

SINGLE GERMLINE  $V_H$  AND  $V_K$  GENES ENCODE  
PREDOMINATING ANTIBODY VARIABLE REGIONS  
ELICITED IN STRAIN A MICE BY IMMUNIZATION  
WITH *p*-AZOPHENYLARSONATE

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Specific selection of B cells for participation in the immune response appears to operate through noncovalent chemical binding of ligands to V regions of surface receptor antibodies (1, 2). Thus, our understanding of cellular selection processes that transpire during the acquisition of humoral immunity has been facilitated by structural and functional analyses of antigen-specific mAbs that are derived from immunized mice (3); V regions of such antibodies are presumably identical to those of surface receptor antibodies of B cells participating in the immune response.

To understand how germline and somatic sources of antibody V region diversity contribute to functional immunity, we have pursued structural and functional analyses of mAbs that are elicited in strain A mice by immunization with protein conjugates of Ars and that bear determinants of a major idiotype, termed the strain A major crossreactive idiotype ( $Id^{CR}$ )<sup>1</sup> (4). Approximately half of the elicited population of strain A antibodies specific for Ars are  $Id^{CR+}$  (4). Sequences of monoclonal  $Id^{CR+}$  variable regions (5–9) have provided structural information that embodies both germline and somatic forms of antibody diversity (10–26). To identify separately each of these forms of diversity and their contribution to the functional repertoire, we have found it necessary to identify and sequence the corresponding germline V gene segments that encode these  $Id^{CR+}$  V regions.

Previously we reported (27) that a defined combination of germline  $V_H$  D and  $J_H$  gene segments is used for the somatic construction of the heavy chain V genes encoding most  $Id^{CR+}$  V regions that are elicited during the secondary immune response. These structures have limited diversity at the heavy chain V segment

This work was supported by grant NP-6N from the American Cancer Society to M. L. Gelter; grants AI-13357 and CA-28900 from the National Institutes of Health to M. L. Gelter; and by a fellowship from the American Cancer Society (PF-2200) to L. J. Wysocki. T. Gridley's present address is The Whitehead Institute, Nine Cambridge Center, Cambridge, MA 02142. A. Grande's present address is New England Biolabs, 32 Tozer Rd., Beverly, MA 01915. Address correspondence to Lawrence J. Wysocki, Dept. of Biology, 56-708, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139.

<sup>1</sup> *Abbreviation used in this paper:*  $Id^{CR}$ , strain A major crossreactive idiotype.

junctions as well, but are extensively mutated. Here we report that the expressed light chain V genes encoding most secondary Id<sup>CR+</sup> V regions are also derived from single germline V<sub>κ</sub> and J<sub>κ</sub> gene segments, lack junctional diversity, but are diversified by somatic mutation. Thus, the major population of V regions elicited during the strain A secondary immune response to Ars is encoded by one or two combinations of V<sub>H</sub>, D, J<sub>H</sub>, V<sub>κ</sub>, and J<sub>κ</sub> gene segments, exhibits limited junctional diversity, and is extensively diversified by somatic mutational alteration of both chains.

### Materials and Methods

*Hybridoma Cell Lines.* The generation and selection of hybridomas that were used in these analyses have been described (25, 27).

*Nucleic Acid Hybridization Analyses.* A V<sub>κ</sub>10 probe comprising 276 bp of the coding sequence and 630 bp of 5' flanking sequence was provided by Drs. Marjorie Shapiro and Martin Weigert, Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA. Two additional probes were derived from a cloned DNA fragment containing the 36–65 V<sub>κ</sub> gene (see Fig. 1): a 115-bp flanking probe, located 1.1 kb 5' of the V<sub>κ</sub> coding region, and a 530-bp probe that includes 144 bp of the 5' coding region of the 36–65 V<sub>κ</sub> gene. Southern hybridization analyses were carried out using nick-translated probes as reported (28), with the modifications that hybridization temperatures were reduced to 45°C and formamide washings were performed at 53°C. Autoradiography was performed by exposing Kodak XAR-5 film for 2 d (530-bp probe and V<sub>κ</sub>10 probe) or for 8 d (115-bp probe) to filters at -70°C with the use of a Kronex Lightning-Plus intensifying screen (DuPont Co., Wilmington, DE).

*Cloning and Sequencing Analysis of V<sub>κ</sub> 36–65.* DNA from 36–65 was digested with Bam HI (New England Biolabs, Beverly, MA), and fragments in the range of 9–12 kb were isolated by electroelution and purified on an elutip-D column (Schleicher & Schuell, Keene, NH). The size-selected DNA was cloned in phage λ EMBL3A (29). *E. coli* NM539 (29) was infected with unamplified recombinant phage and 2 × 10<sup>5</sup> phage plaques were screened by hybridization analysis with the V<sub>κ</sub>10 probe. A 9.8-kb DNA insert from one of eight positive phage isolates was subcloned into pBR322 for restriction site mapping and sequencing analyses. Two Sau 3A fragments, each starting at position 144 within the V<sub>κ</sub> gene, one extending 530 bp in the 5' direction and the other 630 bp in the 3' direction, were subcloned for sequencing analysis into the Bam HI site of plasmid pGEM-4 (Promega Biotech, Madison, WI). Both DNA strands covering the V<sub>κ</sub> coding portion of each subclone were sequenced, starting near the shared Sau 3A end by using the dideoxynucleotide, chain-termination method for one strand (30) and the chemical degradation method for the opposite strand (31).

*Cloning and Sequencing Analysis of the V<sub>κ</sub>Id<sup>CR</sup> Germline Gene.* A/J-strain kidney DNA was digested with Bgl II, phosphatase treated, and fragments in the range of 10–16 kb were isolated, purified, and cloned in phage λ EMBL3A as described above for 36–65. 3 × 10<sup>5</sup> phage plaques, grown directly from unamplified recombinant phage, were screened by hybridization with the 115-bp flanking probe. From 24 isolates that hybridized with this probe under stringent conditions, 12 were chosen for restriction site mapping. DNA from each isolate was cleaved with Sal I (which excises the cloned insert) and additionally with one of 6 restriction endonucleases (New England Biolabs), all of which were specific for hexamer sequences and cleaved infrequently the EMBL3A vector DNA. Fragment digestion patterns from each isolate were compared with one another and with the digestion pattern of purified EMBL3A left and right arms. Four of the phage clones were chosen for further mapping and sequencing analyses. From each isolate, two restriction fragments were subcloned into pGEM-4: a 1-kb Kpn I–Sac I fragment that included the entire V<sub>κ</sub> coding region and in which the Kpn I site was located just 6 bp 5' of the coding region, and a 600-bp Sau 3A fragment originating 144 bp into the V<sub>κ</sub> coding region and extending in the 3' direction. Sequence analyses were initiated at or

near the Kpn I and internal Sau 3A sites by using the dideoxynucleotide, chain-termination method for one strand (30) and the chemical degradation method for the opposite strand (31).

## Results

*Strategy for Identifying the Germline Origin(s) of Expressed  $V_{\kappa}$  Genes.* To determine the germline origin(s) of expressed  $V_{\kappa}$  genes encoding  $\text{Id}^{\text{CR}+}$  antibodies, we followed a protocol analogous to the one used by Siekevitz et al. (25) for the evaluation of expressed  $V_{\text{H}}$  genes encoding these antibodies. An expressed  $V_{\kappa}$  light chain gene from one hybridoma making an  $\text{Id}^{\text{CR}+}$  antibody was cloned. From the cloned  $V_{\kappa}$  gene a probe was isolated that hybridized under stringent conditions with the expressed  $V_{\kappa}$  genes encoding other  $\text{Id}^{\text{CR}+}$  antibodies, and that also produced only a single band upon Southern blot analysis of digested germline DNA. The germline fragment(s) thus identified were cloned and multiple cloned isolates were subjected to restriction site mapping and sequencing analyses to determine the number of different germline  $V_{\kappa}$  gene segments hybridizing with the  $V_{\kappa}$  probe.

*Cloning of an Expressed  $V_{\kappa}\text{Id}^{\text{CR}}$  Gene.* DNAs from  $\text{Id}^{\text{CR}+}$  antibody-producing hybridomas that are generated during the secondary immune response share a common 9.8-kb Bam HI restriction fragment that hybridizes with a  $J_{\kappa}$  probe and that is absent from similarly digested germline DNA (27). Other unshared hybridizing fragments of varying length are also seen. The V region of the light chain polypeptide made by one of these hybridomas, 36–65, has been sequenced (Margolies, M., Massachusetts General Hospital, Boston, MA, personal communication) and was found to be ~93% homologous to the deduced sequence of a  $V_{\kappa}10$  gene that was previously cloned from the DNA of a BALB/c hybridoma (Shapiro, M., Fox Chase Cancer Center, personal communication). This high degree of amino acid sequence homology indicated that nucleic acid sequence homology might be sufficient for detection of the 36–65  $V_{\kappa}$  gene by hybridization with a  $V_{\kappa}10$  probe. With this in mind, we performed Southern hybridization analyses with a  $V_{\kappa}10$  probe (see Materials and Methods) to identify the restriction fragment containing the productively rearranged  $V_{\kappa}$  gene in 36–65. We found that DNAs from 36–65 and from nine other  $\text{Id}^{\text{CR}+}$  antibody-producing hybridomas, derived by fusions performed during the secondary immune response, shared a rearranged 9.8-kb Bam HI fragment that hybridized with the  $V_{\kappa}10$  probe (27). This result strongly suggested that all of the hybridomas in this panel expressed  $V_{\kappa}$  genes that were located on 9.8-kb Bam HI fragments of DNA.

We cloned this somatically rearranged 9.8-kb Bam HI fragment of DNA from 36–65 into phage  $\lambda$ , mapped the cloned fragment, and sequenced the  $V_{\kappa}$  gene. The deduced amino acid sequence of the  $V_{\kappa}$  gene was identical to that of the 36–65 light chain polypeptide V region (Margolies, M., personal communication), including the  $J_{\kappa}$  region. We conclude that the cloned, rearranged  $V_{\kappa}$  gene encodes the 36–65  $V_{\text{L}}$  region.

*Identification of a Germline Restriction Fragment Containing the  $V_{\kappa}$  Gene that Encodes  $\text{Id}^{\text{CR}+}$  mAb  $V_{\text{L}}$  Regions.* To determine whether the other  $\text{Id}^{\text{CR}+}$  antibody-producing hybridomas in our panel were expressing the same  $V_{\kappa}$  gene as 36–65,

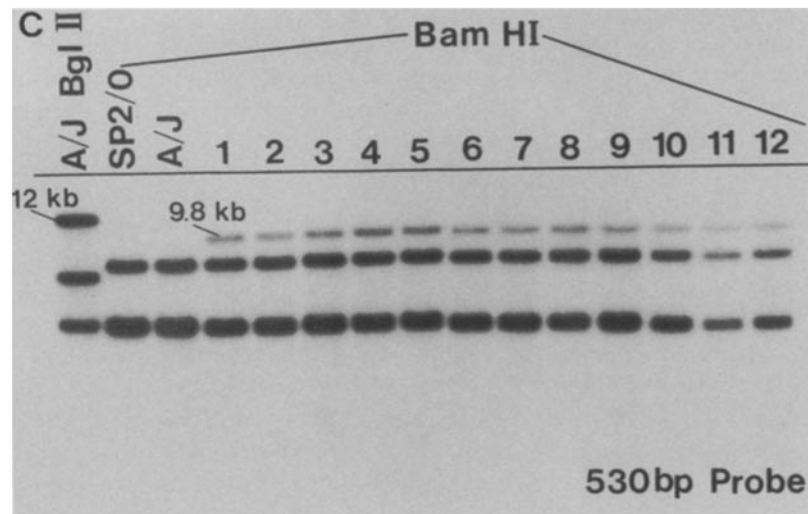
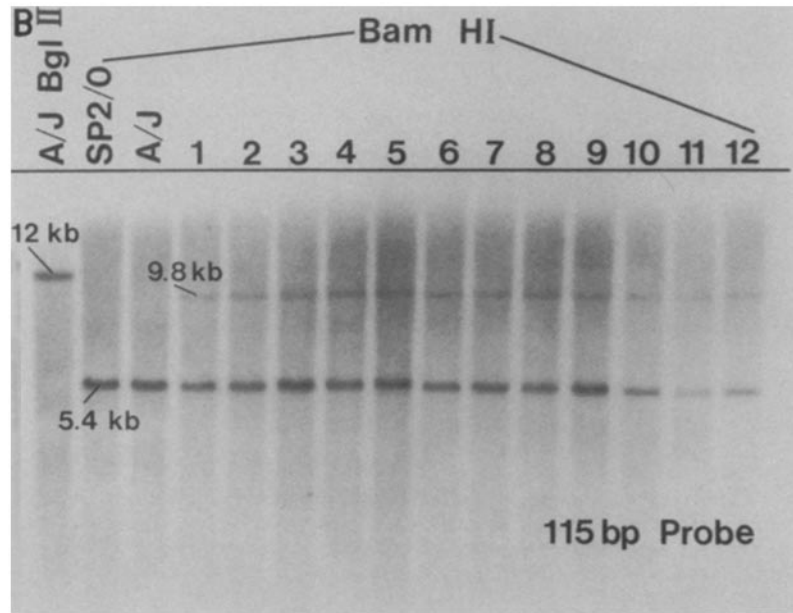
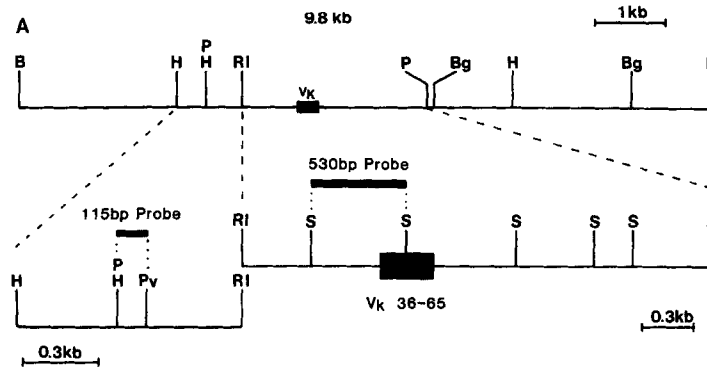
we isolated probes for hybridization analyses from the cloned fragment containing the 36–65  $V_{\kappa}$  gene. One probe, 115 bp in length and located 1.1 kb upstream of the  $V_{\kappa}$  coding sequence (see Fig. 1), proved to be particularly useful because it produced a single band when used in Southern hybridization analyses of germline DNA. The DNAs of 12  $\text{Id}^{\text{CR}+}$  antibody-producing hybridomas that shared the 9.8-kb Bam HI fragment (hybridizing with  $J_{\kappa}$  and  $V_{\kappa}10$ ) were selected for hybridization analyses with the 5'-flanking 115-bp probe. Under stringent conditions of hybridization with this probe (see Materials and Methods), all of the Bam HI-cut DNAs from these lines revealed two bands corresponding to DNA fragments of 9.8 kb and 5.4 kb in length. Germline DNA treated in the same way revealed a single 5.4-kb hybridizing fragment. Cleavage of germline DNA with Eco RI or Bgl II restriction endonucleases also generated single fragments (4.8 kb and 12 kb, respectively) that hybridized with the 5' flanking probe, under conditions in which all of the selected hybridoma DNAs gave a single rearranged, hybridizing fragment. Finally, 9.8-kb fragments, derived from the hybridoma DNAs by cleavage with Bam HI, also hybridized with a 530-bp probe that included the 5' 144 nucleotides of the 36–65  $V_{\kappa}$  coding sequence. This supported our assumption that the rearranged fragments contain the  $V_{\kappa}$  gene itself. These data suggest that the hybridomas sharing the somatically rearranged 9.8-kb Bam HI fragment of DNA are expressing a single germline  $V_{\kappa}$  gene segment.

More recently, we have analyzed the DNAs derived from 23 additional  $\text{Id}^{\text{CR}+}$  antibody-secreting lines, sharing the 9.8-kb rearranged fragment of DNA that hybridizes with a  $J_{\kappa}$  probe. DNAs from each of these cell lines also gave rise to a 9.8-kb Bam HI fragment that hybridized with the 115-bp flanking probe under conditions of high stringency.

*A Single Germline  $V_{\kappa}$  Gene Accounts for the Majority of Expressed  $V_{\kappa}$  Genes Encoding  $\text{Id}^{\text{CR}+}$  Antibodies.* We cloned 12-kb Bgl II restriction fragments of germline DNA in phage  $\lambda$ , and isolated recombinant phage on the basis of hybridization with the  $V_{\kappa}$  probes described above. DNA inserts from 12 independent recombinant phage were mapped with 6 different restriction endonucleases (Bam HI, Hind III, Eco RI, Nco I, Kpn I, Sac I). All 12 mapped identically, strongly suggesting that we had cloned a single DNA fragment with one  $V_{\kappa}$  gene in multiple phage isolates. Portions of DNA inserts from four of these recombinant phage were subcloned and both DNA strands of the  $V_{\kappa}$  coding regions were sequenced (Fig. 2). Each was identical in sequence and identical to the sequence of the expressed 36–65  $V_{\kappa}$  gene. On the basis of these combined molecular analyses, we conclude that expressed  $V_{\kappa}\text{Id}^{\text{CR}}$  genes, located on 9.8-kb Bam HI

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FIGURE 1. Hybridization analyses of DNAs derived from hybridomas making  $\text{Id}^{\text{CR}+}$  antibodies. (A) Restriction endonuclease map of a cloned 9.8-kb Bam HI fragment of DNA containing the expressed  $V_{\kappa}$  gene of 36–65. Probes used for hybridization analyses in B and C are shown. Abbreviations for restriction enzymes are: B, Bam HI; H, Hind III; P, Pst I; RI, Eco RI; Bg, Bgl II. (B) DNAs in lanes 1–4, 9, and 10 were derived from hybridomas P65D6-3, P65C6-2, P65I4-2, P65D6-2, P65J4-1, and P65L9-2, respectively, which were generated by cell fusions performed during the primary immune response (27). DNAs in lanes 5–8, 11, and 12 were derived from hybridomas hVH65-213, hVH65-207, hVH65-210, hVH65-217, hVH65-208, and hVH65-211, respectively, which were obtained by fusions performed during the secondary immune response (27).



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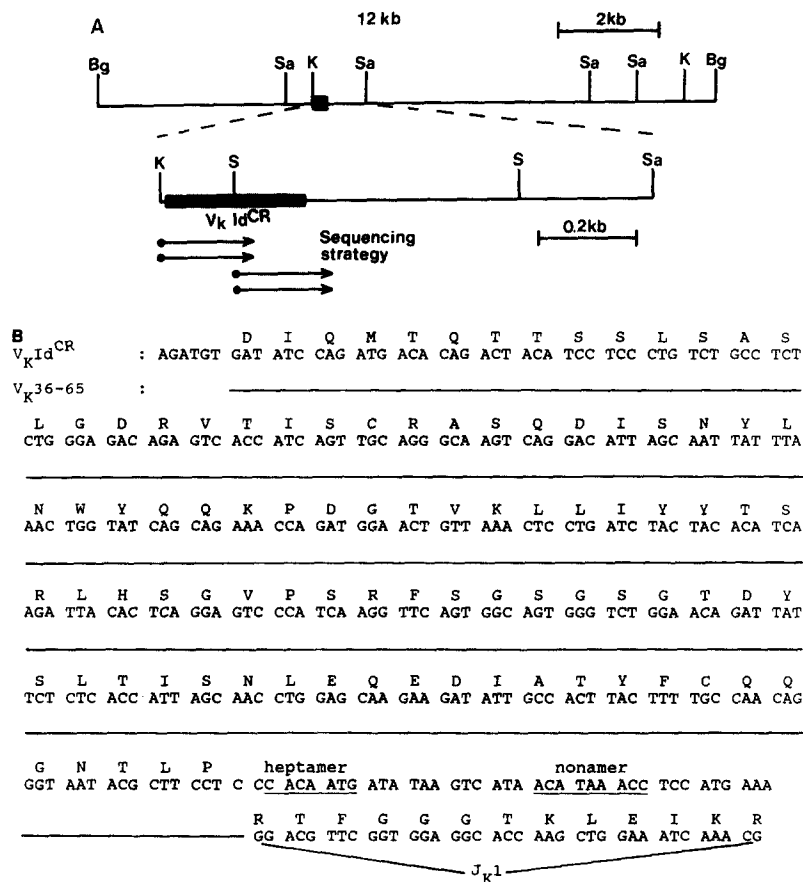


FIGURE 2. Restriction endonuclease map of a 12-kb Bgl II fragment of germline DNA containing  $V_{\kappa}Id^{CR}$  and sequence identity between  $V_{\kappa}Id^{CR}$  and the expressed  $V_{\kappa}$  of 36-65. (A) Abbreviations for restriction enzymes are: Bg, Bgl II; Sa, Sac I; K, Kpn I; S, Sau 3A. (B) Top  $V_{\kappa}$  sequence was obtained from four independent isolates of the 12-kb fragment: clones 1, 2, 10, 15. Below is the sequence of the expressed 36-65  $V_{\kappa}$  gene, where a line indicates identity with the top (germline  $V_{\kappa}Id^{CR}$ ) sequence.

DNA fragments, are derived from a single germline gene segment,  $V_{\kappa}Id^{CR}$ . Uniformity in the lengths of the somatically rearranged fragments containing the expressed  $V_{\kappa}Id^{CR}$  genes of different hybridomas also indicates the predominant use of a single  $J_{\kappa}$  ( $J_{\kappa}1$ ) gene segment.

### Discussion

To evaluate germline and somatic contributions to functional antibody diversity, we have cloned and sequenced the germline  $V_{\kappa}$  gene from which the majority of expressed  $V_{\kappa}$  genes encoding  $Id^{CR+}$  anti-Ars antibodies are derived. Previous results (25) have shown that all V regions of  $Id^{CR+}$  mAbs are apparently encoded by a single  $V_H$  gene segment,  $V_HId^{CR}$  (25). Past results have also revealed that nearly all of the  $Id^{CR+}$  antibody-producing hybridomas derived during the secondary immune response and ~60-70% of those derived during the primary

immune response share a somatically rearranged, 9.8-kb Bam HI fragment of DNA that hybridizes with  $V_{\kappa}10$  and  $J_{\kappa}$  probes (27). The results described here demonstrate that the presence of this fragment reflects the expression of single germline  $V_{\kappa}$  ( $V_{\kappa}Id^{CR}$ ) and  $J_{\kappa}$  ( $J_{\kappa}1$ ) gene segments. This conclusion is drawn from results of Southern blotting, restriction site mapping, and sequencing analyses; Southern blotting analysis of germline DNA, digested with Bam HI, Bgl II, and Eco RI and hybridized with a  $V_{\kappa}$  probe derived from an expressed  $V_{\kappa}Id^{CR}$  gene, produced a single band with each enzyme; 12 independently cloned fragments of germline DNA, each containing a  $V_{\kappa}$  gene that was a candidate for the origin of expressed  $V_{\kappa}Id^{CR}$  genes, produced identical restriction maps after digestion with six different restriction enzymes; four of the germline  $V_{\kappa}$  candidates isolated on independently cloned germline fragments of DNA were identical in sequence. It therefore appears that during the primary immune response, ~60–70% of the participating B cells, synthesizing  $Id^{CR+}$  antibodies, are synthesizing antibodies encoded by a single  $V_H, V_{\kappa}, J_{\kappa}$  combination of gene segments, whereas during the secondary immune response this frequency approaches 100%. This result is supported by serological analyses as well (32). D and  $J_H$  gene-segment usage is also restricted in  $Id^{CR+}$  antibodies elicited during the secondary immune response (27, 33), leading to the conclusion that the majority of these V regions are encoded by one or two combinations of five V gene segments ( $V_H Id^{CR}$ ,  $D_{F116}$ ,  $J_H 2$ ,  $V_{\kappa} Id^{CR}$ ,  $J_{\kappa} 1$ ) (27). The possibility that two D gene segments are used that differ by a single silent nucleotide change has not been formally disproved.

The results reported here, together with those of amino acid sequencing analyses, reveal that light chain junctional and combinational diversities are restricted in  $V_{\kappa}Id^{CR}$ -encoded antibodies; in all cases examined (19/19),  $V_{\kappa}Id^{CR}$  is found to be expressed exclusively with the  $J_{\kappa} 1$  gene segment, and of 15 sequenced  $V_{\kappa}Id^{CR}$  regions, all have an arginine residue bridging the  $V_{\kappa}J_{\kappa}$  juncture at position 96 (5, 6, 8, 34, and Margolies, M., personal communication). The sequence of the  $V_{\kappa}Id^{CR}$  gene expressed by 36–65 reveals that the first nucleotide of this arginine 96 codon (C) is derived from the  $V_{\kappa}Id^{CR}$  gene segment and the remaining GG dinucleotide is derived from the  $J_{\kappa} 1$  gene segment (deduced from the published sequences of germline  $J_{\kappa}$  genes in the BALB/c genome [14]).

The absence of junctional diversity in  $V_{\kappa}Id^{CR}$  light chains is accompanied by limited heavy chain junctional diversity in the predominant population of  $Id^{CR+}$  V regions that are apparently encoded by one combination of V gene segments. These homogeneous structures dominate the secondary immune response to Ars. They differ from one another most consistently at only two junctional amino acid positions (27), one at the  $V_H$ -D boundary (amino acid 100) and the other at the D- $J_H$  boundary (amino acid 107). Many of them are identical at these points as well. They differ elsewhere more extensively, but less consistently by somatic mutational alterations, which are scattered throughout the V region (6–9, 25, 27). Presumably, the mutational differences are introduced after immunization (27, 35). Thus, unmutated versions of these homogeneous V regions are present in the preimmune repertoire of most individuals. This implies that a large fraction of the total preimmune repertoire expressed in this strain is contained within the B cell population ( $\sim 2 \times 10^8$  cells) of every mouse. Models postulating that the preimmune repertoire is generated from an independent assortment of V gene

segments and the random addition of several codons of information (N segments) (36) at the  $V_H$ -D and D- $J_H$  boundaries of every combination would predict the size of the potential preimmune repertoire to exceed by far  $2 \times 10^8$  different V region sequences. Such models would predict that only a small fraction of the potential repertoire should be found within any single individual. The discrepancy between this prediction and the observation of a recurrently expressed V region in most strain A individuals suggests that the size of the potential repertoire may be more limited than that predicted by assuming that each V segment combination is subject to the random addition of nucleotides at the  $V_H$ -D and D- $J_H$  segment boundaries. In further support of this idea, reports from three laboratories have documented evidence for a sequence-specific origin of nucleotides at or near the  $V_H$ -D junction (33, 37-39).

Comparison of the deduced amino acid sequence of the germline  $V_\kappa Id^{CR}$  gene with sequences of light chain V regions, derived from  $Id^{CR+}$  mAbs that are elicited after two or more immunizations (5, 6, 8, 34, and Margolies, M., personal communication), reveals that the latter are often somatically mutated. Thus, in the absence of junctional and combinational diversity, somatic mutation contributes all of the light chain diversity thus far observed in  $V_\kappa Id^{CR}$ -encoded V regions. Taken together with previous results of structural analyses of heavy chain V regions, the light chain data shows that most of the diversity in  $Id^{CR+}$  structures elicited during the secondary immune response is derived from somatic mutation.

The frequency of amino acid substitutions that are introduced somatically in  $V_\kappa Id^{CR}$ -encoded regions is somewhat less than that observed in  $V_H Id^{CR}$ -encoded regions expressed by the same hybridomas (3% vs. 5%).  $V_\kappa Id^{CR}$ -encoded regions, like  $V_H Id^{CR}$ -encoded regions, display a nonrandom distribution of somatically introduced amino acid replacements. This observation has now been reported for antibodies elicited in other immune responses (40, 41). In light chains encoded by  $V_\kappa Id^{CR}$ , ~50% of all somatically introduced amino acid replacements observed thus far occur in only 6 of 108 positions (5, 6, 8, 34, and Margolies, M., personal communication). At some of these positions, recurrent amino acid substitutions are observed in antibodies synthesized by independently derived hybridoma cell lines. This is most notable at position 30 of the first complementarity determining region where a serine to asparagine substitution is found in 6 of 19 sequences. Previous reports from this (27) and other laboratories (40, 42), indicating a role for somatic mutation in the generation of antibodies of improved affinity, have suggested that the recurrence of particular somatically introduced amino acid substitutions may be the indirect consequence of an affinity-selection process. One monoclonal  $Id^{CR+}$  antibody, encoded by  $V_\kappa Id^{CR}$  but which has no detectable affinity for Ars (43), also shares the asparagine substitution at position 30 (34, 44), raising the possibility that this phenomenon may be due in part to a nonrandomness in the somatic mutation process.

The availability of germline sequences for a  $V_H$  and  $V_\kappa$  pair that encode the dominant antibody variable region elicited during both primary and secondary phases of an antihapten immune response should allow us to chart the kinetics, magnitude, distribution, and influence of somatic mutation on a complete antibody variable region.



### Summary

We have cloned and sequenced the predominant germline  $V_{\kappa}$  gene segment expressed by B cells of strain A origin that synthesize antibodies with specificity for Ars. In hybridomas synthesizing anti-Ars antibodies, this  $V_{\kappa}$  gene segment ( $V_{\kappa}Id^{CR}$ ) has been found exclusively associated with the  $J_{\kappa}1$  gene segment without exhibiting junctional sequence variation. Sequence comparisons of the germline  $V_{\kappa}Id^{CR}$  gene with expressed derivatives reveals that the latter frequently contain somatically introduced amino acid replacements. Taken together with results of previous structural analyses (27), these results show that the predominant population of  $Id^{CR+}$  V regions elicited in the secondary immune response is encoded by one or two combinations of V gene segments, has little junctional diversity, and is extensively diversified by somatic mutation in both heavy and light chains.

We thank Marjorie Shapiro and Martin Weigert for the  $V_{\kappa}10$  probe and Tim Manser for critical discussion.

Received for publication 6 February 1987.

*Note added in proof:* Sanz and Capra (45) have recently reported the isolation of a germline  $V_{\kappa}$  gene. The coding portion of this gene is the same as that reported here, suggesting that the two are the same.

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