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Molecular basis of an isogeneic anti-idiotypic response

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The nucleotide sequences of the variable region genes expressed in the heavy and light chains of six isogeneic anti-idiotope antibodies recognizing idiotopes on two closely related antibodies with specificity for the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) were determined. In two independently derived anti-idiotope cell lines the same or strongly homologous V_χ, V_H and D region genes had originally been rearranged. The two lines express long and partly homologous N sequences (presumed to be not of germ line origin) at the border of D, resulting in CDR3s of unusual length. An unusually long CDR3, partly encoded by N sequences, is also present in the heavy chain of a third anti-idiotope antibody. The V_H regions of the three remaining anti-idiotope antibodies originate from a single V_H gene which belongs to the same V_H group as the V_H genes expressed in the other anti-idiotopes. Two of these antibodies, expressing similar V, D and J elements, had been isolated from the same mouse and appear to have diverged from the same B cell precursor by at least two rounds of somatic mutation. Somatic point mutations have occurred in most, if not all anti-idiotope V region sequences. In two instances somatic mutations in J increase the structural homology between anti-idiotopes. The anti-idiotypic response in this system is thus genetically restricted and may depend upon the selection of non-germ line sequences, suggesting an explanation for the low frequency at which anti-idiotope antibodies are expressed in this system.

Key words: V gene sequences/somatic diversification/antibody repertoire/anti-idiotypic antibodies/idiotypic network

Introduction

Complementary V regions of antibodies (idiotypes and anti-idiotypes) co-exist in the immune system and their interaction may play a role in the regulation of the antibody repertoire (Jerne, 1974). Experiments by Schuler *et al.* (1977) and Seppälä and Eichmann (1979) suggest that only a small number of anti-idiotypic B cell clones are activated in isogeneic anti-idiotypic responses of individual mice, although the potential anti-idiotypic repertoire appeared to be large. We decided to investigate the anti-idiotypic repertoire at the structural level using isogeneic monoclonal anti-idiotypic antibodies which had previously been isolated in our laboratory (Reth *et al.*, 1981; Rajewsky *et al.*, 1981; Wildner, 1982).

Results and Discussion

The experimental system

Most of the primary antibody response of C57BL/6 mice against the hapten (4-hydroxy-3-nitro-phenyl)acetyl (NP) is under the control of a single V_H (V_H186.2) and a single V_L ($V_{\lambda 1}$) gene, in combination with various D and J elements (reviewed by Rajewsky and Takemori, 1983). As schematically shown in Figure 1, two members of the antibody family controlled by these genes were selected for the production of monoclonal anti-idiotope antibodies, namely antibodies B1-8 and S43 (Reth *et al.*, 1978). Antibody B1-8 (IgM, λ1; derived from a primary anti-NP response) expresses the germ line V genes V_H186.2 and $V_{\lambda 1}$, whereas the V genes expressed in antibody S43 (IgG2a, λ1; derived from a hyperimmune response to NP) are somatic mutants of the same genes (Bothwell *et al.*, 1981, 1982). The monoclonal anti-idiotope antibodies A25.9.7 (IgG1, κ), A39.40.5 (IgG1, κ), A6/24 (IgG2a, κ) (Rajewsky *et al.*, 1981) and A31.90 (IgG2b, κ) bind to V region determinants (idiotopes) of the B1-8 antibody which are not present on antibody S43. In contrast, antibodies A20/44 and A8/4 (both IgG1, κ) bind to idiotopes which are expressed by the S43 but not the B1-8 antibody (Wildner, 1982) (Figure 1). The primary structure of the V regions of these anti-idiotope antibodies is the subject of this paper.

Two different approaches were chosen to determine the sequences of the heavy and light chain variable regions of anti-idiotope antibodies. In the case of the cell line A25.9.7 the *Eco*RI fragment containing the VDJ gene segment was cloned from a λgt WES phage library. The VDJ region was subcloned in M13mp701 and the V_H gene sequenced with a synthetic primer specific for the J_H gene segment. In all other cases sequences of the variable regions expressed in the anti-idiotope antibodies were obtained by directly sequencing poly(A)$^+$ RNA with specific oligonucleotide primers (see Materials and methods).

The results of the sequence determination, abbreviated in Figure 1 and discussed below, revealed a striking restriction of the anti-idiotypic response in that antibodies A25.9.7 and A31.90 on the one hand and A6/24, A20/44 and A8/4 on the other turned out to be structurally and genetically closely related.

The independently derived anti-idiotope cell lines A25.9.7 and A31.90 have selected the same germ line V_H and V_L genes and express them in somatically mutated form, together with the same D element and N sequences of identical length

A25.9.7 and A31.90 are two independently derived anti-idiotope cell lines. In Figure 2a the nucleotide sequences of the V_H genes expressed by these lines (V genes are given the same designation as the cell lines expressing them) are compared with each other and with the nucleotide sequence of the closely related germ line V_H gene MVAR11 (Blankenstein *et al.*, 1984). The latter gene was isolated from the genome of a hybridoma cell line [B1-8. δV1 (Brüggemann *et al.*, 1982)] which contains two IgH loci, one of the 'a' and one of the 'b' allotype (Sablitzky *et al.*, 1982). The MVAR11 gene belongs to the V_H gene group No. 1 in the classification of Dildrop (1984) and is characterized by its unusual length in that it carries eight nucleotides between codon 98 (AGA, coding for the amino acid arginine and usually at the 3′ end of V_H genes)

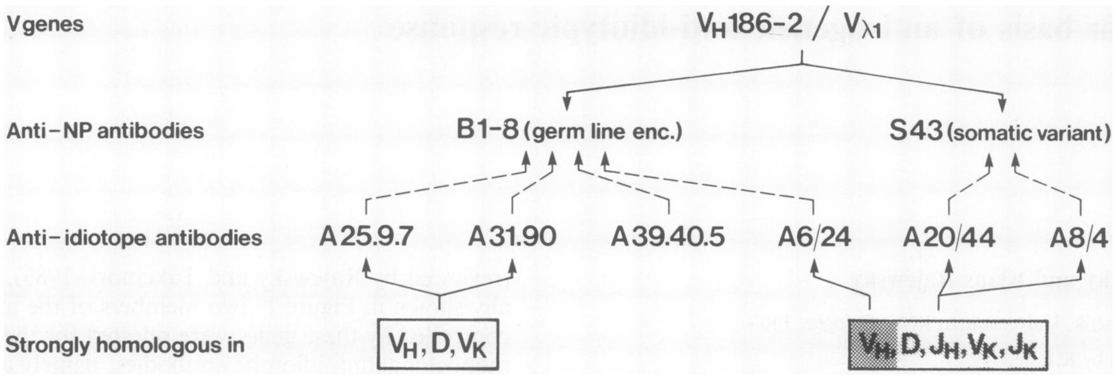

Fig. 1. Schematic drawing of the experimental system. Isogeneic anti-idiotope antibodies were raised against two monoclonal C57BL/6 antibodies with specificity for the hapten (4-hydroxy-3-nitro-phenyl)acetyl (NP), namely antibodies B1-8 and S43 (Reth *et al.*, 1978). The variable regions of antibody B1-8 (IgM, λ1) are encoded by the germ line genes V_H186.2 and $V_{\lambda 1}$, whereas the V genes expressed in antibody S43 (IgG2a, λ) are somatic mutants of the same genes (Bothwell *et al.*, 1981, 1982). The monoclonal anti-idiotope antibodies A25.9.7 (IgG1, κ), A31.90 (IgG2b, κ), A39.40.5 (IgG1, κ) and A6/24 (IgG2a, κ) bind to idiotopes of the B1-8 antibody. In contrast, anti-idiotope antibodies A20/44 and A8/4 (both IgG1, κ) bind to determinants of the S43 antibody. As shown and discussed here, antibodies A25.9.7 and A31.90 are strongly homologous in the V_H, D and V_κ gene segments. Similarly, the V_H genes expressed in antibodies A6/24, A20/44 and A8/4 are closely related. In addition, the latter two express strongly homologous V_κ genes and the same D, J_H and J_κ gene segments.

(a)

```
             1                   5                  10                  15                  20                  25                 30
MVAR11       .   .   .   L   Q   Q   S   G   A   G   L   V   K   P   G   A   S   V   K   L   S   C   K   A   S   G   Y   T   F   T
A25.9.7      Q   V   Q   -   -   -   -   -   -   E   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
A31.90       -   -   -   -   -   -   -   -   -   E   -   -   -   -   -   -   -   -   -   -   -   -   Q   -   -   -   -   -   -   S
MVAR11      ... ... ... CTG CAG CAG TCT GGA GCT GGG CTG GTG AAA CCC GGG GCA TCA GTG AAG CTG TCC TGC AAG GCT TCT GGC TAC ACC TTC ACT
A25.9.7     CAG GTC CAG --- --- --- --- --- --- -A- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
A31.90      --- --- --- --- --- --- --- --- --- -AA --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- --- --- --- --- -G-
```

```
              CDR1                                                                        CDR2
                             35                  40                  45                  50                  55                  60
MVAR11       E   Y   I   I   H   W   V   K   Q   R   S   G   Q   G   L   E   W   I   G   W   F   S   P   G   S   G   S   I   K   Y
A25.9.7      D   -   T   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   Y   -   -   -   -   -   -   -   -
A31.90       D   -   T   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   X   -   Y   -   -   -   D   -   -   R   -
MVAR11      GAG TAT ATT ATA CAC TGG GTA AAG CAG AGG TCT GGA CAG GGT CTT GAG TGG ATT GGG TGG TTT TCA CCT GGA AGT GGT AGT ATA AAG TAC
A25.9.7     --T --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -AC --- --- --- --- --- --- --- ---
A31.90      --C --- -C- --- --- --- --- --- --- --- --- --X --- --- --- --- --- --- --- -X- --- -AC --- --- --- -A- --- --- -GA ---
```

```
                             65                  70                  75                  80                  85                  90
MVAR11       N   E   K   F   K   D   K   A   T   L   T   A   D   K   S   S   S   T   V   Y   M   E   L   S   R   L   T   S   E   D
A25.9.7      -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
A31.90       -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   T   -   -   -   -   D   -   -   -   -   -   -   -   -
MVAR11      AAT GAG AAA TTC AAG GAC AAG GCC ACA TTG ACT GCG GAC AAA TCC TCC AGC ACA GTC TAT ATG GAG CTT ATG AGA TTG ACA TCT GAA GAC
A25.9.7     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- --G ---
A31.90      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- --- --- --- --- --C --- --- --- --- --- --- --- ---
```

```
                             95                 100
MVAR11       S   A   V   Y   F   C   A   R   H   E   D
A25.9.7      -   -   -   -   -   -   -   -   -   -   -
A31.90       -   -   -   -   -   -   -   -   -   A   E
MVAR11      TCT GCG GTC TAT TTC TGT GCA AGA CAC GAA GAC ACAGTG
A25.9.7     --- --- --- --- --- --- --- --- --- --- ---
A31.90      --- --- --- --- --- --- --- --- --- -CC --A
```

(b)

```
                                       CDR3
                 VH                DFl16.1                                            JH
                100                  105            110                  115                 120                 125
germ line    H   E   D   .   Y   Y   Y   G   S   S | .   . | Y   F | D | Y   W   G   Q   G   T   T   L   T   V   S   S
A25.9.7      -   -   -   V   -   -   -   -   -   T | L   V | X   - | - | -   -   -   X   -   -   -   -   -   -   -   -
A31.90       -   A   E   I   -   -   -   -   -   - | -   - | P   - | - | -   -   -   -   -   -   L   V   -   -   -   A
germ line                                                   W   - | A | -   -   -   -   -   -   L   V   -   -   -   A
germ line   CAC GAA GAC .TT TAT TAC TAC GGT AGT AGC|... ...|TAC TTT|GAC|TAC TGG GGC CAA GGC ACC ACT CTC ACA GTC TCC TCA G   #2
A25.9.7     --- --- --- G-A --- --- --- --- --- -C-|TTA GTC|CX- ---|---|--- --- --X XX- --- --- --- --- --- --- --- --- -   #2
A31.90      --- -CC --A A-- --- --- --- --- --- --T|C-T ---|CCG ---|--T|--- --- --T --- --G --T CTG G-- --T --- --T G-- -   #3
germ line                                                   TGG ---|-CT|--- --- --- --- --G --T CTG G-- --T --- --T G-- -   #3
```

Fig. 2. (a) The nucleotide and deduced amino acid sequences (one-letter code) of the V_H genes A25.9.7 and A31.90 are compared with a non-rearranged (germ line) V_H gene MVAR11 (Blankenstein *et al.*, 1984). Nucleotides and amino acids identical to the reference sequence of V_H MVAR11 are indicated by dashes. Ambiguities of the mRNA sequences are indicated by X. The putative joining signal of the V_H gene MVAR11 is underlined. CDR, complementarity-determining-region. **(b)** Sequence comparison of the CDR3 and J_H regions of antibodies A25.9.7 and A31.90 and the corresponding germ line gene segments V_H MVAR11, DFl16.1 (Kurosawa and Tonegawa, 1980) and J_H2 and 3 (Sakano *et al.*, 1980). Boxed sequences are of particular interest and are described in the text.

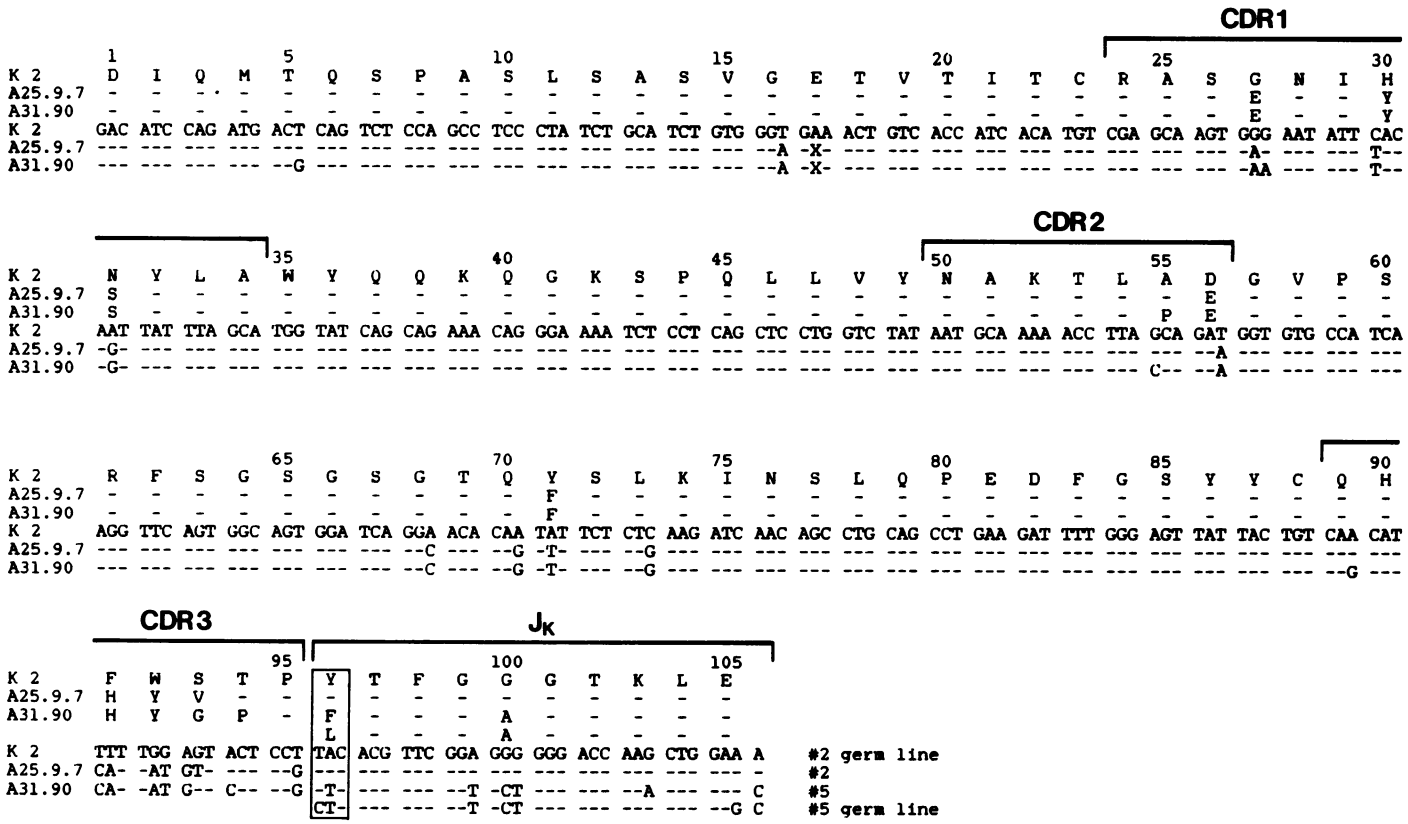

Fig. 3. Sequence comparison of the V_x and J_x gene segments expressed in antibodies A25.9.7 and A31.90 and the BALB/c derived germ line V_x gene K2 (Nishioka and Leder, 1980) and J_x gene segments 2 and 5 (Sakano *et al.*, 1979).

and the putative joining signal CACAGTG (underlined in Figure 2a).

The V_H genes expressed in antibodies A25.9.7 and A31.90 are strongly homologous to MVAR11, also at the 3′ end. V_H A25.9.7 differs from MVAR11 in seven nucleotides, resulting in amino acid substitutions at position 10, 31, 33 and 52. Four of these seven nucleotide differences and all four amino acid substitutions are also found in V_H A31.90, but the latter gene carries further substitutions distinguishing it in 16 positions (12 replacement substitutions) from V_H MVAR11 and in 13 positions (eight replacement substitutions) from V_H A25.9.7. We interpret these results to mean that the same germ line V_H gene had originally been rearranged in the precursor B cells from which the A25.9.7 and A31.90 cell lines are derived. This V_H gene is very similar to MVAR11, and, assigning the latter to the *a* haplotype, might represent its *b* allele. The gene might differ from MVAR11 in the positions in which both genes, V_H A25.9.7 and A31.90, differ from MVAR11, but some of these differences could also be due to somatic mutation in the two anti-idiotope antibodies (the latter would be plausible for the replacement substitutions in codons 31, 33 and 52 which are all located in hypervariable regions). Extensive somatic mutation is obvious in V_H gene A31.90 and has presumably generated most of the differences between this and the A25.9.7 gene. The latter interpretation is supported by the fact that all the amino acids by which the V_H region of A31.90 differs from that of A25.9.7, are not or only rarely found in the same position among the known sequences of group No. 1. Such unique amino acid substitutions are a typical result of somatic mutation (Dildrop, 1984).

The close structural homology between A25.9.7 and A31.90 extends also into those parts of the heavy chain V region which are encoded by D and J_H gene segments and by N sequences (Figure 2b). The latter sequences are thought to be generated somatically in connection with the process of VDJ gene rearrangement (Alt and Baltimore, 1982). Both cell lines express the same D gene segment, namely DFl16.1 (Kurosawa and Tonegawa, 1982). Only the spacer (N) sequences at the junction of D and J_H and D and V_H differ in the two antibodies. However, the same number of nucleotides are inserted at the 5′ end as well as at the 3′ end of the D coding region. In addition, structurally similar or even identical amino acids are encoded by the N sequences. In both antibodies the same nucleotide triplet encodes the amino acid valine at position 110. At position 109 different nucleotide triplets encode the same amino acid (leucine) (boxed in Figure 2b). In positions 102 and 108 the amino acids are structurally related (valine *versus* isoleucine and threonine *versus* serine). Because of the N sequences and the unusual length of the V_H gene the CDR3s of the A25.9.7 and A31.90 heavy chains comprise 16 amino acids each.

In the J_H region it is striking to see that somatic mutation has increased the structural homology between the two antibodies which express different J_H gene segments. The nucleotide sequence of the J_H3 gene segment expressed by the cell line A31.90 differs in two bases (positions 113 and 116) from its germ line counterpart, leading to the exchange of the amino acid alanine by the amino acid aspartic acid in position 113 (boxed in Figure 2b). The same amino acid is encoded in this position by the germ line J_H2 gene segment expressed in antibody A25.9.7.

Sequence comparison of the V_x genes expressed in antibodies A25.9.7 and A31.90 and the BALB/c derived germ line V_x gene K2 (Nishioka and Leder, 1980), shown in Figure

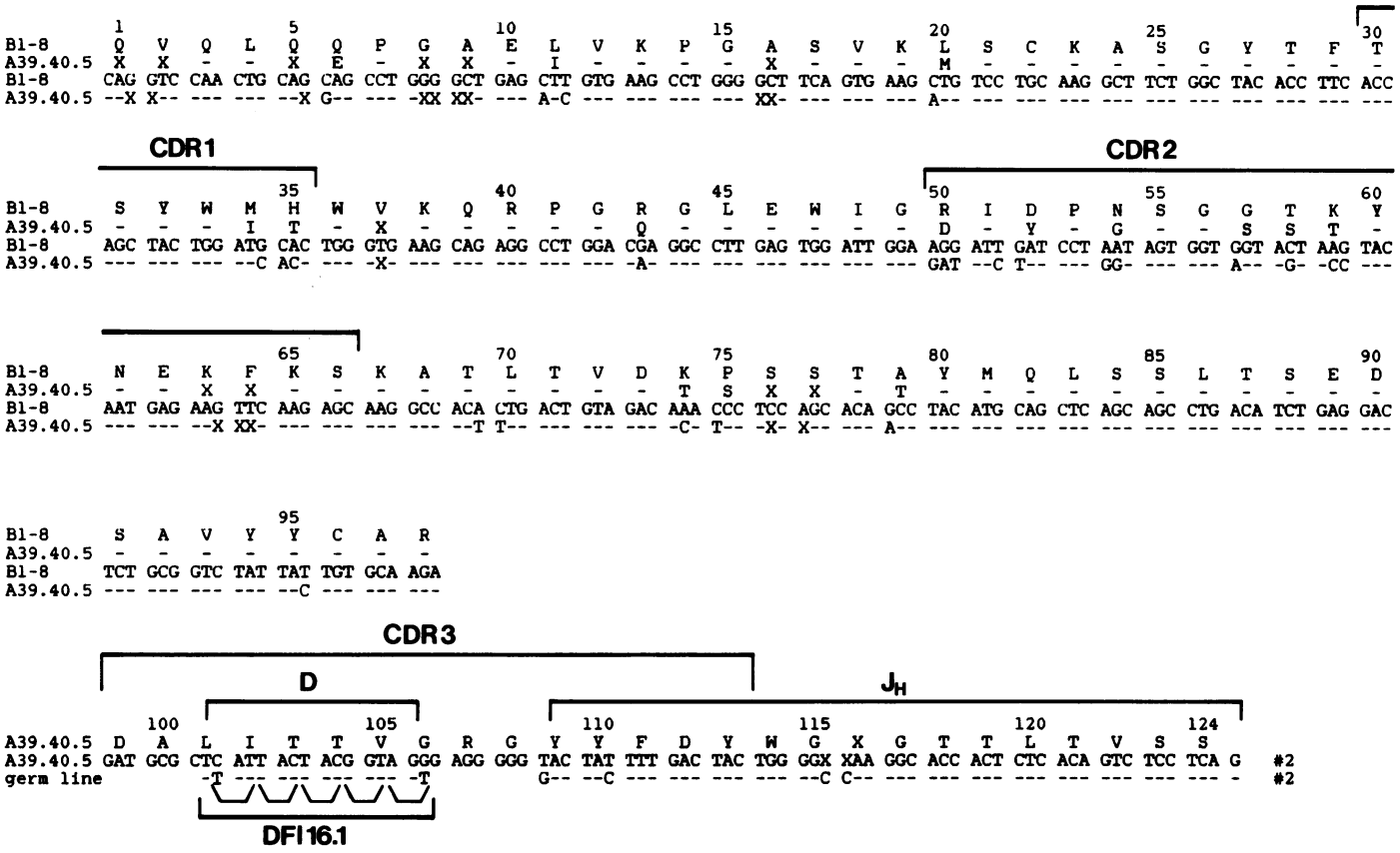

Fig. 4. Nucleotide and amino acid sequences of V_H, D and J_H expressed in antibody A39.40.5 are compared with the germ line V_H gene 186-2 expressed in antibody B1-8 and with the germ line gene segments DFl16.1 and J_H2. The reading frame of the DFl16.1 gene segment in antibody A39.40.5 differs from the usual one.

3, demonstrates the strong sequence homology of all three genes. The V_χ genes A25.9.7 and A31.90 have 15 nucleotide differences in common if compared with K2. Seven of these base exchanges result in amino acid replacements (positions 27, 30, 31, 56, 71, 91 and 92). Some of these differences could again represent allelic differences between a C57BL/6 and a BALB/c derived germ line V_χ gene. It seems obvious that as in the case of V_H, the same V_χ gene had originally been rearranged in the precursors of the A25.9.7 and A31.90 cell lines and that somatic mutation took place in the latter line, generating the six base differences between V_χ A25.9.7 and V_χ A31.90. These point mutations lead to amino acid replacements in positions 55, 93 and 94. Somatic point mutations have also affected the $J_\chi5$ gene segment expressed by the A31.90 line. Of the three mutations seen (Figure 3), two are silent (positions 103 and 105). The third, at the border of CDR3 (position 96), leads to the replacement of leucine by phenylalanine. Strikingly, a structurally related amino acid (tyrosine) is encoded at this position in the germ line encoded $J_\chi2$ gene segment expressed in antibody A25.9.7.

In summary, the two independently derived anti-idiotope antibodies A25.9.7 and A31.90 are structurally closely related. They express the same V_H, D and V_χ germ line genes (with or without somatic mutations) and similar N sequences which together with D generate a CDR3 of unusual length (16 amino acids). Somatic point mutations have occurred predominantly in the A31.90 cell line, affecting both heavy and light chain variable regions. Two such mutations increase the structural homology between the J_H and J_χ regions expressed by the two cell lines.

Antibody A39.40.5, like antibodies A25.9.7 and A31.90, expresses a CDR3 of unusual length

The V_H gene expressed in antibody A39.40.5 belongs to V_H group No. 1 following the classification of Dildrop (1984), like the V_H genes expressed in the two anti-idiotopes described above. This is shown in Figure 4, in which the sequence of V_H A39.40.5 is compared with the sequence of the prototype gene of that group, namely V_H186.2, expressed in antibody B1-8, the target of antibody A39.40.5. The CDR3 of the A39.40.5 heavy chain appears to be partly encoded by DFl16.1, although not in the usual reading frame, and extended by N sequences. Altogether, a CDR3 of 15 amino acids is generated in this fashion, almost as long as the CDR3s of antibodies A25.9.7 and A31.90. The 3′ end of the A39.40.5 VDJ gene is encoded by J_H2 with a silent mutation in codon 110 (Figure 4).

The A39.40.5 V_χ gene, expressed together with $J_\chi5$, is related to the $V_\chi0x1$ gene which encodes the light chain V region of certain antibodies with specificity for the hapten 2-phenyloxazolone (Kaartinen *et al.*, 1983) (Figure 5).

The V_H gene expressed by the anti-idiotope cell line A6/24 binding to antibody B1-8 is closely related to the V_H genes expressed in antibodies A20/44 and A8/4 which bind to antibody S43

In Figure 6 the amino acid sequences encoded by the V_H genes expressed by the anti-idiotope cell lines A20/44, A8/4 and A6/24 are compared with the amino acid sequence of the V_H region of the monoclonal antibody Ac38.205.12 (Dildrop *et al.*, 1984). The V_H region of the latter antibody is most

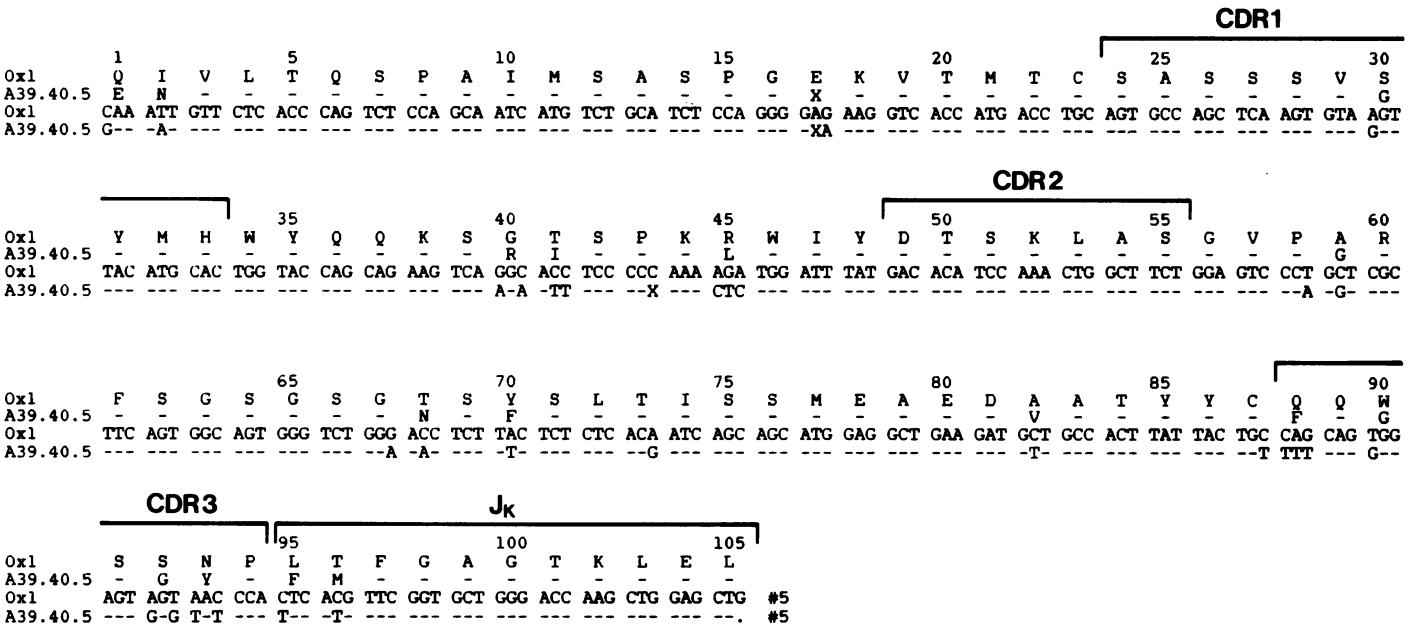

Fig. 5. Sequence comparison of the V_x and J_x5 gene segments expressed in antibody A39.40.5 and the V_x Ox1 gene expressed by certain hybridoma cell lines which produce antibodies against 2-phenyloxazolone (Kaartinen *et al.*, 1983) and to the germ line J_x5 gene segment.

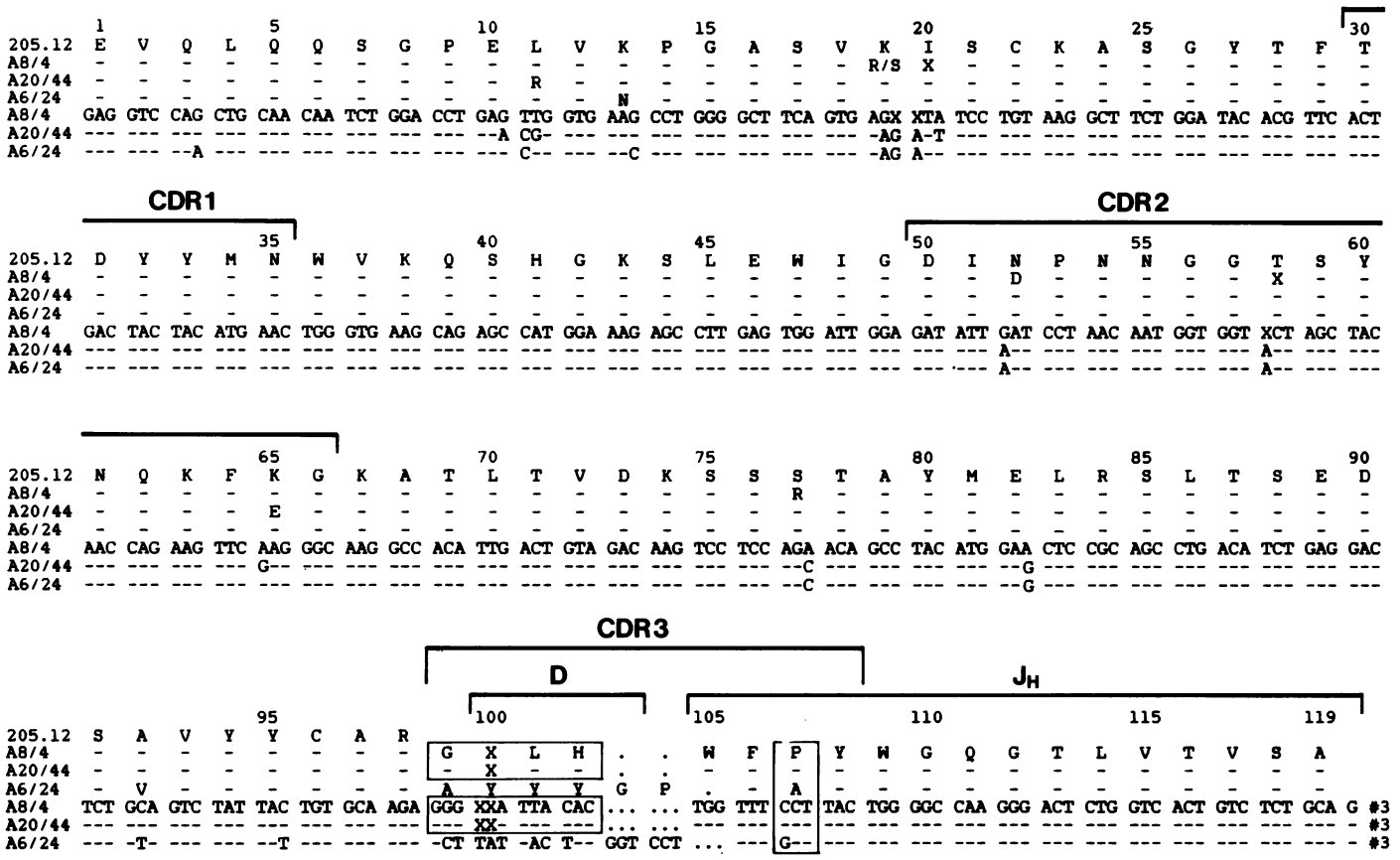

Fig. 6. Sequence comparison of the V_H, D and J_H gene segments expressed in antibodies A8/4, A20/44 and A6/24. In addition, the deduced amino acid sequences of the V_H genes are compared with the amino acid sequenecs of a V_H gene expressed in the hybridoma cell line Ac38.205.12 (designated as 205.12) (Dildrop *et al.*, 1984).

likely encoded by a germ line V_H gene and belongs again to the V_H group No. 1 (Dildrop *et al.*, 1984). The V_H gene A8/4 differs by three replacement substitutions (codons 19, 52 and 77) from the V_H gene expressed in antibody Ac38.205.12. Two replacement substitutions are found in the V_H genes expressed in antibodies A20/44 (codons 11 and 65) and A6/24 (codons 13 and 92). Most likely, the V_H genes expressed in the anti-idiotope antibodies originate from the same germ line

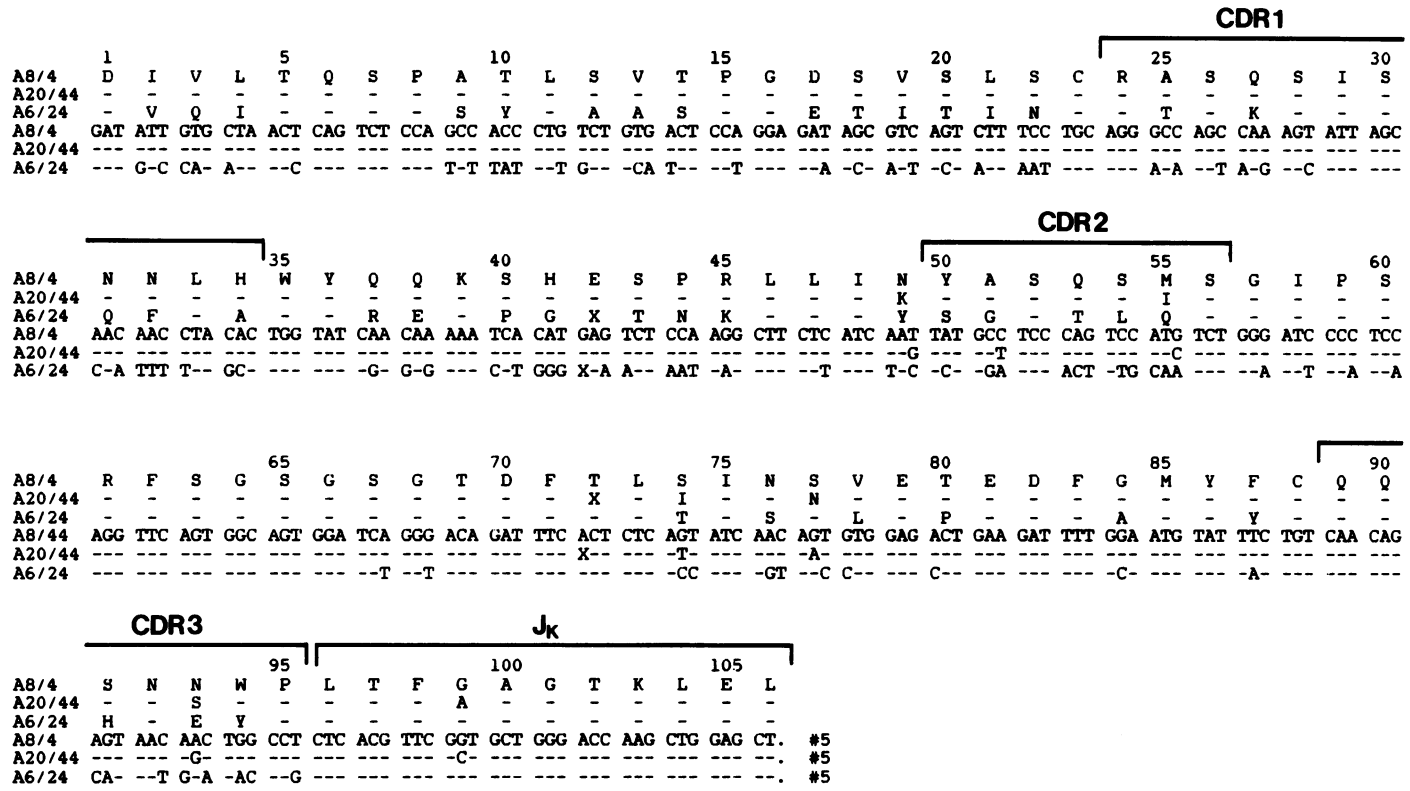

Fig. 7. Sequence comparison of the V_χ and J_χ gene segments expressed in anti-idiotope antibodies A8/4, A20/44 and A6/24.

V_H gene, namely the gene encoding the V_H region of antibody Ac38.205.12 and have diverged from it by somatic point mutations.

The different binding specificities of the anti-idiotope antibodies might be due to the fact that the D gene as well as the V_χ gene expressed by the cell line A6/24 differs from the ones expressed by the cell lines A20/44 and A8/4 (Figures 6 and 7).

The anti-idiotope cell lines A20/44 and A8/4 are derived from the same precursor B cell and carry somatic point mutations in V and J

The anti-idiotope cell lines A20/44 and A8/4 were generated from the spleen cells of a single C57BL/6 mouse (Wildner, 1982). As shown in Figures 6 and 7 the variable regions of heavy and light chains of both antibodies are encoded by strongly homologous gene segments. The nucleotide sequences of the V_H genes differ by only nine nucleotides, leading to five amino acid exchanges at positions 11, 19, 52, 65 and 77.

In both anti-idiotope antibodies the joining of the V_H, D and J_H gene segments generated identical nucleotide sequences coding for four amino acids of the D region (boxed in Figure 6), although at least five of 12 nucleotides are inserted in the joining process. Two nucleotides of codon 100 remained unidentified (X), but both mRNA sequences look identical on the autoradiogram (data not shown). If any, only a fragmentary D coding region is left (the nucleotides AT-TAC in positions 100, 101 and 102 could be derived from DFl16.1). However, the reading frame of the D coding region would be different from the usual one.

The J_H3 gene segments expressed by both anti-idiotope cell lines carry the same point mutation in codon 107, leading to an amino acid exchange from alanine to proline (boxed in Figure 6).

The sequences of the V_χ genes expressed by the two cell lines are shown in Figure 7. The two genes differ by only six nucleotides. Five of these nucleotide substitutions result in amino acid replacements (positions 49, 55, 74, 77 and 93).

A point mutation in the $J_\chi5$ gene segment expressed by the cell line A20/44 results in an amino acid exchange of the germ line encoded glycine by alanine in position 99 (Figure 7).

Southern blot analysis using a specific probe complementary to the J_H3-4 region demonstrated that both cell lines possess two distinct IgH loci which cannot be distinguished between the lines by the restriction enzymes *Eco*RI, *Bam*HI and *Bgl*II (data not shown).

The latter result, the expression of closely related V genes, identical nucleotide sequences of the D region and a common point mutation in the J_H3 gene segment strongly indicate that the two anti-idiotope cell lines are derived from a single precursor B cell whose progeny diverged by somatic mutation. The common exchange in J_H in position 107 suggests that somatic mutation in this B cell clone occurred in more than one step and generation, in line with the more extensive data of McKean *et al.* (1984) and Rudikoff *et al.* (1984).

Implications of the structural data for the anti-idiotypic response and network regulation

The most striking feature of the present data is the structural and genetic restriction of the isogeneic anti-idiotope response. The same germ line V_H gene appears to be rearranged in three of the six anti-idiotypic lines, even though one of them (A6/24) reacts with a different member of the idotypic antibody family than the two others (A20/44 and A8/4). These latter two were picked from a single mouse and found to belong to the progeny of a single B cell precursor. Since only a total of four anti-idiotypic hybridomas could be isolated from that animal (Wildner, 1982), its anti-idiotypic response

was presumably dominated by only a few B cell clones. Of the remaining three anti-idiotope cell lines which we have analyzed, two (A25.9.7 and A31.90) have in all likelihood independently rearranged the same V_H and V_χ genes which were subsequently somatically mutated. The antibodies synthesized by these two lines also express the same D element and carry structurally similar N sequences of unusual and identical length at the 3′ end of the D gene segment. Since the V_H gene expressed in the two antibodies is also of unusual length, they both carry a CDR3 of 16 amino acids in the heavy chain. This structural feature is preserved in the third antibody (A39.40.5) which possesses a CDR3 of 15 amino acids in the heavy chain, although it expresses different V_H and V_χ genes.

One of the rare cases in which such D regions have previously been found is the antibody Kol, a human cryoglobulin (Schmidt *et al.*, 1983). The CDR3 of the Kol heavy chain comprises 17 amino acids. The X-ray crystallographic analysis of this protein by Huber *et al.* (1976) has shown that its D element covers the cleft of the variable domain. It also interacts with the hinge region and the C terminus of the light chain of neighbouring molecules in the crystal. Our finding of D elements of similar length in three out of six anti-idiotope antibodies suggests that the D region-mediated reaction of the Kol protein with itself may be of importance as a model for interactions of antibodies with protein antigens including idiotypic interactions. Serological data indicate that even structurally very closely related anti-idiotopes such as A25.9.7 and A31.90 differ in their anti-idiotypic fine specificity (Brüggemann, 1984; M.Siekevitz, personal communication). If idiotypic interactions involve extended surface areas as in the case of the Kol protein, then this phenomenon might be due to differences of the molecular contacts between the interacting surface areas which might be the same for both anti-idiotopes.

The genetic and structural restriction of the anti-idiotypic response coincides with a low frequency in which anti-idiotope producing cells appear to occur in the mouse, at least in our system. Upon immunization of C57BL/6 mice with antibody B1-8 we have found five anti-idiotope producing hybridomas among >3000 hybrids (Rajewsky *et al.*, 1981), and the response of the mice to antibody S43 was also low (Wildner, 1982). Similarly, limiting dilution analysis of LPS-reactive B cells suggests a very low frequency ($<10^{-5}$) of B cells with specificity for antibody B1-8 (T.Takemori, unpublished data). Rare cells activated upon immunization may have to undergo substantial clonal expansion in order to become detectable. This could explain the fact that all our anti-idiotopes belong to the IgG class and that despite considerable efforts we have failed to generate anti-idiotope antibodies of the IgM class. Extensive clonal expansion would also explain the frequent occurrence of somatic point mutations in the anti-idiotope sequences. At least some of these mutations may contribute to an increased affinity to the target idiotope such as the point mutations in J_H and J_χ of antibody A31.90 (Figures 2 and 3). An obvious clustering of the point mutations in CDRs is not observed, however.

Is the restriction of the anti-idiotypic repertoire responsible for the low frequency of anti-idiotope producing cells and hence the small size of the isogeneic anti-idiotypic response? The vigorous restricted responses to haptens like NP, arsonate and others (reviewed by Rajewsky and Takemori, 1983) demonstrate that the restriction of a response to the expression of a few V and D elements does not necessarily also restrict its size. However, one could argue that the requirement of long CDR3s with selected N sequences in the heavy chain (antibodies A25.9.7, A31.90 and A39.40.5) reduces the frequency of precursor cells below a critical threshold. For antibodies A6/24, A20/44 and A8/4 other arguments of the same type would then have to be made, e.g., that in the V regions of these antibodies certain mutations are required in order to generate anti-idiotypic specificity. In essence, this interpretation implies that at least in this system idiotypic interactions only rarely occur at the level of the germ line encoded antibody repertoire (Rajewsky, 1983). Alternatively, the low isogeneic anti-idiotypic response could be due to active suppression by idiotype which is continuously generated in the mouse at the level of the germ line encoded antibody repertoire. There is some evidence in the literature for this type of suppression, although in the present system it is unlikely to occur because of the low concentration (1–100 ng/ml, depending on the idiotope) at which B1-8 and S43 idiotopes are found in normal C57BL/6 sera (for review, see Rajewsky and Takemori, 1983). Suppression might operate efficiently at the level of newly generated B cells, i.e., the germ line encoded repertoire, whereas anti-idiotypic cells, generated by somatic mutation and thus perhaps representing mature B cells having arisen in immune responses which the animal has undergone in its past, might escape it. Experiments are underway to find out whether in mice idiotypically suppressed for B1-8 idiotopes anti-idiotypic responses can be seen which are not seen in normal mice.

Be this as it may, our results seem to limit the role of the idiotypic network in the generation of the antibody repertoire, assuming of course that they can be extrapolated to other specificities. They argue that within the antibody repertoire either idiotypic self-reactivity is largely eliminated through suppression or, alternatively, that idiotypic complementarity is highly restricted genetically and depends perhaps to a large extent on the selection of non-germ line sequences. It should be kept in mind, however, that the idiotypic network may play its main role in the interplay of B cells with T lymphocytes, an issue not addressed by the present experiments.

Materials and methods

Anti-idiotope cell lines and antibodies

The isolation of the hybridoma lines A25.9.7, A39.40.5, A6/24, A8/4 and A20/44 secreting monoclonal anti-idiotope antibodies and the characterization of the latter antibodies has been described previously (Reth *et al.*, 1981; Rajewsky *et al.*, 1981; Wildner, 1982; see also Beyreuther *et al.*, 1983). All cell lines were established by fusing X63.Ag8.653 myeloma cells (Kearney *et al.*, 1979) with spleen cells from C57BL/6 mice immunized with the monoclonal anti-NP antibodies B1-8 or S43 (Reth *et al.*, 1978), cross-linked with keyhole limpet hemocyanin. The A31.90 line was isolated as described by Rajewsky *et al.* (1981). It secretes a χ chain bearing IgG2b with specificity for antibody B1-8. The reaction of anti-idiotope antibody with the target antibody can in some cases (A39.40.5 and A20/44) be specifically inhibited by the hapten NP-caproic acid and in all cases by NP-bovine serum albumin conjugates.

λ phage library

*Eco*RI-digested DNA of the anti-idiotope cell line A25.9.7 was ligated to phage arms of λgt WES and packed *in vitro* (Leder *et al.*, 1977; Hohn and Murray, 1977). Recombinant phages positive for a 3′ J_H probe (a 0.8-kb *Xba*I-*Eco*RI fragment, which is derived from the 3′ end of the *Eco*RI fragment carrying the J_H locus) were isolated and *Bam*HI-*Eco*RI fragments subcloned into M13mp701 (gift of D.Bentley, Oxford).

M13 sequencing

Sequencing was done as described (Sanger *et al.*, 1980) except that oligonucleotide primers specific for J_H2-4 (see below) were used.

Isolation of poly(A)$^+$ RNA

Total RNA of the anti-idiotope cell lines was isolated as described by Siekevitz

et al. (1983) and poly(A)$^+$ RNA was enriched by passing the total RNA over an oligo(dT) column (Edmonds *et al.*, 1971).

mRNA sequencing

Poly(A)$^+$ RNA was directly sequenced by using synthetic oligonucleotide primers which are specific for certain regions of the mRNA to initiate reverse transcriptase. The cDNA synthesis was specifically stopped by adding dideoxynucleotides and the nucleotide sequence determined (Hamlyn *et al.*, 1978). In order to get rid of most of the ambiguities, sequence reactions were done with [^{32}P]ATP and in a second experiment with [^{32}P]CTP.

Synthetic oligonucleotide primers

The oligonucleotide primers were synthesized with an Applied Biosystems 380 A DNA Synthesizer kindly provided by K.Otto and B.Müller-Hill. The oligonucleotide primers were separated from incomplete synthesis products by polyacrylamide gel electrophoresis and purified as described by Maxam and Gilbert (1977). The Cγ-primer (5′ GGCCAGTGGATAGAC 3′) hybridizes to the 5′ end of all Cγ constant region genes (Kaartinen *et al.*, 1983). The J_H1-primer (5′ CCTG$^{T}_{C}$GCCCCAGAC 3′) hybridizes specifically to the J_H1 gene segment at positions 7–11. The J_H2-4-primer (5′ CCTTG$^{A}_{G}$CCCCAGTA 3′) hybridizes J_H gene segments Nos. 2–4. The V_{NP}-primer (5′ CCAATCCACTCAAG 3′) hybridizes to most of the V_H genes of group 1 (Dildrop, 1984) at positions 45–49. The C_x-primer (5′ TGCAGCATCAGCCC 3′) is specific for the 5′ end of the C_x constant region gene (Max *et al.*, 1981). The V_x-primer (5′ CCTGATCCACTGCCAC 3′) hybridizes at positions 63–68 to the V_x genes expressed in the anti-idiotope antibodies.

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