The synthetic random terpolymer (Glu\textsuperscript{60} Ala\textsuperscript{30} Tyr\textsuperscript{10})\textsubscript{n} (GAT)\textsuperscript{1} elicits, in a number of inbred strains of mice, anti-GAT antibodies that can be characterized by various idiotypic specificities. Most anti-GAT antibodies express the so-called CGAT (1) or pGAT (2) public idiotypic specificities, which correlate with the recognition of Glu-Tyr-containing epitopes (3). They require both the heavy (H) and light (L) chains of the idiotype in order to be expressed (4). Analysis of monoclonal antibodies allowed the definition, within the CGAT\textsuperscript{−} anti-GAT antibodies, of a new family of idiotypic determinants. They are also found in several anti-GA antibodies and are therefore termed GA-1 specificities (5). Finally, a third group of anti-GAT antibodies expressed neither the CGAT (or pGAT) nor the GA-1 idiotopes. Isolation of a collection of anti-GAT monoclonal antibodies from BALB/c, DBA/2, or C57BL/6 origin made it possible to study the V\textsubscript{H} and V\textsubscript{L} anti-GAT repertoire in the different strains and to search for idiotypic structural correlates.

An NH\textsubscript{2}-terminal amino acid sequence of 16 V\textsubscript{H}-V\textsubscript{L} pairs has been recently reported (6), leading to the proposals that both the CGAT (or pGAT) and the GA-1 idiotypes represented the expression of a pauci-gene system. The anti-GAT,CGAT\textsuperscript{+} and the anti-GAT,GA-1\textsuperscript{+} antibodies use V\textsubscript{H} regions that may use the same germline gene(s), whereas these two types of antibodies express discrete sets of V\textsubscript{\kappa} chains. The complete sequences of four BALB/c (7) and one C57BL/6 (8) anti-GAT V\textsubscript{H} regions have been recently determined. We report here on the complete sequence of eight CGAT\textsuperscript{+} anti-GAT monoclonal \kappa chain variable regions. The experimental approach combined amino acid sequencing with an analysis of the nucleotide sequence (7), using mRNA as a template, a synthetic
oligonucleotide as a primer, and a modification (9) of the chain terminator method of DNA sequencing (10).

Materials and Methods

Hybridoma and Anti-GAT Monoclonal Antibodies. The eight monoclonal antibodies were isolated from seven distinct series of hybridoma lines. Experimental conditions have been given previously in detail. The G5, G6 (11), and F17 (12) series were derived from fusion with the NS1 cell line, whereas G7, G8 (11), H51, and H56 (6) were obtained with the X63 Ag8.653 (13) myeloma cell line. Characterization of idiotypic specificities was reported earlier (6, 11). The hybridoma cells were transplanted into BALB/c mice or appropriate F1 hybrids and the resulting solid tumors were removed and stored at −70°C. The corresponding anti-GAT monoclonal antibodies were specifically isolated from ascitic fluids on a GAT-amino-hexyl Sepharose column, as previously reported (12).

Preparation of Peptides from the L Chains. A combination of cleavage at tryptophanyl and glutamyl residues was used to isolate a peptide encompassing residues 36-105 (Kabat numbering; see reference 14) according to Lazure et al. (15) with slight modifications. Mildly reduced and alkylated L chains (10 mg/ml) were dissolved in 0.05 M ammonium bicarbonate-0.2% sodium dodecyl sulfate (SDS), and digested with V8 streptococcus protease (16), using an enzyme/substrate ratio of 1:25 (wt/wt). Incubation proceeded for 24 h at 37°C. The digest was then transferred into dialysis tubing (benzoylated dialysis tubing; Sigma Chemical Co., St. Louis, MO), that retains molecules with a molecular mass >2,000 D. After exhaustive dialysis against a 0.15 M Tris-HCl, 2.10⁻⁴ M EDTA, pH 8.2 buffer, the sample was made 5 M in guanidine HCl, 0.15 M Tris HCl, 2.10⁻⁴ M EDTA, pH 8.2, fully reduced with 20 mM iodoacetic acid, and dialyzed against 0.05 M ammonium bicarbonate, succinylated (17), and dialyzed against 0.1 M acetic acid. The materials were passed through a TSK SW 2000 high performance liquid chromatography (HPLC) column (Beckman Instruments, Inc., Fullerton, CA) equilibrated in 5 M guanidine HCl, 0.1 M acetic acid. The fraction corresponding to the 11,000 D size was made 80% in acetic acid and treated with an equal weight of iodosobenzoic acid. Incubation proceeded for 3 h at room temperature, in the dark, such that tryptophanyl-X peptide bonds were selectively cleaved (15). After dialysis against 0.1 M ammonium bicarbonate, the recovered material was lyophilized, then subjected immediately to amino acid sequencing.

Protein Sequencing. NH₂-terminal amino acid sequences were determined in a Beckman 890 C automatic sequencer, using program No. 122974 operated with 0.3 M Quadrol in the presence of polybrene (Aldrich Europe) (18). The succinylated, iodosobenzoic acid (IBA) cleaved fragments were sequenced using the same conditions, except that the glycyl-glycine dipeptide was added to the polybrene (19). Phenylthiohydantoin (PTH) amino acid derivatives were identified by HPLC, using a C 18 micro-Bondapack column and a Waters apparatus equipped with an automatic injector (WISP; Waters, Paris, France). An aliquot of each PTH derivative was hydrolyzed with iodohydric acid (20) and the resulting amino acid was identified on a 121 M Beckman amino acid analyzer.

RNA Purification and Nucleotide Sequencing. The RNA were extracted directly from the frozen tumors by the LiCl-urea method (21), selected for the poly(A)⁺-containing fraction on an oligo dT-cellulose column, and enriched for the L-coding mRNA by ultracentrifugation on a sucrose gradient (5-20%). Nucleotide sequences were determined towards the 5' end of the mRNA, according to a modification of the original chain terminator method (10), using the mRNA as a template (9) and a synthetic oligonucleotide as a primer for the reverse transcriptase (obtained from Dr. Beard, Life Science, Inc., St. Petersburg, FLA). This oligonucleotide had a sequence d(GTAGAAGGGTGGTAGGT) complementary to an mRNA segment of the 5' end of the C, region (Ser 116-Ser 122). It had been synthesized according to Gait et al. (22) and was a generous gift from Dr. C. Milstein. 2 µg of mRNA and 10 ng of oligonucleotide primer were used per sequence. To minimize possible ambiguities in sequence determination on gels (9), labeling with each of the three α-³²P deoxynucleotides (dCTP, dATP, and dGTP) was performed in separate experiments and each repeated at least twice.
Results

Hybridomas and Monoclonal Antibodies. Main features of the eight hybridomas and their corresponding monoclonal antibodies are given in Table I. All antibodies were raised against the random terpolymer GAT. The five hybridomas of the G series were derived from BALB/c whereas H56 was derived from DBA/2 mice. The F17 and H51 hybridomas were prepared from F1 hybrid mice (C57BL/6 × DBA/2 and BALB/c × DBA/2, respectively), and it was thus not possible to determine the exact genetic origin of the isolated monoclonal chains, except for the F17 γ1 chains, which were shown to be of C57BL/6 origin (8).

General Strategy for Sequence Determination. The sequencing strategy, based on a combination of amino acid and nucleotide sequence, using mRNA as a template, is outlined in Fig. 1. NH2-terminal sequences were already reported for most chains (6) with the exception of F17. On average, the first 43 amino acid residues were identified. The sequence of the G6 κ chains was reinvestigated up to residue 44. Nucleotide sequencing, which proceeded from the beginning of the constant region, was allowed to reach, in four cases, codon 35 (G8C, G8A, G7, and G6), providing therefore a large overlap with the NH2-terminal amino acid sequence. Furthermore, an overlap with the nucleotide sequence for the remaining chains and confirmation for the G7 and G8 A chains were obtained by sequencing a peptide (starting at residue 36; Kabat numbering) that had been isolated by a two-step hydrolysis with V8 staphylococcal protease and IBA. Finally, the complete amino acid sequence was derived for the eight κ chains, from residue 1 to 118, thus encompassing the complete V segments, J segments, and the beginning of the constant region.

Isolation of the “V8-IBA” Peptides. The combination of hydrolysis by V8 protease with cleavage by IBA, under conditions defined by Lazure et al. (15), leads

| Nomenclature | Abbreviation | Full name | Myeloma cell line used for fusion | Mouse strain | Antibody isotypes | Public idiotypic specificities (CGAT or pGAT)* | References |
|--------------|--------------|-----------|----------------------------------|--------------|------------------|---------------------------------------------|------------|
| G5 Bb 2.2    | G5           | NS1       | BALB/c                            | γ1κ          | +                | 2, 3, 11                                    |
| G6 Bd 2.6    | G6           | NS1       | BALB/c                            | γ1κ          | ±                | 2, 3, 11                                    |
| G7 Ab 2.9    | G7           | X63 Ag8.653| BALB/c                            | γ1κ          | +                | 2, 3, 11                                    |
| G8 Ca 1.7    | G8C          | X63 Ag8.653| BALB/c                            | γ1κ          | +                | 2, 3, 11                                    |
| G8 Ad 3.8    | G8A          | X63 Ag8.653| BALB/c                            | γ1κ          | +                | 2, 3, 11                                    |
| F17.170.2    | F17          | NS1       | C57BL/6 × DBA/2                    | γ1κ          | +                | 12, 11                                      |
| H51.5.2      | H51          | X63 Ag8.653| BALB/c × DBA/2                    | γ1κ          | +                | 6                                           |
| H56.406.48   | H56          | X63 Ag8.653| DBA/2                             | μκ           | +                | 6                                           |

* CGAT and pGAT idiotypic specificities may be considered very similar if not identical (11). The CGAT specificities were detected using a guinea pig antiserum against DI-LP anti-GAT antibodies (1), whereas the pGAT specificities were identified by a rabbit antiserum directed against BALB/c polyclonal anti-GAT antibodies (2, 3).
to the isolation of a large peptide extending from residue 36 to 105. Cleavage with V8 protease occurs at glutamic acid residue 105, with an efficiency averaging 50%. After complete reduction and alkylation, both the released peptides and the uncleaved k chains were precipitated. Solubilization was obtained upon succinylation, a treatment that also blocks the k-NH2 terminus. A peptide, with a molecular mass of ~11,000 D, was isolated with HPLC using an SW 2000 column. This material was treated with IBA, which selectively cleaves the peptide bond involving the unique tryptophanyl residue at position 35 with an efficiency of 50–60%. An additional peptide, obtained for the H51 L chain due to an extra Trp residue at position 95, was lost upon dialysis. The peptide beginning with tyrosyl 36 was directly sequenced without further separation, since the NH2-terminal peptide was succinylated. The number of amino acid residues that were successfully identified was largely dependent upon the amount of peptide that was submitted to automatic sequencing, ranging from 13 residues with 20 nmol of G5 to 45 positions with 80 nmol of H51.

mRNA Sequencing. The chain terminator method (10), as applied to the mRNA templates (9), allowed us to identify between 160 (H51 and H56) and 250 (most chains of the G series) nucleotides. Surprisingly, almost no background was seen, due to the possible presence of MOPC-21 Vk in mRNA in samples isolated from G5, G6, and F17, for which the NS-1 cell line was used for fusion.
Very few ambiguous positions (8, ~0.5%) remained after three separate analyses, using either of the three α-32P-deoxynucleotides (dCTP, dATP, or dGTP; see Materials and Methods).

Complete Sequence of Eight GAT-specific V\textsubscript{\kappa} Chains. Nucleotide and amino acid sequence data are given in Fig. 2. It can be observed that for all but one of the 134 amino acid/codon positions that have been determined by both methods, there is a complete agreement between results derived from mRNA and those obtained by protein sequencing. The only discrepancy concerned position 50 of the F17 \kappa chain, for which a lysyl residue was identified as its succinyl derivative, using protein sequencing, whereas codon AGG (arginyl) was consistently determined upon mRNA sequencing.

Organization of the Diversity on the V\textsubscript{\kappa} Gene-encoded Section. As pointed out previously (6), the eight L chains are highly homologous with respect to their \textsubscript{NH\textsubscript{2}}-terminal amino acid sequences (Fig. 2). They clearly belong to the same subgroup (VK-1) as that defined by Potter et al. (23). However, considering that multiple identical substitutions at one given position may witness the expression of a discrete germline gene, it may be that the eight sequences fall into at least two families: One, represented by BALB/c \kappa chains G8C, G5, and G7 may be defined by residues Ile (27 b), Tyr (34), and Arg (50), whereas the second one, composed of the F17, H51, and H56 chains, expresses alternative residues Leu (27 b), His (34), Lys (50), and Leu (83). An additional variability is superimposed on this polymorphism but appears unevenly distributed along the V region (Fig. 3). One amino acid substitution was observed in FR1, 5 in CDR1, but none in FR2 or in CDR2, whereas fourteen substitutions were identified in FR3 and 13 in CDR3. Therefore, out of 33 amino acid substitutions, 27 were located after position 65. Interestingly, the overall pattern looks quite different if one considers silent nucleotide substitutions, although this information was only partial since mRNA sequencing did not cover the beginning of the chains. In fact, very few silent nucleotide substitutions were identified except for F17, which contained 18 such differences that appeared scattered along the variable region.

J\textsubscript{\kappa} Segments and the V-J and J-C Joining. Three J\textsubscript{\kappa} segments were identified in the eight anti-GAT \kappa chains, although unevenly represented. G8A used J\textsubscript{\kappa}5, H51 contained J\textsubscript{\kappa}1, and all other chains expressed J\textsubscript{\kappa}2. Alternative V-J rearrangements yielded four discrete amino acids at position 96 (Fig. 4), in agreement with other systems (24, 25). It may be noted that four BALB/c chains, G8C, G5, G6, and G7 used the same rearrangement, leading to a histidyl residue. The junction between J\textsubscript{\kappa} and C\textsubscript{\kappa} was such that the first two bases of the arginyl codon at position 108 derived from J\textsubscript{\kappa}, whereas the third base (G) pertained to the last residue of the 5' "flanking" region of C\textsubscript{\kappa}. This situation is similar to that reported for the MOPC-21 \kappa chain (9).

Discussion

Most GAT-specific murine antibodies (i.e., ~70-80%) express a major public idiotype, CGAT (1) or pGAT (2). Since it was previously shown that the presence of both the H and the L chains of the idiotype was required for these public specificities to be expressed (4), we have analyzed a collection of monoclonal anti-GAT antibodies derived from different strains of mice to look for structural
FIGURE 2. Combined amino acid and nucleotide sequences of eight anti-GAT, CGAT\(^+\), monoclonal V\(_{	ext{\gamma}}\) chains. Chains of the G series were derived from BALB/c immunized mice; H56.406.48 was derived from DBA/2, F17 from (C57BL/6 × DBA/2)\(F_1\) hybrids, and H51 from (BALB/c × DBA/2)\(F_1\) hybrids. Vertical lines indicate V\(_\gamma\)-J, and J-C, joinings.
correlates of idiotypity in this system. This detailed analysis also provides insight into the origin of antibody diversity.

In previous reports (6, 8), we presented evidence that the CGAT (or pGAT) GAT-specific antibodies used a very limited number of germ line genes for the expression of both the H and the L chains. The determination of the complete amino acid and/or nucleotide sequence of eight $V_\lambda$ GAT-specific monoclonal chains reported in this paper fully substantiates these proposals.

**Origin of the Diversity in the $V_\lambda$, GAT-specific L Chains.** Comparison of the eight $V_\lambda$ amino acid sequences, as determined directly by protein sequencing or indirectly from the mRNA nucleotide sequences, is shown in Fig. 5. Two groups of sequences may be easily distinguished, on the basis of alternate multiple
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FIGURE 4. Amino acids identified at position 96 resulting from various modalities in the V-J joinings.

FIGURE 5. Amino acid sequence of the eight anti-GAT, CGAT monoclonal V, chains, as compared with the NZB myeloma L chain 2205 (15), BALB/c TEPC-105 (15), and partial sequence of the BALB/c SAMM L chain (26). The three chains included for comparison all pertain to the V,1 subgroup (23).

repeats at positions 27 b (Ile/Leu), 34 (Tyr/His), and 50 (Arg/Lys). The first group contains four BALB/c sequences (G8C, G5, G6, and G7). The second group, composed of the F17, H56, and H51 chains, expresses in addition a characteristic Leu at position 83. The alternate multiple repeats may be considered hallmarks of two discrete germline genes. Whether these two genes represent allelic forms, one being expressed in the BALB/c strain (G series) and the other in DBA/2 (H56 chain), cannot be distinguished because the two remaining chains, F17 and H51, were derived from (C57BL/6 × DBA/2) and (BALB/c × DBA/2) hybrid mice, respectively. In addition, all sequences clearly pertain to the V,1 subgroup (23), but, within this family, the four BALB/c representatives (G8C, G5, G6, G7) were closely related to an NZB myeloma sequence (protein 2205; reference 15), whereas the second set (F17, H51, H56) was extremely homologous to TEPC-105, a BALB/c myeloma chain (15). Since the H56 was derived from a DBA/2 mouse, it might suggest that DBA/2, BALB/c, NZB, and possibly C57BL/6 strains possess the same germline gene repertoire, although each strain may use different V, gene subsets to produce anti-GAT antibodies. Finally, one V, sequence, of BALB/c origin (G8A), was found to be extremely homologous to the BALB/c "SAMM" (26) myeloma L chain and may
represent the expression of a third \( \nu \) gene, characterized by a leucyl residue at position 3 and a glutamyl at position 34. Since the SAMM protein was sequenced only as far as residue 35, it cannot be decided whether Lys (50) and Leu (83) may also be regarded as characteristic residues of this possibly third germline gene, or whether they might have arisen upon conversion (27, 28) from the TEPC-105 prototype.

We reported earlier, based on NH2-terminal amino acid sequences, that the GAT-specific \( \nu \) chains were extremely conserved. Now that the complete sequences are determined, the same conclusion holds true, except that a cluster of amino acid substitutions was clearly apparent between residues 83 and 93, i.e., at the end of the third framework and third hypervariable regions. By contrast, only one substitution occurred in FR1, none in FR2 or CDR2, and five in CDR1. This clustering of variations, which seems superimposed on the germ-line patterns described above, is not found in other systems such as arsonate (29), galactane (30), or inulin (31) antibodies. A detailed comparison of the location of substitutions is given in Fig. 6. A similar observation was made for the GAT-specific \( \nu_H \) regions (8). It seems worth noting that two of the L chains that presented maximal substitutions in the FR3/CDR3 area (G8A and F17) were associated with H chains that possessed the highest diversity in the corresponding region (G8A [7], F17 [8]). This would suggest that various combinations of variable stretches of both the H and the L chains in the V-D-J and V-J joining

![Diagram](image-url)  

**Figure 6.** Computation of variable positions within each of the three \( \nu \) chain systems: GAT (this paper), arsonate (Ars [29]), and galactane (Gal [30]). Variability is expressed according to the method defined by Wu and Kabat (32). Numbers of chains compared were 8, 8, and 16 for GAT, Ars, and Gal, respectively. Probable germline polymorphism is indicated (▲) for the GAT \( \nu \) chains.
areas might result in similarly interacting structures at the combining site. It should also be emphasized that both germline polymorphism and somatic variations are presumably superimposed in Fig. 6 (top), and thus amplify the diversity pattern as compared with those obtained for the galactane and arsonate systems. Furthermore, the overall diversity that was observed within the antibodies of the GAT system may also result from the higher heterogeneity of the random copolymer, as compared with the dominant epitopes of the galactane and arsonate antigens.

Silent nucleotide substitutions were found almost exclusively in the F17 chain (Fig. 3). If these differences were proven to be of germline origin, it would force the conclusion that F17 expresses a C57BL/6 chain, different from the BALB/c and DBA/2 structures. However, if these substitutions were of somatic origin, their even distribution all along the chain would contrast sharply with the clustered organization of substitution mutations. This would imply the existence of a particular selective pressure on the COOH-terminal portion of the V\textsubscript{\textalpha} region, which might result in conformational constraints and/or selection by antigen. A similar observation has been made for the H chains of anti-GAT antibodies (8).

**Structural Correlates for the Public CGAT (or pGAT) Idiotypes.** The structural basis of idiotypy has been extensively examined in a number of systems (for a review see reference 33). Apart from the Dex system (34), in which the presence of public idiotypic specificities correlates with no more than two amino acid substitutions in the second hypervariable region of the H chain, public (or private) idiotypic specificities generally seem to correlate with multiple amino acid substitutions. A detailed study of the CGAT system points to the same conclusions, although the system possesses some specific features. The most interesting aspect is that the CGAT (or pGAT) idiotypic specificities are found in all strains of mice that produce anti-GAT antibodies, even though the expression of this repertoire may require in certain strains the attachment of the GAT random copolymer onto a protein carrier (1). Because of this conservation, it was proposed that these specificities could be considered as germline markers (1, 3, 6). The close relatedness of the V regions within each of the CGAT\textsuperscript{+}, GAT-specific V\textsubscript{H} (7) and V\textsubscript{\textalpha} (this paper) families certainly supports this view. It seems, however, that these markers cannot be merely associated to a single V\textsubscript{H}-V\textsubscript{\textalpha} gene product pair, since several, although few, genes are necessary to account for the expression of the entire CGAT\textsuperscript{+} repertoire. At least two V\textsubscript{H} genes, one in BALB/c (7), one in C57BL/6 (8), and two or three V\textsubscript{\textalpha} genes (this paper) seem to be a minimum number so far. Since the expression of these public idiotopes clearly depends upon H-L interactions (4), one must consider that either similar three-dimensional determinants may result from slightly different amino acid sequences, or that they may be contributed to by stretches that are highly conserved between the different chains. In this regard, the situation is not symmetrical when the H and the L chains are considered. The evidence presented in this article clearly points to the high degree of homology that exists between the chains expressed by different strains, even though some strain-specific polymorphism may be present. By contrast, differences were more pronounced whenever H chains pertaining to discrete strains were analyzed. This was clearly the case for the F17 (8) H chain, as compared with the BALB/c counterparts.
This observation might indicate that the L chain plays a major role in the constitution of the idiotope per se, although the necessity of H-L interaction also clearly points to some participation of the H chain in the expression of idiotypic specificities. Wherever the amino acid residues implicated in the expression of the CGAT epitopes are to be found, it may be observed that maximal sequence conservation lies in the first part of both the H (between residues 1 and 54; see reference 7) and L (between residues 1 and somewhere in the middle of FR3; this paper) chains. The final answer will ultimately rely on the precise analysis of the three-dimensional structure of several monoclonal anti-GAT antibodies. The possibility of forming similar idiotypic (or more generally antigenic) determinants with interacting stretches of complementary H and L chains that are homologous but far from identical is certainly gaining more and more supporting evidence.

Summary
A large proportion of (Glu\textsuperscript{60} Ala\textsuperscript{50} Tyr\textsuperscript{10})\textsubscript{n} (GAT)-specific antibodies expresses public idiotypic specificities, termed CGAT (or pGAT), that require the presence of both the heavy and the light chains in order to be expressed. We report in this paper the complete sequence of eight V\textsubscript{\gamma} regions pertaining to eight anti-GAT monoclonal antibodies derived from three strains of mice: BALB/c, DBA/2, and C57BL/6. The methodology used a combination of NH\textsubscript{2}-terminal amino acid and mRNA nucleotide sequencing. All eight sequences analyzed, although highly homologous and all pertaining to the same V\textsubscript{\gamma} I subgroup, allowed definition of three germline genes that are likely to be present in all three strains of mice and also in NZB. It seems likely, however, that any given strain may not necessarily use all three genes for making anti-GAT antibodies. The search for structural correlates of idiotypes could not be framed in a simple picture, but our data suggest that similar idiotopes may result from different interacting primary structures, leading to structural homologies that should be visualized at the three-dimensional level.

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