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Structural features of a murine monoclonal anti-ssDNA autoantibody

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Abstract

Binding properties of a murine (NZB × $NZWF_1$) monoclonal anti-ssDNA autoantibody (BV04-01) was compared with another anti-ssDNA autoantibody (BV16-19). Both monoclonal antibodies preferentially bound ssDNA and poly(dT) relative to poly(dU). Significant binding of synthetic polynucleotides was detectable when the homopolymer exceeded 5 pyrimidines in length. Binding showed an inverse temperature dependence between 2 and 40°C, while variations in ionic strength showed minimal effects. The primary structure of the variable region of the heavy (IgG2b) and light (kappa) chains comprising BV04-01 was determined using primer extension methodology. A polynucleotide synthesized to correspond with unique sequences within heavy chain CDR2 was used as a gene probe in Northern blot analyses.

Key words: Binding properties, polynucleotide, gene probe, homopolymer, autoimmune diseases, monoclonal antibodies.

1. Introduction

Autoanti-nucleic acid antibodies represent important clinical and research entities since they are implicated in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE)[1] and are potentially valuable reagents in cellular and molecular biology[2]. Research efforts defining autoanti-nucleic acid antibodies have historically advanced from characterization of heterogeneous antibody populations[3,4] to monoclonal antibodies[5–8]. This progression has paralleled desires of investigators to better understand fundamental properties of nucleic acid antigenic determinants as well as basic structure-function relationships governing reactivity of autoantibody molecules. Both analyses are impended by an inherent complexity which dictates that investigators must rely upon

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spontaneous generation of autoantibodies instead of induced responses to defined nucleic acid immunogens[2]. Thus, autoepitopes involved in anti-nucleic acid auto-antibody induction are for the most part incompletely defined. Concomitantly, structure-function studies at the immunoglobulin (Ig) protein level are also imperfect in terms of binding specificity.

Studies impacting both facets of the autoantigen and autoantibody problems outlined above are enhanced by an availability of important inbred strains of mice (*e.g.* NZB × NZW F_1, BXSB and MRL − 1 pr/1pr), which spontaneously develop human-like SLE syndromes[9,10]. As a consequence of chemically mediated splenocyte fusions derived from autoimmune mice, monoclonal autoantibodies specific for nucleic acid determinants have been generated[5–8]. Relative to polyclonal antibody populations homogeneous autoimmune Ig reagents represent an important experimental advance facilitating exploration of: 1) a precise definition of nucleic acid autoepitopes through specificity binding studies, and 2) structure-function parameters by correlating variable region primary structures with the potential for tertiary structural determinations through X-ray crystallographic (Fourier) analysis of the protein[11].

Fine specificity analyses of monoclonal anti-DNA autoantibodies are furthered by the level of structural definition of the DNA macromolecule[12]. Both the sequence (nucleotide) and conformational (helical) features of DNA make it an effective specificity probe in immunological reactions[13]. In addition, the autoantibody-DNA bimolecular complex represents an important protein-nucleic acid interaction for which characterization information can be correlated with the existing non-immunoglobulin models. For example, lambda repressor and cro-protein bind the same operator sites on DNA in the B-configuration. Lambda repressor protein has been shown to consist of two domains: the globular amino-terminal and carboxy-terminal domains containing 90 and 100 amino acids, respectively. Through successful structural resolution by crystallization of the amino-terminal domain[14], the mode of lambda repressor binding to DNA is reasonably well defined. A similar protein structure and mechanism of binding has been found with cro-protein and CAP. Such DNA-binding molecules[15], in addition to studies with DNA polymerase I[16], represent valuable reference models by which immunochemists can compare mechanisms of binding with the anti-DNA autoantibody system.

Although some primary structural information has become available for anti-DNA autoantibodies[17] it is difficult to establish rules governing structure-function relationships based on a relatively sparse data base. Deriving primary structures of autoantibodies allows one to categorize variable, hypervariable, and framework regions (genes) utilized by the heavy (H) and light (L) chains. Such information can be correlated with anti-DNA hybridomas (both spontaneous and induced)[18] of different specificities yielding important structural and genetic information.

Similar to lambda repressor, crystallization and X-ray diffraction studies of autoantibodies must be successfully conducted before mechanisms of antibody-DNA interactions can be fully addressed. Such experimental breakthroughs have been preliminarily achieved with an anti-ssDNA autoantibody (BV04-01) derived from a female BW(NZB × NZW F_1) mouse[19]. Papain-derived Fab fragments from hybridoma BV04-01

(IgG2b, K) were crystallized and subjected to X-ray diffraction (Fourier) analyses[11]. Although initial crystal studies were performed without antigen (DNA) bound to the active site, it is known that BV04-01 preferentially binds poly-dT[19]. Thus, it is anticipated that continued studies elucidating structural features of anti-ssDNA autoantibody with and without bound ligand will lead to more understanding of the antibody protein and DNA epitopes.

Finally, thorough analyses of autoantibodies at the protein and molecular levels can be projected to understanding relationships between Ig genes and genetic mechanisms that result in clinical expression of autoantibodies and autoimmune disorders. Thus, structural analyses of autoantibodies provide an important bridge to better understanding autoimmune gene repertoires. Attesting to this end we describe in depth characterization of a murine monoclonal anti-ssDNA autoantibody in terms of its nucleic acid-binding properties and primary structure at both the protein and structural gene levels. Reported studies show that such analyses have led to the development of a CDR nucleic acid probe useful in exploring related autoantibody structural genes and predicting structure-function properties at the protein level.

2. Materials and methods

2.1. *Reagents*

All nucleic-acid antigens used in these studies were previously described[20].

2.2. *Anti-DNA radioimmunoassays*

Solid phase and modified Farr-binding assays were previously described[20].

2.3. *Cell fusion*

Splenocytes from a nine-month old female BW (NZB × NZW F_1) mouse exhibiting serum anti-DNA antibodies were chemically fused with a non-Ig-secreting myeloma cell line SP 2/0-Ag14 as described[7].

Hybridoma cells were grown in selective media and supernatant solutions screened for anti-ssDNA activity in a liquid phase radio-immunoassay[20]. Hybrid cells with appropriate binding activity were cloned in 0.2%(w/v) soft agar. Cloned hybrids were injected intraperitoneally into pristane-primed Balb/c mice to elicit ascites fluid. Monoclonal antibodies BV04-01 (IgG2b, K) and BV16-19 (IgG2a, K) used in these studies were generated using established procedures[7,20].

2.4. *Purification of anti-ssDNA antibodies*

Ascites fluid was removed from the abdominal cavity, and the gamma-globulin fraction precipitated with 50%-saturated ammonium sulfate. After centrifugation at 12,000 × g for

30 min at 4°C the precipitate was dissolved in 10 mM phosphate, pH 8.0, 0.15 M NaCl, 10 mM EDTA and dialyzed exhaustively against the same buffer. Anti-ssDNA antibodies were isolated by affinity chromatography (Sepharose 6B conjugated to ssDNA) and eluted were 10 mM phosphate, pH 8.0, 1.0 M NaCl, and 10 mM EDTA. Affinity-purified antibodies were precipitated with 50%-saturated ammonium sulfate, and stored at 4°C as a slurry.

2.5. *Derivation of primary amino acid structure of anti-DNA autoantibodies*

Primary structural analyses of autoanti-DNA antibodies were conducted in the following manner. First, each anti-DNA autoantibody was purified by affinity chromatography and dissociation conditions developed for each antibody to maximize yields and purity. Purity was ascertained by SDS-PAGE and isoelectric focusing after incubation of each monoclonal antibody with neuraminidase to eliminate influence of charged terminal sialic acid residues on pI determinations. Second, H and L chains were resolved by molecular sieve chromatography after thiol reduction using acetic acid as a dispersing agent. Upon neutralization, isolated polypeptide chains were reacted with specific enzymes to eliminate cyclized (PCA) N-terminal amino acid residues. Both H and L chains were subjected to amino-acid sequencing as described below.

2.6. *Amino-terminal sequence determination*

Antibodies were reduced in 3.5 M guanidine and 50 mM DTT, alkylated with 4-vinyl pyridine and H and L chains resolved through a P-100 column equilibrated in 1 N acetic acid. Amino-terminal sequences were determined at the University of Illinois Genetic Engineering Faculty by automated Edman degradation utilizing an Applied Biosystems Model 470 A gas, liquid protein sequenator. A Waters HPLC equipped with a reverse phase column was used to detect derivatized amino acids released after each cycle.

2.7. *Preparation of total cellular RNA*

RNA from culture-grown hybridoma cells was prepared using the guanidinium-thiocyanate method described by Chirgwin *et al*[21], with modifications to enhance yields from cultured cells[22]. Isolation of poly A^+ mRNA by oligo(dT) column chromatography was sometimes found to be unnecessary for successful sequencing.

2.8. *mRNA sequencing: rationale*

Direct sequencing of mRNAs by the dideoxy chain-termination technique was largely as described[22] with minor modifications. The procedure relies on relatively large amounts of specific immunoglobulin mRNA synthesized by hybridomas, and the ability to prime cDNA synthesis of variable regions using synthetic oligonucleotides specific for invariant constant region domains.

For sequencing of the V_HDJ_H junctions, primers complementary to the C_H1 domains of

the γ constant regions were synthesized using an Applied Biosystems DNA synthesizer or obtained from Dr. Joel Habener (Mass. General Hospital). The primers included those specific for each of the γ subtypes. IgG2a and 2b isotypes are similar enough to prime with the same consensus oligonucleotide.

To derive the primary structure of the V region, several sets of reactions were performed priming sequentially further 5′ on the mRNA, using primers specific for relatively conserved parts of the V domain.

2.9. *Technique*

Total cellular RNA (40 μg) and primer (110 ng) were denatured in buffer (50 mM Tris, pH 8.3, 60 mM NaCl, 6 mM $MgCl_2$ and 20 mM DTT) in a total volume of 32 μl at 65° for 5 min and allowed to anneal, first at 42° and then at room temperature, for 5 min each. The mixture was divided into two tubes, and 2.2 μl radiolabelled nucleotide (α-^{32}P-dCTP or α-^{32}P-dTTP, 400 Ci/mmol; Amersham) to provide $\cong$ 2 μM final concentration and 0.5-2 μl AMV reverse transcriptase (Life Sciences, St. Petersburg, FL) added. After gentle mixing and centrifugation, the mixture from each tube was divided (4 μl/aliquot) into four tubes containing 1 μl of 5X 'd + dd' stocks (Pharmacia; three dNTPs with one dideoxy NTP, final concentrations 50 μM and 10 μM, respectively; except the ddCTP in the labeled C reaction, and ddTTP in the labeled T reaction, were present at 0.4 μm). Reactions proceeded 15 min at 42°, then were 'chased' by adding 1 μl of 500 μM all four dNTPs and incubated 15 min. Reactions were quenched with 100 μl Stop (0.3 M sequencing grade Na acetate, 2 mM EDTA, and 20 μg/ml sonicated herring sperm DNA) and labeled cDNA precipitated with 300 μl ethanol. Pellets were dried by allowing drainage on to a piece of paper and then warming them upright in a 37°C incubator for 15-30 min. Pellets were taken up in 5 μl and loaded in the appropriate lane of a sequencing gel.

In addition to using a chase with excess unlabeled dNTPs[22] to minimize sequence ambiguities due to pauses by the enzyme, all sequencing reactions were done in parallel using ^{32}P-dCTP and ^{32}P-dTTP (the labeled deoxynucleotide, being present at the lowest concentration, is usually responsible for ambiguity). Gels were run at 2000-2400 V until the xylene cyanol marker migrated to 60-65 cm. Top and bottom halves of gels were exposed to X-ray film separately, with intensifying screens used only on bottoms. Exposure varied from overnight to one week, depending on the experiment.

2.10. *Technical considerations*

Unlike dideoxy sequencing using pure single-species DNA templates and the Klenow fragment of DNA polymerase, only one strand was available for sequencing and consequently complete reliability of sequence information at all positions could not be attained. Certain features of the protocol minimized but did not necessarily eliminate ambiguities.

The rate of occurrence of actual errors in base assignment was low. Based on repeated sequencing and reading of the same RNA, the running of multiple different RNA

preparations side by side on gels to provide internal controls, and the use of a known 'baseline' sequence, 186.2, to which the new sequences were compared, the error rate was unlikely to be greater than 1%. In fact it was probably lower; the V_H mRNA sequence of the anti-DNA hybridoma BV04-01 (for which no exact prototypical sequence was available) had only one erroneous base assignment out of approximately 360, and could be used to correct ambiguities in the Maxam-Gilbert sequencing (P. Pace and A. L. M. Bothwell, unpublished) of the cloned rearranged gene.

2.11. *RNA preparation for Northern blots*

Total RNA was prepared from anti-DNA hybridoma cells using the guanidium/cesium chloride method. Modifications included resuspension of the RNA pellet following cesium chloride centrifugation into the original 4M-guanidium extraction buffer and storage of the RNA in distilled water at $-70°C$ after the final ethanol precipitation.

2.12. *Total RNA Northern blot analysis*

Approximately 12 μg of total RNA prepared as described above was denatured in 2M glyoxal and electrophoresed in 1% agarose slab gels in 20 mM $NaPO_4$. RNA integrity was verified by the presence of distinct 28S- and 18S-ribosomal RNA bands after staining with ethidium bromide. RNA was transferred to Gene Screen Plus membrane (transfer buffer was 2X SSC). The membrane was prehybridized in sealed bags in 6X SSC, 5X Denhardts, 0.7% SDS, and 100 ug/ml denatured salmon sperm DNA at 37°C with constant agitation for 12-24 h. Labeled nucleotide probes (specific activity of $1\text{-}6 \times 10^8$ cpm/ug) prepared as described below were boiled for 10 min and added to the prehybridization solution at a concentration of $1\text{-}3 \times 10^6$ cpm/ml. Hybridization was for 15-20 h at 37°C with constant agitation. The membrane was washed twice in 2X SSC, 0.5% SDS at 22°C for 20 min followed by two washes at either 37°C (non-stringent conditions) or 55°C (stringent conditions) in 2X SSC, 0.1% SDS for 30 min. Membrane was air dried for 1 min, immediately wrapped in saran wrap and exposed to Kodak X-Omat AR film with an intensifying screen at $-70°C$.

2.13. *Labelling of probes*

Oligonucleotides complimentary to 18 base pairs of the amino terminal end of γ2A or γ2B H chain constant region were end labeled using T4 kinase and γ-^{32}P. Free nucleotide was removed by either ethanol precipitation or passage through a Sepak C18 cartridge (Millipore) using manufacturers instructions. The oligonucleotide complementary to CDR2 of BV04-01 was cloned into the Sma 1 site of pTZ18U as described in *Results*. Plasmid containing the cloned fragment was cleaved with PvuII and electrophoresed in a 1% agarose gel. The resulting 405 base pair fragment, including the cloned oligonucleotide was purified from the gel slice by electroelution and subsequently labeled with α-^{32}P dATP using a random-primed DNA labeling procedure (BMB). Free nucleotide was removed by gel filtration using a Sephadex G-50 spin column.

3. Results

3.1. *Monoclonal anti-ssDNA autoantibodies*

Binding properties of BW-derived monoclonal anti-DNA autoantibody BV04-01 which reacted preferentially with thermally denatured calf thymus and plasmid DNA were examined to evaluate the molecular basis of anti-ssDNA specificity. Throughout these studies, DNA binding properties of BV04-01 were compared to another BW-derived anti-ssDNA monoclonal autoantibody BV16-19. BV04-01 and BV16-19, although both specific for ssDNA, were unrelated idiotypically[19]. Results presented describe effects of various antigen and reaction parameters on monoclonal antibody/ssDNA binding in solution and/or solid phase RIA, including: (a) deoxyribohomopolymer base composition, (b) oligomer size, (c) temperature, and (d) ionic strength.

3.2. *Nucleotide specificity of anti-ssDNA antibody*

Antibodies BV04-01 and BV16-19 were affinity purified from BALB/c ascites and hybridoma culture supernatants. Electrophoretic analyses of purified antibodies indicated such preparations were free of detectable non-Ig contaminants. Calf thymus DNA antigens (ssDNA and dsDNA) and deoxyribohomopolymers electrostatically complexed with solid phase methylated BSA (mBSA) were titrated with the purified monoclonal antibodies. Both antibody preparations exhibited: (1) preferential binding with denatured DNA, and (2) specificity for pyrimidine-containing synthetic homopolymers [*i.e.* poly(dT)] when tested over a 1000-fold concentration range (figs 1 and 2). Nonspecific antibody binding with mBSA-coated control plates in the absence of nucleic acid was negligible in the concentration range tested.

3.3. *Polynucleotide size dependency and ssDNA binding*

Thymidine oligomers incremental in size up to 20 consecutive residues were used to estimate the limiting antigen size required for stable complex formation with each monoclonal antibody. Phosphorylated (5′) oligodeoxythymidilic acids within this size range were covalently conjugated to BSA at normalized molar oligomer/protein ratios through carbodiimide activation for use as coating reagents in the solid phase RIA. Binding of oligomer conjugates by either BV04-01 or BV16-19 in the solid phase assay became significant when the polymer size exceeded 5 bases (figs 3 and 4). No binding to oligomer containing 1 to 5 residues was detected relative to control titrations of unsubstituted BSA treated with carbodiimide in the absence of antigen.

3.4. *Binding studies with synthetic pyrimidine polymers*

Dephosphorylated oligodeoxythymidilic acids containing chain lengths of 10 and 18 bases [oligo$(dT)_{10}$ and oligo$(dT)_{18}$] were used as test antigens in a modified Farr assay to compare direct and competitive binding reactivities of BV04-01 and BV16-19. Oligomers were end-labeled (5′) with [γ-^{32}P] ATP and separated from unincorporated label by gel filtration

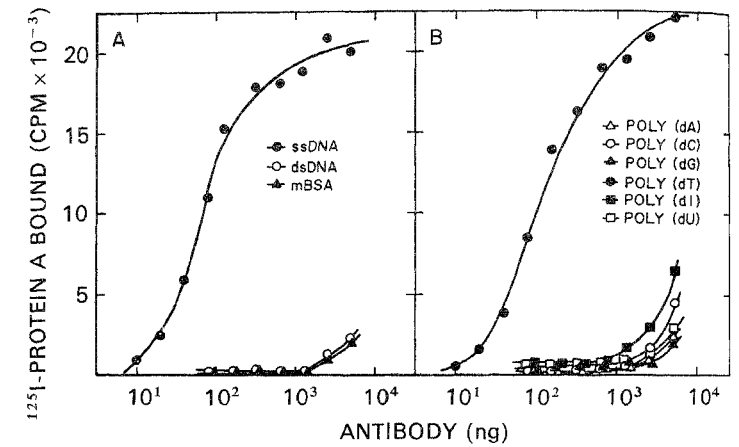

FIG. 1. Binding of monoclonal autoantibody BV04-01 to solid-phase deoxyribohomopolymers. Methylated BSA (mBSA)-coated polystyrene wells were incubated with 50 μl of DNA (1 ug/ml), masked with 1% BSA, and titrated with increasing concentrations of affinity-purified BV04-01. Bound antibody was detected with ^{125}I-protein A (input ~ 50,000 cpm). Control experiments included incubation of buffer rather than DNA in mBSA-coated wells.

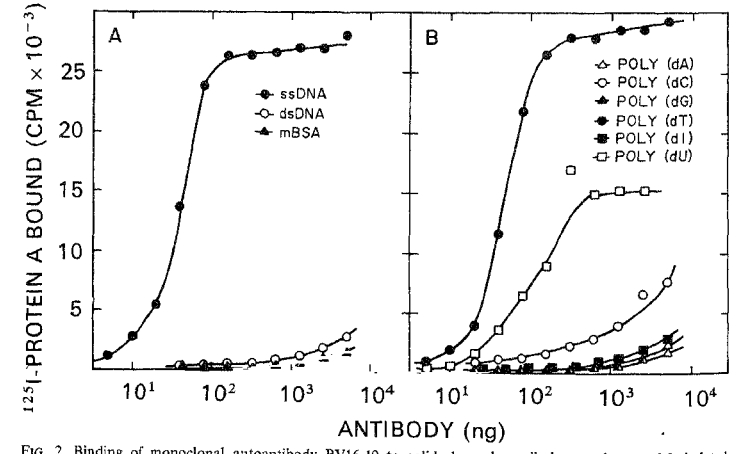

FIG. 2 Binding of monoclonal autoantibody BV16-19 to solid-phase deoxyribohomopolymers. Methylated BSA(mBSA)-coated polystyrene wells were incubated with 50 μl of DNA (1 ug/ml, masked with 1% BSA, and titrated with increasing concentrations of affinity-purified BV16-19. Bound antibody was detected with ^{125}I-protein A (input ~ 50,000 cpm). Control experiments included incubation of buffer rather than in mBSA-coated wells.

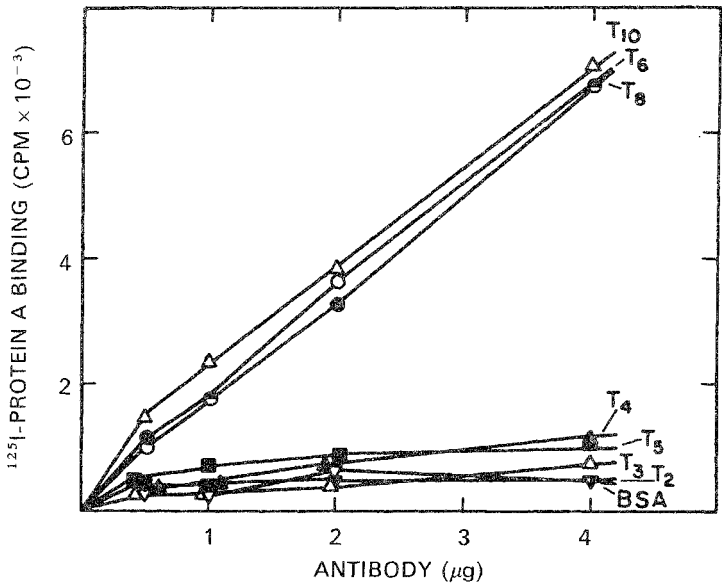

FIG. 3. Dependence of BV04-01 binding on size of oligodeoxythymidylic acid oligomer. Oligodeoxythymidylic acid (T_n, n is the number of bases per epitope) was conjugated to BSA at normalized molar oligomer/protein ratios and coated (1 ug/ml) on polystyrene wells. Wells were masked with 1% BSA and titrated with increasing amounts of affinity-purified BV04-01 Bound antibody was detected with ^{125}I-protein A (input ~ 100,000 cpm) Control experiments included substitution of BSA, treated with coupling agent in the absence of oligomer, for BSA-T_n conjugate.

(Sephadex G-50). Column fractions containing [^{32}P] DNA, as determined by analytical thin-layer chromatography, were > 90% precipitable by specific antibody in the presence of 50% saturated ammonium sulfate. Relative binding constants (K_r) determined from solution titrations of [^{32}P]dT_{10} with BV04-01 and BV16-19 differed about four fold (K_r values of 2 and 0.5 μM, respectively (fig. 5)). K_r values for both autoantibodies were augmented five to ten fold upon increasing the test oligomer length from 10 to 18 nucleotides. Binding of either BV04-01 or BV16-19 with [^{32}P]dT_{10} was inhibitable by poly(dT) and poly(dU) (fig. 6). Nucleic acid concentrations (expressed as molar nucleotides due to polydispersion) required to effect 50% inhibition of [^{32}P]dT_{10} binding (K_i) by BV16-19 [poly(dT)K_i ~ 0.3 uM; poly(dU)K_i ~ 4 uM] and BV04-01 [poly(dT)K_i ~ 0.7 μM; poly(dU)K_i ~ 8 μM] indicated preferential binding (> 10 fold) with poly(dT) relative to poly(dU) for both monoclonal antibodies. Half-maximal inhibition of monoclonal antibody [^{32}P]dT_{10}

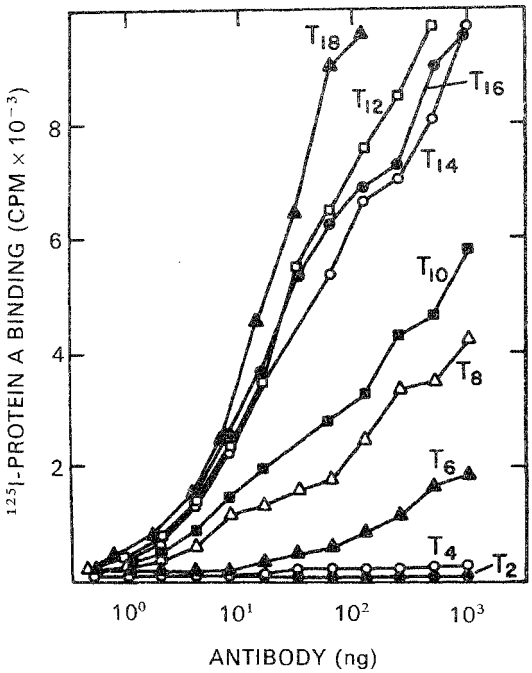

FIG. 4. Dependence of BV16-19 binding on size of oligodeoxythymidylic acid oligomer. Oligodeoxythymidylic acid (T_n, n is the number of bases per epitope) was conjugated to BSA at normalized molar oligomer/protein ratios and coated (1 ug/ml) on polystyrene wells. Wells were masked with 1% BSA and titrated with increasing amounts of affinity-purified BV04-01. Bound antibody was detected with ^{125}I-protein A (input ~ 100,000 cpm). Control experiments included substitution of BSA, treated with coupling agent in the absence of oligomer, and showed negligible binding (< 500 cpm).

binding by poly(dG), poly(dC), or poly (dI) was not attained ($K_r > 10\ \mu M$) within this range. Due to base pairing complementary with [^{32}P]dT_{10}, poly(dA) was not tested.

3.5. *Dependency of anti-DNA binding on temperature and ionic strength*

End-labeled oligo$(dT)_{10}$ was employed in the Farr assay to measure relative effects of reaction temperature and ionic strength on the equilibrium concentration of DNA/anti-

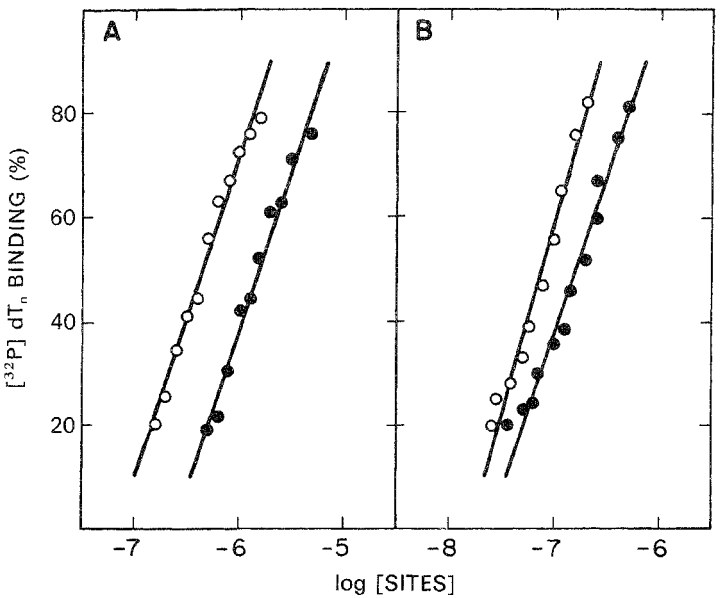

FIG 5. Monoclonal anti-DNA antibody binding with ^{32}P-end labeled oligodeoxynucleotides dT_{10} and dT_{18}. Four picomoles of $[^{32}P]dT_{10}$ (fig. 5A) or $[^{32}P]dT_{18}$ (fig. 5B) (input ~ 40,000 cpm) were incubated with increasing amounts of affinity-purified BV16-19 (○) or BV04-01 (●). Binding values were determined from the percentage of input $[^{32}P]dT_n$ (normalized with respect to molar fragments) precipitable in 50% saturated ammonium sulfate.

DNA complexes. Temperature-dependent binding isotherms were constructed from independent titrations of $[^{32}P]dT_{10}$ with BV04-01 and BV16-19. Relative binding constants (K_r) were determined at 50% binding as a function of temperature. As shown in fig. 7A, oligonucleotide binding by either BV04-01 ($\Delta H \sim -7.5$ Kcal/mole) or BV16-19 ($\Delta H \sim -4.6$ Kcal/mole) was characterized by an inverse temperature dependence between 2 and 40°C.

Studies with DNA-binding proteins have indicated that ionic strength sensitivity reflects the degree to which electrostatic interactions stabilize formed complexes. Stability of polyclonal anti-DNA/DNA complexes in the presence of high salt is dependent on the functional affinity of interacting anti-DNA antibody. The influence of ionic strength on DNA binding by BV16-19 and BV04-01 was determined by titrations of $[^{32}P]dT_{10}$ equilibrated at different NaCl concentrations in the Farr assay. Relative monoclonal antibody/$[^{32}P]dT_{10}$

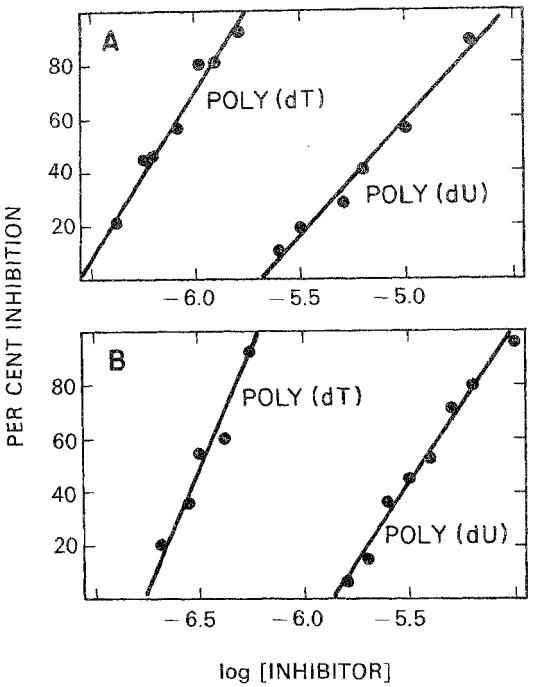

FIG. 6. Inhibition of monoclonal antibody/[^{32}P]dT_{10} binding by pyrimidine deoxyribohomopolymers in modified Farr assay. Binding of [^{32}P]dT_{10} (4 pmoles; input ~ 30,000 cpm) was inhibited by incubation of affinity-purified BV04-01 (fig. 6A) or BV16-19 (fig. 6B) with polydisperse homopolymers (concentrations expressed in molar nucleotides) prior to the addition of [^{32}P]-labeled oligomer. Antibody-bound [^{32}P]dT_{10} was precipitated in 50% saturated ammonium sulfate and quantitated by liquid scintillation counting.

binding constants (K_i) determined from titrations over a 0.15 to 1.5 M NaCl concentration range (fig. 7B) decreased less than two fold with increasing ionic strength, suggesting a minor role for phosphate moieties relative to the heterocyclic base in monoclonal anti-DNA/oligomer complex stability. These data were consistent with results obtained from independent titrations in which excess NaCl was added subsequent to mixing antibody with end-labeled DNA under physiological salt condition (*i.e.* 0.15 M NaCl).

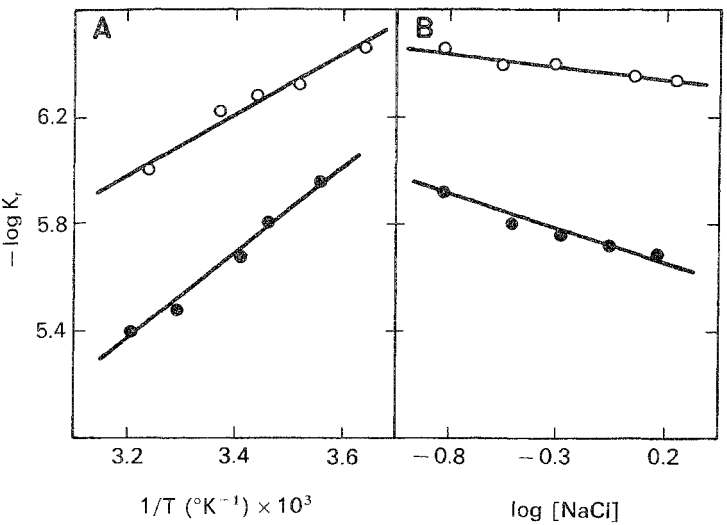

FIG 7. Dependence of anti-DNA antibody binding on temperature and ionic strength. Relative binding constants were determined by titration of [^{32}P]dT_{10} (4 moles) with affinity-purified BV16-19 (-○-○-) or BV04-01 (-●-●-) as a function of temperature (fig. 7a) or under conditions of increasing [NaCl] at 4 (fig. 7b). Active site concentrations required to yield 50% precipitation of input oligomer in the presence of half-saturated ammonium sulfate (K_r) were computed by linear regression of binding isotherms (see fig. 5) constructed for each parameter indicated.

3.6. *Properties of anti-DNA Fab fragments in the Farr assay*

Papain-derived monovalent Fab fragments, of BV04-01, were purified by ssDNA agarose chromatography (fig. 8) and tested for binding in the Farr assay relative to non-digested BV04-01. Because the minimal distance between Fab regions in bivalent IgG ranges 60-100 Å, both Fab and intact IgG should exhibit similar affinities for an oligomer containing <20 base (<100 Å). To confirm this contention, [^{32}P]dT_{18} was titrated with increasing amounts (normalized with respect to molar-active site concentration) of BV04-01 Fab fragments and intact protein in the Farr assay. As shown in fig. 8, binding curves for such titrations were displaced from each other by at least two orders of magnitude. Further studies with [^{32}P]dT_{10} yielded similar differences in K_r values for the two monoclonal proteins. These results were shown to reflect differential solubilities of intact and monovalent antibody [$<5\%$ and $>75\%$ solubility, respectively) in the presence of carrier protein (2 mg/ml) and half-saturated ammonium sulfate.

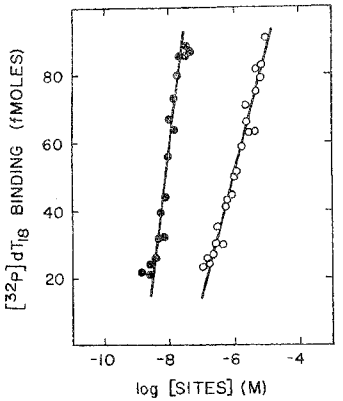

FIG. 8. Comparative binding of BV04-01 and Fab fragments with $[^{32}P]dT_{18}$. One hundred femptomoles of $[^{32}P]dT_{18}$ (input ~ 20,000 cpm) were titrated with increasing amounts (normalized with respect to molar active site concentration) of intact BV04-01 protein (●) or BV04-01 Fab fragments (○). Binding values were determined from the percentage of input $[^{32}P]dT_{18}$ precipitable in 50% saturated ammonium sulfate after incubation with specific antibody.

3.7. *Variable region primary structure of BV04-01*

Based on defined binding and isotypic properties of hybridoma BV04-01 it was important to determine the primary structure of the constitutive polypeptide chains through sequence analyses. Such determinations are imperative to establish structure-function relationships. Figures 9 and 10 show the primary sequences of BV04-01 derived heavy (H) and light (L) chains, respectively.

The nucleotide sequence of the variable (V_H) domain of the heavy chain and the amino acid sequence deduced from the nucleotide sequence was determined using two procedures. First, messenger RNA derived from hybridoma cells was sequenced by the dideoxy primer extension method, with reverse transcriptase and synthetic oligonucleotide primers. Two oligonucleotides were used as primers, one specific for the constant (C_H1) domain and the second constructed complementary to sequences around codon 70 for the V_H domain. In addition, the functional V_H gene was cloned from genomic DNA isolated from the BV04-01 hybridoma. A recombinant-phage library containing DNA which was partially digested with Mbo I was constructed in EMBL-3 vectors. Phage hybridized with J_H probes was isolated by standard procedures. DNA sequences were determined by the methods of Maxam and

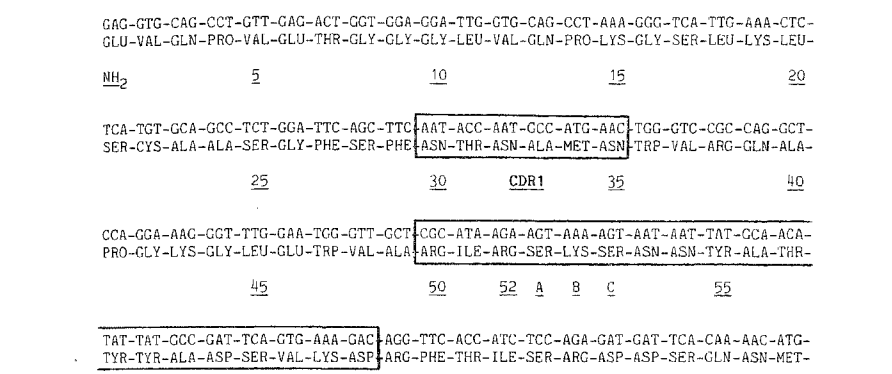

FIG. 9. Nucleic and deduced amino acid primary structures of heavy chain variable region of BV04-01. Complementarity determining regions (CDR1, CDR2 and CDR3) are indicated respectively by enclosure within boxes. Location and types of D and J regions are denoted below the appropriate position in the sequence.

Gilbert. Using similar procedures, the nucleotide sequence and deduced amino acid sequence of the V domain of the light chain was determined (fig. 10).

3.8. *Primary structure analysis of unique CDR of BV04-01*

Having established both the nucleotide and amino acid primary structures of hybridoma BV04-01 it was important to relate the results with existing information.

First, with regards to the complementarity determining regions (CDR) the primary amino acid sequences of the light chain of BV04-01 light chain were analyzed by comparison to sequence computer data bases. In the case of CDR1, twelve Ig light chains were identified with no amino acid differences. CDR2 showed 23 light chains identified with no differences in the matching pattern. Finally a computer-based comparison (PROPHET Computer System of the Division of Research Resources, N.I.H.) of CDR3 showed only one light chain with no

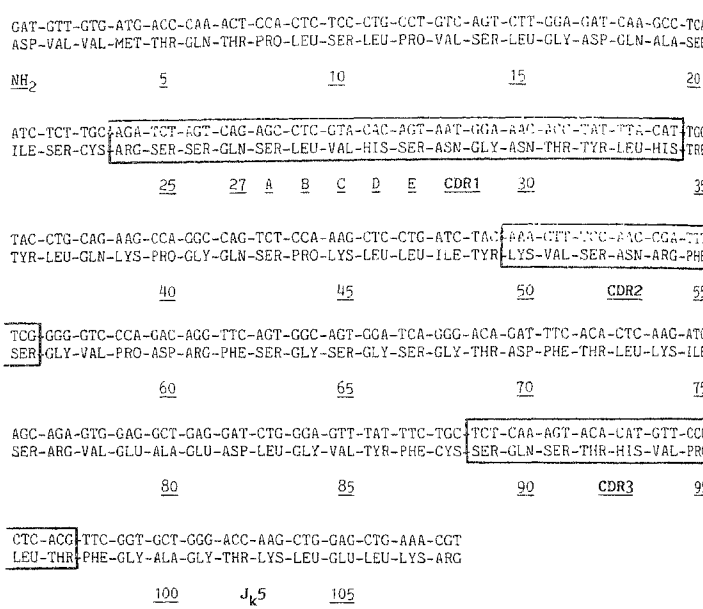

FIG. 10. Nucleotide and deduce amino acid primary structures of light chain variable region of BV04-01. Complementarity determining regions (CDR1, CDR2 and CDR3) are indicated respectively by enclosure within boxes. The J_K5 region is denoted below the appropriate position in the sequence.

differences in the matching pattern. The latter was identified as TEPC-602.

Examination of the heavy-chain amino-acid sequence of BV04-01 in a similar computer search revealed quite different results. In separate analyses of CDR1, CDR2 and CDR3 no heavy chains were identified with no differences in the matching pattern. In particular CDR2 was found to be particularly unique, containing sequences not found in other characterized immunoglobulins.

3.9. *Chemical synthesis of CDR2 heavy-chain nucleic-acid probe*

A polynucleotide corresponding to the sequence of CDR2 of the H chain of BV04-01 was synthesized using the phosphoramidite polynucleotide synthetic scheme. The oligomer contained the sequence shown in fig. 11. The polynucleotide probe was inserted into the SmaI site of the plasmid (PZT18/19R/U) making use of the SmaI tails generated during synthesis, and produced in quantities sufficient for purification and verification of nucleotide

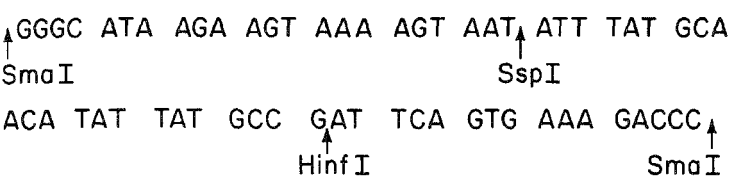

FIG 11 Sequence of synthetic polynucleotide probe corresponding to H chain CDR2 of BV04-01 The original CDR2 sequence was altered by the addition of Sma I tails and an A to T transition at position 24 generating an Ssp I restriction site.

primary sequence. One base (A for T) was alternated on a 50% molar basis during the synthetic process in order to incorporate an Sspl restriction site (fig. 11). Upon purification of the oligomer it was determined (agarose gel) that 50% of the synthesized product was cleaved with the restriction endonuclease Sspl and 50% was resistant to cleavage. Use of the complete probe or portions thereof (37 or 63%) derived *via* the engineered Sspl site allowed for relative hybridization analyses with specific segments of the probe.

3.10. *Hybridization studies with heavy-chain CDR2 probe*

The CDR2 synthetic polynucleotide was radiolabeled as described (*Materials and methods*) and used to probe Northern blots of total cellular RNA obtained from several different anti-DNA antibody hybridoma cell lines. Figure 12A shows an ethidium bromide-stained agarose gel of RNA from 4 cell lines producing antibodies specific to ds or ssDNA.

Hybridization results with the CDR2 polynucleotide probe are shown in fig. 12. Hybridization and subsequent washing conditions were kept non-stringent to allow for potential weak interactions between the probe and partially homologous RNA sequences. In addition, the presence of intact IgG heavy-chain mRNA for each of the cell lines had previously been determined by probing with an end-labeled γ2A and γ2B constant region probe (data not shown). Specific hybridization of the CDR2 probe occurred only with BV04-01 total RNA and not with RNA derived from other cell lines (lane 2, fig. 12B).

3.11. *Crystallization of BV04-01 Fab fragments*

Purified Fab fragments, obtained from BV04-01 by papain digestion, have been crystallized as described[11], and were dialyzed against 50 mM sodium phosphate, with the pH adjusted to the pI of the fragment (7.3 or 7.6). They were crystallized at protein concentrations of 12-20 mg/ml in the presence of 1.7-1.8 M ammonium sulfate[11]. X-ray diffraction data were collected to 2.0-Å resolution. Orientation of the Fab in the unit cell was determined by molecular replacement methods in conjunction with the known structure of McPC 603 Fab.

4. Discussion

Binding properties comparing two BW murine monoclonal anti-ssDNA autoantibodies

(BV04-01 and BV16-19) revealed similar specificity patterns for ssDNA and synthetic polynucleotides (figs 1 and 2). Both BV04-01 and BV16-19 preferentially bound poly(dT), but the latter showed a significant degree of cross reactivity with poly(dU) (fig. 6). Correlation between oligodeoxythymidilic acid size and binding by both anti-ssDNA monoclonal autoantibodies was similar (figs 3–5). Ligand inhibition studies in addition to temperature and ionic strength dependency indicated that BV16-19 was probably of slightly higher affinity than BV04-01 (fig. 7). The latter was consistent with the relatively higher degree of cross reactivity exhibited by BV16-19 (fig. 2). Collectively the binding studies indicated that the pyrimidine base (thymine) was the immunodominant epitope (figs 1 and 2). This was consistent with ionic strength studies which suggested a minor role for phosphate moieties (fig. 7). Of secondary importance but further defining specificity, both BV04-01 and

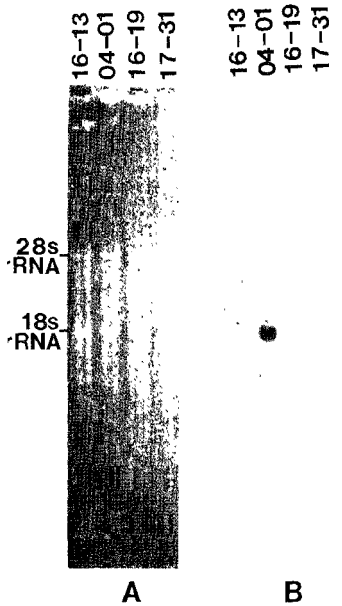

FIG 12. A. Ethiduim bromide-stained agarose gel of total cellular RNA obtained from four different anti-DNA antibody-secreting hybridomas. 16-13 (anti-dsDNA), 04-01 (anti-ssDNA) 16-19 (anti-ssDNA), 17-31 (anti-dsDNA). B. Northern blot of agarose gel from panel A probed with ^{32}P-labeled BV04-01 H chain CDR2 oligonucleotide.

BV16-19 preferentially bound the deoxyribose form of thymidine[23]. Thus, both autoantibodies appeared to exhibit primary specificity for ssDNA and a lower affinity cross reactivity with RNA (fig. 6). The minimum length (pentanucleotide) requirement for binding of the oligomer (figs 3 and 4), further suggested that both autoantibodies preferred the recognized series of nucleotides to be in a particular conformation which was stabilized by the longer homopolymers. This was further emphasized in the preferential binding of poly$(dT)_{18}$ relative to poly$(dT)_{10}$ (fig. 5). It was not determined to what degree antibody induced a desired conformation in the antigen upon binding.

Despite similar binding properties exhibited by both anti-ssDNA monoclonal antibodies, it had been previously determined that BV04-01 and BV16-19 were not idiotypically related[19]. Preliminary comparative amino-terminal sequence data (first 25-30 residues) compiled with H and L chains derived from each protein had revealed significant differences.

Primary structures of the variable regions of the H and L chains of BV04-01 (figs 9 and 10) allowed for a preliminary correlation between structural features and DNA binding (specificity) patterns. Consistent with the primary structure of DNA (*i.e.* base, deoxysugar and phosphate groups) one might anticipate a binding protein featuring aromatic and basic amino acid residues. CDR2 and 3 of BV04-01 heavy chain showed a predominance of aromatic residues (tyrosine and tryptophan) as well as arginine and lysine residues. CDR regions of the light chain (fig. 10) featured similar residues, but in addition histidine moeities are evident. Having succeeded in crystallizing and analyzing the conformation of the Fab fragment derived from BV04-01[24], it was possible to determine if the residues cited above correlated with the structure and function of the autoantibody active site of BV04-01.

Based on Fourier analysis of X-ray diffraction patterns from crystalline Fab fragments of BV04-01, the active site was a large, irregular groove[24] of the general type postulated by Kabat for linear polymers[25]. The groove was lined with amino-acid side chain residues from both the heavy and light chains[24]. Specifically, the light chain contributed two histidine and one tyrosyl residue. The heavy chain provided single tryptophan, tyrosine and lysine residues in addition to two arginines. Thus, in considering the antigen-binding site of BV04-01, its architecture and chemical composition was consistent with the binding of a linear nucleic acid polymer. The principal constituents chemically were aromatic amino-acid residues enriched with positively charged side chains. This structure predicted certain specific intermolecular forces[26]. Steric complementarity was evident in the groove-linear polymer fit evident in BV04-01. The aromatic amino-acid residues implied van der Waals'-London or dispersion forces involving the nucleic acid bases (*e.g.* polydeoxythymidine). Positively charged amino acids lining the groove implied electrostatic (or Coulombic) interactions especially involving the negatively charged phosphate groups in the nucleic acid polymer. Finally, hydrogen bonding is inferred by dipole interactions with amino acids such as arginine, histidine, lysine, tyrosine and tryptophan. The energy associated with hydrogen bonding is of the same order as van der Waals' and ionic interactions.

Light-chain constituents near the groove included residues 27B-34, 49-50, and 89-98. Contributed heavy-chain residues were 47-64 and 98-105. Studies in this report have begun to establish a structure function model based on an anti-ssDNA specific autoantibody. The

structure-function model is important in order to better understand the reactivity of an autoantibody and to elucidate an immunoglobulin involved in important protein-nucleic acid interactions. Central to these studies is the origin of the anti-ssDNA specificity exhibited by BV04-01. Comparative analyses of the primary structure indicated that the light chain of BV04-01 shared significant primary structure homology with several characterized monoclonal antibodies. For example, the first 95 residues (V_k) were identical to that of TEPC 105. However, the adjacent (J_k) segment belonged to a different subclass with a sequence of LTFGAGTKLELKR. The primary structure of the BV04-01 heavy chain differed relatively more with known sequences. Based on this trend the CDR regions were analyzed since they directly reflect binding specificity. As indicated in *Results*, CDR2 of the heavy chain was unique and a synthetic polymer was constructed corresponding to the established sequence (fig. 11).

In Northern blot hybridization studies it was shown that neither BV16-19 (anti-ssDNA) nor BV16-13 and BV17-31 (both anti-dsDNA) shared the unique CDR. The hybridization results with BV16-19 were interesting due to the similar DNA binding properties shared with BV04-01 and documented extensively in this report. The results suggest that the sequence of CDR2 in the heavy chain of BV16-19 will differ significantly from BV04-01.

In summary, these studies document a preliminary attempt to understand basic structure-function relationships in an anti-ssDNA autoantibody. Primary and conformation structural information have been correlated with antigen-binding patterns in a preliminary way. More complete Fourier analyses will be completed in the near future[24].

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