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Structure of a rearranged $\gamma 1$ chain gene and its implication to immunoglobulin class-switch mechanism

(gene cloning/nucleotide sequence determination/repetitive sequence/sister-chromatid-exchange model)

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ABSTRACT **An expressed gene for $\gamma 1$ chain of MC 101 myeloma was cloned from a phage library containing partial *Eco*RI digests of MC 101 DNA. The cloned DNA was analyzed by restriction enzyme cleavage, Southern blot hybridization, R-loop formation, and nucleotide sequence determination. The results indicate that the expressed $\gamma 1$ chain gene comprises at least four germline DNA segments, namely a variable-region gene, a segment of the 5′ flanking region of the μ chain gene (containing J regions), a segment of the 5′ flanking region of the α chain gene, and the $\gamma 1$ chain gene with its flanking regions. The presence of the α chain gene-flanking switch (S) region (S_α region) at the 5′ side of the $\gamma 1$ chain gene-flanking region ($S_{\gamma 1}$ region) indicates that the heavy chain class switch may not be mediated by stepwise linear deletion along the order of the heavy chain constant-region genes (5′-μ-$\gamma 3$-$\gamma 1$-$\gamma 2b$-$\gamma 2a$-α-3′). We propose a sister-chromatid exchange model that explains class switch-associated deletion of heavy chain genes by unequal crossing-over events between sister chromatids.**

Immunoglobulins are divided into five classes, IgM, IgG, IgA, IgD, and IgE, which are defined by their heavy (H) chain constituents, μ, γ, α, δ, and ε, respectively. Each gene locus is itself split into two areas encoding the variable (V) and constant (C) regions (1–5). In the H chain, C region (C_H) genes share a family of V region (V_H) genes. During differentiation of a given B lymphocyte, a single V_H region gene (V_H gene) is first expressed as a part of the μ chain, and at a later stage the expressed H chain switches the C region from μ chain to γ or α chains without alteration of the V_H-region sequence.

Recent studies on the expressed H chain genes clearly demonstrate that two distinct types of DNA rearrangements take place during the differentiation of B lymphocytes (6–9). One type, termed V–J recombination, generates the complete V_H gene by joining the V_H-, D-, and J_H-gene segments (8, 10, 11). In C_H-region genes (C_H genes) only one set of the J-region genes is proposed to be present in the 5′ flanking region of the μ chain gene (μ gene) (7, 9). A second type of recombination mediates the H chain class switch that associates a particular antigen-binding specificity, the completed V region, with a series of different C_H regions. The rearrangement is termed S–S recombination because it joins two switch (S) regions which are located in the 5′ flanking region of each C_H gene (7, 9). To switch from μ to γ chain, the S_μ region recombines with the S_γ region, keeping the V_H gene unaffected.

It has been shown that the S–S recombination is associated with the deletion of the intervening DNA between the V_H gene and the C_H gene (12–18). Based on the order of C_H-gene deletion in various myeloma cells, we have proposed an H chain gene order of 5′-μ-$\gamma 3$-$\gamma 1$-$\gamma 2b$-$\gamma 2a$-α-3′. Recent cloning experiments have directly demonstrated the order of 5′-$\gamma 1$-$\gamma 2b$-$\gamma 2a$-ε-α-3′ (19, 20).

In this paper we report a detailed structure of a rearranged (expressed) $\gamma 1$ chain gene ($\gamma 1$ gene) cloned from a myeloma MC 101 cell. Partial nucleotide sequence determination indicates that the rearranged $\gamma 1$ gene contains a small S_α segment between the S_μ and $S_{\gamma 1}$ segments. The unique structure of this $\gamma 1$ gene implicates the molecular mechanism for the class-switch (S–S) recombination.

EXPERIMENTAL PROCEDURES

Cloning. Charon 4A (21) phage library of partial *Eco*RI digests of MC101 DNA was screened by using $\gamma 1$ chain-cDNA clone pG1-6 (22) as a probe as described (23). Germline V genes were cloned from mouse embryo DNA library (provided by P. Leder, National Institutes of Health) with a V portion of the expressed $\gamma 1$ gene (Ig$\gamma 1$-704) as a probe. Cloning and nucleotide sequence determination of these clones will be described in more detail elsewhere. Cloning experiments were done under P2-EK2 or P3-EK1 conditions.

R Loop. R loops were formed between Ch·M·Ig$\gamma 1$-704, (see *Results*) and partially purified MC 101 mRNA [dT 1 stage (24)] by the procedure of Kaback *et al.* (25). DNA was spread and stained as described (26).

Nucleotide Sequencing. The insert of λgtWES·IgH703 (7) was recloned into pBR322 (27). The recombinant plasmid was designated "pIgH703." Nucleotide sequences were determined, in part, by the isolation of a restriction DNA fragment of pIgH703 (Fig. 3, fragment a) that contains a S_α portion embedded in Ig$\gamma 1$-704. Because this portion does not contain adequate restriction sites except for one site each of *Mbo* II and *Sau* III A, we have adopted a novel trick that can be applied to any DNA fragment. A 2.5-kb *Hind* III–*Hap* II fragment of IgH703 (Fig. 3, fragment b) was partially digested with *Alu* I, and ligated with *Eco*RI linkers (G-G-A-A-T-T-C-C) purchased from Collaborative Research (Waltham, MA). *Alu* I partial digests with *Eco*RI linkers were cut with *Eco*RI, ligated to pBR322 at the *Eco*RI site, and used to transform *Escherichia coli* strain LE392. Transformants were screened (28) with IgH7 (7) as a probe. Among a number of positive clones we selected those that have a *Mbo* II site in the insert. The nucleotide sequences were determined by using *Eco*RI restriction sites incorporated

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Abbreviations: V region, variable region; C region, constant region; H chain, heavy chain; C_H, constant region of the heavy chain; V_H, variable region of the heavy chain; V_H gene, V_H-region gene(s); C_H gene, C_H-region gene(s); S region, switch region; kb, kilobase; bp, base pair(s), $\gamma 1$ gene, $\gamma 1$ chain gene; μ gene, μ chain gene; α gene, α chain gene.

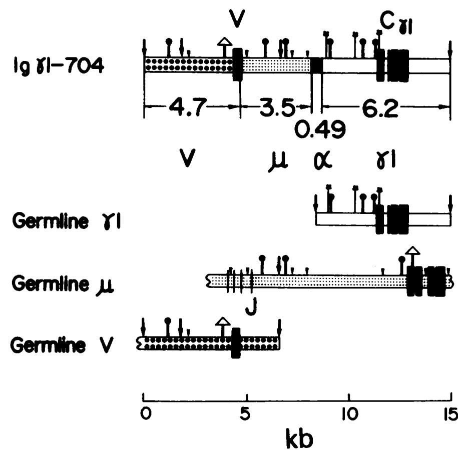

FIG. 1. Structure of the expressed γ1 gene of MC 101 myeloma. The cloned DNA fragments are shown by horizontal squares with the direction of transcription from left to right. Closed squares show the coding region. Restriction sites of the germline γ1- and μ-gene clones are taken from previous reports (7, 30). ↓, *EcoRI*; ▾, *HindIII*; ⬧, *Xba* I; ⇧, *Bgl* II; ↟, *BamHI*.

by the linker or other restriction sites present in pBR322. Sequences were determined by the Maxam and Gilbert procedure (29).

RESULTS

Structure of the Expressed γ1 Gene from MC 101 Myeloma. We have reported cloning and characterization of a 3′ *EcoRI* fragment (8.3 kb) of the expressed γ1 gene of MC 101 myeloma (7). This *EcoRI* fragment, designated "IgH7," contained not only the γ1 gene and its flanking regions but also the 5′ flanking region of the μ gene at its 5′ side. Subsequently, we cloned two complete gene fragments of the γ1 chain from a Charon 4A phage library containing partial *EcoRI* digests of MC 101 DNA. The clones are designated "Ch·M·Igγ1-704" and "Ch·M·Igγ1-706," the inserts of which are called "Igγ1-704" and "Igγ1-706," respectively. The restriction cleavage sites of Igγ1-704 are shown in Fig. 1. Igγ1-704 is 15 kb long and contains three *EcoRI* fragments of 1.8, 4.9, and 8.3 kb. Igγ1-706 is identical to Igγ1-704, except that Igγ1-706 does not contain the 5′ *EcoRI* fragment (1.8 kb). The 3′ *EcoRI* fragment (8.3 kb) is identical to the previous clone, IgH7, as determined by restriction-map comparison and Southern blot hybridization (data not shown). Comparison of restriction maps of Igγ1-704 and the 5′ flanking region of the μ gene (8) indicated that a segment of approximately 3.5 kb was derived from the 5′ flanking region of the μ gene and that the 5′ end of the μ segment seemed to extend to the J_{H3} region.

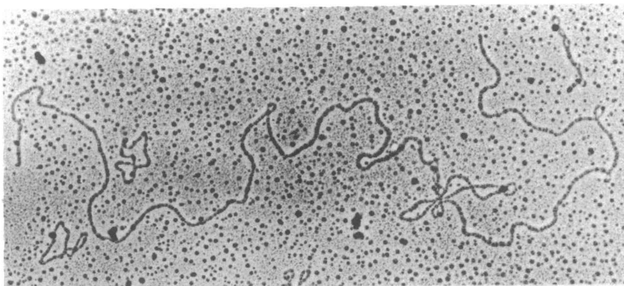

FIG. 2. R loops formed between Igγ1-706 and MC 101 mRNA. Electromicroscopic picture of R loops formed between Ch·M·Igγ1-706 DNA and MC 101 mRNA is shown with schematical interpretation. CH_1, CH_2, CH_3, and V indicate respective coding domains. Solid and broken lines show DNA and mRNA, respectively. Twenty-nine molecules were measured with pA043 plasmid DNA (31) as the length standard.

We tested whether Igγ1-706 encompasses a V-region sequence by R-loop analysis using mRNA of MC 101 myeloma. Electron microscopic observation indicates that Igγ1-706 and MC 101 mRNA can form a structure with four R loops (Fig. 2). Three adjacent R loops of about equal size are separated from a fourth by a long intervening sequence (6.78 ± 0.62 kb). This multiloop structure looks similar to those found in genes for the γ1 and γ2b chains (5, 30), in which each domain and the hinge region are interrupted by the intervening sequence. The sizes of the CH_1, CH_2, and CH_3 domains were 0.37 ± 0.04 kb, 0.32 ± 0.05 kb, and 0.37 ± 0.06 kb, respectively, which agree roughly with the number of nucleotides of each domain (30). The fourth R loop of 0.4 ± 0.06 kb seems to correspond to the V domain, which is located about 6.8 kb 5′ to the γ1 gene. The distance is slightly larger than the sum of the 2.8-kb segment of the 5′ flanking region of the γ1 gene in IgH7 (7) and the 3.5-kb segment derived from the 5′ flanking region of the μ gene

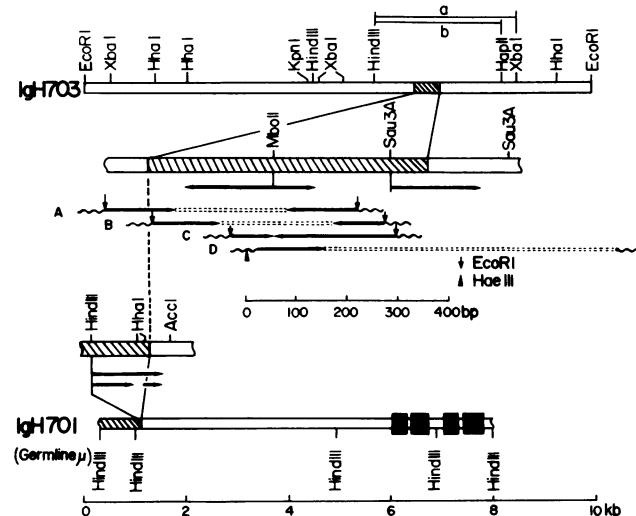

FIG. 3. Strategy for sequencing the germline S_α and S_μ regions surrounding the recombination sites Igγ1-704. A 2.8-kb *HindIII–Xba* I fragment of IgH703 (fragment a), which hybridized most strongly with the rearranged γ1 clone Igγ1-704, was isolated by polyacrylamide gel electrophoresis. The fragment was digested with *Mbo* II or *Sau* III A and labeled at their ends for sequence determination as described (29). Alternatively, subclones of IgH703 containing the portion embedded in Igγ1-704 were obtained. The subclone plasmids were digested with *EcoRI*, and the inserts were isolated by polyacrylamide gel electrophoresis. The *EcoRI* sites of the inserts were labeled with [γ-^{32}P]ATP (Amersham, England), followed by digestion with *Mbo* II. Alternatively, plasmid DNA was digested with *Hae* III, and the largest fragment containing the insert was isolated. The *Hae* III site in pBR322 vector, which is located 18 bp from the *EcoRI* site, was labeled with ^{32}P, followed by the second digestion with the *HindIII* site in pBR322 (26). A 3.8-kb *HindIII* fragment of IgH701 [germline μ-gene clone (7, 32)] was isolated for sequence determination. The range and the restriction sites used in sequence determinations; □, the region present in Igγ1-704; ■, the coding domains.

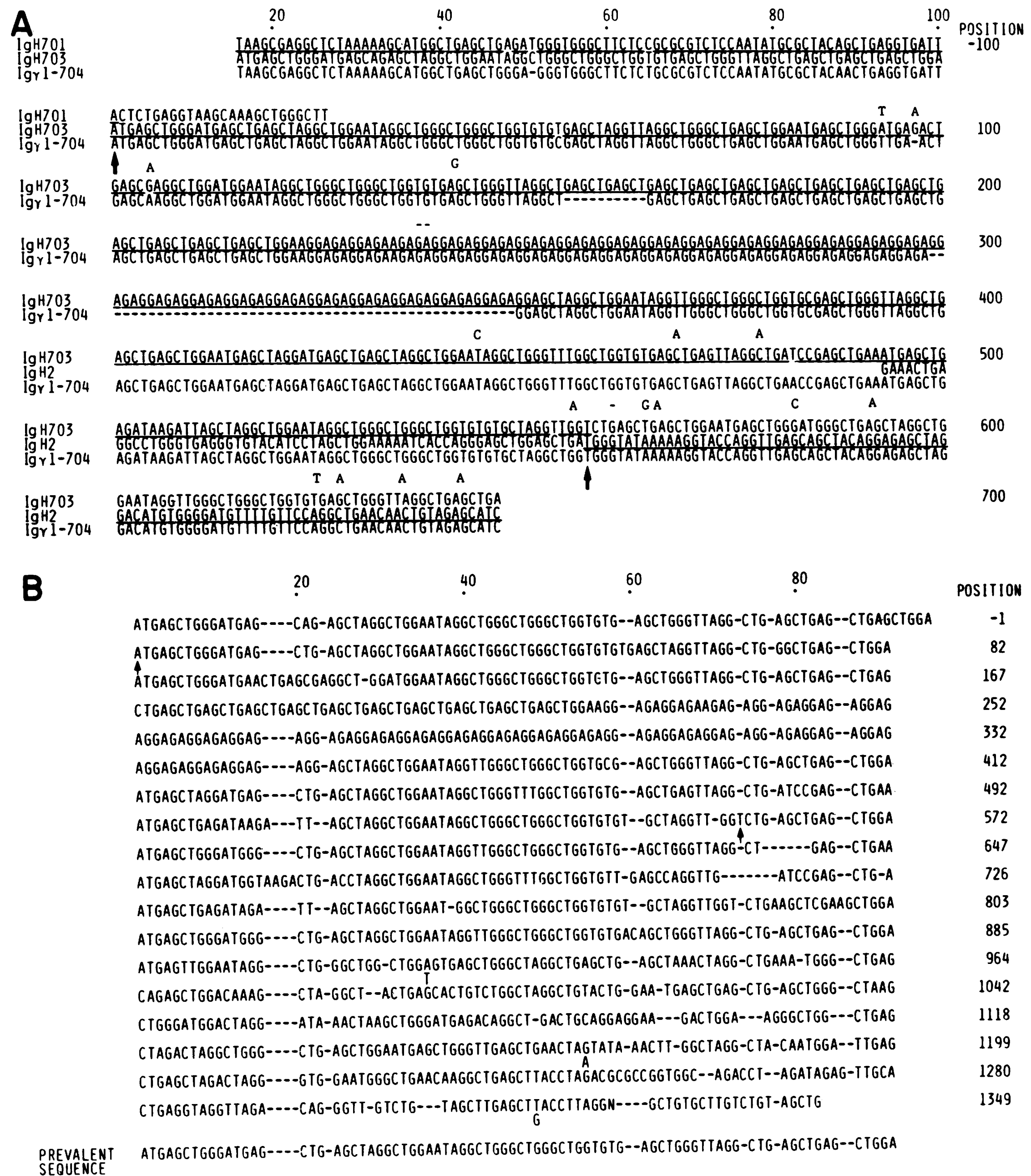

FIG. 4. Comparison of nucleotide sequences surrounding the recombination sites of Igγ1-704 and their germline counterparts. Nucleotide sequences are displayed with the direction of transcription from left to right. (*A*) Underlined sequences are homologous to the expressed γ1 gene (Igγ1-704). The sequence of Igγ1-704 was taken from the data for IgH7 (7) with several corrections. The nucleotides, which are differently assigned by Davis *et al.* (33), are shown in pica letters above the sequence of IgH703. Dashes show deletions. Inserted nucleotides are shown above and between two nucleotides of IgH703. IgH701, the germline μ-gene clone (32); IgH703, the germline α-gene clone; IgH2, the germline γ1-gene clone (7, 30); Igγ1-704, the expressed γ1 gene. (*B*) The sequence of the S_α region (IgH703) is aligned so that the 80-bp repeating units have maximal homology with each other. The 3′ portion (703 bp) of the sequence was taken from the data of Davis *et al.* (33). The position number is as shown in *A*.

(Fig. 1). The location of the V domain was further confirmed by R-loop formation with the middle *Eco*RI fragment (4.9 kb) of Igγ1-704. Such R-loop formation clearly indicates that the clones Igγ1-704 and Igγ1-706 are the expressed γ1 gene in MC 101. The sequence of the V domain (ref. 11; unpublished data) was found to be characteristic of a V_H-framework sequence and the J_{H3} sequence (8).

We have cloned five germline V genes that hybridize with the V-gene segment of Igγ1-704 (unpublished data). The restriction enzyme cleavage map of one of the germline V genes, IgV_H-MC 101-1, is indistinguishable from the 5′ region (4.7 kb) of Igγ1-704 (Fig. 1). As summarized in Fig. 2, Igγ1-704 is composed of at least three germline gene segments: a V_H gene, the μ gene-flanking region (including J_H), and the γ1 gene (including its flanking regions). Furthermore, nucleotide sequence determination of the germline V gene indicates the presence of another segment called "D" (8, 10) between the V gene and the J-region segment (ref. 11; unpublished data).

Presence of the α Gene-Flanking Region in Igγ1-704. The previous analysis (7) suggested that there might be another small germline gene segment between the μ gene-flanking and the γ1 gene-flanking regions. We cloned a possible candidate designated as IgH703 for this germline gene, using a 5′ segment of IgH7 as a probe (7). We have tested the possibility that

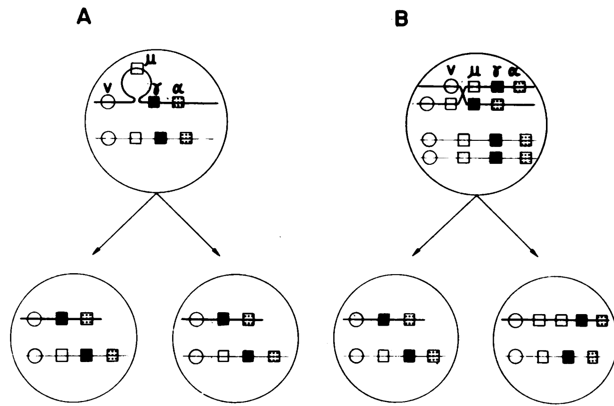

FIG. 5. Possible models for deletion of C_H genes in class switch. (*A*) Looping-out model. (*B*) Sister-chromatid-exchange model.

IgH703 is related to any of the other C_H genes and found that the restriction map of IgH703 looks identical to that of the germline clone of α chain gene (α gene) (6). We have recently cloned a complete α-gene fragment, the 5′ part of which is identical to IgH703 as assayed by restriction-map analysis and Southern blot hybridization (unpublished data). The complete α-gene clone was identified by an α chain-cDNA clone.

The 496-bp S_α Segment Is Located Between the S_μ and $S_{\gamma1}$ Regions. We determined the nucleotide sequences of such regions of the germline μ (IgH701) and α chain (IgH703) gene-flanking segments that are embedded in the expressed γ1 gene of MC 101 (Igγ1-704). These sequences were compared with that of Igγ1-704, which was taken from the data determined for IgH7 (7). The sequence of IgH7 (7) was reexamined and corrected at 16 base pairs (bp). The strategy of sequencing is shown in Fig. 3. Insomuch as we could not find suitable restriction sites in the region of IgH703 that we wished to sequence, we adopted a unique strategy as described that can be applied in general to DNA fragments which do not have suitable restriction sites for sequence determination. These sequences are shown in Fig. 4*A*.

The nucleotide sequence of the 5′ region of Igγ1-704 coincides with that of the germline S_μ region and, abruptly downstream from position 1, begins to agree with that of IgH703. The highly repetitive sequences $(\text{G-A-G-C-T})_2$ and $(\text{G-G-A-G-A})_{10}$ seem to have been deleted from Igγ1-704. Deletion of these repetitive sequences may have taken place either during cloning or propagation of myeloma tumors. The coincidence of the sequences of Igγ1-704 and IgH703 ceases at position 557; thereafter toward the 3′ direction the sequence of Igγ1-704 is identical to that of the γ1 gene-flanking region as has been shown (7). The results unequivocally show that a segment (496 bp) of the S_α region is embedded between the S_μ and $S_{\gamma1}$ regions of the expressed γ1 gene of MC 101.

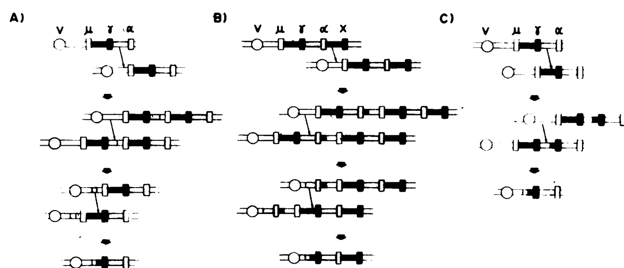

FIG. 6. Possible recombination pathways to and from the expressed γ1 gene of MC 101.

DISCUSSION

Repetitive Sequences in the S_α Region. We have shown that the 5′ flanking regions of the genes of γ1, γ2b, and γ3 chains contain tandem repetition of conserved 49-bp units (unpublished data). These repetitive sequences seem to provide the functionally-defined S region (7) with the unique structural basis. The nucleotide sequence of the 5′ flanking region of the α gene (S_α region) also comprises tandem repetition of relatively conserved 80-bp units as shown in Fig. 4*B*. Davis *et al.* (33) have shown that this portion of the S_α region is actually used for class-switch recombination in two expressed α genes. Their sequences are almost identical to our sequence of IgH703 except for a 10-bp replacement and addition of $(\text{G-G-A-G-G})_2$. Such coincidence of the nucleotide sequences between IgH703 and the α-gene clone of the other group unequivocally confirms that IgH703 is the α-gene clone. They have suggested that the sequence of the S_α region contains an irregularly interspersed repetition of conserved 30-bp units. Their 30-bp unit composes both ends of our 80 bp-unit (Fig. 4*B*). The S_μ region also comprises tandem repetition of conserved 20–30 bp (refs. 8, 9; unpublished data). The prevalent sequences of $S_{\gamma1}$, $S_{\gamma2b}$, $S_{\gamma3}$, and S_α share common short sequences (G-G-G-C-T, G-A-G-C-T, T-G-G-G, etc.) with that of the S_μ region. The results support the idea that the combination of common short sequences and tandem repetitive sequences may play an important role for the recognition of the S–S recombination (9).

Sister-Chromatid-Exchange Model. It is established that the S–S recombination results in the deletion of a DNA segment between two joining sequences (12–18). In addition, the genetic studies using allotype markers have clearly demonstrated that the V_H and C_H genes of a single chromosome are coordinately expressed (i.e., *cis* expression) (34–36). Two alternative models can be proposed to explain the mechanism of the C_H-gene deletion in B lymphocytes as illustrated in Fig. 5. The first model postulates that the S–S recombination takes place on a single chromosome by mutual recognition of two S regions (Fig. 5*A*). The inbetween DNA segment is looped out and lost from the chromosome. This model is referred to as a looping-out model. Such recombination can occur at any stage of the cell cycle in principle. The other model, called "a sister-chromatid-exchange model," explains the deletion of the DNA segment by an unequal crossing-over event between sister chromatids (Fig. 5*B*). According to this model one of the daughter cells contains an additional copy of the C_H gene that is lost in the other daughter cell. Sister-chromatid exchange is unlikely to occur at any other stage of the cell cycle except for the mitotic phase.

It has been shown that a single lymphocyte can give rise to progeny cells synthesizing IgM, IgG, or IgA (37, 38). Inhibition of cell division leads to an increase in the frequency of binucleated cells able to direct synthesis of both IgM and IgG (39, 40). The results suggest that the class switch from IgM to IgG may involve an asymmetric cell division, which is in agreement with the sister-chromatid-exchange model, although they do not necessarily exclude the looping-out model. Because the percentage of cells containing both IgM and IgG relative to cells containing IgG was rather high (10–20%) and increased 2- to 3-fold by inhibition of cell division (40), the switching process appears to take place during cell division, probably during or after replication of DNA.

Implication of the Presence of the S_α Segment in the MC 101 γ1 Gene. It appears as if the presence of the S_α segment between S_μ and $S_{\gamma1}$ segments of a rearranged γ1 gene of MC

101 myeloma (Fig. 3) contradicted the linear arrangement of C_H genes, 5′-μ-γ3-γ1-γ2b-γ2a-α-3′ (12), and the deletion mechanism for the class switch as pointed out by Davis *et al.* (33). However, the generation of such a γ1 gene can be explained by two or three successive unequal crossing-over events. There are various possible pathways to create MC 101 γ1 gene, several examples of which are illustrated in Fig. 6. In one pathway (Fig. 6*A*), the first recombination produces a chromosome with a duplicated segment containing the μ and γ genes. The second crossing-over occurs between the S_μ and S_α regions, resulting in the expression of the μ gene that is linked to a V gene, the S_μ and S_α regions at its 5′ side. The third crossing-over takes place between the S_α and S_γ regions, giving rise to a γ gene that is linked to a V gene, with the S_μ and S_α regions at its 5′ side. In a second pathway (Fig. 6*B*) we postulated another C_H gene at the 3′ side to the α gene. In a third (Fig. 6*C*) we show the shortest pathway.

Inasmuch as we found that the α gene and its 5′ flanking region of MC 101 DNA is indistinguishable from the germline α gene by Southern blot experiments (data not shown), the presence of the S_α segment in Igγ1-704 cannot be explained by translocation of the S_α segment from the α gene of MC 101. Although other explanations by rather rare events such as somatic recombination between homologous chromosomes may be possible, the C_H-gene organization in MC 101 myeloma seems to imply that the sister-chromatid-exchange mechanism operates for switching the H chain class. There are several myelomas whose C_H-gene context appears exceptional to the deletion model. For example, MPC 11 (γ2b chain producer), MOPC 141 (γ2b chain producer) and HOPC 1 (γ2a chain producer) contain the μ gene (13, 14, 17). These apparent exceptions can be explained without difficulty by the sister-chromatid-exchange model, although other mechanisms as described above are again possible.

In contrast to the looping-out model, the sister-chromatid-exchange model allows some lymphocytes to switch in a reverse direction of the C_H-gene order proposed (12) and partially proved (19, 20) that the number of clones switching in the reverse direction may be lower than that switching in the forward direction because the number of the recombinations required for the reverse switch is larger than that for the forward switch. In addition, one of such recombination products could be inviable and, therefore, could not be established among progenies. Nonetheless, Radbruck *et al.* (41) have reported that a variant of myeloma X63 can switch from γ2b to γ1 chain.

The sister-chromatid-exchange model can be directly tested by analyzing the content and context of C_H genes in the progeny of a single B lymphocyte because asymmetric segregation of C_H genes inevitably produces progeny clones with duplicated and deleted C_H genes.

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