

## GENETIC BASIS FOR EXPRESSION OF THE IDIOTYPIC NETWORK

One Unique Ig V<sub>H</sub> Germline Gene Accounts for the Major Family of  
Ab1 and Ab3 (Ab1') Antibodies of the GAT System

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The idiotypic network as proposed by Jerne (1), and first approached by serial immunizations performed in discrete animals (2-5), may be most simply written as a cascade  $Ag \rightarrow Ab1 \rightarrow Ab2 \rightarrow Ab3 \dots$  in which Ag represents the original immunizing antigen, Ab1 the responding antibody (idiotypic set), which, in turn, may elicit the synthesis of Ab2, or antiidiotypic antibodies. Jerne et al. (6) more recently proposed that Ab2 could be divided into Ab2 $\alpha$ , or true antiidiotypic antibodies, which recognize the Ab1 idiotopes, and Ab2 $\beta$ , complementary to the Ab1 paratope, and that thus appear as representing an internal image of the original antigen. The concept of internal image is crucial to account for the emergence, among Ab3 antibodies, of a population of molecules resembling Ab1 in that they may recognize the original antigen (Ag), besides expressing idiotopes of Ab1. They are generally termed Ab1'. In addition, Ab2 $\epsilon$  have also been described as epibodies (7), which are able to recognize both the original antigen and the Ab1 idiotopes.

A major issue concerns the physiological role of the idiotypic network. It may regulate the production of a given family of antibodies as a result of the perturbation introduced by a foreign antigen (1). Idiotypic manipulations leading either to activation or suppression of one member of the cascade have been extensively documented (3-6, 8; see also 9 for a review). More profound may be the idea, centered on the internal image, that the idiotypic network may find its major physiological significance in playing a key role in the emergence of the antibody repertoire, and thus contribute essentially as an internal selective pressure that would allow Ig germline genes to have evolved to recognize a huge array of potential epitopes (1, 3, 4). The first requirement for the idiotypic network to play such a role is to prove that the cascade may spontaneously occur within one single animal. This was first shown in the mouse for the PC system (10). Experimental models have largely made use of inbred strains of mice, and

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573

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of a number of rather simple antigens, allowing the production of antibodies expressing public or crossreactive idiotypes. Among the more extensively studied, the ARS (5), dextran (DEX)<sup>1</sup> (11), PC (12), 4-(hydroxy-4-nitrophenyl)acetyl (NP) (13), DNP (14), levane (15), and GAT (16, 17) have been largely documented, both from regulation and structural standpoints (see 9 and 18 for reviews).

We have been investigating the structural and genetic basis for the entire idiotypic cascade obtained in a syngeneic situation in the BALB/c mouse with GAT, which elicits, in a number of strains of mice, anti-GAT antibodies, the majority of which express public idiotypic specificities, termed C.GAT (16) or pGAT (17). The presence of this idio type correlates strongly with a preferential recognition of the Glu-Tyr-containing epitopes (19), and the expression of the idiotypic specificities requires both the Ab1 H and L chains (20). Hybridomas have been isolated for each of the Ab1, Ab2, and Ab3 levels of this idiotypic cascade in BALB/c mice (21–23). Nucleotide sequencing of H and L mRNA (24, 25) and cDNA, and Southern blot analysis (26, 27) revealed that expression of the pGAT specificities in Ab1 strongly correlated with the presence of conserved V<sub>H</sub> and V<sub>κ</sub> sequences, an observation compatible with the use of a very small number of germline genes. More recently, similar analyses, effected at the Ab3 level, indicated that Ab1' molecules also made use of the same small set of germline genes, both for the H and the L variable regions (28). In this paper, we report the isolation and sequencing of three closely related V<sub>H</sub> germline genes, from which one single gene accounts for expressed V<sub>H</sub> sequences of the GAT Ab1/Ab1' antibodies that were previously reported (24, 26, 28).

## Materials and Methods

### *Hybridomas*

Hybridomas of the Ab1 series (G7 and G8) were derived from fusions using spleen cells of BALB/c mice immunized with GAT coupled to KLH, and the X63-Ag8-653 cell line, as previously described (21).

### *Isolation of Cellular DNA*

BALB/c cellular DNA was extracted from adult liver and hybridomas, according to Gross-Bellard et al. (29).

### *Southern Blot Analysis*

Southern blot analysis (30) was performed on 15 μg samples of EcoRI-digested DNA. Hybridization after transfer to nitrocellulose was made with a J<sub>H</sub> probe in 2× SSC, 10× Denhardt's solution, 0.1% SDS, and 50 μg/ml salmon sperm DNA, at 65°C for 48 h, followed by washings in 2× SSC in Denhardt's solution and 0.2× SSC, at the same temperature.

The P5JH probe was a kind gift of P. Legrain (31), and consists of the Bam HI-Eco RI fragment encompassing the 3' end of the J<sub>H</sub> cluster, containing the J<sub>H</sub>3 and J<sub>H</sub>4 coding fragments. It was originally derived from the 6.2 kb probe pRI-JH (32). The probe was nick-translated to a sp act of 2 × 10<sup>8</sup> cpm/μg, of which 3.6 × 10<sup>7</sup> cpm were used in 8 ml of hybridization buffer.

### *Construction of Genomic Libraries*

*Restricted libraries.* As previous work indicated (26), BALB/c Eco RI restriction fragments that specifically hybridized with an anti-GAT cDNA V<sub>H</sub> probe were identified at about 7.5, 7.0, 4.3, and 3.2 kb. Materials containing 4.3 and 3.2 kb Eco RI restriction

<sup>1</sup> Abbreviations used in this paper: DEX, dextran; NP, (4-hydroxy-3-nitrophenyl)acetyl.

fragments were isolated after centrifugation (41,000 *g* for 4 h at 20°C in a Beckman SW 41 rotor) in a sucrose gradient (5–20%), and ligated at Eco RI sites of pBR328 using T4 ligase. Transformation was performed according to Hanahan (33). Cells were directly plated on Millipore Triton-free nitrocellulose filters (Millipore S. A., Molsheim, France) in standard Petri dishes at high density (~10,000 colonies per filter), as described by Hanahan and Meselson (34).

**Complete library.** BALB/c liver DNA was partially cleaved with Mbo I, and centrifuged in a sucrose gradient (10–40%) in a Beckman SW 27 rotor, at 27,000 *g* for 22 h at 20°C. Fractions sizing between 15 and 20 kb were pooled, inserted at the Bam HI restriction site of EMBL 3 phage as described by Maniatis et al. (35), and encapsidated using a  $\lambda$  DNA in vitro packaging kit (Amersham France).

**GAT-specific  $V_H$  probes.** Libraries were screened either with a nick-translated <sup>32</sup>P-labelled GAT-specific  $V_H$  cDNA (HIV.92, a 230 bp Pst I fragment encoding amino acids 4–81 [26]), or with a 780 bp Hinc II restriction fragment derived from a germline gene (clone H4a-3) starting 174 bp 5' upstream of the leader segment (see Fig. 1). The hybridization was made at 65°C in 4× SET, 2× Denhardt's, 0.2% SDS, and the washings were done in 3× SET (20× SET is 3 M NaCl, 0.04 M EDTA, 0.6 M Tris, pH 8.0), 0.2% SDS followed by 0.5× SET, 0.2% SDS at the same temperature.

**Subcloning into M13 vector.** Double-stranded (RF) mp 8 vector (36) was digested with Sma I and ligated with T4 ligase to recombinant DNA digested with Hae III or Alu I. Colonies of transformed bacteria (JM 101) were screened with either of the above  $V_H$  probes.

**DNA sequencing.** Nucleotide sequences were determined by the dideoxy chain terminator procedure of Sanger et al. (37), using the M13 universal primer (Amersham Corp.), and DNA polymerase Klenow fragment (Bethesda Research Laboratories, Herblay, France).

## Results

**Screening of Genomic Libraries and Characterization of GAT-related  $V_H$  Germline Genes.** On the basis of previous work (26), it was known that GAT-related  $V_H$  germline genes would be identified within Eco RI inserts having a size of 3.2, 4.3, 7.0, and/or 7.5 kb. In addition, mRNA (24) or cDNA (26) nucleotide sequence data revealed the existence of a characteristic 230 bp Pst I fragment located within the coding region (codons 4–81). These two criteria were used to select potential GAT-specific  $V_H$  genes among recombinant clones that gave a strong positive signal with the cDNA HIV.92 probe.

Out of the 7,000 recombinant clones obtained from the 4.3 kb Eco RI-restricted library, three gave a positive signal in stringent conditions, from which one clone, termed H4a-3 met the above requirements. The two other 4.3 kb Eco RI inserts gave fainter signals and did not contain the characteristic 230 bp Pst I fragments. A restriction map of this clone is given in Fig. 1, as well as limits of the probe that was derived from it and used to screen the complete EMBL 3 library. Out of a total of  $1.4 \times 10^6$  nonamplified recombinant clones representing two to three times the mouse genome, 200 positive plaques were harvested, from which the 47 that gave the strongest signals were replated and serially screened. After the fourth screening, clones were divided into two groups, on the basis of the intensity of the hybridization signal. Only the group (composed of 24 members) that gave consistently a very strong signal was then extensively analyzed by restriction mapping. Five clones had the required expected Eco RI and Pst I restriction sites. Two contained a 4.3 kb Eco RI fragment that possessed restriction fragments (Eco RI; Eco RI + Hind III; Hinc II; Hinc II + Stu I; Pst I) that

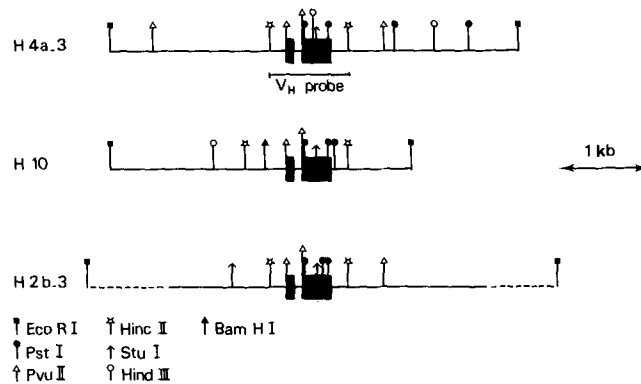


FIGURE 1. Restriction maps of the three V<sub>H</sub> germline Eco RI fragments derived from clones that specifically hybridized with a GAT-specific V<sub>H</sub> probe. H4a-3, H10, and H2b-3 Eco RI fragments were 4.3, 3.2, and 7.6 kb, respectively.

hybridized with the V<sub>H</sub> probe as depicted in Fig. 1, and which were characteristic of the H4a-3 gene. It was thus assumed that these clones were identical to H4a-3. Three had the 3.2 kb Eco RI fragment and the same restriction map in the coding region, described as the H10 prototype in Fig. 1. Within each group, the respective Eco RI fragments were contained in overlapping inserts having lengths between 15 and 20 kb. The 19 remaining clones did not meet completely the aforementioned requirements. Taking into account other restriction sites that could be defined in the H4a-3/H10 family (Stu I, Hinc II, Pvu II), five clones were still considered potential candidate.

Finally, an Eco RI fragment with a size of 7.6 kb, termed H2b-3 (Fig. 1) was identified in one of the nine positive clones from another restricted library that yielded 50,000 clones. The eight remaining positive clones gave only faint signals when high stringency was used for hybridization.

**Nucleotide Sequences.** The H4a-3, the three genes of the H10 group, the H2b-3, and the five potential anti-GAT candidates were sequenced by the Sanger method. Hae III and Alu I subclones were prepared in the M13 vector, and those containing coding and immediate flanking regions were selected and sequenced according to the strategy depicted in Fig. 2. The three genes of the H10 group had identical sequence referred to as the H10 prototype. Sequences of H10, H4a-3, and H2b-3 were highly homologous (Fig. 3) and could be closely related to anti-GAT Ab1 sequences. All three genes exhibited characteristic features of the V<sub>H</sub>II family (38), including organization of the 5' noncoding region, high homology of the leader and coding region, and conservation in length and homology of the first intron (39). The homology between the three genes was >95%, whichever leader, intron, or main coding region was considered. The five remaining clones also pertained to the V<sub>H</sub>II family, but their sequences were only 80–85% homologous to that of the anti-GAT family (data not shown).

**Southern Blot Hybridization of GAT-specific Hybridoma DNA with Specific J<sub>H</sub> Probe.** BALB/c mouse liver DNA and GAT-specific Ab1 hybridoma DNA (G8 Ca 1.7 and G7 Ab 2.9) Eco RI fragments were analyzed by Southern blot hybridization with a specific J<sub>H</sub> probe (Fig. 4). The unrearranged 6.4 kb Eco RI

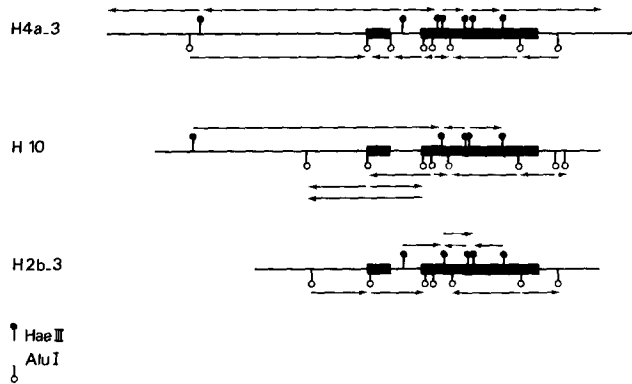


FIGURE 2. Strategy used for M13 subcloning and nucleotide sequence determination (Sanger dideoxy method).

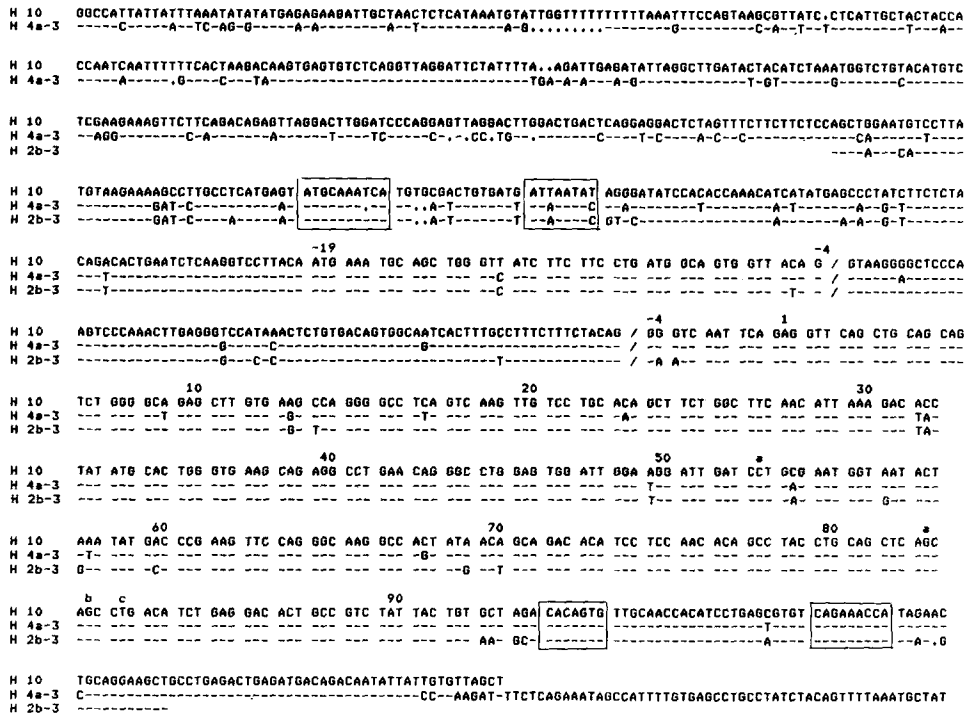


FIGURE 3. Nucleotide sequences of the three V<sub>H</sub> germline genes, partially encompassing 5' and 3' noncoding regions. Numbering of the coding region is that of Kabat et al. (38). Regulatory signals are boxed.

fragment containing all J<sub>H</sub> sequences was present in all tracks, an observation compatible both with the germline organization of liver DNA and the contribution of the parental X63.Ag8.653 cell line DNA (40). Interestingly, another band corresponding to the V-D-J rearrangement was observed at the same position (3.6 kb) for the two hybridoma DNA. As it was previously shown that both G8 Ca 1.7 and G7 Ab 2.9 used J<sub>H</sub>4 (24), this was strongly suggestive that they used the same V<sub>H</sub> germline gene.

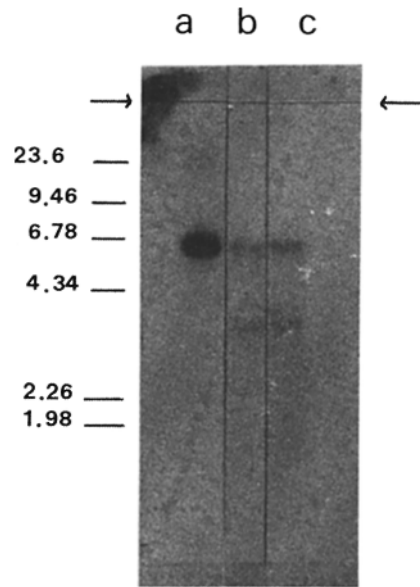


FIGURE 4. Southern blot of Eco RI DNA restriction fragments from (a) adult BALB/c liver, (b) G7 Ab 2.9, and (c) G8 Ca 1.7 GAT-specific hybridomas. Hybridization was performed using the p5 J<sub>H</sub> probe. Arrows indicate the origin.

### Discussion

Most antibodies prepared in various strains of mice against the random terpolymer GAT express a public idiotype (16, 17), which presumably contains a mosaic of idiotopes, to which epitopes 20 and/or 22 may be associated (22). Amino acid, mRNA, and cDNA nucleotide sequence determination effected on a collection of BALB/c anti-GAT hybridomas clearly suggested that the GAT-specific, pGAT<sup>+</sup> antibodies (Ab1) were encoded by a very small number of V<sub>H</sub> and V<sub>K</sub> germline genes (24–27). The H chains seemed to use one or very few germline V<sub>H</sub>, preferentially associated with J<sub>H</sub>4. The D region, using either a partially mutated D.SP2 or FL.16 gene, had interesting features (presence of aromatic and positively charged amino acids) that suggested it might be involved in GAT recognition. When Ab3 antibodies were produced by injecting monoclonal Ab2, a large proportion were found to express the pGAT public specificities (Ab1'), some of which were, in addition, GAT specific. This could be predicted from the essence of the idiotypic network and from the suggestion that an internal image-like structure was present in the D region of the Ab2 mAb (41). The expression of the pGAT idiotypic specificities by Ab1' strictly correlated with the presence of V<sub>H</sub> and V<sub>K</sub> sequences that were very close, if not identical, to that of the Ab1 prototype (G8 Ca 1.7). The presence of these V region sequences was not sufficient, however, to insure GAT recognition, which was shown to be highly dependent upon the structure of the D region (28). If one considers that V germline genes may be selected in evolution as a consequence of a physiological role of the immune network (42), one should expect to induce germline genes more efficiently with Ab2 immunization than with the corresponding antigen.

*General Features of GAT-related V<sub>H</sub> Germline Genes.* We have isolated and characterized seven clones that contained the characteristic Pst I 230 bp fragment predicted from the nucleotide sequence of the coding region and possessing the Eco RI fragments of expected sizes (i.e., 3.2, 4.3, and 7.6 kb) that specifically hybridized in Southern blot analysis with V<sub>H</sub> GAT-specific probes, using stringent conditions.

These clones were assigned to three distinct, strongly homologous sequences, identified as H10, H4a-3, and H2b-3, corresponding to clones containing the expected Eco RI fragments of 3.2, 4.3, and 7.6 kb, respectively. Three clones expressing the H10 sequence were independently isolated and sequenced. Three clones were assigned to the H4a-3 prototype on the basis of a very detailed restriction map. A unique clone containing the H2b-3 gene was isolated. Using the same probe and the same hybridization conditions, 19 other related V<sub>H</sub> genes have been isolated and characterized by restriction mapping analysis. Five of them have been sequenced and differed rather extensively from the anti-GAT-related genes.

Nucleotide sequence of the three clones H4a-3, H10, and H2b-3 (Fig. 3), indicated that they all pertained to the V<sub>H</sub>II family (38), within which they represent a highly homologous subfamily. Strong homology (constantly >95%) begins in the section starting at the initiation codon and extends downstream to the 3' noncoding region. The conservation of the leader segment and that of the first intron is especially striking. The length of this intron has already been reported as characteristic of the family (39, 43). All three genes have an open reading frame in the coding regions, and appear potentially functional, with a possible TATA box (44) starting about 78 bp 5' upstream of the initiation codon, and a highly conserved element (45, 46), reading ATGCAAATCA, which has been recently reported to control cell type specificity of transcription (47).

The nucleotide sequence of the three germline genes were then compared with cDNA (26) or mRNA (24) sequences of Ab1 (G series) and Ab1' (28) (20 and 22 series) monoclonal V<sub>H</sub> sequences (Fig. 5). Main characteristics of Ab1/Ab1' mAb are given in Table I.

*One Single V<sub>H</sub> Germline Gene Accounts for H Chain V Regions of Expressed Anti-GAT (pGAT<sup>+</sup>) Ab1 Antibodies in BALB/c Mice.* The most striking feature noted was that the anti-GAT (Ab1) V<sub>H</sub> cDNA sequence of G8 Ca 1.7 (26) was completely identical to that of the H10 germline gene. As previously reported (24) mRNA nucleotide sequences of G8 Ca 1.7 and G5 Bb 2.2 were also completely identical, demonstrating that the same germline gene was employed in two hybridomas derived from separate fusions. Incidentally, the mRNA sequence previously reported (24) for codon 14 should read CCA, and not CCT, in agreement with results derived from cDNA sequencing (26).

A substitution of A → G was observed between H10 and most of the expressed V<sub>H</sub> at the last position of the V region. This may be explained on the basis of V-D joining, and the inclusion of a G residue as the result of terminal transferase activity, as suggested by Alt and Baltimore (32), regarding the N-diversity.

The two remaining Ab1 anti-GAT antibodies (with a slight uncertainty due to the lack of nucleotide sequence for the first eight codons, which expressed





TABLE I  
Main Characteristics of Ab1 and Ab1' Hybridomas

Hybridoma	Isotype	Anti-GAT activity	pGAT expression
Ab1			
G8 Ca 1.7	$\gamma 1\kappa$	+	+
G5 Bb 2.2	$\gamma 1\kappa$	+	+
G7 Ab 2.9	$\gamma 1\kappa$	+	+
G8 Ad 3.8	$\gamma 1\kappa$	+	+
Ab1'			
22.8	$\mu\kappa$	+/-	+
22.176	$\mu\kappa$	-	+
20.11	$\gamma 1\kappa$	+	+
22.186	$\mu\kappa$	+	+
20.33	$\mu\kappa$	+	+
20.8	$\mu\kappa$	+	+

Data are taken from Leclercq et al. (21) (G series) and Roth et al. (28) (20 and 22 series).

are due to somatic mutations. This observation is similar to situations reported in other systems such as PC (48, 49), ARS (50, 51), Oxazolone (52, 53), and NP<sup>b</sup> (54, 55).

*The Same H10 Germline Gene Is Used in Ab1 and Ab1' V<sub>H</sub> Regions of the Anti-GAT System.* In Fig. 5 is also given the comparison of the H10 sequence with six monoclonal Ab1' V<sub>H</sub> regions. All of these Ab1' had been produced by immunization with monoclonal Ab2 (22, 23). All express the pGAT specificities, and all but two (22.8 and 22.176) were GAT-specific, as determined either by direct fixation or inhibition tests (28, and see Table I). Five of these (22.8, 22.176, 22.186, 20.33, 20.8) had sequences that were completely identical over the region analyzed, which runs from codon 29, 43, or 54 (28). As it was previously shown (24) that, in the GAT system, mutations accumulated preferentially in the COOH-terminal half of the V<sub>H</sub> region; it seems likely that the entire V<sub>H</sub> of these hybridomas express the H10 germline gene without modification. Hybridoma 20.11 had four differences from the germline sequence.

The absence or the low level of somatic mutations observed in Ab1' antibodies may result from several factors: (a) fusions were made with lymphocytes derived from one single immunization. This is consistent with the existence of a majority of  $\mu$  chains, although it should be recalled that fusion was performed 1 mo after immunization. The absence of mutation might thus be simply related to a low rate of division of IgM-expressing B cells. (b) Alternatively, the system may tend not to accumulate mutations because, in Ig-Ig interactions of the idiotypic network, the optimal fit might be already insured by germline gene-encoded antibodies.

The selection of this germline V<sub>H</sub> repertoire is not necessarily associated with GAT recognition, since hybridomas 22.8 and 22.176, which used the H10 germline gene did not bind the GAT antigen (Table I). It was previously shown (28) that GAT recognition in Ab1' was associated with structural features of the D region.

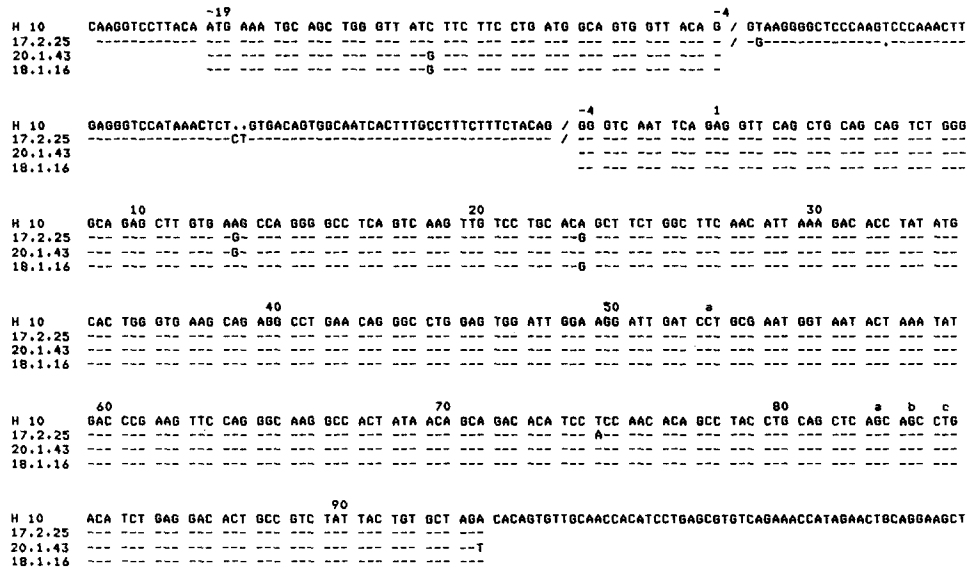


FIGURE 6. Comparison of H10 germline gene with nucleotide sequences of rearranged V<sub>H</sub> gene (17.2.25, ref. 59), and cDNA (20.1.43 and 18.1.16, ref. 60) derived from anti-NP<sup>a</sup>-producing hybridomas in BALB/c mice.

This observation is in line with similar conclusions drawn in the levane (31) and the ARS (56) systems. In contrast, in the NP<sup>b</sup> system, another example for which Ab3 antibodies of the Ab1' type have been analyzed (57), only those antibodies that both expressed the public idotype and were antigen specific used V<sub>H</sub> similar to that used for the Ab1.

We also have evidence that, in the GAT system, V<sub>K</sub> germline genes are used preferentially (Corbet, S., manuscript in preparation). As the expression of the pGAT idiotypic specificities relies on the presence of both the H and the L chains, this clearly suggests a very strict role of a given member of the idiotypic cascade for the restricted expression of an idotype, which is largely dependent on the use of germline gene sequences.

*The Same H10 Germline Gene Encodes V<sub>H</sub> GAT and V<sub>H</sub> NP<sup>a</sup> Sequences.* We reported earlier (58) that a GAT V<sub>H</sub> sequence derived from a C57BL/6 mouse was almost identical to the germline gene V186.2 (54) encoding the V<sub>H</sub> of the NP<sup>b</sup> family. This suggested that the same germline gene could be used, through different V-D-J rearrangements and various H-L pairings, to yield antibodies of discrete specificities. A similar observation was reported in the BALB/c mouse, in which GAT-V<sub>H</sub> and NP<sup>a</sup>-V<sub>H</sub> seemed to derive from the same germline gene (26, 59). Recently, Boersch-Supan et al. (60) reported the nucleotide sequence of two NP<sup>a</sup> V<sub>H</sub> cDNA (20.1.43 and 18.1.16) that were highly homologous to that previously reported (17.2.25) (59). These three sequences are aligned with the H10 sequence in Fig. 6. The overall homology between each of these sequences and H10 was >99%, including the leader segment. In the coding region, 18.1.16, 20.1.43, and 17.2.25 differed from H10 by only one, two, and three nucleotides, respectively, reinforcing the previous hypothesis that NP<sup>a</sup>, and anti-GAT V<sub>H</sub> regions were encoded by the same germline gene. An additional

argument was derived from Southern blot analysis performed on Eco RI-rearranged DNA. Boersch-Supan et al. (60) reported that the hybridization patterns strongly suggested that the same  $V_H$  gene was used in each rearrangement. This  $V_H$  gene was contained in an Eco RI restriction fragment, to which it contributes 2.2 kb 5' upstream of the V coding region, a value very close to that discussed above for G8 Ca 1.7 and G7 Ab 2.9 GAT-specific hybridomas. Detailed 5' restriction mapping of 17.2.25 (59) was also in complete agreement with that of H10. Finally, and as already stressed, three clones that all contained the 3.2 kb Eco RI fragment were isolated and sequenced. All three sequences were identical and are referred to as that of the H10 germline gene. This strongly argues against the possibility of having additional germline genes that would differ from H10 at one, two, or three positions, and accounting for each of the NP<sup>a</sup> reported sequences (18.1.16, 20.1.43, and 17.2.25).

Out of the few positions that differed from the H10 germline sequence (Fig. 6), two were clustered at codons 13 and 23 (and also one at -13 in the leader segment). In all cases, the same nucleotide (G) was found, which may be suggestive of a conversion event (49). We may thus unequivocally conclude that the same germline gene is being used in the NP<sup>a</sup> and GAT systems. This definitive proof will presumably be extended to other  $V_H$  and  $V_L$  gene combinations, since, at the expression level, a number of cases have already been reported in which the same chains are involved in different specificities (61). The random pairing of H and L chains must therefore contribute a major source of antibody diversity, as originally proposed in 1963 by Edelman et al. (62).

### Summary

Ig germline genes have been isolated from recombinant clones prepared in separate libraries constructed from adult BALB/c liver DNA either in pBR328 plasmid or in EMBL 3 phage. Three clones that gave a very strong positive hybridization signal with a  $V_H$  anti-GAT-specific probe were completely characterized and sequenced. All three were >95% homologous, with the exception of the 5' noncoding region, which was only 85% homologous but contained characteristic regulatory signals.

One of these genes, H10, had a sequence that was completely identical to that of a cDNA derived from a GAT-specific BALB/c hybridoma. Southern blot analysis using Eco RI-digested DNA from rearranged GAT-specific hybridomas revealed that the same gene was used for other GAT-specific  $V_H$  regions, including one differing from the H10 sequence by 12 nucleotides, which must have been generated by a somatic mechanism. The same H10 germline gene was also used, in most cases without any nucleotide substitution, in hybridomas of the Ab1' set of the GAT idiotypic cascade, suggesting that immunization with Ab2 (antiidiotypic) antibodies preferentially stimulates the direct expression of  $V_H$  germline genes.

Finally, the previous hypothesis that NP<sup>a</sup> and GAT  $V_H$  genes were derived from the same germline gene was definitively confirmed, both from sequence data and Southern blot analysis.

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