Variation and Homology in the Mu and Gamma Heavy Chains of Human Immunoglobulins

Abstract. *Sequence analysis of an IgM immunoglobulin shows that the variable regions of human μ and γ1 heavy chains may have twice as much homology as their constant regions and that evolutionary divergence of μ and γ1 heavy chain genes occurred not long after the separation of heavy and light chain genes.*

Because of the importance of IgM immunoglobulin in the primary immune response and in certain autoimmune diseases, we have determined the complete amino acid sequence of the Fabμ portion of a human macroglobulin (*1*). This segment consists of the intact, light chain which is linked by a disulfide bond to the NH_2-terminal portion of the μ heavy chain (the Fdμ piece); in IgM antibodies the Fabμ piece contains the specific antigen-combining site. The protein sequenced (Ou) is from a patient with macroglobulinemia and serves as a model for structural study of IgM antibodies just as Bence Jones proteins and myeloma globulins have served as models for

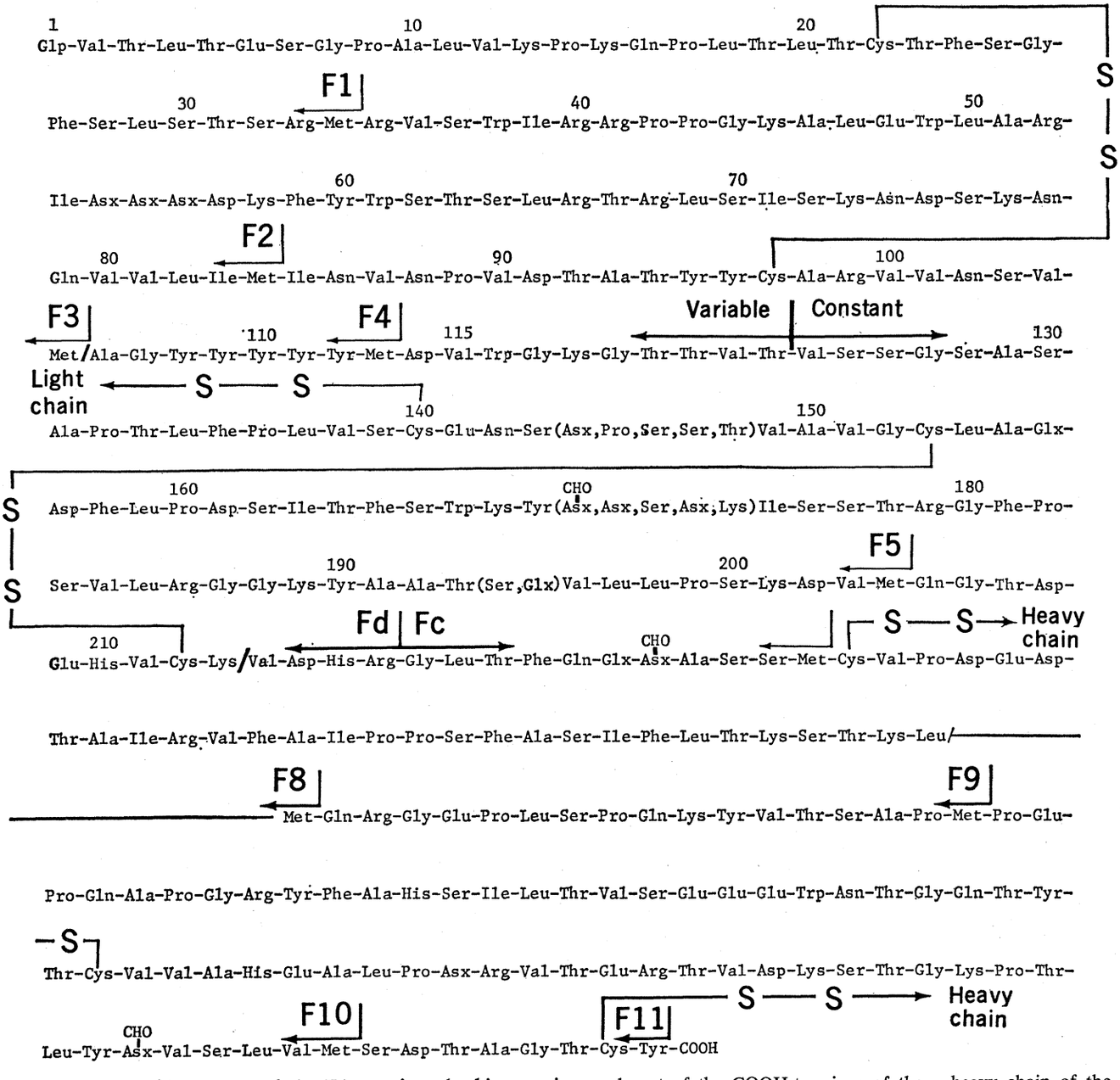

Fig. 1. Amino acid sequence of the Fdμ portion, the hinge region, and part of the COOH-terminus of the μ heavy chain of the IgM immunoglobulin Ou. The CNBr fragments are denoted F1, F2, and so on. The location of three of four interchain disulfide bridges is shown, and that of two intrachain bridges. Carbohydrate (CHO) is identified at three sites in the μ chain and is present at two other sites in the portion of the incomplete sequence in Fcμ. The overlap at F3 and F4 is based on homology to human γ1 chains and that after Lys-213 is still uncertain. The sequence of the CNBr fragment F8 is incomplete. Information on the sequence of the J piece is lacking (*29–31*).

IgG immunoglobulins (*1*). The sequence of the κ light chain is very similar to that of others of the κ_I subgroup and has been published (*2*); the sequence of the first 105 residues of the variable part of this μ heavy chain has been published (*3*). We here report the continuous sequence of 213 residues in the Fd segment of this μ chain, together with the first 40 residues of the Fcμ region (*4*) and the last 88 residues of the COOH-terminus of the Fcμ segment. Whereas the sequence of the variable region (V_H) of this μ chain is closely homologous to that of several human γ1 chains of the same (V_{HII}) variable-region subgroup (*5, 6*), the sequence of the constant region (Cμ) of the μ chain has only about 30 percent homology with that of the constant region (Cγ1) of the human γ1 chain. This is similar to the degree of homology of the constant regions of human heavy and light chains. These results give further support to the concept that two genes code for each immunoglobulin heavy chain (*6–8*): one for the variable region, which may code for the same antibody-specific site in μ and γ chains, and one that codes for the constant region, which is characteristic for each class of heavy chain. Further, they suggest that on an evolutionary time scale, the genes for the constant regions of μ and γ heavy chains diverged almost as long ago as did the genes for the constant regions of light and heavy chains.

The Fabμ and Fcμ fragments were prepared by limited digestion of IgM Ou with trypsin for 1 hour at 60°C and were separated on Sephadex G-200 (*4*). The Fabμ fragment was cleaved with CNBr in 70 percent formic acid, and the CNBr fragments were separated on Sephadex G-100 in 30 percent acetic acid (*9*).

Because of the very large size of the whole μ chain (about 600 residues) and of several of the CNBr fragments, a combination of procedures was needed to assemble the sequence shown in Fig. 1. Thermolysin, chymotryptic, and tryptic peptides were prepared by digestion of the fragments or of the whole μ chain, and the several hundred peptides obtained were sequenced by conventional methods (*9*). Portions of the sequence were determined or confirmed by use of the Beckman protein sequencer (*9*). The resultant sequence (Fig. 1) is complete, except for three small segments, enclosed in parentheses, where the order of the amino acids is not yet determined. Two overlaps are not yet fully established—that between F3 and F4, which is based partly on homology to other heavy chain variable regions (*5*) and the one following Lys-213 at the COOH-terminal end of Fdμ (*10*). By separate procedures, five glycopeptides were isolated from the Ou μ chain (*11*). One of the oligosaccharides is located in the Fdμ region on the asparagine at or near position 170, another is in the hinge region at the beginning of Fcμ, and a third is near the COOH-terminus (Fig. 1).

For purposes of discussion the μ chain sequence can be divided into six regions: (i) the first 100 residues of the variable region, (ii) the remainder of the variable region, (iii) the first homologous segment of the constant region which contains the second disulfide loop of the μ chain, (iv) the hinge region, (v) the incompletely sequenced section of Fcμ, and (vi) the COOH-terminus of Fcμ.

Since our report of the sequence of the NH_2-terminal, 105 residues of the Ou μ chain, comprising the first three CNBr fragments F1, F2, and F3 (*3*), the complete variable sequence has been published for five human γ1 heavy chains, that is, Daw and Cor (*5*), He (*6*) and Eu (*12*), and Nie (*13*). In the first 100 residues, Daw and Cor have about 75 percent identity in sequence with Ou, and He has about 65 percent identity with all three (*14*). The variable region (V_H) of each of these four heavy chains (one μ and three γ1) is of the V_{HII} subgroup (*8*) and shares only about 45 percent identity in amino acid sequence with the variable region of Nie, which is of the V_{HIII} subgroup (*13*), and only about 30 percent identity with the variable region of Eu, which is of the V_{HI} subgroup (*12*). In contrast, all four γ1 heavy chains are believed to have an identical amino acid sequence for all but one (or possibly two) of the 332 residues in their constant regions. Together with more limited data on the NH_2-terminal sequence of other μ, γ, and α chains, these results have led us (*8*) and others (*15*) to conclude that four variable-sequence subgroups are common to heavy chains and that separate genes code for the V_H and C_H regions of heavy chains. As a result, the class character of heavy chains is expressed only in the constant region, and idiotypic differences in sequence are restricted to the variable region where presumably they govern the structure of the specific combining site of antibodies.

Near the end of the variable region heavy chains of different subgroups and different species share a similar sequence that is believed to be close to the antibody-combining site. This is illustrated by the sequence Asp-Thr-Ala-Thr-Tyr-Tyr-Cys-Ala-Arg, which concludes the first 100 residues of the Ou μ chain. An identical sequence is present at the corresponding positions in the Daw and Cor γ1 chains (*5*); only one residue differs in the human γ1 chain Nie (*13*), in the rabbit γ chain (*16*), and in the mouse (MOPC-173) γ2a chain (*17*). Even the human γ1 chain Eu (*12*), which is of subgroup V_{HI}, is identical at two-thirds of the positions in this short sequence. The proximity of this region to the antibody-combining site is indicated by the fact that in rabbit antibodies to DNP (dinitrophenol hapten) the Thr-Tyr sequence contains the tyrosine residue that is very reactive with affinity-labeling reagents (*18*).

The V_H regions of human μ and γ chains differ not only in their specific residues but also in their lengths because of apparent deletions. Here the Ala-Gly-Tyr-Tyr-Tyr-Tyr-Tyr-Met sequence of the F4 fragment of the μ chain is highly distinctive. Although the Cor γ1 chain has an Ala-Gly-Tyr-Met sequence, it lacks the unique pentatyrosine sequence of Ou, which has not been detected in any other immunoglobulin or other protein. This hypervariable region has been implicated as part of the antibody-combining site in rabbit γ chains through affinity-labeling experiments (*18*). Another example of a difference in length is illustrated by the need to insert from four to ten gaps in the γ1 chain sequence relative to the Ou μ chain in order to place the characteristic Val-Thr sequence in register just before the onset of the constant region. Hence, we refer to this as the deletion region. Such deletions may be caused by errors in repair during the joining of V and C genes. Smithies *et al.* (*19*) have pointed to such deletions in immunoglobulin polypeptide chains as evidence for breakage and repair of DNA and suggest that this may be one mechanism through which antibody variability is generated.

The constant region of γ1 chains begins with the sequence Val-Ser-Ser- corresponding to position 115 in the Eu numbering system (Fig. 2). The same starting sequence appears to be-

gin the constant region at position 124 in the Ou μ chain, and there is significant homology between the μ and $\gamma 1$ chains in the first part of the C_H sequence. Whereas the V_H region of $\gamma 1$ chains varies in length from 114 to 118 residues, our results indicate that the V_H region of the Ou μ chain extends for 123 residues and terminates at Thr-123 (Fig. 1). This conclusion is based (i) on the strong homology of V_{HII} subgroup proteins just prior to the onset of the constant region, (ii) on the homology to the $\gamma 1$ constant region shown in Fig. 2, and (iii) on the fact that the sequence around the disulfide bridge at Cys-140, between the light and heavy chains, is the same for at least four different μ chains that we and others (*2, 20*) have studied. Of course, complete sequence analysis of several μ chains (which we are doing) will be required to establish the exact point of division between the V_H and C_H regions of human μ chains.

At the beginning of the constant regions of human κ and λ chains there is a common sequence of Ala-Ala-Pro-Ser-Val-. In one theory of the generation of antibody diversity (*21*) it has been proposed that a common sequence such as this reflects a set of codons in the genes for light chains which acts as a recognition signal for the repair of DNA; alternatively, it may reflect an information point for the joining of the V and C genes for light chains. Although the sequence Ala-Pro-Ser-Val is present in rabbit and guinea pig γ heavy chains, it is lacking in human μ and $\gamma 1$ chains; however, it can be reconstructed by joining together portions of the sequence of the μ and $\gamma 1$ chains at the beginning of the constant regions in Fig. 2; that is, Ala-Pro at positions 131 and 132 in the μ chain and Pro-Ser-Val at positions 123 to 125 in the $\gamma 1$ chain. This vestigial homology probably reflects a common evolutionary origin of the genes for light and heavy chains, as does the

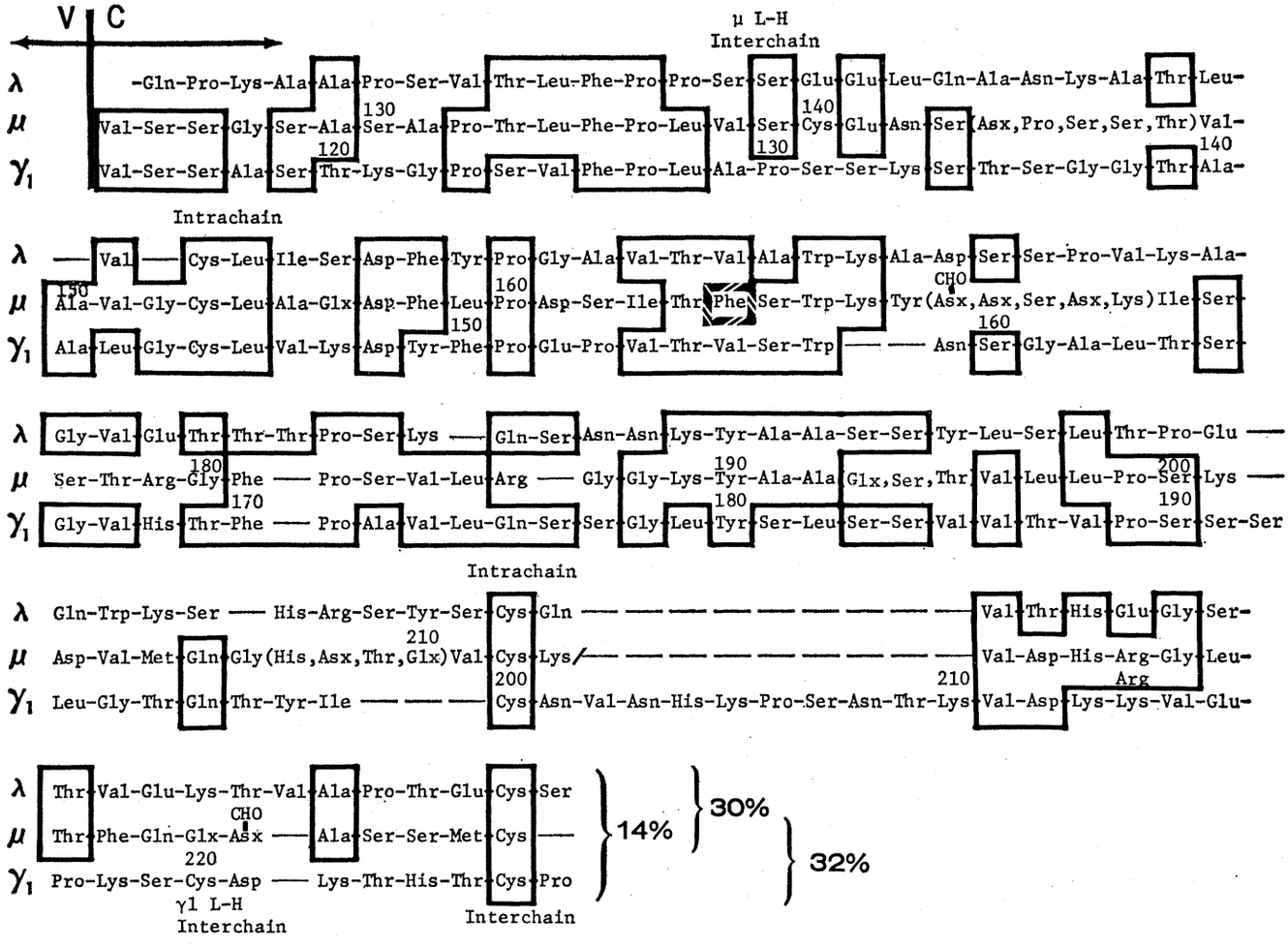

Fig. 2. Comparison of the amino acid sequence of the constant region of the human λ light chain and the first part of the constant region of the human μ and $\gamma 1$ heavy chains. The μ chain (from IgM Ou) sequence was determined in this work, the λ chain sequence was previously reported by our laboratory (*32*), and the $\gamma 1$ chain sequence by others (*5, 6*). The chains have been aligned by placing the intrachain disulfide bridges in register and by inserting gaps to maximize the homology. This procedure also aligns one half-cystine residue in each chain that is involved in an interchain bridge, that is, Cys-212 in the λ chain which links this light chain to a heavy chain, Cys-229 in the μ chain, which links a pair of μ chains, and Cys-226 in the $\gamma 1$ chain, which links a pair of $\gamma 1$ chains. However, the half-cystines that link μ and $\gamma 1$ chains to light chains are far apart, that is, at Cys-140 and Cys-220, respectively. Identical residues at the same positions are enclosed by boxes in solid lines. The numbering system for the μ chain is based on the IgM protein Ou (see Fig. 1) and for the γ chain on the IgG protein Eu (*12*). The sequence shown for the λ light chain (not numbered) comprises the entire constant region from Gln-109 through Ser-213. The percentage figures indicate the degree of homology in sequence for the chains compared, excluding the gaps inserted in the sequence. V, Variable region; C, constant region.

homology elsewhere throughout their primary structure.

The most unexpected finding from sequence analysis of the Ou μ chain is the surprisingly low homology of the constant portions of the human μ and γ1 chains. The homology is greater at the beginning of the constant regions than later in the sequence. The two chains differ significantly in the location of the disulfide bridge to the light chain. In the μ chain this is near the beginning of the constant region at Cys-140, whereas in the human γ1 chain the bridge between light and heavy chains is located some 90 residues further along in the hinge region at Cys-220 (*5, 12*). However, in all other human immunoglobulins studied and in many animal immunoglobulins the bridge between the light and heavy chains is located near the beginning of the constant region of the heavy chain in a sequence strongly homologous to that around Cys-140 in the human μ chain. This has been reported for the following heavy chains: human γ2, γ3, γ4 (*22*), and δ (*23*), rabbit γ (*16*), guinea pig γ2a (*24*), and mouse γ2a and γ2b (*25*).

From the beginning of the C region up to the next intrachain disulfide loop, the μ chain has strong homology to those human and animal γ chains that have been sequenced; however, the homology diminishes rapidly thereafter. One of the major differences is the presence of a complex polysaccharide on the μ chain at Asx-170. Within the first disulfide loop of the C region only a few identical residues can be aligned even when frequent gaps are placed in the human μ and γ1 chains to achieve maximum homology.

The low homology of the human μ and γ1 chains continues throughout the hinge region (*4*). In Fig. 1, the hinge region of the μ chain is represented by the sequence joining Fdμ and Fcμ and continuing beyond the disulfide bridge connecting two μ chains in the monomeric subunit. The hinge region of the human μ chain lacks the proline-rich sequence characteristic of all human and animal γ chains yet studied. For example, in the segment consisting of 41 residues at the beginning of the Fcμ region, there are only three prolines compared to nine in comparable segments of the human γ1 and rabbit γ chains, and 12 in the guinea pig γ2a chain (*5, 12, 16, 24*). The hinge region of the human and animal γ chains contains from two to five interchain disulfide bonds, whereas only one is present in the human μ chain. Finally, a complex polysaccharide containing glucosamine is present just before the interchain disulfide bridge on the μ chain but is absent on the human γ chains (*11*). These factors, that is, the proline content, the number of disulfide bonds, and the carbohydrate content, undoubtedly contribute to a conformational discontinuity in the middle of the heavy chains (*4*). Hence, the hinge region represents an important differentiating characteristic of μ and γ chains and may make a large contribution to their individual biological properties.

Another significant difference is that the human μ chain contains oligosaccharide groups at five sites on the constant region, one in Fdμ, one in the hinge region, two within Fcμ, and one near the COOH-terminus (*11*), whereas the human γ1 and rabbit γ chains have only one. None of the glycopeptides of the μ chain are homologous in sequence or in position with the one in the γ chain.

Although our sequence for the Fcμ region is still incomplete, we have extensive data for the undetermined region represented by CNBr fragments F7 and F8. The sequence of the last 88 residues is complete and includes the heptadecapeptide F9 (*3*), the large CNBr fragment F10, and the COOH-terminal octapeptide F11 (*3*). The latter contains the penultimate half-cystine residue that forms one of the disulfide bridges between the μ chains of the monomeric subunit.

The overall homology in sequence of the constant regions of human μ and γ1 chains is only about 35 percent. For example, in Fig. 2, which compares some 115 positions in the μ and γ1 chains from the start of the constant sequence at Val-Ser-Ser, there are only 33 identities in the 101 pairs of amino acids directly compared, although 18 gaps were placed in one chain or the other to maximize the homology. Even excluding gaps, the homology in this stretch is only 32 percent. There is little homology if the 90 residues of the carboxyl termini of the μ and γ1 chains are compared directly. Although the homology at the COOH-terminus increases to about 35 percent if the last intrachain disulfide bridges in the two chains are aligned, this leaves the last 19 residues of the μ chain with no counterpart in the γ1 chain (*26*). In the same regions the human μ and rabbit γ chains are more similar than expected from the fact that the human and rabbit γ chains are 65 percent identical. This indicates that some sequences have been conserved throughout the evolution of heavy chains.

The evolutionary relationship of the genes for human light and heavy chains is illustrated in Fig. 2, which shows that the μ constant region is remarkably different from the γ1 constant region (they are only 32 percent homologous). Indeed, the constant regions of the μ and λ chains are almost as alike (30 percent homology). This suggests that the constant regions of the μ and γ1 heavy chains diverged during evolution almost as early as did the light and heavy chains.

Maximum homology is achieved only when the half-cystines are aligned to place the intrachain disulfide bridges in register. This supports the hypothesis that all heavy and light immunoglobulin chains originated from an ancestral light chain gene that coded for a primitive immunoglobulin chain of about 110 residues and contained one disulfide loop within the chain (*7, 27–28*). If one accepts an average mutation rate leading to the fixation of one amino acid difference per 100 residues per 10^7 years (*28*), the genes for the constant regions of μ and γ chains must have diverged not long (on an evolutionary time scale) after the separation of primitive light and heavy chain genes but long before the species divergence of primate and rabbit lines. Because the rise in IgM antibody titer precedes that of IgG in the newborn and in the immune response, it has been suggested that IgM preceded IgG immunoglobulin in the course of evolutionary development of antibodies. Until the μ chain sequence is completed and comparisons with the human γ1 and the light chains are made with a computer, the mutation distances cannot be estimated accurately. Even then it may not be possible to conclude whether IgM or IgG came first because they both were derived presumably from a common ancestor.

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References and Notes

1. For the nomenclature of immunoglobulins and for schematic diagrams of the polypeptide chain structure of immunoglobulins illustrating the Fab and Fc pieces, see F. W. Putnam, *Science* **163**, 633 (1969). The variable and constant regions are termed the "V region" and the "C region," respectively, and

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9. The first peak contained fragments bound together by disulfide bridges; the second comprised fragments F1, F2, and F3, which together constitute the first 105 residues in the sequence (*3*) (Fig. 1). The tyrosine-rich peptide F4 was isolated in good yield from the latter part of the Sephadex eluate. The material in the first peak was reduced with 0.2*M* mercaptoethanol in 6*M* guanidine and aminoethylated with ethylenimine to break the disulfide bonds and was reapplied to Sephadex G-100. This separated the κ light chain, fragment F5, and the NH_2-terminal portion of F6. In addition, the whole μ chain was cleaved with trypsin and with thermolysin in separate experiments (*2*). The whole μ chain and the Fcμ fragment were also cleaved with CNBr, reduced in 6*M* guanidine, and alkylated with ethylenimine, and the resultant CNBr fragments were separated on Sephadex. Gel filtration, ion-exchange chromatography, paper electrophoresis, and paper chromatography were used to purify chymotryptic peptides obtained from F5, F6, and Fcμ, tryptic peptides from the whole μ chain and from the CNBr fragments of the whole μ chain and of Fcμ, and thermolysin peptides from the whole μ chain. These were in addition to the tryptic and chymotryptic peptides from F1, F2, and F3 that were earlier sequenced (*3*) and the tryptic and thermolysin peptides that led to the sequence of the κ light chain (*2*). The Beckman sequencer model 890 was used to determine the NH_2-terminal sequence for 10 to 47 steps of various unblocked fragments. The sequence of the tryptic, chymotryptic, and thermolysin peptides was determined by methods already described by us [K. Titani, M. Wikler, F. W. Putnam, *J. Biol. Chem.* **245**, 2142 (1970); T. Shinoda, K. Titani, F. W. Putnam, *ibid.*, p. 4463].
10. Abbreviations for amino acid residues: Lys, lysine; His, histidine; Arg, arginine; Asp, aspartic acid; Asn, asparagine; Asx, aspartic acid or asparagine, identity not established; Thr, threonine; Ser, serine; Glu, glutamic acid; Gln, glutamine; Glx, glutamic acid or glutamine, identity not established; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; Trp, tryptophan; Cys, half-cystine. The first amino acid of the chain is designated Asp-1 and so forth.
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26. We had earlier suggested (*3*) that the 17-residue fragment F9 was homologous to a segment corresponding to residues 338 through 355 in rabbit and human γ chains. However, with this alignment the homology between the μ and γ chains diminishes rapidly after the 17-residue segment. On the other hand, the homology is about 35 percent for a segment of 70 residues if the half-cystines in the last intrachain bridge of the μ and γ1 chains are aligned, that is, Cys-425 in the Eu γ1 chain and the next to the last half-cystine in the μ chain. Yet, in this alignment an additional 19 amino acid residues in the μ chain extend beyond the C-terminal glycine of the γ1 chain. A similar result is obtained when the μ chain is compared with other subclasses of human γ chains and with animal γ chains. This extension has one carbohydrate prosthetic group and one interchain disulfide bridge that are unique to the μ chain.
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Core Binding Energy Difference between Bridging and Nonbridging Oxygen Atoms in a Silicate Chain

Abstract. *The x-ray photoelectron spectra of the oxygen 1s level of olivines contain a single component whereas those of pyroxenes contain two components with an intensity ratio of 2 : 1 and an energy separation of about 1 electron volt. We interpret these two components to be the result of the binding energy differences between nonbridging and bridging oxygen atoms within a silicate chain in the pyroxene structure.*

There have been many photoelectron spectroscopic studies of the shift in the binding energy of core electrons due to various chemical effects such as valence states, bond character, and spin-exchange splitting (*1*). In the second (*2*) of a series of three articles entitled "Molecular Spectroscopy by Means of ESCA" (Electron Spectroscopy for Chemical Analysis) (*2, 3*) Lindberg and his co-workers presented specific correlations between the binding energy of the core electrons of sulfur and the structure of sulfur compounds. We have now observed shifts in the binding energy of the 1*s* level of oxygen atoms caused by the differences in the local environment of oxygen atoms within a silicate chain in the pyroxene structure.

The photoelectron spectra were obtained in a newly designed electrostatic analyzer situated in an oil-free vacuum system with operating pressure in the 1×10^{-8} torr region. Although the spectrometer system is capable of producing 1.35 ev half-width for the photoelectron peak from the $4f_{7/2}$ level of gold with Al$K\alpha_{1,2}$ excitation, its resolution was degraded to 2.2 ev for the experiment reported here in order to enhance the signal-to-noise ratio. The samples were in the form of fine powders (< 400 mesh). An extremely thin layer of the powdered sample was brushed onto an aluminum foil substrate, one side of which was covered with several monolayers of adhesive. Because of the presence of surface contaminants each sample was sputter-cleaned with Ar^+-bombardment at 1.5 kev and 20 μm pressure in an antechamber immediately before measurements. The time interval between the end of Ar^+-bombardment and the beginning of measurements in the high 10^{-8} torr range was approximately 6 minutes.