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Heavy-Chain Mutants Derived from γ_{2b} Mouse Myeloma: Characterization of Heavy-Chain Messenger Ribonucleic Acid, Proteins, and Secretion in Deletion Mutants and Messenger Ribonucleic Acid in γ_{2a} Mutant Progeny†

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ABSTRACT: Mouse myeloma mutants isolated from cell line 45.6 (γ_{2b}) producing structurally altered immunoglobulin heavy (H) chains have been characterized. The mutant 10-1 synthesizes an H chain of 47 000 daltons containing a CH_1 deletion; two mutants, G251 and I17, derived from 10-1 synthesize H chains of 40 000 and 35 000 daltons, respectively. The messenger ribonucleic acids (mRNAs) in these mutants have been shown to be smaller in molecular weight than mRNAs produced in 45.6 cells and lack a portion, but not all, of the CH_1 domain. The H chains of G251 and I17 no longer express IgG subclass-specific determinants, are not secreted, and are structurally altered in the carboxy-terminal portion of the molecule. In vitro the mRNAs of the mutants code for the synthesis of a polypeptide precursor characteristic of secreted proteins; the shortened proteins are apparently glycosylated intracellularly. Somatic cell hybrids between a structurally altered nonsecretor and a drug-marked wild-type myeloma cell secrete only the wild-type protein. Reversion to secretion for G251 or I17 is accompanied by a change in the amino acid composition of the H chain such that γ_{2a} subclass-specific determinants are expressed. Therefore, the primary structure of the H chain is an important factor in determining secretion. The γ_{2a}-secreted chains from G251 and I17 fall into two classes: (1) those synthesizing proteins of ~47 000 daltons producing H-chain mRNAs of ~1.66 kilobases that are deleted for a portion, but not all, of CH_1; (2) those synthesizing γ_{2a} proteins of ~55 000 daltons that are encoded in mRNAs of apparently wild-type size and that have regained CH_1 sequences. The molecular explanations for the production of these alterations is discussed.

Cultured myeloma cells provide a suitable system in which to study the biosynthesis of proteins, mutational events, and gene rearrangements in eukaryotic cells. The myeloma cells are derived from a plasma cell tumor and usually synthesize a single immunoglobulin (Ig) molecule. Both the wild-type and mutant genes and gene products of such cell lines are available for detailed characterization.

The multiple domain structure of the Ig heavy (H) chain protein is reflected in the deoxyribonucleic acid (DNA)[1] encoding it. For example, the active IgG (γ_{2b}) heavy-chain gene of the mouse myeloma has intervening sequences separating the V, CH_1, hinge, CH_2, and CH_3 domains (Maki et al., 1980). In both heavy and light chains, the variable (V) region of the expressed gene is constructed from the rearrangement of discrete DNA sequences (Sakano et al., 1979; Max et al., 1979; Early et al., 1980; Schilling et al., 1980). Each antibody-producing cell appears to be committed to the production of a defined V_L, C_L, and V_H for its entire differentiation from B lymphocyte to plasma cell. However, many different heavy-chain constant region genes may be expressed with one V_H. Initially, a B cell expresses C_μ (Raff, 1976); these immature B cells may further develop to produce another class of heavy chain. This class switch seems to be accomplished by a DNA rearrangement (Davis et al., 1980) presumably associated with the deletion of preceding heavy-chain constant region genes (Honjo & Kataoka, 1978; Cory & Adams, 1980; Rabbits et al., 1980; Coleclough et al., 1980). In addition to normal lymphocytes, myeloma cells in culture also have been shown to switch the subclass of IgG being produced

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[1] Abbreviations used: DNA, deoxyribonucleic acid; cDNA, complementary DNA; RNA, ribonucleic acid; mRNA, messenger RNA; hnRNA, heterogeneous nuclear RNA; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate; poly(A), poly(adenylic acid); SSC, standard saline citrate; EDTA, ethylenediaminetetraacetic acid; VTL, valine, threonine, and leucine.

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(Preud'homme et al., 1975; Leisegang et al., 1978; Morrison, 1979). Study of these myeloma mutants that have switched the class of Ig synthesized may shed light on the normal process of H-chain class switch.

As is true for many other eukaryotic mRNAs, Ig heavy-chain mRNA is derived from a large nuclear precursor RNA molecule that is the primary transcript of the gene (Schibler et al., 1978). In MPC-11, a γ_{2b}-expressing mouse myeloma, the primary transcript of the γ_{2b} gene is 11 000 bases long. This precursor contains noncoding intervening sequences that are removed during RNA processing resulting in the mature 1.8-kilobase (kb) H-chain mRNA that is transported to the cytoplasm.

It is possible to genetically alter expression of the Ig genes so that aberrant Ig heavy-chain proteins are produced. Base substitutions, point mutations, and shifts in reading frame due to insertion or deletions have been shown to occur in Ig genes (Adetugo & Milstein 1977a,b; Baumal et al., 1973). Intervening sequences in genes may also serve as sites of mutation; improper splicing could lead to deletion of whole domains or portion thereof or to insertion and translation of previously untranslated intervening sequences.

A spontaneously occurring mutant (10-1), synthesizing a γ_{2b} heavy-chain Ig with a deletion of a portion of CH_1 has been described (Morrison, 1978). In this paper, we show that the mRNA of mutant 10-1 lacks a portion but not all of the CH_1 domain. Two mutants (I17 and G251) derived from 10-1 also have H-chain mRNA which lacks a portion of the CH_1 domain. These secondary mutants which produce even shorter H chain than 10-1 must have additional mutations elsewhere in the mRNA. Mouse myeloma mutants have been isolated which have switched from the expression of γ_{2b} subclass heavy chain of 45.6 to the expression of γ_{2a} heavy chains. Subclass switch mutants can be isolated either directly from 45.6 or from the short-chain mutants (10-1, I17, G251) with CH_1 deletions. The γ_{2a} producers derived from CH_1-deleted proteins are of two size classes: those which are normal sized and those which are approximately the size of 10-1 (Morrison, 1979). In this paper, we show that shorter mRNAs, with CH_1 deletions, are present in the producers of shortened γ_{2a} proteins. Full-length mRNA containing no detectable deletions in CH_1 was found in γ_{2a} producers synthesizing wild type sized heavy chain, suggesting that in these cells the defective γ_{2b} gene of the parent is no longer expressed but instead that a new constant region is being transcribed.

Peptide analyses of the heavy chains of the mutants G251 and I17 have confirmed their altered structure. In vitro translation studies demonstrate that these shortened heavy chains are not due to partial intracellular degradation, that a precursor form characteristic of secretory proteins is synthesized from the mRNA of the mutants, and that the mutant heavy chains appear to be glycosylated intracellularly. Hybrid cells producing mutant and wild-type heavy chains secrete only wild-type H chains suggesting that the lack of secretion of the mutant protein is a consequence of its altered structure and not of some change in cell function. Mutants that have reverted to the secretion of larger molecular weight heavy chains, isolated from G251 and I17 (Morrison, 1979), are γ_{2a} serologically and therefore altered in structure. Secretion of the H chain in the secondary mutants suggests that it is the primary structure of the H chain which determines if it is secreted.

Materials and Methods

Cells. The 45.6 mouse myeloma cell line (IgG_{2b}) was adapted to tissue culture from the MPC-11 tumor (Laskov & Scharff, 1970). The 4T001 cell line derived from 45.6 and resistant to 5 μg/mL thioguanine and 2.5 mM ouabain was obtained from M. D. Scharff. Spontaneously arising and mutagen-induced mutants were isolated as previously described (Morrison, 1978, 1979). The mouse myeloma cells were grown in Dulbecco's Modified Eagle's Medium (Grand Island Biological Co., Grand Island, New York), supplemented with 20% heat-inactivated horse serum (30 min at 56 °C), nonessential amino acids, glutamine, penicillin, and streptomycin.

Isolation of Myeloma Cell RNA. For preparation of cytoplasmic RNA, the cells were washed once in cold isotonic phosphate-buffered saline and lysed in 0.01 M NaCl, 0.01 M Tris-HCl (pH 7.4), and 0.0015 M $MgCl_2$ containing 1% Nonidet P-40 (Shell Chemical Co., London, England). Nuclei were pelleted at 2000*g* for 10 min, and the RNA was isolated from the cytoplasmic supernatant by cesium chloride centrifugation (Glisin et al., 1974). The isolated RNA had an optical density ratio at 260:280 nm of 2.05–2.1. A^+ RNA was prepared by passage over an oligo(dT)–cellulose (Collaborative Research, Waltham, MA) column. Approximately 2.0–2.5% of the total RNA bound to the column. The A^+ RNA was relatively free of ribosomal RNA as determined by the optical density profiles on sucrose gradients and agarose gels.

Preparation of Labeled Cell Products. Cytoplasmic and secreted Ig's produced by cells were labeled with [^{3}H]- or [^{14}C]-L-valine, -threonine, and -leucine (Schwarz/Mann Radiochemicals, Orangeburg, NY) as previously described (Morrison, 1979). Immune precipitation of labeled Ig was as described below. For preparation of nonglycosylated heavy chains, cells were preincubated for 30 min at 37 °C in Eagle's spinner medium lacking VTL and containing glucosamine at 4.5 mg/mL, then labeled in radioactive medium containing 4.5 mg/mL glucosamine, and processed as above. This level of glucosamine has been shown to inhibit glycosylation (Nakamura & Compans, 1978; Klenk et al., 1972).

Immunoprecipitation of Radioactively Labeled Cell Lysates, Secretions, and in Vitro Translation Products. The labeled immunoglobulins were reacted in antibody excess with a rabbit antiserum specific for MPC-11 light and heavy chains for 1 h at 4 °C; the soluble complexes were precipitated on *Staphylococcus aureus* protein A insolubilized on formalin-fixed bacteria (Kessler, 1975). After being washed, the immune-precipitated material was eluted from the adsorbent by adding 2% $NaDodSO_4$ and heating for 2 min at 100 °C. Alternatively, the soluble immune complexes were precipitated by using sheep anti-rabbit Ig added at equivalence.

Polyacrylamide–$NaDodSO_4$ Gel Analysis. In vitro translated products were analyzed on Tris–glycine–$NaDodSO_4$ polyacrylamide gels (Maizel, 1971). For 7.5% acrylamide gels the bis(acrylamide)/acrylamide ratio was 0.016 while for 17.5% gels the ratio of bis(acrylamide)/acrylamide was 0.004. Varying the ratio of bis(acrylamide) to acrylamide in the gels allowed the drying of the higher percent acrylamide gels without cracking. Analysis of CNBr fragments was done on 12% acrylamide gels containing 8 M urea, 0.1 M sodium phosphate (pH 7), and 0.1% $NaDodSO_4$; the running buffer was the same except urea was omitted. All analytical gels were run as slabs. Slab gels were processed for fluorography as described (Bonner & Laskey, 1974; Laskey & Mills, 1975).

Cell-Free Protein Synthesis. The wheat germ extract was prepared as described (Roberts & Paterson, 1973) except that the preincubation step was omitted. Active batches of wheat germ were prepared from several sources (Bar Rav Mil., Israel, Niblack Foods, Rochester, NY, and General Mills, Battle Creek, MI). High specific activity [^{35}S]methionine (500–1000

Ci/mmol, Amersham, Arlington Heights, IL) was used for translations. Immunoglobulin heavy-chain RNA was best translated under the following conditions: 155–160 mM K^+, 2.5 mM Mg^{2+}, 0.003 mM spermidine, and 30 °C for 60 min.

Recovery of Chains and Fragments and Peptide Mapping. Intact radiolabeled H and L chains were recovered from cylindrical 5% acrylamide gels, as previously described (Morrison, 1979). CNBr digestion was done in 70% formic acid by using CNBr at a concentration of 100 mg/mL. Peptide mapping of tryptic or tryptic plus chymotryptic peptides was done as previously described (Morrison, 1979) by using a pyridine acetate gradient and a Technicon P-2 resin.

Somatic Cell Hybridization. Fusion of TG^ROu^R 4T001 cells and Ou^S mutant cells was effected by using 35% poly(ethylene glycol), pH 8.0, essentially as described by Sharon et al. (1980). After hybridization, cells were grown for 6 h in nonselective medium, centrifuged out, and resuspended in medium containing hypoxanthine–aminopterin–thymidine (HAT) (Szybalski et al., 1962; Littlefield, 1964) and 2.5 mM ouabain. HAT selects against TG^R 4T001. After 40 h, cells were removed from the medium by centrifugation and cloned in soft agarose over rat embryo fibroblasts in HAT medium. After ~10 days of growth, hybrid cell clones were recovered in medium containing hypoxanthine–thymidine (HT) and 2.5 mM ouabain.

Gel Electrophoresis and Analysis of RNA. Myeloma A^+ RNA was electrophoresed on 1.0% agarose gels containing methylmercury hydroxide as described (Bailey & Davidson, 1976). The aminobenzyloxymethyl paper (ABM paper) was prepared, and RNA was transferred from the gels and hybridized as described (Alwine et al., 1977) by using 100 μg/mL poly(A) in the hybridization mix.

Myeloma A^+ RNA was also electrophoresed on 1% agarose gels with formaldehyde by a modification of the technique of Schwinghamer & Shepherd (1980). RNA was blotted from the gels to nitrocellulose filters with 10 × SSC, and the filters were baked at 80 °C in vacuo for 5 h. Filters were blocked and hybridized at 42 °C (Stark & Williams, 1979) and washed with 5 × SSC (fragment A blots) or 2 × SSC at 42 °C.

Agarose Gel Electrophoresis of DNA Fragments. Plasmid DNA or fragments were analyzed on agarose gels containing 0.04 M Tris-HCl (pH 7.0), 1 mM EDTA, and 4 mM sodium acetate with the same concentrations of reagents in the running buffer. Following separation by electrophoresis, the appropriate DNA bands, visualized by ethidium bromide (EtBr) staining, were electroeluted from gel slices in dialysis bags containing electrophoresis buffer. DNA fragments were precipitated with ethanol, extracted 3 times with buffer-saturated phenol and 1 time with chloroform–isoamyl alcohol (25:1), and ethanol precipitated again. To test the purity of the isolated DNA fragments, we fractionated plasmid DNA cut with restriction enzymes on agarose gels, blotted it to nitrocellulose (Southern, 1975), and hybridized it with nick-translated ^{32}P-labeled purified fragment. The pure fragments hybridized only with the appropriate DNA pieces.

Nick Translation Reaction. The $p\gamma_{2b}$ (11) plasmid and DNA fragments from it were nick translated to high specific activity with ^{32}P-labeled dXTPs according to the procedure to Maniatis et al. (1975) using *Escherichia coli* DNA polymerase I (Boehringer-Mannheim). The DNA (0.1–1 μg) was first treated with 1 ng of DNase I for 5–10 min at 37 °C, then the DNA polymerase I was added, and the reaction was incubated at 15 °C for 60 min. The DNA specific activities obtained were 2×10^8–5×10^8 cpm/μg.

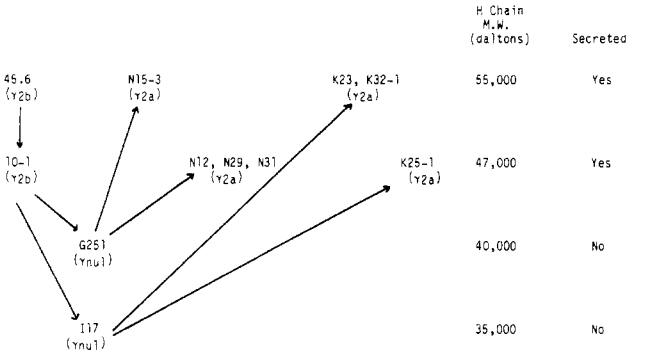

FIGURE 1: Interrelationship of heavy-chain mutants analyzed.

The DNA was nick translated to low specific activity for mapping by omitting DNase I. The specific activity of the DNAs obtained by this procedure was 1×10^7–2×10^7 cpm/μg. The reaction was extracted three times with phenol and with chloroform–isoamyl alcohol (25:1). The [^{32}P]DNA was recovered by ethanol precipitation.

Restriction Enzyme Analysis of $p\gamma_{2b}$ (11). The $p\gamma_{2b}$ (11) plasmid was generously supplied by Drs. R. Perry and K. Marcu. The plasmid was grown as described (Schibler et al., 1978). The plasmid DNA was purified by an ethidium bromide dye–cesium chloride centrifugation procedure (Clewell & Helinski, 1972).

The smaller DNA fragment produced by digestion with the restriction enzyme *Bam*H1 was isolated from agarose. This fragment contains the sequences which code for almost all of the MPC-11 heavy-chain constant region. The DNA fragment was ^{32}P labeled to low specific activity. Single- and double-enzyme digestions were performed and the molecular weights of the resulting fragments determined.

Results

Isolation of H-Chain Mutants. Several mouse myeloma variants producing structurally altered H chains were isolated from the γ_{2b} mouse myeloma 45.6. 10-1 is a spontaneous mutant of 45.6 which synthesizes a heavy chain with a deletion in CH_1; this H chain forms dimers with itself, does not covalently bind light chains, and is secreted (Morrison, 1978). From 10-1, two subsequent mutants were isolated which synthesized smaller heavy chains lacking subclass determinants. Mutant I17, isolated after nitrosoguanidine mutagenesis, synthesizes a heavy chain of ~35 000 daltons which does not form covalent bonds with light chains. A spontaneous mutant of 10-1, G251, synthesizes an H chain of ~40 000 daltons which also does not form H–L disulfide bonds (Morrison, 1979). The mutants G251 and I17 secrete little or none of their H chains. From both G251 and I17 subsequent mutants secreting H chains with γ_{2a} determinants have been isolated (Morrison, 1979). These mutants synthesize heavy chains with a molecular weight either similar to that of 10-1 (e.g., N31-2, N12, N29, and K25) or similar to wild type (e.g., N15, K32-1, and K23). The interrelationship of the heavy-chain mutants is shown in Figure 1. We have investigated the relationship between the size and composition of the mRNA and the proteins whose synthesis they direct.

Ig Heavy-Chain mRNA Size Determinations. Methylmercury–agarose gels provide a system that eliminates all RNA secondary structure (Bailey & Davidson, 1977) and affords high resolution of RNA species. Poly(A^+) mRNAs from the wild type and mutants were size fractionated by electrophoresis on methylmercury–agarose gels, transferred to diazobenzyloxymethyl paper, and then hybridized to either

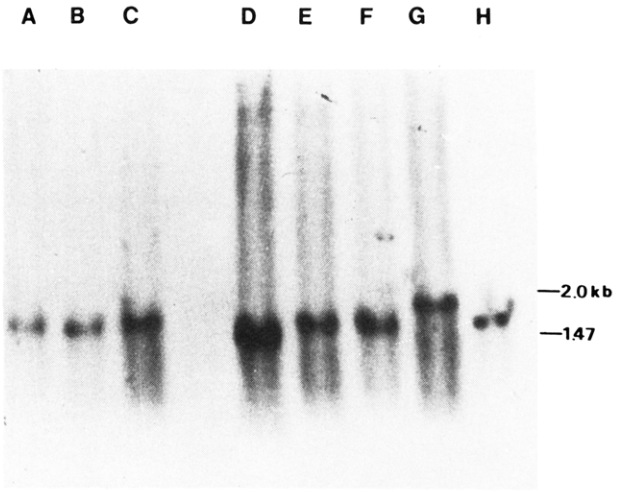

FIGURE 2: Determination of immunoglobulin heavy-chain mRNA sizes. A^+ RNAs were electrophoresed on 1% agarose gels containing methylmercury hydroxide. The RNAs were transferred to diazobenzyloxymethyl paper and then hybridized to ^{32}P-labeled DNA fragment C. High specific activity fragment C was prepared by the nick translation procedure. Lanes: (A) N12 A^+ RNA; (B) K25-1 A^+ RNA; (C) N31-2 A^+ RNA; (D) I17 A^+ RNA; (E) G251 A^+ RNA; (F) 10-1 A^+ RNA; (G) 45-6 A^+ RNA; (H) K25-1 A^+ RNA.

whole pγ_{2b} (11) plasmid DNA or subfractions of it labeled with ^{32}P to high specific activity by nick translation (see Methods). Using this procedure, we obtained an accurate determination of the RNA sizes. In Figure 2 (D–G) the migration of A^+ H-chain mRNAs from 45.6 and the mutants 10-1, G251, and I17 are compared. The 45-6 heavy chain mRNA is found to be 1.88 kb in size, while the 10-1 and G251 heavy-chain mRNAs are 1.66 kb and the I17 heavy-chain mRNA is 1.43 kb in size. We also analyzed all the samples on gels after glyoxal treatment and formamide–formaldehyde treatment with identical results. All cell lines were hybridized with total pγ_{2b} (11) Ig sequence as well as with subfractions of it (not all data shown), and only a single size class Ig H-chain mRNA was seen in each cell.

Because of extensive sequence homology the γ_{2b} probe cross hybridizes with γ_{2a} heavy-chain mRNA (Sikorav et al., 1980); we were therefore able to use the γ_{2b} probe to determine the sizes of the heavy-chain mRNA from the γ_{2a} mutants. The γ_{2b} probe also hybridizes with the mRNA of MOPC-173, an authentic γ_{2a} producer (data not shown). The sizes of all heavy-chain mRNAs (γ_{2b} and γ_{2a}) we have analyzed are summarized in Table I. Mutants K23, K32, and K25 are especially noteworthy since they synthesize H-chain mRNAs of 1.88, 1.88, and 1.66 kb, respectively, in spite of being derived from I17 which makes a 1.43 kb H-chain mRNA.

Deletion Mapping of Mutant Ig Heavy-Chain mRNAs. For determination of the locations of the sequences missing from the mutant heavy-chain mRNAs, mRNA was fractionated on methylmercury–agarose gels and hybridized to nick-translated DNA fragments derived from different regions of the γ_{2b} constant region. Hybridization with a restriction fragment would demonstrate that at least some of the sequences contained in that fragment were present; lack of hybridization with a fragment would demonstrate that most of the sequences contained in that fragment were missing.

The Ig insert of pγ_{2b} (11), a cDNA copy of the Ig mRNA from MPC-11 (γ_{2b}), is 1100 plus 50 bp in length, extends from the sequences that code for amino acid 145, in the CH_1 domain, to the carboxy-terminal end of the γ_{2b} heavy-chain polypeptide, and includes most of the 3′-untranslated region (Tucker et al., 1979b). A restriction map was determined by sequential cleavage of a ^{32}P-labeled *Bam*HI fragment containing almost all of the γ_{2b} (11) insert (see Materials and Methods). The relative positions of the restriction sites we have found for *Bam*HI, *Kpn*I, *Pst*I, and *Xho*I are consistent with the published data and are shown in Figure 3. We could only ascertain the position of one *Pst*I site by our mapping technique (see Materials and Methods). The other *Pst*I site predicted by the sequence (Tucker et al., 1979b) is indicated in parentheses on Figure 3. The published DNA sequences does not, however, show a cleavage site for the enzyme *Hinc*II, which we have found cleaves the *Bam*HI fragment of pγ_{2b} DNA and produces two fragments of 520 and ~1050 nu-

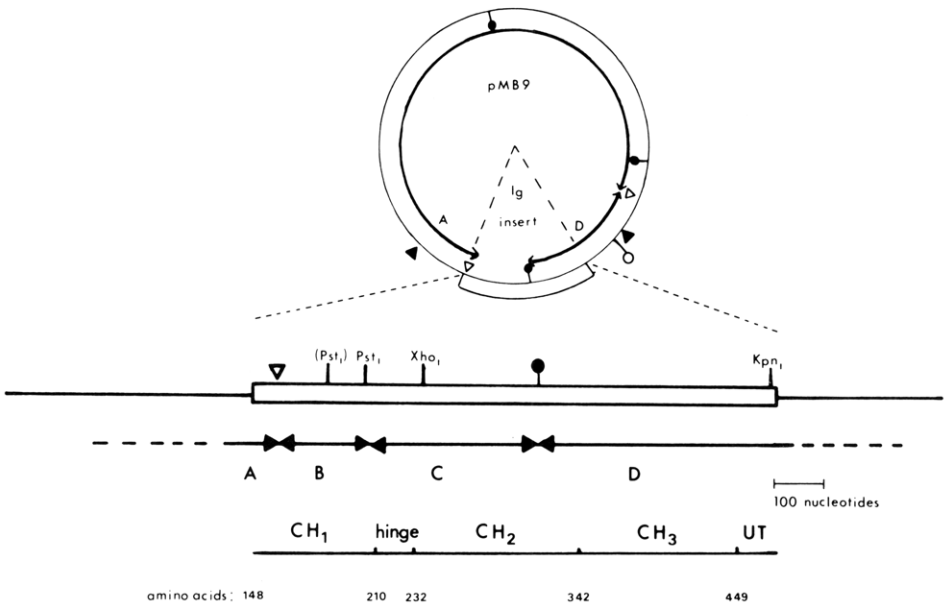

FIGURE 3: Restriction enzyme map of pγ_{2b} (11) plasmid. The *Bam*HI fragment of pγ_{2b} containing most of the Ig sequences was isolated on a gel, nick translated with ^{32}P to low specific activity, and digested with the other indicated enzymes. Sites of digestion were determined by comparison of sizes in single and double digestions. Subsequently, fragments A, B, C, and D were isolated and used for hybridizations shown in Table I and Figure 4. Symbols: (▽) digestion sites of *Bam*HI; (♦) *Hinc*II sites; (▼) *Hha*I sites; (♀) *Hin*dIII sites. The positioning of domains and amino acids is from Tucker et al. (1979a,b). The *Pst*I site was implied from the sequence of Tucker et al. (1979a,b); one *Pst*I site was detected in this work.

Table I: Ig Heavy-Chain mRNA and Protein Sizes and Hybridization Pattern

cell line	mRNA size (kb)	protein sizes (daltons × 10^{-3}) predicted[a]	protein sizes (daltons × 10^{-3}) obsd[b]	hybridization of selected DNA fragments[c] to A^+ RNAs of mutant cell lines A	B	C	D
45.6	1.88	55	55	+	+	+	+
10.1	1.66	46.1	47	+	−	+	+
G251	1.66	46.1	40	+	−	+	+
I17	1.43	37	34	+	−	+	+
K23	1.88	55	55	nd[d]	nd	+	nd
K32-1	1.88	55	55	+	+	nd	nd
N15-3	1.88	55	55	+	+	nd	nd
K25-1	1.66	46.1	45 ± 2	+	−	+	+
N12	1.66	46.1	45 ± 2	+	−	+	+
N31	1.66	46.1	45 ± 2	+	−	+	+
N29	1.66	46.1	nd	nd	nd	nd	nd

[a] Predicted values were calculated with the following assumptions: (1) 447 amino acids for γ_{2b} heavy chain; (2) 120 daltons/amino acid residue; (3) 540 nucleotides of 5′- and 3′-nontranslated segments of the mutant mRNAs do not change; (4) carbohydrate moiety contributes equally to all molecular weight determinations. [b] Ranges of values indicate differences observed in different gel systems that are averaged here. [c] Fragment origins are depicted in Figure 3. Nick-translated fragments were hybridized to the mRNAs electrophoresed in methylmercury gels and transferred to DBM paper or in formaldehyde gels and transferred to nitrocellulose. The purity of all fragments was determined as described under Materials and Methods. [d] Not done.

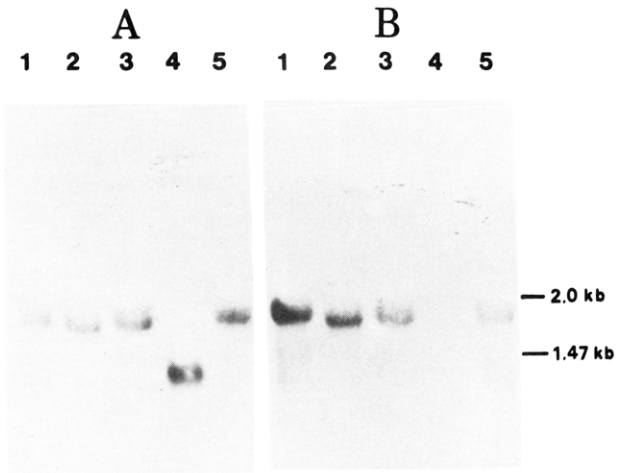

FIGURE 4: Hybridization analysis of mutant Ig heavy-chain mRNAs with region-specific probe. A^+ RNAs were electrophoresed in formamide–formaldehyde gels and transferred to nitrocellulose filters (see Materials and Methods). The filter was cut in half, and one half was hybridized with total $p\gamma_{2b}$ (11) nick translated with ^{32}P and the other half with ^{32}P-labeled B fragment DNA (see Figure 3). Filters were washed, and autoradiography was performed. (Panel A) $p\gamma_{2b}$ (11) total probe hybridized to RNA from lane 1, 45.6; lane 2, K32; lane 3, N15-3; lane 4, I17; and lane 5, 45.6. (Panel B) B fragment probe hybridized to RNA from lane 1, 45.6; lane 2, K32; lane 3, N15-3; lane 4, I17; and lane 5, 45.6.

cleotides. Consistent with our results are the sequencing data of Yamawaki-Kataoka et al. (1980), who found the sequence GTCAAC which would be cleaved by *Hinc*II 17 amino acids from the end of the CH_2 coding region of an embryonic γ_{2b} gene. These data would predict that the distance between the *Bam*HI and *Hinc*II sites in $p\gamma_{2b}$ would be ~500 nucleotides as we have observed. Our results suggest that there is an error in the sequencing of the γ_{2b} cDNA of Tucker et al. (1979b). Several DNA fragments containing Ig sequences corresponding to different regions of the γ_{2b} heavy-chain constant region were isolated. The DNA fragments were labeled to high specific activity and used as hybridization probes. The DNA fragments used are shown in Figure 3 and designated (A–D). A summation of all the hybridizations performed is shown in Table I. The DNA fragments A, C, and D hybridized to the wild-type 45-6 A^+ RNA and to the A^+ RNAs of all the mutants tested. Only fragment B was found to hybridize differentially to the mutant mRNAs. Fragment B hybridized to mRNA from 45.6 (γ_{2b}) and to K32 and N15.3 derived from I17 and G251, respectively, which synthesize full-length γ_{2a} chains (Figure 4) but not to any mutant which synthesizes a shortened H chain (see Table I). Fragment B represents a portion of the CH_1 domain extending from residue 159 to residue 196 (Tucker et al., 1979a,b). These data show that the heavy-chain mRNA of the mutant 10-1 is missing part of the CH_1 domain, in agreement with the peptide map data (Morrison, 1978). The mutants G251 and I17 isolated from 10-1 share part, if not all, of the deletion present in 10-1. Furthermore, the mutants K25-1, N29, and N31-2 (γ_{2a}) that were isolated from the mutants I17 and G251 and which synthesize H chains of ~47 000 daltons also have a deletion in CH_1. Fragment B accounts for ~114 out of 200 bases deleted; about 86 nucleotides or 29 amino acids remain unaccounted for. We were not able to determine if the carboxy terminus of the deletion corresponded to the CH_1–hinge or hinge–CH_2 boundary. Since fragment A hybridizes to all mRNAs, we have demonstrated that the amino-terminal end of the deletion does not correspond to the 5′ domain boundary of CH_1. In addition, the size difference in the mRNAs in the 47 000 molecular weight proteins is 200 nucleotides, not 360, the length of an entire domain (see Discussion).

The procedures we have described have localized the site of deletion within the CH_1 domain in many of the mutants. We have been unable to accurately locate the other site(s) of deletion in I17 due to the shortcomings of this technique, i.e., the dependence on finding small enough fragments within the sequences of interest. The mRNA data do not explain, in addition, the discrepancies in observed vs. predicted protein sizes for G251 and I17 (see Discussion). Therefore, peptide analysis of the heavy chains was undertaken.

Peptide Analysis of Deleted Heavy Chains. G251 has been shown to produce a heavy chain of ~40 000 daltons, yet it has heavy-chain mRNA the same size as 10-1 which synthesizes a heavy chain of ~47 000 daltons. To locate the additional sequences missing from the G251 heavy chain, we labeled G251 cells with ^3H-labeled VTL and immunoprecipitated the immunoglobulins with specific antisera. The precipitates were digested with cyanogen bromide, mixed with CNBr-cleaved Fc from 10.1 labeled with ^{14}C-labeled VTL, and following reduction and alkylation, fractionated by using a Sephadex G-75 superfine column. When the Fc of 10.1 is cleaved by CNBr three fragments identical with those found in 45.6 are generated; (Figure 5A) these fragments correspond to residues 255–316 (fragment II.2), 317–413 (fragment II.1), and 414–447 (fragment II.3) (Francus & Birshtein, 1978). These three fragments derived from 10.1 are clearly resolved on the Sephadex column shown in Figure 5A; the order of elution from the Sephadex column is II.1, II.2, and II.3. The peak seen at fractions 188–198 is from contaminating Fd. The profile from G251 has many peaks because it contains the CNBr fragments from the entire heavy and light chains and any nonspecifically precipitated radioactivity. From Figure 5A it appears that G251 lacks II.1 but contains II.2. For verification of this conclusion, pool 1, containing ^3H-labeled II.1 derived from 10-1 and any G251 peptides of similar size, and pool 3, containing II.2 and any G251 peptides of similar size, were recovered and peptide mapped. When ^3H-labeled

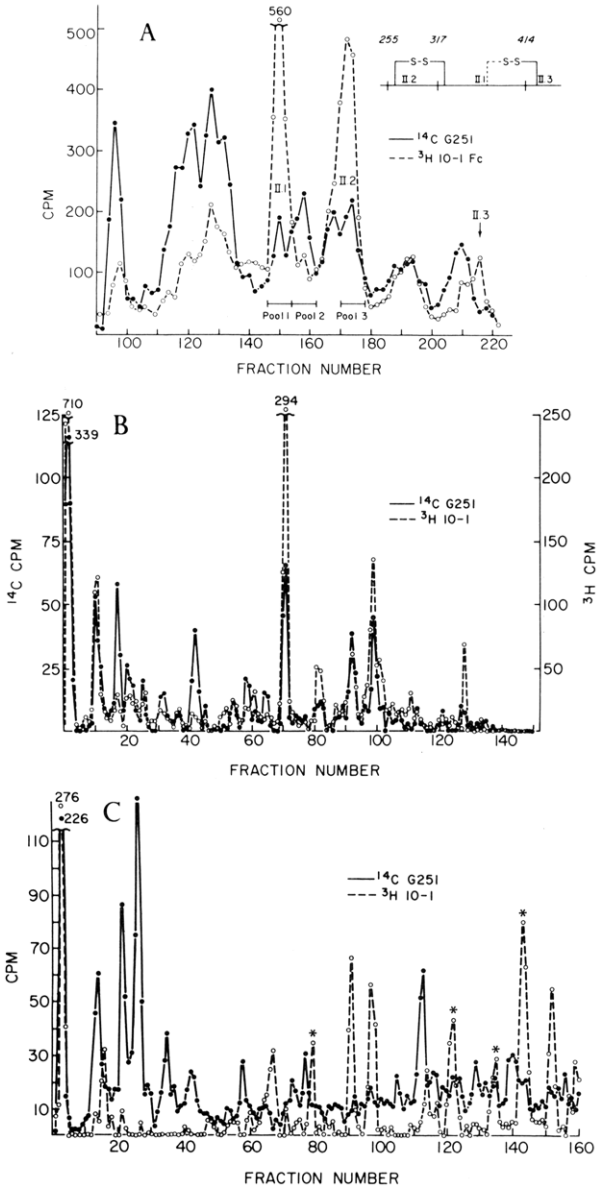

FIGURE 5: Elution profile of CNBr fragments from G251 Ig and 10.1 Fc. (A) ^{3}H-Labeled VTL-labeled 10.1 Ig was immunoprecipitated from cellular secretions and cleaved with CNBr, and the CNBr Fc was isolated by using a Sephadex G150 column equilibrated in 8 M urea and 0.1 M formic acid. ^{14}C-Labeled VTL-labeled Ig was immunoprecipitated from the cytoplasm of G251 cells, cleaved with CNBr, mixed with the ^{3}H-labeled 10.1 CNBr Fc, completely reduced and alkylated, and chromatographed on a column (2.5 × 200 cm) of Sephadex G-75 superfine equilibrated in 8 M urea and 0.1 M formic acid. The position of the peaks was determined by counting aliquots in a liquid scintillation counter. Pools were made as indicated, desalted on Sephadex G-25 in 0.1 M formic acid, and lyophilized to dryness. The nomenclature of the wild-type fragments and CNBr fragmentation pattern of the γ_{2b} Fc is adapted from Francus & Birshtein (1978). (B) Pool 3 from the Sephadex column shown in (A) was recovered, lyophilized to dryness, and digested with trypsin and chymotrypsin, and peptide map analysis was performed as described. (C) Pool 1 from the Sephadex column shown in (A) was recovered, lyophilized to dryness, and digested with trypsin and chymotrypsin, and peptide map analysis was performed as described. When, in a separate experiment, pool 2 was mixed with pool 1 and peptide mapped, fragments derived from G251 cochromatographing with those indicated by asterisks were observed.

II.2 from 10-1 was compared with the material from G251 which cochromatographed with it (pool 3), G251 was found to contain all of the peptides present in the same size fragment from 10-1, indicating that the amino acids from residues 255–316 were present in G251 (Figure 5B). When pool 1

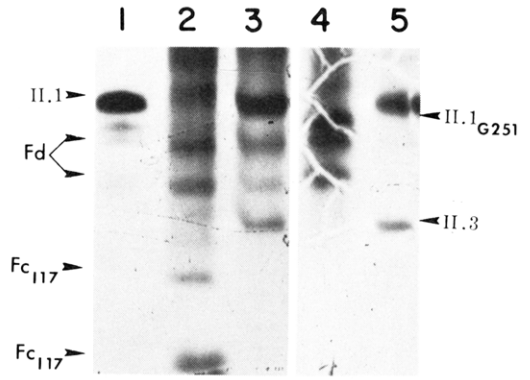

FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoresis of CNBr fragments. ^{14}C-Labeled CNBr-cleaved fragments from 45.6 Fc were isolated by chromatography on a Sephadex G-75 superfine column in 8 M urea and 0.1 M formic acid and used markers. ^{14}C-VTL-labeled immunoglobulins were immunoprecipitated from cytoplasmic lysates of 10-1, G251, and I17, cleaved with CNBr, lyophilized to dryness, and dissolved in 2% $NaDodSO_4$ prior to electrophoresis. Electrophoresis was carried out in 12% acrylamide gels containing 8 M urea. The position of the peaks was determined by fluorography. Fragment II.2, which appears to contain carbohydrate, does not resolve well in this sysem. Lane 1, II.1; lane 2, I17; lane 3, 10-1; lane 4, G251; lane 5, II.1 and II.3.

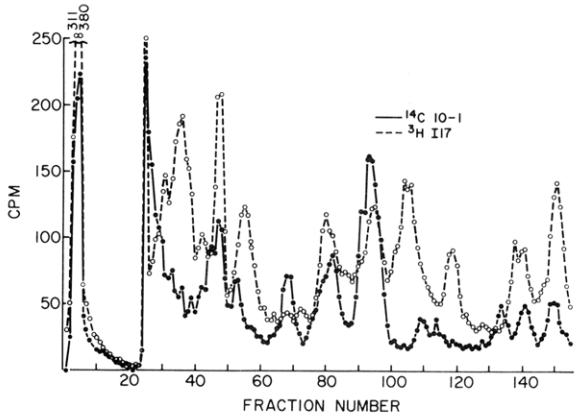

FIGURE 7: Peptide map analysis of heavy chains from I17 and 10-1. Radiolabeled heavy chains were immunoprecipitated from cytoplasmic lysates, recovered from $NaDodSO_4$–acrylamide gels, and peptide mapped following digestion with trypsin plus chymotrypsin as described.

containing ^{3}H-labeled 10-1 II.1 and the material from G251 which cochromatographed with it was peptide mapped, G251 did not contain the peptides present in II.1 (Figure 5C), indicating that the intact fragment from amino acids 317–413 was not present in G251. However, when pool 2 was mixed with pool 1 and peptide mapped, peptides cochromatographing with the peaks indicated by asterisks were observed, indicating that some of the peptide residues present in residues 255–317 in the wild type were present in this smaller fragment. Similarly, when CNBr-cleaved G251 was fractionated on $NaDodSO_4$ gels, a fragment migrating at the same place as II.1 Fc was missing; instead, a new peak, $II.1_{G251}$, not present in an intact Fc was observed (Figure 6). Insufficient radioactivity was present to peptide map II.3, but $NaDodSO_4$ gel analysis indicated that II.3 was missing from G251. These data indicate that the additional deletion in G251 accounting for its smaller protein size begins somewhere in II.1 and extends to the carboxy terminus of the molecule.

I17 also produces a heavy chain which is shorter than that of 10-1. When the H chain of I17 is peptide mapped against that of its parent, 10-1, it is found to lack many of the peptides present in 10-1 but contains several additional peptides not

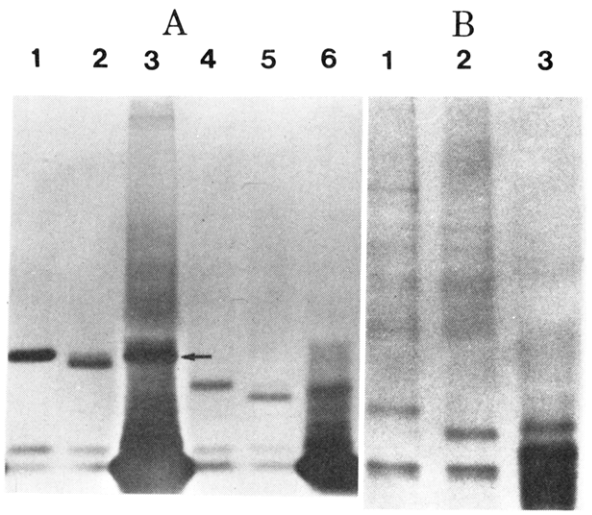

FIGURE 8: Comparison of heavy chain synthesized by cells in the presence and absence of high levels of glucosamine with in vitro translated products. Poly(A)-containing RNA was translated in the wheat germ translation system and then the Ig immunoprecipitated with antiserum specific for MPC-11 light and heavy chains. Heavy and light chains were immunoprecipitated from cells incubated for 3 min in ^{14}C-labeled amino acids. Heavy chains lacking carbohydrate synthesized by cells were prepared as described. The immune-precipitated in vitro translated heavy chains were compared to glycosylation-inhibited heavy chains and normal heavy chains synthesized by cells on 7.5% polyacrylamide–$NaDodSO_4$ gels. The translated light-chain constant region fragment of MPC11 (Kuehl & Scharff, 1974) has been run off the gels. (Panel A) Lane 1, 10-1 cellular; lane 2, 10-1 cellular nonglycosylated; lane 3, 10-1 in vitro translated; lane 4, G251 cellular; lane 5, G251 cellular nonglycosylated; lane 6, G251 in vitro translated. (Panel B) I17 products. Lane 1, cellular; lane 2, cellular nonglycosylated; lane 3, in vitro translated.

present in 10-1 (Figure 7). Thus, I17 appears to contain additional sequences not present in the H chain of 10-1. This conclusion is verified by analysis of I17 on $NaDodSO_4$–polyacrylamide gel electrophoresis after cleavage with CNBr; additional peaks (Fc_{I17}) not present in 10-1 are seen in I17 (Figure 6).

In Vitro Translation Studies. A^+ mRNAs from mutant and wild-type cell lines were translated in vitro by using a wheat germ system, to determine if the smaller heavy chains might result from partial proteolytic degradation. Igs were immunoprecipitated by using the *Staphylococcal* protein A antibody adsorbent procedure and analyzed on polyacrylamide–$NaDodSO_4$ gels. In Figure 8, lanes A3, A6, and B3, the immune precipitates of in vitro translated A^+ mRNA of 10-1, G251, and I17 are shown. When compared to their corresponding intracellularly labeled and immune-precipitated light and heavy chains (lanes A1, A4, and B1), it is clear that the heavy-chain polypeptides in the cells and in vitro heavy-chain polypeptides are approximately the same size. Bands of molecular weight equivalent to wild-type protein are not detected in the translation products of the mutant mRNAs. These data indicate that the short Ig heavy-chain proteins are the primary products of translation of the heavy-chain mRNA. For confirmation that the shortened H chains did not result from proteolysis, A^+ mRNA from the wild-type cell line (45.6) and each mutant in turn were mixed and translated together; wild-type H chain was always observed (data not shown). These results indicate that the smaller heavy chains do not result from cleavage by a cotranslated proteolytic enzyme.

Cytoplasmic A^+ mRNA from wild type and mutants was translated in the wheat germ system to determine if the mutant heavy chains have an in vitro precursor form. The wheat germ system does not remove amino-terminal leader sequences and does not glycosylate proteins (Morrison et al., 1974; Morrison & Lodish, 1975; Morrison & McQuain, 1977). Immunoprecipitated in vitro translated heavy chains were compared on polyacrylamide–$NaDodSO_4$ gels with the intracellular heavy chains synthesized in the absence or presence of high levels of glucosamine to inhibit glycosylation. Since glycosylation does not take place in the wheat germ system, the size of the in vitro products must be compared to the non-glycosylated intracellular heavy chains to identify the presence of larger molecular weight precursors in vitro.

In Figure 8 it can be seen that 10-1, G251, and I17 all have an in vitro precursor form and that all of the heavy chains apparently are glycosylated in the cells. Similar results were found for 45.6 and the γ_{2a} mutants as well (data not shown). From the difference in migration of the in vitro vs. non-glycosylated intracellular chains, we estimated that the size of the precursor sequence for 45.6 and 10-1 is ~18 amino acids, which agrees with the size of the precursor for the MOPC-315 heavy chain previously determined (Jilka & Pestka 1977). Resolution was not sufficient on the gels to determine the exact size of the precursors for G251 and I17. These studies indicate that the nonsecreted H chains contain carbohydrate and are synthesized with a precursor form; they do not rule out subtle alteration in either the sequence of the precursor form or in the structure of the carbohydrate.

Hybridization with Wild-Type Cells. Light chains are secreted normally in all the mutants which we have studied suggesting that no gross cellular alterations which interfere with secretion have occurred. In order to determine if a cellular change affecting only heavy chain secretion had occurred, the mutant G251 (oubain sensitive but able to grow in HAT) was fused with Ou^RTG^R wild-type (4T001) cells and hybrids selected. The intracellular and secreted products of the hybrids were immune precipitated and analyzed on $NaDodSO_4$ gels. While the hybrids produce both wild-type and mutant H chains, only wild-type full-length H chain is secreted (Figure 9). These results suggest that it is the structural alteration in the mutant H chain which prevents its secretion.

Discussion

The analysis of Ig heavy-chain mRNA sizes by denaturing agarose gels indicates that the mutants 10-1, G251, I17, K25-1, N29, and N31-2 produce mRNAs that are smaller than the wild-type (45.6) mRNA. All these smaller mRNAs have deletions within the CH_1 coding region. Mutants K23, K32, and N15-2, derived from I17 and G251, respectively, synthesize full-length heavy-chain mRNA. Translation of the mRNAs of the mutant cells in vitro has shown that the mutant heavy chains are not the result of partial proteolytic degradation of a full-length protein.

Comparisons of observed protein size vs. that predicted from the mRNA size for all the mutants are summarized in Table I. In predicting the Ig heavy-chain mRNA coding potential, we have assumed that the missing sequences are lost from the translated regions of the Ig heavy-chain mRNA. From Table I it can be seen that the heavy-chain protein size of the mutants 10-1, K25, and N31-2 agrees reasonably well with the protein size predicted from the heavy-chain mRNA size.

Mutant G251. In contrast, a comparison of the mutant G251 heavy chain observed and the predicted protein size indicates a discrepancy. The observed protein is 6000 daltons shorter than predicted, corresponding to a loss of ~50 amino acids. Since the heavy-chain mRNA sizes of the mutants 10-1 and G251 are the same and since the mutant G251 was isolated from the mutant 10-1, the data suggest that G251 has the same deletion as the mutant 10-1. This conclusion was

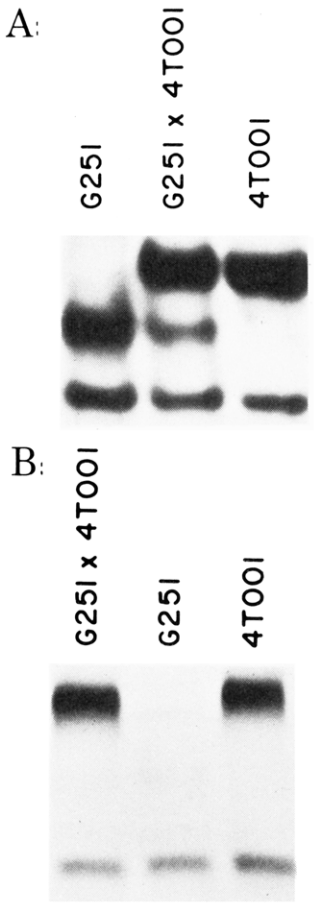

FIGURE 9: Production and nonsecretion of mutant Ig H chain in hybrid cells. Cells from the 4T001 line, resistant to thioguanine and ouabain and derived from 45.6, were fused with the ouabain-sensitive mutant G251, as described under Materials and Methods. Hybrid cells were labeled with ^{14}C-labeled amino acids; supernatants and cytoplasmic material were immunoprecipitated with rabbit anti-mouse Ig and protein A and the immunoprecipitates analyzed on $NaDodSO_4$–polyacrylamide gels. (Panel A) H and L chains immune precipitated from the cytoplasmic lysate of G251, 4T001, and the G251 × 4T001 hybrid. (Panel B) H and L chains immune precipitated from the secretions of G251, 4T001, and the G251 × 4T001 hybrid.

confirmed because G251 H-chain mRNA does not hybridize with the B fragment. G251 has a second alteration which leads to the loss of all amino acids carboxy-terminal to residue number 414 and of other peptides in the region of amino acids 317–413. The mRNA size of the H chain of G251 indicates that the second change is not merely a large RNA deletion. Instead, taking into consideration both the mRNA and the peptide data, it appears that shortened G251 heavy chain results from a combination of CH_1 deletion and a premature termination of mRNA translation. Nonsense mutations could arise either by base substitutions or by frame-shift mutations. An analysis of the published γ_{2b} DNA gene sequences indicates that the addition or deletion of one or two bases could generate >20 nonsense codons within CH_2 which could give rise to premature termination. Kenter & Birshtein (1979) have described a mutant, M311, the γ_{2b} heavy chain of which terminates just prior to the end of CH_2; presumably this mutation has occurred by a deletion of two nucleotides just prior to the CH_2–CH_3 splice site resulting in a nonsense codon. The mRNA of this mutant should be full sized but has not yet been analyzed. A spontaneously occurring frame-shift mutation resulting from a two-base deletion was observed (Adetugbo & Milstein, 1977b) in the P_3K line of MOPC21 (γ_1); this mutant produces a shorter protein than wild type.

Mutant I17. The mRNA coding for the H chain of I17 is smaller than the mRNA coding for 10-1 (Table I). The mRNA of I17 continues to miss the CH_1 domain sequences absent in 10-1; in addition, other sequences are missing from the mRNA, based on its small size. R-loop analyses indicate that I17 mRNA has a second internal deletion near the CH_2–CH_3 boundary (unpublished results); even with such a deletion the mRNA retains sequences which hybridize with fragments C and D (see Table I). The I17 heavy-chain protein predicted from the mRNA is larger (37 000 daltons) than the observed size (34 000 daltons) and is distinguished by the fact that it contains many peptides absent from its parent but is shorter by 13 000 daltons. Thus, I17 has two internal deletions, and apparently associated with the second deletion is the translation of new peptides. These new peptides could result from the translation either of the same nucleotides as are present in 10-1 in a different reading frame or of new information, perhaps an intervening sequence.

Mutant 10-1. The internal deletion of 10-1 could arise from a loss of sequences in the DNA or from subtle mutations in the DNA to produce a defective primary nuclear RNA transcript which is incorrectly processed. Preliminary experiments indicate that gross DNA deletions have not occurred in the mutant 10-1, pointing to splicing errors as the cause of mRNA deletions (C. Brandt, S. L. Morrison, and C. Milcarek, unpublished experiments). Several human heavy-chain disease proteins have been analyzed and shown to have internal deletions beginning or ending near the hinge with various parts of domains deleted (Frangione & Franklin, 1973; Franklin & Frangione, 1975). The mutant IF-2 (Adetugo & Milstein 1977a,b) derived from MOPC-21 produces a smaller heavy-chain protein and a mRNA containing a deletion of the CH_1 domain. However, 10-1 differs from IF-2 because the deletion in IF-2 is exactly one domain in extent (Dunnick et al., 1980). In this paper we have demonstrated that the deletion of 10-1 is not of the entire CH_1 domain but that the amino terminus (5′ side) of CH_1 is still present in the mRNA. However, we have not demonstrated whether the 3′ end of the deletion corresponds to a domain boundary.

γ_{2a} Mutants. The γ_{2a} proteins synthesized by the subclass mutants fall into two classes, those with molecular weights of 55 000 which now contain CH_1 and are encoded by an mRNA of 1.88 kb and those with molecular weights of 47 000 which lack CH_1 and are encoded by an mRNA of 1.66 kb. The molecular weights of the proteins lacking CH_1 are similar to that of 10-1. In all cases the observed protein size and that predicted from the size of the mRNA agree reasonably well. It should be noted that the subclass switch mutants K25-1, K23, and K32 produce H chains and mRNAs which are larger (1.67 and 1.88 kb) than that of their parent I17 (1.43 kb) and in the case of K32, contain sequences not present in I17. The γ_{2a} producers apparently retain a V_H similar to the V_H of 45.6 (the original cell line) because all γ_{2a}'s tested react with anti-45.6 idiotype sera. Analysis of several subclass switch mutants shows that they synthesize H-chain proteins with different isoelectric focusing patterns and presumably different amino acids sequences. The γ_{2b} and γ_{2a} subclass switch then does not occur only at the V_H–C_H boundary; instead, the occurrence of deleted γ_{2a}'s and protein sequence data have suggested that it may occur in regions of sequence homology within domains (Morrison, 1979; Birshtein et al., 1980).

Several mechanisms can account for the expression of a new constant region gene. (1) The $\gamma_{2b} \rightarrow \gamma_{2a}$ subclass switch may be a reflection of the normal C region switching that occurs during the development of the B cell. Normal switching has

been proposed to occur by deletion. If the subclass switch mutants occur by the same mechanism, then a deletion of γ_{2b} genetic information should be seen in the subclass switch mutants. (2) $C\gamma_{2a}$ and $C\gamma_{2b}$ may be expressed on a large hnRNA; subclass switching could result from altered processing. Analyses of the primary hnRNA transcript with γ_{2b}- and γ_{2a}-specific probes would indicate whether coexpression occurs. (3) Unequal sister chromatid exchange could generate a subclass switch. The similarities in sequence of a moderately repetitive gene family like the Ig γ's would facilitate this through a recombination like process. Deletion of the $C\gamma_{2b}$ gene may or may not be detectable in this case; rearrangements of sequences flanking the $C\gamma_{2a}$ might be detectable. Experiments are in progress to distinguish between the models presented.

Secretion Properties of Altered Heavy Chains. The wild-type 45.6 heavy chain, the shorter heavy-chain mutant 10-1, and all the mutants expressing the γ_{2a} subclass were found to secrete their heavy chains. The mutants G251 and I17, lacking subclass-specific determinants, secreted very little or none of their heavy chain. Several possibilities exist to explain the lack of secretion of H chain in I17 and G251: (1) there is a cellular defect which interferes with secretion, (2) the proteins are not glycosylated, (3) there is no hydrophobic leader sequence so that the H chains cannot enter the cisternae of the endoplasmic reticulum, or (4) the altered primary structure of the H chain prevents its secretion. We examined these possibilities. A cellular defect which interferes with H-chain secretion is ruled out by the cell–cell hybridization experiments. These show that in the hybrid, the wild-type H chains continue to be secreted while the mutant H chains are not.

It has been postulated that the hydrophobic leader sequence which is removed in the secretory process acts as a signal for the secretion of some proteins (Blobel & Dobbersterin, 1975a,b). However, both I17 and G251, which are not secreted, direct the translation of heavy chains with precursor sequences. Therefore, although leader sequences may facilitate secretion, their presence does not guarantee the secretion of the immunoglobulin. In addition it has been postulated that the glycosylation of the heavy chain is an important factor in determining its secretion (Eylar, 1965; Melchers, 1973). However, these nonsecreted heavy chains appear to be glycosylated.

Instead it would appear that the primary structure of the protein is important in determining secretion. However, there does not seem to be any simple correlation between the structure of a heavy chain and its secretion. 10-1, which maintains the γ_{2b}-specific determinants, is secreted while I17 and G251, which lack the γ_{2b} determinants, are not secreted. However, in a series of previously reported MPC-11 cell line mutants which lack γ_{2b} determinants and synthesize H chains ranging in molecular weight between 40000 and 50000 daltons, some are secreted while others are not (Birshtein et al., 1974; Weitzman & Scharff, 1976; Cook et al., 1979). A correlation between the formation of H_2L_2 molecules and secretion can be drawn because the previously described γ_{neg} molecules which are secreted form H_2L_2 molecules; neither G251 nor I17 assemble their heavy chains. But H_2L_2 assembly is not strictly determining because 10-1, which is secreted, does not form H_2L_2 molecules; instead 10-1 forms only disulfide-linked H_2. In fact, noncovalent interactions to make noncovalently bound H_2L_2 are also not necessary for secretion of the 10-1 H_2, because 10-1 will continue to secrete its heavy chain as H_2 in the absence of L chain synthesis (Morrison, 1978). Therefore, a complex interaction of factors influencing the conformation and solubility of the heavy chain appear to determine its secretion.

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Antibody Specificity: A 270-MHz Hydrogen-1 Nuclear Magnetic Resonance Study of the Binding of Dinitrophenyl Compounds to the V_L Dimer of Protein 315†

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ABSTRACT: The binding of dinitrophenyl (DNP) compounds to the V_L dimer of protein 315 (IgA/λ_2) results in small perturbations of about ten resonances in the aromatic region of the 270-MHz ^1H NMR spectrum of the protein. From a comparison of the chemical shifts of these resonances with the chemical shifts of resonances which are affected by the binding of DNP compounds to the Fv fragment of protein 315, it is concluded that the conformation of combining site residues in the V_L domain of the Fv fragment is maintained in the V_L dimer. The binding of DNP compounds to the V_L dimer is therefore considered to reflect the retention of structural features important in determining the specificity of the Fv fragment and not the fortuitious creation of a binding site. These findings provide a structural explanation for the observation that antibodies with a λ_2 light chain are contained in the anti-DNP response of BALB/c mice [Cotner, T., & Eisen, H. N. (1978) *J. Exp. Med. 148*, 1388]. The ligand resonances undergo large upfield chemical shift changes following binding to the V_L dimer. These changes are interpreted in terms of ring-current effects from aromatic residues. It is shown that the same conformation of the combining site aromatic residues of the V_L domain is capable of explaining the chemical shift changes of the ligand resonances which are observed on binding to the V_L dimer and to the Fv fragment. The chemical shifts of the aromatic resonances of DNP-aspartate and DNP-glycine, when bound to the V_L dimer, are found to differ. Ring-current calculations show that the positions of the DNP rings may differ by about 1.5 Å relative to Trp-93_L. A difference of about 0.5 Å is calculated for binding to the Fv fragment. It is suggested that the specificity of protein 315 for DNP compounds is determined largely by the size and shape of a predominantly nonpolar combining site.

Many factors which are important in determining the structural basis of antibody specificity and diversity have now been recognized. The comparison of a large number of antibody V-region sequences (Wu & Kabat, 1970) and the determination of the structures of several antibody V domains by X-ray crystallography (Poljak et al., 1973; Schiffer et al., 1973; Segal et al., 1974; Epp et al., 1974; Wang et al., 1979), have confirmed the idea that the structural mechanism used to generate the diversity of antigen binding sites is a conserved framework, the immunoglobulin fold, to which the hypervariable sequences, forming the antigen binding site, are attached. It has therefore been considered feasible to construct models of immunoglobulin combining sites for which no X-ray data exist on the basis of their presumed homology with known structures (Poljak et al., 1974; Padlan et al., 1976). Further aspects of antibody specificity may then be investigated by use of these models.

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