Establishment of a C1sA targeted mouse line and the differential expression of the complement serine protease C1sA and the related gene C1sB in mouse tissues

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Meenu Jassal

Department of Infection, Immunity and Inflammation

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled (Establishment of a C1sA targeted mouse line and the differential expression of the complement serine protease C1sA and the related gene C1sB in healthy mouse tissue) is based on work conducted by the author at the University of Leicester mainly during the period between October 2005 and March 2009.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other university

Signed: Date:

Acknowledgement

In retrospective, I feel that I could never have completed this project successfully without the help I had received from many sources.

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I take pride on myself in being daughter of ideal great parents. I veraciously realize the inadequacy of words at my command to express my feelings of indebtedness for my lovable parents for the affection, remarkable patience, inspiration, indispensible and fruitful suggestions.

Needless to say, errors and omissions are of mine

Abstract

Establishment of a C1sA targeted mouse line and the differential expression of the complement serine protease C1sA and the related gene C1sB in mouse tissues.

Meenu Jassal

The complement system bridges the innate and adaptive immune responses. Complement can be activated via three distinct pathways, the classical, lectin and alternative pathways, all of which play a crucial role in the identification and elimination of pathogenic microorganisms. The classical pathway (CP) is activated mainly by immune complexes (complexes of antigen and immunoglobulin). The lectin pathway (LP) is activated when a carbohydrate recognition complex and associated serine proteases bind to specific carbohydrate patterns on the surface of pathogens. In addition to providing protection from microbial infections, the LP has been shown to be involved in inducing post-ischemic inflammatory tissue loss (ischemia/ reperfusion injury), which significantly contributes to the pathology of ischemic diseases such as, myocardial, renal and GI ischemia.

Separate mouse models of CP and LP deficiency are available, mice deficient in C1q (the CP recognition complex) and MASP-2 (a serine protease essential for LP activation), respectively. However, a model of combined CP and LP deficiency is not available. It has not been possible to generate mice deficient of both C1q and MASP-2, because the genes are located on the same chromosome in close proximity, and mice deficient in C4 (a constituent of both pathways) are not genuinely LP deficient, because the LP can activate C3 in the absence of C4. To establish a mouse strain deficient of both pathways, the gene for murine C1s was targeted the essential serine protease of the CP, with the aim of producing a C1s/MASP-2 double deficient mouse. A C1s deficient line would also be a valuable model to study C1q functional activity in the absence of CP activation. The mouse has two genes for C1s, C1sA and C1sB. The expression profiles for C1sA and C1sB in normal mouse tissue were assessed using qRT-PCR and *in situ* hybridisation, and confirmed that C1sA is widely expressed whereas C1sB expression is restricted to the testis. Thus C1sB is very unlikely to compensate for C1sA in a C1sA deficient mouse line. C1sA was targeted and the germ line transmission of the disrupted allele was confirmed. However, intercrossing of heterozygote mice did not result in the expected generation of homozygous C1sA deficient mice. Analysis of the offspring indicated that homozygous deficiency for C1sA appears to be lethal.

Abbreviations

Δ	Heat aggregated
α	Alpha
β	Beta
µ	Micro
Amp^R	Ampicillin resistance cassette
AP	Alkaline phosphate
AP	Alternative Pathway
APS	Ammonium persulphate
bp	Base pair
BIPC	5-Bromo-4-Chloro-3-Indolyl-Phosphate
BSA	Bovine serum albumin
C1-INH	C1-inhibitor
cDNA	Complementary deoxy-ribonucleic acid
CIPA	Calf intestinal alkaline phosphatase
CP	Classical Pathway
CUB	C1r/C1s-Uegf-bone morphogenetic protein
DAF	Decay-accelerating factor
DEPC-H_2O	Diethyl pyrocarbonate water
DIG	Digoxigenin
DMSO	Dimethyl sulphoxide
dCTP	Deoxycytidine triphosphate
DNA	Deoxyribonucleic acid

DTT	Dithiothreitol
dUTP	Deoxyuridine triphosphate
E.Coli	*Escherichia coli*
ECL	Enhanced chemiluminiscence
EGF	Epidermal growth factor
EDTA	Ethylenediaminetetra acetic acid
EF	Embryonic fibroblasts
ES cells	Embryonic Stem cells
FCS	Foetal calf serum
g	Grams
GAPDH	Glucose-6-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
HCG	Human chorionic gonadotropin
His	Histidin
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgM	Immunoglobulin M
IPTG	Isopropyl – β-D-thiogalactopyronidase
kb	Kilobase
kDa	Kilodalton
KO	Knockout
LB medium	Luria Bertani medium
LIF	Leukemia inhibitory factor
LP	Lectin Pathway
Map19	19 kDa mannose binding lectin associated protein
MASP	Mannose binding lectin serine protein
MAC	Membrane attack complex

MBL	Mannose binding lectin
MCS	Multiple cloning site
MEFs	Mouse embryo fibroblasts
min	Minutes
mRNA	messanger RNA
MW	Molecular weight
NBT	Nitroblue tetrazolium chloride
Neo^R	Neomycin resistance cassette
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PMSG	Pregnant mare serum gonadotropin
PCR	Polymerase chain reaction
Pen/Strep	Penicillin/streptomycin
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse Transcriptase- PCR
sec	Seconds
SDS	Sodium dodecylsulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TAE buffer	Tris acetate EDTA electrophoresis buffer
TBE buffer	Tris borate EDTA electrophoresis buffer
TE buffer	Tris EDTA buffer
TEMED	N,N,N’,N’-Tetramethylethylendiamin
TK	Thymidine kinase
Tm	melting temperature of primer
wt	Wild type
x-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Chapter I

Introduction

I.1. Immune system

The immune system is composed of many independent components within an organism that collectively protect the body against disease by (i) identifying and killing pathogens and (ii) by identifying and killing transformed malignant cells. The cells of immune system can attack a wide variety of virulent organisms, from viruses to parasitic worms, and needs to distinguish them from the organism's own healthy cells and tissues in order to function properly. Detection is complicated as pathogens can evolve vigorously and develop adaptations to escape from the immune response and allow the pathogens to infect their hosts successfully.

Depending on the mode of pathogen recognition and action, the immune system is divided into major branches now known as the innate immune response and the adaptive immune response. The innate immune response responds instantly to pathogen exposure and does not require adaptation. The innate immune defense includes surface barriers such as skin, the phagocytes such as macrophages and granulocytes, and cells that release danger signals like

cytokines and chemokines. The complement system provides humoral protection from pathogens. The innate immune system provides a first line of host defence in vertebrates and invertebrates and represents the most ancient form of defence mechanisms (Hoffman *et al*., 1999). The innate response is usually triggered when microbes are identified by pattern recognition receptors, which bind to components that are conserved among broad groups of microorganisms (Medzhitov R., 2007).

The adaptive immune response is antigen-specific and requires the recognition of specific "non-self" antigens during a process called antigen presentation. Antigen specificity allows for the generation of responses that are tailored to specific pathogens or pathogen-infected cells (Pancer Z *et al*., 2006). The adaptive immunity can be divided into two components: the cell-mediated and the humoral response. Cell-mediated immunity is an immune response that involves the activation of macrophages, natural killer cells (NK), antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen. The humoral adaptive immune response is mediated by secreted antibodies produced in B lymphocytes following exposure to antigens.

There is a strong crosstalk and interactions between the innate and adaptive immunity where the complement system is one of the key players. The complement system is mainly considered as part of innate immunity, but it also supports the adaptive immune response, for example by providing cytotoxic and

bacteriolytic activity to antibodies and immune complexes (Figure 1.1) (Ricklin D *et al*., 2007).

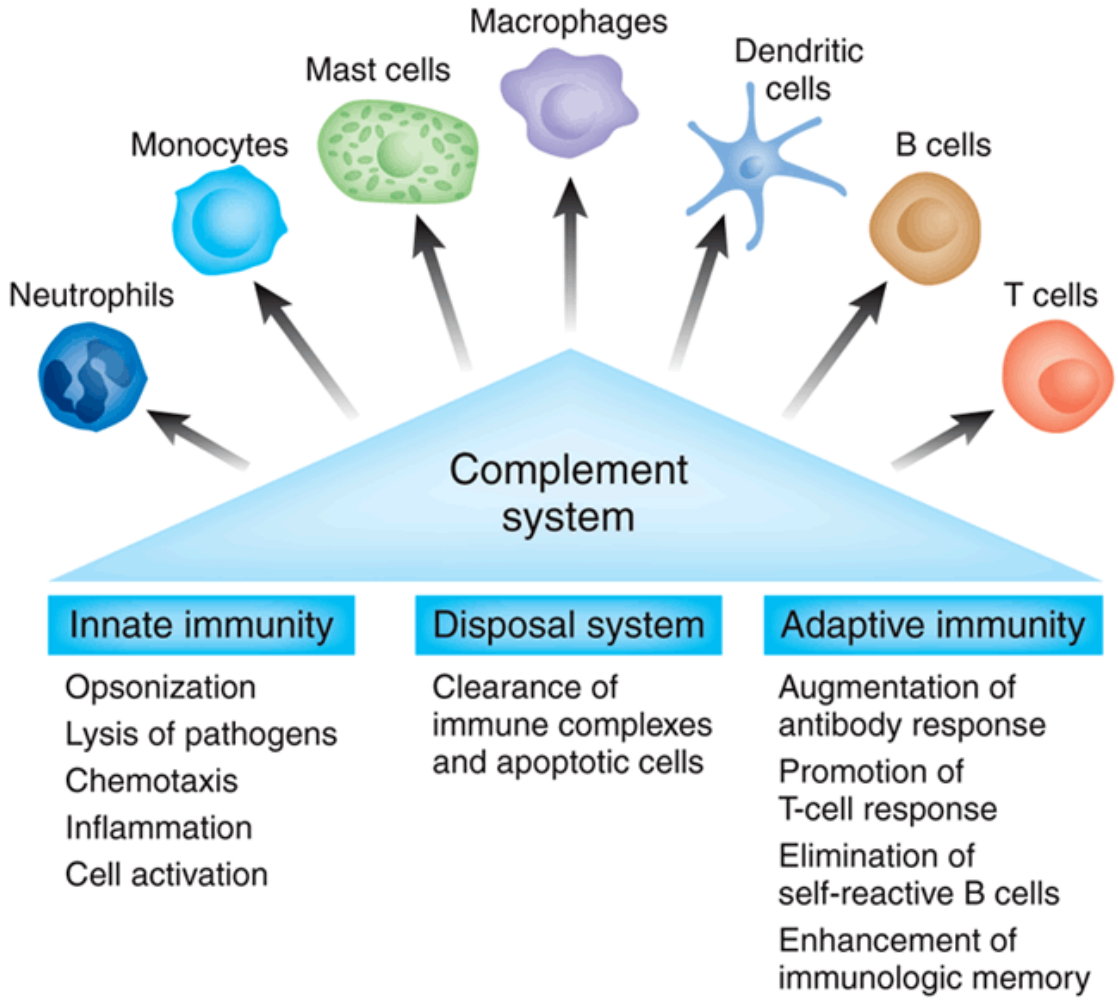

Figure 1.1 Shows Innate and Adaptive immune system are linked together by Complement system (Ricklin D *et al*., 2007)

I.2 Complement system

The complement system is an essential component of the innate immune system and provides a first line of defense against pathogens (Muller-Eberhard 1988; Reid 1983). The complement system was first described by Jules Bordet in the 1890's as a heat liable component of plasma, which he named as Alexion. Paul

Ehrlich gave the heat liable component its present name as ‘Complement’and since then it is known as complement system. The complement system is composed of more than 30 proteins in plasma (most of them circulating as inactive zymogens) and on cell surface receptors (Walport, 2001 and Makrides, 1998). Once activated, proteases in the system cleave specific proteins and initiate an amplifying cascade of cleavages. The end-result of this activation cascade is enormous amplification of the response and activation of the cell-killing membrane attack complex (Liszewski *et al*., 1996 and Parker, 1992).

There are three distinct pathways through which complement may be activated: The Classical pathway (initiated by the binding of C1 (composed of C1q and C1r2 and C1s2)), the Alternative pathway (which is believed to be initiated by the binding of hydrolysed C3 to the surface of a pathogen) and the so called Lectin pathway (which is initiated by a complex formed of carbohydrate recognition molecules in association with a serine protease called MASP-2) (Figure 1.2).

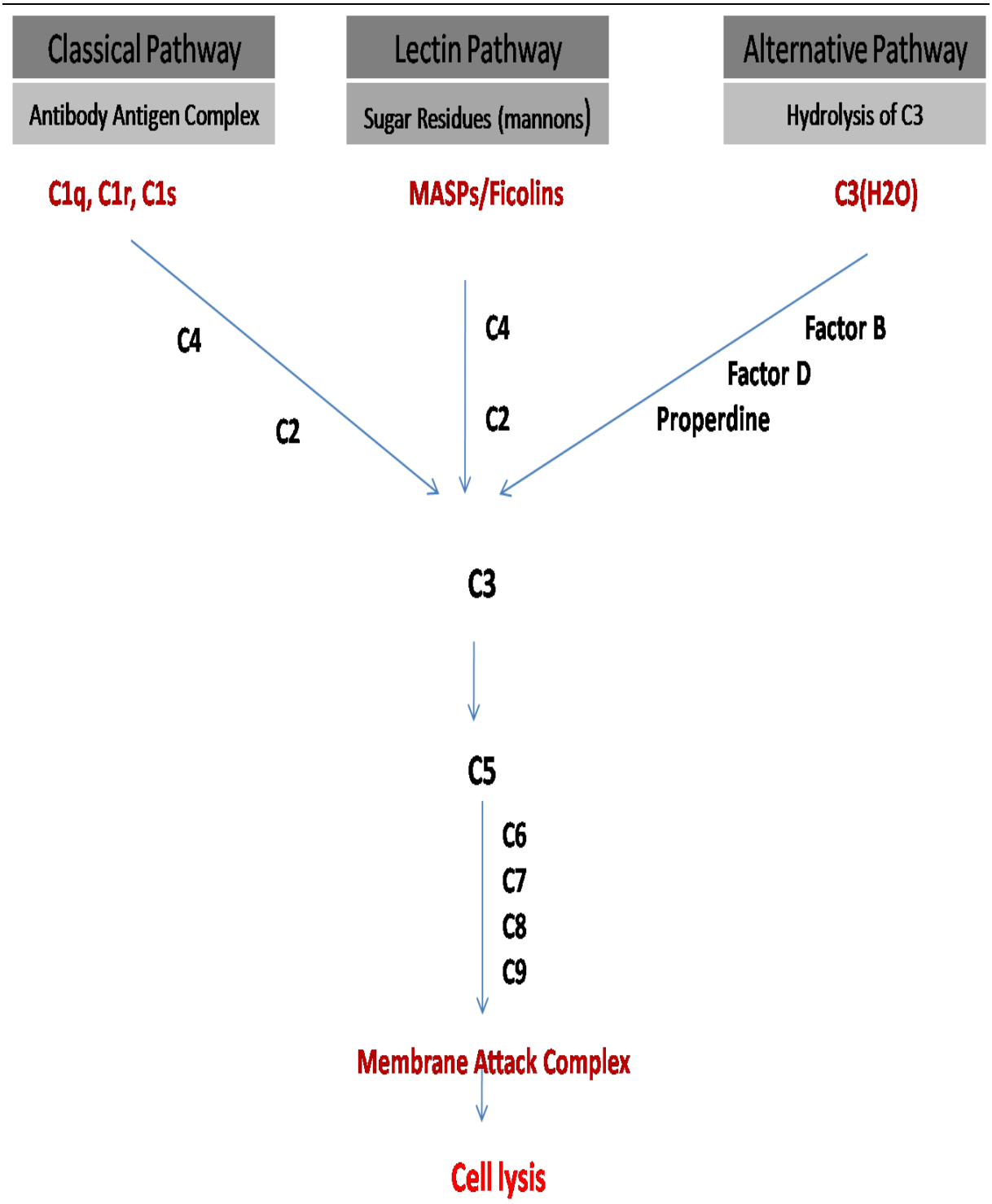

Figure 1.2 The three pathways of complement system. The classical pathway; lectin pathway and alternative pathway

MASP-2 is the effector component of the lectin pathway, as the serine protease C1s is the effector component of the classical pathway (Schwaeble *et al*., 2002).

All the three pathways lead to the activation of C3 (C3 convertase), the main factor of complement and lead to formation of the membrane attack complex. Complement activation is started by a cascade of complex steps involving enzymatic cleavage. Complement activation is followed by number of proinflammatory reactions that leads to generation of toxic radicals (Kirschfink and Mollnes, 2003). The various proinflammatory reactions include chemotaxis, phagocytosis, opsonisation, cell adhesion, lysis, clearance of immune complexes are summarized in figure 1.3. All the three pathways can function in an antibody independent way but the classical pathway can be triggered by antibody-antigen complexes (immune complexes) and can destroy pathogens recognised by adaptive immune system (Ducan and Winter 1998; Perkins *et al* 1991; Sim and Reid 1991). Table 1.1 shows all the known components of complement system.

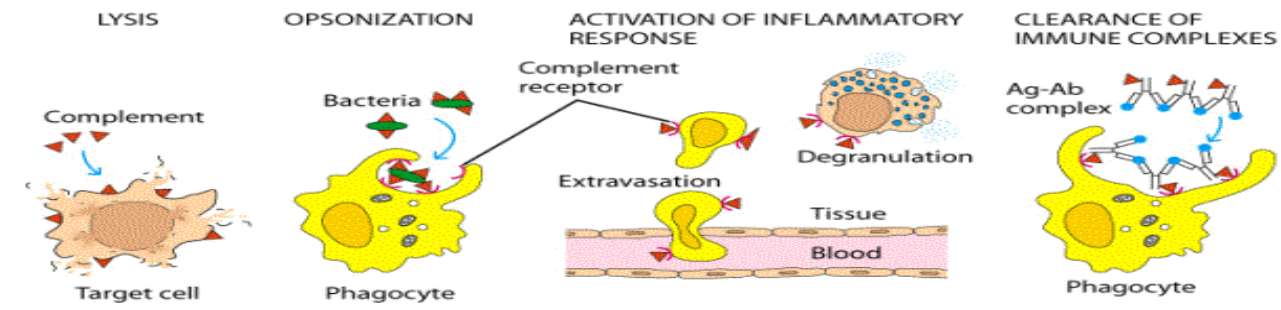

Figure 1.3 Shows the proinflammatory reactions of Complement System activation.

Table 1.1 Components of the Complement system

Components of Complement System	Chromosome location	Molecular Weight kDa	Function of Component in complement activation
Classical Pathway			
C1 Complex			
C1q	Chr. 1p36.3-34.1	460kDa	Bind to the Fc region of Immunoglobins (IgM & IgG)
C1r	Chr. 12p13	83kDa	Activates C1s
C1s	Chr. 12p13	83kDa	Activates and Cleave C4 &C2
C4 component	Chr. 6p21.3	240kDa	
C4a			Anaphylatoxins
C4b			Binds to C2a, forms a part of C3-convertase
C2 component	Chr. 6p21.3	102kDa	
C2a			Is the enzymatically active subcomponent of the classical pathway convertase C4b2a
C2b			--------

Table 1.1 Components of the Complement system

Components of Complement System	Chromosome location	Molecular Weight kDa	Function of Component in complement activation
C3 Component	Chr. p1913.3-13.2	185kDa	
C3a			Inflammation
C3b			May bind to the C3 convertase to form a C5 convertase ($C4b2a(C3b)_n$)
Lectin Pathway			
MBL	Chr. 10	540kDa	Identify polysaccharide on the surface of a pathogen
MASP-1	Chr. 3q27-28	94kDa	Binds to carbohydrates on the microbial surface
MASP-2	Chr. 1p36.3-36.2	76kDa	Cleaves C4 and C2
Ficolins			
L-Ficolin	Chr. 9	35kDa	Activates the lectin pathway by recognisition of acetylated sugar moieties on the surface of pathogens
H-Ficolin	Chr. 1	35kDa	Activates the lectin pathway by recognisition of acetylated sugar moieties on the surface of pathogens

Table 1.1 Components of the Complement system

Components of Complement System	Chromosome location	Molecular Weight kDa	Function of Component in complement activation

M-Ficolin	Chr. 9	-------	Forms complex with the MASP-1 and MASP-2 lectin pathway serine proteases
Alternative pathway			
Factor B	Chr. 6p21.3	92kDa	
Ba			----------
Bb			C3bbb acts as C3 convertase and generates C5 convertase (C3bBb3b)
Factor D	Chr. 19p13.3	24kDa	Cleaves factor B to generate C3 convertase
Properdin	Chr. Xp11.4		Binds to and stabilizes C3 convertase

Table 1.1 Components of the Complement system

Components of Complement System	Chromosome location	Molecular Weight kDa	Function of Component in complement activation
Membrane Attack complex			
C5 component	Chr. 9q32-34	190kDa	
C5a			Acts as anaphylotxin (in Chemotaxis)
C5b			First part of the Membrane Attack Complex (MAC)
C6 component	Chr. 5p13	120kDa	Bind to C5b to form C5b-6 complex
C7 component	Chr. 5p13	110kDa	Binds to C5b-6 to form C5b-7 complex
C8 component	Chr. 1p34(α &β) Chr. 9q (γ)	150kDa	Bind to C5b-7 complex and activates C9 for cell lysis
C9 component	Chr. 5p13	71kDa	Acts as a component involved in pore formation

I.2.1 The classical pathway

The classical pathway of complement is initiated by C1, a 790 kDa multimolecular complex which consists of the recognition subcomponent C1q and two associated serine proteases, C1r and C1s. C1r and C1s form a C1s–C1r–C1r–C1s heterotetramer of approximately 340 kDa in the presence of calcium (Ca^{2+}) (see Figure1.4) within a C1q:$C1r_2$:$C1s_2$ complex (Copper, 1985; Arlaud *et al*., 1998).

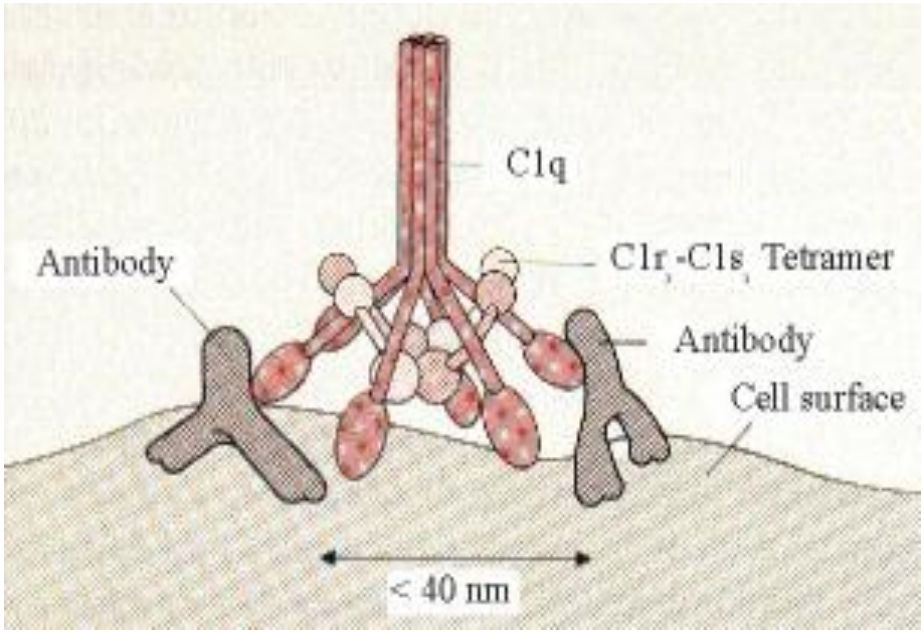

Figure 1.4 Diagrammatic representation of the association of C1q, C1r and C1s to form the first component of the classical pathway, the C1 complex (*http://www.complement-genetics.uni-mainz.de)*

The C1q is 460 kDa hexameric protein, composed of three different polypeptide chains A, B and C (Arlaud *et al*., 2002 and Ghiran *et al*., 2002). Each polypeptide chain is composed of an N-terminal collagenous tail region and a C-terminal globular head domain (Kishore and Reid, 2000). Six subunits of C1q

are attached together in the N-terminal tail region and associate to a bunch of tulip like structure with the globular heads forming the tulip heads and the collagenous stalks forming the tulip stems (figure 1.5).

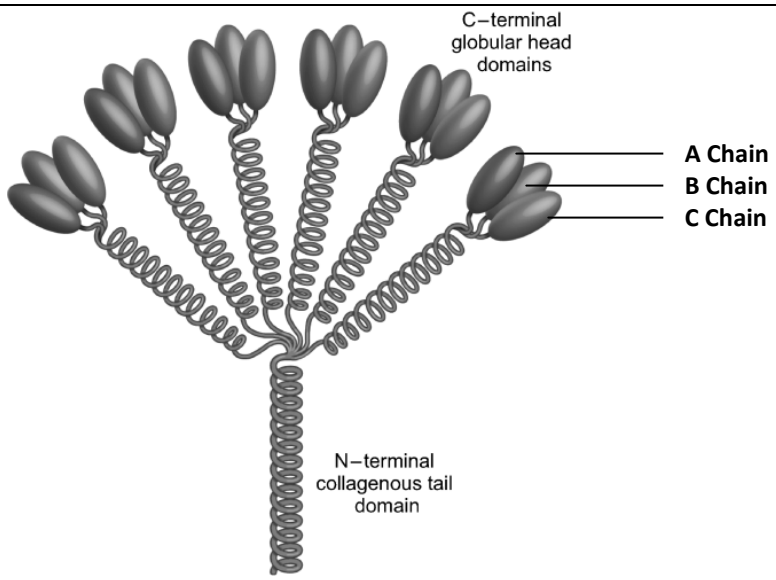

Figure 1.5 Structure of C1q. C1q is composed of the N-terminal collagenous tail regions and the C-terminal globular head domains (modified from Francis *et al*, 2003).

The serine proteases C1r and C1s, are composed of six structural domains, an N-terminal CUB I domain (the name CUB domain was came from the proteins or organisms where these domains were first described i.e. C from Complement, U from Sea Urchin, B from Bone-morphogenetic protein) followed by an EGF (Epidermal Growth Factor) domain, a second CUB domain (i.e. CUB II) and two CCP domains (for Complement Control Protein) i.e. CCP-1 and CCP-2 and the C-terminal serine protease domain. Both serine proteases are converted from their zymogen form into an active enzyme by cleavage into a larger N-terminal

A chain and a smaller B chain (both held together by a disulfate bridge). A cleavage site is located between CCP-2 and the SP-domain.

The binding of C1q to the surface of a pathogen or to the Fc region of immune complexes (IgG and IgM) leads to conformational changes in C1, resulting in the activation of C1s–C1r–C1r–C1s tetramer (Dodds *et al*., 1978). Table 1 shows the active forms of the classical pathway components and their function. Upon activation, C1r cleaves its only substrate C1s. Activated C1s subsequently cleaves complement C4 and C4b bound C2 (Kusumuto H *et al*., 1988). The C1s mediated cleavage of C4 splits C4 into two fragments: C4a and C4b. C4a, a small peptide may act as an anaphylatoxins and promote pro inflammatory cellular response, while the larger fragment C4b has a thioester group that is able to bind covalently to complement activators i.e. pathogen cell surface (Moller K *et al*., 2003; Wallis *et al*., 2007 and Dodds *et al* 1996). C4b binds to complement component C2. C4b bound C2 is the second substrate of C1s which cleaves C2 into two parts: C2a and C2b. A cleavage fragment C2a forms the enzymaticale active part of C4b2a complex which cleaves complement C3 and is the C3 convertase of the classical pathway (Figure 1.6). C4b2a cleaves C3 into C3a and C3b. C3b binds to the cell surface. C3b bound to the C3 convertase forms a C5 convertase (C4b2a3b) and cleaves C5 into two fragments C5a and c5b. C5b initiates the activation of the terminal complement activation cascade leading to the formation of the membrane attack complex.

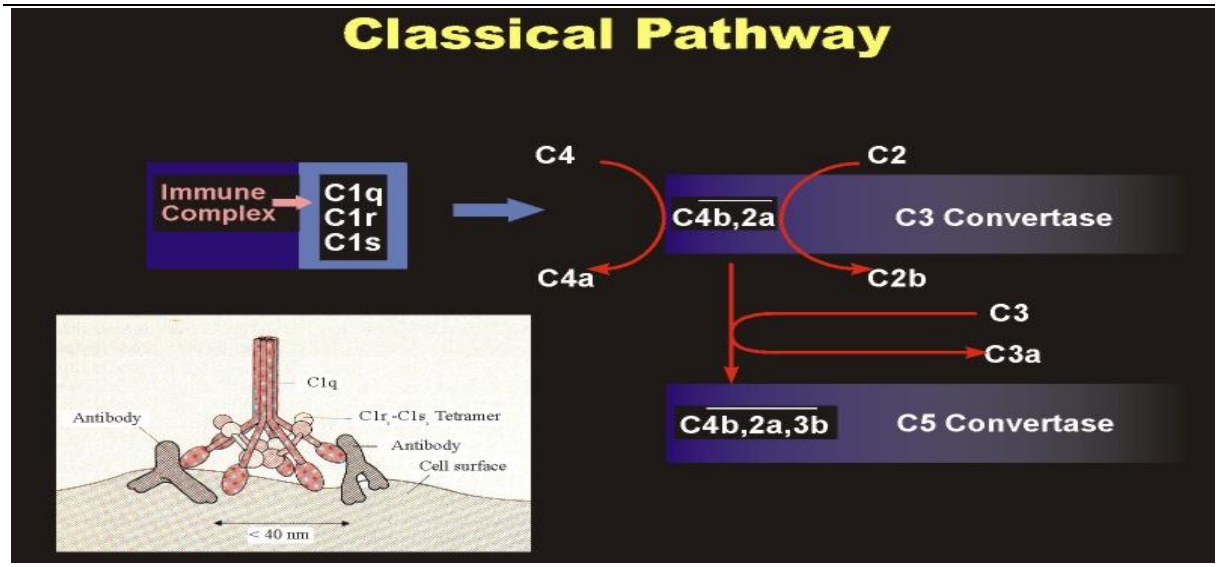

Figure 1.6 Activation of Classical pathway (*http://www.complement-genetics.uni-mainz.de*)

I.2.2 The lectin pathway

The lectin pathway of complement activation is initiated by the recognition of carbohydrate structures on the surface of wide range of microorganisms by the MBLs or Ficolins associated with MASP-2 (Thiel *et al*., 2000; Matsushita *et al* 2000 and Schwaeble *et al* 2002).

MBL belongs to a protein family known as 'Collectins' and is present in serum as a mixture of different oligomers. Similar to the C1q, MBL is composed of multimers of three identical polypeptide chains composed of an N-terminal cystein rich domain, a collegen like domain, a neck region and C-terminal carbohydrate recognition domain (Wallis *et al*., 2005). MBL forms complexes with three different MBL associated serine proteases called (in accordance to the sequence of their discovery) MASP-1, MASP-2 and MASP-3. In addition, the MASP-2 gene encodes small non enzymatic protein known as MAp19

(Schwaeble *et al*., 2002 ; Stover *et al* 1999 and Wallis and Dodd 2000). MASP-1 and MASP-3 are encoded by a single structural MASP-1/MASP-3 gene and generated by an alternative splicing process. Both MASP-1 and MASP-3 share identical N-terminal domain but have a different serine protease domain.

In humans three different types of ficolins are present in serum. Two of these ficolins (L-ficolin and H-ficolin) activates the lectin pathway by direct recognition of acetylated sugar components on the microbial pathogen where as the third ficolin, M-ficolin, is a nonserum ficolin and mainly expressed in leukocytes (neutophiles and monocytes) where it act as a receptor for phagocytosis, forms a complex with serine proteases MASP-1 and MASP-2 and activates the lectin pathway (Liu. Yu. *et al*, 2005). In mouse two types of ficolins are present, ficolin-A which resembles human L-ficolin and also found in serum where as ficolin-B is expressed in phagocytosis but it does not bind to MASP-2 like human M-ficolin and does not activates the lectin pathway (Endo et al,2005).

MBL-MASP-2 and Ficolin-MASP-2 complexes bind to the surface of pathogen and this activates MASP-2 (Figure 1.7). Activated MASP-2 cleaves C4 component into two fragments C4a and C4b. C4b binds to cell surface and C4a is released. Activated MASP-2 also dissociates C2 into two fragments C2a and C2b. C4b and C2a bind to form C3 convertase (C4b2a). The C3 convertase is identical to that formed via classical pathway activation. C3 convertase leads

to the formation of C5 convertase and the entire terminal pathway is similar to the classical pathway.

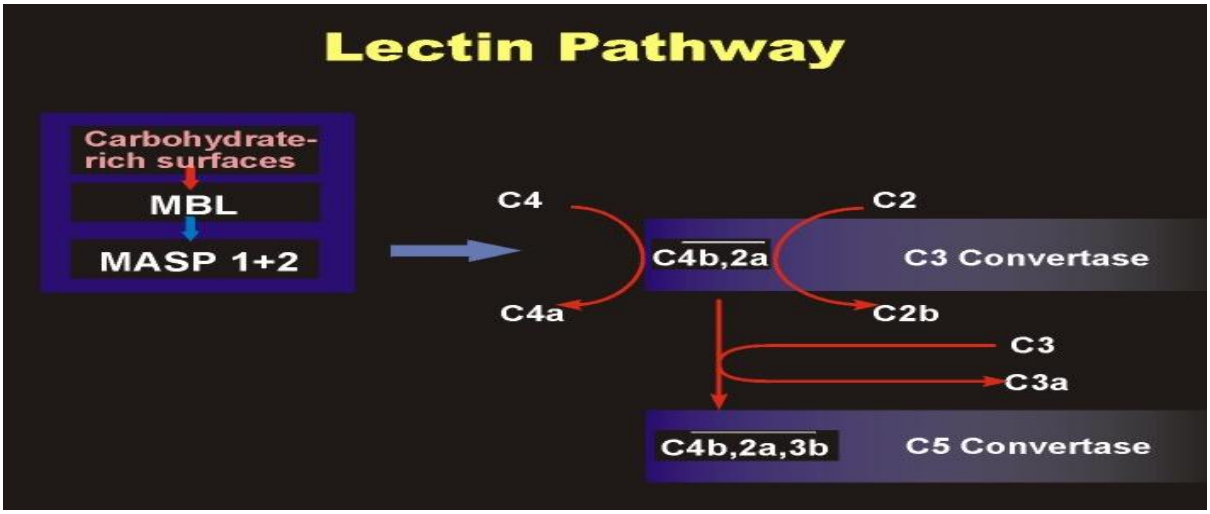

Figure 1.7 Activation of lectin Pathway (*http://www.complement-genetics.uni-mainz.de)*

I.2.3 The alternative pathway

The activation of alternative pathway occurs in absence of pathway specific recognition complexes. Alternative pathway activation is initiated by a spontaneous hydrolysis of C3 to form C3 (H_2O) which can form C3 (H_2O) B complex with the alternative pathway zymogen factor B. This complex C3 (H_2O) B can then be converted into an enzymatically active C3 convertase by factor D. Factor D cleaves C3 (H_2O) B or C3b bound factor B into the two fragments Ba and Bb. C3 (H_2O) Bb or C3bBb is the alternative pathway C3 convertase which cleaves C3 and converts into C3a and C3b. C3b binds covalently to a pathogen surface. The continuous activity of the C3 convertase C3bBb complex leads to the accumulation of C3b fragments in close proximity

of the C3bBb complex which in turn shifts the specificity of this complex towards a substitute to form the alternative complement pathway C5 convertase C3Bb(C3b)n (Farries *et al*., 1988) (Figure 1.8). This C5 convertase cleaves the C5 complement component in the same site as in lectin and classical pathway (C5 convertase C4b2a (C3b)n and initiates the formation of the membrane attack complex and the release of the potent complement anaphylatoxin C5a.

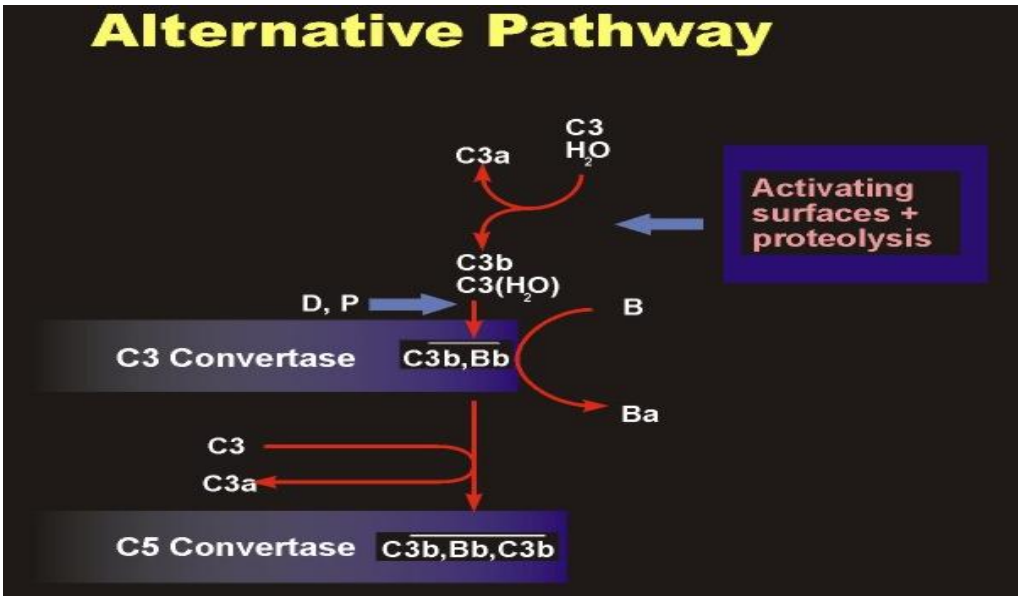

Figure 1.8 Activation of Alternative Pathway (*http://www.complement-genetics.uni-mainz.de)*

The effectiveness of alternative pathway depends on the ability to discriminate between host cells and foreign cells. To prevent self destruction, there are a number of regulatory proteins (MCP, DAF, CR1, factor H and factor I) residing on host tissue which avoid the activation of AP and discussed in more detail in section 1.4 of present chapter and the absence of these regulators on microbial

surface leaves them defenceless (Kraus. D. *et al*, 1998 and Oglesby. Y.J. *et al*, 1992).

I.2.4 Terminal pathway of complement and Membrane attack complex

C5 convertase generated in all the three pathways cleaves C5 into two fragments C5a and C5b and initiates the terminal pathway which finally leads to the formation of membrane attack complex and then cell lysis. C5b forms a complex with the C6 and C7. The binding promotes a structural change in the C7 component exposing the hydrophobic site, which inserts into the lipid bilayer of the microorganism. The C5b67 complex binds to C8 and several C9 molecules to form a membrane attack complex $C5b6789_{(n)}$ which creates a small pore of $10A^{o}$ diameter on the membrane of pathogen (Figure 1.9). Formation of MAC allows the excess intracellular calcium influx of water into the cell and the loss of electrolytes that leads to the osmotic instability of the cells resulting in its lysis or death (Bhakdi *et al*., 1991).

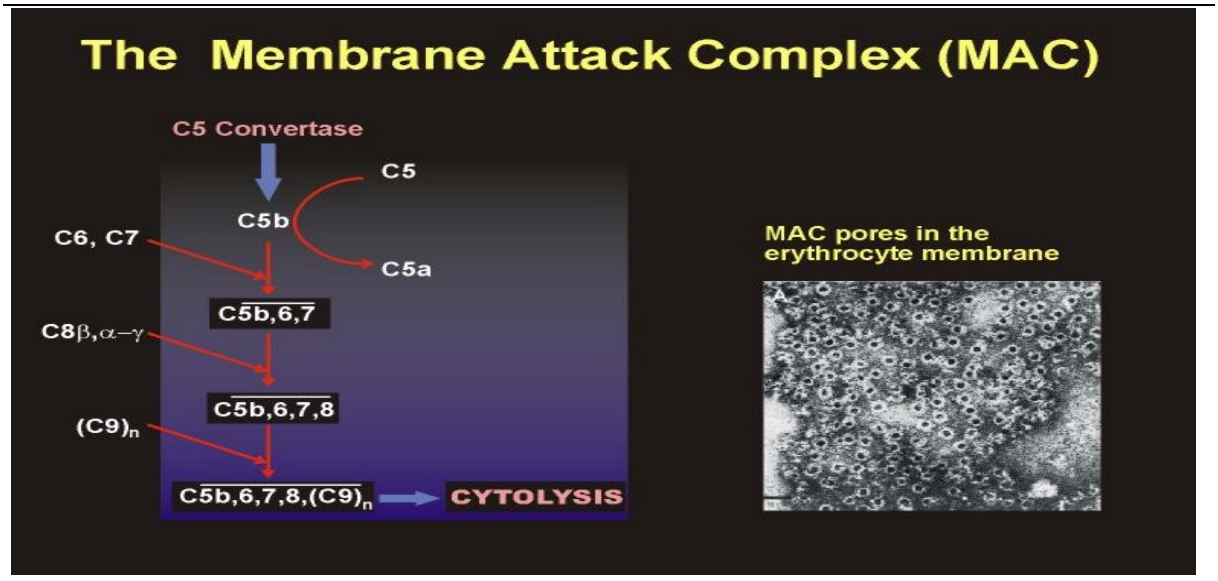

Figure 1.9 Membrane attack complex and the electron micrograph showing the holes formed in the cell membrane of bacteria (*http://www.complement-genetics.uni-mainz.de)*

I.3 Biological effects of complement

There are different ways in which the complement system protects the body against infection. The proteins of the complement system act as a cascade, where each enzyme acts as a catalyst for the next. The biological activities of complement include opsonisation, initiation of inflammatory response, clearance of immune complex and apoptotic cell debris (Janeway *et al*., 2005).

Opsonization: The opsonisation of microorganisms is very important because it promotes the destruction of pathogen by phagocytic cells. This occurs by the specific recognition of bound complement components by complement receptors (CRs). Opsonisation of pathogen is done by C3b or iC3b and to a lesser extent by iC4b and C4b (Mevorach *et al*, 1998). C3b is present on the surface of pathogen which leads to phagocytosis by macrophages and leuckocytes through binding to the complement receptors CR1 (CD35) and CR3 (Whaley and Schwaeble., 1997 and Aoyagi *et al*., 2005).

Inflammation: The anaphylatoxins C3a, C4a and C5a act on specific receptors to produce local inflammatory response such as chemotaxis plus triggering histamine release (which increases vascular permeablity).

Clearing of apoptotic debris: The complement system plays a major role in clearing apoptotic cells. The C terminal domain of C1q binds to the surface of

apoptotic cell and results on the clearance of this complex by macrophages (Taylor *et al*., 2000).

Clearing of Immune complexes(IC): Immune complexes are cleared from circulation by erythrocytes via CR1, complement receptor type 1. These immune complexes are transferred to spleen and liver where IC are degraded by marcophages (Cornacoff. J.B. *et al*, 1983 and Miyaike. J. *et al*, 2002).

Killing of cells due to lysis: Complement system can lead to direct killing of bacteria (gram negative bacteria) by formation of membrane attack complex (MAC) which leads to the formation of pores in the cell wall and destruction of cell membrane and eventually cell lysis.

I.4 Regulation of Complement system

The effect of complement activation would be devastating to host cells without regulation. In order to prevent the host cell damage there are number of regulatory proteins. These proteins include the cell-surface complement regulatory proteins and the fluid phase regulatory proteins. Table 1.2 summarizes the proteins that regulate the complement system.

I.4.1 Cell surface complement regulatory proteins

The cell surface complement regulatory proteins are complement receptor type 1 (CR1), CD59, membrane co-factor protein (MCP), decay-accelerating factor (DAF). CR1 blocks the formation of C3 convertase by binding to C3b and C4b. MCP binds to C3b and C4b, acting as a cofactor for the factor I catalysed cleavage of C3b and C4b (Whaley and Schwaeble, 1997). CD59 is present on the surface of cells and binds to the C8 and C9 (MAC) components preventing the damage to the cell (Rollins *et al*., 1991). DAF is present on the membrane and binds to C3b blocking the formation of C3 and C5 convertase (Medof *et al*., 1984).

I.4.2 Fluid Phase Regulatory proteins

Fluid phase regulatory proteins are present in plasma and include C1 inhibitor (C1 Inh), C4 binding protein (C4bp), factor H and S protein. C1 inhibitor is a serine protease inhibitor (SERPIN) that dissociates $C1r_2s_2$ from C1q (Sim *et al*., 1979) and also blocks any further cleavage (Chen and Boackle, 1998). C4-binding protein blocks the formation of C3 convertase by binding to C4b and also acts as cofactor for cleavage of C4b by factor I (Jurianz *et al*., 1999). Factor H blocks the formation of C3 convertase by binding to C3b and also acts as cofactor for cleavage of C3b by factor I (Turnberg and Botto, 2003). S protein and Clusterin binds to C5b67 complex and prevents the formation of MAC (Jenne and Tschopp, 1989).

Table 1.2 Cell surface and Fluid phase proteins that regulate complement activation

Regulatory Protein	Type of Protein	Pathway effected	Function
Complement Receptor type 1 (CR1)	Cell surface protein	Classical, lectin and alternative pathway	Blocks formation of C3 convertase by binding to C4b and C3b
Membrane Cofactor protein (MCP)	Cell surface protein	Classical, lectin and alternative pathway	Cofactor for factor I catalysed cleavage of C4b and c3b
Decay Acclerating Factor (DAF)	Cell surface protein	Classical, lectin and alternative pathway	Binds to C3b blocking the formation of C3 and C5 convertase
C1 Inhibitor (C1 Inh)	Fluid phase protein	Classical pathway	Serine protease inhibitor (SERPIN) that dissociates $C1r_2s_2$ from C1q
C4 binding protein	Fluid phase protein	Classical and lectin pathway	Blocks the formation of C3 convertase by binding to C4b and also acts as cofactor for cleavage of C4b by factor I
Factor H	Fluid phase protein	Alternative pathway	Blocks the formation of C3 convertase by binding to C3b and also acts as cofactor for cleavage of C3b by factor I

Table 1.2 Cell surface and Fluid phase proteins that regulate complement activation

Regulatory Protein	Type of Protein	Pathway effected	Function
Factor I	Fluid phase protein	Classical, lectin and alternative pathway	Cleaves C3 convertase by using CRI, DAF, C4 binding protein and Factor H
S- Protein	Fluid phase protein	Terminal pathway	Binds to C5b67 complex and prevents the formation of MAC
Clusterin	Fluid phase protein	Terminal pathway	Binds to C5b67 complex and prevents the formation of MAC

I.5 Complement Deficiencies

The complement system is required for the smooth running of the immune system and is involved in the elimination of infectious agents. Complement deficiencies are linked to number of autoimmune diseases and microbial infections.

I.5.1 Classical pathway deficiencies

Deficiency in early components of the classical pathway results in immune complex diseases. Deficiency of complement components C1q and C1s results in a systemic lupus erythematosis (SLE) like syndrome (Fremeaux *et al*., 1996 and Amano *et al*., 2008). This suggests that C1q is vital in the clearance of immune complex by participating in the cleavage of C3 (Bowness *et al*., 1994 and Ohashi and Erickson., 1998). Hereditary C2 deficiency was found in a patient in Sweden which leads to arteriosclerosis and rheumatic disorder (Jönsson *et al*., 2005). C2 deficiency can lead to invasive infection mainly septicaemia and meningitis caused by *S.pneumoniae* (Jönsson *et al*., 2005). Deficiency of C5-9 is less severe, but can lead to infectious disease like meningococcal meningitis and extragenital or disseminated gonococcal infection (Gupta *et al*., 2002). Deficiency of C1 inhibitor causes inherited autosomal disease known as hereditary angioneurotic edema (HAE) (Karmer *et al*., 1991). The absence of C1-INH decreases the level of classical pathway activation and results in the consumption of complement components C4 and C2. Inherited hemolytic-uremic syndrome (HUS) is caused by the deficiency of

factor H (Pichette *et al*., 1994). Deficiency of decay accelerating factor (DAF) leads to illness known as paroxysmal nocturnal hemoglobinuria.

I.5.2 Lectin pathway deficiency

Mannan binding lectin (MBL) deficiency is one of the commonest immunodeficiencies in humans. Low levels of MBL in serum are linked with single nucleotide polymorphisms in the promoter and on exon 1 of MBL2. These alterations (SNPs) causes a single amino acid substitution at codon 52 (substitution of glycine with aspartic acid), codon 54 (substitution of glycine with aspartic acid) and codon 57 (Cysteine with arginine) and lowers the levels of MBL and leading to lack of lectin pathway activation (Wallis and Cheng, 1999 and Garred P, 2003). There are other number of SNPs which influences the levels of MBL in serum, three of those mutations which are common on promoter region are H/L at position -551, X/L at position -221 and P/Q at position +4 (Madsen. H. *et al*., 1995, Turner, 2003). MBL deficiency plays significant role in the development of sepsis or Systemic Inflammatory Response Syndrome (SIRS) in paediatric patients (Fidler *et al*., 2004 and Klein. N.J. 2005). MBL deficiency can lead to cardiovascular disorders like arterial thrombosis (Ohlenschlaeger *et al*., 2004).

A low level of L-ficolin in plasma is associated with recurrent respiratory infection in children (Atkinson *et al*., 2004, Guardia A *et al*., 2003 and Granell *et al*., 2006). Ficolin deficiencies are also associated with other disorders like

IgA nephropathy, systemic lupus erythematosus (Zhang. X.L. and Ali. M.A., 2008).

I.5.3 Alternative pathway deficiency

Alternative pathway deficiencies occur due to lack of or low levels of factor I and factor H which results in a haemolytic-uremic syndrome (Thurman and Holers, 2006). Deficienciey in properdin (Schwaeble and Reid, 1999) and due to mutation in factor D (Sprong. T. *et al*, 2006) leads to meningococcal infection.

I.6 C1s, the protease messenger of the C1 complex

C1s is an extremely specific serine protease that mediates the proteolytic activity of the C1complex and initiates the activation of complement via the classical pathway (Gaboriaud *et al*., 2000).

I.7 Structural organisation of C1s

C1s is a serum protein with a molecular weight of 79 KDa. It consists of a 688 amino acid polypeptide chain (Tosi *et al*., 1987 and Spycher *et al* 1986). Upon activation, C1s is cleaved into two chains: the heavy A- chain and the light B-chain which are held together by a disulphide bridge. The N terminal CUB1 domain is encoded by exon three and exon four, while the CUB2 domain is encoded by exons six and seven (CUB represents C1r/C1s), exon five encodes EGF (Epidermal Growth Factor) domain, CCP1 domain (CCP for complement control protein) is encoded by exons eight and nine while following two exons; exon ten and exon eleven, encode the CCP2 domain. The C-terminal serine protease domain is encoded by exon twelve (figure 1.10). This serine protease domain has high resemblance to the serine protease domain of chymotrypsin (Bork P *et al*., 1993; Campbell I *et al*., 1993 and Reid K *et al* 1986). The cleavage site to convert the C1s zymogen into its enzymatically active form is located between the CCP 2 and the SP domain. There are two N glycosylation sites; one is at the border of the EGF domain and one located on the CCP2 domain (Petillo T *et al* 1995).

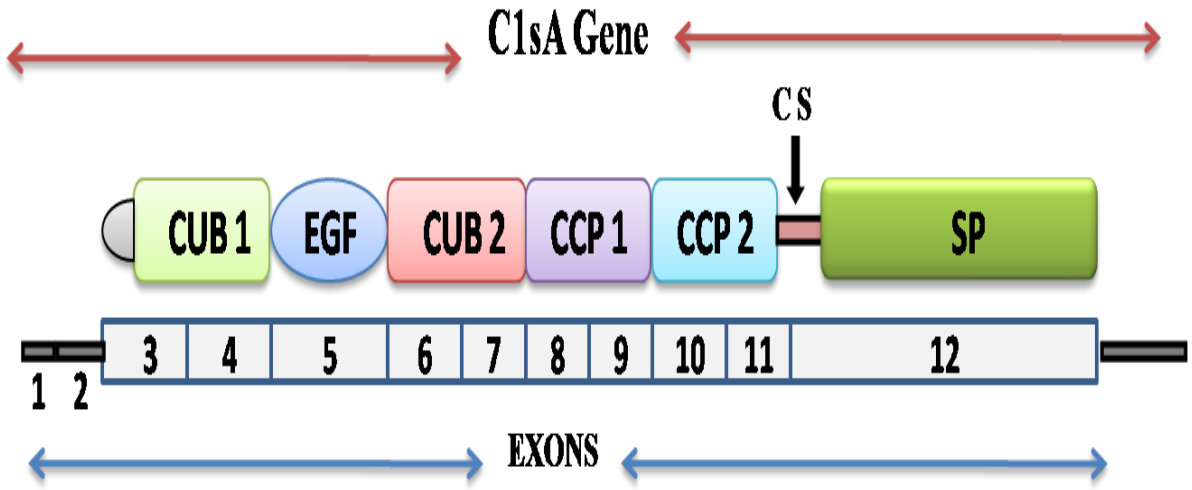

Figure 1.10 Structure of the gene encoding the domain of C1s.
Schematic alignment of C1sA gene structure: Showing CUB 1, EGF, CUB 2, CCP 1, CCP 2 and SP domains and relationship of exons to respective domains. Narrow portions represent leader peptide. Vertical arrow represents activating cleavage site (CS).

I.8 Genetics and the tissue distribution of C1s

Human C1s gene is located on chromosome 12 region p13. It consists of 12 exons (Endo Y *et al*., 1998 and Tosi *et al* 1989). The C1s gene in mouse is duplicated, one set of gene is called C1sA and the other one C1sB (Garnier *et al*., 2003).The mouse C1s genes are located on chromosome 6 position F2 and consists of 12 exons. The murine C1sA gene is homologous to the human C1s gene and is produced in liver where as the C1sB gene is exclusively expressed in testis (male reproductive organs) and therefore is not considered to play a role in the activation via the classical complement pathway.

I.9 C1s in health and disease

The main role of C1s is to cleave C4 and C4b-bound C2 and lead to the formation of complement activation cascade. Figure 1.11 shows the inference of C1s in health and disease.

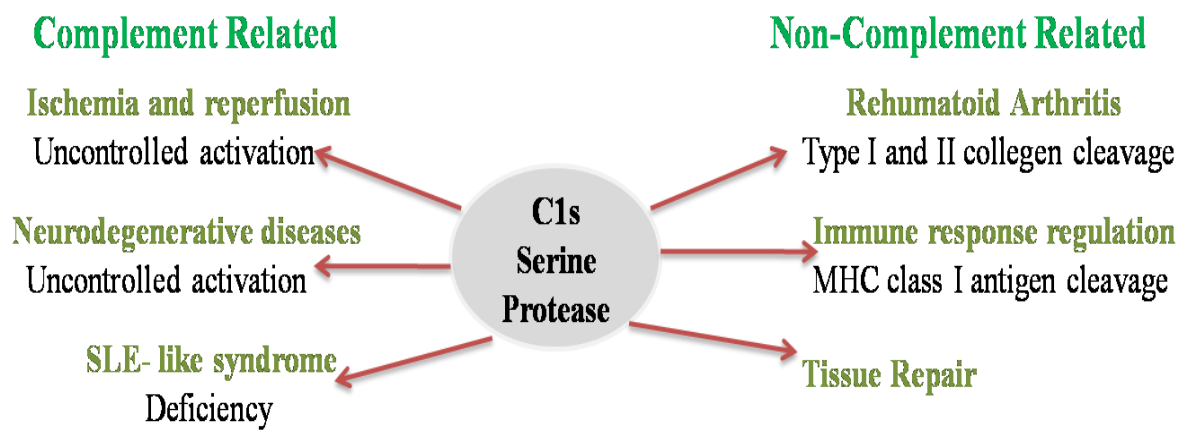

Figure 1.11 The inference of C1s in health and disease. Figure shows the possible processes and diseases in which C1s can participate (modified from Gal *et al*., 2002).

The high or low levels of C1s can contribute to severe pathology. A harmful role of C1s activation has been observed in neurodegenerative diseases such as Alzheimer disease (Jiang *et al*., 1994) and in inflammatory pathologies such as the ischemia reperfusion injury (Weiser *et al*., 1996). Deficiency of C1s causes an autoimmune disease, a systemic lupus erythematosus (SLE) like syndrome (He and Lin., 1998).

I.10 Aims of the thesis

In order to study the interdependence between the three complement activation pathways in vivo and in vitro, the following aims were designed and discussed in this thesis.

- For this reason , C1sA gene was disrupted by gene targeting, which is the effecter enzyme of the CP, equivalent to MASP-2 in the LP, to generate C1sA-/- mice and back-cross the C1sA gene deficient mice with the MASP-2 gene deficient mice so that it will allow us to define the role of alternative pathway in a mouse model deficient of both LP and CP activation. C1sA-/- mice will help us in *in vivo* analysis of the phenotype.
- Analysis of the location and cell types expressing C1sA and C1sB in vivo using *in situ* hybridization.
- Cloning and expression of recombinant mouse C1sA and human C1s for monoclonal antibody production and to reconstitute the phenotype of C1sA-/- mouse.

Chapter II

Material and Methods

II.1. Materials

II.1.1 Reagents and Kits

Reagents and Kits	Supplier
Bio-Rad Silver Stain	Bio-Rad
ECL Western Blotting Substrate	Pierce, USA
Nucleobond AX Kit (for Maxiprep)	Marcherey-Nagel, Germany
Prime-It® II Random Primer labelling Kit	Stratagene
Superscript ™ First-Strand synthesis System for RT-PCR(Reverse Transcriptase kit)	Invitrogen
QIA Quick Gel Extraction Kit	QIAGEN
QIA Quick Nucleotide Removal Kit	QIAGEN
Wizard Plus ® SV miniprep DNA purification System	Promega
Wizard genomic DNA Purification	Promega

II.1.2 Chemicals, media and solutions

Chemicals, media and solutions	Supplier
Acetic Acid	Fisher Scientific
Acetic anhydride	Sigma
Agar	Melford
Agarose (Electrophoresis)	Invitrogen
Ampicillin	Sigma-Aldrich
Ammonium persulphate (APS)	Sigma-Aldrich
Bovine Serum Albumin (BSA)	Promega
Coomassie Brilliant Blue R250	Fluka
(α-^{32}P)dCTP(3000Ci/mmol)	Perkin Elmer
(α-^{35}S)dUTP(1250Ci/mmol)	Perkin Elmer
Deoxynucleotide triphosphate mix(dNTP), 10µM	Promega
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
DNA Marker (1 Kb ladder, 100ng/ µl)	Sigma-Aldrich
Ethidium Bromide	Sigma-Aldrich
Ethylenediaminetetra acetic acid (EDTA)	Sigma-Aldrich
Eosin	BDH laboratories
Formamide	Sigma-Aldrich
Foetal Calf Serum (FCS)	Harlan
Gentamycin	Gibco, Invitrogen

Chemicals, media and solutions	**Supplier**
Glycerol	Fisher Scientific
Graces Insect Medium (Supplemented)	Gibco, Invitrogen
Graces Insect Medium (Unsupplemented)	Gibco, Invitrogen
Haemotoxylin	BDH laboratories
H_2O (double distilled)	Milli Q UF Plus System
Hygromycine-B	Invitrogen
Isopropyl β-D-thiogalactopyranoside (IPTG)	Sigma-Aldrich
Isopentane	Fisher Scientific
Isopropyl alcohol	Sigma-Aldrich
Low fat dry milk powder	Supermarket
β-mercaptoethanol	Sigma-Aldrich
Methanol	Fisher Scientific
$MgCl_2$ (25µM)	AB gene
Nulease free water	Promega
Pefabloc	Roche
Paraformaldehyde	Fisher Scientific
Penicillin/Streptomycin (100U/ml)	Invitrogen
Phenol-chloroform, for RNA purification	Sigma
Prestained 1X SeeBlue-plus2	Invitogen
Quikhyb hybridization Solution	Stratagene

Chemicals, media and solutions	Supplier
Restriction endonucleases	New England Biolabs
RNAse A, DNAse free	Promega
Salmon Sperm DNA (100mg/ml)	Sigma-Aldrich
Sodium dodecyl sulphate (SDS)	Sigma
T4 DNA ligase (20’000 units/µl)	New England Biolabs
T4 ligase buffer	New England Biolabs
TEMED	Sigma-Aldrich
Thermoprime plus DNA polymerase (5units/µl)	AB gene
Tris	Sigma-Aldrich
Triton-100	Sigma-Aldrich
Trizol	Invitrogen
Trypsine-EDTA	Gibco, Invitrogen
Tryptone	M Lab
Tween-20	Sigma-Aldrich
X-gal(5-bromo-4-chloro-3-indolyl-β-D galactoside)	Melford
Yeast Extract	M Lab
The remaining chemicals and solutions not mentioned in the list were obtained from	Sigma-Aldrich and Fisher Scientific

II.1.3 Competent *E.coli.* strains

The *E.Coli.* strains which were used in this study are OneShot® TOP10F' cells and XL-1 blue cells. There is brief description about these cells and their genotype.

II.1.3.1 OneShot® TOP10F'

OneShot® TOP10F cells overexpress the Lac repressor gene (*lac*I^q gene). And are used for blue/white screening to select recombinants that can enzymatically convert X-gal/IPTG to demonstrate expression of the lac promoter.

The genotype of the OneShot® TOP10F a cell is:

F' {*lac*I^q Tn10 (Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ΦΔ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ (ara-leu)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G.

II.1.3.2 XL-1 blue

XL-1 blue cells are resistant to tetracycline, deficient in endonuclesae (*end*A) and are also deficient in the recombination gene (*rec*A). These factors improve the stability of the construct and the XL-1 blue cells are the host used for blue/white screening.

The genotype of the XL-1 blue cells is:

*rec*A1 *end*A1 *gyr*A*96 thl*-1 *hsd*R*17 sup*E*44 rel*A*1 lac* { F' *pro*AB *lac*lqZM15 Tn10 (Tetr)}

II.1.4 Vectors used for cloning

pGEM®-T Easy Vector (Promega) was used for cloning PCR products, Mammalian Expression Vectors pCINeo (Promega) and pSec Tag2/HygroC (Invitrogen) and insect expression vector are used for protein expression and pKO Scrambler NTKV-1901 vector (Stratagene) was used for gene targeting.

II.1.4.1 Vector used for Cloning PCR products

The pGEM®-T Easy Vector (3Kb) was primed by cutting the pGEM®-T Easy Vectors with *Eco*R V with addition of 3´thymidine terminals on both sides. Single 3´-Thymidine overhangs at the insertion site significantly improve the ligation efficiency of PCR products in the plasmids by preventing refolding of the vector and providing a compatible overhang for PCR products generated by *Taq* polymerases. pGEM®-T Easy Vector is usually used for Blue/white screening in order to obtain recombinants.

II.1.4.2 Mammalian expression vectors

II.1.4.2.1 pCI-neo Mammalian Expression Vector

The pCI-neo Mammalian Expression Vector contains the human cytomegalovirus (CMV) immediate-early enhancer/promoter region promote transcription of cloned DNA inserts in mammalian cells. The pCI-neo Vector also contains a selectable marker for mammalian cells (neomycin phosphotransferase gene) that can be used for stable expression of transfected cells with the antibiotic Genamycin. In the pCI-neo Vector (5.4Kb), T7 and T3 RNA polymerase promoters flank the multiple cloning region (Figure 2.1).

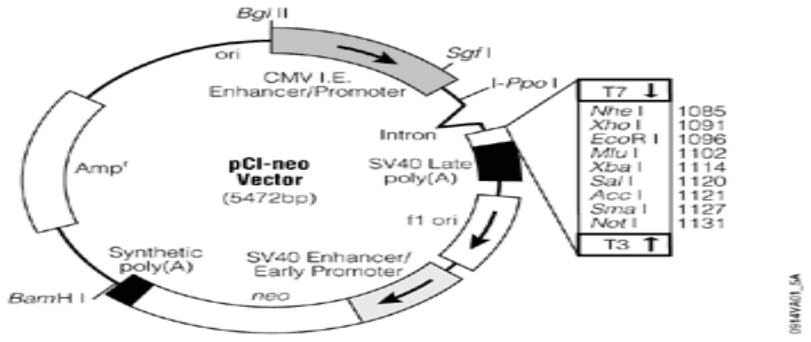

Figure 2.1. Map of pCI-neo Mammalian expression Vector . Neo = neomycin phosphotransferase; CMV I.E. = cytomegalovirus immediate-early. T7 and T3 RNA

polymerase promoters and multiple cloning region sequence. (www.promega.com/vectors)

II.1.4.2.2 pSec Tag2/HygroC Mammalian Expression Vector

pSec Tag2/HygroC is a 5.7Kb expression vector constructed for elevated expression and secretion in mammalian host cells. The vector bears the hygromycin B resistance gene for selection in mammalian cells and also encodes the β-lactamase gene for bacterial selection. Protein expressed from this vector are merged at N-terminal with the murine Ig kappa chain leader sequence, which allows fusion protein secretion (Coloma *et al*, 1992) and fused at the C-terminal to a peptide containing the *c-myc* epitope and six tandem histidine residues for dectection and easy purification (Figure 2.2).

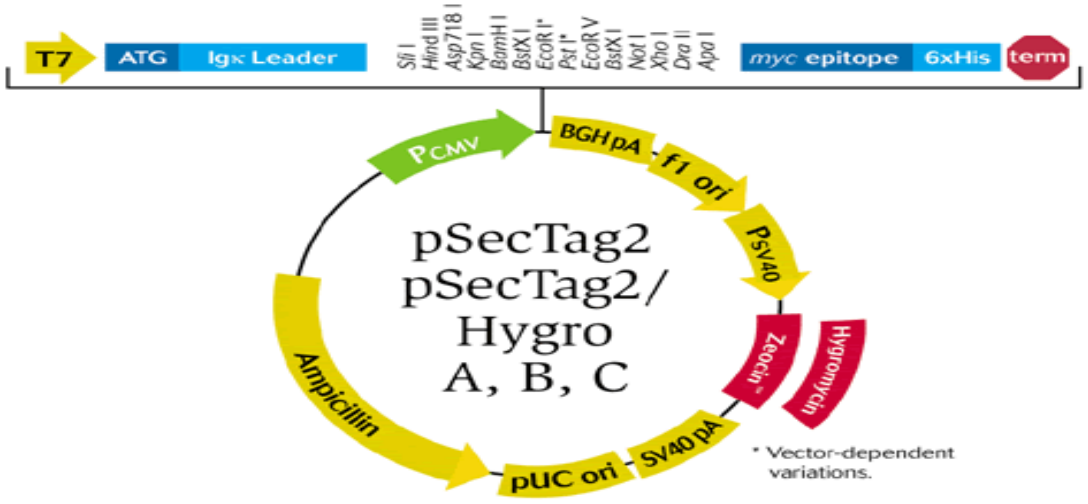

Figure 2.2 pSec Tag2/HygroC Mammalian Expression Vector map
(www.invitrogen.com/vectors)

II.1.4.2.3 Gene-Targeting Vector (pKO Scrambler NTKV-1901 vector)

The pKO Scrambler NTKV 1901 is a 5.6kb gene targeting vector which is designed to have restriction sites within the two polylinker regions (Scrambler A and Scrambler B) with positive *neo* and negative *TK* selection markers. pKO Scrambler NTKV 1901 has a wide range of restriction sites (as shown in Table 2.1). Appropriate 5' and 3' regions of the gene of interest are inserted into Scrambler A and Scrambler B, respectively (Figure 2.3)**.**

Table 2.1 Shows the restriction sites present in pKO Scrambler vector

pKO Scrambler NTKV 1901 polylinker Restriction Sites									
Scrambler A					**Scrambler B**				
Hpal	Bgl ll	Hind lll	Kpn l	Xho l	BamH l	Cla l	EcoR l	Sac ll	Sma l

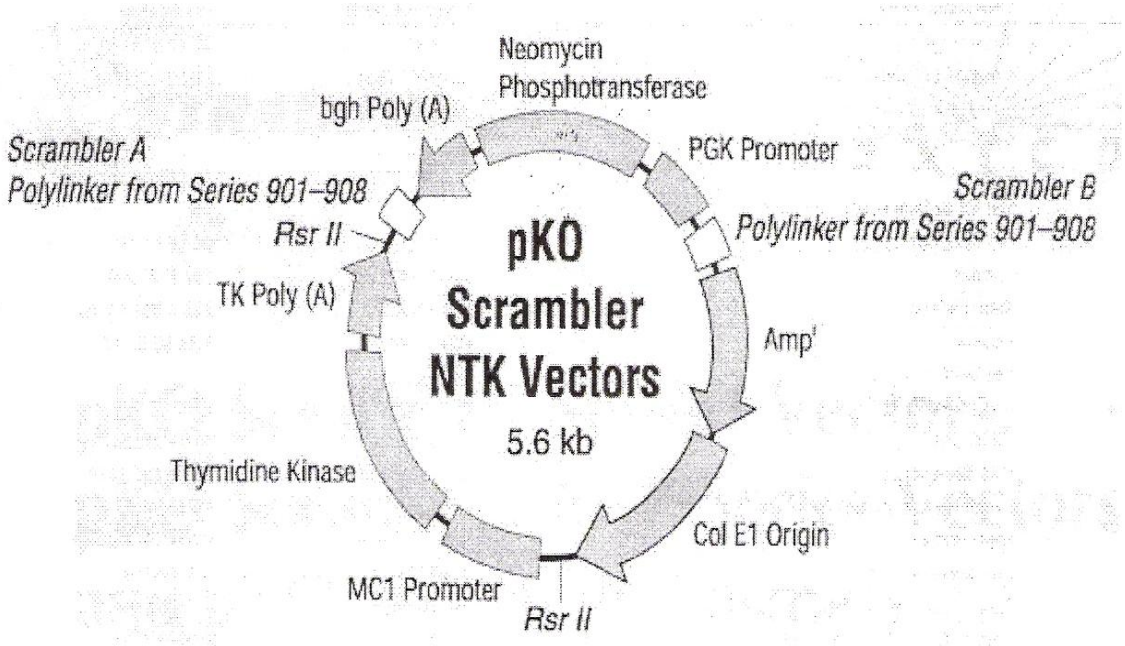

Figure 2.3. pKO Scrambler NTKV-1901 vector map (www.stratagene.com)

II.1.4.2.4. pBlueBacHis2 A Baculovirus transfer vector

The pBlueBacHis2 A vector is baculovirus transfer vector constructed for expression and purification of recombinant proteins in insect cells. Proteins expressed from pBlueBacHis 2 are fused at the N-terminus to a tag of six

tandem histidine residues and an enterokinase cleavage site and the Xpress Tag is an antibody epitope for detection (Figure 2.4).

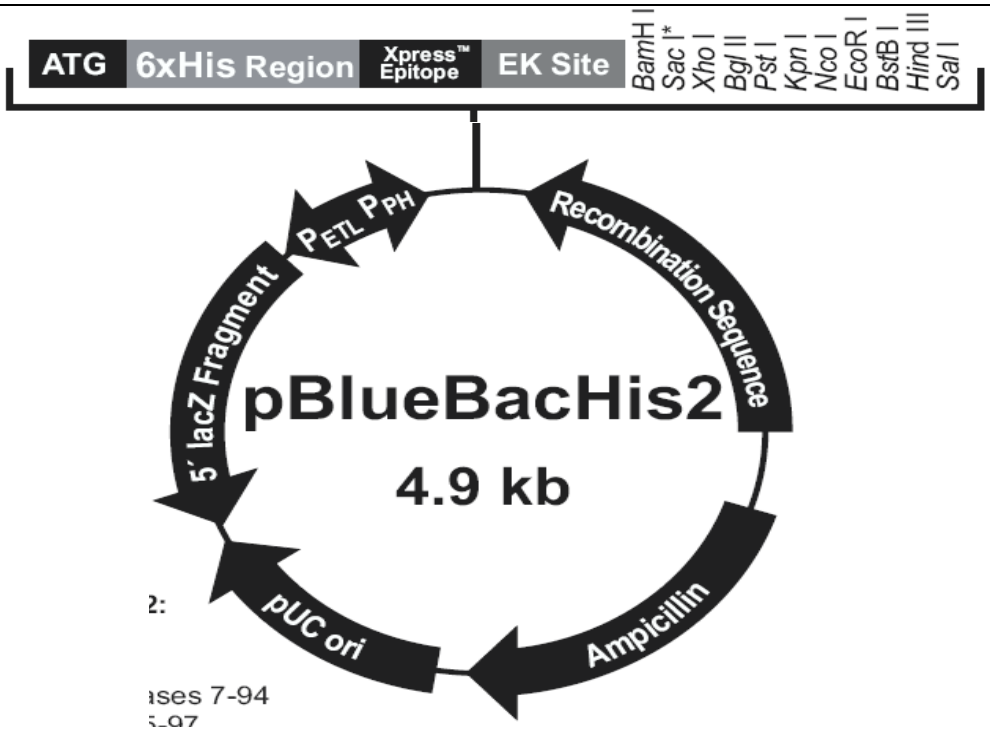

Figure 2.4 pBlueBacHis2 A vector Map.

II.1.5 Antibodies and Serum Used

Antibody	Source	Application
Anti his tagged antibody	Sigma	Western Blotting
Sheep polyclonal anti C1s antibody	Abcam	Western Blotting
Donkey anti-Sheep IgG-peroxidase	Sigma	Western Blotting
Anti Digoxigenin AP Fab fragment	Roche	*In situ* hybridization
Donkey Serum (Blocking Serum)	ABC staining system	*In situ* hybridization

II.1.6 Oligonucleotides

All oligonucleotides used for cloning, sequencing and screening were designed in-house and obtained from MGW Biotech, Germany at a concentration of 100pmol/µl (Table 2.2). All the primers were stored at -20^{o}C and the working concentration of the primers was 5µM

Table 2.2 Sequence of Oligonucleotides used

Oligonucleotide Name	Oligonucleotide Length	Oligonucleotide Tm	5' → 3' Sequence
hcDNA into pCI-Neo			
hC1s_ XbaI	26	64.8 ^{o}C	5'- TCT AGA GCC TAC CAT GTA TGG GGA GA -3'
hC1s_R1	23	58.9 ^{o}C	5'-GGA TGT ATC TGG ATT AGT CCT CA-3'
hcDNA into pSecTag/HygroC			
hC1s_ KpnI	26	66.4^{o}C	5'- GGT ACC CTA CCA TGT ATG GGG AGA TC -3'
hC1s_XhoIR	28	71 ^{o}C	5'-CTC GAG CCT CAC GGG GGG TGC TAT TTT C-3'
mcDNA into pCI-Neo			
mC1sA_XbaI	24	66.1 ^{o}C	5'-TCT AGA GCC CAC CAT GCA TGG GGA-3'
mC1sA_rev2	21	64^{o}C	5'-CCTACGGATTAGTCCTTCCTG-3'
mcDNA into pSecTag/HygroC			
mC1sA_XhoI	23	67.8 ^{o}C	5'-CTC GAG CCC ACC ATG CAT GGG GA-3'
mC1sA_rev3	22	66^{o}C	5'-GAGAACAGTGAGCTTGCTATGC-3'
mcDNA into pbluebac His2A			
mC1sA_Hind	20	57.3 ^{o}C	5'-AAG CTT GCT ATG CCC TTG AG-3'
Gene expression of C1sA			
mC1sA_for1	23	62.4 ^{o}C	5'-CTC CAA CGA AGA ACG GTT TAC GG-3'

Table 2.2 Sequence of Oligonucleotides used

Oligonucleotide Name	Oligonucleotide Length	Oligonucleotide Tm	5' → 3' Sequence
mC1s_rev2	27	68 ^{o}C	5'- TGA ATC TGG TAT TCA CAC CTT GAG TTC -3'
Gene expression of C1sB			
mC1sB_for1	27	61.0 ^{o}C	5'- GAC TTC TCC ATC GAA GAA CAG TTT ACC -3'
mC1s_rev1	22	62.1 ^{o}C	5'-ATC GTC ACC ACC ACT TGG AAG C-3'
***In Situ* hybridization Probe for C1sA**			
mC1sA_fp1	20	59.4 ^{o}C	5'-CTG ACG GCT GCT CAC GTT TT-3'
mC1sA_rp2	21	52.0 ^{o}C	5'-TGA GGT TGA TAA CAA AGA CTT-3'
***In Situ* hybridization Probe for C1sB**			
mC1sB_fp1	20	63.5 ^{o}C	5'-CTG ACG GCC GCT CAC GTT GT-3'
mC1sB_rp2	21	55.9 ^{o}C	5'-TGA GGT TGA TAA CAT GGA GTC-3'
C1sA Gene Targeting			
For A Cassette			
C1sA_KAF1	21	64^{o}C	5'-GCTTGGCAGCAGGTTTAGAGA-3'
C1sA_KAR1	22	66^{o}C	5'-GCTGGAAAAGGGAAAGCTGAGA-3'
For B Cassette			
KBR_Cla1	23	68^{o}C	5'-atcgaTCTGGTCCTCTATGAACACAGCA-3'

Table 2.2 Sequence of Oligonucleotides used

Oligonucleotide Name	Oligonucleotide Length	Oligonucleotide Tm	5' → 3' Sequence
C1sA_BamH2	21	62^{o}C	5'-ggatccTCCAACGAAGAACGGTTTACG-3'
For External Probe			
C1sA_P(SB)_FP2	20	62^{o}C	5'-GAG GAC CAG AGT TCA GTT CC-3'
C1sA_P(SB)_RP2	20	60.0 ^{o}C	5'-ATG GCT CAT GCC AGT AAT CC-3'
For C1sA Genotying			
C1sA_KAF$_4$	23	70^{o}C	5'-GAC ACG GGT TCT CAC ATG TAG GA-3'
C1sA_KAR$_2$	20	62^{o}C	5'-CCG TGT GGT GAT AGG ATG CA-3'
Neo5_R$_2$	21	64^{o}C	5'-GAT CTG GAC GAA GAG CAT CAG-3'
Baculovirus Expression			
Plaque assay Primers			
Forward Primer	24	62^{o}C	5'-TTTACTGTTTTCGTAACAGTTTTG-3'
Reverse Primer	21	58^{o}C	5'-CAACAACGCACAGAATCTAGC-3'
Linker Primers			
XX_link_F	34	100^{o}C	5'-TCGAGCCACCATGGCTCATCATCATCATCATCAT-3'
XX_link_FR	34	100^{o}C	5'-CTAGATGATGATGATGATGATGAGCCATGGTGGC3'

II.2. Methods

II.2.1 DNA based cloning techniques

II.2.1.1 Setting up of LB agar plates

For successful cloning or transformation it was important to make the LB agar plates as described:

1. For 400ml of LB agar medium add 4g of tryptone, 2g of yeast extract, 2g of NaCl and 6g of agar in double distilled water and autoclave.
2. Let the medium come to room temperature (37^{o}C). In the meanwhile arrange 20 x 90mm petri plates in a laminar airflow cabinet cleaned with 70% IMS.
3. Once the medium cools down, add 800µl of ampicillin (stock concentration 50mg/ml) to *LB medium* (Appendix1) to make a final concentration of 50µg/ml and mix.
4. Pour the agar quickly into petri dishes, filling up to about a quarter of an inch of the plate and close lid immediately. Once they solidify, store at 4^{o}C.

II.2.1.2. Preparation of chemically competent *E.coli*. strains

The protocol used for harvesting the competent *E.coli.* strains such as TOP10F' cells and XL-1 blue cells is a modification of the methodology described by Hanahan. D, 1983. The protocol is described below

1. Streak the host strains on *LB agar* (Appendix 1) plates containing 10mM Mg^{2+} and incubate at 37^{o}C overnight.
2. On the following day pick a single colony from the plate and inoculate 5ml of LB medium with that colony in a 50ml sterile falcon tube. Incubate the cultured tube at 30^{o}C in a shaking incubator overnight.
3. On the next day inoculate 100ml of fresh *LB medium* (Appendix 1) with 1ml of the bacterial culture, incubate overnight and let it grow at 37^{o}C in a shaking incubator until it reaches an OD_{550} of 0.7 to 0.8.
4. Collect the cells by centrifugation at 2000g for 10 min and decant the supernatant.
5. Resuspend the cells in 30 ml sterile ice cold *TfbI buffer* (Appendix 1) and incubate on ice for approximately 10mins.

6. Following 10 min incubation on ice and further 10 min centrifugation at 2000g (4^{o}C), the cells are resuspended in 4ml ice cold *TfbII buffer* (Appendix1).
7. Aliquot 100ul fractions into eppendroff tubes and store at -80^{o}C.

II.2.1.3. Ligation of insert into vector

A necessary part of cloning is the ligation of DNA fragments. Covalently joining together two linear DNA fragments is known as ligation and it is proficiently done by using the T4 DNA ligase enzyme from New England Biolabs which is encoded by bacteriophage T4. It catalyzes a joining reaction between DNA molecules involving the 3' - hydroxy and the 5' - phosphate termini. T_4 ligase works best at 37^{o}C. If the DNA of interest has to be cloned into an uncut plasmid by ligation then the plasmid should be pre-digested with specific restriction enzyme producing a linear DNA molecule with overhanging sticky ends for efficient ligation.

II.2.1.3.1 Ligation of PCR product into pGEM®-T Easy vector

Taq polymerase has a non template-dependent terminal transferase activity which adds a single deoxyadenosine to the 3’ ends of the PCR product. The

vector has a single 3’ thymidine overhang that allows PCR inserts to ligate easily with the plasmid. The protocol for ligation is described in below.

1. Briefly centrifuge the tube containing the pGEM®-T Easy vector.
2. Prepare the ligation reaction in following order

Reagents	Ligation Reaction	Negative Control
T4 DNA ligase buffer	1µl	1µl
T4 DNA ligase	1µl	1µl
PCR Product (~60 to 150 ng/ µl)	2µl	
pGEM®-T Easy vector (50ng/ µl)	1µl	1µl
Double distilled H_2O	5µl	6µl
Final volume	10µl	10µl

3. Mix the reagents and incubate overnight at 4°C.

II 2.1.3.2 Optimizing Insert: Vector Molar Ratios

The concentration of PCR product or DNA fragment and vector which are restriction digested are estimated by comparison to DNA standards on an agarose gel. The amount of insert DNA to be added was calculated using the following formula for insert- vector optimization. Insert: vector molar ratio was 3:1(Sambrook *et al*., 1989).

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert: vector molar ratio} = \text{ng of insert}$$

II.2.1.4. Chemical transformation of plasmid into competent *E. coli* cells

The purpose of this technique is to introduce a foreign plasmid (containing the DNA of interest) into bacteria where it will be amplified. This technique is based on the natural function of the plasmid where, the transfer of genetic information (through the plasmid) is vital for the survival of the bacteria. Protocol for transformation is as below:

1. Thaw 50 µl of Top 10 F´ competent cells on ice and add to a pre cooled eppendroff tube. Add 2µl of the ligation reaction and mix it. Incubate on ice for 20min.
2. Heat shock the cells at 37^{o}C for 5 mins and incubate them back on ice for 1min immediately.
3. Add 450µl of LB medium and incubate the reaction in a 37^{o}C shaking incubator for 1hour.
4. After incubation the bacterial culture is plated out on plates containing LB agar and ampicillin (50µg/ml), X-gal (25 µg/ml) and IPTG (100

μg/ml) for the selection of recombinant bacterial colonies by blue white screening.

II.2.1.4.1 Blue white screening for the selection of recombinant clones

Successful cloning of DNA inserts into pGEM-T easy vector is confirmed by blue white screening. pGEM-T easy contains a Lac-Z gene which, codes for β-galactosidase, an enzyme that digests and converts X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactside) into a blue coloured product. For the production of the enzyme by the active transcription of the DNA, IPTG (Isopropyl β-D-thiogalactopyranoside) is delivered to the bacteria through the medium. Within the Lac Z gene is a multiple cloning site (MCS) where the gene of interest can be inserted. Hence, if the insert DNA is present in the MCS of the LacZ gene the expression of LacZ gene becomes disrupted and no functional β-galactosidase will be expressed. This disruption in enzyme activity is observed as white colonies as no functional Lac Z is available to digest X-gal and produce blue colour. Therefore, if the insert is present in the vector the colonies appear white in colour and their absence leads to blue colour colonies on the LB agar plate.

II.2.1.5. Isolation and purification of plasmid DNA

II.2.1.5.1. Culture colonies for isolation of DNA

Select about 8-10 colonies from the transformed plate using a toothpick and add an individual clone in 15ml falcon tube containing 2.5ml of LB medium with 50 μg/mL ampicillin. Incubate the falcon tube at 37^{o}C in an orbital shaker overnight.

II.2.1.5.2. Small scale isolation and purification of plasmid DNA (Minipreps)

Minipreps are used to extract plasmid DNA from bacterial cell suspensions. The plasmids are a relatively small supercoiled molecule whereas bacterial chromosomal DNA is large in size and less supercoiled. This difference in topology helps in selective precipitation of chromosomal DNA and protein from plasmid DNA.

Small scale isolation and purification of plasmid DNA was carried out using the Wizard® Plus SV Minipreps DNA Purification System from Promega. The kit combines two techniques; alkaline lysis and silica resin based DNA purification. Cells are partially lysed using an alkaline solution of the detergent SDS. This allows small plasmid DNA molecules to escape from

the cells, while genomic DNA remains within the cells. When a concentrated potassium acetate solution is added to the cell lysate, cell debris is precipitated, while plasmids and soluble proteins remain in solution. If the cell membrane is dissolved completely, plasmid and soluble proteins remain in solution. If the cell membranes are dissolved completely, sheared genomic DNA may be released, contaminating the plasmid prep. To avoid this, the lysis step is carried out for just enough time for the solution to clear. The adhesion of DNA to a silica matrix is based on the fact that nucleic acids adhere to silica in high but not in low salt conditions. DNA binds to silica in the lysis solution and is eluted from the silica matrix by TE buffer.

II.2.1.5.2.1 Production of Cell Lysate

1. Centrifuge 1ml of overnight bacterial culture for 5min at a speed of 10,000g in a centrifuge. Discard the supernatant and collect the pellet.
2. Resuspend the pellet in 250µl of cell suspension solution by repeatedly pipetting.
3. Add 250µl of cell lysis solution and mix by inverting the tube 3-4 times then add 10µl of alkaline protease solution and mix by inverting the tube and incubate for 5mins at room temperature.

4. The cell lysis reaction was stopped by adding 350µl of neutralization solution and mixing by inverting the tube. The lysate was then centrifuged at 14,000g for 10 mins.

II.2.1.5.2.2 DNA isolation and purification

1. Transfer the cleared lysate to the spin column by decanting. Avoid disturbing or transferring any of the white precipitate with the supernatant.
2. Centrifuge the supernatant at the highest speed in a microcentrifuge for 1 min at room temperature. Remove the spin column from the tube and discard the flow through from the collection tube.
3. Add 750µl of column wash solution and centrifuge for another 1min at the highest speed. Remove the spin column from the tube and discard the flow through from the collection tube.
4. Repeat the wash step by using 250µl of column wash solution. Spin the column for another 2 mins at room temperature.
5. Put the spin column in a fresh sterile 1.5µl eppendorff tube.
6. Elute the plasmid DNA by adding 100µl of double distilled water to the spin column and centrifuge the tube at highest speed for 2 mins.

7. After elution store the plasmid DNA at -20^{o}C.
8. The plasmid identity can then be checked by using restriction digestion.

II.2.1.5.3. Large scale isolation and purification of plasmid DNA (Maxipreps)

In order to obtain a working quality of purified plasmid DNA the Nucleobond AX Kit were used. The principle on which this kit works is that bacteria are harvested from an overnight culture and lysed using the alkaline lysis procedure (see below). The bacterial lysate is cleared and loaded onto the equilibrated column and the plasmid DNA binds to the anion-exchange resin. The column is first washed using equilibration buffer to wash out residual lysate from the filter and obtain maximum recovery of DNA. After subsequent washing, the purified plasmid DNA is eluted in a high-salt buffer and precipitated with isopropanol.

The method involved in the isolation and purification of plasmid DNA is briefly described below

1.100ml of LB medium containing ampicillin was inoculated with the original culture in a ratio of 1/1000 and incubated in an orbital shaker

overnight at 37^{o}C.

2. Centrifuge 50ml of overnight bacterial culture for 15min at a speed of 10,000g, discard the supernatant and collect the pellet.
3. Resuspend the pellet in buffer S1(supplied with kit) containing RNase A, mix well and then add buffer S2 (supplied with kit) to lyse the cells and incubate for 2mins.
4. Add chilled buffer S3 (supplied with kit) for neutralization and incubate for 5 mins on ice.
5. In the meantime neutralize the NucleoBond Xtra column with buffer N1 (supplied with kit).
6. Pour the bacterial lysate into the neutralized NucleoBond Xtra column and let the solution flow through the column drop by drop.
7. Wash the column with buffer N2 (supplied with kit).
8. Elute by using buffer N5 (supplied with kit) and add isopropanol to the eluted plasmid DNA and then centrifuge for 30 min at high speed. Decant the supernatant and collect the pellet and wash with 70% ethanol.
9. Resuspend the pellet in double distilled H_2O and store at -20^{o}C

II.2.1.5.4. Glycerol stocks

For long term storage of positive transformants, bacterial cultures were mixed 1:1 with sterile 50% glycerol in cryotubes and frozen down at -80^{o}C.

II.2.1.5.5. Spectrophotometric determination of DNA concentration

In order to quantify the concentration of purified DNA absorbance of diluted samples were measured at 260 nm. An absorbance of 1.0 corresponds to 50µg/ml of double stranded DNA. Concentration of DNA can be determined by using the formula shown below.

Concentration of DNA (µg/ml) = OD_{260} x 50 x Dilution factor

The ratio of readings at 260nm and 280nm [A 260/ A 280] provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have ratios between 1.8 – 2.0. If there is significant contamination with protein or phenol, the ratio will be lower and an accurate quantitation is not possible.

II.2.1.6. Dephosphorylation of a linearised plasmid

During ligation, DNA ligase will catalyze the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide contains a 5'-phosphate group and the other a 3'-hydroxyl group. Recircularization of

plasmid DNA can therefore be minimized by removing the 5' phosphates from both ends of the linear DNA with calf intestinal alkaline phosphatase (CIAP) (Seeburg *et al*., 1977 and Ullrich *et al*., 1977). As a result, neither strand of the duplex can form a phosphodiester bond.

For dephosphorylization, the following protocol is used as provided by Promega

Reagents	Reaction Mixture
The vector DNA digest (50ng/µl)	Xµl
10X CIAP buffer	10µl
CIAP enzyme (0.05Units)	5µl
dH2O	Yµl
Final volume	100µl

The reaction is then incubated for 30 mins at 37^{o}C. The dephosphorylization reaction is followed by phenol extraction and ethanol precipitation.

II.2.1.7. Restriction digestion

Restriction enzymes cleave DNA at specific sites. Individual restriction enzymes have their unique DNA recognition sequence on a given DNA. To

perform restriction digest with a particular restriction enzyme, the DNA is incubated with the enzyme and a buffer that is appropriate for its optimal performance. To set up a restriction digest with two different enzymes, one common buffer is used. The amount of enzyme required and the procedure varies depending on the DNA samples subjected to digestion. The amount of DNA used is a matter of judgement depending on the concentration of the DNA samples.

Set up the restriction digest as described below

Reagents	Reaction Mixture
DNA (200ng/µl)	2µl (Depending on DNA concentration)
Restriction enzyme 1 (20,000 units/µl)	1µl
Restriction enzyme 2 (20,000 units/µl)	1µl
10X BSA	2µl
Buffer (Compatible to both enzymes)	2µl
dH2O	11µl
Final volume	20µl

All the reagents were added in an eppendorf tube to make up to a final volume of 20µl. The samples were kept for digestion for about 2 hours at the

optimal reaction temperature of the used enzyme which is usually 37ºC. Later on, samples were analyzed using 1% agarose gel electrophoresis in *TAE buffer* (Appendix 1) at 120 volts.

II.2.1.8. Agarose gel electrophoresis

Agarose gel electrophoresis is a method for separating and analyzing DNA depending on size. The purpose of this technique is either to look at the expected size of the DNA (PCR product) and/or quantify it or to isolate a particular band (restriction digestion product). DNA is negatively charged and therefore migrates towards the anode in an electrical field. When DNA samples are loaded into an agarose gel and electrical field is applied the DNA fragments will travel through the gel and they will be separated due to their size. The bigger the size of the DNA fragments the smaller the distance it travels through the gel and vice versa. By increasing the concentration of the agarose gel smaller sizes of DNA fragments can be visualised properly. The DNA can be visualized by the addition of ethidium bromide which binds to the DNA by intercalating between the bases and allows the DNA to be visualized upon the exposure to UV light.

II.2.1.8.1 Preparation of gel

The 1% agarose gel was prepared by dissolving 1g of agarose in *1x TAE buffer* (Appendix 1) containing ethidium bromide. The 1% agarose gel was poured in the gel plate and the comb was placed for the formation of the wells. Once the gel was solidified the comb was removed carefully and the gel tray was placed in the electrophoresis tank with *1X TAE buffer* (Appendix 1). DNA samples to be analysed were mixed with a required volume of *6X loading dye* (Appendix 1) and were loaded into the wells against 10µl of 1 Kb ladder (100ng/ µl). The gel was run at 120V till the dye front reached the end of the gel and was later visualized and analyzed under UV light.

II.2.1.8.2. Extraction and purification of DNA from agarose gel

The QIA Quick Gel Extraction Kit was used for purification of the DNA fragments obtained from the agarose gel. QIAquick Kit contains a silica membrane assembly for binding of DNA in high-salt buffer and elution with low-salt buffer or water. The purification procedure removes primers, nucleotides, enzymes, mineral oil, salts, agarose, ethidium bromide, and other impurities from the DNA samples.

After restriction analysis or after running the PCR product in the agarose gel, the band of expected size was excised from the agarose gel with a clean scalpel and then the protocol given by manufacturers was followed. After purification the DNA concentration was checked on a 1% agarose gel.

II.2.1.9. Southern Blotting of Genomic DNA

The southern blotting or the capillary blotting technique first described by Southern in 1975 is still the most widely used technique for transferring separated nucleic acid fragments from an agarose gel to a solid support.

Briefly the process starts with the restriction enzyme digestion of the genomic DNA and then continues with the separation of the DNA fragments on an agarose gel. After separation the DNA-fragments are transferred to a nylon membrane. The DNA in the gel is denatured with alkali-solution; later capillary transport of the DNA fragments takes place from the gel to the nylon membrane, using the same alkali-solution. The relative positions of the DNA-fragments are preserved during their transfer to the nylon membrane. After blotting the DNA-fragments are bound to the membrane and then the membrane is hybridized with a radio-labelled nuclic acid probe in order to detect specific fragments.

The protocol described below is taken from Sambrook *et al*., 1989.

1. Separate the genomic DNA samples on a suitable neutral agarose gel (Section II.2.1.8.1). An electric field is applied across the agarose gel on which the DNA samples are loaded. At neutral pH the DNA-fragments have a negative charge and will therefore migrate towards the anode (positive charge). The DNA-fragments are separated according to their size; the smaller fragments will migrate faster through the gel than the larger fragments.
2. After electrophoresis visualize the DNA samples in the gel using UV light and photograph with a scale alongside.
3. Process the gel for blotting between each step rinse the gel in distilled water.

(i) Depurination step: Soak the gel in 0.25M HCl solution for 10 mins (until bromophenol blue turns yellow), then briefly rinse the gel with dH_2O. Depurination is not required for the DNA fragments $\leq$ 10Kb in size.

(ii) Denaturation step: Rinse the gel with distilled water briefly before

soaking the gel in 0.5M NaOH/1.5M NaCl solution. Agitate gently for 15-20 mins (until the bromophenol blue dye returns to its original colour).

4. Set up the southern blot or the capillary blot as described in the figure. Fill the tray of a suitable size with the transfer buffer. Make a platform and cover it with a sheet of 3MM Whatman paper which is larger than the platform in all directions. Fold the edges of the paper so that they touch the bottom of the tray.
5. Place the treated gel on the platform and avoid trapping any air bubbles between the gel and the 3MM Whatman paper.
6. Cut a piece of Electran +ve charged membrane from BDH to the size of the gel. Immerse the membrane in distilled water first and then in transfer buffer until it is completely wet. Place the wet nylon membrane on top of the gel and slightly cut right hand corner of the gel and the membrane. Mark the position of the sample wells with a ballpoint pen.
7. Place three sheets of 3MM Whatman paper cut to the size of gel and membrane over the membrane. Avoid any air bubbles.

8. Place a pile of paper towels approximately 5-8cm high, cut just smaller than the 3MM Whatman paper. Place a glass plate on the top of paper towels and a weight on top of the papers. Liquid flows from through the gel and the membrane and into the dry paper towels, transferring DNA from the gel on to the membrane. Allow the transfer to proceed overnight.
9. On the next day carefully dismantle the transfer apparatus and rinse the membrane in 2 x SSC solutions. Fix the DNA to the membrane by baking it in an 80^{o}C incubator for 2-3 hours.
10. Blots can be used immediately for hybridization or you can store them by wrapping in aluminum foil at room temperature.

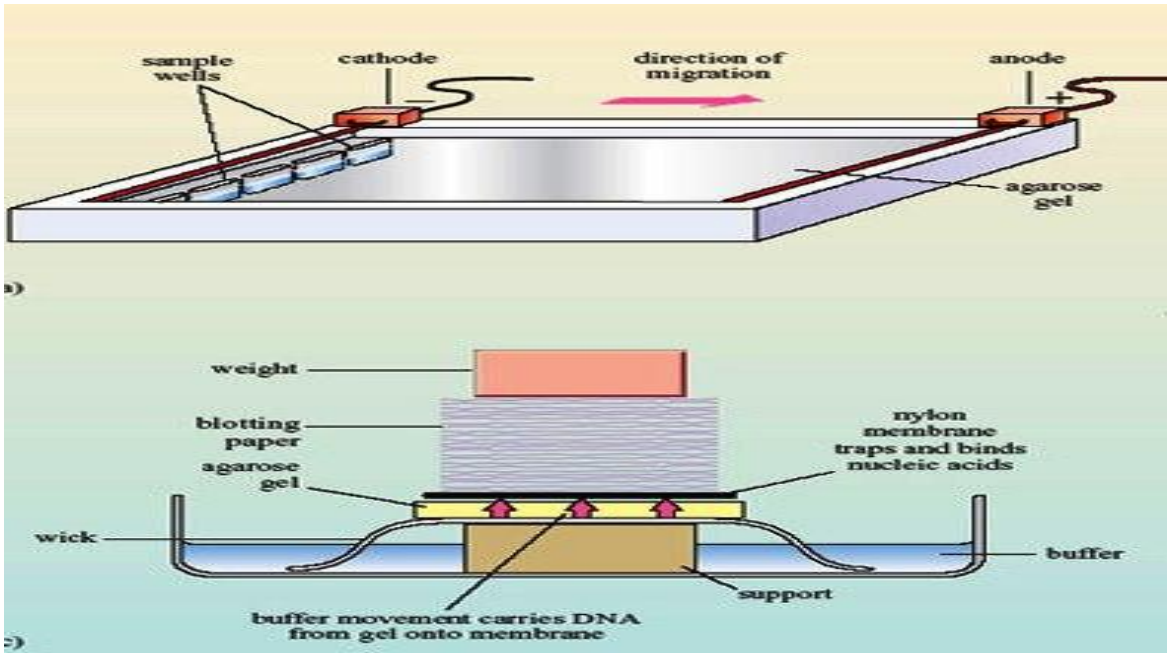

Figure 2.5. Diagrammatic representation of a Southern blotting apparatus.

II.2.1.10. DNA Probe Labelling by Random Priming

The use of a "random primed" DNA sequence to prime DNA synthesis was originally introduced by Feinberg and Vogelstein. The process is based on the ability of random oligonucleotides to hybridise to all possible sites along the denatured template DNA to be labeled in order to generate high specific-activity probes (Feinberg *et al*., 1983 and Feinberg *et al*., 1984). The

complementary DNA strand is synthesized by a Klenow fragment of *E. Coli* DNA Polymerase I, using the random oligonucleotides as primers. By substituting a radiolabeled nucleotide for a non-radioactive equivalent in the reaction mixture, the newly synthesized complementary DNA is made radioactive. This rapid labeling is accomplished with the use of the Klenow fragment, which lacks 5'-3' exonuclease activity. The DNA probe is labeled with (α-^{32}P) dCTP using Prime-It® II Random Primer labelling Kit from Stratagene. The kit contains random oligonucleotides, a Klenow fragment of DNA polymerase I, dATP, dGTP, dTTP and a reaction buffer concentrate. The protocol for random prime labeling is described briefly below.

1. Prepare the reaction as shown below in a clean microcentrifuge tube.

Components	Reaction mixture
DNA template (25µg/ml = 25ng)	1µl
Random oligonuleotide primers	10µl
Distilled H_2O	23µl

2. Incubate the reaction tube in a heat-block which is set at 95^{o}C for 5 mins in order to denature the DNA, briefly centrifuge the tube at room temperature to collect the reaction mixture. Place the tube on ice for 2 mins.

3. Add the following components to the microcentrifuge tube.

Components	Reaction mixture
5X dCTP primer buffer	10µl
(α-^{32}P) dCTP (3000Ci/mmol)	5µl
Exo(-) Klenow enzyme (5U/µl)	1µl

Mix the reaction components.

4. Incubate the reaction at 37^{o}C for 10 mins. The DNA labeling mix allows the labeling of the template DNA to a specific activity of 2 x 10^{9} dpm/µg after only 10 minutes of incubation. Stop the reaction by adding 2µl of stop mix.

5. The DNA labelled probe is purified (in order to remove unincorporated labeled nucleotides) by using ProbQuant G50 MicroSpin column (Amersham Biosciences), vortex columns for 2 mins at 2500rpm with loose cap. Denature the radiolabelled probe at 95^{o}C for 10mins and use for hybridization (Section II.2.1.11.**)**.

II.2.1.11. Probe hybridisation

1. Preheat the hybridisation oven and Stratagene QuikHyb hybridisation buffer to 68^{o}C. Immerse the blotted membrane in 2X SSC in order to make it wet.
2. Roll the blot into a thin tube and put into the hybridisation bottle. Make sure that the DNA side of the blot should face inside.
3. Add 20mls of hybridisation buffer to the hybridisation bottle and pre - hyb for a minimum of 20 mins at 68^{o}C.
4. Boil the labeled probe at 95^{o}C for 10 mins in a heat-block then chill on ice for 2 mins. Add the labeled probe in QuikHyb hybridisation buffer to the hybridisation bottle and incubate at 68^{o}C overnight.
5. Pour off the radioactive hybridisation solution into clean falcon tube and store at 4^{o}C for further use. Wash the membrane with 2x SSC solution twice at 68^{o}C for 5mins then wash with 2x SSC/ 0.1% SDS solution at 68^{o}C for 5mins twice and finally wash the membrane twice with 0.5x SSC/ 01% SDS solution at 68^{o}C.

6. Put the washed membrane on to a piece of 3MM Whatman paper and allow drying. Once dry stick the membrane to paper with tape and put radioactive markers on the four corners.
7. Cover the membrane with saran wrap or cling film and put into x-ray cassette. Lay down a piece of Kodak film with intensifying screen on top and expose the film at -80^{o}C overnight or longer. Develop the film (autoradiograph).

II.2.1.12. Isolation of genomic DNA

Genomic DNA is isolated from the mouse ear snips by using Wizard genomic DNA Purification Kit provided by Promega.

Day I

1. Add 60µl of a 0.5M EDTA (pH 8.0) solution to 250 µl of Nuclei Lysis solution (comes with kit) to each sample in an eppendrof tube and chill on ice.
2. Add 0.5-1cm of fresh or thawed mouse ear snips to a 1.5ml eppendrof tube.
3. Add 310 µl of EDTA/Nucleic Lysis solution to the tube.
4. Add 10 µl of 20mg/ml Proteinase K (Invitrogen).

5. Incubate overnight at 55^{o}C with gentle shaking. Alternatively, perform 3 hour incubation at 55^{o}C with shaking, vortex the sample once per hour if performing 3 hour incubation. Make sure the ear snips is completely digested.

   # Master Mix = (NLS + EDTA + Proteinase K)

Day II

6. Add 1.5 µl of Rnase A solution (4mg/ml) to the nuclear lysate and mix by inverting the tube 2-5 times.
7. Incubate the mixture for 15-30 minutes at 37^{o}C.
8. Allow the sample to cool to room temperature (RT) for 5 minutes before proceeding.
9. Add 100 µl of Protein Precipitation solution to the room temperature (RT) sample and vortex vigorously at high speed for 20 sec.
10. Chill sample on ice for 5 minutes.
11. Centrifuge for 4 minutes at 13,000 rpm. The precipitated protein will form a white pellet.

12. Carefully remove the supernatant containing the DNA (leaving the protein pellet behind) and transfer it to a clean 1.5 ml labelled eppendrof tube.
13. Add 300 µl of room temperature (RT) isopropanol and mix the solution by inversion until the white thread like strand of DNA form a visible mass.
14. Centrifuge for 20 minutes at 13,000rpm at RT. The DNA will be visible as a small white pellet. Carefully remove the supernatant.
15. Add 300 µl of 70% RT ethanol and gently invert the tube several times to wash the genomic DNA.
16. Centrifuge for 20 minutes at 13,000 rpm at RT.
17. Carefully remove the ethanol using a Gilson pipette. The DNA pellet is very loose at this point and care must be used to avoid drawing the pellet into the pipette tip.
18. Invert the tube on clean absorbent paper and air dry the pellet for longer until all ethanol is removed.
19. Add 50 or 100 µl of H_2O (Nuclease free water) and incubate at 65^{o}C for 1 hour. Periodically mix the solution by gently flicking the tube.

Alternatively re-hydrate the genomic DNA overnight at room temperature or at 4^{o}C

20. Store the DNA at 2-8^{o}C.
21. Run a check out gel to determine quality and quantity of genomic DNA.

II.2.1.13. Screening of Genomic DNA

1. Dilute DNA sample(s) 1:10 (depending on original concentration.)
2. Add DNA to PCR tubes or plate (1µl per reaction).
3. Start the basic PCR program (Section 2.2.1.1) on the thermal cycler of use. Once the program is started, PAUSE it to allow the machine to heat up to 94-95°C.

4. Prepare the PCR master mix as described below:

Reaction Components	Volume
DNA Template	1.0µl
$MgCl_2$	1.5µl
10X PCR Buffer	1.5µl
dNTPs (2.5 mM each)	0.3µl
Forward Primer (C1sA_KAF_4)	1.5µl
Reverse Primer (C1sA_KAR_2)	1.5µl
Reverse Primer (Neo5_R_2)	1.5µl
dH2O	6.08µl
Taq Polymerase (5 units/µL)	0.12µl
Total volume	15µl

5. Add master mix to tubes containing DNA samples.
6. Begin PCR by putting tubes in the thermal cycler (once it has reached 94-95°C) and pressing PAUSE again, to allow the reaction to proceed.
7. Once completed, mix 15µl PCR products and 3 µl 6X loading dye to sample prior to gel analysis.

II.2.2. DNA and RNA based PCR Programmes

II.2.2.1. Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a method of amplifiying a specific nucleic acid target sequence present in a complex template to produce a large amount of specific DNA fragment of defined length and sequence in vitro (Saiki *et al.*, 1988).

II.2.2.1.1. Basic PCR

The basic PCR reaction takes place in vitro (inside eppendrof tube), where the primer strand is added from outside in the form of deoxyoligonucleotide and Taq polymerase enzyme is added to helping polymerization. Unlimited supply of amplified DNA is obtained by repeating the reaction, which is made possible by regular denaturation of freshly synthesized double stranded DNA molecules by heating it to 90-98^{o}C. At this high temperature the two strands separate. Taq polymerase is the DNA polymerase which is isolated from *Thermus aquaticus* growing in hot springs. The enzyme acts best at 72^{o}C and the denaturation temperature of 90^{o}C does not destroy its enzymatic activity.

In vitro amplification using polymerase chain reaction (PCR) was performed in a programmable thermal cycler. The PCR mix for reaction of 25µl contained the following reagents and make sure to prepare the master mix on ice.

Different components for the PCR mix

Reagents	Stock Concentration	Volume
Sterile H_2O	-----	13.3µl
PCR Buffer	10x	2.5µl
$MgCl_2$	25nm	2.5µl
dNTP mix	10nm	0.5µl
Primer (Forward)	5µM	2.5µl
Primer (Reverse)	5µM	2.5µl
Taq Polymerase	5U/µl	0.2µl
DNA Template	50ng/µl	1µl
Total		25µl

Temperature and cycling profile of standard PCR

Step	Temperature	Time (minutes)	Number of Cycles
Initial Denaturation	94 °C	5 min	1 cycle
Denaturation	94 °C	1 min	35 cycles
Annealing	55-68 °C	1 min	
Extension	72 °C	1 min	
Final Extension	72 °C	5 min	
Cooling Down	4 °C	∞	

II.2.2.1.2. Touchdown PCR

Touchdown Polymerase Chain Reaction is variation of standard PCR which avoids amplification of non specific sequences by forward and reverse primers. The annealing temperature of the first amplification is adjusted higher than the calculated melting temperature (Don R, 1991). The annealing temperature is lowered by 1-2 °C for each succeeding cycle. At some point temperature accurate for specific priming will be reached and amplification of the sequence will begin (Hecker K, 1996).

In touchdown PCR Extensor Hi Fidelity enzyme is used from AB gene instead of Taq DNA polymerase. The particular enzyme contains Thermoprime plus DNA polymerase with a proofreading activity. Therefore,

extensors can amplify DNA having four time's higher fidelity and double yield than Taq polymerase enzyme.

Different components for the PCR mix

Reagents	Stock Concentration	Volume
Sterile H_2O	-----	31.6µl
PCR Buffer	10x	5µl
dNTP mix	10nm	1µl
Primer (Forward)	5µM	5µl
Primer (Reverse)	5µM	5µl
DNA Template	50ng/µl	2µl
Extensor Hi Fidelity polymerase enzyme	5U/µl	0.4µl
Total		50µl

Temperature and cycling profile of standard PCR

Step	Temperature	Time (minutes)	Number of Cycles
Initial Denaturation	94 ^{o}C	1.30 min	1 cycle
Denaturation	94 ^{o}C	0.15 sec	15 cycles
Annealing	70 ^{o}C (-1 ^{o}C / cycle)	0.30 sec	
Extension	72 ^{o}C	0.30 sec	

Step	Temperature	Time (minutes)	Number of Cycles
Denaturation	94 °C	0.15 sec	25 cycles
Annealing	55 °C	0.30 sec	
Extension	72 °C	0.30 sec	
Final Extension	72 °C	5 min	
Cooling Down	4 °C	∞	

II.2.2.1.3. Gradient PCR

Gradient PCR is mostly done to determine the optimum annealing temperature of the primer to the DNA. This type of PCR involves setting up a temperature gradient across the thermo block, starting at 5ºC below the primer melting point (Tm) and continues increasing till it reaches 5ºC above the Tm. With the help of this technique, in a single PCR reaction, we can find the particular annealing temperature at which the primer amplifies the specific gene sequence.

II.2.2.2 Reverse Transcriptase PCR

Reverse transcription polymerase chain reaction (RT-PCR) is designed for the synthesis of cDNA from total RNA using the enzyme Superscript II ™ Reverse Transcriptase (Invitrogen). This enzyme works by eliminating RNase H activity that degrades mRNA during first strand reaction and helps

in obtaining full length cDNA. Conventionally RT-PCR involves two steps: the RT reaction and PCR amplification. Use of Oligo(dT) primers is a more specific method for priming first strand cDNA synthesis. In order to synthesis first strand cDNA for the experimental purposes the Invitrogen Superscript II ™ Reverse Transcriptase kit was used.

Protocol for RT-PCR using Oligo (dT) primers is as under

1. Briefly centrifuge all the reagents before starting the reaction and make sure you work on ice at all times.
2. Set up the RNA and primer mixture in a sterile 0.5ml PCR tube

Reagents	Volume taken
1µg RNA (along with dH_2O)	10.5µl (eg. 3.8µl RNA + 6.7µl H_2O)
Oligo(dT) (0.5µg/µl)	1.0µl
Total	11.5µl

3. Incubate the reaction mixture at 70^{o}C for 10 mins in PCR machine.
4. Prepare the master mix as described below. Add the reagents in the same order.

Reagents	Volume taken
PCR Buffer (10X)	2µl
MgCl2 (25mM)	2µl
DTT (0.1M)	2µl
dNTP mix (10nM)	1µl
Superscript II ™ Reverse Transcriptase enzyme	1µl
RNaseOUT (RNase inhibitor)	0.5µl
Total	8.5µl

Prewarm the master mix

5. Add the master mix to the reaction tube and incubate for 60 min at 45 ^{o}C.
6. Stop the reaction a 70 ^{o}C for 10 mins and then put the reaction tube on ice.
7. Digest with RNase H (add 1µl) at 37^{o}C for 30 min.
8. Make volume the up to 50µl by adding DEPC treated water and store at -20^{o}C for short term and -80^{o}C for long term storage.

II.2.2.3. Quantitative Real Time-PCR (qRT-PCR)

Quantitative Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real

time) as opposed to the endpoint detection. There are three general methods for the quantitative detection

1. DNA-binding agents (SYBR Green)
2. Hydrolysis probes (TaqMan, Beacons)
3. Hybridisation probes (Light Cycler)

II.2.2.3.1 Real-time Quantification

Real-time PCR was conducted by amplifying 1µl of cDNA with the SYBR Green Supermix. SYBR Green I fluorescence is extremely increased when it binds to double-stranded DNA. During the extension step, more and more SYBR Green I bind to the PCR product, resulting in large increase in the fluorescence. GAPDH (Glucose-6-phosphate dehydrogenase) is used as an endogenous control to normalize the amount of starting material in the tube. A cycling program usually consisting of 35-45 cycles.

Experimental Protocol

Program:	Denat			Type:	None	Cycles:	1
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2° Target Temp (°C)**	**Step Size (C°)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	95	900	20	0	0	0	None

Program:	cycling			Type:	Quantification	Cycles:	40
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2° Target Temp (°C)**	**Step Size (C°)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	95	15	20	0	0	0	None
2	70	30	20	58	0.8	0	None
3	72	15	20	0	0	0	Single

Program:	melt			Type:	Melting Curves	Cycles:	1
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2° Target Temp (°C)**	**Step Size (C°)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	96	2	20	0	0	0	None
2	65	10	1	0	0	0	None
3	96	0	0.1	0	0	0	Continuous

Program:	cool			Type:	None	Cycles:	1
Segment Number	**Temperature Target (°C)**	**Hold Time (sec)**	**Slope (C°/sec)**	**2° Target Temp (°C)**	**Step Size (C°)**	**Step Delay (Cycles)**	**Acquisition Mode**
1	40	30	20	0	0	0	None

Figure 2.6 Shows the quantification protocol for the amplification of cDNA

II.2.2.3.2 Optimization of Real Time PCR

II.2.2.3.2.1 Melting Curve Analysis

To carry out melting curve analysis, the temperature is increased very slowly from a low temperature (65°C) to a high temperature (95°C). Figure 2.7 shows a typical melting curve analysis. The fluorescence intensity is plotted against the temperature.

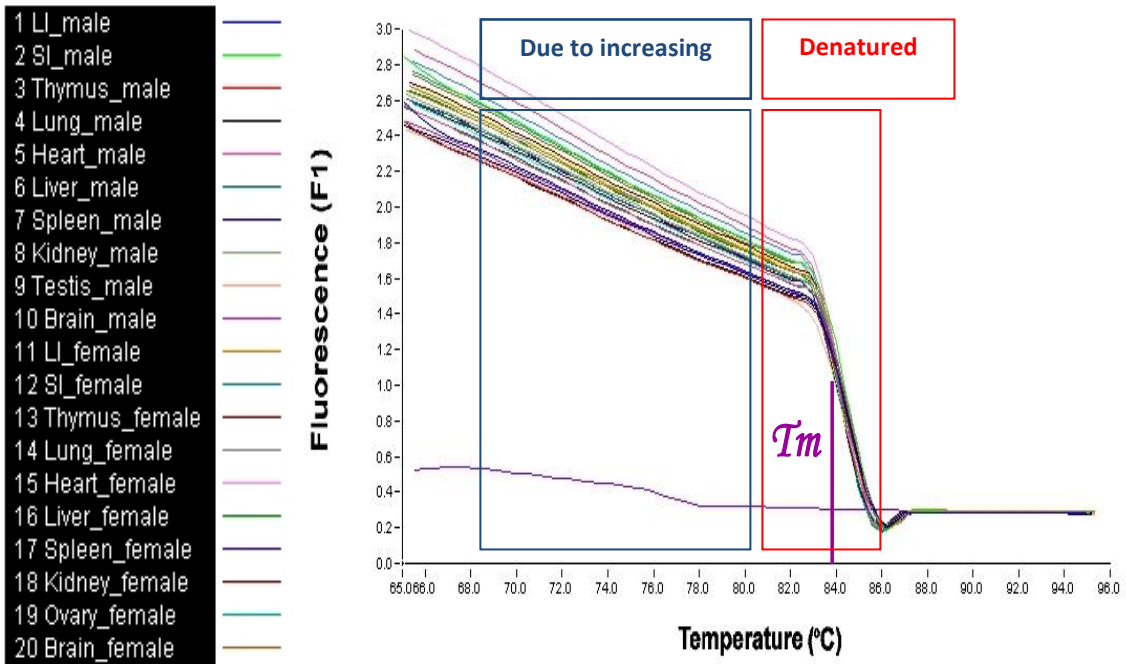

Figure 2.7 Shows a typical melting curve analysis

As shown in figure, the fluorescence decreases as the temperature increases. There are two distinct stages to the curve. The first shown in the blue

outline is due to increasing temperature, and not the melting of the dsDNA. The second part of the curve shown by the red outline is due to denaturation of the dsDNA and release of SGI. The Tm or the melting temperature indicated by the arrow is the point of inflection on the melting curve and is indicative of the temperature at which half of the DNA is double stranded and half is single stranded.

The light cycler software also plots the negative derivative of the rate of change of fluorescence vs. temperature (-dI/dT) (Figure 2.8). In the figure, the Tm is easily identified by the single peak at 84^{o}C.

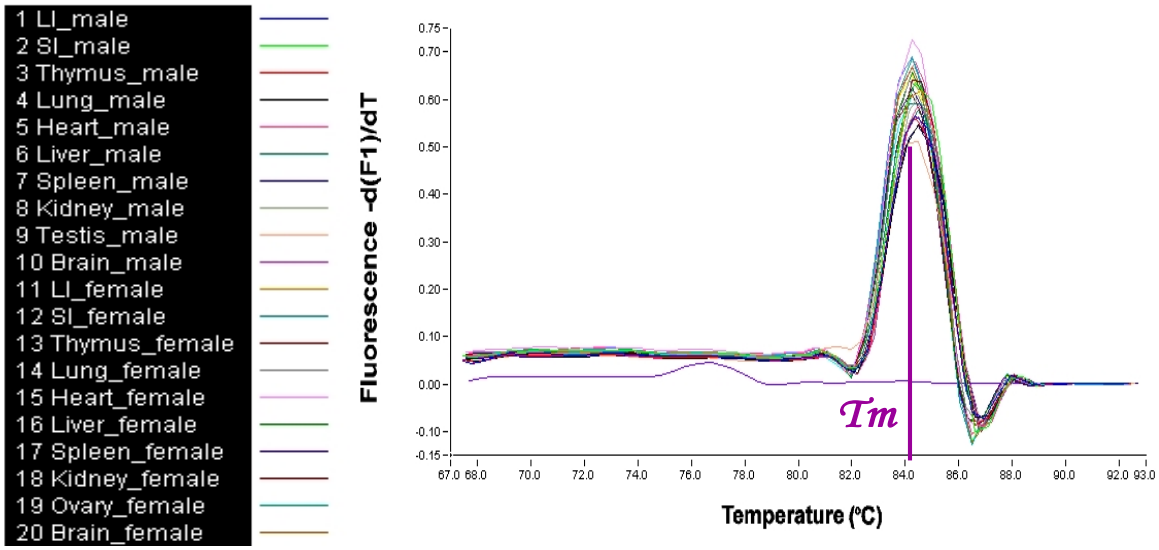

Figure 2.8 Shows the negative derivative of the rate of change of fluorescence vs. temperature (-dI/dT)

II.2.3. RNA based methods

II.2.3.1. RNA isolation from Mouse organs

In order to study the expression pattern and the analysis of the location and cell types expressing C1sA and C1sB in mouse tissue. Total RNA was extracted from different organs like large intestine, small intestine, thymus, lung, heart, spleen, kidney, testis, brain and ovary. The Trizol reagent is used for the isolation of total RNA from tissues. Trizol reagent is a mono-phasic solution of phenol and guanidine isothiocyanate, which helps inactivate RNase. As soon as the organs were dissected from mouse they were placed in a 15ml Falcon tube containing ice cold Trizol reagent.

Steps involved in RNA isolation are

1. **Tissue homogenization:** Tissue samples were homogenised by using homogenizer in 5ml of Trizole reagent. Trizol reagent maintains the integrity of the RNA while distracting cells and dissolving cell components during homogenization. After homogenization centrifuge the homogenate for 15 min at 12000x g and collect the supernatant because it contains the RNA.

2. **Phase Separation:** incubate the homogenized samples for 15 min at 30^{o}C to allow the full dissociation of nucleoproteins. Add 0.2 ml of chloroform per 1ml of Trizol reagent, mix it and centrifuge for 15 min at 12000x g. After centrigugation the homogenate separates into three layers, the lower layer is phenol, middle layer chloroform and a colourless upper aqueous phase contains the RNA.
3. **RNA Precipitation:** Precipitate the RNA from aqueous layer by mixing with 2.5ml of isopropyl alcohol. Incubate for 15 min at 30^{o}C and then centrifuge for 15 min at 12000x g. Discard the supernatant and collect the pellet (which is often invisible).
4. **RNA Wash:** Wash the pellet with 75% ethanol and centrifuge at 7500x g for 5mins.
5. **Dissolving the RNA:** Dry the RNA pellet and dissolve it into DEPC treated water and calculate RNA purity (A_{260}/A_{280} = between 1.6 & 1.8) and RNA concentration (OD_{260} X 40 X Dilution factor = Yµg/ul).

II.2.3.2. Treatment of RNA samples with DNase I

1. Digest 10µg of RNA sample with DNase I and incubate at 37^{o}C for 30 mins

Reagents	Volume
RNA	Xµl
dH_2O (DEPC treated)	Yµl
DNase I	2µl
DNaseI Buffer	20µl
Total	200µl

2. After digestion add 200µl of phenol/ chloroform/isoamyl alcohol and vortex it.
3. Centrifuge for 5 mins at full speed. Remove the upper aqueous layer to a clean eppendrof tube.
4. Ethanol precipitate with 2.5 volume of 100% ethanol and 1/10 volume of 3M NaAcetate and mix them. Store at -20^{o}C for overnight or at least 30 mins.
5. Spin down the mixture at full speed for 10 mins.
6. Discard the supernatant and dry the pellet. Resuspend the RNA pellet in 25µl of DEPC treated water.
7. Calculate the RNA purity (A_{260}/A_{280} = between 1.6 & 1.8) and RNA concentration (OD_{260} X 40 X Dilution factor = Yµg/ul).

II.2.3.3. *In Situ* hybridisation

In situ hybridisation, as the name suggests, is a method of localizing and detecting specific mRNA sequences in morphologically preserved tissues sections or cell preparations by hybridising the complementary strand of a nucleotide probe to the sequence of interest. For *in situ* hybridisation the probe can be labelled in two ways

1. Radiolabelled probes (e.g., [35S] UTP labelled probe) are localized and quantitated in the tissue using autoradiography.
2. Non radiolabelled probes, antigen-labeled bases (e.g., digoxigenin), are localized and quantitated in the tissue.

II.2.3.3.1 In situ hybridisation using ^{35}S-labelled riboprobe

II.2.3.3.1.1 Prehybridisation

This method fixes the tissue with paraformaldehyde, then permeablises it to allow the probe access to the RNA by using Triton-100 and finally acetylates any free-SH groups in the sample to prevent formation of disulphide bridges with the ^{35}S-labelled riboprobe.

Protocol for hybridization is described below.

1. Remove sections from -80^{o}C and allow to air dry.

2. Fix in *4% paraformaldehde/PBS* (Appenxix 1) for 1 hour with gentle shaking at room temperature.
3. Wash in PBS three times with gentle stirring for 10 mins.
4. Place slides in 0.4% Triton-100/PBS solution for 10 mins with constant stirring.
5. Wash in PBS for 10 mins with stirring and then wash in distilled water for 1 min.
6. Incubate slides in 1litre freshly prepared *TEA buffer* (Appendix1) and stir for 1 min and add 2.5ml acetic anhydride to the TEA buffer while stirring and incubate for 10 mins.
7. Wash in PBS for 10 mins with stirring and then wash in distilled water for 1 min.
8. Dehydrate sections by rinsing them in 50% alcohol and then with 70% alcohol, allow them to air dry. Store at -20^{o}C or hybridise the sections

II.2.3.3.1.2 Probe Labelling

^{35}S-labelled riboprobe are generated by *in vitro* transcription using a linearized plasmid containing the template cDNA and a viral RNA

polymerase. The orientation of the template in the plasmid and the restriction map of the vector and the insert should be known.

Protocol for probe labelling is described below

1. Preparation of the template: Take 10-20µg of maxiprep and digest with an enzyme that cuts only once in the multiple cloning site of the vector. To generate a sense probe cut at the 3' end of the insert and for anti-sense probe cut at the 5' end of the insert. Digest the template completely and then purify the template using nucleotide removal kit (Qiagen).
2. Dry down the radioactivity: The concentration of the radiolabeled ^{35}S-UTP is the limiting factor in the reaction. It must exceed 13µM for the reaction to proceed efficiently. Practically this means that at least 13µl of radioactivity are needed in a 10µl reaction volume. The solution is to dry the radioactive UTP α [^{35}S] solution in a Speed Vac, and then add the other components. Aliquot 16µl for full reaction (anti-sense) and 8µl for a half reaction (sense) into a clean 0.5 ml eppendrof tube and dry in the speed Vac for about 20min.

3. Add the following to the dried radioactivity, starting with the template DNA.

Components	Full reaction (α-Sense) (16 µl ^{35}S UTP)	Half reaction (Sense) (8 µl ^{35}S UTP)
Template DNA	4.5µl (700-1000ng)	2.25 µl (350-500ng)
5x Reaction Buffer (supplied with the enzyme)	2µl	1µl
0.1 M DTT	1µl	0.5µl
5 mM GTP/CTP/ATP mix	1µl	0.5µl
RNAse inhibitor	0.5µl	0.25µl
RNA Polymerase	1µl	0.5µl
Total Volume	10µl	5µl

4. Incubate at 37^{o}C for 90 mins.
5. Add 1µl of DNAseI (RNAse-free) and incubate for a further 20 mins.
6. For probes of 400bp or less, add distilled water to an end volume of 50µl and place on ice.
7. Before adding the sample take a 1µl sample of each probe and add to a scintillation vial containing 2ml of scintillation fluid (Perkin Elmer).

8. Purify the probe to remove unincorporated 35S-labelled UTP by using a Sephadex G-50 spin column (Amersham Pharmacia). After purification take 1µl of a sample and label it as with the after sample.
9. Using the 14C programme on the scintillation counter, count the before samples and after samples. Calculate the % incorporation (after/before x 100). Calculate the total activity of the purified probe (after x 50).
10. Dilute the probe to 50 million dmp/ml (50,000 dpm/µl) in hybridization buffer. Add 1M DTT to an end concentration of 10 to 20 mM. Vortex vigorously and store at -20^{o}C until required.

II.2.3.3.1.3 Hybridisation and Washing

1. Remove the pre hybridised sections and the labelled probe from -20^{o}C. Pre warm the hybridisation oven to 68^{o}C. Prepare the hybridisation chamber by filling with 50% formamide.
2. Pipette the 1µl of probe on to a cover slip. Gently place the slide over cover slip. Place the slide with cover slip side on the top in chamber and incubate the slides overnight at 68^{o}C.

3. Prepare 2 liters of 2 x SSC. Remove the hybridisation chamber from the hybridisation oven. Take a slid and dip it in 2 x SSC solution and slide the cover slip to the corner by using forceps and dispose in the radioactive waste.
4. Place *RNAse buffer* (Appendix 1) in a 37^{o}C water bath to pre warm it. Carry on with the following washing steps with constant washing.

Washing Step	Time
Wash in 2 x SSC	20 min
Wash in 1 x SSC	20 min
Wash in pre-warmed RNAse buffer	30 min
Wash in 1 x SSC	20 min
Wash in 0.5 x SSC	20 min
Wash in 0.2 x SSC	20 min
Wash in 0.2 x SSC, 60oC	60 min
Wash in 0.2 x SSC	15 min
Wash in dH_2O	15 min
Wash in 50% ethanol	1 min
Wash in 70% ethanol	1 min

5. Allow the sections to air dry, then place in an autoradiography cassette and expose to Kodak BioMax MR film.

II.2.3.3.2 *In situ* hybridisation using the DIG-labelled riboprobe

The protocol for DIG (Digoxigenin) labelled riboprobes is similar to the probes labelled with ^{35}S. The prehybridisation (Section II.2.3.3.1.1**)** and hybridisation/washing steps (Section II.2.3.3.1.3**)** are similar so the probe labelling and detection steps shown here in brief.

II.2.3.3.2.1 Probe Labelling

DIG-labelled riboprobes are generated by *in vitro* transcription using a linearized plasmid containing the template cDNA and a viral RNA polymerase. The orientation of the template in the plasmid and the restriction map of the vector and the insert should be known.

Protocol for probe labelling is described below

1. Preparation of the template: Take 10-20µg of the maxiprep and digest with an enzyme that cuts only once in the multiple cloning site of the vector. To generate a sense probe cut at the 3’ end of the insert and for the anti-sense probe cut at the 5’ end of the insert. Digest the template

completely and then purify the template using the nucleotide removal kit (Qiagen).

2. Add the following components, starting with the template DNA.

Components	Volume
Template DNA	6.6µl (1µg)
5 x Reaction Buffer	2 µl
0.1 M DTT	2 µl
DIG RNA Mix	2 µl
RNAse inhibitor	2 µl
RNA Polymerase	2 µl
Distilled H_2O	4.4µl
Total	20µl

3. Incubate at 37^{o}C for 90 mins to 2 hours.
4. Add 1µl of DNAseI (RNAse-free) and incubate for a further 20 mins and add 29µl of dH_2O.
5. Purify the probe by using Sephadex G-50 spin column (Amersham Pharmacia).
6. Check the optical density of the samples at wavelength of 260.

7. Add 1M DTT to an end concentration of 10 to 20 mM. Vortex vigorously and store at -20^{o}C until required.

II.2.3.3.2.2 Immunological Detection of DIG-Labelled probe

1. Circle the sections on the slide with PAP-Pen.
2. Block the slides containing tissue sections with 0.05% Triton X-100 and 2% Donkey serum (acts as blocking serum) in *Buffer 1*(Appendix1). Put the slides in a chamber containing water and incubate for 30 mins at 37^{o}C.
3. Wash the slides 3 times in Buffer 1 for 10 mins.
4. Prepare the solution containing anti-DIG-AP (alkaline phosphates - conjugated anti-digoxigenin antibody diluted 1:1000 in Buffer 1) in 0.3% Triton X-100 and 1% Donkey serum in Buffer1.
5. Dry the slides with the help of vaccum pump incubate the slides at 4^{o}C for 16 hours or overnight in a solution containing the anti-DIG-AP antibody (see step 4).
6. Next morning wash the slides in Buffer 1 twice for 10 mins and then wash in Buffer 2 for 10 mins.

7. Prepare the chromogen solution containing 45µl of NBT-Dimethyl Formamide, 35 µl of BCIP-Dimethyl Formamide and 2.4mg Levamisol in *Buffer 2* (Appendix 1). Incubate the slides for 30 mins to 1 hour.
8. Stop the reaction in TE buffer and wash with water.
9. Immerse slides in 50% and 70% ethanol for a while and dry the slides.

II.2.4 Cell Culture techniques

II.2.4.1 Establishment of Sf9 insect cell line

II.2.4.1.1 Initiating a cell culture from frozen stock

The first step was to establish a suitable culture system for insect cells that were to be transfected with the plasmid DNA. The cells are stored frozen in liquid nitrogen and then removed from the liquid nitrogen then they were transported in, and thawed in the medium that they were to be grown in. The following are the required steps for thawing the cells and establishing the insect cell line.

1. Remove the vials of cells from the liquid nitrogen and place in a water bath at 37^{o}C. Apply gentle agitation and thaw the cells quickly until

they are almost thawed, then remove the cells from the water bath, as leaving the cells at this temperature until after they have thawed will cause them to die.

2. Straight away, treat the outside of the cell vial with 70% ethanol to decontaminate it, and then dry the vial. Put the vial on ice.
3. Prepare a 25cm^2 flask by coating the adherent surface with 4ml of complete *TNM-FH insect medium* (Appendix1).
4. Transfer 1ml of the cell suspension into the 4ml of medium in the flask.
5. Put the flask into an incubator at 27^{o}C for about 30-45 minutes. This will allow the cells to attach to the adherent surface.
6. When the cells have attached, the medium needs to be removed gently. This serves two purposes, one is to remove from the medium the DMSO (Dimethyl Sulphoxide), a cryoprotectant added to the freezing medium to prevent the death of cells, and the other is to remove all cellular debris or unhealthy cells that don't adhere to the surface of the flask.

7. The removed medium should then be replaced by 5ml of fresh medium.
8. The medium should be changed again after 24 hours, and this should result in the viability of revived cell cultures being greater than 70%. To change the medium of an adherent cell culture, the flask should be tipped so that all the medium flows to one corner of the flask, and then the medium should be drawn out of the flask using a pipette. Great care needs to be taken to avoid touching the cell monolayer. The medium should then be replaced by pipetting fresh medium down the side of the flask opposite the cell monolayer so that the medium collects at the bottom, then the flask is placed back the right way up. The cells should be kept in incubation until a confluent monolayer is formed, which is when the cells have grown to cover the base of the flask completely, when they should be subcultured.

II.2.4.1.2 Subculturing

Subculturing, also known as passaging, of cell cultures involves diluting cells back to a density that allows them to maintain log phase growth. Adherent cell cultures should be passaged at confluency, and are usually

passaged down to a dilution where the ratio of the volume of cells to the final volume of the medium is 1:5 e.g. 2ml of medium containing cells in 10ml of the final volume of medium.

The method used to remove the adherent cells from the flask is known as sloughing.

1. Firstly, remove all but 5ml of the medium from the flask.
2. Tilt the flask away from you so that the remaining medium flows to one corner of the flask.
3. Using a pipette, draw up the remaining medium and slowly stream it against the cell monolayer, starting at the corner that the medium is in, and gently streaming the medium from side to side working up and away from that corner. As the cells at the bottom start to loosen, the cells stuck above them will be easier to remove.
4. This method of removing the cell monolayer with the least mechanical force being used against the cells leads to a greater viability.

II.2.4.2 Co-transfection of Sf9 cells with Bac-N-Blue ™ DNA and plasmid DNA

II.2.4.2.1 Preparation of cells

Before transfection, the Sf9 cells from the cell culture need to be prepared for the reaction. For all transfections, cells at log phase need to be used, and they must have greater than 98% viability.

1. Seed 2 x 10^6 Sf9 cells in complete TNM-FH medium in a 30mm dish, then rock the dish from side to side to allow the cells to evenly distribute themselves around the dish.
2. Leave the cells to completely attach and form a monolayer on the bottom of the dish. This will take at least 15 minutes.
3. Ensure that the cells have attached by viewing them under a microscope.

II.2.4.2.2 Transfection Procedure

The transfection procedure allows the previously grown Sf9 cells to be infected with the recombinant transfer DNA.

1. Take one of the 1.5ml microcentrifuge tubes that contain 10µl of Bac-N-Blue ™ DNA, and centrifuge it.

2. Add to the tube:

   2µl of recombinant transfer plasmid

   500µl of Grace's Insect Medium without supplements or FBS

   10µl of Cellfectin ® Reagent

Cellfectin ® Reagent allows the recombinant DNA to penetrate the cell membrane of the Sf9 cells. This reagent should be mixed well before use and should always be added last.

3. The transfection mixture is mixed for 10 seconds, then incubated for 15 minutes at room temperature.
4. While the mixture is being incubated, carefully remove the medium from the dishes of cells without disturbing the cell monolayer and then wash the cells using 2ml of Grace's Insect Medium without supplements or FBS. This serves to remove small amounts of serum that can affect the performance of the liposome from the Cellfectin ® Reagent that is critical in the transfection.
5. After it has been washed, the medium again needs to be removed from the cell monolayer, and then the entire transfection mix is added to the dish dropwise into the dish containing the cell monolayer, and the

drops should be distributed evenly to avoid disrupting the cell monolayer.

6. The dishes are then incubated for 4 hours at room temperature on a side-to-side rocker, at a speed of 2 side-to-side motions a minute.
7. When the four hour incubation is over, add 1ml of complete TNM-FH medium to each dish, then place the dishes in a sealed plastic bag and incubate for 72 hours in a 27^{o}C incubator.

II.2.4.2.3. Post Transfection procedure

After the transfection certain steps must be taken to ensure that the transfection has been successful. After the 72 hour period of incubation, budded virus will start to be released into the medium, and this can be tested for recombinant plaques containing infected insect cells. Despite this, the cells may not show signs of infection until 4 or 5 days after they were first incubated, so even if signs of infection are absent at first, the cells should be kept in incubation and inspected daily to confirm any signs of transfection. This is the procedure to test for recombinant plaques:

1. Take 2ml of medium from each transfection dish using a pipette and transfer to a sterile 15ml snap-cap polypropylene tube. This viral stock

can be stored at 4^{o}C until it is needed, and it will be used to identify recombinant virus by plaque assay.

2. 3ml of fresh complete TNM-FH medium is then added to the transfected cells and they are incubated at 27^{o}C for another 48 hours.
3. Cells should be checked between 4-7 days after transfection under a microscope for visual signs of transfection. Signs of viral infection are classed as early, late or very late, and are all identified differently.

Early: Increased cell diameter, often by between 25-50%.

Increased size of cell nuclei, which may appear to fill the cells.

Late: Cells appear to stop growing compared to a cells only control.

The cells start to appear granular. Viral occlusions, where refractive Crystals appear in the cell nucleus. Detachment, where cells loose adherence to the dish.

Very late: Lysis, where cells fill with the virus, then they die and burst, leaving gaps in the monolayer.

4. When a successful transfection has been confirmed, the recombinant virus must be purified by plaque assay.

II.2.4.3 Purification of recombinant virus by plaque assay

In the supernatant from the transfection dish, there will be an active viable virus that will be 90% recombinant, and this needs to be purified to remove any uncut viral DNA or non-homologous recombinant DNA that doesn't contain the required gene. If the recombinant virus has not been purified, then this can result in dilution of the recombinant virus over time. The purification is done by infecting cells with dilutions of the viral stock taken at the beginning of the post transfection procedure, and then isolating plaques from an agarose overlay.

II.2.4.3.1 Plaque Formation

To get the best plaques forming, there must be a good quality cell monolayer, the cells must all be in log phase growth and be evenly distributed around the dish. Viral plaques can be seen as a place in the cell monolayer where that is surrounded by infected cells, and these infected cells can be identified due to their generally larger diameter, larger nuclei and some signs of cell lysis. These are the stages that lead to plaque formation and figure 6 explains the plaque assay:

a) One infectious virus infects one cell.

b) The cell dies and lyses, releasing virons that infect any adjacent cells.

c) These cells then lyse, which results in the release of more virons, which in turn infect more cells, moving away from the original cell.

d) This process continues, with the virus moving outwards and leaving a space in the cell monolayer where all the cell debris from the lysed cells collects.

e) If β-galactosidase is added to the medium, then a blue or blue-green haze will be produced when cells lyse, making plaques easier to identify

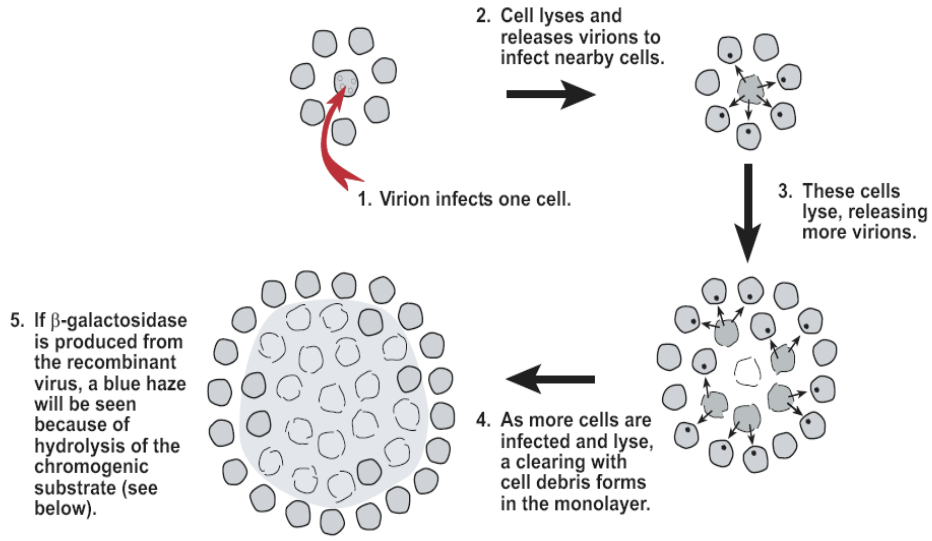

Figure 2.9 Stages involved in Plaque Assay

II. 2.4.3.2. Preparation of cells for plaque assay

Plaque formation will only be identified when a good quality cell monolayer has been formed. A control containing untransfected cells is useful, as it helps assess cell viability and monolayer quality.

1. Sf9 cells at log phase growth in complete TNM-FH medium are seeded in 100mm plates that have been wetted with TNM-FH medium at a density of 5×10^6 cells per 100mm plate. Between 3 and 8 plates are prepared for each viral dilution that will be used.
2. Plates must be rocked on a side-to-side rocking platform at room temperature for 10 minutes at 8 rocking motions per minute. This will distribute the cells evenly around the plates. Orbital shakers must not be used as this will mean the cells will be distributed more around the edges instead of evenly across the plate.
3. After the 10 minutes the rocker is stopped, and the plates need to be left to stand for at least 10 minutes to produce an even monolayer. The rocker must be stopped when it is level so that the monolayer is even.

4. The plates are then removed from the rocker and left for at least 30 minutes at room temperature before use so that the cells have plenty of time to attach.
5. To make sure that the cells have attached, they are examined under a microscope.

II. 2.4.3.3. Diluting the Virus

The viral stock taken after transfection now needs to be diluted before it is added to the cells. The viral stock must first be vortexed vigorously, and then diluted in complete TNM-FH. About 1ml of diluted virus is needed for each plate. The dilutions usually used are 10^{-2}, 10^{-3} and 10^{-4}. To ensure an evenly suspended virus, the viral stock is vortexed again before each dilution is prepared. After the dilutions are made, the viral stock can be returned to storage at 4^{o}C for future use.

II. 2.4.3.4. Viral Infection and Agarose Overlay

The purpose of this procedure is to infect new Sf9 cells with the viral dilutions and then cover the cell monolayer with an agarose overlay. This procedure has to be done with the utmost care to avoid disrupting the cell monolayer.

1. Before the plaque assay is performed, the tissue culture hood that it will be completed under needs to be prepared, along with all the chemicals that are needed for the assay. First, a water bath is decontaminated with alcohol and set at 47^{o}C under the hood.
2. A 100ml bottle containing 50ml of complete TNM-FH medium is kept in the water bath along with an agarose-TNM-FH solution.
3. A 15ml falcon tube containing 5ml of complete TNM-FH medium is also prepared for each plate that will be assayed. This will contain any chromogenic substrate if it is required.
4. A large supply of 5ml and 10ml pipettes should also be ready for use.
5. Only one viral dilution should be done at a time. All the plates that have been set aside for one viral dilution were taken and remove all but 2ml of the medium they are in.
6. Next, 1ml of the corresponding viral dilution is added to the plate drop wise using a sterile pipette, with great care taken not to disturb the cell monolayer.
7. The plates are then incubated at room temperature on a rocking platform at 2 side-to-side motions per minute for 1 hour. The first 3

steps are then repeated for each viral dilution, so care should be taken to ensure all plates are incubated for the same length of time.

8. While the plates are being incubated, the water bath is checked to ensure it is at 47^{o}C. An agarose solution that is too hot will kill the cells, but if it is too cold it will clump and will be difficult to pour into the plate, and could disrupt the monolayer.
9. When the first set of plates have finished the hour long incubation period, they have all the medium completely removed using a sterile pipette, by tipping the plate and drawing the medium from the edge of the plate.
10. Quickly take 5ml of the agarose-medium solution at 47^{o}C and add to one of the tubes containing 5ml of medium at room temperature. The medium is drawn up repeatedly to mix it.
11. The entire mix is then drawn into the pipette and gently streamed against the side edge of the plate, without actually touching the edge with the pipette, so that the mixture runs down the side edge of the plate and across the plate, covering the cell monolayer.

12. The plates must then be left and not touched until the agarose overlay has set.
13. This procedure is repeated with all the other dilutions as they finish the incubation period, but don't move any of the plates until the agarose layer has set.
14. The plates are then sealed in a plastic container with paper towels dampened in EDTA that prevents the plates from drying out, and also prevents the formation of mildew and bacteria on the towels.
15. The container is opened if condensation is formed, as this can damage the cell monolayer.
16. The plates are then incubated at 27^{o}C for 5 or 6 days, or until well-formed plaques have appeared.

II.2.4.4. Verifying recombinant plaques by PCR

Polymerase Chain Reaction (PCR) makes it possible to confirm that any recombinant plaque from the transfection of the Sf9 cells is a pure, recombinant plaque. Through a series of temperature cycles, a thermal cycler replicates a certain section of DNA defined by the primers added to the reaction. As the length of the gene that the cells have been transfected with

is already known, then use gel electrophoresis to ensure that the recombinant DNA is from a pure recombinant plaque and that there is no contamination from wild-type viral DNA.

II.2.4.4.1 Growth of recombinant Virus

Before PCR, the recombinant virus must be extracted from the plaques in the agarose plates and grown in preparation for PCR. This will also allow for any pure recombinant plaques found to be identified and used to make a high-titre stock.

1. The extracted plaques are grown for PCR in 12-well microtitre plates, so usually extractions are taken from ten different recombinant plaques, and the other two wells are controls with wild-type plaques in one and a cells only control in the other. These will also undergo PCR to ensure that the recombinant plaques contain the required gene. Each well of the plate is pre-wet with 2ml of complete TNM-FH medium.
2. 5×10^5 log-phase cells are seeded into each well, and the total volume of each well shouldn't be greater than 3ml.
3. Using a sterile pipette, the agarose layer is penetrated above the plaque and the monolayer containing the plaque is removed.

4. The agarose plug with the plaque is then added to one of the wells in the plate.
5. These steps are then repeated until 10 of the 12 wells are infected. To one of the others, a wild-type plaque is added to the cells, and the other well is left as a cells only control.
6. The plate is then sealed with parafilm and incubated for three days at 27^{o}C.
7. After the three days, the wells are all viewed under a microscope for evidence of infection. Infected cells will appear swollen. Any wells that had been infected with recombinant plaques that contain occlusion bodies are not used for PCR.
8. With all the wells that are going to be used for PCR, the contents of the well are pipetted up and down to loosen the cells in the plate. Then 0.75ml of the medium from each well is removed and put in a microcentrifuge tube, and this is used for PCR (Section II.2.2.1.).
9. The microtitre plates are then returned to incubation until all the cells have lysed, and then the medium is removed and stored at 4^{o}C. One of these will later be used to generate a high titre stock.

II.2.4.5. Generating a high-titre stock

Now that a recombinant virus containing the gene of interest has been found, the cells from the well of the microtitre plate in which the pure recombinant virus was located are harvested. This is the P-1 viral stock. From this a large scale, high titre stock with a known titre can be created and this can be used for protein expression studies.

1. Firstly, 2 x 10^6 log-phase Sf9 cells in 5ml of complete TNM-FH medium are seeded in 25cm^2 flasks.
2. 20µl of the P-1 viral stock is added to each flask, and then they are incubated at 27^oC until all the cells have lysed completely. This usually takes between 5 and 10 days.
3. After the cells have lysed completely, they are removed from the flasks along with the entire medium. This small scale, high titre stock is the P-2 viral stock. From this, 1ml is put in long term storage at –80^oC, and 4ml is kept as a reserve stock at 4^oC.
4. The remaining 5ml of the P-2 viral stock is added to a 500ml suspension culture of Sf9 cells and mixed for 5 minutes.

5. 5ml of this suspension is taken and added to a new 25cm^2 flask, and this is used to monitor the infection process.
6. The 500ml suspension culture is incubated at 27^{o}C with constant stirring for about 7-10 days. Using the 25cm^2 flask, the progress of the infection can be monitored to discover what proportion of the cells has already been lysed. When more than 90% of the cells have undergone lysis, then the cells are harvested.
7. To harvest the cells, the culture is pelleted at 1000 x g for 20 minutes, which causes the cell debris of the lysed cells to collect at the bottom of the container. Then the supernatant is removed and put in a fresh sterile container. This supernatant is the P-3 viral stock, which is both large scale and high titre, and needs to be stored at 4^{o}C. This viral stock is suitable for use in expression studies.

II.2.5. Protein Based Methods

II.2.5.1. Sodium Dodecyl Sulfate Polyacrylamide Gel electrophoresis (SDS-PAGE)

The main function of SDS-PAGE is to assess proteins according to their molecular size under reducing and non reducing conditions. Protein samples

to be separated by SDS-PAGE gel electrophoresis are incubated at 95^{o}C to denature protein in the presence of sodium dodecyl sulfate (SDS), an anionic detergent that can break up hydrophobic molecules but also has a negative charge (sulfate) attached to it, and a reducing agent (DTT or 2-mercaptoethanol). The gel comprises of two different layers, the top one known as stacking gel (pH 6.8) and the lower one known as resolving gel (pH 8.8).

The protocol for SDS-PAGE gel is taken from Sambrook *et al*, 1989.

1. Clean the glass plates and sample with detergent first and then with distilled water and dry them with tissue paper. Assemble the apparatus for gel preparation as described by the manufacturer.
2. Prepare 12 % resolving gel (as shown in table below) and fill the gel mould up to half and overlay the resolving gel with n-butanol. Let the gel set for 30 mins.
3. Once the gel polymerize pour off the n-butanol layer and dry it with whatman paper and let air dry for 2 mins.
4. In the meantime prepare 12% stacking gel (as shown in table below) and fill the mould until top and place comb avoid air bubbles.

5. Arrange samples to be loaded on the gel and denature them at 95^{o}C by adding *5x SDS loading buffer* (Appendix 1).
6. Fill the gel tank with *Tris-glycine buffer* (Appendix1) and place the gels in the tank and load the samples and the protein marker by removing the comb.
7. Run the gel at 120 V for 1 hour or until the samples runs to the bottom of the gel. Dismantle the gel and proceed with further immune assays (II 2.6.) to visualize the gel.

Table 2.3 Solutions for preparing 12% Resolving and Stacking gel for SDS-PAGE

Components	Stacking Gel Solution Volume (15ml)	Resolving gel Solution Volume (5ml)
Distilled Water	4.9 ml	3.4 ml
30% Acrylamide Mix	6.0 ml	0.83 ml
1.5 M Tris (pH 8.8)	3.8 ml	-------
1.0 M Tris (pH 6.8)	-------	0.63 ml
10% SDS	0.15 ml	0.05 ml
10% APS (Ammonium persulphate)	0.15 ml	0.05 ml
TEMED	0.006 ml	0.005 ml

II.2.5.2. Immunoassays

II.2.5.2.1 Western Blotting

Western blotting is the transfer of proteins after they are run on SDS-PAGE gel onto a nitrocellulose membrane for further detection by immunoblotting (Section II 2.5.2). The transfer is induced by electric voltage and the proteins move out towards a membrane.

The protocol for western blotting is described below.

1. As the SDS-PAGE gel (Section II 2.5.1) is close to finishing point.
2. Cut the nitrocellulose membrane (0.2µm pore size) and two sheets of 3MM Whatman filter paper to the size of the gel (7 x 8.5 cm) to be blotted. Prepare 1liter of *PAGE Western transfer buffer* (Appendix 1).
3. Once the gel is run, dismantle the electrophoresis assembly and take the gel out.
4. Pour the transfer buffer into a plastic tray and soak nitrocellulose membrane, filter paper and scotch brite pads before use.
5. Place the transfer cassette in a plastic tray with the black side facing towards bottom. Assemble the gel, membrane, filter paper and the pads in form of sandwich as shown in the figure 2.7. First place the soaked pad on the black side of cassette and then put the filter paper followed by the gel (facing the protein side of the gel on top) then

place the nitrocellulose membrane. Try to avoid any air bubbles and then again place the second filter paper and the pad.

6. Place the sandwich cassette inside electrode assembly and place the assembly in transblot tank such that the nitrocellulose membrane faces the black side of assembly means towards the anode. Place the ice block inside the transblot tank.
7. Fill the transblot tank with the transfer buffer and attach a power supply and run for 1hour at 250mA.

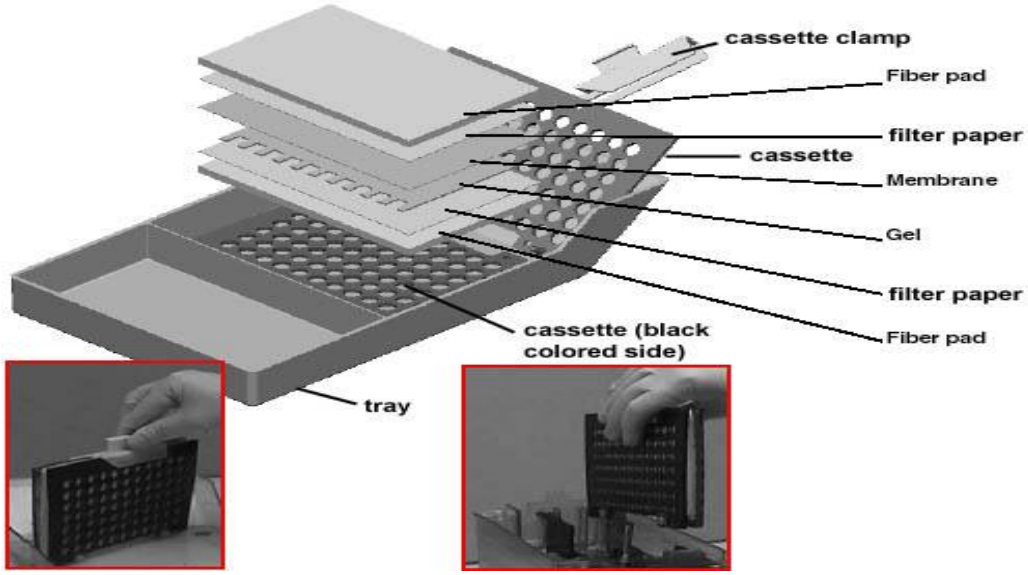

Figure 2.10 Assembly of the gel, membrane, filter paper and sponge pads

II.2.5.3. Immunoblotting

Immunoblotting involves the identification of specific protein on nitrocellulose membrane by using antibodies. Primary and secondary antibodies are used for detection.

Primary antibody is allowed to bind to the specific site on the protein. Secondary antibody, which can recognize the primary antibody, is used after several washes. The commonly used secondary antibodies are horse radish peroxidase (HRP) and alkaline phosphate (AP).

The protocol for immunoblotting is described under

1. Remove the nitrocellulose membrane from the sandwitch cassette once the run is finished.
2. Place the membrane in blocking solution (PBS containing 5% semi skimmed milk powder) and shake in a circular motion at room temperature for at least 30 mins.
3. Discard the blocking solution and incubate the membrane in PBS/5% semi skimmed milk solution in which primary antibody is diluted. Incubate the membrane for 2 hours at room temperature or overnight at 4^0C with gentle shaking.

4. Wash the membrane 3 times for 10 mins in PBS solution containing 0.05% Tween 20.
5. Add secondary antibody which is diluted in PBS/ 5% semi skimmed milk solution for 1 hour with gentle shaking.
6. Then wash the membrane with PBS/ 0.05% Tween 20 three times with shaking at room temperature for 10 mins. Then proceed to immunodetection.

II.2.5.4 Immunodetection

The commonly used method for immunodetection identification of protein is enhanced chemiluminescence (ECL).

1. Place the membrane on top of the sheet of cling film with protein side facing up.
2. Mix the ECL detection reagent A and B in same ratio i.e 1:1(Amersham).
3. Incubate the membrane with the reagents for one min and then quickly drain off the excess reagent by touching the tissue paper.

4. Prepare the cassette with cling film and place the membrane and then tap the membrane along with cling film to the cassette to avoid movement.
5. Place a Koadk X-ray film on top of the membrane and close the cassette for 15 seconds to 5 mins, then quickly develop the membrane.
6. Arrange in a line the developed film with the membrane and mark the location of prestained marker on the film.

II.2.5.5 Dot Blot

A Dot blot is a method to detect proteins as western boltting does but the only difference is that the protein samples are not electrophorised on the gel instead they are spotted directly on the nitrocellulose membrane.

1. On a nitrocellulose membrane draw positions by pencil in order to indicate the position where protein sample is going to be loaded.
2. Using pipette tip sot a 2µl of sample on nitrocellulose membrane and let the membrane air dry.
3. Place the membrane in blocking solution (PBS containing 5% semi skimmed milk powder) for 30 mins.

4. Discard the blocking solution and incubate in primary antibody dissolved in PBS containing *5% semi skimmed milk powder* (Appendix 1) for 1 hour at room temperature with constant shaking.
5. Wash the membrane with *0.05% tween 20* (Appendix 1)in PBS three times for 5 mins each.
6. Incubate with secondary antibody which is also dissolved in PBS containing 5% semi skimmed milk powder for 1 hour at room temperature with constant shaking.
7. Wash the membrane with 0.05% tween 20 in PBS three times for 5 mins each.
8. Detect protein using ECL reagent (Section II 2.5.3) and expose X-ray film and develop the film.

II.2.6 Cryosectioning

II.2.6.1. Freezing tissue in Isopentane

Isopentane is used to freeze tissue for histology. Tissue freezes instantaneously in the isopentane once they are dissected from the mouse and stored at -80^{o}C. The fast cooling rate with isopentane results in reduced

crystal size, which is required for histology.

II.2.6.2. Cryostate Sectioning

2.5 micron sections from different tissues are cut at -25^{o}C using a Bright Cryostat on top of polysine coated microscope glass slides (VWR). The slides containing sections are allowed to dry for 15 mins and then stored at -20^{o}C until they are used for prehybridization (Section II.2.3.3.1.1).

II.2.7 Hematoxylin and Eosin Staining

Dehydrate the slides in 70% ethanol for 1min and then put in distilled water for another 1min. then start with

Hematoxylin staining: Immerse slides in Haematoxylin stain (BDH) for 1min then put them in distilled water for 1min, rinse 2-3 times, and wash the slides under running water. If there is excessive stain then destain the slides in acid ethanol. Get rid of excessive water by tapping the rack containing slides on paper towels.

Eosin Staining: Immerse the slides in Eosin stain for 30sec and rinse them in distilled water. Put slides in pot containing 70% ethanol and then 90% and 100% ethanol. Rinse sildes in xylene solution for 1 min (work in fume cupboard). Let the slides air dry. Take a cover slip and put a drop of

Fluoromount (contains xylene) and place on top of a section avoid any air bubbles.leave them to dry for overnight. Next day observe under microscope.

II.2.8 Mouse Embryonic Fibroblasts and Stem cells

II.2.8.1 Preparing Feeders - Mouse Embryo Fibroblasts (MEFs)

The best feeders to use are mouse embryo fibroblasts (MEFs) derived directly from 14 day old mouse embryos. Set up timed pregnancies and harvest embryos at day 14 p.c. and place in PBS. Remove uterine membranes and fetal liver and then mash the rest of embryo through cell sieve into a 10 cm petri dish. Collect the cell mix in MEF medium and allow fibroblasts to grow out over several days. Change medium frequently and wash cells 3-4 times with PBS every time you change medium. When all debris has gone, make frozen stocks; 2 vials for each confluent 10cm plate. These MEFs must contain a neo^R gene as they must be capable of surviving G418 treatment.

II.2.8.2 Culturing Feeders

Take an aliquot of feeders from the liquid nitrogen and thaw them in a 37^oC water bath. Wipe down with 70% ethanol before putting the vial in the hood.

Take a 12ml Falcon tube and transfer the cells into the tube and add 5ml media mix and spin at 1500 rpm for 3 mins. Discard the supernatant and resuspend the cells in 10 ml of media and transfer cells to a 10 cm diameter tissue culture dish. Subculture until there are sufficient cells to treat with mitomycin C.

II.2.8.3 Mitomycin C treatment of feeder cells

When MEFs are 80% confluent, add mitomycin C to the culture medium and leave in the incubator for 2-3 hours. Wash with PBS 3 times. Mitomycin C works by inserting itself into DNA strands and binding them together. This stops the cells from making genetic material and proteins and thus preventing the cells from growing.

II.2.8.4. Isolation and Culturing ES Cells

Embryonic stem (ES) cells are the pluripotent cells derived from the inner cell mass of day 3.5 blastocysts, which means they are able to differentiate into nearly any type of adult cell and have the prospective to direct the development of a mouse. The embryonic stem cells are isolated from ES cell line between C57BL/6 X 129/SV (Figure2.11). ES cells are cultured directly

on MEFs in ES cell medium in 10% CO_2. Split ES cells when colonies are getting too large or when they reach 70-80% confluency.

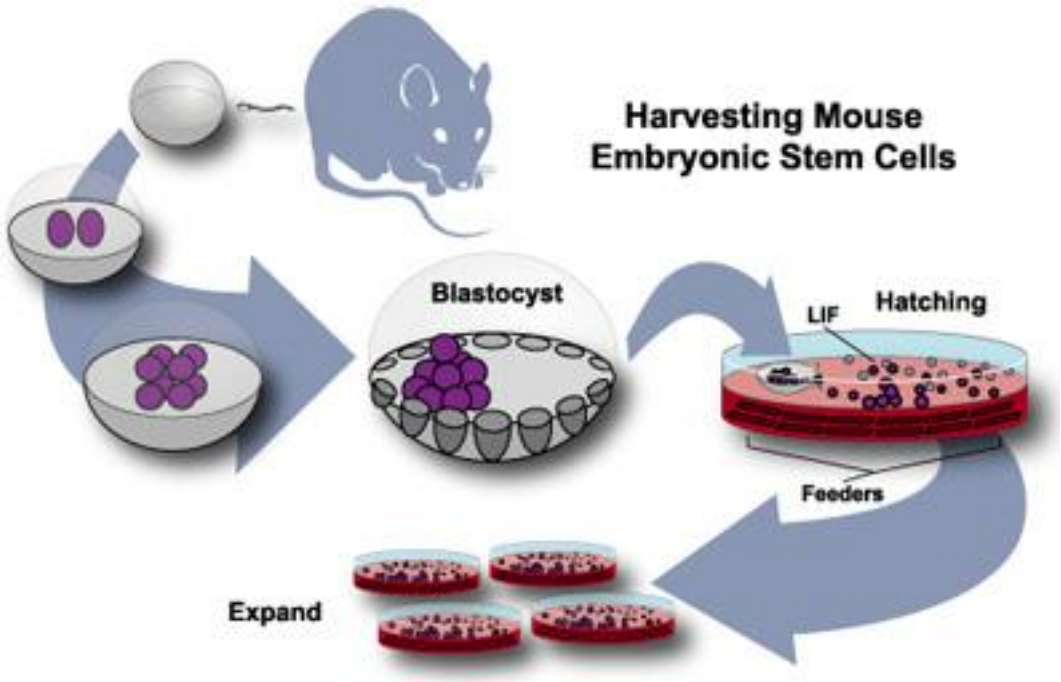

Figure 2.11. Embryonic Stem cell isolation (http://www.openbiosystems.com/GeneTargeting/MEScells/)

II.2.8.5. Electroporation of ES cells and picking of clones

DNA can be transfected into ES cells by applying by high voltage electric pulse to suspension of cells and DNA. The DNA passes through pores in the cell membrane resulting through electric shock.

II.2.8.5.1 Electroporation

It is important that the cells for electroporation are in log phase of growth and are not overgrown. So split the cells two days before the electroporation so that they are growing actively. The day before electroporation add fresh media. On day of electroporation add fresh media to the cells. Harvest 5 x 10^7-10^8 ES cells. Wash in PBS and spin and then resuspend in 1.6 ml PBS. Add 60µg linearized plasmid DNA (make sure DNA is cleaned by Qiagen purified kit). Place 0.8 ml in each cuvette and shock using Biorad electroporator set at 240 volts/500µFD capacitance with the time constant. Leave cuvettes at RT for 5 min, pool both shocks then place in 40 ml of ES medium. Place cells in a dish containing MEFs with 10 ml fresh medium.

II.2.8.5.2 Selection

The vector used for targeting the C1sA gene contains a neomycin resistance cassette, so clones are selected in the presence of G418.

The next day, apply G418 at a concentration of 330µg/ml. Change the ES medium every day and wash the cells with PBS several times to remove debris. Clones should be visible after 7 days of selection. When clones are ready to be picked they appear round with a shiny well-defined border.

II.2.8.5.3 Picking clones

Over 800 clones are picked up from one transfection. Pick independent colonies by viewing under dissection microscope using yellow tips. Place the colony in 96 well plate and add 30µl 1 x trypsin/ EDTA and incubate at 37^{o}C until colony becomes single cell suspension. Transfer to 48 well plate containing MEFs in ES medium with selection.

II.2.9 Mice used for gene targeting

II.2.9.1 Superovulation

Superovulation involves the use of fertility drugs to stimulate the ovaries. 6-8 weeks old C57Bl/6 female mice weighting 24-28g are superovulated by injecting 5U PMSG (pregnant mare serum gonadotropin) which is a FSH hormone in 100µl PBS at 11.00 am on day 0. On Day 2 means 48 hours later inject 5 U of HCG (Human chorionic gonadotropin) in 100 µl. HCG is an Ovulation inducing hormone. These females are then mated to 9 month old C57BL/6 male mice and checked for the presence of vaginal plugs on day 3. Plug positive mice are then caged separately and blastocysts are collected on day 6 and used for injection (Section 2.9.5).

II.2.9.2 Pseudopregnant Female Mice

To produce pseudopregnant female mice (B6CBF1 mice), a C57 female mouse is crossed with a vasectomized or genetically sterile a CBA male mouse to generate B6CBF1 female mice. The females from this strain are usually known as good mothers. Plug positive females mice are used for reimplantation of microinjected blastocysts.

II.2.9.3 Preparing ES cells for injection

The cells which have gone through homologous recombinant event are split 2 days before the injection. Remove growth medium from the wells to be harvested and wash with PBS. Add 1ml of 1x trypsin and incubate for 5 mins at 37^{o}C. Agitate the cells by sucking up and down repeatedly with the help pf of a P1000 Gilson pipette to obtain a single cell suspension. Remove cells into 10 ml universal tube and wash twice with 1 ml PBS. Spin down the cells at 1250 rpm for 4 minutes. Discard the supernatant (PBS/Trypsin) and disrupt the pellet by tapping the tube. Resuspend pellet in 500µl of injection media and use for injection.

II.2.9.4 Isolation Blastocysts for Injection

Dissect the uterus from 1or 2 superovulated females (section II 2.9.1) by cross section through the cervix. Transfer one of the uteri to a sterile perti dish containing the clean M1 medium (Sigma), known as isolation medium. Separate the uterus horns from the cervix. Carefully flush the blastocysts out of both the uterus horns by using 27-gage needle and syringe containing 1ml of M1 medium. Collected blastocysts are transferred to M16 culture medium and stored at 37^{o}C with 5% CO_2.

II.2.9.5 Injection of Blastocysts with ES cells

Transfer the blastocysts from the M16 medium into the isolation medium (M1 medium). Transfer one blastocyst to the injection chamber and load the injection needle with 75-120 ES cells. 12-15 cells are injected into single blastocyst. Successfully transferred blastocysts are kept in culture medium at 37^{o}C with 5% CO_2. The injection is performed Nikon microinjection apparatus shown below (figure 2.12).

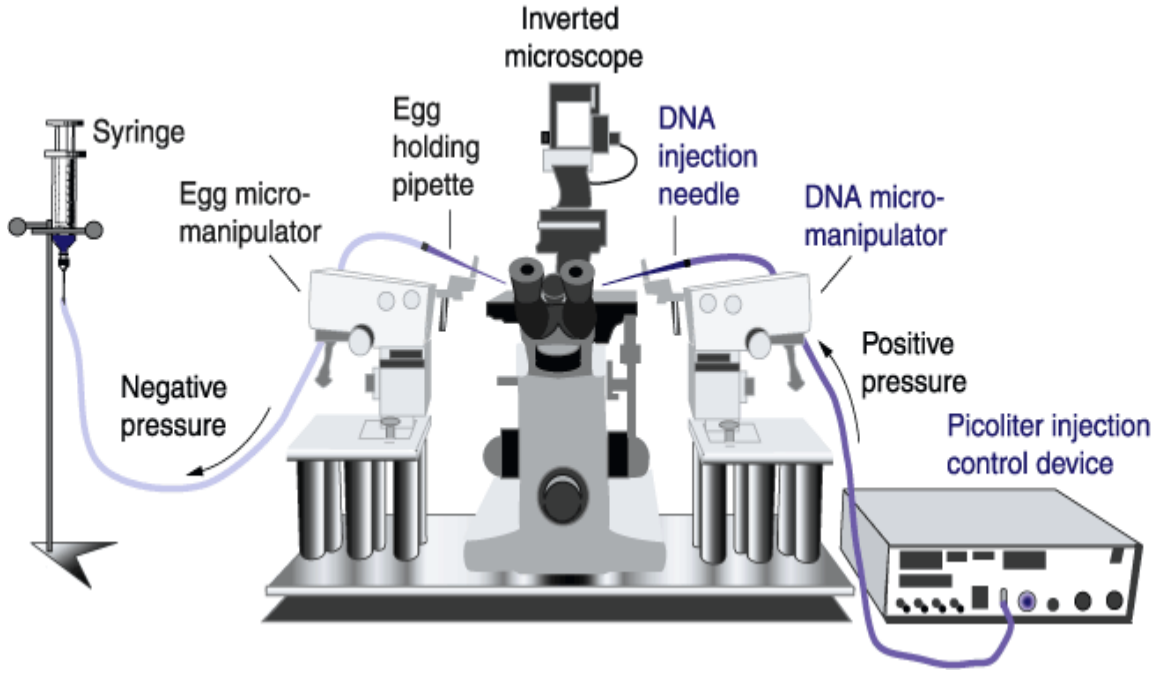

Figure 2.12 Shows the three important parts of the injection apparatus are an inverted microscope with stage magnification of 400X, a micromanipulator-mounted negative pressure holding pipette on left side and a micromanipulator-mounted injection pipette on right side.

II.2.9.6 Blastocyst transfer into uterus

Carefully pull out the uterus horn and gently insert a 27-gage needle into the uterus. Remove the needle and gently insert a glass transfer pipette containing injected blastocysts through the uterus. After blastocyst transfer the pseudopregnant mothers are kept in a quiet place in order to reduce stress so it doesn't affect the mice.

Chimeric offspring's can be identified phenotypically by coat colour (black/brown) and genetically identified by doing PCR (Section II 2.1.11.) on genomic DNA obtained from ear snips (Section II 2.1.10.) and later crossed with wild type female (C57).

Chapter III

Generation of C1sA deficient mouse line

III.1 Results

In order to study the interdependence between the three complement activation pathways *in vivo* and *in vitro*, my research focused on the generation of a gene targeted mouse line deficient of the C1sA gene (C1sA-/-). Among other specific aims the availability of such a C1sA deficient line would allow to define the role of the alternative pathway in a mouse model deficient of both the classical and the lectin activation pathway. Although another mouse strain with classical pathway deficiency is available (the C1q-/- mouse), this strain was unsuitable to intercross with MASP-2-/- mice, as the murine MASP-2 and C1q genes are in very close proximity to each other, too close for a recombination of the two targeted genes to occur (frequent intercrosses failed to produce a strain carrying both deficient genes).

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocysts at day 3.5. They are able to differentiate into nearly any type of

adult cell and tissue and have the potential to direct the development of a mouse. The establishment of a pluripotent stem cell line which can be kept in culture without losing its potential to differentiate into any tissue-specific cell type allowed establishing gene targeted cell clones through homologous recombination using gene targeting. There are two methods in which the gene deficient mice can be generated: One method is called gene targeting and the other method is called gene trapping. To generate a C1sA deficient mouse line the gene targeting method was used, a technique based on homologous recombination of the targeting construct with the target gene.

III.1.1 Gene targeting strategy

Murine C1sA gene is localised on chromosome 6 of the mouse genome at position 6F2 (Contig, AC115911.14.) and comprises of 12 exons (Figure 3.1).

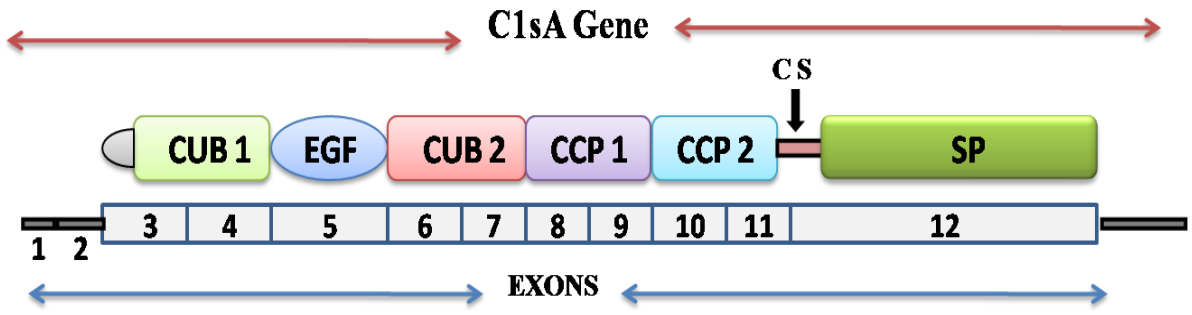

Figure 3.1: Structure of the gene encoding the domain of mouse C1sA.
Schematic alignment of C1sA gene structure: Showing CUB 1, EGF, CUB 2, CCP 1, CCP 2 and SP domains and relationship of exons to respective domains. Narrow portions represent leader peptide. Vertical arrow represents activating cleavage site (CS).

Gene targeting is defined by the introduction of site-specific modification into the mouse genome by homologous recombination and has been successfully used for the production of mutant animals to study gene function in vivo. Gene targeting can be divided into two phases, phase one is construction of targeting construct and gene targeting in ES cells and phase two is generation of gene targeted mice. Homologous recombination of foreign DNA to change the endogenous gene in genomic sequence is not common in mammalian cells. The only competent gene targeting method present upto date uses pluripotent mouse embryonic stem cells. The few ES cells that take up the new gene after selection are allowed to proliferate. Phase 2 starts with the injection of mutant ES cells into blastocyst and implanted into pseudopregnant mother to give birth to an implanted chimeric mouse (which contains both normal and genetically modified cells). The breeding of germline chimeric mice with normal mice allows the generation of a heterozygous mice and intercrossing this heterozygous offspring will eventually lead to the generation of a homozygous gene deficient mouse line. Figure 3.2 shows the general strategy for gene targeting in mice.

A replacement type vector was used to target the C1sA gene in ES cells. The gene replacement vector pKO Scrambler NTKV 1901 was used, containing a

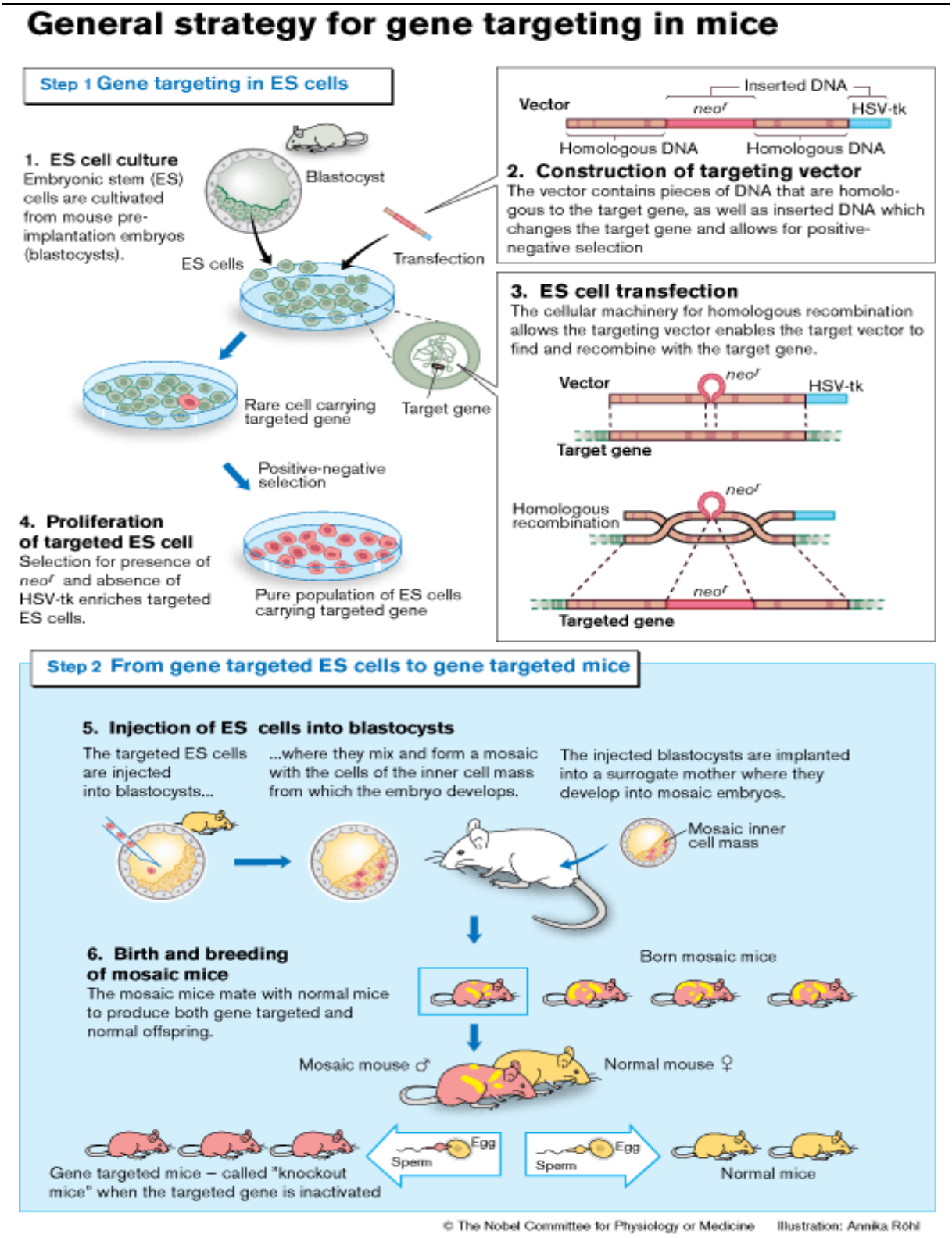

Figure 3.2. General strategy for gene targeting in mice.

short and long arm of DNA (3-10Kb in total) homologous to the genomic target locus which are interupted by a positive selection marker such as the bacterial aminoglycoside phosphotransferase (neo) gene which confers resistance to G418 and an additional negative selection marker like the Herpes Simplex Virus thymidine kinase (tk) at the end of the long homology arm of the vector. Gene targeting vectors are introduced into ES cells by electroporation following linearisation of the targeting construct. For linearisation of the targeting construct, a unique restriction site should be present which is usually the NotI restriction site in pKO Scrambler NTKV 1901.

The most commonly used techniques to identify homologous recombinants are Southern blotting and PCR and used both methods for the identification of homologous recombinants.

III.1.2. Generation of a C1sA targeting construct

The C1sA targeting construct was designed to replace exons 2 and 3 (encoding N terminal CUB1 domain), plus the splice acceptor at the start of exon 4 (encoding CUB1 domain), with the PGK-Neo cassette (gene encoding neomycin resistance) by homologous recombination in 129/Sv ES cells, inserting a frameshift mutation and deleting 1478 bp coding sequence (Figure 1 Appendix 2).

For the generation of C1sA targeting construct, the A cassette (1575 bps, the long arm of homology) and the B cassette (1506 bps, the short arm of homology) were amplified from 129/Sv mouse genomic DNA by using set of specific primers for the A cassette and B cassette of my construct. The primers used for A cassette are C1sA_KAF2 and C1sA_KAR1 and primers for B cassette are C1sA_BamH2 and KBR_ClaI (See section II.1.6). The PCR products named A cassette and B cassette are then cloned into pGEM-T Easy vector separately (see section II.1.4.1 and II.2.1). The A cassette and B cassette fragments were then subcloned into the gene targeting vector pKO Scrambler NTKV 1901. For this purpose the pGEM-TEasy A cassette construct was digested with restriction enzymes BglII & XhoI and an insert of 1575bps was subcloned into the pKO Scrambler A polylinker site of pKO Scrambler NTKV 1901 (which was also digested with the same restriction enzymes) (see Figure 3.3). The next step was the insertion of the B cassette into the pKO Scrambler NTKV 1901. For this purpose, the B cassette, (1506 bps in length) was excised from the pGEM-TEasy B cassette construct by using BamHI and ClaI restriction enzyme and then ligated into the pKO Scrambler B polylinker site of pKO Scrambler NTKV 1901(which was digested with BamHI and ClaI restriction enzymes) (see Figure 3.3). After subcloning, all

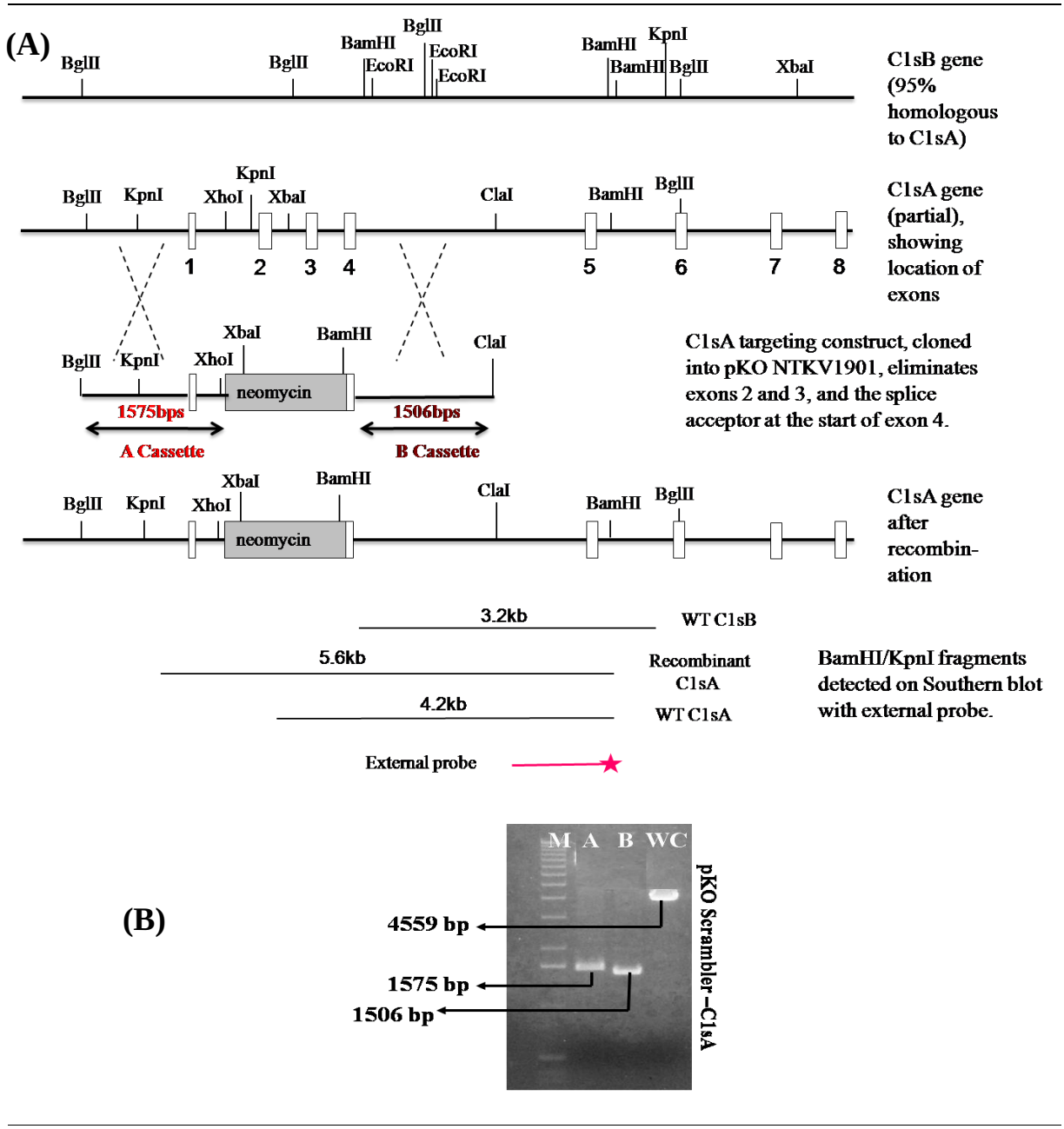

Figure 3.3 Construction of C1sA targeting construct. (A) C1sA gene was disrupted by homologous recombination in embryonic stem cells using a gene targeting vector pKO Scrambler 1901. C1sA gene was disrupted at exon 2 and 3, and the splice acceptor at the start of exon 4 with the PGK-Neo cassette. **(B)** All the fragments of C1sA targeting vector are checked by restriction analysis. Lane A represents A cassette, 1575bps (Digested with BglII and XhoI), Lane B represents B cassette, 1506 bps (Digested with BamHI and ClaI) and Lane WC (A cassette + Neo cassette + B cassette represents the whole construct, 4559 bps (Digested with BglII and ClaI).

fragments were validated by sequencing and restriction analysis (see Figure 3.3 B and section II.2.1.7).

III.1.3 Proliferation of Targeted Embryonic Stem Cells

III.1.3.1. Gene targeting in Embryonic Stem Cells

The pKO Scrambler –C1sA targeting construct was used to transfect the embryonic stem cell line through electroporation (see Section II.2.8.5.) following G418 antibiotic selection, approximately 1800 clones were picked and genomic DNA was isolated for examination of a homologous recombinant event (Figure 3.4).

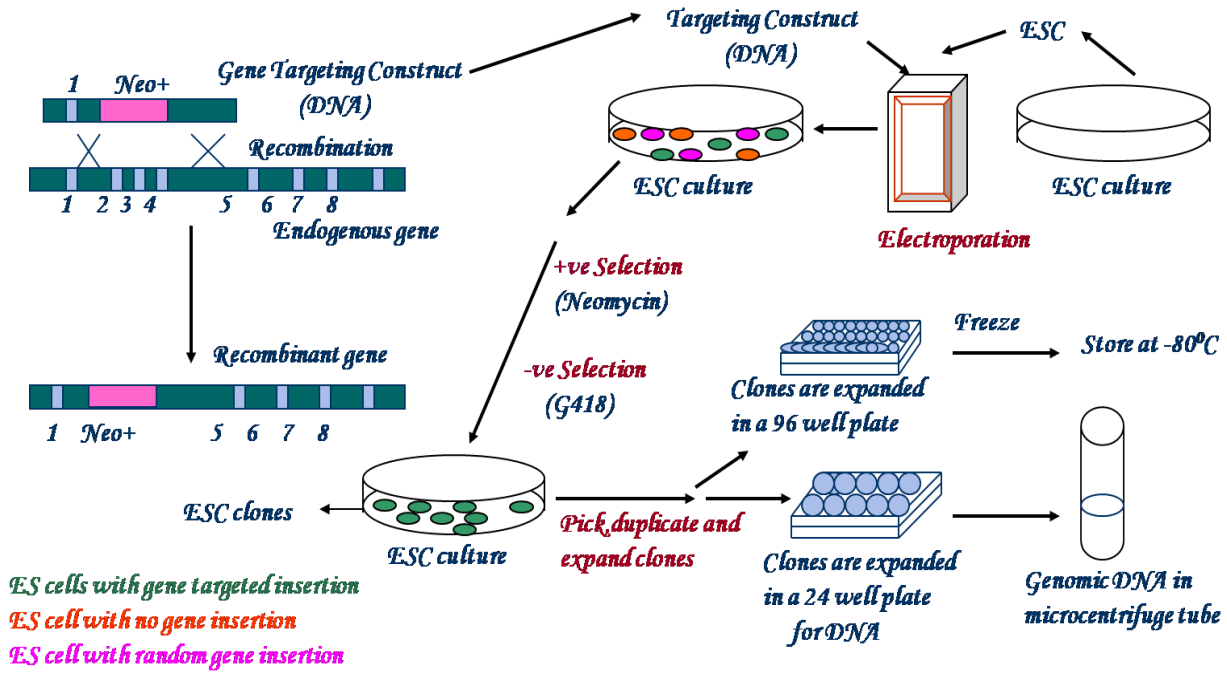

Figure 3.4 Electroporation and selection of clones for homologous recombinants

III.1.3.2. Identification of Homologous Recombinants

Embryonic stem cells that harbour homologous recombination will be genetically heterozygous at the target chromosomal locus. So it is important to have a genotyping strategy that will simply differentiate between the wild type and mutant type in the cells that have undergone a homologous recombinant event. The commonly used methods for the screening are Southern blotting and PCR and the experimental design for screening is described in figure 3.5.

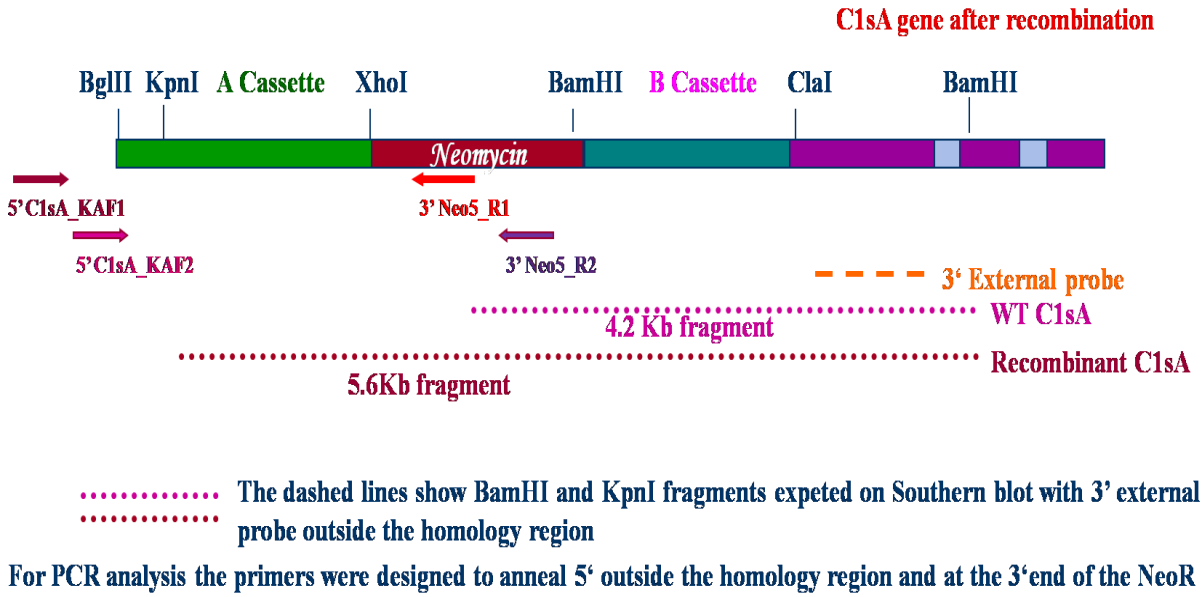

Figure 3.5 Experimental design to identify homologous recombinants by Southern blotting and PCR. A targeting vector is designed to contain a long arm (A cassette in green) and short arm (B cassette in blue) of homology and a positive resistance Neomycin cassette (in red). For Southern blotting the genomic DNA is digested with restriction enzyme BamHI and KpnI and detected with a 3'external probe binding outside the homology region (shown in orange dashed lines). The expected band size for wild type and recombinant C1sA are shown in pink and red colour respectively. For PCR analysis the primers were designed to anneal 5'outside the homology region and at the 3'end of the NeoR.

The 129/Sv genomic DNA sequence used to amplify a C1sA specific 3' external probe used for Southern blotting analysis was shown in figure 3.6 along with the forward primer (C1sA_P (SB) _FP2) and reverse primer (C1sA_P (SB) _RP2) used for external probe.

ATGCTGGAGTTACAGCATGTACCAATTGGGCAGGGTGTTGTATAGTTTGTACGCAAGAAAAGTCATAGGGTTTTCATGGTGGAGCTCCTTA
CAGCGCCGTGGCAAAACACAGCTCTGAGTCAGACTTCAGAAAAGTAGTTTAGATTGTATTCGTAGATTTTAAAATATGGGGCTGGAAAGA
GATGACCCAGCAGTTCAGGGAACTTGCTGTGTTCATA**GAGGACCAGAGTTCAGTTCC**CAGCATCCACATGGCAGCTCACAACTGTTGGT
AACTCCAAAACAAGAGCATCTTATACTCTTTTCTGATCTCAAAGTGCACCAGACCTGCATGTAGTATAAAATATCAATACAATAAATACAT
CCATAAATATTGAAAAACTAAAGTGTGTTAAGATTTATTTTATTATTTTAAATCTTTTATTTATCTCATGTGTGTAGGTGTTTTGCTTGCTAG
CAAGTATGTGCATCACATACATAGGGTATCTGAGGAGTCCACCAGAAGGCATTGGGTCCCTGGAACTGCACTGGAATTATAGCGTTGTGA
GCTGCCTTGTGGGTGCTGGGAATTGAACCTCTGGAATGATAGCACATACTCTTAACCAGTGAGCCATATCTCTACCCCTCCAAACTTCTTTT
ATTAAAATGTCTTACCTGTACATAATAATGGTTTCATTATGACATGTTCATACTCATCTATGTTTTTGTCCAAGTGGCCCTCAGTACATTGTC
TCCTCCTAATCCCCCCTCTCTTCTGTGTTTTGCTTTTCAGTCCTTTTTATTTTTTCATTAGGATTGTGTAAGAGCTTGTCTAGGGGTTACTTAT
AGGTACATCGGCACCTTACCAGTGGCTGCATGCTAAAGATGTCTCTCTTCCTGCAGTAATAAATTAGTTGCCTATAGCGCCTCGGGGTGGG
GTGGAGTCCTCCTGAGTCCTTCCCCATGCCATGACAGGATATCGCCAGGCACAGTCTTGTTCAGATAATAACAGCCGTGGTGACCTAAGAG
TGTCGTGGCCTATCACGCCTGGAAGACAGCATCCCGCAAAACTTCCGTCTGTTCCTCTGGCTCCTACATTCTTTTGTGCATGTCCCCCTCTTT
CATGATGTTCTTGGGGCCTTAGAGTCTTCCTTCCTTCCTCCTTTGCTGTCTCCTACTCTTCCTCTCTCCCTTCCTCCTTCACTTCTTCCTTCCTT
CCAGCTGTCCCTCTGTCTCTTACTCTCTGTCTTGTCTGTCTCTGACTCTGTCTGTCTCTCTTCCTTTCTTTCTTGGAGAAAAGGTTTTGCGTTG
AAGCCCAGGCTGGCTTTGAACTCATGTCTTTCTGTTTCACCTACCTGAATACT**GGGATTACTGGCATGAGCCAT**TATACCTGGCTTGGAG
AGTATTTAAGCAACAAACCTTTTCTGGTTTAGTTTGTTTGTTTGTGCTTTATCCTTATAGATTTGGCTTCCATCGTTCTTCCTTCTTGGTCTTT
GTTCTTCACCTTGAGTTTTCTCTGCTTTTTGCAGACATAAATGAATGCACAGATTTTACAGATGTCCCCTGTAGCCACTTCTGCAATAACTTC
ATTGGT GATACTTCTGCTCCTGTCCCCCAGAATACTTCCTCCACGATGACATGAGGAATTGTGGAG

Figure 3.6 shows the sequence used for the generation of 3' external probe.
The primers used for probe are shown in different colours. The forward primer C1sA_P (SB) _FP2 is shown in pink colour and the reverse primer C1sA_P (SB) _RP2 is shown in red colour. The brown coloured sequence represents a part of B cassette and yellow represents exon 5.

III.1.3.2.1 Screening of Homologous Recombinants by Southern Blotting

The detection of homologues recombination events by southern blotting analysis depends on the selection of suitable restriction enzymes that will generate restriction fragments that are distinctive to the wild type and mutant allele. It also requires a careful selection of an informative DNA hybridisation probe. Around 1800 transfected ES cell colonies were picked and examined for homologous recombination events by southern blotting (see Section II 2.1.9). In brief, genomic DNA was isolated and digested using the restriction enzymes BamHI and KpnI at 37^{o}C overnight in order to make sure that restriction digestion was complete (see Figure 3.5). Digested genomic DNA samples were separated by gel electrophoresis in a 0.8% agarose gel (see Figure 3.7A). A 1143bp fragment was amplified from 129/Sv mouse genomic DNA to be used as a 3' external probe by PCR using primers C1sA_P (SB)_FP2 and C1sA_P(SB)_RP2 (see Section II. 1.6, figure 3.6). The amplified PCR product was then ligated into pGEMT-Easy vector and excised from vector by EcoRI restriction digestion of the plasmid including the genomic probe. The 3' external probe is labelled radioactively using P^{32} α-dCTP (see Section II.2.1.10.) and then hybridized to the blotted DNA (see Section II.2.1.11.). From 1800 isolated colonies only clone, number 61, showed to have undergone a homologous recombination event.

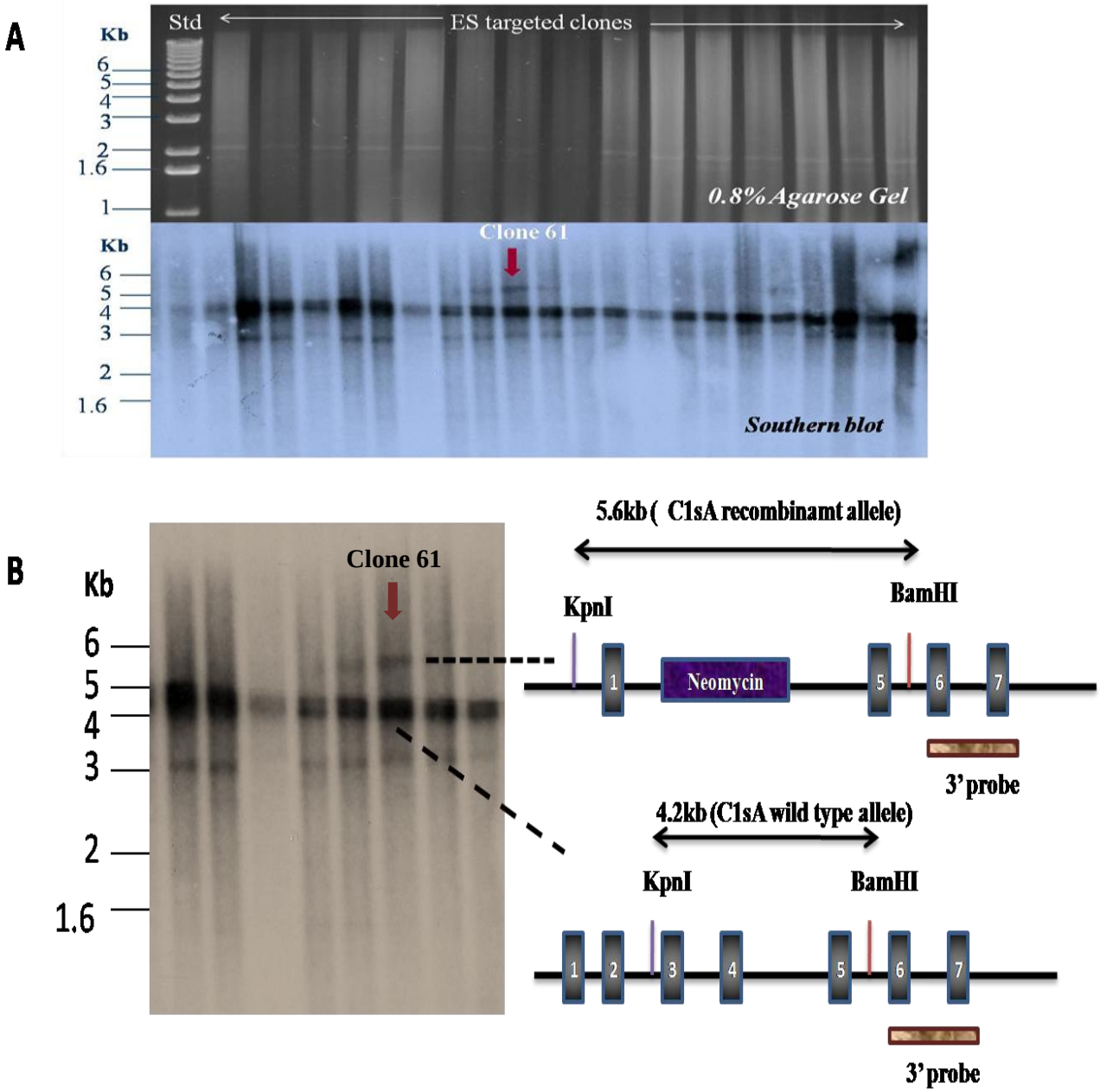

Figure 3.7 Screening of targeted clone by Southern blotting. (A) BamHI and KpnI restriction digested genomic DNA isolated from clones are electrophoresis on a 0.8% agarose gel and then DNA is blotted on nitrocellulose membrane and hybridized with radioactive 3' probe and exposed to x-ray film after washing at -80^{o}C and then developed. **(B)** Shows clone 61 on southern blot. Recombinant C1sA allele shows a band of 5.6 kb and wild type allele shows a band of 4.2 kb.

The southern blot signals that identified this homologous event are shown in figure 3.7. This candidate clone 61 was then further characterised by PCR (see Section III.1.3.2.2).

III.1.3.2.2 Screening of homologous recombinant by PCR

In order to confirm that clone 61 has undergone homologous recombination and to confirm the Southern blotting results the PCR technique approach was used to detect homologous recombinant. Whether or not a genomic multiplex PCR delivers clear and reproducible results depends on the design of the oligonucleotide primer pairs to generate predicted PCR products to detect and discriminate between wild type allels from the targeted allel. For a multiplex PCR screen, one of oligonucleotides should anneal to a flanking genomic sequence that is not present on the targeting vector [therefore designed the 5'primer which is complementary to the 5' genomic region outside the homology sequence and combined it with a 3' primer complementary to the 3' end of the neomycine cassette (see Figure 3.5)]. The 3' primer will only therefore amplify a PCR product if the genomic DNA bears a neomycin cassette as result of homologous recombination (see Figure 3.8). The candidate clone 61 was therefore analysed further using touchdown multiplex PCR using the following primer sets. The primers used to compose a C1sA multiplex PCR were: C1sA_KAF1, C1sA_KAF2, Neo5_R1 and Neo5_R2. Four different set of

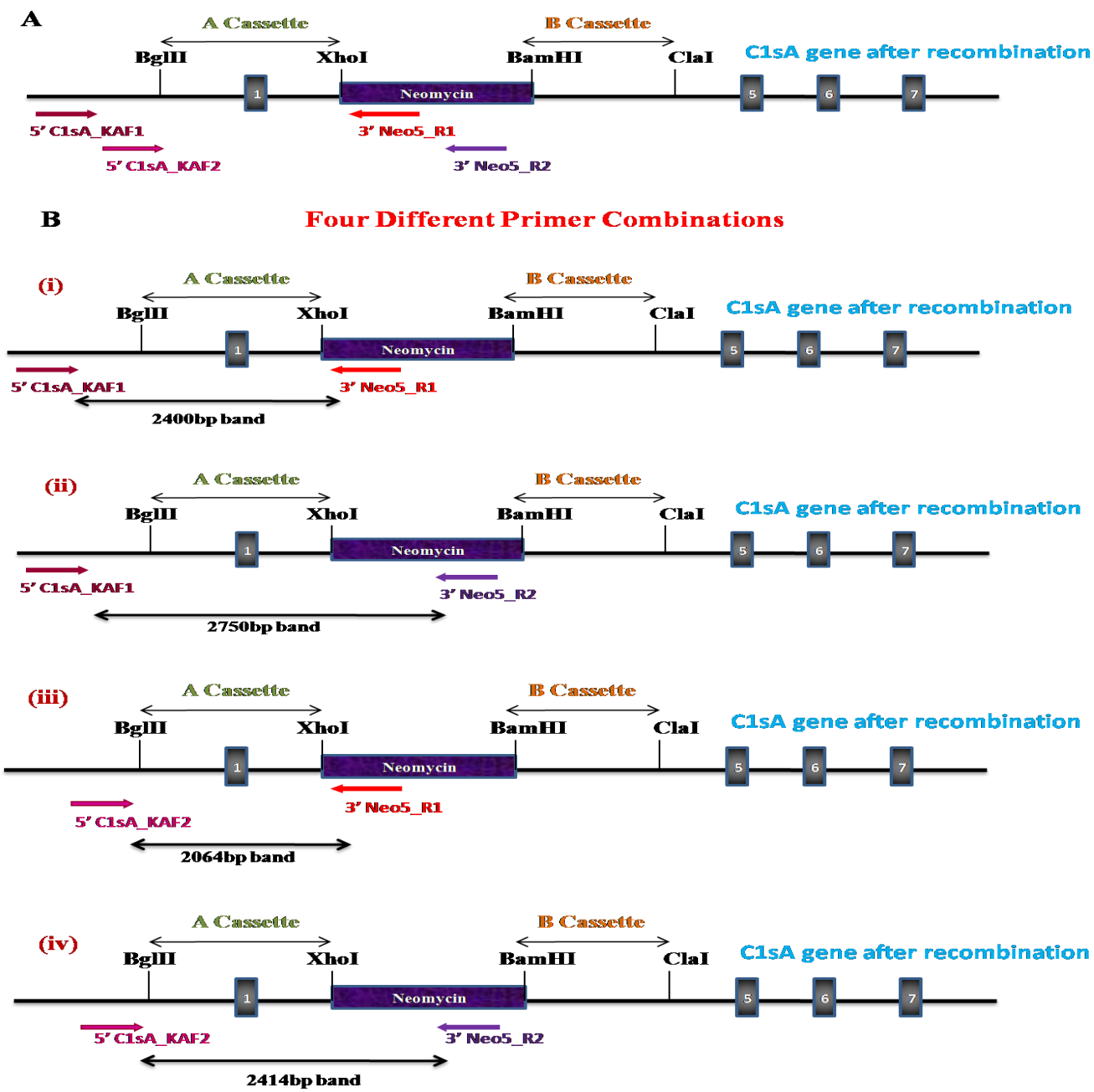

Figure 3.8 Schematic representation of experimental design to identify homologous recombinants by PCR. (A) C1sA gene after recombination along with the 5' forward and 3' reverse primers. **(B)** Four different set of primer combinations used for PCR analysis to identify homologous recombinant.

(i) C1sA_KAF1 & Neo5_R1 (2400bps)
(ii) C1sA_KAF1 & Neo5_R2 (2750bps)
(iii) C1sA_KAF2 & Neo5_R1(2064bps)
(iv) C1sA_KAF2 & Neo5_R2 (2414bps)

PCR reactions were carried out using four different combinations of forward and reverse primers. The combinations were C1sA_KAF1 & Neo5_R1 (amplifying a fragment of 2400bps), C1sA_KAF1 & Neo5_R2 (amplify a product of 2750bps), C1sA_KAF2 & Neo5_R1 (a product of 2064bps) and C1sA_KAF2 & Neo5_R2 (amplifying a product of 2414bps) shown in figure 3.8 and 3.9. The 129/Sv genomic DNA sequence used for designing the PCR primers was shown in figure 2 (Appendix 2). The results obtained from PCR confirmed that clone 61 is a homologous recombinant clone which has undergone homologous recombination and can be used for blastocyst injection. This also confirms that the homologous recombination event is in the correct place.

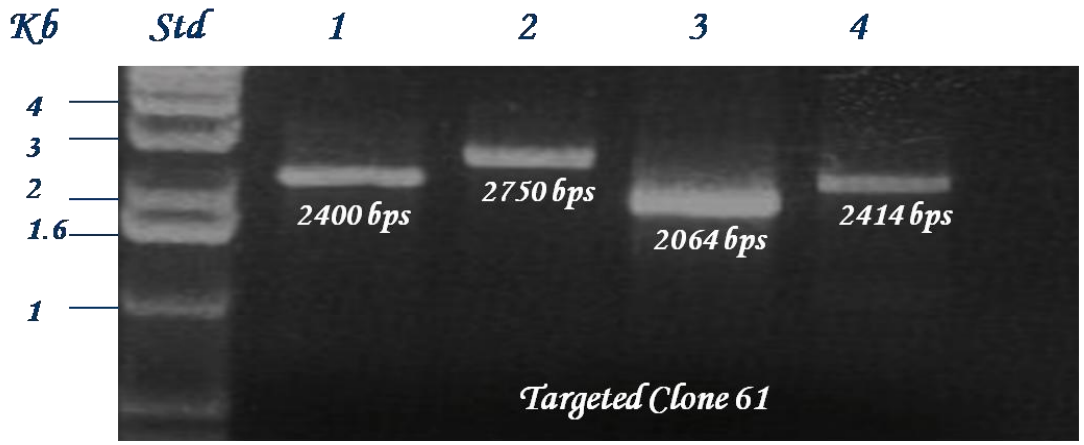

Figure 3.9 Screening of homologous recombinant by PCR. Clone 61 is counterchecked by using different set of primer combinations Lane 1 C1sA_KAF1 and Neo5_R1 primer, Lane 2 C1sA_KAF1and Neo5_R2, Lane 3 C1sA_KAF2 and Neo5_R1, Lane 4 C1sA_KAF2 and Neo5_R2.

III.1.3.3. Generation of C1sA Knockout mice

Generation and breeding of C1sA chimeric mouse and the desired C1sA gene targeted mouse line were carried out in the University of Leicester Biomedical Services Facility situated at the Leicester Royal Infirmary site.

III.1.3.3.1 Microinjection and Reimplantation of Blastocysts

Once I confirmed that clone 61 had undergone a homologous recombinant event by southern blotting and PCR, the targeted ES cell , clone 61, was expanded and prepared for the microinjection into blastocysts obtained from pregnant C57BL/6 female (Section II.2.9.1). About 10-15 cells, obtained from clone 61 were injected into blastocyst (see Figure 3.10).

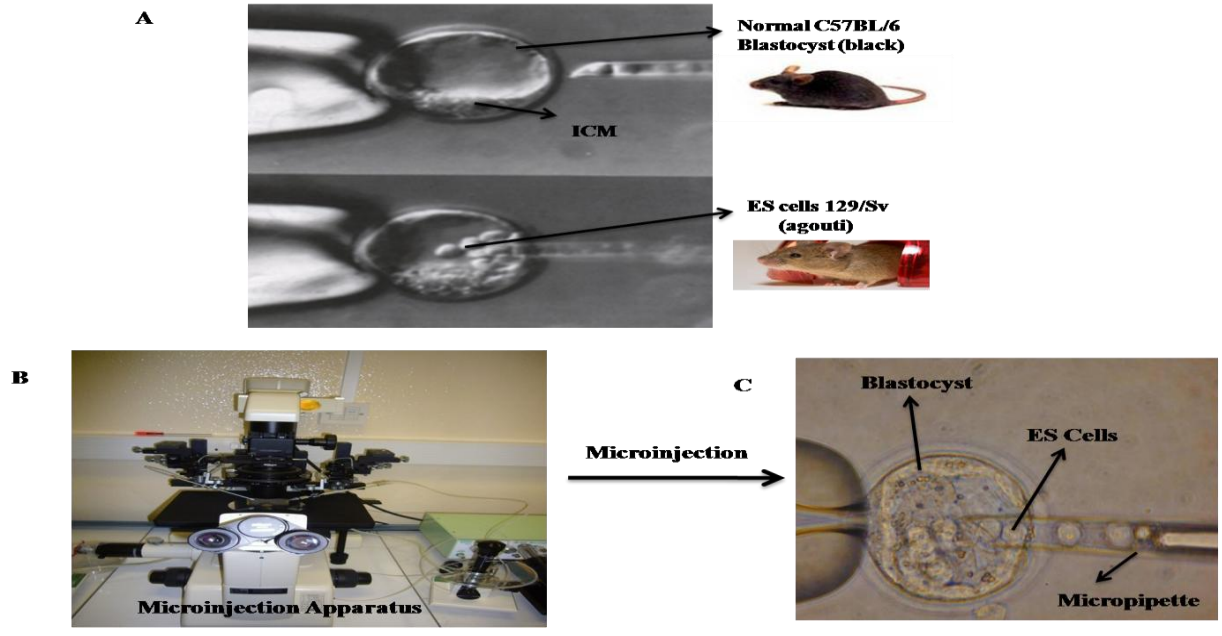

Figure 3.10 Microinjection of Blastocyst. (A) Blastocysts are obtained from pregnant C57BL/6 females and the ES cells obtained from 129/Sv mice. **(B)** Shows microinjection apparatus (See section II.2.9.5) and **(C)** Shows the procedure of microinjection. ES cells fuse directly with cells from the inner cell mass of the embryo with very high efficiency.

After microinjection blastocysts are reimplanted into uterus (see Figure 3.11) of B6CBA /F1 pseudopregnant female (cross between C57 female and genetically sterile CBA male) and pups are born after a 17-day gestation period.

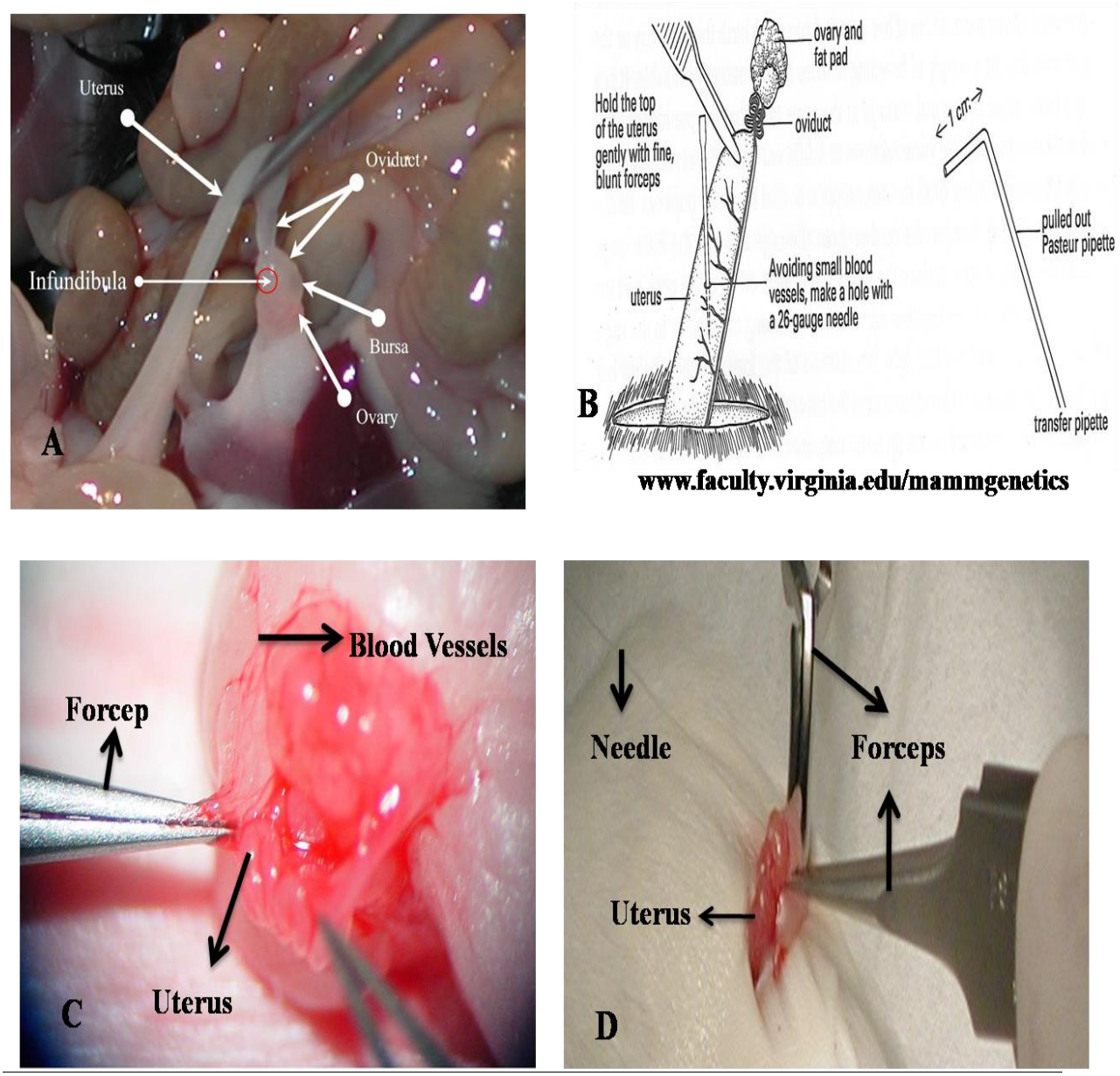

Figure 3.11 Reimplantation of blastocyst into uterus. (A) Various parts of female mice reproductive organs. **(B, C, D)** Describe how to transfer blastocysts into uterus.

III.1.3.3.2 Generation of C1sA targeted chimeric mouse and germline transmission of the targeting event

After a 17 day gestation period, eight pups were born out of which seven were wild type mice and one was a male chimeric mouse (see Figure 3.12). Chimeric mice are usually identified by their coat colour 10-14 days after birth. Chimeras are most frequently of male sex as the original 129 ES cell line is derived from a male mouse. To confirm that the mouse is purely chimeric, genotyping was done using the multiplex PCR described overleaf (see Section II.2.1.11 and III.1.3.3.3).

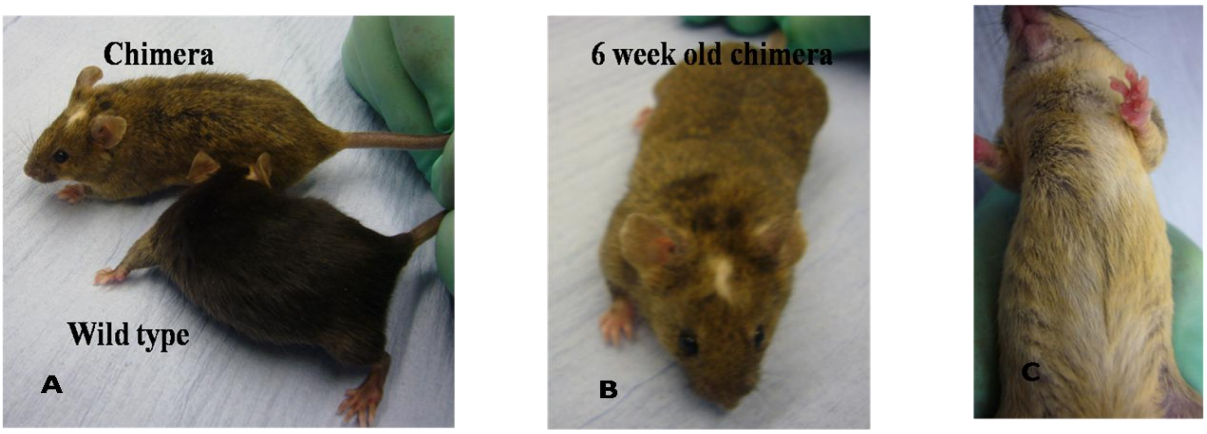

Figure 3.12 Six week old Chimera. (A) Chimeric mice and wild type. **(B, C)** Shows brown white coat colour pattern which is characteristic feature of chimeric mouse.

A Six week old chimeric mouse is mated with 2-3 C57 black female for germline transmission in F1 generation. Germline transmission is detected by the "Agouti" coat colour in the pups against black colour pups. After crossing

the chimeric mouse, a female agouti mouse (C-38) was born (F1 generation) (see Figure 3.13 A). To proceed to F2 generation, this C-38 agouti female was backcrossed to C57 wild type male mouse. In F2 generation, eight pups are born out of which three were black and five were agouti (according to coat colour) (see Figure 3.13 B). To confirm genetically that the mice are wild type or heterozygous a multiplex PCR was carried out on genomic DNA of all of them (see Section II.2.1.11 and III.1.3.3.3).

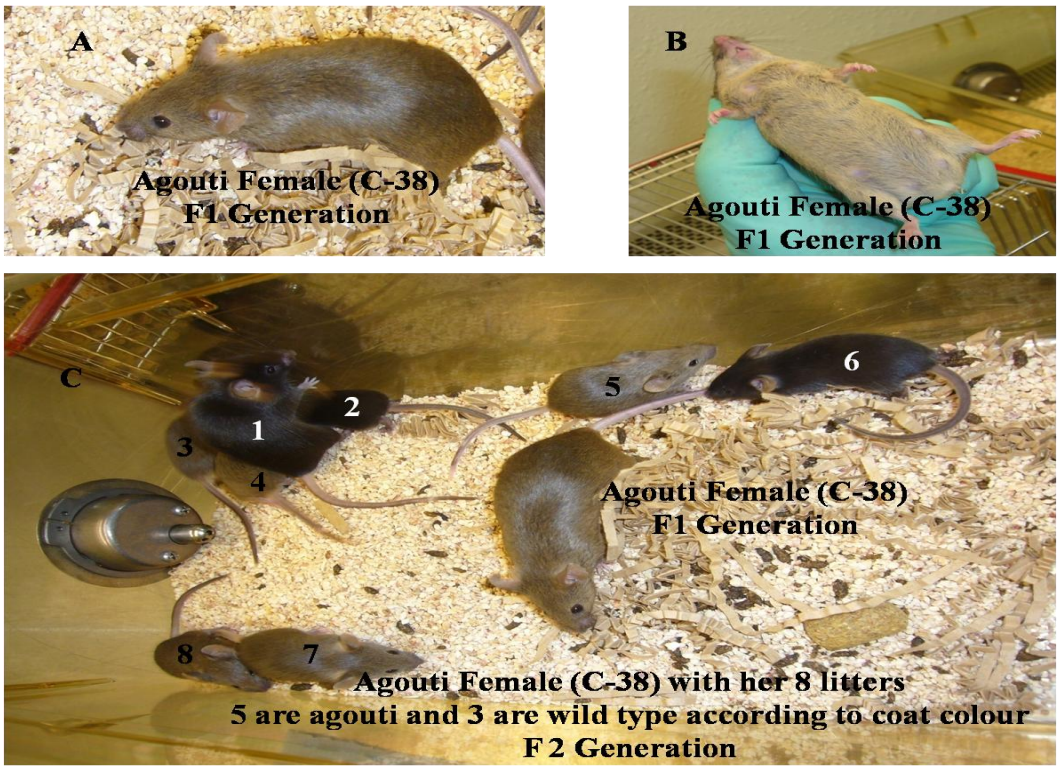

Figure 3.13 F1 and F2 C1sA-/- generation. (A, B) Agouti female mouse (C-38), F1 generation. **(C)** Agouti female mouse with her eight litters (five are agouti and three are black), F2 generation.

III.1.3.3.3 Genotyping of C1sA-/- mice

Although it is possible to identify the chimeric (F0 generation) and the (F1 &F2 generation) through agouti coat colour, it is essential to confirm the presence and transmission of the targeted allele in the genomic DNA of ear snip samples of individual mice by multiplex PCR and Southern blotting (see Section II.2.1.11). For genotyping three oligonucleotides were designed. One oligonucleotide will anneal to a flanking genomic DNA sequence which is not present in the targeting construct and will therefore prime both the disrupted allele and wild type when paired with the oligonucleotide specific for the wildtype and distrupted allele. The primer chosen for our approach is forward primer C1sA_KAF4 (see sequence in Materials and Methods). The other two primers are reverse primers here described as C1sA_KAR2 and Neo5_R2. Reverse primer C1sA_KAR2 is positioned in the wild type genomic sequence which is replaced by the neomycin cassette in the targeting construct whereas the other reverse primer Neo5_R2 is only hybridising to a sequence in the neomycin cassette (which replaces parts of the C1sA gene in the targeting construct) (see Figure 3.14). The primer pair C1sA_KAF4 and C1sA_KAR2 will amplify the wild type allele and produce a PCR band of 245bps in length. The primer pair C1sA_KAF4 and Neo5_R2 will amplify a band of 787bps in length and only if the neomycin cassette of the disrupted allele is present. This

means that the DNA of a homozygous gene targeted mouse will amplify a band of 787bps whereas that of a homozygous genomic wild type mouse will amplify a band of 245bps only.

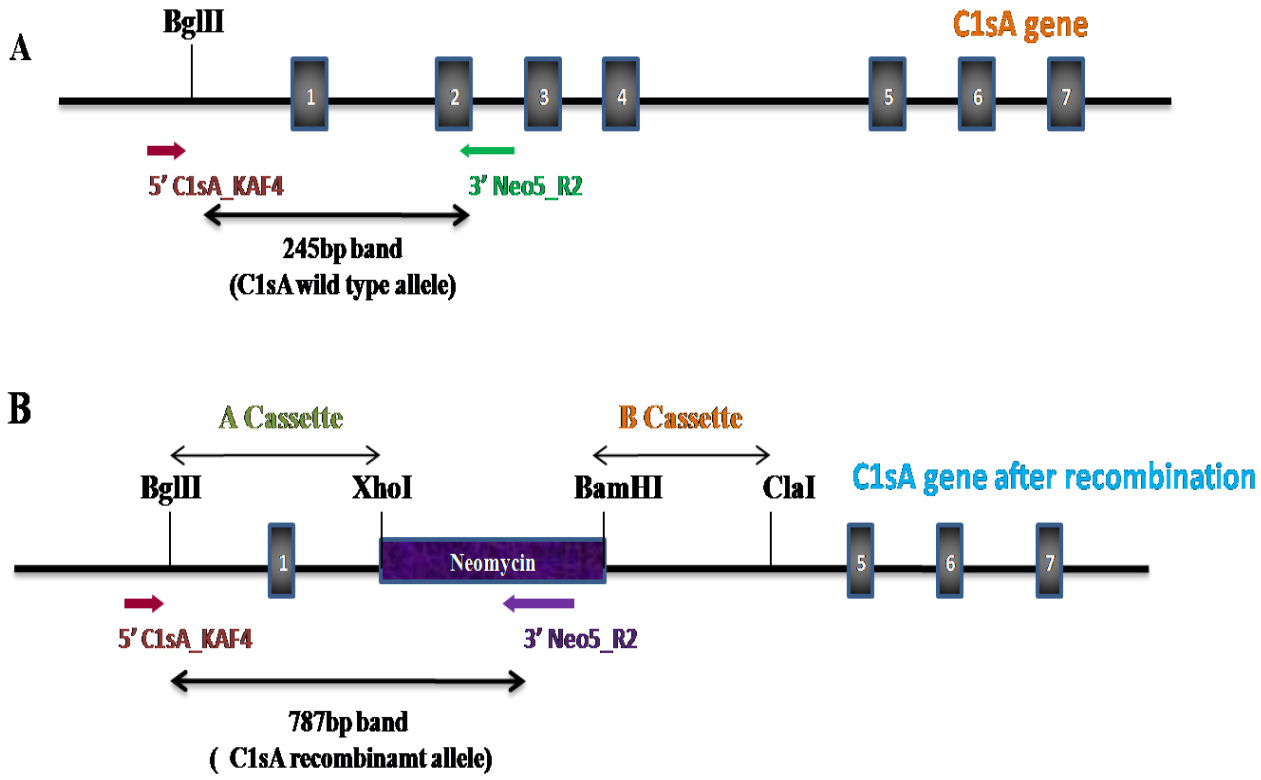

Figure 3.14 Primer design for genotyping. (A) Primer design for C1sA wild type allele. Primer pair C1sA_KAF4 &C1sA_KAR2 amplify a band of 245bp size. **(B)** Primer design for C1sA recombinant allele. Primer pair C1sA_KAF4 and Neo5_R2 amplifies a band of 787bp size.

The genomic DNA of a heterozygous mouse will show two bands in this multiplex PCR corresponding to 245bps and 787bps. Figure 3.15 shows the genotyping result of the F1 generation and Figure 3.16 shows the genotyping results of F2 generation.

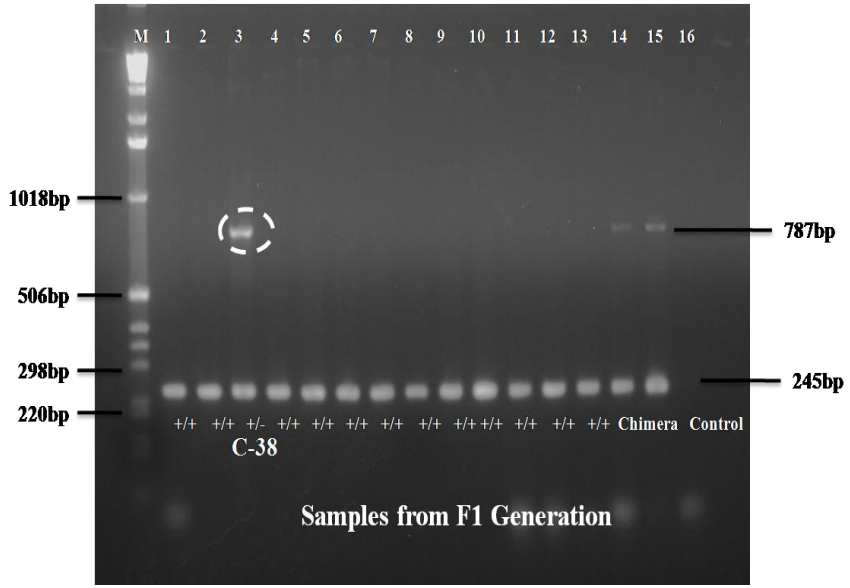

Figure 3.15 Genotyping results from F1 generation. Lane M represents 1Kb DNA ladder. Lane 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 13 represents DNA from individual homozygous for the wild-type allele (Primer combination C1sA_KAF4 and C1sA_KAR2 results in band size of 245bp). Lane 3 represents DNA from C-38 individual heterozygous for the C1sA polymorphism (Primer combination C1sA_KAF4 and Neo5_R2 results in band size of 787bp). Lane 14 and 15 represents DNA from Chimera (Primer combination C1sA_KAF4 and Neo5_R2 results in band size of 787bp).

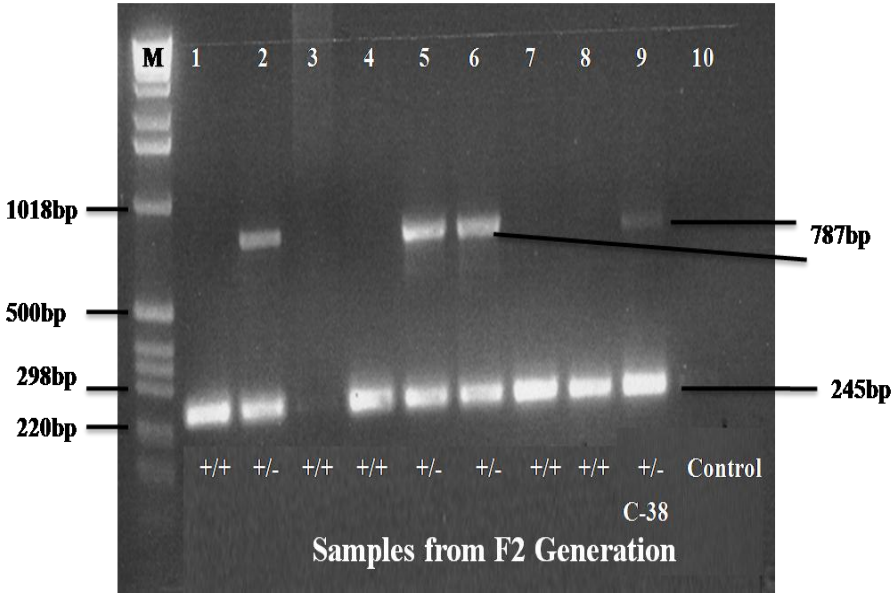

Figure 3.16 Genotyping results from F2 generation. Lane M represents 1Kb DNA ladder. Lane 1, 3, 4, 7, and 8 represents DNA from individual homozygous for the wild-type allele (Primer combination C1sA_KAF4 and C1sA_KAR2 results in band size of 245bp). Lane 2, 5, 6 represents DNA from individual heterozygous for the C1sA polymorphism (Primer combination C1sA_KAF4 and Neo5_R2 results in band size of 787bp). Lane 9 represents DNA from C-38 heterozygous mouse in F1 generation, used as positive control (Primer combination C1sA_KAF4 and Neo5_R2 results in band size of 787bp).

Surprisingly there was no C1sA-/- homozygous gene deficient mice after intercrossing the C1sA-/+ heterozygous mice. It was confirmed again that the clone 61 had the homologous recombinant event by restriction digest analysis using appropriate enzymes and by sequencing the genomic DNA from clone 61.

In order to verify the genotyping, the PCR products were sent for sequencing and also performed restriction digestion using specific XhoI enzyme on genotyped PCR product to confirm that the heterozygous mice are real heterozygotes and to confirm genotyping primers amplifying right product (Section III.1.3.3.4.1). To make sure that the genotyping approach is providing us with correct data phenotypic analysis were performed on the transgenic mouse serum using C1sA specific antibodies (Section III.1.3.3.4.2) but disappointingly only found wild type and heterozygous serum and the results agrees with the genotyping.

From all these results it could be concluded that there might be two reasons why C1sA-/- homozygous mice are not in existence, the first hypothesis is that gene deficient mice gene is lethal in uteri and the other hypothesis is that gene for gene deficient mice cannot be transmitted by sperm (Section III.1.3.3.4).

III.1.3.3.4 Verification of the results obtained from genotyping

III.1.3.3.4.1 Restriction digest analysis of genotyped PCR products

To check that the 245bp wild type band seen in the heterozygous mice samples was a specific C1sA product and not a C1sB product, a restriction analysis of the PCR products was done by using XhoI enzyme. C1sA contains XhoI site in the amplified region, which is absent in the corresponding region of C1sB.

The PCR reaction was carried out using the primer pair C1sA_KAF4 and C1sA_KAR2 which results in band size of 245bp. As in the C1sA targeting construct exon 2 was eliminated along with exon 3and 4 to insert the neomycin cassette, the XhoI enzyme is located between the exon 1 and exon 2 on the mouse C1sA gene (figure 3.3 and the reverse primer C1sA_KAR2 is situated in exon 2 and will only amplify wild type allele (figure 3.17). So the question arises here if the heterozygotes are truely heterozygotes they will give a band of 165 bps and 80bps after digestion with the enzyme XhoI (figure 3.17). As it can be concluded from the restriction digest results that wild type band corresponds to C1sA allele and not to C1sB, hence there is no homozygous mice generated after intercrosses.

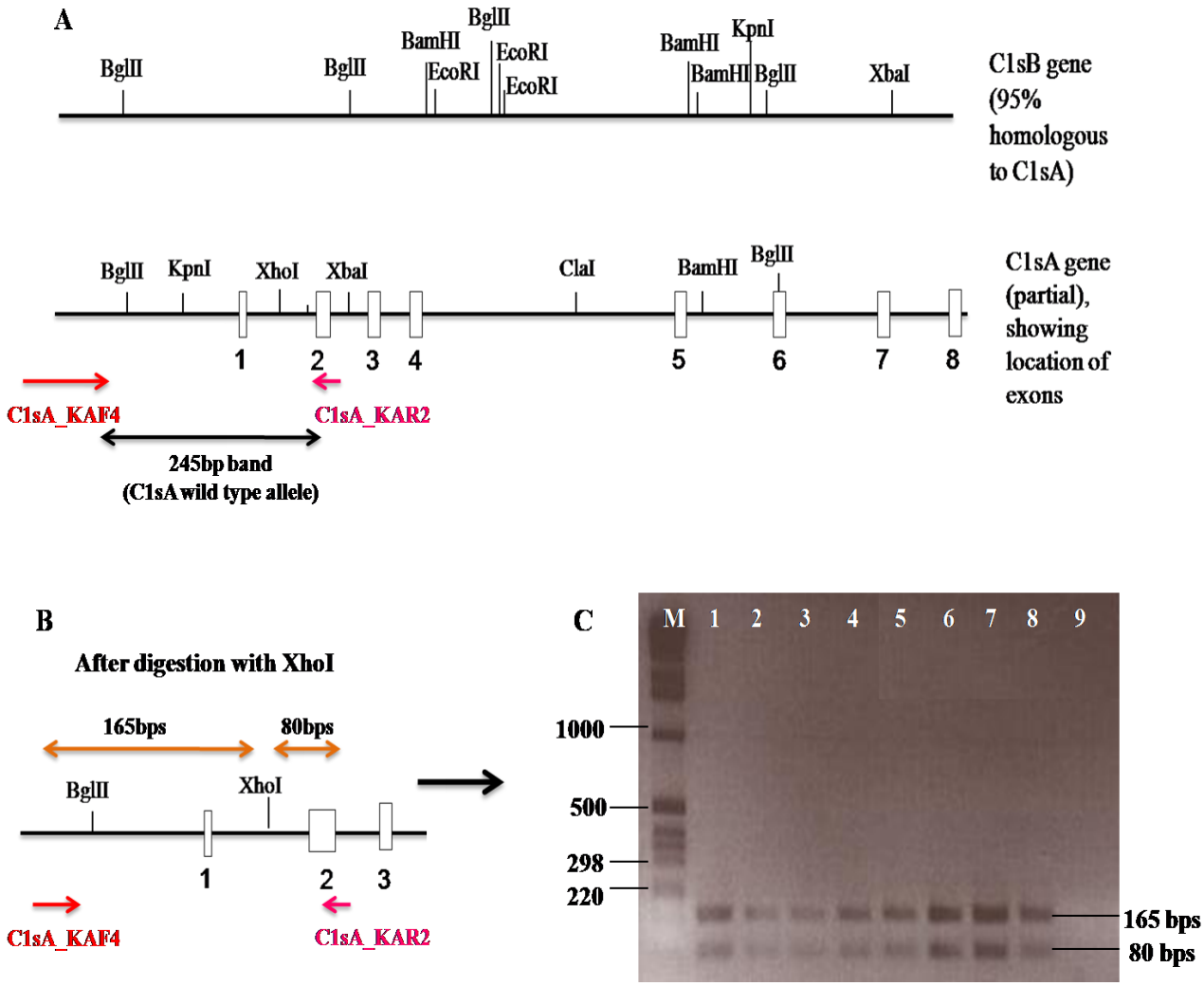

Figure 3.17 Restriction analysis of genotyped PCR product. (A) C1sA gene showing the location of XhoI enzyme and the position of primers used for genotyping (C1sA_KAF4 and C1sA_KAR2 results in band size of 245bp). The corresponding C1sB gene also shown **(B)** shows the corresponding size of fragments generated after XhoI digestion (165 bps and 80 bps). **(C)** 2% gel electrophoresis image showing the bands of 165 bps and 80 bps after digestion of genotyped heterozygous and wild type mice PCR products. Lane M represents 1Kb DNA ladder, lane 1-6 the heterozygous samples and lane 7 & 8 the wild type samples and lane 9 is negative control.

III.1.3.3.4.2 Analysis of transgenic mouse serum

Phenotypic characterisation of heterozygous mice was done to determine the phenotype weather they are heterozygous or homozygous mice. The homozygous mice will not give the band corresponding to C1sA as the mice should be deficient of C1sA allele whereas the heterozygous mice will give a C1sA band showing that C1sA allele is present in the heterozygous mice. In order to perform the phenotypic analysis a serum was collected from the transgenic mice (figure 3.18 A). The serum was run through 12% SDS-PAGE gel electrophoresis and Western blot analysis was performed using C1s specific antibodies, the primary antibody was sheep anti human polyclonal C1s antibody and the secondary antibody was donkey peroxidase labelled anti-Sheep IGg. The antibodies recognises and detects the expected 85kDa protein band on non reducing gel (figure 3.18 B) and a 58kDa heavy chain and 28kDa light chain protein band on reducing gel (figure 3.18 C) representing the C1sA. The results from Western blotting confirmed that C1sA protein was present in the serum collected from transgenic mice and there were even not single mice which showed the lack of C1sA protein thus concluded from this experiment that mice generated from intercrosses between heterozygous mice fail to generate a homozygous mice.

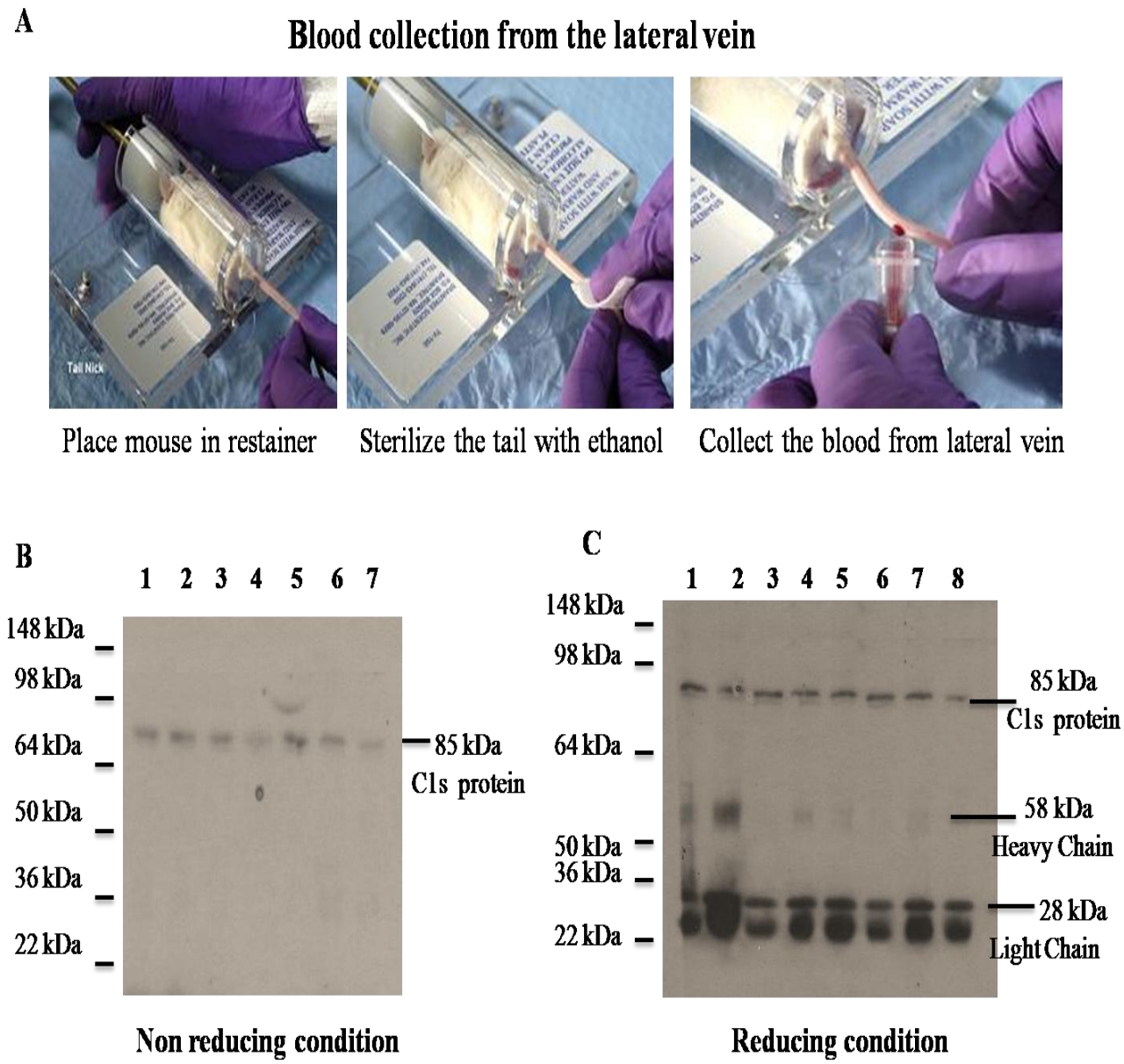

Figure 3.18 Phenotypic analyses on transgenic mouse serum. (A) Schematic illustration of blood collection from the lateral vein of mouse. **(B)** Western blot analysis show a band of 85kDa when serum was run on 12% non-reducing SDS-PAGE gel. Sample 1 represents wild type and sample 2-7 represents heterozygous mouse **(C)** Western blot analysis showing a band of 58kDa (heavy chain) and 28 kDa (light chain) on 12% reducing gel. Sample 1 is wild type and samples 2-8 are heterozygous mouse.

After confirming the genotyping results by doing above mentioned experiments, there comes a stage where four possible hypotheses can arise which might be able to describe us why it is unable to obtain homozygous C1sA mice after intercrossing the C1sA heterozygous mice. The hypotheses are described below briefly and in section III.1.3.3.5 in detail.

Hypothesis 1: states that there might be a genotyping error means that miscalling the C1sA gene deficient mice as C1sA heterozygous mice and the expected ratio will be 3:1. 91offsprings were genotyped after intercross between the heterozygous mice. Chi square analysis was performed. The Chi square analysis showed a significant difference between the numbers of observed and expected heterozygous mice and states that the hypothesis is not in line with the genotyping data.

Table 3.1 Stratified genotype of all the intercrosses between C1sA-/+ mice in presence of 3:1 ratio

Genotype	**Observed**	**Expected**
C1sA+/+	34	23
C1sA-/+	57	68
C1sA-/-	0	0
Total	91	91
$\chi 2$ test valve 7.04	$p < 0.01$	

Hypothesis 2: states that the generation of C1sA-/- mice is lethal in uterus and give rise to a ratio of 2:1 (discussed in detail in section III.1.3.3.5).

Hypothesis 3: states that the C1sA-/+ males are infertile and cannot transmit the C1sA deficient gene. The expected ratio in this case is 1:1(discussed in detail in section III.1.3.3.5).

Hypothesis 4: states if the intercrosses between heterozygous mice give rise to the expected results (25% wildtype, 25% homozygous and 50% heterozygous mice), discussed in detail in section III.1.3.3.5.

III.1.3.3.5 Heterozygous and wild type ratio in presence of homozygous mice lethality (2:1 ratio instead of 1:2:1 expected Mendelian ratio)

Mendel's law of segregation states that each trait of a species is represented in the somatic cells by a pair of units, now known as genes, which are segregated during meiosis so that each gamete receives only one gene for each trait. In any monohybrid crossing, the possible ratio for the phenotypic expression of a particular dominant trait is 3:1, whereas the genotypic ratio of pure dominants to hybrids to pure recessives is 1:2:1.

According to the above mentioned law of segregation, a 1:2:1 ratio was expected after intercrossing two C1sA-/+ heterozygous mice but unfortunately may be due to embryonic death or lethality (meiotic drive) or due to segregation distortion an unusual segregation ratio of 1:2 with only

wild type (C1sA+/+) mice and heterozygous (C1sA-/+) mice but no homozygous (C1sA-/-) mice as illustrated in figure 3.19.

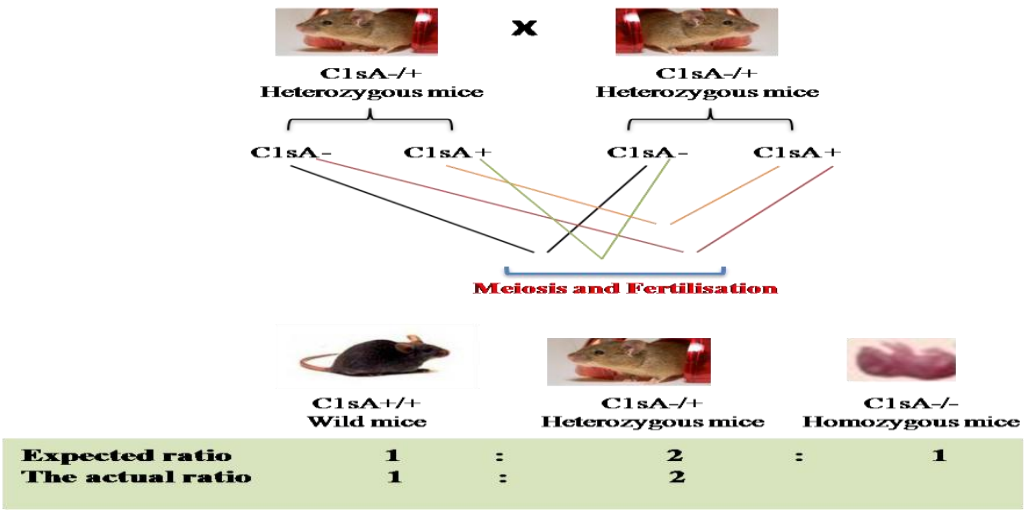

Figure 3.19 A 1:2 ratio results after intercrossing C1sA-/+ mice giving only wild type and heterozygous mice

The tail DNA genotyping revealed the absence of mice homozygous for C1sA mutation from intercrosses between C1sA-/+ heterozygotes and the ratio (1:2) deviates from the expected Mendelian ratio of 1:2:1(Table 3.1).

Table 3.2 Genotyping of all the intercross between C1sA-/+ mice

Number of mice	Genotype	Number	Percentage
91	C1sA+/+	34	37.3%
	C1sA-/+	57	63%
	C1sA-/-	0	0

Table 3.2 Genotyping of all the intercross between C1sA-/+ mice

Number of mice	Genotype	Number	Percentage
91	Male (♂)	44	48.3%
	Female (♀)	47	52%

91 offsprings were genotyped from an intercross between C1sA-/+ mice that yielded only heterozygous and wild type mice. In the absence of lethality, 25% of offspring were expected to be C1sA-/-. Chi square analysis showed a highly significant difference between the numbers of mice observed and those expected based only on Mendelian inheritance assuming no lethal effects of the deficiency ($p < 0.0001$) in Table 3.2.

Table 3.3 Stratified genotype of all the intercross between C1sA-/+ mice (1:2:1 ratio)

Genotype	Observed	Expected
C1sA+/+	34	23
C1sA-/+	57	45
C1sA-/-	0	23
Total	91	91
$\chi 2$ test valve 31.46	$p < 0.0001$	

Although the heterozygous intercrosses yielded no homozygous mice, noticed an abnormal segregation ratio distortion, defined as a significant

different approach from 1:2:1expected Mendelian ratio. Mysteriously, it is noticed that a segregation distortion preferred *C1sA-/+* mice over wildtype C1sA+/+ mice. All the generation of intercrosses between *C1sA-/+* mice give rise to 63% *C1sA-/+* mice and 37% *C1sA+/+* mice (n = 27), in the presence of homozygous lethality (χ^2 = 0.44, *P* = 0.6) (Table 3.3). Chi-squared analysis was used to determine the statistically significant valve comparing values to expected Mendelian ratio (1:2) in the presence of homozygous lethality (Table 3.3).

Table 3.4 Intercross between C1sA-/+ mice and significant values in the presence of homozygous letality (1:2 ratio)

Genotype	**Male ##**	**Female #**	**Total***
C1sA+/+	17(39%)	17(36%)	34(63%)
C1sA-/+	27(61%)	30(63%)	57(37%)
C1sA-/- Total	0 44	0 47	0 91
Cumulative χ^2 P Value	0.82 0.4	0.78 0.4	0.44 0.6

Thus the intercross between C1sA-/+ mice exhibit segregation distortion consistant with in homozygous lethality. Another possibility that can be considered was that C1sA- sperm are infertile, which would lead to a 1:1

ratio of heterozygote's and wild type mice with no homozygous mice as illustrated below in figure 3.20.

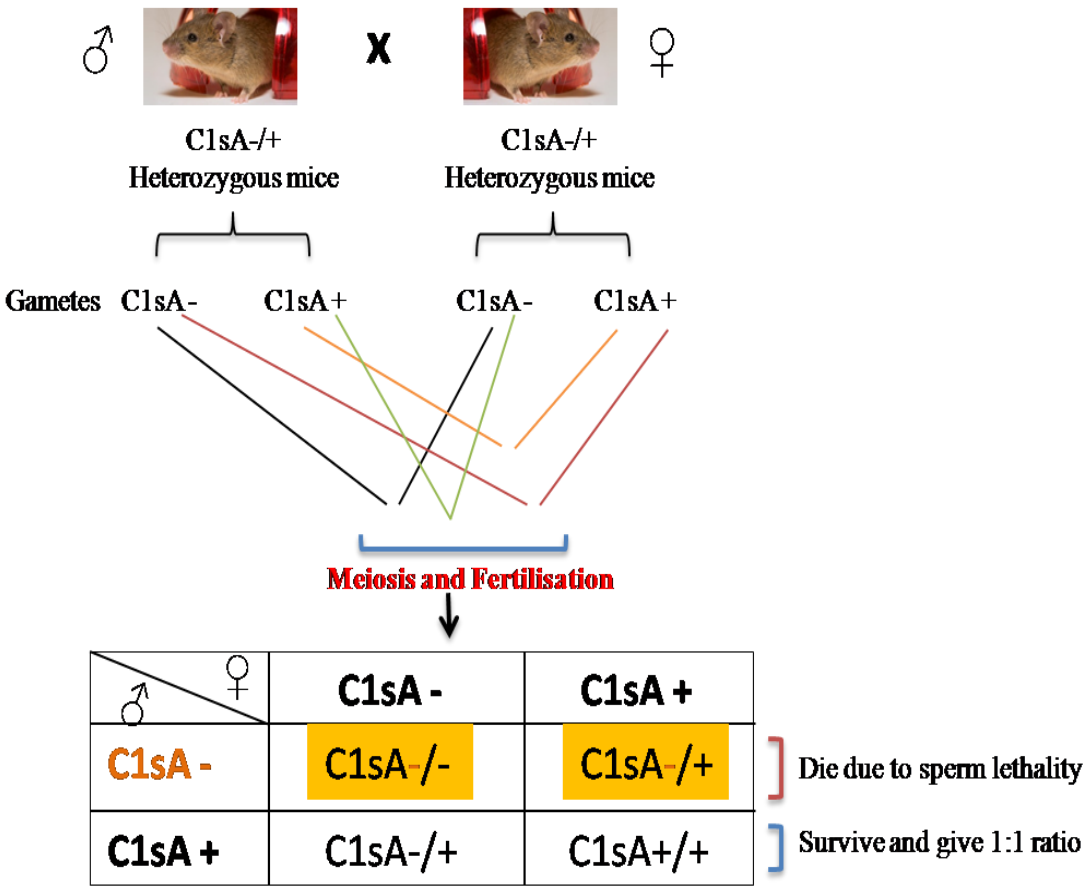

Figure 3.20 1:1 Ratio due to gene not transmitted by sperm

This hypothesis is inconsistent with the observed 1:2 ratio.

Further, crossing the C1sA-/+ (heterozygous) males with wild type females gave the expected 1:1 ratio of heterozygous and wild type progeny, illustrating that C1sA- sperms are viable.

In the end it can be concluded that my genotyping data is entirely consistent with the lethality of C1sA-/- gene deficient mice.

As evident from the data, no C1sA-/- mice survived after intercross and Chi- square (x2) analysis showed a highly significant difference between the numbers of mice observed and those expected based on normal Mendelian ratio of inheritance (1:2:1) $p< 0.0001$ whereas the observed ratio was entirely consistent with 1:2 ratio (wild type: heterozygous) ratio expected if the homozygous state is lethal ($p> 0.5$).

Chapter IV

Analysis of the location of C1sA and C1sB biosynthesis and identification of the cell types expressing C1sA and C1sB

IV.1 Results

C1r and C1s are the serine proteases of the first component of the classical activation pathway of the complement system. In the mouse, the genes for C1s and C1r genes are duplicated and present in two gene clusters (C1sA and C1rA and C1sB and C1rB). C1sA and C1rA are mainly expressed in the liver and are considered to be the orthlogous of the human complement serine proteases C1s and C1r respectively. The C1sA and the C1rA genes encode the relevant classical pathway serine protease of the mouse. In contrast, C1sB and C1rB were previously shown to be exclusively expressed in testis tissue (Garnier *et al.*, 2003).

In order to confirm the sites of biosynthesis and the cell types expressing C1sA and C1sB, two male and female mice were dissected to obtain organs, such as the Large Intestine, the Small Intestine, the Thymus, the Lung, the Heart, the

Spleen, the Kidney, the Testis, the Brain and the Ovary. One set of organs was used for the extraction of total RNA (using Trizol reagent from Invitrogen (Section II.2.3)) for the subsequent synthesis of cDNA (Section II.2.2.2), to be used as templates in Real time PCR analysis. The other set of organs was used for in situ hybridization analysis.

IV.1.1. Analysis of C1sA and C1sB gene expression by quantitative RT-PCR (qRT-PCR)

C1sA and C1sB cDNA is analysed by quantitative PCR using a Light Cycler machine (Roche) to monitor the incorporation of SYBR Green I into the PCR products in real time. Primers were chosen in regards of divergences between the highly conserved C1sA and C1sB genes to avoid any similar detection.The primers used to amplify C1sA gene in the various cDNAs obtained from various mouse organs were mC1sA_F1 and mC1s_rev2 (amplifying a PCR product of 278bps, see Figure 4.1 A and C) and primers used to express C1sB gene were mC1sB_F1 and mC1s_rev1 (amplifying a 316 bp PCR product, see Figure 4.1 B and C).

(A)

gcccaccatg catggggaga tcctgtcccc taactatccc caggcgtacc ccaatgacgt cgtgaaatct tgggacattg aagtcccaga
ggggtttggg attcacctct actttaccca tgtggacata gagccgtcag agagctgtgc atacgactca gtgcagataa tctcaggagg
catcgaggaa gggagactct gtggccagaa gaccagcaag agccccaact cccccattat agaagagttt cagttcccat acaataaact
tcaggtggtc tttacctcag acttctccaa cgaagaacgg tttacggget ttgcagcata ctacactgcc atagacataa atgaatgcac
agattttaca gatgtcccct gtagccactt ctgcaataac ttcattggtg gatacttctg ctcctgtccc ccagaatact tcctccacga
tgacatgagg aattgtggag tcaactgtag tggagatgtg ttcactgccc tgattgggga gatctcaagt cccaattatc ccaatccata

cccgga**gaac tcaaggtgtg aataccagat tcag**ctgcag gagggcttcc aagtggtggt gacgatgcaa agagaagatt ttgatgtgga gcctgctgac tcagagggga actgccctga cagtttaact

(B)

gcccaccatg catggggaga tcctgtcccc taactatccc caggcgtacc ccaatgacgt cgtgaaatct tgggacattg aagtcccaga ggggtttggg attcacctct actttaccca tgtggacata gagccgtcag agagctgtgc atacgactca gtgcagataa tctcaggagg catcgaggaa gggagactct gtggccagaa gaccagcaag agccccaact cccccattat agaagagttt cagttcccat acaataaact tcaggtggtc tttacctca**g acttctccat cgaagaacag tttaccggct** ttgcagcata ctacactgcc atagacataa atgaatgcac agattttaca gatgtcccct gtagccactt ctgcaataac ttcattggtg gatacttctg ctcctgtccc ccagaatact tcctccacga tgacatgagg aattgtggag tcaactgtag tggagatgtg ttcactgccc tgattgggga gatctcaagt cccaattatc ccaatccata cccggagaac tcaaggtgtg aataccagat tcaactgcag gagg**gcttcc aagtggtggt gacgat**gcag agagaagatt ttgatgtgga gccctgctgac tcagagggga actgccctga cagtttaact

(C)

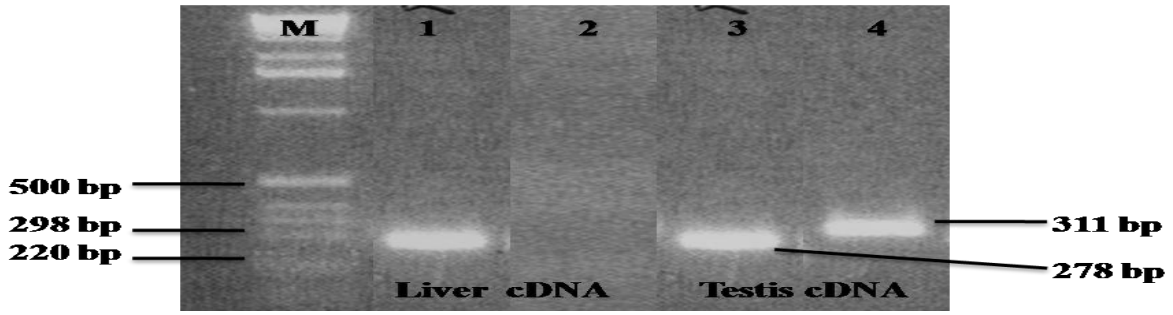

Figure 4.1 Expression of C1sA and C1sB. (A) Putative sequence used for C1sA specific primers; mC1sA_F1(Blue) & mC1s_rev2 (pink) and **(B)** putative sequence used for C1sB specific primers; mC1sA_F1(Green) and mc1s_rev1(Purple). C1sA and C1sB sequence is 90% similar with few difference in bases. The differences are shown in red colour. **(C)** Touchdown PCR on C1sA and C1sB gene. Lane 1 shows that C1sA is expressed in Liver (using C1sA specific primers) while lane 2 C1sB is not expressed in liver (using C1sBspecific primers), lane 3 and 4 shows expression of C1sA and C1sB in testis (using C1sA and C1sB specific primers).

Fourty cycles of amplification were performed; initially the annealing temperature was gradually reduced from initially 70°C to 58°C during the first 15 cycles and was kept constant at 58°C thereafter (Section II.2.2.3.1). The fluorescence signal was detected at the end of each cycle, and results are

analyzed using the Fit Points option in the LDCA software supplied with the machine (Wittwer. C. T. *et al*., 1997). The melting curve analysis was used to verify the specificity of the products (Ririe. K. M. *et al*., 1997). Standard curves were established for each analysis using serial dilutions (1, 1/5, 1/25 and 1/125) of the pooled cDNA.

Results obtained from qRT-PCR analysis of mouse tissue also confirmed that C1sA expression is most abundant in the liver obtained from both male and female, followed by the lung, the spleen, the gonads, the thymus, the large intestine, the heart, the kidney, the small intestine and the brain. C1sA expression is higher in female gonads compared to male (Figure 4.3). C1sB is highly expressed in male reproductive tissue whereas the expression of C1sB mRNA was undetectable in other tissues tested including liver, lung, spleen, thymus, large intestine, heart, kidney, small intestine and brain (Figure 4.6). GAPDH was used as an endogenous control to normalize the amount of starting material in each tube (Zhong and Simon., 1999). Figure 4.2 shows the normalised values, mean and standard deviations for both C1sA and C1sB mRNAs.

(A) Normalised values for C1sA gene expression

	1st run		2nd run		3rd run		Mean		SD	
Tissue	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Liver	1.526	2.305	7.911	4.624	5.396	3.298	6.654	3.409	1.778106	1.163272
Lung	2.116	1.044	2.291	1.272	1.325	0.724	1.911	1.013	0.51456	0.275033
Spleen	0.863	0.696	1.112	0.707	0.383	0.340	0.786	0.581	0.370892	0.20857
Gonads	0.365	1.100	0.348	1.251	0.385	0.455	0.366	0.935	0.018484	0.422751
Thymus	0.409	0.216	0.252	0.096	0.128	0.071	0.263	0.128	0.140987	0.077092
Large intestine	0.329	0.099	0.235	0.093	0.143	0.045	0.236	0.079	0.092988	0.029488
Heart	0.085	0.315	0.111	0.358	0.045	0.144	0.080	0.272	0.033441	0.113519
Kidney	0.061	0.105	0.019	0.140	0.032	0.047	0.037	0.097	0.021268	0.047249
Small intestine	0.059	0.206	0.025	0.190	0.012	0.043	0.032	0.146	0.024689	0.090074

(B)Normalised values for C1sB gene expression

	1st run		2nd run		3rd run		Mean		SD	
Tissue	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Liver	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Lung	0.003	0.047	0.000	0.002	0.000	0.004	0.000	0.003	0.000	0.001
Spleen	0.059	0.066	0.000	0.025	0.003	0.027	0.001	0.026	0.002	0.001
Gonads	2.052	0.039	1.214	0.015	1.228	0.009	1.221	0.012	0.010	0.005
Thymus	0.018	0.072	0.019	0.012	0.009	0.006	0.014	0.009	0.007	0.004
Large intestine	0.001	0.003	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Heart	0.000	0.000	0.001	0.002	0.000	0.000	0.000	0.001	0.000	0.001
Kidney	0.003	0.026	0.002	0.006	0.002	0.009	0.002	0.007	0.000	0.002
Small intestine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Brain	0.005	0.005	0.000	0.004	0.000	0.001	0.000	0.002	0.000	0.002

Figure 4.2 Normalised values for C1sA and C1sB gene

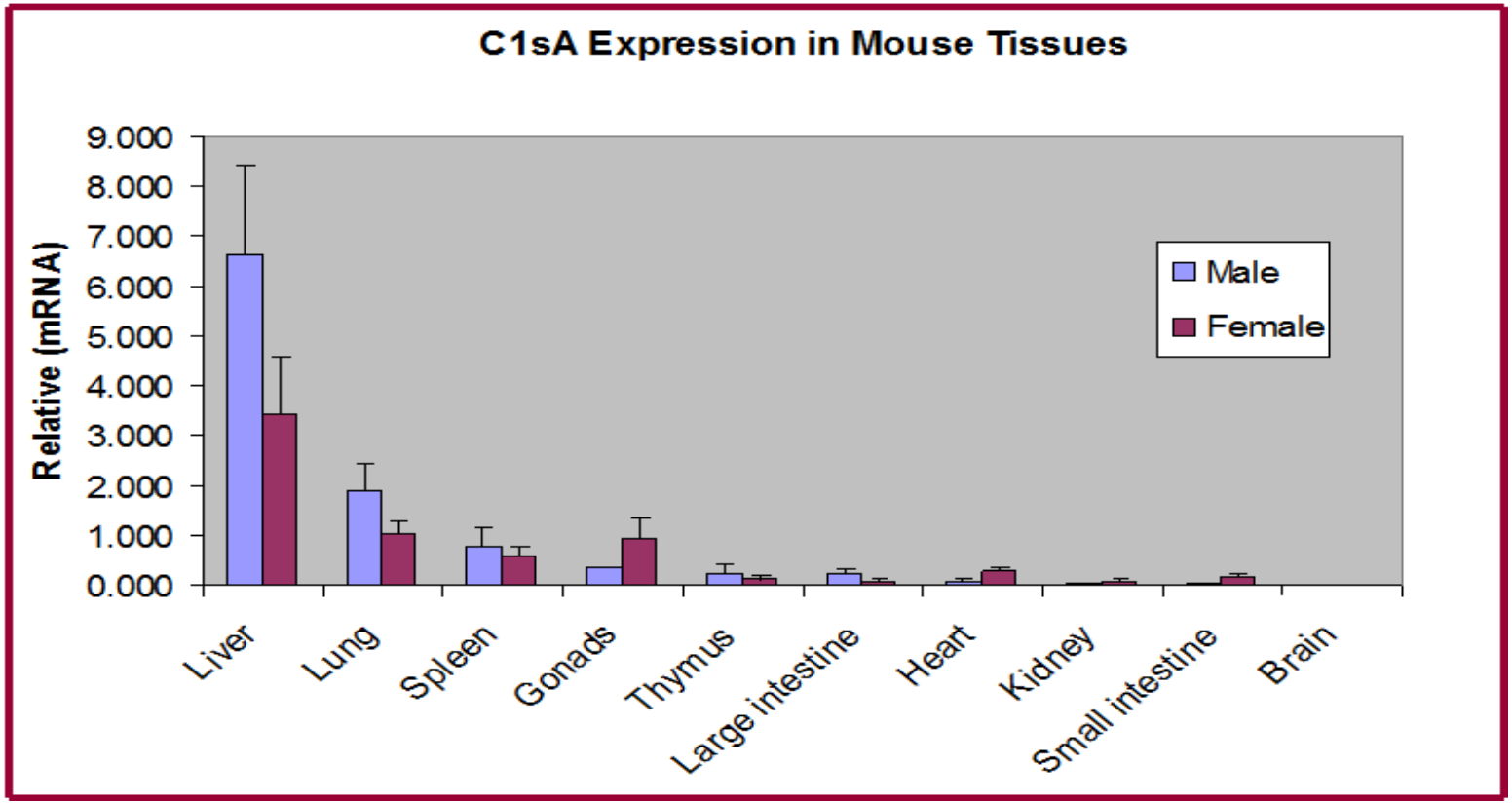

Figure 4.3 The qRT-PCR analysis of C1sA expression in various mouse tissues. cDNA is analyzed by real-time PCR using a Light Cycler. Each bar represents the mean ± S.D. of three separate experiments. The ratio was calculated as *C1sA* gene expression/GAPDH gene expression.Error bars represent the SD.

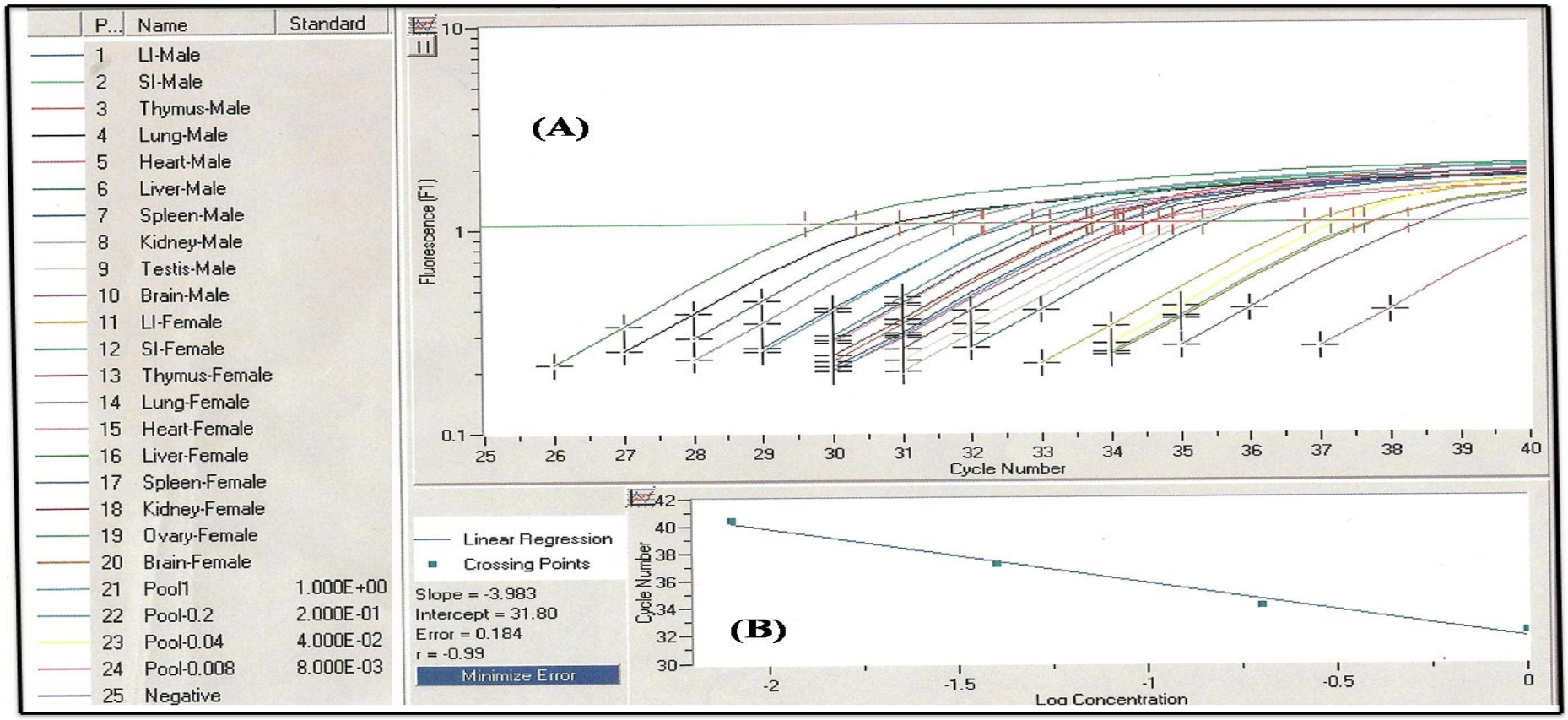

Figure 4.4 (A) Amplification plot of C1sA cDNA. Fivefold serial dilutions of pooled cDNA is amplified by real-time RT-PCR. (B) The standard curve is generated by plotting cycles at threshold fluorescence (Ct) against the logarithmic values of standard RNA amounts. A quantity of standard RNA is expressed as dilution factors (1, 0.5, 0.25, and 0.125). Correlation coefficients (r) and amplification efficiencies (E) are shown.

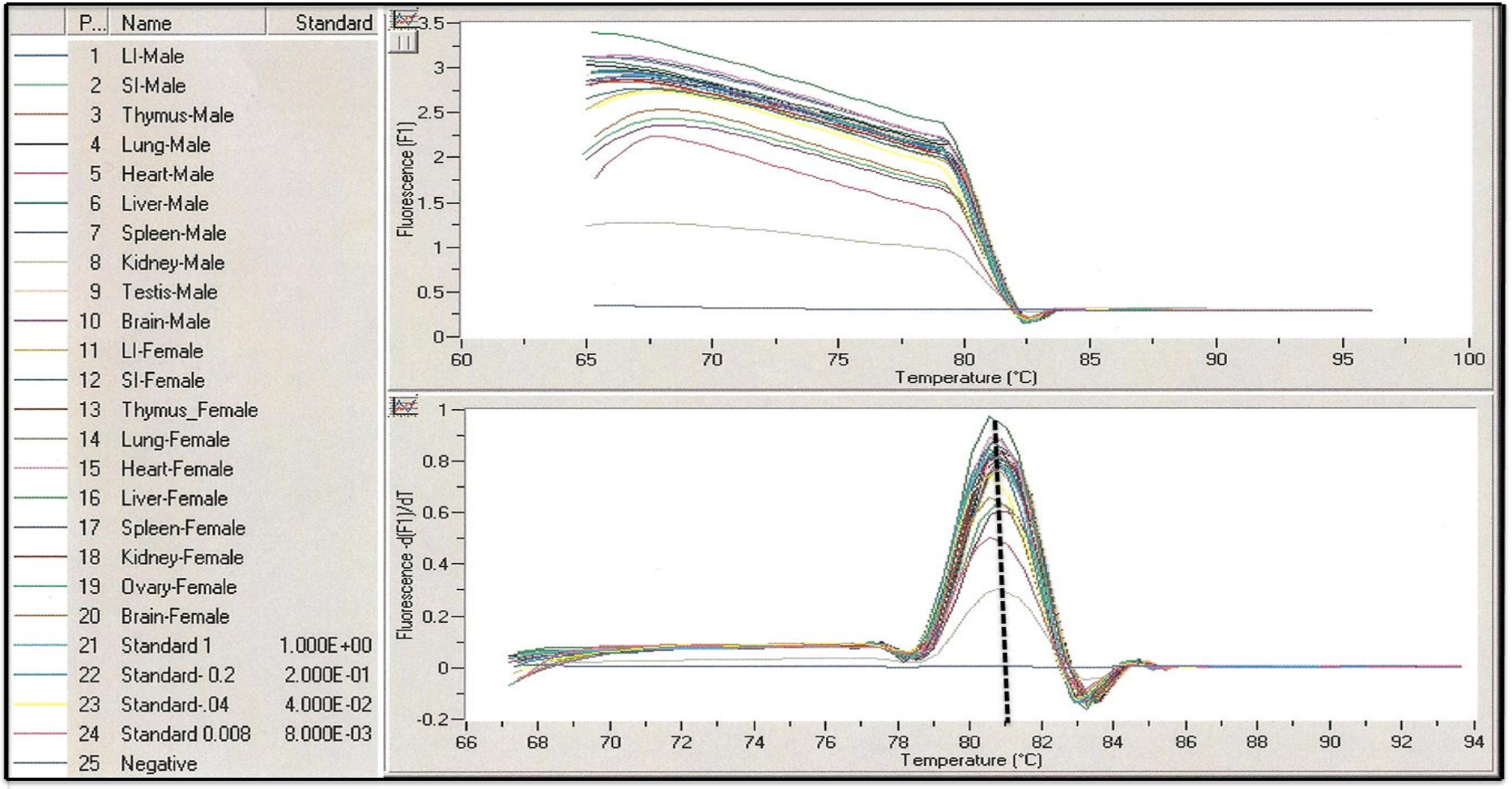

Figure 4.5 Melting curve analysis of C1sA after 40 cycles. The light cycler software also plots the negative derivative of the rate of change of fluorescence vs. temperature (-dI/dT) .The Tm is easily identified by the single peak at 81^{o}C.

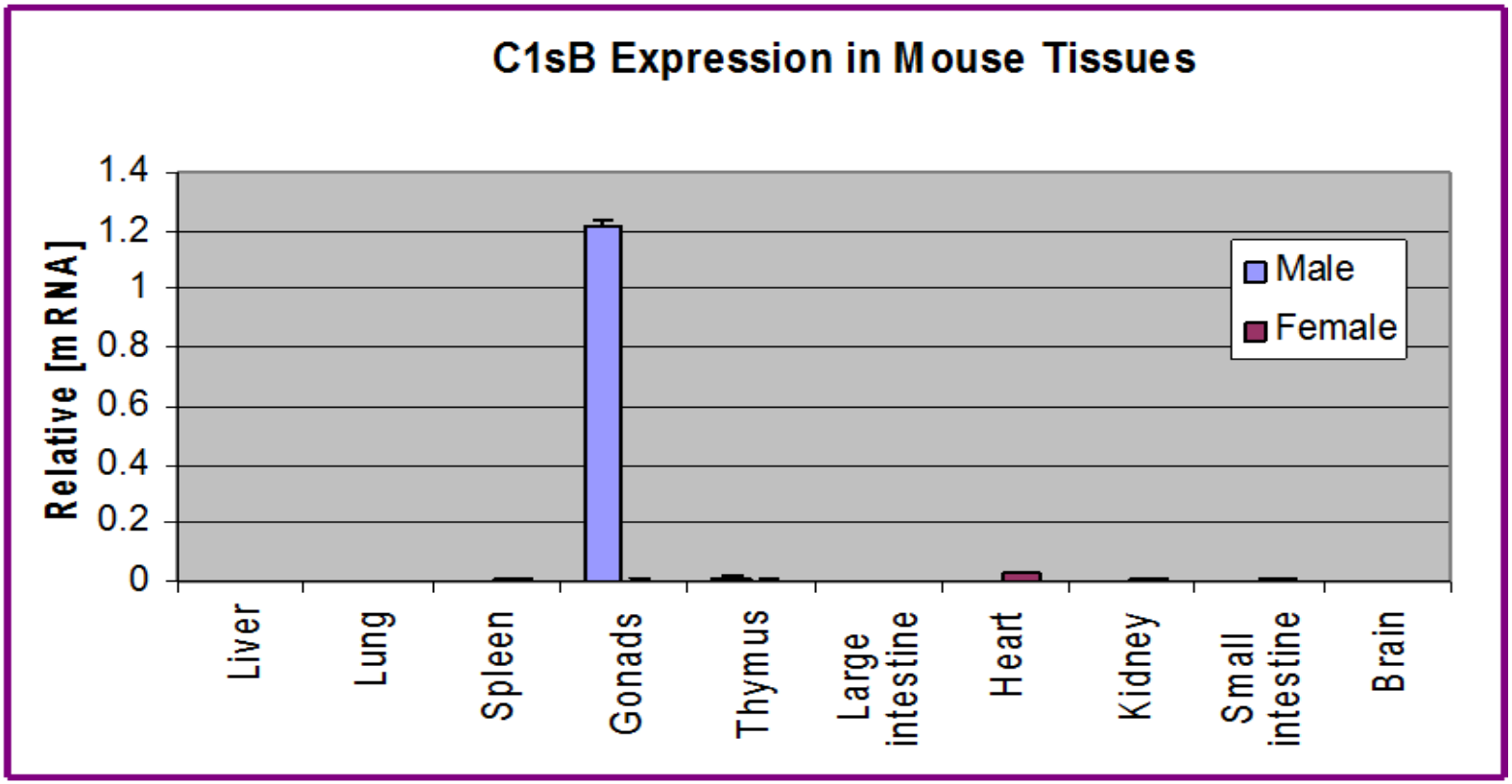

Figure 4.6 The qRT-PCR analysis of C1sB expression in various mouse tissues. cDNA is analyzed by real-time PCR using a Light Cycler. Each bar represents the mean ± S.D. of three separate experiments. The ratio was calculated as *C1sB* gene expression/GAPDH gene expression.Error bars represent the SD.

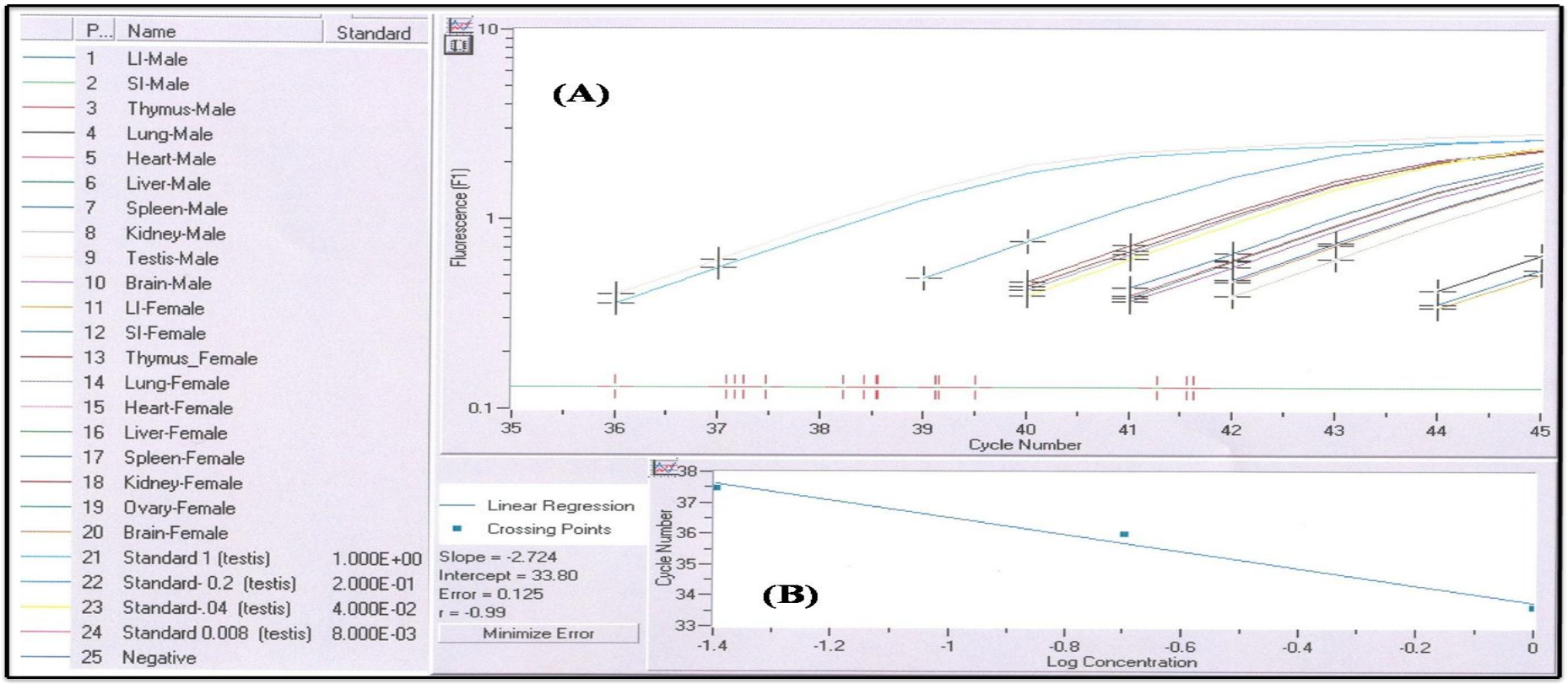

Figure 4.7 (A) Amplification plot of C1sB cDNA. Fivefold serial dilutions of pooled cDNA is amplified by real-time RT-PCR. (B) The standard curve is generated by plotting cycles at threshold fluorescence (Ct) against the logarithmic values of standard RNA amounts. A quantity of standard RNA is expressed as dilution factors (1, 0.5, 0.25, and 0.125). Correlation coefficients (r) and amplification efficiencies (E) are shown.

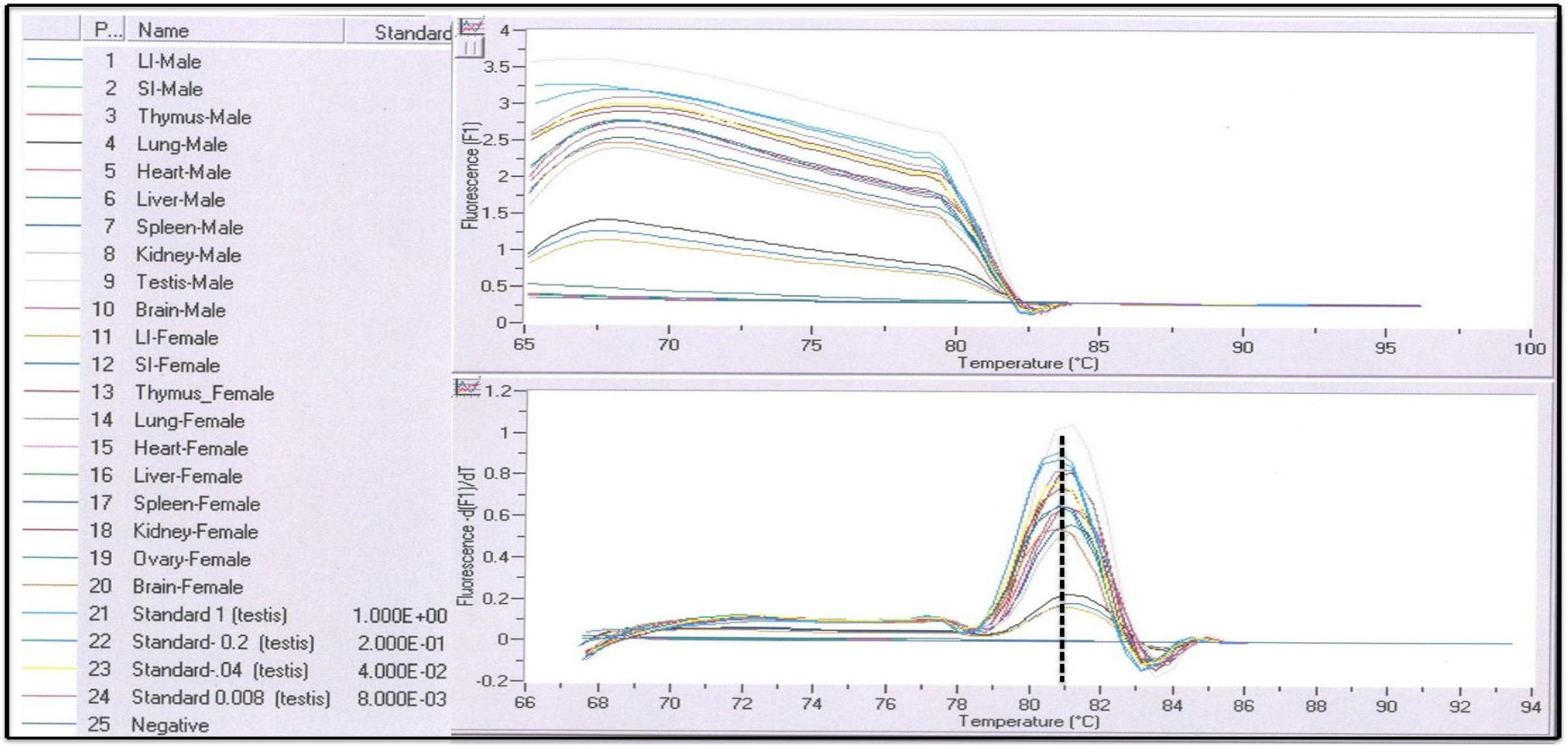

Figure 4.8 Melting curve analysis of C1sB after 40 cycles. The light cycler software also plots the negative derivative of the rate of change of fluorescence vs. temperature (-dI/dT) .The Tm is easily identified by the single peak at 81°C.

IV.1.2. The sites of C1sA andC1sB biosynthesis determined by *in situ* hybridisation

In situ hybridisation, as the name suggests, is a method to localize and detect specific mRNA sequences in morphologically preserved tissue sections or cell preparations by hybridising the complementary strand of a nucleotide probe to the sequence of interest. An *in situ* hybridisation probe can be alternatively labelled in two ways:

1. Radiolabelled probes (e.g., [^{35}S] UTP labelled probe) (which also allows a relative quantification of mRNA expression in the tissue using autoradiography.)
2. Non-radiolabelled probes, antigen-labelling (e.g., digoxigenin), is detected using digoxigenin specific antibodies with the limitations in quantification known from Western blot analysis.

In order to identify the location of C1sA and C1sBin combination biosynthesis in various mouse tissues, both labelling techniques were used.

The C1sA is most abundantly expressed mostly in liver while the C1sB is exclusively expressed in male reproductive tissue as confirmed by our results also of the quantitative real time PCR analysis.

IV.1.2.1 Generation of *In situ* hybridisation probes using ^{35}S labelling (Isotopic Detection) and Digoxygenin (Non-isotopic Detection)

To identify the sites of mRNA expression, ^{35}S-labeled and DIG labelled antisense RNA probes were used for our *in situ* hybridisation study. Briefly in order to generate specific sense and antisense RNA probe template cDNA was generated by PCR and cloned into pGEMTeasy vector. The cDNA templates were sequenced in order to confirm the identity and orientation of PCR generated template for single RNA transcription.

For C1sA probe, a fragment of 336bp was amplified from mouse genomic DNA (M_2K/O DNA) using set of primers mC1sA_fp1 and mC1sA_rp2 and for generation of C1sB specific probe, a fragment of 336 bp was amplified from mouse genomic DNA using primers mC1sB_fp1 and mC1sB_rp2 (Figure 4.9 C). Primers were chosen from regions of maximum dissimilarity, the red shaded area in the sequence (figure 4.9) represents the dissimilarity between C1sA and C1sB. The sequence used for the generation of C1sA and C1sB probes is shown in figure 4.9 A and B respectively.

(A) C1sA Sequence

cctgtg gagtacccac cgagcccttt caagtgcatc agaggatatt tggaggacaa cctgcaaaga ttgaaaattt tccctggcaa gtcttcttta atcaccccag agctagtggg gctcttatta atgagtactg ggtg**ctgacg gctgctcacg ttttg**gagaa aatctccgac ccttaatgt atgtcgggac catgtctgtg aggacaactc tcttggaaaa tgcccagaga ctctacagta aacgtgtctt tattcatccc agctggaaga aagaagatga cccaaacaca cggacaaatt ttgacaatga cattgccctg gtgcagctga aagaccctgt gaaaatggga cccaagtct cccccatctg cctaccaggc acctcctcag agtacaacgt ctcaccgggt gacatggggc tgatctcagg gtggggcagt acagaaaaga **aagtctttgt tatcaacctc a**gaggggcaa aggtaccagt

(B) C1sB Sequence

tctgtg gagtacccac cgagcccttt caagtgcagc agaagatatt cggaggacaa cctgcaaaga ttgaaaattt tccctggcaa gtcttcttta atcaccccac agctggtggg gctcttatta atgagtactg ggtg**ctgacg gccgctcacg ttgt**ggagaa aaactccgac ccttcaatgt atgccgggat cacggctttg agactggctg acttggaaaa tgcccagagg ctctacacca agcgtgtgat tattcatccc ggctggaagg aagacgatga cctaaaccca cggacaaatt ttgacaatga cattgccctg gtccagctga aagaccctgt gaaaatggga cccaagtttt cccccatctg cctaccaggc acctcctcag agtacaacct ctcaccgggt gacatggggc tgatctcagg gtggggcaga acagaaaaga **gactccatgt tatcaacctc** agaggggcaa aaa

(C)C1sA and C1sB amplified Probe

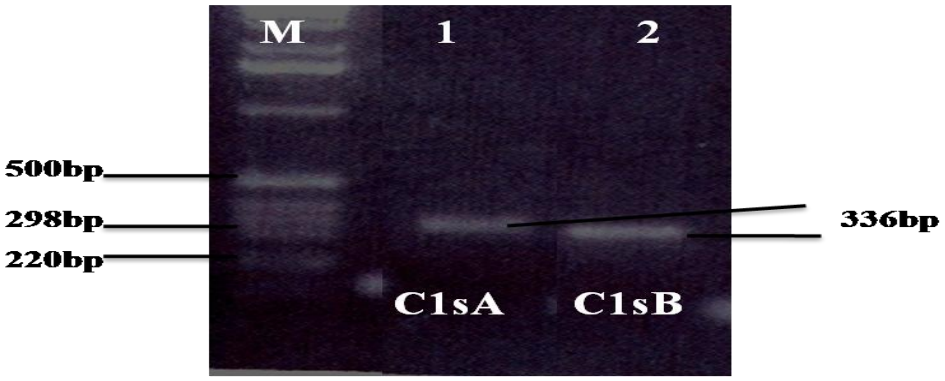

Figure 4.9 (A) Putative sequence used for C1sA specific primers; mC1sA_fp1(pink) & mC1s_rp2 (blue) and **(B)** Sequence used for C1sB specific primers; mC1sA_fp1(purple) and mc1s_rp2 (green). C1sA and C1sB sequence is 90% similar with few difference in bases. The differences are shown in red colour. **(C)** C1sA and C1sB probes amplidied by touchdown PCR. Lane 1 shows 336bp amplified C1sA probe (using C1sA specific primers) while lane 2 336 bp C1sB probe (using C1sBspecific primers). Same probes were used for isotopic and nonisotopic in situ hybridization.

RNA probes, Antisense and sense probes were transcribed from the pGEMT-Easy cloned template using the protocol described by Melton *et al*., 1984 (Section II.2.3.3). T7 and SP6 RNA polymerase are used.

IV.1.2.2. *In situ* hybridisation using ^{35}S-labeled probes (Radiolabel probes)

In situ hybridisation was used to locate the presence of C1sA and C1sB in mouse tissues. Twenty micron thick tissue sections were sectioned using a

cryostat. The tissues were mounted on poly lysine coated slides from VWR. The slides were pre hybridised before hybridisation with ^{35}S-labeled sense and antisense probes. In vitro transcription and [α^{35}S] UTP labelling was described in detail in section II.2.3.3 of methods and materials. For the generation of antisense probe, the template cloned into pGEMT-Easy vector was linerarised by using SalI restriction enzyme and T7 RNA polymerase was used for transcription. For sense probe, the template was cut with NcoI restriction enzyme and for transcription SP6 RNA polymerase was used. Hybridisation using ^{35}S labelled probe is described in detail in section II.2.3.3 of materials and methods. Following the hybridisation and washing steps, slides were exposed to Kodak Bio Max MR X-ray films for 48 hours to detect the signal. Figure 4.10 shows the expression of C1sA in liver, spleen thymus, large intestine tissues after hybridizations. Abundant C1sA mRNA expression was found in liver tissue. The spleen sections hybridised with the C1sA sense probe showed the same results as the spleen sections hybridised with the C1sA antisense probe (Figure 4.10 C). This may due to not washing the slides sufficiently after hybridization and a high background signal seems to be detected which could be due to be due to overexposure of the tissue sections. Moreover it is evident from the sections hybridized with DIG- labelled C1sA sense probe that there are no positive signals in the spleen control sections (Figure 4.11C).

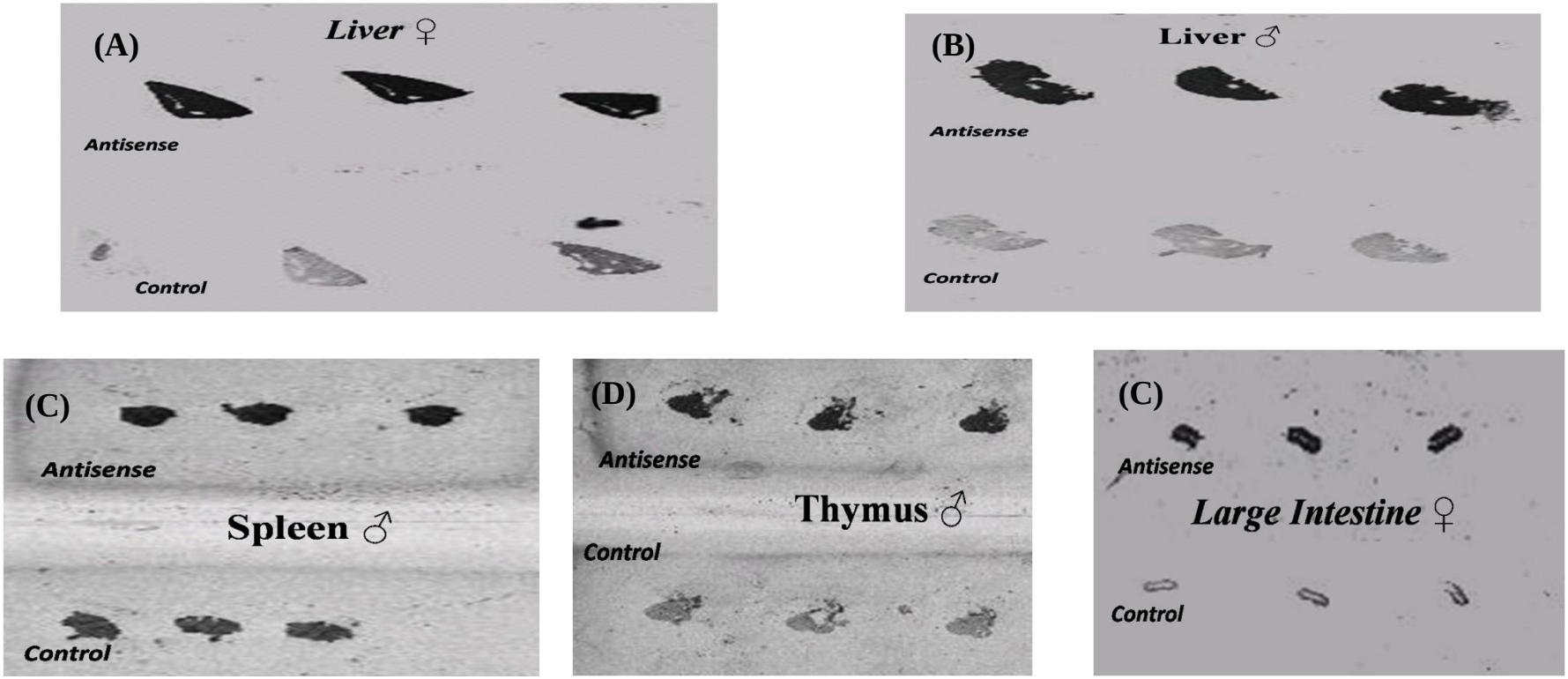

Figure 4.10 Localisation of C1sA mRNA by *in situ* hybridisation in mouse tissue. Cryostat sections (20 µm) were hybridised with ^{35}S-labeled probes and exposed to Kodak Bio Max MR film for 48 hours. A-B shows the mouse liver♀, liver♂, spleen♂, thymus ♂ and large intestine♀ tissues hybridised with C1sA antisense and sense probes. C1sA is largely expressed in liver.

IV.1.2.3. *In situ* hybridisation using DIG labelled probes.

Detection of the sites of mRNA expression, by using non radioactive probes for *in situ* hybridisation has emerged as a reliable technique to study the gene expression at the cytological level. Antisense and sense probes were generated in the same procedure as discussed to generate ^{35}S labelled probes. Twenty micron thick cryostat tissue sections were cut and hybridised with C1sA mRNA antisense and sense probes labelled with digoxygenin (DIG), which is subsequently detected with an anti-DIG antibody coupled to alkaline phosphate (Section II.2.3.3).

For the generation of antisense probe, both C1sA and C1sB, the template cloned into pGEMT-Easy vector was linerarised by using SalI restriction enzyme and T7 RNA polymerase was used for transcription. For C1sA and C1sB sense probe, the template was cut with NcoI restriction enzyme and for transcription SP6 RNA polymerase was used. Hybridisation using DIG labelled probe is described in detail in section II.2.3.3 of materials and methods. After hybridisation and washing steps the slides were incubated with an anti-DIG antibody coupled to alkaline phosphatise and finally the enzymatic reaction is carried out by applying a chromogen solution containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to detect the signal. The sections are then counterstained with haematoxylin and eosin stains and analysed under light microscope and photographs of stained tissues sections

showing C1sA and C1sB expression were taken. Expression of C1sA mRNA was clearly detected in all the sections hybridised with the C1sA antisense probe (figure 4.11 b) for spleen, (figure 4.12 a) for liver, (figure 4.13 b) for ovary and expression of C1sAB mRNA was clearly detected in the testis sections hybridised with the C1sB antisense probe (figure 4.15 a) for testis whereas other tissues doesn't show any C1sB expression (figure 4.14).

Regarding the spleen cell types expressing C1sA, *in situ* hybridisation with antisense C1sA mRNA probe demonstrates strong signals within the white pulp of the spleen (figure. 4.11b). With respect to the cells expressing C1sA message were localised within the T cell region or T lymphocytes (also known as peri arteriolar lymphoid sheath) and B cell region or B lymphocyte follicles of the white pulp (figure. 4.11b) however, not all cells appeared to express the same level of the C1sA mRNA. The splenic sections hybridised with the C1sA sense probe did not show any positive signals (figure 4.11c). In brief the white pulp consists of central T lymphocyte masses or PALS, which contain networks of fibroblastic reticular cells (FRCs) surrounded by a marginal network of B cell follicles or follicular dendritic cells (FDCs).

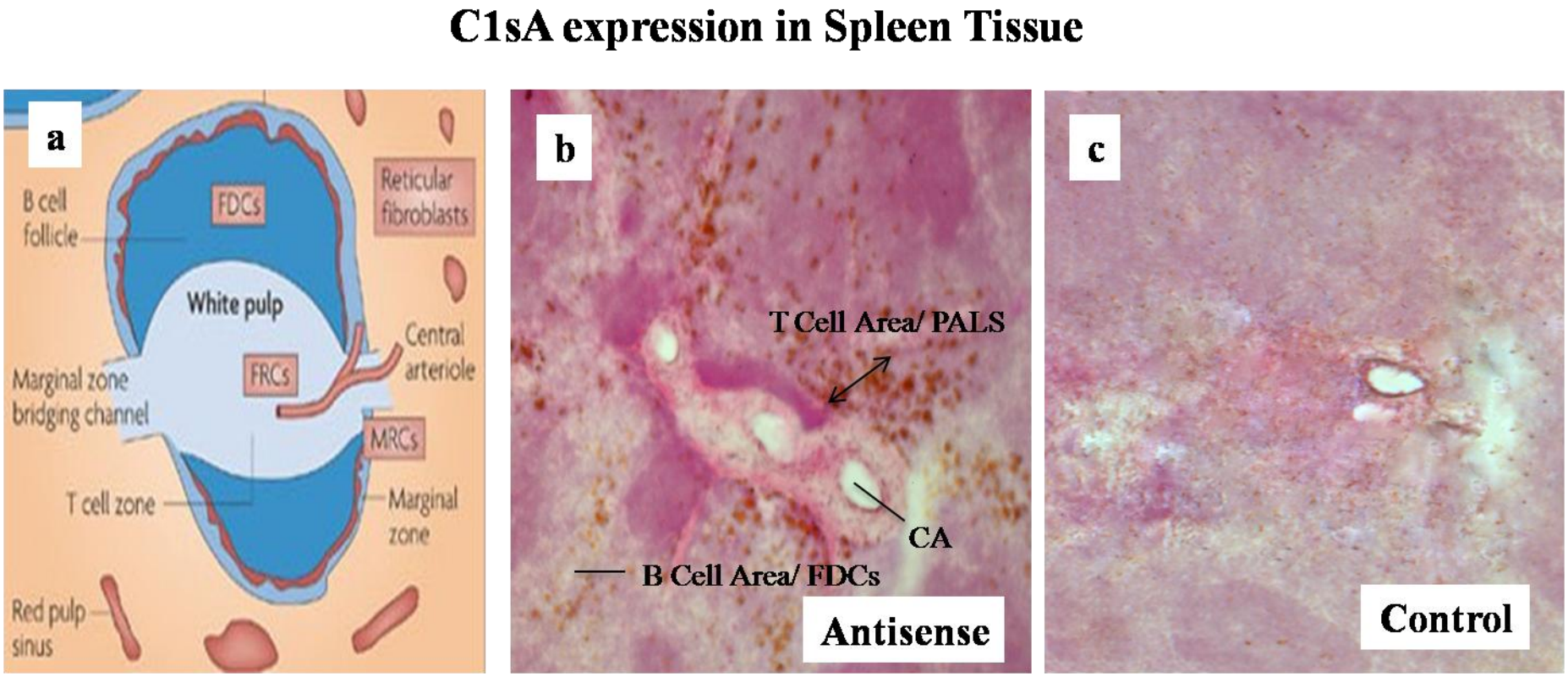

Figure 4.11 Sections of mouse spleen tissue hybridised with DIG-labelled C1sA probe. (a) Illustration of the cells organised in the spleen (b) Magnification view of spleen tissue after hybridisation with C1sA antisense probe (x200). (c) Magnification view of spleen tissue after hybridisation with C1sA sense probe (x200). CA represents centeral arteriol, PALS represent periarteriolar lymphoid sheath, FDCs represents follicular dendritic cells.

Histological examination of the liver tissue obtained from the wild type mice showed that the antisense C1sA mRNA probe predominantly labelled all hepatocytes of the liver (figure. 4.12 a). As expected, the C1sA sense RNA probe did not hybridise to the liver tissue from mice (figure 4.12 b). Interestingly the first component of classical pathway of complement system C1q is expressed in Kupffer cells which are specialised macrophages located in the liver (Morgan. B.P and Gasque. P., 1997) where as other components of complement system such as C3, MASP-2 and MBL are mainly expression in hepatocytes of the liver (Morris. K.M., *et al* 1982; Schwaeble. W. *et al*, 2002 Wanger. S *et al* 2003 and Bouwman. L.H. *et al*, 2005).

To verify the cell types approachable to the C1sA expression in the ovary tissue obtained from the wild type, distribution of the C1sA mRNA was studied histologically by *in situ* hybridisation using C1sA mRNA antisense and sense probes. Expression of the C1sA mRNA was observed all over the ovary tissue but a strong hybridization signal was mainly detected in the granulose luteum cells and theca luteum cells of the corpus luteum (figure 4.13b) while no hybridisation signal was detected in tissue hybridised with C1sA sense probe (figure 4.13c).

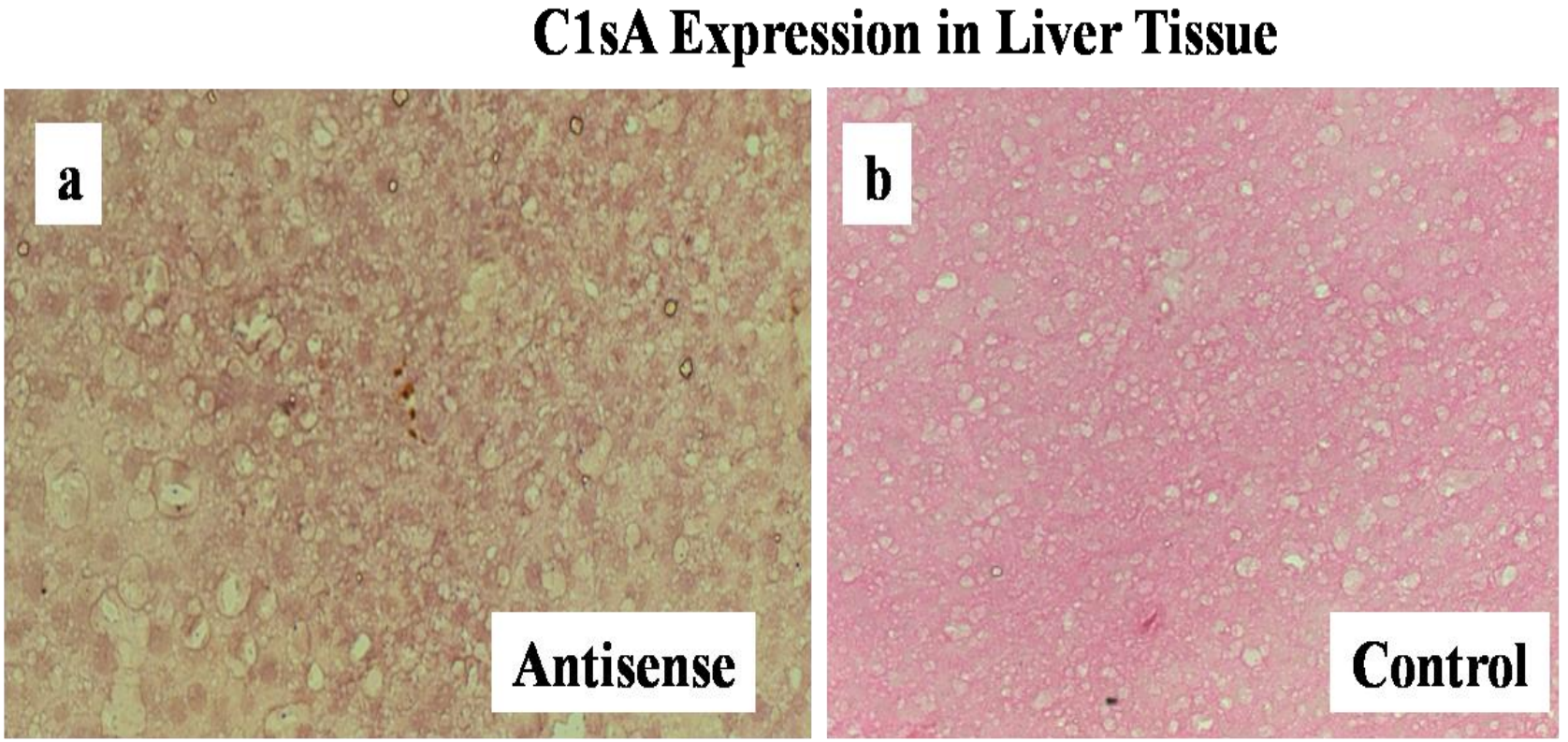

Figure 4.12 Sections of mouse liver tissue hybridised with DIG-labelled C1sA probe. (a) 200x magnification view of liver tissue after hybridisation with C1sA antisense probe. (b) 200x magnification view of liver tissue after hybridisation with C1sA sense probe.

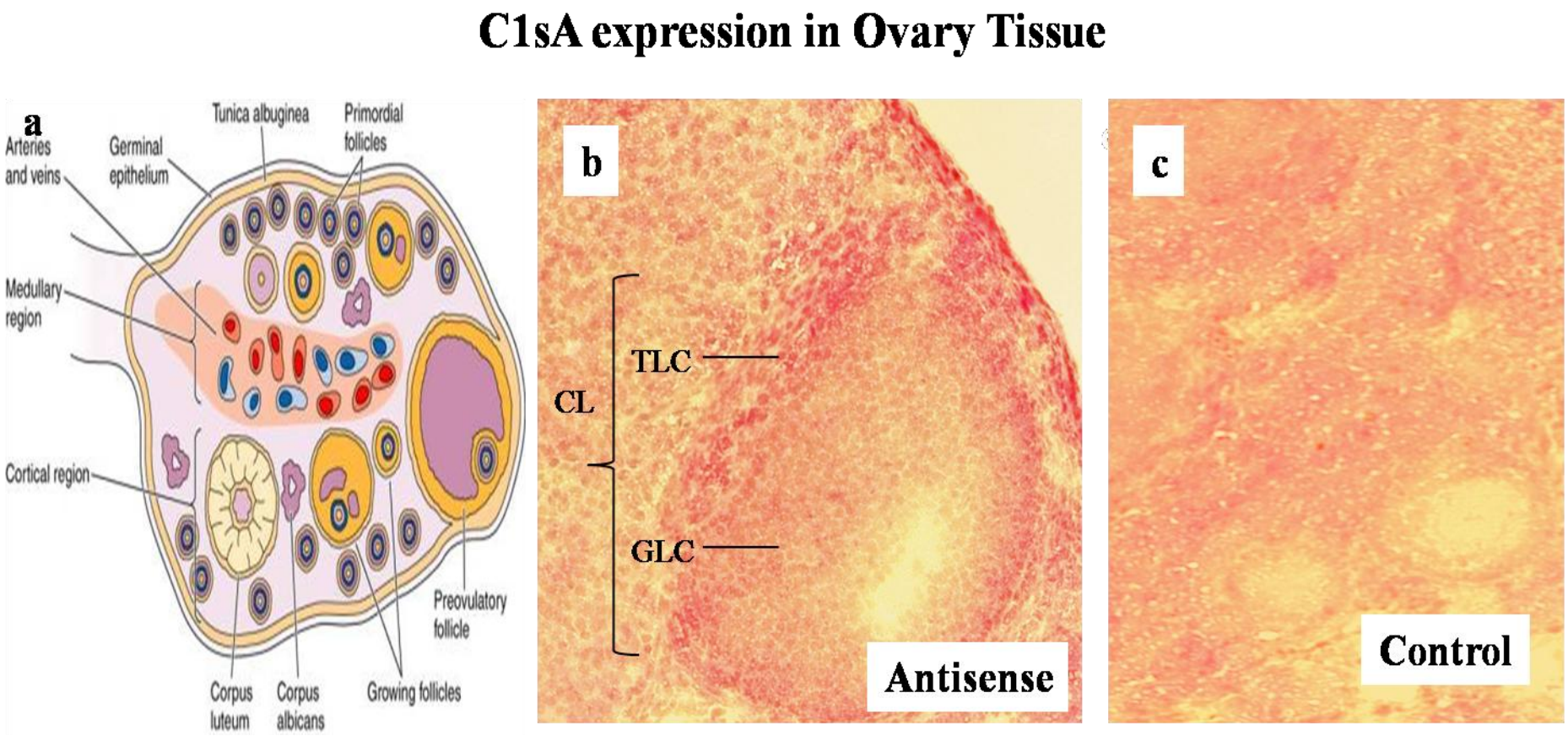

Figure 4.13 Sections of mouse ovary tissue hybridised with DIG-labelled C1sA probe. (a) General histology of Ovary (b) 200x magnification view of ovary tissue after hybridisation with C1sA antisense probe and shows C1sA expression. (c) 200x magnification view of ovary tissue after hybridisation with C1sA sense probe. CS represents Cortical Stroma, CL represents Corpus Luteum, TLC represents Theca Luteum cells and GLC represents Granulose Luteum cells.

The expression of C1sB was examined in mouse testis tissue only. Quantitative analyses and *in situ* hybridisation showed C1sB to be testis-specifically, with no expression detected in other tissues (Figure 4.6 and figure 4.14). Moreover, C1sB mRNA is specifically expressed in semniferous tubules particularly in Sertoli cells (nurse cells) and spermatozoa cells (figure 4.15a). The low level expression of C1sB mRNA was also observed in the Leydig cells. No positive signal was detected in tissues hybridised with sense probe (figure 4.15b).

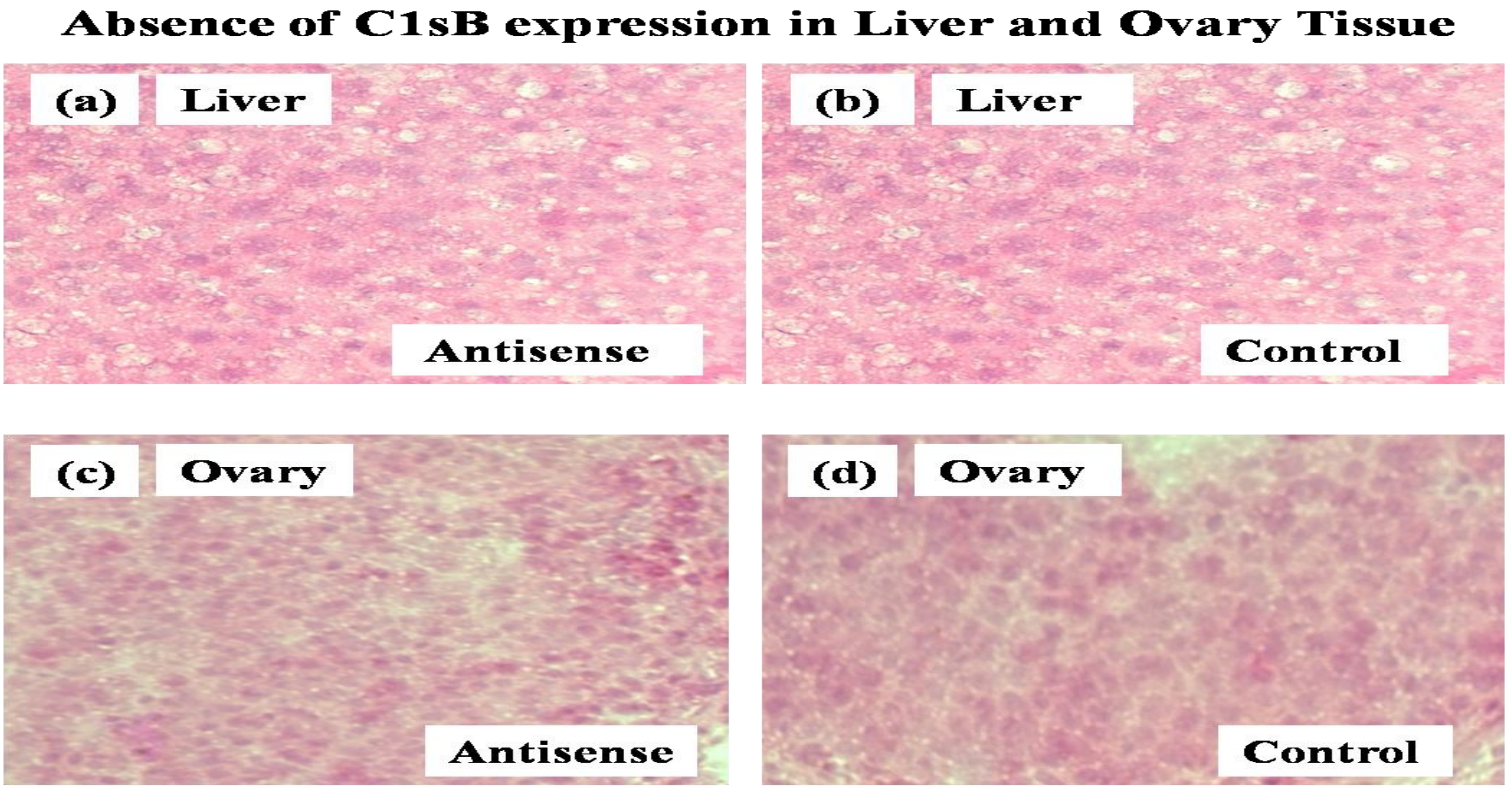

Figure 4.14 Sections of mouse liver and ovary tissue hybridised with DIG-labelled C1sB probe. There is no C1sB mRNA expression in liver and ovary as confirmed by RT-PCR (Fig. 4.6). (a and c) shows 200x magnification view of liver and ovary tissue after hybridisation with C1sB antisense probe respectively whereas (b and d) shows 200x magnification view of liver and ovary tissue after hybridisation with C1sB sense probe respectively.

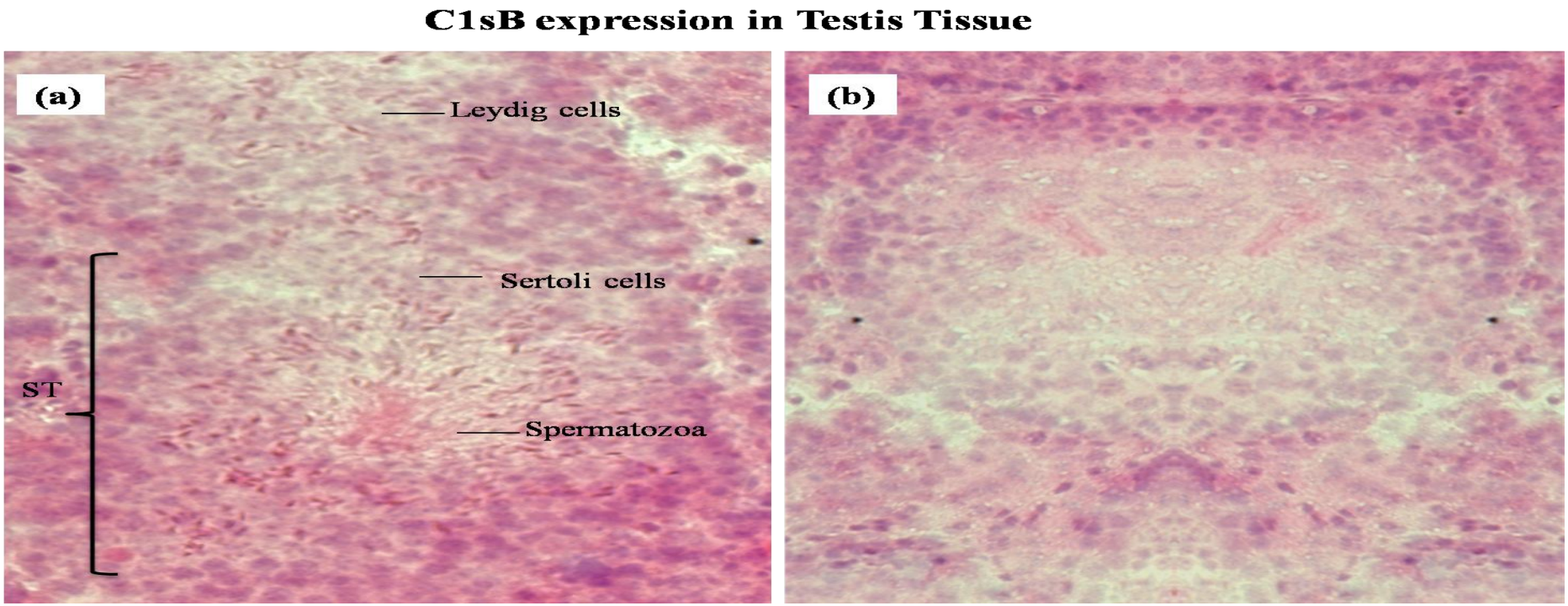

Figure 4.15 Sections of mouse testis tissue hybridised with DIG-labelled C1sB probe. (a) 200x magnification view of testis tissue after hybridisation with C1sB antisense probe shows expression of C1sB. C1sB is expressed in testis tissue only as confirmed by qRT-PCR (Fig. 4.6). (b) 200x magnification view of testis tissue after hybridisation with C1sB sense probe. ST represents Seminiferous Tubules.

Chapter V

Cloning and expression of recombinant mouse C1sA and human C1s

V.1 Results

The generation of novel tools such as monoclonal antibodies against human C1s and mouse C1sA requires a large amount of purified protein to be present for immunization process. So for the production of recombinant protein the first step was the cloning of human C1s and mouse C1sA into expression vectors, followed by protein production in eukaryotic cells and finally the purification of recombinant protein.

V.1.1 Cloning of human C1s and mouse C1sA into the expression vectors

V.1.1.1 Generation of human and mouse C1sA expression construct

The vectors used for cloning of the human and the mouse C1s cDNAs were pCI-Neo for intracellular protein expression (leader peptide was excluded from the C1sA gene), pSecTag/Hygro C for extracellular protein expression. A third vector was used for expression of C1s in an insect cell expression system, pbluebacHis2A. The human C1s and mouse C1sA cDNA sequences are shown in figure 5.1 A &B.

aaatatgccg cggggttctc atcatcatca tcatcatggt atggctagca tgactggtgg acagcaaatg ggtcgggatc tgtacgacga
tgacgataag gatcgatggg gatccgagct cgagatctgc agctggtacc ctaccatgta tggggagatc ctgtccccta actatcctca
ggcatatccc agtgaggtag agaaatcttg ggacatagaa gttcctgaag ggtatgggat tcacctctac ttcacccatc tggacattga
gctgtcagag aactgtgcgt atgactcagt gcagataatc tcaggagaca ctgaagaagg gaggctctgt ggacagagga
gcagtaacaa tccccactct ccaattgtgg aagagttcca agtcccatac aacaaactcc aggtgatctt taagtcagac ttttccaatg
aagagcgttt tacggggttt gctgcatact atgttgccac agacataaat gaatgcacag attttgtaga tgtcccttgt agccacttct
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gggatgtatt cactgcactg attggggaga ttgcaagtcc caattatccc aaaccatatc cagagaactc aaggtgtgaa
taccagatcc ggttggagaa agggttccaa gtggtggtga ccttgcggag agaagatttt gatgtggaag cagctgactc
agcgggaaac tgccttgaca gtttagtttt tgttgcagga gatcggcaat ttggtcctta ctgtggtcat ggattccctg ggcctctaaa
tattgaaacc aagagtaatg ctcttgatat catcttccaa actgatctaa cagggcaaaa aaagggctgg aaacttcgct
atcatggaga tccaatgccc tgccctaagg aagacactcc caattctgtt tgggagcctg cgaaggcaaa atatgtcttt
agagatgtgg tgcagataac ctgtctggat gggtttgaag ttgtggaggg acgtgttggt gcaacatctt tctattcgac ttgtcaaagc
aatggaaagt ggagtaattc caaactgaaa tgtcaacctg tggactgtgg cattcctgaa tccattgaga atggtaaagt
tgaagaccca gagagcactt tgtttggttc tgtcatccgc tacacttgtg aggagccata ttactacatg gaaaatggag
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gtctgtggag tccccagaga acccctttgaa gaaaaacaga ggataattgg aggatccgat gcagatatta aaaacttccc
ctggcaagtc ttctttgaca acccatgggc tggtggagcg ctcattaatg agtactgggt gctgacggct gctcatgttg tggagggaaa
cagggagcca acaatgtatg ttgggtccac ctcagtgcag acctcacggc tggcaaaatc caagatgctc actcctgagc
atgtgtttat tcatccggga tggaagctgc tggaagtccc agaaggacga accaattttg ataatgacat tgcactggtg
cggctgaaag acccagtgaa aatgggaccc accgtctctc ccatctgcct accaggcacc tcttccgact acaacctcat ggatggggac
ctgggactga tctcaggctg gggccgaaca gagaagagag atcgtgctgt tcgcctcaag gcggcaaggt tacctgtagc
tcctttaaga aaatgcaaag aagtgaaagt ggagaaaccc acagcagatg cagaggccta tgttttcact cctaacatga
tctgtgctgg aggagagaag ggcatggata gctgtaaagg ggacagtggt ggggcctttg ctgtacagga tcccaatgac
aagaccaaat tctacgcagc tggcctggtg tcctgggggc cccagtgtgg gacctatggg ctctacacac gggtaaagaa
ctatgttgac tggataatga agactatgca ggaaaatagc acccccgtg agg

Figure 5.1 (A) Shows the human C1s nucleotide sequence. The shaded region in pink colour represents the forward primer (hC1s_KpnI) and the green colour represents the reverse primer (hC1s_XhoIR). The region shaded in grey colour represents C1s gene. Start codon is shown in blue colour

aaatatgccg cggggttctc atcatcatca tcatcatggt atggctagca tgactggtgg acagcaaatg ggtcgggatc tgtacgacga tgacgataag gatcgatggg gatccgagct cgagcccacc atgcatgggg agatcctgtc ccctaactat ccccaggcgt accccaatga cgtcgtgaaa tcttgggaca ttgaagtccc agaggggttt gggattcacc tctactttac ccatgtggac atagagccgt cagagagctg tgcatacgac tcagtgcaga taatctcagg aggcatcgag gaagggagac tctgtggcca gaagaccagc aagagcccca actcccccat tatagaagag tttcagttcc catacaataa acttcaggtg gtctttacct cagacttctc caacgaagaa cggtttacgg gctttgcagc atactacact gccatagaca taaatgaatg cacagatttt acagatgtcc cctgtagcca cttctgcaat aacttcattg gtggatactt ctgctcctgt cccccagaat acttcctcca cgatgacatg aggaattgtg gagtcaactg tagtggagat gtgttcactg ccctgattgg ggagatctca agtcccaatt atcccaatcc atacccggag aactcaaggt gtgaatacca gattcagctg caggagggct tccaagtggt ggtgacgatg caaagagaag attttgatgt ggagcctgct gactcagagg ggaactgccc tgacagttta acttttgctt caaaaaacca acagtttggt ccttactgtg gtaatgggtt ccctggaccg ctaactatta gaacccagag caatactctc ggcattgtct ttcaaactga cctaatgggg caaaagaagg gttggaagct tcgttaccat ggagatccca tctcctgtgc caaaaaaatc actgccaatt ctacttggga gcccgataag gcaaaatacg tgtttaaaga tgtggtgaag ataacctgtg tggacggatt tgaagttgtg gagggacatg ttagctctac ttcatactat tccacttgtc aaagcgatgg acagtggagc aactccggtc taaaatgtca acctgtgtat tgtggaattc cagatcccat tgcgaatggt aaagttgaag agccagagaa tagtgtgttt ggtactgttg ttcactacac gtgtgaagag ccatattact acatggaaca tgaagaaggt ggggagtatc gatgtgctgc aaatgggaga tgggtaaatg accagctggg tattgagctt ccaagatgta ttccagcctg tggagtaccc accgagccct ttcaagtgca tcagaggata tttggaggac aacctgcaaa gattgaaaat tttccctggc aagtcttctt taatcacccc agagctagtg gggctcttat taatgagtac tgggtgctga cggctgctca cgttttggag aaaatctccg acccttaat gtatgtcggg accatgtctg tgaggacaac tctcttggaa aatgcccaga gactctacag taaacgtgtc tttattcatc ccagctggaa gaaagaagat gacccaaaca cacggacaaa ttttgacaat gacattgccc tggtgcagct gaaagaccct gtgaaaatgg gacccaaagt ctcccccatc tgcctaccag gcacctcctc agagtacaac gtctcaccgg gtgacatggg gctgatctca gggtggggca gtacagaaaa gaaagtcttt gttatcaacc tcagaggggc aaaggtacca gtaacctctt tagaaacctg caaacaggtg aaagaggaga accccactgt aaggcctgag gactatgttt tcactgacaa catgatctgt gctggcgaga aaggtgttga cagctgtcac ggggacagcg gcggggcttt tgctttccag gtccccaatg tcacggtccc caaattctat gtagctggct tggtgtcttg ggggaaaagg tgtgggactt atggggttta cacaaaggta aagaattacg tggactggat cctgaaaact atgcaggaga atagtgggcc caggaaggac taatccgtag ggcgacaact cccctcaagg gcatagcaag ctt

Figure 5.1 (B) Shows the mouse C1sA nucleotide sequence. The shaded region in orange colour represents the forward primer (mC1sA_XhoI) and the pink colour represents the reverse primer (mC1sA_HindIII). The region shaded in grey colour represents C1sA gene. Start codon is shown in blue colour and stop codon in green colour

In order to express human C1s in pCI-neo expression vector using an N-terminal his tag for purification, a 2kb fragment was amplified from liver cDNA by PCR using primers hC1s_XbaI and hC1s_R1. The 2kb PCR product was then cloned into pGEMTeasy vector (figure 5.2), excised with XbaI and NotI and ligated into pCI-neo (figure 5.3 A). For the expression of mouse C1sA in the pCI-neo using N-terminal his tag for purification, a 2kb fragment was amplified from liver cDNA by PCR using primers mC1sA_XbaI and mC1sA_rev2. The 2kb PCR amplified fragment was cloned into pGEMTeasy vector (figure 5.2), restriction digested with XbaI and NotI and ligated into pCI-neo (figure 5.3 A). Afterwards, the His tag was inserted into both constructs by digesting with XbaI and XhoI enzymes and ligating in the N-terminal six histidine linker which is prepared by annealing oligonucleotides XX_link_F and XX_link_F.

In order to express human C1s and mouse C1sA in the pSecTag/HygroC expression vector using C-terminal his tag for purification, 2kb fragments were amplified from liver cDNA by PCR. Primers used for mouse C1sA were mC1sA_XhoI and mC1sA_rev3 and for human C1s primers used for amplification were hC1s_KpnI and hC1s_XohIR. The 2kb PCR product was then cloned into pGEMTeasy vector (figure 5.2) and then excised with KpnI and XhoI in case of human and restriction digested with XhoI and ApaI in case of

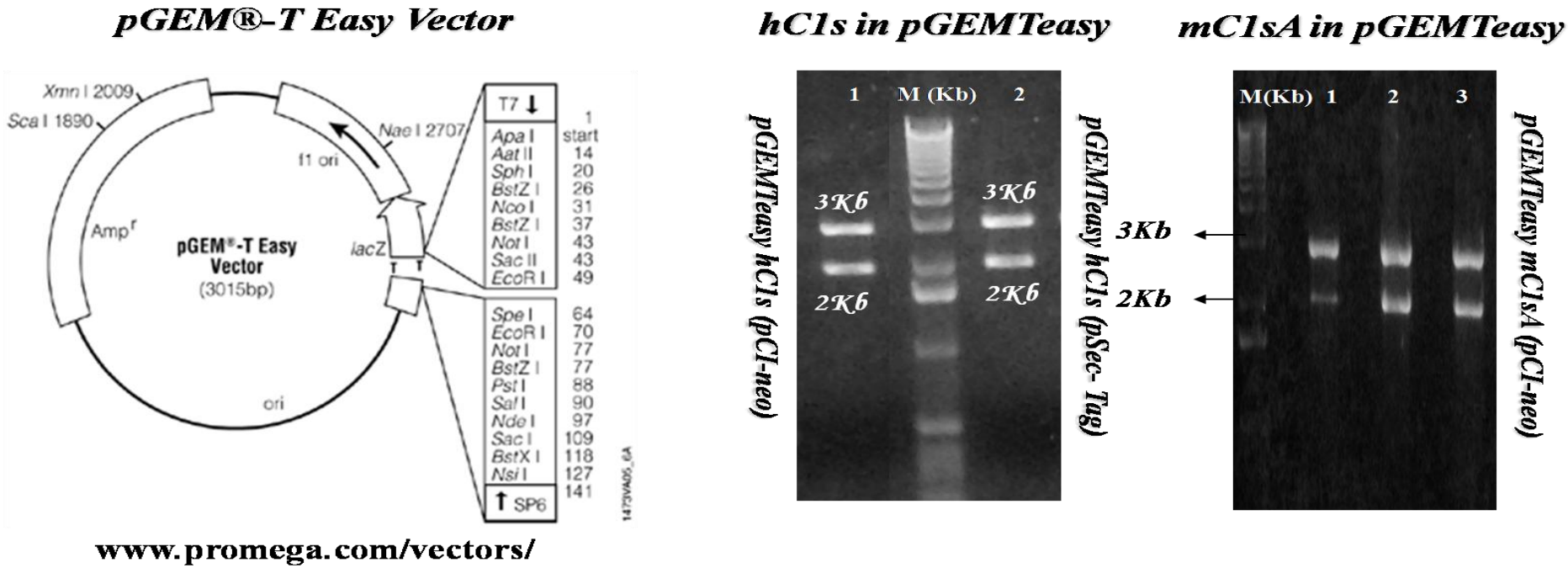


Figure 5.2 Ligation of human and mouse C1sA into pGEMT-Easy vector. A 2Kb fragment was amplified from human C1s and mouse C1sA liver cDNA and subsequently ligated into pGEMT-Easy vector (3Kb). A 1% agarose gel is showing a 2kbfrom both mouse C1sA and human C1s gene.

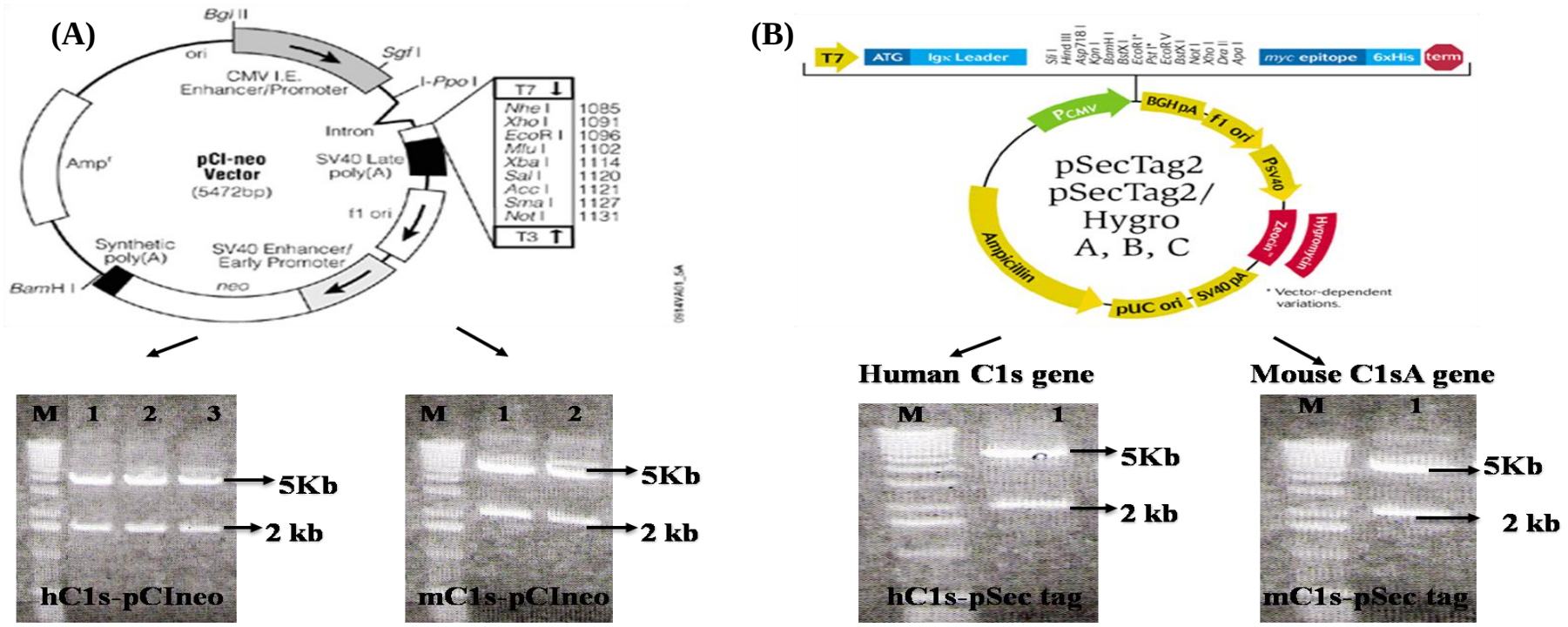


Figure 5.3 Generation of human and mouse C1sA expression constructs. (A) The 2kb PCR product cloned into pGEMTeasy vector was excised with XbaI and NotI and ligated into pCI-neo vector. **(B)** The 2kb PCR product was then cloned into pGEMTeasy vector and then excised with KpnI and XhoI in case of human and excised with XhoI and ApaI in case of mouse and then ligated into pSecTag/HygroC.

mouse and then sub-cloned into pSecTag/HygroC (figure 5.3B). The pSecTag/HygroC vector already contains 6X his tag.

V.1.1.2 Cell transfection (Expression of human C1s and mouse C1sA in CHO cells)

CHO cells were transfected with human C1s in pCI-neo and pSecTag2/HygroC as well as mouse C1sA cloned into same expression vectors for the generation of cell lines for stable expression of recombinant proteins, (Figure 5.4) provides the details about how the transfecton was preformed.

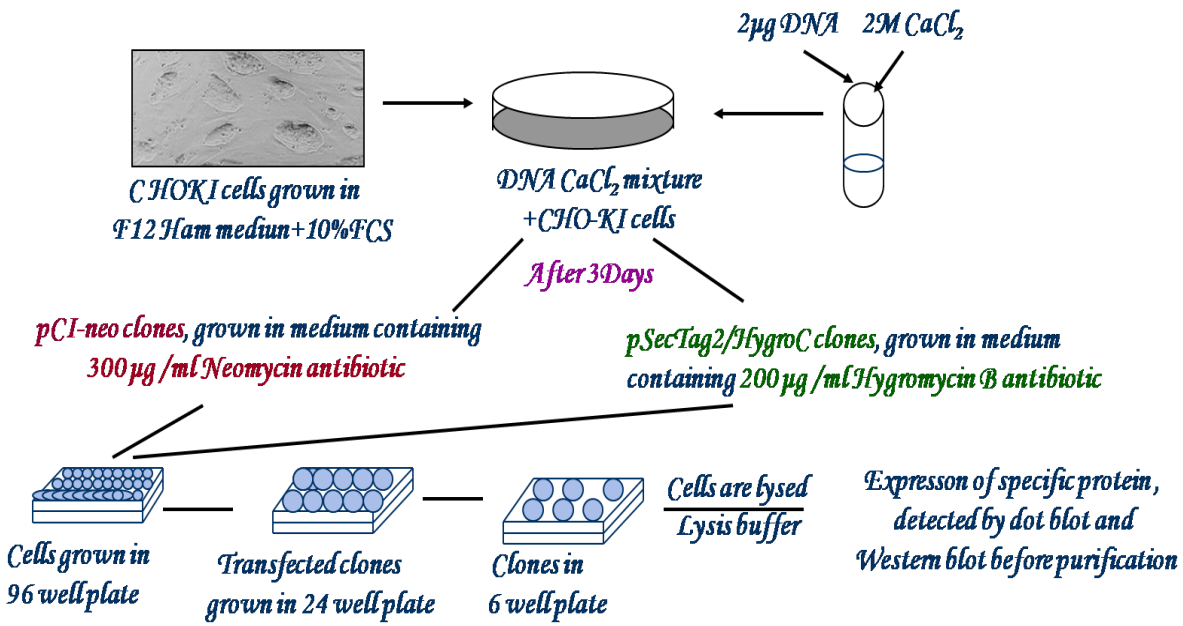
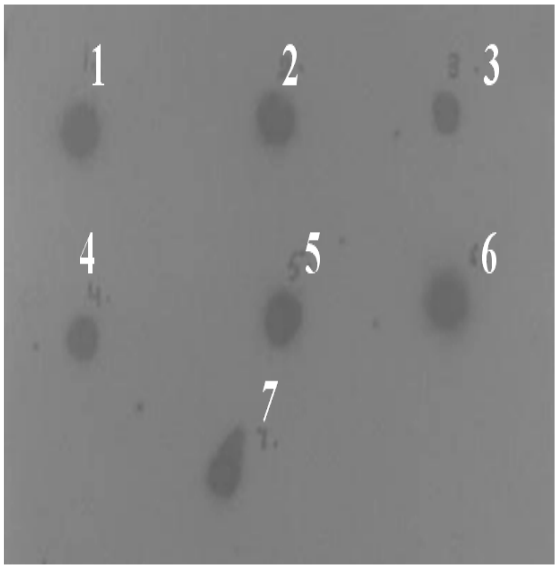


Figure 5.4 Stable expression of hC1s and mC1sA in CHO cells

After 4weeks under gentamicin and hygromycin-B selection, stably transfected CHO-K1 cells plus expression plasmid were lysed with 1ml of lysis buffer and clear cell lysate was harvested by centrifugation. The cell lysate was evaluated

for protein expression by Dot blot using monoclonal mouse anti polyhistidine peroxidase antibody. The human C1s and murine C1sA bands were successfully expressed as indicated by the dot blot figure 5.5. The negative control, CHO-KI cells without transfection, which is missing in the dot blot below has been tested experimentally with the same antibody and didnt show any expression.



1. hC1s_pCINeo
2. hC1s_pSecTag (clone 2)
3. mC1sA_pSecTag (clone 3)
4. mC1sA_pSecTag(clone4)
5. mC1sA_pCINeo (clone 3)
6. mC1sA_pCINeo (clone 4)
7. +ve control (Tbb CRT with his Tag)

Figure 5.5 Dot blot screening of clones for the expression of Human C1s and mouse C1sA using monoclonal mouse anti polyhistidine peroxidase antibody.

V.1.1.3 Purification of recombinant C1sA

Human C1s and mouse C1sA recombinant protein with histidine tags were purified using MagneHis^{TM} protein purification system. Cell lysate was obtained in the same way as described before and purified according to conditions described in instruction manual. Flow through (without any protein), washing (I, II &III) and elution fractions were collected and protein evaluation was done by

SDS-PAGE and then Western Blot using monoclonal mouse anti polyhistidine peroxidase antibody. The human C1s and mouse C1sA bands were compared with molecular weight standard after loading and running the 12% SDS-PAGE gel. Bands were seen between the BSA (98 kDa) and the glutamic anhydrogenase (64 kDa). The expected molecular weight (MW) of human and mouse C1s protein is 85 kDa (figure 5.6).

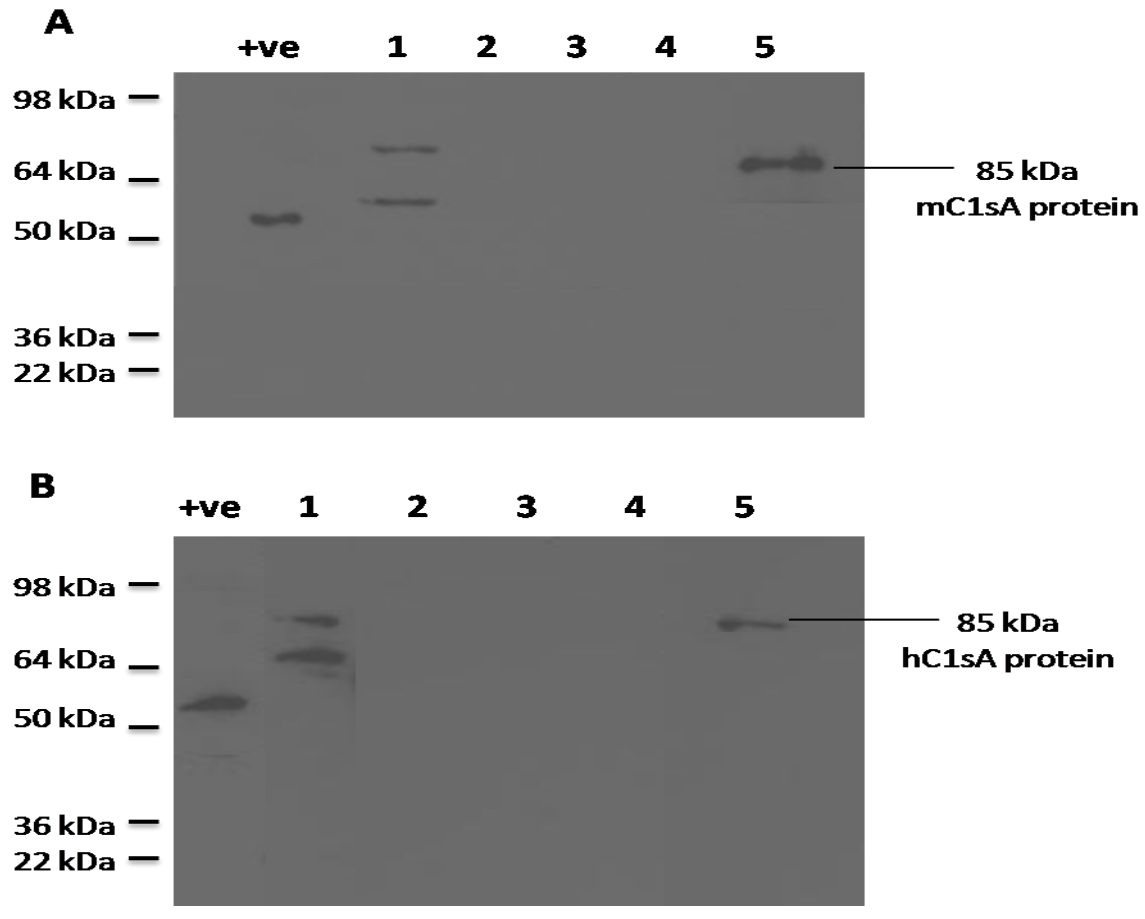


Figure 5.6 Western blot analyses using monoclonal mouse anti polyhistidine peroxidase antibody was done on mouse C1sA protein **(A)** and human C1s protein **(B)** after purification. Lane +ve is positive control for his tag antibody, Lane 1 represents unpurified protein, Lane 2-4 washes and lane 5 purified C1s protein.

There was successful protein expression after purification but the quantity of protein expressed is too low to use it for further studies like monoclonal antibody production. The protein was even expressed in large scale but unfortunately the concentration of protein expression in terms of µg/ L of culture medium were so low. So changed to baculovirus expression system (see section V.1.2).

V 1.2 Baculovirus expression system

The Baculovirus expression system (BES) has been extensively used system in research for expression of recombinant protein over past fifteen years.

The gene of interest is first cloned into a plasmid transfer vector in this case, pbluebac His 2A, so that it can be transferred into the baculovirus. The pbluebac His 2A transfer vector contain the same sequence that flanks the polyhedron in baculovirus so that recombination between the viral DNA and the transfer vector can facilitate the insertion of GOI into the viral genome after co-transfection of insect cells (SF9cells) resulting in the construction of a recombinant virus DNA. Figure 5.7 shows the schematic representation of recombinant virus production.

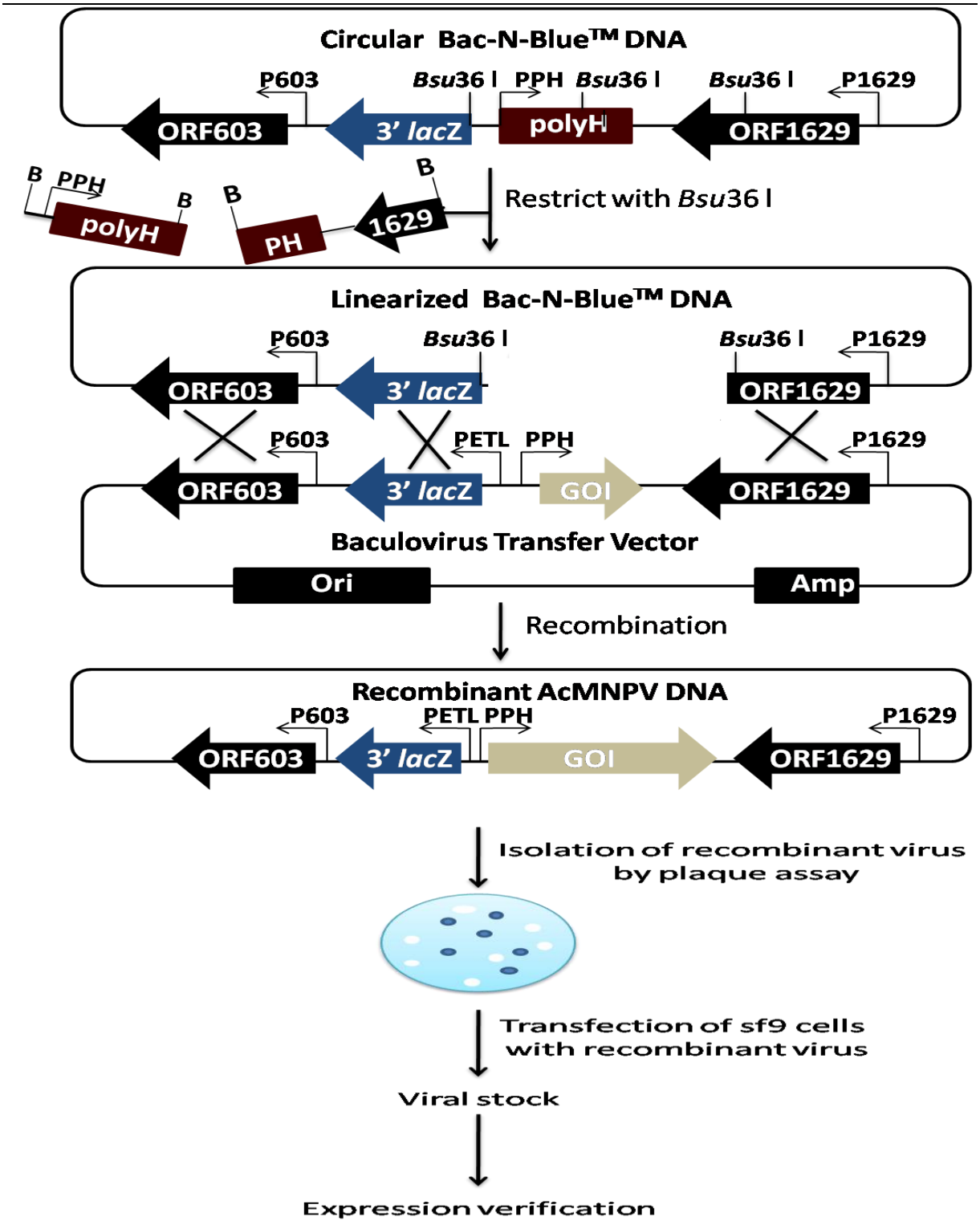


Figure 5.7 Schematic representation of recombinant virus production

V.1.2.1 Cloning of human and mouse C1sA into the pbluebac His 2A expression vectors

In order to express the human C1s in pbluebac His 2A expression vector (which allows to add an N-terminal His tag) a 2Kb fragment was excised from the pSecTag/HygroC vector with restriction enzymes KpnI and XhoI and subcloned in the pbluebac His 2A vector cut with the same restriction enzymes (figure 5.8 A). Once the clone was ready it was analysed by restriction digest and sequencing.

For the expression of mouse C1sA in pbluebac His 2A expression vector with N-terminal his tag, a 2kb fragment was amplified from an already existing clone in pSecTag/HygroC by PCR using forward primer mC1sA_XhoI and reverse primer mC1sA_HindIII. The PCR product of 2kb was then cloned into pGEMTeasy vector and excised with XhoI and Hind III restriction enzymes and then sub-cloned into pbluebac His 2A (figure 5.8 B). The pbluebac His 2A vector already encodes six histidine residues (6X his tag) to facilitate purification of the recombinant expression product. After subcloning, the expression construct was characterised using restriction mapping and sequencing.

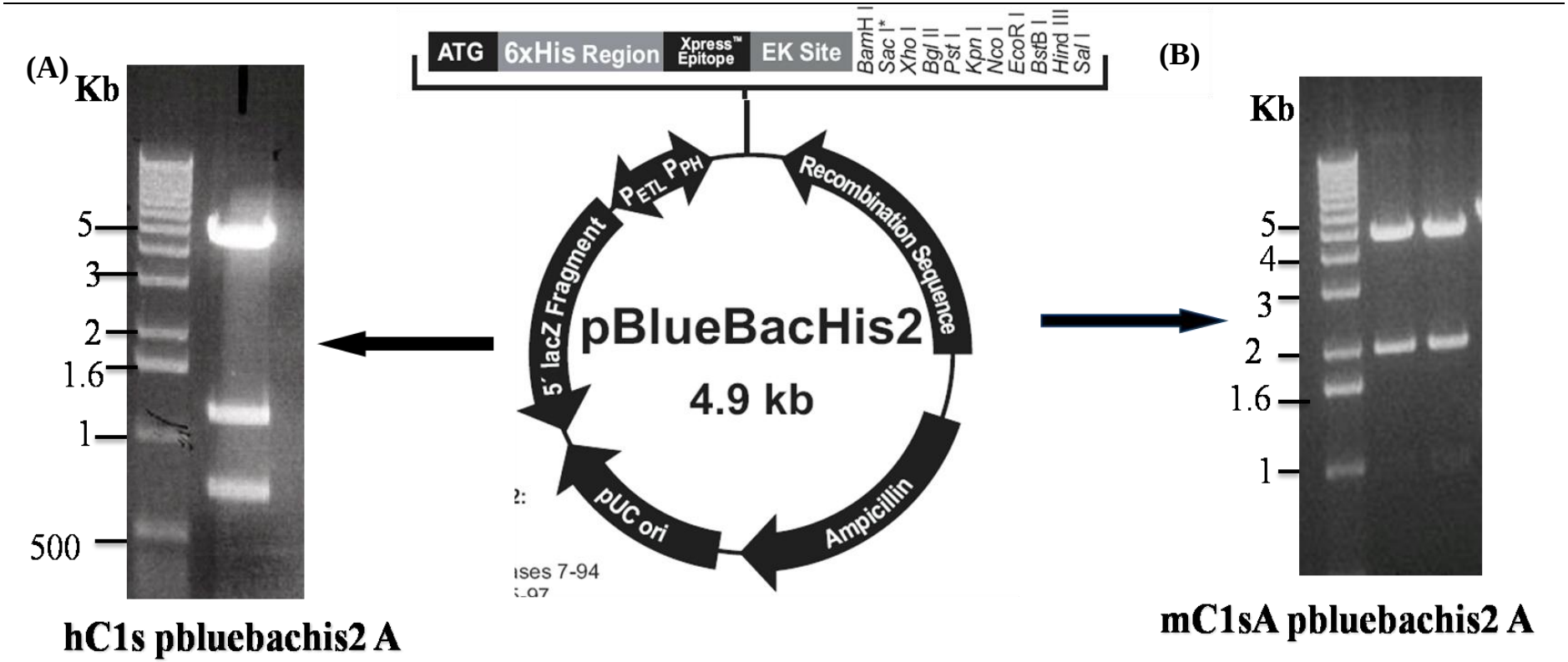
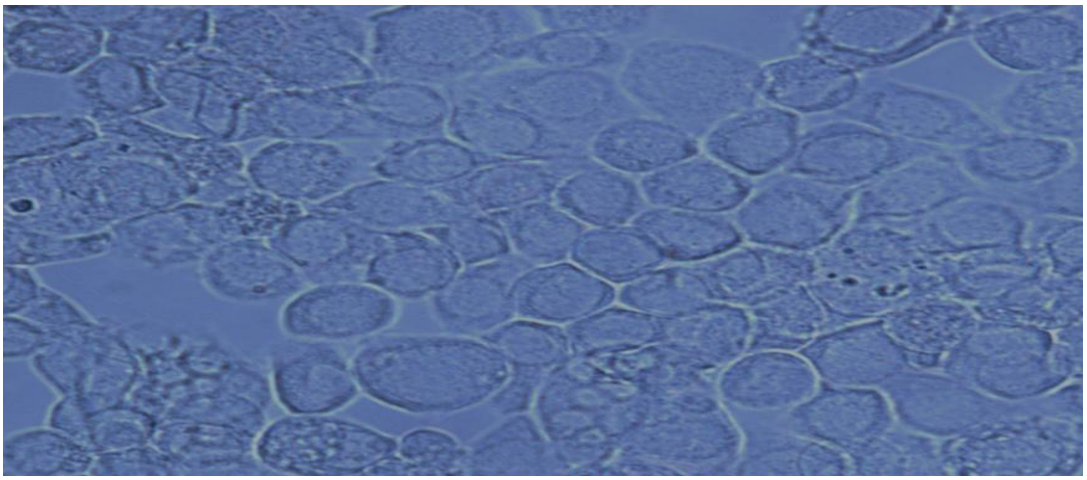


Figure 5.8 Cloning of Human C1s and Mouse C1sA into pbluebacHis 2A. (A) A fragment of 2kb hC1s is cloned into pbluebacHis 2A vector. The 1% agarose gel image shows the two fragments, upper one of 1198bps and lower one of 724bps, after digestion with BglII and ApaI restriction enzyme. **(B)** A 2kb fragment of mouse C1sA is cloned into pbluebacHis2A vector.

V 1.2.2 Cell transfection (Expression of mouse C1sA in Sf9 cells)

Sf9 cells, an insect ovarian cell line from *Spodoptera frugiperdan* cells, have been extensively used to express recombinant proteins were transfected with the mouse C1sA expression construct (in the cloned into pbluebac His 2A expression vector) in order to generate of cell lines for stable expression of recombinant C1sA. For this, Bac-N-blue DNA and pbluebac his2A-mC1sA transfer vector are mixed with the Cellfectin Reagent (Invitrogen). A positively-charged liposome-DNA complex forms and binds to the negatively-charged plasma membrane. The liposomes fuse with the cell membrane and the DNA is taken up by the cells. After incubation at 27 ºC for 72 hours, budded virus particles were released into the medium and the transfection medium was harvested for further analysis to generate the viral stock (recombinant plaques) for transfection. Figure 5.9 shows Sf9 cells before and after transfection. After transfection, cells show increased cellular diameters and an increased size of the cell nuclei.

Normal Sf9 cells



72 hours infected cells

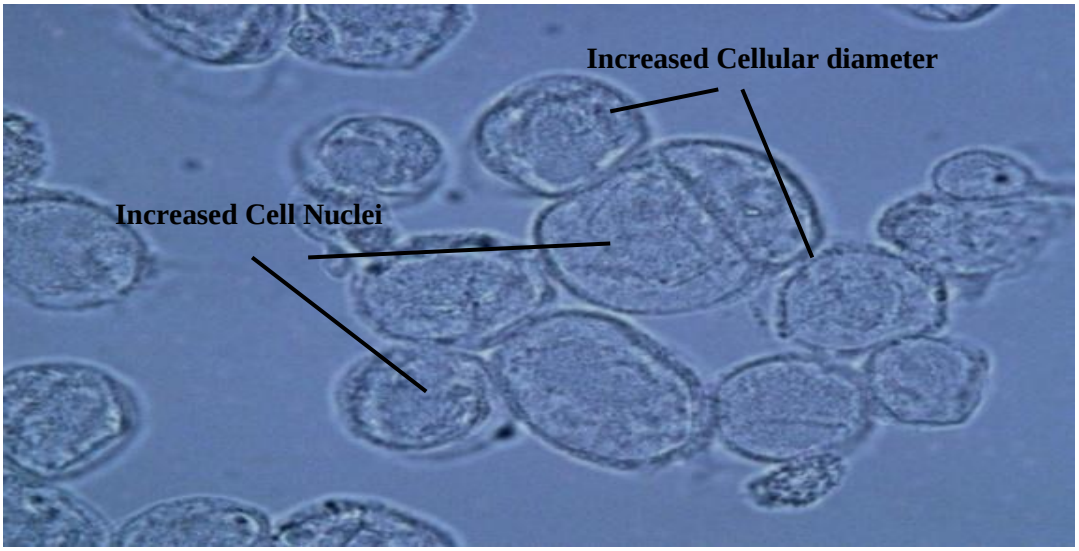


Figure 5.9 Shows Sf9 cells before and after (72 hours) transfection

V.1.2.3. Plaque assay

Purification of recombinant baculovirus was achieved by using a plaque assay. Purification of recombinant plaques is achieved by infecting Sf9 cells with dilutions of the transfection medium harvested 72 hours after transfection. It is important to purify recombinant virus from any non-recombinant viral DNA. Contamination of recombinant DNA with uncut (occ^+) DNA will lead to the dilution of recombination virus over time (wild type or uncut virus infects and replicates at higher efficiency than recombinant virus). 10 fold serial dilutions of transfection viral stock were made in complete TNM-FH medium. Dilutions up to 10^{-2}, 10^{-3} and 10^{-4} were performed. The plaque assay procedure involves infecting a Sf9 cell monolayer with viral dilutions and overlaying the monolayer with agarose. Figure 5.10 shows blue areas where the cells have been killed. Each plaque is generated by one original infectious virus particle. Chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (x-gal) is mixed into the medium. X-gal will be converted by β-galactosidase of the vector and form a blue /green plaques. Hydrolosis of chromogenic substance doesn't occur until the cells lyse releasing β-D-galactoside.

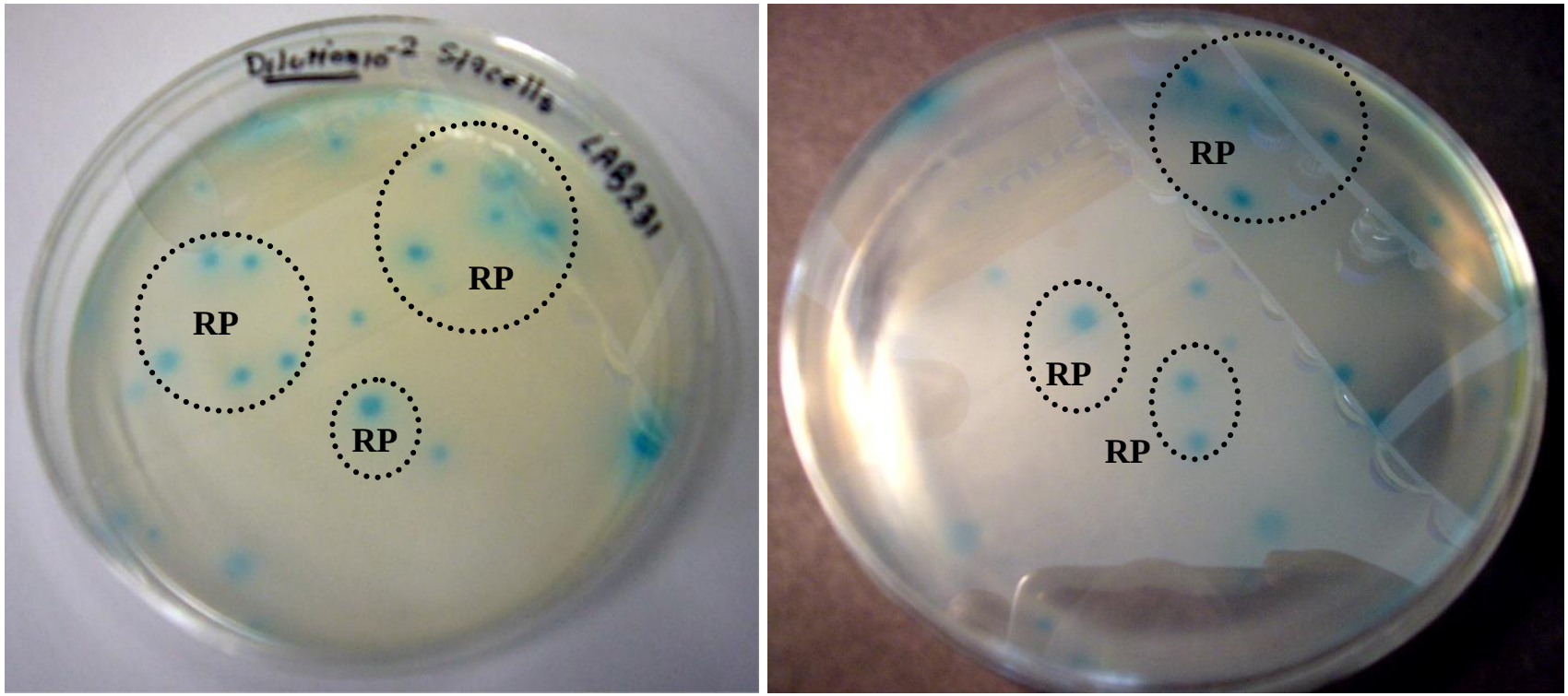


Figure 5.10 Blue clones represent a recombinant plaque. Each plaque is the result of the presence of one original infectious virus particle. Circles represent the recombinant plaque area. RP: recombinant plaque.

V.1.2.4. PCR Analysis of Recombinant virus

Polymerase chain reaction was used to verify the presence of an insert in a putative recombinant virus and confirm the isolation of a pure, recombinant plaque. PCR analysis is a quick and efficient way to rule out false positives at an early stage. Since the size of the foreign gene insert is known, the expected size of the PCR product can be determined and contaminations with wild type virus discovered. In order to perform a PCR analysis, I amplified recombinant plaques to isolate viral DNA which then was used for the PCR analysis and for the generation of the P-1 viral stock which is further used to produce a large scale, high titer stock (HTS). Briefly, seed log phase Sf9 cells into a 12 well microtiter plate, pick 10 recombinant plaques from the agarose plate and one wild type plaque and leave the last wells as cells only controls. Incubate the 12 well microtiter plates at 27^{o} C for 5-6 days until all the cells lyse but on day 3 take approximately 0.75 ml of suspension from each well for DNA extraction (see detailed protocol in Materials and Methods). PCR analysis was performed on extracted DNA using Baculovirus specific forward and reverses primers. Figure 5.11 shows a typical PCR analysis of recombinant viral clones. The expected size of PCR fragments could be calculated by adding on the size of the insert, 2000bps is the insert size in this case, and the size of the DNA contributed by

the transfer vector, 338 bps contributed bypbluebachis2A. In my case the expected calculated size of PCR fragment was 2338base pairs.

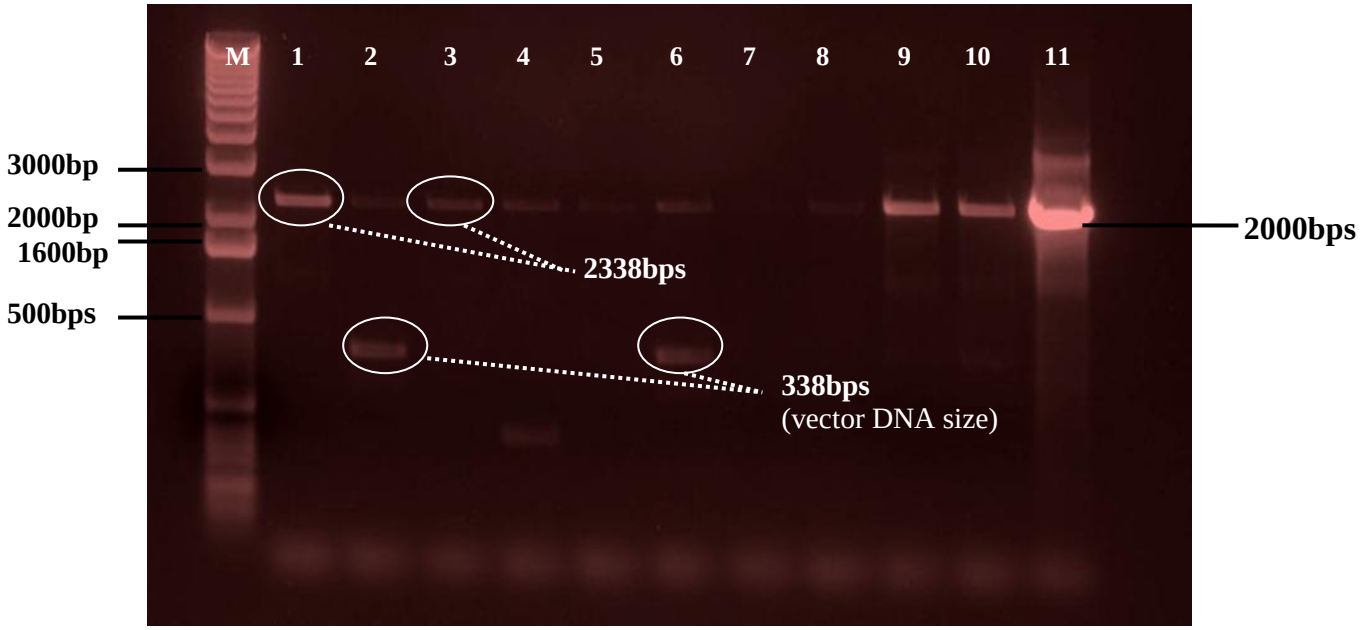


Figure 5.11 PCR analysis of recombinant plaque. Lane 1-10 represents the recombinant clones and lane 11 is the control. Lane 1 and 3 is a pure, recombinant plaque of 2338bps and which is expected where as Lane 2, 4 and 6 demonstrates that putative recombinant plaque is actually a mixture of recombinant and wild type virus.

V.1.2.5. Preparing High-Titer Viral Stocks

After identification of a pure recombinant virus, go back to the P-1 viral stock (low titer stock) and use that for generation of high titer viral stock. Briefly seed two 25 cm^2 flasks with Sf9 cells in complete TMN-FH medium and use 20ul of P-1 viral stock to infect the cells. After 5-10 days (the cells are 100% lysed). Collect the medium this is going to be P-2 viral stock and used to infect large

amount of medium for the production of high titer stock. After 10 days collect the medium which is a high titer stock. Figure 5.12 shows how the Sf9 cells look after P-2 viral infection. Cells show cytopathic effect caused by baculovirus-infected Sf9 cells, which is characterized by reduced cell numbers due to cell lysis and irregular shaped cells caused by virus replication and subsequent cell death.

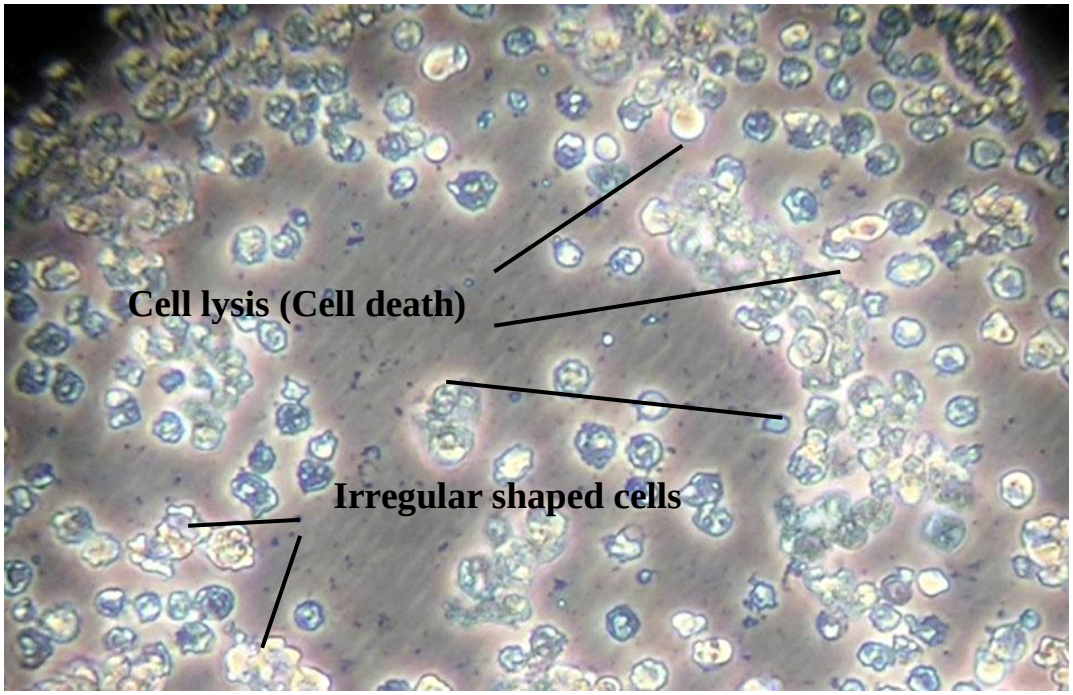


Figure 5.12 Shows Sf9 cells at 10 days post infection with baculovirus and the characteristic features of baculovirus infection.

V.1.2.6 Detection of recombinant m-C1sA by Dot blot

P-2 viral stock was used to detect the production of recombinant mC1sA protein. Supernatant from cell monolayers known as P-2 viral stock was blotted onto nitrocellulose membrane. The nitrocellulose membrane was then incubated with monoclonal mouse anti polyhistidine peroxidase antibody. Figure 5.13 shows the dot blot result.

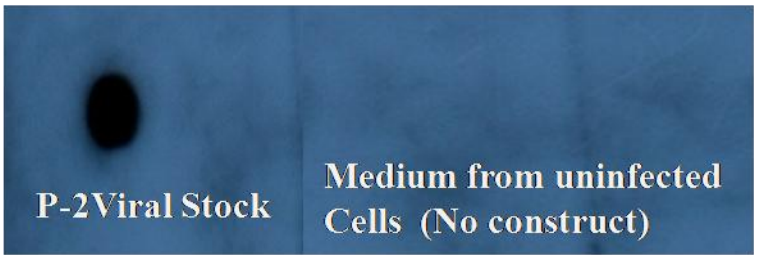


Figure 5.13 Dot blot image of P-2 viral stock evaluated with monoclonal mouse anti polyhistidine peroxidase antibody. The blot image indicates the presence of recombinant mC1sA.

V.1.2.7 Multiplicity of infection and time course expression of recombinant mC1sA protein.

The time course experiment enables us to establish the optimal multiplicity of infection (MOI) and the time of harvest for the recombinant protein. A wide range of multiplicity of infections can be used in order to determine the best kinetics of infection for huge protein expression. An MOI of 5 and 10 was used for the protein expression in our experiment. A wide range of time points were established in order to find out the mouse C1sA protein expression. Briefly plate

two 6 well plates with a cell density of 10^6 cells in each well and label wells with five different time points i.e. 24 hours, 48 hours, 72 hours, 96 hours and 120hours and keeping the last well as control without any infection. One plate was for MOI of 5 and the other one for 10. The cell pellet and the supernatant from each well were collected at their respective times. Harvest the cell pellet and supernatant from control well at 48 hour time point. Once all the time points collected. The cell pellets were lysed by using lysis buffer (0.1% Triton X-100 in PBS) and after lysis proceed to analysis of time point samples by running them on SDS-PAGE and blotting on nitrocellulose membrane for western blotting. The nitrocellulose membrane was then incubated with monoclonal mouse anti polyhistidine peroxidase antibody and also with C1s specific antibodies, primary antibody was sheep polyclonal C1s antibody and the secondary antibody was Donkey peroxidase labelled anti-Sheep IGg. The Western blot results for MOI of 5 and 10 at different time points can be seen in figure 5.14 and figure 5.15.The mC1sA protein bands were compared with molecular weight standard after loading and running the 10.0% SDS-PAGE gel. Bands were seen between the BSA (98 kDa) and the glutamic anhydrogenase (64 kDa). The expected MW of human and mouse C1s protein is 85 kDa. The mouse C1sA heavy chain band is 58kDa and the light chain band is 28kDa.

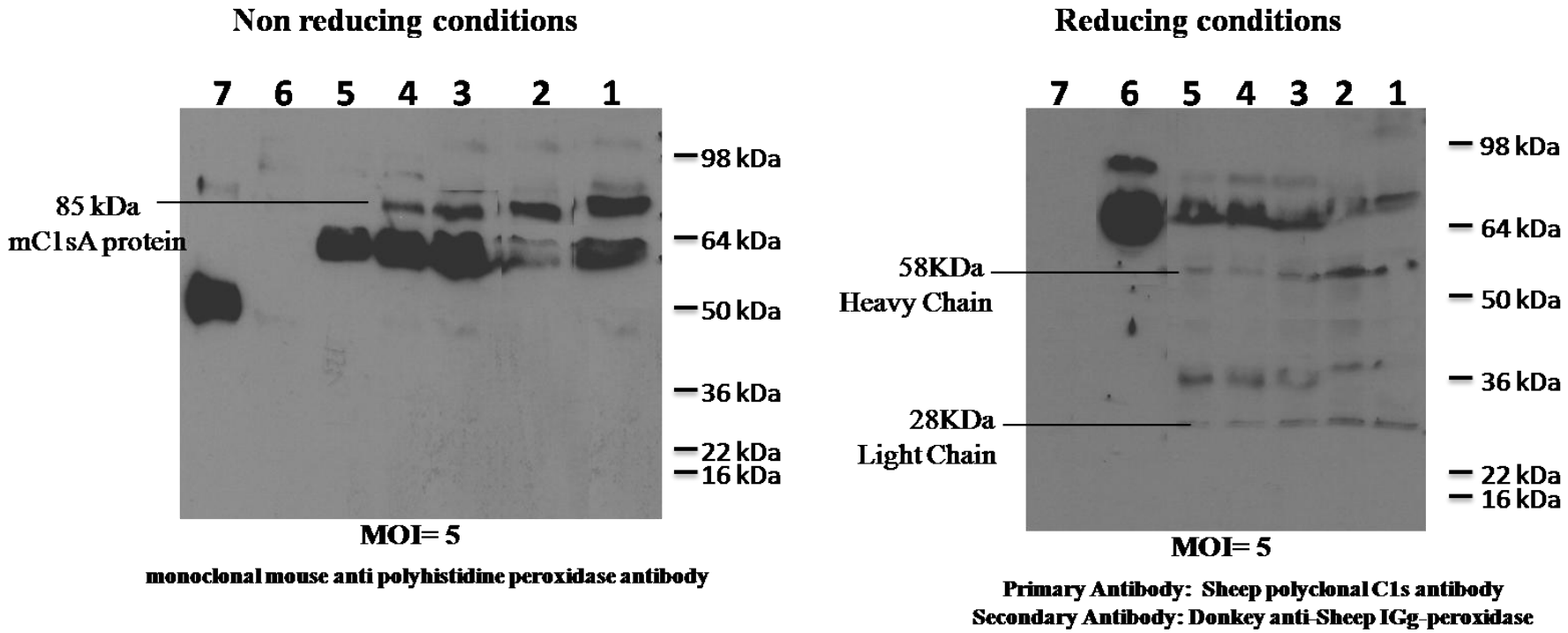


Figure 5.14 Protein expression of mouse C1sA for MOI 5. Bands were seen between the BSA (98 kDa) and the glutamic anhydrogenase (64 kDa). The expected MW of mouse C1s protein is 85 kDa. The Heavy C1sA chain is 58 KDa and light Chain is 28 KDa. Lane 1 cell lysate from 24hrs, lane 2 from 48hrs, lane 3 from 72hrs, lane 4 from 96 hrs, lane 5 from 120hrs, lane 6 uninfected cell lysate from 48hrs, lane 7 in case of his tag antibody was MASP-2 protein and lane 6 in case of C1sA specific antibody was a mouse serum and lane 7 uninfected cell lysate from 48 hrs. Antibodies used were mouse anti polyhistidine peroxidase antibody and C1sA specific antibodies, primary anti body was sheep polyclonal C1s antibody and the secondary antibody was Donkey anti-Sheep IGg-peroxidase.

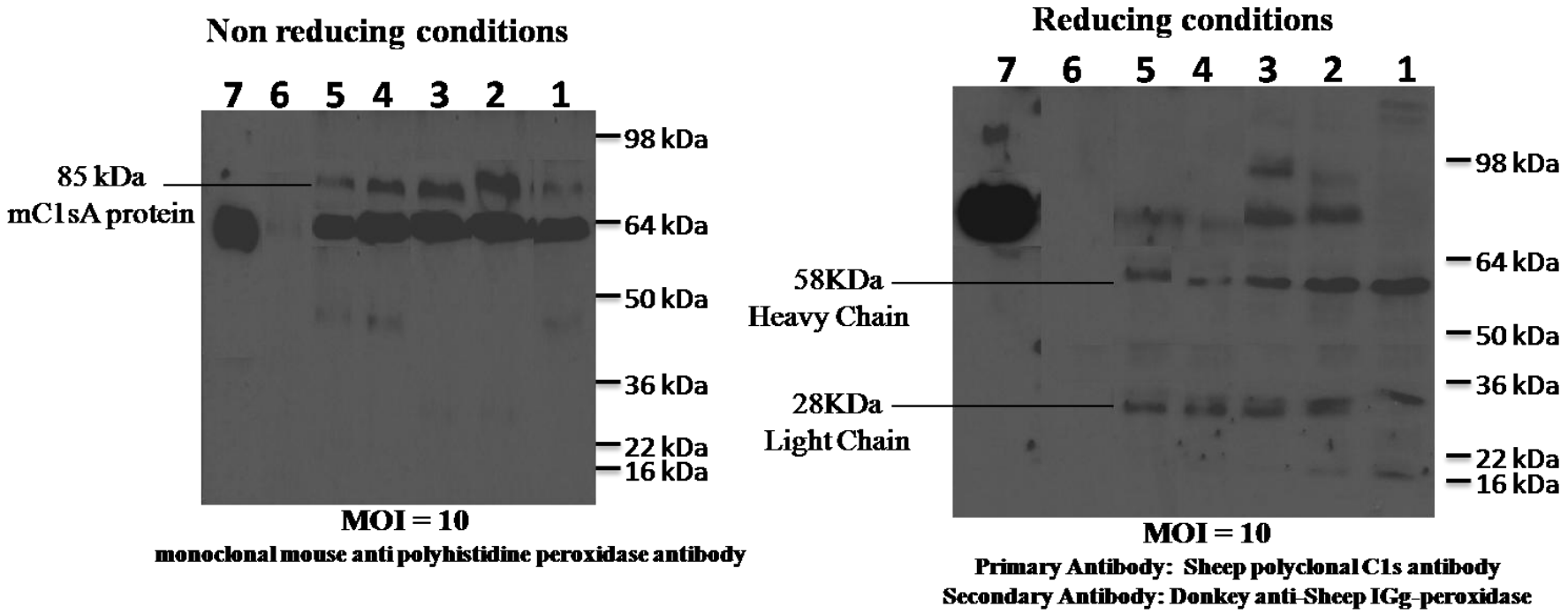


Figure 5.15 Protein expression of mouse C1sA for MOI 10. Bands were seen between the BSA (98 kDa) and the glutamic anhydrogenase (64 kDa). The expected MW of mouse C1s protein is 85 kDa. The Heavy C1sA chain is 58 KDa and light Chain is 28 KDa. Lane 1 cell lysate from 24hrs, lane 2 from 48hrs, lane 3 from 72hrs, lane 4 from 96 hrs, lane 5 from 120hrs, lane 6 uninfected cell lysate from 48hrs, lane 7 in case of his tag antibody was MASP-2 protein and in case of C1sA specific antibody was a mouse serum. Antibodies used were mouse anti polyhistidine peroxidase antibody and C1sA specific antibodies, primary anti body was sheep polyclonal C1s antibody and the secondary antibody was Donkey anti-Sheep IGg-peroxidase.

His GraviTrap columns were used to purify the recombinant mouse C1sA protein at MOI of 5 at time point 48hrs and 96hrs. The purified protein was analysed on 12%SDS-PAGE gel and confirmed by western blot using C1sA specific antibodies, primary antibody was sheep polyclonal C1s antibody and the secondary antibody was Donkey peroxidase labelled anti-Sheep IGg. The Recombinant C1sA protein runs at a band of molecular weight 85kDa and the heavy chain runs at approximately 58kDa and light chain runs at a molecular weight of 28kDa (Figure 5.16).

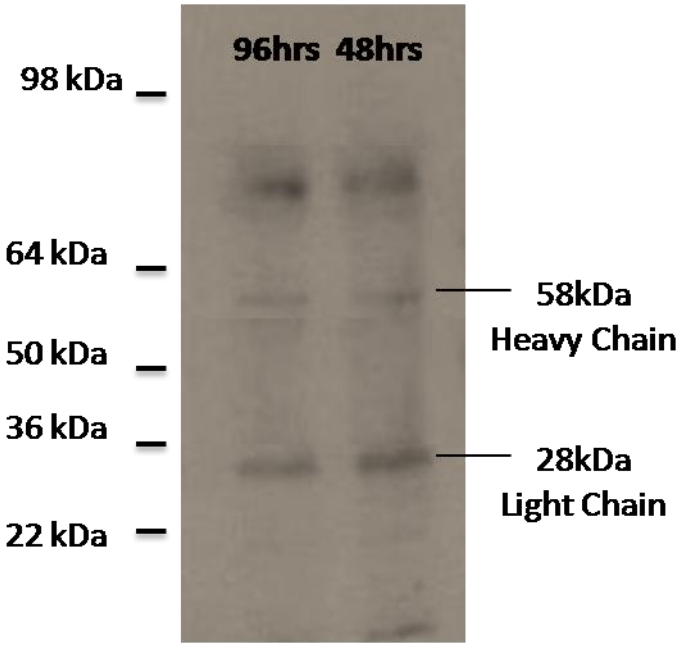


Figure 5.16 Western blot analysis using C1sA specific antibodies was done on purified C1sA protein.

Once confirmed at what time point and MOI the protein is expressing then large scale protein production will be done. The purified protein will be used in the generation of monoclonal antibodies and will be used to reconstitute the classical pathway functional activity in C1sA-/- mouse plasma.

Chapter VI

Discussion

Complement activation is an important process within the host immune defence. It is required for efficient phagocytosis, an effective inflammatory response and the recruitment of effector cells to the sites of injury and/or inflammation. It also plays a key role in the removal of debris and apoptotic cells.

The complement system cross -links the innate and adaptive immune responses. Activation of classical pathway (CP) is largely dependent on the binding of C1 to immune complexes, whereas the lectin and alternative pathways (LP & AP) may be activated through antibody-independent mechanisms, initiated by direct binding of complement components to the surface of a pathogen (Atkinson, 2006). It has been shown that complement activation, in particular the CP, plays an important role in the pathogenesis of autoimmune disease such as SLE, RA and nephritis. There is also mounting evidence that complement activation, in particular activation via

the LP, is responsible for the tissue damage that follows ischemia (i.e. reperfusion injury)(Stahl, G. *et al*., 2003).

To assess the phenotype of the LP and CP combined deficiency, aimed to establish a mouse strain deficient of both LP and CP activation. Theoretically, there ia already a mouse line available that should be deficient of both, CP and LP activation, a mouse line with a targeted deficiency of C4. However, new data demonstrate that C4 deficient mice are not deficient in the lectin pathway, only in CP - functional activity, as the lectin pathway can activate C3 in a C4-independent bypass activation route involving MASP-2 and MASP-1(Prof. W. Schwaeble and N. Lynch personal communication, Selander. B. *et al*., 2006). To establish a mouse line deficient of the CP and the LP, first tried to cross the MASP-2 deficient mouse line with a C1q deficient mouse line but the establishment of a line deficient of C1q and MASP-2 proved to be unsuitable as the C1q and MASP-2 genes are very close to each other and within the same chromosome region (less than 100cM away from each other, which makes a recombination event rather unlikely). Therefore, the establishment of a mouse line deficient of the CP effector enzyme C1sA was considered to provide a feasible alternative, as the C1sA gene is located on chromosome 6 which would allow to intercross

this gene targeted line with MASP-2 deficient mice (the MASP-2 gene is located on mouse chromosome 4 and generate mice deficient of MASP-2 and C1sA and for the future investigation of C1sA deficiency in diverse experimental models of infectious and non-infectious disease. For this reason, the actual aim of my research project was to generate a C1sA deficient mouse strain.

Gene deficient mice have been extensively used in the study of various infectious diseases. With the generation of C1sA gene deficient mice it would be able to assess the role of CP and LP in host defence by using bacterial and fungal infection models and also be able to define the contribution of both pathways in ischemia/reperfusion injury using myocardial infarction and renal reperfusion models.

In the mouse, the genes for C1s and C1r genes are duplicated in two gene clusters (C1sA and C1rA and C1sB and C1rB). The C1sA gene encodes the relevant classical pathway serine protease while the C1sB gene is not expressed in any other tissues but testis (Garnier. G. *et al*., 2003). By disrupting the C1sA gene makes classical pathway of activation inactive as the C1sB doesn’t contribute towards the classical pathway functional activity.

In line with the generation of a C1sA deficient mouse line, I analysed the tissue specificity of C1sA and C1sB expression and determined cell types expressing C1sA and C1sB in vivo. I also succeeded in generating recombinant C1sA protein.

VI.1 Generation of C1sA deficient mouse line

In order to establish a C1sA deficient mouse line I established a C1sA targeting construct which would disrupt the C1sA gene by replacing the exons 2 and 3 of the C1sA gene (including the splice acceptor at the start of exon 4) with a neomycin resistance gene. Due to extremely lengthy procedures involved in the generation of a gene targeted mouse line, my work has so far lead to the generation of mice heterozygous for a disrupted allele for the C1sA gene while the backcross on pure C57BL is still progressing. The germ line transmission and the correct integration of the disruption construct in the targeted gene has been confirmed by Southern blotting, PCR analysis and genomic sequencing of the targeted and the wild type alleles.

The fundamental construction of the targeting vector in my research targeting strategy is based on established procedures. In general, a long 5' homology arm was chosen and combined with a shorter 3' homology arm to

keep the size of the targeting construct below 5kb and to facilitate the PCR-based genotyping. The targeting vector also provides a negative selection marker, thymidine kinase (tk), which is located next to the 5' arm. This negative selection marker ensures that only such cells that integrated the targeting construct survive, as the tk gene would be lost in the recombination event. In order to facilitate the homologous recombination event in ES cells the positive selection marker PGK marker is used ,which makes it likely to target genes that are both expressed and non-expressed in ES cells, is situated adjacent to the short arm corresponding.

The Southern blotting is designed to monitor a successful targeting event of the murine C1sA gene in the ES cell line. Only one of 1800 targeted cell clones showed homologous recombination by the Southern blot analysis (shown in figure 3.8) when hybridised with a 3'external probe located downstream from the sequence used to build the targeting construct on the C1sA gene.

The targeted allele was detected by a restriction fragment length polymorphism after digestion of the genomic DNA with the restriction endonuclease KpnI and BamHI while the non targeted wild type allele was detected by a fragment of 4.2bps and the targeted allele was detected by a

fragment of 5.6bps which hybridised with the external C1sA specific cDNA probe (shown in figure 3.8). The single clone 61 showing a targeting event in one of the C1sA alleles. In order to transmit the targeted allele into the germline, the ES cell clone 61 was used for microinjections into blastocysts of B6CBA /F1 mice (black for colour) to generate chimeras. Wild type female C57BL mice were bred with chimeric male anticipating that the targeted SV129 ES cells would also differentiate into spermatocytes and transmit the targeted allele into the germline to generate heterozygous (mice with targeted allele) and wild type mice. The litters obtained from the cross were genotyped using a multiple PCR using three different PCR primers (for sequences please refer to materials and Methods, page 45). A C1sA germ line transmission of our targeted allele in heterozygous mice by was detecting a fragment of 787bps for gene deficient allele and a fragment of 245bps representative of the wild type allele (see figure 3.15 and 3.16). After number of intercrosses between C1sA-/+ (heterozygous) mice were remain unsuccessful in generating a C1sA-/- homozygous mice, every time only got wild type and heterozygous litters .Various molecular and phenotypic experiments were performed to verify that the results coming from genotyping were accurate or giving us false picture as the heterozygous mice

appeared after each intercross were healthy and fertile and indistinguishable from wild type mice. In order to verify the genotyping results, the genotyped PCR products were send for sequencing and did restriction digest analysis to confirm that primers designed for genotyping were amplifying the right product (Section III.1.3.3.4.1in results). Also performed phenotypic experiment by collecting serum from the heterozygous and wild type mice obtained after intercrosses, but the phenotypic results also match the genotyping results confirming that there were no homozygous C1sA mice (Section III.1.3.3.4.2). The observed frequency of heterozygotes and wild type mice from our breeding pairs was tested with the expected Mendalian ratio of 1:2:1 as stated by law of segregation but does not agree with our results instead have a ratio of 1:2 which occurs due to segregation distortion or due to meiotic drive resulting in homozygous lethality. Statistical Chi-square analysis was performed to prove that (Section III.1.3.3.5).

From what is known about the functional activities of the human C1s serine protease and the phenotype of C1q-/- mice, we anticipate that C1sA-/- mice are likely to be healthy and fertile (Botto. M., 1998 and Botto.M. *et al*., 1998). At last it can be postulated that the deficiency of C1sA gene has no lethal effects as numerous cases of human C1s deficiency have been

reported (Inoue. N. *et al*., 1998 and Endo. Y. *et al*., 1999). But from the results obtained after intercrosses, we come to conclusion that the deficiency of C1sA gene in homozygosity may be lethal in *utero*. So the above drawn conclusion is in line with our statistical results as obtaining a ratio of 1:2 instead of 1:2:1 ratio which can only occur due to lethality.

VI.2 Analysis of the location of C1sA and C1sB biosynthesis and identification of the cell types expressing C1sA and C1sB

In the current study, it is verified that the murine C1s serine protease genes are duplicated forming two different gene clusters i.e. C1sA and C1sB, as demonstrated by Garnier. G. *et al*., 2003.

Gene mapping and genomic sequencing data show that human C1s and C1r serine protease genes are each encoded by a single structural gene and are primarily expressed, but not exclusively, in hepatic cells (Morris, K. *et al*., 1982). To localise the biosynthesis of C1sA and C1sB, a gene expression profile was established using both *in situ* hybridisation and time resolved PCR of cDNAs from different organs, including the liver, the lung, the spleen, the gonads, the thymus, the large intestine, the heart, the kidney, the small intestine and the brain from both male and female mice. The

quantitative real time PCR results showed that C1sA is expressed in almost all the tissues except the gonards (Figure 4.3) whereas C1sB is exclusively expressed in testis tissue (Figure 4.6). Mouse C1sA is therefore the homologue of human C1s and its expression profile is well in line with that of human C1s serine protease previously shown to be mainly produced by the hepatic cells (Ramadori. G., *et al* 1986; Morris. K.M., *et al* 1982; Xuebin. Q., *et al* 2006 and Tosi. M., *et al* 1989). Studies on hamster C1s (Sakiyama. H., *et al* 1991) and baboon C1s (Ramadori. G., *et al* 1986). The expression of the human complement serine proteases and complementary regulatory proteins (DAF and MCP) in female and male reproductive system has recently been well studied. MCP, DAF, and CD59 protect extra embryonic tissues from complement damage initiating from maternal and fetal blood. (Oglesby. T. J., 1998, Bozas. S.E., *et al* 1993, Anderson. D. J., *et al* 1993 and Rooney. I. A., *et al* 1993).

In situ hybridization (ISH) is a successful tool for the localisation of gene expression at the cytological level. It is a well-designed synthesis of histology and molecular biology. The principle of *in situ* hybridisation is the particular binding of a labelled probe to complementary sequences of target

nucleic acids in a parformaldehyde fixed tissue sample and then detection and localisation of mRNA by using cytological methods.

Riboprobes were *in vitro* transcribed from cloned C1sA and C1sB nucleotide sequences with SP6/T7 RNA polymerases in the presence of DIG-UTP and were used for hybridisation and were immunologically detected using alkaline phosphatse conjugated anti-dioxigenenin antibodies (Anti-DIG-AP).

Regards to the cellular location, *in situ* hybridisation analysis of spleen sections from normal female and male mice using DIG-labelled C1sA probe (Figure 4.11 A) and using ^{35} S-labelled probe (Figure 4.10 C) confirms a presence of the C1sA expressing cells throughout the white pulp. The C1sA signals are mostly localized in T lymphocytes and B lymphocytes. *In situ* hybridisation analysis on liver tissue using radioactive and non radioactive probes showed the C1sA (see Figure 4.10 A and Figure 4.11C) mRNA being expressed in hepatocytes whereas C1sB mRNA was undetectable. In the ovary, C1sA mRNA is mainly expressed in corpus luteum cells (TLC and GLC cells) (Figure 4.12 A) while no C1sB mRNA could be detected. As shown in Figure 4.12 E C1sB mRNA was abundantly expressed in testis

semniferous tubules particularly in Sertoli cells (nurse cells) and spermatozoa cells, while C1sA mRNA was undetectable.

VI.3 Production of recombinant mouse C1sA

The baculovirus expression system has already been used effectively to produce number of integral multidomain proteins including the human complement proteins C1r and C1s (Gal. P., *et al* 1998 and Luo. C. *et al*., 1992). Mouse cDNA coding for a 688 amino acid C1sA protein was cloned into the pbluebacHis2A vector and expressed in the Spodoptera frugiperda (Sf9) insect cells using the baculovirus expression system. In the present work, the Bac-N-Blue ^{TM} Baculovirus Expression system (O'Reilly, D.R., *et al* 1992) was used to express recombinant mouse C1sA. The establishment of baculovirus transfer vector system and purified protein after co-transfection with Bac-N-Blue ^{TM} DNA demonstrated that the system is suitable to generate stably transfected sf9 cell line producing high quantities of recombinant mouse C1sA. In addition, the quantity of protein produced is usually higher than the amount of protein produced by mammalian expression system (Veronique, R., *et al* 1998) and the main advantage of using Bac-N-Blue TM expression system that it produces high yield of endogenous proteins. The baculovirus expression system is a most regularly

used technique to produce proteins in eukaryotic cells efficiently and successfully. It takes advantage of a strong polyhedrine promoter and polyhedrine ORF thus allowing the high level expression of recombinant protein (Gearing and Possee, 1990).

C1sA protein is expressed in the cell pellet and detergent lysis buffer is used to lyse the cells effectively and extract intracellular protein. The amount of protein expressed was high. The cell lysate was evaluated for protein expression by Dot blot and Western blot using monoclonal mouse anti polyhistidine peroxidase antibody. The hC1s and mC1sA bands were compared with molecular weight standard after loading and running the 10.0% SDS-PAGE gel. Bands were seen between the BSA (98 kDa) and the glutamic anhydrogenase (64 kDa). The expected MW of human and mouse C1s protein is 85 kDa. The mouse C1sA heavy chain 58kDa and light chain 28kDa were analysed by using C1sA specific antibodies, primary antibody was sheep polyclonal C1s antibody and the secondary antibody was Donkey peroxidase labelled anti-Sheep IGg. Affinity chromatography technique is used for the protein purification using nickel charged sepharose columns.

The recombinant protein expressed will help in the production of specific monoclonal antibodies against mouse native C1sA and will be used to reconstitute the classical pathway functional activity in mouse plasma.

Chapter VII

Future Work

VII.1 Generation of C1sA deficient mouse line

Once the C1sA (C1sA-/-) mouse line is fully established it will provide us with a very informative mouse model of classical pathway deficiency. This mouse line will be most valuable to help us in studying the interdependence between the three complement activation pathways in vivo and in vitro. It will also allow us to define the role of the alternative pathway and its initiation mechanisms once established a mouse model deficient of both the classical and the lectin activation pathway by crossing the MASP2-/- mouse line with the C1sA-/- line. Other objectives which can be accomplished using C1sA-/- mouse are:

1. To assess the contribution of CP and LP activation to host defence in experimental models of bacterial and fungal infections such as models of pneumococcal lung infection (a collaborative work with Prof. Peter Andrew, Leicester) polymicrobial CLP in collaboration with Prof. Daniela Männel, Regensburg, Germany and infection with *Candida*

albicans and *Aspergillus fumigates* in collaboration with Prof. Kenneth B.M. Reid and Dr. Howard Clark.

2. To assess the contribution of CP and LP activation in ischemia/reperfusion injury in models of myocardial infarction and gut infarction in collaboration with Dr. Gregory Stahl, Boston, USA; renal reperfusion and kidney transplantation in collaboration with Prof. Steven Sacks, MRC Centre of Transplantation, Guys Hospital London and MCO model of stroke in collaboration with Prof. B. Paul Morgan, Cardiff.
3. To assess the contribution of CP and LP activation in murine models of immune complex nephritis in collaboration with Prof. Mohamed R. Daha and Dr. Anja Roos, Leiden, Netherlands.
4. To define the interactions and functional co-operations between the LP, CP and AP; *in vivo* and *in vitro* will be carried out in the Prof. Wilhelm Schwaeble's laboratory in Leicester.

This model of classical pathway deficiency is unique in so far as it will allow us to separate the role of the CP of complement activation from that of the CP recognition subcomponent C1q, which is known to mediate roles within the immune response independent of CP specific complement

activation. In addition, this C1sA deficient strain will allow us to establish a line of combined LP and CP deficiency a line deficient of C1sA and MASP-2.This is not achievable by crossing MASP-2 deficient animals with C1q-/- mice, because of the close proximity of both the C1q and the MASP-2 gene (less than 100 centimorgan away from each other). Likewise, the C4-/- mouse line is not deficient of both CP and LP as our recent work has demonstrated a LP specific activation route of complement C3 in C4-/- plasma.

VII.2 Establishment of C1sA specific antibodies

The next step is the production of C1sA specific antibodies. The main reason for the expression of recombinant proteins was there use in immunization of rabbits (polyclonal antibody) and rat (monoclonal antibodies) to produce specific anti-sera. Once the antibodies are produced they will be used in animal experiment and can be used for western blotting and ELISA.

VII.3 Establishment of a human minigene construct for transgenic expression of human C1s in C1sA deficient mice

After generation of a C1sA gene deficient mouse, the C1sA deficiency in C1sA-/- mouse will be replaced by human minigene construct of C1s gene which transgenically expresses the human C1s in the C1sA-/- mouse. This

transgenic line will be generated by inserting human C1s minigene construct into the fertilized oocytes of C1sA-/- mouse through microinjection. The purpose of establishing a mice with human C1s gene is to test the activity of mouse C1sA monoclonal antibodies once they generated.

VII.4 Alternative gene-targeting strategies

Though gene targeting technology by knocking out the gene of interest provides a significant tool in scientific research but still some restrictions do exist. 20% of gene deficient mice (knockout mice) are lethal or have deleterious effect on growing embryo that means the genetically changed embryos cannot grow into adult mice.

The other approach to overcome this deleterious effect is to generate a conditional gene targeting (conditional knockouts), which allows the deletion or modification of a gene of interest in a spatial and temporal manner. The commonly use conditional gene targeting systems are Cre-lox or Flip-FRT.

If the crossing between two C1sA heterozygous mice will remain unsuccessful then may switch to a conditional gene targeting starategy.

Chapter VIII

Appendix 1

VIII.1 Buffers and solutions

Buffers and solutions	
Competent cell preparation buffers	
TfbI solution	
30mM potassium-acetate 50mM $MnCl_2$ 100mM KCl 10mM $CaCl_2$ 15% glycerol pH 7.4	Mix 10ml of 3M potassium acetate, 50ml of 1M $MnCl_2$, 50ml of 2M KCl, 10ml of 1M $CaCl_2$ and 150 ml of glycerol with 830 ml of dH_2O. Filter sterilises and store at -20^{o}C.
Tfb II solution	
10mM Na-MOPS 75mM $CaCl_2$ 10mM KCl 15% glycerol pH 7.4	Mix 10 ml of 1M Na-MOPS buffer (pH 7.0), 75 ml of 1M $CaCl_2$, 5 ml of 2M KCl and 150 ml of glycerol with 860 ml of dH_20. Filter sterilises and store at -20^{o}C.

In situ hybridization buffers	
10x PBS	Mix 55g Na_2HPO_4, 18g $NaH_2PO_4.2H_2O$ and 450g NaCl with 4.5l dH_2O. Adjust pH to 6.8 and volume to 5l.
4% paraformaldehyde/PBS	Warm 500ml of dH2O to 50-55^oC in the microwave. Working in the fume cupboard, weigh out 40g of paraformaldehyde and add to the pre-warmed water. Place on a stirrer and add 10M NaOH drop-wise, until the solution is clear. In a separate beaker, prepare 500ml of 2 x PBS (100ml of 10 x PBS and 400ml of water). Mix the two solutions and adjust the pH to 7.3-7.4. Keep for 1-2 weeks at 4^oC.
Na Carbonate Buffer, pH 10.2	Prepare by mixing 3.2ml of 1M $NaHCO_3$ and 4.8ml of 1 M Na_2CO_3 in a 50 ml Falcon tube. Add water to 40 ml, and check that the pH is about 10.2. Aliquot and freeze.
Hybridization Buffer 600mM NaCl 10mM Tris 1mM EDTA	For 50 ml, mix 6 ml 5M NaCl, 500µl 1M Tris HCl, pH 7.5, 100µl 0.5 M

1x Denhardt's reagent 10% w/v dextransulphate 100mg/ml salmon sperm DNA 50% formamide	EDTA, 1ml 50 x Denhardt's reagent, 5 g of dextran sulphate, 500 µl of sonicated salmon sperm and 25 ml of formamide. Fill with dH_2O to 50 ml and warm to 37^oC to dissolve. Store at -20^oC.
RNAse buffer	Mix 29.2 g of NaCl, 2ml of 0.5M EDTA and 10ml of 1M Tris Cl, pH 8.0 in about 800ml of water. Adjust pH back to 8.0, volume to 1L, and add 2ml of 10mg/ml RNAseA solution. Store in fridge and re-use.
TEA Buffer	Add 13.3 ml of TEA (triethanolamine) to about 800 ml dH_2O; adjust pH to 8.0 with conc. HCl, then volume to 1L.
Buffer 1, pH 7.5	15.76g of 100mM Tris-HCl and 8.77g of 150mM NaCl to make 1L.
Buffer 2, pH 9.5	15.76g of 100mM Tris-HCl, 5.84g of 100mM NaCl and 10.16g of 50mM $MgCl_2$ to make 1L.
SDS-PAGE and Western Blot Buffer	
Tris-Glycine Buffer	25 mM Tris base 192 mM Glycine 0.1% SDS

	pH 8.3
SDS Loading Buffer	100 mM Tris-HCl 4% SDS 5% β Mercaptoethanol 10% Glycerol 0.2% (w/v) Bromophenol blue pH 6.8
SDS-Transfer Buffer (pH 8.3)	20 mM Tris base 150 mM Glycine 0.038% SDS 20% (v/v) Methanol Read pH without adding methanol
Blocking Solution	1g of semi-skimmed milk in 20 ml of 1x PBS.
PBS Tween 0.05%	For 200 ml, 180 ml of 1 x PBS and 90µl of Tween 20 (cut off tip of 1000µl tip, to pipette Tween 20).
Southern Blotting Buffers	
Transfer Buffer	For 2 liters, 10.0 M NaOH and 5.0M NaCl
Neutralization Buffer	For 2 liters, 0.3 M Tris HCl and 3.0 M NaCl

20x SSC, pH 7	3 M NaCl 300 mM $Na_3C_6H_5O_7.H_2O$
Other Buffers	
Injection Media	To make up 20ml 15.2ml of DMM(High Glucose) 4.0ml of FCS (Lab Tech) 0.4ml of L-Glutamine 0.2ml of NaPyruvate 0.2ml of Pen/Strep
Complete TNM-FH Medium	Complete TNM-FH medium is Grace's Insect Medium with supplements (lactalbumin hydrolysate, L-glutamine, TC-yeastolate) and 10% fetal bovine serum (FBS). Grace's Insect Medium supplemented from Gibco™, add 55ml of FBS. Mix well. Add Pen/Strep.Filter-sterilize the solution through a 0.2µ filter into a sterile container. Store at 4°C and warm to 27°C before use.
Agarose-TNM-FH Solution	To make 50 mls, add 25ml of Grace's Insect Medium (2X) supplemented with FBS, 12.5ml sterile water and 12.5ml melted 4% Agarose. Mix well

	and place the container in 40^{o}C water bath until use.
PEG/NaCl Solution	For 100ml, 20g of 20% PEG 8000 and 5.84g NaCl in 100ml water and autoclave for 20 mins(15lbs/sq.in).
X-gal solution	Add 100mg of X-gal in 2 ml dimethylformamide (DMF) and store at -20oC in amber bottle.
LB Medium	For 500 ml, 2.5g of NaCl, 2.5g of yeast extract, 5g of tryptone and add 500ml of double distilled water and autoclave the medium
LB Amp Medium for LB plates	For 400 ml, 4g of tryptone, 2g of yeast extract, 2g of NaCl, 6g of agar and add 400ml of double distilled water and autoclave. After autoclave add 800µl of 50mg/ml Ampicilline for 400ml of LB medium.
50 X TAE Buffer	For 2 liters; add 484g of Tris, add 1l of nanopure H_2O, 200ml of 0.5M EDTA and 114.2ml of glacial acetic acid. After dissolving check the pH and add rest of nanopure H_2O to make buffer.

1 X TAE Buffer	For 10 liters, add 200ml of 50X TAE add 200µl of ethidium bromide and add 10 liters of distilled water.

Appendix 2

GTTCATTACTATTTAAAATTTTTCTTTTAGATTTAATTATTTGATTTTATGTGTATGAGCATTTTGCCTGCATATATGTATGTGTTCGACATGAATGCCTGGTGGCCATAGAGACCAGAAGGTGTCAGGTTTCCTGGAAC

CAGAATTACATCGAAAGTTGTGAGCCAGTGAGATGGCTCAGTGGTTAAGTACACTGACTGCTCTTCCAAAGGTCCTGAGTTCAAATCCCAGCAACCATGTGGTGGCTCACAACCATCCGTAATGAGATCTGATGCACT

CTTCTGGTGTGTCTGAAGACAGCTACAGTGTACTTACATATAATAATAATAAACAAATCTTTAAAAAAGAAAAGAAAAAAATGTTGTGAGCCACCATGCCAGTGCTGGGACTTGAACCTGGGTCCTCTGGAAGAACA

GCAGTGCTCTTCACCTCAGAACCATCTTTTCATGTCTTTGTTAATAAATAATTTCCAAATGGACTTAGAATGATGCTTGAGGGCAAATGTATTAGAATTGGAGAAACAACATTAGGCAAGCCTTTTTGGATCTTAGGTA

GCCCAGGTTGACCTCCAAATGACTATATAAAGAAAGACTGTCCCTGATTTTCTGATAGCTCCTGGCTTCACTTCCTAAGTACTGAGGTCATAGCTGTGCATCAAATTAAACAAGAAACAAATGTACTATACAGAACTA

GCCAGCCTGATATTCTCTATGTAGTCCAGGCTAGACTCAGATTCTCAGAAATTCTTCTCCTGGACTTACAGGCATTAACCACCTTGCCCAGCCTTGAGATGGGACTTTCGGGACGAAGAAACTTTGAGTGCACATGCCG

TCTAGGTGTAAATTGTCTCGAAGGCAGGATGAGTTGCATTTATGTATTAATTAAAGAAAGAATTGCATTGGCATGTTCTCAGAAGACAGCTTGCAGGAACTGTTGGTTCACCTATGATGTGGGTCCCAGGGGATGTAG

AGTTCATGTCTTTCTGCCTGGAGGTAGGTACCTTTCTAAGCTGAGCTATCTTGCCTCCTAGAGCCTCCAGACAATGGCTTCTACTTCTGATGACTCTCTTCCTTTGATTCATGAATCACACAGAGGGTTGGACTTTCTGA

GTTTAGTTTTCGTCCCTCCTGTTAAGCCTTCCCTCCTTAAGAAGATAAATCACCTGACTTCCTCAAAAGCTCTGAAACACACCCTTTGGCTGTAAGAGAGAATTAGGCAATCTCAAAATTCCCCATTCCCCCCTTTTGCT

CTGGACCTCTCAGCCCAGCAAAGCTGGTGCAGTTCTTGTTTTAAACGCAGAGGGCACTCTAAGATCAAGGCTGATTACTCTTAACTAGATTGTGATCTCCCAGCCCCCTCTGTTTGGAACAGGGAATTTTGGACATGCA

AAAACACACTCCCTTAGGGAATCTTTAGAGCAAACAGAACAGGGAGGTGGGGAGGAGCTTCCTGCAAAGGCCACTCCCTCTGGAGCTGCAGGGGCTTAACTGCATGACCAGAGGCAGGAGAGGAGGCTGGCCCACT

TGTTCCCATCAGCTCCTGAAGGTAACAACTTTAAGAGCAAGCCTTAAAGAGAGAAGCTTTGATGAAGGAAGAGGGAAAGACAAGGAACCATGTACAGTAAGGTTTGGAGCAAAGATGGAGGGTTGGAAGGAGCTCA

GCGAGAGGTCAGAATACAGACACGGGTTCTCACATGTAGGACCCAGCCCCACCCCACAACAGCCTCATCCTTTCACACAGCAGTGTTTCAGGGGTCAGAGGCAATTTGTGAAAGACTTTTCTGGGAACTATGGGAAG

ATACCTTGCTCTTTAAGCAAAGGACAAAACAAACCAACTCGAGGGGCGCGCCCCCAGCTGGTTCTTTCCGCCTCAGAAGCCATAGAGCCCACCGCATCCCCAGCATGCCTGCTATTGTCTTCCCAATCCTCCC

CCTTGCTGTCCTGCCCCACCCCACCCCCCAGAATAGAATGACACCTACTCAGACAATGCGATGCAATTTCCTCATTTTATTAGGAAAGGACAGTGGGAGTGGCACCTTCCAGGGTCAAGGAAGGCACGGG

GGAGGGGCAAACAACAGATGGCTGGCAACTAGAAGGCACAGTCGAGGCTGATCAGCGAGCTCTAGAGAATTGATCCCCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGG

AGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGATCCGCCACACCCAGCCGGCCACAGTCGATGAA

TCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCATCGCCATGGGTCACGACGAGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTG

ATGCTCTTCGTCCAGATCATCCTGATTGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATT

GCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAA

GGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCCTGCAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAACCGGGCGCCCCTGCGCTGACAGCCGGAACACGGCGGC

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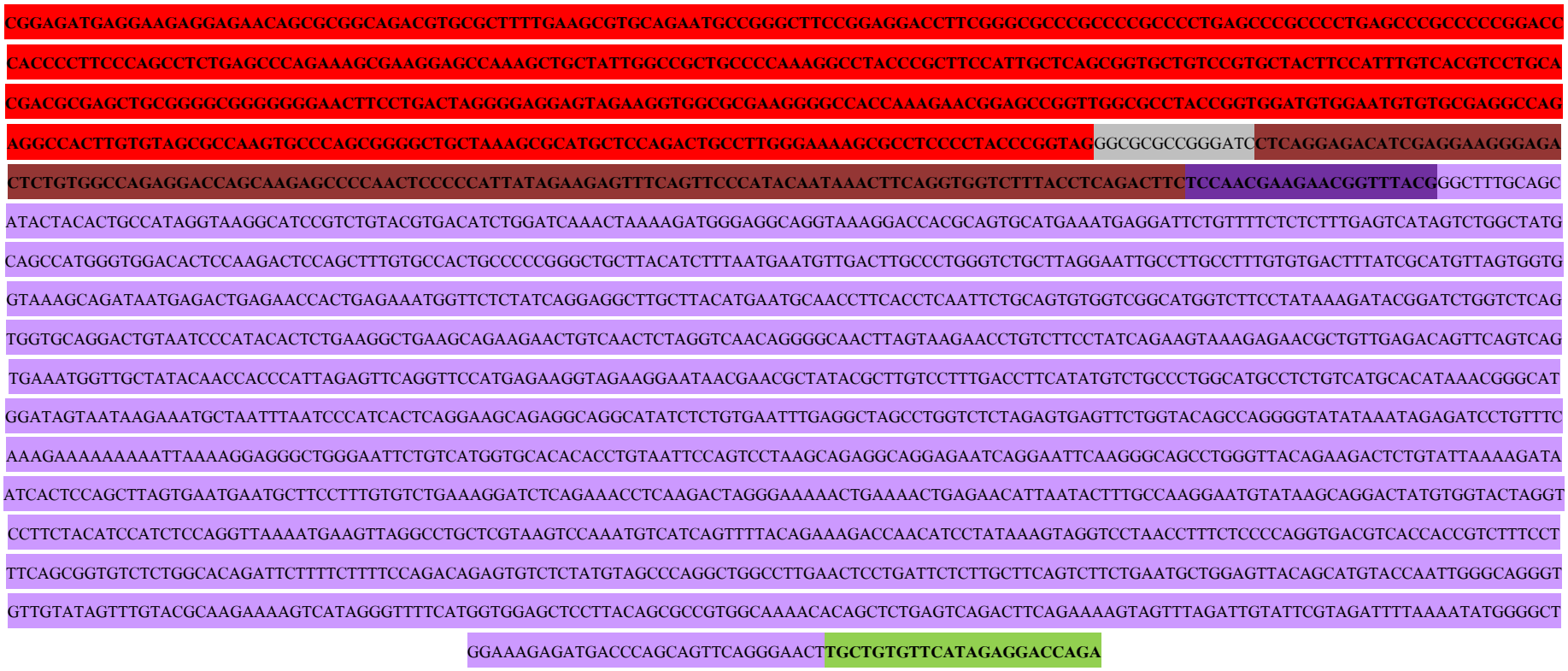

Figure 1 This figure shows the C1sA sequence used for the generation of C1sA deficient mouse line. The sequence for the longer arm (A Cassette) is shown in brown shaded area and for shorter arm (B Cassette) is shown in light purple colour. The sequence for bgh Poly (A) (pink colour), Neomycin (yellow colour) and PGKP promoter (red colour) is also shown. The exon 4 is shaded in maroon colour. Cloning site used for A Cassette are also shown BglII (light blue colour) and XhoI (dark blue colour) and the primers used for B Cassette : forward primer C1sA_BamH2 (dark purple colour) and reverse primer KBR_ClaI (green colour).

GCACATTAACTCCACAGGCAGGCCCATGAGCCCCATCCTGTCCCACACTTGGAGCTGGATGAAGTGGGCCCTACTGCAG**GCTTGGC**

AGCAGGTTTAGAGATACATAGCCCTCCATGTTTCTTCCCAGGGATACCCTTCTTTTAAAGAATGATATTCAGTAGCCAAAGGGCAGT

GTGCTAATGGGGGGATGATGCCCCCATATCTCTCTGTCTCTCTCCCCCTCTCTCTCTGTCTCTCTGTCTCTGTCTGTCTGTCTGTC

TCTGTCTCACTCTGTCTCTCTGTCTCTCTCTGTCTCTGTCTCTCTCTGTCTCTCTTTCTGTGTATGAGGGGGAGGGGGAGGAGGAGGAG

GAGGAAGAGCAACGTTCATTACTATTTAAAATTTTTCTTTTAGATTTAATTATTTGATTTTAT**GTGTATGAGCATTTTGCCTGCA**TA

TATGTATGTGTTCGACATGAATGCCTGGTGGCCATAGAGACCAGAAGGTGTCAGGTTTCCTGGAACCAGAATTACATCGAAAGTTGT

GAGCCAGTGAGATGGCTCAGTGGTTAAGTACACTGACTGCTCTTCCAAAGGTCCTGAGTTCAAATCCCAGCAACCATGTGGTGGCTC

ACAACCATCCGTAATGAGATCTGATGCACTCTTCTGGTGTGTCTGAAGACAGCTACAGTGTACTTACATATAATAATAATAAACAAA

TCTTTAAAAAAGAAAAGAAAAAAATGTTGTGAGCCACCATGCCAGTGCTGGGACTTGAACCTGGGTCCTCTGGAAGAACAGCAGTG

CTCTTCACCTCAGAACCATCTTTTCATGTCTTTGTTAATAAATAATTTCCAAATGGACTTAGAATGATGCTTGAGGGCAAATGTATTA

GAATTGGAGAAACAACATTAGGCAAGCCTTTTTGGATCTTAGGTAGCCCAGGTTGACCTCCAAATGACTATATAAAGAAAGACTGTC

CCTGATTTTCTGATAGCTCCTGGCTTCACTTCCTAAGTACTGAGGTCATAGCTGTGCATCAAATTAAACAAGAAACAAATGTACTATA

CAGAACTAGCCAGCCTGATATTCTCTATGTAGTCCAGGCTAGACTCAGATTCTCAGAAATTCTTCTCCTGGACTTACAGGCATTAACC

ACCTTGCCCAGCCTTGAGATGGGACTTTCGGGACGAAGAAACTTTGAGTGCACATGCCGTCTAGGTGTAAATTGTCTCGAAGGCAGG

ATGAGTTGCATTTATGTATTAATTAAAGAAAGAATTGCATTGGCATGTTCTCAGAAGACAGCTTGCAGGAACTGTTGGTTCACCTAT

GATGTGGGTCCCAGGGGATGTAGAGTTCATGTCTTTCTGCCTGGAGGTAGGTACCTTTCTAAGCTGAGCTATCTTGCCTCCTAGAGCC

TCCAGACAATGGCTTCTACTTCTGATGACTCTCTTCCTTTGATTCATGAATCACACAGAGGGTTGGACTTTCTGAGTTTAGTTTTCGTC

CCTCCTGTTAAGCCTTCCCTCCTTAAGAAGATAAATCACCTGACTTCCTCAAAAGCTCTGAAACACACCCTTTGGCTGTAAGAGAGA

ATTAGGCAATCTCAAAATTCCCCATTCCCCCCTTTTGCTCTGGACCTCTCAGCCCAGCAAAGCTGGTGCAGTTCTTGTTTTAAACGCA

GAGGGCACTCTAAGATCAAGGCTGATTACTCTTAACTAGATTGTGATCTCCCAGCCCCCTCTGTTTGGAACAGGGAATTTTGGACAT

GCAAAAACACACTCCCTTAGGGAATCTTTAGAGCAAACAGAACAGGGAGGTGGGGAGGAGCTTCCTGCAAAGGCCACTCCCTCTGG

AGCTGCAGGGGCTTAACTGCATGACCAGAGGCAGGAGAGGAGGCTGGCCCACTTGTTCCCATCAGCTCCTGAAGGTAACAACTTTA

AGAGCAAGCCTTAAAGAGAGAAGCTTTGATGAAGGAAGAGGGAAAGACAAGGAACCATGTACAGTAAGGTTTGGAGCAAAGATGG

AGGGTTGGAAGGAGCTCAGCGAGAGGTCAGAATACAGACACGGGTTCTCACATGTAGGACCCAGCCCCACCCCACAACAGCCTCAT

CCTTTCACACAGCAGTGTTTCAGGGGTCAGAGGCAATTTGTGAAAGACTTTTCTGGGAACTATGGGAAGATACCTTGCTCTTTAAGC

AAAGGACAAAACAAACCAACTCGAG GGCGCGCCCCCAGCTGGTTCTTTCCGCCTCAGAAGCCATAGAGCCCACCGCATCCCCAGC

ATGCCTGCTATTGTCTTCCCAATCCTCCCCCTTGCTGTCCTGCCCCACCCCACCCCCCAGAATAGAATGACACCTACTCAGACAATGC

GATGCAATTTCCTCATTTTATTAGGAAAGGACAGTGGGAGTGGCACCTTCCAGGGTCAAGGAAGGCACGGGGGAGGGGCAAACAAC

AGATGGCTGGCAACTAGAAG**GCACAGTCGAGGCTGATCAG**CGAGCTCTAGAGAATTGATCCCCTCAGAAGAACTCGTCAAGAAG

GCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTT

CAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGATCCGCCACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGG

CCATTTTCCACCATGATATTCGGCAAGCAGGCATCGCCATGGGTCACGACGAGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTG

GCGAACAGTTCGGCTGGCGCGAGCCC**CTGATGCTCTTCGTCCAGATC**ATCCTGATTGACAAGACCGGCTTCCATCCGAGTACGTGC

TCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGAT

GGAT

Figure 2 The putative sequence used for designing the primers used for screening of homozygous recombinants. The primers used are C1sA_KAF1 (shown in pink), C1sA_KAF2 (shown in purple), Neo5_R1 (shown in brown) and Neo5_R2 (shown in green). A cassette is shown in blue colour. Neomycin resistance is shown in yellow colour.

Chapter IX

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