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A γ2b–γ2a Hybrid Immunoglobulin Heavy Chain Produced by a Variant of the MPC 11 Mouse Myeloma Cell Line†

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ABSTRACT: The IgG2b-producing MPC 11 mouse myeloma cell line has yielded a number of variants which synthesize heavy chains characteristic of a different immunoglobulin subclass, IgG2a, as shown initially by serology, peptide maps, and assembly profiles. Primary structural analysis of the immunoglobulin synthesized by one variant, ICR 9.9.2.1, showed that the Fc fragment was most probably identical with that of MOPC 173, an IgG2a protein of known sequence, and different from the parental γ2b Fc fragment. We report here our studies on the protein synthesized by a second γ2a-producing variant, ICR 11.19.3. The Fc fragment of ICR 11.19.3 differed from that of ICR 9.9.2.1 by comparative peptide mapping and was shown by partial sequence determination to contain a long C-terminal stretch of γ2a sequence and a short stretch of γ2b sequence at the amino terminus. Identification of additional residue positions which discriminate between the two subclasses have localized the junction of γ2b and γ2a sequences in ICR 11.19.3 between residues N-308 and N-331, some 8–32 residues N terminal to the C_H2/C_H3 domain boundary. This junction comprises 24 amino acids which, with one possible exception, are identical between γ2b and γ2a subclasses. Our isolation and characterization of CNBr fragments from the N-terminal region of the parental MPC 11 heavy chain allowed us to describe an additional γ2b constant region fragment. The ICR 11.19.3 variant protein contained a homologous fragment which seemed to be identical, thus confirming the presence of γ2b sequence in this region. From these studies, we conclude that ICR 11.19.3 synthesizes a γ2b–γ2a hybrid immunoglobulin heavy chain.

A number of variants synthesizing altered immunoglobulin heavy chains have been isolated from the MPC 11 mouse myeloma cell line. Some variants synthesize short heavy chains of 50 000 and 40 000 M_r (Birshtein et al., 1974), compared to the parental size of 55 000 M_r. Other variants have discontinued synthesis of heavy chains containing the parental γ2b serological markers and now make heavy chains with the serological, peptide, and assembly characteristics of a second subclass, γ2a (Preud'homme et al., 1975; Koskimies & Birshtein, 1976; Francus et al., 1978; Liesegang et al., 1978; Morrison, 1979). We have been especially interested in this latter group of variants since they reflect the activation and expression of previously silent genetic information. Some γ2a-producing variants have appeared in the MPC 11 cell line either spontaneously or after mutagenesis with ICR-191 or Melphalan, while others arose upon recloning of certain primary variants. Secondary variants synthesizing γ2a heavy chains of 55 000 M_r have been derived both from two primary variants synthesizing heavy chains of 50 000 M_r and from the single primary variant which synthesizes a large γ2a heavy chain of 75 000 M_r.

The γ2a variant proteins share the parental idiotype, indicating the retention of at least part of the original variable region gene (Francus et al., 1978). However, they can be distinguished by electrophoretic mobility, peptide maps, and assembly patterns. Several variant proteins could be grouped on the basis of similarities in these parameters. One group consists of the immunoglobulins synthesized by two primary variants derived after Melphalan mutagenesis and one derived after ICR-191 treatment. A second group consists of the immunoglobulins synthesized by four secondary variants derived by recloning primary variants which synthesize short heavy chains of 50 000 M_r. The variant protein synthesized by the ICR 11.19.3 cell line, the subject of this paper, is included in this group (Scheme I). Still other variant proteins did not seem to fall into either of these two groups.

To approach the formidable task of determining the structural defects in these variant proteins, we decided to

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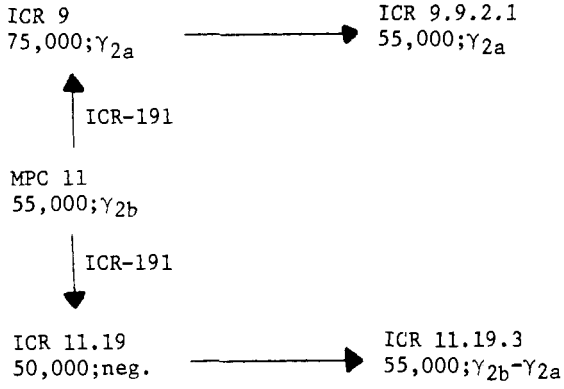
Scheme I: Derivation of Two Secondary γ2a-Producing Variants from the MPC 11 Cell Line (Koskimies & Birshtein, 1976)[a]



[a] The numbers are the molecular weights of the heavy chains as determined by NaDodSO$_4$-polyacrylamide gel electrophoresis. The notation of γ2b, γ2a, etc. shows the presence of subclass-specific serological markers by using commercially available antisera or rabbit antisera prepared in our laboratory. The particular variants described are those referred to in this paper, which have been the focus of detailed structural study. Many other variants have been isolated (Francus et al., 1978).

isolate and characterize the CNBr fragments of the parent γ2b heavy chain, focusing initially on the Fc region[1] (Figure 1), which comprises two-thirds of the constant region. Three CNBr fragments II.1, II.2, and II.3 (Figure 1) were isolated and, together with intact Fc, were individually subjected to partial sequence determination. Since the γ2a sequence was previously determined by Fougereau et al. (1976) (Figure 1), these studies permitted the description of several residue positions which discriminate between the homologous γ2b and γ2a heavy chains (Francus & Birshtein, 1978).

The first γ2a-producing variant we chose to study was ICR 9.9.2.1, a secondary variant derived from ICR 9, a primary variant which synthesizes the long γ2a heavy chain of 75 000 M_r (Scheme I) (Koskimies & Birshtein, 1976). Though especially interesting because of its long heavy chain, the primary variant protein is difficult to study since it is not secreted from the cell, and structural studies upon the small amount of isolatable material will require radiolabeling technology. The secondary variant protein ICR 9.9.2.1 is secreted and can be isolated in quantities suitable for primary structural analysis by classical techniques. The results (Francus & Birshtein, 1978) of immunoelectrophoresis of papain digests, NaDodSO$_4$-polyacrylamide gel electrophoretic analysis of Fc CNBr fragments, and comparative ion-exchange chromatography of radiolabeled tryptic and chymotryptic Fc peptides indicated that the Fc fragment of ICR 9.9.2.1 was most probably identical with that of MOPC 173, an IgG2a protein of known sequence, and distinct from that of MPC 11 (Figure 1). The amino acid analysis and partial sequence determination of Fc CNBr fragments of ICR 9.9.2.1 confirmed this finding. We have chosen to begin detailed structural studies on another variant protein, ICR 11.19.3, because it is a member of a group of four variant proteins that are distinct from ICR 9.9.2.1 in electrophoretic charge, assembly patterns, and peptide maps. In this report, we demonstrate that the heavy chain of ICR

[1] Abbreviations used: Fab and Fc fragments of immunoglobulin are generated by papain cleavage at the hinge region of the H_2L_2 molecule. The Fab includes the N-terminal half of the molecule and the Fc includes the C-terminal half of the heavy chain (see Figure 1). The IgG2b immunoglobulin synthesized by the MPC 11 cell line contains a γ2b heavy chain constant region. The IgG2a immunoglobulin synthesized by the MOPC 173 cell line contains a γ2a heavy chain constant region.

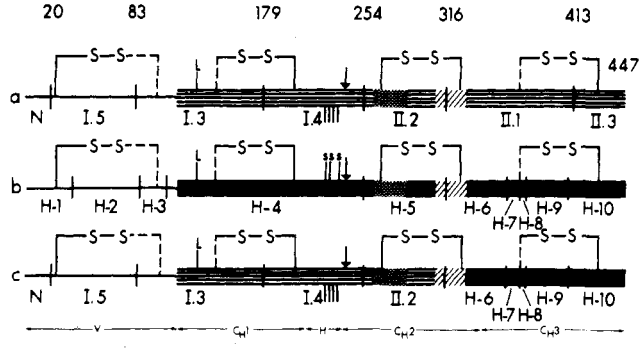


FIGURE 1: Schematic of the structural organization of three myeloma chains. (a) MPC 11, IgG2b; (b) MOPC 173, IgG2a; (c) ICR 11.19.3, a γ2b–γ2a hybrid chain. Vertical lines mark methionine residues and demarcate CNBr fragments. The complete sequence of MOPC 173 has been determined by Fougereau et al. (1976). The isolation of MPC 11 Fc CNBr fragments was described by Francus & Birshtein (1978), and the isolation of the MPC 11 N-terminal CNBr fragments comes from this paper. A description of ICR 11.19.3 rests on the data described in this report. See Chart I and Table II. The shaded areas represent two large stretches of sequence that seem identical in γ2b and γ2a chains with the exception of possible interchanges at N-282 (γ2a-Asp; γ2b-Asn) and N-314 (γ2a-Asn; γ2b-Asp). These segments are defined by primary structural analysis of the MOPC 173 protein and by our studies and the predicted protein sequence established by Tucker et al. (1979a,b) from the sequence of the DNA coding for the MPC 11 protein. Different kinds of shading are used to indicate that these are two nonidentical segments: i.e., not an internal repeat. Of course, other smaller stretches of identical residues are present, but those shaded are the longest and are especially noteworthy. An arrow marks the site of papain cleavage dividing the molecule into Fab (N-terminal) and Fc (C-terminal) fragments. The bottom line shows the general organization of the immunoglobulin gene. Intervening sequences are present at every boundary.

11.19.3 is a γ2b–γ2a hybrid molecule.

Experimental Procedures

The origins of ICR 9.9.2.1 and ICR 11.19.3 are shown in Scheme I. The following procedures have been described previously (Francus et al., 1978; Francus & Birshtein, 1978): maintenance of cell lines, preparation of radiolabeled secreted immunoglobulins, comparative ion-exchange chromatography of peptides, isolation of immunoglobulins from ascites fluid, preparation of CNBr, Fab, and Fc fragments, amino acid analysis, and sequence determination. Tryptic digestions were carried out in 0.05 M ammonium acetate, pH 8. Ion-exchange chromatography was also used to prepare nonradiolabeled tryptic peptides. The column of the cationic-exchange resin (Type-P Chromobeads, Technicon) and the pyridine–acetate gradient (Francus & Birshtein, 1978) were the same as those used for comparative ion-exchange chromatography of radiolabeled peptides. Aliquots of fractions were evaporated to dryness and then redissolved in 10 μL of pyridine–acetate buffer. Samples were applied to Whatman 3M paper, and the paper was stained with cadmium–ninhydrin reagent (Heathcote & Haworth, 1969) to detect fractions containing peptides. The blocked N-terminal residue of fragment N from ICR 11.19.3 was removed by using the enzyme pyroglutamyl aminopeptidase as described by Podell & Abraham (1978).

Nomenclature. The nomenclature for CNBr fragments derived from MOPC 173 is that used by Fougereau et al. (1976), and our numbering of residue positions is the same as theirs. Homologous fragments from variant proteins are identified by an asterisk until they are proved to be identical with the corresponding fragment from MOPC 173. The naming of CNBr fragments from MPC 11 has come from our method of preparation. The H_2L_2 molecule is cleaved by CNBr into two major pieces, separable by gel filtration: pool

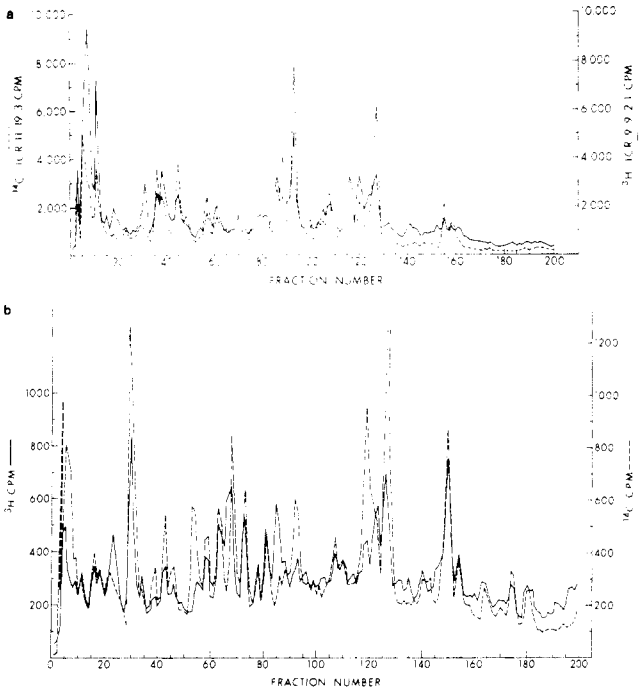


FIGURE 2: Comparative peptide maps of (a) Fab and (b) Fc fragments of ICR 9.9.2.1 (^{3}H) and ICR 11.19.3 (^{14}C). As described under Experimental Procedures, Fab and Fc fragments were prepared from radiolabeled secreted immunoglobulins by using ^{3}H- or ^{14}C-labeled valine, threonine, and leucine. These fragments were subjected to sequential digestion with trypsin and chymotrypsin and separated by ion-exchange chromatography. Fractions were counted in the liquid scintillation counter.

I, the N-terminal portion of the molecule, and pool II, the C-terminal section. Individual CNBr fragments are isolated after total reduction and alkylation and gel filtration and are named in order of elution. The nomenclature of the cell lines is described by Francus et al. (1978). The prefix ICR in ICR 9.9.2.1 and ICR 11.19.3 indicates that the variant was obtained after mutagenesis with ICR-191.

Results

Comparison of Fab and Fc Fragments of ICR 9.9.2.1 and ICR 11.19.3. Since the comparative peptide maps of the heavy chains of ICR 9.9.2.1 and ICR 11.19.3 showed several differences (Francus et al., 1978), experiments were carried out to determine whether these differences were confined to either the Fab or Fc regions. Radiolabeled Fab and Fc fragments of both immunoglobulins were compared by peptide maps. Both Fab and Fc maps showed differences (Figure 2). Since the Fc region of ICR 9.9.2.1 has previously been shown to be indistinguishable from that of a known γ2a myeloma chain (Francus & Birshtein, 1978), this finding implied that ICR 11.19.3 might not contain a complete γ2a constant region.

Identification of γ2b-Specific Residues in the Fc Fragment of ICR 11.19.3. The Fc fragment was prepared from immunoglobulin isolated from the ascites fluid of mice bearing tumors produced by ICR 11.19.3 and then subjected to automated sequential degradation. Chart I shows the results of these studies and compares them to the corresponding sequence determined for MPC 11 and ICR 9.9.2.1. At positions N-237 and N-248, ICR 11.19.3 contains γ2b distinctive residues. This evidence suggested that ICR 11.19.3 might contain a stretch of γ2b residues despite its lack of γ2b-specific serological markers (Francus et al., 1978).

To extend these observations, we isolated CNBr fragments from the C-terminal half of the molecule and analyzed them

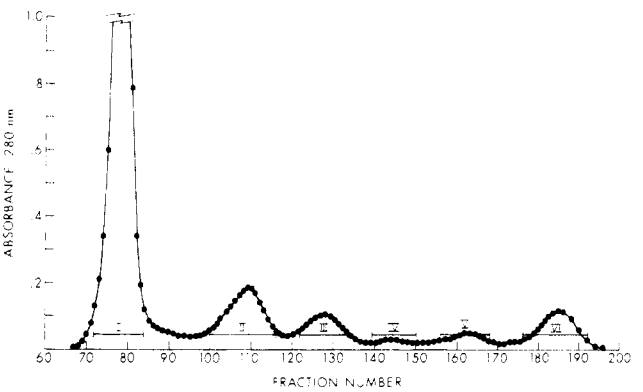


FIGURE 3: Elution profile of the CNBr digest of ICR 11.19.3 H_2L_2 on a column (2.5 × 200 cm) of Sephadex G-100, equilibrated in 8 M urea and 0.1 M formic acid. Fraction volume was ~5 mL.

by amino acid composition and automated sequential degradation. Figure 3 shows the elution profile of the CNBr digest of ICR 11.19.3 (H_2L_2). Pool I contains most of the N-terminal half of the molecule and will be discussed later. After complete reduction of disulfide bridges and radioalkylation, and by use of the methods described previously (Bourgois & Fougereau, 1970), fragments (Figure 1) H-5*, H-6-7*, and H-10* were isolated from pool II, fragments H-5* and H-6* were isolated from pool III, fragment H-9* was isolated from pool IV, and fragments H-7* and H-10* were isolated from pool V. (A fragment corresponding to the pentapeptide H-8 of MOPC 173 has not yet been isolated from ICR 11.19.3. This peptide might be found in pool VI, which we did not examine further.) The amino acid compositions of these fragments are shown in Table I, and the results of automated sequential degradation of fragments of H-5*, -6*, -9*, and -10* are shown in Chart I. The C-terminal fragments of H-6*, -7*, -9*, and 10* were γ2a-like since they seemed identical with the corresponding fragments from MOPC 173 (IgG2a,κ) (Fougereau et al., 1976) and ICR 9.9.2.1 and thus different from the parental MPC 11 (IgG2b,κ) at several positions (Francus & Birshtein, 1978) (Figure 2), but fragment H-5* contained amino acid residues at positions N-258 and N-260 characteristic of the parental γ2b chain. These data confirmed that the Fc region of ICR 11.19.3 was a hybrid γ2b–γ2a constant region.

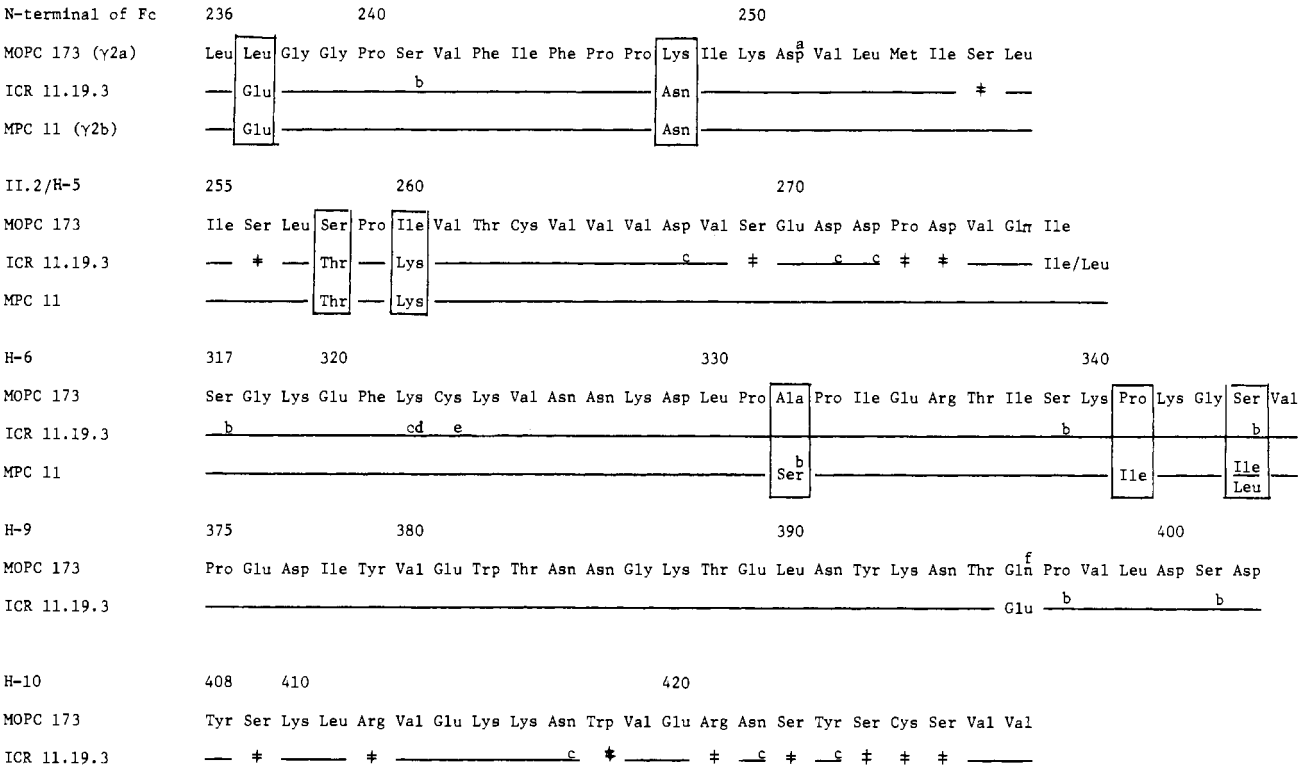
Isolation of Peptides from MPC 11 Containing Additional γ2b-Specific Residues. The preceding sequence analyses localized the junction of γ2b and γ2a sequences between N-260 (in II.2*/H-5*) and N-332 (in H-6*) (Figure 1) because four discriminating positions within N-236–277 (II.2*/H-5*) were γ2b specific (N-237, -248, -258, and -260) while the first discriminating residue in H-6* (N-332) was γ2a specific. A more precise definition of the junction required the identification of additional residues within this segment of 73 amino acids which could distinguish γ2b and γ2a subclasses. We therefore subjected fragment II.2 from MPC 11 (Figure 1) to tryptic digestion and isolated by ion-exchange chromatography two peptides from regions not previously sequenced in the γ2b chain. The amino acid compositions of these peptides and the corresponding peptides for MOPC 173 are shown in Table II. The presence of homoserine in peptide $T^{C\,term}$ places it at the C terminal of II.2 (N-304–316), and the composition of peptide T^{CHO} identifies it as the carbohydrate-containing peptide immediately adjacent to the C-terminal peptide (N-296–303) (Kehoe et al., 1974). Comparison of amino acid compositions of the peptides from the two subclasses allowed the identification of two additional subclass-discriminating residues, both of which could be localized on the basis of the known sequence: residue N-302 in peptide

Table I: Amino Acid Compositions of CNBr Fragments from the Fc Region of ICR 11.19.3 Compared to Those of Homologous Fragments from MPC 11 and MOPC 173[a]

	ICR 11.19.3 II.2 H-5	MPC 11 II.2 γ2b	MOPC 173 H-5 γ2a	ICR 11.19.3 H-6	MOPC 173 H-6	ICR 11.19.3 H-7	MOPC 173 H-7	ICR 11.19.3 H-9	MOPC 173 H-9	ICR 11.19.3 H-10	MOPC 173 H-10
SCM[c]	1.2	0.83	1	0.96	1	0.35	1			0.78	1
Asp	8.0	8.2	9	3.0	3			6.1	7	3.2	3
Thr	6.1	6.4	5	1.6	1	2.6	3	2.6	3	2.7	2
Ser	4.7	5.1	6	2.9	3			1.6	2	4.2	7
Hse	0.53	0.86	1	0.82	1	0.91	1	1.0	1		
Glu	8.7	8.4	8	5.4	5	1.2	1	4.2	4	3.8	3
Pro	3.6	3.6	3	5.1	7			2.1	2	2.0	1
Gly	1.4	0.91		1.9	2			2.1	2	2.2	2
Ala	1.7	1.4	2	1.6	2			0.35		0.62	
Val	9.6	9.5	11	3.6	4	1.1	1	2.0	2	4.2	5
Ile	3.3	4.6	4	1.6	2			0.95	1	0.39	
Leu	2.2	2.7	3	2.0	2	1.0	1	2.0	2	2.1	2
Tyr	1.3	1.1	1	1.1	1			2.5	3	1.8	2
Phe	1.0	1.0	1	0.97	1			1.0	1	1.1	1
His	2.7	2.8	3	0.82						3.1	3
Lys	2.3	1.6		5.1	6	2.0	2	2.0	2	4.1	4
Arg	2.0	2.1	2	2.1	2					2.9	3
Trp			2							+[b]	1

[a] Compositions for CNBr fragments from MOPC 173 are from Fougereau et al. (1976). [b] From automated sequential degradation. [c] SCM, *S*-(carboxymethyl)cysteine.

Chart I: Automated Sequential Determinations of Intact Fc and Fc CNBr Fragments from ICR 11.19.3 Compared with Corresponding Regions Previously Determined for MOPC 173 (Fougereau et al., 1976) and MPC 11 (Francus & Birshtein, 1978)



[a] In our hands, N-251 of MOPC 173 was shown to be Asp. [b] The residue at this position was detected by a single back-hydrolysis. [c] Detected by thin-layer chromatography only. [d] Valine was also detected at this position. [e] Glutamic acid was also detected in this position. [f] In ICR 9.9.2.1, a variant whose Fc seems identical with that of MOPC 173, this position is a glutamic acid. (‡) The residue at this position could not be identified. Boxes mark discriminating positions. The MOPC 173 sequence is taken from Fougereau et al. (1976) with the exceptions noted previously (Francus & Birshtein, 1978). Most of the MPC 11 sequence was determined previously (Francus & Birshtein, 1978). However, additional identifications of the amino acids have been made through studies in our laboratory and, more recently, from the DNA sequence of the MPC 11 γ2b constant region gene (Tucker et al., 1979a,b). The particular residue positions are the following. (1) N-241, previously reported as leucine, is predicted to be serine from the DNA sequence. Sequence studies on a second γ2b protein have also shown serine; however, repeated determinations of MPC 11 show the presence of some leucine. (2) N-269, previously reported as tryptophan, is predicted to be serine from the DNA sequence. (3) N-332, previously not identified, has been shown to be serine by back-hydrolysis of the phenylthiohydantoin derivative from MPC 11 and from a second γ2b chain and is predicted to be serine from the DNA sequence. (4) N-336 is predicted to be arginine from the DNA sequence, and we have confirmed it from isolation of the corresponding tryptic peptide from ICR 11.19.3. (5) N-339, previously not identified, was shown to be serine from the DNA sequence. (6) N-342 and N-343 have been identified from our sequence studies and are consistent with the predictions of the DNA sequence.

Table II: Tryptic Fragments from Fragment II.2 of MPC 11[a] and II.2*/H-5* of ICR 11.19.3[b]

	T^{CHO}			$T^{C\ terminal}$		
	ICR 11.19.3	MPC 11	MOPC 173[c]	ICR 11.19.3	MPC 11	MOPC 173[c]
SCM						
Asp	2.2	1.9	2	1.3	1.2	1
Thr	0.94	1.0	1	1.2	1.1	
Ser	1.0	1.1	1	1.2	1.2	1
Hse				0.45	0.92	1
Glu	1.2	1.2	1	2.0	2.3	2
Pro				0.75	1.0	1
Gly	0.60			0.65		
Ala	0.22			0.48		1
Val	0.41			1.4	1.2	2
Ile	0.71	0.86		0.68	0.89	1
Leu	0.26		1	0.77	0.92	1
Tyr	1.2	1.2	1			
Phe						
His				0.85	1.2	1
Lys						
Arg	0.63	0.78	1			
Trp				ND	ND	1

[a] Tryptic peptides from MPC 11 II.2 were isolated by ion-exchange chromatography using a cationic-exchange resin and a pyridine–acetate gradient, as described under Experimental Procedures. [b] Tryptic peptides from ICR 11.19.3 II.2*/H-5* were first separated by gel filtration on Sephadex G-50, equilibrated in 0.05 M NH_4OH. Pools were made and subjected to amino acid analysis as shown in the table. Further purification by ion-exchange chromatography did not alter the amino acid composition. However, we feel that the 0.48 residue of alanine in peptide $T^{C\ term}$ is a contaminant, along with glycine, since both were found in peptide T^{CHO} as well, which was isolated and analyzed at the same period. [c] Taken from Kehoe et al. (1974).

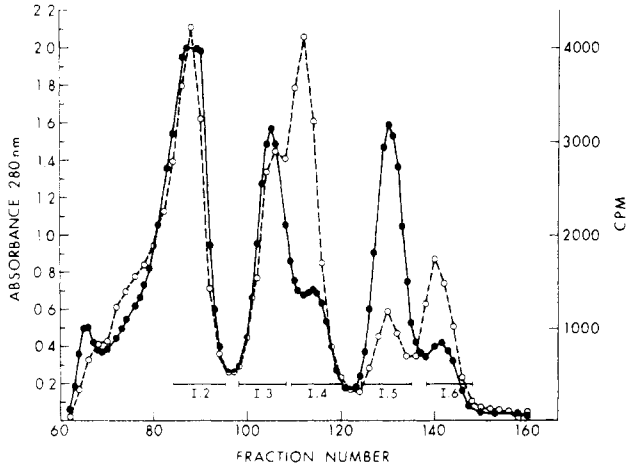


FIGURE 4: Elution profile of pool I of MPC 11 after total reduction and radioalkylation on a column (2.5 × 200 cm) of Sephadex G-75, equilibrated in 8 M urea and 0.1 M formic acid. Fraction volume was ~5 mL. 0.1 mL of every other tube was taken for counting.

T^{CHO} (γ2a-Leu; γ2b-Ile) and residue N-307 in peptide $T^{C\ term}$ (γ2a-Ala; γ2b-Thr).

Localization of the Junction Point between γ2b and γ2a Sequences in ICR 11.19.3. Fragment II.2* H-5* from ICR 11.19.3 was then subjected to tryptic digestion, and peptides T^{CHO}* and $T^{C\ term}$* were purified by a combination of gel filtration on Sephadex G-50 and ion-exchange chromatography. The compositions of peptides T^{CHO} and $T^{C\ term}$ from ICR 11.19.3 are also shown in Table II. Both peptides show the presence of the γ2b-characteristic residues. We thus conclude that residues N-237–N-307 are γ2b-like in ICR 11.19.3 while residues N-332 to the C terminal are γ2a-like. The junction between γ2b and γ2a sequences is therefore localized between

Table III: CNBr Fragments from the N-Terminal Half of the MPC 11 Heavy Chain[a]

	N	I.5	I.3	I.4	I.4 from ICR 11.19.3	I.4 C-terminal tryptic
SCM		0.57	2.9	4.9	4.8	
Asp	1.1	6.5	8.0	6.1	6.2	1.0
Thr	1.2	7.0	8.9	5.7	6.6	
Ser	1.8	5.6	12.6	8.5	9.9	
Hse	0.92	0.74	0.69	0.94	0.41	1.0
Glu	4.1	3.0	7.0	4.9	5.2	
Pro	1.0	3.0	9.8	14.8	14.4	
Gly	2.3	8.4	7.5	3.6	4.6	
Ala	1.4	4.7	4.8	3.2	3.0	
Val	3.0	1.4	7.7	5.4	5.2	0.97
Ile		2.1	3.8	4.5	3.8	
Leu	1.8	1.9	7.1	3.7	3.2	0.97
Tyr		5.4	3.7			
Phe		2.8	3.0	2.2	1.6	
His		1.3	2.0	2.2	2.0	
Lys	0.44	4.0	4.7	6.1	6.1	
Arg	0.89	1.3	1.4			
Trp		+[b]				

[a] This is a single representative amino acid analysis. The compositions were normalized in the following way: N = 20; I.5 = 62 (includes two Trp); I.3 = 97; I.4 = 77. [b] The presence of tryptophan was determined by automated sequential degradation.

Chart II: N-Terminal Sequences of Fab CNBr Fragments of MPC 11

N[a]	PCA Val Gln Leu
I.5	Ser Cys Lys Ala Ala Gly Tyr Thr Phe Thr Asn Tyr
I.3	Glu $\frac{Ile}{Leu}$ ‡ ‡ ‡ Thr ‡ Glu Asp ‡ Ala
I.4	‡ ‡ ‡ Val Thr ‡ Pro ‡ ‡ Thr Trp Pro

[a] N is from ICR 11.19.3.

N-308 and N-331. No further delineation can occur at the protein level since this segment of 24 amino acids seems identical in both γ2a and γ2b subclasses with the sole exception of a possible Asp (γ2b)/Asn (γ2a) interchange at residue N-314. The DNA sequence of MPC 11 (Tucker et al., 1979a) predicts aspartic acid. When isolated from ICR 11.19.3, peptide $T^{C\ term}$, which comprises residues N-304–316, shows similar elution properties to the MPC 11 parental peptide on ion-exchange chromatography, suggesting that it, too, might contain aspartic acid at this position.

Isolation of CNBr Fragments Comprising the N-Terminal Half of the MPC 11 Heavy Chain. The identification of the γ2b segment in ICR 11.19.3 was based on four subclass-specific residues in the amino-terminal segment of the Fc fragment. We wanted to examine the N-terminal portion of the heavy chain constant region to confirm the presence of the γ2b sequence in this region as well. To do this first required that we identify parental CNBr fragments from this region. Accordingly, MPC 11 was subjected to CNBr cleavage, and pools I (100 000 M_r) and II (29 000 M_r) were separated by gel filtration on a column of Sephadex G-100 equilibrated in 8 M urea and 0.1 M formic acid (Francus & Birshtein, 1978). Pool II has been previously shown to contain the C-terminal half of the molecule (Francus & Birshtein, 1978). Pool I was subjected to conditions of total reduction and radioalkylation and was fractionated by gel filtration on a column of Sephadex G-75, equilibrated in 8 M urea and 0.1 M formic acid. The elution profile is shown in Figure 4. Pools I.2 and I.6 were

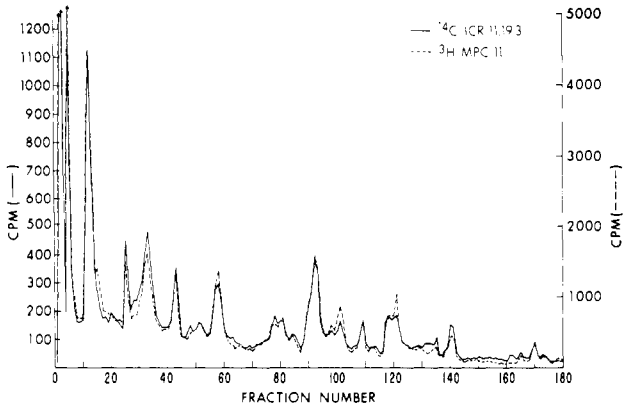


FIGURE 5: Comparative peptide map of I.4 fragments from MPC 11 and ICR 11.19.3. See Experimental Procedures and the legend to Figure 2.

shown by amino acid composition and automated sequential degradation to derive from the light chain of MPC 11 (Smith, 1973). Pools I.3 and I.4 were further separated by ion-exchange chromatography on DEAE-Sephadex using a linear gradient generated with a starting buffer of 0.1 M Tris-HCl, pH 8, and a final buffer of 0.1 M Tris-Cl, pH 8, containing 0.6 M NaCl. The amino acid compositions of I.3, I.4, and I.5 are shown in Table III. The high number of cysteine and proline residues made I.4 a candidate for the hinge-region peptide. Each fragment was then subjected to automated sequential degradation, and the results are shown in Chart II. By comparison with known sequences of immunoglobulin heavy chains (Kabat et al., 1976), fragments I.5, I.3, and I.4 commence at positions N-21, N-84, and N-180, respectively (MOPC 173 numbering) (Figure 1). Thus, fragment I.5 derives only from the variable region and contains two hypervariable regions, fragment I.3 contains the third hypervariable region and the junction between variable and constant regions, and fragment I.4 derives only from the constant region and would be expected to contain the hinge region.

Isolation of the N-Terminal CNBr Fragment of the MPC 11 Heavy Chain. That fragment of I.5 began at position N-21 implied the existence of another CNBr fragment(s) comprising the N-terminal 20 residues (Figure 1). Such a fragment would be released upon CNBr cleavage of the whole molecule but might have gone undetected since the initial gel filtration column using 8 M urea and 0.1 M formic acid as a buffer necessarily was monitored by absorbance at 280 nm and the fragment(s) might well have lacked any tryptophan and tyrosine. Consequently, we searched for such a fragment by making pools of the eluant from the gel filtration column from immediately past pool II to the beginning of the salt peak. These pools were then desalted and the absorbance was monitored at 215 nm. The absorbing material was then separated once again by gel filtration on a column of Sephadex G-50, equilibrated in 0.05 M NH_4OH. Pools were made and analyzed, and a fragment, N, was found which contained ~20 amino acids and whose N terminus was blocked. The composition of this putative N-terminal fragment is shown in Table III. A homologous fragment with identical amino acid composition was isolated from ICR 11.19.3. Treatment of the fragment with pyroglutamyl aminopeptidase, followed by automated sequential degradation, gave the N-terminal sequence in Chart II.

Comparison of the Hinge-Region Fragments of MPC 11 and ICR 11.19.3. The preliminary data on amino acid composition and N-terminal sequence analysis showed I.4 to contain the hinge region and to contain only constant region sequences. To confirm its identity and to place it in the heavy chain, we subjected it to tryptic digestion and isolated the C-terminal tetrapeptide. Its composition is shown in Table III. The sequence of this peptide was Asp-Val-Leu(Hse) and was identical with residues N-251–254 in the Fc fragment of MPC 11 (Chart I). Fragment I.4 is thus placed immediately adjacent to fragment II.2, spanning residue positions N-180–N-254 (Figure 1). One should note that the methionine which serves to separate I.3 from I.4 is also a subclass-discriminating residue (Figure 1).[2]

To examine the N-terminal portion of the heavy chain of ICR 11.19.3, we subjected pool I (Figure 3) to conditions of total reduction and radioalkylation, followed by gel filtration, and found that the elution profile was very similar to that of MPC 11 (Figure 4), implying the presence of a similar set of CNBr fragments. Fragment I.4 was further purified by ion-exchange chromatography, and its amino acid composition, as shown in Table III, seems identical with that of MPC 11. ^{14}C-Labeled immunoglobulin was prepared from cell cultures of ICR 11.19.3, and ^{3}H-labeled immunoglobulin was prepared from cell cultures of MPC 11. Radiolabeled I.4 fragments were isolated as indicated above, and comparative peptide mapping was done. The maps in Figure 5 show that, by this criterion, the I.4 fragments of ICR 11.19.3 and MPC 11 seem indistinguishable. Figure 1 shows the schematic for the structure of the ICR 11.19.3 heavy chain.

Discussion

The most important finding of these studies is that the immunoglobulin heavy chain produced by ICR 11.19.3 is a hybrid protein. Other examples of eucaryotic recombinant protein chains exist: e.g., Lepore ($\delta\beta$), anti-Lepore ($\beta\delta$), and Kenya ($\gamma\beta$) hemoglobins (Weatherall & Clegg, 1979), hybrid IgG4–IgG2 (Natvig & Kunkel, 1974) and IgG3–IgG1 (Kunkel et al., 1969; Werner & Steinberg, 1974) human immunoglobulins, and γ2b–γ2a NZB mouse immunoglobulin (Warner et al., 1966). Recently, molecular biological studies have shown that hemoglobin Lepore is the product of the fusion of δ and β genes which presumably arose from unequal crossing over during meiosis (Flavell et al., 1978). The characterization of the $\delta\beta$ fused gene from the Lepore cells ruled out the possibility that the mutant globin was the result of abnormal processing of δ and β hnRNA transcripts. The mechanism(s) that accounts for any of the immunoglobulin hybrid proteins has not yet been defined. Since ICR 11.19.3 arose during the subculturing of a cell line, one can eliminate meiotic crossing-over as a possibility. Alternate mechanisms include errors in processing at the RNA level, unequal mitotic recombinational events, transposable elements, and activation of a previously unexpressed germ-line gene.

Disorders in splicing cannot account for ICR 11.19.3. The sequence of a germ-line gene coding for the γ2b constant region has recently been determined (Tucker et al., 1979b) and shows that as in γ1 (Sakano et al., 1979) and α (Early et al., 1979) genes, domains are separated by intervening sequences. Such an intervening sequence occurs within the codon for lysine at position N-342 separating the C_H2 and C_H3 domain. The junction between γ2b and γ2a sequences in ICR 11.19.3 lies within the C_H2 domain, some 8–32 residues N terminal to the domain boundary.

[2] Pool I from ICR 9.9.2.1 does not yield fragments I.3 and I.4, implying that the methionine at position N-179 is absent and that the variant heavy chain is γ2a-like at least through this site. This is consistent with the peptide map differences observed between the Fab fragments of ICR 9.9.2.1 and ICR 11.19.3 (Figure 2a).

A plausible mechanism for generating ICR 11.19.3 is a recombinational event. The junction between γ2b and γ2a sequences in ICR 11.19.3 encompasses some 24 amino acids, all of which seem identical between γ2b and γ2a chains with the exception of a possible Asp/Asn interchange at position N-314. Although the DNA sequence coding for the γ2a heavy chain is not yet known, this segment potentially provides a large region of sequence identity that could be the focus for recombinational events such as mitotic recombination, sister-chromatid exchange, or errors in DNA replication that lead to deletion of gene segments.

As mentioned, ICR 11.19.3 is one of a group of four variants, all of which are likely to make γ2b–γ2a hybrid immunoglobulin heavy chains. The proteins made by these variants have the same mobility on agarose gel electrophoresis and migrate differently from MPC 11 and other γ2a-producing variant proteins (Francus et al., 1978). In addition, they show a characteristic assembly pattern and have similar heavy chain peptide maps (Francus et al., 1978). More recently, Oi and Herzenberg, Stanford University (unpublished data), have shown that in contrast to other γ2a myeloma proteins, this group of variant proteins lacks a specific γ2a allotypic marker defined by a monoclonal antibody, a finding which is consistent with the proposed presence of a γ2b segment and the absence of the corresponding γ2a sequence. However, peptide maps have shown small differences between these variant proteins (Francus et al., 1978), and it will be interesting to examine them to see whether and how they might differ from ICR 11.19.3. It is possible, for example, that some could have recombined at a different nearby site: a comparison of γ2b (Tucker et al., 1979a) and γ2a (Fougereau et al., 1976) sequences shows a second sizable segment of amino acid sequence identity spanning residues N-261–281 (Figure 2). Whether other γ2a variant proteins, apart from this group, are likewise recombinant chains with the crossover point located in another portion of the molecule is not yet known. We do know that these proteins also have some peptide map differences (Francus et al., 1978). Additional γ2a-variant proteins, isolated by Morrison (1979), have considerable molecular weight differences as well.

Variant proteins of this type can be valuable reagents in probing different features of the immune response. For example, using ICR 11.19.3 and other characterized variants of MPC 11, we have described the location in the C_H2 domain of a binding site for the γ2b Fc receptor on mouse macrophages (Diamond et al., 1979). In addition, the fact that ICR 11.19.3 bears the standard BALB/c γ2a allotypic markers (deWitt and Bosma, unpublished data; Lieberman, unpublished data) permits us to locate them in the heavy chain C terminal to residue N-332.

ICR 11.19.3 might also have differences elsewhere in the molecule. A study of the variable region is especially warranted since Rajewsky and colleagues (Liesegang et al., 1978) have noted a change in idiotype of a γ2a-variant protein derived from MPC 11. In addition, Scharff and colleagues (Cook & Scharff, 1977; Cook et al., 1979) have observed the appearance of changes in both variable and constant regions of immunoglobulin variants. Although ICR 11.19.3 has been shown to have the same idiotype as MPC 11 (Francus et al., 1978), nonetheless, structural differences may well be present.

Our development of the isolation procedures which we have described for the N-terminal CNBr fragments will enable us to examine the variable region for possible changes. One special feature of the MPC 11 variable region is the location of methionine; it is present at residues N-20 and N-83 and absent from position N-34. Such a pattern is similar to that seen in the cross-reacting idiotype of antibenzene arsonate antibodies from A/J mice (Capra & Nisonoff, 1979) although MPC 11 does not seem to bind this antigen. The isolation procedures for the CNBr fragments of MPC 11 are straightforward and easily applied to these numerous variant proteins. We do not know if this distinctive variable region might be correlated with the ease in generating variants.

How changes in cultured myeloma cells which generate variant immunoglobulins are related to normal cellular events is still unknown. During the course of the maturation of the immune response, heavy chain class changes occur within cells expressing the same idiotype and have been postulated to arise from sequential translocations of variable and constant regions. Thus far, we have seen only γ2b to γ2a subclass switches. However, the potential for generating the known array of different subclasses in vitro seems feasible, and systems of this type may be suitable models for normal mechanisms. The γ2b–γ2a switch is consistent with the gene order proposed by Honjo & Kataoka (1978), based on nucleic acid hybridization studies of myeloma DNA. Whether recombinant proteins, such as ICR 11.19.3, reflect mistakes in translocation and to what degree hybrid proteins may be expressed during the normal immune response are still in question. Such hybrid constant regions could be coded for by separate germ-line genes distinct from both γ2b and γ2a constant region genes.

Unique to our system is the ability to induce or select for this type of recombinational event. Four variants synthesizing recombinant proteins have been generated in the same way as ICR 11.19.3 (Figure 1) (Koskimies & Birshtein, 1976; Francus et al., 1978). We would like to know whether the generation of these variants is dependent on an initial deletion event and whether ICR-191 is central to inducing such a deletion. We would also like to know whether other γ2a variant proteins, which differ structurally from ICR 11.19.3, may be generated by similar mechanisms.

Several new tools to help us understand how these immunoglobulin variants are generated are now available. The sequence of the DNA coding for the MPC 11 γ2b constant region has recently been determined (Tucker et al., 1979a). Probes for various immunoglobulin genes—γ2b, among them (Marcu et al., 1978; Schibler et al., 1978; Yamawaki-Kataoka et al., 1979)—have been prepared. We have characterized a number of variants and have devised procedures to study the variant proteins. The combined approaches of protein and nucleic acid chemistry will help us to decipher the genetic mechanisms involved in generating these variant proteins in hopes of learning more about the regulation of the normal immune response.

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Use of Oligodeoxynucleotide Primers To Determine Poly(adenylic acid) Adjacent Sequences in Messenger Ribonucleic Acid. 3′-Terminal Noncoding Sequence of Bovine Growth Hormone Messenger Ribonucleic Acid†

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ABSTRACT: Twelve synthetic oligodeoxynucleotide primers of the general sequence d(pT_8-N-N′) were tested in a reverse transcriptase reaction for specific initiation of complementary deoxyribonucleic acid (cDNA) synthesis at the poly(adenylic acid) junction of a messenger ribonucleic acid (mRNA) template. Only the sequence d(pT_8-G-C) functioned as a specific primer of cDNA synthesis with an enriched fraction of bovine growth hormone mRNA from the anterior pituitary gland and produced unique fragments in a dideoxy sequencing reaction. The nucleotide sequence obtained by this method extended into the protein coding region of bovine growth hormone mRNA and was confirmed by chemical sequencing of the cDNA initiated with [5′-^{32}P]d(pT_8-G-C). The 3′-untranslated region of bovine growth hormone mRNA is 104 nucleotides in length and contains regions of significant homology with both rat and human growth hormone mRNAs, including the region surrounding the common AAUAAA hexanucleotide. The method presented here for selection of the d(pT_8-N-N′) primer complementary to the poly(A) junction of mRNA is of general applicability for nucleotide sequence analysis of partially purified mRNAs.

An understanding of the biosynthesis and function of a specific mRNA requires a knowledge of its primary structure. Advances in both DNA and RNA nucleotide sequence analysis in the past several years have greatly stimulated studies of specific genes and gene transcripts at the molecular level. The development of two rapid DNA sequencing methods involving termination of growing DNA chains (Sanger et al., 1977) and chemical cleavage of terminally labeled DNA (Maxam & Gilbert, 1977) has contributed most significantly to DNA structural analysis of cloned gene sequences. Occassionally, however, it is possible to isolate significant quantities of specific mRNA molecules from which sequence information may be obtained directly without prior cloning of the mRNA sequences. Indeed, a number of investigators have now adapted

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