Ligand Recognition by Anti-DNA Autoantibodies. Affinity, Specificity, and Mode of Binding†

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ABSTRACT: Understanding the molecular basis of DNA recognition by anti-DNA autoantibodies is a key element in defining the role of antibody·DNA complexes in the pathogenesis of the autoimmune disorder systemic lupus erythematosus. As part of our efforts to relate anti-DNA affinity and specificity to antibody structure, and ultimately to disease pathogenesis, we have generated a panel of eight anti-DNA mAbs from an autoimmune MRL MpJ-*lpr*/*lpr* mouse and have assessed the binding properties of these antibodies. We find that none of our anti-DNA mAbs bind to RNA and only one low-affinity mAb cross-reacts with non-DNA antigens, albeit weakly. None of the mAbs in our panel bind double-stranded DNA exclusively. Antibodies that recognize single-stranded DNA can be categorized into two groups based on their affinity and apparent mode of binding. One group possesses relatively high affinity for oligo(dT) and may recognize single-stranded DNA ligands by accommodating thymine bases in hydrophobic pockets on the antigen binding site. The second group binds more weakly, apparently recognizes single-stranded DNA nonspecifically, and in some cases also binds double-stranded DNA. Although different mechanisms are used for binding single- and double-stranded ligands, the mode of DNA recognition appears conserved within groups of antibodies.

Anti-DNA autoantibodies (anti-DNA)[1] are a hallmark of the autoimmune disorder systemic lupus erythematosus (SLE) (Tan, 1989). A small population of these autoantibodies is believed to mediate the renal damage associated with SLE through formation of anti-DNA·DNA immune complexes that localize to substructures within kidney tissue (Vlahakos et al., 1992; Foster et al., 1993). Both direct evidence and indirect evidence indicate that for the subset of pathogenic anti-DNA, the affinity for DNA or DNA-containing antigens *in vivo* is correlated with renal pathogenesis (Koffler et al., 1971; Foster et al., 1993; Katz et al., 1994). Consequently, defining a link between antigen recognition (i.e., affinity, specificity, and mode of DNA binding) and pathogenesis is a central goal of SLE research (Koffler et al., 1967, 1971; Tsao et al., 1990; Foster et al., 1993). On a practical level, understanding the relationship between binding and pathogenesis will be invaluable in guiding the development of new agents to treat SLE, like low molecular weight antagonists that inhibit DNA binding (Ben-Chetrit et al., 1988), and may also furnish a means to distinguish, *a priori*, pathogenic anti-DNA from those that are benign (Ohnishi et al., 1994). Furthermore, this information will provide insight about how the antibody combining site, a relatively uncharacterized locus with respect to nucleic acid binding, affects molecular recognition of single-stranded and double-stranded DNA ligands (Eilat & Anderson, 1994).

The origins of lupus anti-DNA are not fully understood (Schwartz & Stollar, 1985; Isenberg et al., 1994). However, several groups have demonstrated that in some strains of lupus-prone mice, defects in the Fas gene (or Fas ligand) allow anti-DNA B cells that are normally deleted to survive (Nagata & Golstein, 1995), and Fas mutations also impair apoptosis in some human autoimmune syndromes (Fisher et al., 1995). Clonal expansion and affinity maturation of these anti-DNA driven by DNA or DNA-containing molecules then produce a large group of anti-DNA, most of which are benign (Marion et al., 1992; Mohan et al., 1993; Radic & Weigert, 1994). In this regard, the anti-DNA autoimmune response resembles a normal immune response against exogenous antigens like bacteria and viruses.

While several hundred lupus anti-DNA mAbs from both human and murine sources have been reported (Radic & Weigert, 1994), the general features of DNA epitopes, the parameters that distinguish high- and low-affinity anti-DNA, and the molecular mechanisms used for DNA recognition are not well-defined (Eilat & Anderson, 1994). As part of our efforts to relate anti-DNA affinity and specificity to antibody structure, and ultimately to renal pathogenesis, we have generated a panel of eight anti-DNA mAbs from an autoimmune MRL MpJ-*lpr*/*lpr* mouse (MRL-*lpr*), and we present here a preliminary assessment of these antibodies. A novel aspect of this work is that in addition to employing techniques and concepts developed to study antibody·antigen interactions, we have examined our mAbs with experiments developed to study DNA binding proteins (Rhodes, 1989).

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[1] Abbreviations: anti-DNA, anti-DNA autoantibodies; SLE, systemic lupus erythematosus; CDRs, complementarity determining regions; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; mAbs, monoclonal antibodies; $KMnO_4$, potassium permanganate; nIgG, normal mouse IgG.

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This point is significant because only about four lupus anti-DNA mAbs, HEd10 (Lee et al., 1982; Cygler et al., 1987), BV04-01 (Herron et al., 1991; Rumbley et al., 1993; Stevens et al., 1993; Tetin et al., 1993; Swanson et al., 1994), BV17-45 (Ballard et al., 1984), and Je1241 (Braun & Lee, 1987), have been characterized in this manner [several anti-DNA mAbs experimentally induced in normal mice have been characterized in this fashion; cf. Runkel and Nordheim (1986), Sanford and Stollar (1990), Chmielewski and Schultz (1991), and Polymenis and Stollar (1994)]. We discuss the results from our work in terms of both DNA antigenicity and protein·DNA recognition.

MATERIALS AND METHODS

Monoclonal Antibody Preparation. An unmanipulated 8-week-old female MRL-*lpr* mouse was obtained from Jackson Laboratories (Bar Harbor, ME) and housed in the University of Michigan Unit for Laboratory Animal Medicine in a pathogen-free environment. Serum samples were screened by ELISA (*vide infra*) every 4 weeks for the presence of anti-DNA. At 26 weeks of age, high levels of both anti-ssDNA and anti-dsDNA were detected, and the mouse was sacrificed by CO_2 asphyxiation. The spleen was removed and fused with nonsecreting myeloma Sp2/0 cells in a 5:1 ratio in PBS containing 15% DMSO and 42% PEG 4000 at 37 °C for 30 s (Oi & Herzenberg, 1980). The fusion mixture was slowly diluted with serum-free media, and unstimulated peritoneal cells in HAT media were added. The hybridomas were then seeded in 96-well microtiter plates and allowed to grow at 37 °C. After 21 days, 620 viable hybridomas were observed (65% of 960 starting wells), 137 of which produced mAbs that bound heat-denatured calf thymus DNA in direct ELISA measurements. All 620 viable hybridomas were also screened by ELISA against native calf thymus DNA, and 6 mAbs tested positive (these 6 mAbs were part of the group of 137 that bind ssDNA). All 137 hybridomas were subcloned by limiting dilution and reassayed for anti-DNA reactivity. As is common with fusions involving older autoimmune mice, most of the hybridomas died while others stopped producing antibody (presumably due to gradual chromosome loss and segregation of the genes required for antibody synthesis; Margulies et al., 1976), leaving eight viable anti-DNA-producing cell lines. The isotype of the anti-DNA produced by each hybridoma was determined using an isotyping kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions.

Large quantities of mAbs for characterization were produced in ascites by interperitoneal injection of $\sim 10^7$ hybridoma cells into pristane-primed retired female BALB/c breeders. Ascites fluid was clarified by centrifugation and then chromatographed over protein A−agarose (Pierce, Rockford, IL) at 4 °C using the ImmunoPure buffer system (Pierce) according to manufacturer's specifications. Eluted immunoglobulin was exchanged into DNA binding buffer (50 mM K_2HPO_4, pH 8, 150 mM NaCl, and 1 mM EDTA) using Centricon 30 microconcentrators (Amicon, Beverly, MA). The IgG fraction (1−2 mg) was then chromatographed over ssDNA−agarose (GIBCO-BRL, Gaithersburg, MD; 2 mL bed volume) equilibrated with DNA binding buffer at 4 °C. After being washed with 10 column volumes of binding buffer (flow rate ∼0.15 mL/min), mAbs 4B2, 7B3, and 10F4 were eluted with DNA elution buffer (binding buffer plus 2 M NaCl), whereas 8D8, 9F11, 11F8, 15B10, and 15D8 were eluted with a step gradient of urea (to 4 M dissolved in elution buffer). Samples of purified 11F8 were chromatographed a second time on the ssDNA−agarose column, and changes in the K_d (as judged with the gel shift assay; *vide infra*) were not observed. These results suggest that elution with the low concentrations of urea used in the second affinity chromatography step is non-denaturing.

Anti-DNA samples were purified to >98% purity by high-performance ion-exchange chromatography on DEAE. Briefly, the mAbs were exchanged into TRIS buffer (20 mM TRIS·HCl, pH 7.5), and about 1−2 mg of protein was loaded onto a Protein Pak DEAE 5PW column (Waters, Marlborough, MA) equilibrated with TRIS buffer at 4 °C. The protein was eluted with a linear gradient of TRIS buffer (pH 8) containing 1 M NaCl at a flow rate of 1 mL/min. The desired fractions were pooled, concentrated, and exchanged into binding buffer. Protein concentrations were determined using the BCA protein assay reagent (Pierce) using normal mouse IgG of known concentration as a standard.

F(ab) Preparation. Crude IgG was isolated from clarified ascites fluid by protein A chromatography as described above. Intact IgG was digested with immobilized papain using the ImmunoPure F(ab) preparation kit (Pierce) according to manufacturer's specifications. Isolated F(ab) was exchanged into DNA binding buffer and chromatographed on a ssDNA−agarose matrix. F(ab) purified using this strategy was >95% pure by SDS−PAGE. The p*I* of the F(ab) was determined by comparison to p*I* markers (Pharmacia, Piscataway, NJ) on IEF 3−9 gels using the PhastGel system (Pharmacia).

Nucleic Acids. Calf thymus DNA (10 mg; Calbiochem, San Diego, CA) was dissolved in TRIS buffer (60 mM TRIS, pH 8.0, 100 mM NaCl, and 2 mM $CaCl_2$, to 10 mL total volume), deproteinized by chloroform/isoamyl alcohol extraction, and precipitated with ethanol. The DNA was then briefly incubated with micrococcal nuclease (40 units/mg of DNA) and purified by gel filtration on BioGel A1.5M (BioRad, Hercules, CA) as previously described (Papalian et al., 1980). Single-stranded DNA and RNA homopolymers, poly(dA)·poly(dT) and poly(dG)·poly(dC) were purchased from Pharmacia and used without further purification. Oligodeoxyribonucleotides were chemically synthesized and 5′-end-labeled with [γ-^{32}P]ATP as previously described (Glick et al., 1992; Swanson et al., 1994).

ELISA. Immulon II microtiter plates (Dynatec, Chantilly, VA) were coated with heat-denatured calf thymus DNA (100 °C for 12 min, then 0 °C for 20 min, 10 μg/mL) or polynucleotides (10 μg/mL) in TBS (10 mM TRIS, pH 7.4, 150 mM NaCl) for 18 h at room temperature. To prepare wells containing only dsDNA, wells coated with calf thymus DNA were treated with S1 nuclease (0.2 unit/mL) in nuclease buffer (100 mM sodium acetate, pH 4.6, 100 mM NaCl, 0.1 mM $ZnCl_2$, and 0.043% glycerol) at 37 °C for 2 h (Eaton et al., 1983). After being blocked with PBS containing 3% BSA for 18 h at room temperature, the wells were washed with PBS containing 0.1% Tween-20 (PBS-Tween). Anti-DNA samples for assay were diluted in PBS containing 1% BSA and 0.05% Tween-20 (PBT), added to the appropriate wells, and incubated for 2 h at room temperature. After the wells were washed with PBS−Tween, goat anti-mouse Ig−alkaline phosphatase conjugate (Boehringer-Mannheim) di-

luted 1:1000 in PBT was added and incubated for 2 h. After washing, bound anti-DNA was visualized by the addition of *p*-nitrophenyl phosphate (Sigma-104, 1 mg/mL) in Na_2CO_3 (100 mM, pH 9.6). The absorption at 405 nm was measured using a microtiter plate reader (Biotek Instruments, Winooski, VT).

Reactivity to Sm, Sm/nRNP, SS-A, SS-B, histone, and Scl-70 was determined by direct ELISA using a commercially available kit (INCSTAR, Stillwater, MN). Briefly, anti-DNA samples (50 μL of 5 μg/mL solutions) were added to wells that were precoated with antigen (as provided by the manufacturer) and incubated for 30 min at 25 °C. After the wells were washed, bound anti-DNA was detected using a goat anti-mouse Ig−alkaline phosphatase conjugate and visualized by addition of *p*-nitrophenyl phosphate as described above. Positive controls provided by the manufacturer were run in parallel. Background reactivity of mAbs to wells containing no antigen did not exceed an OD_{405} value of 0.1 AU after 2 h.

Binding of cardiolipin (CL; Fluka, Ronkonkoma, NY), fibronectin (FN; Fluka), laminin (Lam; ICN, Costa Mesa, CA), heparin sulfate (HS; Fluka), phosphatidylserine (PS; Fluka), and collagen type IV (ColIV; Fluka) was assessed by direct ELISA. Briefly, solutions of CL, PS in ethanol (100 μL of 50 μg/mL solutions) were added to Immulon II microtiter plates and evaporated over 18 h at 25 °C (Smeenk et al., 1988). Lam, ColIV, HS (50 μL of solutions that are 2, 5, and 50 μg/mL, respectively, in PBS), and FN (50 μL of a 10 μg/mL solution in 0.1 M sodium carbonate, pH 9.6) were coated 18 h at 25 °C (Gay et al., 1985; Lake et al., 1985; Faaber et al., 1986; Sabbaga et al., 1989). After being blocked with PBS containing 3% BSA, anti-DNA (50 μL of 5 μg/mL solutions) were incubated with the antigens for 2 h at 25 °C. Bound antibody was detected as described above. Monoclonal antibodies specific for FN, ColIV, Lam, and HS were obtained commercially and served as a positive control in these experiments. Background reactivity of mAbs to wells containing no antigen did not exceed an OD_{405} value of 0.08 AU after 2 h.

Gel Shift Assay and DNA Footprinting. Anti-DNA affinity for oligonucleotide ligands 5′-end-labeled with ^{32}P was measured by gel shift assay in TBE buffer (pH 7.4) containing NaCl (150 mM) as previously described (Stevens et al., 1994). The dependence of binding on buffer ionic strength was assessed by performing a series of binding titrations as a function of NaCl concentration. Footprinting with $KMnO_4$, diethyl pyrocarbonate, and dimethyl sulfate was performed as previously described (Swanson et al., 1994).

Fluorescence Quenching Experiments. Fluorescence measurements were performed on a Perkin-Elmer LS-50 luminescence spectrometer equipped with a thermostated cell block maintained at 23 °C. All measurements were carried out at least 3 times in phosphate buffer (50 mM Na_2HPO_4, 150 mM NaCl, pH 8.0) as previously described (Kelly et al., 1976; Kowalczykowski et al., 1986; Kim et al., 1992). Briefly, mAb solutions (~0.2 μM) were excited either at 278 or at 295 nm (slit width = 2.5 nm), and the emission at 345 nm (slit width = 15 nm) was monitored for changes in fluorescence as a function of varying DNA ligand concentration. To minimize inner filter effects, the optical density at both excitation wavelengths was kept under 0.1 AU (Tetin et al., 1993). The binding site size was approximated by measuring the number of molar equivalents of nucleotide necessary to achieve saturation of DNA as indicated by fluorescence quenching (Kelly et al., 1976). The molar concentration of bases for each ligand was determined using a value of $8.1 \times 10^3\ M^{-1}\ cm^{-1}$ as the extinction coefficient for phosphate (Kim et al., 1992). Fluorescence quenching values were obtained from the data by integrating both the fluorescence emission of the protein in the absence of DNA and the emission signal of the mAb·DNA complexes after each addition of ligand. The amount of quenching after each addition of ligand was calculated using eq 1:

$$\frac{f_F - f_B}{f_F} \tag{1}$$

where f_F is the integrated fluorescence intensity in the absence of DNA and f_B is the integrated fluorescence intensity after each addition of DNA.

RESULTS

Isolation and Purification of Monoclonal Anti-DNA. We have screened a panel of hybridomas generated from a 26-week-old unmanipulated MRL-*lpr* mouse for the presence of spontaneously occurring autoantibodies that bind to the ssDNA or dsDNA epitopes of calf thymus DNA. All of the mAbs identified in the ELISA screen after somatic cell hybridization recognize ssDNA, and several of these mAbs also cross-react with dsDNA. However, none of the mAbs bind dsDNA alone. Our final panel after subcloning consists of six anti-ssDNA and two mAbs that bind both ssDNA and dsDNA. Overall, these results are consistent with previous work indicating that anti-ssDNA comprise the bulk of lupus anti-DNA, mAbs that bind both ssDNA and dsDNA are less common, and anti-DNA that bind only dsDNA are rare (Tan, 1989; Voss, 1988).

Purification of milligram quantities of mAbs from ascites fluid was achieved by affinity chromatography on protein A−agarose followed by affinity chromatography using an ssDNA−agarose matrix. Additional purification of each mAb by preparative ion-exchange chromatography on a DEAE column affords samples >98% pure as judged by analytical ion-exchange chromatography and SDS−PAGE. Each mAb is more acidic than its corresponding F(ab) fragment (data not shown), and the p*I* values of the IgG2a F(ab)s are generally higher than those generated from the IgG3 or IgG2b mAbs. Moreover, F(ab)s generated from the anti-DNA mAbs that bind dsDNA (10F4 and 4B2, IgG2a) possess higher p*I* values than all of the other anti-DNA. These observations suggest the IgG2a mAbs may possess a greater number of cationic residues than the other anti-ssDNA in our panel.

Specificity of Monoclonal Anti-DNA. While monoclonal antibodies generally do not cross-react with structurally unrelated antigens, the reactivity of anti-DNA mAbs can be wider, including recognition of both cellular and nuclear materials (Eilat, 1985). Precisely defining anti-DNA specificity is important because recognition of antigens other than DNA may be involved in both the evolution of anti-DNA and their pathogenicity *in vivo* (Pankewycz et al., 1987). Although several studies have addressed this issue, there is conflicting evidence on the nature and extent of anti-DNA cross-reactivity (Andrzejewski et al., 1981; Edberg & Taylor,

Table 1: Nucleic Acid Binding Specificity[a]

mAb	isotype[b]	p*I*	ssDNA[c]	dsDNA[d]	poly(dT)	poly(dC)	poly(dA)	poly(dI)	poly(U)	poly(C)	poly(G)
15D8	IgG2b	8.0	+++	- - -	+++	+	- - -	- - -	- - -	- - -	- - -
9F11	IgG2b	7.5	+++	- - -	+++	+	- - -	- - -	- - -	- - -	- - -
15B10	IgG2b	7.5	+++	+	+++	+	- - -	- - -	- - -	- - -	- - -
4B2	IgG2a	7.3	+++	++	+++	+++	- - -	+++	- - -	- - -	- - -
10F4	IgG2a	7.8	+++	- - -	+++	++	- - -	+	- - -	- - -	- - -
7B3	IgG2a	7.2	++	- - -	+++	+++	- - -	+++	- - -	- - -	+
8D8	IgG2a	7.2	+++	- - -	+++	- - -	- - -	+++	- - -	- - -	- - -
11F8	IgG3	7.5	+++	- - -	+++	- - -	- - -	- - -	- - -	- - -	- - -

[a] Binding to RNA and DNA antigens was determined by direct ELISA as described under Materials and Methods. Data represent the mean of triplicate measurements taken after 2 h at 405 nm. The background reactivity of mAbs to blank wells did not exceed 0.07 AU. (- - -), <2× background; (+), 2−5× background; (++), 5−10× background; (+++), >10× background. Positive controls were all >10-fold above background. [b] All mAbs possess κ light chains. [c] Heat-denatured calf thymus DNA. [d] Native calf thymus DNA.

1986; Madaio et al., 1987; Costello & Green, 1988; Smeenk et al., 1988; Brinkman et al., 1990; Ohnishi et al., 1994). To determine the extent that cross-reactivity is a feature of our anti-DNA, we assessed the ability of these mAbs to bind non-nucleic acid antigens identified previously as possibly having cross-reactive epitopes (Gay et al., 1985; Lake et al., 1985; Faaber et al., 1986; Sabbaga et al., 1989). These molecules include nuclear proteins, ribonucleoproteins, phospholipids, proteoglycans, and extracellular matrix components present within the normal glomerular basement membrane (see Materials and Methods). Only mAb 7B3 cross-reacts with any of these antigens (laminin, heparin sulfate, phosphatidylserine, and cardiolipin), albeit to a limited extent (between 2- and 10-fold above background). These data suggest that the specificity of our anti-DNA is narrow.

To gain a better understanding of the specificity that our anti-DNA display for nucleic acid ligands, their reactivity toward several different polynucleotides was assessed by direct ELISA. The data indicate that each mAb strongly reacts with poly(dT), which is consistent with previous observations that poly(dT) contains immunodominant epitopes (Table 1) (Stollar et al., 1962; Andrzejewski et al., 1981; Lee et al., 1982; Munns & Freeman, 1989; Voss, 1988). Several mAbs, including 9F11, 15B10, 15D8, and 11F8, apparently only recognize poly(dT), whereas the others bind one or more of the other polydeoxyribonucleotides. In contrast, none of the mAbs bind significantly to polyribonucleotides, a result that is consistent with previous studies (Andrzejewski et al., 1981; Lee et al., 1982; Voss, 1988). These findings suggest either that anti-DNA can discriminate between the two different sugar moieties or that more complicated secondary or tertiary structures exist in these polymers that limit access to the nucleotide bases in RNA relative to DNA (Arnott, 1970). Given that our anti-DNA bind oligo(dU), the latter alternative seems more likely (*vide infra*).

Relative Affinity of Anti-DNA for Oligonucleotide Ligands. Although our initial experiments reveal some information about the specificity of our anti-DNA, direct ELISA measurements cannot provide a reliable assessment of the relative differences in affinity between different antigens (Pesce & Michael, 1992; Sanford & Stollar, 1992). To address this point, we investigated binding to small DNA oligomers using a gel shift assay which directly measures binding in solution (Carey, 1991). We selected 21-base-long oligomers for the test antigens because they are long enough to retain the features of polymeric DNA (i.e., they should contain at least one contiguous epitope), but they are too short to be bridged by both antibody combining sites simultaneously, thereby avoiding the possibility of artifacts caused by multivalent recognition. The reactivity patterns determined by ELISA generally reflect the gel shift data; all of the mAbs have the highest relative affinity for dT_{21}, regardless of isotype (Table 2). However, the relative affinity for dT_{21} varies with the isotype, with IgG2b mAbs having the highest affinity. dA_{21} is not recognized by any of our mAbs, and the affinity toward either dG_{15} or dC_{21} varies among the different antibodies, especially in the IgG2a subclass. Although the IgG2b and IgG3 anti-DNA do not bind to poly(dI) by ELISA, the gel shift data show that the order of base specificity for the IgG2b and IgG3 mAbs is dT ≫ dG ≫ dC ≥ dA.

Table 2: Relative Affinity of Anti-DNA MAbs for dN_{21}[a]

mAb	isotype	dT_{21}	dG_{15}[b]	dC_{21}	duplex[c]
7B3	IgG2a	4300	- - -	11000	- - -
8D8	IgG2a	96	16600	- - -	- - -
4B2	IgG2a	817	26400	4270	7170
10F4	IgG2a	1470	7130	45500	∼77000
9F11	IgG2b	1	480	- - -	- - -
15B10	IgG2b	2.9	750	- - -	- - -
15D8	IgG2b	5.5	1860	- - -	- - -
11F8	IgG3	44	3230	- - -	- - -

[a] Relative affinity was measured using a gel shift assay as previously described (Stevens et al., 1994). Briefly, the fraction of free DNA was quantified by densitometry from triplicate experiments, and the protein concentration required to reach half-saturation of the ligand was determined by linear interpolation. The values presented in the table are normalized to 9F11 (0.3×10^{-9} M, per IgG binding site). 9F11 is the tightest binder so that the larger values for the other mAbs represent weaker binding. While binding of more than one antibody to a single oligomer is possible, the formation of multiple anti-DNA·DNA complexes is not observed in the gel shift assay, and the relative affinities of the mAbs and their corresponding F(ab) fragments (per antigen binding site) are within error (data not shown). (- - -), no binding detected at the highest concentration tested (<20 μM). [b] dG_{21} is not synthetically accessible. [c] Duplex = ligand **1**.

Other groups have observed similar trends in the specificity of monoclonal anti-DNA. For example, using fluorescence quenching measurements, Tetin et al. find that BV04-01 (IgG2b, κ) binds dT_6 5-fold tighter than it binds dG_6 (K_d = 0.13 vs 0.71 μM, respectively) and does not exhibit appreciable affinity for either dC_6 or dA_6 (Tetin et al., 1993). Similarly, Lee reports that HEd10 (IgG2a, κ) displays much higher affinity toward oligo(dT) than either oligo(dC) or oligo(dA) (K_d = 80 nM vs >2 μM, respectively; Lee et al., 1982). Both BV04-01 and HEd10 are derived from (NZBx-NZW)F_1 mice. The data presented here indicate these trends are also true for anti-DNA derived from MRL-*lpr* mice and

Ligand	Designation
$\begin{matrix} S\text{-}T\,G\,C\,G\,A\,A\,T\,T\,C\,G\,C\,T\text{-}S \\ S\text{-}T\,C\,G\,C\,T\,T\,A\,A\,G\,C\,G\,T\text{-}S \end{matrix}$	**1**
$A_{1\text{-}8}\ T_9 T_{10} T_{11} T_{12} T_{13}\ A_{14\text{-}21}$	**2**

FIGURE 1: Sequence of model DNA ligands. Synthesis of these sequences has been described previously (Glick et al., 1992). The disulfide cross-link in **1** increases its thermal stability without disrupting the native helical geometry (Glick et al., 1992; Wang et al., 1994).

suggest that our panel is representative of the large group of anti-DNA previously generated from lupus-prone mice.

In addition to single-stranded homopolymers, the affinity for dsDNA was assessed with a disulfide cross-linked duplex (**1**, Figure 1). This construct was chosen because a dodecamer cannot bind at both antigen binding sites simulataneously (*vide supra*), and the disulfide cross-links prevent the duplex from adopting alternate conformations (Marky et al., 1983) without compromising the native helical geometry (Glick et al., 1992; Wang et al., 1994). Although our anti-DNA may possess sequence selectivity and may not bind duplex **1** optimally (*vide infra*), this sequence nevertheless provides the basic geometric requirements of B-DNA (Wing et al., 1980) and is therefore a good starting point with which to examine our anti-dsDNA. The data in Table 2 indicate that only two of the IgG2a anti-DNA, 4B2 and 10F4, possess appreciable affinity for dsDNA. These anti-DNA also bind oligo(dT), although the relative affinity for ssDNA is much weaker than seen with the IgG2b anti-DNA.

To address the contribution of ion pair formation in stabilizing DNA complexes with our anti-DNA, we employed a gel shift assay to measure K_d values to each mAb as a function of [Na^+] (Record et al., 1976; Weeks & Crothers, 1992). Assuming that the released cations were originally bound to the phosphates on DNA, and neglecting anion effects, the number of released cations represents the number of phosphates that have exchanged a sodium ion for a positively charged residue on the antibody (Lohman et al., 1980). Binding of dT_{21} by the IgG2b mAbs (e.g., 15D8) and by 11F8 does not appear to be affected by changes in ionic strength, whereas the other IgG2a mAbs (e.g., 4B2) show a small dependence in K_d upon varying [Na^+] (Figure 2). These data suggest that ion pairs may not form in complexes between the IgG2b and IgG3 mAbs and dT_{21}, whereas one salt bridge apparently forms in complexes with the IgG2a anti-DNA. Furthermore, these results are consistent with the ELISA data showing that our mAbs do not bind to negatively charged polymers like cardiolipin, heparin sulfate, and phosphatidylserine. We also measured the affinity of 4B2 for duplex **1** as a function of [Na^+]. These experiments suggest one cation is released upon formation of the 4B2·dsDNA complex (data not shown), which is the same number observed in the binding of 4B2 to dT_{21}. Similar analysis could not be performed for mAb 10F4 because binding was too weak to measure at high [Na^+].

Fluorescence Measurements, Binding Site Size, and Affinity for 5-mers. DNA recognition can result in the quenching of the intrinsic fluorescence of tryptophan and/or tyrosine residues in DNA binding proteins (Kelly et al., 1976). For anti-DNA, these phenomena have been attributed to changes in the microenvironment upon ligand binding and can reflect both a static and a dynamic mechanism of

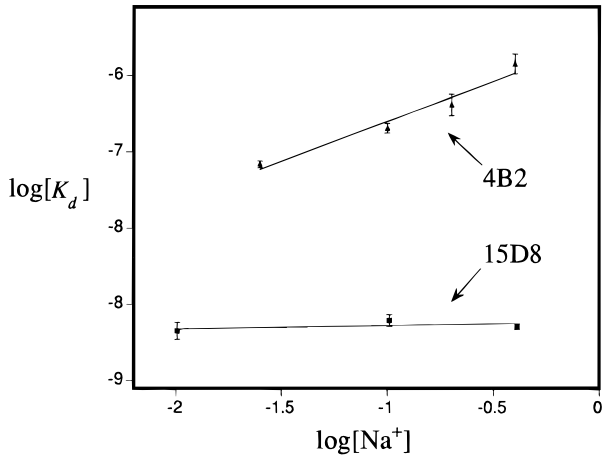

FIGURE 2: Cation release upon binding dT_{21}. Apparent K_d values were measured as a function of [Na^+] as described under Materials and Methods. The number of sodium cations released upon binding is calculated by solving the equation $\log K_d = Z\psi \log [Na^+] + \log K^0$ for Z using a value of 0.71 for ψ and the slope derived from a plot of log K_d vs log [Na^+] (Record et al., 1976). Plots of log K_d vs log [Na^+] are shown for 15D8 ($Z\psi = 0.04$, $r = 0.98$) and 4B2 ($Z\psi = 0.79$, $r = 0.98$), and represent anti-DNA that apparently release zero (IgG2b mAbs) or one cation (IgG2a mAbs), respectively, in complexes with dT_{21}. The plot for 11F8 shows a slight dependence in [Na^+] upon binding, but the slope is less than that observed with the IgG2a mAbs. Similar results are obtained using dT_5 with both 9F11 and 11F8; however, binding by the IgG2a mAbs is too weak to measure so this analysis could not be performed. MAb 4B2 also apparently releases one sodium ion upon binding duplex DNA (data not shown).

quenching (Lee et al., 1982; Tetin et al., 1993). The intrinsic fluorescence of each mAb in our panel is quenched when titrated with poly(dT) (Figure 3). This effect is observed at excitation wavelengths of either 278 nm or 295 nm, implicating tryptophan, and perhaps tyrosine, residues in binding (Kelly et al., 1976; Kim et al., 1992). In control experiments using poly(dA), fluorescence quenching is not observed, indicating that complex formation is required for this effect. Also, when normal mouse IgG is titrated with poly(dT), no quenching is found, indicating that nonspecific binding is not responsible for the changes in fluorescence intensity. Lastly, while poly(dG) and poly(dC) bind to our mAbs (see Table 1), they do not induce fluorescence quenching. These results suggest that the other DNA homopolymers may not bind in the same manner as poly(dT) and that the difference in affinity may reflect these different modes of recognition.

The number of consecutive nucleotides occluded upon binding has been determined for many different ssDNA binding proteins using fluorescence quenching methods [cf. Kelly et al. (1976) and Kim et al. (1992)]. This analysis is possible if the affinity for a polynucleotide is sufficiently high to approximate a linear reduction in fluorescence intensity as a function of nucleotide concentration. After a stoichiometric amount of ligand is added, all of the available protein binding sites are saturated, and titrating with additional ligand has no effect on the protein fluorescence. Performing this experiment with our mAbs using poly(dT) as the ligand reveals that about five nucleotides are occluded upon binding (Figure 3). These data are in general agreement with the site size determined for both BV04-01 and HEd10 (Lee et al., 1982; Voss, 1988) and are consistent with the dimensions of antibody binding sites.

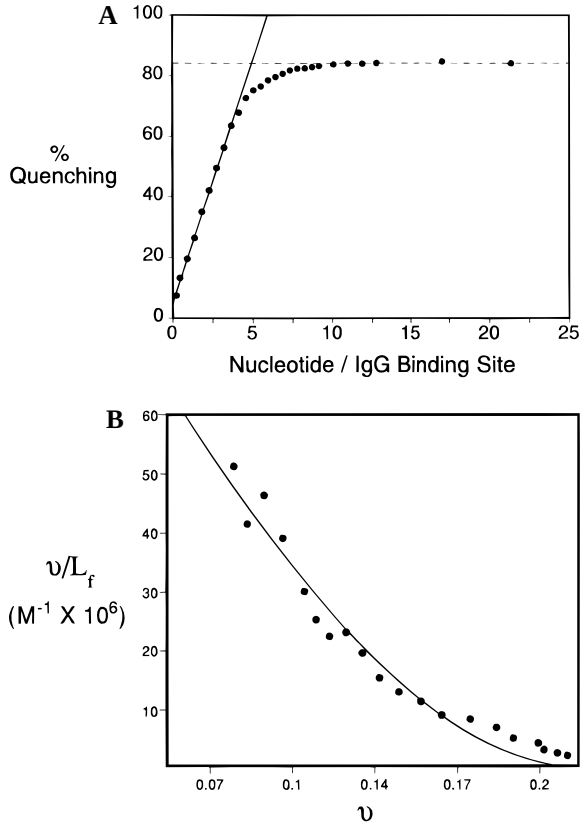

FIGURE 3: Fluorescence quenching titration of 9F11 with poly(dT). MAb 9F11 (0.2 μM) was excited at 278 nm, and the emission was monitored at 345 nm as a function of DNA concentration (0−0.05 μM). All measurements are background-corrected. Similar results are obtained with the other mAbs (data not shown). (A) Calculation of the number of bases occluded upon binding. The initial slope was determined from the best fit to the first 10 data points (solid line) and extrapolated to the point of intersection with a horizontal line drawn through the data points measured after saturation (dashed line). The *x*-axis value where the two lines intersect reflects the number of nucleotides occluded in the binding site. That the intersection of the two lines reflects the binding stoichiometry (and the number of bases occluded upon binding) can be derived from the equilibrium expression of the binding reaction (Woodbury & von Hippel, 1983). Briefly, at the intersection, $n = (P_t + K_d)/D_{int}$, where n = the binding stoichiometry, P_t is the total protein concentration, K_d is the apparent dissociation constant for binding to the high molecular weight polymer [in this case poly(dT)], and D_{int} is the value of the intercept. When $P_t \gg K_d$ (as it is here, see Table 2), $n = P_t/D_{int}$, which can be obtained from the graph. Similar results are obtained using 10-fold lower and higher protein concentrations (data not shown). Due to their relatively low affinity, mAbs 4B2, 7B3, and 10F4 do not yield a distinct saturation point. Based on the data for these antibodies, we can only estimate that from four to six consecutive nucleotides are occluded upon binding. (B) Scatchard plot of data. This plot was generated using the equations described by McGhee and von Hippel where ν is the number of nucleotides per IgG binding site (McGhee & von Hippel, 1974). Note that several data points obtained after saturation in plot A are not shown here.

Since our mAbs "cover" (Kelly et al., 1976) about five consecutive nucleotides upon binding, dN_{21} must contain multiple overlapping $d(N)_5$ segments, and statistical effects contribute to the relative affinities presented in Table 2 (Kelly et al., 1976). Therefore, we determined the affinity of our mAbs for dT_5, dG_5, and dC_5 using gel shift assays. Significant binding is observed only with dT_5 and only for about half of the mAbs (Table 3). For each mAb, the apparent K_d value for dT_5 is lower than the apparent affinity observed for dT_{21}. In fact, for many of these mAbs, their affinity for dT_5 is similar to that measured for an anti-DNA mAb obtained by immunizing normal mice with a short ssDNA epitope (Chmielewski & Schultz, 1991), which supports the proposal that anti-DNA generated by immunization may be similar to spontaneously occurring lupus anti-DNA (Krishnan & Marion, 1993).

Table 3: Absolute Affinity of Anti-DNA MAbs for dN_5 (μM)[a]

mAb	dT_5	dU_5	dC_5	dG_5
15D8	0.59 ± 0.07	2.3 ± 0.035	- - -	- - -
9F11	0.08 ± 0.016	0.30 ± 0.02	- - -	- - -
15B10	0.23 ± 0.03	1.7 ± 0.03	- - -	- - -
4B2	- - -	- - -	- - -	- - -
10F4	- - -	- - -	- - -	- - -
7B3	- - -	- - -	- - -	- - -
8D8	5.7 ± 0.9	- - -	- - -	- - -
11F8	0.94 ± 0.08	6.6 ± 0.8	- - -	- - -

[a] Absolute apparent K_d values were measured using a gel shift assay as previously described (Stevens et al., 1994). The fraction of free DNA was quantified by densitometry, and the values from triplicate experiments were fit by nonlinear least-squares regression to the single-site binding isotherm (Stevens et al., 1994). The error given in the table represents the error to the fit. To verify the gel shift data, we measured the absolute affinity of 9F11, 11F8, and 15D8 for dT_5 by fluorescence quenching and find that the K_d values obtained by both methods agree to within about 35% (data not shown). For those mAbs that bind dT_5, the apparent K_d values for $dT_5 \approx dT_6$, and the affinity for longer oligomers (e.g., 11-mers) is that expected based on statistical considerations (data not shown; Kelly et al., 1976). In addition, the affinity of each mAb for dT_4 is ≥2-fold lower than that for dT_5, and binding to dT_3 is too weak to be measured by direct gel shift assays. These data suggest that binding site size and the number of consecutive nucleotides that participate in binding to these mAbs are similar (Kelly et al., 1976). (- - -), no binding detected at the highest protein concentration tested (20 μM).

To determine if the C5 methyl group of thymine helps in stabilizing anti-DNA·oligo(dT) complexes, we synthesized the 2′-deoxyuridine analogs of the oligo(dT) ligands and performed binding titrations using the gel shift assay and fluorescence spectroscopy (Table 3). The fluorescence spectra of our anti-DNA are quenched by the addition of dU_{21}, suggesting that the mode of recognition of oligo(dU) is similar to oligo(dT). However, the affinity for dU_5 measured by gel shift assay is about 5-fold lower than that for dT_5, for both 9F11 and 11F8, which suggests a role for the C5 methyl group in the complex (similar results have also been obtained with HEd10; Lee et al., 1982). One explanation for the increased affinity for oligo(dT) relative to oligo(dU) is that the C5 methyl groups of some thymine bases in dT_5 make favorable van der Waals contacts upon binding. As described below, this hypothesis is consistent with the results of $KMnO_4$ footprinting experiments.

We also investigated 4B2 complexes of poly(dG)·poly(dC), poly(dA)·poly(dT), and duplex **1** by fluorescence binding titrations and find that these ligands do not afford changes in the fluorescence spectra of 4B2 upon binding (data not shown). One explanation for these data is that binding occurs at a site other than the antigen binding cleft. However, dT_{21} competes with **1** in complexes with anti-DNA and *vice versa*, indicating that both ligands are bound in the same site (data not shown). Alternatively, this observation may indicate that for 4B2, the mode of binding dsDNA is different from the mode of ssDNA recognition. Quenching of the chromophore in ssDNA binding may involve direct contact with ligand or conformational changes in the antigen binding site associated with complexation that alters the microenvironment of chromophore (Tetin et al., 1993).

Binding dsDNA may utilize a different set of residues (Marion et al., 1992; Radic & Weigert, 1994) or require less movement to accommodate the ligand.

Since the fluorescence spectrum of 4B2 is unchanged upon titration with dsDNA, the size of the dsDNA epitope recognized by this mAb cannot be determined by this method and must await further structural studies. However, by measuring the helix−coil transition of poly(dA)·poly(dT) in the presence of different concentrations of anti-dsDNA Jel241, Braun and Lee estimate that each binding site on this mAb recognizes about 6 base pairs, which is consistent both with the dimensions of the known antibody combining site size and with the binding site size obtained here for ssDNA ligands (Braun & Lee, 1987). This point is significant because it has been proposed, based on analysis of somatic mutations (*vide infra*), that anti-dsDNA occlude about 12 base pairs per site upon binding (Marion et al., 1992; Radic & Weigert, 1994).

Defining the Autoreactive Epitopes on DNA Antigens. Potassium permanganate selectively oxidizes the C5−C6 double bond of thymine bases that are not Watson−Crick hydrogen-bonded and can be used to probe the accessibility of this base in protein·DNA complexes, including those with anti-DNA (Swanson et al., 1994). Since each of our mAbs has a preference for binding poly(dT) and a binding site size of about 5 nucleotides in length, we examined their interaction with a 21-base-long oligomer containing a central dT_5 segment flanked by a nonbinding region (dA_8) to aid in sequencing (oligomer **2**, Figure 1). When compared to normal mouse IgG as a control, the pattern of permanganate reactivity of **2** is marginally increased to a uniform extent when complexed to any of the IgG2a mAbs (Figure 4). However, the reactivity of the thymine bases is quite different when bound by the IgG2b and the IgG3 mAbs. Quantitative measurements of the difference in probability of modification show that in the presence of 9F11 and 11F8 the thymine bases in **2** exhibit hyperreactivity and reduced reactivity relative to normal mouse IgG. Furthermore, the asymmetry of the $KMnO_4$ reactivity pattern suggests that d(A_8-T_5-A_8) is bound with a specific polarity; however, the orientation of the DNA (5′ to 3′ or 3′ to 5′) in the antibody binding site cannot be ascertained from these data alone.

Those positions that are hypermodified (e.g., T_{11} and T_9 for 9F11) may result from a DNA conformation in which the C5−C6 double bond of the base projects away from the binding site where it is exposed to $KMnO_4$ modification (Swanson et al., 1994). In contrast, if the base is oriented in a pocket so that the C5 methyl groups face the antigen binding site or the DNA adopts a conformation in the complex that shields the base relative to the free DNA, the C5−C6 double bonds may be rendered less accessible to $KMnO_4$ modifiction (e.g., T_{10} and T_{12} for 9F11). The minimal increases in permanganate sensitivity seen in DNA ligands bound by the IgG2a mAbs coupled with their relatively low affinity suggest that DNA binding may be nonspecific (Record & Spolar, 1990).

Footprinting duplex **1** (Figure 1) complexed to mAbs 4B2 and 10F4 with $KMnO_4$, dimethyl sulfate, diethyl pyrocarbonate, and hydroxyl radical does not result in a detectable pattern of protection or provide evidence of conformational changes in the ligand (data not shown). Similar results are also obtained with oligo(dA)·oligo(dT) and oligo(dG)·oligo(dC), both of which bind with micromolar affinity. The lack

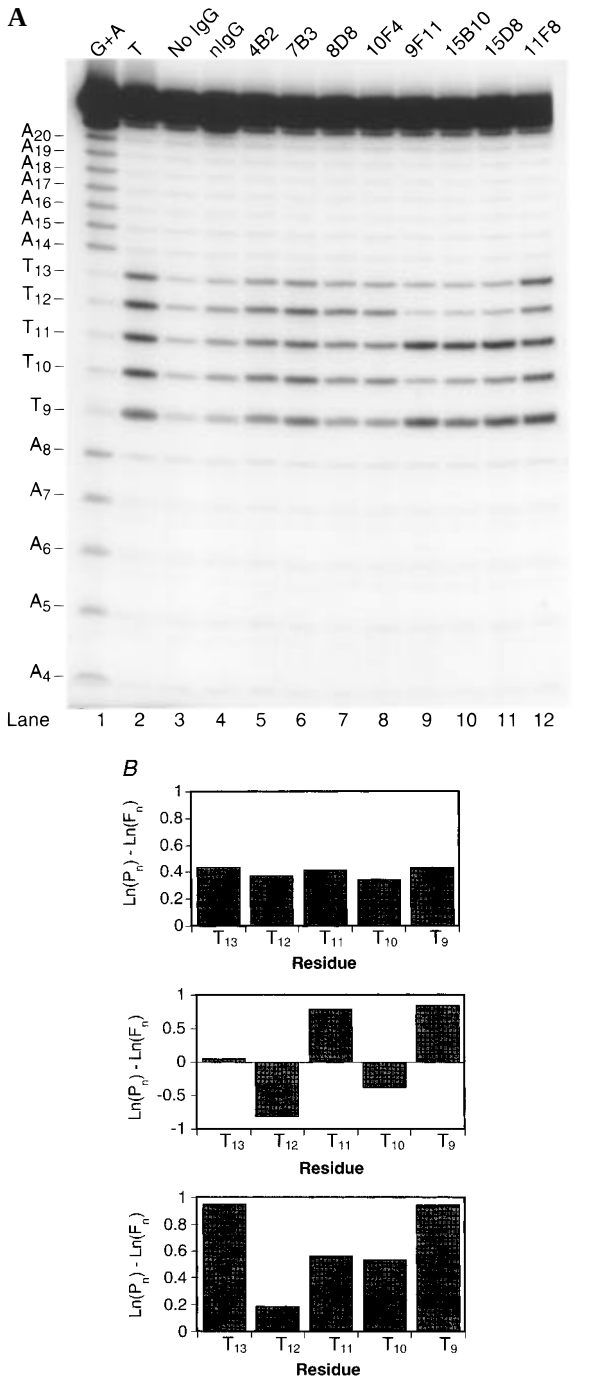

FIGURE 4: Potassium permanganate footprinting. Anti-DNA or normal mouse IgG (nIgG) (10−20 μM) was complexed to d(A_8-T_5-A_8) (0.5 nM), reacted with $KMnO_4$ for 30 s, and then treated as previously described (Swanson et al., 1994). Equivalent amounts of DNA (as judged by liquid scintillation counting) were applied to the gel. (A) Autoradiograph of cleavage products separated by denaturing gel electrophoresis. Lanes 1 and 2, G+A and T sequencing reactions, respectively; lanes 3 and 4, $KMnO_4$ modification in the absence (lane 3) or presence (lane 4) of nIgG; lanes 5−12, $KMnO_4$ modification in the presence of mAbs 4B2 (lane 5), 7B3 (lane 6), 8D8 (lane 7), 10F4 (lane 8), 9F11, (lane 9), 15B10 (lane 10), 15D8 (lane 11), and 11F8 (lane 12). (B) Difference probability maps for mAbs 4B2 (top), 9F11 (middle), and 11F8 (bottom). The difference in $KMnO_4$ modification at each thymidine is represented by bars and is calculated by subtracting the logarithm of the probability of modification at each position in the free DNA (F_n) from the same position in the complex (P_n) according to Rhodes (1989). A larger negative value of $\ln(P_n) - \ln(F_n)$ indicates greater protection from modification.

of a distinct footprint suggests either that the targets of the chemical probes are equally accessible in both the bound and free DNA or that the binding is not specific enough to yield clear footprints. Because the dsDNA ligands used here may not contain the optimal sequence for binding, the latter alternative may be more likely. In support of this hypothesis, La Baer and Yamamoto report a single base-pair substitution in the consensus sequence of the glucocorticoid receptor decreases the affinity only 5-fold relative to the wild-type sequence, yet completely abolishes a distinct footprint (La Baer & Yamamoto, 1994).

DISCUSSION

Two approaches are generally employed to define the molecular basis by which lupus anti-DNA recognize their DNA targets. In the first, comparative sequence alignments are used to uncover the somatic mutations that arise during the anti-DNA autoimmune response (Marion et al., 1992; Radic & Weigert, 1994), and in some cases, these predictions are tested through site-directed mutagenesis (Radic et al., 1993; Katz et al., 1994). The underlying hypothesis behind this approach is that somatically mutated residues are selected to increase the affinity for DNA. However, there are several limitations to this strategy. First, somatic mutations may not affect binding affinity or specificity. For example, of 17 somatically mutated residues found in an anti-azophenyl arsonate antibody, only 3 affected the affinity for *p*-azophenyl arsonate (Sharon, 1990). Second, sequence alignments cannot determine *a priori* whether a somatic mutation affects binding by direct contact with antigen, by altering a particular CDR conformation that changes the complementarity of the antigen binding site, or through long-range effects (Schillbach et al., 1993). Finally, the contribution of germline-encoded residues to binding cannot be readily evaluated by analysis of somatic mutations, and autoantibodies encoded by nearly unmutated germline genes can bind DNA (Shefner et al., 1991; Foster et al., 1992; Radic et al., 1993).

A second approach to investigate molecular recognition of DNA by lupus anti-DNA mAbs has relied on binding measurements, mostly using immunochemical assays with high molecular weight test antigens. From these studies, we have learned that anti-DNA can be classified as specific for ssDNA or dsDNA or cross-reactive between the two, and that certain DNAs [e.g., oligo(dT)- and d(G/C)-rich sequences] contain immunodominant epitopes (Stollar et al., 1962; Munns & Freeman, 1989; Voss, 1988). To complement immunochemical studies of lupus anti-DNA, efforts in several laboratories have focused on application of equilibrium-based affinity measurements (Lee et al., 1982; Ballard et al., 1984; Braun & Lee, 1987; Stevens et al., 1993; Tetin et al., 1993; Stevens et al., 1994; Swanson et al., 1994), spectroscopic experiments (Lee et al., 1982; Braun & Lee, 1987; Tetin et al., 1993), DNA footprinting (Swanson et al., 1994), site-directed mutagenesis (Rumbley et al., 1993; Katz et al., 1994; Radic et al., 1994), and X-ray crystallography (Cygler et al., 1987; Herron et al., 1991) to investigate anti-DNA affinity and specificity.

Yet the ideal approach to elucidate the protein residues involved in DNA binding, the epitopes on DNA ligands that are bound, and the interactions that mediate recognition of these determinants is to combine genetic analysis, mutagenesis, and high-resolution binding measurements. This strategy is commonly employed to investigate DNA binding proteins like transcription factors (Pabo & Sauer, 1992), and has been used successfully to study one anti-DNA mAb, BV04-01 (Herron et al., 1991; Rumbley et al., 1993; Stevens et al., 1993; Tetin et al., 1993; Swanson et al., 1994; *vide infra*). As the first step in our efforts to further illuminate the relationships between DNA binding and renal pathogenesis in SLE, we have generated a panel of eight anti-DNA mAbs and have characterized them with the aim of developing a molecular understanding of their binding properties.

Anti-ssDNA. Antibodies that bind ssDNA constitute the largest population of anti-DNA, and their reactivity and mode of binding are better understood than anti-dsDNA (Voss, 1988; Eilat & Anderson, 1994). Most of what is known about anti-ssDNA is derived from studies of mAbs HEd10 and BV04-01, with the BV04-01·d(pT_3) crystal structure, footprinting studies of BV04-01·ssDNA complexes, and mutagenesis experiments being most noteworthy (Lee et al., 1982; Cygler et al., 1987; Herron et al., 1991; Rumbley et al., 1993; Stevens et al., 1993; Tetin et al., 1993; Swanson et al., 1994). Studies with HEd10 and BV04-01 shows that (a) the binding site for ssDNA is a long, relatively deep cleft, (b) base stacking of thymine bases between aromatic residues in this cleft stabilizes binding of ssDNA ligands, (c) ion pair formation in complexes with ssDNA is limited, and (d) hydrogen bonding to the phosphate backbone as well as to loci on the DNA bases occurs upon binding.

The four tightest binding mAbs in this study, 9F11, 15B10, 15D8, and 11F8, comprise one group of anti-ssDNA and share many similarities with BV04-01 and HEd10. These similarities include specificity for oligo(dT), relative insensitivity of K_d upon varying [Na^+], the involvement of tryptophan and tyrosine residues in binding, and a binding site size that is about five nucleotides long. Potassium permanganate footprinting experiments demonstrate that all of the mAbs in this group bind ssDNA analogously. Specifically, in the presence of 9F11, 15B10, and 15D8, two thymine bases on d(A_8-T_5-A_8) are protected from oxidation, and two bases are hypermodified. When bound to 11F8, all five thymine bases on d(A_8-T_5-A_8) are modified with four of them being oxidized significantly more than the fifth. These observations can be explained if the less reactive thymine bases are bound in subsites, possibly sandwiched between two aromatic residues as seen in X-ray and modeling studies of BV04-01 and predicted for HEd10 (Cygler et al., 1987; Herron et al., 1991). That 11F8 protects one less base from modification than the other three mAbs may account for its lower affinity to oligo(dT) (i.e., this mAb may possess one less subsite in the CDRs; see Table 3), but the overall mechanism of binding appears to be conserved among this group of four mAbs.

Taken together, these data suggest that our "high affinity" anti-ssDNA may have a limited repertoire of motifs to bind ssDNA. This hypothesis is supported by molecular modeling studies of more than 40 F(ab)s encoded by different variable genes and derived from several autoimmune mouse strains (Barry et al., 1994; Eagan et al., 1995). Specifically, the antigen binding site on high-affinity anti-ssDNA appears to be a long deep cleft, whereas the CDRs on both lower affinity anti-ssDNA and anti-DNA that bind dsDNA are a flat open surface. Unlike other ssDNA binding proteins, ligand recognition by these mAbs does not appear to be accompanied by significant cation release (Kowalczykowski

et al., 1981). Therefore, the driving force for complexation may involve hydrophobic effects such as van der Waals contacts and the entropic benefit of releasing bound water molecules from the protein surface (Record & Spolar, 1990, and references cited therein).

MAbs 4B2, 7B3, 8D8, and 10F4 comprise a second group of anti-DNA and possess lower affinity for oligo(dT) relative to the first group of mAbs. In addition, 4B2 and 10F4 also bind dsDNA. MAbs in this second group apparently form one ion pair in complexes with oligo(dT) and show no distinct pattern of $KMnO_4$ protection which suggests that their antigen binding sites lack pockets to accommodate thymine bases. The CDRs of these mAbs may possess a topology similar to the flat binding surface observed in the X-ray structure of Je172, which is a mAb obtained by *immunization* of a normal mouse with dsDNA (Mol et al., 1994; X-ray structures of lupus anti-dsDNA have not been reported). In fact, both the affinity and the footprinting data suggest that binding of ssDNA ligands by this second group of mAbs may simply result from nonspecific association. *In vitro* selection experiments to obtain tighter binding, and to identify more specific DNA ligands for all of our mAbs, are currently in progress. Although this work is not yet complete, preliminary results indicate that our mAbs are sequence-specific binders and that thymidine is overrepresented in the selected DNAs.

Our anti-ssDNA share a number of features with other ssDNA binding proteins, like the T4 bacteriophage gene 32 protein and the *Escherichia coli* single-strand binding protein (SSB) (Kowalczykowski et al., 1981; Karpel, 1990; Lohman & Bujalowski, 1990). These similarities include specificity and affinity for poly(dT) and involvement of tryptophan and tyrosine residues in binding. Furthermore, structural studies of the bacteriophage f1 gene V ssDNA binding protein suggest that flexible loops within a framework of β-sheets, similar to antibody CDRs, may be involved in recognition of ssDNA (Skinner et al., 1994). However, there are several important differences between these other ssDNA binding proteins and our anti-DNA mAbs, such as a strong dependence of K_d upon $[Na^+]$ and different modes of DNA recognition. In addition, the affinity of mAbs like 9F11 and 11F8 for dT_5 (the binding site size for our anti-DNA) is tighter than the affinity of the SSB and gene 32 proteins for oligo(dT) fragments corresponding to their cognate binding site sizes. In this regard, the SSB and gene 32 proteins may more closely resemble our lower affinity mAbs like 10F4.

Anti-dsDNA. A molecular understanding of anti-dsDNA recognition remains elusive, in part due to the lack of high-resolution binding and structural data for anti-dsDNA·-dsDNA complexes. Indirect evidence from comparative sequence alignments (Marion et al., 1992; Radic & Weigert, 1994) and competition binding experiments using both native and chemically modified DNA duplexes (Braun & Lee, 1986; Stollar et al., 1986) suggests that lupus anti-dsDNA recognize epitopes on the bases in both the major and minor groove, as well as the phosphate backbone of dsDNA.

MAb 4B2 binds duplex DNA with micromolar affinity. In competition experiments, we showed that dsDNA and ssDNA bind in the same locus. However, ligand-induced fluorescence quenching is not observed upon binding duplex DNA, which suggests that this mAb recognizes ssDNA and dsDNA in different ways. Binding of dsDNA by 4B2 is apparently stabilized by the formation of one ion pair, similar to that reported for anti-dsDNA BV17-45 (Ballard et al., 1984), but less than the four ion pairs reported to form in dsDNA complexes with anti-dsDNA Je1241 (Braun & Lee, 1987), which binds dsDNA more weakly than both 4B2 and BV17-45. This point is significant because it has been inferred that the number of ion pairs formed upon binding correlates with affinity (Radic et al., 1993).

The low absolute affinity of 4B2 for dsDNA, together with the finding that distinct protection patterns cannot be obtained in footprinting experiments, suggests that dsDNA may not be the relevant antigen for this mAb. However, this mAb does not cross-react with RNA or non-nucleic acid antigens including anionic polymers like cardiolipin. Alternatively, these data may indicate that 4B2 recognizes dsDNA with some degree of sequence selectivity and that the ligands used in our studies do not contain this epitope. Sequence specificity often results from protein secondary structures that insert into the major groove of dsDNA, forming a network of hydrogen bonds to the bases. In fact, sequence-specific dsDNA binding proteins that use a β-sheet architecture for recognition are known (Pabo & Sauer, 1992). In this regard, Stollar, Marion, and Weigert have proposed that anti-dsDNA might straddle the phosphate backbone, allowing the CDR loops to project into the major and minor grooves (Stollar et al., 1986; Marion et al., 1992; Radic & Weigert, 1994). Whether lupus anti-DNA can indeed recognize dsDNA in a sequence-specific manner is not known. As mentioned above, *in vitro* selection experiments are currently underway to address this question.

To summarize, these studies provide new insight about molecular recognition of DNA by lupus anti-DNA autoantibodies. The data suggest that the range of anti-DNA reactivity in our panel is narrow and that these mAbs may be grouped according to shared modes of ligand recognition. If the nature of DNA binding is conserved among larger groups of anti-DNA, it may be possible to design antagonists for the subset of pathogenic anti-ssDNA and anti-dsDNA autoantibodies. Such molecules in principle would be more selective than the nonspecific immunosuppressive agents currently used to treat SLE. In recent work, we have sequenced the variable region genes of our mAbs and have examined their properties *in vivo*. Of particular interest, anti-ssDNA mAb 11F8 is perhaps the most pathogenic anti-DNA mAb to be identified whereas 9F11, which is similar to 11F8 *in vitro*, is essentially benign (Swanson et al., submitted for publication). These reagents therefore provide us with a unique opportunity for future studies of anti-DNA structure, binding, and pathogenicity.

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