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Mouse Immunoglobulin Heavy Chains Are Coded by Multiple Germ Line Variable Region Genes

(amino-acid sequence/sequenator/BALB/c mouse/hapten binding/selection)

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ABSTRACT **The N-terminal 20 residues of 13 heavy immunoglobulin chains from myeloma proteins of the BALB/c mouse are compared with the same residues of 15 other heavy chains described in the literature. Sixteen of 28 sequences are different from one another. These proteins fall into four major sets, with 18 of the proteins in the largest set being further divisible into at least five subsets. This pattern of diversity suggests there are at least eight germ line genes coding for the variable regions of mouse heavy chain. Many of the immunoglobulins from which these heavy chains are derived exhibit binding activity for various haptens. The differing hapten specificities are closely correlated with distinct primary amino-acid sequences.**

An understanding of the genetic mechanism responsible for antibody diversity is one of the most intriguing problems in modern immunology, for its resolution may provide insights into the problems of information storage and expression in other complex systems (1, 2). Two alternative hypotheses for antibody diversity have been proposed—the *germ line* theory suggests that diversity arises in antibody genes during the evolution of the species, whereas the *somatic* theories propose that antibody diversity is created anew during the development of each individual by somatic mutation (1–3). Insights into the nature of this problem have come, in part, through the patterns observed from the detailed amino-acid sequence analysis of homogeneous myeloma immunoglobulins. The immunoglobulin molecule is comprised of two different polypeptides, light and heavy chains (4). All immunoglobulin chains can be divided into two segments, the variable (V) and the constant (C) regions, which code, respectively, for the antigen-binding and the various effector functions of the antibody molecule (5). There are three families of immunoglobulin genes, which are unlinked in the mammalian genome. Two code for light chains [lambda (λ) and kappa (κ)] and the third codes for heavy chains (1). The *V* and *C* genes of a given family appear to be coded by separate germ line genes (6). Myeloma proteins from the inbred BALB/c mouse have proven to be a fruitful system for the analysis of amino acid sequence patterns because tumors can be induced at will and are readily transplanted from one individual to a second (7). In addition, these inbred mice are presumably genetically homogeneous, in contradistinction to the genetic polymorphism present in most outbred populations such as humans and rabbits.

Abbreviations: V and C, variable and constant regions of immunoglobulin chains.

Two important patterns relevant to theories of antibody diversity have emerged from the sequence studies on myeloma proteins. First, the V regions from most immunoglobulin families can differ so extensively that all immunologists agree that they are coded by multiple germ line *V* genes. Thus, all theories of antibody diversity are multigenic, the question at issue is how multigenic. Second, when the V regions from large numbers of myeloma light or heavy chains are compared, three to four sections of extreme sequence variability are noted, which are designated "hypervariable regions" (8, 9). X-ray crystallographic studies have established that antibody polypeptides fold so that hypervariable regions constitute the walls of the antigen-binding crevice (10, 11, 32). The presence of hypervariable regions that make up the antigen-binding site has divided somatic theories into two groups. Both agree that the total antibody diversity is coded in part by germ line *V* genes and in part by *V* genes somatically derived from the germ line by mutation and stepwise selection for superior antigen binding sites. One class of somatic theories argues that the selection for somatic variants occurs predominantly, if not exclusively, in the hypervariable regions (2), whereas, a second group of somatic theories argues that somatic variation can occur throughout the entire V region (3).

Previous studies from our laboratories have assessed the diversity present in myeloma proteins from the two light chain families of the BALB/c mouse, λ and κ (12–16). In this paper we extend these studies to include an analysis of the N-terminal 20 amino-acid residues of 28 myeloma heavy chains derived from BALB/c mice. These sequences lie outside the hypervariable regions of the heavy chain and will, accordingly, place different constraints on the various theories of antibody diversity outlined above.

METHODS AND RESULTS

Mouse myeloma proteins were isolated from serum of tumor-bearing BALB/c mice by immunoabsorption for those examples with known specificities; J558 and MOPC 104E on dextran B-1355S–polyacrylamide gel (17), W3129 and W3434 on Sephadex G-75, and S23 on dinitrophenyl (Dnp) Sepharose (18). J606 and Y5606 were precipitated from serum by dialysis against 0.01 M NH_4HCO_3 (19). Y5476, Y2020, Y5444, W3082, and S176 were isolated by the methods described by Potter (20). The purified proteins were partially reduced and alkylated and heavy and light chains were separated as described by Bridges and Little (21). The heavy chains were desalted, lyophilized, and approximately 10 mg per run was loaded on the sequencer.

Tumor Number	Ig Class	Light Chain Type	1 5 10 15 20	Activity
Prototype V_{HI}			EVQLQESGPSLVKPSQ TLSL	
M460	IgA	κ	——————————*——	Dnp
S23	IgA	κ	——————————	Dnp
M315[a]	IgA	λ	D————G————S——	Dnp
Prototype V_{HII}			EVQL QESGPELVKPGASVKM	
J558	IgA	λ	——*——*——————	1,3D
M104E	IgM	λ	——————————	1,3D
Prototype V_{HIII}			EVKL LESGGGLVQPGGSLKL	
Y5476	IgA	κ	——————————	L
W3434	IgA	κ	——————————	1,6D
W3129	IgA	κ	——VI*————*——	1,6D
M173[c]	IgA	κ	————————P——L——	U
H2020	IgA	λ	—Q—V————*——	U
Y5444	IgG2a	λ	—M—V————————*—	U
S10[d]	IgA	κ	——————————	1,6G
X24[d]	IgA	κ	——————————*—	1,6G
X44[d]	IgA	κ	——————————	1,6G
T191[d]	IgA	κ	——————————	1,6G
J539[d]	IgA	κ	——————————	1,6G
J1[d]	IgA	κ	———I————————	1,6G
H8[e]	IgA	κ	———V————————R—	PC
T15[e]	IgA	κ	———V————————R—	PC
S107[e]	IgA	κ	———V————————R—	PC
M603[e]	IgA	κ	———V————————R—	PC
M167[e]	IgA	κ	———VV———————R—	PC
Y5606	IgG3	λ	D—Q—V————*——Z——	TMA
M21A[b]	IgA	κ	D—Q—VQ————————M——	U
M406[b]	IgA	κ	D———Q————————	AM
W3082	IgA	κ	———E————————M——	L
J606	IgG3	κ	———E————————M——	L
Prototype V_{HIV}			EVQLQZSGTVLARPGSSLKM	
S176 major	IgA	λ	S——————————————	5AU
S176 minor	IgA	λ	——————————————	5AU

FIG. 1. N-terminal sequences of BALB/c heavy chains. The one letter code of Dayhoff (28) is: A, Ala; B, Asx; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro, Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp, X, ?; Y, Tyr; Z, Glx; Ig indicates immunoglobulin; Dnp designates dinitrophenyl; 1,3D indicates α-1→3 dextran; 1,6D designates α-1→6 dextran; U indicates unknown specificity; L designates levan; AM designates *N*-acetyl-D-mannosamine; 1,6G indicates β-1→6 galactan; PC indicates phosphorylcholine; TMA designates trimethylamine; and 5AU is 5-acetyluracil. [a] From reference 29. [b] Quoted in ref. 7 and represents sequences. derived from a single sequentor run (J. M. Kehoe and J. D. Capra, personal communication). [c] From 30. [d] From 31. [e] From 24. * indicates uncertainty about amide or residue assignment. M104E indicates MOPC 104E; M406 indicates MOPC 406; the remainder of the complete names for the proteins is given in ref. 16.

Each chain was subjected to 20 steps of automated Edman degradation on an updated Beckman 890A protein sequenator employing a standard Quadrol or DMBA program. Samples were dried under N_2 and converted to the phenylthiohydantoin amino acids (PTH) by treatment with 1 M HCl–1 mM ethanethiol for 10 min at 80°. Following extraction in ethylacetate, 5% of each sample was run on a Hewlett Packard gas chromatograph equipped with dual glass 7.5% DC-560 columns. Glutamine–glutamic acid and asparagine–aspartic acid were resolved by thin-layer chromatography on silica gel (benzene:acetic acid, 9:1) or by silylation of the phenylthiohydantoin derivatives followed by gas chromatography. The amino acids were then regenerated by hydrolysis in hydriodic acid for 18 hr at 120° (22) and analyzed on a Durrum D-500 amino-acid analyzer. Throughout these experiments phenylthiohydantoin norleucine was added to the sequencer tubes prior to conversion, to normalize for any losses due to handling. The methodologies used have been described in detail in a previous publication (16). Yields of approximately 50% of the theoretical values were noted at step 1 and repetitive yields averaged 90% for all chains.

TABLE 1. *Percent homology between mouse and human prototype sequences for the N-terminal 20 residues*

	*MV_{HII}	MV_{HIII}	MV_{HIV}	*HV_{HI}	HV_{HII}	HV_{HIII}
MV_{HI}	65	45	55	40	65	45
MV_{HII}		60	75	70	50	60
MV_{HIII}			60	45	50	85
MV_{HIV}				55	40	60
HV_{HI}					40	50
HV_{HII}						50

* MV_{HI} and HV_{HI} respectively denote the prototypes from mouse and human V_H regions. The human prototype sequences are derived from the data given in ref. 26.

DISCUSSION

Selection of Proteins. The heavy chains used in this analysis were generally selected because the immunoglobulins from which they were derived exhibited specific binding for one or more haptens (Fig. 1).

Patterns of Diversity. The amino terminal 20 residues of 13 heavy chains are compared with 15 sequences taken from the literature (Fig. 1). Sixteen of the 28 sequences differ by one residue or more. By sequence homology, these sequences can be divided into four major sets, which contain, respectively, 22, 3, 2, and 1 proteins. Prototype sequences can be derived for each of these sets by selecting the major residue alternative expressed at each position (Fig. 1) and these are respectively designated prototypes V_{HIII}, V_{HI}, V_{HII}, and V_{HIV}.§ The four prototype sequences differ from one another by 25–55% of their residues (Table 1). Prototype set V_{HIII} can be broken down into at least four subsets that are defined by two or more linked residues. For example, the heavy chains from W3082 and J606 have a glutamic acid at position 5 and a methionine at position 18 that distinguish these proteins from the remaining proteins of this set (Fig. 1). In this fashion, the heavy chains from H8, T15, S107, M603, and M167 as well as Y5606 and M21A can also be divided into two additional subsets by the presence of linked amino acids (Fig. 1).

The variable region of the heavy chain (V_H region) is about 120 residues in length and hence the N-terminal 20 residues represent about 1/6 of the V_H region. Accordingly, a rough

§ The largest set of heavy chains is designated V_{HIII} because the chains show the greatest homology with the human heavy chains belonging to the V_{HIII} subgroup (see ref. 26). We have refrained from using the term V region subgroup for two reasons. First, with the accumulation of large amounts of sequence data it has become apparent that the definition of what constitutes a subgroup is uncertain. For example, within many subgroups, additional groupings are defined using smaller numbers of linked residues. Are these new groupings additional V region subgroups? Second, the term V region subgroup has had the genetic implication that it is encoded by at least one germ line gene. Yet each of the theories of antibody diversity incorporates very different ideas about what types of sequence patterns define additional germ line *V* genes. Thus we feel the term subgroup is undefinable, has confusing genetic implications, and should be replaced by a more neutral term such as "set."

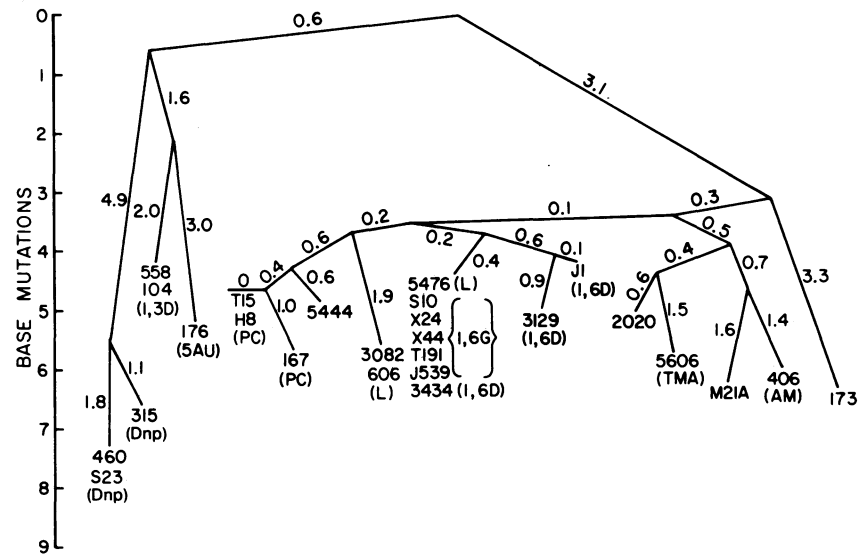

FIG. 2. A genealogic tree of heavy chains from BALB/c myeloma proteins. This tree was constructed using only the N-terminal 20 residues. Many of the immunoglobulins from which these V_H regions were derived bind to one or more haptens. These specificities are shown below the tumor number. Abbreviations: Dnp, dinitrophenyl; PC, phosphorylcholine; 1,6D, α-1→6 dextran; 1,3D, α-1→3 dextran; L, Levan; TMA, trimethylamine (7).

estimate of the number of amino-acid differences between two V_H regions can be determined by multiplying the difference in diversity shown over the N-terminal 20 residues by six (this is an underestimate because no hypervariable regions are included in this region). Thus, proteins in different sets will probably differ by 48 residues or more, whereas proteins within a set can, from this estimate, differ by as many as 24 or more residues. The only two nearly complete mouse V_H sequences from different sets, M315 and T173, are consistent with the above analysis in that they differ by 10 residues in the first 20 and by 62/104 residues and two sequence gaps.

Theories of V Gene Diversity. A more sophisticated examination of these proteins can be carried out by a genealogic analysis (1). The sequences within any set can be related to one another by an evolutionary tree which depicts the minimal number of genetic events (i.e., single base substitutions) required to generate this set of sequences from a single ancestral sequence (gene). This procedure permits a step by step analysis of the single base substitutions required to generate diversity in a given set of sequences. One can then ask what events are compatible only with germ line evolution and what events might be explained by somatic evolution. The genealogic analysis in Fig. 2 shows that the relationships of these sequences to one another are somewhat more complex at the nucleotide than at the protein level because certain single amino-acid differences are coded by two base substitutions. In spite of this, four distinct branches are noted on the genealogic tree, which correspond to the four sets of sequences described previously, V_{HI} through V_{HIV}. The V_{III} branch is divided into five or more sub-branches by the presence of linked nucleotide bases in certain of the sequences (e.g., T15-167-5444; 3082-606; 5476-3129-J1; 2020-5606-M21A-406; and 173—see Fig. 2). Obviously additional data will be required (more and longer sequences) to more sharply delineate this genealogic tree, particularly with regard to the sub-branch structure of V_{HIII}.

The somatic theory which argues for somatic variation only in hypervariable regions (2) and the germ line theory would agree on the conclusions to be drawn from this genealogic tree; namely, each of the V regions that differ from one another is coded by distinct germ line *V* genes that arose by gene duplication, sequence divergence (mutation, recombination, etc.) and selection during the evolution of the species. Thus both of these theories would agree that the diversity noted in these V_H regions must be coded by at least 16 germ line V_H genes. If, however, it is postulated that replacements in the N-terminal 20 residues result from somatic mutation and selection, two questions must be asked. First, how many single base substitutions are reasonable to postulate during the somatic development of a given lymphocyte line? Second, how many parallel (or identical) mutations may occur in lymphocyte lines in separate individuals? If somatic diversification occurs by ordinary random mutation followed by selection, then it would appear likely that relatively few (i.e., 1–5) mutations occur per lymphocyte line because of the need to select and expand out each mutant clone of lymphocytes (2). In addition, no selective forces adequate to explain significant parallel mutation have been postulated to occur somatically, thus each set of sequences with multiple linked residues is probably derived from a different germ line gene (2). Accordingly, from the viewpoint of random somatic mutation that can occur throughout the entire *V* gene, at least eight germ line V_H genes must be postulated for this group of V_H regions, one for each distinct branch and sub-branch of the genealogic tree. A discussion of other somatic theories is beyond the scope of this report.

The mouse heavy chain sequences examined here appear to exhibit less diversity than the mouse κ family and far more diversity than the mouse λ family. This generalization should be qualified, however, because most of these V_H sequences were derived from immunoglobulins selected because they exhibited binding activity for various haptens. Thus, these heavy chains certainly constitute a selected subset of BALB/c heavy chain myeloma pool. The degree of selection that occurred in examining primarily V_H regions derived from myeloma proteins with binding activity is entirely unknown.

It will be very important to analyze the diversity exhibited by unselected V_H regions to obtain a more reliable estimate of the diversity present in the BALB/c pool of myeloma heavy chains. In addition, mouse heavy chains blocked at their N-termini comprise about 78% of the normal serum immunoglobulin (23). Thus it will be particularly valuable to analyze the extent of diversity present in these blocked sequences.

Normal Pooled Mouse Heavy Chains Appear to be More Restricted in Sequence Than the Pool of Myeloma Heavy Chains. A recent study reports that normal pooled mouse heavy chains differ in two respects from the sequences reported here. First, as mentioned earlier, the normal mouse H chains have a blocked α-amino group in 78% of the proteins (23). Only one of 20 myeloma H chains we examined was blocked (HOPC-1). Second, the pool of unblocked mouse H chains obtained from the serum appeared to have a single residue at the 95% level at most of the N-terminal 28 positions. This is in striking contrast to the myeloma data we have reported here. For example, in the pool of myeloma heavy chains, aspartic acid is seen in 14% of the sequences at position 1; glutamine is seen in 32% of the sequences at position 3; the alternatives at position 5 are evenly spread among a number of residues. Thus, the myeloma pool of heavy chains reveals a heterogeneity not detectable by sequencing the normal pool, because the myeloma population, not surprisingly, expands individual sequences that are normally represented infrequently or not at all in the serum pool. Hence one must be cautious about drawing conclusions concerning the nature and extent of V region diversity from studies on normal pooled sequences.

It appears that the myeloma immunoglobulins are themselves highly selected in that 5% or more of them bind to a few determinants (7). This is unexpected in view of the enormous functional heterogeneity of the normal immune response. Accordingly, both the normal and myeloma pools could be interpreted not to represent the true V region diversity that is coded in the BALB/c genome.

One obvious difference between the myeloma pool and the normal serum pool is the fact that the artificial induction of myeloma proteins in BALB/c mice selects primarily IgA proteins, whereas the normal serum pool is mostly of the IgG type (7). Obviously, an analysis has yet to be carried out of the extent and nature of amino-acid sequence diversity in the V_H regions associated with BALB/c γ heavy chains. In any case, a critical question arises. Are the V_H regions associated with $C\gamma$ regions distinct from those associated with C_α regions? If the V_H regions from γ and α myeloma proteins are equally diverse, then the normal and myeloma heavy chain pools express the same family of germ-line V_H genes. If the V_H regions from γ myeloma proteins are less diverse than those from their α counterparts, then selection must occur in the association of certain germ-line V_H genes with given C_H genes.

Some Mouse V_H Regions Are Similar to Their Human Counterparts, Whereas Others Are Quite Distinct. A rough comparison of the similarity of mouse and human heavy chains at their N-termini can be made by comparing the prototype sequences of the mouse and human V_H sets (Table 1). The mouse and human prototypes are quite distinct from one another (30–60%) apart from the mouse and human V_{HIII} sequences (about 15% difference). The comparison of the single nearly complete human V_{HIII} sequence (Nie) with two mouse V_H regions (M173-39% and M315-61%) suggests that the group of mouse sequences to which M173 belongs is more closely related to the human V_{HIII} sequences than other mouse V_H regions are, although they are not as closely related as suggested by the N-terminal data. The fact that many mouse V_H prototypes are as closely related to one another as to their human counterparts suggests that multiple V_H genes existed in the mouse-human ancestor (Table 1). Thus some of the V_H genes in human and mouse may have been derived from a common V_H gene in the human–mouse ancestor, whereas others appear to have diverged from distinct genes in this ancestor. The heterogeneity of the myeloma heavy chains at the N-terminus compared with their normal counterparts also suggests that from the analysis of normal pooled sequences it is not possible to conclude that species- or phylogenetically associated residues are coded by a major portion of the germ-line V genes.¶

The Hapten Binding Properties of Certain Myeloma Proteins Appear to Correlate with Their Heavy Chain Sequences. Six groups of myeloma proteins with binding activity for different haptens are shown in Fig. 1. The binding activity for a specific hapten appears to correlate with the sequence of the N-terminal 20 residues (Figs. 1 and 2). Indeed, each of the major branches on the genealogic tree with just a few sequences correlates with a single hapten binding activity (e.g., dinitrophenyl–V_{HI}, α-1→3 dextran–V_{HII}, and 5-acetyluracil–V_{HIV}). The binding specificities for heavy chains in the V_{HIII} branch generally correspond to the linked sets of proteins or subbranches that occur within this branch (e.g., the phosphorylcholine and levan binding heavy chains). Other studies suggest that the correlation of specificity with distinct amino-acid sequences is much greater with the heavy than with the light chain sequences (24).

The Correlation of Particular Antigen Binding Specificities with Particular N-Terminal Sequences Suggests That Selection for Function Occurs Outside As Well As Within Hypervariable Segments of V Regions. This observation renders unlikely theories of antibody diversity that argue that special hypermutational mechanisms operate only in the hypervariable regions or that the hypervariable regions are coded by separate episome-like genes which are integrated into a framework V gene. Clearly, mutation and selection occur through the entire V gene. The relative contributions of germ line versus somatic diversification to this process remain uncertain.

Heavy Chains May Have a Precursor with Additional Residues at the N-Terminus. The amino-acid sequence analysis of S176 revealed a mixture of two sequences, probably identical to one another but for a single extra residue at the N-terminus (Fig. 1). A recent report has demonstrated that a myeloma light chain synthesized *in vitro* has an additional 10–15 residues on the N-terminus (25) which are, presumably, cleaved off by a post-translational event *in vivo*. Accordingly, we suggest that heavy chains also have a precursor form that is modified post-translationally. The presence of the normal heavy chain in this tumor product suggests that the extra N-terminal residue cannot be accounted for by a mutational event at the DNA level.

¶ Species- or phylogenetically associated residues are those found at a particular position in most of the serum immunoglobulin chains of one species and which are distinct from the homologous residues of a second species (23, 27).

Are There Any Differences in the Set of V_H Regions Associated with λ As Compared to k Light Chains? None of the heavy chains associated with λ chains has a lysine at position 3, a residue found in the heavy chains associated with 18 of 21 κ type molecules (Fig. 1). Eight of nine heavy chains derived from λ type molecules have glutamine at position 3. On the other hand, the V_H regions from M315 and M460 as well as Y5606 and M21A are quite similar, yet in each case one molecule is of the λ and the other of the κ type. Obviously a great deal more sequence data must be available before conclusions can be drawn regarding V_H associations with V_λ and V_κ regions.

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