# Construction and Characterization of a Quadruplex DNA Selective Single-Chain Autoantibody from a Viable Motheaten Mouse Hybridoma with Homology to Telomeric DNA Binding Proteins†

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*Received June 17, 1998; Revised Manuscript Received September 9, 1998*

ABSTRACT: An autoantibody produced by a hybridoma derived from a viable motheaten mouse was isolated and found to have moderately high binding affinity for nucleic acids and specific preference for quadruplex DNAs. Polymerase chain reaction primers were designed to link the cloned parental antibody variable region fragments together in a subcloning vector. This single-chain variable fragment construct was then subcloned into the T7 promoter-driven expression vector pET22b(+). The construct contains (N- to C-terminal) a *pelB* leader sequence, variable heavy chain, glycine-serine polylinker, variable light chain, and biotin mimic peptide "strep-tag" sequence (*pelB*−$V_H$−linker−$V_L$−strep-tag). The ca. 29 kDa protein was expressed, exported to the periplasmic space of NovaBlue (DE) *Escherichia coli*, and purified by streptavidin affinity chromatography by binding the fused strep-tag peptide. The specificity of the purified single-chain variable fragment (scFv) for quadruplex and duplex DNAs was evaluated by a radioimmunofilterbinding assay. It retained about 10-fold higher affinity for quadruplexes relative to duplex DNA, a reduction of ca. 4-fold from the relative preferences of the parent IgG. The complementary-determining regions contain sequences that are homologous to or conservatively divergent from the key DNA-binding helix−turn−helix-forming motifs of Myb/RAP1 family telomeric DNA-binding proteins (*1*−*3*). The presence of this antibody in the autoimmune repertoire suggests a possible linkage between autoimmunity, telomeric DNA binding proteins, and aging.

The application of powerful cloning methods based on the polymerase chain reaction (PCR)[1] has allowed direct access to a large number of immunoglobulin heavy- and light-chain sequences while increasing the speed of cloning (*4*). Larrick et al. (*5*) have demonstrated that the variable (V) genes of a hybridoma could be amplified by PCR using degenerate primers. The key to this technique was to develop "universal primers" that hybridized to conserved regions in the 5′ and 3′ regions of the V genes, while also incorporating restriction site sequences, so the V-genes could be force-cloned into an expression vector (*6*, *7*). Many "universal primer" families have now been identified to allow cloning of V-genes from human (*8*), mouse (*9*), or rat (*10*) hybridomas.

The Fv is the smallest unit of an antibody that retains the complete antigen binding site. Until recently, it was only available by proteolysis (*11*), but now it can be produced in soluble form in *Escherichia coli*. Several investigators have noticed that the sequence variability of the $V_H$ and $V_L$ chains produces variations in intramolecular interactions that result in a range of stabilities in the absence of antigen. This has led to the development of novel ways to stabilize them (*12*), such as the single-chain Fv (scFv), which consists of the variable domains of the antibody connected by a peptide linker, as first reported by Bird et al. (*13*) and Huston et al. (*14*). Single-chain antibodies have several attributes that make them very popular and versatile: they are small, easy to engineer, stable at low concentrations, and rapidly penetrate into and clear from tissues (*15*, *16*). ScFvs typically have binding affinities equivalent to, or within 1 order of magnitude of, the parent monoclonal antibodies (*17*).

Autoimmune mice often produce antibodies against both single- and double-stranded (ss and ds) DNA; e.g., New Zealand Black × New Zealand $F_1$ (NZB × NZW $F_1$) and Murphy-Roths lymphoproliferator (MRL-*lpr*/*lpr*). The occurrence of anti-dsDNA antibodies has been called the hallmark of systemic lupus erythematosis (SLE) (*18*). Anti-DNA antibodies have only recently been found in viable motheaten (VME) autoimmune mice (*19*). In subsequent analyses it was found that several of the VME mouse antibodies studied by Westhoff et al. (*19*) bound to quadruplex DNAs (*20*−*22*; manuscript in preparation).

† Supported by grants from the National Institutes of Health (GM47431), North Carolina Biotechnology Center, and North Carolina Agricultural Research Service.

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[1] Abbreviations: AP, alkaline phosphatase; $B_{max}$, maximal amount bound; CDR, complementarity-determining region; ELISA, enzyme-linked immunosorbent assay; Fab, antigen binding fragment; Fv, variable region fragment; IgG, immunoglobulin G; IgM, immunoglobulin M; PCR, polymerase chain reaction; *pelB*, signal peptide that directs translation products to the *Escherichia coli* periplasmic space; rTP, replication telomeric protein; RIFB, radioimmunofilter binding assay; scFv, single-chain variable fragment; SLE, systemic lupus erythematosis; strep-tag, biotin mimic peptide; $V_H$, variable heavy-chain fragment; $V_L$, variable light-chain fragment; VME, viable motheaten (C57BL/6J-*Hcph*$^{me-v}$/*Hcph*$^{me-v}$) strain.

10.1021/bi981434y CCC: $15.00 
Published on Web 10/29/1998

Guanine-rich DNA sequences that occur in three major regions of eukaryotic chromatin, telomeres, gene promoter regions, and immunoglobulin switch regions, readily form quadruplexes [reviewed by Williamson (*23*)]. There has been much speculation that these quadruplex formation reactions might play functionally significant roles in scenarios such as protecting chromosomal ends, mediating interactions between telomeres on separate chromosomes, regulating gene expression, V(D)J "switch" recombination, and mediating viral packaging (*23*−*27*).

The present report describes the cloning of a VME-derived quadruplex-specific antibody from cells isolated by Brown et al. (*20*−*22*) and the design, production, and characterization of the corresponding scFv. This tool is currently being used to investigate the possibility that quadruplex DNAs actively participate in biological events.

## EXPERIMENTAL PROCEDURES

*Parent Antibody Cloning.* Total hybridoma RNA was isolated in preparation for cDNA production. Hybridomas were grown in 250 mL Corning slow speed stirring vessels in RPMI-1640 medium supplemented with 20 mM glutamine, 10 mM HEPES (pH 7.4), and 5% FBS under air at 37 °C with continuous stirring. Total RNA was prepared from 225 mL (ca. $9.2 \times 10^7$ cells) of log-phase hybridomas by the guanidine isothiocyanate procedure (*28*).

The Mouse Ig-Prime kit (Novagen) was used to clone the immunoglobulin variable sequence region DNAs that encode the mouse anti-quadruplex DNA antibody; cDNA was synthesized following the protocol supplied with the kit. Primers that were used to reverse-transcribe the cDNA preparations took advantage of the 3′ conserved regions adjacent to DNAs that encode the hypervariable CDRs of the heavy- and light-chain immunoglobulin proteins.

PCR (*29*) was used to amplify specific variable region sequences of the cDNA (*30*). Amplification reactions were accomplished following the protocol supplied with the Novagen Mouse Ig-Prime kit. Only the amplified PCR products were subcloned into pT7Blue T-vector (Novagen) and transformed into NovaBlue *E. coli* cells for proliferation and evaluation of ligation efficiency, using blue/white screening as described in the product literature. The $V_H$ and $V_L$ sequences (Figure 5) were determined by automated DNA sequence analysis at the University of Georgia Molecular Genetics Facility using primers that were complementary to known plasmid-derived flanking sequences. Sequences can be obtained from GenBank (accession numbers: $V_H$, AF089740; $V_L$, AF089741; scFv, AF089742).

*ScFv Expression Vector Construction.* From sequence information obtained for the variable regions, PCR primers were designed and synthesized at the Molecular Genetics Facility (NCSU) to facilitate linkage of the 3′ end of the variable heavy-chain DNA to the 5′ end of the variable light-chain DNA with a glycine−serine [$(G_4S)_3$] polylinker. The primers contained "tails" (nonoverlapping regions) that encode for additional restriction sites, the biotin-like strep-tag, and a glycine−serine-encoding polylinker (Figure 2). $V_H$ or $V_L$ DNA was added to the amplification reaction, containing 10 mM Tris·HCl (pH 8.3), 3.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTP mix, 25 pmol of the appropriate scFv primers (Figure 2), and 1.25 units of Taq DNA polymerase (Boehringer-Mannheim). The thermal cycle protocol consisted of denaturing duplex DNA for 1 min at 94 °C, annealing primers to the $V_H$ or $V_L$ fragments for 1 min at 55 °C, followed by extension of the primers for 2 min at 72 °C. Forty cycles were performed, followed by an additional 10 min at 70 °C. The PCR products were size-selected and gel-purified before ligation into pGEM-T Easy Vector (Promega) and transformed into JM109 *E. coli* for plasmid isolation and verification (Figure 3).

Purified $V_H$ plasmids were digested with *Ngo*MI and *Sal*I, while the purified $V_L$ plasmids were digested with *Sal*I and *Hin*dIII (Promega) to generate restriction sites for unidirectional ligation into the *Ngo*MI/*Hin*dIII-digested pGEM3Z-*pelB* vector. The pGEM3Z-*pelB* contains the *pelB* leader sequence from the pET22b(+) plasmid (Novagen). Ligation reactions were carried out in 10 $\mu$L volumes containing ca. 25 ng of the digested vector (ca. 3 kb), 25 ng of each insert ($V_H$ and $V_L$, 419 or 422 bp, respectively), 2 units of T4 ligase (Promega), 10 mM ATP, 10 mM Tris−acetate (pH 7.5), 10 mM magnesium acetate, and 50 mM potassium acetate. The samples were incubated overnight at 23 °C and then the ligation products were transformed into JM109 *E. coli*.

After the success of the unidirectional ligation was verified by restriction endonuclease analyses, the plasmid product was cut with *Xba*I and *Hin*dIII and the scFv fragment was gel-purified and ligated into the pET22b(+) vector for protein expression. This expression vector was transformed into NovaBlue (DE) *E. coli* cells (Novagen) and sequenced to verify the identity of the scFv insert and reading frame (Figure 4).

*Expression and Purification of scFv.* *E. coli* NovaBlue (DE) cells containing the scFv expression vector were grown at 30 °C until mid-log phase (i.e., $A_{600} \sim 0.6$). To induce expression of the scFv, IPTG was added to cultures to a final concentration of 75 mM and they were incubated at 30 °C overnight with shaking (250 rpm). The protein was harvested by the freeze/thaw method of Johnson and Hecht (*31*).

A column containing 2.5 mL of streptavidin agarose (Gibco−BRL) was equilibrated with 250 mL of wash buffer, 50 mM Tris-HCl (pH 7.4), and 50 mM NaCl. Approximately 2 mL of protein supernatant derived from 500 mL of growth medium, following freeze−thaw and $(NH_4)_2SO_4$ precipitation (31% w/v), was added to the column matrix and then the mixture was incubated for 6 h at 25 °C or overnight at 4 °C, while rocking. The column was washed with 25 mL of wash buffer or until no more protein eluted. Two milliliters of 5 mM diaminobiotin (Sigma) was then added to the column and it was rocked for 30 min to 1 h to facilitate displacement of the strep-tag portion of the scFv from the matrix by diaminobiotin. The elutant was collected and the column was washed with 30 mL of wash buffer. It was regenerated with 100 mM Tris-HCl (pH 8), followed by reequilibration with 50 mL of the wash buffer and then stored at 4 °C. The bicinchoninic acid (BCA) assay (Pierce) was used to determine the concentration of elutant (*32*).

*Analysis of scFv Protein.* A standard SDS−PAGE 12% stacking gel stained with silver (Pierce) was used to visualize the purification procedure of the scFv. In addition, a duplicate gel was used in a Western immunoblot analysis. The proteins on the gel were transferred to nitrocellulose paper via electroblotting and blocked overnight with 0.5% BSA and 0.5% nonfat dry milk dissolved in TBST [25 mM

Tris-HCl (pH 7.4), 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween] buffer. The blot was probed with 2 $\mu$g/mL streptavidin-conjugated AP (Jackson Immunologicals Inc.). Strep-tag-containing scFv protein was detected by incubating the immunoblot with the chromogenic substrates nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in a buffer solution of 100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris·HCl (pH 9.5).

*ELISA Assays.* In addition, ELISAs were used to show that the scFv contained both Fab and streptavidin elements and that they were present throughout the purification procedure. Fractions (150 $\mu$L) from the chromotagraphy samples were incubated in wells of polystyrene plates overnight at 25 °C. The wells were then blocked with 0.5% BSA and 1% nonfat dry milk in TBST for 4 h. Anti-mouse IgG (Fab-specific)−AP (Sigma), and in separate experiments streptavidin−AP (2 $\mu$g/mL), were added to the wells of the polystyrene plate (150 $\mu$L) for scFv detection. Afterward, *p*-nitrophenyl phosphate (5 $\mu$g/mL in 50 mM Tris·HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) was added to each well. The presence of the scFv was detected by absorbance readings obtained at 405 nm at 5 min intervals over the course of 1 h.

*Filter-Binding Assays.* RIFBs were performed in sterile siliconized 0.5 mL eppendorf tubes as described (*20*−*22*). After quantitation by the BCA assay, purified scFv was diluted with 50 mM Tris-HCl (pH 8), 10 mM EDTA, and 50 mM NaCl (TES) to a concentration of 2 pmol/$\mu$L. Aliquots of diluted scFv (10 $\mu$L) were incubated for 90 min at 25 °C in a total volume of 110 $\mu$L with 0−1 nmol of $^{32}$P 5′-end-labeled nucleic acid complexes (in 100 pmol increments). The conformations of the DNAs were verified by CD (*20*−*22*). Following incubation, reactions were transferred to individual wells of Multiscreen-HA (0.45 $\mu$m nitrocellulose) microtiter plates (Millipore) that had been presoaked with TBST for 30 min. Reaction contents were incubated in the plate for 30 min at 25 °C and then aspirated through the filter by applying a vacuum. All filters were washed once with TBST, followed by six washes with TBS (25 mM Tris·HCl, pH 7.4, 137 mM NaCl, and 2.7 mM KCl) buffer, and then air-dried at room temperature for 20 min. Individual filters were punched out into plastic scintillation vials and counted on a Packard TriCarb scintillation counter. Binding data were analyzed as described (*21*, *22*).

## RESULTS

*Parent Antibody Cloning.* The parent antibody, *me*$^{v}$IIB4 (formerly called *me*$^{v}$ α-$Q_1$; *20*), was isolated from VME-derived hybridomas that were previously described (*20*−*22*). This antibody shows a higher specificity for quadruplex DNA than single-, double-, and triple-stranded nucleic acid structures. Our goal was to clone and determine the sequences of the genes that encode the binding site fragments of this antibody and then construct an scFv for expression in *E. coli*.

The Mouse Ig-Prime kit (Novagen) was used to clone the variable heavy and light chains of the parent antibody ($V_H$ and $V_L$). This kit contained a group of universal primers that hybridize with the constant regions on either side of the $V_H$ and $V_L$ genes. These primers amplified IgM, IgG, $\kappa$, and $\lambda$ variable genes separately, so the isotype of the antibody could also be determined. Messenger RNA was isolated

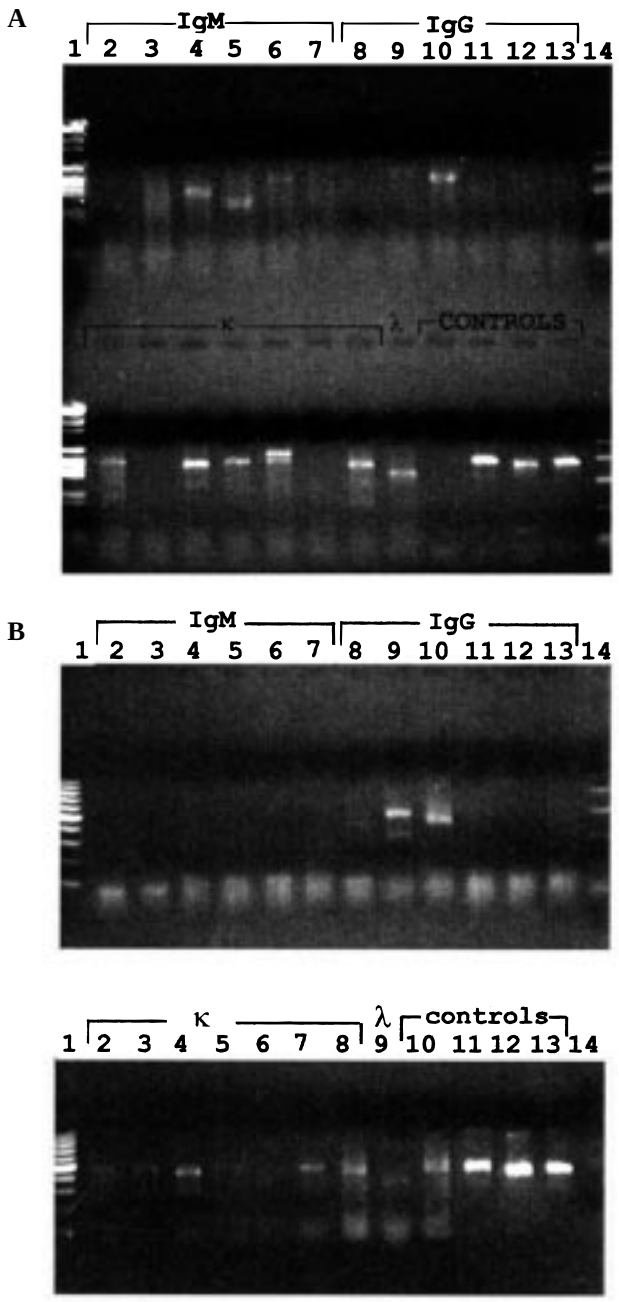


FIGURE 1: (A) PCR amplification of $V_H$ and $V_L$ genes. The original cDNA library and the universal primer set provided in the Mouse Ig-Prime kit (Novagen) were used to PCR amplify variable heavy and light chain genes. (Upper lanes) Lane 1, pGEM molecular weight markers (Promega) (2645, 1605, 1198, 676, 517, 460, 396, 350, 222, 179, 126, 75, 65, 51, and 36 bp); lanes 2−7, IgM PCR products; lanes 8−13, IgG PCR products; and lane 14, PCR molecular weight markers (Promega) (1000, 750, 500, 300, 150, and 50 bp). (Lower lanes) Lane 1, pGEM molecular weight markers; lanes 2−8, $\kappa$ PCR products; lane 9, $\lambda$ PCR products; lanes 10−13, positive controls from the Novagen kit; and lane 14, PCR molecular weight markers. (B) PCR amplification II: $V_H$ and $V_L$ genes. The second cDNA library, which was created 3 months after the first library, and the universal primer set provided in the Mouse Ig-Prime kit (Novagen) were used to PCR-amplify variable heavy and light chain genes. (Upper gel) Lane 1, pGEM molecular weight markers (Promega); lanes 2−7, IgM PCR products; lanes 8−13, IgG PCR products; and lane 14, PCR molecular weight markers (Promega). (Lower gel) Lane 1, pGEM molecular weight markers; lanes 2−8, $\kappa$ PCR products; lane 9, $\lambda$ PCR products; lanes 10−13, positive controls from the Novagen kit; and lane 14, PCR molecular weight markers.

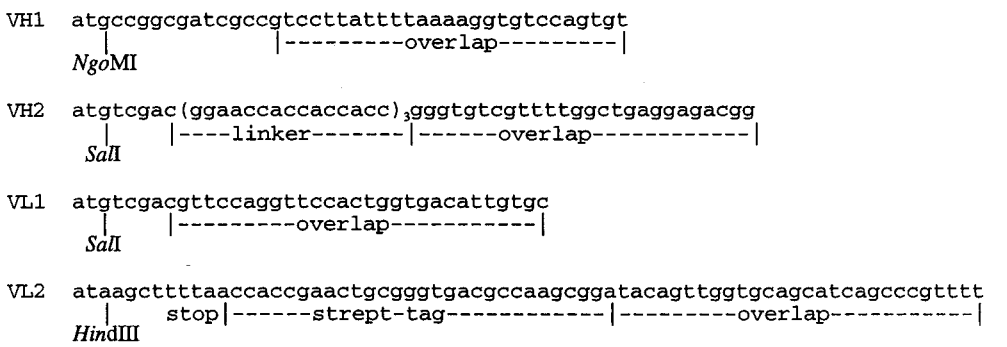


FIGURE 2: Designed PCR primers for linkage of variable chain encoding DNA fragments. These primers were used in PCR reactions to amplify the $V_H$ and $V_L$ genes and prepare them for linkage. Each primer adds a restriction endonuclease cleavage site and either a polylinker or strep-tag encoding sequence. Each primer has an overlap region (23−28 bp) to ensure that false priming does not occur. The *Sal* I sites found at the 3′ end of the $V_H$ fragment and 5′ end of the $V_L$ fragment serve as the point of linkage. The *Hin*dIII and *Ngo*MI sites were added to facilitate cloning. The strep-tag was included for use in affinity purification.

Table 1: Scatchard and Nonlinear Regression Analyses of ScFv DNA Binding Data[a]

| | Scatchard analyses | | | nonlinear regression | | |
|---|---|---|---|---|---|---|
| substrate[b] | $K_d$ (M) | $K_a$ ($M^{-1}$) | $B_{max}$ ($M^{-1}$) | $K_d$ (M) | $K_a$ ($M^{-1}$) | $B_{max}$ ($M^{-1}$) |
| TET4 | $6.65 \times 10^{-7}$ | $1.43 \times 10^{6}$ | 0.009755 | $9.73 \times 10^{-7}$ | $1.03 \times 10^{6}$ | 0.002108 |
| TG4 | $9.37 \times 10^{-7}$ | $1.07 \times 10^{6}$ | 0.1427 | $1.92 \times 10^{-6}$ | $5.20 \times 10^{5}$ | 0.1689 |
| G4 | $1.10 \times 10^{-6}$ | $9.09 \times 10^{5}$ | 0.007049 | $2.56 \times 10^{-6}$ | $3.90 \times 10^{5}$ | 0.01099 |
| CG5 | $8.60 \times 10^{-6}$ | $1.16 \times 10^{5}$ | 0.03382 | $3.35 \times 10^{-6}$ | $2.99 \times 10^{5}$ | 0.01851 |
| HS | $2.45 \times 10^{-5}$ | $4.08 \times 10^{4}$ | 0.2693 | $4.34 \times 10^{-5}$ | $2.30 \times 10^{4}$ | 0.4348 |
| HSM | $2.42 \times 10^{-5}$ | $4.12 \times 10^{4}$ | 0.2589 | $7.86 \times 10^{-5}$ | $1.27 \times 10^{4}$ | 0.3684 |

[a] Analyzed with substrates as described (*21*). [b] Quadruplexes, TET4, TG4, and G4; duplexes, CG5 and HS; single strands, HSM.

from the monoclonal hybridoma that carried the genes which encoded the parent antibody, to produce a cDNA library. The library was used in the PCR reactions with the universal primer set to generate immunoglobulin sequences, and the resulting DNAs were analyzed by agarose gel electrophoresis (Figure 1A).

Interestingly, this experiment was repeated with the same monoclonal hybridomas after 3 months of growth in tissue culture and the results had changed (Figure 1B). Comparison of the two sets of results indicated that a class switch of the antibody from IgM to IgG had occurred. This conclusion was confirmed by PCR, ELISA, and Western blot analyses (data not shown).

*Design of the scFv.* From the $V_H$ and $V_L$ sequence information, PCR primers were designed to link these regions together. At the same time, adding unique restriction enzyme sites, a polylinker and a strep-tag-encoding sequence were incorporated into the scFv-encoding fragment (Figure 2). The 5′ primer of the heavy chain contained an *Ngo*MI restriction site to enable cloning into the pGEM3Z-*pelB* plasmid. These steps are shown in Figures 3 and 4. The 3′ primer of the heavy chain contained a glycine−serine-encoding linker sequence (45 nucleotides) to help ensure the proper conformation of the scFv (*14*) and a *Sal*I restriction site to allow linkage to the light-chain DNA. The 5′ primer of the light chain also had a *Sal*I restriction site for ligation to the heavy-chain DNA fragment during the three-component ligation reaction. The 3′ primer of the heavy chain contained the strep-tag-encoding sequence, which was designed for easier detection and purification (*33*), an ochre translational termination codon (TAA), and a *Hin*dIII site for cloning into the pET22b(+) expression vector, which was transformed into NovaBlue(DE) *E. coli* cells.

*Expression and Purification of scFv.* Growth and induction were accomplished at reduced temperature to minimize inclusion body formation, which occurs readily above 30 °C (*34*). After translation, the scFv was directed to the periplasmic space of the cell by the *pelB* signal peptide (*35*). This signal peptide should be cleaved off in the periplasmic space, so as not to interfere with folding of the scFv. The soluble scFv was released from the cell periplasm by repeated cycles of freezing and thawing (*31*). This method will release 50−90% of the overexpressed protein without lysing the cell.

To verify the identity and subcellular location of the protein, *E. coli* culture supernatants and subfractions were analyzed via ELISA for both the strep-tag and mouse Fab protein subdomains (data not shown). The culture supernatant showed some reactivity to both probes, but the impure protein supernatant showed an approximately 4-fold higher reactivity in both reactions. This indicates that some scFv leaks out of the cells, but most of it remains in the periplasm until the freeze/thaw steps. Fractions obtained by affinity purification of the impure supernatant over a streptavidin column (to which the strep-tag should bind) also showed reactivity to the two probes, adding to the evidence that intact scFv is produced. Since the C-terminal strep-tag was detected, we conclude that the entire scFv was being expressed in the correct reading frame.

Purification of the scFv (ca. 28 kDa) was accomplished on a streptavidin column that bound the C-terminal biotin-mimicking strep-tag peptide. The scFv was eluted from the column with the biotin analogue diaminobiotin. Purification of the scFv was monitored by both SDS−polyacrylamide gels and Western immunoblots, which were probed with

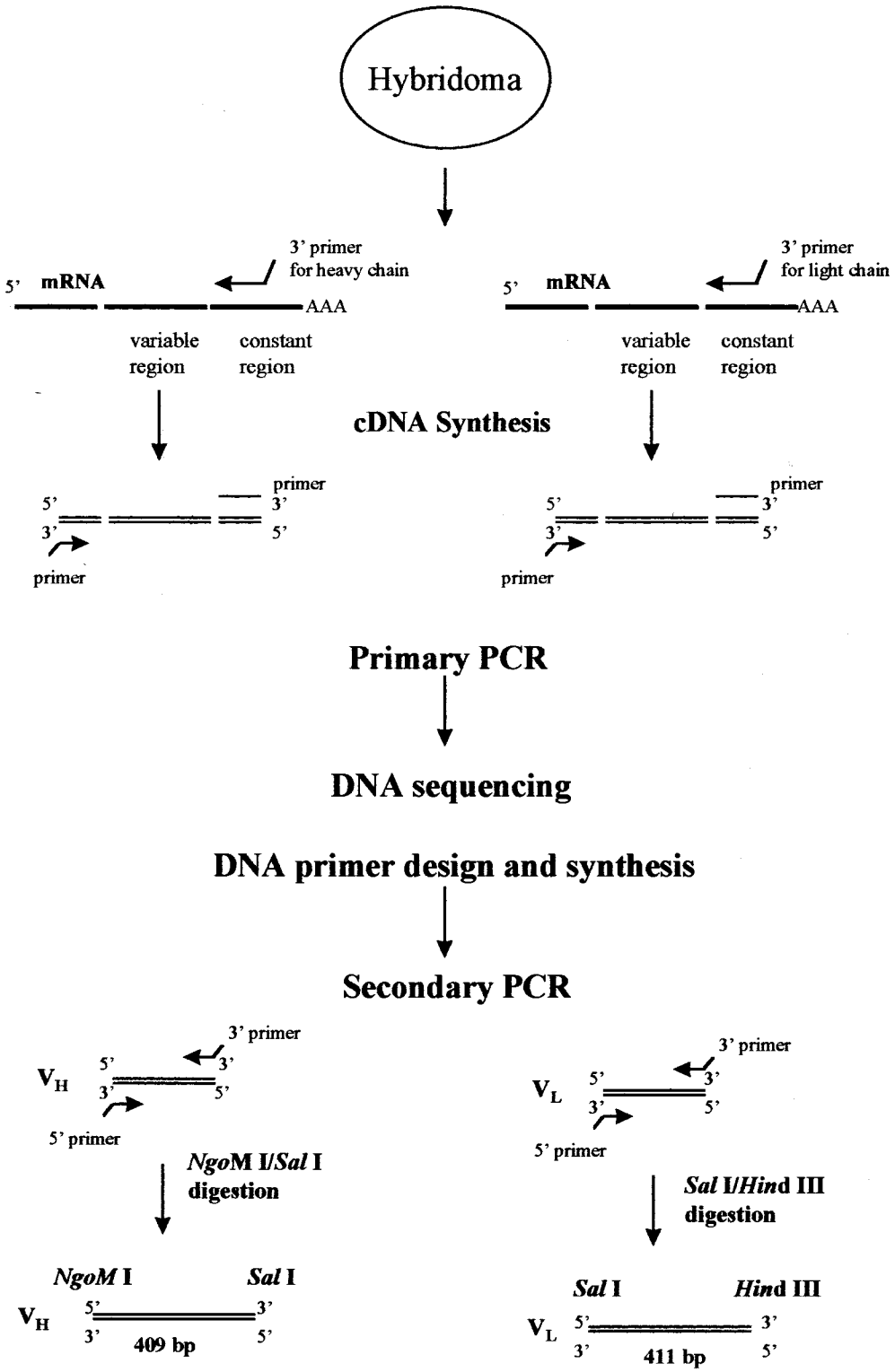


FIGURE 3: Summary of steps leading from hybridoma cells to engineered $V_H$ and $V_L$ PCR products. Messenger RNA was isolated from monoclonal hybridoma cells that produced anti-quadruplex antibodies. The cDNA library was made by using the appropriate 3′ primer from the Mouse Ig-Prime kit. 5′ primers for specific classes of mouse antibodies were used in conjunction with the library to amplify the heavy- and light-chain variable genes that encode the antibody. Based on results from DNA sequence analyses, primers were designed to link the heavy and light variable genes together. During the PCR reactions, these primers provided templates for the addition of specific restriction endonuclease cleavage sites to the heavy- and light-chain gene fragments. These sites were cleaved with the appropriate restriction endonuclease in preparation for the linkage reactions.

streptavidin−alkaline phosphatase (AP) conjugate (data not shown). Western blot analyses were also used to verify the presence of the scFv by using a streptavidin immunoconjugate. Bands detected with the streptavidin probe corresponded to approximately 28 and 56 kDa. This led us to believe that the scFv was produced, adequately purified, and moderately prone to dimerization. Together, the SDS−polyacrylamide gel and the Western blot data agreed with the ELISA (DNA binding specificity) results, strongly suggesting that the scFv was intact and folded correctly.

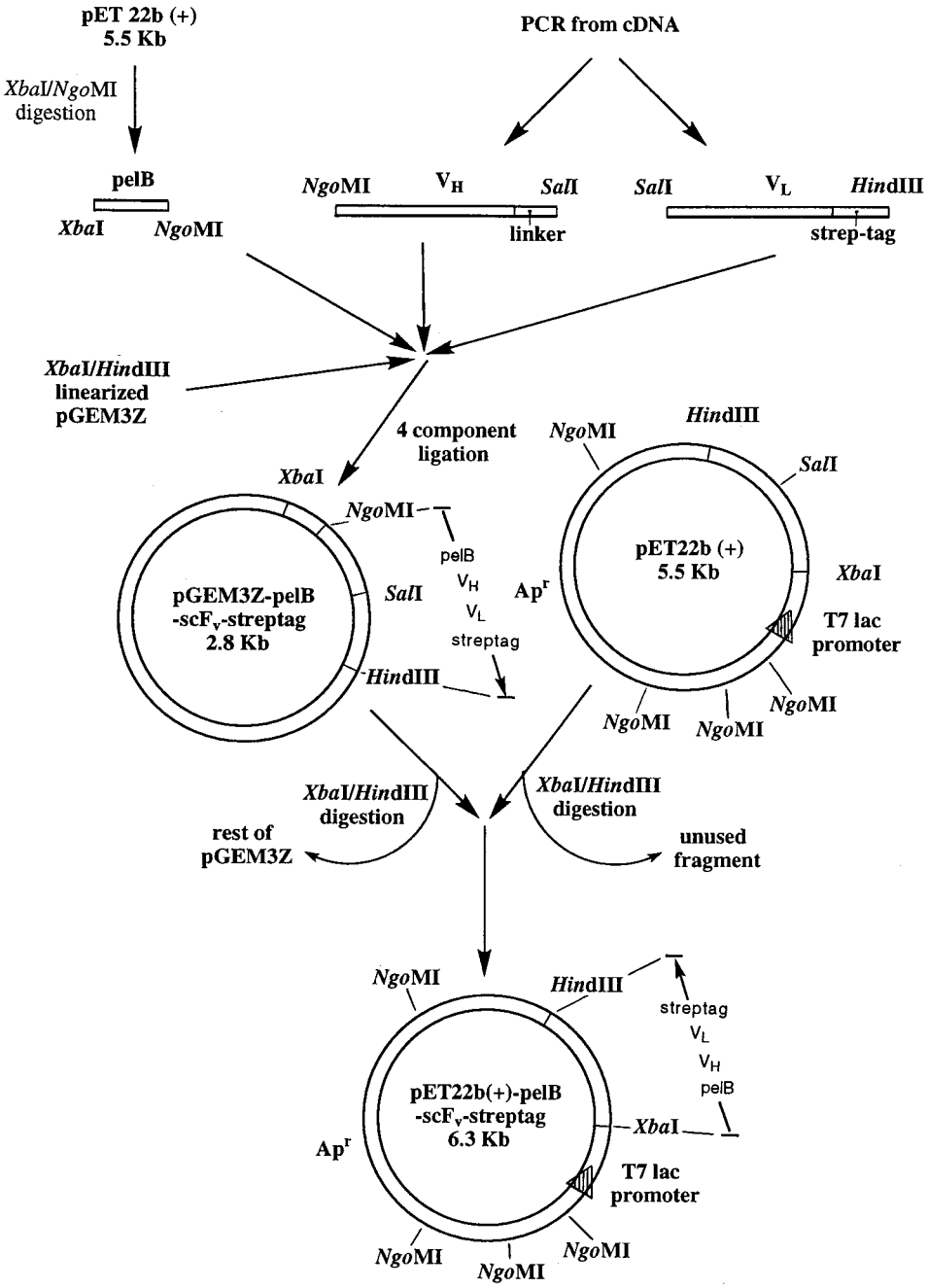


FIGURE 4: Summary of reactions leading to linkage and cloning of $V_H$ and $V_L$ gene fragments. A 3-way ligation of the $V_H$ and $V_L$ gene fragments into the pGEM3Z-*pelB* vector produced the scFv gene. A glycine-serine encoding polylinker linked the two genes together, while a strep-tag encoding sequence at the end of the $V_L$ gene encodes a fragment that serves to facilitate purification. The scFv gene was cleaved out of the pGEM3Z-*pelB* vector and cloned into an expression vector, pET22b(+).

*Nucleic Acid Binding Preference of the scFv.* To confirm that the scFv binds nucleic acids and determine if the preference of the parent antibody for quadruplex DNA was retained, a radioimmunofilter binding assay (RIFB) was used. The following structures were tested: three quadruplexes, TET4, TG4, and G4; two ds DNAs, CG5 and HS; and one ss DNA, HSM. These structures were described and characterized by circular dichroism and NMR (*20*−*22*). The parent antibody bound quadruplexes with 10−40-fold higher affinity than other DNA structures (*20*−*22*). This assay was used to determine whether the scFv retained this property.

Both Scatchard and nonlinear regression analyses were performed for each nucleic acid tested. Table 1 lists the binding constants $K_d$, $K_a$, and maximal amounts of bound substrates ($B_{max}$) that were derived from these analyses. From these results, we concluded that the scFv binds quadruplexes with higher affinities than ds or ss DNAs; however, the binding affinities are reduced by about 10-fold relative to those observed with intact *me*$^{v}$IIB4 IgG. The specific preference for quadruplex relative to ds DNA was reduced by about 4-fold, resulting in a ca. 10-fold preference for quadruplex instead of the 40-fold difference observed for the intact IgG.

## DISCUSSION

*Anti-Quadruplex scFv Production.* Hundreds of anti-DNA antibodies from immunized and unimmunized sources have been reported in the literature, but anti-DNA antibodies from

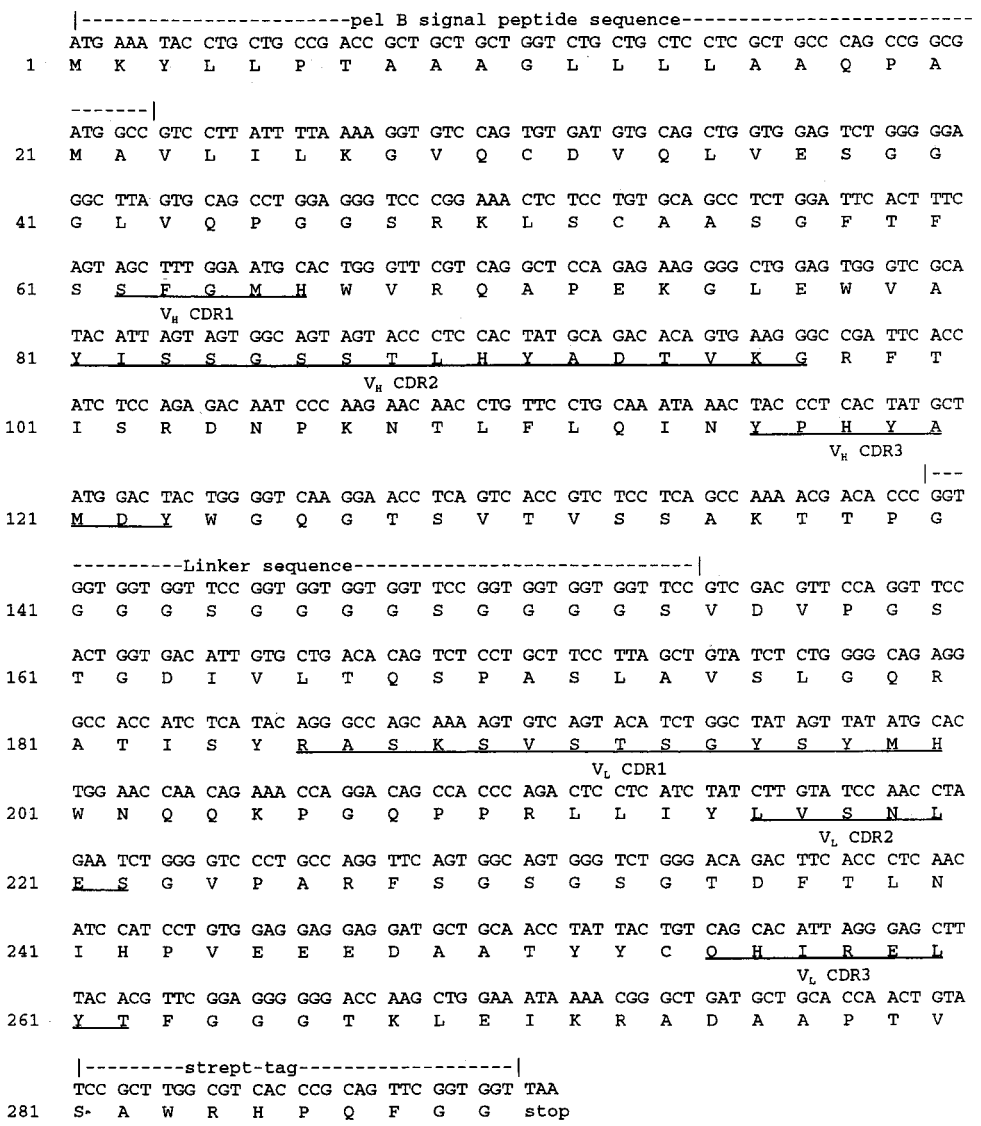


FIGURE 5: DNA and protein sequences of the scFv gene and gene product. After DNA sequence analysis, the sequence of the scFv, including the signal peptide and strep-tag sequences, were translated into the appropriate amino acids using DNA Strider 1.0. The underlined amino acid residues represent the CDR regions of both the heavy and light chains.

VME mice have only recently been reported (*19*). Twelve anti-DNA antibodies have been characterized and sequenced and were all found to be IgMs with specificities for both ss and ds DNA, but higher affinities for the latter. A broad range of defined DNA structures were not used in those studies to characterize the specificities of the antibodies.

The evidence suggests that the parent anti-DNA quadruplex antibody derived from *me*$^{v}$IIB4 hybridomas was originally an IgM but underwent isotype switching to an IgG in cell culture. The antibody also showed specificity for DNA, with a higher affinity for quadruplexes than for a range of other DNA structures (*20*−*22*). The heavy- and light-chain genes of this parent antibody were used to construct the scFv that was developed in this study. The main purposes for developing the scFv were to reduce the size of the antibody ($IgG_3$, 165 kDa, to scFv, 28 kDa), produce it more cost-efficiently (i.e., from *E. coli* instead of tissue culture cells), and improve its purity through use of the strep-tag/streptavidin affinity purification system.

*Autoimmunity and Gene Usage.* By comparing the sequence of the quadruplex-specific scFv (Figure 5) with other autoantibodies, one gains some tentative insights into their production. Recently, Reichlin et al. (*36*) demonstrated that lupus anti-DNA autoantibodies can bind to proteins of ribonucleoprotein (RNP) antigens, raising the possibility that non-DNA structures could induce antibodies that bind DNA and that DNA may not be the "true" antigen that is bound with the highest affinity. This would make the antibody bi- or multispecific. Many monoclonal anti-DNA autoantibodies that have been studied are clearly bispecific or multispecific in that they also react with a number of seemingly unrelated antigens such as phospholipids (*37*), rheumatoid factor (*38*), and cardiolipin (*39*). These cross-reactions are likely to be due to commonly shared epitopes such as the phosphodiester linkages, which are, for example, separated by three carbon atoms on both DNA and cardiolipin. Homology studies of the scFv sequence demonstrated significant homology with anti-cancer, anti-collagenase IV, anti-estrogen receptor, and other anti-DNA antibodies. This might indicate that non-DNA structures were in fact responsible for induction of the quadruplex DNA-specific antibody and that the antibody is bi-specific.

Specific amino acids in the CDR regions of anti-DNA antibodies are thought to determine DNA affinity. Anti-DNA antibodies have been found to be very diverse with respect to their conformational specificity for DNA (ds vs ss). Arginine, lysine, and histidine are likely to participate in charge−charge interaction with the phosphate residues of the nucleic acid backbone, whereas arginine and asparagine can also engage in stable hydrogen bonds with the heterocyclic bases (*40*, *41*). Tyrosine, which is prominent in certain anti-DNA autoantibodies, may facilitate DNA binding by interacting with the thymidine bases (*42*).

The CDR3 region of the heavy chain is thought to be the most important region for DNA/antibody interaction. This region is the most variable, resulting from higher recombination frequencies during V(D)J recombination. It has been shown that mutations at numerous arginine sites can contribute to DNA binding by antibodies. The importance of arginines in the CDR3 region for DNA binding is implied by their significantly higher frequency in anti-DNA relative to other antibodies (*43*). However, arginines are underrepresented in other binding sites (*44*). Interestingly, the scFv does not have any arginines in the CDR3 of the heavy chain, but it does have *three tyrosines and an asparagine*, two other commonly implemented DNA-binding amino acids. In addition, another study of anti-DNA antibodies from VME mice reported no significant increases in the number of arginines in the CDR3 regions (*19*). So, perhaps arginines in CDR3 regions are not as essential for anti-DNA−DNA interactions as previously thought.

The light chains of anti-DNA autoantibodies have some sequence requirements and structural restrictions, but they are not unique to autoantibodies or to anti-nucleic acid antibodies, in particular (*45*). The light chain of the scFv was found to be 100% homologous with a previously described aberrant $\kappa$ chain. Other light chains of antibodies, such as anti-cancer, anti-DNA, and anti-estrogen receptor, have also shown homology to GenBank sequences. Therefore, the anti-quadruplex light chain does not demonstrate uniqueness to anti-DNA antibodies. However, the light chains do modulate the affinity and specificity of the antibody−DNA interaction, even if the heavy chain can bind DNA alone (*46*).

Anti-DNA antibodies usually have long light-chain CDR1 regions: the anti-quadruplex antibody has one arginine, two tyrosines, and five serines in the 15 amino acid $V_L$ CDR1 region. Arginine and asparagine are common in the long $V_L$ CDR3 in anti-DNA antibodies, but *tyrosine and serine* can also bind DNA. Position 96 (tyrosine 261 in Figure 5) is an important feature of the light chain. It is a point of antigen (DNA) contact, since it projects out of the CDR3 loop (*47*). All described anti-DNA autoantibodies that have an arginine at position 96 bind dsDNA, although not all anti-dsDNA antibodies have an arginine at position 96 (*48*). The anti-quadruplex antibody has a tyrosine instead of an arginine at position 96. This amino acid might interact particularly favorably with coplanar guanines or between stacked G-quartets. The CDR2 has little, if any, impact on DNA binding, because of the large protruding CDR1 (*44*). The CDR2 of the anti-quadruplex antibody contains few, if any, potential DNA-binding amino acids; therefore alluding to minimal participation in DNA interaction. One possible important role of the light chain is to prevent developing B cells, which express heavy chains with potential for high-affinity autoreactivity, from being eliminated or inactivated (*49*), or allow the antibody to escape tolerance mechanisms by combinations with light chains that prevent autoreactivity (*50*). The anti-quadruplex antibody has an aberrant light chain that may allow it to escape these tolerance mechanisms in this manner.

Table 2: Comparison of ScFv Antibody Amino Acid Contents with Those of Telomere-Binding Proteins and Fragments

| | | | Myb-like three α-helix domain fragments[c] | | | | five-protein sample (range)[d] | |
|---|---|---|---|---|---|---|---|---|
| amino acid | scFv[a] | rTF[b] | RAP1 SC (1) | Myb2 | engrailed | TBF1 | high | low |
| (A) Highly Hydrophobic | | | | | | | | |
| Ile (I) | 4.1 | 5.2 | 5.3 | 7.7 | 5.6 | 3.3 | 7.7 | 0 |
| Val (V) | **7.8**[e] | **6.7** | **7.0** | 5.8 | 0 | 4.9 | 9.8 | 2.9 |
| Leu (L) | 7.8 | 6.3 | 5.3 | 7.7 | 9.3 | 11.5 | 12.8 | 5.8 |
| Phe (F) | 3.3 | **5.8** | 5.3 | 0 | 5.6 | 0 | 5.9 | 2.1 |
| Met (M) | 1.2 | 1.1 | 0 | 0 | 0 | 4.9 | 2.9 | 0 |
| (B) Less Hydrophobic | | | | | | | | |
| Ala (A) | 7.0 | 5.2 | 1.8 | 1.9 | 7.4 | 4.9 | 14.9 | 5.8 |
| Gly (G) | **10.3** | 3.8 | 1.8 | 7.7 | 1.9 | 8.2 | 12.5 | 5.0 |
| Cys (C) | 1.2 | 0.7 | 0 | 1.9 | 0 | 0 | 6.2 | 0.7 |
| Trp (W) | 1.6 | 1.3 | 0 | 5.8 | 1.9 | 4.9 | 4.7 | 0 |
| Tyr (Y) | **4.9** | **4.5** | **5.3** | 1.9 | 1.9 | 3.3 | 7.8 | 2.1 |
| Pro (P) | **5.3** | 2.7 | 3.5 | **5.8** | 1.9 | 3.3 | 5.0 | 1.6 |
| Thr (T) | **7.8** | **5.4** | **10.5** | 1.9 | 3.7 | 4.9 | 8.2 | 2.0 |
| Ser (S) | **11.9** | **7.8** | **7.0** | 1.9 | 7.4 | 4.9 | 7.8 | 1.9 |
| (C) Highly Hydrophilic | | | | | | | | |
| Asn (N) | 2.5 | **7.2** | 0 | 5.8 | **9.3** | 4.9 | 10.1 | 2.1 |
| Gln (Q) | **5.0** | 3.4 | **7.0** | 3.8 | **7.4** | **4.9** | 4.1 | 0.7 |
| (D) Acidic | | | | | | | | |
| Asp (D) | 3.7 | **7.8** | 7.0 | 1.9 | 0 | 1.6 | 11.3 | 0 |
| Glu (E) | 3.7 | **4.5** | 7.0 | **9.6** | **11.1** | **11.5** | 9.3 | 1.6 |
| (E) Basic | | | | | | | | |
| His (H) | 2.5 | 3.3 | 8.8 | 5.8 | 0 | 0 | 7.1 | 0.8 |
| Lys (K) | 4.1 | **13.9** | 5.3 | **11.5** | 7.4 | **16.4** | 17.3 | 2.0 |
| Arg (R) | 4.1 | 2.7 | **12.3** | **9.6** | **16.7** | 4.9 | 8.5 | 1.0 |

[a] Percent relative to content of $V_L$ and $V_H$ fragments only, excluding the *pelB*, strep-tag, and peptide linker sequences. [b] Percent relative to content of entire *Euplotes* rTF sequence (calculated from Table 3 of ref *1*; also see ref *52*). [c] Percent relative to contents of the respective three α-helix domain fragments listed (calculated from Table 2 of ref *3*). [d] Percent relative to the high and low extremes of the ranges of contents of lysozyme, cytochrome *c*, ferredoxin, insulin, and the α chain of hemoglobin (calculated from Table 4.3, ref *51*). [e] Boldface type highlights figures that are notably higher than average for the five-protein sample.

*Amino Acid Sequence Preferences.* The parentally derived $V_H + V_L$ sequence (Figure 5) was compared with a small random sampling of protein sequences and some selected telomere-binding proteins and fragments (Table 2; *51*). The scFv was found to be highly enriched in serine (11.9%) and to contain slightly high amounts of threonine (7.8%), arginine (4.1%), lysine (4.1%), glutamine (5%), and (less so) histidine (2.5%), a high amount of tyrosine (4.9), and significant amounts of phenylalanine (3.3%), tryptophan (1.6%), and asparagine (2.5%). High amounts of serines, tyrosines, and basic residues enrich the antigen combining sites, resembling the sequence preferences of large tracts in the G-rich DNA specific "replication telomere proteins" (rTPs) from *Euplotes* and *Oxytricha* (*1*, *52*) and the yeast quadruplex DNA-inducing protein RAP1 (*3*). These results might provide a basis for factors that link the predispositions for binding telomeric DNAs and in the case of RAP1 the induction of quadruplex DNAs. Of particular interest are the presence

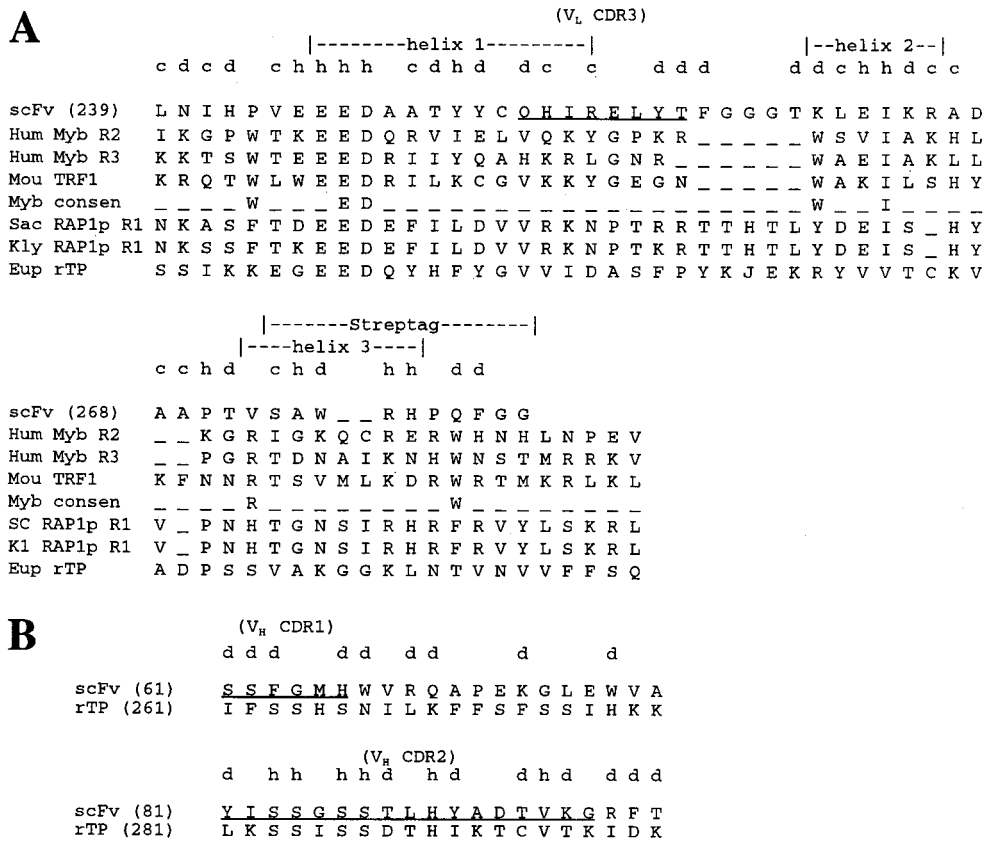


FIGURE 6: Protein sequence of the scFv aligned with DNA binding domains found in telomere binding proteins. (A) Two three-helix bundles in the DNA binding domain of human Myb (R1, R2), single Myb repeats in human and mouse telomere repeat binding factors (TRF1), the first Myb repeat (R1) of *Saccharomyces cerevisiae* (Sac) and *Kluyveromyces lactis* (Kly) RAP1p, and the Myb consensus (adapted from Smith and De Lange, *2*). An analogous sequence from the "replication telomere protein" (rTP) of *Euplotes crassus* (*1*, *52*) is also shown. Lower-case letters refer to exact homology (h) with one or more of the other sequences, conservative substitution (c), and possible DNA-binding residues (d) of the scFv. (B) Alignment of serine enriched fragments of the scFv and rTP.

of unusually large amounts of the hydroxylated residues S, T, and Y and the contrast in basic amino acid content between that of the scFv (lower in K/R) and those of rTP and RAP1. The lower basic amino acid content might lead to more specific binding to the more hydrophobic quadruplex, while higher basic amino acid content might result in a higher overall affinity but a decrease in specificity for quadruplex DNAs.

Sequence analysis (Figure 6) demonstrates homologies between the antibody CDRs and sequences of MYB/RAP1 family telomere binding proteins. Figure 6A shows an alignment between the region in the vicinity of the light chain CDR3 and the key helix−turn−helix DNA-binding motifs of the MYB family proteins from humans and mice, RAP1 proteins from two different yeast species, and rTP from *Euplotes* (*1*, *2*). Alignments were based on the conserved MYB consensus residues ED (in helix 2) and I (in helix 3). Note the reasonably high degree of exact homology (h) and conservative divergence (c). In addition, possible DNA-binding residues (d) in the scFv are labeled to facilitate comparison with possible functional conservation relative to the other sequences. Figure 6B shows an alignment between the region in the vicinity of $V_H$ CDRs 1 and 2 of the scFv and a homologous sequence in *Euplotes* rTP CDR2 is very homologous, while CDR1 is slightly scrambled but very similar to the aligned region in rTP. Large amounts of aromatic and amide-containing amino acids are found in both the scFv and Myb proteins, suggesting common denominators for recognition of G-rich DNA sequences. The quadruplex-binding capability of this scFv is correlated with lower basic amino acid content and more serines, threonines, tyrosines, and valines.

Table 3: Comparison of $K_a$s for the Parent and ScFv Antibodies Derived from Scatchard and Nonlinear Regression Analyses[a]

| | Scatchard analysis | | nonlinear regression $K_a$ ($M^{-1}$) | |
|---|---|---|---|---|
| substrates[b] | parent $IgG_3$ | scFv | parent IgG | scFv |
| TET4 | $2.28 \times 10^7$ | $1.43 \times 10^6$ | $1.66 \times 10^7$ | $1.03 \times 10^6$ |
| TG4 | $5.95 \times 10^6$ | $1.07 \times 10^6$ | $5.38 \times 10^6$ | $5.20 \times 10^5$ |
| G4 | $5.41 \times 10^6$ | $9.09 \times 10^5$ | $3.30 \times 10^6$ | $3.90 \times 10^5$ |
| CG5 | $7.66 \times 10^5$ | $1.16 \times 10^5$ | $8.26 \times 10^5$ | $2.99 \times 10^5$ |

[a] Analyzed as described (*21*). [b] Quadruplexes, TET4, TG4, and G4; duplex, CG5.

*Potential Applications.* The anti-quadruplex IgG *me$^v$*IIB4 and scFv were intended in part for use as in vivo immunoprobes. Therefore, the binding affinities of the scFv for a variety of nucleic acid structures were determined by RIFB assays in the same manner as for the parent antibody (*20*, *21*). A comparison of the $K_a$ values decreased from that of the intact IgG to the scFv antibody fragment by a factor of about 10; however, the same hierarchy of binding affinities was retained (Table 3).

This decrease in binding affinity might be expected, due to the manipulation of the original antibody structure. Previous studies have shown that the measured affinity was

3−6-fold lower for scFvs relative to the Fab fragment (*13*, *14*, *17*). It has been suggested that these small decreases in scFv affinity may be due to the introduction of the synthetic linker (*14*). It is thought that connecting the heavy- and light-chain genes with a synthetic linker affects protein folding by allowing the protein to "breathe" more than an intact IgG antibody. Therefore, the linker might be expected to decrease the affinity of the protein for its antigen.

Only antibodies that express high dissociation constants ($10^{-8}$ to $10^{-10}$ M) have traditionally been considered biologically useful (e.g., as in vivo probes). Virtually without exception, such antibodies were the result of repeated immunizations (*53*). In contrast, antibodies produced in the initial response to a variety of antigens have affinities in the micromolar range ($10^{-5}$ to $10^{-7}$ M) (*54*, *55*). This affinity interaction defined a demonstrable threshold ($\sim 10^{-5}$ M) for significant antibody/antigen interactions. Therefore, antibodies with affinities $\leq 10^{-5}$ M are considered immunologically relevant (*56*). The scFv had a binding affinity of $< 10^{-6}$ M and also showed a difference in binding affinity of 10-fold between quadruplexes and dsDNA. This binding affinity is significant and should allow the scFv to be used as a selective immunoprobe.

This study also demonstrates that quadruplex DNA-specific IgGs are found in autoimmune mice. The homology between the scFv CDR sequences and Myb/RAP1 family telomeric DNA binding proteins suggests an intriguing link between aging and autoimmunity. Four points should be mentioned in this regard. First, the previously demonstrated homology of the Myb domains in telomere-binding proteins (even in helix 3) is relatively loose, yet the antigen-binding domain of the antibody almost completely fulfills the minimal requirements of the consensus, certainly as well as most of the other family members. Second, the antibody has a strong affinity for DNA, specifically quadruplex DNA, which is most often cited as having possible importance in the context of telomeric G-string overhangs (e.g., ref *57*). Third, the telomeric DNA-binding protein RAP1 is homologous to Myb and induces quadruplex formation in vitro (*58*). Is it a coincidence that the scFv is homologous to Myb and binds quadruplex DNAs with higher affinity than other forms of DNA? Finally, these sequences are found in an autoantibody. While it seems unlikely that the antibody was intended to fulfill the biological role of a telomeric DNA-binding protein, the possibility remains that the autoimmune role of the antibody might be the result of functionally significant telomeric interactions. In turn, this correlation might provide a link between autoimmunity and premature aging (*59*).

## ACKNOWLEDGMENT

We thank Dr. Kai Koo and Professor Harold Swaisgood (NCSU Food Science Department) for supplying plasmids and helpful advice in the planning stages and Dr. David Presutti, Jeff Kuhn, Donna Newman, and Dr. Nick Watkins (NCSU Biochemistry Department) for technical assistance.

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BI981434Y