rate of 1 ml per min. The eluent was collected in approximately 20 ml fractions. Fractions containing lactoferrin were pooled into 100 ml aliquots, concentrated by ultrafiltration and stored at -20°C in small aliquots.

2.3.4.2 N-terminal sequence analysis of bovine lactoferrin

50 mg of bovine lactoferrin was dialysed against 0.1 M NH_4CO_3 overnight, lyophilised and resuspended in 50% (v/v) formic acid. Approximately 1 μmole of lactoferrin was loaded onto a prewashed filter and subjected to automated N-terminal sequencing on a gas phase protein sequencer (Applied Biosystems Inc., 320A).

2.3.4.3 Isolation and N-terminal sequence of the C-terminal 50 kDa tryptic fragment from bovine lactoferrin

2.3.4.3.1 Preparation of immobilised trypsin on Sepharose CL-6B

Trypsin (Sigma type III, 2x crystallised) (50 mg) in 1 ml of dH_2O was covalently attached to 12.5 g CDI activated, aminocaproic acid substituted Sepharose in the presence of CMC at pH 5.4 as described in 2.3.3.2. The trypsin-Sepharose was stored at 4°C in 0.1% (w/v) thiomersal in 0.1 M Tris-HCl pH 7.2, 0.05 M CaCl_2 . Trypsin activity was assayed by measuring the rate of hydrolysis of $\text{N}\alpha$ -benzoyl-DL-arginine-p-nitroanilide (BAPNA, Sigma Chemical Co., B-4875). Briefly, 1.8 ml of 0.1 M Tris-HCl pH 7.2, 0.05 M

CaCl_2 was mixed with 1.0 ml 1 mg/ml BAPNA in dimethylsulphoxide. 0.2 ml of sample was added and trypsin activity was followed by the increase in absorbance at 405 nm.

2.3.4.3.2 Partial digestion of lactoferrin with immobilised trypsin

Electrophoretically pure iron-saturated bovine lactoferrin (60 mg) was diluted with an equal volume of 0.1 M Tris-HCl pH 8.0. Trypsin-Sepharose (2 ml) was added and the sample was mixed gently for several hours at room temperature. The reaction was stopped by centrifugation to remove the trypsin-Sepharose (MSE microfuge, 13,000 rpm, 5 minutes, 4°C). The supernatant was carefully removed and set on ice.

2.3.4.3.3 Separation of partial tryptic fragments by preparative gel electrophoresis and isolation by electroelution

Partially digested lactoferrin (60 mg) was diluted with an equal volume (4 ml) of SDS-PAGE sample buffer and heated to 100°C for 5 min. A fluorescent marker was prepared by mixing 100 μl partially digested lactoferrin with 5 μl 1% (w/v) fluorescein isothiocyanate and incubating at 37°C for 5 min. The fluorescent sample was diluted with an equal volume of SDS-PAGE buffer and heated at 100°C for 5 min. Both samples were cooled on ice and loaded onto a 10% (w/v) polyacrylamide-SDS gel. The fluorescent marker was loaded in a side well and the bulk sample loaded into a single large well. Electrophoresis on preparative polyacrylamide gels was at 75 mA for 13.5 hours at room temperature. The marker track was excised from the gel and exposed to ultraviolet light to observe the fluorescent labelled products. The region of the gel corresponding to the 50 kDa C-terminal polypeptide was excised and sliced into 5 mm³ cubes. A 'homemade' electroelution apparatus was assembled using a funnel made from a 500 ml Erlenmeyer flask. Stacking gel (~30 ml) was cast in the neck of the upturned funnel. The stacking gel formed a plug in the neck of the funnel to support the diced gel fragments. The gel cubes were sealed in stacking gel and the flask was flooded with electrophoresis buffer. The peptide was collected in a dialysis bag attached to the bottom of the flask by electrophoresis at 50 mA for 7 hours at room temperature. The electroeluted peptide was concentrated by ultrafiltration (YM-5 membrane, Amicon) and dialysed overnight against several changes of dH₂O. A small sample of the dialysed product was set

aside for characterisation by gel electrophoresis and protein assay. The remaining sample was freeze dried and resuspended in 500 μ l sdH₂O. Approximately 2 mmoles (about 100 μ g of peptide) in 20 μ l 50% formic acid was loaded onto a prewashed filter for automated N-terminal sequencing.

2.3.4.3.4 Tryptic mapping of bovine lactoferrin and N-terminal sequencing of selected peptides

Bovine lactoferrin (50 mg) was carboxymethylated with iodoacetic acid as described by Gurd (1972) and then dialysed exhaustively against deionised water. Lactoferrins are basic proteins (IEP ~ 8), containing many lysine and arginine residues. In order to decrease the number of peptides resulting from digestion with trypsin, the lysine residues in the carboxymethylated protein were blocked with maleic anhydride as described by Butler and Hartley (1972). The sample was digested with trypsin as described by Greene and Bartelt (1977). After dialysis against dH₂O, the tryptic digest was freeze-dried and resuspended in 1 ml of buffer A (0.1% (v/v) trifluoroacetic acid in dH₂O). Acid insoluble products were removed by centrifugation (10 min, 13,000 rpm, room temperature, MSE microfuge). Acid soluble peptides were separated by reverse phase high performance liquid chromatography (RP-HPLC) (Spectra Physics instrument). A tryptic map of the acid soluble fraction was obtained by loading a small sample (50 μ l) onto a C-18 Vydec column equilibrated in buffer A at 1 ml/min. Peptides were eluted from the column by a linear gradient from 100% buffer A to 100% buffer B (0.1% trifluoroacetic acid in dH₂O: isopropanol: acetonitrile (1:1:1 (v/v/v))) over 90 minutes at 1 ml/min. The column was washed with 100% isopropanol and equilibrated with buffer A before further samples were loaded. A preparative sample (250 μ l) of acid soluble tryptic peptides was loaded onto the C-18 column and eluted using identical conditions to those described above. Peptides, corresponding to peaks of absorbance at 230 nm, were collected in 1.5 ml plastic centrifuge tubes and evaporated to dryness under vacuum (Speedvac, Savant instruments). Dessicated peptides were stored at -20°C until required for N-terminal sequence analysis. Peptides were resuspended in 20 μ l 0.1% (v/v) trifluoroacetic acid in dH₂O and subjected to automated gas phase N-terminal sequencing.

2.3.5 Preparation and purification of antbovine lactoferrin antibodies

2.3.5.1 Immunisation of rabbits with bovine lactoferrin

Antibodies to bovine lactoferrin were raised in New Zealand white rabbits. Electrophoretically pure bovine lactoferrin, 1 mg/ml in phosphate buffered saline (PBS, 10 mM NaH₂PO₄ pH 7.2, 0.15 M NaCl) was emulsified in an equal volume of Freund's complete adjuvant (Sigma Chemical Co., Mo). The emulsified samples were used to initiate an antibody response in rabbits. Lactoferrin in PBS was used for all 'booster' injections. A humoral antibody response to bovine lactoferrin was produced by the following regime of injections:

- | | |
|---------|--|
| Day 1. | 1 ml of emulsion intradermally in 4-6 sites on the back of the rabbit.
1 ml of emulsion intramuscularly (I.M.) in one hind leg. |
| Day 7. | Repeat day 1, but use other hind leg for I.M. injection. |
| Day 21. | 0.1 mg lactoferrin in 0.5 ml PBS injected subcutaneously (S.C.) on the back. |
| Day 22. | 0.5 mg lactoferrin in 0.5 ml PBS injected S.C. on back. |
| Day 23. | 1 to 5 mg of lactoferrin in 0.5 ml PBS injected S.C. on back. |
| Day 28. | Collect blood from rabbits by cardiac puncture at seven day intervals as required. |

2.3.5.2 Collection of blood and preparation of serum

Blood was harvested from rabbits at weekly intervals by cardiac puncture. Approximately 50 ml of blood was obtained from each animal and transferred into plastic SS-34 centrifuge tubes. The blood was allowed to clot at room temperature for at least one hour. The clots were dislodged from the walls of the tubes and the tubes were left at 4°C overnight to allow the clots to retract. The serum was separated by centrifugation (17,000 rpm, SS-34 rotor, 10 minutes, 4°C), and decanted into clean centrifuge tubes (SS-34, Sorvall). Complement was inactivated by heating the serum to 54°C for 10 minutes. The serum was centrifuged again to remove any heat precipitated products

(17,000 rpm, SS-34 rotor, 10 min, 4°C). Serum was stored at -20°C. Small aliquots were assayed for the presence of anti-bovine lactoferrin antibody by the double-immunodiffusion test.

2.3.5.2.1 Preparation of double-immunodiffusion assay plates

Double-immunodiffusion assays were performed as described by Oucetlony (1962). Ethanol washed microscope slides were precoated with molten agarose (0.1% (w/v) in PBS), and dried at 60°C. 2.5 ml of molten agarose (1% (w/v) in PBS) was carefully pipetted onto each precoated slide on a level surface. The agarose was allowed to solidify at room temperature. The slides were then incubated at 4°C for 1 hour to harden the agarose. Wells were made in the chilled agarose with a well punch (Gelman) and the plugs removed with gentle suction. The plates were stored at 4°C in a humidified environment until required. Approximately 5 µg of bovine lactoferrin (antigen) and 5 µl immune sera (antibody) were pipetted into adjacent wells on plates. The plates were incubated overnight at 4°C in a humidified environment to allow precipitation lines to appear.

2.3.5.3 Ammonium sulphate fractionation of globulins

The gamma-globulin fraction of rabbit serum was partially purified by ammonium sulphate fractionation. Complement depleted serum was diluted with an equal volume of PBS (10 mM NaH₂PO₄ pH 7.2, 0.15 M NaCl). Saturated ammonium sulphate solution (pH 7.0) was added slowly to give a final concentration of 40% ammonium sulphate saturation. Proteins were allowed to precipitate at 4°C for 30 minutes and collected by centrifugation (17,000 rpm, SS-34 rotor, 10 minutes, 4°C). The supernatants were discarded and the pellets resuspended in 2.5 volumes (relative to the original serum volume) of PBS. Saturated ammonium sulphate was added to 40% (w/v) saturation. The tubes were incubated and precipitated protein collected by centrifugation as described above. The pellets were resuspended in 0.5 volumes of PBS and dialysed against PBS to remove ammonium sulphate. The protein concentration of the dialysed gamma-globulin fractions was determined by A²⁸⁰ measurements and they were stored at -20°C.

2.3.5.4 Purification of anti-bovine lactoferrin gamma-globulin on bovine lactoferrin Sepharose

Approximately 20 ml of bovine lactoferrin Sepharose was packed into a small Pharmacia liquid chromatography column and equilibrated with degassed phosphate buffer (PB, 10 mM NaH_2PO_4 pH 7.2) at room temperature. Buffer was pumped downwards through the column at 1 ml/min with a peristaltic pump (LKB). The absorbance of the eluent was measured at 280 nm with an ISCO UV monitor and flow cell. Approximately 200 mg of gamma-globulin fraction (ammonium sulphate precipitate) in 10 ml of buffer was diluted with an equal volume of degassed PB. The diluted sample was loaded onto the column at a flow rate of 5 ml/hr. 2.5 ml fractions were collected manually at 30 minute intervals. When loading was complete, the column was washed with degassed PB at the same flow rate (5 ml/hr) until the absorbance at 280 nm began to decrease. At this point the flow rate was increased to 0.2 ml/min for the rest of the column run. 1.2 ml fractions were collected at 6 minute intervals with an automated fraction collector (ISCO). When the absorbance at 280 nm returned to baseline, the buffer was changed to degassed phosphate buffered saline (10 mM NaH_2PO_4 pH 7.2, 0.15 M NaCl). Elution with this buffer continued until the absorbance at 280 nm again returned to baseline. The column was then washed with degassed 0.1 M Tris-HCl pH 8.3, 0.5 M NaCl. After collecting six fractions the direction of buffer flow was changed to upwards through the column and elution was continued until the A^{280} returned to baseline. Antibody bound specifically to the column was then eluted by washing with 3.0 M KCNS in PB until the A^{280} returned to baseline. The packed column was washed with degassed PB and stored at 4°C in PB + 0.1% (w/v) thiomersal.

2.3.5.4.1 Spot precipitin test of column fractions

To determine which column fractions contain anti-bovine lactoferrin antibody, spot precipitin tests were performed as follows. 20 μl of each fraction to be tested was diluted with 50 μl PB in a Durham tube. 5 μg of bovine lactoferrin (1 mg/ml in PB) was added with gentle mixing. The tubes were incubated at room temperature for at least one hour. The presence of a white immunoprecipitate at the bottom to the tube indicated the presence of anti-bovine lactoferrin antibodies in a column fraction. Fractions containing lactoferrin specific antibodies were pooled and dialysed overnight against

several changes of PB at 4°C. The affinity purified antibodies were assayed for activity and protein content and stored at -20°C in small aliquots.

2.3.5.4.2 Determination of anti-lactoferrin antibody activity by immunotitration

The anti-lactoferrin antibody activity of pooled, dialysed column fractions was determined by immunotitrations as outlined below.

For each assay, sixteen sucrose gradients were prepared in 0.5 ml tall-form microfuge tubes as follows: 100 µl of 1 M sucrose, 1% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100 in PB, 100 µl of 0.5 M sucrose, 1% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100 in PB and finally 100 µl 1% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100 in PB. A constant amount of gamma-globulin fraction (10-50 µg) was added to each tube. ¹²⁵I-labelled lactoferrin was added over a range of 0 to 14.10 µg at 0.94 µg increment to the sixteen tubes. The tubes were incubated at 37°C for 1 hour and then overnight at 4°C. The immunoprecipitated protein was collected by centrifugation in SS-34 (Sorvall) centrifuge tubes, (8 microfuge tubes per SS-34 tube on a cushion of water), in a swingout rotor (13,000 rpm, HB-4 rotor, 10 min, 4°C). Immunoprecipitated ¹²⁵I-labelled lactoferrin collects at the bottom of the tubes and the 'free' ¹²⁵I-labelled lactoferrin remains in the supernatant above the sucrose gradient. The tubes were frozen rapidly in liquid air and cut in half with side cutters. The two halves were transferred to disposable plastic tubes for counting in a γ counter.

2.3.5.5 Electroimmunodiffusion assay for bovine lactoferrin

The lactoferrin concentration of various solutions was measured by electroimmunodiffusion assay (Rocket electrophoresis) as described by Laurell (1966). Ethanol washed glass plates (250 x 100 mm) were precoated with 0.1% (w/v) agarose in distilled water, and allowed to dry. A 1% (w/v) agarose (Sigma type I, low EEO) solution was prepared in 1x Rocket electrophoresis buffer (374 mM Glycine, 187 mM Tris, 31.5 mM sodium barbitone, 5.56 mM diethylbarbituric acid, 1.5 mM sodium azide, pH 8.6-8.8). Agarose was melted by heating to 100°C in a water bath and the temperature subsequently maintained at 55°C. Rabbit anti-bovine lactoferrin gamma-globulin (ammonium sulphate fraction) was added to the molten agarose to a final concentration of 0.001% (w/v). 50 ml of molten agarose

was carefully poured onto a warm precoated glass plate on a level surface. When solidified, the agarose was hardened by incubating the plate at 4°C for one hour. Wells were made in the agarose using a 2.5 mm diameter gel punch and a homemade template. Agarose plugs were removed from the wells by gentle suction. Agarose plates were mounted on a water cooled horizontal electrophoresis apparatus (LKB multiphor). The reservoirs were flooded with 1x Rocket buffer and the circuit completed with filter paper wicks (Whatman #1) soaked in 1x Rocket buffer.

Standard lactoferrin was prepared from electrophoretically pure bovine lactoferrin. Samples and standards (ranging from 0.1 to 0.5 µg lactoferrin) were loaded into the wells with a 5 µl glass Hamilton syringe. Electrophoresis was at 2.5 V/cm for 16-18 hours at 16°C. After electrophoresis, agarose plates were washed gently with deionised water. A sheet of filter paper (Whatman #1) cut approximately 5 cm larger than the dimension of the plate was soaked in distilled water and carefully laid over the agarose plate. The plate was incubated at 42°C for several hours until completely dry. The filter paper was wet with deionised water and carefully lifted from the plate. The plate was rinsed in deionised water to remove paper debris and then stained. Proteins were visualised by staining with Coomassie Brilliant Blue R250.

The amount of lactoferrin was calculated by estimating the area inside the rocket shaped peaks. The area under the rocket (which is proportional to the amount of lactoferrin) is equal to the height of the rocket multiplied by the width at half height.

2.3.6 Affinity purification of bovine lactoferrin by chromatography on rabbit anti-bovine lactoferrin gamma-globulin Sepharose

Bovine lactoferrin was isolated from colostrum whey by affinity chromatography on rabbit anti-bovine lactoferrin gamma-globulin Sepharose CL-6B.

2.3.6.1 Preparation of resins

(a) Anti-bovine lactoferrin gamma-globulin Sepharose

Aminocaproic acid substituted resin (15 g) was reacted with 37.5 mg of affinity purified rabbit anti-bovine lactoferrin gamma-globulin in the presence of CMC at pH 5.4 as described earlier (2.3.3).

In order to determine the nature of the binding of lactoferrin to this 'affinity' resin, two trial resins were prepared. Lactoferrin is a basic protein and hence will be positively charged at physiological pH. It is likely that lactoferrin will interact with the carboxyl termini of the free spacer arms on the Sepharose support. To determine whether lactoferrin binding to the gamma-globulin resin was simply due to an ion exchange effect, a trial column was packed with 2 g of CDI activated aminocaproic acid-substituted Sepharose CL-6B and washed with PB.

Lactoferrin may also have other interactions with the carbon chain of the spacer arm or the Sepharose support when the negatively charged terminus of the spacer arm is blocked. A second resin was prepared in which the carboxyl group of the spacer arm was blocked with a small uncharged molecule.

(b) Methylamine Sepharose

Aminocaproic acid substituted Sepharose CL-6B (2 g) was allowed to react with methylamine in the presence of CMC at pH 5.4 as described earlier. Methylamine was added in five fold excess over that required to fully saturate all free carboxyl groups on the resin. The reaction was allowed to proceed reacting for two days on a rotator and the resin was washed and transferred to an automated titrator vessel. The resin was titrated to pH 8.0 with 1 M NaOH to determine the degree of methylamine substitution on the resin. Approximately 90% of the titratable (carboxyl) groups had been blocked with methylamine. The resin was packed into a small column and washed with PB.

2.3.6.2 Trial isolation of lactoferrin on substituted resins

Three small columns, packed with either methylamine Sepharose, aminocaproic acid Sepharose or anti-bovine lactoferrin gamma-globulin

Sepharose were washed with degassed PB. Bovine lactoferrin (1-5 mg in PB) was loaded onto the columns which were then washed with PB. The columns were eluted by washing sequentially with several column volumes of PB, PBS, 0.5 M NaCl in PB and finally 3.0 M KCNS in PB. Fractions (1 ml) were collected from the columns and the absorbance at 280 nm was determined. Lactoferrin recovered from the antibody column was dialysed against 0.01 M NaHCO₃ pH 7.0 and the absorbance spectrum between 200 and 700 nm was determined.

2.3.6.3 Isolation of lactoferrin from bovine colostrum whey by affinity chromatography

Bovine lactoferrin was isolated from colostrum whey by affinity chromatography on rabbit anti-bovine lactoferrin gamma-globulin Sepharose. 15 g of anti-bovine lactoferrin gamma-globulin substituted Sepharose was packed into a small column and washed with degassed PB. Bovine colostrum whey (126 ml) was dialysed against PB and loaded onto the equilibrated column. The sample was loaded at 5 ml/hr with a peristaltic pump. Fractions were collected at regular intervals with a Golden Retriever Fraction Collector (ISCO). When loading was complete, the pump speed was increased to 0.2 ml/min and the column washed with degassed PB until the A²⁸⁰ returned to baseline. The column was washed with PBS and then with 0.5 M NaCl in PB, until the A²⁸⁰ returned to baseline. The direction of buffer flow was changed to upwards through the column and a protein peak was eluted with 3.0 M KCNS in PB. Fractions containing lactoferrin were identified by the spot precipitin test as outlined earlier (2.3.5.4.1), pooled, and dialysed against PB. The dialysed samples were stored at 4°C for subsequent determination of protein concentration and analysis by gel electrophoresis.

2.3.7 Animal studies

2.3.7.1 Preliminary study

2.3.7.1.1 Case History

A cull cow (#87) was obtained from the Massey University Dairy Research Unit. The animal was a four year old Fresian-Jersey cow, with two previous seasons of milking. The animal was six months pregnant at the beginning of

the experiment. The tissue sampling was initiated on the last day of regular milking which is referred to as day zero of involution.

2.3.7.1.2 Tissue Sampling

The 435 kg cow was anaesthetised with an injection of 9 ml Rompun in her hindquarter, followed by a topical injection of local anaesthetic into the udder (Nopain^R). Wedges of mammary tissue (approximately 100 g) were surgically removed from alternate quarters at five day intervals, for a total of six biopsies. On day 10 of the sampling regime, that is, after two biopsies had been taken, it was noted that local infections were present at the sites of the first two biopsies. To avoid the possibility of systemic infection, the cow was treated with antibiotics (penicillin and streptomycin). By day 14 of the regime the infections were no longer apparent.

2.3.7.1.3 Handling and storage of the tissue

Approximately 1 g of the tissue was removed from the excised mammary biopsy and stored on ice in buffered saline (Hepes pH 7.4, 0.15 M NaCl, 10 mM D-glucose) for transport back to the laboratory. The remaining tissue was frozen rapidly in liquid nitrogen and later fragmented into 1 to 2 g pieces and stored at -70°C.

2.3.7.1.4 *In vitro* labelling of newly synthesized mammary proteins with ³⁵S-methionine

The mammary tissue stored in buffered saline was diced into 1-2 mm cubes by drawing between two sterile razor blades. The tissue fragments were transferred to sterile 1.5 ml microfuge tubes and washed 10 times with ice cold buffered saline to remove all debris. The tissue fragments were collected by rapid centrifugation (MSE microfuge, 13,000 rpm, 1-2 seconds). Newly synthesized proteins were labelled *in vitro* by incubation of the tissue fragments in the presence of a radiolabelled amino acid. Aliquots of tissue fragments (approximately 50 -100 mg) were added to media in cell culture dishes (Nuclon) with a sterile inoculation loop. Each incubation dish contained 500 µl culture medium ((modified from Folley and French, 1949; 1950) 2 mM NaH₂PO₄·2H₂O, 18 mM NaHCO₃, 0.1 M NaCl, 10 mM Hepes pH 7.4, 10 mM D-glucose, 0.0005% (w/v) phenol red). All incubations were performed in triplicate. To prevent microbial growth in the

incubation media, one of two antimicrobial systems was added. Either gentamycin (50 µg/ml final concentration) or a mixture of kanamycin (0.1 mg/ml), streptomycin (0.1 mg/ml), penicillin (100 units/ml) and fungizone (0.25 mg/ml) was added to inhibit microbial growth.

The measurement of newly synthesized protein was initiated by the addition of ^{35}S -L-methionine (50 µCi, 11.13 mCi/mmol). The cell culture dishes were incubated for six hours at 37°C in a humidified environment.

2.3.7.1.5 Sampling and estimation of total secreted protein synthesis

Lactoferrin is a secreted protein and will be present in the incubation medium surrounding cells synthesizing the protein. The synthesis of total secreted protein was measured by determining the amount of radiolabelled TCA precipitable products in the incubation medium. Aliquots of incubation medium (30 µl) were removed at intervals of one hour. These aliquots were centrifuged to remove particulate matter (MSE microfuge, 13,000 rpm, 1 minute). 20 µl of supernatant was diluted in 1 ml of ice cold water and mixed with 0.5 ml bleaching solution (1 M NaOH, 0.3% H_2O_2 (v/v)). Proteins were precipitated by the addition of 1 ml ice cold 25% TCA (w/v). The samples were mixed thoroughly and incubated on ice for one hour. The precipitated products were collected on glass fibre filter discs (Whatman GF/A or GF/C) using a vacuum-assisted multiwell sampling manifold (Multipore). The filters were washed twice with 2 ml ice cold 10% (w/v) TCA to remove unincorporated label and finally with 2 ml 95% ethanol to speed up the drying process. The washed filters were carefully placed in the neck of scintillation vials (Whetton) and dried under a heat lamp. The dry filters were pushed to the bottom of the vials and submerged in 3 ml liquid scintillation fluid (4 g PPO, 0.1 g POPOP, 333 ml triton X-100, 667 ml redistilled toluene). β emission from the incorporated radiolabelled amino acid was measured by scintillation counting (Beckman LS 8000, programme 4, unmodified). After six hours of incubation, the final sample was taken for estimation of protein synthesis and the reactions were terminated by placing the culture dishes on ice. The contents of the culture dishes were transferred to sterile 1.5 ml microfuge tubes and centrifuged (MSE microfuge, 13,000 rpm, 5 minutes, room temperature). The supernatant was carefully removed from each tube and stored in sterile microfuge tubes at -20°C until required for further analysis.

2.3.7.1.6 Estimation of lactoferrin synthesis by immuno-precipitation and gel electrophoresis

Bovine lactoferrin secreted during the incubation of the mammary cells was immunoprecipitated with rabbit anti-bovine lactoferrin gamma-globulin. Aliquots (50 - 100 μ l) of supernatant (after six hours of incubation of mammary tissue in the presence of 35 S-L-methionine) were diluted in phosphate buffer saline containing 1% (w/v) Triton X100 and 1% (w/v) sodium deoxycholate (PBS + TX100 + DOC). Affinity purified rabbit anti-bovine lactoferrin gamma-globulin (90 μ g; specific activity = 1 mg of gamma-globulin immunoprecipitated 345 μ g bovine lactoferrin), was mixed with the diluted supernatants. No carrier lactoferrin was added to the samples. The immunoprecipitation reactions were incubated for 1 to 3 hours at 37°C. Precipitated protein was collected by centrifugation (MSE microfuge, 13,000 rpm, 5 minutes, 4°C). The pelleted material was washed three times for 15 minutes with ice cold PBS + TX100 + DOC. The washed pellets were finally resuspended in SDS-PAGE sample buffer and denatured at 100°C for five minutes. Samples were separated by electrophoresis on 10% (w/v) polyacrylamide-SDS gels and visualised by staining with Coomassie Brilliant Blue R250 (Sigma) as described earlier.

The destained gels were photographed and also scanned for absorbance at 580 nm (ISCO gel scanner). Each sample track was sliced into 2 mm sections with a multiblade cutter. Sections were transferred to scintillation vials and solubilised by incubating overnight at 60°C in 1 ml 99% (v/v) H_2O_2 , 1% (w/v) NH_4OH . Liquid scintillation fluid (9 ml) was added and each vial was mixed thoroughly. Radiolabelled immunoprecipitated products were quantitated by liquid scintillation counting (Beckman LS 8000, programme 4 unmodified).

2.3.7.1.7 Determination of lactoferrin content of tissue samples

The total lactoferrin content of frozen mammary tissue samples was estimated by rocket electrophoresis. The lactoferrin content of each tissue sample should reflect any changes in the amount of lactoferrin in the gland during the early stages of involution.

Frozen mammary tissue (2 to 3 g) was crushed in a liquid nitrogen cooled steel mortar. The pulverised material was transferred to centrifuge tubes (SS-34, polypropylene) containing 15 ml of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. The tissue was further disrupted with an Ultraturrax tissue homogeniser (10 N probe, full speed, 30 seconds). Cell debris was removed by centrifugation (SS-34, 10,000 rpm, 10 minutes, 4°C). The supernatant was filtered through glass wool to remove floating fatty material. The filtrate was stored at either 4°C or -20°C until required. The concentration of lactoferrin in the tissue homogenate was determined by electroimmuno-diffusion assay (Rocket electrophoresis) as described in section 2.3.5.5. Total protein concentration of the homogenates was measured by the Biuret method and the value obtained was used to normalise the rocket electrophoresis data.

2.3.7.2 Second Animal Study

Six dairy cows (#9, 173, 198, 200, 202, 222) were obtained from Massey University Dairy Cattle Research Unit. All were non-pregnant Jersey or Fresian/Jersey cross cull cows ranging from 5 to 11 years of age.

2.3.7.2.1 Sampling of the 'dry' secretion

On the last day of regular milking, referred to here as day 0 of involution, the animals were milked and small samples of the secretion were taken for analysis. The animals were milked at five day intervals during the involution process. All milking was performed by milking machines. The mammary glands were completely emptied at each sampling. This procedure was chosen to reduce the risk of infection (mastitis) in the stimulated gland. When milking begins, the stimulated mammary gland 'lets down' milk and if the gland is not completely emptied there is an increased risk of mammary infection (Annabel Whetton, Massey University N^o4 dairy farm manager, personal communication).

2.3.7.2.2 Treatment and analysis of lacteal samples

Small samples (approximately 50 ml) of mammary secretions were collected from each cow and centrifuged to remove fat and cell debris (SS-34, 10 minutes, 7,500 rpm, 10°C).

The supernatant (skim milk) was drained through glass wool and stored in small aliquots at 4°C for short periods or at -20°C for long term storage. Lactoferrin concentration of the skim milk samples were measured by electroimmunodiffusion assay as described earlier (2.3.5.5).

2.3.7.2.3 Selection and Case History of Cow #198

Of the six cows that entered the experiment only one was chosen to take a tissue sample from. Cows #9, 202 and 173 were removed from the program due to mammary infections (mastitis). Cow #222 gave virtually no milk on day 10 of the sampling regime and was omitted from further sampling. Cow #200 was inadvertently sent to the freezing works on day 12 of the sampling program. By day 14 of the experiment only cow #198 remained healthy and continued to be sampled. Cow #198 was a seven year old Fresian-Jersey cross with three previous milking seasons at the Massey University Dairy Cattle Research Unit. During this period cow #198 had no recorded cases of mastitis. The animal had been treated with 'Dry Cow Therapy' at the end of the milking season.

The final sample of mammary secretion was collected twenty four days after the termination of regular milking. Later that day the cow was transported to Waitaki Freezing Company works at Feilding for slaughter the following day.

2.3.7.2.4 Collection and handling of the mammary biopsy

Cow #198 was slaughtered by exanguination following stunning twenty-five days after the initiation of the sampling regime. The entire mammary gland was removed from the animal within 15 to 20 minutes of slaughter. Approximately 200 g of tissue was carefully excised from the mammary gland and frozen in liquid nitrogen. A second biopsy, of approximately 1 g, was removed from the mammary gland and stored in ice cold buffered saline. This sample was used for *in vitro* labelling of newly synthesized proteins with ³⁵S-L-methionine and immunoprecipitation of lactoferrin as described in sections 2.4.2.4. to 2.4.2.5 inclusive. The tissue frozen in liquid nitrogen was later stored at -70°C until required, either for estimation of total lactoferrin in the mammary tissue as described in 2.4.2.6, or for the isolation of total cellular RNA (Chapter 3).

2.3.7.2.5 Estimation of lactoferrin synthesis by immunoprecipitation

The incorporation of ^{35}S -L-methionine into lactoferrin by mammary tissue was also measured by immunoprecipitation and direct counting of the radiolabelled precipitated products, rather than by SDS-gel electrophoresis of immunoprecipitates as described in section 2.3.7.1.6. Sucrose gradients were prepared in 1.5 ml plastic microfuge tubes. 300 μl of 1 M sucrose in PBS + 1% (w/v) Triton X100 + 1% (w/v) sodium deoxycholate was overlayed with 300 μl of 0.5 M sucrose in the same buffer. Finally 300 μl PBS + 1% (w/v) Triton X100 + 1% (w/v) sodium deoxycholate was added. Incubation media from the six hour incubation of mammary tissue in the presence of ^{35}S -L-methionine was prepared as described in 2.3.7.1.5. 100 μl of supernatant was carefully pipetted on top of the sucrose gradient. Cold lactoferrin (2.5 μg) was added to aid immunoprecipitation. Lactoferrin was precipitated by the addition of affinity purified rabbit anti-bovine lactoferrin gamma-globulin in ten-fold excess over that required to precipitate all the carrier lactoferrin. Each immunoprecipitation was performed in duplicate. The reactions were incubated at 37°C for three hours, followed by overnight incubation at 4°C. Immunoprecipitable material was collected by centrifugation through the sucrose gradient (HB-4 rotor, 13,000 rpm, 10 minutes, 4°C). The supernatants were carefully removed and the pellets were washed three times with 1 ml 1% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate in PBS. Precipitates were collected by centrifugation (MSE microfuge, 13000 rpm, 1 minute, 4°C). the washed pellets were resuspended in 0.5 ml of 0.1 M NaOH, and transferred to scintillation vials. The tubes were washed with a further 0.5 ml of 0.1 M NaOH and the washings pooled in separate scintillation vials. 9 ml of scintillation fluid was added to each vial. After thorough mixing, radioactivity was determined by scintillation counting (LS 8000, program 4 unmodified).

2.4 Results and Discussion

2.4.1 Isolation of Bovine Lactoferrin

Bovine lactoferrin was isolated from defatted colostrum by stepwise NaCl elution from CM-Sephadex followed by gradient elution from a column of the same material (section 2.3.4.1). Figure 2.2 shows the elution profile of protein from the second cation exchange chromatography column. The protein eluted as a single, symmetrical peak. The fractions (20 ml) containing protein were pooled into 100 ml aliquots and stored at -20°C . Approximately 2.7 g of lactoferrin was isolated from the 10 l of defatted colostrum.

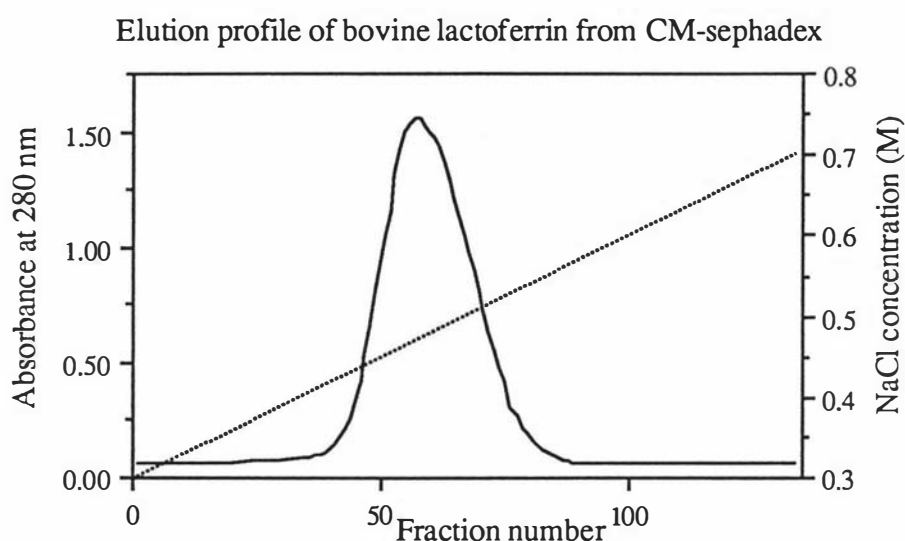
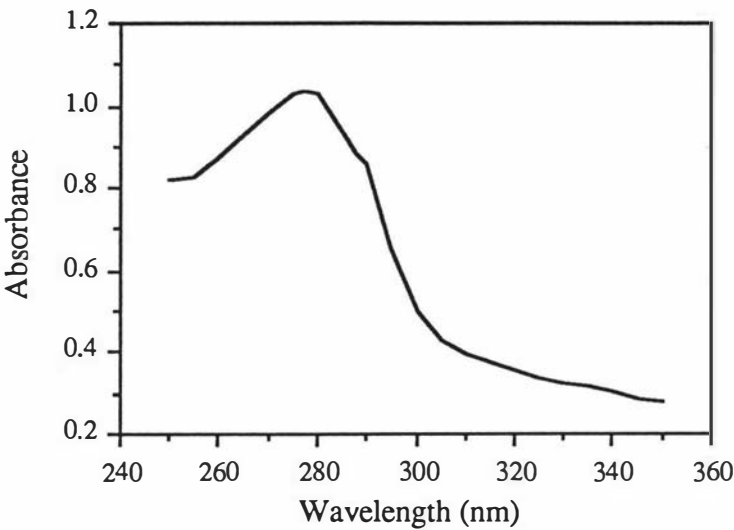


Figure 2.2 Elution profile of bovine lactoferrin from CM-Sephadex. The solid line represents the absorbance at 280 nm. The dotted line represents the linear salt gradient used to elute the protein from the column. Each fraction contained 20 ml of eluate.

Figure 2.3 shows a typical absorbance spectrum of the protein eluted from the Sephadex CM-50 column. The absorbance data for lactoferrin purified by CM-Sephadex chromatography is given in Table 2.2.

Absorbance spectrum of bovine lactoferrin eluted from CM-Sephadex

A.



B.

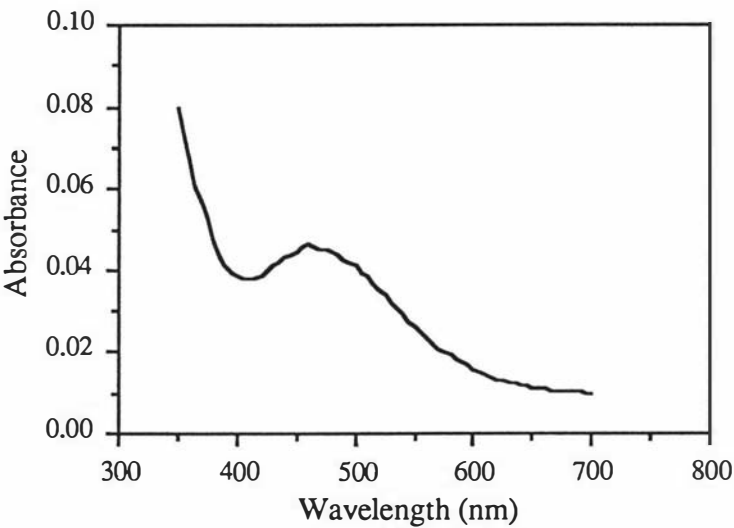


Figure 2.3 Absorbance spectrum of bovine lactoferrin isolated from colostrum by cation exchange chromatography. A. Absorbance from 250 nm to 350 nm. B. Absorbance from 350 nm to 700 nm.

Table 2.2 Spectral data for bovine lactoferrin purified by cation exchange chromatography from defatted colostrum.

A ₂₈₀	A ₄₁₀	A ₄₆₅	A ₂₈₀ /A ₄₆₅	A ₄₁₀ /A ₄₆₅
0.68	0.0244	0.0316	21.5	0.77

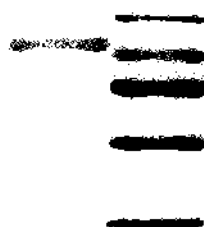
The absorbance spectrum obtained for the bovine lactoferrin sample is similar to that of other members of the transferrin family. In particular the absorbance maxima at approximately 465 nm and the absorbance minima at 410 nm are characteristic of iron-saturated transferrins (Aisen and Leibman, 1972). The ratio of the absorbance at 280 nm to that at 465 nm is an indicator of the purity of lactoferrin and of its degree of iron saturation. Pure bovine lactoferrin has an A^{280}/A^{465} ratio of approximately 20 and a A^{410}/A^{465} ratio of 0.7 to 0.8. The spectral data shown in figure 2.3 and table 2.2 indicates a high degree of purity and iron-saturation for the bovine lactoferrin isolated from defatted colostrum.

A small sample of the eluted protein was analysed by electrophoresis on a 10% (w/v) polyacrylamide-SDS gel (figure 2.4). The protein bands were visualised by staining with Coomassie Brilliant Blue R-250. The single densely stained band with a mobility corresponding to approximately 80 kD indicates that the protein preparation was homogeneous. The molecular weight of the protein band, calculated from the $\log_{10}$ MW versus mobility plot shown in figure 2.5 is approximately 83,000 Daltons. This value is somewhat higher than the molecular weight for bovine lactoferrin reported by others (78.5 kDa, Aisen and Leibman, 1972; 77.1 kDa, Castellino *et al.*, 1970; 76 kDa, Querinjean *et al.*, 1971).

2.4.2 N-terminal sequence of bovine lactoferrin

Intact bovine lactoferrin was subjected to automated N-terminal sequencing as described in section 2.3.4.2. Table 2.3 shows the N-terminal amino acid sequence data obtained. Also shown in the figure are the N-terminal sequences reported for other members of the transferrin family. The amino acid residues conserved throughout the transferrin family are also conserved in the sequence obtained for bovine lactoferrin.

1 2



Gel details: 10% (w/v) polyacrylamide-SDS gel stained with Coomassie Brilliant Blue.

Loading details:

1. Bovine lactoferrin eluted from CM-Sephadex column.
2. Molecular weight markers (Protein standards are phosphorylase B (92.5 kDa), transferrin (80 kDa), bovine serum albumin (68 kDa), catalase (57.5 kDa), citrate synthetase (50 kDa), and malate dehydrogenase (37 kDa)).

Figure 2.4 SDS-polyacrylamide gel electrophoresis of bovine lactoferrin isolated from bovine colostrum whey by cation exchange chromatography.

Log molecular weight versus electrophoretic mobility plot

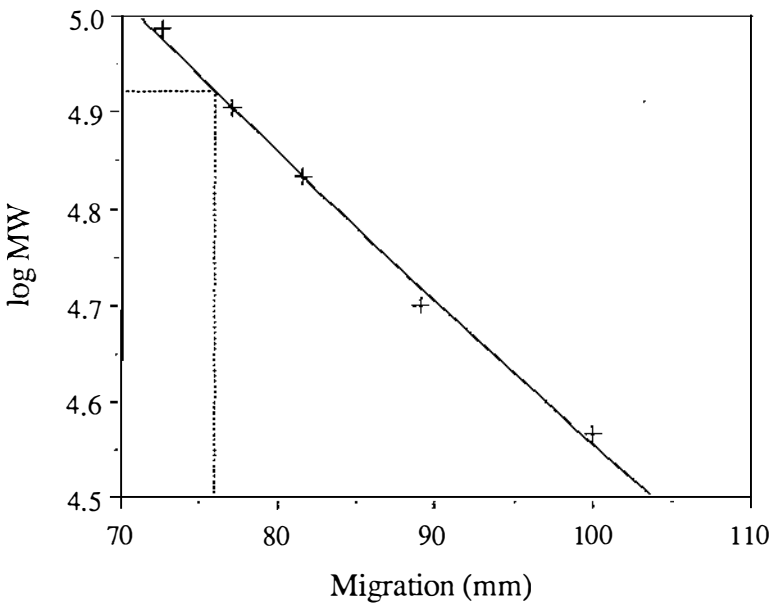


Figure 2.5 Log molecular weight versus mobility on SDS-PAGE plot to determine the molecular weight of bovine lactoferrin. The dotted line indicates the relative mobility of bovine lactoferrin. Protein standards were phosphorylase B (92.5 kDa), transferrin (80 kDa), bovine serum albumin (68 kDa), catalase (57.5 kDa), citrate synthetase (50 kDa).

Table 2.3 The N-terminal amino acid sequence of bovine lactoferrin compared to other members of the transferrin family.

Bovine lactoferrin	APRKNVFWXTISQPEWFKXRR...
Human lactoferrin	GRRRRSVQWCAVSNPEATKCFQ...
Mouse lactoferrin	AKATTVRWCAVSNSEEEKCLR...
Human serum transferrin	VPDKTVRWCAVSEHEATKQQS...
Chicken ovotransferrin	APPKSVIRWCTISSPEEKKONN...
Human melanotransferrin	GGMEVRFWCATSDPEQHKQGN...

Notes: X are probably cysteine residues. No signal was obtained from the sequencer at these cycles. Unprotected cysteine groups are completely destroyed by the chemistry used in the sequencing reactions. Boxes indicate amino acids which are invariant throughout the transferrin family.

2.4.3 Preparation and isolation of the C-terminal 50 kDa polypeptide from bovine lactoferrin

2.4.3.1 Immobilisation of trypsin of Sepharose CL-6B

Trypsin was covalently attached to Sepharose beads as outlined earlier (2.3.4.3.1). The trypsin activity associated with the resin and with the washings from the resin was measured by the hydrolysis of the chromogenic substrate BAPNA as described in 2.3.4.3.1. The data is presented in table 2.4.

Table 2.4 Trypsin activity measured by the rate of hydrolysis of the chromogenic substrate BAPNA.

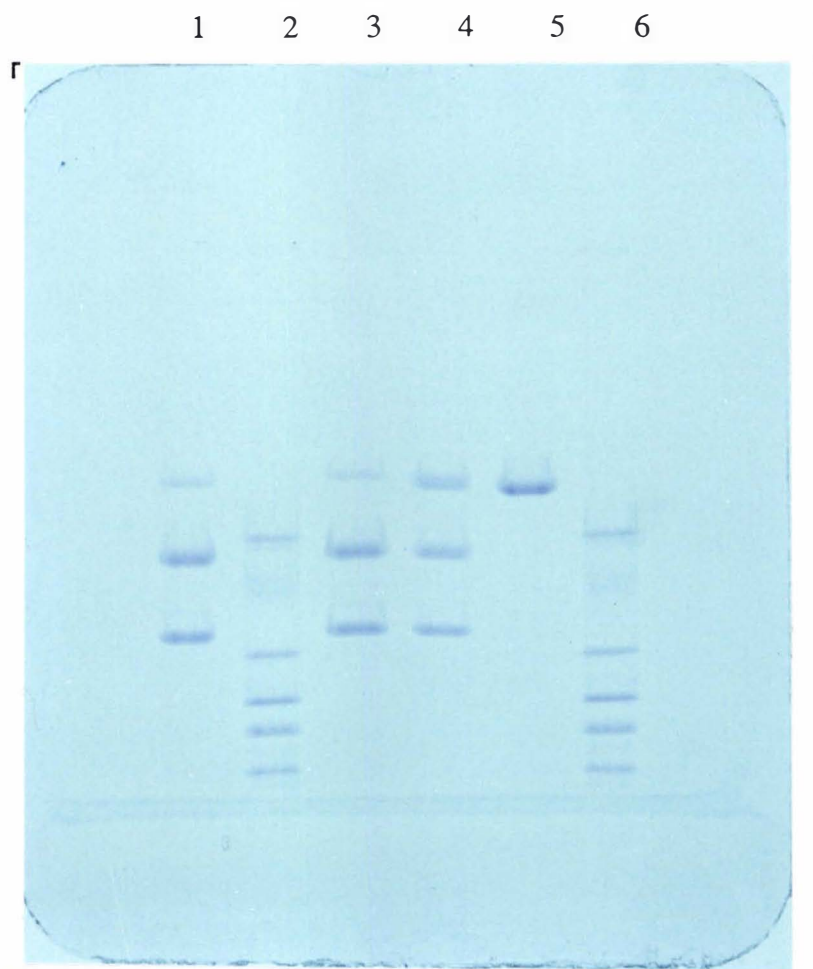
Sample	Background rate of BAPNA hydrolysis ($\Delta A^{405} \cdot \text{ml}^{-1} \cdot \text{min}^{-1}$)	Rate of BAPNA hydrolysis after addition of sample ($\Delta A^{405} \cdot \text{ml}^{-1} \cdot \text{min}^{-1}$)
Trypsin-Sepharose	0.00	0.79
Washings from trypsin-Sepharose	0.00	0.00

The data in table 2.4 indicates that trypsin had been immobilised on Sepharose and maintained its catalytic activity. The absence of trypsin in the washings from the resin indicates complete or near complete attachment of the ligand to the Sepharose support. Having ascertained that the enzyme immobilised on the resin was active, the trypsin-Sepharose was used to partially digest samples of iron saturated bovine lactoferrin.

2.4.3.2 Digestion of lactoferrin with immobilised trypsin and the isolation of the 50 kDa C-terminal peptide

Iron loaded bovine lactoferrin was partially digested with immobilised trypsin as outlined in 2.3.4.3.2. The progress of hydrolysis of lactoferrin was followed by gel electrophoresis. Figure 2.6 illustrates a typical electrophoretic pattern of bovine lactoferrin digested with immobilised trypsin. After 9.5 hours of gentle mixing at room temperature the 80 kDa lactoferrin band was almost completely converted into 50 kDa and 30 kDa polypeptides. The peptides were separated by preparative gel electrophoresis

on 10% (w/v) polyacrylamide-SDS gels. A fluorescein isothiocyanate labelled sample of the partially digested lactoferrin was loaded into a marker track on the side of the preparative gel. The position of the peptides after electrophoresis was determined by examining the marker track under ultraviolet light. Figure 2.7 A shows a marker track from a preparative gel exposed to ultraviolet light. The fluorescent labelled peptides were clearly visible when exposed to ultraviolet light on a transilluminator. Small sections of preparative gel corresponding to each of the three major components (80, 50, and 30 kDa bands) were cut from the gel. These sections were placed in the wells of an analytical 10% (w/v) polyacrylamide-SDS gel and subjected electrophoresis to determine the molecular weight of the peptides. Figure 2.7 B illustrates the typical electrophoretic pattern from the preparative gel sections. Having determined that the fluorescent markers indicated the presence of the three polypeptides in the gel, the 50 kDa peptide was isolated from the preparative gel by electroelution. The 50 kDa fragment of lactoferrin was eluted into a dialysis bag, dialysed against 0.01 M NH_4HCO_3 and lyophilised. The lyophilised material was resuspended in sterile dH_2O . Figure 2.8 illustrates a silver stained 10% (w/v) polyacrylamide-SDS PHAST gel of the 50 kDa polypeptide isolated from the preparative gel. The major component in the electroeluted sample is an intensely stained band which migrates to the same position as the 50 kDa C-terminal tryptic peptide of bovine lactoferrin.



Gel details: 10-15% (w/v) polyacrylamide-SDS PHAST gel stained with Coomassie Brilliant Blue.

Loading details:

- 1 and 3. Bovine lactoferrin digested with immobilised trypsin for 9.5 hours.
- 2 and 6. Molecular weight markers (see figure 2.4 for details).
- 4. Bovine lactoferrin digested with immobilised trypsin for 1.0 hour.
- 5. Bovine lactoferrin prior to digestion with trypsin.

Figure 2.6 PHAST gel electrophoresis of bovine lactoferrin before and after limited proteolysis with immobilised trypsin.

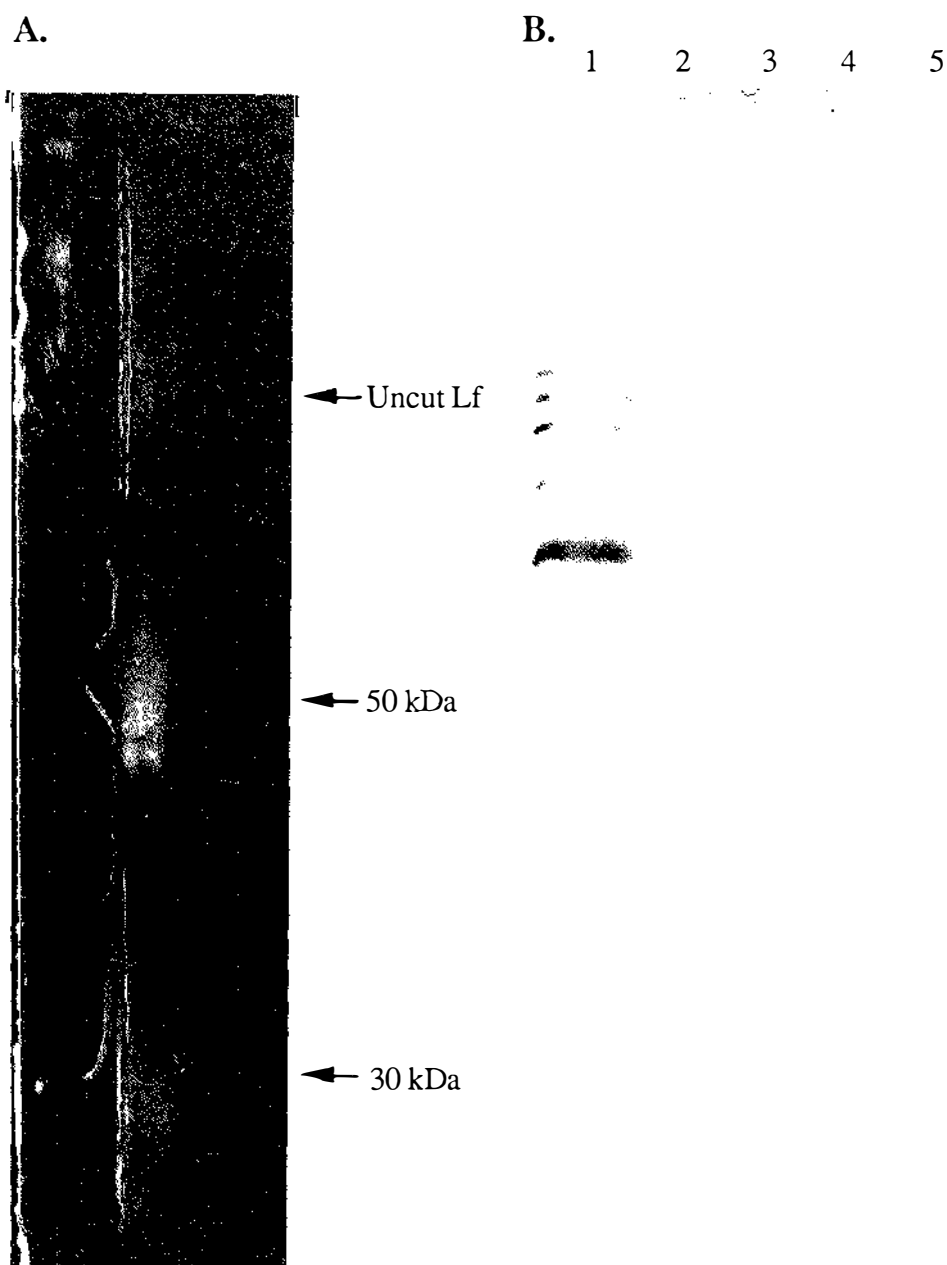


Figure 2.7 Analysis of partially digested lactoferrin by gel electrophoresis.

A. Gel details: 10% (w/v) polyacrylamide-SDS preparative gel.

Loading details: Bovine lactoferrin partially digested with immobilised trypsin and stained with FITC and photographed on a Transilluminator.

B. Gel details: 10% (w/v) polyacrylamide-SDS analytical gel stained with Coomassie Brilliant Blue.

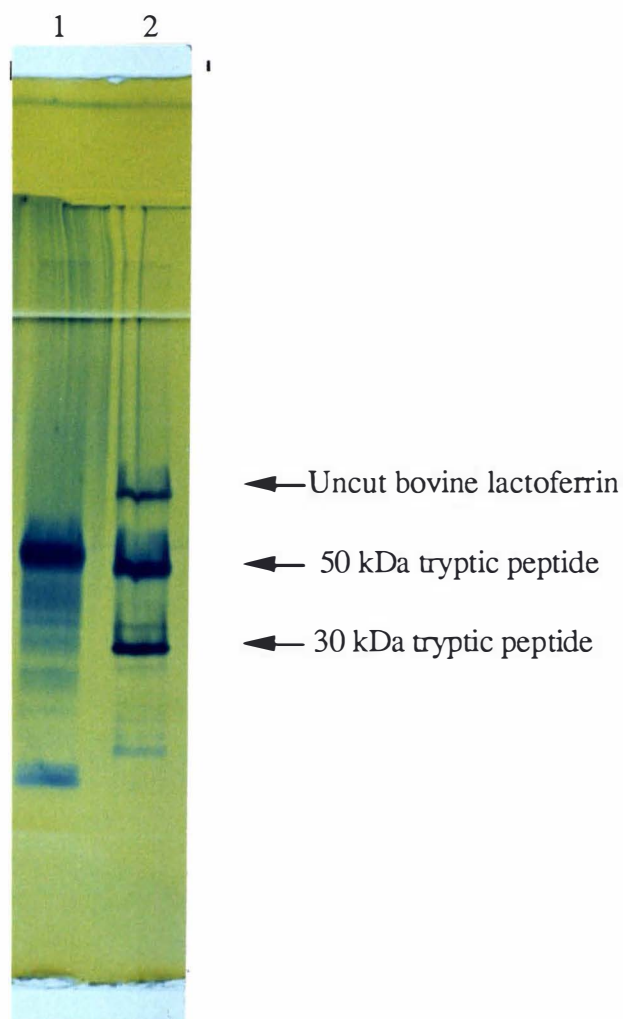
Loading details: 1. Molecular weight markers (see figure 2.4 for details).

2. 30 kDa lactoferrin tryptic peptide excised from the preparative gel.

3. 50 kDa lactoferrin tryptic peptide excised from the preparative gel.

4. Uncut bovine lactoferrin excised from the preparative gel.

5. Bovine lactoferrin partially digested with immobilised trypsin.



Gel details: 10-15% (w/v) polyacrylamide-SDS PHAST gel stained with silver nitrate.

Loading details:

1. Electroeluted 50 kDa bovine lactoferrin tryptic peptide isolated from a preparative polyacrylamide gel.
2. Bovine lactoferrin partially digested with immobilised trypsin.

Figure 2.8 Polyacrylamide-SDS gel electrophoresis of the C-terminal 50 kDa tryptic peptide of bovine lactoferrin isolated by preparative gel electrophoresis.

Approximately 100 µg of purified 50 kDa peptide in 50% formic acid was subjected to automated N-terminal sequencing as described in 2.3.4.3.3. The N-terminal sequence data from the 50 kDa peptide is presented in table 2.5.

Table 2.5 The N-terminal amino acid sequence of the 50 kDa tryptic peptides from bovine lactoferrin. The sequence of the corresponding region of human lactoferrin is listed above the bovine lactoferrin peptides. The boxes indicate the residues that are identical in all the members of the transferrin family. See appendix III for the alignment of the complete bovine lactoferrin amino acid sequence with other members of the transferrin family.

		290		300		310
Human Lf	. . GKDKSPK	FLFGSPSCQK	DLLEK	DSAIGFSRVPPRIDSG . .		
50 kD Bovine Lf Peptide A.		SFQ	FLFGSPFGQTD	LLLEK	DSALGFLRIPSKVD . .	
50 kD Bovine Lf Peptide B.		SRSE	FLFGSPFGQTD	LLLEK	DSALGFLRIPSKVD . .	

Two peptides were sequenced simultaneously. These two peptides were present in virtually equimolar amounts in the trypsin digested lactoferrin. The sequence of the two peptides was identical but staggered by two cycles. The most likely explanation for this observation is cleavage at one of two basic residues separated by a single amino acid (serine) residue. Either of these basic amino acids could have been recognised as the site of hydrolysis by trypsin. The sequence of the N-terminal of the 50 kDa peptide from bovine lactoferrin was compared to the entire human lactoferrin sequence (Metz-Boutigue *et al.*, 1984). The N-terminal of the 50 kDa bovine lactoferrin peptide aligned to position 282 in the human lactoferrin sequence. The bovine and human sequences share approximately 75% identity over the 33 amino acids sequenced. Examination of the tertiary structure of human lactoferrin (Prof. E.N. Baker, personal communication) showed that this sequence corresponds to an external loop on the N-terminal lobe of the protein. It is reasonable to assume, due to the high degree of sequence homology in transferrin family, that the gross tertiary structures of human and bovine lactoferrins will be similar. This has been supported by recent X-ray crystallographic studies on bovine lactoferrin which suggest that the tertiary structure of human and bovine lactoferrin are similar (Prof. E.N. Baker and Dr. B.F. Anderson, personal communication). Further, it is also reasonable to assume that the basic residues on this external loop form a hypersensitive site for trypsin degradation. Incubation of iron-loaded human

lactoferrin in the presence of trypsin also results in the hydrolysis of the protein to give two large (50 kDa and 30 kDa) peptides (Brock *et al.*, 1976; Brines and Brock, 1983).

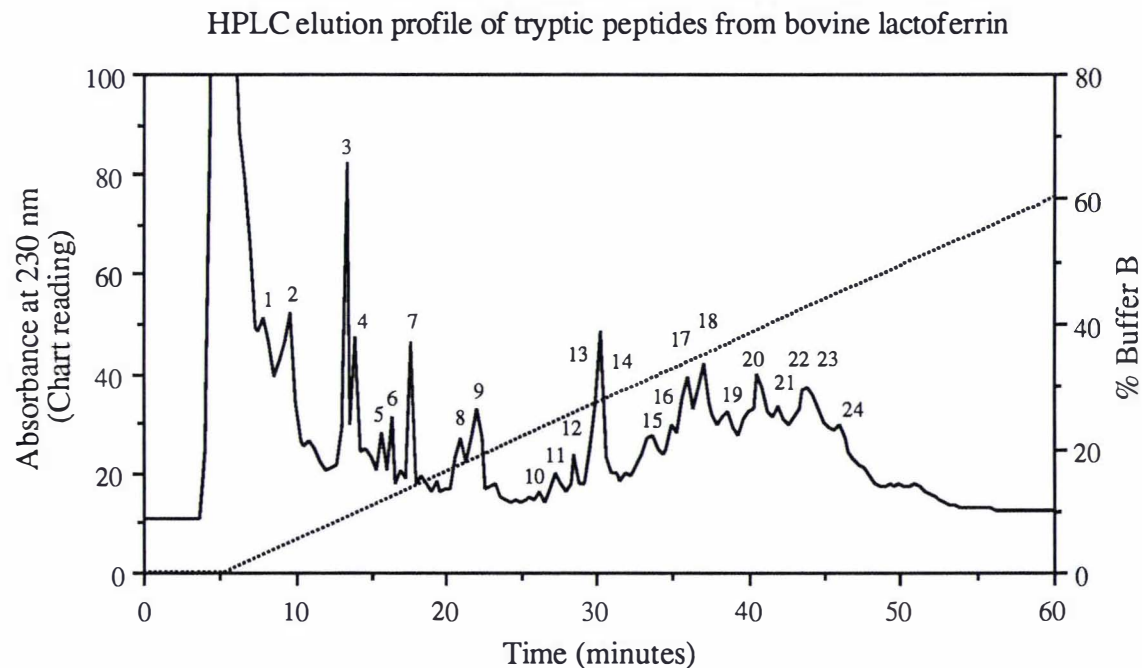
2.4.3.3 Tryptic mapping and sequencing of selected peptides from bovine lactoferrin

Bovine lactoferrin was carboxylmethylated, reacted with maleic anhydride and then digested with trypsin. The resultant acid-soluble peptides were separated by reverse phase HPLC as described in 2.3.4.3.4. Figure 2.9 shows the tryptic map generated by the separation of the peptides on a C-18 Vydac column. Several column fractions were sequenced by automated gas phase sequencing. Table 2.6 shows the amino acid sequence data obtained from the selected column fractions that were sequenced.

Table 2.6 The amino acid sequence of selected acid soluble tryptic peptides from carboxymethylated, maleonated bovine lactoferrin separated by reverse phase HPLC.

Fraction number	Sequence
3	KVNR
4	YTR
7	KVNR and EDFR
8	KSCHTGLGR
11	KPVTEAQSCHLAVGPNHAVVSR
12	KPVTEAQSCHLAVGPNHAVVSR
14	WQWR
15	APVDAFKECHLAQVPSHAVVAR
17	APVDAFKECHLAQVPSHAVVAR
18	YLTTLKNLR and AFALECLTR

Fractions 7 and 18 contained two discrete peptides which sequenced simultaneously. The predominant sequences from fractions 11 and 12 were identical as were the sequences from fractions 15 and 17. The presence of



Column: C-18 Vydac HPLC apparatus: Spectra Physics Absorbance range: 0-2.0 Chart Speed: 20 cm/hr

Gradient: 5 minutes at 100% buffer A. Buffer A= 0.1% TFA in H₂O

90 minutes at 100% buffer A to 100% buffer B. Buffer B= 0.1% TFA in H₂O/isopropanol/acetonitrile (1/1/1 (v/v/v)).

Sample: 250 μ l of acid (0.1% TFA in H₂O) soluble tryptic peptides from carboxymethylated bovine lactoferrin treated with maleic anhydride.
(Approximately 5 mg of peptides). Notes: Numbers indicate fractions collected manually. The dotted line represents the buffer gradient.

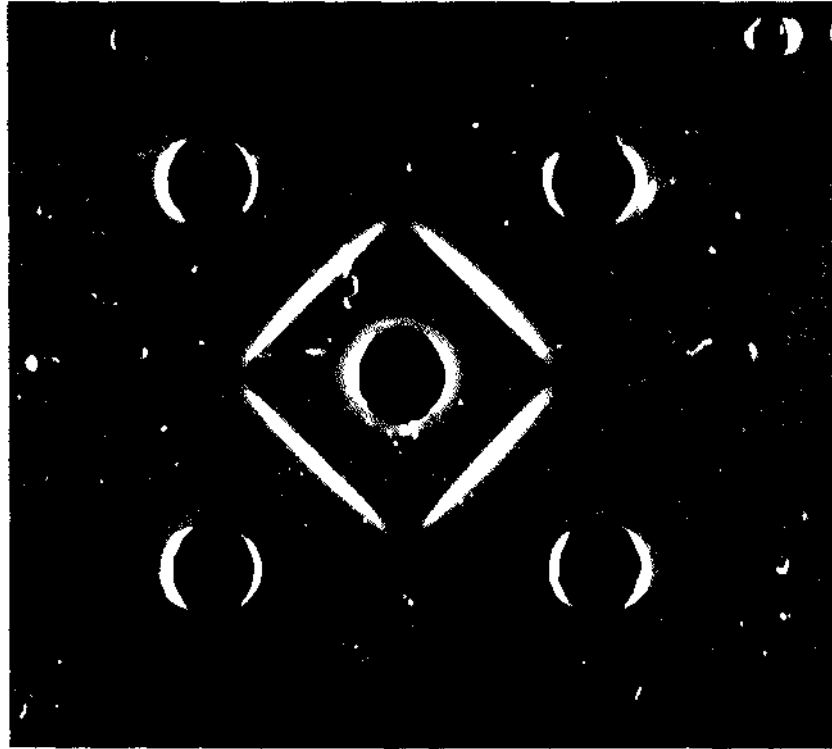
Figure 2.9 Tryptic map of bovine lactoferrin after incubation with iodoacetic acid and maleic anhydride.

the same peptides in two separate fractions was probably due to the peptides associating with minor peptide components in the sample. Most of the peptide sequences obtained could be aligned to the human lactoferrin amino acid sequence. Several of the shorter peptides, namely 3,4,7 and 14, could only be placed once the entire amino acid sequence for bovine lactoferrin had been deduced from the messenger RNA sequence (see Chapter 3).

2.4.4 Isolation and purification of anti-bovine lactoferrin antibodies

Antibodies to bovine lactoferrin were isolated from immune rabbit sera as described earlier (2.3.5.1 to 2.3.5.3). The presence of antibody to bovine lactoferrin in the serum of rabbits was determined by immunoprecipitation assay on agarose plates. Figure 2.10 shows a typical double immunodiffusion assay plate following incubation overnight at 4°C. The presence of the white precipitin lines in the agarose indicates the presence of antibody to lactoferrin in the serum samples. The total gamma-globulin fraction was further purified by affinity chromatography on lactoferrin-Sepharose as described in 2.3.5.4. Figure 2.11 illustrates the typical elution profile of gamma-globulin from the affinity resin. Column fractions were assayed for anti-bovine lactoferrin activity by the spot-precipitation method as outlined in 2.3.5.4.1. The only fractions which formed a precipitate upon the addition of lactoferrin were from the final 3.0 M KCNS in PB elution, as illustrated diagrammatically in figure 2.11.

The activity of various antibody fractions was measured by immunotitration as described in 2.3.5.4.2. The amount of lactoferrin immunoprecipitated by a known amount of antibody was quantitated using ^{125}I -labelled bovine lactoferrin. Figure 2.12 shows a typical immunotitration curve and clearly illustrates the 'breakpoint'. The 'breakpoint' is reached when the amount of lactoferrin added exceeds the capacity of the antibody to precipitate the protein. That is, as the amount of radiolabelled lactoferrin exceeds the 'breakpoint', the excess radiolabelled protein will remain in the supernatant and will not be precipitated. In the example given in figure 2.12, the 'breakpoint' is reached at approximately 4 μg of ^{125}I -labelled lactoferrin. Once this amount of lactoferrin has been added, radiolabelled protein is detected in the supernatant.



Gel details: 1% (w/v) agarose in phosphate buffered saline (PBS).

Loading details:

Upper wells. 7.5 μ l of serum from Rabbit #1 harvested 54 days after the initiation of immunisation with bovine lactoferrin.

Lower wells. 7.5 μ l of serum from Rabbit #2 harvested 54 days after the initiation of immunisation with bovine lactoferrin.

Centre well. 7.5 μ g of bovine lactoferrin in PBS.

Figure 2.10 Double immunodiffusion assay plate (Ouchterlony test) used to determine the presence of anti-lactoferrin antibody in rabbit serum.

Affinity purification of antilactoferrin antibody on bovine lactoferrin sepharose

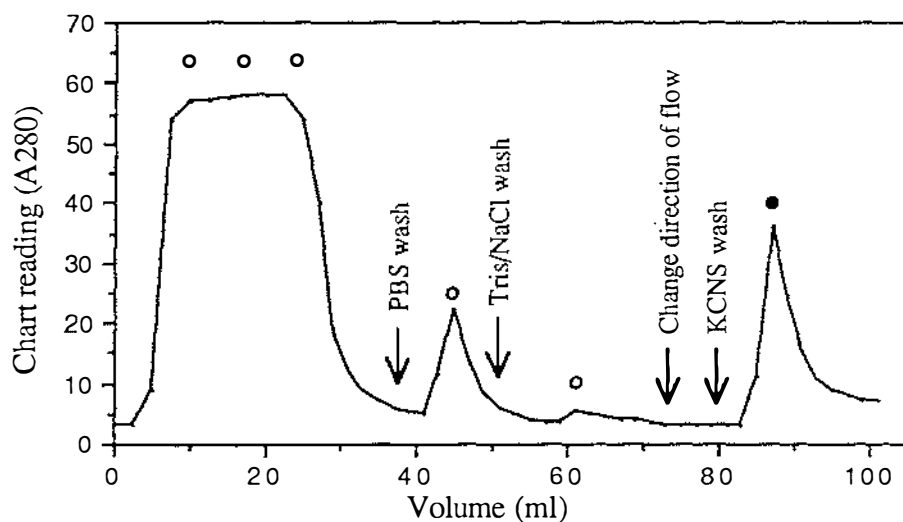


Figure 2.11 The elution profile of anti-bovine lactoferrin antibodies from bovine lactoferrin Sepharose.

Key: ○ = No immunoprecipitate formed when bovine lactoferrin was added to a sample of the column fraction directly below the symbol.

● = Immunoprecipitate formed when bovine lactoferrin was added to a sample of the column fraction directly below the symbol.

Immunotitration of affinity purified rabbit anti-bovine Lf gamma globulin

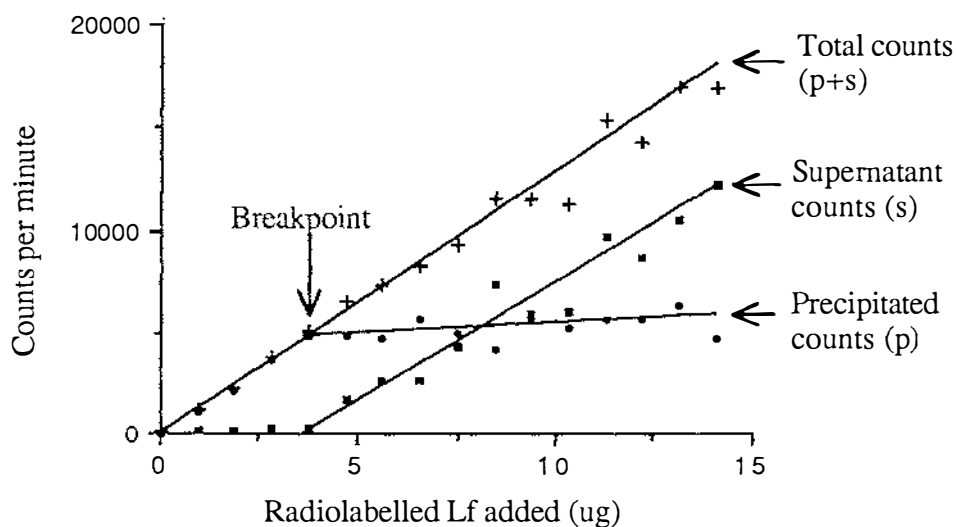


Figure 2.12 Immunotitration of affinity purified anti-bovine lactoferrin gamma-globulin. 12.3 μg of gamma-globulin was added to each tube. 0.94 μg increments of ^{125}I -labelled lactoferrin was added to consecutive tubes. The breakpoint occurs at approximately 3.6 μg of added ^{125}I -lactoferrin. That is, 1 mg of affinity purified gamma-globulin immunoprecipitates 292.7 μg of ^{125}I -lactoferrin.

Table 2.7 lists typical data for the purification and recovery of antibody activity following the isolation of rabbit anti-bovine lactoferrin gamma-globulin on lactoferrin-Sepharose. Anti-lactoferrin antibody activity was only detected in the fractions eluted with 3.0 M KCNS. The recovery of protein from the affinity column was routinely between 95-100%. The recovery of antibody activity, that is the total activity of the affinity purified fraction compared to the activity in the total gamma-globulin fraction, always exceeded 140%. This high recovery may be due to the under-estimation of the anti-lactoferrin antibody activity in the total gamma-globulin fraction. The presence of non-specific antibody in the total gamma-globulin fraction may interfere with the precipitation of ^{125}I -lactoferrin. If the anti-lactoferrin antibody activity in the total gamma-globulin fraction is underestimated, then the apparent recovery would be high.

Affinity purified, quantitated antibody was stored in small aliquots at -20°C . The monospecific antibody was used to immunoprecipitate radiolabelled lactoferrin following *in vitro* labelling of newly synthesized mammary proteins (2.3.7.1.6). The affinity purified antibody was also covalently attached to Sepharose (2.3.6.1). Immobilised antibody was also used for a single step purification method to isolate bovine lactoferrin from colostrum whey (2.3.6.3).

2.4.4.1 Affinity purification of bovine lactoferrin on anti-lactoferrin gamma-globulin Sepharose

Affinity purified anti-lactoferrin antibody was covalently attached to Sepharose as described earlier (2.3.6.1). Small trial columns were prepared to determine the nature of the interaction of lactoferrin with the affinity resin. Small quantities of pure lactoferrin (1 mg) were loaded on to and eluted from mini-columns packed with either aminocaproic acid Sepharose, methylamine Sepharose or anti-lactoferrin gamma-globulin Sepharose. Figure 2.13 illustrates the elution profiles of bovine lactoferrin from these columns.

Table 2.7 Purification of anti-bovine lactoferrin gamma-globulin by chromatography on lactoferrin Sepharose.

Rabbit	Amount of gamma-globulin loaded onto the column (mg)	Specific activity of loaded protein ($\mu\text{g Lf/mg gamma-globulin}$) ^a	Anti-Lf gamma-globulin recovered from the column (mg) ^b	Specific activity of recovered protein ($\mu\text{g Lf/mg gamma-globulin}$) ^a	% Recovery of antibody activity ^c	Fold purification ^d
1	228.6	12.76	18.45	292.7	185	22.9
2	175.3	16.7	17.26	280	165	16.8

Notes: **a.** Specific activity of samples is measured in the μg of lactoferrin immunoprecipitated by 1 mg of gamma-globulin. Calculated from immunotitration curves (see figure 2.12).

b. Anti-lactoferrin gamma-globulin recovered from the column by elution with 3.0 M KCNS in PB (see figure 2.11).

c. Percent recovery of anti-bovine lactoferrin antibody activity.

For example: Rabbit 1. Total activity loaded (T) = protein loaded (mg) x activity of loaded protein ($\mu\text{g Lf/mg gamma-globulin}$)
 $= 228.6 \times 12.76 = 2916.4$

Total activity recovered (R) = protein recovered by elution with 3.0 M KCNS in PB (mg) x activity of recovered protein ($\mu\text{g Lf/mg gamma-globulin}$).
 $= 18.45 \times 292.7 = 5400.3$

Percent Recovery = $R/T \times 100 = 5400.3/2916.4 \times 100 = 185 \%$

d. Fold purification = Specific activity of recovered antibody / specific activity of antibody applied to the column.

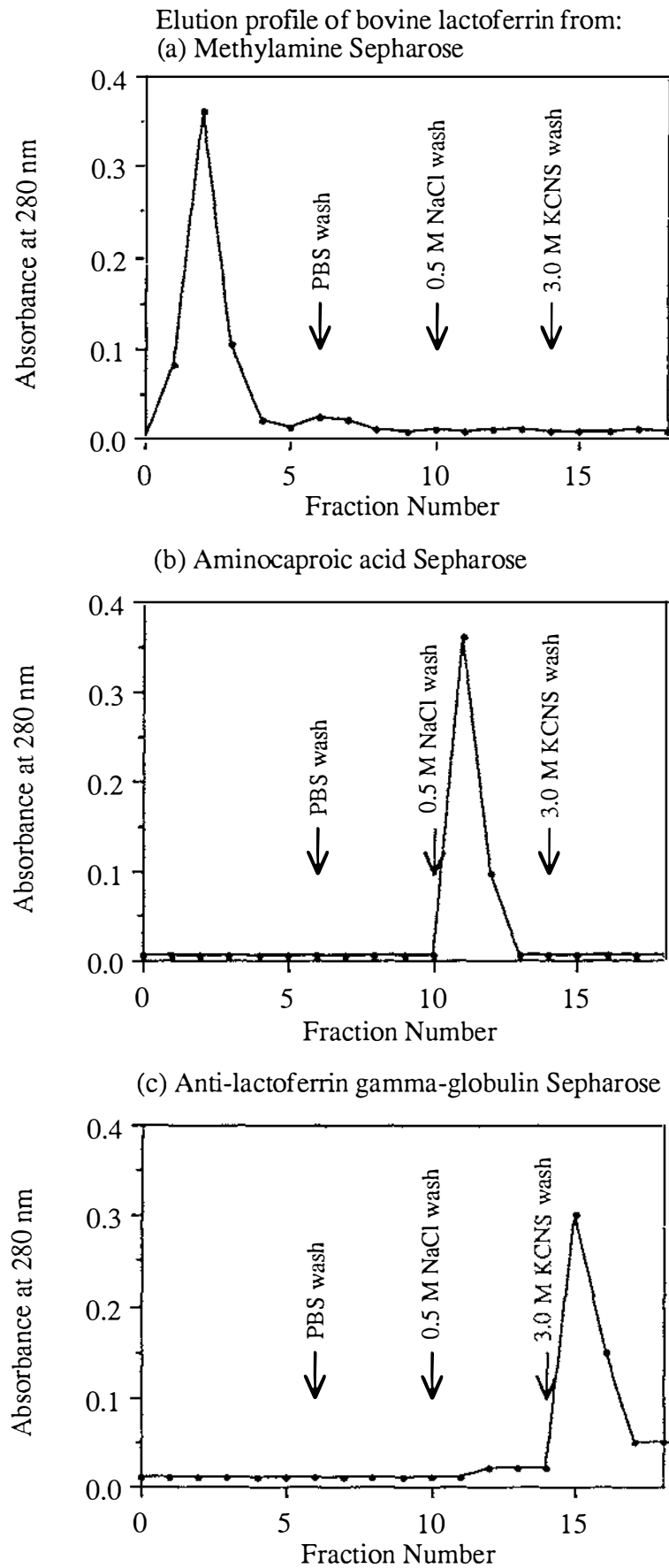


Figure 2.13 Elution profiles of bovine lactoferrin from trial columns. See text for details.

Lactoferrin did not interact with the methylamine Sepharose and was washed straight through the column. This indicates that lactoferrin does not interact with either the Sepharose beads or the carbon side chain of the aminocaproate spacer arms attached to them.

As expected the lactoferrin did bind to the unsubstituted amino-caproic acid Sepharose. The salmon pink protein was eluted from the resin by washing with 0.5 M NaCl in PB. The negatively charged carboxyl groups of the amino caproic acid spacer arms acts as cation exchangers and retain the positively charged protein on the resin. In this respect the interaction of lactoferrin with the amino caproic acid Sepharose was similar to that of lactoferrin with the CM-Sephadex used to purify large quantities of lactoferrin from bovine colostrum (2.3.4.1). Lactoferrin also bound to the anti-lactoferrin gamma-globulin Sepharose. Lactoferrin was loaded in PB and formed a bright red narrow band at the top of the column. The red band dispersed when the column was washed with 0.5 M NaCl in PB but the protein remained bound to the column. A likely explanation for this phenomenon is that the lactoferrin, loaded in a low salt buffer, is interacting with both the antibody and the unsubstituted carboxylate groups on the aminocaproic acid spacer arms. Washing with 0.5 M NaCl would disrupt the interactions between lactoferrin and the carboxylate groups but would not disrupt the interactions between the antibody and lactoferrin. The lactoferrin released from the ionic interactions with the unsubstituted spacer arms is then bound by antibody elsewhere on the column. Lactoferrin was eluted from the antibody column with 3.0 M KCNS in PB. The high concentration of this mild chaotropic agent disrupts the binding of the antibody to its antigen.

Lactoferrin eluted from the column was dialysed against 0.01 M NaHCO₃ to remove the KCNS. The conditions used to elute the lactoferrin from the resin (3.0 M KCNS in PB) caused the release of iron from the protein. Iron-saturated lactoferrin has a characteristic absorbance spectrum in the visible region. As indicated earlier, (2.4.1) absorbance data can be used to estimate the purity and degree of iron-saturation of lactoferrin. The dialysed lactoferrin samples were saturated with iron and the absorbance spectrum between 250 and 700 nm was determined. Figure 2.14 shows the absorbance spectrum of the lactoferrin retrieved from the antibody column compared to the absorbance spectrum of the sample loaded. The absorbance spectra for the two samples are markedly different in the visible region of the spectrum.

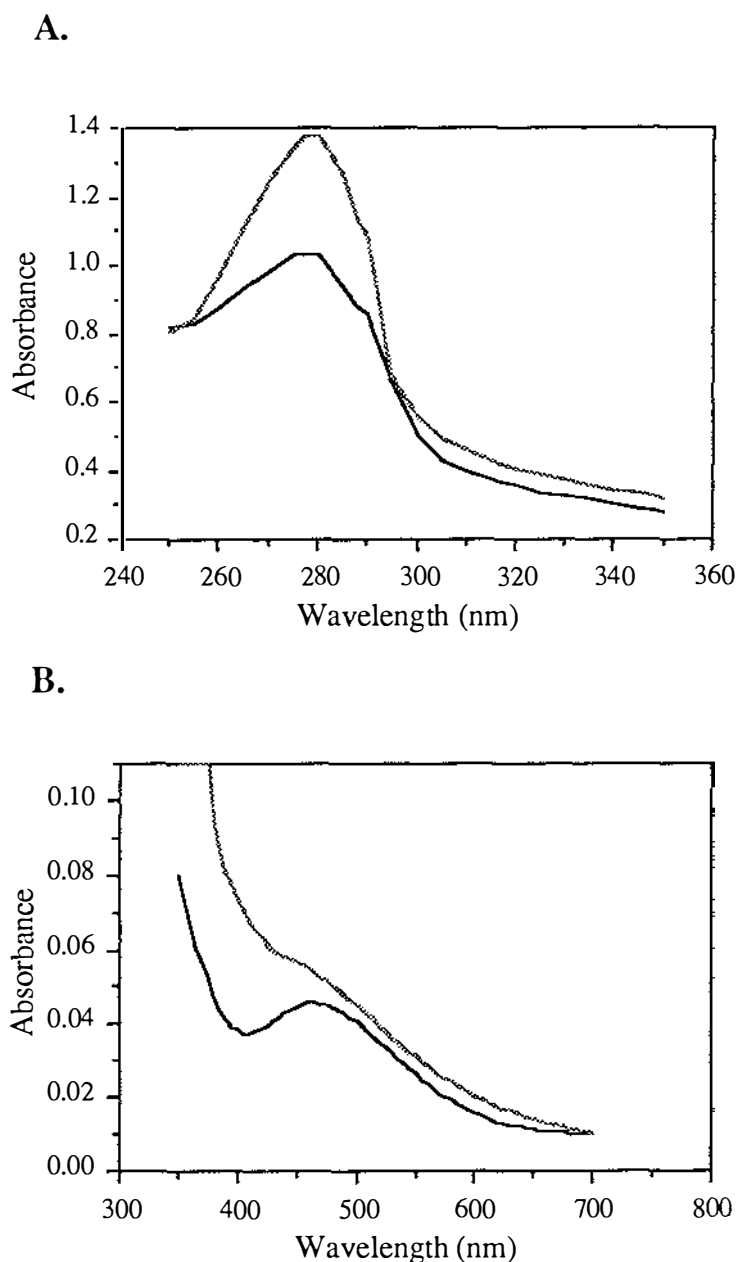


Figure 2.14 Absorbance spectra of pure bovine lactoferrin before and after chromatography on antibovine lactoferrin gamma-globulin Sepharose. A. Absorbance spectra between 250 nm and 350 nm. B. Absorbance spectra between 350 nm and 700 nm. The solid line indicates the absorbance spectra of pure lactoferrin prior to interaction with the column. The grey line indicates the absorbance spectra of lactoferrin eluted from the affinity resin.

The trough at 410 nm, characteristic of the iron binding site, was completely missing from the eluted sample. The A^{280}/A^{465} ratio, an indicator of the purity of lactoferrin, was similar for the loaded and eluted samples (22.7 and 25.6 respectively). The A^{410}/A^{465} ratio, an indicator of the integrity of the iron binding site, was markedly different for the two samples. The A^{410}/A^{465} ratio of the lactoferrin sample loaded onto the column was 0.78,

which is within the expected range for pure bovine lactoferrin (0.7 - 0.8). The A^{410}/A^{465} ratios of the eluted material was 1.33, which was well outside the expected range. The spectral data derived from figure 2.14 is summarised in table 2.8. The change in the absorbance spectrum of the eluted protein indicates perturbation of the iron binding sites of lactoferrin.

Table 2.8 Spectral data for bovine lactoferrin purified by affinity chromatography on anti-lactoferrin gamma-globulin Sepharose.

Sample	A^{280}/A^{465}	A^{410}/A^{465}
Lactoferrin loaded onto the affinity column	22.7	0.78
Lactoferrin eluted from the affinity column	25.6	1.33

2.4.4.2 Purification of lactoferrin from bovine colostrum by affinity chromatography on anti-bovine lactoferrin gamma-globulin Sepharose

Bovine lactoferrin was isolated from colostrum by affinity chromatography as outlined in 2.3.6.3. Bovine colostrum, dialysed against PB, was loaded onto a anti-bovine lactoferrin antibody column. Figure 2.15 shows a typical elution profile of protein from the antibody column. Fractions containing protein were dialysed and analysed by gel electrophoresis. Figure 2.16 shows a SDS-PAGE gel of fractions from the elution profile illustrated in figure 2.15. The gel electrophoresis profile of fractions from the antibody column clearly illustrates the purification of lactoferrin from bovine colostrum. The protein band in the 3.0 M KCNS peak (Fraction E) comigrates with the pure bovine lactoferrin marker. Although purification of lactoferrin from bovine colostrum has been achieved by this one step procedure, there remains the problem of the disruption of the iron-binding site. A less disruptive procedure for eluting the bound protein from the column is required. Broxmeyer *et al.* (1986) described the isolation of lactoferrin from human neutrophils by chromatography on immobilised monoclonal antibody.

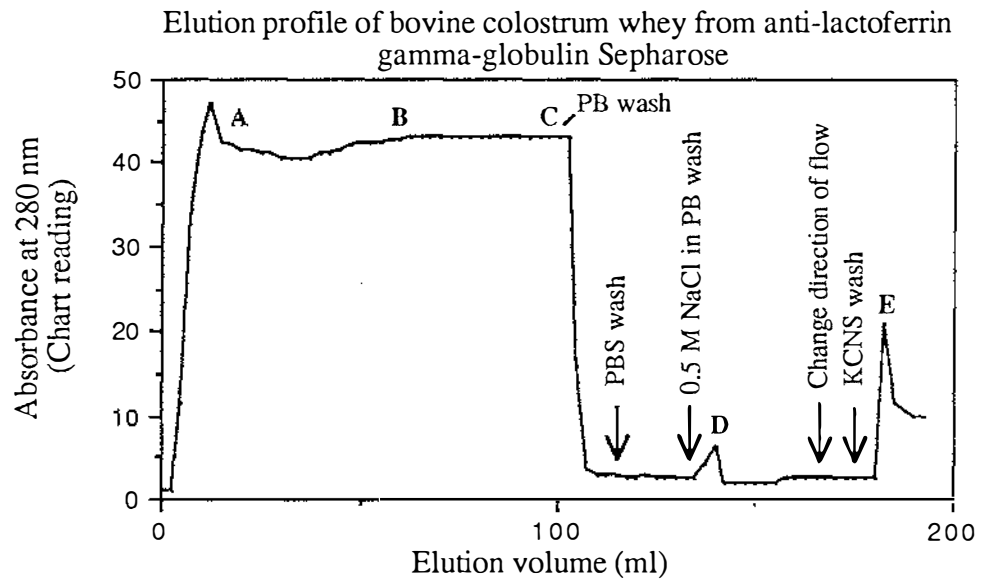
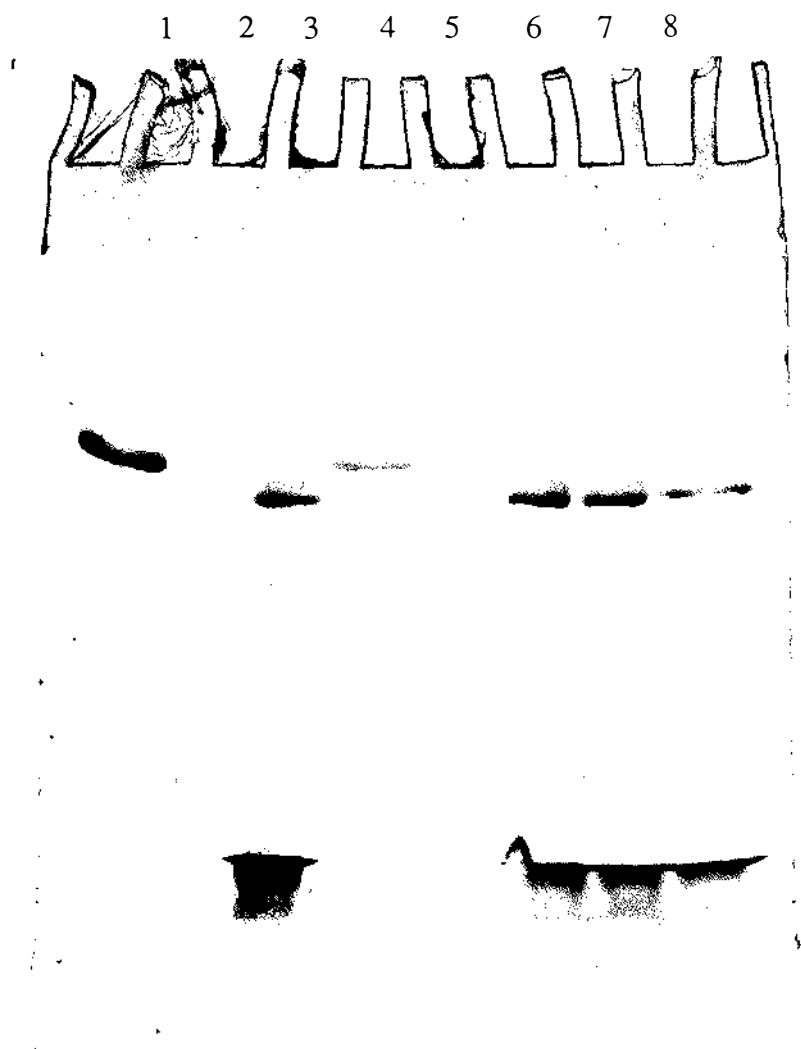


Figure 2.15 Elution profile of bovine colostrum whey from anti-bovine lactoferrin gamma-globulin Sepharose. The letters in bold indicate the fractions that were analysed by gel electrophoresis (see figure 2.15).

Lactoferrin was eluted from the monoclonal column with 0.1 M phosphate buffer pH 2.7. Kawakami *et al.* (1987) have described the isolation of both human and bovine lactoferrin from raw skin milks by affinity chromatography using immobilised monoclonal antibodies. Lactoferrin was eluted with a solution of 0.2 M acetate and 0.15 M NaCl at pH 2.7.



Gel details: 10% polyacrylamide-SDS gel stained with Coomassie Brilliant Blue.

Loading details: 1. Bovine lactoferrin.

2. Molecular weight markers (β -galactosidase (116 kDa), phosphorylase B (92.5 kDa), bovine serum albumin (68 kDa), hen egg albumin (45 kDa), carbonic anhydrase (29 kDa).

3. Bovine colostrum loaded onto the affinity column.

4. Fraction E (see figure 2.14), 3.0 M KCNS in PB wash.

5. Fraction D, 0.5 M NaCl in PB wash.

6. Fraction C, PB wash.

7. Fraction B, flow through.

8. Fraction A, flow through.

Figure 2.16 SDS-polyacrylamide gel electrophoresis of fractions from the chromatography of bovine colostrum whey on anti-bovine lactoferrin gamma-globulin Sepharose.

2.4.5 Animal studies

2.4.5.1 Preliminary Study, Cow #87

Mammary tissue biopsies were dissected from an involuting cow at regular intervals following the termination of regular milking. Fresh biopsy tissue was labelled *in vitro* with a radiolabelled amino acid as described earlier (2.3.7.1.4). The *de novo* synthesis and secretions of protein from mammary tissue fragments was followed by measuring the amount of radiolabelled trichloroacetic acid insoluble products in the incubation media. Figure 2.17 illustrates the incorporation of ^{35}S -methionine into TCA precipitable products by mammary tissue fragments and shows the incorporation of radiolabel was linear over the incubation period.

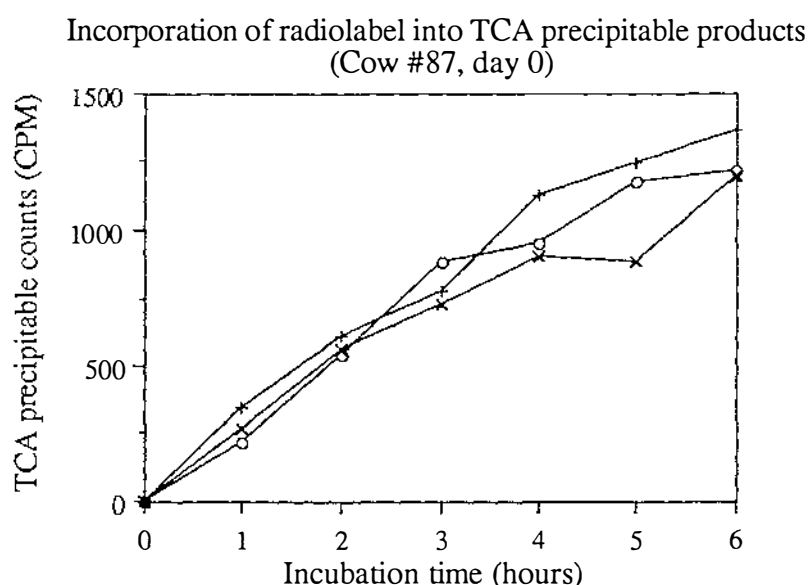


Figure 2.17 Incorporation of ^{35}S -methionine into trichloroacetic acid precipitable products by mammary tissue fragments during a six hour incubation period. Incubations were performed in triplicate.

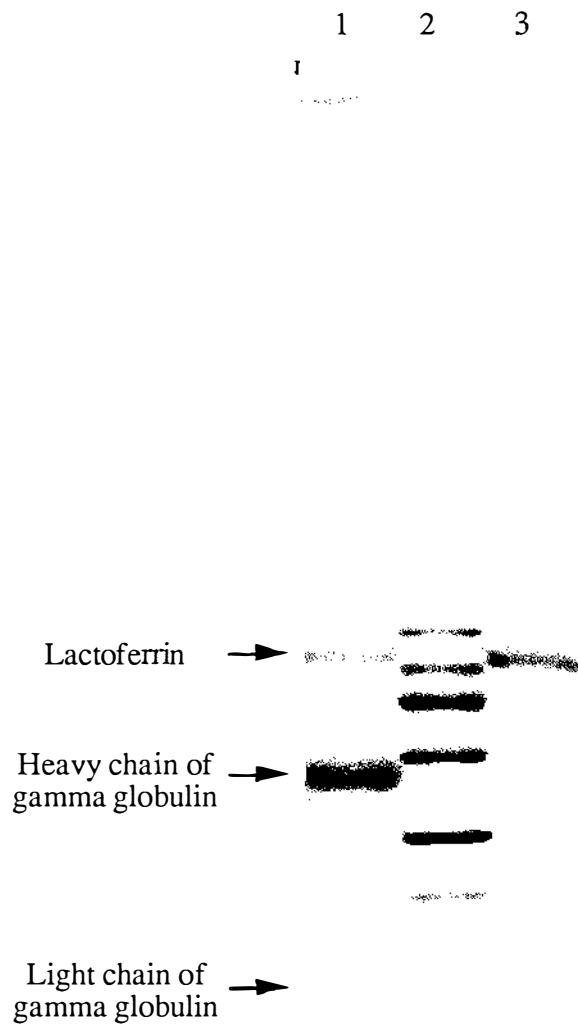
Lactoferrin was immunoprecipitated from the incubation media with affinity purified rabbit anti-bovine lactoferrin gamma-globulin (2.3.7.1.6). Immunoprecipitates were separated by electrophoresis on 10% (w/v) polyacrylamide-SDS gels. Following visualisation of the protein bands by staining with Coomassie brilliant blue, the gels were photographed and also scanned for absorbance at 580 nm. Figure 2.18 shows a typical polyacrylamide-SDS gel of an immunoprecipitate formed from the incubation

media surrounding mammary tissue fragments. The protein precipitated by the antibody had identical electrophoretic mobility to that of pure bovine lactoferrin.

The proportion of lactoferrin synthesis was determined by slicing the immunoprecipitate track into 2 mm sections, solubilising each section, and determining the amount of radioactive label by liquid scintillation counting. Figure 2.19 illustrates the absorbance profile of a typical lactoferrin immunoprecipitate after electrophoresis on a SDS-polyacrylamide gel. Superimposed on this plot is the radioactivity (CPM) associated with each 2 mm section from this track. Figure 2.19 clearly illustrates that the only radioactivity in the lactoferrin immunoprecipitate comigrates with the absorbance peak of the Coomassie Brilliant Blue stained lactoferrin band.

Lactoferrin synthesis relative to total secreted protein synthesis was estimated from the ratio of the counts associated with the immunoprecipitated lactoferrin in the gel to those in the total TCA precipitate. The relative amount of lactoferrin synthesis was estimated in each of the tissue biopsies taken during the course of involution. Figure 2.20 depicts the change in the relative rate of lactoferrin synthesis following the termination of regular milking. Lactoferrin synthesis increased in the first two weeks of involution from 0.35% to 0.8% of the total secreted protein synthesized by the mammary tissue fragments. During the subsequent ten day period the relative rate of lactoferrin synthesis decreased to approximately 0.4% of the total secreted protein synthesis.

The amount of lactoferrin present in mammary tissue samples was determined by electroimmunodiffusion assay of tissue homogenates. Frozen mammary tissue was crushed in a steel mortar and homogenised in TE (pH 7.6). The homogenates were centrifuged to remove fat and cellular debris and were assayed to determine the concentration of lactoferrin and total protein. Figure 2.21 A illustrates a typical electroimmunodiffusion (rocket electrophoresis) plate following electrophoresis and visualisation of protein immunoprecipitate peaks after staining with Coomassie Brilliant Blue. Homogeneous bovine lactoferrin was used to produce a standard curve. Figure 2.21 B shows a typical standard curve for bovine lactoferrin by electroimmunodiffusion assay. The area of the rocket shaped peaks, determined by triangulation, was directly proportional to the amount of lactoferrin loaded. Over the range of 0 to 0.5 μg lactoferrin the standard curve was linear.



Gel details: 10% (w/v) polyacrylamide-SDS gel stained with Coomassie Brilliant Blue.

Loading details: 1. Radiolabelled bovine lactoferrin immunoprecipitated from six hour incubation supernatant with affinity purified anti-lactoferrin gamma-globulin.

2. Molecular weight markers. (Protein standards are phosphorylase B (92.5 kDa), transferrin (80 kDa), bovine serum albumin (68 kDa), catalase (57.5 kDa), citrate synthetase (50 kDa), and malate dehydrogenase (37 kDa)).

3. Bovine lactoferrin purified by CM-Sephadex chromatography.

Figure 2.18 SDS-polyacrylamide gel electrophoresis of radiolabelled lactoferrin immunoprecipitated with affinity purified anti-bovine lactoferrin gamma-globulin.

Overlay plot of absorbance at 580 nm and counts per gel slice

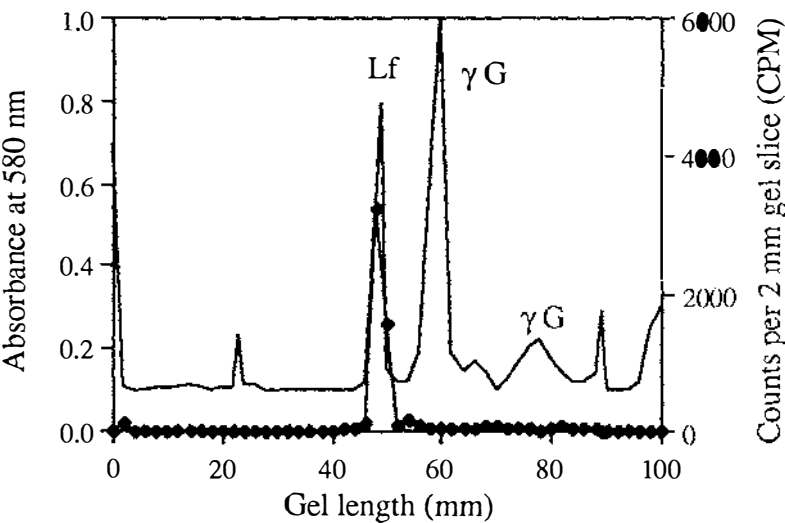


Figure 2.19 Superimposed profiles of absorbance maxima and radioactivity present in a gel after electrophoresis of an immunoprecipitate of radiolabelled bovine lactoferrin. The absorbance profile was determined by a light densitometer after staining the gel with Coomassie Brilliant Blue. The radioactivity (indicated in the diagram by diamond-shaped points) was determined by liquid scintillation of gel slices.

Lactoferrin synthesis in mammary tissue during involution

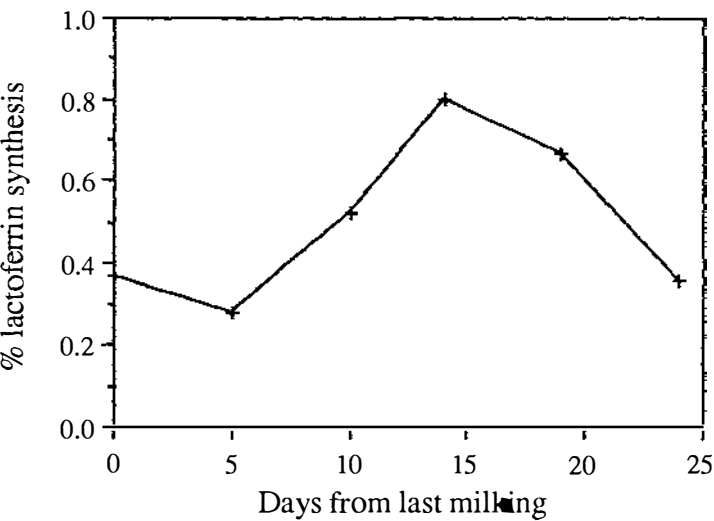


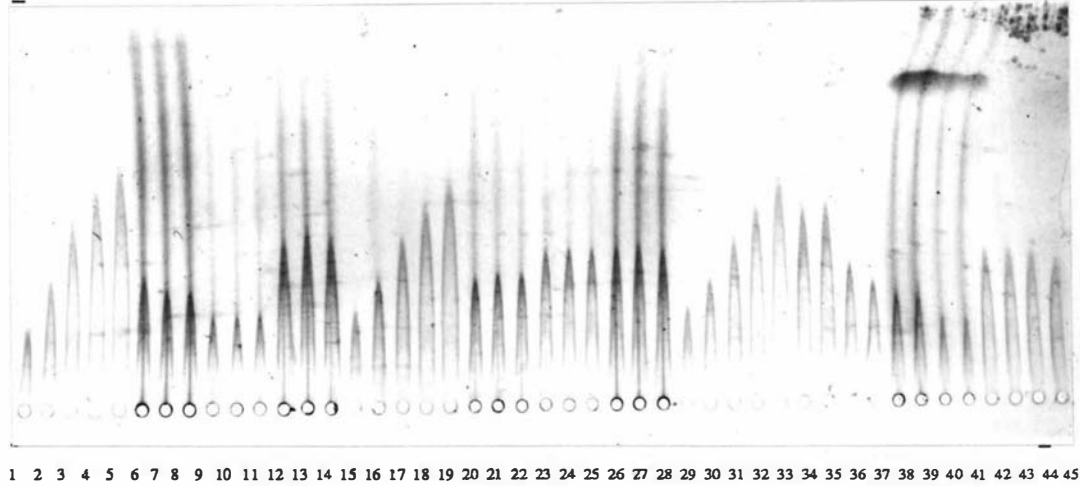
Figure 2.20 Lactoferrin synthesis* in mammary gland biopsies taken after the termination of regular milking. (* Relative to total secreted protein synthesis. See text for details.)

Loading details for electroimmunoassay plate (figure 2.21 A):

Well	Sample
1, 15, 29	1 µl of pure bovine lactoferrin (0.1 µg)
2, 16, 30	2 µl of pure bovine lactoferrin (0.2 µg)
3, 17, 31	3 µl of pure bovine lactoferrin (0.3 µg)
4, 18, 32	4 µl of pure bovine lactoferrin (0.4 µg)
5, 19, 33	5 µl of pure bovine lactoferrin (0.5 µg)
6, 7, 8	5 µl of homogenate prepared from cow #87, day 0 *
9, 10, 11	5 µl of homogenate prepared from cow #87, day 5 (1/5 diluted)
12, 13, 14	5 µl of homogenate prepared from cow #87, day 10 (1/5 diluted)
20, 21, 22	5 µl of homogenate prepared from cow #87, day 14 (1/5 diluted)
23, 24, 25	5 µl of homogenate prepared from cow #87, day 19 (1/10 diluted)
26, 27, 28	5 µl of homogenate prepared from cow #87, day 24 (1/2 diluted)
34, 35	5 µl of of sample loaded onto a CM-Sephadex column (1/50 diluted)
36, 37	2.5 µl of of sample loaded onto a CM-Sephadex column (1/50 diluted)
38, 39	5 µl of of washings from a CM-Sephadex column (1/10 diluted)
40, 41	2.5 µl of of washings from a CM-Sephadex column (1/10 diluted)
42, 43, 44, 45	2.5 µl of pure bovine lactoferrin (0.25 µg)

* Homogenates were prepared from mammary tissue isolated from cow #87 on the day indicated in the table (see text for details (2.3.7.1.7)). Samples were diluted with 10 mM Tris·Cl pH 7.4, 1 mM EDTA as required.

A.



Electroimmunodiffusion assay plate. 1% (w/v) agarose in 1x Rocket buffer (see text for details) containing 0.001% (w/v) anti-bovine lactoferrin gamma-globulin. Electrophoresis was at 2.5V/cm at 15°C for 18.5 hours. Protein peaks were visualised by staining with Coomassie Brilliant Blue. Loading details: See facing page.

B.

Standard curve of bovine lactoferrin measured by Rocket electrophoresis

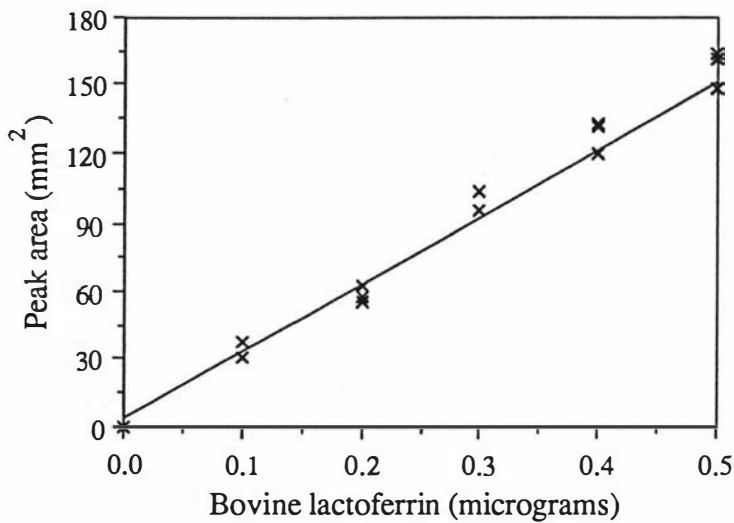


Figure 2.21 Estimation of lactoferrin concentration by electroimmuno-diffusion assay (Rocket electrophoresis).

Standard curves were used to estimate the lactoferrin concentration of the tissue homogenates. The total protein concentration of each tissue homogenate was measured by the Biuret method and used to normalise the lactoferrin concentration data. The lactoferrin concentration of the tissue homogenates was expressed as the μg lactoferrin per mg of total protein. Figure 2.22 illustrates the change in lactoferrin concentration in mammary tissue following the termination of regular milking. The concentration of lactoferrin in the tissue homogenates increased from 5 to 70 μg of lactoferrin per mg of total protein over the first 19 days of involution. The concentration of lactoferrin in homogenates prepared from mammary tissue isolated on day 24 was considerably less than from tissue isolated on day 19. Over this five day period the concentration of lactoferrin appeared to decrease from 70 to 10 μg lactoferrin per mg total protein.

The concentration of lactoferrin in mammary tissue biopsies

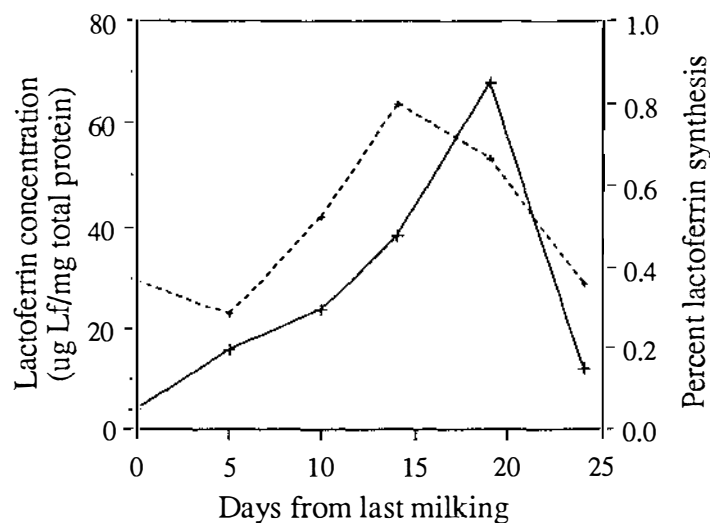


Figure 2.22 The concentration of lactoferrin in homogenates prepared from the mammary tissue biopsies removed from cow #87 after the termination of regular milking. The dotted line represents the percent lactoferrin synthesis of total secreted protein synthesis (see figure 2.19).

Figure 2.22 also illustrates the change in lactoferrin synthesis as a percentage of total secreted protein synthesis (see figure 2.19). The percent lactoferrin synthesis profile was included to illustrate the similar trend in the two profiles. The decrease in the concentration of lactoferrin in the tissue homogenates follows the decrease in the relative synthesis of lactoferrin in the tissue samples.

2.4.5.2 Second Animal Study, Cow #198

In an independent study, six non-pregnant dairy cows were selected from the Massey University Dairy Cattle Research Unit herd. Lacteal secretions were collected from these animals at regular intervals following the termination of regular milking. During the course of the sampling regime three of the cows developed mastitis and were eliminated from the study. By day 14 of the sampling regime only one cow, cow #198, remained in the study. The concentration of lactoferrin in the whey samples collected from this animal was measured by electroimmunodiffusion assay. Figure 2.23 illustrates the change in lactoferrin concentration in the lacteal samples from cow #198. On the final day of regular milking the concentration of lactoferrin in the whey sample was approximately 2 mg/ml. This lactoferrin concentration is higher than would be expected in normal milk. The normal range of lactoferrin concentration in bovine milk is 0.1 to 0.35 mg/ml (Smith and Schanbacher, 1977). The ten fold increase above normal milk concentration may reflect the changing functional status of the gland. That is, the involution process may have been initiated prior to termination of regular milking.

The concentration of lactoferrin in the secretions from the involuting gland increased markedly during the course of the sampling regime. Twenty four days after the last regular milking, the concentration of lactoferrin in the secretion from the mammary gland was 20 mg/ml. This result supports data presented by other workers. Smith and Schanbacher (1977) have reported that after twenty to thirty days of involution the concentration of lactoferrin in the dry secretion was routinely 20 to 30 mg/ml.

On day 25 of the sampling regime cow #198 was slaughtered and the entire mammary gland was removed from the carcass. Mammary tissue was dissected from the gland and frozen in liquid nitrogen for later analysis. A small sample of tissue was dissected and stored in ice cold buffered saline. This sample was used to estimate lactoferrin synthesis by the incorporation of a radiolabelled amino acid into protein.

Lactoferrin concentration in the lacteal secretion of cow #198 during involution

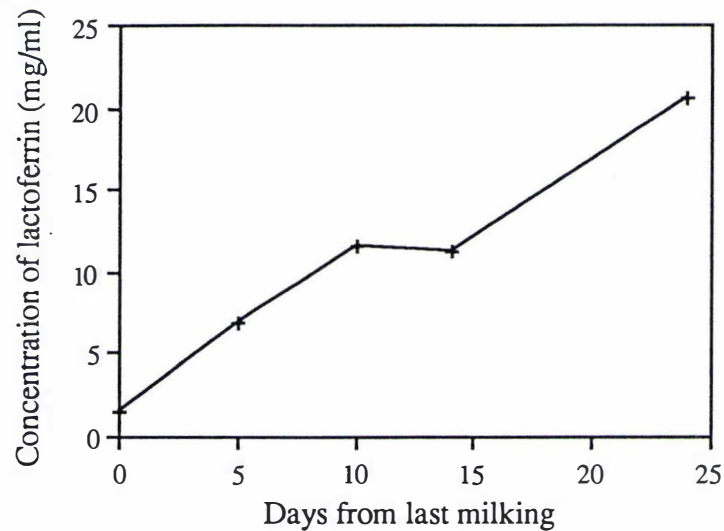


Figure 2.23 The concentration of lactoferrin in the lacteal secretion from cow #198 following the termination of regular milking.

The incorporation of radiolabel by mammary tissue fragments into TCA precipitable products was linear over the six hour incubation period (data not shown). Lactoferrin synthesis was estimated by immunoprecipitation with monospecific polyclonal anti-lactoferrin antibody. Immunoprecipitated lactoferrin was separated by electrophoresis on 10% (w/v) polyacrylamide-SDS gels. The radioactivity associated with the lactoferrin band was estimated by slicing the gel into 2 mm sections, solubilising the gel and assaying for β -emissions by liquid scintillation. Lactoferrin synthesis in mammary tissue from cow #198 twenty five days after the termination of regular milking was 3.25% of the total secreted TCA precipitable radiolabelled products.

Lactoferrin synthesis in this tissue was also estimated by direct counting of the immunoprecipitated products. Lactoferrin precipitated by monospecific antibody was centrifuged through sucrose gradients and washed with non-ionic detergents in PBS to remove non-specific trapped protein (section 2.3.7.2.5). The immunoprecipitates were solubilised in 0.1 M NaOH and transferred to scintillation vials. The radioactivity present in the immunoprecipitate was determined by liquid scintillation. Table 2.9 shows the data from duplicate immunoprecipitation experiments. The proportion of newly synthesized lactoferrin was 5.7% of the total TCA precipitable material secreted.

Table 2.9 Lactoferrin synthesis in bovine mammary tissue estimated by direct counting of immunoprecipitated radiolabelled protein.

Incubation	Sample	Immuno-precipitable counts (CPM)	Total secreted counts (CPM)	% Lactoferrin of total counts
1	A	2192.85	42886	5.1
	B	3110.26	42886	7.3
2	A	3147.41	66641	4.7
	B	3694.19	66641	5.5
Average percent lactoferrin synthesis				5.7

The difference in the percent lactoferrin synthesis between the two methods (3.25% versus 5.7%, gel slicing and direct counting methods respectively) may be due to the different counting efficiencies of the two methods. The gel slicing and the direct counting of the immunoprecipitates may have quite different counting efficiencies due to the different composition of the liquid scintillation cocktails. Also, the direct counting of the immunoprecipitate may over-estimate the percent lactoferrin synthesis due to non-specific trapping of radiolabelled products. These differences aside, there remains a large difference between the percent lactoferrin synthesis observed in the two animal studies. Twenty-four days after the termination of regular milking the relative lactoferrin synthesis, estimated by the gel slicing method, was 0.4% and 3.25% for cows #87 and #198 respectively. On day 14, the highest point of lactoferrin synthesis in the mammary tissue from cow #87, the percent lactoferrin synthesis of total secreted protein synthesis was only 0.8%. The difference in lactoferrin synthesis may be due to the intrusive nature of the first animal study. The removal of large (100 g) biopsies at regular intervals, the localised infection that ensued following the first two biopsies, and the treatment with antibiotics may have interfered with the normal course of involution.

The concentration of lactoferrin in the mammary tissue from cow #198 was measured by rocket electrophoresis of tissue homogenates, and was found to be 65 µg lactoferrin per milligram total protein. This concentration of lactoferrin is very similar to that from mammary tissue from cow #87 nineteen days after the termination of milking. Table 2.10 shows lactoferrin

and total protein concentration of mammary homogenates from cows #87 and #198.

Table 2.10 Comparison of the lactoferrin and total protein concentrations of mammary homogenates from cows #87 and #198.

	Cow #87	Cow #198
Days from last milking	19	24
Weight of tissue homogenised (g)	2.29	1.81
Protein concentration of the homogenate (mg/ml)	6.5	6.09
Lactoferrin concentration ($\mu\text{g/ml}$)	455	385
Lactoferrin concentration ($\mu\text{g Lf/mg total protein}$)	70	65
Lactoferrin concentration ($\mu\text{g Lf/g mammary tissue}$)	2.98	3.19

The data presented in table 2.9 clearly shows that the amount of lactoferrin present in the two mammary biopsies is fairly similar despite the relative lactoferrin synthesis in the two tissue samples being quite different, (approximately 3.25% and 0.7% lactoferrin of total secreted protein synthesis for cow #198 (day 24) and cow #87 (day 19) respectively). Although more data is required before the relevance of this finding can be explained, it is interesting to speculate that the source of the lactoferrin is different in the two tissues. The localised infection that followed the removal of mammary biopsies from cow #87 is likely to have increased the number of neutrophils in the tissue. The degranulation of these cells would release lactoferrin into the surrounding extracellular fluid.

Regardless of the differences in relative lactoferrin synthesis and the apparent anomaly in the amount of lactoferrin present in the two mammary glands, lactoferrin synthesis has been demonstrated in involuting bovine mammary tissue.

Although more experimental data is required to determine the nature of the increase in lactoferrin in the 'dry' secretion, lactoferrin synthesis in the involuting mammary tissue has been demonstrated and is likely to be a contributing factor. The *de novo* synthesis of bovine lactoferrin clearly

illustrates the presence of messenger RNA coding for lactoferrin in the mammary tissue biopsies taken from the two cows.

2.5 Summary

This chapter has outlined the isolation and partial characterisation of bovine lactoferrin isolated from normal colostrum. Amino acid sequence of tryptic peptides and the mature protein was found to be homologous to other members of the transferrin family. Antibodies specific for bovine lactoferrin, have been used as the basis of an assay system for the protein. The same antibodies were employed to identify the synthesis of lactoferrin in involuting bovine mammary biopsies.

The following chapter details the isolation of messenger RNA from frozen mammary tissue samples and the subsequent creation of complementary DNA (cDNA) libraries. DNA sequences coding for bovine lactoferrin were isolated from these libraries and characterised.

Chapter Three

Molecular cloning of the cDNA coding for bovine lactoferrin

3.1 Introduction

The development of recombinant DNA technology has lead to an information explosion in the biological sciences. The isolation of enzymes that can specifically modify DNA has enabled precise *in vitro* manipulation of DNA sequences. Perhaps one of the most significant advances came with the discovery of reverse transcriptases which are capable of copying RNA sequences into DNA sequences. Used in conjunction with other DNA modifying enzymes, the RNA-directed DNA polymerases have enabled biochemists to determine the nucleotide sequence of RNA species that code for proteins. Recombinant DNA technology applied in this way has greatly increased the speed at which the amino acid sequence of proteins can be determined.

Recombinant DNA technology was employed in this study to determine the nucleotide sequence of the messenger RNA coding for bovine lactoferrin. The amino acid sequence of bovine lactoferrin was predicted from the nucleotide sequence of the cDNA.

3.2 Methods and materials

3.2.1 Materials

All general reagents were of analytical grade or higher and supplied by the same manufacturers listed in 2.2. Molecular biology reagents and reagent kits were from Bestheda Research Laboratories (BRL), New England Biolabs (NEB), Amersham, or Bohringer Manheim. The suppliers of particular reagents are noted in the description of the methods as they were required.

3.2.2 General methods for the isolation and manipulation of RNA

Due to the extreme sensitivity of RNA to ribonuclease (RNase) digestion, special precautions were taken to avoid the introduction of this enzyme into RNA preparations. All glassware was baked for at least two hours at 250°C. All plasticware was autoclaved. All solutions were treated with diethylpyrocarbonate (DEPC) to a final concentration of 0.1% (v/v) and incubated for thirty minutes at room temperature prior to autoclaving. Plastic centrifuge tubes were immersed in a 0.1% (v/v) solution of DEPC in sdH₂O for thirty minutes and then autoclaved. Gloves were worn during all manipulations of RNA to prevent the introduction of ribonuclease from the fingers of the investigator.

3.2.2.1 Isolation of total cellular RNA

Total cellular RNA was isolated from frozen bovine mammary tissue as described by Chirgwin *et al.* (1979) and modified by Dr. B. Mansfield (personal communication).

Approximately 1 g of frozen bovine mammary tissue was cooled in liquid nitrogen, and crushed in a liquid nitrogen cooled steel mortar. The crushed tissue was immediately transferred while still frozen to a sterile 50 ml plastic centrifuge tube (SS-34, Sorvall) containing approximately 25 ml of denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 10 mM β -mercaptoethanol, and 0.5% (w/v) sodium n-lauryl sarcosine (NLS). The crushed tissue was homogenised with an Ultraturrax tissue homogeniser (10 N probe, full speed, thirty seconds). Cell debris was removed from the homogenate by centrifugation (11950 g, 10 minutes, 10°C). The supernatant was carefully layered onto 4 ml of 5.7 M CsCl, 100 mM EDTA in DEPC-treated SW-41 centrifuge tubes. RNA was collected by centrifugation (Beckman Ultracentrifuge, SW-41Ti rotor, 33,000 rpm, 15°C, 25 hours). When the centrifugation was complete the rotor was allowed to coast to a stop with no braking. The supernatant was carefully pipetted from the tubes to below the original level of the caesium chloride. The remaining supernatant was carefully decanted and the tubes were allowed to drain on tissue paper. The inverted tubes were cut at the original level of the caesium chloride to prevent contamination by denatured proteins on the sides of the tube. The

small pellets of RNA were resuspended in 200 μ l of denaturing solution (without NLS) and transferred to sterile, capped, 10 ml centrifuge tubes. The cut tubes were rinsed twice with 200 μ l of denaturing solution without NLS. The washings were pooled with the resuspended RNA pellets and the volume was adjusted to approximately 5 ml with denaturing solution containing NLS. The resuspended pellets were layered onto 4 ml of caesium chloride solution in SW-41 tubes and centrifuged as described above. The RNA pellet resulting from the second ultracentrifugation step was resuspended in denaturing solution without NLS as described above. The RNA was precipitated by the addition of 0.1 volumes of DEPC-treated 2 M potassium acetate and 2.5 volumes of absolute ethanol at -20°C . Nucleic acids were precipitated overnight at -20°C , and collected by centrifugation (Sorvall HL-8 rotor, 3000 rpm, 30 minutes, -10°C). The supernatants were decanted from the tubes and the pellets were washed twice with 70% (v/v) ethanol, 30 mM sodium chloride at -20°C for thirty minutes. The precipitated nucleic acids were collected by centrifugation following each wash (HL-8 rotor, 3000 rpm, 30 minutes, -10°C). The ethanol washed tubes were inverted and allowed to drain on tissue paper at 4°C for thirty minutes. The pellets were resuspended in 1 ml of 1% (w/v) NLS, 20 mM EDTA pH 7.0 and then washed with phenol to remove contaminating proteins. 2 ml of redistilled phenol (saturated with 1% (w/v) NLS, 20 mM EDTA pH 7.0) was mixed with the resuspended nucleic acids for five minutes on a vortex mixer. Chloroform: isoamyl alcohol (2 ml of 24:1 (v/v)) was added and the tubes were mixed for a further five minutes. The phases were separated by centrifugation (HL-8 rotor, 1000 rpm, 5 minutes, 10°C) and the aqueous (upper) phases were transferred to a fresh tube. The lower organic phases, containing denatured proteins, were back-extracted by adding 1 ml of 1% (w/v) NLS, 20 mM EDTA pH 7.0, mixing for five minutes and centrifuging as described above. The aqueous phases were pooled and re-extracted with phenol, chloroform: isoamyl alcohol as described earlier. The RNA in the aqueous phase was precipitated with ethanol as outlined above. RNA was collected by centrifugation, washed twice with 70% (v/v) ethanol, 30 mM sodium chloride, dried in a vacuum desiccator and resuspended in 200 μ l of 10 mM Tris-HCl pH 7.6, 1 mM EDTA. RNA samples were stored at -20°C either as ethanol precipitates or in aqueous solution.

3.2.2.2 Separation of poly A⁺ RNA from total cellular RNA

The poly A⁺ fraction of the total cellular RNA was isolated by affinity chromatography on oligo (dT)-cellulose as outlined by Maniatis *et al.* (1982), adapted from Edmonds *et al.* (1971) and Aviv and Leder (1972). HEPES was used in place of Tris in all buffers. Solutions containing Tris can not be treated with DEPC as this compound will acylate Tris.

3.2.2.3 Spectrophotometric analysis of RNA preparations

An estimation of the quantity and purity of the RNA isolated was made by spectrophotometric measurements of diluted RNA samples. Quantification was based on the assumption that 1.0 A²⁶⁰ unit represents 40 µg/ml of single-stranded RNA. The purity of the RNA preparations was estimated from the ratio of the absorbances at 260 and 280 nm. A solution of pure nucleic acid has an A²⁶⁰/A²⁸⁰ ratio of approximately 2.0 whereas a solution of pure protein has an A²⁶⁰/A²⁸⁰ ratio of approximately 0.6. Intermediate ratios indicate contamination of the RNA preparation with protein.

Samples were diluted with sterile 10 mM Tris-HCl pH 7.6, 1 mM EDTA and the absorbances were determined with a Cecil UV/VIS spectrophotometer or a Hewitt Packard Diode Array spectrophotometer.

3.2.2.4 Gel electrophoresis of RNA samples

Samples containing ribonucleic acids were analysed by gel electrophoresis in horizontal agarose gels containing 2.2 M formaldehyde as described by Maniatis *et al.* (1982). Instead of staining the gels after electrophoresis, ethidium bromide (approximately 20 µg) was added to each of the samples prior to loading. The excess dye migrates towards the cathode and out of the gel. Enough ethidium bromide remains associated with the ribonucleic acids to enable clear visualisation of the ribosomal RNA bands when the gel is exposed to ultraviolet light after electrophoresis.

3.2.2.5 Northern transfer of RNA from agarose gels to nitrocellulose

RNA samples separated by gel electrophoresis were transferred to nitrocellulose filters as described by Maniatis *et al.* (1982). Nitrocellulose filters were hybridised with ^{32}P -labelled cDNA probes as described in 3.2.3.

3.2.3 General methods used in the isolation and manipulation of DNA

All glassware, plasticware and solutions required for the isolation and manipulation of DNA were autoclaved prior to use.

All standard manipulations of DNA were as described in the following laboratory manuals; Maniatis *et al.* (1982) [Molecular Cloning: A Laboratory Manual]; Ausubel *et al.* (1989) [Current Protocols in Molecular Biology (Volumes 1 and 2)]; and Berger and Kimmel (1987) [Methods in Enzymology: A Guide to Molecular Cloning (Volume 152)]. Techniques outlined by these manuals include the isolation of plasmid DNA, the purification of plasmid DNA by phenol/chloroform extraction, ethanol precipitation of DNA from aqueous solutions, agarose gel electrophoresis of DNA and staining with ethidium bromide, the transfer of DNA from agarose gels to nitrocellulose (Southern blotting), and the maintenance and growth of the various bacterial strains required in this study. Table 3.1 lists the strains of *E. coli* used in this study and their relevant genotypes.

Restriction endonuclease digestion of DNA samples was in accordance with the suppliers instructions. Where digestion of the DNA with two or more restriction enzymes with different salt requirements was required, TA buffer was used instead of the buffers supplied with the enzymes. TA buffer was described by O'Farrel *et al.* (1980) and consists of 33 mM Tris-acetate pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM DTT and 0.01% (w/v) bovine serum albumin.

Table 3.1 Strains of *Escherichia coli* K12 used in this study.

Strain	Genotype
DH1	<i>supE44, hsdR17, recA1, endA1, gyrA96, thi-1, relA1</i>
DH5 α	<i>supE44, ΔlacU169(ϕ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1</i>
XL1-Blue	<i>supE44, hsdR17, recA1, endA1, gyrA46, thi, relA1, lac⁻F'[proAB⁺, lacI^q, lacZΔM15Tn10(<i>tet^r</i>)]</i>
Y1088*	<i>supE, supF, metB, trpR, hsdR⁻, hsdM⁺, tonA21, strA, ΔlacU169 (proC::Tn5) (pMC9)</i>
Y1089*	<i>ΔlacU169 proA⁺, ΔlonA, araD139, strA, hflA 150, (chr::Tn10) (pMC9)</i>
Y1090*	<i>ΔlacU169 proA⁺, ΔlonA, araD139, strA, supF, (trpC22::Tn10) (pMC9)</i>
Y1090 r-m ⁺	<i>F', hsdR(rk⁻, mk⁺), ΔlacU169 proA⁺, ΔlonA, araD139, strA, supF, [trpC22::Tn10(<i>tet^r</i>)] (pMC9)</i>

* Young and Davis, (1983).

DNA restriction fragments used for either cloning or labelling with ^{32}P were isolated from low gelling temperature agarose (SeaPlaque agarose, FMC BioProducts, Rockland, ME) gels in 1x TAE (0.04 M Tris-acetate, 0.001 M EDTA). DNA bands were visualised with long wavelength ultraviolet light and excised from the gel with scalpel blades. The DNA was purified from the gel slices by association with glass beads (Gene Clean kit, Bio 101) according to the manufacturers instructions.

DNA restriction fragments to be used as probes were labelled with [α - ^{32}P] dCTP by the random primer method using kits supplied by Bethesda Research Laboratories (BRL). Hybridisation of radiolabelled DNA fragments to DNA or RNA on nitrocellulose filters was performed as described by Maniatis *et al.* (1982). Briefly, nitrocellulose filters to be hybridised were first prehybridised at 68°C for two hours in a solution containing 1x Denhardt's (50x Denhardt's reagent; 5 g Ficoll (Type 400, Sigma), 5 g polyvinylpyrrolidone, 5 g bovine serum albumin (Fraction V, Sigma) in 500 ml of dH₂O), 0.5% (w/v) SDS, 0.01% (w/v) Herring sperm DNA, and made to the required volume with 6x SSC (20x SSC; 3.0 M NaCl, 0.3 M sodium citrate, pH 7.0). Prehybridised filters were placed in either

roller bottles or sealed in plastic bags containing 10 ml of hybridisation solution (1x Denhardt's, 0.5% (w/v) SDS, 1 M sodium chloride, 2 mM EDTA, and 50 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ pH 6.5). The radiolabelled cDNA probes were heated to 100°C, cooled on ice and added to the hybridisation vessels. Hybridisation was at 68°C for at least sixteen hours. Non-specific radiolabel was removed by washing the hybridised nitrocellulose filters twice in 6x SSC, 1% (w/v) SDS for one hour at 68°C and then twice in 6x SSC, 0.5% (w/v) SDS at 68°C for thirty minutes. The final wash, in either 1x or 2x SSC was for exactly thirty minutes at 68°C. The washed filters were placed on cardboard supports, wrapped in plastic film and exposed overnight to X-ray film (either Kodak XAR or Fuji). X-ray film was developed in an automated X-ray film developer (Kodak).

3.2.3.1 Synthesis of double-stranded cDNA

Complementary DNA (cDNA) synthesis was performed by a variety of methods. The first method utilised a cDNA synthesis kit supplied by Bethesda Research Laboratories (BRL). The BRL system is a modification of the method described by Gubler and Hoffman (1983), and combines the first and second strand reactions into a single tube protocol as outlined by D'Alessio *et al.* (1987). The first strand cDNA is synthesized by the RNA-directed DNA polymerase Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase. Following partial degradation of the messenger RNA template with RNase H, the second strand of the cDNA is synthesized with DNA polymerase I. After the addition of synthetic linkers, the double-stranded cDNA was cloned into the EcoRI restriction endonuclease site of the bacteriophage λ gt11.

The second method of cDNA synthesis attempted used the Avian Myeloblastosis Virus (AMV) reverse transcriptase to synthesize the first strand. A homopolymeric tail of guanosine residues was added to the 3' hydroxyl terminal of the first strand cDNA with the enzyme terminal deoxynucleotidyl transferase. The second strand synthesis was then attempted by two alternative procedures. In the first method, a homopolymeric oligonucleotide consisting of twenty cytosine residues, d(pC)_{20} , was used to prime the synthesis of the second strand. The d(pC)_{20} oligonucleotide hybridised to the 'G-tail' at the 3' end of the first strand cDNA and *E.coli* DNA polymerase I completed the synthesis of the second

strand. Double-stranded cDNA produced by this method was cloned directly into the SmaI restriction endonuclease site of the plasmid vector pGEM-2™. The second method for producing double-stranded cDNA from the 'G-tailed' first strand products was by the polymerase chain reaction (PCR). PCR, first described by Saiki *et al.* (1985), is a means of rapidly amplifying specific DNA sequences. Short DNA primers flanking the sequence to be amplified are used to direct the DNA polymerase to the appropriate region. Thermostable DNA polymerases are used to allow repeated cycles of heating to denature the DNA duplex following the annealing of primers and synthesis of the target sequence.

3.2.3.1.1 cDNA synthesis using the BRL cDNA Synthesis System

Double-stranded cDNA was prepared from involuted bovine mammary poly A⁺ RNA using the BRL cDNA Synthesis System. The protocols recommended by the manufacturer were followed and are outlined in figure 3.1.

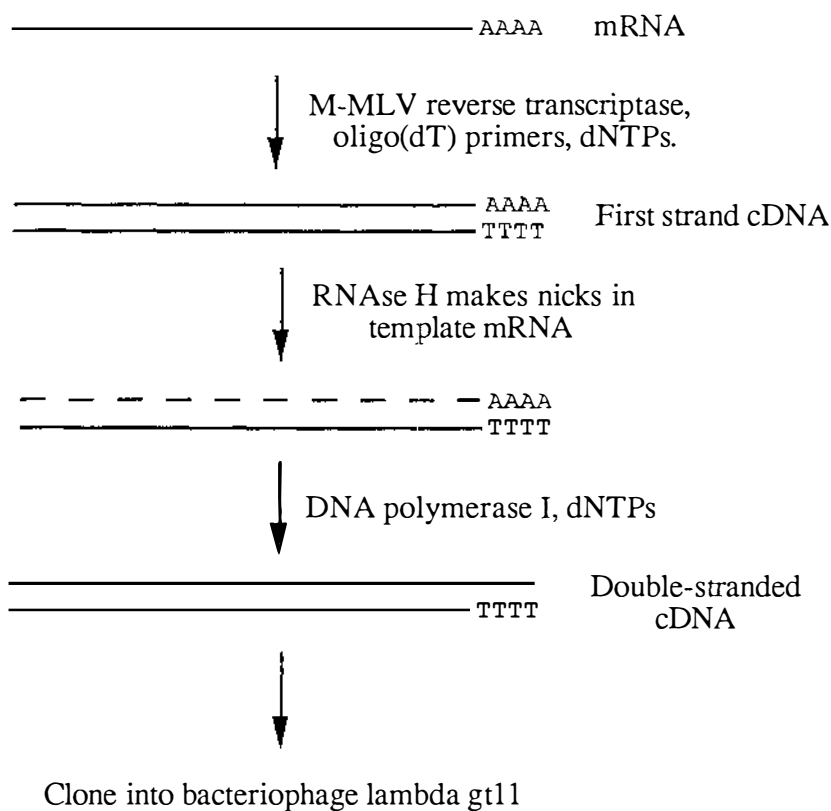


Figure 3.1 cDNA synthesis using M-MLV reverse transcriptase and RNase H.

The final reaction conditions in the first strand reaction mix were as follows; 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 500 μM of each dATP, dCTP, dGTP, and dTTP, 50 μg/ml oligo (dT)₁₂₋₁₈, approximately 100 μg/ml bovine mammary poly A⁺ RNA, and 10,000 units/ml of cloned M-MLV reverse transcriptase in a total volume of 40 μl. The reaction mix was incubated for one hour at 37°C. The second strand reaction was performed in the same tube by the addition of reactants so that the final reaction conditions in the second strand reaction were as follows; 25 mM Tris-HCl pH 8.3, 100 mM KCl, 5 mM MgCl₂, 250 μM of each dATP, dCTP, dGTP, and dTTP, 5 mM DTT, 250 units/ml E. coli DNA polymerase I and 8.5 units/ml RNase H in a total volume of 320 μl. The second strand synthesis was at 16°C for two hours.

The double-stranded cDNA was extracted twice with phenol/chloroform to remove proteins and then precipitated overnight at -20°C with ethanol. cDNA synthesized by this method was cloned into the bacteriophage λgt11.

The synthesis of the first and second strand cDNA products was monitored by the incorporation of [α-³²P] dCTP into acid precipitable products. The only deviation from the recommended procedure was in the assessment of the second strand reaction products. Instead of adding radiolabelled dCTP to the entire second strand reaction, 20 μl of second strand reaction mix was transferred to a separate tube containing 1 μl [α-³²P] dCTP (3000 mCi/mmol). The tracer reaction of the second strand synthesis was used to avoid radiolabelling all the cDNA products. The double-stranded cDNA was often stored for periods of time before cloning and the disintegration of the incorporated radioisotope may have damaged the DNA.

3.2.3.1.2 cDNA synthesis using AMV reverse transcriptase

An alternative method of cDNA synthesis was attempted using AMV reverse transcriptase to synthesize the first strand. The protocols employed are outlined in figure 3.2.

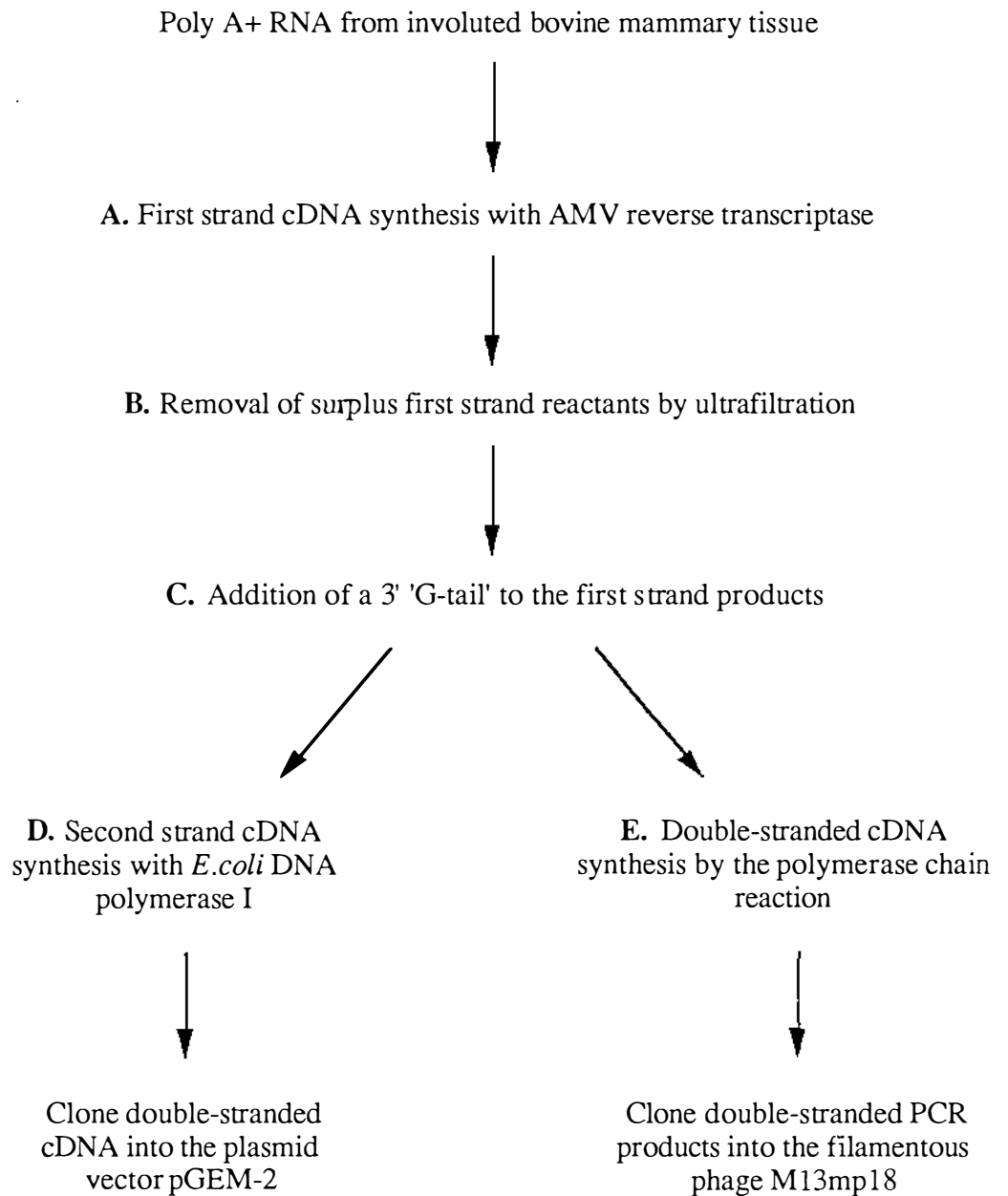


Figure 3.2 Outline of cDNA synthesis using AMV reverse transcriptase to synthesize the first strand cDNA.

A. First strand cDNA synthesis

First strand synthesis was performed according to Ausubel *et al.* (1989) using the RNA directed DNA polymerase AMV reverse transcriptase. 1 μ g of poly A⁺ RNA in 5.5 μ l of DEPC-treated sdH₂O was denatured by heating to 65°C for 3 minutes and then placed on ice. 0.5 μ l of placental ribonuclease inhibitor, RNasin (Promega), was added. The other reactants were added to the RNA sample on ice as follows:

6 μ l	Poly A ⁺ RNA/RNasin
2 μ l	10x 1st Strand AMV buffer
2 μ l	10 mM dCTP
2 μ l	10 mM dGTP
2 μ l	10 mM dATP
2 μ l	10 mM dTTP
2 μ l	0.5 mg/ml oligo (dT) ₂₀
2 μ l	AMV reverse transcriptase.

The final reaction conditions of the first strand cDNA synthesis were 50 mM Tris-HCl pH 8.3, 8 mM MgCl₂, 30 mM KCl, 1 mM of each dATP, dCTP, dGTP and dTTP, 1 μ g oligo (dT)₂₀ primer, 25 units ribonuclease inhibitor RNasin, 1 μ g bovine mammary poly A⁺ RNA, and 64 units of AMV reverse transcriptase. A 2 μ l aliquot of the first strand reaction was transferred to a tube containing 1 μ l [α -³²P] dGTP (3000 mCi/mmol). Both tubes were incubated at 42°C for one hour. The reactions were terminated by the addition of 1 μ l and 5 μ l of 0.25 M EDTA pH 7.5 to tracer and main reactions respectively. The tracer reaction was diluted to 100 μ l by the addition of sdH₂O. The incorporation of radiolabel into acid precipitable DNA products was determined as described in 3.2.3.1.3.

B. Purification of first strand cDNA by ultrafiltration

The main reaction was diluted to 1 ml with 0.5 mM Tris-HCl pH 7.6, 0.05 mM EDTA and transferred to a Centricon-30 ultrafiltration unit. The buffer and excess first strand reactants were removed by ultrafiltration. This step was necessary to remove the excess oligo (dT)₂₀ primer prior to second strand cDNA synthesis by the anchored PCR method. Any oligo (dT)₂₀ remaining in the reaction mix would prime DNA synthesis in the polymerase chain reaction. The high molecular weight products were isolated by centrifugation (SS-34 rotor, 5000 rpm, 5°C, 30 min). The concentrated

products were washed three times with 1 ml 0.5 M Tris-HCl pH 7.6, 0.05 mM EDTA and centrifuged as above. The first strand cDNA products were collected in the cap of the inverted ultrafiltration unit by centrifugation (2000 rpm, SS-34 rotor, 5°C, 5 min). The concentrated sample was transferred to a sterile 1.5 ml plastic centrifuge tube, a small aliquot was set aside and the remaining volume was reduced to approximately 20 µl by vacuum desiccation (Speedvac, Savant Instruments).

C. Addition of the 3' Guanosine tract

A tract of guanosine residues was added to the 3' end of the first cDNA using terminal deoxynucleotidyl transferase (TdT). The volume of the first strand products was adjusted to 23 µl with sdH₂O. The other reactants were added on ice as follows: 1 µl of 0.5 mM dGTP (prepared by diluting 1 µl 10 mM dGTP and 1 µl [α -³²P] dGTP in 18 µl sdH₂O), 6 µl 5x BRL terminal deoxynucleotidyl transferase tailing buffer and 1.36 µl terminal deoxynucleotidyl transferase. The final reaction conditions in the tailing reaction were approximately as follows; 0.2 mM DTT, 17 mM dGTP, 0.5 µCi [α -³²P] dGTP, 2 mM CoCl₂, 100 mM potassium cacodylate (pH 7.2) and 15 units of terminal deoxynucleotidyl transferase. After incubation at 37°C for thirty minutes, the reaction was terminated by incubation at 65°C for fifteen minutes, and then transferred to ice. A 2 µl aliquot was removed and diluted to 20 µl with sdH₂O. Duplicate 5 µl aliquots of this diluted tailing reaction were spotted onto glass fibre filters (Whatman GF/A). The incorporation of radiolabel was estimated as for the cDNA products and is described in section 3.2.3.1.3.

D. Second Strand cDNA Synthesis with *E. coli* DNA polymerase I

Double-stranded cDNA was prepared from the 'G-tailed' first strand cDNA products using *E. coli* DNA polymerase I. As no special precautions were taken to avoid the introduction of ribonuclease during the purification of the first strand products, it is unlikely that the messenger RNA template survived the ultrafiltration and tailing reaction. Consequently, a homopolymeric cytosine oligonucleotide (d(pC)₂₀) was added to prime the synthesis of the second strand cDNA.

20 μ l of the tailed first strand products was mixed with 1 μ g of d(pC)₂₀ in 1 μ l of sdH₂O in a sterile 1.5 ml capped plastic centrifuge tube. The tube was heated to 80°C for five minutes and then cooled slowly to room temperature to allow the d(pC)₂₀ oligonucleotide to anneal to the 'G-tail' at the 3' end of the first strand products. The other components of the second strand reaction were added such that the final reaction conditions were as follows; 25 mM Tris-HCl pH 8.3, 100 mM KCl, 5 mM MgCl₂, 5 mM DTT, 0.2 mM of each dATP, dCTP, dGTP and dTTP, and 80 unit of *E. coli* DNA polymerase I in a total volume of 300 μ l. The second strand reaction was incubated at 16°C for two hours. The reaction was terminated by the addition of 20 μ l of 0.5 M EDTA pH 8.0. The double-stranded cDNA was extracted twice with phenol/chloroform, precipitated with ethanol and subsequently cloned into the plasmid vector pGEM-2™.

E. Synthesis of double-stranded cDNA by the polymerase chain reaction

The synthesis of double-stranded cDNA specific for the 5' end of the bovine lactoferrin mRNA was accomplished by anchored polymerase chain reaction (PCR).

The PCR reactions were performed using commercially prepared kits (GeneAmp™ DNA Amplification Reagent kit with Amplitaq™ recombinant *Taq* DNA polymerase from Perkin-Elmer-Cetus) according to the manufacturers instructions. Briefly, first strand 'G-tailed' cDNA was diluted ten-fold with sdH₂O. Negative controls, with diluted first strand cDNA lacking the 3' 'G-tail' were prepared. The PCR reactions were prepared in sterile PCR tubes as follows:

53.5 μ l	sterile water
10 μ l	10x PCR buffer
16 μ l	1.25 mM dNTPs
5 μ l	100 ng/5 μ l d(pC) ₂₀ primer
5 μ l	100 ng/5 μ l JT12 or JT15 primer
10 μ l	diluted first strand cDNA

The reaction tubes were heated to 100°C for five minutes to denature the DNA and then set on ice. 0.5 μ l of cloned *Thermus aquaticus* (*Taq*) DNA polymerase was added. The final PCR reaction conditions were as follows; 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin,

0.2 mM of each dATP, dCTP, dGTP and dTTP, 100 ng of d(pC)₂₀ primer, 100 ng of either JT12 or JT15 primer, and 2.5 units of *Taq* DNA polymerase. After the addition of *Taq* DNA polymerase the reactants were overlaid with two drops of mineral oil to prevent evaporation during the repeated heating cycles. The tubes were placed in an automated DNA thermal cycler (Perkin-Elmer-Cetus) and incubated as follows:

- 94°C for one minute to denature double-stranded DNA;
- 55°C for two minutes to allow DNA and primers to anneal;
- 72°C for three minutes for extension of DNA from primers by *Taq* polymerase.

The temperature profile was repeated for thirty cycles and was followed by an incubation at 4°C until the products were analysed by agarose gel electrophoresis.

When further amplification of a sample was required, a 10 µl aliquot of the PCR products was used as the template for DNA synthesis in place of the 'G-tailed' first strand cDNA. The other reaction conditions were as listed above. The primers used to initiate DNA synthesis in the PCR reactions were all synthetic oligonucleotides and their sequences are given in table 3.2.

Table 3.2 The nucleotide sequence of the synthetic oligonucleotide primers used for the enzymatic amplification of the 5' end of the cDNA coding for bovine lactoferrin.

Oligo-nucleotide	Length (Bases)	Sequence (5' to 3')
d(pC) ₂₀	20	CCCCCCCCCCCCCCCCCCCC
JT 12	24	CCGGCCCGCCTCAAACACCATGCC
JT 15	23	CCTCAAACACCATGCCACCATCC

The oligonucleotide d(pC)₂₀ hybridises to the 3' 'G-tail' added to the first strand cDNA and primes synthesis of the DNA strand with the same sequence as the messenger RNA. Oligonucleotides JT12 and JT15 are complementary to sequences near the 5' end of the bovine lactoferrin messenger RNA. The two specific primers were selected from nucleotide sequence close to the 5'-most sequence obtained by conventional cDNA cloning techniques (See clone PM 8, section 3.3.5.2.2). Primer JT 15

overlaps the 5' sequence of the of JT 12 and was used in an attempt to increase the specificity of the PCR reaction. That is, the first thirty cycles of PCR were with primers d(pC)₂₀ and JT 12. The second thirty cycles, or reamplification, were with primers d(pC)₂₀ and JT 15.

3.2.3.1.3 Analysis of radiolabelled cDNA products

The synthesis of first and second strand cDNA products was followed by the incorporation of radiolabelled deoxyribonucleotides into acid insoluble products (section 3.2.3.1.3 A).

A. Direct counting method

Small aliquots of the radiolabelled DNA samples were diluted approximately twenty-fold with TE pH 7.6. Duplicate 5 µl aliquots of the diluted samples were spotted onto glass fibre filter disks (GF/A or GF/C, Whatman). One of the duplicate filters was allowed to air dry. This filter was used to estimate the total radioactivity in the sample. The other filter was washed three times for five minutes each by submerging in ice-cold 10% (w/v) TCA, 0.05% (w/v) PPI. The filter was then washed with ice-cold 95% ethanol. The washed filter was dried under an infrared heat lamp. The washed filter was used to estimate the amount of radiolabel incorporated into acid insoluble products. Both filters were placed in scintillation vials and submerged in 3 ml scintillation fluid (4 g PPO, 0.1 g POPOP, 333 ml triton X-100, 667 ml redistilled toluene).

B. Gel electrophoresis of radiolabelled cDNA products

Radiolabelled cDNA was analysed by electrophoresis on alkaline agarose gels according to Maniatis *et al.* (1982). Agarose gels (1.4% (w/v)) were prepared in 1 x gel buffer (30 mM NaCl, 2 mM EDTA pH 7.5) and poured into a vertical gel apparatus (GE 4, Pharmacia). The gel electrophoresis tank was filled with 1x electrophoresis buffer (30 mM NaOH, 2 mM EDTA). Approximately 100 ng of BRL 1 kb ladder was radiolabelled with [α -³²P dCTP] in the presence of T4 DNA polymerase. Radiolabelled DNA samples were diluted with an equal volume of 2x sample buffer (20 mM NaOH, 20% (v/v) glycerol, 0.02% (w/v) bromophenol blue). Samples were loaded and electrophoresis was at 75 mA, at 16°C for approximately 3 hours or until the

dye front had migrated approximately two-thirds the length of the gel. The gels were dried onto DE 81 paper (Whatman) at 80°C for one hour and exposed to X-ray film overnight.

3.2.3.2 Cloning double-stranded cDNA

Double-stranded cDNA, synthesized by the three methods described earlier, was cloned into one of the following vectors; the bacteriophage λ gt11, the plasmid vector pGEM-2™ or the filamentous bacteriophage M13.

3.2.3.2.1 Cloning cDNA into bacteriophage λ gt11

Double-stranded cDNA synthesized using the BRL cDNA Synthesis System was cloned into λ gt11 as described by Huynh *et al.* (1985). The cloning strategy is summarised in figure 3.3.

λ gt11 was chosen as the cloning vector as clones carrying lactoferrin DNA sequences were to be identified using the mono-specific anti-lactoferrin antibodies prepared earlier (see chapter two). The cloning site in this bacteriophage is within the gene coding for β -galactosidase. Consequently, recombinant clones will produce a fusion protein with β -galactosidase when this gene is induced. The presence of specific DNA sequences can be identified by using antibodies raised against the protein of interest. During the course of this work two human lactoferrin cDNA clones became available. As screening cDNA libraries with homologous cDNA probes is generally more fruitful than using immunochemical techniques, the antibody approach was abandoned in favour of the nucleic acid hybridisation technique.

A. Methylation of the EcoRI restriction sites in the cDNA

The EcoRI restriction endonuclease sites in the cDNA sample were protected by specific methylation of the DNA. Ethanol precipitated cDNA (1 to 5 μ g) was resuspended in 16.5 μ l of sdH₂O. Methylase buffer, S-adenosyl-L-methionine (SAM) and EcoRI methylase were added to the resuspended cDNA. The final reaction conditions of the 22 μ l methylation reaction were; 0.1 M Tris-HCl pH 8.0, 0.1 M NaCl, 1.0 mM EDTA, 0.08 mM S-adenosyl-

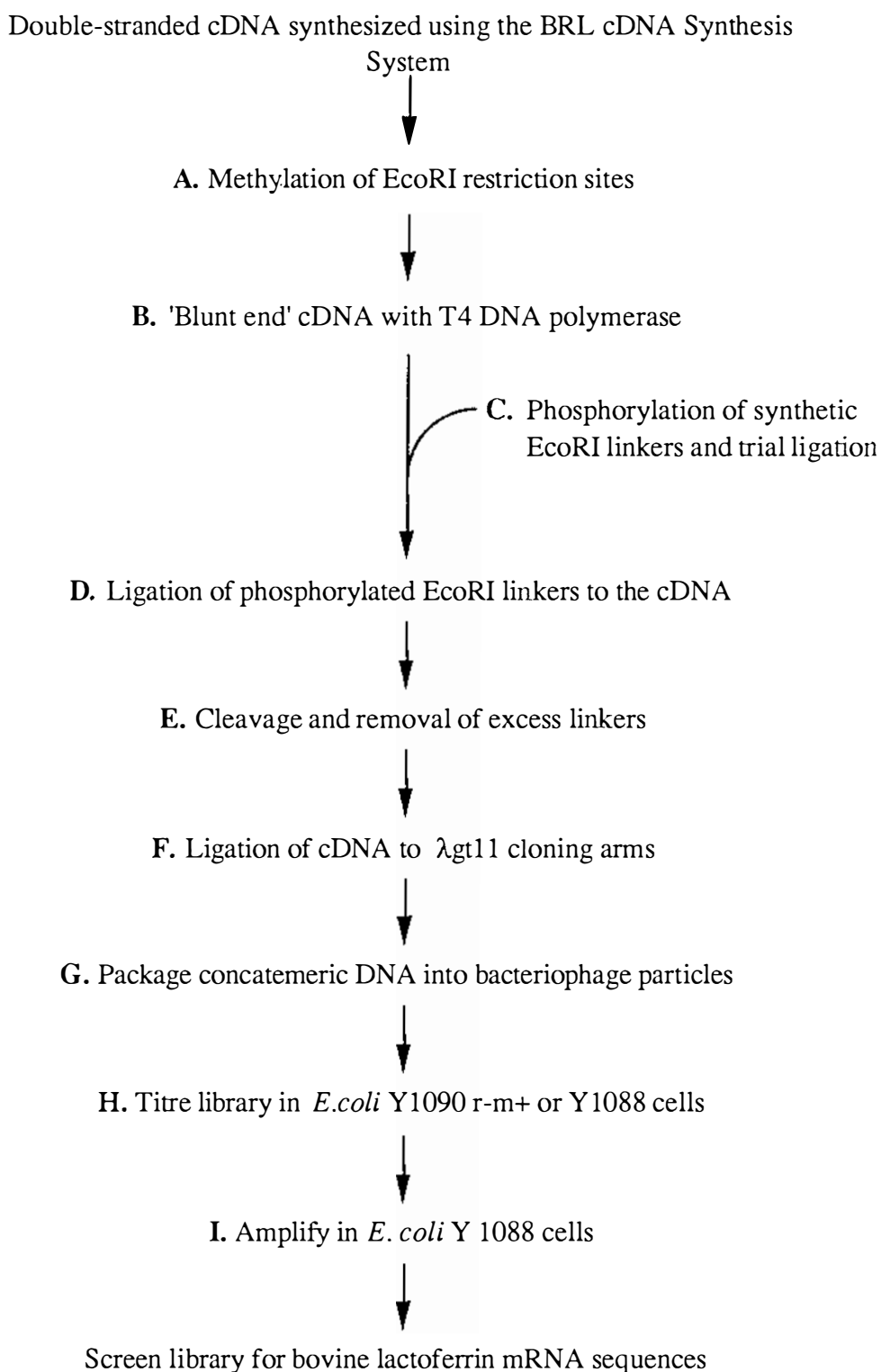


Figure 3.3 Outline of the strategy for cloning double-stranded cDNA into the bacteriophage λ gt11.

L-methionine, and 30 units of EcoRI methylase. The reaction proceeded for one hour at 37°C and was terminated by incubation at 70°C for ten minutes.

B. Blunt ending the cDNA with T4 DNA polymerase

Prior to the ligation of EcoRI linkers, the methylated cDNA was incubated in the presence of T4 DNA polymerase to ensure that the double-stranded cDNA was blunt ended. The following reagents were added to the 22 µl of methylated cDNA; 1.25 µl of 0.2 M MgCl₂, 1.25 µl of a 2.25 M mixture of dATP, dCTP, dGTP, dTTP and 3.5 µl (3.5 units) of T4 DNA polymerase. The reaction was incubated at 24°C for ten minutes. The reaction was terminated by the addition of 12 ml of 50 mM EDTA pH 8.0. The blunt ended, methylated cDNA was extracted twice with phenol/chloroform and ethanol precipitated overnight at -20°C.

C. Phosphorylation and trial ligation of EcoRI linkers

The EcoRI linkers purchased from BRL lacked 5' phosphate groups and required phosphorylation prior to ligation to the cDNA sample. The linkers were phosphorylated in the presence of [γ -³²P] ATP so that ligation to the cDNA could be followed. The final reaction conditions of the EcoRI linker phosphorylation reaction were as follows; 4 µg EcoRI linkers, 66 mM Tris·HCl pH 7.5, 10 mM MgCl₂, 15 mM DTT, 34 µCi [γ -³²P] ATP, 1 mM ATP, and 10 units of T4 polynucleotide kinase in total volume of 20 µl. The reaction was incubated for one hour at 37°C. Phosphorylated linkers were stored at -70°C until required.

Before ligation to cDNA, a trial ligation of the phosphorylated linkers was performed. The final reaction conditions of the duplicate 10 µl trial ligation reactions were as follows; 66 mM Tris·HCl pH 7.5, 10 mM MgCl₂, 15 mM DTT, 1 mM ATP, and 0.5 units of T4 DNA ligase. A third reaction was prepared which contained no ligase and which acted as a negative control. The reactions were incubated at 22°C for sixteen hours. One of the ligated samples was digested with EcoRI for one hour at 37°C to determine that the ligated linkers could be cut. The three samples were diluted with an equal volume of deionised formamide, heated at 90°C for three minutes, and cooled on ice. The denatured samples were then loaded onto a vertical 20% (w/v) polyacrylamide gel containing 7 M urea in 1x TBE (0.089 M Tris·HCl pH

8.8, 0.089 M boric acid, 0.002 M EDTA). Electrophoresis was at 1500 V for forty-five minutes. The gel was fixed in 5% (v/v) methanol, 5% (v/v) glacial acetic acid for thirty minutes and then dried onto blotting paper at 80°C under vacuum. The dried gel was exposed to X-ray film overnight.

D. Addition of phosphorylated EcoRI linkers to the cDNA

The phosphorylated EcoRI linkers were ligated to the methylated, blunt ended, cDNA. The final reaction conditions in the 20 µl ligation were; 66 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 15 mM DTT, 1 mM ATP, 1 unit of T4 DNA ligase, 0.2 µg ³²P-labelled phosphorylated EcoRI linkers and approximately 5 µg of methylated, blunt ended cDNA. The ligation reaction was incubated at 12°C overnight.

E. Cleavage and removal of excess linkers

After incubation overnight, the ligation reaction was supplemented with an equal volume (20 µl) of 50 mM Tris-HCl pH 7.5, 10 mM MgSO₄, 200 mM NaCl. The ligase activity was destroyed by heating to 70°C for ten minutes. The sample was cooled on ice and 1 unit of EcoRI restriction endonuclease was added to the diluted ligation reaction. After incubating for one hour at 37°C, the EcoRI activity was destroyed by heating to 70°C for ten minutes. The digested sample was diluted to 1 ml with sdH₂O and transferred to a Centricon-30 ultrafiltration unit. The cDNA sample was concentrated by centrifugation (5000 rpm, SS-34 rotor, thirty minutes, 4°C). The filtrate, containing the cleaved linker fragments, was transferred to a sterile 1.5 ml plastic centrifuge tube. The concentrated sample was washed three times with 1 ml aliquots of sdH₂O, and re-concentrated by centrifugation as described above. The concentrated cDNA-linker sample was collected in the cap of the inverted ultrafiltration unit by centrifugation (2000 rpm, SS-34 rotor, two minutes, 4°C), and transferred to a sterile 1.5 ml plastic centrifuge tube. The tubes containing the cDNA sample and the washing from the ultrafiltration unit were placed in separate glass scintillation vials. The β-emission from the radiolabelled EcoRI linkers was estimated by Cerenkov counting (Beckman LS 8000 liquid scintillation counter, Program 1).

F. Ligation of cDNA to λ gt11 arms

Half of the concentrated cDNA-linker sample (approximately 20 μ l) was supplemented with approximately 1 μ g of EcoRI-cut λ gt11 cloning arms which had been treated with calf alkaline phosphatase to remove the 5' terminal phosphate groups. The mixture was ethanol precipitated overnight at -20°C. The precipitated nucleic acids were resuspended in 4 μ l of 10 mM Tris-HCl pH 7.5, 10 mM MgCl₂ by gentle mixing and repeated centrifugation (MSE microfuge, 13,000 rpm, 1-2 seconds) for approximately one hour. To aid dissolution of the DNA, the sample was warmed to 42°C for ten minutes several times during the one hour resuspension procedure. 1.1 μ l of a cocktail containing 5 μ l 10 μ M ATP, 5 μ l 0.1 M DTT, and 1 μ l T4 DNA ligase was added to the resuspended DNA. The final reaction conditions in the ligation reaction were approximately 8 mM Tris-HCl pH 7.5, 8 mM MgCl₂, 1 mM ATP, 10 mM DTT, 0.2 units of T4 DNA ligase, ~2 μ g cDNA, and 1 μ g λ gt11 arms. The ligation reaction was incubated overnight at 12°C. A 0.5 μ l sample of the ligation mix was analysed by agarose gel electrophoresis. The remaining ligation reaction was diluted with 5 μ l of 8 mM Tris-HCl pH 7.5, 8 mM MgCl₂, 1 mM ATP, 10 mM DTT and incubated for a further twenty-four hours at 12°C. A second sample of the ligation reaction (approximately 0.75 μ l) was analysed by agarose gel electrophoresis.

G. Packaging of concatemeric DNA into bacteriophage particles

An aliquot (5 μ l) of the reaction mixture from ligation of the cDNA to the λ gt11 arms ligation reaction was added to 50 μ l of packaging extract (Promega Packagene mix). The sample was incubated for two hours at 22°C, and then supplemented with 500 μ l of phage dilution buffer (PDB, 100 mM NaCl, 20 mM Tris-HCl pH 7.4, 10 mM MgSO₄) and 25 μ l of chloroform. After gentle mixing, the sample was incubated overnight at 4°C.

H. Titre of the λ gt11 cDNA library

Overnight cultures of *E. coli* Y1090 r^{m+} or *E. coli* Y1088 were grown in Luria-Bertani broth (LB) supplemented with 10 mM MgSO₄ and 0.2% (w/v) maltose. The cells were collected by centrifugation, resuspended in one half

volume of 10 mM MgSO₄ and incubated for at least one hour at 4°C.

Aliquots of the packaged λ gt11 library were diluted 100 and 10,000-fold with phage dilution buffer (PDB). 100 μ l of diluted packaging reaction was mixed with 200 μ l of resuspended *E. coli* cells and incubated at 37°C for thirty minutes to allow the phage to attach to the bacteria. 3 ml of molten top agar (0.7% (w/v) agar in LB), 50 μ l 20 mg/ml X-gal in diethylformamide, and 20 μ l 24 mg/ml IPTG in sdH₂O were mixed with the incubated samples. The samples were immediately plated on LB plates (1% (w/v) agar in LB) and incubated overnight inverted in a humidified environment at 42°C.

I. Amplification of the λ gt11 libraries

The rest of the packaged λ gt11 library, stored at 4°C over chloroform, was plated on LB plates as described above. The plates were incubated overnight at 42°C and then for one hour at 4°C to harden the agar. Approximately 8 ml of chilled SM (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 100 mM MgSO₄ and 0.01% (w/v) gelatine) was carefully pipetted onto the surface of the agar. The plates were incubated overnight at 4°C to allow the bacteriophage to disperse into the liquid medium above the agar. The SM was carefully removed from the plates and transferred to 50 ml sterile centrifuge tubes containing 200 μ l chloroform. The amplified libraries were stored at 4°C over chloroform or at either -20°C in 50% (v/v) glycerol or at -70°C in 20% (v/v) glycerol.

3.2.3.2.2 Cloning cDNA into the plasmid vector pGEM-2™

Double-stranded cDNA synthesized from first strand 'G-tailed' cDNA by *E. coli* DNA polymerase I was ligated into the plasmid pGEM-2™. Approximately half of the phenol-washed, ethanol-precipitated double-stranded cDNA products were ligated into SmaI-cut, phosphatased pGEM-2™. The ligation reaction was incubated overnight at 12°C. Log-phase *E. coli* XL 1 cells were made competent by treatment with ice-cold 50 mM CaCl₂ (Maniatis *et al.*, 1982). Circularised plasmid was introduced into competent XL 1 cells by the heat shock method (adapted from Maniatis *et al.*, 1982). Aliquots of the ligation reactions were diluted in 200 μ l of competent XL 1 cells. After resting on ice for thirty minutes, the cells were heat shocked at 42°C for ninety seconds. After a five minute recovery period on ice, the transformation mixes were diluted to 1 ml with LB containing 10 mM

MgSO₄, 10 mM MgCl₂ and 20 mM glucose. The cells were grown at 37°C for one hour with rapid shaking. This unchallenged growth period was to allow the cells containing the pGEM-2™ plasmid to start producing β-lactamase prior to plating on LB agar plates containing ampicillin. Aliquots of the transformation reaction were spread on LB agar plates containing 100 µg/ml ampicillin.

3.2.3.2.3 Cloning cDNA into the filamentous bacteriophage M13

Double-stranded cDNA synthesized from first strand 'G-tailed' cDNA by the polymerase chain reaction was cloned into the filamentous bacteriophage M13. Briefly, PCR products were ligated into double-stranded M13 DNA cut with SmaI. The ligated DNA was transformed into competent bacteria as described in 3.2.3.2.1. The transformed bacteria were plated in top agar (0.7% (w/v) agar in LB) containing X-gal and IPTG on LB plates.

3.3 Results and Discussion

3.3.1 Isolation of total cellular RNA

The isolation of total cellular RNA from involuting bovine mammary tissue was performed as described in 3.2.2.1. The purity and quantity of the RNA isolated was determined spectrophotometrically. Figure 3.4 illustrates a typical absorbance spectrum of total cellular RNA isolated from bovine mammary tissue. The absorbance maxima at 260 nm is characteristic of nucleic acids in aqueous solution. Table 3.3 illustrates the absorbance data for selected total cellular RNA preparations.

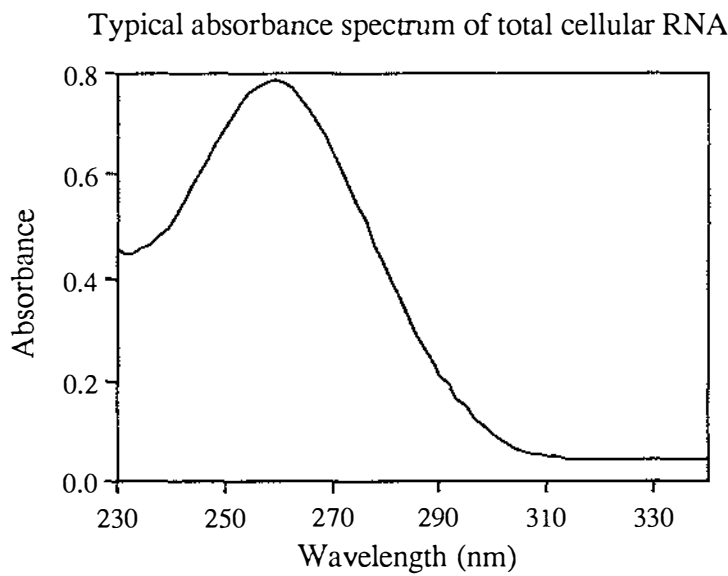


Figure 3.4 Typical absorbance spectrum of total cellular RNA isolated from bovine mammary tissue.

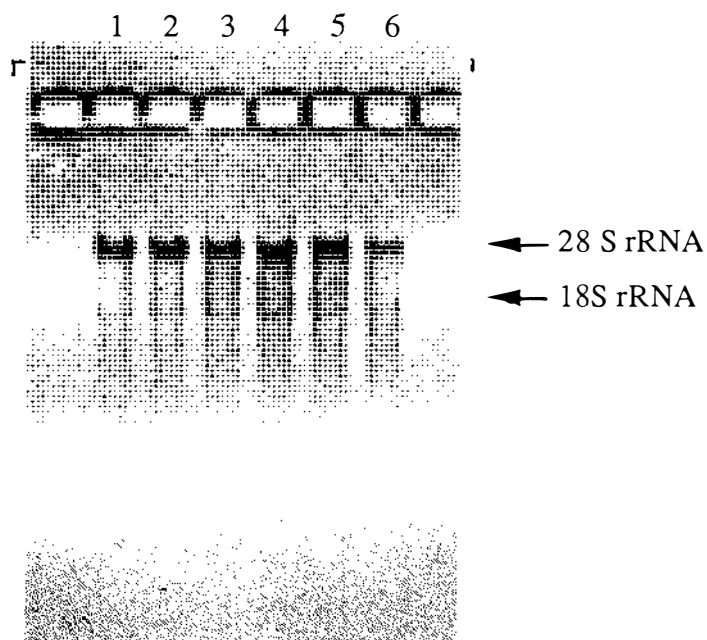
Table 3.3 Absorbance data for total cellular RNA isolated from involuting bovine mammary tissue (cow #198, day 25). Samples were assayed in duplicate. Sample 2 (a) was measured from the spectrum shown in figure 3.4.

Sample	A260	A280	A260/A280	Yield (µg RNA)
1 (a)	1.113	0.570	1.95	3580
(b)	1.152	0.585	1.97	3710
2 (a)	0.781	0.399	1.96	2515
(b)	0.809	0.414	1.95	2600

The data presented in table 3.3 clearly indicates that the RNA isolated from involuting mammary tissue was essentially free of contaminating protein.

The integrity of the RNA, that is, the absence of degradation by ribonucleases, was determined by denaturing agarose gel electrophoresis of RNA samples as described in 3.2.2.4. Figure 3.5 illustrates a typical denaturing agarose gel electrophoresis profile of total cellular RNA isolated from involuting mammary tissue. The presence of high molecular weight ribosomal RNA species in the samples suggests that the samples were essentially free of nuclease degradation. Figure 3.6 illustrates the effect of partial ribonuclease digestion on the electrophoretic profile of the total cellular RNA. Ribonuclease degradation of the ribosomal RNA results in the disappearance of the discrete 28S and 18S rRNA bands that are seen in 'intact' total cellular RNA samples.

Total cellular RNA, separated by gel electrophoresis, was transferred to nitrocellulose filters by capillary blotting (northern transfer). The nitrocellulose filters were probed with radiolabelled human or bovine cDNA sequences. A typical northern blot analysis of total cellular RNA isolated from mammary tissue can be seen in figure 3.10 (page 134, lane 1). Figure 3.10 illustrates an ethidium bromide-stained agarose gel and the corresponding autoradiograph of the northern blot of a sample of total cellular (and poly A⁺ and poly A⁻) RNA isolated from involuting bovine mammary tissue. The 3' end of the bovine lactoferrin cDNA (clone PM 1, see section 3.3.4.2) was radiolabelled by the random primer method and allowed to hybridise to complementary sequences on the nitrocellulose filter. Radiolabelled cDNA which was bound non-specifically was removed by washing with low ionic strength buffers. The washed filter was exposed to X-ray film as described in 3.2.2.5. The presence of high molecular weight ribosomal RNA bands (on the ethidium bromide-stained gel) provided a convenient means of estimating the size of the messenger RNA which hybridised to the lactoferrin cDNA sequence. Figure 3.7 depicts a plot of log₁₀ (number of bases) versus mobility, used to estimate the length of the mRNA coding for bovine lactoferrin. The members of the transferrin family are all monomeric proteins of approximately 700 amino acids in length. The messenger RNA coding for these proteins must be at least 2,100 bases long, not including any 5' or 3' untranslated sequences. The estimated length for the bovine lactoferrin

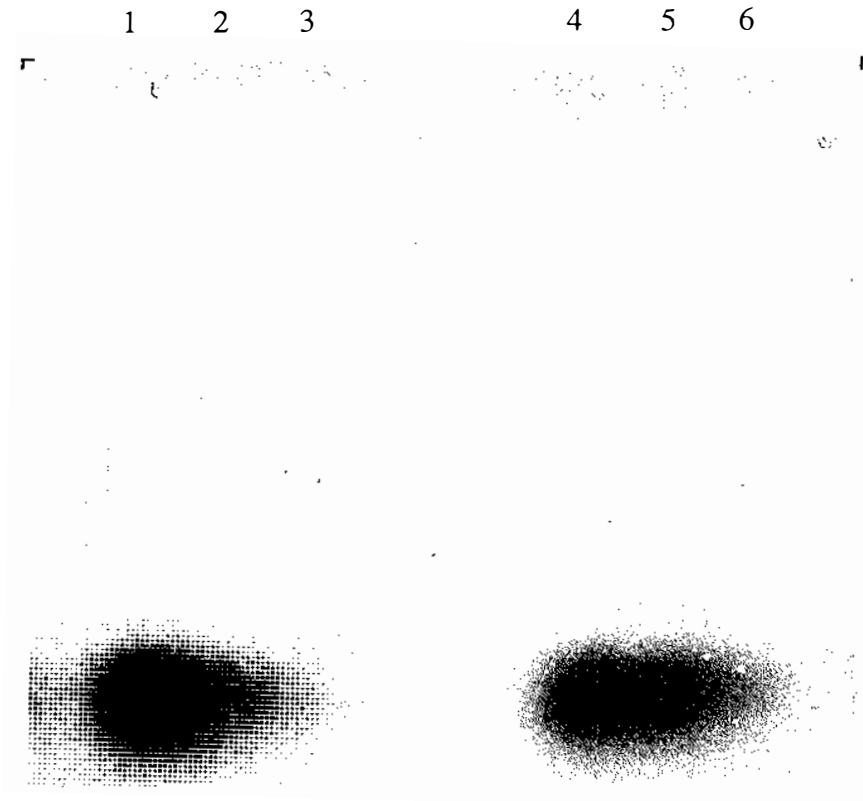


Gel details: 1.5% (w/v) agarose gel containing 2.2 M formaldehyde and stained with ethidium bromide.

Loading details: 10 μ g of total cellular RNA isolated from involuting mammary tissue (cow #87) was applied to each lane.

1. Day 24.
2. Day 19.
3. Day 14.
4. Day 10.
5. Day 5.
6. Day 0.

Figure 3.5 Gel electrophoresis of total cellular RNA isolated from involuting bovine mammary tissue.



Gel details: 1.5% (w/v) agarose gel containing 2.2 M formaldehyde and stained with ethidium bromide.

Loading details: All samples are total cellular RNA from cow #87, day 14 of involution. Samples in lanes 1, 2 and 3 were partially digested with ribonuclease.

1 and 4. 10 μ g of RNA.

2 and 5. 5 μ g of RNA.

3 and 6. 2.5 μ g of RNA.

Figure 3.6 Denaturing agarose gel electrophoresis of total cellular RNA samples to illustrate the effect of ribonuclease digestion.

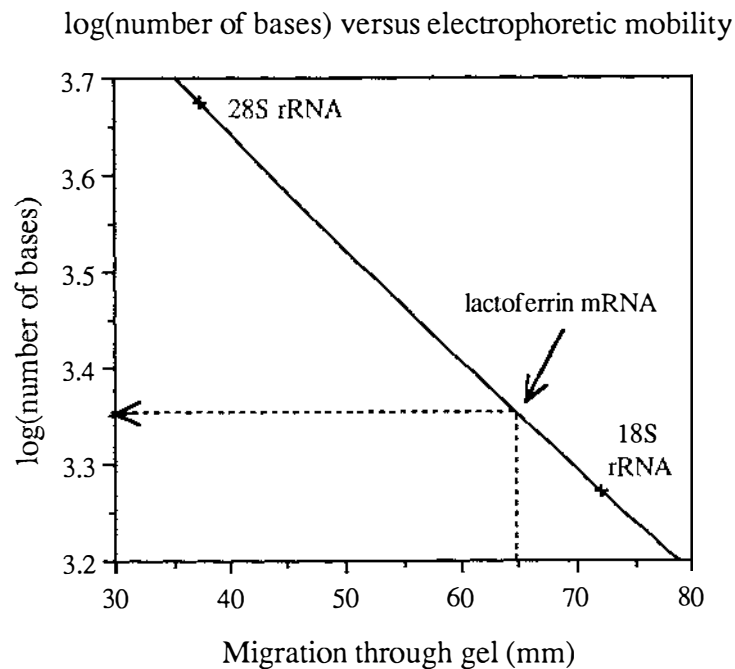


Figure 3.7 $\text{Log}_{10}(\text{number of bases})$ versus mobility plot to determine the length of the mRNA coding for bovine lactoferrin. The bovine lactoferrin cDNA (PM 1) hybridised to a band at approximately 65 mm from the top of the gel. This corresponded to an mRNA molecule of approximately 2,290 bases ($10^{3.36} = 2,290$).

mRNA of 2,290 bases is therefore sufficient to contain the entire coding region for the protein.

Total cellular RNA was isolated from each of the mammary tissue samples taken from the two cows that were used in this study (#87 and #198). Figure 3.8 A illustrates an ethidium bromide-stained formaldehyde-agarose gel of total cellular RNA isolated at various stages of involution. Figure 3.8 B illustrates an autoradiograph of the same gel after transfer of the nucleic acids to a nitrocellulose filter by capillary blotting and hybridisation with radiolabelled PM 1 DNA. The ethidium bromide-stained gel (figure 3.8 A) indicates that, with exception of the sample from cow #87 day 25 (lane 7), approximately the same amount of RNA was loaded into each track. The northern blot of this gel, hybridised with radiolabelled bovine lactoferrin cDNA (clone PM 1), indicates the change in the relative amounts of lactoferrin mRNA in each biopsy. Although the total amount of RNA varies between samples (as indicated by the ethidium bromide-stained gel), there appears to be an increase in the amount of mRNA coding for lactoferrin in the

A. Gel details: 1.5% (w/v) agarose gel containing 2.2 M formaldehyde.

Loading details: All samples are total cellular RNA isolated from bovine mammary tissue at different stages of involution. Approximately 10 µg of RNA was loaded into each track.

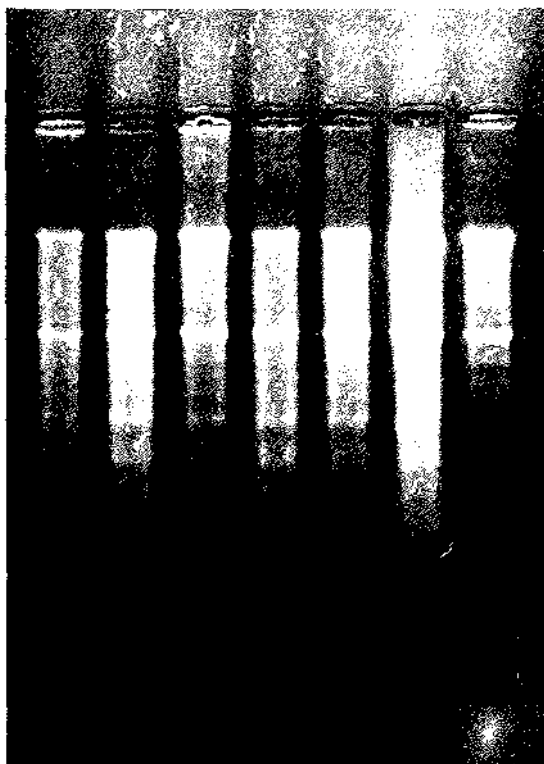
1. Cow #87, day 0.
2. Cow #87, day 5.
3. Cow #87, day 10.
4. Cow #87, day 14.
5. Cow #87, day 19.
6. Cow #87, day 24.
7. Cow #198, day 25.

B. Northern blot of gel A probed with radiolabelled bovine lactoferrin cDNA (PM 1).

Figure 3.8 Denaturing agarose gel electrophoresis and northern blot analysis of bovine mammary RNA isolated at different stages of involution.

A. Ethidium bromide-stained gel

1 2 3 4 5 6 7



B. Northern blot probed with PM 1 DNA

1 2 3 4 5 6 7



mammary tissue as involution progresses. For example, if the northern blot results from cow #87, days 5 and 19 (lanes 2 and 5, respectively) are compared, there is a notable increase in the amount of mRNA coding for lactoferrin in the later tissue biopsy. The intensity of the ribosomal RNA bands on the ethidium bromide-stained gel indicates that there was approximately the same amount of RNA loaded in these lanes. Therefore, the increase in signal from the northern blot probed with PM 1 cDNA indicates an increase in the relative amount of lactoferrin present in the later sample.

3.3.2 Isolation of Poly A⁺ RNA from total cellular RNA

Poly A⁺ RNA was isolated from involuted bovine mammary total cellular RNA by affinity chromatography on oligo(dT)-cellulose as described in 3.2.2.2. Figure 3.9 A illustrates the typical elution profile of total cellular RNA from oligo(dT)-cellulose. The poly A⁺ RNA fraction was then loaded onto the affinity column for a second passage through the column in an effort to increase the purity of the poly A⁺ fraction. The elution profile of RNA from the second affinity chromatography step is shown in figure 3.9 B. Table 3.4 shows selected data for the purification of poly A⁺ RNA from total cellular RNA isolated from the mammary tissue from cow #198.

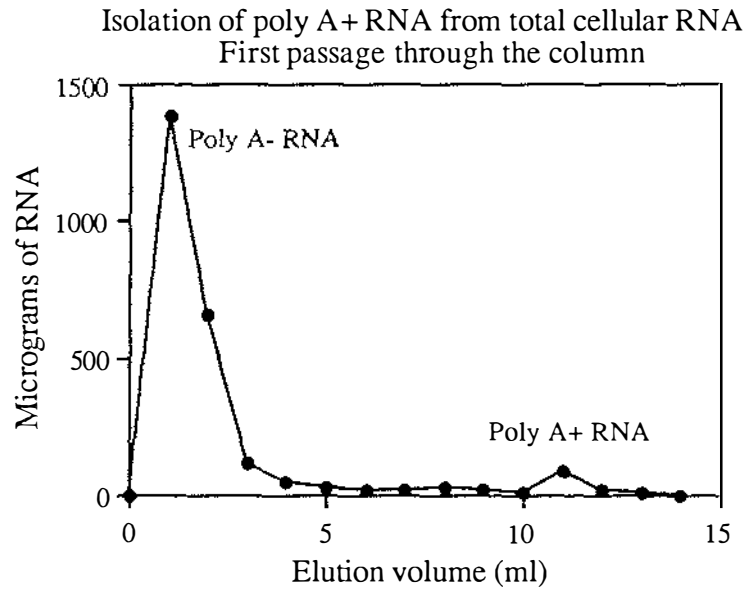
Table 3.4 Purification data for poly A⁺ RNA isolated from total cellular RNA by affinity chromatography on oligo(dT)-cellulose.

Sample	First passage through column			Second passage through column		
	Poly A ⁺ RNA isolated (μg)	Percent Poly A ⁺ RNA of tcRNA	Percent recovery from column	Poly A ⁺ RNA isolated (μg)	Percent Poly A ⁺ RNA of tcRNA	Percent recovery from column
1	154.69	4.59	ND	51.92	1.77	82
2	104.28	4.26	88	44.44	1.82	90.3

ND = not determined.

The data in table 3.4 shows that the poly A⁺ RNA represents a small fraction of the total cellular RNA isolated from mammary tissue. The messenger (or

A.



B.

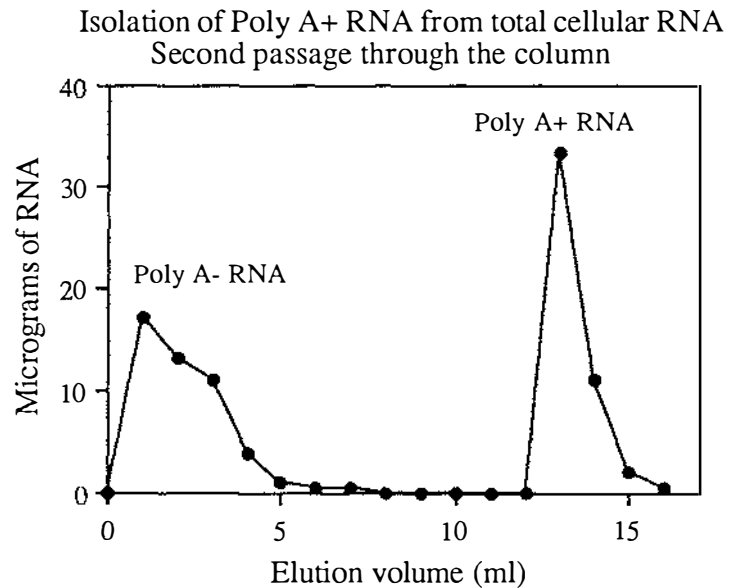
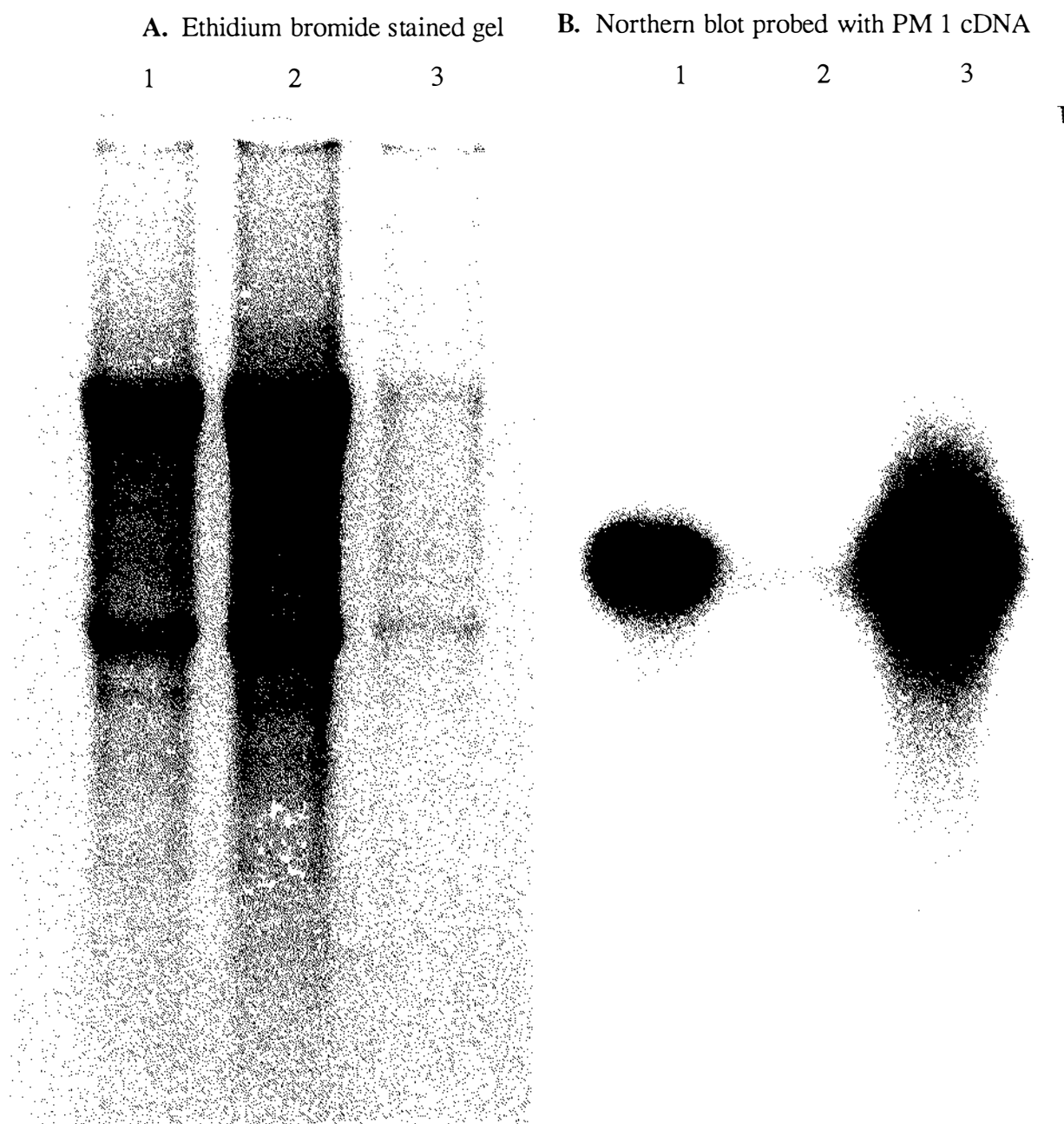


Figure 3.9 Isolation of poly A⁺ RNA from total cellular RNA by affinity chromatography on oligo(dT)-cellulose. The poly A⁺ peak from the first column run was chromatographed a second time in order to further purify the sample.

poly A⁺) RNA fraction of mammalian cells is typically only 1 to 5% of the total cellular RNA. The high percent recovery from the affinity column indicates that most of the RNA loaded onto the column was recovered in the subsequent elution steps.

Samples of total cellular, poly A⁺, and poly A⁻ RNA were analysed by denaturing agarose gel electrophoresis and were transferred to nitrocellulose filters. The filters were probed with the 3'-most 800 base pairs of the bovine lactoferrin cDNA (refer to clone PM 1, section 3.3.4.2). Figure 3.10 shows a denaturing agarose gel and the corresponding northern blot of total cellular, poly A⁺, and poly A⁻ RNA. The poly A⁺ sample shown in this figure had only one passage through the oligo(dT)-cellulose affinity chromatography column. This may explain the presence of small amounts of ribosomal RNA visible in the ethidium bromide-stained agarose gel. The second passage of the sample through the affinity column removed these rRNA contaminants from the poly A⁺ RNA (data not shown). The concentration of bovine lactoferrin messenger RNA sequences in the poly A⁺ sample is clearly illustrated. The intensity of the signal on the autoradiograph of the northern blot is much greater for the poly A⁺ RNA than the total cellular RNA. This is despite the fact that there was approximately three times more total cellular RNA loaded onto the gel than poly A⁺ RNA. The depletion of messenger RNA coding for bovine lactoferrin from the poly A⁻ RNA fraction is also clearly illustrated. 7.2 µg of total cellular RNA and 10.6 µg of poly A⁻ RNA were loaded into adjacent lanes on the denaturing gel. The signal on the autoradiograph from the total cellular RNA is much more intense than the signal from the poly A⁻ RNA sample.



A. Gel details: 1.5% (w/v) agarose gel containing 2.2 M formaldehyde and stained with ethidium bromide.

Loading details:

1. 7.2 μg of total cellular RNA.
2. 10.6 μg of poly A⁻ RNA.
3. 2.65 μg of poly A⁺ RNA.

B. Northern blot of gel A probed with ³²P-labelled PM 1 cDNA.

Figure 3.10 Northern blot analysis of total cellular, poly A⁺ and poly A⁻ RNA.

3.3.3 Synthesis of double-stranded cDNA using the BRL cDNA Synthesis System

Complementary DNA synthesis from poly A⁺ RNA isolated from involuted bovine mammary tissue was attempted by three different methods as outlined in 3.2.3. The first method of cDNA synthesis was with a BRL cDNA Synthesis System and is described in section 3.2.3.1.1. First strand cDNA synthesis from poly A⁺ RNA was primed with oligo (dT)₁₂₋₁₈ oligonucleotides and catalysed by M-M⁺LV reverse transcriptase. Second strand synthesis was with *E.coli* DNA polymerase I in the presence of RNase H which partially degrades the RNA template. The first and second strand synthesis was followed by the incorporation of [α -³²P] dCTP into acid precipitable products. Table 3.5 shows radiolabel incorporation data from selected attempts to synthesize cDNA using the BRL cDNA synthesis system.

cDNA synthesis using the BRL cDNA Synthesis System had limited success as shown in table 3.5. The expected copying efficiency of the messenger RNA into first strand DNA is around 30% (BRL cDNA Synthesis System instruction manual). Only one of the syntheses (#4) listed in table 3.5 had a copying efficiency close to the expected copying efficiency. The generally low copying of mRNA into first strand cDNA products may be due to a variety of factors. Firstly, the quality of the template RNA has a major influence on the ability of the reverse transcriptase to copy the the RNA sequence into first strand cDNA products. Not only does the RNA have to be free of nuclease degradation but it must also be free of any contaminants such as proteins and chemicals used in the purification procedure. The poly A⁺ RNA sample used to direct first strand synthesis in attempt #4 (table 3.5) had been resuspended and ethanol precipitated twice before being resuspended in DEPC-H₂O for cDNA synthesis. This procedure may have diluted out or washed away the contaminants that were inhibiting the other first strand cDNA syntheses.

An alternative reason is that the tracer reaction does not give an accurate indication of the cDNA synthesis in the main, unlabelled reaction. This is of particular note in synthesis #3 from table 3.5. The first strand synthesis tracer reaction indicated that approximately 0.5 μ g of DNA products had been

Table 3.5 Radiolabel incorporation data for first and second strand cDNA synthesis using the BRL cDNA Synthesis System.

First strand cDNA synthesis was directed by bovine mammary poly A⁺RNA.

cDNA synthesis	Amount of Poly A ⁺ RNA used (μg)	Incorporation of ³² P into 1 st strand products (%) ¹	Yield of 1 st strand cDNA (μg) 2	Copying efficiency (%) 3	Incorporation of ³² P into 2 nd strand products (%) ¹	Yield of 2 nd strand cDNA (μg) 4	Yield of double stranded cDNA (μg) ⁵
#1	10	3.0	0.794	7.9	21.7	1.147	2.29
#2	9.4	1.3	0.342	3.6	12.0	0.632	1.27
#3	10	1.8	0.466	4.7	58.3	3.079	6.16
#4	5	6.8	1.792	35.8	26.1	1.380	2.76

Calculations: 1 Incorporation of ³²P into cDNA products (%) = TCA precipitable CPM per μl/total CPM per μl x 100.

2 Yield of first strand cDNA. Specific activity (CPM per pmol dCTP) = Total CPM per 5 μl/pmol dCTP per 5 μl (166 pmol dCTP per 5 μl).

$$\text{Yield (μg DNA)} = \frac{\text{TCA ppt CPM per 5 μl} \times (345/10) \times (4 \text{ pmol per pmol dCTP}) \times 4}{(\text{CPM per pmol dCTP}) \times (3030 \text{ pmol dNTP per μg DNA})}$$

3 Copying efficiency (%) = (Yield of first strand cDNA (μg)/Amount of mRNA used to direct reaction (μg)) x 100

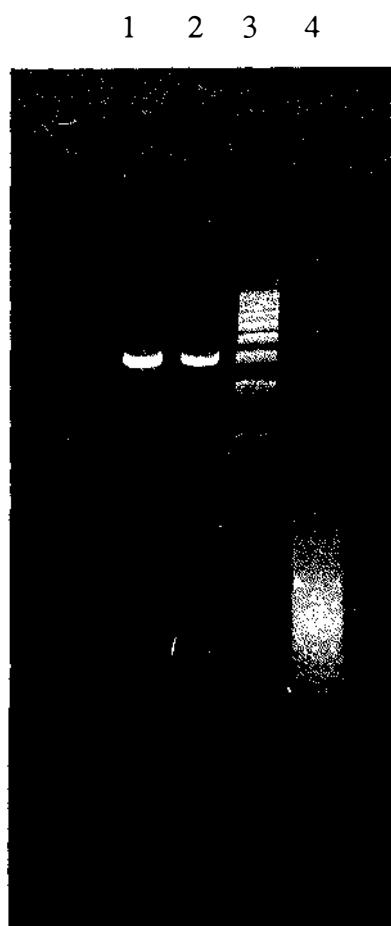
4 Yield of second strand cDNA (μg) = As for yield of first strand cDNA.

5 Yield of double stranded cDNA (μg) = 2 x yield of second strand cDNA.

synthesized in the main reaction. The second strand tracer reaction indicated that approximately 3.1 μg of second stranded DNA had been synthesized in the main reaction. This would amount to a total of around 6.2 μg double-stranded DNA synthesized from 0.5 μg of first strand cDNA. Clearly there was an error in one, if not both, of the estimations of the cDNA synthesis reactions. In order to determine which of the two tracer reactions gave the more reliable estimate of the amount of cDNA synthesized, a small aliquot of the unlabelled double-stranded cDNA from synthesis #3 was analysed by agarose gel electrophoresis. Figure 3.11 an agarose gel of double-stranded cDNA products from synthesis #3 (table 3.5). Approximately one-twelfth of the total unlabelled cDNA was loaded onto the gel. The intensity of the smear of the ethidium bromide-stained DNA in the cDNA lane suggests that the actual yield of double-stranded cDNA lay between the two estimates from the tracer reactions. If the first strand tracer reaction was correct then a total of around 1 μg of unlabelled double-stranded cDNA would have been produced. This amount of DNA would not have given a stain as intense as that shown in figure 3.11.

A possible explanation for the low estimate from the first strand tracer reaction is the inhibition of M-MLV reverse transcriptase by the [α - ^{32}P] dCTP used. This is of particular importance in the case described above (cDNA synthesis #3) because the radiolabel used in these tracer reactions had already gone through at least two half-lives before use. The presence of sulphur analogs resulting from the disintegration of ^{32}P may have adversely affected the M-MLV reverse transcriptase.

The first and second strand cDNA products were analysed by alkaline agarose gel electrophoresis. Figure 3.12 illustrates an autoradiograph of a typical alkaline agarose gel of radiolabelled cDNA products. Both the first and second strand products extend from a few hundred to around 7,000 base pairs in length. The presence of discrete bands in the cDNA tracts is probably due to the presence of abundant messenger RNA species in the poly A⁺ RNA sample used to direct the first strand cDNA synthesis.



Gel details: 0.7% (w/v) agarose gel stained with ethidium bromide.

Loading details: 1. 20 ng plasmid standard.

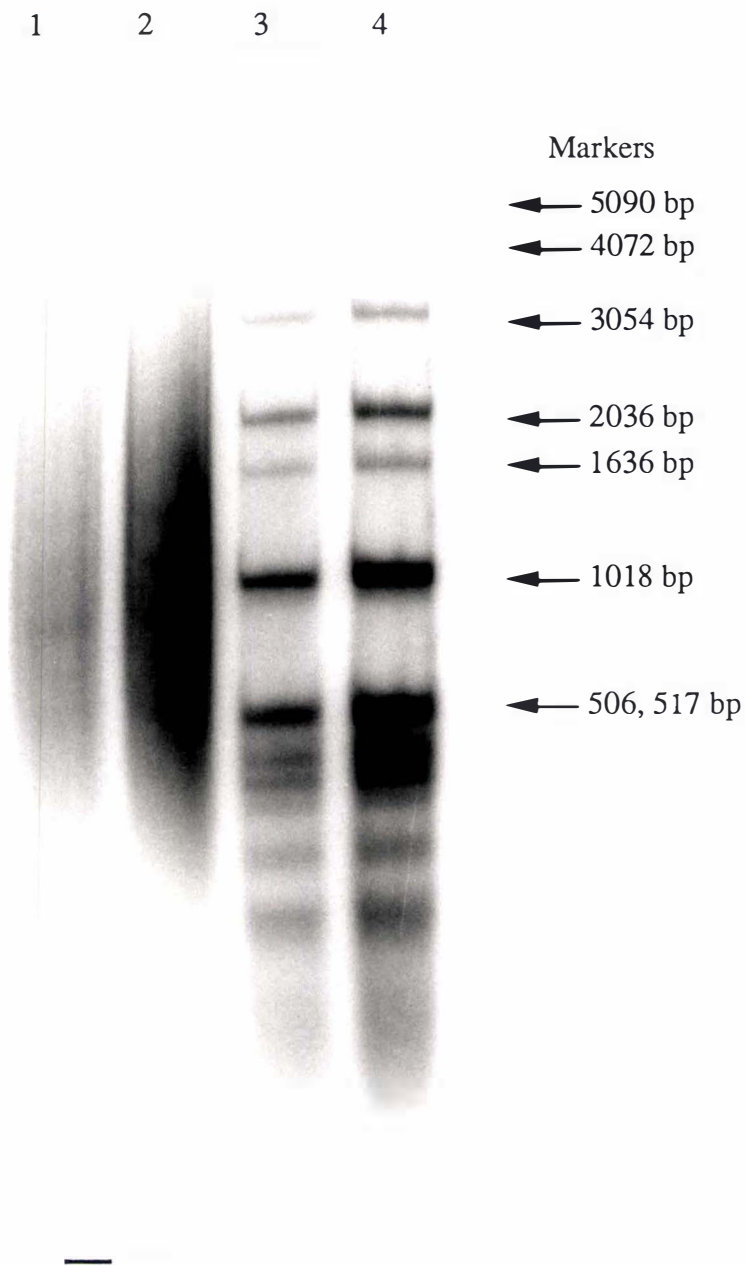
2. 10 ng plasmid standard.

3. BRL 1 kb ladder

4. Double-stranded cDNA sample from synthesis #3 (refer to table 3.5)

Approximately one-twelfth of the total double-stranded cDNA produced in this reaction.

Figure 3.11 Agarose gel electrophoresis of double-stranded cDNA synthesized from bovine mammary poly A⁺ RNA.



Gel details: 1.5% (w/v) agarose gel in an alkaline buffer (30 mM NaOH, 2 mM EDTA).

Loading details: 1 ^{32}P -labelled second strand cDNA products.

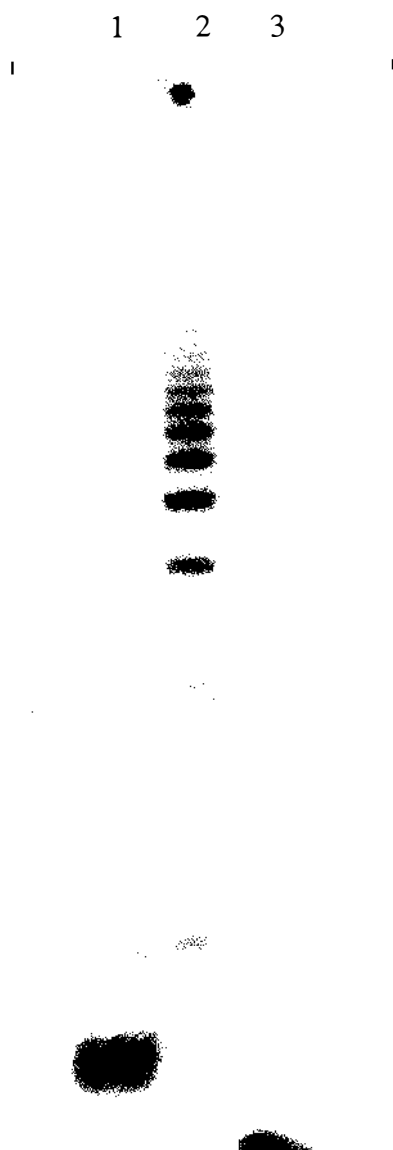
2. ^{32}P -labelled first strand cDNA products from synthesis #1 (refer to table 3.5).

3 and 4. ^{32}P -labelled BRL 1 kb ladder.

Figure 3.12 Autoradiograph of an alkaline agarose gel after electrophoresis of radiolabelled first and second strand cDNA products.

3.3.3.1 Cloning double-stranded cDNA into the bacteriophage λ gt11

Double-stranded cDNA was cloned into the cloning vector λ gt11 as described in section 3.2.3.2.1. Synthetic EcoRI linkers were covalently attached to the methylated, blunt-ended cDNA which facilitated cloning into the unique EcoRI site in λ gt11. The EcoRI linkers were phosphorylated in the presence of [γ - 32 P] ATP. A trial ligation of the phosphorylated EcoRI linkers was performed before they were ligated to the cDNA. The ligated linkers were separated by polyacrylamide gel electrophoresis in the presence of urea and visualised by autoradiography. Figure 3.12a illustrates the result of a trial ligation of phosphorylated EcoRI linkers. In the absence of T4 DNA ligase there is a discrete band on the X-ray film which represents the single radiolabelled EcoRI linker unit. In the presence of T4 DNA ligase the phosphorylated linkers were joined to form oligomers, which range from two to approximately twenty linker units in length. When digested with EcoRI, the ligated linkers were cleaved to yield a discrete band on the autoradiograph. Satisfied that the phosphorylated linkers could be ligated and then digested with EcoRI, the phosphorylated linkers were ligated to the blunt-ended, methylated double-stranded cDNA. Following ligation, the sample was digested with EcoRI to remove excess linkers. This EcoRI digestion also served to provide the cDNA with EcoRI cohesive ends. The small linker fragments resulting from the EcoRI digestion were removed from the cDNA sample by ultrafiltration. Table 3.6 shows typical data for the removal of 32 P-labelled EcoRI linker fragments after the digestion of the cDNA and linker ligation reaction with EcoRI.



Gel details: 20% (w/v) polyacrylamide gel containing 7 M urea.

Loading details: 1. 0.1 μg ^{32}P -labelled BRL EcoRI linkers incubated overnight with T4 DNA ligase and then digested with EcoRI.

2. 0.1 μg ^{32}P -labelled BRL EcoRI linkers incubated overnight with T4 DNA ligase.

3. 0.1 μg ^{32}P -labelled BRL EcoRI linkers incubated overnight without T4 DNA ligase.

Figure 3.12a Autoradiograph of a polyacrylamide gel after electrophoresis of the products from the trial ligation of phosphorylated EcoRI linkers.

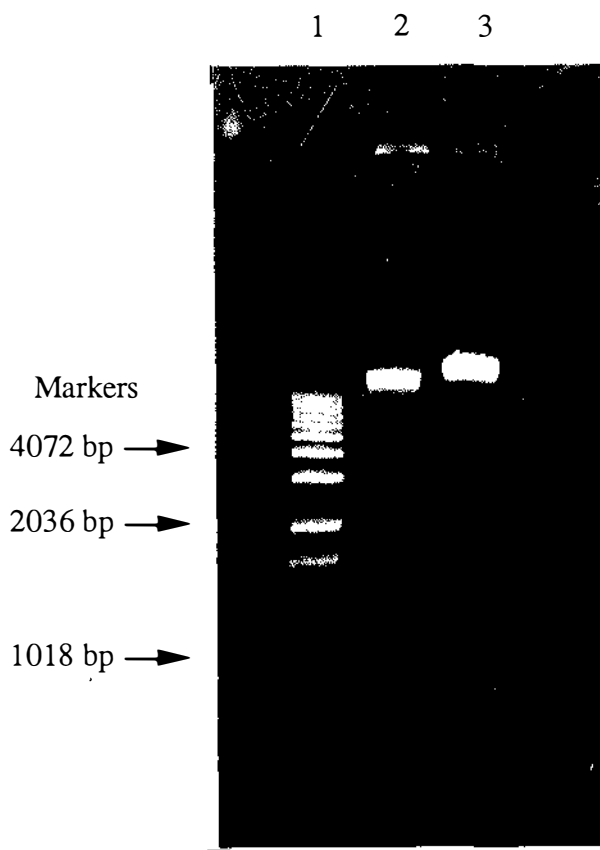
Table 3.6 Typical data for the removal of ³²P-labelled linker fragments from the cDNA:linker ligation reaction after digestion with EcoRI.

Sample	CPM (Cerenkov counting)	Volume of sample (μl)	CPM/μl
Filtrate from wash 1	108635.89	1000	108.6
Filtrate from wash 2	7054.32	1000	7.0
Filtrate from wash 3	775.10	1000	0.8
Filtrate from wash 4	361.70	1000	0.4
Retentate (cDNA:linkers)	3403.10	40	85.1

The data in table 3.6 clearly illustrates the removal of ³²P-labelled DNA fragments from the cDNA sample. After washing the cDNA:linker sample four times with 1 ml aliquots of sterile water the counts in the filtrate had decreased approximately three hundred fold. The radioactivity in the washed cDNA sample indicated the presence of EcoRI linkers ligated to the blunt ended, high molecular weight cDNA.

The cDNA with EcoRI cohesive ends was ligated into commercially-prepared λgt11 cloning arms cut with EcoRI and lacking 5' phosphate groups. The 5' phosphate groups had been enzymatically removed to prevent joining of the λgt11 arms during the ligation reaction. Dephosphorylation of the vector arms reduces the proportion of non-recombinant λgt11 in the resulting library. The ligation of the cDNA into the λgt11 cloning arms was followed by agarose gel electrophoresis of small aliquots of the ligation reaction. Figure 3.13 shows an ethidium bromide-stained gel of aliquot of a λgt11:cDNA ligation reaction. The λgt11 cloning arms in the marker track are clearly visible as two discrete bands. The discrete bands are absent in the λgt11:cDNA ligation sample. This indicates that the ligation was successful. Although not clearly visible in the reproduction of figure 3.13, there was a faint high molecular weight smear of DNA in the λgt11:cDNA ligation sample. This smear was absent in the other lanes of the gel and probably indicated the presence of concatemeric λgt11 DNA.

The ligated λgt11:cDNA sample was packaged to form viable λ particles using a commercially-prepared λ packaging extract. Aliquots of the packaged ligation reaction were introduced into *E. coli* Y1088 or Y1090 r⁺m⁻ cells and



Gel details: 0.7% (w/v) agarose gel stained with ethidium bromide.

Loading details:

1. BRL 1 kb ladder.
2. Approximately 0.2 μg of EcoRI digested $\lambda\text{gt}11$ cloning arms (BRL).
3. Bovine mammary cDNA (synthesis #3, refer to table 3.5) ligated into $\lambda\text{gt}11$ cloning arms (approximately one-tenth of the ligation reaction).

Figure 3.13 Agarose gel electrophoresis of cDNA ligated into the bacteriophage cloning vector $\lambda\text{gt}11$.

plated on LB plates in the presence of X-Gal and IPTG. IPTG acts as an inducer of the lac operon and X-Gal is a chromogenic substrate for the lacZ gene product β -galactosidase. The EcoRI cloning site of λ gt11 is in the coding region of the lacZ gene. When cDNA is introduced into the EcoRI site, the lacZ gene is disrupted and no longer codes for a functional protein. This results in the formation of clear plaques in the lawn of *E. coli* in the presence of X-Gal and IPTG. If the λ gt11 cloning arms join without the insertion of foreign DNA, the lacZ gene is restored and a functional lacZ gene product will result. These non-recombinant λ gt11 form blue plaques in a lawn of *E. coli* in the presence of X-Gal and IPTG. The titre of the λ gt11 cDNA libraries was estimated from the number of plaques that formed in the lawn of *E. coli*.

Bovine mammary cDNA λ gt11 library #1 was created from cDNA synthesized in synthesis #3 (see table 3.5) and had a titre of approximately 2.8×10^4 plaque forming units (pfu) per μ g of λ gt11 cloning arms. The fraction of non-recombinant clones (blue plaques) was approximately 1% of the total titre. The titre of this library was less than expected.

To determine whether the low titre was due to the cloning system or to the insert DNA, a trial packaging reaction was performed using 0.5 μ g of λ gt11 cloning arms ligated to 0.1 μ g of EcoRI-cut control DNA supplied with the cloning kit. The ligated control reaction was packaged under the same conditions as the cDNA library and plated on LB agar plates. The titre of the control λ gt11 library was 7.2×10^5 pfu/ μ g λ gt11 cloning arms, with approximately 1.1% non-recombinant clones. This value is close to the range expected (1×10^6 to 1×10^7 pfu/ml) from the manufacturers specifications. The low titre of the bovine mammary cDNA λ gt11 library #1 was therefore most likely due to the poor quality or quantity of the insert used.

Bovine mammary cDNA λ gt11 library #1 was amplified as described in section 3.2.3.2.1. The final titre of the amplified library was approximately 2.65×10^8 pfu/ml with approximately 20% non-recombinant clones. The increased proportion of non-recombinant clones in the amplified library (~1% and ~20% non-recombinant clones in the unamplified and amplified libraries, respectively) is due to the preferential amplification of lambda phage without cloned insert DNA.

3.3.4 Screening λ gt11 cDNA libraries for bovine lactoferrin sequences

The amplified bovine mammary cDNA λ gt11 library #1 was screened with human lactoferrin cDNA sequences to identify clones containing bovine lactoferrin sequences. Approximately 5×10^4 clones were screened at a density of around 5×10^3 pfu per plate. Duplicate nitrocellulose phage lifts were taken from each plate as described in 3.2.3. One of the duplicate filters was probed with the human lactoferrin cDNA clone PHL-41. The other filters were probed with the human lactoferrin cDNA clone PHL-44. As illustrated in figure 3.14, these two partial human lactoferrin cDNA clones overlap and together contain the entire coding region for the mature protein.

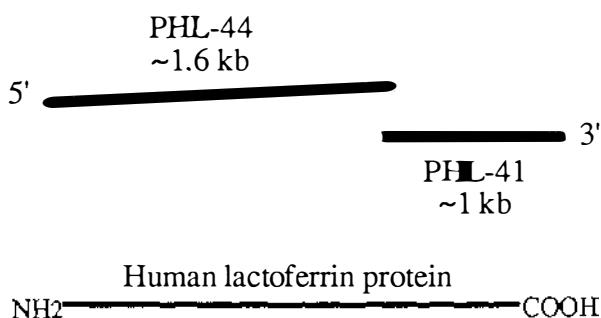


Figure 3.14 Schematic diagram to illustrate the relationship of the two human lactoferrin cDNA clones used to screen the bovine lactoferrin cDNA libraries. cDNA clone PHL-44 is approximately 1.6 kb in length and encodes the amino-terminal end of the human lactoferrin protein. This clone also has a small region of 5' untranslated sequence. Clone PHL-41 is approximately 1 kb in length and encodes the the C-terminal end of human lactoferrin. This clone contains the 3' untranslated region from the translation stop codon to the polyadenylate tail. The 5' end of PHL-41 overlaps the 3' end of PHL-44 by approximately 100 bp. Both cDNA clones were supplied cloned into the plasmid vector pUC 9.

Figure 3.15 shows a typical autoradiograph of a nitrocellulose filter phage lift from bovine mammary cDNA λ gt11 library #1. The intense dark spots are due to the hybridisation of the radiolabelled cDNA clone PHL-41 and indicate

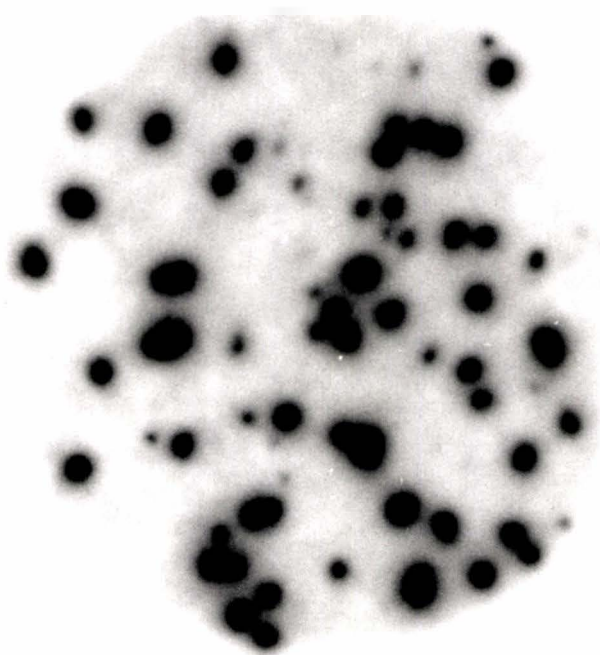


Figure 3.15 Autoradiograph of a nitrocellulose phage lift probed with ^{32}P -labelled PHL-41 cDNA.

the position of homologous sequences on the filters. The high number of 'positive' clones on each filter reflects the high proportion of messenger RNA molecules coding for lactoferrin in the poly A⁺ RNA sample used to direct first strand cDNA synthesis. Approximately 2% of the phage on the primary filters hybridised to the human lactoferrin cDNA clone PHL-41. The percentage of clones that hybridised to clone PHL-44 was ~0.5%.

The autoradiographs were aligned to orientation marks on the master plates. Several positive clones were picked from the agar plates and eluted into SM media. The titre of each isolate was determined and the clones were then plated at a density of approximately 200 pfu per plate. Duplicate filter lifts were taken from the second round plates and hybridised as above. Individual, well isolated plaques that hybridised to the human lactoferrin sequences were picked, eluted into SM and stored at 4°C over chloroform. Table 3.7 shows typical isolation data for twelve λ gt11 clones selected by the filter hybridisation technique described above. Isolates 11 and 12 are both negative controls picked from the agar plates.

The λ gt11 isolates from the second round screening were titred and then plated on LB agarose plates for DNA isolation by the plate lysate method (section 3.2.3).

3.3.4.1 Analysis of insert DNA from λ gt11 isolates

λ gt11 DNA was digested with restriction endonucleases and the digests were separated by electrophoresis on agarose gels. The size fractionated DNA was transferred to nitrocellulose filters and probed with the two partial human lactoferrin cDNA clones PHL-41 and PHL-44. Figure 3.16 shows an ethidium bromide-stained agarose gel and the corresponding Southern blots of three λ gt11 clones isolated from bovine mammary cDNA library #1. The λ gt11 DNA was digested with EcoRI. This enzyme cuts at the cloning site and should release the cDNA insert. Uncut DNA isolated from clones λ gt11Lf 1.2, 1.3 and 1.4 hybridised strongly to both PHL-41 and PHL-44. When digested with EcoRI, each clone gave a different restriction pattern. The approximate sizes of the EcoRI fragments that hybridised to the human lactoferrin cDNA clones are listed in table 3.8.

Table 3.7 Hybridisation data for isolates from bovine mammary cDNA λ gt11 library #1. Duplicate nitrocellulose filter lifts were probed with human lactoferrin clones PHL-41 and PHL-44.

Isolate	First round screening		Second round screening	
	Hybridised to PHL-41	Hybridised to PHL-44	Hybridised to PHL-41	Hybridised to PHL-44
λ gt11Lf 1.1	+	+	+	+
λ gt11Lf 1.2	+	+	+	+
λ gt11Lf 1.3	+	+	+	+
λ gt11Lf 1.4	+	+	+	$\pm$
λ gt11Lf 1.5	+	+	+	-
λ gt11Lf 1.6	+	+	+	-
λ gt11Lf 1.7	+	+	+	-
λ gt11Lf 1.8	+	+	+	-
λ gt11Lf 1.9	+	+	+	-
λ gt11Lf 1.10	+	+	+	-
λ gt11Lf 1.11	-	-	-	-
λ gt11Lf 1.12	-	-	-	-

Notes: + indicates hybridisation to the partial human lactoferrin cDNA clones. - indicates no hybridisation to the partial human lactoferrin cDNA clones. $\pm$ indicates very weak hybridisation to the partial human lactoferrin cDNA clones.

Table 3.8 The approximate length of the EcoRI fragments released from the λ gt11 clones illustrated in figure 3.15.

Clone	Length of EcoRI fragments (bp) that hybridise to	
	PHL-41	PHL-44
λ gt11Lf 1.2	500,~100	~100
λ gt11Lf 1.3	600,~100	~100
λ gt11Lf 1.4	~100	~100

Notes: The size of the ~100 bp fragment is approximate only. The low molecular weight markers were not visible in this region.

A. Gel details: 1.2% (w/v) agarose gel stained with ethidium bromide.

Loading details: 1. BRL 1 kb ladder.

2 and 3. Clone λ gt11Lf 1.2.

4 and 5. Clone λ gt11Lf 1.3.

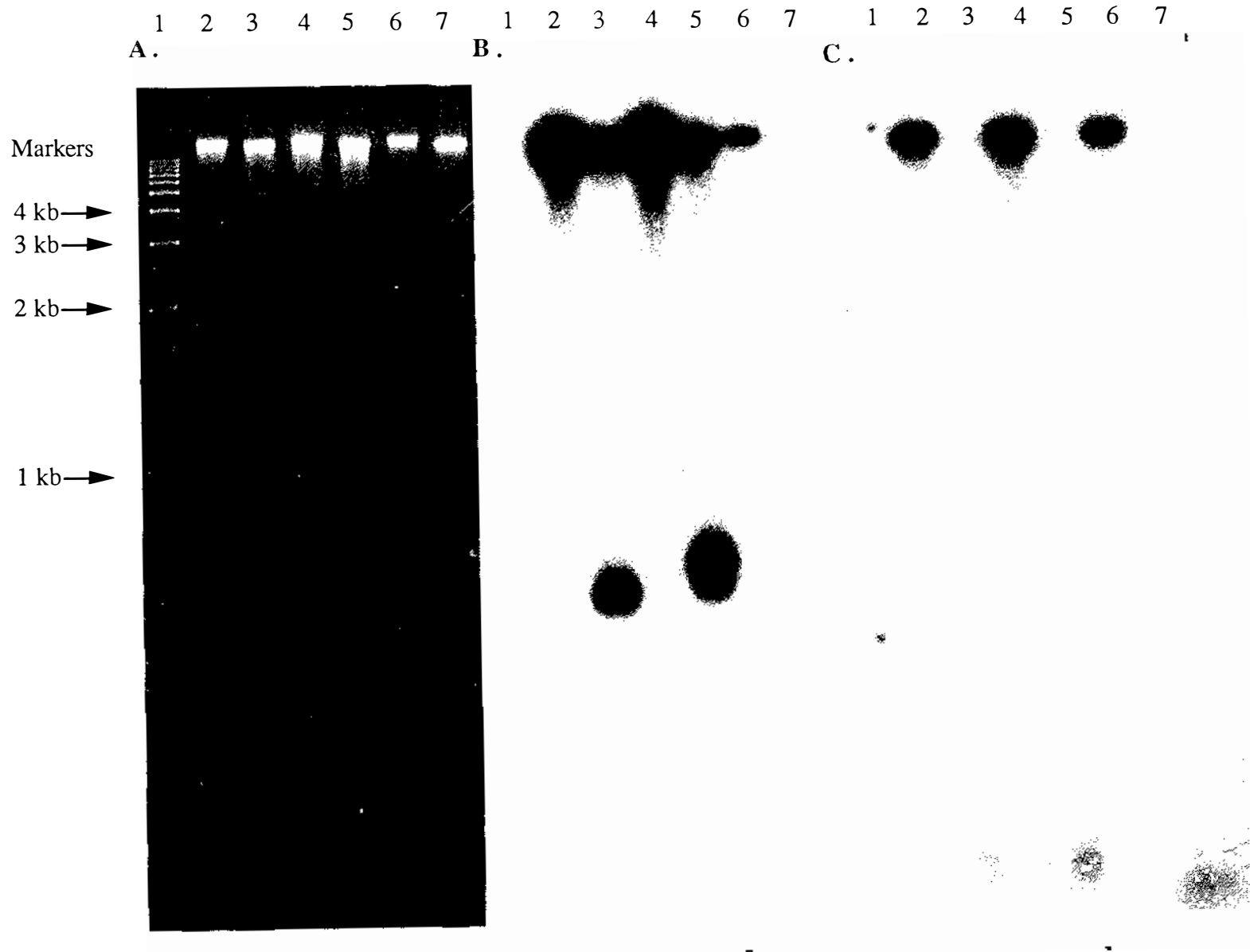
6 and 7. Clone λ gt11Lf 1.4.

DNA in lanes 3, 5 and 7 was digested with EcoRI.

B. Southern blot of gel A probed with ^{32}P -labelled human lactoferrin cDNA 3' clone PHL-41.

C. Southern blot of gel A probed with ^{32}P -labelled human lactoferrin cDNA 5' clone PHL-44.

Figure 3.16 Southern blot analysis of clones isolated from bovine mammary cDNA λ gt11 library #1. DNA isolated from clones λ gt11Lf 1.2, 1.3 and 1.4 was digested with EcoRI and probed with PHL-41 and PHL-44.



The results from the Southern blot illustrated in figure 3.16 and summarised in table 3.8, suggests that there are EcoRI restriction sites within the cloned DNA. In an attempt to determine the complete length of the cloned insert DNA, the λ gt11 isolates were digested with MluI. This 'rare cutter' restriction enzyme cuts λ gt11 DNA on either side of the EcoRI cloning site as depicted diagrammatically in figure 3.17. The insert DNA is released from the vector with approximately 2 kb of λ gt11 DNA. The size of the insert, providing that it does not contain MluI restriction sites, can be calculated by subtracting the length of λ gt11 DNA fragments from the total length of the DNA fragment that hybridises to the human cDNA probes.

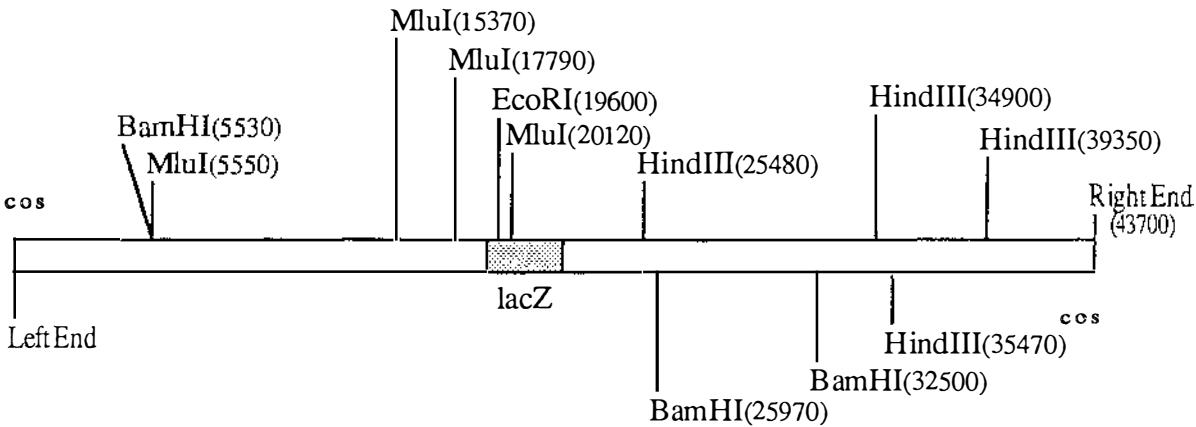


Figure 3.17 Partial restriction map of λ gt11 DNA illustrating the position of the MluI sites on either side of the unique EcoRI site.

DNA prepared from the twelve λ gt11 clones isolated from the second round plates was digested with MluI, separated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed with PHL-41 and PHL-44 (see figure 3.18).

As expected, DNA isolated from the negative control clones, λ gt11Lf 1.11 and 1.12, did not hybridise to either PHL-41 or PHL-44. However, DNA isolated from λ gt11Lf 1.1 did not hybridise to either probe. A possible explanation for this is that a 'false positive' clone was picked from the second round plate.

Human lactoferrin clone PHL-41 hybridised to a 2.6 kb MluI fragment from clone λ gt11Lf 1.4 and to a 3.2 kb MluI fragment from clones λ gt11Lf 1.2, 1.3, 1.5, 1.7, 1.8, 1.9, and 1.10. The insert size in the later clones is therefore close to 1.2 kb, while the size of the cDNA insert in clone λ gt11Lf 1.4 is approximately 0.6 kb. DNA from clone λ gt11Lf 1.6 was analysed by gel electrophoresis as an uncut marker and therefore the size of the insert in this clone can not be determined from this figure.

Only clones λ gt11Lf 1.2, 1.3 and 1.4 hybridised to human lactoferrin cDNA probe PHL-44. The MluI fragments that hybridised to PHL-44 are the same size as those that hybridised to PHL-41. That is, a 2.6 kb fragment from λ gt11Lf 1.4 and a 3.2 kb fragment from clones λ gt11Lf 1.2 and 1.3. These results reveal an anomaly between the hybridisation and restriction site analysis data. When digested with MluI, clones λ gt11Lf 1.5, 1.7, 1.8, 1.9, and 1.10 had similar restriction and hybridisation profiles as clones λ gt11Lf 1.2 and 1.3 with the exception that the former clones did not hybridise to PHL-44. That is, all of these clones appeared to have inserts of approximately the same size, but they did not all hybridise equally. Furthermore, clone λ gt11Lf 1.4 hybridised to both the 3' and 5' human lactoferrin cDNA probes (PHL-41 and PHL-44 respectively) but the insert DNA appeared to be considerably shorter than several clones that hybridised only to the 3' probe. The cDNA insert in clone λ gt11Lf 1.4 was estimated to be approximately 0.6 kb, around half the size of the inserts in clones λ gt11Lf 1.5, 1.7, 1.8, 1.9, and 1.10.

In an attempt to resolve some of these anomalies, clone λ gt11Lf 1.10 was digested with EcoRI, size fractionated by agarose gel electrophoresis and transferred to nitrocellulose by capillary blotting. Figure 3.19 illustrates an ethidium bromide-stained agarose gel and the corresponding autoradiograph of the Southern blot of DNA isolated from clone λ gt11Lf 1.10 digested with EcoRI. The Southern blot was probed with radiolabelled PHL-41. Clone λ gt11Lf 1.10 did not hybridise to the 5' human lactoferrin cDNA PHL-44 (data not shown). Undigested λ gt11Lf 1.10 DNA hybridised strongly to the 3' human lactoferrin cDNA PHL-41. A band at approximately 800 bp hybridised strongly to PHL-41 in the lane containing EcoRI digested λ gt11Lf 1.10 DNA. Just visible in this lane is a band that hybridised at approximately 1,200 bp. This weak signal on the autoradiograph may indicate the presence of a partial product resulting from the incomplete digestion of the λ clone

A. Gel details: 1% (w/v) agarose gel stained with ethidium bromide.

Loading details: 1. BRL 1 kb ladder.

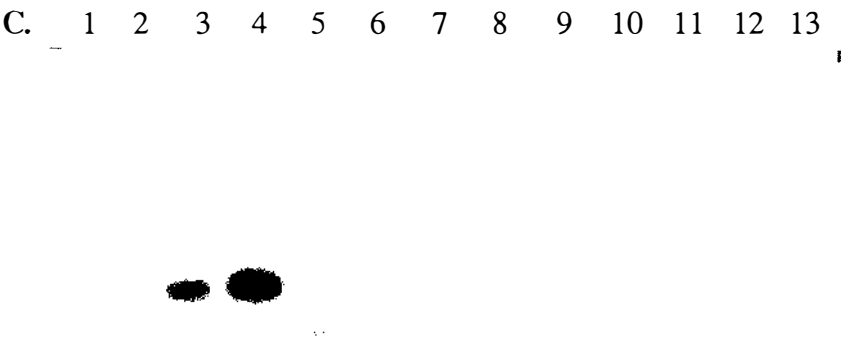
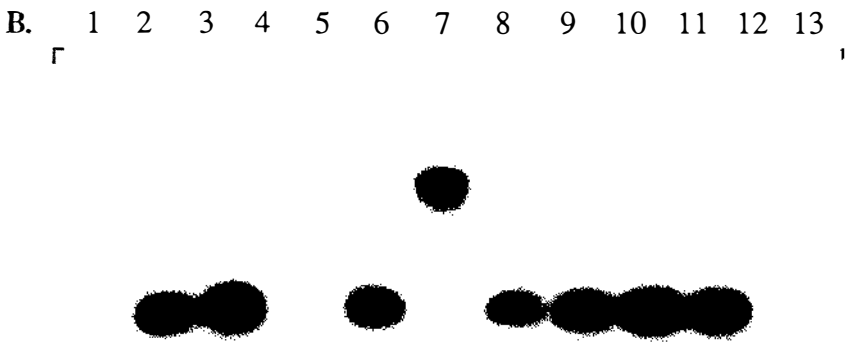
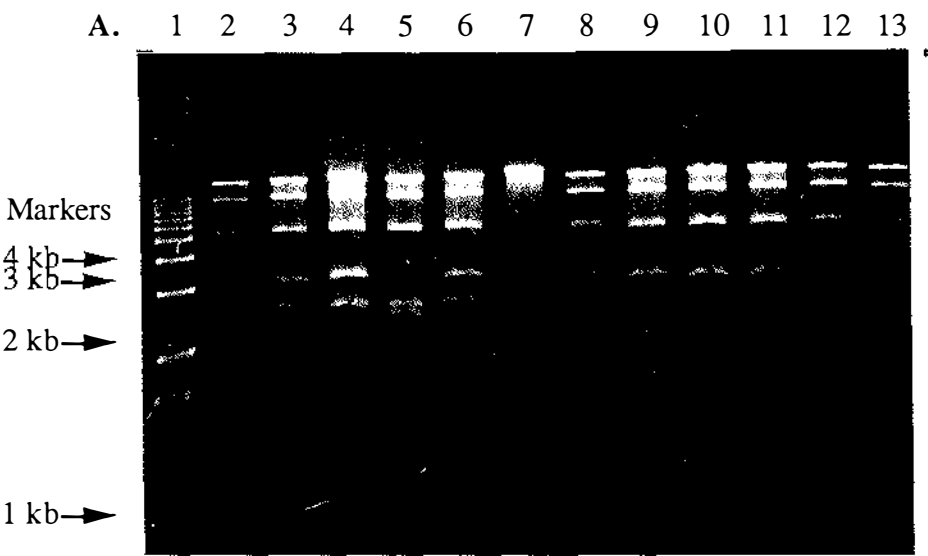
DNA was isolated from the following clones and digested with MluI. DNA in lane 7 (λ gt11Lf 1.6) was not digested.

2. λ gt11Lf 1.1.
3. λ gt11Lf 1.2.
4. λ gt11Lf 1.3.
5. λ gt11Lf 1.4.
6. λ gt11Lf 1.5.
7. λ gt11Lf 1.6 (uncut).
8. λ gt11Lf 1.7.
9. λ gt11Lf 1.8.
10. λ gt11Lf 1.9.
11. λ gt11Lf 1.10.
12. λ gt11Lf 1.11.
13. λ gt11Lf 1.12.

B. Southern blot of agarose gel illustrated in A probed with radiolabelled PHL-41 cDNA.

C. Southern blot of agarose gel illustrated in A probed with radiolabelled PHL-44 cDNA.

Figure 3.18 Agarose gel electrophoresis and corresponding Southern blots of λ gt11 isolates digested with MluI and probed with either PHL-41 or PHL-44.

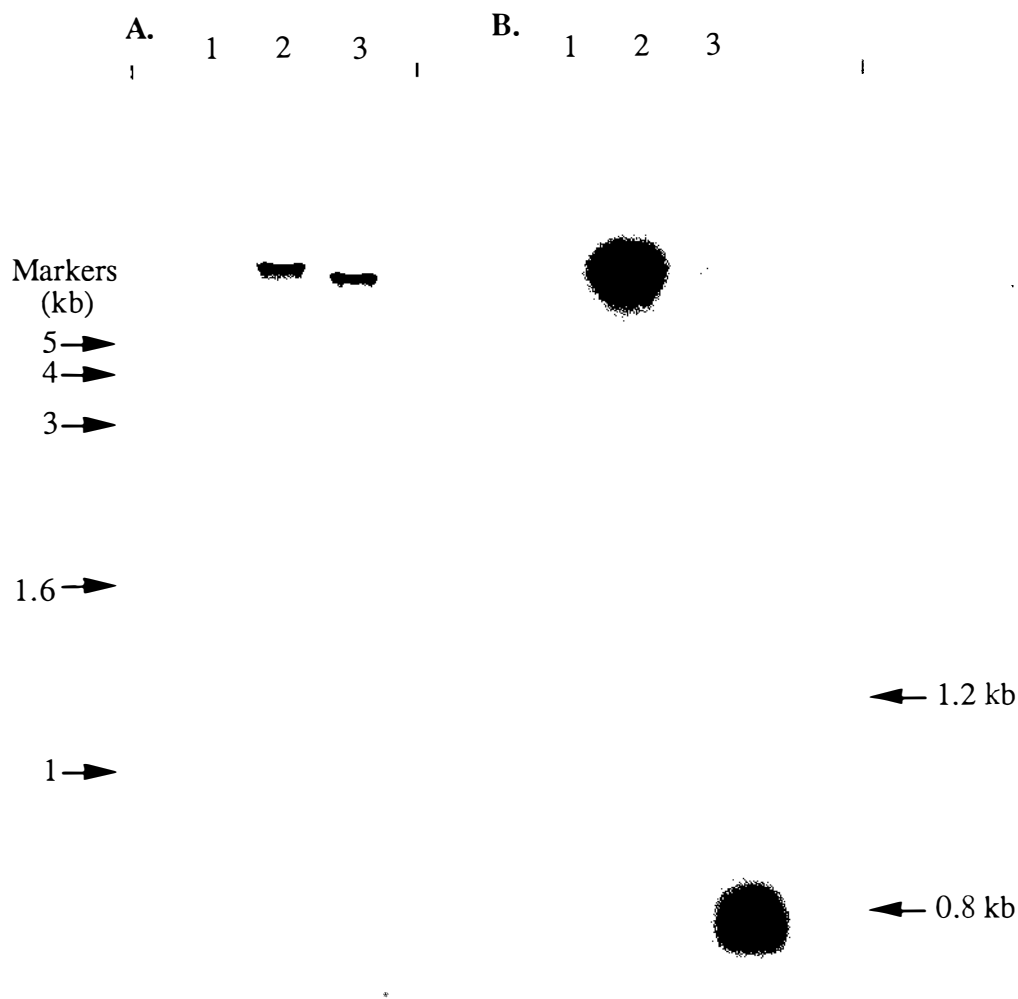


with EcoRI. The presence of this partial digestion product supports the assumption made earlier that the cDNA coding for bovine lactoferrin contains at least one internal EcoRI restriction site. The length of the 'partial' EcoRI fragment coincides with that estimated for the complete cDNA insert from the MluI restriction data (~1.2 kb).

Although this result supports the hypothesis of EcoRI restriction sites within the insert, it does little to solve the enigma of the different hybridisation profiles. The EcoRI fragment from clone λ gt11Lf 1.10 that hybridised to PHL-41 was approximately 800 bp long. The EcoRI fragments that hybridised to PHL-41 from λ gt11Lf 1.2 and 1.3 were close to 500 and 600 bp respectively.

One of several phenomena may be responsible for these findings. Firstly, each of these clones may have different lengths of 3' poly A tracts. This may have arisen from the oligo(dT) oligonucleotide used to prime the first strand cDNA synthesis hybridising in different positions along the poly A tail at the 3' end of messenger RNA. Alternatively, the oligo(dT) primer may have hybridised to an 'A-rich' region 5' to the poly A tail in the messenger RNA, and thus truncated the resulting cDNA. Likewise, one or more of these clones may be artifacts due to aberrant ligation or to deletion during the cloning procedure. It must also be noted that these clones may represent a population of related, but distinct messenger RNA species in the involuting bovine mammary gland. At this point none of the above explanations could be ruled out.

One of the clones, λ gt11Lf 1.10, was characterised further by subcloning and DNA sequencing.



A. Gel details: 1% (w/v) agarose gel stained with ethidium bromide.

Loading details: 1. BRL 1 kb ladder.

2. Undigested λ gt11Lf 1.10 DNA.

3. λ gt11Lf 1.10 DNA digested with EcoRI.

B. Southern blot of gel illustrated in A, probed with radiolabelled PHL-41 cDNA.

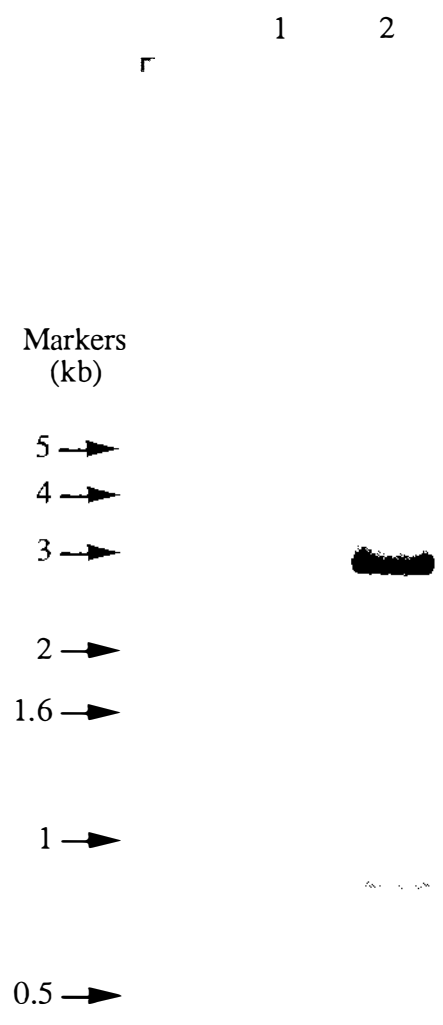
Figure 3.19 Southern blot analysis of clone λ gt11Lf 1.10 isolated from bovine mammary cDNA λ gt11 library #1. The DNA was digested with EcoRI and the Southern plot was probed with radiolabelled PHL-41 cDNA.

3.3.4.2 Subcloning and sequencing the 800 base pair EcoRI fragment from λ gt11Lf 1.10

Approximately 500 ng λ gt11Lf 1.10 DNA was digested with EcoRI and then co-precipitated with approximately 300 ng of EcoRI digested, phosphatased pGEM-2™ DNA. The resuspended DNA was ligated and transformed into competent *E. coli* DH1 cells. This 'shotgun' cloning approach was used as the isolation of the small (800 bp) fragment from the large λ vector proved to cumbersome. Plasmid DNA was isolated from several bacterial colonies that grew in the presence of ampicillin. The DNA was digested with EcoRI and separated by agarose gel electrophoresis. Of the twelve clones that were analysed, only one had an 800 bp insert when digested with EcoRI. This clone was called PM 1. Figure 3.20 shows an ethidium bromide-stained agarose gel of PM 1 DNA digested with EcoRI.

Figure 3.21 illustrates an ethidium bromide-stained agarose gel and the corresponding Southern blot, probed with PHL-41, of PM 1 DNA digested with various restriction endonucleases. Digestion of PM 1 DNA revealed the presence of a PstI site within the 800 bp cloned insert. This enzyme was used to further fragment the cloned cDNA for subcloning prior to DNA sequencing.

PM 1 DNA was subcloned into M13mp18 and M13mp19 for sequencing by the dideoxy chain termination method described by Sanger *et al.* (1977) (Sequenase Version 2 sequencing kit, United States Biochemicals). Figure 3.22 illustrates the sequencing strategy used to determine the complete DNA sequence of the cDNA clone PM 1.



Gel details: 1% (w/v) agarose gel stained with ethidium bromide.
Loading details: 1. BRL 1 kb ladder.
2. PM 1 DNA digested with EcoRI.

Figure 3.20 Agarose gel electrophoresis of PM 1 DNA digested with the restriction endonuclease EcoRI.

A. Gel details: 1.2% (w/v) agarose gel stained with ethidium bromide.

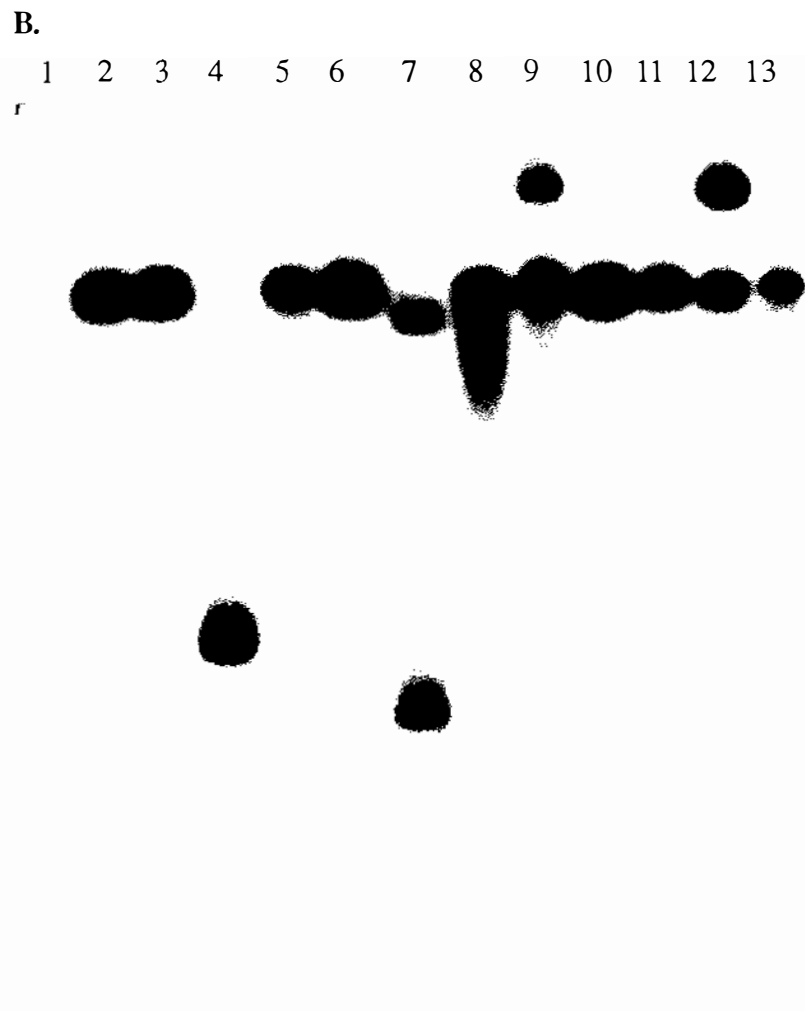
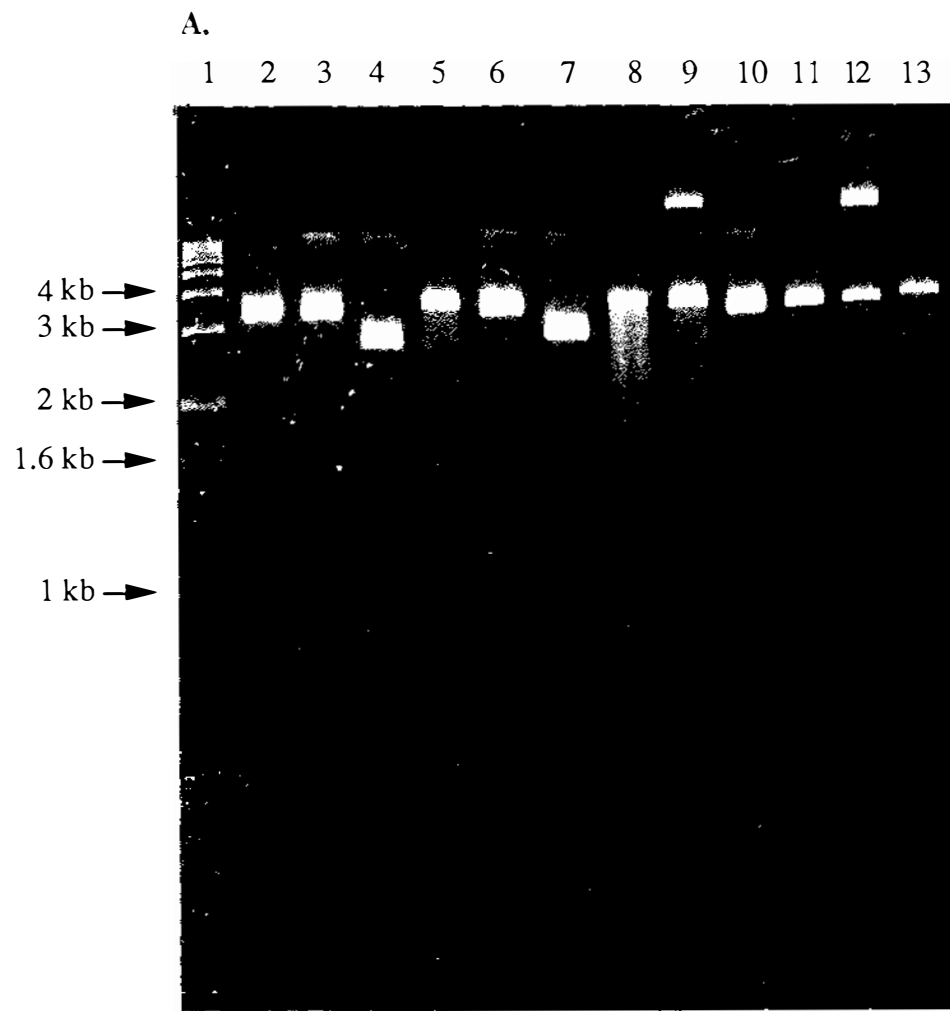
Loading details: 1. BRL 1 kb ladder.

Lanes 2 to 13 contain DNA from clone PM 1 digested with the following restriction endonucleases:

2. AvaI.
3. BamHI.
4. EcoRI.
5. HincII.
6. HindIII.
7. PstI.
8. PvuI.
9. SacI.
10. SalI.
11. SmaI.
12. SphI.
13. XbaI.

B. Southern blot of agarose gel illustrated in A, probed with radiolabelled PHL-41 DNA.

Figure 3.21 Agarose gel electrophoresis and corresponding Southern blot of PM 1 DNA digested with various restriction endonucleases.



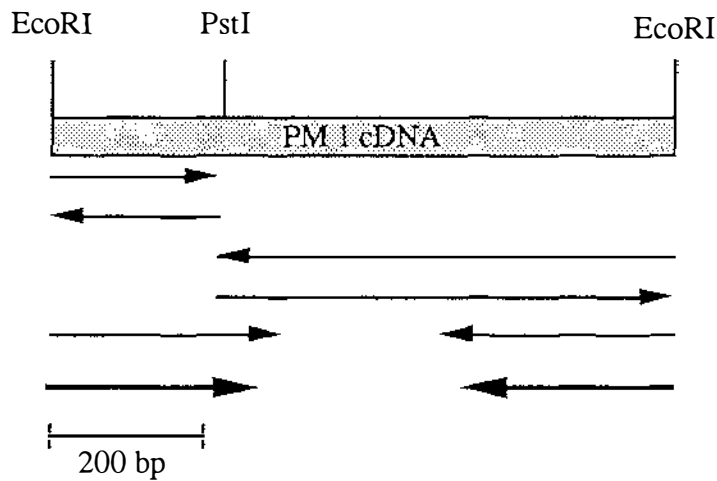


Figure 3.22 Sequencing strategy used to determine the complete nucleotide sequence of cDNA clone PM 1. The thin arrows represent the direction and extent of single-stranded sequencing (in M13mp18 or 19). The thick arrows represent the direction and extent of sequencing in the double-stranded plasmid vector pGEM-2™.

Figure 3.23 shows the complete nucleotide sequence of cDNA clone PM 1. The predicted amino acid sequence is listed below the nucleotide sequence. The deduced amino acid sequence shares sequence identity with with two of the tryptic peptides from bovine lactoferrin determined earlier in this study (refer to table 2.6, HPLC fractions 7, 11 and 12). The position of the two tryptic peptides is indicated by the hatched underlining. The amino acid sequence also has a high degree of sequence homology with the C-terminal of other transferrins. For example, the deduced amino acid sequence of clone PM 1 shares approximately 73% amino acid sequence identity with the corresponding region of human lactoferrin.

Figure 3.23 The nucleotide sequence and predicted amino acid sequence of cDNA clone PM 1.

GAATTCTTTAGTCAGAGCTGTGCCCCCTGGGGCTGACCCGAAATCCAGACTCTGTGCCTTG
-----+-----+-----+-----+-----+-----+60
CTTAAGAAATCAGTCTCGACACGGGGACCCCGACTGGGCTTTAGGTCTGAGACACGGAAC
E F F S Q S C A P G A D P K S R L C A L

TGTGCTGGCGATGACCAGGGCCTGGACAAGTGTGTGCCCAACTCTAAGGAGAAGTACTAT
-----+-----+-----+-----+-----+-----+120
ACACGACCGCTACTGGTCCCGGACCTGTTTACACACGGGTTGAGATTCTCTTCATGATA
C A G D D Q G L D K C V P N S K E K Y Y

GGCTATACCGGGGCTTTTCAGGTGCCTGGCTGAGGACGTTGGGGACGTTGCCTTTGTGAAA
-----+-----+-----+-----+-----+-----+180
CCGATATGGCCCCGAAAGTCCACGGACCGACTCCTGCAACCCCTGCAACGGAAACACTTT
G Y T G A F R C L A E D V G D V A F V K

AACGACACAGTCTGGGAGAACACGAATGGAGAGAGCACTGCAGACTGGGCTAAGAACTTG
-----+-----+-----+-----+-----+-----+240
TTGCTGTGTGACACCCTCTTGTGCTTACCTCTCTCGTGACGTCTGACCCGATTCTTGAAC
N D T V W E N T N G E S T A D W A K N L

AATCGTGAGGACTTCAGGTTGCTCTGCCTCGATGGCACCAGGAAGCCTGTGACGGAGGCT
-----+-----+-----+-----+-----+-----+300
TTAGCACTCCTGAAGTCCAACGAGACGGAGCTACCGTGGTTCCTTCGGACACTGCCTCCGA
N R E D F R L L C L D G T R K P V T E A

CAGAGCTGCCACCTGGCGGTGGCCCCGAATCACGCTGTGGTGTCTCGGAGCGATAGGGCA
-----+-----+-----+-----+-----+-----+360
GTCTCGACGGTGGACCGCCACCGGGGCTTAGTGCGACACCACAGAGCCTCGCTATCCCGT
Q S C H L A V A P N H A V V S R S D R A

GCACACGTGAAACAGGTGCTGCCACCAGCAGGCTCTGTTTGGGAAAAATGGAAAAAC
-----+-----+-----+-----+-----+-----+420
CGTGTGCACTTTGTCCACGACGAGGTGGTTCGTCGAGACAAACCCTTTTACCTTTTTTG
A H V K Q V L L H Q Q A L F G K N G K N

TGCCCCGACAAGTTTGTGTTGTTCAAATCTGAAACCAAAAACCTTCTGTTCAATGACAAC
-----+-----+-----+-----+-----+-----+480
ACGGGCGCTGTTCAAAACAAACAAGTTTAGACTTTGGTTTTTGGAAAGACAAGTTACTGTTG
C P D K F C L F K S E T K N L L F N D N

ACTGAGTGTCTGGCCAACTTGGAGGCAGACCAACGTATGAAGAATATTTGGGGACAGAG
-----+-----+-----+-----+-----+-----+540
TGA CTCACAGACCGGTTTGAACCTCCGTCTGGTTGCATACTTCTTATAAACCCCTGTCTC
T E C L A K L G G R P T Y E E Y L G T E

TATGTCACGGCCATTGCCAACCTGAAAAATGCTCAACCTCCCCGCTTCTGGAAGCCTGC
-----+-----+-----+-----+-----+-----+600
ATACAGTGCCGGTAACGGTTGGACTTTTTTACGAGTTGGAGGGGCGAAGACCTTCGGACG
Y V T A I A N L K K C S T S P L L E A C

GCCTTCCTGACGAGGTAAAGCCTGCAAAGAAGCTAGCCTGCCTCCCTGGGCCTCAGCTCC
-----+-----+-----+-----+-----+-----+660
CGGAAGGACTGCTCCATTTCGGACGTTTCTTCGATCGGACGGAGGGACCCGGAGTCGAGG
A F L T R *

TCCCTGCTCTCAGCCCCAATCTCCAGGCGCGAGGGACCTTCTCTCCCTTCCTGAAGTCG
-----+-----+-----+-----+-----+-----+720
AGGGACGAGAGTCGGGGTTAGAGGTCCGCGCTCCCTGGAAGGAGAGGGAAGGACTTCAGC

```

GATTTTGGCCAAGCTCATCAGTATTTACAATTCCCTGCTGTCATTTTAGCAAGAAATAAA
-----+-----+-----+-----+-----+-----+-----+780
CTAAAAACGGTTCGAGTAGTCATAAATGTTAAGGGACGACAGTAAAATCGTTCTTTATTT

ATTACAAATGCTGTTGATTTTCATTCCCTAATAAATAACCG
-----+-----+-----+-----+-----+820
TAATGTTTACGACAACATAAAGTAAGGGATTTTTTTTGGC

```

*Notes: *indicates the position of the putative termination codon TAA. The solid underlined sequences represent the polyadenylation signal and the polyadenylate tail near the 3' terminus of the cDNA clone. The polyadenylation signal (AATAAA) is approximately thirty bases 5' to the poly A tail. The sequence in bold at the 3' terminus of the cDNA indicates the 5' end of the EcoRI linker used to clone the molecule into λ gt11. Amino acid sequences showing identity with tryptic peptides from bovine lactoferrin are shown by hatched underlining. See text for further discussion.*

3.3.4.3 Screening λ gt11 library #1 for cDNA sequences 5' to PM 1

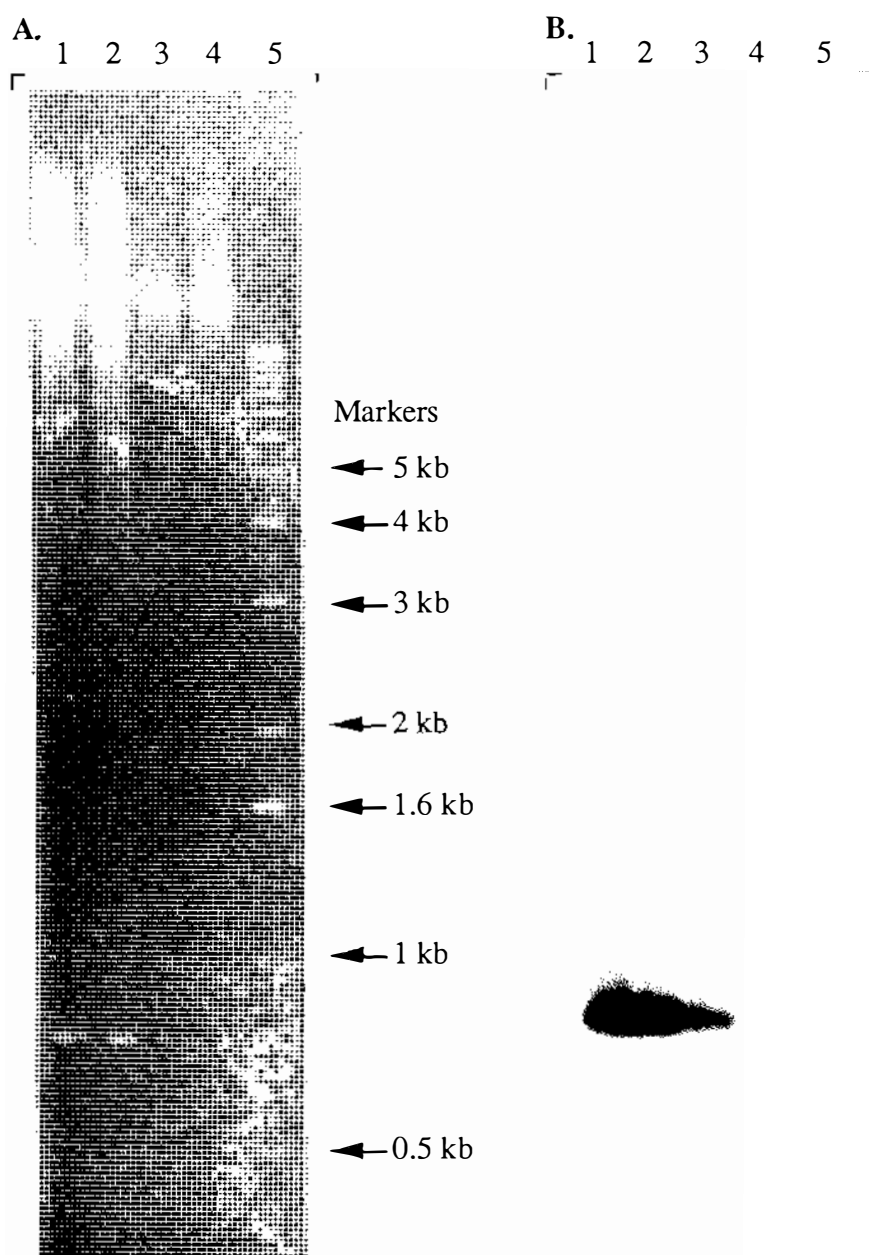
Bovine mammary cDNA λ gt11 library #1 was screened with the bovine lactoferrin 3' cDNA clone PM 1 and the human 5' cDNA clone PHL-44. Phage were plated at a density of approximately 4000 pfu per plate. Duplicate nitrocellulose filter lifts were taken from each of the twelve plates. One filter was hybridised with ^{32}P -labelled PM 1 DNA and the other was hybridised with labelled PHL-44 DNA. Clones were selected and isolated on their ability to hybridise to PHL-44. Several clones that hybridised to both probes were chosen for further analysis. Clones that hybridised to PM 1 but not PHL-44 were not selected. Table 3.9 illustrates the hybridisation profiles of fifteen clones isolated from second round plates.

Table 3.9 Hybridisation data for isolates from bovine mammary cDNA λ gt11 library #1. Duplicate nitrocellulose filter lifts were taken from second round plates and probed with lactoferrin cDNA clones PM 1 and PHL-44.

Isolate	Hybridised to PM 1	Hybridised to PHL-44
λ gt11Lf 2.1	-	+
λ gt11Lf 2.2	-	+
λ gt11Lf 2.3	-	+
λ gt11Lf 2.4	+	+
λ gt11Lf 2.5	+	+
λ gt11Lf 2.6	-	+
λ gt11Lf 2.7	-	+
λ gt11Lf 2.8	-	+
λ gt11Lf 2.9	-	+
λ gt11Lf 2.10	-	+
λ gt11Lf 2.11	-	+
λ gt11Lf 2.12	-	+
λ gt11Lf 2.13	-	+
λ gt11Lf 2.14	-	+
λ gt11Lf 2.15	-	-

Notes: + indicates hybridisation to the cDNA clones. - indicates no hybridisation to the cDNA clones.

DNA was isolated from the λ gt11 clones listed in table 3.9 and digested with EcoRI. The resulting DNA fragments were separated by agarose gel electrophoresis and blotted onto nitrocellulose filters. The filters were hybridised with the bovine lactoferrin cDNA clone PM 1 and the human lactoferrin cDNA clone PHL-44. Figure 3.24 illustrates the ethidium bromide-stained gel and the corresponding Southern blots of EcoRI digested λ gt11 DNA probed with PHL-44. Clones λ gt11Lf 2.12, 2.13 and 2.14 released an approximately 750 base pair fragment when digested with EcoRI. The insert DNA hybridised to PHL-44 (see figure 3.24) but not to PM 1 (data not shown). Of the other λ gt11 clones that were isolated from this round of screening (refer to table 3.9), clones λ gt11Lf 2.1, 2.2, 2.3, 2.7, 2.8, 2.9 and 2.10 had identical hybridisation profiles to clones 2.12, 2.13 and 2.14 (data not shown). That is, when digested EcoRI, DNA from these clones released an approximately 750 bp fragment which hybridised to PHL-44 but not to



A. Gel details: 1% (w/v) agarose stained with ethidium bromide.

Loading details: Lanes 1 to 4 contain λ gt11 DNA digested with EcoRI.

1. λ gt11Lf 2.12.
2. λ gt11Lf 2.13.
3. λ gt11Lf 2.14.
4. λ gt11Lf 2.15.
5. BRL 1 kb ladder.

B. Southern blot of gel illustrated in A probed with radiolabelled PHL-44 cDNA.

Figure 3.24 Agarose gel electrophoresis and Southern blot analysis of cDNA clones isolated from bovine mammary gland cDNA λ gt11 library #1.

PM 1 DNA. When digested with EcoRI, DNA isolated from λ gt11Lf 2.4 and 2.5 released two fragments. One fragment, approximately 400 bp in length, hybridised to PHL-44 but not to PM 1 DNA. The other EcoRI fragment, approximately 500 bp in length, hybridised to radiolabelled PM 1 DNA and not to PHL-44 (data not shown). The hybridisation profile of clones λ gt11Lf 2.4 and 2.5 supported the notion that there was at least one EcoRI site within the cDNA that codes for bovine lactoferrin. There remained the possibility, however, that clones λ gt11Lf 2.4 and 2.5 were the result of cloning artefacts that were produced by the ligation of two cDNA molecules with EcoRI cohesive ends into a single λ gt11 vector. For this reason, it was decided to continue with the characterisation of the 750 bp insert that was released from λ gt11Lf 2.13 DNA when digested with EcoRI.

3.3.4.4 Subcloning and sequencing of the 750 base pair EcoRI fragment from λ gt11Lf 2.13

DNA isolated from clone λ gt11Lf 2.13 was digested with EcoRI and separated by electrophoresis on a low gelling temperature agarose gel. The approximately 750 base pair fragment was excised from the gel and purified by adherence to glass beads. The isolated DNA fragment was subcloned into the plasmid vector pGEM-2™ (hereafter termed PM 2) and into the filamentous phage vectors M13mp18 and mp19. Figure 3.25 illustrates an ethidium bromide-stained agarose gel of PM 2 DNA digested with EcoRI and BamHI. This figure also shows PM 1 and λ gt11Lf 2.13 DNA digested with EcoRI. The cloned DNA was sequenced by the dideoxyribonucleotide chain terminator method of Sanger *et al.* (1977). Figure 3.26 shows the sequencing strategy used to determine the entire nucleotide sequence of the DNA insert from λ gt11Lf 2.13.



Gel details: 1% (w/v) agarose gel stained with ethidium bromide.

Loading details: 1. PM 1 DNA digested with EcoRI.

2. PM 2 DNA digested with EcoRI and BamHI.

3. PM 2 DNA digested with EcoRI.

4. λ gt11Lf 2.13 DNA digested with EcoRI.

5. BRL 1 kb ladder.

Figure 3.25 Agarose gel electrophoresis of cloned PM 2 DNA digested with various restriction endonucleases.

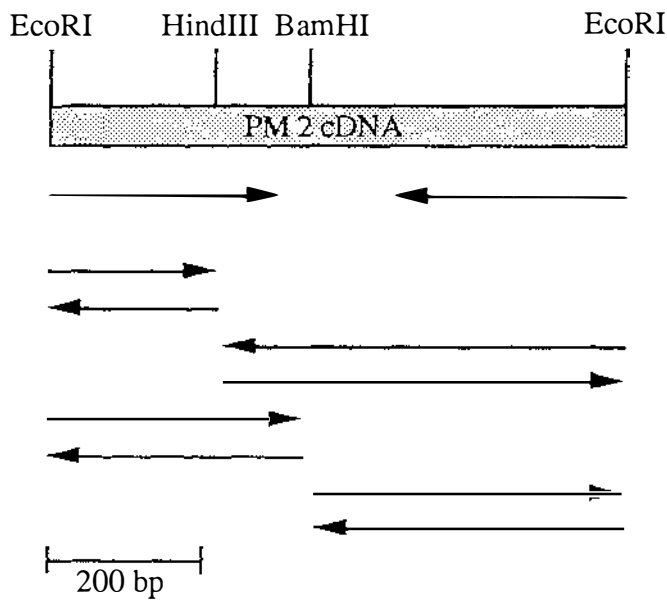


Figure 3.26 Sequencing strategy used to determine the complete nucleotide sequence of cDNA clone PM 2. The thin arrows represent the direction and extent of single-stranded sequencing (in M13mp18 or 19).

The nucleotide sequence of PM 2 is shown in figure 3.27. The predicted amino acid sequence of bovine lactoferrin cDNA clone PM 2 is listed below the nucleotide sequence. The amino acid sequence deduced from the nucleotide sequence shares sequence identity with two of the tryptic peptides from bovine mammary lactoferrin. The position of the two peptides is illustrated by the hatched underlining in figure 3.27. The sequence of the 5' most peptide is identical to the amino acid sequence of HPLC fractions 15 and 17 (see table 2.6, chapter two). The 3' most peptide shares sequence identity with one of the peptides sequenced in HPLC fraction 18 (see table 2.6, chapter two). The deduced amino acid sequence of PM 2 shares sequence identity with other members of the transferrin family. For example, the predicted amino acid sequence of clone PM 2 shares approximately 71% amino acid sequence identity with the corresponding region of human lactoferrin.

Figure 3.27 The nucleotide sequence and predicted amino acid sequence of cDNA clone PM 2.

GAATTCGGGCAGGACGGGGCTGGAGACGTGGCTTTTGTAAAGAGACGACAGTGTGTTGAG
-----+-----+-----+-----+-----+-----+60
CTTAAGCCCGTCTCTGCCCCGACCTCTGCACCGAAAACAATTTCTCTGCTGTCACAACTC
 Q D G A G D V A F V K E T T V F E

AACTTGCCAGAGAAGGCTGACAGGGACCAGTATGAGCTTCTCTGCCTGAACAACAGTCGG
-----+-----+-----+-----+-----+-----+120
TTGAACGGTCTCTTCCGACTGTCCCTGGTCATACTCGAAGAGACGGACTTGTTGTCAGCC
N L P E K A D R D Q Y E L L C L N N S R

GCGCCAGTGGATGCGTTCAAGGAGTGCCACCTGGCCCAGGTCCCTTCTCATGCTGTGCTG
-----+-----+-----+-----+-----+-----+180
CGCGGTACCTACGCAAGTTCCTCACGGTGGACCGGGTCCAGGGAAGAGTACGACAGCAC
A P V D A F K E C H L A Q V P S H A V V

GCCCGAAGTGTGGATGGCAAGGAAGACTTGATCTGGAAGCTTCTCAGCAAGGCGCAGGAG
-----+-----+-----+-----+-----+-----+240
CGGGCTTCACACCTACCGTTCCTTCTGAACCTAGACCTTCTGAAGAGTCGTTCCGCGTCCTC
A R S V D G K E D L I W K L L S K A Q E

AAATTTGGAACCAAGTCTCGGAGCTTCCAGCTCTTTGGCTCTCCACCCGGCCAGAGG
-----+-----+-----+-----+-----+-----+300
TTTAAACCTTTTTTGTTCAGAGCCTCGAAGGTCGAGAAACCGAGAGGTGGGCGGTCTCC
K F G K N K S R S F Q L F G S P P G Q R

GACCTGCTGTTCAAAGACTCTGCTCTTGGGTTTTTGAGGATCCCCTCGAAGGTAGATTCTG
-----+-----+-----+-----+-----+-----+360
CTGGACGACAAGTTTCTGAGACGAGAACCCAAAACTCCTAGGGGAGCTTCCATCTAAGC
D L L F K D S A L G F L R I P S K V D S

GCGCTGTACCTGGGCTCCCGCTACTTGACCACCTTGAAGAACCTCAGGGAACCTGCGGAG
-----+-----+-----+-----+-----+-----+420
CGCGACATGGACCCGAGGGCGATGAACTGGTGGAACTTCTTGGAGTCCCTTTGACGCCTC
A L Y L G S R Y L T T L K N L R E T A E

GAGGTGAAGGCGCGGTACACCAGGGTCGTGTGGTGTGCCGTGGGACCTGAGGAGCAGAAG
-----+-----+-----+-----+-----+-----+480
CTCCACTTCCGCGCCATGTGGTCCCAGCACACCACACGGCACCTGGACTCCTCGTCTTC
E V K A R Y T R V V W C A V G P E E Q K

AAGTGCCAGCAGTGGAGCCAGCAGAGCGGCCAGAACGTGACCTGTGCCACGGCGTCCACC
-----+-----+-----+-----+-----+-----+540
TTCACGGTCGTACCTCGGTCTGTCGCCGGTCTTGCACTGGACACGGTGCCGCAGGTGG
K C Q Q W S Q Q S G Q N V T C A T A S T

ACTGACGACTGCATCGTCCTGGTGCTGAAAGGGGAAGCAGATGCCCTGAACTTGGATGGA
-----+-----+-----+-----+-----+-----+600
TGACTGCTGACGTAGCAGGACCACGACTTTCCCTTCGTCTACGGGACTTGAACCTACCT
T D D C I V L V L K G E A D A L N L D G

GGATATATCTACACTGCGGGCAAGTGTGGCCTGGTGCCTGTCCTGGCAGAGAACCGGAAA
-----+-----+-----+-----+-----+-----+660
CCTATATAGATGTGACGCCCGTTACACCGGACCACGGACAGGACCGTCTCTTGGCCTTT
G Y I Y T A G K C G L V P V L A E N R K

```
TCCTCCAAACACAGTAGCCTAGATTGTGTGCTGAGACCAACGGAAGGGTACCTTGCCGTG
-----+-----+-----+-----+-----+-----+720
AGGAGGTTTGTGTCATCGGATCTAACACACGACTCTGGTTGCCTTCCCATGGAACGGCAC
S  S  K  H  S  S  L  D  C  V  L  R  P  T  E  G  Y  L  A  V

GCAGTTGTCAAGAAAGCAAATGAGGGGCTCACATGGAATTC
-----+-----+-----+-----+761
CGTCAACAGTTCTTTTCGTTTACTCCCCGAGTGTACCTTAAG
A  V  V  K  K  A  N  E  G  L  T  W  N
```

Notes: The sequence in bold at the 5' terminus of the clone represents the EcoRI linker used to clone the molecule into λgt11. The position of tryptic peptides from bovine lactoferrin are shown by the hatched underlining.

The cDNA clone PM 2 lies 5' to clone PM 1 as depicted diagrammatically in figure 3.28. The 5' end of clone PM 1 and the 3' end of clone PM 2 do not appear to be contiguous. Comparison to other members of the transferrin family indicated that clones PM 1 and PM 2 were separated by approximately 40 amino acids (approximately 120 bp).

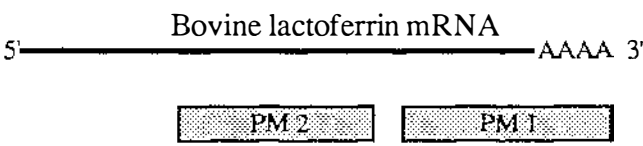


Figure 3.28 The relationship of cDNA clones PM 1 and PM 2 to the mRNA coding for bovine lactoferrin.

Clones PM 1 and PM 2 were both subcloned EcoRI fragments released from the unique EcoRI site in λgt11. The EcoRI sites at the 5' end of PM 1 and the 3' end of PM 2 lack the characteristic linker sequence that is present at the opposite ends of these clones. The EcoRI linkers that were used in the cloning of the double-stranded cDNA into λgt11 have the sequence 5' CCGAATTCGG 3'. Consequently EcoRI sites that were added in the original cloning steps will have either two cytosines or two guanosines immediately before or after the EcoRI site. EcoRI sites which lack these characteristic nucleotides must have originated from sequence within the cloned cDNA. The EcoRI sites at the 5' end of PM 1 and the 3' end of PM 2 do not have the characteristic linker sequence, and therefore must be internal sites within the cDNA coding for bovine mammary lactoferrin. This evidence supports the hypothesis presented earlier that λgt11 clones Lf 1.2, 1.3, 1.4 and 1.10 contain internal EcoRI sites.

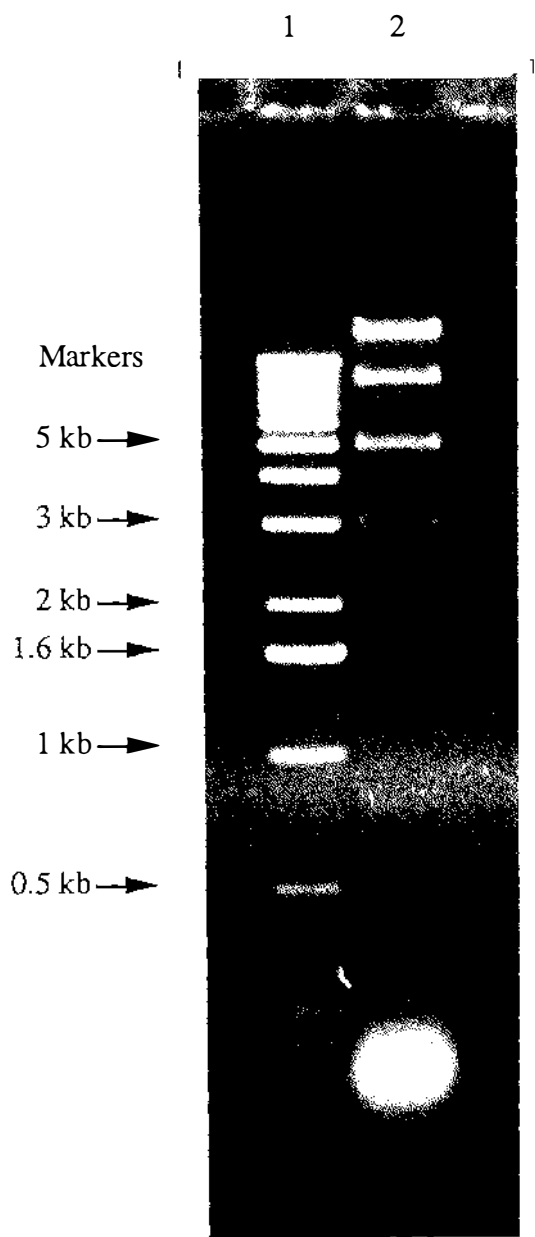
Several attempts were made to isolate the approximately 120 bp EcoRI fragment that connected PM 1 and PM 2. Efforts to gel-purify the small EcoRI fragment from λ gt11 clones proved fruitless. Again, use was made of the MluI restriction sites on either side of the EcoRI cloning site in λ gt11.

3.3.4.5 Subcloning and sequencing of a 120 bp EcoRI fragment from λ gt11Lf 1.3

The cDNA clone λ gt11Lf 1.3 was selected for isolation of the 120 bp DNA fragment. PM 1 hybridised to PHL-41 but not to PHL-44 and PM 2 hybridised to PHL-44 but not to PHL-41. The clone λ gt11Lf 1.3 hybridised to both PHL-41 and PHL-44, indicating that this clone contains sequence that connects PM 1 and PM 2.

Approximately 1 μ g of λ gt11Lf 1.3 DNA was digested with MluI. The DNA fragments were precipitated with ethanol and resuspended in 20 μ l of sterile water. The cohesive termini were filled in using the large fragment of *E.coli* DNA polymerase I (Klenow fragment). The products were separated by electrophoresis on low gelling temperature agarose. Figure 3.29 illustrates the ethidium bromide-stained gel of MluI-cut, Klenow-repaired λ gt11Lf 1.3 DNA. The 3.2 kb fragment was isolated from the gel by adherence to glass beads and subcloned into the plasmid vector pGEM-2™ (referred to hereafter as Lf 1.3-M3.2). Figure 3.30 shows an ethidium bromide-stained gel of the cloned, blunt ended 3.2 kb MluI fragment from λ gt11Lf 1.3 (Lf 1.3-M3.2) digested with either EcoRI or KpnI and PstI. The figure also illustrates the restriction map deduced from the gel data.

Sequence data from clones PM 1 and PM 2 show a KpnI restriction site near the 3' end of PM 2 and a PstI restriction site near the 5' end of PM 1 (see Appendix II for a complete restriction map of the cDNA coding for bovine lactoferrin). If there were no other internal PstI or KpnI sites, digestion of the cloned 3.2 kb MluI fragment with these enzymes should have released a 430 bp fragment that contains the missing 120 bp EcoRI fragment. However, as the data presented in figure 3.30 indicates that there is either a PstI or a KpnI restriction site between the 5' end of PM 1 and the 3' end of PM 2.

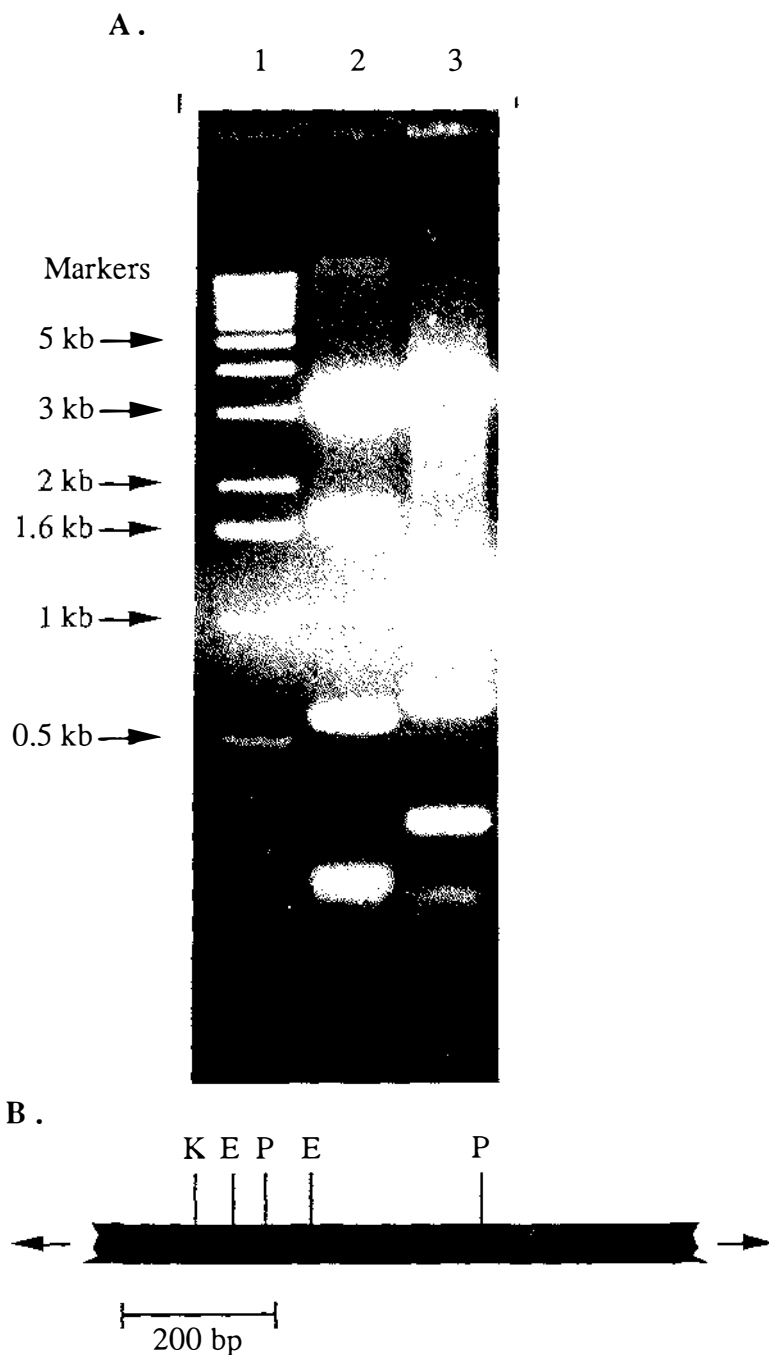


Gel details: 1% (w/v) agarose gel stained with ethidium bromide.

Loading details: 1. BRL 1 kb ladder.

2. λgt11Lf 1.3 DNA digested with MluI and 'end filled' with Klenow.

Figure 3.29 Agarose gel electrophoresis of DNA isolated from λgt11Lf 1.3 digested with MluI and then 'end filled' with Klenow.



A. Gel details: 1% (w/v) agarose gel stained with ethidium bromide.

Loading details: 1. BRL 1 kb ladder.

2. Cloned Lf 1.3-M3.2 DNA digested with EcoRI.

3. Cloned Lf 1.3-M3.2 DNA digested with KpnI and PstI.

B. Partial restriction map of the insert DNA in clone Lf 1.3-M3.2. Key:

K=KpnI, P=PstI and E=EcoRI (internal EcoRI sites, not the λ gt11 cloning site).

Figure 3.30 Agarose gel electrophoresis and partial restriction map of clone Lf1.3-M3.2.

The 120 bp EcoRI fragment was subcloned into the sequencing vector M13mp18. The sequencing strategy used to determine the complete nucleotide sequence of the 120 bp EcoRI fragment (hereafter termed PM 3) is illustrated in figure 3.31. Figure 3.32 depicts the nucleotide sequence and the deduced amino acid sequence of PM 3.

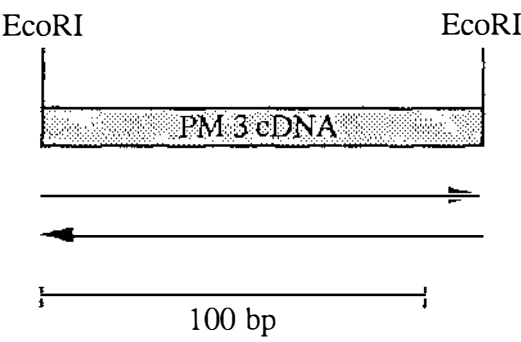


Figure 3.31 Sequencing strategy used to determine the complete nucleotide sequence of cDNA clone PM 3. The thin arrows represent the direction and extent of single-stranded sequencing (in M13mp18 or 19).

Figure 3.32 The nucleotide sequence and predicted amino acid sequence of the cDNA clone PM 3.

```
GAATTCTCTGAAAGACAAGAAGTCGTGCCACACCGCCGTGGACAGGACTGCAGGCTGGAA
-----+-----+-----+-----+-----+-----+60
CTTAAGAGACTTTCTGTTCTTCAGCACGGTGTGGCGGCACCTGTCCTGACGTCCGACCTT
  N  S  L  K  D  K  K  S  C  H  T  A  V  D  R  T  A  G  W  N

CATCCCCATGGGCCTGATCGTCAACCAGACAGGCTCCTGCGCATTTGATGAATTC
-----+-----+-----+-----+-----+115
GTAGGGGTACCCGGACTAGCAGTTGGTCTGTCCGAGGACGCGTAACTACTTAAG
  I  P  M  G  L  I  V  N  Q  T  G  S  C  A  F  D  E  F
```

The predicted amino acid sequence of PM 3 shown in figure 3.32 has sequence homology with other members of the transferrin family. The deduced amino acid sequence of clone PM 3 shares approximately 84% amino acid sequence identity with the corresponding region of human lactoferrin.

The combined sequence of cDNA clones PM 1, PM 2 and PM 3 account for approximately two-thirds of the mature bovine lactoferrin protein sequence. Northern blot hybridisation data and comparison to other transferrin sequences indicates that the length of the messenger RNA coding for bovine

lactoferrin is approximately 2.3 kb. The combined length of the three cDNA clones isolated is 1685 base pairs (excluding bases added by linker sequences). This leaves approximately 600 bp of 5' bovine lactoferrin sequence unaccounted for. In an attempt to isolate full length clones the bovine mammary cDNA λ gt11 library was screened with the 5' most 830 bp of PHL-44 and PM 1. Several clones were isolated by this strategy and characterised by restriction mapping, Southern blot analysis and DNA sequencing. No additional 5' sequence information was acquired by this approach. An alternative strategy was designed to obtain DNA closer to the 5' end of the cDNA coding for bovine lactoferrin. The lack of clones with 5' sequence in the λ gt11 library may have been due to secondary structure in the mRNA used to prime the first strand cDNA. The presence of secondary structure in the mRNA can lead to premature termination of the reverse transcriptase reaction, leading to cDNA molecules which lack 5' sequence information. In an attempt to overcome this potential problem, another cDNA library was constructed using AMV reverse transcriptase to prime the first strand cDNA synthesis. The advantage of this enzyme, over the MMLV reverse transcriptase, is that AMV reverse transcriptase can remain active at higher temperatures. First strand cDNA synthesis at higher temperatures may reduce the amount of secondary structure in the template mRNA and prevent premature termination by reverse transcriptase.

3.3.5 cDNA synthesis using AMV reverse transcriptase and cloning into plasmid vectors

3.3.5.1 Generation of first strand, 'G-tailed' cDNA from bovine mammary poly A⁺ RNA

First strand cDNA was synthesized from bovine mammary messenger RNA using AMV reverse transcriptase as described in section 3.2.3.1.2. The synthesis of first strand products was followed by the incorporation of [α -³²P] dCTP into acid-precipitable products in a tracer reaction. Approximately 4% of the radiolabel was incorporated into acid-insoluble products. In the first strand syntheses described earlier, the yield of cDNA was calculated from the incorporation of [α -³²P] dCTP in a tracer reaction. In this experiment the dilution of the 2 μ l first strand tracer reaction with 1 μ l of [α -³²P] dCTP resulted in the calculation of the yield of DNA in the main,

undiluted reaction being grossly inaccurate. That is, the dilution of the tracer reaction with radiolabel was much greater in this experiment than in the earlier first strand cDNA syntheses (1/3-diluted and 1/20-diluted, respectively). Although not quantitative, the incorporation of radiolabel into acid-precipitable products served to illustrate the synthesis of cDNA.

The excess first strand reactants were removed from the main (18 μ l) reaction by ultrafiltration in a Centricon-30 cell. The first strand cDNA products were 'G-tailed' using terminal dideoxynucleotidyl transferase. The tailing reaction was spiked with [α - 32 P] dGTP to follow the addition of guanosine residues to the 3' termini of the first strand cDNA. Approximately 5% of the radiolabelled dGTP was incorporated into acid insoluble products, indicating that the tailing reaction has been successful.

The 'G-tailed' first strand cDNA was used as a template for second strand cDNA synthesis by two alternative methods. The first method relied on hybridisation of an oligomeric deoxycytosine twenty-mer (d(pC)₂₀) to the 'G-tail' to prime synthesis of the second strand using *E. coli* DNA polymerase I (refer to section 3.2.3.1.2). Double-stranded cDNA prepared by this method was cloned into the plasmid vector pGEM-2™ (refer to section 3.2.3.2.2). The second method employed the polymerase chain reaction (PCR) to synthesize the second strand cDNA. The oligomeric deoxycytosine twenty-mer (d(pC)₂₀) was used to 'anchor' the 5' end and one of two bovine lactoferrin-specific oligonucleotide primers were used to initiate DNA synthesis from the other strand (refer to section 3.2.3.1.2). Double-stranded cDNA prepared by the polymerase chain reaction was cloned into M13 for sequence analysis (refer to section 3.2.3.2.3).

3.3.5.2 Identification of pGEM-2™ clones containing bovine lactoferrin specific sequences by colony hybridisation

Double-stranded cDNA synthesized from 'G-tailed' first strand products using *E. coli* DNA polymerase I was ligated into the plasmid vector pGEM-2™. The recombinant plasmids were transformed into competent *E. coli* XL 1 cells as described in 3.2.3.2.2. As pGEM-2™ does not allow 'blue/white' selection for non-recombinant clones, an estimate of the number of non-recombinant clones in the library was made by plating an aliquot of a control ligation reaction containing no insert. The total number of colonies that grew

on LB agar containing 100 µg/ml ampicillin was approximately 2.7×10^4 . After subtraction of the number of non-recombinant clones, estimated from the control ligation with no insert added, the number of recombinant clones in the library was approximately 2.35×10^4 . This library was called bovine mammary cDNA pGEM-2™ library #1.

Bovine mammary cDNA pGEM-2™ library #1 was screened, without prior amplification, by the colony hybridisation method (Maniatis *et al.*, 1982). Single nitrocellulose filter lifts were taken from the LB agar plates containing 100 µg/ml ampicillin. The filters were treated with alkali to disrupt the bacteria and washed exhaustively to remove excess bacterial protein bound to the nitrocellulose.

In an attempt to isolate clones that contained sequence information 5' to clone PM 2, the plasmid library was screened with the 5' most 830 bp EcoRI-SmaI fragment of human lactoferrin cDNA clone PHL-44 (hereafter termed PHL-44.830). Figure 3.33 depicts the restriction map of human lactoferrin cDNA clone PHL-44 and illustrates the strategy used to isolate PHL-44.830. Figure 3.34 illustrates an ethidium bromide-stained gel of cloned PHL-44 DNA digested with EcoRI and PstI and then with SmaI. The 830 bp EcoRI-SmaI fragment (PHL-44.830) was isolated from a low gelling temperature gel and purified by adherence to glass beads.

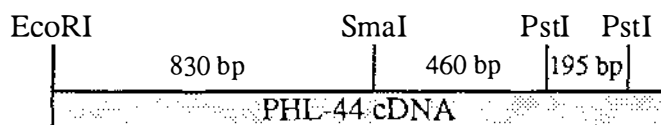
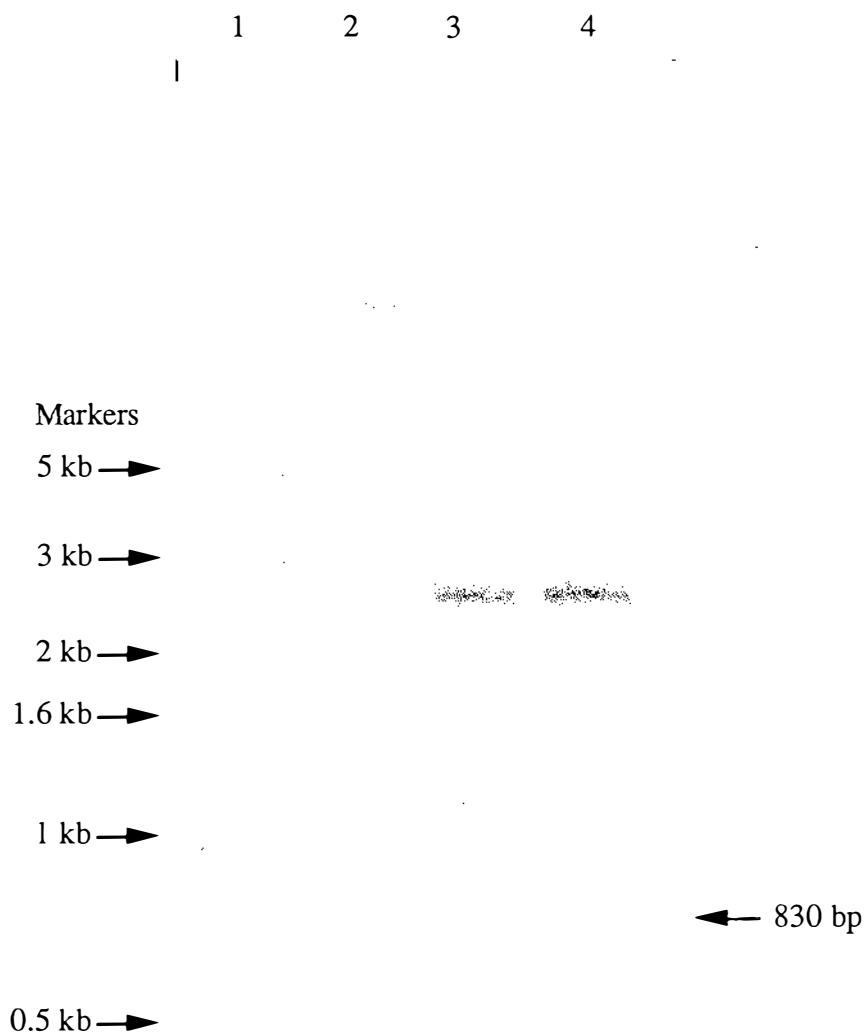


Figure 3.33 Partial restriction map of human lactoferrin cDNA clone PHL-44 illustrating the restriction sites used to generate the cDNA probe PHL-44.830.



Gel details: 1% (w/v) agarose gel stained with ethidium bromide.

Loading details: 1. BRL 1 kb ladder.

2. Undigested PHL-44 DNA.

3. PHL-44 DNA digested with EcoRI and PstI.

4. PHL-44 DNA digested with EcoRI, PstI and SmaI.

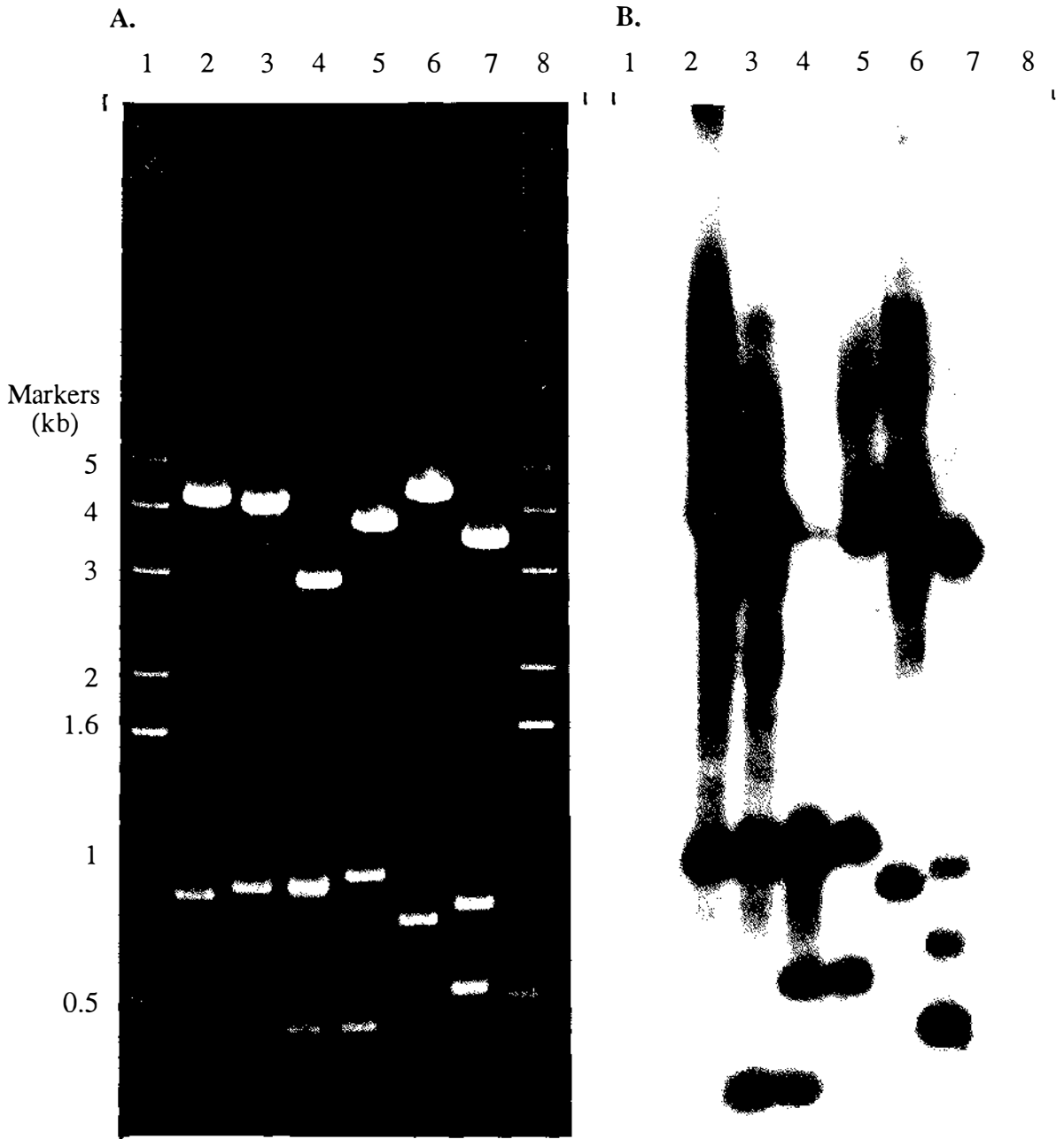
Figure 3.34 Agarose gel electrophoresis of human lactoferrin cDNA clone PHL-44 digested with EcoRI, PstI and SmaI.

Bovine lactoferrin specific sequences were identified by hybridisation to the 830 bp 5' end of the human lactoferrin cDNA (clone PHL-44.830). Twelve clones that hybridised to the human lactoferrin cDNA probe were isolated from the primary plates. The hybridisation of the human lactoferrin probe to the primary filters was very weak. *In situ* amplification of the bacteria on the nitrocellulose filters may have increased the signal from positive clones and may have allowed more clones to be picked from the first round screen.

The twelve positive colonies were individually resuspended in LB broth and plated on LB agar plates containing 100 µg/ml ampicillin. A second round of colony hybridisation was used to isolate the lactoferrin cDNA containing clones. The second round filters were also probed with radiolabelled PHL-44.830 DNA. Only two of the original twelve clones picked from the primary plates hybridised to the human cDNA probe on the second round of screening. The two clones that hybridised to PHL-44.830 were isolated from the second round plates and transferred to LB broth containing 100 µg/ml ampicillin. Plasmid DNA was isolated from overnight cultures and analysed by restriction mapping and Southern blot analysis. Gel electrophoresis of plasmid DNA digested with various restriction endonucleases indicated that the two pGEM-2™ clones had different sized inserts. These two clones were called PM 7 and PM 8. Characterisation of clones PM 7 and PM 8 will be discussed in detail in sections 3.3.5.2.1 and 3.3.5.2.2 respectively.

3.3.5.2.1 Restriction mapping and sequence analysis of pGEM-2™ clone PM 7

Plasmid DNA isolated from pGEM-2™ clone PM 7 was digested with various restriction enzymes and separated by electrophoresis on a 1% (w/v) agarose gel. Figure 3.35 illustrates an ethidium bromide-stained agarose gel and the corresponding Southern blot of PM 7 DNA digested with restriction endonucleases. The Southern blot was probed with a full length human lactoferrin cDNA PCR product. This lactoferrin cDNA probe was synthesized from cloned template DNA by PCR using human lactoferrin specific oligonucleotide primers (Amos 1 and Amos 2) that were kindly donated by K.M. Stowell (Massey University). The resultant 2.3 kb PCR product (data not shown) contains the entire coding region of the human lactoferrin mRNA (K.M. Stowell, personal communication).



A. Gel details: 1% (w/v) agarose gel stained with ethidium bromide.
 Loading details: 1 and 8. BRL 1 kb ladder.
 Lanes 2 to 7 contain PM 7 DNA digested with the following restriction enzymes:

2. BamHI	3. EcoRI
4. BamHI and EcoRI	5. HincII
6. HindIII	7. PstI

B. Southern blot of gel A, probed with radiolabelled full length human lactoferrin cDNA.

Figure 3.35 Agarose gel electrophoresis and Southern blot of pGEM-2™ clone PM 7.

The human lactoferrin cDNA PCR product was gel-purified from low gelling temperature agarose by adherence to glass beads (Geneclean kit, Bio 101, La Jolla, CA.) and labelled with [α - 32 P] dCTP by the random primer method (BRL random primer kit).

Figure 3.36 illustrates the restriction map of PM 7 (and the sequencing strategy used to determine the nucleotide sequence of the insert DNA). The size of the cDNA insert in PM 7, estimated from restriction endonuclease data illustrated in figure 3.35, was approximately 2.1 kb. The presumed position of restriction endonuclease sites in PM 7 corresponded to the position of the same restriction sites in bovine lactoferrin cDNA clones PM 1, PM 2, and PM 3. This indicated that PM 7 may be a near full length clone coding for bovine lactoferrin.

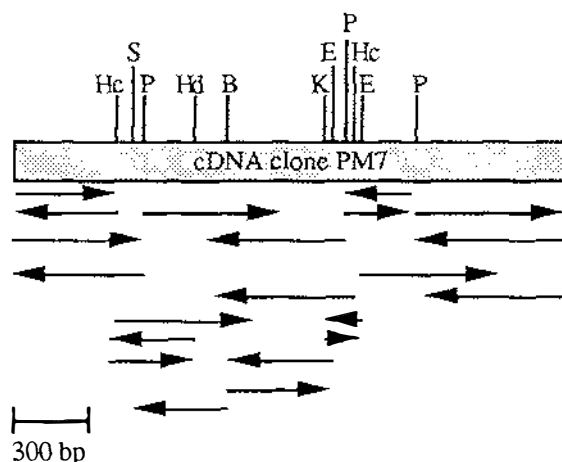
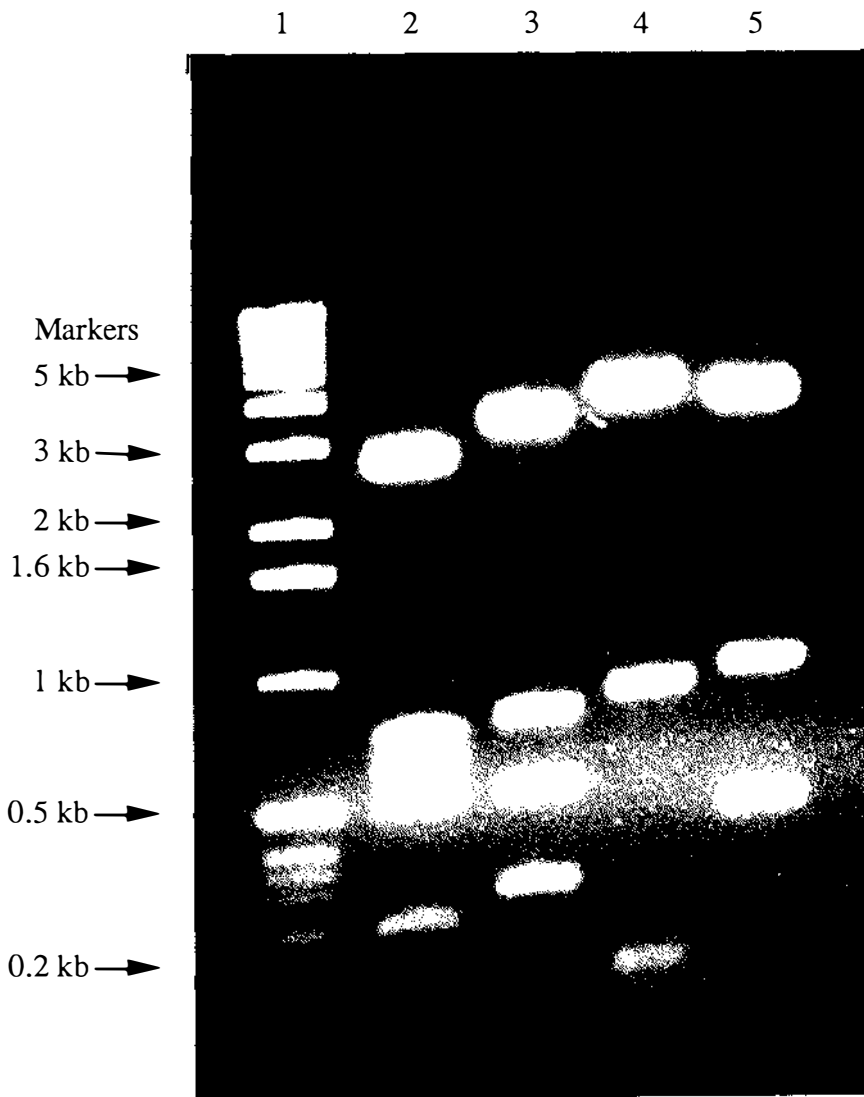


Figure 3.36 Predicted restriction map of clone PM 7. This figure includes the sequencing strategy used to determine the nucleotide sequence of PM 7. The arrows represent the direction and extent of single-stranded sequencing (in M13mp18 or 19). Key: B=BamHI, E=EcoRI, Hc=HincII, Hd=HindIII, K=KpnI, P=PstI, S=SmaI.

The complete nucleotide sequence of clone PM 7 was determined by the dideoxynucleotide chain termination technique (Sanger *et al.*, 1977). Figure 3.37 illustrates an ethidium bromide-stained gel of PM 7 DNA digested with various restriction enzymes used to subclone fragments of PM 7 into M13 for sequence analysis. Figure 3.36 depicts the sequencing strategy used to determine the nucleotide sequence of PM 7. Figure 3.38 illustrates the nucleotide sequence of clone PM 7 and the deduced amino acid sequence.



Gel details: 1% (w/v) agarose gel stained with ethidium bromide.

Loading details:

1. BRL 1 kb ladder.

Lanes 2 to 5 contain PM 7 DNA digested with the following restriction endonucleases:

2. EcoRI and PstI.

3. PstI.

4. EcoRI.

5. HincII.

Figure 3.37 Agarose gel electrophoresis of PM 7 DNA digested with various restriction endonucleases prior to subcloning into M13 for sequence analysis.

Figure 3.38 The nucleotide sequence and predicted amino acid sequence of cDNA clone PM 7.

```
ccccccccccccccccccccccggcctttgccttggaatgtatccggggccatcgcgga
-----+-----+-----+-----+-----+-----+60
ggggggggggggggggggggggggccggaaacggaaccttacataggcccggtagcgctt
          A F A L E C I R A I A E

gaaaaaggcggatgctgtgaccttgatggtggcatggtgtttgaggcgggcccgggacctt
-----+-----+-----+-----+-----+-----+120
ctttttccgcctacgacactgggacctaccaccgtaccacaaactccgcccggccctggg
K K A D A V T L D G G M V F E A G R D P

ctacaaactgcggccagtagcagcagagatctatgggacgaaagagtctcccaaacccta
-----+-----+-----+-----+-----+-----+180
gatgtttgacgccggtcatcgctcgtctctagataccctgctttctcagaggggtttgggt
Y K L R P V A A E I Y G T K E S P Q T H

ctattatgctgtggccgctcgtgaagaagggcagcaactttcagctggaccagctgcaagg
-----+-----+-----+-----+-----+-----+240
gataatacgacaccggcagcaacttcttcccgctcgttgaaagtcgacctggctcgacgttcc
Y Y A V A V V K K G S N F Q L D Q L Q G

ccggaagtcctgccatacgggccttggcaggtccgctgggtggatcatccctatgggaat
-----+-----+-----+-----+-----+-----+300
ggccttcaggacggtatgcccggaaacggtccaggcgaccacctagtagggataccctta
R K S C H T G L G R S A G W I I P M G I

ccttcgccccgtaacttgagctggacagagtcaactcgagccccctccaggagctgtggctaa
-----+-----+-----+-----+-----+-----+360
ggaagcgggcatgaaactcgacctgtctcagtgagctcggggagggtccctcgacaccgatt
L R P Y L S W T E S L E P L Q G A V A K

attcttctctgccagctgtgttccctgcattgatagacaagcataccccaacctgtgtca
-----+-----+-----+-----+-----+-----+420
taagaagagacggctcgacacaagggacgtaactatctgttcgtatggggttggacacagt
F F S A S C V P C I D R Q A Y P N L C Q

actgtgcaagggggagggggagaaaccagtgctgcctgctcctcccggaaccatacttcgg
-----+-----+-----+-----+-----+-----+480
tgacacgttccccctccccctcttggtcacacggacgaggagggcccttggatgaagcc
L C K G E G E N Q C A C S S R E P Y F G

ttattctgggtgccttcaagtgtctgcaggacggggctggagacgtggcTTTTGTTAAAGA
-----+-----+-----+-----+-----+-----+540
aataagaccacggaagtTCACAGACGTCCTGCCCCGACCTCTGCACCGAAAACAATTTCT
Y S G A F K C L Q D G A G D V A F V K E

gacgacagtgtttgagaacttgccagagaaggctgacagggaccagtatgagcttctctg
-----+-----+-----+-----+-----+-----+600
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T T V F E N L P E K A D R D Q Y E L L C

cctgaacaacagtcgggGCCAGTGGATGCGTTCAAGGAGTGCCACCTGGCCCAGGTCCC
-----+-----+-----+-----+-----+-----+660
ggaacttgTTGTCAGCCCGCGGTACCTACGCAAGTTCCTCACGGTGGACCGGGTCCAGGG
L N N S R A P V D A F K E C H L A Q V P
```

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-----+-----+-----+-----+-----+-----+720
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S H A V V A R S V D G K E D L I W K L L

CAGCAAGGCGCAGGAGAAATTTGGA~~AAAA~~ACAAGTCTCGGAGCTTCCAGCTCTTTGGCTC
-----+-----+-----+-----+-----+-----+780
GTCGTTCCGCGTCTCTTTAAACCTTTTTTGTTCAGAGCCTCGAAGGTCGAGAAACCGAG
S K A Q E K F G K N K S R S F Q L F G S

TCCACCCGGCCAGAGGGACCTGCTGTTC~~AA~~AGACTCTGCTCTTGGGTTTTTGAGGATCCC
-----+-----+-----+-----+-----+-----+840
AGGTGGGCCGGTCTCCCTGGACGACAAGTTTCTGAGACGAGAACCCAAAACTCCTAGGG
P P G Q R D L L F K D S A L G F L R I P

CTCGAAGGTAGATTCGGCGCTGTACCTGGGCTCCCGCTACTTGACCACCTTGAAGAACCT
-----+-----+-----+-----+-----+-----+900
GAGCTTCCATCTAAGCCGCGACATGGACCCGAGGGCGATGAACTGGTGGAACTTCTTGGA
S K V D S A L Y L G S R Y L T T L K N L

CAGGGAAACTGCGGAGGAGGTGAAGGCGCGGTACACCAGGGTCGTGTGGTGTGCCGTGGG
-----+-----+-----+-----+-----+-----+960
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R E T A E E V K A R Y T R V V W C A V G

ACCTGAGGAGCAGAAGAAGTGCCAGCAGTGGAGCCAGCAGAGCGGCCAGAACGTGACCTG
-----+-----+-----+-----+-----+-----+1020
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P E E Q K K C Q Q W S Q Q S G Q N V T C

TGCCACGGCGTCCACCACTGACGACTGCATCGTCCTGGTGCTGAAAGGGGAAGCAGATGC
-----+-----+-----+-----+-----+-----+1080
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A T A S T T D D C I V L V L K G E A D A

CCTGAACTTGATGGAGGATATATCTACACTGCGGGCAAGTGTGGCCTGGTGCCTGTCCT
-----+-----+-----+-----+-----+-----+1140
GGA~~CT~~TGAACCTACCTCCTATATAGATGTGACGCCCCGTTACACCGGACCACGGACAGGA
L N L D G G Y I Y T A G K C G L V P V L

GGCAGAGAACCGGAAATCCTCCAACACAGTAGCCTAGATTGTGTGCTGAGACCAACGGA
-----+-----+-----+-----+-----+-----+1200
CCGTCTCTTGGCCTTTAGGAGGTTTGTGTATCGGATCTAACACACGACTCTGGTTGCCT
A E N R K S S K H S S L D C V L R P T E

AGGGTACCTTGCCGTGGCAGTTGTCAAGAAAGCAAATGAGGGGCTCACATGGAATTCTCT
-----+-----+-----+-----+-----+-----+1260
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G Y L A V A V V K K A N E G L T W N S L

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K D K K S C H T A V D R T A G W N I P M

GGGCCTGATCGTCAACCAGACAGGCTCCTGCGCATTTGATGAATTCTTTAGTCAGAGCTG
-----+-----+-----+-----+-----+-----+1380
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G L I V N Q T G S C A F D E F F S Q S C

TGCCCCCTGGGGCTGACCCGAAATCCAGACTCTGTGCCTTGTGTGCTGGCGATGACCAGGG
-----+-----+-----+-----+-----+-----+1440
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A P G A D P K S R L C A L C A G D D Q G

CCTGGACAAGTGTGTGCCCAACTCTAAGGAGAAGTACTATGGCTATACCGGGGCTTTTCAG
-----+-----+-----+-----+-----+-----+1500
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L D K C V P N S K E K Y Y G Y T G A F R

GTGCCTGGCTGAGGACGTTGGGGACGTTGCCTTTGTGAAAAACGACACAGTCTGGGAGAA
-----+-----+-----+-----+-----+-----+1560
CACGGACCGACTCCTGCAACCCCTGCAACGGAACACTTTTTGCTGTGTGTCAGACCCCTCTT
C L A E D V G D V A F V K N D T V W E N

CACGAATGGAGAGAGCACTGCAGACTGGGCTAAGAACTTGAATCGTGAGGACTTCAGGTT
-----+-----+-----+-----+-----+-----+1620
GTGCTTACCTCTCTCGTGACGTCTGACCCGATTCTTGAACCTTAGCACTCCTGAAGTCCAA
T N G E S T A D W A K N L N R E D F R L

GCTCTGCCTCGATGGCACCAGGAAGCCTGTGACGGAGGCTCAGAGCTGCCACCTGGCGGT
-----+-----+-----+-----+-----+-----+1680
CGAGACGGAGCTACCGTGGTCCCTTCGGACACTGCCTCCGAGTCTCGACGGTGGACCGCCA
L C L D G T R K P V T E A Q S C H L A V

GGCCCCGAATCACGCTGTGGTGTCTCGGAGCGATAGGGCAGCACACGTGAAACAGGTGCT
-----+-----+-----+-----+-----+-----+1740
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A P N H A V V S R S D R A A H V K Q V L

GCTCCACCAGCAGGCTCTGTTTGGGAAAAATGGAAAAACTGCCCGGACAAGTTTTGTTT
-----+-----+-----+-----+-----+-----+1800
CGAGGTGGTTCGTCCGAGACAAACCCTTTTTTACCTTTTTTTGACGGGCCTGTTCAAAACAAA
L H Q Q A L F G K N G K N C P D K F C L

GTTCAAATCTGAAACCAAAAACCTTCTGTTCAATGACAACACTGAGTGTCTGGCCAAACT
-----+-----+-----+-----+-----+-----+1860
CAAGTTTACACTTTTGGTTTTTGGGAAGACAAGTTACTGTTGTGACTCACAGACCGGTTTGA
F K S E T K N L L F N D N T E C L A K L

TGGAGGCAGACCAACGTATGAAGAATATTTGGGGACAGAGTATGTCACGGCCATTGCCAA
-----+-----+-----+-----+-----+-----+1920
ACCTCCGTCTGGTTGCATACTTCTTATAAACCCCTGTCTCATACAGTGCCGGTAACGGTT
G G R P T Y E E Y L G T E Y V T A I A N

CCTGAAAAATGCTCAACCTCCCCGCTTCTGGAAGCCTGCGCCTTCCTGACGAGGTAAAG
-----+-----+-----+-----+-----+-----+1980
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L K K C S T S P L L E A C A F L T R *

CCTGCAAAGAAGCTAGCCTGCCTCCCTGGGCCTCAGCTCCTCCCTGCTCTCAGCCCCAAT
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CTCCAGGCGCGAGGGACCTTCTCTCCCTTCTGAAAGTCGGATTTTTGCCAAGCTCATCA
-----+-----+-----+-----+-----+-----+2100
GAGGTCCGCGCTCCCTGGAAGGAGAGGGAAGGACTTCAGCCTAAAAACGGTTCGAGTAGT

```
GTATTTACAATTCCCTGCTGTCATTTTAGCAAGAAATAAAATTACAAATGCTGTTGATTT
-----+-----+-----+-----+-----+-----+-----+2160
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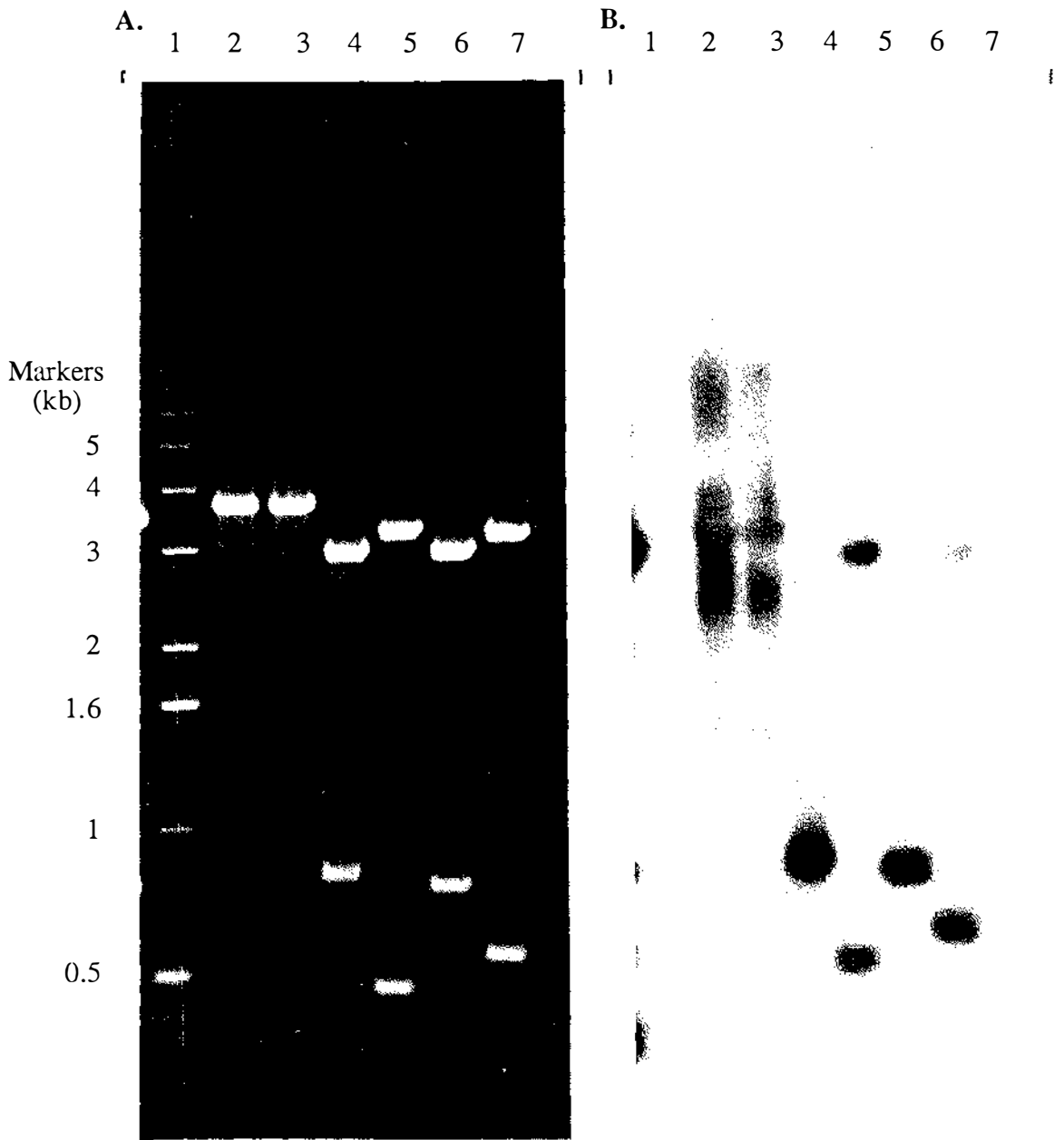
TCATTCCTTAAAAAAA
-----+-----2177
AGTAAGGGATTTTTTTT
```

*Notes: The sequence in bold at the 5' end of clone PM 7 represents the 'G-tail' that was added to the 3' end of the first strand cDNA product. The position of the bovine lactoferrin tryptic peptides sequenced earlier are shown by the hatched underlining. The position of the putative stop codon (UAA, ochre) is shown by the *. The putative polyadenylation signal (AAUAAA) is indicated in the 3' untranslated region by the double underlining.*

The nucleotide sequence of clone PM 7 shares 100% sequence identity with clones PM 1, PM 2 and PM 3. Bovine lactoferrin cDNA clone PM 7 extends 480 bp 5' to the terminus of PM 2. The deduced amino acid sequence of PM 7 shares approximately 71% sequence identity with the corresponding region of human lactoferrin.

3.3.5.2.2 Restriction mapping and sequence analysis of pGEM-2™ clone PM 8

Plasmid DNA isolated from bovine lactoferrin cDNA clone PM 8 was physically mapped by restriction endonuclease digestion and Southern blot analysis. Figure 3.39 illustrates an ethidium bromide-stained agarose gel of PM 8 DNA digested with various restriction enzymes and the corresponding Southern blot probed with full length human lactoferrin PCR cDNA (see section 3.3.5.2.1 for details of the probe). Figure 3.40 depicts the expected restriction map of clone PM 8 determined from data illustrated in figure 3.39. The complete nucleotide sequence of PM 8 was determined. Figure 3.40 also illustrates the sequencing strategy used to determine the sequence of pGEM-2™ clone PM 8. Figure 3.41 illustrates the nucleotide and predicted amino acid sequence of cDNA clone PM 8. The amino acid sequence of clone PM 8 shares approximately 65% sequence identity with the corresponding region of human lactoferrin.



A. Gel details: 1% (w/v) agarose gel stained with ethidium bromide.

Loading details: 1. BRL 1 kb ladder.

Lanes 2 to 7 contain PM 8 DNA digested with the following restriction endonucleases:

2. BamHI.

3. EcoRI.

4. BamHI and EcoRI.

5. HincII.

6. HindIII.

7. PstI.

B. Southern blot of gel A, probed with radiolabelled full length human lactoferrin cDNA.

Figure 3.39 Agarose gel electrophoresis and Southern blot analysis of clone PM 8.

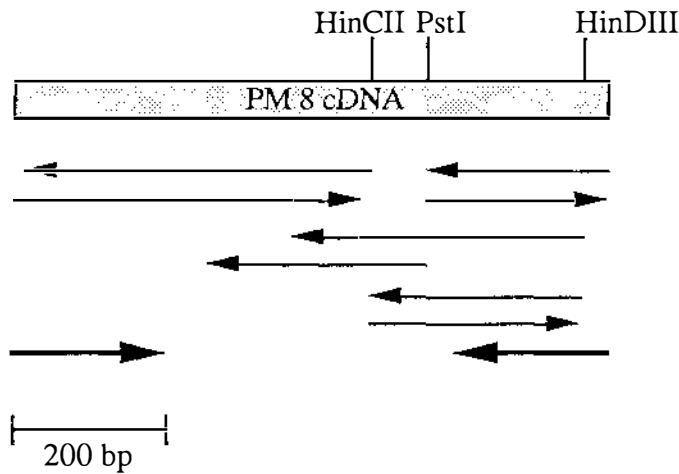


Figure 3.40 Predicted restriction map of cDNA clone PM 8 and the sequencing strategy used to determine the nucleotide sequence of PM 8. The thin arrows represent the direction and extent of single-stranded sequencing (in M13mp18 or 19). The thick arrows represent the direction and extent of sequencing in the double-stranded plasmid vector pGEM-2™.

Figure 3.41 The nucleotide sequence and predicted amino acid sequence of cDNA clone PM 8.

```

ccccccccccccccccccagaagctgggtgctccctctatcacctgtgtgaggagggcctt
-----+-----+-----+-----+-----+-----+60
ggggggggggggggggggtcttcgacccacgaggagatagtggacacactcctcccgaa
          K  L  G  A  P  S  I  T  C  V  R  R  A  F

TGCCTTGGAATGTATCCGGGCCATCGCGGAGAAAAAGGCGGATGCTGTGACCTGGATGG
-----+-----+-----+-----+-----+-----+120
ACGGAACCTTACATAGGCCCGGTAGCGCCTCTTTTCCGCCTACGACACTGGGACCTACC
A  L  E  C  I  R  A  I  A  E  K  K  A  D  A  V  T  L  D  G

TGGCATGGTGTGTTTGAGGCGGGCCGGGACCCCTACAACTGCGGCCAGTAGCAGCAGAGAT
-----+-----+-----+-----+-----+-----+180
ACCGTACCACAACTCCGCCCGGCCCTGGGGATGTTTGACGCCGGTCATCGTCTCTCTA
G  M  V  F  E  A  G  R  D  P  Y  K  L  R  P  V  A  A  E  I

CTATGGGACGAAAGAGTCTCCCCAAACCCACTATTATGCTGTGGCCGTCGTGAAGAAGGG
-----+-----+-----+-----+-----+-----+240
GATACCTGCTTTCTCAGAGGGGTTTGGGTGATAATACGACACCGGCAGCACTTCTTCCC
Y  G  T  K  E  S  P  Q  T  H  Y  Y  A  V  A  V  V  K  K  G

CAGCAACTTTCAGCTGGACCAGC:TGCAAGGCCGGAAGTCCTGCCATACGGGCCTTGGCAG
-----+-----+-----+-----+-----+-----+300
GTCGTTGAAAGTCGACCTGGTCGACGTTCCGGCCTTCAGGACGGTATGCCCGGAACCGTC
S  N  F  Q  L  D  Q  L  Q  G  R  K  S  C  H  T  G  L  G  R

GTCCGCTGGGTGGATCATCCCTATGGGAATCCTTCGCCCGTACTTGAGCTGGACAGAGTC
-----+-----+-----+-----+-----+-----+360
CAGGCGACCCACCTAGTAGGGATACCCTTAGGAAGCGGCATGAACTCGACCTGTCTCAG
S  A  G  W  I  I  P  M  G  I  L  R  P  Y  L  S  W  T  E  S
```

```

ACTCGAGCCCCCTCCAGGGAGCTGTGGCTAAATTCTTCTCTGCCAGCTGTGTTCCCTGCAT
-----+-----+-----+-----+-----+-----+420
TGAGCTCGGGGAGGTCCCTCGACACCGATTAAAGAAGAGACGGTCGACACAAGGGACGTA
  L  E  P  L  Q  G  A  V  A  K  F  F  S  A  S  C  V  P  C  I

TGATAGACAAGCATACCCCAACCTGTGTCAACTGTGCAAGGGGGAGGGGGAGAACCAGTG
-----+-----+-----+-----+-----+-----+480
ACTATCTGTTTCGTATGGGGTTGGACACAGTTGACACGTTCCCCCTCCCCCTCTTGGTCAC
  D  R  Q  A  Y  P  N  L  C  Q  L  C  K  G  E  G  E  N  Q  C

TGCCTGCTCCTCCCGGAACCATACTTCGGTTATTCTGGTGCCTTCAAGTGTCTGCAGGA
-----+-----+-----+-----+-----+-----+540
ACGGACGAGGAGGGGCCCTTGGTATGAAGCCAATAAGACCACGGAAGTTCACAGACGTCCT
  A  C  S  S  R  E  P  Y  F  G  Y  S  G  A  F  K  C  L  Q  D

CGGGGCTGGAGACGTGGCTTTTGTAAAGAGACGACAGTGTGAGAACTTGCCAGAGAA
-----+-----+-----+-----+-----+-----+600
GCCCCGACCTCTGCACCGAAAACAATTTCTCTGCTGTACAACTCTTGAACGGTCTCTT
  G  A  G  D  V  A  F  V  K  E  T  T  V  F  E  N  L  P  E  K

GGCTGACAGGGACCAGTATGAGCTTCTCTGCCTGAACAACAGTCGGGCGCCAGTGGATGC
-----+-----+-----+-----+-----+-----+660
CCGACTGTCCCTGGTCATACTCGAAGAGACGGACTTGTGTCAGCCCGCGGTACCTACG
  A  D  R  D  Q  Y  E  L  L  C  L  N  N  S  R  A  P  V  D  A

GTTCAAGGAGTGCCACCTGGCCCAGGTCCCTTCTCATGCTGTCTGCGTGGCCGAAGTGTGGA
-----+-----+-----+-----+-----+-----+720
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F  K  E  C  H  L  A  Q  V  P  S  H  A  V  V  A  R  S  V  D

TGGCAAGGAAGACTTGATCTGGAAGCTTCTCAGCAAGGCGCAGGAAAAAAAAAAAAAAAA
-----+-----+-----+-----+-----+-----+778
ACCGTTCCTTCTGAACTAGACCTTCGAAGAGTCGTTCCGCGTCCTTTTTTTTTTTTTTT
  G  K  E  D  L  I  W  K  L  L  S  K  A  Q

```

Notes: The characters in bold at the 5' end of cDNA clone PM 8 indicate the 'G-tail' added to the 3' end of the first strand cDNA prior to the synthesis of the second strand.

The underlined sequence at the 3' end of the clone indicates the polyadenylate sequence used to prime the synthesis of the first strand cDNA. This polyadenylate sequence is most likely an artefact produced by the annealing of the oligo(dT) primer to an A-rich region in the messenger RNA coding for bovine lactoferrin. See text for further discussion.

The hatched underlined sequence illustrates the position of peptide sequence determined from mature bovine lactoferrin.

As indicated in figure 3.41, the 3' end of clone PM 8 has a polyadenylate tail characteristic of the 3' end of mature messenger RNA species. The cDNA insert in clone PM 8 is only 778 bp long and encodes sequence near the 5' terminus of the bovine lactoferrin mRNA. Consequently, this polyadenylate tail is most likely a cloning artefact produced by the annealing of an oligo(dT) primer to an adenine-rich tract within the the coding region of the bovine

lactoferrin mRNA. Figure 3.42 depicts one possible way in which this cloning artefact may have occurred.

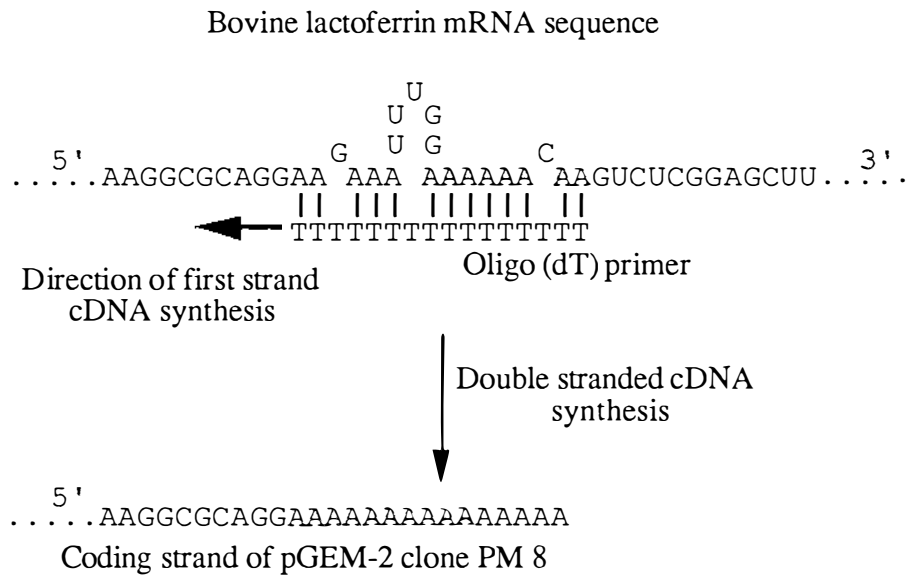


Figure 3.42 A possible scheme for the cDNA synthesis of pGEM-2™ clone PM 8.

Several lines of evidence support the theory that PM 8 resulted from a cloning artefact and that it does not actually represent a distinct mRNA species. Firstly, the sequence near the 3' end of PM 8 lacks the usual polyadenylation signal seen in most mRNAs. Most eukaryotic messenger RNAs have the signal sequence AAUAAA approximately 15 to 30 bases 5' to the poly A tail (Proudfoot, 1982). Futhermore, the sequence 709 base pairs 5' to the polyadenylate tail is identical to the nucleotide sequence of PM 7 (25 bp to 734 bp in clone PM 7). Finally, the predicted amino acid sequence of clone PM 8 concurs with the sequence of two bovine lactoferrin tryptic peptides (HPLC fractions 8 and 15,17; see chapter two, table 2.6). See figure 3.41 for the position of the two tryptic peptides (hatched underlines) within the deduced amino acid sequence for PM 8.

3.3.5.3 Cloning and sequencing of double-stranded cDNA produced by the polymerase chain reaction

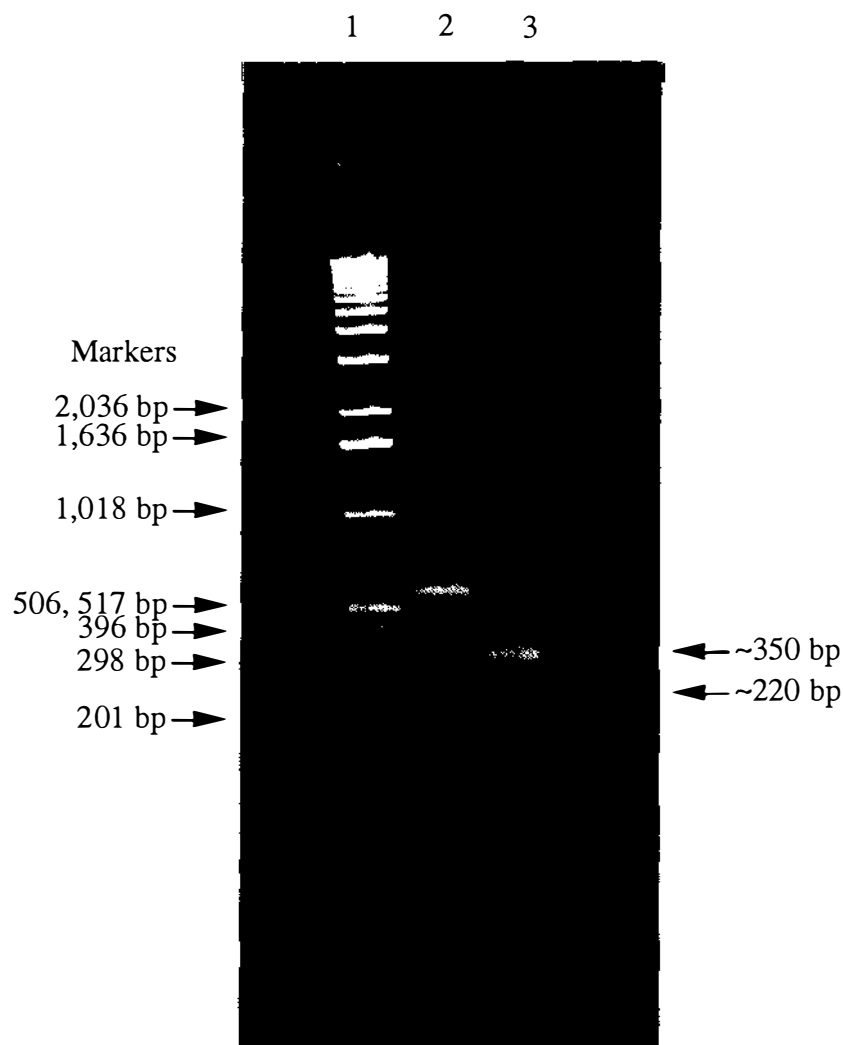
Double-stranded cDNA was prepared from first strand 'G-tailed' products by the polymerase chain reaction (PCR) as described in 3.2.3.1.2. The first thirty cycles were performed in the presence of d(pC)₂₀ and JT 12. The products from this reaction were analysed by agarose gel electrophoresis

(data not shown). The cDNA products appeared as a very diffuse smear on the gel when stained with ethidium bromide and observed under ultraviolet light. The products of the first round of PCR ranged from approximately one hundred to over three thousand base pairs in length.

A small aliquot (approximately one-tenth) of the first round PCR was reamplified using a nested primer (JT 15) in an attempt to increase the specificity of the PCR reaction. An aliquot of the second round of PCR was examined by agarose gel electrophoresis. Figure 3.43 illustrates the electrophoretic profile of the second round PCR products on an ethidium bromide-stained agarose gel. Figure 3.43 (lane 2) also illustrates the PCR products amplified when cDNA clone PM 8 was used as the template. In this track there are two bands, one at approximately 550 bp and another at approximately 200 bp. The lower of the two bands is of the size expected from the amplification of PM 8 DNA with d(pC)₂₀ and JT 15. The higher molecular weight band (~550 bp) is most likely an artefact produced by the non-specific annealing of either of the primers used to direct the amplification. This high molecular weight band was not characterised further.

In the PCR reaction directed by first strand 'G-tailed' cDNA, there were two clearly visible bands at approximately 350 and 220 bp. There was another, more diffuse, band visible at approximately 600 bp. The ~350 bp and the ~220 bp bands (figure 3.43, lane 3) were isolated from low gelling temperature agarose by adherence to glass beads and cloned into the sequencing vector M13mp18. Clear plaques were chosen at random. DNA isolated from these plaques was sequenced by the dideoxynucleotide chain termination method. The PCR product of approximately 600 bp (figure 3.43, lane 3) was not characterised further.

Figures 3.44 and 3.45 illustrate the sequence data obtained from two clones (PCR 1 and PCR 2 respectively) isolated at random from the PCR products cloned into M13. Clone PCR 1 was isolated from the ligation products of the high molecular weight (350 bp) band.



Gel details: 1% (w/v) agarose gel stained with ethidium bromide.

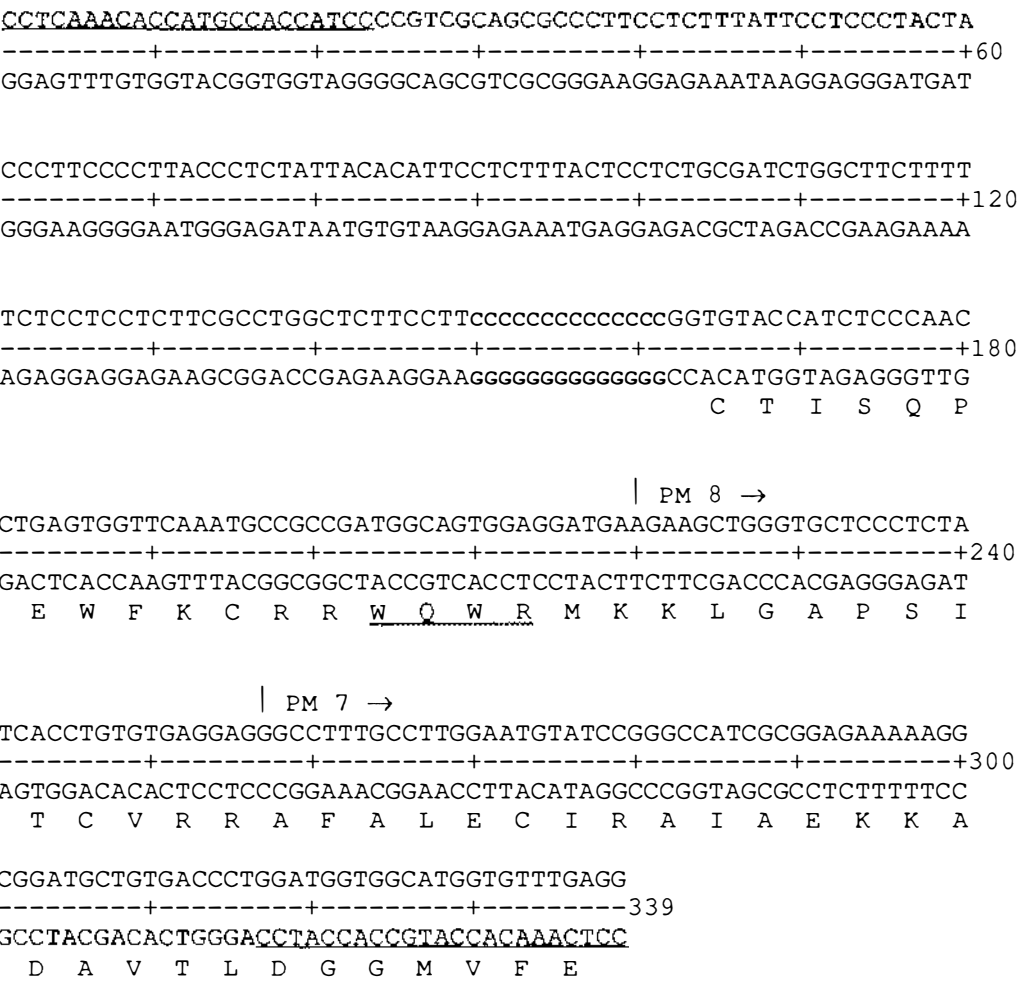
Loading details: 1. BRL 1 kb ladder.

2. PCR products from the amplification of cloned PM 8 DNA using primers JT 15 and d(pC)₂₀ (one-tenth of the PCR reaction).

3. PCR products from the amplification of 'G-tailed' first strand cDNA using primers JT 12 and d(pC)₂₀ for the first thirty cycles of PCR, and then reamplified for thirty cycles using primers JT 15 and d(pC)₂₀ (one-tenth of the PCR reaction).

Figure 3.43 Agarose gel electrophoresis of anchored PCR products.

Figure 3.44 The nucleotide sequence and predicted amino acid sequence of the cloned cDNA PCR product PCR 1.



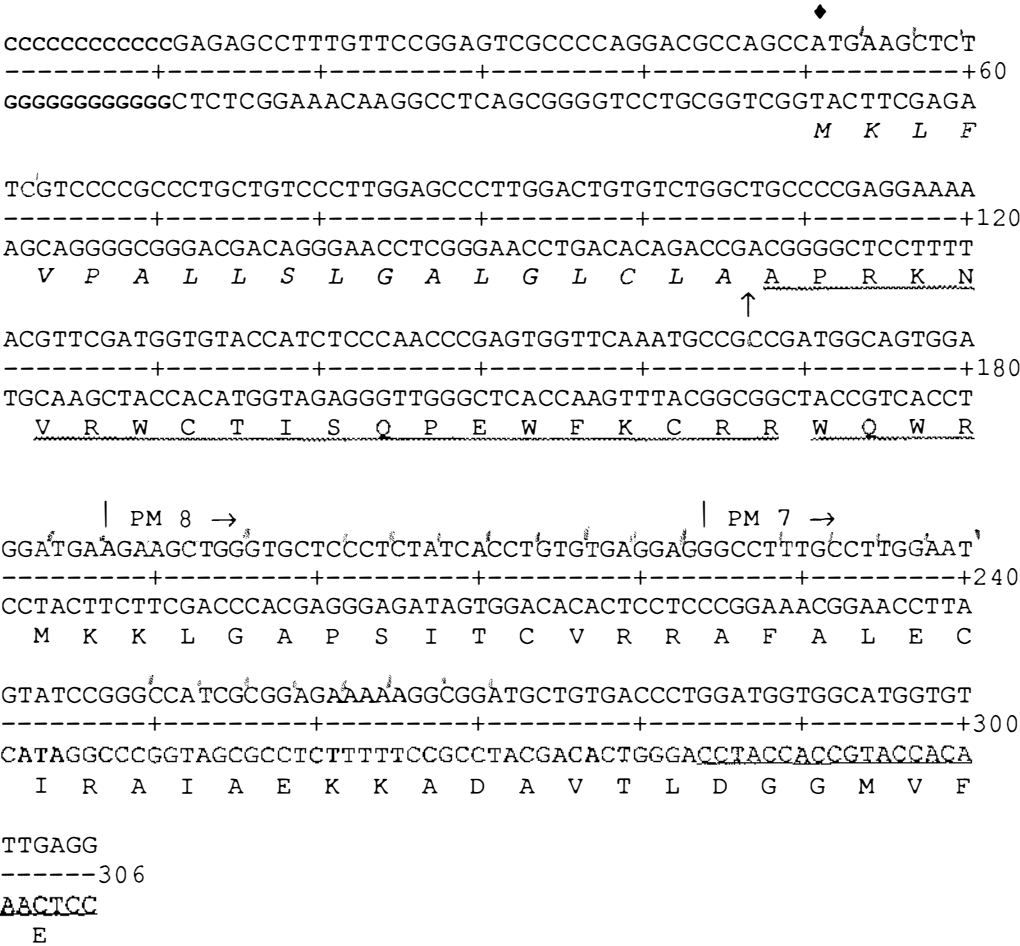
Notes: The outlined characters indicate the 'G-tail' added to the 3' end of the first strand cDNA. The characters underlined at the 5' and 3' ends of clone PCR 1 represent the sequence of primer JT 15 used to amplify the 5' bovine lactoferrin cDNA sequence. The sequence 5' to the 'G-tail' is most likely a PCR induced artefact. See text for further discussion. The hatched underlined characters indicate the position of peptide sequences determined from mature bovine lactoferrin. The position of the 5' termini of clones PM 7 and PM 8 are shown (eg. | PM 7 →).

The 5' 148 bp of clone PCR 1 appears to be a cloning artefact. The translated nucleotide sequence does not correspond to known bovine lactoferrin amino acid sequence. The presence of the 'G-tail' in the middle of the clone and the presence of primer JT 15 at both ends of the clone also indicate a cloning artefact. The sequence 3' to the 'G-tail' corresponds to known bovine lactoferrin mRNA sequence. The 176 bp sequence 3' to the 'G-tail' overlaps the sequence of clones PM 7 and PM 8 by 84 and 121 base pairs respectively and extends 55 bp 5' to the bovine lactoferrin mRNA sequence previously determined (that is, the 5' end of clone PM 8).

The amino acid sequence deduced from PCR 1 overlaps the mature bovine lactoferrin N-terminal (see chapter two, table 2.3). The sequence of PCR 1 is missing the first eight amino acids of the mature protein. The complete amino acid sequence of bovine lactoferrin can be determined by combining the predicted amino acid sequence of the overlapping clones (PM 7 and PCR 1) with the N-terminal amino acid sequence of the mature protein.

In an attempt to find sequence that extends further 5' to the known sequences, another clone was selected at random and sequenced as described earlier. Clone PCR 2 was isolated from the ligation products of the lower molecular weight (~220 bp) band (see figure 3.43). The nucleotide sequence of clone PCR 2 is shown in figure 3.45. This figure also shows the predicted amino acid sequence of clone PCR 2.

Figure 3.45 The nucleotide sequence and predicted amino acid sequence of cloned cDNA PCR product PCR 2.



Notes: The ♦ indicates the position of the putative ATG initiation codon. The amino acids shown in *italics* indicate the postulated N-terminal hydrophobic leader sequence. This 19 amino acid peptide is cleaved, at the position indicated (↑), prior to the secretion of the mature protein. The characters in **bold** at the 5' end of the molecule illustrate the 'G-tail' that was added to the 3' end of the first strand cDNA. The characters underlined at the 3' end of clone PCR 2 represent the sequence of the PCR primer JT 15. The hatched underlined characters indicate the position of peptide sequences determined from mature bovine lactoferrin. The 5' termini of PM 7 and PM 8 are shown (eg. | PM 7 →).

Clone PCR 2 overlaps sequence obtained earlier for the bovine lactoferrin mRNA. More importantly, the sequence of PCR 2 includes the initiation codon and 38 bases of 5' untranslated sequence. The deduced amino acid sequence contains a putative 19 residue signal peptide which would direct the nascent polypeptide into the lumen of the rough endoplasmic reticulum.

3.4 Discussion and Summary

The interpretation of the sequence data obtained in this chapter will be discussed in detail in the subsequent chapter. In this section attention will be paid to the molecular techniques employed to determine the complete mRNA nucleotide sequence coding for bovine lactoferrin.

The combined sequence of the cDNA clones listed in this chapter overlap to encompass the entire coding region of the mRNA coding for bovine lactoferrin. The combined sequence is listed in appendix I. Since the publication of this sequence (Mead and Tweedie, 1990), another group has reported the molecular cloning of the cDNA coding for bovine salivary gland lactoferrin (Pierce *et al.*, 1991). The sequence reported by these authors is approximately 99% identical to the nucleotide sequence listed in appendix I. The differences in the two sequences are likely to be either sequencing errors or they may represent normal sequence variations arising between different populations of cows.

Several clones that hybridised to the human lactoferrin cDNA probes were isolated from the λ gt11 library which, on Southern blot analysis, did not appear to fit into the map for the mRNA coding for bovine lactoferrin. For example, clone λ gt11Lf 1.4 hybridised to both PHL-41 and PHL-44 but appeared to be shorter than clones that hybridised to only PHL-41 (for example λ gt11Lf 1.10; refer to section 3.3.4.1). Subcloning and partial nucleotide sequence analysis (data not shown) indicated that these clones resulted from cloning artefacts that occurred during the construction of the cDNA library. Several phenomena may be responsible for these artefacts. For example, annealing of the oligo(dT) primer to 'A-rich' sequence 5' to the polyadenylate tail may result in 3'-truncated cDNA clones (for example, PM 8). Although several of these clones were partially characterised it is not possible to state the true nature of the cloning events that lead to the formation of these clones. Consequently, it is conceivable, that these clones may represent a distinct, but closely related family of proteins in the involuting bovine mammary gland.

The isolation of clones from the λ gt11 library that had EcoRI restriction endonuclease sites at each end, but lacked the characteristic sequence of the EcoRI linker used in cloning, indicated that there had been incomplete

methylation of the cDNA prior to addition of the linkers. Double-stranded cDNA was incubated in the presence of EcoRI methylase and a methyl donor (S-adenosyl-methionine) in order to protect the EcoRI sites within the cDNA. After the addition of the linkers, the cDNA was digested with EcoRI to remove the excess linkers and to expose EcoRI cohesive ends. If the methylation reaction failed to protect all the EcoRI sites within the cDNA, these sites would have been recognised and cut by the enzyme in the subsequent steps. Bovine lactoferrin clones PM 1 and PM 2 are thought to have arisen from this cloning step. Other investigators have reported this problem. For example, when trying to isolate the cDNA coding for the amyloid A4 precursor (indicated in Alzheimer's disease) researchers noted that insufficient methylation of cDNA resulted in truncated amyloid A4 precursor clones (Muller-Hill and Beyreuther, 1989).

cDNA clones isolated from the λ gt11 library did not include sequence near the 5' end of the bovine lactoferrin mRNA. Several factors may be responsible for the lack of full-length cDNA clones in the library. Firstly, insufficient methylation of the cDNA during the cloning steps may have lead to digestion of the cDNA during EcoRI digestion to expose EcoRI cohesive ends. Secondly, the quality of the mRNA used to prime the synthesis of the first strand cDNA is critical to the isolation full-length clones. If the template mRNA is partially degraded the resulting cDNA will not be full-length. Although the template mRNA used in this study appeared intact by northern blot analysis, it is impossible to rule out the possibility that partial degradation of the mRNA was a cause for the truncated cDNA clones isolated from the λ gt11 library. Thirdly, secondary structure in the mRNA can lead to truncated cDNA. The presence of secondary structure in the mRNA can lead to the reverse transcriptase 'stalling' on the mRNA. At this point, the endogenous RNase H, which recognises RNA:DNA hybrids and cleaves the RNA, may digest the mRNA, resulting in truncated first strand cDNA.

Anchored PCR was used in an attempt to isolate cDNA clones that encoded the 5'-end of the bovine lactoferrin mRNA. PCR is a powerful technique which allows the rapid amplification of rare DNA sequences. Frohman and co-workers (1988) described a technique for the rapid amplification of cDNA ends (RACE). The method used in this study was adapted from the method reported by these workers.

Several attempts were made to produce full-length first strand 'G-tailed' cDNA. Pancreatic ribonuclease inhibitor (RNasin) was added to the reactions to inhibit the degradation of the template mRNA. In addition, various methods were attempted in an effort to decrease the potential amount of secondary structure in the mRNA. These included, denaturation at 65°C followed by quenching on ice, pretreatment of the mRNA with methyl mercury hydroxide to methylate the RNA (data not shown) and incubation of the first strand cDNA reaction at higher temperatures than usual (that is, at 45°C). In addition, AMV reverse transcriptase was used in place of M-MLV reverse transcriptase as the former can withstand higher temperatures.

Cloning and sequencing of anchored PCR products elucidated the sequence at the 5' end of the bovine lactoferrin mRNA. However, the addition of a 'G-tail' at the 5' end of the first strand cDNA did lead to problems when sequencing these clones. The tailing reaction was necessary to provide an 'anchor' of the PCR reaction, but it also resulted in gel compression problems when sequencing the PCR products. Another problem associated with 'G-tailing' the first strand cDNA was the production of PCR artefacts such as PCR 1. Clone PCR 1 had the same specific primer (JT 15) at each end and the 'G-tail' in the centre (refer to section 3.3.5.3).

Summary

This chapter has outlined the cloning and sequencing of cDNA clones that encompass the entire coding region of the bovine lactoferrin mRNA. Although a full-length cDNA clone was not isolated, overlapping regions of the clones isolated in this study shared 100% sequence identity. Furthermore, the predicted amino acid sequence concurred with the sequence of the tryptic peptides of bovine lactoferrin isolated in this study.

The features of the composite bovine lactoferrin mRNA and amino acid sequences will be discussed in detail in the next chapter.

Chapter Four

General Discussion

4.1 Introduction

This chapter will focus primarily on the interpretation of the sequence data obtained in the previous chapters.

4.2 Nucleic acid sequence data

The complete mRNA sequence coding for bovine mammary lactoferrin has been determined by cDNA cloning techniques. This sequence is listed in appendix I. The sequence is a composite of the nucleic acids sequences of cDNA clones PM 7 and PCR 2. Appendix II lists the computer generated restriction map of the composite bovine lactoferrin cDNA sequence.

The length of the mRNA coding for bovine lactoferrin was estimated to be approximately 2.3 kb by northern blot analysis of bovine mammary RNA (see chapter three, figure 3.10). The combined length of the cDNA encoding bovine lactoferrin is 2364 bp and is in accord with the length of the mRNA determined by northern hybridisation. The sequence presented in appendix I contains a putative open reading frame of 2124 bp corresponding to a 708 amino acid propeptide. The open reading frame is flanked by a 38 bp 5' untranslated region and a 192 bp 3' untranslated region. The characteristics of the untranslated regions and the putative open reading frame will be discussed separately in the following sections.

4.2.1 The 5' untranslated region of the bovine lactoferrin mRNA

The 5'-most cDNA clone isolated in this study, PCR 2, contained a 38 bp 5' untranslated region upstream from the putative ATG translation start site. As indicated above, the total composite length of the cDNAs isolated in this study was 2364 bp, which is approximately the same as the length of the mRNA estimated by northern blot hybridisation experiments. The precise length of the 5' noncoding "leader" sequence, that is the number of bases from the m⁷G cap site to the putative ATG, remains unsolved. Virtually all eukaryotic mRNAs have a 5'-terminal cap structure consisting of a

cleavage and polyadenylation of the mRNA (Wigley *et al.*, 1990). The enzyme that adds the poly(A) tail is a classical poly(A) polymerase and a specificity factor is required for selectivity of AAUAAA-containing RNA species (Wigley *et al.*, 1990). The presence of a poly(A) tail has been shown to both stimulate translation and stabilize the mRNA (Wilkins, 1990). There is only one polyadenylation signal present in the 3' untranslated region of the bovine lactoferrin cDNA.

The second feature of note in the 3' noncoding region is the presence of a potential mRNA destabilising sequence. Caput *et al.* (1986) reported the presence of a putative regulatory sequence (UUAUUUAU) in the 3'-untranslated region of mRNA molecules encoding inflammatory regulators. The messenger RNAs encoding regulatory cellular proteins such as oncogenes, lymphokines, cytokines and transcriptional activators have a very short half-life in the cell. The 3'-untranslated region of these mRNA molecules contain a common AUUUA pentamer which imparts cytoplasmic instability (Malter, 1989). Malter (1989) identified a cytoplasmic protein that binds specifically to the AUUUA pentamer sequence. Malter proposed that the binding of this specific cytoplasmic factor targeted the mRNA for rapid cytoplasmic degradation. The presence of a single AUUUA sequence in the 3'-untranslated region of the bovine lactoferrin mRNA (2290-2294 bp, appendix I) may reflect a requirement for strict control of the expression of bovine lactoferrin. Interestingly, lactoferrin has been implicated as a growth factor (Nichols *et al.*, 1987) and as a regulator of inflammation and immunity (Nemet and Simonovits, 1985). The presence of a regulatory sequence, commonly found in inducible growth regulators, may reflect the involvement of lactoferrin in the regulation of cell growth and the inflammation process. Although the 3'-noncoding regions of some oncogene and cytokine mRNAs have only one AUUUA pentamer (Cosman, 1987), it is not clear if the presence a single 'pentamer' is sufficient to allow recognition of the AUUUA-specific mRNA binding protein (Malter, 1989).

4.2.3 The putative open reading frame

The bovine lactoferrin mRNA has a putative open reading frame of 2124 bp. The features of the amino acid sequence deduced from this open reading frame will be discussed in subsequent sections. As indicated earlier in this thesis (chapter one), the members of the transferrin family appear to have

evolved from an ancient gene duplication event. The two halves of the putative open reading frame share extensive nucleic acid sequence homology. The two 'halves' of the open reading frame, corresponding to the N- and C-terminal lobes of the protein, were aligned using the UWGCG package programmes FASTA and WORDSEARCH (Devereux *et al.*, 1984). The percent nucleic acid sequence identity between the two 'halves' of the coding region was approximately 53%.

4.3 Amino acid sequence data

Amino acid sequence data for bovine lactoferrin were obtained by both automated N-terminal amino acid sequencing (chapter two) and the translation of the nucleic acid sequence of cloned cDNAs (chapter three).

4.3.1 The pre-lactoferrin sequence

The putative open reading frame contains a nineteen amino acid leader sequence at the N-terminal of the protein. This predominantly hydrophobic sequence is characteristic of secreted proteins. The role of the leader sequence is to target the nascent polypeptide into the lumen of the rough endoplasmic reticulum. The leader sequence is cleaved from the protein prior to secretion from the cell.

The deduced bovine lactoferrin leader sequence has the characteristic signal for cleavage by the endoplasmic reticulum signal peptidase [Ala (-1); Cys (-3)] (von Heijne, 1983). In common with the leader sequence of other secreted proteins, the signal peptide in bovine lactoferrin contains predominantly hydrophobic residues.

4.3.2 The amino acid sequence of mature bovine lactoferrin

The complete amino acid sequence of bovine lactoferrin has been determined from the nucleic acid sequence of the mRNA. The deduced sequence is identical to the amino acid sequence of the N-terminal of the mature protein and of the tryptic peptides determined in this study. The features of the amino acid sequence will be discussed in the following sections.

(i) Similarity to other members of the transferrin family

The deduced amino acid sequence shares extensive sequence identity with the other members of the transferrin family. Appendix III illustrates the alignment of the members of the transferrin family. The protein sequences were aligned to further illustrate the extensive amino acid identity observed between the two lobes of each protein. Of particular importance are the highly conserved iron-binding ligands. These are illustrated in appendix III by asterisks.

Table 4.1 lists the amino acid sequence identity and similarity observed between mature bovine lactoferrin and some of members of the transferrin family. This table clearly illustrates that bovine lactoferrin exhibits extensive sequence homology with other members of the transferrin superfamily of iron-binding proteins.

(ii) N-linked glycosylation sites

N-linked glycosyl groups are added to proteins at sites distinguished by the tripeptide sequence NXS or NXT. There are five potential glycosylation sites in the putative open reading frame encoding bovine lactoferrin. In comparison, human lactoferrin contains only two covalently attached sugar chains. Although there are five potential sites in the bovine protein sequence, it remains to be determined whether all of these sites are utilised in the mature protein. Tsuji and co-workers (1989) have reported the presence of multiple molecular forms of lactoferrin in bovine milk. In this study no alternative protein sequences were observed that could account for the different molecular forms of lactoferrin. It is possible, however, that the alternative molecular forms represent differences in glycosylation patterns on the peptide backbone.

(iii) The secondary anion binding site

To bind metal ions, all transferrins require the concomitant binding of an anion. In physiological conditions this anion is usually carbonate. Several workers have illustrated that other anions can replace carbonate in the co-ordination of metals ions at the iron binding site (see chapter one).

Table 4.1 Percent amino acid sequence identity and similarity between bovine lactoferrin and several members of the transferrin family.

Protein	Percent identity with bovine lactoferrin	Percent similarity with bovine lactoferrin
Human lactoferrin ¹	69.7	82.0
Mouse lactoferrin ²	64.0	77.5
Human transferrin ³	62.3	75.4
Horse transferrin ⁴	63.4	75.6
Pig transferrin ⁵	61.9	75.7
Rabbit transferrin ⁶	61.7	74.2
Chicken ovotransferrin ⁷	54.0	69.1
Human melano-transferrin ⁸	42.4	61.6

Notes: Sequence alignments were made using the UWGCG program BESTFIT (Devereux et al., 1984). References: 1. Human lactoferrin (Accession number X52941) Powell and Ogden (1990); 2. Mouse lactoferrin (J03298) Pentecost and Teng (1987); 3. Human transferrin (M12530) Yang et al. (1984). 4. Horse transferrin (M69020) Carpenter, M.A. unpublished data (1991); 5. Pig transferrin (X12386) Baldwin and Weinstock (1988); 6. Rabbit transferrin (X58533) Banfield D.K., Chow, B.K., Funk, W.D., Robertson, K.A., Umelas, T.M., Woodworth, R.C. and MacGillivray, R.T. unpublished data (1991); 7. Chicken ovotransferrin (X02009) Jeltsch and Chambon (1982); 8. Human melanotransferrin (M12154) Rose et al. (1986). Appendix IV depicts the sequence comparisons between the members of the transferrin family.

The release of iron from lactoferrin also requires the presence of ions. Kretchmar and Raymond (1988) reported that as the ionic strength of the medium approached zero the rate of release of iron from lactoferrin also approached zero. In addition, Williams *et al.* (1982) reported that non-synergistic anions binding in secondary sites influenced the release of iron from lactoferrin. The results of the present study indicate a site, potentially capable of binding large anions, such as citrate, exists in the C-terminal lobe of bovine lactoferrin.

The putative secondary anion binding site in the C-terminal lobe of bovine lactoferrin is illustrated diagrammatically in the following figures (4.2 to 4.5).

The nature of the proposed secondary anion site will be explained graphically using the structure of human lactoferrin to highlight the salient points. The high degree of sequence homology between human and bovine lactoferrin suggests that the overall folding pattern of the two proteins will be similar. Moreover, preliminary tertiary structure analysis of bovine lactoferrin has indicated that the molecular structure of the two proteins are similar (Dr. M. Haridas and Prof. E.N. Baker, personal communication). Figure 4.2 depicts an α -carbon ribbon structure diagram of human lactoferrin. The reader will be guided to the region of interest by the next two figures. Figure 4.3 illustrates the ribbon structure of the C-terminal lobe of human lactoferrin. The side chains providing iron-binding ligands and the carbonate anion co-ordinated with the metal are shown in red. The metal atom is depicted by the small sphere in the centre of the structure. Figure 4.4 represents a small section of the C-terminal lobe of human lactoferrin. Again the iron atom is represented by the small sphere in the centre of the diagram and the iron-binding ligands are shown in red. The side chains involved in the postulated secondary anion site in bovine lactoferrin are shown in orange. Figure 4.5 depicts the predicted α -carbon ribbon structure of bovine lactoferrin in the same region of the C-terminal lobe depicted in figure 4.4. Amino acid sequence data suggests that the iron binding ligands are the same in human and bovine lactoferrin. The side chain and carbonate ligands co-ordinated with the metal are shown in orange. The ligands involved in the postulated secondary anion-binding site are shown in green. The side chains of interest are the those attached to α -carbon atoms (CA) 415, 546 and 547 (see figure 4.5). Table 4.2 lists the residues in human and bovine lactoferrin at these positions.

Table 4.2 Comparison of the amino acid side chains of human and bovine lactoferrin thought to be involved in the secondary anion site in bovine lactoferrin.

Residue number	Human lactoferrin		Bovine lactoferrin	
	Side chain	Charge	Side chain	Charge
415	Tyr	0	Arg	+1
546	Lys	+1	Lys	+1
547	Asp	-1	Asn	0

Notes: Residue numbers are from human lactoferrin. Charge refers to the net charge of the side chain group at physiological pH.

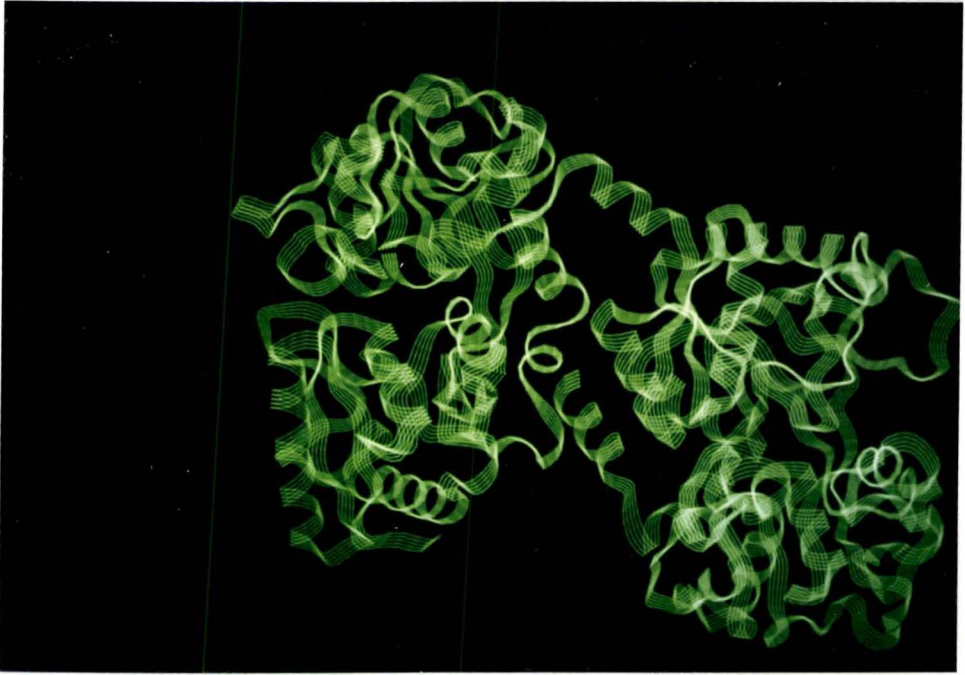


Figure 4.2 α -carbon ribbon structure of human lactoferrin. The N-lobe and the C-lobe are on the left and right hand sides of the diagram respectively.

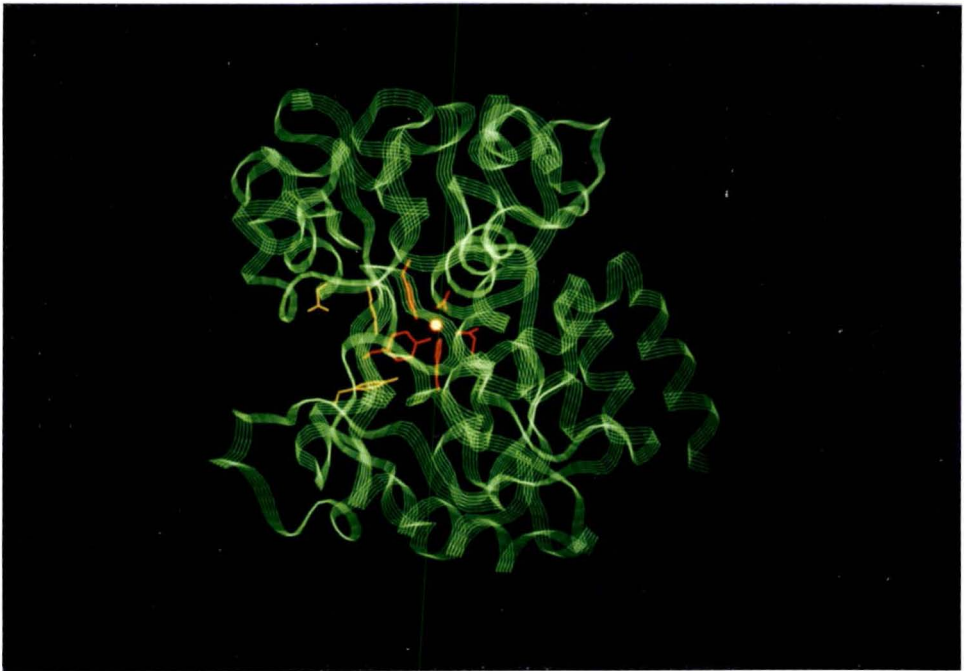


Figure 4.3 α -carbon ribbon structure of the C-terminal lobe of human lactoferrin. The metal atom is indicated by the small sphere in the centre of the illustration.

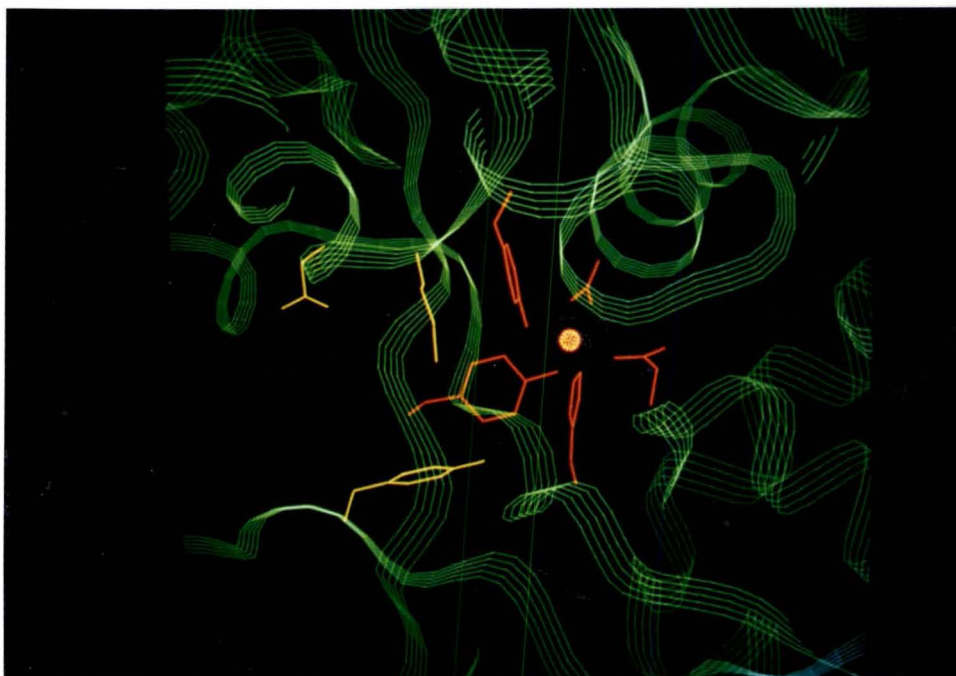


Figure 4.4 Close-up of the iron-binding site in the C-terminal lobe of human lactoferrin. This α -carbon ribbon structure diagram also indicates some neighbouring side chains thought to be involved in forming a secondary anion-binding site in bovine lactoferrin (orange-coloured side chains). The metal-binding ligands are shown in red.

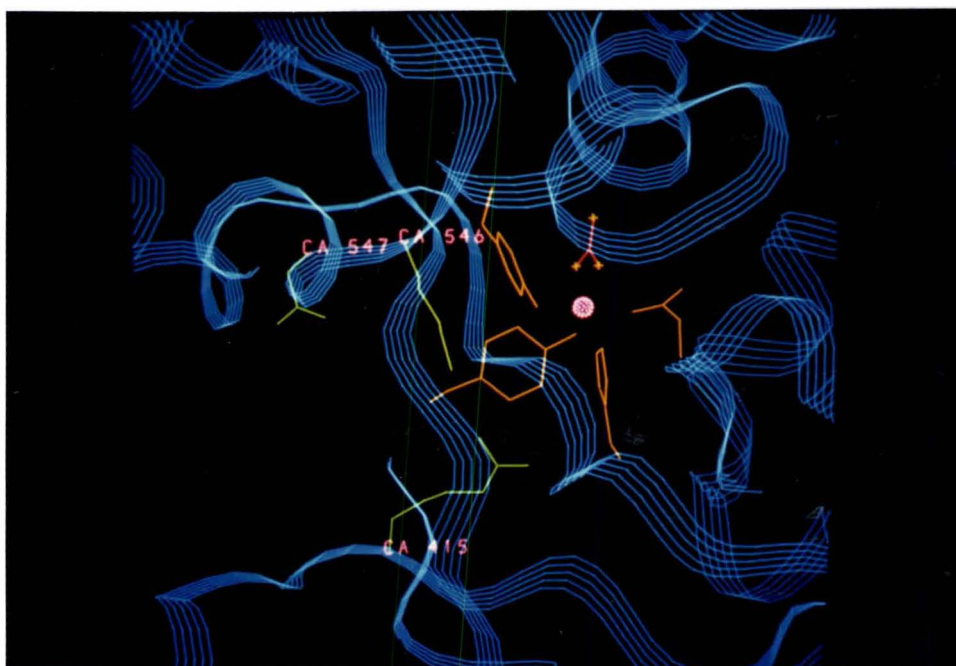


Figure 4.5 Close-up of the proposed iron-binding site of the C-terminal lobe of bovine lactoferrin. The iron-binding ligands are shown in orange. The residues forming the putative secondary anion-binding site are shown in green. The structure illustrated here is based on that of human lactoferrin.

In this model, the two basic residues (Arg⁴¹⁵ and Lys⁵⁴⁶) in bovine lactoferrin, face one another across the cleft that separates the C1 and C2 domains. The structure of bovine lactoferrin has not been solved to a resolution that allows identification of individual side chains. For this reason, the position of the side chains in the corresponding positions in bovine lactoferrin was modelled on the position of the residues in human lactoferrin. The position of the side chains was 'predicted' using the computer program HYDRA, and were fitted to maximise hydrogen bond contacts.

It is proposed that an anionic species, such as citrate, could interact with these positively charged groups. Citrate is not a synergistic anion, that is, it can not replace carbonate in forming a stable transferrin-iron-anion ternary complex (Schlabach and Bates, 1975). However, citrate does interact specifically with bovine lactoferrin and binds in a position close enough to the iron atom to effect the ESR-signal from the co-ordinated metal (Prof. E.N. Baker, personal communication). The binding of an anion at this secondary anion-binding site may perturb the structure of the iron-binding site and facilitate the release of the metal. This secondary anion site is apparently unique to the C-terminal lobe of bovine lactoferrin. Although the lysine at position 546 is conserved in both human and bovine lactoferrin, the tyrosine at position 415 in human lactoferrin is an arginine in bovine lactoferrin. In human lactoferrin the lysine residue (Lys⁵⁴⁶) forms a salt bridge with a neighbouring aspartic acid side chain (Asp⁵⁴⁷). In bovine lactoferrin this acidic residue is replaced by a neutral asparagine side chain (Asn⁵⁴⁷). Asn⁵⁴⁷ in bovine lactoferrin may be glycosylated as it is contained within a putative glycosylation attachment signal sequence (NDT).

The significance of this putative secondary anion-binding site is not clear. As indicated above, anions binding to the protein at this position may facilitate the release of iron. It is tempting to speculate that this secondary anion-binding site may account, at least in part, for the reversal of the bacteriostasis effect of lactoferrin in the presence of high concentrations of citrate. Several groups have reported that the presence of citrate decreases the ability of bovine lactoferrin to inhibit the growth of coliform bacteria (see section 1.4.1). *In vitro* mutagenesis experiments, for example the creation of a similar secondary anion-binding site in human lactoferrin, may help to determine the significance of this site.

4.4 Summary

The primary objective of this study, namely the isolation of cDNA clones for bovine lactoferrin and the characterisation of the primary sequence of the protein, has been realised. Sequence information obtained by determining and translating the nucleotide sequence of the mRNA encoding bovine lactoferrin has been corroborated by partial amino acid sequence data collected by traditional protein chemistry techniques. Northern blot analysis, using radiolabelled bovine lactoferrin cDNA probes, has been used to demonstrate an increase in expression of lactoferrin mRNA during the involuting of the bovine mammary gland. This may be the source of the high concentration of lactoferrin found in the involuting gland.

4.5 Suggestions for future work

There are several questions which have arisen from this work which could be fruitfully addressed in future studies. Some of these will be discussed briefly in the following sections.

(i) Verification of the sequence of the mRNA encoding bovine lactoferrin

The sequence presented in this thesis is a composite of at least two cDNA clones. Consequently, despite the extensive overlaps, one must consider the possibility that the clones presented in this study are, in fact, from separate members of a closely related family of proteins. The isolation of a single full length bovine lactoferrin cDNA clone would help resolve this minor dilemma. In defense of this study, the evidence from both amino acid and nucleic acid sequence data supports the notion that the sequence reported is that of bovine mammary gland lactoferrin.

As mentioned earlier in this chapter, primer extension studies could be used to determine the precise length of the 5' untranslated region of the bovine lactoferrin mRNA.

(ii) Expression of bovine lactoferrin in the mammary gland

cDNA clones isolated and sequenced in this study have been used for *in situ* hybridisation experiments to determine the nature of lactoferrin expression in the mammary gland (Dr. Dick Wilkins and co-workers, MAF Ruakura Research Station, Hamilton, New Zealand). *In situ* hybridisation studies may be used to determine the sites of lactoferrin synthesis and changes in response to various stimuli such as involution and infection of the mammary gland. Mammary biopsies, taken at different stages during the functional cycle of the gland, could be sectioned, mounted and probed with labelled bovine lactoferrin probes. Bovine lactoferrin cDNA cloned into the plasmid vector pGEM-2™ (PM 7 and PM 8) would be particularly useful for this technique. The pGEM vectors allow production of single-stranded 'riboprobes'. These would provide valuable controls for *in situ* hybridisation experiments. Such *in situ* hybridisation experiments may reveal the cell types in the mammary gland which are synthesizing lactoferrin. Not only mammary tissue, but other tissues could be examined by this technique. For example, bone marrow biopsies could be examined by *in situ* hybridisation to determine which neutrophil precursors are synthesizing lactoferrin messenger RNA.

(iii) Expression of bovine lactoferrin in transfected cell lines

Introduction of the bovine lactoferrin cDNA into a suitable eukaryotic expression vector and transfection of a suitable tissue culture cell line would enable *in vitro* mutagenesis experiments to be carried out on this protein. The ability to specifically manipulate proteins *in vitro* and study both the physical and biological effects of the altered protein is an exciting development in biochemistry. This technology is being employed, in the laboratory where this work was carried out, to further characterise human lactoferrin (Stowell *et al.*, 1991). Application of this technology to the study of bovine lactoferrin would advance our understanding of transferrins in general.

(iv) Isolation and characterisation of the bovine lactoferrin gene

Bovine lactoferrin cDNA clones will be used to characterise the lactoferrin gene. Preliminary experiments using Southern blot analysis have determined that the bovine lactoferrin gene spans approximately 35 kb of genomic DNA (this study, data not shown). This is similar to the reported size of the human serum transferrin gene (33.5 kb; Schaeffer *et al.*, 1987).

The bovine lactoferrin cDNA clones produced during this study have been used to isolate genomic DNA sequences encoding the lactoferrin gene (H.B. Bain and J.W. Tweedie, personal communication). Characterisation of the bovine lactoferrin gene will allow more accurate definition of the phylogenetic links between bovine lactoferrin and the other members of the transferrin family.

Characterisation of the promoter region at the 5' end of the bovine lactoferrin gene will be of particular interest. Comparison with the promoter regions of other transferrin genes may help identify transcription factor binding motifs in the DNA that account for the tissue specificity and inducibility of expression of this protein. Attaching the bovine lactoferrin promoter region to a reporter gene, such as chloramphenicol acyl transferase (CAT) or luciferase, and introducing these constructs into suitable cell lines will help identify the DNA motifs that control the expression of the gene. The effects of selectively deleting transcriptional activator binding motifs from the promoter region can be followed using reporter gene technology. Precise mapping and characterisation of the bovine lactoferrin promoter region will undoubtedly advance our knowledge of the expression of the transferrin gene family in general. Tissue-specific expression and hormonal inducibility are two areas of study that are awaiting further clarification.

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Appendices

Appendix I. The nucleotide sequence of the cDNA coding for bovine mammary gland lactoferrin and the predicted amino acid sequence of the protein.

```

GAGAGCCTTTGTTCCGGAGTCGCCCCAGGACGCCAGCCATGAAGCTCTTCGTCCCCGCC
-----+-----+-----+-----+-----+-----+60
CTCTCGGAAACAAGGCCTCAGCGGGGTCCTGCGGTCTCGAGAAGCAGGGGCGGG
                                M K L F V P A L

TGCTGTCCCTTGGAGCCCTTGGACTGTGTCTGGCTGCCCCGAGGAAAAACGTTTCGATGGT
-----+-----+-----+-----+-----+-----+120
ACGACAGGGAACCTCGGGAACCTGACACAGACCGACGGGGCTCCTTTTTGCAAGCTACCA
    L S L G A L G L C L A A P R K N V R W C

GTACCATCTCCCAACCCGAGTGGTTCAAATGCCGCCGATGGCAGTGGAGGATGAAGAAGC
-----+-----+-----+-----+-----+-----+180
CATGGTAGAGGGTTGGGCTCACCAAGTTTACGGCGGCTACCGTCACCTCCTACTTCTTCG
    T I S Q P E W F K C R R W Q W R M K K L

TGGGTGCTCCCTCTATCACCTGTGTGAGGAGGGCCTTTGCCTTGAATGTATCCGGGCCA
-----+-----+-----+-----+-----+-----+240
ACCCACGAGGGAGATAGTGGACACACTCCTCCCGGAAACGGAACCTTACATAGGCCCGGT
    G A P S I T C V R R A F A L E C I R A I

TCGCGGAGAAAAAGGCGGATGCTGTGACCCTGGATGGTGGCATGGTGTGTTGAGGCGGGCC
-----+-----+-----+-----+-----+-----+300
AGCGCCTCTTTTTCCGCTACGACACTGGGACCTACCACCGTACCACAAACTCCGCCCGG
    A E K K A D A V T L D G G M V F E A G R

GGGACCCCTACAAACTGCGGCCAGTAGCAGCAGAGATCTATGGGACGAAAGAGTCTCCCC
-----+-----+-----+-----+-----+-----+360
CCCTGGGGATGTTTGACGCCGGTCATCGTCGTCTCTAGATACCCTGCTTTCTCAGAGGGG
    D P Y K L R P V A A E I Y G T K E S P Q

AAACCCACTATTATGCTGTGGCCGTCGTGAAGAAGGGCAGCAACTTTCAGCTGGAACAGC
-----+-----+-----+-----+-----+-----+420
TTTGGGTGATAATACGACACCGGCAGCACTTCTTCCCGTCGTTGAAAGTCGACCTGGTCG
    T H Y Y A V A V V K K G S N F Q L D Q L

TGCAAGGCCGGAAGTCCTGCCATACGGGCCTTGGCAGGTCCGCTGGGTGGATCATCCCTA
-----+-----+-----+-----+-----+-----+480
ACGTTCCGGCCTTCAGGACGGTATGCCCAGAACCGTCCAGGCGACCCACCTAGTAGGGAT
    Q G R K S C H T G L G R S A G W I I P M

TGGAATCCTTCGCCCCGTAAGTCTGAGCTGGACAGAGTCACTCGAGCCCCCTCAGGGAGCTG
-----+-----+-----+-----+-----+-----+540
ACCCTTAGGAAGCGGGCATGAACTCGACCTGTCTCAGTGAGCTCGGGGAGGTCCCTCGAC
    G I L R P Y L S W T E S L E P L Q G A V

TGGCTAAATTCTTCTCTGCCAGCTGTGTTCCCTGCATTGATAGACAAGCATACCCCAACC
-----+-----+-----+-----+-----+-----+600
ACCGATTTAAGAAGAGACGGTCGACACAAGGGACGTAAGTATCTGTTCGTATGGGGTTGG
    A K F F S A S C V P C I D R Q A Y P N L

```

TGTGTCAACTGTGCAAGGGGAGGGGAGAACCAGTGTGCCTGCTCCTCCCGGAACCAT
 -----+-----+-----+-----+-----+-----+660
 ACACAGTTGACACGTTCCCCCTCCCCCTCTTGGTCACACGGACGAGGAGGGCCCTTGGTA
 C Q L C K G E G E N Q C A C S S R E P Y

 ACTTCGGTTATTCTGGTGCCTTCAAGTGTCTGCAGGACGGGGCTGGAGACGTGGCTTTTG
 -----+-----+-----+-----+-----+-----+720
 TGAAGCCAATAAGACCACGGAAGTTCACAGACGTCCTGCCCCGACCTCTGCACCGAAAAC
 F G Y S G A F K C L Q D G A G D V A F V

 TTAAAGAGACGACAGTGTGTTGAGAACTTGCCAGAGAAGGCTGACAGGGACCAGTATGAGC
 -----+-----+-----+-----+-----+-----+780
 AATTTCTCTGCTGTCACAACTCTTGAACGGTCTCTTCCGACTGTCCCTGGTCATACTCG
 K E T T V F E N L P E K A D R D Q Y E L

 TTCTCTGCCTGAACAACAGTCGGGCGCCAGTGGATGCGTTCAAGGAGTGCCACCTGGCCC
 -----+-----+-----+-----+-----+-----+840
 AAGAGACGGACTTGTGTCAGCCCGCGGTACCTACGCAAGTTCCTCACGGTGGACCGGG
 L C L N N S R A P V D A F K E C H L A Q

 AGGTCCCTTCTCATGCTGCTGCTGGCCCGAAGTGTGGATGGCAAGGAAGACTTGATCTGGA
 -----+-----+-----+-----+-----+-----+900
 TCCAGGGAAGAGTACGACAGCACCGGGCTTACACCTACCGTTCCTTCTGAACTAGACCT
 V P S H A V V A R S V D G K E D L I W K

 AGCTTCTCAGCAAGGCGCAGGAGAAATTTGGAACCAAGTCTCGGAGCTTCCAGCTCT
 -----+-----+-----+-----+-----+-----+960
 TCGAAGAGTCGTTCCGCGTCCTCTTTAAACCTTTTTTGTTCAGAGCCTCGAAGGTCGAGA
 L L S K A Q E K F G K N K S R S F Q L F

 TTGGCTCTCCACCCGGCCAGAGGGACCTGCTGTTCAAAGACTCTGCTCTTGGGTTTTTGA
 -----+-----+-----+-----+-----+-----+1020
 AACCGAGAGGTGGGCCGGTCTCCCTGGACGACAAGTTTCTGAGACGAGAACCCAAAACCT
 G S P P G Q R D L L F K D S A L G F L R

 GGATCCCCCTCGAAGGTAGATTCTGGCGCTGTACCTGGGCTCCCGCTACTTGACCACCTTGA
 -----+-----+-----+-----+-----+-----+1080
 CCTAGGGGAGCTTCCATCTAAGCCGCGACATGGACCCGAGGGCGATGAACTGGTGGAACCT
 I P S K V D S A L Y L G S R Y L T T L K

 AGAACCTCAGGGAACTGCGGAGGAGGTGAAGGCGCGGTACACCAGGGTCGTGTGGTGTG
 -----+-----+-----+-----+-----+-----+1140
 TCTTGGAGTCCCTTTGACGCCTCCTCCACTTCCGCGCCATGTGGTCCCAGCACACCACAC
 N L R E T A E E V K A R Y T R V V W C A

 CCGTGGGACCTGAGGAGCAGAAGAAGTGCCAGCAGTGGAGCCAGCAGAGCGGCCAGAACG
 -----+-----+-----+-----+-----+-----+1200
 GGCACCCTGGACTCCTCGTCTTCTTCACGGTCGTCACCTCGGTCTCGCCGGTCTTGC
 V G P E E Q K K C Q Q W S Q Q S G Q N V

 TGACCTGTGCCACGGCGTCCACCACTGACGACTGCATCGTCCTGGTGCTGAAAGGGGAAG
 -----+-----+-----+-----+-----+-----+1260
 ACTGGACACGGTGCCGAGGTGGTGAAGTGTGACGTAGCAGGACCAGACTTTCCCTTC
T C A T A S T T D D C I V L V L K G E A

 CAGATGCCCTGAACTTGGATGGAGGATATATCTACACTGCGGGCAAGTGTGGCCTGGTGC
 -----+-----+-----+-----+-----+-----+1320
 GTCTACGGGACTTGAACCTACCTCCTATATAGATGTGACGCCCGTTACACCGGACCACG
 D A L N L D G G Y I Y T A G K C G L V P

CTGTCCTGGCAGAGAACCGGAAATCCTCCAAACACAGTAGCCTAGATTGTGTGCTGAGAC
 -----+-----+-----+-----+-----+-----+1380
 GACAGGACCGTCTCTTGGCCTTTAGGAGGTTTGTGTCATCGGATCTAACACACGACTCTG
 V L A E N R K S S K H S S L D C V L R P

 CAACGGAAGGGTACCTTGCCGTGGCAGTTGTCAAGAAAGCAAATGAGGGGCTCACATGGA
 -----+-----+-----+-----+-----+-----+1440
 GTTGCCCTTCCCATGGAACGGCACCGTCAACAGTTCTTTCGTTTACTCCCCGAGTGACCT
 T E G Y L A V A V V K K A N E G L T W N

 ATTCTCTGAAAGACAAGAAGTCGTGCCACACCGCCGTGGACAGGACTGCAGGCTGGAACA
 -----+-----+-----+-----+-----+-----+1500
 TAAGAGACTTTTCTGTTCTTCAGCACGGTGTGGCGGCACCTGTCCTGACGTCCGACCTTGT
 S L K D K K S C H T A V D R T A G W N I

 TCCCCATGGGCCTGATCGTCAACCAGACAGGCTCCTGCGCATTTGATGAATTCTTTAGTC
 -----+-----+-----+-----+-----+-----+1560
 AGGGGTACCCGACTAGCAGTTGGTCTGTCCGAGGACGCGTAAACTACTTAAGAAATCAG
 P M G L I V N Q T G S C A F D E F F S Q

 AGAGCTGTGCCCCTGGGGCTGACCCGAAATCCAGACTCTGTGCCTTGTGTGCTGGCGATG
 -----+-----+-----+-----+-----+-----+1620
 TCTCGACACGGGGACCCGACTGGGCTTTAGGTCTGAGACACGGAACACACGACCGCTAC
 S C A P G A D P K S R L C A L C A G D D

 ACCAGGGCCTGGACAAGTGTGTGCCCAACTCTAAGGAGAAGTACTATGGCTATACCGGGG
 -----+-----+-----+-----+-----+-----+1680
 TGGTCCCGGACCTGTTACACACGGGTTGAGATTCCCTCTTCATGATACCGATATGGCCCC
 Q G L D K C V P N S K E K Y Y G Y T G A

 CTTTCAGGTGCCTGGCTGAGGACGTTGGGGACGTTGCCTTTGTGAAAAACGACACAGTCT
 -----+-----+-----+-----+-----+-----+1740
 GAAAGTCCACGGACCGACTCCTGCAACCCCTGCAACGGAAACACTTTTTGCTGTGTCAGA
 F R C L A E D V G D V A F V K N D T V W

 GGGAGAACACGAATGGAGAGAGCACTGCAGACTGGGCTAAGAACTGAATCGTGAGGACT
 -----+-----+-----+-----+-----+-----+1800
 CCCTCTTGTGCTTACCTCTCTCGTGACGTCTGACCCGATTCTTGAACCTAGCACTCCTGA
 E N T N G E S T A D W A K N L N R E D F

 TCAGGTTGCTCTGCCTCGATGGCACCAGGAAGCCTGTGACGGAGGCTCAGAGCTGCCACC
 -----+-----+-----+-----+-----+-----+1860
 AGTCCAACGAGACGGAGCTACCGTGGTCTTCGGACACTGCCTCCGAGTCTCGACGGTGG
 R L L C L D G T R K P V T E A Q S C H L

 TGGCGGTGGCCCCGAATCACGCTGTGGTGTCTCGGAGCGATAGGGCAGCACACGTGAAAC
 -----+-----+-----+-----+-----+-----+1920
 ACCGCCACCGGGGCTTAGTGCGACACCACAGACCTCGCTATCCCGTCGTGTGCACTTTG
 A V A P N H A V V S R S D R A A H V K Q

 AGGTGCTGCTCCACCAGCAGGCTCTGTTTGGGAAAAATGGAAAAACTGCCCGGACAAGT
 -----+-----+-----+-----+-----+-----+1980
 TCCACGACGAGGTGGTCGTCCGAGACAAACCTTTTTACCTTTTTTGACGGGCCTGTTCA
 V L L H Q Q A L F G K N G K N C P D K F

 TTTGTTTGTTCAAATCTGAAACAAAAACCTTCTGTTCAATGACAACACTGAGTGTCTGG
 -----+-----+-----+-----+-----+-----+2040
 AAACAAACAAGTTTAGACTTTGGTTTTTGAAGACAAGTTACTGTTGTGACTCACAGACC
 C L F K S E T K N L L F N D N T E C L A

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CCAAACTTGGAGGCAGACCAACGTATGAAGAATATTTGGGGACAGAGTATGTCACGGCCA
-----+-----+-----+-----+-----+-----+2100
GGTTTGAACCTCCGTCTGGTTGCATACTTCTTATAAACCCCTGTCTCATAAGTGCCGGT
  K  L  G  G  R  P  T  Y  E  E  Y  L  G  T  E  Y  V  T  A  I

TTGCCAACCTGAAAAAATGCTCAACCTCCCCGCTTCTGGAAGCCTGCGCCTTCCTGACGA
-----+-----+-----+-----+-----+-----+2160
AACGGTTGGACTTTTTTACGAGTTGGAGGGGCGAAGACCTTCGGACGCGGAAGGACTGCT
  A  N  L  K  K  C  S  T  S  P  L  L  E  A  C  A  F  L  T  R

GGTAAAGCCTGCAAAGAAGCTAGCCTGCCTCCCTGGGCCTCAGCTCCTCCCTGCTCTCAG
-----+-----+-----+-----+-----+-----+2220
CCATTTTCGGACGTTTCTTCGATCGGACGGAGGGACCCGGAGTCGAGGAGGGACGAGAGTC
  *
CCCCAATCTCCAGGCGGAGGGACCTTCCTCTCCCTTCCTGAAGTCGGATTTTTTGCCAAG
-----+-----+-----+-----+-----+-----+2280
GGGGTTAGAGGTCCGCGCTCCCTGGAAGGAGAGGGAAGGACTTCAGCCTAAAAACGGTTC

CTCATCAGTATTTACAATTCCTGCTGTCATTTTAGCAAGAAATAAAATTACAAATGCTG
-----+-----+-----+-----+-----+-----+2340
GAGTAGTCATAAATGTTAAGGGACGACAGTAAATCGTTCTTTATTTAATGTTTACGAC

TTGATTTTCATTCCCTAAAAAAA
-----+-----+-----+-----2364
AACTAAAAGTAAGGGATTTTTTTT

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*Notes: The initiation codon, ATG (shown by the symbol ♦) begins at nucleotide 39. The nineteen amino acid leader sequence is shown in italics. The sequence of the mature (secreted) protein begins at amino acid 20. The five potential N-linked glycosylation sites (either NXS or NXT) are underlined. The symbol * indicates the position of the stop codon TAA. The polyadenylation signal (AATAAA) is shown in small bold and is approximately 30 bp upstream of the polyadenylate tail. The putative mRNA destabilisation signal (ATTTTA) is indicated by the dotted underline.*


```

                                     B
                                     s
                                     T
                                     S MTt
                                     C B t Hash C Bl cBS C B
H      iT      R      Av s      yGiepl ATPMXv a2 osc      AvMs
nf      s      li p      LsnI41 vallhi n8 Rar      linp
fi      a      uJ G      TufI51 aqeyoJ I6 IJF      uJlC
II      I      II I      IIIIII IIIIII II III      IIII
                                     /      // /      /      /      //
TGGGAATCCTTCGCCCCGTA CTTGAGCTGGACAGAGTCACTCGAGCCCCCTCCAGGGAGCTG
-----+-----+-----+-----+-----+-----+540
ACCCCTTAGGAAGCGGGCATGAACTCGACCTGTCTCAGTGAGCTCGGGGAGGTCCCTCGAC
G I L R P Y L S W T E S L E P L Q G A V

                                     N
MC      T      CsP
bv      s      Avpv
oi      p      liBu
IJ      E      uJII
II      I      IIII
                                     ///
TGGCTAAATTCTTCTCTGCCAGCTGTGTTCCCTGCATTGATAGACAAGCATACCCCAACC
-----+-----+-----+-----+-----+-----+600
ACCGATTTAAGAAGAGACGGTTCGACACAAGGGACGTA ACTATCTGTTCGTATGGGGTTGG
A K F F S A S C V P C I D R Q A Y P N L

T
t
h      H
l      i      D      B      SND S
l      n      M      rB      AsMNNclrMcS
l      c      n      ds      vascradnrm
I      I      l      Ir      aJpiiFIIlFa
I      I      I      II      IIIIIIVIIII
                                     ///// ///
TGTGTCAACTGTGCAAGGGGGAGGGGGAGAACCAGTGTGCCTGCTCCTCCCGGAACCAT
-----+-----+-----+-----+-----+-----+660
ACACAGTTGACACGTTCCCCCTCCCCCTCTTGGTCACACGGACGAGGAGGGCCCTTGGA
C Q L C K G E G E N Q C A C S S R E P Y

                                     N      B C      M E C      B
B l      S P      s v      a s v      s
a a      f s      m i      e p i      m
n I      e t      A J      I 3 J      A
I V      I I      I I      I I I      I
ACTTCGGTTATTCTGGTGCCTTCAAGTGTCTGCAGGACGGGGCTGGAGACGTGGCTTTTG
-----+-----+-----+-----+-----+-----+720
TGAAGCCAATAAGACCACGGAAGTTCACAGACGTCCTGCCCGACCTCTGCACCGAAAC
F G Y S G A F K C L Q D G A G D V A F V
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      T
      t
      h
      l      E
M  G  l      s      S      C      S      C
s  s  l      p      y      v      vluB      Av
e  u  I      3      L      i      aa9s      li
I  I  I      I      T      J      II6r      uJ
      I      I      IVII      II
      /      /
TTAAAGAGACGACAGTGTGTTGAGAACTTGCCAGAGAAGGCTGACAGGGACAGTATGAGC
-----+-----+-----+-----+-----+780
AATTTCTCTGCTGTGCACAAACTCTTGAACGGTCTCTTCCGACTGTCCCTGGTCATACTCG
K  E  T  T  V  F  E  N  L  P  E  K  A  D  R  D  Q  Y  E  L

      E  S  E
      A  HNS      c  S  aCBec
      BhNHBalf      F      o  c  uvsco
      aaahseaa      o      R  r  9iaoR
      nIrarIIN      k      I  F  6JJNI
      IIIIIIVI      I      I  I  IIIII
      /  ///      //
TTCTCTGCCTGAACAACAGTCGGGCGCCAGTGGATGCGTTCAAGGAGTGCCACCTGGCCC
-----+-----+-----+-----+-----+840
AAGAGACGGACTTGTTGTCAGCCCGCGGTACCTACGCAAGTTCCTCACGGTGGACCGGG
L  C  L  N  N  S  R  A  P  V  D  A  F  K  E  C  H  L  A  Q

      E
      C
      H  o  S      N      S  H      S      i
      aAO  NPaS      l      aCa      Ba  M  n
      evlFlpuc      a      M  uve      F  buD  b  d
      Ia0iau9r      I      w  9iI      o  v3p  o  I
      II9nIM6F      I      o  6JI      k  IAn  I  I
      IIIIVIII      I      I  III      I  III  I  I
      /  /  ///      /
AGGTCCCTTCTCATGCTGTCTGGCCCGAAGTGTGGATGGCAAGGAAGACTTGATCTGGA
-----+-----+-----+-----+-----+900
TCCAGGGAAGAGTACGACAGCACCGGGCTTCACACCTACCGTTCCTTCTGAACTAGACCT
V  P  S  H  A  V  V  A  R  S  V  D  G  K  E  D  L  I  W  K

      C      T      B  C      C  B
      Av  D      H      s      s  Av      Av  s
      li  d      h      p      m  li      li  t
      uJ  e      a      E      A  uJ      uJ  X
      II  I      I      I      I  II      II  I
      /      /
AGCTTCTCAGCAAGGCGCAGGAGAAATTTGGAACCAAGTCTCGGAGCTTCCAGCTCT
-----+-----+-----+-----+-----+960
TCGAAGAGTCGTTCCGCGTCTCTTTAAACCTTTTTTGTTCAGAGCCTCGAAGGTCGAGA
L  L  S  K  A  Q  E  K  F  G  K  N  K  S  R  S  F  Q  L  F
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      E
      C
      o S
    H S
C      G Ca St AONPa      B H
v      dMEMveNcy vllpu      Ps i      M M A
i      inasiIcrL a0au9      lp n      l n l
J      IlepJliFT I9IM6      eM f      y l w
I      IIIIIIIII IIVII      II I      I I I
      / / // // //
TTGGCTCTCCACCCGGCCAGAGGGACCTGCTGTTCAAAGACTCTGCTCTTGGGTTTTTGA
-----+-----+-----+-----+-----+1020
AACCGAGAGGTGGGCCGGTCTCCCTGGACGACAAGTTTCTGAGACGAGAACCCAAAAACT
G S P P G Q R D L L F K D S A L G F L R

      B
      s
      p
    S      E      CNB1B
BB Na      H      H cBS      vla2sS      F
asDlu      AT      MiT      Ha Rosc      a
mtpa3      la      nnf      he sRar      ian8pp      a
HYnIA      wq      lfi      aI aIJF      JII6Ci      u
IIIVI      II      III      II IIII      IVIIII      I
      / // / //
GGATCCCCTCGAAGGTAGATTTCGGCGCTGTACCTGGGCTCCCGCTACTTGACCACCTTGA
-----+-----+-----+-----+-----+1080
CCTAGGGGAGCTTCCATCTAAGCCGCGACATGGACCCGAGGGCGATGAACCTGGTGGAAC
I P S K V D S A L Y L G S R Y L T T L K

      B      E
      s M      cBS B
uD      b M MM      HT HR osc c
3d      o n nn      hh ps Rar e
6e      I l ll      aa ha IJF f
II      I I II      II II III I
      / /
AGAACCTCAGGGAAACTGCGGAGGAGGTGAAGGCGCGGTACACCAGGGTCGTGTGGTGTG
-----+-----+-----+-----+-----+1140
TCTTGGAGTCCCTTTGACGCCTCCTCCACTTCCGCGCCATGTGGTCCCAGCACACCACAC
N L R E T A E E V K A R Y T R V V W C A

      E      E      E
      c      c S F H M
      o B S      c      c S F H M
B AONPs a      oM NC o tG nCa Ma
sD M vllpuDu      Pb lv P Myd Euve ae
as n a0au3d9      lo ai 1 wLi a4iI eI
Ja l I9IM6e6      5I IJ 5 oTI eHJI II
II I IIVIIII      II VI I III IIII II
      / // // / / /
CCGTGGGACCTGAGGAGCAGAAGAAGTGCCAGTCAGTGAGCCAGCAGCGGCCAGAACG
-----+-----+-----+-----+-----+1200
GGCACCTGGACTCCTCGTCTTCTTCACGGTCGTACCTCGGTCTGTCGCCGGTCTTGC
V G P E E Q K K C Q Q W S Q Q S G Q N V

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T
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sDs h
asp a
JaC I
III I

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o cf
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I FN
I II

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a
N
I

TGACCTGTGCCACGGCGTCCACCACTGACGACTGCATCGTCCTGGTGCTGAAAGGGGAAG
-----+-----+-----+-----+-----+-----+-----+1260
ACTGGACACGGGTGCCGCAGGTGGTGACTGCTGACGTAGCAGGACCACGACTTTCCCTTC
T C A T A S T T D D C I V L V L K G E A

B
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C
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M
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l
I

F F
o a
k u
I I

E H
Cc a SN
voHeBcl
iRaIara
JIeInFI
IIIIIIIV
/// /

CAGATGCCCTGAACTTGGATGGAGGATATATCTACACTGCGGGCAAGTGTGGCCTGGTGC
-----+-----+-----+-----+-----+-----+1320
GTCTACGGGACTTGAACCTACCTCCTATATAGATGTGACGCCCCTTCACACCGGACCACG
D A L N L D G G Y I Y T A G K C G L V P

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c S
o c
R r
I F
I I

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v M
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l J e
I I I
I I I

B
Bs
sm
aA
II

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D
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o
P
I

CTGTCCTGGCAGAGAACCGGAAATCCTCCAAACACAGTAGCCTAGATTGTGTGCTGAGAC
-----+-----+-----+-----+-----+-----+1380
GACAGGACCGTCTCTTGGCCTTTAGGAGGTTTGTGTCATCGGATCTAACACACGACTCTG
V L A E N R K S S K H S S L D C V L R P

B
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lRK
a asp
n Ian
I VII

B B
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asp
JaC
III

M
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l
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C B1
v a2
i n8
J I6
I II

lE
ac
Io
IR
II

CAACGGAAGGGTACCTTGCCGTGGCAGTTGTCAAGAAAGCAAATGAGGGGCTCACATGGA
-----+-----+-----+-----+-----+-----+1440
GTTGCCTTCCCATGGAACGGCACCGTCAACAGTTCTTTTCGTTTACTCCCCGAGTGTACCT
T E G Y L A V A V V K K A N E G L T W N

U
b
a
T 1 B B C
s 1 c S sD FS P v
p 0 e p as of s i
E 5 f i Ja ke t J
I I I I I II II I I
/
ATTCTCTGAAAGACAAGAAGTCGTGCCACACCGCCGTGGACAGGACTGCAGGCTGGAACA
-----+-----+-----+-----+-----+1500
TAAGAGACTTTCTGTTCTTCAGCACGGTGTGGCGGCACCTGTCCTGACGTCCGACCTTGT
S L K D K K S C H T A V D R T A G W N I

NS H S H
B laCa a i CN B E T
sDNSauve u D n vl s FH c s
asctI9iI 3 p c ia p sh o p
JaoyI6JI A n I JI C pa R E
IIIIIIII I I I IV I II I I
/// / /
TCCCCATGGGCCTGATCGTCAACCAGACAGGCTCCTGCGCATTTGATGAATTCTTTAGTC
-----+-----+-----+-----+-----+1560
AGGGGTACCCGGACTAGCAGTTGGTCTGTCCGAGGACGCGTAAACTACTTAAGAAATCAG
P M G L I V N Q T G S C A F D E F F S Q

B
s
S pE
t C BB1c SC H A
y Av ss2oBMcv P i l M M
L li aa8Rgwri l n w l w
T uJ JJ6IloFJ e f N y o
I II IIIIIII I I I I I
/ // //
AGAGCTGTGCCCTGGGGCTGACCCGAAATCCAGACTCTGTGCCTTGTGTGCTGGCGATG
-----+-----+-----+-----+-----+1620
TCTCGACACGGGGACCCGACTGGGCTTTAGGTCTGAGACACGGAACACACGACCGCTAC
S C A P G A D P K S R L C A L C A G D D

E B
c s
E oS EH p
cBS OaCacaBPS 1 C B S C
osc luvloesfc 2 D RS v sMnc v
Rar 09iwRIplr 8 d sc i ascr i
IJF 96JNIIIGMF 6 e aa J JpiF J
III IIIIIIIII I I II I IIII I
/ // // /
ACCAGGGCCTGGACAAGTGTGTGCCCAACTCTAAGGAGAAGTACTATGGCTATACCGGGG
-----+-----+-----+-----+-----+1680
TGGTCCCGGACCTGTTTCACACACGGGTTGAGATTCTTCTTCATGATACCGATATGGCCCC
Q G L D K C V P N S K E K Y Y G Y T G A

E B t
 Nc pS C E M M h
 B loMuc vDc a a l
 a aRn1r ido e e l
 n I1l0F JeN I I l
 I VIII III I I I
 / / /
 CTTTCAGGTGCCTGGCTGAGGACGTTGGGGACGTTGCCTTTGTGAAAAACGACACAGTCT
 -----+-----+-----+-----+-----+1740
 GAAAGTCCACGGACCGACTCCTGCAACCCCTGCAACGGAAACACTTTTTGCTGTGTCTAGA
 F R C L A E D V G D V A F V K N D T V W

 B
 H s
 i p E
 n 1H C c H
 f 2gS P M BvD o MiT
 I 8if s w sid 5 nnf
 I 6Ae t o rJe 7 lfi
 I III I I III I III
 // //
 GGGAGAACACGAATGGAGAGAGCACTGCAGACTGGGCTAAGAACTTGAATCGTGAGGACT
 -----+-----+-----+-----+-----+1800
 CCCTCTTGTGCTTACCTCTCTCGTGACGTCTGACCCGATTCTTGAACCTTAGCACTCCTGA
 E N T N G E S T A D W A K N L N R E D F

 E M T S F E
 Nc S C a s C t Cn c S
 T BMB loMc v Me p B vD y Avu o c
 a gwa aRnr i nI 4 b id L li4 R r
 q lon I1lF J lI 5 v Je T uJH I F
 I III VIII I III I I II I III I I
 / / //
 TCAGGTTGCTCTGCCTCGATGGCACCAGGAAGCCTGTGACGGAGGCTCAGAGCTGCCACC
 -----+-----+-----+-----+-----+1860
 AGTCCAACGAGACGGAGCTACCGTGGTCTTCGGACACTGCCTCCGAGTCTCGACGGTGG
 R L L C L D G T R K P V T E A Q S C H L

 H
 S H i D F A
 B aCaN Hn r B n f BM
 s uvel ifT a s u l BsaP B
 p 9iIa nIf I m 4 I baem b
 C 6JII fIi I A H I vAIl v
 I IIIV III I I I I III I
 // // //
 TGGCGGTGGCCCCGAATCACGCTGTGGTGTCTCGGAGCGATAGGGCAGCACACGTGAAAC
 -----+-----+-----+-----+-----+1920
 ACCGCCACCGGGGCTTAGTGCGACACCACAGAGCCTCGCTATCCCGTCGTGTGCACTTTG
 A V A P N H A V V S R S D R A A H V K Q

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      T      T
      t E    t
      h c    h
F      l o    C A      S l
An     M l P   v l    MNcl
lu     w l l   i w    scr l
w4     o l 5   J N    piFI
NH     I I I   I I    I I I I
II     I I I   I I    I I I I
                        ///
AGGTGCTGCTCCACCAGCAGGCTCTGTTTGGGAAAAATGGAAAAAACTGCCCGGACAAGT
-----+-----+-----+-----+-----+-----+-----+1980
TCCACGACGAGGTGGTTCGTCGAGACAAACCTTTTTACCTTTTTTGACGGGCCTGTTCA
  V  L  L  H  Q  Q  A  L  F  G  K  N  G  K  N  C  P  D  K  F

      D
      r
      D a      E B
      d I      a a
      e I      e l
      I I      I I

TTTGTGTTGTTCAAATCTGAAACCAAAAACCTTCTGTTCAATGACAACACTGAGTGTCTGG
-----+-----+-----+-----+-----+-----+2040
AAACAAACAAGTTTAGACTTTGGTTTTTGGGAAGACAAGTTACTGTTGTGACTCACAGACC
  C  L  F  K  S  E  T  K  N  L  L  F  N  D  N  T  E  C  L  A

      H      S      M T      H
C a      E      M      M      t      a sG      Ca B
vHeM      c      a      S      b      y      e pd E ve s
iaIn      o      e      s      o      L      I 4i a iI p
JeIl      P      I      p      I      T      I 5I e JI C
IIII      I      I      I      I      I      I  I I I I I I
//////      /

CCAAACTTGGAGGCAGACCAACGTATGAAGAATATTTGGGGACAGAGTATGTCACGGCCA
-----+-----+-----+-----+-----+-----+2100
GGTTTGAACCTCCGTCTGGTTGCATACTTCTTATAAACCCCTGTCTCATACAGTGCCGGT
  K  L  G  G  R  P  T  Y  E  E  Y  L  G  T  E  Y  V  T  A  I

      B      C B
      c      S      M F      v s      H M
      e      p      n a      i p      h n
      f      i      l u      J C      a l
      I      I      I I      I I      I I

TTGCCAACCTGAAAAATGCTCAACCTCCCCGCTTCTGGAAGCCTGCGCCTTCCTGACGA
-----+-----+-----+-----+-----+-----+2160
AACGGTTGGACTTTTTTACGAGTTGGAGGGGCGAAGACCTTCGGACGCGGAAGGACTGCT
  A  N  L  K  K  C  S  T  S  P  L  L  E  A  C  A  F  L  T  R

      E S      HB
      C      C C      BBcSa CapB      C      C
v      AvMNv      ssocu veusDMAv      M      G      DM      v
i      liahi      aaRr9 iIlp dnl i      n      s      dn      i
J      uJeeJ      JJIF6 JI0CeluJ      l      u      el      J
I      I I I I      I I I I I I I I I      I      I      I I      I
                        ///      //      /      //      /

GGTAAAGCCTGCAAAGAAGCTAGCCTGCCTCCCTGGGCCTCAGCTCCTCCCTGCTCTCAG
-----+-----+-----+-----+-----+-----+2220
CCATTTCGGACGTTTCTTCGATCGGACGGAGGGACCCGGAGTCGAGGAGGGACGAGAGTC

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      E
      c
      o   S
E   c S   AONP a
oMc   HT   vllpMu           M           A
Rnr   hh   a0aum9           n           l
IlF   aa   I9IMe6           l           u
III   II   IIVIII           I           I
      /   /// /

CCCCAATCTCCAGGCGCGAGGGACCTTCCTCTCCCTTCCTGAAGTCGGATTTTTGCCAAG
-----+-----+-----+-----+-----+-----+2280
GGGGTTAGAGGTCCGCGCTCCCTGGAAGGAGAGGGAAGGACTTCAGCCTAAAAACGGTTC

      E
      Cc           T           T
      vo           s           s
      i5           p           p
      J7           E           E
      II           I           I
      //

CTCATCAGTATTTACAATTCCCTGCTGTCATTTTAGCAAGAAATAAAATTACAAATGCTG
-----+-----+-----+-----+-----+-----+2340
GAGTAGTCATAAATGTTAAGGGACGACAGTAAAATCGTTCCTTTATTTTAATGTTTACGAC

TTGATTTTCATTCCCTAAAAAAAAA
-----+-----+-----+-----2364
AACTAAAAGTAAGGGATTTTTTTT
```

Enzymes that do cut the cDNA coding for bovine lactoferrin:

AflIII	AhaII	AluI	AlwI	AlwNI
AvaI	AvaII	BalI	BamH	BanI
BanII	BbvI	BbvII	BceFI	BglI
BglII	Bpu10I	BsaIBsaAI	BsaJI	BsmAI
Bsp1286I	BspCI	BspGI	BspMI	BspMII
BsrI	BstXI	BstYI	Bsu36I	CviJI
DdeI	DpnI	DraIII	DrdI	DrdII
DsaI	EaeI	EarI	Eco57I	EcoNI
EcoO109I	EcoPI	EcoP15I	EcoRI	EcoRII
Esp3I	FauI	FinI	Fnu4HI	FokI
FspI	GdiII	GsuI	HaeI	HaeII
HaeIII	HgaI	HgiAI	HgiEII	HhaI
HincII	HindIII	HinfI	HinfIII	HphI
KpnI	MaeI	MaeII	MaeIII	MboII
MlyI	MmeI	MnlI	MseI	MspI
MwoI	NarI	NciI	NcoI	NheI
NlaIII	NlaIV	NspBII	PflMI	PleI
PmlI	PpuMI	PstI	PvuII	RsaI
Sau96I	Sau3AI	ScaI	ScrFI	SfaNI
SfeI	SmaI	SpiI	SspI	StyI
StyLTI	StySPI	TaqI	TaqII-2TfiI	ThaI
Tsp45I	TspEI	Tth111I	Tth111II	Uba1105I
XcmI	XhoI	XmnI		

Enzymes that do not cut the cDNA coding for bovine lactoferrin:

AatII	AccI	AflII	AgeI	ApaI
ApaLI	AseI	AvrII	BcgI	BclI
BsaBI	BsiI	BsmI	BspHI	BssHII
BstBI	BstEII	Cfr10I	CfrAI	ClaI
DraI	EciI	Eco47III	EcoAI	EcoBI
EcoDI	EcoDXXI	EcoEI	EcoKI	EcoR124I
EcoR	124/3I	EcoRV	EspI	FseI
HpaI	McrI	MfeI	MluI	NaeI
NdeI	NotI	NruI	NsiI	NspI
PshAI	PvuI	RleAI	RsrII	SacII
SalISfii	SgrAI	SnaI	SnaBI	SpeI
SphI	SplI	SstI	StuI	StySBI
StySJI	StySQI	TaqII-1	Uba1108I	XbaI
XmaIII				

Appendix III. The amino acid sequences of members of the transferrin family. The N- and C-terminal lobes are aligned to illustrate the homology between the two lobes of each protein. The numbering system used in this illustration is relative to human lactoferrin. The conserved residues are presented in inverse type and illustrate the high degree of sequence homology within the transferrin family. The iron binding ligands are indicated by the asterisks.

	1	10	20	30	40	50
hLF	GRRRSVQ	WCAVSNPEAT	KQFQWRNMRKYR	---	GPPV	SGLKRDSPICQIAIAE
mLF	AKATTVR	WCAVSNSEED	KLRWQNMERYG	---	GPPL	SEVKKSSSTRQIAIVT
hLF	APRKTVR	WCAVSNPEW	FKERRQWRMKLG	---	AP	SITVRRFALEIRIAIE
sTF	VPDKTVR	WCAVSEHEAT	KQSFDRHMKSVIP	SDGP	SYA	SVKASYLDIRIAAA
oTF	APPKSVIR	WCAVSSPEEK	KNNLRDLTQQR	---	I	SLTEVQKATYLDIKAIAN
MTF	GGMEVVR	WCAVSDPEQ	HKGGMISEAFREAG	---	QP	SLLSVRGTSAHGVQLIAA
	341	350	360	370	380	
hLF	RRARVVR	WCAVGEQEL	RKNQWSGLSE	---	GSVTE	SSASTTDEIALVVK
mLF	SKARVVR	WCAVGEQEL	RKNQWSGLSE	---	GRVTE	SSASTTDEIALVVK
hLF	RYTRVVR	WCAVGEQEL	RKNQWSGLSE	---	QNVTE	SSASTTDEIALVVK
sTF	ECKPYVR	WCAVGEQEL	RKNQWSGLSE	---	GKIEV	SSASTTDEIALVVK
oTF	RENRIQR	WCAVGEQEL	RKNQWSGLSE	---	GDVEV	SSASTTDEIALVVK
MTF	LPPYLRV	WCAVGEQEL	RKNQWSGLSE	---	LPEIQ	SSASTTDEIALVVK
	60	70	80	90		
hLF	NRADAVT	LIQEF	YDAGLAPYK	LRVVAEIVT	GTGTE	-----RQPRTHYVAVA
mLF	NRADAVT	LIQEF	YDAGLAPYK	LRVVAEIVT	GTGTE	-----EQPRTHYVAVA
hLF	KKADAVT	LIQEF	YDAGLAPYK	LRVVAEIVT	GTGTE	-----ESPQTHYVAVA
sTF	NRADAVT	LIQEF	YDAGLAPYK	LRVVAEIVT	GTGTE	-----EDPQTHYVAVA
oTF	NRADAVT	LIQEF	YDAGLAPYK	LRVVAEIVT	GTGTE	-----EGSTTHYVAVA
MTF	QADAVT	LIQEF	YDAGLAPYK	LRVVAEIVT	GTGTE	-----EVGTTHYVAVA
	390	400	410	420	430	
hLF	GEADAVT	LIQEF	YDAGLAPYK	LRVVAEIVT	GTGTE	-----CGLVFLAENKSSQSSDPDPNCYDRPVEGYLATA
mLF	GEADAVT	LIQEF	YDAGLAPYK	LRVVAEIVT	GTGTE	-----CGLVFLAENKSSSKSNGLD-CYNRPVEGYLATA
hLF	GEADAVT	LIQEF	YDAGLAPYK	LRVVAEIVT	GTGTE	-----CGLVFLAENKSSSKHSSLD-CYLRPTEGYLATA
sTF	GEADAVT	LIQEF	YDAGLAPYK	LRVVAEIVT	GTGTE	-----CGLVFLAENKSSKSSD-CEDTPEAGYFATA
oTF	GEADAVT	LIQEF	YDAGLAPYK	LRVVAEIVT	GTGTE	-----CGLVFLAENKSSKSSD-TDERPASIFATA
MTF	EQADAVT	LIQEF	YDAGLAPYK	LRVVAEIVT	GTGTE	-----CGLVFLAENKSSKSSD-DSSNSHYVAVA
	100	110	120	130	140	
hLF	WKKGG	---	SFQLNDLQELK	CHTGLR	ETAEWNVF	DEILRPFL---NWTGPPEPIE
mLF	WKNSS	---	NFHLNQLQELR	CHTGLR	ETAEWNVF	DEILRPYL---NWNPPASLE
hLF	WKKGS	---	NFQLDQLQERK	CHTGLR	ETAEWNVF	DEILRPYL---SWTESLEPLQ
sTF	WKKDS	---	GFQINQLQERK	CHTGLR	ETAEWNVF	DEILRPYL---PEPRKPLE
oTF	WKKGT	---	EFTYNDLQERK	CHTGLR	ETAEWNVF	DEILRPYL---RLSVMGCDVL
MTF	WRRSS	---	HVTIDTLQERK	CHTGLR	ETAEWNVF	DEILRPYL---RPSKCDVL
	440	450	460	470	480	
hLF	WRRSDT	---	SLTWNSVREKK	CHTAYDE	TAEWNVF	DEILRPYL---RPSKCDVL
mLF	WRRSDT	---	SLTWNSVREKK	CHTAYDE	TAEWNVF	DEILRPYL---RPSKCDVL
hLF	WRRSDT	---	SLTWNSVREKK	CHTAYDE	TAEWNVF	DEILRPYL---RPSKCDVL
sTF	WRRSDT	---	SLTWNSVREKK	CHTAYDE	TAEWNVF	DEILRPYL---RPSKCDVL
oTF	WRRSDT	---	SLTWNSVREKK	CHTAYDE	TAEWNVF	DEILRPYL---RPSKCDVL
MTF	WRRSDT	---	SLTWNSVREKK	CHTAYDE	TAEWNVF	DEILRPYL---RPSKCDVL

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150      160      170      180      190
hLF  AAVARFFASVFGADKGQFP-NLRRLAETGENK--EAFSSQEPYFSYSEAFK
mLF  EAVSKFFSKSVFGAQKDRFP-NLSSSAETGANK--GASSPEEPYSGYAEALR
hLF  GAVAKFFBASVFCIDRQAYP-NLQQLKEEGENQ--GACSSREPWFYGYSEAFK
sTF  KAVANFFGSAFACADGTDFF-QLQQLPE-----GCGSTLNWFYGYSEAFK
oTF  QAVAKFFBASVFGATIEQ---KLRRQKEDPKTK--GARN--PYSGYSEAFH
MTF  KAVANFFGGSVFGAGETSYSESLQQLREDSSSGEGV--DKSPLERYDYSEAFR

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490      500      510      520      530
hLF  CKFDEYFQSCAFGSD-PRS--NLALALDEDEQGENK--VPNSNERHYGYTEAFR
mLF  CKFNFFQSCAFGAD-PKS--NLALALDEDEKGENK--APNSKERYQGYTEALR
hLF  CAFDEFFQSCAFGAD-PKS--RLALALDEDDQGLDK--VPNSKEKHYGYTEAFR
sTF  CRFDEFFREGCAFSGSK-KDS--SLKLEMS--GLNLSEPNKKEGYGYTEAFR
oTF  CNFDEYFREGCAFSGSP-PNS--RLQQLQESGIPPEK--VASSHEKWFYGYTEALR
MTF  TAVSEFFNASVIVNNPKNYPSSSLALALVEDEQGRNK--VGNSSQERYGYTEAFR

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200      210      220      230      240
hLF  LKDGAEIVAFIRESTVFEDLSD-----EAERDEYELLSPUNTEKPTDKFKD
mLF  LRDNAEIVAFTRGSTVFEELPN-----KAERDQYKLLSPUNTWKPTTEYKE
hLF  LQDGAIEIVAFVKETTVFENLPE-----KADRQYELLQLNNSFAPWDAFK
sTF  LKDGAEIVAFVKHSTIFENLAN-----KADRQYELLQLNTEKPTDEYKD
oTF  LKDGKEIVAFVKHTTVNENAPD-----LN-DEYELLQLIGSQPTDNYKT
MTF  LAEGAIEIVAFVKHSTVLENTDGKTLPSWGQALLSQDFELLRLIGSFADWTEWRQ

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540      550      560      570      580
hLF  LAENEIEIVAFVKDVTVLQNTDGNNEAWAKDLKLADFALLQLIGKEKPTTEARS
mLF  LAEKAIEIVAFVKDSTVLQNTDGNTEEWARNLKLKDFELLQLIDTEKPTTEAKN
hLF  LAEDVEIVAFVKNDTVWENTNGESTADWAKNLNREDFALLQLIGTEKPTTEAQ
sTF  LVE-KIEIVAFVKHTVLENTNGKNPDWAKNLNEKDYELLQLIGTEKPTQEYAN
oTF  LVE-KIEIVAFIQHSTVEENTGGKNKADWAKNLQMDDFELLQLIGTEKPTMDYRE
MTF  LVENAEIVAFVRHTTVFDNTNGHNSEPWAAELRSEDYELLSPNGAFADTSQFAA

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```

250      260      270      280      290
hLF  HLAQVESHAVTAFSVNGKEDAIWNLLRQATEKFEKD---KSPKFQLFGSPS---
mLF  HLAQVESHAVTAFSTNDKEEAIWELLRQSTKEFEKK---QASGFQLFAEPS---
hLF  HLAQVESHAVTAFSDGKEDLIWKLLSKATEKFEKN---KSRSEQLFGSPS---
sTF  HLAQVESHAVTAFSMGGKEDLIWELLNQATEHFEKD---KSKEFQLFSEPH---
oTF  NHAQVAAHAVTAFODN-KVEDIWSFLSKATSDFEVD---TKSDFHLLFGPPGKKDP
MTF  HLAQVFAHAVTAFADT-DGGLIFRLNNEGURLF-SH---EGSSSQMFSEAY---

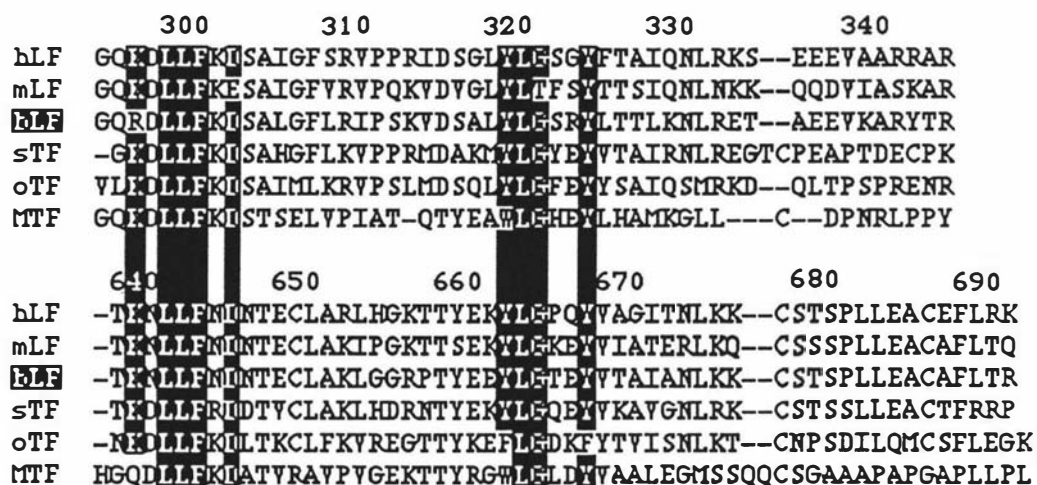
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590      600      610      620      630
hLF  HLAAAFNHAVTSE-MD-KVERLKQVLLHQIAKFERNGSDCPDKFCLFQEE----
mLF  HLAAAFNHAVTSE-TD-KVEVLQQVLLDQVQFERNGQRCPEGFCLEFQEK----
hLF  HLAAAFNHAVTSE-SD-RAAHVKQVLLHQIALFEKNGKNCPDKFCLFQEE----
sTF  HLAAAFNHAVTSE-KD-KEACVHKDLRQQHLEFSNVTDCSGKFCLFRESE----
oTF  KLAQVETHAVTSE-PE-KANKIRDLLEKQKFEVNGSEK-SKFMTEFESE----
MTF  KLAQVETHAVTSE-PDTNIPTVYGLLDKADLFEDDHKNK-G-EKMTDESNY---

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*



Abbreviations: hLF, human lactoferrin; mLF, mouse lactoferrin; **bLF**, bovine lactoferrin; sTF, human serum transferrin; oTF, hen egg transferrin; MTF, human melanotransferrin (p97 antigen).

This diagram was modified from that of Professor E.N. Baker (personal communication).

Appendix IV. Amino acid sequence comparisons between members of the transferrin family. Sequence alignments were made using the UWGCG program BESTFIT (Devereux et al., 1984).

A. Percent sequence identity between members of the transferrin family.

bLf	hLf	mLf	hTf	pTf	hoTf	rTf	oTf	xTf	
100	69.7	64.0	62.3	61.9	63.4	61.7	54.0	45.4	bLf
	100	70.6	62.1	62.6	62.3	62.4	53.0	47.0	hLf
		100	57.9	59.0	57.1	58.0	49.5	47.5	mLf
			100	71.5	73.4	79.0	52.6	46.6	hTf
				100	73.5	72.9	53.0	49.0	pTf
					100	73.2	53.8	48.5	hoTf
						100	52.6	46.8	rTf
							100	47.1	oTf
								100	xTf

B. Percent sequence similarity between members of the transferrin family.

bLf	hLf	mLf	hTf	pTf	hoTf	rTf	oTf	xTf	
100	82.0	77.5	75.4	75.7	75.6	74.2	69.1	62.5	bLf
	100	81.8	75.1	76.5	74.6	75.7	69.7	66.1	hLf
		100	57.9	74.3	71.1	71.6	65.6	63.0	mLf
			100	84.8	82.6	87.5	67.8	63.5	hTf
				100	81.8	83.2	69.0	64.2	pTf
					100	81.6	67.9	66.4	hoTf
						100	67.4	63.3	rTf
							100	63.1	oTf
								100	xTf

References: hLf = human lactoferrin (Accession number X52941) Powell and Ogden (1990); mLf = mouse lactoferrin (J03298) Pentecost and Teng (1987); hTf = human transferrin (M12530) Yang et al. (1984). hoTf = horse transferrin (M69020) Carpenter, M.A. unpublished data (1991); pTf = pig transferrin (X12386) Baldwin and Weinstock (1988); rTf = rabbit transferrin (X58533) Banfield D.K., Chow, B.K., Funk, W.D., Robertson, K.A., Umelas, T.M., Woodworth, R.C. and MacGillivray, R.T. unpublished data (1991); oTf = chicken ovotransferrin (X02009) Jeltsch and Chambon (1982); xTf = xenopus transferrin (X54530) Moskaitis et al. (1990).