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Nucleotide sequences of immunoglobulin μ heavy chain deletion mutants

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ABSTRACT

Mutants of an IgM producing hybridoma cell line were isolated which produce μ heavy chain fragments. Two such mutants were found to have internal deletions in the μ gene and the nucleotide sequence of the deletion endpoints was determined. No evidence was found for a role of the heavy chain switch region in the formation of these deletions. The implications of these mutants in defining the requirements of immunoglobulin gene expression are discussed.

INTRODUCTION

The immunoglobulin (Ig) genes in myeloma and hybridoma cells appear to be particularly susceptible to mutation. Depending on the cell line and type of mutant considered, the mutation rate has been calculated to be 10^{-4}-10^{-7} per cell per generation (1-4). An appreciable fraction of the mutations are gross rearrangements such as insertions (5) or deletions (6-7), and have large effects on gene expression or on protein structure. Functional expression of the immunoglobulin genes requires specific DNA rearrangements: genes for the variable regions are assembled by juxtaposing V, D, and J DNA segments; the heavy chain class switch might be effected by DNA deletion (reviewed in references 8-10). We would like to know whether sequences which are involved with normal Ig gene rearrangements in some way promote the occurrence of deletion mutations. It is also of interest to correlate the structure of such mutations with their effects on gene function. To these ends we report here the DNA sequences at the endpoints of two deletion mutations which occurred in the μ heavy chain gene.

MATERIALS AND METHODS

Bacteria, phage plasmids and cell lines. Escherichia coli K803 is an $r_k^- m_k^-$ sup E sup F strain and was obtained from G. Mattheyssens. The phage vector

λgtWES-B (11) was obtained from P. Leder. The plasmid pH76μ17 (12) bears a nearly full length cDNA copy of a μ mRNA and was obtained from J. Adams. A pbr322 plasmid carrying the Cμ1-Cμ2 Hind III fragment was obtained from K. Marcu. The hybridoma cell line PC7 which makes IgM specific for the hapten phosphorycholine has been described (4). The mutants were derived from the cell lines PC704, PC705 and PC700ul.1, which are subclones of PC7 and make wildtype IgM specific for phosphorylcholine.

Enzymes. Restriction enzymes were purchased from Boehringer Mannheim Canada, and Bethesda Research Laboratories. DNA polymerases were purchased from Boehringer. T_4 DNA ligase was purchased from New England Biolabs. Proteinase K was from Merck Co. RNAase T_1 and RNAase A were obtained from Sigma Chemical Co. Terminase was a gift from A. Becker and M. Gold.

Isotopes. ^{14}C-leucine for labeling Ig, [^{32}P] dCTP (3000 Ci/mmol) for nick translation and [^{32}P] dATP (3000 Ci/mmol) for DNA sequencing were purchased from Amersham and New England Nuclear.

Other materials. Agarose was obtained from Seakem. Nitrocellulose paper Type BA85 was obtained From Schleicher and Schuell.

IgM analysis. The methods for detecting IgM by the lysis of Staphylococcus protein A-coupled red cells and for analysing biosynthetically labeled IgM by SDS-PAGE have been described (4). For the IgM ELISA, IgM(k) from the hybridoma Sp603 (3) was purified by its binding to TNP-sepharose. IgM(k) was adsorbed to plastic dishes by incubating the purified IgM(k) at 1 μg/ml for 90' at 37°, after which unbound IgM was removed and remaining "sites" were blocked by overnight incubation with 1% albumin. Bound IgM could be detected by first incubating with rat monoclonal anti-μ antibodies, prepared as a dilution of hybridoma culture supernatants (13). Bound anti-μ was then incubated with a preparation of alkaline phosphatase-linked rabbit anti-rat IgG (Zymed), which was then assayed with p-nitrophenyl phosphate (4). Samples at the appropriate dilutions were incubated in the wells in the presence of a limiting amount of rat monoclonal anti-μ. The reduction of bound anti-rat IgG thus gives a measure of μ content.

Preparation of probes. The DNA segments used as specific probes for μ RNA and DNA are shown in figure 3. Plasmid DNA bearing these segments was prepared (14), digested with the indicated restriction enzymes, and electrophoresed in 5% polyacrylamide. The appropriate bands were excised and DNA was recovered by electroelution (15). Probes were labeled by nick translation (16).

RNA analysis. Total cytoplasmic RNA was prepared as described (17). RNA

(4μg) from each cell line was treated with glyoxal, electrophoresed through a 1% agarose gel, transferred to nitrocellulose and hybridized to the indicated probe (18).

DNA analysis. DNA was extracted from approximately 2 x 10^8 cells with SDS and proteinase K (19). DNA (20μg) was digested to completion with the indicated restriction enzymes, fractionated by electrophoresis in agarose, transferred to nitrocellulose filters, and hybridized to the radioactive probes (20).

Cloning of mutant μ genes. DNA from the deletion mutants was digested with Eco RI. The DNA bearing the μ gene of mutant #128 was enriched by electrophoresis (21). For mutant #208, μ DNA was enriched by centrifugation (22). DNA was ligated (23) with purified arms (24) of the vector λgtWES-B, and packaged according to Becker and Gold (25). Phage were plated on K803 and the plaques containing μ gene bearing phage were detected, as described (26), with a mixture of probes A and D (#128) or probe B (#208). Phage were then prepared (27) and DNA extracted (28).

DNA sequencing. The indicated fragments of the cloned genes were inserted in the DNA of phage M13mp9 (29) and DNA was sequenced by the dideoxy method of Sanger, et al. (30).

RESULTS

Origin of the Mutants. The mutants derive from a hybridoma cell line (PC7) which secretes IgM specific for the hapten phosphoryl choline (PC). The mutant selection method has been described in detail (4). Briefly, a reactive derivative of the PC hapten was attached covalently to the surface of the hybridoma cells. The cells were then incubated in the presence of complement. Under conditions of limited diffusion, the PC-specific IgM apparently binds preferentially to the PC on the surface of the cell from which it was secreted, where it activates complement, killing that cell. Thus, cells secreting normal amounts of normal IgM were lysed, whereas IgM-defective mutants survived. After several cycles of enrichment for mutants the surviving cells were cloned by limiting dilution, and culture fluid was tested for the presence of IgM using a protein A anti-IgM lysis test which detects material bearing antigenic determinants of the μ heavy chain (4). Where the culture fluid was positive in this test, secreted Ig was analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Cells which synthesized μ chains of faster than normal mobility were considered potential deletion mutants and were studied further. Mutants #109 and #128

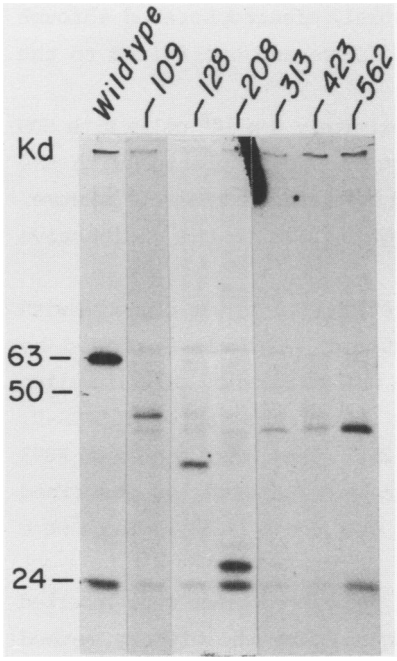

Figure 1. Analysis of unglycosylated μ chains. Unglycosylated [^{3}H]leucine-labeled μ was prepared by incubating cells 5 hrs. in the presence of tunicamycin (10 μg/ml). The anti-μ precipitated material was reduced and analyzed by SDS-PAGE. After electrophoresis, the gel was soaked in salicylate to enhance the sensitivity of the autoradiography.

were isolated from an unmutagenized wildtype clone PC705 (4). Mutant #208 was isolated from the wildtype clone PC704 after mutagenesis with ethylmethanesulfonate (4). Mutants #313, #423, and #562 were isolated from the wildtype clone PC700Oul.1, after treatment with the mutagen ICR 191, as described (31). The mutants #423 and #562 could not be distinguished by the tests described here, and might derive from the same mutational event. For comparison, we have included the mutant #1, which makes no μ chain (4), and as shown below lacks the Cμ gene.

Size of the μ Chain Fragments. To measure the polypeptide molecular weight of the μ chain fragments the mutants were incubated with ^{14}C-leucine in the presence of tunicamycin to inhibit glycosylation. The labeled material was then incubated with a rabbit anti-IgM serum and the precipitate was solubilized, reduced and analysed by SDS-PAGE (Fig. 1). The molecular weights of the μ chains of mutants #109, #128, and #208 were estimated previously (4) to be 47,000, 39,000, and 27,000 daltons respectively, compared to 63,000 daltons for wildtype μ. The μ chains of the mutants #313, #423, and #562 are estimated here to have a molecular weight of 44,000 daltons. These molecular weights would correspond to deletions of 145 amino acids (#109), 220 amino acids (#128), 330 amino acids (#208), and 170 amino

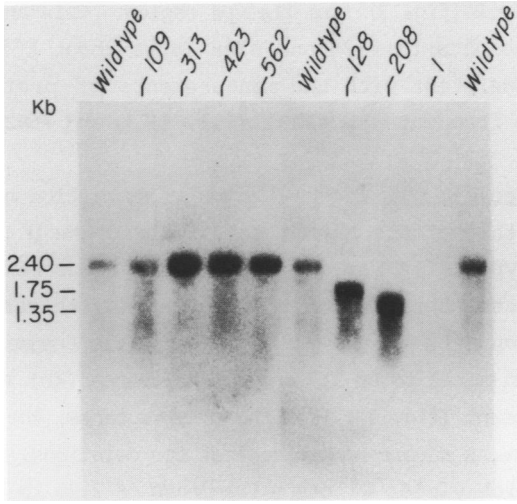

Figure 2. Analysis of μ specific RNA. RNA was prepared from the indicated cell lines, electrophoresed in 1% agarose and probed with segment B (fig. 3).

acids (#313, #423, #562).

Analysis of μ Specific RNA We tested whether the μ specific RNA of these mutants was also smaller than normal size. Total cytoplasmic RNA was subjected to gel electrophoresis (fig. 2) and μ specific sequences were

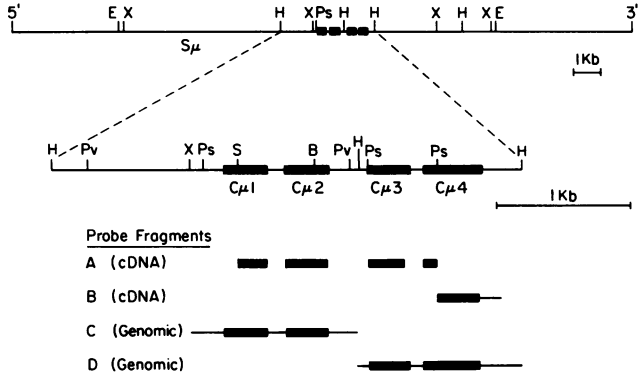

Figure 3. Structure of μ constant region. The restriction enzyme map is derived from several sources (32-34,54). B, Bam HI; E, Eco RI; H, Hind III; Ps, Pst I; Pv, Pvu II; S, Sma I; X, Xba I. The segments used as probes include the indicated regions. The exons encoding the four domains (Cμ1, Cμ2, Cμ3, Cμ4) of the μ constant region are indicated as black boxes. The region involved in the heavy chain class switch is indicated as Sμ.

detected with probe B (fig. 3) from the Cμ4 region. This analysis indicates that RNA from the mutants #128 and #208 lacks about 650 and 1050 bases respectively, consistent with the measurements of protein size. In contrast the RNA from mutants #109, #313, #423, and #562 is of apparently normal size.

Analysis of μ Specific DNA Figure 3 gives a restriction map of the μ gene. To test whether the mutants harbor deletions in the μ gene, the DNA of mutant and wildtype cells was digested with various restriction enzymes, sized by gel electrophoresis and then annealed with various μ specific probes. Digestion with Eco RI yielded μ specific fragments from #128 and #208 which were estimated to be 1.3 and 2.6 kilobases (kb) shorter than the normal 13 kb fragment (fig. 4). For the other mutants, the Eco RI fragments were of normal size.As a more sensitive test for deletions, DNA was digested with Hind III which yields wildtype fragments of 2.25 kb (Cμ1-Cμ2) and 1.2 kb (Cμ3-Cμ4), spanning the μ constant region. DNA blots were probed with fragment C (Cμ1-Cμ2) and fragment B (Cμ4) (Fig. 5). DNA from the mutant #208 showed no detectable homology with the Cμ1-Cμ2 probe; mutant #128 gave a fragment of normal size which reacted weakly with this probe. Both these mutants gave novel size fragments when their DNA was annealed to the Cμ4 probe. These results suggest that for both mutant #128 and #208 the Hind III site in the Cμ2-Cμ3 intron is deleted and that the deletions are 1.2 kb and 2.5 kb, respectively, consistent with the size estimates from figure 4. Thus, these results suggested that the deletions are completely included between the Hind III sites flanking the μ constant region. The apparently faster mobility of the Cμ3-Cμ4 fragment of #313 was not reproduced in later experiments. The mutants #109, #423, #562 are indistinguishable from normal, and we conclude that any deletions or insertions in these mutant DNAs must be less than 100 bases.

As shown in figure 6, digestion with Xba I gave a fragment from #128 which was 1.2 kb smaller than wildtype, suggesting that the Xba I site 5' of Cμ1 is intact in #128. Mutant #208 gave a fragment about 3.6 kb larger than wildtype, indicating that this Xba I site is absent in #208.

Digestion of wildtype DNA with Pst I gives two μ specific fragments of 1.2 and 0.56 kb, when probed with fragment A (fig. 7). Mutant #128 also gives a 0.56 kb fragment. From the DNA blot experiment, we cannot distinguish whether the 5' Pst I site of this fragment comes from the left (5' of Cμ1) or the right (3' of Cμ2) of the deletion, but this result indicates that at least one of these Pst I sites should be present in mutant

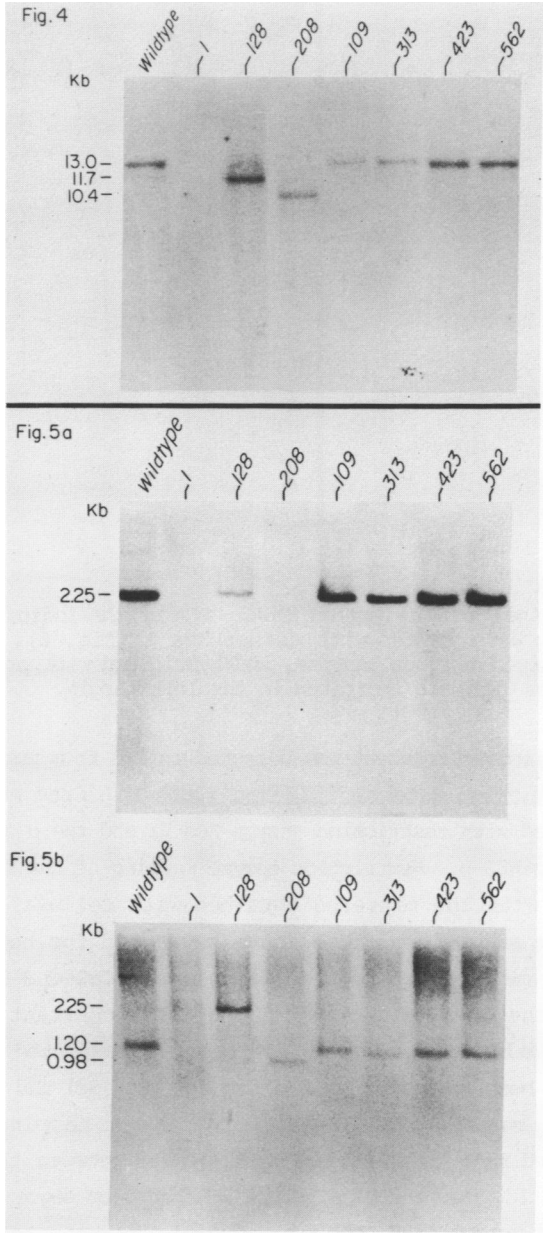

Figure 4 & 5. Analysis of mutant DNA. DNA of the indicated wildtype and mutant cell lines was digested with either Eco RI (fig. 4), or Hind III (fig. 5), electrophoresed through agarose, and hybridized with a mixture of probes A and B (fig. 4), probe C (fig. 5a), or probe B (fig. 5b). Fragment sizes in kilobases (kb) are indicated in the left margin.

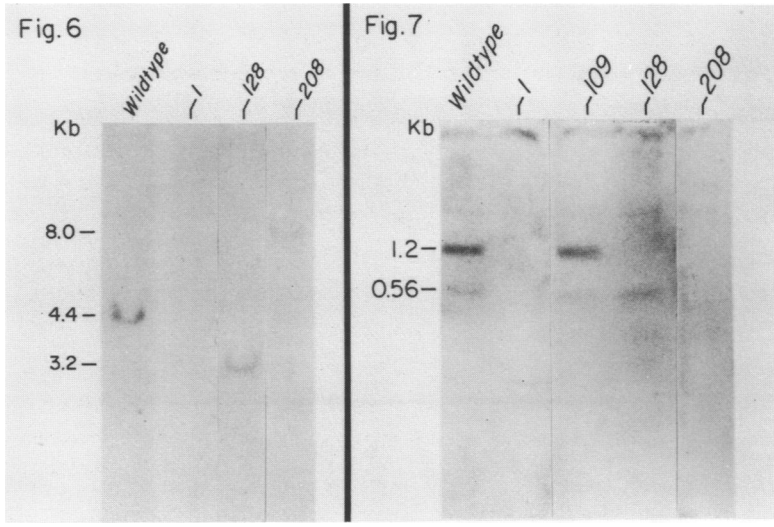

Figure 6 & 7. Analysis of mutant DNA. DNA of the indicated wildtype and mutant cell lines was digested with either Xba I (fig. 6), or Pst I (fig. 7), electrophoresed through agarose, and hybridized with probe A. Fragment sizes in kilobases (kb) are indicated in the left margin.

#128. Neither wildtype fragment was detected in DNA from mutant #208.

Cloning of the Deletion Mutants #128 and #208 DNA from mutants #128 and #208 was digested with restriction enzyme Eco RI and the μ specific fragment was cloned in λgtWES-B. Restriction enzyme mapping of the cloned fragments was consistent with the patterns obtained with cellular (uncloned) DNA (figs. 4-7). The mapping described above suggested for both mutants that the deletion removes the Hind III site in the Cμ2-Cμ3 intron and lies totally between the Hind III sites which flank the constant region (fig 3; fig 8a). For both mutants the 3' endpoint was expected to lie in the Cμ region which has been sequenced by Goldberg, et al. (32) and by Kawakami, et al. (33). For mutant #208, restriction enzyme mapping of the cloned fragment indicated that the 5' endpoint should lie between the Hind III and Pvu II sites; this segment of wildtype DNA has been sequenced by Greenberg et al. (34). For mutant #128, the 5' endpoint was expected to be outside sequenced regions so that in this case it was necessary to sequence the corresponding wildtype fragment. The indicated Hind III and Hind III - Pst I fragments were subcloned in M13mp9 for sequencing with the dideoxy nucleotide method (30). The mobility of the Hind III fragments was

indistinguishable from the mobility of the corresponding fragments of cellular (uncloned) DNA indicating that cloning had not resulted in gross deletions or insertions (less than 100 bases; results not shown).

Sequencing of Mutant #128. The Hind III fragments bearing Cμ from #128 and Cμ1-Cμ2 from wildtype were subcloned in M13mp9 so that the 5' end of the Cμ gene adjoined the primer binding site. As indicated above, the Xba I site was expected to be close to the 5' endpoint of the #128 deletion. In order to move this Xba I site closer to the primer binding site, the double stranded DNAs from M13-#128 and from M13-wildtype were digested with Eco RI and Xba I. The single stranded ends were filled with Klenow polymerase, ligated with T4 DNA ligase and transfected to obtain phage with the truncated insert. The sequence results are shown in figure 8. The 3' end of the wildtype sequence overlaps that published by Goldberg et al. (32) for 50 nucleotides. The #128 sequence is identical to the wildtype for 74 nucleotides, as measured from the midpoint of the Xba I site, after which it coincides with the sequence found 1194 bases away in the Cμ2-Cμ3 intron.

Sequencing of Mutant #208. The Cμ bearing Hind III fragment from mutant #208 was subcloned in M13mp9 so that the 5' end of the gene adjoined the primer binding site. In order to sequence from the 3' end of the gene, the Hind III - Pst I fragment was cloned in the opposite orientation so that the Pst I site (3' end of the gene) adjoined the primer binding site. The sequence read from the Hind III site overlaps that read in the opposite direction from the Pst I site for 35 bases. The #208 sequence corresponds to that reported by Greenberg et al. (34) for 221 bases after which it coincides with the sequence found in the Cμ3-Cμ4 intron (fig. 8).

Analysis With Monoclonal Anti-μ Reagents. IgM from the mutants #128 and #208 was used in an earlier study (13) which mapped the epitopes recognized by a series of monoclonal μ specific IgGs. We have used these monoclonal IgGs to analyse the fragments produced by the mutants #109, #313, #423, and #562 (Table 1). These results suggest that the mutants #109, #313, #423, and #562 lack material from the carboxy terminus of wildtype μ chain.

DISCUSSION

The clonal selection theory (35) proposed that Ig producing cells should make antibodies of only one specificity; that is, cells should have only one functionally rearranged gene for the heavy and light chain. Normal IgM producing B-cells appear to have two rearranged μ genes (36); presumably only one μ gene is functional. The deletion mutants studied here

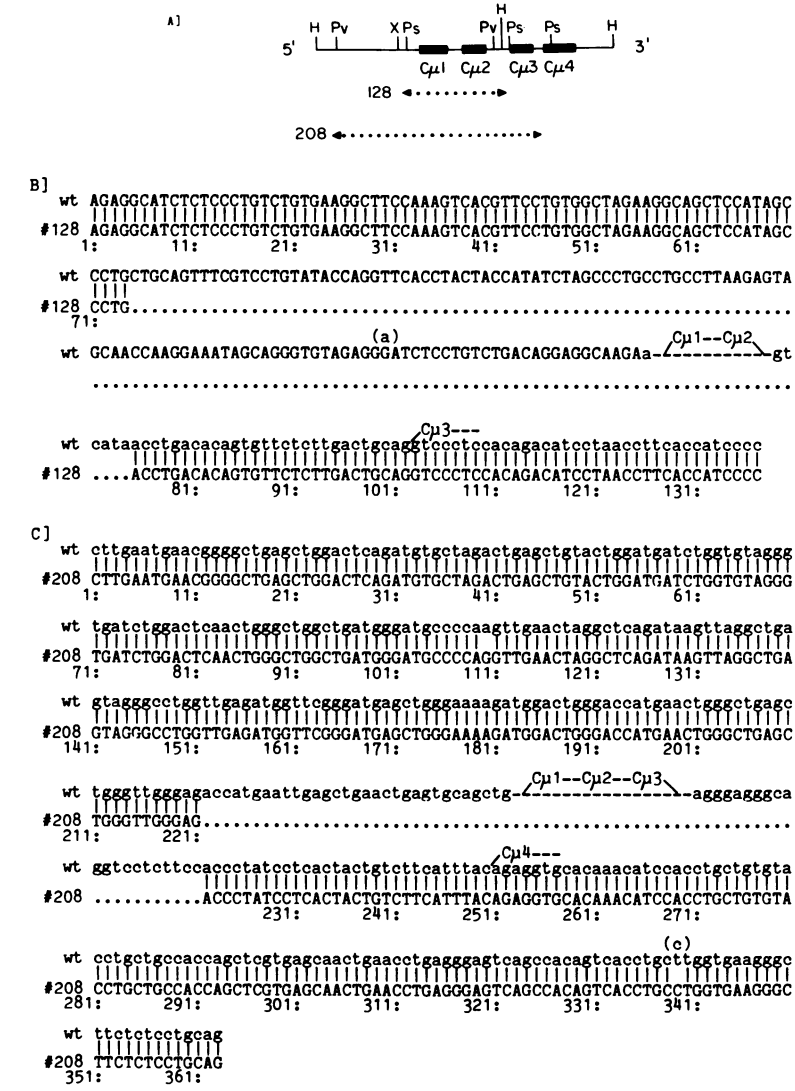

Figure 8. Nucleotide sequence of the deletion mutants.
A) The extent of the deletions in mutants #128 and #208 is indicated by the dotted lines.
B,C) In each case the upper sequence is from the wildtype (wt); the lower is from the mutant. Where the sequences agree is indicated by |. The sequences determined here are in upper case letters, those taken from other work (32-34) are in lower case. Part B gives the sequence for the mutant #128, numbered from the midpoint of the Xba I site, 5' of Cμ1. The wildtype and mutant sequences agree up to position 74. As indicated, after this point of divergence the mutant sequence corresponds to that reported for the intron between Cμ2 and Cμ3 (31,33). The wildtype sequence from position 146

to position 195 agrees with that reported by Goldberg, et al. (32) except as indicated at position 170. Part C gives the sequence from the mutant #208, numbered from the midpoint of the Hind III site 5' of Cμ1. As indicated this sequence corresponds to that published by Greenberg, et al. (34), up to position 221. The sequence from position 222 to 363 corresponds to that reported for the region 5' of the Pst I site in Cμ4 (32,33). The published sequences also differ at position corresponding to nucleotide 339 in mutant #208: T in ref. 32, and C in ref. 33.

and earlier (7) have one μ specific restriction fragment and it is distinct in size from the normal fragment. These results suggest that the wildtype hybridomas used in these studies have only one functional and no nonfunctional μ genes.

Switching from expression of the μ to other heavy chain constant regions is associated with deletions which join the newly expressed constant region to the variable region (9,10). The switch sites (fig. 3) defined by such deletions might participate in other rearrangements. Thus, cell lines expressing the μ heavy chain have also undergone a small deletion in the switch region (12,37). The cloning of the μ gene as recombinant DNA has usually resulted in a deletion of material in the switch region (discussed in reference 38). Finally, in many myelomas, translocations have occurred between the chromosome bearing the (inactive) heavy chain locus and that bearing the myc oncogene, and in some cases these translocations have been mapped within the switch region of the α heavy chain (reviewed in reference 39). For such reasons it might be expected that the switch region would be a hotspot for deletion formation. However, the overall distribution of the deletions analysed here for mutants #128 and #208 and earlier (7) does not support this expectation.

Other features of DNA structure could play a role in causing deletions. From their analysis of bacterial deletions, Albertini et al.(40) and Marvo, et al. (41) have argued that short repeats increase the likelihood of deletion formation. Eckhardt, et al (42) have suggested that the fusion of the γ2b and γ2a genes occurred at sites of particularly close homology. On the other hand, deletions in SV40 mutants (43) and in drosophila mutants (44) were not associated with significant repeats at the deletion sites. With regard to the μ gene deletions analysed here, it is perhaps of interest that for both mutants at least one deletion endpoint has the sequence ACC and that in both cases the sequence GACC is generated at the new junction. From their analysis of retrovirus sequences, Van Beveren et al., (45) have suggested that the sequence GACC might be associated with DNA

Table 1

Cell Line	Deletion	Monoclonal anti-μ Specific for domain Cμ1 (R33/24)	Cμ2 (b7.6)	Cμ3 (1M41)	Cμ4 (C2.23)	Cμ4 (M8/7)
PC7	none	5	5	3	5	2
Igml2	Cμ1	Ø	4	3	5	2
#128	Cμ1-Cμ2	Ø	Ø	3	3	4
#208	Cμ1-Cμ2-Cμ3	Ø	Ø	Ø	2	5
#109		4	2	Ø	Ø	Ø
#313		3	2	Ø	Ø	Ø
#423		4	6	4	Ø	Ø
#562		4	5	4	Ø	Ø

For this solid phase ELISA, IgM was adsorbed to 96 well plates as described in Methods. The concentration of IgM in culture supernatant of the indicated cell lines was detected by its capacity to compete with the adsorbed IgM for binding the rat monoclonal anti-μ's. The particular rat hybridoma cell line used here is indicated in parentheses. To each well was added culture supernatant serially diluted in 3 fold steps (dilution range tested 3^{0}-3^{5}). The monoclonal anti-μ's were then added to each well and the mixture incubated to permit binding of the rat anti-μ's to the IgM. The plates were then washed, and bound anti-μ was detected by adding anti-rat IgG coupled to alkaline phosphatase which in turn was detected by its capacity to hydrolyse p-nitrophenylphosphate. The table lists for each cell line the number of wells in which culture supernatant caused significant inhibition of binding by the monoclonal anti-μ's to the adsorbed IgM.

rearrangements. The μ gene mutations analysed here apparently arose as simple deletions; that is, there is no evidence of accompanying insertions or other rearrangements, as have been reported in other cases (discussed in reference 41).

Mutants #109, #313, #423, and #562 make proteins which appear to lack the carboxyterminus of the μ heavy chain; their DNA appears not to have an extensive deletion and their RNA is of normal size. Their μ chain fragments might arise from proteolytic digestion, or from nonsense or frameshift mutations. The μ chain of #109 is apparently longer than that of #423 and #562, but it lacks an antigenic determinant present on the shorter chains. These results suggest that #109 is a frameshift mutation. However, it should be emphasized that we do not know to what extent the antigenic sites are determined only by nearby amino acids.

The structure of the deletion mutants also gives information on the location of DNA sequences required for RNA transcription. The deletion mutant igm-43, described in an earlier study (7), has a deletion which extends from the switch region through the Cμ1 exon. The deletion of mutant #208 described here overlaps the igm-43 deletion at Cμ1 and extends to the

intron between Cμ3 and Cμ4. Together these mutants argue that the DNA segment between the switch region and the Cμ3-Cμ4 intron does not play an important role in determining the magnitude of gene expression. A transcription enhancer sequence has been identified in the J-Cμ intron (55,56). The mapping of this enhancer indicates that it should be present in all the μ gene deletion mutants analysed here and earlier (7), and is thus consistent with the observation that these truncated μ genes are transcribed at approximately normal levels. Several of the mutants analysed here make μ chain fragments apparently lacking the carboxyterminal Cμ3-Cμ4 part of the μ heavy chain. As noted previously (7), such mutants do not have an extensive deletion. The failure to find such deletions suggested that there is a site 3' of Cμ4, perhaps the poly A addition site, which is critical for μ gene expression. In many tumor cell lines producing γ or α heavy chains, the region 3' of Cμ4 is deleted, and we suppose that this putative critical 3' site would be present 3' of all heavy chain genes.

The mutants #128 and #208 lack segments of DNA which encode the domains Cμ1-Cμ2 and Cμ1-Cμ2-Cμ3, respectively, and produce proteins which apparently lack only these same regions. Normally the 5' site at J would be spliced to the 3' site at Cμ1. In these mutants which lack this 3' splice site, splicing is apparently to the 3' site of Cμ3 (#128) and of Cμ4 (#208). The mutant splicing patterns are not detected as protein or RNA fragments in wildtype cells. Nor are the fragments characteristic of mutant #208 detected in mutant #128. These results are consistent with a mechanism in which RNA splicing is not very sensitive to the overall structure of the primary transcript, and which proceeds by joining the nearest 5' and 3' sites. There are several other reports where intron structure has been altered without large effects on the level of gene expression (50-52). In contrast it has been observed that deletions far from the splice sites can have large effects on splicing activity (53). It might be of interest for the analysis of molecular function or for therapeutic purposes to construct immunoglobulins in which part or all of the constant region is replaced by part or all of other constant regions. That the mutant immunoglobulin genes are expressed efficiently suggests that genetic engineering by interchanging exons might be straightforward.

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