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NUCLEOTIDE SEQUENCES ENCODING MEMBRANE DOMAINS ARE CONSERVED AMONG IMMUNOGLOBULIN GAMMA SUBCLASS GENES

SUMIKO NAKAI[1], VERNON OI[2], LEONARD A. HERZENBERG[2], HIDEO YAMAGISHI[3] and TASUKU HONJO[1]

[1]Department of Genetics, Osaka University Medical School, Kitaku, Osaka 530, Japan, [2]Department of Genetics, Stanford University Medical Center, Stanford, California 94305, U.S.A., and [3]Department of Biophysics, Faculty of Science, Kyoto University, Kyoto 606, Japan

ABSTRACT

Two $C_\gamma 2a$ mRNA (1.8 kb and 4.0 kb) were identified in the 2PK3 B-lymphoma cell line which synthesizes both membrane and secreted IgG2a heavy chains. The 1.8 kb mRNA was shown previously to code for secreted $C_\gamma 2a$ heavy chians. Though membrane IgG2a heavy chains have been shown to be about 10,000 daltons larger than secreted IgG2a heavy chains (55,000 daltons), the length of the 4.0 kb mRNA is longer than necessary to code for this polypeptide chain. Using the mRNA from 2PK3 cells, two membrane exons (0.1 kb and 1.1 kb) separated by a 0.5 kb intron, have been identified in the 3′ flanking region of the $C_\gamma 2a$ gene by R-loop analysis and mRNA hybridization experiments. The identification of these structural gene sequences and their similarity to the two exon-structure coding for the carboxy-terminus of membrane IgM heavy chains suggest that membrane IgG2a antigen-receptors, like IgM receptors, are generated by alternate splicing of messenger RNA precursors. A portion of both $C_\gamma 2a$ membrane exons is conserved among all the C_γ genes as revealed by heteroduplex analysis. This suggests to us that there is a membrane form for all IgG subclasses that act as antigen-receptors on B-lymphocytes.

The integral membrane forms of IgM and IgD are the predominant immunoglobulin receptors on the majority of B-lymphocytes. Following antigen stimulation, a small subpopulation of antigen-specific B cells differentiate into a pool of memory B cells. There is overwhelming evidence that these memory B cells express new immunoglobulin isotypes (*e.g.*, IgG subclasses) as membrane antigen-receptors (18).

Recently several laboratories have presented evidence for the existence of a membrane form of IgG heavy chain molecules that is larger (about 10,000 daltons) than the secreted form of the same immunoglobulin isotype (17, 32). This putative IgG receptor also has been shown to have hydrophobic qualities similar to known integral membrane proteins and to membrane IgM and IgD receptors (31). We present evidence demonstrating the presence of two mRNAs, a 4.0 kb and a 1.8 kb mRNA, in the B-lymphoma cell line, 2PK3, which synthesizes significant amounts of membrane IgG2a molecules. The 1.8 kb RNA is the expected size for an mRNA transcript coding for secreted IgG2a heavy chain molecules. The 4.0 kb RNA, we propose to be the membrane IgG2a heavy chain mRNA. By mRNA hybridization experiments and R-loop analysis, we have located two membrane (M) exons, 1.3 kb and 1.9 kb 3′ of the known $C_\gamma 2a$ gene. This structure is reminiscent of the membrane exon-structure of the C_μ gene (3, 5, 21).

The entire immunoglobulin heavy chain gene complex has been elucidated by molecular clon-

ing techniques as tandem genes in the order 5′-C_μ-(4.5 kb)-C_δ-(55 kb)-C_γ3-(34 kb)-C_γ1-(21 kb)-C_γ2b-(15 kb)-C_γ2a-(14.5 kb)-C_ε-(12 kb)-C_α-3′ (6, 12, 16, 20, 23, 24, 26). The C_γ genes are not only clustered within this gene family but also have been shown to be closely related to each other. The four C_γ genes have essentially identical cistronic structures in terms of the location and length of each of their intervening and structural sequences (2, 7, 19, 28, 34, 35). Their nucleotide sequences bear obvious homology to one another. It is extremely likely that the C_γ genes have evolved from a common ancestral gene. In fact, comparison of the nucleotide sequences of the four C_γ genes indicates that segments of these genes have been exchanged by recombination during the volution of this gene family (15, 35).

In this paper we extend this comparative analysis to the 3′ flanking region of each C_γ genes, to include the region where we located the two membrane exons of the C_γ2a gene. We have analyzed electron micrographs of heteroduplexes between all possible pairs of the C_γ genes. This revealed two conserved regions in the 3′ flanking reigon of each C_γ gene. These two regions overlap the sequences identified as the two membrane exons of the C_γ2a gene. We conclude that each C_γ gene has at least two membrane exons which are conserved, at least in part, among the four C_γ genes. Furthermore, we conclude that each IgG subclass can act as integral membrane antigen-receptor.

MATERIALS AND METHODS

DNA and RNA

Phage DNAs containing C_γ genes used in this study (Table 1) were prepared as described previously (7, 11, 23, 34). RNA from the B-lymphoma, 2PK3 (17), the hybridoma cell line, IgE-53-569 (22), and the myeloma, HOPC1 (8) were prepared also as described previously. RNAs were either once or twice enriched for poly(A)RNA with oligo-dT cellulose.

Electron Microscopy

Recombinant phage DNAs containing genomic C_γ gene inserts in the same orientation relative to the phage arms were used for heteroduplex analyses. Heteroduplexes were formed using a modification (33) of the formamide technique described by Davis *et al.* (4). R-loops were formed between the 19.4 kb insert of Igγ2a-11 (23) and 2PK3 mRNA according to the procedure of Kaback *et al.* (10). Electron micrographs were taken with a Hitachi HU12A electron microscope at ×10,000 magnification and images were enlarged an additional ×10. DNA lengths were measured with a digigramer, Mutoh Model G, using pBR322 and fd DNA as size markers.

mRNA Hybridization

Glyoxal-treated RNAs were analysed by electrophoresis in 1.1% agarose gels. RNA was transferred from the gel to nitrocellulose membranes as described by Thomas (27). Hybridization with appropriate probes was done using the same conditions as described for Southern blot hybridization (9, 25). Nick-translations of probes were done as described by Maniatis, *et al.* (7, 13).

Other Materials

Sources of restriction endonucleases were described previously (7). α-^{32}P-dCTP (2,000–3,000 Ci/mmol) was purchased from Radiochemical Center (Amersham, England).

RESULTS

Our initial examination of poly(A)RNA from the B-lymphoma, 2PK3, revealed two hybridization bands of 1.8 kb and 4.0 kb. The 1.8 kb

Table 1 *List of Recombinant Phages*

Clone	Immunoglobulin gene	Insert length(kb)	Reference
IgH2	C_γ1	6.6	(7)
Ch·Igγ1-3	C_γ1	14.4	(23)
IgH22	C_γ2b	6.6	(32)
Ch·Igγ2b-2	C_γ2b	17.3	(23)
Ch·Igγ2a-11	C_γ2a	20.0	(23)
Ch·Igγ2a-32	C_γ2a	18.7	(23)
Ch·Igγ3-30	C_γ3	18.0	(11)

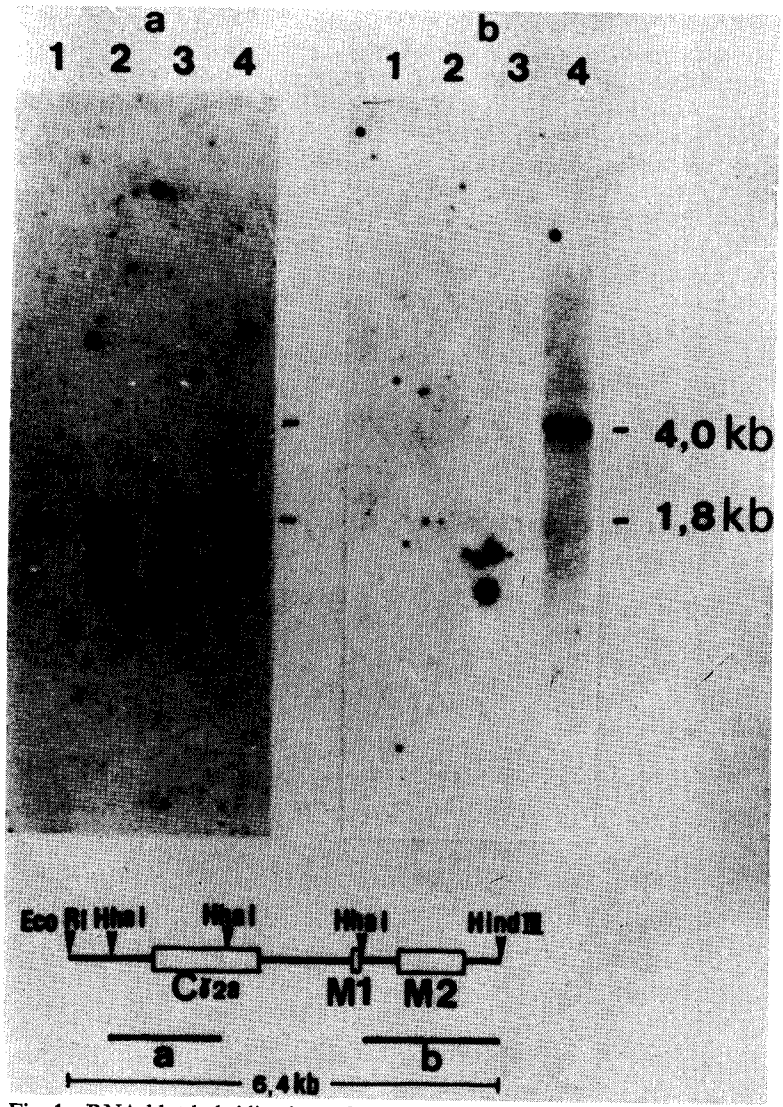

Fig. 1 RNA blot hybridization. RNAs were separated in 1.1% agarose gels and blotted to nitrocellulose filters as described in Materials and Methods. Lane: 1, 0.5 μg mRNA from the IgE-producing hybridoma cell line (IgE-53-569) that was twice-enriched by oligo-dT cellulose affinity chromatography; 2, 5.0 μg of HOPC1 (IgG2a) myeloma mRNA; 3, 2.5 μg of HOPCl myeloma mRNA; 4, 0.5 μg mRNA from 2PK3 (membrane IgG2a-producing lymphoma) that was twice-enriched by oligo-dT cellulose affinity chromatography. Probe a (1.75 kb) which contains a major portion of the structural domains of the $C_\gamma 2a$ gene and probe b (2.1 kb) which contains a part of the first and all of the second membrane domain are shown in the restriction map.

mRNA corresponds to the expected size for an mRNA transcript coding for secreted IgG2a heavy chain message. In fact, only a 1.8 kb mRNA band is present in the IgG2a-producing myeloma, HOPC1 (Fig. 1). The 4.0 kb mRNA is larger than expected for a 65,000 dalton membrane heavy chain. This is in contrast with data presented by Early *et al.* (5) showing membrane IgM heavy chain mRNA to be only 2.7 kb.

3′ Flanking Sequences Are Included in the 4.0 kb mRNA

We first identified the location of the sequences

included in the 4.0 kb mRNA by mRNA hybridization experiments. Poly(A)RNAs were separated by electrophoresis in an agarose gel and transferred to nitocellulose filters. When we used as a DNA probe, sequences containing the 5′ half of the $C_\gamma 2a$ genomic sequence which included both the CH1 and CH2 exons, as well as 600 bases 5′ to CH1 (probe a), both 4.0 kb and 1.8 kb bands hybridized as shown in Fig. 1. On the other hand, only the 4.0 kb mRNA was revealed by hybridization with a DNA fragment containing the 3′ flanking region of the $C_\gamma 2a$ gene (probe b). These results indicate that 3′ flanking sequences of the $C_\gamma 2a$ gene are transcribed as part of the mRNA coding for membrane IgG2a heavy chains.

Localization of $C_\gamma 2a$ Membrane Exons

R-loops formed between 2PK3 mRNA and a germline $C_\gamma 2a$ clone, Ch·Igγ2a-11 were analysed by electron microscopy to locate the sequences included in the membrane mRNA. The relative positions and lengths of the CH1, CH2 and CH3 exons revealed by R-looping (Fig. 2) are as expected from the known nucleotide sequence of the $C_\gamma 2a$ gene and the Ch·Igγ2a-11 phage DNA. In addition, two R-loops of 0.1 kb and 1.1 kb, interrupted by a 0.5 kb intervening sequence, are formed in the 3′ flanking region of the $C_\gamma 2a$ gene. The smaller exon (0.1 kb) is located 1.3 kb 3′ to the CH2–CH3 R-loop. A schematic diagram showing the location of the two membrane exons and introns also is included in Fig. 2.

The length (4.0 kb) of the membrane IgG2a heavy chain mRNA is longer than expected from the R-loop data. Assuming all parts of the secreted IgG2a heavy chain mRNA (1.8 kb) is

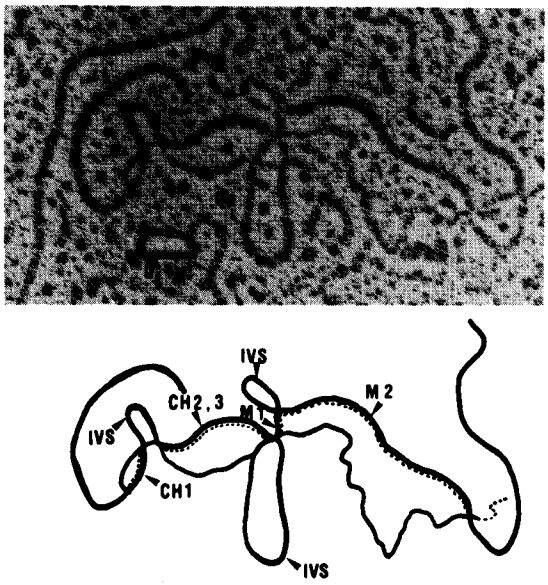

Fig. 2 Electron micrograph of R-loops formed between the $C_\gamma 2a$ gene and 2PK3 mRNA. R-loops were formed between Ch·Igγ2-11 and 2PK3 mRNA. The horizontal bar represents 0.1 μm. Interpretation of the micrograph and the duplex lengths measured (in kb) are illustrated. Seventy-five molecules were examined and measured.

Table 2 *Homology between γ Subclass CH Genes*

Regions	$C_\gamma1$–$C_\gamma2b$	$C_\gamma1$–$C_\gamma2a$	$C_\gamma2b$–$C_\gamma2a$
CH1	87*	87	87
IVS1 5′	75	66	73
3′	46	43	80
H	37	40	69
IVS2	54	52	90
CH2	74	74	94
IVS3	48	51	87
CH3	67	71	75
UT	73	71	88

* Numbers are in % matched base (15, 33). IVS, intervening sequence; UT, untranslated sequence

expressed also in the membrane mRNA, its expected length is the sum of 1.8 kb plus the lengths of the two membrane exons (1.2 kb), *i.e.*, 3.0 kb. This difference could be due to either the presence of another undetected exon, or extensive polyadenylation of the message, or an artifact of the glyoxal-agarose gel determination of mRNA size. Complete nucleotide sequencing of the membrane exons and their flanking sequences, which is in progress, will answer this question.

Sequences Overlapping the Two $C_\gamma2a$ Membrane Exons Are Conserved among All C_γ Genes

$C_\gamma1$ *vs.* $C_\gamma2b$: The sequence homology between the CH1 and CH2 exons of the $C_\gamma1$ and $C_\gamma2b$ genes are 87% and 74%, respectively (Table 2). The expected location and length of the heteroduplexes formed between these exons are shown in Fig. 3A. This is an electron micrograph of a heteroduplex molecule between a germline $C_\gamma1$ clone, IgH2, and a germline $C_\gamma2b$ clone, IgH22. In addition, a third homologous region of about 0.3 kb is located in the 3′ flanking region, about 1.4 kb from the 3′ end of the coding region of the $C_\gamma1$ and $C_\gamma2b$ genes.

$C_\gamma1$ *vs.* $C_\gamma3$: Five conserved regions are seen in a heteroduplex molecule between Ch·Igγ1-3, another $C_\gamma1$ germline DNA clone, and a germline $C_\gamma3$ clone, Ch·Igγ3-30. The first conserved region (from the left in Fig. 3B) of about 0.5 kb, located about 3 kb 5′ of the start of the coding regions of the $C_\gamma1$ and $C_\gamma3$ genes, is part of the S region which shares homologous repetitive sequences between the C_γ genes (6, 11). The second and third homologous regions from the left were identified as the region encoding the CH1 and CH2 exons, respectively (2, 15, 35). There are two additional conserved regions of about 0.3 kb and 0.1 kb in the 3′ flanking region, separated by a 0.6 kb non-homologous region. The larger region is 5′ to the smaller and located 1.0 kb from the 3′ end of the coding region of the $C_\gamma1$ and $C_\gamma3$ genes. This observation is in general agreement with the report by Tyler and Adams (29).

$C_\gamma1$ *vs.* $C_\gamma2a$: We identified two conserved regions having sizes and locations similar to those conserved between the $C_\gamma1$ and $C_\gamma3$ genes when comparing the $C_\gamma1$ and $C_\gamma2a$ genes (Fig. 3C). The larger homologous region is about 0.4 kb long and is located 1.1 kb from the 3′ end of the coding region of the $C_\gamma1$ and $C_\gamma2a$ genes. The smaller homologous region is about 0.1 kb long and located 0.6 kb from the 3′ end of the larger homologous region. As expected from sequence homology (Table 2), heteroduplexes were seen in locations corresponding to the CH1 and the CH2 and CH3 exons of $C_\gamma1$ and $C_\gamma2a$ (Fig. 3C). Another small conserved region of 0.5 kb is located in the S region.

$C_\gamma2b$ *vs.* $C_\gamma2a$: The heteroduplex formed between the $C_\gamma2b$ and $C_\gamma2a$ clones is quite different than all the other pairs. As shown in Fig. 3D, a germline $C_\gamma2b$ clone, Ch·Igγ2b-2 and a germline $C_\gamma2a$ clone, Ch·Igγ2a-32 formed a complete duplex between about 1.2 kb 5′ and 3 kb 3′ to the coding regions of $C_\gamma2b$ and $C_\gamma2a$ genes. There are four more conserved regions that occur within 6 kb from coding regions which cover almost 40% of the 5′ flanking region (data not shown).

$C_\gamma3$ *vs.* $C_\gamma2b$: A heteroduplex between the $C_\gamma3$ gene clone, Ch·Igγ3-30 and a germline $C_\gamma2b$ gene clone, Ch·Igγ2b-2 (Fig. 3E) also revealed two conserved regions in the 3′ flanking region which have sizes and locations similar to those described above (Fig. 3C). Similar homologous regions in the 3′ flanking region also were shown previously in a heteroduplex between the $C_\gamma3$ and

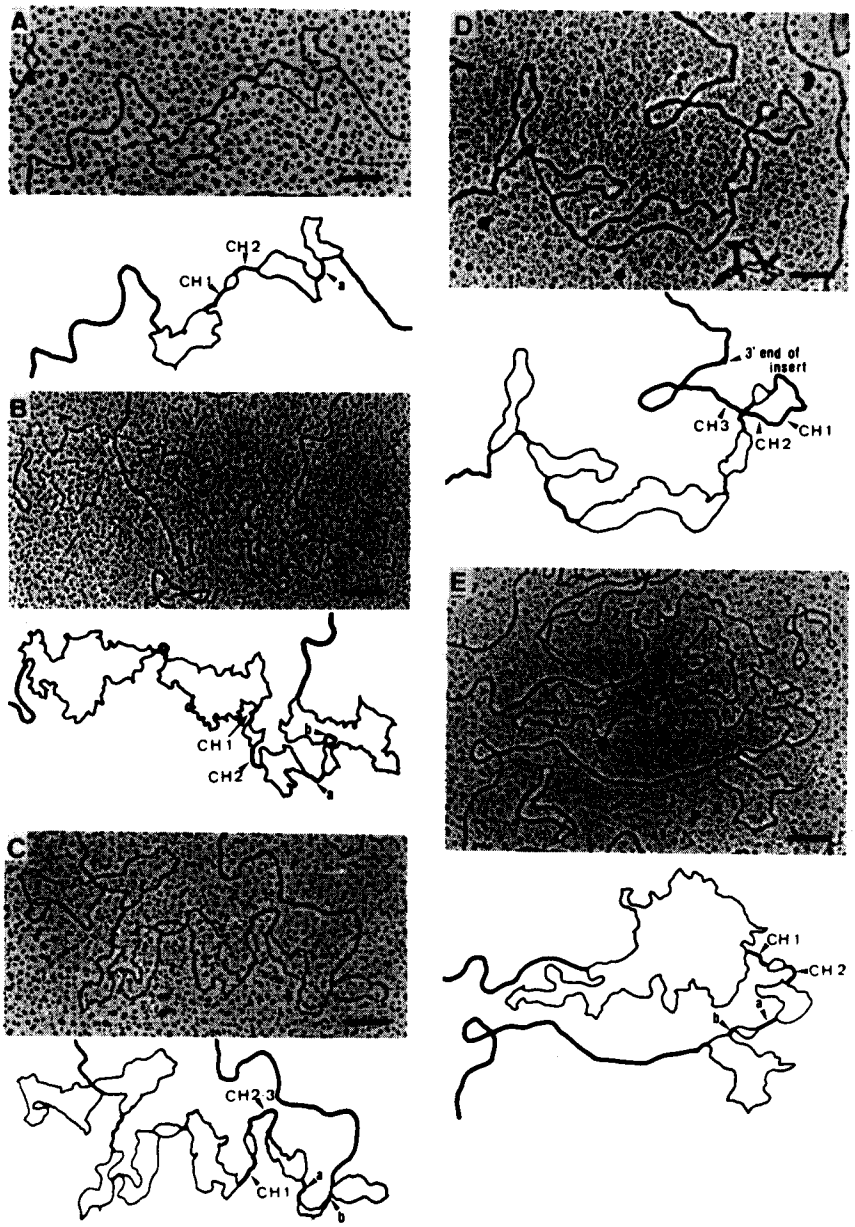

Fig. 3 Heteroduplexes between germline C_γ genes and their interpretations. The horizontal bar represents 0.2 μm.

A: $C_\gamma 1$ *vs.* $C_\gamma 2b$. Lengths (kb) of duplexed regions: CH1, 0.4 ± 0.1; CH2, 0.3 ± 0.1; a, 0.3 ± 0.1. Lengths (kb) of non-homologous regions: CH1–CH2, 0.5 ± 0.1; CH2–a, 1.8 ± 0.3. Thirty-three molecules were measured.

B: $C_\gamma 3$ *vs.* $C_\gamma 1$. Lengths (kb) of duplexed regions: CH1, 0.3 ± 0.1; CH2, 0.3 ± 0.1; a, 0.3 ± 0.1; b, 0.1 ± 0.1. Lengths (kb) of non-homologous regions: CH1–CH2, 0.4 ± 0.1; CH2–a, 1.4 ± 0.1; a–b, 0.6 ± 0.1. One third of the $C_\gamma 3$ molecules showed a stem-loop structure 0.5–1.0 kb long, 0.5–4.5 kb 5′ to the CH1 domain (not seen in this micrograph). Nineteen molecules were measured.

C: $C_\gamma 1$ *vs.* $C_\gamma 2a$. Lengths (kb) of duplexed regions: CH1, 0.4 ± 0.1; CH2–CH3, 0.8 ± 0.1; a, 0.4 ± 0.1; b, 0.1 ± 0.1. Lengths (kb) of non-homologous regions: CH1–CH2, 0.5 ± 0.1; CH3–a, 1.1 ± 0.2; a–b, 0.6 ± 0.1. Twenty-three molecules were measured.

D: $C_\gamma 2b$ *vs.* $C_\gamma 2a$. The length of duplexed region which includes the 3′ flanking region

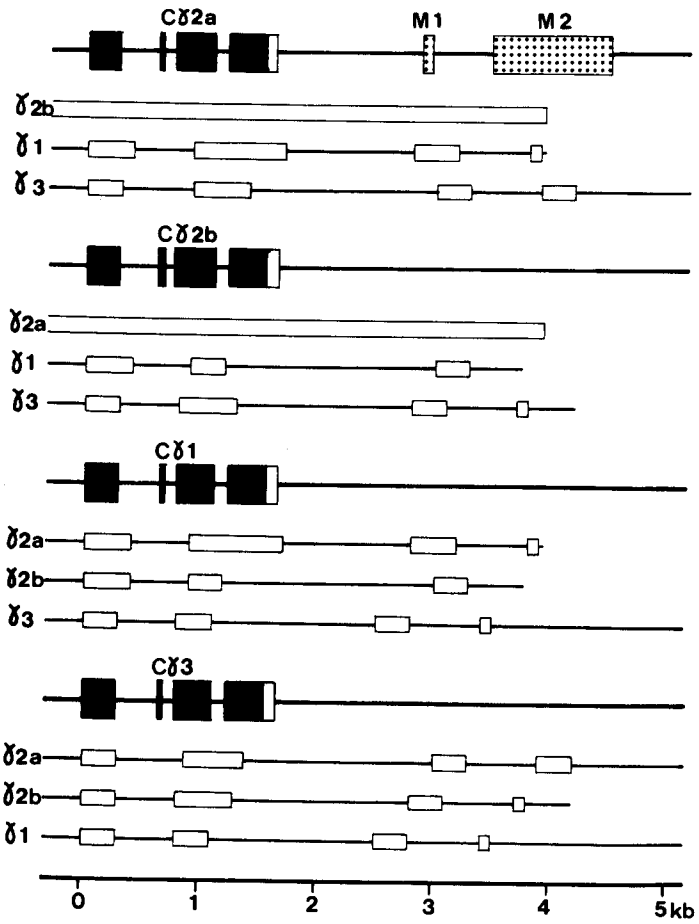

Fig. 4 Summary of heteroduplexes of C_γ genes. The solid rectangles indicate the location of the structural sequences in each C_γ gene. The lower three lines in each group show the heteroduplexed regions (open rectangles) formed between the pairs of C_γ genes indicated at the left. M1 and M2 are the membrane exons identified by R-loop analysis (Fig. 2).

C_γ2a clones (26).

A summary of our heteroduplex analyses is schematically presented in Fig. 4. This illustrates the length and location of the conserved segments in the 3′ flanking regions of the C_γ genes. It is clear that the 3′ flanking region encompasses two regions of 0.3 kb and 0.1 kb which are conserved among all the C_γ genes and overlaps the two membrane exons identified by R-loop analysis of membrane mRNA with the germline C_γ2a gene. The smaller R-loop, which we call M1 (for membrane exon 1), seems to be the 3′ part of the larger conserved region in the 3′ flanking region. Only the 5′ part of the larger R-loop, which we call M2 (for membrane exon 2), is conserved among the C_γ genes.

of both C_γ genes and the short arm of Charon 4A DNA is 15.2 ± 0.8kb. The length of the duplexed insert is 4.3 kb since the length of the short arm of the phage DNA is 10.9 kb. Nineteen molecules were measured.

E: C_γ3 *vs.* C_γ2b. Lengths (kb) of duplexed regions: CH1, 0.3 ± 0.1; CH2, 0.5 ± 0.1; a, 0.3 ± 0.1; b, 0.1 ± 0.1. Lengths (kb) of non-homologous regions: CH1–CH2, 0.5 ± 0.1; CH2–a, 1.5 ± 0.2; a–b, 0.6 ± 0.1. One third of these heteroduplexes showed inverted-repeat sequences as described in Fig. 3B. Additional duplexed regions (0.3–1.6 kb) with variable stability were found scattered between 2 and 6 kb 5′ to the CH1 domain (not seen in this micrograph). Twenty-five molecules were measured.

We have recently determined partial nucleotide sequences of the M1 exons of the $C_\gamma 1$, $C_\gamma 2b$ and $C_\gamma 2a$ genes and found a region with 80% nucleotide sequence homology (Y. Yamawaki-Kataoka, unpublished data). Taken together, it is clear that the C_γ genes have two membrane exons, about 1.3 kb and 1.9 kb 3′ to the CH3 domain and that the nucleotide sequences of the membrane exons are conserved, at least in part, among all C_γ genes.

DISCUSSION

The existence of specific membrane exons for each IgG subclass supports earlier findings of membrane IgG molecules on memory B cells (18) and on a small population of virgin B cells (1). These structural sequences resemble the membrane exon-structure coding for membrane IgM heavy chains which suggests that alternate splicing of messenger RNA precursors generates membrane and secreted IgG heavy chains.

The membrane $C_\gamma 2a$ heavy chains have been reported to be about 65,000 daltons (17). The length of the membrane $C_\gamma 2a$ mRNA (4.0 kb) is longer than necessary to code for a protein molecule of this size. Another polypeptide involved in immunoglobulin structure also has extensive untranslated sequences. J-Chain mRNA has been found to be longer than necessary to code for a 15,000 dalton polypeptide chain (14).

Further nucleotide sequence analysis of the $C_\gamma 2a$ membrane exons will demonstrate whether membrane IgG molecules are transmembrane proteins. Its structure may elucidate its role in mediating antigen-induced transmembrane proliferative signals.

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Note added in proof. Tyler B. M. *et al.* (30) have recently made similar observations to ours using a cDNA clone constructed from 2PK3 mRNA.

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