

## Deletions in immunoglobulin mu chains

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**Eight mutant hybridoma lines are described, which synthesize short immunoglobulin  $\mu$  chains. Four internal deletions were mapped by Southern blot analysis. They are shown to remove DNA from either part or all of the first, and first and second, constant  $\mu$  exons. The sizes of the deletions range between 0.6 and 5 kb, leaving an equal or unequal number of splice signals. Shorter  $\mu$  RNA of one size was found irrespective of whether an exon was completely or only partially deleted. These results preclude exclusive 3' (constant region) to 5' (variable region) directional splicing of the  $\mu$  RNA. No important signals seem to reside in the deleted DNA stretches affecting the transcription or the correct RNA splicing of the remaining exons. The internal  $\mu$  protein deletions revealed unusual covalent light chain attachment demonstrating functional homology between the first (normally used) and fourth  $\mu$  constant domain. The other  $\mu$  protein deletions (10, 11, and 12 kd) involved neither gross DNA nor RNA lesions and are considered to be due to premature chain termination. Since secretion is found in most of the mutant IgM-producing lines, no single one of the four  $\mu$  constant domains (including the C-terminal one which contains the so-called secretory piece) is necessary for secretion.**

**Key words:** immunoglobulin M/domain deletions/RNA splicing/light chain attachment/secretion

### Introduction

In an attempt to understand how a mammalian gene operates, we chose to analyze IgM by studying mutant cell lines defective at various levels of production. Intracellular IgM exists mainly in a monomeric form consisting of two heavy ( $\mu$ ) and two light (here  $\kappa$ ) chains, assembles into pentamers of 900 kd mol. wt. shortly before secretion, is heavily glycosylated, and requires glycosylation for effective secretion (Hickman and Kornfeld, 1978). It exists also as a membrane form with a distinct C-terminal sequence (Kehry *et al.*, 1980) originating from a differential splicing event (Rogers *et al.*, 1980; Early *et al.*, 1980b). The DNA and protein sequences have been determined for both  $\mu$  (Kehry *et al.*, 1979; Kawakami *et al.*, 1980) and  $\kappa$  light chains (Svasti and Milstein, 1972; Hamlyn *et al.*, 1981; Max *et al.*, 1981; Altenburger *et al.*, 1981). Both genes become active only after an intra-chromosomal rearrangement step, bringing the variable and constant regions into proximity (Early *et al.*, 1980a; Sakano *et al.*, 1980; Brack *et al.*, 1978). The secretory  $\mu$  chain consists of five protein domains which are reflected in the DNA structure as five separate exons (Gough *et al.*, 1980). Somatic

mutation has recently been found to operate in immunoglobulin genes, increasing the antibody repertoire (Bernard *et al.*, 1978; Selsing and Storb, 1981; Crews *et al.*, 1981; Bothwell *et al.*, 1981).

The great advantages of applying the mutational approach to this system are that  $\mu$  and  $\kappa$  genes give rise to functional protein from only one of the homologous chromosomes, 12 and 6, respectively, (Hengartner *et al.*, 1978) and that IgM of single cells can easily be detected using modifications of the Jerne plaque technique (Jerne and Nordin, 1963).

A selection procedure and the mutants derived from two independent and non-mutagenized selections have been described (Köhler and Shulman, 1980). In short, variants of a hybridoma line secreting an anti-trinitrophenyl-specific IgM were selected by covalently coupling the hapten to the surface of the wild-type (WT) cells and incubating them in the presence of complement. WT cells thus commit suicide whereas variant cells secreting altered IgM are enriched. A panel of variants was derived including cells which make little or no  $\mu$  or  $\kappa$  chains, whose IgM has lower affinity for the hapten, has lost its lytic activity, or is unable to make pentameric IgM. A major group of variants was found to synthesize smaller  $\mu$  chains, the structural basis of which are described in this paper.

### Results

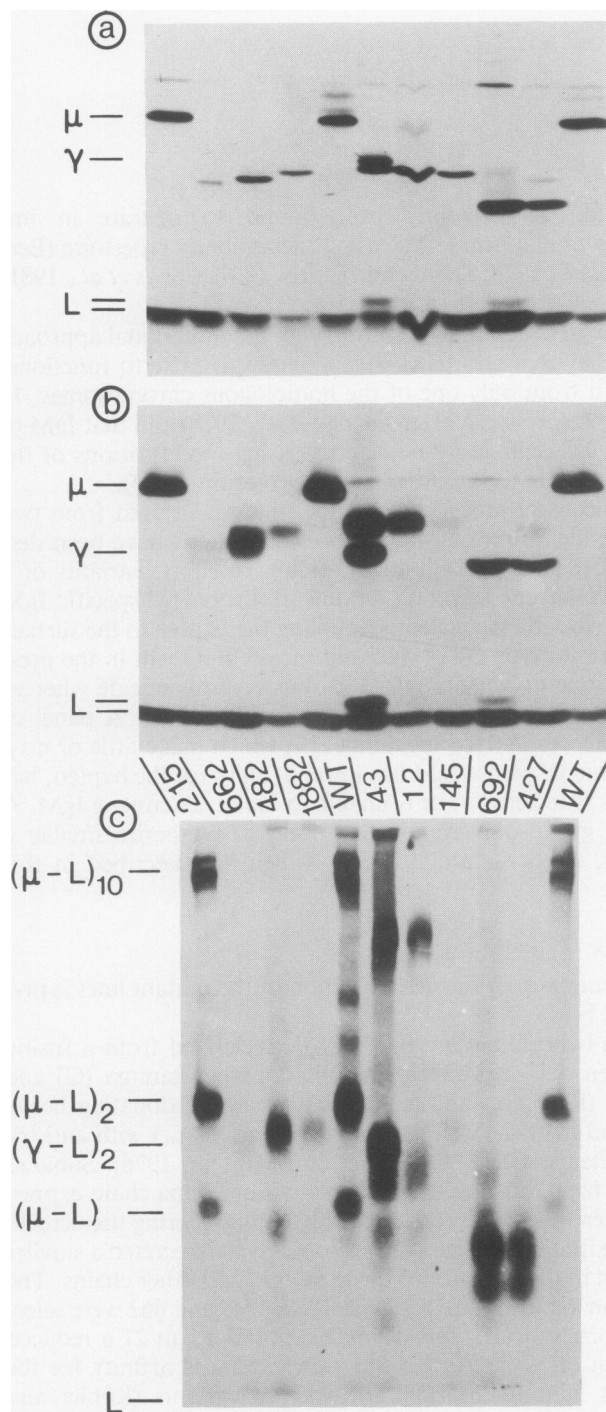
A summary of the derivatization of the mutant lines is presented below.

The hybridoma line Sp6/HLGK is derived from a fusion between X63-Ag8 myeloma cells donating gamma (G) and kappa (K) chains and a Balb/c lymphocyte donating heavy (H) and light (L) chains. It secretes an IgM ( $\kappa$ ) with anti-trinitrophenyl specificity (Köhler and Milstein, 1976). Sublines which have lost the X63-Ag8 gamma or kappa chain expression were selected by repeated subcloning. During this screening the mutant line igm 43 was found which secreted a smaller  $\mu$  chain in the presence of the three (G,L,K) other chains. The deletion variants igm 427, 482, 1882, 145, and 662 were selected from Sp6/HL. Igm 10 makes no, and igm 21 a reduced amount of,  $\mu$  chain, igm 215 shows reduced affinity for the hapten, igm 12 and 692 are deletion variants (Köhler and Shulman, 1980). Igm 12, 692, 215, 21, and 10 were selected from a Sp6/HLK line which still secretes small amounts of X63  $\kappa$  chain, visible only for 692 in Figure 1.

#### Deletions of mutant $\mu$ proteins

The  $\mu$  protein deletions of the independently derived mutant cells are shown in the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) slabs run under reducing conditions (Figure 1a). The cells were treated with tunicamycin to obtain unglycosylated chains and intra- and extracellular material was precipitated with rabbit anti-mouse IgM. The size of the deletion in igm 12 and 43 was identical (10 kd), as was the deletion in igm 427 and 692 (20 kd). The deletion of igm 1882 and 145 was 11 kd, that of igm 482 was 12 kd, and of igm 662 13 kd. The reducing and non-reducing SDS-PAGE (Figure 1 b and c) of intracellular glycosylated IgM again revealed

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**Fig. 1.** Size fractionation of  $\mu$  protein. SDS-PAGE under reducing (a, b) and non-reducing (c) conditions. Unglycosylated, tunicamycin-treated samples are shown in a. Intracellular samples of non-tunicamycin treated cultures are shown in b and c. WT corresponds to Sp6/HL WT chains, 215 is a mutant with 10-fold lower affinity (Köhler and Shulman, 1980) and is used here as normal IgM control. The other numbers indicate  $\mu$ -deletion variants. Igm 43 and 692 synthesize, in addition to the mutant IgM, an IgG and light chain, respectively, derived from the fusion partner X63-Ag8.

similar patterns for the pairs igm 12/43, igm 1882/145, and igm 692/427 at mol. wts. of 600, 160, and 66 kd, for the non-reduced molecules. For igm 482 and 662, apparent mol. wts. of  $\sim$ 160 and 150 kd, were found. Double bands for glycosylated, intracellular  $\mu$ , as clearly seen for 662 in Figure

1b, sometimes can also be visualised for the WT  $\mu$  chain and may be due to differential glycosylation (Sidman, 1981). To determine the nature of these deletions in more detail, Southern and Northern blot analysis of WT *versus* mutant DNA and RNA were performed.

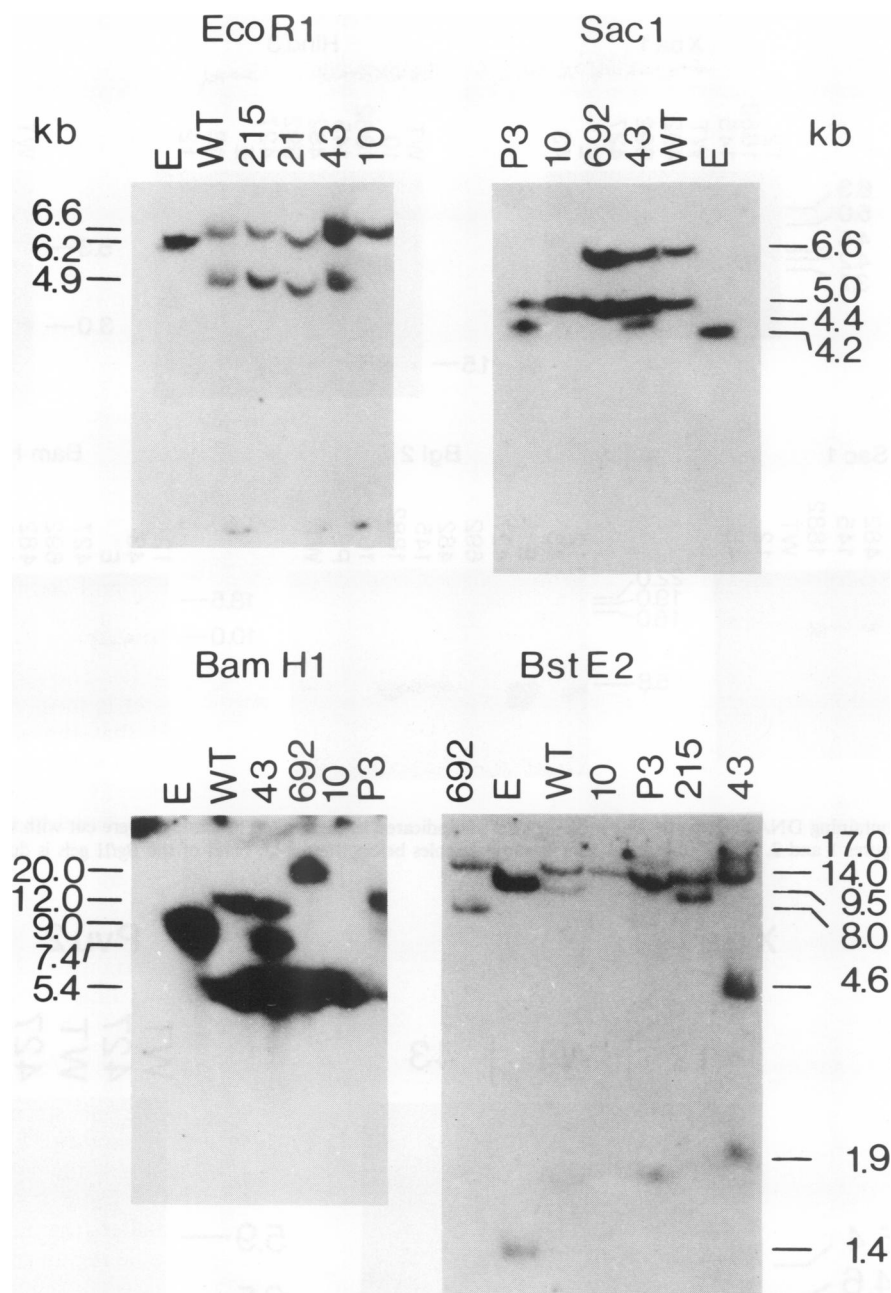
#### DNA characterization

*Sp6 variable region is joined to J3.* The DNA region covered by the J probe pJ-11 (Marcu *et al.*, 1980) is indicated in Figure 7. The *EcoRI* restriction pattern of DNA derived from the various cell lines shows that Sp6 has rearrangements involving two J loci, since they differ in size from the embryonic (E) 6.2 kb band (Figure 2, E *versus* WT). Igm 10 synthesizes no heavy chain, igm 215 synthesizes IgM of lower affinity, igm 21 synthesizes a reduced amount of IgM (Köhler and Shulman, 1980). The lower band of 4.9 kb is the functionally rearranged  $V_{H6}$  gene since the upper one of 6.6 kb is also present in the heavy chain-negative igm 10 line. Altered IgM expression in igm 215 and igm 21 cannot be attributed to gross changes in the vicinity of the variable region, as is also evidenced by *HindIII*, *XbaI*, and *BamHI* restriction enzyme digests (not shown). Upon cutting with *SacI*, a third band becomes visible in igm 43, which also synthesizes the  $\gamma 1$  chain of the X63-Ag8 parent, (P3). The igm 10 band co-migrates with the P3 silent gene and is most probably derived from P3-X63-Ag8. To confirm that the actively rearranged Sp6 variable gene is the one which is missing in igm 10 but present in WT, igm 43 and 692 were also subjected to digestion with *BamHI*. Their DNA was expected to give a smaller (igm 43) and a larger (igm 692) fragment than the active 12-kb band in WT due to internal DNA deletions (see next paragraph) which indeed were observed. The band remaining at the 12 kb position in igm 43 is due to the  $\gamma 1$  (P3) rearrangement. The *BamHI* enzyme cuts to the right of J2 (see Figure 7), so that rearrangements to either J1 or J2 would remain unnoticed, i.e., an unaltered embryonic 9-kb band should be revealed. No embryonic band is seen, indicating that none of the rearrangements observed use J1 or J2. The *BstEII* restriction enzyme is the most informative one, since it cuts between J3 and J4 and gives the embryonic band (9.5 kb) for Sp6 and igx 215. This shows that J3 is used for Sp6- and J4 for both P3-derived rearrangements. The loss of the embryonic band and the appearance of smaller fragments in igm 43 and igm 692 confirm that the variable region of the Sp6  $\mu$  gene ( $V_{H6}$ ) is indeed joined to J3.

Although  $V_{H6}$  is joined to J3 and the variable region of the P3  $\gamma 1$  heavy chain is joined to J4, we were unable to discriminate these rearrangements, both present in igm 43, with five out of eight restriction enzymes used (see the *EcoRI* and *BamHI* digest in Figure 2). This may indicate that  $V_{H6}$  and  $V_{H6}P3$  are related to each other.

*Mapping of the deletions in the  $\mu$  constant gene.* Southern blot analyses of DNA of the mutant lines using a variety of restriction enzymes are summarized in Figure 3. The cDNA probe pAB $\mu$ -1 which reveals  $\mu$ -specific bands was obtained from D. Baltimore, and included the fourth, and part of the third,  $\mu$  constant domains (Alt *et al.*, 1980), see Figure 7.

The *EcoRI* digest of embryonic DNA (Figure 3,E) shows a  $\mu$  gene-containing fragment of 13 kb. The size of this fragment is not altered in WT  $\mu$  DNA (k-1 is a light chain variant of Sp6 – used here as a control) or in the variant line igm 662. Deletions were however seen in the mutant lines igm 43 (5.6 kb), igm 12 (1.6 kb), and igm 692 (4 kb). No embryonic size band remained in these deletion mutants. Thus, only one



**Fig. 2.** Size fractionation of J containing DNA fragments. The probe pJ-11 is indicated in Figure 7. The samples were digested with various restriction enzymes and are E (Balb/c embryonal DNA); WT (Sp6/HL), igx 215 (a low affinity variant); igm 21 (a low synthesis variant); igm 10 (synthesizing no  $\mu$  chain); P3 (the parental line to Sp6/HL), igm 43, 692 deletion variants, see Figure 3.

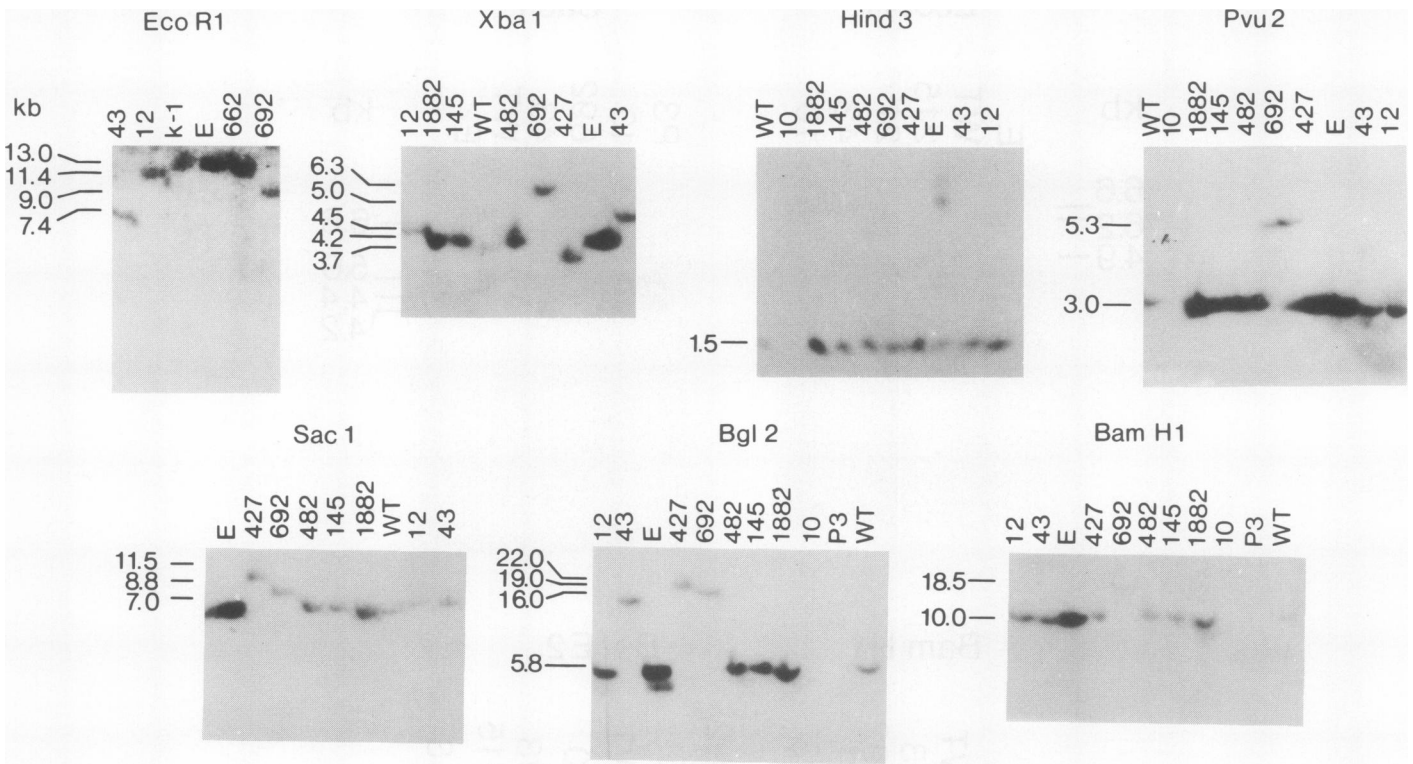
$\mu$  gene is present in the parental Sp6 line facilitating the analysis. Digests with XbaI reveal that igm 43, 12, and 692 show larger fragments than WT DNA whereas igm 427 shows a deletion of  $\sim 0.5$  kb, which remained hidden in an EcoRI comparison (data not shown). The HindIII digest of all lines (igm 662 not shown) gives rise to only one fragment of  $\sim 1.5$  kb. This result shows that none of the mutant lines had a deletion of  $>100$  bases in the third or fourth domain. Although igm 662, 1882, 145, and 482 had deletions of about one domain in size at the protein level, no DNA deletion of this size was revealed with any enzymes used (for igm 662 see Figure 4).

The altered  $\mu$ -containing EcoRI and XbaI restriction fragments obtained from igm 12, 43, 692, and 427 have to be

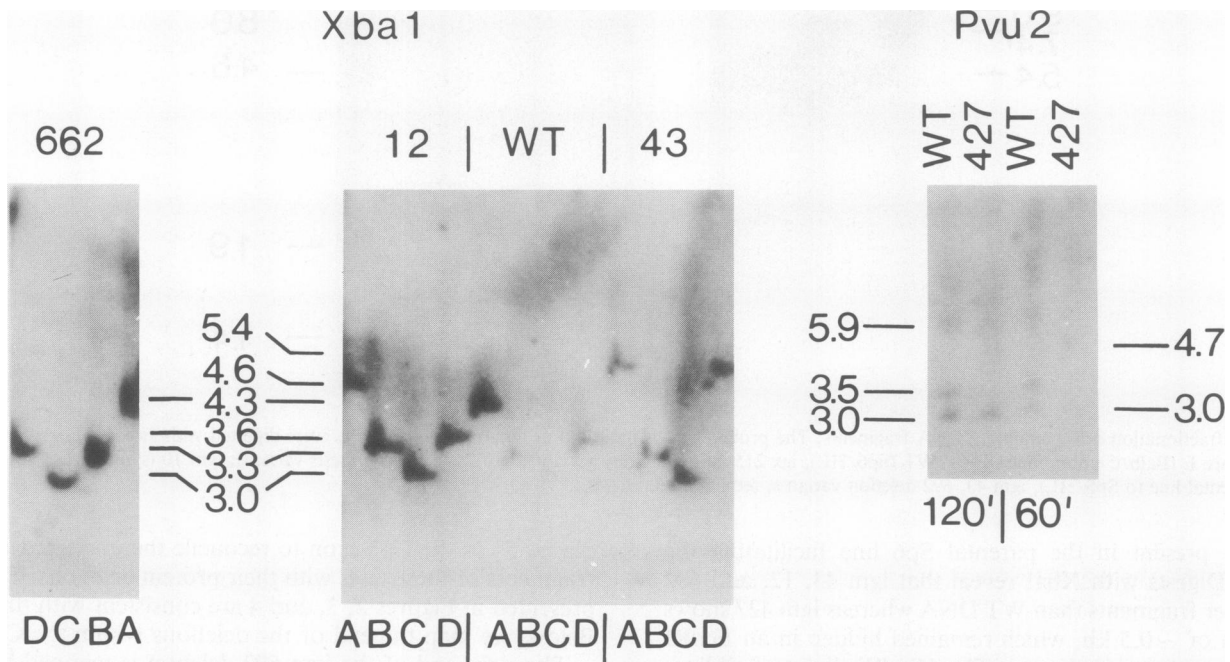
placed 5' to the  $C\mu 3$  exon to reconcile the unaltered HindIII fragments of these lines with their protein deletions. The data presented in Figures 2, 3, and 4 are consistent with this view. Below, we map the end of the deletions within the  $C\mu$  gene.

The right end of the igm 692 deletion is mapped between the PvuII and the HindIII sites in the  $C\mu 2/3$  intron, since the PvuII fragment is altered, whereas the HindIII fragment is not. The size of the PvuII fragment (5.3 kb instead of 3.0 kb) is consistent with a deletion of 4 kb as found in the EcoRI digest. This removes the first and second domains as shown by the absence of the BamHI, SacI, and BglII sites (compare Figure 7).

Igm 427 has the BamHI site but has lost the SacI and BglII sites. Since the PvuII site between  $C\mu 2$  and 3 is common to



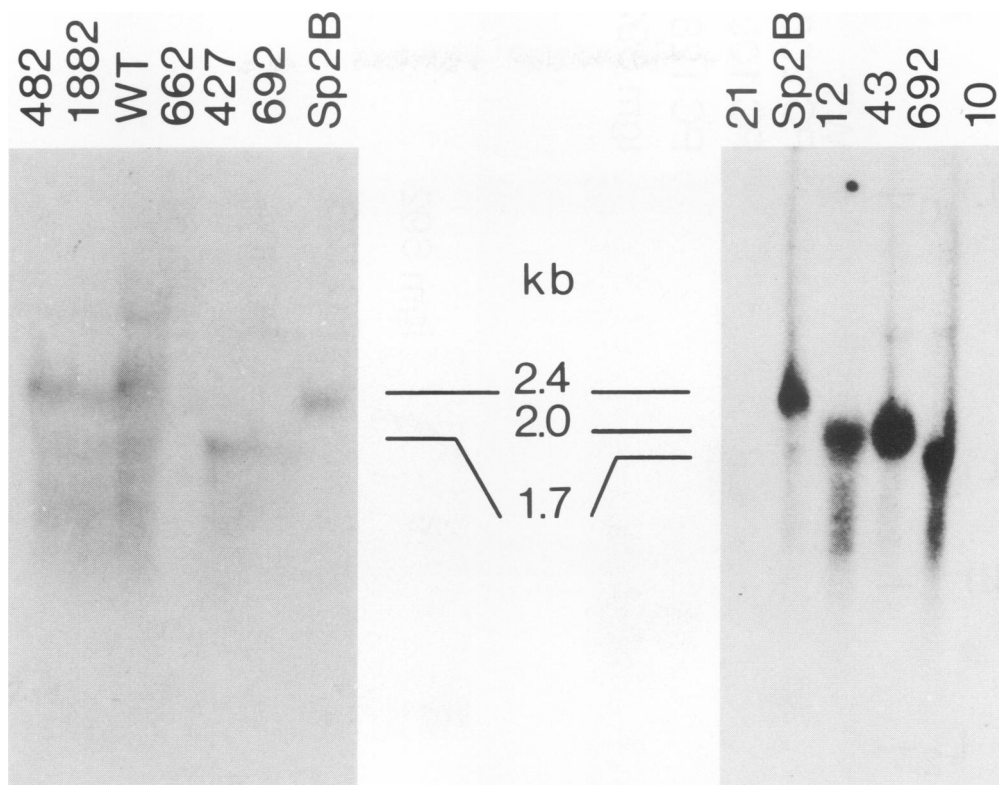
**Fig. 3.** Size fractionation of  $\mu$  containing DNA fragments. The probe pAB $\mu$ -1 is indicated in Figure 7. The samples were cut with various restriction enzymes. The samples are explained in Figures 1 and 2. The double band seen in some samples below the 5.8-kb band of the *Bgl*II gels is due to a blotting artefact.



**Fig. 4.** Size fractionation of  $\mu$  containing DNA fragments. The DNAs shown on the two gels on the left were first cut with the *Xba*I (A) and then with *Bam*HI (B), *Pvu*II (C), and *Bgl*II (D). Only *Bgl*II in igm 43 is unable to generate a shorter piece. The DNA shown on the gel on the right, was cut with limiting amounts of the *Pvu*II restriction enzyme for 60 and 120 min. The 3.5-kb DNA fragment present in WT is absent in the mutant igm 427, which shows a new 4.7-kb band. Probe pAB $\mu$ -1 was used to visualize the bands, see Figure 7.

igm 427 and WT, a partial digest with this enzyme has been used to probe for the loss of the *Pvu*II site at the beginning of *C $\mu$  2* (Figure 4). In WT DNA, a 3.0-kb *Pvu*II fragment of fully digested DNA is seen in addition to a 3.5-kb band contain-

ing the 0.5-kb *Pvu*II fragment to the left of the pAB $\mu$ -1 probe (Figure 7). In igm 427, the 3.5-kb band is missing, indicating the absence of the *Pvu*II site at the beginning of the second *C $\mu$  exon*. A new band of 4.7 kb appears in the mutant. The



**Fig. 5.** Size fractionation of cytoplasmic RNA. Probe pAB $\mu$ -1 was used to visualize the RNA. Sp2 B is an unrelated IgM producing hybridoma, known to produce higher mol. wt.  $\mu$  chain (Köhler *et al.*, 1978). Here no higher mol. wt. RNA is seen. It has served therefore as a normal  $\mu$  RNA control. Igm 21 is a low synthesis variant, igm 10 makes no  $\mu$  chains (Köhler and Shulman, 1980). The other numbers indicate RNA samples from the deletion variants.

corresponding WT fragment using the *PvuII* sites outside the  $C\mu$  exons would be 5.4 kb. Thus, a deletion of 0.7 kb is observed in igm 427, in agreement with the 0.5-kb deletion measured after *XbaI* treatment.

In igm 43 all restriction sites (Figure 3) in the  $\mu$  constant coding DNA are present, with the exception of the *BglII* site at the end of the first  $\mu$  constant exon. The absence of this site is confirmed by a double digest (Figure 4). After *XbaI* treatment igm 662, 12, and WT DNA give rise to smaller fragments when further digested with *BamHI*, *PvuII*, and *BglII*. Therefore, these sites are present in all three *XbaI* DNA fragments. In igm 43 the *BglII* enzyme left the *XbaI* DNA fragment unaltered, indicating the absence of the *BglII* site. The deletion of igm 43 thus starts between the *SacI* and the *BglII* site in the  $C\mu$  1/2 intron and stretches  $\sim$  5 kb into the large V/C intron.

The deletion of igm 12 is the least precisely located one. Its deletion is 1.6 kb long, as deduced from the *EcoRI* digest, involving the *XbaI* site left of the  $\mu$  constant gene but leaving the *BglII* site at the end of the first  $\mu$  constant exon intact (Figure 3, compare also the double digestions *XbaI* and *BglII*, *PvuII*, *BamHI* in Figure 4). The summary in Figure 7 indicates the possible end of each of these deletions with a broad line.

#### The size of $\mu$ RNA

Northern blots (Figure 5) using unfractionated cytoplasmic RNA showed a major  $\mu$  specific band of 2.4 kb for all lines except igm 10 (no band), igm 12 and 43 (2.0 kb), and igm 427 and 692 (1.7 kb). Sp2 B is another  $\mu$  secreting line (Köhler *et al.*, 1978). It is interesting that igm 21, described as a  $\mu$  low producer (Köhler and Shulman, 1980) also seems to have less

cytoplasmic  $\mu$  RNA as a percentage of total RNA, though the weak band of WT size is not well reproduced in the picture.

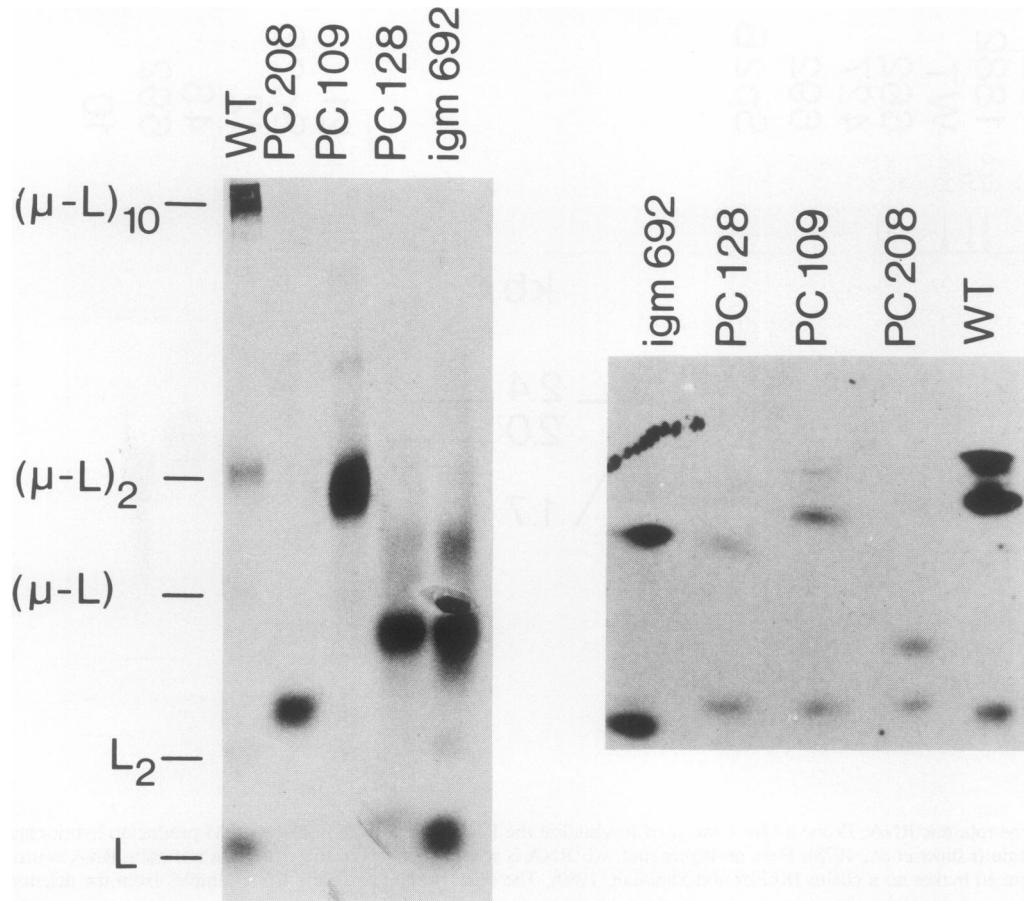
#### Covalent light chain attachment

Light chains bind through a disulphide bridge to  $C\mu$  1 at residue 140 (Kehry *et al.*, 1979). In a previous paper (Köhler and Shulman, 1980), it was shown that the igm 12  $\mu$  chain, which lacks only the  $C\mu$  1 domain does not covalently bind light chain. Surprisingly, the igm 692  $\mu$  chain which lacks  $C\mu$  1 and 2, covalently binds the light chain (Figure 6). In order to map the site of light chain attachment, we introduce a new set of variants: PC 109 a C-terminal deletion similar to the one of igm 662 or 1882/145, PC 128 a deletion of  $C\mu$  1 and 2 like igm 692/427, and PC 208 a deletion of  $C\mu$  1, 2, and 3 giving rise to the smallest ( $\sim$  30-kd) fragment  $\mu$  chain so far observed. These deletion variants were isolated from the hybridoma line PC 700 using a similar suicide enrichment technique. Their derivation and characterization will be described in separate papers. We demonstrate that all these variants (PC 208 was of special interest) covalently bind light chains, as shown by cutting out the main band seen in the non-reducing gel (Figure 6, left) and rerunning this material under reducing conditions (Figure 6, right). It is not surprising that PC 109 (Figure 6) and igm 662, 1882, 145, 482 (data not shown) covalently bind light chains since these variants all have an intact  $C\mu$  1 domain. More interestingly, PC 128 and igm 692 which lack  $C\mu$  1+2 and PC 208 which lacks  $C\mu$  1+2+3, covalently bind the light chain.

#### Discussion

Our selection of mutant IgM secreting cell lines has yielded





**Fig. 6.** Covalent light chain attachment of various mutant IgMs. The major bands of WT  $[(\mu-L)_{10}]$  and of mutant IgM producing lines after non-reducing SDS-PAGE (left) were cut out and rerun under reducing conditions (right). All IgMs after reduction show a band characteristic for light chain. The samples are WT (Sp6/HL), igm 692 (a deletion variant thereof, lacking the  $C\mu$  1 and 2 domains), PC 109, 128, and 208 are  $\mu$ -deletion variants of an anti-phosphorylcholine specific IgM secreting hybridoma line lacking a C-terminal piece,  $C\mu$  1 and 2, and  $C\mu$  1, 2, and 3, respectively.

(among other variants) a collection of  $\mu$  deletion proteins. These will be discussed in connection with RNA splicing models, heavy-light chain protein interactions and secretion of the mutant molecules.

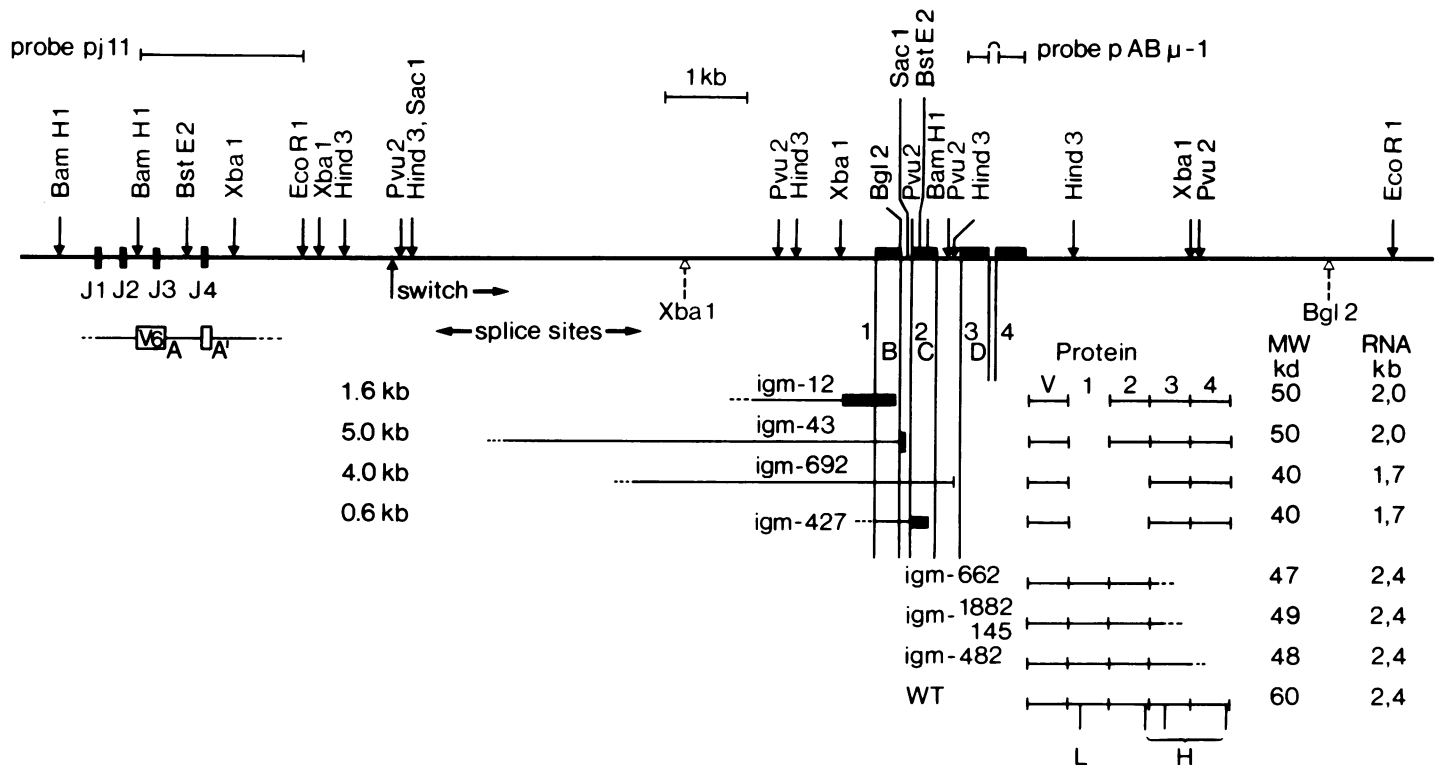
The properties of the deletion mutants are summarized in Figure 7. The restriction enzyme map we used has been taken from published nucleotide sequences of this region (Kawakami *et al.*, 1980; Goldberg *et al.*, 1981; Sakano *et al.*, 1980). The scarcity of restriction sites in the V- $C\mu$  intron is due to the fact that cloned DNA, because of repetitive sequences, often shows deletions in this area (Marcu *et al.*, 1980; Sakano *et al.*, 1980). The DNA-deletion endpoints were mapped with varying precision. For igm 12 the deletion may start anywhere between the 3' end of the  $C\mu$  1 *Bgl*II site and the missing *Xba*I site and extend 1.6 kb into the large V/C intron (the uncertainty is indicated by a broad line). The sizes of the deletions were deduced from the restriction fragments seen in Figures 2, 3, and 4 and are accurate to about  $\pm 10\%$ . We assume that the deletion of igm 12 interferes with the splicing site 1 (Figure 7) in order to explain the mRNA and protein similarities with igm 43. In igm 43 the deletion starts in the 99 nucleotides between the *Sac*I and the *Bgl*II site, which is 16 nucleotides into exon  $C\mu$  1. It is, therefore, not clear whether or not splice site B has been removed by the deletion. Igm 43 has the largest deletion consisting of  $\sim 5$  kb.

The mutant pair igm 692 and igm 427 is more clearly defin-

ed. The igm 692 deletion starts somewhere in the 45 nucleotides between the *Pvu*II and the *Hind*III restriction sites in the  $C\mu$  2/3 intron and takes out  $\sim 4.0$  kb to the 5' side. Therefore, the splice sites 1, B, 2, and C are removed. In igm 427 only a 0.6-kb deletion was found, which begins somewhere in the 199 nucleotides between the *Pvu*II and the *Bam*HI sites of the  $C\mu$  2 exon. Here the splice sites 1, B, and 2 have been removed, although the removal of splice site 1 is only inferred from the size of the deletion and the similarity of the mRNAs and proteins of igm 427 and 692.

We think that these deletions impose some restrictions on the mechanism of RNA splicing (Sharp, 1981). First, the removal of exon  $C\mu$  1 (igm 43) or  $C\mu$  1 and 2 (igm 692) and up to 4 kb of the V/C intron does not prevent the proper splicing of the remaining exons. This speaks against the importance of the overall secondary structure of  $\mu$  RNA for the splicing process. Secondly, whether or not the deletion starts in an intron (igm 692 and possibly igm 43) or in the corresponding exon (igm 427 and possibly igm 12) similar RNAs and proteins are observed. These results preclude sole directional 3' (constant)  $\rightarrow$  5' (variable) splicing at least for the splice site 3 to C of the  $\mu$  RNA. This combination does not generate a possible functional new splice site. The splice 2  $\rightarrow$  B and 1  $\rightarrow$  A would lead to a new splice site which may be used in a second splicing event.

On the other hand, exclusive directional 5' (variable)  $\rightarrow$  3'



**Fig. 7.** The restriction enzyme map of the Balb/c  $\mu$ -gene region. Summary of the results of the  $\mu$  deletion variants at the DNA, RNA, and protein level. The probes used are indicated at the top. Restriction sites were extracted from published nucleotide sequences of the region. The joining regions (J1, J2, J3, J4) precede the four  $\mu$  constant regions shown as filled boxes. The switch region is indicated by arrows. The restriction sites *Xba*I and *Bgl*II (broken arrows) were derived from this work and placed by size measurements only. Below the embryonic gene, the rearranged Sp6  $\mu$  gene is indicated, where the variable region is joined to J3. Splice donor sites are lettered A–D, splice acceptor sites are numbered 1–4. The DNA deletions of the mutant lines are shown as lines, their length is given to the left and the uncertainty of their end points, is given by the broad lines. The mol. wt. measurements of the unglycosylated  $\mu$  proteins are given, as well as the size of their RNA. The WT  $\mu$  protein is drawn to show the variable domain (V) and the four constant domains. Disulphide bridges to light chain (L) and heavy chains (H) are indicated. The exact boundaries of the protein deletions are not known.

(constant) splicing would give rise to the observed results. Directional 5'  $\rightarrow$  3' splicing would explain the splicing pattern of the aberrantly rearranged  $\kappa$  light chain of MPC-11 (Choi *et al.*, 1980; Seidman and Leder, 1980; Schnell *et al.*, 1980) only if the new splice site which is generated after splicing the precursor piece (P) to the variable (V) region (lacking a splice donor site) were re-used to give rise to the observed P-C<sub>x</sub> fragment RNA.

All the large DNA deletions start in the 8-kb intron between V and C which is thought to facilitate deletion processes due to repetitive DNA sequences used for the immunoglobulin class switch (Nikaido *et al.*, 1981). Since the exons of the  $\mu$  constant region are in one reading frame, a property not necessarily found in other split genes (Sharp, 1981), and since whole domains in protein are lost whether the DNA deletion starts in an intron or exon, it may not be surprising to find phenotypic repeats by analysing only eight deletion mutants. It is, however, surprising that none of the C-terminal deletions (four described here and four others recently isolated) are due to DNA deletions, suggesting a requirement for 3'  $\mu$  RNA sequences.

The switch from  $\mu$  and  $\delta$  observed in the B1-8 line (Neuberger and Rajewsky, 1981) and the deletion of the first constant domain of a  $\gamma$ 1 (P3-MOPC 21) producer (Dunnick *et al.*, 1980) have been shown to be due to DNA deletions starting in the V-C intron. The fact that deletions preferentially occur in the switch box regions preceding the immunoglobulin heavy chain genes may explain the  $\gamma$ 2b to  $\gamma$ 2a switch observed in the MPC-11 line (Morrison, 1979) and some of the human heavy

chain disease molecules (Franklin and Frangione, 1975). Interestingly, the variable region of the MOPC 21  $\gamma$ 1 chain was shown to be associated with  $\gamma$ 2b,  $\gamma$ 2a, and  $\alpha$  constant regions in a series of related mutants (Radbruch *et al.*, 1980). Co-expression of different subclasses was observed and reversions were 10 times more frequent than the forward mutation. All these features could be explained if deletions bring the different constant heavy chain genes closer together so that one transcription unit is formed, which can then be differentially spliced as shown for the  $\mu$ - $\delta$  transcriptional unit (Maki *et al.*, 1981).

In addition to the deletion mutants described here, two others, derived from the hybridoma line PC 700 producing an anti-phosphorylcholine IgM, are being analysed. They appear to be, respectively, DNA deletions of the C $\mu$  1 and 2 domains for mutant PC 128 and of domains C $\mu$  1, 2, and 3 for mutant PC 208 (M. Shulman, in preparation). Their protein size is compatible with this interpretation (Figure 6) and indeed PC 128 IgM was indistinguishable from either igm 427 or 692 by electrophoresis in reducing or non-reducing SDS gels and by its reactivity pattern to 12 monoclonal rat anti-mouse  $\mu$  antibodies (Potash *et al.*, in preparation). The reactivity pattern of these monoclonal antibodies mapped igm 662, 1882, 145, 482 and PC 109 to be C-terminal, an assignment agreeing well with their normal size  $\mu$  DNA, their binding of light chains, and their inability to form pentamers (Figure 1). However, we were surprised when we analysed the capacity of the internal deletion mutants to bind light chains covalently. Igm 12 had been shown in a previous paper (Köhler and Shulman, 1980)

not to bind light chains covalently, whereas igm 692 did. We suggested, therefore, that the igm 12 protein lacked and that the igm 692 retained the  $C_{\mu}$  1 domain, where the  $\mu$ -L disulphide bridge normally resides (Kehry *et al.*, 1979). Here we show that igm 12 indeed lacks  $C_{\mu}$  1, but that igm 692 lacks  $C_{\mu}$  1 and 2. Not only igm 692, igm 427, or PC 128, which all have deletions of the first and second  $C_{\mu}$  domains, but also PC 208, which has lost  $C_{\mu}$  domains 1, 2, and 3, can bind light chains covalently (Figure 6). This indicates that  $C_{\mu}$  4 can form a disulphide bridge to the light chain (PC 208). Either  $C_{\mu}$  3 or  $C_{\mu}$  4 might be responsible for the light chain binding in igm 692, 427, and PC 128.  $C_{\mu}$  2 is not able to form this bridge. This indicates functional homology of  $C_{\mu}$  1 and  $C_{\mu}$  4 and questions the sole importance of nucleotide sequence homologies (Goldberg *et al.*, 1981), where  $C_{\mu}$  1 and  $C_{\mu}$  4 were the second worst pair of six possible comparisons.

Secretion is found in mutants displaying deletions in any of the four constant domains (Figure 7; note PC 208 and igm 482). Thus no single  $C_{\mu}$  domain is essential for secretion. Pentamer ( $H_{10}L_{10}$ ) or monomer ( $H_2L_2$ ) formation are also not necessary since in igm 427, 692, and PC 128 mostly hemimers ( $H_1L_1$ ) are secreted. The three low secretors, igm 662, 1882, and 145 which form monomers and lack  $C_{\mu}$  4 and unknown stretches of  $C_{\mu}$  3, exhibit enhanced intracellular degradation (Sidman *et al.*, 1981). This observation together with the fact that they do not bind a monoclonal antibody specific for  $C_{\mu}$  3, although they differ only marginally in size as compared to igm 482 (Figure 1) which binds this antibody and is well secreted, indicates that igm 1882 and 145 and possibly also igm 662 are frameshift variants. The addition of out-of-frame amino acids may account for their enhanced degradation and low secretion properties.

Drastic changes in the structure of IgM or IgG (Dunnick *et al.*, 1980) do not affect secretion; thus it is puzzling that  $\mu$  or  $\gamma$  chains cannot be secreted unless bound to light chains (Burrows *et al.*, 1979). Light chain requirement for secretion is preserved in two mutants affecting either the first constant domain or some portion of the C-terminal region in gamma-producing cell lines (Morrison, 1978; Wilde and Milstein, 1980). However, deletions including stretches of the variable region of human heavy chain disease molecules have been shown to lead to secretion of free heavy chains (Seligmann *et al.*, 1979). This may indicate structures in the variable region of H chains which, unless covered by L chains, hinder secretion.

## Materials and methods

### Selection of mutant cell lines

The selections were performed by covalently coupling the hapten to the cell surface and incubating the cells in methylcellulose in the presence of guinea-pig complement. WT cells thus commit suicide whereas cells producing more or less lytic IgM are preferentially enriched. This technique is described in detail (Köhler and Shulman, 1980). The only change in the protocol was to use rabbit complement in three of the four selections performed with Sp6/HL. All deletion variants described in this paper, except igm 482 and 427, came from independent selections.

The cell lines were grown in Dulbecco's modified Eagles medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum,  $5 \times 10^{-5}$  M 2-mercaptoethanol, and 100 units/ml penicillin and streptomycin.

### Radiolabelling of Ig and its analysis

To obtain unglycosylated protein chains,  $10^7$  cells were washed in leucine-free medium and grown in 2 ml thereof for 30 min at 37°C in the presence of 10  $\mu$ g/ml of tunicamycin (Sigma, St Louis, MO). Tritiated leucine (Amersham, UK) was added to 150  $\mu$ Ci/ml (153 Ci/mmol) and the cells grown for a further 5 h. Cells were lysed by addition of 1% Nonidet P-40 (NP-40) and nuclei spun out. Igs in cell extract and supernatant were precipitated using a

rabbit anti-mouse Ig serum and Protein A-Sepharose (Pharmacia Uppsala, Sweden). To obtain glycosylated intracellular Ig chains a similar protocol was used, omitting tunicamycin, with a 2-h incubation with radiolabelled leucine and removal of the cell supernatant before lysis of the cells.

The precipitates were analysed by SDS-PAGE. For the second dimension, radioactive bands of the first non-reducing SDS-PAGE were cut and fixed with scotch tape for the reducing SDS-PAGE. It is important to keep the gel-strips dry, until covered with the denaturation mix in 1% agarose (Köhler *et al.*, 1978). The reducing gels were enhanced using the NEN enhance mixture (New England Nuclear, Dreieich, FRG); the non-reducing gels were soaked in 1 M sodium salicylate for 30 min. Gels were exposed for 1–14 days on Kodak X-Omat RP films.

### Preparation of cellular DNA and its analysis

Between  $10^6$  and  $4 \times 10^8$  cells were grown, washed twice in saline, and resuspended in 10 ml of 10 mM Tris pH 8, 1 mM EDTA, and 150 mM saline. The cells were lysed by adding SDS to 0.2%. Proteinase K digestion (100  $\mu$ g/ml, 37°C overnight) was followed by phenol extraction, ethanol precipitation, dialysis, and RNase A treatment (50  $\mu$ g/ml, 37°C, 2–3 h). Further proteinase K treatment (50  $\mu$ g/ml, 37°C for 6 h), phenol extractions, and dialysis followed. The protocol is essentially the one described by Maki *et al.* (1981). 20  $\mu$ g of DNA were used for the restriction enzyme digestions. The enzymes were purchased from Boehringer, Mannheim, FRG; Bethesda Research Laboratories, Bethesda, MD; and Biolabs, Beverly, MA. They were used according to the manufacturers' specifications under conditions sufficient to completely digest even 3–10 times more DNA. Half of the amount of *Pvu*II necessary to cut the DNA shown in Figure 4 (right) in 1 h was used to generate a partial digest. 20  $\mu$ g of DNA were applied to 1% agarose gels. Blotting was performed according to Southern (Southern, 1975) and bands were revealed using 1–4  $\times 10^7$  c.p.m. of nick-translated  $\mu$  DNA at 0.5–4  $\times 10^8$  c.p.m./ $\mu$ g DNA. The probes were  $\mu$ -specific cDNA pAB $\mu$ -1 obtained from D. Baltimore, (Alt *et al.*, 1980) and a J-specific probe pJ-11 from K. Marcu, (Marcu *et al.*, 1980).

### Preparation and analysis of cytoplasmic RNA

Cytoplasmic RNA was extracted from 5–10  $\times 10^8$  cells using the hot phenol procedure (Maki *et al.*, 1981) after removing nuclei obtained by lysing cells with 0.5% NP-40. About 20  $\mu$ g of unfractionated RNA in 50  $\mu$ l sample buffer (50% formamide, 2.2 M formaldehyde in gel buffer: 20 mM morpholino propane sulfonic acid, 5 mM sodium acetate, 1 mM EDTA) were applied on 1% agarose gels containing the same buffer (Lehrach *et al.*, 1977). Gels were run overnight in gel buffer at 15 V. Ribosomal RNAs stained separately with ethidium bromide were used as size markers. The gel was blotted onto nitrocellulose filters and stained in a similar way to the DNA gels. The probe pAB $\mu$ -1 (Alt *et al.*, 1980) used was specific for the  $\mu$  constant part (see Figure 7).

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