Sequences of 12 monoclonal anti-dinitrophenyl spin-label antibodies for NMR studies

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ABSTRACT   Eleven monoclonal antibodies specific for a spin-labeled dinitrophenyl hapten (DNP-SL) have been produced for use in NMR studies. They have been named AN01 and AN03–AN12. The stability constants for the association of these antibodies with DNP-SL and related haptens were measured by fluorescence quenching and ranged from 5.0×10^4 M^{-1} to $>1.0 \times 10^8$ M^{-1}. cDNA clones coding for the heavy and light chains of each antibody and of an additional anti-DNP-SL monoclonal antibody, AN02, have been isolated. The nucleic acid sequence of the 5' end of each clone has been determined, and the amino acid sequence of the variable regions of each antibody has been deduced from the cDNA sequence. The sequences are relatively heterogeneous, but both the heavy and the light chains of AN01 and AN03 are derived from the same variable-region gene families as those of the AN02 antibody. AN07 has a heavy chain that is related to that of AN02, and AN09 has a related light chain. AN05 and AN06 are unrelated to AN02 but share virtually identical heavy and light chains. Preliminary NMR difference spectra comparing related antibodies show that sequence-specific assignment of resonances is possible. Such spectra also provide a measure of structural relatedness.

Many different magnetic resonance techniques can be used to gain structural information about antibodies in solution (1). The combining sites of antibodies specific for spin-labeled molecules are particularly accessible to study by nuclear magnetic resonance (NMR). The spin-label broadens the NMR signals of nearby (<17 Å) protons in a strongly distance-dependent manner. A simple NMR difference spectrum, antibody alone minus antibody with bound spin-label, is dominated by resonances from protons that are near the electron spin. Anglister *et al.* (2) used this effect to gain information about the amino acid composition of the binding site of AN02, a monoclonal antibody raised against a spin-labeled dinitrophenyl hapten (DNP-SL). Growth of the AN02-producing cell line in medium containing selected deuterated amino acids results in virtually complete incorporation of these deuterated amino acids into the antibody. This selective deuteration permits the assignment of resonances to specific amino acid types. Considerable simplification of spectra is afforded through the use of partially deuterated amino acids to remove splitting of the resonances. Measurement of the broadening effect of the spin-label on specific resonance signals at various binding-site occupancies allows the calculation of the distance between these protons and the electron spin. A complication in this type of distance measurement arises if the spin-label adopts more than one conformation relative to the protein (3). An electron paramagnetic resonance spectrum of AN02 with the spin-label hapten shows the spin-label to be tumbling at the same rate as would be expected for AN02 (2). The distances from the spin-label of seven AN02 tyrosines were measured by

varying the amount of spin-label in the binding site (4). Such distance measurements were also made for resonances arising from alanine, isoleucine, leucine, threonine, and valine (G.S.R., D.J.L., and H.M.M., unpublished data). Recombination of heavy and light chains with different deuterations has allowed identification of the chain of origin of these tyrosine signals (5). Nuclear magnetization transfer measurements were used to identify resonance signals from two tryptophans in AN02 that must be <5 Å from the hapten (6).

The NMR lineshapes provide information about the binding-site dynamics. The linewidths of resonance signals originating from residues on the surface of AN02 indicate that these residues are moving much faster than the entire Fab molecule. The linewidths of peaks corresponding to the tyrosine residues for which distance measurements were made varied from 5 to 15 Hz. The sharpness of signals from binding-site protons greatly simplifies analysis of NMR spectra from a protein as large as an Fab fragment. The change in linewidth of a resonance in the AN02 NMR spectrum upon binding of a diamagnetic hapten was used to measure the off-rate for this hapten (4).

Given the available distance information about amino acids near the spin-label in AN02, sequence-specific assignment of resonance signals will enable construction of a working model of the combining site. We believe that such sequence-specific assignments can be obtained in part by using monoclonal antibodies differing from AN02 by a small number of amino acids. We have thus constructed a panel of 12 monoclonal antibodies that bind the DNP-SL hapten. We have also isolated and sequenced cDNA clones for both chains of each of these antibodies.* These clones enable creation of related antibodies through site-directed mutagenesis, genetic mixing and matching of heavy and light chains, and construction of hybrid variable (V) regions. Preliminary data given below demonstrate NMR difference spectra between closely related antibodies to be a viable technique to obtain sequence-specific assignments.

The Fab fragment of the AN02 antibody forms cocrystals with the DNP-SL hapten that diffract to high resolution (D.J.L., H.M.M., and R. O. Fox, unpublished data). The solution of the AN02 crystal structure will provide an opportunity to compare NMR and x-ray structural information for antibody–hapten complexes.

MATERIALS AND METHODS

Immunization of Mice. Six- to 8-week old female BALB/c mice were given intraperitoneal injections of the DNP-SL hapten coupled to 250 μg of bovine serum albumin (DNP-SL-BSA) in a 1:1 emulsion with complete Freund's adjuvant. The DNP-SL-BSA complex was prepared by the method

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Abbreviations: DNP-SL, spin-labeled dinitrophenyl; C, constant; D, diversity; J, joining; V, variable.
*The sequences reported here are being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession nos. J03832 and J03833).

described for DNP-SL-keyhole limpet hemocyanin (7). After 2 weeks the mice were boosted with subcutaneous injections of 250 μg of DNP-SL-BSA in complete Freund's adjuvant. Two weeks later, sera from these mice were screened by ELISA for anti-DNP-SL activity. Mice with the highest anti-DNP-SL titers were then injected with 70 μg of soluble DNP-SL-BSA in aqueous solution via the tail vein. The spleens were removed for fusion 3 days later.

ELISA. DNP-SL-lipid (7) at 2.5 mg/ml in $CHCl_3$ was diluted 1:100 with ethanol and coated on the wells of a 96-well polystyrene plate by evaporation. The wells were washed five times with 0.3% gelatin in phosphate-buffered saline and flicked dry. Appropriately diluted serum or supernatant was added to the wells and incubated for 1 hr. The plates were washed as before and specific antibody binding was detected with β-galactosidase-conjugated goat anti-mouse immunoglobulin (Bethesda Research Laboratories) followed by an appropriate enzyme substrate.

Preparation of Hybridomas. Exponentially growing Sp2/0 cells (2 × 10^7) were mixed at a 1:5 ratio with immune spleen cells. The cells were washed three times in serum-free RPMI-1640 medium and gently resuspended in 1 ml of RPMI-1640 containing 40% (wt/vol) polyethylene glycol (PEG; M_r 3500–3700; Baker) and 10% (vol/vol) dimethyl sulfoxide; this solution was added dropwise at 37°C over 1 min. The cells were incubated at 37°C for 2 min and then diluted slowly with 5% PEG in RPMI-1640. The cells were pelleted by centrifugation and resuspended in 5 ml of serum-free RPMI-1640. The suspension was then brought to 100 ml with RPMI-1640 containing 20% (vol/vol) fetal bovine serum (HyClone, Logan, UT), 2 μg of azaserine per ml, and 0.2 mM hypoxanthine, and the cells were seeded in 96-well plates. The cells were fed every 3–4 days, and supernatants were screened by ELISA for anti-DNP-SL activity after the appearance of colonies. Colonies testing positively were immediately cloned and later recloned until all new subclones tested positively. All cell lines were subsequently adapted for growth in medium containing 1% fetal bovine serum to minimize the presence of protonated amino acids during deuteration experiments for NMR.

cDNA Cloning. RNA was isolated from each cell line by the method of Chirgwin *et al.* (8), and poly(A)^+ RNA was selected with oligo(dT)-cellulose (Collaborative Research, Waltham, MA). A complementary strand of DNA was synthesized by reverse transcriptase (Life Sciences, St. Petersburg, FL) using an oligo(dT) primer (Pharmacia). The RNA was removed by heating to 90°C and digestion with RNase A (Sigma). The single-stranded cDNA was poly(G)-tailed by terminal deoxynucleotidyltransferase (Pharmacia) using dGTP (Pharmacia). Second-strand synthesis was primed with an oligo(dC) primer that contained a *Not* I restriction site on its 5' end (D. W. Denney and T. St. John, personal communication). The DNA was then methylated with *Eco*RI methylase and ligated to *Eco*RI linkers (New England Biolabs). The methylated DNA was digested with *Eco*RI, size-selected by electrophoresis in an agarose gel, and ligated into *Eco*RI-treated λgt10. The resulting phage library was screened with hybridization probes that had been labeled with [α-^32P]dCTP (New England Nuclear) by the random hexamer method (9). The probes consisted of DNA encoding the constant (C) region of γ heavy chain or κ or λ light chain. Positive phage were purified, and the cDNA inserts were sized by electrophoresis and then were subcloned as *Not* I–*Eco*RI fragments into pUC18N, mp18N, and mp19N. These are standard pUC and M13 mp vectors that have been modified to contain a *Not* I site in the polylinker.

DNA Sequencing. All clones were sequenced by the dideoxy method with 2'-deoxyadenosine 5'-[α-[^35S]thio]triphosphate (New England Nuclear) as label (10, 11). Two 60-cm gradient gels, one run for 30,000 V·hr and one run for 8500

V·hr, were sufficient to sequence the entire V region with the use of a single primer. The antisense strands were sequenced by using the standard M13 primers, and the coding strands were sequenced with the following primers, which are complementary to regions on the 5' end of the appropriate constant region: 5' d(CAGGGTCACCATGGA) 3' (γ), 5' d(CACGAC-TGAGGCACC) 3' (κ), and 5' d(GCTCCTCAGAGGAGGGC) 3' (λ). Multiple independent clones were sequenced for >75% of the chains.

Preparation of DNP-SL. The synthesis of the spin-labeled hapten has been described (7). The chemical formula can also be found in Anglister *et al.* (2).

NMR Spectra. Samples were prepared for NMR as described (2). NMR spectra were obtained in Fourier-transform mode on a General Electric NMR spectrometer operating at 500 MHz (proton). Approximately 6000 scans of 16,000 data points with a sweep width of ±4000 Hz were taken for each spectrum. Residual water protons were presaturated, and chemical shifts are given relative to a tetramethylsilane standard.

RESULTS

Eleven new anti-DNP-SL monoclonal antibody-producing cell lines were generated from the spleens of five mice. The isotypes, subgrouping according to Kabat *et al.* (12), joining (*J*)-region usage, and stability constants of these antibodies are shown in Table 1. All antibodies are of the IgG class and have stability constants for DNP-SL ranging from 5.0 × 10^4 M^{-1} to 7.3 × 10^7 M^{-1}. The previously described (2) monoclonal anti-spin-label antibody was given the name AN02, so we have named these 11 new antibodies AN01 and AN03–AN12. Balakrishnan *et al.* (7) referred to an antibody named AN01, but it later proved identical to AN02. We have thus used the name AN01 for one of the new antibodies.

The deduced amino acid sequences of the heavy- and light-chain V regions of each antibody are shown in Figs. 1 and 2, respectively. The nucleic acid sequences are being contributed to the GenBank data base* (13), and copies are available upon request. This panel of anti-DNP-SL antibodies is not dominated by sequences originating from one V-region gene family. There are subgroups, however, that do show a high degree of similarity among themselves. Fig. 3 indicates which heavy and light chains have highly similar DNA sequences. Both the heavy and the light chains of AN01 and AN03 utilize V regions derived from the same families as those of AN02. The heavy chain of AN07 and the light chain of AN09 are similarly related to AN02. These heavy and light chains show no preferred diversity (D)- or J-region usage. All gene segments of the AN05 and AN06 antibodies are similar if not identical in origin.

In addition to clones coding for the expressed antibody proteins, cDNA clones coding for an aberrant γ or an aberrant κ chain were found. The aberrant γ clones were always identical in sequence, as were the aberrant κ clones, although they were found in several libraries constructed from independent hybridoma cell lines. The κ-chain sequence was identical to the sequence found and characterized in several fusion partners of common lineage with Sp2/0 (S. Levy, personal communication). The aberrant γ-chain sequence consisted of a portion of the intron between the *J* and *C* coding regions spliced to the $C_\gamma I$ exon. Examination of the *J–C* intron sequence listed in the GenBank data base† (13) revealed a splice donor site, 5' d(AAATG/GTAAGCC) 3' (14), at sequence positions 4189–4200 that would generate the observed sequence. The 5' end of each of these clones

Table 1. Characteristics of the monoclonal antibodies

Name	KH	KL	H	L	J_H	J_L	Stability constant, M^{-1}						
							DNP-SL	DNP-SG	DNP-G$_2$	DNP-SA	DNP-D$_2$	DNP-en$_2$	DNP-G
AN01	Ia	VI	γ1	κ	J_H2	$J_\kappa4$	5.1×10^4						
AN02	Ia	VI	γ1	κ	J_H3	$J_\kappa5$	7.5×10^6	1.8×10^7	3.1×10^6	2.0×10^7	2.7×10^5	6.0×10^6	4.5×10^6
AN03	Ia	VI	γ1	κ	J_H2	$J_\kappa5$	6.2×10^4	Low	Low				
AN04	IIa	V	γ1	κ	J_H3	$J_\kappa1$	6.8×10^6	4.4×10^7	2.2×10^6	2.9×10^6	Low		
AN05	IIc	II	γ1	κ	J_H2	$J_\kappa5$	9.5×10^6	9.2×10^6	Low	1.2×10^7			
AN06	IIc	II	γ1	κ	J_H2	$J_\kappa5$	1.2×10^7	2.7×10^7	Low	2.6×10^7			
AN07	Ia	I	γ1	λ	J_H3	$J_\lambda1$	2.5×10^7	$>5 \times 10^7$	4.9×10^5	9.7×10^5	Low		
AN08	IIa	V	γ1	κ	J_H4	$J_\kappa2$	7.3×10^7	3.0×10^7	1.7×10^6	2.4×10^6	Low		
AN09	IIa	VI	γ2a	κ	J_H3	$J_\kappa5$	1.6×10^7	$>1 \times 10^8$	4.0×10^7	$>1 \times 10^8$	2.7×10^6		
AN10	III	I	γ1	λ	J_H4	$J_\lambda1$	4.9×10^7						
AN11	IIa	V	γ1	κ	J_H1	$J_\kappa2$	5.0×10^5						
AN12	IIa	V	γ1	κ	J_H3	$J_\kappa2$	2.1×10^6						

Subgrouping [according to Kabat *et al.* (12)] for the heavy (KH) and light (KL) chain, isotype for the heavy (H) and light (L) chain, J-region usage for the heavy (J_H) and light (J_L) chain, and stability constant for various DNP haptens are shown for each antibody. The haptens were as follows: DNP-TEMPO-ethylenediamine (DNP-SL), DNP-TEMPO-glycine (DNP-SG), DNP-bis(glycine) (DNP-G$_2$), DNP-TEMPO-aspartic acid (DNP-SA), DNP-bis(aspartic acid) (DNP-D$_2$), DNP-bis(ethylenediamine) (DNP-en$_2$), DNP-glycine (DNP-G). TEMPO is 2,2,6,6-tetramethyl-1-oxypiperidin-4-yl. All haptens were synthesized by amine linkage of the substituents to the 2 and 4 positions of 2,4-difluoro-1,5-dinitrobenzene except DNP-glycine, which was purchased from Sigma.

extended to the *Eco*RI site at positions 3559–3564 of the J–C intron. This termination site is probably an artifact of our size selection and inadequate methylation during construction of the libraries. These transcripts are probably nonfunctional, as they contain several in-frame stop codons. The presence of this clone in a library expressing a γ2a heavy chain indicates that it is not solely due to missplicing of the expressed transcript. The aberrant γ sequence constituted ≈10% of γ clones screened from all libraries.

Sequencing of multiple clones revealed occasional differences between clones encoding the same polypeptide chain. Of ≈25,000 overlapping sequence measurements, 4 single-base-pair substitutions were detected. The errors appeared to be random and indicated a small but nonnegligible error rate

(on the order of 1 error per 6000 base pairs) in our cloning procedures.

A 7-base-pair insertion was observed in one of the clones coding for the AN04 κ chain. The correct sequence at the insertion site, as determined from two independent clones, consisted of a contiguous repeat of the 7-base-pair sequence 5' d(CAGCCTG) 3'. In the abnormal clone this sequence was repeated three times, suggesting a slippage or "hiccup" of the polymerase during chain elongation. Error due to the slipping of a polymerase between direct repeats has been postulated as a mechanism of somatic mutation in immunoglobulins (15).

A series of abnormalities was also observed in the leader sequence of AN02 heavy chain clones near the initiating

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      -15          -5      1            10          20          30    35 36
       I            I      I            I           I           I     I  AB
ANO2  MRVLILLWLFTAFPGILSDVQLQESGPGLVKPSQSQSLTCTVTGYSITSDYAWN WI
ANO1  .K..S..Y.L..I...........................L....S.........G.Y.. ..
ANO3  .K..S..Y.L..I...........................L....S.........G.Y.. ..
ANO7  ........C.....................D.......L...............G.S.H ..
ANO5  ME.HW.F.F...SVTA.VH.QF.P.Q..AE.A..GA.VKMS.KAS..TF..YWMH .V
ANO6  ME.HW.F.F...SVTA.VH.Q..P.Q..AE.A..GA.VKMS.KAS...F.RYWMH .V
ANO4  MGWSW.F.F.LSGTA.VHCQI..KQ...E....GA.VKIS.KAS...F.DY.IN .V
ANO9  MGWSY.I.F.VATATDVH.Q....QP.AE....GA.VK.S.KAS..TF..YWMH .V
AN11       MSW.F.F.LSGTA.VH.E....Q...E..R.GA.VKMS.KAS..TF..YVMH .V
AN12  MEWNWVV.F.LSLTA.VYAQG.M.Q..AE....GA.VK.S.KTS.FTFR.S.IG .L
ANO8  MEWLWN..F.MA.AQS.QAQI..VQ...E.K..GETVRIS.KAS..TF.TAGIQ .V
AN1O  MNFGFS.IF.VLVLK.VQCE.K.V...G.....GG.LK.S.AAS.FTFS.YAMS .V

      40          52 53      60          70          82 83      90
       I            ABC                  I           I  ABC      I
ANO2  RQFPGNKLEWMGYMS  YSGSTRYNPSLRSRISITRDTSKNQFFLQLKSVTTEDTATYF
ANO1  .............IN  .D.RNN.....KN................K..........Y
ANO3  .............IN  .D.NNN.....KN................K.N.........Y
ANO7  .....H........IH  .....N.....K.................N..........Y
ANO5  K.R..QG...I..INP  NT.Y.V..QKFKDKATL.A.K.SSTAYM..S.L.SD.S.V.Y
ANO6  K.R..QG...I..INP  ST.Y.E..QKFKDKATL.A.K.SSTAYM..S.L.S..S.V.Y
ANO4  K.K..QG...I.WIYP  G..NNK..EKFKGKATL.I...SSTVYI..S.L.S....V..
ANO9  ..R..QG...I.EINP  SN.R.N..EKFK.KATLNV.K.SSTAYM.IS.L.S..S.V.Y
AN11  K.K..QG...I..INP  .NDG.K..EKFKGKATL.S.K.SSTAYIE.S.L.S..S.V.Y
AN12  K.K..QS...IAWIYA  GT.G.S..QKFTGKARL.V...SSTAYM.FS.L.....S.I.Y
ANO8  QKM..KG.K.I.WINT  R..VPK.AEDFKG.FAFSLE...ASTAY...ISNLRND...A..
AN1O  ..T.ERR...VASI.   SGYI.Y.PD.VKG.FT.S..NAR.ILV..MS.LRS....M.Y

      95   100         105  110
       I    ABCDEFGHIJK I    I
ANO2  CARGWP            LAYWGQGTQVSVSE
ANO1  ...EDDGYYI        FD.....STLT..S
ANO3  ...EGYGYF         FD......TLT..S
ANO7  ...VIYYYGSSYV     WF.......L.T..A
ANO5  ..YYGSS           YFD......TLT..S
ANO6  ..HYGRS           YFD......TLT..S
ANO4  .V.YGYDG          FG......L.T..A
ANO9  ...R.GSYVGG       F.......NM.T..A
AN11  ...FGYYGR         YWYFDV..A..T.T..S
AN12  ...WD.INRG        F.......L.T..A
ANO8  .G.TDYYGST        YYAMD......SS.T..S
AN1O  ...WGHRYDVL       D......S.T..S
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FIG. 1. Deduced amino acid sequences of the V regions of the heavy chains of the anti-DNP-SL monoclonal antibodies AN01–AN12. Numbering system is according to Kabat *et al.* (12). Standard one-letter amino acid symbols are used.
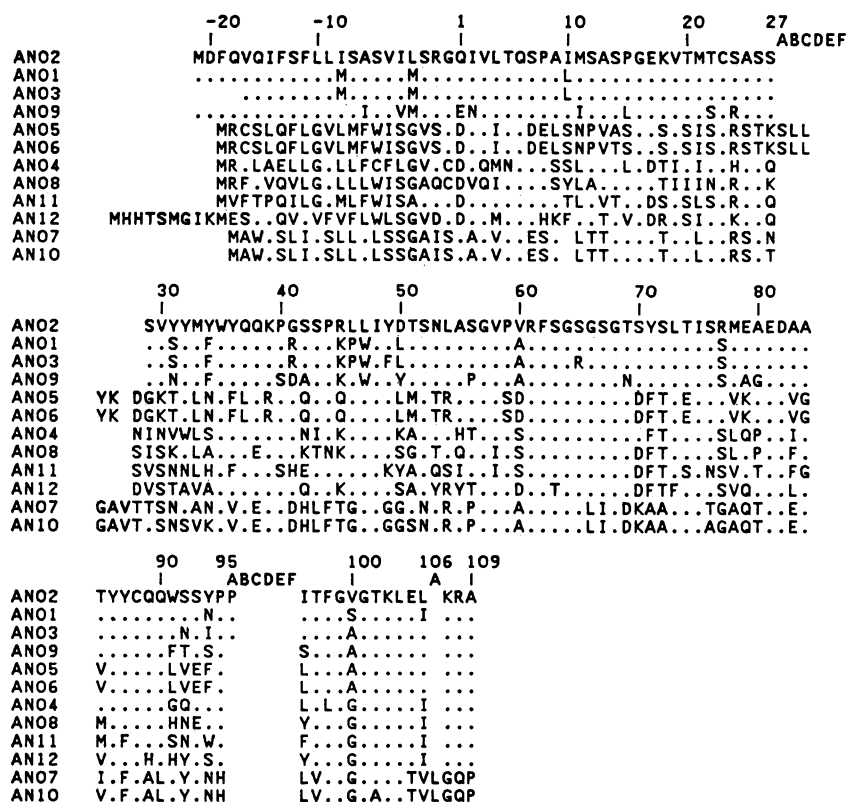
```
                   -20        -10         1         10        20    27
                    I          I          I         I         I      I ABCDEF
        ANO2   MDFQVQIFSFLLISASVILSRGQIVLTQSPAIMSASPGEKVTMTCSASS
        ANO1   .............M.....M.............L...................
        ANO3   .........M.....M.............L...................
        ANO9   ................I..VM...EN.........I....L......S.R...
        ANO5   MRCSLQFLGVLMFWISGVS.D..I..DELSNPVAS..S.SIS.RSTKSLL
        ANO6   MRCSLQFLGVLMFWISGVS.D..I..DELSNPVTS..S.SIS.RSTKSLL
        ANO4   MR.LAELLG.LLFCFLGV.CD.QMN...SSL...L.DTI.I..H..Q
        ANO8   MRF.VQVLG.LLLWISGAQCDVQI....SYLA.....TIIIN.R..K
        AN11   MVFTPQILG.MLFWISA...D........TL.VT..DS.SLS.R..Q
        AN12   MHHTSMGIKMES..QV.VFVFLWLSGVD.D..M...HKF..T.V.DR.SI..K..Q
        ANO7       MAW.SLI.SLL.LSSGAIS.A.V..ES. LTT....T..L..RS.N
        AN1O       MAW.SLI.SLL.LSSGAIS.A.V..ES. LTT....T..L..RS.T


                   30        40        50        60        70        80
                    I         I         I         I         I         I
        ANO2   SVYYMYWYQQKPGSSPRLLIYDTSNLASGVPVRFSGSGSGTSYSLTISRMEAEDAA
        ANO1   ..S..F......R...KPW..L.........A..............S.......
        ANO3   ..S..F......R...KPW.FL.........A....R..........S.......
        ANO9   ..N..F.....SDA..K.W..Y.....P...A..........N.......S.AG....
        ANO5   YK DGKT.LN.FL.R..Q..Q....LM.TR....SD.........DFT.E...VK...VG
        ANO6   YK DGKT.LN.FL.R..Q..Q....LM.TR....SD.........DFT.E...VK...VG
        ANO4   NINVWLS.......NI.K....KA...HT...S...........FT....SLQP..I.
        ANO8   SISK.LA...E...KTNK....SG.T.Q..I.S.........DFT....SL.P..F.
        AN11   SVSNNLH.F...SHE......KYA.QSI..I.S.........DFT.S.NSV.T..FG
        AN12   DVSTAVA.......Q..K....SA.YRYT...D..T.......DFTF...SVQ...L.
        ANO7   GAVTTSN.AN.V.E..DHLFTG..GG.N.R.P...A.....LI.DKAA...TGAQT..E.
        AN1O   GAVT.SNSVK.V.E..DHLFTG..GGSN.R.P...A.....LI.DKAA...AGAQT..E.


                   90    95          100   106 109
                    I      ABCDEF      I     A  I
        ANO2   TYYCQQWSSYPP      ITFGVGTKLEL KRA
        ANO1   .........N..      ....S.....I ...
        ANO3   ........N.I..      ....A...... ...
        ANO9   ......FT.S.      S...A...... ...
        ANO5   V.....LVEF.      L...A...... ...
        ANO6   V.....LVEF.      L...A...... ...
        ANO4   ......GQ...      L.L.G.....I ...
        ANO8   M.....HNE..      Y...G.....I ...
        AN11   M.F...SN.W.      F...G.....I ...
        AN12   V...H.HY.S.      Y...G.....I ...
        ANO7   I.F.AL.Y.NH      LV..G....TVLGQP
        AN1O   V.F.AL.Y.NH      LV..G.A..TVLGQP
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FIG. 2. Deduced amino acid sequences of the V regions of the light chains of the anti-DNP-SL monoclonal antibodies AN01–AN12. Numbering system is according to Kabat *et al.* (12).

ATG. Of four independent cDNA clones sequenced, three different sequences were found in this region (Fig. 4), one of which was identical to the sequence of this region from a genomic clone of the AN02 heavy chain. Plasmids containing the non-germ-line versions of this gene appear to lyse the host bacteria before saturating growth conditions are reached.

DISCUSSION

In Fig. 5, spectra a and b show the aromatic regions in an NMR difference spectrum, unoccupied Fab minus Fab occupied with a low concentration of DNP-SL, for AN05 and AN06, respectively. The signals in these spectra originate from protons in close proximity to the spin-label (<10 Å). There is only one sequence difference between AN05 and AN06 variable regions that involves aromatic residues. AN05 has a tyrosine at position 94 of the heavy chain, whereas AN06 has a histidine at this position. Spectrum c is the double difference spectrum obtained by subtracting spectrum b from spectrum a. This spectrum clearly reveals four negative signals, which are likely to arise from the tyrosine-94 protons of AN05, and two positive signals, which are candidates to

arise from the histidine-94 protons of AN06. The upfield chemical shift of the positive features is unusual for histidine residues but not without precedent (16). Further experiments, such as selective deuteration, are necessary before a detailed interpretation of these spectra can be made.

The simplicity of the double difference spectrum, c, in Fig. 5 shows that the binding sites of AN05 and AN06 have nearly identical conformations. This result illustrates how simple NMR difference spectra can provide a useful measure of the structural relatedness between antibodies of highly similar sequence. This approach may also be of use for other families of closely related proteins for which a limited number of structures are known.

Both AN05 and AN06 have a yellow color after elution from a protein A-Sepharose column. This coloring could not be removed by extensive dialysis. Preliminary experiments show this color to be due to bound riboflavin, presumably from the culture medium. AN05 and AN06 also bind flavin mononucleotide and flavin adenine dinucleotide. This binding of DNP-SL and these flavins is competitive.

AN01 and AN03 are similar enough in sequence that NMR difference spectra, in combination with deuteration, should yield some sequence-specific assignments. AN01 and AN03
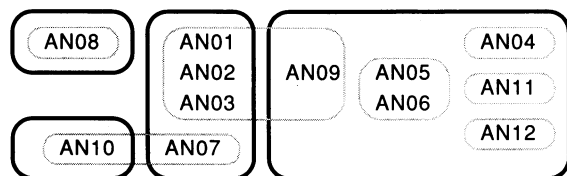
FIG. 3. AN01–AN12 grouped according to those that have closely related chains. Dark lines enclose antibodies whose heavy-chain nucleic acid sequences are >75% identical. Lighter lines enclose antibodies whose light-chain nucleic acid sequences are >85% identical. A different criterion was used for heavy chains because this distinction provided the clearest discrimination between sequences.

```
              -17   -16   -15   -14   -13   -12
    1.  GCC   TAA   AAG   ATG   AGA   GTG   CTG   ATT   CTT
    2.  CAG   CCT   CCA   GTG   AGA   GCG   ATG   ATT   CTT
    3.  CAG   CCT   CCA   GTG   AGA   GCG   CTG   ATT   CTT
```

FIG. 4. Nucleic acid sequences of the leader regions of different cDNA clones encoding the AN02 heavy chain. The codons are numbered relative to the last residue of the leader peptide (−1). The sequences 3' to those shown were identical among all clones and coded for an otherwise normal heavy chain. Sequence 1 is identical to the sequence for this region found in a genomic clone of the AN02 heavy chain. Sequence 2 was found in two independent cDNA libraries constructed from different preparations of RNA. Sequence 3 did not contain an apparent initiating ATG codon. These differing leaders do not arise from an identifiable splicing event (14).
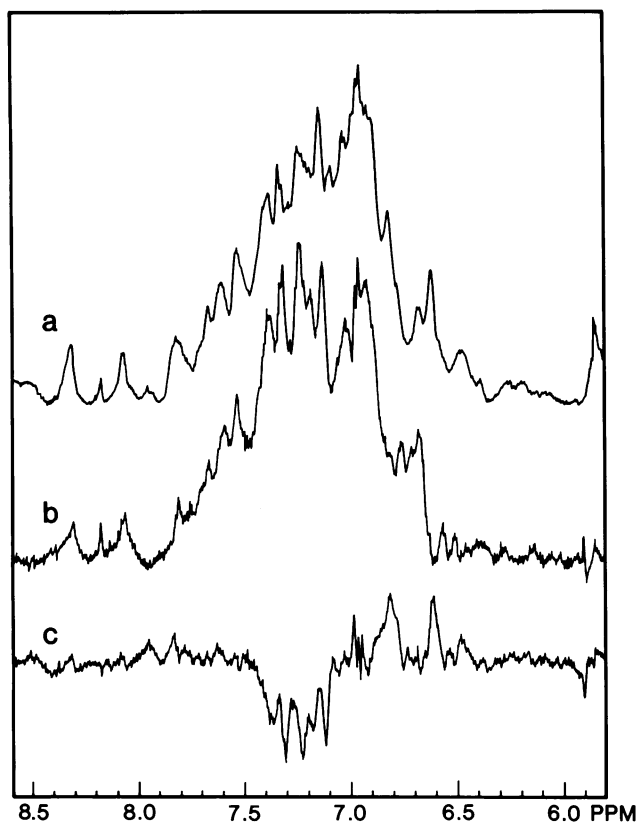
FIG. 5. Aromatic regions of the difference spectra, unoccupied Fab minus Fab with 4% (mol/mol) bound spin-label, for AN06 (spectrum a) and AN05 (spectrum b). Spectrum c is the double difference spectrum a minus b.

differ enough from AN02 that direct comparison of NMR spectra is unlikely to yield sequence-specific assignments for AN02. The number of amino acid differences in the V regions and the use of different leader sequences indicate that both chains of AN02 are derived from related but different germ-line genes as those of AN01 and AN03. AN02 has 100-fold higher affinity for DNP-SL than do AN01 and AN03. Site-directed mutagenesis should allow identification of residues important for high-affinity binding in these related antibodies. Cloning and sequencing of the germ-line genes that gave rise to the AN02 V regions should enable the construction of mutants for investigation of structural pathways selected during somatic mutation.

The remaining antibodies use unique combinations of heavy and light chains. The diversity found in these anti-DNP-SL antibodies provides a rich source for structural and functional studies. The creation of new antibodies with recombinant techniques further increases the diversity of this system. Mixing and matching heavy and light chains combinatorially increases the number of related antibodies within a subgroup and provides for chain-specific assignments. Restriction endonuclease sites can be used to create hybrid chains. For example, restriction sites in the third framework

region of the heavy chain allow switching of the D-J combination in cases where similar V, but different D and J, regions are used.

NMR studies provide kinetic information in a time range particularly relevant to hapten binding (H.M.M., T. E. Frey, and J. Anglister, unpublished data). Construction of mutants that affect the on-rates or the off-rates of specific haptens should reveal structural features relating to chemical kinetics (17).

The NMR techniques used to study the combining sites of monoclonal anti-spin-label antibodies can clearly be extended to other proteins with modest affinities for spin-labeled ligands. Modification of ligands with spin-labels, in combination with amino acid deuteration and recombinant DNA techniques, promises to allow detailed analyses of protein binding sites by NMR.

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