Molecular Characteristics of Antibodies Bearing an Anti-DNA-associated Idiotype

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Summary

Anti–double-stranded DNA antibodies are the hallmark of the disease systemic lupus erythematosus and are believed to contribute to pathogenesis. While a large number of anti-DNA antibodies from mice with lupus-like syndromes have been characterized and their variable region genes sequenced, few human anti-DNA antibodies have been reported. We describe here the variable region gene sequences of eight antibodies produced by Epstein-Barr virus (EBV)-transformed B cells that bear the 3I idiotype, an idiotype expressed on anti-DNA antibodies and present in high titer in patients with systemic lupus. The comparison of these antibodies to the light chains of $3I^+$ myeloma proteins and serum antibodies reveals that EBV transformation yields B cells producing antibodies representative of the expressed antibody repertoire. The analysis of nucleotide and amino acid sequences of these antibodies suggests the first complementarity determining region of the light chain may be important in DNA binding and that paradigms previously generated to account for DNA binding require modification. The understanding of the molecular genetics of the anti-DNA response requires a more complete description of the immunoglobulin germ line repertoire, but data reported here suggest that somatic diversification is a characteristic of the anti-DNA response.

Systemic lupus erythematosus (SLE) is an autoimmune disorder with a wide spectrum of clinical manifestations. The glomerulonephritis of SLE is thought to be caused, in part, by antibodies to double-stranded DNA (dsDNA)[1] (1–3). Antibodies to dsDNA are unique to this disease. Their presence correlates with disease activity and they can be eluted from the kidneys of patients with lupus nephritis (1). Recent studies have shown that anti-DNA mAbs perfused through kidneys in vitro or injected into mice in vivo can lead to glomerular pathology similar to that seen in SLE (4, 5). Anti-DNA antibodies are thought to cause disease either by binding directly to glomerular basement membrane antigens or by depositing as immune complexes in the glomeruli.

To understand the molecular genetic origin of anti-dsDNA antibodies and the structural basis for DNA binding, we undertook an idiotypic analysis of anti-DNA antibodies. The antibodies reported here all bear the 3I idiotype. This idiotype is present on κ light chains of anti-DNA antibodies (6). High titered expression of $3I^+$ antibodies is present in ~80% of SLE patients with anti-DNA activity. $3I^+$ Igs are deposited in the skin and kidneys of SLE patients indicating that $3I^+$ antibodies include a pathogenic subset (7). Nonetheless, like other autoantibody-associated idiotypes, the 3I idiotype is also present on antibodies without DNA binding specificity (8, 9). From studies on $3I^+$ myeloma proteins, we have hypothesized that in this idiotype system, DNA binding is acquired by somatic mutation of germ line variable region genes that do not, in their germ line configuration, display specificity for DNA.

Previously, our laboratory has shown that $3I^+$ B cell lines derived from lupus patients and from a myeloma patient with a $3I^+$ myeloma protein preferentially utilize members of the V_K1 gene family to encode their light chains (10). To further characterize $3I^+$ Igs and attempt to define the structural basis for idiotype and the motifs contributing to DNA binding, we decided to obtain sequence data from a large number of antibodies. The nucleotide and deduced amino acid sequences of eight $3I^+$ Igs produced by B cell lines from SLE and myeloma patients are reported. In addition, we report partial amino acid sequence analysis from several $3I^+$ myeloma proteins and a consensus sequence from polyclonal $3I^+$ anti-DNA antibodies isolated from the serum of a SLE patient. The mutational analysis of these antibodies is at-

[1] *Abbreviations used in this paper:* dsDNA, double-stranded DNA; R/S, replacement to silent; FW, framework.

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tempted in order to elucidate the molecular genetic origins of idiotype-positive anti-DNA antibodies and the role of antigen in eliciting their production.

Materials and Methods

Generation of Anti-DNA Antibodies. Anti-DNA antibodies were generated by EBV transformation of bone marrow (myeloma patient), peripheral blood, or spleen cells (three SLE patients) as described in detail previously (10). Isotype and idiotype determinations were made by ELISA and have been described previously (10) (Table 1).

RNA Preparation. Total RNA was prepared from 10^8 cells using guanidinium thiocyanate extraction (10). Determination of variable region gene utilization for both heavy and light chains has been described (10).

Cloning the Productively Rearranged V_K and V_H Genes. C_K primer 5'GTTCCAGATTTCAACTGCTC3', $C\mu$ primer 5'GAGGGGGAAAAGGGTTGGGGC3', $C\gamma$ primer 5'GCCAGGGGGAAGACCGATGG3', or $C\alpha$ primer 5'GGCTCAGCGGGAAGACCTTG3' was used to reverse transcribe 10 μg of total RNA into cDNA. The complete PCR has been described in detail elsewhere (10). The primers for the V_H and V_K regions were oligonucleotides specific for the 5' leader sequence or first framework sequences of each appropriate variable region gene family. V_K1, 5'ACATGAGGGTCCCCGCTCAG3', or 5'GACATCCAGTTGACCCAGTC3' (H2F, I-2a); V_K3; 5'ATGGAAACCCCAGCGCAGCT3'; V_H1, 5'CAGGTGCAGCTGGTGCAGTC3; (IC4) V_H3, 5'CAGGTGCAGCTGGTGGAGTCT3'; V_H4, 5'CAGGTGCAGCTGCAGGAGTC3'; and V_H5, 5'ATGGGGTCAACCGCCATCCT3'. 30 PCR cycles were performed under the following conditions: denaturation (94°C, 1 min), annealing (48°C, 1 min), and extension (72°C, 3 min). Further amplification of PCR products was performed after elution of DNA from a 2.5% low melt agarose gel (NuSieve GTG; FMR, BioProducts, Rockland, ME). The amplified products were blunt end ligated to either dephosphorylated pGEM or pBluescript SK^+. Competent DH5 cells (pGEM) or XL-1 Blue cells (Bluescript SK^+) were transformed with the ligation mixtures. DNA was prepared from V_K-positive colonies and DNA was sequenced using Sequenase (Amersham Corp., Arlington Heights, IL) (11, 12). Some PCR products were directly sequenced, as previously described (10).

Cloning of Germ Line V_K1 Genes. Complete EcoRI digests of genomic DNA from bone marrow–derived cells of patient HIC were cloned into the EcoRI site of Charon 16A (13) and $>10^6$ phage were screened using a V_K1-specific DNA coding region probe as described elsewhere (10). V_K1-positive phages were purified, subcloned into pGEM, and sequenced using the standard Sequenase protocol.

An MboI partial library from spleen cells of patient DIL was constructed in Charon 40. More than 10^6 phage were screened using the V_K1 probe described above. V_K1-positive phage were purified, subcloned into pBluescript, selected with a CDR1 oligo 5'TTAGTAACTATTTAAATTGG3', and sequenced as above.

Myeloma Sera and SLE Sera. Myeloma were obtained from three sources: Dr. Jean-Louis Preud'homme (Poitiers, France), Dr. Peter Wiernick (Albert Einstein College of Medicine, Bronx, NY), and Dr. Alan Solomon (University of Tennessee, Knoxville, TN). SLE serum from one patient was obtained from the clinic at the Bronx Municipal Hospital Center. All were screened for 3I reactivity by ELISA and dsDNA binding by Western blot analysis (14) (Table 2).

IgG from SLE Patient. Purified IgG was obtained from serum by molecular sieve chromatography using a Sephacryl 300 column (Pharmacia Fine Chemicals, Uppsala, Sweden) with 0.01 M Tris, 0.001 M EDTA, 0.5 M NaCl, pH 7, as running buffer. DNA binding antibodies were precipitated with dsDNA and then 3I-reactive antibodies were obtained by affinity chromatography (6).

NH_2-terminal Amino Acid Sequences of 3I-reactive Light Chains from Myeloma Proteins and SLE Serum. 10 ml of sera from each of six patients with a 3I-reactive myeloma protein was precipitated with 50% ammonium sulphate and the precipitate resuspended and displayed on an analytic IEF gel to determine the isoelectric point of the myeloma protein. The myeloma protein was then purified by preparative IEF. The purity of each protein was determined by SDS-PAGE and analytic IEF gel.

Myeloma proteins and $3I^+$ serum antibodies were reduced using 6 M urea and either 0.01 M dithiothreitol or 2% mercaptoethanol in 0.1 M Tris, pH 8.0, at 37°C for 2 h and alkylated using 0.05 M iodoacetic acid (15). Heavy and light chains were separated by gel filtration on a Sephacryl 200 column (Pharmacia Fine Chemicals) in 0.05 M Tris, 0.001 M EDTA, 0.1 M NaCl 1% SDS, pH 6.8. The alkylated light chains were subjected to automated sequence analysis (B890M; Beckman Instruments, Fullerton, CA). The initial protein loading was between 5 and 10 nmol and was sequenced in the presence of 2 mg polybrene. The amino acids were identified by two independent reverse phase HPLC methods (16). Protein sequences were then compared with known Ig light chains from the Dayhoff Protein Sequence Bank and were analyzed for the presence of invariant residues of the V_K families (17).

Results

$3I^+$ Light Chains of EBV-transformed B Cell Lines. The molecular characterization of anti-dsDNA antibodies necessitated that we obtain nucleotide sequences of the light and heavy chain variable regions of the $3I^+$ antibodies produced by EBV-transformed cell lines. Six of the monoclonal lines and one previously reported, 2A4, (18) express a V_K1-encoded light chain (Fig. 1). While there is a high degree of homology among the V_K1 sequences, it is not so striking

Table 1. *EBV-transformed Cell Lines*

Cell line	Patient	Isotype	DNA binding	V_K	V_H
III-3R	DIL (SLE)	μ	+	1	3
III-2R	DIL	μ	+	1	1
IC4	HIC (myeloma)	μ	+	1	4
I-2a	DIL	γ	+	1	3
1X7RG1	DIL	α	+	1	3
2A4	HIC	γ	+	1	4
R3.5H5G	RIO (SLE)	γ	–	1	1
II-1	DIL	μ	+	3	5
H2F	HER (SLE)	γ	+	4	3

Characteristics of EBV-transformed B cell lines denoting patient origin, isotype of antibody produced, DNA binding, and V_K and V_H family utilization.

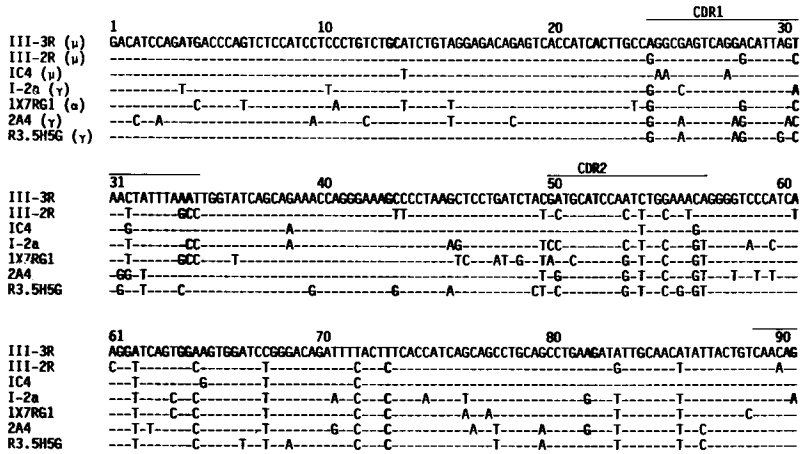

Figure 1. Nucleotide sequences of seven human V_K1 $3I^+$ light chain variable regions. The sequence of 2A4 has been previously reported (18) and is included for comparison.

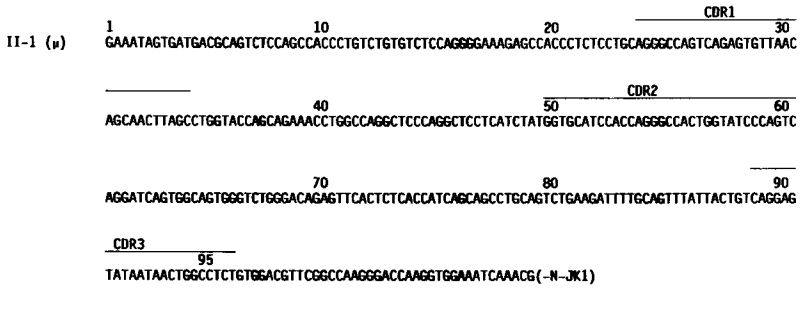

Figure 2. Nucleotide sequence of the II-1 light chain variable region.

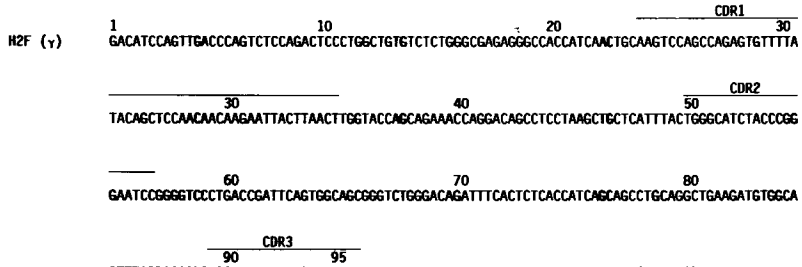

Figure 3. Nucleotide sequence of the H2F light chain variable region.

	FW1	CDR1	FW2	CDR2	FW3	CDR 3
III-3R	DIQMTQSPSSLSASVGDRVTITC	QASQDISNYLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRISGSGSGTDFTFTISSLQPEDIATYYC	QQYDNLP
III-2R	----------	R---G-----A	--------V------	A--T-QS	-----F----------L---------V-----	QKYNSAP
IC4	----------	-T-----K---	----------	------R	-----F--G----------	QQYDTLP
I-2a	---L-----F----------	R-----R---T	----------V---	P--T-QS	-----F-------E--L--I------F-----	QQLISYP
1X7RG1	---I-H----------	R---G-----A	-F---------S-L-	NP-S-QS	-----F----------L--NN-----F-----	QQYHSYP
2A4	-LK--------P-----T-----	R---S-NRF--	----------	G--S-QS	-FS--F-------E--L--R------F-----	QQSYSTP
R3.5H5G	----------	R---S-GSF-H	--------G-----S	A--S-QS	-----F----------L---------F-----	QQSYSTL
II-1	E-V-----AT--V-P-E-A-LS-	R---SVNSN-A	-------Q--R----	G--TRA-	-I-V---------E---------S--F-V---	QEYNMWP
H2F	---L----D--AV-L-E-A--N-	KS--*NNK---T	-------QP------	W--TR-S	---D-F----------L------A--V-V---	QQYYSTP

Figure 4. Derived amino acid sequences of $3I^+$ V_K genes showing conservation of sequence in framework regions. H2F has a six-amino acid insertion in CDR1 between the glutamine and the asparagine residues. *A six-amino acid insertion in CDR1: SVLYSS.

as to suggest a common germ line gene. One cell line, II-1, expresses a V_K3-encoded light chain (Fig. 2). The remaining line, H2F uses a V_K4-derived light chain (Fig. 3). The V-J junctions of each of the five rearranged lines from patient DIL and of the two lines from patient HIC differ, indicating that the B cells within each set are not clonally related.

The deduced amino acid sequences of the V_K1 light chains reveal conserved framework (FW) regions (Fig. 4).

		FW1	CDR1	FW2	CDR2	FW3
Vk1	MAR	DIQMTQSPSSLSASVGDRVTITC	RASQGITDALA	WYQQKPGKAPKLLLH	EASRLTR	G
	SCH	------F----------S---	----NVYTFL--	----------IY	---N-EG	GVPS-FSG-gv
	GOU	A--------------------N	----D-RND-G	----------Iy	gAQP	
	SNA	?---------------E-A--S-	----SVS-YLT/a	-T---		
Vk3	REN	E-VL-Q--Gt--A-P-E-AtLSS	-A-sDVD			
	TEN	E-V-----AT--V-P-G-A-LSc	RASQNVNs-LA	SYQQKP--a		
	HIC	-LV-----AT--V-P-QTA-LSC	RASQGVSSDLV	WY----GSAPSLLI		

Figure 5. Partial protein sequences of the light chains of six $3I^+$ myeloma proteins and the light chains of $3I^+$ anti-DNA antibodies purified from the serum of an SLE patient (SNA). Lower case letters denote probable amino acids.

This is especially true for FW2. Interestingly, the FWs of the V_K3- and the V_K4-encoded light chains are also highly homologous to the V_K1 protein sequences, providing a potential explanation for their 3I reactivity. The partial protein sequences of the $3I^+$ myeloma proteins are remarkably similar to the deduced amino acid sequences of the $3I^+$ antibodies (Fig. 5). Similarly, the consensus sequence of the light chains of $3I^+$ serum anti-dsDNA antibodies from the lupus patient, SNA, exhibits very strong homology to the light chains of the myeloma proteins and to the predicted amino acid sequences of the light chains of the EBV-transformed lines (Fig. 5). The analysis of these primary amino acid sequences reveals many regions of homology, yet no unique region that might represent the $3I^+$ idiotope.

All the antibodies except R3.5H5G bind dsDNA. Many DNA binding antibodies and other DNA binding proteins are enriched for arginine, asparagine, and glutamine residues. In anti-DNA antibodies, these residues are present in CDRs and presumably are contact residues for antigen (19–23). Here we find an unusually high number of these residues in CDR1. A random sampling of V_K1 light chains shows that on average 2.8 such residues are present in CDR1 (17). The light chains of the eight dsDNA binding antibodies reported here contain an average of 3.75 (range, 3–5). The R3.5H5G antibody is the only antibody that does not exhibit any specificity for dsDNA and it possesses only two such residues in CDR1. Although most of the myeloma proteins also bind dsDNA (see Table 2), the incidence of arginine, glutamine, and asparagine residues in the myeloma proteins is not so striking as in the antibodies from the EBV-transformed B cell lines.

Comparison of Expressed 3I Sequences to Their Putative Germline Genes. The human V_K1 gene family is the largest of the four κ gene families, containing ~30 genes (24–26). Our previous study of the 2A4 cell line revealed ~45 nucleotide differences between the most homologous V_K1 germ line gene and the 2A4 light chain nucleotide sequence, suggesting that no published V_K1 gene was the germ line gene template for 2A4 (18). This observation prompted us to construct genomic libraries from both the lupus patient DIL and myeloma patient HIC with the hope of obtaining new V_K1 genes that might be the germ line genes from which our expressed antibodies are derived.

We screened an HIC bone marrow library and a DIL spleen library with a V_K1 probe and isolated >30 clones hybridizing to a V_K1 fragment (10). To identify those V_K1 genes that had not been previously reported and that might be the template for our $3I^+$ antibodies, we screened the V_K1 clones with an oligonucleotide probe highly homologous to the CDR1 sequence of two of the three IgM antibodies. This CDR sequence was not present in any previously reported V_K1 gene and so was likely to identify new V_K1 genes in our libraries. Using this probe, we isolated from the HIC library two new functional germ line genes and one pseudogene, as well as two new pseudogenes from the DIL library. The germ line gene DILp1 is the only one of the genes we cloned with strong homology to any of the $3I^+$ sequences reported here. (Fig. 6). This V_K1 gene is quite unusual; codon 88, GGT, encodes glycine, whereas all V_K genes reported to date encode cysteine at this codon. As shown in Fig. 1, all the expressed $3I^+$ light chain variable regions encode cysteine at codon 88 (TGT).

We then compared the light chain nucleotide sequences of the V_K1 $3I^+$ antibodies to the DILp1 gene or to the most homologous germ line sequence available in the literature (Table 3). The III-2R IgM line derived from DIL is the most homologous to DILp1, differing by 17 nucleotides. Two others, I-2a and 2A4, are more homologous to DILp1 than to any other reported V_K1 gene but differ by 32 and 37 nucleotides, respectively (Table 3). Three V_K1 cell lines, III-3R, IC4, and IX7RG1, show most homology to the germ line gene HK137 (Table 3). In fact, the III-2R line is also more homologous to HK137, differing by 15 nucleotides, than to DILp1; however, it displays more homology in FW regions to DILp1 than to HK 137 (25, 27). The V_K1 sequence from the non-DNA binding R3.5H5G cell line is most homologous to the germ line gene b' (Table 3).

Calculation of amino acid replacement to silent changes (R/S) (Table 3) indicates that almost all the V_K1 antibodies

Table 2. *$3I^+$ Myeloma Proteins*

Protein	V_K gene family	DNA binding
MAR	V_K1	–
SCH	V_K1	+
GOU	V_K1	+
REN	V_K3	–
TEN	V_K3	+
HIC	V_K3	+

Characteristics of $3I^+$ myeloma proteins including V_K usage and DNA binding.

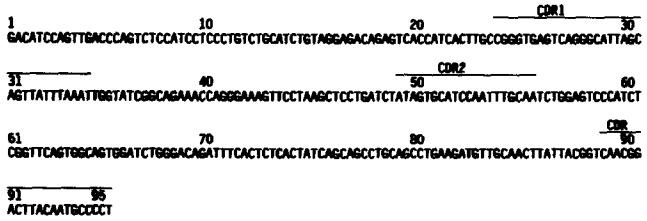

Figure 6. Nucleotide sequence of the V_K1 germline gene DILp1.

have an R/S ratio in the CDRs that is >2.9, the ratio predicted by random mutation. If III-2R is presumed to derive from the DILp1 germ line gene, then only 1X7RG1 shows a lower than random R/S ratio in the CDRs. All have an R/S ratio <2 in FW regions, suggesting that there may be some pressure for conservation of FW residues. A similar low R/S ratio in FW has been demonstrated in antibodies to foreign antigen (28).

The V_K3-rearranged gene encoding the II-1 light chain shows 98% homology with an autoantibody encoding germline gene, V_K328 (29). Although V_K328 is often used to encode rheumatoid factors, II-1 has no rheumatoid factor activity (data not shown). Both the base pair substitutions in the CDR regions lead to replacement changes, hence, the R/S ratio is 2/0, although the total number of mutations is too few to deduce antigen selection. The R/S ratio in FW regions, 2:1, is consistent with random mutation and the absence of selective pressures (Table 3).

H2F, which is encoded by a V_K4-rearranged gene, shows a 98% identity with the only reported V_K4 germ line (30). The three nucleotide changes in the CDRs lead to a single replacement substitution, and three nucleotide substitutions in the FWs (two in one codon) account for two amino acid changes (Table 3).

Analysis of the replacement mutations can provide information regarding the selective forces driving the immune response, and the structural basis for DNA binding. (Tables 4, 5, and 6). Table 4 displays the amino acid changes of three cell lines relative to the DILp1 germ line gene. In CDR1, cell lines 2A4 and I-2a acquire an arginine or asparagine at residue 30. Cell lines III-2R, 2A4, and I-2a acquire an arginine or asparagine at residue 31 compared to the DILp1-encoded sequence (Table 4). It is important to note that two of the lines, III-2R and I-2a, lose an asparagine at residue 34, so only 2A4 and I-2a show a net accumulation of arginine or asparagine in CDR1. All three lines presumptively encoded by DILp1 lose an asparagine in both CDR2 and CDR3 at residues 53 and 93 as well as an arginine at residue 90. The arginine is replaced by glutamine or lysine, two additional amino acids implicated in DNA binding. The asparagines, however, are replaced by serine or threonine, and are, therefore, replacements that might decrease affinity for DNA. III-2R acquires an asparagine at residue 92 in CDR3.

The $3I^+$ light chains possibly encoded by the HK137 gene are similarly analyzed in Table 5. III-3R and IC4 acquire an asparagine at residue 34 in CDR1. In CDR2, IC4 and III-3R acquire additional asparagines at residue 53; IC4 acquires a second arginine at residue 56, and 1X7RG1 acquires an asparagine at residue 50. In CDR3, three lines, III-3R, 1X7RG1, and IC4, lose a glutamine at residue 90, although III-3R acquires asparagine at residue 93. The sequence of the R3.5H5G antibody shows a loss of an asparagine at residue

Table 3. *Comparison of V_K Sequences to Most Homologous Germ Line Gene*

Germ line gene	Cell line	Percent homology	R/S ratios: FWs Total	FWs R	FWs S	CDRs Total	CDRs R	CDRs S	R/S per domain: FW1	FW2	FW3	CDR1	CDR2	CDR3
DILp1	III-2R(μ)	96	8	3	5	9	9	0	1:0	1:0	1:5	3:0	2:0	4:0
	2A4(γ)	87	25	13	12	12	11	1	5:2	2:0	6:10	5:0	2:0	4:1
	I-2a(γ)	89	19	8	11	13	12	1	1:0	3:2	4:9	5:0	2:0	5:1
HK137	III-3R(μ)	91	13	5	8	13	10	3	0:1	2:1	3:6	3:2	4:1	3:0
	IC4(μ)	92	10	5	5	13	12	1	0:3	2:2	3:0	5:1	4:0	3:0
	III-2R(μ)	95	10	4	5	5	3	2	0:2	3:0	1:4	0:1	1:0	2:1
	1X7RG1(α)	95	9	5	4	6	3	3	2:2	1:2	2:0	0:1	2:0	1:2
b′	R3.5H5G(γ)	92	11	3	8	11	8	3	1:1	2:2	0:5	4:1	0:1	4:1
V_K328	II-1(μ)	98	3	2	1	2	2	0	0:0	0:0	2:1	1:0	0:0	1:0
V_K4	H2F(γ)	98	3	3	0	3	1	2	3:0	0:0	0:0	1:2	0:0	0:0

Replacement (R) to silent (S) ratios in the light chains of the $3I^+$ antibodies. The ratio of mutations that result in an amino acid replacement to those that are silent are tabulated for the FWs and the CDRs.

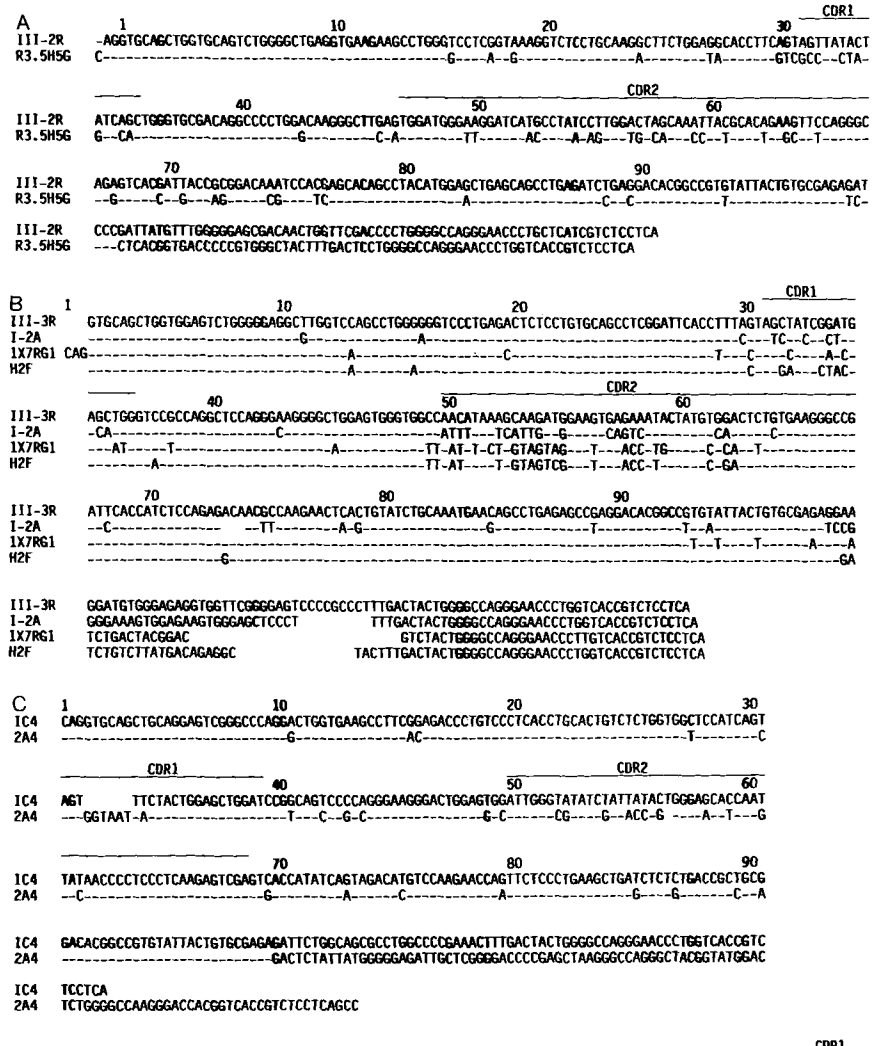

Figure 7. (*A*) Nucleotide sequences of V_H1-encoded heavy chains. (*B*) Nucleotide sequences of V_H3-encoded heavy chains. (*C*) Nucleotide sequences of V_H4-encoded heavy chains. The sequence of 2A4 has been previously reported (18) and is included for comparison. (*D*) Nucleotide sequence of V_H5-encoded heavy chain.

92 in CDR3 when compared to the sequence encoded by the b′ germ line gene (Table 6).

Pathogenic anti-DNA antibodies are thought to be cationic (31). Among the V_K1 light chains, there is no trend to acquire a more positive net charge. Although cationic anti-dsDNA antibodies are preferentially sequestered in the kidney, most of the V_K1 sequences expressed in our panel of anti-DNA B cells are more negatively charged than the deduced amino acid sequence of the germ line genes.

Two of the $3I^+$ sequences are not encoded by V_K1 genes. The II-1 light chain derives from a V_K3 gene. It differs from the presumed germ line–encoded amino acid sequence by two amino acids in FW regions and two in CDRs (Table 7). The expressed antibody reflects the acquisition of an asparagine in CDR1 and the loss of a glutamine in CDR3. Similarly, the V_K4-encoded H2F light chain differs by only three amino acids from the putative germ line template (Table 8). The single substitution in a CDR replaces an alanine with a threonine. The amino acid substitutions in II-1 and H2F are too few to argue for or against antigen selection.

$3I^+$ Antibodies Are Encoded by Four V_H Gene Families. The V_H gene segments encoding the expressed heavy chains of the $3I^+$ antibodies are derived from four families: V_H1, V_H3, V_H4, and V_H5. The nucleotide sequences, including that of 2A4 (18), are shown in Fig. 7. The predicted protein sequences of the heavy chains are presented in Fig. 8. If the appropriate germ line genes have all been cloned, our sequence data suggest that the heavy chains are also somatic mutants of their germline counterparts. Table 9 presents the replacement to silent substitution ratios in FW and CDR regions of each antibody compared to the sequence encoded by the most homologous published V_H germ line gene. In general,

Table 4. *Comparison of 3I⁺ V_K1 Antibodies to DILp1*

	Amino acid	DILp1	III-2R	2A4	I-2A
FW1	2	I	–	L	–
	3	Q	–	K	–
	4	L	M	M	–
	10	S	–	–	F
	12	S	–	P	–
	18	R	–	T	–
CDR1	25	V	A	A	A
	28	G	–	S	D
	30	S	–	N	R
	31	S	N	R	N
	32	Y	–	F	–
	34	N	A	–	T
FW2	37	R	Q	Q	Q
	43	V	–	A	A
	46	L	–	–	V
CDR2	50	S	A	G	P
	53	N	T	S	T
FW3	58	V	–	F	–
	59	P	–	S	–
	70	D	–	E	E
	76	S	–	R	I
	83	V	–	F	F
	88	G	C	C	C
CDR3	90	R	K	Q	Q
	91	T	Y	S	L
	92	Y	N	–	I
	93	N	S	S	S
	94	A	–	T	Y

Table 5. *Comparison of 3I⁺ V_K1 Antibodies to HK137*

	Amino acid	Hk137	III-3R	III-2R	1X7RG1	IC4
FW1	4	M	–	–	I	–
	6	Q	–	–	H	–
CDR1	24	R	Q	–	–	Q
	25	A	–	–	–	T
	28	G	D	–	–	D
	31	N	–	–	–	K
	34	A	N	–	–	N
FW2	36	F	Y	Y	–	Y
	43	A	–	V	–	–
	46	S	L	L	–	L
	48	I	–	–	L	–
CDR2	50	A	D	–	N	D
	51	A	–	–	P	–
	53	S	N	T	–	N
	55	Q	E	–	–	E
	56	S	T	–	–	R
FW3	62	F	I	–	–	–
	65	S	–	–	–	G
	73	L	F	–	–	F
	76	S	–	–	N	–
	77	S	–	–	N	–
	83	F	I	V	–	I
CDR3	90	Q	–	K	–	–
	92	N	D	–	H	D
	93	S	N	–	–	T
	94	Y	L	A	–	L

similar to what was seen in the light chains, there is a lower than random R/S ratio in FW regions and a higher than random R/S ratio in CDRs. When the actual amino acid substitutions are examined, a net accumulation of asparagine, arginine, or glutamine is seen in five antibodies and a net loss in four (Tables 10–13). Similarly, four anti-DNA antibodies show a net gain in positive charge, and three show a net loss. One anti-DNA antibody shows no change in charge and R3.5H5G, which does not bind DNA, also shows no change in charge.

CDR3 Regions Differ among Anti-DNA Antibodies. Fig. 9 shows that a variety of D segments are used by these EBV lines. In fact, three lines, III-3R, III-2R, and 2A4, express D-D fusions, a previously reported feature of autoantibodies (18, 32). Other lines express D regions that have yet to be reported and differ from each other as well as from the reported D region genes.

J_H usage is also not restricted among 3I⁺ or DNA binding EBV lines, although J_H4 is used in seven of the nine lines (Fig. 9). All J_H4^+ antibodies have a single silent nucleotide change from the published J_H4 sequence (CAG instead of CAA) (33). This change may represent a polymorphism within the population similar to what others have reported for J_H6 (34). As in the light chain sequences, there is no common junction evident among antibodies deriving from a single individual. We, therefore, cannot establish any genealogy by examining the heavy chain genes.

Discussion

Structural analyses of antibodies provide information about the basis for both idiotype expression and antigenic specificity. Molecular genetic studies can provide insights about the B

Table 6. *Comparison of R3.5H5G to b'*

	Amino acid	b'	R3.5H5G
FW1	11	V	L
CDR1	28	G	S
	30	S	G
	32	W	F
	34	A	H
FW2	43	A	G
	49	Y	S
CDR3	91	A	S
	92	N	Y
	94	F	T
	95	P	L

Table 7. *Comparison of II-1 to V_K328*

	Amino acid	V_K328	II-1
CDR1	30	S	N
FW3	60	G	V
	62	F	I
CDR3	90	Q	E

Table 8. *Comparison Mutations in H2F to V_K4*

	Amino acid	V_K4	H2F
FW1	3	V	Q
	4	M	L
CDR1	34	A	T

cell producing a given antibody and the role that antigen or other selective pressures play in the antibody response. To further our understanding of potentially pathogenic anti-dsDNA antibodies, we elected to study a large number of antibodies bearing the $3I^+$ anti-DNA-associated idiotype. We also wished to study genealogies of $3I^+$ B cells to correlate changes in amino acid sequence with changes in DNA binding. While we were unable to obtain B cell genealogies, we have analyzed multiple B cell lines derived from two individuals as well as single B lines from an additional two individuals.

We have determined the light and heavy chain variable region gene sequences of nine EBV-transformed $3I^+$ clonal B cell lines. All except one antibody binds to dsDNA. Because elevated titers of $3I^+$ antibodies are associated in SLE patients with glomerulonephritis and since we have documented that $3I^+$ antibodies are present in the kidneys of patients with SLE, the antibodies that bind to dsDNA are presumed to include representatives of a pathogenic subset present in a large percent of patients with SLE.

Previously, we addressed questions regarding general molecular characteristics of EBV-transformed $3I^+$ B cell lines (10). We found that $3I^+$ antibodies were encoded mainly by V_K1 light chain genes regardless of patient origin. We believe EBV transformation does not introduce experimental bias in creating this V_K restriction for two reasons. First, these lines express light chains with amino acid sequences that are homologous to the sequence of $3I^+$ polyclonal serum antibodies from a SLE patient and to a number of $3I^+$ myeloma proteins. Second, other reports of V_K gene usage in EBV-transformed B cells show a variety of expressed V_K genes (34, 35). It is likely, therefore, that the data reflect a disease-associated, as well as idiotype-associated, V region restriction as has been proposed by Shen et al. (36) in chronic lymphocytic leukemia.

We wished to determine if sequence analysis could reveal residues or regions among the V_K1, V_K3, and V_K4 antibodies that constitute the 3I epitope or that participate in DNA binding. Our results indicate that the $3I^+$ V_K1 antibodies are closely related on both the nucleotide and the protein level. In fact, the data show strong conservation of sequence in FW regions among all the antibodies. This conservation is evident in the partial protein sequences of the $3I^+$ myeloma light chains as well. The greater homology in FWs than in CDRs supports the hypothesis that the 3I determinant(s) lies within the framework regions of the light chain. In general, it has been difficult to map antibody idiotypes; in the 3I system also, more definitive analysis using peptide scanning and site directed mutagenesis will be required to localize the idiotype.

Sequence analysis of anti-DNA antibodies from various mouse strains has led to the hypothesis that the presence of particular amino acids in the CDRs is associated with the ability to bind to DNA. A number of amino acids, including asparagine, glutamine, and arginine, have been postulated to interact with the major and minor grooves of dsDNA (19). These have been implicated in the antigenic specificity of anti-DNA antibodies. Amino acid sequence analysis of mouse anti-DNA antibodies has led to the interesting observation that there is an increase in the number of arginines and tyrosines over that of previously sequenced mouse antibodies (20, 21, 37). It has also been suggested that DNA binding may select for arginine in position 98 of mouse heavy chain variable regions (38). In the heavy chain variable region of human anti-DNA antibodies, tyrosine residues at position 32 and/or 33 as well as Ser-Thr-Asn-Tyr-Asn in CDR2 have been postulated to form the DNA binding pocket in anti-DNA antibodies (37).

Our data show that the number of arginine, glutamine,

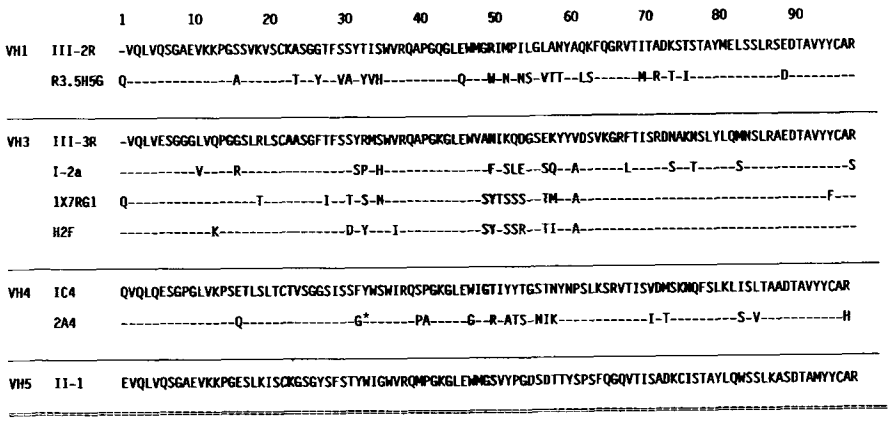

Figure 8. Derived heavy chain amino acid sequences of EBV-transformed cell lines. 2A4 has a two-amino acid insertion in CDR2. *NY.

asparagine, and tyrosine residues in CDR1 of the κ chain variable region is distributed as follows: in the eight DNA binding antibodies, four contain five putative DNA-interacting amino acids and four antibodies contain four such residues, while R3.5H5G, the one antibody that shows no dsDNA binding, contains only two such residues (Fig. 4). Additionally, the total number of asparagine residues in CDRs of the entire light chain variable region of the DNA binding antibodies is higher than in the one non-dsDNA binding antibody. Four antibodies contain four asparagine residues, three contain two, and one contains one. R3.5H5G, which does not bind dsDNA, encodes no asparagine residues. Examination of a random population of previously sequenced human V_K1, V_K3, and V_K4 antibodies reveals that they possess fewer asparagine residues than the $3I^+$ DNA binding antibodies we report here (mean, 1.33; *n*, 12) (17).

While it has been suggested that tyrosine residues in CDRs of the heavy chain in mouse may be significant in DNA binding, we do not find any significant increase in tyrosine residues in our set of anti-DNA antibodies. In fact, R3.5H5G, the one $3I^+$ antibody that does not bind dsDNA, contains two juxtaposed tyrosine residues within CDR1 of the heavy chain.

In searching the sequences of our antibodies for ATP binding site motifs, Gly-X-Gly-Lys-Thr, Gly-$(X)_4$-Gly-Lys-Thr-$(X)_6$-Ile/Val, or Gly-X-Gly-X-X-Gly/Ser, we find stretches of Gly residues interspersed with Ser residues within FW regions. These regions appear, however, to be conserved among all the V κ families and not to be specific for V regions used to form anti-DNA antibodies.

While the three-dimensional structure of a folded polypeptide chain is determined by its primary sequence, similar

Table 9. *Comparison of V_H Sequences to Most Homologous Germline Genes*

Germ line gene	Cell line	Percent homology	R/S ratios						R/S per domain				
			FWs			CDRs							
			Total	R	S	Total	R	S	FW1	FW2	FW3	CDR1	CDR2
51P1*	III-2R(μ)	96	3	1	2	8	5	3	0:2	0:0	1:0	1:1	4:2
H16BR‡	III-3R(μ)	90	18	7	11	7	5	2	1:4	2:1	4:6	3:0	2:2
71-4§	IC4(μ)	97	10	7	3	3	3	0	3:0	1:3	3:0	1:0	2:0
56P1*	I-2a(γ)	94	3	3	0	9	7	2	0:0	0:0	3:0	2:1	5:1
V_H26§	1X7RG1(α)	89	18	8	10	12	10	2	4:2	0:3	4:5	3:0	7:2
71-2§	2A4(γ)	92	15	5	10	7	7	0	2:3	2:3	1:4	1:0	6:0
20P3*	R3.5H5G(γ)	94	12	6	6	9	7	2	3:0	1:2	2:4	2:1	5:1
251§	II-1(μ)	98	4	2	2	5	5	0	1:1	0:1	1:0	2:0	3:0
V_H26§	H2F(γ)	90	16	6	10	11	9	2	2:4	1:1	3:5	2:1	7:1

Replacement (R) to silent (S) ratios in the heavy chains of the $3I^+$ antibodies. The ratios of mutations that result in an amino acid replacement to those that are silent are tabulated for the FWs and the CDRs.

* Rearranged gene of fetal origin.

‡ Germ line pseudogene.

§ Closest homologous germline gene.

Table 10. *Replacement Mutations in 3I⁺ V_H1 Sequences*

	Amino acid	51P1	III-2R
CDR1	33	A	T
CDR2	50	G	R
	52	I	M
	55	F	L
	57	T	L
FW3	74	E	K
	Amino acid	20P3	R3.5H5G
FW1	24	A	T
	30	T	V
CDR1	31	G	A
	34	M	V
FW2	46	E	Q
CDR2	57	G	V
	59	N	T
	62	Q	L
	63	K	S
FW3	85	R	S
	98	T	R

Table 11. *Replacement Mutations in 3I⁺ V_H3 Sequences*

	Amino acid	VH26	H2F	1X7RG1
FW1	1	E	-	Q
	5	L	V	V
	13	Q	K	-
	19	R	-	T
	28	T	-	I
CDR1	31	S	D	T
	33	A	Y	S
	35	S	-	N
FW2	37	V	I	-
CDR2	50	A	Y	Y
	51	I	-	T
	53	G	S	S
	54	S	R	-
	56	G	S	S
	57	S	T	T
	58	T	I	M
	61	G	A	A
FW3	75	S	A	A
	78	T	S	S
	95	Y	-	F
	98	K	R	R

folds can be formed by very different sequences. It is likely that there are multiple ways different amino acids combine to form an antigen binding pocket with affinity for dsDNA antigenic epitopes. Different idiotypic systems may display different motifs, and even within an idiotypic system different motifs may be present. The specificity of a particular amino acid will depend on the conformation and orientation of the protein backbone. Such observations may help explain the apparent acquisition of dsDNA binding in the V_K1-encoded antibodies as a result of presumed somatic mutation despite no actual increase in the number of amino acids capable of contacting DNA. It is also important, however, to consider that DNA need not be the eliciting antigen and some amino acid substitution may be present to provide increased binding to an unidentified antigen.

Based on the S107-U4 paradigm in which an antibacterial antibody, S107, underwent a single amino acid substitution in CDR1 and acquired binding to dsDNA, we have hypothesized that somatic mutation in vivo may also lead to DNA binding (39). In fact, all anti-dsDNA antibodies derived from individuals with SLE or lupus-prone mice that have been sequenced so far appear to show somatic mutations (21, 22, 40, 41). An analysis of the somatic mutations present in the expressed V region sequences can help determine if the antibody response is antigen driven or is a consequence of polyclonal activation, a major question in the study of the anti-DNA response. If somatic mutations occur randomly throughout the V region yielding a replacement to silent mutation ratio of 2.9:1, then the antibodies may reflect B cell or T cell polyclonal activation. If, however, mutations are clustered in CDRs or if the R/S ratio in CDRs is >2.9:1, then antigen selection may play a role in expanding particular B cells clones. The somatic mutations seen in some anti-dsDNA antibodies previously appear to lead to the acquisition of DNA binding, and the unmutated germ line genes may be presumed to encode antibodies with little or no affinity for dsDNA. For example, the sequence of the 2A4 anti-dsDNA antibody displays substitutions that lead to a more cationic antibody and one containing more putative DNA binding residues in CDRs (18).

We have performed an analysis of the presumed somatic mutations present in the 3I⁺ light chains sequences reported here. Because all the human V_K genes have not yet been cloned and sequenced, we attempted to isolate additional V_K1 germline genes. Of the five we sequenced, two were

Table 11. *(continued)*

	Amino acid	56P1	I-2a
CDR1	32	Y	S
	33	A	P
CDR2	50	V	F
	53	Y	L
	54	D	E
	57	N	S
	58	K	Q
FW3	68	F	L
	84	N	S
	98	R	S
	Amino acid	H16BR	III-3R
FW1	9	stop	G
CDR1	32	S	Y
	33	W	R
	35	H	S
FW2	38	C	R
	42	E	G
CDR2	50	D	N
	53	C	Q
FW3	83	V	M
	91	M	T
	92	T	A
	97	V	A

Table 12. *Replacement Mutations in 3I⁺ V_H4 Sequences*

	Amino acid	71-4	IC4
FW1	6	Q	E
	29	S	I
	30	G	S
CDR1	32	Y	F
FW2	40	P	S
CDR2	50	Y	T
	54	S	T
FW3	73	T	M
	83	S	I
	85	V	L
	Amino acid	V71-2	2A4*
FW1	16	E	Q
	29	V	I
CDR1	33	S	N
FW2	43	P	A
	49	W	G
CDR2	52	Y	R
	54	Y	D
	55	Y	T
	58	S	N
	59	T	I
	60	N	K
FW3	73	V	I

* Previously published data.

pseudogenes. This finding is consistent with the reported high frequency of pseudogenes among human V region genes. We believe that all the 3I⁺ light chains we have described have undergone somatic mutation. Studies on selected lines have shown the absence of CDR nucleotide sequences in the patient's germline DNA, confirming the somatic acquisition of the CDR nucleotide sequences. It is clear, however, that the description of human V region genes is far from complete and that there is substantial allelic polymorphism in the population; therefore, the mutational analysis remains speculative. When 3I⁺ antibody genes are compared to the most homologous germline gene sequences available, they exhibit between 89% and 96% homology to the closest V_K1 germ line gene. Most of the V_K1-encoded 3I⁺ antibodies display high R/S ratios in CDRs, suggesting substantial changes in antigen binding from their germ line–encoded counterparts. The analysis of these antibodies suggests that antigen selection drives the anti-DNA response, and it is possible that the parental B cell may be initially triggered by an antigen only distantly related to DNA. II-1 exhibits 98% homology the nearest V_K3 germline gene, and H2F exhibits 98% homology to the single reported V_K4 germline gene. For these two antibodies, the number of mutations is too small to speculate on whether they represent antigen selection or not.

Our previous studies revealed that the V_H gene utilization among this panel of EBV cell lines was quite heterogeneous (10). Among the antibodies described here, four of the six V_H families are represented: V_H1, V_H3, V_H4, and V_H5. The V_H1 and V_H3 families are the largest families, including >30 genes in each, whereas V_H4 and V_H5 are significantly smaller (6–10 genes and 2–3 genes, respectively) (42). The distribution of V_H gene usage by our panel of antibodies probably reflects on the size of each family and shows no restriction.

The D regions expressed by this group of lines are quite unusual. Only one of the eight lines newly reported, III-3R, expresses an already identified D gene. III-3R, III-2R, and

Table 13. *Replacement Mutations in 3I⁺ V_H5 Sequences*

	Amino acid	251	II-1
FW1	30	T	S
CDR1	31	S	T
	34	T	I
CDR2	50	I	S
	51	I	V
	59	R	T
FW3	75	S	C

2A4 express D-D fusions, a previously reported feature of autoantibodies (18, 32). The remaining six antibodies express D segments of unknown origin. It is possible that these D segments are highly diversified through somatic mechanisms or, alternatively, derive from germline Ds not yet described. J_H usage is possibly restricted as seven of nine lines utilize J_H4.

Most of the expressed V_H genes are 89–98% homologous to previously published functional germ line genes, germ line pseudogenes, or rearranged genes derived from human fetal tissue (presumably germ line in sequence). It is apparent that the data presented in Table 9, showing replacement to silent substitution ratios for both FWs and CDRs, indicate that, similar to what is seen in the light chains, the heavy chains show strong conservation in FW sequences and high CDR substitution rates. However, unlike the light chains, there appear to be few arginine, asparagine, and glutamine replacements, suggesting perhaps that DNA binding by these EBV lines is more a function of the light chain than the heavy chain.

Van Es et al. (43) described an IgG anti-DNA antibody from an EBV-transformed cell line derived from an SLE patient encoded by V_KIIIb and V_H4 genes. Similar to the antibodies we report, this antibody appears to display more replacement mutations in CDRs than in FW regions. Also, like the antibodies we report, only one out of six presumed replacement mutations in the heavy chain CDRs enriches for a potential DNA binding amino acid, while the light chain replacement mutations enrich for arginine and asparagine.

Our earlier studies on myeloma proteins showed that 3I⁺ IgM antibodies are non-DNA binding, while the most 3I⁺ IgG proteins bind to dsDNA (14). This observation on the association of DNA binding with the IgG isotype is in contrast to studies on the EBV lines that we report here, where both IgM and IgG antibodies bind DNA although with varying affinities (10). While IgM myeloma proteins are often encoded by unmutated germ line genes, the IgM antibodies produced by our EBV lines are most probably mutated. This may be due to antigen selection for mutated IgMs or perhaps to a mechanism of hypermutation in SLE.

These studies demonstrate some of the difficulties in structural and genetic studies of antibody responses. While our initial study of a single anti-dsDNA antibody suggested antigen selection and amino acid substitutions leading to an increased affinity for DNA, the analysis of the additional seven DNA binding antibodies reported here suggests that selection for a more cationic antibody or for DNA binding residues in CDRs is not an obvious feature of all or even most of the antibodies. It is probable, however, that multiple forces act on the anti-DNA response and that the selection of the expressed repertoire is determined by forces such as idiotype as well as antigen, DNA, or otherwise. The analysis of a large number of human anti-DNA antibodies, including multiple antibodies from individual patients, shows that there is no single paradigm for explaining structural or molecular genetic features of these autoantibodies. The structural basis of the 3I idiotype cannot be localized from our amino acid sequences. Idiotypic determinants may well be conformation dependent and the accumulation of sequence data alone may be insufficient for idiotype localization. The CDR1 domain of the light chain may well play a critical role in the binding of these antibodies to dsDNA, although this motif alone may not be sufficient. Site-directed mutagenesis of these antibody genes will be necessary to confirm this hypothesis. The analysis of presumed somatic mutations in these antibodies is complex. Human V region genes have not been analyzed extensively enough to make mutational analysis more than suggestive. Nevertheless, the preliminary analysis of these antibodies shows that mutations do not always lead to an increase in dsDNA binding residues or in antibody charge. More information on anti-DNA sequences and the human V region germ line repertoire, including the extent of allelic polymorphism, is necessary in order to understand fully the origins of an autoantibody response and to elucidate the forces that determine the expressed antibody repertoire in SLE.

VH1	III-2R	DPDYVWGSDWFDPWGQGTLVIVSS(DUNK-JH5)
	R3.5H5G	SPLTVTPVGYFDSWGQGTLVTVSS(DUNK-JH4)
VH3	III-3R	GRWERWFGESPPFDYWGQGTLVTVSS(DUNK-DXP'1-N-JH4)
	I-2a	PGKVEIQWELPFDYWGQGTLVTVSS(DUNK-JH4)
	1X7A61	DLTTDVYWGQGTLVTGVSS(DUNK-JH4)
	H2F	DLSYDRGYFDYWGQGTLVTVSS(DUNK-JH4)
VH4	IC4	DSAWPRNFDYWGQGTLVTVSS(DUNK-JH4)
	2A4	DSINGEIARGPRAXGQGYGMDVWGQGTTVTVSSA(DM2-LR3-JH6)
VH5	II-1	RDYNYGYFDPWGQGTLVTVSS(DUNK-JH4)

Figure 9. Junctional nucleotide sequences of heavy chains. Several D regions could not be identified in the literature and are indicated as unknown (UNK).

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