Molecular Characterization of a Somatically Mutated Anti-DNA Antibody Bearing Two Systemic Lupus Erythematosus-related Idiotypes

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Abstract

We report the molecular characterization of 2A4, an IgG, DNA-binding antibody bearing the 3I and F4 idiotypes which are associated with anti-DNA antibodies in serum of patients with systemic lupus erythematosus (SLE). The antibody is produced by an EBV-transformed B cell line derived from a patient with multiple myeloma whose myeloma protein is also an IgG, 3I-reactive, F4-reactive, DNA-binding immunoglobulin, although the 2A4 antibody does not itself represent the myeloma protein. The 2A4 heavy chain is encoded by a V_H4 gene, a D–D gene fusion and the J_H6 gene; the light chain is derived from a V_k1 gene and the J_k2 gene. This is the first human antibody shown to have a CDR3 encoded by a D–D fusion. DNA sequence analysis of the 2A4 V_H gene together with a Southern blot of genomic DNA probed with a 2A4 V_H-specific oligonucleotide strongly suggest it to be somatically mutated. The data provide evidence that human autoantibodies can be products of somatically mutated genes and suggest that the 2A4 antibody may reflect the selective pressure of antigen. (*J. Clin. Invest.* 1990. 85:1401–1409.) anti-DNA antibody • Epstein-Barr virus-transformed B cell • somatic mutation • systemic lupus erythematosus

Introduction

Many sources are contributing to our understanding of the molecular genetic origins of human autoantibodies. Although the data are not yet conclusive, it appears that the germline immunoglobulin genes used to encode autoantibodies exist in nonautoimmune individuals and can be used to encode non-self-reactive antibodies (1–4). There is still considerable controversy, however, whether autoantibodies are encoded by unmutated germ line genes and produced by circulating B cells that are not specifically activated by antigen (2, 5–8), or whether they are encoded by somatically mutated genes which because of increased affinity for antigen are selected during a secondary B cell response (9–11).

We have been studying anti-DNA antibodies, the serologic hallmark of the disease systemic lupus erythematosus (SLE).[1] Analyses of anti-DNA antibodies from genetically unrelated SLE patients have demonstrated the existence of cross-reactive idiotypes (12–14) suggesting that anti-DNA antibodies are encoded by a restricted set of germline genes. The heterogeneity of serum anti-DNA antibodies precludes a detailed study of their fine specificity or of the amino acid sequences of their variable regions. Without such information no conclusions can be drawn with respect to the relationship of high-affinity DNA-binding antibodies to germline variable region genes. We have previously exploited the availability of human myeloma proteins to explore the role of somatic mutation in the production of anti-DNA antibodies (11, 15). We examined myeloma proteins for the expression of two anti-DNA-associated idiotypes, 3I and F4. The 3I idiotype is present on κ light chains of anti-DNA antibodies, and the F4 idiotype is present on the heavy chain variable region. We found that within the 3I idiotype system, DNA binding is associated predominantly with IgG antibodies and is a feature of only a very few IgM antibodies (15). We further found that coexpression of 3I and F4 reactivity is highly associated with DNA binding. Although 3I reactivity is present on both IgM and IgG antibodies, F4 reactivity is almost exclusively found on IgG antibodies (11).

Hybridoma anti-DNA antibodies have been obtained from fusions of peripheral blood lymphocytes of SLE patients to a human drug-marked myeloma cell line (16–18). The antibodies generated in this fashion are almost exclusively of the IgM isotype. Although these appear to reflect the unmutated germ line gene sequences, they are not representative of the high-affinity IgG antibodies that are characteristically found in lupus sera and are presumed to represent the pathogenic autoantibodies (19, 20). We decided to transform bone marrow B cells from a patient with a 3I- and F4-reactive, DNA-binding IgG myeloma in the hope of obtaining a B cell line producing a 3I- and F4-reactive anti-DNA antibody. Previous studies have shown that patients with myeloma possess in their bone marrow B cells at various stages of maturation expressing the idiotype of the myeloma protein (21, 22). These B cells may be precursors of the malignant plasma cell before expression of a fully malignant phenotype (21, 23) or they may be related to the malignant plasma cell through an idiotypic network (22). Using EBV transformation to immortalize the cells, we obtained both 3I-reactive IgM and IgG-producing lines and 3I- and F4-reactive, IgG-producing lines. Some of the lines were DNA binding.

We now report the cloning and sequencing of the heavy and light chain genes of 2A4, a 3I- and F4-reactive line that encodes a DNA-binding IgG antibody that is not identical to the myeloma protein. It uses a member of the V_H4 family, DLR3-DIR2 and J_H6 to encode the heavy chain V region and a V_{K1} and J_{K2} to encode the light chain V region. Both the heavy and light chain V region sequences show many differences from the most homologous germline genes yet identified. Whereas the germ line V_H from which the 2A4 V_H is most likely derived is already cloned and sequenced (24), to date no

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1. *Abbreviations used in this paper:* CDR, complementarity-determining region; FR, framework region; SLE, systemic lupus erythematosus.

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germline V_K sequence bearing sufficient homology to the gene that encodes the 2A4 light chain has been reported. A high replacement/silent mutation ratio in the heavy chain complementarity-determining regions (CDRs), compared with that in the framework regions (FRs), suggests that the 2A4 antibody has arisen in an antigen-selected response.

Methods

Generation of EBV-transformed B cell lines. Lymphocytes were obtained by Ficoll-Hypaque centrifugation of bone marrow cells of patient Hic with multiple myeloma. The patient's DNA-binding myeloma protein is an IgG antibody and expresses both the F4 and the 3I idiotypic determinants.

Lymphocytes 10^6/ml were suspended in RPMI media (Mediatech, Washington, DC), supplemented with 10% FCS (Hyclone Laboratories, Logan, UT), 1% glutamine, 1% penicillin streptomycin, 1% sodium pyruvate, 1% nonessential amino acids, and 0.01 M Hepes. Aliquots of 1 ml were transformed with 200 μl of supernatant from a dense culture of the EBV-secreting marmoset cell line B95.8 (American Type Culture Collection, Rockville, MD) and 0.5 μg of cyclosporin A. After 2 wk, bulk culture supernatants were screened by ELISA for idiotype expression. Plates (Probind, Falcon Labware, Lincoln Park, NJ) coated with pure F4 or 3I anti-idiotype at a concentration of 10 μg/ml in PBS (0.02 M $NaPO_4$, 0.15 M NaCl, pH 7.2) at 4°C overnight, were washed and blocked with PBS containing 5% FCS, 3% BSA for 1 h and then incubated with 100 μl culture supernatant at 37°C for 1 h. Plates were washed and incubated with peroxidase conjugated affinity-purified goat anti-human immunoglobulins (Cappel Laboratories, Westchester, PA) at a 1/750 dilution in PBS 1% BSA followed by 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)] substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Purified idiotype-positive myeloma proteins and media alone were used as controls. Bulk cultures expressing either the F4 or 3I idiotype were then subcloned into round-bottom plates (Corning Glass Works, Corning, NY) in medium supplemented with 10% FCS and 20% supernatant from the murine macrophage cell line J774.2 (25) at a dilution such that less than one-third of the wells yielded viable clones. Clones were rescreened for idiotype expression.

Assay for Ig isotype. Clonal lines were screened for immunoglobulin isotype by ELISA. Plates (Probind) were coated overnight at 4°C with each of goat anti-human IgM, IgG, and IgA (Meloy Laboratories, Inc., Springfield, VA) at a concentration of 10 μg/ml in PBS. Plates were blocked with PBS containing 5% FCS/3% BSA and incubated with cell supernatant followed by peroxidase-conjugated goat anti-human immunoglobulin (Cappel Laboratories) at a concentration of 1/750 in PBS/1% BSA and then 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)] substrate. Myeloma proteins of known isotype and media alone were used as controls.

Assays for DNA binding. Cell lines were grown in serum-free medium and tested for DNA binding by filter assay (Millipore Corp., Bedford, MA). Supernatant from a serum-free culture was quantitated for IgG content by standardization with known quantities of purified IgG. For the filter assay IgG was incubated for 30 min at 37° with nick-translated ^{32}P-labeled DNA made double-stranded by passage through a nitrocellulose filter (HAWP 45, Millipore Corp.). The mixture was then passed through a nitrocellulose filter, and the filter was assayed for bound radioactivity.

Cloning and sequencing of 2A4 heavy and light chain genes. RNA was extracted from 10^9 cells of the 2A4 clone and the 4B4 clone by standard techniques using 4M guanidium thiocyanate followed by layering on a 5.7 M cesium chloride gradient. Polyadenylated RNA was purified on an oligo-dT column (Collaborative Research Inc., Bedford, MA). Preliminary mRNA sequencing of heavy and light chains was performed using the dideoxy termination method (26). The V_H was found to belong to the V_H4 family and the V_K to the V_K1 family. A cDNA library of each clone was generated in λgt10 using 5' Cγ and Cκ probes to generate the first strand cDNA. 5×10^5 plaques from each library were screened with a 5' V_H4-specific probe (obtained from Drs. J. Berman and F. Alt, Columbia University) (27) and with an oligonucleotide probe directed to a conserved 20-basepair region of the leader sequence of the V_K1 gene family 5' GCAGGAGCCCCAGGAGCTGA 3' (28). Appropriate clones were plaque purified and subcloned into pGEM vectors (Promega Biotec, Madison, WI.) for sequencing. Dideoxy chain termination sequencing of both strands was performed using a standard kit (Sequenase, United States Biochemical Corp., Cleveland, OH).

Southern blot analysis. Cells from wells positive for idiotype expression were screened for clonality by Southern blotting with J_K and J_H probes. Genomic DNA was extracted from cells. 10 μg of DNA was digested with EcoRI and Bam HI restriction enzymes and electrophoresed at 50 V overnight. DNA was blotted onto nitrocellulose and probed overnight at 42°C with human J_H (obtained from Dr. F. Alt) and J_K probes (obtained from Dr. P. Leder, Harvard University) radiolabeled with ^{32}P by nick-translation. Blots were then washed in 2× SSC 1% SDS at 68°C and autoradiographed. Those cloned cell lines showing no more than two rearrangements of both J_H and J_K loci were selected for further characterization.

Southern blot analysis using oligonucleotide probes. To determine if the heavy chain-variable region genes utilized by the cell line 2A4 is a somatic mutant of germline genes, Southern analysis using an oligonucleotide probe to the CDR2 region of the heavy chain 5' ATCGATACCAGTGGGAACAT 3' was performed. Briefly, 30–50 μg of genomic DNA prepared from Hic bone marrow or from the cell line 2A4 was digested with BamHI. DNA was fractionated on a 0.8% agarose gel. After electrophoresis, DNA was transferred from the gel to gene screen (New England Nuclear Research Products, Boston, MA) overnight. Subsequently, filters were baked, pretreated, with 5× SSC, 20 mM PO_4, 7% SDS, 10× Denhardt's, 1 mg/ml salmon sperm DNA, hybridized (12.5% dextran sulfate) to the oligonucleotide probe at Tm-5°C, washed 1 h in 3× SSC, 20 mM PO_4, 5% SDS, 10× Denhardt's followed by 1 h in 1× SSC 1% SDS, and autoradiographed as previously described (29).

Results

EBV-transformed B cell lines were readily obtained. The characteristics of these cells lines will be reported elsewhere. Several 3I- and F4-reactive clones were obtained. Although some of these lines represent independent clones as demonstrated by Southern blot analysis, not all represent independent clones as bulk cultures were maintained several weeks before cloning. The 2A4 cell line was selected for molecular study because of expression of both the 3I and the F4 idiotype, DNA binding, and IgG isotype, (Tables I and II) all characteristics of pathogenic antibodies found in serum and kidneys of SLE patients (19, 20). Clonality of the line was confirmed by Southern blotting using J_H and J_K probes to identify immunoglobulin gene rearrangements. Two heavy chain gene rearrangements and two light chain gene rearrangements were identified (Fig. 1). A second 3I- and F4-reactive DNA IgG binding clone, 4B4, was also analyzed. It had identical J_H and J_k rearrangements. These rearrangements both differ from the immunoglobulin gene rearrangements that are responsible for the myeloma protein (data not shown) so the 2A4/4B4 antibody does not represent the Hic myeloma protein.

Cloning of heavy and light chain genes. Preliminary mRNA sequencing of 2A4 revealed that the 2A4 heavy chain is encoded by a member of the V_{H4} gene family and that the light chain is encoded by a member of the V_{K1} family. Probes specific for these gene families were therefore used to screen cDNA libraries from the 2A4 and 4B4 lines. The sequence of the entire heavy and light chain variable regions including the leader sequence of each line was obtained. Inasmuch as the

Table I. 2A4 Expresses 3I and F4 Reactivity

	OD 405	
	3I	F4
Negative control	0.140±0.021	0.136±0.053
Positive control	0.620±0.219	0.391±0.141
2A4	0.448±0.016	0.442±0.013

1 μg of 3I or F4 antibody was adsorbed to microtiter wells. 2A4 antibody or purified IgG myeloma proteins (1 μg/ml) ($3I^-$ $F4^-$ and $3I^+$ $F4^+$) were added, followed by antibody to human Ig and substrate.

sequences obtained from the two lines were identical, we will discuss the 2A4 line. The 2A4 sequences were compared with known V_{H4} and V_{K1} germline gene sequences. Nine germline sequences have thus far been reported for the V_H4 gene family (24, 27, 30). The 2A4 heavy chain is most homologous to the V71-2 germline gene reported by Lee et al. (24) (Fig. 2). Analysis of the ratio of replacement to silent mutations is shown in Table III. 15 base changes occur in FRs; 5 are replacement mutations and 10 are silent. All nine mutations in CDRs are replacement mutations. The derived protein sequence for the 2A4 heavy chain is shown in Fig. 3. The heavy chain variable region differs from the derived V71-2 sequence by 12 amino acids, four of which represent nonconservative changes resulting in a net accumulation of two positive charges.

The D region of 2A4 appears to be a fusion of two human D region genes, DLR3 and a DIR gene upstream of DM2. The DIR gene is a member of a newly described D gene family. This family is characterized by 12–23 spacers rather than the usual 12–12 D region spacers. These unusual spacers may allow for D–D joining (31). 2A4 has 15 of 18 nucleotides in common with the DLR3 and 16 of 20 nucleotides in common with the DIR2 gene. The 2A4 D region has an initial 5 nucleotides that may represent N sequences, 18 nucleotides from DLR3, an inserted cytosine, then 20 nucleotides from the DIR2 gene followed by another six bases that may represent N sequences (Fig. 4).

The DLR3 sequence has base changes leading to the substitution of a methionine for a valine, a glycine for a valine,

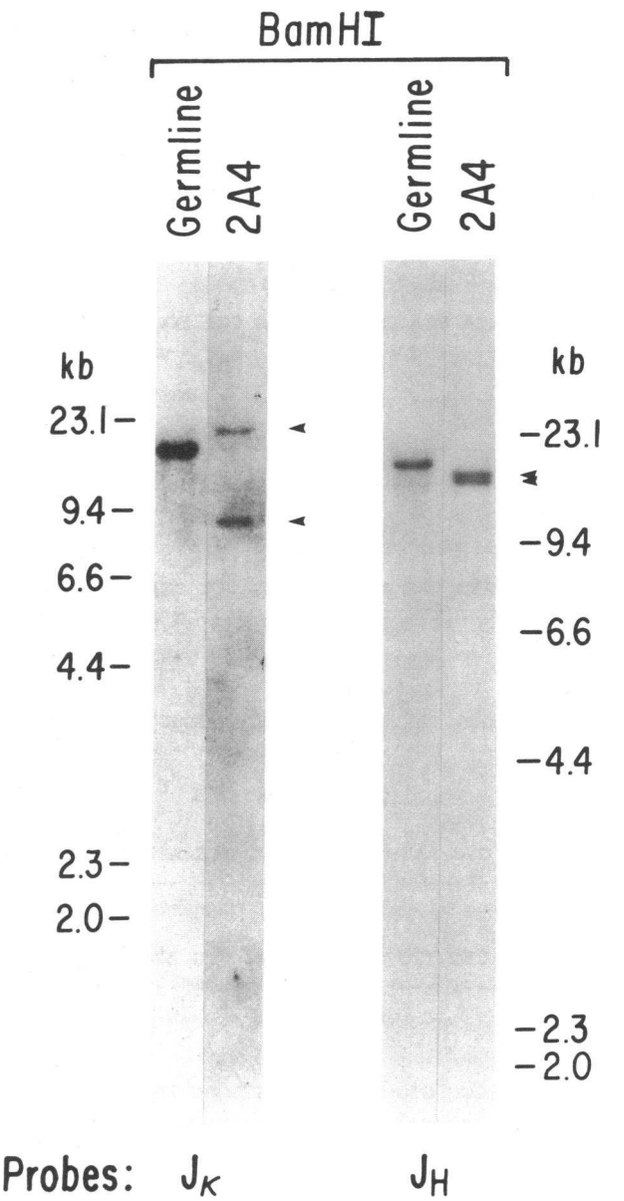

Figure 1. Southern analysis of germline DNA and heavy and light chain rearrangements of the 2A4 DNA digested with BamHI and probed with J_H and J_K probes. There are two heavy chain and two light chain rearrangements identified by the arrows. Germline J_K is 12 kb and germline J_H is 17 kb. Samples were electrophoresed on two different gels.

Table II. 2A4 Binds DNA by Millipore Filter Assay

	Dilution	Bound radioactivity
		cpm
2.5 μg/ml normal IgG	1:1	277
	1:2	395
	1:5	394
2.5 μg/ml 2A4 IgG	1:1	1,612
	1:2	1,263
	1:5	1,153
Medium alone		16

2A4 supernatant or purified normal human IgG diluted in serum-free media was incubated with ^{32}P-labeled double-stranded DNA and passed through a nitrocellulose filter. Bound radioactivity represents anti-DNA activity.

and a glutamic acid for a valine. These substitutions result in the net accumulation of one negative charge more than in the sequence encoded by the DLR3 germline sequence. The insertion of a cytosine as the first base in the DIR2 sequence leads to the substitution of an arginine for a glycine. In addition, there is a substitution downstream replacing a glutamine with a lysine and thus generating a net of two positive charges in the second half of the D region.

In an attempt to prove that the 2A4 antibody is indeed the product of somatic mutation, we synthesized an oligonucleotide probe identical to the CDR2 of the 2A4 heavy chain. This oligonucleotide was used to probe DNA from the 2A4 cell line and Hic bone marrow genomic DNA. The oligonucleotide

```
        Leader
        -19
V71-2   ATG AAA CAC CTG TGG TTC TTC CTC CTC CTG GTG GCA GCT CCC
2A4     --- --- --t --- --- --- -GG --- --- --- --- --- --- ---

                                  Framework 1
                                    1
V71-2   AGA TGG GTC CTG TCC         CAG GTG CAG CTG CAG GAG TCG
2A4     --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                 10                                        20
V71-2   GGC CCA GGA CTG GTG AAG CCT TCG GAG ACC CTG TCC CTC ACC
2A4     --- --- --g --- --- --- --- --a C-- --- --- --- --- ---

                                           CDR1
                                         30
V71-2   TGC ACT GTC TCT GGT GGC TCC GTC AGC AGT GGT AGT TAC TAC
2A4     --- --- --- --- --- --t --- A-- --- --- --- -A- --- ---

                Framework 2
                         40
V71-2   TGG AGC TGG ATC CGG CAG CCC CCA GGG AAG GGA CTG GAG TGG
2A4     --- --- --- --- --t --- --- G-c --- --- --- --- --- G-c

                CDR2
        50                                          60
V71-2   ATT GGG TAT ATC TAT TAC AGT GGG AGC ACC AAC TAC AAC CCC
2A4     --- --- CG- --- G-- AC- --- --- -A- -T- --G --- --- ---

                         Framework 3
                                   70
V71-2   TCC CTC AAG AGT CGA GTC ACC ATA TCA GTA GAC ACG TCC AAG
2A4     --- --- --- --- --- --g --- --- --- A-- --- --- --- ---

                80                                            90
V71-2   AAC CAG TTC TCC CTG AAG CTG AGC TCT GTG ACC GCT GCG GAC
2A4     --- --a --- --- --- --- --- --- --- --- --- --c --a ---

                                         CDR3 (D region)
                                         100
V71-2   ACG GCC GTG TAT TAC TGT GCG AGA
2A4     --- --- --- --- --- --- --- --- GAC TCT ATT ATG GGG GAG

                                                     J region
J 6                                                  TAC GGT ATG
2A4     ATT GCT CGG GGA CCC CGA GCT AAG GGC CAG GGC --- --- ---

                Framework 4                                    Constant

J 6     GAC GTC TGG GGG CAA GGG ACC ACG GTC ACC GTC TCC TCA GCC
2A4     --- --- --- --c --- --- --- --- --- --- --- --- --- ---
```

Figure 2. The DNA sequence of 2A4 heavy chain is compared with the sequence of the germline V71-2 and J6. Replacement mutations are indicated by upper case letters and silent mutations by lower case letters. There are 14 replacement mutations and 10 silent mutations in the V gene. These sequence data are available from EMBL/GenBank/DDBJ under accession number M33061.

hybridizes to a band representing the rearranged V_H4 gene from the 2A4 cell line, but does not hybridize to any band representing a V_H4 germ line gene in the Southern blot of the patient's own (Hic) genomic DNA (Fig. 5). The heavy chain CDR2 must therefore contain sequences that vary from germline sequences present in the patient's own DNA.

For the V_k1 gene family, 15 of approximately 20–30 germline sequences have been reported of which nine are functional genes (28, 32). The 2A4 light chain gene has 45 base differences from the Hk101 gene and 44 from the Hk 102 gene (Fig. 6). The human V_{K1} gene family is a large family and from comparison of the 2A4 sequence to known V_{k1} sequences it appears unlikely that the appropriate germline gene has yet been isolated and sequenced. Thus an analysis of replacement and silent mutations cannot be performed for the light chain of 2A4. However, the J segment sequence of 2A4 differs from germline J_K2 by four base changes resulting in two replacement and two silent mutations.

Discussion

The B cell response to antigen is a complex one. The primary response to antigen is mediated by antibodies of the IgM iso-

```
        Framework
         1                                   10
V71-2   GLN VAL GLN LEU GLN GLU SER GLY PRO GLY LEU VAL LYS PRO
2A4     --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                           20
V71-2   SER GLU THR LEU SER LEU THR CYS THR VAL SER GLY GLY SER
2A4     --- GLN --- --- --- --- --- --- --- --- --- --- --- ---

                CDR 1                          Framework 2
            30                                         40
V71-2   VAL SER SER GLY SER TYR TYR TRP SER TRP ILE ARG GLN PRO
2A4     ILE --- --- --- ASN --- --- --- --- --- --- --- --- ---

                                    CDR 2
                                    50
V71-2   PRO GLY LYS GLY LEU GLU TRP ILE GLY TYR ILE TYR TYR SER
2A4     ALA --- --- --- --- --- GLY --- --- ARG --- ASP THR ---

                                                       Framework 3
                     60                                          70
V71-2   GLY SER THR ASN TYR ASN PRO SER LEU LYS SER ARG VAL THR
2A4     --- ASN ILE LYS --- --- --- --- --- --- --- --- --- ---

                                           80
V71-2   ILE SER VAL ASP THR SER LYS ASN GLN PHE SER LEU LYS LEU
2A4     --- --- ILE --- --- --- --- --- --- --- --- --- --- ---

                           90
V71-2   SER SER VAL THR ALA ALA ASP THR ALA VAL TYR TYR CYS ALA
2A4     --- --- --- --- --- --- --- --- --- --- --- --- --- ---

            CDR 3 (D region)
V71-2   ARG 100
2A4     --- ASP SER ILE MET GLY GLU ILE ALA ARG GLY PRO ARG ALA

                        J region            Framework 4
J6                      TYR GLY MET ASP VAL TRP GLY GLN GLY THR
2A4     LYS GLY GLN GLY --- --- --- --- --- --- --- --- --- ---

                                  Constant
J6      THR VAL THR VAL SER SER ALA
2A4     --- --- --- --- --- --- ---
```

Figure 3. The derived protein sequences of the V71-2 and 2A4 heavy chains are compared. These sequence data are available from EMBL/GenBank/DDBJ under accession number M33060.

type that are present in the preimmune repertoire and generally reflect germline gene sequences. As the immune response develops and B cells interact with both antigen and activated T helper cells, B cells with higher affinity for antigen are generated by somatic mutation and are selected by antigen for clonal expansion (33). This hypermutation process is most frequently seen in IgG antibodies. It has long been debated whether autoantibodies found in autoimmune diseases are the products of nonspecific B cell activation and therefore reflect the low-affinity, unmutated IgM antibodies found in the preimmune repertoire (8) or whether they arise in the course of an immune response to a specific antigen and reflect the higher-affinity mutated, IgG antibodies of the secondary response (11). Molecular analysis of autoantibodies is one approach to resolving this problem.

We have previously demonstrated that anti-DNA antibodies found in patients with SLE share cross-reactive idiotypes (11, 12). This observation suggests that these antibodies are encoded by a restricted number of germline genes. The 3I monoclonal anti-idiotype recognizes K chains of anti-DNA antibodies in 85% of SLE patients with anti-DNA activity (34). The F4 anti-idiotype recognizes heavy chains on anti-DNA antibodies in 60% of SLE patients. Studies of myeloma proteins and SLE sera have revealed that coexpression of both 3I and F4 idiotypes is highly associated with DNA binding activity (11). Furthermore, our studies of myeloma proteins revealed that DNA binding is present almost exclusively on antibodies of the IgG isotype and is associated in the 3I idiotype system with cationic immunoglobulins (15). These studies have led us to hypothesize that DNA binding activity may reflect the accumulation of somatic mutations that occurs after exposure to antigen and T cell factors. For this reason, we wished to obtain the sequence of DNA binding antibodies bearing SLE related idiotypes and compare them with known germline gene sequences.

Myeloma proteins frequently display autoreactivity. In addition, they often express autoantibody associated idiotypes. It is not known why the repertoire of malignant B cells is skewed towards autoreactivity compared with the repertoire of serum immunoglobulin. It is also not understood why individuals

```
Germline DLR3    AG CAT ATT GTG GTG GTG ATT GCTATTCC
2A4             G-C TC- --- A-- -G- -A- --- --
                N sequence

Germline DIR    CACCCAGGA GGC CCC AGA GCT CAG GGC GCC CCG
2A4                   C-g --a --- c-- --- A-- --- CAG GGC
                      N sequence                  N sequence
```

Figure 4. The complete 2A4 D sequence is compared with the germline DLR3 and DIR2 genes. The 2A4 D region sequence has 5 nucleotides of N sequence followed by 15 of 18 nucleotides homologous to DLR3, an inserted C and 16 of 20 nucleotides homologous to DIR2 followed by another 6 nucleotides of N sequence. Upper case letters indicate replacement mutations; lower case letters indicate silent mutations. These sequence data are available from EMBL/GenBank/DDBJ under accession number M33061.

with autoreactive myeloma proteins, like family members of SLE patients, appear to have no stigmata of autoimmune disease despite their circulating autoantibodies. Nevertheless, myeloma proteins have been used as a model for autoantibodies. While the Hic myeloma protein from this same patient is a 3I- and F4-reactive anti-DNA antibody, its light chain is most likely is encoded by a V_K3 rather than a V_K1 gene (35). The Hic myeloma protein therefore differs from the 2A4 antibody. Furthermore, the myeloma protein has lower affinity for DNA, perhaps accounting in part for the lack of SLE-related symptoms in the patient. We have previously found by protein sequence analysis that 3I-reactive light chains from different patients can be encoded by V_K genes of more than one gene family (35). The presence of nontransformed B cells in the bone marrow of patients with myeloma that are idiotypically related to the myeloma cells have been reported by several laboratories (21–23). Although it has been suggested that these are precursors to the myeloma cell, it is possible that they represent multiple independent clones activated by an idiotypic network. This study shows that 3I reactivity can be encoded by more than one light chain gene in an individual patient and lends support to the hypothesis that there may be multiple independent B cell clones in a myeloma patient all producing antibodies expressing the myeloma idiotype. It is likely that in this patient with a 3I-reactive myeloma protein, 3I-reactive nonmalignant B cells might be generated through the presence of an idiotypic network. It is not clear that all these B cells secrete immunoglobulin in vivo.

The human immunoglobulin gene repertoire is large and includes 50 to several hundred variable region genes in each

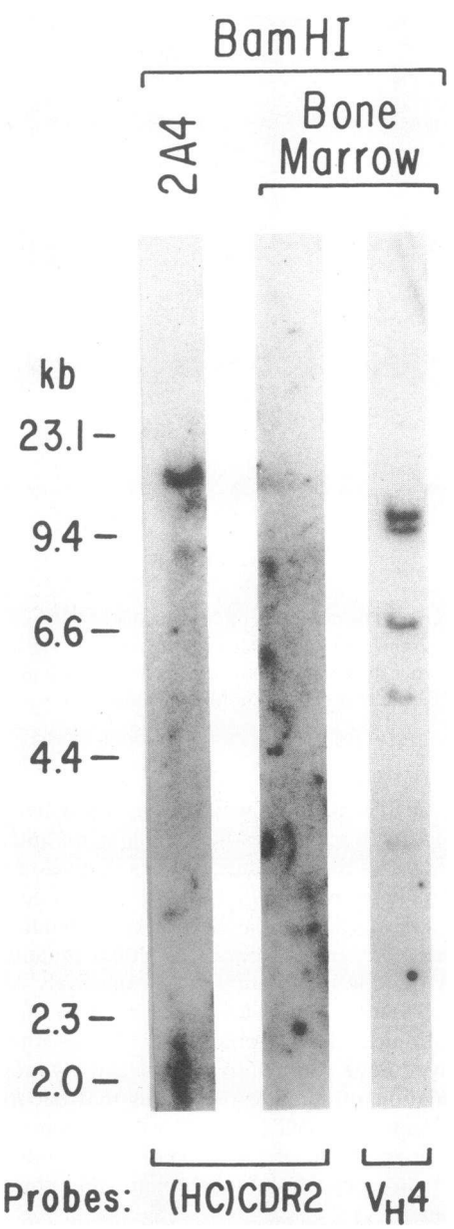

Figure 5. Southern analysis of 2A4 DNA and Hic germline DNA probed with a 2A4 V_H CDR2-specific oligonucleotide. The oligonucleotide probe hybridizes to the 2A4 heavy chain DNA but not to Hic germline DNA. DNA is present in Hic germline blot as indicated by hybridization to V_{H4} germline bands.

Table III. Replacement Mutations in the 2A4 Heavy Chain V Region

	V71-2	2A4
FR1	Glu (−)	*Gln (P)
	Val (NP)	Ile (NP)
CDR1	Ser (P)	Asn (P)
FR2	Pro (NP)	Ala (NP)
	Trp (NP)	Gly (P)
CDR2	Tyr (P)	*Arg (+)
	Tyr (P)	*Asp (−)
	Tyr (P)	Thr (P)
	Ser (P)	Asn (P)
	Thr (P)	Ile (NP)
	Asn (P)	*Lys (+)
FR3	Val (NP)	Ile (NP)

Comparison of amino acid differences between the derived protein sequence of germline V71-2 (24) and the 2A4 heavy chain. Nonconservative changes are indicated by an asterisk. The 2A4 V region has a net gain of two positive changes compared with V71-2. Abbreviations: P, polar; NP, nonpolar.

	Leader													
	-19													
Hk101	ATG	GAC	ATG	AGG	GTC	CCC	GCT	CAG	CTC	CTG	GGG	CTC	CTG	CTG
2A4	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Hk102	---	---	---	---	---	---	---	---	---	---	---	---	---	---
									Framework 1					
									1					
Hk101	CTC	TGT	TTC	CCA	GGT	GCC	AGA	TGT	GAC	ATC	CAG	ATG	ACC	CAG
2A4	---	--G	C--	-G-	A--	---	---	---	---	C--	A--	---	---	---
HK102	---	--G	---	---	---	---	-A-	---	---	---	---	---	---	---
				10										20
Hk101	TCT	CCA	TCC	TCA	CTG	TCT	GCA	TCT	GTA	GGA	GAC	AGA	GTC	ACC
2A4	---	---	--A	--C	---	C--	---	---	--T	---	---	-C-	---	---
HK102	---	--T	---	A-C	---	---	---	---	---	---	---	---	---	---
			CDR1											
										30				
Hk101	ATC	ACT	TGT	CGG	GCG	AGG	CAG	GGT	ATT	AGC	AGC	TGG	TTA	GCC
2A4	---	---	--C	---	--A	--T	---	A-C	---	-A-	--G	-TT	---	AAT
HK102	---	---	--C	---	--C	--T	---	A--	---	--T	---	---	--G	---
	Framework 2													
						40								
Hk101	TGG	TAT	CAG	CAG	AAA	CCA	GAG	AAA	GCC	CCT	AAG	TCC	CTG	ATC
2A4	---	---	---	---	---	---	-G-	---	---	---	---	CT-	---	---
HK102	---	---	---	---	---	---	-G-	---	---	---	---	CT-	---	---
		CDR2						Framework 3						
		50										60		
Hk101	TAT	GCT	GCA	TCC	AGT	TTG	CAA	AGT	GGG	GTC	CCA	TCA	AGG	TTC
2A4	---	-G-	---	---	---	---	---	---	---	T--	T-T	---	---	--T
HK102	---	-A-	--C	---	---	---	G--	---	---	---	---	---	---	---
								70						
Hk101	AGC	GGC	AGT	GGA	TCT	GGG	ACA	GAT	TTC	ACT	CTC	ACC	ATC	AGC
2A4	--T	---	---	---	---	---	---	--G	---	---	---	---	---	--A
HK102	---	---	---	---	---	---	---	--A	---	---	---	---	---	---
													CDR 3	
				80										90
Hk101	AGC	CTG	CAG	CCT	GAA	GAT	TTT	GCA	ACT	TAT	TAC	TGC	CAA	CAG
2A4	--T	---	--A	---	--G	---	---	---	---	--C	---	--T	---	--A
HK102	---	---	---	---	--T	---	---	---	---	---	---	---	---	---
									Framework 4					
						95A	J2				100			
Hk101	TAT	AAT	AGT	TAC	CCT		TAC	ACT	TTT	GGC	CAG	GGG	ACC	AAG
2A4	AG-	T--	---	AC-	---	CCG	G--	---	---	---	---	---	---	---
HK102	---	---	---	--T	T--									
					108									
Hk101	CTG	GAG	ATC	AAA	CGT									
2A4	G--	--a	---	---	--a									

Figure 6. 2A4 light chain DNA sequence compared with HK101 and HK102 genes (28, 32). There are 45 differences from HK101 and 44 from HK102. These sequence data are available from EMBL/GenBank/DDBJ under accession number M33060.

variable region gene locus (27, 28). There may be little polymorphism among individuals (27, 36). The human V_{H4} gene family is a relatively small family with approximately 6–10 different members as demonstrated by restriction enzyme analysis. Nine different V_H4 germline genes, sequenced from two individuals, are described in the literature (24, 27). Analysis of the Hic and 2A4 genomic Southern blots by using a mutant specific oligonucleotide proves that the 2A4 V_H gene is somatically mutated as the 2A4-specific probe does not hybridize to the patient's own germline DNA. The DNA sequence of the 2A4 V_H gene segment is highly homologous to the V 71-2 germline gene (24). There are 24 basepair differ-

ences of which 10 result in silent mutations. Only replacement mutations are seen in the CDRs, suggesting that these mutations are part of an antigen selected response. Furthermore, these mutations produce a more positively charged variable region consistent with our previous demonstration within the 3I idiotype system that DNA binding is associated with cationic antibodies (1) and that 3I-reactive, F4-reactive DNA-binding antibodies are, in general, cationic (11). The mutations also lead to the substitution of a glutamine for a glutamate in framework 1, an asparagine for a serine in CDR1, an arginine for a tyrosine and an asparagine for a serine in CDR2, a glutamine for a lysine and an arginine for a glycine in CDR3. Arginine can form two hydrogen bonds with guanine in double-stranded DNA and glutamine and asparagine can hydrogen bond to adenine and so, as postulated by Seeman et al. (37), these amino acids may be important in generating anti-DNA activity. The V_H4 gene family has been reported in one other anti-DNA antibody C6B2 (18). This V_H4 sequence also appears to be encoded by the V71-2 germline gene. It is not known whether the differences between V71-2 and C6B2 represent polymorphisms of the germline gene or whether C6B2 is also encoded by a somatically mutated V_H4 gene.

The 2A4 heavy chain possesses an unusually long D region which is generated by the joining of two distinct D region gene segments. Although DlR genes have been reported in two previous immunoglobulin heavy chain rearrangements (31), to our knowledge this is the first human antibody to be encoded by a D–D fusion. Eilat et al. (38) have shown that D regions in anti-DNA antibodies may be used out of frame and that they may be mutated to generate arginine residues (38). Sanz and Capra (39) have postulated that murine autoantibodies frequently contain abnormally long D regions generated by unusual genetic events such as D–D joining or by inverted D regions (39). It may be that similarly unusual D regions may be common in human autoantibodies.

The human V_K1 family is a large gene family comprising at least 20 members. Of these, nine potentially functional genes and six pseudogenes have thus far been sequenced. The 2A4 light chain is most homologous to the H_{K101} and H_{K102} (28, 32) genes. However, the many base differences between the 2A4 light chain and the V_K1 germline sequences suggest that the 2A4 light chain V region probably derives from a yet uncharacterized germline gene.

The nature of the mutations present in the 2A4 antibody suggest that the antibody has been exposed to the selective forces of antigen and perhaps T cells as well. It will be of interest to compare this antibody to one encoded by the unmutated germ line genes to know how the affinity for DNA is changed. Such analyses have been performed in other antibody systems (33, 40). Perhaps the analysis of B cell genealogies from a single patient or of a large panel of idiotypic antibodies from a number of patients can help resolve the question of the role of DNA as an antigen in the production of DNA binding antibodies.

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