# Mouse Myeloma Cells That Make Short Immunoglobulin Heavy Chains: Pleiotropic Effects on Glycosylation and Chain Assembly

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ABSTRACT Two variants in immunoglobulin heavy chain production, derived from the MPC 11 mouse myeloma cell line, make short heavy (H) chains with identical precise deletions of the $C_H3$ domain. The $C_H3$ domain is expressed in the H chain mRNA from both variants. Although in vitro translation of this mRNA produces one H chain species, deleted heavy chains are secreted as heavy–light (HL) and $H_2L_2$ moieties in contrast to MPC 11, which secretes only $H_2L_2$. The heavy chains of HL apparently contain more carbohydrate ($CHO^+$) than do the H chains of $H_2L_2$, and inhibition of N-linked glycosylation results in the secretion of relatively more $H_2L_2$. Here we present evidence suggesting that (*a*) the absence of the $C_H3$ domain has led to conformational changes in these molecules, (*b*) these changes permit posttranslational glycosylation, and (c) unrestrained glycosylation can frequently yield unusual $CHO^+$ structures that make complete assembly unlikely.

The mouse myeloma cell line, MPC 11, has been the source of many variants affecting immunoglobulin heavy (H)[1] chain production. One such variant, M 311, synthesizes a short H chain of 40,000 mol wt as compared with the parental MPC 11 γ2b H chain of 55,000 mol wt (1, 2). We have shown by primary structural analysis that the smaller sized Ig H chain is due to precise deletion of the $C_H3$ domain (3). Comparison of the amino acid sequence obtained from the COOH-terminal peptide of M 311 and the DNA sequence of the γ2b constant region gene suggested that deletion of two adenines from a stretch of five produced a nonsense codon and a consequent premature termination of H chain synthesis (3). The lesion in the H chain was accompanied by other phenotypic alterations affecting its glycosylation and assembly. For example, a significant amount of cytoplasmic M 311 H chain was shown to be nonglycosylated in contrast to parental H chains, all of which bear carbohydrate (4). In addition, M 311 secretes both $H_2L_2$ (L, light) and HL species while the parent cell line secretes only the fully assembled molecule (4).

Another variant, independently derived from MPC 11, ICR 4.68.13.9 (13.9), also synthesizes a short (40,000 mol wt) H chain and large amounts of HL (2). Here we report that both M 311 and 13.9 synthesize a single H chain with apparently identical COOH-terminal deletions. The relationship between alterations in assembly and glycosylation patterns is explored.

## MATERIALS AND METHODS

*Purification of Immunoglobulin Paraprotein and Isolation of CNBr fragments:* Immunoglobulins M 311 and 13.9 were purified as described (5). $H_2L_2$ and HL were separated on a column of Ultrogel AcA 44 (LKB Instruments, Washington, DC), equilibrated in 0.15 M $NH_4HCO_3$ (3). Both M 311 and 13.9 $H_2L_2$ and HL were submitted to CNBr cleavage in 70% formic acid (5). The cleavage products, pools I′ and II′, were separated as described (5). Separation of CNBr fragments from pool I′ and pool II′ was carried out as described (5, 6).

*Amino Acid Analysis:* Peptides were hydrolyzed in 0.5 ml 6 N HCl and 15 μl 1 M phenol at 100°C for 20 h under vacuum (20 μm). Homoserine lactone was decyclized as described (7). Samples were then analyzed on a Durrum D-500 amino acid analyzer.

*SDS PAGE:* 5% SDS PAGE was performed as described (8). 10–20% SDS polyacrylamide Tris-Cl gradient gels were prepared according to the method of Maizel (9).

[1] *Abbreviations used in this paper*: H, heavy (chain); L, light (chain); mRNA, messenger RNA; Tm, tunicamycin.

*Cell Culture and Biosynthetic Labeling of Myeloma Cells:* Myeloma cells were grown as suspension cultures in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY), supplemented with 20% horse serum, nonessential amino acids, glutamine, penicillin, and streptomycin.

To biosynthetically label, we washed cells and resuspended them to $3 \times 10^6$/ml in Dulbecco's modified Eagle's medium without Na-pyruvate and all other amino acids (Gibco Laboratories, formula No. 78-0632), and supplemented with 10% fetal calf serum and 25 $\mu$Ci of $^{14}$C-amino acids (valine, threonine, and leucine [New England Nuclear, Boston, MA]).

*Inhibition of Glycosylation:* Inhibition of glycosylation was achieved by radiolabeling as described above except that cells were treated with 2 $\mu$g/ml of tunicamycin (Tm) (1 mg/ml in DMSO) for 1–2 h at 37°C before labeled amino acids were added (10). Control wells received the same volume of DMSO without Tm. Tm was a gift from Dr. R. Hamill, Eli Lilly, Indianapolis.

*Immunoprecipitation (11):* *Staphylococcus aureus* (IgSorb; Enzyme Center, Boston, MA) was washed and stored as a 10% suspension (wt/vol) at −70°C. 1 ml of labeled myeloma secretions was treated with 5 $\mu$l of rabbit anti-mouse IgG2 antisera on ice for 1 h. 50 $\mu$l of washed *S. aureus* was added and kept on ice for 30 min and the immune precipitate was collected by centrifugation. The resulting pellet was washed three times in *S. aureus* washing buffer minus ovalbumin. The washed precipitate was resuspended in 20–25 $\mu$l of 2% SDS (Bio-Rad Laboratories, Richmond, CA) and boiled for 2 min.

*RNA Isolation and Analysis:* Total cytoplasmic RNA was prepared essentially according to the guanidinium hydrochloride method (12). To prepare RNA for in vitro translation, we found that a phenol/chloroform extraction followed by an ether extraction was necessary. Poly A containing RNA was isolated by affinity chromatography on an oligo dT cellose column (13).

RNA was sized in an electrophoretic denaturing system using formamide/formaldehyde. Gels were 1.0% agarose in a buffer containing 10 mM 3-(*N*-Morpholino)propane-sulphonic acid (MOPS) (Leonard Garfinkel, personal communication), 5 mM Na-acetate, pH 7.0, 1 mM EDTA, and 2.2 M formaldehyde (Fisher Scientific Co., Pittsburgh, PA; 37% wt/wt). Running buffer was 10 mM MOPS, 5 mM Na-acetate, pH 7.0, 1 mM EDTA. Samples were prepared in a solution of 1× running buffer, 2.2 M formaldehyde, and 50% formamide (MC&B Mfg. Chemists, Inc., Norwood, OH). Up to 7 $\mu$g of total cytoplasmic RNA or 2 $\mu$g of polyA$^+$ RNA was loaded per gel track and electrophoresed at 7 V/cm for 3 h. RNA was transferred from the agarose gel to a nitrocellulose filter (14).

Probes were nick-translated to a specific activity of $10^8$ cpm/$\mu$g (15). When restriction fragments were used as the probes, DNase was withheld from the reaction mixture (15) and a specific activity of $10^7$ cpm/$\mu$g was obtained.

*Preparation of $C_H3$ DNA Fragment from $\gamma$2b:* The plasmid containing the complementary DNA for the $\gamma_{2b}$ gene, p$\gamma$2b (11)[7] (16) was digested with Sac I which generates a fragment of 300 base pairs containing the $C_H3$ domain. The digest was electrophoresed in a Tris-borate polyacrylamide gel, and the fragment containing the $C_H3$ domain was isolated by electroelution (17).

*Probe for the Variable Region of the MPC 11 H Chain Gene:* A gift of Dr. Kenneth Marcu, State University of New York Stony Brook, this was a 2.1 kilobase pair Hind III fragment of RBL216, a lambda vector containing the MPC 11 expressed $\gamma$2b gene (18). The Hind III fragment was inserted into the Hind III site of pBR322.

*RNA Hybridization:* Filters were prehybridized in 0.90 M NaCl, 0.90 M Na-citrate (3× standard saline-citrate buffer, SSC), 0.02% polyvinylpyrrolidone, 0.02% BSA, 0.02% Ficoll, 0.1% SDS, 20 $\mu$g/ml polyadenylic acid, 50 $\mu$g/ml sheared salmon sperm DNA, 50% formamide at 42°C for 4 h. Hybridization was carried out in a fresh change of the above buffer with the addition of $10^5$ cpm per track of RNA and 10% (wt/vol) of dextran sulfate in a volume of 1 ml per track for a minimum of 8 h at 42°C. The nitrocellulose filters were then put through four consecutive washes of 0.1× SSC and 0.1% SDS. Washes were done at 50°C for 15 min each. Filters were prepared for rehybridization by rinsing four times in boiling 0.01× SSC–0.01% SDS. Autoradiography was done at −70°C using a DuPont Lightening-Plus intensifying screen (E. I. DuPont de Nemours & Co., Inc., Newtown, CT).

*In Vitro Translation:* The wheat germ cell-free protein synthesizing system was used as previously described (19). Dog pancreatic microsomal membranes, prepared as described (20), were added to the cell-free system at a final concentration of 2 $A_{280}$/ml.

## RESULTS

Comparison by SDS PAGE of the H chains secreted from 13.9 to those secreted by M 311 showed that both short H chains are heterogeneous, co-migrating at an average molecular weight of 40,000, as compared with the 55,000 $M_r$ H chain produced by the parental cell line, MPC 11 (2). The simplest way to account for the decreased molecular weight in 13.9 is by a C-terminal deletion like that found in M 311. When CNBr fragments were generated from 13.9 Ig molecules and subjected to gel filtration, the elution profile was altered from that of MPC 11 but similar to that obtained from M 311 (3). Like M 311, 13.9 lacked the C-terminal parental fragments, II.1 and II.3 (Fig. 1), derived from the C-terminal segment of the $C_H2$ domain and the entire $C_H3$ domain. Instead, 13.9 had a new fragment (II.C) that contained no homoserine (Fig. 1 and Table I). Cyanogen bromide digestion cleaves methionine residues to produce a homoserine lactone at the new C-terminal position in each resulting peptide. The only fragment lacking homoserine is the COOH-terminal fragment of the intact H chain. The absence of homoserine from the amino acid composition of CNBr fragment II.C indicated II.C to be the COOH-terminal fragment in 13.9 H chains. The amino acid composition of this fragment was identical to that of II.C from M 311 (Table I). We therefore infer that the amino acid sequence of the COOH-terminal segment of 13.9 is identical to that obtained for CNBr fragment II.C from M 311 HL molecules. Thus 13.9, like M 311, has a precise $C_H3$ domain deletion that could result from a deletion of two adenine bases leading to immediate premature termination (Fig. 1*B*). To substantiate this hypothesis, we undertook analysis of messenger RNA (mRNA) by Northern blot hybridization.

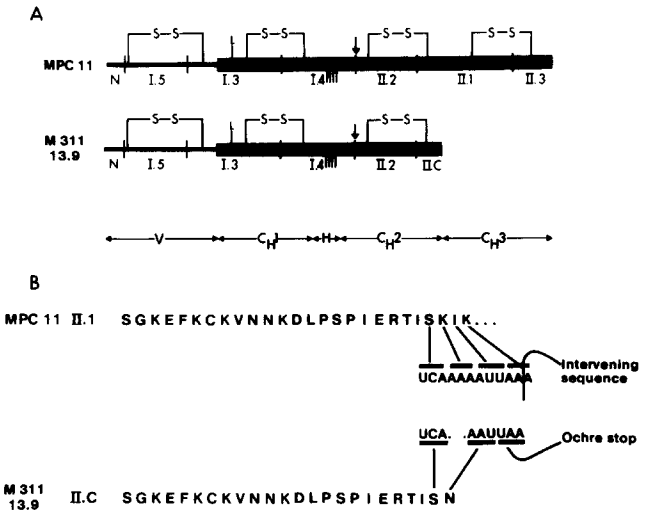


FIGURE 1 Carboxyl-terminal deletion in M 311 and 13.9 H chains. (*A*) Schematic of CNBr fragments of H chains of MPC 11, M 311, and 13.9. The arrow marks the separation by CNBr cleavage of N-terminal Pool I fragments (I.5, I.3, and I.4, coupled to a large segment of L chain) from C-terminal Pool II fragments (II.2, II.1, and II.3, for MPC 11; II.2 and II.C for M 311 and 13.9). CNBr fragments within Pool I and Pool II remain linked by disulfide bridges. (*B*) Comparison of the sequence of CNBr fragment II.1 from MPC 11 and II.C from M 311 HL. The amino acid composition of II.C from 13.9 is identical to that of II.C from M 311 and thus the sequences are presumed to be identical. The nucleotide sequence for MPC 11 is that reported for the $\gamma$2b DNA sequence (25). A deletion of two adenine bases would account for the observed substitution of asparagine for lysine and immediate premature termination of M 311 and 13.9 H chains.

## Analysis of mRNA from Variant Cells

To show that the $\gamma_{2b}$-$C_H3$ domain sequences were expressed in the RNA from these variant cell lines, we hybridized poly $A^+$ RNA with $p\gamma_{2b}$ (11)[7]-$C_H3$, a probe that contained only the $C_H3$ domain (see Material and Methods). This probe was specific for the $\gamma$2b gene, hybridizing relatively poorly with $\gamma$2a genes. The probe hybridized to messenger RNA from variant cells, (1.2 kilobase pairs, 17.5S; in agreement with the published size of MPC 11 H chain mRNA [16]) (Fig. 2). Although equal quantities of mRNA were applied to each track, M 311 RNA had a relatively less intense signal than that of the other mRNAs. When the same nitrocellulose filter was erased and rehybridized with a restriction fragment containing the MPC 11 variable region sequence, M311 mRNA showed a similar reduction in signal (Fig. 2*B*). Thus, intact heavy chain specific mRNA was present in smaller quantities in the M 311 preparation. We conclude that the $\gamma$2b-$C_H3$ domain is expressed in the mRNA of both M 311 and 13.9, consistent with the hypothesis that these variants arose by a frameshift mutation.

TABLE I

*Amino Acid Compositions of Fragment II.C from 13.9 and M311*

| Amino acid | 13.9* | M311‡ |
|---|---|---|
| CMCys | 0.84 | 1 |
| Asp | 4.1 | 4 |
| Thr | 1.0 | 1 |
| Ser | 2.2 | 3 |
| Hser | — | — |
| Glu | 2.1 | 2 |
| Pro | 1.9 | 2 |
| Gly | 1.1 | 1 |
| Ala | — | — |
| Val | 1.1 | 1 |
| Ile | 1.7 | 2 |
| Leu | 0.90 | 1 |
| Tyr | — | — |
| Phe | 1.0 | 1 |
| His | — | — |
| Lys | 3.8 | 4 |
| Arg | 1.0 | 1 |

* Fragment II.C was isolated from $H_2L_2$.
‡ Based on the sequence established for II.C of M311 (3).

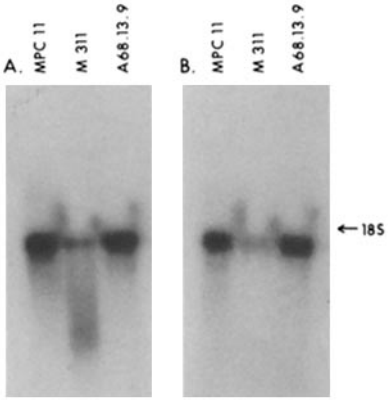


FIGURE 2 The $C_H3$ domain is expressed in variant H chain mRNA. Poly $A^+$ RNA was isolated from MPC 11, M 311, and 13.9 and size fractionated by formaldehyde-formamide gel electrophoresis on 1.0% agarose gels in a 10 mM MOPS buffer system (Material and Methods). 2 $\mu$g of poly $A^+$ RNA was loaded per track. RNA was transferred to nitrocellulose and (*A*) hybridized with the $\gamma$2b-$C_H3$ restriction fragment probe, or (*B*) filter A was washed to remove $\gamma$2b-$C_H3$-labeled probe and rehybridized with the $V_{MPC\,11}$ restriction fragment probe. Size markers were 28S and 18S eucaryotic and 23S and 16S bacterial ribosomal RNA (not shown).

## In Vitro Translation of Variant mRNA

M 311 and 13.9 secrete approximately equal amounts of HL and $H_2L_2$, in contrast to the parental cell line MPC 11, which secretes predominantly $H_2L_2$. To examine whether the heavy chains associated with HL and $H_2L_2$ represented genetically distinct H chains, we isolated total cytoplasmic RNA from variant cell lines and then translated it in the wheat germ system. The immunoprecipitated translation products were analyzed by gel electrophoresis (Fig. 3, data are shown for 13.9). The finding of a single heavy chain band implies that a single primary translation product is responsible for both HL and $H_2L_2$ forms of Ig produced by 13.9 and M 311.

## Unusual Glycosylation Perturbs Assembly

An alternative explanation to account for the substantial amounts of dimeric HL secreted from the variant cell lines is that a posttranslational event, such as glycosylation, alters the H chain structure, thus inhibiting it from completely assembling into $H_2L_2$. Treatment of cell cultures with Tm will inhibit asparagine-linked carbohydrate addition while not significantly affecting Ig secretion (21), making it possible to test this hypothesis. The amount of secreted $H_2L_2$ relative to HL is greater when produced in the presence of Tm than that seen in the absence of Tm (Fig. 4). Though MPC 11 was slightly affected by Tm treatment, the perturbation was not as visible since 90–95% of its H chain is normally assembled into $H_2L_2$. This result suggests that the presence of an N-linked glycosylation product in M 311 and 13.9 diminishes the relative amount of secreted $H_2L_2$. Furthermore, previous studies (22) have shown that this shift does not result from intracytoplasmic degradation of nonglycosylated heavy chain.

## H Chains of Secreted HL and $H_2L_2$ Are Differentially Glycosylated

To directly ascertain whether the H chains associated with

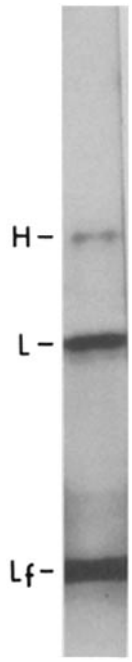


FIGURE 3 A single primary translation product is responsible for M 311 and 13.9 H chains. Total cytoplasmic RNA from 13.9 was translated in a wheat germ extract system, immunoprecipitated, and analyzed by 10–20% SDS PAGE (Material and Methods).

HL and $H_2L_2$ could be distinguished, radiolabeled HL and $H_2L_2$ secreted from M 311 and 13.9 were isolated by gel filtration chromatography (Material and Methods). When the mobilities of the H chains were compared by gel electrophoresis, the H chains of HL and $H_2L_2$ both showed multiple bands (Fig. 5). The predominant band for H of $H_2L_2$ had the lower apparent molecular weight implying that additional carbohydrate could account for the H chains of HL, consistent with the results of Tm treatment described above. Since there is only a single primary translation H chain product made by M 311 and 13.9 mRNA, this result supports the notion that differential glycosylation may influence Ig assembly in these variant cells.

## Comparison of Cytoplasmic and Secreted H Chains

It had been previously demonstrated that in vivo most of the M 311 H chain remained nonglycosylated during the first few minutes after translation, but with time, the cytoplasmic H chain pool became increasingly glycosylated (4). The same phenomenon is seen in 13.9 cells. After these cells were biosynthetically labeled for 10 h, analysis of the immunoprecipitated cytoplasmic H chain by gel electrophoresis showed

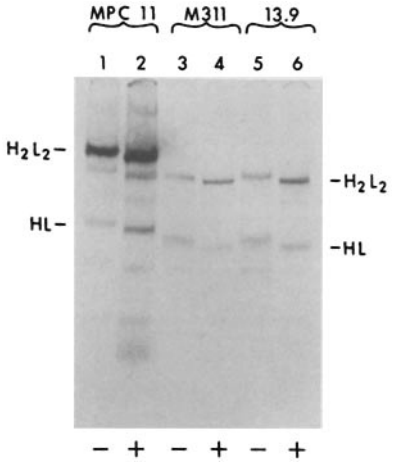


FIGURE 4 Inhibition of glycosylation promotes assembly of short H chains. MPC 11, M 311, and 13.9 cells were labeled for 10 h with $^{14}C$ Val, Thr, and Leu. Secreted Igs were immunoprecipitated and examined by SDS PAGE (5%). + and − indicate the presence or absence of Tm in the labeling cultures.

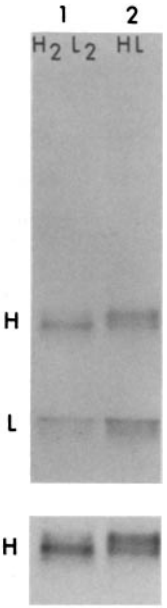


FIGURE 5 The H chains of HL and $H_2L_2$ are differentially glycosylated. M311 cells were biosynthetically labeled, and HL and $H_2L_2$ were separated by gel filtration chromatography as described in Material and Methods. The isolated Ig variant molecules were reduced and electrophoresed on a 10–20% polyacrylamide gradient gel in the presence of SDS (Material and Methods). The gel was autoradiographed for 7 d (*upper*) and 14 d (*lower*).

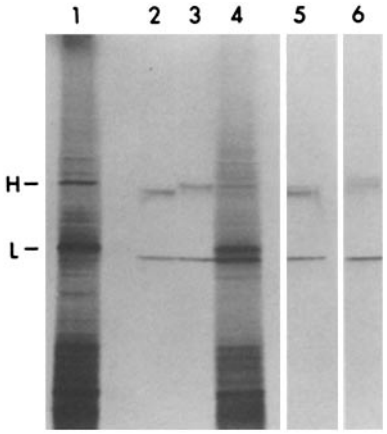


FIGURE 6 Comparison of cytoplasmic and secreted variant H chains analyzed by 10–20% gradient SDS PAGE. Total cytoplasmic RNA from 13.9 was translated in a wheat germ extract system in the absence (lane *1*) and presence (lane *4*) of dog pancreatic membranes. Biosynthetically labeled (10 h) and immunoprecipitated Ig produced by 13.9; (lane *2*) cytoplasmic, in the presence of Tm; (lane *3*) cytoplasmic, in the absence of Tm; (lane *5*) secreted, in the presence of Tm; and (lane *6*) secreted, in the absence of Tm.

two H chain bands in a ratio of about 90/10 (upper to lower bands) (Fig. 6, lane *3*). Similar analyses of shorter periods of labeling—15 and 30 min and 3 h—show progressive movement of H chain from the lower to upper bands (data not shown). To show that this molecular weight change is due to glycosylation, we biosynthetically labeled 13.9 cells in the presence of Tm and analyzed them by gel electrophoresis. Cytoplasmic H chains from cells treated with Tm migrate as a single band coincident with the lower cytoplasmic H chain band observed in untreated cells (Fig. 6, lanes *2* and *3*). Our studies here indicate that after 10 h of biosynthetic labeling, the predominant cytoplasmic H chain form in 13.9, like M 311, is glycosylated. The glycosylated cytoplasmic H chain has a mobility identical to that of H chain synthesized by in vitro translation in the presence of microsomal membranes (Fig. 6, lane *4*).

Additional glycosylation events occur upon secretion. Secreted H chains from M 311 and 13.9 gave a heterogeneous pattern of three to four bands when analyzed by gel electrophoresis (Fig. 6, lane *6*). Treatment of MPC 11 with Tm yielded one secreted H chain band of faster mobility (data not shown). In contrast, M 311 and 13.9 expressed two secreted H chain bands after Tm treatment, (Fig. 6, lane *5*). The lower of these two H chain bands is likely to represent the completely nongycosylated H chain form because it comigrates with cytoplasmic H chain immunoprecipitated from cells that had been radiolabeled in the presence of Tm (Fig. 6, compare lanes *2* and *5*). By inference, the upper band likely contains O-linked sugar moieties, which are known to be unaffected by Tm treatment (23, 24). These results suggest that late steps of glycosylation, including presumptive O-linked sugars, occur late in the secretory pathway. It remains unclear whether the secreted H chain is drawn specifically from either or both $CHO^+$ or $CHO^-$ cytoplasmic H chain pools.

## Comparison of Glycosylation Sites on the H Chains of MPC 11, M 311, and 13.9

Observation of multiple bands for secreted Ig H chain from 13.9 and M 311 suggests the existence of multiple glycosylation sites. The amino acid sequence constellation of Asn-X-Ser/Thr has been shown to be a requirement for addition of asparagine-linked carbohydrate (24). One asparagine-linked glycosylation site for MPC 11 H chain was predicted from

the complete DNA sequence (25) and confirmed by subsequent studies (26, 27). It lies in the C-terminal region of the molecule, in the peptide corresponding to CNBr fragment II.2 (Fig. 1*A*). To confirm the presence of this glycosylation site in the variants, we isolated fragment II.2 from M 311 $H_2L_2$ and submitted it to tryptic digestion. The resulting tryptic peptides were separated by gel filtration and the pools were submitted to amino acid analysis. A tryptic peptide was identified with the amino acid molar ratios of Glu, Asp2, Tyr, Ser, Leu, Arg, which corresponded to the predicted glycosylated peptide. The sugar composition (derived by Dr. J. Hakimi, Albert Einstein College of Medicine, using gas chromatography) was fucose 1.15, mannose 3.0, galactose 1.68, and GlcNAc 3.74, where mannose was used to normalize the ratios), and was as expected for complex type N-linked sugars (24). It can be concluded that like MPC 11, the M 311 H chain contains an N-linked sugar in the $C_H2$ domain.

Other glycosylation sites for MPC 11 and the short H chain variants were reported to be associated with the N-terminal portion of the molecule—most likely within CNBr fragment I.3[2] (Fig. 1; see references 26, 27). Cyanogen bromide digestion of the MPC 11 Ig molecule generates two major peptide fragments, Pool I (which contains fragment I.3) and Pool II (which contains fragment II.2), approximately equivalent to Fab and Fc, respectively (Fig. 1, reference 9). When the CNBr fragments associated with Pool I of parental and variant H chains were compared by amino acid analysis, no differences were found (28). Therefore, any differences in migration of Pool I fragments of MPC 11, M 311, and 13.9 should reflect differences in carbohydrate. In Fig. 7, two major bands are seen for Pool I from both MPC 11 and M 311 $H_2L_2$; however, while the lower bands co-migrate, the upper ones do not. After treatment with Tm, no apparent change in Pool I bands is seen (Fig. 7). The results obtained for 13.9 Pool "I" were identical to those found for M 311. These data suggest that there are glycosylation sites in the Fab portion of these Igs not predicted from the DNA sequence of the gene coding for the MPC 11 H chain. In addition, the sugar moiety present in at least one site in this segment of MPC 11 differs from that seen for M 311 or 13.9.

## DISCUSSION

M 311 and 13.9 are two variant cell lines independently isolated from MPC 11, both of which produce short H chains of 40,000 mol wt. When compared with the parental $\gamma$2b heavy chain, both variant H chains were found to have comparable C-terminal deletions comprising the $C_H3$ domain. The C-terminal CNBr fragment of M 311 is identical in primary sequence to a corresponding segment of a parental H chain CNBr fragment with the exception of a lysine to asparagine substitution at the C-terminal residue (3). The amino acid composition of the corresponding C-terminal peptide in 13.9 is identical with that obtained for M 311 and suggests that the same genetic mechanism generated these variants: a −2 frameshift mutation, producing a nonsense codon that leads to premature termination (3). Northern blot hybridization showed that $\gamma$2b-$C_H3$ sequences are expressed in the short variants, supporting this hypothesis.

[2] Using the gel filtration protocol outlined by us in previous studies, Weitzman and Portmore showed radiolabel to be associated with Pool I.3 in Fig. 3, reference 27. However, Pool I.3 was improperly named Pool I.4 in their manuscript.

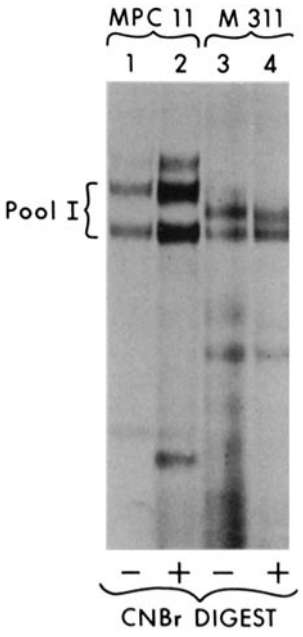


FIGURE 7 Glycosylation sites in the Fab portion of MPC 11 and M 311. Cells were biosynthetically labeled and secretions were immunoprecipitated (as in Materials and Methods), released from *S. aureus* by addition of 70% formic acid, digested with CNBr as described to generate Pools I and II, and separated by electrophoresis on 5% SDS-PAGE under nonreducing conditions. + and − indicate treatment of the cultures with Tm during the labeling procedure.

The deletion of the $C_H3$ domain is accompanied by additional alterations in heavy chain glycosylation and assembly. For example, M 311 assembles and secretes an unusually large amount of dimeric HL as compared with the MPC 11 cell line (4). This observation has now been extended to 13.9, a secondary variant cell line which expresses a H chain with the same apparent genetic mutation as found in the M 311 H chain. In contrast, Laskov et al. (29) have shown that myeloma cells producing normal-sized H and L chains do not accumulate significant amounts of HL in the cytoplasm or secretions.

We considered two possible explanations for the presence of excess HL in variants making short H chains: (*a*) primary structural differences between the H chains of HL and $H_2L_2$, or (*b*) an assembly blockage due to unusual glycosylation. The first hypothesis was tested by in vitro translation of the RNA isolated from the variants which showed that a single H chain is produced. Accumulation of HL in these variants, therefore, does not reflect production of a second genetically distinct H chain.

Rather, the assembly process appears to be perturbed by two factors acting in synergy; the absence of the $C_H3$ domain and aberrant glycosylation. It is quite possible that the differences in sugar moieties between MPC 11 and M 311 (and presumably 13.9) have been initiated by the loss of the $C_H3$ domain, which in turn confers conformational changes throughout the molecule. In support of this, it has been shown that alteration of protein conformation can lead to changes in glycosylation pattern both for site attachment and subsequent sugar processing (22, 24, 28, 29). Indeed, previous studies have shown that in MPC 11 cells, H chains that have completed translation can no longer be glycosylated while both nascent and completed M 311 (and 13.9) H chains can be glycosylated (4, 22, 30, 31). It has also been shown that the assembly of M 311 H chain into a complete $H_2L_2$ molecule proceeds more slowly than that of MPC 11 (4). X-ray diffraction studies of the IgG molecule indicate that the $C_H1$ and $C_L$ domains interact as do the $C_H3$ domains by noncovalent forces. The $C_H2$ domains, however, do not interact: the site through which the $C_H2$ domains would interact is obstructed

by the presence of carbohydrate (32, 33). It is possible that H chain that is progressively glycosylated will finally attain a CHO+ structure that is too large to be accommodated between the two H chains and that will disrupt the orderly progression of interchain disulfide bond formation. These H chains will ultimately appear as HL half molecules. Consistent with this suggestion is the finding that H chains isolated from HL molecules carry higher MW sugar structures.

Analysis of secreted heavy chains produced by both M 311 and 13.9 variant cell lines showed multiple bands indicating extensive glycosylation heterogeneity (Figs. 5 and 6). Inhibition of N-linked glycosylation with Tm reduced the number of secreted H chains to two, rather than one as seen for cytoplasmic H chains (Fig. 6). This suggests that a subpopulation of secreted H chains may contain carbohydrates that are unaffected by Tm treatment (e.g., O-linked sugars). Since Tm treatment resulted in H chains that migrated more rapidly, these studies support the conclusion that almost all secreted variant H chains are glycosylated. This differs from an earlier study which reported that 30% of H chains secreted by M 311 remain nonglycosylated (4). In that previous study, conclusions based on data for cytoplasmic H chains were assumed to apply to secreted H chains. Additionally, small molecular weight differences contributed by carbohydrate could not be discriminated with the gel electrophoresis system used.

Weitzman et al. (26) have shown that there are two sugar attachment sites in the MPC 11 H chain, one located in the $C_H2$ domain (fragment II.2) and one in the N-terminal segment (fragment I.3) (Fig. 2) (27). Only the former is predicted from the DNA sequence to contain a site for N-linked sugar (25). In M 311, three sugar moieties were found, two of which were presumed to be located at sites homologous to those found in MPC 11, i.e., fragments II.2 and I.3 (26, 27). We have confirmed the presence of sugar on the M 311 II.2 CNBr fragment: amino acid analysis and sugar composition of the $CHO^+$ tryptic peptide have identified this sugar attachment site as the asparagine-linked type. On the basis of differences in mobility on P6 gel chromatography after pronase treatment, Weitzman and co-workers (26, 27) had concluded that none of the $CHO^+$ moieties present on M 311 was identical to the MPC 11 parental forms. Neuraminidase treatment of the carbohydrate moieties associated with the M 311 H chain suggested that sialic acid is not responsible for $CHO^+$ heterogeneity (26). Comparison of the sugar structures in the H chains of MPC 11 and M 311 was carried out by digestion with endo-*N*-acetylglucosaminidase-D (26). The MPC 11 H chain carried a minor large glycopeptide resistant to digestion and a major smaller structure sensitive to digestion. In contrast, the M 311 H chain carried a relatively large endoglycosidase-resistant glycopeptide as the predominant species. Our studies of MPC 11, M 311, and 13.9 show that the N-terminal segment of the molecule, generated by CNBr cleavage (Pool I, see Fig. 1), has two bands when analyzed by SDS PAGE, reflecting the presence of differentially glycosylated forms (Fig. 7). This pattern is unchanged after treatment of cells with Tm, suggesting that an O-linked sugar resides in the N-terminal of the parental chain. Taken together, these analyses imply that the variant H chains have one N-linked sugar in the II.2 CNBr fragment and two other sugar moieties associated with the Fd region. (L chains are not glycosylated.)

Precedent for differential glycosylation is shown by human H chains (34). In this case, membrane and secreted $\delta$-type immunoglobulins are found as two primary translation products which are subsequently glycosylated to give four discrete forms. McCune et al. (34) have suggested that this phenomenon may reflect structural features of the H chains that are incompatible with uniform attachment of core oligosaccharides. A similar example of differential glycosylation is the bovine pancreatic RNases, A and B: although of presumptive identical amino acid sequences, RNase B is glycosylated and RNase A is not (35, 36).

Interestingly, the original MPC 11 tumor secreted large amounts of HL dimer (37) while the MPC 11 myeloma cells adapted to culture did not (38). The unusual glycosylation pattern expressed in the variant cells may represent a reversion to the original tumor phenotype. This explanation is supported by the observation that differential glycosylation occurs only in the secreted Ig. The variant cell lines characterized here will be good models for investigation of the subcellular localization and post-translation modification of Ig protein during secretion.

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