AN INVESTIGATION OF THE BINDING OF HUMAN MONOCLONAL ANTIBODIES TO AUTOANTIGENS BY THE MODIFICATION AND EXPRESSION OF CLONED AUTOANTIBODY cDNA.

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ABSTRACT

Autoantibodies to a wide variety of antigens are associated with Systemic Lupus Erythematosus (SLE). High affinity IgG antibodies to double-stranded DNA (anti-dsDNA) are thought to be particularly closely related to tissue damage and disease activity in SLE. Sequence analysis of murine and human anti-DNA antibodies has shown that these binding properties are derived from the accumulation of replacement mutations and basic residues in the complementarity determining regions (CDRs) of such antibodies. This thesis describes the effects of particular sequence motifs, often sites of somatic mutation and/or containing arginine residues, on the binding to dsDNA and other autoantigens of a high affinity IgG anti-dsDNA mAb, B3.

Using a transient expression system whole IgG containing B3 heavy chain (V_H) paired with different light chain sequences (V_λ), all derived from human germline λ gene, 2a2 but with different patterns of somatic mutations, were produced. As predicted by computer-generated models, some mutations to V_λCDR arginine residues enhanced binding to DNA whilst others blocked DNA binding. These CDR motifs also affect binding to histones and Sm antigen. It was concluded that it is not the presence of arginine residues in the CDRs but their precise position that is important in determining autoantigen-binding affinity. The V_H also plays a role as pairing of the same 2a2-derived V_λ with the V_H of a different anti-dsDNA antibody, 33.H11, gave reduced ability to bind DNA in comparison to B3V_H.

To study the pathogenic effects of these sequence motifs, a stable expression system was used to produce two cell lines, one producing wild type B3 and the other producing a B3 variant in which a V_λCDR1 arginine critical to DNA binding had been reverted to a serine. The quantities of IgG produced were sufficient for such assays and also showed the same DNA binding affinities as the transient system IgG.

This thesis is dedicated to my husband Tony and the rest of my family without whose continual support and encouragement this work would not have been possible.

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DECLARATION

The work described in this thesis was carried out by the author unless otherwise stated.

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ABBREVIATIONS

ANA — Anti-nuclear antibodies

Anti-dsDNA — Antibodies to double stranded DNA

bp — Base pairs (of DNA)

BIC — Bicarbonate buffer

BSA — Bovine serum albumin

C — Complement component (as in C1q, C2 etc.)

CDR — Complementarity-determining region

C_H — Constant domain of heavy chain

C_L — Constant domain of light chain

C Region — Constant region

DNA — Deoxyribonucleic acid

DNase — Deoxyribonuclease

ds — Double stranded

EDTA — Ethylenediaminetetra-acetic acid

ELISA — Enzyme-linked immunosorbent assay

Fab — Antigen binding fragment of antibody

Fc — Crystallizable fragment of antibody

FR — Framework region

GBM — Glomerular basement membrane

H-CDR — CDR of heavy chain

H-FR — FR of heavy chain

HCLV — Heavy chain loss variant

HS — Heparan sulphate

Id — Idiotype

Ig	Immunoglobulin
IPTG	Isopropylthiogalactoside
J	Joining region gene
Kb	Kilobase
La	La antigen
LAC	Lupus anticoagulant
LB	Luria- Bertani
L-CDR	CDR of light chain
L-FR	FR of light chain
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
mIg	Membrane-bound immunoglobulin
MW	Molecular weight
NAA	Natural autoantibodies
OD	Optical density
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
phOx	2-phenyl-5-oxazolone
pG1D1	Heavy chain expression vector (AERES Biomedical, London, UK)
pG1D210	Heavy chain expression vector (AERES Biomedical, London, UK)
pLN10	Light chain expression vector (AERES Biomedical, London, UK)
RA	Rheumatoid arthritis
Ro	Ro antigen
RNA	Ribonucleic acid
RNase	Ribonuclease

scFv	Single chain Fv fragment of Antibody
SD	Standard deviation
SEC	Sample/enzyme/conjugate dilution buffer
SCID	Severe Combined Immunodeficiency
SLE	Systemic Lupus Erythematosus
SPR	Surface plasmon resonance
ss	Single stranded
TAE	Tris Acetate EDTA buffer
TBE	Tris Borate EDTA buffer
TE	Tris EDTA buffer
V_H	Variable domain of heavy chain
V_L	Variable domain of light chain
V Region	Variable region
wt	Wild type

CHAPTER ONE

Introduction

1.1 Role of Antibodies in the Immune System.

1.1.1 Antibodies: Structure and function.

The experimental work of Emil Von Behring and Shibasaburo Kitasato in 1890, provided the first description of antibodies (Kuby, 1997). Antibodies (Ab), also called immunoglobulins (Ig), are a group of antigen-binding proteins present on the B cell membrane and secreted by plasma cells. The function of an antibody is to recognise foreign cells or molecules that have entered the body by binding to their antigen (any molecule that can be specifically recognised by the adaptive elements of the immune system) and to then either neutralise or facilitate elimination of the foreign body. Antibody molecules do not bind to the whole of a foreign molecule or pathogen. Instead, due to their specificity, each antibody molecule binds to one of the many molecules (antigens) on the pathogen's surface. Each antibody binding-site binds to a restricted part of the antigen called an antigenic determinant or epitope. The antigenic specificity of the immune system enables it to distinguish subtle differences among antigens. Antibodies can differentiate between two molecules that differ by only a single amino acid (Kuby, 1997; Roitt *et al.*, 1996). The way in which a sufficient diversity of molecules is generated to bind all the different antigens encountered in a lifetime is explained in section 1.3.3.

The specificity and effector functions of an antibody are determined by its structure. An antibody or immunoglobulin molecule consists of two identical heavy (H) chains (MW 50,000–70,000) and two identical light (L) chains (MW 25,000) linked by disulphide bridges. Any L chains may combine with any H chain but in any one immunoglobulin molecule both L chains are identical as are the H chains. Sequence analysis of Igs first became feasible with the discovery of multiple myeloma (cancer of plasma cells) (Wu *et al.*, 1970). In patients with multiple myeloma, plasma cells in the neoplastic clone continue to proliferate in an unregulated way and produce specific antibody (referred to as myeloma protein) indefinitely. As a result, a huge excess of myeloma-derived antibody forms in their bloodstream compared to the levels of antibodies produced by other plasma cells. Since every cell in a myeloma is identical, a myeloma is a huge source of homogenous antibody protein (ideal for sequence analysis). Sequence analysis of myeloma proteins showed that the carboxyl terminal part of each chain is constant for all antibodies of a given isotype. These are called the C_L (Constant: light chain) region and the C_H (Constant: heavy chain) region (Wu *et al.*, 1970). The C_L of most vertebrates

including humans have been shown to exist in two distinct forms or isotypes, kappa (κ) and lambda (λ). In humans, 60% of the L chains are κ and 40% are λ (Schable *et al.*, 1993; Williams *et al.*, 1993) whereas in mice 95% are κ whilst only 5% are λ (Kofler *et al.*, 1992). A single antibody molecule contains either kappa or lambda chains but never both. λ chains are further divided into subtypes due to amino acid substitutions in their amino acid sequences. Mice have three subtypes (λ1, λ2 and λ3) whilst humans have four (Kuby, 1997).

The C_H of most vertebrates including humans have been shown to exist in five distinct forms; μ, γ, α, δ and ε. These are isotypes. The H chains of a given antibody determine the class of that antibody; IgM, IgG, IgA, IgD and IgE, respectively. Each class can pair with κ or λ chains. Similarly to L chains, subtypes of each chain isotype exist. IgG has four subtypes (γ1, γ2, γ3 and γ4) and consequently four classes (IgG1, IgG2, IgG3 and IgG4). IgA also has two subclasses but none have been described for IgM, IgD and IgE (Kuby, 1997).

The amino-terminal parts of the chains however show much sequence variability between different antibody molecules and are known as the variable regions (V_H and V_L). Within the variable regions there are three areas of hyper variability, the Complementarity-determining regions (CDRs) (Wu *et al.*, 1970). These are separated by Framework regions (FRs), longer stretches of sequence (β-pleated sheet structure) that are more conserved between different antibodies. From crystallographic and computer modelling studies, it has been deduced that FRs maintain spatial orientation of the antigen-binding pocket whilst the CDRs determine antigen specificity, although some exceptions exist (Barry *et al.*, 1994; Herron *et al.*, 1991). Each variable region consists of three CDRs and four FRs (Khalil *et al.*, 1999). The antigen-binding site is therefore encoded by just the amino-terminal domains of these chains. If digested with papain the antibody is separated into two Fab fragments and one Fc fragment. However only Fab fragments are able to bind antigen as they contain the V regions whilst the Fc fragment does not (see Figure 1.1).

The primary function of an antibody is to bind antigen. In some cases this has a direct effect, for example neutralising toxins or viral particles by coating them and preventing their subsequent binding or penetration of target host cells. However the specific

interaction of antibody and antigen via its variable (Fab) region is insignificant unless the antibody can also perform secondary effector functions. Although the C_L and C_H regions (Fc) do not determine the antigen-specificity of the antibody they mediate the effector functions of the molecule. For example, antigen-binding by certain isotypes of antibody (IgM, IgG1, IgG2 and IgG3 only) activates the complement system. Binding of these antibody isotypes to antigen triggers a conformational change in the Fc region subsequently exposing a binding site for the first complement component in the classical complement pathway. Activation of complement can result in opsonisation of the micro-organism or foreign body (thus promoting engulfment by phagocytes bearing complement receptor) and/or formation of a membrane attack complex that directly attacks the cell membrane of the source of the antigen, causing subsequent lysis. Complement can also help induce localised inflammatory responses (for example, by stimulating extravasation and chemotaxis of leucocytes at site of inflammation) whilst also facilitating the clearance or solubilisation of immune (antigen-antibody) complexes. Additionally, antibody can aid clearance of certain antigens by cross-linking antigen thus forming clusters (agglutination) that are more readily ingested by phagocytic cells, particularly since many phagocytes possess receptors specific for the Fc portion of the antibody (Fc receptors) (Kuby, 1997; Roitt *et al.*, 1996).
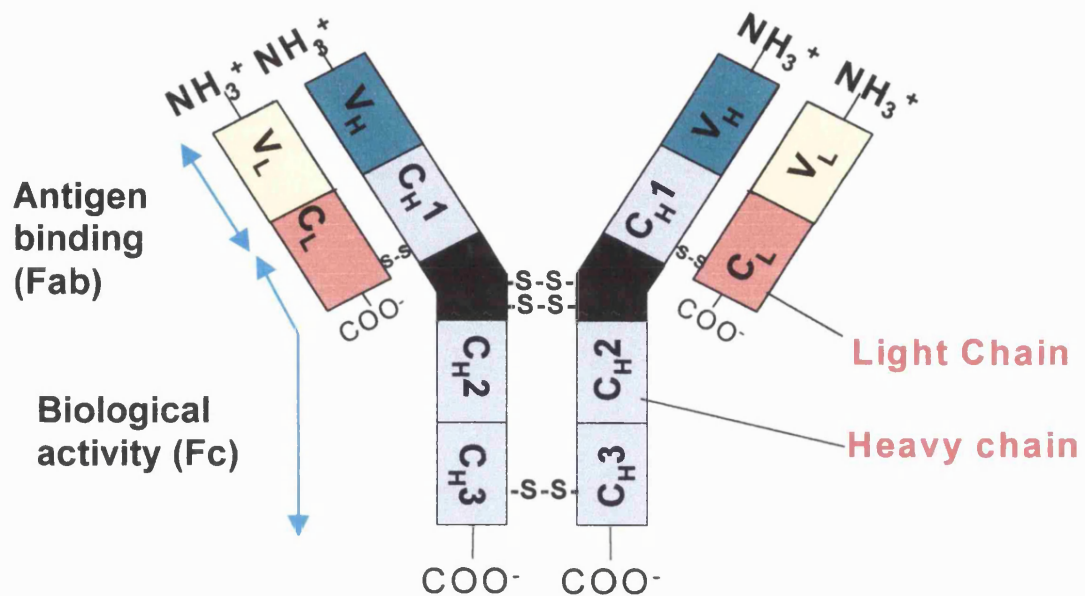
Figure 1.1   **Schematic diagram of the basic structure of an immunoglobulin molecule**

An antibody or immunoglobulin molecule consists of two identical heavy (H) chains (MW 50,000–70,000) and two identical light (L) chains (MW 25,000) linked by disulphide bridges. The carboxyl (COO⁻) terminal part of both chains is constant for all antibodies of a given isotype and is called the C_L (Constant: light chain) region and the C_H (Constant: heavy chain) region. There is one C_L region and three C_H regions. The amino (NH_3^+) terminal parts of the chains however show much sequence variability between different antibody molecules and are known as the variable regions (V_H and V_L). If digested with papain the antibody is separated into two Fab fragments and one Fc fragment. However only Fab fragments are able to bind antigen as they contain the V regions whilst the Fc fragment does not. The Fc fragment is responsible for the biological activity of the immunoglobulin molecule (Kuby, 1997).

1.1.2   Role of T helper lymphocytes in the production of antibodies

The immune system is made up of two main components: humoral immunity and cell-mediated immunity. Antibodies secreted by B cells are the major effector molecules of humoral immunity. In the humoral branch of the immune system when a naïve B cell, which has not previously encountered antigen, first encounters the antigen for which its membrane-bound antibody/immunoglobulin (mIg) is specific, the B cell will bind to the antigen. However before the B cell can become activated   (i.e. proliferate and differentiate), additional interactions with both molecules on the surface of activated CD4+ T helper (T_H) lymphocytes and also with cytokines produced by these T_H cells are required (Kuby, 1997; Roitt *et al.*, 1996).

Once an antigen cross-links the specific B cell mIg, the antigen is internalised by receptor-mediated endocytosis. This event causes levels of MHC (Major Histocompatibility complex) Class II molecules and co-stimulatory molecule, B7 (ligand for T cell CD28) to be upregulated on the surface of the B cell. The internalised antigen is degraded and some peptides are packaged with MHC Class II molecules to be presented on the membrane of the B cell. T_H cells can only recognise antigen in the presence of self-MHC. Activation of a T_H cell can occur when its membrane-bound T cell receptor (TCR) encounters the peptide for which it is specific whilst the B cell surface molecule B7 simultaneously contacts its ligand on the T cell surface (CD28). As a consequence, the T cell begins to express CD40L, which binds to CD40 on the B cell surface thus triggering the B cells to begin to express cytokine receptors so that they may interact with cytokines produced by the activated T_H cell. When stimulated by activated T_H cell membranes, B cells can only proliferate but cannot differentiate into either memory B cells or effector cells (plasma cells) without cytokines. Memory B cells have a longer life span and continue to express membrane-bound antibody with the same specificity as the original naïve B cell. Plasma cells do not express membrane-bound antibody; instead they produce the antibody in a form that can be secreted (Roitt *et al.*, 1996).

Together with the cell-mediated immune response, involving the differentiation of CD8+ T cytotoxic (T_c) cells into effector cytotoxic T lymphocytes (CTLs) and the recruitment of phagocytic cells (also mediated by cytokines released by activated CD4+ T_H cells), the antibody (humoral) immune response provides an adaptable and effective defence system.

1.1.3 Autoantibodies and Autoimmunity

Autoimmunity is the response of the immune system against self-components. Normally self-tolerance mechanisms protect an individual from potentially self-reactive lymphocytes. Antibodies that bind to self-antigens (i.e. antigens present in the body of the host) are termed autoantibodies. Originally it was believed that all self-reactive lymphocytes were eliminated during development and that failure to eliminate these lymphocytes resulted inevitably in autoimmune disease. However, the immune system does produce some antibodies spontaneously, without being immunised against the relevant antigens and these so-called natural autoantibodies (NAA) are commonly found in healthy individuals (Fritzler *et al.*, 1985), first degree relatives of patients with autoimmune diseases (Isenberg *et al.*, 1985) and among elderly people (Tomer *et al.*, 1988). This implies that these natural autoantibodies are not always harmful. What, however is the purpose of these natural autoantibodies? The two main possibilities are, firstly, that NAA are an epiphenomenon and by-products of an immune system that is continuously processing huge amounts of antibodies. The second is that the immune system would not allow the persistence of immune molecules that are unnecessary to the normal functions of the organism. The latter notion is supported by the fact that several physiological and regulatory roles have been attributed to these NAA (George *et al.*, 1996). They may fulfil a biological regulatory role acting as carriers for catabolic products by binding to degraded tissue components, followed by their opsonisation and phagocytosis (Lutz *et al.*, 1987).

Autoimmune disease in humans can be divided into two broad categories; organ-specific or systemic autoimmune disease. In an organ-specific disease, the immune response is directed to a target antigen unique to a single organ or gland, so that the effects are largely localised to that organ. For example, patients with Hashimoto's thyroiditis have autoantibodies and sensitised T_H cells that target thyroid antigens including thyroglobulin and thyroid peroxidase, which are both involved in iodine uptake. Consequently, binding of these thyroid antigens disrupts iodine uptake and leads to hypothyroidism. However, in a systemic autoimmune disease, the immune response is directed to a wide range of target antigens thus involving many tissues and organs. Consequently, since neither antigen nor disease is tissue specific, it is often difficult to determine the direct cause of the pathogenesis of the disease.

In some systemic diseases, certain autoantibodies are believed to cause the tissue damage seen. Normally the complexing of antibodies with antigen facilitates the clearance of antigen by phagocytic cells since these cells bear Fc receptors. However if these immune complexes are not cleared and large amounts of immune complexes remain, this can lead to tissue-damaging type III hypersensitivity reactions. The magnitude of the reaction depends on the quantity of immune complexes as well as their distribution in the body. A reaction and subsequent tissue damage can develop wherever the complexes are deposited (e.g. on the glomerular basement membrane of the kidneys) (Kuby, 1997).

Tissue damage occurs when the formation of immune complexes causes the activation of the complement system's effector molecules. The complement system is composed of a group of serum proteins that exist in an inactive proenzyme state. Following initial activation, the various complement components interact in a highly regulated enzymatic cascade, to generate reaction (split) products. These complement-split products serve as immune effector molecules that cause localised vasodilation and chemotactically attract neutrophils. Much of the damage to tissue in which immune complexes have deposited is caused by the release of lytic enzymes by neutrophils attempting to phagocytose the immune complexes. The C3b complement component acts as an opsonin, coating immune complexes. A neutrophil binds to a C3b-coated immune complex by means of a receptor specific for C3b. As the complex is attached to the basement-membrane surface, phagocytosis is impeded, allowing lytic enzymes to be released during the unsuccessful attempts of the neutrophil to ingest the adhering immune complex. Further activation of the membrane-attack mechanism of the complement system can also contribute to tissue damage (Kuby, 1997; Roitt *et al.*, 1996).

Therefore the question is why are some autoantibodies pathogenic whilst others are not? To answer this question researchers have tried to identify the differences between the two types of autoantibody. Pathogenic antibodies differ from natural autoantibodies in a number of ways. Pathogenic autoantibodies are normally IgG isotype, and have a high affinity for a particular autoantigen whilst natural autoantibodies tend to be of the IgM isotype and polyreactive with low affinity to autoantigens. Autoantibodies are invariably found in patients with systemic autoimmune diseases of which Systemic Lupus Erythematosus (SLE) is the best example. In order to study these autoantibodies in more detail they need to be extracted from the patient's blood. However such

antibodies are heterogeneous, comprising a mixture of immunoglobulins each recognising one or more antigenic epitopes. To study the structural characteristics of a particular autoantibody (in relation to its binding and pathogenic abilities), those derived from a single clone in which every cell has the same genomic DNA rearrangement and secretes the same immunoglobulin are required. These are called monoclonal antibodies.

1.1.4 Monoclonal Antibodies.

Direct biochemical purification of a monoclonal antibody from a polyclonal antibody preparation is extremely complex. However, a method for preparing monoclonal antibodies was devised by Kohler *et al.* (1975). Through the fusion of a normal, activated, antibody producing B cell with a myeloma cell (a cancerous plasma cell), a hybrid cell (called a hybridoma) was produced. This hybridoma consequently had the immortal growth properties of the myeloma and the ability to secrete the antibody produced by the B cell. The resulting clones of hybridoma cells, which secreted large quantities of monoclonal antibody were then able to be cultured indefinitely as cell lines. This method was originally used to produce murine monoclonal antibodies (Kohler *et al.*, 1975) but then later adapted to produce human monoclonal antibodies (Olsson *et al.*, 1980, Teng *et al.*, 1983). Hybridoma cell lines of human anti-DNA antibodies have been produced from patients with SLE by various groups using this method (Ehrenstein *et al.*, 1995; Ravirajan *et al.*, 1998; Winkler *et al.*, 1992).

1.2 Systemic Lupus Erythematosus and the role of antibodies to double stranded DNA (anti-dsDNA)

1.2.1 Aetiology of SLE

Systemic Lupus Erythematosus (SLE) is an autoimmune rheumatic disease. The term "lupus erythematosus" was first used in 1851 by the Frenchman Cazenave (Amital *et al.*, 1999) and its systemic nature has been accepted for the past 100 years (Isenberg *et al.*, 1997).

The incidence rate of SLE in the UK is approximately four cases per 10^5 people every year (Johnson *et al.*, 1995). As is the case for many other autoimmune diseases, women are more commonly affected than men (1 in 10 patients are male). The age of onset is, on average, between the ages of 20 and 40 years (Alarcòn-Segovia *et al.*, 1999). Various groups across Europe and USA have shown that the prevalence of SLE varies between racial groups (Johnson *et al.*, 1995, Hochberg, 1990). In women, the highest prevalence is found in Afro-Caribbeans (approximately 206 cases per 10^5) compared to 91 cases per 10^5 in Asians and 36 cases per 10^5 in Caucasians. In men, the corresponding figures were 9.3 per 10^5, 2.6 per 10^5 and 3.4 per 10^5 for these three ethnic groups respectively (Johnson *et al.*, 1995). The overall probability of survival in patients with SLE has improved drastically over the last few decades. However for some patients SLE can be fatal relatively early on in the disease. Furthermore, SLE tends to be a more serious disease in non-Caucasians. The exact cause of SLE is not known. However it is clearly a multi-factorial disease influenced by genetic, environmental and hormonal factors.

Monozygotic twins are identical in their germline genes. Therefore a higher concordance rate than dizygotic twins gives a measure of the heritability of the phenotype under study. Reichlin *et al.* (1992) showed that monozygotic twins have a higher concordance rate for SLE than dizygotic twins, (approximately 25% versus 3% respectively). Furthermore, 10-12% of all SLE patients have a first- or second-degree relative with the disease (Alarcòn-Segovia *et al.*, 1999). These findings indicate that genetic factors have an important role in the aetiology of SLE.

SLE is believed to result from the effects of a number of gene interactions. However it is possible that some individuals may possess all these genes but still not develop SLE. Much research is required to investigate how different genes play a different role in

SLE, either through disease susceptibility, modification of other genes or through their effect on disease expression. Some genes linked to SLE are present in the Major Histocompatibility complex (MHC) also known in humans as the Human Leukocyte Antigen System A (HLA). Tissue typing of SLE patients has consistently shown an association between SLE and both the HLA class I antigens, A1 and B8 and the class II antigen, DR3. These antigens are common in the normal healthy population but their frequency is significantly increased amongst patients with SLE (Worrall *et al.*, 1990). All are carried on chromosome six (Walport *et al.*, 1982). The importance of the associations between these particular HLA genes and SLE is uncertain, but one possibility is that they reflect linkage disequilibrium (i.e. two alleles are inherited together with a higher frequency than would normally be expected) with other loci that determine risk factors. In this context, the HLA Class III genes (situated between the Class I and Class II genes in the MHC) that encode complement polymorphisms C2, C4a and C4b may be candidates. In addition to the expressed polymorphic variations, null (silent) alleles have been described for C2 and C4 loci. Consequently, it was shown that SLE patients were more likely than normal controls to possess null alleles of C2, C4a or C4b. It was also shown that in these patients the majority of alleles encoding C2 and C4 alleles are all encoded on haplotypes bearing HLA-DR3. However an increased prevalence of the C4 null alleles has been seen in SLE patients who lack HLA-DR3, suggesting that complement abnormalities may predispose to SLE, independently of any linkage with particular HLA genes (Batchelor *et al.*, 1987).

The importance of complement in the aetiology of SLE has been demonstrated in studies of patients who suffer from rare hereditary deficiencies of complement proteins (Davies *et al.*, 1993; Walport, 1993). Complement deficiencies found in SLE patients are largely restricted to those affecting the components of the classical complement pathway, particularly those that occur earlier in the cascade. A hierarchy of disease prevalence and severity according to the missing protein has been described. Almost all patients with C1q deficiency have a severe SLE-like disease with many autoantibodies characteristic of the disease. 75% of patients with C4 deficiency have SLE whilst 33% with C2 deficiency have the disease (Walport, 1993). The C1q gene is on chromosome one whilst MHC, C2 and C4 genes are on chromosome six, therefore it is unlikely that each of these associations is due to linked genes on two different chromosomes (Walport, 1993). To investigate how complement deficiency predisposes to SLE, Davies and colleagues (Davies *et al.*, 1992, 1993) have used radiolabelled immune

complexes to show that splenic uptake of immune complexes is complement-dependent and much reduced in patients with SLE particularly those with acquired complement deficiency compared to healthy controls. Furthermore normal splenic uptake of immune complexes was restored to a patient with homozygous C2 deficiency by repletion with the lacking complement protein (Davies *et al.*, 1993). In SLE, it is widely thought that immune complexes are directly associated with tissue damage. The failure to localise immune complexes and their antigens in the spleen of SLE patients may lead to the abnormal persistence of either exogenous or self-antigens in the circulation potentially leading to an abnormal cellular or humoral immune response (Davies, 1996).

Furthermore it has also been shown that Fc-receptor-dependent handling of immune complexes by macrophages is defective in patients with SLE and that ligation of immune complexes by Fc receptors is critical for their efficient binding and retention by the macrophages in the liver (Davies *et al.*, 2002). Much recent interest has focussed on genetically determined polymorphisms of Fcγ receptor type IIA (FcγRIIA) and FcγRIII. Although controversial, evidence indicates that a variant of FcγRIIA (R131) may constitute a disease-susceptibility gene for SLE in some populations (Salmon *et al.*, 1996). In other populations it only appears to influence the pattern and severity of the disease (Manger *et al.*, 1998; Manger *et al.*, 2002; Norsworthy *et al.*, 1999). There is also some evidence suggesting that polymorphic variants of FcγRIII may similarly be implicated in disease (Salmon *et al.*, 1992).

Further evidence for a genetic component in the aetiology of SLE, has come from studies of lupus-prone mouse strains. Several mouse models of lupus displaying different phenotypes and genotypes have collectively led to the identification of more than 20 genomic intervals associated with the susceptibility to SLE or SLE-related phenotypes (Theofilopoulos *et al.*, 1999). Wakeland and colleagues have identified the genomic positions of three recessive loci (*Sle1*, *Sle2* and *Sle3* on murine chromosomes one, four and seven respectively) that are strongly associated with SLE-susceptibility in the NZB/NZW-related NZM2410 lupus-prone strain by selecting for the development of nephritis and the presence of anti-dsDNA through a series of backcrosses (Morel *et al.*, 1994). In order to determine the nature of the component phenotypes each locus contributes and how they interact to produce the immunopathology of the susceptible parental strains, Wakeland and colleagues adopted a congenic dissection approach. The basic principle of this approach is to convert a polygenic system into a series of

monogenic systems in individual congenic strains. Two strains are congenic if they are genetically identical except at a single genetic locus or region. Any phenotypic differences that can be detected between congenic strains are related to the genetic region that distinguishes the strains. Three congenic strains B6.NZM*Sle1*, B6.NZM*Sle2*, B6.NZM*Sle3* were created, each carrying a different susceptibility interval on a resistant genetic background (mouse strain C57BL/6 [B6]). These three strains were used to characterise the primary phenotypes associated with the *Sle* loci. *Sle1* mediates the loss of tolerance to nuclear antigens. *Sle2* lowers the activation threshold of B cells leading to the production of polyclonal IgM antibodies. *Sle3* mediates a T cell dysregulation that is associated with polyclonal IgG production and a decrease in activation-induced cell death in CD4+ T cells (Morel *et al.*, 2000).

Using bi- and tricongenic strains containing various combinations of the *Sle*-containing genomic intervals, Wakeland and colleagues showed that *Sle1* was the key locus for the development of fatal lupus since the combination of *Sle1* with either *Sle2* or *Sle3* led to development of systemic autoimmunity with variably penetrant glomerulonephritis leading to kidney failure whilst other locus combinations without *Sle1* failed to mediate fatal disease. The tricongenic strain resulted in fully penetrant, fatal glomerulonephritis. Therefore these loci contain the minimal set of genes sufficient to reconstitute a fully penetrant SLE phenotype in these mice (Morel *et al.*, 2000). These results are believed to be relevant to human SLE since these susceptibility loci are syntenic with regions found in human chromosomes (Morel *et al.*, 2000).

SLE is more prevalent in females thus suggesting that SLE may be related to hormonal factors. In murine studies, various groups have shown a disease-accelerating effect of oestrogens whilst in similar studies, testosterone has been shown to have a protective effect (Roubinian *et al.*, 1979). Prolactin, a peptide hormone that stimulates mammary growth and differentiation has also been implicated in the aetiology of SLE. However the association of hyperprolactinemia and SLE is a subject of debate. The variation between reports generates from the varying classification of disease activity. For example, Buskila *et al.* (1996) reports that serum prolactin is not elevated in patients with active disease. However Miranda *et al.* (1998) found that when looking specifically at SLE patients with lupus nephritis, the individuals with severe renal activity had higher serum prolactin levels than those patients with mild renal activity.

However this is a chicken and egg question. Could the high disease activity and the drugs used to treat it have caused prolactin levels to rise?

It has been suggested that normal individuals may have the genetic predisposition to develop SLE should the correct triggering environmental factor come along. UV light for example, particularly wavelengths in the ultraviolet (UV-B) range (280-320 nm) can not only provoke an abnormal cutaneous reaction but also the initiation or activation of systemic disease activity in SLE patients (Millard *et al.*, 2001). Little is known about other possible environmental or occupational exposures in relation to SLE disease risk. There is limited evidence that heavy metals may induce autoimmune kidney disease or lupus-like syndromes whilst there are several epidemiological studies into the potential role of silica dust or certain chemicals found in solvents, hair dyes or tobacco smoke. Furthermore various drugs and certain food stuffs (alfalfa sprouts) have been shown to activate lupus-like syndromes (Cooper *et al.*, 1998). An early clinical observation was that the onset of SLE followed a previous infection although this has been difficult to prove. It has been hypothesised that SLE may be triggered by an abnormal immune response to a common pathogen. Viruses have been implicated in the aetiology of SLE, particularly the Epstein-Barr virus since autoantibodies to this virus have been shown to cross react with autoantigens (Sabbatini *et al.*, 1993).

1.2.2 Clinical Presentation of SLE

Systemic Lupus Erythematosus (SLE), as its name suggests, can affect a wide variety of organs and tissues throughout the body. The skin and joints are the organs most frequently involved. Many patients also have activity in the brain or peripheral nervous system, lungs, heart or kidney disease. The classification of SLE is made on clinical grounds with the support of laboratory tests. To aid diagnosis, the American College of Rheumatology (Table 1.1) designated eleven criteria. To be classified as having SLE, a patient must either have serially, or simultaneously, satisfied four or more of these criteria (Hochberg, 1997; Tan *et al.*, 1982). The clinical features shown vary greatly from patient to patient. SLE can be fatal with the most frequent cases of death divided similarly between active SLE, infections (due to use of immunosuppressive treatments) and cardiovascular disease (Cervera *et al.*, 1999).

CRITERION	DEFINITION
1. Malar Rash	Butterfly rash
2. Discoid Rash	Erythemous raised patches
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight
4. Oral Ulcers	Oral or nasopharyngeal ulceration, usually painless
5. Arthritis	Nonerosive arthritis, involving two or more peripheral joints
6. Serositis	Pleuritis or Pericarditis
7. Renal disorder	Persistent proteinuria greater than 0.5g per day or greater than 3+
8. Neurologic disorder	Seizures or Psychosis in absence of offending drugs or other metabolic derangement
9. Haematologic disorder	Haemolytic anaemia or Leukopenia (<4 000/mm^3 total on two or more occasions) or Lymphopenia (<1 500/mm^3 total on two or more occasions) or Thrombocytopenia (<100 000/mm^3 total in absence of offending drugs)
10. Immunologic disorder	Presence of anti-DNA or anti-Sm or anti-phospholipid antibodies, based on i) abnormal serum level of IgG and IgM anti-cardiolipin antibodies ii) positive for lupus anticoagulant iii) false positive serologic test for syphilis known to be positive for at least six months
11. Anti-Nuclear Antibody	Abnormal titre of ANA at any point in time and in the absence of drugs known to be associated with "drug-induced" lupus

Table 1.1 The American College of Rheumatology Criteria for the Diagnosis of Systemic Lupus Erythematosus.

Adapted from (Tan *et al.,* 1982) and (Hochberg, 1997)

The activity of the disease fluctuates over time and also between organs and tissues. Previously known symptoms may worsen and together may constitute what is commonly known as a "flare" of the disease. High dose steroids and immunosuppressive treatments are often used to treat these flares, however these treatments can often produce unpleasant side effects such as sterility. Consequently, SLE is a disabling and potentially fatal disease that tends to affect women in the prime years of their lives. The treatment or prevention measures available at this moment in time are limited, especially in view of potentially severe side effects. In order to improve our treatment there is a continuing need to increase our understanding of the pathogenesis of the disease. Due to the systemic nature of this disease, autoantibodies are among the main candidates for investigation.

Many different components of the immune system are believed to be involved in the pathogenesis of SLE. These include T cells (section 1.2.7.1), cytokines (section 1.2.7.2), complement (section 1.2.1) and removal of apoptotic cells (sections 1.2.6). However although these other components will be discussed, much of this thesis will concentrate on the role played by autoantibodies in the pathogenesis of SLE.

1.2.3   Autoantibodies in SLE

It was only in 1957 that antibodies to DNA were first isolated from the sera of patients with SLE by four different groups (Cepillini *et al.*, 1957; Meischer *et al.*, 1957; Robbins *et al.*, 1957, Seligmann, 1957). Over the next two decades, a broad spectrum of autoantibodies was identified in patients with SLE and their prevalence in the serum of SLE patients is shown in Table 1.2, adapted from (Rahman *et al.*, 2002). It was originally believed by some that autoantibody production was due to polyclonal B cell hyperactivity causing a random profile of abnormal antibody reactions. However, analyses of autoantibody specificities in patients with SLE suggest that of the approximately 2000 mammalian intracellular proteins detectable currently, relatively few are targeted by lupus autoantibodies (Gharavi *et al.*, 1988).

As discussed below, it is the group of autoantibodies that bind to double stranded DNA (dsDNA) that have generated the most interest since they are believed to be most closely involved with the pathogenesis of the disease (Isenberg *et al.*, 1997). Anti-dsDNA antibodies are almost specific to patients with SLE, serum levels of IgG anti-dsDNA correlate with disease severity in some but not all patients, particularly with

ANTIBODY	% OF PATIENTS
ANA	94
dsDNA	60
ssDNA	-
Histone	-
Sm	9
RNP	21
Ro	32
La	12
Cardiolipin	25 (IgG) 13 (IgM)
Lupus anti-coagulant	14
Fc IgG (RF)	20
Reduced C3	40
Anti-thyroglobulin	10
Anti-thyroid microsomes	13

Table 1.2   Approximate prevalence of antibodies detectable in the serum of patients with SLE.

The right hand column shows the autoantibody and C3 profile of SLE based on the first 300 patients attending UCH/Middlesex Lupus Clinic between 1978-2000 (Rahman *et al.*, 2002).

lupus nephritis (Koffler *et al.*, 1971a; Swaak *et al.*, 1979; ter Borg *et al.*, 1990) and they have been shown to deposit in the inflamed tissues of patients with SLE and also lupus prone murine models, especially the kidney (Ehrenstein *et al.*, 1995; Koffler *et al.*, 1967; Winfield *et al.*, 1977).

1.2.4   Relationship between anti-dsDNA levels and disease activity in SLE patients

Anti-dsDNA assays are a very useful diagnostic tool for SLE as between 60 and 83 % of SLE patients test positive for anti-dsDNA at some point in their illness (ter Borg *et al.*, 1990). The most frequently used assays to detect anti-DNA antibodies are the Farr assay, Enzyme-linked Immunosorbent assay (ELISA) and the *Crithidia luciliae* assay. A brief outline of each assay is outlined in Table 1.3.

In 1966, Tan *et al.* discovered that just prior to disease exacerbation the anti-DNA antibody levels in the serum rose in some SLE patients. Once techniques were developed that could distinguish between anti-dsDNA and anti-ssDNA antibodies, many groups reported that anti-dsDNA antibodies were more commonly found in SLE patients with active disease rather than inactive disease and were almost never found in patients who did not have SLE (Pincus *et al.*, 1969; Schur *et al.*, 1968). No such correlation with disease activity has been found with anti-ssDNA antibodies and many healthy individuals and individuals with diseases other than SLE have antibodies to ssDNA (Diamond *et al.*, 1992; Hahn, 1998; Koffler *et al.*, 1971a). These early reports of there being a significant correlation between anti-dsDNA titres (but not anti-ssDNA) in the sera of SLE patients and severity of the disease, particularly with lupus nephritis have since been confirmed by various large cohort studies (Cervera *et al.*, 1999; Schur *et al.*, 1968; ter Borg *et al.*, 1990).

Swaak *et al.* (1979) showed that in many patients with renal disease, a sharp fall in anti-dsDNA serum levels during exacerbation of the flare was preceded by a rise prior to the flare suggesting that autoantibodies are deposited in an organ such as the kidney. Consequently, Bootsma *et al.* (1995) investigated whether these flares could be prevented by treating the SLE patient with a corticosteroidal drug, prednisone (normally only given to patients during a flare) as soon as a rise in anti-dsDNA levels was seen. The study involved 156 SLE patients. A randomised half were given conventional treatment (i.e. treatment with increased dose prednisone and cytotoxic drugs only given during flares/relapses) whilst the remaining half were administered an increased daily

ASSAY	PRINCIPLE	DETECTION OF ANTI-DNA		ADVANTAGES	LIMITATIONS
		High-Affinity Anti-DNA	Low-Affinity Anti-DNA		
Farr	Radiolabelled ssDNA or dsDNA is added to sample to be tested for anti-DNA antibodies. Resulting radioactive immune complexes are precipitated by ammonium sulphate and results expressed as % of radioactivity in precipitate.	Positive	Negative	Excellent assay for the detection of high affinity antibodies.	
ELISA	Microtitre plates are coated with ssDNA or dsDNA. Test sample containing primary antibody is added to coated plates. Primary antibody is detected by an enzyme-linked secondary antibody. Addition of a substrate that produces a colour in the presence of the enzyme is then added. The Ig concentration is determined by the optical density read by a spectrophotometer.	Positive	Positive	Rapid, simple, quantitative and reproducible assay. Can determine antibody isotype and light chain usage depending on specificity of secondary antibody. Can detect both high and low affinity antibodies.	Assay specificity enhanced by by treatment of sera or supernatant with DNase to dissociate immune complexes.
Crithidia Luciliae	Anti-dsDNA are detected by their ability to bind the kinetoplast of Crithidia luciliae, a protozoan organism with a double-stranded circular DNA structure that is not associated with histone proteins. Test samples are incubated with the organism on a glass slide with fluoresceinated anti-Ig antibody. Detection of fluorescent kinetoplast indicates presence of anti-dsDNA antibodies only.	Positive	Positive	Most specific test for anti-dsDNA antibodies. Can determine distinct anti-dsDNA subsets depending on specificity of secondary antibody.	Not as sensitive as ELISA

Table 1.3.   Assays for the detection of Anti-DNA antibodies.

Adapted from Khalil et al., (1999) and Bell et al., (1997)

prednisone dose when a rise in anti-dsDNA levels was confirmed, despite the absence of a relapse of the disease. During the study period (May 1991 – November 1993), seven major relapses occurred in the conventional group whilst only two occurred in the prednisone group. It was concluded that treatment with prednisone as soon as a significant rise in anti-dsDNA occurs does prevent relapse in most cases. However, despite these results, most clinicians do not give patients with SLE immunosuppressive steroids as soon as a rise in anti-dsDNA is seen prior to relapse. This is due to the increased numerous adverse side effects (including weight gain, Cushingoid appearance, gastric complaints, hypertension etc.) of these drugs.

The predictive value of tests for anti-dsDNA antibodies in SLE patients is controversial. Not all patients show a high correlation between high anti-dsDNA levels and active SLE. Some patients have active SLE disease but no anti-dsDNA (Maddison *et al.*, 1979; McCarty *et al.*, 1993) and others have high anti-dsDNA levels but inactive disease (Schur *et al*, 1968; Swaak *et al.*, 1979). Furthermore, some patients with high anti-dsDNA levels do not have SLE but have myeloma proteins, which bind DNA (Davidson *et al.*, 1987). There are several reasons why these discrepancies may have occurred. Firstly, interpretation of the relationship between anti-DNA levels and lupus nephritis may be misleading without renal histological data. For example, in the study by Okamura *et al.* (1993) the incidence of clinically active nephritis was 55% although 95% had renal histological abnormalities. Furthermore, three patients had severe renal histology but presented no symptoms. Secondly, many patients in these studies had been treated with immunosuppressive drugs that induce rapid changes in anti-DNA serum levels and renal histological features, consequently affecting any studies made between the two factors. Data obtained from untreated patients as in the study by Okamura *et al.* (1993) is therefore very important. Thirdly, the sensitivity and specificity of the assays used to detect anti-dsDNA levels varied between studies.

Not all anti-dsDNA are believed to be pathogenic. There appears to be a particular subset of anti-dsDNA that are more pathogenic than the rest. This subset has certain characteristics, the first being the isotype of the anti-dsDNA. Okamura *et al.* (1993) studied the correlation between renal histology and anti-ssDNA and anti-dsDNA levels (IgG and IgM) in forty untreated SLE patients. IgG antibody levels to dsDNA and ssDNA were significantly higher in patients with severe nephritis. IgG anti-dsDNA showed a strong correlation with the renal histological score whilst IgG anti-ssDNA

only showed a very weak correlation. IgM antibody levels to dsDNA and ssDNA were not correlated with renal histology.

The second characteristic of this subset of pathogenic anti-dsDNA is their electrostatic charge. Patients with lupus nephritis have been reported to have more positively charged anti-dsDNA in their serum than patients with inactive disease. Furthermore, levels of positively charged anti-dsDNA IgG correlated more closely with disease activity than total anti-dsDNA IgG (Suzuki *et al.*, 1993, Harada *et al.*, 1994).

From these studies it can be deduced that levels of anti-dsDNA reflect disease activity, particularly lupus nephritis in some SLE patients but not all and that this is specific to antibodies of certain isotype and binding ability. Autoantibodies to alternative antigens may also be important in the pathogenesis of SLE, such as antibodies to nucleosomes, histones, Sm or cytoplasmic antigens. For example, Maddison *et al.* (1979) reported two SLE patients with sera containing antibodies to soluble cytoplasmic antigen, Ro. A reduction in these antibodies was associated with increasing severity of lupus nephritis in these patients and anti-Ro antibodies were found in eluates of the patient's kidneys, strongly suggesting that Ro-anti-Ro immune complexes participate directly in progressive renal disease. Anti-Smith antibodies (anti-Sm) are specific to patients with SLE, although they are only found in 5-30% (depending on ethnic background) of SLE cases and no exact pathogenic mechanism has been determined. However, these antibodies may constitute up to 20% of the immunoglobulin repertoire of these patients (Maddison *et al.*, 1979). The main antigenic targets of anti-Sm antibodies are spliceosomal components, which like nucleosomes, are released from apoptotic cells (Casciola-Rosen *et al.*, 1996). In this thesis I shall mainly focus on the subset of positively charged, high avidity, IgG that fix complement and show preference for dsDNA since they are the most likely autoantibody candidates to be involved in pathogenesis in SLE. I shall also consider the role of other antigens such as histones, nucleosomes, Sm antigen and Ro antigen due to the growing evidence for their involvement in the pathogenesis of SLE.

1.2.5 Renal deposition of anti-dsDNA in lupus nephritis

It is believed that autoantibodies are deposited in the kidneys of patients with lupus nephritis as many of these patients experience a sharp fall in anti-dsDNA serum levels

during exacerbation of a renal flare, preceded by a rise prior to the renal flare (Swaak *et al.*, 1979). This observation had previously been suggested by Koffler *et al.* (1967) who used immunofluoresence techniques to show that the kidneys of patients who had died from lupus nephritis contained glomerular deposits of IgG and complement but not IgM. The glomerular antibody was eluted from the kidney and tested for ANA activity. The ANA activity of glomerular IgG when compared to an equal amount of serum IgG from the same patient was much higher and this ANA activity could be inhibited by the addition of dsDNA. Furthermore, eluates from non-SLE patients with inflamed kidneys showed no ANA activity. Koffler *et al.* (1967) concluded that in SLE patients, anti-dsDNA may cause glomerulonephritis by depositing in the glomeruli and causing complement activation.

This association of anti-DNA with lupus nephritis has also been confirmed in murine studies. For example, administration of murine anti-DNA into non-autoimmune mice produced nephritis (Vlahakos *et al.*, 1992a) and transgenic mice only expressing the secreted form of an anti-DNA antibody also develop nephritis (Tsao *et al.*, 1992). When human anti-dsDNA hybridomas were transplanted into SCID mice some deposited in the kidneys and induced proteinuria. One of these antibodies not only produced glomerular deposits in the kidneys but also electron dense deposits and thickening of the basement membrane (viewed using electron microscopy) (Ravirajan *et al.*, 1998). Mouse and human anti-DNA have also been shown to cause increased proteinuria in a perfused rat kidney system through direct interaction with glomeruli (and subsequent fixation of complement in some cases) (Raz *et al.*, 1989).

During glomerular ultrafiltration in the kidney, the barrier restricting the passage of plasma proteins into the urine is the glomerular capillary wall, which consists of the fenestrated endothelium, the glomerular basement membrane (GBM) and the foot processes (podocytes). The fenestrae of the endothelium allow the direct contact of the blood with the GBM and hardly restrict the passage of molecules. The GBM however does restrict the passage of molecules according to both size and charge (Raats *et al.*, 2000). In kidneys of lupus patients and murine models of the disease, deposition of anti-dsDNA IgG can be seen at multiple sites including the subendothelial and subepithelial spaces, in the mesangium and along the basement membranes and tubules (Hahn, 1998). To explain how anti-dsDNA reached the glomeruli in lupus nephritis, (Koffler *et al.*, 1971b) proposed that they were deposited in the form of DNA-anti-DNA immune

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complexes. Immune complex deposition in these areas would result in varying degrees of cell proliferation, fibrosis and enhanced permeability of the GBM for macromolecules (proteinuria) eventually resulting in renal failure (Raats *et al.*, 2000).

Various investigations into the mechanism of pathogenesis by anti-DNA antibodies in lupus nephritis agree that formation of glomerular immune complexes is the initial event of the inflammatory process, however they do not agree on the nature and origin of these immune complexes.

1.2.6 The role of anti-DNA in the pathogenesis of lupus nephritis.

1.2.6.1 Circulating immune complex hypothesis

It was originally believed that immune complex deposition is caused by the passive entrapment in the glomerular basement membrane (GBM) of immune complexes of DNA and anti-DNA (DNA-anti-DNA) that have been pre-formed in the circulation. So the question is, are these DNA-anti-DNA present in the bloodstream of patients with SLE?

Initially reported levels of free DNA present in the circulation were inconsistent due to contamination with DNA derived either from leukocytes (Steinman, 1975) during the clotting process or from bacteria, particularly in stored blood samples (McCoubrey-Hoyer *et al.*, 1984). However once measurement of free DNA in plasma and not serum was accepted it was found that the amount of free DNA circulating in the blood of both healthy individuals and SLE patients is relatively low (McCoubrey-Hoyer *et al.*, 1984).

Sano *et al.* (1981) showed that DNA could be extracted only from the gamma globulin fraction of blood from patients with active SLE and was characterised by a low molecular weight (approximately 100-200 bp) (McCoubrey-Hoyer *et al.*, 1984; Sano *et al.*, 1982). When run on an electrophoresis gel, DNA purified from SLE plasma was found to occur in discrete bands, corresponding to the sizes 200, 400, 600 and 800 bp, closely resembling the characteristic 200bp ladder found with oligonucleosomal DNA. Furthermore the DNA extracted from the plasma of SLE patients could be precipitated by anti-histone antibodies, thus suggesting that circulating anti-DNA antibodies are bound to nucleosomes (the basic components of chromatin) rather than naked DNA. Nucleosomes are protein-DNA complexes consisting of 166 to 240 base pairs of DNA

wound round an octameric complex of several different types of histone: 2A, 2B, 3 and 4. The whole DNA-octameric complex is stabilised by binding to a single molecule of histone 1 (H1) (Rumore *et al.*, 1990).

As the experimental evidence highlighted above suggests, the formation of immune complexes containing nucleosomes is important in the pathogenesis of SLE. Therefore nucleosomes must be widely available. So the next question is; where do the nucleosomes present in the circulation originate in patients with SLE?

Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages in patients with SLE is well established (Herrmann *et al.*, 1998). Therefore it has been speculated that apoptosis provides a source of nuclear autoantigens to trigger autoimmunity, especially since potential nuclear antigens (for example, nucleosomal DNA, Ro, La and RNP particles) are clustered in high concentrations within surface blebs of apoptotic cells or in apoptotic bodies (Casciola-Rosen *et al.*, 1994, Herrmann *et al.*, 1998). Normally cells undergoing apoptosis are quickly removed *in vivo* by noninflammatory engulfment phagocytosis. This fast and efficient removal leads to a low number of late stage apoptotic cells persisting in the circulation and undergoing secondary necrosis. However if the removal of these apoptotic cells is delayed, proteins in the apoptotic material may accumulate in the lymphoid tissues and act as driving antigens for the formation of autoantibodies. In the later stages of apoptosis, these nuclear autoantigens may be cleaved enzymatically thus leading to the presentation of hidden epitopes to which the immune system has not achieved tolerance (Casciola-Rosen *et al.*, 1999).

Cocca *et al.* (2002) illustrated that the clearance of apoptotic cells may be slowed in patients with SLE. They showed that a murine autoantibody can bind directly to blebs on apoptotic cells, due to the presence of certain ligands, and consequently prevent another molecule, annexin V from recognising ligands on the surface and binding. If autoantibody binding can prevent annexin V from binding then it is possible that autoantibody binding to blebs may slow apoptotic cell clearance further when other molecules recognising factors on surface of apoptotic cells like phagocytes, complement etc. are prevented from reaching their receptors and aiding apoptotic cell removal.

In conclusion it appears that DNA is found in the circulation of patients with SLE, some complexed to antibody and some in nucleosomes. These nucleosomes are believed to be important antigens for both B cells and T cells. The contribution of T_H cells specific for nucleosomes is considered in section 1.2.7.

There are three main theories to explain the binding of anti-DNA and/or anti-nucleosome antibodies to the kidney in SLE. Firstly that intracellular antigens such as DNA or nucleosomes are released into the circulation and subsequently bind to certain sites within tissues such as the glomerulus and serve as antigenic determinants (or "planted antigen") to which anti-nuclear antibodies can bind (Lefkowith et al., 1996). Alternatively, anti-DNA or anti-nucleosome antibodies may bind to cellular or extracellular components via DNA-histone or DNA-nucleosome bridges (Kramers et al., 1994). A third alternative hypothesis is that anti-DNA or anti-nucleosomes may cross-react with other glomerular antigens in the absence of DNA or nucleosomes (Faaber et al., 1986; Mostoslavsky et al., 2001).

1.2.6.2 DNA bound to GBM acts as "planted antigen" for anti-DNA antibodies

The "planted antigen" hypothesis states that anti-DNA antibodies form immune deposits by binding to autoantigens, such as DNA, that have been previously bound to renal tissue (Lefkowith et al., 1996). Various groups have shown that both DNA and nucleosomes have affinity for the GBM and for glomeruli (Coritsidis et al., 1995).

It is unlikely that DNA itself is the "planted antigen" in the GBM since it is unlikely that it will easily bind to the GBM due to the repulsion between molecules of the same negative charge that both carry. The sugar phosphate backbone is negatively charged in DNA whilst the GBM carries an overall negative charge due to the presence of the anionic glycosaminoglycan side chains, especially Heparan sulphate (HS) of the HS proteoglycans (HSPGs) (Kramers et al., 1994). Free DNA may bind to the mesangium and act as "planted antigen" for anti-DNA antibodies as evidence shows that DNA can interact with type IV collagen (Bernstein et al., 1995) and type V collagen, (Gay et al., 1985) which are found in this part of the kidney. This notion is interesting, as when rat kidney was perfused in vivo with DNA alone followed by anti-DNA, binding in the glomerulus was mainly restricted to the mesangium (Termaat et al., 1992).

1.2.6.3 The importance of DNA-histone complexes in lupus nephritis

As previously mentioned nucleosomes consist of histones bound to eukaryotic DNA. Histones are highly conserved polycationic proteins. It has been hypothesised that the positively charged histones bind to the negatively charged HS in the GBM whilst the DNA part of the nucleosomes would be bound by anti-DNA antibodies. Thus nucleosomes may act as a bridge between anti-DNA antibodies and the negatively charged heparan sulphate in the GBM.

It was initially thought that anti-dsDNA could bind HS directly as Faaber *et al.* (1986) reported cross-reactivity of anti-DNA antibodies from both serum and glomerular eluates with HS. However, it was later shown using *ex vivo* binding studies that HS cross-reactivity is a property of anti-DNA complexed with nucleosomal antigens (i.e. DNA and histones) (Termaat *et al.*, 1990a).

A mechanism for the binding of anti-DNA antibodies to HS was first proposed by Schmiedeke *et al.* (1989). It was shown that various histone fractions bind both to HS *in vitro* and to the GBM in renal perfusion studies. Therefore it was proposed that histones may act as a site for deposition of DNA and that anti-DNA antibodies bind HS through a DNA-histone complex.

However all the evidence above was carried out *in vitro*, therefore the question was, could this binding also occur *in vivo*?

The answer is yes. Sequential renal perfusion studies of histones, DNA and a murine anti-DNA mAb with high avidity for dsDNA demonstrated localisation of the antibodies in the GBM. Binding to the GBM was reliant on the presence of histones as when DNA alone was perfused prior to the anti-DNA mAb, only mesangial deposition was seen. No direct glomerular binding was seen with the perfusion of anti-DNA mAb alone, histones and anti-DNA mAb or DNA, histones and a control mAb that did not bind dsDNA. However, it is unlikely that DNA or histones circulate in an uncomplexed free form in the body (McCoubrey-Hoyer *et al.*, 1984). A more physiologically relevant study involving the renal perfusion of nucleosome-containing immune complexes resulted in similar GBM binding (Kramers *et al.*, 1994). In the same study, the importance of HS in this binding to the GBM was evaluated by the renal perfusion of heparinase to remove HS from the GBM. A significant reduction in binding to the GBM

by the nucleosome-containing immune complexes was seen, although binding was not abolished (Kramers *et al.*, 1994).

Much evidence suggests that nucleosomes or nucleosome-containing immune complexes deposit in the kidneys of patients with lupus nephritis and murine models, by binding to HS. Firstly, in biopsies taken from lupus nephritis patients, immunohistochemical staining for HS was absent but staining for HS proteoglycan (HSPG) core protein was present in the GBM. In contrast, kidney from healthy control individuals showed homogenous staining for HS and HSPG-core protein (Van Den Born *et al.*, 1993).

Secondly, in murine models of lupus nephritis-like disease, it has been shown that as disease activity and proteinuria increases, staining for HS decreases although the total amount of HS present does not. It was presumed that deposits of immunoglobulin were masking the HS from the stain. *In vitro* studies revealed that autoantibodies complexed to nucleosomal antigens could inhibit the binding of an anti-HS monoclonal antibody to HS. Also, an inverse correlation between HS staining and deposition of IgG in the GBM and albuminuria was seen. It was concluded that the immunoglobulins masking the HS from the stain were nucleosome-containing IgG complexes and that binding to the HS was most likely to be via the positively charged histone part of the nucleosome (van Bruggen *et al.*, 1995).

Termaat *et al.* (1990b) measured anti-HS reactivity in patients with lupus nephritis and found that anti-HS levels rose prior to and during a renal flare and then fell rapidly. Anti-HS reactivity was only found in sera positive for anti-DNA by Farr assay but the anti-HS titre was not directly related to the reactivity measured in the Farr assay. The authors concluded that this indicates that only a subpopulation of anti-DNA can bind to HS. In a subsequent study, Kramers *et al.* (1993) showed that the presence, but not the titre, of anti-HS correlates with clinical features of nephritis.

Biopsies from patients with lupus nephritis and from patients with non-SLE glomerulonephritis were screened with nucleosome-specific mAb for the presence of nucleosomes (van Bruggen *et al.*, 1996). Nucleosomes were only detected from patients with lupus nephritis but not in any of the biopsies from patients with non-SLE

glomerulonephritis (van Bruggen *et al.*, 1997). This was the first evidence to prove that nucleosomes are present in immune deposits in the kidney.

From the evidence derived from studies of both patients with lupus nephritis and murine models of the disease, it can be concluded in my opinion that DNA-histone or nucleosome immune complexes and their affinity for HS in the GBM play an important role in the pathogenesis of lupus nephritis. However, some patients with renal SLE have no detectable anti-HS at all (Kramers *et al.*, 1993). Therefore although this mechanism is likely to play a role in the pathogenesis of lupus nephritis, other mechanisms that do not rely on HS are likely to be involved.

1.2.6.4 Cross-reactivity of anti-DNA antibodies with renal antigens

Nephritogenic anti-DNA antibodies are highly polyreactive. A comparison of anti-DNA derived from the circulation of normal individuals, patients with active SLE and from renal eluates of patients with lupus nephritis showed that anti-DNA from the kidney eluates were the most polyreactive and for example reacted with polynucleotides, phospholipids and the SmRNP complex (Sabbaga *et al.*, 1990). The cross-reactivity of anti-DNA with cardiolipin and other negatively charged phospholipids suggested that anti-DNA might react with renal tissue antigens that share epitopes with DNA (Shoenfeld *et al.*, 1983). This idea was supported by the fact that exogenously administered mouse mAb to DNA formed immune deposits in glomeruli and this binding was not inhibited by DNase treatment (Madaio *et al.*, 1987).

In addition, mutagenesis work has shown that binding to DNA is not the sole determinant of pathogenesis. For example, mutants of anti-DNA with decreased binding to DNA are still pathogenic (Putterman *et al.*, 1996) whilst small numbers of amino acid changes in the variable region of a murine anti-DNA antibody, R4A alter the site of deposition of this antibody, converting one that deposits in renal glomeruli to one that deposits in tubules when injected into SCID mice. It appears that the change in dsDNA binding affinity is associated with the gain of a new antigenic cross reactivity (Katz *et al.* 1994). What is the identity of the glomerular antigen with which anti-DNA cross-react? Is it an intracellular antigen like DNA, a cell surface protein or an extracellular component? The main candidates include important constituents of the kidney GBM and mesangium, such as laminin (Sabbaga *et al.*, 1989) and alpha-actinin (Mostoslavsky *et al.*, 2001). Laminin is a component of the GBM of the extracellular matrix in the

kidney. Eilat *et al.* (1999) reported that a 21-mer peptide derived from the globular region of laminin was a target for human and murine monoclonal and polyclonal anti-DNA lupus antibodies. When a panel of mouse and human monoclonal antibodies were analysed, a correlation was found between the cross-reactivity with the laminin-derived peptide and the nephritogenic ability in SCID and Rag1-deficient mice. Furthermore prolonged survival and suppression of renal disease was seen in MRL-*lpr/lpr* mice when treated with the laminin peptide (Ben Yehuda *et al.*, 1995).

Another antigen recently implicated in several experimental glomerulopathies is alpha-actinin (α-actinin). α-actinin is a 100-kDa actin binding protein and is an important component of glomerular podocytes and mesangial cells in the kidney as it plays a crucial role in maintaining the structural integrity of glomerular filtration components (Mostoslavsky *et al.*, 2001, Deocharan *et al.*, 2002). The identification of alpha-actinin as a potential cross-reacting antigen was made by two separate groups, both of whom were aiming to identify any differences between a pathogenic and non-pathogenic subset of anti-DNA antibodies and whether a particular antigen was involved in SLE pathogenesis. When each group studied the *in vivo* pathogenicity of murine anti-DNA mAb in immune deficient mice, no consistent genetic or immunochemical differences were found between the pathogenic and non-pathogenic subsets of these anti-DNA mAbs. However, the pathogenic subset was found to cross-react with a 100kDa protein, later identified as α-actinin whilst the non-pathogenic subset did not (Deocharan *et al.*, 2002; Mostoslavsky *et al.*, 2001).

Cross reactivity of anti-DNA or immune complexes and the subsequent binding to glomerular antigens may lead to pathogenesis either by causing a disruption in the normal physiological function of the kidney or by triggering local inflammation or tissue damage or both. However instead of binding to constitutively expressed renal antigens, anti-DNA may cross react with antigens that are only expressed by the kidney as a result of inflammation. These antigens are termed neoantigens. This point is depicted by the pattern of deposition of two murine monoclonal anti-DNA antibodies. When hybridomas secreting these antibodies were injected intraperitoneally, a greater inflammatory response and wider renal deposition range was seen than when the purified antibodies were injected intravenously. Therefore perhaps the inflammatory response triggered by the hybridomas produced neoantigens to which the anti-DNA bound (Vlahakos *et al.*, 1992b).

In addition to binding to extracellular antigens, direct binding to the cell membrane of kidney cells is also believed to be important. Anti-DNA antibodies from patients with SLE (Yanase *et al.*, 1997) and a subset of murine monoclonal anti-DNA antibodies (Zack *et al.*, 1996) have been shown to bind to membranes of living cells *in vitro*, penetrate cells via the myosin in the cell membranes in an energy dependent fashion and then bind to cytoplasmic or nuclear structures. The consequences of cell penetration are unknown but could influence cell proliferation, protein synthesis and apoptosis (Hahn, 1998).

In conclusion, it seems likely that there are several mechanisms by which anti-DNA can cause tissue damage. It seems unlikely that the circulating immune complex hypothesis is correct. However whether the anti-HS activity of DNA-histone immune complexes contributes more to pathogenesis in lupus nephritis than the cross-reactivity of these anti-nuclear antibodies with renal antigens is difficult to determine. Alternatively both mechanisms may be involved in lupus nephritis, but in different areas of the kidney. For example, nucleosomes or nucleosome complexes bound via their positively charged histone region to HS may be more important in the deposition of immune complexes in the GBM whilst DNA or anti-DNA immune complexes may be more important in immune complex deposition in the mesangium.

In my opinion it is likely that both mechanisms to a certain extent occur in lupus nephritis although different mechanisms may operate in different patients thus explaining the variation in the patterns of deposition between patients.

1.2.7 Overview of the role of T cells and cytokines in SLE

Having described the evidence linking autoantibodies to tissue damage in SLE, it is important to place this in context by reviewing other mechanisms that may be important in the pathogenesis of this disease. The importance of complement (section 1.2.1) and the deficient removal of apoptotic cell debris (section 1.2.6) have already been discussed. However T lymphocytes and cytokines also play important roles.

1.2.7.1 Role of T cells in SLE

Murine and human SLE result not only from abnormal functions of B cells i.e. the production of pathogenic autoantibodies, but also from the abnormal functions of T cells. T cell abnormalities are crucial in the pathogenesis of the disease because T cells regulate B cell function and the production of most antibodies is T-cell dependent. B cells alone are unable to secrete the adequate quantities of pathogenic immunoglobulin to induce disease.

Therefore much research has been directed towards the properties of the T_H CD4+ cells (subset that activates B cells) found in human and murine SLE (Hahn et al., 1997). The importance of T_H CD4+ cells in SLE was first illustrated by Wofsy et al. (1985) who showed that treating NZB/NZW F_1 mice with a mAb specific for the LT34 T cell marker (equivalent to CD4 in humans) caused a significant reduction in anti-DNA titres, renal disease and proteinuria and prolonged their survival. It has also been shown that double negative T cells (CD4⁻ and CD8⁻) are largely expanded in murine models and patients with SLE and that these cells provide help to B cells to produce cationic IgG anti-DNA antibodies (Datta et al., 1987).

In both patients with SLE and murine models of the disease but not in normal subjects or patients with remission, it has been found that the select population of B cells that produce the anti-DNA pathogenic antibodies are expanded by special autoimmune T_H cells. Although their antigenic specificities were not known, the T_H cells were cloned by taking advantage of their unique ability to induce the production of pathogenic anti-DNA antibodies when co-cultured with B cells. Only 15% of about 800 clones derived from T cells that are activated in vivo in patients or mice with lupus nephritis have this ability (Datta et al., 1997).

The T cell receptors (TCRs) of these pathogenic T_H cells are heterogeneous although sequence analysis has shown that the V-D-J or V-J junctional region (CDR3 loops) always have one or more negatively charged residues generated by N-nucleotide additions. These results suggested for the first time that these pathogenic T_H cells are specific for some reciprocally charged autoantigenic peptide (Datta et al., 1997). It was later found that the majority of these pathogenic T_H cells are specific for nucleosomal antigens and are detectable prior to the production of pathogenic autoantibodies. Therefore nucleosomes are thought to be one of the major immunogens that initiate

cognate interactions between autoimmune T_H and B cells for the production of pathogenic anti-DNA autoantibodies in SLE (Desai-Mehta *et al.*, 1995; Mohan *et al.*, 1993). Nucleosomes are displayed by apoptotic cells in healthy individuals without eliciting an immune response. This is because the spontaneous expansion of nucleosome-reactive T cells is specific to SLE (Kaliyaperumal *et al.*, 1996; Lu *et al.*, 1999). Subsequently certain peptide autoepitopes in nucleosomes that were recurrently recognised by autoimmune T cells of patients with SLE and of murine models, were identified (Kaliyaperumal *et al.*, 1996; Kaliyaperumal *et al.*, 2002; Lu *et al.*, 1999).

However in these studies, it was deduced that although the presence of these autoreactive T_H cell subsets was involved in the pathogenesis of SLE, additional events are required for the specialised function of helping in autoantibody production because when SLE patients in remission were studied it was found that although they possessed this subset of autoimmune T_H cells they possessed no anti-DNA antibodies. Therefore for example, an individual who has an inadequate number of anti-DNA antibody-producing B cells is unlikely to develop significant titres of pathogenic anti-DNA antibodies regardless of whether autoimmune nucleosome-specific T_H cells are present or not (Lu *et al.*, 1999).

Another possible T cell signalling disorder that has been reported in patients with SLE is the reduced activation of a subset of CD8+ T cells (by CD4+ T cells) that is responsible for suppressing B cell hyperactivity, thus helping to augment the B cell hyperactivity seen in patients with SLE (Silva et al., 2001).

1.2.7.2 Role of cytokines in SLE

Cytokines are soluble molecules that mediate interactions between cells. Some cytokines have pre-dominantly anti-inflammatory effects such as IL-10 which reduces the activation of various other cytokines (e.g. pro-inflammatory TNFα, IL-1, IL-12) whilst other cytokines are mostly pro-inflammatory. Many cytokines have been implicated in regulating disease activity and in the involvement of various organs in patients with SLE. It is difficult to interpret the role played by cytokines in SLE since the biological activity and detectable levels of a cytokine can be affected by a number of factors including relative levels of inhibitors or receptors specific for the cytokine or even levels of other cytokines. For example, in the case of TNF-α, although patients with SLE have high levels of TNFα compared to patients with Rheumatoid arthritis

(RA), patients with SLE have a lower ratio of TNFα:TNF-R. TNF-R is a soluble inhibitor of TNFα. One function of TNFα is to stimulate the release of acute phase proteins from the liver such as serum amyloid protein (SAP) or C-reactive protein (CRP). CRP and SAP are involved in the clearance of apoptotic cell debris and levels of CRP are often low in patients with SLE. Thus, reduced biological activity of TNFα may contribute to deficient removal of such debris thus exposing potential autoantigens to the immune system (Gabay et al., 1997). Further evidence for a possible protective effect of TNFα against SLE in humans comes from the fact that a small minority of patients treated with anti-TNFα for Rheumatoid arthritis develop anti-dsDNA antibodies. A few of these patients have developed clinical SLE (Maini et al., 2002).

Evidence from the murine model, NZB/NZW F_1 further supports the important role of TNFα in SLE. Jacob et al. (1988) reported that treating NZB/NZW F_1 mice with recombinant TNFα significantly delays the development of nephritis (and proteinuria) and also prolongs survival. However research by Brennan et al. (1989) showed that this effect was not only dose-dependent but was also dependent on the age of the mice at time of administration of TNFα.

Similar to the gene loci of some complement proteins the TNFα gene locus is also linked to the MHC and is classed as HLA-Class III. Consequently various haplotypes in mice and humans are associated with different levels of expression of TNFα due to linkage disequilibrium with the HLA-Class I and II genes. For example, a high TNFα:TNF-R ratio has been seen in patients with SLE with haplotypes DR3 and DR4 which are associated with a lower incidence of lupus nephritis. Whilst a decreased TNFα:TNF-R ratio as seen in patients with SLE with haplotypes DQw1 and DR2, is associated with lupus nephritis (Dean et al., 2000).

Another cytokine associated with SLE is interleukin-10 (IL-10). SLE patients produce abnormally high levels of IL-10 (Lacki et al., 1997) and serum levels have been shown to correlate with disease activity and anti-dsDNA titres (Houssiau et al., 1995). Twin studies have suggested that approximately 75% of the variation in IL-10 production is genetically determined (Westendorp et al., 1997). It appears that patients with SLE are more responsive to some actions of this cytokine but not to others. For example, IL-10 appears to stimulate B cell maturation and proliferation and subsequent autoantibody

production in SLE although it does not regulate the production of pro-inflammatory cytokines as it does in healthy individuals.

When the peripheral blood mononuclear cells (PBMCs) from a patient with SLE were transferred into SCID mice, the mice developed high titres of anti-dsDNA antibodies that were subsequently abolished when the mice were treated with an anti-IL10 mAb (Llorente et al., 2000). An open pilot study was also performed in which a murine anti-IL10 mAb (B-N10) was administered to six patients with active, steroid-dependent SLE for 21 days, with a monthly follow-up for six months (Llorente et al., 2000). In all six patients, disease activity scores (SLEDAI) were improved, although improvements were mainly restricted to skin and joint symptoms and anti-dsDNA titres were unaffected. Only one patient had a history of renal disease. The treatment had no effect on renal disease activity in this patient. Cutaneous symptoms may have been affected by the administration of B-N10 because IL-10 is directly released by keratinocytes in response to ultraviolet light. This would explain the rapid improvements seen with the administration of B-N10 on cutaneous symptoms in particular (Llorente et al., 2000). Larger studies are now being carried out to assess the long-term therapeutic potential of this mAb in the treatment of patients with SLE.

The imbalance of many other cytokines and their receptors have been implicated as contributing to the development of pathogenesis in SLE patients, for example, IL-6, TGF-β (Dean et al., 2000). This subject area is complicated by the fact that depending on the phenotype of the disease, different cytokine profiles are found in different patients.

In conclusion, it is clear that cytokines, T cells and a wide range of factors (including macrophages and complement) in addition to autoantibodies play a role in the pathogenesis of SLE. However this thesis aims to target and develop further understanding of the specific area of the structure-function relationship of those autoantibodies considered to be most closely related to disease pathogenesis in SLE. In order to do this, it is first necessary to describe the molecular mechanisms involved in the production of an antibody binding site. This will be done in section 1.3, and the principles described will be applied to the analysis of human and murine anti-DNA antibody in section 1.4.

1.3 Immunoglobulin genes: Recombination and Repertoire

1.3.1 *Germline arrangement of gene segments encoding immunoglobulin chains*

The structure of antibodies has been described in section 1.1.4. The mammalian immune system generates approximately 10^8 to 10^{11} different antibody specificities allowing an animal to respond to an enormous number of potential antigens (Berek *et al.*, 1988a). Consequently for several decades, immunologists endeavoured to envisage a genetic mechanism that might generate such diversity whilst also conserving essential biological effector functions. In 1965, Dreyer *et al.* suggested the "two genes, one polypeptide" hypothesis which predicted that each H and L chain of an Ig molecule are encoded by separate genes and that each chain is encoded by one of a small group of C region genes combining with one of a much larger and more diverse group of V region genes.

Sequence analysis of cloned mouse V_λ (Tonegawa *et al.*, 1977; Tonegawa *et al.*, 1978), V_κ (Sakano *et al.*, 1979; Schilling *et al.*, 1980) and V_H (Schilling *et al.*, 1980) showed that the variable region of the H chain is encoded by three fused gene segments in the order V-D-J (variable–diversity–joining) whilst the L chain variable region is encoded by two fused gene segments in the order V-J (variable–joining).

In L chains, the V segment encodes most of the variable region except for most of FR4, which is encoded by the J segment. In H chains, the V segment encodes most of the H chain region except for most of CDR3, which is encoded by the D and J segments. Thus CDR3 has the potential to be much more variable than the other regions. FR4 of the H chain is encoded by the J_H segment. The C gene encodes for the entire C region of each chain (Tonegawa, 1983).

DNA sequencing analysis of murine germline gene segments and their flanking regions revealed the presence of conserved palindromic heptamer sequences (CACTGTG) and conserved nonamer sequences (GGTTTTTGT) near the ends of the V, D and J segments in the germline. These heptamer and nonamer sequences are separated by an intervening sequence or spacer of 12 or 23 base pairs. One "heptamer-spacer-nonamer" unit is located 3' to each V gene segment, 5' to each J gene segment and on both sides of each D gene segment. The length but not the sequence of the spacer is conserved (Tonegawa, 1983). Early *et al.* (1980) observed that the intervening 12bp and 23bp spacers

correspond respectively to one and two turns of the DNA helix and were referred to as one-turn signal sequences and two-turn signal sequences. All V_κ, J_λ (Sakano *et al.*, 1979; Tonegawa, 1983) and D (Kurosawa *et al.*, 1982) spacers are one-turn signal sequences whilst almost all J_κ, V_λ, V_H and J_H spacers are two-turn signal sequences (Sakano *et al.*, 1979; Tonegawa, 1983). As shown in Figure 1.2, the arrangement of the spacers is such that heptamer-nonamer sequences separated by a one-turn spacer can only join with heptamer-nonamer sequences separated by a two-turn spacer (the so-called "one-turn/two-turn joining rule") thus explaining why some arrangements were allowed (e.g. V-D, D-J, $V_\lambda J_\lambda$) whilst others were not (e.g. V-V, $V_\lambda J_\kappa$ or V_H-J_H without a D in the middle) (Early *et al.*, 1980). This work was all carried out in mice, however once the first human V_H gene (Matthyssens *et al.*, 1980), J_H (Ravetch *et al.*, 1981), D_H (Siebenlist *et al.*, 1981), V_κ (Bentley *et al.*, 1980), V_λ (Brockly *et al.*, 1989), J_κ (Hieter *et al.*, 1980) and J_λ (Vasicek *et al.*, 1990) genes were cloned, it was found that the same principle applied to human Ig gene segments.

The heptamer-nonamer sequences have been termed recombination signal sequences (RSS) since they directly flank each germline V, D and J segment and seem to signal exactly where the germline DNA should be broken during rearrangement. These sequences function as signals for the recombination process and are only found in the loci of Ig genes and T cell receptors (Schatz *et al.*, 1992).

1.3.2 The mechanism of immunoglobulin variable region DNA rearrangements.

Current evidence suggests that recombination of variable-region gene segments is a multi-step process involving V(D)J recombinase as well as two proteins RAG-1, RAG-2 and the enzyme terminal deoxynucleotidyl transferase [Tdt]) (Alt *et al.*, 1987). V-D-J rearrangement occurs at the junctions between the RSSs and coding sequences. In brief, V(D)J recombinases recognise the RSSs and catalyse the formation of double strand breaks between both of the coding region/heptamer junctions. Double-strand break repair (DSBR) enzymes then catalyse the ligation of the coding sequences with each other and the signal sequences with each other. However joining of the ends of Ig gene segments is imprecise and contributes to antibody diversity. Sakano *et al.* (1979) showed that mouse κ chains derived from the same V_κ and J_κ genes differed in sequence at the V_κ/J_κ junction whilst Kurosawa *et al.* (1982) showed the same occurred at VDJ junctions in H chains. This junctional diversity at the V-J and V-D-J coding joints is generated by a number of mechanisms including the removal and/or addition of

```
C A C A G T G —    ┌──────┐   — A C A A A A A C C        A C A A A A A C C —   ┌──────┐   — C A C A G T G
                   │ 23bp │                                                    │ 12bp │
G T G T C A C —    │ 23bp │   — T G T T T T T G G        T G T T T T T G G —   │ 12bp │   — G T G T C A C
                   └──────┘                                                    └──────┘
   Heptamer                      Nonamer                      Nonamer                        Heptamer
```

Two-turn RSS

One-turn RSS

λ-chain DNA

V_λ J_λ C_λ

5′ ⋯ 3′

κ-chain DNA

V_κ J_κ C_κ

5′ ⋯ 3′

Heavy-chain DNA

V_H D_H J_H C_H

5′ ⋯ 3′
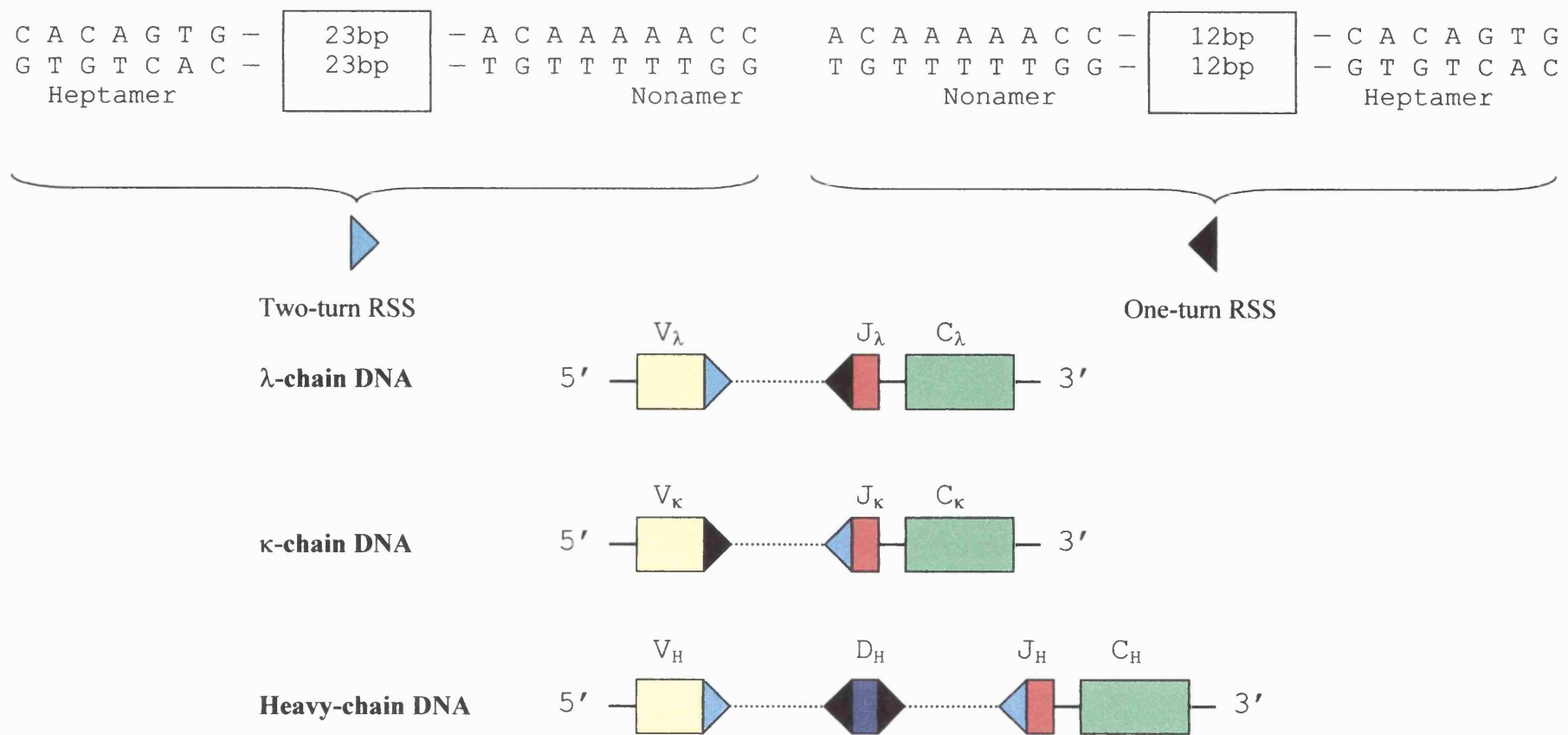
Figure 1.2   Schematic diagram to show positions of recombination signal sequences (RSS) relative to variable region gene segments.
The arrangement of RSSs is such that heptamer-nonamer sequences separated by a one-turn spacer can only join with heptamer-nonamer sequences separated by a two-turn spacer ("one-turn/two-turn joining rule") thus explaining why some combinations are possible (V-D, D-J, $V_\lambda J_\lambda$) whilst others are not (e.g. V-V, $V_\lambda J_\kappa$ or V_H-J_H without a D in the middle). Adapted from {Kuby 1997 237 /id}.

so-called N-nucleotides and is believed to be mediated by the enzyme, TdT (Alt *et al.*, 1987).

However as a result of this joining flexibility, non-productive rearrangements may occur. Non-productive rearrangements are when gene segments are joined out of phase so that the triplet reading frame for translation is disrupted. The resulting VJ or VDJ unit from a non-productive rearrangement contains stop codons, which halt translation. Productive rearrangements are only made when gene segments are joined in phase and the triplet reading frames are maintained thus leading to VJ or VDJ units that can be translated into a complete variable region polypeptide. If one allele rearranges non-productively then a B cell can rearrange the other allele and produce a productive rearrangement. However if a productive rearrangement is not made for both a H and L chain in a B cell then the B cell dies by apoptosis (Schatz *et al.*, 1992). Like all somatic cells, B cells are diploid. However B cells only express the re-arranged H chain genes from one chromosome and the re-arranged L chain genes from one chromosome. This mechanism is called allelic exclusion and ensures that functional B cells cannot contain more than one functional $V_L J_L$ and $V_H D_H J_H$. Therefore it also ensures the antigenic specificity of the B cell since if all the alleles were expressed, the B cell would be polyreactive. It is presumed that once productive $V_L J_L$ and $V_H D_H J_H$ units have been produced, the recombination process is stopped to ensure that the H and L chains on the homologous chromosomes are not expressed (Alt *et al.*, 1987).

One model to account for allelic exclusion suggests that once a productive rearrangement has been made the resultant protein acts as a signal for the recombination machinery to be turned off. The presence of the H chain signals for H chain recombination to stop and for κ chain recombination to start. If a productive κ rearrangement is made, a κ chain is produced and serves to switch off L chain recombination. However if a productive κ arrangement is not made on either chromosome then λ chain recombination begins. If a productive λ rearrangement is not made then the B cell dies from apoptosis (Alt *et al.*, 1987). However the products of a productive H and L chain rearrangement may not always shut down further rearrangement. To investigate this phenomenon, (Prak *et al.*, 1995) used a new mouse model in which an unrearranged J_κ gene was replaced with a rearranged $V_\kappa J_\kappa$ gene. This L chain replacement ($V_\kappa R$) has a single rearranged gene and therefore the $V_\kappa R$ model simulates the genotype of a normal B cell with a functional $V_\kappa J_\kappa$ L chain on one allele.

This model was used to look precisely at how the rearranged L chain gene influences L chain rearrangement. This model showed that some B cells only had the $V_\kappa R$ L chain rearrangement whilst others had additional rearrangements. Some B cells rearranged a second L chain locus whilst others made a second rearrangement of the active $V_\kappa R$ locus and previously productive genes were deleted. This concept of the L chain of a B cell changing but the H chain remaining the same is called "receptor editing". (The receptors in this case are referring to surface Ig of B cells that bind to antigen). Receptor editing allows a B cell to change the sequence and consequently the binding properties of an antibody very quickly. A process analogous to receptor editing called "receptor revision" has been shown to replace the H chain but not the L chain of an antibody (Nemazee et al., 2000; Wilson et al., 2000).

In the germline downstream from the J_H segments are the C_H gene segments. The C_H gene segments are organised into a series of coding exons and non-coding introns. Each exon encodes a separate domain of the H chain constant region. The conservation of important biological effector functions is maintained by the limited number of H chain constant region gene segments. The gene segments are arranged in the germline in the order μ, δ, γ, ε and α. This sequential order is no accident as it is related to the order of appearance of each related Ig class in the course of an immune response (Kuby, 1997). During B cell maturation, antibodies can undergo a process known as H chain class switching. In an antibody the $C\mu$ constant region is the first constant region to be expressed (i.e. as IgM) but can be replaced by downstream constant regions (e.g. IgG, IgD, IgE, IgA). This phenomenon allows clonally derived B cells to maintain the same V region specificity in association with different H chain constant regions specifying different effector functions (Alt et al., 1987).

1.3.3 Antibody diversity

Multiple germ-line gene segments exist and their contribution to the generation of antibody diversity is furthered by their random rearrangement in B cells ([V-(D)-J] combinatorial diversity). The hypervariability of V_HCDR3 is further increased by the addition and removal of nucleotides at the ends of the gene segments (junctional diversity), as described in section 1.3.2 (Tonegawa, 1983).

All of the antibody diversity mechanisms discussed so far occur during the formation of variable regions by gene rearrangement in the pre-B cell (early B cell precursor that

does not express functional membrane-bound Ig and is therefore not sensitive to antigen). These mechanisms in the pre-B cell cannot account for the changes on the binding properties of antibody expressed by B cells after Ig gene rearrangement has occurred. During the course of an antigen-specific immune response, the affinity of the serum antibodies increases with time; a phenomenon referred to as affinity maturation. This affinity maturation is produced by alteration in the sequence of the rearranged V region genes by a mechanism (unique to B cells) called somatic hypermutation (Berek et al., 1987; Berek et al., 1988b).

During an immune response, B cells migrate to the germinal centres where antigen is presented to them in the presence of T_H cells, stimulating B cell division. (If a B cell is activated by interacting with antigen for which its membrane-bound antibody is specific, the cell undergoes proliferation [clonal expansion] and differentiation, generating a population of antibody-secreting cells and memory cells). There in the germinal centres the rearranged Ig V region genes undergo somatic hypermutation. In somatic hypermutation, a high frequency of individual nucleotides in rearranged VJ or VDJ units are replaced with alternative bases, thus potentially altering the amino acid sequence of the antibody chain and therefore potentially altering the specificity of the antibody. No other genes within the cell are affected (Wagner et al., 1996). Mutations at some positions in the sequence will either adversely affect the basic structure of the antibody molecule or reduce its affinity for antigen. The effect of either of these cases would be to reduce the ability of the surface Ig to bind to the antigen and cells which accumulate such mutations will not be stimulated to proliferate and die out. In contrast, other B cells may accumulate mutations that would lead to an increased affinity of their surface Ig for the driving antigen. Those B cells with the highest affinity surface Ig for the driving antigen would consequently proliferate faster thus eventually dominating the germinal centre. This is termed antigen-driven clonal expansion (Berek et al., 1987; Berek et al., 1988b).

Further evidence for the hypothesis of antigen driven clonal expansion was provided by Schlomchik et al. (1987) through studies involving a panel of murine monoclonal antibodies to IgG_{2a} (rheumatoid factors [RF]). Sequence analysis showed that mAb derived from a single mouse were from a small number of expanded clones. Clonally related antibodies can be recognised by their possession of identical VDJ joint sequences. The potential for junctional diversity is very high and therefore it is

extremely unlikely that two B cells would contain the same sequence unless they were descendants of the same clone. In these mAbs, the V regions had a high ratio of Replacement (R) mutations (alters amino acid sequence of antibody) to Silent (S) mutations (does not alter amino acid sequence of antibody) in the hypervariable CDRs (but not the FRs). Since the structure of the antigen-binding site is mainly determined by the V region CDRs, replacement mutations in CDRs are more likely to alter antigen binding properties and to be selected by antigen. Schlomchik *et al.* (1987) suggested that a high replacement : silent mutation ratio in the CDRs but not the FRs of an mAb indicated that the development of a particular antibody sequence was antigen-driven.

Statistical analysis can be applied to the distribution of replacement and silent mutations in an antibody sequence to determine whether this pattern of somatic mutation is a consequence of antigen-drive. Two statistical methods exist at present: a binomial distribution method (Chang *et al.*, 1994) and a multinomial distribution method (Lossos *et al.*, 2000). The binomial method involves the use of a mathematical formula for calculating the probability (*p*) that a particular pattern of mutations (in the Ig V gene CDRs or FRs) could have developed by chance only i.e. in the <u>absence</u> of antigen drive. The formula is:

$$p = [n! \, / \, k! \, (n-k)!] \, x \, q^k \, x \, (1-q)^{n-k}$$

Where: *n* = total number of observed mutations

n! = n x (n-1) x (n-2) x (n-3)..................x2 x1

k = number of observed replacement (R) mutations in the CDRs or FRs

q = probability that an R mutation will localise to CDR or FR

(q = proportion of bases in CDRs x *Rf* for CDR [or] proportion of bases in FRs x *Rf* for FR)

Rf = replacement frequency inherent to a particular CDR or FR sequence

(The *Rf* of CDRs is typically higher than the *Rf* of FRs and can be derived from published tables or by using computer programme InhSusCalc v 1.0).

The second statistical approach, devised by Lossos *et al.* (2000) is based on multinomial distribution and is thought to be more accurate since unlike the binomial method it takes into account all four possible outcomes of a particular mutation i.e. R and S mutations in the CDRs and/or FRs of the gene. Calculations by the multinomial method can be

made using the method provided by Lossos et al (2000) and the JAVA applet provided at http://www-stat.stanford.edu/immunoglobin. When Lossos *et al.* (2000) compared the multinomial and binomial methods side by side there were some discrepancies in the results leading to opposite statistical conclusions about antigen selection. Section 1.4 and table 1.8 show the probabilities that particular V_H or V_L sequences of the anti-DNA antibodies analysed (calculated according to Lossos *et al.*, 2000) resulted from antigen-driven accumulation of somatic mutations.

The precise mechanisms of somatic hypermutation are not fully understood however it is known that mutations are not targeted randomly along the length of the V gene and that mutations tend to occur at particular consensus sequences (A/G-G-C/T) in the CDRs known as mutational hotspots whilst other residues have been noted to be rarely targeted (i.e. coldspots) (Wagner *et al.*, 1996). Since hypermutation is a non-random process and is likely to be more useful for some parts of an antibody molecule (CDRs) rather than others (FRs) to be mutated during antibody affinity maturation, it was postulated that V gene sequences may have evolved so that hotspots are strategically located. Wagner *et al.* (1996) tested this hypothesis using the amino acid serine. Serine is encoded by two triplet codons that conform to the hotspot consensus (AGC and AGT) and one that does not (TCN i.e. TCA, TCC, TCG and TCT). Therefore if serine codons in CDRs are more frequent targets than serine codons in FRs, it would be expected that codon usage would have evolved to favour AGC/AGT triplets in the CDRs and TCN in the FRs. This was the case.

Hypermutation and isotype switching are believed to be linked since they occur at a similar point in cell development and IgG antibodies tend to carry more somatic mutations than IgM of the same specificity (Tillman *et al.*, 1992).

1.3.4 Human Variable region gene repertoire.

As described in the previous sections, the antigen-binding site of an antibody is encoded by the variable regions (in particular by the CDRs) of the H and L chains. Sequence analysis of the variable regions of particular mAbs that share the same binding or pathogenic characteristics can be used to determine if these antibodies have any particular characteristics in common. Due to the recent publication of the maps of the entire human V_κ, V_λ and V_H gene loci it is now possible to begin to determine definitively whether these mAb show preferential usage of V_H or V_L gene segments or

whether they possess a certain pattern of somatic mutations (Cook *et al.*, 1994; Schable *et al.*, 1993; Williams *et al.*, 1996).

1.3.5 Genetics of the Immunoglobulin variable germline gene repertoire

Ig κ and λ chains and H chains are encoded by separate multigene families that are found on chromosomes 2, 22 and 14 respectively in humans. All functional $V_κ$, $V_λ$ and V_H genes have now been mapped to these three different chromosomes (Cook *et al.*, 1994; Schable *et al.*, 1993; Williams *et al.*, 1996).

1.3.6.1.1 Human Heavy (H) chain immunoglobulin germline gene locus

The human H chain locus on chromosome 14 contains approximately 50 functional V_H gene segments, (Cook *et al.*, 1994) about 30 D_H segments, (Corbett *et al.*, 1997) and six J_H segments (Ravetch *et al.*, 1981).

1.3.6.1.2 Human V_H locus

The human Ig V_H locus at chromosome 14q23.3 is 1100kb in length and the 50 functional V_H gene segments are interspersed among a similar number of pseudogenes (Cook *et al.*, 1995). A V_H segment is defined as functional if it contains an open reading frame (ORF) and is arranged in a functional Ig gene. Pseudogenes are nucleotide sequences that are stable components of the genome but are incapable of being expressed. Pseudogenes are thought to have been derived by mutation of active ancestral genes (Kuby, 1997). On the basis of sequence homology, the functional V_H genes are classified into seven families. Each V_H gene bears 80% homology to the rest of its family at nucleotide sequence level. Members of some families are highly related (V_H4) whereas members of others are more divergent (V_H5), although members of all seven different V_H families are interspersed throughout the locus (Cook *et al.*, 1995). There are also 24 V_H genes on chromosome 15 and 16 however as these have no D, J or C segments, they are unlikely to be functional. There is no evidence that interchromosomal rearrangement with D, J and C segments on other chromosomes occurs (Cook *et al.*, 1995).

In a single B cell, only one functional V_H segment will be rearranged adjacent to one D_H, which is also adjacent to one J_H to encode the V_H domain. However all these gene segments are not equally likely to be utilised in the rearranged human V_H domain. Brezinschek *et al.* (1997) have analysed rearrangements in a single human peripheral B

cell using polymerase chain reaction (PCR) and have shown that some genes are more likely to be used than others. The genes that belong to the larger families V_H1, V_H3 and V_H4 are the most commonly expressed in humans (Cook *et al.*, 1995).

The exact dimensions of the V_H locus and the number of functional segments vary from person to person. In particular, the V_H locus has a certain amount of polymorphism. Two types of polymorphism affect the germline repertoire: the insertion or deletion of V_H segments and the occurrence of different alleles of the same segment. Allelic polymorphism is limited however insertion/deletion polymorphism is fairly common. 75% of people have an extra group of genes located between gene segments 3-30 and 4-31. These extra gene segments are listed in VBASE (Tomlinson *et al.*, 1992).

1.3.6.1.3   Human D_H locus

When Corbett *et al.* (1997) determined the complete nucleotide sequence of the human Ig D segment locus on chromosome 14q32.3, a total of 27 D segments organised into four tandem repeat units were identified. V_HCDR3 vary greatly due to versatility of the D_H segments. Various hypotheses exist to explain this extensive versatility. For example, Sanz (1991) described the occurrence of D-D recombination (regardless of the 12/23 rule) and the incorporation of additional elements such as DIR segments (longer sequences interspersed amongst the functional D_H segments or the "minor" D segments (a cluster of D segments on chromosome 15) (Cook *et al.*, 1995). However when the complete sequence of the human Ig D segment locus was systematically compared with a database of rearranged sequences, no evidence was found to suggest that DIR segments (longer sequences interspersed amongst the functional D segments that are flanked by multiple 12 and 23 bp spacer RSS), D-D joins or use of "minor" D segments produced alignments better than those produced by randomly generated sequences (Corbett *et al.*, 1997). This discrepancy may be due to the stringent conditions applied by Corbett *et al.* (1997). However this does not explain the fact that DIR segments have been shown to encode HCDR3 in previous studies (Brezinschek *et al.*, 1995).

1.3.6.1.3   Human J_H locus

The human J_H locus contains nine J-like gene segments. Six of the nine germline J-like genes appear to be active and easily account for most of the known human H chain amino acid sequences whilst three are pseudogenes (Ravetch *et al.*, 1981).

1.3.6.2 Light (L) chain immunoglobulin germline genes

1.3.6.2.1 Human kappa (κ) locus

The κ light chain locus is situated on chromosome 2 in humans and consists of approximately 40 functional V_κ genes in seven families (Schable *et al.*, 1993) and five J_κ genes. There are no D_L gene segments. The V_κ locus has been mapped to chromosome 2p11-12. Like the V_H locus, members of all seven different V_κ families (40 functional genes) are interspersed throughout the locus amongst 36 pseudogenes. Some extra V_κ genes have been located on chromosomes 1 and 22 although these are pseudogenes. Allelic polymorphism is rare and where present, alleles only differ by one or two nucleotides (Schable *et al.*, 1993). Only one human C_κ gene segment and five J_κ segments exist. Therefore no alternative isotypes of the κ chain exist.

1.3.6.2.2 Human Lambda (λ) locus

Completion of the human λ light chain locus map on chromosome 22q11.2 has shown that the V_λ genes are arranged in three distinct clusters, each containing members of different V_λ families. Sequence analysis has shown that there are approximately 30 functional V_λ genes that belong to ten families. As in the V_H locus, the larger V_λ1, 2 and 3 families are more commonly expressed in humans (Williams *et al.*, 1996). Just as in the human V_H and V_κ loci, allelic polymorphism is relatively rare and where found alleles tend to only differ by one or two nucleotides (Williams *et al.*, 1996).

The J_λ and C_λ genes are all located on chromosome 22. Unlike the C_κ locus, the C_λ locus contains seven different genes, four of which are functional and three of which are pseudogenes. Consequently λ chains like H chains can exist as one of several isotypes. Each of the four functional C_λ genes is preceded by a single J_λ gene segment of which J_λ2 and J_λ3 are identical therefore mAb sequences can include only one of three different J_λ sequences (Vasicek *et al.*, 1990).

1.3.6 The importance of defining the human immunoglobulin (Ig) gene repertoire

It is now quite easy to determine the location and nature (replacement or silent) of mutations in an antibody by comparing it with the published germline gene that it most closely resembles. VBASE, a database of all known human Ig V region genes enables rapid identification of the most similar germline gene. Each variable region sequence can be aligned to the closest germ-line counterpart in the VBASE Sequence Directory

using DNAplot software, thus allowing subsequent analysis of nucleotide homology and distribution of somatic mutations (Tomlinson *et al.*, 1996). It is theoretically possible that the antibody may have been derived from a gene or allele unique to the particular person from whom it was derived and consequently this gene is not in VBASE. However this is unlikely since it is believed that the entire human loci have been mapped and the presence of multiple alleles at a specific genetic locus (polymorphism) is relatively rare (Cook *et al.*, 1994; Schable *et al.*, 1993; Williams *et al.*, 1993).

Through identification of the pattern of somatic mutations and the particular gene segments used to encode an antibody, the effects of mechanisms such as recombination, somatic hypermutation and antigen drive can be studied.

1.4 Sequence Analysis of Monoclonal Anti-DNA Antibodies

The antigen-binding site of an antibody is determined by the variable region of the H and L chains. In particular the CDRs have been shown by crystallography to contain the majority of sites where the antigen makes contact with the antibody (detailed in section 1.5). Consequently sequence analysis of an antibody tends to focus on sequence changes in the CDRs and the effect that these changes have on the binding ability and specificity of the antibody.

1.4.1 Source of antibodies for analysis

To study the direct effects of the structural characteristics of a particular autoantibody on its binding and pathogenic abilities, large amounts of that antibody and also the DNA encoding it need to be obtained. Hybridoma or Epstein-Barr virus transformed cell lines are good sources since they not only constitutively secrete large quantities of mAb but also the mRNA encoding the antibodies can be extracted from the cell, converted to cDNA and then amplified using polymerase chain reaction (PCR). Such cell lines have been produced from both murine models of SLE and from patients with SLE although due to difficulties inherent in the procedure, it has been much easier to produce murine-derived cell lines than lines derived from humans.

Human monoclonal anti-DNA antibodies have also been produced using repertoire cloning by phage display (Barbas *et al.*, 1995). This approach is described in full in section 1.6.2.1. There are advantages and disadvantages to using repertoire cloning. The advantages are that it avoids the need to make cell lines and also it can be used to produce several anti-DNA antibodies at one time. The disadvantage of this technique is that since the V_H/V_L combinations are randomly generated it is possible that they do not truly represent H/L chain combinations found in antibodies, which would occur *in vivo*. (See section 1.6.2.2 for further discussion of this point). A mAb derived from a hybridoma does contain the exact H/L chain combination that originally occurred in that cell *in vivo*.

1.4.2 Sequence analysis of monoclonal anti-DNA antibodies from murine models of SLE

The sequences of over 300 murine monoclonal anti-DNA antibodies have been reported. These antibodies were derived from many different murine models of SLE of

which there are two main types. The first are known as autoimmune strains. These strains develop autoantibodies and clinical features similar to those of SLE. F_1 hybrids of New Zealand Black (NZB) mice and New Zealand white (NZW) mice ([NZB/NZW]F_1) spontaneously develop autoimmune disease closely paralleling the symptoms of human SLE. At two to four months of age, these mice spontaneously develop a range of autoantibodies (including antibodies against nuclear proteins and DNA, glomerulonephritis caused by the deposition of immune complexes in the kidney and die by 18 months. Interestingly, as in human SLE, the incidence of autoimmunity in the (NZB/NZW) F_1 hybrids is much greater in females (Howie *et al.*, 1968).

In the strain MRL *lpr/lpr* which is characterised by the homozygous presence of a mutation termed *lpr* (for lymphoproliferation) the mice develop massive lymphoid organ enlargement, a range of autoantibodies (including anti-dsDNA and anti-Sm) and accelerated severe lupus-like disease in early life (50% die from glomerulonephritis within six months) (Andrews *et al.*, 1978). The *lpr* mutation is in fact a defective *fas* gene. The protein encoded by the *fas* gene is a cell surface protein that interacts with its ligand to produce a signal that leads to apoptotic death of those cells carrying the fas-protein on their surface. Fas-induced cell death is involved in the clonal deletion of self-reactive lymphocytes. Consequently a defect in the fas gene may lead to the persistence of autoreactive lymphocytes as found in SLE or lupus-like syndromes in mice.

The second main type of lupus-prone murine models are those in which autoimmune dysfunctions similar to that of human SLE have been experimentally induced in normal mice through immunisation with proteins/DNA complexes or antibodies some of which carry public idiotypes associated with autoantibodies. For example, normal, healthy mice immunised with monoclonal (Mendlovic *et al.*, 1988) or polyclonal (Tincani *et al.*, 1993) antibodies that bear the public idiotype 16/6 have been seen to produce autoantibodies and develop glomerulonephritis. However this model is controversial, as it has been difficult to reproduce in other laboratories (Isenberg *et al.*, 1991).

However despite the different origins of these murine monoclonal anti-DNA antibodies, sequence analysis has led to the identification of recurrent characteristics amongst them (Radic *et al.*, 1994). Firstly, anti-DNA antibodies from various different models tend to use the same V_H and V_κ genes. In an extensive review of information from the sequence analysis of over 300 monoclonal anti-DNA antibodies derived from various murine

models, Radic *et al.* (1994) found that 65% of all the V_H sequences were derived from just one V_H family, J558. A further 22% were derived from a second V_H family (known as 7183) with the remainder (13%) derived from various other families. Use of V_κ families was much less restricted. Although J558 is the largest murine V_H family, it is unlikely that this fully explains the apparent preferential usage of this family to encode anti-DNA antibodies since it was found that there is also preferential usage of individual genes within the V_H families encoding anti-DNA antibodies. For example, ten J558 genes and three 7183 genes were found to encode a third of the published V_H sequences. Moreover just eleven V_κ genes were found to encode just under a third of published V_κ sequences. Given the large number of V genes in the mouse genome, it is unlikely that the preferential usage of certain genes to encode anti-DNA antibodies occurs by chance (Kofler *et al.*, 1992).

Secondly, sequence analysis of anti-DNA antibodies from various murine models has highlighted the importance of clonal expansion and antigen driven accumulation of somatic mutations in determining the ability of these antibodies to bind dsDNA. Marion and colleagues analysed the sequences of 117 monoclonal anti-DNA antibodies from eleven different (NZB x NZW)F_1 mice (Marion *et al.*, 1992). In some cases, IgG produced by a single mouse were derived from the same expanded B cell clone whereas the majority of IgM were not. Clonally related IgM and IgG hybridomas were obtained from two individual mice. Within a single clone, more mutations were found in the IgG than the IgM, particularly in the CDRs. This increase in mutations was associated with increased ability to bind dsDNA (Marion *et al.*, 1992).

Similar results were seen when Shlomchik *et al.* (1987) produced four monoclonal anti-DNA antibodies from an MRL *lpr/lpr* mouse. All four mAb (3H9, 1A1, 2F2 and 4H8) were derived from the same expanded B cell clone, but were not identical due to the presence of somatic mutations. All four of the antibodies had a high percentage of replacement mutations in their CDRs compared to the FRs, thus providing substantial evidence that these anti-DNA antibodies developed in these MRL *lpr/lpr* mice due to antigen driven clonal expansion.

Thirdly, sequence analysis of these murine anti-DNA antibodies has led to the speculation that certain amino acids are present in the CDRs of anti-DNA antibodies at a higher frequency than in antibodies with other antigen specificities (Radic *et al.*,

1994). In the 117 anti-DNA mAb analysed by Marion *et al.* (1992) it was found that as the immune response matured from IgM to IgG, more mutations accumulated leading to an increased ability to bind dsDNA rather than just ssDNA. The mutations that led to high avidity binding to dsDNA were those that increased the number of arginine residues in the CDRs, especially V_HCDR3.

Shlomchik *et al.* (1987) found that one of the mAb derived from the MRL *lpr/lpr*, 3H9 only differs from the other clone members by several different somatic mutations and by its unique ability to bind dsDNA. Since clonally related B cells inherit the same V region genes, the binding difference must be due to the somatic mutations that occurred during clonal expansion. In particular one of these somatic mutations involved the replacement of a glycine with an arginine residue.

Radic *et al.* (1994) also noted that in many murine anti-dsDNA IgG antibodies, antigen-driven accumulation of somatic mutations has led to higher frequencies of certain amino acids, including arginine, asparagine and lysine being present at various positions in the CDRs. It has been suggested that the structures of these amino acids allow them to form electrostatic interactions and/or hydrogen bonds with the negatively charged DNA phosphodiester backbone. Arginine and lysine are positively charged and therefore could form charge interactions directly with this backbone. Asparagine is uncharged. However both asparagine and arginine have the ability to function as either a donor or recipient of hydrogen bonds with nucleotides within the helix (Radic *et al.*, 1994).

In conclusion, sequence analysis of murine monoclonal anti-DNA antibodies has shown that there is preferential use of V_H and $V_κ$ genes to encode these antibodies and that there is antigen-driven accumulation of somatic mutations in the CDRs leading to the prevalence of certain amino acid residues, in particular arginine, in the CDRs.

1.4.3 The importance of studying human monoclonal anti-DNA antibodies

It is important to determine whether the principles that apply to monoclonal anti-DNA antibodies from murine models, described above, also apply to human monoclonal anti-DNA antibodies since there are several reasons why there may be differences between antibodies from the different species. Firstly, the murine anti-DNA antibodies are generated from mice that have a disease that is only similar but not identical to human SLE.

Secondly, the murine Ig germline gene repertoire is very different to the human Ig germline gene repertoire. For example, only 5% of murine antibodies have λ chains opposed to 40% of human antibodies. As a result, no conclusions as to whether there is preferential usage of λ genes in anti-DNA antibodies can be drawn from studying murine anti-DNA antibodies, particularly since the human repertoire contains three V_λ gene families to which the murine repertoire has no equivalent (Ignatovich et al., 1997). Furthermore, the human repertoire is believed to only have 50 functional V_H gene segments (Cook et al., 1994) whilst the murine repertoire is believed to have anything from 300 to 1000 V_H gene segments (Kofler et al., 1992). The differences between the two species do suggest that mice rely on an increased number of V_H gene segments to gain further antibody diversity whilst humans may rely on their greater ability to utilise V_λ gene segments. It is questionable whether the murine antibodies produced are a true reflection of those produced in patients with SLE and so similar investigations of human monoclonal anti-dsDNA antibodies are essential before any conclusions can be drawn.

1.4.4 Sequence analysis of monoclonal anti-DNA antibodies derived from patients with SLE

The first human monoclonal anti-DNA antibodies were published in 1982 (Shoenfeld et al., 1982). Over the past twenty years, many more groups have produced human anti-DNA antibodies. Only 66 of these have been published with sequence data. These 66 anti-DNA antibodies fall into four categories. Tables 1.4 to 1.7. have been compiled by myself and other members of our group (Rahman et al., 2002) to display the results of sequence analysis of these four categories of antibodies in order to see whether certain sequence features show trends within the categories.

The first antibodies to be discovered tended to be human IgM which bind DNA but are not specific for DNA (Table 1.4) thus representing the category of NAA described in section 1.1.3, rather than the pathogenic antibodies found in patients with SLE. Table 1.5 shows the characteristics of human IgM anti-DNA that due to their monospecificity for DNA or the presence of idiotypes previously shown to be present on antibodies associated with lupus nephritis, are believed to be more relevant to SLE pathogenesis than those in table 1.4. Table 1.6 details the group of antibodies thought to be most associated with pathogenesis in SLE, the human monoclonal IgG and IgA anti-DNA antibodies. The two IgA antibodies were put into this group as they are more likely to resemble IgG than IgM since both IgG and IgA appear after isotype switching and are

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therefore subject to similar degrees of mutation and selection. The final group shown in table 1.7 details the properties of human Fab and scFv anti-DNA produced by phage display. These antibodies are in a separate table from those in table 1.6 since due to the nature of repertoire cloning using phage display, it is not known whether the H/L chain combinations in these antibodies would actually occur *in vivo* (see section 1.4.1).

Through sequence analysis of these human anti-DNA antibodies, it is possible to determine whether the same principles that apply to murine anti-DNA also apply to human anti-DNA antibodies. Therefore when analysing the sequences of human monoclonal anti-DNA antibodies it is necessary to answer the following questions;

1. Is there preferential utilisation of specific gene segments to encode human anti-DNA antibodies?
2. Is there an accumulation of somatic mutations in the CDRs?
3. Is there evidence of antigen driven clonal expansion?
4. Is there an accumulation of basic residues in the CDRs?

Sequence Analysis of Monoclonal Anti-DNA Antibodies

mAb	Origin	VH	DH	JH	VH Homology	VL family	VL gene	JL	VL homology	Reference
18.2	SLE PBL	3-23	D3-10	5	100%	NP	NP	NP	N	1
1.17	SLE PBL	3-23	D3 10	5	100%	NP	NP	NP	N	1
21.28	SLE PBL	1-03	D3 10	4	100%	NP	NP	NP	N	1
BE10	Leprosy	1-03	NM	4	100%	NP	NP	NP	N	1
C6B2	Sickle cell spleen	4-61	D3 10	3	97.5%	NP	NP	NP	N	2
Kim 4.6	Healthy tonsil	3-30	D3 10	6	100%	Vλ1	1b	Jλ3	100%	3
A10	Healthy PBL	6-01	D7-27	3	99%	NP	NP	NP	N	4
A431	Healthy PBL	6-01	NM	4	98%	NP	NP	NP	N	4
L16	Fetal liver	6-01	D7-27	3	100%	NP	NP	NP	N	4
ML1	Fetal spleen	6-01	NM	4	100%	NP	NP	NP	N	4
POP	CLL PBL	3-23	D2-2	4	96%	VκIII	L6	Jκ5	99%	5
Ab47	Healthy PBL	7-4.1	NP	NP	98%	NP	NP	NP	N	6
BEG2	Fetal liver	4-61	NM	5	99%	NP	NP	NP	N	7
B19.7	Fetal bone marrow	3-23	NM	4	100%	NP	NP	NP	N	8
WRI176	SLE spleen	3-23	D5-5	3	100%	VκIII	L16	Jκ5	99%	9
RT79	SLE spleen	4-34	D2-2	3	100%	VκII	A3/A19	Jκ5	99%	10
RT72	SLE spleen	4-b	NM	5	100%	VκI	L12	Jκ1	98%	11
RT84	SLE spleen	4-34	NM	6	100%	NP	NP	NP	NP	11
9500	SLE PBL	3-33	D6-13	1	100%	VκI	L8	Jκ4	98%	12
9604	SLE PBL	3-21	D4-11	4	96%	Vλ3	3m	Jλ2	98%	12
C119	SLE PBL	3-23	D5-5	3	96%	VκIII	L6	Jκ4	100%	12
C471	SLE PBL	3-64	D2-2	4	99.6%	VκIII	L2	Jκ2	10%	12
B122	Healthy PBL	1-18	DIR4/D3-10	4	97%	VκI	L12	Jκ2	98%	12
B6204	Healthy PBL	3-23	DIR1/2C	4	97%	VκIII	A27	Jκ1	99.7%	12
A5	Healthy PBL	3-23	DHFL16 inverted	6	100%	Vλ3	3p	Jλ2	97%	13
KSUN	SLE PBL	4-59	NM	4	100%	Vλ9	9a	NP	100%	14

Table 1.4   Sequence characteristics of human polyreactive IgM which bind DNA

Adapted from Rahman *et al.* (2002)

mAb	Origin	V$_H$	D$_H$	J$_H$	V$_H$ Homology	Evidence for antigen-drive in CDRs*	V$_L$ family	V$_L$ gene	J$_L$	V$_L$ homology	Evidence for antigen-drive in CDRs*	Reference
III-2R	SLE Spleen	1-69	NM	5	96%	No	Vκ1	A20	Jκ1	100%		15
III-3R	SLE Spleen	3-07	D3-10	4	99%		Vκ1	18	Jκ4	99%		15
IC-4	Myeloma bone marrow	4-59	NM	4	97%	No	Vκ1	18	Jκ4	96%	No	15
II-1	SLE Spleen	5-51	NM	4	98%		Vκ3	L16	Jκ1	98%		15
NE1	SLE PBL	4-34	D3-10	6	10%		Vκ1	L5	Jκ4	99.7%		16
NE13	SLE PBL	4-34	D3-10	6	100%		Vκ1	L5	Jκ4	100%		16
Kim 11.4	Healthy Tonsil	4-39	NM	5	98%		Vλ1	1C	Jλ2/3	98%		17
B8807	RA PBL	3-23	D1-7	5	100%		Vκ1	A30	Jκ5	99.6%		12
B8815	RA PBL	1-03	NM	3	100%		Vκ3	L6	Jκ4	99%		12

Table 1.5   **Sequence characteristics of human IgM anti-DNA antibodies characterised by monospecificity or presence of clinically relevant idiotypes**

Adapted from Rahman *et al.* (2002)

mAb	Origin	V_H	D_H	J_H	V_H Homology	Evidence for antigen-drive in CDRs *	V_L family	V_L gene	J_L	V_L homology	Evidence for antigen-drive in CDRs *	Reference
IgG Antibodies												
32.B9	SLE PBL	3-23	D2-15	6	97%	No	Vλ8	8a	Jλ2	98%		18
33.H11	SLE PBL	3-07	NM	4	85%	Yes	Vλ2	2a2	Jλ2	99.40%		18
33.F12	SLE PBL	3-11	D6-13	3	98%		VκIII	A27	Jκ2	99.70%		18
33.C9	SLE PBL	4-39	NM	3	94%	No	VκI	L12	Jκ4	98%		18
35.21	SLE PBL	3-74	D1-7/D1-14	4	95%	Yes	NP	NP	NP	NP		18
19 E7	SLE PBL	3-30	DHFL16	6	99%		VκIII	L6	Jκ3	99.7%		18
T14	SLE PBL	4-34	D3-10	4	96%	No	VκIII	A27	Jκ1	99%		19
2A4	Myeloma bone marrow	4-61	D1-14/D2-21	6	92%		VκI	O2	Jκ2	94%	No	15, 20
1-2a	SLE Spleen	3-30	NM	4	94%		VκI	L8	Jκ4	95%	Yes	15
H2F	SLE PBL	3-11	NM	4	99%		VκIV	B3	Jκ1	99%		15
D5	SLE PBL	4-34	D3-10	5	94%	No	VκIII	A27	Jκ5	96%	No	10
B3	SLE PBL	3-23	NM	4	94%	Yes	Vλ2	2a2	Jλ2/3	93%	Yes	21
KS3	SLE Spleen	4-34	NM	6	94%	Yes	Vλ2	2a2	Jλ3	96%	No	22, 23
SD6	Healthy PBL	3-30	D3-22	6	97%	No	Vλ2	2a2	Jλ2	96%	No	22, 23
9702	SLE PBL	1-46	DIR5C/D7-27	5	98%		VκIII	A27	Jκ1	99%		12
B8801	RA PBL	3-11	DIR4C	6	96%	Yes	VκI	A30	Jκ4	97%	Yes	12
R149	SLE PBL	1-69	NM	6	97%	Yes	VκII	A30	Jκ2	99.4%		24
KSUG	SLE PBL	3-23	NM	4	95%	No	Vλ3	3m	Jλ1	94%	No	14
IgA Antibodies												
412.67	SLE PBL	3-21	D1-14	4	96%	Yes	VκII	A27	Jκ1	98%		25
1X7RG1	SLE Spleen	3-48	NM	4	?	No	VκI	L1	Jκ2	95%		15

Table 1.6   Sequence characteristics of human monoclonal IgG and IgA anti-DNA antibodies

Adapted from Rahman *et al.* (2002)

Sequence Analysis of Monoclonal Anti-DNA Antibodies

mAb	Origin	V_H	D_H	J_H	V_H Homology	Evidence of antigen-drive in CDRs *	V_L family	V_L gene	J_L	V_L homology	Evidence of antigen-drive in CDRs *	Reference
SI-1	SLE PBL	3-23	NM	4	98%		VκIII	A27	Jκ1	98%		26
SI-13	SLE PBL	3-23	NM	4	92%		Vλ2	2a2	Jλ2/3	92%		26
SI-22	SLE PBL	3-30	NM	6	98%		Vλ1	1e	Jλ2/3	99%		26
SI-32	SLE PBL	3-23	NM	4	98%		Vλ3	3I	Jλ2/3	99.6%		26
SI-39	SLE PBL	1-02	NM	6	95%		VκIII	A27	Jκ1	98%		26
SI-40	SLE PBL	3-30	NM	6	99%		Vλ1	1e	Jλ2/3	96%		26
NNA2	Healthy PBL	6-01	NM	5	92%		Vλ7	7a	Jλ2/3	95%		26
AD4-37	SLE PBL	3-30	NM	3	99.3%		Vλ3	3r	Jλ2/3	99.2%		27
AD4-18	SLE PBL	5-51	D3-10	5	92%		Vλ1	1e	Jλ2/3	85%		27
DNA1	SLE Bone marrow	3-30	D2-2/D5-24	6	97.0%	No	VκIII	A19	Jκ1	95%	No	28
DNA4	SLE bone	5-51	D2-2	6	93%	No	VκIII	A27	Jκ1	99%		28

Table 1.7   Sequence characteristics of human anti-DNA antibodies produced by phage display

Adapted from Rahman *et al.* (2002))

List of References for Tables 1.4-1.7

1	Dersimonian *et al.*, (1987)
2	Hoch *et al.*, (1987)
3	Siminovitch *et al.*, (1989)
4	Logtenberg *et al.*, (1989)
5	Spatz *et al.*, (1990)
6	Sanz *et al.*, (1989)
7	Watts *et al.*, (1991)
8	Guillaume *et al.*, (1990)
9	Blanco *et al.*, (1994)
10	Stevenson *et al.*, (1993)
11	Kalsi *et al.*, (1995)
12	Rioux *et al.*, (1995)
13	Hohmann *et al.*, (1995)
14	Song *et al.*, (1998)
15	Manheimer-Lory *et al.*, (1991)
16	Hirabayashi *et al.*, (1993)
17	Daley *et al.*, (1993)
18	Winkler *et al.*, (1992)
19	van Es *et al.*, (1991)
20	Davidson *et al.*, (1990)
21	Ehrenstein *et al.*, (1994)
22	Paul *et al.*, (1992)
23	Paul *et al.*, (1993)
24	van Es *et al.*, (1992)
25	Li *et al.*, (2000)
26	Barbas *et al.*, (1995)
27	Roben *et al.*, (1996)
28	Seal *et al.*, (2000)

Abbreviations used in Tables 1.4-1.7

PBL	Peripheral blood lymphocytes
CLL	Chronic lymphocytic leukaemia
RA	Rheumatoid Arthritis
NM	No match found
NP	Sequence not published

*   Evidence of antigen-drive in CDRs was considered positive if the probability that the observed distribution of somatic mutations arose in the CDRs by chance alone was less than 0.05, as determined using the method of (Lossos *et al.*, 2000)

1.4.4.1 Is there evidence for preferential immunoglobulin gene utilisation in human monoclonal anti-DNA antibodies?

Sequence analysis of monoclonal anti-DNA antibodies, enables two things to be asked regarding gene utilisation. Firstly, are the genes used to encode these antibodies the same as those commonly rearranged by B cells or is there evidence of preferential utilisation of specific gene segments? Secondly, is there evidence that receptor editing has been involved in the production of these anti-DNA antibodies? Secondary rearrangements of the L chain loci tend to use J genes such as $J_\kappa 4$ and $J_\kappa 5$ that are distal to the V_κ genes (Dorner et al., 1998). Thus if these particular J_κ segments encode a significant number of these anti-DNA antibodies then there is evidence that receptor editing is involved in their production.

1.4.4.1.1 Utilisation of heavy chain genes

It is important to identify which gene segments are rearranged to encode autoantibodies since preferential usage of particular gene segments could play a role in the development of autoimmune B cells. It is important to look at non-productive rearrangements as well as productive rearrangements.

Productive rearrangements do make surface Ig receptors. Therefore the distribution of Ig gene segments in productive rearrangements is not only influenced by V(D)J recombination, but also by subsequent positive and negative selection and potential receptor editing during B cell development. Non-productive rearrangements do not make surface Ig receptors and are not influenced by such selection processes. Therefore by looking at the non-productive repertoire, the impact of V(D)J recombination can be assessed (Dorner et al., 2001, Farner et al, 1999).

When both productive and non-productive rearrangements of V_H genes in peripheral B cells from two normal healthy donors were analysed by Lipsky and colleagues, it was found that the majority of both types of rearrangement involved genes from the largest V_H families, $V_H 1$, $V_H 3$ and $V_H 4$. Ten individual V_H genes within these families ($V_H 3$-23, 4-29, 4-39, 3-07, 3-30, 1-18, 3-30.3, DP-58, 4-34 and 3-09) were found to be used by approximately 60% of these normal peripheral B cells. One particular $V_H 3$ family member, $V_H 3$-23 was used by approximately 13% of these normal peripheral B cells (Brezinschek et al., 1997). When V_H gene utilisation in peripheral B cells (IgM$^+$ only) from a patient with SLE were subsequently studied, no significant differences were seen

from the pattern of usage found in the normal individuals. However there was one exception to this finding as a large proportion of the productive rearrangements involved the V_H3-11 gene in this particular SLE patient (Dorner et al., 1999). Such an over-representation of this particular gene was not seen in the healthy repertoire (Brezinschek et al., 1997). The sequences of the eight V_H3-11 rearrangements obtained from the patient with SLE showed that six were the result of the same expanded B cell clone although previous studies had shown no evidence of over-utilisation of V_H3-11 (Dorner et al., 1999).

When a different group analysed H chain gene utilisation in single peripheral B cells (IgG^+ only) between one normal (209 B cells) and two SLE (156 B cells) donors, no statistically significant differences were seen between the three B cell repertoires (de Wildt et al., 2000). In particular, no over-utilisation of gene segment V_H3-11 was seen. This suggests that the over-utilisation of this gene segment in the B cell repertoire derived from the patient with SLE in the previous study by Dorner et al. (1999) is perhaps not indicative of all B cell repertoires in patients with this disease.

There are two criticisms of these studies. Firstly the number of patients in the studies by both groups was extremely small. Secondly none of the patients with SLE from whom the B cells were derived were typical cases. For example, Dorner et al. (1999) derived B cells from a 54 year old male patient with SLE who despite repeated testing had no anti-dsDNA antibodies. The patients chosen by de Wildt et al. (2000) fulfilled the classification criteria for the "so-called" mixed connective tissue disease (MCTD) rather than SLE.

Sequence analysis of the monoclonal anti-dsDNA antibodies shown in tables 1.4 – 1.7 indicate that the majority utilised genes from the largest V_H families, V_H1, V_H3 and V_H4 and that V3-23 is the most commonly rearranged gene segment. Furthermore, three anti-dsDNA IgG from three different patients used the gene V3-11 which is interesting since Dorner et al. (1999) found an expanded clone with this particular gene segment also in their SLE patient.

Both de Wildt et al. (2000) and Dorner et al. (1999) found that the most commonly used J_H gene accounting for approximately 50% of J_H gene use in both patients with SLE and

in healthy individuals was J_H4. Again the analyses in tables 1.4-1.7 are consistent, as the most frequently utilised gene was J_H4.

de Wildt *et al.* (1999) found that D_H utilisation was similar in both the healthy and SLE patient repertoires and was dominated by a few segments with D3 and D6 family members being the most regularly seen. The most frequent D segment utilised in the patients with SLE is D3-10. A wide range of D_H gene segments were used in tables 1.4 - 1.7 but similarly to de Wildt *et al.* (2000) the most frequently used gene was D3-10. In conclusion, the patterns of H chain gene utilisation in monoclonal anti-dsDNA antibodies found in tables 1.4-1.7 are concordant with those patterns found in previous studies of gene utilisation in B cells in general for both healthy people and patients with SLE.

1.4.4.1.2 Utilisation of light chain genes

When de Wildt *et al.* (2000) investigated H chain gene utilisation in single peripheral IgG^+ B cells between one normal and two SLE donors as detailed above, L chain gene utilisation was simultaneously studied. Again no statistically significant differences were seen in the pattern of L chain gene segment usage between the healthy and SLE B cell repertoires.

Conversely, when Lipsky and colleagues looked at L chain utilisation in the same B cells as investigated above in their group's study of H chain gene utilisation they found that in the productive repertoire of their patient with SLE there was a significant over-representation of particular V_L and J_L segments which are commonly used as a result of receptor editing. For example, B3 (the most J_κ-proximal gene segment) was significantly over-represented in the productive SLE repertoire compared to the normal repertoire and was frequently rearranged to $J_\kappa5$. Furthermore, when B3 was rearranged to $J_\kappa5$, it contained less mutations than when rearranged to $J_\kappa1$-4. Those V_κ segments that have significantly lower mutational frequencies are likely to be the products of secondary rearrangements since receptor editing is thought to occur after somatic hypermutation has been initiated. Thus it was concluded that that some of the over-representation of B3 in the productive repertoire of this SLE patient was the result of receptor editing. The prevalence of $J_\kappa5$ in this repertoire is also an indicator of receptor editing (Dorner *et al.*, 1998). The results of the sequence analysis carried out by de

Wildt *et al.* (2000) did not show such evidence for enhanced receptor editing in the repertoire of patients with SLE compared to normal subjects.

In tables 1.4-1.7, 65% of the antibodies had κ chains and 35% had λ chains. Two thirds of the κ chains use genes, which are amongst those identified as being the most commonly used genes in the studies highlighted above. However there is no evidence for receptor editing since only one antibody used the gene segment B3 and only five used $J_\kappa 5$. Almost all (83%) of the λ chains in these tables use genes, which are amongst those identified as being the most commonly used genes in the studies highlighted above with the most commonly used gene being 2a2. 2a2 is the most commonly arranged V_λ gene in the productive rearrangements in healthy individuals (Farner *et al.*, 1999).

The patterns of L chain gene utilisation from tables 1.4-1.7 are in accordance with previous studies and show that there is no significant difference between these patterns of utilisation and those found in healthy individuals. Thus it can be concluded that there is no preferential usage of particular gene segments to encode anti-DNA antibodies. Unlike the findings of Dorner *et al.* (1998) no evidence for enhanced receptor editing was found.

1.4.4.2 *Somatic mutation in human monoclonal anti-DNA antibodies and evidence for antigen drive.*

Tables 1.4 – 1.7 show the percentage homology (at the nucleotide level) to the closest germline gene for the V_H and V_L of these antibodies. The homologies of the IgG and IgA antibodies in table 1.6 were much lower than those IgM in tables 1.4 and 1.5. The V_H were more mutated than the V_L. However the antibodies produced by repertoire cloning in table 1.7 showed very little mutation even the V_H. All of the V_H and V_L in tables 1.5 and 1.6 that had homologies to their germline gene lower than 98% were analysed statistically using the multinomial method described by Lossos *et al.* (2000) (see section 1.3.3). Those antibodies whose pattern of somatic mutations were shown to have been antigen-driven are highlighted in the tables. The majority of V_H/V_L from IgG and IgA in table 1.6 that were statistically analysed showed evidence for antigen drive. Only two IgM were statistically analysed, as the rest were more than 98% homologous to their germline gene. These two IgM did show evidence for antigen drive but mainly in the FR region. In all cases, there was more evidence for antigen drive in V_H than V_L.

Only two Fab in table 1.7 could be statistically analysed. Neither showed evidence for antigen driven accumulation of somatic mutations in their CDRs. This is similar to the conclusion of Barbas *et al.* (1995) who reported that the high affinity anti-DNA Fab fragments derived originally from a patient with SLE showed minimal somatic hypermutation in the V genes.

In conclusion, the group of antibodies identified as being the most closely involved in the pathogenesis of SLE, the high affinity human anti-dsDNA IgG antibodies, were found to have a higher frequency of somatic mutations when compared to IgM, particularly in V_H more than V_L. Statistical analysis showed that the accumulation of somatic mutations in the CDRs of the majority of the anti-dsDNA IgG and IgA in table 1.6 was significantly affected by antigen drive. However in contrast the anti-DNA Fab antibodies in table 1.7 showed a low frequency of somatic hypermutation and no evidence for antigen drive.

1.4.5 Antigen-driven accumulation of arginine, asparagine and lysine residues in anti-DNA antibodies

Now it has been established that the accumulation of somatic mutation in the CDRs is antigen driven, the final question to be answered from sequence analysis of human anti-DNA antibodies is whether this antigen drive results in an accumulation of basic residues, particularly arginine (R) but also asparagine (N) and lysine (K) in the CDRs as previously suggested from sequence analysis of murine anti-DNA antibodies (Radic *et al.*, 1994).

The CDRs form most of the contacts between antibody and antigen. However it is important to note that this is not an absolute rule. As shown by MacCallum *et al.* (1996), some CDR residues rarely contact antigen whilst some FR residues often contact antigen. MacCallum *et al.* (1996) analysed 26 antibody Fab and Fv crystal structures that were each complexed with an antigen and determined the sites at which the antigen most commonly made contact with the antibody. Consequently, MacCallum *et al.* (1996) created a new definition termed "contact regions". These contact regions were the sites of the H and L chain variable regions at which the antibody was most likely to contact an antigen. The ranges of the CDRs and contact sites are shown in Table 1.8.

RANGE OF AMINO ACID RESIDUES WITHIN EACH CDR / CONTACT REGION		
	CDR AS DEFINED BY Wu and Kabat (1970)	CONTACT REGION AS DEFINED BY MacCallum *et al.* (1996)
L1	L24-L34	L30-L36
L2	L50-56	L46-L55
L3	L89-L97	L89-L96
H1	H31-H35b	H30-H35b
H2	H50-H65	H47-H58
H3	H95-H102	H93-H101

Table 1.8   Range of amino acid residues in each CDR / Contact region.

MacCallum *et al.* (1996) analysed 26 antibody Fab and Fv crystal structures that were each complexed with an antigen and determined the sites at which the antigen most commonly made contact with the antibody. Consequently, MacCallum *et al.* (1996) created a new definition termed "contact regions". These contact regions were the sites of the H and L chain variable regions at which the antibody was most likely to contact an antigen. The ranges of the CDRs and contact sites are shown in the table above.

Analysis of the human anti-DNA antibodies in tables 1.5 and 1.6 showed that the majority of somatic mutations tended to occur within the contact regions H1, H2, V_κL1 of the IgG and IgA antibodies, less frequently in V_λL1, L2 and L3 and very rarely in IgM. Furthermore there appeared to be an excess of somatic mutations resulting in an accumulation of arginine, asparagine and lysine residues in the contact regions of the IgG and IgA anti-DNA antibodies but not the IgM, particularly in V_HH1, V_HH2 and V_κL1. It was very difficult to assign sites of somatically mutated residues to the V_HH3 due to the extensive variability in this region due to junctional and D region diversity. In conclusion, antigen-driven accumulation of somatic mutations in the IgG and IgA anti-dsDNA antibodies leads to the accumulation of R, N and K residues in the contact regions and thus may increase the affinity of these antibodies for negatively charged antigens such as dsDNA. This is not the case for IgM where somatic mutation does not play a major role in determining the affinity of the antibody for antigen.

However, it is important to note that not all antibodies with arginine, asparagine or lysine residues in their CDRs bind dsDNA. Instead it is the actual positions of the arginine residues within the variable region of the H and L chains that determine the binding affinity of the antibody. For example, antibodies RT-79 and D5 are both derived from the gene, V_H4-34. Despite the fact that RT-79 V_HCDR3 contains five arginine residues whilst D5 V_HCDR3 only contains two, D5 binds dsDNA with high affinity whilst RT-79 only binds ssDNA (Stevenson *et al.*, 1993).

The sequence analysis of human monoclonal anti-dsDNA mAb shows that they differ from murine anti-dsDNA in that there is no good evidence for preferential use of particular V_H or V_L genes. However like murine anti-dsDNA antibodies, human IgG anti-dsDNA are highly mutated and patterns of mutation suggest that antigen has driven the accumulation of these mutations. This non-random accumulation leads to a high content of arginine, lysine and asparagine residues at the contact sites of these antibodies in both species. However the roles of individual amino acid residues in determining the binding affinity of an antibody cannot be determined by simple sequence analysis. To extend the hypotheses deduced from sequence analysis, various groups have produced three-dimensional images of the antibodies interacting with their antigen through either X ray crystallography or computer-generated modelling. Such images can be used to pinpoint the specific amino acid residues in the antigen-binding site of individual antibodies that are most likely to make contact with the antigen.

1.5 Investigation of anti-DNA antibodies by modelling three-dimensional structure

1.5.1 Crystal structures of anti-DNA antibodies

Very few crystal structures of anti-DNA antibodies have been published. This is mainly due to the fact that purification of sufficient quantities of a single mAb for X-ray crystallography analysis is very difficult. The most informative of those published is a co-crystal of a murine monoclonal anti-DNA antibody (BV04-01) that binds ssDNA, with a trinucleotide of deoxythymidic acid [d(pT)$_3$]. Co-crystals show the precise locations at which amino acid residues of the antibody make contacts with the antigen and also changes in conformation of an antibody, if any, on binding to an antigen. For example, a co-crystal of the interaction between a Fab and a ligand, vitamin K developed by Amzel et al. (1974) highlighted that the majority of contacts made between an antibody and an antigen are in the CDRs.

When complexed with d(pT)$_3$, BV04-01 undergoes conformational changes in order to incorporate the trinucleotide. The co-crystal also showed that the trinucleotide is held in a large irregular groove between the V_H and V_L regions on the surface of the antibody and that all three CDRs of the H and L chains contribute to this binding site. Only one arginine residue in V_HCDR2 at position 52, was seen to form an electrostatic contact with the phosphodiester backbone of the trinucleotide. Although other arginine and lysine residues were present in the binding site, they were unable to directly interact with the antigen due to either participating in ionic bonds in order to stabilise the topography of the antigen binding site or due to not being in the appropriate locations to contact the phosphodiester backbone (Herron et al., 1991).

From the crystal structures already known, it has been possible to derive computer-modelling programmes that predict the antibody-antigen interaction just from the amino acid sequence (Martin et al., 1991).

1.5.1 *Computer modelling of interactions of anti-DNA antibodies with DNA*

Barry *et al.* (1994) modelled six murine monoclonal anti-DNA antibodies. The accuracy of such models was highlighted by the fact that when crystal structures for some of these same anti-DNA antibodies were compared to the computer-generated models the differences between the two at the majority of sites were within 0.1nm. The antigen-binding site of these antibodies when complexed with ssDNA were shallow clefts on the surface of the molecule whereas dsDNA was believed to bind to the surface of the antibody without the need for such a cleft.

Kalsi *et al.* (1996) used computer modelling to investigate interaction between the human monoclonal anti-DNA antibody, B3 and dsDNA (see Figure 1.3). In contrast to Barry *et al.* (1994) the antigen-binding site of B3 contained a groove on the surface of the molecule between the H and L chain. The combining site could contain a complete turn of the dsDNA helix along the V_H-V_L axis. The interaction between dsDNA and the antibody, B3 was stabilised by the presence of three arginine residues situated on the periphery of the groove. These three arginines were L27a (in V_LCDR1), L54 (in V_LCDR2) and H53 (in V_HCDR2). The arginines at L27a and H53 were derived from somatic mutations.

These computer models can therefore be used to identify particular amino acid residues that potentially affect the interaction of anti-DNA antibodies with DNA. However they do not show whether the sequence features that appear to affect the binding of the anti-DNA antibody also affect the pathogenic properties of the antibody. Therefore once those amino acid residues critical for ensuring a high affinity antibody-DNA interaction are identified from a model, site-directed mutagenesis can be used to alter such residues to test their effect on binding and/or pathogenic properties. This is of course only possible if the wild type and mutated antibodies can be expressed as antibody molecules and their properties compared. A number of different systems to express autoantibody DNA sequences have been developed, as detailed in section 1.6.
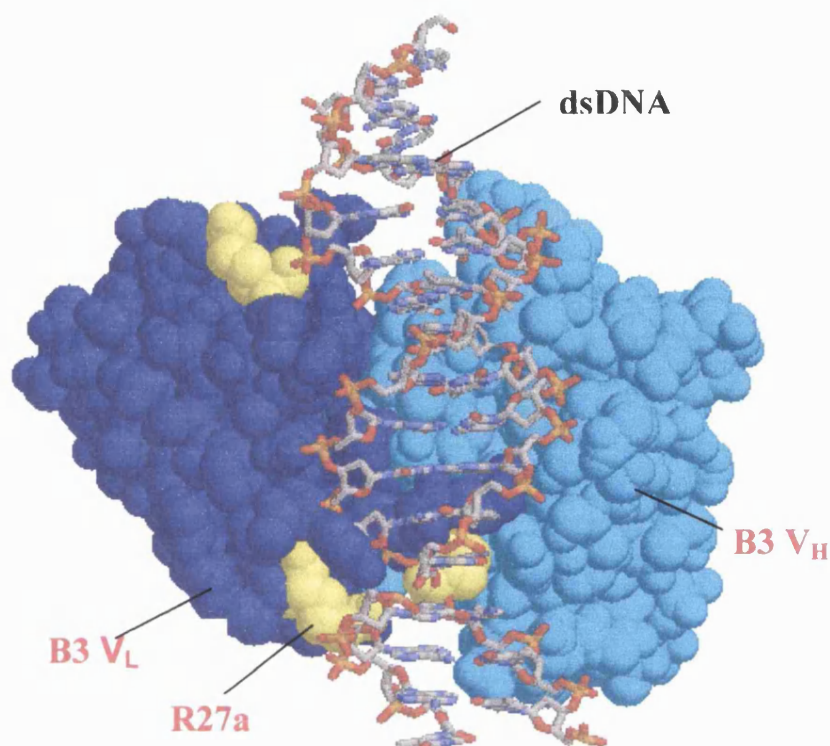
Figure 1.3   Computer generated model of B3V$_H$/B3V$_\lambda$ with dsDNA.

The double helix of the DNA molecule is shown in the centre of the figure. B3V$_H$ is shown in light blue whilst B3V$_\lambda$ is shown in dark blue. The arginine residues at the periphery of the site, predicted by the model to stabilise the interaction with the dsDNA helix, are shown in yellow. The only arginine to be derived from a somatic mutation is in V$_\lambda$CDR1 at position 27a (R27a). Modelled by Dr Sylvia Nagl.

1.6 Expression Systems for Anti-DNA Antibodies

1.6.1 The importance of expression of anti-DNA antibodies in vitro

The expression of anti-DNA antibodies and antibody fragments from cloned DNA *in vitro* enables researchers to further investigate the structure/function relationship of these antibodies. The total yield of Ig from *in vitro* expression of cloned DNA is often poor in comparison to that produced by hybridoma cell lines. However such expression systems allow the researcher to determine the sequence being expressed in order to test the effects of specific sequence motifs on the binding and/or pathogenic properties of the antibody. Monoclonal anti-DNA antibodies from patients with SLE and murine models with lupus-like disease have been expressed in such systems to test various hypotheses ranging from the effects of single point mutations to the effects of exchanging entire H or L chains.

All expression systems involve cloning V_H and/or V_L sequences of a selected antibody into an expression vector containing the appropriate amount of C_H and/or C_L as well as the components required to enable the plasmid vector to express Ig protein once transfected into either a bacterial or eukaryotic cell. Expression systems using yeast (Horwitz *et al.*, 1988; Wood *et al.*, 1985) or insect cells (Hasemann *et al.*, 1990) have been described, however only bacterial and eukaryotic systems have been used to express anti-DNA antibodies. Whole Ig molecules have been produced using expression systems. However since the antigen-binding site of an antibody is encoded by only the variable region, smaller fragments of antibodies have been expressed that still bind to antigen, for example, scFv or Fab molecules. The different types of expression system and their advantages and disadvantages are described below, along with the conclusions that have been derived from such experiments.

1.6.2 Bacterial expression systems

Single chain Fv molecules (scFv) are the simplest of the expression products. They consist of the V_H and V_L domains joined by a small flexible linker fragment that is usually glycine- and serine-rich. The flexibility of this linker enables the V_H and V_L to adopt various conformations to enable them to produce an antigen-binding site. scFv are produced by cloning V_H and V_L into an expression plasmid in which they are separated by an oligonucleotide sequence encoding the linker. One drawback of scFv is that there

is no C region DNA in the scFv expression vector, so the scFv consisting only of the variable regions does not resemble any molecule found in nature. However some scFv have been shown to have similar binding properties to the mAb from which they were derived (Brigido *et al.*, 1991; Polymenis *et al.*, 1994).

In some scFv expression systems, the V_H and V_L DNA are ligated into phagemids rather than plasmid vectors. Phagemids are plasmid-phage hybrids that enable scFv to be expressed as a fusion protein connected covalently to a surface protein of the phage particle. This method is known as phage display. If a library of V_H or V_L sequences is transfected into a phagemid, a library of different phage particles that display a repertoire of different V_H/V_L combinations as scFv is produced. Those scFv combinations with the ability to bind DNA are detected via panning a library of phage-displayed antibodies with DNA immobilised either on a plastic plate or on paramagnetic beads. The phage is allowed to bind to the immobilised DNA after which the bound material is eluted. The eluted phage is then re-amplified and several additional cycles of binding and amplification are performed under increasingly stringent conditions in order to enrich for phage clones, which have the ability to bind DNA (Roben *et al.*, 1996). This is process is termed repertoire cloning.

Bacterial expression can also be used to produce Fab fragments rather than scFv. Fab can either be expressed in a soluble form or on the surface of a phage particle thus allowing repertoire cloning, as described above. A Fab expression vector contains V_H and V_L followed 3' by a C_H1 sequence (i.e. DNA encoding the first H chain constant region) and a C_L sequence, respectively. Lower yields of Fab relative to scFv yields are produced but Fab fragments are the preferred structure for crystallisation studies if sufficient expression product can be produced and purified through large-scale bacterial cultures (Hasemann *et al.*, 1990).

One problem with using repertoire cloning is that it is uncertain whether the random H/L chain combinations in the Fab produced represent combinations found *in vivo*. To investigate this issue, Roben *et al.* (1996) used anti-idiotype antibodies. Idiotypes are antigenic determinants found in the variable region of antibodies. Few anti-idiotypes identify the gene segment used to encode the antibody chain, however antibodies with the same particular idiotype should in theory have the same amino acid sequence at the part of the sequence that encodes the idiotype. Therefore two antibodies which have the

same H chain idiotype and the same L chain idiotype are likely to have very similar H and L chain sequences.

Roben *et al.* (1996) obtained IgG anti-dsDNA Fab from the PBL of two clinically active SLE patients by repertoire cloning. One of these Fab, AD4-37, carried the H chain idiotype B6 and the L chain idiotype λIIIa. Using anti-idiotype antibodies in an inhibition ELISA to test the serum of the patient from whom AD4-37 was derived, it was found that both of these idiotypes did occur on the same anti-DNA antibody.

Although much research has focussed on the role of the V_HCDR3 of anti-DNA antibodies, bacterial expression systems have also been used to show that light chains also contribute to the DNA binding affinity of murine (Brigido *et al.*, 1991; Jang *et al.*, 1998; Polymenis *et al.*, 1994) and human (Mockridge *et al.*, 1996) anti-DNA antibodies. The systems described in these studies all illustrated the importance of not the presence but the precise position of residues such as arginine, lysine and asparagine on DNA-binding.

Fab and scFv both lack the full complement of C region domains and are therefore not ideal for use in functional assays. Therefore although these antibody fragments are useful in determining the binding properties of the V region of an antibody, they cannot be used to investigate the effects of site-directed mutagenesis on the pathogenicity of these antibodies *in vivo*. The only way to investigate the pathogenic properties of anti-DNA antibodies is therefore through the production of whole Ig molecules. As yet, it has not been possible to produce whole Ig molecules using bacterial expression systems. Even if whole Igs were to be expressed in bacterial systems, it is unlikely that they would be fully functional. To become functional, proteins need to fold into their native conformation as well as undergo post-translational modifications such as glycosylation for which bacteria do not possess the majority of enzymes required. (Hasemann *et al.*, 1990). Therefore, although more difficult and time-consuming, eukaryotic expression systems are used to produce functional whole Ig molecules.

1.6.3 Eukaryotic expression systems

A number of techniques have been described for the expression of anti-DNA antibodies in mammalian cells and these fall into two groups; those created for transient expression and those involving the selection of stably transfected cell lines.

In transient expression systems, the foreign genes of interest that are inserted onto the plasmid vector are not integrated into the genome of the cell. Therefore the cell only produces the expression product for a short amount of time since the foreign genes are soon lost from the cell. In general, three days post-transfection, the cells are harvested and the expression products of the introduced genes are assayed. The disadvantage to this system is that only small amounts of expression product are produced.

In contrast, in stable expression systems a small proportion of the cells will retain the transfected foreign genetic information for a considerable period of time since it has been incorporated into the genome of the host cell. This new DNA is passed on to all subsequent cell generations. However it is essential to select those cells that have successfully integrated the DNA and that are expressing the highest amount of product. Markers for drug resistance are often used to isolate this minority of high producing cells since only those that have taken up the plasmid will survive in the selective media used.

The H chain expression vectors used in eukaryotic expression systems contain the V_H DNA sequence 5' to the appropriate C_H DNA sequence (i.e. encoding the entire H chain constant region and the hinge region). The L chain expression vectors contain the V_L DNA sequence 5' to the appropriate κ or λ C_L DNA sequence. The H and L chain expression vectors are simultaneously transfected into eukaryotic cells and whole fully glycosylated Ig molecules are expressed. Alternatively, the V_H, C_H, V_L and C_L DNA sequences may all be cloned into the same expression vector. The advantage of this is that more cells may acquire the DNA required to make both chains therefore increasing the yield of Ig produced. However, the simultaneous transfection of separate H and L chain expression vectors does make it easier to produce a wide range of Igs with different H/L chain combinations.

Alternatively, H chain expression vectors may be transfected into H chain loss variants, which are hybridoma cell lines that secrete L chains but have lost their ability to secrete H chain. This method has been used to produce whole functional anti-DNA Ig molecules (Katz *et al.*, 1994; Radic *et al.*, 1993).

1.6.3.1 Transient expression of whole IgG molecules in eukaryotic cells

Both human and murine anti-DNA antibodies have been produced in the form of whole IgG *in vitro* by transfecting both H and L chain expression vectors into eukaryotic cells that do not normally produce Ig. COS-7 cells (immortal African green monkey kidney cell line) have been used for this purpose. COS-7 cells are permissive for the replication of many plasmids that contain a short sequence (340 bp) including the replication origin of the monkey papova virus, SV40 (Simian Virus 40). This is possible due to the presence of an origin-defective SV40 mutant in COS-7 cells, which allows them to produce the functional large T antigen of the SV40 virus constitutively. To stimulate replication, the T antigen binds to the SV40 origin. The SV40 origin is not found in these COS-7 cells but any plasmid vector containing it will be actively replicated in COS-7 cells. Once transfected, foreign DNA cloned into these plasmids is expressed. However expression in this system is only transient and can only be used over a short period of 3-6 days (Gluzman, 1981).

This method was used by Zack *et al.* (1995) to express the murine anti-DNA antibody, 3E10 as whole IgG in COS-7 cells. The resultant IgG showed the same binding properties as the original mAb, as it bound both ssDNA and dsDNA. Using this system in conjunction with site-directed mutagenesis, it was found that mutations in all V$_H$CDRs altered the affinity for DNA. Specifically, the loss of an arginine in CDR3 reduced affinity whereas gain of an asparagine in CDR1 increased it.

Members of our group have previously used a transient system in which DNA sequences from human anti-DNA antibodies, B3 and WRI176 were expressed as whole IgG1 in COS-7 cells (Rahman *et al.*, 1998). By co-transfecting COS-7 cells with separate L and H chain expression vectors whole IgG1 were produced from each of the four H/L chain combinations. The yield of antibody was low but it was possible to show binding to DNA in a direct ELISA in the antibodies with homologous H/L chain pairs. In those antibodies with the H and L chains derived from different antibodies, no binding to DNA was seen. This suggested that, as predicted by computer models of

these antibodies, both chains are involved in conferring these antibodies with the ability to bind DNA.

1.6.3.2 Stable expression of whole IgG molecules in eukaryotic cells

In many situations the quantities of expression product generated by transient expression of cloned genes in COS-7 cells will provide all the data required to answer the biological aims of the experiment. However certain affinity assays such as BIAcore (surface plasmon resonance) or functional tests in mice to detect the pathogenicity of antibodies, require purified antibody. During the purification process, much antibody protein is lost. As a result, larger quantities of antibody are required than the yield produced in a transient expression system. Such quantities can be produced through sustained expression of the chosen antibody using stable cell lines. However, establishing permanent stably transfected cell lines can be time-consuming and difficult.

The main purpose of producing whole Ig molecules using expression systems is that if sufficient quantities are produced, the pathogenic properties of sequence alterations can be determined. Katz *et al.* (1994) injected young SCID mice intraperitoneally with either R4A hybridoma cells or one of the H chain loss variants expressing whole IgG with the altered R4A H chain. All mice carrying the R4A hybridoma developed proteinuria with glomerular deposition of the antibody in the kidneys. The mutant cell line producing IgG with the highest anti-dsDNA ability showed some glomerular deposition but deposited to a greater extent in the tubules, suggesting that the change in affinity for dsDNA may also be associated with the acquisition of a novel antigenic cross-reactivity. In conclusion, these results indicate that the ability to bind dsDNA does not mean that an antibody is pathogenic.

HCLVs cannot be used to investigate of the DNA-binding role of L chains in these antibodies, whereas stable cell lines can be used to study the role of both the H and L chains. As yet, human monoclonal anti-DNA antibodies have only been stably expressed in eukaryotic cells by one group (Li *et al.*, 2000). In this system F3B6 human/mouse heteromyeloma cells were transfected simultaneously with expression vectors containing H or L chain DNA of the human IgA monoclonal anti-DNA antibody 412.67. The antibody was expressed as IgG rather than IgA, because the expression vector contained a cloned C_γ sequence (not C_α). Neomycin was used as a selecting agent as the L chain vector carried a neomycin resistance gene *neo*. Therefore only those cells

successfully transfected with the L chain expression vector survived the neomycin treatment. No other selecting agents were necessary as in transfectants expressing H chain alone, the accumulation of H chain molecules would lead to cell death. Those cells expressing both H and L chain whole IgG were detected by ELISA and the IgG purified by ammonium sulphate precipitation and then on a column. The quantities of whole Ig produced were far greater than those produced by transient expression systems thus enabling further binding assays to be carried out, such as inhibition assays which require a greater yield of Ig than that produced by transient expression. Li *et al.* (2000) used this stable expression system to show that for 412.67 to bind dsDNA the presence of two V_HCDR3 arginine residues was essential although the presence of either arginine singly was sufficient for ssDNA binding.

A major difficulty in studying a mAb derived from an individual patient is always that it is uncertain as to whether that antibody is typical of pathogenic anti-DNA antibodies found in other patients. However as the pattern of V_H/V_L gene usage is not significantly different in patients with SLE in comparison to that seen in healthy individuals, it is likely that representative anti-DNA antibodies for all SLE patients are those in which both the V_H and V_L sequences are encoded by the most commonly rearranged genes (Brezinschek *et al.*, 1997, Ignatovich *et al.*, 1999). 412.67V_L is derived from a commonly used gene (A27) however 412.67V_H is not. Furthermore 412.67 is an IgA antibody whereas the isotype of the antibodies most thought to be involved in the pathogenesis of disease are IgG and it is unknown whether 412.67 is pathogenic *in vivo*. Therefore although the sequence-related conclusions above are applicable to this antibody 412.67, it could be questioned whether they would be applicable to pathogenic human anti-DNA antibodies, as 412.67 is not typical of these antibodies.

1.7   CONCLUSION

In conclusion it appears that high affinity anti-dsDNA IgG are particularly closely related to tissue damage and disease activity in patients with SLE. Sequence analysis has shown that these isotype and binding properties are associated with antigen-driven accumulation of multiple somatic mutations in the V_H and V_L CDRs of these antibodies. The effect of these somatic mutations has been to create increased numbers of certain residues, particularly arginine. The importance of these residues has been highlighted using site-directed mutagenesis and various expression systems. However there remains a need to investigate sequence-related binding and pathogenic properties of a human anti-DNA antibody that is considered representative of those antibodies most closely related to disease pathogenesis in patients with SLE. A monoclonal antibody of this type would need to be IgG isotype, known to be pathogenic *in vivo* and be derived from commonly used genes. Such an antibody is the human monoclonal anti-DNA antibody, B3 that was derived by members of our group from a patient with active SLE (Ehrenstein *et al.*, 1994).

B3 is relevant to studying SLE for a number of reasons. It has an IgG isotype, a high affinity for dsDNA and its V_H and V_L are encoded by the two most commonly rearranged human V_λ and V_H genes, $V_\lambda 2a2$ and $V_H 3-23$. Furthermore when injected into SCID mice, B3 deposited in the kidneys and the mice developed proteinuria (Ehrenstein *et al.*, 1995).

Sequence analysis has shown that there is evidence for antigen-driven accumulation of somatic mutations in both $B3V_H$ and $B3V_\lambda$. As described in section 1.5.2 our group has published a computer-generated model of the B3/dsDNA complex (Kalsi *et al.*, 1996). The model suggests that binding is stabilised by the interaction of dsDNA with three arginine residues on the periphery of the binding site. One of these arginine residues (R27a in V_LCDR1) is the product of a somatic mutation of the germline gene, 2a2. Previously our group has used a COS-7 transient expression system to express the V_H and V_L of B3 as whole IgG molecules (Rahman *et al.*, 1998).

Therefore one of the main aims of this thesis was to further this work by using the same transient expression system to determine whether particular sequence motifs, especially R27a are important in enabling B3 to bind DNA as predicted by the model.

As described before, the yield of whole immunoglobulin protein produced by transient expression systems is insufficient for use in functional assays to determine the effects of sequence features on the pathogenicity of an antibody. For this purpose, the second main aim of this thesis was to express large amounts of whole antibodies by stably transfecting mammalian cells with the same expression vectors as those used for transient expression.

The expression systems used in this thesis were originally developed by researchers at AERES Biomedical, MRC Collaborative Unit, Mill Hill, London, UK. The main advantage of these particular techniques is that the same expression vectors as those used for the transient system can be used in a stable expression system in CHO cells.

AIMS OF THIS THESIS

1. To use the COS-7 transient expression system to investigate the importance of somatic mutations and the presence of particular amino acid residues on the binding properties of the human monoclonal anti-DNA antibody, B3 by:

    a) exchanging V_H or V_L regions between antibodies

    b) site-directed mutagenesis of R27a predicted by a computer model to be important in DNA binding

    c) exchanging CDR regions between V_λ

2. To develop a system for the stable expression of cloned variable region cDNA derived from human monoclonal anti-DNA mAb as whole IgG molecules in CHO*dhfr*⁻ cells

CHAPTER TWO

Materials and Methods

CHAPTER TWO. MATERIALS AND METHODS

All chemicals used were supplied by VWR International (Leicester, UK) unless otherwise stated. The constituents of all growth media and buffers used are listed in the text of this chapter and also in Appendix C.

2.1 Human monoclonal antibodies

B3, (Ehrenstein *et al.*, 1994) 33.H11, (Winkler *et al.*, 1991) and UK-4 (Menon *et al.*, 1997) are all human IgG1 monoclonal antibodies (mAbs) derived by the fusion of peripheral blood lymphocytes (PBL) from patients with Systemic Lupus Erythematosus (SLE) with cells of the mouse heteromyeloma line CB-F7. 33.H11 was a kind gift from Dr Thomas Winkler (Erlangen, Germany). All three antibodies were derived from lymphocytes from different patients. The clinical and serological features of the patients from whom the mAb were derived are shown in Table 2.1.

The binding properties of the mAbs are shown in Table 2.2. B3 binds both dsDNA and ssDNA, 33.H11 is specific for dsDNA, whilst UK-4 binds negatively charged (but not neutral) phospholipids, but does not bind DNA. The V_λ regions of all three antibodies are derived from the same germline gene, 2a2. $B3V_H$ is encoded by V3-23, $33.H11V_H$ by V3-07 and $UK-4V_H$ by V3-74. These are all members of the V_H3 family and share strong sequence homology.

Feature	Patient		
	K.H.	D.H.	SLE2
Rash	+	-	NS
Arthritis	+	+	NS
Central nervous system	-	+	NS
Serositis	-	+	NS
Renal	-	+	+
Thrombocytopaenia	-	-	NS
History of thrombosis	-	stroke*	NS
Miscarriages	-	-	NS
Migraines	-	+	NS
Anti-nuclear antibodies	+	+	NS
Anti-dsDNA antibodies	+	+	+
Anti-Cardiolipin IgG	-	+	NS
Anti-Cardiolipin IgM	-	-	NS
Lupus anti-coagulant	-	-	NS

Table 2.1 **Clinical and serological features of patients from whom mAb were derived**

B3 was derived from PBL of a 59-year-old Caucasian lady with SLE (K.H.) (Ehrenstein *et al.*, 1994).

UK-4 was derived from a 49-year-old Asian lady with SLE (D.H.) (Menon *et al.*, 1997).

33.H11 was derived from a SLE patient (SLE patient 2) (Winkler *et al.*, 1991).

(Unlike B3 and UK-4, 33.H11 was not produced by our own group and was a kind gift from Dr. Winkler. 'NS' indicates where it was "not stated" in the relevant journal paper whether the patient from whom 33.H11 was derived, had those particular features or not).

* intracerebral haemorrhage

Antigen	mAb		
	B3	**33.H11**	**UK-4**
dsDNA (ELISA)	+	+	-
Crithidia luciliae	+	-	-
ssDNA (ELISA)	+	-	-
Histones	+	NT	-
Nucleosomes	+	NT	NT
Heparan sulphate	-	NT	NT
Chondroitin sulphate	-	NT	NT
Laminin	-	NT	NT
Collagen type IV	-	NT	NT
α-actinin*	+	NT	NT
Cardiolipin*	+	NT	+
Neutral phospholipids	NT	NT	-

Table 2.2   Properties of the original IgG1/λ monoclonal antibodies (mAb)

The table shows the binding properties of the three original monoclonal antibodies (mAb) used in this thesis. The binding properties of B3, UK-4 and 33.H11 are as stated in the original publication of these antibodies (Ehrenstein *et al.*, 1993, Menon *et al.*, 1997 and Winkler *et al.*, 1991 respectively).

'NT' indicates where the mAb was not tested for its ability to bind that particular antigen

* Indicates where the binding ability of B3 for a particular antigen was not stated in the original journal paper but has since been tested in our laboratory by ELISA

2.2 Assembly of original expression constructs

The following expression constructs were prepared by Dr Anisur Rahman prior to the beginning of my Ph.D.: $B3V_H/pG1D1$, $33.H11V_H/pG1D1$, $B3V_\lambda/pLN10$, $33.H11V_\lambda/pLN10$ and $UK-4V_\lambda/pLN10$ (see Figures 2.1 and 2.2). The methods used are described fully in two papers by our group (Rahman *et al.*, 2001; Rahman *et al.*, 1998). pG1D1 and pLN10 are expression vectors and were both kind gifts from Dr C.A. Kettleborough and Dr T. Jones at AERES Biomedical, MRC Collaborative Unit, Mill Hill, London. Due to commercial sensitivity, no published references are available for these vectors.

Each expression vector contains the human cytomegalovirus (HCMV) promoter to drive transcription of the recombinant immunoglobulin gene, the SV40 origin of replication to give high levels of transient expression in COS-7 cells and either the bacterial *neo* gene (in pLN10) or mouse *dhfr* gene (in pG1D1 or pG1D210) coding sequences driven by the SV40 early promoter to act as dominant selectable markers during stable transformation. However the SV40 promoter that drives the *dhfr* is crippled by the presence of a defective SV40 promoter-enhancer sequence so that expression is poor thus allowing for the selection of high expression level clones using comparatively low levels of methotrexate. Each vector also contains an ampicillin resistance gene driven by an internal promoter to enable it to be cultured in *E.coli*. pG1D1 is a heavy chain expression vector as it also contains a cloned PCR fragment that encodes the human γ1 constant region ($C_\gamma1$) whilst pLN10 is a λ light chain vector as it contains a cloned PCR fragment of DNA encoding the human λ_2 constant region (C_λ).

The V_H region sequences of both B3 and 33.H11 were each cloned separately into expression vector pG1D1 5' to the $C_\gamma1$ human DNA sequence to produce the constructs, $B3V_H/pG1D1$ and $33.H11V_H/pG1D1$ (see Figure 2.1). Each of the V_λ region sequences were ligated into expression vector pLN10 5' to the C_λ human DNA sequence to produce the constructs, $B3V_\lambda/pLN10$, $33.H11V_\lambda/pLN10$ and $UK-4V_\lambda/pLN10$ (see Figure 2.2).

In both pLN10 and pG1D1, the immunoglobulin variable (V) region sequence is immediately followed by a splice donor (SD) site whilst the immunoglobulin constant (C) region sequence is immediately preceded by a splice acceptor (SA) site. The DNA between these SA and SD sites is treated like an intron by mammalian cells and is not represented in the expressed heavy or light chain peptide. The purpose of these splice sites was to facilitate the transfer of the V region cassette (V region sequence and immunoglobulin leader sequence) into the expression vectors. The SA site was from the original vector whilst the SD was not. The SD site and a *BamHI* site were added to the 3' end of the V region DNA by PCR (as detailed previously in Rahman *et al.*, 2001; Rahman *et al.*, 1998) prior to being incorporated into the final expression vector as part of a *HindIII/BamHI* insert with the V region cassette. Without the SA and SD sites the V region and C region DNA would need to be immediately adjacent to each other in the vector and therefore the restriction site for the V region cassette would need to be within the V region sequence. As a consequence, for some sequences this may not be possible without changing the expressed amino acid sequence of the V region, which is undesirable.

Figure 2.1   Vector map of recombinant heavy chain expression vector, B3V$_H$/pG1D1

pG1D1 contains the human cytomegalovirus (HCMV) promoter to drive transcription of the recombinant immunoglobulin gene, the SV40 origin of replication to give high levels of transient expression in COS-7 cells and the mouse *dhfr* gene coding sequence driven by the SV40 early promoter to act as a dominant selectable marker during stable transformation. The SV40 promoter that drives the *dhfr* is crippled by the presence of a defective SV40 promoter-enhancer sequence so that expression is poor thus allowing for the selection of high expression level clones using comparatively low levels of methotrexate. pG1D1 also contains an ampicillin resistance gene (AmpR) driven by an internal promoter to enable it to be cultured in *E.coli* and a cloned PCR fragment that encodes the human γ1 constant region (C$_\gamma$1). The V$_H$ region sequences of both B3 and 33.H11 were each cloned separately into expression vector pG1D1 5' to the C$_\gamma$1 human DNA sequence to produce the constructs, B3V$_H$/pG1D1 and 33.H11V$_H$/pG1D1. The immunoglobulin variable (V) region sequence is immediately followed by a splice donor (SD) site whilst the immunoglobulin constant (C) region sequence is immediately preceded by a splice acceptor (SA) site. The DNA between these SA and SD sites is treated like an intron by mammalian cells and is not represented in the expressed heavy or light chain peptide. The purpose of these splice sites was to facilitate the transfer of the V region cassette (V region sequence and immunoglobulin leader sequence) into the expression vectors.

KEY

→ Promoter

▬► Gene

➡ Antibody DNA sequence

▬ Intron between SA and SD not represented in final expression product

▮ Immunoglobulin leader sequence

SA   Splice acceptor site

SD   Splice donor site

AmpR   Ampicillin resistance gene

dhfr   Dihydrofolate reductase gene

HCMV   Human cytomegalovirus

Most restriction sites are shown in black. Those written in red indicate that they were used for cloning described in this thesis.

Figure 2.2   Vector map of recombinant light chain expression vector pLN10

pLN10 contains the human cytomegalovirus (HCMV) promoter to drive transcription of the recombinant immunoglobulin gene, the SV40 origin of replication to give high levels of transient expression in COS-7 cells and the bacterial *neo* gene coding sequence driven by the SV40 early promoter to act as a dominant selectable marker during stable transformation. pLN10 also contains an ampicillin resistance gene (AmpR) driven by an internal promoter to enable it to be cultured in *E.coli* and a cloned PCR fragment that encodes the human λ2 constant region ($C_\lambda 2$). The V_λ region sequences of B3, UK-4 and 33.H11 were each cloned separately into expression vector pLN10 5' to the C_λ human DNA sequences to produce the constructs, B3V_H/pLN10, UK-4V_H/pLN10 and 33.H11V_H/pG1D1. The immunoglobulin variable (V) region sequence is immediately followed by a splice donor (SD) site whilst the immunoglobulin constant (C) region sequence is immediately preceded by a splice acceptor (SA) site. The DNA between these SA and SD sites is treated like an intron by mammalian cells and is not represented in the expressed heavy or light chain peptide. The purpose of these splice sites was to facilitate the transfer of the V region cassette (V region sequence and immunoglobulin leader sequence) into the expression vectors.

Hpa I 7095

EcoRI 1

Sac I 1133

HinD III 1225

NruI 6224

HCMV promoter

Recombinant pLN10
7447 bp

Light chain
variable region

BamHI 1581

SD 1586

Intron

BssH II 5766

NeoR

SA 1706

Light chain
constant region

Eag I 5275

Bcl I 5210

Bgl II 5205

NeoR prom

AmpR prom

EcoRI 2260

Sfi I 4818

SV40 origin

AmpR

Xmn I 2659

Sca I 2778

Pvu I 2890

KEY

→ Promoter

⬛ Gene

➡ Antibody DNA sequence

🟦 Intron between SA and SD not represented in final expression product

🟧 Immunoglobulin leader sequence

SA — Splice acceptor site

SD — Splice donor site

AmpR — Ampicillin resistance gene

NeoR — Neomycin resistance gene

HCMV — Human cytomegalovirus

Most restriction sites are shown in black. Those written in red indicate that they were used for cloning described in this thesis.

2.3 Assembly of variant B3V$_\lambda$ constructs for expression.

2.3.1 Site-directed mutagenesis of B3V$_\lambda$.

The aim of mutagenesis was to produce a variant of B3V$_\lambda$ in which the positively charged arginine (R) residue at position 27a had been altered to serine (S), which is both uncharged and present at that position in the unmutated sequence of 2a2. The site-directed mutagenesis was carried out by Dr Anisur Rahman and the method used was the megaprimer method described by Seraphin *et al.* (1996). The two successive PCR reactions involved are described fully in Rahman *et al.* (2001). The product of the second PCR was purified and ligated into the plasmid *pGEM®-T Easy* vector system (Promega, Southampton, UK) by TA cloning. Sequencing in this plasmid confirmed the presence of the desired mutation.

2.3.2 Transfer of mutagenised B3V$_\lambda$ sequences from the pGEM®-T Easy vector system (Promega, Southampton, UK) to the expression vector, pLN10.

2.3.2.1 Small-scale extraction of recombinant mutagenised-B3V$_\lambda$/pGEM®-T Easy from Escherichia coli (E.coli).

DH5α-strain *E.coli* containing the recombinant mutagenised-V$_\lambda$/pGEM®-T Easy vector (Promega, Southampton, UK) was stored in media containing 15% (v/v) glycerol at -70°C (as recommended by Sambrook *et al.* (2001). Under sterile conditions (i.e. next to a Bunsen burner flame), a sterilised wire loop was used to streak the glycerol-stored cultures onto a Luria-Bertani (LB) agar plate (5g Bacto-tryptone, 2.5g Yeast extract, 5g Sodium chloride, 7.5g Bacto-agar, made up to 500ml with double distilled water, adjusted to pH 7.0 using 5.0 M Sodium Hydroxide and autoclaved) supplemented with 50µg/ml ampicillin and incubated at 37°C in a dry incubator overnight. The plates were transferred to the refrigerator and kept at 4°C. 5ml LB medium (5g Bacto-tryptone, 2.5g Yeast extract, 5g Sodium chloride, made up to 500ml with double distilled water, adjusted to pH 7.0 using 5.0 M Sodium Hydroxide and autoclaved) supplemented with 100µg/ml ampicillin in a 50ml polypropylene tube was inoculated with a colony using a sterile wire loop and incubated at 37°C in a shaking incubator overnight. 5ml of LB medium (supplemented with 100µg/ml ampicillin) that had not been inoculated were also incubated under the same conditions. This aliquot was a control to ensure that irrelevant bacteria did not contaminate the LB medium.

Recombinant vector was extracted from overnight *E. coli* bacterial cultures using the QIAGEN QIAprep® Miniprep kit (Crawley, West Sussex, UK) according to the manufacturer's instructions. The DNA minipreps were resuspended in Tris-EDTA (T.E.) buffer (10mM Tris-HCl, 1 mM EDTA, pH 7.5) and stored at -20°C.

2.3.2.2 Restriction digest of recombinant pGEM®-T Easy vector containing B3Vλ variant PCR fragments, with Bam HI/HindIII or Bam HI/SacI.

In order to transfer the $B3V_\lambda$ variant PCR fragments ($B3V_\lambda a$ and $B3V_\lambda b$) into pLN10, both pLN10 and the recombinant *pGEM®-T Easy* vectors containing the variant $B3V_\lambda$ PCR fragments were first digested with *HindIII/BamHI* or *SacI/BamHI*. Best results were obtained using *HindIII/BamHI* for $B3V_\lambda a$ and *SacI/BamHI* for $B3V_\lambda b$. The restriction digests were carried out with 1u enzyme/μg/hr for 1 hour at 37°C as recommended by the manufacturer (Promega, Southampton, UK).

2.3.2.3 Separation of DNA fragments by agarose gel electrophoresis.

In order to separate the DNA fragments resulting from the above restriction digests, the products were run on a 0.7% agarose gel (0.7% agarose solution in Tris Acetate EDTA (TAE) buffer [40mM Tris-acetate, 1mM EDTA]) in an electrophoresis tank containing TAE buffer, for 30-60 minutes at approximately 100V. The running buffer used was TAE buffer. The DNA bands were visualised under long wavelength ultraviolet light by adding ethidium bromide to the gel at a final concentration of 0.2μg/ml.

To determine the molecular weights of the DNA bands, DNA molecular weight ladders were also run alongside the DNA samples on the gel. The sizes of the expected bands determined which DNA ladders were used however the standard markers used were the 1Kb DNA ladder (Invitrogen, Paisley, UK) and the λ/*Hind III* marker (Promega, Southampton, UK). Photographs of the gel were taken to provide a permanent record of the gel.

2.3.2.4 Purification of DNA from agarose gels

The following bands were excised from the gel: pLN10 (approx. 7kb band), B3V$_\lambda$a (355bp) and B3V$_\lambda$b (445bp). DNA was extracted from the agarose gel slices using either the QIAEX II kit or the QIAquick® spin kit (Qiagen, Crawley, West Sussex, UK) as recommended by the manufacturer. In each case, once purified from the agarose, part of the DNA was run on a 0.7% agarose gel with the 1Kb DNA molecular weight marker (Invitrogen, Paisley, UK) in order to calculate from the relative band intensities the amount of DNA available for the subsequent reactions.

2.3.2.5 Ligation of linearised pLN10 with B3V$_\lambda$a or B3V$_\lambda$b PCR fragments.

The ligation of the B3V$_\lambda$ variant PCR fragment into pLN10 was carried out using T4 DNA ligase enzyme and buffers (Promega, Southampton, UK) according to the manufacturer's instructions: The reaction mix contained a 1:1 molar ratio of vector:insert DNA (a typical reaction used 100-200ng of vector DNA), 1u T4 DNA ligase in a total volume of 20μl with water (supplemented with the appropriate ligase buffer as supplied by the manufacturer). Two vector only controls were set up for each ligation in which the insert DNA was replaced by the appropriate volume of sterile ddH$_2$O. In order to ensure that the T4 DNA ligase was functional, one of the controls did not contain T4 DNA ligase. The ligation mixes were incubated overnight at 4°C and then stored at -20°C until transformed into competent *E. coli* cells.

2.3.2.6 Production of fresh competent DH5α-strain E.coli cells.

Bacterial cultures were prepared from glycerol stocks of DH5α-strain *E. coli* as described in section 2.3.2.1 except that no antibiotic was added to agar plates or LB medium. This culture was then grown overnight in a shaking incubator (250rpm) at 37°C. 100μl of this overnight culture were added to 100ml LB medium in a 500ml sterile (autoclaved) conical flask and then returned to the shaking incubator (250rpm) for approximately 3 hours.

After 2 hours and then every 30 minutes after that, the growth of the cell culture was checked. The 100ml culture was removed from the incubator when the culture was in

the exponential log phase of the growth curve (i.e. clouds of bacteria swirling in the medium were just visible when held up to the light and when the absorbance reading of the culture when measured on the spectrometer at 600nm was between 0.3 and 0.8).

The culture was then divided between two 50ml sterile falcon tubes and spun down at 1750g for 10 minutes in a Sorvall RTH-750 rotor at 4°C. The supernatant was discarded and the pellet in each tube was resuspended in 10ml ice cold 100mM calcium chloride solution. The preparations were spun down again at 1750g for 10 minutes in a Sorvall RTH-750 rotor at 4°C. The supernatant was discarded and each pellet was resuspended in 2ml ice cold 100mM calcium chloride solution. The cell suspensions were put on ice and used immediately for transformation with recombinant vector. This protocol was derived from Sambrook *et al.* (2001).

2.3.2.7 Transformation of competent *Escherichia coli* with recombinant vector

For each separate ligation reaction, 200μl of the competent cells were transferred to ice cold 1.5ml microcentrifuge tubes and kept on ice. 5μl of each ligation mix were added to 200μl of these competent cells. The DNA/cell preparations were gently mixed, left on ice for 30 minutes and then subjected to a heat shock by incubating the samples for 90 seconds at 42°C in a water bath. The DNA/cell preparations were then returned to ice for a further two minutes in order to recover from the heat shock. 1ml of LB medium (no antibiotic) was added to each tube and then the samples were incubated in a shaking incubator (250rpm) at 37°C for one hour. The cell suspensions were centrifuged for 10 minutes at 3500g at room temperature in a microcentrifuge and the pellets resuspended in 100μl LB medium each. These samples were then spread onto a LB agar plate containing 50μg/ml ampicillin. Once dry, the plates were left upside down overnight at 37°C in a dry incubator.

The DH5α-strain of *E. coli* is unable to grow in the presence of the antibiotic ampicillin, however the vector pLN10 contains an ampicillin resistance gene. Therefore only those bacterial cells that incorporated the vector should have survived to produce a colony on the plate. The next day the plates were removed from the incubator and the number of colonies counted. A number of colonies were "picked" using a sterile wire loop and transferred into 1ml LB cultures containing 50μg/ml ampicillin. These cultures were

transferred to a shaking incubator (250rpm) at 37°C overnight. From the overnight cultures, glycerol stocks were made by adding 0.7ml sterile autoclaved 100% glycerol to 0.3ml overnight culture in a fresh sterile 1.5ml microcentrifuge tube. The tube was vortexed to mix the contents and then immediately transferred to the -80°C freezer for storage. This protocol was derived from Sambrook *et al.* (2001).

DNA was extracted from the remainder of the overnight culture using the QIAGEN QIAprep® Miniprep kit (as described in section 2.3.2.1) and then digested with *BamHI/HindIII* or *BamHI/SacI* (1u enzyme/µg/hr for 1 hour at 37°C as recommended by the manufacturer, Promega, Southampton, UK) to check that the ligation was successful.

Only those glycerol stocks of the clones that produced the correct size bands on the agarose gel were kept. Each of these glycerol stocks represented a clone containing the final expression light chain vector, pLN10 containing the sequence encoding the B3V$_\lambda$a or B3V$_\lambda$b regions.

2.4 Cloning of light chain expression vector CDR hybrids.

As an alternative to targeting single amino acids within the CDRs of the IgG variable region using site-directed mutagenesis, it was decided that swapping entire CDRs between various light chains all derived from the same germline gene would further highlight those regions most heavily involved in binding to various antigens.

The following cloning steps were used to produce hybrids of B3/UK-4, 33.H11/B3 and UK-4/33.H11 λ chain expression vectors. The method below depicts the steps that I used to produce the hybrids BU (B3V$_\lambda$ CDR1, UK-4V$_\lambda$ CDR2 and CDR3) and UB (UK-4V$_\lambda$ CDR1, B3V$_\lambda$ CDR2 and CDR3). The other hybrids 33B (33.H11V$_\lambda$ CDR1, B3V$_\lambda$ CDR2 and 3), B33 (B3V$_\lambda$ CDR1, 33.H11V$_\lambda$ CDR2 and CDR3), 33U (33.H11V$_\lambda$ CDR1, UK-4V$_\lambda$ CDR2 and 3) and U33 (UK-4V$_\lambda$ CDR1, 33.H11V$_\lambda$ CDR2 and CDR3) were all produced using the same method, but by my colleague Lesley Mason.

2.4.1 Restriction digest of parent single light chain expression vectors.

Plasmid DNA for the B3V$_\lambda$ expression vector and UK-4 V$_\lambda$ expression vector was prepared as described in section 2.3.2.1 and then digested with *PvuI* and *KpnI* (Promega, Southampton, UK) as recommended by the manufacturer's instructions (1u enzyme/µg/hr for 1 hour at 37°C). *KpnI* was chosen because it cuts only once and at the same place in each of B3, UK-4 and 33.H11 but does not cut pLN10. *PvuI* was chosen because it cuts the vector pLN10 but not any of the three inserts (see Figure 2.3).

Kpn I cuts both vectors at position 106 bp in FR2 of the V$_\lambda$ variable region sequence whilst *PvuI* cuts both vectors at position 2890 bp in the expression vector pLN10 DNA downstream of the constant region. Therefore each vector was digested into two linear bands; one of approximately 1.5 kb (containing V$_\lambda$ CDR2 and 3) and the other, of approximately 5.9 kb (containing V$_\lambda$ CDR1), as shown in Figure 2.3. The DNA fragments were purified from the agarose gel using the protocol described in section 2.3.2.4 and then run on a 0.7% agarose/TAE gel to determine the amount of DNA present as described in section 2.3.2.3.

Figure 2.3   **Restriction enzyme digest method used to exchange V$_\lambda$CDR cloned DNA sequences.**

a)   Vector map of recombinant light chain expression vector, pLN10 showing the positions of the two restriction sites used to exchange V$_\lambda$CDR cloned DNA sequences, *KpnI* and *PvuI* highlighted in red.

KEY

→	Promoter
⮕	Gene
⮕	Antibody DNA sequence
▭	Intron between SA and SD not represented in final expression product
▭	Immunoglobulin leader sequence
SA	Splice acceptor site
SD	Splice donor site
AmpR	Ampicillin resistance gene
NeoR	Neomycin resistance gene
HCMV	Human cytomegalovirus

b)   Products of *KpnI/PvuI* restriction digest of recombinant pLN10 containing B3 or UK-4 V$_\lambda$ cloned DNA sequences.

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a)
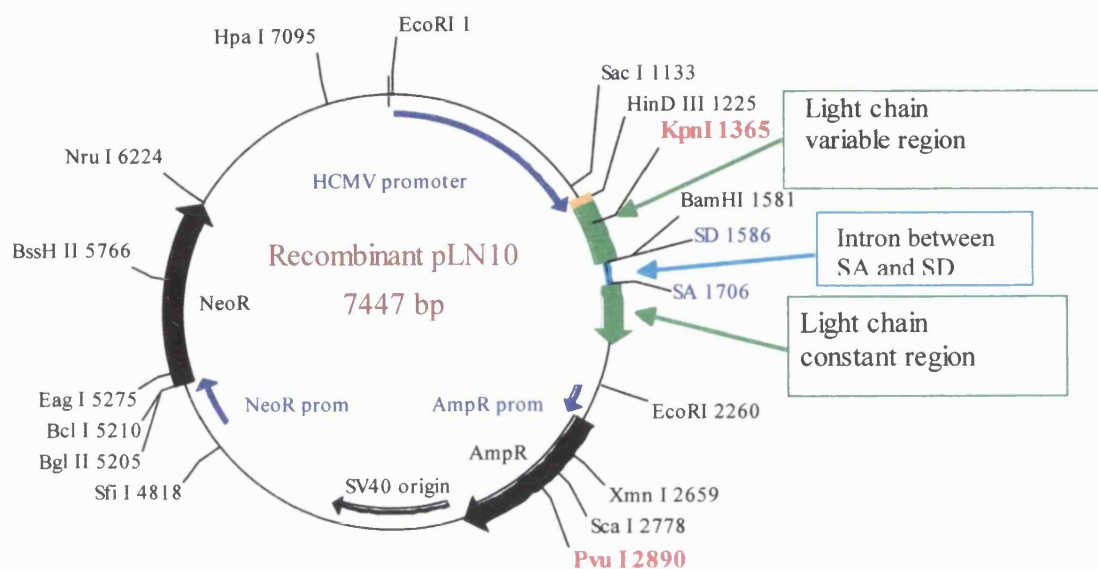
b)
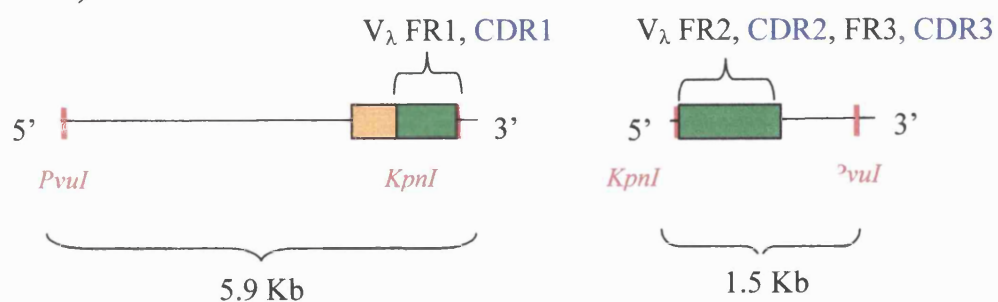
2.4.2 Ligation of 5.9 kb DNA fragments (containing CDR1 of V_λ of chosen IgG) and 1.5 kb DNA fragments (containing CDR2 and CDR3 of V_λ of other chosen IgG).

The 1.5 kb fragment contains CDR2 and CDR3 of the IgG V_λ region and also part of the downstream expression vector containing the λ constant region sequence whilst the 5.9 kb fragment contains the V_λCDR1 and the rest of the vector. Therefore the objective was to ligate the B3V_λ vector-5.9 kb fragment with the UK-4V_λ vector-1.5 kb fragment and *vice versa*. The ligation and subsequent transformation into competent *E. coli* cells were carried out as described in sections 2.3.2.5-2.3.2.7.

2.4.3 Restriction digest to identify those clones containing the correct hybrid V_λ CDR regions.

To ensure that each construct contained the correct CDRs, two different restriction digests were carried out; *AatII* digest and a *HindIII/AvaI* digest. The restriction digests were performed using Promega (Southampton, UK) enzymes and buffers as recommended in the manufacturer's instructions (1u enzyme/µg/hr for 1 hour at 37°C). A *KpnI/PvuI* or a *BamHI/HindIII* digest was not sufficient as the UK-4 and B3 V_λ fragments were of identical sizes. Due to the different pattern of somatic mutations in the V_λ of B3 and UK-4, the bands produced from an *AatII* or a *HindIII/AvaI* restriction digest were sufficiently different to distinguish which CDRs each hybrid possessed. Figure 2.4 shows the *AatII*, *AvaI* and *HindIII* sites in both B3V_λ/pLN10 and UK-4V_λ/pLN10).

Figure 2.4 **Restriction digest maps of a) B3V$_\lambda$/pLN10 and b) UK-4V$_\lambda$/pLN10 recombinant light chain expression vectors**

The restriction digest maps opposite are of recombinant light chain expression vectors, a) B3V$_\lambda$/pLN10 and b) UK-4V$_\lambda$/pLN10 and show the positions of the restriction sites used to determine which cloned DNA sequence V$_\lambda$ CDRs were present in each clone.

A *KpnI/PvuI* or a *BamHI/HindIII* digest was not sufficient as the UK-4 and B3 V$_\lambda$ fragments were of identical sizes. Instead two separate restriction digests were carried out; an *AatII* and a *HindIII/AvaI* restriction digest. Due to the different pattern of somatic mutations in the V$_\lambda$ of B3 and UK-4, the bands produced from an *AatII* or a *HindIII/AvaI* restriction digest were sufficiently different to distinguish which CDRs each hybrid possessed. The *AatII* sites of the first digest are shown in blue whilst the *AvaI* and *HindIII* sites of the second digest are shown in red in both B3V$_\lambda$/pLN10 and UK-4V$_\lambda$/pLN10.

KEY

➔	Promoter
➔	Gene
➔	Antibody DNA sequence
⊏⊐	Intron between SA and SD not represented in final expression product
⌐	Immunoglobulin leader sequence
SA	Splice acceptor site
SD	Splice donor site
AmpR	Ampicillin resistance gene
NeoR	Neomycin resistance gene
HCMV	Human cytomegalovirus

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a)

b)

2.5   DNA sequencing and site-directed mutagenesis of 33.H11V$_H$/pG1D1 construct.

2.5.1   Sequencing of expression vector (pG1D1) containing 33.H11V$_H$ sequence.

DNA sequencing of the expression vector (pG1D1) containing 33.H11V$_H$ sequence was carried out using the Sanger dideoxynucleoside chain termination method (Sanger et al., 1977) using the T7 Sequenase (de-aza) kit, version 2.0 (Amersham), according to the manufacturers instructions. The primers used for sequencing were Hug1 and Neo A.

Hug1   5' TTGGAGGAGGGTGCCAG 3'

(binds to pG1D1 90bp 3' of V$_H$ region cassette)

Neo A   5' CTCCATAGAAGACACCG 3'

(binds to pG1D1 30bp 5' of V$_H$ region cassette)

The reactions were run on a polyacrylamide gel (60ml acrylamide:bis-acrylamide, 19:1 (w/v) gel solution (Amresco, Anachem, Luton, Bedfordshire, UK), 300µl 10% ammonium persulphate (w/v) and 30µl TEMED (N, N, N', N'-tetramethylethylenediamine solution) (Sigma, Poole, UK) in a sequencing tank containing Tris Borate EDTA (TBE) buffer (45mM Tris-borate, 1mM EDTA). The gel was exposed to X-ray film (Kodak, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) in a film cassette for 24 – 48 hours and developed using an automatic developer (Xograph Imaging System X4, Bedfordshire, UK).

2.5.2   Site-directed mutagenesis of 33.H11 V$_H$ expression vector using the QuikChange™ Site-directed Mutagenesis Kit (Stratagene, California, USA).

Through sequence analysis of 33.H11V$_H$/pG1D1, it was deduced that expression may be improved by reverting a proline (derived from an earlier PCR error) to a leucine by reverting a single nucleotide change from a C to a T in FR2 of the V$_H$ sequence. This single nucleotide change was carried out using the QuikChange™ Site-directed Mutagenesis Kit (Stratagene, California, USA) according to the manufacturers instructions.

The mutagenic primers used in this protocol were designed individually according to the desired mutation. The primers were made and purified by fast polynucleotide liquid chromatography (FPLC) by Genosys, Cambridge, UK. The primers used were:

RE1MFOR

5' CAA CGT CAA GAA CTC ACT GTA TCT GCA AAT GAA CAG CC 3'

RE1MBACK

5' GGC TGT TCA TTT GCA GAT ACA GTG AGT TCT TGA CGT TG 3'

The dsDNA template used was the whole 33.H11V$_H$/pG1D1 plasmid. The dsDNA was prepared using the QIAGEN QIAprep $^®$ Miniprep kit (Crawley, West Sussex) as detailed in section 2.3.2.1. The DNA was linearised using a *HindII* restriction digest and the product was run on a 0.7% agarose gel in order to estimate the concentration of dsDNA in the preparation. As recommended by the manufacturers, a series of sample reactions (5, 10, 20 and 50ng) using various concentrations were set up while keeping the amount of primer constant.

To confirm that the point mutation of a C to a T (proline to leucine) had been successful, DNA sequencing of the mutagenised expression vector was carried out as before (see section 2.5.1). The primer used was the 3' primer JH4b FOR as shown below in Figure 2.5.


```
                          pG1D1              33.H11V_H region
                    ┌──────────────┐   ┌──────────────────┐
33.H11V_H/pG1D1   3' AAC CTA GGT GAG TGG ACT CCT CTG CCA CTG GTC 5'

Primer:
JH4b FOR          5' TTG GAT CCA CTC ACC TGA GGA GAC GGT GAC CAG 3'
```
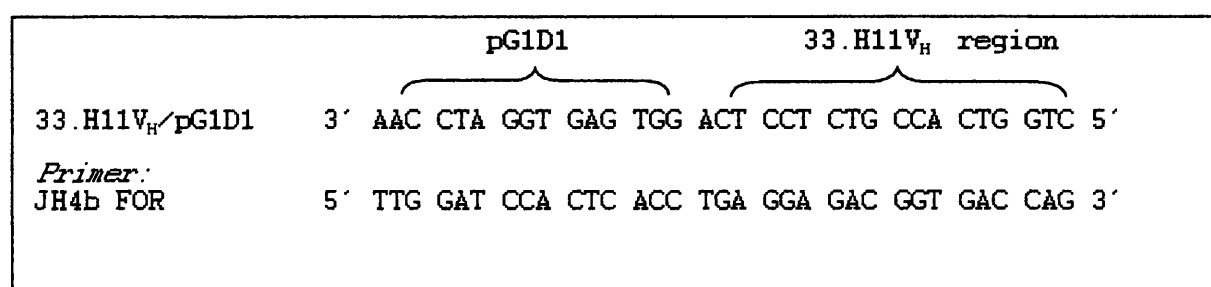
Figure 2.5   **Design of JH4b FOR for DNA sequencing of 33.H11V$_H$**

This primer binds to the last six codons of the 33.H11 V$_H$ region and to the first five codons in pG1D1 3' to the insert.

2.6   Transfer of B3V_H sequence from expression vector pG1D1 to pG1D210.

2.6.1   Background

The original heavy chain expression vector, pG1D1 was used successfully by our research group and by its developers (AERES Biomedical, Mill Hill, London, UK) until the year 2000. At that point AERES Biomedical developed a new vector, pG1D210. Again due to commercial sensitivity, no published reference is available for this vector. This vector contained two potential improvements. The first was the presence of the HCMVi enhancer 5' to the insert, which had previously been shown to enhance expression (Chapman *et al.*, 1991).

Secondly, AERES Biomedical had discovered that the SA site in pG1D1 was not 100% efficient. Using mass spectrometry it was shown that in some but not all proteins derived from COS-7 cells transfected with pG1D1, the intron between the SD site and the SA site was not spliced out and consequently some heavy chains contained an extra 22 amino acid region between V_H and C_H. This extra 22 amino acid region was only detected because this sequence encodes a glycosylation site. (Normally a difference of 22 amino acids would not be detected by mass spectrometry). In the improved vector, pG1D210, the SA and SD sites were removed such that the C_H sequence followed directly after the V_H (see Figure 2.6 for pG1D210 vector map). Although neither our research group nor AERES had noted any functional problems associated with the presence of this intron, we decided that ideally our V_H sequences should be transferred into the new pG1D210 vector to take advantage of these potential improvements. All of the expression experiments in this thesis were carried out with the original pG1D1 vector. However for future reference the whole IgG expression levels and anti-DNA binding activity of B3V_H/pG1D1 and B3V_H/pG1D210 are compared in this thesis, firstly to identify whether the HCMVi enhancer did improve expression and secondly to ensure that the vector used did not affect IgG binding activity.

Figure 2.6   **Vector map of recombinant heavy chain expression vector, B3V$_H$/pG1D210**

pG1D210 contains the human cytomegalovirus (HCMV) promoter to drive transcription of the recombinant immunoglobulin gene, the SV40 origin of replication to give high levels of transient expression in COS-7 cells and the mouse *dhfr* gene coding sequence driven by the SV40 early promoter to act as a dominant selectable marker during stable transformation. The SV40 promoter that drives the *dhfr* is crippled by the presence of a defective SV40 promoter-enhancer sequence so that expression is poor thus allowing for the selection of high expression level clones using comparatively low levels of methotrexate. pG1D210 also contains an ampicillin resistance gene (AmpR) driven by an internal promoter to enable it to be cultured in *E.coli* and a cloned PCR fragment that encodes the human γ1 constant region (C$_\gamma$1). The V$_H$ region sequence of B3 was cloned into expression vector pG1D1 immediately 5' to the C$_\gamma$1 human DNA sequence to produce the constructs, B3V$_H$/pG1D1 and 33.H11V$_H$/pG1D1.
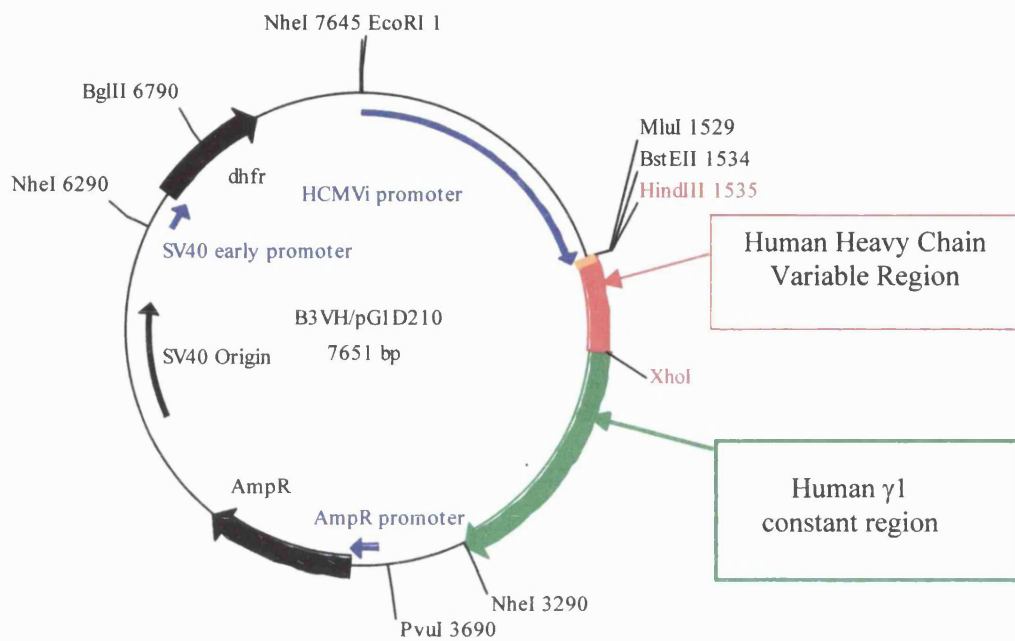
KEY

→	Promoter
➤	Gene
▮	Human heavy chain variable region
➤	Human γ 1 constant region DNA sequence
▬	Immunoglobulin leader sequence
SA	Splice acceptor site
SD	Splice donor site
AmpR	Ampicillin resistance gene
dhfr	Dihydrofolate reductase gene
HCMV	Human cytomegalovirus

Most restriction sites are shown in black. Those written in red indicate that they were used for cloning described in this thesis.

2.6.2 Production of a XhoI site in pG1D1/B3VH by PCR.

The expression vector pG1D210 has a multiple cloning site that contains the restriction sites, *HindIII* and *XhoI*. As there are no SA and SD sites in pG1D210, to transfer a fragment that contained the Ig leader sequence and the B3V$_H$ region sequence into pG1D210, a *XhoI* site was introduced into the end of the B3V$_H$ sequence without changing the amino acid sequence by PCR, as described below. The primers were made and purified by fast polynucleotide liquid chromatography (FPLC) by Genosys, Cambridge, UK. The primers used were:

CIH BACK   5' GAG CTA AGC TTG CCG CCA CCA   3'

IBH FOR   5' CTC ACC GCT CGA GAC GAT GAC CAG 3'

The primer CIHBACK binds to the sequence of pG1D1 5' to the HindIII site (i.e. outside the leader region whilst the primer IBHFOR anneals to the last six codons of B3 V$_H$ insert in pG1D1, as shown in Figure 2.7.

```
B3VH              L   V   T   V   S   S  pG1D1

             5' CTG GTC ACC GTC TCC TCA GCC TCC 3'
XhoI         5'                 C TCG AG          3'
Primer;
IBH FOR      3' GAC CAG TAG CAG AGC TCG CCA CTC 5'
```

Figure 2.7   Primer design to incorporate new *XhoI* site into B3V$_H$.

A *XhoI* site was introduced into the end of the B3V$_H$ sequence without changing the amino acid sequence, by PCR.

The dsDNA template used was the whole plasmid B3V$_H$/pG1D1. The dsDNA was prepared using the QIAGEN QIAprep $^{\circledR}$ Miniprep kit (Crawley, West Sussex) as detailed in section 2.3.2.1. *Taq* polymerase enzyme and buffers (Promega, Southampton, UK) were used as recommended in the manufacturer's instructions. The PCR mixture contained 5 u *Taq* polymerase, 10μg template DNA, 1μl dNTP (a mixture of dATP, dCTP, dGTP and dTTP in which the concentration of each was 25mM), 1μl of 1μM stock forward primer, 1μl of 1μM stock back primer, in a final volume of 100μl

with water (supplemented with the appropriate reaction buffer and $MgCl_2$ supplied by the manufacturer).

A second PCR mixture was set up as a negative control in which the plasmid DNA was replaced by ddH_2O. PCR was carried out in a thermal cycler (Eppendorf, Helena Biosciences, Sunderland, UK) using the following programme: initial denaturation of DNA at 94°C for 5 minutes, then 30 cycles of [94°C for one minute, 42°C for one minute (annealing temperature) and 72°C for one minute] and then a final elongation step at 72°C for five minutes.

The presence of a PCR product of the expected approximate size of 434bp was confirmed by gel electrophoresis (see section 2.3.2.3) and DNA extracted from the agarose as described in section 2.3.2.4.

2.6.3 HindIII/XhoI restriction digest of pG1D210 and new B3V$_H$ PCR fragment.

Both pG1D210 and the new B3V$_H$ PCR fragment were then digested with *HindIII* and *XhoI* (Promega, Southampton, UK) according to the manufacturer's instructions (1u enzyme/μg/hr for 1 hour at 37°C).

The entire volume of each digestion product was then run on a 0.7% agarose gel (see section 2.3.2.3). The linearised heavy chain expression vector pG1D210 7217bp band and the 434bp B3V$_H$ band were excised and extracted from the agarose as described in section 2.3.2.4.

2.6.4 Ligation of B3V$_H$ into pG1D210.

The B3V$_H$ digested PCR fragment was ligated into the pG1D210 vector in a final volume of 20μl, using T4 DNA ligase enzyme and buffers (Promega, Southampton, UK) as recommended by the manufacturer's instructions. The same method was used as described in section 2.3.2.5.

The ligation mixes were transformed into competent *E. coli* cells (see sections 2.3.2.5-2.3.2.7) and the colonies produced screened for the correct sized bands (7217bp and 434bp) by *HindIII/XhoI* restriction digest. To ensure that no mutations had been

introduced during the PCR step, the pG1D210 heavy chain expression vector containing B3V$_H$ region sequence (B3V$_H$/pG1D210) was sequenced using the same method and primers (Hug1 and NeoA) as before (see section 2.5.1).

2.7 Production of Supervectors.

As described in section 2.2, the expression vectors pLN10 and pG1D1 can be used in both a transient expression system (see section 2.9) and a stable expression system (see section 2.11). The heavy chain expression vector pG1D1 contains the dihydrofolate reductase gene (*dhfr*) whilst the light chain expression vector pLN10 contains the neomycin resistance gene (*neo*). Therefore in CHO*dhfr⁻* (selective) growth medium B (MEM α-Medium (α-MEM) without ribonucleosides and deoxyribonucleosides (32571-029, Invitrogen, Paisley, UK) supplemented with 10% (v/v) Ultra low IgG Foetal calf serum (FCS) (16250-078, Invitrogen, Paisley, UK)*, 50 Units/ml penicillin/ 50 µg/ml streptomycin (15140-122, Invitrogen, Paisley, UK) and 0.5mg/ml G418 (Invitrogen, Paisley, UK) only those CHO cells with a functional *dhfr* gene and a functional *neo* gene would survive. Consequently, the surviving cells would also have the ability to produce IgG heavy chains and light chains. However, although this system has been shown to work in the past, there was a risk of excess production of heavy chain compared to light chain since co-transfection with *dhfr* may be comparatively more productive than co-transfection with *neo*. Therefore, the two vectors were combined to produce "supervectors". From the plasmid vector pLN10, an *EcoRI* fragment containing the HCMV promoter, the λ constant region gene and the λ variable region gene (of chosen autoantibody) was transferred into the vector pG1D1/B3V$_H$.

Five supervectors (SV) were produced in which B3 heavy chain was paired with the following light chains: B3 (SVBL), B3a (SVBLX2), B3b (SVBLX4), 33.H11 (SVBN) and UK-4 (SVBM). The same protocol was used to produce all of the supervectors. For clarity the method below depicts the production of SVBL, as an example of the technique used.

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*Throughout the work in this thesis with CHO*dhfr⁻* cells, Ultra low IgG FCS (Invitrogen, Paisley, UK) was used. Although this did not appear to effect the selection of clones (selection of pure foci) possessing the functional *dhfr* gene, I would recommend that in future experiments, dialysed foetal calf serum that has been tested for the absence of ribonucleosides and deoxyribonucleosides is used.

From the recombinant vector B3Vλ/pLN10, a cassette containing the HCMV promoter, the Cλ region sequence and the B3Vλ region sequence was cut out of the vector using an *EcoRI* restriction digest whilst the recombinant vector B3V$_H$/pG1D1 was linearised using an *EcoRI* restriction digest. The restriction digests were performed using Promega (Southampton, UK) enzymes and buffers as recommended in the manufacturer's instructions (1u enzyme/μg/hr for 1 hour at 37°C).

The digested light chain DNA was run on a 0.7% agarose gel as detailed in section 2.3.2.3. The light chain expression cassette (approximately 2260bp) was then excised and extracted from the gel as detailed in section 2.3.2.4.

The linearised pG1D1/B3V$_H$ was treated with Calf Intestinal Alkaline Phosphatase (CIAP) using Promega (Southampton, UK) enzymes and buffers as recommended by the manufacturer's instructions (0.01u enzyme per μg of 6917bp DNA for 30 minutes at 37°C and then 10 minutes at 90°C) to prevent it from re-ligating to itself. CIAP catalyses the dephosphorylation or hydrolysis of 5'-phosphate groups from DNA. Therefore by removing the phosphate groups from both of the 5' termini of the linearised heavy chain vector, the vector is unlikely to religate. This significantly increases the chance of obtaining colonies that contain the heavy chain vector and the insert (light chain sequence) following the ligation and transformation of this DNA into *E.coli*. The 5' overhang dephosphorylated linearised heavy chain vector was purified as before (see section 2.3.2.4).

The light chain cassette was ligated into the linearised pG1D1/B3V$_H$ using T4 DNA ligase enzyme and buffers (Promega, Southampton, UK) according to the manufacturer's instructions, as detailed before in section 2.3.2.5. After the overnight incubation at 4°C, the ligation mixes were stored at -20°C until transformed into competent *E. coli* cells as before (see section 2.3.2.7).

The DNA extracted from the "supervector" colonies was screened using a *HindIII* (Promega, Southampton, UK) restriction digest as recommended by the manufacturer. If the light chain sequence was inserted into the plasmid in the same direction as the B3V$_H$ sequence the bands expected were approximately 6913bp and 2264bp. However if the

light chain sequence was inserted into the plasmid in the opposite direction to $B3V_H$ sequence the bands expected were approximately 6724bp and 2453bp.

This method is outlined in Figure 2.8.

Figure 2.8   **Cloning method used to construct the supervectors by combining the light chain and heavy chain expression vectors**

a)   *EcoRI* restriction sites in recombinant light chain expression vector, pLN10 containing V_λ cloned DNA sequences

b)   *EcoRI-* digested light chain cassette containing HCMV promoter, immunoglobulin leader sequence, light chain variable region DNA sequence and constant region DNA sequence

c)   Ligation of light chain cassette vector into *EcoRI*-linearised heavy chain vector to produce final supervector, containing all components required to produce whole IgG1.

KEY

→   Promoter

➡   Gene

▮   Light chain variable region DNA sequence

⬚   Light (λ) chain constant region DNA sequence

▮   Heavy chain variable region DNA sequence

⬚   Heavy chain (γ1) constant region DNA sequence

▮   Intron between SA and SD not represented in final expression product

▮   Immunoglobulin leader sequence

SA   Splice acceptor site

SD   Splice donor site

AmpR   Ampicillin resistance gene

dhfr   Dihydrofolate reductase gene

HCMV   Human cytomegalovirus

a) Recombinant pLN10 7447 bp

Hpa I 7095
EcoRI 1
Sac I 1133
HinD III 1225
BamHI 1581
SD 1586
SA 1706
EcoRI digest
HCMV promoter
Nru I 6224
BssH II 5766
NeoR
Eag I 5275
Bcl I 5210
Bgl II 5205
Sfi I 4818
NeoR prom
AmpR prom
AmpR
EcoRI 2260
SV40 origin
Xmn I 2659
Sca I 2778
Pvu I 2890

b) HCMV promoter
EcoRI 1
EcoRI 2260

Ligation of *EcoRI* digested
lambda fragment into *EcoRI*
linearised recombinant
pG1D1 / B3 V$_H$

c) SUPERVECTOR 9177 bp

AmpR promoter
EcoRI 2
Pvu I 8556
AmpR
Hind III 1225
Light chain
variable region
HCMV promoter
Light chain
constant region
SV40 origin
EcoRI 2261
Mlu I 2311
Nhe I 6680
SV40 early promoter
HCMV promoter
dhfr
Bgl II 5947
HinD III 3489
Heavy chain
variable region
Hpa I 5235
SD 3926
BamH I 3928
SA 3990
Age I 4097
Heavy chain
constant region

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2.8 Preparation of DNA for transfection into eukaryotic cells.

2.8.1 Large-scale extraction of plasmid DNA from E. coli.

The small-scale extraction of plasmid DNA from *E. coli* as described before in section 2.3.2.3 did not yield sufficient amounts of DNA for use in eukaryotic expression experiments. Therefore, the QIAGEN® Plasmid Maxi Kits (Crawley, West Sussex, UK) were used to produce much larger quantities of plasmid DNA, using the protocol recommended in the manufacturer's instructions.

The DNA was resuspended in Tris-EDTA (TE) buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5). Once resuspended the DNA was stored at -20°C.

2.8.2 Quantification of dsDNA concentration.

dsDNA concentrations were quantified in two ways (as recommended in Sambrook *et al.*, 2001); either by running the linearised DNA samples along side molecular weight markers of known DNA concentration on an agarose gel and comparing the intensity of the bands or by using ultraviolet absorbance spectrophotometry. The amount of ultraviolet light absorbed by a solution of DNA is directly proportional to the amount of DNA in the sample. Absorbance was measured at 260nm, at which wavelength an absorbance (A_{260}) of 1.0 corresponds to 50µg of double-stranded DNA per ml.

Ultraviolet absorbance was also measured at the wavelength 280nm in order to check the purity of the DNA preparation. The ratio of the absorbance at 260nm and 280 nm (A_{260}/A_{280}) of a pure sample of DNA would be 1.8. A ratio of less than 1.8 indicated that the preparation was contaminated with protein.

2.8.3 Ethanol Precipitation of DNA.

In the presence of salt (monovalent cations such as Na^+) and at a temperature of -20°C or less, absolute ethanol efficiently precipitates nucleic acids. Therefore 0.1 volume of 3M Sodium Acetate, pH 5.2 and 2.5 volumes of 100% ethanol were added to the DNA preparation in a 1.5ml microcentrifuge tube. This was placed in a −80°C freezer for one hour to precipitate the DNA.

To collect the precipitate the preparation was firstly spun in a microcentrifuge for 10 minutes at 13000g at 4°C. The supernatant was discarded and the pellet washed with 1ml 70% ice-cold ethanol (i.e. 1ml of 70% ethanol was added to pellet and then vortexed gently). The solution was centrifuged again for 10 minutes in a microcentrifuge at 13000g at 4°C and then air dried on the bench for 10-30 minutes. Care was taken to ensure that no ethanol remained as this could affect subsequent procedures such as ligations, restriction digests or transfections. This protocol was derived from Sambrook *et al.* (2001).

Plasmid DNA intended for transfection into eukaryotic cells was resuspended in sterile autoclaved ddH$_2$O at a concentration of 2µg/µl under sterile conditions in a tissue culture hood. The DNA was stored at -20°C.

2.9 Transient Expression System in COS-7 cells.

The COS-7 cell line was a gift from Mrs Alison Levy, AERES Biomedical, MRC Collaborative Unit, Mill Hill, London and was originally derived from American Type Culture Collection, Ref No. CRL 1651. All protocols used for the culture and transfection of COS-7 cells were obtained from AERES Biomedical.

2.9.1 Defrosting COS-7 cells aliquots stored in liquid nitrogen.

When using COS-7 cells in a transient expression system, efficiency is optimised by transfecting the cells at as low a passage number as possible. Therefore a master cell bank of the COS-7 cell line stored in liquid nitrogen was essential.

COS-7 aliquots in this bank were stored in COS-7 freezing medium (Dulbecco's Modified Eagle Medium (DMEM) [41966-029 Invitrogen, Paisley, UK] supplemented with: 10% (v/v) Foetal calf serum (FCS) [10099-133, Invitrogen, Paisley, UK], 580µg/ml L-glutamine [25030-024, Invitrogen, Paisley, UK], 50 units/ml penicillin/50 µg/ml streptomycin [15140-122, Invitrogen, Paisley, UK] and 10% DMSO (v/v) [D-5879, Sigma, Poole, UK]). Although during freezing DMSO is cryoprotective, when defrosted DMSO is toxic to the cells. Therefore once defrosted at 37°C, the cell aliquot

was immediately added to 10ml fresh, pre-warmed COS-7 growth medium I (Dulbecco's Modified Eagle Medium (DMEM) [41966-029 Invitrogen, Paisley, UK] supplemented with: 10% (v/v) Foetal calf serum (FCS) [10099-133, Invitrogen, Paisley, UK], 580μg/ml L-glutamine [25030-024, Invitrogen, Paisley, UK] and 50 units/ml penicillin/50 μg/ml streptomycin [15140-122, Invitrogen, Paisley, UK]) and spun down at 250g for 10 minutes at room temperature in a Sorvall RTH-750 rotor. The medium was discarded whilst the pellet was resuspended in 10ml COS-7 growth medium I and transferred to a 75cm^2 culture flask containing 15ml medium.

2.9.2 Maintenance of COS-7 cells in culture.

COS-7 cells were grown in (pre-electroporation) COS-7 Growth Medium I in either 80cm^2 or 175cm^2 tissue culture flasks. All work carried out with these cells was performed under sterile conditions in a sterile tissue culture hood. Furthermore, all buffers and solutions used for this work were kept in the tissue culture laboratory and only used for this purpose.

The COS-7 cells were maintained in continuous culture by changing the growth medium every three to four days as follows:

The 80cm^2/175cm^2 tissue culture flask containing the COS-7 cells was removed from the incubator and placed in the hood. A light microscope was used to view the confluency and morphology of the cells. The old medium was removed by aspiration and discarded. The cells were then washed by adding 8ml of sterile Hank's Buffer Solution containing no magnesium and no calcium (HBS) (14175-053, Invitrogen, Paisley, UK) to the side of the flask with no cells adhered to it. The flask was agitated gently so that all the cells were washed with the Hanks' buffer solution. The wash buffer was removed by aspiration and 3ml of filter-sterilised trypsin/versene solution (at a final concentration of 0.25% trypsin [15090-046 Invitrogen, Paisley, UK] (v/v) in Versene (0.2g/L EDTA in PBS) [15040, Invitrogen, Paisley, UK]) was added to the side of the flask with no cells adhered to it. The flask was laid on its side so that all the cells were covered with the Trypsin/Versene solution and incubated in the 37°C incubator for two minutes. At the end of this time, the flask was removed from the incubator and each side of the flask was tapped firmly. A light microscope was used to ensure that at least 90% of the cells had detached from the side of the flask. 9ml of pre-electroporation

COS-7 growth medium I were added to the flask and the contents were mixed gently. The trypsin reaction was not allowed to proceed for more than two minutes since this could damage the cells. Once the cells were detached from the side of the flask the pre-electroporation COS-7 growth medium I containing foetal calf serum was added. The foetal calf serum in the growth medium stopped the action of the trypsin on the cells.

The cell solution was split between three new flasks of the same size as the original, to each of which fresh pre-warmed COS-7 Growth medium I was added to a final volume of either 25ml for a 175cm^2 flask or 20ml for a 80cm^2 flask. The flasks were then placed horizontally (with the caps slightly open) in a 37°C incubator in which the concentration of CO_2 was 5%. This procedure was carried out every three to four days or when the cells were confluent.

2.9.3 Freezing down COS-7 cells for storage in liquid nitrogen.

To create a cell bank the cells were frozen down at as low a passage number as possible and at a concentration of 1×10^6 to 1×10^7 cells/ml when they were growing well preferably in exponential phase.

The cells were trypsinised as detailed above (see section 2.9.1), spun down at 250g for 10 minutes at room temperature in a Sorvall RTH-750 rotor and then resuspended in an appropriate volume of COS-7 pre-electroporation growth medium I. 10% DMSO (v/v) (D-5879, Sigma, Poole, UK) by volume was then added. DMSO is toxic to the cells and once added, the cells were immediately placed at −80°C and then were left overnight. The frozen aliquots were then transferred to liquid nitrogen.

2.9.4 Preparation of COS-7 cells for electroporation

The COS-7 cell line was maintained as detailed in section 2.9.2 and used in a transfection at as low a passage number as possible. The cells were split 24 hours prior to transfection to ensure exponential growth as follows:

24 hours prior to the electroporation, the COS-7 cells were trypsinised (as described in section 2.9.2) and then divided equally between three 175cm^2 flasks, each containing 25ml of fresh, pre-warmed COS-7 growth medium I. The cells were incubated

overnight at 37°C in 5% CO_2. The number of flasks of COS-7 cells that were prepared was dependent on the number of electroporations that were planned (approximately one 175cm^2 flask per electroporation).

The next day whilst still growing exponentially, the COS-7 cells were harvested as follows. The COS-7 cells were trypsinised and pelleted at 250g at room temperature in a RTH-750 Sorvall rotor. The cells were then washed by resuspending the pellets in 20ml of sterile autoclaved 0.15M Phosphate-buffered saline, pH 7.4 (PBS) (Invitrogen, Paisley, UK) and then re-pelleting the cells at 250g for 5 minutes in RTH-750 Sorvall rotor at room temperature. The cells were resuspended in sufficient PBS to produce a cell concentration of 1x10^7 cells/ml. Each flask should have contained approximately 1x10^7 cells although this was confirmed using a Trypan blue viable cell count, as described in section 2.9.5.

2.9.5 Trypan blue viable cell count.

Trypan blue is one of several stains that can be used in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes whilst dead (non-viable) cells do. The following protocol (derived from Sambrook *et al.* (2001) was used:

The cells were re-suspended in Hank's Balanced Salt solution (HBSS). 0.5ml of 0.4% Trypan blue solution (w/v) was transferred to a 5ml bijou tube. 0.3ml of HBSS and 0.2ml of the cells suspension were then added to the bijou, therefore producing a dilution factor of five. The preparation was mixed thoroughly and then allowed to stand for 5 to 15 minutes. (If cells are exposed to Trypan blue for extended periods of time, viable cells as well as non-viable cells may begin to take up the dye).

A haemocytometer was used to count the cells. Each square of the haemocytometer with the cover slip in place represented a total volume of 0.1mm^3 or 10^{-4}cm^3. Since 1cm^3 is equivalent to 1ml, the subsequent cell concentration per ml and the total number of cells were determined as follows:

CELLS per ml = the average count per square x dilution factor x 10^4 (count 10 squares)

TOTAL CELLS = cells per ml x the original volume of fluid from which cell sample was removed

This procedure was repeated in order to ensure accuracy.

2.9.6 Transfection of COS-7 cells with recombinant expression vectors by electroporation.

Recombinant expression vector DNA was removed from storage at –20°C and thawed at room temperature. 5µl of heavy chain and 5µl of λ light chain expression vector DNA (each at 2µg/µl) were added to a sterile autoclaved 1.5ml microcentrifuge tube. Alternatively, 10µl (2µg/µl) of single expression vector containing both the heavy and light chain sequence ("supervector") was added to a sterile autoclaved 1.5ml microcentrifuge tube. A separate tube was used for each supervector or combination of heavy and light chain vectors. 7 x 10^6 washed COS-7 cells (700µl) were then added to each microcentrifuge tube and gently mixed with the DNA by slowly pipetting the contents of the tube up and down three times. The contents of each tube were then carefully pipetted into a Gene Pulser® (0.4cm gap) cuvette. To ensure that no arcing could occur during the electroporation, it was ensured that there was neither liquid on the side of the cuvette nor air bubbles nor clumps of cells in the cuvette.

Using the Bio-Rad Gene Pulser®apparatus a 1900 Volt, 25µFarad capacitance pulse was delivered to each cuvette. A "no DNA" negative control was also carried out by electroporating COS-7 cells in the absence of any DNA.

The transfected COS-7 cells were allowed to recover at room temperature for 10 minutes. The cells from each cuvette were then gently pipetted into a 100mm diameter tissue culture dish containing 8ml of fresh pre-warmed (post-electroporation) COS-7 growth medium II (Dulbecco's Modified Eagle Medium (DMEM) [41966-029 Invitrogen, Paisley, UK] supplemented with: 10% (v/v) Ultra low IgG Foetal calf serum (FCS) [16250-078, Invitrogen, Paisley, UK], 580µg/ml L-glutamine [25030-024, Invitrogen, Paisley, UK] and 50 units/ml penicillin/50 µg/ml streptomycin [15140-122, Invitrogen, Paisley, UK]). The cells were incubated in 5% CO_2 at 37°C for 72 hours.

2.9.7 Treatment of COS-7 cell supernatants with DNaseI (RNase –free).

After 72 hours post-electroporation the supernatants were removed from the cells and transferred to sterile 15ml falcon tubes. The supernatants were spun for 10 minutes at 700g in a Sorvall RTH-750 rotor at room temperature in order to pellet all the cell debris present. Following centrifugation, the COS-7 cell supernatants were immediately transferred to fresh 15ml falcon tubes and treated with RNase-free DNaseI (776 785, Roche, Lewes, East Sussex, UK) as follows:

DNaseI (RNase-free) was added to the COS-7 cell supernatants to produce a final concentration of 7.5u DNaseI per ml of supernatant (i.e. 6μl of 10u/μl DNaseI was added to 8ml supernatant). The tubes were inverted to mix and then incubated at 37°C in a dry incubator for one hour. After the one-hour incubation, the action of the DNaseI was halted by adding EDTA, pH 8.0 to the supernatant to produce a final concentration of 15mM. The function of the EDTA is to chelate the magnesium ions in the solution required by the DNAseI to function. Sodium azide was added to the supernatants (final concentration 0.5mM) to prevent fungal growth and the supernatants were then stored at 4°C until required.

The treatment of the supernatants with DNaseI (RNase-free) was essential as when the cells were electroporated 80% of the cells died, thus releasing their contents (including DNA) into the supernatant. The whole IgG antibody molecules that were produced due to the transfection may then have bound this DNA, forming immune complexes. This would make them undetectable by the ELISA as had been demonstrated previously by Rahman *et al.* (1998). Therefore the DNaseI was used to digest this DNA thus breaking down the immune complexes leaving the antibody molecules free to be detected by ELISA.

2.9.8 Concentration of whole IgG in Supernatants.

Due to the relatively low concentrations of IgG produced by the transient expression system, the IgG levels in the COS-7 supernatants were concentrated prior to ELISA analysis. The supernatants were concentrated by using *Centricon-YM30* centrifugal concentrators (30 000 MW cut-off) (Amicon Bioseparations, Millipore, UK) according to the manufacturer's instructions.

2.10 Analysis of COS-7 supernatant IgG by immunoassays.

2.10.1 Detection of total whole IgG molecules in COS-7 Supernatant by ELISA.

The protocol described here is routinely used in our laboratory to detect the amount of whole IgG present in the supernatants from both hybridoma cell lines and transfected cells. The method was originally derived from AERES Biomedical, Mill Hill, London UK and is also cited by Rahman *et al.* (1998).

A "Sandwich" ELISA (Enzyme-linked Immunosorbent assay) was used to detect the total whole IgG molecule concentration of each supernatant. A 96 well *Maxisorp* ELISA plate was marked vertically into two halves; the test half and the control half. The test halves of the plates were coated with 400ng/ml goat anti-human IgG (Fc fragment specific) (I8885, Sigma, Poole, UK) in Bicarbonate buffer (BIC) (0.5M sodium bicarbonate, 0.05M sodium dihydrogencarbonate, pH 9.6). The control halves of the plates were coated with BIC buffer only. The plates were incubated overnight at 4°C wrapped in clingfilm. The plates were washed three times with 0.15M Phosphate-buffered saline, pH 7.4 (PBS) (Invitrogen, Paisley, UK) supplemented with 0.1% Tween 20 (Sigma, Poole, UK) (PBS / 0.1% Tween 20). 100μl PBS containing 2% bovine serum albumin (BSA) (Sigma, Poole, UK) was then added to the wells to block non-specific binding of the IgG to the plastic. Plates were incubated for 1 hour at 37°C and then washed three times with PBS / 0.1% Tween 20.

Concentrated COS-7 supernatants were serially diluted in Sample/enzyme/conjugate-dilution (SEC-dilution) buffer (100mM Tris-HCl, 100mM, 0.02% Tween 20 and 0.2% Bovine serum albumin, BSA) and added to the wells on the plate so that for each well in the test half there was a well in the control half containing the same supernatant at the same dilution. Purified human IgGλ (I4014, Sigma, Poole, UK) of known concentration (usually 100ng/ml or 25ng/ml) was diluted serially and loaded in the same way to allow the construction of a standard curve relating optical density to IgG concentration. The plates were incubated for one hour at 37°C and then washed three times with PBS / 0.1% Tween 20.

To detect bound antibody, 50μl goat anti-human λ alkaline phosphatase conjugate (A2904, Sigma, Poole, UK) (diluted 2500-fold in SEC dilution buffer) was added to the

wells and incubated for one hour at 37°C. After one hour, the plates were washed three times with PBS / 0.1% Tween 20 and once with BIC buffer. One p nitrophenyl phosphate substrate tablet (104-105, Sigma, Poole, UK) was dissolved in 5ml BIC buffer supplemented with a final concentration of 2mM $MgCl_2$. 50μl of this solution were added to each of the wells on the plate. The plates were then incubated at 37°C to allow a yellow colour to develop in each well. The optical density of the reaction was read after 30 minutes and 60 minutes at 405nm (reference 490nm) using an ELISA plate reader (Labsystems, Cheshire, UK). The final reading was calculated by subtracting the OD value of each control well from the OD value of the same sample in the corresponding test well. This ensures that only conjugate bound directly to IgG in the supernatant that had been captured by the anti-Fc IgG, would contribute to the result.

The binding of the whole IgG molecules in the supernatants to the plate depended on the presence of the Fc region whereas detection by the alkaline phosphatase conjugate depended on the presence of the λ light chain, therefore this method only detected whole IgGλ antibodies.

2.10.2 Detection of anti-dsDNA activity in the COS-7 Supernatants by ELISA. (Protocol A)

Direct ELISAs were used to detect the anti-dsDNA activity of the whole IgG antibody molecules in the supernatant. The protocol described here was routinely used in our laboratory to detect the amount of anti-dsDNA activity of supernatants from both hybridoma cell lines and transfected cells. The method was originally described by Dar *et al.* (1988). A 96 well *Maxisorp* ELISA plate was marked vertically into two halves, the test half and the control half. The test half of the plate was coated with 50μl of 500μg/ml unpurified (crude) calf thymus dsDNA (D1501, Sigma, Poole, UK) dissolved in citrate buffer (0.15M Sodium chloride, 0.015M Sodium citrate, pH 8.0). The control half of the plate was coated with citrate buffer only. The plates were incubated for 2 hours at 37°C and then washed twice with PBS / 0.1% Tween 20. No blocking was required.

The concentrated COS-7 supernatants were serially diluted in SEC dilution buffer to produce concentrations of 15%, 20%, 30%, 40%, 60% and 80%. A sample of human serum or hybridoma supernatant (known to contain anti-dsDNA IgG) was serially diluted and loaded in the same way onto the plate, as a positive control. The plates were incubated for 90 minutes at 37°C and then washed four times with PBS/0.1% Tween 20.

To detect bound antibody, a goat anti-human IgG alkaline phosphatase conjugate (A3150, Sigma, Poole, UK) was diluted to 1/1000 in SEC buffer and 50μl was then added to each well. The plates were incubated for one hour at 37°C and then washed three times with PBS / 0.1% Tween 20 and once with BIC buffer. Bound antibodies were detected using the same substrate and ELISA plate reader as described for the human IgG ELISA.

2.10.3 Detection of anti-ssDNA activity in the COS-7 Supernatants by ELISA.
(Protocol A)

To further investigate whether any of the mutations had resulted in modified antigenic specificity the whole IgG molecules produced were also tested for binding to ssDNA by direct ELISA. The anti-ssDNA ELISA was carried out using the same protocol as used for the anti-dsDNA ELISA. However the plates were coated with 50μl of 1mg/ml ssDNA instead of dsDNA and the positive standard was human plasma or hybridoma supernatant known to contain anti-ssDNA IgG. The ssDNA was produced by boiling a solution of unpurified (crude) dsDNA in ddH$_2$O (Sigma, Poole, UK) in a 100°C water bath for 30 minutes. The protocol described here was routinely used in our laboratory to detect the amount of anti-ssDNA activity of supernatants from both hybridoma cell lines and transfected cells.

2.10.4 Detection of anti-dsDNA activity in the COS-7 Supernatants by ELISA.
(Protocol B)

During the course of my Ph.D., a slightly different anti-dsDNA protocol was adopted in our laboratory. This protocol is a modification of the assay of Katz *et al.* (1994). The main difference between the two protocols is the preparation of the dsDNA used to coat the plate. Previously crude calf thymus DNA (Sigma, Poole, UK) was used, however in

this second protocol the DNA used was purified prior to the ELISA by another member of our group, Dr Arti Sharma.

To prepare the pure DNA, calf thymus DNA (Sigma, Poole, UK) was dissolved in TE buffer (pH 7.5) on a rotator at room temperature overnight. The DNA solution was then subjected to a phenol: chloroform: isoamyl alcohol (25:24:1) extraction and ethanol-precipitated (as before, see section 2.8.3). The dried pellet of DNA was resuspended in TE buffer (pH 7.5) and then sonicated at high frequency waves for one minute. ssDNA was removed by passing the solution through a 0.45μg Millex-HA filter that contained methyl-cellulose esters. In theory this protocol is better than the previous one as the sonication of the DNA produces a more reproducible coating of the plate whilst there is less chance of the binding detected being due to ssDNA rather than dsDNA. Despite this, there were however no differences seen between the results obtained from each protocol.

A 96 well *Maxisorp* ELISA plate was marked vertically into two halves, the test half and the control half. The test half of the plate was coated with 50μl of 10mg/ml purified dsDNA dissolved in PBS, pH 7.4, whilst the control half was coated with PBS, pH 7.4 only. The plates were incubated overnight at 4°C and then washed three times with PBS / 0.1% Tween 20. Non-specific binding to the plate was blocked by adding 150μl of 2% casein in PBS to each well. The plates were then incubated at 37°C for one hour. The plates were not washed. Instead the casein was discarded and the plates were blotted onto tissue.

The concentrated COS-7 supernatants were serially diluted in the PBS/0.1% Tween 20 to produce concentrations of 20%, 30%, 40%, 60%, 80% and 100%. As a positive control for experiments described in chapter three of this thesis, human serum (known to contain anti-dsDNA IgG) was serially diluted from 1/400 to 1/32400 onto the plate. However as a positive control for experiments described in chapters four and five of this thesis, hybridoma supernatant known to contain anti-dsDNA IgG at a known concentration (125ng/ml) was serially diluted onto the plate. The plates were incubated for one hour at 37°C and then washed six times with PBS/0.1% Tween 20. Bound antibodies were detected using the same method as described for the previous human anti-DNA ELISA (see section 2.10.3).

2.10.5 Detection of anti-ssDNA activity in the COS-7 Supernatants by ELISA. (Protocol B)

As for the first protocol, to further investigate whether any of the mutations had resulted in modified antigenic specificity the whole IgG molecules produced were also tested for binding to ssDNA by direct ELISA. The anti-ssDNA ELISA was carried out using the same protocol as used for the anti-dsDNA ELISA (protocol B) although the plates were coated with 50µl of 1mg/ml ssDNA (prepared as in section 2.10.3 but from the purified dsDNA). The protocol described here is routinely used in our laboratory to detect the amount of anti-ssDNA activity of supernatants from both hybridoma cell lines and transfected cells.

2.10.6 Line immunoassay.

In order to screen the binding characteristics of the IgG produced in the transient expression systems further and to identify those antigens requiring further analysis by ELISA, an enzyme immunoassay was carried out using the INNO-LIA® ANA Update kit (Innogenetics, Ghent, Belgium). The kit was originally designed for the detection and identification, in human serum, of autoantibodies against the following nuclear and cytoplasmic antigens: Sm (SmB and SmD), RNP (RNP-70k, RNP-A, RNP-C), Ro52 and Ro60 (SSA), La/SSB, centromere (Cenp-B), Scl-70 (DNA topoisomerase I), Jo-1, ribosomal protein P, and histones.

The kit was used as described in the manufacturer's instructions except that 200µl of the concentrated COS-7 supernatant was added to the sample diluent in each test trough rather than the 10µl of sera as recommended. This was because the concentration of whole IgG in our COS-7 supernatants was expected to be lower than that found in patient serum.

2.10.7 Detection of anti-histone activity in the COS-7 Supernatants by ELISA.

The protocol described here is routinely used in our laboratory to detect the amount of anti-histone activity of supernatants from both hybridoma cell lines and transfected cells (Ehrenstein et al., 1995). Direct ELISAs were used to detect the anti-histone activity of the whole IgG antibody molecules in the supernatant. A 96 well Maxisorp ELISA plate

was marked vertically into two halves, the test half and the control half. The test half of the plate was coated with 10µg/ml of Calf thymus histones (H5505, Sigma, Poole, UK) dissolved in 0.1M glycine/NaOH, 0.1M NaCl, pH 9.0. The control half of the plate was coated with 0.1M glycine/NaOH, 0.1M NaCl, pH 9.0 only. The plates were incubated overnight at 4°C and then washed five times with distilled water.

Non-specific binding to the plate was blocked by adding 150µl of 2% casein in PBS to each well. The plates were then incubated at 37°C for one hour. The plates were not washed. Instead the casein was discarded and the plates were blotted onto tissue.

The concentrated COS-7 supernatants were serially diluted in PBS/0.1% Tween 20 to produce concentrations of 20%, 30%, 40%, 60%, 80% and 100%. Hybridoma supernatant (known to contain anti-histone IgG) was serially diluted from 1/100 to 1/3200 onto the plate, as a positive control. The plates were incubated for one hour at 37°C and then washed three times with PBS/0.1% Tween 20. Bound antibodies were detected using the same method as described for the human anti-DNA ELISA.

2.10.8 Treatment of supernatants with ethidium bromide.

To investigate whether the antibodies were binding directly to histones or whether the positive results were due to the presence of bound DNA, a sample of supernatants were treated with ethidium bromide prior to the anti-histone ELISA and line immunoassay. A range of concentrations between 10-400 µg/ml ethidium bromide was used as described by previous authors (Lai et al., 1992, Budhram-Mahadeo et al., 1998). Ethidium bromide is known to intercalate with the bases of DNA and so it was postulated that its binding to the DNA would render the IgG unable to bind the DNA.

2.11 Stable expression system in Chinese Hamster Ovary (CHO) cells.

2.11.1 CHOdhfr⁻ cell line

The CHOdhfr⁻ mutant line used was the DXB11 line, which contains a single point mutated allele, the other dhfr allele having been deleted and therefore lacks a functional dihydrofolate reductase (dhfr) gene. The enzyme dihydrofolate reductase (DHFR) is responsible for the intracellular reduction of dihydrofolic acid to tetrahydrofolic acid,

which is an important co-factor in the synthesis of nucleic acid precursors. To survive, the CHO*dhfr⁻* cell line required exogenous adenine, glycine, proline and thymidine for survival (Urlaub *et al.*, 1980). All of these nutritional requirements were provided via non-selective medium.

However if these CHO *dhfr⁻* cells were successfully transfected with the supervectors (see section 2.10) which contain a functional *dhfr* gene (see Figure 2.8), the cells were transformed to a *dhfr⁺* phenotype. This allowed them to grow in⁻ selective medium that was depleted of ribonucleosides and deoxyribonucleosides. (The selective medium still contained proline though as all CHO cells are proline-dependent even in the presence of *dhfr* gene). Consequently, CHO *dhfr⁻* cells were used to provide an expression system in which the genes required to produce IgG could be co-transfected with the *dhfr* gene.

Methotrexate is a competitive inhibitor of the intracellular activity of the DHFR enzyme. Progressive selection of cells that are resistant to increasing concentrations of methotrexate leads to amplification of the *dhfr* gene, with concomitant amplification of IgG-encoding sequences that flank the *dhfr* sequences (Schimke, 1984). In those resistant cells where amplification has occurred, there is not only sufficient free DHFR enzyme to generate intracellular tetrahydrofolate required for cell biosynthesis but also these resulting cell lines express high levels of immunoglobulin.

All protocols used for the culture and transfection of CHO*dhfr⁻* cells were obtained from AERES Biomedical, Mill Hill, UK.

2.11.2 Maintenance of CHO cells in culture.

The CHO*dhfr⁻* cell line was grown in CHO*dhfr⁻* (non-selective) medium A (MEM α-Medium (α-MEM) with ribonucleosides and deoxyribonucleosides (32571-028, Invitrogen,Paisley, UK) supplemented with 10% (v/v) FCS (10099-133, Invitrogen, Paisley, UK) and 50 Units/ml penicillin/ 50 µg/ml streptomycin (15140-122, Invitrogen, Paisley, UK)) in a 175cm² flask. All work carried out with these cells was performed under sterile conditions in a sterile tissue culture hood. Furthermore, all buffers and solutions used for this work were kept in the tissue culture laboratory and only used for this purpose.

The CHO cells were maintained in continuous culture by changing the growth medium every three to four days using a similar trypsin method as that used for the COS-7 cells (see section 2.9.2). The only difference was that once the trypsin/versene was added to the flask, the cells were incubated at room temperature for two minutes and not at 37°C.

2.11.3 Preparation of CHO cells for electroporation

The CHO cell line was maintained as described in section 2.11.2 and transfected at as low a passage number as possible. Therefore a master cell bank of the CHO cell line stored in liquid nitrogen was essential. CHO cells were defrosted and frozen down using identical protocols to those used for COS-7 cells (see section 2.9.1 and 2.9.3)

The cells were split 24 hours prior to transfection to ensure exponential growth as follows; 24 hours prior to the electroporation, flasks containing confluent CHO cells were trypsinised (see section 2.11.1) and then divided equally between three 175cm^2 flasks, each containing 25ml of fresh, pre-warmed CHO*dhfr*⁻ (non-selective) growth medium A. The cells were incubated overnight at 37°C in 5% CO_2. The number of flasks of CHO cells that were prepared was dependent on the number of electroporations that were planned (approximately one flask per electroporation).

Using the same protocol as for the COS-7 cells (see sections 2.9.4 and 2.9.5), the next day whilst still growing exponentially, the CHO cells were harvested and resuspended in sufficient PBS to produce a cell concentration of 1×10^7 cells/ml.

2.11.4 Transfection of CHO*dhfr*⁻ cells by electroporation.

The CHO*dhfr*⁻ cells were electroporated (using the same method as that used for the COS-7 cells, see section 2.9.6) with 10µl (2µg/µl) of the expression vector containing both the heavy and light chain sequence together ("supervector"). A "no DNA" negative control was also carried out by electroporating CHO*dhfr*⁻ cells in the absence of any DNA. The transfected CHO*dhfr*⁻ cells from each cuvette were pipetted into a 100mm diameter tissue culture dish containing 8ml of pre-warmed CHO*dhfr*⁻ (non-selective) growth medium A and incubated in 5% CO_2 at 37°C overnight.

2.11.5 Firefly Luciferase reporter assay.

To ensure that each set of stable expression electroporations had been successful, an aliquot of 7×10^6 CHO$dhfr^-$ cells was also electroporated (under the same conditions as described in section 2.11.4) with 10μg vector containing the firefly luciferase gene (pGL3-control vector, Promega, Southampton, UK).

Following electroporation the transfected cells were treated as described for the rest of the electroporated CHO$dhfr^-$ cells except 72 hours post-electroporation the cells were harvested. The cells were lysed and then assayed for firefly luciferase activity in a luminometer using Promega (Southampton, UK) Firefly luciferase reporter assay buffers and reagents as recommended by the manufacturer (100μl diluted firefly luciferase reagent was added to 20μl [from total of 50μl] cell lysate, incubated for 15 seconds and the reaction read for 30 seconds in a luminometer [TD20/20, Turner Designs Inc., California, USA]).

2.11.6 Selection of transfected CHO_dhfr_ cells following electroporation.

Following overnight growth of the transfected CHO$dhfr^-$ cells, the cells were trypsinized and pelleted in a bench top centrifuge as before (section 2.11.2). The cells were resuspended in 100ml of pre-warmed CHO$dhfr^-$ (selective) growth medium B. The cells were divided equally between ten 100mm-diameter tissue culture dishes and incubated in 5% CO_2 at 37°C for 10-14 days. The CHO$dhfr^-$ (selective) growth medium B was changed every 3-4 days. After 10-14 days, all the cells on the "no DNA" negative control dish were dead and foci of transfected cells were clearly visible on all the test culture dishes. A focus can be seen by the naked eye without the aid of a microscope.

To "pick" the foci, 1mm squares of sterile autoclaved 1MM filter paper were immersed in 0.25% Trypsin/Versene solution. Meanwhile, the CHO$dhfr^-$ (selective) growth medium B was decanted from the culture dish and the cells washed with 5ml of PBS. The PBS was removed from the dish and then using sterile forceps, the squares of 1MM Whatman filter paper (saturated in 0.25% Trypsin/Versene solution) were carefully placed onto individual foci of cells. The forceps were washed in PBS between the picking of each individual focus of cells to prevent cross-contamination.

The squares were left on the foci for ten seconds before transferring them into individual wells of a 24-well tissue culture plate containing 1ml of pre-warmed CHO*dhfr* (selective) growth medium B. Approximately 48 foci were picked for each transfection. A different pair of forceps was used for each cell line that had been transfected with a different expression vector(s) again to prevent any cross-contamination.

The "picked" cells were allowed to grow in CHO*dhfr* (selective) growth medium B until almost confluent (usually after 7-14 days), whilst remembering to change the medium every 7 days or as required. There was great variation in the cell growth rates seen with individual clones of cells and this was accommodated in this and the following steps.

Once almost confluent, the medium from the individual wells was tested for antibody production using the Whole IgG ELISA (see section 2.10.1). Those clones producing the highest levels of antibody were selected for expansion in CHO*dhfr* (selective) growth medium B until the cells were growing in 175cm^2 tissue culture flasks. In order to identify those clones worthy of further analysis and development, the antibody production rates of individual clones were then determined as described in section 2.11.7.

2.11.7 Assay of Antibody Production in transfected CHOdhfr cells.

Following the growth of selected CHO*dhfr* cells to just below confluent level in CHO*dhfr* (selective) growth medium B, the cells were trypsinised and spun down in a bench top centrifuge (250g for 5 minutes at room temperature). The cells were then resuspended in 20ml of pre-warmed CHO*dhfr* (selective) growth medium B and a viable cell count was carried out (see section 2.9.5). The cells were pelleted again as before and resuspended in sufficient pre-warmed CHO*dhfr* (selective) growth medium B to produce a viable cell count of 1 x 10^6 cells/ml. 1ml (i.e. 1 x 10^6 cells) was then added to a 100mm diameter tissue culture dish containing a further 9ml of pre-warmed CHO*dhfr* (selective) growth medium B. The cells were grown to near confluency by incubating the cells for 3 days in 5% CO$_2$ at 37°C. After three days, the supernatant of these cells was decanted and the concentration of intact whole IgG antibody assayed

using an ELISA, as in section 2.10.1. The cells from each 100mm diameter tissue culture dish were trypsinised and counted using a Trypan blue (see section 2.9.5).

From the new viable cell count and the antibody concentration in the decanted supernatants, it was then possible to calculate the level of antibody production in $ng/10^6$ cells/day. A cell bank was created of those cell lines producing the highest amounts of whole IgG and further selection was carried out using methotrexate amplification.

2.11.8 Amplification of transfected CHO*dhfr⁻* cells following electroporation and selection.

Following the growth of the selected CHO*dhfr⁻* cells in CHO*dhfr⁻* (selective) growth medium B in a $175cm^2$ flask, the cell lines producing the highest levels of IgG were trypsinised and spun down at 250g for two minutes at room temperature in a RTH-750 Sorvall rotor. The cells were resuspended in 20ml of CHO*dhfr⁻* (selective) growth medium B to produce a final viable cell count of 1 x 10^6 cells/ml. 0.5ml (i.e. 5 x 10^5 cells) of this solution was diluted in a further 49.5ml of pre-warmed 1 x 10^{-9}M amplification medium (i.e. CHO*dhfr⁻* (selective) growth medium B supplemented with 1 x 10^{-9}M methotrexate [Amethopterin A6770, Sigma, Poole, UK]). The resulting culture was then divided equally between five 100mm-diameter tissue culture dishes, (i.e. 1 x 10^5 cells/culture dish). This dilution was required in order to produce pure foci that could be easily identified from one another on the plate.

In addition, 0.5ml aliquots of the above cells were also plated out in amplification medium supplemented with 1 x 10^{-8}M methotrexate. This second higher concentration of methotrexate was used in case the initial 1 x 10^{-9}M concentration was too low for the cell line to produce single discrete foci.

The cells were incubated at 37°C in 5% CO_2 for 10-14 days. The amplification medium (supplemented with the appropriate concentration of methotrexate) was changed every 3-4 days. After 10-14 days, foci of transfected cells were easily visible in most, but not all of the culture dishes. If all the dishes, at a particular methotrexate concentration failed to produce any discrete visible foci, the dishes were incubated for a further 7 days to allow any slow growing foci to appear and were then discarded.

Approximately 48 foci were picked for each transfection using the same method as before (see section 2.11.5) and were then transferred into individual wells of a 24-well tissue culture plate containing 1ml of pre-warmed CHO*dhfr⁻* amplification medium. The "picked" cells were allowed to grow in the amplification medium until almost confluent (usually after 7-14 days), whilst the medium was changed every 7 days or as required. Once almost confluent, the medium from the individual wells was tested for antibody production as before, using the Whole IgG ELISA (see section 2.10.1).

Those clones producing the highest levels of antibody were selected for expansion in CHO*dhfr⁻* (selective) growth medium B until the cells were growing in 175cm^2 tissue culture flasks. The antibody production rates of individual clones (see section 2.11.6) were then determined in order to identify those clones worthy of further analysis and development.

A cell bank was created of the selected amplified cell lines and a second round of methotrexate amplification using a higher concentration of methotrexate was considered. Normally the concentration of methotrexate used in a second round of amplification should be no greater than 10-fold the concentration of methotrexate that was used to derive the amplified cells in the first round. When creating a cell bank, selective pressure was maintained when freezing cells i.e. concentration of methotrexate was kept the same on freezing as that used in culture.

2.11.9 Production of CHOdhfr⁻ control line-containing pG1D210 only (no V$_\lambda$ region sequence).

The ultimate aim of producing cell lines that would stably produce whole IgG was to use them to produce IgG not only for functional tests *in vitro* but also in tests to determine the pathogenicity of this IgG *in vivo*. If the pathogenic effects of these cell lines were to be investigated *in vivo*, a negative control cell line that had undergone the same procedures and stresses as the IgG-producing cell lines but that would not produce IgG would be required. This was achieved by transfecting the CHO*dhfr⁻* cells with empty (i.e. no V$_H$ or V$_\lambda$ encoding regions) expression vector pG1D210. Since neither V$_H$ nor V$_\lambda$ sequences were present in the vector then no IgG could be made. This

control cell line was treated exactly as the IgG producing cell lines. The clones were treated (i.e. selected and amplified with methotrexate) exactly the same as the test IgG-producing cell lines.

Non-transfected CHO*dhfr*⁻ cells were potentially insufficient as a control for these *in vivo* experiments for two reasons. Firstly it could be argued that the presence of the expression plasmid DNA inside the cells, regardless of the IgG produced, could possibly be toxic and cause pathogenic effects to the mice. Secondly, the non-transfected cells CHO*dhfr*⁻ do not contain a functional DHFR gene as they do not contain the expression vector containing the *dhfr* gene as do the test cell lines. Therefore this cell line may not grow as well *in vivo* due to the fact that the concentrations and availability of the deoxyribonucleosides and ribonucleosides required for growth might not be sufficient and these CHO*dhfr*⁻ cells would not be able to assimilate these essential nutrients.

CHAPTER THREE

The role of sequence features in B3V$_\lambda$ in the binding of B3 to DNA.

CHAPTER THREE.

THE ROLE OF SEQUENCE FEATURES IN B3V$_\lambda$ IN THE BINDING OF B3 TO DNA.

3.1 Introduction and aims of this chapter

The experiments described in this chapter investigated sequence-related DNA-binding properties of a human anti-DNA antibody that is considered representative of those antibodies most closely related to disease pathogenesis in patients with SLE. The antibody selected was the human monoclonal anti-DNA antibody, B3. B3 has an IgG1 isotype, a high affinity for dsDNA, its V$_H$ and V$_L$ are encoded by the two most commonly rearranged human V$_\lambda$ and V$_H$ genes, V$_\lambda$2a2 and V$_H$3-23, it is known to be pathogenic *in vivo* in SCID mice and it was derived from a patient with active SLE (Ehrenstein *et al.*, 1994). The properties of B3 and the clinical and serological features of the SLE patient from whom the mAb was derived are described in more detail in section 2.1.

Previously our group has used a COS-7 transient expression system to express the V$_H$ and V$_L$ of the human monoclonal anti-DNA antibody, B3 as whole IgG1 molecules (Rahman *et al.*, 1998). The principal aim of the experiments described in this chapter was to use this same COS-7 transient expression system to produce a variety of IgG1 molecules, each with the same B3 heavy chain but paired with a different light chain in order to investigate the role of sequence motifs in the light chain of B3 in determining its ability to bind DNA.

3.2 Properties of autoantibody V$_\lambda$ to be expressed in the eukaryotic transient expression system

The transient expression system was used to produce five different IgG1 molecules, each containing the same B3 heavy chain combined with a different light chain. The light chain sequences were similar in that they were derived from the same germline λ gene 2a2, however they contained different patterns of somatic mutations. The combinations tested were as follows: B3V$_H$/B3V$_\lambda$ (wild type), B3V$_H$/33.H11V$_\lambda$, B3V$_H$/UK-4V$_\lambda$, B3V$_H$/B3V$_\lambda$a and B3V$_H$/B3V$_\lambda$b.

3.2.1 Sequence analysis of B3V$_\lambda$, 33.H11V$_\lambda$ and UK-4V$_\lambda$

33.H11 is an anti-DNA mAb (Winkler *et al.*, 1992) and UK-4 is an anti-phospholipid mAb (Menon *et al.*, 1997). The properties of 33.H11 and UK-4 and the clinical and serological features of the SLE patients from whom the mAbs were derived are described in detail in section 2.1. Similarly to B3V$_\lambda$ both 33.H11V$_\lambda$ and UK-4V$_\lambda$ are derived from the lambda germline gene 2a2, so all three antibodies have very similar V$_\lambda$ sequences, differing only at sites of somatic mutation. The amino acid sequences of 2a2, B3V$_\lambda$, 33.H11V$_\lambda$ and UK-4V$_\lambda$ are shown aligned in Figure 3.1.

Both B3 and UK-4 V$_\lambda$ have many somatic mutations and have been shown to contain clusters of replacement mutations in the CDRs, which is consistent with antigen-driven selection. The probabilities that the observed patterns of mutations in these light chains could have developed by chance alone in the absence of antigen drive, was calculated using the method of Lossos *et al.* (2000), as described in section 1.3.3. For B3V$_\lambda$, the p values for CDRs (P_{CDR}) and FRs (P_{FR}) respectively, are 0.023 and 0.0074. For UK-4V$_\lambda$, the p values for CDRs (P_{CDR}) and FRs (P_{FR}) respectively, are 0.016 and 0.035. All P values are less than 0.05. Thus, the patterns of mutation in both CDRs and FRs of these light chains strongly support the idea that they developed due to antigen-driven selection.

33.H11V$_\lambda$ has fewer mutations. The original published sequence of 33.H11V$_\lambda$ (Winkler *et al.*, 1992) shows three replacement mutations all in the CDRs. This could suggest antigen-driven selection although the small number of mutations makes this difficult to conclude. It is important to note that the 33.H11V$_\lambda$ sequence expressed here has two further mutations that differ to the original published sequence. The mutations (proline to leucine at position 44 and asparagine to threonine at position 53) arose during PCR manipulations in our laboratory. As the aim of the experiments described in this chapter was to investigate the effect of pairing various 2a2-derived V$_\lambda$ with B3V$_H$ on binding affinity, the fact that the expressed 33.H11V$_\lambda$ differed from the original was not considered important here.

Sequence analysis of both human and murine monoclonal anti-DNA antibodies has previously shown that antigen-driven accumulation of somatic mutations leads to the

accumulation of R, N and K amino acid residues in the CDRs and consequently may increase the affinity of these antibodies for negatively-charged antigens such as dsDNA (Radic *et al.*, 1994; Rahman *et al.*, 2002). B3V_λ contains two adjacent arginine residues in CDR1 both produced by somatic mutations. 33.H11V_λ contains two arginine residues in CDR3, neither of which is germline encoded. One is the result of a somatic mutation whilst the other is formed by the junction between 2a2 and $J_\lambda 2$. UK-4V_λ has a single somatic mutation to arginine in CDR3.

```
        FR1                              CDR1                FR2                    CDR2
        2       10         20          24  27             34  35   40                   50
        |       |          |           |   |abc           |   |    |                    |
2a2     SALTQPASVSG.SPGQSITISC TGTSSDVGGYNYVS       WYQQHPGKAPKLMIY    EVSNRPS

B3V_λ   -----------.----------  ---RR------F--        ---------------    ---H---

B3V_λa  -----------.----------  ---R-------F--        ---------------    ---H---

B3V_λb  -----------.----------  ---R----S--F--        ---------------    ---H---

33.H11V_λ-----------.---------- ------I-------        --------L-----     D--T---

UK-4V_λ -----------.---------- ---SN---S-----        ---L---E----L--    DAIK---
                                              ←————————→              ←—————————→
                                              CDR-L1                    CDR-L2
```

```
        FR3                           CDR3
        60          70         80     89     95          100
        |           |          |      |      |a    J_λ2  |
2a2     GVSNRFSGSKSGNTASLTISGLQAEDEADYYC SSYTSSST   VVFGGGTKLTVLG

B3V_λ   ---T---A----S--------------------  ---S-TTR    ------------

B3V_λa  ---T---A----S--------------------  ---S-TTR    ------------

BLV_λb  ---T---A----S--------------------  ---S-TTR    ------------

33.H11V_λ--------------------------------  ---R----    R-----------

UK-4V_λ ---------E----------------G----    ----NR..    -F----------
                                           ←————————→
                                           CDR-L3
```

Figure 3.1   Amino acid sequences of expressed V$_\lambda$ regions compared to their closest germline λ gene, 2a2

The amino acid sequences of B3V$_\lambda$ (wild type), B3V$_\lambda$a, B3V$_\lambda$b, 33.H11V$_\lambda$ and UK-4V$_\lambda$ regions are numbered according to Wu and Kabat (1970).

Amino acids are indicated according to their one letter code as listed in Appendix A. Dots have been inserted to facilitate the alignment. A dash indicates sequence identity with that of 2a2. Antigen contact sites as defined by MacCallum *et al.* (1996) are shown by red arrows.

FR = Framework region

CDR = Complementarity-determining region

3.2.2 Production of B3V$_\lambda$ variants for expression

As described in section 1.5.2 our group has published a computer-generated model of the B3/dsDNA complex (see Figure 1.3) (Kalsi *et al.*, 1996). The model suggests that binding is stabilised by the interaction of dsDNA with three arginine residues on the periphery of the binding site. One of these arginine residues is at position 27a (R27a) in B3V$_\lambda$ CDR1 and is the product of a somatic mutation of the λ germline gene, 2a2. Consequently in this chapter the transient expression system was also used to determine whether R27a is important in enabling B3 to bind DNA, as predicted by the model.

To study the contribution of R27a to the binding ability of B3, variants of B3V$_\lambda$ (B3V$_\lambda$a and B3V$_\lambda$b) that differ from the wild type by either one or two amino acids were used. In B3V$_\lambda$a the only alteration is a point mutation reverting the arginine at position 27a (R27a) to a serine (S) found in the germline λ gene 2a2. B3V$_\lambda$b contains an additional second mutation that was produced presumably by a PCR error. This mutation converts a germline gene-encoded glycine at position 29 (G29) to a serine (S). As this G to S alteration occurs as a somatic mutation in UK-4V$_\lambda$, it was decided that it would be interesting to test for any additional effects of this second mutation on binding to DNA. These point mutations are hereafter referred to as R27aS and G29S respectively.

Figure 3.2 shows the sites of site-directed mutagenesis in the V$_\lambda$ CDR1 of the wild type B3V$_\lambda$ and its variants B3V$_\lambda$a and B3V$_\lambda$b. However the full amino acid sequences of B3V$_\lambda$a and B3V$_\lambda$b are shown in Figure 3.1 aligned to 2a2, B3V$_\lambda$, 33.H11V$_\lambda$ and UK-4V$_\lambda$.The two B3 light chain variants (B3V$_\lambda$a and B3V$_\lambda$b) were generated by Dr Anisur Rahman using site-directed mutagenesis. The method used was the modified megaprimer method described by Seraphin and Kandels-Lewis (1996) as outlined in (Rahman *et al.*, 2001). This method involved two successive PCR amplifications. The product of the second PCR was approximately 600bp long. This was then ligated into the plasmid, *pGEM®-T Easy* (Promega, Southampton, UK) and the presence of the desired mutation(s) confirmed by sequencing by Dr Anisur Rahman.

The mutagenised B3V$_\lambda$ sequences were ligated into pLN10 using a *Hind III/BamHI* or *SacI/BamHI* restriction digest as described in section 2.2. Successful ligations were confirmed by digesting B3V$_\lambda$a/pLN10 with *HindIII/BamHI* (producing bands at 7091

bp and 355 bp) and B3V$_\lambda$b/pLN10 with *SacI/BamHI* (producing bands at 7000 bp and 445 bp) as shown in Figure 3.3.

```
                   CDR1
                   24      27a      29           34
                   |        |        |            |
2a2 λ germline gene    TGTSSDVGGYNYVS

B3Vλ (wild type)       TGTRRDVGGYNFVS

B3Vλa                  TGTRSDVGGYNFVS

B3Vλb                  TGTRSDVGSYNFVS
```

Figure 3.2   **Sites of site-directed mutagenesis in the CDR1 of the wild type B3V$_\lambda$ and its variants B3V$_\lambda$a and B3V$_\lambda$b.**

The amino acid sequences of the L-CDR1 of the wild type B3V$_\lambda$ and its variants B3V$_\lambda$a and B3V$_\lambda$b are shown aligned with the germline λ gene, 2a2 and are numbered according to Wu and Kabat (1970).

Amino acids are indicated according to their one letter code as listed in Appendix A. The amino acid changes produced by site-directed mutagenesis are shown in red. B3V$_\lambda$a differs from B3V$_\lambda$ (wild type) by only one point mutation which reverts the arginine at position 27a (R27a) to a serine (S) as found in the germline λ gene 2a2. B3V$_\lambda$b contains an additional second mutation that converts a germline gene-encoded glycine at position 29 (G29) to a serine (S).

	A	B	C	D	
7126 bp →					← 7091 bp
					← 7000 bp
1636 bp →					
506, 517 bp →					← 445 bp
396 bp →					← 355 bp
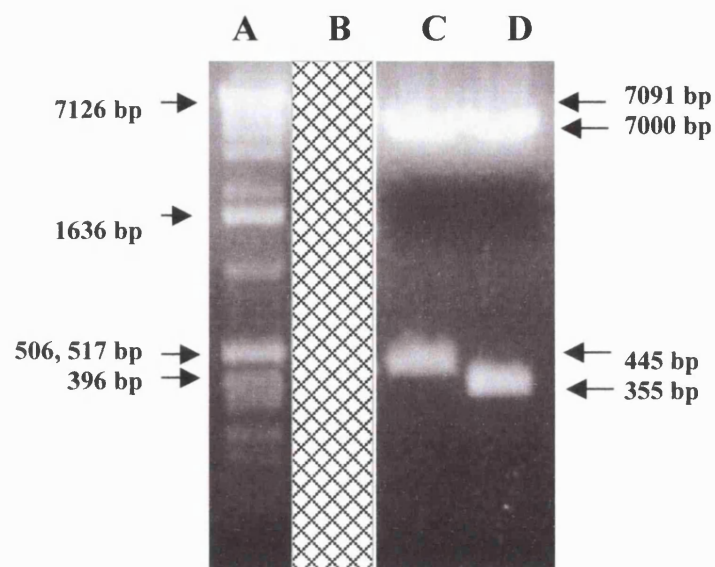
Figure 3.3   **Restriction digest of recombinant vectors containing B3V$_\lambda$ variants on 0.7% agarose gel.**

Lane A: 1kb DNA molecular weight marker (Gibco)

Lane B   No DNA

Lane B: *SacI/BamHI* restriction digest of B3V$_\lambda$b

Lane C: *Hind III/BamHI* restriction digest of B3V$_\lambda$a

3.3 Expression of whole IgG1 molecules in COS-7 cells

The following heavy/light chain combinations were expressed in COS-7 cells; B3V$_H$/B3V$_\lambda$, B3V$_H$/B3V$_\lambda$a, B3V$_H$/B3V$_\lambda$b, B3V$_H$/33.H11V$_\lambda$ and B3V$_H$/UK-4V$_\lambda$. Three expression experiments were carried out for each combination.

The concentration of IgG1 in each COS-7 cell supernatant was determined from the optical density (*OD*) detected in the whole IgG1 ELISA. This was done by comparison with the standard curve of *OD* against concentration derived from the positive control sample of known concentration. This procedure was carried out for every COS-7 cell supernatant of every electroporation. A representative standard curve is shown in Figure 3.4. For each combination, similar yields of antibody were produced in each of the three expression experiments. The mean yields obtained are shown in Table 3.1.

In each experiment, the negative control sample in which COS-7 cells were electroporated without any plasmid DNA contained no detectable IgG1. The yields of IgG1 produced by heavy/light chain combination B3V$_H$/UK-4V$_\lambda$ were consistently much higher than that produced by the other combinations. The reason for this is not clear although variability of yield from different constructs has been previously observed in this expression system (Kettleborough *et al.*, 1991; Rahman *et al.*, 1998) as well as in other expression systems (Mockridge *et al.*, 1996).
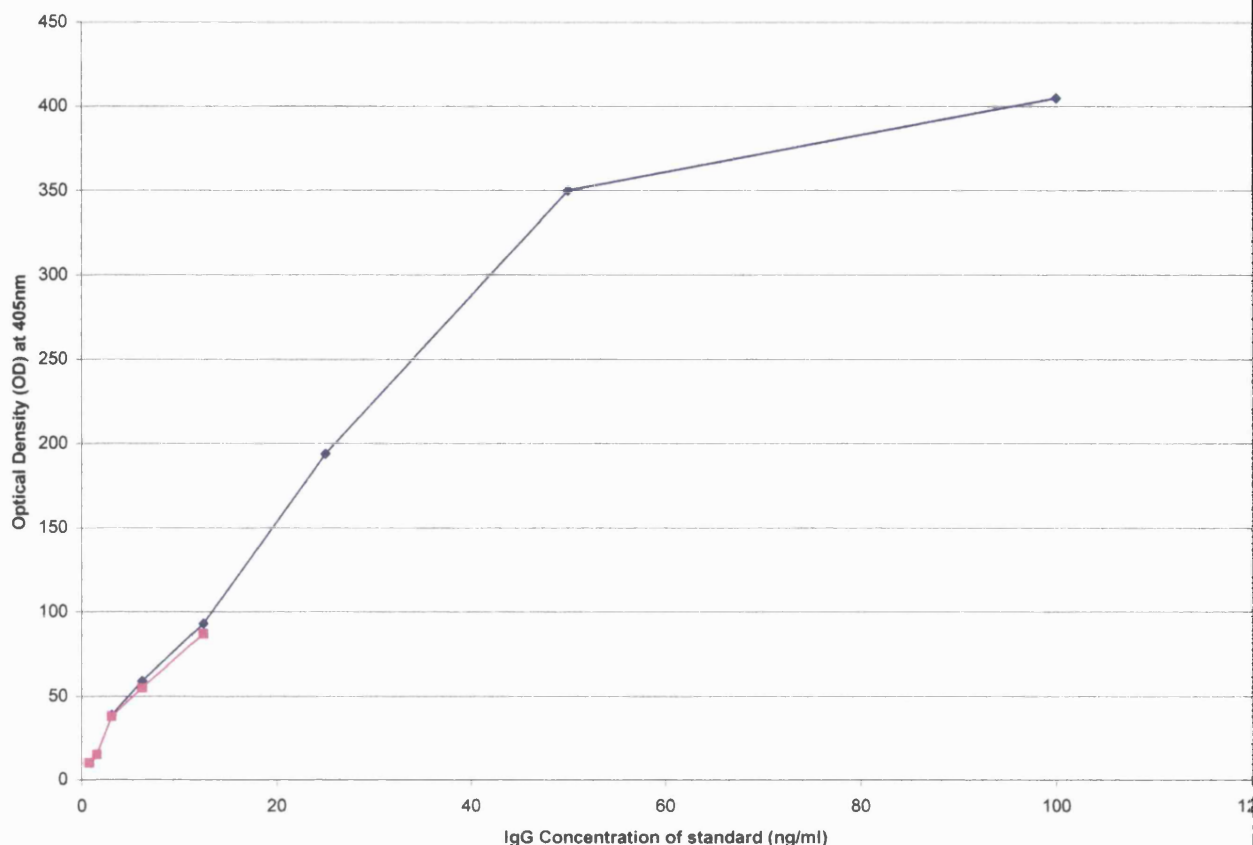
Figure 3.4   **A representative standard curve of optical density (at 405nm) against concentration of whole IgG (ng/ml) of positive control of known IgG concentration.**

The concentration of IgG1 in each COS-7 cell supernatant was determined from the optical density (*OD*) detected in the whole IgG1 ELISA. This was done by comparison with the standard curve of *OD* against concentration derived from the positive control sample of known IgG concentration that was run tested alongside the test supernatants on every plate. This procedure was carried out for every COS-7 cell supernatant of every electroporation. The same standard was run on each plate at two different starting concentrations, one sample was added at 100ng/ml whilst the other was added at 25ng/ml. When loaded onto the ELISA plate, the standards (at their starting concentrations) as well as the test supernatants were serially diluted down the plate. Therefore *OD* readings were obtained at a range of dilutions All of these OD readings were read against the standard curve to calculate the concentration for each supernatant at each dilution and an average total IgG calculated.

Combination		Mean IgG concentration (ng/ml) ± SD
Heavy chain	Light chain	
B3	B3 (wild type)	10 ± 2.5
B3	B3a	34 ± 3.7
B3	B3b	58 ± 5.2
B3	33.H11	16 ± 3.0
B3	UK-4	300 ± 25

Table 3.1   Mean whole IgG concentration of the COS-7 cell supernatants expressing each heavy/light chain combination.

For each combination, similar yields of antibody were produced in each of the three expression experiments. The right hand column shows the mean and standard deviation of the three experiments.

3.4 Anti-DNA activity of expressed whole IgG1 heavy/light chain combinations

Binding of the five heavy / light chain combination IgG1 to dsDNA is shown in Figure 3.5 and anti-ssDNA binding is shown in Figure 3.6. The electroporations were carried out and tested by ELISA for anti-dsDNA binding on three separate occasions. In each case, similar results were found in all three expression experiments and the figures show results of a single representative experiment. However the standard deviations (SD) between the optical density (*OD*) readings of the three experiments were calculated for all the points on each curve (see legend of Figures 3.5 and 3.6).

In both Figures 3.5 and 3.6, the negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was patient serum known to contain anti-DNA IgG although the exact anti-dsDNA IgG concentration was unknown and therefore could not be included in Figures 3.5 and 3.6. The same aliquot of patient serum was used for all the ELISAs and the standard deviation of the positive control between the *OD* readings of the three experiments was always less than 0.04 in both the anti-dsDNA and anti-ssDNA ELISAs.

The strongest binding was seen with the B3V$_H$/B3V$_\lambda$ (wild-type) chain combination. The reversion of the arginine residue to a serine residue at position 27a (R27aS) in V$_\lambda$CDR1 led to a reduction in binding to dsDNA such that approximately double the concentration of IgG1 was required to produce the same *OD* reading. The introduction of a second (G29S) mutation reduced binding to DNA further.

Figure 3.6 shows that binding of these heavy/light chain combinations to ssDNA gave similar results although the overall *OD* readings were consistently lower than those seen in the anti-dsDNA ELISA.

Despite being tested at a range of concentrations between twice and 35 times higher than those which gave maximal DNA binding for the other combinations, B3V$_H$/UK-4V$_\lambda$ showed no binding to neither dsDNA nor ssDNA. In contrast, the B3V$_H$/33.H11V$_\lambda$ combination bound to both dsDNA and ssDNA. However, the trends seen suggest that

the binding of B3V$_H$/33.H11V$_\lambda$ was less than that of B3V$_H$/B3V$_\lambda$, similar to that of B3V$_H$/B3V$_\lambda$a and stronger than B3V$_H$/B3V$_\lambda$b.

Figure 3.5 Detection of anti-dsDNA activity of whole IgG in COS-7 cell supernatants by ELISA.

The graph shows binding of IgG in COS-7 cell supernatants containing each B3 heavy chain/λ gene 2a2-derived light chain combination to dsDNA. The electroporations were carried out and tested by ELISA for anti-dsDNA binding on three separate occasions. The above graph shows the results of a representative experiment. However the standard deviations (SD) between the optical density (OD) readings of the three experiments were calculated for all the points on each curve as follows: SD < 0.18 OD units for all points on curve B3V$_H$/B3V$_\lambda$(wild type). Similarly, at all points, SD < 0.18 for B3V$_H$/B3V$_\lambda$a, SD < 0.29 for B3V$_H$/B3V$_\lambda$b, SD < 0.12 for B3V$_H$/33.H11V$_\lambda$ and SD < 0.045 for B3V$_H$/UK-4V$_\lambda$. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was patient serum known to contain anti-dsDNA IgG although the exact anti-dsDNA IgG concentration was not tested and therefore could not be included in this graph. The same aliquot of patient serum was used for all the ELISAs and the SD of the positive control between the OD readings of the three experiments was less than 0.04.
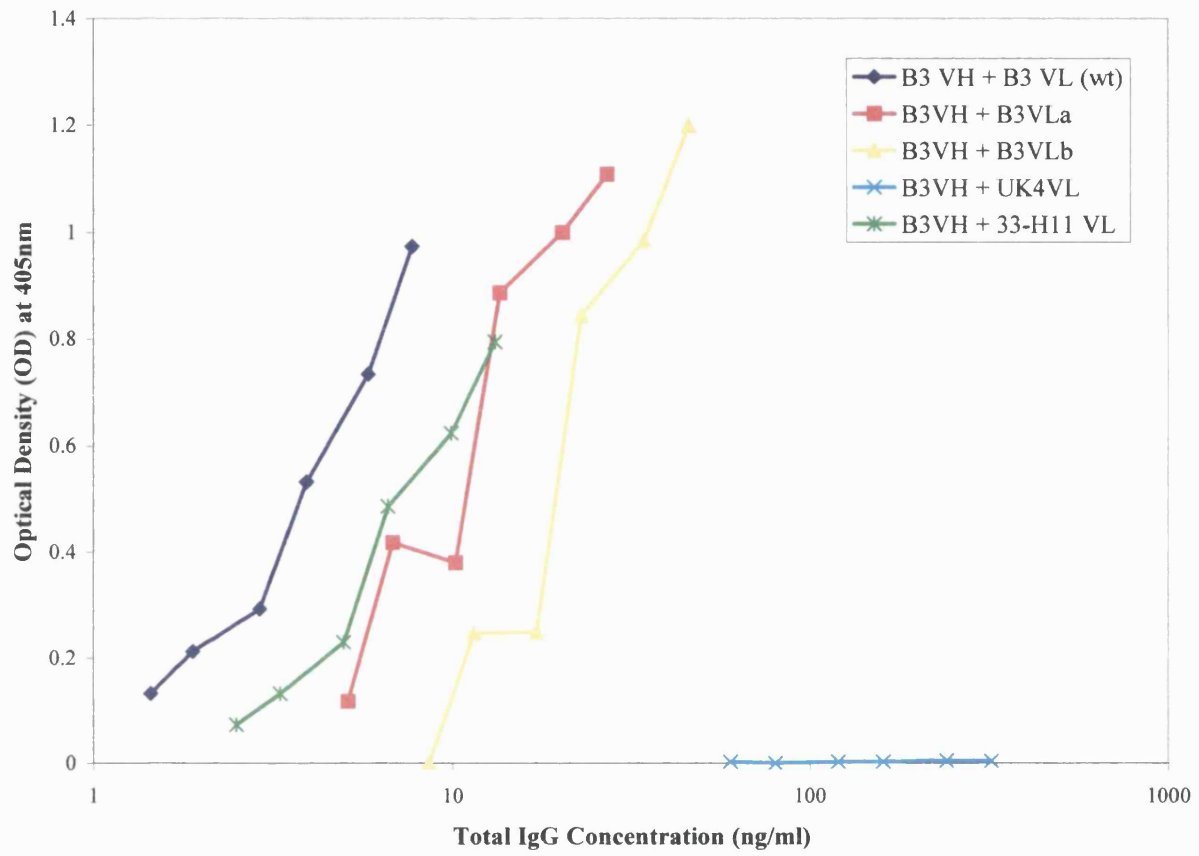
Figure 3.6 Detection of anti-ssDNA activity of whole IgG in COS-7 cell supernatants by ELISA.

The graph shows binding of IgG in COS-7 cell supernatants containing each B3 heavy chain/λ gene 2a2-derived light chain combination to ssDNA. The electroporations were carried out and tested by ELISA for anti-dsDNA binding on three separate occasions. The above graph shows the results of a representative experiment. However the standard deviations (SD) between the optical density (*OD*) readings of the three experiments were calculated for all the points on each curve as follows: SD < 0.037 *OD* units for all points on curve B3V$_H$/B3V$_\lambda$(wild type). Similarly, at all points, SD < 0.099 for B3V$_H$/B3V$_\lambda$a, SD < 0.35 for B3V$_H$/B3V$_\lambda$b, SD < 0.094 for B3V$_H$/33.H11V$_\lambda$ and SD < 0.002 for B3V$_H$/UK-4V$_\lambda$. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was patient serum known to contain anti-ssDNA IgG although the exact anti-ssDNA IgG concentration was not tested and therefore could not be included in this graph. The same aliquot of patient serum was used for all the ELISAs and the SD of this positive control between the *OD* readings of the three experiments was less than 0.04.
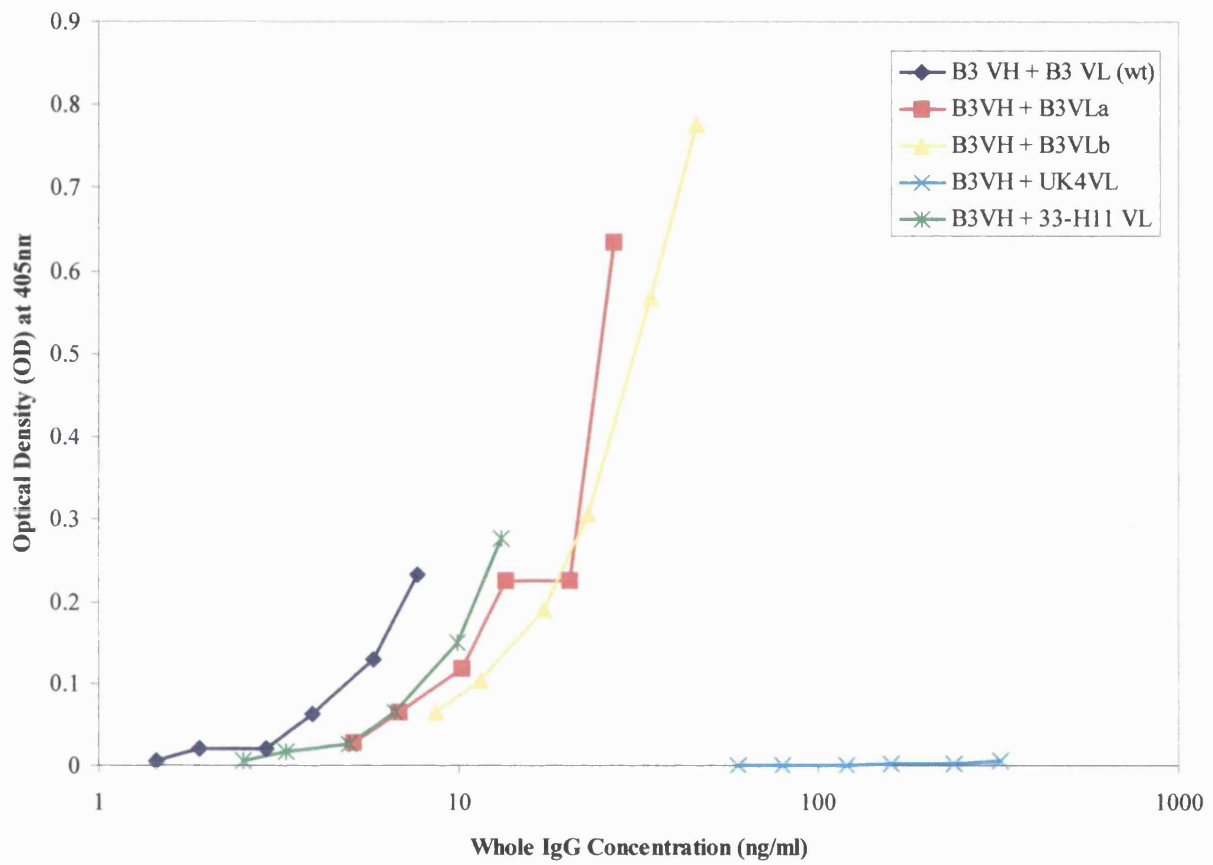
3.5 Computer modelling of three-dimensional structures of the interaction of the heavy/light chain combinations with dsDNA

The computer models of the three-dimensional structures of these heavy/light chain combinations shown in Figures 3.7, 3.8 and 3.9 were kindly modelled by Dr Sylvia Nagl (Bloomsbury Centre for Structural Biology, University College London). The computer models were generated using the minimal perturbation procedure (as described by Martin *et al.* (1991) using the previously published model of B3 (Kalsi *et al.*, 1996) shown in Figure 1.3, as a template. This original computer model of the B3/dsDNA complex suggested that the dsDNA double helix would lie in a groove on the surface of B3 between the V$_H$ and V$_\lambda$ and that this interaction was stabilised by the presence of three arginine residues at the periphery of the antigen-binding site (R27a and R54 of the light chain and R53 of the heavy chain).

The effects of replacing V$_\lambda$ with 33.H11V$_\lambda$ and UK-4 V$_\lambda$ are shown in Figures 3.7 and 3.8 respectively. The differences in amino acid sequence between these light chains and that of B3 are highlighted in red.

According to the computer model shown in Figure 3.7, the B3V$_H$/33.H11V$_\lambda$ combination is likely to bind DNA. The model predicts that this interaction is stabilised by the presence of an arginine residue at position 92 of 33.H11V$_\lambda$ (R92). In contrast, the computer model shown in Figure 3.8 predicts that the B3V$_H$/UK-4V$_\lambda$ combination is likely to be a poor binder to DNA. This is because the groove in the B3V$_H$/UK-4V$_\lambda$ model is blocked by various residues from the light chain including a bulky positively charged arginine residue at position 94 (R94). In addition, the serine residue at position 29 introduces a possible destabilising electrostatic interaction with the phosphate backbone of DNA.

Figure 3.9 shows the effect of reverting the arginine at position 27a in B3V$_\lambda$a to a serine. The positively charged arginine (R27a) in the upper diagram shown in blue interacts directly with the negatively charged phosphate backbone of the DNA molecule. However this specific electrostatic interaction is lost when R27a is replaced with a serine. S27a, shown in green in the lower diagram is unable to interact with the DNA molecule. As a consequence this R27aS reversion was expected to reduce DNA

binding. As noted in the B3V$_H$/UK-4V$_\lambda$ complex, the glycine to serine mutation at position 29 of the B3V$_\lambda$b introduces a possible destabilising electrostatic interaction with the phosphate backbone of DNA, thus resulting in a further reduction in ability to bind DNA in B3V$_\lambda$b.
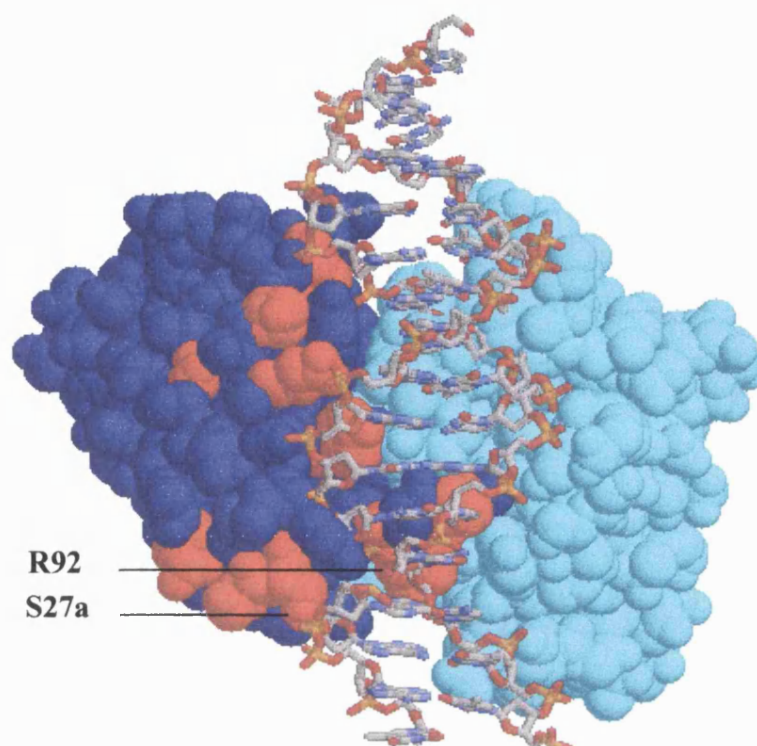
Figure 3.7   Computer-generated model of B3V$_H$/33.H11V$_\lambda$ with dsDNA.

The computer model predicts that the 33.H11V$_\lambda$ is able to create a DNA binding site in combination with B3V$_H$. B3V$_H$ is shown in light blue whilst 33.H11V$_\lambda$ is shown in dark blue. The V$_\lambda$ residues that differ from those found at the same positions in B3V$_\lambda$ are shown in red. The double helix of the DNA molecule is shown in the centre of the figure. Unlike B3V$_\lambda$, 33.H11V$_\lambda$ does not have an arginine residue at position 27a in CDR1 and instead has the germline-derived serine (S27a). However the presence of an arginine residue at position 92 (R92) in 33.H11V$_\lambda$ CDR3 may compensate for this as the model predicts that R92 can interact with the backbone of DNA. The positions of both S27a and R92 are marked in the computer model.

Modelled by Dr Sylvia Nagl.
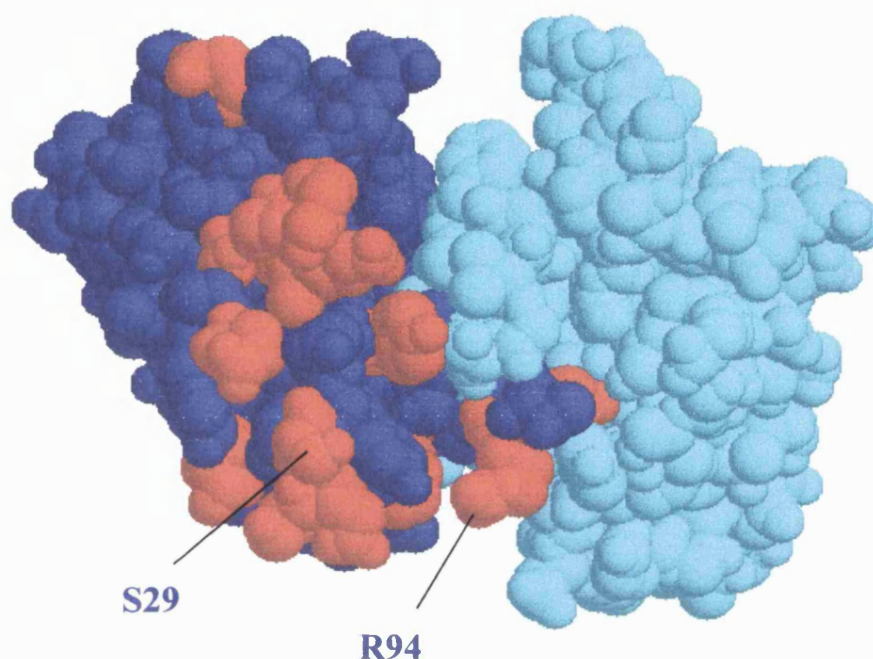
Figure 3.8   Computer generated model of B3V$_H$/UK-4V$_\lambda$.

B3V$_H$ is shown in light blue whilst UK-4V$_\lambda$ is shown in dark blue. The V$_\lambda$ residues that differ from those found at the same positions in B3V$_\lambda$ are shown in red. No complex with DNA could be modelled. The model predicts that UK-4V$_\lambda$ is unable to bind DNA in combination with B3V$_H$ due to steric hindrance resulting from the introduction of an arginine residue at position 94 (R94) and a serine residue at position 29 (S29) in UK-4V$_\lambda$ by somatic mutation.

Modelled by Dr Sylvia Nagl.
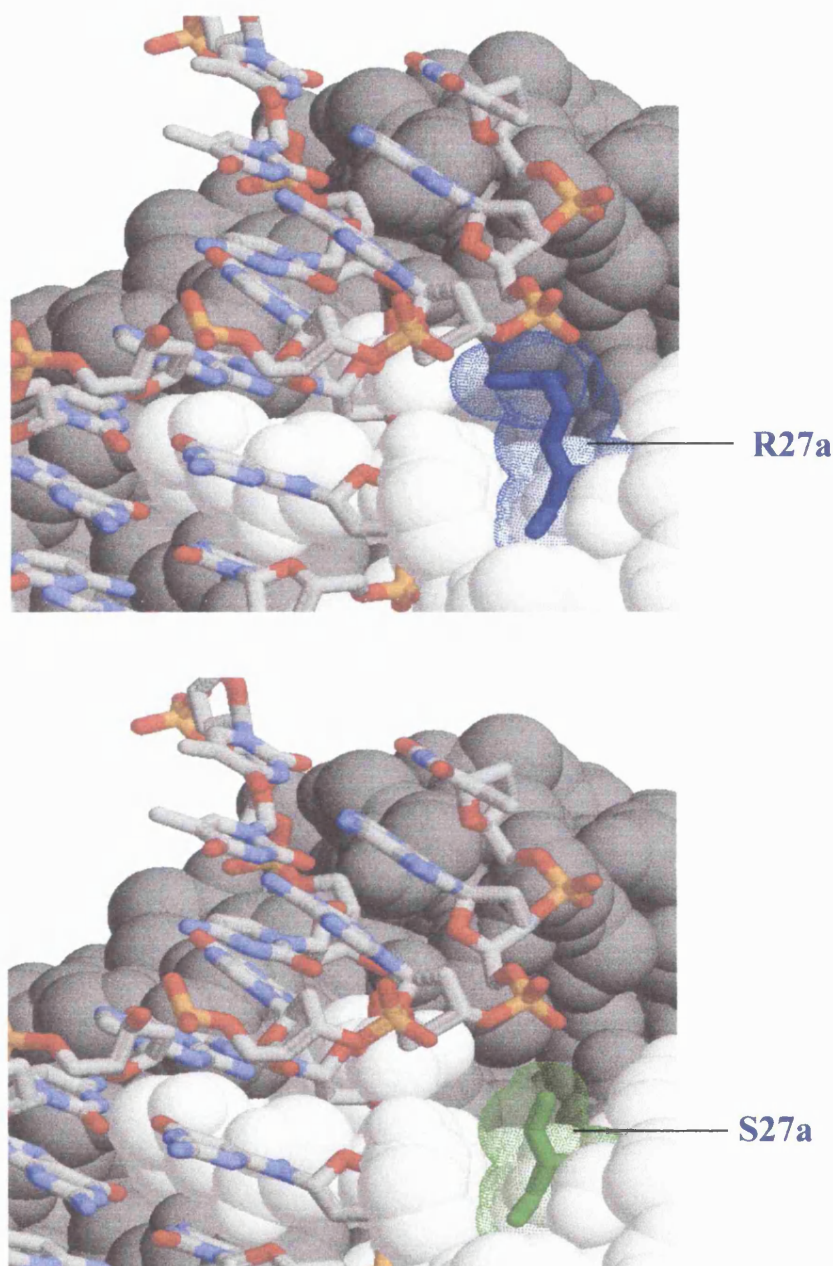
Figure 3.9   Computer-generated model to show the interaction of the amino acid residue at position 27a of V$_\lambda$CDR1 with dsDNA helix in B3V$_H$/B3V$_\lambda$.

The upper diagram shows the interaction between an arginine residue at position 27a of B3V$_\lambda$CDR1 (R27a) (shown in blue) and the dsDNA helix in B3V$_H$/B3V$_\lambda$. The lower diagram shows the same region of B3V$_H$/B3V$_\lambda$a. The serine residue at position 27a (S27a) of B3V$_\lambda$a (shown in green) is unable to interact with the DNA molecule.

Modelled by Dr Sylvia Nagl.

3.6   Discussion of results

The results described in this chapter show that whole human IgG1 molecules that bind both ssDNA and dsDNA can be produced through the transient expression of cloned autoantibody DNA sequences in eukaryotic cells. The total yield of IgG1 varied between 10ng/ml to 250ng/ml depending on the heavy/light chain combination. Low yields of expressed IgG have been reported before not only by our group (Rahman *et al.*, 1998) but also by Zack *et al.* (1995) who produced only 30ng/ml of IgG through the use of a similar expression system in COS-7 cells. The fact that the yield of IgG varies between different constructs has also been previously seen when this expression system was used to humanise a mouse mAb by CDR grafting (Kettleborough *et al.*, 1991).

B3V$_H$/B3V$_L$ (wild type) bound both ssDNA and dsDNA, which is consistent with the previously reported properties of this mAb, (Ehrenstein *et al.*, 1994) as well as the computer model of the interaction between a dsDNA helix and B3, in Figure 1.3 (Kalsi *et al.*, 1996).

The results of the ELISA tests correlated with the predictions of the computer models in showing that the ability of B3 to bind dsDNA is dependent upon the pattern of somatic mutations in the CDRs of the λ germline gene, 2a2. The arginine residue at position 27a in CDR1 is able to form a specific electrostatic interaction with the phosphate backbone, which is critically lost by mutation to serine, thus leading to a reduction in DNA binding ability by the variant light chain when compared to the wild type. When a second mutation was introduced, in which a glycine was replaced by a serine at position 29 of CDR1, additional unfavourable electrostatic interactions were created leading to a further decrease in binding to DNA. This highlights the fact that amino acid residues other than arginine, lysine and asparagine, can affect an antibody's binding ability for DNA.

S29 is also present in UK-4V$_\lambda$ CDR1. This light chain also has an asparagine at position 27a rather than an arginine. As predicted by the computer model of B3V$_H$/UK-4V$_\lambda$ (Figure 3.6), the pattern of somatic mutations in UK-4V$_\lambda$ prevents any binding to DNA, even when paired with B3 heavy chain. From the computer model, it can be deduced that the introduction of the arginine residue at position 94 in addition to the changes at

positions 27a and 29 prevent DNA from binding. This finding is in agreement with previous studies that have also shown that it is not the mere presence of arginine residues in the CDRs that confers the ability to bind DNA but their precise positions. For example, Li *et al.* (2000) found that although the presence of arginine residues in the H-CDR3 of stably expressed mAb 412.67 contributed to DNA-binding, an arginine residue in L-CDR3 did not. As in our system these effects were explained using a computer model of 412.67 Fv. This model showed that the H-CDR3 arginines were exposed on the surface of the antibody and therefore available to bind DNA whilst the inward orientation of the L-CDR3 arginine from the surface of the antigen-binding site prevented it from binding to DNA.

Despite the fact that 33.H11V$_\lambda$ contains a serine at position 27a rather than an arginine it is able to bind DNA when in combination with B3 heavy chain. The computer model of the B3V$_\lambda$/33.H11V$_\lambda$ combination predicts that the arginine at position 92 in the CDR3 is able to interact with the dsDNA backbone. Therefore it is possible that the presence of arginine residues in CDR3 of 33.H11V$_\lambda$ could compensate for their absence in CDR1.

In conclusion, the results shown here suggest that in a 2a2 encoded light chain such as B3V$_\lambda$ which has undergone extensive somatic mutation, DNA-binding ability is determined by the pattern of these somatic mutations. Both single amino acid changes as well as multiple sequence alterations can effect DNA binding of this antibody, especially where these alterations involve arginine residues that have been predicted by the computer models to directly interact with the dsDNA helix.

Various groups have previously used computer models to produce three-dimensional images of anti-DNA antibodies (mainly murine) interacting with DNA. These too, like ours, have been used to predict sites of contact between dsDNA and certain amino acid residues at the antigen-binding site (Radic *et al.*, 1994). Using site-directed mutagenesis as in our system, the validity of these predictions has been tested. In many cases the targets of mutagenesis have been arginine residues and as in our system the number of arginine residues did not always strictly correlate with the ability of the antibody to bind DNA (Radic *et al.*, 1993). For example, Radic and colleagues used heavy chain loss variants (based on murine IgG anti-dsDNA mAb, 3H9) to show that although the

addition of a single arginine residue, in most cases, resulted in an increased affinity of between 7- and 10-fold for ssDNA and 5- and 8-fold for dsDNA, the mutation of a leucine in 3H9 HCDR2 to an arginine (L64 to R64), completely eliminated binding to ssDNA and dsDNA. The authors suggested that this may be due to the formation of a salt bridge of R64 with a neighbouring aspartic acid (D65) that may produce an Ig conformation incompatible with DNA binding (Radic *et al.*, 1993).

Katz *et al.* (1994) used site-directed mutagenesis to alter amino acid residues in the V$_H$ sequence of a murine anti-dsDNA antibody, R4A. The data showed that at many sites the loss of basic amino acid residues reduced DNA binding. However in contrast to the results of Radic and colleagues, the V$_H$ sequence that resulted in the highest ability to bind DNA contained two fewer arginine residues than R4A, highlighting the fact that charge interaction is not the sole determinant of binding to DNA. These two arginines were both lost from R4A V$_H$FR3, suggesting that framework regions not only perform a scaffolding function but also affect binding specificity. This has previously been shown by other groups. For example, Tramontano *et al.* (1990) showed that FR2 residue 71 is a major determinant of the position and conformation of the V$_H$CDR2. Furthermore, Radic *et al.* (1994) predicted from a computer model of a murine anti-dsDNA antibody that V$_H$FR3 is positioned so as to contribute contacts with the minor groove of the DNA.

Members of our group have used a bacterial expression system to produce Fab with different H/L chain combinations derived from the same different human mAb as used in this thesis (Kumar *et al.*, 2000; Kumar *et al.*, 2001). This system involved the transformation of *E. coli* with plasmid expression vectors containing the appropriate V$_\lambda$-C$_\lambda$ and V$_H$-C$_H$ DNA for each antibody. Following the culture of these bacteria, Fab were harvested from the periplasm of the E.coli and then purified. Analysis of the binding properties of the hybrid chain Fabs expressed demonstrated that the anti-DNA binding ability of two human monoclonal anti-DNA IgG antibodies, B3 and 33.H11 relied heavily on the presence of their L chains. These results are consistent with the binding results and computer generated models of these H/L chain combinations expressed in eukaryotic systems, as presented in this chapter. Expression levels of 5-9mg of Fab protein per litre of culture were achieved. This was comparable with the amount of scFv antibody fragment produced by another group using *E.coli* expression (30μg-15mg),

however Fab fragments remain the preferred structure for crystallisation studies which is the long term objective of our group regarding bacterial expression of these particular anti-DNA antibodies.

In this particular set of 2a2-derived antibodies tested in this thesis, it appears that those arginine residues critical to DNA binding are those in B3V$_\lambda$ CDR1 and 33.H11V$_\lambda$ CDR3 and that these may have an additive effect in enhancing binding to DNA whilst an arginine in UK-4V$_\lambda$ CDR3 blocks DNA binding. Therefore the next step was to use this transient system to investigate these hypotheses further. As described in chapter four, this was achieved by swapping CDRs between light chains thus producing sequences in which CDR1 was derived from one 2a2-derived antibody and CDRs 2 and 3 were derived from another 2a2-derived antibody.

CHAPTER FOUR

The influence of V_H and V_λ sequence motifs on binding of human monoclonal antibodies to DNA, histones, SmD and Ro antigen.

CHAPTER FOUR.

THE INFLUENCE OF V_H AND V_λ SEQUENCE MOTIFS ON BINDING OF HUMAN MONOCLONAL ANTIBODIES TO DNA, HISTONES, SmD ANTIGEN AND Ro.

4.1   Aims of this chapter

The aims of the experiments described in this chapter were as follows:

1) To optimise the transient expression system by addressing issues, which had arisen from the experiments, described in chapter three.

2) To investigate whether the sequence motifs identified in chapter three as being critical to DNA binding (particularly arginine residues in $B3V_\lambda$ CDR1 and 33.H11V_λ CDR3) have an additive effect in enhancing binding to DNA and whether the suggested effect of the arginine in UK-4V_λ CDR3 to block DNA binding is dominant.

3) To determine the effect of these sequence motifs on binding to a wider range of nuclear autoantigens

4) To investigate the effect of changing the heavy chain from B3 to that of another anti-DNA mAb, 33.H11

4.2   Optimisation of transient expression system

4.2.1   Cell viability of COS-7 cells following transfection with anti-DNA V_H/V_λ DNA

The first issue to be dealt with in this transient expression system was the variation in immunoglobulin expression levels between the heavy/light chain combinations. Those with a higher DNA-binding ability appeared to be expressed at much lower levels than those such as $B3V_H$/UK-4V_λ, which does not bind DNA at all (even when diluted to levels comparable to those of the heavy/light chain combinations that do bind DNA). It was hypothesised that this was due to the fact that the heavy/light chain combinations with higher DNA binding abilities may be toxic to the COS-7 cells that are producing the IgG1 thus reducing the levels of expressed whole IgG1. This is particularly relevant to B3 since when administered to SCID mice it was found that this antibody was able to penetrate cells and bind to the nuclei within (Ehrenstein *et al.*, 1995). Anti-DNA from

SLE patients (Yanase *et al.*, 1997) and a subset of murine monoclonal anti-DNA antibodies (Vlahakos *et al.*, 1992; Zack *et al.*, 1996) have also previously been shown to bind to membranes of living cells *in vitro*, penetrate cells via the myosin in the cell membranes in an energy dependent fashion and then bind to cytoplasmic or nuclear structures. The consequences of cell penetration are unknown but could influence cell proliferation, protein synthesis and apoptosis (Hahn, 1998).

To test this hypothesis, a Trypan blue viable cell count was used to determine whether the number of living COS-7 cells differed according to which H/L combination they had been transfected with. Figure 4.1 shows the mean total number of viable cells remaining three days post-electroporation compared to the mean whole IgG1 concentration of each corresponding COS-7 cell supernatant. This procedure was carried out on two separate occasions three days post-electroporation and as shown by the standard error bars in Figure 4.1, similar results were seen in each of the experiments.

The highest number of viable COS-7 cells seen after three days is seen in the sample that was electroporated in the absence of DNA as a negative control. Possible reasons for this are that the DNA transfected into the cells is toxic to the COS-7 cells to some extent or that all of the IgG1 are attacking the cells. Alternatively the cells may grow and divide more slowly if having to divide their energy between growing and also expressing and assembling whole IgG. If the latter were the case then it would be expected that this would have been reported previously elsewhere, which it is not. However in the stable expression system (see chapter five of this thesis), the negative control cell line that was treated under the same conditions as the IgG-expressing CHO cells but does not express IgG since it was originally transfected with empty vector (i.e. expression vector containing no variable region DNA sequences) did noticeably grow faster than those cell lines expressing IgG1, although no specific measurements were taken of this factor.

Therefore in future electroporations, an aliquot of cells could be transfected with empty vector as an additional control to determine whether it is the transfection of the cells with DNA or whether it is the production of immunoglobulins that slows the cell growth compared to the no DNA control in the transient system.

Statistical analysis shows that there is no significant correlation between the number of viable COS-7 cells and the whole IgG concentration of the various $B3V_H/2a2$-derived V_λ combinations (p=0.75, n=2 using Spearman's rank test with 95% confidence level). These results therefore do not support the hypothesis that those H/L combinations that can bind DNA kill or slow the growth of the COS-7 cells more than those H/L combinations that do not bind DNA.
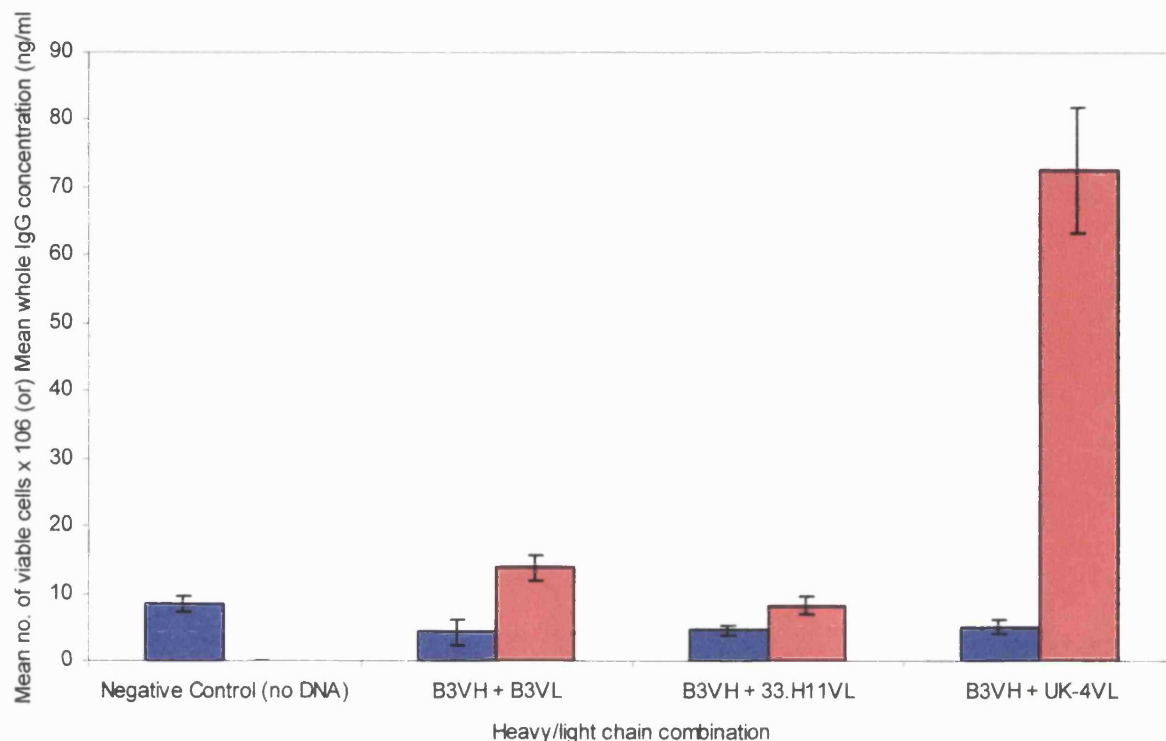
Figure 4.1 Comparison of cell viability and level of whole IgG expression of COS-7 cells transfected with different heavy/light chain expression vectors three days post-electroporation.

■   Mean total number of viable cells three days post-electroporation (x 10^6)

■   Mean whole IgG concentration of COS-7 cell supernatant (ng/ml)

This experiment was carried out on two separate occasions. The bar chart shows the mean total number of viable cells remaining three days post-electroporation compared to the mean whole IgG concentration of each corresponding COS-7 cell supernatant. The error bars shown on the graph correspond to the standard error about the means to show any variation in results between the two experiments. Statistical analysis shows that there is no significant correlation between the number of viable COS-7 cells and the whole IgG concentration of the various $B3V_H/2a2$-derived V_λ combinations (p=0.75, n=2 using Spearman's rank test with 95% confidence level).

4.2.2 Effect of treatment of COS-7 supernatants with (RNase-free) DNaseI

When electroporated, approximately 80% of the cells die, thus releasing their contents (including DNA) into the supernatant. Therefore an alternative explanation for the variation in IgG1 yield could be that as both $B3V_H/B3V_\lambda$ and $B3V_H/33.H11V_\lambda$ bind DNA, it is possible that the heavy/light chain combination IgG1 may form immune complexes with the DNA in the supernatant thus rendering them undetectable in ELISA.

It was previously found that by treating the COS-7 cell supernatants with DNaseI prior to ELISA, the immune complexes were broken down thus leaving the IgG1 molecules free to be detected in the supernatants (Rahman *et al.*, 1998). It was therefore important to investigate whether varying the amount of DNaseI present could alter the amount of free antibody detected.

The original 7.5u DNaseI per ml of supernatant were added to one half of each supernatant collected from the COS-7 cells and the other half was treated with 37.5u DNaseI per ml of supernatant (an increase of five-fold). Figure 4.2 shows the effect of increasing the amount of DNaseI added to the COS-7 cell supernatants by five-fold on the mean total IgG1 concentration of each heavy/light chain combination detected by ELISA This procedure was carried out on two separate occasions, three days post-electroporation and for each H/L chain combination as shown by the standard error bars in Figure 4.2, similar results were seen in each of the experiments.

The whole IgG1 concentrations of COS-7 cell supernatants that were not DNaseI-treated were too low to be detected by the ELISA and are therefore not shown in the chart. The negative control was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG1 nor anti-DNA activity on testing by ELISA.

As shown in Figure 4.2, statistical analysis shows that the difference between the mean concentration of IgG1 detected in the supernatants treated with 7.5u DNaseI compared to those treated with 37.5u DNaseI was not statistically significant for all of the H/L

chain combinations (p>0.05, n=2 for all H/L chain combinations as calculated using the Student's t test with confidence level of 95%).

Therefore it was concluded that 7.5u DNaseI per ml of supernatant was sufficient to enable the ELISA to detect the IgG1 concentration of the collected supernatants.

These results do not support the hypothesis that the apparently low concentrations of some of the H/L chain combination antibodies were due to binding to DNA in the supernatant.
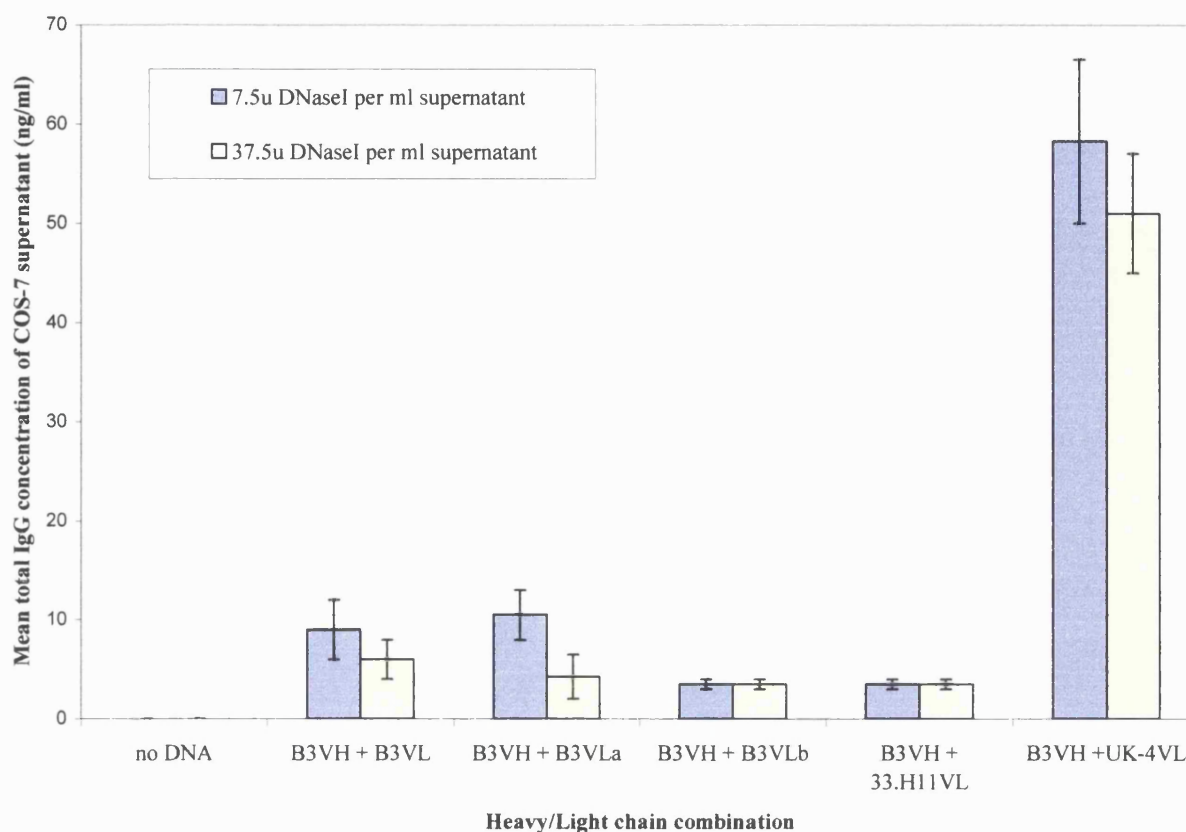
Figure 4.2 Effect of treatment of COS-7 cell supernatants with different levels of (RNase-free) DNaseI on total IgG concentration detected by ELISA.

The bar chart shows the effect of increasing five-fold the amount of DNaseI added to the COS-7 cell supernatants from 7.5u enzyme per ml supernatant (shown in blue) to 37.5u enzyme per ml supernatant (shown in yellow) on the mean total IgG concentration detected by ELISA. This experiment was carried out on two separate occasions. The IgG concentrations shown for each DNaseI treatment of each heavy/light chain combination are the means of these two experiments. The error bars shown on the graph correspond to the standard error about the mean to show any variation in results between the two experiments. The whole IgG concentrations of COS-7 cell supernatants that were not DNaseI-treated were too low to be detected by the ELISA and are therefore not shown in the chart. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. Statistical analysis shows that the difference between the mean concentration of IgG1 detected in the supernatants treated with 7.5u DNaseI compared to those treated with 37.5u DNaseI was not statistically significant for all of the H/L chain combinations ($p > 0.05$, $n = 2$ for all H/L chain combinations as calculated using the Student's t test with confidence level of 95%).

4.2.3   Length of period of expression of whole IgG1 by COS-7 cells

In the last set of transient expression experiments, as detailed in chapter three, the supernatants of the transfected COS-7 cells were collected after three days and the cells were then discarded. The cells were not kept longer as it was presumed that as expression in this system is only transient, the COS-7 cells would only produce significant amounts of IgG1 for approximately three days. However due to the amount of work involved in the preparation of the expression vector DNA and the transfection of the COS-7 cells, it was decided that it would be worthwhile investigating whether the transfected cells do express IgG1 for longer than three days.

Therefore following the removal of the supernatant 72 hours post-transfection, the cells were incubated in fresh media at 37°C, 5% CO_2 for a further three days. At the end of this second three-day period, the supernatants were DNaseI-treated, concentrated and the concentration of their IgG1 determined by ELISA, as before. Figure 4.3 shows the mean concentration of whole IgG1 (detected by ELISA) in the supernatants of COS-7 cells expressing both a low-expressed (B3V_H/B3V_λ) and a high-expressed (B3V_H/UK-4V_λ) chain combination, both three days and six days post-electroporation. This experiment was carried out on two separate occasions. The error bars shown on the graph correspond to the standard error about the mean to show any variation in results between the two experiments. No further statistical analysis has been carried out on this particular experiment since the fact that IgG is still produced in comparable amounts at six days as on three days addresses the initial objective of this experiment. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG1 nor anti-DNA activity on testing by ELISA.

Figure 4.3 shows that regardless of whether COS-7 cells were transfected with either a high (B3V_H/UK-4 V_λ) or a low (B3V_H/B3 V_λ) expressed combination, both sets continued to express whole IgG1 for at least six days post-electroporation.

As a consequence, supernatants were collected both after three days and six days of culture of transfected COS-7 for all the following transient expression experiments. However due to the fact that there was a decrease in the amount of IgG1 expressed between three and six days, the cells were not kept any longer.
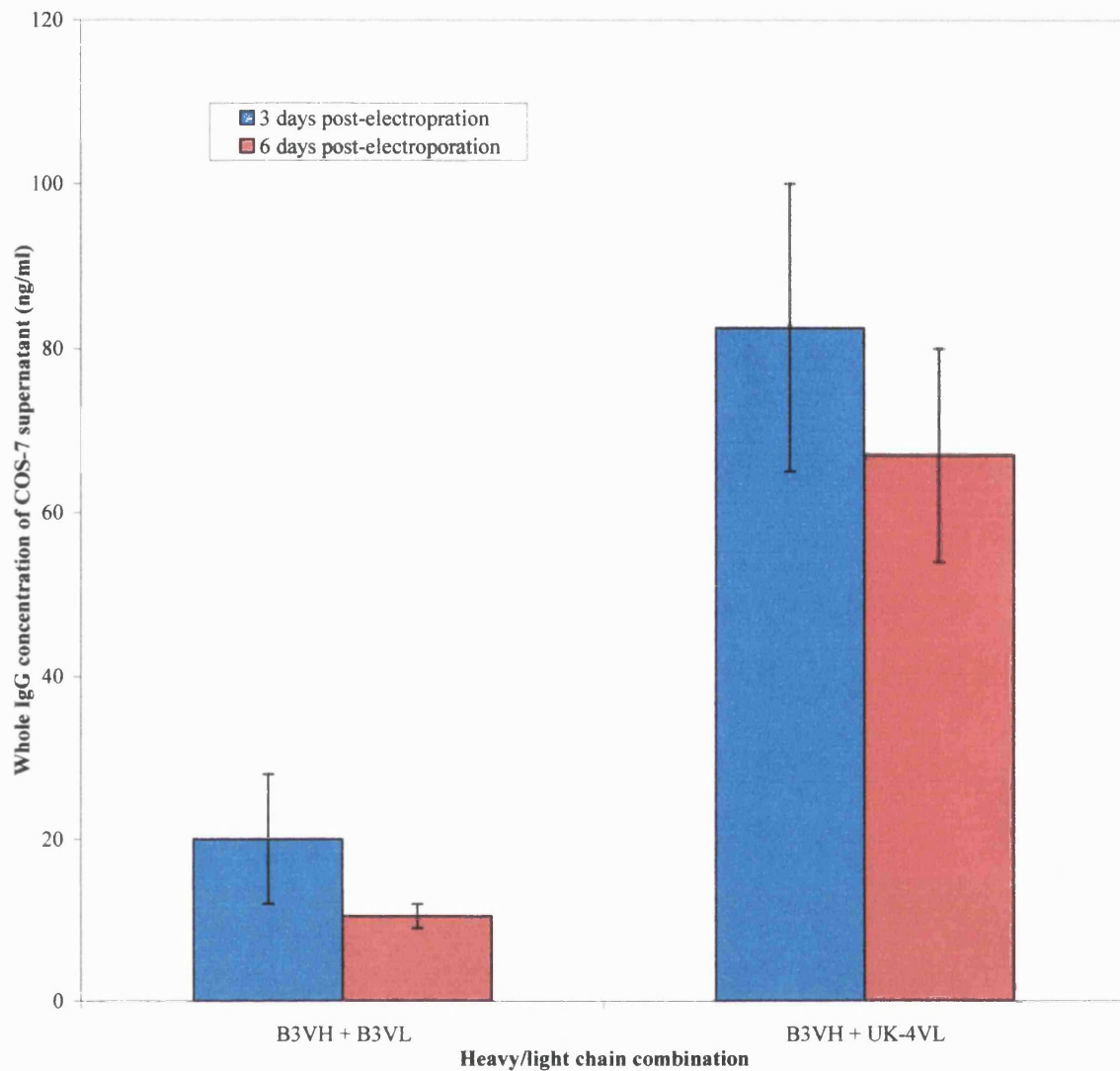
Figure 4.3 Whole IgG concentration of COS-7 supernatants three days and six days post-electroporation, detected by ELISA.

The bar chart shows the concentration of whole IgG (detected by ELISA) in the supernatants of COS-7 cells expressing both a low-expressed (B3V_H/B3V_λ) and a high-expressed (B3V_H/UK-4V_λ) chain combination, both three days (shown in blue) and six days (shown in red) post-electroporation. This experiment was carried out on two separate occasions. The error bars shown on the graph correspond to the standard error about the mean to show any variation in results between the two experiments. No further statistical analysis has been carried out on this particular experiment since the fact that IgG is still produced in comparable amounts at six days as on three days addresses the initial objective of this experiment. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA.

4.2.4 Freeze/thaw experiment to investigate the stability of the expressed whole IgG1 in storage

Due to the large number of assays, COS-7 supernatants collected from the transfected cells were stored either at 4°C or -20°C for an interval of between a few days to a few weeks, prior to being tested in an ELISA. Therefore it was essential to ensure that the whole IgG1 in the COS-7 supernatants were stable under these conditions.

The total immunoglobulin concentration of supernatants from five sets of transfected COS-7 cells (each expressing a different heavy/light chain combination) were determined using ELISA. The COS-7 supernatants were tested at different time points. Firstly they were tested immediately following concentration with the *Centricon* centrifugal filters and the addition of sodium azide to prevent bacterial or fungal growth (as described in chapter 2). They were then tested after one month of storage at either 4°C or -20°C and then again after two months of storage at 4°C or -20°C. Figure 4.4 compares the effect of storage at 4°C and -20°C over two months on the total whole IgG1 concentration of each heavy/light chain combination detected by ELISA.

This set of experiments was carried out twice and similar trends were obtained from both. The IgG1 concentrations shown for each heavy/light chain combination at each time point at each temperature are the means of these two experiments. The error bars shown on the graph indicate any variation in results between the two experiments. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG1 nor anti-DNA activity on testing by ELISA.

Overall the concentration of IgG1 in each COS-7 supernatant did not fluctuate greatly. After one month at 4°C, the IgG1 concentrations of all the COS-7 supernatants had not differed greatly from the original concentration. Following a further month of storage, the IgG1 concentrations of those stored at 4°C dropped slightly although not as much as those stored at -20°C. Statistical analysis showed that the difference between the mean concentration of IgG detected in those supernatants stored at -20°C were significantly lower in several of the H/L chain combination samples after two months but not one month (for example after two months, $p < 0.05$ for samples $B3V_H/B3V_\lambda$ ($p = 0.0299$, $n = 2$)

and B3V_H/B3V_λa (p=0.0373, n=2), as calculated by the Student's t test, with confidence level of 95%).

Although the number of tests was low, these experiments suggested that the expressed IgG1 were stable at 4°C for at least two months but not at -20°C. It is likely that the repeated freeze-thawing would have been the cause of the reduction in stability of the COS-7 cell supernatant IgG1 seen in those samples stored at -20°C.

Figure 4.4 Mean total whole IgG concentration detected by ELISA following storage of COS-7 supernatants at either 4°C or -20°C for two months.

The chart compares the effect of storage at 4°C and -20°C over two months on the total whole IgG concentration of each heavy/light chain combination detected by ELISA. This experiment was carried out on two separate occasions. The IgG concentrations shown for each heavy/light chain combination at each time point at each temperature are the means of these two experiments. The error bars shown on the graph correspond to the standard error about the mean to show any variation in results between the two experiments. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA.

■   Initial whole IgG concentration

□   Whole IgG concentration after 1 month stored at 4°C

□   Whole IgG concentration after 2 months stored at 4°C

□   Whole IgG concentration after 1 month stored at -20°C

◻   Whole IgG concentration after 2 months stored at -20°C

Statistical analysis showed that the difference between the mean concentration of IgG detected in those supernatants stored at -20°C were significantly lower in several of the H/L chain combination samples after two months but not one month (for example, $p < 0.05$ for samples $B3V_H/B3V_\lambda$ ($p = 0.0299$, $n = 2$) and $B3V_H/B3V_\lambda a$ ($p = 0.0373$, $n = 2$), as calculated by the Student's t test, with confidence level of 95%).
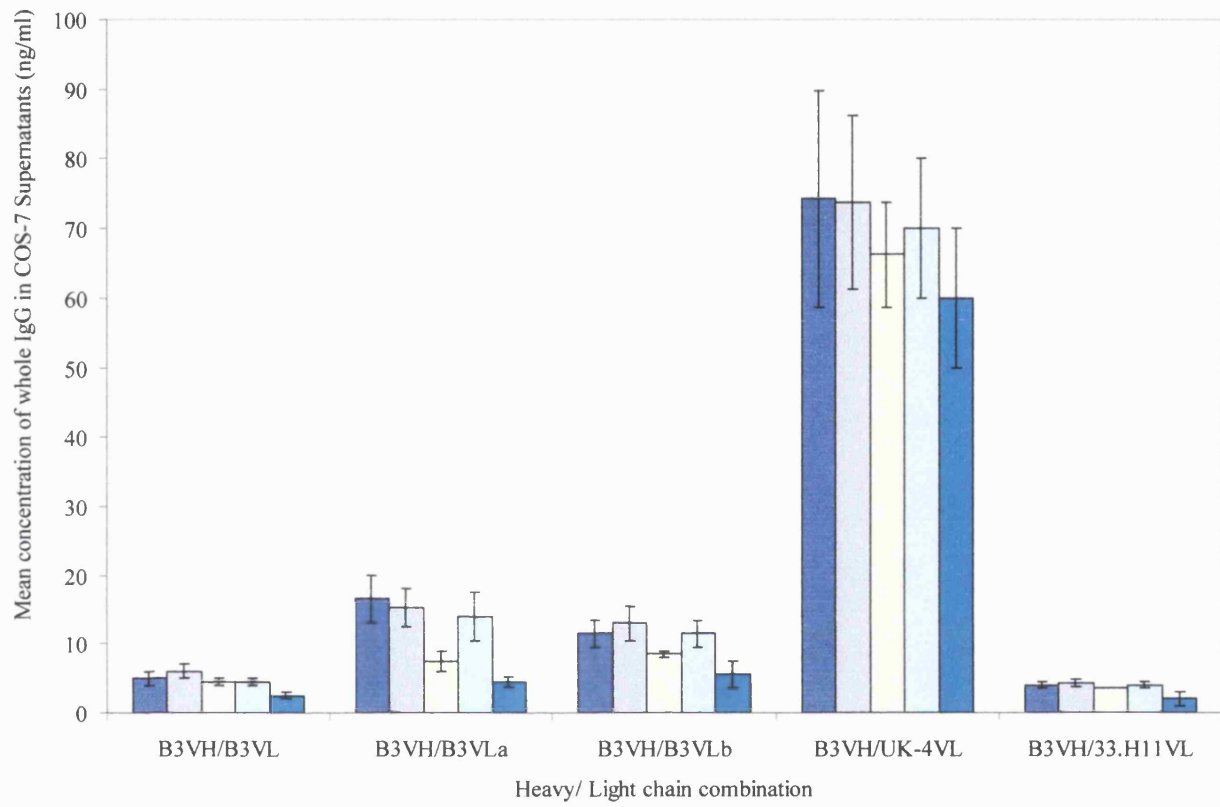
4.3 Transfer of B3V$_H$ cloned PCR fragment from pG1D1 to pG1D210 and the properties of the new recombinant B3V$_H$/pG1D210.

As explained in more detail in section 2.6.1, B3V$_H$ cloned PCR fragment was transferred from the original heavy chain expression vector, pG1D1 to a new vector, pG1D210. Although all the experiments in this thesis used the original vector pG1D1, it was thought important for future reference to compare the performance of pG1D210 and pG1D1 (both containing B3V$_H$ sequence) in the transient expression system for two reasons. Firstly, to ensure that the extra intron between V$_H$ and C$_H$ had not influenced our binding results and secondly for the purpose of future experiments using this system to determine whether the HCMVi enhancer does improve expression levels, as reported by the manufacturers AERES Biomedical, Mill Hill, London (*personal communication*).

4.3.1 Transfer of B3V$_H$ cloned PCR fragment from pG1D1 to pG1D210

As explained in section 2.6, B3V$_H$ cloned PCR fragment was transferred from the original heavy chain expression vector, pG1D1 to a new vector, pG1D210. Through the design of specific primers, a *XhoI* site was introduced into B3V$_H$/pG1D1 so that the B3V$_H$ region DNA sequence (but not the SD, SA sites or the problematic intron) could be extracted from pG1D1 and transferred to pG1D210 with *HindIII* and *XhoI*. Once ligated into pG1D210, a *HindIII/XhoI* restriction digest was used to verify the presence of B3V$_H$ DNA sequence in the new vector. Those clones containing recombinant B3V$_H$/pG1D210 produced bands of 7217bp and 434bp when digested with *HindIII/XhoI*, as shown in Figure 4.5. DNA sequencing ensured that no additional mutations had been introduced into the sequence due to PCR error.
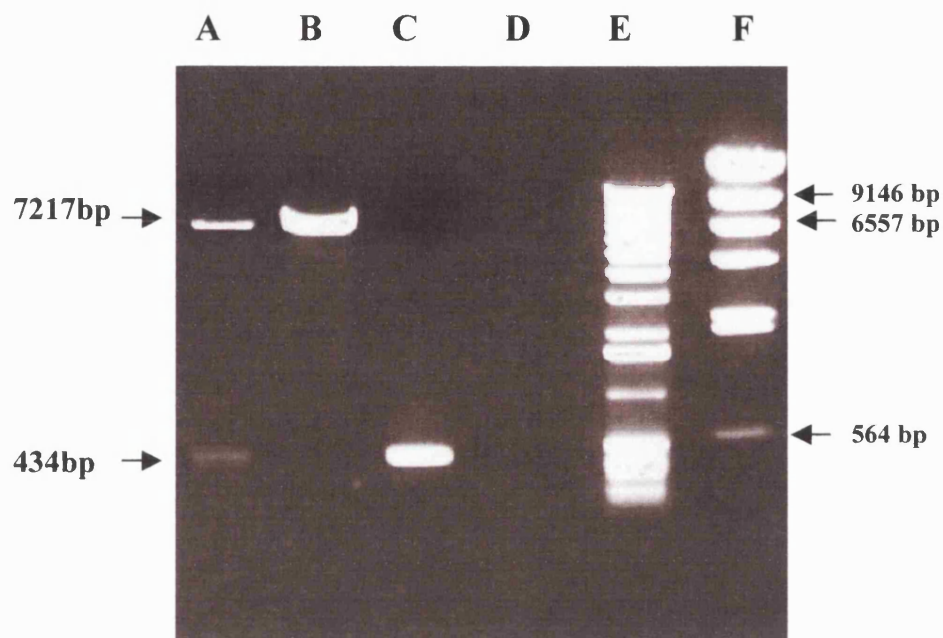
Figure 4.5	Restriction digests of B3V_H/pG1D210 on a 0.7% agarose gel.
Lane A	*XhoI/HindIII* digest of B3V_H/pG1D210
Lane B	*HindIII* single cut digest of original pG1D210
Lane C	*XhoI/HindIII* digested B3V_H PCR fragment
Lane D	Blank lane
Lane E	λ/*HindIII* DNA molecular weight marker (Promega, Southampton, UK)
Lane F	1Kb DNA molecular weight marker (Invitrogen, Paisley, UK)

4.3.2 Total IgG1 expressed using recombinant B3V$_H$/pG1D1 compared to recombinant B3V$_H$/pG1D210

The total IgG1 concentration (detected by ELISA) of the supernatants of COS-7 cells transfected with either the B3V$_H$/B3V$_\lambda$ or B3V$_H$/UK-4V$_\lambda$ combinations in which the B3V$_H$ was in pG1D1 were compared to those in which the B3V$_H$ was in pG1D210, as shown in Figure 4.6. This experiment was carried out twice. The IgG1 concentration that is shown for each heavy chain vector used to express each heavy/light chain combination is the mean of these two experiments and the error bars indicate the variation in results between the two experiments. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG1 nor anti-DNA activity on testing by ELISA.

As illustrated in Figure 4.6 when transfected in parallel with the same light chain expression vectors, the COS-7 cells transfected with B3V$_H$/pG1D210 expressed slightly higher levels of IgG1 than those transfected with B3V$_H$/pG1D1 as predicted. However, most likely due to the small sample size, statistical analysis shows that there is no significant difference between the levels of IgG expressed when COS-7 cells were transfected with pG1D1 heavy chain expression vector compared to COS-7 cells transfected with pG1D210 heavy chain expression vector. This is the case for both the H/L chain combinations tested i.e. B3V$_H$/B3V$_\lambda$ ($p=0.4$, $n=2$ calculated using Student t-test at 95% confidence level) and B3V$_H$/UK-4V$_\lambda$ ($p=0.2678$, $n=2$ calculated using Student t-test at 95% confidence level).
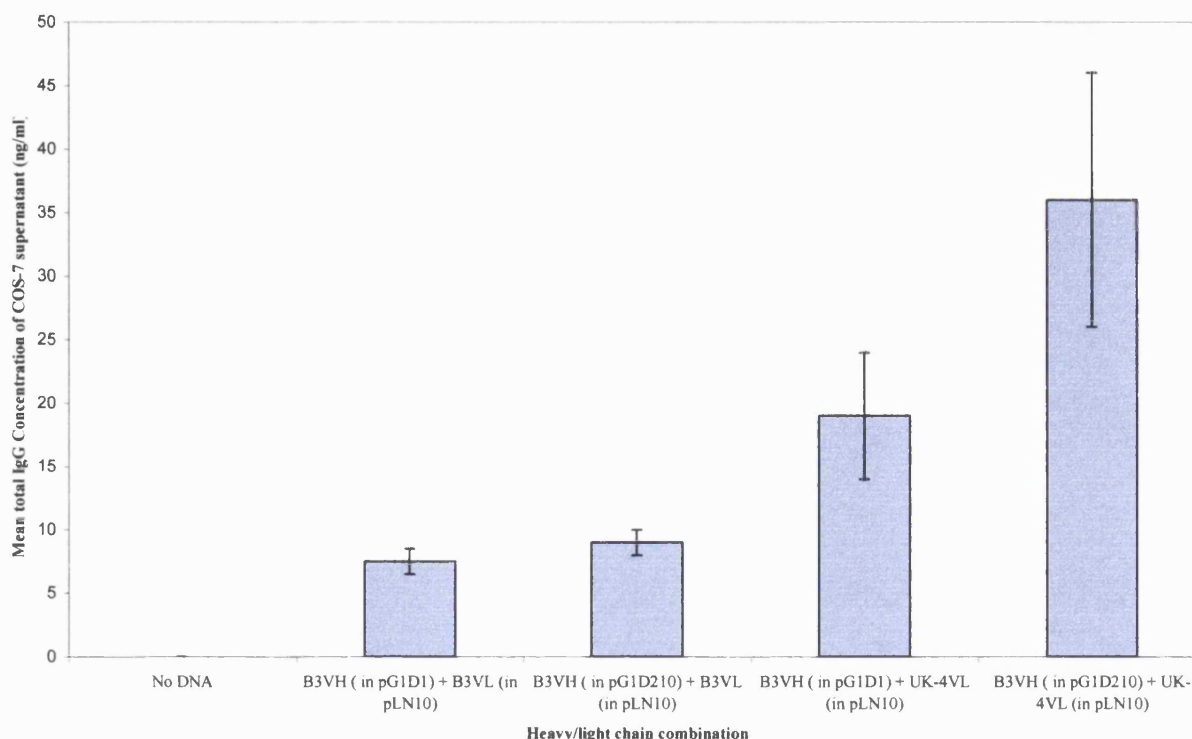
Figure 4.6   Effects of the two heavy chain expression vectors, pG1D1 and pG1D210 on IgG expression levels.

The bar chart compares the total IgG concentration (detected by ELISA) of the supernatants of COS-7 cells transfected with either the $B3V_H/B3V_\lambda$ or $B3V_H/UK-4V_\lambda$ combinations in which the $B3V_H$ is in either pG1D1 or pG1D210. This experiment was carried out twice. The IgG concentration that is shown for each heavy chain vector used to express each heavy/light chain combination is the mean of these two experiments. The error bars shown on the graph correspond to the standard error about the mean to show any variation in results between the two experiments. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. Statistical analysis shows that there is no significant difference between the levels of IgG expressed when COS-7 cells were transfected with pG1D1 heavy chain expression vector compared to COS-7 cells transfected with pG1D210 heavy chain expression vector. This is the case for both the H/L chain combinations tested i.e. $B3V_H/B3V_\lambda$ ($p=0.4$, $n=2$ calculated using Student t-test at 95% confidence level) and $B3V_H/UK-4V_\lambda$ ($p=0.2678$, $n=2$ calculated using Student t-test at 95% confidence level).

4.3.3 Anti-dsDNA binding ability of IgG1 expressed using recombinant B3V$_H$/pG1D1 compared to IgG1 expressed using recombinant B3V$_H$/pG1D210

Although the heavy chain expression vector used and consequently the possibility of expression of the extra intron (in pG1D1-derived IgG1) were not expected to affect the DNA binding ability of the IgG1 constructs, it was checked anyway to show that the results in this thesis have not been affected by the potentially inefficient splice acceptor site in pG1D1.

As illustrated in Figure 4.7, the anti-dsDNA binding ability of B3V$_H$/B3V$_\lambda$ or B3V$_H$/UK-4V$_\lambda$ combination IgG in COS-7 cell supernatants is not affected by whether recombinant pG1D1 or pG1D210 was used as the heavy chain expression vector.

The electroporations were carried out and tested by ELISA for anti-dsDNA binding on two separate occasions. Figure 4.7 shows the results of a representative experiment, however the standard deviations (SD) between the optical density (OD) readings of the two experiments were calculated for all the points on each curve and are shown in the legend. The low standard deviations indicate that similar results were seen in each of the experiments. The negative control was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-dsDNA IgG at a known concentration.

Figure 4.7 Anti-dsDNA binding ability detected by ELISA of B3V$_H$/2a2-derived V$_\lambda$ IgG in COS-7 cell supernatants transfected with recombinant pLN10 and either recombinant pg1D1 or pG1D210.

The graph shows that the anti-dsDNA binding ability of B3V$_H$/B3V$_\lambda$ or B3V$_H$/UK-4V$_\lambda$ combination IgG in COS-7 cell supernatants is not affected by whether recombinant pG1D1 or pG1D210 was used as the heavy chain expression vector. The electroporations were carried out and tested by ELISA for anti-dsDNA binding on two separate occasions. The above graph shows the results of a representative experiment. However the standard deviations (SD) between the optical density (OD) readings of the two experiments were calculated for all the points on each curve as follows: SD < 0.13 OD units for all points on curve B3V$_H$ (in pG1D1)/B3V$_\lambda$(wild type). Similarly, at all points, SD < 0.14 for B3V$_H$ (in pG1D210)/B3V$_\lambda$, SD < 0.006 for B3V$_H$ (in pG1D1)/UK-4V$_\lambda$ and SD < 0.028 for B3V$_H$ (in pG1D210)/UK-4V$_\lambda$. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-dsDNA IgG at a known concentration. The same aliquot of hybridoma supernatant was used for all the ELISAs and the SD of the positive control between the OD readings of the two experiments was always less than 0.13. The positive control of the representative experiment is shown on the graph above.
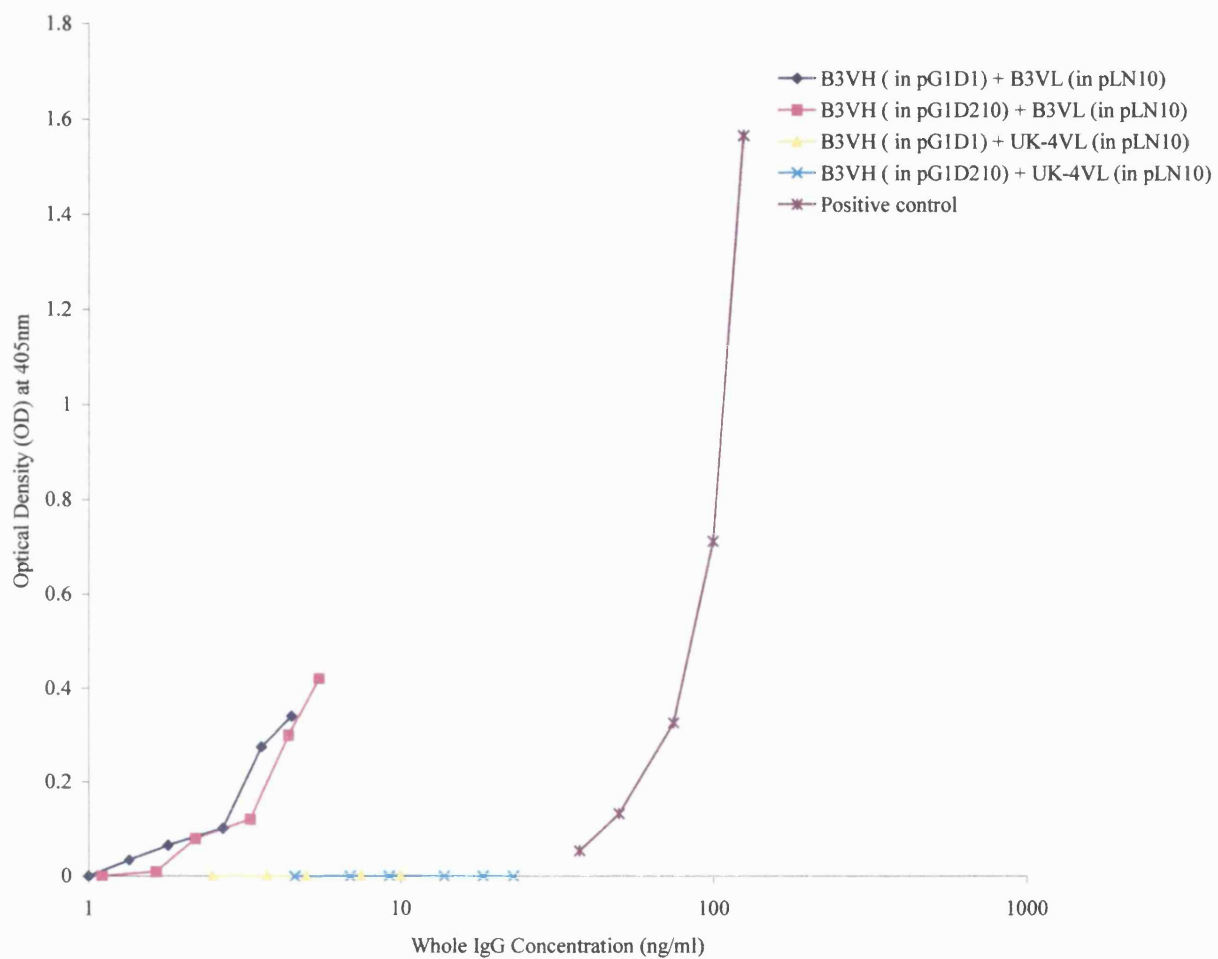
4.4 Properties of autoantibody V_H to be expressed in the eukaryotic transient expression system

4.4.1 Sequence analysis of $B3V_H$ and $33.H11V_H$

One of the main aims of this chapter was to investigate the effect of changing the heavy chain from B3 to that of another anti-DNA mAb, 33.H11. The heavy chain amino acid sequences of the two anti-DNA antibodies, B3 and 33.H11 are shown aligned in Figure 4.8 to the germline genes from which they were derived, V3-23 and V3-07, respectively. $B3V_H$ and $33.H11V_H$ both contain multiple somatic mutations and the pattern of these mutations is again consistent with an antigen-driven process. The P_{CDR} values as calculated using the Lossos Multinomial method (Lossos *et al.*, 2000) of $B3V_H$ and $33.H11V_H$ are 0.03 and 0.00008 respectively whilst the P_{FR} values are 0.02 and 0.0056. These values are consistent with antigen drive. $B3V_H$ contains a replacement mutation from serine to arginine at position 54 in V_HCDR2 whilst $33.H11V_H$ contains several arginine, asparagine and lysine residues derived from somatic mutations, particularly in 33.H11 V_HCDR1 and V_HCDR2.

4.4.2 Transfection of COS-7 cells with 33.H11 V_H / 2a2 V_λ expression vectors.

Prior to the beginning of my Ph.D., $33.H11V_H$ was cloned into the heavy chain expression vector, pG1D1 as detailed in section 2.2 by Dr Anisur Rahman. However, despite the fact that $33.H11V_H$ sequence was cloned into the expression vector pG1D1 and then co-transfected into COS-7 cells with the same light chain expression vectors as those used in chapter three, using identical methods to those used for $B3V_H$, no whole IgG could be detected in the supernatants of these transfected COS-7 cells. Consequently the $33.H11V_H$/pG1D1 expression vector used was sequenced to ensure that no sequence alterations had occurred during the cloning steps required to transfer the V_H sequence into the final heavy chain expression vector.

The sequence of 33.H11 in pG1D1 contained a nucleotide change from a C to a T in FR3 of the V_H sequence (at position 238 in the DNA sequence of $33.H11V_H$). This point mutation resulted in the replacement of a leucine residue with a proline residue (at position 78 in the amino acid sequence of $33.H11V_H$). This residue is highlighted in the

33.H11V_H DNA sequence in Figure 4.8. Proline is the most rigid of the twenty naturally occurring amino acids since its side chain is covalently linked with the main chain nitrogen. Due to proline's unique conformation, its presence has the potential to disrupt the secondary structure of an antibody protein (Stryer, 1995). It was therefore hypothesised that the additional proline in 33.H11V_H/pG1D1 FR3 was causing a change in the conformation of the heavy chain so great that it could not form a whole immunoglobulin molecule with any light chain in the transient expression system.

Once this mutation was reverted to a leucine using site-directed mutagenesis, the change was confirmed by DNA sequencing. Figure 4.9 shows the section of DNA sequencing gel that displays the 33.H11V_H FR3 before and after the proline to leucine reversion.

Once the proline residue had been reverted to the original leucine, 33.H11 heavy chains were able to be expressed in conjunction with the 2a2-derived V_λ chains, using the transient expression system as shown in section 4.4. Therefore these results supported the above hypothesis, that the mutation of a leucine to a proline in 33.H11V_H/pG1D1 FR3 due to PCR error had caused a change in the conformation of the heavy chain so great that it could not form a whole immunoglobulin molecule with any light chain in the transient expression system. It has also been reported previously that certain residues in the FRs can affect the conformation of an antibody. For example, Tramontano *et al.* (1990) showed that FR2 residue 71 is a major determinant of the position and conformation of the V_HCDR2.

```
       FR1                                        CDR1

       1        10        20        30           31
       |        |         |         |            |ab
3-23   EVQLLESGGGLVQPGGSLRLSCAASGFTFS            S..YAMS

B3     ----V----------------T-----T              T..----


3-07   EVQLVESGGGLVQPGGSLRLSCAASGFTFS            S..YWMS

33.H11 ------------------------I--               N..F--T
                                        ◁──────────▷
                                            CDR-H1


       FR2               CDR2

           40                52        60
           |                | abc      |
3-23   WVRQAPGKGLEWVS    AISG..SGGSTYYADSVKG

B3     --------------    T---..R-S-----G--Q-


3-07   WVRQAPGKGLEWVA    NIKQ..DGSEKYYVDSVKG

33.H11 -------------     --N-..G--KRS------
                    ◁────────────────▷
                           CDR-H2


       FR3                                 CDR3   J$_H$

           70        82        90             100
           |         |abc       |
3-23   RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK          NWFDPWGQGTLVTVSS

B3     ---L------S-------S-------------          PNVGSG    WS--S-----------


3-07   RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR          YFDYWGQGTLVTVSS

33.H11 --------V---P-------------------          LV       -C-------------
                                        ◁───────▷
                                           CDR-H3
```
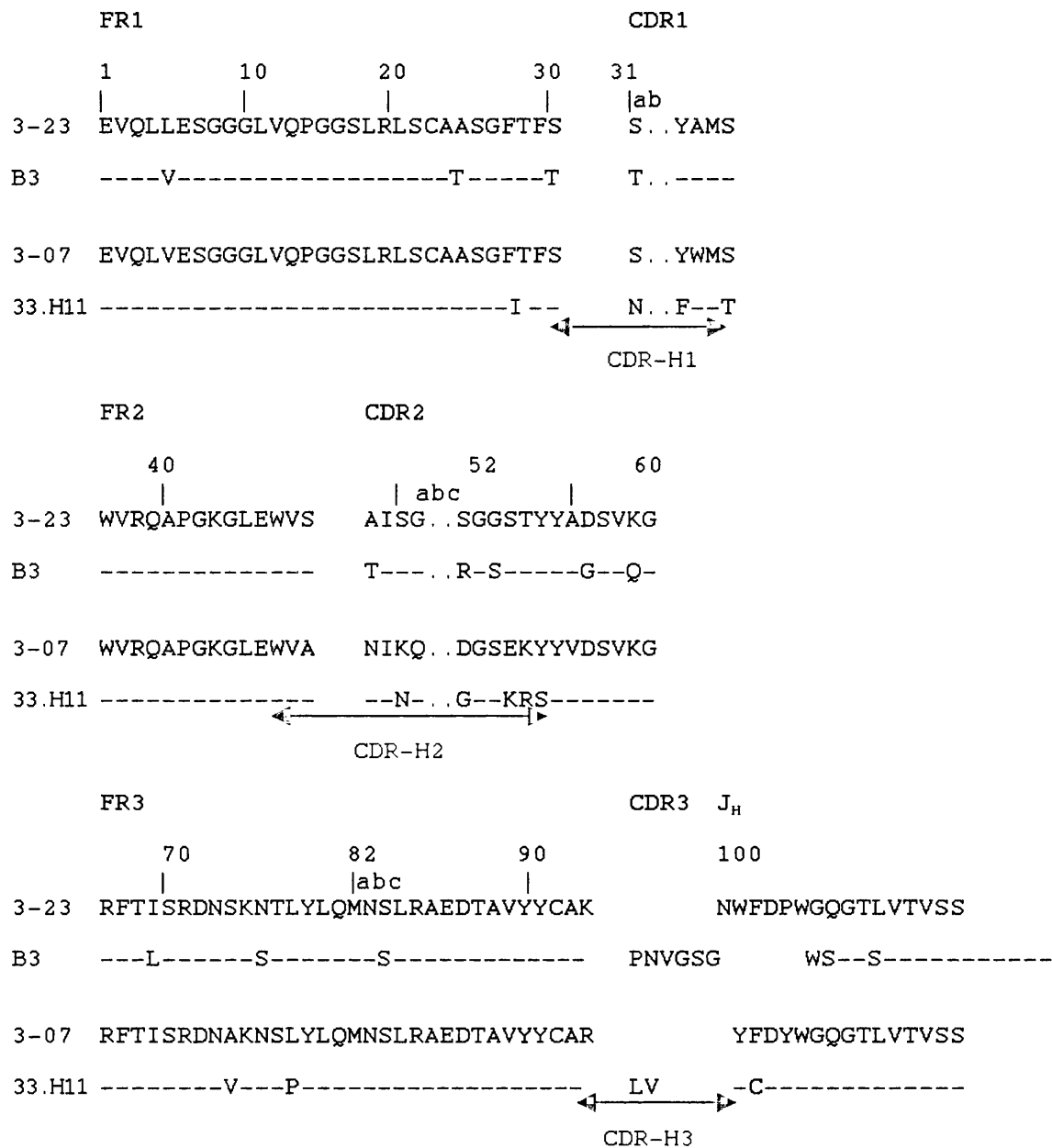
Figure 4.8	Amino acid sequences of expressed V$_H$ regions compared to their closest germline gene

The amino acid sequences of B3V$_H$ and 33.H11V$_H$ regions in pG1D1 are aligned with their closest germline gene (V3-23 and V3-07, respectively) and numbered according to Wu and Kabat (1970).

Amino acids are indicated according to their one letter code as listed in Appendix A. Dots have been inserted to facilitate the alignment. A dash indicates sequence identity with that of 2a2. Antigen contact sites, as defined by MacCallum *et al.* (1996) are shown by red arrows.

The proline residue in 33.H11 H-FR3 shown in blue is not the product of a somatic mutation but occurred during the PCR steps involved in transferring the 33.H11V$_H$ into the expression vector, pG1D1. This residue was later reverted to the original leucine using site-directed mutagenesis (see section 4.3).

FR = Framework region, CDR = Complementarity-determining region

C at position 238 →

← T at position 238

33.H11V_H/pG1D1 before site-directed mutagenesis

33.H11V_H/pG1D1 after site-directed mutagenesis to revert C at position 238 to T
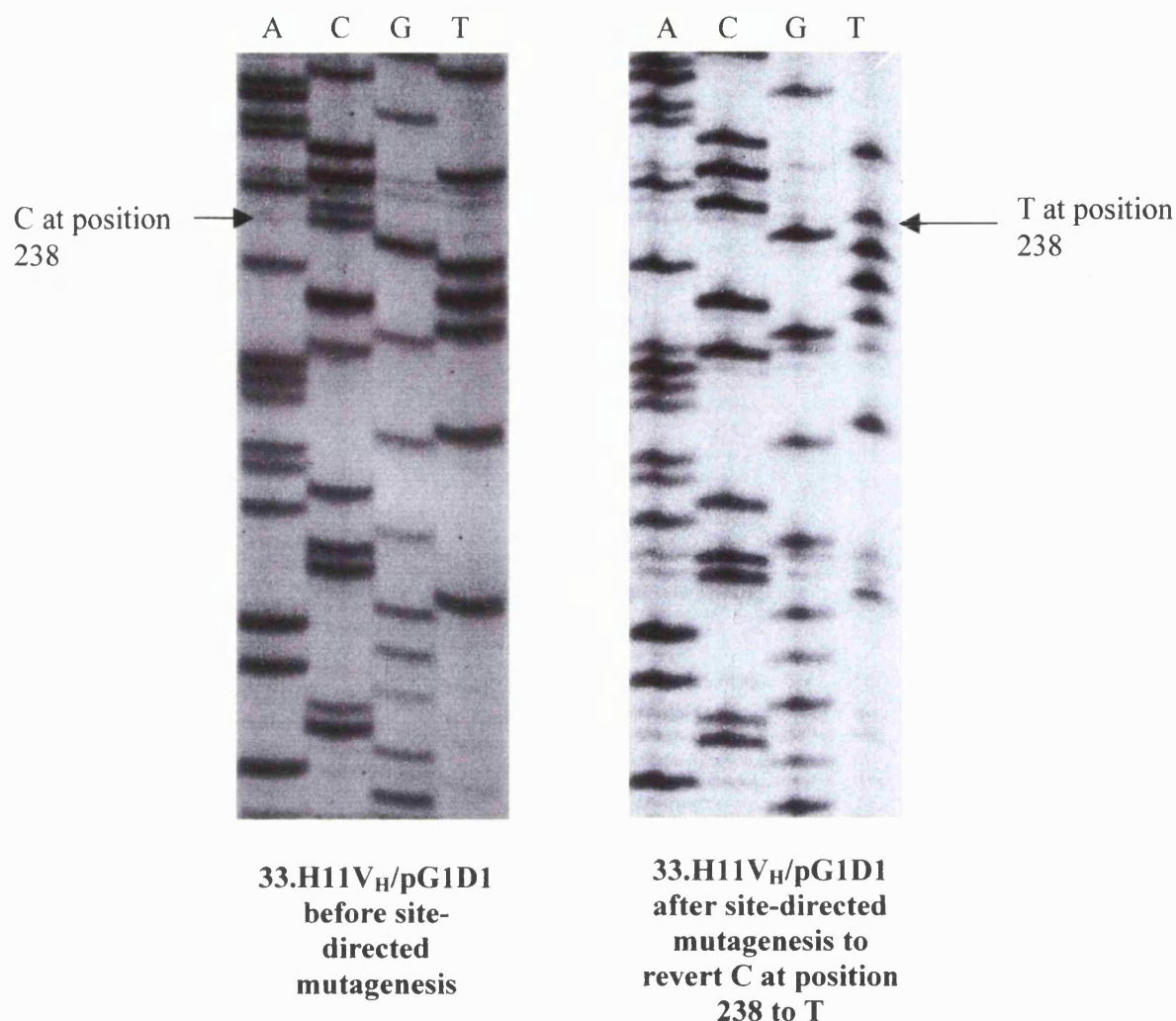
Figure 4.9 **DNA sequencing gels showing the sequence of 33.H11V_H/pG1D1 before and after site-directed mutagenesis**

The sections of sequencing gels above show the DNA sequence of 33.H11 in pG1D1 from position 227 to 269. The gel on the left shows the sequence of 33.H11V_H/pG1D1 prior to site-directed mutagenesis whilst the gel on the right shows 33.H11V_H/pG1D1 after site-directed mutagenesis was used to revert the C to a T at position 238 in the DNA sequence of 33.H11V_H FR3. This point mutation resulted in the replacement of a leucine residue with a proline residue (at position 78 in the amino acid sequence of 33.H11V_H).

4.5 Production of autoantibody hybrid 2a2-derived V_λ

In order to investigate whether the particular light chain sequence motifs identified in chapter three are in fact critical to DNA binding, entire CDRs were swapped between B3, 33.H11 and UK-4 light chains. I produced the hybrids BU (B3 CDR1, UK4 CDR2 and CDR3) and UB (UK4 CDR1, B3 CDR2 and CDR3) whilst the other hybrids 33B (33.H11 CDR1, B3 CDR2 and 3), B33 (B3 CDR1, 33.H11 CDR2 and CDR3), 33U (33.H11 CDR1, UK4 CDR2 and 3) and U33 (UK4 CDR1, 33.H11 CDR2 and CDR3) were all produced using the same method, but by my colleague, Lesley Mason.

Initially the recombinant expression vectors containing the V_λ sequence of B3, UK-4 or 33.H11 were digested with *PvuI/KpnI* to produce a 5.9 Kb (contains V_λ CDR1) and a 1.5 Kb fragment (contains V_λ CDR2 and CDR3) each. The 5.9Kb fragment of one recombinant vector was then ligated to the 1.5 Kb fragment of a different recombinant vector to produce the hybrids. To ensure that each construct contained the correct V_λ CDRs, both an *AatII* digest and a *HindIII/AvaI* digest were carried out. The sizes of the bands produced are shown in Figures 4.10 and 4.11. The figures show that the BU-hybrid V_λ sequence gives the same pattern as B3V_λ on digestion with *AatII*, but the same pattern as UK-4V_λ when digested with *HindIII/AvaI*. The converse is true for the UB-hybrid V_λ. Thus these digests confirm that the hybrids contain part of B3V_λ and part of UK-4V_λ and furthermore which particular clones contain which CDRs of each mAb, as they were designed to do.

Figure 4.10 *AatII* **restriction digest of BU and UB hybrid V_λ on a 0.7% agarose gel.**

a) Expected bands

b) Actual bands on 0.7% agarose gel

Lane A	1kb DNA molecular weight marker (Gibco)
Lane B	No bands shown (the sample run in this lane was not relevant to this particular set of results and is therefore hidden)
Lane C	BU-hybrid V_λ in pLN10
Lane D	B3V_λ in pLN10
Lane E	UK-4V_λ in pLN10
Lane F	UB-hybrid V_λ in pLN10

Due to their small size it was very difficult to distinguish the smaller bands.

a)

5780 bp →

1352 bp →
995 bp →

357 bp →

186 bp →
83 bp
53 bp

C　　D　　E　　F

b)

A　B　C　D　E　F

6108 bp →
5090 bp

1636 bp →

1018 bp →

344 bp →

← 5780 bp

← 1352 bp
← 995 bp

← 357 bp

← 186 bp
　83 bp
　53 bp
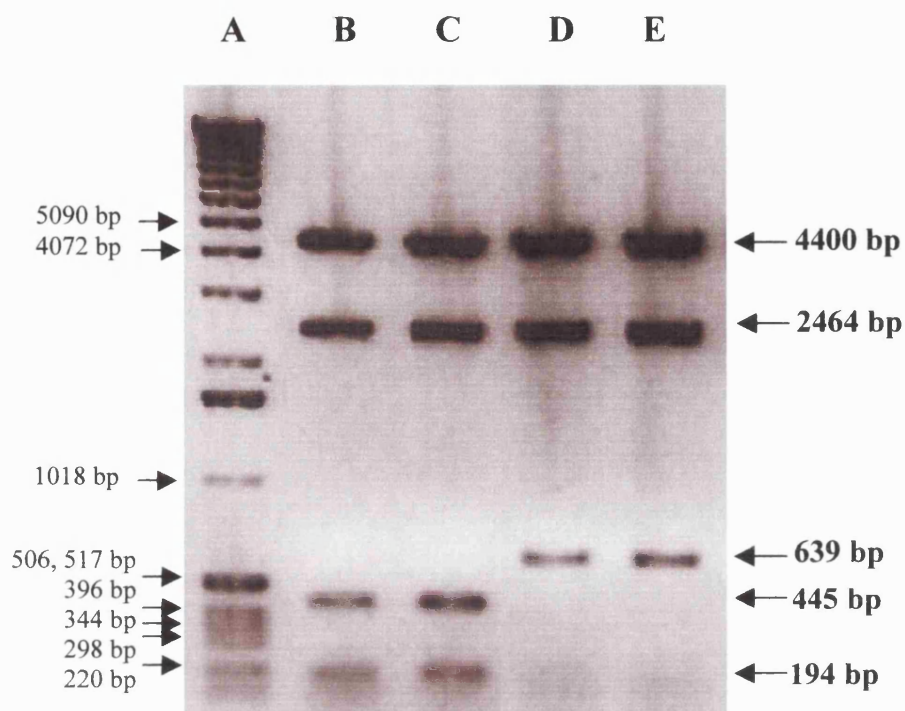
Figure 4.11 *AvaI/HindIII* **restriction digest of BU and UB hybrid V_λ on a 0.7% agarose gel.**

Lane A   1kb DNA molecular weight marker (Gibco)

Lane B   BU-hybrid V_λ in pLN10

Lane C   UK-4V_λ in pLN10

Lane D   UB-hybrid V_λ in pLN10

Lane E   B3V_λ in pLN10

4.6   Expression of whole IgG1 molecules in COS-7 cells

The following light chains were expressed in combination with both $B3V_H$ and $33.H11V_H$ in COS-7 cells; $B3V_\lambda$, $B3V_\lambda a$, $B3V_\lambda b$, $33.H11V_\lambda$, UK-$4V_\lambda$, $B33V_\lambda$ hybrid, $33BV_\lambda$ hybrid, BUV_λ hybrid, UBV_λ hybrid, $U33V_\lambda$ hybrid and $33UV_\lambda$ hybrid. Three or more expression experiments were carried out for each combination.

The mean whole IgG1 yields (± standard deviation) (after concentration of the supernatant) obtained in the expression experiments are shown in Table 4.1. The negative control sample supernatant was derived from COS-7 cells that were electroporated without plasmid DNA and contained no detectable IgG1. For the majority of combinations, the whole IgG1 yields for each V_H/ V_λ combination were similar between each of the expression experiments. However, IgG1 yields of some of the light/heavy chain combinations varied considerably. This could have been for a number of reasons, such as variable post-electroporation COS-7 cell growth, or transfection efficiency. As in the transient expression experiments reported in chapter three of this thesis, all combinations with $B3V_H$ and constructs containing UK-$4V_\lambda$ CDR3 produced noticeably higher yields of whole IgG1 than the other combinations. Again there is no clear reason for this. In contrast the IgG1 yields for the $33.H11V_H/2a2$-derived V_λ combinations varied less and were all in the same approximate range of 10-32 ng/ml, except for the $33.H11V_H/B33$ hybrid V_λ construct whose IgG1 yield was lower.

Heavy Chain	2a2-derived Light chain	Mean IgG concentrations (ng/ml)	Standard Deviation (SD)
B3	B3	12.8	± 2.9
B3	33.H11	6.8	± 2.4
B3	UK-4	69.3	± 30.0
B3	BU-hybrid	102.4	± 65.3
B3	UB-hybrid	29.2	± 8.8
B3	33U-hybrid	99.3	± 44.2
B3	U33-hybrid	4.8	± 1.6
B3	B33-hybrid	5.2	± 3.8
B3	33B-hybrid	23.0	± 7.0
B3	B3a	16.0	± 11.3
B3	B3b	19.0	± 17.0
33.H11	B3	19.0	± 5.4
33.H11	33.H11	15.5	± 2.5
33.H11	UK-4	25.7	± 5.1
33.H11	BU-hybrid	58.0	± 28.1
33.H11	UB-hybrid	50.0	± 24.6
33.H11	33U-hybrid	40.2	± 20.3
33.H11	U33-hybrid	23.0	± 9.2
33.H11	B33-hybrid	19.2	± 2.9
33.H11	33B-hybrid	23.3	± 5.5
33.H11	B3a	23.7	± 4.7
33.H11	B3b	25.0	± 1.0
no DNA	no DNA	0	0

Table 4.1 **The mean whole IgG1 yields produced for each heavy/light chain construct during the expression experiments, detected by ELISA.**

For each electroporation, the whole IgG1 yields for each V_H/V_L combination were detected by ELISA. The mean whole IgG1 yields (± standard deviation) (after concentration of the supernatant) obtained in the expression experiments are shown above. The negative control sample supernatant was derived from COS-7 cells that were electroporated without plasmid DNA and contained no detectable IgG1.

4.7 Anti-DNA activity of expressed whole IgG1 heavy/light chain combinations

The dsDNA and ssDNA binding ability of each of the B3V_H/2a2-derived-V_λ and 33.H11/2a2-derived V_λ combinations were tested by ELISA and are shown in Figures 4.12 to 4.15. Each expression experiment (i.e. electroporation and subsequent anti-DNA ELISAs) was carried out three times and in each case, similar results were seen in each. Figures 4.12 to 4.15 show the DNA binding results of a single representative experiment. However the standard deviations (SD) between the optical density (OD) readings of the three experiments were calculated for all the points on each curve. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant known to contain anti-DNA IgG at a known concentration. The same aliquot of hybridoma supernatant was used for all the ELISAs and the standard deviation of the positive control between the OD readings of the three experiments was always less than 0.1 in both the anti-dsDNA and anti-ssDNA ELISAs.

The B3V_H/B33 hybrid V_λ combination consistently showed the strongest binding to dsDNA. This combination was followed closely by the wild type B3V_H/B3V_λ (wild type), and the B3V_H/U33 hybrid V_λ. As reported before in chapter three of this thesis, the single reversion of an arginine to a serine in CDR1 led to a slight reduction in binding to dsDNA as did the presence of the 33.H11V_λ chain. dsDNA binding was further reduced in those combinations lacking both the 33.H11V_λ CDR2 and CDR3 and the B3V_λ CDR1, (i.e. B3V_H/33B and B3V_H/UB) such that approximately double the concentration of IgG1 was required to obtain the same OD reading as that of the highest dsDNA binding combination, B3V_H/B33. Figure 4.14 shows that binding to ssDNA gave similar results for these different combinations, although the overall OD readings were lower.

Despite being tested at a range of concentrations between four and fifty times higher than those which gave the highest binding to dsDNA for the other combinations, those combinations containing the UK-4V_λ CDR2 and CDR3 showed no binding to dsDNA

regardless of the origin of the V_λ CDR1 present (i.e. B3V_H/UK-4V_λ, B3V_H/BU V_λ and B3V_H/33U V_λ).

When the 33.H11V_H was expressed with the same 11 lambda chains there was significantly less binding to dsDNA and ssDNA despite the IgG1 yields being in the same concentration range or higher than those obtained using B3V_H. In fact, the only construct to show significant binding to dsDNA or ssDNA in combination with 33.H11V_H was the 33.H11V_H/B33-hybrid V_λ combination. There was an indication of slight binding to dsDNA and ssDNA by the 33.H11V_H/33.H11V_λ combination, however the *OD* achieved was very low (see Figures 4.13 and 4.15). It can be concluded that B3V_H confers a higher ability to bind dsDNA and ssDNA than 33.H11V_H when paired with the same set of light chains.

Figure 4.12 Anti-dsDNA activity of B3 heavy chain/2a2-derived λ chain IgG in COS-7 cell supernatants, detected by ELISA.

The graph shows binding of IgG in COS-7 cell supernatants containing each B3 heavy chain/λ gene 2a2-derived light chain combination to dsDNA. The electroporations were carried out and tested by ELISA for anti-dsDNA binding on three separate occasions. The above graph shows the results of a representative experiment (after one-hour incubation with substrate). However the standard deviations (SD) between the optical density (OD) readings of the three experiments were calculated for all the points on each curve as follows: SD < 0.2 OD units for all points on curve $B3V_H/B3V_\lambda$(wild type). Similarly, at all points, SD < 0.027 for $B3V_H/B3V_\lambda a$, SD < 0.056 for $B3V_H/B3V_\lambda b$, SD < 0.15 for $B3V_H/33.H11V_\lambda$, SD < 0.033 for $B3V_H/UK-4V_\lambda$, SD < 0.04 for $B3V_H/BU$-hybrid V_λ, SD < 0.043 for $B3V_H/UB$-hybrid V_λ, SD < 0.047 for $B3V_H/33B$-hybrid V_λ, SD < 0.18 for $B3V_H/B33$-hybrid V_λ, SD < 0.051 for $B3V_H/33U$-hybrid V_λ and SD < 0.045 for $B3V_H/U33$-hybrid V_λ. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-dsDNA IgG at a known concentration. The same aliquot of hybridoma supernatant was used for all the ELISAs and the SD of the positive control between the OD readings of the three experiments was always less than 0.1. The positive control of the representative experiment is shown on the graph above.
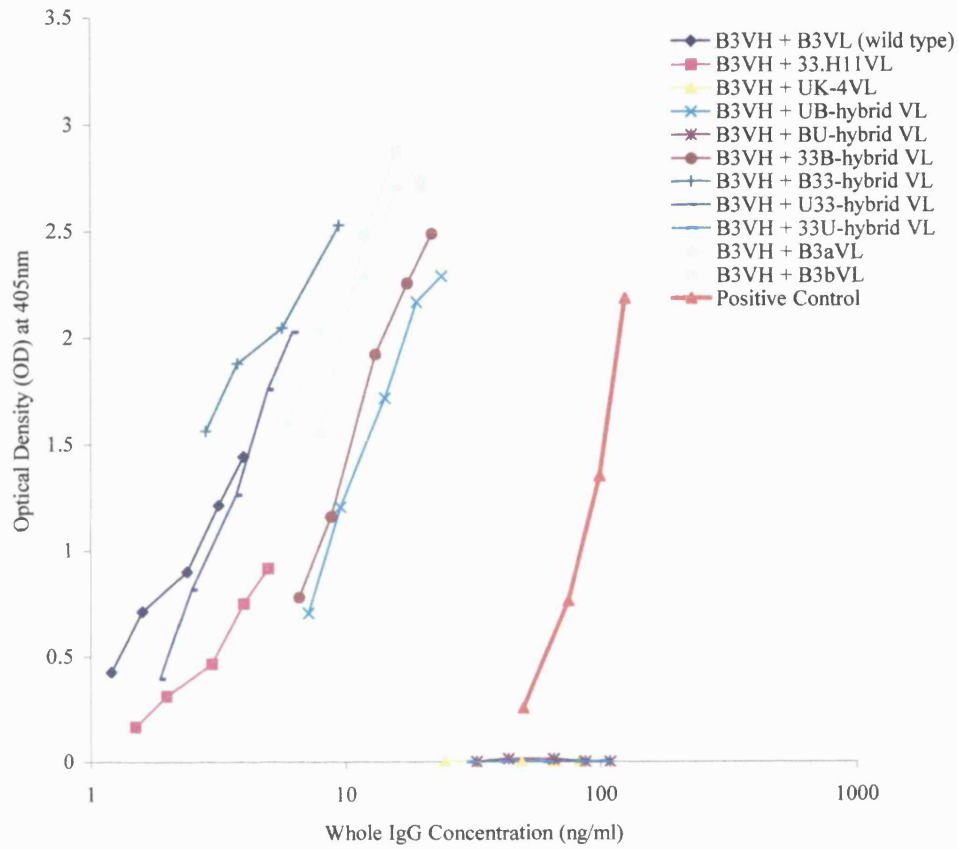
Figure 4.13 Anti-dsDNA activity of 33.H11 heavy chain/λ gene 2a2-derived light chain IgG in COS-7 cell supernatants, detected by ELISA.

The graph shows binding of IgG in COS-7 cell supernatants containing each 33.H11 heavy chain/λ gene 2a2-derived light chain combination to dsDNA. The electroporations were carried out and tested by ELISA for anti-dsDNA binding on three separate occasions. The above graph shows the results of a representative experiment (after one-hour incubation with substrate). However the standard deviations (SD) between the optical density (OD) readings of the three experiments were calculated for all the points on each curve as follows: SD < 0.005 OD units for all points on curve 33.H11V_H/B3V_λ(wild type). Similarly, at all points, SD < 0.022 for 33.H11V_H/B3V_λa, SD < 0.0028 for 33.H11V_H/B3V_λb, SD < 0.017 for 33.H11V_H/33.H11V_λ, SD = 0 for 33.H11V_H/UK-4V_λ, SD < 0.0021 for 33.H11V_H/BU-hybrid V_λ, SD < 0.0042 for 33.H11V_H/UB-hybrid V_λ, SD < 0.0064 for 33.H11V_H/33B-hybrid V_λ, SD < 0.1 for 33.H11V_H/B33-hybrid V_λ, SD < 0.0064 for 33.H11V_H/33U-hybrid V_λ and SD < 0.1 for 33.H11V_H/U33-hybrid V_λ. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-dsDNA IgG at a known concentration. The same aliquot of hybridoma supernatant was used for all the ELISAs and the SD of the positive control between the OD readings of the three experiments was always less than 0.19. The positive control of the representative experiment is shown on the graph above. The graph above is plotted on a different y-axis to the graph in figure 4.12 as the OD readings are very low for this data set.
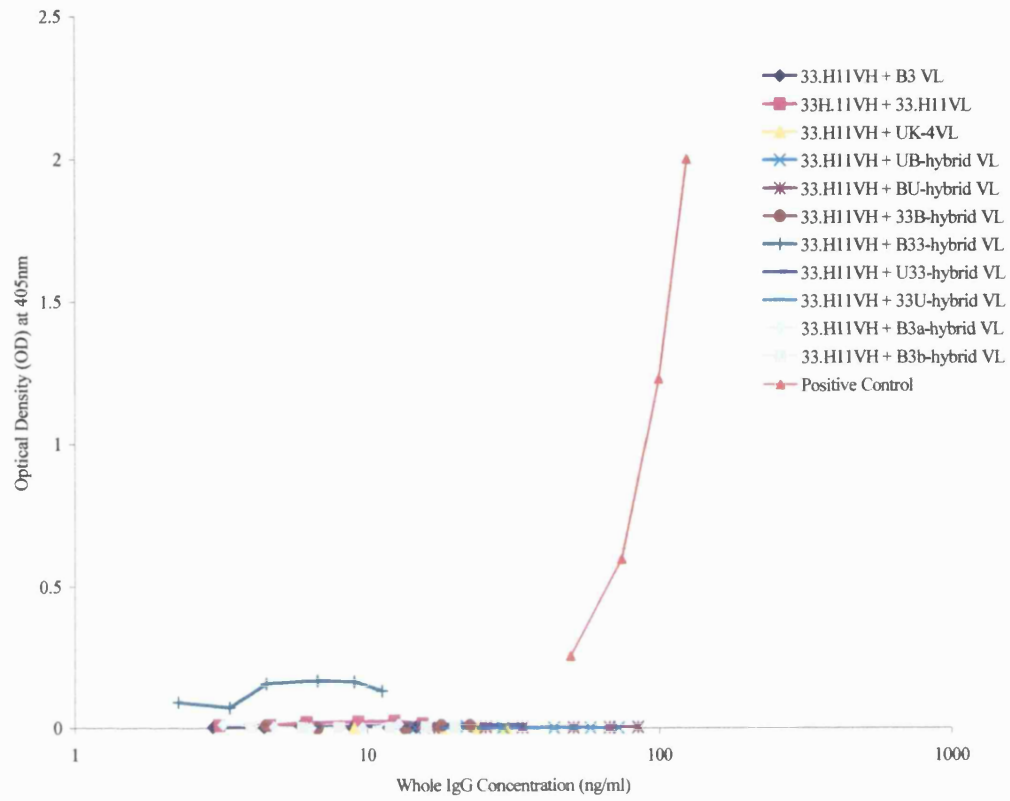
Figure 4.14 Anti-ssDNA activity of B3 heavy chain/2a2-derived λ chain IgG in COS-7 cell supernatants, detected by ELISA.

The graph shows binding of IgG in COS-7 cell supernatants containing each B3 heavy chain/λ gene 2a2-derived light chain combination to dsDNA. The electroporations were carried out and tested by ELISA for anti-dsDNA binding on three separate occasions. The above graph shows the results of a representative experiment (after one-hour incubation with substrate). However the standard deviations (SD) between the optical density (OD) readings of the three experiments were calculated for all the points on each curve as follows: SD < 0.23 OD units for all points on curve B3V_H/B3V_λ(wild type). Similarly, at all points, SD < 0.24 for B3V_H/B3V_λa, SD < 0.19 for B3V_H/B3V_λb, SD < 0.024 for B3V_H/33.H11V_λ, SD < 0.0042 for B3V_H/UK-4V_λ, SD < 0.05 for B3V_H/BU-hybrid V_λ, SD < 0.11 for B3V_H/UB-hybrid V_λ, SD < 0.05 for B3V_H/33B-hybrid V_λ, SD < 0.038 for B3V_H/B33-hybrid V_λ, SD < 0.004 for B3V_H/33U-hybrid V_λ and SD < 0.13 for B3V_H/U33-hybrid V_λ. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-dsDNA IgG at a known concentration. The same aliquot of hybridoma supernatant was used for all the ELISAs and the SD of the positive control between the OD readings of the three experiments was always less than 0.1. The positive control of the representative experiment is shown on the graph above.
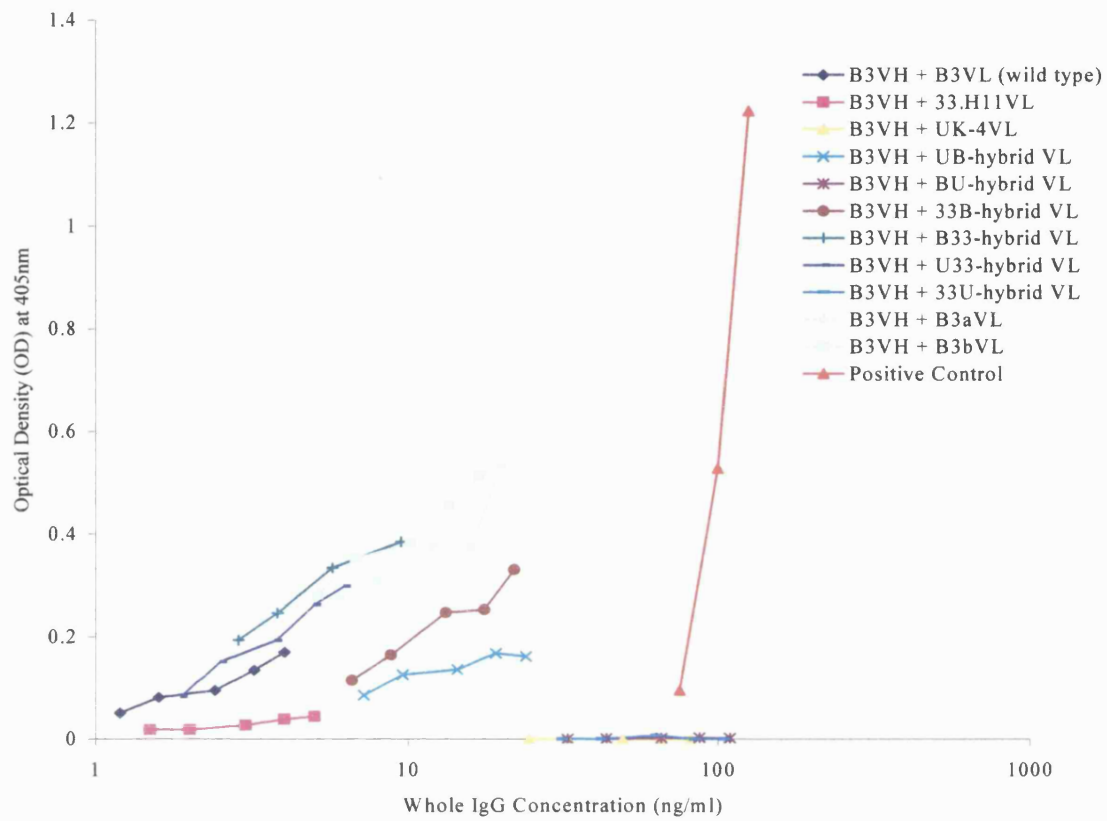
Figure 4.15 Anti-ssDNA activity of 33.H11 heavy chain/λ gene 2a2-derived light chain IgG in COS-7 cell supernatants, detected by ELISA.

The graph shows binding of IgG in COS-7 cell supernatants containing each 33.H11 heavy chain/λ gene 2a2-derived light chain combination to ssDNA. The electroporations were carried out and tested by ELISA for anti-dsDNA binding on three separate occasions. The above graph shows the results of a representative experiment (after one-hour incubation with substrate). However the standard deviations (SD) between the optical density (*OD*) readings of the three experiments were calculated for all the points on each curve as follows: SD < 0.001 *OD* units for all points on curve 33.H11V_H/B3V_λ(wild type). Similarly, at all points, SD < 0.021 for 33.H11V_H/B3V_λa, SD < 0.0035 for 33.H11V_H/B3V_λb, SD < 0.03 for 33.H11V_H/33.H11V_λ, SD < 0.021 for 33.H11V_H/UK-4V_λ, SD < 0.01 for 33.H11V_H/BU-hybrid V_λ, SD < 0.0078 for 33.H11V_H/UB-hybrid V_λ, SD < 0.001 for 33.H11V_H/33B-hybrid V_λ, SD < 0.053 for 33.H11V_H/B33-hybrid V_λ, SD < 0.0021 for 33.H11V_H/33U-hybrid V_λ and SD < 0.012 for 33.H11V_H/U33-hybrid V_λ. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-dsDNA IgG at a known concentration. The same aliquot of hybridoma supernatant was used for all the ELISAs and the SD of the positive control between the *OD* readings of the three experiments was always less than 0.096. The positive control of the representative experiment is shown on the graph above. The graph above is plotted on a different y-axis to the graph in figure 4.14 as the *OD* readings are very low for this data set.
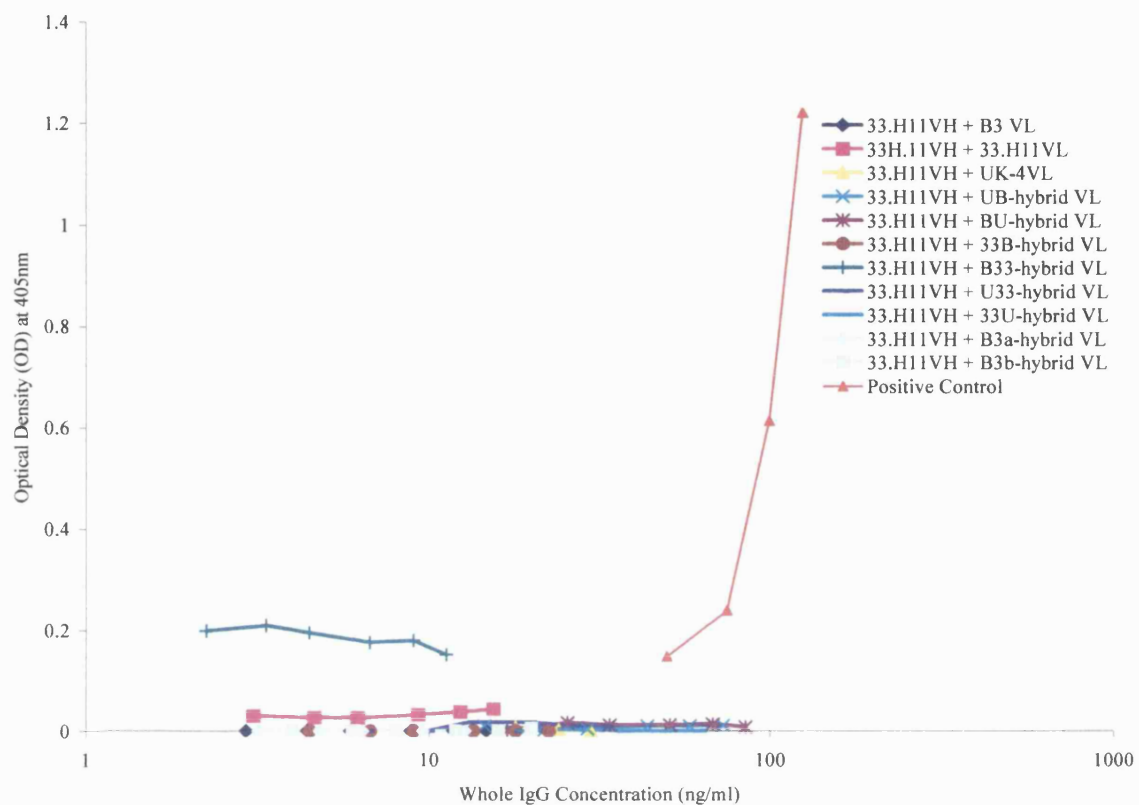
4.8   Line immunoassay of expressed whole IgG1 heavy/light chain combinations

Whereas, in the previous chapter, only binding to ssDNA and dsDNA was assayed, in this chapter binding to a much larger range of nuclear antigens relevant to autoimmune rheumatic disease was also tested. Initially a screening test for binding to a range of antigens was carried out using a line immunoassay. This enabled exclusion of binding to a number of these antigens.

The principle of the line assay was that the autoantibodies in a sample were tested for binding to autoantigens that were present on a nylon strip. The strips provided by the kit were coated with recombinant antigens (SmB, RNP-70k, RNP-A, RNP-C, Ro52, La/SSB, Cenp-B, Topo-I, Jo-1), synthetic peptides (SmD and ribosomal P) and natural proteins (Ro60, histones) as discrete lines on a nylon membrane. This binding was detected using an enzyme to digest a chromogenic substrate in the same manner as in the ELISAs. The presence of antibodies to a particular autoantigen on the strip was detected if a dark brown line appeared at the point on the strip corresponding to that autoantigen. COS-7 supernatant from the no DNA negative control was used as the negative control in the line assay. The positive control used was provided with the kit and consisted of a mixture of human plasma and sera, containing antibodies against a large range of nuclear antigens relevant to autoimmune rheumatic disease. Furthermore the first line on each strip is the sample addition control line. A dark brown line at this point signified the presence of IgG antibodies in the supernatant. If the supernatant did not contain antibodies able to bind any of the autoantigens on the strip, the labelled anti-human antibody supplied with the kit did not bind to autoantigen/autoantibody immune complexes and as a result only the control line and a low background colour developed. Each COS-7 supernatant containing each heavy/light chain combination was tested with its own strip and these strips are shown in Figure 4.16. The binding characteristics of the IgG1 produced in the transient expression systems are also summarised in Table 4.2.

Overall, the expressed IgG1 only bound to SmD, Ro-60, Scl-70 (DNA topoisomerase I) and histones. This screening assay gave either positive or negative results for binding. The strength of binding could not be measured quantitatively.

All of the $B3V_H$/2a2-derived V_λ combinations, except for those containing the UK-4 CDR2 and 3, bound histones in the line immunoassay. The combinations that bound to histones were the combinations also shown to bind to dsDNA and ssDNA in the anti-DNA ELISA. All those $B3V_H$ combinations containing the $B3V_\lambda$ CDRs 2 and 3 were observed to bind to SmD regardless of the V_λ CDR1 origin. However, in contrast to the binding of histones and DNA, the presence of the 33.H11V_λ CDR3 did not necessarily confer the ability to bind SmD. Although $B3V_H$/33.H11V_λ did bind to SmD, $B3V_H$/B33-hybridV_λ and $B3V_H$/U33-hybrid V_λ did not. Again, despite being tested at a range of concentrations between four and fifty times higher than those that did bind SmD for the other combinations, those combinations containing the UK-4V_λ CDR2 and 3 showed no binding to SmD regardless of the origin of the CDR1 present.

The results of binding to SSA/Ro60 are particularly interesting, in that antibodies containing UK-4V_λ CDR3 are capable of binding to this antigen. The blocking effect of this CDR, which is apparently dominant in binding to dsDNA, ssDNA and histones, does not affect binding to SSA/Ro60. Binding to Ro was not investigated further in this thesis due to limitations of time. When the $B3V_H$ was replaced by the 33.H11V_H with the same 2a2-derived lambda chains, no binding to any of the line immunoassay antigens was observed, despite the IgG1 yields being in the same concentration range or higher than those obtained using $B3V_H$.

Figure 4.16   Line immunoassay of expressed whole IgG1 heavy/light chain combinations.

The presence of antibodies to a particular autoantigen on the strip is considered positive if a dark brown line appeared at the point on the strip corresponding to that autoantigen. COS-7 supernatant from the no DNA negative control was used as the negative control in the line assay. The positive control used was provided with the kit and consisted of a mixture of human plasma and sera, containing antibodies against a large range of nuclear antigens relevant to autoimmune rheumatic disease. Furthermore the first line on each strip is the sample addition control line. A dark brown line at this point signified the presence of IgG antibodies in the supernatant. If the supernatant did not contain antibodies able to bind any of the autoantigens on the strip, the labelled anti-human antibody supplied with the kit did not bind to autoantigen/autoantibody immune complexes and as a result only the control line and a low background colour developed. Each COS-7 supernatant containing each heavy/light chain combination was tested with its own strip.

Letters A-N show the position of the antigens on the immunoassay strip whilst numbers 1-13 indicate which H/L chain combination IgG are in each COS-7 supernatant tested.

Key:

A	Control line	H	SSA/Ro60
B	SmB	I	SSB/La
C	SmD	J	Cenp-B
D	RNP-70k	K	Topo-I/Scl-70
E	RNP-A	L	Jo-1/HRS
F	RNP-C	M	Ribosomal P
G	SSA/Ro52	N	Histones

1	Positive control	8	B3V_H/B3V_λa
2	B3V_H/B3V_λ	9	B3V_H/B3V_λb
3	B3V_H/33BV_λ-hybrid	10	B3V_H/UK-4V_λ
4	B3V_H/UBV_λ-hybrid	11	B3V_H/33UV_λ-hybrid
5	B3V_H/33.H11V_λ	12	B3V_H/BUV_λ-hybrid
6	B3V_H/B33V_λ-hybrid	13	No DNA control
7	B3V_H/U33V_λ-hybrid	14	Positive control

COMBINATION	ANTIGEN			
	SmD	SSA/ Ro60	Topo-I/ Scl-70	Histones
B3V$_H$ + B3VL (wild type)	+	-	+	+
B3V$_H$ + 33.H11V$_L$	+	+	+	+
B3V$_H$ + UK-4V$_L$	-	+	-	-
B3V$_H$ + UB-hybrid V$_L$	+	-	+	+
B3V$_H$ + BU-hybrid V$_L$	-	+	-	-
B3V$_H$ + U33-hybrid V$_L$	-	+	+	+
B3V$_H$ + 33U-hybrid V$_L$	-	+	-	-
B3V$_H$ + B33-hybrid V$_L$	-	+	+	+
B3V$_H$ + 33B-hybrid V$_L$	+	-	+	+
B3V$_H$ + B3a V$_L$	+	+	+	+
B3V$_H$ + B3b V$_L$	+	+	+	+
All 33.H11V$_H$/2a2-derived V$_L$	-	-	-	-
No DNA (negative control)	-	-	-	-

Table 4.2   Summary of the binding characteristics of the whole IgG1 produced in the transient expression system when screened against a range of nuclear and cytoplasmic antigens using the INNO-LIA® ANA Update kit (Innogenetics, Ghent, Belgium).

When screened against a range of nuclear and cytoplasmic antigens, at least one of the heavy/light chain combination IgG1 produced in the transient expression system bound to the four autoantigens shown in the table above. No binding by any of the transiently expressed heavy/light chain combination IgG1 was seen towards the rest of the autoantigens tested in the line immunoassay. The negative control used was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation. The positive control used was provided with the kit and consisted of a mixture of human plasma and sera, containing antibodies against a large range of nuclear antigens relevant to autoimmune rheumatic disease. "+" represents binding of the IgG heavy/light chain combination to the antigen whilst "–" represents no binding.

N.B. All those V_H/V_L combinations shown to bind histones in the Line Immunoassay also bound DNA in the direct ELISA.

4.9   Treatment of COS-7 Supernatants with Ethidium Bromide to remove DNA

Ethidium bromide can be used to intercalate with DNA so as to prevent it from participating in a reaction, thus excluding the possibility that an observed protein-protein interaction is an artefact arising from the fact that both proteins bind DNA (Budhram-Mahadeo *et al.*, 1998; Lai *et al.*, 1992).

In this experiment, the purpose of using ethidium bromide was to confirm that any positive result should be due to direct binding of the antibodies in the supernatant to histones and not due to bound DNA.

When the supernatants of B3V_H/B3V_λ were treated with concentrations of ethidium bromide varying from 10-400μg/ml prior to the line immunoassay, binding to histones of each H/L chain combination was not altered, as shown in Figure 4.17. These results suggest that the antibodies do bind histones independently from dsDNA.

Figure 4.17 **Line immunoassay of expressed whole IgG1 heavy/light chain combinations following treatment with varying concentrations of ethidium bromide**

The presence of antibodies to a particular autoantigen on the strip is considered positive if a dark brown line appeared at the point on the strip corresponding to that autoantigen. COS-7 supernatant from the no DNA negative control was used as the negative control in the line assay. The positive control used was provided with the kit and consisted of a mixture of human plasma and sera, containing antibodies against a large range of nuclear antigens relevant to autoimmune rheumatic disease. Furthermore the first line on each strip is the sample addition control line. A dark brown line at this point signified the presence of IgG antibodies in the supernatant. If the supernatant did not contain antibodies able to bind any of the autoantigens on the strip, the labelled anti-human antibody supplied with the kit did not bind to autoantigen/autoantibody immune complexes and as a result only the control line and a low background colour developed. Each COS-7 supernatant containing each heavy/light chain combination was tested with its own strip.

Letters A-N show the position of the antigens on the immunoassay strip whilst numbers 1-7 indicate the concentration of ethidium bromide added to each test supernatant containing $B3V_H/B3V_\lambda$ chain combination IgG, during the line immunoassay.
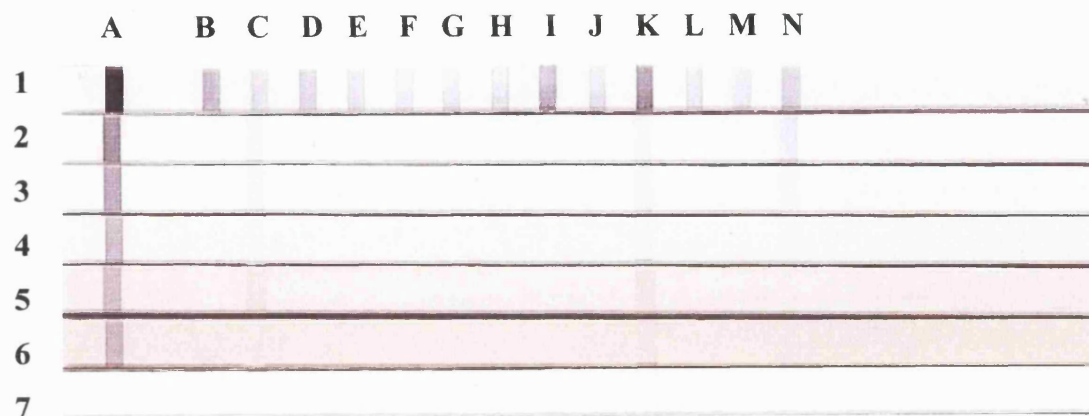
Key:

A	Control line	H	SSA/Ro60
B	SmB	I	SSB/La
C	SmD	J	Cenp-B
D	RNP-70k	K	Topo-I/Scl-70
E	RNP-A	L	Jo-1/HRS
F	RNP-C	M	Ribosomal P
G	SSA/Ro52	N	Histones

1	Positive control	
2	$B3V_H/B3V_\lambda$	(no ethidium bromide)
3	$B3V_H/B3V_\lambda$	+ 10 µg/ml ethidium bromide
4	$B3V_H/B3V_\lambda$	+ 50 µg/ml ethidium bromide
5	$B3V_H/B3V_\lambda$	+ 200 µg/ml ethidium bromide
6	$B3V_H/B3V_\lambda$	+ 400 µg/ml ethidium bromide
7	No DNA control	

4.10   Anti-histone   activity   of   expressed   whole   IgG1   heavy/light   chain combinations

The results of the line immunoassay showed that when paired with $B3V_H$ the pattern of somatic mutations in the 2a2-derived light chains determined whether the H/L combination bound to histones. Consequently binding to histones was investigated more precisely using direct ELISA, in view of the possible pathogenic relevance of this antigen.

The binding to histones of each of the $B3V_H$/2a2-derived V_λ combinations is shown in Figure 4.18. Although none of the 33.H11V_H/2a2-derived V_λ. combinations bound histones in the line assay, binding to histones was also assayed by ELISA for these combinations, as shown in Figure 4.19. The electroporations were carried out and tested by ELISA for anti-histone binding on three separate occasions and both figures show the results of a representative experiment. However the standard deviations (SD) between the optical density (*OD*) readings of the three experiments were calculated for all the points on each curve. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-histones IgG1 at a known concentration. The same aliquot of hybridoma supernatant was used for all the ELISAs and the SD of the positive control between the *OD* readings of the three experiments was always less than 0.15.

As previously seen for binding to dsDNA and ssDNA, the presence of UK-4V_λ CDR2 and 3 in light chains UK-4 (wild type), BU and 33U appeared to prevent binding to histones. The other light chains tested all bound histones, when paired with $B3V_H$, but the relative ability of the combinations to bind to histones differed from their relative ability to bind DNA. The $B3V_H$/$B3V_\lambda$ combination consistently showed the strongest binding to histones when compared to the rest of the H/L chain combinations, at the same IgG concentration. However, whereas the presence of $B3V_\lambda$ CDR1 is most important in binding to DNA, the presence of $B3V_\lambda$ CDR2 and 3 is more important in binding to histones. The R to S reversion in CDR1 of $B3V_\lambda$ reduced binding to histones when compared to the wild type $B3V_\lambda$, as shown by the downward shift of the curve for

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B3V$_\lambda$a in Figure 4.18. Replacement of B3V$_\lambda$ CDR1 with CDR1 of UK-4V$_\lambda$ or 33.H-11V$_\lambda$.(hybrids UB and 33B) reduced binding to a similar extent and these three curves occupy similar positions on the graph in Figure 4.18. Thus, the CDR1 arginine motif, which is important in binding to dsDNA, also seems to have some relevance in binding to histones.

The replacement of B3V$_\lambda$ CDR2 and 3 by 33.H11V$_\lambda$ CDR2 and 3 (hybrids B33, U33 and wild type 33.H11 light chain) led to a much larger reduction in binding to histones. At the same approximate concentration of whole IgG1, the *OD* reading for these combinations was reduced by a factor of three, when compared with the values obtained using the wild type B3 V$_H$ sequence.

The differences between the results obtained in the anti-DNA and anti-histone ELISAs are best illustrated by considering hybrids B33 and U33. In combination with B3 heavy chain, these hybrids give very good at binding to DNA, but are poor at binding to histones. This suggests that the presence of B3V$_\lambda$ CDR2 and 3 is more important in binding to histones than in binding to DNA.

As shown in Figure 4.19, when 33.H11V$_H$ was expressed with the same 2a2-derived lambda chains, no binding to histones was observed above background levels despite the IgG1 yields being in the same concentration range or higher than those obtained using B3V$_H$.

Figure 4.18 Anti-histone activity of B3 heavy chain/2a2-derived λ chain IgG in COS-7 cell supernatants, detected by ELISA.

The graph shows binding of IgG in COS-7 cell supernatants containing each B3 heavy chain/λ gene 2a2-derived light chain combination to histones (calf thymus). The electroporations were carried out and tested by ELISA for anti-histone binding on three separate occasions. The graph opposite shows the results of a representative experiment (after one-hour incubation with substrate). However the standard deviations (SD) between the optical density (*OD*) readings of the three experiments were calculated for all the points on each curve as follows: SD < 0.0455 *OD* units for all points on curve B3V_H/B3V_λ(wild type). Similarly, at all points, SD < 0.22 for B3V_H/B3V_λa, SD < 0.19 for B3V_H/33.H11V_λ, SD < 0.0086 for B3V_H/UK-4V_λ, SD < 0.0086 for B3V_H/BU-hybrid V_λ, SD < 0.22 for B3V_H/UB-hybrid V_λ, SD < 0.11 for B3V_H/33B-hybrid V_λ, SD < 0.13 for B3V_H/B33-hybrid V_λ, SD < 0.0091 for B3V_H/33U-hybrid V_λ and SD < 0.22 for B3V_H/U33-hybrid V_λ. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-histones IgG at a known concentration. The same aliquot of hybridoma supernatant was used for all the ELISAs and the SD of the positive control between the *OD* readings of the three experiments was always less than 0.15. The positive control of the representative experiment is shown on the graph above.
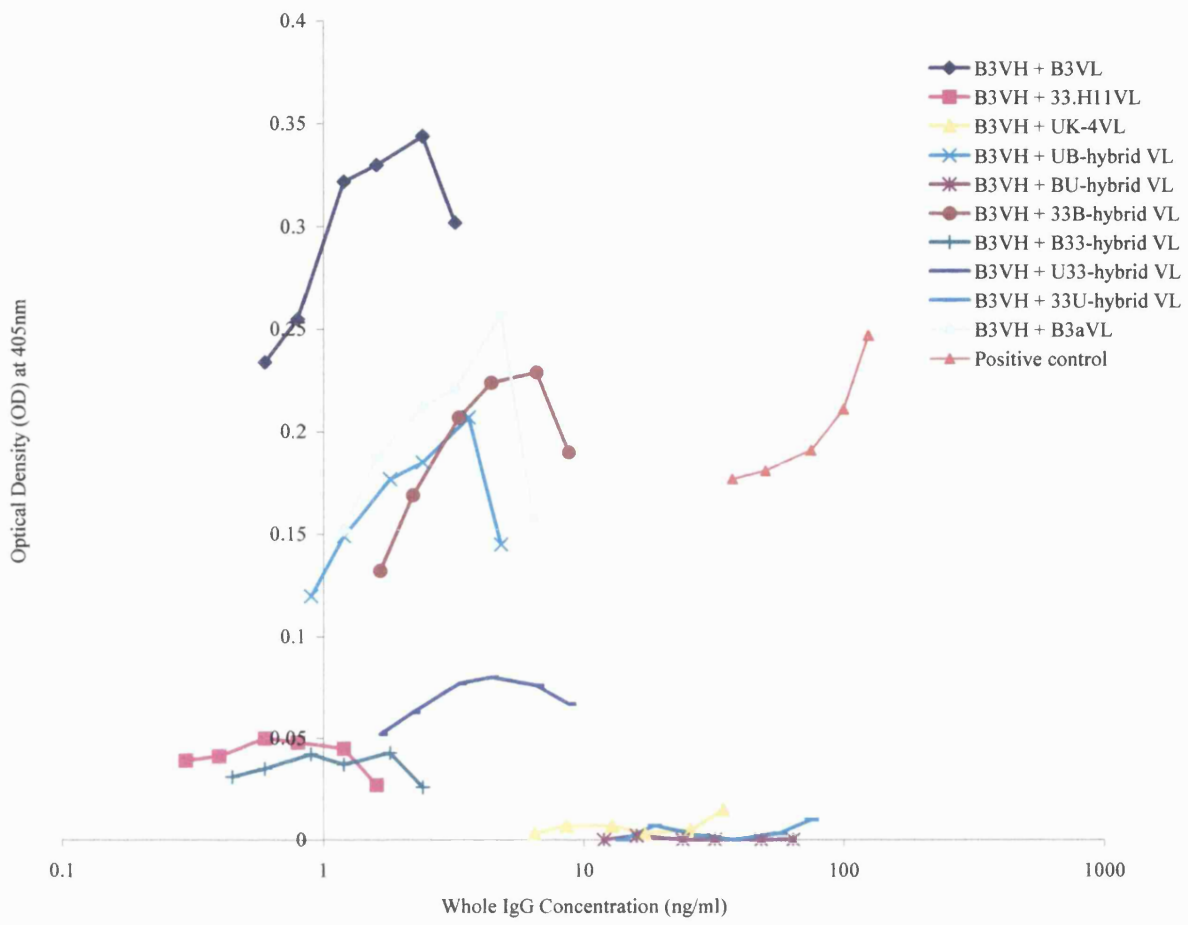
Figure 4.19 Anti-histone activity of 33.H11 heavy chain/2a2-derived λ chain IgG in COS-7 cell supernatants, detected by ELISA.

The graph shows binding of IgG in COS-7 cell supernatants containing each 33.H11 heavy chain/λ gene 2a2-derived light chain combination to histones (calf thymus). The electroporations were carried out and tested by ELISA for anti-dsDNA binding on three separate occasions. The graph opposite shows the results of a representative experiment (after one-hour incubation with substrate). However the standard deviations (SD) between the optical density (OD) readings of the three experiments were calculated for all the points on each curve as follows: SD < 0.003 OD units for all points on curve 33.H11V_H/B3V_λ(wild type). Similarly, at all points, SD < 0.0021 for 33.H11V_H/B3V_λa, SD < 0.0067 for 33.H11V_H/33.H11V_λ, SD < 0.0044 for 33.H11V_H/UK-4V_λ, SD < 0.0035 for 33.H11V_H/BU-hybrid V_λ, SD < 0.012 for 33.H11V_H/UB-hybrid V_λ, SD < 0.0028 for 33.H11V_H/33B-hybrid V_λ, SD < 0.005 for 33.H11V_H/B33-hybrid V_λ, SD < 0.0058 for 33.H11V_H/33U-hybrid V_λ and SD < 0.0057 for 33.H11V_H/U33-hybrid V_λ. The negative control in each case was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation and contained neither IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-histones IgG at a known concentration. The same aliquot of hybridoma supernatant was used for all the ELISAs and the SD of the positive control between the OD readings of the three experiments was always less than 0.15. The positive control of the representative experiment is shown on the graph above.

Influence of V_H and V_λ Sequence motifs on binding to Autoantigens.
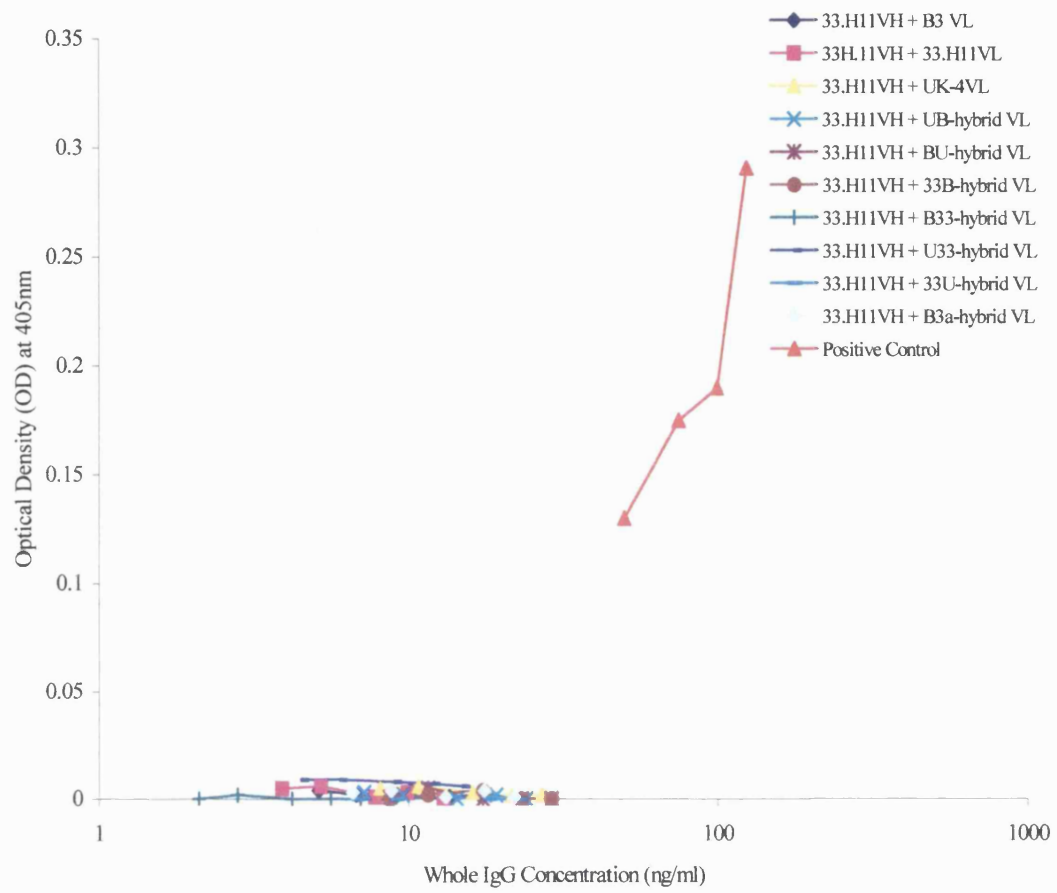
4.11 Computer modelling of three-dimensional structures of the interaction of the heavy/light chain combinations with dsDNA

The strongest binding to dsDNA was obtained using the hybrid light chain B33. In the models (made by Dr Sylvia Nagl) reported previously in chapter three of this thesis, it was shown that Arg 27a in B3 V_λ CDR1 and Arg 92 in 33.H11 V_λ CDR3 could both make contacts with dsDNA. The strong binding found with the chain B33 which, unlike either of the parent light chains, contains both these arginines might therefore be explained if both Arg 27a and Arg 92 could be shown to contact DNA. Figures 4.20[a] and 4.20[b] show models of the complex of dsDNA with B3V_H/B33 hybrid V_λ. These models do indeed predict that both these arginines interact with the dsDNA molecule in this complex.

Figures 4.20[c] and 4.20[d] show models of B3V_H/BU hybrid V_λ and B3V_H/UB hybrid V_λ respectively. The first does not bind DNA due to the presence of the large blocking arginine group (shown in yellow) at position 94 of UK-4 V_λ. When this group is removed from UK-4 V_λ, by replacing CDR2 and 3 with those of B3 V_λ (Figure 4.20d), the resultant light chain UB would be predicted by the model to support binding to DNA. The presence of the UK-4 V_λCDR1 region would not prevent this binding. The results predicted by these models are exactly those found in the ELISA assays. UB hybrid V_λ supported binding to dsDNA whereas BU hybrid V_λ did not.

Figure 4.20 Computer models of B3V$_H$/ hybrid germline gene 2a2-derived V$_\lambda$ chain combinations.

The molecular surfaces of the antibody chains are shown in dark blue (light chain) and light blue (B3V$_H$); dsDNA is depicted in stick mode and coloured by element. Key arginine residues predicted to enhance DNA binding are shown in grey and red. The B3V$_H$/hybrid germline gene 2a2-derived V$_\lambda$ chain combinations depicted are:

a) B3V$_H$/B33-hybrid V$_\lambda$ (contains B3V$_\lambda$ CDR1, 33.H11V$_\lambda$ CDR2 and 3)

b) Detailed view of predicted stabilizing interactions between arginine at position 27a (R27a) and arginine at position 92 (R92) and the DNA backbone in B3V$_H$/B33-hybrid V$_\lambda$

c) B3V$_H$/BU-hybrid V$_\lambda$ (contains B3V$_\lambda$ CDR1 and UK-4V$_\lambda$ CDR2 and CDR3). The predicted blocking residue Arg 94 is shown in yellow.

d) B3V$_H$/UB-hybrid V$_\lambda$ (contains UK-4V$_\lambda$ CDR1 and B3V$_\lambda$ CDR2 and CDR3).

Figures modeled by Dr Sylvia Nagl.
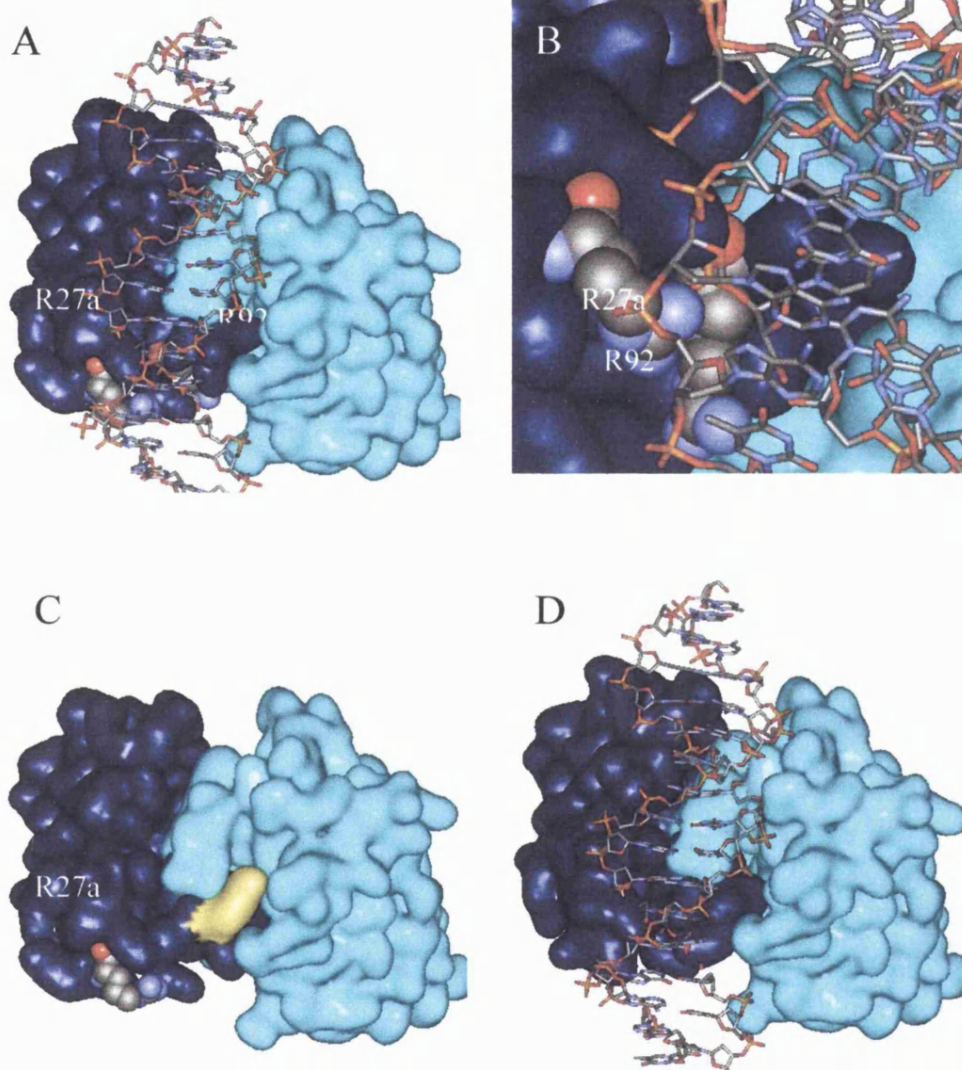
4.12 Discussion of results

Before this set of transient experiments were begun, a series of experiments were used to optimise this system. It was found that the COS-7 cells in this system continue to express whole IgG1 for at least six days and therefore I would recommend that in the future supernatants are collected three and six days post-electroporation to maximise IgG1 yields. Furthermore, for future reference it was found that IgG1 in supernatants were more stable if kept at 4°C rather than -20°C most probably due to the detrimental effects of the freeze-thaw process involved in the latter. The IgG1 stored at 4°C were relatively stable for at least two months.

As in chapter three, the IgG1 yields of those H/L combinations that have a high ability to bind DNA are lower than those that do not, such as those IgG1 with the UK-4V_λ CDR2 and CDR3. It was investigated whether this was due to the H/L combinations with higher DNA binding ability being toxic to the COS-7 cells that are producing the IgG1 thus reducing the levels of expressed whole IgG1. However no significant differences were found between the number of viable cells three days post-electroporation between the different H/L chain combinations. It was also concluded that IgG1 yields detected in ELISA were not being hindered by the formation of immune complexes with DNA left in the supernatant, as an increase in the amount of DNaseI added to the supernatants did not result in higher yields being detected.

In chapter three, this transient expression system was used to show that particular sequence motifs in the CDRs of certain human anti-DNA monoclonal antibodies are very important in determining their ability to form a DNA-binding site. The data reported in this chapter confirm and extend the results and hypotheses discussed in chapter three.

The computer model of the B3V_H/UK-4V_λ complex (Figure 3.6) suggested that this complex was unable to bind dsDNA due to the presence of an arginine residue at position 94 (R94) in UK-4 V_λ CDR3. The results shown here support this hypothesis since all B3V_H combinations bound dsDNA and ssDNA except for those with light chains containing UK-4 V_λ CDR2 and 3 regardless of the V_λ CDR1 origin. Furthermore, the UK-4 V_λ CDR1 region was able to contribute to a functional binding

site when combined with either B3 or 33.H11 V_λ CDR2 and 3 (in hybrids UB and U33). The high binding of hybrid U33 to dsDNA is especially striking. In this case, CDR swapping has clearly converted a non-binding wild-type light chain (UK-4) into a DNA-binding light chain (U33). This result strongly supports the hypothesis that the V_λ CDR3 regions of UK-4 and 33.H11 exert opposite effects on DNA binding, despite the fact that arginine residues are present at only slightly different positions in the two molecules (R92 in 33.H11 and R94 in UK-4).

The computer models suggested that binding to dsDNA is enhanced by the presence of R92 in 33.H11 V_λ CDR3 and R27a in B3 V_λ CDR1. This hypothesis was supported by the finding that, in combination with B3V_H, the light chain that consistently showed the highest binding to both dsDNA and ssDNA was the chain containing both of these CDR motifs (i.e. B33). Conversely, dsDNA binding was reduced twofold in combinations lacking both of these particular CDR motifs (33B and UB). The contribution of these CDR motifs to DNA binding was demonstrated further by the fact that 33.H11V_H only bound DNA when paired with the B33V_λ hybrid chain.

These CDR motifs also affect binding to other autoantigens thought to be important in SLE, such as histones and SmD. The binding to these different antigens is affected in different ways by these CDR motifs. As with DNA, the UK-4 R94 CDR motif blocked binding to all of the autoantigens tested in the immunoassay except Ro whilst all the combinations that bound DNA in the anti-DNA ELISA also bound to histones. However the relative ability of these combinations to bind to histones differed from their relative ability to bind DNA.

The B3 V_λCDR1 R27a motif does appear to play a role in binding to histones, but the presence of B3 V_λCDR2 and 3 seems to be more important. The 33.H11 V_λCDR3 R92 motif does not enhance binding to histones, since this binding was increased threefold when 33.H11 V_λCDR2 and CDR3 were replaced by B3 V_λCDR2 and CDR3.

The presence of B3V_λ CDR2 and CDR3 also appears to confer binding to SmD regardless of V_λCDR1 origin, whilst in contrast to the binding of histones and DNA, the presence of the 33.H11V_λCDR2 and CDR3 motif does not necessarily confer ability to

bind SmD. The exact identity of the amino acids important in binding to these antigens cannot be ascertained, since docking models of the antibody-histone and antibody–SmD complexes are not available. It is possible, however, that the positively charged R92 residue in 33.H11 V_λCDR3 may inhibit binding of positively charged histones while enhancing binding to DNA. Large protein antigens, such as histones, are especially likely to make contacts with apical residues in the CDR3 loops, such as those at positions 92-94 (MacCallum *et al.*, 1996).

The fact that the light chains UK-4V_λ, 33U and BU will all support binding to Ro60/SSA in combination with B3V_H is important. It shows that the inability of these light chains to support binding to other antigens is not due to their causing a major change in structure so that the heavy/light chain combinations containing UK-4V_λ CDR3 cannot function as antibodies at all. These results also imply that these antibodies bind the Ro antigen at a site distinct from the DNA binding site, which probably does not involve the surface cleft. It would be interesting to investigate this further using techniques such as ELISA, BIAcore or computer modelling.

When the same range of 11 2a2 derived light chains were paired with the heavy chain of a different anti-DNA mAb, 33.H11, the ability of the IgG1 to bind dsDNA and ssDNA was reduced greatly. Using a bacterial expression system to express cloned antibody cDNA sequences in the form of Fab, Kumar *et al.* (2000) have also shown that dsDNA binding is reduced when the heavy chain of B3 is replaced by that of 33.H11. Our results show that both heavy and light chains of these antibodies can have an affect on DNA binding ability, as reported previously by various groups for both murine and human anti-DNA monoclonal antibodies. Radic *et al.* (1991) showed that the heavy chain of the murine monoclonal anti-DNA antibody 3H9 was dominant in determining ability to bind DNA, but that the nature of the accompanying light chain was important in controlling fine specificity. Mockridge *et al.* (1996) demonstrated that both heavy and light chain features were important in DNA binding of the human monoclonal anti-DNA antibody D5.

Therefore in the case of B3, replacement of either the heavy chain or the light chain can lead to a dramatic reduction in the ability to bind DNA. The original B3/dsDNA computer model is in accordance with this as it predicts that the heavy chain of B3 is

also important in creating the antigen-binding site. In particular the V_HCDR2 arginine shown in yellow at the periphery of the site along with the other two light chain arginines is predicted to be essential in stabilising the B3/dsDNA interaction. It is important to note that not all heavy chains will combine with the B3 light chain to form a DNA binding site. Previously our group has shown that when B3V_λ is paired with the V_H of a different antibody, WRI176, it is able to combine to produce a whole IgG1 molecule but that this combination cannot bind either dsDNA or ssDNA (Rahman et al., 1998).

The V_H regions of B3 and 33.H11 are derived from different germline genes, V_H3-23 and V_H3-07, respectively. Therefore it is possible that V_H3-23, prior to any somatic mutation, may be more predisposed to higher DNA binding than V_H3-07.

Alternatively, the somatic mutations in B3V_H may enhance binding to dsDNA more than those in 33.H11V_H. Both V_H sequences are extensively mutated, compared to their germline genes, with clustering of replacement mutations in the CDRs suggestive of antigen drive.

MacCallum et al. (1996) proposed a set of rules to predict which amino acids within an antibody sequence are most likely to form contacts with antigen (see Figures 3.1 and 4.8). Both heavy chains contain somatic mutations at some of these particular contact sites. For example, in both B3 and 33.H11, the residue at position 53, which is located in the centre of V_HCDR2, is a product of somatic mutation. At position 53, serine is mutated to a positively charged arginine in B3V_H and to a neutral glycine in 33.H11V_H. The positively charged arginine mutation in B3V_H may contribute to the increased DNA binding seen with this chain, particularly since it is located towards the centre of the combining site and was identified as a site of antigen-antibody contact in original B3/dsDNA model (Figure 1.3) (Kalsi et al., 1996). In 33.H11V_H, however, the lack of an arginine residue at position 53 may be balanced by an extra somatic mutation from a negatively charged glutamic acid to a positively charged lysine at another centrally located antigen contact site, position 56 in V_HCDR2.

The presence of arginine at position 53 in V_H was also noted to affect binding to DNA in studies of the murine anti-dsDNA antibody 3H9 (Radic et al., 1993). 3H9V_H contains

three somatic mutations in CDR2, one being an arginine at position 53. When this R53 was reverted to glycine, binding to dsDNA was virtually eliminated whilst reversion of the other somatic mutations in CDR2 had little or no effect on binding.

Therefore in conclusion, I have demonstrated that both heavy chain and light chains contribute to the production of the antigen-binding site in the antibodies considered in this thesis. Arginines in the CDRs play a major role in binding to DNA, but the actual positions of these residues are crucial. I have also identified particular sequence motifs that are very important in determining the ability of an antibody to bind not only DNA but other autoantigens known to be important in SLE, such as histones and SmD. Furthermore, these CDR sequence motifs affect binding to different antigens in different ways.

CHAPTER FIVE

Stable expression of whole IgG1 from cloned anti-DNA antibody DNA sequences in CHO*dhfr*⁻ cells.

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CHAPTER FIVE.

STABLE EXPRESSION OF WHOLE IgG1 FROM CLONED ANTI-DNA ANTIBODY DNA SEQUENCES IN CHO*dhfr⁻* CELLS

5.1 Aims of this chapter

The ability to bind DNA *in vitro* does not automatically confer an antibody with the ability to cause tissue damage in SLE. Some murine and human monoclonal anti-DNA antibodies do not have any pathogenic effects when tested *in vivo* (Ehrenstein *et al..*, 1995; Madaio *et al..*, 1987; Ravirajan *et al..*, 1998). Consequently, the next step is to test the effects of those CDR motifs identified in the previous two chapters as being important in binding DNA, on the pathogenicity of these IgG1 *in vivo*. Unfortunately, the quantities of IgG1 produced by the transient expression system are insufficient for this type of work. Therefore this chapter describes the use of the same expression constructs (combined to make supervectors) to produce stable cell lines expressing some of the heavy/light combinations reported in the two previous chapters, in preparation for such *in vivo* experiments.

5.2 Assembly of recombinant supervectors for expression

As described previously in section 2.11, the expression vectors pLN10 and pG1D1 used in the transient expression system can also be used in the stable expression system. However when using the single chain expression vectors there is a theoretical risk of excess production of heavy chain compared to light chain since co-transfection with *dhfr* (in heavy chain expression vector only) may be comparatively more productive than co-transfection with *neo* (in light chain expression vector only). Therefore, the two vectors were combined to produce "supervectors". From the plasmid vector pLN10, an *EcoRI* fragment containing the light chain cassette (the HCMV promoter, the λ constant region gene and the λ variable region gene of the chosen autoantibody) was transferred into the *EcoRI*-linearised recombinant vector pG1D1/B3V$_H$. Using an *EcoRI* restriction digest, the incorporation of the inserted light chain cassette was verified by the presence of a 6917bp band and a 2260bp band when run on an agarose gel, as shown in Figure 5.1. Five supervectors were produced in total, all containing the same B3 heavy chain but with different light chains. All the light chains were derived from the same germline

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gene but contained a different pattern of somatic mutations. Table 5.1 shows the nomenclature used for the supervectors.

SUPERVECTOR	HEAVY CHAIN	LIGHT CHAIN
SVBL	B3	B3
SVBLX2	B3	B3a
SVBLX4	B3	B3b
SVBM	B3	UK-4
SVBN	B3	33.H11

Table 5.1   Nomenclature used for each of the supervectors

In the stable expression system, it is several weeks before it can be determined whether the transfected CHO$dhfr^-$ cells are able to express whole IgG1 molecules. Therefore prior to their transfection into the stable system SVBL and SVBLX2 were transfected into the transient system. The supervectors were found to be functional and the expression levels produced by COS-7 cells transfected with supervectors were slightly higher than those transfected with the single chain vectors, as shown in Figure 5.2. However statistical analysis shows that the mean IgG1 concentrations are not significantly different between the supernatants from the COS-7 cells transfected with single chain expression vector compared to those transfected with supervector. For example, B3V$_H$/B3V$_\lambda$ (p=0.3288, n=2 calculated according to Student t-test, with 95% confidence level) and B3V$_H$/B3V$_\lambda$a (p=0.2155, n=2 calculated according to Student t-test, with 95% confidence level).

In addition, to ensure the electroporation conditions of each set of stable expression transfections were optimal, an aliquot of CHO$dhfr^-$ cells was also electroporated (under the same conditions as the expression vectors) with a vector containing the firefly luciferase gene (pGL3-control vector, Promega, Southampton, UK). By assaying the firefly luciferase reporter activity of the cell lysate of this aliquot 72 hours post-transfection, it was determined whether electroporation conditions had been optimal on that day. In all cases, the luciferase reporter assay activity was high and so the transfected CHO$dhfr^-$ cells were maintained in culture and the stable cell lines developed. If the luciferase reporter assay results had been negative or low, the electroporation conditions would have been assessed and the transfections would have been repeated.
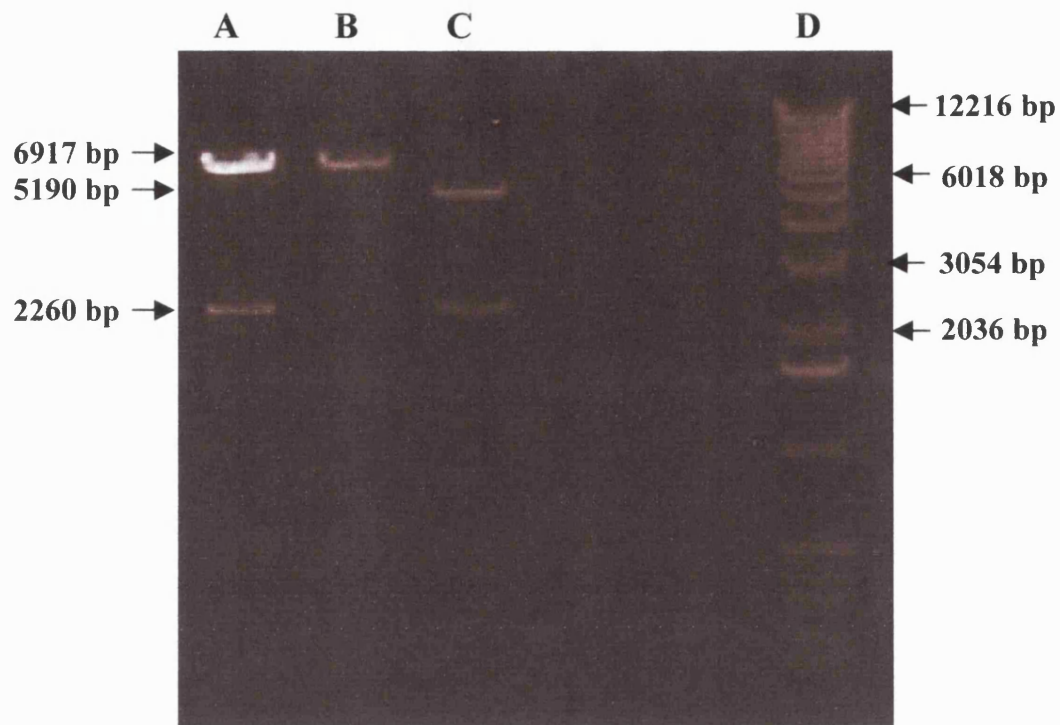
Figure 5.1   *EcoRI* restriction digest of recombinant supervector (SV), parent recombinant heavy chain vector (pG1D1) and parent recombinant light chain vector (pLN10)

Lane A   Supervector SVBL

Lane B   Recombinant B3V$_H$/pG1D1 vector

Lane C   Recombinant B3V$_\lambda$/pLN10 vector

Lane D   1Kb DNA Molecular weight marker
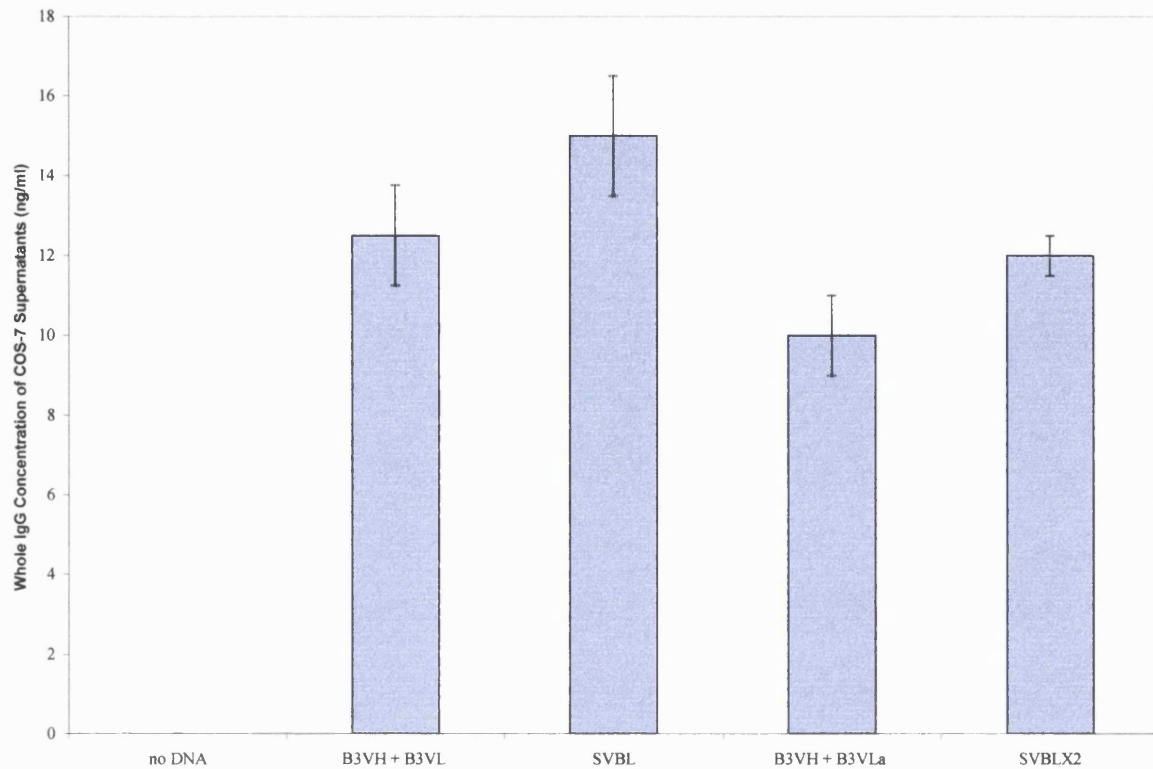
Figure 5.2   **IgG expression levels of COS-7 cells transfected with either single chain expression vectors or supervectors.**

Constructs: B3VH + B3VL (single chain expression vectors)

SVBL (supervector containing both B3V$_H$ + B3V$_\lambda$)

B3VH + B3VLa (single chain expression vectors)

SVBLX2 (supervector containing B3V$_H$ + B3V$_\lambda$a)

This experiment was carried out on two separate occasions. The bar chart shows the mean whole IgG concentration of each supernatant of COS-7 cells transfected with either single chain expression vectors or supervectors. The error bars shown on the graph correspond to the standard error about the mean to show any variation in results between the two experiments. Statistical analysis shows that the mean IgG1 concentrations are not significantly different between the supernatants from the COS-7 cells transfected with single chain expression vector compared to those transfected with supervector. For example, B3V$_H$/B3V$_\lambda$ (p=0.3288, n=2 calculated according to Student t-test, with 95% confidence level) and B3V$_H$/B3V$_\lambda$a (p=0.2155, n=2 calculated according to Student t-test, with 95% confidence level).

5.3 Stable expression of whole IgG1 molecules in CHOdhfr⁻ cells

Three stable cell lines were produced, CHO-B3, CHO-B3 [R27aS] and CHO-(negative control). Both CHO-B3 and CHO-B3 [R27aS] cell lines produce whole IgG1 with the same heavy chain (B3) but different light chains. CHO-B3 produces IgG1 with the wild type B3 light chain (CHO-B3) whilst CHO-B3 [R27aS] produces IgG1 with the B3 light chain containing the arginine to serine reversion in CDR1. As expected, the CHO-(negative control) cell line, transfected with empty expression vector (i.e. contains no heavy or light chain variable region DNA sequences) produced no detectable IgG1.

Two rounds of methotrexate amplification were carried out in order to maximise the amount of antibody that could be produced and harvested from cell lines, CHO-B3 and CHO-B3 [R27aS]. The CHO-(negative control) cell line was also selected and amplified with methotrexate in exactly the same way as the test IgG1-producing cell lines. This was because when the pathogenic effects of the IgG1-producing cell lines are investigated *in vivo*, a negative control cell line that has undergone the same procedures and stresses as the IgG1-producing cell lines would be required.

The amount of IgG1 produced was determined by whole IgG ELISA, as before. With the first methotrexate amplification (1×10^{-9} M methotrexate) the amount of IgG1 produced, increased by 8-12 times and with the second methotrexate amplification (1×10^{-7} M) it increased by a further two- to three-fold. The CHO-B3 (R27aS) cell line consistently had a slightly higher level of expression than the wild type cell line. As expected, the control CHO*dhfr⁻* cell line that was transfected with empty expression vector (i.e. contains no heavy or light chain variable region DNA sequences) produced no detectable IgG1. The exact IgG1 production rates for each cell line are shown in Table 5.2.

5.4   Anti-dsDNA activity of whole IgG1 stably expressed in CHO*dhfr⁻* cells

The dsDNA binding activity of each of the B3V$_H$/B3-derived-V$_\lambda$ IgG1 combinations stably expressed by the transfected CHO*dhfr⁻* cells (CHO-B3 and CHO-B3 [R27aS]) are shown in Figure 5.3. Each expression experiment (i.e. electroporation and subsequent anti-dsDNA ELISA) was carried out three times and in each case, similar results were seen in each. Figure 5.3 shows the results of a representative experiment (after a 30-minute incubation with the substrate). However the standard deviations (SD) between the optical density (*OD*) readings of the three experiments were calculated for all the points on each curve. The negative controls used in the ELISAs were CHO*dhfr⁻* media and also supernatant from the CHO-(empty vector) cell lines. Neither contained IgG nor anti-dsDNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-dsDNA IgG at a known concentration. The same aliquot of hybridoma supernatant was used for all the ELISAs and the SD of the positive control between the *OD* readings of the three experiments was always less than 0.2. The positive control of the representative experiment is shown in the graph.

The IgG1 produced by both cell lines, CHO-B3 and CHO-B3 [R27aS] were shown to bind dsDNA. As indicated previously in the transient expression system, the introduction of the single arginine to serine reversion in V$_\lambda$CDR1 significantly reduced binding such that double the concentration of IgG1 was required to produce the same *OD* reading for dsDNA.

Methotrexate Concentration of CHO*dhfr* Amplification media	IgG Production Rate (ng/10^6 cells/day)		
	CHO-B3 stable cell line	*CHO-B3 (R27aS) stable cell line*	*CHO-(negative control cell line)*
Nil	4	10	0
1 x 10^{-9} M	60	80	0
1 x 10^{-7} M	130	250	0

Table 5.1. **The effect of methotrexate amplification on the IgG production rate of the different stable cell lines.**

The effects of two rounds of methotrexate amplification on the IgG expression levels of the three stable cell lines CHO-B3, CHO-B3 [R27aS] and CHO-(negative control) are shown above. Both CHO-B3 and CHO-B3 [R27aS] cell lines produce whole IgG with the same heavy chain (B3) but different light chains. CHO-B3 produces IgG with the wild type B3 light chain (CHO-B3) whilst CHO-B3 [R27aS] produces IgG with the B3 light chain containing the arginine to serine reversion in CDR1. The CHO-(negative control) cell line (transfected with empty expression vector i.e. contains no heavy or light chain variable region DNA sequences) produces no detectable IgG.
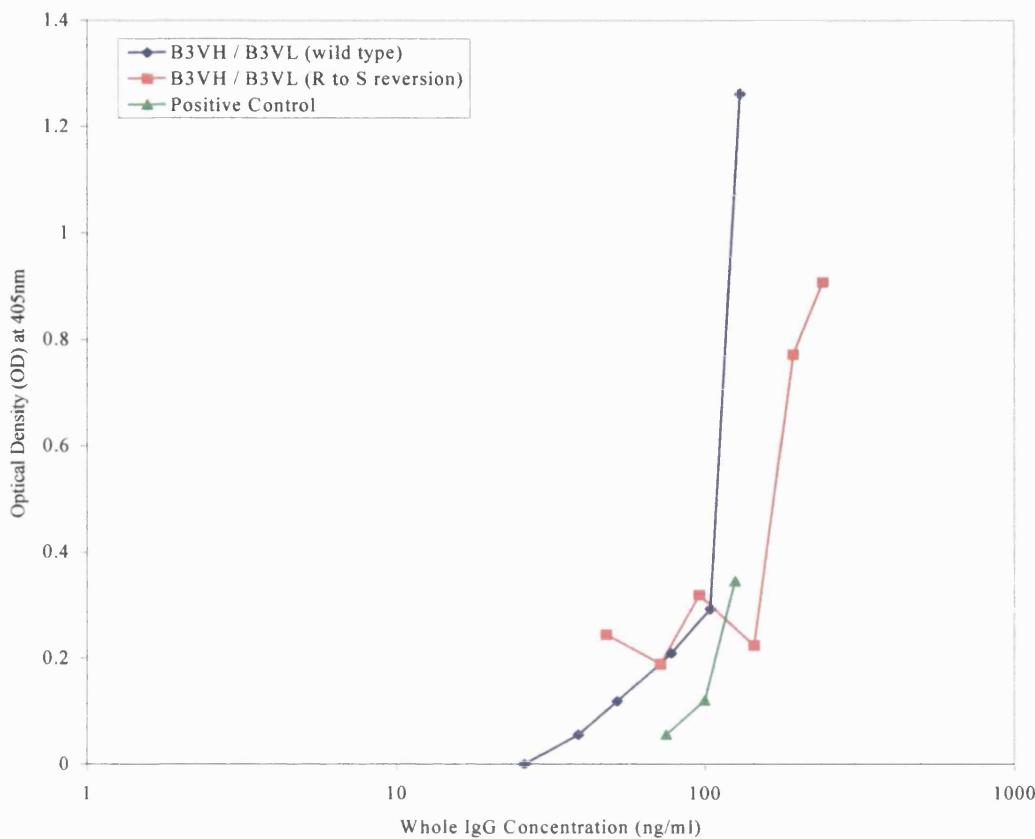
Figure 5.3   Anti-dsDNA activity of IgG produced by stable CHO*dhfr* cell lines, detected by ELISA.

The graph shows binding of IgG produced by stable CHO*dhfr* cell lines, CHO-B3 and CHO-B3 [R27aS] to dsDNA. The electroporations were carried out and tested by ELISA for anti-dsDNA binding on three separate occasions. The above graph shows the results of a representative experiment (after only a 30-minute incubation with the substrate). However the standard deviations (SD) between the optical density (*OD*) readings of the three experiments were calculated for all the points on each curve as follows: SD < 0.25 *OD* units for all points on curve CHO-B3 (wild type) whilst similarly, at all points, SD < 0.12 for CHO-B3 [R27aS]. Both CHO*dhfr* media and supernatant from the CHO-(empty vector) cell lines were used as negative controls in the ELISAs. Neither contained IgG nor anti-DNA activity on testing by ELISA. The positive control in each case was hybridoma supernatant containing anti-dsDNA IgG at a known concentration. The same aliquot of hybridoma supernatant was used for all the ELISAs and the SD of the positive control between the *OD* readings of the three experiments was always less than 0.2. The positive control of the representative experiment is shown in the graph.

5.5   Line immunoassay of whole IgG1 stably expressed in CHO*dhfr* cells

Similarly to the previous chapter, binding of the IgG1 produced by the stable cell lines was assayed against a large range of nuclear antigens relevant to autoimmune rheumatic disease using a line immunoassay. Each of the supernatants of the cell lines, CHO-B3, CHO-B3 [R27aS] and CHO-(negative control) was tested on its own nylon strip (coated with discrete lines of autoantigens). The presence of antibodies to a particular autoantigen on the strip was detected if a dark brown line appeared at the point on the strip corresponding to that autoantigen. These strips are shown in Figure 5.4. The binding characteristics of the IgG1 produced in the transient expression systems are also summarised in Table 5.3.

As shown in Figure 5.4 and Table 5.3, the binding characteristics of the IgG1 produced in the stable expression system were identical to those seen with similar heavy/light chain combinations in the transient expression systems when screened against a range of nuclear and cytoplasmic antigens (see section 4.6).

Supernatant from the CHO-(empty vector) cell lines was used as the negative control in the line assay. The positive control used was as described in section 4.6.

Figure 5.4   **Line immunoassay of expressed whole IgG1 heavy/light chain combinations from COS-7 cells transfected with either single chain expression vectors or supervectors**

The presence of antibodies to a particular autoantigen on the strip is considered positive if a dark brown line appeared at the point on the strip corresponding to that autoantigen. COS-7 supernatant from the no DNA negative control was used as the negative control in the line assay. The positive control used was provided with the kit and consisted of a mixture of human plasma and sera, containing antibodies against a large range of nuclear antigens relevant to autoimmune rheumatic disease. Furthermore the first line on each strip is the sample addition control line. A dark brown line at this point signified the presence of IgG antibodies in the supernatant. If the supernatant did not contain antibodies able to bind any of the autoantigens on the strip, the labelled anti-human antibody supplied with the kit did not bind to autoantigen/autoantibody immune complexes and as a result only the control line and a low background colour developed. Each COS-7 supernatant containing each heavy/light chain combination was tested with its own strip.

Letters A-N show the position of the antigens on the immunoassay strip whilst numbers 1-6 indicate the supernatant being tested.

Key:

A	Control line		H	SSA/Ro60
B	SmB		I	SSB/La
C	SmD		J	Cenp-B
D	RNP-70k		K	Topo-I/Scl-70
E	RNP-A	L	Jo-1/HRS	
F	RNP-C	M	Ribosomal P	
G	SSA/Ro52		N	Histones

1	Positive control	
2	No DNA control	
3	$B3V_H/B3V_\lambda$	(Supernatant of COS-7 cells transfected with single chain expression vectors)
4	SVBL	(Supernatant of CHO cells transfected with $B3V_H/B3V_\lambda$ Supervector)
5	$B3V_H/B3V_\lambda a$	(Supernatant of COS-7 cells transfected with single chain expression vectors)
6	SVBLX2	(Supernatant of CHO cells transfected with $B3V_H/B3V_\lambda a$ Supervector)

COMBINATION	ANTIGEN			
	SmD	SSA/ Ro60	Topo-I/ Scl-70	Histones
B3V$_H$ + B3V$_L$ (Transient expression)	+	-	+	+
B3V$_H$ + B3V$_L$ (Stable expression)	+	-	+	+
B3VH + B3V$_L$a (Transient expression)	+	+	+	+
B3V$_H$ + B3V$_L$a (Stable expression)	+	+	+	+
No DNA (negative control) (Transient expression)	-	-	-	-
CHO-(empty vector) cell line (Stable expression)	-	-	-	-

Table 5.2.   **Summary of the binding characteristics of the whole IgG1 produced in both the stable and transient expression systems when screened against a range of nuclear and cytoplasmic antigens using the INNO-LIA$^®$ ANA Update kit (Innogenetics, Ghent, Belgium).**

When screened against a range of nuclear and cytoplasmic antigens, at least one of the heavy/light chain combination IgG1 produced in the stable and transient expression system bound to the four autoantigens shown in the table above. No binding by any of the expressed heavy/light chain combination IgG1 was seen towards the rest of the autoantigens tested in the line immunoassay. The binding characteristics of the IgG1 produced in the stable expression system were identical to those seen with similar heavy/light chain combinations in the transient expression systems when screened against a range of nuclear and cytoplasmic antigens. Supernatant from the CHO-(empty vector) cell lines was used as the negative control in the line assay whilst the negative control in the transient expression system was supernatant from COS-7 cells to which no plasmid DNA had been added during electroporation. The positive control used was provided with the kit and consisted of a mixture of human plasma and sera, containing antibodies against a large range of nuclear antigens relevant to autoimmune rheumatic disease.

"+" represents binding of the IgG heavy/light chain combination to the antigen whilst "–" represents no binding.

5.6 Discussion of results

The results described in this final chapter show that I have successfully developed a stable expression system to produce whole human recombinant anti-DNA IgG1 molecules from cloned PCR products encoding anti-DNA antibody sequences. This system has the potential to produce significant amounts of antibody whose sequence-related functional properties can be studied both in *in vitro* and *in vivo* assays.

The total yield of IgG1 varied between 130 and 250 ng per 10^6 cells per day depending on the heavy/light chain combination expressed. This is very low when compared to the amounts produced by hybridoma cell lines. However larger yields could possibly be obtained with these cell lines by transferring them into larger scale *in vitro* culture systems. In fact at present, one of the stable cell lines is being tested in a hollow fibre system by a company in collaboration with our group and their preliminary data show that they can produce up to one milligram of purified antibody by this method. With large amounts of purified antibody such as that generated using such methods, not only could these antibodies be studied *in vivo* but also more accurate information about changes in affinity for DNA could be obtained in assays such as surface plasmon resonance or inhibition ELISA.

The DNA binding properties of the antibodies produced in this stable expression system were concordant with those seen with the transient system as the introduction of the single arginine to serine reversion in B3V$_\lambda$CDR1 significantly reduced binding to dsDNA when assayed by ELISA.

Various groups have previously used stable expression systems to produce functional murine anti-DNA antibodies from cloned DNA sequences (Radic *et al*.., 1993). In each case, site-directed mutagenesis was used to alter the number and position of arginine residues in the V$_H$ sequence of a murine anti-dsDNA antibody. Heavy chain expression vectors containing the mutated sequences were transfected into heavy chain loss variant hybridoma cell lines in order to produce a range of antibodies whose individual binding properties would differ solely due to the alterations made to the V$_H$ sequence. Similar to the results shown in this chapter with our system, the data showed that changes involving arginine residues affected the DNA binding ability of the antibodies.

The main purpose of producing whole immunoglobulin molecules using expression systems is that if sufficient quantities are produced, the pathogenic properties of sequence alterations can be determined. Katz *et al..* (1994) were the only group to use the expressed murine antibodies to investigate the effects of such changes on their pathogenicity. Young SCID mice were implanted intraperitoneally with either hybridoma cells producing the wild type anti-DNA antibody, R4A, or one of the heavy chain loss variants expressing whole IgG with the altered R4A heavy chain. All mice carrying the R4A hybridoma developed proteinuria with glomerular deposition of the antibody in the kidneys. However the mutant cell line producing IgG with the highest anti-dsDNA affinity showed some glomerular deposition but deposited to a greater extent in the tubules. Furthermore this particular recombinant antibody had fewer arginine residues than the wild type R4A. These results suggest that the ability to bind dsDNA does not mean that an antibody will bind to glomeruli and that there is not a simple relationship between contact of arginines, binding to DNA and pathogenicity.

To date human anti-dsDNA antibodies have not been expressed using heavy chain loss variants. As the particular amino acid residue that I was specifically interested in was in the light chain (R27a), HCLVs could not be used for the experiments reported in this chapter. The stable expression of whole human anti-DNA antibodies has only been reported by one other group. Li *et al.* (2000) have expressed the variable regions of the human IgA anti-DNA antibody, 412.67 in a F3B6 human/mouse heteromyeloma cell line. Although the DNA sequence was cloned originally from an IgA mAb, the expression vectors contained gamma and not alpha DNA. As a result, the expressed product was IgG isotype. By modification and expression of cloned antibody DNA sequences, these authors showed that the presence of arginine residues in V_HCDR3 of 412.67 were particularly important in conferring the ability to bind DNA. However it is unknown as to whether these arginine residues affect the pathogenicity of 412.67 or even if the original wild type antibody is pathogenic.

Therefore the stable expression of B3 reported here is only the second report of stable expression of a human anti-DNA antibody. However B3 has an advantage over 412.67 in that the original B3 hybridoma cell line is known to be pathogenic, causing proteinuria in SCID mice (Ehrenstein *et al..*, 1995).

CHAPTER SIX

Conclusions and ideas for future work.

CHAPTER SIX.   CONCLUSIONS AND IDEAS FOR FUTURE WORK

The work described in this thesis furthers our understanding of the importance of somatic mutations and the presence of particular amino acid residues on the binding properties of human anti-DNA antibodies. Computer models have been used in conjunction with a eukaryotic transient expression system to demonstrate that both heavy and light chains contribute to the production of the antigen-binding site in the human anti-DNA IgG1 antibodies, B3 and 33.H11. Arginines in the CDRs play a major role in binding to DNA. However it is the actual positions of these residues that are crucial to conferring binding ability to the antibody. Particular sequence motifs have been identified that are very important in determining the ability of an antibody to bind not only DNA but other autoantigens known to be important in SLE, such as histones, and SmD. Furthermore, these CDR sequence motifs affect binding to different antigens in different ways.

Although the direct binding ELISAs used in this thesis were sufficient for testing the binding ability of the range of different heavy/light chain combinations expressed, these assays did not determine the affinity of the IgG1. Thus a future aim is to determine the effects of the important sequence motifs identified on the affinity of the expressed IgG1, by using further assays such as inhibition ELISAs or surface plasmon resonance (SPR) (e.g. BIAcore). In inhibition ELISAs, the affinity of immunoglobulins in a test sample is determined according to the amount of antigen that has to be added to the test sample in order to inhibit binding to the same antigen coated to the plate. In surface plasmon resonance (e.g. BIAcore), affinity is measured through the calculation of kinetic rate constants detected for the binding and dissociation of an antibody when it is passed over a chip coated with antigen.

The amounts of antibodies produced from COS-7 cells were not adequate for carrying out inhibition ELISAs or SPR since both of these assays require purified immunoglobulin. During purification, much immunoglobulin protein is lost, so larger quantities of immunoglobulin are required than those produced by the transient system. Such quantities can be produced using stable cell lines. Therefore a further major achievement of this thesis was to set up stable cell lines that stably express between 130 and 250 ng per 10^6 cells per day after two methotrexate amplifications along with a

negative control line that does not express IgG. At present, large amounts of IgG are being purified from these stable cell lines through the use of a large scale, hollow fibre *in vitro* culture system. With the large amounts of purified antibody generated using this method, it will be possible in the future to obtain more accurate information regarding the importance of the R27aS mutation in determining the difference in DNA affinity of CHO-B3 compared to CHO-B3[R27aS] using assays such as SPR or inhibition ELISAs. Furthermore such quantities of purified antibodies could be used to investigate the direct effects of such specific changes in human anti-DNA antibody sequences on the pathogenicity of these antibodies *in vivo*.

An alternative to using large quantities of purified antibody to investigate the pathogenicity of these antibodies *in vivo* is to implant the actual stable cell lines directly into SCID (severe combined immunodeficient) mice. Each method has its own advantages and disadvantages. For example, with purified antibody, the half life is short so you can demonstrate deposition but the mice will probably not develop tissue damage. Alternatively, with cells, the mice are exposed to antibody for longer (since the antibody is being produced continually) however one has to allow for effects of the cells themselves.

In fact another member of our group, Lesley Mason, is already carrying this out in our laboratory. To investigate whether the reduction in DNA binding by B3 seen with the single amino acid reversion of V_λCDR1 R27a to S was paralleled by an effect on the pathogenicity of this antibody, the stable cell lines CHO-B3 and CHO-B3 [R27aS] were implanted in SCID mice. In brief, pristane-primed eight-week-old SCID mice were intraperitoneally implanted with 10^6 CHO cells. Two separate experiments were carried out. In each experiment, mice were implanted with either CHO cells expressing the B3 wild type antibody (CHO-B3) or CHO cells expressing the B3 antibody with the R27aS reversion (CHO-B3 [R27aS]). In the first experiment, a control group of mice were implanted with untransfected CHO cells whilst in the second experiment the control group were implanted with the CHO-(negative control) stable cell line containing no variable region DNA sequences and therefore unable to produce IgG. Each experiment also included mice that received the pristane injection only. Each control or test "group" consisted of four to five mice.

In both experiments, mice implanted with CHO-B3 showed significantly higher levels of proteinuria than mice implanted with the CHO-B3 [R27aS] line. Furthermore in both experiments those mice implanted with the CHO-B3 line became ill and died before those mice implanted with the CHO-B3 [R27aS] line or those in the control groups. However no evidence of SLE-related pathology could be seen in any of these mice once sacrificed.

This is the first time that the direct effects of specific changes in a human anti-DNA antibody sequence on the pathogenicity of an antibody have been observed. The main conclusion from the implantation of the two stable cell lines by Lesley Mason into the SCID mice was that the R27aS reversion in the mutant cell line appeared to reduce the pathogenicity of the antibody B3. In both experiments, those mice implanted with the CHO-B3 line died earlier and had more proteinuria than those implanted with the CHO-B3 [R27aS] line. It is unlikely that the proteinuria seen is due to the mere presence of the stable cell lines in the SCID mice as both of these groups of mice died earlier and showed higher levels of proteinuria than those mice in the control groups. Furthermore the replacement of the untransfected CHO cells in the first experiment with the empty expression vector cell line in the second showed that the simple presence of the plasmid DNA in the mice was not the cause of the disease seen. It is also unlikely that the differences between the two cell lines are due to differences in the antibody expression levels as both the *in vitro* production rate and the serum human IgG1 levels of the mice over the first three weeks post-implantation were higher in the CHO-B3 [R27aS] line. Therefore if antibody expression levels were the determining factor for the development of pathogenesis then it would have been expected that the mice implanted with CHO-B3 [R27aS] line would show a more severe disease. Moreover, the mice did not die due to tumour growth, as tumour growth did not differ between CHO-B3 and CHO-B3 [R27aS]. No firm evidence of binding or deposition of the secreted antibodies was found in the kidneys of the SCID mice. However this may be a result of the very small amounts of antibody found in the mice.

The work described in this thesis is only the second report of stable expression of a human anti-DNA antibody, although this is the first time that the direct effects of specific changes in a human anti-DNA antibody sequence on the pathogenicity of an antibody have been observed *in vivo*.

In the future, now that the stable expression system has been set up to express anti-DNA antibodies, it should be possible to set up more stable cell lines to express different heavy/light chain combinations. This will enable further investigation of the direct effects of specific changes in human anti-DNA antibody sequences on the pathogenicity of these antibodies *in vivo*.

The expression vectors used in this thesis have enabled me to manipulate immunoglobulin genes and subsequently express them as whole antibodies in order to gain a valuable insight into the effects of particular sequence motifs on the binding abilities of these monoclonal autoantibodies. The design of the vectors has allowed me to co-transfect cells with two expression vectors (one containing only the heavy chain and the other containing only the light chain DNA sequences) or to transfect cells with supervectors that enable the expression of both immunoglobulin chains from a single vector. Both types of vectors have various different advantages and disadvantages. For example, through the use of the single chain expression vectors in the transient expression system, a large number of combinations of heavy and light chains could be expressed and their binding ability assessed very quickly. However when using these single chain expression vectors in the stable expression system, there was a theoretical risk of excess production of heavy chain compared to light chain since co-transfection of the heavy chain with *dhfr* may be comparatively more productive than co-transfection of the light chain with *neo* in the CHO*dhfr⁻* selective growth medium used. Therefore, the two vectors were combined to produce "supervectors". The combination of both the heavy and light chain sequences into the same vector also increases the chance that any cell will take up both the heavy and light chain DNA. However a disadvantage of this method was that constructing a supervector for each heavy/light chain combination to be tested, was time-consuming.

Nevertheless there is still a risk that there will be an imbalance in heavy/light chain expression due to the design of the supervectors. This is because the construction of the supervectors involves a light chain expression cassette (containing the HCMV promoter, the λ constant region gene and the λ variable region gene of chosen autoantibody) being transferred into the heavy chain expression vector directly upstream from the heavy chain HCMV promoter. As a result, the light chain and heavy chain are being expressed in sequence. When two promoters in sequence are used to express two

genes in this way it is very likely that the second gene will be expressed at a lower level than the first. Therefore it is possible that the heavy chain in our supervectors will be expressed at lower levels than the light chain.

This is important, as a possible criticism of the system used in this thesis could be that an imbalance in heavy or light chains could affect the binding abilities of the IgG1 expressed. It has previously been shown that a heavy chain fusion protein could bind dsDNA in the absence of any light chain. For example, (Lecerf et al., 1998)used a bacterial expression system to produce eleven fusion proteins. Each fusion protein contained a V_H domain derived from healthy human peripheral B cells (but no V_L domain), which was fused with a B domain of *Staphylococcus* protein A. The purpose of the protein A was to facilitate secretion of soluble protein, assay and purification. Once purified, the protein A was cleaved from the V_H domain using thrombin. Five of the eleven V_H domains were able to bind ssDNA whilst two could bind dsDNA, when tested by both ELISA and surface plasmon resonance.

However in my opinion, it is unlikely that the results reported in this thesis are due to potential artefacts in the immunoassays due to either light or heavy chain over-expression. If binding were solely due to the concentration of a particular heavy chain, then it would be expected that no difference would be seen between the heavy/light chain combinations containing an identical heavy chain, regardless of the light chain present. This was not the case, for example, when the same heavy chain ($B3V_H$) was paired with a range of different light chains, some combinations showed substantial binding to particular autoantigens whilst others showed no binding at all. Therefore the differences seen in binding could not have been determined by the amount of heavy chain present as all combinations were transfected with the same quantity of the same heavy chain expression vector.

Similarly, when the same range of light chains was paired with a different heavy chain ($33.H11V_H$), the binding abilities of those combinations containing the same light chains but a different heavy chain also varied. For example, $B3V_H/B3V_\lambda$ could bind DNA whilst $33.H11V_H/B3V_\lambda$ could not bind DNA and although many of the $B3V_H/2a2$-derived V_λ combinations were able to bind histones, none of the $33.H11V_H/2a2$-derived

V_λ could. As a result, the differences seen in binding are unlikely to have been determined solely by the amount of light chain present.

However, an imbalance in the amounts of heavy and light chains produced in an expression system is not ideal. Therefore in future experiments using this transient or stable expression, I would suggest that the supernatants of the transfected cells were assayed by ELISA for the concentration of detectable heavy chain versus detectable light chain. This would show if there was an excess in the amount of light chain expressed as opposed to the amount of heavy chain expressed. If this were the case, a possible solution could be to co-transfect the supervector with a single chain expression vector containing the same heavy chain as the supervector. However this is not ideal, as it would still be difficult to balance accurately the expression of heavy chain versus light chain in the transfected cells. A preferred solution would be to insert an internal ribosome entry site (IRES) into the supervector.

An IRES is a sequence that allows the ribosomal machinery to initiate translation from a secondary site, within a single transcript. The hypothesis of the scanning ribosome mechanism in translation of mRNA to protein states that the initial contact between protein components (initiation factors and the 40S ribosomal subunit) and mRNA usually occurs at the 5' end of mRNA. The discovery of the 5'-terminal cap structure of eukaryotic mRNA and the description of proteins with the ability to bind both to the cap structure and to ribosomal subunits provided a plausible mechanism by which the 40S ribosomal subunits could be attracted to the 5' end of mRNA. Therefore the 5' cap structures are believed to play an important role in the initial ribosome entry or binding step (Stryer, 1995). However studies on the control of eukaryotic translation initiation have shown that cap-independent recruitment of the 40S ribosomal subunit to internal mRNA sequences called IRES can occur (Hellen et al., 2001; Vagner et al., 2001). If an IRES-sequence was inserted into our supervectors between the light chain and heavy chain cassettes, then expression of the two chains separated by the IRES should be proportional as heterologous genes supported by an IRES-element are located in the same RNA transcript.

As mentioned above, it has previously been shown that a heavy chain fusion protein could bind dsDNA in the absence of any light chain (Lecerf et al., 1998). If single

immunoglobulin chains are able to bind to antigens then in theory there is the potential that over-expression of either the light or heavy chain by the stable cell lines in this thesis could have affected the *in vivo* experiment carried out by another member of our group. For example, in humans there exists a disease called light-chain deposition disease (LCDD) which entails large amounts of monoclonal, amorphous, light chains deposition in multiple organs particularly the kidney often causing proteinuria and renal insufficiency (Randall et al., 1976). Consequently if the SCID mice implanted with the stable cell lines were over-expressing the light chains or heavy chains then it is possible that this is the reason that neutrophils but no direct tissue damage is seen. But this is unlikely, as it would be expected that if there were such a problem there would be no difference between the two IgG-producing cell lines.

B3 is a highly relevant antibody to study in the context of lupus as it has been derived from a patient with active lupus, it has a IgG1 isotype and it has high affinity for dsDNA. Although B3 is derived from an individual patient it is likely that it is typical of pathogenic anti-DNA antibodies found in other patients with SLE as its V_L and V_H are encoded by $V_\lambda 2a2$ and $V_H 3\text{-}23$ respectively. $V_\lambda 2a2$ and $V_H 3\text{-}23$ are the two most commonly rearranged V_λ and V_H genes, in both healthy individuals and SLE patients (Brezinschek et al., 1997; Ignatovich et al., 1999). Therefore the conclusions drawn from this thesis are likely to be relevant to a significant proportion of human anti-DNA antibodies found in patients with SLE. The long term aim of the work carried out in this thesis will be to utilise the expression system techniques now developed, to increase our understanding of the structure-function relationship of autoantibodies in SLE. This will be done by looking further at the effects of specific amino acid alterations in the sequence of these antibodies on not only binding to specific antigens but also on pathogenicity *in vivo* (e.g. in SCID mice). For example, it is already known that the strength of DNA binding does not always correlate to whether an antibody is pathogenic *in vivo*. Therefore it may be possible to use the expression system and constructs developed in this thesis to determine which sequence motifs and/or antigen specificities are most closely associated with pathogenesis and thus which sequence motif/antigen a future therapeutic strategy should focus on. The long-term therapeutic potential of this work is to eventually use the conclusions made to design an effective antagonist to block the effects of these autoantibodies in patients with SLE.

As anti-DNA antibodies are widely thought to be involved in the pathogenesis of patients with SLE particularly lupus nephritis, major attempts by pharmaceutical companies are already being made to either block or neutralise the effects of these antibodies. For example, La Jolla Pharmaceuticals (San Diego, USA) has developed an antigen-specific approach for the treatment of SLE called LJP 394 or Riquent™ (Jones et al., 1995; Linnik et al., 2002; Lorenz, 2002; McNeeley et al., 2001). LJP 394 is intended to deplete circulating anti-dsDNA antibodies and tolerise the B cells that produce anti-dsDNA antibodies. It provides tetravalent presentation of B cell epitopes in the form of four 20-mer synthetic, double-stranded oligonucleotide (dsDNA) B cell epitopes on a non-immunogenic organic platform (Jones et al., 1995). It does not contain T-cell epitopes, so that B cells which engage the drug on their surfaces will, die due to lack of T cell help. Pre-clinical *in vivo* studies showed that by administering 300μg LJP-394 (twice weekly for 2 to 5.5 months) to male BXSB mice (murine model of SLE involving spontaneous production of anti-dsDNA antibodies and significant renal inflammation secondary to immune complex deposition), anti-dsDNA levels were successfully lowered relative to controls with no observed non-specific effects (Jones et al., 1995).

Since these pre-clinical results, LJP 394 has undergone a variety of clinical research trials, including two dose regimen trials where anti-dsDNA levels were lowered by up to 50%, depending on the dose administered (Furie et al., 2001). An international, multicentre, randomised, double-blind, placebo-controlled Phase II/III trial was then carried out in order to determine the potential of LJP 394 to delay the time to renal flare in SLE patients. In this 76-week study, four weekly treatments with LJP 394 reduced circulating anti-dsDNA levels by 25±3% from baseline, whereas levels were increased in patients treated with placebo. Treatment with LJP 394 in patients with high affinity antibodies to LJP 394 prolongs the time to renal flare when compared with placebo group whilst the LJP 394-treated group also required less treatment with high-dose corticosteroids and/or cyclophosphamide. Furthermore the drug appeared to be well tolerated (Linnik et al., 2002). The results of an international phase III randomised, controlled clinical trial to determine if treatment with 100mg/week of LJP 394 can delay time to renal flare in patients with SLE and a history of renal disease are currently being compiled by La Jolla Pharmaceuticals (San Diego, USA). One potential problem with this particular approach is that LJP 394 only seems effective on those patients that

possess high affinity antibodies to the drug. However not all patients with SLE do have these antibodies and therefore the drug is of no use to them.

Although LJP 394 is effective in causing the depletion of anti-DNA levels in certain patients with SLE, it only has limited effects on reducing the disease activity in these patients. This suggests that rather than aiming to deplete all anti-DNA antibodies, future therapeutic strategies must aim to specifically deplete or block the subset of anti-DNA antibodies that cause pathogenesis in patients with SLE. In order to approach this goal, this pathogenic subset of antibodies firstly needs to be distinguished. The antigen or antigens to which these antibodies bind in order to contribute to the pathogenesis of SLE must then be identified. One therapeutic strategy would then be to design molecules that can specifically recognise and block the interaction of these pathogenic antibodies with antigen. In order to achieve this, a detailed understanding of the interaction between the antibody and the antigen is required at the molecular level. Not only does the work described in this thesis take us closer to a full understanding of this interaction, it also describes systems that are now established and ready to be used for future investigation into the structure-function relationship of the autoantibodies found in patients with SLE. The eukaryotic expression system will be particularly appropriate for this since it can be used to modify and express antibody sequences, which can then be tested in both assays of binding and assays of pathogenicity.

APPENDICES

Appendix A   One Letter Code For Amino Acids

ONE LETTER CODE	AMINO ACID
A	Alanine
C	Cysteine
D	Aspartic Acid
E	Glutamic Acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

Appendix B   Publications arising from work described in this PhD thesis

Rahman, A., Haley, J., Radway-Bright, E., Nagl, S., Low, D. G., Latchman, D. S., and Isenberg, D. A. (2001). The importance of somatic mutations in the V_λ gene 2a2 in human monoclonal anti-DNA antibodies. *J.Mol Biol* **307** 149-160.

Rahman, A., Haley, J., and Latchman, D. S. (2002). Molecular expression systems for anti-DNA antibodies. *Lupus* **11** 824-832

Rahman, A., Giles, I., Haley, J., and Isenberg, D. A. (2002). Systematic analysis of sequences of anti-DNA antibodies - Relevance to theories of origin and pathogenicity. *Lupus* **11** 807-823

Giles, I., Haley, J., Nagl, S., Isenberg, D. A., Latchman, D. S., and Rahman, A. (2003). A systematic analysis of sequences of human antiphospholipid and anti-β_2Glycoprotein I antibodies reveals the importance of somatic mutations and certain sequence motifs. *Semin Arthritis and Rheumatism* In Press

Haley, J., Mason, L. J., Nagl, S., Giles, I., Latchman, D. S., Isenberg, D. A., and Rahman, A. (2003). Somatic mutations to arginine residues affect the binding of human monoclonal antibodies to DNA, histones and SmD. *Mol. Immunol* Submitted

Mason, L. J., Haley, J. D. Isenberg, D. A., and Rahman, A. (2003). Stable expression of recombinant human anti-dsDNA antibodies to investigate the relationships between antibody sequence and pathogenicity. *J. Rheum.* Submitted.

First Authors are underlined.

APPENDIX C   GROWTH MEDIA AND BUFFERS

a) Bacterial growth media

Luria-Bertani (LB) medium

For 500ml medium:

    5g   Bacto-tryptone

    2.5g Yeast extract

    5g   Sodium chloride

This was made up to 500ml with double distilled water.

The pH was adjusted to pH 7.0 using 5.0 M Sodium Hydroxide and then autoclaved.

When antibiotic was added to the medium, the final concentration used was 100μg/ml ampicillin. Ampicillin was stored in 1ml aliquots as a stock solution of 50mg/ml in sterile water at -20°C in light-tight containers.

To make LB agar plates for the growth of bacteria, 1.5g of Bacto-agar was added to each 100ml LB medium.

NZY+ Broth

For one litre of medium:

    10g of NZ amine (casein hydrolysate)

    5g of Yeast extract

    5g of Sodium chloride

This was made up to one litre with double distilled water.

The pH was adjusted to pH 7.5 using 5.0 M Sodium Hydroxide and then autoclaved.

The following supplements were added to the media prior to use:

    12.5ml of 1M $MgCl_2$

    12.5ml of 1M $MgSO_4$

    10ml of a 2M filter-sterilised glucose solution (or 20ml of 20% (w/v) glucose)

b) Growth media for eukaryotic cells

COS-7 Cells

(Pre-Electroporation) COS-7 Growth Medium I

Dulbecco's Modified Eagle Medium (DMEM) (41966-029 Invitrogen, Paisley, UK)
Supplemented with:

> 10% (v/v) Foetal calf serum (FCS) (10099-133, Invitrogen, Paisley, UK)
>
> 580μg/ml L-glutamine (25030-024, Invitrogen, Paisley, UK)
>
> 50 units/ml penicillin/50 μg/ml streptomycin (15140-122, Invitrogen, Paisley, UK)

(Post-Electroporation) COS-7 Growth Medium II

Dulbecco's Modified Eagle Medium (DMEM) (41966-029 Invitrogen, Paisley, UK)
Supplemented with:

> 10% (v/v) Ultra low IgG Foetal calf serum (FCS) (16250-078, Invitrogen, Paisley, UK)
>
> 580μg/ml L-glutamine (25030-024, Invitrogen, Paisley, UK)
>
> 50 units/ml penicillin/50 μg/ml streptomycin (15140-122, Invitrogen, Paisley, UK)

COS-7 Freezing Medium

Dulbecco's Modified Eagle Medium (DMEM) (41966-029 Invitrogen, Paisley, UK)
Supplemented with:

> 10% (v/v) Foetal calf serum (FCS) (10099-133, Invitrogen, Paisley, UK)
>
> 580μg/ml L-glutamine (25030-024, Invitrogen, Paisley, UK)
>
> 50 units/ml penicillin/50 μg/ml streptomycin (15140-122, Invitrogen, Paisley, UK)
>
> 10% DMSO (D-5879, Sigma, Poole, UK).

CHOdhfr⁻ cells

*N.B. Throughout the work carried out in this thesis with CHO*dhfr⁻* cells, the foetal calf serum used was Ultra low IgG Foetal calf serum (FCS) (16250-078, Invitrogen, Paisley, UK). Although this did not appear to effect the selection of clones (selection of pure foci) possessing the functional *dhfr* gene, I would recommend that in the future Dialysed Foetal calf serum that has been tested for the absence of ribonucleosides and deoxyribonucleosides is used.

CHOdhfr⁻ (Non-selective) Growth Medium A

MEM α-Medium (α-MEM) with ribonucleosides and deoxyribonucleosides (32571-028, Invitrogen,Paisley, UK)

Supplemented with:

> 10% (v/v) FCS (10099-133, Invitrogen, Paisley, UK)
>
> 50 units/ml penicillin/50 μg/ml streptomycin (15140-122, Invitrogen, Paisley, UK)

CHOdhfr⁻ (Selective) Growth Medium B

MEM α-Medium (α-MEM) without ribonucleosides and deoxyribonucleosides (Invitrogen, Paisley, UK, 32571-029)

> Supplemented with: 10% (v/v) Ultra low IgG Foetal calf serum (FCS) (16250-078, Invitrogen, Paisley, UK)
>
> 50 units/ml penicillin/50 μg/ml streptomycin (15140-122, Invitrogen, Paisley, UK)

CHOdhfr⁻ freezing media

When creating a cell bank of CHO cells, the growth media and selective pressure (i.e. methotrexate concentration) used was identical to the media that the cells were cultured in immediately prior to freezing except that the freezing media contained 10% DMSO (D-5879, Sigma, Poole, UK).

c) Buffers

Tris Acetate EDTA (TAE buffer)

40mM Tris-acetate

1mM EDTA.

Tris Borate EDTA (T.B.E. buffer)

45mM Tris-borate

1mM EDTA.

Tris-EDTA (T.E.) buffer, pH 7.5

10mM Tris-HCl (pH 7.5)

1 mM EDTA

Phosphate buffered saline (PBS) pH 7.4

One PBS tablet (Invitrogen, Paisley, UK) was added per 500ml of sterile water and then autoclaved.

To make PBS/0.1% Tween, 1ml of "Tween 20" (Sigma, Poole, UK) was added to 1l of autoclaved PBS.

ELISA buffers

   i) ELISA citrate buffer, pH 8.0

       0.15M Sodium chloride

       0.015M Sodium citrate

   ii) ELISA sample, enzyme and conjugate dilution buffer (SEC)

       100mM Tris-HCl

       100mM Sodium chloride

       0.02% Tween 20

       0.2% BSA (Sigma, Poole, UK)

iii) ELISA Bicarbonate (BIC) buffer, pH 9.6

       0.5M sodium bicarbonate

       0.05M sodium dihydrogencarbonate

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