Abstract: In collaboration with Michael Levitt we have constructed semiempirical/semitheoretical structural models of the twelve anti-nitroxide spin label antibodies previously sequenced in this laboratory. These models are in striking qualitative accord with the NMR difference spectra in showing the presence of a large number of aromatic amino acids (especially tyrosines) in the combining site region, i.e., within about 20 Å of the unpaired electron. The high motional freedom of the tyrosine residues (i.e., > 10-12 residues in AN01-AN03) inferred from the NMR indicates that these residues play no significant role in maintaining a rigid three-dimensional structure of these proteins. We have determined the germline sequences of AN02 and AN07 and find similar numbers of tyrosine residues in the combining site regions. It is likely that the tyrosine and other residues in the combining site regions that are highly mobile, and distributed over a large surface (± 20 Å x 20Å) are mostly designed for protein antigen recognition (not small haptens) including (idiotype-anti-idiotype regulation (1,1)).
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Line-Shape Analysis of NMR Difference Spectra of an Anti-Spin-Label Antibody†

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ABSTRACT: Specifically deuteriated Fab fragments of the anti-spin-label antibody AN02 were prepared. NMR difference spectra were obtained, in which the spectrum of Fab with some fraction of the binding sites occupied with spin-label hapten was subtracted from the spectrum of Fab with no spin-label. The peak heights were analyzed as a function of the fractional occupation of the binding site, using a computer program that calculates a best fit to the observed spectra. This method treats all of the peaks in the spectra simultaneously. Analyzing all peaks at once allows for the interdependencies in the spectra arising from overlap of positive and negative signals from different peaks. The fitting program calculates line widths for the peaks arising from protons in the binding site region. Almost all of the line widths calculated for the spectrum of the Fab complex with diamagnetic hapten dinitrophenylglycine were found to be narrower than the line widths of the corresponding resonances in the spectrum of Fab with an empty binding site. The distances of the binding site region protons from the unpaired electron of the hapten were also obtained from this calculation. Two tyrosine protons were found to be close (<7 Å) to this electron. These line-width and distance results are discussed with respect to the structure and dynamics of the antibody binding site.

In an effort to improve the understanding of antibody binding site structure, we have pursued an NMR study of the anti-spin-label antibody AN02. In previous work, we used biosynthetic deuteration and protein chemistry with NMR to obtain information about the amino acid makeup of the binding site region (Anglister et al., 1984a; Frey et al., 1984) and to assign resonances to the heavy and light chains (Anglister et al., 1985). Nuclear magnetization transfer was used to identify the resonances of protons in close proximity to the hapten (Anglister et al., 1987). The effect of the paramagnetic hapten on the protein NMR spectrum was used to measure the distance of certain antibody protons from the unpaired electron of the hapten (Anglister et al., 1984b).

The effect of the hapten on the AN02 resonance spectrum was described, and a technique for extracting distance information was discussed. This technique involves titrating the binding site of the Fab with paramagnetic hapten and subtracting the NMR spectrum taken at each titration point from the spectrum of the Fab with no hapten bound. The change in peak height of each resonance was analyzed as a function of fractional occupation of the binding site. Several tyrosine resonances displayed the theoretically expected behavior.

The technique presented in our earlier paper analyzed one signal at a time, required that the line width of the proton resonance be known, and assumed that the resonance signals were homogeneous and Lorentzian. Of course, this is not satisfactory for spectra with multiple, overlapping resonances. Further difficulties arise with line-width determination when resonances are very broad or very narrow, due to low signal to noise and digitization, respectively. In the present work, we find that simulation and computer fitting of the observed spectra can be used successfully to extract the desired line-width and distance information.

MATERIALS AND METHODS

The preparation and purification of the AN02 antibody have been described. Fab fragments were prepared and purified as previously described. The affinity of AN02 for spin-label...
The Fab fragments used for the spectra in this paper were biosynthetically deuterated in the tryptophan and phenyl-
alanine residues, and in the 2- and 6-positions of the tyrosine 
rings. The only proton resonances seen in the spectral region 
of 6.0–9.0 ppm are therefore from the 3- and 5-protons of 

\[ \text{tyrosine and possibly from histidine. We have shown that Fab} \]

with an unoccupied binding site, the line width of the same 
resonances is extremely broad. The fitting algorithm used finds 
the parameters giving a

\[ \text{minimum in the sum of the squares of the deviations from the} \]

calculated amplitudes from the observed data. The only free 
parameters in the model are the line width of the signal in the 
Fab with an unoccupied binding site, the line width of the same 
resonance in the with DNP complex, and the distance of the 
protons from the unpaired spin in the with spin-label complex.

Thus, there are 3 parameters per proton to be fit in each data 
set (30 for the data set discussed in this paper). There is one 
observed amplitude per peak per titration point in the data 
set (108 points). Note that peak B is a composite of 2 signals 
(Anglister et al., 1985) and there are therefore 9 peaks and 
10 protons. The fit is still overdetermined even for this many 
parameters, but the derived values for the two signals from 
peak B are given with less confidence. Further complicating 
the interpretation of peak B is the possibility of a third signal 
forming a shoulder on the high chemical shift side in difference 
spectra with high spin-label occupancy.

Some assumptions are implicit in the analysis of these NMR 
spectra. The scaling of the spectra assumes that at least one 
line width in one spectrum can be measured accurately. For 
the present calculations, the lines labeled H and G in the 
without minus with 100% spin-label spectrum were used for 
scaling all of the without minus with spin-label spectra. These 
special peaks were selected on the basis of the following 
criteria. (i) The location of the base line is clear. In these 
difference spectra, the summing of negative signals in regions 
with many resonances can affect the shape of the base line 
even when any one of these negative signals is too broad to 
make a noticeable effect. (ii) There is no overlap with other 
resonances. (iii) The protons are sufficiently near the unpaired 
electron that the contribution of the negative signal in the 
difference spectrum is completely obliterated at 100% occu-
pancy. (Note that when proton signals of different width are 
subtracted the width at half-height of the resulting feature is 
not the line width of either proton signal.) (iv) The lines are 
narrow enough to provide good signal to noise but broad 

\[ \text{enough to provide enough data points to minimize error from} \]

the discontinuous nature of the digital spectrum. The mea-

\[ \text{sured line widths should also yield relative peak amplitudes} \]

that are consistent with those observed in the spectra. Using 
similar criteria, we used line H to scale the with DNP minus 
with spin-label titration.

A second set of assumptions concerns the spectral position 
of resonances in the Fab spin-label complex. The chemical 
shifts of several tyrosine resonance change with the occupation 
of the binding site. Since these resonances are extremely broad 
in the spectrum of the Fab spin-label complex, it is not possible 
to determine the chemical shift directly. These shifts are 
therefore assumed to be the same as those observed in the 
complex with reduced spin-label. The chemical shifts of 
resonances in the reduced spin-label complex are very similar 
to those in the DNP complex, and so this likely to be a good 
approximation (Anglister et al., 1985).

Another question arises when treating the with DNP minus 
with spin-label spectra. The system was analyzed as a two-

\[ \text{state system involving only the DNP complex and the spin-} \]

label complex. The concentrations of DNP and spin-label were 
kept high enough to saturate all the binding sites, and the 
concentration of Fab was high enough to make the concentra-
tion of free Fab negligible. The Appendix shows that under 
these conditions a three-state NMR line-shape problem is 
reduced to the two-state treatment used.
Changes make the amplitudes of pairs of lines interdependent. This narrowing of line width in the with DNP spectrum is observed by comparing Figure 1a and Figure 2a. These different scaling of the two titrations are shown. The spectra for the third and fifth highest occupancy are (a) 100%, (b) 20.03%, (c) 4.96%, and (d) 2.57%.

Figure 1 shows the simulated and observed spectra for several of the difference spectra in the without minus with spin-label titration. All spectra are to the same scale. Figure 2 shows results for the with DNP minus with spin-label titration. The spectra in Figures 1 and 2 are to different scales. Only four of the six recorded spectra in each data set are complex. Examination of the spectra in Figures 1 and 2 shows results for the proton tyrosine resonances and the line widths derived from the best fit to the titration data are shown in the table. Table II shows results for the with DNP minus with spin-label difference spectra. Identical labels for the protons in the two tables indicated that the protons are from the same amino acid. The method used to interrelate signals in the two spectra has been discussed previously (Anglister et al., 1984). Note that the line widths of a given proton are not the same in the two types of spectra and are almost always narrower in the DNP complex. Examination of the spectra in Figures 1 and 2 shows that the line shapes calculated are in most cases in good agreement with the observed spectra, so that this difference in calculated line width is not likely to be an artifact of the different scaling of the two titrations.

This narrowing of line width in the with DNP spectrum is interesting. If some paramagnetic impurity is less able to penetrate the protein when hapten is bound, a narrowing effect affect the other's amplitude in one or both of the titrations. The negative feature from line F moves through the position of the positive signal from line E in the without minus with spin-label titration, thus affecting its amplitude. The fitting algorithm allows for these interdependencies. It is more difficult to model the small signal at slightly higher chemical shift than line A and its effect on the observed amplitude of line A. This is probably not a tyrosine signal (or even necessarily from a single source). Both histidine and unexchanged peptide amide protons would show signals in this spectral region. Since the number of protons giving rise to this signal cannot be determined, it cannot be scaled properly with respect to the tyrosine signals in the calculation.

Table I lists spectral parameters derived from the without spin-label minus with spin-label spectra. The chemical shifts of the protein tyrosine resonances and the line widths derived from the best fit to the titration data are shown in the table. Table II shows results for the with DNP minus with spin-label difference spectra. Identical labels for the protons in the two tables indicated that the protons are from the same amino acid. The method used to interrelate signals in the two spectra has been discussed previously (Anglister et al., 1984). Note that the line widths of a given proton are not the same in the two types of spectra and are almost always narrower in the DNP complex. Examination of the spectra in Figures 1 and 2 shows that the line shapes calculated are in most cases in good agreement with the observed spectra, so that this difference in calculated line width is not likely to be an artifact of the different scaling of the two titrations.

This narrowing of line width in the with DNP spectrum is interesting. If some paramagnetic impurity is less able to penetrate the protein when hapten is bound, a narrowing effect

<table>
<thead>
<tr>
<th>Proton</th>
<th>Chemical Shift (ppm)</th>
<th>Line Width (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7.126</td>
<td>9.4</td>
</tr>
<tr>
<td>B</td>
<td>6.864</td>
<td>6.9</td>
</tr>
<tr>
<td>C</td>
<td>6.857</td>
<td>7.7</td>
</tr>
<tr>
<td>D</td>
<td>6.810</td>
<td>5.1</td>
</tr>
<tr>
<td>E</td>
<td>6.799</td>
<td>8.4</td>
</tr>
<tr>
<td>F</td>
<td>6.722</td>
<td>4.8</td>
</tr>
<tr>
<td>G</td>
<td>6.093</td>
<td>12.1</td>
</tr>
<tr>
<td>H</td>
<td>6.487</td>
<td>10.8</td>
</tr>
<tr>
<td>I</td>
<td>6.435</td>
<td>10.2</td>
</tr>
<tr>
<td>J</td>
<td>6.595</td>
<td>17.0</td>
</tr>
</tbody>
</table>

*The chemical shifts and line widths individually do not have any significance beyond ±0.01 ppm and ±1 Hz. The shapes of overlapping signals are more sensitive to the relative values of line positions and widths than these limits, as indicated by the number of significant figures used in the table. *BROAD.

<table>
<thead>
<tr>
<th>Proton</th>
<th>Chemical Shift (ppm)</th>
<th>Line Width (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7.082</td>
<td>5.8</td>
</tr>
<tr>
<td>B</td>
<td>6.865</td>
<td>6.0</td>
</tr>
<tr>
<td>C</td>
<td>6.865</td>
<td>2.9</td>
</tr>
<tr>
<td>D</td>
<td>6.784</td>
<td>2.8</td>
</tr>
<tr>
<td>E</td>
<td>6.795</td>
<td>4.2</td>
</tr>
<tr>
<td>F</td>
<td>6.706</td>
<td>3.3</td>
</tr>
<tr>
<td>G</td>
<td>6.738</td>
<td>8.25</td>
</tr>
<tr>
<td>H</td>
<td>6.135</td>
<td>1.6*</td>
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<td>I</td>
<td>6.349</td>
<td>5.15</td>
</tr>
<tr>
<td>J</td>
<td>6.575</td>
<td>11.0</td>
</tr>
</tbody>
</table>

*The chemical shifts and line widths individually do not have any significance beyond ±0.01 ppm and ±1 Hz. The shapes of overlapping signals are more sensitive to the relative values of line positions and widths than these limits, as indicated by the number of significant figures used in the table. *BROAD.
might be observed. We have accumulated spectra in the presence and absence of dissolved oxygen and see no difference in line width, ruling out the most likely candidate for the broadening agent. A second possibility is that there is more mobility for tyrosine residues not in contact with the hapten when hapten is bound. We have previously stated that the large number of resonances showing change in chemical shift might indicate a significant change in average conformation. On the other hand, there is clearly an immobilizing effect on the tyrosine giving rise to resonance G, along with the very large change in chemical shift seen for this resonance.

Table III shows the change in chemical shift undergone by each Fab tyrosine resonance upon DNP binding. This table also shows the calculated distance of each of these protons from the paramagnetic center of the hapten. Distances of less than 7 Å cannot be resolved because of the magnitude of the off rate of the hapten (Anglister et al., 1984), and are all labeled as <7. The change in chemical shift and the distance from the spin are the two parameters with strongest implications for structural models of the antibody. An L or H is included in the last column to indicate a light- or heavy-chain origin for the resonance (Anglister et al., 1985). The distances given here are in agreement with those reported previously (Anglister et al., 1984), except for line D. The overlap in line D with line C is likely to be a source of error in the previous calculations.

The value for the distance of line G from the paramagnetic center could not be determined. This is due to the large change in chemical shift undergone by this signal during the without minus with spin-label titration. The negative signal from the partially occupied spectrum moves out from under the positive signal at very low occupancy and is exchange broadened independent of any paramagnetic effect. A distance value for protons giving rise to signal G cannot be obtained from the with DPN minus with spin-label titration due to the extremely poor signal to noise for this line caused by its large line width.

**DISCUSSION**

It can be seen from the simulated spectra (Figures 1 and 2) that the computer analysis used in this work gives a good approximation to the observed spectra. The simulation of the spectra allows the analysis of overlapping signals. The rate of appearance of a resonance in a titration is dependent on the line width, but line-width measurements are not very accurate when determined directly from the spectra. Spectral simulation forces the line widths to be mutually consistent. The derived line widths also provide a quantitative confirmation of the qualitative observation that resonances in the DNP complex are narrower than in the free Fab, as discussed under Results.

The spectra discussed here yield results with structural implications. We have previously discussed a computer model of the antibody and used it to assign likely tryptophan residues to specific resonances (Anglister et al., 1987). The assignment of tyrosine resonances is not so straightforward. There are two heavy-chain tyrosines (33 and 50) which are candidates for signals B and H, but the large number of tyrosine residues makes this assignment very provisional. Further complicating the situation is the lack of an obvious candidate for resonance G. This is the tyrosine resonance that undergoes a large change in chemical shift upon hapten binding. One reason for the lack of a candidate is that the model was computed for an empty binding site, and the change in chemical shift could be due to conformational change in the Fab. There are at least four light-chain tyrosine residues that could be candidates under these assumptions, including the three tyrosines in the second hypervariable loop of the light chain.

The type of analysis described here is being applied to titrations done with several different deuteriations. It is hoped that a collection of these measurements will lead to an understanding of the solution structure of this antibody and, in conjunction with the crystallographic and mutagenesis studies under way, will lead to an improved understanding of antibody–hapten binding in terms of both structure and kinetics.

**ACKNOWLEDGMENTS**

We thank Dr. Michael Levitt for devising the computer model discussed in this paper.

**APPENDIX**

**Magnetization Transfer Due to Chemical Exchange.** The chemical components of the system are Fab, spin-label (SL), DNP-diglycine (DNP), and the complexes of Fab with spin-label (FabSL) and of Fab with DNP (FabDNP). Two reversible reactions are assumed:

\[
\text{FabSL} \leftrightarrow \text{Fab} + \text{SL} \quad (1)
\]

\[
\text{FabDNP} \leftrightarrow \text{Fab} + \text{DNP} \quad (2)
\]

Thus, we assume that FabSL can only be converted to FabDNP through the intermediate free Fab.

Let \(M_s\) be the macroscopic magnetization association with a specific proton in the Fab fragment when spin-label is bound and \(M_D\) be the magnetization of the same proton when DNP is bound. Let \(M_0\) be the magnetization of this proton when the binding site is empty. The changes of the macroscopic magnetization due to chemical exchange can then be written as follows:

\[
M_s = -k_{so}M_S + k_{os}M_0 \quad (3)
\]

\[
M_D = -k_{do}M_D + k_{od}M_0 \quad (4)
\]

\[
M_0 = -k_{os}M_s + k_{do}M_D + k_{so}M_0 + k_{od}M_D \quad (5)
\]

where \(k_{so}\) is the first-order rate constant for dissociation of the Fab–spin-label complex and \(k_{os}\) is the pseudo-first-order rate constant for reassociation.

Assume experimental conditions where there is always an excess of spin-label and/or DNP such that the binding sites of the Fab fragments are almost always occupied. Under these circumstances, the concentration of free Fab is very small, and we may set \(M_0 = 0\). We then obtain the following expression for \(M_0\):

\[
M_0 = \frac{k_{so}M_S + k_{do}M_D}{k_{os} + k_{od}} \quad (6)
\]

**Table III: Results with Relevance to Model Building**

<table>
<thead>
<tr>
<th>proton</th>
<th>change in chemical shift (without spin-label to with DNP in ppm)</th>
<th>distance* (Å)</th>
<th>chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.044</td>
<td>12.9</td>
<td>H</td>
</tr>
<tr>
<td>B</td>
<td>-0.001</td>
<td>12.3</td>
<td>H</td>
</tr>
<tr>
<td>B</td>
<td>&lt;7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.026</td>
<td>11.5</td>
<td>L</td>
</tr>
<tr>
<td>D</td>
<td>0.004</td>
<td>9.0</td>
<td>E</td>
</tr>
<tr>
<td>E</td>
<td>0.016</td>
<td>13.7</td>
<td>H</td>
</tr>
<tr>
<td>F</td>
<td>-0.045</td>
<td>10.85</td>
<td>L</td>
</tr>
<tr>
<td>G</td>
<td>0.352</td>
<td>ND</td>
<td>L</td>
</tr>
<tr>
<td>H</td>
<td>0.086</td>
<td>&lt;7</td>
<td>H</td>
</tr>
<tr>
<td>I</td>
<td>0.020</td>
<td>16.0</td>
<td>L</td>
</tr>
</tbody>
</table>

*The calculated distances are derived from averages of the inverse sixth power of the electron spin-proton spin distance and may represent averages over different molecular conformations. *Not determined.
Substitution of this expression for $M_0$ in eq 3 and 4 allows these equations to be rewritten as

$$M_S = -k_{SD}M_S + k_{DS}M_D$$  \hspace{0.5cm} (7)$$

$$M_D = -k_{DS}M_D + k_{SD}M_S$$  \hspace{0.5cm} (8)$$

where

$$k_{SD} = \frac{k_{DS}k_{DD}}{k_{DD} + k_{DS}}$$  \hspace{0.5cm} (9)$$

$$k_{DS} = \frac{k_{DD}k_{DS}}{k_{DD} + k_{DS}}$$  \hspace{0.5cm} (10)$$

Thus, the equations describing the change of magnetization due to chemical exchange have the form of a two-site exchange reaction, as though the reaction

$$\text{FabSL} + \text{DNP} \rightleftharpoons \text{FabDNP} + \text{SL}$$  \hspace{0.5cm} (11)$$

took place with rate constants $k_{SD}$ and $k_{DS}$.

In the form of the Bloch equations described in Wien et al. (1972), the parameter $\tau$ is used, and for the present problem

$$\tau = 1/k_{SD} + 1/k_{DS}$$  \hspace{0.5cm} (12)$$

In order to put this equation into useful form, we can express the time $\tau$ in terms of the true first-order off rate constants $k_{SO}$ and $k_{DO}$ the fractional occupation of the binding site by spin-label and DNP, namely, $f_S$ and $f_D$. To do this, first note that

$$k_{OS} = k'_{OS}[\text{SL}]$$  \hspace{0.5cm} (13)$$

$$k_{OD} = k'_{OD}[\text{DNP}]$$  \hspace{0.5cm} (14)$$

where $k'_{OS}$ and $k'_{OD}$ are the true second-order rate constants for the reverse reactions in eq 1 and 2. The equilibrium constant for reaction 1 is

$$K_S = \frac{[\text{Fab}][\text{SL}]}{[\text{FabSL}]} = \frac{k_{SO}}{k'_{OS}}$$  \hspace{0.5cm} (15)$$

and for reaction 2

$$K_D = \frac{[\text{Fab}][\text{DNP}]}{[\text{FabDNP}]} = \frac{k_{DO}}{k'_{OD}}$$  \hspace{0.5cm} (16)$$

By combining eq 15 and 16 with eq 12, we obtain the following expression:

$$\tau = \frac{1}{f_Dk_{DO}} + \frac{1}{k_{SO}/f_S}$$  \hspace{0.5cm} (17)$$

or

$$\tau = r_D/f_D + r_{SO}/f_S$$  \hspace{0.5cm} (18)$$

where $r_D$ and $r_{SO}$ are the lifetimes for the two haptens in the combining site. (Note that for the two-site exchange case, where there's no DNP-diglycine, $r = 1/k_{SO}/f_S$. The effect of the DNP-diglycine is effectively to slow down the rate of entry of the spin-label into the binding site.)

Registry No. DNP-glycine, 1084-76-0.

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

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Contributed by Harden M. McConnell, January 19, 1988

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 $5 \times 10^4$ 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 tryptophan 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 crystals 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

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. J03732 and J03833).
ences between clones encoding the same polypeptide chain, indicates that it is not solely due to missplicing of the
of this clone in a library expressing a
selection and inadequate methylation during construction of A 7-bpse-pair insertion was observed in one of the clones
intron. This termination site is probably an artifact of our size procedures.

extended to the EcoRI site at positions 3559-3564 of the J-C
intron. This termination site is probably an artifact of our size

Table 1. Characteristics of the monoclonal antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>KH</th>
<th>KL</th>
<th>H</th>
<th>L</th>
<th>JH</th>
<th>Jk</th>
<th>DNP-SL</th>
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<th>DNP-SA</th>
<th>DNP-D2</th>
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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 (JH) and light (Jk) chain, and stability constant for various DNP haptons are shown for each antibody. The haptons were as follows: DNP-TEMPO-ethylenediamine (DNP-SL), DNP-TEMPO-glycine (DNP-SG), DNP-bis(glycine) (DNP-D2), DNP-TEMPO-aspartic acid (DNP-SA), DNP-bis(glycine)acid (DNP-D2), DNP-bis(γ-epoxy)isobutyric acid (DNP-EN). DNP-glycine (DNP-G) . TEMPO is 2,2,6,6-tetramethyl-1-oxypiperidin-4-yl. All haptons were synthesized by amino linkage of the substituents to the 2 and 4 positions of 2,4-difluoro-1,3-diminoresorcinol except DNP-glycine, which was purchased from Sigma.

A 7-base-pair insertion was observed in one of the clones coding for the AN04 heavy 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 slippage 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

| Numbering system is according to Kabat et al. [12]. Standard one-letter amino acid symbols are used. | Fig. 1. Deduced amino acid sequences of the V regions of the heavy chains of the anti-DNP-SL monoclonal antibodies AN01-AN12. |
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 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 specttra 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 deuterium, should yield some sequence-specific assignments. AN01 and AN03...
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 hapten 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.

We are pleased to thank Dan Denney for the constant assistance and guidance that made the molecular cloning aspects of this work possible. We thank Prof. I. L. Weissman for use of his laboratory during the early stages of this work. This work was supported by Office of Naval Research contract N00014-86-K-0388. D.J.L. is a student in the Medical Scientist Training Program (GM07365-11). G.S.R. is a recipient of a Jane Coffin Childs fellowship.
Crystallization of the Fab Fragment of a Monoclonal Anti-Spin-Label Antibody With and Without Bound Hapten

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ABSTRACT

The Fab fragment of a monoclonal antibody, AN02, specific for a spin-labelled dinitrophenyl hapten was crystallized both with and without bound hapten. Both crystals formed in phosphate buffered saline (150mM NaCl, 10mM Na₂PO₄, .02% NaN₃, pH 7.4) at 4°C and diffract to at least 2.2 Å resolution. Fab with bound hapten crystallizes in space group P6₁22 or P6₅22 with cell dimensions a=b=73 Å and c=373 Å. Unoccupied Fab also crystallizes in space group P6₁22 or P6₅22 but with cell dimensions a=b=75 Å and c=376 Å.
The structural change that an antibody undergoes upon binding of antigen is interesting in terms of antibody diversity and function. Independent crystal structures of an antibody both with and without antigen have not yet been solved. We report here the crystallization of the Fab fragment of AN02, a monoclonal antibody specific for a spin-labelled dinitrophenyl hapten, with and without hapten (for the chemical structure of this hapten see Anglister et al. (1984a)).

In addition to allowing assessment of the effect of hapten binding on antibody structure, solution of the AN02 crystal structures will enable a comparison of x-ray and NMR structural information. The bound spin-label hapten broadens NMR signals of nearby (<17Å) protons in a strongly distance dependent manner. An NMR difference spectrum, Fab alone minus Fab with bound spin-label, is dominated by resonances from protons near the electron spin. Culturing the antibody producing cells in media containing specific deuterated amino acids results in virtually complete incorporation of the deuterated amino acids into the antibody. The spin-label's broadening effect, in combination with deuteration, has enabled identification of NMR signals from amino acids in the hapten binding site and measurement of the distance between many of these residues and the electron spin (Anglister et al., 1984a;1984b;1987).

The genes coding for the AN02 heavy and light chains have recently been cloned (Leahy et al., 1988), and a panel of AN02 mutants is currently being constructed using site directed mutagenesis. The ability to use NMR, x-ray, and recombinant DNA techniques makes AN02 and its mutants a rich source for study of structural and functional aspects of the antibody combining site.

The Fab fragment was prepared as described previously for NMR studies (Anglister et al., 1984a). Briefly, whole antibody was isolated from tissue culture supernatants with a protein A-sepharose column. The antibody was concentrated by vacuum dialysis and digested with papain. The Fab fragment was separated from the Fc fragment and smaller digestion products by chromatography on a G-75 sephadex column followed by a protein A-sepharose column. The product was concentrated to 20 mg/ml and dialysed into phosphate buffered saline (150mM NaCl, 10mM Na2PO4, .02% NaN3, pH 7.4). To prepare samples of
Fab with hapten, an excess of solid hapten was added to the Fab and the mixture gently rotated at room temperature for four hours. The sample was then spun in a microfuge at 15,000 rpm for 20 min and the hapten saturated Fab supernatant removed. From UV absorption spectra we estimate that less than .01% of the Fab in these samples is free of hapten. The Fab samples were centrifuged a second time for 20 min at 15,000 rpm in a microfuge, and the solution aliquotted into 5 mm glass NMR tubes. The samples were then maintained at 4°C.

Crystals formed after approximately two weeks with different tubes containing different sized crystals. Most crystals grew as hexagonal barrels with the longest dimension along the six-fold axis. Either many small crystals coated the NMR tubes and did not appear to grow significantly after first observation, or a few crystals appeared which then continued to grow to a much larger size. In cases where a few large crystals appeared, crystals grew to an average size of 0.5x0.5x0.7mm. One crystal grew to approximately 2x2x3mm.

Crystals were mounted in quartz capillaries and screened precession photographs (μ=15°) of the hk0, h0l, and 11l planes were taken using rotating anode radiation. Crystals of AN02 Fab both with and without hapten crystallized in space group P6_122 or P6_522. The cell dimensions for Fab alone are a=b=75 Å and c=376Å, and the cell dimensions for Fab with the spin-label hapten bound are a=b=73Å and c=373 Å. Both crystals withstand x-rays well and diffract to greater than 2.2 Å resolution, making them suitable for x-ray crystallographic studies.

Acknowledgements
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REFERENCES


SPECIFICITIES OF GERM LINE ANTIBODIES

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The past few years have seen remarkable progress in research on the structure and function of antibodies. In our own work we have shown that it is possible to obtain extensive significant information on the composition and structure of antibody combining sites using NMR, together with nitroxide spin-label haptens (Anglister et.al., 1984a, 1985 and 1987; Frey et .al., 1984). This derived information includes the amino acid composition of the combining site region, that is, the number of tyrosines, alanines, etc. that are within ~ 20 Å of the odd electron on the paramagnetic hapten. In antibody molecules there are typically 40-50 amino acids in this combining site region. We have shown that NMR titration data can be used to estimate distances between individual protons on amino acid side chains and the odd electron (Anglister et.al., 1984b; Frey et. al., 1988). These measured distances extend out to about 20 Å, and in to distances of the order of 3-5 Å. Shorter distances can sometimes be estimated from nuclear magnetization transfer experiments. The NMR data also provide a powerful and convenient means of obtaining the on-off kinetics of hapten-antibody reactions, using resonance signals from the hapten as well as from the protein.

Our NMR studies are being carried out on 12 monoclonal anti-dinitrophenyl nitroxide spin label antibodies that we have prepared and sequenced (cDNA) (Leahy et.al., 1988). Theoretical models for the Fab fragments of all of these antibodies have been made in collaboration with Dr. Michael Levitt (unpublished). A major purpose of our current work is to compare these theoretical structure models and the NMR data. An essential requirement for this work is the production of antibody mutants that can be used to obtain rigorous NMR assignments.

Figures 1 and 2 show schematically the sequence similarities of the Fab fragments of the antibodies AN01-AN12. Some pairs of these antibodies have
sequences that are very similar in both heavy and light chains (AN01,2,3), or (AN05,6), some pairs have similarities in only the heavy chains (AN04,11,12), and other pairs have similarities in only the light chains (AN07-ANI0). Clearly many sequences can accommodate the same hapten. Since germline antibody genes doubtless evolved long before dinitro-phenyl was first synthesized, we have developed an interest in the question of whether the germline antibodies would also recognize the dinitrophenyl nitroxide spin label hapten. If the germline antibodies did not recognize this hapten, then we would conclude that their specificity/affinity was largely developed through somatic mutation. If the germline antibodies did recognize the dinitrophenyl nitroxide spin label hapten with essentially the same specificity/affinity, then we would conclude that the specificity of these antibodies was essentially accidental, and we might then speculate on the nature of the primordial antigen and how its structure might be related to dinitrophenyl.

MATERIALS AND METHODS

Southern Blot Analysis

Balb-c genomic DNA was isolated from the livers of Balb-c mice as described in Maniatis et. al. (1982). AN02 genomic DNA was similarly isolated from AN02 antibody producing hybridoma cells. Each of these DNAs were digested with a series of restriction enzymes and analyzed by the method of Southern (1975). The probes used in the hybridization were prepared from the variable regions of the light and heavy chain coding sequences of an AN02 containing plasmid. The light chain probe was a 1000 base pair fragment made by digesting the AN02 genomic kappa chain clone with BgIII and PpuMI. This probe begins approximately 800bp 5' to the variable region. The heavy chain probe was a 600bp fragment isolated as an XbaI and EcoRI fragment from an AN02 heavy chain clone which extends to within 40 base pairs of the end of the variable region. The probes were labelled using Pharmacia's oligo labelling kit with a-32P dCTP(New England Nuclear) as label. The hybridizations were carried out as described in Maniatis (1982) at 42 degrees Celsius with formamide concentrations ranging from 35% to 50%.
**Germline gene cloning and sequencing**

Balb-c liver DNA was digested with the appropriate restriction enzyme, size selected on an agarose gel and isolated using an International Biotechnology Unidirectional Electroelutor. Libraries were made by ligating these fragments into either lambda ZAP or lambda NM590. Recombinant phage DNA was packaged as described in Maniatis (1982) and the resultant libraries were screened using either the heavy or light chain probe. Positive clones were purified and subcloned into pUC322 from lambda NM590 or excised from lambda ZAP into its pBluescript phagemid. The plasmid clones were mapped with 15 to 35 combinations of single and double restriction digests. The clones were subcloned again into M13 vectors and sequenced by the didideoxy method with α-35S dATP as label. (New England Nuclear)

**RESULTS**

**Rearrangement of Balb-c DNA**

Genetic rearrangement as indicated by restriction fragment length polymorphism is evidenced by the results of the Southern blot experiments shown in figures 3 and 4. In the case of the heavy chain for AN02, the DNA sequence of the variable region was known to share 97.6% sequence identity with a heavy chain Balb-c germline sequence named SB32 and with the variable region of a gene isolated from an IgM producing hybridoma LB8 published by Dzierzak et.al. (1986). The hybridization of fragments obtained by digestion with the enzymes EcoRI, HindIII, and XbaI shows a pattern consistent with what had been seen previously, namely hybridization to a 2.4 kb EcoRI fragment, an 7.5 kb HindIII fragment, and a 7.0 kb XbaI fragment. The AN02 genomic heavy chain gene is known to be contained on a 4.6 kb EcoRI and a 1.9 kb XbaI fragment (G. Rule unpublished data). These fragments are seen to hybridize as would be expected. Our assumption then was that a Balb-c germline variable region gene which was contained on 2.4 kb EcoRI and 7.0 kb XbaI fragments was rearranged by the processes of recombination and somatic mutation such that it rested on 4.6 kb EcoRI and 1.9 kb XbaI...
fragments in the AN02 genome. The results for digestion with HindIII are less clear, since the size of the restriction fragment which contains the AN02 genomic heavy chain gene is not known. The patterns seen in the double digestion with XbaI/HindIII and XbaI/KpnI, indicate that the single band in the XbaI lane results from more than one germline variable region.

In the case of the light chain a large number of fragments cross hybridize with the AN02 light chain probe indicating that the AN02 kappa variable gene comes from a large family of related germline genes. Most of the hybridizing bands seen in the Balb-c lanes are matched by equivalent bands in AN02. The major difference is that a 3.7 kb HindIII fragment hybridizes with approximately twice as much intensity in Balb-c as in AN02. This pattern would be expected if only one of the allelic light chain containing chromosomes were recombined during the differentiation of the AN02 producing B lymphocyte and if the rearranged germline variable gene were situated at the 3’ end of the family of cross reacting variable regions. Further evidence that the AN02 light chain germline is contained on a 3.7 kb HindIII fragment comes from performing the hybridization under more stringent conditions. Only the bands at 3.5, 3.7, and 3.9 kb appear, and the 3.7 kb band is the most intense.

Cloning and sequencing of germline genes

To determine whether the SB32 gene was indeed the germline gene for AN02 or if a gene with even more sequence identity exists, the 7.0 kb XbaI as well as the 7.5 kb HindIII fragments were ligated into lambda ZAP and lambda NM590 respectively. Multiple positive clones were purified and sequenced from both libraries and in each case a germline gene was obtained with greater than 99% sequence identity to the heavy chain variable region of the antibody AN07 and only 92% sequence identity to AN02. Since the antibody AN07 has a lambda type light chain, the complete germline configuration of AN07 could be deduced. The inability to find the SB32 germline gene or any other AN02-like gene in the XbaI or HindIII libraries upon sequencing 14 independent clones indicated that there may have been a systematic problem with the vectors or hosts used in the cloning process. To better understand the clonability of these variable genes, as well as to obtain partial sequence data for other germline
variable regions, a library was constructed which contained EcoRI/XbaI Balb-c DNA fragments of approximately 600 base pairs in length. Two AN02-like germline genes were sequenced from this library in the five clones which have been purified. One of these was the SB32 germline, the other was a gene that was only 87% similar to AN02.

For the light chain germline gene libraries were made in lambda NM590 with HindIII fragments of approximately 3.5, 3.7, and 3.9 kb. Positive clones were purified and sequenced in each case. Two light chain germline genes were found on the 3.5 kb fragments which were 94% similar to the AN02 light chain, one was found on the 3.9 kb fragment that was 96% similar, and one was found on the 3.7 kb fragment which had more than 98% sequence identity. This is assumed to be the germline gene for the light chain variable region of AN02.

Construction of antibody genes in the recombinant germline state.

In order to investigate the binding properties of the IgM class antibody which was found on the surface of the lymphocyte clone that eventually mutated into AN02 or AN07, it is necessary to first make genetic constructs which reflect the sequence of this germline antibody and then express these constructs in a suitable system. Taking the variable regions described above as the true germline variable genes, the remainder of the recombinant antibody genes can be found in the germline joining and diversity sequences which have been well characterized (Kabat et. al. 1987). For AN07 the diversity segment DFL16.1 and the joining segments JH3 and JL1 were used. For AN02 the diversity segment was too short to determine unambiguously while the joining segments JH3 and JK5 were used. The inferred protein sequence of the heavy and light chain cDNA clones for AN02 and AN07 together with that of the corresponding germline variable regions, diversity segments and joining regions recombinated in the proper reading frame are shown in figures 5 and 6. In each case more than 97% of the protein residues for each antibody can be assigned to their germline states. The ambiguities that exist occur at the junctions between the variable regions, joining, and diversity segments. At splice junctions it is impossible to determine which germline splice partner donated the germline sequence since mutation after recombination could
convert one sequence into the other. Compounding this problem in the heavy chains is the mechanism of nucleotide insertion which can place random nucleotides into the V-D and D-J junctions during recombination. However, given that the observed mutation rate in antibody genes during somatic diversification is approximately 0.5% (Gearhart et al. 1983), the small set of ambiguous nucleotides is highly likely to have been the same in the germline as in the mature antibody gene. In the worst case, AN02 with 11 ambiguous nucleotides, the probability is still approximately 95% that the inferred protein sequence for all of these codons correctly represent the germline.

**Future studies.**

Site specific mutagenesis has already been used to change the AN02 light chain cDNA clone into the germline gene sequence defined above. Progress is currently underway to construct the other germline genes from the cDNA clones by either site directed mutagenesis or where suitable restriction sites exist, by replacement with the germline DNA itself. These constructs will then be used in an expression system from which mutant protein can be obtained. Studies will then center on characterizing these proteins in terms of their binding affinities and kinetics for various DNP derivatives.

**DISCUSSION**

The results of the germline sequencing for AN07 indicate that very little somatic mutation occurred for this antibody. Only two residue changes exist between the known germline genes and the cDNA clones. One of these is removed from the complementary determining regions and is not considered to be a factor. The other occurs in CDR 1 of the light chain. Modelling studies suggest that this residue is removed from the binding pocket and will not be in contact with the bound hapten. Thus we expect the germline antibody and AN07 to have the same affinity for the DNP hapten.

The AN02 germline has a total of 11 residue differences from the sequence of AN02, however only 3 of these occur in the hypervariable loops. Although two of these occur in residues of the second hypervariable loop of the heavy
chain they are well removed from our model of the binding pocket and are unlikely to have any significant effect on the binding of the hapten. The residue change in CDR 2 of the light chain, Ser in the germline to Tyr in AN02 at position 31, can also be considered unimportant since NMR studies of tyrosines have implicated only one tyrosine, Tyr34L, as being in contact with the hapten (Rule et.al., in preparation). We believe that the antibody in its germline state will have a similar affinity and show similar kinetics to the mature antibody as far as the DNP hapten is concerned.

The plausible assumption that the germline antibodies bind DNP hapten leads us to the conclusion that the DNP specificities of these antibodies are essentially accidental since the germline genes could have only experienced evolutionary pressure in response to naturally occurring antigens. The question then arises as to what the primordial antigen might have been. For example, on the molecular scale, was it big or small? Our NMR studies of the antibodies AN01, AN02, and AN03 show a remarkably large concentration of aromatic residues, especially tyrosines, in the combining site region. In our most studied molecule, AN02, there are 8-10 tyrosine residues in this region (Anglister et. al., 1984), many more than could possibly all be in contact with the hapten. Further, as will be discussed elsewhere (Rule et. al., in preparation), the sharp proton resonance signals from these residues, with one notable exception, show almost no change on hapten binding, leading us to believe they play no functional role in the binding of DNP hapten. Kinetics studies of antibody-hapten binding also support the view that no significant change in protein structure takes place on hapten binding. We therefore suggest that the primordial antigen recognized by these germline genes was large, with a size of the order of a protein antigen. Studies of other antibody-protein complexes (Amit et. al., 1986; Sheriff et. al., 1987) typically show that the antibody recognition area is large, on the order of 700 square angstroms of interface. Furthermore, AN02 has been shown to be a cryoglobulin (Theriault et. al., in preparation), indicating a possible role for the binding site aromatic residues in idiotype-antiidiotype regulation, the idiotype here being self protein. These ideas will be developed in fuller detail elsewhere.
ACKNOWLEDGEMENTS

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REFERENCES


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Figure 1. Deduced amino acid sequences of the V regions of the light chains of the anti-DNP-SL monoclonal antibodies ANO1-AN12. Numbering system according to Kabat et al. (1987). From Leahy et al. (1988).
Figure 3. Southern blot results using the light chain probe. ANO2 hybridoma and Balb-c genomic DNA were digested with HindIII and EcoRI. Fragment sizes were internally referenced by including HindIII digested lambda DNA in the gel and as a radiolabelled probe. Shown are experiments in which the hybridizations were carried out in 50% and 35% formamide.
Figure 4. Southern blot results using the heavy chain probe. AN02 hybridoma and Balb-c genomic DNA were digested with the same enzymes. Fragment sizes were internally referenced by including HindIII digested lambda DNA in the gel and as a radiolabelled probe.