STRUCTURE AND FUNCTION OF MURINE IMMUNOGLOBULIN M FROM SERUM AND CELL MEMBRANE

Thesis by

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To my mother, Edith

and my husband

Larry

Many thanks to those who worked and talked with me, Sandy Ewald, Minnie McMillian, and John Frelinger;

My appreciation goes to my advisor, Lee Hood, for his enthusiasm about science and his humor and receptiveness in listening to me;

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Abstract

Immunoglobulin M (IgM) molecules are secreted into the bloodstream by plasma cells as soluble pentamers and also exist as monomeric integral membrane receptor proteins on the surface of B lymphocytes. Investigation of the structure of membrane and secreted μ chains has provided an understanding of the basis for the existence of IgM molecules in two very different physical environments.

The complete amino acid sequence of a μ chain secreted by the murine myeloma MOPC 104E has been determined. When the μ chains of mouse, human and dog are compared, there is a striking gradient of increasing amino acid sequence homology from the NH_2-terminus to the COOH-terminus of the μ chain, reflecting a functional conservation of structure. There are five sites of carbohydrate attachment in the mouse μ chain constant region, one of which is present 14 residues from the carboxyterminus. The secreted μ chain (μ_s) contains no stretches of unchanged amino acids long enough to allow it to exist as an integral membrane protein.

IgM molecules synthesized by the murine B lymphoma, WEHI 279, have been characterized. The cells synthesize internal precursors to secreted μ chains which contain incompletely glycosylated complex carbohydrate moieties but in all other respects are identical to secreted μ chains. WEHI 279 membrane IgM is monomeric and contains mature complex carbohydrate structures. The μ_m and μ_s chains differ in the structure of their COOH-terminal regions. The carbohydrate moiety and methionine residue present in the COOH-terminal 19 amino acids of μ_s chains are absent in μ_m chains. In addition, four COOH-terminal amino acids which are different from the carboxyterminus of μ_s chains are released by carboxypeptidase treatment of μ_m chains. Based on these protein and other nucleic acid data, we believe μ_m chains possess an uncharged and hydrophobic C-membrane terminal domain which allows monomeric IgM molecules to be integral receptor proteins in the B cell plasma membrane.

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INTRODUCTION

Antibody molecules constitute an important part of an organism's major defense system against disease and foreign substances. This system of humoral immunity exhibits remarkable specificity, diversity, memory and exquisite control. Despite its importance, knowledge of the detailed molecular events governing an individual's immune response to a foreign antigen is still sparse. One of the most fascinating and as yet unanswered questions in the intricate process of antibody production is how a specific antibody secreting cell, a B lymphocyte, is stimulated by an antigen to divide and differentiate into a clone of antibody producing plasma cells. More than ten years ago, it was found that a B lymphocyte possesses antibody molecules on its cell surface which are available for interaction with a complementary antigen (literature review in 1). This then established the specificity of the B cell triggering process. The predominant class of antibodies present as integral membrane proteins on the cell surface of B lymphocytes is called Immunoglobulin M (IgM) (1). The IgM class of antibodies is also secreted by plasma cells as a soluble molecule and circulates in the bloodstream, where it binds and clears antigen, fixes complement, and functions in the humoral immune system. In the bloodstream IgM exists as a covalently linked pentamer, yet on the B cell surface where it plays a crucial role in antigen induced differentiation, IgM is present as a monomer. My research has used the well-characterized immune system of the mouse to investigate the structural basis which allows IgM molecules to exist in two very different environments: as water soluble pentamers in the bloodstream and as monomers in the hydrophobic plasma membrane. Delineation of the structural differences between membrane bound and secreted IgM molecules may provide an understanding of the basis for their different effector functions and perhaps lend insight into the process of B lymphocyte triggering by antigen. I will 1) review antibody structure, 2) summarize what is currently known about

the B cell differentiation process in the mouse and 3) review the work done on this problem by other investigators over the last nine years.

Antibody Structure (2)

Antibody molecules are composed of two different polypeptide chains, heavy (H) and light (L) chains which are held together by disulfide bonds and form a monomeric immunoglobulin unit, H_2L_2. Each chain is divided into a variable (V) or antigen binding region and a constant (C) region. There are five different classes of H chain which differ in their constant region structures: γ, μ, α, ε and δ. The five corresponding classes of immunoglobulins are designated IgG, IgM, IgA, IgE and IgD, respectively. The V regions of the H and L chains fold together to form the antigen combining portion of the antibody. The constant region of a γ heavy chain, for example, is composed of three structural domains (based on X-ray chrystallographic analyses, structural studies, and amino acid sequences) (3), Cγ1, Cγ2 and Cγ3, which carry out the effector functions of the IgG molecule. Effector functions are carried out by the antibody following antigen binding. Cγ1 folds with the C_L domain and is separated from the Cγ2 and Cγ3 domains (formed by the homologous association of the two γ chains) by a region of great structural flexibility in the γ chain, the hinge. The basic structure of an IgG molecule is illustrated in Figure 1.

The structure of serum IgM differs in several important respects from that of IgG. Mu chains contain four constant region domains, one additional domain than is present in γ chains. Mu chains have no hinge region but possess a 20 amino acid carboxyterminal extra-domain segment after the Cμ4 domain (3). The molecular weight of μ chains is 72,000 and they are moderately glycosylated (13% carbohydrate by weight) (4). This is the highest molecular weight of

Figure 1. Structure of the IgG molecule.

The heavy (H) and light (L) chains are shown as they interact to form the functional domains of an IgG molecule. Variable (V) regions are stippled and the hinge region is shaded. Disulfide bridges between the chains are indicated by heavy black lines. The portions of the molecule produced by papain cleavage in the hinge region are designated as the Fab (antibody binding) and Fc (crystallizable) fragments (adapted from reference 2).

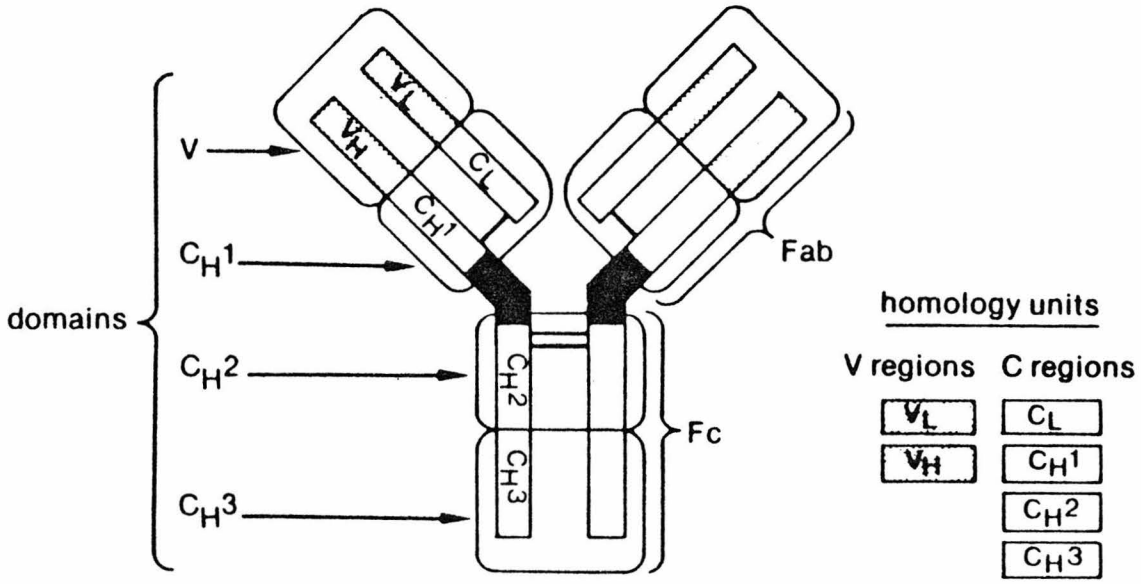
V
C_H1
C_H2
C_H3
domains
V_L
V_H
C_L
C_H1
C_H2
C_H3
Fab
Fc
homology units
V regions
C regions
V_L
V_H
C_L
C_H1
C_H2
C_H3

FIGURE 1.

any of the different heavy chain classes (e.g., γ chains have a molecular weight of 55,000), and mu chains are approximately three times larger than light chains. Serum μ chains have five sites of carbohydrate attachment in the constant region (5, 6). The NH_2-terminal three carbohydrates are complex type and the COOH-terminal two are high mannose. Noteworthy is the fact that the most COOH-terminal carbohydrate moiety is attached to the 20 amino acid extra-domain region of the μ chain.

The COOH-terminal region of the μ chain plays an important role in the existence of pentameric IgM $(\mu_2 L_2)_5$ (IgG exists only as a monomer). Prior to secretion, IgM monomers polymerize covalently by forming disulfide bonds between the penultimate cysteine residues in the μ chains (7). A third polypeptide chain denoted joining (J) chain, also is present in the IgM pentamer (one per pentamer), and is thought to play a role in facilitating IgM secretion by catalyzing the polymerization of monomers (8, 9).

IgM is also unique in that it is the most ancestral of the five immunoglobulin classes. Antibody molecules synthesized by organisms with very primitive humoral immune systems are composed of H chains similar to the μ chain of higher mammals (10). In addition, during the development of the immune system IgM is the first class of antibodies expressed — both as a membrane bound receptor and as a soluble pentamer (11). Thus, IgM is a fascinating immunoglobulin on which to perform structural and evolutionary studies.

Murine B Lymphocyte Differentiation

In the fetus, lymphoid cell precursors are found in the liver (12, 13, 14). (Undifferentiated lymphoid precursors or stem cells in the adult are derived from the bone marrow and migrate to the spleen where they undergo differentiation steps similar to those of fetal liver stem cells.) The earliest B cell stage is

detected in the fetal liver at 11 days gestation (12). This large dividing pre-B cell contains cytoplasmic μ chains, no light chains, and has no IgM on the cell surface (12, 15, 16, 17). The large pre-B cell differentiates further into a small pre-B cell that is nondividing and again contains cytoplasmic μ chains (15). Additional steps (which include initiation of light chain synthesis) then give rise to a small nondividing B lymphocyte displaying membrane bound monomeric IgM on its cell surface (1). This, then, is the B cell which may be triggered by antigen to divide and differentiate into plasma cell clones secreting IgM, IgG, IgA, etc.

Most B lymphocytes in the adult spleen also display IgD on their cell surfaces (8). Membrane IgM appears before IgD on differentiating lymphocytes (19). Recent studies show that IgD may allow paucivalent antigens to more effectively stimulate the resting B lymphocyte (20), although the exact role played by IgD in initiation of the humoral response remains unclear. Structural information on δ heavy chains is currently being obtained in several laboratories.

The cellular changes accompanying B lymphocyte differentiation to IgM secreting plasma cells are also unknown, but clearly initiation of J chain synthesis is required (21). At the level of antibody genes, recent findings have shown that the commitment of a pre-B cell to μ chain synthesis includes a rearrangement of DNA so that a given V_H gene segment becomes situated 5' to the Cμ gene (22). In addition, changes accompanying B lymphocyte differentiation to plasma cells also frequently involve DNA rearrangement; the differentiation of a B lymphocyte synthesizing membrane IgM to a plasma cell synthesizing IgG or IgA requires that the V_H gene segment adjacent to the expressed Cμ gene be moved adjacent to the Cγ or Cα gene. This pattern of gene rearrangement occurs in IgA and IgG secreting myelomas (23).

Clearly, B cell differentiation and triggering are complicated processes. An ideal starting point for studying antigen interaction with B lymphocytes is to

study the antigen receptor, monomeric membrane IgM. By investigating this receptor, we hope to understand the changes involved as the differentiating B cell begins to synthesize and secrete pentameric IgM and ceases synthesis of cell surface receptor IgM.

History of Work Done on Membrane IgM

The existence of monomeric IgM on the surface of murine splenic B lymphocytes was first documented in 1973 by Vitetta and coworkers (1). The role of membrane IgM in B cell triggering was subsequently clarified by many investigators. Although antigen binding to cell surface IgM is not the only signal which is required for B cell activation (24), antigen interaction is extremely important in determining the specificity and the memory of an antibody response. The general structure of human serum IgM has been known for several years (25), and an obvious question was how the membrane and secreted forms of IgM could have such similar overall structures, yet exist in such different physical environments and carry out different effector functions.

Early studies focused on the logical hypothesis that IgM molecules were attached to the cell surface via their μ chains, leaving the antigen binding V domains available for interaction with molecules in the external environment. Therefore, membrane μ (μ_m) chains were isolated and compared with secreted μ (μ_s) chains. Membrane μ chains could be dissociated from the cell surface only by detergents, implying that membrane IgM is an integral membrane protein (26, 1). A subtle molecular weight difference between μ_m and μ_s chains was also apparent, μ_m being larger than μ_s by approximately 1500 daltons (27). Attempts at examining the carbohydrate structure of μ_m chains by incorporation of radiolabeled sugars showed conflicting results due to the slow turnover rate of membrane IgM in resting splenic B lymphocytes (28, 29). Anderson and coworkers found that μ_m chains were not

glycosylated with terminal sugars as were μ_s chains (29). Yet, Vitetta and Uhr showed significant incorporation of galactose, fucose and glucosamine into surface IgM (28). We now know that the carbohydrate structures of μ_m chains are fully glycosylated, although μ_m lacks a glycosylation site present in μ_s chains (30, 31). Low specific activity radiolabeled sugars and isolation procedures for membrane IgM may have been a problem in these previous experiments (28). Serological studies also suggested that certain antigenic determinants present on secreted IgM were absent on membrane IgM bound to the B cell surface. These differences were serologically localized to the Fc region of membrane IgM, implying that a more COOH-terminal portion of the μ_m chain is responsible for structural differences between membrane and secreted IgM (1, 32). Detergent binding studies also showed that membrane IgM bound small but significant amounts of detergent when compared with secreted IgM (33, 34).

The μ_m chains used in this early work were derived from resting B lymphocytes isolated from the spleen. There are many difficulties inherent in structural studies on such B cells and their membrane IgM molecules. These difficulties include very small quantities of protein, low protein turnover rates, a heterogeneous population of μ_m molecules (different V_H's), a heterogeneous population of spleen cells in different developmental stages, and relative to plasma cells, poor incorporation of radiolabeled amino acids and sugars. For the above reasons, many investigators now have turned to studying B cell tumors as a source of membrane IgM.

In the murine system of inbred strains, experimentors have found a spectrum of B cell tumors which seem to be arrested at various stages in the differentiation pathway from pre-B cells to plasma cells (36, 37). These differentiation stages can be distinguished morphologically and serologically on the basis of characteristic cell surface proteins. The cells are grown in continuous culture, divide, and synthesize homogeneous antibody molecules. Nonetheless, one must be cautious in

interpreting experiments performed on tumor cells. The developmental stages may simply represent, for example, blockage of a biosynthetic step (e.g., J chain synthesis) required for IgM secretion. Extensive characterization of the cells is necessary before valid conclusions can be drawn from experiments involving tumor lines. In particular, we have found that B-cell lymphomas (the tumor equivalent of a nonsecreting small B lymphocyte) synthesize an internal form of μ_s chains (pre-secreted μ) in addition to μ_m chains (30, 31). Solubilized whole cells then yield a population of μ chains consisting of μ_s and μ_m molecules which need to be completely separated from one another for use in structural studies. In particular, this situation has caused two independent research groups who performed similar experiments on different B cell tumors to draw conflicting conclusions regarding the structural nature of the COOH-terminus of μ_m chains. By digesting μ_m chains with carboxypeptidase, Feinstein and coworkers found that the COOH-terminus of μ_m chains was identical to that of μ_s chains (38) (which possess a COOH-terminal Tyr residue). On the other hand, Grey and coworkers found that several hydrophobic amino acids other than Tyr were released from μ_m chains by carboxypeptidase (39). Our findings indicate, though, that the two results can be reconciled by considering the fact that μ_m and μ_s chains are indeed different at their COOH-termini and that many B cell lines synthesize both a membrane bound and an internal presecreted form of μ chains (30, 40, 41, 42). Peptide mapping studies on μ_m chains derived from a B-cell lymphoma also showed that small peptide differences exist between μ_m and μ_s chains. Unfortunately, these differences were situated on a high background due to the design of the experiment (multiple labeled amino acids and incomplete enzyme digestion) (35).

Other investigators have more recently characterized the IgM molecules synethsized by other B cell tumor lines. Our structural findings have been complemented by these more indirect studies. A brief summary to date of published

and unpublished findings is as follows. B cell lines do synthesize both μ_m and μ_s chains in varying proportions depending upon the individual cell line (30, 40, 41, 42). μ_m chains are clearly larger in molecular weight than μ_s chains. The molecular weight difference between μ_m and μ_s chains is attributable to a difference in the primary amino acid structures of μ_m and $\mu_s$chains. In particular, μ_m chains synthesized in the presence of the glycosylation inhibitor tunicamycin are 1500 daltons larger than nonglycosylated μ_s chains (30, 34, 43, 44). In addition, translation of μ_m and μ_s mRNA in vitro results in the synthesis of a μ_m polypeptide which is larger in molecular weight than the μ_s polypeptide (43, 44, 45). μ_m possesses a different and longer C-terminal domain which contains a hydrophobic region that may be capable of interacting with the lipid bilayer (31, 34, 46). This altered C-terminal domain explains all of the observed differences between μ_m and μ_s chains. The two chains differ in the number of carbohydrate attachment sites, μ_m lacking a high mannose carbohydrate structure present in the C-terminal region of μ_s chains (30, 31). The 20 additional amino acids in μ_m chains plus lack of a high mannose carbohydrate structure is reflected in the 1500 dalton molecular weight difference between mature μ_m and μ_s chains (30, 43, 44, 46). Finally, the μ_s chains have a COOH-terminal Tyr residue (4, 6), while μ_m chains have a different C-terminal domain containing COOH-terminal Val (31). This is consistent with the inability to label a Tyr residue in μ_m chains on the inside of the plasma membrane (47). The biochemical changes which occur in μ_m chains will be discussed in greater detail in the following chapters and conclusion.

General Approach of Research

When this project was started four years ago, virtually nothing was known about the structure of mouse μ_s chains (the sequence of human μ_s chains was

determined in 1973 by Putnam and coworkers (25)). Since large quantities of μ_s chains for structural determinations are obtainable from mouse myelomas, our strategy was as follows. The structural comparisons we wished to make between μ_s and μ_m chains were at the level of the primary amino acid sequence. Therefore, once we had detailed structural information on μ_s chains, comparative studies with μ_m chains should be relatively straightforward. It was decided to then characterize the cyanogen bromide (CnBr) fragment structure of the μ_s chains synthesized by the mouse myeloma line MOPC 104E (a plasmacytoma induced in BALB/c mice by mineral oil injection). This particular μ_s chain was interesting for several reasons: i) MOPC 104E (M104E) IgM specifically binds the simple hapten α-1,3-dextran, an antigenic constitutent of many bacterial cell surfaces; ii) a particular dextran idiotype had been defined using M104E IgM; iii) IgM biosynthesis and secretion had been well studied in M104E cells (4, 48, 49) and; iv) M104E μ_s chains were used by a large number of investigators as a "reference standard" μ chain for overall structural characteristics such as molecular weight and glycosylation. Two years of classical and not so classical protein chemistry (which included devising procedures for handling the relatively insoluble M104E μ_s chain and its CNBr fragments) brought the structural work to 80% completion. During the next year when the primary structure was completed and carbohydrate attachment sites were identified, a rabbit antiserum to M104E μ_s chains was made and work was started on characterizing membrane IgM molecules. We had originally planned on large scale isolation of membrane μ chains from mouse splenic B cells. But at this time, many new B cell tumor lines became available, and because these lines offered advantages for studying membrane IgM, we began characterizing the WEHI 279 B-cell lymphoma (37), a chemically induced tumor found in the thymus of NZC mice [an inbred strain derived from (NZB x BALB/c)F_1 mice]. These WEHI 279 tumor cells produce the parental BALB/c allele of μ chains and were a gift from Dr. N. Warner.

Our approach was to 1) analyze the primary amino acid sequence of μ_m chains using direct radiolabeled sequencing of CNBr fragments and comparative peptide mapping and 2) label sugars and assess the glycosylation levels and sites on μ_m chains. In these studies, we naturally intended to focus on the COOH-terminal region of the μ_m chain. Initial experiments on WEHI 279 cells revealed the existence of a rapidly labeled internal pool of μ chains, a membrane bound μ chain synthesized by these cells and little if any (10% of the total) μ chain secreted into the culture fluid. Thus, the cells seemed representative of small B lymphocytes from the spleen.

The majority of μ chains in the internal (μ_i) pool was found to consist of percursors to μ_s chains. These pre-secreted μ chains lacked terminal sugars on their complex carbohydrate structures, and since secretion was minimal, apparently turned over nonproductively inside the cells. We then realized that WEHI 279 μ_s chains (with a WEHI 279 V_H region) were needed for making structural comparisons with WEHI 279 μ_m chains at the level of peptide mapping and cyanogen bromide fragmentation. The hybridoma technology was able to supply the solution. Dr. W. Raschke at the Salk Institute kindly sent us a hybrid cell line created by the fusion of WEHI 279 and MPC11 myeloma cells. The MPC11 x W279.2 (MxW) cells secrete pentameric IgM composed of WEHI 279 μ_s chains and were used subsequently for comparison of the polypeptide structures of μ_m, μ_i and μ_s chains. The hybridoma was amplified as ascites in BALB/c mice, and 80% of the V_H region amino acid sequence of the WEHI 279 μ_s chain was determined in order to quantitate the different V region amino acids.

At this point, we were sent a replacement clone of WEHI 279 cells which was used for <u>all</u> further studies on μ_m and μ_i chains. This new clone is able to polymerize and secrete the internal pre-secreted μ chains more efficiently than the original WEHI 279 cells. The more recent clone secretes 15-fold more IgM,

and 46% of the secreted IgM is fully polymerized into pentamers. As suggested by M. Koshland and coworkers, the ability to secrete mature IgM pentamers probably reflects the availability of J chains in the cell (21, 50). In support of this idea, Mather and Koshland have found that original WEHI 279 cells produced little J chain; MPC11 cells were found to synthesize large amounts of J chain as were MxW cells (21); recent clone WEHI 279 cells synthesized 100-fold more J chain than the original WEHI 279 cells (50). We believe that this change in the cell line in no way affects the studies on μ_m isolated from both cell lines. Since the V region CNBr fragments of μ_m chains from both lines are identical, the μ_m chains of the two lines should be identical.

The following chapters are a chronology of findings first about μ_s structure, second, how the different μ chains synthesized by WEHI 279 B lymphoma cells were localized and identified, and finally the structural characterization of the WEHI 279 μ chains (with the complement of recombinant DNA technology) to show that μ_m and μ_s chains possess different primary amino acid sequences at their COOH-termini.

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CHAPTER 1

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Amino acid sequence of a mouse immunoglobulin μ chain

(automated sequencer/immunoglobulin domain/myeloma tumor MOPC 104E/immunoglobulin evolution/carbohydrate attachment sites)

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ABSTRACT **The complete amino acid sequence of the mouse μ chain from the BALB/c myeloma tumor MOPC 104E is reported. The C_μ region contains four consecutive homology regions of approximately 110 residues and a COOH-terminal region of 19 residues. A comparison of this μ chain from mouse with a complete μ sequence from human (Ou) and a partial μ chain sequence from dog (Moo) reveals a striking gradient of increasing homology from the NH_2-terminal to the COOH-terminal portion of these μ chains, with the former being the least and the latter the most highly conserved. Four of the five sites of carbohydrate attachment appear to be at identical residue positions when the constant regions of the mouse and human μ chains are compared. The μ chain of MOPC 104E has a carbohydrate moiety attached in the second hypervariable region. This is particularly interesting in view of the fact that MOPC 104E binds α-(1→3)-dextran, a simple carbohydrate. The structural and functional constraints imposed by these comparative sequence analyses are discussed.**

Immunoglobulins are comprised of two polypeptides, light (L) and heavy (H) chains, and may be divided into five major classes, IgM, IgG, IgA, IgD, and IgE, which are defined by their corresponding heavy chains, μ, γ, α, δ, and ϵ, respectively. The IgM molecule is the most primitive immunoglobulin in that it is the first to appear in vertebrate evolution (1). Moreover, IgM is the first immunoglobulin to appear during the ontogenetic development of the immune system (2). Serological, immunochemical, and evolutionary data suggest that IgM immunoglobulins have been more highly conserved than other classes of immunoglobulins (1). For these reasons, the IgM molecule is an excellent model protein for evolutionary analysis.

Each immunoglobulin chain is divided into an NH_2-terminal variable (V) region and a COOH-terminal constant (C) region. The immunoglobulin polypeptides are divided into consecutive homology regions approximately 110 residues in length. The L chain has two homology regions and the human μ chain has five homology regions in addition to a COOH-terminal segment composed of 19 residues (3). The basic unit of the immunoglobulin molecule is two light and two heavy chains (H_2L_2), in which pairs of homology regions fold together to form globular domains. The L chain and the NH_2-terminal portion of the H chain generate two domains each, and the remaining COOH-terminal regions of the two μ chains fold into three domains. From the NH_2 to the COOH terminus of the μ chain, these domains are designated V, $C_\mu1$, $C_\mu2$, $C_\mu3$, and $C_\mu4$, respectively.

The V domains recognize foreign structures (antigens) and the C domains carry out a variety of effector functions that ultimately lead to the destruction or elimination of foreign antigens. The IgM molecule has at least three special effector functions associated with its COOH-terminal domains. First, the IgM molecule serves as a cell-surface receptor that can trigger the progenitors of antibody-producing B cells to differentiate upon interaction with complementary antigen (4). Thus the IgM molecule can be an integral membrane protein (5, 6), which is displayed on the membrane as a 7S monomer, (μ_2L_2) (7, 8). Second, cells that have been stimulated to differentiate into antibody-producing cells can secrete IgM molecules as 19S pentamers, $(\mu_2L_2)_5$, composed of 10 light and 10 heavy chains held together by a third type of polypeptide designated the J chain (9). It is not known whether the membrane-bound and secreted μ chains have the same polypeptide structure. Finally, after the IgM molecule binds antigen, the $C_\mu4$ domain activates the effector pathways of the complement system (10).

In this paper we report the amino acid sequence of the secreted μ chain derived from the mouse myeloma tumor MOPC 104E. The mouse μ chain is compared with a human μ chain whose sequence has been completely determined (11) and a dog μ chain whose sequence has been partially determined (12, 13). As previously observed when the human μ sequence and the dog μ partial sequence were compared, there is a striking gradient of sequence homology, with the COOH-terminal homology regions significantly more conserved than the NH_2-terminal homology regions (12). Several structural features of the μ chain are highly conserved, including the placement of disulfide bridges and the locations of tryptophan residues and carbohydrate moieties in the constant region. The structure of the secreted μ chain places several interesting constraints on the nature of the membrane-bound μ chain of the IgM receptor molecule.

MATERIALS AND METHODS

Isolation of μ Chain. The IgM-secreting tumor MOPC 104E (μ, λ) was grown intraperitoneally in (BALB/c × DBA/2) F_1 mice. The IgM molecules were precipitated from ascites fluid with ammonium sulfate and purified by gel filtration on a column of ACA 22 (LKB). The purified IgM was completely reduced with dithiothreitol and alkylated with iodoacetamide in 6 M guanidine·HCl (14), and the heavy and light chains were separated by gel filtration on a column of ACA 34 (LKB) in 3 M guanidine and 0.2 M ammonium bicarbonate. The complete amino acid sequence of the λ light chain of MOPC 104E has been determined (15, 16).

Isolation of Cyanogen Bromide Fragments. Purified μ chains were cleaved with cyanogen bromide in 70% (wt/vol) formic acid for 20–22 hr at 4°C (17). Cyanogen bromide peptides were separated by gel filtration on a column of ACA 54 (LKB) in 3 M guanidine and 0.2 M ammonium bicarbonate.

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Abbreviations: H and L, heavy and light chains of immunoglobulins; V and C, variable and constant regions of immunoglobulin chains; CHO, carbohydrate.

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Protein and peptide purity was monitored at all stages of purification by electrophoresis in the presence of sodium dodecyl sulfate on 10% or 18% polyacrylamide gels (18).

Compositional and Sequence Analysis. Amino acid compositions and carbohydrate analyses for amino sugars were determined on samples hydrolyzed in 6 M HCl (110°C for 24 hr and 3 hr, respectively) by using a Durrum D-500 amino acid analyzer. Automated sequence analyses were performed on a modified Beckman sequenator (19, 20). Samples were loaded by using Polybrene (Aldrich), and phenylthiohydantoin derivatives were identified by high-pressure liquid chromatography (Waters Associates) as described (21).

Peptide Fragments. Fc fragments of IgM were prepared according to the method of Shimizu *et al.* (22) and purified by gel filtration on ACA 22 and ACA 34. Cyanogen bromide peptides were succinylated (23) for trypsin digestion limited to arginine residues. Cyanogen bromide peptides also were cleaved at tryptophan residues by the procedure of Ozols *et al.* (24). Smaller peptides were produced from cyanogen bromide fragments by digestion with trypsin, chymotrypsin, and thermolysin. The resulting peptides were separated in two dimensions on paper by chromatography and high-voltage electrophoresis (25) and where appropriate their amino acid sequences were determined in the automated sequenator in the presence of Polybrene (see ref. 21).

RESULTS

Sequence Strategy. The methionine residues of the MOPC 104E μ chain were cleaved with cyanogen bromide and 10 peptides were isolated by gel filtration. The amino acid sequences of these peptides were determined at their NH_2 termini on the automatic sequenator. The sequences of selected peptides produced by tryptophan, thermolysin, trypsin, and chymotrypsin cleavages also were determined to complete the primary structure of each cyanogen bromide fragment. Fig. 1 schematically illustrates the order of the cyanogen bromide fragments. Only fragment CN1 was not isolated. Two cyanogen bromide fragments that resulted from incomplete cleavage of the methionine residues at positions 20 (CN1-2) and 568 (CN8-9) were isolated. Thus, cyanogen bromide fragments covering the entire μ chain have been obtained and their sequences have been completely determined.

The 10 methionine fragments of the mouse μ chain can be unambiguously aligned in a linear order without resorting to homology comparisons with the human μ chain. Sequence analysis of the NH_2-terminal 38 residues of the intact μ chain assigned cyanogen bromide fragments 1, 2, and 3, respectively, to the NH_2 terminus (Fig. 1). An Fc fragment, obtained by trypsin digestion of the intact IgM in 5 M urea (22), shows that the COOH-terminal sequence of CN5 and the NH_2-terminal sequence of CN6 are contiguous (Fig. 1). In addition, a cyanogen bromide digest of the Fc fragment yielded CN6, CN7, and CN8-9. Because CN4 is not in the Fc fragment, it must reside between CN3 and CN5 (Fig. 1). Because CN8-9 does not have a COOH-terminal homoserine residue it must be the COOH-terminal fragment. The only remaining fragment, CN7, is then located between CN6 and CN8-9 (Fig. 1). These assignments are supported by the previous isolation and sequence analysis of pepsin peptide fragments overlapping CN4 to CN5 and CN7 to CN8 (27). Thus direct sequence overlaps are available for all methionine residues except those between CN6-CN7 and CN3-CN4. There is the possibility that additional methionine fragments between CN3-CN4 and CN6-CN7 may have been lost in the isolation procedures. This possibility appears unlikely because the mouse μ chain in these two regions can be aligned without sequence gaps against a completely sequenced human μ chain (Fig. 2).

Disulfide Bridges. The positions of the 14 cysteine residues of the mouse μ chain are indicated in Fig. 1. The assignments of two disulfide bridges (residues 22 to 97; residues 153 to 213) have been established through the isolation of cyanogen bromide peptides from μ chain with intact intrachain bridges followed by analysis on reducing and nonreducing 18% polyacrylamide gels. In addition, the methionine fragments of the Fc region, CN6, CN7, and CN8, are linked by intrachain disulfide bridges. All 14 cysteine residues in the mouse μ chain

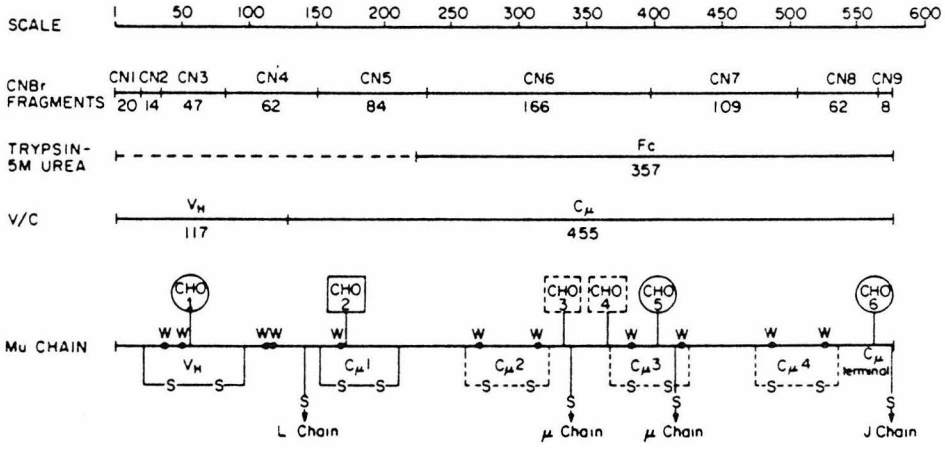

FIG. 1. Schematic drawing of the μ heavy chain of IgM MOPC 104E showing (*i*) sites of cleavage by CNBr and the respective fragments (CN1 to CN9), (*ii*) the point of cleavage by trypsin in 5 M urea giving the Fc fragment, (*iii*) the point of division of the variable (V_H) and constant (C_μ) regions (3), (*iv*) the intrachain disulfide bridges, (*v*) the six homology regions (V_H, $C_\mu 1$ to $C_\mu 4$, C_μ terminal), and (*vi*) the locations of the six oligosaccharides (CHO 1 to CHO 6). The high-mannose oligosaccharides are circled and complex type oligosaccharides are boxed. Two oligosaccharides are enclosed in broken lines to denote that the assignment of complex type was made from homology with the human μ oligosaccharides (26). The linkages of three of the intrachain bridges are indicated by broken lines because they have been assigned only by assuming homology with the human μ sequence Ou (11). W indicates tryptophan residues. To facilitate homology comparisons, the scale corresponds to the amino acid sequence positions of the human myeloma μ chain Ou (11). Because there are a number of gaps and insertions between the two sequences, the numbers indicating total amino acid residues in each 104E μ chain fragment do not necessarily correspond to the numbers obtained by counting the amino acid residues in the homologous Ou sequence.

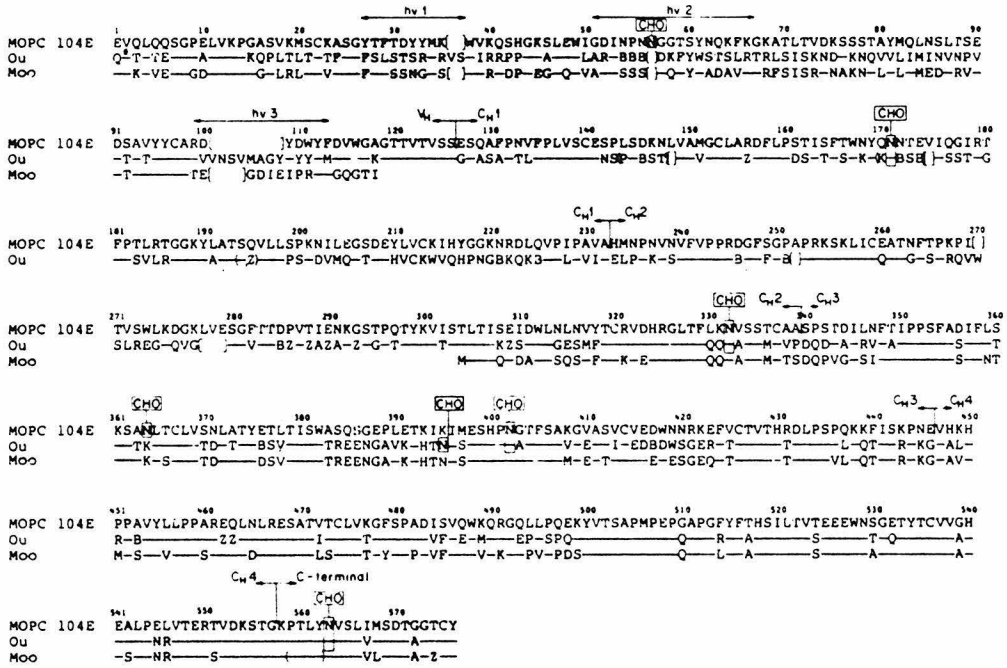

FIG. 2. The amino acid sequences of μ chains from mouse MOPC 104E, human Ou (11), and canine Moo (12, 13). (The one-letter code for amino acids is given in ref. 28.) Only the NH_2-terminal and Fc sequences of the dog μ chain are available. A straight line indicates identity with the MOPC 104E sequence. Deletions are indicated by [ ]. The sites of carbohydrate attachment are boxed. Positions of carbohydrate attachment have not been determined for the dog μ chain. The homology unit boundaries have been assigned by an analysis of immunoglobulin three-dimensional structure as indicated in Table 1 (3). Three hypervariable regions (hv 1, hv 2, and hv 3) are indicated by arrows over appropriate V region segments. Q* indicates pyrrolidone carboxylic acid.

are in positions homologous to the 14 cysteine residues of the human μ chain. Thus we assume the same cysteine residues will be involved in interchain bridges and intradomain bridges as indicated in Fig. 1. Accordingly, the cysteine residue at position 140 is probably bridged to the L chain and the cysteines at 337, 414, and 575 most likely form bridges with other μ chains or with the J chain (16, 29).

Oligosaccharide Assignments. The 104E μ chain has six sites of carbohydrate attachment, five in the constant region and one in the variable region (Fig. 1). All six oligosaccharides contain glucosamine and are attached to asparagine residues. The sites of carbohydrate attachment were identified as blank cycles in automatic sequenator runs. Subsequent enzymatic limit digests of the carbohydrate-containing cyanogen bromide fragments produced small peptides that were analyzed for the presence of glucosamine. Four of the five C region oligosaccharides of the mouse μ chain are in positions identical to those of the human μ chain (Figs. 1 and 2). The fifth C region oligosaccharide is attached to a nonhomologous position 31 residues NH_2-terminal to the site in Ou (positions 395 and 364 in human and mouse, respectively).

DISCUSSION

The COOH-Terminal Portion of the C_μ Region Appears More Highly Conserved Than the NH_2-Terminal Portion. In Fig. 2 the complete sequence of the μ chains of mouse and human are listed along with the V region and Fc sequence data from the dog μ chain. Several insertions, deletions, and sequence inversions, some of which may be technical artifacts in analysis (28), are noted in comparing these sequences. Using the criteria of Beale and Feinstein (3), we have identified in the mouse C_μ, as in its human counterpart, four homology regions of approximately 110 residues and an additional COOH-terminal region 19 residues in length (Fig. 2). The mouse μ chain, like the human μ chain, has no hinge region separating any of the C region domains (3).

Two amino acid residues, cysteine and tryptophan, are highly conserved in the C_μ regions (Figs. 1 and 2). All 12 cysteine residues are conserved in the mouse and human C_μ regions, as are the 8 that can be compared in the dog sequence. The mouse C_μ region has six tryptophan residues, each of which is homologous to one of the seven tryptophan residues in the human C_μ region (Fig. 2). All five tryptophans in the partial sequence of the dog C_μ region are in the same positions as their mouse and human counterparts. In contrast, the human and mouse C_μ regions have six and five methionine residues, respectively, only two of which are conserved. Because of their conservation, it is reasonable to conclude that cysteine and tryptophan residues play an important role in the domain structure of immunoglobulins.

Table 1 is a compilation of the homology comparisons between the various homology regions of mouse, human, and dog. There is a striking gradient of homology from the NH_2-terminal to the COOH-terminal region. The $C_\mu 1$ homology unit of mouse shows 48% homology with human μ as contrasted with 78% and 89% homologies in the $C_\mu 4$ and the COOH-terminal regions, respectively. The existence of sequence gaps in the NH_2-terminal homology regions (two in $C_\mu 1$ and three in $C_\mu 2$) but not in the COOH-terminal homology regions of the μ chain (Fig. 2) suggests that the NH_2-terminal regions have diverged more. Thus, the COOH-terminal portion of the μ chain is significantly more conserved than the NH_2-terminal portion. If individual domains do carry out separate functions, these observations imply that there are strong selective constraints on

Table 1. Homology matrix of the constant region domains of mouse, human, and dog μ chains

Comparison	V	C_H1	C_H2	C_H3	C_H4	C-Terminus
Mouse 104E vs. Human Ou	38% 116/116	48% 106/106	59% 106/106	53% 107/107	78% 111/111	89% 19/19
Dog Moo vs. Human Ou	38% 112/112		69% 36/106	79% 107/107	79% 111/111	89% 19/19
Mouse 104E vs. Dog Moo	44% 108/108		56% 36/106	56% 107/107	72% 111/111	79% 19/19

The μ chains were divided into four homology units and a COOH-terminal region according to an analysis of the three-dimensional structures of several immunoglobulins not of the μ class: C_H1 = residues 127–232; C_H2 = residues 233–339; C_H3 = residues 340–446; C_H4 = residues 447–557; and C terminus = residues 558–576 (3). Sequence gaps (deletions or insertions) are not counted in homology comparisons. In the lower right of the square for each region and comparison is listed the number of amino acids compared in the homology calculation divided by the total number of amino acids in that homology unit. The number of sequence identities in each unit was used to calculate the percent homology, which is given in the upper left of each square.

the more COOH-terminal domains. These constraints may be related to the requirements for the polymerization of pentamers in the secreted IgM molecule, the structural requirements imposed on an integral membrane receptor with regard to membrane attachment and the triggering of differentiation, or the activation of the complement pathway, which has been localized to the$C_\mu4$ domain (10).

Sites of Carbohydrate Attachment to the C_μ Region Are Similar in Human and Mouse μ Chains. The μ chain of mouse has five sites of carbohydrate attachment in the constant region (Fig. 1). Four of these five carbohydrate attachment sites are at identical positions in the mouse and human (26) C_μ regions (Fig. 2). The carbohydrate moiety on residue 171 is of the complex type and those on residues 402 and 563 are of the high-mannose type (W. J. Grimes, personal communication), in agreement with the carbohydrate types on the human μ chain (26). Compositional analyses on the remaining two oligosaccharides are not yet complete. Each carbohydrate moiety is attached to the asparagine residue of a three-residue recognition sequence, -Asn-X-Ser/Thr, in which X may be any amino acid and Ser/Thr indicates either serine or threonine. Substitution of a methionine for a serine at position 397 in mouse μ has eliminated the recognition sequence for the carbohydrate attachment site at position 395 in human μ. In the mouse μ chain we find a carbohydrate moiety at residue 364, where there exists a carbohydrate recognition sequence that is absent in the dog and human μ chains. Other recognition sequences that are apparently not glycosylated also occur in the μ chain (74–76 in human Ou, 263–265 and 347–349 in mouse MOPC 104E). Thus, the elimination and formation of an attachment site implies either that this region of the protein is on the exterior of the molecule and is therefore accessible to glycosylating enzymes or that a carbohydrate moiety is required (structurally, functionally, or both) in this region of the μ chain.

The carbohydrate moieties of human and mouse μ chains are present in the $C_\mu1$, $C_\mu2$, and $C_\mu3$ domains as well as the COOH-terminal region (Figs. 1 and 2). A variety of functions have been suggested for the carbohydrate on heavy chains, including the solubilization of the heavy chain, the facilitation of the secretory process, the promotion of the assembly of pentamers, and the contribution of structural features for a variety of specific effector functions (26, 30–34). Whatever their function, the conservation of the locations of carbohydrate attachment during the 75 million years that mouse and human C_μ genes have diverged from one another suggests that strong selective forces are maintaining the recognition sequences.

The V Domain of MOPC 104E, an α-(1→3)-Dextran Binder, Has a Carbohydrate Moiety in Its Antigen-Binding Site. A carbohydrate moiety has been identified at asparagine 57, which lies in the middle of the second hypervariable region of the MOPC 104E μ chain. There are several unusual features of the V region carbohydrate site. First, the usual recognition sequence is missing; this high-mannose carbohydrate moiety is attached to the asparagine of an Asn-Gly-Gly-Thr sequence. Although the majority of asparagine-linked carbohydrate moieties are associated with a recognition sequence, the sequence is clearly not an absolute requirement for the attachment of carbohydrate to asparagine residues (35). Second, the IgM molecule secreted by the MOPC 104E tumor binds a simple carbohydrate, α-(1→3)-dextran (36). Thus, we have localized a carbohydrate moiety to the antigen-binding site of the heavy chain of an immunoglobulin that binds a defined hapten. This observation raises the possibility that carbohydrate–carbohydrate interactions may constitute a portion of the binding energy for the hapten, α-(1→3)-dextran. In view of what we know about the specificities of protein and carbohydrate interactions, however, this possibility appears unlikely (37). Third, a second myeloma protein, J558(α, λ), which also binds α-(1→3)-dextran, appears to have a carbohydrate moiety at precisely the same position (unpublished observations). It is likely that this carbohydrate moiety also occurs in the heavy chains of normally induced serum antibodies to α-(1→3)-dextran (unpublished observations). The role, if any, for carbohydrate in the V domains of immunoglobulins remains an interesting, but unanswered question.

Structure of the μ Chain Places Constraints on the IgM Molecule as an Integral Membrane Receptor. The monomeric IgM molecule is an integral membrane protein that serves as a cell-surface receptor for antigen on B cells that can trigger the B cells to differentiate into antibody-producing cells (4). The membrane (m) and secreted (s) IgM molecules are serologically crossreactive, implying that they share structural features or may even be identical. Conflicting data suggest both that the COOH terminal portion of the μ_m chain is identical to μ_s (38) and that these regions are different (39).

If the μ_m and μ_s chains are identical in amino acid sequence, the μ_m chain cannot be transmembrane in its orientation. The α-helix is the most stable polypeptide secondary structure within a membrane (40, 41). This configuration requires about 21 uncharged residues to span the membrane. Alternatively, the extended β configuration requires two paired stretches of 9 uncharged amino acids to span the membrane. We have compared the three mammalian μ chains over their COOH-terminal two homology units, which presumably contain the site of membrane attachment. The longest sequence of uncharged amino acids in any of these molecules is 14 residues in length (positions 469–482 in the human and dog μ chains) (Fig. 2). However, the mouse μ chain has a charged lysine residue at position 477, reducing the stretch of uncharged amino acids in this region to 8 residues. In addition, this region contains a cysteine at position 474 that is presumably joined to cysteine-536 to form the $C_\mu4$ intradomain disulfide bridge. If one assumes that all mammals will attach their μ chains to membranes in a similar fashion, there is no uncharged sequence shared by the COOH-terminal regions of the mouse, human, or dog μ chains that could span the membrane in either the α-helical or the extended unpaired β configuration (41, 42). We conclude that, if the μ_m and μ_s chains are identical, the IgM molecule cannot be a transmembrane protein that meets the above specifications. Moreover, tyrosine-labeling experiments on inside-out vesicles are consistent with the supposition that the IgM molecule is not transmembrane (43). If the μ_m chain is not a transmembrane polypeptide, then the IgM molecule may associate with the membrane by virtue of a specific receptor molecule, an association analogous to the interaction of IgE with its membrane receptor on mast cells (44). In this regard, both IgM and IgE have an additional constant region domain when compared with IgG and IgA molecules (3).

We have preliminary evidence that suggests the μ_m and μ_s chains differ in their primary amino acid sequences. Any primary structural differences could be generated by mRNA splicing mechanisms, posttranslational proteolysis, or the occurrence of duplicated μ chains genes. It is possible to determine the chemical structure of μ_m and define precisely the relationship between μ_m and μ_s chains. Moreover, we have isolated a genomic μ clone, whose nucleotide sequence can be determined. Such studies should allow us to distinguish unambiguously among the three models for μ_m and μ_s expression cited above.

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CHAPTER 2

This paper will be submitted to Biochemistry.

Complete Amino Acid Sequence of a Mouse Mu Chain: Homology among Heavy Chain Constant Region Domains†

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Footnotes

[1]Abbreviations used: L, light chain; H, heavy chain; 104E, MOPC 104E myeloma; BBS, borate buffered saline; EDTA, ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulfate; TPCK, tosylphenylchloroketone; Pth, phenylthiohydantoin; V_H, immunoglobulin heavy chain variable region; C_H, immunoglobulin heavy chain constant region; J, joining chain.

ABSTRACT: The complete amino acid sequence of the mouse μ chain secreted by the MOPC 104E myeloma tumor has been determined. There are four constant region domains in the μ chain which exhibit internal amino acid sequence homology and a 20-residue C-terminal segment which plays a role in the polymerization of pentameric IgM molecules. There are six sites of carbohydrate attachment in the MOPC 104E μ chain. Three complex type and two high mannose oligosaccharides are located in the μ chain constant region. In addition, a unique small carbohydrate structure is attached to an asparagine residue in the MOPC 104E second hypervariable region. The general type and location of carbohydrate moieties in the μ chain constant region is completely conserved between mouse and human μ chains. Homology in the location of carbohydrate structures on different classes of heavy chains is discussed. The amino acid sequence homologies among individual domains in the μ, γ, ε and α heavy chains reveal that some interesting genetic events have occurred during the evolution of constant region domains.

Immunoglobulin molecules are composed of two polypeptide chains, light (L)[1] and heavy (H) chains. The five major classes of immunoglobulins, IgM, IgG, IgA, IgD, and IgE, are distinguished by the structures of their corresponding heavy chains, μ, γ, α, δ, and ε, respectively. Each heavy chain may be divided into a variable (V_H) or antigen binding region and a constant (C_H) region which performs the various effector functions of the immunoglobulin molecule. Heavy chain constant regions may be further subdivided into homology regions or domains approximately 110 residues in length (Beale & Feinstein, 1976). The μ chain has four C_H domains which are designated $C_\mu 1$, $C_\mu 2$, $C_\mu 3$, and $C_\mu 4$ from the NH_2- to the COOH-terminus. There is an additional C-terminal segment in the μ chain which is composed of 20 amino acid residues (Calame et al., 1980).

The basic unit of the immunoglobulin molecule consists of two H and two L chains (H_2L_2) which are covalently linked together through disulfide bridges. In this tertiary structure, pairs of homology regions fold together to form discrete functional globular domains. IgM molecules are unique among the different classes of immunoglobulins in that they exist in two different polymeric forms which perform different effector functions in the immune system. First, the IgM molecule is a cell-surface receptor which functions to trigger the differentiation of precursor B lymphocytes to antibody-producing plasma cells (Warner, 1974). This membrane-bound form of the IgM molecule is a monomeric (μ_2L_2) immunoglobulin embedded in the plasma membrane of B lymphocytes (Vitetta et al., 1971). Second, antibody-producing plasma cells secrete IgM molecules which are assembled into pentamers $(\mu_2L_2)_5$ by the addition of a joining (J) chain (Della Corte & Parkhouse, 1973). These soluble IgM pentamers then circulate in the bloodstream where they function to bind and neutralize foreign antigens and to fix complement. The μ chain polypeptides which compose the membrane-bound and secreted forms of the IgM molecule are known to differ in the amino acid sequence of their C-terminal segments (Kehry

et al., 1980; Early et al., 1980; Rogers et al., 1980). IgM molecules are also unique in being the first immunoglobulins to appear in vertebrate evolution (Marchalonis, 1972) and the first to appear during the development of the immune system (Lawton et al., 1975). The above features make the IgM molecule an interesting protein to structurally characterize and to use for investigating the evolution of immunoglobulin heavy chain classes.

We present here a complete structural analysis of a secreted μ chain from the mouse myeloma tumor MOPC 104E. DNA sequencing studies on a cloned mouse μ chain gene complement the protein sequence by delineating the exact boundaries between C_μ region domains (Calame et al., 1980). We discuss the composition of the six oligosaccharide moieties attached to the MOPC 104E μ chain and the homologies in the sites and types of carbohydrate attached to the different classes of heavy chains. We also give an analysis of an amino acid sequence homology comparison among the C_H domains of μ, γ, α and ε chains. The C-terminal segments of secreted μ and α chains function specifically in the formation of IgM and IgA polymers. Some interesting features in the evolution of C_H region domains from a primordial immunoglobulin molecule have been found.

Experimental Procedures

Isolation of MOPC 104E μ Chain. The IgM-secreting myeloma MOPC 104E (μ, λ), subsequently abbreviated 104E, was obtained from Dr. M. Potter and was passaged subcutaneously in (BALB/c x DBA/2)F_1 mice. Ascites fluid was collected from mice injected intraperitoneally with 104E cells, centrifuged to remove cells, and the supernatant fluid was used for purification of the IgM immunoglobulin. Proteins were precipitated from ascites fluid (40 ml) by two cycles of treatment with 50% saturated ammonium sulfate at 4°C. The final precipitate was dissolved in 0.15 M sodium borate, 0.14 M NaCl, 0.02% NaN_3 (BBS) and the pentameric

IgM molecules were purified by gel filtration on a column (5 x 100 cm) of ACA 22 (LKB) equilibrated in BBS. IgM molecules were eluted just after the void volume and the disulfide bridges were completely reduced and alkylated as follows. Appropriate fractions were pooled, precipitated with 50% saturated ammonium sulfate at 4°C, and the precipitate was dissolved in 6 M guanidine-HCl, 0.25 M Tris-HCl pH 8.5, 0.14 M EDTA. Reduction and alkylation were carried out essentially as described (Konigsberg, 1972) using 0.01 M dithiothreitol and 0.025 M iodoacetamide (3X recrystallized). When iodo(1-^{14}C)-acetamide was employed for alkylation, the reaction was allowed to proceed for 30 min followed by the addition of non-radioactive iodoacetamide to 0.025 M. After dilution of the guanidine concentration to 4 M by the addition of 0.4 M ammonium bicarbonate, heavy and light chains were separated by gel filtration on a column (5 x 100 cm) of ACA 34 (LKB) equilibrated in 3 M guanidine-HCl, 0.2 M ammonium bicarbonate, 0.02% NaN_3.

Isolation of Cyanogen Bromide Fragments. Purified μ chains in 3 M guanidine HCl, 0.2 M ammonium bicarbonate were concentrated (Millipore Molecular Separator), acidified by gradual addition of 88% formic acid, and desalted on a column (2.5 x 40 cm) of Bio-Gel P-2 in 50% formic acid at 4°C. Pooled μ chains were concentrated by partial lyophilization, the concentration of formic acid was adjusted to 70%, and the protein was cleaved by cyanogen bromide (50 mg/ml) in the dark with constant stirring at 4°C (Gross, 1967). After 20-22 h, the reaction mixture was diluted with 11 volumes of water and lyophilized. The peptides were dissolved sequentially in 8 M guanidine-HCl, 3 M guanidine-HCl and 0.2 M ammonium bicarbonate, and finally 0.4 M ammonium bicarbonate. The solutions were combined, and the cyanogen bromide peptides were separated by gel filtration on a column (3.5 x 140 cm) of ACA 54 (LKB) equilibrated in 3 M guanidine-HCl, 0.2 M ammonium bicarbonate, 0.02% NaN_3. Fractions were monitored by reading the absorbance at 280 nm, and aliquots were counted for radioactivity when the cysteine residues

were ^{14}C-alkylated. The cyanogen bromide fragments were found to be of sufficient purity for sequence analyses after the one-step gel filtration on the ACA 54 column (see Figure 1). For direct sequence determinations, pooled fragments were either neutralized and desalted on a column (2.5 x 40 cm) of Bio-Gel P-2 in 20% formic acid or dialyzed (Spectra/Por 3) against three changes of 5% formic acid at 4°C followed by lyophilization.

Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970) was routinely used to monitor the purification of IgM and μ chains (10% polyacrylamide gels) and cyanogen bromide fragments (18% polyacrylamide gels). Guanidine was removed from the samples prior to electrophoresis by dialysis (Spectra/Por 3) against 5% formic acid followed by lyophilization. Gels were stained with 0.025% Coomassie Brilliant Blue in 25% isopropanol, 10% acetic acid and destained in 10% acetic acid.

Composite gels (1% agarose and 2.5% polyacrylamide) were run by a modification of the procedure of Peacock and Dingman (1968). Gels contained 0.5 M urea, 0.2 M sodium phosphate, pH 7.2, and 0.1% SDS. Running buffer contained 0.5 M urea, 0.1% SDS, 0.75 M sodium phosphate, pH 7.2 and sample buffer was composed of 2% SDS, 10% glycerol, 0.05 M sodium phosphate, pH 6.8. Gels were 10 cm long and were electrophoresed at 3.5 ma/gel for 16 h, stained for 1 h with 0.25% Coomassie Brilliant Blue in methanol:acetic acid:water, 5:1:5, and destained in the same solution without Coomassie Brilliant Blue, followed by equiliberation in 10% acetic acid.

Tryptic, Chymotryptic and Thermolysin Digestion. Cyanogen bromide fragments were dialyzed (Spectra/Por 3) exhaustively against 0.2 M ammonium bicarbonate, lyophilized and dissolved in a small volume of 0.2 M ammonium bicarbonate. TPCK-trypsin (Worthington) or chymotrypsin (Sigma) was added in 0.001 N HCl or thermolysin (Sigma) was added in 0.2 M ammonium bicarbonate

(freshly prepared enzyme stock) at an enzyme:substrate ratio of 1:100. Enzyme addition was performed three times at 1-h intervals. Incubation was carried out at 37°C for a total period of 2.5 h, and the digestion was terminated by lyophilization.

Peptides were separated in two dimensions by chromatography followed by high voltage electrophoresis on 3 MM paper (Whatman) as described previously (Katz et al., 1959). Ninhydrin positive spots on analytical and preparative fingerprints were excised, eluted by two extractions into 0.5 M NH_4OH and lyophilized.

Cleavage at Arginine or Tryptophan Residues. For specific cleavage at arginine residues, cyanogen bromide fragments were dialyzed exhaustively (Spectra/Por 3) against 5% formic acid at 4°C, lyophilized and dissolved in 12 ml 6 M guanidine-HCl, 0.01 M Tris pH 9.5. Amino groups on the protein were succinylated by gradual addition of finely powdered succinic anhydride (40-fold excess by weight over protein) in a pH-stat (Klapper & Klotz, 1972). When reversible blockage of lysine residues was desired, the protein was reacted with citroconic anhydride or maleic anhydride under similar conditions. In some instances the protein was radiolabeled by reacting with ^{14}C-succinic anhydride. Reagents were removed by exhaustive dialysis against 0.1 M NH_4OH, followed by lyophilization. Trypsin digestion was performed as described in the previous section. In some cases, peptides were separated on a column of Sephadex G-75 or G-50 (Sigma) equilibrated in 0.2 M ammonium bicarbonate or ACA 54 (LKB) equilibrated in 3 M guanidine, 0.2 M ammonium bicarbonate, 0.02% NaN_3. The absorbance at 280 nm and, when appropriate, the absorbance at 230 nm were monitored. The maleylation or citraconylation was reversed by acidifying (pH 3.5) the protein overnight. Trypsin cleavage at lysine residues was then performed as described in the previous section.

For specific cleavage at tryptophan residues (Ozols et al., 1977), cyanogen bromide fragments were dialyzed exhaustively (Spectro/Por 3) against 5% formic

acid at 4°C, succinylated, lyophilized and dissolved in equal volumes of 88% formic acid and anhydrous heptafluorobutyric acid (Pierce). Cyanogen bromide was added to 350 mg/ml. The reaction vessel was vented into water in a fume hood, and the tryptophan cleavage reaction performed essentially as described by Ozols et al. (1977). After 24 h, the reagents were evaporated under a stream of N_2 and the peptides lyophilized after addition of 10 ml water. In some cases, peptides were then separated on a column of Sephadex G-75 or G-50 (Sigma) equilibrated in 0.2 M ammonium bicarbonate. The tryptophan cleavage reaction also modified tyrosine residues in an unknown fashion which greatly altered the chromatographic behavior of the modified phenylthiohydantoin (Pth)-tyrosine derivative obtained by sequence analysis.

Fc Digestion. IgM Fc fragments were produced by a modification of the method of Shimizu et al. (1975). Whole IgM in BBS was concentrated, desalted on a column (3 x 15 cm) of Sephadex G-25 (Sigma) equilibrated in freshly prepared 5 M urea, 0.1 M Tris pH 8, 0.15 M NaCl, 0.01% NaN_3. After concentration of the desalted IgM molecules, a stock solution of 2 mg/ml TPCK-trypsin (Worthington) in 0.1 M Tris-HCl, pH 8.0, 0.1 M $CaCl_2$, 0.15 M NaCl was added to give an enzyme: substrate ratio of 1:100 and incubated at 25°C for 18 h. The efficiency of pentameric Fc production was monitored by removing aliquots at specific times after addition of enzyme. Proteins were then electrophoresed without reduction on composite 1% agarose, 2.5% polyacrylamide-SDS gels (Peacock & Dingman, 1968) or the proteins were reduced and analyzed on 10% polyacrylamide-SDS gels (Laemmli, 1970). For preparative isolation of Fc fragments, lima bean trypsin inhibitor (LBI) (LBI:trypsin, 1:3, w/w) was added to inhibit further digestion. The mixture was dialyzed against BBS at 4°C to remove urea, and the pentameric Fc fragments were purified on a column of ACA 22 (LKB) equilibrated in BBS. The μ chain Fc fragments were obtained from the pentameric Fc fragments after complete

reduction and alkylation of disulfide bridges followed by purification on a column of ACA 34 (LKB) equilibrated in 3 M guanidine-HCl, 0.2 M ammonium bicarbonate, 0.02% NaN_3.

Mild Acid Cleavage. For cleavage predominantly at aspartic acid-proline peptide bonds, completely reduced and alkylated 104E μ chain was dissolved in 10% acetic acid and 7 M guanidine-HCl, adjusted to pH 2.5 with pyridine and incubated at 45°C for 108 h with occasional mixing. The resulting fragments were dialyzed against 3 M guanidine-HCl, 0.2 M ammonium bicarbonate at 4°C and separated by gel filtration on a column (2.5 x 100 cm) of ACA 34 (LKB) equilibrated in 3 M guanidine-HCl, 0.2 M ammonium bicarbonate.

Amino Acid Analysis. Peptide samples were flushed with N_2 and evacuated three times, sealed under vacuum and hydrolyzed at 100°C for 20 h in constant boiling 6 N HCl. When quantitation of glucosamine was required, hydrolysis was for 3 h. Amino acid analysis was performed on a Durrum D-500 analyzer.

Carbohydrate Determinations. Individual cyanogen bromide fragments were rechromatographed on Bio-Gel P-6 in 0.1 N acetic acid to remove galactose and glucose contamination. Alditol derivatives were separated and quantitated by gas chromatography (Kim et al., 1967).

Sequence Determination. All large fragments and peptides were sequenced by loading on a modified Beckman sequenator with automatic Pth-conversion using trifluoroacetic acid (Hunkapiller & Hood, 1978; Wittmann-Liebold, 1973; Wittmann-Liebold et al., 1976). Samples were loaded into a polybrene-coated cup (containing 20 nmole glycylglycine and pre-run for 5-7 cycles) in trifluoroacetic acid (Pierce) and 10-20% water. The CN3 fragment was loaded in dilute NH_4OH and small peptides generated by enzymatic cleavages were dissolved in water. The first step was coupled twice. Pth-amino acid derivatives were identified by high performance liquid chromatography (Waters Associates, DuPont) as described

(Hunkapiller & Hood, 1978; Johnson et al., 1979).

DNA sequences were determined by the method of Maxam and Gilbert (1977), using the G (alternative), A > C, T + C, and C reactions as described (Rogers et al., 1980).

Computer Comparison of C_H Amino Acid Sequences. Homology comparisons of individual C_H domains were performed for the following heavy chains: MOPC 104E μ (Kehry et al., 1979; this paper), GAL μ (Watanabe et al., 1973), OU μ (Putnam et al., 1973), MOO μ (Wasserman & Capra, 1978; McCumber et al., 1979), ND ε (Bennich & Von Bahr-Lindstrom, 1974), MOPC 21 γ_1 (Adetugbo et al., 1977), EU γ_1 (Edelman et al., 1969), NIE γ_1 (Ponstingl & Hilschmann, 1972), rabbit γ (Fruchter et al., 1970), guinea pig γ_1 (Cebra et al., 1977), MOPC 173 γ_{2a} (Fougereau et al., 1976), MPC11 γ_{2b} (Tucker et al., 1979a,b), guinea pig γ_2 (Trischmann & Cebra, 1974; Cebra et al., 1977), VIN γ_4 (Pink et al., 1970), ZUC γ_3 (Wolfenstein-Todel et al., 1976), HER γ_3 (Michaelsen et al., 1977), TRO α_1 (Kratzin et al., 1978), BUR α_1 (Low et al., 1976), BUT α2-2 (Torano & Putnam, 1978), LAN α2-1 (Tsuzukida, 1979), MOPC 47A α (Robinson & Appella, 1977), MOPC 315 α and MOC 511 α (Robinson & Appella, 1977). Domains were defined on the basis of intron locations in the μ and γ chain genes (Calame et al., 1980; Tucker et al., 1979) and domain homology in the α and ε chains (Beale & Feinstein, 1976). Sequences were compared using a computer program as described (Loh et al., 1979).

Results

Sequence Strategy. Cyanogen bromide cleavage of the MOPC 104E (104E) μ chain produced nine peptides (CN1-CN9) which were separated by gel filtration (Figure 1). Determination of the NH_2-terminal sequence of each peptide showed that each peak gave a single homogeneous amino acid sequence. Therefore, the one-step gel filtration procedure illustrated in Figure 1 separates each

of the 104E cyanogen bromide peptides from one another. Amino terminal sequence homology of the 104E C_H region cyanogen bromide peptides to the human μ chain sequence (Putnam et al., 1973) permitted determination of a presumptive alignment of peptides (top portion of Figure 1). This order was confirmed by additional sequence determinations which completed the sequence of each fragment and in all but two cases directly overlapped the fragments. Fragment CN1 was not isolated, but since CN1 is the NH_2-terminal V_H region fragment, this sequence had been previously determined on the intact μ chain (Barstad et al., 1978). Fragment CN1 was found to migrate between fragments CN1-2 and CN2 on the ACA 54 column by chromatographing ^3H-leucine and tyrosine labeled 104E μ chain cyanogen bromide fragments (not shown). Incomplete cleavage of the methionine-serine bonds at positions 20 and 568 by cyanogen bromide resulted in the isolation of two partial fragments, CN1-2 and CN8-9, respectively, as indicated in Figure 1.

The complete amino acid sequence of the 104E μ chain is shown in Figure 2, along with the data used to determine the sequence. The amino acid and carbohydrate composition of the 104E μ chain and the cyanogen bromide fragments as derived from the amino acid sequence is given in Table I. There are three amino acid differences from the previously published sequence (Kehry et al., 1979); position 130 (alanine to serine) was miscalled due to contaminating sequences and residues 233 (histidine to glutamic acid) and 545 (glutamic acid to histidine) were identified by using an improved analytical method. In addition, we have determined the type of oligosaccharide attached to each of the six carbohydrate sites in the 104E μ chain.

Sequence of Cyanogen Bromide Fragments and Alignment. (a) Sequence of CN1 and CN2. Amino terminal sequence analysis of the first 38 residues of the intact 104E μ chain (Barstad et al., 1978) established the order of fragments CN1, 2, and 3 (Figure 1). A sequenator run on the entire CN1-2 fragment verified

its identity as residues 1 to 34. The sequence of CN2 as residues 21 to 34 also was completely determined in a single sequenator run (Figure 2).

(b) Sequence of CN3. The CN3 fragment extends from lysine$_{34}$ to methionine$_{82}$ and is 47 residues in length. [To facilitate homology comparisons, the numbering of the 104E μ chain corresponds to the amino acid sequence of the human μ chain OU (Putnam et al., 1973) and the total amino acid residues in each 104E μ chain fragment are not necessarily obtained by counting the assigned residue numbers (Kehry et al., 1979).] Quantitation of an NH_2-terminal sequence determination on CN3 is shown in Figure 3. To complete the sequence, the CN3 fragment was digested with trypsin, and the resulting peptides were separated on paper by chromatography and high-voltage electrophoresis (Katz et al., 1959). Amino acid compositions of the isolated tryptic peptides are given in Table II. Sequence determination of the peptide CN3T7 provided the overlap data which established the sequence of CN3 (Figures 1, 2 and 3). The CN1 and CN3 fragments are the only μ chain cyanogen bromide fragments which contain no cysteine residues. The second hypervariable region is located in CN3 and includes a carbohydrate moiety attached to asparagine$_{57}$ (see below).

(c) Sequence of CN4. The CN4 fragment spans the variable-constant region junction from glutamine$_{83}$ to methionine$_{151}$ and is 62 residues in length. The sequence overlap between CN3 and CN4 has not been rigorously established, but because this variable region sequence can be aligned without sequence gaps with those of other heavy chains (Kabat et al., 1976), the CN3 and CN4 fragments are probably contiguous. The NH_2-terminal sequence of CN4 was determined to the variable-constant region junction. The remaining sequence was obtained and confirmed by determining the sequences of the major peptide produced by tryptophan cleavage (Ozols et al., 1977), and the tryptic and thermolysin peptides CN4Th3, CN4Th7, CN4Th9, CN4Th10, and CN4T8 (Figure 2). The one inserted

residue at the end of this fragment (position 148) also has been confirmed independently by Milstein et al. (1975) through an analysis of the corresponding cysteine-containing peptide.

(d) Sequence of CN5. The CN5 fragment extends from glycine$_{152}$ to methionine$_{234}$ and constitutes the major portion of the C_H1 domain. This 84-residue fragment also contains the first complex carbohydrate moiety in the constant region. Determination of the NH_2-terminal sequence plus the sequences of the two arginine fragments (CN5RI and CN5RII) and the fragment generated by cleavage at the tryptophan residue (CN5W) completed the sequence of the CN5 fragment (Figure 2). The Fc fragment produced by trypsin digestion of the IgM molecule in the presence of 5 M urea (Shimizu et al., 1975) provided the COOH-terminal sequence of CN5 and the overlap between CN5 and CN6. The first 30 amino acids of the Fc fragment contain seven proline residues which are in the boundary region between C_H1 and C_H2. This proline-rich stretch in the μ chain then bears a striking resemblance to the proline-rich hinge regions of γ and α heavy chains (Aldersberg, 1976).

(e) Sequence of CN6. The CN6 fragment encompasses the entire C_H2 domain and half of the C_H3 domain in its 166 residues. Extending from asparagine$_{235}$ to methionine$_{397}$, CN6 contains two complex carbohydrate moieties, one in C_H2 and one in C_H3 (see below and Figure 2). The amino acid sequence of the first half of CN6 was established by Edman degradations of the NH_2-terminus and of the two arginine fragments (CN6RI and CN6RII). Cleavage of the one aspartic acid-proline peptide bond in the fully reduced and alkylated 104E μ chain by mild acid produced a fragment with an NH_2-terminus at proline$_{286}$. The sequence of this acid-derived fragment was then overlapped with the NH_2-terminus of the CN6RII arginine fragment. The remaining COOH-terminal sequence of CN6 was determined by isolating CN6RII, followed by demaleylating or decitraconylating

the lysine residues and digesting the fragment with trypsin. Four predominant amino termini resulted from this procedure: CN6RIIa, beginning at the NH_2-terminus of CN6RII, glycine$_{326}$; CN6RIIb, produced in low yield due to incomplete cleavage of the peptide bond between lysine$_{331}$ and glycosylated asparagine$_{332}$; CN6RIIc, beginning at serine$_{362}$; and CN6RIId, the result of a chymotryptic cleavage at phenylalanine$_{348}$. The sequence was confirmed and completed by the isolation of the thermolysin and trypsin plus chymotrypsin peptides, CN6TC4, CN6Th7 and CN6TC6 (Figure 2). The overlap between CN6 and CN7 has not been determined, but the sequences of CN6 and CN7 are contiguous when aligned by homology with two human μ chains and a dog μ chain (Watanabe et al., 1973; Putnam et al., 1973; Wasserman & Capra, 1978). Therefore we believe that no additional cyanogen bromide fragment is located between CN6 and CN7.

(f) Sequence of CN7. The CN7 fragment, which is 109 residues in length (glutamic acid$_{398}$ to methionine$_{506}$), spans the remaining portion of C_H3 and the first two-thirds of the C_H4 domain (Figure 2). The one high mannose carbohydrate moiety in CN7 is attached to asparagine$_{402}$ in the C_H3 domain, making C_H4 the only μ chain constant region domain which has no covalently attached oligosaccharide moiety. In addition to an NH_4-terminal sequence, the sequences of the two tryptophan fragments (CN7WI and CN7WII) and the sequences of the overlapping thermolysin and tryptic peptides, CN7Th10, CN7T6, CN7T7, CN7Th13, CN7Th14, CN7Th15 and CN7T8 completed the covalent structure of the CN7 fragment (Figure 2).

(g) Sequence of CN8, CN8-9 and CN9. The sequence of a peptide which overlaps CN7 and CN8 has been determined by Milstein et al. (1975). NH_2-terminal sequenator runs on CN8 and CN8-9, beginning at proline$_{507}$, established the sequence through tyrosine$_{562}$. Cleavage at the single arginine residue in CN8-9 allowed determination of the remaining sequence of the C-terminal segment (to tyrosine$_{576}$).

Sequence determination on the arginine fragment of CN8 indicated that at least two-thirds of this peak lacked residues 569 to 576 (Figure 2). Isolation and sequence analysis of the CN9 fragment from a cyanogen bromide digestion of ^{14}C-alkylated 104E μ chains confirmed the cyanogen bromide cleavage between methionine$_{568}$ and serine$_{569}$. The high mannose carbohydrate attached to asparagine$_{563}$ in the C-terminal segment was identified from a chymotryptic and thermolysin peptide (CN8-9CTh4).

DNA Sequences. Recently, several C_μ gene clones have been obtained, both from BALB/c germline chromosomal DNA and from MOPC 104E μ chain messenger-complementary DNA (Calame et al., 1980). As is shown in Table III, DNA sequence analysis of these clones provided data covering approximately 60% of the 104E μ chain constant region (260 out of 450 residues) (Calame et al., 1980; Rogers et al., 1980; Rogers, unpublished results). These DNA sequences confirm the protein sequence at all positions except for two discrepancies (asparagine$_{347}$ to threonine and threonine$_{379}$ to asparagine). Since the cloning of this gene involved no in vitro copying of the DNA, it is unlikely that the differences are due to a cloning artifact, unless a mutation has occurred during replication in E. coli. These residues also are clearly identified in the automatic sequenator runs. Since most of these sequence determinations were performed on peptide mixtures containing two or three sequences, we would like to point out that the sequence presented here does indeed conflict with the DNA sequence data. These sequence differences may represent a low level of natural polymorphism for the C_μ gene.

Carbohydrate Moieties. Six carbohydrate moieties are attached to asparagine residues in the 104E μ chain. One site is located in the V_H region on asparagine$_{57}$ (Figure 2). The residues which are glycosylated were initially identified as blank cycles in the automatic sequenator runs. The precise location of each carbohydrate

was subsequently confirmed by the recovery of glucosamine from a short-term (3 h) HCl hydrolysis on a small peptide in addition to the presence of an extra aspartic acid residue which was not identified in the sequenator runs. The constant region cyanogen bromide fragments CN5, CN7 and CN8 each contain one oligosaccharide. The carbohydrate compositions of these purified peptides and of two small pronase glycopeptides from the CN6 fragment are shown in Table II. These compositions establish that the first three constant region oligosaccharides attached to residues 171, 332, and 364 are of the complex type. In addition, the CN5 and CN6 fragments which contain these complex type carbohydrates are characteristic glycopeptides in that these are the only 104E μ chain cyanogen bromide fragments which contain galactose residues, as judged by extrinsic labeling with galactose oxidase and NaB^3H_4 (Kehry et al., 1980).

Carbohydrate compositions on the CN7 and CN8 oligosaccharides (Table II) indicate that these two COOH-terminal carbohydrates are high mannose oligosaccharides. The carbohydrate moiety attached to fragment CN3 at $asparagine_{57}$ in the V_H region contains some glucosamine by ninhydrin analysis (Table I) and small amounts of mannose, galactose, and an unidentified sugar derivative (Table II). This must be a unique carbohydrate structure, not of the high mannose or complex type, consisting of one glucosamine and one mannose residue with a small proportion of the $asparagine_{57}$ residues having galactose and/or another sugar moiety.

Discussion

Complete μ Chain Sequence. The complete amino acid sequence of the MOPC 104E μ chain has been determined (Figure 2). The μ chain may be divided into a V_H and a C_μ region. The C_μ region can be further subdivided into four domains of approximately 110 residues whose boundaries have previously been delineated by comparisons of internal heavy chain amino acid homology with the

three-dimensional structure of the immunoglobulin molecule (Beale & Feinstein, 1976). In addition, a 20-residue C-terminal segment is located COOH-terminal to the $C_\mu 4$ domain in the secreted μ chains. There are five sites of carbohydrate attachment in the secreted μ chain constant region, with only the $C_\mu 4$ domain lacking carbohydrate moieties. When the amino acid sequences of mouse, human and dog μ chains are compared, there is an increasing gradient of sequence homology from the NH_2-terminal V_H domain to the C-terminal segment (Kehry et al., 1979).

Immunoglobulin heavy chain domains are also known to be discrete coding elements at the level of the heavy chain gene and are separated from one another by short intervening DNA sequences (Early et al., 1979; Calame et al., 1980; Tucker et al., 1979a,b). The coding region for the C-terminal segment of the secreted μ chain, though, is contiguous with that of the $C_\mu 4$ domain (Calame et al., 1980). The precise domain junctions which have been determined by DNA sequence analysis of BALB/c μ genomic and complementary DNA clones (Calame et al., 1980) are in remarkable agreement with those determined form the μ chain amino acid sequence (Beale & Feinstein, 1976; Kehry et al., 1979). For the secreted μ chain, there are five domain boundaries which have been described (Beale & Feinstein, 1976), and four of these boundaries match those determined from the μ gene DNA sequence or are only shifted by one amino acid (Calame et al., 1980). The remaining boundary between $C_\mu 1$ and $C_\mu 2$ in the μ chain gene is shifted by just three amino acids from the boundary previously described from the protein structure.

Carbohydrate Moieties in the μ Chain. Two high mannose and three complex type carbohydrate moieties are attached to asparagine residues in the μ chain constant region. Both types of oligosaccharide structures are composed of a core carbohydrate containing two N-acetylglucosamine and five mannose residues (Kornfeld & Kornfeld, 1976). The high mannose structures are synthesized by the addition of mannose residues to the core to form a branched structure.

Complex type carbohydrates are also highly branched and are formed by the sequential additions of glucosamine, galactose, fucose and N-acetyl neuraminic acid to the core structure (Kornfeld & Kornfeld, 1976). In addition, a unique small carbohydrate structure which is not of the high mannose or complex type is located in the 104E second hypervariable region.

As noted previously, four of the five C_{μ} region carbohydrates are attached to identical positions in the mouse and human μ chains (Kehry et al., 1979). Each oligosaccharide is attached to the asparagine residue of an Asn-X-Ser/Thr recognition sequence (Marshall, 1972), where X may be any amino acid. Since three additional recognition sequences which are not glycosylated are present in OU and 104E μ chains (Kehry et al., 1979), clearly the conservation of carbohydrate attachment sites suggests they are important structural or functional features of the IgM molecule. Interestingly, the DNA sequence discrepancy which could change position 379 to an asparagine residue would create a recognition sequence for the glycosylation of that asparagine residue. In the mouse μ chain there is an oligosaccharide which is attached to $asparagine_{364}$ (Kehry et al., 1979). This oligosaccharide is in a different location ($asparagine_{395}$) in the human μ chain. This change in carbohydrate location is the result of a change in amino acid sequence between mouse and human μ chains. Thus, if the DNA sequence of the mouse μ chain indicates that residue 379 is asparagine, the selective pressure in this region to form a recognition sequence for glycosylating enzymes is remarkably high.

The general type of carbohydrate moiety, complex or high mannose, is completely conserved between mouse and human μ chains (Shimizu et al., 1971). Proceeding from the C_H1 to the C-terminal domain, the first three oligosaccharides attached to the μ chain are of the complex type, containing terminal galactose and N-acetyl neuraminic acid residues. The remaining two oligosaccharides, including the one in the C-terminal region, are high mannose carbohydrate structures composed

of glucosamine and mannose. Apparently, the glycosylating enzymes involved in the synthesis of these distinct types of oligosaccharides efficiently discriminate among multiple recognition sites on the same molecule. The complex and high mannose oligosaccharides then presumably have different structural or functional roles in the IgM molecule which require a precise localization.

At first glance, though, carbohydrate structures attached to heavy chains of different classes seem to differ in type and location (Torano et al., 1977). For example, most human γ chains possess one oligosaccharide N-glycosidically linked to an asparagine residue in the C_H2 domain (Kornfeld et al., 1971; Beale & Feinstein, 1976). Human α chains differ remarkably in glycosylation even between the α_1 and α_2 subclasses. The α_2 chains have one or two (depending upon the α_2 allele) N-linked carbohydrate structures located in the C_H1 and hinge domains and two in the C_H2 domain, one of which is identical in the α_1 chain. Both α_1 and α_2 chains have one N-linked carbohydrate in the C-terminal segment and only α_1 chains have five O-linked (to serine residues) oligosaccharides in the hinge region (Torano et al., 1977; Tsuzukida et al., 1979). Human ε chains, like μ chians, contain only N-linked oligosaccharides, three of which are located in the C_H1 domain, one in the C_H2, and two in the C_H3 domain (Bennich & von Bahr-Lindstrom, 1974). The one oligosaccharide in γ chains (in the $C_\gamma2$ domain) is in a homologous position to an N-linked carbohydrate in the C_H3 domain of μ and ε chains (Beale & Feinstein, 1976), suggesting that these domains either have a common evolutionary origin or that they perform similar functions. The one common feature shared by all heavy chain classes is that the last domain ($C_\gamma3$, $C_\alpha3$, $C_\mu4$ or $C_\varepsilon4$) lacks a carbohydrate moiety entirely. In the case of γ and ε chains, a carbohydrate moiety may interfere with Fc receptor-immunoglobulin interactions in this COOH-terminal domain.

The one other exception to this lack of homology in the location and type of heavy chain carbohydrate structures is the high mannose oligosaccharide situated in the C-terminal segment of both μ (asparagine$_{563}$) and α (asparagine$_{459}$) chains (Putnam, 1974). This carbohydrate is located on homologous residues in human α, human μ, and mouse μ chain C-terminal segments. In addition, the amino acid sequence homology between human α and μ chain C-terminal segments is a striking 70% (14/20 identical residues). Since IgM and IgA are the only immunoglobulin classes which form polymers with the J chain, and since only α and μ chains possess the 20-residue C-terminal segment, this region must be very important in the polymerization process and in the maintenance of the polymer structure. This high amount of homology in the C-terminal segments of α and μ chains is even more impressive when one considers that the degree of C_H region homology between μ and α chains (40% by amino acid sequence) is such that cloned C_μ and C_α gene probes will not cross-hybridize (P. Early, personal communication). The penultimate cysteine residue in α and μ chains is involved in the formation of covalent linkages to the J chain and to other heavy chains in the polymer (Della Corte & Parkhouse, 1973; Mestecky & Schrohenloher, 1974; Mestecky et al., 1974; Mendez et al., 1973). In addition, we envision that the highly conserved C-terminal high mannose oligosaccharide plays a structural or functional role in the IgM and IgA polymer. The potential importance of the C-terminal segment in polymer formation is strengthened by the fact that membrane IgM molecules, which exist only as monomeric immunoglobulins, lack the C-terminal segment and carbohydrate moiety present in secreted μ and α chains (Kehry et al., 1980).

The composition of the C-terminal high mannose oligosaccharide is different from that of the other oligosaccharides in the μ chain. Quantitation of the ^{3}H-mannose incorporated into 104E μ chain cyanogen bromide fragments correlates well with the number of carbohydrate moieties attached to each fragment (Kehry

et al., 1980). The CN7 and CN5 fragments, each with one carbohydrate, and the CN6 fragment with two carbohydrates are labeled with ^{3}H-mannose in a 1:1:2.5 ratio (CN7:CN5:CN6). But, quantitation of ^{3}H-mannose labeled CN8 plus CN8-9 fragments shows that only 0.5 carbohydrate moiety is attached to the C-terminal segment. There are two possible explanations for the fractional amount of mannose in the C-terminal oligosaccharide. Either this carbohydrate structure contains half as many mannose residues as a mature high mannose oligosaccharide, or only a portion of the μ chains are glycosylated at this position. We favor the latter explanation in view of our amino acid sequence data on the arginine fragment of CN8. In the automatic sequenator, the asparagine residue to which a carbohydrate moiety is attached is not extracted out of the sequenator cup and therefore shows up as a blank cycle in the analytical system. In the sequence of the CN8-9 arginine fragment, asparagine$_{563}$ was a blank cycle, as expected for a position of carbohydrate attachment. But in the arginine fragment of CN8, position 563 showed approximately 0.7 residue of asparagine, indicating that a portion of the 104E μ chains are unglycosylated in the C-terminal region. Since the fractional mannose incorporation into the CN8 fragment also is reproducibly seen in other secreted μ chains and in internal precursors to secreted μ chains (Kehry et al., 1980), it is probably significant at the structural or functional level.

Domain Homologies Among Heavy Chains. (a) Mu Chain Homologies. When the mouse, dog and human C_{μ} sequences are compared, there is an increasing homology from the $C_{\mu}1$ domain to the C-terminal segment (Kehry et al., 1980). The mouse and human μ chains are 61% homologous in the C_{μ} region, ranging from 48% identical residues (in addition to two sequence gaps in $C_{\mu}1$ and three gaps in $C_{\mu}2$) in the $C_{\mu}1$ domain to 90% identical residues in the C-terminal segment (no sequence gaps in $C_{\mu}3$, $C_{\mu}4$ and the C-terminal segment). Similarly, the mouse and dog C_{μ} regions are 59% homologous with a range of 50% identical residues

in the $C_\mu 1$ domain (two sequence gaps in $C_\mu 1$ and two gaps in $C_\mu 2$) to 80% identical residues in the C-terminal segment (again, no sequence gaps after the $C_\mu 2$ domain). The amino acid sequence differences between 104E μ chains and those of human OU and dog MOO appear to be clustered in eight regions of 12 to 20 amino acids in length (residues 161-180, 210-225, 260-279, 330-347, 383-397, 410-425, 437-449, and 484-498). In all but one of these nonhomologous regions, the dog MOO and human OU chains exhibit significantly more homology to one another than either does with the mouse 104E μ chain (Kehry et al., 1979). One of the more striking examples is residues 383-397, where mouse 104E and human OU μ chains differ in 13/15 positions while dog MOO and human OU differ in only 1/15 residues. Since DNA studies on the mouse C_μ gene suggest that the C_μ gene is present in only one copy per haploid genome (Early et al., 1980; Calame et al., 1980; Cory & Adams, 1980), the differing extents of homology among the mouse, dog, and human μ chains are not due to the existence of μ chain subclasses in the mouse. The difference probably reflects a closer evolutionary relationship between dogs and humans than between mice and dogs or humans.

We have previously shown that among mouse, human and dog μ chains, the $C_\mu 4$ domain is the most highly conserved of the four μ chain constant region domains (Kehry et al., 1979). Interestingly, the binding site for the first component of the complement cascade has been localized to a region in $C_\mu 4$, residues 468-491 joined to 515-546 (Hurst et al., 1975). These two stretches of amino acids are at least 75% identical in the sequences of the mouse, human, and dog μ chains. This complement binding region contributes significantly to the high degree of $C_\mu 4$ sequence conservation among the three species, and presumably reflects the structural and/or functional constraints related to complement fixation that have been imposed upon this distinct region during species evolution.

(b) Homologies Among μ, γ, α, and ε Chains. An interesting question in the evolution of the different heavy chain classes is how additional domains have evolved (four C_ε and C_μ domains), or how regions have been deleted (no hinge region in μ or ε chains). The intervening DNA sequences between the domains in heavy chain genes provide an excellent mechanism for rearranging, duplicating, or deleting individual domains. An examination of the homologies among all of the completely sequenced C_H domains may reveal the evolutionary origins of these C_H domains. In particular, these comparisons may be used to identify a primordial domain or pair of domains from which all heavy chain genes evolved by gene duplication.

For example, in an analysis of the divergence exhibited by mouse γ chain protein and nucleic acid sequences, Honjo has found that the C_H1 domains of γ_1, γ_{2a} and γ_{2b} chains have diverged singificantly less from one another than any of the other C_γ domains (Yamawaki-Kataoka et al., 1980). In addition, the C_H2 domains of γ_{2a} and γ_{2b} chains also diverge very little, indicating that the C_H1 and C_H2 gene segments of γ_1, γ_{2a} and γ_{2b} chains have recombined prior to the complete divergence of these subclasses in the mouse.

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TABLE I: Amino Acid Composition of Tryptic Peptides from 104E μ CN3.[a]

	CN3T1[b]	CN3T2	CN3T3	CN3T4	CN3T5	CN3T6	CN3T7
CMCys							
Asx			4.9 (5)			1.0 (1)	
Thr			1.0 (1)			1.9 (1)	1.1 (1)
Ser		1.0 (1)	1.7 (2)				2.8 (3)
Glu		0.9 (1)	1.8 (2)				
Pro			1.1 (1)				
Gly		1.1 (1)	2.8 (3)		0.9 (1)		
Ala						1.0 (1)	1.2 (1)
Val	0.9 (1)					1.0 (1)	
Ile			1.3 (2)				
Leu			1.0 (1)			1.0 (1)	
Tyr			1.0 (1)				0.9 (1)
Phe				0.9 (1)			
Glc-NH_2[e]			0.2				
His		0.9 (1)					
HSer[c]							0.6 (1)
Lys	1.1 (1)	1.1 (1)	1.0 (1)	1.1 (1)	1.1 (1)	1.0 (1)	
Arg							
Trp[f]	+						
Total residues	3	5	20	2	2	7	7
Yield (%)[d]	30	35	15	60	70	75	20
Residue Numbers[g]	38-40	41-45	46-64	65-66	67-68	69-75	76-82

Legend to Table I

[a] Values reported are amino acid residues. Amino acids present at a level of less than 0.2 residue are omitted. Values in parentheses represent the nearest integral number of residues present in the sequence.

[b] The name of each peptide indicates the cyanogen bromide fragment from which it was derived, followed by the enzyme used and the contiguous order in the fragment. T = trypsin; Th = thermolysin; C = chymotrypsin.

[c] Includes homoserine as well as homoserine lactone.

[d] Yields are based on nanomoles of peptides isolated compared with nanomoles of fragment originally digested with enzyme.

[e] The presence of glucosamine was determined by a separate hydrolysis in 6 N HCl at 110° for 3 h.

[f] u.v. positive peptide.

[g] The numbering does not correspond to the number of amino acids due to the introduction of gaps for sequence alignment.

TABLE II. Amino Acid Composition of CNBr Fragments from 104E u.[a]

	CN1	CN2	CN3	CN4	CN5	CN6	CN7	CN8	CN9	Whole u Total
Cys		1		2	2	4	3	1	1	14
Asp		1	2	5	3	7	3	1	1	23
Asn			4	3	5	10	5	2		30
Thr		2	4	4	7	21	6	9	2	54
Ser	2	2	6	10	5	18	9	4	1	56
Glu	2		1	3	4	7	7	7		31
Gln	3		2	2	4	2	6			19
Pro	2		1	3	5	13	11	5		40
Gly	2	1	5	2	7	7	4	5	2	35
Ala	1	1	2	4	4	8	7	2		30
Val	3		2	7	5	11	11	6		45
Met										8
Ile			2		6	12	2	2		24
Leu	2		2	5	9	15	8	5		46
Tyr		3	2	4	4	3	2	3	1	22
Phe		1	1	3	3	8	4	2		22
Trp			2	2	1	3	2	1		11
His			1		1	1	4	3		10
HSer	1	1	1	1	1	1	1	1		
Lys	2	1	7	1	4	11	9	2		37
Arg				1	4	4	5	1		15
CHO moiety[b]			1		1	2	1	1		6
						A	B			
Fuc[c]					0.5	3.3	1.3	0.1	0.2	
Man			0.5		3.0	5.2	5.5	2.1	1.5	
Gal			0.2		2.4	7.6	4.1	0.7	1.0	
Glc-NH_2					5.3	3.5	5.4	1.0	1.1	
NANA					0.3	0.7			0.1	
X[d]			0.2							
TOTAL	20	14	47	62	84	166	109	62	8	572
Residue Numbers[e]	1-20	21-34	35-81	83-151	152-234	235-397	398-506	507-568	569-576	
Molecular Weight[c]	2150	1600	5180	6895	9860	18,105	12,230	6815	900	64,200

Legend to Table II

[a] Values reported are amino acid residues from the completed sequence.

[b] Number of carbohydrate attachment sites in each cyanogen bromide fragment. Locations: CN3, residue 57; CN5, residue 171; CN6: A, residue 332, B, residue 364; CN7, residue 402; CN8, residue 563.

[c] Carbohydrate residues determined by gas chromatography of alditol acetates. Values reported are mole carbohydrate/mole glycopeptide. The two glycopeptides in CN6 were obtained by pronase digestion of CN6 followed by chromatography on Biogel P-10. A, first peak from P-10 column; B, second peak from P-10 column. Abbreviations: Fuc, Fucose; Man, Mannose; Gal, Galactose; Glc-NH_2, glucosamine; NANA, N-acetylneuraminic acid.

[d] Unidentified gas chromatographic alditol acetate, present only in CN3.

[e] Molecular weight in daltons calculated from the sequence of amino acids in the fragment; does not include carbohydrate.

[g] See legend to Table I.

TABLE III. Sequenced Portions of C_μ Gene

Codons (inclusive)	Clone	Ref.
127 - 153	μA1	*
218 - 250	μA1	*
300 - 323	μ12	Calame et al., 1980
336 - 388	μA1	*
432 - 466	μA1	*
432 - 470	μ12	*
531 - 556	μ6	Rogers et al., 1980
542 - 576	μ12	Calame et al., 1980

Numbering is as for Ou. For a description of the clones and maps showing sequencing strategies, see Calame et al. (1980) and Rogers et al. (1980). μA1 = SpμA1; this is a subclone from ChSpμ7, which contains chromosomal DNA from BALB/c sperm. μ6 = p104Eμ6, μ12 = p104Eμ12; these contain complementary DNA from MOPC 104E mRNA. The sequences of codons 218 - 250 and 542 - 576 were obtained from two strands; the remaining sequences were obtained from only one of the two strands.

*Unpublished results.

FIGURE 1: Separation of MOPC 104E cyanogen bromide fragments. Completely reduced and alkylated 104E μ chains (35 mg) were cleaved with cyanogen bromide and the fragments separated by gel filtration on a column of ACA 54 (LKB) as described in Experimental Procedures. Fraction volume was 5 ml. The fragments are labeled (CN1 to CN9) according to their position in the μ chain sequence beginning at the NH_2-terminus. Agg. is the excluded column peak which consists of aggregated fragments and large uncleaved peptides. At the top of the figure is a schematic drawing of the 104E μ chain (to scale) showing the linear order and sizes of the cyanogen bromide fragments (CN1 to CN9). Two incomplete cleavage products, CN1-2 and CN8-9, result from partial cleavage of the methionine residues at positions 20 and 568, respectively. CHO denotes the sites of carbohydrate attachment. The complex type carbohydrates are indicated by boxes and the high mannose carbohydrates are indicated by circles.

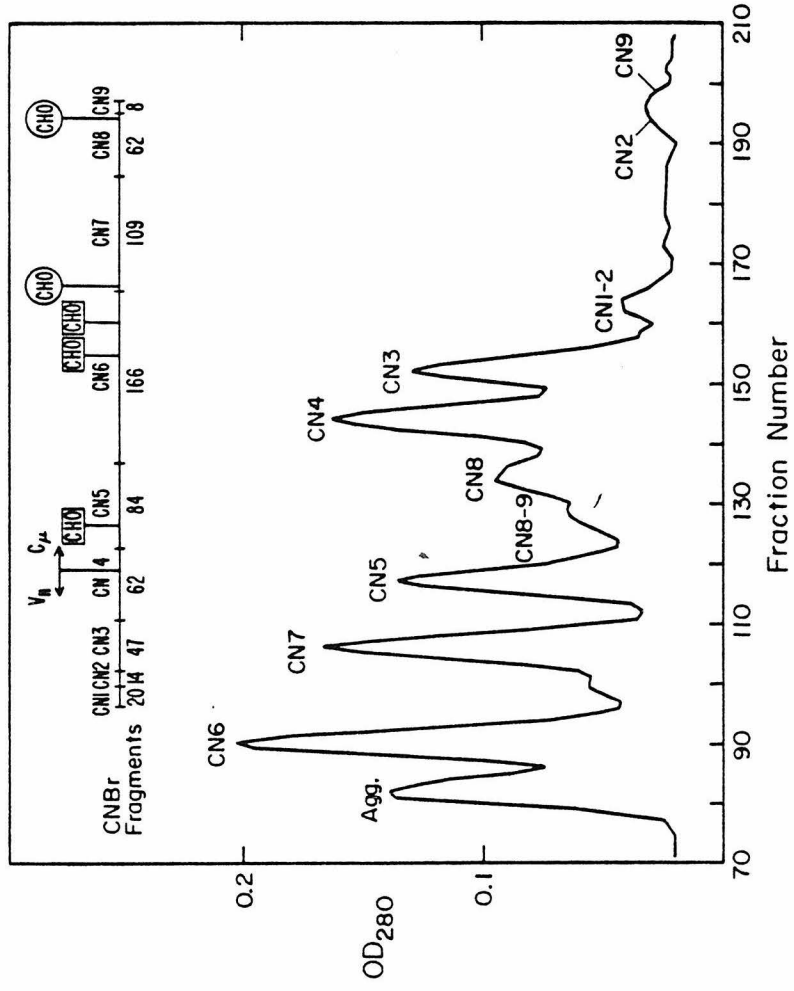
CNBr Fragments
CN1 CN2 CN3
20 14 47
CN 4
62
CHO
CN5
84
CHO
CHO
CHO
CN6
166
CHO
CN7
109
CHO
CN8
62
CN9
8
Agg.
CN6
CN7
CN5
CN4
CN3
CN8
CN8-9
CN1-2
CN2
CN9
0.2
0.1
OD280
70
90
110
130
150
170
190
210
Fraction Number

FIGURE 1.

FIGURE 2: Complete sequence of the MOPC 104E μ chain. The one-letter code for amino acids is given in Dayhoff, (1976). Tryptic (T), chymotryptic (C), and thermolysin (Th) peptides are indicated. Sequences of peptides produced by cleavage at methionine (CN), arginine (R) and tryptophan (W) residues are also indicated in the left-hand margin. All peptides are identified by the cyanogen bromide fragment number from which they were derived. Residues sequenced automatically are indicated by $\rightarrow$. CHO denotes the sites of carbohydrate attachment to asparagine residues. The constant region domain boundaries are from DNA sequence determinations on a cloned BALB/c μ gene (Calame et al., 1980).

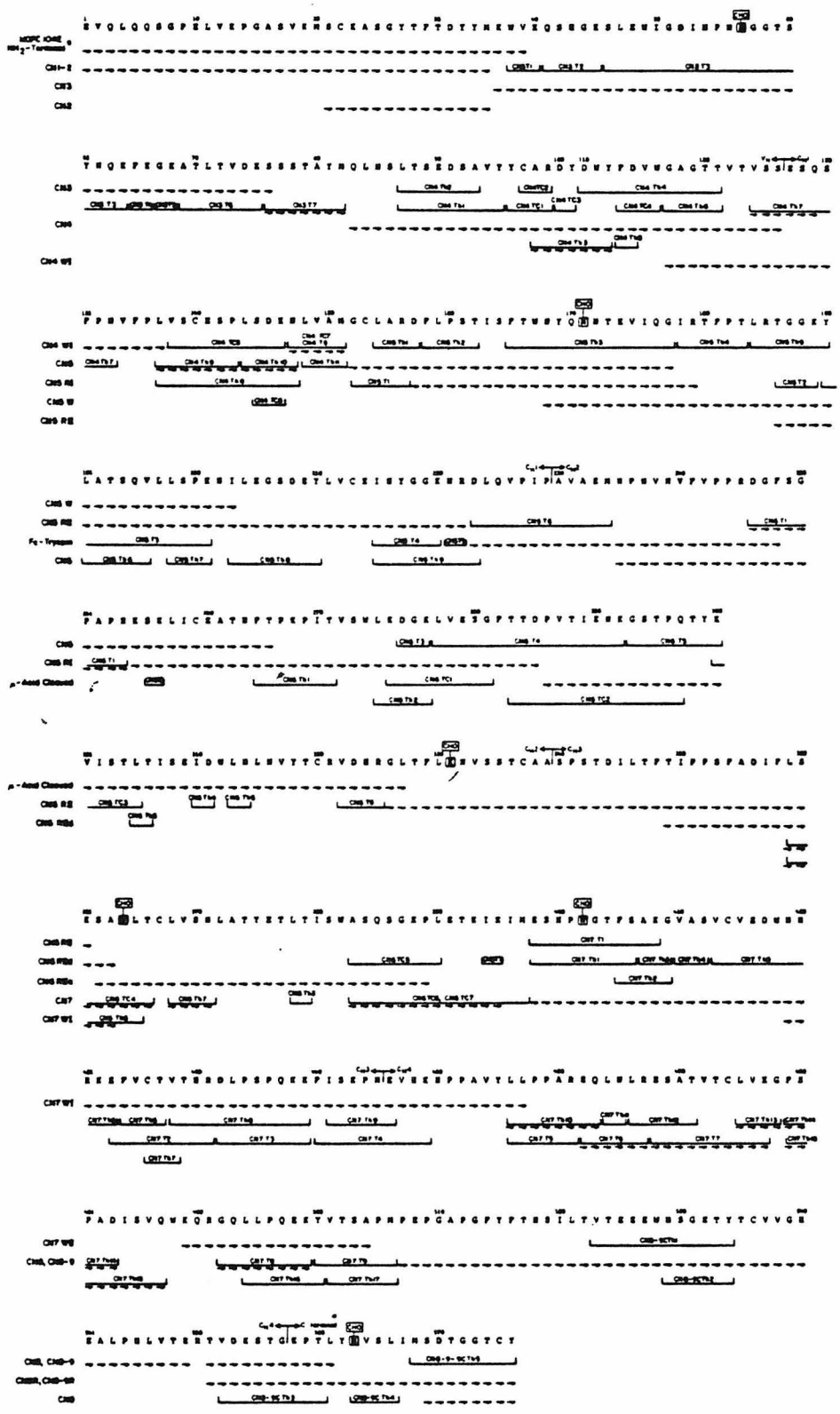

FIGURE 2.

FIGURE 3: Yields of phenylthiohydantoin amino acids and sequence of MOPC 104E CN3 from an NH_2-terminal sequenator analysis. The CN3 fragment was purified and the amino terminal sequence was determined as described in Experimental Procedures. Aliquots of each cycle were analyzed by high performance liquid chromatography; peak heights were converted to nanomole yield for each derivative by comparison with a standard phenylthiohydantoin amino acid mixture. Assigned residues are indicated by a larger filled circle and scale changes are as indicated by -\\- on the graphs. Residue number 1 corresponds to residue 35 in the intact 104E μ chain.

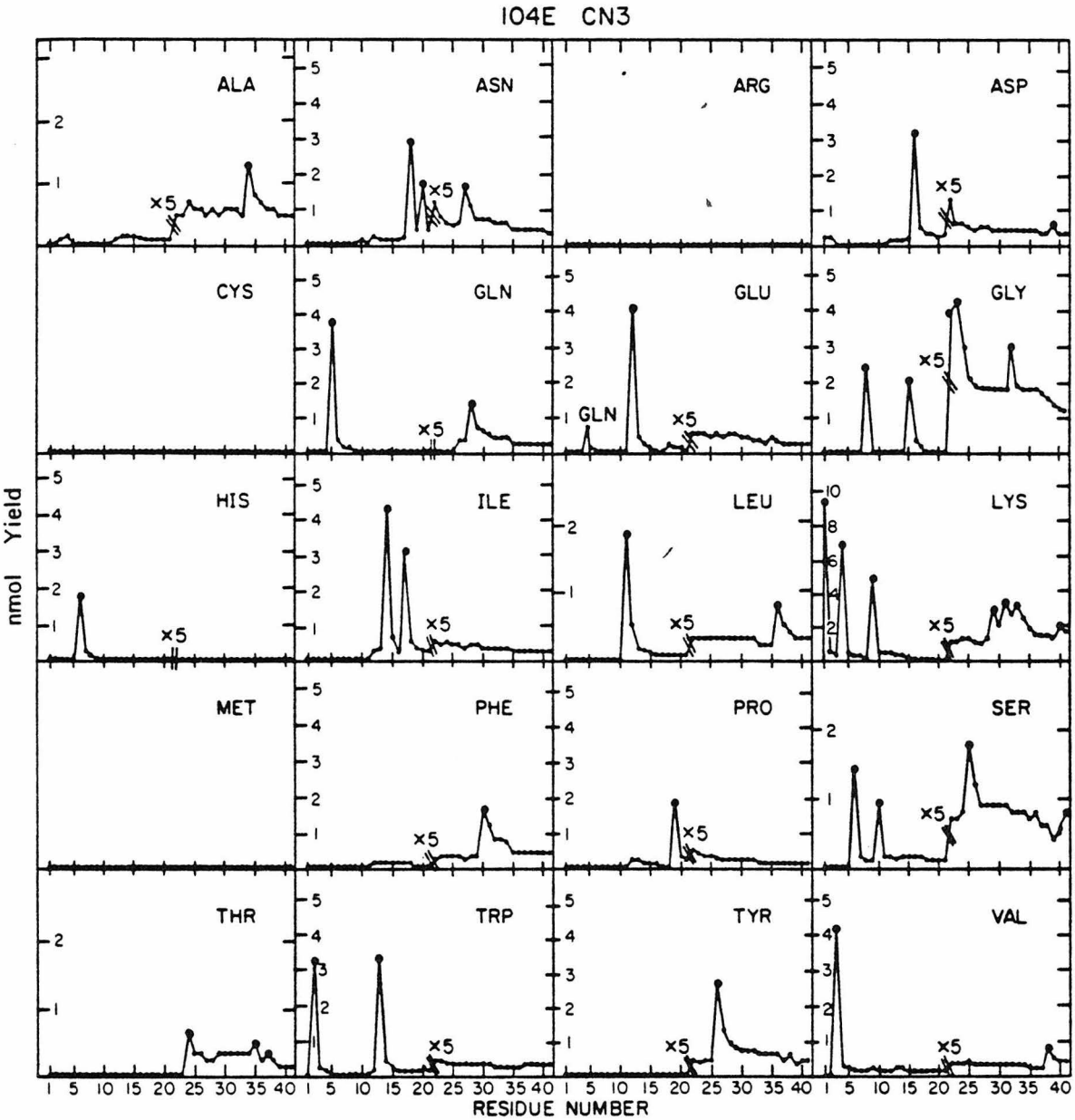
104E CN3
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
GLN
×5
nmol Yield
RESIDUE NUMBER
1 10 20
Lys-Trp-Val-Lys-Gln-Ser-His-Gly-Lys-Ser-Leu-Glu-Trp-Ile-Gly-Asp-Ile-Asn-Pro-Asn-
21 30 40
Asn-Gly-Gly-Thr-Ser-Tyr-Asn-Gln-Lys-Phe-Lys-Gly-Lys-Ala-Thr-Leu-Thr-Val-Asp-Lys-Ser

FIGURE 3.

CHAPTER 3

CHARACTERIZATION OF MULTIPLE IMMUNOGLOBULIN MU CHAINS SYNTHESIZED BY TWO CLONES OF A B-CELL LYMPHOMA

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ABSTRACT

We have identified three species of μ chain synthesized by mouse WEHI 279 lymphoma cells that differ in cellular location, size and charge - μ_i (internal), μ_m (membrane) and μ_s (secreted). Lactoperoxidase and/galactose oxidase labeling experiments localize the μ_m chain to the plasma membrane and the μ_i chain to the cytoplasm. Pulse-chase experiments demonstrate that the μ_i pool contains precursors to both μ_m and μ_s chains. Comparative peptide mapping studies and cell labeling in the presence of tunicamycin suggest the μ_m chain is 2000 daltons larger than the μ_s chains with the difference due to covalent polypeptide alterations. The WEHI 279 lymphoma has differentiated while in culture to a more advanced stage in the pathway of B-cell differentiation.

INTRODUCTION

B cells pass through an orderly series of differentiation stages. The stem cell differentiates into a pre-B cell which expresses cytoplasmic μ chains, but not light (L) chains (1, 2). The pre-B cell then undergoes additional changes and becomes a B lymphocyte which synthesizes light chains as well as μ chains. This nondividing B lymphocyte synthesizes monomeric IgM molecules (μ_2L_2) that are placed on the plasma membrane to act as antigen receptors (3). Upon stimulation with antigen, subsequent differentiation steps occur, and the B cell acquires the ability to secrete pentameric IgM molecules held together by disulfide bridges between monomers and the J or joining chain (4) [$(\mu_2L_2)_5J$]. The terminal stage of B lymphocyte differentiation is the plasma cell which secretes large quantities of the IgM pentamer. Fortunately, various tumor cell lines synthesizing homogeneous immunoglobulin molecules appear to correspond to these distinct stages of differentiation (5, 6). For example, the B-cell lymphoma and the plasmacytoma or myeloma tumor correspond to the B lymphocyte and the plasma cell, respectively.

The plasma cell membrane and the blood constitute very different chemical environments for IgM molecules and, accordingly, the membrane-bound and secreted IgM molecules, although similar, are apparently not identical proteins (7, 8, 9, 10, 11, 12, 13). The IgM molecule is anchored in the B-cell plasma membrane via the C-terminus of the μ chain (14, 15). Therefore, specific structural adaptations in the membrane IgM (mIgM) molecule which facilitate its interaction with the lipid bilayer should occur in this region. We have initiated an analysis of secreted (μ_s) and membrane-bound (μ_m) mu chains by comparing the IgM molecules synthesized by an IgM-secreting myeloma tumor with the membrane-bound molecules synthesized by a B-cell lymphoma. We have determined the complete amino acid sequence of the μ_s chain secreted by the BALB/c mouse plasmacytoma,

MOPC 104E (M104E) (16). We have compared these μ_s chains with the μ_m chains derived from the B-cell lymphoma WEHI 279 (W279). The W279 lymphoma was isolated and initially characterized by Dr. Noel Warner and colleagues (17). It originated in the NZC inbred mouse strain derived from a (BALB/c x NZB)F_1 mouse. The W279 IgM molecules bear the allotype of the BALB/c parent. The W279 cells originally had Fc receptors, high levels of mIgM molecules and secreted negligible amounts of IgM molecules. This suggests that they represented lymphocytes transformed at an early stage of B-cell development. A more recent clone (W279.1/12) of the original W279 cell line exhibits increased secretion of pentameric IgM molecules and decreased levels of mIgM molecules, representing a clone of lymphocytes transformed at a more advanced stage of B-cell development. Hybrid cells created by the fusion of W279 lymphoma cells and MPC11 myeloma cells secrete large quantities of W279 μ_s chains assembled in the pentameric IgM form.

In this paper we demonstrate three discrete pools of μ chains are produced by the W279.1/12 B-cell lymphoma, μ_i (internal), μ_s (secreted) and μ_m (membrane). These molecules are distinguished by their cellular location, charge, structure and size. The W279 cell line has differentiated while in culture to a more advanced IgM-secreting stage of B-cell differentiation.

MATERIALS AND METHODS

Cells. The WEHI 279 (W279) uncloned cell line was a gift from Dr. Noel Warner. The WEHI 279.1/12 clone, sent to us by Dr. Vernon Oi, was isolated by Dr. Warner's laboratory as a later clone of the W279 tumor cells with greater proliferative potential than the original WEHI 279 cell line. This clone will be denoted W279.1 hereafter. W279.1 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with nonessential amino acids (GIBCO) containing 20% heat inactivated fetal calf serum, and 5×10^{-5} M β-mercaptoethanol in

a humidified atmosphere of 90% air, 10% CO_2. W279 cells were grown in the above medium containing only 10% fetal calf serum. The MPC11xW279 (MxW) hybridoma was generated by a fusion between the MPC11 myeloma and WEHI 279 lymphoma cells (18). Hybridoma cells were grown in DMEM supplemented with 10% fetal calf serum.

Labeling procedures. Cells were labeled for the indicated periods with ^{35}S-methionine (Amersham, 900-1200 Ci/mmole), ^{35}S-cysteine (New England Nuclear, 700 Ci/mmole) or ^{3}H-tyrosine and leucine (New England Nuclear, 40 mCi/mmole and 60 mCi/mmole, respectively) in DMEM devoid of methionine or tyrosine and leucine at cell densities between 2 x 10^6/ml and 1 x 10^7/ml. Intact cells and cell lysates were labeled with Na^{125}I and lactoperoxidase according to Marchalonis, Cone and Santer (19) or with NaB$^{3}H_4$ and galactose oxidase by the method of Gahmberg and Hakomori (20). Cell lysates were prepared by washing cells once in cold medium and then suspending the cells in ice cold lysing buffer (0.5% Triton X-100, 0.14 M NaCl, 0.01 M Tris-HCl, pH 7.4, 2 mM phenylmethyl sulfonylfluoride [PMSF]) at a concentration of 4 x 10^7 cells/ml for 20 min on ice. Supernatant fractions were prepared by centrifuging the cell lysates for 20 min at 1500 x g or 5 min at 12,000 x g to remove nuclei.

When cells were labeled in the presence of tunicamycin (a gift from Dr. Robert Hamill, Eli Lilly Co.), the cells were first preincubated in 5 μg/ml, 1.5 μg/ml or 0.5 μg/ml tunicamycin for 60 min at 37°C. Cell labeling was then performed in the presence of 5 μg/ml, 1.5 μg/ml or 0.5 μg/ml of tunicamycin as described above.

Immunoprecipitation. Cell lysates prepared from 2 x 10^6 cells were pre-incubated with 50 μl of a washed 10% suspension of formalin-fixed, heat-killed Staphylococcus aureus, Cowan I strain (SACI) (21) for 20 min on ice in precipitating buffer (0.5% Triton X-100, 0.5% sodium dodecyl sulfate [SDS], 0.14 M

NaCl, 0.01 M Tris-HCl, pH 7.4, 2 mM PMSF). The SACI was removed by centrifugation at 1500 x g for 10 min, and the cleared lysate reacted with 10 µl of specific rabbit anti mouse µ (M104E µ) antiserum for 1 hr on ice. Fifty µl of the washed SACI suspension were added and the mixture incubated for an additional 20 min on ice. Precipitates were washed three times with washing buffer (0.5% Triton X-100, 0.1% SDS, 0.14 M NaCl, 0.01 M Tris-HCl, pH 7.4) and the precipitated molecules released by heating the SACI in boiling water for 5 min in 50 to 100 µl of the loading buffer appropriate to the gel analysis.

Gel analyses. Analytical composite agarose-polyacrylamide gels were poured and run according to a modification of Dingman and Peacock (22). Gels were composed of 0.5 M urea, 0.2 M sodium phosphate, pH 7.2, 0.1% SDS, 1% agarose, 2.5% acrylamide, and 1.2% bisacrylamide. The loading buffer contained 2% SDS and 0.05 M sodium phosphate at pH 6.8. Preparative 10% polyacrylamide-SDS gels were prepared according to Laemmli (23) with a two centimeter stacking gel. The loading buffer contained 4% SDS, 0.05 M Tris-HCl, pH 6.8, 15% glycerol and 4% β-mercaptoethanol. Analytical two-dimensional gel analysis was performed according to O'Farrell (24). The loading buffer contained 1.5% pH 3.5-10 ampholines (LKB), 0.5% pH 4-6 ampholines (LKB), 9 M urea, 0.1% SDS, 2% Triton X-100 and 5% β-mercaptoethanol. Gels were processed for fluorography by the method of Bonner and Laskey (25).

Peptide mapping. W279.1 μ_m chains were preparatively isolated by running immunoprecipitates of IgM molecules on 10% polyacrylamide-SDS gels. The µ chains were eluted from the appropriate 1 mm gel slices by incubation with 0.5 ml of 0.5% SDS at room temperature for 24 hr. W279.1 and MxW μ_s chains were isolated from culture medium by the same procedure. Carrier pig IgG (0.25 mg) was added to each eluted and pooled μ_m or μ_s sample and the chains were reduced and alkylated as follows. The pooled µ chains were lyophilized, redissolved in

9% SDS, 0.5 M Tris-HCl, pH 8.5, 0.01 M EDTA, boiled for 5 min, flushed with argon for 5 min, and dithiothreitol was added to a concentration of 20 mM. The samples were then boiled an additional 2 min, sealed under argon and incubated at 37°C for 1-1/2 hr. Alkylation with 50 mM iodoacetamide (3X recrystallized) was performed for 1 hr at room temperature in the dark. The reagents were removed by desalting on Sephadex G-25 (Pharmacia) equilibrated with 0.5% SDS and 0.05 M Tris-HCl, pH 7.5, and the proteins were precipitated with 25% trichloroacetic acid, washed twice with cold 20% trichloroacetic acid, twice with ethanol:ether (1:2) and once with ether. The precipitate was air-dried and dissolved in 200 µl 0.2 M ammonium bicarbonate, digested with TPCK-trypsin (Worthington) at room temperature (total of 150 µg for 22 hr), followed by digestion with chymotrypsin (Worthington) at 37°C (total of 200 µg for 28 hr). The samples were quick frozen and lyophilized to terminate the reaction, dissolved in 45 µl of 0.5 M phosphate buffer (pH 1.8):acetone (2:1) and the peptides separated by high performance liquid chromatography as described (26).

RESULTS

W279.1 cells synthesize three forms of µ chains - μ_i, μ_m and μ_s. The W279.1 cells synthesize three forms of µ chains which we have designated as μ_i (internal µ), μ_m (membrane µ) and μ_s (secreted µ). These µ chains differ in their cellular location, size, charge and level of glycosylation.

W279.1 cells were radiolabeled by growth in ^{35}S-cysteine for 16 hr. The cells were solubilized in non-ionic detergent and the lysate was specifically immunoprecipitated with a rabbit antibody directed against mouse µ chains. When these precipitates are analyzed without reduction on composite agarose-polyacrylamide gels (Fig. 1A), the largest species are about 200,000 daltons, the characteristic size of cell-surface monomeric (μ_2L_2) IgM molecules. The other major species

is about 100,000 daltons, the size appropriate for assembled (μL) half molecules. A similar analysis of IgM molecules from the cell medium (Fig. 1B) reveals that about half are assembled in the pentameric form. Thus, W279.1 cells clearly synthesize both membrane and secreted IgM molecules.

In order to further characterize the μ chains, W279.1 cells were labeled with ^{3}H-tyrosine and leucine for 8 hr. IgM molecules from the cell lysate were immunoprecipitated, reduced, separated by two-dimensional gel electrophoresis and analyzed by fluorography. Two major species of μ chains are evident (Fig. 2). The two species have an apparent molecular weight difference of about 4,000. They also differ in isoelectric point; the smaller species encompasses a range of isoelectric points from pH 6.5 to pH 5.6 and the larger a range from pH 5.8 to pH 5.2. A similar two-dimensional gel of the W279.1 secreted IgM molecules revealed that the μ_s chains are similar in pI to their μ_m counterparts but are smaller by about 3,000 daltons (data not shown). All three μ chains exhibited a cyanogen bromide cleavage pattern characteristic of C_μ region peptides derived from μ_s chains synthesized by the myeloma tumor M104E (27). Thus, W279.1 cells contain three distinct types of μ chains which differ in molecular weight, charge and location. As is shown below, these correspond to the previously defined μ_i, μ_m and μ_s chains.

The larger μ chain is on the plasma membrane. The location of the two species of μ chains contained in the cell lysate was determined using two methods of radiolabeling. Intact cells were treated with neuraminidase and galactose oxidase followed by labeling with NaB^3H_4 (20). The labeled cells were solubilized in non-ionic detergent, and the IgM molecules were immunoprecipitated, reduced and analyzed on two-dimensional gels. Only the larger, more acidic μ chains were labeled under these conditions, showing that this species of μ chain is on the plasma membrane and contains galactose residues accessible to labeling with extrinsic

reagents (Fig. 3A). The unglycosylated light chain was not labeled and serves as an internal control (data not shown). The labeled cell-surface IgM was also analyzed without reduction on 5% polyacrylamide-SDS gels. All of the IgM molecules labeled under these conditions possess a molecular weight of 200,000 (Fig. 4), indicating that these μ chains are present on the plasma membrane as monomeric (μ_2L_2) IgM molecules. Radioiodination of intact cells with lactoperoxidase and $Na^{125}I$ (19) also labeled only the larger and more acidic species of μ chain, confirming their location on the cell surface (Fig. 3B). In contrast, radioiodination of a cell lysate, which destroys the protective barrier of the cell membrane, labels both μ species equally (data not shown). These observations suggest that the larger μ species is the membrane-bound μ chain (μ_m) and that the smaller species is located within the W279.1 cells and represents an internal pool of μ chain (μ_i).

The W279.1 secreted IgM molecules contain μ_s chains. To identify the type of μ chains in IgM molecules from the culture fluid of W279.1 cells, we compared the tyrosine-labeled tryptic and chymotryptic peptides of W279.1 μ chains from the medium with μ_s chains secreted by the MxW hybridoma cells. The cell fusion has induced a high level of pentameric IgM secretion by these hybridoma cells (18) and the IgM pentamers are composed of W279 μ_s chains (27). W279.1 and MxW cells were labeled with ^{3}H-tyrosine for 16 hr, the μ_s chains isolated from the culture medium of both cells and from the lysate of W279.1 cells, and cleaved with trypsin and chymotrypsin as described in Materials and Methods. The resulting peptides were separated by high performance liquid chromatography (26). As shown in Fig. 5A, the tyrosine peptides of the two secreted μ chains are identical. The IgM molecules found in the culture medium from W279.1 lymphoma cells are therefore composed of μ_s chains. In contrast, μ_m chains contain several distinct tyrosine peptides not found in μ_s chains (Fig. 5B). The μ_m chains therefore differ in structure from μ_s chains. These differences could reflect carbohydrate

differences, protein differences, or both. The important point is that the μ_s and μ_m chains can be distinguished from one another structurally.

The μ_i species contains precursors of μ_m and μ_s chains. In order to determine the relationship of the μ_i chains to the μ_m and μ_s chains, we pulse-labeled W279.1 cells with ^{35}S-methionine for 15 minutes, chased the label by washing and resuspending the cells in unlabeled medium containing a 10-fold excess of unlabeled methionine and examined the distribution of radiolabel in the μ_i and μ_m pools for 4 hr after the pulse. Figures 6A-6F display the portion of each two-dimensional gel which contains the μ chains, aligning the gels on a small invariant spot (indicated with an arrow). Figure 6A shows that during the 15 min pulse, only the μ_i pool is labeled. Moreover, these gels show that there are two sizes of μ chains within the μ_i pool. The two species of μ_i chains are equally labeled in 15 min (Fig. 6A). Over the 4 hr chase period, the smaller species progressively disappears (Figs. 6B-6F). Some of the label in the μ_i pool is chased into μ_m chains (Fig. 6B-6F). By this method, it is not possible to determine directly the fate of the two species of the μ_i pool. However, it is clear that the μ_i pool must contain precursors to the μ_m chain.

During a short pulse and chase, only small amounts of IgM molecules are found in the medium. However, when the chase period is extended, labeled secreted IgM molecules are easily isolated. When cells are incubated for 1 hr in ^{35}S-methionine, mainly the μ_i pool is labeled, but the two sizes within the pool are difficult to discern (Fig. 7A). As before, a portion of the label in the μ_i pool is chased into the μ_m chains and remains as μ_m chains over long periods (Fig. 7A-7E). At the same time, label from the μ_i pool chases into μ_s chains in the medium (Fig. 7F), demonstrating clearly that precursors to both μ_m and μ_s chains are present in the intracellular pool.

Tunicamycin studies demonstrate that two species of μ polypeptide chains are synthesized by W279.1 cells. In order to further characterize the μ polypeptide chains from W279.1 cells, proteins were labeled by incubation of the cells in ^{35}S-methionine in the presence and absence of tunicamycin, an antibiotic which inhibits glycosylation of asparagine residues (28, 29). When IgM molecules were immunoprecipitated from lysates of tunicamycin-treated cells and analyzed on 10% polyacrylamide-SDS gels, two bands in the size appropriate for unglycosylated μ chains, 67,000 daltons and 69,000 daltons, were observed (Fig. 8, lane C). The precipitates shown are from cells incubated with 5 μg/ml of tunicamycin. Identical results were obtained with either 1.5 μg/ml or 0.5 μg/ml of tunicamycin, indicating that the two bands are not an artifact of tunicamycin toxicity to cells.

Since W279.1 cells synthesize both membrane and secreted IgM molecules, this observation suggests that the two size classes represent the two species of μ chain, μ_s and μ_m. This interpretation is further supported by analysis of the mRNA isolated from W279.1 cells and other lymphoma cells (30, 31). Cytoplasmic polyA+ mRNA was isolated, sized on agarose gels and hybridized to a cloned μ cDNA. Two classes of μ chain mRNA were found which differed in size (2.7 and 2.4 kilobases) and appeared to encode the μ_m and μ_s chains, respectively (30). When W279.1 cells were labeled for short periods in the absence of tunicamycin, two size classes of μ chains could be identified (Fig. 8, lane D). These two bands correspond to the smaller, more basic μ chains seen on two-dimensional gels (Figs. 2 and 6A).

When W279.1 cells are labeled in the presence of tunicamycin, IgM molecules can also be isolated from the medium. These unglycosylated μ chains were isolated and their size analyzed. The smaller of the two bands was present in proportionally greater amounts than the larger one (Fig. 8, lane B). Since the pentameric secreted form of IgM predominates in medium from these cells, it

appears likely that the smaller form is the μ chain corresponding to secreted IgM molecules. This idea is strengthened by the fact that the μ chains from tunicamycin-treated M104E cells correspond in size to the smaller of the two bands in W279.1 cells (Fig. 8, lane A). Taken together, these results suggest that the larger band represents unglycosylated μ_m chains and the smaller band represents unglycosylated μ_s chains.

W279 cells have differentiated while in culture and thus exhibit different stages in the B-cell differentiation pathway. We initially began characterizing the original uncloned W279 cell line. Because these W279 cells exhibited poor growth characteristics with a relatively low degree of overall cell viability, we obtained a later subclone, W279.1, which exhibited greater proliferative potential. The original W279 cells were initially characterized with respect to the size of the IgM molecules the cells synthesized. W279 cells were radiolabeled by growth in ^{3}H-tyrosine and leucine for 16 hr and the IgM molecules precipitated from cell lysates and culture medium. These precipitates were analyzed without reduction on composite agarose-polyacrylamide gels (Fig. 9). The two major species of IgM molecules isolated from W279 cell lysates were similar in size and quantity to those isolated from W279.1 cells (compare Figs. 9A and 1A). Equivalent quantities of cell lysates and medium (in cell numbers) were analyzed in Figs. 1 and 9. In comparison to IgM molecules isolated from cell lysates, IgM molecules in the medium from W279 cells were present in negligible amounts (compare Figs. 9B and 1B). At least 15-fold less IgM was secreted into the medium by W279 than by W279.1 cells. Thus, the greater level of secreted pentameric IgM molecules in the W279.1 clone suggests that this clone has differentiated in culture to become a more mature B cell than the parental W279 cells.

DISCUSSION

W279.1 cells synthesize three species of μ chains, μ_i, μ_m and μ_s, which differ in their cellular locations. The galactose oxidase and lactoperoxidase labeling experiments on intact W279.1 cells suggest that the μ_m chains are integral membrane proteins inserted into the B-cell plasma membrane and assembled as monomeric (μ_2L_2) IgM molecules of 200,000 daltons. The μ_m chains appear to be fully glycosylated by virtue of the fact they have terminal galactose residues that can be labeled with galactose oxidase and NaB^3H_4.

The W279.1 cells secrete IgM molecules into the medium, and 50% of these are assembled as secreted IgM pentamers. The μ_s chains from these secreted molecules can readily be distinguished from μ_m chains by comparative peptide map analysis.

The W279.1 cells have an intracellular pool of μ chains (μ_i) whose complex carbohydrate moieties lack their terminal galactose residues. Furthermore, the μ_i chains have a more basic pI than their μ_m and μ_s counterparts which probably reflects the lack of charged sialic acid residues on the μ_i chains. Thus, the μ_i chains appear to be lacking the terminal sugars of their complex carbohydrates. Pulse-chase studies with ^{35}S-labeled methionine suggest that the μ_i pool contains precursors to both the μ_m and μ_s chains. Following extended labeling, the majority of the μ_i chains appear to be precursors to μ_s chains in that structural comparisons of peptides suggest that the predominant species of μ_i chains are very similar to the μ_s chains (27).

The μ chains are glycosylated in two stages. The steps required for asparagine linked glycosylation of complex carbohydrates in proteins have been carefully documented (32, 33, 34, 35). The process occurs in two distinct phases. A mannose-rich carbohydrate core is transferred en bloc from dolicholphosphate

to the asparagine residue in a recognition sequence Asn-X-Ser or Thr. This mannose-rich core is partly degraded and then reformed by the addition of neutral sugars (N-acetyl glucosamine, galactose and fucose) and charged sialic acid. The net effect of the second stage of glycosylation is to increase the apparent molecular weight of the glycoprotein in the presence of SDS and, because of the sialic acid, to decrease its pI value. Our analysis of W279.1 μ chain synthesis and processing suggests that the complex carbohydrates in μ chains are glycosylated according to this scheme. First, the μ_i pool is labeled when W279.1 cells are incubated in ^{3}H-mannose (27). Because μ chains have only asparagine-linked carbohydrate moieties (16), this observation suggests that a mannose-rich core has been attached to the μ_i chains. Second, when a lysate of W279 cells was labeled with NaB^3H_4 and galactose oxidase after treatment with neuraminidase, μ_m chains but not the μ_i chains were labeled (data not shown), indicating that the complex carbohydrates attached to μ_i chains contain no galactose residues. This observation and the pI difference between μ_i and μ_s chains suggests that the terminal sugar moieties have not yet been added to the mannose-rich cores in μ_i chains. Thus, μ_i chains have undergone the first step but not the second step of complex carbohydrate synthesis. Moreover, μ chains synthesized in the presence of tunicamycin, a specific inhibitor of the synthesis of oligosaccharide-dolichol-phosphate (28, 29), incorporate less than 10% of the mannose found in control μ chains (C. Sibley, in preparation), have the same range of isoelectric points as μ_i chains (data not shown) but are lower in apparent molecular weight by about 6000 (Fig. 8). This is exactly what one would predict if the μ_i chains lacked the terminal sugar residues of mature complex carbohydrate moieties but contained the mannose core sugars.

Taken together with the pulse-chase and labeling experiments, these results are consistent with the idea that μ chains are glycosylated during synthesis by addition of core carbohydrate and that the progression from the μ_i pool to

μ_m and μ_s chains represents the addition of the terminal neutral and charged sugars on the complex carbohydrate structures. If this is correct, the fate of the two size classes from the μ_i pool should differ. Both forms should become larger in apparent molecular weight and more acidic as glycosylation proceeds from the first to the second stage. However, the μ_m precursor should remain cell-associated even after it is fully glycosylated. By analogy with other immunoglobulins, the secreted form should be externalized very rapidly after the second stage of glycosylation is completed (36). Our biochemical analyses of the larger, acidic form isolated from cells suggest that it does consist principally of the membrane-bound μ chain (27). This reasoning explains why little of the secreted form remains in the cell after its glycosylation is completed. Therefore, the largest μ chain associated with W279.1 cells after a long labeling period represents μ_m chains.

The μ_m chain is 2000 daltons larger than the μ_s chain. Two lines of evidence suggest that this molecular weight difference reflects polypeptide sequence differences and not differing levels of glycosylation. First, tunicamycin blocks the addition of asparagine-linked carbohydrate moieties to polypeptide chains. Mu chains from W279.1 cells synthesized in the presence of tunicamycin consist of two size classes that differ from one another by 2000 daltons. Second, ^{3}H-tyrosine-labeled μ_s and μ_m chains compared by peptide mapping after digestion with trypsin and chymotrypsin reveal μ_s and μ_m chains have several different tyrosine peptides. These data suggest the μ_m and μ_s chains differ in their primary amino acid sequence. A final point is that W279.1 cells synthesize two size classes of μ chain mRNA, 2.7 kilobases and 2.4 kilobases, which apparently synthesize μ_m and μ_s chains, respectively (30). These two types of μ chain mRNAs are identical except for the portion of the mRNAs that encodes the C-terminal segment of the μ chain. These coding differences are consistent with a μ_m chain approximately 2000 daltons larger than the μ_s chain.

The W279 and W279.1 cells represent lymphocytes at different stages of B-cell differentiation. The original W279 cells clearly secrete little, if any, IgM molecules. In contrast, the W279.1 clone appears to secrete significant amounts of pentameric IgM. Dr. Warner and his colleagues have demonstrated that increased section of pentameric IgM molecules correlates with the more advanced stages of B-cell differentiation (6) (Fig. 10). This observation suggests that the W279.1 clone is composed of a more mature B cell population than the original uncloned W279 cells. This supposition is further confirmed by the observation that in comparison to the original W279 cell line, W279.1 cells possess increased concentrations of Ia antigens and PCA (plasma cell antigen) and decreased concentrations of membrane IgM (M. Daley and N. Warner, personal communication). These features also are characteristic of more mature B cells (6).

A question which then arises is whether W279.1-like cells are present in the uncloned W279 population or has the differentiation of W279 cells occurred over a period of two years in culture? The W279 cells were originally isolated, characterized and later cloned in Dr. Warner's laboratory. A comparison of the original W279 cells with the later W279.1 cells, which were cloned and grown under different culture conditions, reveals that the W279.1 cells secrete significantly more pentameric IgM molecules and contain fewer membrane IgM molecules than the originally isolated W279 cells. Commensurate with their ability to secrete significantly increased amounts of pentameric IgM molecules, W279.1 cells also have 100-fold greater levels of J chain than their W279 counterparts (E. Mather and M. Koshland, personal communication). Accordingly, these two isolates of W279 cells appear to have differentiated with respect to one another. We conclude that the differences in culture conditions have caused the W279.1 cells to differentiate. Similar observations have been made for myeloid tumors (37-39).

These observations suggest that we have analyzed W279 cells at three distinct stages of differentiation from a B lymphocyte to a mature plasma cell (Fig. 10). A second B-cell lymphoma, WEHI 231, appears to have all of the characteristics of original W279 cells, namely both represent an early stage of B-cell differentiation. The W279.1 cells have differentiated to represent a later intermediate stage, and the myeloma M104E or MxW cells represent the terminal stage of B-cell differentiation. Thus the differentiation process may be induced in a single B-cell lymphoma. Perhaps these stages of B-cell tumors will be useful in delineating the regulatory mechanisms that mediate the shift in synthesis of μ_m to μ_s chains.

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Figure 1. Composite agarose-polyacrylamide gel electrophoresis of IgM molecules synthesized by W279.1 cells. Cells were radioactively labeled by incubation in ^{35}S-cysteine. IgM was isolated without reduction from medium and lysates. Its size was assessed on composite agarose-polyacrylamide gels as described in the Materials and Methods. Arrows indicate the migration of molecular weight standards run in parallel gels: 104E, MOPC 104E pentameric IgM of 1×10^6 daltons; IgG, pig γ globulin of 150,000 daltons; BSA, bovine serum albumin of 65,000 daltons. A. Cell lysate from W279.1 cells. The molecular weights of the two predominant species are 200,000 and 100,000, corresponding to (μ_2L_2) IgM monomers and (μL) half monomers, respectively. B. Medium from W279.1 cells.

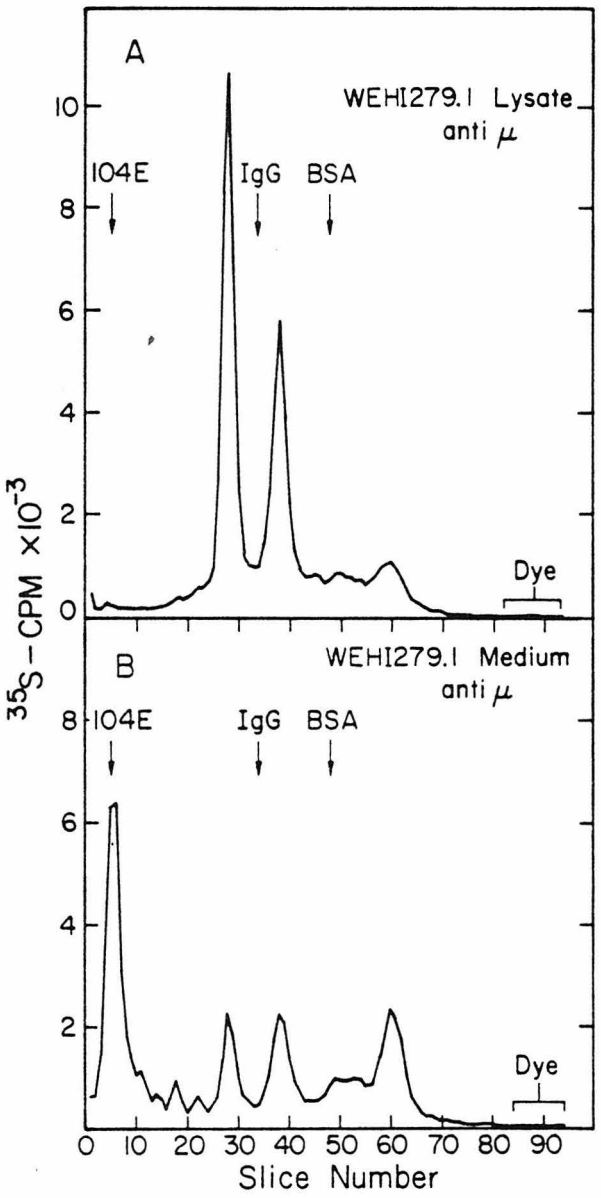
A
WEHI279.1 Lysate
anti μ
104E
IgG
BSA
Dye
B
WEHI279.1 Medium
anti μ
104E
IgG
BSA
Dye
35S-CPM ×10-3
Slice Number

FIGURE 1.

Figure 2. Two-dimensional gel analysis of μ chains from a W279.1 cell lysate. W279.1 cells were radiolabeled with ^{3}H-tyrosine and leucine and analyzed on two-dimensional gels as described in Materials and Methods. Light indicates light chain. The 50,000 dalton molecules can be eliminated by washing the immunoprecipitates with a buffer containing 1% deoxycholate, 1% Triton X-100 and 0.15% SDS.

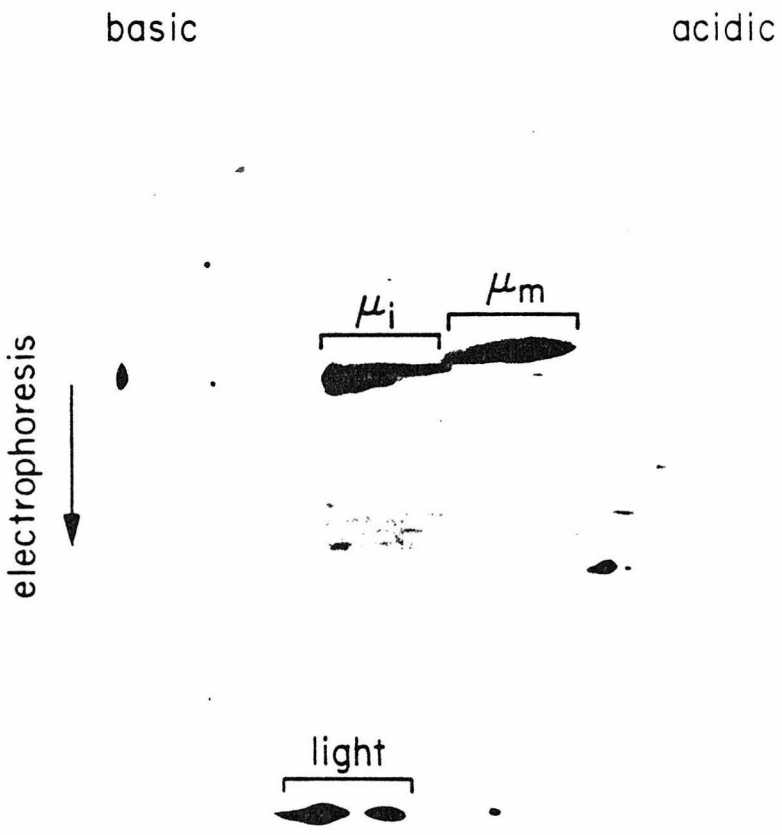

FIGURE 2.

Figure 3. Two-dimensional gels of extrinsically labeled W279.1 cells.

A. Intact W279.1 cells were labeled with NaB^3H_4 and galactose oxidase (20), cell lysates prepared, immunoprecipitated and analyzed on two-dimensional gels.

B. Intact W279.1 cells were labeled with $Na^{125}I$ and lactoperoxidase, cell lysates prepared, immunoprecipitated and analyzed by two-dimensional gel electrophoresis as described in Materials and Methods. Only the portion of the gel containing μ chains is shown in each case.

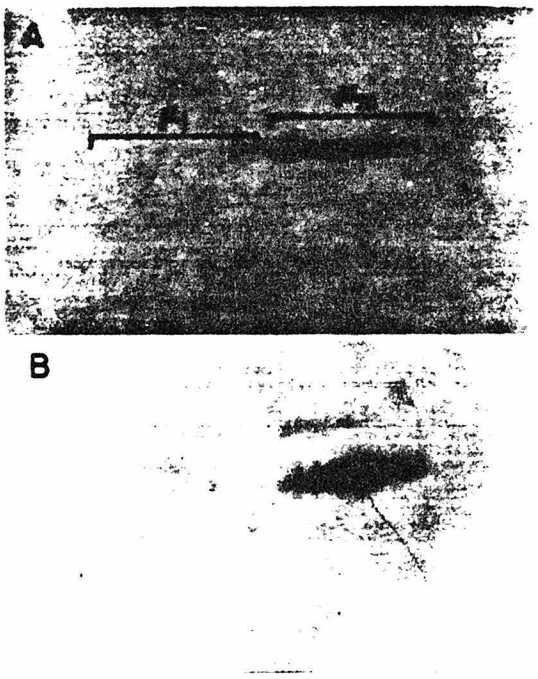

FIGURE 3.

Figure 4. SDS-polyacrylamide gel analysis of membrane IgM molecules. Intact W279.1 cells were labeled with NaB^3H_4 and galactose oxidase (20). IgM molecules were isolated from cell lysates without reduction and the size assessed on 5% polyacrylamide gels as described in Materials and Methods. 7S IgM marks the position of the 200,000 dalton monomeric IgM molecule.

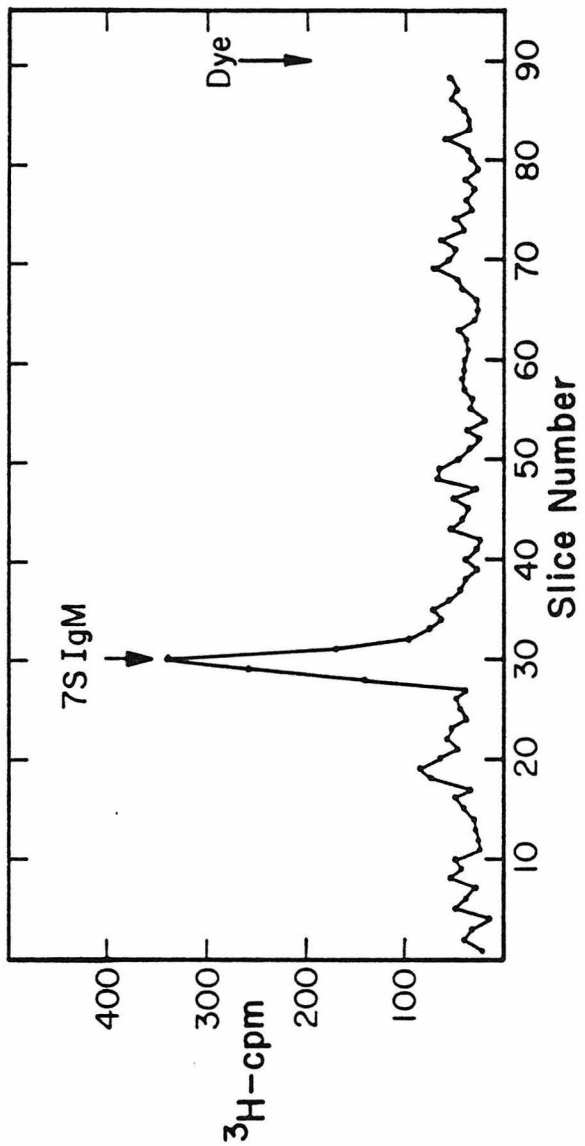
7S IgM
Dye
400
300
200
100
^{3}H-cpm
10
20
30
40
50
60
70
80
90
Slice Number

FIGURE 4.

Figure 5. Comparison of tyrosine peptides from W279.1 μ_s, μ_m and MxW μ_s chains. Mu chains were isolated from the culture medium of W279.1 and MxW cells and from the lysates of W279.1 cells incubated for 16 hr in the presence of ^{3}H-tyrosine. Reduction, alkylation, and digestion with trypsin and chymotrypsin were performed as described in Materials and Methods. Peptides were separated on a DuPont ODS C-18 high performance liquid chromatography column (26) and 0.5 min (0.5 ml) fractions were collected, dried and counted for radioactivity. Peptide differences are crosshatched. (.....) gradient of acetone used for elution of the peptides. A. (——) MxW μ_s chains; (----) W279.1 μ_s chains. B. (——) MxW μ_s chains; (----) W279.1 μ_m chains.

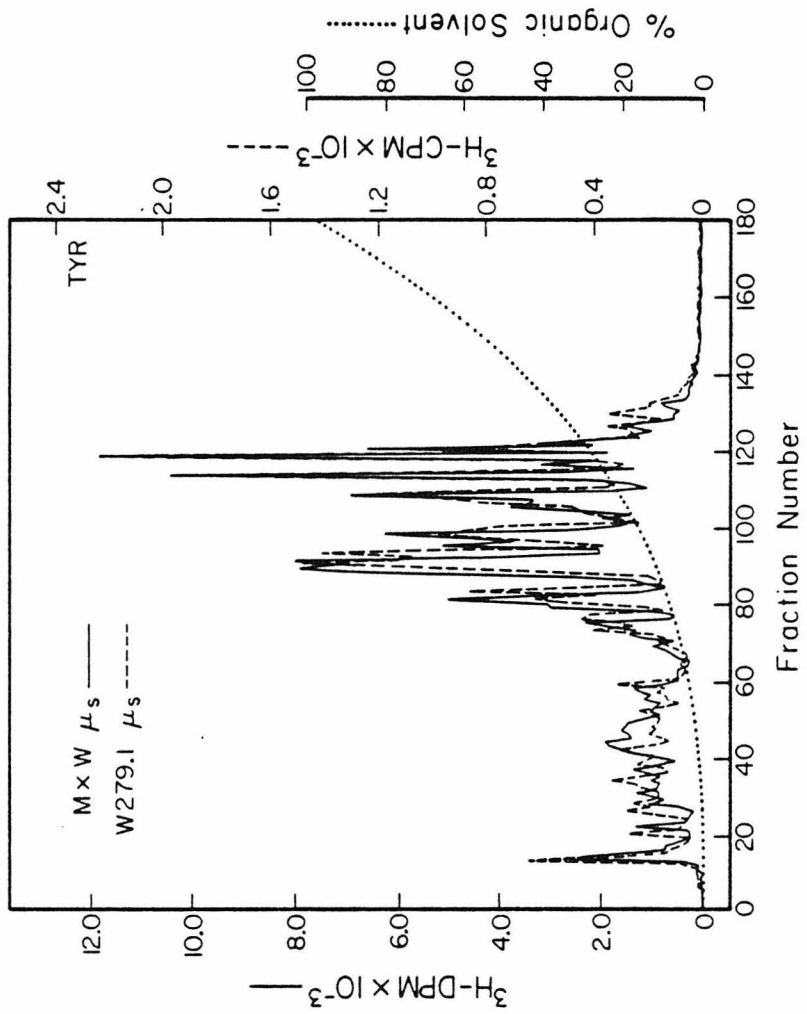
TYR
M x W μ_s ——
W279.1 μ_s - - - -
^{3}H-DPM x 10^{-3} ——
^{3}H-CPM x 10^{-3} - - - -
% Organic Solvent .....
Fraction Number

FIGURE 5A.

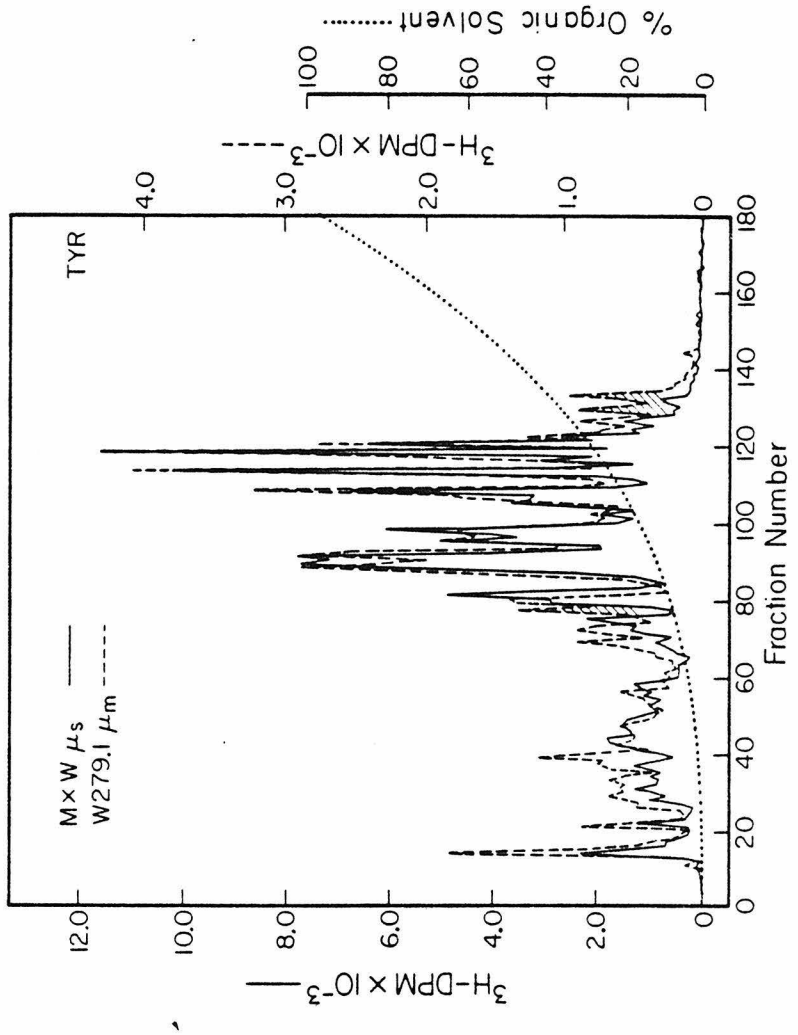
TYR
M×W μ_s ——
W279.1 μ_m - - - -
^{3}H-DPM × 10^{-3} ——
^{3}H-DPM × 10^{-3} - - - -
% Organic Solvent .....
Fraction Number
0
2.0
4.0
6.0
8.0
10.0
12.0
1.0
2.0
3.0
4.0
20
40
60
80
100
120
140
160
180

FIGURE 5B.

Figure 6. Two-dimensional gel analysis of a short pulse chase of the μ_i pool into μ_m in W279.1 cells. W279.1 cells were labeled for 15 min with ^{35}S-methionine and chased for 4 hr by resuspending them in growth medium without radiolabel. Samples were taken at the indicated times for lysis and immunoprecipitation analysis of radioactive μ chains remaining in the cells. A. At the end of the labeling period, t = 0. B. After 30 min in growth medium. C. After 45 min. D. After 1 hr. E. After 2 hr. F. After 4 hr. The μ_i and μ_m chains are designated on gel A. Gels were aligned with reference to the invariant spot designated with Δ. Only that region of the gel containing μ chains is shown.

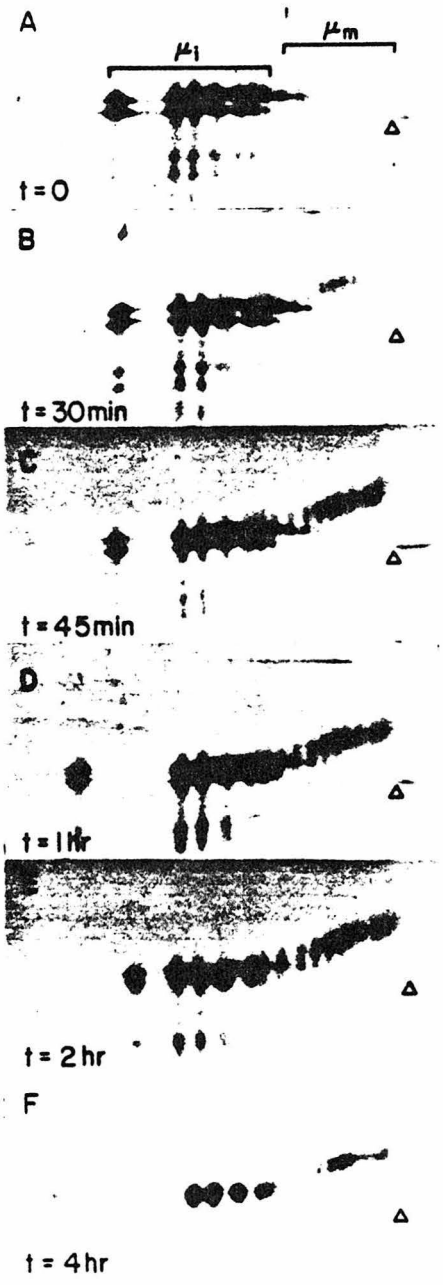

FIGURE 6.

Figure 7. Two-dimensional gel analysis of a long pulse chase of μ_i into μ_m and μ_s chains in W279.1 cells. W279.1 cells were labeled for 1 hr with ^{35}S-methionine and chased for 20 hr by resuspending them in growth medium without radiolabel. Samples were taken for lysis and immunoprecipitation analysis of radioactive μ chains remaining in the cells or in the medium. A. Cell lysate at the end of labeling period, t = 0. B. Cell lysate after 4 hr. C. Cell lysate after 6 hr. D. Cell lysate after 8 hr. E. Cell lysate after 20 hr. F. Radioactive μ chains found in the medium after a 20 hr chase. The μ_i and μ_m chains are designated on gel A. Gels were aligned with reference to the invariant spot designated with Δ. Cell equivalent amounts of lysate were used for the analysis at each timepoint. Only that region of the gel containing μ chains is shown.

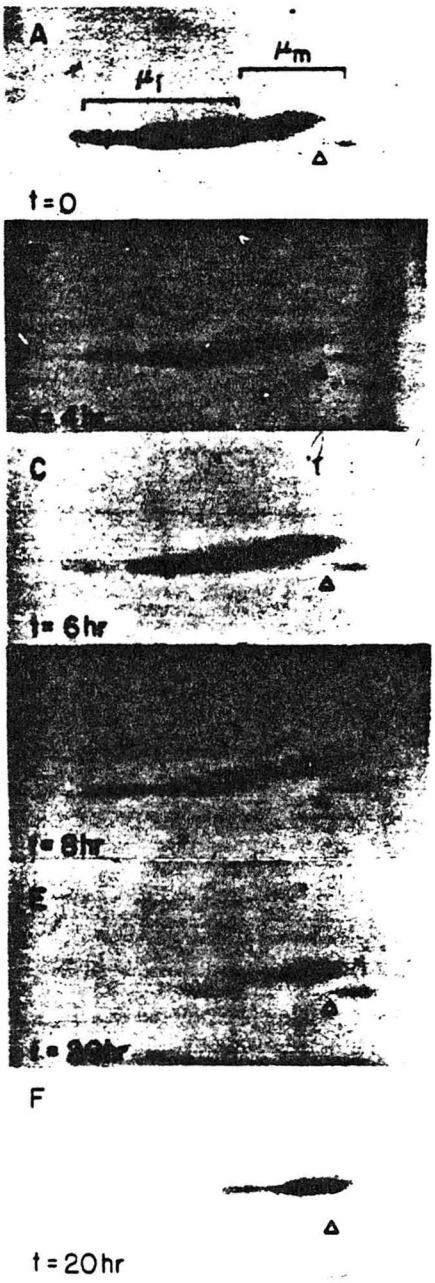

FIGURE 7.

Figure 8. SDS-polyacrylamide gel analysis of μ chains synthesized by W279.1 and M104E cells in the presence and absence of tunicamycin. M104E and W279.1 cells were radiolabeled with ^{35}S-methionine in the presence or absence of tunicamycin. Samples were immunoprecipitated and analyzed on 10% SDS polyacrylamide slab gels as described in Materials and Methods. Lane A: Cell lysate from M104E cells labeled for 4 hr in the presence of 5 μg/ml tunicamycin. Lane B: Medium from W279.1 cells labeled for 4 hr with 5 μg/ml tunicamycin. Lane C: Cell lysate from W279.1 cells labeled for 4 hr with 5 μg/ml tunicamycin. Lane D: Cell lysate from W279.1 cells labeled for 15 min without tunicamycin. Lane E: Cell lysate from W279.1 cells labeled for 4 hr without tunicamycin. Arrows indicate the position of fully glycosylated M104E μ_s chains. The unglycosylated μ chain bands in lanes B and C are not aligned since the lanes are from different polyacrylamide gels which each contained internal control lanes.

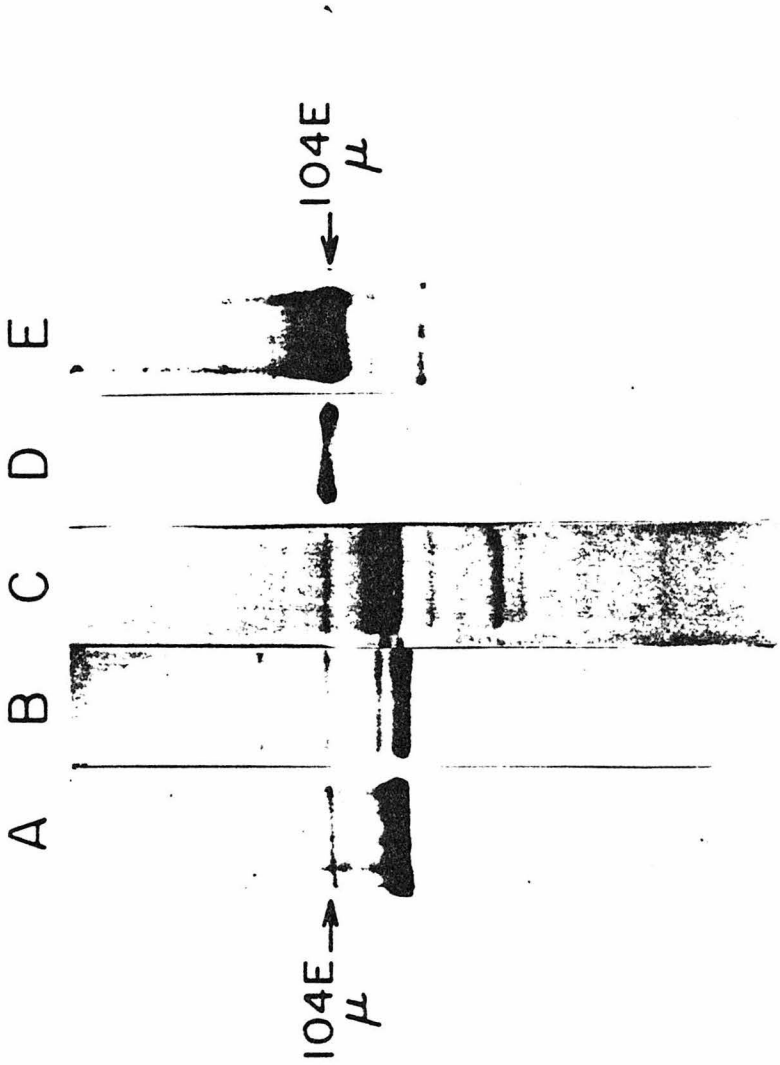

FIGURE 8.

Figure 9. Composite agarose-polyacrylamide gel analysis of IgM molecules synthesized by original W279 cells. Cells were labeled by incubation in ^{3}H-tyrosine and leucine. Isolation and gel electrophoresis are as described in the legend to Fig. 1 and in Materials and Methods. A. Cell lysate from W279. B. Medium from W279. Equivalent quantities (in cell numbers) of cell lysate and medium were analyzed here and in Fig. 1.

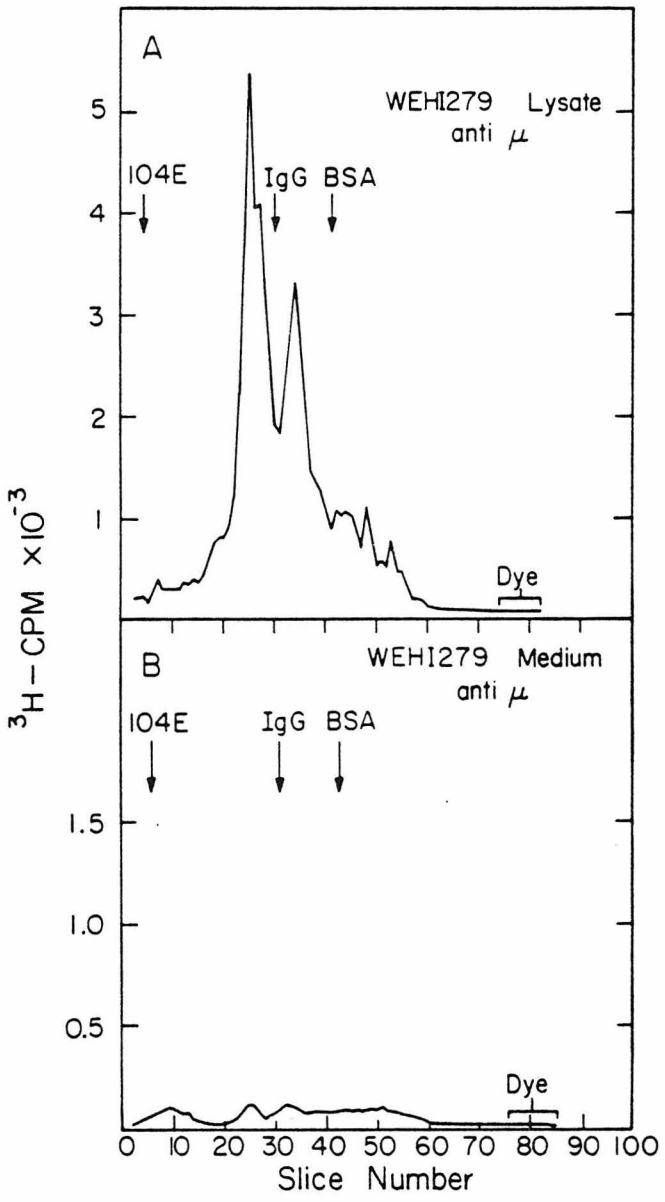
A
WEHI279 Lysate
anti μ
104E
IgG
BSA
Dye
B
WEHI279 Medium
anti μ
104E
IgG
BSA
Dye
^{3}H-CPM ×10^{-3}
Slice Number
0 10 20 30 40 50 60 70 80 90 100
1 2 3 4 5
0.5 1.0 1.5

FIGURE 9.

Figure 10. Differentiation stages of IgM secreting cells. The schematic drawing illustrates the various stages that have been characterized in the development of stem cells to mature plasma cells. Internal μ chains and IgM molecules, membrane IgM monomers and secreted IgM pentamers are illustrated. The different circle diameters used to represent the pre-B cell and the plasma cell indicate that the cells of these two stages are larger than the others. The B-cell lymphomas W279 and W231 correspond to a nonsecreting B cell. The W279.1 lymphoma cells represent an intermediate IgM-secreting B cell stage, and M104E and MxW cells correspond to terminally differentiated plasma cells containing little if any membrane IgM molecules.

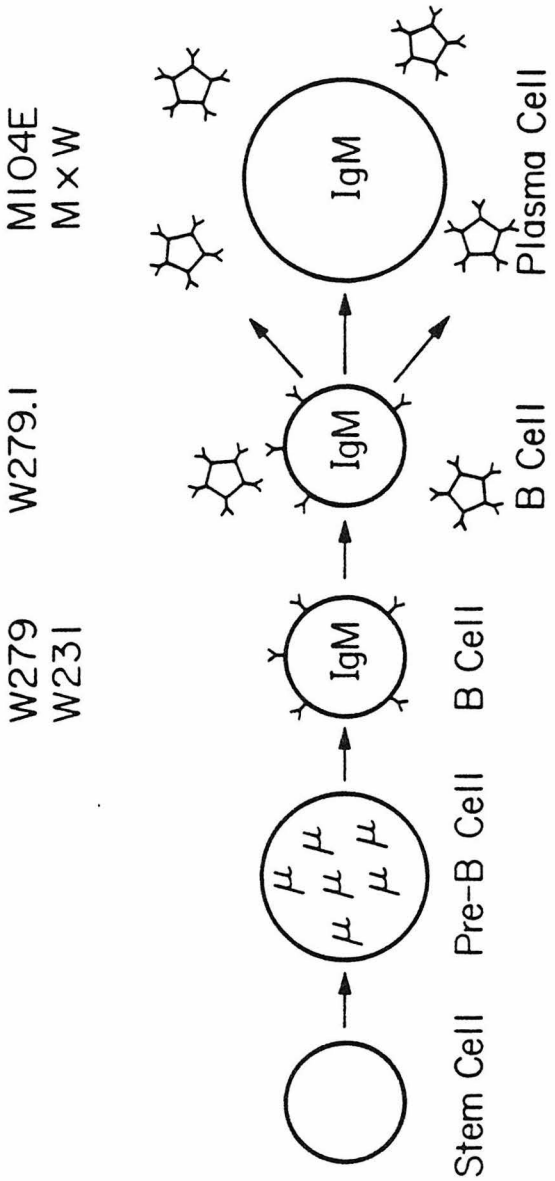
W279
W231
W279.1
M104E
M x W
IgM
IgM
IgM
μ μ μ μ
Stem Cell
Pre-B Cell
B Cell
B Cell
Plasma Cell

FIGURE 10.

CHAPTER 4

The Immunoglobulin μ Chains of Membrane-Bound and Secreted IgM Molecules Differ in their C-Terminal Segments

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Summary

The B lymphocyte synthesizes two forms of IgM molecules during its development from a stem cell to a mature antibody-secreting plasma cell. The monomeric receptor IgM molecule is affixed to the plasma membrane and serves to trigger the later stages of B-cell differentiation, whereas the pentameric secreted IgM molecule is an effector of humoral immunity. The structural differences between membrane-bound and secreted IgM molecules are reflected in the differences between their heavy or mu chains. We have previously determined the complete amino acid sequence of a murine secreted mu (μ_s) chain (Kehry et al., 1979). In this study, we have compared the structures of the secreted and membrane-bound mu (μ_m) heavy chains by peptide mapping, microsequence, and carboxypeptidase analyses. These studies demonstrate that the μ_m and μ_s chains are very similar throughout their V_H, $C_\mu 1$, $C_\mu 2$, $C_\mu 3$ and $C_\mu 4$ domains. The μ_m and μ_s chains differ in the amino acid sequence of their C-terminal segments. These studies in conjunction with those carried out on the μ_m and μ_s mRNAs (Rogers et al., 1980) and the C_μ gene (Early et al., 1980) suggest that the μ_m and μ_s chains from a given B cell are identical but for their 41 and 20 residue C-terminal segments, respectively. The amino sequence of the 41 residue C-membrane terminal segment predicted from the corresponding μ_m mRNA is in perfect agreement with all of the protein studies reported in this paper with the exception that the carboxy-terminal amino acid, lysine, is posttranslationally removed from the mature μ_m polypeptide. Thus, these distinct C-terminal segments are produced by RNA splicing from a single nuclear transcript.

Introduction

In the maturation of B lymphocytes to antibody-secreting plasma cells, discrete stages of differentiation may be identified (Warner, 1974). The pre-B cell synthesizes only internal mu chains (Burrows, Le Jeune and Kearney, 1979). In addition to other changes, induction of the synthesis of light chains accompanies the formation of the small B lymphocyte containing membrane IgM molecules as integral membrane receptors (Vitetta, Baur and Uhr, 1971). Additional steps of differentiation and proliferation then occur which form a clone of IgM secreting plasma cells. Thus, the IgM molecule can exist as a membrane-bound receptor on the surface of B lymphocytes or is secreted by plama cells as a hydrophilic serum antibody. The secreted IgM molecule is a pentamer composed of five monomeric IgM subunits plus a joining (J) chain [$(\mu_2 L_2)_5 J$] (Della Corte and Parkhouse, 1973) whereas the membrane-bound IgM molecule is a monomer ($\mu_2 L_2$). We were interested in characterizing the structural features which distinguish these alternative forms of a single class of antibody molecules. Since the μ heavy chain affixes the IgM molecule to the plasma membrane, the first step in the comparison of the membrane-bound (μ_m) and secreted (μ_s) chains was the determination of the complete covalent structure of a μ_s chain synthesized by the mouse plasmacytoma MOPC 104E (Kehry et al., 1979). This study revealed that the μ_s chain has five homology units or domains, one variable (V) and four constant (C) region domains, (V_H, $C_\mu 1$, $C_\mu 2$, $C_\mu 3$ and $C_\mu 4$) followed by a nonhomologous hydrophilic C-terminal segment of 20 amino acid residues.

Several lines of evidence suggest that the μ_m and μ_s chains differ in structure. IgM molecules are solubilized from the cell surface only by detergents (Melcher, Eidels and Uhr, 1975), and therefore are by definition integral membrane proteins. Detergent binding studies have shown that membrane IgM molecules

and isolated μ_m chains bind small but significant amounts of detergent whereas secreted IgM and μ_s chains bind negligible amounts (Melcher and Uhr, 1977; Vassalli et al., 1979; Parkhouse, Lifter and Choi, 1980). Thus, μ_m chains possess a structural region capable of interacting with the hydrophobic plasma membrane. Molecular weight comparisons of μ_m and μ_s chains by SDS-polyacrylamide gel electrophoresis and by ultracentrifugation have shown that the μ_m chain is larger than the μ_s chain by approximately 1500 daltons (Melcher and Uhr, 1973; Bergman and Haimovich, 1978). Although μ_m and μ_s are glycoproteins, μ_m and μ_s chains synthesized in the presence of tunicamycin are devoid of carbohydrate. Analyses of these nonglycosylated molecules indicate that the molecular weight difference is due to differences in polypeptide structure (Vassalli et al., 1979; A. Williamson, personal communication; J. Haimovich, personal communication; Sibley et al., 1980). Translation of μ_m and μ_s mRNA derived from B lymphoma cells also results in the synthesis of a μ_m polypeptide which is larger in molecular weight than the μ_s polypeptide (A. Williamson, personal communication; J. Haimovich, personal communication). In addition, there seem to be carbohydrate differences between μ_m and μ_s chains (Bergman and Haimovich, 1978; Bergman, Haimovich and Melchers, 1977). At least some of the above differences are localized to the COOH-terminal region of μ_m and μ_s chains. This localization has been demonstrated serologically by antibody competition experiments (Fu and Kunkel, 1974) and structurally by peptide mapping of Fc fragments (Yuan, Uhr and Vitetta, 1980) and by treatment of μ_m chains with carboxypeptidase (Williams, Kubo and Grey, 1978). Peptide mapping studies on μ_m and μ_s chains derived from a single cell line (Yuan, Uhr and Vitetta, 1980) and on μ_m and μ_s chains from a B-cell lymphoma and the IgM-secreting hybrid of the B lymphoma with a plasmacytoma (Raschke, Mather and Koshland, 1979) have indicated that structural differences between μ_m and μ_s chains are small. However, there has been disagreement on whether

the COOH-terminal amino acids of μ_s and μ_m chains are different or identical (A. Williamson, personal communication; Bergman and Haimovich, 1978; McIlhinney, Richardson and Feinstein, 1978).

Previous studies on μ_s and μ_m mRNAs and the C_μ gene (Rogers et al., 1980; Early et al., 1980) report that the μ_m and μ_s chains are identical in their four C_μ domains but differ in their C-terminal segments. In this paper we present a structural analysis on three types of μ chains derived from a cell line. The mouse B-cell lymphoma, WEHI 279 (W279), synthesizes two species of μ chains, μ_m and μ_s, in addition to a μ internal form (μ_i) which contains precursors of both μ_m and μ_s chains (Sibley et al., 1980). These W279 μ chains contain identical variable (V) regions. Fusion of W279 cells with the myeloma cell line MPC 11 generates MPC11 x W279.2 (MxW) hybrid cells that secrete pentameric IgM molecules composed of W279 μ_s chains. These three species of W279 μ chains, μ_i, μ_m and μ_s, differ in molecular weight and charge. The definition of their structural differences and similarities at the carbohydrate and protein levels is the subject of the present report.

Results

Localization of Three Species of W279 μ Chains

Three species of μ chains can be defined in W279 lymphoma and MxW hybridoma cells based on cellular location: μ_m (membrane), μ_i (internal) and μ_s (secreted). Two different cell-surface labeling techniques have demonstrated that μ_m chains are present on the plasma membrane of W279 cells as a 200,000 dalton IgM monomer (Sibley et al., 1980). In contrast, the MxW hybridoma cells have very low levels of cell-surface μ chains and secrete large quantities of IgM pentamers (W. Raschke, unpublished observations). Both W279 and MxW cells contain an internal pool of μ chains (μ_i). In MxW cells, the μ_i pool consists only of precursors to μ_s chains,

while pulse-chase experiments indicate that the μ_i pool of W279 cells contains precursors for both μ_m and μ_s chains (Sibley et al., 1980). In long-term (16 hr) "steady state" labelings (shown below), the μ_i pool in W279 cells is predominantly composed of pre-μ_s chains.

These μ chains can be distinguished by several chemical criteria. The pI of the μ_s chains is similar to that of μ_m chains and both are markedly more acidic than the μ_i chains (Figure 1, Table 1). In addition, μ_m chains are larger in apparent molecular weight than μ_s chains by 4000 daltons (Figure 1, Table 1). This molecular weight difference is not due entirely to differences in the levels of glycosylation because in the presence of the glycosylation inhibitor, tunicamycin, W279 lymphoma cells synthesize two distinct μ polypeptide chains differing in molecular weight by ~1500 daltons (Table 1). The smaller of the two μ polypeptides corresponds in molecular weight to precursor μ_s chains synthesized by MxW cells in the presence of tunicamycin (Table 1). Thus, the B-cell lymphoma synthesizes two μ chains, μ_m and μ_s, which apparently differ in size by 15 to 20 amino acid residues.

The μ_i Chains Are Incompletely Glycosylated

Complex carbohydrate moieites are constructed from a branched mannose and glucosamine core structure by the addition of terminal N-acetyl glucosamine, galactose, fucose, and sialic acid residues (Kornfeld and Kornfeld, 1976). The μ_s chains have three complex carbohydrate moieties (Kehry et al., 1979). Treatment of a detergent solubilized lysate of W279 cells with galactose oxidase and NaB^3H_4 labels μ_m chains but not μ_i chains (Figure 2). Therefore, we conclude that the μ_i chains are missing terminal galactose residues and presumably the other terminal residues such as sialic acid. In contrast, the μ_m chains have terminal galactose residues and presumably are fully glycosylated. The basic pI of the μ_i chains as compared with the μ_m chains can then be explained by the

lack of terminal sialic acid residues on the complex carbohydrate structures of the μ_i chains. Therefore, the final stages of glycosylation have not been completed on these precursor μ_i chains.

The Sites of Carbohydrate Attachment Are Similar in μ_i and μ_s Chains

The cyanogen bromide fragments from the constant region of μ_s chains can be individually resolved by gel filtration (Kehry et al., 1979) as shown in Figure 3 for MOPC 104E (M104E) μ_s chains. Fragments CN1-2, 2, 3 and 4 contain variable region sequences, whereas fragments CN4, 5, 6, 7, 8, 8-9 and 9 contain C_μ sequences with the numbering extending from the NH_2- to the COOH-terminus (CN1 to CN9, respectively). The 20 residue C-terminal segment is contained in fragments CN8, 9 and 8-9. Peptides with two numbers, such as fragment CN8-9, indicate an incomplete methionine cleavage by cyanogen bromide (due to an adjacent serine residue) with the resulting peptide containing two methionine fragments. The M104E μ_s chain contains five C_μ region carbohydrate moieties. Fragments CN5 and CN6 contain complex carbohydrate moieties (boxed in the schematic drawing of the μ_s chain in Figure 3), whereas fragments CN7 and CN8 contain high mannose or simple (circled) carbohydrate moieties. The excluded column peak (Agg.) consists of aggregated fragments and large uncleaved peptides. A small peak migrating on the gel filtration column between CN6 and CN7 has been found only in M104E μ_s chains and consists of a combination of V_H and C_μ region partial cyanogen bromide cleavage products. We have used the M104E μ_s chain as the standard μ_s chain for assessing the glycosylation of W279 μ chains.

W279 cells were labeled with ^{3}H-mannose and the labeled μ_i chains were preparatively isolated as the smaller molecular weight μ peak on 10% polyacrylamide-SDS gels. These μ_i chains were cleaved with cyanogen bromide and the fragments compared by gel filtration with μ_s chains that had been secreted by ^{3}H-mannose-labeled M104E cells and similarly prepared (Figure 4).

The schematic drawing at the top of Figure 4 illustrates the order of cyanogen bromide fragments in the W279 μ chain and the sites of carbohydrate attachment in the mouse μ_s chain C_μ region (see Figure 3). Several points are of interest. First, the μ_i and μ_s chains have very similar ^{3}H-mannose-labeled cyanogen bromide profiles. Therefore the majority of W279 μ_i chains consists of precursors to μ_s chains. Both chains have identical numbers of ^{3}H-mannose-labeled C_μ region peptides, and corresponding peptides in μ_i and μ_s chains incorporate ^{3}H-mannose in identical proportions. Second, the peptides with simple carbohydrate moieties, CN7 and CN8, migrate to identical positions for the μ_i and μ_s chains. Third, the peptides with complex carbohydrate moieties, CN5 and CN6, are smaller in molecular weight in the μ_i (denoted CN5' and CN6') than in the μ_s chains. This observation is consistent with the earlier conclusions drawn from the galactose oxidase studies that the μ_i chains lack the terminal sugars of the complex carbohydrate moieties. These C_μ region peptides should then be smaller in molecular weight for the μ_i chains. Moreover, partial radiolabeled sequence analysis of CN5', CN6' and CN7 fragments from μ_i chains demonstrates that they are identical at their NH_2-termini to their μ_s chain counterparts (Kehry et al., 1979) (Figure 5, Table 2). Because these three cyanogen bromide peptides are contiguous (Figures 3 and 4), the smaller size of the CN5' and CN6' peptides cannot be explained by shifts in the locations of the corresponding methionine residues unless one postulates that additional small C_μ region peptides are generated by cyanogen bromide cleavage of the μ_i chains. Cyanogen bromide fragmentation studies on ^{3}H-amino acid-labeled μ_i chains (to be discussed later) do not reveal any such additional peptides. The above studies suggest that the cyanogen bromide glycopeptides in the majority of μ_i and μ_s chains are identical, apart from differences in the levels of glycosylation for complex carbohydrate moieties.

The μ_i and μ_s Chains Appear To Be Very Similar in Primary Structure

W279 μ_i and MxW μ_s chains were labeled with ^{3}H-tyrosine and ^{3}H-leucine, purified, cleaved with cyanogen bromide and the fragments analyzed by comparative gel filtration (data not shown). The V_H region cyanogen bromide peptides of these μ_i and μ_s chains have been identified by NH_2-terminal amino acid sequence analyses (see below) and were identical in size. Because the W279 μ chain has an unusual methionine distribution in the V_H region, this size correspondence supports the hypothesis that the MxW hybridoma cells secrete μ_s chains with a W279 V_H region.

The μ_i and μ_s chains were labeled with either ^{3}H-tyrosine, ^{3}H-phenylalanine, ^{35}S-cysteine, or ^{35}S-methionine and digested with trypsin and chymotrypsin. The resulting peptides were analyzed by high performance liquid chromatography (HPLC). This method of peptide separation involves the interaction of the amino acid residues with the derivatized packing followed by elution with a continuous exponential gradient of acetone (McMillan et al., 1979). The peaks in the early fractions are somewhat variable due to the shallowness of the gradient in this region. In addition, the difficulty in achieving complete digestion with chymotrypsin produces some smaller variable peaks probably representing partial enzymatic cleavage products. The μ_i and μ_s chains labeled with tritiated tyrosine, phenylalanine, cysteine, or methionine exhibited very similar tryptic and chymotryptic patterns. Figure 6 illustrates the comparison of the peptides from ^{35}S-methionine-labeled μ_i and μ_s chains. The major peaks in the μ_i and μ_s chains elute at identical positions, and these chains are therefore very similar to one another, supporting the idea that the μ_i chains represent primarily an internal pool of pre-μ_s chains.

We have incorporated various ^{3}H-labeled amino acids into the μ_i and μ_s chains for partial amino acid sequence analyses. In addition, we isolated sufficient quantities of W279 μ_s chains from mice inoculated with the MxW hybridoma tumor

so that we could determine directly the sequence of most of the W279 variable region. The amino acid sequence data for each cyanogen bromide fragment of the μ_i chains and the V region and COOH-terminal cyanogen bromide fragments for the μ_s chains are compared with the amino acid sequence of the M104E μ_s chain in Table 2. These data are summarized in a diagrammatic fashion in Figure 7. Several conclusions can be drawn. First, we have determined about 80% of the amino acid sequence of the W279 μ_s V_H region (Table 2). The W279 V_H region has an unusual amino acid sequence that readily allows it to be distinguished from many other mouse V_H regions (Kabat, Wu and Bilofsky, 1976). Second, the V_H regions of the MxW μ_s and W279 μ_i chains are identical at all the positions where they can be compared (Table 2). Because the W279 V_H region is unusual in its sequence, we feel there is a high probability that the W279 μ_i and μ_s V_H regions will be identical throughout their sequences. This conclusion also is supported by the peptide map data presented above. Fusion of the W279 lymphoma cell line with the myeloma cell line therefore induces the secretion of large quantities of μ_s chains containing the W279 V_H region. Third, the C_μ region cyanogen bromide peptides from the W279 μ_i and MxW μ_s chains have identical NH_2-terminal sequences for all the positions where they can be compared (Table 2). In addition, the W279 C_μ region is identical to that of M104E μ_s chains (Table 2). These partial sequence comparisons, together with peptide map comparisons discussed above, lead to the conclusion that the majority of the μ_i chains and the μ_s chains are probably identical in amino acid sequence. Thus, within our limits of detection, the predominant μ_i chain species represents precursors to μ_s chains in that the μ_i chains differ only in the glycosylation levels of their complex carbohydrate moieties.

The μ_m and μ_s Chains Have Complex Carbohydrate Moieties Located in Identical Regions

After treatment of intact W279 cells with galactose oxidase and NaB^3H_4, the labeled μ_m chains were isolated, cleaved with cyanogen bromide and compared by gel filtration to M104E μ_s chains which were similarly treated (Figure 8). Only the fragments CN5 and CN6 contain complex carbohydrate moieties (see Figures 3 and 4), and only fragments CN5 and CN6 contain radiolabeled galactose residues (Figure 8). These peptides migrate at identical positions for the W279 μ_m chains and the M104E μ_s chains. We therefore conclude that complex carbohydrate moieties are located in identical regions in the μ_m and μ_s chains.

The μ_m Chain Lacks the COOH-Terminal High Mannose Carbohydrate Moiety Present on Pre-μ_s Chains and μ_s Chains

W279 cells were labeled with ^{3}H-mannose to label all of the carbohydrate moieties. The labeled μ_m chains were isolated, cleaved with cyanogen bromide, and the fragments compared by gel filtration with μ_s chains that had been similarly labeled and prepared from M104E cells (Figure 9). Of the four carbohydrate containing fragments found in μ_s chains, three are present in the μ_m chains, fragments CN5, CN6, and CN7. These fragments are identical in molecular weight and in the proportions of ^{3}H-mannose incorporated into their respective carbohydrate moieties. However, the position where an expected μ_m CN8-9 fragment should migrate is lacking mannose, and therefore, the high mannose carbohydrate moiety (see Figures 3 and 4). This conclusion is supported by comparing ^{3}H-mannose-labeled μ_i (pre-μ_s) and μ_m chains after digestion with trypsin and chymotrypsin (Figure 10). The high performance liquid chromatographs in Figure 10 show that the μ_m chains are lacking one ^{3}H-mannose-labeled peptide (crosshatched) that is present in μ_i chains. As we noted earlier, the μ_s and μ_i chains have the same number of ^{3}H-

mannose-labeled cyanogen bromide fragments (Figure 4) and trypsin plus chymotrypsin peptides (data not shown). The missing carbohydrate residue in μ_m chains probably reflects the absence of the carbohydrate recognition sequence present in the C-terminal segment of μ_s chains.

The μ_m Chain Differs from the μ_s Chain in Its C-Terminal Segment

To localize any amino acid sequence differences between μ_m and μ_s chains, the ^{3}H-amino acid-labeled cyanogen bromide fragments of μ_m and μ_i chains were compared by gel filtration (Figure 11). In particular, ^{3}H-tyrosine was included in the μ_i and μ_m chains because it labels the COOH-terminal cyanogen bromide fragment of μ_s chains, CN9 (see Figure 4; Kehry et al., 1979). The order and identity of these W279 cyanogen bromide fragments (shown in Figure 4) were deduced from a comparison of the NH_2-terminal radiolabeled sequences of the μ_i cyanogen bromide fragments and the corresponding sequences of the myeloma M104E μ_s chain (Table 2). As expected, the CN5' and CN6' fragments from the μ_i chains are smaller in molecular weight than the CN5 and CN6 fragments from the completely glycosylated μ_m chains. The COOH-terminal cyanogen bromide fragment, CN9, is absent in the μ_m chains, although two-fold larger aliquots of the last 40 column fractions from the cyanogen bromide digest of μ_m chains were counted for radioactivity. The CN9 fragment is present in the μ_i chains, supporting the idea that μ_i chains are mainly composed of the internal precursor μ_s chains before secretion. Identical results also have been obtained by comparing ^{3}H-tyrosine-labeled cyanogen bromide fragments of μ_i and μ_m chains from a second B-cell lymphoma, WEHI 231 (R. Douglas, unpublished observations).

Since we reproducibly achieve 50% cleavage of methionine-serine bonds by cyanogen bromide, the absence of the CN9 fragment from the μ_m chains suggests that the μ_m chains lack the methionine residue nine amino acids from the

COOH-terminus of the μ_s chains. This supposition is substantiated by a comparison of W279 μ_m and MxW μ_s chains labeled with ^{35}S-methionine, digested with trypsin and chymotrypsin and analyzed by HPLC (Figure 12). The HPLC peptide maps indicate that the μ_m chains lack a major methionine peptide (crosshatched) that is present in the μ_s chains. A similar comparison of tryptic and chymotryptic peptides from μ_m and μ_s chains labeled with ^{3}H-phenylalanine (Figure 13) also shows that μ_m chains have two phenylalanine peptides not present in μ_s chains. Verification of the missing high mannose carbohydrate moiety and methionine residue in the C-terminal segment of μ_m as compared with μ_s (μ_i) chains using two independent analytical techniques, suggests that the μ_m and μ_s chains possess different COOH-terminal amino acid sequences.

The μ_m Chains Differ in Amino Acid Sequence from the μ_s Chains at Their COOH-Termini

The amino acids present at the COOH-termini of W279 μ_m, μ_i and MxW μ_s chains were investigated by digestion of the radiolabeled μ chains with carboxypeptidases A and B. The μ chains were labeled with either ^{3}H-tyrosine, leucine, valine, phenylalanine or lysine, purified, the amounts of tritium in each amino acid pool quantitated and the labeled μ chains combined into μ_m, μ_i and μ_s pools. Digestion of W279 μ_m, μ_i and MxW μ_s chains with carboxypeptidases A and B was followed by separation and quantitation of the released amino acids on an amino acid analyzer. The time course of release of the amino acids can be used to determine a COOH-terminal amino acid sequence. As is shown in Figure 14, different amino acids were released from μ_m and μ_s chains. The rapid release of tyrosine from MxW μ_s chains in Figure 14C is expected since tyrosine is the COOH-terminal amino acid in μ_s chains (Kehry et al., 1979). Background release of valine and phenylalanine is also observed for MxW μ_s chains which correlates with the low level release of these

amino acids from unlabeled M104E μ_s chains digested with carboxypeptidase (not shown). In addition, tyrosine is the predominant amino acid released from W279 μ_i chains (Figure 14B). This confirms the previous peptide maps and sequence determinations on μ_i chains that indicate μ_i chains are predominantly incompletely glycosylated precursors to μ_s chains. In contrast, carboxypeptidase digestion of W279 μ_m chains identifies the COOH-terminal sequence as Lys-Val-COOH (Figure 14A). Amino acids other than tyrosine which correspond to those released from μ_m chains were also released from μ_i chains in lower yield. Since this same sequence can be attributed to a polypeptide present as 10-15% of the μ_i chains, we propose that the lysine-valine COOH-terminal sequence represents precursors to μ_m chains in the W279 internal pool of μ chains. Similarly, the rapid release of tyrosine from 20% of the μ_m chains indicates that μ_s chains are present as a contaminant in the μ_m pool which was purified by isolation of the highest molecular weight μ chain from 10% polyacrylamide gels. The above finding that μ_m chains possess a different COOH-terminal amino acid sequence from μ_s chains is consistent with our previous results which indicate that all of the amino acid and carbohydrate differences between μ_m and μ_s chains can be localized to the C-terminal segment.

Discussion

W279 Cells Synthesize Two Distinct Species of μ Chains, μ_s and μ_m

These μ chains differ in electrophoretic mobility, localization in the cells, and covalent structure. The μ_m chains have been localized to the W279 cell plasma membrane by galactose oxidase and lactoperoxidase labeling (Sibley et al., 1980). The μ_s chain is secreted by MxW cells as a pentamer $[(\mu_2 L_2)_5]$ in the presence of a J (joining) chain (Raschke, Mather and Koshland, 1979; M. Koshland and E. Mather, personal communication). Pulse-labeling studies (Sibley et al., 1980)

and carboxypeptidase experiments indicate that the internal μ pool contains precursors for μ_m and μ_s chains that are lacking the terminal sugars (e.g., galactose, sialic acid and presumably fucose) in their complex carbohydrate moieties. Carboxypeptidase and carbohydrate labeling studies suggest that in B-cell lymphomas the μ_s precursor appears to predominate in the μ_i pool (Figure 15). Perhaps the induction of J chain synthesis is an important factor in the differentiation process which converts B cells to terminally differentiated, IgM-secreting plasma cells (Raschke, Mather and Koshland, 1979).

The W279 μ_m and μ_s Chains Are Identical but for Their C-Terminal Segments

Gel filtration comparisons of cyanogen bromide fragments suggest that the μ_m and μ_s chains are identical but for their C-terminal segments (Figure 15). The extensive similarity of the μ_m and μ_s chains is supported by the comparison of tryptic and chymotryptic peptides labeled with a variety of different amino acid residues. In addition, the placement of high mannose and complex carbohydrate moieties appears identical except for the high mannose carbohydrate moiety which is present in the C-terminal segment of μ_s chains and is absent in μ_m chains (Figure 15). This observation implies that the corresponding Asn-X-Ser/Thr carbohydrate recognition sites are preserved throughout the C_μ region of both μ_m and μ_s chains.

The 40-Residue C-Terminal Segment Sequence Predicted from Nucleic Acid Studies is in Complete Accord with our Protein and Carbohydrate Findings on the μ_m Chain

The amino acid sequences of the C-terminal segment of the μ_s chain (Kehry et al., 1979) and the predicted C-membrane terminal segment of the μ_m chain (Rogers et al., 1980; Early et al., 1980) are given in Figure 16. The following points are derived from a comparison of the two amino acid sequences. i) The COOH-terminal sequence of the μ_m chain is -Lys-Val by carboxypeptidase analysis. This observation is consistent with the COOH-terminal sequence predicted by the DNA sequence

if we postulate that the COOH-terminal-most lysine residue has been removed by posttranslational proteolysis. This assumption is reasonable because COOH-terminal lysine residues also are removed by posttranslational proteolysis in γ1 (Honjo et al., 1979) and γ2b (Tucker et al., 1979; Yamawaki-Kataoka et al., 1980) chains. ii) Tryptic and chymotryptic peptide maps of ^{3}H-phenylalanine-labeled μ_s and μ_m chains indicate that μ_m chains have two phenylalanine peptides not found in μ_s chains. This is precisely what is expected from the predicted μ_m C-terminal segment sequence, taking into account the fact that three of the five phenylalanine residues in the C-terminal segment will yield free phenylalanine with this type of enzymatic digestion (Figure 16). iii) There is no methionine residue in the predicted μ_m C-terminal segment. Our comparative studies on cyanogen bromide fragments and ^{35}S-methionine-labeled tryptic and chymotryptic peptides also indicate that μ_m chains lack a COOH-terminal methionine residue. iv) The recognition sequence for asparagine-linked carbohydrate moieties, Asn-X-Ser/Thr, is missing in the predicted sequence of the μ_m C-terminal segment. Accordingly, the absence of the COOH-terminal high mannose carbohydrate moiety in μ_m chains can be readily explained. v) The μ_m C-terminal segment is 20 residues longer than its μ_s counterpart. This is in excellent agreement with the size difference predicted by tunicamycin studies. vi) The μ_m C-terminal segment is missing a half cystine residue found in the μ_s chain one residue from the COOH-terminus. Although we have not demonstrated the absence of this penultimate cystine residue in μ_m chains, this residue is essential for the formation of disulfide-linked secreted IgM pentamers (Mestecky and Schrohenloher, 1974). In this regard, membrane IgM molecules are only capable of forming covalent monomers on the B cell surface, as was shown by our labeling studies with galactose oxidase (Sibley et al., 1980). vii) The μ_m C-terminal segment contains a stretch of 25 uncharged amino acid residues which is comparable in hydrophobicity index (Segrest and

Feldmann, 1974) to the membrane-associated regions in glycophorin (Tomita and Marchesi, 1975), phage M13 coat protein (Wickner, 1976) and HLA heavy chains (J. Strominger, unpublished). The μ_m chain is therefore an integral membrane protein possessing hydrophobic C-terminal segment capable of binding detergents (Vassalli et al., 1979; Melcher and Uhr, 1977; Parkhouse, Lifter and Choi, 1980). Thus the predicted amino acid sequence of the μ_m C-terminal segment is fully consistent with all of the chemical and biological observations on μ_m chains.

The Membrane-Bound IgM Molecule Represents a Structurally Well-Characterized Eukaryotic Membrane Receptor

Membrane receptors transduce signals between the external and internal environments of cells and as such constitute an extremely important class of biological effector molecules. Studies on the structure of most eukaryotic membrane receptors have been limited by the very small amounts of material generally available (Devreotes, Gardner and Fambrough, 1977; Carpenter and Cohen, 1979; Gill, 1976; Cuatrecassas et al., 1975; Andres, Jeng and Bradshaw, 1977). Our studies on the IgM receptor molecule and its corresponding gene and mRNA (Rogers et al., 1980; Early et al., 1980) are important in two respects. First, we have characterized in detail the structure of the IgM receptor molecule. We have demonstrated that μ_m chains are very similar to μ_s chains throughout the V_H and C_μ domains. The sites of carbohydrate attachment within the four C_μ domains appear identical in μ_m and μ_s chains. These chains, however, clearly differ in the amino acid sequence of their C-terminal segments. Indeed, sequence analyses of the μ_m and μ_s mRNAs indicate that the C-terminal segments of the μ_m and μ_s chains consist of 41 and 20 residues, respectively. The protein studies of μ_m chains are in complete agreement with the C-membrane-terminal segment sequence predicted from the mRNA sequences (Figure 16), and therefore this C-membrane-

terminal segment is actually contained in μ_m chains. These studies indicate that the μ_m chain is a transmembrane polypeptide which spans the plasma membrane employing a 25-residue stretch of uncharged and hydrophobic residues near its COOH-terminus (Figure 16) (see Rogers et al., 1980). Second, studies on the μ_m and μ_s mRNAs as well as the C_μ gene suggest that the distinct μ_m and μ_s C-terminal segments are generated by RNA splicing from the RNA transcripts of a single C_μ gene (Rogers et al., 1980; Early et al., 1980). Thus, RNA splicing allows IgM molecules and perhaps other immunoglobulins to be expressed as integral membrane receptors, or alternatively, as soluble effector molecules of humoral immunity.

Experimental Procedures

Cells

The WEHI 279 (W279) cells (a gift from Dr. N. Warner) were grown in stationary suspension cultures at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal calf serum (fcs), nonessential amino acids (Gibco), and 5×10^{-5} M β-mercaptoethanol. We subsequently obtained a later clone of W279 cells (W279.1/12) isolated by Dr. Warner and sent to us by Dr. V. Oi. These two cell lines correspond to slightly different stages in B lymphocyte development (Sibley et al., 1980). W279 cells were used for the experiments illustrated in Figures 4, 5 and 9, some of the sequences in Table 2, and the cell fusion with MPC11 to generate MPC11xW279 cells (described below). The W279.1/12 clone was used for all remaining experiments. Our previous characterization of these two cell lines (Sibley et al., 1980; N. Warner, personal communication) indicates that μ_m and μ_s chains isolated from the two clones are equivalent, and we refer only to W279 cells in all descriptions. The MPC11xW279 (MxW) hybrid cells were generated by a fusion between MPC11 and WEHI 279 cells (Raschke, Mather and

Koshland, 1979). MPC11xW279.2 cloned cells were stable for IgM production for periods of up to 6 months and were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum. MOPC 104E (M104E) myeloma cells were passaged subcutaneously as a solid tumor in (BALB/c x DBA/2)F_1 mice. The MPC11 x W279.2 (MxW) hybrid cells were grown as solid tumors in BALB/c mice. The cells were grown in ascites form for the production of milligram quantities of M104E or W279 secreted IgM or for isolation of M104E cells to be radiolabeled.

Labeling

W279 and MxW cells were biosynthetically labeled by washing with fresh medium and resuspending at cell densities of 2×10^6/ml in labeling medium [DMEM containing 5% dialyzed heat-inactivated fcs, 0.3 mg/ml glutamine, 5×10^{-5} M β-mercaptoethanol and nonessential amino acids (Gibco)] lacking the amino acid or amino acids to be labeled, plus 50 μCi/ml of a ^{3}H-amino acid (New England Nuclear). Cells were incubated in a humidified atmosphere lacking CO_2 for 16 hr at 37°C. M104E ascites fluid was diluted 1:2 with Hanks Balanced Salt Solution (HBSS) (Gibco), cells purified over Ficol-Hypaque, the buffy coat washed three times with HBSS, 5% heat-inactivated fcs, and resuspended in labeling medium at 2×10^6 M104E cells/ml. Labeling conditions were identical for all cell lines. Following the 16 hr labeling period, the cells were harvested by centrifugation at 150 x g for 10 min and the medium was removed, made 1 mM in phenylmethyl sulfonylfluoride (PMSF), 1×10^{-4} M β-mercaptoethanol and stored frozen at -70°C. Cells were washed once in cold DMEM, 20% fcs and resuspended in lysis buffer (0.01 M Tris-HCl pH 7.4, 0.14 M NaCl, 0.5% Triton X-100, 1 mM PMSF, 1×10^{-4} M β-mercaptoethanol) at a concentration of 2.5×10^7 cells/ml and incubated for 20 min at 4°C. The mixture was centrifuged at 1800 x g at 4°C for 10 min to remove nuclei. Supernatants were removed, aliquoted and stored frozen at -70°C.

When tunicamycin was employed, cells were preincubated in 2 μg/ml tunicamycin for 1 hr prior to the addition of ^{35}S-methionine. Cells were labeled in the presence of tunicamycin for 1 hr.

Extrinsic labeling of galactose residues with NaB^3H_4 and galactose oxidase was performed by the method of Gahmberg and Hakomori (1973). Purified M104E IgM molecules or W279 whole cell lysates prepared as above were labeled, desalted on Sephadex G-25 equilibrated in 0.01 M Tris-HCl pH 7.4, 0.14 M NaCl, 0.5% Triton X-100 and stored frozen at -70°C.

Immunoprecipitation

Lysates were preincubated with washed formalin-fixed S. aureus (Kessler, 1976) [50 μl 10% suspension of S. aureus per 100 μl cell lysate] for 20 min at 4°C. After centrifugation at 1800 x g at 4°C for 5 min to remove the S. aureus, the supernatant was made 0.5% in SDS and incubated with rabbit anti M104E μ serum (rabbit anti μ), [10 μl rabbit anti μ per 100 μl cell lysate] for 1-1/2 hr on ice. Immune complexes were precipitated by addition of washed S. aureus [100 μl 10% suspension] for 20 min at 4°C. Precipitates were washed three times in washing buffer [0.01 M Tris-HCl pH 7.4, 0.14 M NaCl, 0.5% Triton X-100, 0.1% SDS] and the proteins eluted in 50 μl to 100 μl sample buffer [50 mM Tris-HCl pH 6.8, 2% β-mercaptoethanol, 2% SDS] per 100 μl cell lysate by incubating in a boiling water bath for 2 min. For samples to be analyzed on 10% polyacrylamide-SDS gels, glycerol plus pyronin Y were added to a final glycerol concentration of 15%. Culture medium was precipitated according to the same protocol except that SDS was omitted from all the buffers and 20 μl rabbit anti μ was used per 1.0 ml medium.

Samples to be analyzed by isoelectric focusing were precipitated by 25% trichloroacetic acid (TCA) at 4°C for 1 hr and the precipitates washed at 4°C twice with 20% TCA, twice with EtOH:ether, 1:2, once with ether, and air dried.

Gel Electrophoresis

The μ_i, μ_m and μ_s chains were isolated from preparative 10% polyacrylamide-SDS gels (Laemmli, 1970) with a 2 cm stacking gel. Proteins were eluted from 1 mm slices by incubation of each 1 mm slice in 0.5 ml 0.5% SDS for 24 hr. Radioactivity was determined for an aliquot of each sample by liquid scintillation counting and the appropriate fractions were pooled.

Two-dimensional gel analysis was performed according to O'Farrell (1975). Isoelectric focusing gels were 5% polyacrylamide, 0.28% bisacrylamide, 9.2 M urea, 2% Triton X-100, 2% ampholines pH 3.5-10, 0.13% ampholines pH 5-7, 0.13% ampholines pH 7-9, 0.13% ampholines pH 4-6, and 0.07% ampholines pH 3.5-5 (LKB). TCA precipitates were dissolved in 50 µl sample buffer (9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, 0.4% ampholines pH 3.5-10, 1.6% ampholines pH 5-7) at least 1 hr before loading. Gels were stained and fixed in 25% isopropanol, 20% sulfosalicyclic acid, 0.025% Coomassie Brilliant Blue, destained in 7.5% acetic acid, 5% methanol and fluorographed according to Bonner and Laskey (1974).

Cyanogen Bromide Cleavage

Unlabeled M104E and MxW μ_s chains were purified as previously described (Kehry et al., 1979). In order to determine the sequence of MxW CN3b, the isolated μ_s chains were succinylated (Klapper and Klotz, 1972) prior to cyanogen bromide cleavage. This procedure blocked the NH_2-terminus of the μ_s chains (e.g., fragment CN3a).

The μ_i, μ_m or μ_s chains pooled from appropriate slices of 10% polyacrylamide-SDS gels were reduced and alkylated as follows. Carrier pig IgG and M104E IgM (3 mg each) were added, the pooled µ chains concentrated by lyophilization and brought to a final concentration of 9% SDS, 0.5 M Tris-HCl pH 8.5, boiled for 5 min, flushed with N_2 for 5 min, dithiothreitol added to a concentration

of 20 mM, boiled 2 min, sealed under N_2 and incubated at 37°C for 1-1/2 hr. Alkylation with 50 mM iodoacetamide (3X recrystallized) was performed for 1 hr at room temperature in the dark. Reagents were removed by desalting on Sephadex G-25 equilibrated with 0.05 M Tris-HCl pH 7.4, 0.5% SDS. Proteins were TCA precipitated as described under Immunoprecipitation, and the air dried precipitate was dissolved in 88% formic acid and combined with 30 mg M104E μ_s chains in 70% formic acid. Cyanogen bromide was added (50 mg/ml) and cleavage was performed for 20 hr at 4°C in the dark with constant stirring (Gross, 1967). Fragments were separated by gel filtration as previously described (Kehry et al., 1979) on a column of ACA54 (LKB) (3.5 x 140 cm) equilibrated with 3 M guanidine-HCl, 0.2 M ammonium bicarbonate, 0.02% NaN_3. 5 ml fractions were collected and aliquots were counted in Aquasol (New England Nuclear) in a liquid scintillation counter (Beckman LS 9000).

Peptide Mapping

0.25 mg of carrier pig IgG was added to each gel-purified μ_i, μ_m or μ_s sample intended for peptide mapping. Chains were completely reduced and alkylated as described above under Cyanogen Bromide Cleavage, TCA precipitated and air dried. Samples were dissolved in 200 µl 0.2 M ammonium bicarbonate, digested with TPCK-trypsin (Worthington) at room temperature [total of 150 µg for 22 hr], followed by digestion with chymotrypsin (Worthington) at 37°C [total of 200 µg for 28 hr]. The peptides were frozen and lyophilized to terminate the reaction, dissolved in 45 µl 0.5 M phosphate buffer (pH 1.8):acetone, 2:1 and the peptides separated as described by high performance liquid chromatography on a DuPont ODS C-18 column (McMillan et al., 1979). Fractions were collected at 0.5 min intervals (0.5 ml), dried in a vortex evaporator, redissolved in 0.25 ml 0.01% SDS and assayed for radioactivity in a liquid scintillation counter.

Sequence Analysis

Cyanogen bromide fragments from the ACA54 column were prepared for sequence determinations by pooling, dialyzing exhaustively against 5% formic acid in low molecular weight cutoff dialysis tubing (Spectra/Por 3) at 4°C, and lyophilizing. Pools contained 1 to 3 mg carrier M104E cyanogen bromide fragments.

Automated sequence analyses on the milligram quantities of MxW μ_s chains and CN3 fragments were performed on a modified Beckman sequenator (Hunkapiller and Hood, 1978; Wittmann-Liebold, 1973; Wittmann-Liebold, Graf-funder and Kohls, 1976). Samples were loaded by using Polybrene (Aldrich), and phenylthiohydantoin (Pth) derivatives were identified by high performance liquid chromatography (HPLC) (Water Associates) as described (Hunkapiller and Hood, 1978; Johnson, Hunkapiller and Hood, 1979). Automated sequence analyses of radiolabeled cyanogen bromide fragments were performed on the Caltech sequenator (Hunkapiller and Hood, 1980). Samples were loaded in trifluoroacetic acid (Pierce) and 20% H_2O by using Polybrene (Aldrich). The Pth derivatives were separated by HPLC (DuPont), the peaks corresponding to the radiolabeled Pth-amino acids were fraction collected, dried, and counted for radioactivity in Liquifluor-toluene (McMillan et al., 1977).

Carboxypeptidase A and B Digestions

Carboxypeptidase A (Worthington) and carboxypeptidase B (Sigma) were employed at an enzyme to substrate ratio of 1:40. Carboxypeptidase A was prepared as described (Ambler, 1972) and carboxypeptidase B in 0.1 M NaCl was thawed and diluted 1:10 in 0.2 M N-ethylmorpholine acetate pH 8.6 just prior to use. A known number of counts of isolated radiolabeled μ chain was reduced and alkylated as for peptide maps and dissolved in 0.2 M N-ethylmorpholine acetate pH 8.6 at a carrier pig IgG concentration of 2.5 mg/ml. An aliquot was removed for a substrate

control, and the remainder was digested with an equimolar mixture of carboxypeptidases A and B at 37°C. Aliquots were removed at indicated times, frozen immediately on dry ice and lyophilized twice. For substrate controls, equivalent amounts of enzymes were boiled in 0.1 N acetic acid for 15 min, added to the substrate and incubated at 37°C for the same length of time as the longest reaction time point. Released amino acids were separated on a Durrum D-500 amino acid analyzer (92% of sample loaded). The ninhydrin coil temperature was turned to the minimum setting and 0.5 min fractions were collected from the time of injection and assayed for radioactivity in a liquid scintillation counter. Radiolabeled amino acid standards were analyzed by the same procedure. Total counts in each peak were quantitated (substrate controls contained no peaks) by comparison with the starting radioactivity and the number of residues of each amino acid in the W279 μ_s chain (Kehry et al., 1979).

Antiserum

Rabbit anti-mouse M104E μ chain was produced by repeated immunization of an NZW rabbit with 300 μg partially reduced and aklylated M104E μ chain in complete Freunds adjuvant. Blood was clotted, centrifuged at 6000 x g, and the serum aliquotted and stored frozen at -70°C. Anti μ serum was used without any further treatment.

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Table 1. Molecular Weights and Isoelectric Points of W279 μ Chains

Cells and μ Chain Species	Molecular Weight[a] - Tunicamycin	Molecular Weight[a] + Tunicamycin	pI Range
W279			
μ_m	82	-	5.69-5.17
pre-μ_m	78	69	5.89-5.58
pre-μ_s	76.5	67.3	6.22&6.10
MxW			
μ_s	78[b]	-	5.83-5.33
pre-μ_s	76.5	67.3	6.22&6.10

[a]Molecular weight in daltons x 10^{-3}.

[b]Molecular weight range of 77,000 to 80,600 daltons.

TABLE 2. Amino Terminal Sequence Analyses of W279 Mu Chain CNBr Fragments

M104E μ_s[a] CN1-2	E V Q L Q Q S G P E L V K P G A S V K M S C K A S G Y T F T D Y Y M K W V
W279 μ_i CN3a	- V - L - - - - P - L V - P - - - L - (L) - - - V
MxW μ_s CN3a[b]	E V Q L Q E S G P S L V K P S Q T L S L T C S V T G D S I T S G Y R W N I
M104E μ_s CN3	I G D I N P N N G G T S Y N Q K F K G K A T L T V D K S S S T A Y M Q
W279 μ_i CN3b	- Y - - Y - - - - Y Y - P - L - - - - - - - - -
MxW μ_s CN3b	G Y I S Y S G S T Y Y N P S L K S R I S I T R D T S K N Q Y Y L Q L G
M104E μ_s CN4	D V W G A G T T V T V S S E S Q S F P N V F P L V S
W279 μ_i CN1-2'	- Y - - - - - - V - V - - - - - - F P - V F P L V -
M104E μ_s CN5	G C L A R D F L P S T I S F T W N Y Q N N T E V I Q
W279 μ_i CN5'	- - L - - - F L P - - - - F - - - Y - - - - - V - -
M104E μ_s CN6	N P N V N V F V
W279 μ_i CN6'	- P - V - V F V
M104E μ_s CN7	E S H P N G T F S A K G V A S V C V E D W N N R K E F V C T V T
W279 μ_i CN7[c]	-(Y)- P - - -(F)- A K - V(A)- V - V -(A)- - - -( )-( )V - - V -
M104E μ_s CN8-9	P E P G A P G F Y F T H S I L T V T E E E W N S G E T Y T C V V G H E A L A P H L V T E R
W279 μ_i CN8-9[d]	P - P - - P - F Y F - - - - L - V - . . . . . - Y - -(V)(V)- . . -( )-( )-( )(V)- - -
MxW μ_s CN8-9	P - P - - P - F Y F - -
MxW μ_s CN8	P - P - - P - F Y F - -

Footnotes to Table 2

[a]MOPC 104E sequence from Kehry et al., 1979.

[b]The sequence of CN3a was determined on intact μ_s chain; the sequence of CN3b was determined on CN3 derived from μ_s chains succinylated prior to CNBr cleavage. Location of CN3b in the V region was determined by alignment and homology with invariant V_{HI} sequences (Kabat, Wu and Bilofsky, 1976). ( ) indicates residues with low yields. A key to the single letter amino acid code is provided in Dayhoff (1976). Fragments differing in molecular weight from 104E μ_s chain fragments are denoted by a ' (prime).

[c]Two exceptions to the identity of the W279 μ_i and 104E μ_s C region sequences have bee noted in CN7. Both residues (Tyr at position 399 and Ala at position 417) were recovered in low yield, indicating they were not a part of the major CN7 sequence. We have not identified a possible contaminating sequence which would account for the two discrepancies.

[d](.....) sequence not analyzed.

Figure 1. Three Species of μ Chains Synthesized by W279 Cells

Composite tracing of two-dimensional 10% polyacrylamide-SDS gel fluorographs of ^{3}H-tyrosine and leucine labeled W279 μ chains. The tracing represents the alignment of three different two-dimensional gels using stained marker M104E μ_s chains as an internal standard. Crosshatching of the μ and light chain spots indicates the complete blackening of the X-ray film. μ_i, μ-internal; μ_m, μ-membrane; μ_s, μ-secreted. The apparent molecular weight of the μ chains in the electrophoretic system is approximately 78,000 daltons; light chains 22,000 daltons.

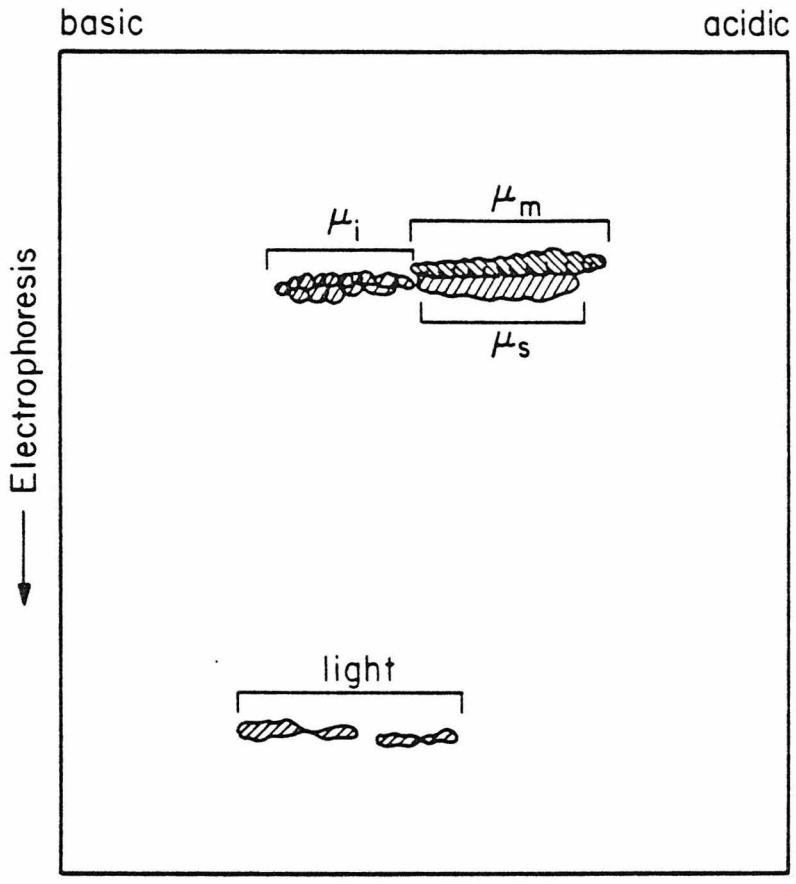


FIGURE 1.

Figure 2. Galactose Residues in W279 μ_m Chains Labeled with NaB^3H_4 and Galactose Oxidase

Two-dimensional gel analysis of a W279 cell lysate labeled with NaB^3H_4 and galactose oxidase as described in Experimental Procedures. After removal of the free radioactivity, the lysate was precipitated with rabbit anti μ. The basic spot at the center left of the gel labels nonspecifically when galactose oxidase is not included in the labeling reaction and is precipitated under our conditions by rabbit anti μ (data not shown). The pI ranges of μ_i and μ_m chain are indicated.

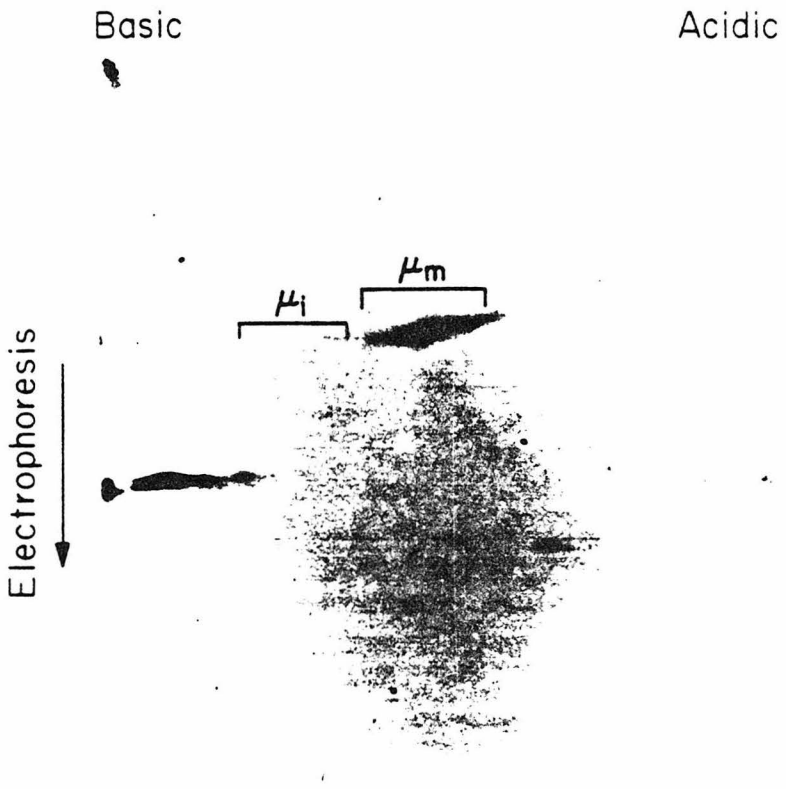


FIGURE 2.

Figure 3. Separation of M104E μ_s Chain Cyanogen Bromide Fragments

Completely reduced and aklylated M104E μ_s chains (35 mg) were cleaved with cyanogen bromide and separated by gel filtration on a column of ACA54 (LKB) as described in Experimental Procedures. Fraction volume was 5 ml. The fragments are labeled (CN1 to CN9) in the order in which they appear in the μ chain sequence. At the top of the figure is a schematic drawing to scale of the M104E μ_s chain showing linear order and sizes of the cyanogen bromide fragments (CN1 to CN9) and the attachment sites of complex (boxes) and simple (circles) carbohydrate moieties. Agg. denotes aggregate peak.

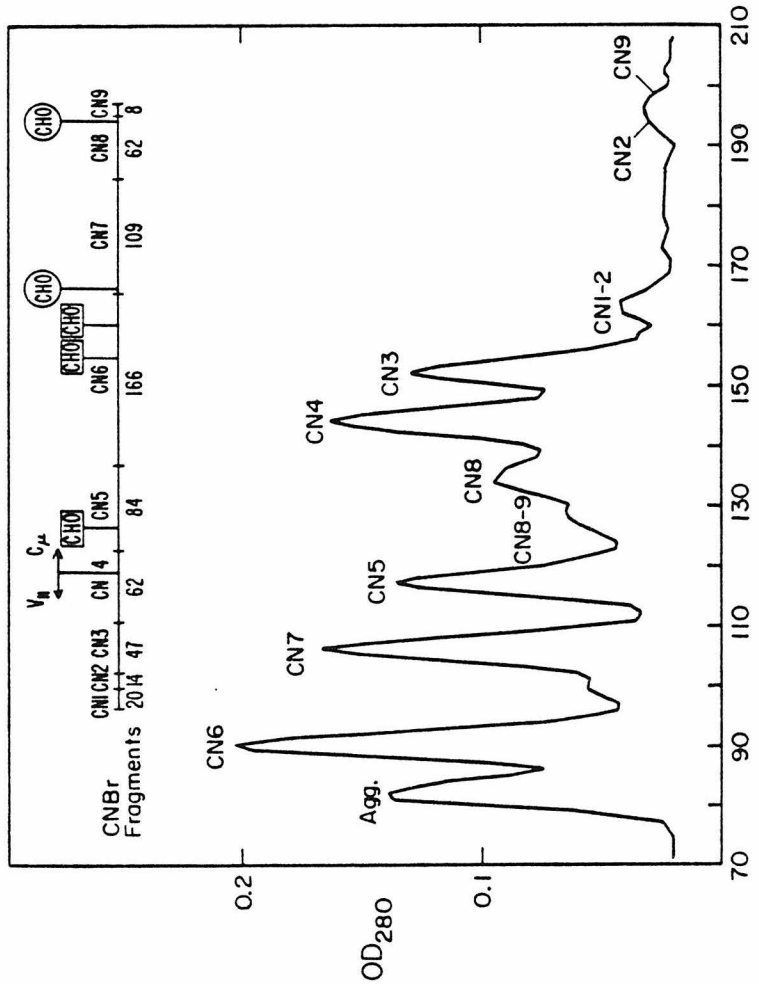

CNBr Fragments
CN1 CN2 CN3
20 14 47
CN 4
62
CHO
CN5
84
CHO
CHO
CN6
166
CHO
CN7
109
CHO
CN8
62
CN9
8
V_{β}
C_{μ}
CN6
CN7
CN5
CN4
CN3
CN8
CN8-9
CN1-2
CN2
CN9
Agg.
OD_{280}
0.2
0.1
70
90
110
130
150
170
190
210
Fraction Number

FIGURE 3.

Figure 4. Comparison of ^{3}H-Mannose Labeled Cyanogen Bromide Peptides of μ_i and μ_s Chains by Gel Filtration

W279 or M104E cells were incubated with ^{3}H-mannose for 16 hr or 8 hr, respectively, in low glucose (1000 mg/ml) Dulbecco's Modified Eagle's Medium. The μ_i, μ_m and μ_s chains were preparatively isolated as in Experimental Procedures. No ^{3}H-mannose was incorporated into light chains. Cyanogen bromide cleavage in the presence of carrier M104E μ_s chains and gel filtration on a column of ACA54 was performed as described in Experimental Procedures. The R_f's of the fractions from the different column runs were then normalized to the internal standard M104E μ_s chain cyanogen bromide fragments. Normalized fractions from the internal standards were then superimposable (not shown). The peak running with the excluded volume consists of aggregated material. Free salt is eluted around fraction 285, and column runs were eluted 10 fractions past this point. Each fraction volume is 5 ml. At the top of the figure is a schematic drawing to scale of the W279 μ_s chain showing the sites of cleavage by cyanogen bromide, the respective fragments (CN1-2' to CN9) and the locations of the five oligosaccharides (CHO1 to CHO5). The complex oligosaccharides are boxed and the high mannose oligosaccharides are circled. Fragments were ordered by comparing their NH_2-terminal sequences to those of M104E μ_s chains (Kehry et al., 1979) and V_{HI} sequences (Kabat, Wu and Bilofsky, 1976). Numbering of the fragments is based on M104E μ_s chain cyanogen bromide fragments. W279 μ_i (----); M104E μ_s (——). The μ_i fragments differing in molecular weight from corresponding M104E μ_s chain cyanogen bromide fragments are denoted by a ' (prime).

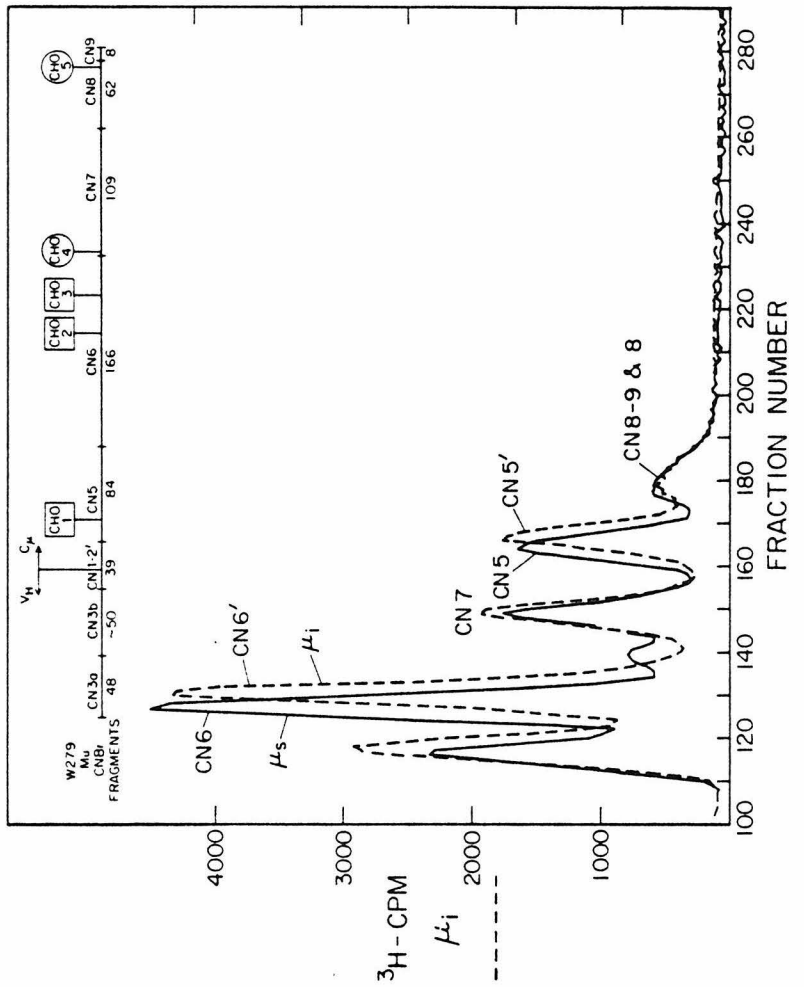

W279
Mu
CNBr
FRAGMENTS
CN3a
CN3b
CN1-2'
CN5
CN6
CN7
CN8
CN9
CHO 1
CHO 2
CHO 3
CHO 4
CHO 5
48
~50
39
84
166
109
62
8
CN6
CN6'
μ_s
μ_i
CN7
CN5
CN5'
CN8-9 & 8
^{3}H - CPM
μ_i
^{3}H - CPM
μ_s
FRACTION NUMBER

FIGURE 4.

Figure 5. Radiolabeled NH_2-Terminal Sequence of W279 μ_i CN5'

W279 μ_i CN5' labeled with ^{3}H-Phe, Pro, Val, Tyr, and Leu was loaded on the Caltech sequenator in trifluoroacetic acid-H_2O as described in Experimental Procedures. Cold carrier M104E CN5 (2 mg) was included. The Pth-amino acids at each step were separated by HPLC and counted for radioactivity. The yield of Leu at step 3 is lower than that at step 8 due to failure of the automatic conversion function for the first 5 steps in the sequenator. Steps 1 to 5 were converted manually, reducing the yield. The sequence is given in Table 2.

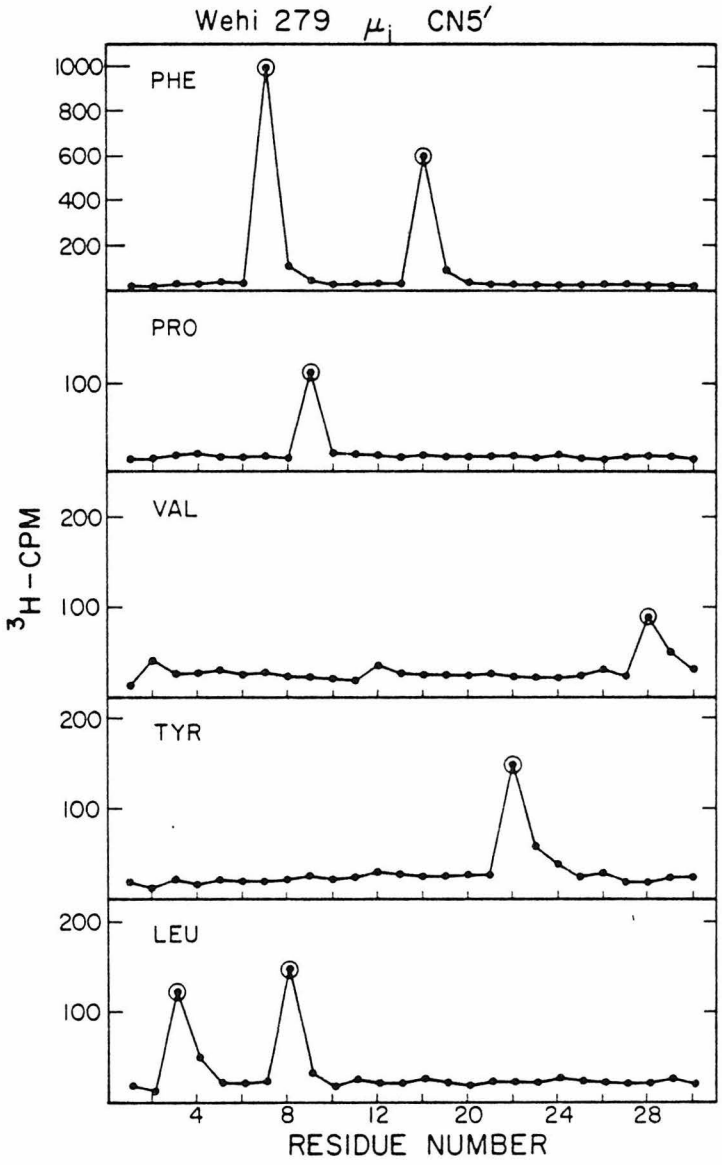

Wehi 279 μ_i CN5′
PHE
PRO
VAL
TYR
LEU
1000
800
600
400
200
100
^{3}H-CPM
4
8
12
20
24
28
RESIDUE NUMBER

FIGURE 5.

Figure 6. Comparative Peptide Mapping of ^{35}S-Met Labeled μ_i and μ_s Chains

^{35}S-Met labeled W279 μ chains were combined with 0.25 mg pig IgG and cleaved with trypsin followed by exhaustive digestion with chymotrypsin as described in Experimental Procedures. Peptides were separated by HPLC (McMillan et al., 1979). Fraction volume is 0.5 ml (0.5 min/fraction). (.....) gradient of acetone used to elute peptides. ^{35}S-Met labeled W279 μ_i (-----); ^{35}S-Met labeled MxW μ_s (——).

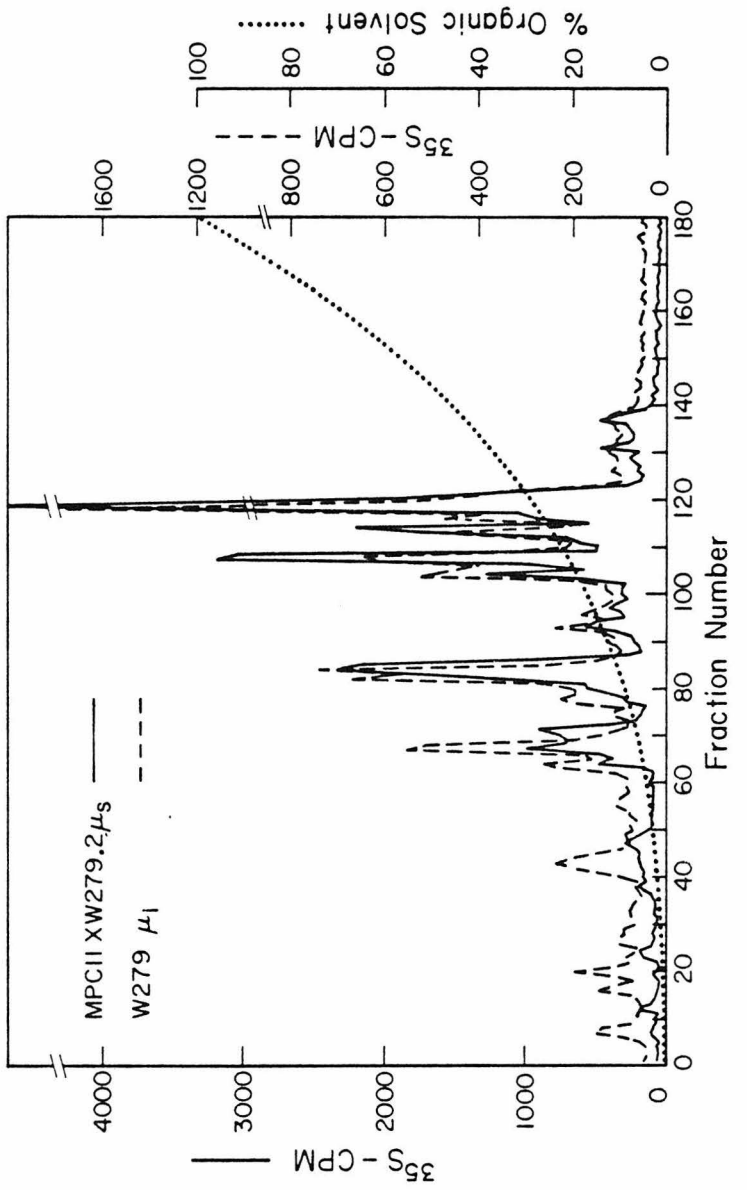

MPC11 XW279.2μs
W279 μl
35S - CPM
35S - CPM
% Organic Solvent
Fraction Number
0
1000
2000
3000
4000
200
400
600
800
1200
1600
20
40
60
80
100
120
140
160
180

FIGURE 6.

Figure 7. Location and Order of W279 Cyanogen Bromide Fragments

Schematic drawing of the W279 μ_i chain showing the sites of cleavage by cyanogen bromide and the respective fragments in the V_H and C_μ regions as compared to M104E μ_s chains. The location of methionine residues in the W279 V_H region is very different from that in the M104E V_H region. The sequence of W279 μ_i CN9 has not been determined. Its identity to M104E CN9 is based on the alignment of a tyrosine-labeled cyanogen bromide fragment with the gel filtration pattern of M104E μ_s. Therefore, CN9 has not been included in the diagram. Crosshatched regions indicate the regions where a radiolabeled sequence has been determined for W279 μ_i chains (^{3}H-Phe, Pro, Val, Tyr and Leu for all μ_i fragments, ^{3}H-Lys and Ala for CN7 only). * The radiolabeled sequence of MxW μ_s chains was also determined for these cyanogen bromide fragments. ‡ Includes the sequence of MxW CN8 and CN8-9. See Table 2.

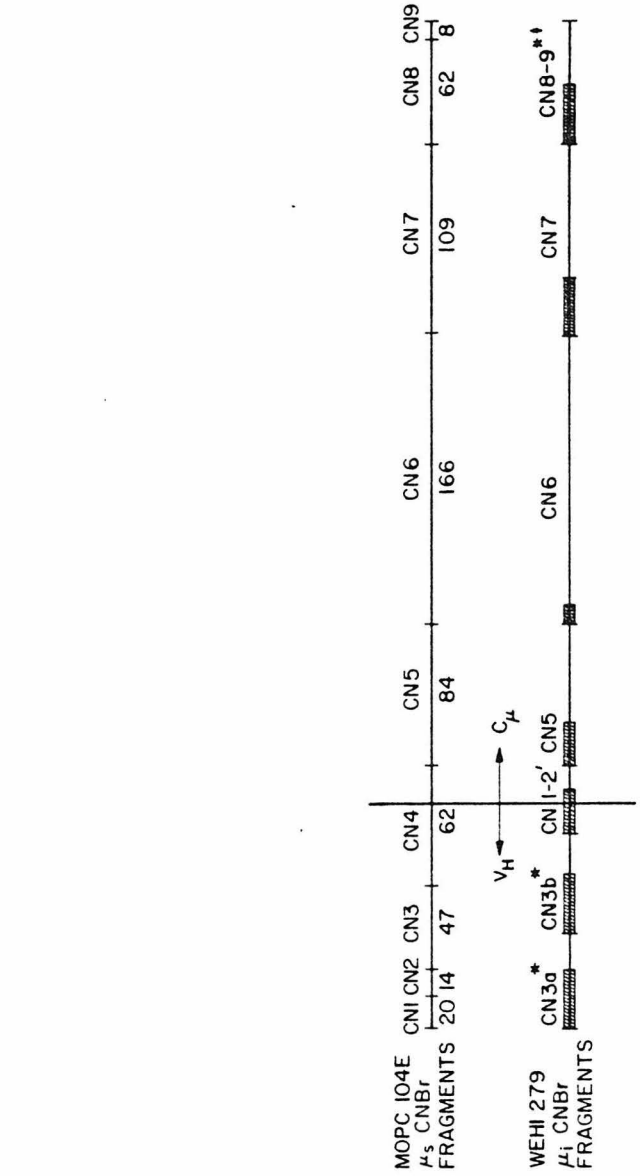

MOPC 104E
μ_s CNBr
FRAGMENTS
CNI CN2 CN3 CN4 CN5 CN6 CN7 CN8 CN9
20 14 47 62 84 166 109 62 8
V_H
C_μ
WEHI 279
μ_i CNBr
FRAGMENTS
CN3a* CN3b* CN 1-2' CN5 CN6 CN7 CN8-9*'

FIGURE 7.

Figure 8. Comparison of NaB^3H_4-Galactose Oxidase Labeled Cyanogen Bromide Peptides of μ_m and μ_s by Gel Filtration

Galactose residues of cell surface W279 μ_m chains were labeled with NaB^3H_4 as described in Experimental Procedures. Pentameric M104E IgM was similarly labeled in solution. The μ_m and μ_s chains were isolated, cleaved with cyanogen bromide and separated as described. The column runs were normalized to internal standard M104E μ_s cyanogen bromide fragments. The peak running with the excluded volume consists of aggregated material. Free salt is eluted around fraction 285. Fraction volume is 5 ml.

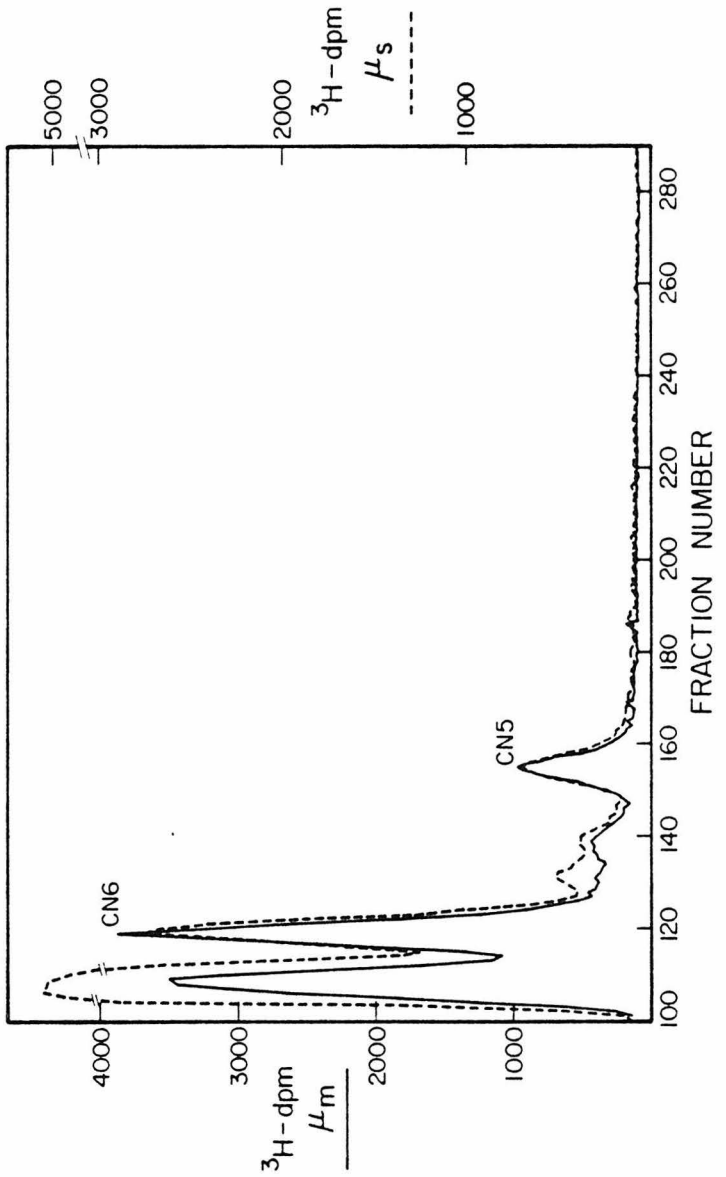

5000
3000
2000
1000
^{3}H-dpm
μ_s
4000
3000
2000
1000
^{3}H-dpm
μ_m
CN6
CN5
100
120
140
160
180
200
220
240
260
280
FRACTION NUMBER

FIGURE 8.

Figure 9. Comparison of ^{3}H-Mannose Labeled Cyanogen Bromide Peptides of μ_m and μ_s Chains by Gel Filtration

See legend to Figure 4. ^{3}H-Mannose cyanogen bromide fragments of W279 μ_m (---) and M104E μ_s (——).

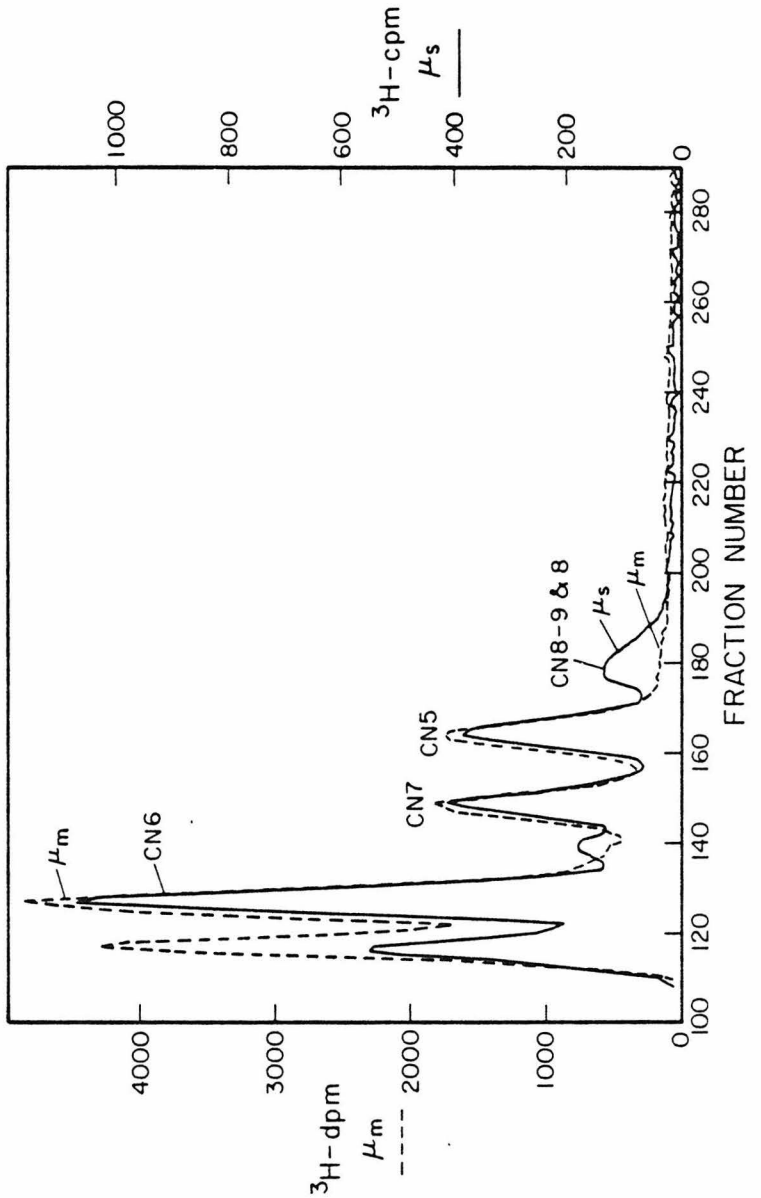

^{3}H-dpm
μ_m
^{3}H-cpm
μ_s
FRACTION NUMBER
μ_m
CN6
CN7
CN5
CN8-9 & 8
μ_s
μ_m

FIGURE 9.

Figure 10. Glycopeptides of μ_m and μ_i Chains Are Different

^{3}H-Mannose labeled W279 μ chains were prepared as described, cleaved with trypsin and chymotrypsin and the glycopeptides separated by HPLC. Fraction volume is 0.5 ml (0.5 min/fraction). (....) gradient of acetone used to elute peptides. ^{3}H-Mannose labeled W279 μ_i (----); ^{3}H-mannose labeled W279 μ_m (——). The major peptide difference is indicated by crosshatching of the peak. The flow-through peak from the column which is seen only in μ_i is not included as a peptide difference.

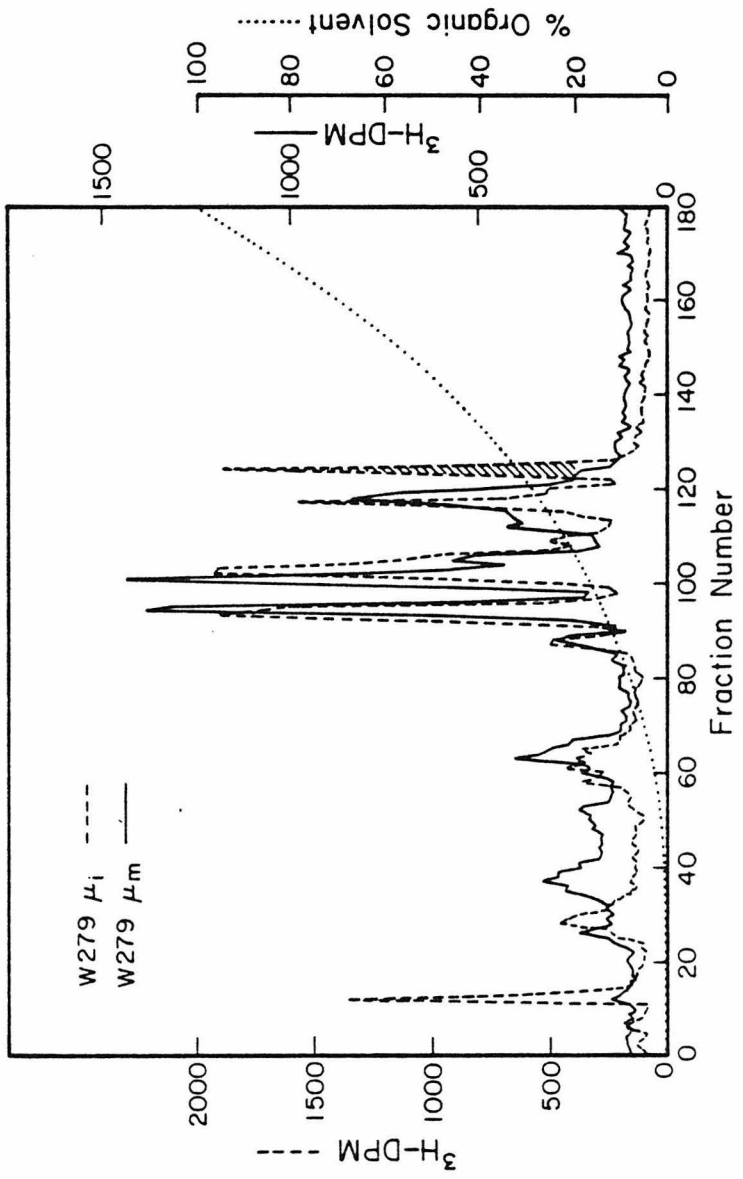
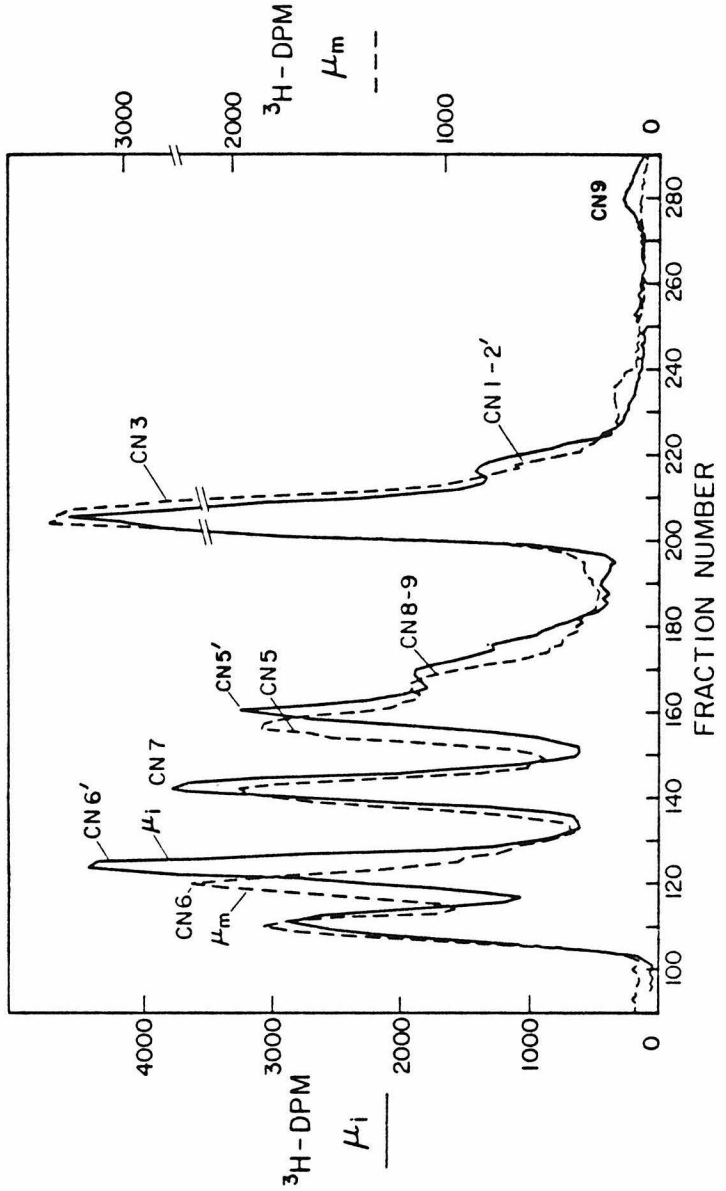

% Organic Solvent .....
^{3}H-DPM ——
^{3}H-DPM ----
W279 μ_i ----
W279 μ_m ——
Fraction Number

FIGURE 10.

Figure 11. Comparison of ^{3}H-Amino Acid Labeled Cyanogen Bromide Peptides of μ_i and μ_m Chains by Gel Filtration

W279 cells were incubated with either ^{3}H-Tyr and Leu (μ_m) or ^{3}H-Tyr, Leu, Ala, and Val (μ_i). The μ_i and μ_m chains were isolated, reduced, alkylated, cyanogen bromide cleaved and the peptides separated by gel filtration as described. Aliquots of isolated μ_i and μ_m chains used for the cyanogen bromide cleavage were rerun on two-dimensional SDS gels to check the purity. The μ_m and μ_i chains were always 90% free of cross-contamination. The individual column runs were aligned by normalization to internal standard M104E μ_s chain cyanogen bromide fragments. The peak running with the excluded volume consists of aggregated material. Free salt is eluted around fraction 285 (just after CN9). Fraction volume is 5 ml. ^{3}H-amino acid cyanogen bromide fragments of W279 μ_m (---) and μ_i (——). 0.2 ml aliquots were counted for μ_i and 0.4 ml aliquots for μ_m through fraction 250; 0.7 ml aliquots were counted for fractions 251-290. Cyanogen bromide fragments differing in molecular weight from M104E μ_s cyanogen bromide fragments are denoted by a ' (prime). CN8 and CN8-9 in μ_i and CN8-9 in μ_m are not distinct peaks due to the migration of a V region incomplete Met-Ser cleavage product (CN3b-CN1-2', detected by sequence determination) between CN5 and CN8-9.


^{3}H-DPM
μ_m

3000
2000
1000
0
CN 3
CN 1-2'
CN 9
CN 8-9
CN 5'
CN 5
CN 7
CN 6'
μ_i
CN 6
μ_m
4000
3000
2000
1000
0
^{3}H-DPM
μ_i
100
120
140
160
180
200
220
240
260
280
FRACTION NUMBER

FIGURE 11.

Figure 12. Comparative Peptide Mapping of ^{35}S-Met Labeled μ_m and μ_s Chains

See legend to Figure 6. ^{35}S-Met labeled W279 μ_m (---); ^{35}S-Met labeled MxW μ_s (——). Peptide differences are indicated by crosshatching of the peaks.

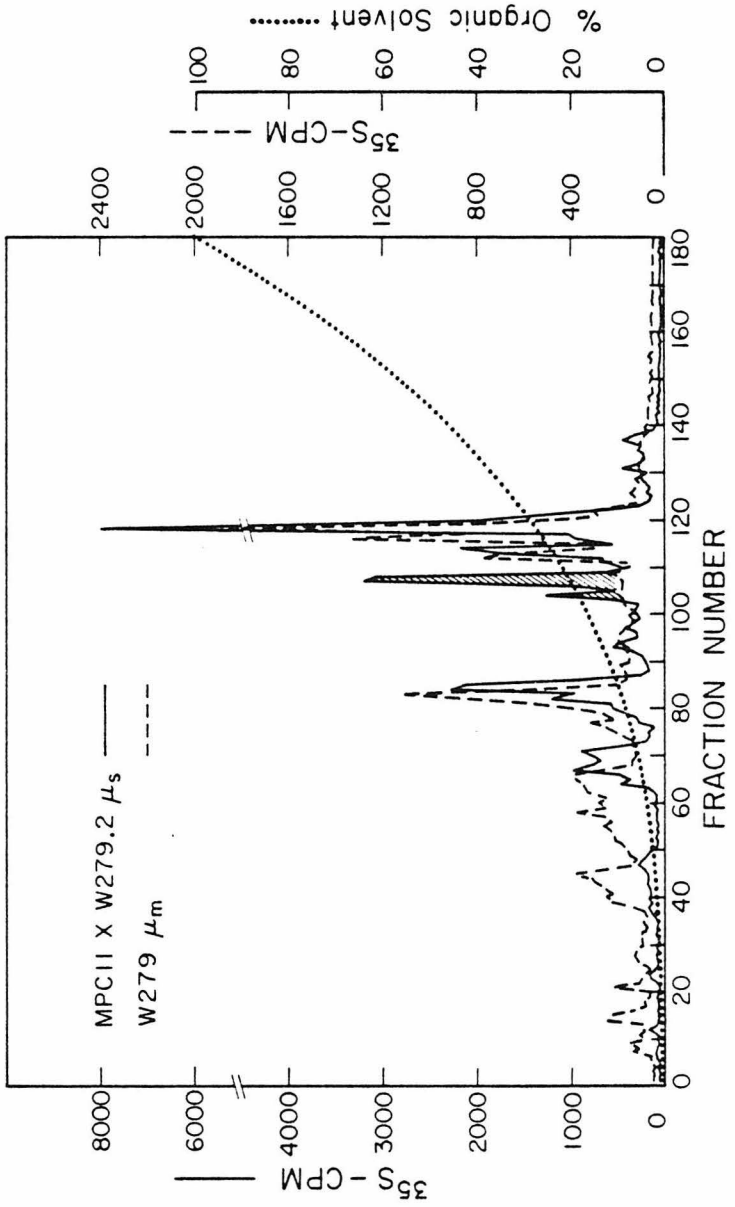

MPC11 X W279.2 μ_s ——
W279 μ_m ----
^{35}S - CPM ——
^{35}S-CPM ----
% Organic Solvent .....
FRACTION NUMBER
0
1000
2000
3000
4000
6000
8000
400
800
1200
1600
2000
2400
20
40
60
80
100
120
140
160
180

FIGURE 12.

Figure 13. Comparative Peptide Mapping of ^{3}H-Phe Labeled μ_m and μ_s Chains See legend to Figure 6. ^{3}H-Phe labeled W279 μ_m (---); ^{3}H-Phe labeled MxW μ_s (——). Peptide differences are indicated by crosshatching of the peaks.

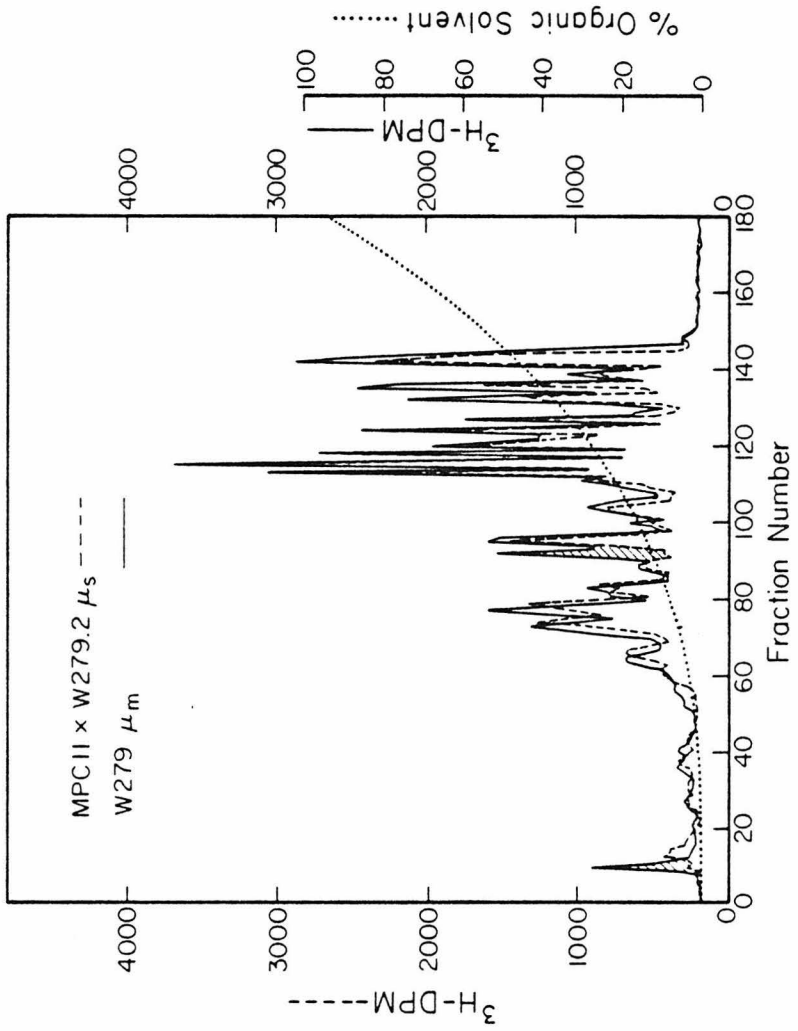

MPC11 × W279.2 μ_s ----
W279 μ_m ——
^{3}H-DPM-----
^{3}H-DPM ——
% Organic Solvent .....
Fraction Number
0
20
40
60
80
100
120
140
160
180
1000
2000
3000
4000
20
40
60
80
100

FIGURE 13.

Figure 14. Release of Amino Acids from μ_m, μ_i and μ_s Chains upon Digestion with Carboxypeptidases A and B

W279 μ_i and μ_m chains and MxW μ_s chains labeled with ^{3}H-Tyr, Val, Lys, Phe and Leu were digested with carboxypeptidases A and B for varying times as described in Experimental Procedures. The μ chains labeled individually with each amino acid were pooled for the digestion, so that the quantitation of residues released is based on radioactivity in the original undigested μ chain, the proportion of the total sample removed and loaded on the amino acid analyzer for each time point, and the number of residues of each amino acid present in the W279 μ chain [22 Tyr, 45 Val, 31 Lys, 22 Phe, and 46 Leu (Kehry et al., 1979)]. Controls incubated for 2 hr on μ_m, μ_i and μ_s chains consisted of an undigested aliquot to which boiled carboxypeptidases A and B were added. No amino acids were released in any of the control incubations. A. Release of COOH-terminal amino acids from W279 μ_m chains. B. Release of COOH-terminal amino acids from W279 μ_i chains. C. Release of COOH-terminal amino acids from MxW μ_s chains; these chains were not labeled with ^{3}H-Lys and Leu.

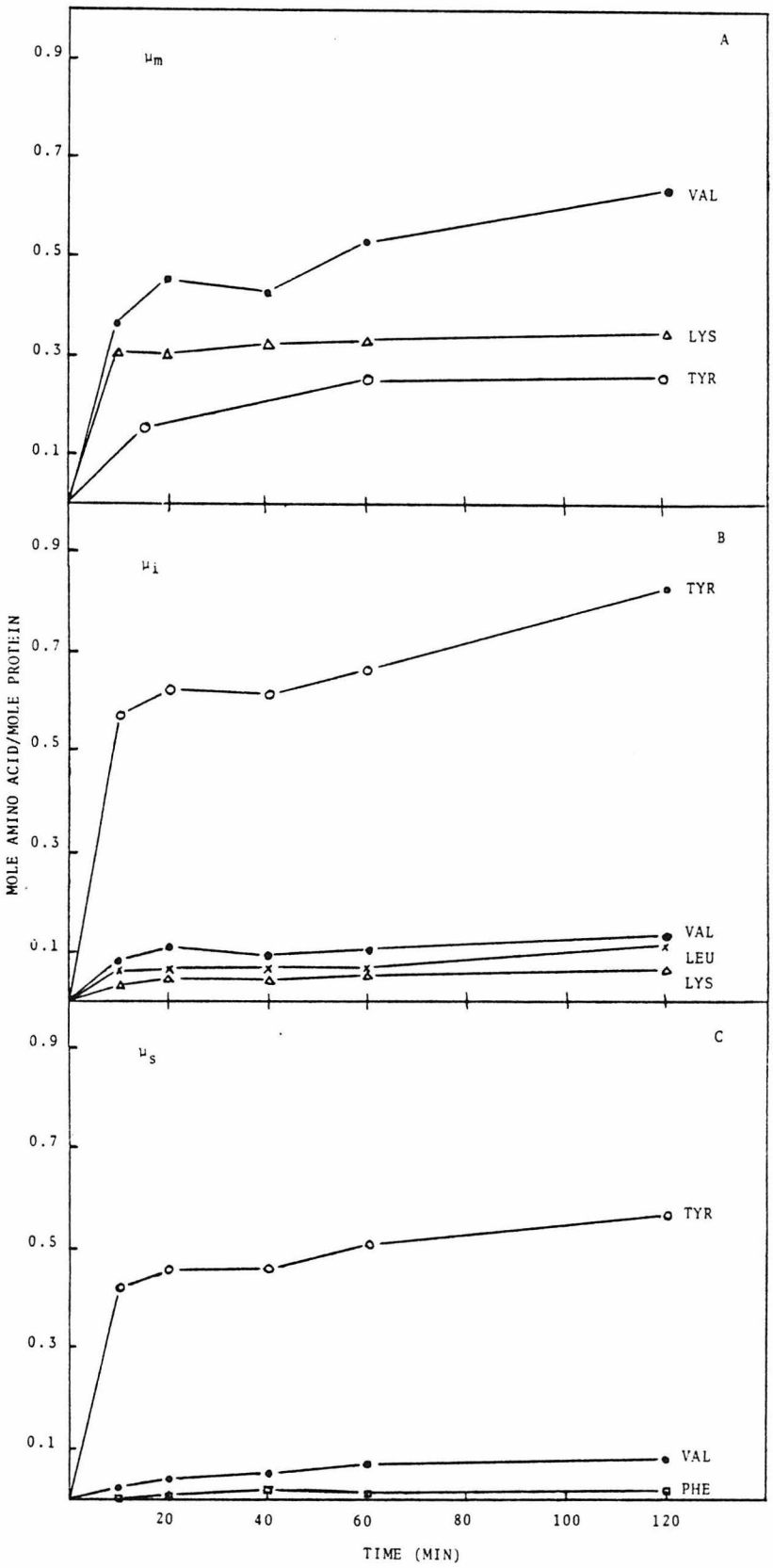

A
μ_m
VAL
LYS
TYR
B
μ_i
TYR
VAL
LEU
LYS
C
μ_s
TYR
VAL
PHE
0.9
0.7
0.5
0.3
0.1
MOLE AMINO ACID/MOLE PROTEIN
20
40
60
80
100
120
TIME (MIN)

FIGURE 14.

Figure 15. Schematic Drawing of the Structure of μ_m, μ_s and μ_i Chains

The V_H and four C_μ domains are represented for the μ_m (left), μ_s (center), and μ_i or precursor μ_s (right) chains. The C-terminal (C_t) segment of μ_m chains is shown as being longer and different in amino acid sequence (crosshatching) from the C_t segments of μ_s and μ_i chains. CHO indicates the sites of carbohydrate attachment in the μ chains with boxes representing complex type carbohydrate moieties (dashed lines showing complex type carbohydrates lacking terminal sugars) and ovals representing high mannose carbohydrate moieties.

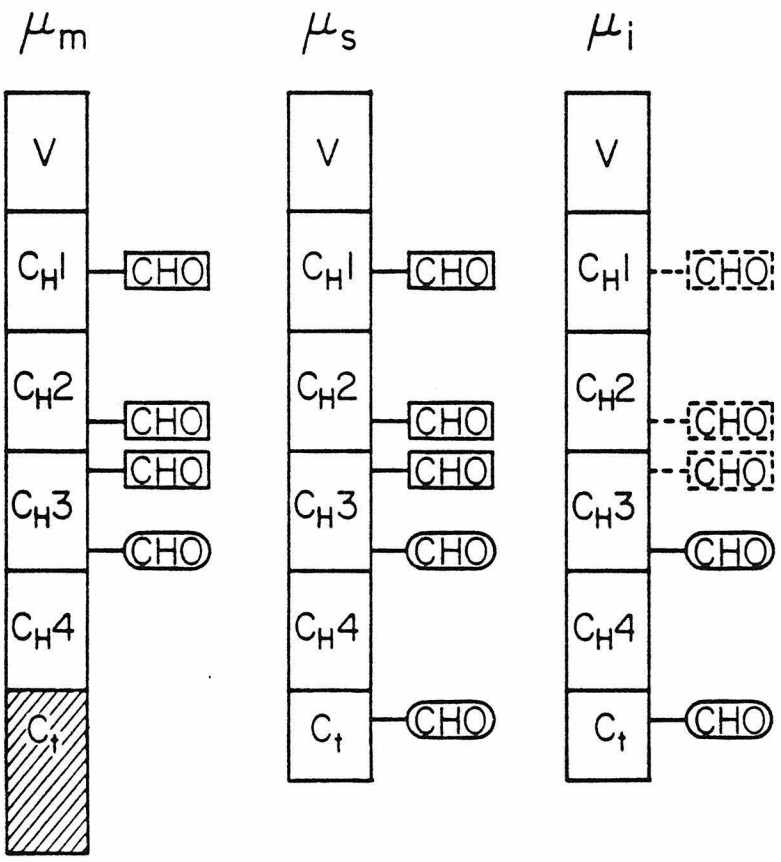


FIGURE 15.

Figure 16. Amino Acid Sequences of the C-Terminal Regions of μ_m and μ_s Chains

The amino acid sequence of the C-terminal region of μ_s chains (Kehry et al., 1979) and the predicted amino acid sequence of the C-terminal membrane segment of μ_m chains (Early et al., 1980; Rogers et al., 1980) are compared. The junction between the $C_\mu 4$ domain and the C-terminal domain is delineated. Boxed residues are described in the text. CHO indicates the site of carbohydrate attachment. A key to the single letter amino acid code is provided in Dayhoff (1976).

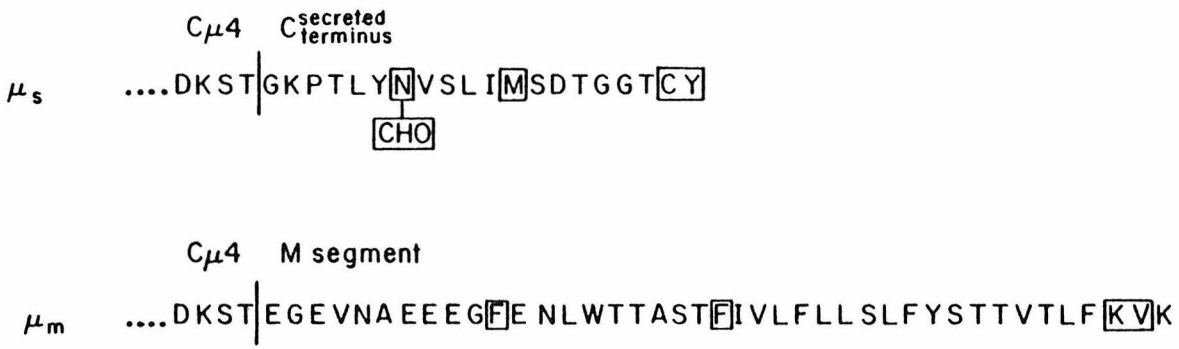

Cμ4
C secreted terminus
μs
....DKST|GKPTLYNVSLIMSDTGGTCY
CHO
Cμ4
M segment
μm
....DKST|EGEVNAEEEGFE NLWTTASTFIVLFLLSLFYSTTVTLFKVK

FIGURE 16.

CONCLUSION

IgM Structures and Functions

The structure of secreted IgM is well adapted to the functions performed by this 19S IgM molecule. The tertiary structure of the pentamer seems to be largely determined by the structure of the secreted μ (μ_s) chain. As is summarized in Chapter 1, μ_s chains are composed of four C region domains in addition to a 20 amino acid COOH-terminal domain (1). Primary amino acid sequence homology among μ_s chains from different species reveals that the more COOH-terminal regions are strikingly conserved (2). In accordance with this high degree of structural similarity among such diverse species as mouse, human and dog, the $C_\mu 4$ and C-terminal regions perform specific effector functions of the IgM molecule.

The C-terminal domain plays a clear role in polymer formation. Only one other class of heavy chains contains a 20 amino acid C-terminal region. This is the α chain, the heavy chain present in IgA (3). IgA is the only other antibody class which is polymerized (usually found as dimer or trimer secretory IgA). The COOH-terminal 20 amino acids of human μ and α chains are 65% homologous (3, 4) including the location of a high mannose carbohydrate. In both α and μ chains, the penultimate cysteine residue is involved in formation of the covalent disulfide bridges between the heavy chains of monomers and between heavy and J chains. IgM and IgA are also the only antibody molecules which are polymerized with the J chain (5).

The highly conserved $C_\mu 4$ domain of IgM pentamers plays an important part in activation of the classical complement pathway after antigen binding. A region has been localized in $C_\mu 4$ which is thought to be the site involved in the binding and activation of the first component of complement (6). It is notable that antigen binding by a single IgM pentamer is sufficient to trigger the entire complement cascade (7), culminating in the lytic destruction of foreign cells (8, 9). IgG molecules may also activate complement, but only when at least two monomers

are aggregated by antigen binding (7). Thus, 19S IgM is an efficient "pre-aggregated" effector antibody.

In addition, certain types of antigens elicit predominantly IgM responses. This so-called "T-independent" antigenic stimulation occurs primarily when the immunogen consists of large multivalent complexes, most commonly polysaccharides, for example constituents of bacterial cell walls (10). The polyvalent IgM made in response to such a multivalent antigen is therefore appropriately designed to efficiently eliminate the foreign substance from the body.

The function of the five carbohydrate moieties attached to each μ_s chain in the Cμ region is unclear at present. Perhaps the oligosaccharide moieties contribute to the solubility of the IgM molecule in the bloodstream. Certainly secreted μ_s chains are relatively insoluble when compared with other serum proteins and γ, α or light chains. Perhaps, too, the carbohydrate contributes to the overall structural conformation of the IgM molecule, even rendering it resistent to proteolytic digestion (11, 12). In addition, the carbohydrate could determine plasma clearance of IgM or its intracellular transport.

The only other antibody class which contains an additional C_H region domain is the IgE class (1). Again, the significance of four C_H domains in IgM and IgE molecules is unclear. Since individual immunoglobulin domains probably do perform discrete functions (13), we would expect IgM and IgE molecules to have a common function. One similarity is that both IgM and IgE molecules can be found attached to the surfaces of cells. IgM is bound to the membrane of resting B lymphocytes and IgE binds to the surface of mast cells via an Fc receptor (14). In both cases, the V regions are exposed to the external mileau for the purpose of antigen binding (13, 14). Indeed, the longer C_H region may allow the cell surface IgE and IgM molecules to extend outwards from the cell membrane, thereby facilitating antigen interaction. In addition, the lack of a hinge region in IgM molecules

may serve to enforce a rigidity which could be important in the functioning of the membrane receptor IgM molecule.

This brings us to the specific structural adaptations incorporated into the membrane IgM molecule. Logically, the most efficient structure for membrane receptor molecules which have the ability to recognize foreign substances and elicit the production of specific antibodies is the antibody molecule itself. What other macromolecule could be designed to convey such exquisite specificity and capacity for memory to the B cell surface? Membrane IgM incorporates all the structural features which characterize the major antigen binding and effector functions of the IgM pentamer. Only in one aspect do membrane and secreted IgM molecules differ structurally. The region governing polymerization is absent in membrane μ (μ_m) chains (15, 16, 17). Instead, μ_m chains possess a C-membrane terminal region COOH-terminal to the $C_\mu 4$ domain. This extra stretch of 40 amino acids seems well suited for interaction with the hydrophobic lipid bilayer. A central region of 25 uncharged amino acids is flanked by groups of charged residues (16, 17). The uncharged amino acids in μ_m chains are capable of forming an alpha helix structure sufficient in length to span the lipid bilayer (18, 19), and these amino acids may serve as two membrane "anchors" for each monomeric IgM molecule. Charged residues present on the immediate interior or exterior of the plasma membrane may further stabilize membrane attachment by interacting with charged lipid heads and prevent the newly synthesized μ_m chain from passing through to the interior of the endoplasmic reticulum membrane.

All but one of the sites of carbohydrate attachment are identical in μ_s and μ_m chains (15). The fifth oligosaccharide, attached to the C-terminal region in μ_s chains, is absent in μ_m chains due to the absence of an Asn-X-Ser or Thr recognition sequence in the C-membrane terminal domain (16, 17). The function

of the fifth carbohydrate must then in some way relate to the polymerization and/or structure of the pentamer, since polymeric IgA molecules also have an identical carbohydrate moiety in the C-terminal region of the α chains (3, 20). The remaining carbohydrates on the μ chain must therefore be required directly or indirectly for maintaining IgM structure related to antigen binding and may be required for carrying out effector functions.

IgM Expression: The C_μ Gene

B cells, then, are remarkably efficient in their mechanism of switching from membrane to secreted IgM expression. Regulation may occur at multiple levels: transcriptional, mRNA processing, translational, and/or post translational. Transcriptional control is exerted in the initiation of μ chain synthesis (including allelic exclusion) by a pre-B cell (16, 17). This step is probably related to the complex DNA rearrangements which occur in moving the V_H gene closer to the C_μ gene (16, 17). Knowledge of the control steps involved is sparse.

Since the C_μ gene is present in only one copy per haploid genome (16, 21), each μ gene primary transcript has the option of being processed for either μ_s or μ_m chain expression. In the germline C_μ gene, the coding regions for the $C_\mu 4$ and secreted C-terminal domains are contiguous (16, 22). 3' to the secreted C-terminal coding region is an 1800 nucleotide intervening sequence followed by a region of DNA encoding the C-membrane terminus (16). Expression of μ_m chains simply entails removing the intervening nucleic acid sequences from the primary RNA transcript between the end of the $C_\mu 4$ region to the beginning of the C-membrane terminal region (17). Removal of the secreted C-terminal sequence is mediated by an RNA splicing site located in the coding region of the secreted C-terminal domain (16, 17). Therefore, μ_s mRNA expression is obtained by removing RNA sequences 3' to the secreted C-terminal domain. Plasma cells have differentiated so as to process the majority of μ gene transcripts in the

latter fashion. Presumably, this differentiation involves expression of specific RNA splicing enzymes.

Many B cell tumor lines, though, seem to synthesize equal quantities of μ_m and μ_s mRNA (23, 24). Yet in some of these cells, μ_s chains are synthesized and turn over at a much more rapid rate than μ_m chains (25). Clearly, translation of the μ_s and μ_m mRNA pools is not equally efficient. In addition, synthesis of J chain is partially limiting for IgM polymerization and secretion. We are now able to offer an explanation for how antigen stimulated B cells secrete IgM without initially requiring RNA transcription (26), and how they may develop into IgM secreting blast cells in the absence of DNA synthesis (27). Activation of J chain mRNA translation would allow rapid maturation and polymerization of the pre-secreted pool of μ chains inside these cells. In addition, the relative stabilities of μ_m and μ_s mRNA following antigenic stimulation may change to favor μ_s synthesis. Changes accompanying large-scale IgM secretion, though, would require transcription of additional copies of μ_s mRNA (26).

Finally, posttranslational modifications, in particular glycosylation, may also regulate the rate of IgM maturation and secretion. Carbohydrate must represent an important aspect in IgM structure and/or function, since exact locations of oligosaccharide residues have been conserved in mouse and human μ chains throughout evolution (2). Treatment of plasma cells with the glycosylation inhibitor, tunicamycin, produces an 80% inhibition of IgM secretion by these cells (28). It is not unreasonable that maturing plasma cells might in part increase the level of IgM secretion by increasing the availability of oligosaccharide precursors or glycosylating enzymes.

Future Questions on B Lymphocyte IgM

Many important aspects of B cell IgM and the control of IgM synthesis

remain to be clarified. This does not even include acquiring knowledge about the molecular mechanisms underlying B cell triggering. Additional studies can be directed towards elucidating the structure and function of the proteins and of the nucleic acids involved in carrying out the B cell differentiation process.

Experiments on B lymphocytes at the protein level address the functional role of IgM in the cell. Do membrane-bound IgM molecules actually have a transmembrane orientation in the plasma membrane? Attempts should be made to label and identify regions of the μ_m chains which may be exposed to the cytoplasm. A related problem is the degree to which the IgM molecule is exposed on the cell surface. Do portions of the $C_\mu 3$ or $C_\mu 4$ domains interact with $C_\mu 3$ or $C_\mu 4$ domains in other IgM monomers as in the pentamer structure, or are there any C_μ domain interactions with unidentified effector molecules? Although not simply interpreted, crosslinking experiments may be used to identify regions of the μ_m chain which specifically interact with the lipid bilayer, with membrane proteins on the cell surface, or with cytoplasmic proteins. Ideally, antigen activated cells would yield more interesting receptor IgM protein associations which are significant in the immune response. The availability of inducible cell lines will be useful in future studies on the topography of membrane IgM molecules in activated cells (29).

The identity of μ_i chains in early pre-B cells is also unknown. Does this μ_i pool consist of pre-μ_m molecules, pre-μ_s molecules or both? The tools, including protein and nucleic acid chemistry, are currently available for investigating this point.

One of the most important considerations, and I believe often overlooked, is that results obtained on B cell tumor lines should be confirmed on normal B cells from spleen, lymph nodes and blood. Tumor cell lines are known to be polyploid and they possess abortive gene rearrangements. Thus, especially in questions

regarding the nature of immunoglobulin genes and their transcription, normal B cell RNA and DNA must also be examined.

Nucleic acid chemistry is already well enough advanced in the murine system to approach several problems in B cell development. Defining the control mechanisms involved in μ_s vs. μ_m mRNA processing would greatly advance knowledge about changes occurring in developing B cells. The subject of C_H switching (expression of a given V_H region with the C_μ, C_δ, C_γ, or C_α genes) also can be examined in greater detail. What is initially responsible for specifically turning on μ gene transcription? What steps are involved in processing and removing the interdomain intervening sequences in antibody mRNAs?

Perhaps even more approachable is the problem of defining when given genes are expressed in the lineage from stem cells to plasma cells. When are J chain genes and light chain genes actively expressed and when does the expression of the $C_{\mu m}$ and C_δ genes cease? Are, in fact, membrane IgM and IgD molecules expressed in plasma cells at low levels? For example, MOPC 104E myeloma cells process 10% of their μ gene transcripts to form μ_m mRNA (16, 17). Is this μ_m gene expression by terminally differentiated plasma cells normal and are these messengers translated and the proteins utilized for specific purposes? Examining nuclear and cytoplasmic mRNA populations from B cells at different developmental stages for the presence of sequences complementary to J, light, μ and δ chain genes may begin to define developmental expression of immunoglobulins. There are obviously many problems remaining to be explored on B cell receptor immunoglobulin molecules.

Triggering B Cell Differentiation

What, then, has our structural information on B cell receptor IgM molecules contributed to the understanding of B cell triggering by antigen? Characterization

of the IgM receptor is only the first step in studying B cell triggering. Now that we know membrane IgM is indeed an integral component of the lymphocyte plasma membrane, we can look for the involvement of IgM in transmembrane signaling processes. This is an enormous step forward. If cell surface IgM had been only peripherally associated with an accessory membrane protein, that secondary protein would have required characterization in order to understand its role in antigen triggering. Therefore, the path is now clear for concentrating on the primary interactions of membrane μ chains with other molecules in the B cell membrane.

The molecular mechanisms involved in antigen-B cell (and T cell) interaction and the mechanisms of triggering of B cell differentiation and proliferation remain wide open to speculation — and to experimentation. Certainly insights into the triggering process will be obtained when we know: 1) What molecules on the T and B cell surfaces and even on macrophages interact with one another? 2) What direct receptor interactions are stimulated by the polyclonal B cell activators, such as LPS, PPD, and lectins? Is this nonspecific form of B cell activation similar to antigen induced triggering? 3) What role in triggering is played by the membrane IgD molecule? Since the majority of IgD molecules are membrane bound [extremely low levels are present in the bloodstream (30)], IgD must function primarily as a cell surface receptor. Do IgD molecules interact specifically with membrane IgM or with other molecules that also interact with IgM? And so on. The next few years should offer exciting opportunities for making new discoveries about immunoglobulins and B lymphocytes.

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APPENDIX

SUPPLEMENTARY FIGURES

Included in the appendix are my supporting and unpublished results consisting of sequence determinations, representative SDS-polyacrylamide gels, preparative and analytical gel filtration and HPLC peptide maps. These data are not presented with the intention of drawing any new conclusions, but merely for purposes of documentation. A complete description of each figure and a statement as to the relevance of the data are provided. All of the figures except for the sequence determinations are presented in chronological order which parallels the preceding chapters.

Figure 1. Purification of MOPC 104E 19S IgM by gel filtration.

Proteins were precipitated from 40 ml MOPC 104E ascites fluid (grown in BALB/c x DBA/2 mice) at 4°C in two successive cycles by a final concentration of 50% saturated ammonium sulfate each time. The precipitate was dissolved in 13 ml borate buffered saline (BBS) and chromatographed on a column of ACA22 (LKB) (5 x 100 cm) in BBS. Fraction volume was 12 ml. Bar indicates 19S IgM fractions pooled. Excluded volume consists primarily of IgM in an aggregated form. Small peak following the 19S IgM peak consists of α-2 macroglobulin (MW 800,000) and is a major contaminant of the 19S IgM. The arrow indicates position of salt elution. The pooled 19S IgM is rendered at least 95% pure by this one step procedure, as assessed by 10% polyacrylamide-SDS gel electrophoresis (1).

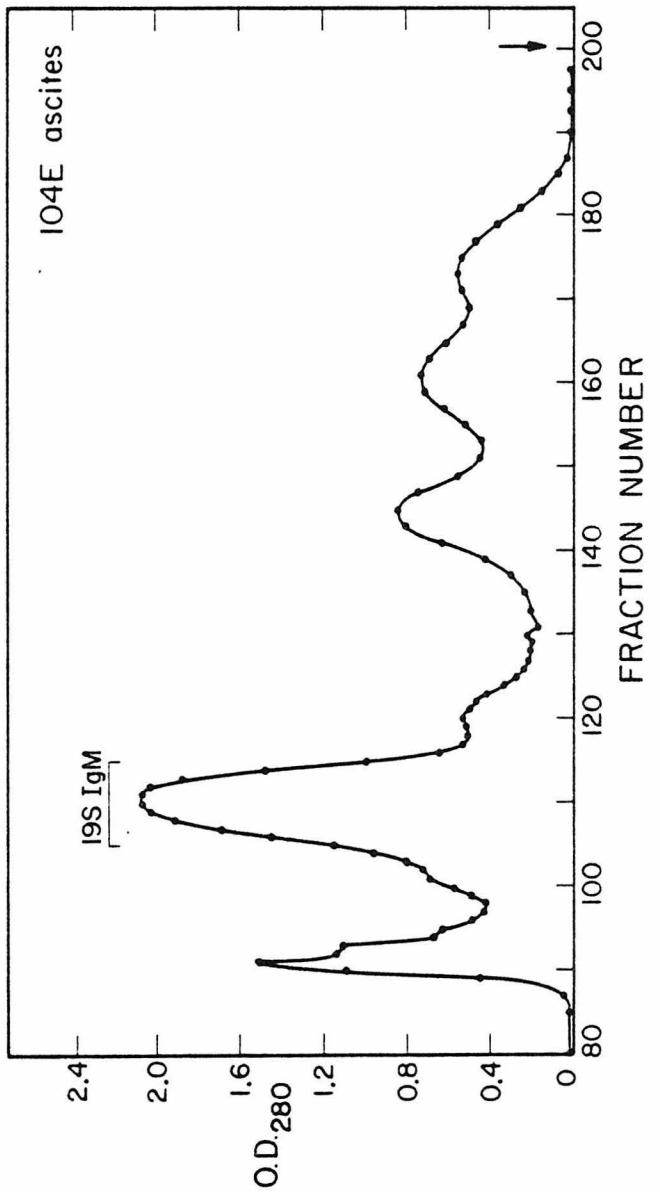

104E ascites
19S IgM
O.D.280
2.4
2.0
1.6
1.2
0.8
0.4
0
80
100
120
140
160
180
200
FRACTION NUMBER

FIGURE 1.

Figure 2. Separation of H and L chains of MOPC 104E 19S IgM by gel filtration.

Pooled 19S IgM in BBS was precipitated at 4°C by 50% saturated ammonium sulfate and the precipitate dissolved in 6 M guanidine-HCl, 0.25 M Tris-HCl pH 8.5, 0.15 M NaCl, 0.02 M EDTA. Disulfide bonds were completely reduced with 0.01 M dithiothreitol (DTT) for 1-1/2 hr at 37°C under N_2. Free thiol groups were then alkylated with 0.025 M iodoacetamide (3X recrystallized) overnight in the dark at 4°C. Guanidine concentration was diluted to 4 M by addition of 0.4 M ammonium bicarbonate, and μ and light chains were separated on a column of ACA34 (LKB) (5 X 100 cm) in 3 M guanidine-HCl, 0.2 M ammonium bicarbonate, 0.02% NaN_3. Fraction volume was 10 ml. Bars indicate μ and light chain fractions pooled. The arrow shows the position of salt elution. Contaminating α-2 macroglobulin subunits (MW 90,000) were removed by this procedure. The pooled μ chain is at least 95% pure as assessed on 10% polyacrylamide-SDS gels (1).

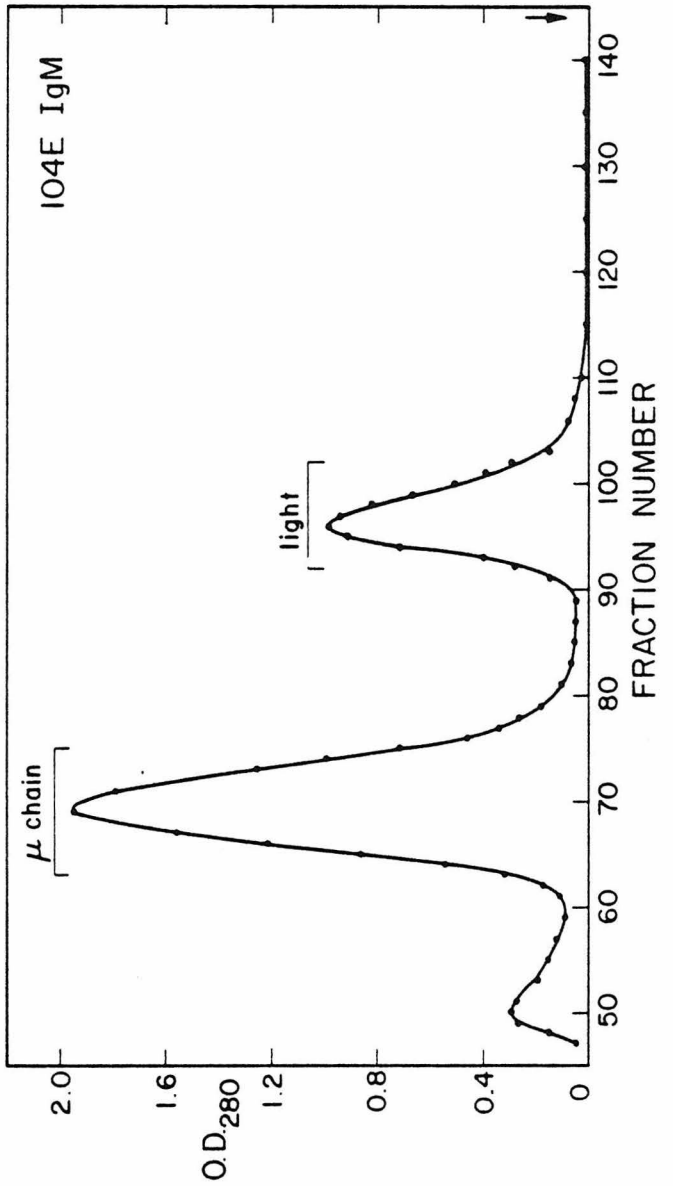

104E IgM
μ chain
light
O.D.280
2.0
1.6
1.2
0.8
0.4
0
50
60
70
80
90
100
110
120
130
140
FRACTION NUMBER

FIGURE 2.

Figure 3. Separation of ^{14}C-Cys CNBr fragments of MOPC 104E μ_s chains.

IgM was purified and disulfide bonds reduced as described in Figures 1 and 2. The free thiol groups were then alkylated with 50 μCi iodo (1-^{14}C) acetamide (Amersham) for 30 min in the dark at 4°C. Iodoacetamide (3X recrystallized) was added to 0.025M and the reaction incubated overnight. Heavy and light chains were separated as described in Figure 2. Pooled ^{14}C-Cys alkylated μ chains were concentrated (Millipore Molecular Separator) neutralized with 88% formic acid and desalted on a column of Bio Gel P-2 (2.5 x 40 cm) in 50% formic acid at 4°C. After concentration by partial lyophilization and adjustment of the formic acid concentration to 70%, the μ chains were cleaved with CNBr (50 mg/ml) for 20 hr in the dark at 4°C with constant stirring. After 11-fold dilution with water and lyophilization, peptides were dissolved sequentially in 8M guanidine-HCl, 3M guanidine-HCl 0.2M ammonium bicarbonate, and 0.4M ammonium bicarbonate (total volume = 12 ml) and chromatographed on a column of ACA54 (LKB) (3.5 x 140 cm) eluted in 3M guanidine-HCl, 0.2 M ammonium bicarbonate, 0.02% NaN_3. Fraction volume = 5 ml. Fifty μl aliquots were counted for radioactivity after reading the OD_{280} of the CNBr peptides. Total radioactivity in each peak is proportional to the number of alkylatable cysteine residues in each fragment. Note that CN3 contains no cysteine residues and is not labeled. CN2 and CN9 were first discovered from this CNBr digestion. The OD_{280} readings were not carried any further than fraction 190 because no peptides were known to chromatograph past CN1-2. Tube numbers 198 and 209 (CN2 and CN9, respectively) were desalted on Sephadex G-10 (Sigma) in 5% formic acid, lyophilized, and the sequences determined. A third peptide, labeled CN3', was also found by this procedure which migrated between CN3 and CN1-2. CN3' was pooled from fractions 157-160 and the complete amino acid sequence determined. The sequence is listed in Figure 10 and is not present in any μ chain. There is also no homology to any immunoglobulin

Figure 3 - Continued

chain. Alignment of this sequence which generates 50% related (not identical) residues can be made with the human J chain sequence, residues 84-116 (2). No information is available on mouse J chain, although the gene has recently been cloned (3).

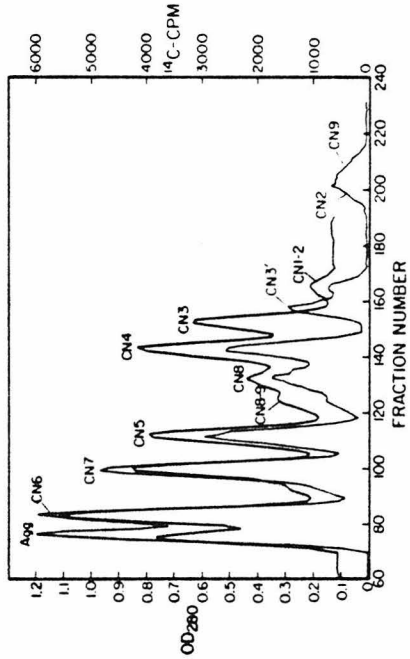

OD280
14C-CPM
FRACTION NUMBER
A99
CN6
CN7
CN5
CN4
CN3
CN8
CN8-9
CN3'
CN1-2
CN2
CN9

FIGURE 3.

Production of Rabbit Antiserum Against MOPC 104E μ Chain

1. Ear bled 45 ml, clotted, retracted and centrifuged. This is normal preimmune serum, 24 ml.

2. Antigen is partially reduced and alkylated MOPC 104E μ chain, sonicated as a fine suspension, 2 mg/ml in Hanks Balanced Salt Solution (HBSS); kept frozen at -20°C.

   2 weeks after pre-bleed: i.m. injection in right thigh with 1 ml emulsification of 0.5 ml CFA and 0.5 ml HBSS (0.15 ml 2 mg/ml μ + 0.35 ml HBSS). 300 μg immunogen total.

3. 1 week after first injection, injected identically in left thigh.

4. 5 weeks after second injection boosted s.c. on multiple sites on back with 1 ml emulsification identical to that used in first injection.

5. Test bled 10 days after boost.

6. Repeated cycle of boosting and bleeding 10 days later at 5 week (minimum) intervals.

Figure 4. Scanning electron micrographs of WEHI 279 cells.

Fixed original clone WEHI 279 cells were attached to poly-L-lysine coated coverslips and prepared for scanning electron microscopy (4). Scale bar is 1 μm.

A. Magnification, X 10,000.

B. Magnification, X 12,000.

The B cell surface is obviously not a smooth sphere, and the density distribution of IgM receptors on the microvilli is unknown.

A

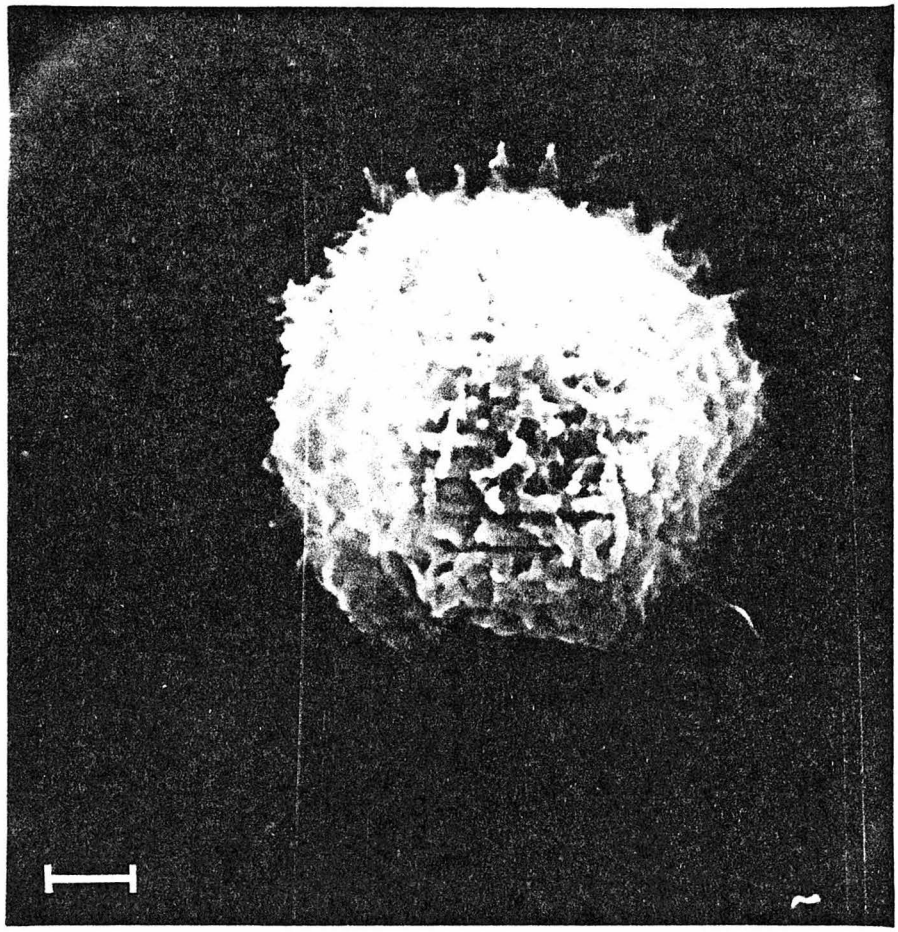


FIGURE 4A.

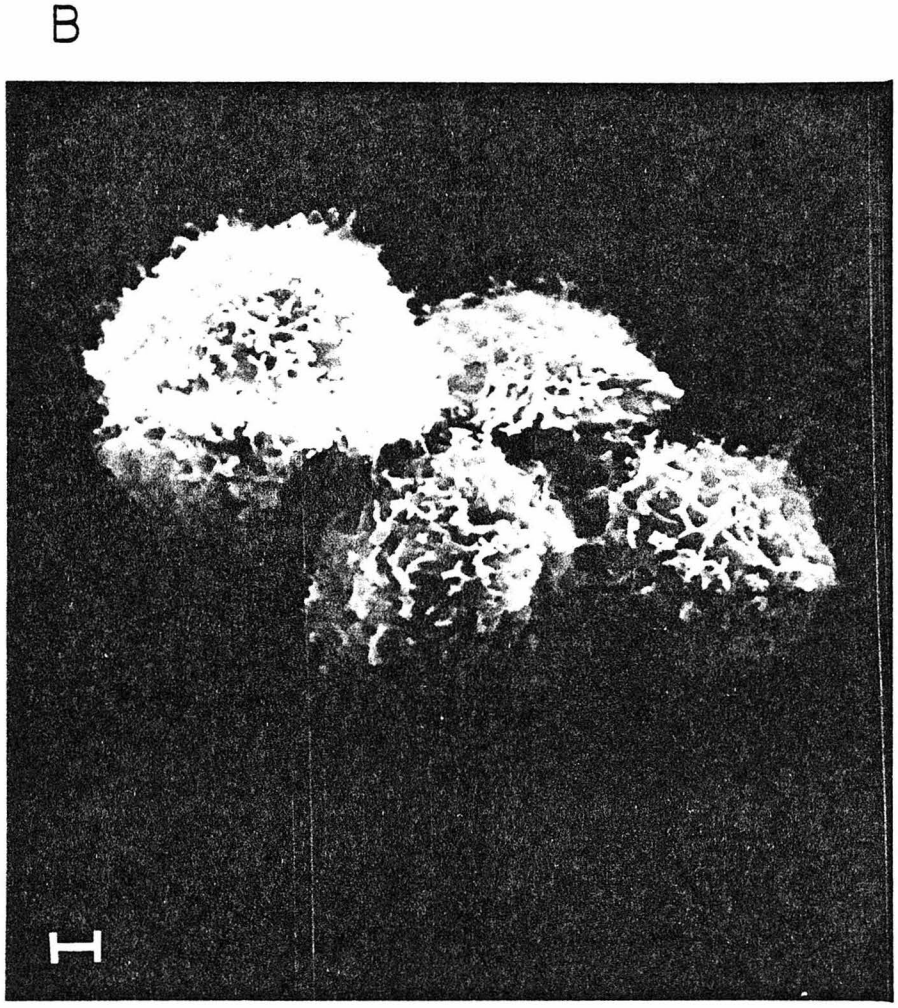


FIGURE 4B.

Figure 5. 10% polyacrylamide gel electrophoresis of WEHI 279 IgM.

WEHI 279 cells were incubated in the presence of ^{3}H-valine for 16 hr (5). Cells were lysed in the presence of 1 mM phenylmethyl sulfonylfluoride (PMSF), and IgM was immunoprecipitated from 100 µl of lysate by incubation with 10 µl rabbit anti MOPC 104E µ chain followed by 100 µl 10% suspension of formalin-fixed Staphalococcus aureus (6). Proteins were eluted in a boiling water bath and electrophoresed on 10% polyacrylamide-SDS gels (1). Gels were sliced into 1 mm sections, the proteins eluted by incubation of each slice in 0.5 ml 0.01% SDS overnight and counted for radioactivity. The bar indicates WEHI 279 light chains and the arrow shows the position of the dye pyronin Y. This is a representative one-dimensional separation of μ_i and μ_m chains, and the valine labeled μ_i and μ_m chains prepared from this lysate were used in carboxypeptidase experiments (7). The peaks around fractions 37-44 are nonspecifically precipitated with the rabbit antiserum, can be removed under more stringent immunoprecipitation conditions (including deoxycholate), and based on two-dimensional gel electrophoresis are partly composed of actin molecules.

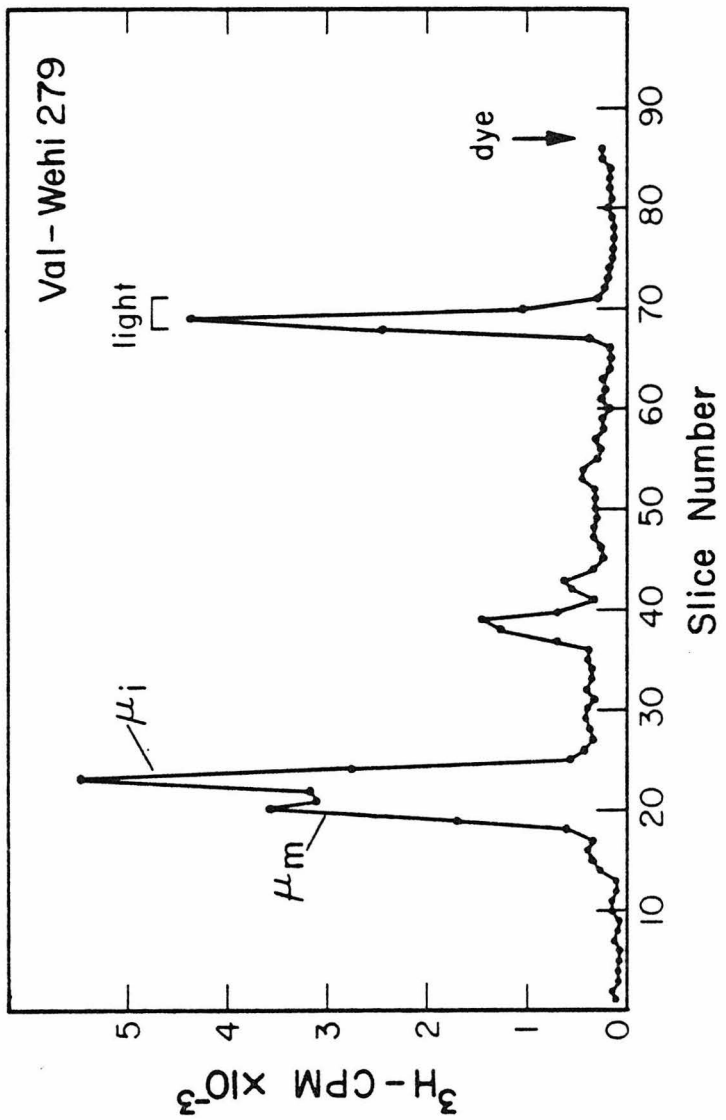

Val-Wehi 279
light
dye
μ_i
μ_m
^3H-CPM x10^{-3}
Slice Number
0
1
2
3
4
5
10
20
30
40
50
60
70
80
90

FIGURE 5.

Figure 6. Two-dimensional gel electrophoresis of WEHI 279 μ chains.

WEHI 279 or MPC11 x W279 cells were incubated in the presence of ^{3}H-tryosine and leucine for 16 hr (5). Cells were lysed, medium collected, and IgM was immunoprecipitated by incubation with rabbit anti μ followed by fixed S. aureus (6). Proteins were eluted from the S. aureus and electrophoresed on 10% polyacrylamide-SDS gels (1) which were sliced into 1 mm sections. The proteins were eluted from the gel slices by incubation of each slice in 0.5 ml 0.5% SDS. Eluted μ chains were pooled, aliquots removed, and the remaining μ chains cleaved with CNBr. To check for purity in separation of the WEHI 279 μ_i and μ_m species, the aliquots were electrophoresed on two-dimensional gels (8) which were processed by fluorography (9). The isoelectric focusing gels contained 5% polyacrylamide, 0.28% bis-acrylamide, 9.2M urea, 2% Triton X-100, 2% pH 3.5-10 ampholines, 0.13% pH 5-7 ampholines, 0.07% pH 3.5-5 ampholines, 0.13% pH 7-9 ampholines, and 0.13% pH 4-6 ampholines (LKB). The focusing gels were equilibrated 30 min, quick frozen and stored at -70°C. Thirty minutes before running the second dimension, gels were thawed and equilibrated in fresh SDS-sample buffer (8). Electrophoresis on 10% polyacrylamide-SDS gels was carried out in the direction of the arrow. Gels were aligned by superimposing the stained carrier MOPC 104E μ_s chains in each gel. Only the region of the two-dimensional gels surrounding the μ chains is shown; the remainder of each gel was blank. Small black dots were made with a needle and used to align the gel and the fluorograph. The regions where μ_i, μ_m and μ_s run are indicated by the bars. Note that less than 5% cross contamination of μ_i and μ_m chains is seen.

A. WEHI 279 μ_i chain. 4 day exposure.

B. WEHI 279 μ_m chain. 4 day exposure. A band corresponding to μ_i is discernible in an 8 day exposure.

Figure 6 - Continued

C. MPC11 x W279 μ_s chain. 3 day exposure. Note that the pI of the μ_s band is shifted slightly to the basic end relative to μ_m. We attribute the shift to inefficient glycosylation of these μ_s chains by the hybrid cell line. When these μ_s chains were cleaved with CNBr (see Figure 9), the CNBr fragments containing complex carbohydrates (CN5 especially) were slightly smaller in molecular weight than those of μ_m or MOPC 104E μ_s (similar instead to WEHI 279 μ_i). This confirms the idea that the hybridoma μ_s chain is not reliably glycosylated.

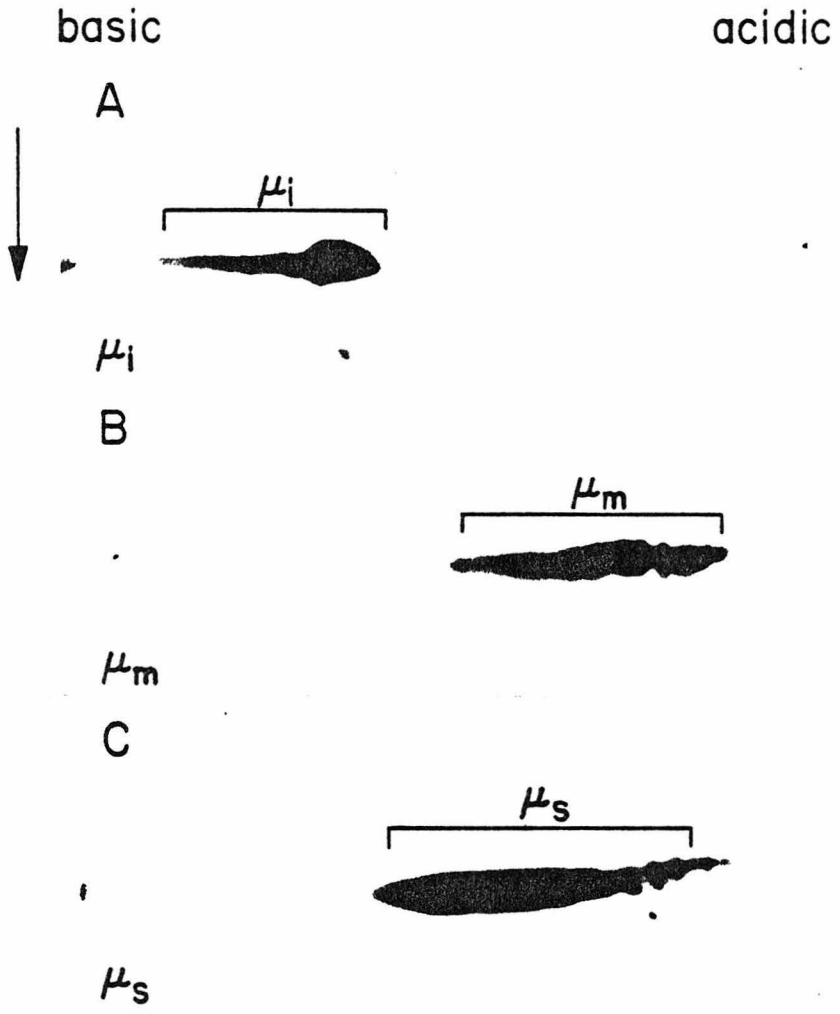


FIGURE 6.

Figure 7. Size of cell associated and secreted MPC11 x W279.2 IgM molecules.

MPC11 x W279.2 cells were incubated in the presence of ^{35}S-Cys for 16 hr (5). Cells were lysed and medium was collected in the presence of 1 mM PMSF. IgM was precipitated by addition of rabbit anti μ followed by S. aureus (6). Proteins were eluted without reduction in a boiling water bath and electrophoresed on 1% agarose, 2.5% acrylamide composite SDS gels (5, 10). Gels were sliced into 1 mm sections, the proteins eluted by incubating each slice in 0.5 ml 0.01% SDS for 24 hr and counted for radioactivity. Positions of pyronin Y dye and of stained marker proteins electrophoresed on a parallel gel are indicated: 104E, 19S MOPC 104E IgM (MW 1 x 10^6); IgG, pig γ globulins (MW 150,000); BSA, bovine serum albumin (MW 65,000).

A. 100 μl cell lysate from MPC11 x W279.2 cells shows the presence of two predominant peaks with MWs of 200,000 and 100,000 corresponding to intracellular 7S monomeric IgM (H_2L_2) and HL monomers, respectively. Note that there is no significant polymerized pool of 19S molecules inside the cells. Polymerization must occur just prior to secretion.

B. 250 μl medium from MPC11 x W279.2 shows the predominance of secreted 19S IgM (40%), 7S IgM (10%), and HL monomers (25%). Additional minor peaks are seen which probably represent intermediate stages in polymerization. Note that less than half of the molecules are covalent pentamers. The remaining species may have dissociated under the stringent S. aureus elution conditions (2% SDS) or may have been secreted incompletely polymerized due to a limiting number of J chain molecules synthesized by the hybrid cells.

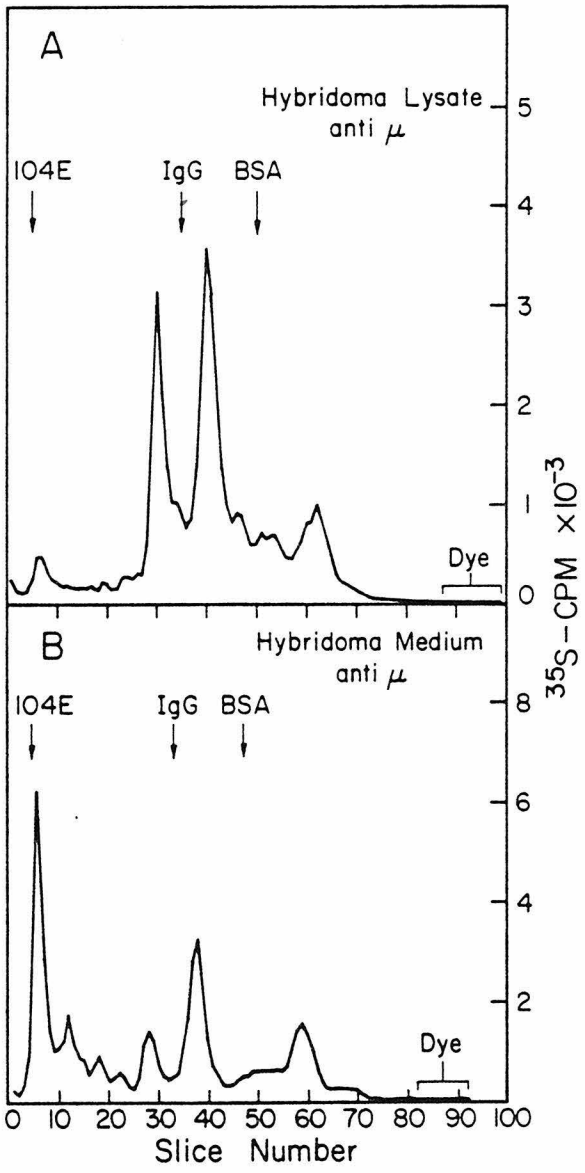

A
Hybridoma Lysate
anti μ
104E
IgG
BSA
Dye
B
Hybridoma Medium
anti μ
104E
IgG
BSA
Dye
0 10 20 30 40 50 60 70 80 90 100
Slice Number
35S-CPM ×10-3

FIGURE 7.

Figure 8. Peptide map comparisons of MPC11 x W279.2 μ_s chains to WEHI 279 μ_i, μ_m and μ_s chains.

μ chains were labeled by incubating cells in the presence of either ^{3}H-mannose, ^{35}S-Met, ^{3}H-Phe, ^{3}H-Tyr or ^{35}S-Cys for 16 hr (5). Cells were lysed, medium collected and μ chains were purified on 10% polyacrylamide gels, reduced, alkylated and digested exhaustively with typsin followed by chymotypsin (7). Peptides were separated by high performance liquid chromatography on a DuPont ODS C-18 column using a $y = x^3$ gradient for elution (11). (. . . . .) indicates the percent organic solvent (acetone) used for elution. 0.5 min (0.5 ml) fractions were collected directly into scintillation vials, dried, redissolved in 0.25 ml 0.01% SDS, and counted for radioactivity. Peptide differences are crosshatched.

Set A. ^{3}H-mannose labeled WEHI 279 μ_i (blue), μ_m (red) and μ_s (black) chains. Five glycopeptides are expected for μ_i and μ_s chains; mature carbohydrate structures in μ_s chains are seen to change in migration relative to μ_i chains. μ_m chains have one less glycopeptide than either μ_i or μ_s chains. Quantitation of mannose residues per complex or high mannose oligosaccharide cannot account for these relative peak areas. ^{3}H may therefore have been incorporated into other sugars, e.g., N-acetylglucosamine, or the peptides overlap and are generating a more complicated pattern than is revealed by this one-dimensional separation.

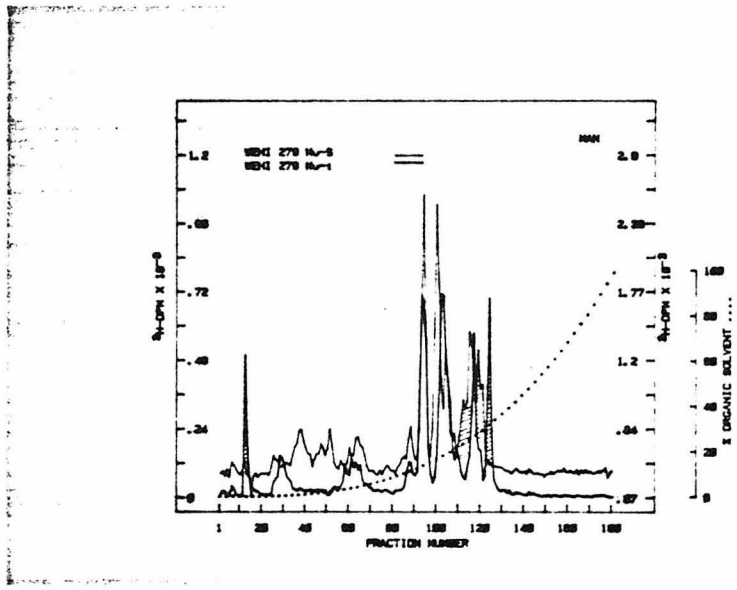
Set B. ^{35}S-Met labeled MPC11 x W279.2 μ_s (black), WEHI 279 μ_i (blue), μ_m (red), and μ_s (green) chains. Seven methionine peptides are expected for WEHI 279 μ_i and μ_s chains and one is glycosylated. μ_m chains have one less methionine peptide than either μ_i or μ_s chains. If relative peak heights are significant, WEHI 279 μ_s chains may also consist of μ_m-like chains or some μ_m chains which have been proteohytically cleaved from the cell surface and lack the C-terminal region.

Figure 8 - Continued

Set. C. ^{3}H-Phe labeled MPC11 x W279.2 μ_s (——), WEHI 279 μ_i (- - -), μ_m (- - -) and μ_s (- - -) chains. μ_m chains have two additional phenylalanine peptides when compared with either μ_i or μ_s chains, consistent with the postulated μ_m chain COOH-terminal sequence. The two μ_s species are seen to be virtually identical. Twenty phenylalanine peptides are expected for μ_i and μ_s chains and one is glycosylated. The large number of phenylalanine residues precludes any quantiation.

Set D. ^{3}H-Tyr labeled MPC11 x W279.2 μ_s (black), WEHI 279 μ_i (blue), μ_m (red), and μ_s (green) chains. Sixteen tyrosine peptides are expected for μ_i and μ_s chains and one is glycosylated. The patterns are very similar for all the μ chain species, although μ_m is somewhat different from μ_s, consistent with a gain and loss of Tyr containing peptides.

Set E. ^{35}S-Cys labeled MPC11 x W279.2 μ_s (black), WEHI 279 μ_i (blue), μ_m (red), and μ_s (green) chains. Fourteen cysteine peptides are expected for μ_i and μ_s chains and one is glycosylated. The patterns are similar for all the μ chain species but the propensity of the cysteine peptides to elute very early in the gradient prevents any quantitative comparisons.



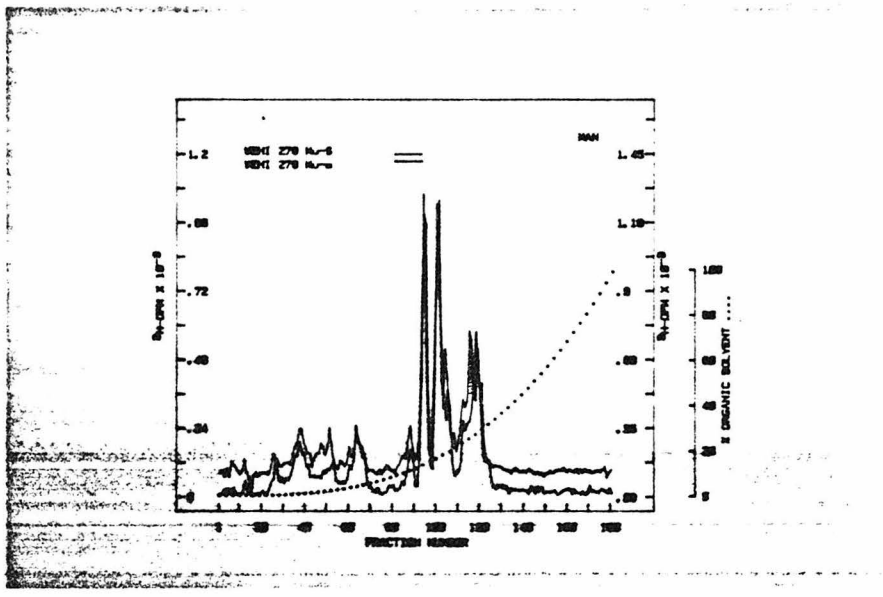
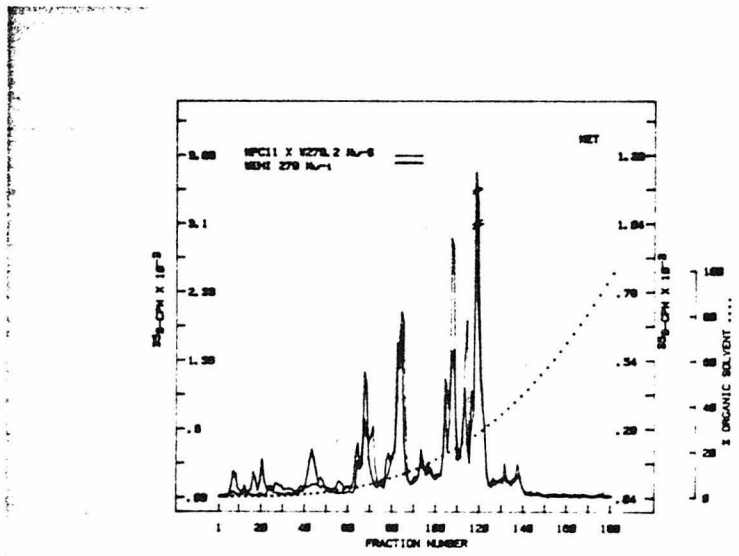


FIGURE 8., SET A



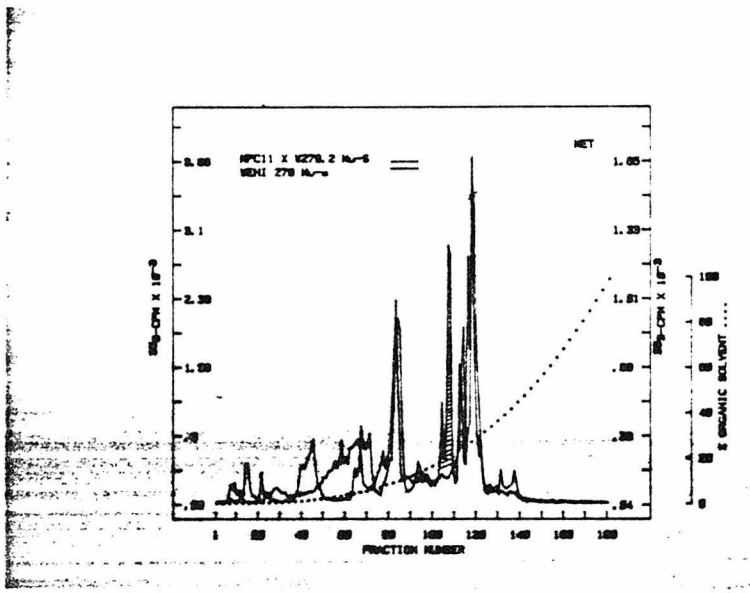


FIGURE 8., SET B

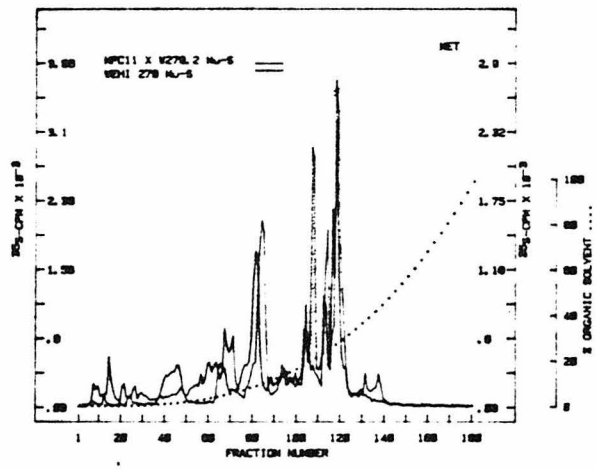
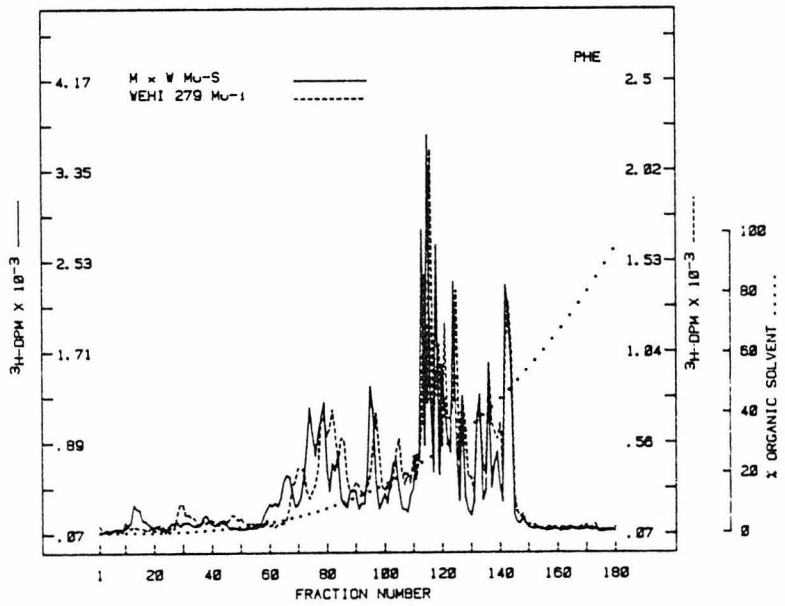


FIGURE 8., SET B



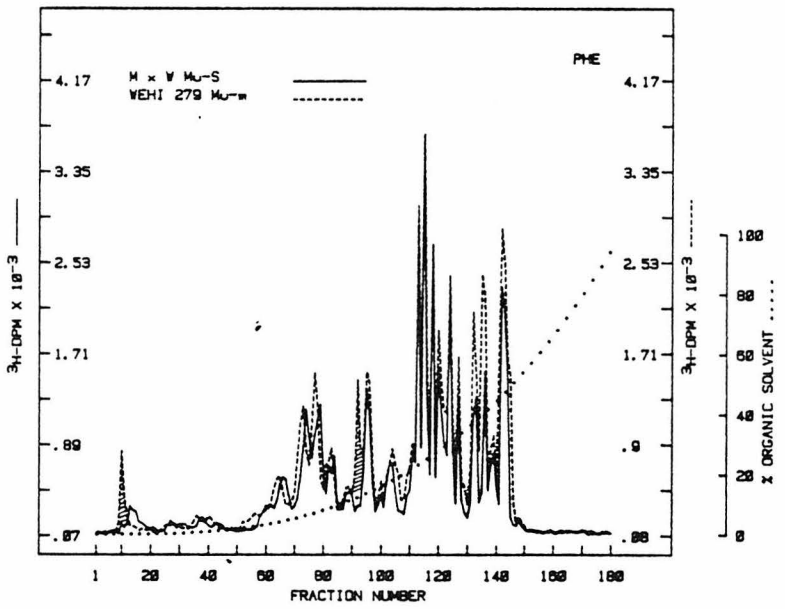


FIGURE 8., SET C

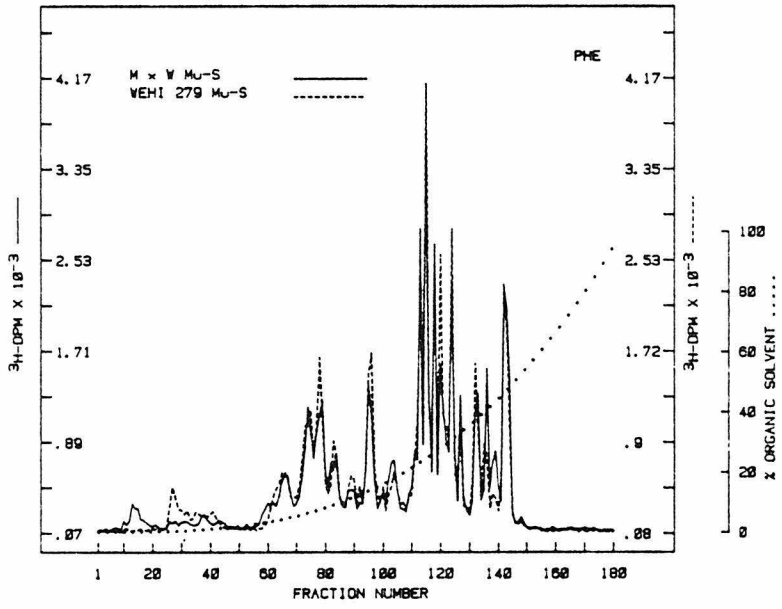
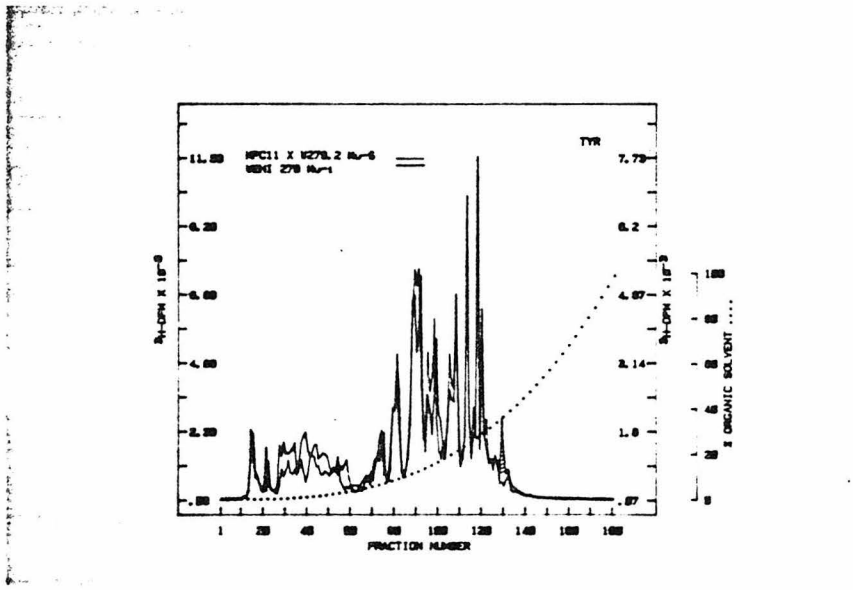
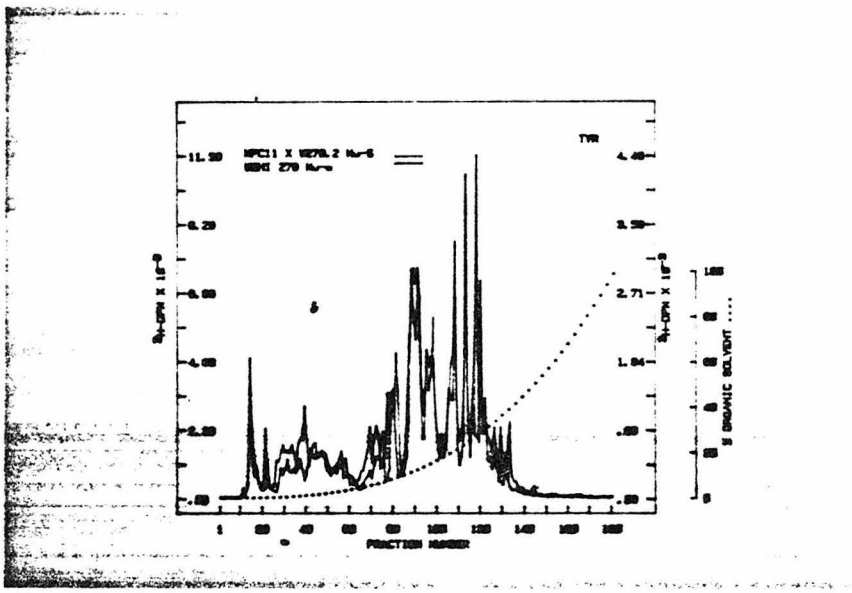


FIGURE 8., SET C





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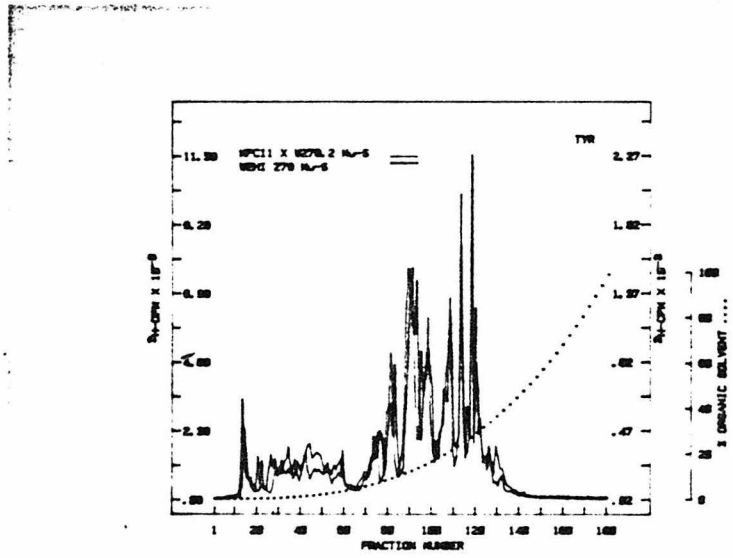
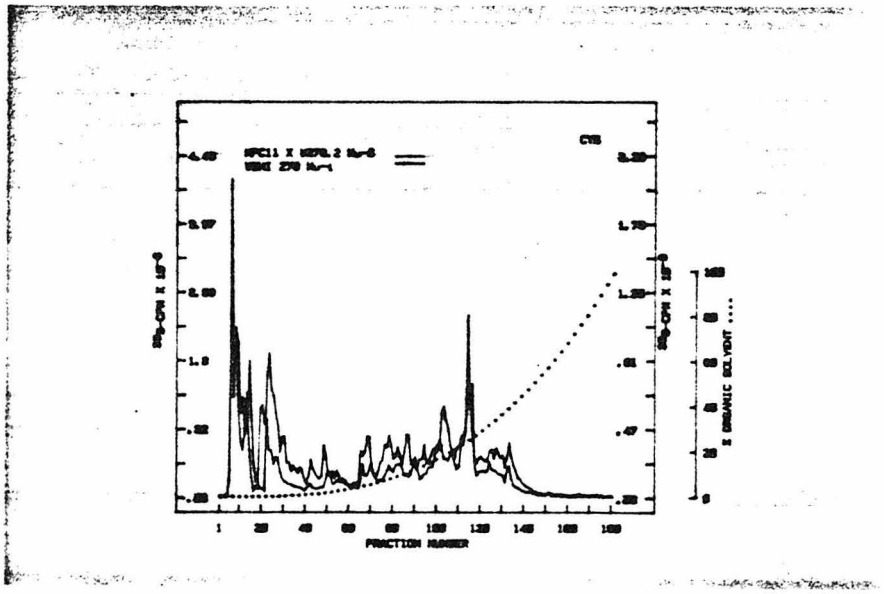


FIGURE 8., SET D



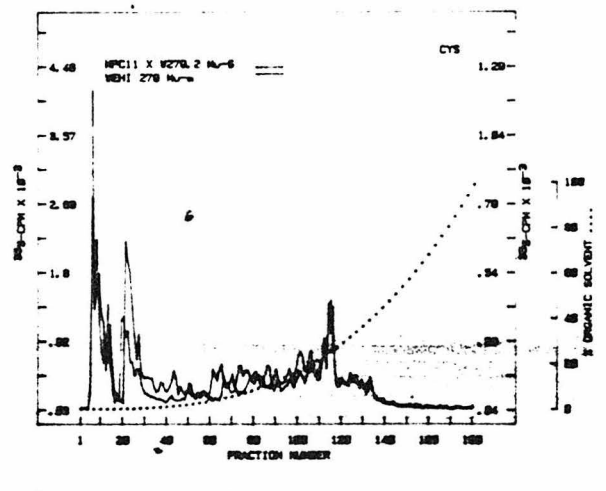


FIGURE 8., SET E

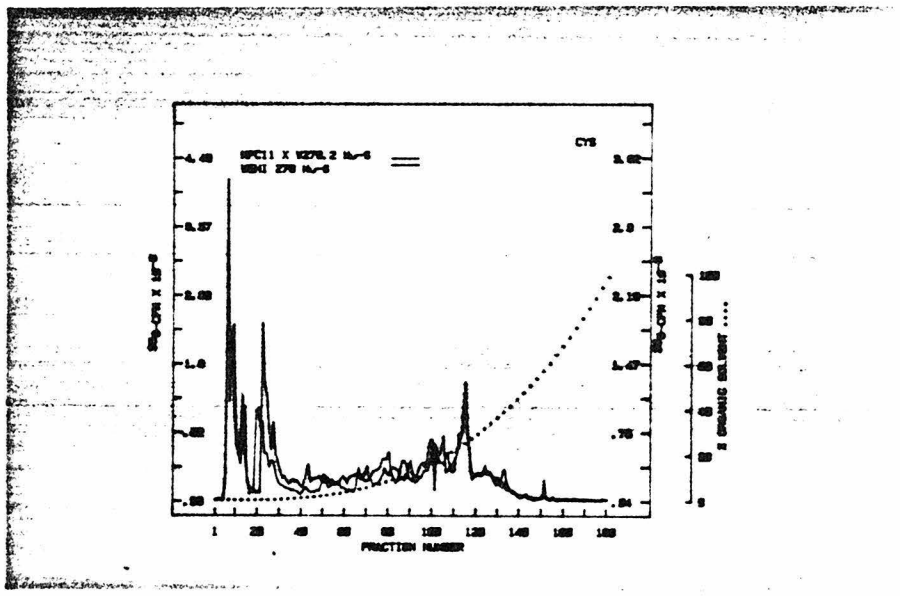


FIGURE 8., SET E

Figure 9. CNBr fragments of MPC11 x W279.2 μ_s chains.

μ_s chains were radiolabeled by incubating cells in the presence of either ^{3}H-Tyr and Leu or ^{3}H-Phe, Pro and Val for 16 hr (5). Medium was collected and μ_s chains were purified on 10% polyacrylamide gels, reduced, alkylated, combined with 30 mg carrier MOPC 104E μ_s chains in 70% formic acid and cleaved with CNBr for 20 hr in the dark at 4°C with constant stirring (7). After removal of reagent, peptides were dissolved sequentially in 8M guanidine-HCl, 3M guanidine 0.2M ammonium bicarbonate, and 0.4M ammonium bicarbonate (total volume $\leq$ 7 ml) and chromatographed on a column of ACA54 (LKB) (3.5 x 140 cm) eluted in 3M guanidine, 0.2M ammonium bicarbonate, 0.02% NaN_3. Fraction volume = 5 ml. Aliquots were counted for radioactivity after reading the OD_{280} of the MOPC 104E μ_s CNBr peptides. Profiles may be aligned exactly with previous and subsequent column runs by normalizing the R_fs of the carrier MOPC 104E μ_s CNBr fragments.

Milligram quantities of the MPC11 x W279.2 μ_s chains were obtained by intraperitoneal injection of 2 x 10^7 MPC11 x W279.2 cells into BALB/c mice. Ascites fluid was produced in BALB/c or (BALB/c x DBA/2) F_1 mice. μ chains were isolated as described and illustrated in Figures 1 and 2. CNBr fragments are numbered as in reference 7.

A. ^{3}H-Tyr, Leu labeled μ_s chain CNBr fragments derived from the μ_s chains shown in Figure 6C. CN6 and CN5 have mobilities similar to the corresponding μ_i fragments, indicating their incomplete glycosylation. 0.25 ml aliquots were counted through fraction 250, 0.5 ml was aliquoted thereafter. Fractions pooled for microsequence determinations (7) (see Figure 11) were: CN3, 199-209; CN8, 177-186; CN8-9, 171-175.

B. ^{3}H-Phe, Pro and Val labeled μ_s chain CNBr fragments. Note the different incorporation of these radiolabeled amino acids (as compared

Figure 9 - Continued

with Tyr and Leu) into CN3, CN1-2' and especially into the CN3b-1-2' partial migrating between CN5 and CN8-9. CN9 is not labeled with phenylalanine, proline or valine. 0.1 ml aliquots were counted through fraction 190. 0.2 ml was aliquoted thereafter. Fractions pooled for microsequence determinations (7) (see Figure 11) were: CN3, 160-168; CN8, 139-147; CN8-9, 131-136.

C. Cold CNBr fragments of MPC11 x W279.2 μ_s chains. Fractions 191-202 were pooled for sequence determination of CN3a and CN3b. CN3 was also isolated from an identical CNBr cleavage of μ_s chains with succinylated NH_2 groups (12). This allowed determination of only the CN3b sequence (see Figure 10).

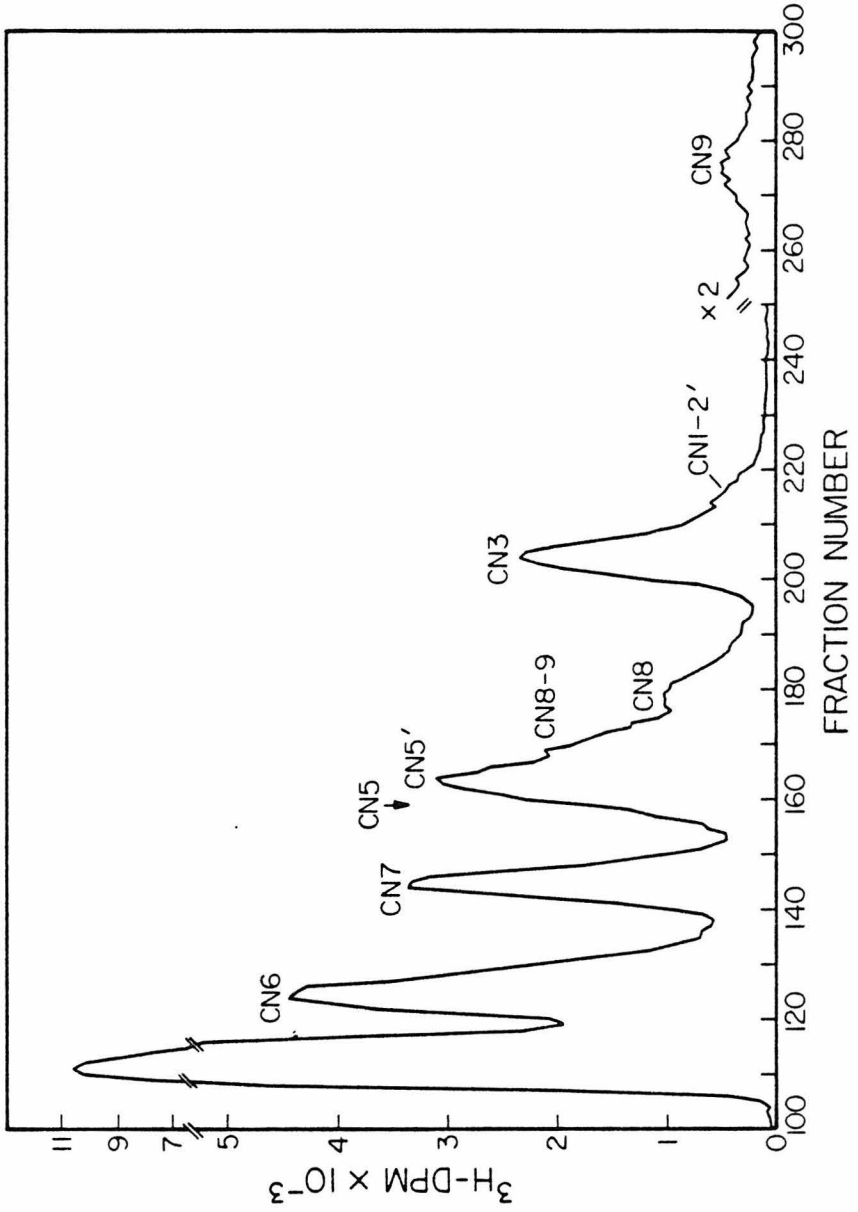

^{3}H-DPM x 10^{-3}
FRACTION NUMBER
CN6
CN7
CN5
CN5'
CN8-9
CN8
CN3
CN1-2'
x 2
CN9

FIGURE 9A.

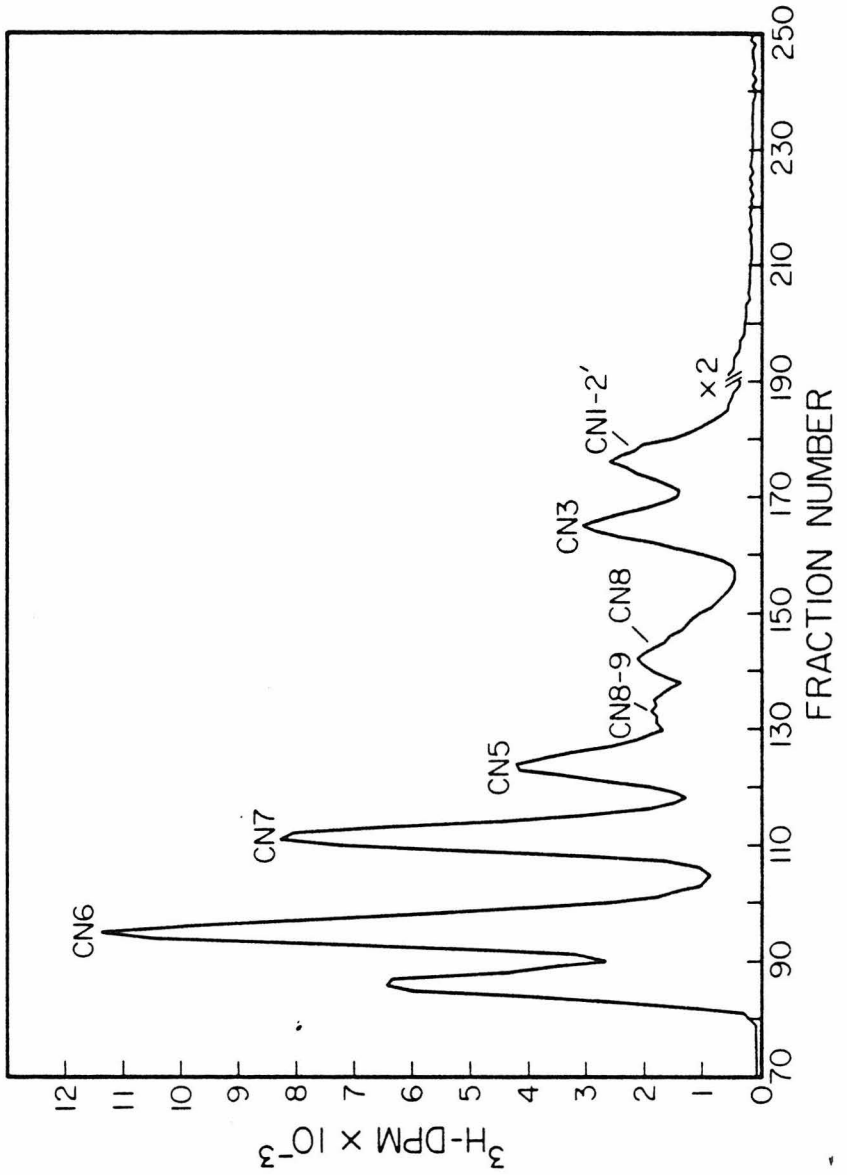


FIGURE 9B.

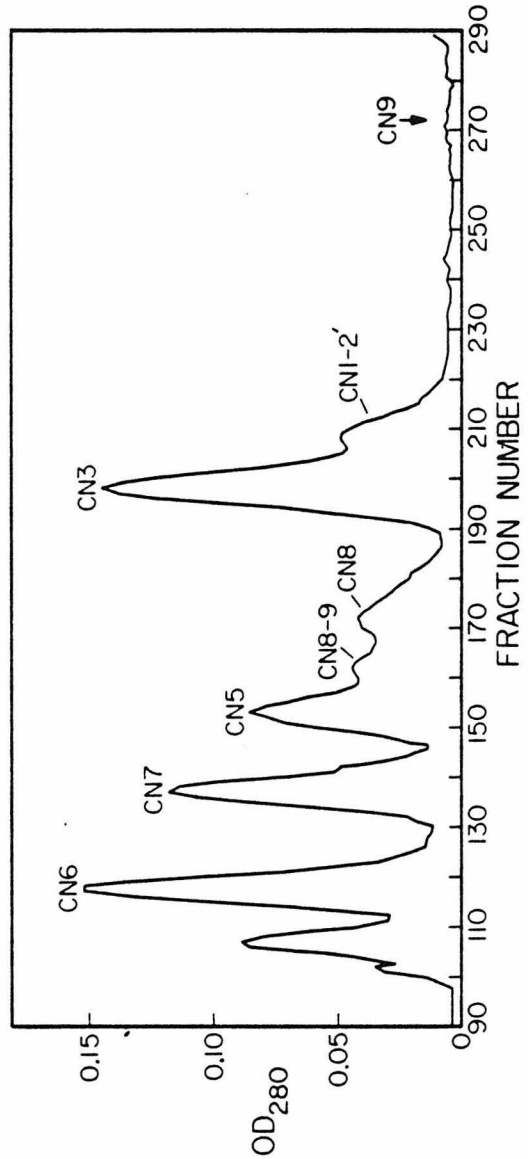

CN6
CN7
CN5
CN8-9
CN8
CN3
CN1-2'
CN9
0.15
0.10
0.05
0
OD_{280}
90
110
130
150
170
190
210
230
250
270
290
FRACTION NUMBER

FIGURE 9C.

Figure 10. Sequenator runs on MOPC 104E μ_s chain CNBr fragments and fragments generated from CNBr fragments by cleavage at tryptophan, arginine or lysine residues.

These sequenator runs establish the entire sequence of the MOPC 104E μ_s chain but for the NH_2-terminal 20 residues (13) and the exceptions noted in reference 14. Relative peak heights on an arbitrary scale (proportional to nanomole yield) are plotted for each automatic sequenator cycle for each of the phenylthiohydantoin (Pth)-amino acids separated by high performance liquid chromatography (HPLC) (15, 16). Scale changes and residue(s) assigned at each step are indicated on the graphs. Peptides are named as in references 14 and 17. Sequenator runs labeled A, C, D, E, I, O, Q, R, S, T, U, V, and W were performed by M. Kehry, those designated F, G, H, J, K, L,M, N, and P were performed by J. Fuhrman and B by J. Schilling. Also included are the 3 sequenator runs establishing 80% of the WEHI 279 V_H amino acid sequence (U, V, and W).

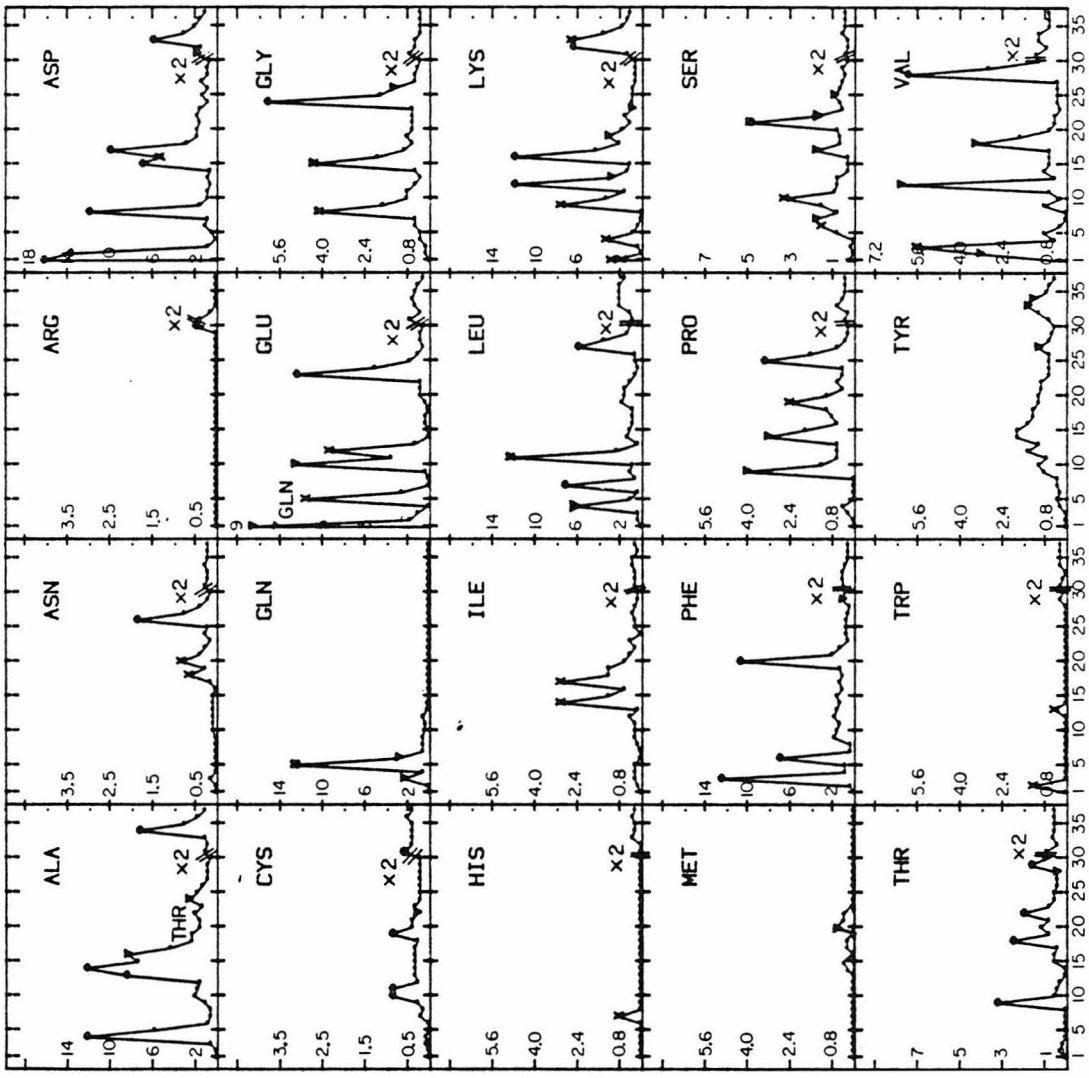

104E CN3' o
104E CN3 x
104E CN1-2 v
RESIDUE #1 CORRESPONDS TO RESIDUES
?, 35, AND 1 IN THE Mu SEQUENCE
-\\- INDICATES CHANGE IN SCALE TO
THAT INDICATED ON THE GRAPH.
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
THR
GLN
x2
RESIDUE NUMBER

FIGURE 10A

Amino Acid Sequence of MOPC 104E CN3'

1 10 20
Asp Asp Phe Ala Gln Phe Leu Asp Thr Cys Cys Lys Ala Ala Asp Lys Asp Thr Cys Phe

21 30
Ser Thr Glu Gly Pro Asn Leu Val Thr Arg Cys Lys Asp Ala

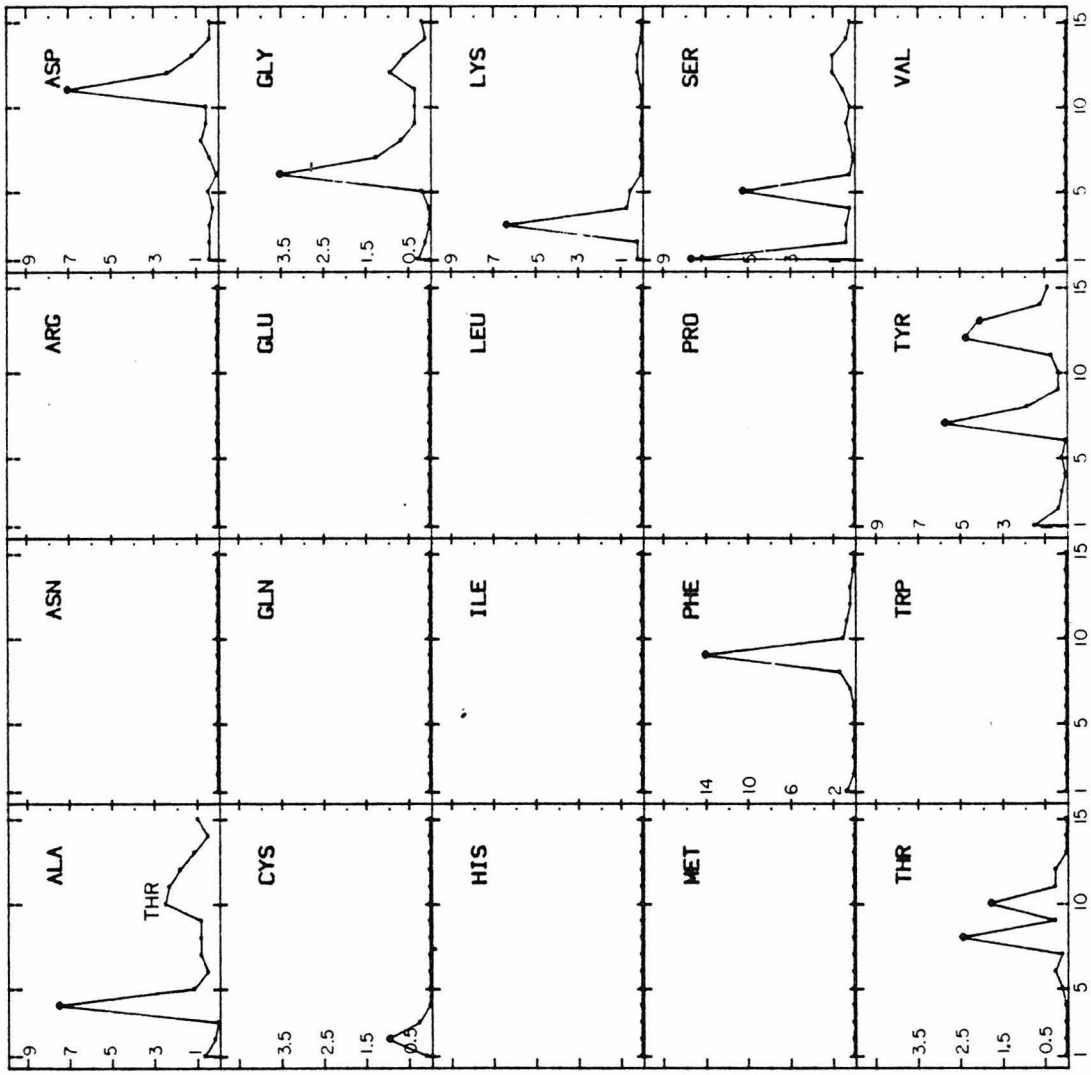

104E CN2
RESIDUE #1 CORRESPONDS TO RESIDUE #21 IN THE Mu SEQUENCE.
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS:
ASN, ARG, GLN, GLU, HIS, ILE, LEU, MET, PRO, TRP, VAL
ALA
THR
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
RESIDUE NUMBER

FIGURE 10B

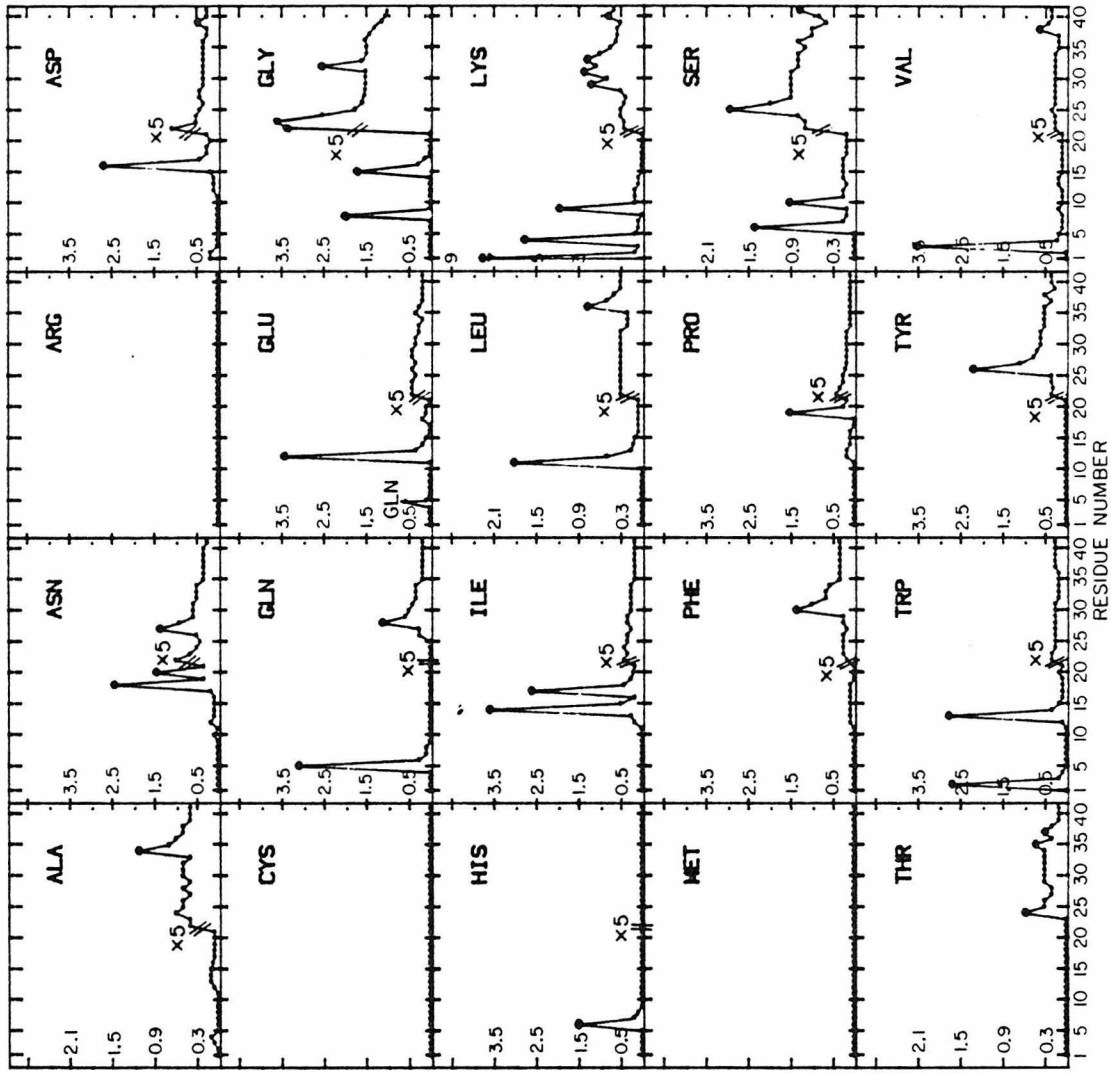

184E CN3
RESIDUE #1 CORRESPONDS TO RESIDUE #35 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS:
ARG, CYS, MET
-\\- INDICATES CHANGE IN SCALE TO THAT INDICATED ON THE GRAPH.
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
GLN
×5
RESIDUE NUMBER

FIGURE 10C

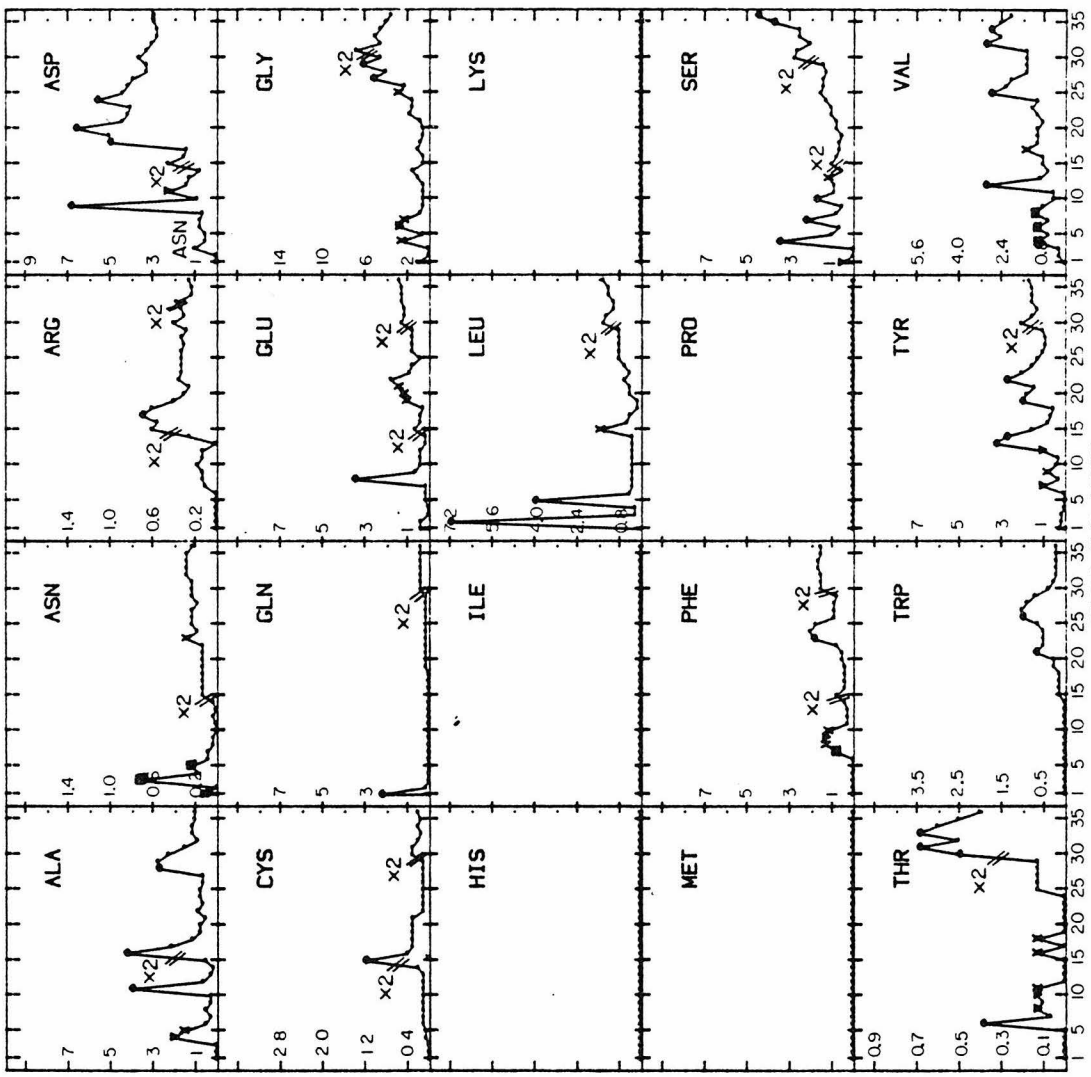

104E CN4 o
104E CN8 x
104E CN2 ▽
104E CN6 □
RESIDUE #1 CORRESPONDS TO RESIDUES
83, 507, 21, 235 IN THE Mu CHAIN
THE FOLLOWING AMINO ACIDS EXHIBITED
ONLY BACKGROUND PEAKS.
HIS, ILE, LYS, MET, PRO
-\\- INDICATES CHANGE IN SCALE TO
THAT INDICATED ON THE GRAPH.
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
RESIDUE NUMBER

FIGURE 10D

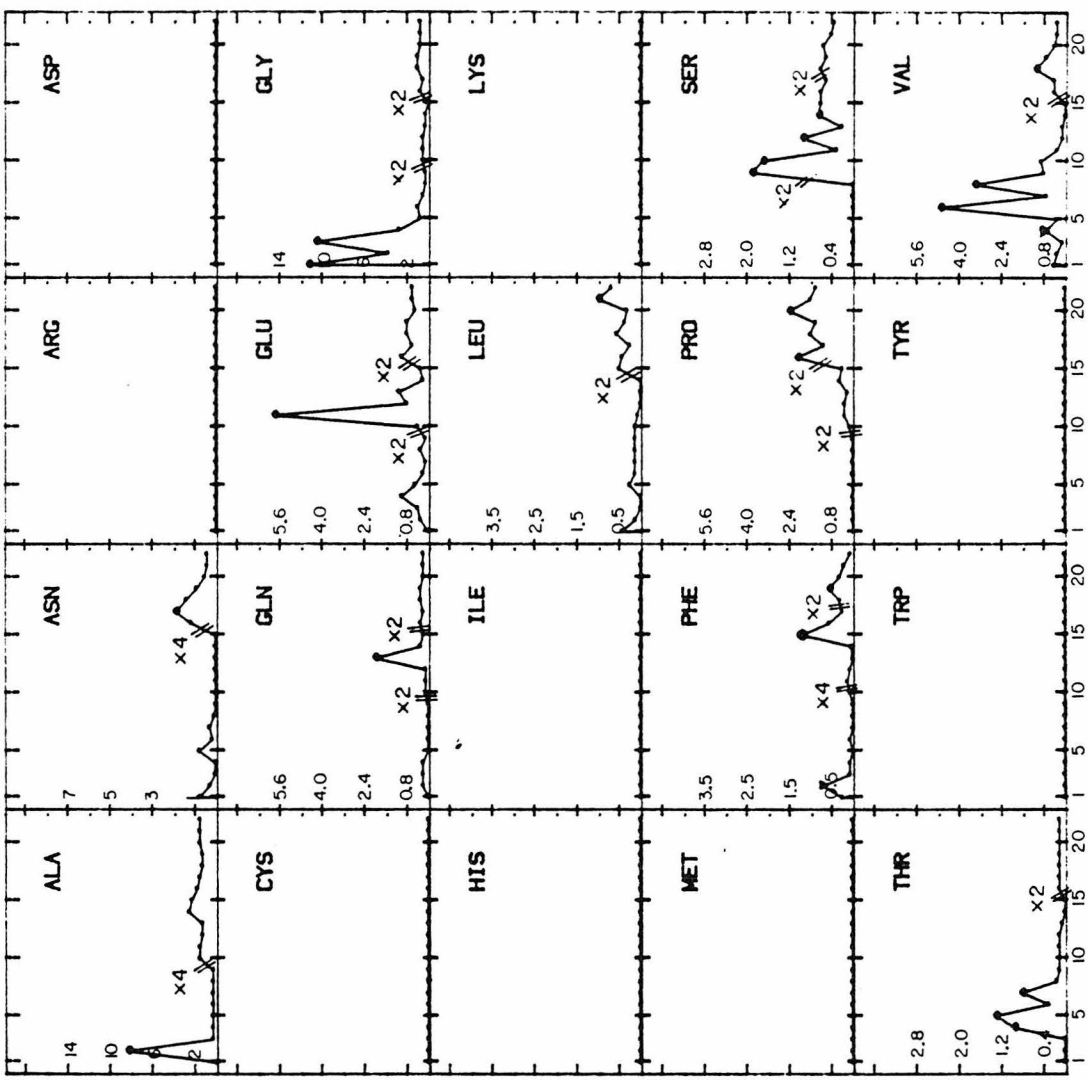

104E CN4 W-I o
104E CN4 W-II v
RESIDUE #1 CORRESPONDS TO RESIDUES 117 AND 112 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS.
ARG, ASP, CYS, HIS, ILE, LYS, MET, TRP, TYR
-\\- INDICATES CHANGE IN SCALE TO THAT INDICATED ON THE GRAPH.
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
RESIDUE NUMBER

FIGURE 10E

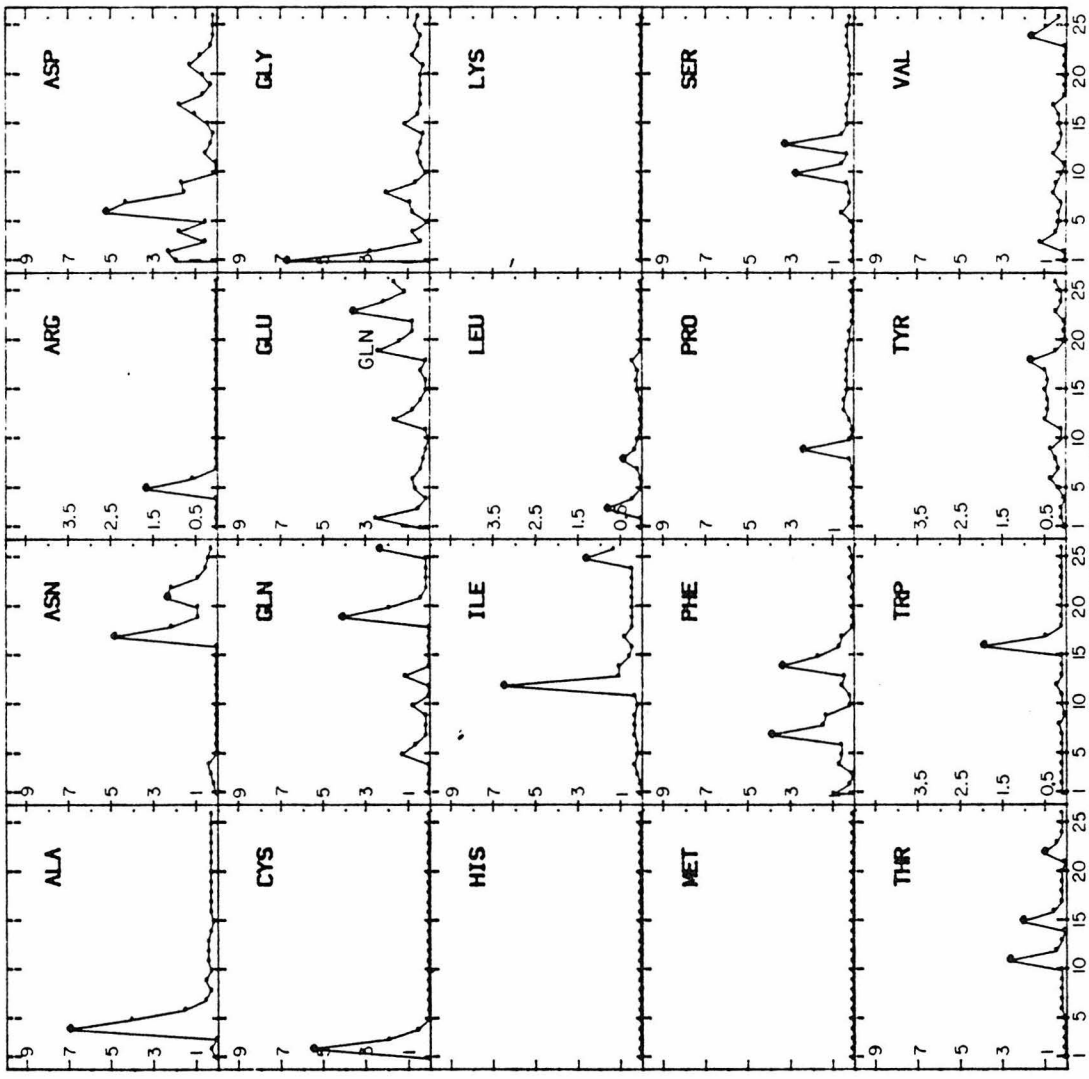

104E CNS
RESIDUE #1 CORRESPONDS TO RESIDUE #152 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS.
HIS, LYS, MET
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
GLN
RESIDUE NUMBER

FIGURE 10F

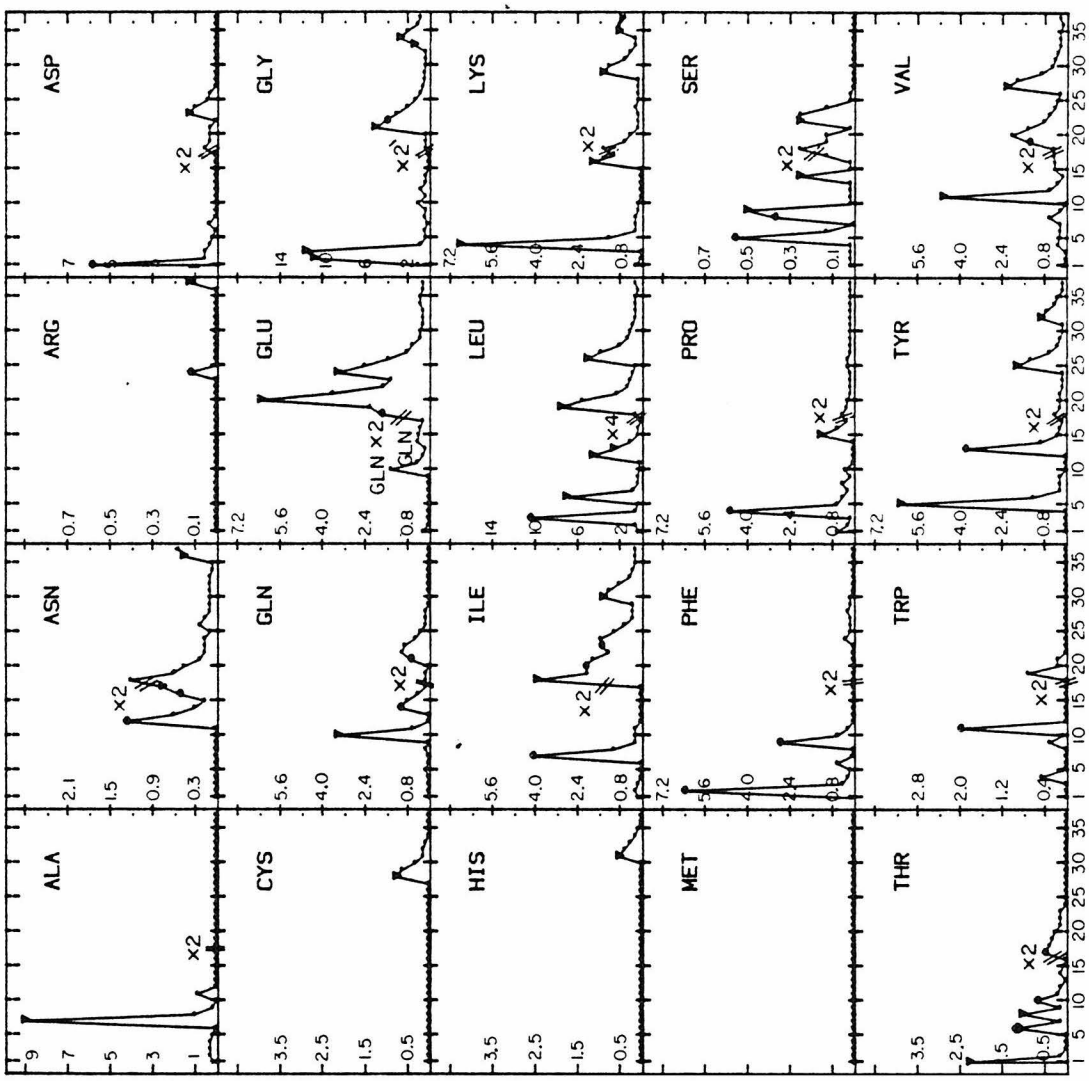

104E CN5 R-I o
104E CN5 R-II ▾
RESIDUE #1 CORRESPONDS TO RESIDUES
157 AND 186 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED
ONLY BACKGROUND PEAKS:
MET
-\\- INDICATES CHANGE IN SCALE TO
THAT INDICATED ON THE GRAPH.
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
GLN x2
RESIDUE NUMBER

FIGURE 10G

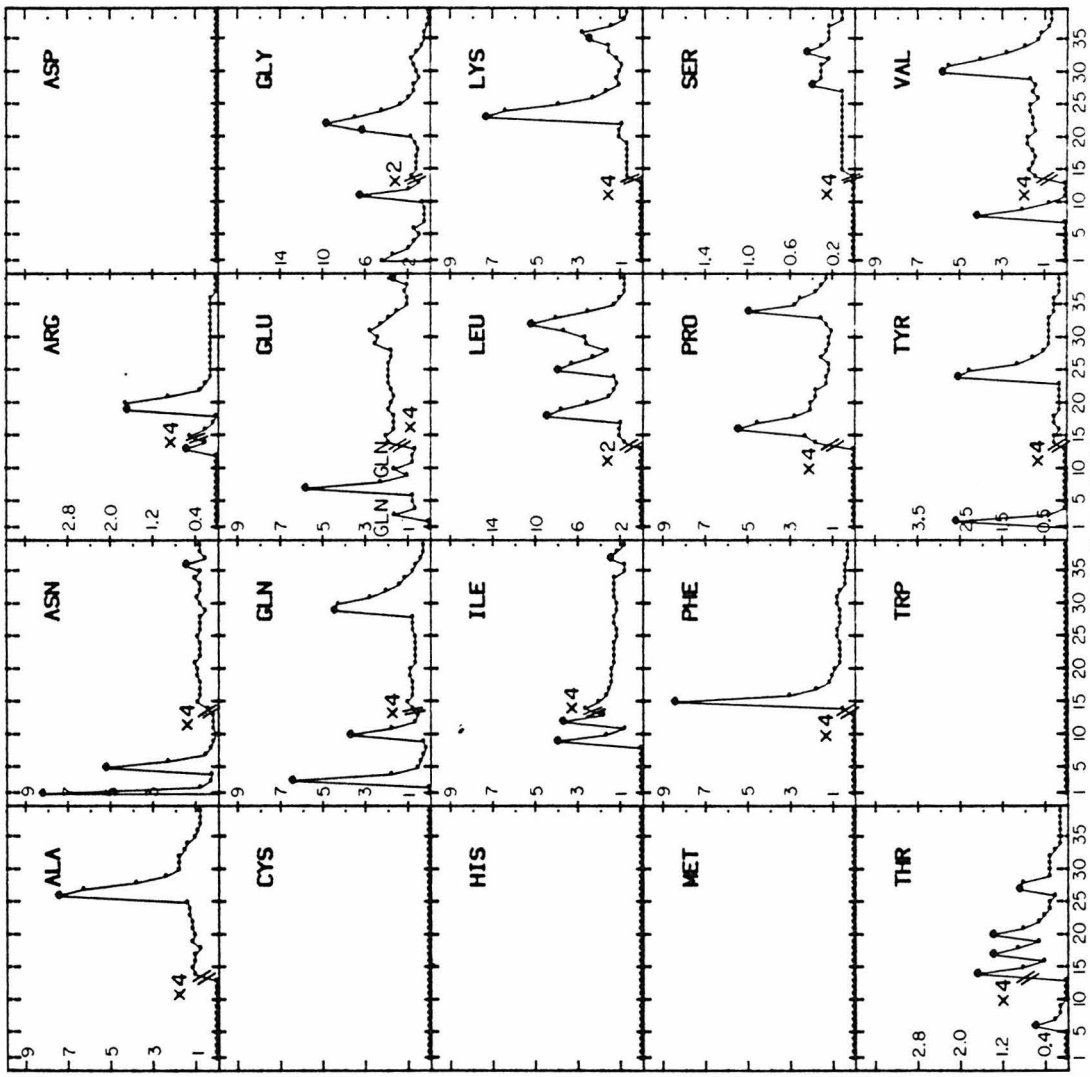

104E CN5 W
RESIDUE #1 CORRESPONDS TO RESIDUE #168 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS, ASP, CYS, HIS, MET, TRP
-\\- INDICATES CHANGE IN SCALE TO THAT INDICATED ON THE GRAPH.
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
RESIDUE NUMBER

FIGURE 10H

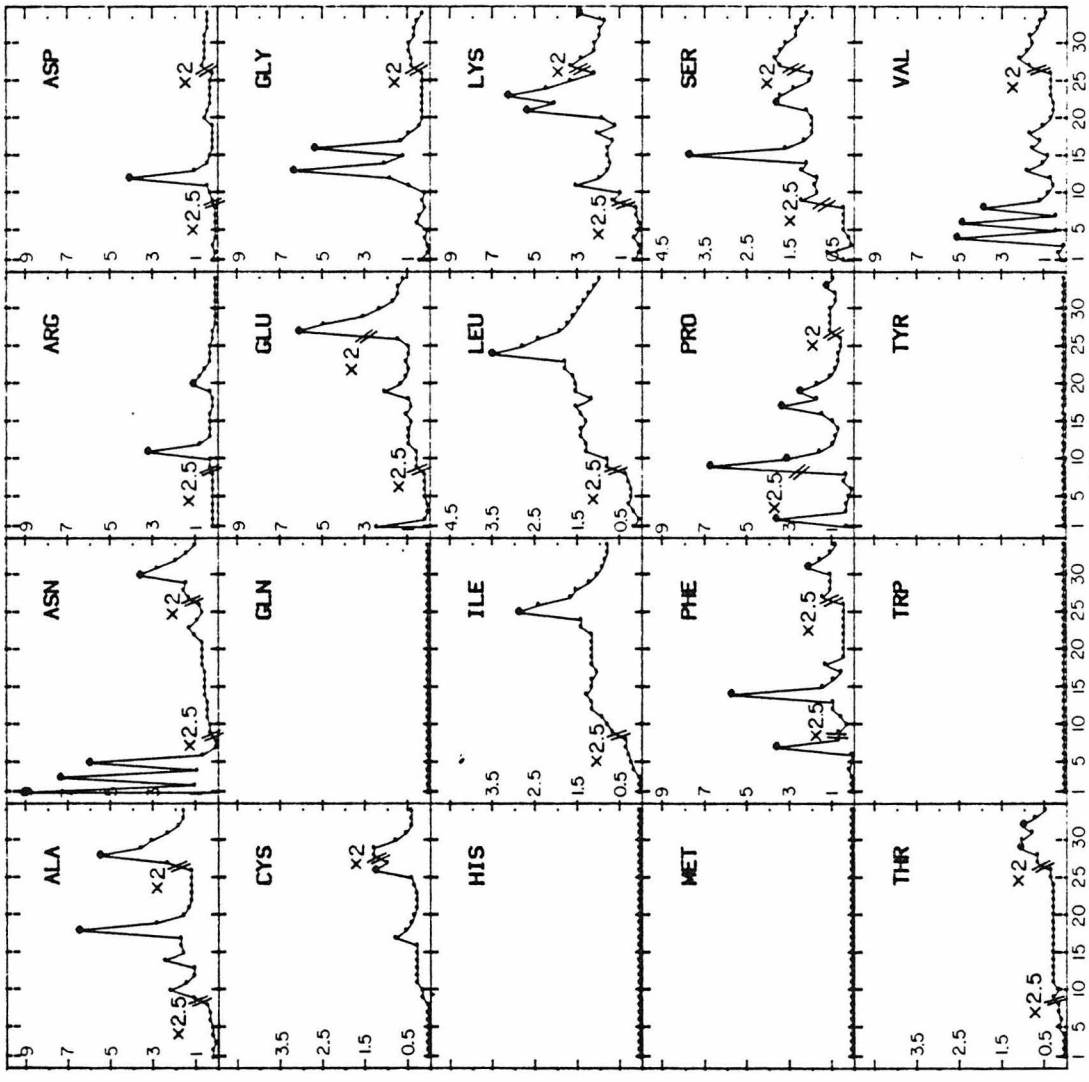

104E CN6
RESIDUE #1 CORRESPONDS TO RESIDUE #235 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS:
GLN, HIS, MET, TRP, TYR
-\\- INDICATES CHANGE IN SCALE TO THAT INDICATED ON THE GRAPH.
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
RESIDUE NUMBER

FIGURE 10I

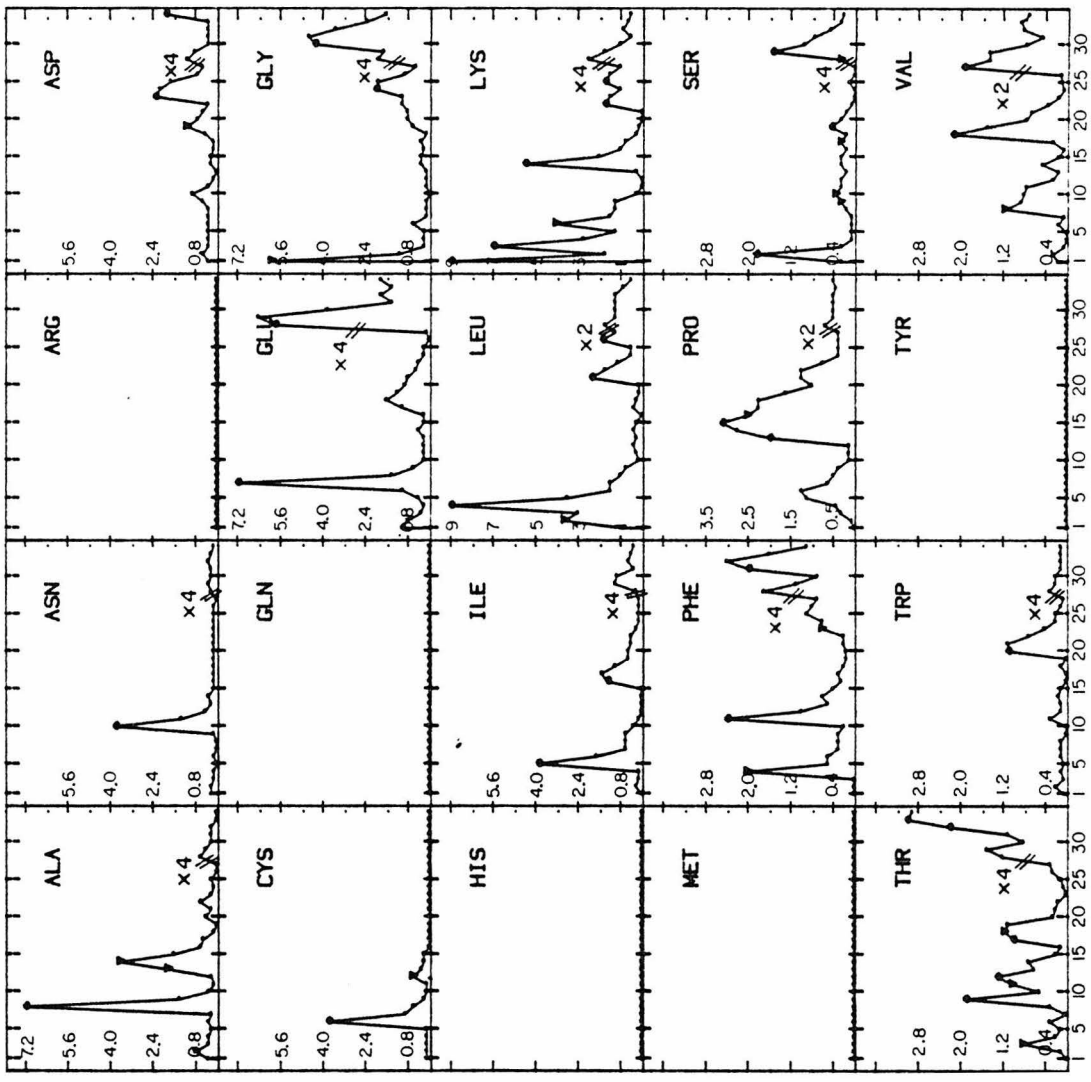
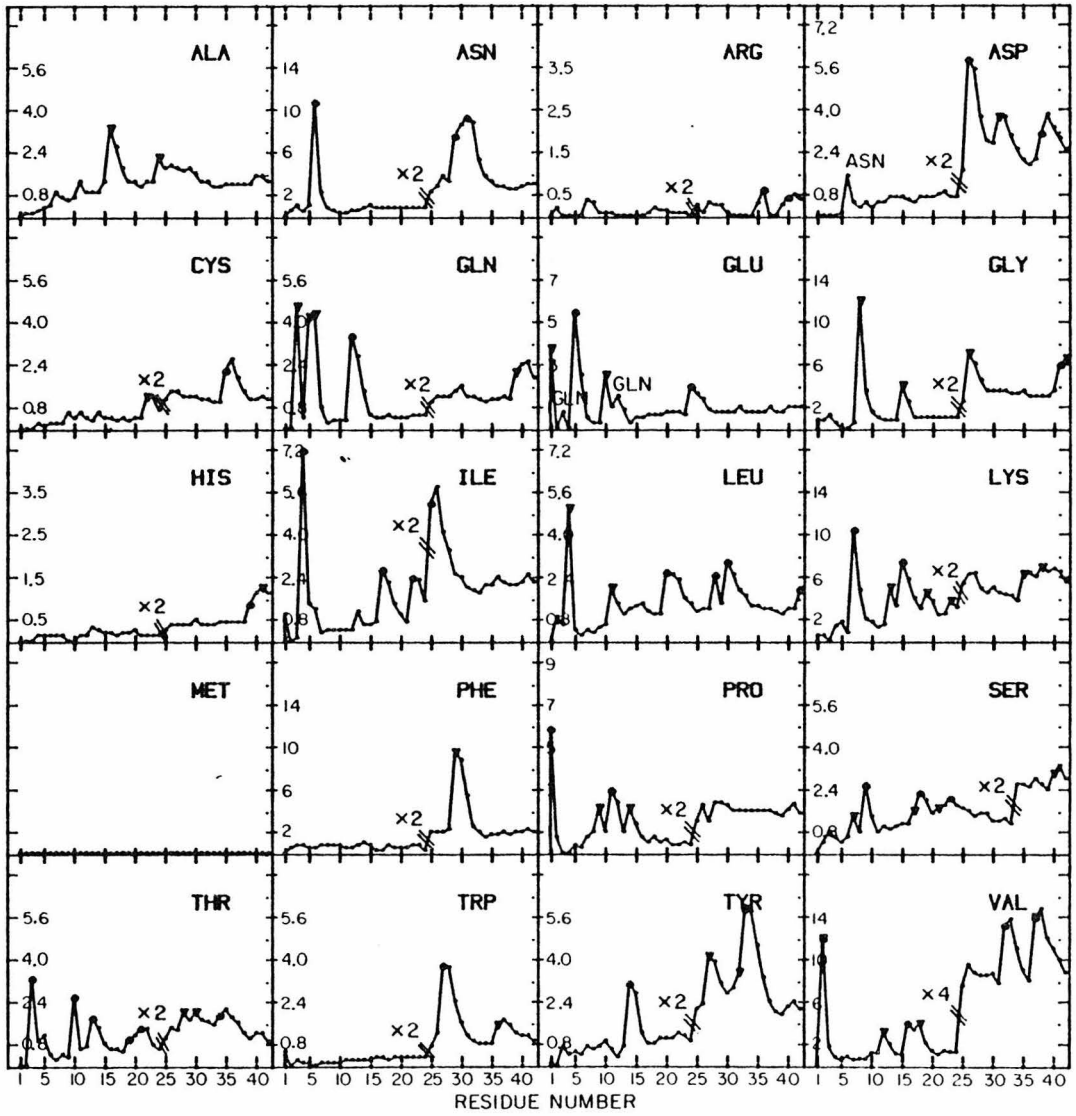

104E CN8 R-I o
104E CN6 R-II v
RESIDUE #1 CORRESPONDS TO RESIDUES
255 AND 328 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED
ONLY BACKGROUND PEAKS.
ARG, GLN, HIS, MET, TYR
-\\- INDICATES CHANGE IN SCALE TO
THAT INDICATED ON THE GRAPH.
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
RESIDUE NUMBER

FIGURE 10J



104E CNB ASP-PRO ACID CLEAVED o
104E Mu AMINO TERMINUS ▽

RESIDUE #1 CORRESPONDS TO RESIDUES 1 AND 286 IN THE Mu SEQUENCE

THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS:
MET

-\\- INDICATES CHANGE IN SCALE TO THAT INDICATED ON THE GRAPH.

FIGURE 10K

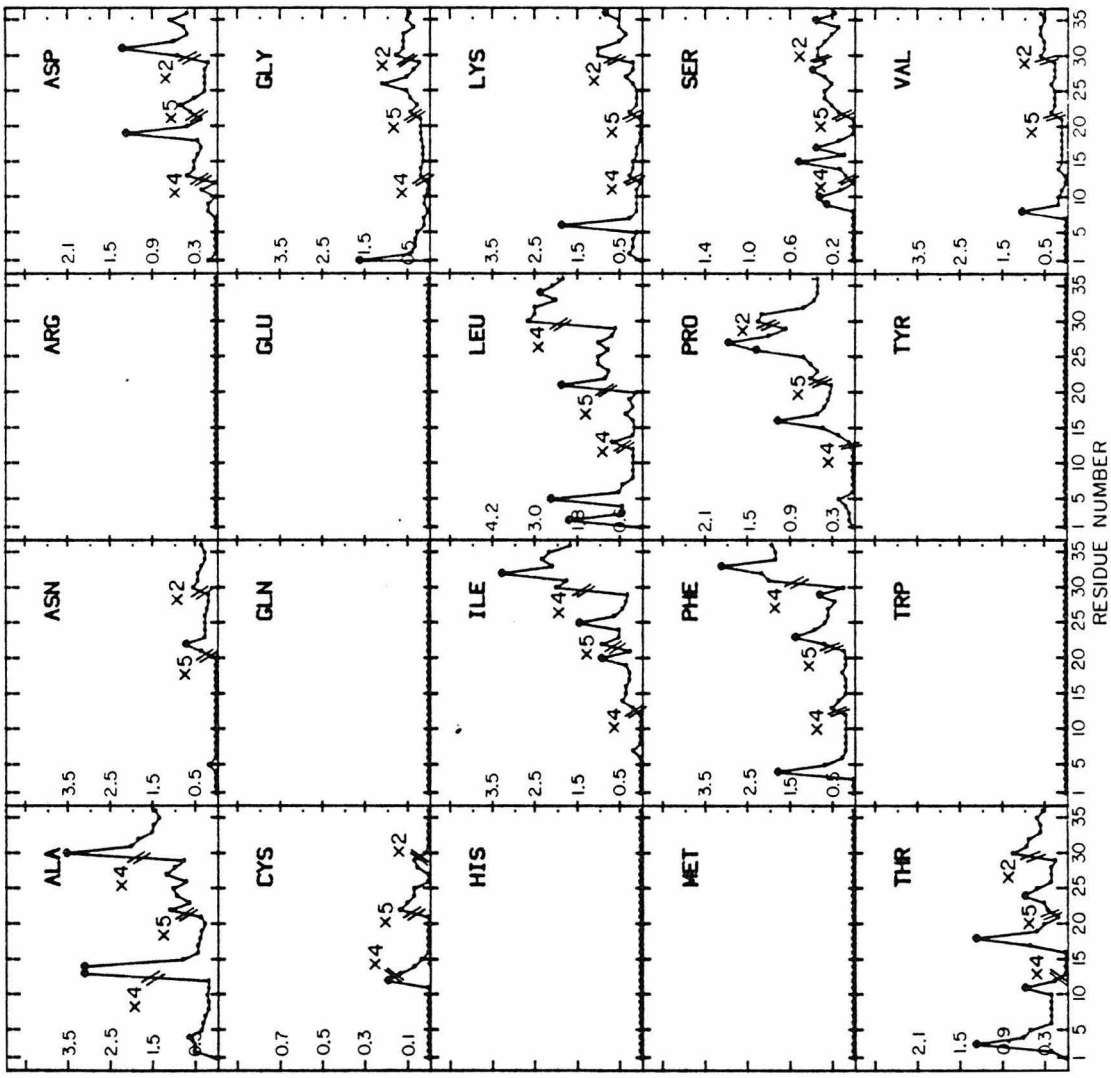

104E CN6 RII
RESIDUE #1 CORRESPONDS TO RESIDUE #326 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS:
ARG, GLN, GLU, HIS, MET, TRP, TYR
-\\- INDICATES CHANGE IN SCALE TO THAT INDICATED ON THE GRAPH
ALA
ARG
ASN
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
RESIDUE NUMBER

FIGURE 10L

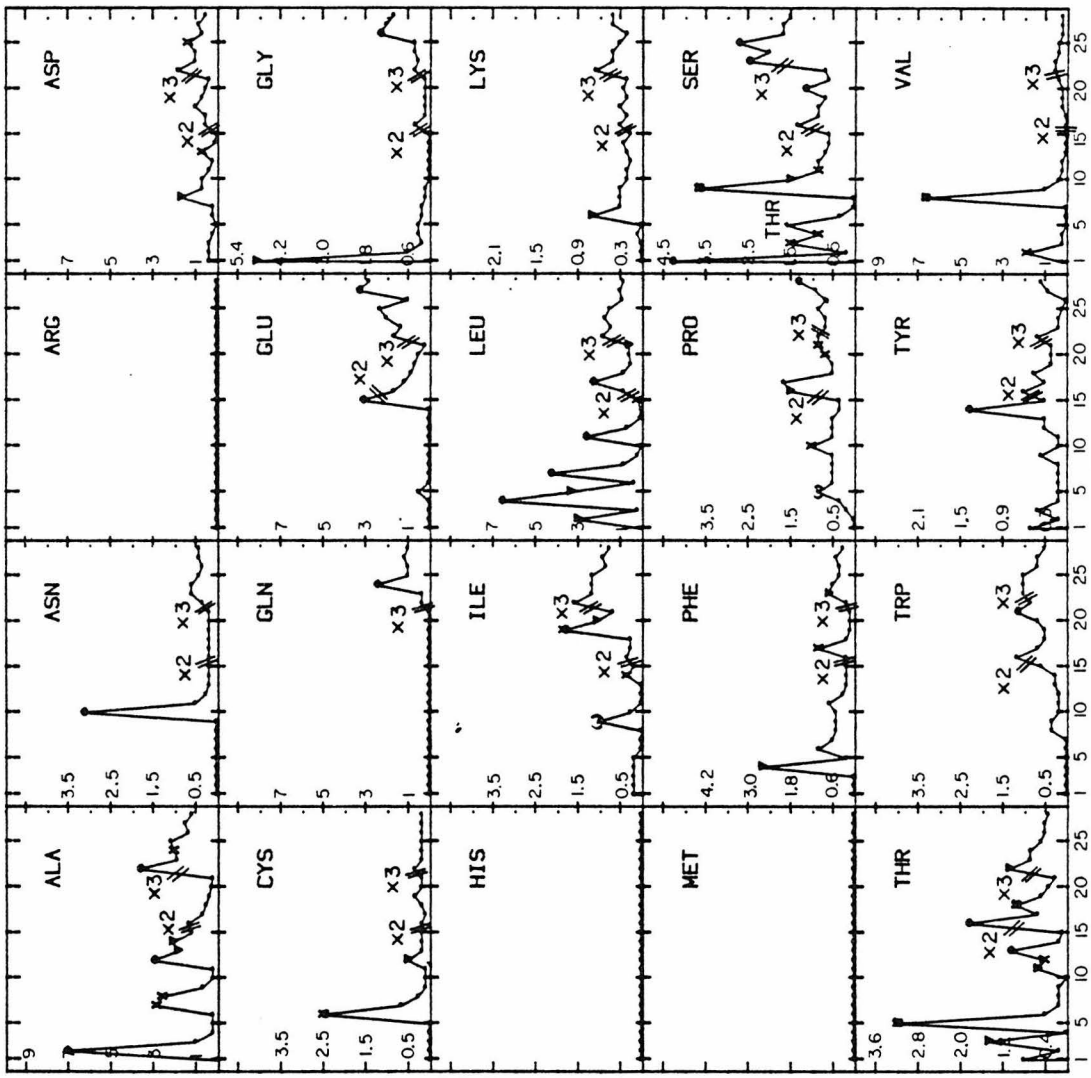
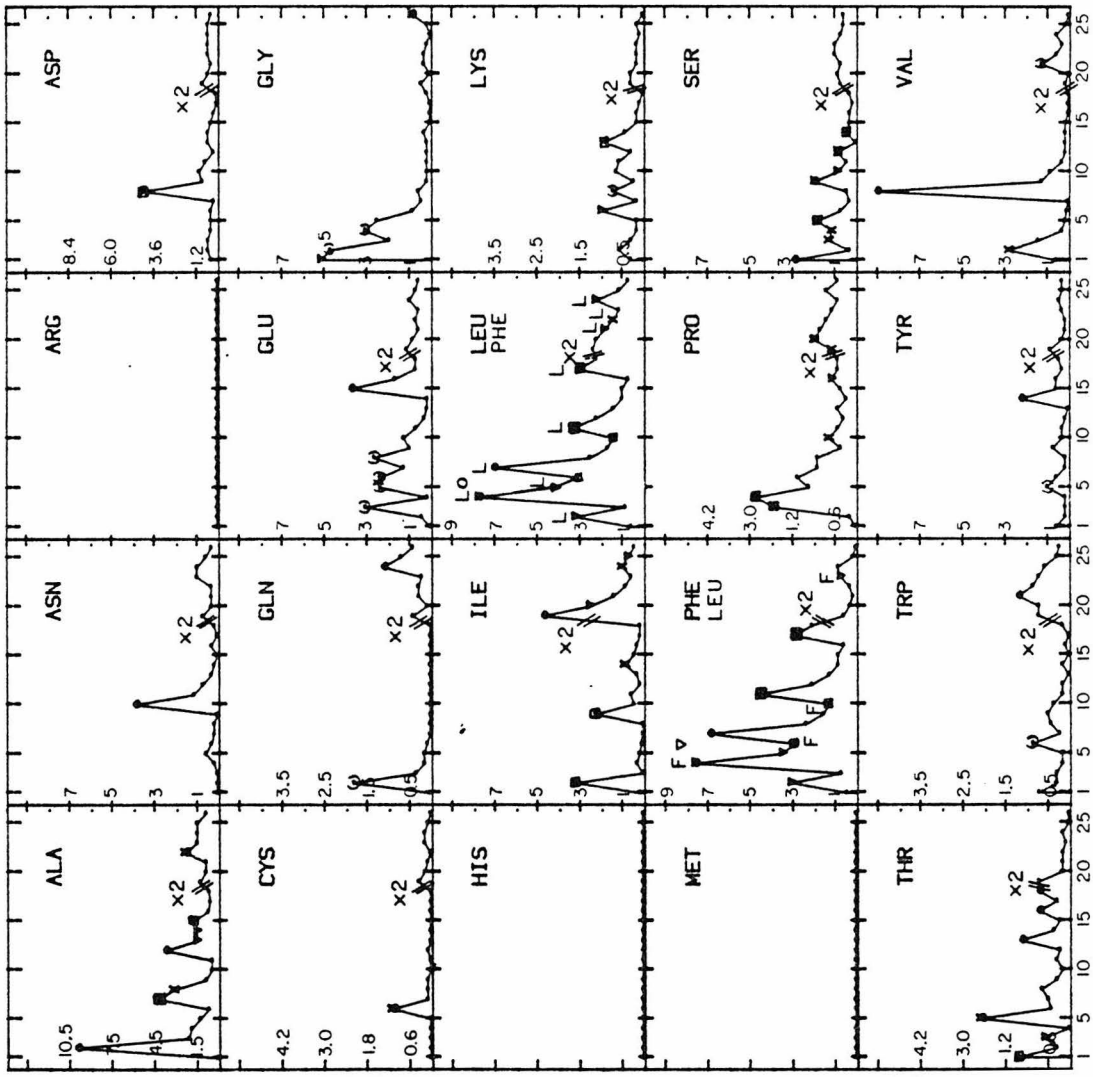

104E CN6 R-II₀ o
104E CN6 R-II₁ x
104E CN6 R-II₂ ▽
UNIDENTIFIED ()
RESIDUE #1 CORRESPONDS TO RESIDUES
362, 332, 326 IN THE Mᵤ SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED
ONLY BACKGROUND PEAKS:
ARG, HIS, MET
-\\- INDICATES CHANGE IN SCALE TO
THAT INDICATED ON THE GRAPH
ALA
ARG
ASN
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
RESIDUE NUMBER

FIGURE 10M



104E CN8 R-IIc o
104E CN6 R-IIb x
104E CN6 R-IIa ▽
104E CN6 R-IId □
UNIDENTIFIED ◇

RESIDUE #1 CORRESPONDS TO RESIDUES
362, 332, 326 IN THE Mu SEQUENCE

THE FOLLOWING AMINO ACIDS EXHIBITED
ONLY BACKGROUND PEAKS:
ARG, HIS, MET

-\\- INDICATES CHANGE IN SCALE TO
THAT INDICATED ON THE GRAPH.

FIGURE 10N

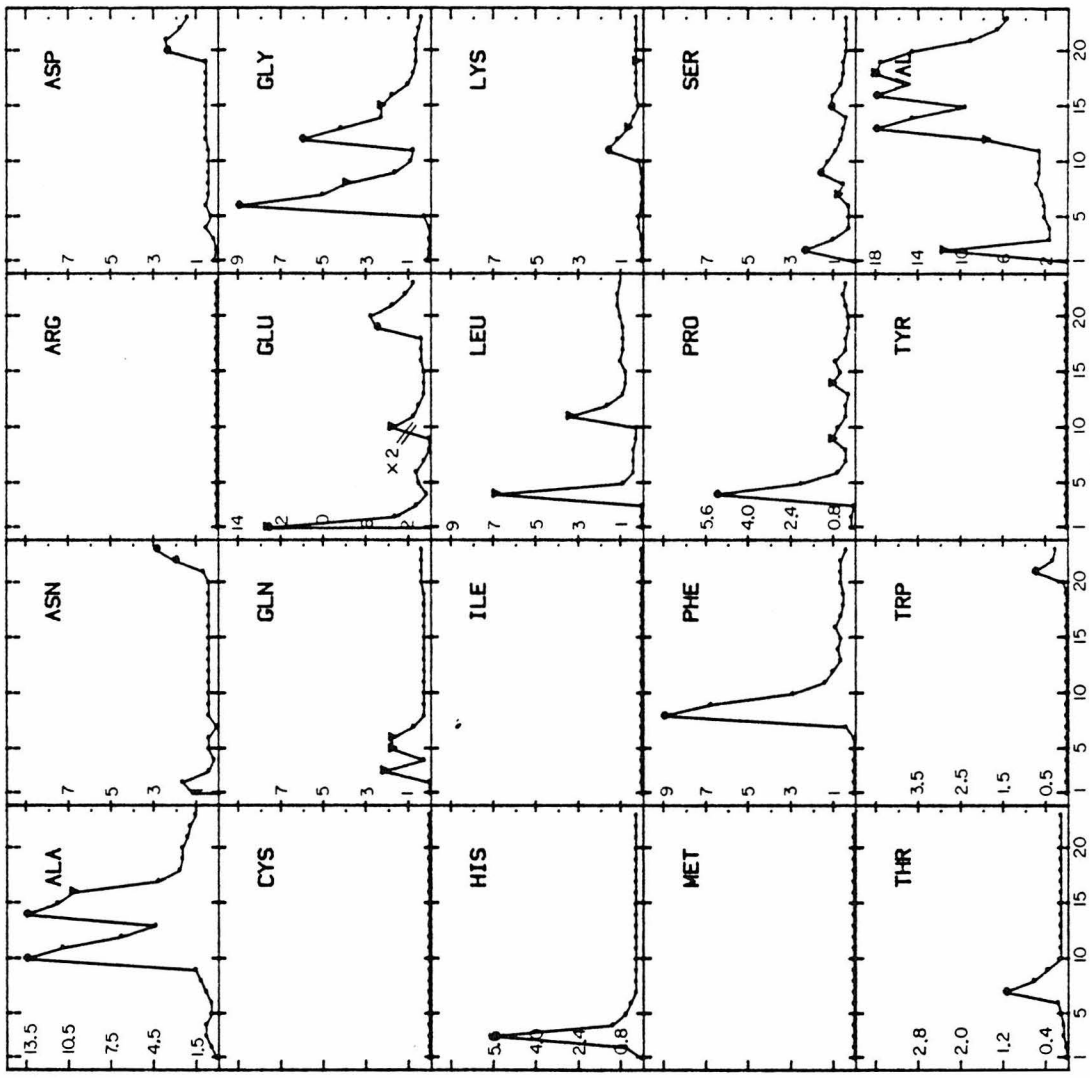
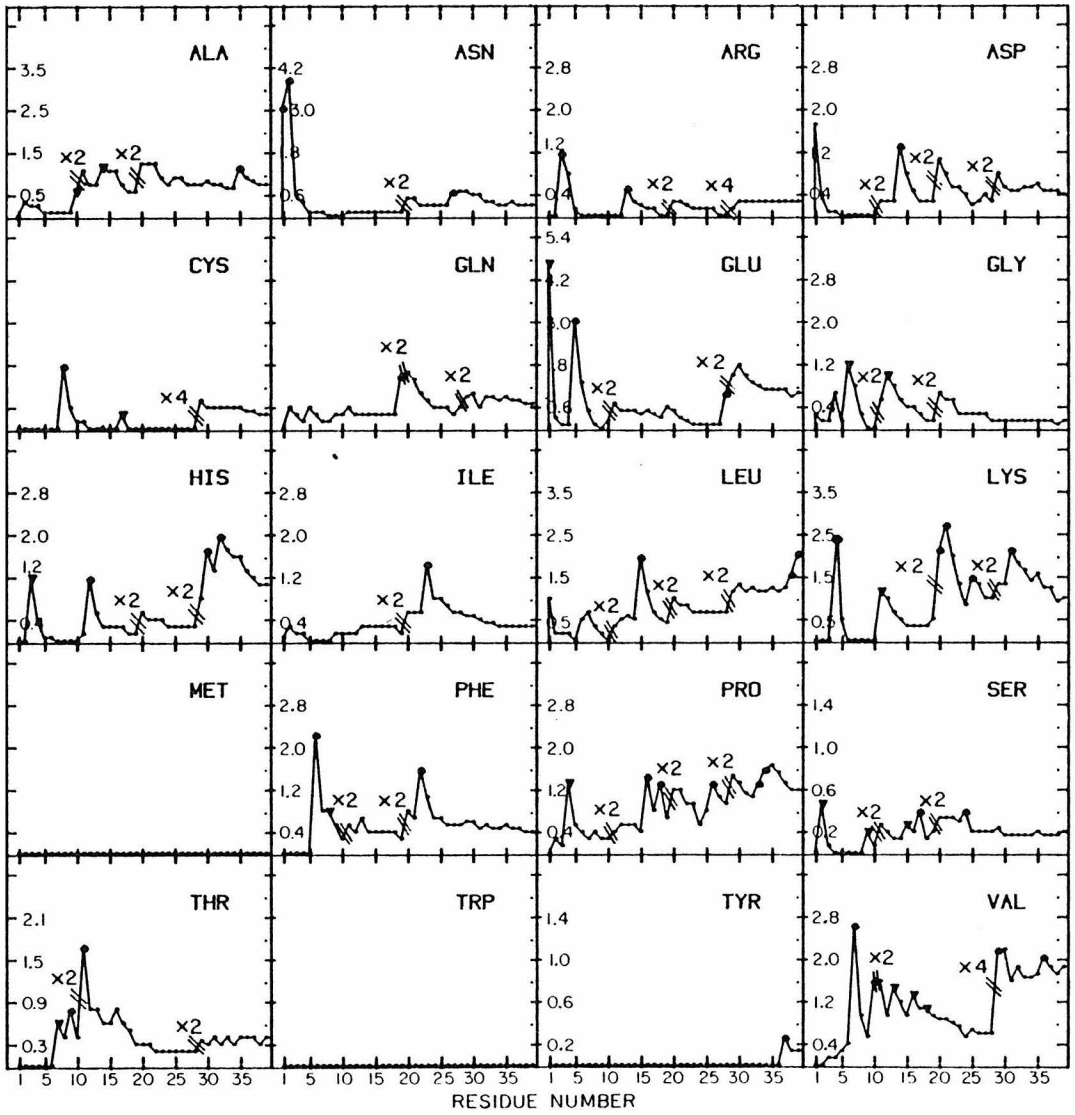

104E CN7 o
104E AMINO TERMINUS ▾
RESIDUE #1 CORRESPONDS TO RESIDUES
398 AND 1 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED
ONLY BACKGROUND PEAKS:
ARG, CYS, ILE, MET, TYR
ALA
ARG
ASN
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
×2
RESIDUE NUMBER

FIGURE 10O



104E CN7 W-I o
104E CN7 ▽

RESIDUE #1 CORRESPONDS TO RESIDUES 419 AND 398 IN THE Mu SEQUENCE

THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS:
MET, TRP

-\\- INDICATES CHANGE IN SCALE TO THAT INDICATED ON THE GRAPH.

FIGURE 10P

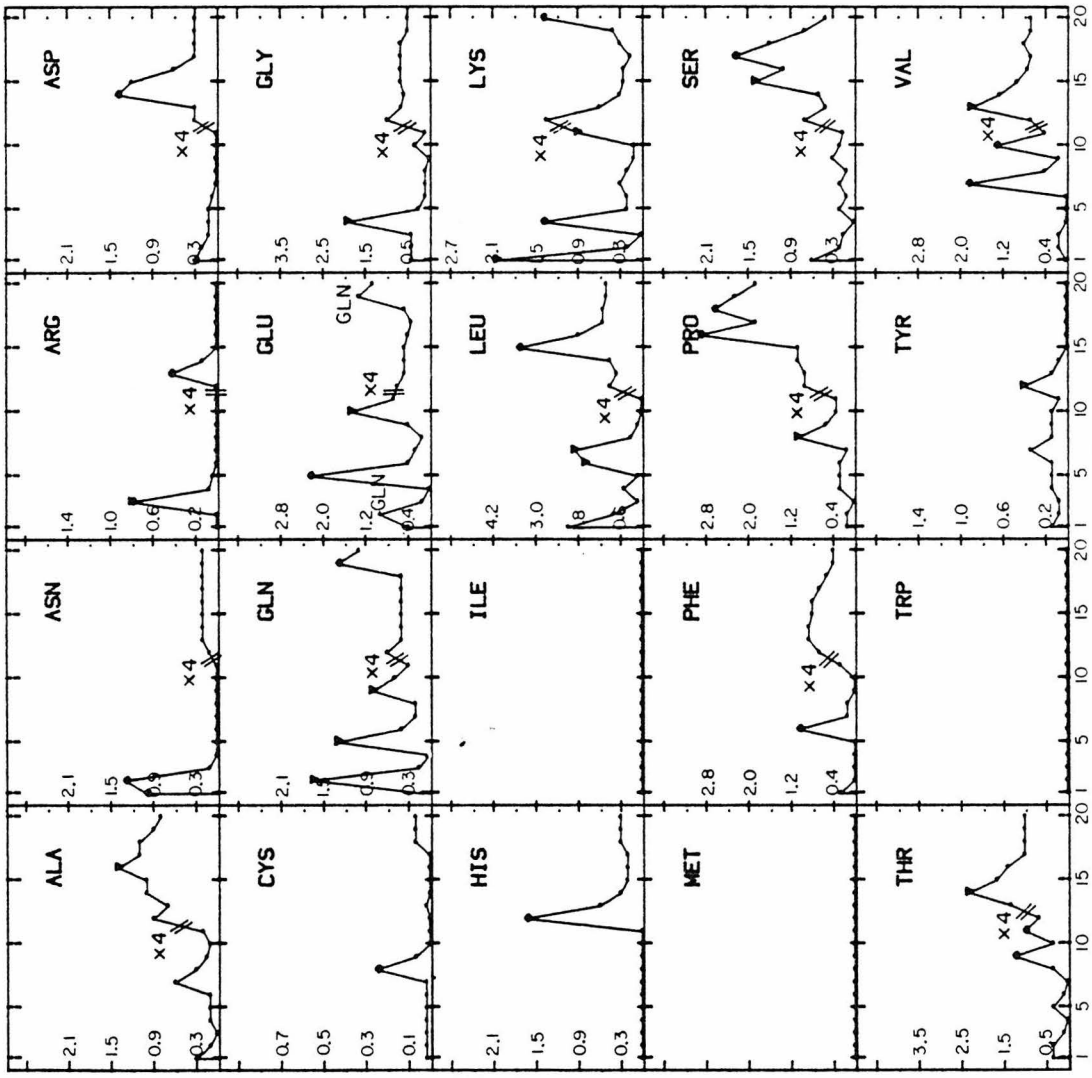

104E CN7 W-I o
104E CN7 W-II v
RESIDUE #1 CORRESPONDS TO RESIDUE #S 419 AND 489 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS.
ILE, MET, TRP
-\\- INDICATES CHANGE IN SCALE TO THAT INDICATED ON THE GRAPH.
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
GLN
x4
RESIDUE NUMBER

FIGURE 10Q

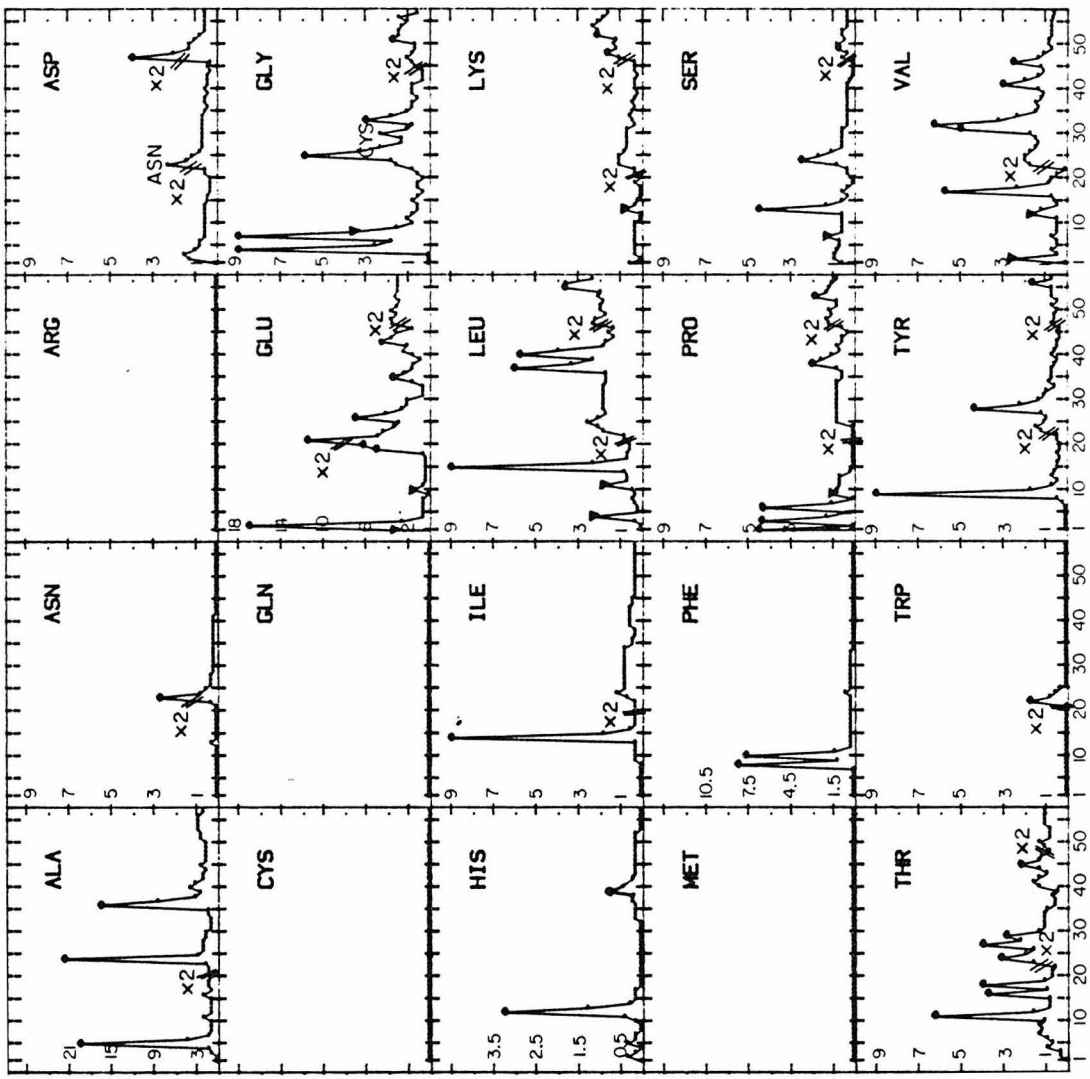

104E CN8-9 o
104E AMINO TERMINAL ▾
RESIDUE #1 CORRESPONDS TO RESIDUE #507 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS:
ARG, CYS, GLN, MET
-\\- INDICATES CHANGE IN SCALE TO THAT INDICATED ON THE GRAPH.
ALA
ARG
ASN
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
×2
RESIDUE NUMBER

FIGURE 10R

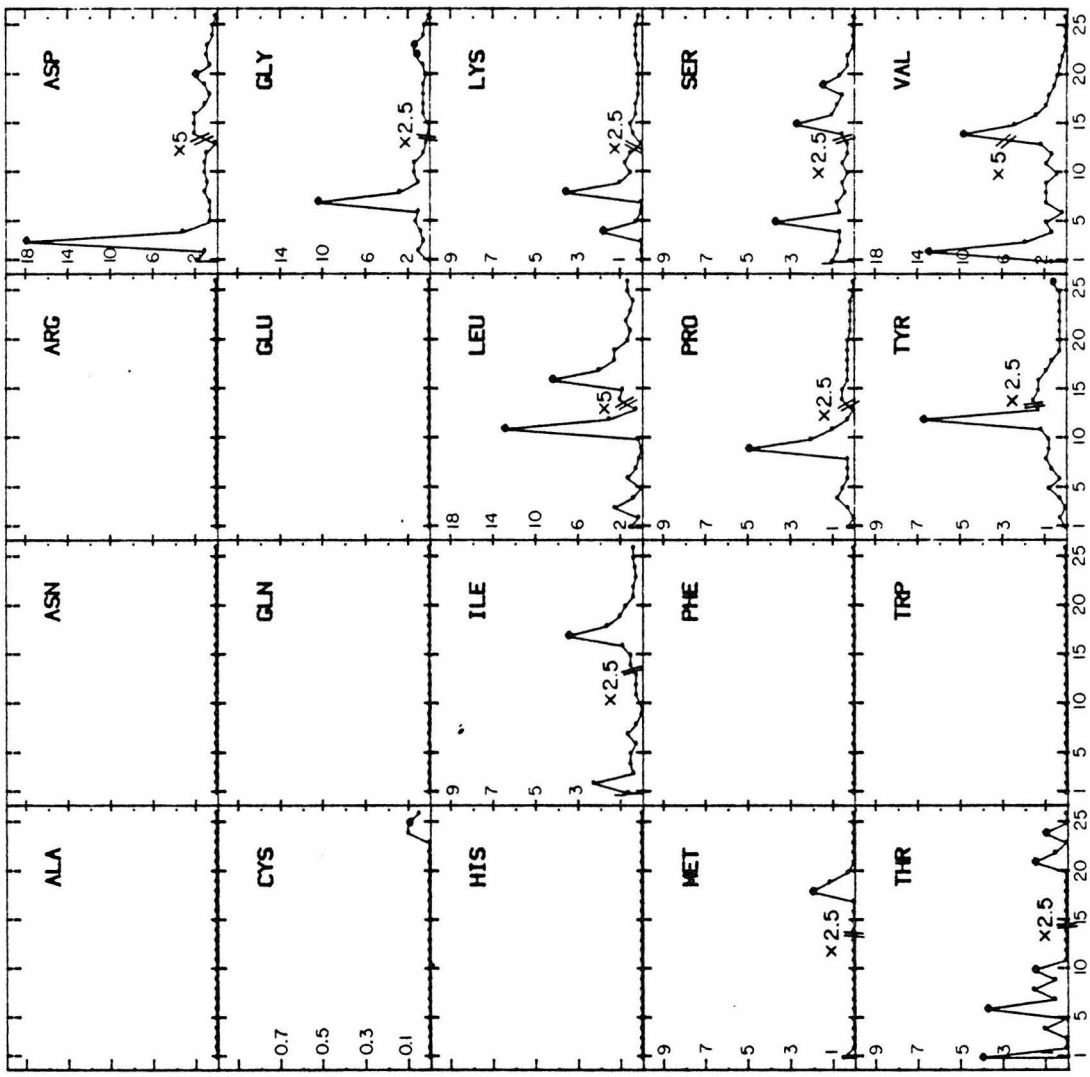

104E CN8-9 R
RESIDUE #1 CORRESPONDS TO RESIDUE #551 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS:
ALA, ASN, ARG, GLN, GLU, HIS, PHE, TRP
-\\- INDICATES CHANGE IN SCALE TO THAT INDICATED ON THE GRAPH.
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
RESIDUE NUMBER

FIGURE 10S

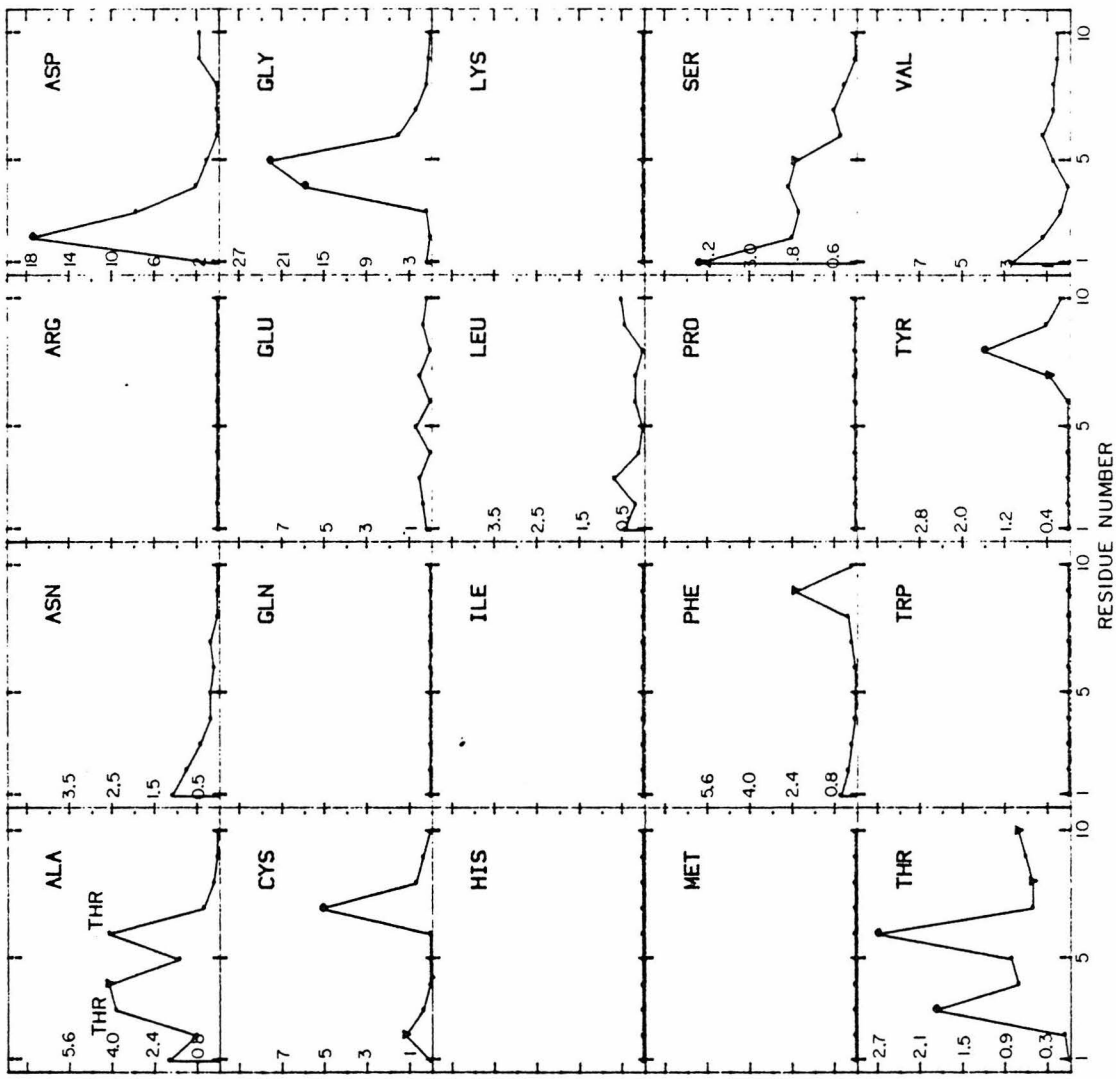

104E CN9 o
104E CN2 v
RESIDUE #1 CORRESPONDS TO RESIDUE NOS. 569 AND 21 IN THE Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS: ARG, GLN, HIS, ILE, LYS, MET, PRO, TRP
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
THR
THR
RESIDUE NUMBER

FIGURE 10T

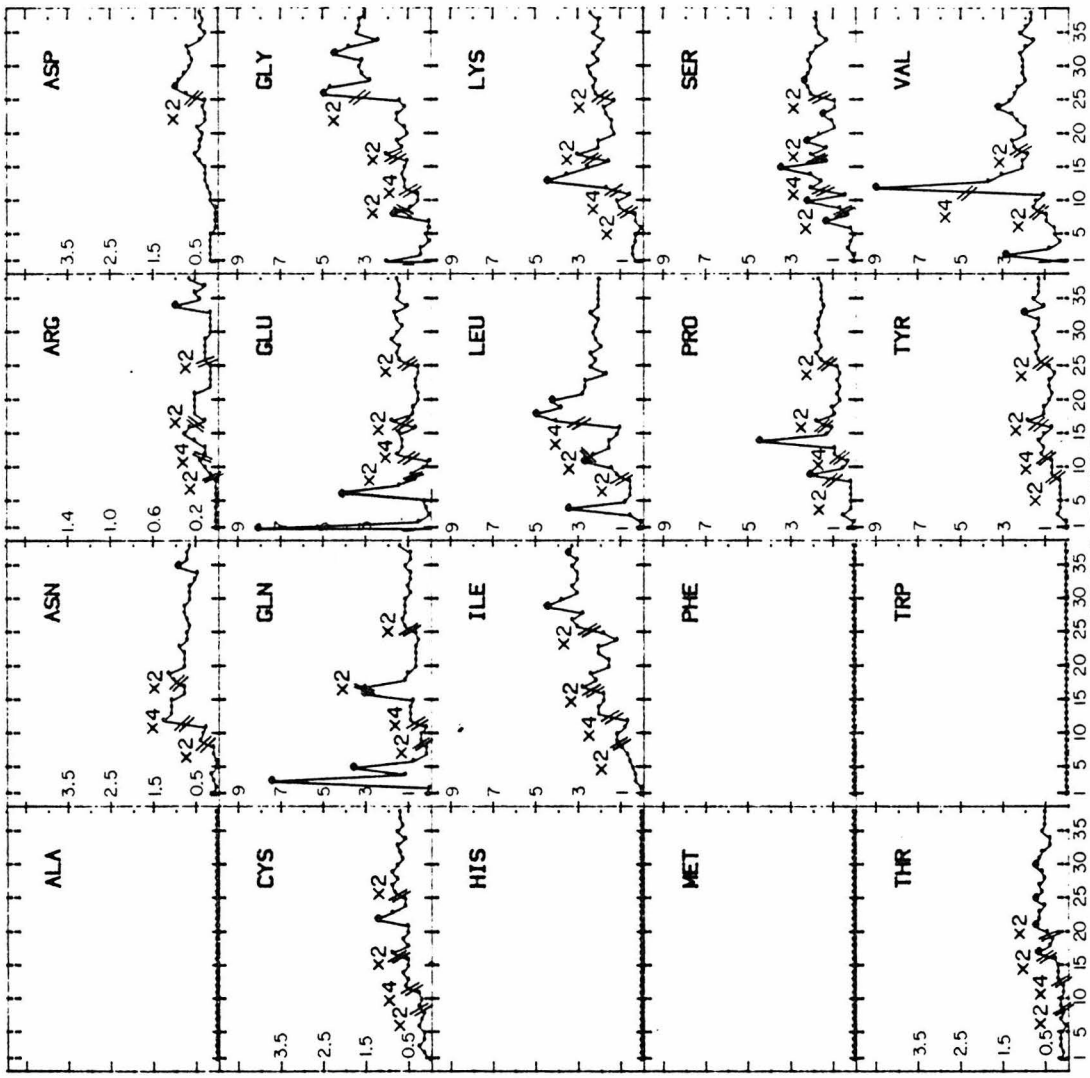

MPC11 X W279.2 MU
RESIDUE #1 CORRESPONDS TO RESIDUE #1 IN THE WEHI 279 MU SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS:
ALA, HIS, MET, PHE, TRP
-\\- INDICATES CHANGE IN SCALE TO THAT INDICATED ON THE GRAPH.
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
RESIDUE NUMBER

FIGURE 10U

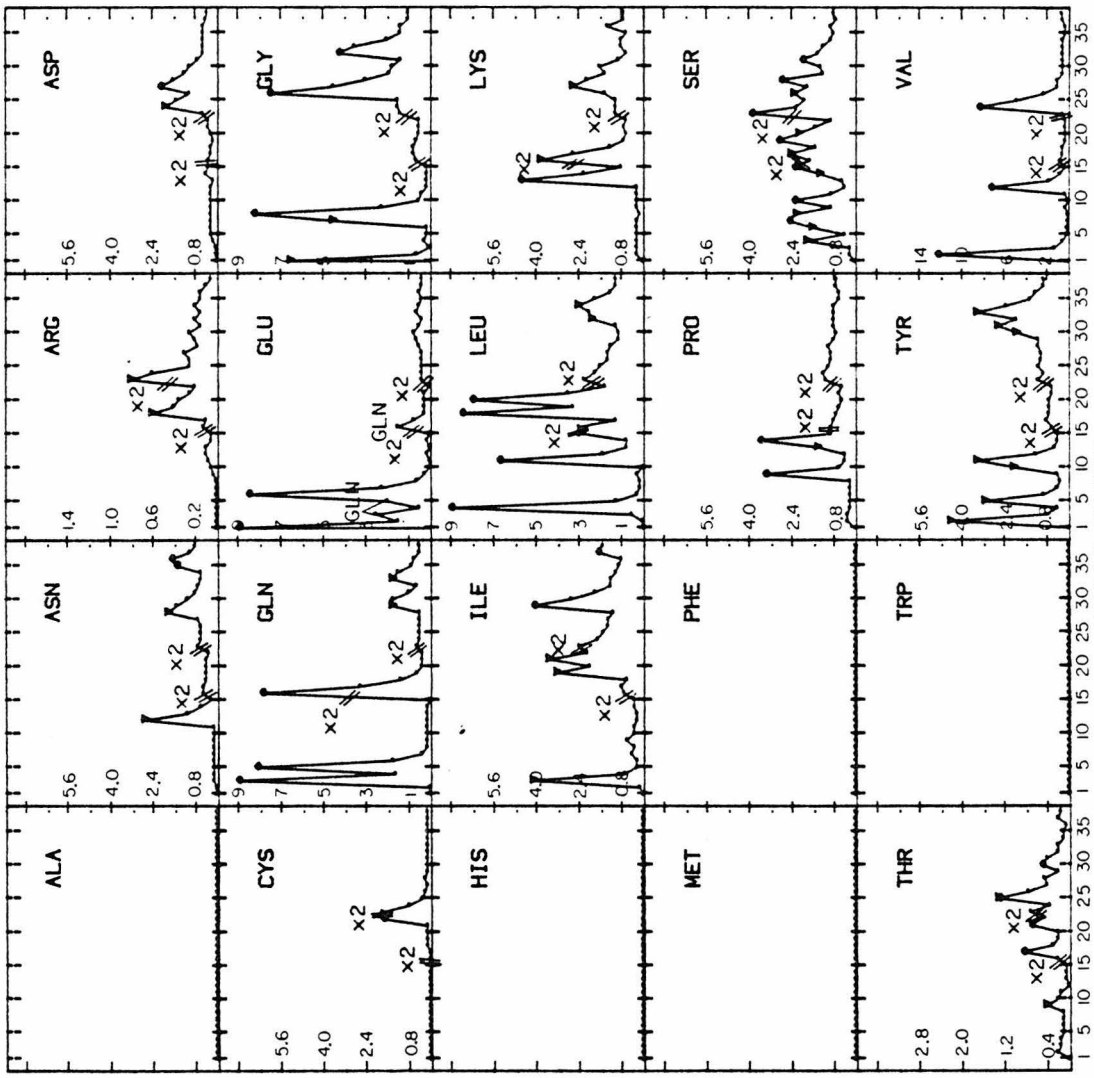

MPC11 X W278.2 CN3a o
MPC11 X W278.2 CN3b v
RESIDUE #1 CORRESPONDS TO RESIDUES
1 AND 49 IN THE WEHI 279 Mu CHAIN
THE FOLLOWING AMINO ACIDS EXHIBITED
ONLY BACKGROUND PEAKS:
ALA, HIS, MET, PHE, TRP
-\\- INDICATES CHANGE IN SCALE TO
THAT INDICATED ON THE GRAPH
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
RESIDUE NUMBER

FIGURE 10V

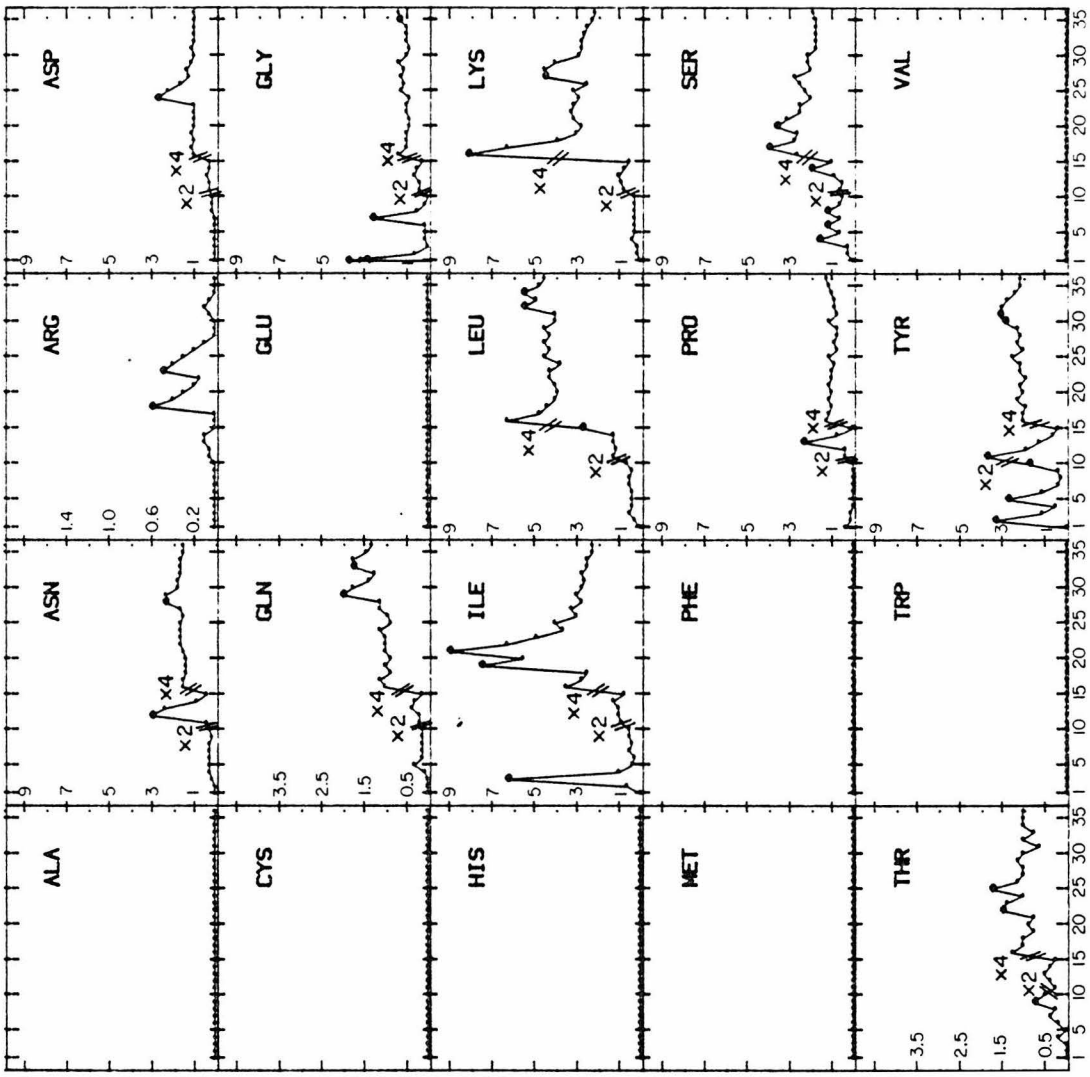

MPC11 X W279.2 CN3b
RESIDUE #1 CORRESPONDS TO RESIDUE #49 IN THE WEHI 279 Mu SEQUENCE
THE FOLLOWING AMINO ACIDS EXHIBITED ONLY BACKGROUND PEAKS:
ALA, CYS, GLU, HIS, MET, PHE, TRP, VAL
-\\- INDICATES CHANGE IN SCALE TO THAT INDICATED ON THE GRAPH.
ALA
ASN
ARG
ASP
CYS
GLN
GLU
GLY
HIS
ILE
LEU
LYS
MET
PHE
PRO
SER
THR
TRP
TYR
VAL
RESIDUE NUMBER

FIGURE 10W

Figure 11. Sequenator runs on radiolabeled MPC11 x W279.2 μ_s and WEHI 279 μ_i chain CNBr fragments.

These sequenator runs establish the identity of the WEHI 279 C_μ and MOPC 104E C_μ CNBr fragments (7). Fractions containing CNBr fragments from the ACA54 column were pooled, dialyzed 3X against 5% formic acid at 4°C, lyophilized and loaded on the Caltech sequenator in trifluoroacetic acid (TFA), 15% water. For each step, Pth-amino acids were separated by HPLC, the labeled residues fraction collected and counted for radioactivity (18). Scale changes and residue(s) identified at each step are indicated. Peptides are named as in reference 7. See reference 7 and Chapter 4 for the corresponding sequences.

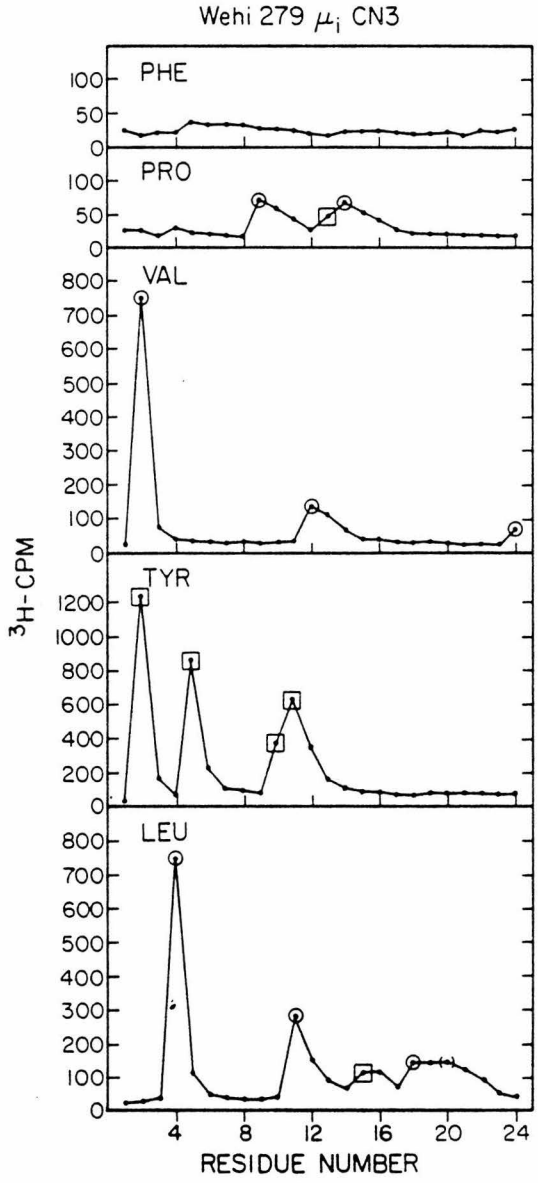


FIGURE 11

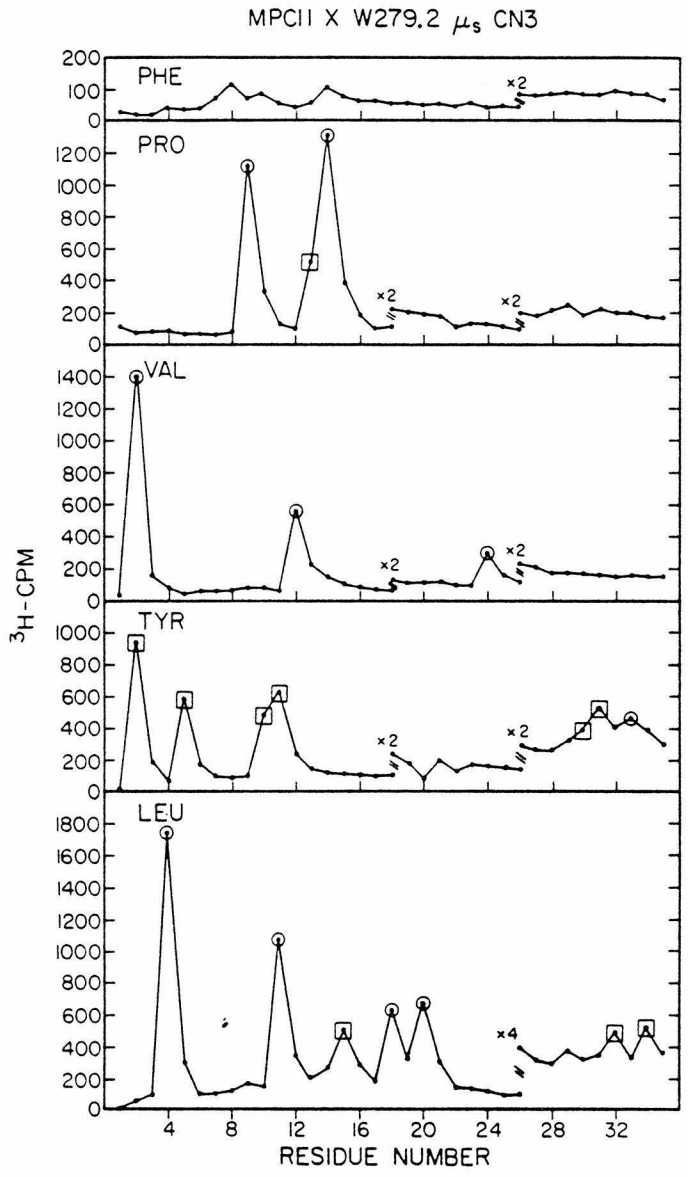

MPC11 X W279.2 μs CN3
PHE
PRO
VAL
TYR
LEU
×2
×4
^{3}H-CPM
RESIDUE NUMBER
4
8
12
16
20
24
28
32

FIGURE 11

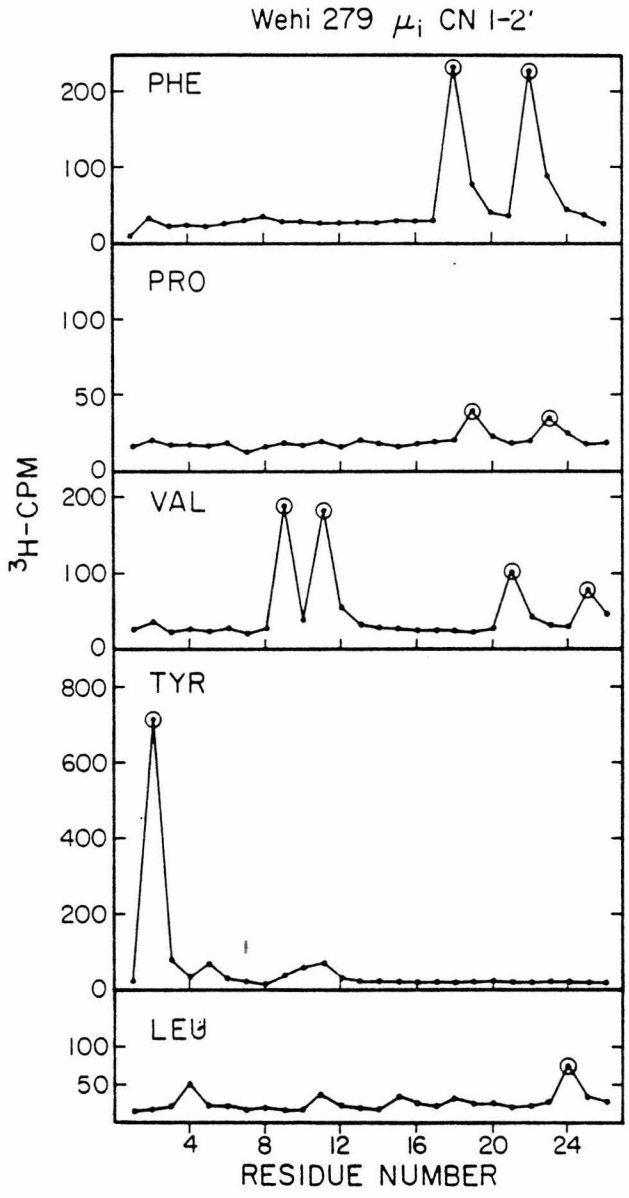

Wehi 279 μ_i CN 1-2'
PHE
PRO
VAL
TYR
LEU
200
100
0
100
50
0
200
100
0
800
600
400
200
0
100
50
^{3}H-CPM
4
8
12
16
20
24
RESIDUE NUMBER

FIGURE 11

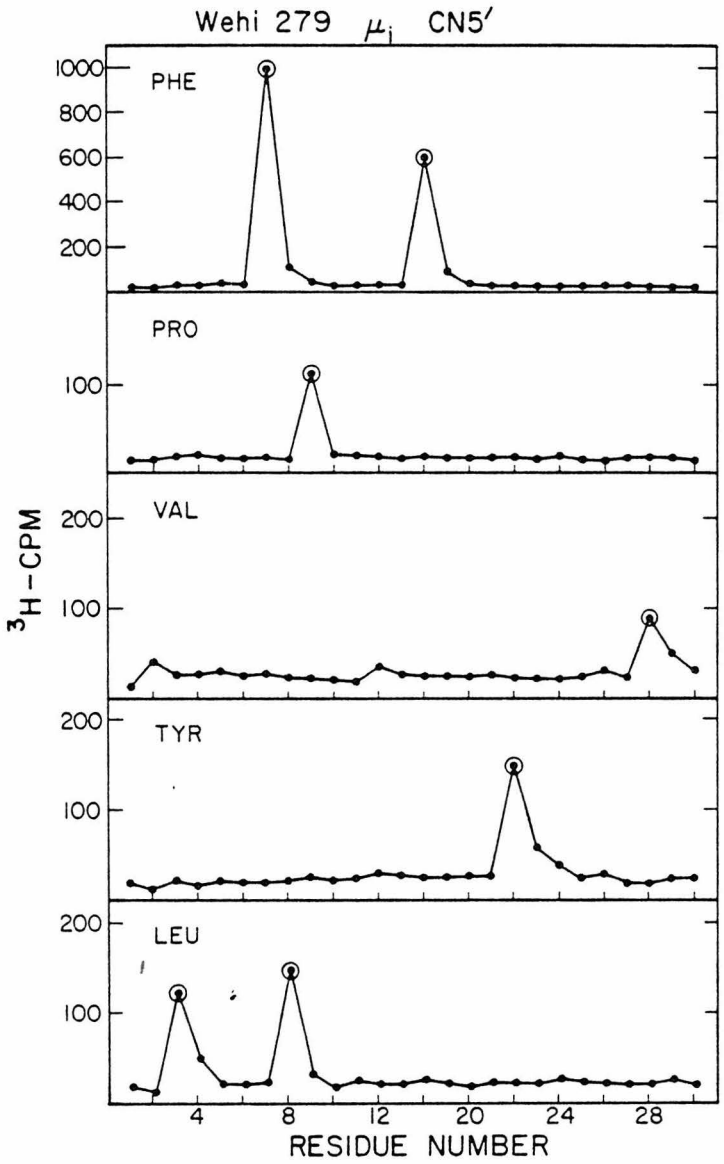

Wehi 279 μ_i CN5′
PHE
PRO
VAL
TYR
LEU
1000
800
600
400
200
100
200
100
200
100
200
100
^{3}H-CPM
4
8
12
20
24
28
RESIDUE NUMBER

FIGURE 11

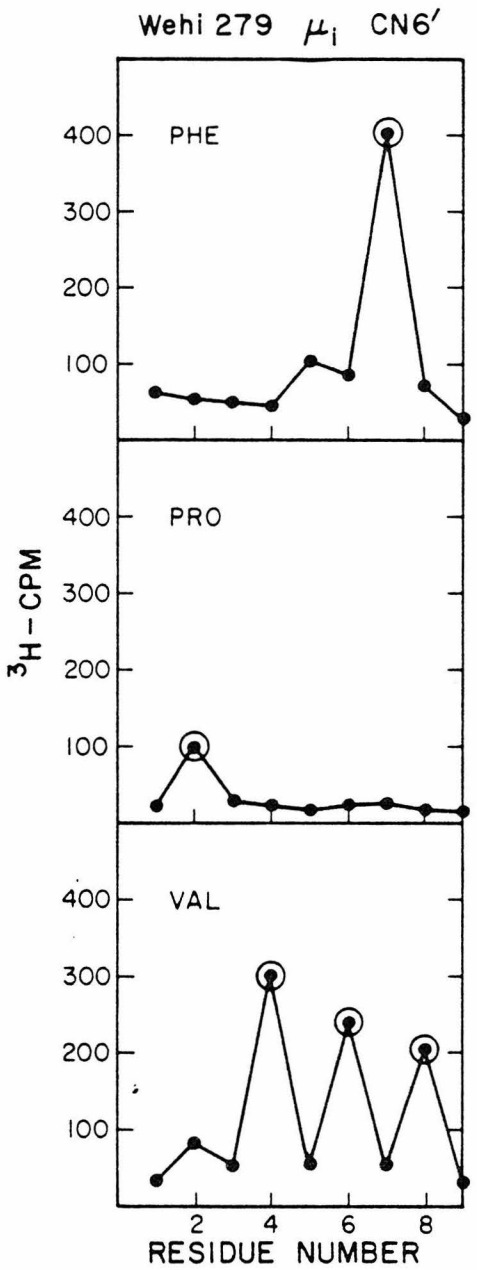

Wehi 279 μ_i CN6′
PHE
PRO
VAL
400
300
200
100
^{3}H-CPM
2
4
6
8
RESIDUE NUMBER

FIGURE 11.

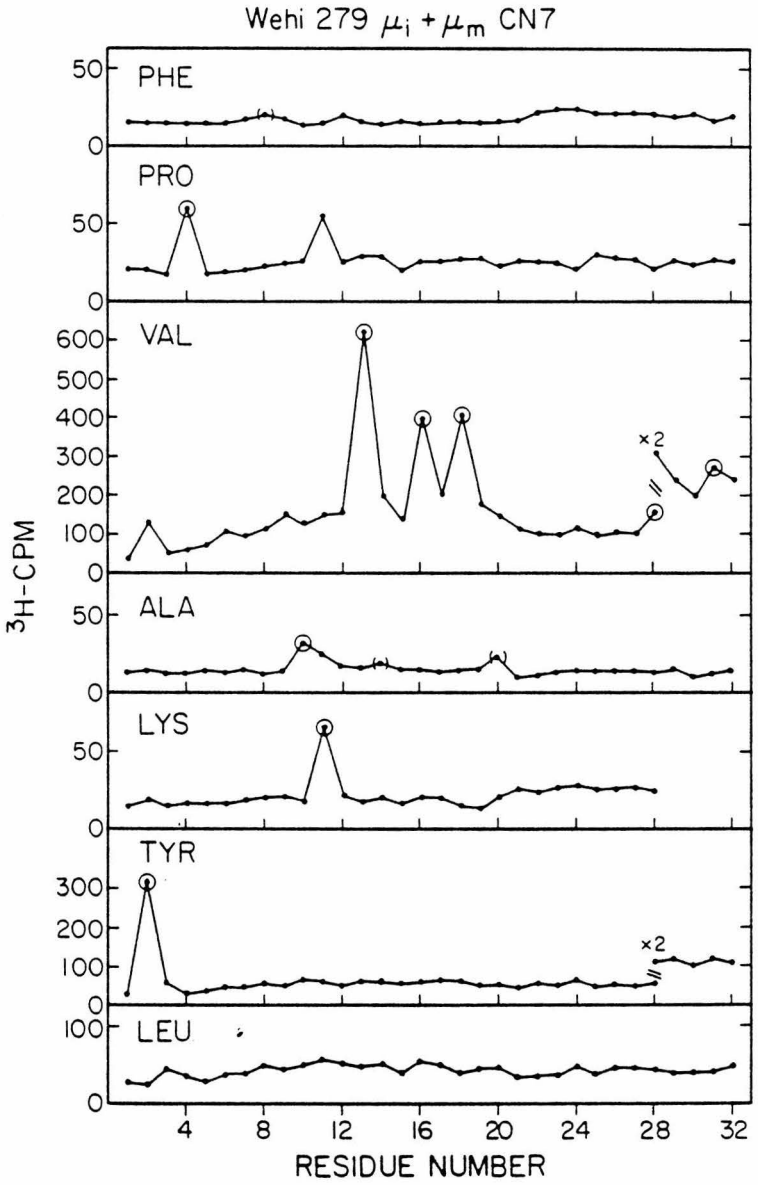

Wehi 279 $\mu_i + \mu_m$ CN7
PHE
PRO
VAL
ALA
LYS
TYR
LEU
×2
×2
^{3}H-CPM
RESIDUE NUMBER
0
50
100
200
300
400
500
600
4
8
12
16
20
24
28
32

FIGURE 11

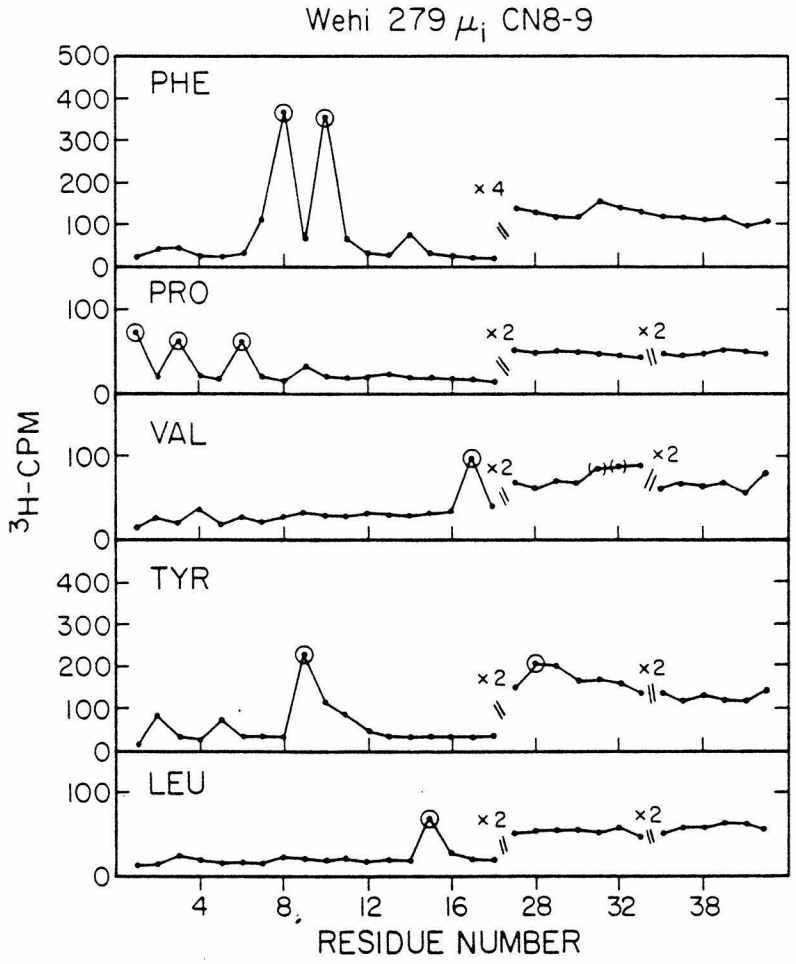


FIGURE 11

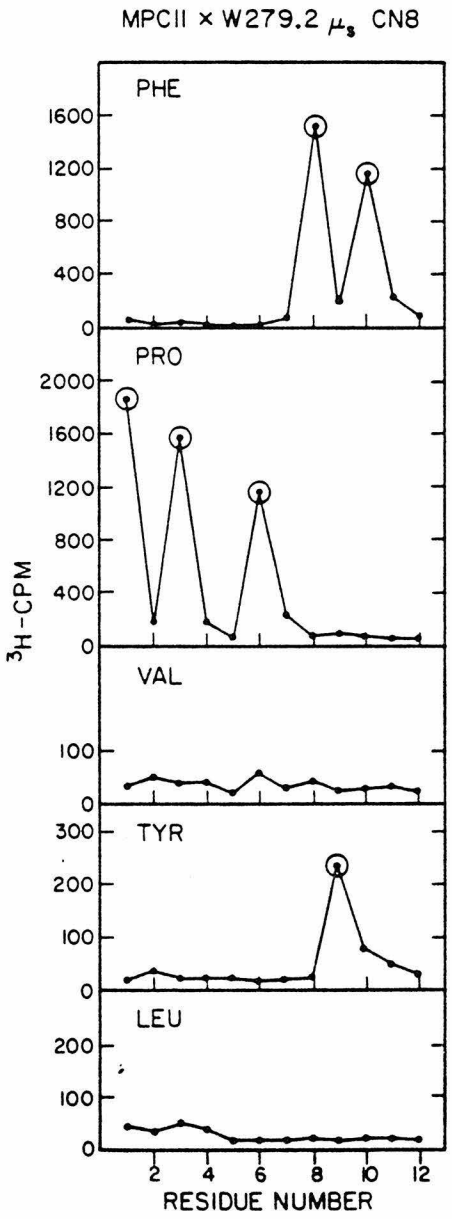


FIGURE 11

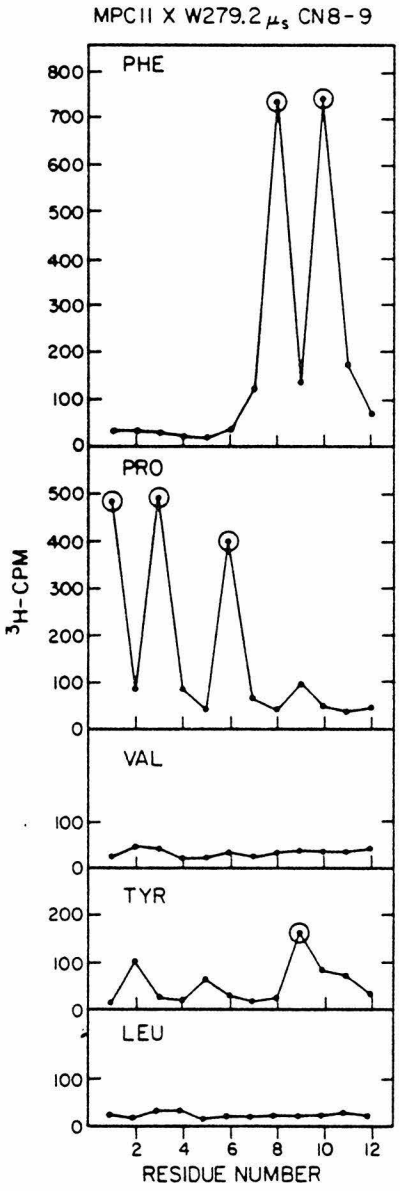

MPC11 X W279.2 μs CN8-9
PHE
PRO
VAL
TYR
LEU
^{3}H-CPM
RESIDUE NUMBER

FIGURE 11

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