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

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ABSTRACT
Two C\textsubscript{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\textsubscript{2a} heavy chains. 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\textsubscript{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\textsubscript{2a} membrane exons is conserved among all the C\textsubscript{2} 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 (15).

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\textsubscript{2a} gene. This structure is reminiscent of the membrane exon-structure of the C\textsubscript{2} 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\textsubscript{2}a(4.5 kb)-C\textsubscript{2}a(55 kb)-C\textsubscript{3}(34 kb)-C\textsubscript{1}(21 kb)-
C\textsubscript{2}b(15 kb)-C\textsubscript{2}a(14.5 kb)-C\textsubscript{2}a(12 kb)-C\textsubscript{3}-3' (6,
12, 16, 20, 23, 24, 26). The C\textsubscript{2} genes are not
only clustered within this gene family but also
have been shown to be closely related to each
other. The four C\textsubscript{2} genes have essentially identi-
cical 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\textsubscript{2}
genesis have evolved from a common ancestral
gene. In fact, comparison of the nucleotide
sequences of the four C\textsubscript{2} genes indicates that
segments of these genes have been exchanged by
recombination during the evolution of this
gene family (15, 35).

In this paper we extend this comparative
analysis to the 5' flanking region of each C\textsubscript{2}
genome, to include the region where we located
the two membrane exons of the C\textsubscript{2}a gene. We
have analyzed electron micrographs of heter-
duplexes between all possible pairs of the C\textsubscript{2}
genome. This revealed two conserved regions in
the 5' flanking region of each C\textsubscript{2} gene. These
two regions overlap the sequences identified as
the two membrane exons of the C\textsubscript{2}a gene. We
conclude that each C\textsubscript{2} gene has at least two mem-
brane exons which are conserved, at least in part,
among the four C\textsubscript{2} 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\textsubscript{2} genes used in this
study (Table I) were prepared as described previ-
ously (7, 11, 23, 34). RNA from the B-lym-
phoma, 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\textsubscript{2} 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 \textit{et al.} (4). R-loops were
formed between the 19.4 kb insert of Ig2a-11
(23) and 2PK3 mRNA according to the pro-
cedure of Kaback \textit{et al.} (10). Electron micro-
graphs were taken with a Hitachi HU12A
electron microscope at \( \times 10,000 \) magnification
and images were enlarged an additional \( \times 10 \).
DNA lengths were measured with a digramer,
Mutoh Model G, using pBR322 and fd DNA
as size markers.

mRNA Hybridization
Glyoxal-treated RNAs were analyzed by electro-
phoresis in 1.1% agarose gels. RNA was trans-
ferred 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 Mariatis,
\textit{et al.} (7, 13).

Other Materials
Sources of restriction endonucleases were des-
cribed previously (7). \( \alpha-\text{32P-dCTP} (2,000-
3,000 \text{Ci/mmol}) \) was purchased from Radio-
chemical Center (Amersham, England).

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

<table>
<thead>
<tr>
<th>Table 1 List of Recombinant Phages</th>
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<tr>
<td>Clone</td>
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</tr>
<tr>
<td>IgH2</td>
</tr>
<tr>
<td>Ch-Igj1-1-3</td>
</tr>
<tr>
<td>IgH22</td>
</tr>
<tr>
<td>Ch-Ig2b-2-2</td>
</tr>
<tr>
<td>Ch-Ig2a-11</td>
</tr>
<tr>
<td>Ch-Ig2a-32</td>
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<tr>
<td>Ch-Ig2-30</td>
</tr>
</tbody>
</table>
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 HOPCI (IgG2a) myeloma mRNA; 3, 2.5 µg of HOPCI 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 C2a 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, HOPCI (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 nitrocellulose filters. When we used as a DNA probe, sequences containing the 5' half of the C2a 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 C2a gene (probe b). These results indicate that 3' flanking sequences of the C2a gene are transcribed as part of the mRNA coding for membrane IgG2a heavy chains.

Localization of C2a Membrane Exons

R-loops formed between 2PK3 mRNA and a germline C2a clone, Ch-Ig2a-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 C2a gene and the Ch-Ig2a-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 C2a 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

![Diagram of R-loops formed between 2PK3 mRNA and a germline C2a clone, Ch-Ig2a-11.](image)

**Fig. 2** Electron micrograph of R-loops formed between the C2a gene and 2PK3 mRNA. R-loops were formed between Ch-Ig2-11 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

<table>
<thead>
<tr>
<th>Regions</th>
<th>CH1</th>
<th>CH1'</th>
<th>CH2</th>
<th>CH2'</th>
<th>CH3</th>
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<tr>
<td></td>
<td>C2b-C2b</td>
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<td>73</td>
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<td>71</td>
<td>88</td>
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</table>

* 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 C2a Membrane Exons Are Conserved among All Cγ Genes

C2b vs. C2a: The sequence homology between the CH1 and CH2 exons of the Cγ1 and Cγ2b 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 Cγ1 clone, IgH2, and a germline Cγ2b 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 Cγ1 and Cγ2b genes.

Cγ1 vs. Cγ2: Five conserved regions are seen in a heteroduplex molecule between CH-1γγ1-3, another Cγ1 germline DNA clone, and a germline Cγ3 clone, CH-1γγ3-30. 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 Cγ1 and Cγ3 genes, is part of the S region which shares homologous repetitive sequences between the Cγ 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 Cγ1 and Cγ3 genes. This observation is in general agreement with the report by Tyler and Adams (29).

Cγ1 vs. Cγ2a: We identified two conserved regions having sizes and locations similar to those conserved between the Cγ1 and Cγ3 genes when comparing the Cγ1 and Cγ2a 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 Cγ1 and Cγ2a 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 Cγ1 and Cγ2a (Fig. 3C). Another small conserved region of 0.5 kb is located in the S region.

Cγ2b vs. Cγ2a: The heteroduplex formed between the Cγ2b and Cγ2a clones is quite different than all the other pairs. As shown in Fig. 3D, a germline Cγ2b clone, CH-Ig2b-2 and a germline Cγ2a clone, CH-Ig2a-32 formed a complete duplex between about 1.2 kb 5' and 3 kb 3' to the coding regions of Cγ2b and Cγ2a 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).

C3 vs. C2b: A heteroduplex between the Cγ3 gene clone, CH-Ig3-30 and a germline Cγ2b gene clone, CH-Ig2b-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 Cγ3 and
Fig. 3  Heteroduplexes between germine C₃ genes and their interpretations. The horizontal bar represents 0.2 μm.
A: C₁ vs. C₂b. 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: C₃ vs. C₁. 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 C₃ 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: C₁ vs. C₂a. 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±0.3; a-b, 0.6±0.1. Twenty-three molecules were measured.
D: C₂b vs. C₂a. The length of duplexed region which includes the 3′ flanking region
C\textsubscript{2}a 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\textsubscript{2} 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\textsubscript{2} genes and overlaps the two membrane exons identified by R-loop analysis of membrane mRNA with the germline C\textsubscript{2}a 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\textsubscript{2} genes.

E: C\textsubscript{2}a vs. C\textsubscript{2}b. Lengths (kb) of dupplexed 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 dupplexed 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.
Sequential expression of immunoglobulin on developing mouse B lymphocytes: a systematic survey that suggests a model for the generation of immunoglobulin isotype diversity. *J. Immunol.*, 120, 2041–2049


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