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Structural Basis For Human Anti-dna Antibody Specificity

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STRUCTURAL BASIS FOR HUMAN ANTI-DNA ANTIBODY SPECIFICITY

by

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ABSTRACT

Anti-DNA antibodies are hallmarks of SLE both in human and inbred lupus prone mice. The role of these antibodies in disease pathogenesis, particularly in glomerulonephritis has been documented. It is now well accepted that the genetic potential to generate anti-DNA antibodies also exists in the normal B cell immune repertoire. This genetic potential has been revealed by detection of anti-DNA antibodies in serum of normal individuals and by immortalizing DNA reactive B cells as hybridomas and as transformed cell lines. There is growing evidence that anti-DNA antibodies from the normal B cell repretoire and disease-associated anti-DNA antibodies share V region gene structures. Evidence for structural similarities includes the expression of normal monoclonal anti-DNA antibody idiotypes by lupus serum anti-DNA antibodies. Further direct evidence has been provided by sequence analysis of anti-DNA antibody genes. These studies showed that anti-DNA antibodies of either origin use no singular or simple mechanism for the generation of their specificity for DNA. It has been shown that VH and VL chain Ig germline genes in different combination can encode both normal and SLE anti-DNA antibodies and many of these genes can also be used by non DNA-binding antibodies. SLE anti-DNA antibodies are often somatically mutated and enriched with arginine residue(s) which could increase their affinity for DNA. In addition, the Ig V region of some anti-DNA antibodies are enriched with amino acids such as tyrosine, asparagine and glutamine which may also facilitate DNA-binding of these

antibodies It has also been suggested that heavy chains make a major contribution to DNA-binding in some anti-DNA antibodies based on the presence of common idiotype markers or motifs. One such motif YYGS, resides in the CDR3 region of KIM4.6 a natural human anti-DNA antibody heavy chain and is also expressed in more than 20% of human and murine anti-DNA antibodies and in the VH CDR2 region of some murine IgG anti-DNA antibodies.

The current study has explored the structural basis for DNA binding of human anti-DNA antibodies and in particular has focussed on the examination of the role of the heavy chain, the diversity region and the YYGS motif in conferring DNA specificity to the natural human monoclonal IgM anti-DNA antibody KIM4.6.

This study describes the generation and molecular characterization of Variants of KIM4.6 hybridoma which have lost their DNA binding property. The Ig V genes of three anti-Sm/RNP antibodies which either react with DNA or use genes related to KIM4.6 were also characterized. Gene manipulation techniques and a phage expression system or an *in vitro* transcription and translation system was used to further directly explore the role of YYGS motif in the DNA specificity of the KIM4.6 anti-DNA antibody.

This study revealed that: 1) the KIM4.6 heavy chain confers DNA specificity in this antibody, 2) the KIM4.6 D region YYGS motif is a necessary but not sufficient structural determinant in KIM4.6 reactivity to ds DNA but not ss DNA.

DEDICATION

To my parents

and

To those closest to me

Zahra, Zohreh and Mozhdeh

ACKNOWLEDGMENT

"He who taught me a word, I am forever in his debt."

"A Middle Eastern Philosopher"

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ABBREVIATIONS

BGG	bovine gamma globulin
BML	bone marrow lymphocyte
BSA	bovine serum albumin
bp	base pair
C	constant (region)
cDNA	complementary deoxyribonucleic acid
CDR	complementarity determining region
CITE	cap-independent translation enhancer
CNS	central nervous system
cpIII	coat protein III
cpVIII	coat protein VIII
cpm	count per minute
D	diversity (region)
ddNTP	dideoxynucleotide triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ds	double stranded
DTT	dithiothreitol
EBV	Epstein-Barr virus
ELISA	enzyme linked immunosorbent assay
EMCV	Encephalomyocarditis virus
Fab	fragment antigen binding
Fc	fragment crystalline
FCS	fetal calf serum
Fv	fragment variable (light and heavy)
FW	framework
GL	germline
H	heavy (chain)
HGM	hybridoma growth medium
HIV-1	Human Immunodeficiency Virus-1
Id	idiotype
Ig	immunoglobulin (IgM, IgG, IgA)
IPTG	isopropyl β-D-thiogalactosidase
J	joining (region)
L	light (chain)
LB	Luria Bertani
LL	liver lymphocyte
MMLV-RT	Moloney Murine Leukemia Virus-reverse transcriptase
NS	normal serum

OD	optical density
PBL	peripheral blood lymphocyte
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLL	poly L-Lysine
PS	patient serum
RBC	red blood cell
RF	rheumatoid factor
RNA	ribonucleic acid
RNP	ribonucleoproteins
RT	room temperature
SCA	sickle cell anemia
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	standard error
SL	splenic lymphocyte
SLE	systemic lupus erythematosus
Sm	Smith antigen
ss	single stranded
STP	single tube protein
TCA	trichloroacetic acid
TL	tonsillar lymphocyte
V region	variable region
v:v	volume:volume
VH	variable heavy
VL	variable light
WT	wild type
κ	kappa
λ	lambda

Chapter 1

Introduction

Since the first description of antibodies to DNA in the serum of the patients with systemic lupus erythematosus (SLE) more than 30 years ago, these autoantibodies have received considerable attention. Antibodies to the right handed helical form of DNA (B-DNA) are unique to SLE and considered reliable diagnostic and prognostic marker of SLE. Despite extensive investigation which has provided many new insights, the origin and role of anti-DNA antibodies in pathogenesis of SLE is still not clear.

Section 1.1 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown etiology defined by its clinical features and by the presence of a wide range of autoantibodies reactive to a variety of cellular components such as DNA, histones and ribonucleoproteins (Pennebaker *et al.* 1977, Tan 1989, Harley & Scofield 1991 and Mohan *et al.* 1993). Indeed many clinical features of the disease appear to correlate with certain autoantibodies (Arnett & Reveille 1992) and in some cases participation of certain autoantibodies in the pathogenesis of some clinical manifestations has been shown (Koffler *et al.* 1967 and Winfield *et al.* 1977). Antibodies to double-stranded (ds) DNA and to the ribonucleoprotein Sm are highly specific for the diagnosis of SLE.

The prevalence of SLE varies throughout the world. In North America and northern Europe, the prevalence of SLE is about 40 per 100,000 population (Hochberg 1991) while it has been reported in 21 per 100,000 population in Japan (Nakae *et al.* 1987). Over 90% of cases occur in women. This excess is especially noteworthy in the 15 to 64-year age group (Hochberg 1990). A greater incidence and prevalence of SLE has been reported in American blacks compared to American Caucasians (Fessel 1974).

The common pathologic process in SLE is blood vessel inflammation. The most common manifestation of SLE is arthritis. SLE arthritis is episodic, oligoarticular and migratory. Joint deformity can be seen as a result of joints soft tissue damage. The classic butterfly malar erythema occurs in one third of the patients. The other cutaneous manifestation of SLE is the patchy maculopapular rash on the areas exposed to the sun. Vasculopathy can result in painful and atrophic finger pads, usually accompanied by Raynaud's phenomenon. Less than a third of SLE patients have serious renal or central nervous system (CNS) involvements. Kidney and CNS abnormalities are the major prognostic determinants. In general, virtually any organ may be involved in SLE.

There is no cure for SLE and the treatment is often directed to individual disease manifestations (Mills 1994). A better understanding of the pathogenesis of SLE will provide more information about the nature and management the disease.

Numerous murine models of SLE have been studied. The most important

models are: NZB/B1 (Bielschowsky *et al.* 1959), NZB/NZW F_1 (Burnet & Holmes 1965), MRL/lpr (Murphy & Roths 1976), BXSB (Murphy & Roths 1979). Each model differs from others in genetic, immunologic and clinical manifestations of autoimmunity. Information from studies of these animals has contributed to understanding human disease. IgG anti-DNA antibodies are the most important antibodies made by NZB/NZW F_1, MRL/lpr and BXSB and contribute to glomerulonephritis. Many of these disease associated anti-DNA antibodies have been molecularly characterized and this provided insights into the structural basis for DNA reactivity of these antibodies (reviewed in Radic & Weigert 1994)

Section 1.2 Anti-DNA Antibodies

Anti-DNA antibodies in serum, a hallmark of SLE were first reported in 1957 (Ceppellini *et al.* 1957, Miescher & Strassle 1957, Robbins *et al.* 1957 and Seligmann 1957). The importance of these antibodies in disease and tissue injury particularly in nephritis were soon recognized (Rothfield & Stollar 1967) and certain subsets of anti-DNA antibodies were described. These antibodies can also be used as reagents to identify specific structures and structural changes in nucleic acids in general (Stollar 1994a).

1.2.1 Subsets of anti-DNA antibodies

Two major subsets of anti-DNA antibodies have been identified: "natural" and "pathogenic". Table 1.1 summarizes the general properties of these two groups. While anti-DNA antibodies are highly specific to SLE, it has been

Table 1.1

Subsets of anti-DNA antibodies[1]

"Natural" Antibodies	"Pathogenic" Antibodies
IgM[2]	IgG[3]
Mostly low affinity for ss DNA	High affinity for ds DNA
Low affinity cross reactivity to:	High affinity binding to:
Cell component Sm/RNP Cytoskeleton Histones Laminin Fc of IgG Phospholipids	Cell component DNA/histone Phospholipids Heparan sulfate Laminin Cationic charge
Bacteria Polysaccharides Proteins Phospholipids	Found in immune complexes in injured tissues
Few or no mutations in VH and VL segment genes	Mutations in VH and VL segment genes
Derived from normal repertoire	Derived from SLE repertoire

[1] Adopted from Hahn 1992

[2] Also can be non complement fixing IgG

[3] Complement fixing IgG and also some IgM

documented that healthy individuals (human and mouse) have the potential to make antibodies to DNA as part of their normal repertoire (Hasselbacher & LeRoy 1974, Fish & Ziff 1982, Cairns *et al.* 1984 and Schwartz & Stollar 1985). These antibodies can be revealed by somatic cell hybridization (Cairns *et al.* 1984) or by *in vitro* EBV transformation of normal human lymphocytes (Lydyard *et al* 1990). These "natural" antibodies are generally IgM, cross reactive with single-stranded (ss) DNA and usually contain little or no somatic mutations in their variable regions. Antibodies to ss DNA can indeed be elevated in diseases other than SLE such as infections, chronic inflammation and aging (Mackworth-Young & Schwartz 1988 a, · Jenberg *et al.* 1994). While anti-ds DNA antibodies are specific for SLE, some IgM or IgG antibodies reactive to ds DNA have been produced by activating resting B cells of both normal mice and humans *in vitro*.

"Pathogenic" anti-DNA antibodies which are associated with active disease are of IgG class. The antibodies derived from lupus prone mouse strain (MRL/*lpr*) have the ability to transfer disease to healthy strains of mice by forming immune complexes at glomerular and vascular sites (Vlahakos *et al.* 1992a and 1992b). "Pathogenic" anti-DNA antibodies have been eluted from lesions in kidneys of SLE patients (Koffler *et al.* 1967). Four different mechanisms have been suggested by which "pathogenic" antibodies to DNA can fix to the tissue and result in the tissue damage (Hahn & Tsao 1992): 1) formation of circulating immune complexes which are trapped in glomerular basement membrane, skin and blood vessels; 2) binding directly to the implanted DNA or DNA-histone complexes in the target tissues; 3)

binding to the polyanions in the proteoglycans of the basement membranes based on their charge; and 4) binding to antigens present in basement membranes other than DNA or DNA-histone complex such as laminin, phospholipids or heparan sulfate.

These "pathogenic" antibodies have high affinity for ds DNA, however, most react with ss DNA as well. It has been shown that most of these "pathogenic" anti-DNA antibodies contain numerous somatic mutations and have undergone antigenic selection (Diamond *et al.* 1992). Studies on hybridomas derived from lupus-prone mice NZB/NZW mice shown that both IgM and IgG anti-DNA antibodies within individual mice may be produced by B cells derived from the same precursor clones (Tillman *et al.* 1992). It is therefore possible that "pathogenic" antibodies in SLE may be selected from an expanded B cell pool of "natural" antibody producing lymphocytes. Initial polyclonal stimulation of "natural" B cells combined with immunoregulatory defects (Singh 1993) could lead to "pathogenic" high affinity autoimmune responses in SLE.

1.2.2 Anti-DNA response

Mammalian DNA is not immunogenic in a variety of animal species tested (Schwartz & Stollar 1985) but in contrast bacterial DNA can induce an anti-DNA antibody response in mice (Gilkeson *et al.* 1989a and 1989b). It is possible that anti-ss DNA response might arise from stimulation with foreign, rather than self DNA. The antibodies derived in this way are probably representative of "natural" anti-DNA antibodies. Thus cross reactivity of some anti-ss DNA with bacterial

antigens has been shown (Schwartz & Stollar 1985). Sequence similarities between anti-DNA and anti-bacterial antibodies also exists (Kofler *et al.* 1987). Anti-ss DNA antibodies have been shown to selectively react with particular bases (more often G or T) and poly(dT) oligonucleotides (Stollar 1973).

Antibodies to ds DNA can react with the exposed features of the DNA helix, the sugar-phosphate backbone or different conformations of ds DNA (Stollar 1994b). It has also been suggested that DNA in SLE patients may be altered structurally to become more antigenic (Hahn & Tsao 1992). However this assumption does not explain the *in vitro* reactivity of SLE anti-DNA antibodies with non-altered non-SLE DNA.

Another possibility for the induction of anti-DNA response is that DNA-histone complexes, as found in the content of nucleosomes, may act as specific autoantigens (Bell *et al.* 1990). It has also been shown that in comparison to normal individuals, nucleosomes are present in higher concentrations in the plasma of SLE patients (Rumore & Steinman 1990 and Isenberg *et al.* 1994). Nucleosomes have been shown to be released from normal mouse and human lymphocyte cultures by apoptotic cell death. The anti-histone antibodies that arise in SLE serum appear to react more readily to these histone molecules exposed in intact nucleosomes. Intact nucleosomes are therefore a good candidate for the specific induction of anti-DNA and anti-histones antibodies. It has been further proposed that nucleosomes could induce anti-DNA and anti-histone antibodies via their property of stimulating non-specific polyclonal B cell activation (Bell *et al.* 1990 and Dawson & Bell 1991).

Section 1.3 V Gene Usage of Human Anti-DNA Antibodies

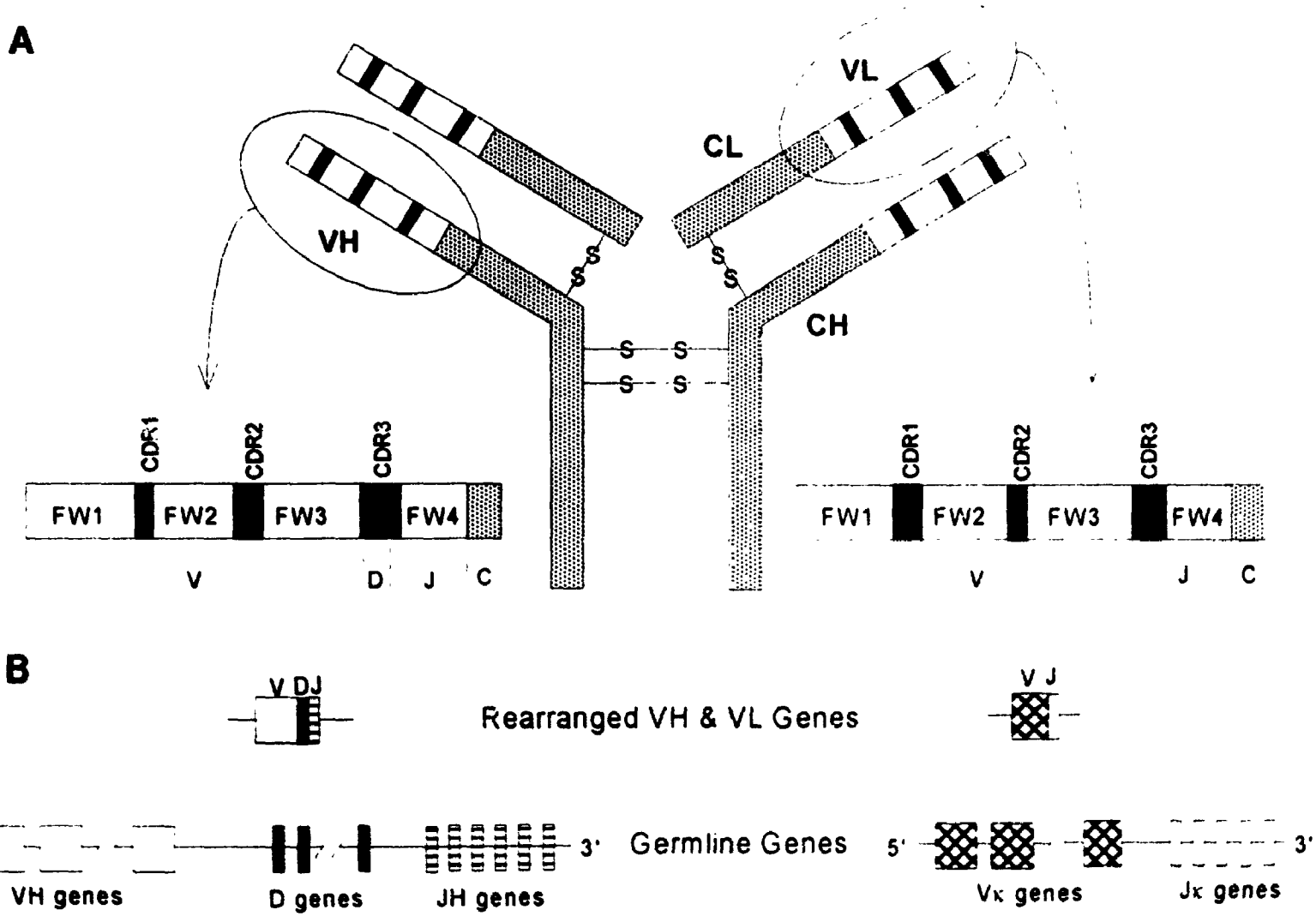
New technologies for rapid cloning and sequencing of immunoglobulin genes have provided considerable information on V gene usage of human anti-DNA antibodies. This information has increased the state of knowledge on the genetic origin of human anti-DNA antibodies.

1.3.1 Organization of human immunoglobulin genes

A schematic structure of an antibody molecule and the relevant genetic elements are shown in Figure 1.1. Each antibody molecule is composed of two light (L) and two heavy (H) chains. Each chain consists of two regions: a constant (C) region and a variable (V) region. The constant region of the heavy chain determines the isotype of an antibody molecule (IgM, IgD, IgG, IgA or IgE) and mediates the effector functions of the molecule such as binding to Fc receptors and activation of the complement cascade. The variable regions of the heavy chain (VH) and the light chain (VL) create the antigen binding site. The variable region contains three complementarity determining regions (CDR), also called hypervariable regions and four frameworks regions (FW). The framework regions maintain the overall structure of the V region and are less polymorphic than the CDRs. CDR regions of VL and VH form the antigen binding site of the antibody molecule.

The first and second CDR regions and the first three frameworks of the heavy chain are encoded by VH segment genes. The human VH repertoire consists of approximately 50 functional V segment genes (Tomlinson *et al.* 1994).

Figure 1.1 Schematic structure of an antibody molecule (A) and its variable region genes organization in human (B). CH: constant heavy chain; CL: constant light chain; VH: variable heavy chain; VL: variable light chain; V: V segment in (A) and V segment gene in (B); D: D segment in (A) and D segment gene in (B); J: J segment in (A) and J segment gene in (B); FW: framework region; CDR: complementarity determining region; -S-S-: indicative of interchain disulfide bonds.


A
VL
CL
VH
CH
S
CDR1
CDR2
CDR3
FW1
FW2
FW3
FW4
V
D
J
C
B
V DJ
Rearranged VH & VL Genes
V J
5'
3'
Germline Genes
VH genes
D genes
JH genes
Vκ genes
Jκ genes

Based on their sequence homology these genes are classified into six families. The assembly of the antibody molecule begins with the rearrangement of one of these VH segment genes to a diversity (D) and a joining (J) segment genes. The D and 5' portion of the J region genes encode the heavy chain third CDR (CDR3) region. The human heavy chain locus is estimated to have approximately 30 D genes (Ichihara *et al.* 1988). FW4 is encoded by J segment genes. There are six functional JH genes in the human VH locus (Ravetch *et al.* 1981) During the VDJ rearrangement non germline encoded N sequences may be inserted at the VD or DJ junctions by a terminal deoxytransfrase enzyme (TdT) (Desiderio *et al.* 1984). The N nucleotide additions at the VD junction can change the reading frame of the D region gene and create more diversity. The rearranged VDJ is initially transcribed with a μ constant gene and encodes the IgM heavy chain. During the secondary immune response VDJ can rearrange with a different constant gene which can result in isotype switching.

The VL region gene is composed of two gene segments: a V segment gene and a J segment gene. The light chain V and J genes are located at two genetic loci: a κ chain genes locus and λ chain gene locus. Vκ and Vλ segment genes are classified into four and seven groups respectively (Kabat *et al.* 1991). A V segment gene encodes the first three frameworks of the light chain and first and second CDR regions. VL CDR3 and FW4 regions are encoded by VJ junction and J genes respectively. The VJ genes of the κ locus are the first to rearrange and if this rearrangement is not productive then the λ gene rearranges to produce the light

chain of the antibody molecule (Paul 1989).

1.3.2 Generation of diversity in antibodies

The diversity of the antibodies used in the primary immune response results from 1) the rearrangement of heavy chain VDJ and light chain VJ from many available genes in heavy and light chain gene loci; 2) junctional diversities of heavy and light chains created by different types of joining and/or N nucleotide additions; and 3) the random pairing of light and heavy chains. The primary response of B cells generally results in the production of low affinity, polyspecific IgM antibodies.

After secondary exposure to antigen, B cells produce antibodies with increased affinity for the antigen. This affinity maturation is achieved by somatic mutation which is another mechanism for generation of diversity in antibodies (Kocks & Rajewsky 1989 and French *et al.* 1989). These antibodies have usually undergone isotype switching and predominantly are of IgG isotype.

1.3.3 Families of the V segment genes used in human anti-DNA antibodies

Table 1.2 shows some of the established characteristics of 41 human monoclonal anti-DNA antibodies. The isotype of these antibodies are IgM (24), IgG (11) and IgA (6) isotypes. It is clear that IgM anti-DNA antibodies may utilize any of 6 VH families. However, it seems that all the IgG anti-DNA antibodies reported thus far are encoded by genes from either VH3 or VH4 families. Nine of 17 anti-DNA antibodies (IgM, IgG and IgA) in VH3 family utilize the VH26 germline gene. Except for one (POP), all VH26 encoded IgM anti-DNA antibodies are identical to

Table 1.2

Characteristics of human anti-DNA antibodies

Antibody	Class	Origin	Binding to DNA	VH	Germline	Identity %[1]	D	JH	VL	Germline	Reference[2]
21/28	M	SLE[3] PBL[4]	ss	1	HA2	93	DXP'1	4	κ	-	Dersimonian 1989
8E10	M	Leprosy PBL	ss	1	HA2	93	U	4	κ III	Humkv325	Dersimonian 1989
BUD 114.4 11	M	Normal TL[5]	ss	1	hv1L1	99.7	DK1	4b	κ IV	Humk18	Mahmoudi 1993
III2R	M	SLE SL[6]	ss	1	51P1	96	U	5	κ I	HK137	Manheimer 1991
424.F6.24	A	SLE PBL	ss	1	VI-3b	93.9	DN1	4b	κ III	Humkv325	Kasaian 1994
C6B2	M	SCA[7] SL	ss > ds	2	58P2	93.8	DXP'1	3	-	-	Hoch 1987
TH3	M	Leprosy PBL	ss	2	?	-	DXP'1	4	κ III	Humkv325	Dersimonian 1989
447.8H	A	SLE PBL	ss	2	MC2	96.6	D21-7	4b	κ III	Humkv325	Kasaian 1994
1/17[8]	M	SLE PBL	ss	3	VH26	100	DXP'1	5	κ	-	Dersimonian 1987
18/2	M	SLE PBL	ss & ds	3	VH26	100	DXP'1	5	κ	-	Dersimonian 1987
B19.7	M	Fetal BML[9]	ss	3	VH26	100	U	4	λ	-	Young 1990
BUD 45.12.8	M	Normal TL	ss	3	VH26	100	DXP4	4b	κ III	Humkv325	Mahmoudi 1993
III3R	M	SLE SL	ds	3	H16BR	90	DXP'1	4	κ I	HK137	Manheimer 1991
KIM4.6	M	Normal TL	ss & ds	3	VH1.9III	100	DXP'1	6	λ I	Humlv117	Cairns 1989
POP	M	Leukemia PBL	ss	3	VH26	95.5	DLR4	4	κ I	kalc6	Spatz 1990
1-2A	G	SLE SL	ds	3	56P1	94	U	4	κ I	DILp1	Manheimer 1991
19.E7	G	SLE PBL	ss & ds	3	hv3019b9	98.9	U	6	κ III	Vg	Winkler 1992
32.B9	G	SLE PBL	ss & ds	3	VH26	96.6	DLR2	6	λ VIII	λ8	Winkler 1992
33.H11	G	SLE PBL	ss & ds	3	VH1.9III	86	U	4	λ II	-	Winkler 1992
33.F12	G	SLE PBL	ss & ds	3	22-2B	96	DN1	3	κ III	Humkv325	Winkler 1992
35.21	G	SLE PBL	ss & ds	3	H11	94.9	DM1/2	4	λ	-	Winkler 1992
H2F	G	SLE PBL	ds	3	VH26	90	U	4	κ IV	Humk18	Manheimer 1991
1X7RG1	A	SLE SL	ds	3	VH26	89	U	4	κ I	HK137	Manheimer 1991
412.67.F1.3	A	SLE PBL	ss & ds	3	WHG16G	96.6	DM2	4b	κ III	Humkv325	Kasaian 1994
412.66.F1	A	SLE PBL	ss	3	VH26	95.9	DXP4	4b	κ I	kalc6	Kasaian 1994

BEG-2	M	Fetal LL[10]	ss & ds	4	V71-2	-	U	5	λ	-	Watts	1991
IC4	M	Myeloma BML	ds	4	V71-4	97	U	4	κ I	HK137	Manheimer	1991
NE-1	M	SLE PBL	ss & ds	4	VH4.21	100	U	6	κ I	Vb'	Hirabayashi	1993
NE-13	M	SLE PBL	ss & ds	4	VH4.21	100	U	6	κ I	Vb'	Hirabayashi	1993
RT79	M	SLE SL	ss > ds	4	VH4.21	100	U	3	κ II	-	Stevenson	1993
2A4	G	Myeloma BML	ds	4	V71-2	92	DM2	6	κ I	DILp1	Davidson	1990
33.C9	G	SLE PBL	ss & ds	4	1.9II	90.6	U	3	κ I	HK102	Winkler	1992
D5	G	SLE PBL	ss & ds	4	VH4.21	93.8	DXP'1	5	κ III	HumIGVQ	Stevenson	1993
T14	G	SLE PBL	ss & ds	4	VH4.21	94.8	DXP'1	4	κ III	Humkv325	van Es	1991
448.9G.F1	A	SLE PBL	ss & ds	4	V71-2	93.3	DLR2	4b	λ I	YM-1	Kasaian	1994
II-1	M	SLE SL	ds	5	VH251	98	U	4	κ III	Humkv328	Manheimer	1991
A10	M	Normal PBL	ss	6	VH6	98.9	Q52	3	κ	-	Logtenberg	1989
A431	M	Normal PBL	ss	6	VH6	97.9	U	4	λ	-	Logtenberg	1989
L16	M	Fetal LL	ss	6	VH6	100	Q52	3	λ	-	Logtenberg	1989
ML1	M	Fatal SL	ss	6	VH6	100	U	4	κ	-	Logtenberg	1989
AB47	M	Normal PBL	ss	?7	?	-	U	4	λ	-	Sanz	1989

[1] % nucleotide identity
[2] Only the main reference is listed
[3] SLE: systemic lupus erythematosus
[4] PBL: peripheral blood lymphocytes
[5] TL: tonsillar lymphocytes
[6] SL: splenic lymphocytes
[7] SCA: sickle cell anemia
[8] 1/17 and 18/2 are sister clones
[9] BML: bone marrow lymphocytes
[10] LL: liver lymphocytes

the germline while the VH26 encoded IgG and IgA anti-DNA antibodies are somatically mutated. Some of these VH26 encoded antibodies express an idiotype 16/6 (Dersimonian *et al.* 1987 and Chen *et al.* 1988). Among the genes from the VH4 family used for anti-DNA antibodies, the VH4.21 germline gene is the most predominant. VH4.21 germline gene encodes 5 out of 10 VH4 anti-DNA antibodies. This gene was initially found to be utilized by antibodies with cold agglutinin activity (Pascual *et al.* 1991a and 1992a) and encodes an idiotype designated as 9G4. It has been shown that VH4.21 constitutes 10.8% of the normal repertoire of bone marrow B cells at any given point in time (Pascual & Capra 1992b). As expected, the VH4.21 encoded IgG anti-DNA antibodies (D5 and T14) are somatically mutated and VH4.21 IgM antibodies (NE-1, NE-13 and RT79) contain VH segments identical to the germline gene. It has been proposed that the recombination of the VH4.21 gene segment with the appropriate D segment gene can encode antibodies with specificity for DNA (Stevenson *et al.* 1993).

Thirty of 41 anti-DNA antibody light chains are encoded by κ genes. IgM, IgG and IgA anti-DNA antibodies may contain κ or λ light chain. Except for the *Humkv325* germline gene which encodes the VL segment of eight of these anti-DNA antibodies, no particular Vκ or Vλ member is predominant.

1.3.4 Anti-DNA antibodies and germline V genes

The VH gene segments encoding ten human anti-DNA antibodies (~25%) are identical to the assigned germline gene sequences. All these antibodies are of the IgM isotype and five of them react with both ss and ds DNA. The light chains

from five of these antibodies have been molecularly characterized. The VL segment of these five antibodies BUD 45.12.8, KIM4.6, NE-1, NE-13 and RT79 show 99.7, 100, 99.6, 100 and 99 % identities with the assigned germline genes which result in 1, 0, 0, 0 and 1 substitutions at the amino acid level respectively. Interestingly KIM4.6, NE-1 and NE-13 antibodies (with 100% germline encoded VH and VL segment) react with both ss and ds DNA. This suggests therefore that the diversity region of these antibodies may be important for DNA specificity.

1.3.5 Anti-DNA antibodies and somatic mutations

All IgG and IgA anti-DNA antibodies show evidence of somatic mutation (van Es *et al.* 1991, Manheimer-Lory *et al.* 1991, Winkler *et al.* 1992, Stevenson *et al.* 1993 and Kasaian *et al.* 1994).

T14 IgG anti-DNA antibody VH and VL genes differ by 14 and 15 nucleotide from the VH4.21 and *Humkv325* germline genes. These differences are concentrated in the CDR regions and yield a number of positively charged arginine and histidine residues at the amino acid level (van Es *et al.* 1991). The presence of several arginine and lysine residues in the somatically mutated D5 antibody is also speculated to be important for binding to DNA (Stevenson *et al.* 1993).

The molecular characteristics of the V genes encoding six human IgG anti-DNA antibodies were described by Winkler et al. Out of a total of 35 mutations in the CDR regions of their antibodies, 15 were arginine, asparagine or lysine residues which are suggested to play a role in binding to DNA. Arginine and asparagine residues have a balanced distribution over the CDR1, CDR2 and CDR3

(Winkler *et al.* 1991).

It has been speculated that arginine residues may interact with the phosphate of the DNA backbone or form hydrogen bonds with guanine and cytidine groups in ds DNA and asparagine and glutamine binds to the adenine and guanine groups in the major and minor groove of ds DNA respectively (Seeman *et al.* 1976). Indeed arginine residues have been shown to play an important role in determining the specificity of anti-DNA antibodies in MRL/*lpr* and NZB x NZW mice (Shlomchik *et al.* 1990).

Manheimer-Lory *et al.* have analyzed the molecular features of eight anti-DNA antibodies and concluded that somatic mutations in these antibodies do not always lead to an increase in ds DNA binding residues or in antibody charge (Manheimer-Lory *et al.* 1991). Five IgA anti-DNA antibodies all showing somatic mutations in both VH and VL genes have been described by Kasaian et al. They showed that among these IgA anti-DNA antibodies the number of arginine substitutions did not directly correlate with the degree of DNA binding (Kasaian *et al.* 1994).

1.3.6 Anti-DNA antibodies D region

18 of the sequenced human anti-DNA antibody D genes (44%) have not been assigned to a known D germline gene. DXP'1 is the most common germline D gene used by these antibodies (9 of 41 or 22%). The frequency of the B cell clones containing sequences with close homology to the DXP'1 gene segment has been estimated to be 8% (Huang *et al.* 1992 and Stewart *et al.* 1992). Although this

frequency is higher than what is expected for a single D gene (~3%), it is significantly less than the 22% frequency seen among anti-DNA antibodies. Based on the presence of a tyrosine-tyrosine-glycine-serine (YYGS) motif sequence encoded by the DXP'1 D germline gene in the D regions of the IgM anti-ss and ds DNA antibody KIM4.6 and some other anti-DNA antibodies, Cairns et al have suggested that the YYGS motif sequence may play an important role in DNA specificity in some anti-DNA antibodies (Cairns *et al.* 1989a). This motif is also expressed in the VH CDR2 or D region of some murine IgG anti-DNA antibodies with nephritogenic properties (Eilat *et al.* 1988 and 1991, Kofler *et al.* 1988, Tsao *et al.* 1990 and Katz *et al.* 1993).

The high content of tyrosine residues in the D region of some anti-DNA antibodies may also play an important role in DNA binding. The first crystal structure of an anti-DNA antibody with bound ligand revealed an interaction in which a thymine base is stacked between tyrosine and tryptophane aromatic rings (Herron *et al.* 1991)

Section 1.4 Expression of Antibody Fab Molecules on the Phage Surface

Filamentous phage was first used to display on its surface small peptides fused to the minor coat protein III (cpIII) (Smith 1985). The 406 amino acid long minor phage coat protein (cpIII) is a product of Gene III of filamentous phage. cpIII is expressed prior to the phage extrusion and is accumulated on the inner

membrane facing into the periplasm of the infected *E.coli*. Two functions are ascribed to cpIII: infectivity and normal (nonpolyphage) morphogenesis. It has been shown that the N-terminal domain of cpIII is required for viral infectivity; and the C-terminal domain which is incorporated into the virion, plays a role in generating normal phage particles (Crissman & Smith 1984). cpIII is located at the trailing end of phage particle (opposite to the end that is extruded first from the host). Two sites of cpIII were used for the fusion of the foreign protein: between the two domains of cpIII in the flexible spacer (Smith 1985) or close to or at the N-terminal of cpIII (Parmley & Smith 1988 and Cwirla *et al.* 1990). If the protein is fused to the N-terminal domain of cpIII, the phage remains infective. However, if the N-terminal is excised and the fusion made to the second domain, the phage is no longer infective and wild type cpIII needs to be provided by the helper phage (Barbas III *et al.* 1991a and 1992a, Hoogenboom & Winter 1992 and Marks *et al.* 1992). The cpIII fusion can be encoded on a plasmid containing a phage origin of replication (phagemid). Only five molecules of cpIII are present in each virion and the cpIII fusion should compete for incorporation with wild type cpIII. It has been estimated that in cpIII helper phage, phagemid expression systems, less than one cpIII fusion is present on each phage particle and these have been termed monovalent phage systems (Barbas III *et al.* 1991a). Even in these systems, it is possible to increase the number of cpIII fusions on a phage surface by using helper phages that lack cpIII and only the cpIII fusions from phagemid are present for incorporation. The major filamentous phage coat protein VIII (cpVIII) with 2700

copies per phage (Crissman & Smith 1984) can also be used to display peptides Obviously the phage population is multivalent and up to 24 antibody fragments can be incorporated per phage particle (Winter *et al.* 1994).

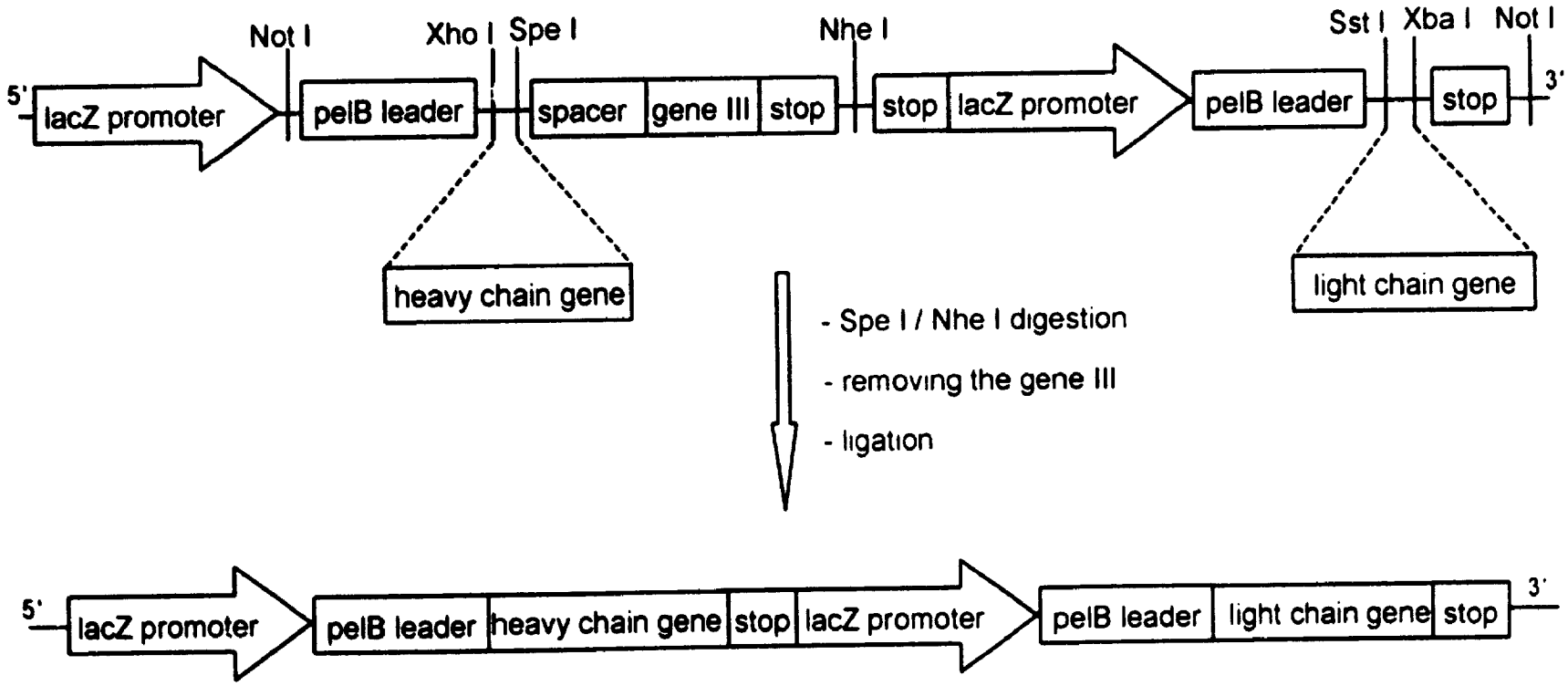
It has been shown that folded antibody fragments can also be displayed on phage. The antibody molecules can be displayed as single chain Fv fragments, in which VH and VL chains are connected by a flexible spacer (Huston *et al.* 1988) or as Fab fragments, in which one chain is fused to cpIII or cpVIII and the other chain is secreted into the periplasm (Barbas III *et al.* 1991a and Hoogenboom *et al.* 1991).

1.4.1 pComb3 expression system

The pComb3 expression system was described by Barbas III *et al.* in 1991. This phagemid system was designed for monovalent display of combinatorial Fab libraries on the surface of the filamentous phage M13. pComb3 is a pBluescript phagemid based vector which has been modified using the lacZ promoter, ribosome binding site, *pelB* leader sequence and sites for directional cloning of antibody heavy and light chain genes (Kang *et al.* 1991). A graphic map and a full restriction map of this vector are included in the Appendix A. Xho I and Spe I sites are provided for cloning of the heavy chain genes and Sst I and Xba I sites are designed for cloning of the light chain genes. 3' to the heavy chain gene cloning site a Gly-Gly-Gly-Gly-Ser spacer encoding sequence is provided which is followed by a cpIII encoding sequence (Figure 1.2). The heavy chain/cpIII sequence and the light chain sequence are placed under control of separate lac promoter/operator

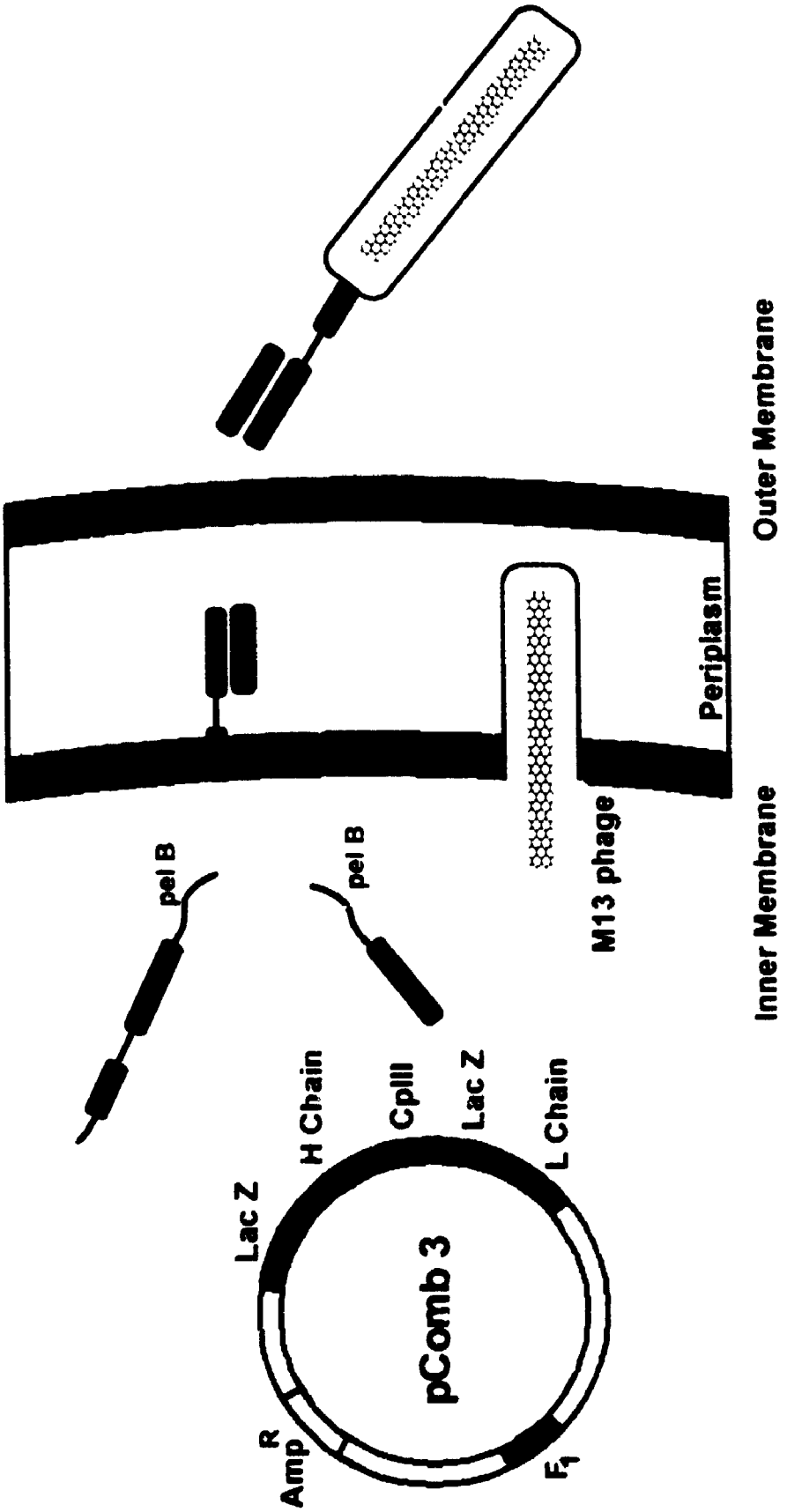
Figure 1.2 pComb3 phagemid constructs for expression of surface and soluble forms of antibody Fab molecule. The top part of the figure shows the location of the cloning sites for heavy and light chain genes, separate lacZ promoters for each gene, gene III (cpIII gene), *pelB* and spacer sequences This form of pComb3 construct results in surface expression of Fab molecules. The lower part of the figure shows the steps required for preparing pComb3 constructs for expression of soluble Fab molecules

pComb3 construct for surface expression of Fab molecules



pComb3 construct for soluble expression of Fab molecules

Figure 1.3 The composition of the pComb3 expression system. The heavy chain/cpIII and the light chain expression is controlled by lac promoter/operator sequences. The chains are directed to the periplasm by *pelB* signal sequences which are subsequently cleaved. The heavy and the light chains are then assembled in the *E.coli* periplasm. During phage extrusion through the cell mambrane, the Fab molecule is incorporated into the phage coat via cpIII.


Outer Membrane
Periplasm
Inner Membrane
M13 phage
pel B
pel B
Lac Z
H Chain
CpIII
Lac Z
L Chain
pComb 3
Amp R
F1

sequences and the produced protein is directed to the *E. coli* periplasmic space by *pelB* leader sequences for functional assembly on the inner membrane (Figure 1.3). Superinfection with the M13 helper phage leads to the expression of the wild type cpIII. Once packaged, the extruded helper phage particles carry both wild type cpIII which is necessary for infectivity and Fab/cpIII which is displayed for selection. Electron microscopy of phage expressing a human anti-tetanus toxoid Fab revealed specific single labeling at one end of the phage (Barbas III *et al.* 1991b).

It is possible to produce soluble Fab molecules by removing the cpIII encoding sequence from the pComb3 phagemids containing heavy and light chain genes.

1.4.2 Expression of antibodies using pComb3 expression system

The expression of antibody Fab molecules using the pComb3 system has been successfully used to isolate anti-tetanus toxoid antibodies (Barbas III *et al.* 1991b), antibodies to hepatitis B surface Ag (Zebedee *et al.* 1992), antibodies to HIV-1 virus gp120 (Barbas III *et al.* 1992b and 1993a), antibodies to respiratory syncytial virus F glycoprotein (Barbas III *et al.* 1992c), antibodies that coordinate metals (Barbas III *et al.* 1993b) and thyroid-specific human autoantibodies (Portolano *et al.* 1993).

This system has also been used for random mutagenesis of the cloned heavy and light chain genes and subsequent selection of the clones with improved affinity for the progesterone-3-oxim-BSA hapten conjugate (Gram *et al.* 1992).

Unfortunately since there is not enough data available on the sequences of

the antibodies screened by this system, the question of the possible bias toward expression of certain specific VH or VL genes cannot be addressed.

This system which will be discussed in Section 2.3 was employed for the combinatorial Fab expression of wild type and manipulated antibody genes in order to determine the binding specificity of the expressed Fab molecules for DNA.

Section 1.5 Protein Synthesis in a Coupled *In vitro* Transcription and Translation System

An *in vitro* transcription and translation system was used as an alternative tool for production of wild type and manipulated antibody heavy chains in this study (Section 2.4).

It has been shown that the synthesis of proteins *in vitro* from prokaryotic or eukaryotic sources yields sufficient product to meet the needs of mapping, regulatory and biochemical experiments. Transcription of cDNA clones by bacteriophage T7 RNA polymerase coupled to translation in the micrococcal nuclease treated rabbit reticulocyte lysate has been shown to result in a high fidelity and efficiency synthesis of proteins which are in a biologically active form (Craig *et al.* 1992).

It has been shown that the translation of the uncapped RNA can be improved by inserting the encephalomyocarditis virus (EMCV) untranslated region (UTR) between the T7 promoter and the target gene. This Cap-Independent Translation Enhancer (CITE) functions as an internal entry point for initiation of translation by

eukaryotic ribosomes and increases (by ~ 10-fold) the translation efficiency of synthetic RNA by rabbit reticulocyte lysates (Elroy-Stein *et al.* 1989).

Section 1.6 Scope of the Project

As discussed above, anti-DNA antibodies are hallmarks of the autoimmune disease SLE and play a major role in the pathogenesis of some of the disease manifestations and in particular the occurrence of glomerulonephritis. Despite considerable investigation over past 30 years, it is still not clear why the disease-associated anti-DNA antibodies appear in SLE and not in normal individuals. Recent attempts to investigate the genetic origin of these antibodies have provided many new insights. It has been shown that VH and VL chain germline genes in numerous different combinations can encode DNA specificity and many of these genes can also be utilized by non DNA-binding antibodies. Natural IgM anti-DNA antibodies generally use V genes in the germline configuration, while V region genes used by IgG anti-DNA antibodies are often somatically mutated. It has been found that the V regions of some of anti-DNA antibodies are enriched with arginine, tyrosine, asparagine and glutamine residues which may facilitate DNA-binding of antibodies in a manner similar to what has been found for other DNA binding proteins. The potential importance of the D region and the D region YYGS motif as a major contributor to DNA binding has also been suggested.

The purpose of this study was to further investigate the structural basis for DNA binding of anti-DNA antibodies. The study in particular focussed on examining

the hypothesis that the heavy chain diversity region and in particular its YYGS motif conferred DNA specificity to the natural human monoclonal IgM anti-DNA autoantibody KIM4.6.

This study describes the generation and molecular characterization of Variants of KIM4.6 hybridoma which have lost their DNA binding property.

Anti-Sm autoantibodies which target a complex of uridine rich RNA and several specific proteins while uncommon, are like antibodies to native DNA highly specific for SLE. The expression of anti-DNA and anti-Sm antibody responses appear to be regulated differently since they are often unlinked in SLE. Further, in autoimmune SLE mice anti-Sm autoantibodies appear to emerge stochastically. There are also earlier studies suggesting a common origin for anti-DNA and anti-Sm antibody V region genes. Analysis of the V region genes encoding several natural IgM anti-Sm/RNP antibodies developed in our lab, offered a further opportunity to examine and compare the molecular characteristics of this relationship. Two of these antibodies were chosen because in addition to Sm/RNP, they also reacted with ssDNA. The other anti-Sm/RNP antibody was chosen because the family of Ig VH and VL genes used were shown to be related to those used by the KIM4.6 anti-DNA antibody.

The phage expression system and *in vitro* transcription and translation system were used to directly explore the influence on the DNA binding property resulting from the manipulation of V region genes which had either maintained (KIM4.6) or lost (Variants) their ability to encode for binding to DNA.

These experiments and the results will be described and discussed in detail in the following chapters.

Chapter 2

Materials and Methods

Section 2.1 Selection and Characterization of the Variant Hybridomas

2.1.1 Selection of the Variant hybridomas

The IgMλ monoclonal antibody producing human hybridoma KIM4.6 was established through the fusion of tonsillar lymphoid cells from a healthy 7 years old girl with the IgG2κ producing lymphoblastoid cell line GM4672 (Cairns *et al.* 1984). KIM4.6 antibody binds to both single-stranded (ss) and double-stranded (ds) DNA (Cairns *et al.* 1984 and 1985). KIM4.6 antibody expresses on its λ light chain a 4.6.3 idiotype (Id) (Cairns *et al.* 1989b).

KIM4.6 hybridoma cells from a frozen stock were thawed and grown in culture. Briefly, after thawing at 37°C, the hybridoma cells (>10^7) were added to 10 ml of hybridoma growth medium (HGM). This medium was composed of: 1 mM sodium pyruvate, 2 mM L-glutamine, 125 U/ml penicillin-streptomycine, 0.5% 10mM non-essential amino acids, 1 M N-2-hydroxyethylpiperazin N-2-ethanesulfate (HEPES) buffer pH 7.2, 0.5 mg/ml gentamicin, 5% NCTC-109, 12% heat inactivated (56°C for 30 minutes) fetal calf serum (FCS) in RPMI 1640, pH 7.4. Then the cells were pelletted by centrifugation at 200 X g for 5 minutes and resuspended in 10 ml HGM containing 1% Nutridoma-HU (Boehringer Mannheim Biochemicals,

Indianapolis, IN). Cells were plated in 24 well culture plates (Costar, Cambridge, MA) at $1x10^6$ cells/well and cultured in a humid chamber at 37°C with 5% CO_2. Approximately 2/3 of the HGM medium was removed and replaced every 2 or 3 days. After two weeks, the KIM4.6 culture supernatant was tested by enzyme linked immunosorbent assay (ELISA) for the presence of IgMλ anti-ss and anti-ds DNA antibody (Sections 2.1.2, 2.1.3 and 2.1.4) and λ light chain 4.6.3 Id expression according to previously described method (Cairns *et al.* 1989b). IgMλ 4.6.3 Id(+) anti-ss and anti-ds DNA producing hybridoma cultures were then subcloned at 5 cells/well, and tested again for secretion of 4.6.3 Id(+) antibodies that bind ss and ds DNA. Positive hybridomas were subcloned again, but at one cell/well. Supernatants from these hybridomas were screened to select those IgMλ 4.6.3 Id(+) hybridoma clones which lost binding to both ss and ds DNA. These selected clones are referred to as Variants.

2.1.2 Detection of immunoglobulin in culture supernatants

The presence of immunoglobulin in culture supernatants was determined by ELISA. Microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with 50 µl of Fc specific $F(ab')_2$ goat anti-human IgM (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) at 10 µg/ml in carbonate-bicarbonate buffer pH 9.6 (15 mM $NaCO_3$ and 35 mM $NaHCO_3$) or with carbonate-bicarbonate buffer alone. Coated plates were incubated overnight at 4°C. The plates were then washed three times with washing buffer (0.1% bovine serum albumin (BSA) in 0.1 M Tris.Cl pH 7.4) containi g 0.05% v:v Tween 20 (Fisher Scientific, Fair Lawn, NJ).

The wells then were blocked with 250 µl of 2% BSA in 0.1 M Tris.Cl pH 7.4 for 2 hours at room temperature (RT). After a single wash in washing buffer, 50 µl of each culture supernatant and 50 µl of each control were added to the wells in duplicate and incubated at 4°C overnight. The plates then were washed 5 times in washing buffer. 50 µl of 1/2500 diluted alkaline phosphatase conjugated Fc specific $F(ab')_2$ goat anti-human IgM (Zymed Laboratories Inc., San Francisco, Ca) in washing buffer was added to the wells and incubated for 3 hours at RT. The wells were washed again 5 times in washing buffer and incubated at 37°C with 50 µl of 1 mg/ml p-nitrophenyl phosphate (Sigma Chemical CO., St. Louis, MO) in diethanolamine buffer (1 mM $MgCl_2$ in 9.7% diethanolamine, pH 9.8) as the substrate. The enzymatic reaction was stopped after 30 minutes with 3N NaOH (25 µl/well). The optical densities (OD) were determined at 405 nm using a Titertek Multiskan (Flow Laboratories Inc., McLean, VA) after 10 minutes.

In this ELISA, positive controls included IgM producing hybridoma supernatants, pooled human IgM (Cooper Biomedical, Inc., Malvern, PA) and diluted human sera. Negative controls were HGM and washing buffer.

2.1.3 Detection of the λ light chain

Microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with 50 µl of Fc specific $F(ab')_2$ goat anti-human IgM (Jackson ImmunoResearch Laboratories Inc.) at 10 µg/ml in carbonate-bicarbonate buffer or with carbonate-bicarbonate buffer alone. Coated plates were incubated overnight at 4°C. The plates were then washed three times with washing buffer (0.1% BSA in 0.1M

Tris.Cl pH 7.4) containing 0.05% v:v Tween 20 (Fisher Scientific). The wells then were blocked with 250 μl of 2% BSA, 0.1 M Tris.Cl pH 7.4 for 2 hours at RT. After a single wash in washing buffer, 50 μl of each culture supernatant and 50 μl of each control were added to the wells in duplicate and incubated at 4°C overnight. The plates were washed 5 times in washing buffer and 50 μl of alkaline phosphatase conjugated goat anti-human λ chain specific (Sigma Chemical Co.) at 1/5000 dilution in 1% BSA, 0.5% bovine gamma globulin (BGG), 0.1M Tris.Cl pH 7.4 was added to each well. The plates were incubated at RT for 3 hours, washed 5 times with washing buffer and incubated at 37°C with 50 μl of 1 mg/ml p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8. The reaction was stopped after 30 minutes with 3N NaOH (25 μl/well) and after 10 minutes the optical densities were determined using a Titertek Multiskan at 405 nm.

Positive controls in this ELISA consisted of hybridoma supernatants containing λ chain antibodies. The negative controls were HGM, 1% BSA, 0.5% BGG in 0.1M Tris.Cl pH 7.4, and hybridoma supernatants containing κ chain antibodies.

2.1.4 Anti-single and anti-double stranded DNA antibody ELISA

Microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with 50 μl/well poly L-Lysine (PLL) (Sigma Chemical Co.) at 50 μg/ml in 0.1 M Tris.Cl pH 7.4. After 1 hour incubation at RT, the wells were washed 3 times in 0.1 M Tris.Cl pH 7.4 and coated with 50 μl/well of ss or ds calf thymus DNA (Millipore Corporation, Freehold, NJ) at 10 μg/ml in 0.1 M Tris.Cl pH 7.4 or with 0.1 M Tris.Cl

pH 7.4 alone. The plates were incubated overnight at 4°C and afterwards washed three times with washing buffer (0.1% BSA in 0.1 M Tris.Cl pH 7.4) containing 0 1% v:v Tween 20. The wells then were blocked with 250 µl of 2% BSA, 0.1 M Tris.Cl pH 7.4 for 2 hours at RT. After one wash with washing buffer containing 0.1% v:v Tween 20, fifty µl of each culture supernatant and 50 µl of each control were added to the wells and incubated at 4°C overnight. The plates were then washed 5 times in washing buffer. 50 µl of 1/2500 diluted alkaline phosphatase conjugated Fc specific $F(ab')_2$ goat anti-human IgM (Jackson ImmunoResearch Laboratories Inc.) in 1% BSA, 0.5% BGG, 0.1 M Tris.Cl pH 7.4, was added to the wells and incubated for 3 hours at RT. The wells were then washed 5 times in washing buffer containing 0.1% v:v Tween 20 and incubated for 30 minutes at 37°C with 50 µl of 1 mg/ml p-nitrophenyl phosphate in diethanolamine buffer. The reaction was stopped with 3N NaOH (25 µl/well). After 10 minutes, the optical densities (OD) were determined at 405 nm using a Titertek Multiskan.

Positive controls were anti-ss and ds DNA KIM4.6 hybridoma supernatant and diluted SLE serum. Negative controls consisted of HGM, 1% BSA 0.5% BGG 0.1M Tris.Cl pH 7.4 and diluted normal human serum.

2.1.5 IgM quantitation ELISA

An IgM quantitative ELISA for those IgMλ 4.6.3 Id(+) hybridoma clones (Variants) which lost binding to both ss and ds DNA was preformed as described in Section 2.1.2, except that: 1) the Variants' supernatants were diluted in HGM and tested in triplicate and 2) pooled human IgM (Cooper Biomedical, Inc.,

Malvern, PA) diluted in HGM (final concentrations ranging from 0.02 to 2 μg/ml) was assayed in order to generate the IgM standard curve. The IgM concentration in the supernatants was determined by extrapolation from the standard curve.

2.1.6 Anti-Sm/RNP ELISA

The supernatants of the Variants were tested for Sm/RNP reactivity by ELISA. Plates were coated with Sm/RNP (Immunovision, Springdale, AR) at 5 μg/ml in coating buffer (carbonate-bicarbonate buffer) or coating buffer alone and incubated at 4°C overnight. After 3 washes with washing buffer containing 0.5% v:v Tween 20, wells were blocked with 250 μl of 2% BSA, 0.1 M Tris.Cl pH 7.4 for 2 hours at RT. After a single wash in washing buffer, 50 μl of each Variant culture supernatant and 50 μl of each control were added to the wells in duplicate and incubated at 4°C overnight. The plates then were washed 5 times in washing buffer. 50 μl of 1/2500 diluted alkaline phosphatase conjugated $F(ab')_2$ goat anti-human IgM Fc specific (Jackson ImmunoResearch Laboratories Inc.) in 1% BSA, 2%BGG, 0.1 M Tris.Cl pH 7.4, was added to the wells and incubated overnight at 4°C. The wells then were washed 5 times in washing buffer and incubated at 37°C with 50 μl of p-nitrophenyl phosphate (1mg/ml) (Sigma Chemical CO.) in diethanolamine buffer. The reaction was stopped after 30 minutes with 3N NaOH (25 μl/well). After 10 minutes the optical densities (OD) were determined at 405 nm using a Titertek Multiscan.

The positive control was supernatant from an anti-Sm/RNP hybridoma and negative controls were HGM, 1% BSA 2%BGG 0.1 M Tris.Cl pH 7.4 and normal

human ser ı.

2.1.7 Rheumatoid Factor (RF) ELISA

The supernatants of the Variants were tested for RF reactivity by ELISA. Microtiter plates were coated overnight at 4°C with 50 µl of the human IgG (Cooper Biomedical), the purified Fc fragment of human IgG (Jackson Laboratories), or the purified Fab fragment of human IgG (Jackson Laboratories) each at a concentration of 10 µg/ml in carbonate-bicarbonate buffer. Control wells were coated only with carbonate-bicarbonate buffer. After two washes with saline containing 0 05% v:v Tween 20 and one wash with saline, the wells were blocked with 250 ml of 3% BSA in saline for 2 hours at RT. Plates were washed once in saline containing 0.05% v:v Tween 20 ar 1 once with saline and then 50 µl of the each Variant supernatant and 50 µl of the each control were added to the wells in duplicate and incubated overnight at 4°C. The plates were then washed 4 times in saline containing 0.05% v:v Tween 20 and once in saline. 50 µl of 1/2500 diluted alkaline phosphatase conjugated Fc specific $F(ab')_2$ goat anti-human IgM (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in 3% BSA, saline, 0.05% v:v Tween 20, was added to the wells and incubated overnight at 4°C. The plates were washed 4 times in saline containing 0.05% v:v Tween 20 and once in saline and incubated at 37°C with 50 µl of 1 mg/ml p-nitrophenyl phosphate in diethanolamine buffer. The reaction was stopped after 30 minutes with 3N NaOH (25 µl/well). Ten minutes later the optical densities (OD) were determined at 405 nm using a Titertek Multiscan.

Positive controls included hybridoma supernatants having RF reactivity. Negative controls were HGM and 3% BSA, 0.05% v:v Tween 20 in saline.

2.1.8 Cold agglutination assay

The supernatants of the Variants were tested for the ability to agglutinate cord or adult group O red blood cells. One drop of phosphate buffered saline (PBS) washed 2% cord or adult red blood cells (provided by the Hematology Laboratory, University Hospital, London, Ontario) were mixed with 3 drops of each of the Variants' supernatants and the controls. After 2 hours of incubation at 4°C cells were examined and the degree of agglutination was estimated microscopically according to the criteria of Dacie and Lewis (Dacie & Lewis 1984 and Stevenson *et al.* 1989).

The positive control was serum from a patient with cold agglutinin disease (provided by the Hematology Laboratory, University Hospital, London, Ontario). The negative controls included normal human sera and previously tested cold agglutinin negative hybridoma supernatants.

Section 2.2 Molecular Characterization of the Immunoglobulin Variable Regions

2.2.1 Isolation of total RNA from the Variants

Variants hybridomas were cultured in HGM to yield a total of approximately 10^8 cells Cells were pelletted by centrifugation at 180 X g and washed 3 times in RPMI 1640. Cell pellets were kept frozen in liquid nitrogen prior to total RNA

isolation. Total cellular RNA was extracted from these cells by the guanidinium thiocyanate and cesium chloride ultracentrifugation modified method (Sambrook *et al.* 1989). Briefly, cells were resuspended in 8 ml of guanidinium thiocyanate homogenization buffer (4.0 M guanidinium thiocyanate, 0.1 M Tris.Cl pH 7.5, 1% 2β-mercaptoethanol) and passaged repeatedly through 18 and then 23-gauge needles. Then each of the samples was layered onto a 4 ml cushion of 5.7 M CsCl, 0.01 M EDTA pH 7.5 in a 13 ml polyallomer centrifuge tube (Beckman Instruments, Inc., PaloAlto, CA). Samples then were centrifuged for 20 hours at RT at 144200 X g in a SW41 rotor in Beckman L8-M ultracentrifuge (Beckman Instruments, Inc , PaloAlto, CA). The RNA pellet was washed 2 times with 70% ethanol and allowed to dry. The pelleted RNA was resuspended in 500 μl of TE buffer (10 mM Tris.Cl pH 7.4, 1 mM EDTA pH 8.0) pH 7.6. A 10 μl aliquot of RNA preparation was used to determine the amount and purity of RNA by reading optical densities at 260 and 280 nm. The remaining RNA was precipitated with 1/10 volume of 3 M sodium acetate pH 5.2 and 2.5 times volume of ice-cold 95% ethanol. The pellet was washed with 70% ethanol, dried and resuspended to a concentration of 5 μg/μl in diethylpyrocarbonate (DEPC) treated sterile distilled water.

2.2.2 Variants' Ig VH and Ig VL cDNA synthesis

The total RNA was used to generate single-stranded cDNA by reverse transcription employing Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) (Gibco/BRL Life Technologies, Inc., Gaithersburg, MD). The reaction mixture contained 2.5 μl of 10 mM deoxynucleotide triphosphates (dNTPs), 10 μl

5 X reverse transcription buffer (250 mM Tris.Cl pH 6.8, 375 mM KCl, 15 mM $MgCl_2$ and 50 mM dithiothreitol), 24.5 µl sterile water, 5 µl total RNA (5 µg/µl) and 5 µl 20 µM appropriate 3' antisense primer. 3' antisense "Cµ short" and "Cλ short" primers (Appendix B) were used for the reverse transcription of Variants' heavy chain and light chain mRNA respectively. The reaction mixture was heated for 2 minutes at 68°C, followed by addition of 0.5 µl (500 U) MMLV-RT. After 45 minutes of incubation with this enzyme at 45°C samples were placed on ice or stored at -20°C for immediate or further use respectively.

2.2.3 Amplification of Variants' Ig VH and Ig VL cDNA

The polymerase chain reaction (PCR) was used to amplify Ig VH and VL cDNAs. For the VH chain cDNA amplification, each of the 6 VH family leader sense primers (Appendix B) and the "Cµ short" antisense primer were employed. For the VL chain cDNA amplification, each of 7 Vλ framework sense primers and also a "VλK46 leader/framework" sense primer (Appendix B) and the "Cλ short" antisense primer were used. Each amplification reaction contained 10 µl 10 X PCR buffer (590 KCl, 117 mM Tris.Cl pH 8.3, 14.7 mM $MgCl_2$, 0.1% gelatin and 2.5 mM each dNTP), 2.5 µl of 20 µM 5' sense primer, 2.5 µl of 20 µM 3' antisense primer, 77.5 µl sterile water and 5 µl cDNA. The reaction mixture was boiled for 2 minutes before addition of 1.5 U AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). Samples were then placed in a cyclothermal apparatus (Perkin Elmer Cetus, Norwalk, CT). Cycling times were 1 minute at 94°C, 1 minute at 50°C, 1 minute at 72°C for 30 cycles followed by 7 minutes at 72°C for final extension. The amplified

PCR products were size fractionated by 1 4% agarose gel electrophoresis (Sambrook *et al.* 1989) in order to confirm that they contain VH and VL cDNAs of expected sizes.

2.2.4 Cloning the amplified Variants' cDNA fragments

PCR amplified cDNA fragments were extracted with phenol:chloroform (1:1) followed by chloroform:isoamyl alcohol (24:1). They were then precipitated with 3 M sodium acetate/95% ethanol and resuspended in sterile water. VH and VL chain amplified DNA fragments were digested with Xho I/EcoR I and Sst I/Xho I (Gibco/BRL Life Technologies, Inc., Gaithersburg, MD) respectively. Restriction enzyme digested fragments were purified using Geneclean II kit (BIO 101, Inc., La Jolla, CA) according to the manufacturer's instructions. The purified cDNAs were ligated to appropriate restriction enzyme digested and Geneclean purified pGEM-11Zf(-) plasmid (Promega Corporation, Madison, WI). *Epicurian coli XL1-Blue* cells (Stratagene, La Jolla, CA) were transformed with recombinant plasmids and plated on MacConkey agar (BDH, Darmstadt, Germany) containing 100 µl/ml ampicillin. Individual weakly or non-lactose fermenting bacterial colonies were transferred from the plates to the tubes containing 3 ml of Luria-Bertani (LB) medium with 100 µl/ml ampicillin for overnight culture at 37°C. Plasmid DNA was isolated from the cultures by the alkaline lysis method (Sambrook *et al.* 1989) followed by Geneclean purification. The DNA inserts were liberated from the plasmid DNA by double-digestion (Xho I /EcoR I for the heavy chain and Sst I/Xho I for the light chain) and then screened by electrophoresis on 1.4% agarose gel to select the DNA inserts

of the correct sizes.

2.2.5 Other Ig VH and Ig VL cDNA synthesis, amplification and cloning

Three other established human hybridomas (BUD 45.12.8, BUD 94.91.8 and BUD 114.4.11) which originated from tonsillar B cells of a normal child (Carruthers 1991) were chosen for molecular characterization of their immunoglobulin genes. BUD 45.12.8 and BUD 114.4.11 hybridomas were chosen because they produced antibodies which in addition to Sm/RNP also reacted with ssDNA. BUD 94.91.8 anti-Sm/RNP antibody was chosen because the family of Ig VH and VL genes used in this antibody were shown to be related to those used by the KIM4.6 anti-DNA antibody. After isolation of total RNA from BUD hybridomas, reverse transcription and PCR of Ig V region genes were performed as described in Sections 2.2.2 and 2.2.3. Primers used in reverse transcription and PCR are listed in Table 2.1. The VH and VL amplified DNA fragments of the BUD antibodies were digested with appropriate restriction enzymes (Appendix B) and cloned into compatible cloning sites of pGEM-11Zf(-) plasmid.

2.2.6 Sequencing

Ig V region genes were sequenced by dideoxy-mediated chain termination method using a genetic variant of bacteriophage T7 DNA polymerase (Tabor & Richardson 1987), Sequenase Version 2.0 Kit (United States Biomedical Corp, Cleveland, Ohio). 3-5 μg of Geneclean purified double-stranded plasmids containing correct size inserts were first denatured in 0.2 M NaOH, 0.2 mM EDTA at 37°C for 30 minutes (Lim & Pene 1988). The mixture was then neutralized by

Table 2.1

Primers used for reverse transcription and PCR of BUD clones[1]

	Reverse Transcription antisense primer	PCR antisense primer	PCR sense primers
BUD 45.12.8 VH	Cμ long	Cμ short	6 VH Leader primers
BUD 94.91.8 VH	Cμ long	Cμ short	6 VH Leader primers
BUD 114.4.11 VH	Cμ long	Cμ long	6 VH Leader primers
BUD 45.12.8 VL	Cκ long	Cκ short	4 Vκ Leader primers
BUD 94.91.8 VL	Mc3'κ	Mc3'κ	4 Vκ Leader primers
BUD 114.4.11 VL	Cλ long + Cλ long2	Cλ short	7 Vλ Framework primers

[1] See Appendix B for detailed information about these primers

adding 0.1 volumes of 3 M sodium acetate pH 4.8, and then the DNA was precipitated with 2.5 volumes of 95% ethanol (15 minutes at -70°C). After washing the DNA pellet with 70% ethanol, the pellet was dissolved in 7 μl of distilled water, 2 μl of Sequenase reaction buffer (provided in the kit) and 1 μl (0.5 pmol) of either M13 forward or M13 reverse primers (Appendix B). This mixture in capped tube was heated to 65°C for two minutes and then cooled to RT over a period of about 30 minutes in order to allow annealing of the primer to the target sequences. The labeling step was performed for 5 minutes at RT after the addition of 1 μl 0.1 M dithiothreitol (DTT), 2 μl diluted labeling mix (provided in the kit), 1 μl [α-^{35}S]dATP 10 μCi/μl (Amersham Corp., Arlington Heights, IL), and 2 μl of 1:8 diluted Sequenase Version 2.0 T7 DNA polymerase and pyrophosphatase enzymes in enzyme dilution buffer (all provided in the kit). Meanwhile tubes labeled A, C, G, T were filled with 2.5 μl of the ddATP, ddCTP, ddGTP and ddTTP termination mixes (provided in the kit) respectively and pre-warmed at 37°C. 3.5 μl of the labeling mixture was transferred to the A, C, G and T tubes and incubated at 37°C for 5 minutes. Four μl of stop solution (provided in the kit) was then added to each tube. Samples were stored at -20°C (no longer than one week) until ready to load to the sequencing gel.

The standard 8 M urea, 8% polyacrylamide sequencing gel was casted into the 35 X 50 cm Sequi-Gen Nucleic Acid Sequencing Cell (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. After pre-running the gel for one hour at 45°C, samples (denatured at 95°C for 2 minutes) were loaded

into the gel and run for 5 to 14 hours at 50°C (approximate voltage of 2000 V). The gel then was fixed in 10% acetic acid and 10% methanol for 15 minutes to remove hygroscopic urea and then transferred to a Whatman 3 mm filter paper. The gel was dried at 80°C for 1 hour using Bio-Rad Gel Dryer, Model 583 (Bio-Rad Laboratories, Richmond, CA). The gel was exposed at RT to XAR Kodak X-ray film (Eastman Kodak Company, Rochester, NY).

The nucleotide sequences usually included at least a 50 base pair overlap from both directions. If this overlap could not be achieved, an internal primer (Appendix B) was designed and employed in the sequencing reaction to obtain the sequence overlap. The nucleotide sequence was confirmed by sequencing DNA from at least two recombinant plasmids isolated from different bacterial colonies.

2.2.7 Nucleotide sequence analysis

Nucleotide sequences were analyzed with the SeqEd, FastA, Fetch, Gap, Map, Mapsort, PlasmidMap, Isoelectric and Publish programs of the Genetics Computer Group Sequence Analysis Software Package, Version 7.3.1-UNIX, September 1993 (Genetics Computer Group, Inc., Madison, WI) and BLASTN program (Altschul *et al.* 1990) through National Center for Biotechnology Information (NCBI) E-Mail server (blast@ ncbi.nlm.nih.gov).

The Ig VH and VL region nucleotide sequences were submitted to the European Molecular Biology Laboratories (EMBL) database using Authorin, Release 1.2, Version 0.5 (IntelliGenetics, Inc., Mountain View, CA).

Section 2.3 Expression and Detection of Antibody Fab Molecules on the Surface of Phage

2.3.1 Ig V region cDNA synthesis and PCR amplification

To generate KIM4.6 and Variant (V3) VH and VL region DNA fragments containing appropriate restriction sites for cloning into the pComb 3 expression vector, reverse transcription and PCR were again performed as explained in Sections 2.2.2 and 2.2.3. Primers used in the reverse transcription of KIM4.6 and V3 VL and VH RNA were "Cλ long" and "Cμ long" (Appendix B) respectively. For KIM4.6 VL and V3 VL PCR a "VλK46 leader/framework" sense primer and the "Cλ long" (Appendix B) antisense primer were used. For KIM4.6 VH PCR "VHK46L Leader" sense primer and "Cμ long" antisense primer were used (Appendix B). "VH4 Leader" and "Cμ long" primers were used for V3 VH PCR. Using these primers Sst I/Xba I and Xho I/Spe I restriction sites were introduced to VH and VL DNA amplified fragments respectively.

2.3.2 Cloning the amplified DNA fragments

pComb 3 phagemid expression vector (Barbas III *et al.* 1991a) was used for cloning of VL and VH genes. To achieve this, the VL gene was first cloned followed by cloning of the VH gene into the same vector. For VL gene cloning, both pComb 3 and amplified VL DNA were digested with Sst I and Xba I restriction enzymes (Gibco/BRL Life Technologies, Inc., Gaithersburg, MD). Digested DNA molecules were purified from the cut DNA bands of 1.4% DNA agarose gel using Geneclean. Digested and purified VL DNA was ligated into the digested and purified pComb3

using T4 DNA Ligase (Boehringer Mannheim Biochemica) according to the manufacturer's instructions. *Epicurian coli XL1-Blue* cells (Stratagene, La Jolla, CA) were transformed with recombinant phagemids and plated on MacConkey agar (BDH, Darmstadt, Germany) containing 100 µl/ml ampicillin. Individual weakly or non-lactose fermenting bacterial colonies were transferred from the plates to the tubes containing 3 ml of Luria-Bertani (LB) medium with 100 µl/ml ampicillin for overnight culture at 37°C. Phagemid DNA was isolated from the cultures by the alkaline lysis method (Sambrook *et al.* 1989) followed by Geneclean purification of DNA. The inserts were liberated from the phagemid DNA by Sst I/Xba I digestion and examined on 1.4% agarose gel to select inserts of the correct size. One of the phagemids containing the correct size VL insert (pComb 3 + VL) was selected for the next step ie. cloning the VH gene. In this step, "pComb 3 + VL" and V3 VH amplified DNA fragment were digested with Xho I and Spe I restriction enzymes (Gibco/BRL Life Technologies, Inc.). "pComb 3 + VL" and V3 VH digested DNA molecules were then purified and ligated as explained above. Bacterial transformation and screening were performed as previously described. The final selected pComb 3 construct which contained V3 VL and V3 VH was mapped with appropriate restriction enzymes which were chosen by using Map and Mapsort programs of the Genetics Computer Group Sequence Analysis Software Package. This mapping was done to confirm that the constructs carry the expected genetic structures in the correct orientations .

The same procedures were also employed to obtain pComb 3 constructs

containing: 1) KIM4.6 VL and VH, 2) V3 VL and Chimeric VH (see Section 2.5.1 for generation of the Chimeric VH) and 3) V3 VL and V3 VH with an insert encoding YYGS motif (see Section 2.5.2 for generation of the V3 VH with an insert encoding YYGS motif).

2.3.3 Phage preparation

Transformed *Epicurian coli XL1-Blue* cells with pComb 3 constructs were grown in LB medium with 100 μl/ml ampicillin and 10 μl/ml tetracycline at 37°C to an OD_{600} of 0.4. Next, cells were incubated with 1mM isopropyl β-D-thiogalactosidase (IPTG) for 1 hour at 37°C. Helper phage VCS-M13 (Stratagene, La Jolla, CA) was then added at a helper phage/cell ratio of 10-20:1 and the culture was grown for a further 1 hour at 37°C. At this time kanamycin (final concentration of 70 μl/ml) was added and the culture was grown for another 6 hours at 37°C. The bacterial cells were pelletted, and the supernatants containing phage particles were assayed.

2.3.4 Detection of antibody Fab molecule on the phage surface

Antibody Fab molecule on the phage surface was detected by ELISA (Figure 2.1a). Microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with 50 μl of IgG goat anti-human λ at 10 μg/ml in carbonate-bicarbonate buffer or 50 μl of IgG goat anti-human κ at 10 μg/ml in carbonate-bicarbonate buffer or with carbonate-bicarbonate buffer alone. After 2 hours incubation at RT, the plates were washed three times with washing buffer (0.1% BSA in 0.1M Tris.Cl pH 7.4). The wells then were blocked with 250 μl of 2% BSA, 0.1 M Tris.Cl pH 7.4 for 2

Figure 2.1 The schematic design of ELISA for Fab expressing phage particles. a) In the test for detection of antibody Fab molecules, the expressed λ light chain (red) is captured on the goat anti human λ coated plates The biotinylated sheep anti-M13 antibody (blue) detects the bound phage particles. This phage binding occurs via the phage surface incorporated heavy chain (green) in association with captured λ light chain. Thus, ELISA O.D. readings are indicative of the presence of phage surface expressed Fab molecules. b) In the test for DNA specificity of Fab molecules expressed on the phage surface, the ss or ds DNA reactive Fabs are captured on the ss or ds DNA coated plates and are detected by biotinylated anti-M13 phage antibody

a) Test for Fab expression

Biotinylated sheep

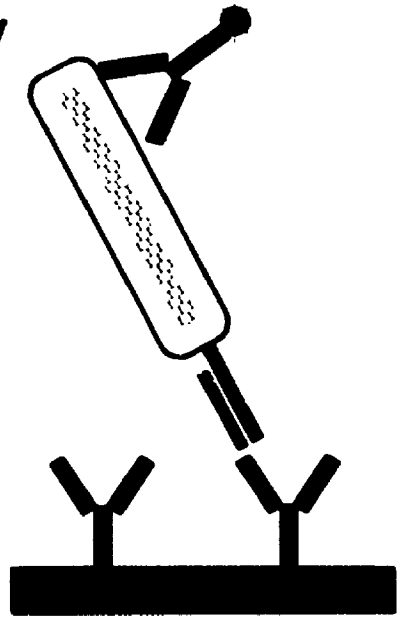
anti-M13 phage antibody

Phage expressing

Fab molecule

Goat anti-human λ

coated plates



b) Test for DNA specificity

Biotinylated sheep

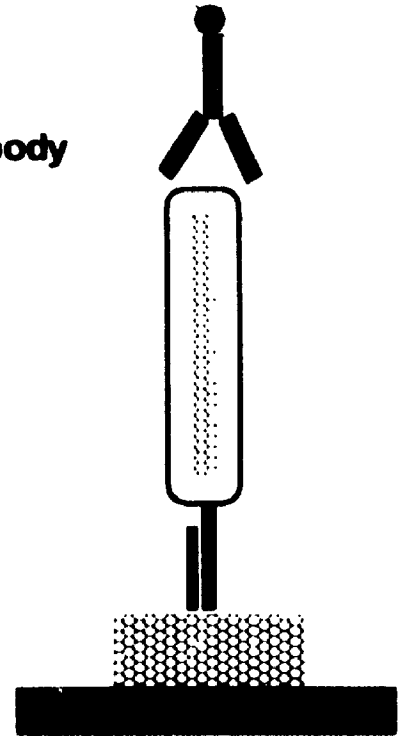
anti-M13 phage antibody

Phage expressing

Fab molecule

ss or ds DNA

coated plates



hours at RT After a single wash in washing buffer, 50 µl of culture supernatants containing phage particles and 50 µl of each control were added to the wells in duplicate and incubated at RT for 2 hours The plates then were washed 5 times in washing buffer. 50 µl of biotinylated sheep antibody to M13 phage (5 Prime to 3 Prime, Inc., Boulder, CO) at 1/2000 dilution in washing buffer was added and incubated at RT for 2 hours The wells then were washed 3 times in washing buffer and incubated at RT for 1 5 hour with streptavidin-alkaline phosphatase conjugate (5 Prime to 3 Prime, Inc., Boulder, CO) at 1/1000 dilution in washing buffer After washing 5 times with washing buffer the plates were incubated at 37°C with 50 µl of 1mg/ml p-nitrophenyl phosphate in diethanolamine buffer The reaction was stopped after 30 minutes with 3N NaOH (25 µl/well) After 10 minutes the optical densities at 405 nm were determined using a Titertek Multiskan.

Controls were LB medium, LB medium containing wild type M13 phage and LB medium containing M13 phage expressing only V3 VH chain.

2.3.5 Anti-single and anti-double stranded DNA ELISA for phage particles

Phage surface expressed Fab molecules were tested for ss and ds-DNA activity by ELISA (Figure 2.1b). Microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with 50 µl/well poly L-Lysine (PLL) (Sigma Chemical Co.) at 50 µg/ml in 0.1 M Tris.Cl pH 7.4 After 1 hour incubation at RT, the wells were washed 3 times in 0.1 M Tris.Cl pH 7.4. Then the plates were coated with 50 µl/well of ss or ds calf thymus DNA (Millipore Corporation) at 10 µg/ml in 0.1 M Tris.Cl pH 7.4 and incubated overnight at 4°C or with 0.1 M Tris.Cl pH 7.4 alone.

Next day, the plates were washed three times with washing buffer (0.1% BSA in 0.1M Tris.Cl pH 7 4) containing 0.1% v:v Tween 20. The wells then were blocked with 250 µl of 2% BSA, 0.1 M Tris.Cl pH 7.4 for 2 hours at RT. After one wash with washing buffer containing 0.1% v:v Tween 20, 50 µl of the culture supernatants and controls were added to the wells and incubated at 4°C overnight. The plates were then washed 5 times in washing buffer and the captured phage was detected using biotinylated sheep anti-M13 phage antibody.

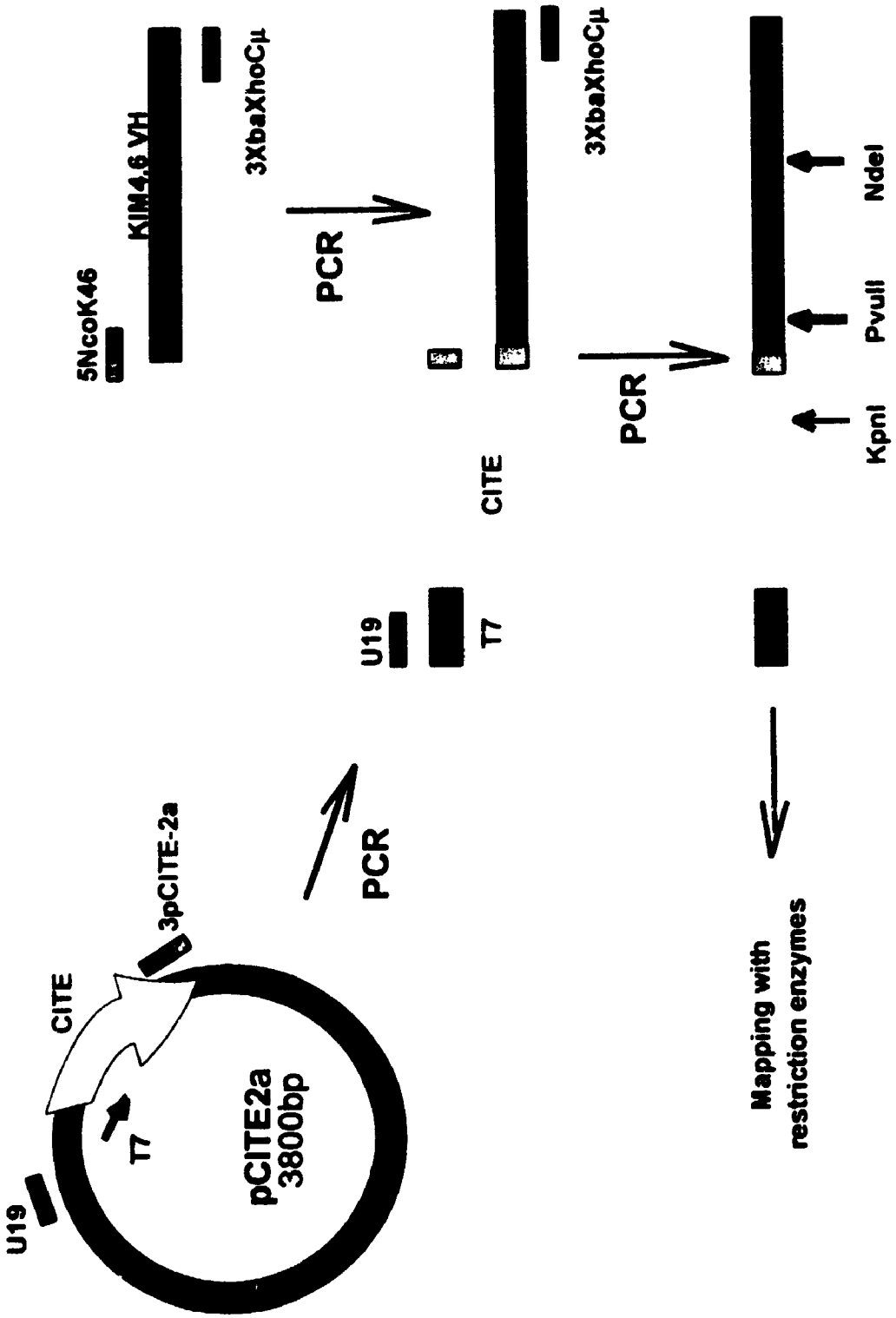
Controls were LB medium, LB medium containing wild type M13 phage and LB medium containing M13 phage expressing only V3 VH chain.

Section 2.4 *In Vitro* Transcription and Translation of Antibody Heavy Chain Molecules

2.4.1 Preparation of the DNA templates

pCITE-2a vector (Novagen, Inc., Madison, WI) carries a segment of the encephalomyocarditis virus (EMCV) RNA 5' non-encoding region, which functions as an internal entry point for initiation of translation by eukaryotic ribosomes (Parks *et al.* 1986). This Cap-Independent Translation Enhancer (CITE) sequence dramatically increases the *in vitro* translation efficiency by rabbit reticulocyte lysates (Elroy-Stein *et al.* 1989). The CITE sequence is located immediately downstream from a T7 promoter in pCITE-2a vector. pCITE-2a was PCR amplified using "U19" sense and "3pCITE-2a" antisense primers (Appendix C) to generate a "T7 promoter + CITE sequence" DNA fragment (Figure 2.2).

Figure 2.2 Construction of "T7 promoter + CITE sequence + KIM4.6" DNA template The orange boxes indicate 12 base pair nucleotides which were introduced by "3pCITE-2a" and "5NcoK46" primers to the templates resulting from the first two PCRs. Using this 12 base pair overlap "T7 promoter + CITE sequence" and KIM4.6 VH constructs were ligated in the third PCR.


U19
T7
CITE
3pCITE-2a
pCITE2a
3800bp
PCR
U19
T7
5NcoK46
KIM4.6 VH
3XbaXhoCμ
PCR
CITE
3XbaXhoCμ
PCR
KpnI
PvuII
NdeI
Mapping with
restriction enzymes

KIM4.6 VH region gene was PCR amplified using "5NcoK46" sense and "3XbaXhoCμ" antisense primers (Appendix C). "5NcoK46" primer contains the ATG initiation codon in a "Kozak consensus" (A/GCCAUGG) context The "5NcoK46" primer was designed in a way that the initiation codon would be in the correct frame with the downstream VH sequence. The "T7 promoter + CITE sequence" and KIM4.6 VH amplified DNA fragments were purified from DNA agarose gel using Geneclean.

Taking advantage of the identical 12 base pairs nucleotide which were introduced by "3pCITE-2a" and "5NcoK46" primers to the "T7 promoter + CITE sequence" 3' end and KIM4.6 VH 5' end respectively, these two purified DNA fragments were PCR ligated to each other. "U19" sense and "3XbaXhoCμ" antisense primers were employed in a PCR reaction to amplify the "T7 promoter + CITE sequence" and KIM4.6 VH DNA templates.

The resulting "T7 promoter + CITE sequence + KIM4.6 VH" DNA was purified from the agarose gel. This DNA construct was mapped with appropriate restriction enzymes which were chosen by using Map and Mapsort programs of the Genetics Computer Group Sequence Analysis Software Package. This mapping was performed to confirm that the constructs carry the expected genetic structures in the correct orientations.

The same procedures were also employed to obtain "T7 promoter + CITE sequence + Chimeric VH" (see Section 2.5.1 for generation of the Chimeric VH) and "T7 promoter + CITE sequence + KIM4.6 VH without the YYGS motif encoding

sequence" (see Section 2.5.3 for generation of KIM4.6 VH without the YYGS motif encoding sequence).

2.4.2 VH chain peptide synthesis

The Single Tube Protein (STP) system (Novagen, Inc., Madison, WI) was used for *in vitro* transcription and translation of VH DNA templates. This system includes T7 RNA polymerase in combination with rabbit reticulocyte lysate so that transcription and translation can be carried out in a coupled reaction (Craig *et al.* 1992). STP reaction was performed on 2µl (~0.5 µg) DNA templates according to maunfacturer's instructions. The VH chain translated peptide was labeled with 2µl of 10 µCi/µl L-[^{35}S] Methionine (NEN, Du Pont Company, Wilmington, DE) during this reaction.

A tube with no added DNA template and another tube with *E.coli* β-galactosidase gene cloned in pCITE-3b(+) (provided in the Novagen STP kit) were included as controls.

2.4.3 Radioactive amino acid incorporation assay

2 µl of the radioactively labeled translated product was transferred to a tube containing 100 µl of 1 N NaOH/2% H_2O_2 and incubated for 10 minutes at 37°C to hydrolyze tRNA and remove the red color. Then the peptide was precipitated with 0.9 ml 25% trichloroacetic acid (TCA) (BDH Inc., Toronto, ONT). The sample was filtered on Whatman GF/A filter paper and the filter rinsed 3 times with 5% TCA. The filter was air dried and counted in the presence of "Ready Solv HP" scintillation buffer (Beckman, Fullerton, CA) to obtain count per minute (cpm) of each sample.

Total counts per minute (cpm) in the reaction was determined as follow: 2 μl of the translated product was added to 18 μl water and 2 μl of this was spotted directly onto a dry filter and counted after air drying. % Incorporation of [^{35}S] Methionine was calculated from this formula:

% Incorporation = net cpm incorporation/total cpm in reaction X 100%

where:

net cpm incorporation = cpm of the sample - cpm of the tube with no DNA.

2.4.4 Gel analysis of the produced VH peptide

4 μl of each translated peptide sample was incubated for 5 minutes with 1 μl RNase, DNase-free (Boehringer Mannheim Biochemica) at RT and then added to 20 μl of sodium dodecyl sulfate (SDS) sample buffer (75 mM Tris.Cl pH 6.8, 2% SDS, 10% glycerol, 5% 2 β-mercaptoethanol). The mixture was heated to 95°C for 3 minutes and loaded to a "Mini-PROTEAN II" SDS polyacrylamide 12% resolving (37.5:1 acrylamide:bisacrylamide), 4% stacking gel (37.5:1 acrylamide :bisacrylamide) (SDS-PAGE) (Bio-Rad Laboratories, Richmond, CA). After 30 minutes running at 200 V constant voltage, the peptides and "Rainbow" molecular weight markers (Amersham Corp., Arlington Heights, IL) were transferred to nitrocellulose paper, air dried and exposed at RT to XAR Kodak X-ray film (Eastman Kodak Company, Rochester, NY).

2.4.5 Anti-single and double stranded DNA assay for the VH peptide

Immulon2 strip wells (Dynatech Laboratories Inc., Alexandria, VA) were coated with 50 μl/well poly L-Lysine (PLL) (Sigma Chemical Co.) at 50 μg/ml in 0.1

M Tris.Cl pH 7.4. After 1 hour incubation at RT, the wells were washed 3 times in 0.1 M Tris.Cl pH 7.4. Then the wells were coated with 50 µl/well of ss or ds calf thymus DNA (Millipore Corporation) at 10 µg/ml in 0.1 M Tris.Cl pH 7.4 and incubated overnight at 4°C. Control wells were coated with 0.1 M Tris.Cl pH 7.4. The wells were washed three times with washing buffer (0.1% BSA in 0.1M Tris.Cl pH 7.4) containing 0.1% v:v Tween 20 and blocked with 300 µl of 2% BSA, 0.1 M Tris.Cl pH 7.4 for 3 hours at RT. After one wash with washing buffer containing 0.1% v:v Tween 20, 200 µl of the samples diluted 1/10 in 1% BSA were added to the wells and incubated for 2 hours at RT. The wells were washed 6 times with washing buffer and counted in the presence of "Ready Solv HP" scintillation buffer (Beckman).

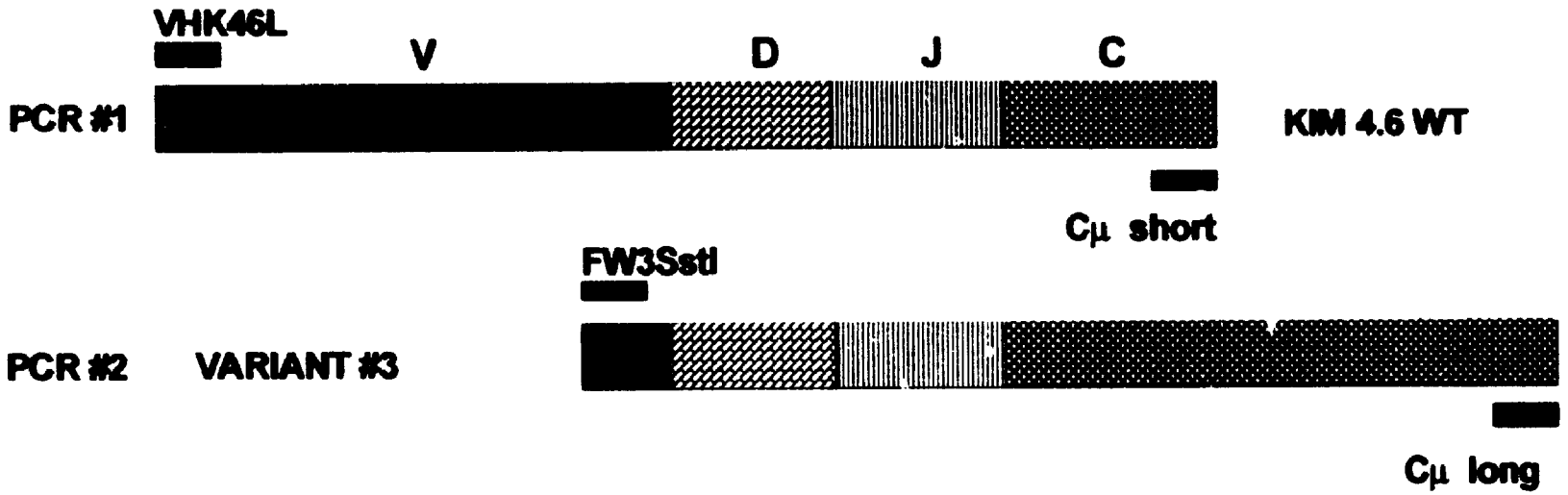
Section 2.5 Immunoglobulin V gene Manipulation

2.5.1 Production of the Chimeric VH gene

KIM4.6 VH was PCR amplified using "VHK46L" sense primer and "Cµ short" antisense primer (Appendix B). Variant #3 (V3) VH was PCR amplified using "FW3SstI" sense and "Cµ long" antisense primer (Appendix B). A third PCR was performed with "VHK46L" sense primer and "Cµ long" antisense primer using the purified DNA fragments from the first two PCRs as templates (Figure 2.3).

The final product was mapped, cloned and sequenced as described in previous Sections.

Figure 2.3 Construction of the Chimeric VH gene. In the first and second separate PCR reactions KIM4.6 VH (WT) DNA template (using "VHK46L" and "Cμ short" primers) and V3 VH DNA template (using "FW3SstI" and "Cμ long" primers) were amplified. The third PCR was performed on the DNA templates generated in the first and second PCR using "VHK46L" and "Cμ long" primers. This can result in the generation of two different products. a KIM4.6 wild type construct and a Chimeric VH construct.



PCR #3

First Possible Product : **KIM 4.6 WT**

Second Possible Product : **Chimeric VH (KIM 4.6 V + Variant DJ)**

2.5.2 Grafting the YYGS encoding sequence into the Variant #3 (V3) VH diversity region

The V3 VH was PCR amplified in two separate reactions. "VH4 Leader" sense and "3MT1" antisense (Appendix C) primers were used in the first PCR and "5MT1" sense (Appendix C) and "Cμ SpeI" antisense primers were used in the second PCR. "5MT1" and "3MT1" primers contain sense and antisense encoding sequence for YYGS respectively. The 3' end of the first PCR amplified DNA fragment contained 12 identical base pairs to the 5' end of the second PCR amplified product (due to the design of "5MT1" and "3MT1" primers). The third PCR was performed with the "VH4 Leader" sense and "Cμ SpeI" antisense primers using agarose gel purified DNA fragments of first and second PCRs as templates (Figure 2.4). The final product was cloned and sequenced to check the accuracy of the YYGS encoding sequence insertion.

2.5.3 Removing the YYGS encoding sequence from the KIM4.6 VH

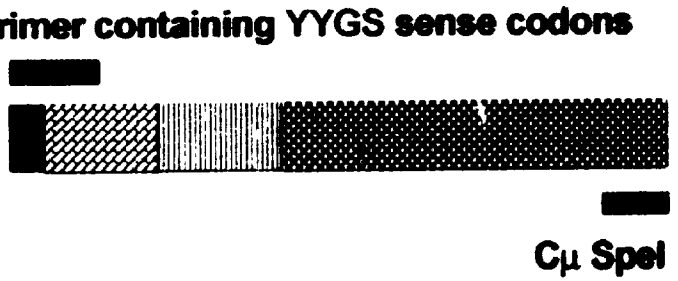
"T7 promoter + CITE sequence + KIM4.6 VH" DNA fragment (Section 2.4.1) was used as template in the following two PCRs. The first PCR contained "U19" sense and "3MT2" antisense (Appendix C) primers. The second PCR contained "5MT2" sense (Appendix C) and "3XbaXhoCμ" antisense primers. "5MT2" and "3MT2" primers were used to loop out the YYGS encoding sequence. The 3' end of the first PCR amplified DNA fragment contained 12 identical base pairs to the 5' end of the second PCR amplified product (due to the design of "5MT2" and "3MT2" primers). The third PCR was performed with "U19" sense and "3XbaXhoCμ"

Figure 2.4 Grafting the YYGS encoding sequence into the V3 VH D region. In the first and second PCR the YYGS encoding sequence (green) was introduced to the V3 VH templates using 3MT1 and 5MT1 primers These two templates with 12 bp overlap (YYGS encoding sequence) were ligated in the third PCR to generate a V3 VH construct containing the YYGS encoding sequence in the D region.


V3 VH

PCR #1 and #2:


VH4 Leader
3MT1 primer containing YYGS antisense codons


5MT1 primer containing YYGS sense codons
Cμ SpeI


VH4 Leader
PCR #3
Cμ SpeI

antisense primers using agarose gel purified DNA fragments from the first and second PCRs as templates. The final product was mapped with appropriate restriction enzymes which were chosen by using Map and Mapsort programs of the Genetics Computer Group Sequence Analysis Software Package. This mapping was performed to confirm that the constructs carry the expected genetic structures in the correct orientations

2.5.4 Changing the KIM4.6 VH reading frame at the beginning of the diversity region

KIM4.6 VH gene was PCR amplified in two separate reactions "VHK46L" sense and "3MT3" antisense (Appendix C) primers were used in the first PCR and "5MT3" sense (Appendix C) and "Cμ SpeI" antisense primers used in the second PCR. One nucleotide has been removed from each "5MT3" and "3MT3" primers in sense and antisense configuration respectively. The 3' end of the first PCR amplified DNA fragment contained 12 identical base pairs to the 5' end of the second PCR amplified product (due to the design of "5MT3" and "3MT3" primers). The third PCR was performed with "VHK46L" sense and "Cμ SpeI" antisense primers using agarose gel purified DNA fragments of first and second PCR as templates.

2.5.5 Changing the KIM4.6 VH reading frame at the beginning of the V region

The KIM4.6 VH gene was PCR amplified using "5MT4" sense (Appendix C) and "Cμ SpeI" antisense primers. The "5MT4" primer contained an extra nucleotide

in the beginning of the KIM4 6 V region Therefore the reading frame would shift and the protein product of this gene would be different from the KIM4 6 VH peptide

2.5.6 Production of the KIM4.6 VH gene for cloning in the reverse orientation

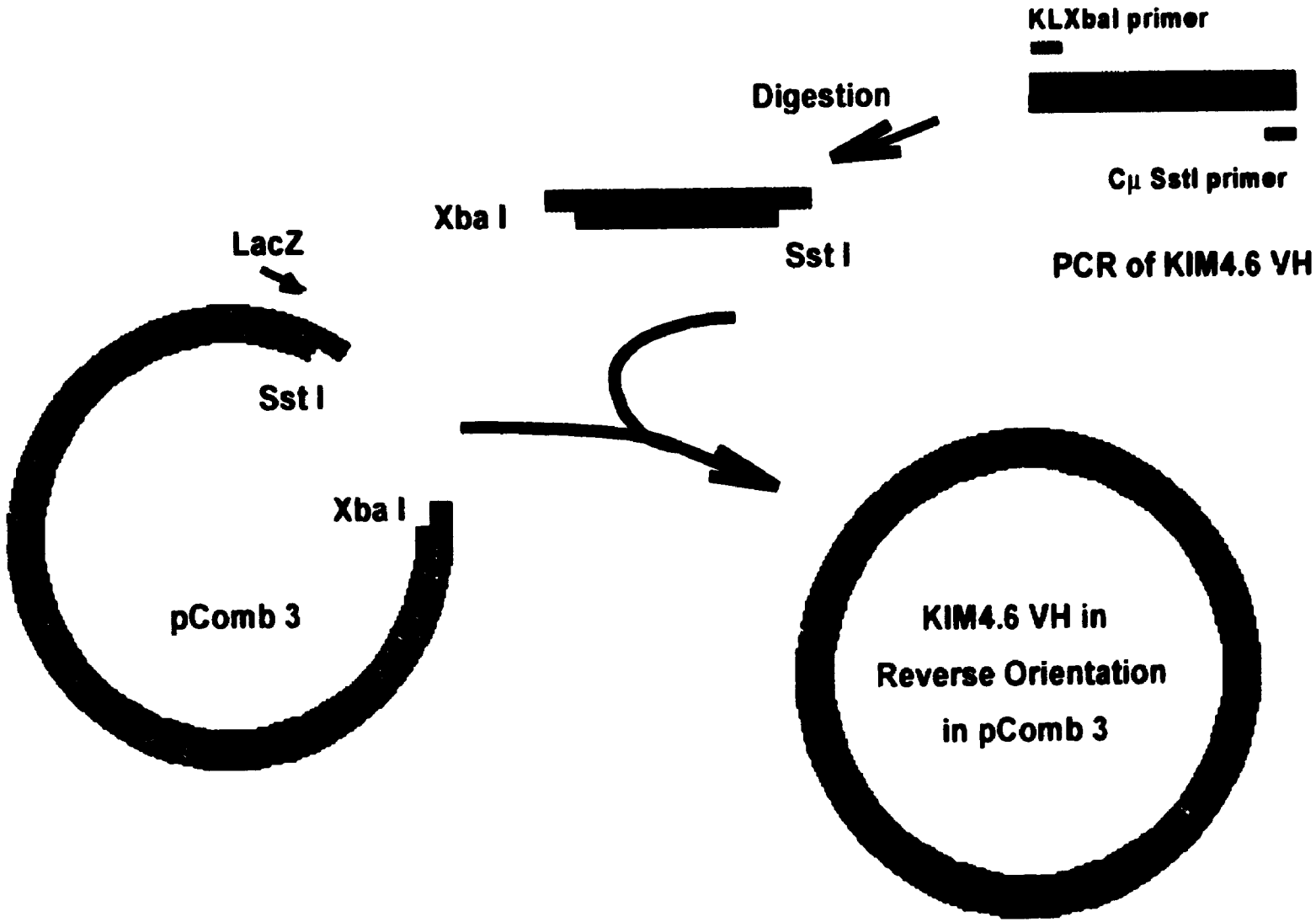
KIM4.6 VH gene was PCR amplified using "KLXbal" sense and "CH1 SstI" antisense primers (Appendix C) This PCR product was digested with Sst I/Xbal restriction enzymes and cloned into the pComb 3 phagemid Sst I/Xbal cloning site in a reverse orientation (Figure 2.5)

Section 2.6 Statistical Analysis of Data

The mean of the replicates was calculated by summing the result of the individual replicates and dividing by the number of replicates.

The two tailed student's T-test and Tukey multiple comparison test were used to determine the significance of differences between groups. Differences with a *P* value more than 0.05 were considered to be not significant.

Figure 2.5 Cloning the KIM4.6 VH gene in the reverse orientation in pComb3 expression vector. KIM4.6 VH was PCR amplified using "KLXbaI" and "Cμ SstI" primers. Because of the special design of the restriction sites for these primers, cloning of this KIM4 6 VH fragment into the Sst I / Xba I site of pComb3 would be in a reverse orientation and subsequently an anti-sense RNA will be produced


KLXbaI primer
Digestion
Cμ SstI primer
Xba I
Sst I
PCR of KIM4.6 VH
LacZ
Sst I
Xba I
pComb 3
KIM4.6 VH in
Reverse Orientation
in pComb 3

Chapter 3

Results

Section 3.1 Selection and Characterization of the Variant Hybridomas

3.1.1 Selection of the non DNA-binding hybridomas

KIM4.6 hybridoma from a frozen stock was revived by culturing in 2 ml wells and was confirmed by ELISA to be positive for the production of 4.6.3 Id IgMλ antibody which bound both ss and ds DNA. The first subcloning (5 cells/well) from one of these wells yielded clones which secreted antibodies characteristic of KIM4.6. The second subcloning (1 cell/well) resulted in the growth in 600 wells. The supernatants from all but three of 600 wells had IgMλ antibodies reactive with ss and ds DNA. The exceptions were supernatants 646 B4-1 (V3), 646 C3-1 (V6) and 746 E7 (V8) which contained IgMλ antibodies lacking specificity for ss or ds DNA (Table 3.1). These three anti-DNA negative antibodies expressed the 4.6.3 Id of the parent KIM4.6.

3.1.2 Antigenic specificity of the Variants

Variant 646 B4-1 (V3), 646 C3-1 (V6) and 746 E7 (V8) supernatants were tested against a panel of autoantigens. None of the three Variants nor KIM4.6 antibody had rheumatoid factor or cold agglutinin activity (Tables 3.2 and 3.3). Neither the Variants nor the KIM4.6 bound to Sm/RNP affinity purified antigen

Table 3.1

Anti-ss and ds DNA ELISA of the Variant antibodies[1]

	OD 405 nm	
	ss DNA	ds DNA
Variant #3[2]	0.00	0 00
Variant #6[2]	0.00	0.00
Variant #8[2]	0.00	0 00
KIM4.6[2]	1.02	0.89
SLE serum 1/2000 dilution	1.91	1 78
normal serum 1/1000 dilution	0.05	0.04
1% BSA, 0.5% BGG in 0.1 M Tric.Cl	0.00	0.00
HGM[3]	0.00	0.00

[1] Anti-ss and ds DNA ELISA has been described in section 2.1.4.
[2] All the hybridoma supernatants were tested at the same IgM concentrations (5.0 μg/ml)
[3] HGM: hybridoma growth medium

Table 3.2

Rheumatoid factor activity of the Variant antibodies[1]

	OD 405 nm		
	Hum. IgG[2]	Fc Hum. IgG[3]	Fab Hum. IgG[4]
Variant #3[5]	0.03	0.02	0.02
Variant #6[5]	0.03	0 02	0.02
Variant #8[5]	0.04	0.02	0.03
KIM4 6[5]	0.03	0.02	0.03
BUD 94 91.8[5]	1.41	0.39	1.95
HGM[6]	0.01	0.00	0.00
3% BSA, 0.05% v/v Tween in saline	0.00	0.00	0.00

[1] Rheumatoid factor ELISA has been described in section 2.1.7.
[2] Hum. IgG: Human IgG
[3] Fc Hum. IgG: Fc fragment of human IgG
[4] Fab Hum. IgG: Fab fragment of human IgG
[5] All the hybridoma supernatants were tested at the same IgM concentrations (2.0 μg/ml)
[6] HGM: hybridoma growth medium

Table 3.3

Cold agglutinin activity of the Variant antibodies[1]

		OD 405 nm			
		Cord RBC		Adult RBC	
		Neat	1/50 dilution	Neat	1/50 dilution
Variant #3[2]		-[3]	-	-	-
Variant #6[2]		-	-	-	-
Variant #8[2]		-	-	-	-
KIM4.6[2]		-	-	-	-
PS[4]	Neat	++	+	++++	++++
	1/50 dilution	+	-	++++	++
NS[5]	Neat	-	-	-	-
	1/50 dilution	-	-	-	-

[1] Cold agglutination assay has been described in section 2.1.8.
[2] All the hybridoma supernatants were tested at the same IgM concentrations (2.0 µg/ml)
[3] The red cell agglutination was scored according to Dacie and Lewis criteria (Dacie 1984)
[4] PS: Serum of a patient with cold agglutinin disease
[5] NS: Normal human serum

Table 3.4

Sm/RNP activity of the Variant antibodies[1]

	OD 405 nm
	Sm/RNP
Variant #3[2]	0.01
Variant #6[2]	0.01
Variant #8[2]	0.01
KIM4.6[2]	0.02
BUD 94.91.8[2]	1.85
NS[3]	0.03
HGM[4]	0.00
1% BSA, 2% BGG in 0.1 M Tris.Cl pH 7.4	0.00

[1] Anti-Sm/RNP ELISA has been described in section 2.1.6.
[2] All the hybridoma supernatants were tested at the same IgM concentrations (2.0 μg/ml)
[3] NS: Normal human serum
[4] HGM: hybridoma growth medium

(Table 3.4). At present, the antigenic specificity of the Variant antibodies remains unknown.

3.1.3 VL region nucleotide sequence of the Variants

The VL cDNAs of the Variants were PCR amplified in separate reactions using each of 7 Vλ framework sense primers and also a VλK46 leader/framework sense primer (Section 2.2.3). DNA gel electrophoresis and ethidium bromide staining of each PCR product showed that the VL of the Variants (V3, V6 and V8) were only amplified by Vλ1 family primers (Vλ1 framework and VλK46 leader/framework). Figure 3.1 shows the ethidium bromide stained DNA agarose gel of PCR amplified V3 VL chain gene. For each of the Variants, the amplified fragment (from the reaction containing VλK46 leader/framework) was purified from the gel, cloned and sequenced as described in Sections 2.2.4 and 2.2.6.

Nucleotide sequence of the VL region of the three Variants (V3, V6, V8) revealed that this region in all of the Variants was identical to the VL of the KIM4.6 parent (Cairns *et al.* 1989a) (Figure 3.2). An exception was found in V6 VL which had a single nucleotide difference (G instead of an A) in CDR2 at position 153. This nucleotide difference is silent at the amino acid level. The VL of the Variants as well as the VL of KIM4.6, are encoded by a Vλ1 unmutated germline gene *Humlv117* (Siminovitch *et al.* 1989) (both including 1 nucleotide of *Humlv117* 3' intron sequence for position 296) and Jλ3 gene. The Jλ of both the parent and the Variants is identical to Jλ3 germline gene except for one nucleotide substitution (A -> G) at position 300.

Figure 3.1 Ethidium bromide stained DNA agarose gel of PCR amplified Variant #3 VL chain gene. Variant #3 VL cDNA was amplified in separate reactions using VλK46 leader/framework and each of seven Vλ framework primers (Appendix B). Lane 1, PCR product using VλK46 leader/framework primer; Lane 2-8 PCR products using Vλ1, Vλ2/5, Vλ3, Vλ4, Vλ6, Vλ7, Vλmisc; Lane 9, negative control (no DNA added); Lane M, 123 DNA marker. Variant #3 VL chain gene was amplified by Vλ1 family primers (Vλ1 framework and VλK46 leader/framework) at the expected size (~370 bp).

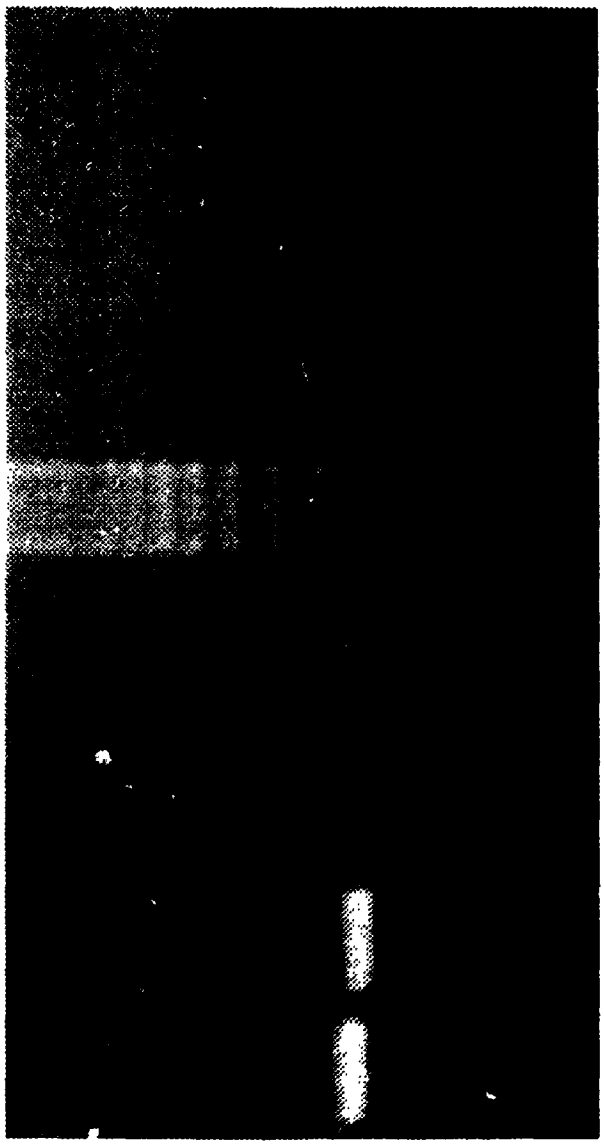
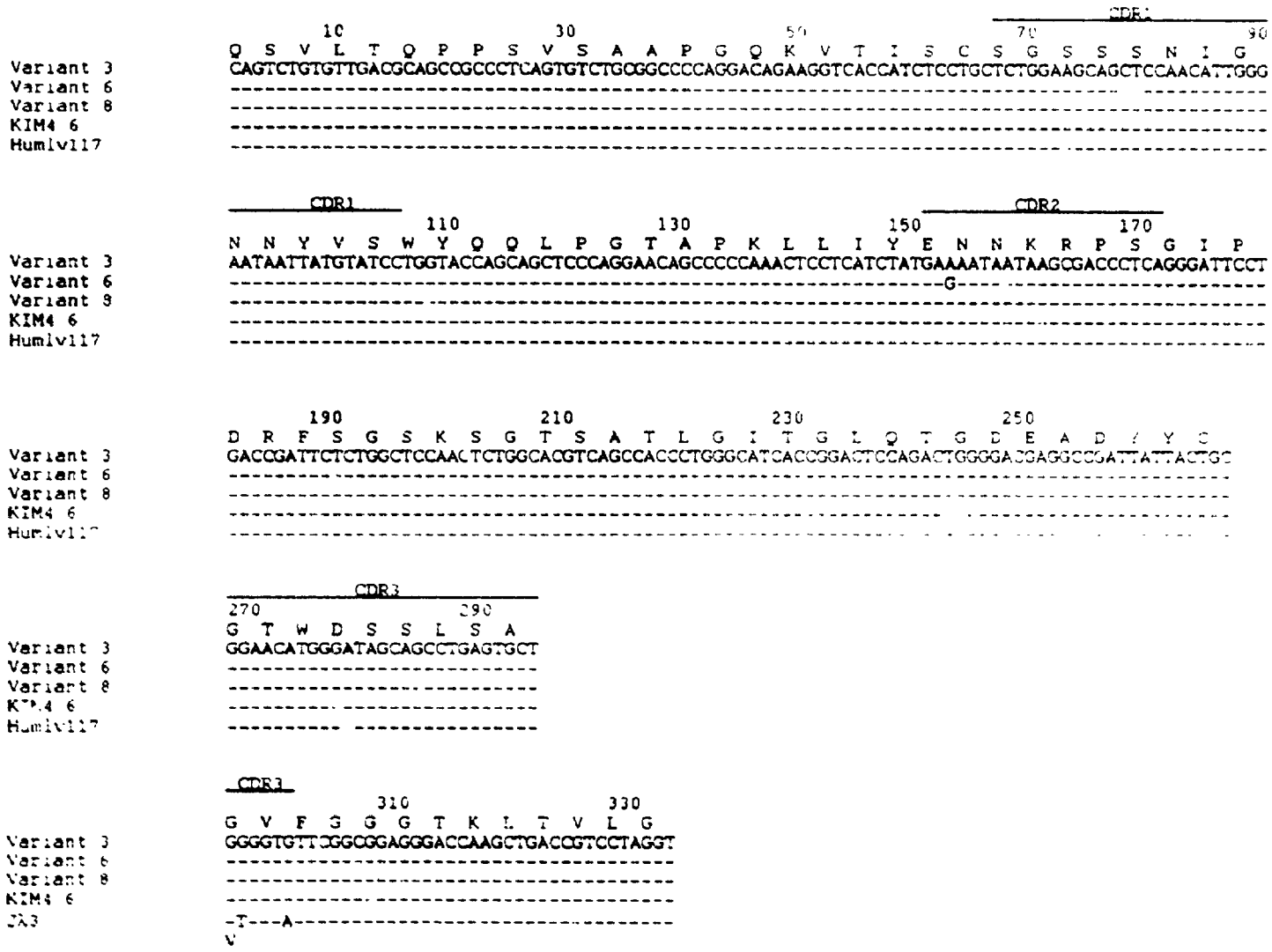

1
2
3
4
5
M
6
7
8
9

Figure 3.2 VL chain nucleotide sequence comparison of non DNA-binding Variant antibodies (#3, #6 and #8) to the VL of DNA-binding KIM4.6 antibody and the *Humlv117* and Jλ3 germline genes. The numbers indicate the nucleotide positions. Identity in the nucleotide sequence is indicated by a dash. The complementarity determining regions (CDR) are indicated above the number scale. Amino acids are designated by one letter codes and shown above and below (differences only) nucleic acid sequences Abbreviations for the amino acids are as follow: 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. Variants VL nucleotide sequence is available through EMBL/GenBank databases under accession number Z35495.


CDR1
10 30 50 70 90
Q S V L T Q P P S V S A A P G Q K V T I S C S G S S S N I G
Variant 3 CAGTCTGTGTTGACGCAGCCGCCCTCAGTGTCTGCGGCCCCAGGACAGAAGGTCACCATCTCCTGCTCTGGAAGCAGCTCCAACATTGGG
Variant 6 ----------
Variant 8 ----------
KIM4 6 ----------
Humlv117 ----------
CDR1
CDR2
110 130 150 170
N N Y V S W Y Q Q L P G T A P K L L I Y E N N K R P S G I P
Variant 3 AATAATTATGTATCCTGGTACCAGCAGCTCCCAGGAACAGCCCCCAAACTCCTCATCTATGAAAATAATAAGCGACCCTCAGGGATTCCT
Variant 6 ----------G----------
Variant 8 ----------
KIM4 6 ----------
Humlv117 ----------
190 210 230 250
D R F S G S K S G T S A T L G I T G L Q T G D E A D Y Y C
Variant 3 GACCGATTCTCTGGCTCCAAGTCTGGCACGTCAGCCACCCTGGGCATCACCGGACTCCAGACTGGGGACGAGGCCGATTATTACTGC
Variant 6 ----------
Variant 8 ----------
KIM4 6 ----------
Humlv117 ----------
CDR3
270 290
G T W D S S L S A
Variant 3 GGAACATGGGATAGCAGCCTGAGTGCT
Variant 6 ----------
Variant 8 ----------
KIM4 6 ----------
Humlv117 ----------
CDR3
310 330
G V F G G G T K L T V L G
Variant 3 GGGGTGTTCGGCGGAGGGACCAAGCTGACCGTCCTAGGT
Variant 6 ----------
Variant 8 ----------
KIM4 6 ----------
JA3 -T---A----------
V

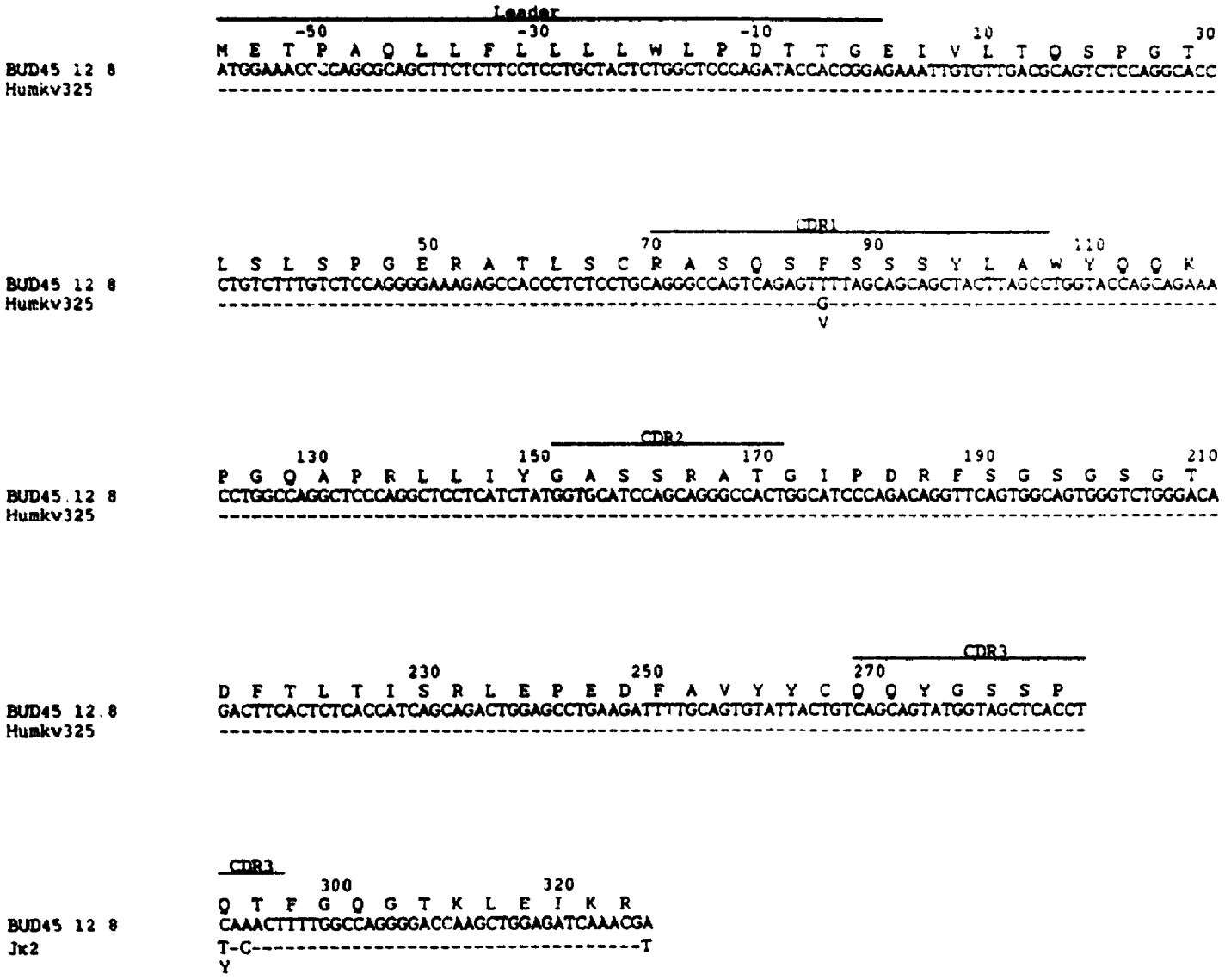

Leader
-50
-30
-10
10
30
M E T P A Q L L F L L L L W L P D T T G E I V L T Q S P G T
BUD45 12 8 ATGGAAACCCCAGCGCAGCTTCTCTTCCTCCTGCTACTCTGGCTCCCAGATACCACCGGAGAAATTGTGTTGACGCAGTCTCCAGGCACC
Humkv325 ----------
CDR1
50
70
90
110
L S L S P G E R A T L S C R A S Q S F S S S Y L A W Y Q Q K
BUD45 12 8 CTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTTTTAGCAGCAGCTACTTAGCCTGGTACCAGCAGAAA
Humkv325 ----------G----------
V
CDR2
130
150
170
190
210
P G Q A P R L L I Y G A S S R A T G I P D R F S G S G S G T
BUD45.12 8 CCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGTCTGGGACA
Humkv325 ----------
CDR3
230
250
270
D F T L T I S R L E P E D F A V Y Y C Q Q Y G S S P
BUD45 12 8 GACTTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGTATGGTAGCTCACCT
Humkv325 ----------
CDR3
300
320
Q T F G Q G T K L E I K R
BUD45 12 8 CAAACTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
Jk2 T-C----------T
Y

Figure 3.3 Ethidium bromide stained DNA agarose gel of PCR amplified Variants #3, #6 and #8 VH chain genes. VH chain genes of all the Variants were amplified most strongly by the VH4 leader primer and their size was ~ 500 bp as expected.

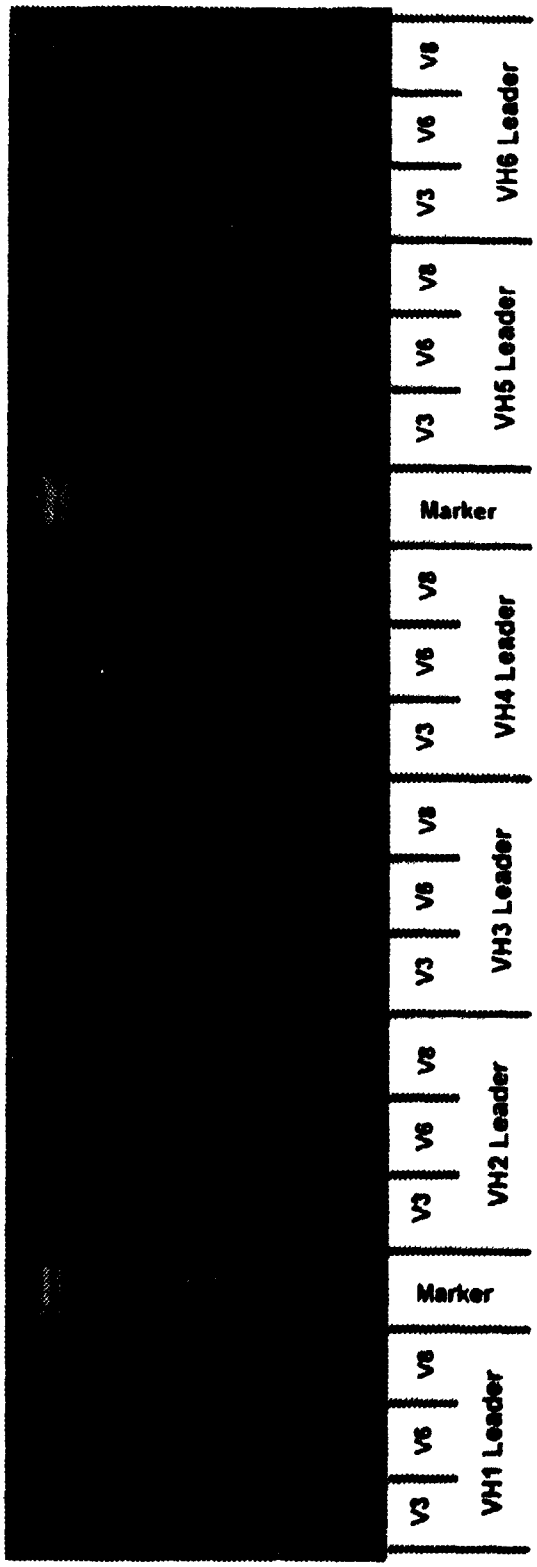

V3
V5
V8
VH1 Leader
Marker
V3
V6
V8
VH2 Leader
V3
V5
V8
VH3 Leader
V3
V6
V8
VH4 Leader
Marker
V3
V6
V8
VH5 Leader
V3
V6
V8
VH6 Leader

Figure 3.4 VH chain nucleotide and deduced amino acid sequence comparison of non DNA-binding Variant antibodies (#3, #6, #8) to the VH4.21 and JH5 germline genes. The diversity (D) gene of the Variants is unique and could not be compared to known D germline gene sequences. The numbers indicate the nucleotide positions. Identity in the nucleotide sequence is indicated by a dash. The complementarity determining regions (CDR) are indicated above the number scale. Amino acids are designated by one letter codes and shown above and below (differences only) nucleic acid sequences. Variants VH chain nucleotide sequence is available through EMBL/GenBank databases under accession number Z35492.

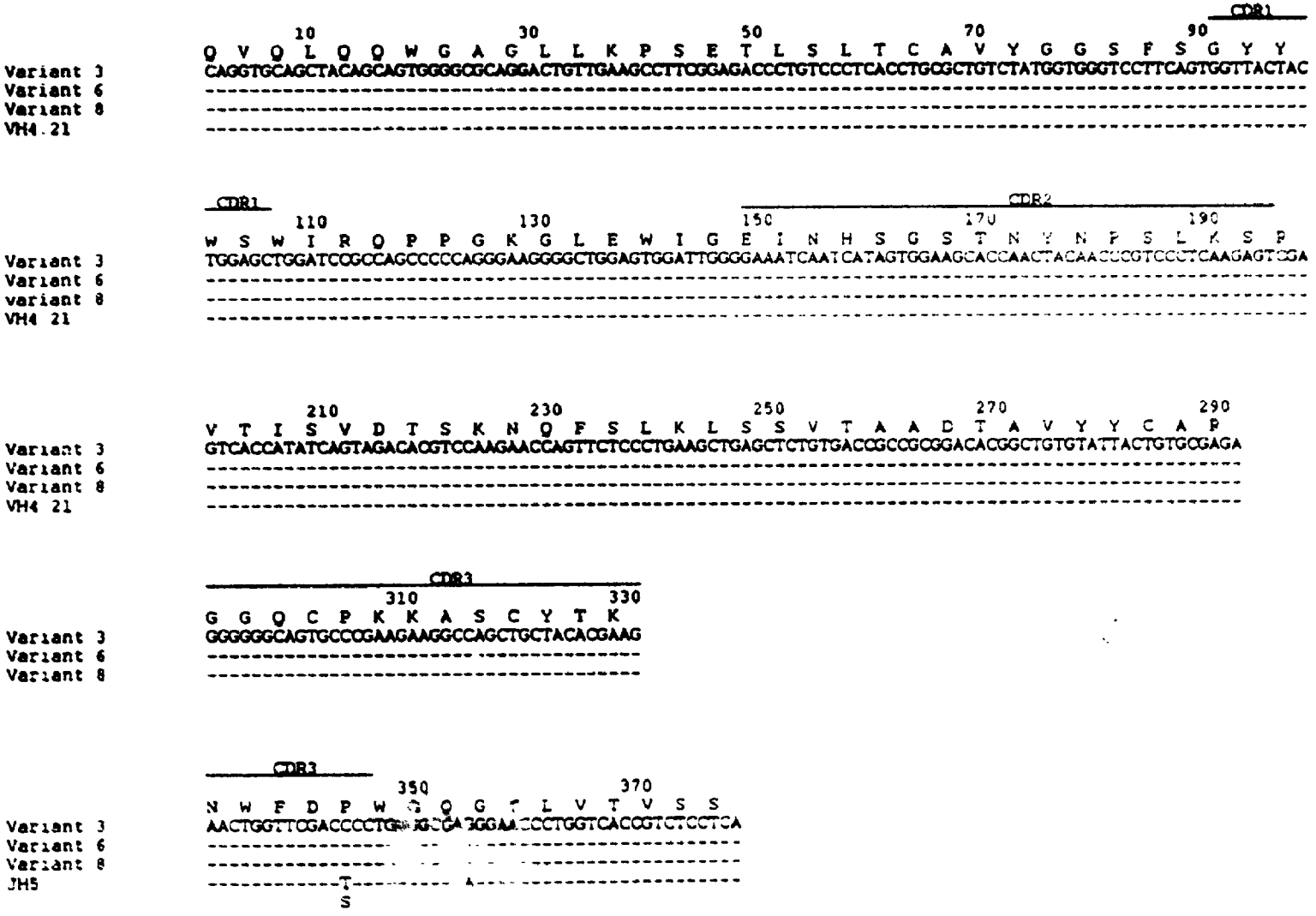

CDR1
10 30 50 70 90
Q V Q L Q Q W G A G L L K P S E T L S L T C A V Y G G S F S G Y Y
Variant 3 CAGGTGCAGCTACAGCAGTGGGGCGCAGGACTGTTGAAGCCTTCGGAGACCCTGTCCCTCACCTGCGCTGTCTATGGTGGGTCCTTCAGTGGTTACTAC
Variant 6 ----------
Variant 8 ----------
VH4.21 ----------
CDR1
CDR2
110 130 150 170 190
W S W I R Q P P G K G L E W I G E I N H S G S T N Y N P S L K S R
Variant 3 TGGAGCTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGGAAATCAATCATAGTGGAAGCACCAACTACAACCCGTCCCTCAAGAGTCGA
Variant 6 ----------
variant 8 ----------
VH4 21 ----------
210 230 250 270 290
V T I S V D T S K N Q F S L K L S S V T A A D T A V Y Y C A R
Variant 3 GTCACCATATCAGTAGACACGTCCAAGAACCAGTTCTCCCTGAAGCTGAGCTCTGTGACCGCCGCGGACACGGCTGTGTATTACTGTGCGAGA
Variant 6 ----------
Variant 8 ----------
VH4 21 ----------
CDR3
310 330
G G Q C P K K A S C Y T K
Variant 3 GGGGGGCAGTGCCCGAAGAAGGCCAGCTGCTACACGAAG
Variant 6 ----------
Variant 8 ----------
CDR3
350 370
N W F D P W G Q G T L V T V S S
Variant 3 AACTGGTTCGACCCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
Variant 6 ----------
Variant 8 ----------
JH5 ----------T---------- A----------
S

Table 3.5

Comparison of anti-DNA antibodies encoded by VH4.21 germline gene with non DNA-binding Variants

	Iso-type	Origin	DNA Binding	Nucleotide Identity with VH4.21 Germline	VDJ Net[1] Charge	CDR3 Region			JH	Light Chain Family and Germline Gene	JL	Reference
						Net Charge[2]	# of Tyr/Arg[3]	D Germline Gene				
T14	IgG	SLE PBL	ss+ds	94.8%	5.82	1.78	7/1	DXP'1	4	κIIIb Humkv325	Jκ1	van Es 1991
D5	IgG	SLE PBL	ss+ds	93.8%	3.83	2.78	2/2	DXP'1	5	κIIIa HumIGKVQ	Jκ5	Stevenson 1993
RT79	IgM	SLE PBL	ss>ds	100%	5.74	3.78	0/5	Unknown	3	κII -	Jκ5	Stevenson 1993
NE-1	IgM	SLE PBL	ss+ds	100%	5.83	3.87	0/4	Unknown	6	κI Vb'	Jκ4	Hirabayashi 1993
NE-13	IgM	SLE PBL	ss+ds	100%	5.83	3.87	0/4	Unknown	6	κI Vb'	Jκ4	Hirabayashi 1993
Variants	IgM	NTL[4]	-	100%	4.46	2.51	0/1	Unknown	5	λ I Humlv117	Jλ3	Mahmoudi 1994

[1] Net charges of the peptides have been calculated at pH 7.5 using the Isoelectric program of the Genetics Computer Group Sequence Analysis Software Package, Version 7.3.1-UNIX (Genetics Computer Group, Inc., Madison, WI).
[2] Net charge in this column is the entire CDR3 region net charge.
[3] Tyr/Arg: number of tyrosine and arginine residues.
[4] NTL: normal tonsillar B cells.

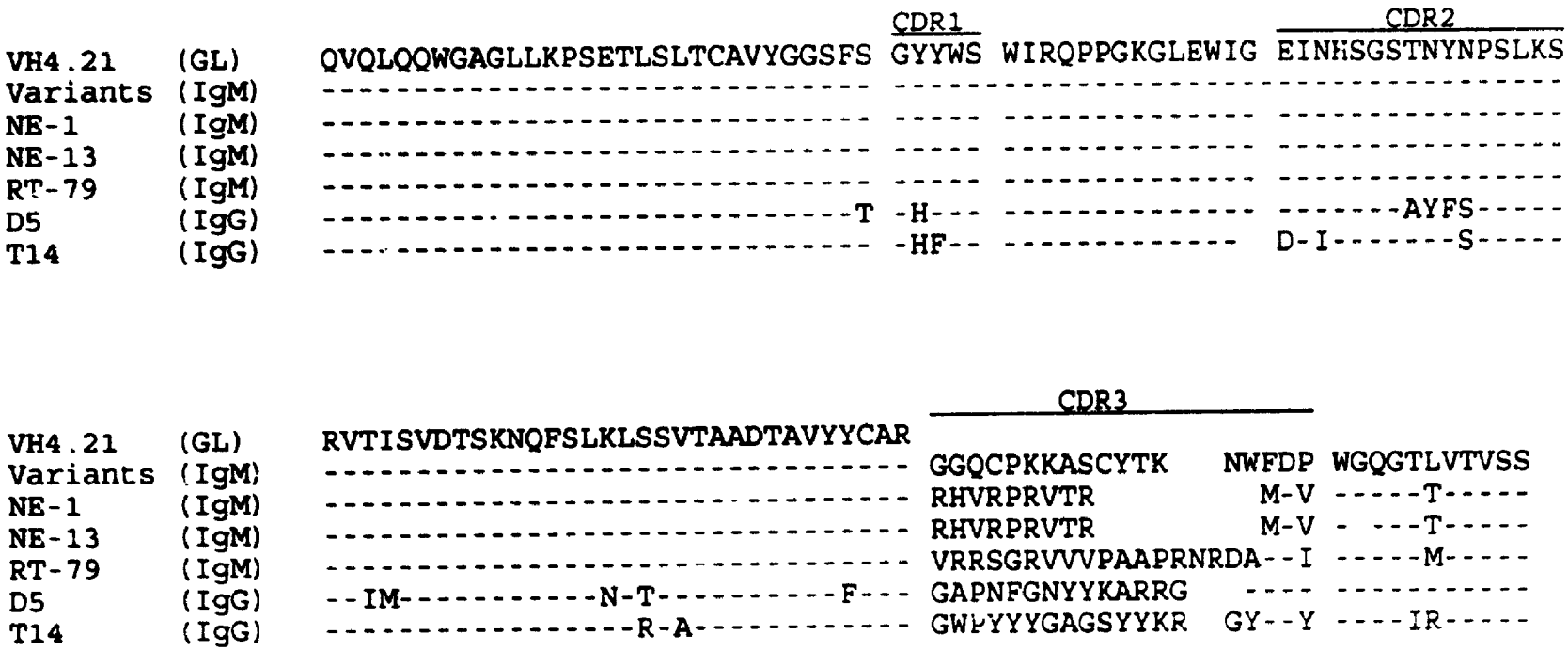


Figure 3.5 Deduced amino acid sequence comparison of VH4 21 germline (GL) gene segment, the VH of the non DNA-reactive Variants and the VH of five anti-DNA antibodies. NE-1, NE-13 (Hirabayashi 1993) RT-79. D5 (Stevenson 1993) and T14 (van Es 1991). Amino acids are designated by one letter codes Dash lines are indicative of identity. Gaps in CDR3 regions were introduced during the alignment of these sequences

(Hirabayashi *et al* 1993), RT-79 and D5 (Stevenson *et al.* 1993). The characteristics of these five anti-DNA antibodies are listed in Table 3.5 . They all bind to ss and ds DNA (RT-79 antibody has weak activity against ds DNA). The VH segments of NE-1, NE-13 and RT-79 antibodies are identical to the VH4.21 germline gene. The VH segments of T14 and D5 show 94.8% and 93.8% homology to the VH4.21 germline gene due to 15 and 18 nucleotide substitutions respectively. These substitutions result in 7 amino acid replacements in T14 and 11 in D5 (Figure 3.5). The amino acid replacements in T14 are distributed as follow: 2 in CDR1, 3 in CDR2 and 2 in framework (FW) 3. In the D5 antibody the amino acid replacements are located as follow: 1 in FW1, 1 in CDR1, 4 in CDR2 and 5 in FW3. In both T14 and D5 the CDR1 amino acid replacements result in changing the SGYY sequence (the inverted sequence of YYGS) which is encoded by VH4.21 germline gene. The VH CDR3 of these five anti-DNA antibodies but not of the Variants are enriched in arginine and/or tyrosine residues. The Variant antibodies are rich in the basic amino acid lysine in this region which helps to provide a net positive charge in CDR3.

Section 3.2 Molecular Characterization of the Anti-Sm/RNP Antibodies

The V region genes of three other established human hybridomas (BUD 45.12.8, BUD 94.91.8 and BUD 114.4.11) which originated from the tonsillar B cells of a normal child (Carruthers 1991) were chosen for molecular characterization. As

Table 3.6

Characteristics of human anti-Sm/RNP antibodies

	BUD 45.12.8	BUD 94.91 8	BUD 114.4.11
Antigenic Specificity[1]:			
Sm/RNP	+	+	+
ss DNA	+	-	+
ds DNA	-	-	-
RF	-	+	-
Ro	-	+	+
La	-	+	-
Cardiolipin	-	-	-
Heavy Chain:			
VH Family	VH III	VH III	VH I
VH Germline	VH26c	hv3019b9	hv1L1
% Identity	100	100	99.7
D Gene	DXP4	DXP4	DK1 or DM1
JH Gene	JH4b	JH6	JH4b
Net Charge[2]:			
VDJ	1.64	0.73	5.73
CDR3	-0.08	-3.08	0.92
Light Chain:			
VL Family	κ III	λ IIIb	κ IV
VL Germline	Humkv325	hsiggll150	Humk18
% Identity	99.7	100	99.4
JL Gene	Jκ2	Jλ2/3	Jκ3
Net Charge[2]:			
VJ	1.65	-2.35	0.64

[1] Carruthers 1991

[2] Net charge of the peptides have been calculated at pH 7.5 using Isoelectric programs of the Genetics Computer Group Sequence Analysis Software Package, Version 7.3.1-UNIX (Genetics Computer Group, Inc., Madison, WI).

previously noted BUD 45.12.8 and BUD 114.4.11 hybridomas bound to Sm/RNP and ss DNA. The anti-Sm/RNP hybridoma BUD 94.91.8 and KIM4.6 anti-DNA hybridoma use related VH and VL genes.

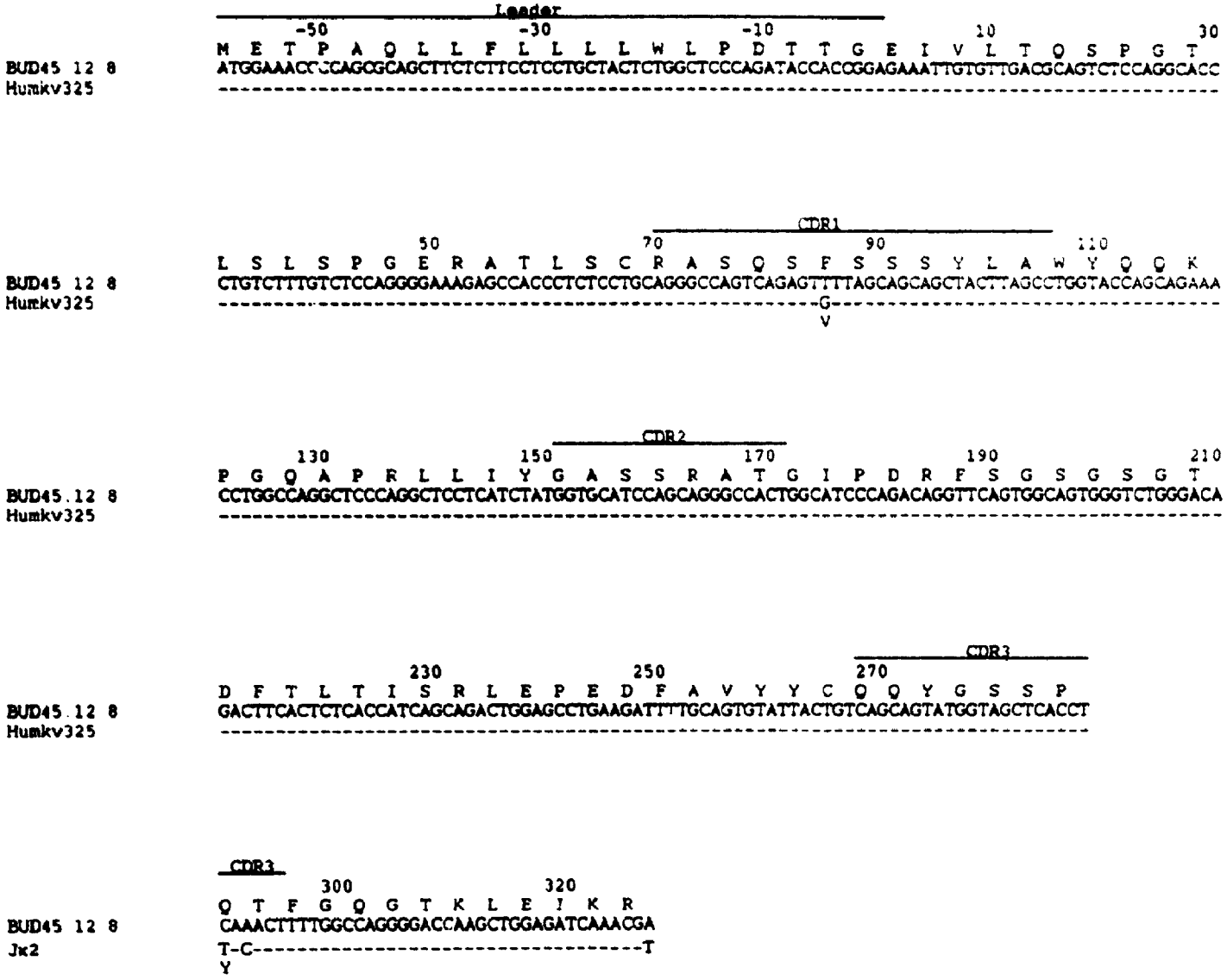
Table 3.6 shows the established characteristics of these BUD clones (Carruthers & Bell 1992) and summarizes the molecular features of their heavy and light chains. These will be discussed individually in the following sections.

3.2.1 BUD 45.12.8 VL region nucleotide sequence

The Ig light chain V gene of BUD 45.12.8 contains a κIIIb gene rearranged with a Jκ2 gene (Figure 3.6). The κIIIb V segment gene is identical to *Humkv325* germline gene (Radoux *et al.* 1986) except for a T to G substitution at position 85. This substitution results in the replacement of a valine for a phenylalanine in CDR1. This replacement was not found in other *Humkv325* encoded genes (GenBank and EMBL databanks and Kabat *et al.* 1991). Glutamine is substituted for a tyrosine at the first codon of Jκ2 gene (Hieter *et al.* 1982). BUD 45.12.8 has an A substitution for a T at the end of Jκ2 gene which is silent at the amino acid level and may represent an allelic polymorphic site.

Humkv325 germline gene has been shown to be associated with IgM rheumatoid factor antibodies (Chen *et al.* 1989, Kipps *et al.* 1989 and Ezaki *et al.* 1991) and is also expressed in κ-bearing CLL B cells that express the CD5 surface antigen (Kipps *et al.* 1990), and in the adult CD5+ B cell subset (Inghirami *et al.* 1991). Interestingly, this VL gene has also been used by some anti-DNA antibodies: IgG T14 (van Es *et al.* 1991), IgG 33.F12 (Winkler *et al.* 1992) and IgM

Figure 3.6 VL chain nucleotide and deduced amino acid sequence comparison of anti-Sm/RNP antibody BUD 45.12.3 to *Humkv325* and Jκ2 germline genes. The numbers indicate the nucleotide positions. Identity in the nucleotide sequence is indicated by a dash. The complementarity determining regions (CDR) are indicated above the number scale. Amino acids are designated by one letter codes and shown above and below (differences only) nucleic acid sequences. BUD 45.12.8 VL chain nucleotide sequence is available through EMBL/GenBank databases under accession number Z46345.


Leader
-50
-30
-10
10
30
M E T P A Q L L F L L L L W L P D T T G E I V L T Q S P G T
BUD45 12 8
ATGGAAACCCCAGCGCAGCTTCTCTTCCTCCTGCTACTCTGGCTCCCAGATACCACCGGAGAAATTGTGTTGACGCAGTCTCCAGGCACC
Humkv325
CDR1
50
70
90
110
L S L S P G E R A T L S C R A S Q S F S S S Y L A W Y Q Q K
BUD45 12 8
CTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTTTTAGCAGCAGCTACTTAGCCTGGTACCAGCAGAAA
Humkv325
G
V
CDR2
130
150
170
190
210
P G Q A P R L L I Y G A S S R A T G I P D R F S G S G S G T
BUD45.12 8
CCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGTCTGGGACA
Humkv325
CDR3
230
250
270
D F T L T I S R L E P E D F A V Y Y C Q Q Y G S S P
BUD45.12 8
GACTTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGTATGGTAGCTCACCT
Humkv325
CDR3
300
320
Q T F G Q G T K L E I K R
BUD45 12 8
CAAACTTTTGGCCAGGGGACCAAGCTGGAGATCAAACGA
Jκ2
T-C
T
Y

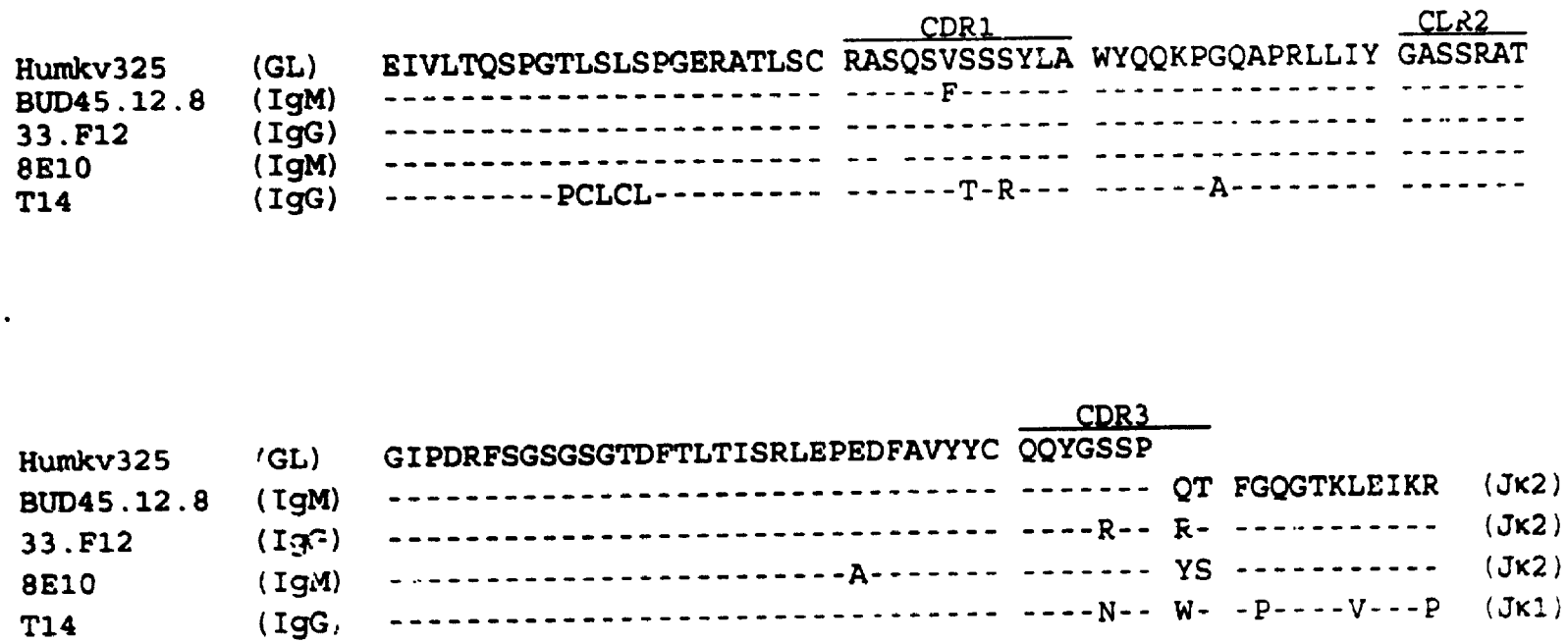


Figure 3.7 Deduced amino acid sequence comparison of *Humkv325* germline gene (GL) VL chain of BUD 45 12 8 anti-Sm/RNP antibody (also binds to ssDNA) and VL chains of 33 F12 (Winkler 1992), 8E10 (Dersimonian 1989) and T14 (van Es 1991) anti-DNA antibodies. Amino acids are designated by one letter codes and dash lines are indicative of identity.

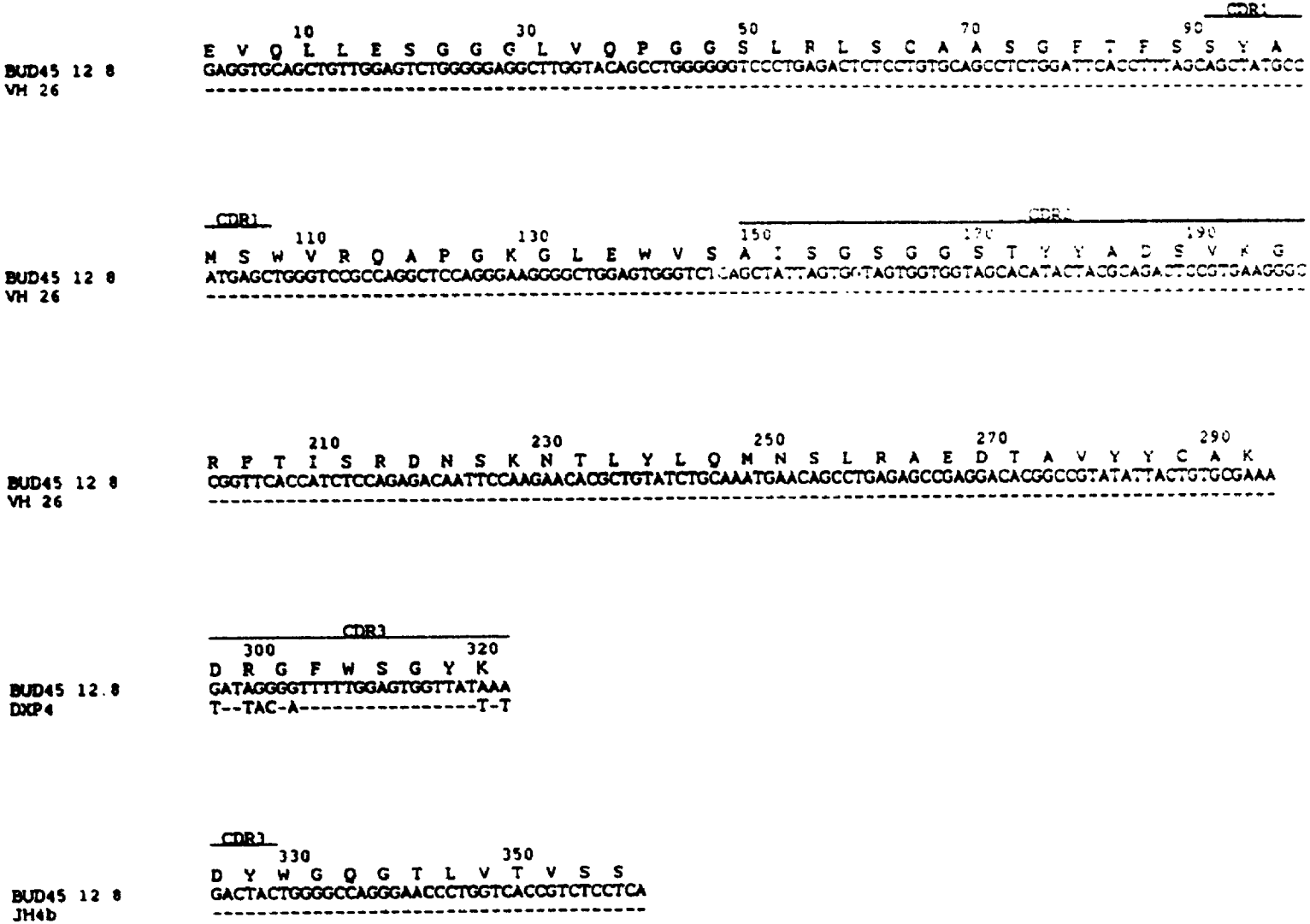
8E10 (Dersimonian *et al.* 1989). IgG T14 VL contains more somatic mutations in comparison to the other anti-DNA antibodies whose VL are encoded by *the Humkv325* germline gene (Figure 3.7). The VL used by BUD 45.12.8 IgM (anti-Sm/RNP and anti-ss DNA) and 8E10 IgM (reacts with both DNA and *Mycobacterium leprae)* differ by one amino acid from the encoded germline sequence in CDR1 and FW3 respectively (Figure 3.7). 33.F12 IgG anti-DNA antibody has two amino acid replacements (both to arginine residues) from *Humkv325* and Jκ2 germline genes in CDR3 region.

3.2.2 BUD 45.12.8 VH region nucleotide sequence

The Ig heavy chain gene of BUD 45.12.8 contains a VH III gene rearranged with a DXP4 and JH4 gene (Figure 3.8). The VH segment nucleotide sequence is identical to *VH26c* (also known as 18/2) germline gene (Chen *et al.* 1989). *VH26c* is a new designation for *VH26* germline gene (Matthyssens & Rabbitts 1980) after resequencing and revising the original sequence (Chen *et al.* 1989). BUD 45.12.8 D region is likely encoded by the DXP4 (Ichihara *et al.* 1988) germline gene with 5 and 2 nucleotide substitutions in the 5' and 3' ends respectively. BUD 45.12.8 JH is identical to a truncated (8 nucleotide from 5' end) JH4b germline gene (Yamada *et al.* 1991). JH4b is considered the prototypic sequence of JH4 (Yamada *et al.* 1991 and Schroeder *et al.* 1987). The original JH4 sequence was reported in a single individual (Ravetch *et al.* 1981).

VH26 (=*VH26c*) is also used to encode 2 of 4 other molecularly characterized human anti-Sm antibodies (RSP-1 and A73). The VH segment gene

Figure 3.8 VH chain nucleotide and deduced amino acid sequence comparison of anti-Sm/RNP antibody BUD 45.12.8 to *VH26* (=*VH26c*), DXP4 and JH4b germline genes. The numbers indicate the nucleotide positions Identity in the nucleotide sequence is indicated by a dash. The complementarity determining regions (CDR) are indicated above the number scale. Amino acids are designated by one letter codes and shown above the nucleic acid sequences. BUD 45.12.8 VH chain nucleotide sequence is available through EMBL/GenBank databases under accession number Z46382.


CDR1
10
30
50
70
90
E V Q L L E S G G G L V Q P G G S L R L S C A A S G F T F S S Y A
BUD45 12 8
GAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCAGCTATGCC
VH 26
CDR1
CDR2
110
130
150
170
190
M S W V R Q A P G K G L E W V S A I S G S G G S T Y Y A D S V K G
BUD45 12 8
ATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGC
VH 26
210
230
250
270
290
R F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A K
BUD45 12 8
CGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAA
VH 26
CDR3
300
320
D R G F W S G Y K
BUD45 12.8
GATAGGGGTTTTTGGAGTGGTTATAAA
DXP4
T--TAC-A----------T-T
CDR3
330
350
D Y W G Q G T L V T V S S
BUD45 12 8
GACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
JH4b

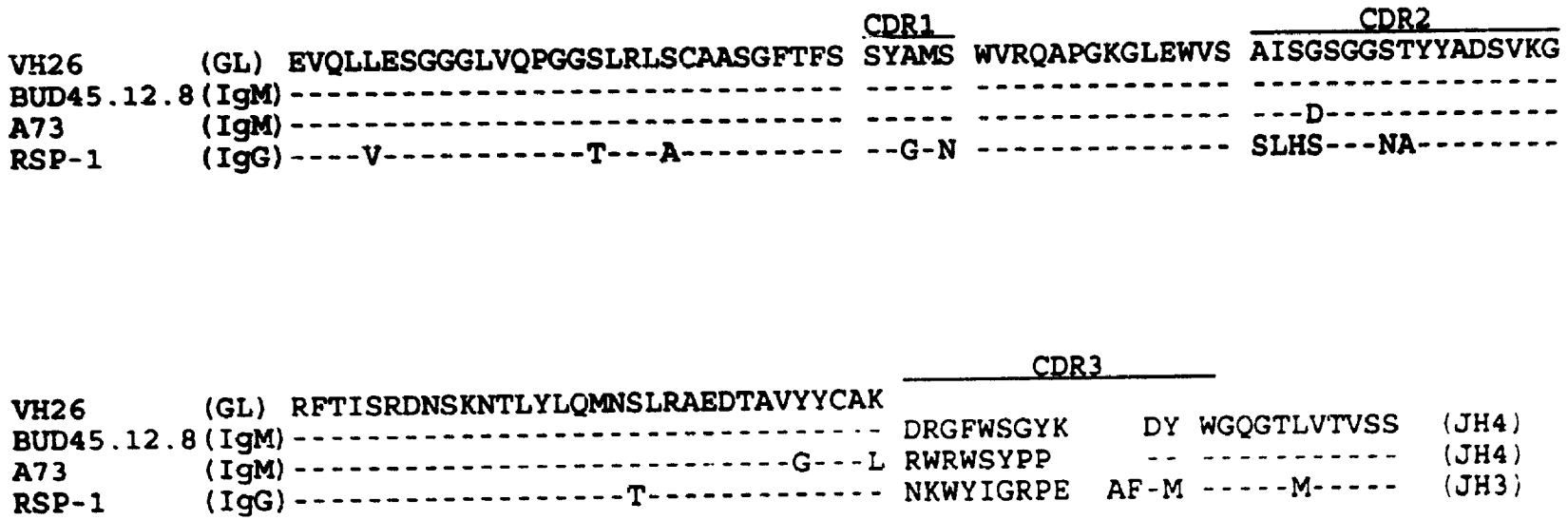


Figure 3.9 Deduced amino acid sequence comparison of *VH26* (=*VH26c*) germline gene (GL) and VH chains of BUD 45.12.8, A73 (Young 1990) and RSP-1(Isenberg et al, personal communication) anti-Sm antibodies. Amino acids are designated by one letter codes and dash lines are indicative of identity. Gaps in CDR3 regions were introduced during alignment of these sequences.

```
                                                          CDR1                          CDR2
VH26          (GL) EVQLLESGGGLVQPGGSLRLSCAASGFTFS SYAMS WVRQAPGKGLEWVS AISGSGGSTYYADSVKG
BUD45.12.8 (IgM) ---------------------------- ----- ---------- ----------------
18/2          (IgM) ---------------------------- ----- ---------- ----------------
1/17          (IgM) ---------------------------- ----- ---------- ----------------
B19.7         (IgM) ---------------------------- ----- ---------- ----------------
```

```
                                                                CDR3
VH26          (GL) RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK
BUD45.12.8 (IgM) ---------------------------- DRGFWSGYK      DY WGQGTLVTVSS (JH4)
18/2          (IgM) ---------------------------- GQVLYYGSGSY HWF-P ---------- (JH5)
1/17          (IgM) ---------------------------- GQVLYYGSGSY HWF-P ---------- (JH5)
B19.7         (IgM) ---------------------------- RAPKLTTKGY   YF-- ---------- (JH4)
```

Figure 3.10 Deduced amino acid sequence comparison of *VH26* (=*VH26c*) germline gene (GL), VH chain of BUD 45.12.8 anti-Sm/RNP antibody (also binds to ssDNA) and VH chains of 18/2, 1/17 (Dersimonian 1989) and B19.7 (Guillaume 1990) anti-DNA antibodies. Amino acids are designated by one letter codes and dash lines are indicative of identity. Gaps in CDR3 regions are introduced during alignment of these sequences.

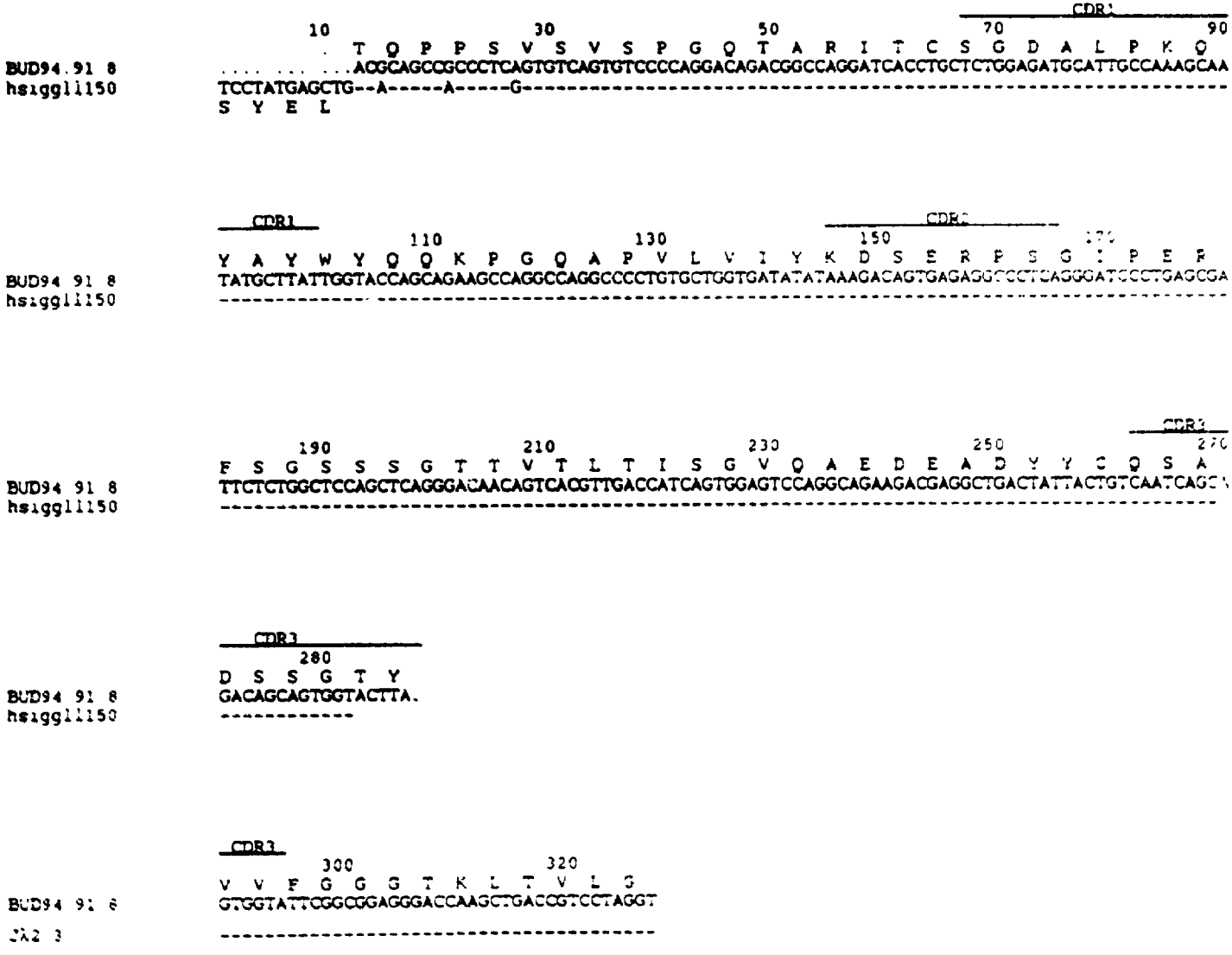
of the human SLE derived IgG anti-Sm and anti-cardiolipin antibody RSP-1 (Isenberg et al, personal communication) contains 16 nucleotide substitutions from the *VH26* germline gene. These substitutions result in 12 replacements at the amino acid level (Figure 3.9). The VH segment gene of human SLE derived IgM anti-Sm/RNP antibody A73 (Young *et al.* 1990 and Guillaume *et al.* 1990) contains 6 mutations from the *VH26* germline gene which result in one and two amino acid replacements in CDR2 and FW3 respectively (Figure 3.9).

Among the anti-DNA antibodies whose Ig VH and VL have been published (Table 1.2), eight are encoded by *VH26*. Interestingly, the VH segments of three IgM anti-DNA antibodies 18/2, 1/17 (Dersimonian *et al.* 1987) and B19.7 (Guillaume *et al.* 1990) are identical to the VH segment of BUD 45.12.8 and the *VH26* germline gene (Figure 3.10). The major difference between the heavy chains of 18/2 and 1/17 anti-ss and anti-ds DNA antibodies and BUD 45.12.8 anti-Sm/RNP and anti-ss DNA antibody is in the CDR3 region. The D regions of 18/2 and 1/17 are encoded by the DXP'1 germline gene and contain the YYGS motif sequence. Both BUD 45.12.8 and B19.7 anti-ss DNA antibodies, but not 18/2 or 1/17 anti-ss and anti-ds DNA antibodies contain one arginine in their D region.

3.2.3 BUD 94.91.8 VL region nucleotide sequence

The Ig light chain gene of BUD 94.91.8 contains a λIIIb gene rearranged with a Jλ2/3 gene (Figure 3.11). All seven 5' sense λ framework primers (Table 2.1 and Appendix B) were used for amplification of BUD 94.91.8 VL gene, and since the amplified product was produced only in the tube containing λ1 framework primer,

Figure 3.11 VL chain nucleotide and deduced amino acid sequence comparison of anti-Sm/RNP antibody BUD 94.91.8 to *hsiggll150* and Jλ2/3 germline genes. The numbers indicate the nucleotide positions. Identity in the nucleotide sequence is indicated by a dash. The complementarity determining regions (CDR) are indicated above the number scale. Amino acids are designated by one letter codes and shown above and below (differences only) nucleic acid sequences. BUD 94.91.8 VL chain nucleotide sequence is available through EMBL/GenBank databases under accession number Z46346.


CDR1
10
30
50
70
90
T Q P P S V S V S P G Q T A R I T C S G D A L P K Q
BUD94.91 8
hsigg11150
TCCTATGAGCTG--A-----A-----G----------
S Y E L
CDR1
CDR2
110
130
150
Y A Y W Y Q Q K P G Q A P V L V I Y K D S E R P S G I P E R
BUD94 91 8
hsigg11150
TATGCTTATTGGTACCAGCAGAAGCCAGGCCAGGCCCCTGTGCTGGTGATATATAAAGACAGTGAGAGGCCCTCAGGGATCCCTGAGCGA
CDR3
190
210
230
250
270
F S G S S S G T T V T L T I S G V Q A E D E A D Y Y C Q S A
BUD94 91 8
hsigg11150
TTCTCTGGCTCCAGCTCAGGGACAACAGTCACGTTGACCATCAGTGGAGTCCAGGCAGAAGACGAGGCTGACTATTACTGTCAATCAGC
CDR3
280
D S S G T Y
BUD94 91 8
hsigg11150
GACAGCAGTGGTACTTA.
CDR3
300
320
V V F G G G T K L T V L G
BUD94 91 8
GTGGTATTCGGCGGAGGGACCAAGCTGACCGTCCTAGGT
JA2 3

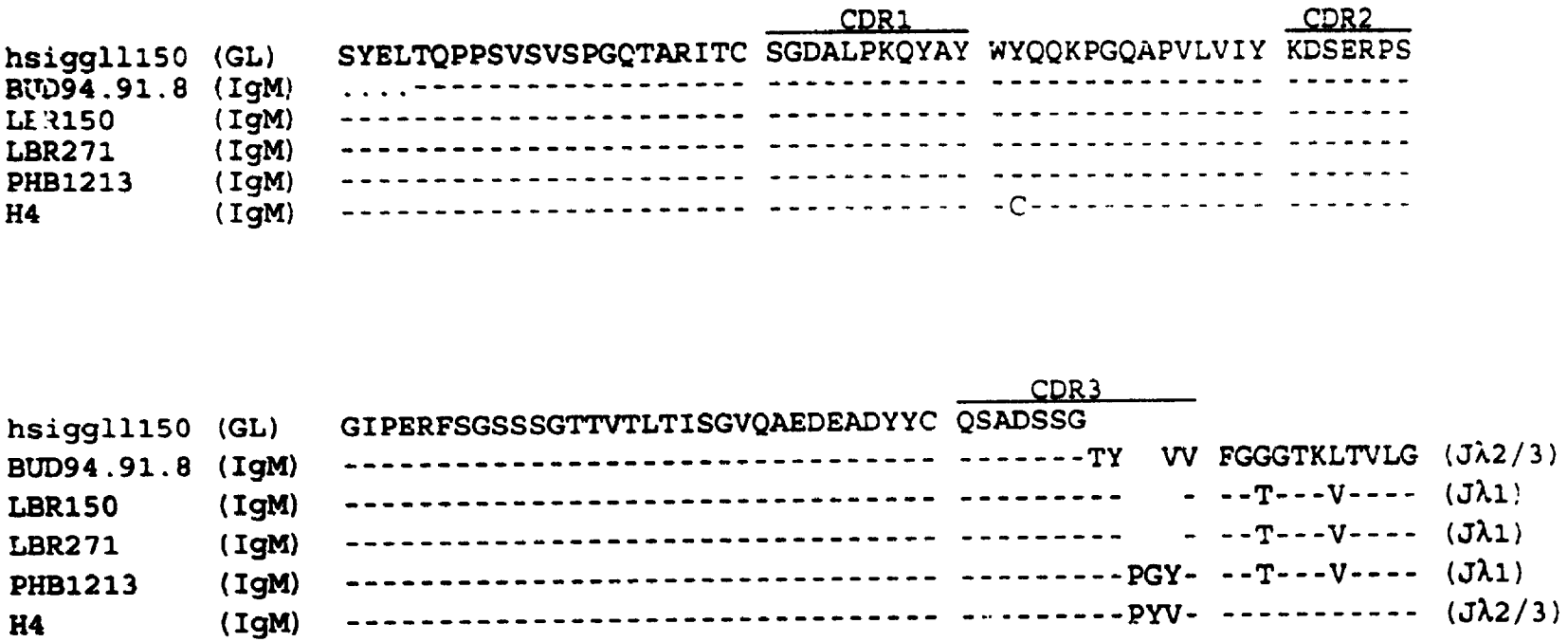


Figure 3.12 Deduced amino acid sequence comparison of *hsiggl1150* germline gene (GL), VL chain of BUD 94.91.8 anti-Sm/RNP antibody and VL chains of LBR150, LBR271, PHB1213 (Fang 1994) and H4 (Wong 1993) anti-rabbit IgG antibodies. Amino acids are designated by one letter codes and dash lines are indicative of identity. Gaps in CDR3 regions were introduced during the alignment of these sequences.

BUD 94.91.8 VL was assigned primarily to the Vλ1 family. After sequencing and comparison of the deduced amino acid sequence to published sequences (Eulitz *et al.* 1991), BUD 94.91.8 was reassigned to λIIIb family. Recently sequences of two λIIIb germline genes have been published (Fang *et al.* 1994). The BUD 94.91.8 VL gene sequence, except for the three nucleotides introduced by the λ1 framework primer, is identical to *hsiggll150* λIIIb germline gene sequence. Four other IgM antibodies are known to be encoded by *hsiggll150* germline gene: LBR150, PHB1213, LBR271 (Fang *et al.* 1994) and H4 (Wong *et al.* 1993). Except for the H4 VL gene which contains one substitution in the FW2 region, the VL gene sequence of three other antibodies are identical to the germline sequence. All of these IgM antibodies are monospecific for rabbit IgG and do not react with human IgG whereas BUD 94.91.8 is a polyspecific antibody which reacts with human IgG. The VL region of H4 is identical to the VL region of BUD 94.91.8 except for a substitution (tyrosine -> cysteine) in FW2 and two extra amino acids (tyrosine and proline) at the VJ junction (Figure 3.12).

Jλ2 and Jλ3 have identical sequences (Vasicek & Leder 1990) and can only be distinguished by sequencing the rearranged constant λ region. Therefore the germline gene encoding for the J region of BUD 94.91.8 is referred to as Jλ2/3.

3.2.4 BUD 94.91.8 VH region nucleotide sequence

The Ig heavy chain gene of BUD 94.91.8 contains a VH III gene rearranged with a DXP4 and JH6 gene (Figure 3.13). The VH segment nucleotide sequence is identical to *hv3019b9* (also known as DP-50) germline gene (Olee *et al.* 1991 and

Figure 3.13 VH chain nucleotide and deduced amino acid sequence comparison of anti-Sm/RNP antibody BUD 94.91.8 to *hv3019b*, DXP4 and JH6 germline genes. The numbers indicate the nucleotide positions. Identity in the nucleotide sequence is indicated by a dash. The complementarity determining regions (CDR) are indicated above the number scale. Amino acids are designated by one letter code and shown above and below (differences only) nucleic acid sequences. BUD 94.91.8 VH chain nucleotide sequence is available through EMBL/GenBank databases under accession number Z46379.

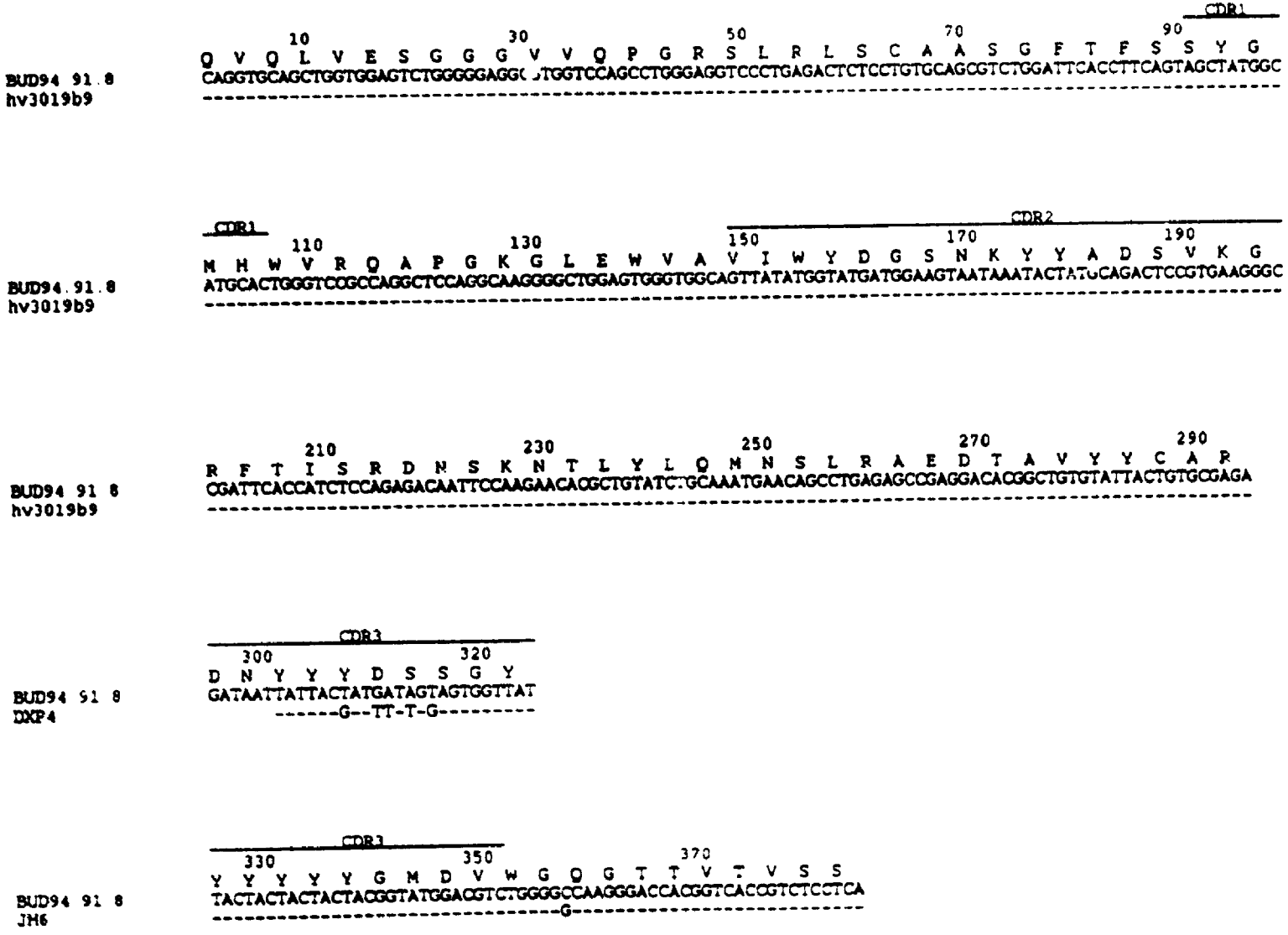
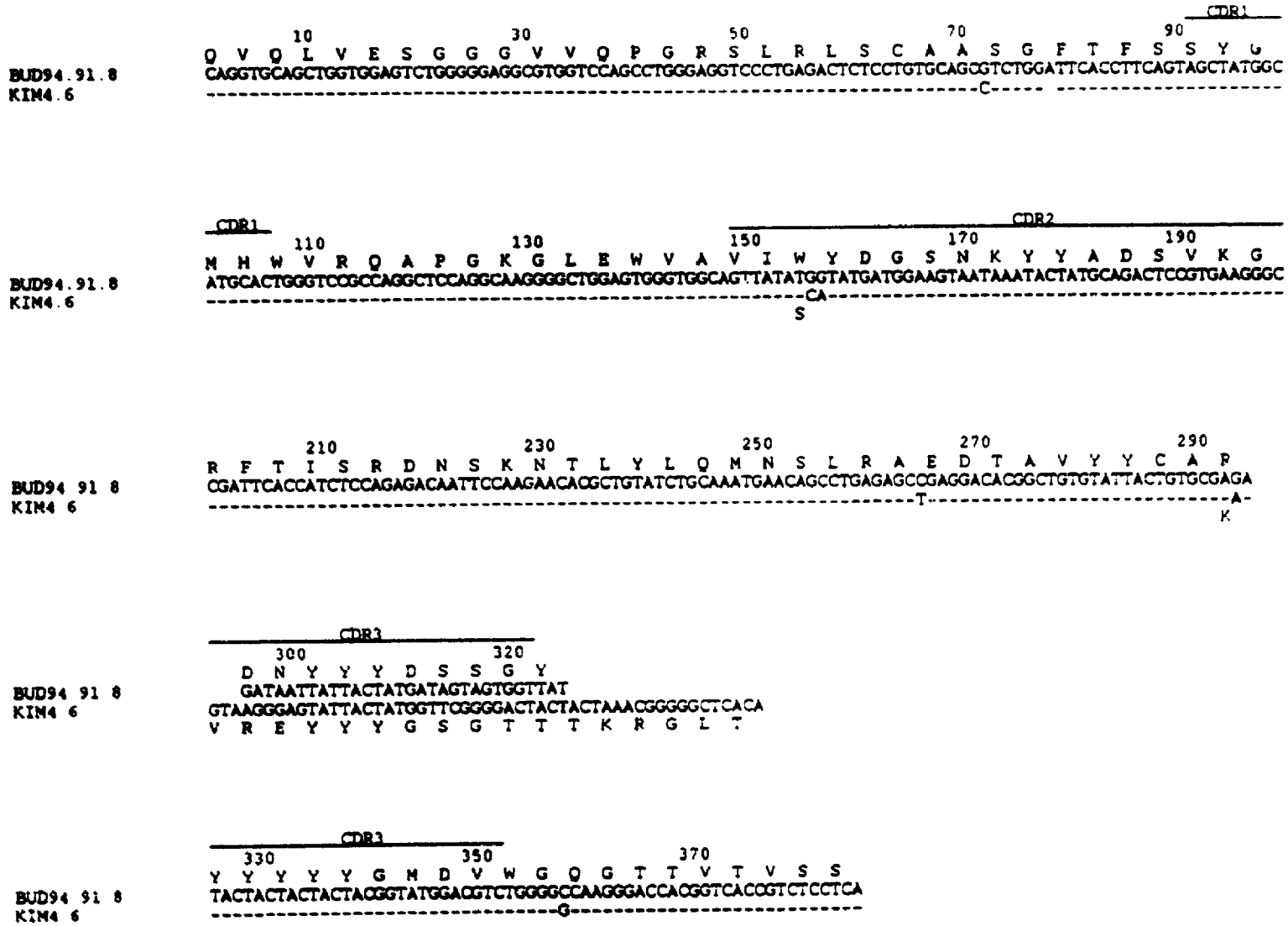

CDR1
10
30
50
70
90
Q V Q L V E S G G G V V Q P G R S L R L S C A A S G F T F S S Y G
BUD94 91 8
CAGGTGCAGCTGGTGGAGTCTGGGGGAGGC GTGGTCCAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCGTCTGGATTCACCTTCAGTAGCTATGGC
hv3019b9
CDR1
CDR2
110
130
150
170
190
M H W V R Q A P G K G L E W V A V I W Y D G S N K Y Y A D S V K G
BUD94.91.8
ATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATATGGTATGATGGAAGTAATAAATACTATGCAGACTCCGTGAAGGGC
hv3019b9
210
230
250
270
290
R F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A R
BUD94 91 8
CGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCGAGA
hv3019b9
CDR3
300
320
D N Y Y Y D S S G Y
BUD94 91 8
GATAATTATTACTATGATAGTAGTGGTTAT
DXP4
------G--TT-T-G---------
CDR3
330
350
370
Y Y Y Y Y G M D V W G Q G T T V T V S S
BUD94 91 8
TACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
JH6
----------G----------

Figure 3.14 VH chain nucleotide and deduced amino acid sequence comparison of anti-Sm/RNP antibody BUD 94.91.8 (non DNA-binding) and KIM4.6 anti-DNA antibody. The numbers indicate the nucleotide positions. Identity in the nucleotide sequence is indicated by a dash. The complementarity determining regions (CDR) are indicated above the number scale. Amino acids are designated by one letter codes and shown above and below (differences only) nucleic acid sequences.


CDR1
10 30 50 70 90
Q V Q L V E S G G G V V Q P G R S L R L S C A A S G F T F S S Y G
BUD94.91.8 CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCGTCTGGATTCACCTTCAGTAGCTATGGC
KIM4.6 -----C-----
CDR1
CDR2
110 130 150 170 190
M H W V R Q A P G K G L E W V A V I W Y D G S N K Y Y A D S V K G
BUD94.91.8 ATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATATGGTATGATGGAAGTAATAAATACTATGCAGACTCCGTGAAGGGC
KIM4.6 -----CA----------
S
210 230 250 270 290
R F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A R
BUD94 91 8 CGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCGAGA
KIM4 6 -----T----------A-
K
CDR3
300 320
D N Y Y Y D S S G Y
BUD94 91 8 GATAATTATTACTATGATAGTAGTGGTTAT
KIM4 6 GTAAGGGAGTATTACTATGGTTCGGGGACTACTACTAAACGGGGGCTCACA
V R E Y Y Y G S G T T T K R G L T
CDR3
330 350 370
Y Y Y Y Y G M D V W G Q G T T V T V S S
BUD94 91 8 TACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCCTCA
KIM4 6 ----------G----------

Tomlinson *et al.* 1992). The BUD 94.91.8 D region is likely encoded by the DXP4 (Ichihara *et al.* 1988) germline gene with 5 nucleotide substitutions. The first six nucleotides in this region are not encoded by DXP4 germline gene. These are probably N nucleotide additions. It is also possible that first two (GA) of these six nucleotides are from the intron sequence at the 3' end of *hv3019b9* germline gene. BUD 94.91.8 JH is identical to JH6 germline gene (Yamada *et al.* 1991) except for a G -> C substitution at position 357. This substitution likely represents a polymorphism rather than a mutation and therefore BUD 94.91.8 JH is probably a germline sequence.

hv3019b9 germline gene is considered to be a 1.9III-like gene (Olee *et al.* 1991). These two genes differ by five nucleotides which result in 2 replacements at the amino acid level. BUD 94.91.8 VH segment (*hv3019b9*) and IgM anti-DNA antibody KIM4.6 VH segment (1.9III) sequences are thus very similar (Cairns *et al.* 1989a). As well as having closely related VH segments, KIM4.6 and BUD 94.91.8 JH regions are both encoded by JH6 germline genes. The major difference between these two antibodies is in their D region genes. KIM4.6 D region is encoded by the DXP'1 germline gene and contains a YYGS motif sequence and two arginine residues. BUD 94.91.8 D region is encoded by DXP4 gene and contains a YYDS partial motif (Figure 3.14). The net charges of BUD 94.91.8 and KIM4.6 VH excluding their CDR3 region are almost identical (3.74 for BUD 94.91.8 and 3.73 for KIM4.6 at pH 7.5). However the corresponding net charges of BUD 94.91.8 and KIM4.6 CDR3 regions (-3.08 and 0.92 at pH 7.5 respectively) affect the overall

net charge of the VDJ peptides. The overall net charges of BUD 94.91.8 and KIM4.6 VH peptides are 0.73 and 4.70 respectively.

3.2.5 BUD 114.4.11 VL region nucleotide sequence

The Ig light chain gene of BUD 114.4.11 contains a κIV gene rearranged with a Jκ3 gene (Figure 3.15) The κIV V segment gene is identical to *Humk18* germline gene (Klobeck *et al.* 1985) except for two substitutions in the last codon. These two substitutions (C -> G and T -> A) result in an alanine for proline replacement at the amino acid level. BUD 114.4.11 J region is identical to Jκ3 germline gene except for a silent substitution (T -> A) at position 342.

3.2.6 BUD 114.4.11 VH region nucleotide sequence

The Ig heavy chain gene of BUD 114.4.11 contains a VH I gene rearranged with a DK 1 and JH4 gene (Figure 3.16). The V segment nucleotide sequence is 99.7% homologous to *hv1l1* germline gene (Olee *et al.* 1992). BUD 114.4.11 D region can be assigned to DK1 or DM1 (Ichihara *et al.* 1988) germline genes. If it is assigned to DK1 germline gene then one has to assume that the first eight nucleotides in the D region of this antibody are N additions. Assignment to DM1 means that there are five substitutions in BUD 114.4.11 D region. BUD 114.4.11 JH is encoded by a truncated (8 nucleotide from 5' end) JH4b germline gene (Yamada *et al.* 1991). The only substitution is at position 310. This nucleotide (A at position 310) can be a residue of DK1 or DM1 diversity germline genes since the next nucleotide in both of these germline genes is an A (Figure 3.16).

hv1l1 germline gene has not been reported to be used by any anti-DNA or

Figure 3.15 VL chain nucleotide and deduced amino acid sequence comparison of anti-Sm/RNP antibody BUD 114.4.11 to *Humk18* and Jκ3 germline genes. The numbers indicate the nucleotide positions. Identity in the nucleotide sequence is indicated by a dash. The complementarity determining regions (CDR) are indicated above the number scale. Amino acids are designated by one letter codes and shown above and below (differences only) nucleic acid sequences. BUD 114.4.11 VL chain nucleotide sequence is available through EMBL/GenBank databases under accession number Z46347.

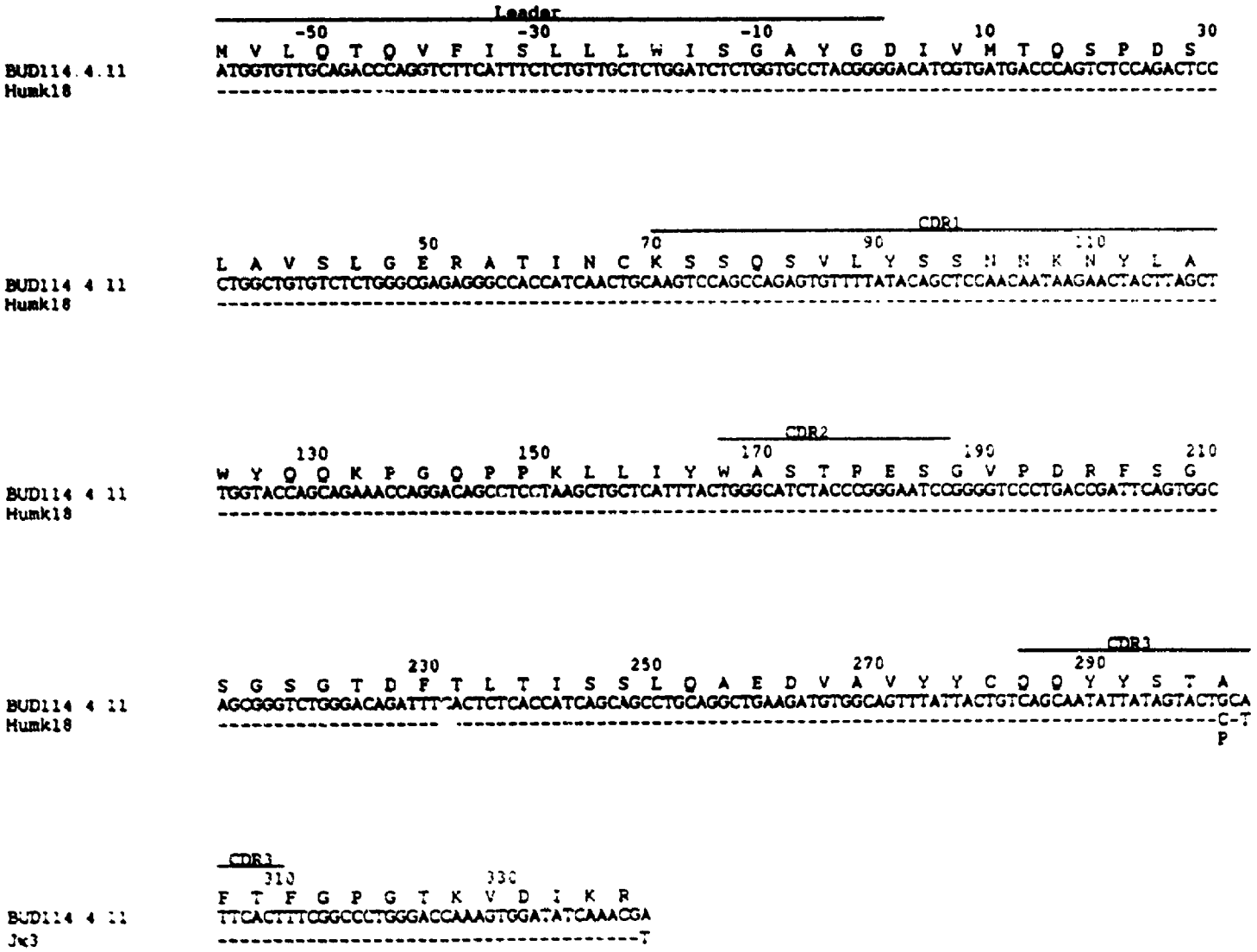
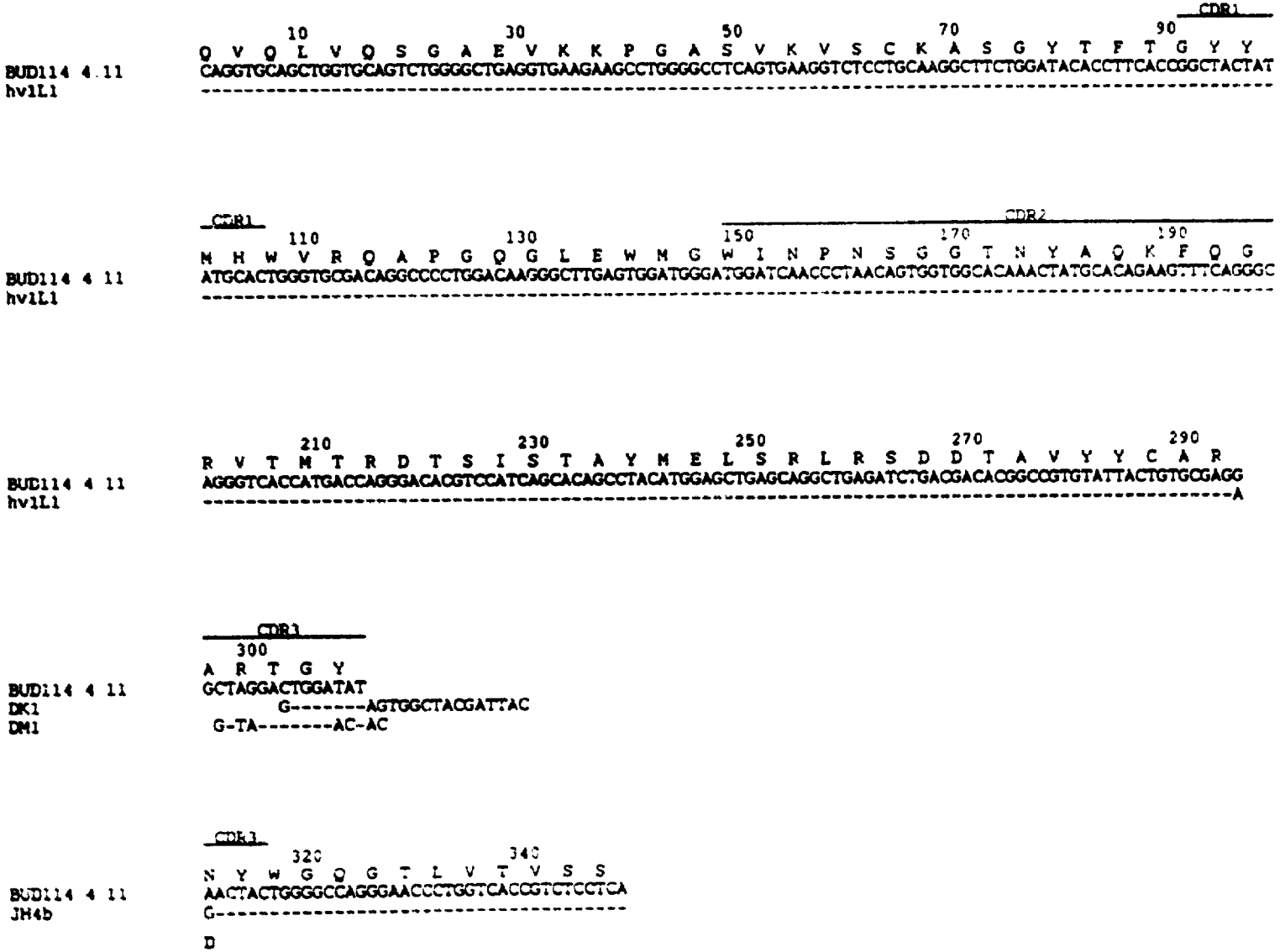

Leader
-50 -30 -10 10 30
M V L Q T Q V F I S L L L W I S G A Y G D I V M T Q S P D S
BUD114.4.11 ATGGTGTTGCAGACCCAGGTCTTCATTTCTCTGTTGCTCTGGATCTCTGGTGCCTACGGGGACATCGTGATGACCCAGTCTCCAGACTCC
Humk18 ----------
CDR1
50 70 90 110
L A V S L G E R A T I N C K S S Q S V L Y S S N N K N Y L A
BUD114 4 11 CTGGCTGTGTCTCTGGGCGAGAGGGCCACCATCAACTGCAAGTCCAGCCAGAGTGTTTTATACAGCTCCAACAATAAGAACTACTTAGCT
Humk18 ----------
CDR2
130 150 170 190 210
W Y Q Q K P G Q P P K L L I Y W A S T R E S G V P D R F S G
BUD114 4 11 TGGTACCAGCAGAAACCAGGACAGCCTCCTAAGCTGCTCATTTACTGGGCATCTACCCGGGAATCCGGGGTCCCTGACCGATTCAGTGGC
Humk18 ----------
CDR3
230 250 270 290
S G S G T D F T L T I S S L Q A E D V A V Y Y C Q Q Y Y S T A
BUD114 4 11 AGCGGGTCTGGGACAGATTTCACTCTCACCATCAGCAGCCTGCAGGCTGAAGATGTGGCAGTTTATTACTGTCAGCAATATTATAGTACTGCA
Humk18 ---------- ----------C-T
P
CDR3
310 330
F T F G P G T K V D I K R
BUD114 4 11 TTCACTTTCGGCCCTGGGACCAAAGTGGATATCAAACGA
Jκ3 ----------T

Figure 3.16 VH chain nucleotide and deduced amino acid sequence comparison of anti-Sm/RNP antibody BUD 114.4.11 to *hv1L1*, DK1, DM1 and JH4b germline genes. The numbers indicate the nucleotide positions. Identity in the nucleotide sequence is indicated by a dash. The complementarity determining regions (CDR) are indicated above the number scale. Amino acids are designated by one letter codes and shown above and below (differences only) nucleic acid sequences. BUD 114.4.11 VH chain nucleotide sequence is available through EMBL/GenBank databases under accession number Z46348.


CDR1
10
30
50
70
90
Q V Q L V Q S G A E V K K P G A S V K V S C K A S G Y T F T G Y Y
BUD114 4.11
CAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTCTCCTGCAAGGCTTCTGGATACACCTTCACCGGCTACTAT
hv1L1
CDR1
CDR2
110
130
150
170
190
M H W V R Q A P G Q G L E W M G W I N P N S G G T N Y A Q K F Q G
BUD114 4 11
ATGCACTGGGTGCGACAGGCCCCTGGACAAGGGCTTGAGTGGATGGGATGGATCAACCCTAACAGTGGTGGCACAAACTATGCACAGAAGTTTCAGGGC
hv1L1
210
230
250
270
290
R V T M T R D T S I S T A Y M E L S R L R S D D T A V Y Y C A R
BUD114 4 11
AGGGTCACCATGACCAGGGACACGTCCATCAGCACAGCCTACATGGAGCTGAGCAGGCTGAGATCTGACGACACGGCCGTGTATTACTGTGCGAGG
hv1L1
A
CDR1
300
A R T G Y
BUD114 4 11
GCTAGGACTGGATAT
DK1
G-------AGTGGCTACGATTAC
DM1
G-TA-------AC-AC
CDR1
320
340
N Y W G Q G T L V T V S S
BUD114 4 11
AACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
JH4b
G----------
D

anti-Sm antibody.

Section 3.3 DNA Specificity of the Phage Surface Expressed Antibody Fab Molecules

3.3.1 Construction of the pComb3 expression vector containing Variant #3 (V3) VL and VH

V3 VL and VH were cloned into the pComb3 phagemid expression vector in two steps as described in Section 2.3.2. First the V3 VL was cloned into the vector and one of the phagemids containing the correct size V3 VL insert (656 bp) (pComb3 + V3 VL) was selected. In the next step V3 VH was cloned into the "pComb3 vector + VL" construct. The final "pComb3 + V3 VL + V3 VH" construct (5298 bp) was mapped with five sets of restriction enzymes (Figure 3.17 and 3.18). This mapping confirmed that the constructs carry the expected genetic structures in the correct orientation.

3.3.2 Construction of the pComb3 expression vector containing KIM4.6 VL and VH

To obtain a positive control for DNA reactivity in the phage expression system cloning of KIM4.6 VH DNA fragment into "pComb3 + V3 VL" (V3VL and KIM4.6 VL sequences are identical) was attempted. Despite numerous attempts this cloning was not successful. Additional experiments were performed in order to determine why the VH gene of KIM4.6 could not be cloned into pComb3 expression vector. These experiments will be discussed in Section 3.5.

Figure 3.17 The expected size of the fragments resulting from restriction mapping of pComb3 vector containing V3 VH and VL. Five sets of restriction enzymes were used to confirm that the constructs carry the expected genetic structures in the correct orientations. VH and VL genes are shown in green and red respectively. The approximate sites for the enzymes are shown in the figure. The expected sizes (bp) of the fragments resulting from each set of digestion reaction is shown in the yellow boxes.

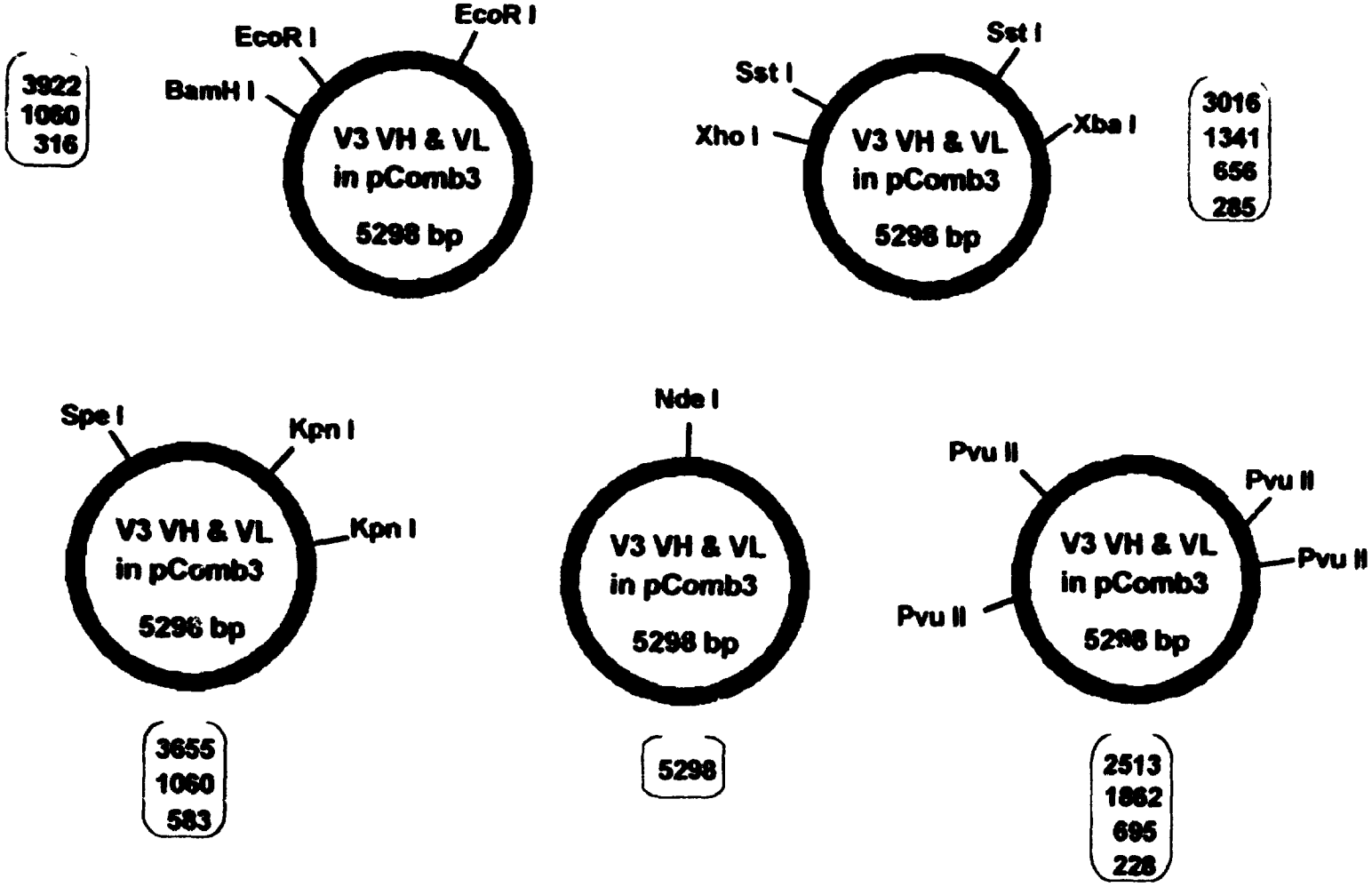
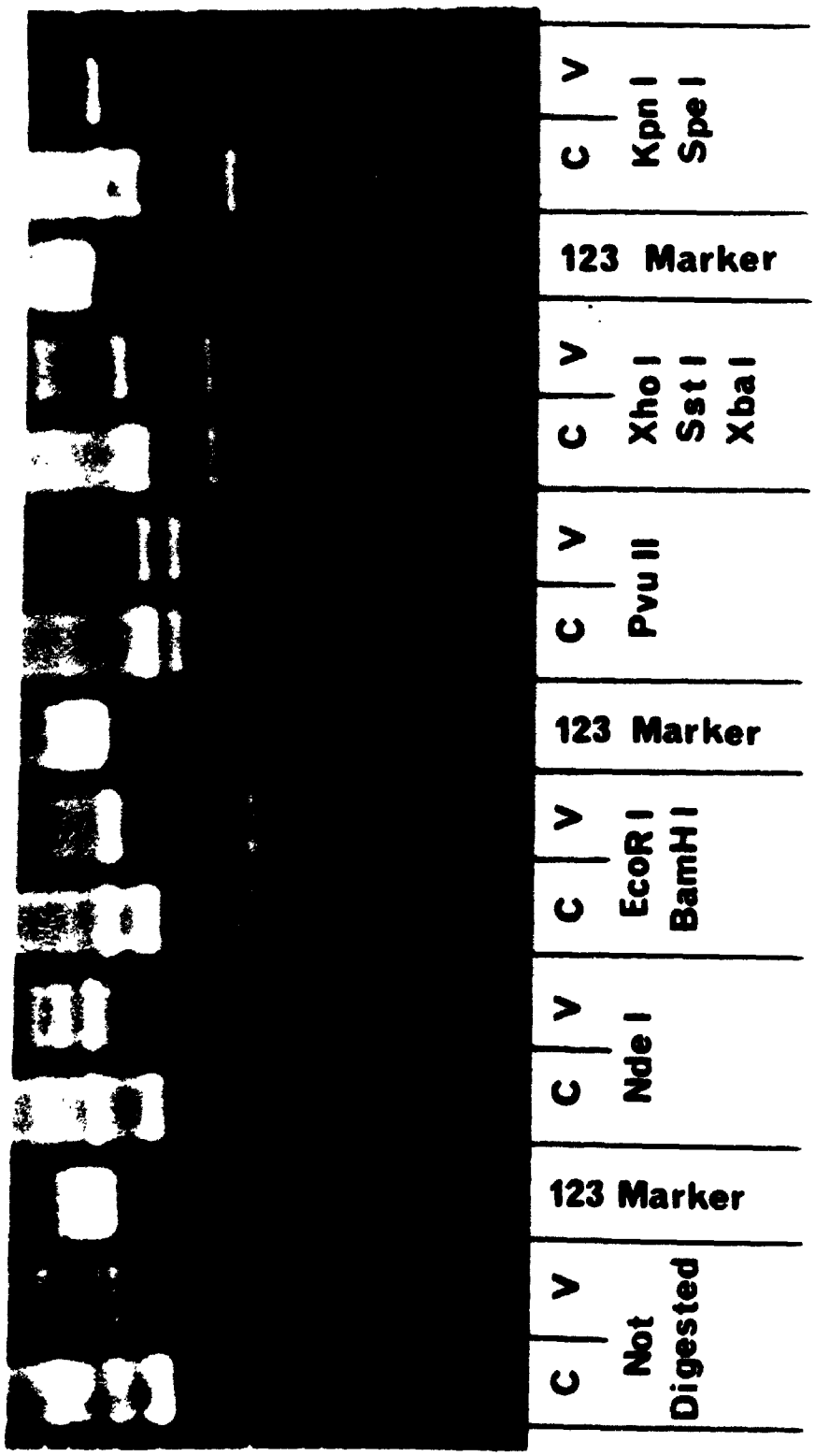

EcoR I
EcoR I
BamH I
V3 VH & VL
in pComb3
5298 bp
3922
1060
316
Sst I
Sst I
Xho I
Xba I
V3 VH & VL
in pComb3
5298 bp
3016
1341
656
285
Spe I
Kpn I
Kpn I
V3 VH & VL
in pComb3
5298 bp
3655
1060
583
Nde I
V3 VH & VL
in pComb3
5298 bp
5298
Pvu II
Pvu II
Pvu II
Pvu II
V3 VH & VL
in pComb3
5298 bp
2513
1862
695
228

Figure 3.18 Ethidium bromide stained DNA agarose gel of mapping pComb3 constructs. Lanes designated with letter C correspond to digested "pComb3 + Chimeric VH + V3 VL" construct and lanes designated with letter V correspond to digested "pComb3 + V3 VH + V3 VL" construct. The size of the fragments resulting form each restriction digestion reaction match with the expected sizes. The expected sizes for "pComb3 + V3 VH + V3 VL" is shown in Figure 3.17 and the expected sizes for "pComb3 + Chimeric VH + V3 VL" shown in figure 3.19. Lanes designated as 123 Marker refer to DNA molecular weight markers in increments of 123 base pairs. The lowest visible band in the marker lanes corresponds to 123 base pairs.


C
V
Not Digested
123 Marker
C
V
Nde I
C
V
EcoR I
BamH I
123 Marker
C
V
Pvu II
C
V
Xho I
Sst I
Xba I
123 Marker
C
V
Kpn I
Spe I

3.3.3 Construction of the pComb3 expression vector containing V3 VL and Chimeric VH

Construction of the Chimeric VH has been described in Section 2.5.1. This procedure should result in the generation of two different products: a KIM4.6 WT VH gene and a Chimeric VH gene (KIM4.6 VH segment + V3 DJ) as shown in Figure 2.3. The purified product from the second round of PCR was cloned into the "pComb3 + V3 VL" construct. Ten phagemids containing the correct size VH insert were selected and mapped. This mapping showed that all these phagemids contain the Chimeric VH insert. Restriction mapping of one of the "pComb3 + Chimeric VH + V3 VL" constructs has been compared to the restriction mapping of "pComb3 + V3 VH + V3 VL" construct in Figure 3.18. Figure 3.19 shows the expected fragment sizes resulting from the mapping of "pComb3 + Chimeric VH + V3 VL". The Chimeric VH was sequenced to determine the exact switch point between two templates (Figure 3.20). As predicted, the switch occurred in the FW3 region of KIM4.6 at the beginning of the FW3SstI primer sequence. The FW3SstI primer introduces two nucleotide substitution to the KIM4.6 VH FW3 which result in one replacements at the amino acid level: a leucine for an aspartic acid (at position 90). In addition, since the last two amino acid residues of the Chimeric VH are encoded by V3 VH, an arginine has been substituted for a lysine as the last amino acid of the KIM4.6 FW3. The lysine to arginine replacement is expected to favor DNA reactivity. Except for these three nucleotide changes in FW3, the Chimeric VH gene contains the KIM4.6 VH segment and D and J regions of the

Figure 3.19 The expected size of the fragments resulting from restriction mapping of pComb3 vector containing Chimeric VH and V3 VL. Five sets of restriction enzymes were used to confirm that the constructs carry the expected genetic structures in the correct orientations. VL gene is shown in red. Chimeric VH gene is shown in orange and green (orange: KIM4.6 VH segment gene and green: V3 DJ and Cμ genes). The approximate sites for the enzymes are shown in the figure. The expected sizes (bp) of the fragments resulting from each set of digestion reaction is shown in the yellow boxes.

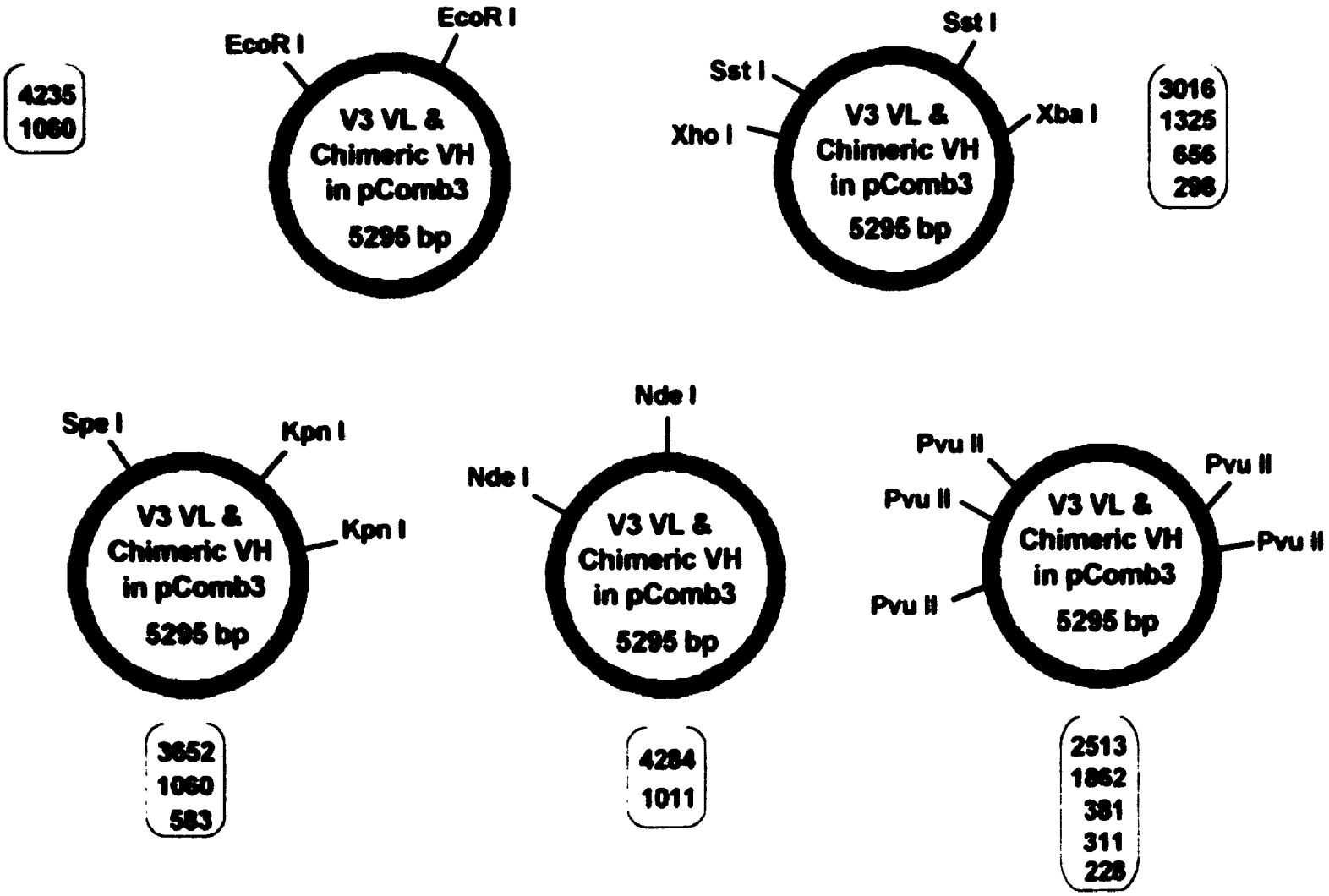
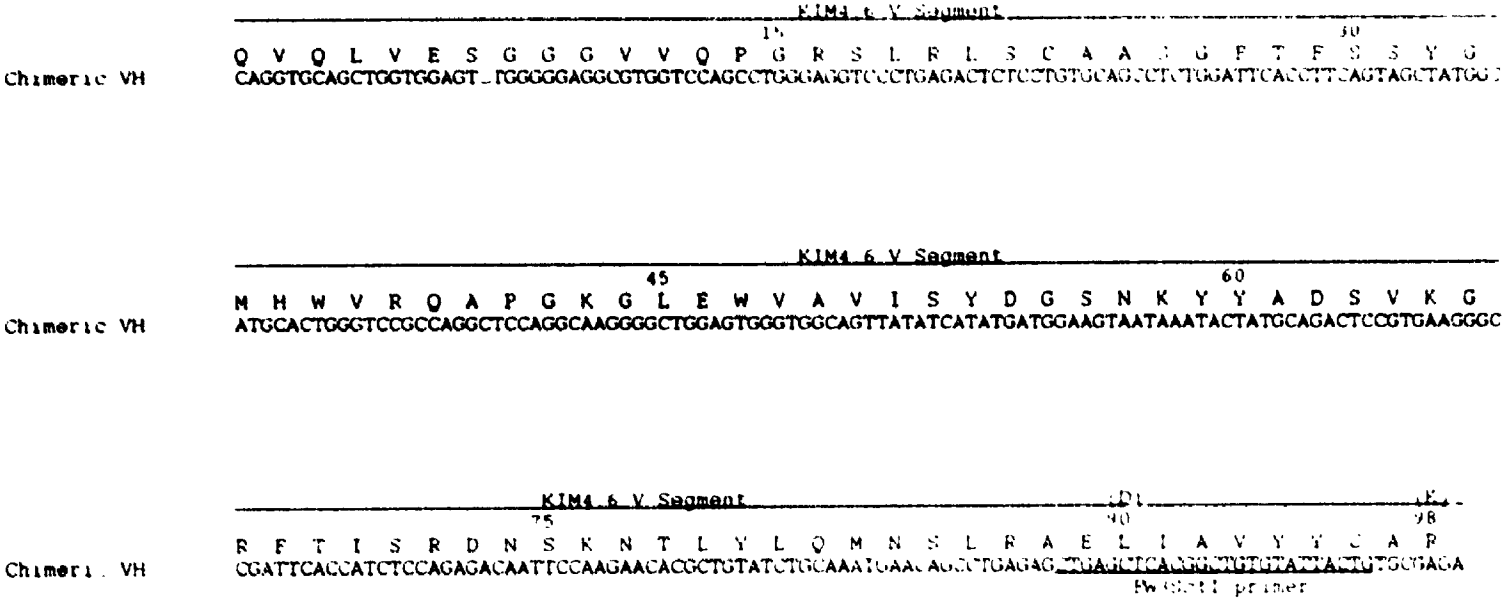

4235
1060
EcoR I
EcoR I
V3 VL &
Chimeric VH
in pComb3
5295 bp
Sst I
Sst I
Xho I
Xba I
V3 VL &
Chimeric VH
in pComb3
5295 bp
3016
1325
656
298
Spe I
Kpn I
Kpn I
V3 VL &
Chimeric VH
in pComb3
5295 bp
3652
1060
583
Nde I
Nde I
V3 VL &
Chimeric VH
in pComb3
5295 bp
4284
1011
Pvu II
Pvu II
Pvu II
Pvu II
Pvu II
V3 VL &
Chimeric VH
in pComb3
5295 bp
2513
1862
381
311
228

Figure 3.20 Chimeric VH chain nucleotide and deduced amino acid sequences. The Chimeric VH construct contains the KIM4 6 VH segment and D and J regions of the Variant #3 (V3) which are indicated above the number scale The sequence of the FW3SstI primer which was used for V3 DJC$_{\mu}$ PCR amplification, is underlined This primer introduces one amino acid change to KIM4 6 FW3 at position 90 (D -> L) Amino acid lysine at position 98 has been replaced with an arginine (the last two amino acids of Chimeric VH FW3 are encoded by V3 VH). KIM4 6 amino acid residues for positions 90 and 98 are shown in brackets. The amino acid sequence of Chimeric VH is shown by one letter codes above the nucleic acid sequence Numbers are indicative of amino acid positions.


KIM4.6 V Segment
15
30
Q V Q L V E S G G G V V Q P G R S L R L S C A A S G F T F S S Y G
Chimeric VH
CAGGTGCAGCTGGTGGAGT TGGGGGAGGCGTGGTCCAGCCTGGGAGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTAGCTATGG
KIM4.6 V Segment
45
60
M H W V R Q A P G K G L E W V A V I S Y D G S N K Y Y A D S V K G
Chimeric VH
ATGCACTGGGTCCGCCAGGCTCCAGGCAAGGGGCTGGAGTGGGTGGCAGTTATATCATATGATGGAAGTAATAAATACTATGCAGACTCCGTGAAGGGC
KIM4.6 V Segment
JD1
JK1
75
90
98
R F T I S R D N S K N T L Y L Q M N S L R A E D T A V Y Y C A R
Chimeric VH
CGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAG
Fw(Sst I primer
Variants D region
110
G G Q C P K K A S C Y T K
Chimeric VH
GGGGGGCAGTGCCCGAAGAAGGCCAGCTGCTACACGAAG
Variants J region
120
N W F D P W G Q G T L V T V S S
Chimeric VH
AACTGGTTCGACCCCTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA

Variant (V3).

3.3.4 Construction of the pComb3 expression vector containing V3 VL and V3 VH with the insert encoding the YYGS motif

The YYGS encoding sequence (TAC TAT GGT TCG) was grafted between the encoding sequence for the two lysine residues in the D region of the V3 VH as described in Section 2.5.2. The VH of V3 with the insert encoding the YYGS motif gene was cloned into the "pComb3 + V3 VL" construct. The entire VH portion of the final product was sequenced to confirm the accuracy of the YYGS encoding sequence insertion. Except for the D region which contained the YYGS encoding sequence (Figure 3.21), the sequence was identical to the V3 VH sequence.

3.3.5 Presence of the antibody Fab molecule on the phage surface

Phage particles expressing Fab molecules were prepared from pComb3 constructs as described in Section 2.3.3. Each preparation was tested by ELISA to confirm the presence of the Fab molecules on the phage surface. Table 3.7 shows that phage preparations from all "pComb3 + V3 VL + V3 VH", "pComb3 + V3 VL + Chimeric VH" and "pComb3 + V3 VL + V3 VH with the insert encoding for YYGS motif' constructs contain phage surface expressed Fab molecules. As described in Section 2.3.4 and shown in Figure 2.1 this ELISA detects the phage particles which are captured on the anti-human λ coated wells. Since the V3 λ light chain is bound to the phage particles via phage surface incorporated heavy chain, the positive readings are indicative of the presence of phage surface expressed Fab molecules.

Figure 3.21 Nucleotide sequence of the D region of the Variant 3 grafted with the YYGS motif encoding sequence. The nucleotide and deduced amino acid sequences are shown. The encoding sequence for YYGS motif (TAC TAT GGT TCG) is grafted between the sequence encoding the two lysine residues in the D region of the Variant 3.

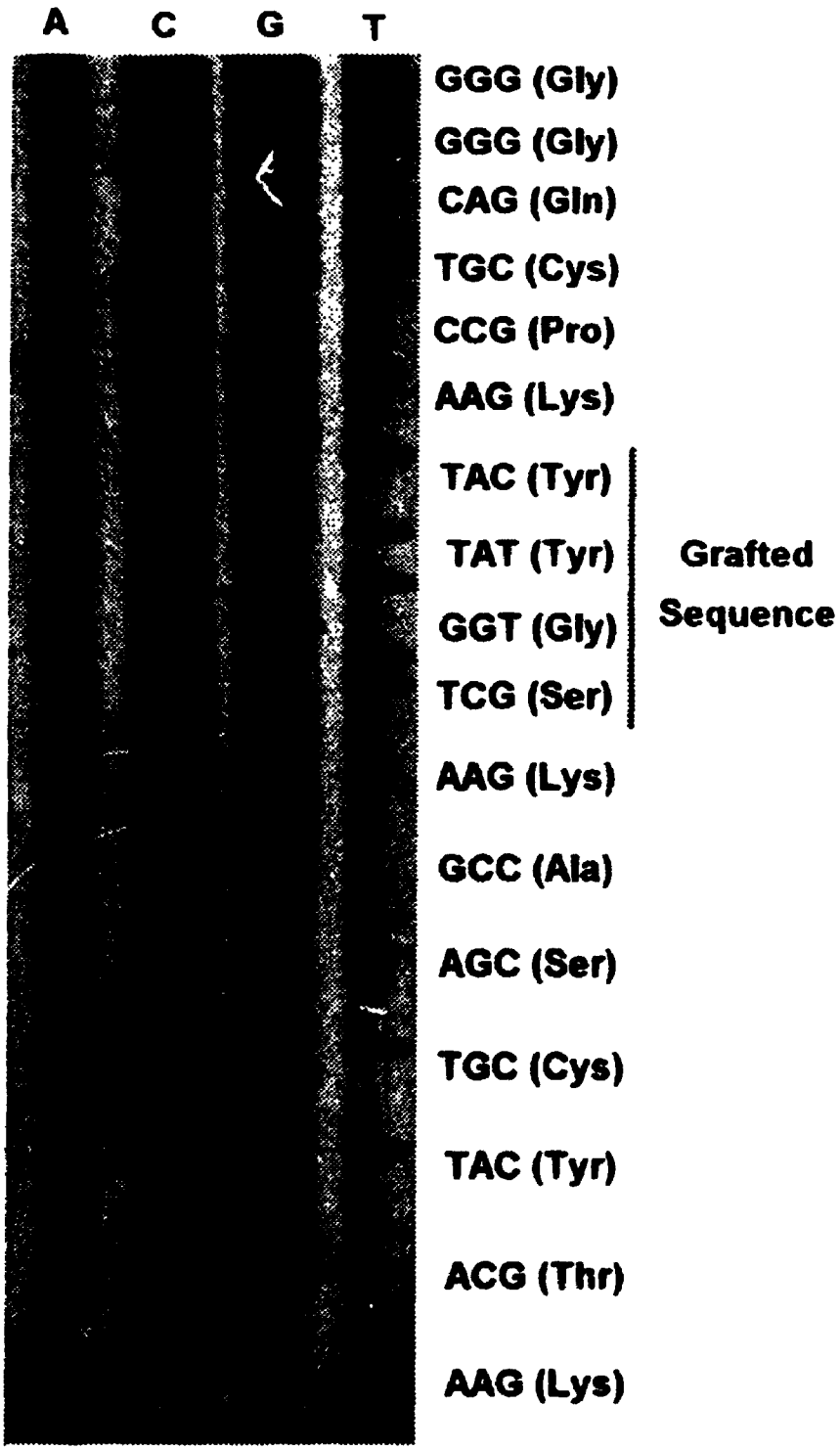

A
C
G
T
GGG (Gly)
GGG (Gly)
CAG (Gln)
TGC (Cys)
CCG (Pro)
AAG (Lys)
TAC (Tyr)
TAT (Tyr)
GGT (Gly)
TCG (Ser)
Grafted Sequence
AAG (Lys)
GCC (Ala)
AGC (Ser)
TGC (Cys)
TAC (Tyr)
ACG (Thr)
AAG (Lys)

Table 3.7

Detecting the presence and determining the DNA specificity of the Fab molecules expressed on the phage surface[1]

	OD 405 nm			
	Presence of the phage expressed Fab molecules		DNA specificity of phage expressed Fab molecules	
	goat anti-human λ[2]	goat anti-human κ[2]	ss DNA[2]	ds DNA[2]
V3 VL + V3 VH on phage surface[3]	0.48	0.02	0.01	0.00
V3 VL + Chimeric VH on phage surface[3]	0.43	0.01	0.02	0.01
V3 VL + V3 VH with the YYGS motif on phage surface[3]	0.45	0.01	0.02	0.01
LB medium	0.01	0.00	0.00	0.00
LB medium containing wild type phage	0.02	0.01	0.01	0.02
V3 VH only on phage surface[3]	0.02	0.02	0.01	0.01
V3 VH + BUD45.12.8 VL on phage[3,4]	0.02	0.39	ND[5]	ND

[1] These assays have been described in Sections 2.3.4 and 2.3.5.
[2] Ag coated on the plate.
[3] In LB medium.
[4] BUD 45.12.8 VL is a κ light chain.
[5] ND: Not determined.

3.3.6 DNA specificity of the phage particles expressing Fab molecules

Fab expressing phage preparations from all "pComb3 + V3 VL + V3 VH", "pComb3 + V3 VL + Chimeric VH" and "pComb3 + V3 VL + V3 VH with the insert encoding for YYGS motif" constructs were tested for ss and ds DNA reactivity. As shown in Table 3.7 none of these Fab molecules bind to ss nor ds DNA. Therefore the insertion of the YYGS motif between two charged lysine residues in the D region of the V3 VH does not change the DNA specificity of the Variant which remains negative for ss and ds DNA reactivity.

Section 3.4 DNA Specificity of the *In Vitro* Transcribed and Translated Antibody Heavy Chain Molecules

3.4.1 Construction of the templates

"T7 promoter + CITE sequence + KIM4.6 VH", "T7 promoter + CITE sequence + KIM4.6 VH without the YYGS motif encoding sequence" and "T7 promoter + CITE sequence + Chimeric VH" DNA templates were constructed as described in Sections 2.4.1, 2.5.1 and 2.5.3. These constructs were mapped using KpnI, PvuII and NdeI restriction enzymes (Figure 2.2) to confirm that they all carry the expected genetic structures in the correct orientation (data not shown).

3.4.2 *In vitro* production and labeling of the VH chain peptides

^{35}S labeled KIM4.6 VH, KIM4.6 VH without YYGS motif and Chimeric VH peptides were produced using "T7 promoter + CITE sequence + KIM4.6 VH", "T7 promoter + CITE sequence + KIM4.6 VH without the YYGS motif encoding

sequence" and "T7 promoter + CITE sequence + Chimeric VH" DNA templates as described in Section 2.4.2. Radioactive amino acid % incorporation values for each of these products have been listed in Table 3.8. This value is comparatively lower for the Chimeric VH since this peptide has one less methionine (3) compared to the other two VH peptides. The lower content of methionine in Chimeric VH is a result of the replacement of KIM4.6 DJ with the DJ of the Variants in Chimeric VH. The higher value for the β-galactosidase peptide is possibly due to the higher concentration of the β-galactosidase DNA template which was provided in the STP kit.

3.4.3 SDS polyacrylamide gel analysis of the labeled VH peptides

The presence of the translated peptides of correct molecular weights was determined by running the translated products on SDS polyacrylamide gel. The length of KIM4.6 VH, KIM4.6 VH without the YYGS motif encoding sequence and Chimeric VH gene constructs are 477, 465 and 454 respectively. As expected the approximate molecular weights of the translated peptides are 17 KD (Figure 3.22).

3.4.4 DNA specificity of the VH peptides

KIM4.6 VH, KIM4.6 VH without the YYGS motif and Chimeric VH peptides were tested for ss and ds DNA reactivity as described in Section 2.4.5 and the results are shown in Figure 3.23. KIM4.6 VH bound to both ss and ds DNA whereas the Chimeric VH which contains the Variants' DJ regions instead of KIM4.6 DJ did not bind to ss nor ds DNA. The YYGS deleted KIM4.6 VH had a significant (>50%) reduced binding to ds DNA when compared with the wild type KIM4.6 VH

Table 3.8

Radioactive amino acid % incorporation of STP derived peptides[1]

	% incorporation[2]
KIM4.6 VH	11.6
YYGS deleted KIM4.6 VH	11.2
Chimeric VH	8.8
β-galactosidase	17.6
No DNA added tube	0.0

[1] Radioactive amino acid incorporation assay has been described in Section 2.4.3.

[2] % incorporation has been calculated as described in Section 2.4.3.

Figure 3.22 SDS-PAGE of STP-translated VH peptides. KIM4.6 VH (Lane 1), YYGS deleted KIM4.6 VH (Lane 2) and Chimeric VH (Lane 3) peptides which were labeled with ^{35}S methionine, are separated by SDS-PAGE to confirm the production of the expected size peptides.

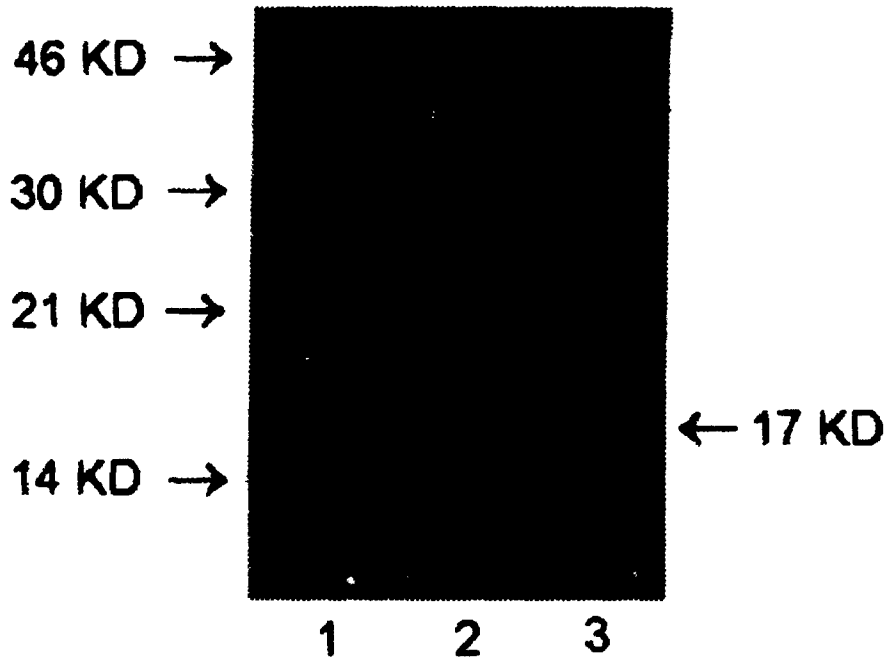
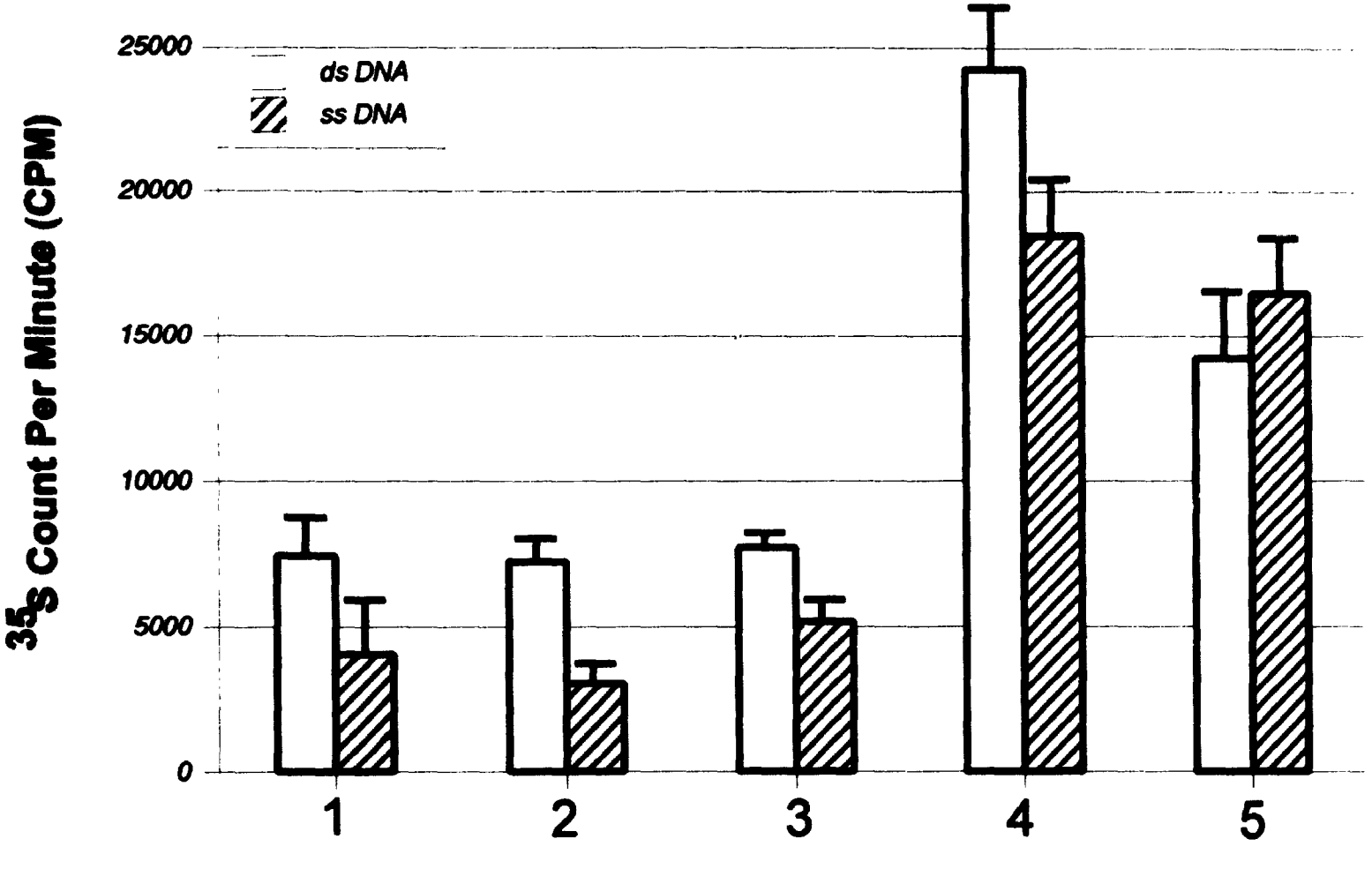

46 KD →
30 KD →
21 KD →
14 KD →
← 17 KD
1
2
3

Figure 3.23 Reactivity of STP-translated peptides with ss and ds DNA. Lane 1 represents the negative control which had no DNA template added to the STP system. Lane 2 shows the counts for STP-derived β-galactosidase peptide. Lane 3 represents the counts for STP-derived Chimeric VH. Lane 4 shows the binding of KIM4.6 VH. Lane 5 represents the binding of KIM4.6 VH without YYGS motif. Results are expressed as mean and standard error (SE). In each lane binding for ss and ds DNA are shown by cross hatched and open columns respectively.


25000
20000
15000
10000
5000
0
ds DNA
ss DNA
35S Count Per Minute (CPM)
1
2
3
4
5

($P = 0.001$). The ss DNA binding values of the KIM4.6 VH and the YYGS deleted KIM4.6 VH do not show a significant difference ($P = 0.582$).

Section 3.5 Cloning of KIM4.6 VH gene in pComb3 phagemid vector

Despite numerous attempts, cloning of KIM4.6 VH DNA fragment into "pComb3 + V3 VL" (V3VL and KIM4.6 VL sequences are identical) was not successful. There are several possible explanations for this problem: 1) Anti-DNA KIM4.6 Fab is lethal for the bacterial cells expressing it, 2) The mRNA of KIM4.6 VH interferes with cell machinery, 3) KIM4.6 VH gene is not compatible for cloning into the pComb3 vector. The success in cloning the Chimeric VH (KIM4.6 VH segment + V3 DJ) led to a more concerted effort to clone the KIM4.6 DJ segments. The reading frame of KIM4.6 VH gene was changed at the beginning of the DJ segment and at the beginning of the V region. Attempts to clone both of these new constructs were not successful. This suggests that a problem exists prior to protein expression and due to either mRNA of KIM4.6 corresponding to the DJ segment and/or to the incompatibility of the sequence of this region with the flanking sequences in the vector. This was confirmed by showing that the KIM4.6 VH gene could be cloned in the reverse orientation in pComb3 vector where both the mRNA produced and the orientation of cloning of the KIM4.6 VH gene have been changed. However, the possible lytic effect of KIM4.6 DJ peptide through binding to exposed bacterial DNA cannot be excluded.

Chapter 4

Discussion

The current study has explored the structural basis for DNA binding of human anti-DNA antibodies and in particular has focussed on the structural basis for DNA binding of the natural monoclonal IgM anti-DNA antibody KIM4.6. The hypothesis that the YYGS motif in KIM4.6 CDR3 is directly responsible for its DNA binding property is addressed in these experiments.

Nucleotide sequence analysis of non-DNA binding Variants pointed to the importance of KIM4.6 heavy chain in its DNA binding since the loss of DNA reactivity in the Variants occurred despite the presence of the KIM4.6 light chain. Phage expressed Chimeric VH (KIM4.6 V and DJ of the Variants) in association with KIM4.6 VL did not bind to ss or ds DNA. This indirectly points to the importance of KIM4.6 DJ region in its binding to DNA. Furthermore, I have shown here that *in vitro* transcribed and translated KIM4.6 VH peptide alone (not associated with VL), bound to both forms of DNA. However, deletion of the YYGS motif from KIM4.6 VH resulted in a significant decrease in KIM4.6 binding to ds DNA but not ss DNA. It is possible that the intercalation of ds DNA bases between the aromatic rings of the tyrosine residues in the YYGS motif and the downstream adjacent tyrosine residues may be responsible for the enhancement in ds DNA binding of KIM4.6. However, the stacking of base pairs of ds DNA between aromatic rings in the anti-DNA antibody combining site has not been yet

investigated.

KIM4.6 VH net positive charge may act as an electrostatic attractant for DNA, since BUD 94.91.8 anti-Sm/RNP antibody which shares VH structures (homologous VH segment, a D region YYDS sequence similar to YYGS motif and tyrosine-rich CDR3 region) with KIM4.6, but differs in VH charge, does not bind to DNA. This is further supported by the presence of cationic VH charges in BUD 45.12.8 and BUD 114.4.11 anti-Sm/RNP antibodies which also bind to ss DNA.

Section 4.1 Analysis of the VH and VL Nucleotide Sequences of the Variant Antibodies

Spontaneous Variants of the anti-DNA antibody producing hybridoma KIM4.6 arose in 1/200 culture wells during KIM4.6 subcloning at 1 cell/well. All three isolated Variants, produced IgMλ and expressed the light chain 4.6.3 Id of KIM4.6. In contrast to KIM4.6, the Variant antibodies did not bind to ss or ds DNA when tested by ELISA at equal to or higher IgM concentration than KIM4.6 anti-DNA antibody.

VL nucleotide sequencing of the three Variant antibodies revealed that except for a single nucleotide difference in Variant #6 (V6) which is silent at the amino acid level, this region in all non DNA-binding Variants was identical to the VL of KIM4.6 DNA-binding parent. This indicates that the λ light chain of KIM4.6 (*Humlv117* and Jλ3) which expresses the 4.6.3 Id is not directly responsible for binding to DNA. This agrees with the previous observation that the 4.6.3 Id+, λ1

light chain from the BL2 cell line does not bind DNA (Cairns 1989b). Moreover KIM4.6 is the only reported monoclonal anti-DNA antibody which expresses this light chain. However, it cannot be excluded that the *Humlv117* λ1 light chain when combined with the Variants' VH chain but not KIM4.6 VH chain, inhibits the DNA binding ability of the Variant antibody. Such modification of the binding site could for example include hydrogen bonding between the VH and VL chain preventing DNA access to the cationic VH CDR3 of the Variants. It has been reported that the disruption of the single hydrogen bond between VH and VL chains of the murine S107 anti-phosphocholine antibody (a dominant response in BALB/c mice to immunization with pneumococcal polysaccharide) resulted in the conversion of this antibody to an anti-DNA antibody (Giusti 1987). This was a consequence of one amino acid substitution (an alanine for glutamic acid) in VH CDR1 which could have permitted exposure of the S107 VH CDR3 region containing the YYGS motif, to DNA.

The spontaneously arising Variant hybridomas described here expressed a different VH chain than their parent KIM4.6. The VH chain in the Variants is encoded by VH4.21, a unique D and JH5 genes. KIM4.6, however is encoded by VH1.9III, DXP'1 and JH6 (Cairns 1989a). The Variants VH chain genes are also different from the V71-2, DK1 and JH4 genes used in the KIM4.6 hybridoma fusion partner GM 4672 (Denomme 1993). The possibility that the Variants originated from a different fusion event is highly unlikely for two reasons: 1) the monoclonality of KIM4.6 hybridoma parent has been previously demonstrated (Cairns 1984) and

2) the VL region genes of the Variants including their V/J junction are identical to KIM4.6 VL. The molecular mechanisms responsible for the generation of the new VH chain in KIM4.6 is unknown. VH gene segment replacement in the VDJ gene by a 5' VH segment gene (Reth 1986) cannot be under consideration since the KIM4.6 VH as well as the D and J segment have been replaced. A new VDJ rearrangement on the same allele is unlikely since according to the map of the human IgH chain locus (Ravetch 1981, Sato 1988, Walter 1990 and 1991 and Pascual 1991b) the D genes and the JH5 gene should be deleted on the allele which underwent VH1.9III, DXP'1 and JH6 rearrangement in KIM4.6. The only plausible explanation for the observed change in the KIM4.6 VH region, therefore is that the new rearrangement of VH4.21, with a unique D and JH5 in the Variants took place on the previously excluded allele in the KIM4.6 hybridoma. Berinstein et al have shown that a mature B cell can rearrange a second light chain gene at a different allele even though it had originally produced a functional surface Ig product (Berinstein 1989).

It is very interesting that the VH chains in the Variants were found to be replaced by VH chains encoded by the VH4.21 germline gene which has been reported to be preferentially rearranged in 10.8% bone marrow B cells (Pascual 1992b). The ten fold increase in usage of VH4.21 by bone marrow B cells compared to the expected use has been speculated to be, a result of either its chromosomal location, and/or accessibility of VH4.21 to the recombinase machinery, or a result of antigen selection. It is not clear whether the VH region

switch in KIM4.6 Variants is a consequence of any of the above phenomena or has occurred by chance.

The VH4.21 gene was initially found to be utilized by cold agglutinin antibodies and it has been shown that even in the absence of somatic mutation the VH4.21 gene segment can encode anti-i and anti-I specificities (Pascual 1991a and 1992a). The VH segments of two cold agglutinin antibodies, FS-5 and FS-7, are identical to VH4.21 germline gene (Pascual 1992a). The D region of FS-5 is encode by a D21-9 germline gene while the D region of FS-7 is unique. The JH segments of FS-5 and FS-7 are both encoded by JH4 germline gene. The VL chains of FS-5 and FS-7 are encoded by Vκ I K16 and Vκ I HK102 germline genes respectively. The Variant antibodies, however, did not have cold agglutinin activity (Table 3.3), possibly because of a difference in the D and/or VL regions of these antibodies compared to FS-5 and FS-8.

Five of the 41 sequenced human anti-DNA antibodies reported are encoded by a VH4.21 germline gene (Table 1.2). Some characteristics of five VH4.21 encoded anti-DNA antibodies are compared with our non DNA-binding Variants in Table 3.5. Interestingly the VH segment of three of these anti-DNA antibodies are identical to the VH4.21 germline gene. The VH CDR3 of these five anti-DNA antibodies in contrast to the Variants, however, are enriched in arginine and/or tyrosine residues.

Radic et al using mutagenesis experiments on a murine anti-DNA antibody, have shown that substitution of an arginine by a glycine in the CDR3 heavy chain

resulted in the loss of DNA binding of the parent antibody. This change in reactivity could be due to a conformational shift in CDR3 or result from a change in the electrostatic interaction between the antibody and DNA (Radic 1993). It has been proposed that arginine residues resulting from somatic mutations in VH (in T14 and D5) or VH CDR3 arginine residues (RT79, NE-1 and NE-13) play an important role in DNA specificity (van Es 1991, Stevenson 1993 and Hirabayashi 1993). However, the enrichment in another basic amino acid, lysine, which also provides a considerable VH CDR3 positive charge but less hydrogen-binding potential than arginine, in the Variants does not result in DNA binding.

An analysis of the nucleotide sequence of non DNA-binding Variants provides evidence that the VL chain of the KIM4.6 anti-DNA antibody is not sufficient for its property of DNA binding. This implies that KIM4.6 VH chain and possibly its D region play a key role in DNA binding of this antibody. A comparison of the VH of the Variants to the VH of VH4.21 derived anti-DNA antibodies provides further evidence for the importance of arginine residues in DNA specificity of this group of antibodies, since there is an increase in VH arginine residues in these VH4.21 derived anti-DNA antibodies compared to the VH4.21 of the Variants which lost DNA binding.

Section 4.2 Analysis of the Ig V Region Nucleotide Sequences of Anti-Sm/RNP Antibodies

The Ig V region genes of three human monoclonal IgM anti-Sm/RNP

antibodies (BUD 45.12.8, BUD 94.91.8 and BUD 114.4.11) were sequenced. There are reports on the V region sequence of only four other human anti-Sm antibodies: 4B4 (Sanz 1989 and Dang 1993), A73 (Young 1990), RSP-1 and RSP-4 (Isenberg et al, personal communication).

Table 3.6 summarizes some characteristics of these BUD clones. Overall the VH chains of six out of seven sequenced human anti-Sm antibodies are encoded by genes of the VH III family. The VH chain of BUD 114.4.11 is the only known example of a VH chain of a human anti-Sm antibody which is encoded by a non VH III gene family. Further sequence studies of human anti-Sm antibodies will be required to determine whether the high frequency of VH III genes in these antibodies is significant.

BUD 45.12.8 and BUD 114.4.11 which both bind to ss DNA in addition to Sm/RNP have positive VH and VL net charges which may facilitate their reactivity to negatively charged ss DNA molecules.

BUD 45.12.8 VL chain is encoded by *Humkv325* and Jκ2 germline genes. *Humkv325* germline gene has been shown to be associated with IgM rheumatoid factor antibodies (Chen 1989). BUD 45.12.8 does not have rheumatoid factor activity when tested by ELISA (Carruthers 1991). BUD 45.12.8 VL contains two amino acid substitutions (valine -> phenylalanine in CDR1 and tyrosine -> glutamine at the VJ junction) which can not be found in the *Humkv325* encoded antibodies with rheumatoid factor activity (Kipps 1989 and Ezaki 1991). *Humkv325* is also used by eight anti-DNA antibodies (Table 1.2). The *Humkv325* VL segments

in all of these latter anti-DNA antibodies (3 IgM, 2 IgG and 3 IgA isotype) are somatically mutated. Four out of eight of these antibodies (2 IgG and 2 IgA) contain at least one somatic mutation to an arginine residue in their *Humkv325* VL segment.

BUD 45.12.8 VH chain is encoded by *VH26*, DXP4 and JH4b germline genes. The *VH26* germline gene has been used by 2 other anti-Sm antibodies (A73 and RSP-1) which contain several somatic mutations in this segment (Figure 3.9). The amino acid substitutions in these two anti-Sm antibodies does not seem to have a specific pattern.

The VH regions of eight other published anti-DNA antibodies are also encoded by a *VH26* germline gene. The VH segments of three of these anti-DNA antibodies (18/2, 1/17 and B19.7) are identical to the VH segment of BUD 45.12.8. The VH amino acid sequence of these antibodies are compared to the VH amino acid sequence of BUD 45.12.8 in Figure 3.10. The major difference in the VH chain of these antibodies resides in their CDR3 region. The CDR3 regions of BUD 45.12.8 and B19.7 anti ss-DNA antibodies contain one arginine residue and at least one other basic amino acid lysine. The CDR3 regions of 18/2 and 1/17 anti-ss and -ds DNA antibodies which are encoded by the DXP'1 germline gene lack any arginine or lysine residues but are enriched with tyrosine residues and also express the YYGS motif. This points out that in this group of anti-DNA antibodies the VH CDR3 plays an important role in DNA binding. Three different mechanisms may possibly explain the DNA reactivity: 1) the presence of arginine and lysine residues; 2) the expression of the YYGS motif; and 3) the presence of tyrosine

residues.

The BUD 114.4.11 VL chain is encoded by *Humk18* and Jκ3 germline genes. The *Humk18* germline gene also encodes the VL chain of an IgG anti-ds DNA antibody, H2F (Manheimer-Lory 1991). The BUD 114.4.11 VL segment is identical to the *Humk18* germline gene except for two substitutions in the last codon which results in an alanine for proline replacement. The H2F VL segment shows a 98% identity with the *Humk18* germline gene. An arginine for phenylalanine replacement in VL CDR3 of H2F compared to BUD 114.4.11 seems to be the most important difference between these two light chains.

BUD 114.4.11 VH chain is encoded by *hv1l1*, DK1 or DM1 and JH4b germline genes. BUD 114.4.11 is the only anti-Sm antibody whose VH is not encoded by a VH III family gene. Further the *hv1l1* germline gene has not been reported to be used by any anti-DNA antibody. BUD 114 4.11 has a 5 amino acid D region which can be assigned to DK1 or DM1 germline genes. Neither the DK1 nor DM1 germline gene can account for the VH CDR3 arginine residue (Figure 3.16) which possibly is a result of N nucleotide additions and/or somatic mutations. BUD 114.4.11 has a considerably high positive VDJ net charge which may possibly facilitate its DNA reactivity.

BUD 94.91.8 VL chain is encoded by *hsiggll150* and Jλ2/3 germline genes. The VL segment of BUD 94.91.8 is identical to *the hsiggll150* germline gene except for the three nucleotide introduced by the λ1 framework primer. This germline gene has been reported to be used by four IgM rheumatoid factors monospecific for

rabbit IgG: H4 (Wong 1993), LBR150, PHB1213 and LBR271 (Fang 1994). The VL segments of LBR150, PHB1213 and LBR271 antibodies are identical to *hsiggII150* germline gene. The H4 VL segment contains a tyrosine -> cysteine substitution in the FW2 region (Figure 3.12). The major difference between BUD 94.91.8 VL and the VL chains of these antibodies is in their VJ junction and/or their J region. In contrast to these antibodies, BUD 94.91.8 has been found to react with human IgG by ELISA (Tables 3.2 and 3.6). Since there is little difference between the VL chain of BUD 94.91.8 and the VL chains of these antibodies and since there is no specific pattern in their VJ junctions, it seems likely that the VH of BUD 94.91.8 is responsible for its anti-human IgG specificity.

The VH chain of BUD 94.91.8 is encoded by *hv3019b9*, DXP4 and JH6 germline genes. The *hv3019b9* germline gene is a 1.9III-like gene (Olee 1991). The 1.9III VH germline gene segment encoding for KIM4.6 anti-DNA antibody differs from the VH segment of BUD 94.91.8 by two amino acids: a serine in the CDR2 region and a lysine in FW3 region of KIM4.6 VH segment are substituted by a tryptophane and an arginine in the BUD 94.91.8 VH segment (Figure 3.14). The lysine -> arginine replacement is expected to favor DNA reactivity. The tryptophan residue in the CDR2 region of BUD 94.91.8 VH region is intact in *hv3019b9* encoded VH of IgG anti-DNA antibody 19.E7 (Winkler 1992) and possibly does not have an inhibitory effect on DNA binding. As well as having closely related VH segments, KIM4.6 and BUD 94.91.8 JH regions have identical JH6 encoded J regions. The major difference between these two antibodies is in their D region.

The D region of KIM4.6 is encoded by the DXP'1 germline gene (Cairns 1989a) and contains the YYGS motif while BUD 94.91.8 is encoded by the DXP4 germline gene and contains a YYDS motif-like sequence. The VH CDR3 regions of both these antibodies are also further enriched in tyrosine residues (8 in KIM4.6 and 9 in BUD 94.91.8).

The lack of basic amino acids in BUD 94.91.8 VH CDR3 region and the presence of two aspartic acid residues result in a considerable negative VH CDR3 net charge (-3.08 at pH 7.5) in this antibody . The net charge of VH CDR3 of KIM4.6 antibody is 0.92 at pH 7.5. The VDJ net charges of BUD 94.91.8 and KIM4.6 at pH 7.5 are 0.73 and 4.70 respectively. The net negative charge of BUD 94.91.8 VL region (-2.35 at pH 7.5) result in an overall negatively charged Fv in this antibody while the Fv of KIM4.6 is cationic. This difference in net charge may explain the different DNA binding property of these antibodies.

Molecular analysis of DNA-binding KIM4.6 and non DNA-binding BUD 94.91.8 antibodies points to the importance of the cationic VH CDR3 region of KIM4.6 in DNA-binding. It seems that the enrichment in tyrosine residues and/or the presence of the YYDS motif-like sequence of BUD 94.91.8 VH CDR3 cannot in the absence of a positive electrostatic potential confer DNA specificity. Radic et al have shown that replacement of an arginine by a glycine at position 96 in the VH of the 3H9 murine anti-DNA antibody results in a loss of DNA binding (Radic 1993). They have suggested that since this replacement results in the loss of positive electrostatic potential, the change in the electrostatic interaction between the

antibody and DNA may be significant enough to reduce the DNA binding to below the detectable level (Radic 1993).

It is also possible that the arginine residues in KIM4.6 VH CDR3 region directly interact with DNA. It has been suggested the arginine residues may interact with the phosphate of the DNA backbone or form hydrogen bonds with guanine and cytidine groups in DNA (Seeman 1976).

Section 4.3 Expression of the Fab Molecules on Phage Surface "A tool for study of the structural basis for anti-DNA antibody specificity"

Altered proteins can be studied by manipulating the genes, expressing the peptides in an organism and screening for new properties. These protein engineering techniques have been used to explore the structure and function of proteins; for example for identification of the amino acid residues involved in catalytic functions (Winter 1982), or identification of the residues which play an important role in protein folding (Matouschek 1990).

It has also been shown that folded antibody fragments can be displayed on phage. The antibody molecules can be displayed as single chain Fv fragments, in which VH and VL chains are connected by a flexible spacer (Huston 1988) or as Fab fragments, in which one chain is fused to cpIII or cpVIII and the other chain is secreted into the periplasm (Barbas III 1991a and Hoogenboom 1991).

The pComb3 phagemid expression system was employed in this study for

expression of wild type and manipulated antibody genes. This phagemid system has been designed for monovalent display of combinatorial Fab libraries on the surface of filamentous phage M13 (Barbas III 1991a). It has been shown that the expression of antibody Fab molecules in the pComb3 system can be used to isolate antibodies to different self and non-self antigens (Barbas III 1991b, Barbas III 1992b, Barbas III 1992c, Zebedee 1992 and Portolano 1993). This system has also been used for random mutagenesis of the cloned heavy and light chain genes and subsequent selection of the clones with improved affinity for the progesterone-3-oxim-BSA hapten conjugate (Gram 1992).

In this study, three different antibody Fab molecules were separately expressed on the phage surface. The VL chains of these three Fab products were the product of the *Humlv117* /JI3 genes, identical to the VL chains of KIM4.6 wild type and the Variants. This VL chain was expressed in association with three different VH: 1) Variants (V3) VH to reconstitute a non DNA-binding Fab in this system, 2) Variants (V3) VH containing a YYGS motif sequence in the D region, 3) Chimeric VH.

The YYGS was inserted between two lysine residues of the wild type V3 VH (Section 3.3.4) for two reasons: 1) basic lysine residues provide a local positive electrostatic potential and 2) since these lysine residues and subsequently the YYGS insertion is located in the middle of the VH CDR3 loop, YYGS will likely have the least effect on the CDR3 region conformation.

The Chimeric VH as described previously (Section 3.3.3) contains the

KIM4.6 VH segment (except for two substitutions in FW3: aspartic acid -> leucine and lysine -> arginine) and the DJ of the Variants (V3).

The expression of these three Fab molecules was confirmed by ELISA (Section 3.3.5 and Table 3.7). However, none of these Fab molecules bound to ss or ds DNA. The lack of DNA-binding by the Chimeric VH + KIM4.6 VL Fab which contains all the components of KIM4.6 wild type Fab except for its DJ region provides additional evidence for the importance of the DJ region in the DNA specificity of KIM4.6. It is noteworthy to mention that even the lysine -> arginine substitution is expected to favor the DNA specificity.

The failure of the V3 VH with the YYGS insertion + KIM4.6 VL Fab to react with ss or ds DNA demonstrates that the insertion of the YYGS motif in this location of V3 VH is not sufficient for binding to DNA.

To obtain a positive control for DNA reactivity in the phage expression system cloning of KIM4.6 VH DNA fragment into "pComb3 + KIM4.6 VL" was attempted. Despite numerous attempts this cloning was not successful. This therefore limits the interpretation of the results on DNA binding properties of the expressed Fabs.

Apparently other investigators have had the same problem of cloning genes from the VH III family. These problems may be solved by using the new generation of pComb 3 phagemid vector (Silverman 1994, personal communication).

Section 4.4 In Vitro Transcription and Translation

"An alternative tool for production of wild type and manipulated antibody VH chains"

Original and altered forms of KIM4.6 VH were synthesized in a coupled in vitro transcription and translation system. Transcription of cDNA clones by bacteriophage T7 RNA polymerase coupled to translation in the micrococcal nuclease treated rabbit reticulocyte lysate has been shown to result in a high fidelity and efficiency synthesis of proteins which are in a biologically active form (Craig 1992).

Based on the previously discussed findings that the VH chain and in particular the D region play the major role in DNA reactivity of KIM4.6, this part of the study was designed to examine the role of KIM4.6 VH and its D region YYGS motif in DNA-binding. In this study, three different VH chains were produced using in vitro transcription and translation: 1) KIM4.6 wild type VH, 2) Chimeric VH, and 3) KIM4.6 VH without the YYGS D region motif. The YYGS motif is located in a considerably long (26 amino acids) CDR3 loop of KIM4.6 VH (Figure 3.14) and its removal probably does not result in a significant conformational change in the KIM4.6 VH.

KIM4.6 VH, KIM4.6 VH without the YYGS motif and Chimeric VH in vitro generated peptides were tested for ss and ds DNA reactivity. The ability of KIM4.6 VH to bind to both ss and ds DNA provides additional indirect evidence for the importance of KIM4.6 VH in conferring DNA specificity. The possible influence of

the KIM4.6 VL in the reduction or enhancement of DNA binding of KIM4 6 VH remains to be studied. The Chimeric VH which contains the Variants' DJ regions instead of KIM4.6 DJ did not bind to ss nor ds DNA. This confirms the result of the ELISA for DNA specificity of phage surface expressed Chimeric VH + KIM4.6 VL Fab molecules and again points to the importance of the KIM4.6 DJ region in DNA reactivity.

The fact that the KIM4.6 VH without the YYGS motif exhibited a significant reduction (>50%, $P = 0.001$) of binding to ds DNA when compared with the wild type KIM4.6 VH demonstrates that the YYGS motif is an essential but not the only structural determinant in KIM4 6 responsible for reactivity to ds DNA. This reduction in binding to ds DNA: 1) can be attributed to the absence of the YYGS motif which may interact directly with ds DNA, for instance through the aromatic rings of the tyrosine residues. Noteworthy in this respect is the finding that the crystal structure of an anti-DNA antibody with bound ss DNA ligand showed a thymine base stacked between the aromatic rings of tyrosine and tryptophan residues (Herron 1991). 2) can be a result of a VH CDR3 conformational change which subsequently prevents the accessibility of other important structures (like VH CDR3 arginine residues) to DNA. X-ray crystallographic studies of KIM4 6 antibody should provide a definite answer for these possibilities.

ss DNA binding of the KIM4.6 VH and the YYGS deleted KIM4 6 VH do not show a significant difference ($P = 0.582$). This indicates that different mechanisms may exist for reactivity to ds and ss DNA. It is possible that KIM4.6 VH specificity

for ss DNA is a result of interaction(s) of structure(s) other than the YYGS motif with the epitopes of ss DNA; for example arginine residues may interact with purine or pyrimidine bases (Seeman 1976). However, it is possible that the YYGS motif interacts only with ds DNA specific epitopes. It has been shown that H241, a murine monoclonal anti-DNA antibody can recognize specific base sequences in the major groove of a helical base-paired oligonucleotide (Stollar 1986). These assumptions can explain the observation that removal of the YYGS motif from KIM4.6 VH did not affect its ss DNA binding but significantly reduces its activity with ds DNA.

Section 4.5 Conclusions and Future Directions

This study has demonstrated the importance of the heavy chain and in particular within it DJ region containing YYGS motif in binding to DNA by the human monoclonal anti-DNA antibody KIM4.6. An analysis of the V region gene nucleotide sequences of the non-DNA binding Variants revealed that the KIM4.6 heavy chain confers DNA specificity to this antibody since the loss of DNA reactivity in the Variants occurred despite the presence of the KIM4.6 light chain but with replacement of KIM4.6 heavy chain with a new VDJ rearrangement. The importance of KIM4.6 VH in specificity for DNA was further directly supported by showing DNA-binding of the in vitro synthesized KIM4.6 VH. The fact that phage expressing KIM4.6 Fab molecules whose DJ regions were replaced with the DJ region of the Variants (Chimeric VH), did not bind to ss or ds forms of DNA points

to the importance of these regions of KIM4.6 in DNA-binding

The YYGS motif does not seem to be a sufficient structure for introducing DNA-binding to the non DNA-binding Variant antibodies since grafting the YYGS motif into the D region of phage expressed Variants does not result in reactivity with ss or ds forms of DNA. The presence of a YYDS motif-like sequence in the D region of a non DNA-binding antibody also indicates that in addition to the YYGS motif, other conditions such as a positive electrostatic potential may also be required for DNA reactivity.

However, the finding that the KIM4.6 VH without the YYGS motif had a significant reduction of binding to ds DNA when compared with the wild type KIM4.6 VH demonstrates that the YYGS motif is a necessary but not the sufficient structural determinant in KIM4.6 for reactivity to ds DNA. Since the removal of the YYGS motif does not affect the KIM4.6 VH binding to ss DNA, it is quite possible that the YYGS motif interacts only with ds DNA specific epitopes. Other structures of KIM4.6 CDR3 region such as arginine and/or lysine residues may account for KIM4.6 reactivity with ss DNA.

The finding of the significant contribution of YYGS to ds DNA binding in the natural KIM4.6 antibody invites a speculation that this motif, which is frequently found on pathogenic SLE monoclonal anti-DNA antibodies, may also contribute to their DNA binding properties. This latter speculation, as well as determining the frequency of expression and function of this motif on *in vivo* occurring SLE anti-DNA antibodies, has yet to be investigated.

Sequence analysis of anti-Sm/RNP and anti ss-DNA antibodies BUD 45.12.8 and BUD 114.4.11 indicate that there is more than one molecular mechanism for DNA reactivity of each group of anti-DNA antibodies. In general, the VH CDR3 regions and the presence of residues such as arginine, tyrosine, the presence of YYGS motif in some of the anti-DNA antibody D region and the Fv net charge all appear to be important determinants for DNA specificity.

Expression of the wild type and manipulated anti-DNA antibody genes especially in transgenic mice has the potential to provide valuable information for identification of the necessary structures for DNA-binding. X-ray crystallographic studies to solve individual anti-DNA antibodies with and without their ligand will provide definite answers for many questions and hypotheses involving the structural basis of anti-DNA antibodies for DNA specificity. A better understanding of the origin of anti-DNA antibodies may help in the design and development of new more specific therapeutic strategies for regulating anti-DNA antibody response and disease in SLE.

APPENDIX A

Graphic and full restriction maps of pComb3 phagemid vector

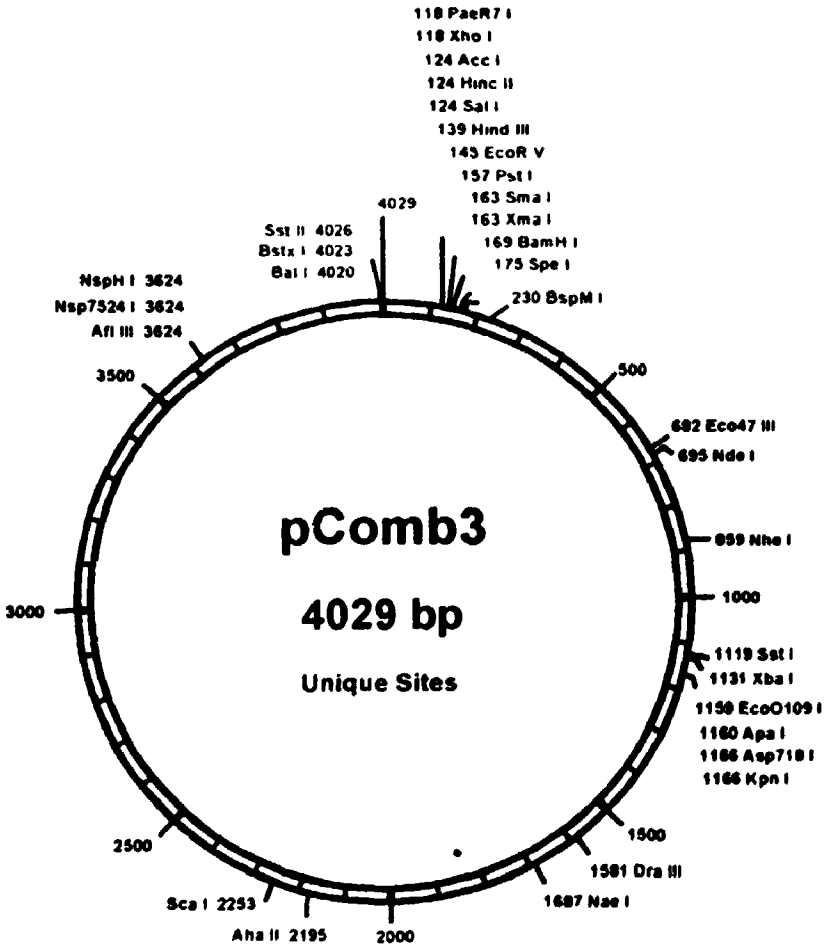
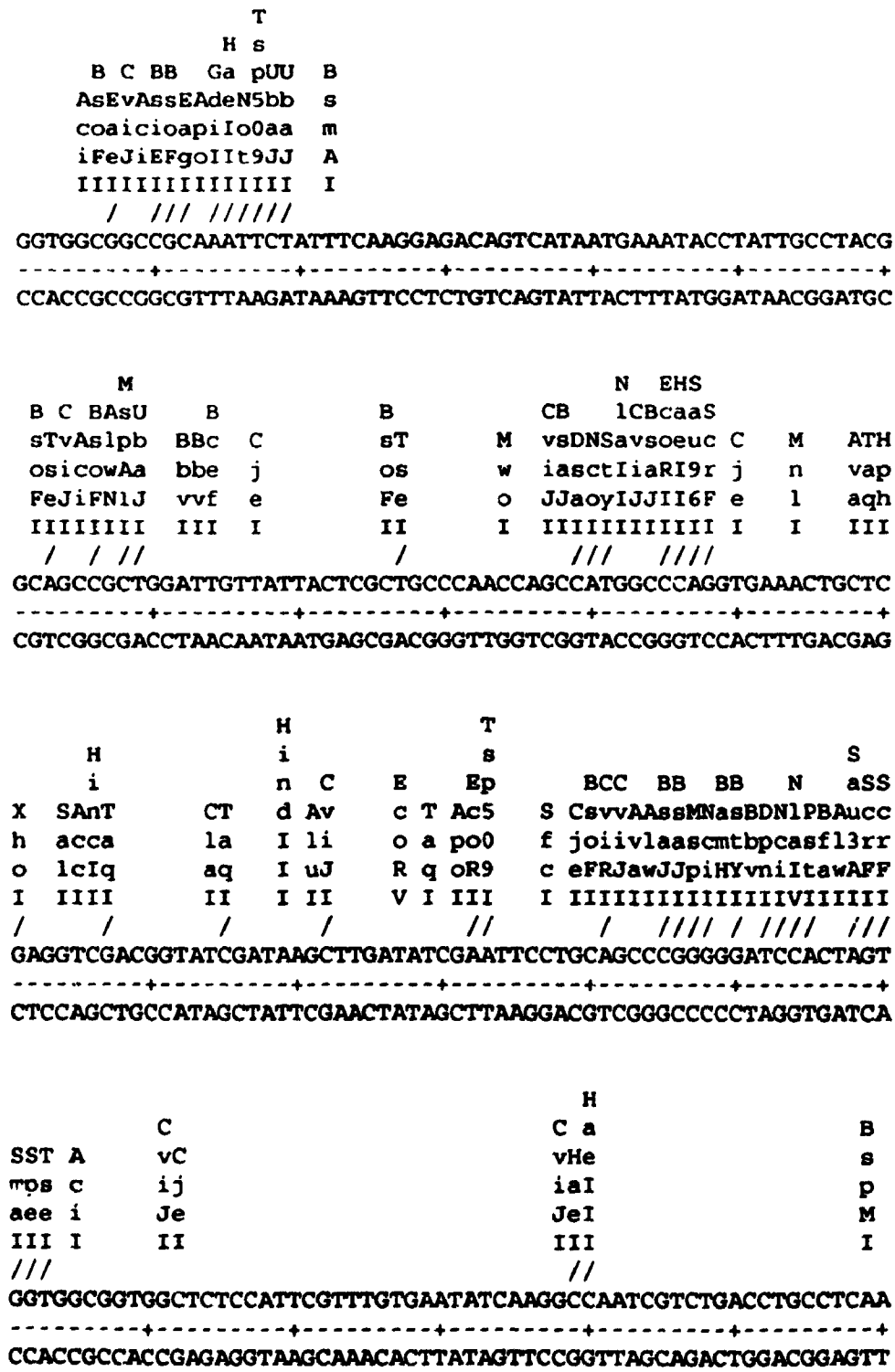
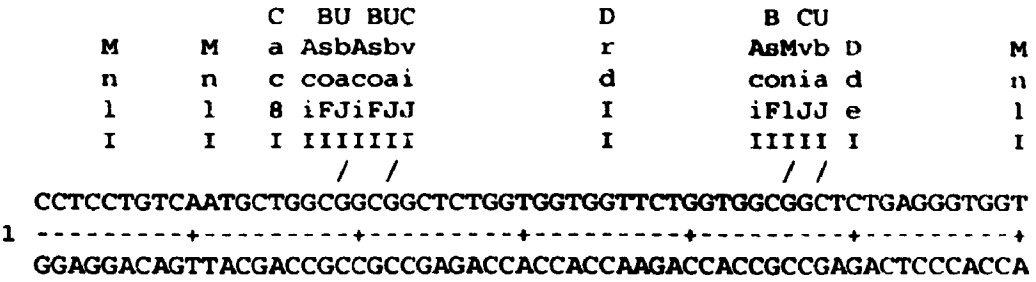


Figure A.1 Graphic map of pComb3 phagemid vector. Unique restriction sites of the vector are shown in this figure. Xho I / Spe I : Ig heavy chain cloning site and Sst I / Xba I : Ig light chain cloning site.

APPENDIX A (continued)

Full restriction map of pComb3 phagemid vector (4029 bp)

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/ / / /
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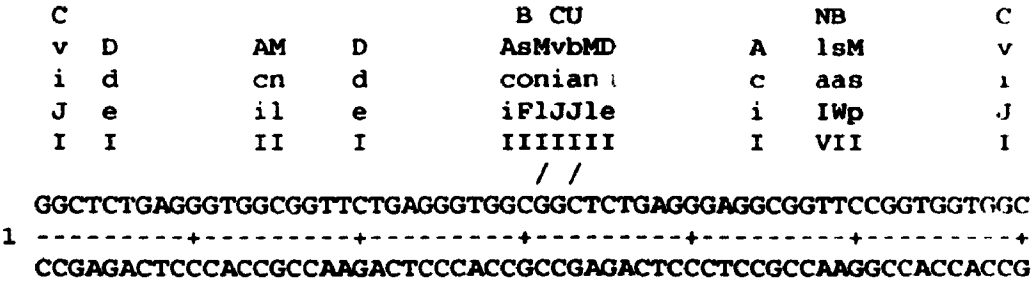
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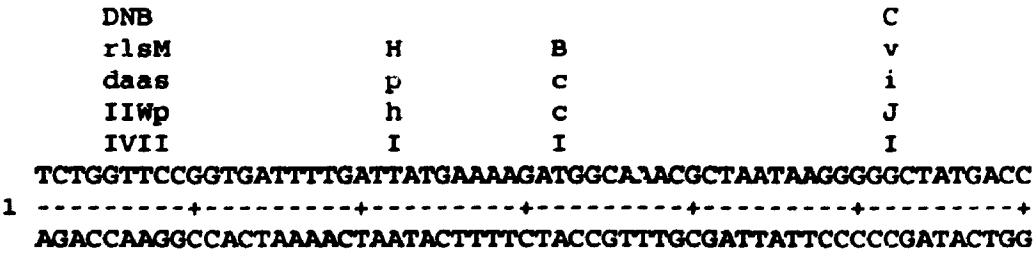
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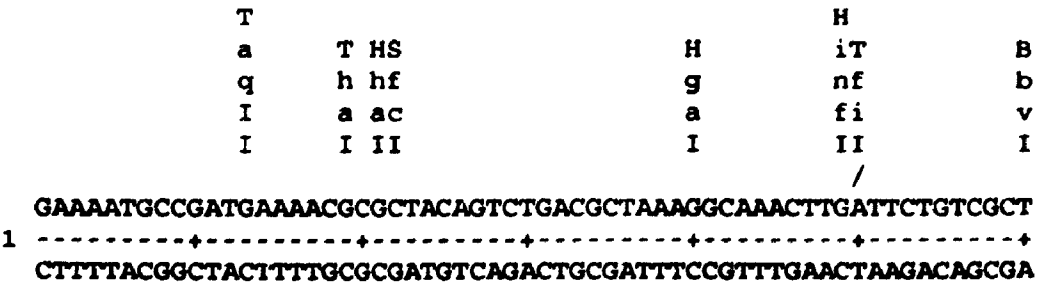
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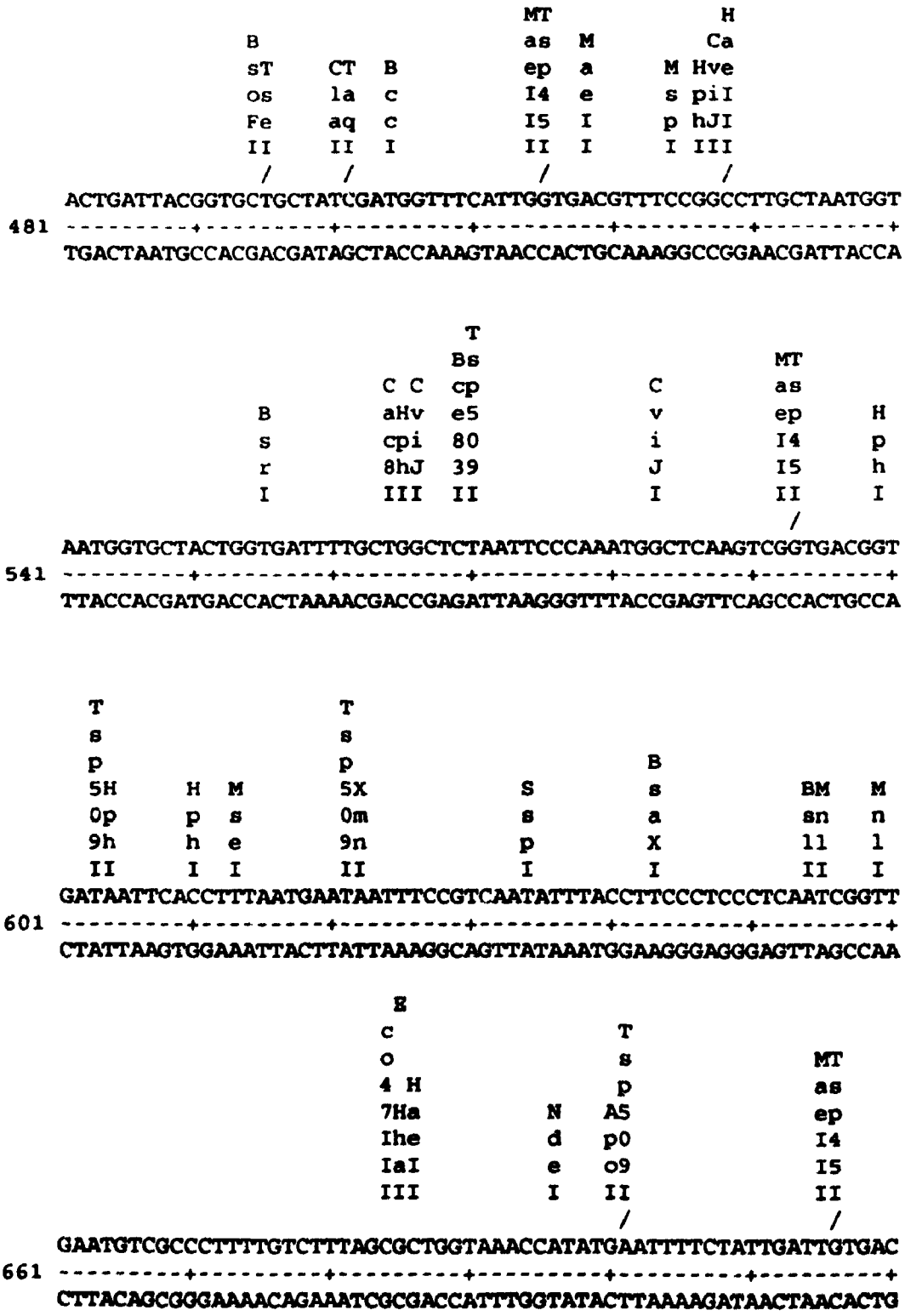
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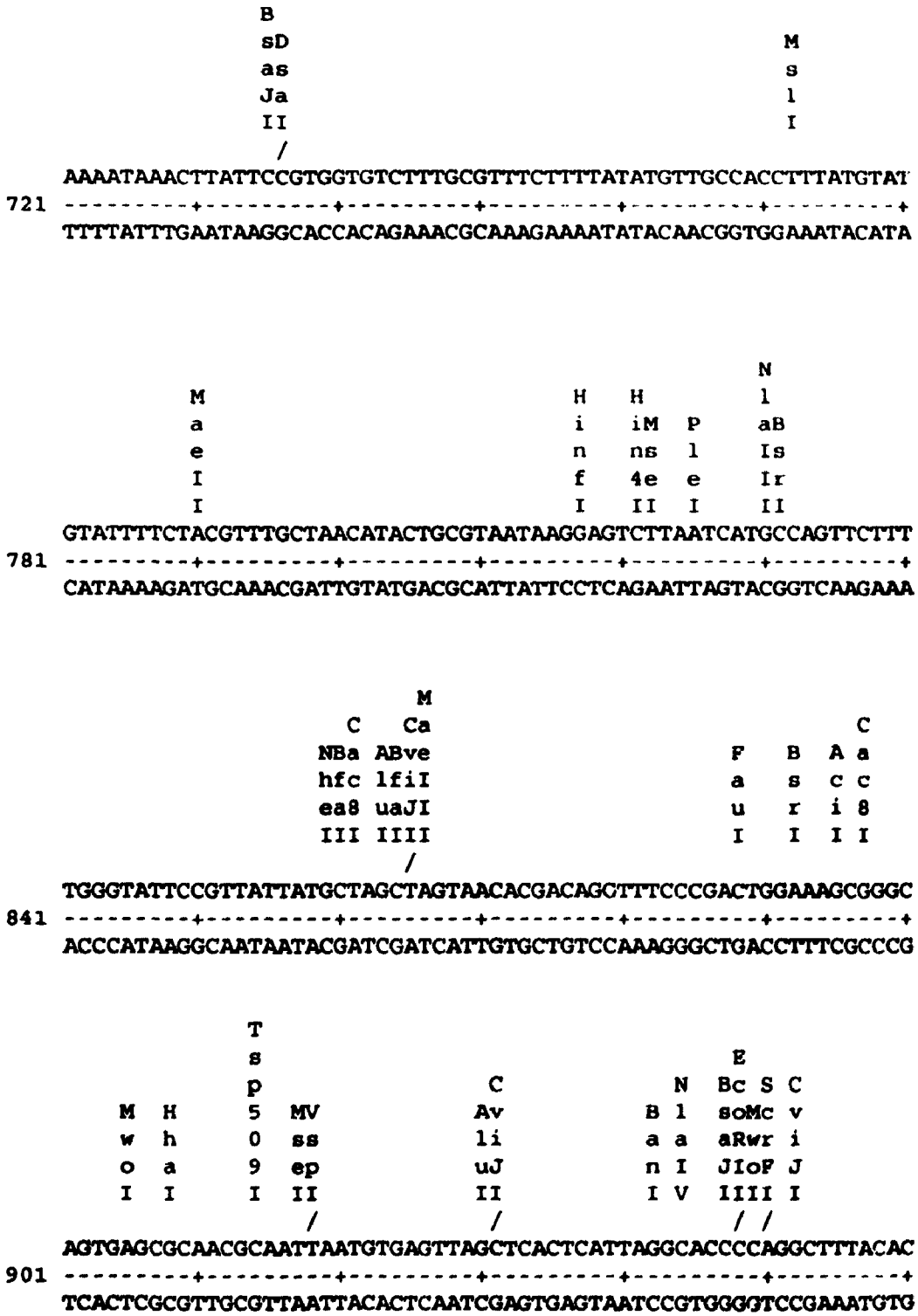
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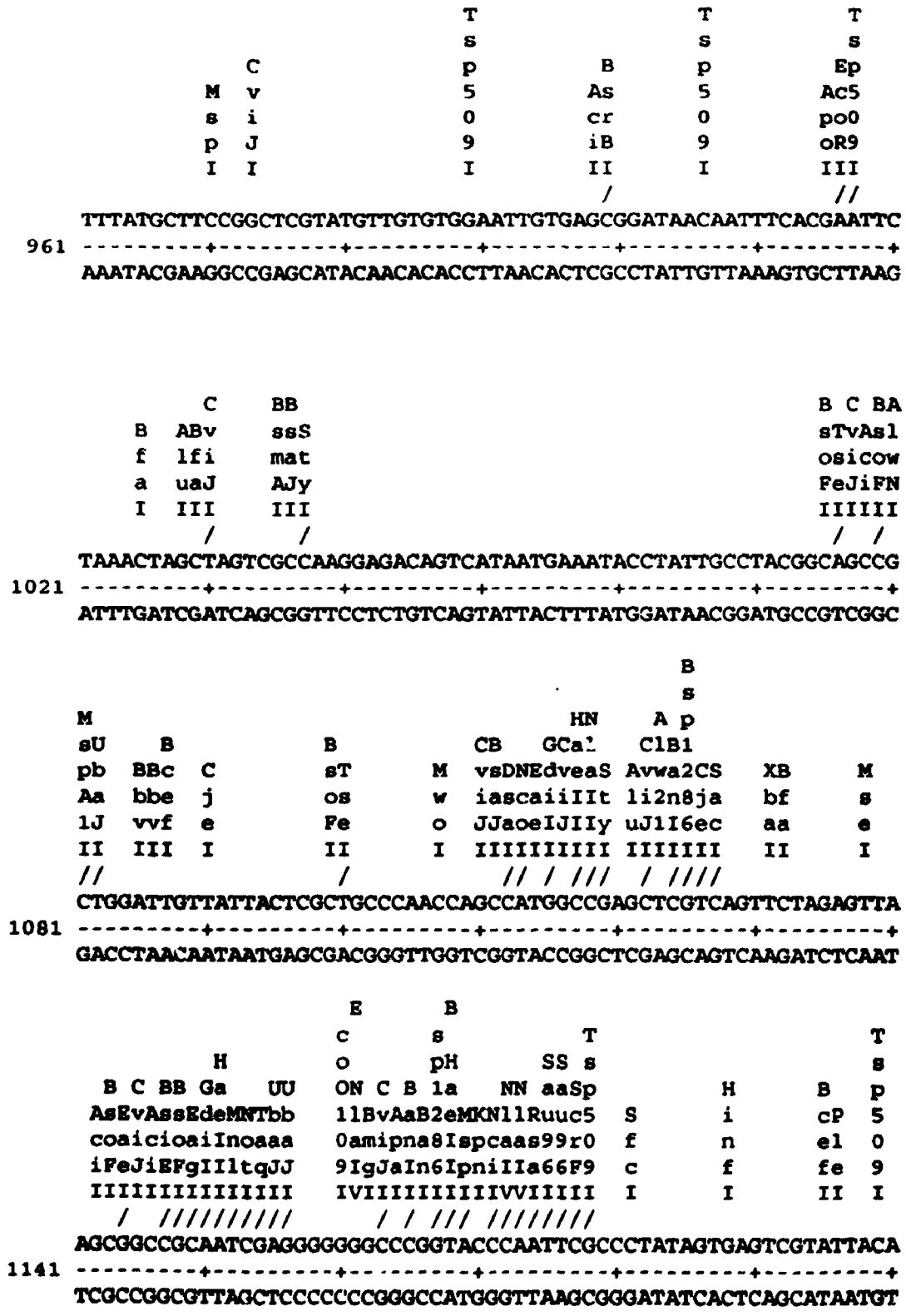
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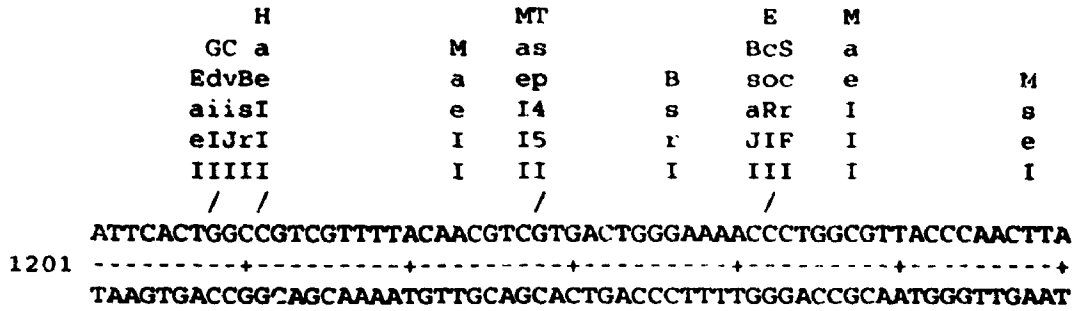
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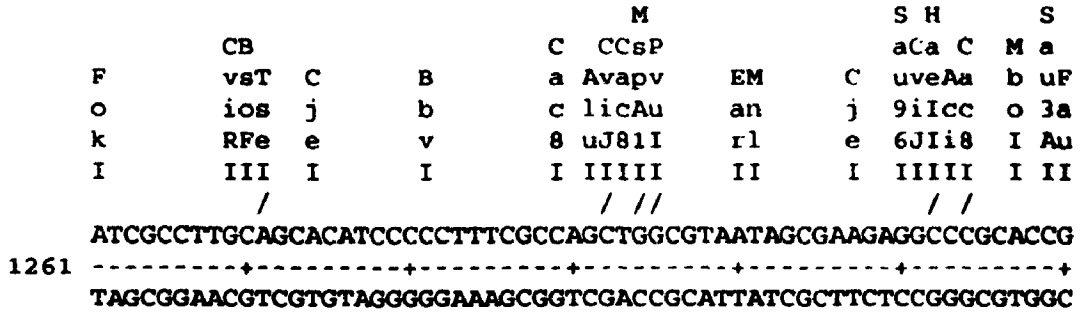
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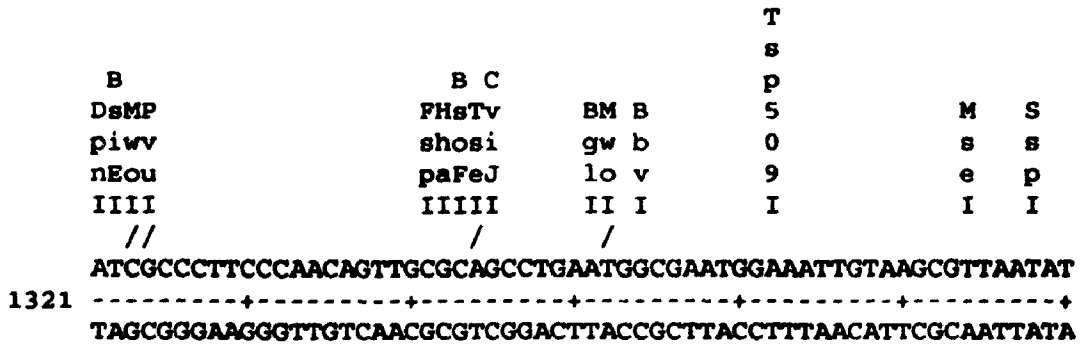
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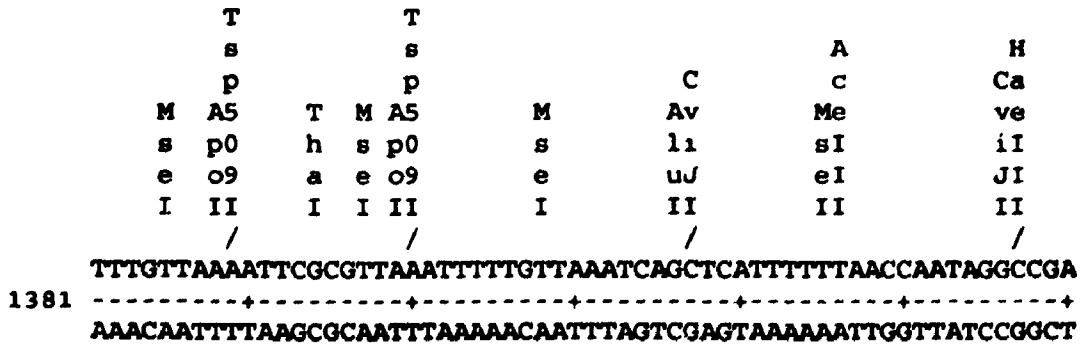
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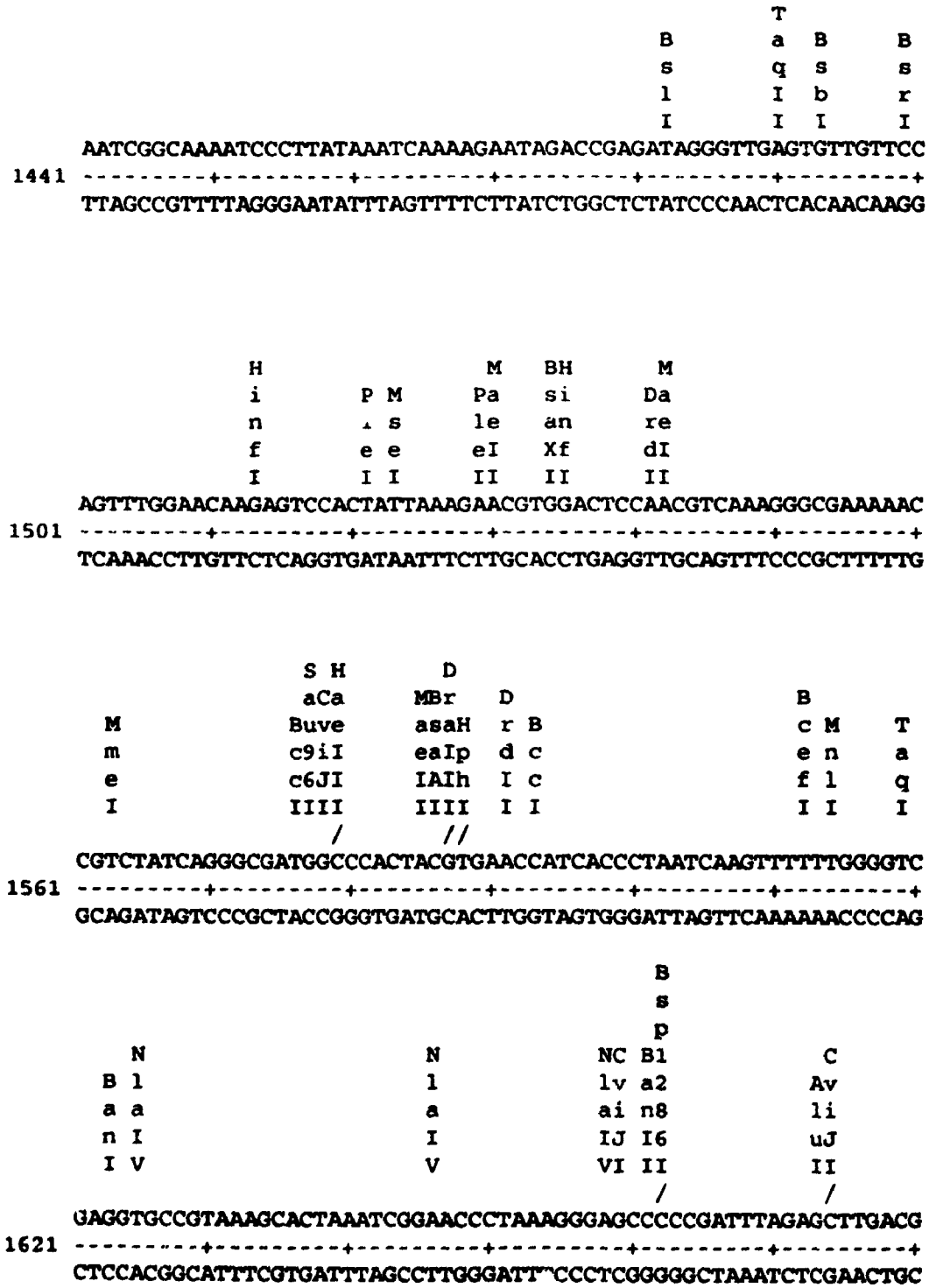
T
B a B B
s q s s
l I b r
I I I I
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H M BH M
i P M Pa si Da
n s le an re
f e e eI Xf dI
I I I II II II
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1501 ---------+---------+---------+---------+---------+---------+
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S H D
aCa MBr D B
M Buve asaH r B c M T
m c9iI eaIp d c e n a
e c6JI IAIh I c f l q
I IIII IIII I I I I I
/ //
CGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTC
1561 ---------+---------+---------+---------+---------+---------+
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B
s
p
N N NC B1 C
B l l lv a2 Av
a a a ai n8 li
n I I IJ I6 uJ
I V V VI II II
/ /
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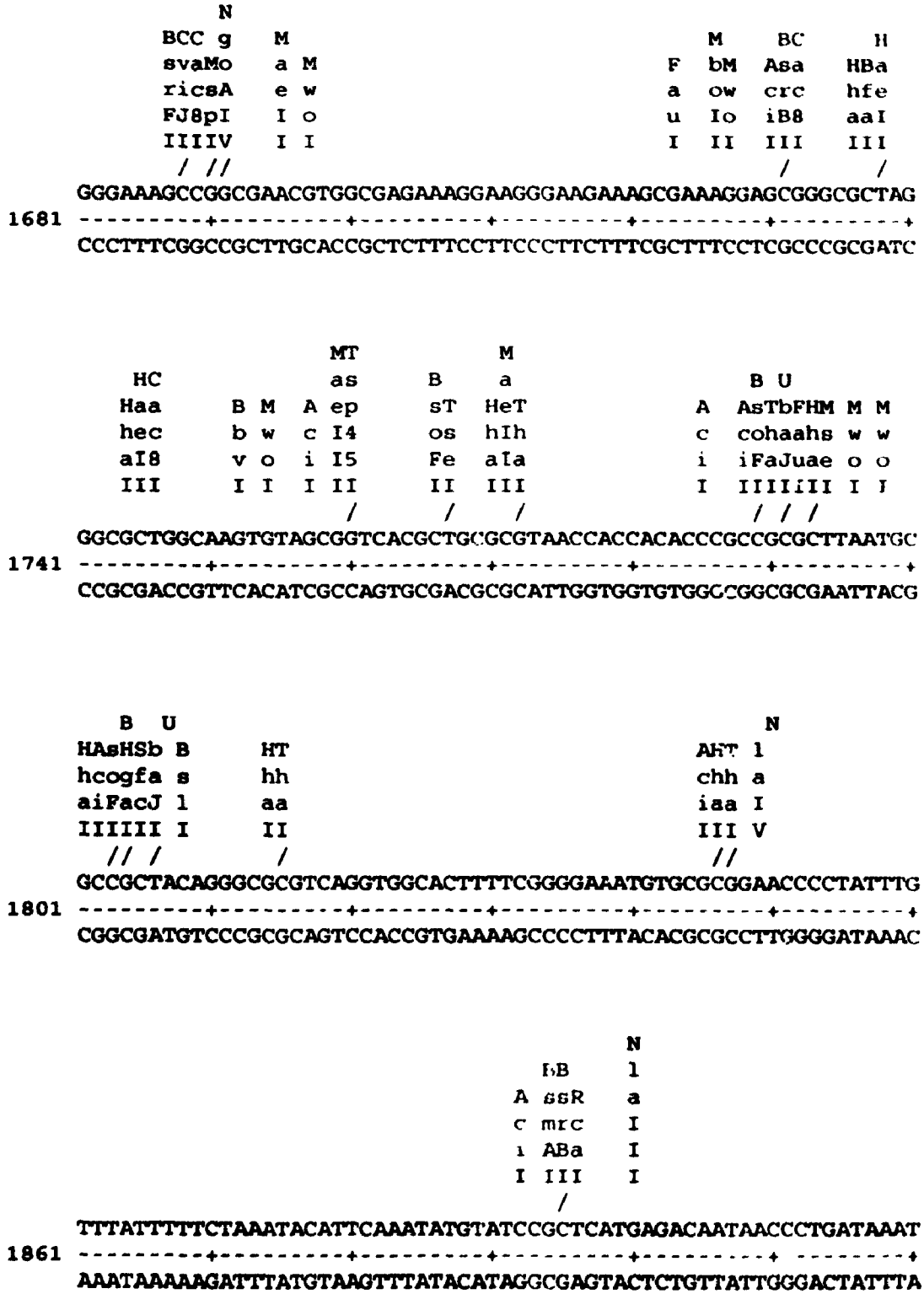
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1801 ---------+---------+---------+---------+---------+---------+
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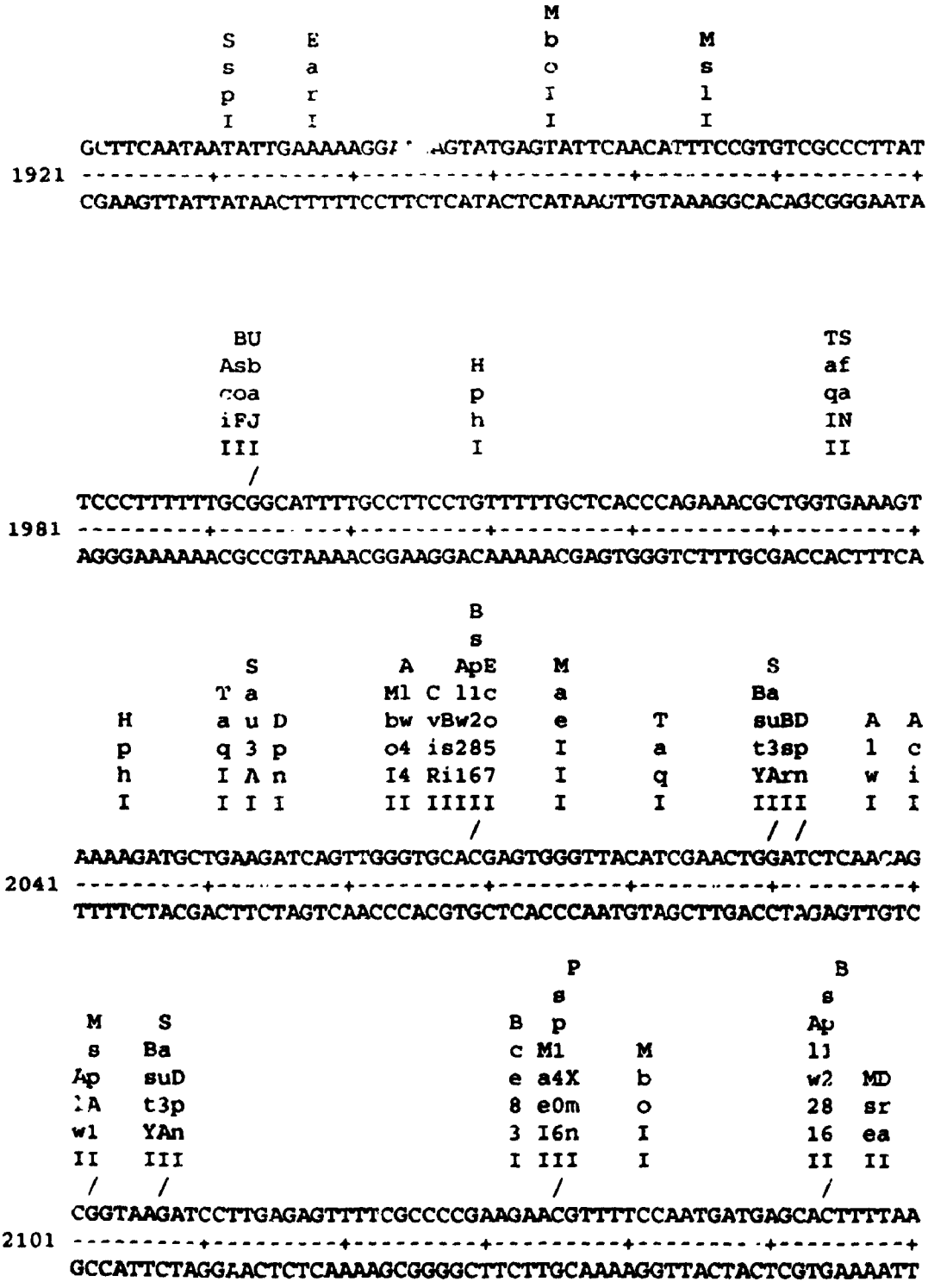
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S E b M
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p r I l
I I I I
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BU TS
Asb H af
coa p qa
iFJ h IN
III I II
/
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1981 ---------+---------+---------+---------+---------+---------+
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s
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T a Ml C 11c a Ba
H a u D bw vBw2o e T suBD A A
p q 3 p o4 is285 I a t3sp l c
h I A n I4 Ri167 I q YArn w i
I I I I II IIIII I I IIII I I
/ / /
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s s
M S B p Ap
s Ba c M1 M 11
Ap suD e a4X b w2 MD
1A t3p 8 e0m o 28 sr
w1 YAn 3 I6n I 16 ea
II III I III I II II
/ / / /
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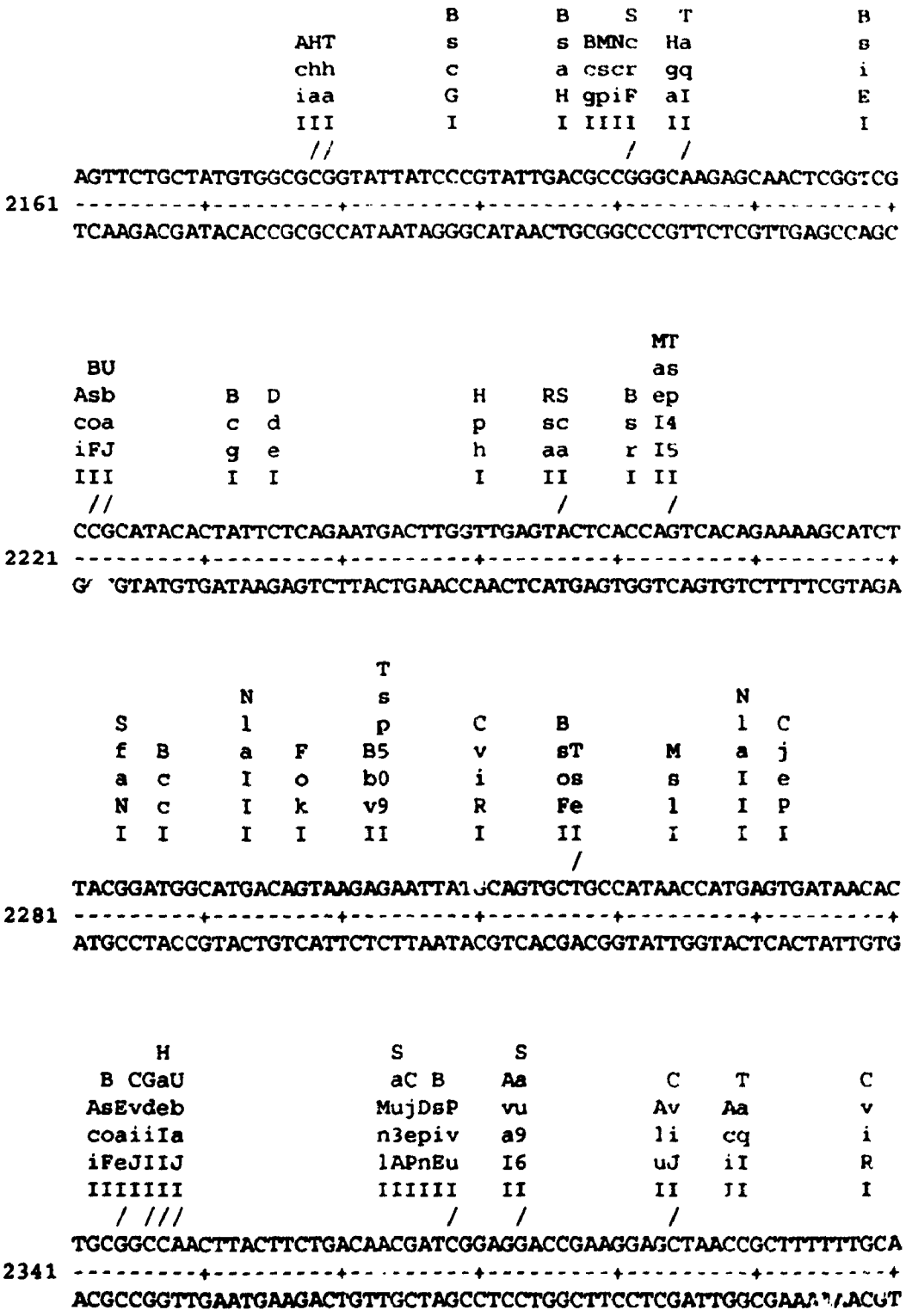
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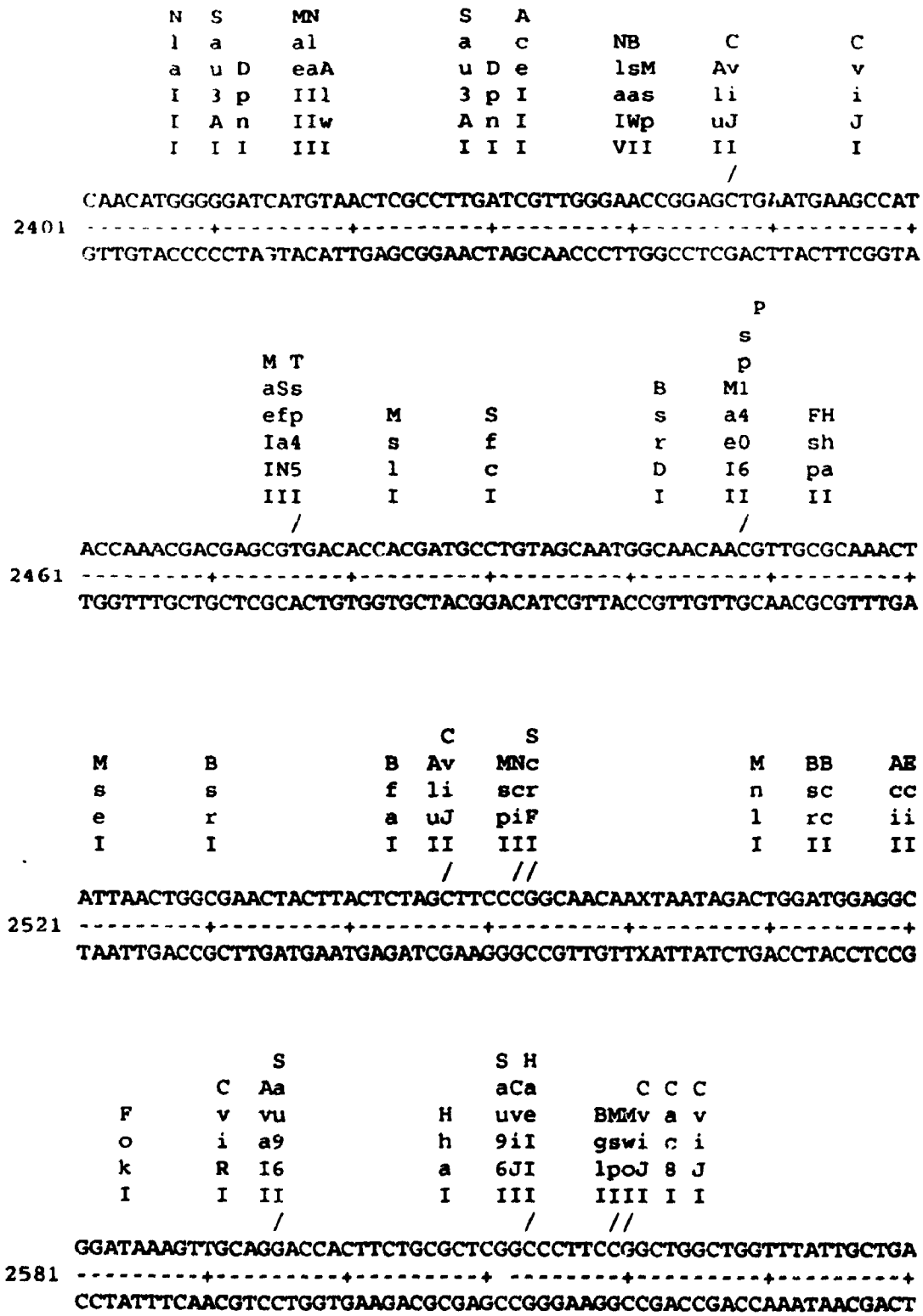
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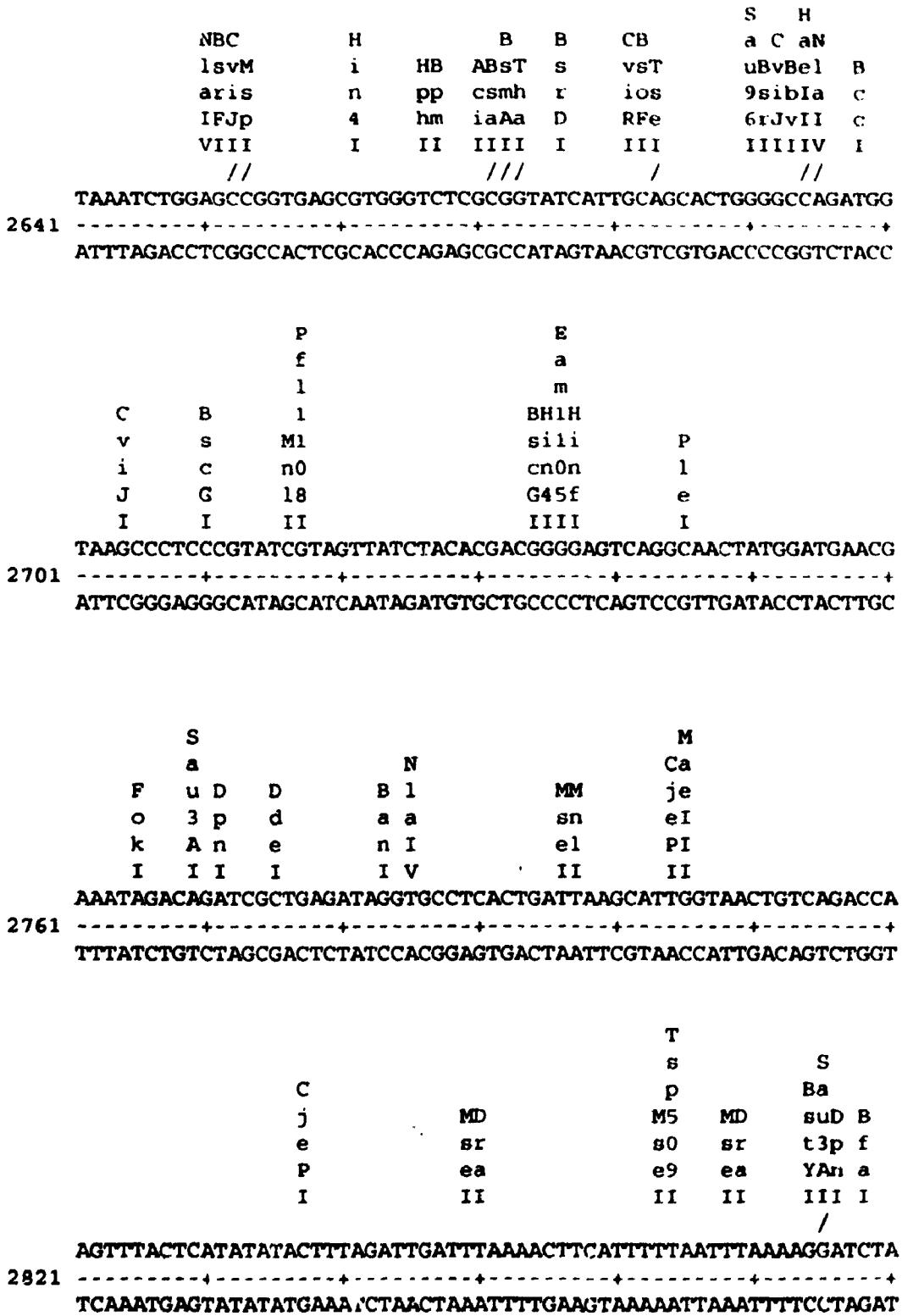
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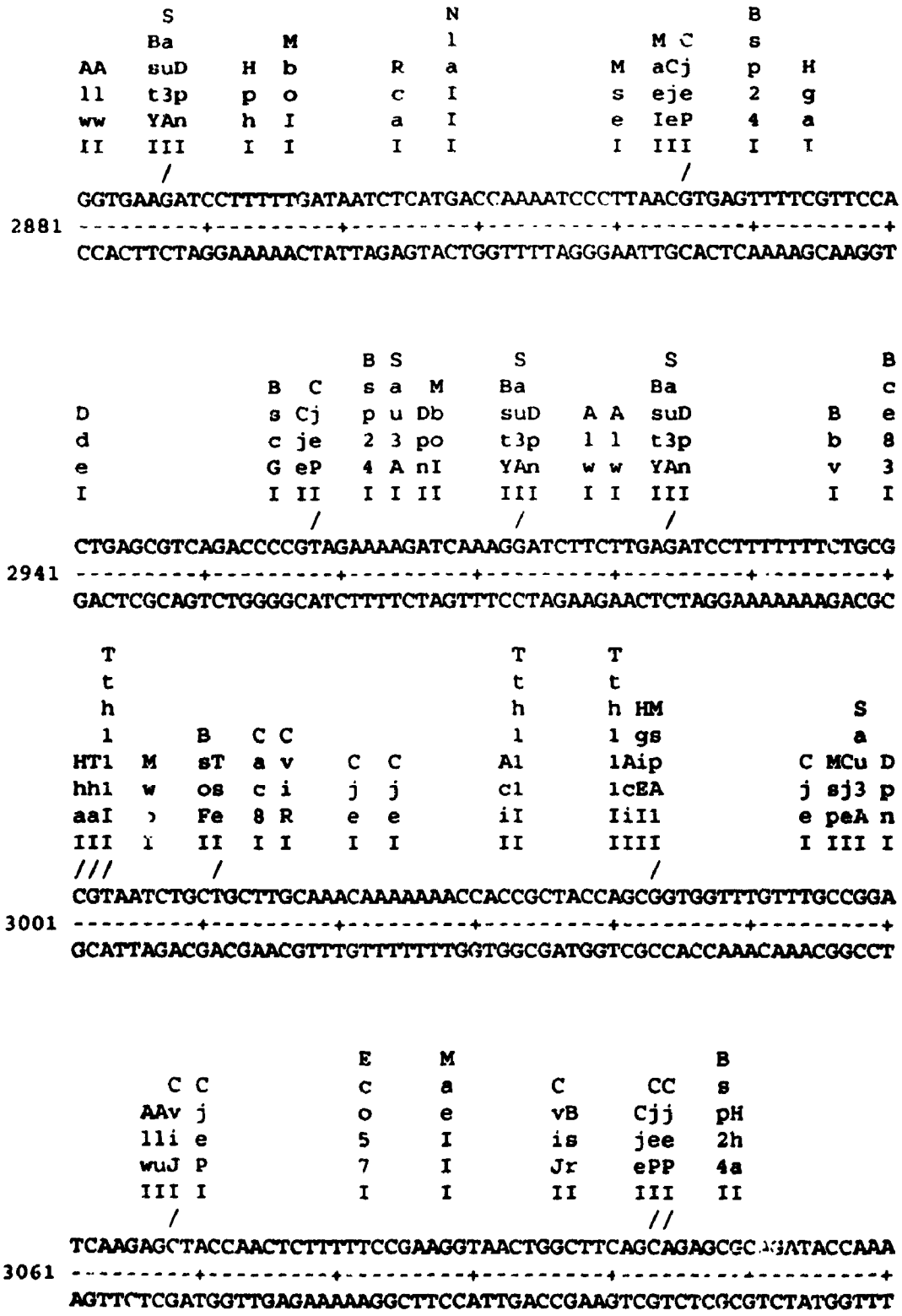
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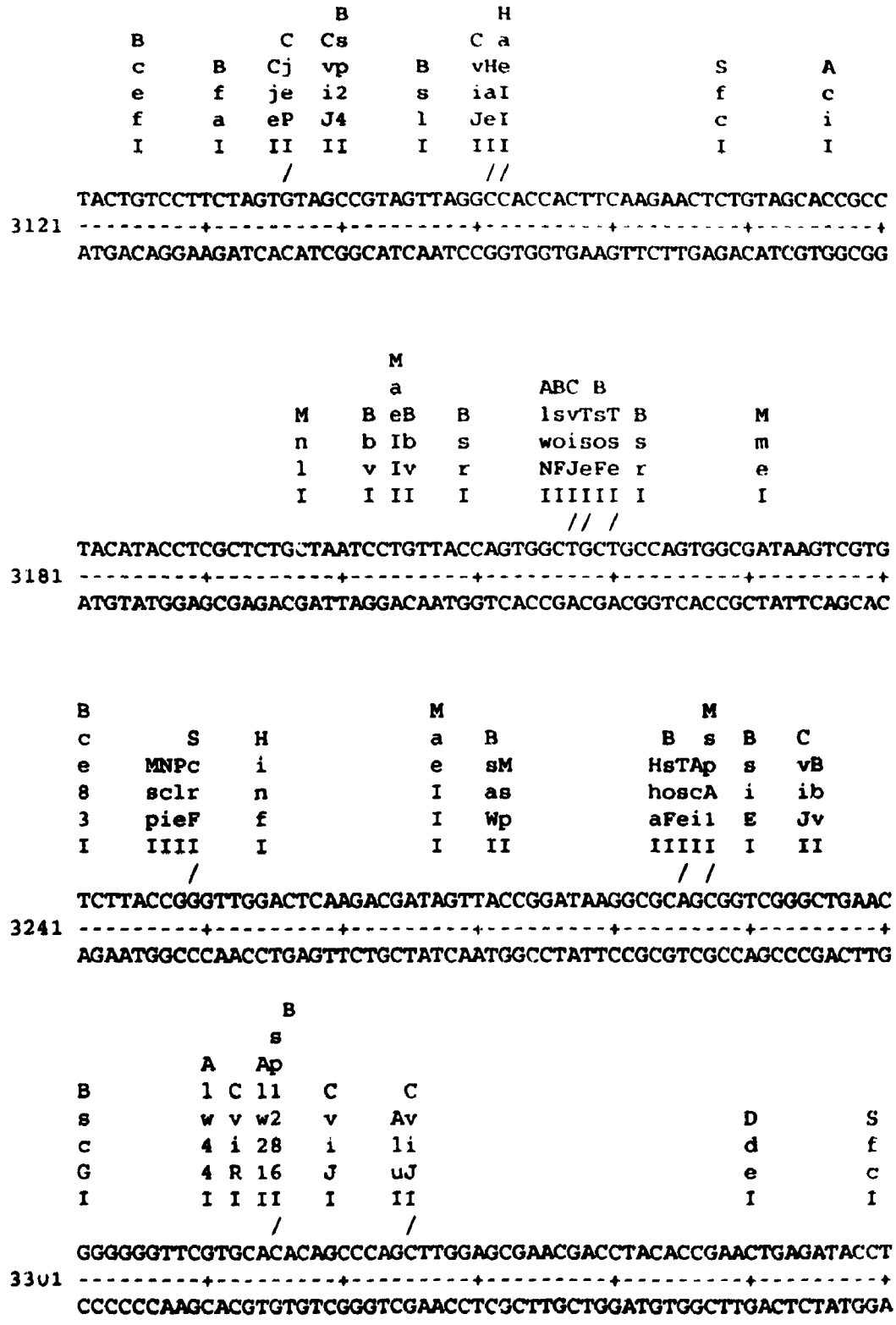
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3181
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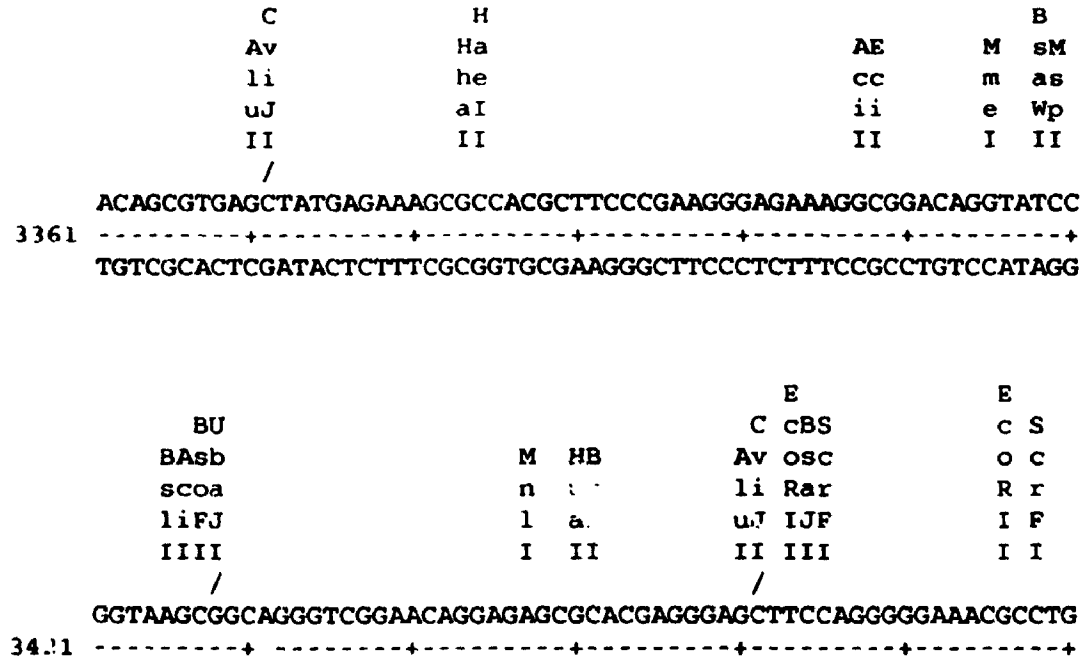
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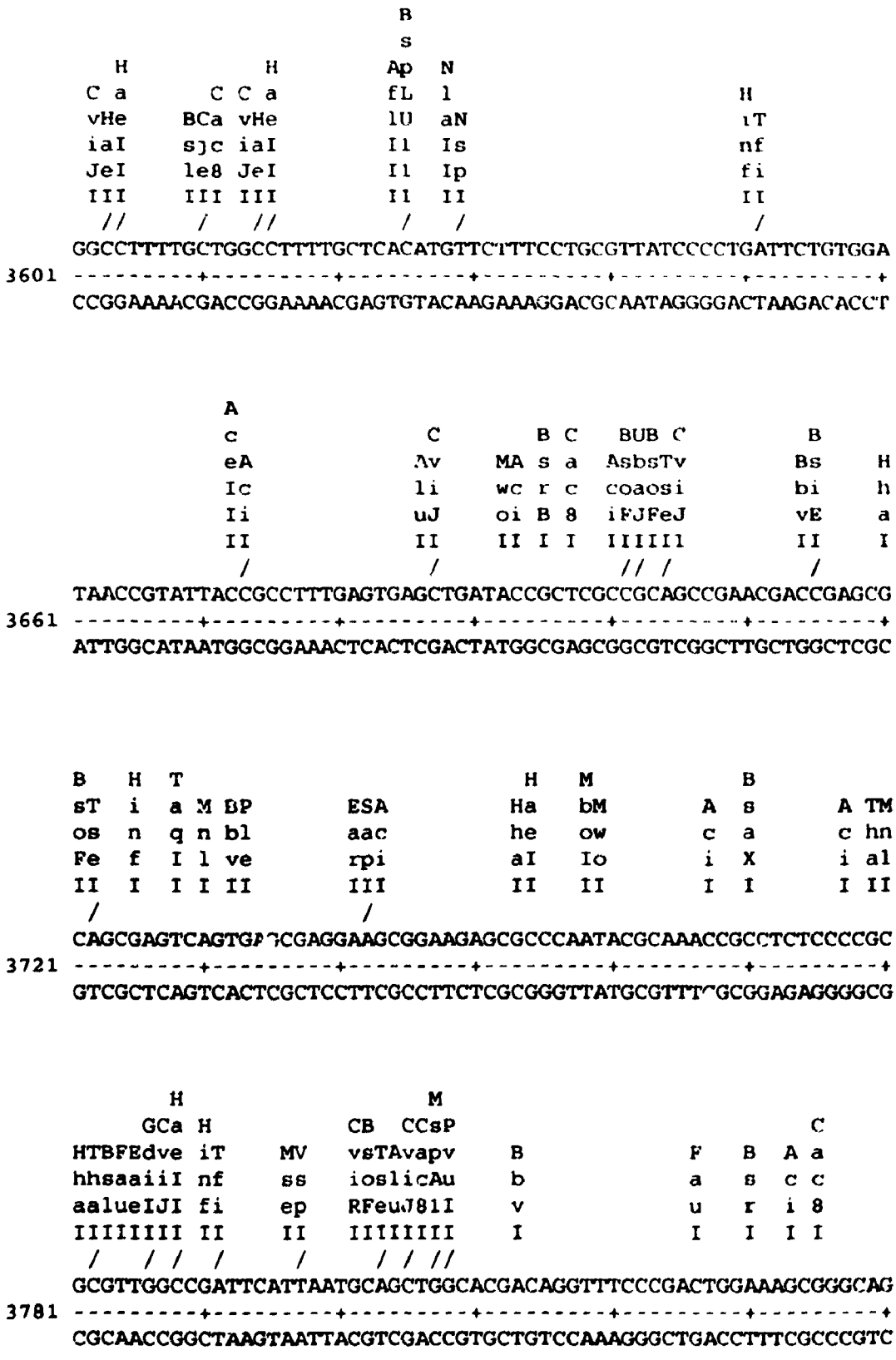
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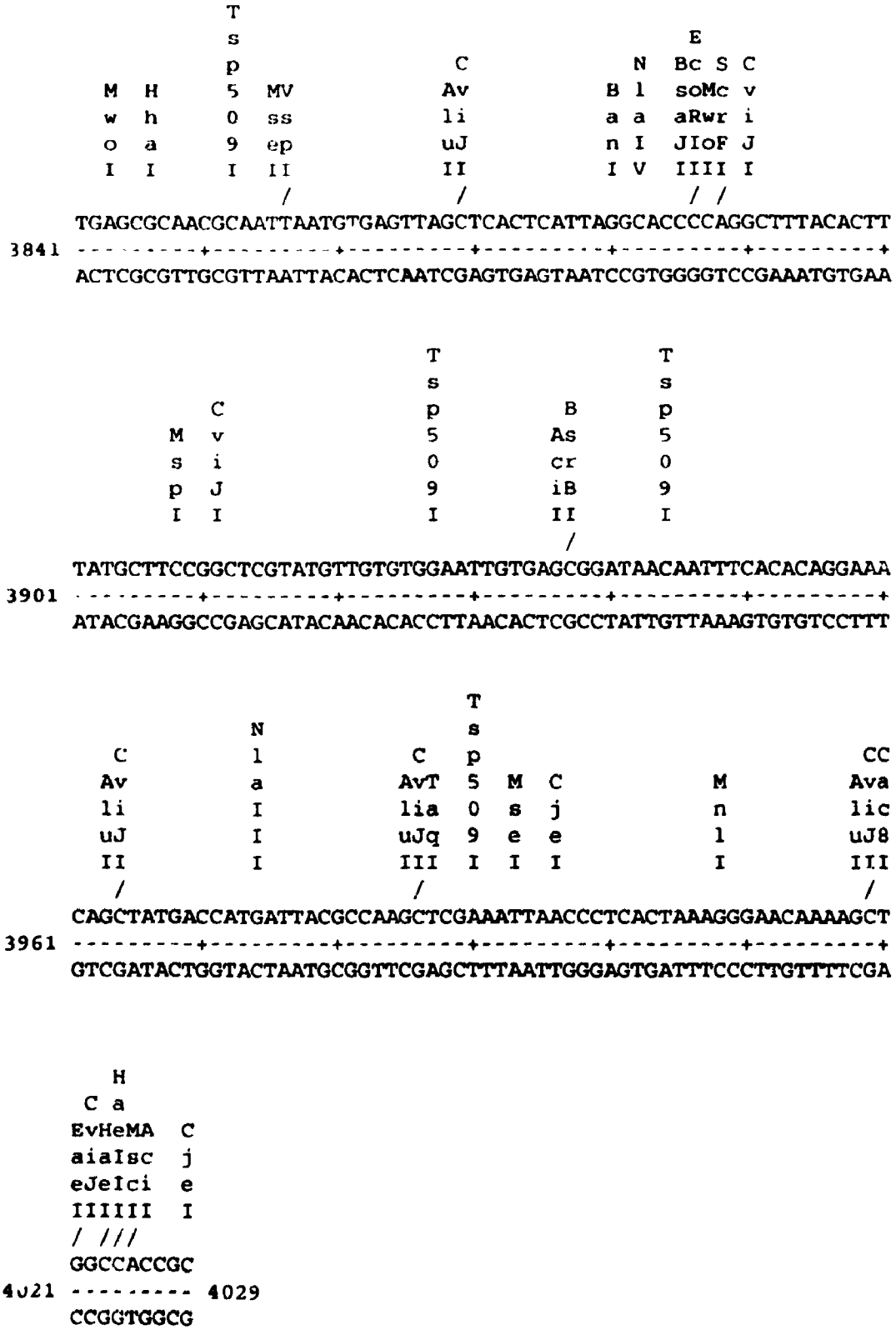
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GGCCACCGC
4021
CCGGTGGCG
4029

AccI	AceIII	AciI	AflIII	AluI	AlwI
Alw21I	Alw44I	AlwNI	ApaI	ApoI	AvaI
AvaII	BamHI	BanI	BanII	BbvI	BccI
Bce83I	BcefI	BcgI	BfaI	BglI	BmgI
BpmI	BsaI	BsaAI	BsaHI	BsaJI	BsaWI
BsaXI	BsbI	BscGI	BsiI	BsiEI	BslI
BsmAI	BsoFI	Bsp24I	Bsp1286I	BspLU11I	BspMI
BsrI	BsrBI	BsrDI	BsrFI	BstYI	Cac8I
CjeI	CjePI	ClaI	CviJI	CviRI	DdeI
DpnI	DraI	DraIII	DrdI	DrdII	DsaI
EaeI	EagI	Eam1105I	EarI	EciI	Eco47III
Eco57I	EcoO109I	EcoRI	EcoRII	EcoRV	FauI
FokI	FspI	GdiII	HaeI	HaeII	HaeIII
HgaI	HgiEII	HhaI	Hin4I	HincII	HindIII
HinfI	HphI	KpnI	MaeII	MaeIII	MboII
MmeI	MnlI	MscI	MseI	MslI	MspI
MspA1I	MwoI	NciI	NcoI	NdeI	NgoAIV
NheI	NlaIII	NlaIV	NotI	NspI	Pfl1108I
PleI	Psp1406I	PstI	PvuI	PvuII	RcaI
RsaI	SacI	SalI	SapI	Sau96I	Sau3AI
ScaI	ScrFI	SfaNI	SfcI	SmaI	SpeI
SspI	StyI	TaqI	TaqII	TfiI	ThaI
TseI	Tsp45I	Tsp509I	Tth111II	UbaJI	VspI
XbaI	XhoI	XmnI			

Enzymes that do not cut pComb3 phagemid vector:

AatII	AflII	AgeI	ApaBI	AscI	AvrII
BaeI	BbsI	BclI	BglII	Bpu10I	Bpu1102I
BsaBI	BseRI	BsgI	BsmI	BsmBI	BsmFI
BspEI	BspGI	BsrG'	BssHII	Bst1107I	BstEII
BstXI	Bsu36I	EcoNI	FseI	HpaI	MluI
MunI	NarI	NruI	NsiI	NspV	PacI
PflMI	PmeI	PmlI	PshAI	Psp5II	RleAI
RsrII	SacII	SexAI	SfiI	SgfI	SgrAI
SnaBI	SphI	SrfI	Sse8387I	StuI	SunI
SwaI	Tth111I	XcmI			

APPENDIX B

Primers for reverse transcription, PCR and sequencing of immunoglobulin genes

Name	Length	Sense/Antisense	5' to 3' Sequence	Restriction site	Ref *
Light chain constant region primers					
Cλ short	30	A	CTCGAG AGT GAC CGA GGG GGC AGC CTT GGG	Xho I	a
Cλ long	34	A	G CAT TCTAGA CTA TTA TGA ACA TTC TGT AGG GGC	Xba I	b
Cλ long2	34	A	G CAT TCTAGA CTA TTA TGA GCA TTC TGC AGG GGC	Xba I	b
Mc3'κ	35	A	CCT TCTAGA TTA CTA ACA CTC ATT CCT GTT GAA GC	Xba I	b
Cκ short	27	A	GGC AGT TCTAGA TTT CAA CTG CTC ATC	Xba I	c
Cκ long	33	A	GCT CAG TCTAGA ATG GGT GAC TTC GCA GGC GTA	Xba I	c
Heavy chain constant region primers					
Cμ short	30	A	TCTAGA G GAATTC TC ACA GGA GAC GAC GGG	Xba I/EcoR I	a
Cμ long	27	A	GTG CTG ACTAGT GCA GAC GAC GTG TTC	Spe I	b
Cμ SpeI	21	A	CTC ACTAGT GAC GAG GGG GAA	Spe I	c
JCμ	25	A	GAT GAGCTC CCT GAG GAG ACG ACG GTG A	Sst I	c
Variable λ light chain					
Leader/framework primer					
VλK46	24	S	GAGCTC CAG TCT GTG TTG ACG CAG	Sst I	a
Framework primers					
Vλ1	25	S	TCT GAGCTC ACG CAG CCG CCC TCA G	Sst I	b
Vλ2/5	25	S	TCT GAGCTC ACT CAG CCT GCC TCC G	Sst I	b
Vλ3	24	S	TCG GAGCTC CTG ACT CAG CCA CAC	Sst I	b
Vλ4	25	S	TCT GAGCTC ACT CAG GAC CCT GTT G	Sst I	b
Vλ6	24	S	AAT GAGCTC CTG ACT CAG CCC CAC	Sst I	b
Vλ7	24	S	CAG GAGCTC GTC ACT CAG GAG CCC	Sst I	b
Vλmisc	24	S	CAG GAGCTC CTG ACT CAA TCG CCC	Sst I	b
Variable κ light chain					
Leader primers					
Vκ1L	26	S	GG GAGCTC ATG GAC ATG AGG GTC CCC	Sst I	d
Vκ2L	26	S	GG GAGCTC ATG AGG CTC CCT GCT CAG	Sst I	d
Vκ3L	25	S	GG GAGCTC ATG GAA ACC CCA GCG CA	Sst I	d
Vκ4L	26	S	GG GAGCTC ATG GTG TTG CAG ACC CAG	Sst I	d
Variable heavy chain					
Leader primers					
VH1L	26	S	TC CTCGAG ATG GAC TGG ACC TGG AGG	Xho I	d
VH2L	29	S	CCA CTCGAG TGC TAC TGA CT(CG) TCC CGT CC	Xho I	d
VH3L	26	S	TC CTCGAG GAG TTT GGG CTG AGC TGG	Xho I	d
VH4L	26	S	TC CTCGAG CTG GTG GCA GCT CCC AGA	Xho I	d
VH5L	24	S	A CCG CTCGAG TCG CCC TCC TCC TG	Xho I	d
VH6L	21	S	TGT CTCGAG CCT CAT CTT CCT	Xho I	d
VHK46L	24	S	CTCGAG GCT CTT TTA AGA GGT GTC	Xho I	a
Framework primers					
VH I	24	S	CTC CTCGAG TCT GGG GCT GAG GTG	Xho I	b
VH IIA	24	S	TTA CTCGAG TCT GGT CCT GGG CTG	Xho I	b
VH IIB	24	S	TTA CTCGAG TCT GGT CCT GCG CTG	Xho I	b
VH III 2	24	S	CTG CTCGAG TCT GG(GA) GGA G(GA)C (TG)TG	Xho I	b
VH IV	24	S	CTG CTCGAG TCT GGA GCA GAG GTG	Xho I	b
VH V	24	S	CTG CTCGAG TCT GGA GCA GAG GTG	Xho I	b
VH VI	24	S	CTG CTCGAG TCA GGT CCA GGA CTG	Xho I	b
FW3SstII	25	S	CT GAGCTC ACG GCT GTG TAT TAC TG	Sst I	c
Sequencing primers					
M13 forward	17	S	GTT TTC CCA GTC ACG AC		e
M13 reverse	16	A	TTC ACA CAG GAA ACA G		e

*References (Ref)

a= Cairns 1989a b= Denomme 1994 c= Kabat 1991 d= van Es 1991 e= United States Biomedical Corp.

APPENDIX C

Primers for immunoglobulin gene manipulation and expression

Name	Length	Sense/Antisense	5' to 3' Sequence	Restriction site
Primers used for the amplification of "T7 promoter + CITE sequence" DNA fragment				
U19	19	S	GTT TTC CCA GTC ACG ACG T	
3pCITE-2a	17	A	CCATGG TAT TAT CAT CG	Nco I
Primers used for the generation of VH DNA fragments used for in vitro transcription and translation				
5NcoK46	28	S	ATA ATA CCATGG TT GCT CTT TTA AGA GG	Nco I
3XbaXhoCμ	28	A	TCTAGA C CTCGAG GGG GAA AAG GGT TGG	Xba I/Xho I
Primers used for grafting the YYGS encoding sequence into the V3 VH diversity region				
5MT1	27	S	TAC TAT GGT TCG AAG GCC AGC TGC TAC	
3MT1	27	A	CGA ACC ATA GTA CTT CGG GCA CTG CCC	
Primers used for removing the YYGS encoding sequence from the KIM4 6 VH				
5MT2	18	S	GGG ACT ACT ACT AAA CGG	
3MT2	30	A	AGT AGT AGT CCC ATA CTC CCT TAC TTT CGC	
Primers used for changing the KIM4.6 VH reading frame at the beginning of the D region				
5MT3	18	S	GCG AAA GTA GGG AGT ATT	
3MT3	25	A	CCA TAG TAA TAC TCC CTA CTT TCG C	
Primer used for changing the KIM4.6 reading frame at the beginning of the V region:				
5MT4	25	S	CTCGAG TGC TCT TTT AAG AGG TGT C	Xho I
Primers used for production of the KIM4.6 VH gene for cloning in the reverse orientation				
KLXbal	24	S	CTC TCTAGA CTT TTA AGA GGT GTC	Xba I
Cμ SstI	22	A	TC GAGCTC G ACG AGG GGG AAA A	Sst I

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