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Repetitive Hinge Region Sequences in Human IgG3: Isolation of an 11,000-Dalton Fragment

(heavy chain disease/unique DNA fragment/partial duplications/crossing-over)

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ABSTRACT **The heavy chain (γ3) of the IgG3 subclass of human immunoglobulins has a molecular weight of 60,000, instead of the 50,000 value reported for γ1, γ2, and γ4 heavy chains. By use of protein Omm, a γ3 heavy chain disease protein, it was possible to isolate and analyze the extra fragment. Protein Omm had a molecular weight of 40,000, glycine as its sole NH_2-terminal, and contained only the hinge region and the C_H2 and C_H3 domains. CNBr cleavage at Met 252 (γ1 numbering) yielded the hinge fraction (Fh fragment). On the basis of the molecular weight of Fh (11,000), its amino-acid composition, its partial sequence, and its unexpectedly low number of tryptic peptides, it is postulated that the extra fragment in γ3 heavy chains represents a series of similar or identical duplications of sections of the previously reported γ3 hinge region. In addition, there are striking homologies with the hinge region of α1 and α2 heavy chains, one of which also has duplications. The relationship of these hinge structures in different immunoglobulins supports the concept that this region is coded by a unique, small piece of DNA, which has evolved in parallel manner with the immunoglobulin genes by partial duplications and/or crossing-over.**

The heavy chains of IgG (γ) consist of four homology regions or "domains," each of which is made up of about 110 amino-acid residues, and is characterized by a single, highly conserved intrachain disulfide bridge (1). The amino-terminal domain, also known as the variable region (V_H), participates in the formation of the antibody-combining site. The constant region, containing three domains (C_H1, C_H2, and C_H3), is identical for molecules belonging to a given subclass and has approximately 90% homology from one γ chain subclass to another (2, 3). Between the C_H1 and C_H2 domains, in the center of the γ chain, is a region of unique sequence known as the "hinge" or interdomain, which has no homology with any known H or L chain domain. In the different subclasses of γ chains, it contains a large number of proline residues, and a varying number of cysteine residues involved in the disulfide bridges linking the two heavy chains (4, 5).

Previous studies of a γ3 myeloma protein (Kup) demonstrated the presence of five cysteine residues in a 33-residue hinge fragment (6). The elucidation of the nearly complete sequence of this stretch of the molecule indicated an insertion of an extra fragment at the beginning of the hinge, the length of which was not known due to the lack of the overlap peptide between the Fd fragment and the hinge region. However, it was found that the yield of some of the peptides in this study was higher than that obtained from the hinge region of a γ3 heavy chain disease (HCD) protein with an internal deletion (Zuc) (6). This fact, coupled with the observation that the molecular weight of the γ3 heavy chain was 58,000–60,000, compared to about 50,000 for the γ1, γ2, and γ4 heavy chains (7, 8), led to the early suggestion (9) and later demonstration (10) that the difference was due to a larger hinge region consisting of about 100 amino-acid residues.

This report presents the results of the isolation and characterization, from an unusual γ3 HCD protein, of a cyanogen bromide fragment that includes the hinge region. Its molecular weight was 11,000, and it contained about 14 cysteine and 27 proline residues. Based on the isolation of all the radioactively labeled cysteine-containing tryptic peptides from this fragment, it appears that this hinge region consists of a series of unequal duplications involving the various cysteine peptides. In addition to previously noted homologies to the hinge regions of the other three γ chain subclasses (4), it bears a striking degree of resemblance to the hinge of the α1 and α2 heavy chains, one of which also has been shown to have short duplications (11).

MATERIALS AND METHODS

Isolation and Immunologic Studies. The serum of patient Omm contained two closely related γ3 proteins. One appears to include the entire heavy chain and the other is a molecule that consists of the entire Fc region and the hinge. It is not known whether it represents a synthetic product or is derived from the larger one by proteolytic digestion. Both fractions were recovered by starch zone electrophoresis and then separated from each other by Sephadex chromatography. Immunoelectrophoresis and Ouchterlony double diffusion in agar were performed according to standard techniques (12, 13). Antisera to γ chains, Fd, Fab, and Fc fragments, and light chains were similar to those used previously (14).

Physicochemical Studies. All studies were carried out on the small protein. Molecular weight determinations were performed in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate, using a modified Weber–Osborn technique (15). Samples were reduced with mercaptoethanol in some

Abbreviations: Nomenclature of immunoglobulins, their chains, and fragments follows the recommendations of the World Health Organization [*WHO Bull.* (1965) **33**, 721; (1966) **35**, 953; (1968) **38**, 151; (1969) **41**, 975]. Myeloma proteins are designated by the first three letters of the patient's name. HCD, heavy chain disease.

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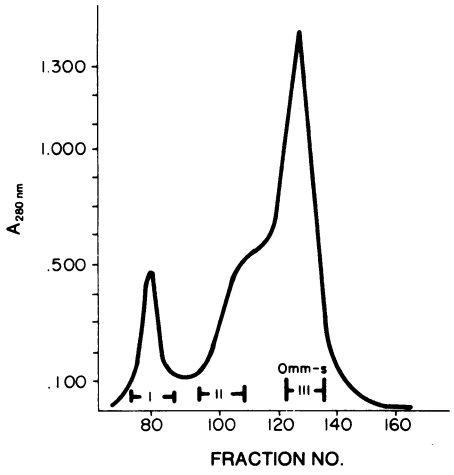

FIG. 1. Gel filtration of γ-globulin fraction of Omm serum on Sephadex G-200 (4.0 × 200 cm) in 0.3 M NaCl, containing 1% butanol. Fractions of 6 ml were collected at a flow rate of 25 ml/hr. Fractions were pooled as indicated.

instances prior to application to the gels. Molecular weight determinations of radioactive cyanogen bromide (CNBr) fragments were performed either by filtration on Sephadex G-50 (16), or, to avoid some of the errors introduced by this technique, by equilibrium sedimentation. The sedimentation equilibrium technique (17) was performed at 30,000 rpm and 20° in a Beckman model E analytical ultracentrifuge equipped with interference optics. The molecular weight determinations were done in solutions from 0.3 to 0.8 mg/ml, and extrapolated to zero concentration.

Dansyl chloride was used to determine the NH_2-terminal amino-acid residue (18), and the dansyl derivative was identified on thin-layer polyamide plates (19, 20).

Chemical typing and diagonal maps were performed as described (5, 21, 22). Peptides with proline as the amino-terminal residue were detected by dipping the paper strip into 0.2% isatin dissolved in 4% acetic acid in acetone and drying at 100° for 5 min. Proline peptides stained bright blue by this method. For complete reduction and alkylation, protein was dissolved in 0.5 M Tris–6 M guanidine–1 mM EDTA, pH 8.6 at a concentration of 20 mg/ml. Reduction was performed with 5 mM dithiothreitol, and alkylation with [^{14}C]iodoacetic acid (0.7 mCi/mmol). Cyanogen bromide digestion was carried out overnight with a 5-fold excess (w/w) of CNBr to protein in 70% formic acid. Reduced and alkylated CNBr fragments were separated on a 200 × 4-cm Sephadex G-75 column in 5% formic acid. Tryptic peptides were identified and isolated by the fingerprint technique, using electrophoresis on paper at pH 3.5, followed by chromatography with butanol–acetic acid–water buffer (4:1.5) in the second dimension (23). Labeled peptides were detected by autoradiography. For large-scale isolation of peptides, completely reduced and [^{14}C]alkylated CNBr fragments were digested with trypsin (enzyme:substrate ratio, 1:50) in 0.2 M NH_4HCO_3, pH 8.5, at 37° for 15 hr. After initial high-voltage paper electrophoresis at pH 3.5, the peptides were isolated by electrophoresis at pH 6.5 and 2.1, as described (20), and by chromatography (23).

Amino-acid analysis was performed on samples hydrolyzed under reduced pressure for 21 hr at 110°, using a Beckman 120C automated amino-acid analyzer. Amino-acid sequence was determined by the Edman degradation, with dansyl chloride (19, 20), and by the Beckman 180C automated sequencer (24).

TABLE 1. *Amino-acid composition[a] of Omm-s and Fh*

	Omm-s	Fh
Lys	27.2	6.4
His	8.4	1.3
Arg	12.2	4.5
Cys	17.7[b]	11.1[c]
Asp	31.9	6.5
Thr	25.6	8.3
Ser	28.0	6.5
Glu	43.4	6.4
Pro	50.3	26.4
Gly	17.3	3.6
Ala	10.4	1.4
Val	28.3	2.2
Met[d]	3.1	0.5
Ile	6.0	
Leu	29.4	5.4
Tyr	12.1	
Phe	11.9	2.5
Trp[e]	4	

[a] Compositions are reported as mol of amino acid per mol of protein, based on a molecular weight of 40,000 for Omm-s and 11,000 for Fh.

[b] Determined as cysteic acid.

[c] Determined as *S*-carboxymethylcysteine. Recovery is not complete.

[d] Determined as methionine sulfone in Omm-s and homoserine lactone in Fh.

[e] Destroyed during hydrolysis. Number assumed on basis of Eu (γ1) sequence (1).

RESULTS

Physicochemical Characteristics of Protein Omm-s. Separation of the γ-globulin fraction of Omm serum on Sephadex G-200 resulted in three peaks, labeled I, II, and III (Fig. 1). Peak I material contained small amounts of IgG and aggregates; the precise nature of the γ3-related protein recovered as peak II is currently under study. Peak III material, which will be called Omm-s, for "short," reacted strongly with anti-Fc serum and not with antisera to L chains, or to Fab and Fd fragments. Its sedimentation coefficient was 3.9 S, and the molecular weight of the reduced and alkylated protein was 40,000. Carbohydrate analysis of Omm-s, kindly performed by Dr. L. Rosenberg, yielded 4.1% hexose, 2.1% hexosamine, and 1% sialic acid. Chemical typing of the partially reduced and alkylated protein showed it to be of the γ3 subclass of IgG, and diagonal map electrophoresis indicated the presence of cystine bridges characteristic of the C_H2 and C_H3 domains (25). The amino-acid composition of the oxidized Omm-s protein is shown in Table 1. Its NH_2-terminus was Gly and the amino-acid sequence of the first ten residues obtained in the sequencer was: Gly-Asp-Thr-Thr-His-?-Cys-Pro-Arg-Cys.

Isolation and Characterization of the "Hinge" by CNBr Cleavage. Omm-s protein was completely reduced and [^{14}C]-alkylated, treated with CNBr, and separated on a Sephadex G-75 column in 5% formic acid (Fig. 2). The first small radioactive peak consisted of aggregates and residual undegraded protein. The second peak, with a high ratio of $A_{280\ nm}$/cpm,

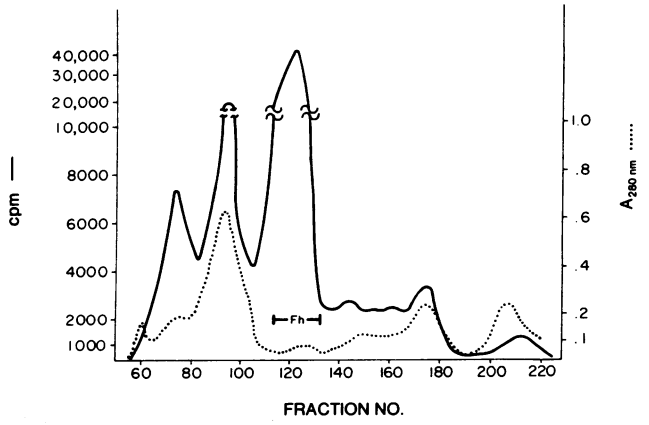

FIG. 2. Gel filtration of CNBr-treated, completely reduced and [^{14}C]carboxymethylated material of peak III (Omm-s) from Fig. 1. The 200 × 4-cm column contained Sephadex G-75 equilibrated with 5% formic acid. Fractions of 6 ml were collected at a flow rate of 25 ml/hr. The third peak contained the hinge, and will be called Fh fragment.

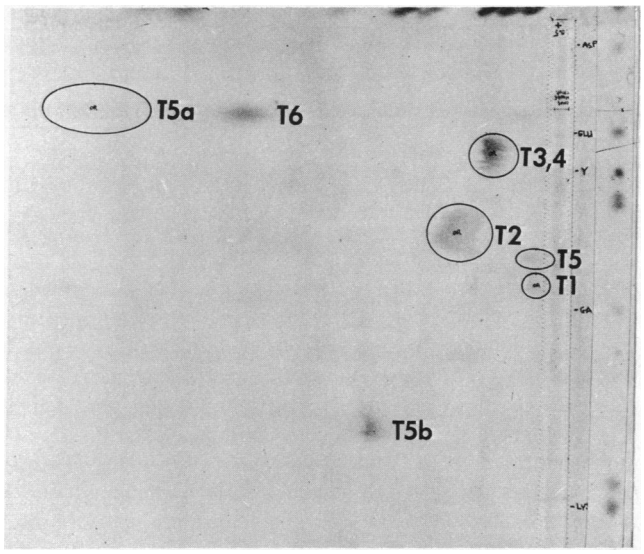

FIG. 4. Fingerprint of trypsin digest of Fh. Electrophoresis at pH 3.5 was performed first with the anode at the top of the picture, and descending chromatography was then performed from right to left. Markers: epsilon-Dnp-lysine, Y; aspartic acid, ASP; glutamic acid, GLU; glycyl-alanine, GA; lysine, LYS. Trypsin, T. Radioactive peptides are circled. Peptide T1, 24,000 cpm; T2, 78,000 cpm; T3,4, 150,000 cpm; T5a, 18,000 cpm. Chlorine stain of this paper revealed no ninhydrin-negative spots (32).

had Ileu as the amino-terminal residue and contained the radioactive peptides belonging to the C_H2 and C_H3 domains (25). The third peak, with low A_{280} nm, had Gly as its sole amino-terminal residue and revealed only the radioactive peptides characteristic of the hinge on chemical typing. Material under this peak was pooled, lyophilized, and used for subsequent physicochemical studies. This peak will be referred to as the hinge, or Fh fragment.

The molecular weight of Fh was determined to be 11,000 by Sephadex gel filtration and by sedimentation equilibrium. Manual Edman degradation of Fh demonstrated Gly-Asp-Thr-Thr-?-Thr-Cys-Pro to be the amino-terminal sequence, and the sequence obtained in the automated sequencer was Gly-Asp-Thr-Thr-His-Thr-Cys-Pro-Arg-Cys-Pro-Glu-Pro-?-Ser, thus clearly showing that Fh is derived from the amino-terminal end of the Omm-s molecule (see above). This sequence is similar to that previously published for another γ3 chain (Kup) (6) (Fig. 3), and completes the sequence around the first cysteine. An extra Thr residue was detected in this fragment which was absent from the analogous peptide in Kup H chain (6). The amino-acid analysis of Fh is shown in Table 1. The values are expressed as the number of residues per 11,000-dalton fragment. No carbohydrate was detected. Also shown in Table 1 is the amino-acid composition of the oxidized Omm-s molecule, based on a molecular weight of 40,000. Since the recovery of *S*-carboxymethylcysteine is usually low, it is not possible to obtain the correct cysteine content from the analysis of Fh. On the other hand, the total number of cysteine residues determined as cysteic acid in the oxidized Omm-s is more accurate. The presence of 17.7 cysteic acid residues, four of which are in the C_H2 and C_H3 domains, suggests that 14 cysteine residues are present in Fh.

Fingerprint analysis of the trypsin-digested Fh fragment (Fig. 4) yielded only five peptides (T5a and T5b are related to T5; see below), instead of 12 expected based on the total number of arginine and lysine residues. This finding raised the

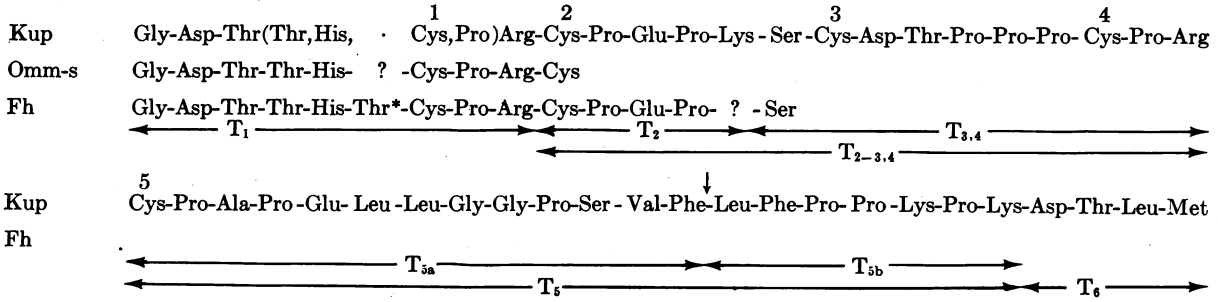

FIG. 3. Amino-acid sequence of tryptic peptides from the hinge region of an IgG3 myeloma protein (Kup) (6) and the partial sequence of Omm-s and Fh as determined by the automated sequencer. Numbers 1–5 indicate the five cysteine residues in the Kup hinge. Tryptic peptides whose composition corresponds to the Kup sequence are also shown. Trypsin, T. *Vertical arrow* indicates site of probable chymotrypsin cleavage. Note the presence of an extra Thr* residue in the sequence of Fh. Ambiguous residues are indicated by question marks. Note that the sequence around cysteines 1 and 4 is identical, and that cysteines 2, 3, and 4 are repeated several times in Fh (not shown) (see *text*).

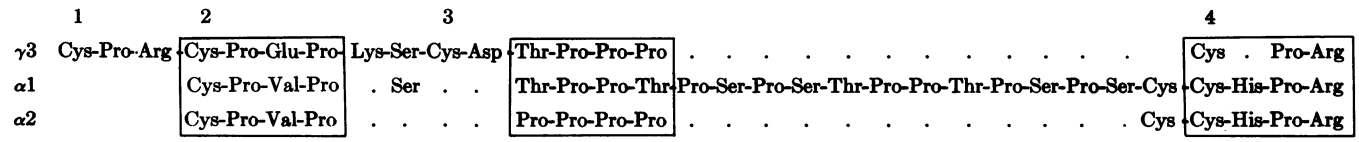

FIG. 5. Comparison of a segment of the hinge region of γ3, α1, and α2 immunoglobulin heavy chains (6, 11). Boxes indicate homologous regions (dots have been introduced to maximize homologies).

possibility that the large number of cysteines might be due to a series of duplications of the four cysteine-containing peptides previously demonstrated in the γ3 hinge (20, 26). To evaluate this possibility, all high-yield tryptic peptides from the completely reduced and alkylated Fh fragment were isolated by high-voltage electrophoresis. Their compositions, yields, and mobilities are listed in Table 2, and their position based on the previously established sequence (6) is also shown in Fig. 3. It should be noted that no cysteine-containing peptides other than those previously described were isolated, clearly ruling out the possibility of additional cysteine-containing peptides in the extended hinge, and pointing to a series of duplications of similar or identical peptides. Peptide T2–3, 4 contains Cys 2, 3, and 4, probably as the result of incomplete tryptic cleavage, while T5a and 5b are probably the result of chymotryptic cleavage of peptide T5. However, the unexpectedly high yield of T5b (Table 2), as well as the total number of Leu and Val residues in Fh (Table 1), remain to be explained. In an attempt to obtain a more precise picture of the nature of the duplication, the labeled peptides from two fingerprints and from a paper electrophoresis of the trypsin-digested Fh fragment were eluted and counted. Fig. 4 shows the results of one of these studies.

DISCUSSION

Previous studies of immunoglobulin heavy chains, except for μ chains (28, 29), have identified a cysteine-rich region in the middle of the chain, linking the two heavy chains by one or more disulfide bridges. The number and arrangement of the cysteine residues are variable and characteristic for each class and subclass. The unique structure of this region, coupled with observations in proteins having deletions (30), has given rise to the suggestion that this region deserves special consideration in attempts to explain the genetic regulation of H chain synthesis. Particular attention has been focused on the γ3 heavy chains, with the recognition of at least five cysteine residues in its hinge, and the discovery that it is approximately 11,000 daltons heavier than the other γ chains. These findings suggest that there may be an extended hinge region in this subclass (7–10). The isolation and determination of the sequence of this extra piece from the intact γ3 chain have proven to be formidable projects, and to date, only the molecular weight and amino-acid composition have been reported (31). However, the availability of an unusual γ3 HCD protein Omm-s, whose hinge appears to be identical to that of the normal γ3 chain, has permitted the ready isolation of the extended hinge (Fh fragment) by simple chemical techniques.

TABLE 2. *Amino-acid composition of tryptic peptides of Fh*[a]

Peptide[b]	T1[c]	T2	T3,4	T2-3,4	T5	T5a	T5b	T6
Lys		1.1		1.1	1.7		2.0	
His	0.9							
Arg	0.9		1.1	1.1				
CMCys-SO_2[d]	0.7	0.8	1.8	2.2	0.8	0.8		
Asp	1.0		1.1	1.4				1.0
Thr	3.0		1.0	1.0				0.9
Ser			1.0	1.1	1.0	1.2		
Glu		1.0		1.1	1.1	1.1		
Pro	0.9	1.8	3.9	5.5	5.6	2.9	2.9	
Gly	1.0				2.2	2.2		
Ala					1.0	1.0		
Val					1.0	0.8		
Met[e]								+
Ile								
Leu					3.1	2.1	1.1	1.0
Tyr								
Phe					2.1	0.9	1.0	
Yield (nmol)	77	152	610	6	12	18	71	34
Mobility (pH 6.5)[f]	0.09	0.38	0.53	0.68	N[g]	0.38	0.67	0.50

[a] Compositions are reported as mol of amino acid per mol of peptide.
[b] Hydrolysis for 21 hr. No corrections were made for destruction during hydrolysis.
[c] T, tryptic peptide (see Fig. 4).
[d] Carboxymethylcysteine sulfone.
[e] As homoserine lactone, present (+).
[f] Mobilities are expressed as fractions of the distance between epsilon-Dnp-lysine and aspartic acid (27).
[g] N, neutral.

On the basis of a molecular weight for Fh of 11,000 and the results of amino-acid analyses and recovery of radioactivity, 12 tryptic and approximately an equal number of radioactive CMCys peptides should have been obtained. Since less than half that number was recovered, the conclusion that there must be a series of duplications seems inescapable. Because the complete sequence of Fh is lacking, the values of 27 proline and 14 cysteine residues are tentative. When compared to the enzymatic Fh fragment studied by Michaelsen (31), the lower content of cysteine and proline, as well as several other minor differences in composition, may reflect differences at the amino- and carboxy-terminal ends of the two fragments and differences in methods of preparation of the hinges studied. Indeed, it is not currently known whether Omm-s is a proteolytic degradation product of an intact IgG3 molecule in peak II (Fig. 1), or is a protein synthesized *de novo*.

Attempts to obtain the sequence of Omm-s and Fh, manually or by automated analysis, yielded an unambiguous sequence for only the first 15 steps, and did not progress far enough to allow us to propose a precise structure for the hinge of $\gamma 3$ H chain. However, the sequences around Cys 1 and 4 are quite similar and, hence, probably the result of a duplication (Fig. 5). On the basis of recovered radioactivity associated with each of the cysteine-containing peptides, it seems likely that Cys 2, 3, and 4 are duplicated several times, (see legend, Fig. 4). The invariable recovery of Cys 3 and 4 in a single peptide, and a small amount of a peptide containing Cys 2, 3, and 4, suggest that these two cysteine-containing peptides are repeated as a pair rather than having each one repeated individually. However, other possibilities cannot be excluded.

The precise arrangement of the disulfide bridges cannot be assessed from currently available data. While the hinge disulfide bonds in the other subclasses of γ chains are involved solely in interchain bridges, there is a distinct possibility that some of the Omm-s extra cysteines form intrachain bridges (26), free sulfhydryl groups, or both. The existence of an intrachain bond extending beyond the hinge cannot be excluded from our study, since the molecule begins at the hinge and was reduced and alkylated before CNBr cleavage. In view of the existence of duplications in the hinge of IgA1 (11), it seems appropriate to compare the structure of the $\gamma 3$ hinge region not only with those of the four subclasses of γ chains (4), but also with those of the $\alpha 1$ and $\alpha 2$ chains. When aligned as in Fig. 5, there are striking homologies between the sequences surrounding Cys 2, 3, and 4 of the $\gamma 3$ chain and the bracketed regions in the $\alpha 1$ and $\alpha 2$ chains.

The occurrence of repetitive sequences in the hinge of several human immunoglobulin heavy chains raises the possibility of gene duplications and/or crossing-over, or other genetic mechanisms, acting on this part of the molecule. A detailed structural comparison of the interdomain regions of immunoglobulin molecules from different species may ultimately provide some understanding of the evolutionary development and the complex genetic control of the different regions of these polypeptide chains.

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