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Genetics

Linkage of the four γ subclass heavy chain genes

(immunoglobulin genes/multigene family/evolution/tandem duplication)

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ABSTRACT **The genes for the heavy-chain constant regions of the four γ subclass immunoglobulins were identified in a set of overlapping mouse DNA fragments representing about 100 kilobase pairs (kb) of the mouse genome that was cloned from bacteriophage λ libraries of BALB/c mouse embryo DNA. R-loop mapping studies show that the genes are located 5′-Cγ$_3$-34 kb-Cγ$_1$-21 kb-Cγ$_{2b}$-15 kb-Cγ$_{2a}$-3′ and lie in the same transcriptional orientation. Two DNA segments, one of 19 kb and another of 15 kb, that surround the Cγ$_{2b}$ and Cγ$_{2a}$ genes, respectively, show considerable homology and implicate a tandem duplication mechanism in the evolution of this gene cluster.**

Immunoglobulins are composed of light and heavy chains, each of which contains variable (V) and constant (C) portions. Genes encoding these proteins are created during differentiation by the somatic recombination between sequences flanking one of many V genes and those flanking a C gene. This process has been shown to occur with slight variations in each of the three independent immunoglobulin gene families—i.e., λ (1, 2) and κ (3, 4) light chain genes and heavy chain genes (5–8). However, heavy chain genes differ from light chain genes in an important aspect. Whereas Vκ or Vλ genes are expressed only with the appropriate light chain C gene, a given heavy chain V gene can be expressed with more than one heavy chain C gene class. In addition, it appears that within a single lymphocyte there is the capacity to switch the class of C gene expressed while maintaining expression of the same V region (9–11). Recent studies from this laboratory (5, 7) and others (8, 12) have shown that this is accomplished by yet another type of recombination. Analysis of a cloned complete V + Cγ$_{2b}$ gene suggests that recombination between sequences that lie between V and Cμ and sequences flanking the Cγ$_{2b}$ gene creates an expressable V + Cγ$_{2b}$ gene from what was a complete V + Cμ gene. Thus, it is believed that switch recombination is necessary for expression of other heavy chain genes. The expression of δ is a notable exception and is regulated by the differential processing of a V-Cμ-Cδ-bearing precursor mRNA (ref. 13 and our unpublished results).

Phylogenetic studies (14) indicate that all of the heavy chain C loci evolved from a common precursor gene. The various subclasses of Cγ appeared recently—possibly after the divergence of various species of mammals. Protein sequencing studies suggest that mouse IgG$_1$ and all human subclasses have a common ancestor (15) which differs from that of the human IgG$_2$ subclasses. Whereas mouse Cγ$_1$ and Cγ$_2$ genes may have diverged prior to the divergence of species, the Cγ$_{2a}$ and Cγ$_{2b}$ genes must have arisen as the result of a very recent event. Comparisons of nucleotide sequences of these genes show substantial conservation of sequences, even in codon selection (16). Whereas protein and nucleotide sequence studies indicate that these genes share strong homologies, genetic studies indicate that the Cγ genes are closely linked. No recombination between these genes has been detected in several thousand crosses (17).

In this paper, we describe the arrangement of the four Cγ subclass genes within a 100-kilobase pair (kb) segment of mouse DNA. Analysis of a set of overlapping recombinant phage clones has made it possible to determine the linkage arrangement and transcriptional orientation of these genes. In addition, strong flanking sequence homologies between Cγ$_{2b}$ and Cγ$_{2a}$ genes suggest that a direct, tandem duplication of a large block of DNA created these two Cγ genes from a common precursor.

MATERIALS AND METHODS

Bacteria and Phages. λ Charon 4A and *Escherichia coli* DP50 (Su II$^+$, Su III$^+$) were obtained from F. Blattner of the University of Wisconsin, Madison (18). *E. coli* 803 (r_k^-, m_k^-, Su III$^+$) was originally from K. and N. E. Murray of the University of Edinburgh. Lysogens used for preparation of packaging mixtures, BHB 2688 [N205 *recA*$^-$ (λ *imm*434 *b*2 *red*3 *Eam*4 *Sam*7)/λ] and BHB 2690 [N205 *recA*$^-$ (λ *imm*434 *cIts b*2 *red*3 *Dam*15 *Sam*7)/λ] were obtained from B. Hohn of the Friedrich Miescher Institute (19).

Preparation of Mouse Embryo Libraries. Bacteriophage λ libraries were prepared as described (5). *Eco*RI* libraries were prepared from mouse embryo DNA that had previously been methylated with *Eco*RI methylase (New England BioLabs) under conditions described (20). *Eco*RI* partial digestion was done in 2 mM $MgCl_2$/25 mM Tris·HCl, pH 8.5. DNA fractionated on 10–40% exponential sucrose gradients was ligated to Charon 4A "arms" (21) and packaged in phage coats (19) as described.

Isolation of Clones. Screening of libraries with nick-translated probes (1) was by the Benton and Davis technique (22) as modified (19).

Electron Microscopy. Procedures used for formation of single-stranded DNA·mRNA hybrids, formation of DNA heteroduplexes, two-step R-loop (double-stranded DNA·RNA hybrid) technique, and formation of R-hybrids have been described (23). In the two-step R-loop technique the R-loop mixture is completely denatured by heating and then incubated at 57°C for 4–6 hr. The R-hybrid technique is essentially the same as the two-step R-loop technique except that two different cloned DNAs are mixed with mRNA.

Southern Blots. Transfer of restriction endonuclease-digested DNA from gels to nitrocellulose filters was done by the procedure described by Southern (24). Hybridization and washing of filters were done as described (25).

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Abbreviations: V and C, variable and constant regions of immunoglobulins; kb, kilobase pair(s).

Other Procedures. Purification of γ chain mRNAs of γ_1 (MOPC21), γ_{2a} (MOPC173), γ_{2b} (MOPC141), and γ_3 (J606) was previously described (26).

All cloning experiments were carried out under P3–EK2 conditions in accordance with the National Institutes of Health guidelines for recombinant DNA research issued in June, 1976.

RESULTS

Linkage of the four Cγ genes

The complete *Eco*RI restriction map of the Cγ gene cluster is depicted in Fig. 1, showing the linked cluster of Cγ genes. $C\gamma_3$ and $C\gamma_1$ are 34 kb apart, $C\gamma_1$ and $C\gamma_{2b}$ 21 kb apart, and $C\gamma_{2b}$ and $C\gamma_{2a}$ 15 kb apart. All genes are aligned in the same order of transcription. This was determined by analysis of a set of bacteriophage λ clones and the correlation of their restriction maps to data obtained by Southern blotting of mouse embryo DNA. We have previously discussed the isolation of clones connecting the $C\gamma_1$ and $C\gamma_{2b}$ genes. Below we discuss those clones containing $C\gamma_3$ and $C\gamma_{2a}$ and the regions common to them and $C\gamma_1$ and $C\gamma_{2b}$ clones.

Southern blots of mouse embryo DNA hybridized to the nick-translated, 6.8-kb, γ_{2b}-containing *Eco*RI fragment reveal four hybridizable fragments. Two fragments of 6.8 and 6.6 kb contain the γ_1 and γ_{2b} genes (5). The remaining two of 22.5 and 18.5 kb were cloned from *Eco*RI complete digests of mouse embryo DNA with the $C\gamma_{2b}$ gene probe after enrichment of the proper size fraction by preparative agarose gel electrophoresis (for the 18.5-kb fragment) or by sucrose gradient fractionation (for the 22.5-kb fragment). Electron microscopic studies were done with heavy chain mRNA from the myelomas J606 (an IgG_3 producer) or MOPC173 (an IgG_{2a} producer). Hybridization of $C\gamma_3$ mRNA with the 18.5-kb fragment from MEP311 revealed that the $C\gamma_3$ gene is carried by this fragment 8.6 kb from the 5′ *Eco*RI site and 6.4 kb from the 3′ *Eco*RI site. Similar analysis of DNA from the clone ME768 shows that the 22.5-kb *Eco*RI fragment contains the $C\gamma_{2a}$ gene and that the gene is located 1.4 kb from the 5′ *Eco*RI site of the fragment.

The size of the γ_{2a}- and γ_3-bearing *Eco*RI fragments approaches the maximal size that can be inserted into the bacteriophage λ vector, Charon 4A (22.5 kb). To obtain clones that contained DNA flanking these *Eco*RI fragments it was necessary to fractionate the cellular DNA with a method other than partial *Eco*RI digestion. For this purpose embryo DNA was partially digested with *Eco*RI* (an auxiliary activity of *Eco*RI, which in low ionic strength buffers cleaves at the sequence A-A-T-T), and a bacteriophage λ *Eco*RI* library was constructed.

Screening of this library with nick-translated DNA fragments from the $C\gamma_3$ region results in the isolation of a number of $C\gamma_3$-containing clones. R-loop analysis and restriction site mapping show that one of these (ME736) carries the $C\gamma_3$ gene 0.7 kb from the 5′ end of the insert and contains additional *Eco*RI fragments of 3.6 and 0.4 kb. The organization of the *Eco*RI sites shown in Fig. 1 (5′-$C\gamma_3$-3.6 kb-0.4 kb-3′) is confirmed by comparison to another *Eco*RI* partial clone, ME535, and mapping of other restriction sites (Fig. 1 and data not shown).

The clones MEP10A, MEP10E, and MEP10 were isolated from an *Eco*RI partial library and contain insert DNA from the 5′ side of the $C\gamma_1$ gene. In addition to the 6.8-kb $C\gamma_1$ *Eco*RI fragment, MEP10 contains a 7.6-kb fragment. MEP10E contains a single 11.5-kb *Eco*RI fragment, and MEP10A contains four *Eco*RI fragments of 10.5, 3.6, 1.0, and 0,4 kb. Electron microscopic examination of heteroduplexes of these clones reveals that the 7.6-, 11.5-, and 10.5-kb fragments share the same 3′ and 5′ sequences but contain deletions. Restriction site mapping of

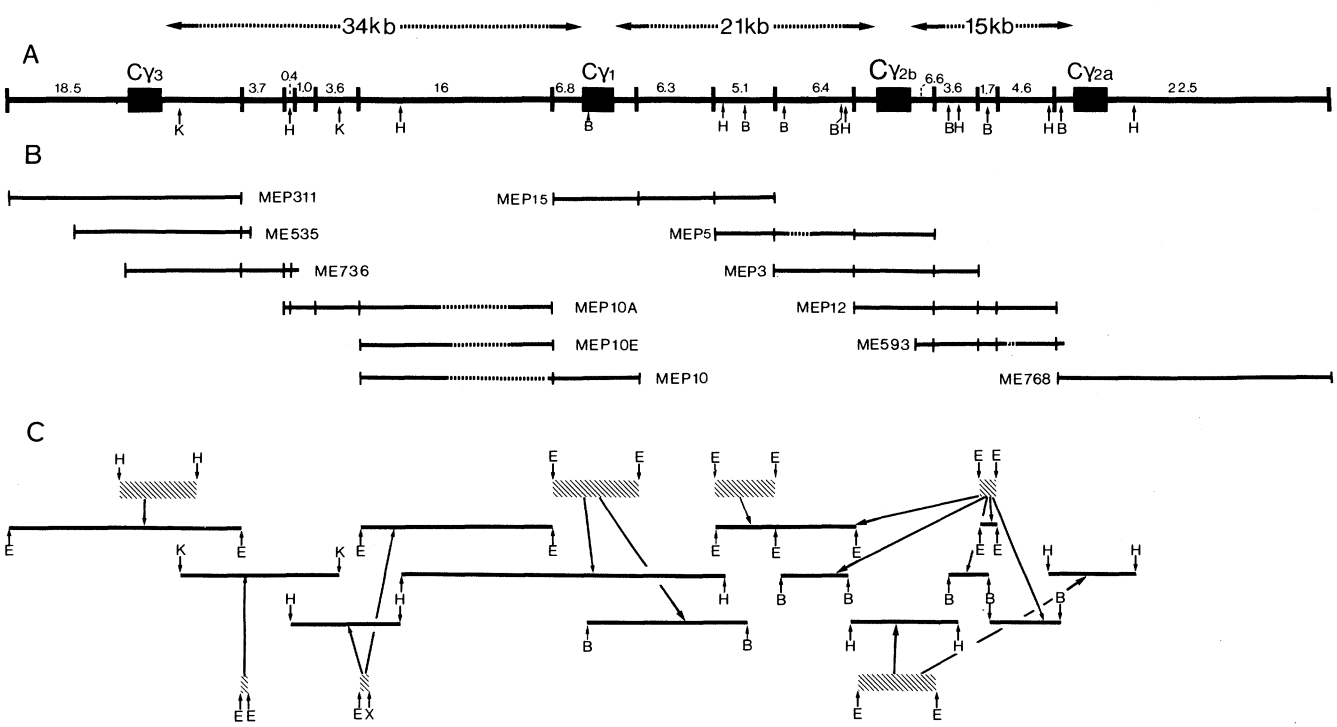

FIG. 1. (*A*) Linkage arrangement of the four Cγ subclass genes. The complete *Eco*RI cleavage map is also indicated; the size of each *Eco*RI fragment is indicated in kb. Other restriction sites are shown by arrows: B, *Bam*HI; H, *Hin*dIII; K, *Kpn* I. Distances between the γ genes are also indicated in kb. The map is drawn so that the 5′-3′ orientation of each gene is from left to right. (*B*) Cloned mouse DNA fragments. The inserts carried by 12 Charon 4A clones are arranged to show their overlapping regions. Broken lines indicate deletions found in some clones. (*C*) Summary of Southern blotting results of mouse embryo DNA. Probes used are indicated by hatched bars and the fragments hybridizing to them by solid lines. Arrows indicating restriction sites are labeled as above; E, *Eco*RI; X, *Xba* I.

MEP10A shows the order of the *Eco*RI fragments to be 5′-0.4 kb-1.0 kb-3.6 kb-10.5 kb-3′.

The 400-base pair fragments of ME736 and MEP10A are identical. Both fragments are similarly cleaved by the enzyme *Hin*dIII, and in Southern blotting experiments (data not shown), both hybridize to the nick-translated 400-base pair fragment isolated from MEP10A. Additional evidence that ME736 and MEP10A constitute an overlap comes from comparison between restriction sites in the phages and those in the embryo DNA shown by Southern blotting. Alignment of the restriction maps of the two clones at the *Eco*RI sites allows the prediction that a *Kpn* I fragment of 13 kb will overlap the sequences contained in these two clones. Such a fragment is detected in Southern blots of embryo DNA probed with a 300-base pair *Eco*RI–*Eco*RI fragment isolated from ME535 (Fig. 1).

Because MEP10A, MEP10E, and MEP10 all contain deletions, we determined the embryonic size of the large *Eco*RI fragment by blotting embryo DNA and hybridizing to a fragment common to all three of these clones, a 1.3-kb *Eco*RI–*Xba* I fragment (Fig. 1). The "true" embryonic size of this fragment is found to be 16 kb. Reconciliation of the data determined from the recombinant clones with that obtained by Southern blots of cellular DNA allows us to conclude that the $C\gamma_3$ and $C\gamma_1$ genes are 34 kb apart.

Sequences common to those carried by MEP12 (containing $C\gamma_{2b}$) and ME768 (containing $C\gamma_{2a}$) were found in the clone ME593. This clone, obtained after screening the *Eco*RI* library with a 1.7-kb *Eco*RI fragment from MEP12, has extensive homology to MEP12 and extends 1 kb beyond its rightmost—i.e., 3′—*Eco*RI site. In heteroduplex experiments with ME768 this 1-kb region hybridized to sequences immediately to the 5′ side of the $C\gamma_{2a}$ gene. The correlation of the regions of homology between MEP12, ME593, and ME768 allowed us to conclude that the $C\gamma_{2b}$ and $C\gamma_{2a}$ genes are separated by 15 kb.

Correspondence between cloned and genomic DNA

As mentioned above, the clones MEP10A, MEP10E, and MEP10 all contain *Eco*RI fragments that differ in size but are related to each other at both their 5′ and 3′ ends. We presume that these regions have suffered deletion upon being cloned in *E. coli*. Two other regions are represented in this series of clones by *Eco*RI fragments that vary in size. The fragment immediately preceding the $C\gamma_{2b}$ gene is 6.4 kb in MEP3 and 4.9 kb in MEP5. Similarly, the 4.6-kb fragment of MEP12 is found in ME593 as one of 4.2 kb.

To determine the embryonic size of the fragments carrying deletions and to locate any other sites that are sensitive to deletion, we did a series of Southern blots of cellular DNA. The six probes shown in Fig. 1*C* hybridize to a series of overlapping fragments covering the entire distance between $C\gamma_3$ and $C\gamma_{2a}$. Reconciliation of these fragment sizes with those found in cloned DNA or predicted from the overlap of two clones shows that only three regions are sensitive to deletion. Also, we are able to determine that the 6.4-kb fragment of MEP3 and the 4.6-kb fragment of MEP12 closely approximate those sizes found in the embryo. As mentioned previously, the *Eco*RI fragment preceding the $C\gamma_1$ gene is 16 kb.

The determination of restriction sites surrounding the $C\gamma_{2b}$ and $C\gamma_{2a}$ genes in embryo DNA was done with two probes that strongly crosshybridize with other fragments. The hybridization of the $C\gamma_{2b}$ *Eco*RI fragment with the $C\gamma_{2a}$-containing fragments has been discussed previously. Areas flanking these genes also crosshybridize (as discussed below) and justify the use of the 1.7-kb *Eco*RI fragment of MEP12 to estimate the size of fragments immediately preceding the $C\gamma_{2b}$ gene.

Crosshybridization of $C\gamma_{2b}$ and $C\gamma_{2a}$ flanking sequences

It is widely assumed that the $C\gamma$ subclass genes arose by gene duplication and subsequent mutational divergence. A striking support for this paradigm is seen when sequences flanking the $C\gamma_{2a}$ and $C\gamma_{2b}$ genes are compared. A 1.7-kb *Eco*RI fragment isolated from a clone carrying the $C\gamma_{2b}$-$C\gamma_{2a}$ intergenic region was hybridized to Southern blots of *Bam*HI- and *Eco*RI-digested DNA. As shown in Fig. 2, the probe hybridizes not only with an *Eco*RI fragment of 1.7 kb but also with one of 6.4 kb. When *Bam*HI-digested DNA was examined three hybridizable bands were detected. Two of these, fragments of 3.3 kb and 6.2 kb, correspond to fragments that overlap the probe and the third to a crosshybridizing 5.6-kb *Bam*HI fragment. A similar result is observed in experiments in which the probe is a 0.8-kb *Eco*RI-*Xba* I fragment that lies about 4.2 kb closer to the $C\gamma_{2a}$ gene. In this case, however, the crosshybridization to the 6.4-kb *Eco*RI fragment is weaker than that observed in the previous experiment.

The crosshybridizing regions lie in the area preceding the $C\gamma_{2b}$ gene. Southern analysis of fragments in the clones MEP3 and MEP5 (Fig. 1) indicates that the 6.4-kb *Eco*RI fragment immediately to the 5′ side of the $C\gamma_{2b}$ gene carries the sequences detected by crosshybridization (data not shown).

The extent of this homology in the flanking sequences is revealed by analysis of heteroduplexes formed between $C\gamma_{2b}$- and $C\gamma_{2a}$-bearing clones. A hybrid formed between MEP3 and ME768 shows that a 6.9-kb region, starting at ME768s 5′ *Eco*RI site and extending through the $C\gamma_{2a}$ gene, is almost completely homologous to the $C\gamma_{2b}$-containing clones (Fig. 3). This experiment defines the extent of the homology between the 3′ sides of the $C\gamma_{2b}$ and $C\gamma_{2a}$ genes. Similar experiments define a section

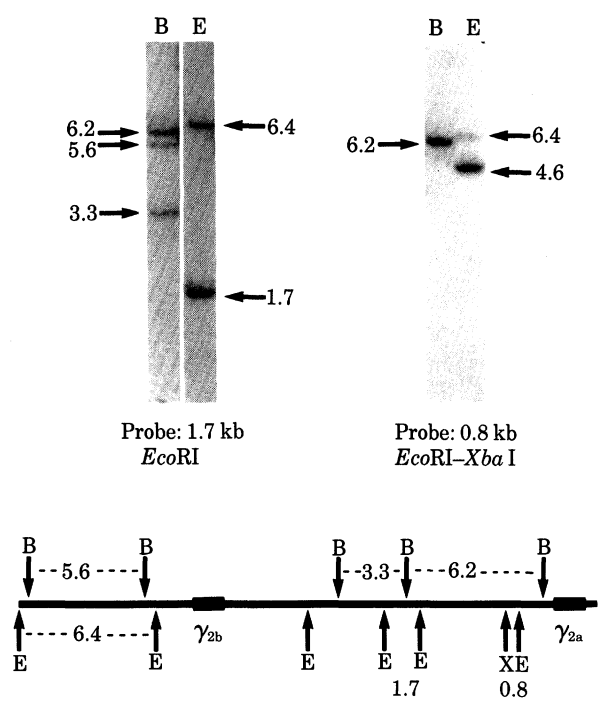

FIG. 2. Homology between $C\gamma_{2b}$ and $C\gamma_{2a}$ flanking sequences. Autoradiograms showing the results of Southern blotting experiments of mouse DNA cleaved with either *Bam*HI (B) or *Eco*RI (E). DNA was fractionated on 0.8% agarose gels and hybridized with either the 1.7-kb *Eco*RI or the 0.8-kb *Eco*RI–*Xba* I fragment. The schematic interpretation below shows the disposition of *Eco*RI and *Bam*HI cleavage sites in the $C\gamma_{2b}$-$C\gamma_{2a}$ region. X, XbaI; E, *Eco*RI; B, *Bam*HI. All fragment sizes are indicated in kb.

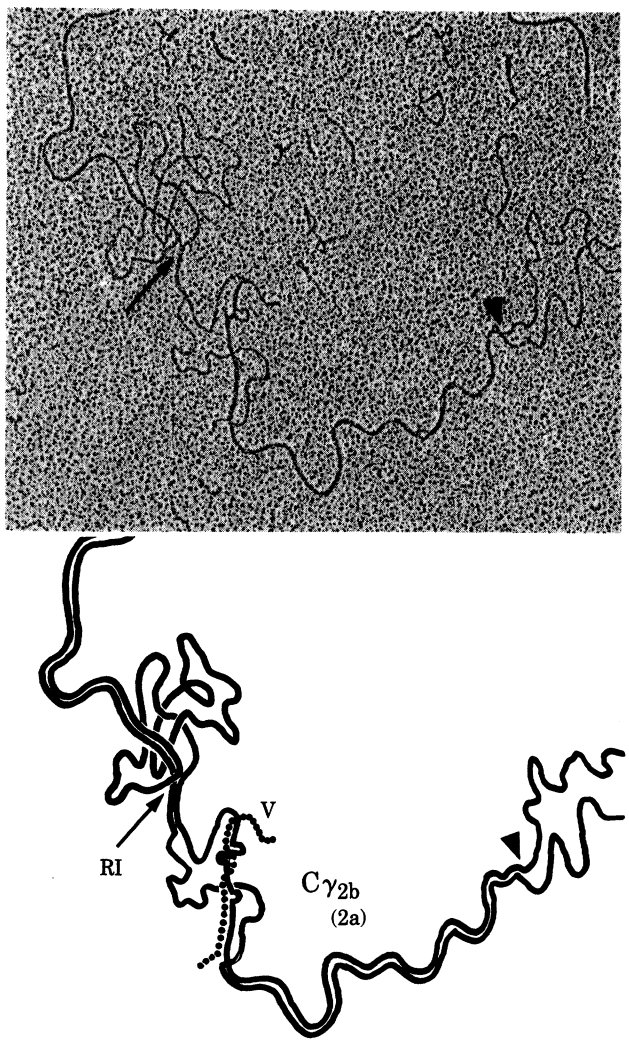

FIG. 3. Electron micrograph and schematic interpretation of R-hybrid observed upon using MOPC141 mRNA ($C\gamma_{2b}$ mRNA) and DNA from the clones MEP3 and ME768. Arrows indicate the points where homology between these clones begins and ends and an *Eco*RI site of ME768. $C\gamma_{2b}$ mRNA on the hybrid serves as a marker to locate the gene. The three-exon structure of the $C\gamma_2$ gene is apparent. Other hybrids that do not contain RNA are completely closed in the region displaced by the mRNA (not shown).

of about 3 kb that displays strong homology between the areas 5′ to the $C\gamma_{2b}$ and $C\gamma_{2a}$ genes. The two hybridizing sections are 5.7–8.7 kb and 3.9–6.9 kb 3′ to the $C\gamma_1$ and $C\gamma_{2b}$ genes, respectively (the shaded region shown in Fig. 4). The DNA sequences located between the two areas of strong homology, denoted by zigzag lines in Fig. 4, also bear some homology. However, heteroduplexes formed between these regions are not easily amenable to analysis in the electron microscope. These hybrids often show duplex structure between these regions, but the heteroduplexes are not consistent from one molecule to the next. We assume that a weak homology exists between these two areas, but we are not able to map these homologies with accuracy. The Southern blotting experiments shown in Fig. 2 confirm that homology between these regions does, in fact, exist.

Within the limits of our measurements, the end of the $C\gamma_{2b}$ "region" (segment A–B in Fig. 4) corresponds to the beginning of the $C\gamma_{2a}$ "region" (segment B–C).

DISCUSSION

We have established the linkage arrangement of the four $C\gamma$ genes and determined that they all lie in the same transcriptional orientation. The organization of the genes is as follows: 5′-$C\gamma_3$-34 kb-$C\gamma_1$-21 kb-$C\gamma_{2b}$-15 kb-$C\gamma_{2a}$, in agreement with that suggested from studies of deletion patterns of various plasmacytomas (27–31). All of these genes have exon-intron structures similar to the structure described by Sakano *et al.* (32) for $C\gamma_1$; each of the three domains and the hinge region are encoded in separate exons.

In three locations we found evidence that the cloned DNA fragments had undergone deletion, probably during growth in *E. coli*. Interestingly, the deletions we observe all lie in areas immediately preceding the $C\gamma_1$, $C\gamma_{2b}$, and $C\gamma_{2a}$ genes. These regions have been implicated as the areas in which recombination between $C\mu$ flanking sequences must occur to allow expression of the complete V-$C\gamma$ gene. In a study of this recombination process (7) we reported that the $C\mu$ flanking region that participates in switch recombination is also subject to deletion in the cloning process and that this region contains many repeats of a similar five-base sequence. Davis *et al.* (33) report that repeated units lie before the $C\alpha$ gene. The deletions suggest that the $C\gamma_1$, $C\gamma_{2b}$, and $C\gamma_{2a}$ flanking regions might also contain repeated sequences. One explanation for these similarities between the various C genes is that the repeated units are participants in the recombination that accompanies heavy chain class switch.

The analysis of large cloned sections of eukaryotic DNA has shown that genes encoding α-globin-like and β-globin-like (for review, see ref. 34) and ovalbumin-like proteins (35) are closely linked. Here we report that the $C\gamma$ subclass genes are similarly linked. Unlike the globin system, in which paris of coordinately expressed genes are found adjacent to each other, a single $C\gamma$ gene is expressed in a lymphocyte at the expense of the others. Indeed, others have shown that C_H genes located 5′ to the expressed gene are deleted in the $C\mu$-$C\gamma$ heavy chain switch recombination (27–31). Therefore, forces that maintain close linkage of the $C\gamma$ genes may be different from those that govern the globin and ovalbumin systems. In these systems the necessity for coexpression may explain the close linkage of genes. Heavy chain loci, on the other hand, may be closely linked to each other and to the $C\mu$ gene to facilitate the switch recombination necessary for their expression.

Within the limits of the techniques described here (i.e., crosshybridization observed by Southern blotting and examination of heteroduplex structures in the electron microscope),

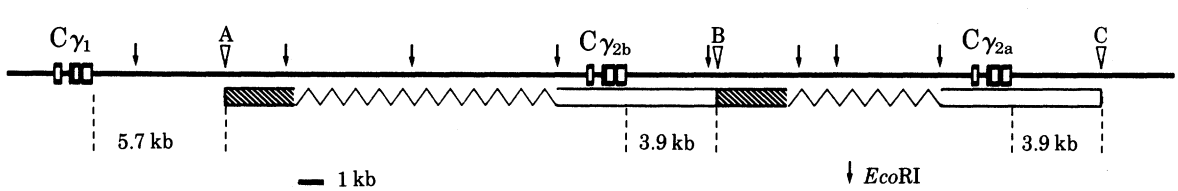

FIG. 4. $C\gamma_{2b}/C\gamma_{2a}$ flanking sequence homologies. Hatched and open boxes indicate homologies observed between the 5′ and 3′ flanking sequences, respectively, of $C\gamma_{2a}$ and $C\gamma_{2b}$. The zigzag lines represent areas of weak homology, the exact locations of which are not mapped.

the flanking sequences of the $C\gamma_{2b}$ and $C\gamma_{2a}$ genes appear to be quite similar. The correspondence of the 3′ end of the $C\gamma_{2b}$ region with the 5′ end of the $C\gamma_{2a}$ region (point B in Fig. 4) suggests duplication of a large DNA block. That the duplicated unit is so large seems to belie models that propose that the various $C\gamma$ subclasses arose by rearrangement in evolution of small DNA sections encoding single domains (36). Rather, we would suggest that a recent unequal-crossover event generated the duplication of a $C\gamma_2$ precursor gene and that mutational divergence modified the duplicated segments into the contemporary $C\gamma_{2b}$ and $C\gamma_{2a}$ genes.

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