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主論文

DELETION OF IMMUNOGLOBULIN HEAVY CHAIN GENES ACCOMPANIES THE CLASS SWITCH REARRANGEMENT

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ABSTRACT

The organization of immunoglobulin heavy chain genes has been investigated by Southern blot hybridization of restriction DNA fragments using cloned $\gamma 1$, $\gamma 2b$ and μ genes as probes. The organization of the heavy chain genes (μ, $\gamma 1$, $\gamma 2b$ and $\gamma 2a$) was compared in newborn mouse (BALB/c) and myelomas producing IgM, IgG3, IgG1, IgG2b, IgG2a and IgA. The $\gamma 1$ gene was present in DNAs from IgM-,IgG3- and IgG1-producing myelomas but absent in DNAs from IgG2b-, IgG2a- and IgA-producing myelomas. The $\gamma 2b$ gene was found in DNAs from IgM-, IgG3, IgG1- and IgG2b-producing myelomas but deleted in DNAs from IgG2a- and IgA-producing myelomas. The $\gamma 2a$ gene was present in DNAs from all the myelomas tested except IgA-producing myelomas. The μ gene was present in DNAs from IgM- and IgG2b-producing myelomas but absent in DNAs from IgG3-, IgG1-, IgG2a and IgA-producing myelomas. The heavy chain genes expressed in most myelomas were rearranged in different forms on different homologous chromosomes. In some myelomas we were unable to show the rearrangement of the expressed heavy chain genes with the restriction enzymes used in the present study. The deletion of the heavy chain genes takes place upstream from the heavy chain gene expressed in each myeloma assuming that the heavy chain genes are arranged in the order μ-$\gamma 3$-$\gamma 1$-$\gamma 2b$-$\gamma 2a$-α. The presence of the μ gene in two $\gamma 2b$-producing myelomas is exceptional. These results are consistent with the allelic deletion model proposed by Honjo and Kataoka (*Proc. Natl. Acad. Sci. USA*, **75**, 2140–2144, 1978) except that the previous model predicted the deletion of the heavy chain genes only from one of the allelic pair of chromosomes. We have discussed possible reasons for the previous overestimation of γ genes using uncloned cDNA.

KEY WORDS deletion of genes / class switch / myelomas / Southern blot / μ, $\gamma 1$, $\gamma 2b$ and $\gamma 2a$ genes

The organization of immunoglobulin genes appears unique in several respects; 1) the variable (V) and constant (C) regions of the immunoglobulin polypeptide are encoded by separate genes in the germline of each organism (3, 14, 19, 20) and 2) these genes are brought together by somatic recombination (1, 11, 15). Furthermore, in a given lymphocyte a C gene of one allele is expressed while the C gene of the other allele is not expressed (allelic exclusion). Dur-

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ing differentiation of a lymphocyte a V region sequence of the heavy (H) chain is successively joined with different C region sequences (class switch). The switch follows the sequence from μ to γ or α.

Previously we showed that the copy numbers of the specific C_H genes in myeloma DNA were reduced depending on the class or subclass of the C_H gene expressed in the myeloma (5). We have proposed the model that the DNA segment locating between a V gene and the C_H gene expressed is excised out of the chromosome to recombine the two genes. Such recombination seemed to occur only on one of the two allelic chromosomes. We have also proposed that H chain genes are aligned on one chromosome in the order V genes, unknown spacer, μ, γ3, γ1, γ2b, γ2a and α. Such alignment together with the successive recombination of V and C_H genes can explain the class switch phenomenon. This model is called allelic deletion model.

Elsewhere we have shown that the deletion of the specific C_H genes takes place only on the expressed member of the allelic pair of chromosomes using a myeloma induced in the F_1 mouse between C57BL and BALB/c which have different allotypes in the H chain C regions (Yaoita and Honjo, submitted). The results are in agreement with the allelic deletion model (5) except that the 5′ flanking region of the μ gene of the unexpressed chromosome is rearranged in DNA from the F_1 myeloma.

To further examine the model we have studied the organization of C_H genes in many myelomas by Southern blot hybridization of restriction DNA fragments using the cloned H chain genes as probes (6–8, 22). The results clearly indicate that the C_H genes are deleted in agreement with the proposed order of the C_H genes (5). The deletion of the C_H genes, however, seems to take place on both chromosomes. We have demonstrated that the sites of the rearrangement are different between an allelic pair of chromosomes. These results can be explained by the successive allelic deletion events on an allelic pair of chromosomes, one on an allele to be expressed and a different form of deletion on the other allele to be unexpressed.

During preparation of this manuscript two other groups published similar experiments using the γ2b probe (12) and the μ and α probes (2). Deletion of the DNA segment locating between two joining sequences was also confirmed in the λ type light chain gene by Sakano *et al.* (13).

MATERIALS AND METHODS

Materials

Restriction endonucleases were purchased from New England Biolabs (Baverly, Massachusetts, U.S.A.) except that Bgl I and II, Bam HI, and Eco RI were purified according to the methods of Greene *et al.* (4) and Yoshimori (24), respectively. *Escherichia coli* DNA polymerase and pancreatic DNase were obtained from Boehringer Mannheim GmbH (Mannheim, West Germany). [α-^{32}P]TTP (specific radioactivity, 2000–3000 ci/mmole) was purchased from The Radiochemical Centre (Amersham, England).

Myelomas

Myeloma tumor lines were supplied by Dr M. Potter (NIH), Dr T. Kishimoto (Osaka University), Dr S. Migita (Kanazawa University) and Dr P. Leder (NIH) and propagated by subcutaneous injection as described by Sibinovic *et al.* (16). Solid tumors were harvested, immediately frozen in liquid N_2, and stored at −80°C. Female BALB/c and male C57BL/6 were mated to produce F_1 mice. MOPC 320 were propagated in F_1 mice. Proteins produced by myelomas were described previously (16). MOPC 315 was induced in F_1 mice between C57BL and BALB/c and shown to produce IgA of BALB/c allotype (16).

Preparation of DNA

Whole newborn mice, mouse livers or myeloma tumors were homogenized in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.15 M NaCl with a Potter homogenizer at 0°C. Sodium dodecylsulfate to 0.3% and $NaClO_4$ to 0.5 M were added to a whole homogenate. The homogenate was extracted with 0.5 volume each of water-saturated phenol and a chloroform-isoamyl alcohol mixture (24 : 1). The water phase was separated by centrifugation and 1.2 g of CsCl was added to one ml of the water phase. After centrifugation at 30,000 rpm for 48 hr, fractions with high viscosity were collected and dialyzed against 0.1×SSC (1×SSC: 0.15 M NaCl, 0.015 M Na citrate) for 48 hr. Alternatively, the water phase was dialyzed against 0.1×SSC for 24 hr, digested with RNase (100 μg/ml) in a dialysis bag for 24 hr at room temperature and then extracted with phenol. After dialysis against 0.1 × SSC for 24 hr, 1.27 g of CsCl was added to one ml of the water phase and DNA was banded by centrifugation and collected as

described above. DNA concentration was calculated assuming that one mg/ml DNA gives 20 A_{260}. The contamination of RNA was negligible when checked by agarose gel electrophoresis.

Southern Blotting

DNA (5–15 μg) was digested with 15–50 units of various restriction enzymes at their optimal conditions for 7 hr at 37C°. Digested DNA was directly applied to an 0.5% agarose (type I, Sigma Chemical Co., St. Louis, Missouri, U.S.A.) gel and electrophoresed. Bacteriophage λ DNA digested with Eco RI or Hind III was included in the gel as size standards. The gel was stained with ethidium bromide and DNA was transferred to a nitrocellulose filter (Schleicher and Schuell, Inc., Keene, New Hampshire, U.S.A.) according to the method of Southern (17).

Hybridization

DNA fixed on filters was hybridized with [^{32}P] labeled probes indicated as described previously (6). The concentration of probes and the time of the incubation were determined to reach a Cot value for 90% annealing. After hybridization filters were rinsed with 2×SSC and then washed in 0.1×SSC -0.1% SDS (four times) for 40 min each at 65°C. Filters were finally rinsed with 2×SSC, dried and exposed to X-ray film using a DuPont Cronex lightening plus intensifier screen at −80°C.

Hybridization Probes

We used cloned genomic DNA fragments IgH2 (6), IgH22 (22) and IgH701 (8; T. Kawakami and T. Honjo, unpublished data) as the γ1, γ2b and μ gene probes, respectively. Complete nucleotide sequences of these clones were determined. γ2a chain cDNA cloned in a bacterial plasmid pBR322 (pG2a-14) was used as the γ2a chain probe (T. Honjo, unpublished data). The pG2a-14 contains about 200-base pair long cDNA. Fragments of the genomic clones (indicated in Fig. 1) and pG2a-14 were labeled with [α-^{32}P]TTP by nick translation (10). The specific activities of the probes were 150–250 cpm/pg.

RESULTS

Restriction Maps of the γ1, γ2b and μ Genes and Probes

The Eco RI fragments containing the γ1, γ2b and μ genes have been cloned from DNA of newborn mouse (BALB/c) and designated as IgH2 (6.6 kb), IgH22 (6.6 kb) and IgH701 (12.8 kb), respectively (6–8, 22). Detailed maps of restriction endonuclease cleavage sites within

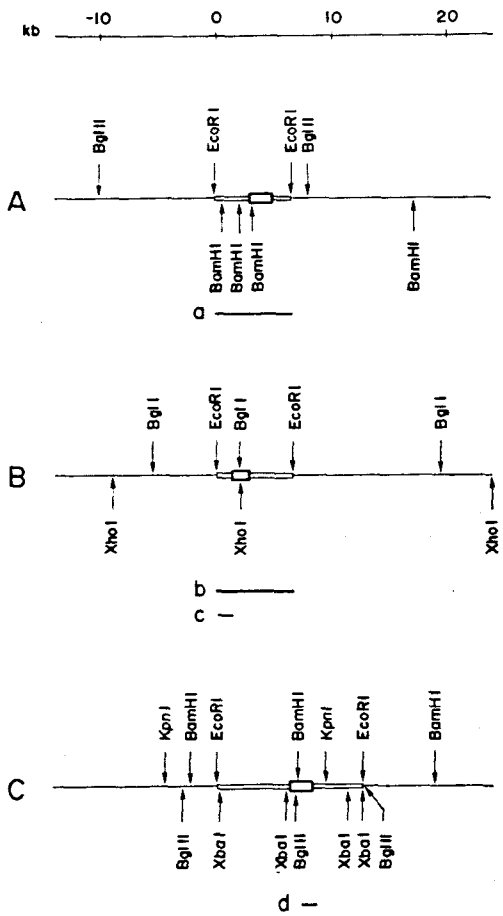

Fig. 1 Restriction enzyme cleavage sites around the γ1, γ2b and μ genes. DNA is displayed from left to right with the direction of transcription. Narrower rectangles show the cloned DNA fragments. Wider rectangles show locations of structural genes. A, γ1 gene and its flanking region. A Bam HI site 5′ to the cloned fragment is not known; B, γ2b gene and its flanking region; C, μ gene and its flanking region. DNA fragments used for probes are indicated by horizontal bars below the restriction maps; a, 6.6 kb Eco RI fragment containing the γ1 gene (IgH2); b, 6.6 kb Eco RI fragment containing the γ2b gene (IgH22); c, 1.35 kb Bam HI fragment (5′ fragment) of IgH22; d, 1.2 kb Hind III fragment that contains the CH3 and CF4 domains of the μ gene (8).

these clones and complete nucleotide sequences of the structural portions have been determined (6, 22; T. Kawakami and T. Honjo, submitted). We have determined restriction sites of Bam HI and Bgl II adjacent to the cloned $\gamma 1$ gene fragment by digestion of mouse DNA with several combinations of two restriction enzymes, followed by Southern blot hybridization of restriction DNA fragments (Fig. 1A). Similarly, restriction sites of Bgl I and Xho I adjacent to the cloned $\gamma 2b$ gene fragment (Fig. 1B) and restriction sites of Kpn I, Bgl II and Bam HI adjacent to the cloned μ gene fragment (Fig. 1C) were determined. We used the Eco RI fragment of the $\gamma 1$ gene (probe a), the Eco RI fragment of the $\gamma 2b$ gene (probe b), the 1.35 kb Bam HI fragment of the $\gamma 2b$ gene clone (probe c) and the 1.2 kb Hind III fragment of the μ gene clone (probe d) as the probes for the $\gamma 1$, $\gamma 2b$-$\gamma 2a$, 5′ $\gamma 2b$ and μ genes, respectively (Fig. 1).

Deletion and Rearrangements of the $\gamma 1$ Gene

BALB/c myeloma and newborn mouse DNAs were digested with Eco RI, Bam HI or Bgl II, and $\gamma 1$ gene fragments were detected by Southern blot hybridizaion. As shown in Fig. 2, Eco RI digestion of newborn mouse DNA produced a dark 6.6 kb band which is equivalent to the cloned $\gamma 1$ gene fragment (IgH 2). Similarly, digestion with Bam HI produced three fragments (14, 1.5 and 1.3 kb) of the $\gamma 1$ gene in agreement with the restriction map shown in Fig. 1. Digestion of newborn mouse DNA with Bgl II produced a dark band (18 kb) of the $\gamma 1$ gene. The $\gamma 1$ gene fragments characteristic of

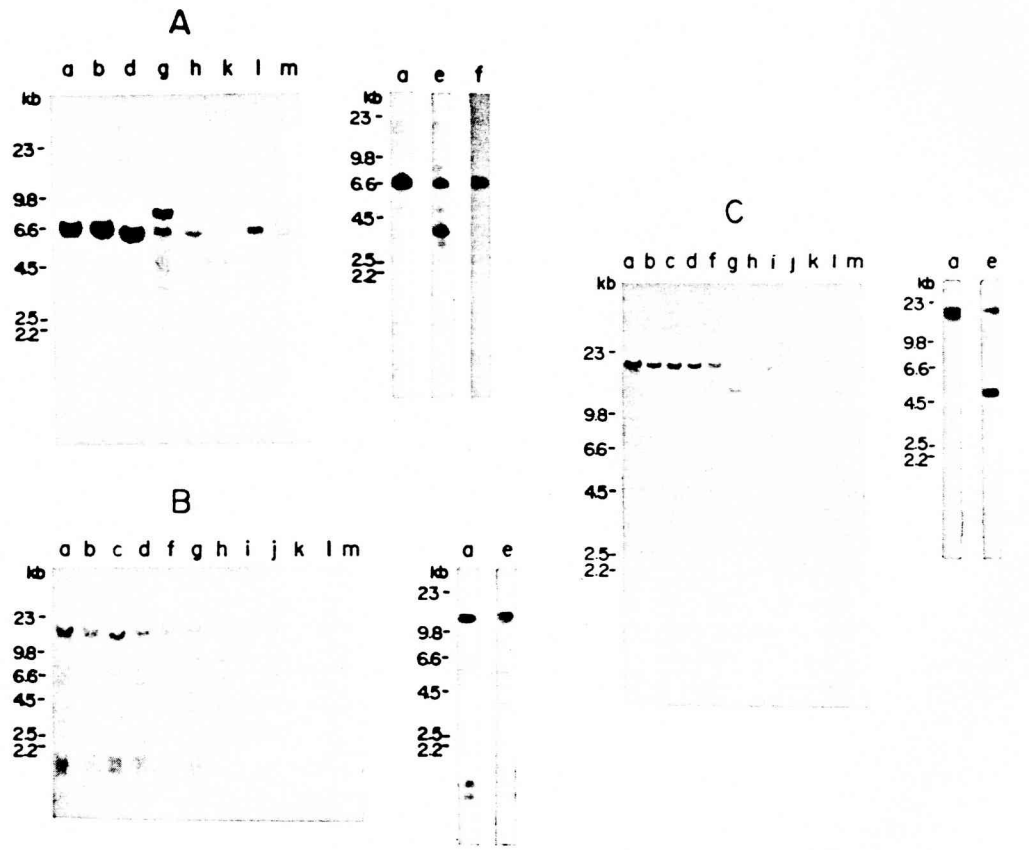

Fig. 2 Hybridization of myeloma DNAs with the $\gamma 1$ gene probe. DNAs (15 μg) of various myelomas were digested with Eco RI (A), Bam HI (B) or Bgl II (C) and the $\gamma 1$ gene fragments were detected by Southern blot hybridization using the $\gamma 1$ gene probe (the probe a, Fig. 1). Origins of DNA are as follows (The class of H chain produced is shown in parentheses.): a, newborn mouse; b, MOPC 104E (μ); c, ABPC 22 (μ); d, FLOPC 21 ($\gamma 3$); e, MOPC 31C ($\gamma 1$); f, MOPC 70A ($\gamma 1$); g, MC 101 ($\gamma 1$); h, MPC 11 ($\gamma 2b$); i, MOPC 141 ($\gamma 2b$); j, MPC 11 ($\gamma 2b$); k, RPC 5 ($\gamma 2a$); l, MOPC 511 (α); m, TEPC 15 (α)

newborn mouse DNA were observed when DNAs of μ and $\gamma 3$ chain-producing myelomas were digested with Eco RI, Bam HI or Bgl II and hybridized to the $\gamma 1$ gene probe (Fig. 2, lanes b, c, d).

On the other hand, extra bands of 8.3 kb and 4.0 kb were observed in addition to the 6.6 kb band when DNAs of $\gamma 1$ chain-producing myelomas MC 101 and MOPC 31C, respectively, were digested with Eco RI (Fig. 2A, lanes e, g). The 8.3 kb band of MC 101 DNA digested with Eco RI was cloned and its nucleotide sequence was determined, showing that the 8.3 kb fragment has a new DNA segment (2 kb) in place of the 5'-terminal 300 base pair segment of the $\gamma 1$ gene fragment cloned from newborn mouse DNA (8). Digestion of MC 101 and MOPC 31C DNAs with Bam HI produced the same band as found in newborn mouse DNA (Fig. 2B, lanes e, g). Digestion of MOPC 31C DNA with Bgl II produced a new fragment of 5 kb in addition to the fragment characteristic of newborn mouse DNA (Fig. 2C, lane e). Digestion of MC 101 DNA with Bgl II produced a single fragment of 13.5 kb (Fig. 2C, lane g). These results indicate that the $\gamma 1$ genes of two chromosomes underwent different types of rearrangements in MOPC 31C and MC 101 myelomas. MOPC 70A (another $\gamma 1$ chain producer), when digested with Eco RI, Bam HI or Bgl II, yielded the $\gamma 1$ gene fragments characteristic of newborn mouse DNA (Fig. 2, lane f). We cannot exclude the possibility that the $\gamma 1$ gene of MOPC 70A DNA has a rearrangement that we were unable to find.

When DNAs of $\gamma 2b$, $\gamma 2a$ and α chain-producing myelomas were digested with Eco RI, Bam HI or Bgl II, only faint bands characteristic of newborn mouse DNA were observed (Fig. 2, lanes h, i, j, k, l, m). These faint bands are presumably ascribed to the DNA of the host animal in which these myelomas were grown, because the faint bands are characteristic of the host cells rather than the myeloma cells (Fig. 3). Bgl II digestion can distinguish the $\gamma 1$ genes of the BALB/c and C57BL alleles (Fig. 3, lanes 1 and 2). DNA from MOPC 315 myeloma (IgA producer induced in a F_1 mouse between BALB/c and C57BL) which was grown in BALB/c mice yielded the faint $\gamma 1$ gene fragment of the BALB/c allele (Fig. 3, lane 3). DNA from MOPC 320 myeloma (IgA producer induced in a C57BL mouse) which was grown in F_1 mice between BALB/c and C57BL yielded the faint $\gamma 1$ gene fragments of both C57BL and BALB/c alleles with equal intensity (Fig. 3, lane 4). These results indicate that $\gamma 1$ genes are deleted from both chromosomes of $\gamma 2b$, $\gamma 2a$ and α chain-producing myelomas.

Deletion and Rearrangements of the $\gamma 2b$ and $\gamma 2a$ Genes

Similar experiments were done using the $\gamma 2b$-$\gamma 2a$ gene probe (probe b) which hybridized with not only the $\gamma 2b$ but also $\gamma 2a$ gene (21). As shown in Fig. 4, Eco RI digestion of newborn mouse DNA produced a weak 23 kb band that corresponds to the $\gamma 2a$ gene fragment (12, Yaoita and Honjo, submitted) and a dark 6.6 kb

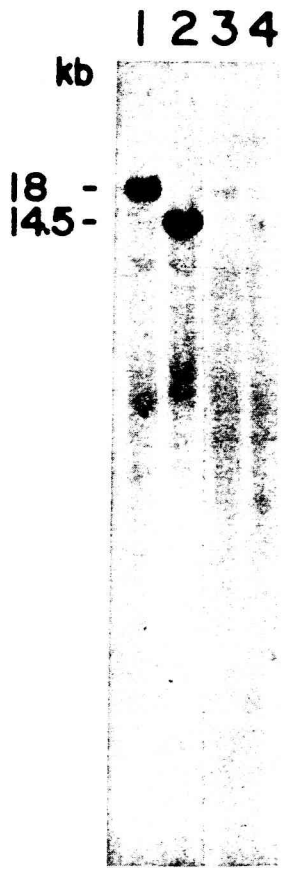

Fig. 3 Hybridization of IgA producing myeloma DNAs with the $\gamma 1$ gene probe. DNA (30 μg) was digested with Bgl II and hybridized with the $\gamma 1$ gene probe (probe a, Fig. 1). Origins of DNA are indicated on each column as follows (the classes of H chains produced are shown in parentheses): 1, BALB/c liver; 2, C57 BL/6 liver; 3, MOPC 315 myeloma (α); 4, MOPC 320 myeloma (α)

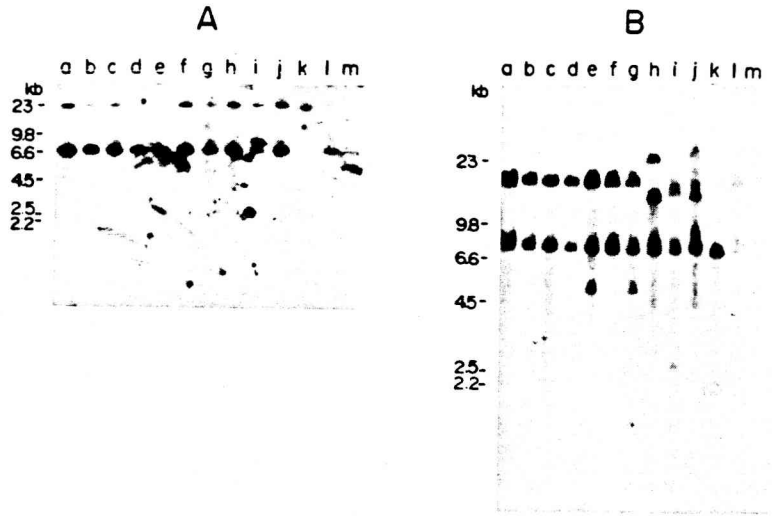

Fig. 4 Hybridization of myeloma DNAs with the γ2b-γ2a gene probe. DNAs (15 μg) of various myelomas were digested with Eco RI (A) or Bgl I (B), and the γ2b and γ2a gene fragments were detected by Southern blot hybridization using the γ2b-γ2a gene probe (probe b, Fig. 1). Origins of DNA are indicated on each column by the alphabet as described in the legend to Fig. 2.

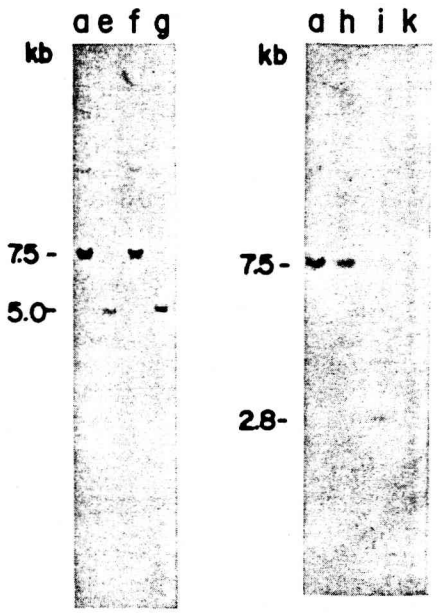

Fig. 5 Hybridization of myeloma DNAs with the 5′ γ2b gene probe. DNA (5 μg) was digested with Bgl I and hybridized with the 5′ γ2b gene probe (probe c, Fig. 1). Origins of DNA are indicated on each column by the alphabet as described in the legend to Fig. 2.

band that is equivalent to the cloned γ2b gene fragment (Fig. 4A, lane a). Digestion with Bgl I produced two fragments of 17.5 kb and 7.5 kb which correspond to the 3′ and 5′ portions of the γ2b gene, respectively (Fig. 4B, lane a). The 7.5 kb band also contains the γ2a gene fragment (data not shown).

When DNAs of μ and γ3 chain-producing myelomas were digested with Eco RI or Bgl I and hybridized to the γ2b-γ2a gene probe, fragments characteristic of newborn mouse DNA were observed (Fig. 4, b, c, d). As shown in Fig. 4B (lanes e, g) Bgl I-digests of MOPC 31C and MC 101 DNAs contained an additional band (5 kb) that hybridized with the 5′ γ2b gene probe (Fig. 5, lanes e, g). These rearrangements are due to the deletion of a 2.5 kb segment (T. Kataoka and T. Honjo, unpublished data).

Rearrangements of the γ2b gene were also observed in DNAs from the γ2b chain-producing myelomas, MOPC 141 and MPC 11. When digested with Eco RI, MOPC 141 DNA produced a new band of 7.8 kb in addition to the 6.6 kb band (Fig. 4A, lane i). Recently we have cloned the 7.8 kb fragment of MOPC 141 DNA and shown that the cloned fragment is the rearranged γ2b gene which has a new DNA segment flanking the 5′ end of the μ gene at the 5′ end of the γ2b gene cloned from newborn mouse DNA (N. Takahashi, T. Kataoka and T. Honjo, submitted). The 6.6 kb band was always less intense than the 7.8 kb band which was of

about half an intensity of the 6.6 kb band observed in newborn mouse DNA. We have drawn a tentative conclusion that the 6.6 kb band of MOPC 141 DNA is due to contaminant host cell DNA and the γ2b gene is deleted from one of an allelic pair of chromosomes of MOPC 141. When digested with Bgl I, the 3′ γ2b gene fragment (17.5 kb) observed in newborn mouse DNA disappeared and a pair of new fragments appeared in DNAs of MOPC 141 (15.5 and 2.8 kb) and MPC 11 (25 and 14.5 kb) (Fig. 4B, lanes h, i, j). As shown n Fig. 5 (lanes h, i), the 5′ γ2b gene probe hybridized to the 2.8 kb fragment of MOPC 141 DNA and the 7.5 kb fragment of MPC 11 DNA. Therefore, the 15.5 kb and 2.8 kb fragments of MOPC 141 are the 3′ and 5′ fragments of the γ2b gene, respectively. The 7.5 kb fragment of MOPC 141 DNA seems to be the γ2a gene. The two new fragments (25 and 14.5 kb) of MPC 11 DNA seem to be the 3′ fragments of the γ2b genes of two homologous chromosomes. These results indicate that the γ2b genes of MOPC 141 and MPC 11 DNAs are rearranged in different forms on an allelic pair of chromosomes and that such rearrangements have taken place not only at their 5′ regions of the γ2b gene but also at their 3′ regions.

When DNA of a γ2a chain-producing myeloma RPC 5 was digested with Eco RI or Bgl I and hybridized with the γ2b-γ2a probe, the γ2b gene bands were not observed and only the 23 kb Eco RI-fragment and the 7.5 kb Bgl I-fragment were observed (Fig. 4, lane k). These bands are shown to be the γ2a gene fragments using the γ2a cDNA cloned in a plasmid as a probe (data not shown). Hybridization with the 5′ γ2b gene probe (probe c) indicates that the γ2b gene is deleted from RPC5 DNA (Fig. 5, lane k). Digestion of α chain-producing myeloma DNAs with the two enzymes yielded only the faint bands characteristic of newborn mouse DNA (γ2b-γ2a probe), indicating that the γ2b gene is missing in α chain-producing myelomas (Fig. 4, lanes l, m). Furthermore, it is evident that the γ2a gene is present in μ, γ3, γ1, γ2b and γ2a chain-producing myelomas whereas α chain-producing myelomas have lost the γ2a genes from both alleles. The above conclusion was also supported by the experiments with Xho I (data not shown).

Deletion and Rearrangements of the μ Gene

When digested with Eco RI, the newborn mouse DNA produced a 12.8 kb fragment that hybridized with the μ gene probe (probe d) as shown in Fig. 6A, lane a. Digestion of newborn mouse DNA with Kpn I and Bam HI yielded 13.5 and 12 kb fragments, respectively, which hybridized to the μ gene probe (Fig. 6B and 6C, lane a). DNAs of all the three μ chain-producing myelomas (MOPC 104E, ABPC 22 and TEPC 183) produced rearranged μ gene fragments when digested with either Eco RI or Kpn I (Fig. 6A and 6B, lanes b, c and n). Digestion of MOPC 104E DNA with Eco RI and Kpn I produced 11 and 14 kb fragments of the μ gene, respectively. The intensity of each band seems to be reduced to about a half of the bands observed in newborn mouse DNA, suggesting that MOPC 104E DNA may have lost the μ gene from one of an allelic pair of chromosomes. MOPC 104E DNA has a rearrangement at the 5′ region to the μ gene of the remaining allele since the Kpn I fragment comprises DNA locating to the 5′ side of the μ gene (see restriction map, Fig. 1C). The 3′ region seems to be intact because the Bam HI fragment remains unchanged (Fig. 6C, lane b). When ABPC 22 DNA was digested with Eco RIor Bam HI, fragments characteristic of newborn mouse DNA were observed but the intensity of each band is reduced to about a half of the band observed in newborn mouse DNA. The results suggest that ABPC 22 DNA may have lost the μ gene from one of an allelic pair of chromosomes (Fig. 6A and 6C, lane c). Digestion of ABPC 22 DNA with Kpn I produced new μ gene fragment of 16 kb (Fig. 6B, lane c). When TEPC 183 DNA was digested with Eco RI, a pair of new μ gene fragments (11 and 7.7 kb) were observed in addition to the fragment characteristic of newborn mouse DNA, indicating that the μ gene on one allele of TEPC 183 DNA has a rearrangement within the Hind III fragment used as a probe. Digestion with Kpn I produced a pair of new μ gene fragments of 16 kb and 10.5 kb, indicating the μ gene on the other allele is also rearranged. Digestion with Bam HI produced the fragment characteristic of newborn mouse DNA. Since DNAs from the three myelomas have the rearranged Kpn I fragments and the intact Bam HI fragments, the rearrangement must lie to the 5′ side of the μ gene. Rearrangements took different forms between an allelic pair of chromosomes.

DNAs from the other myelomas tested except for MPC 11 and MOPC 141 contained only faint bands characteristic of newborn mouse DNA, which were presumably derived from

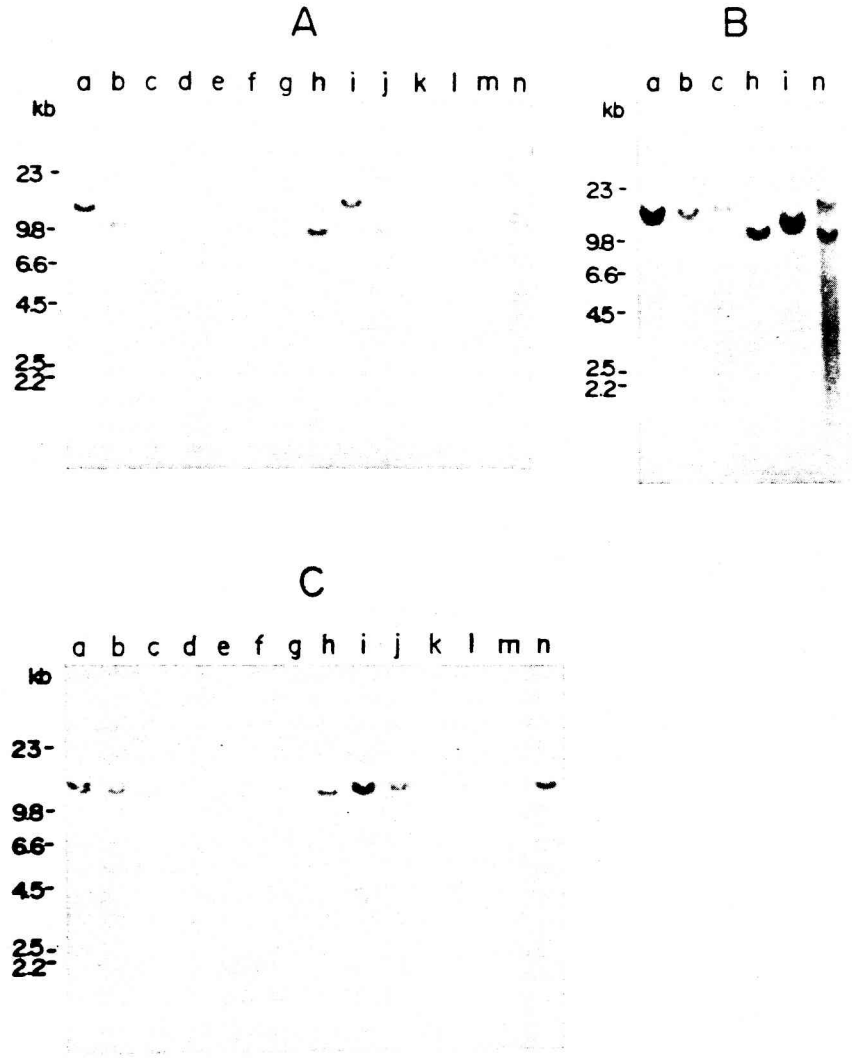

Fig. 6 Hybridization of myeloma DNAs with the μ gene probe. DNAs (5 μg) of various myelomas were digested with Eco RI (A), Kpn I (B), or Bam HI (C) and the μ gene fragments were detected by Southern blot hybridization using the μ gene probe (probe d, Fig. 1). Origins of DNA are indicated on each column by the alphabet as described in the legend to Fig. 2 except for n, TEPC 183 (μ chain producer).

host cell DNA. Unexpectedly, DNAs of γ2b chain-producing myelomas MPC 11 and MOPC 141 contained the μ gene fragments that had been rearranged at the region flanking the 5′ end of the μ gene.

Summary of Deletions and Rearrangements in BALB/c Myelomas

The results mentioned above are summarized in Table 1. The μ chain-producing tumors contain all the γ genes tested as well as the μ gene which has different forms of rearrangements on an allelic pair of chromosomes. The γ3 and γ1 chain-producing tumors contain the γ1, γ2b and γ2a genes whereas they have lost the μ genes from both alleles. We have not tested whether or not the γ3 gene is rearranged. In the γ1 chain-producing myelomas except for MOPC 70A the γ1 genes on two alleles have different forms of rearrangements. The 5′ side of the γ2b gene is rearranged in the γ1

Table 1 *Restriction DNA Fragments of μ, γ1, γ2b and γ2a Genes in Myelomas*

Origin of DNA	μ			γ1			γ2b		γ2a	
	EcoRI	KpnI	BamHI	EcoRI	BamHI	BglII	EcoRI	BglI	EcoRI	BglI
a) Newborn mouse	12.8 / 12.8	13.5 / 13.5	12 / 12	6.6 / 6.6	14, 1.5, 1.3 / 14, 1.5, 1.3	18 / 18	6.6 / 6.6	17.5, 7.5 / 17.5, 7.5	23 / 23	7.5 / 7.5
b) MOPC104E	11/D	14/D	E/D	E/E	E/E	E/E	E/E	E/E	E/E	E/E
c) ABPC22	E/D	15/D	E/D	—	E/E	E/E	E/E	E/E	E/E	E/E
n) TEPC183	11, 7.7 / E	16 / 10.5	E/E	—	—	—	—	—	—	—
d) FLOPC21	D/D	D/D	D/D	E/E	E/E	E/E	E/E	E/E	E/E	E/E
e) MOPC31C	D/D	D/D	D/D	4.0/E	E/E	5/E	E/E	17.5, 5 / 17.5, 5	E/E	E/E
f) MOPC70A	D/D	D/D	D/D	E/E	E/E	E/E	E/E	E/E	E/E	E/E
g) MC101	D/D	D/D	D/D	8.3/E	E/E	13.5/13.5	E/E	17.5, 5 / 17.5, 5	E/E	E/E
h, j) MPC11	10/D	11/D	E/D	D/D	D/D	D/D	E/E	25, 7.5 / 14.5, 7.5	E/E	E/E
i) MOPC141	13.5 / 13.5	12/12	E/E	—	D/D	D/D	7.8/D	15.5, 2.8 / D	E/E	E/E
k) RPC5	D/D	D/D	D/D	D/D	D/D	D/D	D/D	D/D	E/E	E/E
l) MOPC511	D/D	D/D	D/D	D/D	D/D	D/D	D/D	D/D	D/D	D/D
m) TEPC15	D/D	D/D	D/D	D/D	D/D	D/D	D/D	D/D	D/D	D/D

Restriction DNA fragments of two alleles are shown in kilobase. E, embryonic form or the same as newborn mouse DNA. D, deletion

chain-producing myelomas. We did not find any rearrangement of the γ1 gene in DNA from MOPC 70A. The γ2b chain-producing myelomas have the γ2a genes, the rearranged γ2b and μ genes. They have lost the γ1 genes completely. The γ2a chain-producing myeloma contains the γ2a genes, whereas the myeloma does not have the μ, γ1 and γ2b genes at all. The α chain-producing tumors have lost all of the genes tested completely.

Aside from the presence of the μ gene in γ2b chain-producing myelomas, the deletion profile of C_H genes in myelomas is in agreement with the order of the C_H genes proposed previously (5). The expressed genes in myeloma cells seem to be rearranged in different manners on an allelic pair of chromosomes. The C_H genes that lie to the 5′ side of the expressed gene seem to be deleted from both chromosomes. The chromosomes of the representative myeloma of each class were schematically shown in Fig. 7.

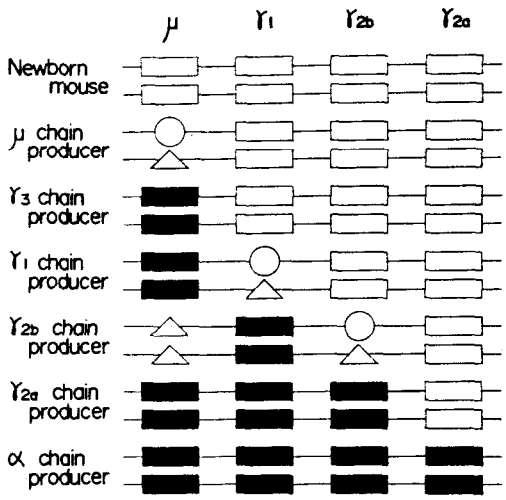

Fig. 7 Summary of the C_H gene deletions in various myelomas. Typical deletion profiles are represented schematically. Each line shows one allele. Open and closed rectangles indicate 'intact' and deleted genes, respectively. The word 'intact' simply means that the change is not detected in the present study. Circles indicate rearranged genes. Triangles indicate rearranged or deleted genes. The γ3 gene that locates between the μ and γ1 genes is omitted.

DISCUSSION

Deletion of C_H Genes

In many BALB/c myelomas the expressed C_H genes of two alleles have undergone different forms of rearrangements, resulting in the dele-

tion of the C_H genes locating left to the exressed gene. The C_H genes are deleted from both alleles with the order predicted by our model (5) except for the presence of the μ gene in γ2b chain-producing myelomas, which will be discussed later. Similar conclusions were drawn by other investigators (2, 12). In some myelomas the expressed C_H gene on one of the homologous chromosomes seems to be deleted. Elsewhere we have reported that a γ2b chain-producing myeloma (BKC F_1#15) induced in a F_1 mouse has deleted the μ and γ1 genes only from the expressed member of an allelic pair of chromosomes (Y. Yaoita and T. Honjo, submitted). All the results including BKC F_1 #15 and BALB/c myelomas can be explained by two successive allelic deletion events; the one occurs on the allele that is to be expressed and the other one may occur on the other allele that is to be unexpressed.

It is possible that the allelic deletion on the unexpressed allele is induced so that rearrangement inactivates the C_H genes on that allele. Alternatively the deletion on the unexpressed allele could be due to aberrant genetic events peculiar to myelomas. Several forms of rearranged or mutated κ type L chain genes were isolated from κ chain-producing myeloma (9, 18). It is well known that myeloma cells have variety of chromosomal abnormalities (23). Such abnormalities may have been accumulated during serial transfer and propagation of myeloma tumors since their induction. It may be relevant to this assumption that BKC F_1 #15 had been propagated *in vivo* only for 3 years before frozen, while most of BALB/c myelomas had been *in vivo* for 6–17 years before frozen (16). Studies on fresh lymphocytes are requir ed to examine whether or not deletion of C_H genes is confined to one allele. It is also possible that a C_H gene on the other allele may be expressed in rare case.

Exception to Deletion Model

We found several myelomas in which the pattern of the C_H gene deletion cannot be explained by the proposed order of the C_H genes. For example, two γ2b chain-producing myelomas out of three retain the μ gene but delete the γ1 gene. MPC 11 DNA also contains another γ2a-like gene which is rearranged (Y. Yaoita and T. Honjo, unpublished data). There are several possibilities to explain these exceptions. The first is chromosomal abnormalities often found in myelomas (23). For example, MPC 11 and MOPC 141 myelomas may have extra chromosomes that contain the μ gene but not the γ1 and γ2b genes. Second, the μ and γ1 genes once excised out of the chromosome may be incorporated into another chromosome. Since the 5′ flanking region of the μ gene is directly linked to the 5′ flanking region of the γ2b gene in the rearranged γ2b gene from MOPC 141 DNA (N. Takahashi, T. Kataoka and T. Honjo, submitted), the μ gene is likely to be removed from the expressed member of the allelic pair of chromosomes in MOPC 141 myeloma. Third, the μ gene was never excised out of the chromosome and the V genes of MPC 11 and MOPC 141 were transferred to the proximity of the γ2b gene by the mechanism different from the deletion. The last possibility can be tested by cloning large fragments of DNA, containing several γ genes.

Background due to Contaminant Host Cells

We have interpreted the faint bands observed in myeloma DNA, which are characteristic of newborn mouse (BALB/c) DNA, are ascribed to contaminant DNA derived from host cells. This interpretation is based not only on the low intensity of the band but also on the fact that we observed faint bands of identical intensity which were derived from both C57BL and BA LB/c alleles when a C57BL myeloma was grown in F_1 mice between C57BL and BALB/c (Fig. 3). Inversely, we observed a faint band of the BALB/c allele alone when a F_1 myeloma MOPC 315 was grown in BALB/c mice (Fig. 3). A significant 6.6 kb band observed in Eco RI-digested DNA is partly due to cross-hybridization of the γ gene probe (21). we have recently cloned several 6.6 kb-long Eco RI fragments which have variable extents of homology with either γ1 or γ2b genes (T. Kataoka, N. Takahashi and T. Honjo, unpublished data).

Possible Reasons for Overestimation of γ Genes

When the γ genes were quantitated previously by hybridization kinetic analysis using uncloned cDNA (5), we detected at least 0.4 copy of the γ genes per haploid for those myeloma DNAs which are now shown to have lost the γ genes completely. We also detected 0.9–1.4 copies of the γ genes for those DNAs which seem to contain one copy per haploid. These overestimation of the γ genes may be ascribed to several factors; 1) contaminant host cell

DNA, 2) cross-hybridization of the γ gene probes, 3) contribution of V region sequence because of the shorter 3′ untranslated sequence than expected and 4) impurity of the cDNA probes employed.

Critical Evaluation of Southern Blotting Data

In order to interpret Southern blotting data properly we have done several control experiments. It is well known that the efficiency of DNA transfer is lower for larger DNA fragments, especially larger than 20 kb. It is also difficult to quantitate the copy number of the gene by the intensity of the bands. As shown in Fig. 8A, we can detect such a tiny amount of the γ1 gene (6.6 kb) as 1.8 pg in one lane. Assuming 15 μg of DNA is employed, the above amount is equal to 0.05 copy per haploid genome. We have shown that we can distinguish the difference of one copy per haploid from two copies on the same blot although our conclusion does not depend on the precise quantitation of the gene copy. We have also shown that our technique can easily detect one copy of a fragment of DNA as large as 45 kb (Fig. 8B). Furthermore, we used at least three types of restriction enzymes to avoid the chance that the restriction fragment containing the C_H genes is too large to be detected by Southern blotting.

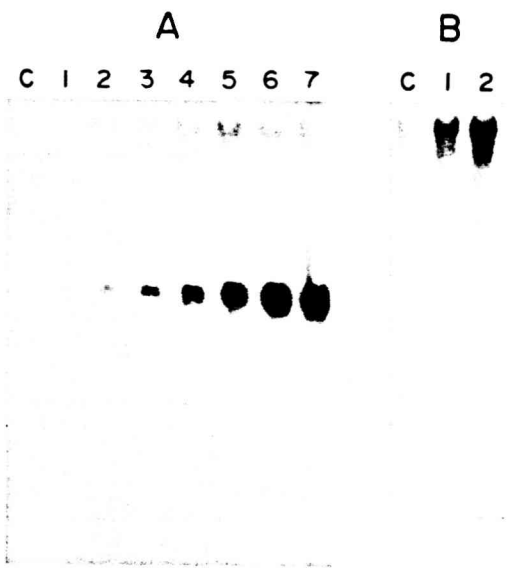

Fig. 8 Hybridization of λgtWES·IgH2 and its insert with the γ1 gene probe. Various amounts of λgtWES·IgH2 (B) or its 6.6 kb insert (A) were mixed with a constant amount (15 μg) of *Escherichia coli* DNA digested with Eco RI. electrophoresed and detected by Southern blot hybridization using the probe a of Fig. 1. The amounts of DNA used are: A: c, 0 pg; 1, 1.8 pg; 2, 3.6 pg: 3, 7. 2 pg; 4, 18 pg; 5, 36 pg; 6, 72 pg; 7, 144 pg. B: c, 0 pg; 1, 250 pg; 2, 500 pg

Rearrangements at the 3′ Portions of the Expressed Genes

We found that the expressed gene had rearrangements not only at its 5′ side but also at its 3′ side in some myelomas (MOPC 31C, MC 101, MOPC 141, MPC 11 and BKC F_1 #15). Rearrangements at the 3′ side also took different forms between two alleles (MPC 11). MO PC 31C and MC 101 DNAs (γ1 chain producers) have rearrangements at the 5′ region to the γ2b gene which, we interpret, corresponds to the 3′ region to the γ1 gene according to the proposed order of the C_H genes (5). Our preliminary study indicates that the rearrangements of MOPC 31C and MC101 DNAs are due to deletion of S region (8) locating to the 5′ side of the γ2b gene (T. Kataoka and T. Honjo, unpublished data). It is difficult to understand why the 3′ rearrangement is necessary to express the C_H gene. It may be possible that the 3′ rearrangement of the C_H gene takes place so that the class switch stops at the gene. Otherwise, a V gene together with a J region moves farther down to a C_H gene remaining at the 3′ side of the expressed gene.

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Expression of lymphocyte surface IgE does not require switch recombination

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Immunoglobin heavy (H) chains are composed of a variable (V_H) and a constant (C_H) region. The latter is encoded respectively by eight distinct genes for the classes and subclasses in mice—C_μ, C_δ, $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, C_ε and C_α genes—arrayed in that order on the chromosome[1]. During differentiation of a single B lymphocyte, a given V_H region is first expressed as a μ chain, followed by the switch of the C_H region to other classes such as δ, γ, ε and α. The molecular genetic basis for this phenomenon, called heavy chain class switch, has been elucidated recently by cloning and characterization of immunoglobulin genes of mouse myelomas secreting various classes of immunoglobulin[2–4]. By this model, DNA rearrangement, called S–S recombination, brings a V_H gene, located originally 5′ to the C_μ gene, close to another C_H gene by deletion of an intervening DNA segment[5–9]. The S–S recombination occurs between S regions located in the 5′ flanking region of each C_H gene. The nucleotide sequences of S regions comprise tandem repetitive sequences sharing short common sequences[10–14]. In contrast, the expression of surface IgD in $\mu^+\delta^+$ lymphomas and in normal $\mu^+\delta^+$ lymphocytes seems not to involve DNA rearrangement in the region between the C_μ and C_δ genes[15,16]. The simultaneous expression of the C_μ and C_δ genes with a single V_H gene may be mediated by two alternative routes of RNA processing of a primary nuclear transcript containing the V_H, C_μ and C_δ genes. We have now studied the organization of C_H genes in sorted $\mu^+\varepsilon^+$ B lymphocytes and found that they retain C_μ, C_δ, C_γ and C_ε genes, suggesting that the simultaneous expression of the C_μ and C_ε genes is mediated by an RNA splicing mechanism. We propose that class switching requires at least two steps of differentiation, the first step involving activation of differential splicing and the second the DNA rearrangement.

During experiments to define the new mouse C_H locus encoding IgE (*Igh-7*)[17], we found that the *Igh*-congeneic strain SJA/9 (Igh^a), which has a SJL background, cannot produce a detectable amount of IgE in the sera even after infection with *Nippostrongylus brasiliensis*, which is known to stimulate polyclonal IgE production. Furthermore, we found that the increase of IgE-bearing B cells after *N. brasiliensis* infection occurs equally in SJA/9 and SJL mice (K.O. *et al.*, in preparation). Approximately 10% of the spleen cells of the *N. brasiliensis*-infected mice carry IgE on their surface, providing a unique opportunity for identifying sufficient IgE-bearing B lymphocytes for molecular genetic analyses. The advantage of SJA/9 mice is that a low IgE level in sera minimizes the binding of IgE to Fc receptors of ε-negative lymphocytes, thus avoiding the contamination of ε-negative cells into sorted ε^+ B cells. This strategy—taking advantage of a low level of a certain isotype in sera—was successfully used to purify IgG2a-bearing B cells from allotype-suppressed mice by fluorescence activated cell sorter[18].

The IgE-bearing B cells were isolated from spleen cells of *N. brasiliensis*-infected SJA/9 mice using the fluorescence-activated cell sorter. Only the brightest top 9% of the stained cells were collected and their purity examined under the fluorescent microscope, which is less sensitive and gives a lower limiting value of staining. As shown in Table 1, at least 86% of the sorted cells were brightly stained with anti-ε antibody. Most of the ε-bearing cells also carried the μ chain on their surface.

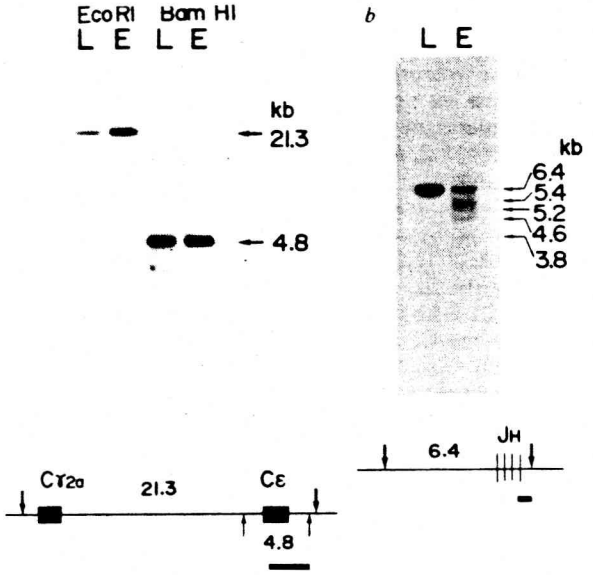

Fig. 1 Analysis of *Eco*RI and *Bam*HI fragments of ε-bearing cell and SJA/9 liver DNAs using cloned mouse C_ε and J_H genes as probes. DNA was isolated from sorted ε^+ B cells according to the method of S. Gattoni (personal communication). About 8×10^6 cells were lysed in 0.5 ml of 0.1% SDS, 10 mM EDTA 10 mM Tris-HCl pH 8.0, 10 mM NaCl, 200 μg ml^{-1} proteinase K for 90 min at 30°C with gentle shaking. An equal amount of neutralized heated (60 °C) phenol was added to the lysate and tilted for 15 min. A few millilitres of ethanol were added to the water phase and DNA was wound up at the interphase with a glass rod. DNA was washed in a large amount of ethanol and dissolved in 100 μl H_2O. About 6 μg DNA was obtained. Liver DNA was isolated as described before. The fragments obtained by *Eco*RI and *Bam*HI digestions of ε-bearing cell DNA (E) and SJA/9 liver DNA (L) were fractionated by electrophoresis in a 0.5% agarose gel, transferred to nitrocellulose filters and hybridized to the cloned mouse C_ε (*a*) and J_H (*b*) probes which are indicated by wide bars below the restriction maps. In *b*, only *Eco*RI digests were analysed. Each lane contains ~1 μg DNA. Probes were labelled by nick-translation and hybridized as described[9]. In the restriction maps closed rectangles indicate structural genes. Numbers show lengths (kb) of fragments. ↓, *Eco*RI; ↑, *Bam*HI.

However, they were not stained with anti-δ, anti-γ2a or anti-γ1 antibody (data not shown). As a control, about 8% of the ε-depleted cells and 10% of the whole spleen cells were stained with anti-ε antibody. The results indicate that the sorted cells are the essentially pure population of $\mu^+\varepsilon^+$ B cells.

To confirm that IgE on the surface of $\mu^+\varepsilon^+$ B cells is endogenously synthesized, ε^+ B cells were treated with trypsin (2.5 mg ml^{-1}) for 30 min at 37°C to strip off all cell-surface immunoglobulins, then after culturing, newly synthesized immunoglobulins on the surface were re-examined by fluorescence staining. As expected, 2 and 5 h after the trypsin treatment, 84 and 97%, respectively, of cells were stained with anti-ε. Furthermore, the sorted ε^+ B cells of SJA/9 were shown to secrete IgE when T cells of SJL were provided (K.O. *et al.*, in preparation).

We have extracted DNA from the sorted $\mu^+\varepsilon^+$ cells and examined the C_H gene organization in ε-bearing cells using the Southern blotting technique. When DNA of ε^+ B cells was digested with *Eco*RI, blotted and hybridized with the C_ε gene probe, it produced a 21.3-kilobase (kb) fragment identical to that produced in SJA/9 liver DNA (Fig. 1*a*). Similarly, *Bam*HI digestion of DNAs of ε^+ B cells and SJA/9 liver yielded an identical fragment (4.8 kb) hybridizing with the C_ε probe. As the *Eco*RI fragment (21.3 kb) encompasses the whole region between the $C_{\gamma 2a}$ and C_ε genes, the above results indicate that the C_ε gene does not rearrange in the IgE-bearing lymphocytes, unlike the IgE-secreting hybridoma and myeloma[19,20].

On the other hand, the J_H gene fragment of the IgE-bearing

lymphocyte DNA drastically reduced the intensity as compared with that of SJA/9 liver DNA, and appeared blurred, in agreement with the interpretation that a large number of different rearrangements have generated many new *Eco*RI fragments of different lengths in polyclonal B cells[21] (Fig. 1*b*). Each of several faint bands (5.4, 5.2, 4.6 and 3.8 kb) having less than a few per cent of the intensity of the germ-line band in liver DNA, may represent rearranged J_H genes for anitbodies against *N. brasiliensis* antigens *per se*.

When DNA of IgE-bearing cells was digested with *Eco*RI, blotted and hybridized with the $C_{\gamma 2b}$–$C_{\gamma 2a}$ probe, it produced the 6.6- and 21.3-kb fragments which correspond to the germ-line forms of the $C_{\gamma 2b}$ and $C_{\gamma 2a}$ genes, respectively (Fig. 2*a*). The germ-line $C_{\gamma 2b}$ and $C_{\gamma 2a}$ gene fragments (9 and 6.2 kb, respectively) were also detected in DNA of IgE-bearing cells when digested with *Hin*dIII. Similarly, *Eco*RI or *Hin*dIII digestion of IgE-bearing cell DNA produced the germ-line forms of the $C_{\gamma 1}$ and $C_{\gamma 3}$ genes (data not shown). In fact, the faint 23-kb *Hin*dIII fragment is the germ-line $C_{\gamma 1}$ gene detected by cross-hybridization of the $C_{\gamma 2b}$ probe (Fig. 2*a*).

*Eco*RI digestion of the ε^+ B-cell DNA produced the germ-line form of the C_μ gene fragment (13 kb) (Fig. 2*b*). Inasmuch as the 13-kb C_μ fragment contains the whole S_μ region, there is no doubt about the absence of the DNA rearrangement in the S_μ region. *Bam*HI digestion of the ε^+ B-cell and SJA/9 liver DNAs yielded the 11.5-kb fragment hybridizing with the C_μ probe. As the 11.5-kb *Bam*HI fragment encompasses the C_δ as well as the C_μ gene, the results indicate that the C_δ gene is not rearranged in the ε^- B cells.

The intensity of each C_H gene band was quantified at the corresponding size by hybridizing the same filters used in the above experiments with the β-globin and ribosomal RNA gene probes. The ratio of the intensity of each C_H gene band to the β-globin and ribosomal RNA gene bands as determined by densitometer tracings remained constant regardless of the origin of DNA, that is, from liver or ε^- B cells. These results suggest that the organization of the C_H gene in the IgE-bearing cells is probably the same as the germ-line gene except that the J_H is rearranged. These data indicate that IgE expression in $\mu^+\varepsilon^+$ B cells does not involve the S_μ–S_ε rearrangement although it is well established that the intervening DNA segment is deleted

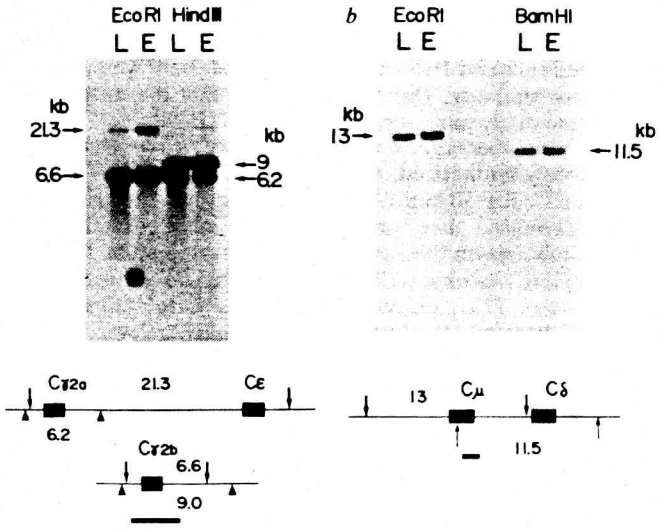

Fig. 2 Analysis of *Eco*RI, *Hin*dIII and *Bam*HI fragments of ε-bearing cell and SJA/9 liver DNAs using cloned mouse $C_{\gamma 2b}$ and C_μ genes as probes. Experimental conditions are as described in Fig. 1 legend. The fragments produced by *Eco*RI, *Hin*dIII and *Bam*HI digestion of ε-bearing cell DNA (E) and SJA/9 liver DNA (L) were electrophoresed and blotted to nitrocellulose filters. The restriction maps surrounding probes used [$C_{\gamma 2b}$ (*a*) and C_μ (*b*)] are shown below. Each lane contains ~1 μg DNA. ▲, *Hin*dIII; ↓, *Eco*RI; ↑, *Bam*HI.

Table 1 Characterization of sorted ε-bearing cells

Cells	Surface immunoglobulin-positive cells (%) μ	ε
Whole spleen cells	46	9.6
Sorted cells		
Sorted ε^+ B cells	77	86
The remaining cells	45	8.3

SJA/9 mice were infected with *N. brasiliensis* by subcutaneous injection of third-stage larvae (750 per mouse). The mice were killed 2 weeks later and their spleens gently teased to obtain lymphocytes. Spleen lymphocytes were stained with guinea pig anti-murine IgE sera[17] and fluorescein isothiocyanate-labelled rabbit anti-guinea pig IgG1 antibodies. Stained spleen cells were sorted on the FACS III (Becton-Dickinson). An aliquot of the sorted cells and spleen cells were stained with biotin-conjugated rabbit anti-murine μ antibodies and rhodamine isothiocyanate-labelled avidin after photobleaching. As stained cells were counted under the fluorescent microscope, the values shown are lower limits.

by the S–S recombination in various immunoglobulin-secreting B-cell lymphomas, hybridomas and myelomas including IgE secreters[5–9].

Given these results we propose that differentiation of IgM-bearing B lymphocytes to IgE-secreting plasma cells may proceed by at least two biochemical steps, as shown in Fig. 3. The first step (step I) promotes differentiation of IgM-bearing B lymphocytes into IgM–IgE-bearing B lymphocytes, which involves the activation of differential RNA processing of a single large RNA transcript containing V_H, C_μ, C_δ, C_γ and C_ε gene sequences. The large transcript may be spliced into μ or ε mRNA by specific enzymes and/or specific assisting molecules such as low molecular weight RNA[22,23]. The size of the primary transcript is estimated to be ~180 kb from the C_H gene organization[1]. Naturally, the μ and ε mRNAs share an identical V_H region sequence. As we handled a mixed populaton of $\mu^+\varepsilon^+$ lymphocytes, we were unable to determine whether the same V_H sequence was associated with the C_μ and C_ε sequences in a single cell. However, IgD and IgM molecules were shown to bear the identical V_H region in a $\mu^+\delta^+$ lymphoma[16]. We presume that step I does not involve any major DNA rearrangement.

IgM–IgE-bearing B lymphocytes differentiate into IgE-secreting B cells or plasma cells by step II, which involves S_μ–S_ε recombination and simultaneous DNA deletion as established previously[2–9]. Obviously, a similar mechanism should apply to the switch from IgM-bearing cells to IgD-, IgG- or IgA-secreting plasma cells. It is not clear whether DNA rearrangement accompanies the differentiation from IgM-bearing B cells to IgM-secreting plasma cells. Interestingly, most of the IgM-

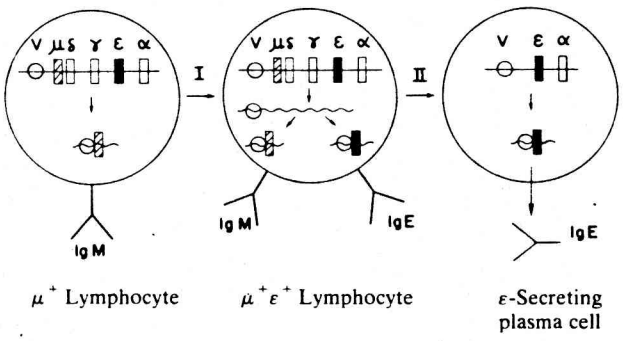

Fig. 3 Two steps of differentiation from μ^+ lymphocytes to ε-secreting plasma cells. Step I involves the activation of differential splicing. Alternate splicing of a long transcript containing V_H, C_μ, C_γ and C_ε sequences will produce mRNA encoding either μ or ε chain with the same V-region sequence. Step II involves DNA deletion. See the text.

secreting myelomas and hybridomas seem to have a deletion in the S_μ region[7,24,25]. We think it reasonable that deletion of the S_μ region facilitates the transcription of the C_μ gene and promotes IgM secretion as the extremely G+C-rich S_μ region[11] may hinder efficient transcription.

There are no data concerning the length of the primary transcript of μ mRNA in IgM-bearing B cells. They may transcribe the whole C_H gene locus from the beginning. If so, step I is mediated by the activation of a new differential splicing system. Alternatively, the primary transcript in IgM-bearing B cells may contain only the V_H and C_μ sequences. In this case, step I requires at least two new biochemical events—the transcription of a much larger RNA and the activation of a new differential splicing system. To avoid the premature termination of transcription, lymphocytes may have to introduce some biochemical changes in the C_H gene locus such as demethylation[26]. In fact, the C_δ gene is demethylated in $\mu^+\delta^+$ hybridoma but not in μ^+ lymphoma[27].

This model favours the hypothesis that the splicing as well as recombination mechanism is class specific. Otherwise, the isotype expression in B cells should be transient and multiple (more than three isotypes per cell) until they become plasma cells. Several lines of evidence suggest that the expression of a certain V_H sequence is closely associated with a specific C_H isotype. CBA/N mice have genetic defects which make them incapable of producing anti-phosphorylcholine antibody of any classes other than IgE whereas anti-phosphorylcholine antibody of IgM and IgG is very common in most mouse strains[28,29]. A lymphoma cell line I.29 has been shown consistently to switch from μ to α[30]. Such results seem to indicate that the S–S recombination is catalysed by the class-specific enzyme(s).

IgM–IgE-bearing lymphocytes accumulated in spleens of *N. brasiliensis*-infected SJA/9 mice are capable of differentiating into IgE-secreting plasma cells when T cells of SJL are provided (K.O. *et al.*, in preparation). As SJA/9 mice can synthesize normal amounts of IgM, IgG and IgA, the defect of SJA/9 seems to reside in IgE-specific regulatory T cells[31]. Furthermore, it is probably at step II that the T cells affect B-cell differentiation.

After completion of this manuscript we learned that Perlmutter and Gilbert[32] had found the C_μ gene in γ_1-bearing B cells purified from normal spleen using antibody-coated Petri dishes.

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Deletion of immunoglobulin heavy chain genes from expressed allelic chromosome

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We have studied the organization of immunoglobulin heavy-chain genes in a γ2b-chain (BALB/c allotype)-producing myeloma BKC F_1 # 15 induced in a F_1 mouse between C57BL and BALB/c. Southern blot hybridization studies using cloned μ, γ1 and γ2b-chain genes as probes demonstrate that the μ- and γ1-chain genes of the expressed chromosome are deleted while these genes of the unexpressed chromosome are retained. The γ2b-chain gene of the expressed allele is rearranged while that gene of the unexpressed allele seems unchanged, as do the γ2a-chain genes. These results support the allelic deletion mechanism in heavy-chain class switch and the order of H chain genes.

DREYER and Bennett[1] proposed a model that a variable region (V) and a constant region (C) of the immunoglobulin polypeptide chain are encoded by two separate genes in the germ-line, which are eventually brought together by somatic recombination. Recent isolation and characterization of immunoglobulin light (L)- and heavy (H)-chain genes have convincingly borne out the predictions of the Dreyer–Bennett hypothesis[2-11]. Analyses of L-chain genes have demonstrated that the V–C recombination takes place between a germ-line V_L gene and a J_L region that is a few thousand nucleotides away from the C_L gene[12,13]. It is believed that the intervening sequence separating the J and C genes is removed at the RNA level by splicing[14].

Possible mechanisms have been proposed to account for the V–C recombination, which can be classified into deletion, copy-insertion, excision–insertion and inversion models[15-19]. A model for the immunoglobulin gene organization and reorganization should explain such unique phenomena known in lymphocytes as allelic exclusion and class switch. In a given lymphocyte a C_H gene of one allele is expressed and that of the other allele is suppressed (allelic exclusion). A V_H-region sequence is successively joined with different C_H-region sequences during differentiation of a lymphocyte (class switch).

Previously we showed that the copy numbers of the specific C_H genes in myeloma DNA were reduced depending on the class or subclass of the C_H gene expressed in the myeloma[20]. Our data indicate that the DNA segment located between a V_H gene and the C_H gene expressed is excised out of the chromosome to recombine the two genes. Such recombination seemed to occur only on one of the two allelic chromosomes. We have also proposed that H-chain genes are aligned on one chromosome in the order: V_H genes, unknown spacer, μ, γ3, γ1, γ2b, γ2a and α (ref. 20). Such alignment, together with successive deletion, can explain the class-switch phenomenon. This model is called the allelic deletion model. The deletion of C_H genes, in accord with the above order of the C_H genes, was recently confirmed by Rabbitts *et al.*[21]. The deletion of the DNA segment located between two adjoining sequences was also confirmed in the κ-type L-chain gene by Sakano *et al.*[12].

Elsewhere we have reported cloning of the rearranged γ1-chain gene from a γ1 chain-producing myeloma MC 101 and compared its structure with H-chain genes cloned from newborn mouse DNA[22]. Such analyses indicate that the rearranged γ1-chain gene contains at its 5′ flanking region a newly introduced segment that was located originally at the 5′ flanking region of the μ-chain gene in newborn mouse DNA. The recombination site is not the putative J region but a novel region called *S* region. These results are in agreement with the prediction of the deletion model, although they do not necessarily prove the model.

In this report we describe direct evidence that the deletion of the specific H-chain genes takes place only on the expressed chromosome using a myeloma induced in a F_1 mouse between C57BL and BALB/c which have different allotypes in the H-chain C regions.

Probes and restriction maps of the μ-, γ1- and γ2b-chain genes

We have already cloned the *Eco*RI fragments containing the γ1-, γ2b and μ-chain genes from newborn mouse DNA and determined their nucleotide sequences[4,8,9,22-24]. We used the 1.2-kilobase pair *Hin*dIII fragment of the cloned μ-chain gene,

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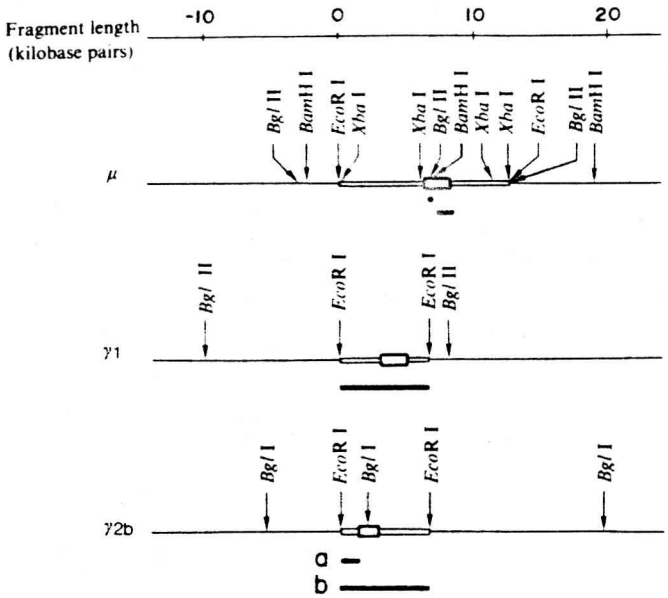

Fig. 1 Restriction enzyme cleavage sites around the μ-, γ1- and γ2b-chain genes. DNA is displayed from left to right with the direction of transcription. Narrower rectangles show the cloned DNA fragments. Wider rectangles show locations of structural genes. DNA fragments used for probes are indicated by horizontal bars below the restriction maps. μ-chain gene probe, 1.2-kilobase pair *Hin*dIII fragment that contains the CH3 and CH4 domains of the μ-chain gene clone[21]; γ1-chain probe, 6.6-kilobase pair *Eco*RI fragment containing the γ1-chain gene[8]; γ2b-chain gene probe a, 1.35-kilobase pair, *Bam*HI fragment (5′ fragment) of the γ2b-chain gene clone[22]; γ2b-chain gene probe b, 6.6-kilobase pair *Eco*RI fragment containing the γ2b-chain gene[22].

the cloned *Eco*RI fragment (6.6 kilobase pairs) of the γ1-chain gene, the cloned γ2b-chain gene and the 1.35-kilobase pair *Bam*HI fragment of the cloned γ2b-chain gene as probes for hybridization (Fig. 1). Although the whole γ2b-chain gene clone (γ2b probe b) has extensive homology with the γ2a-chain gene[23,25], the 1.35-kilobase pair *Bam*HI fragment (γ2b probe a), which corresponds to the 5′ fragment of the cloned γ2b-chain gene, does not cross-hybridize with the γ2a-chain gene under the stringent washing conditions used in the present study. Detailed maps of restriction endonuclease cleavage sites within these clones have been determined[8,22-24]. We have determined the restriction sites of *Bgl*II adjacent to the cloned γ1-chain gene fragment by digestion of mouse DNA with several combinations of two restriction enzymes, followed by Southern blot hybridization of the restriction DNA fragments. Similarly, we have determined the restriction sites of *Bgl* I adjacent to the cloned γ2b-chain gene fragment and the sites of *Bam*HI and *Bgl* II adjacent to the cloned μ-chain gene fragment. These restriction sites were confirmed by Southern blot hybridization using the 5′ or 3′ fragment of the cloned DNA as a probe. Figure 1 summarizes the results of these analyses and the known sites of restriction endonucleases used in the present study. The fragments used for probes are also shown.

Allelic deletion of the μ- and γ1-chain genes

To prove the deletion of the C_H gene from one chromosome, it is essential to distinguish the C_H genes on different chromosomes. BALB/c and C57BL mice have different allotypes in H-chain C regions[26], suggesting that their C_H genes may have different nucleotide sequences. We searched for restriction endonucleases that produce the C_H-gene fragments of different lengths between BALB/c and C57BL alleles. Among restriction endonucleases tested (*Pvu*II, *Bam*HI, *Eco*RI, *Hin*dIII, *Sma*I, *Pst*I, *Kpn*I, *Xba*I *Bgl*II) we found that *Bgl*II can distinguish the γ1-chain genes of BALB/c and C57BL alleles. As for the γ2b-chain gene, we have tested *Xho*I, *Ava*I, *Sal*I, *Xba*I, *Bgl*II, *Bam*HI, *Bst*EII and *Bgl*I, and found that *Xho*I or *Bgl*I digestion produces different γ2b-chain gene fragments between BALB/c and C57BL DNAs. The μ-chain genes of C57BL and BALB/c alleles were distinguished by digestion with *Xba*I, *Eco*RI or *Kpn*I, while digestion with *Bam*HI or *Bgl* II did not distinguish the two alleles.

Only a few myelomas are available, which were induced in the F_1 mouse between BALB/c and C57BL (ref. 27). BKC F_1 # 15, that produces the γ2b-chain protein with the BALB/c allotype, is suitable for our purpose since the allelic deletion model predicts the deletion of the γ1- and μ-chain genes from the BALB/c chromosome of this myeloma.

When BALB/c and C57BL DNAs were digested with *Xba*I, blotted and hybridized with the μ-chain probe, they produced the 4.8 and 2.8-kilobase pair bands, respectively (Fig. 2*a*, lanes 1

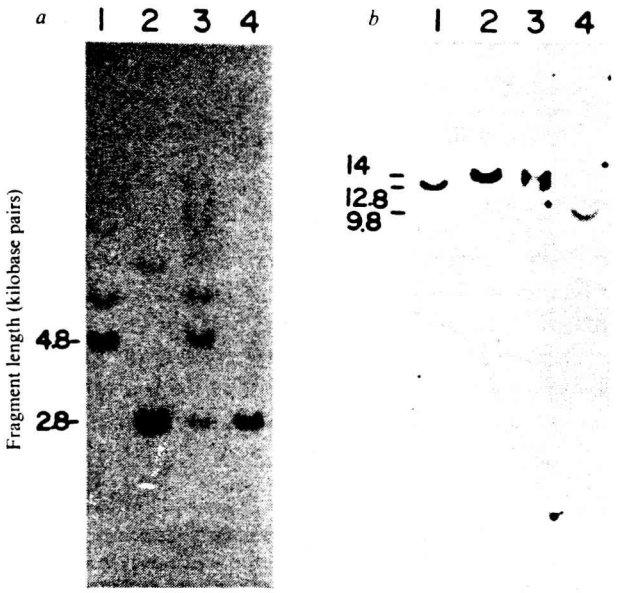

Fig. 2 Hybridization of mouse liver and F_1 myeloma DNAs with the μ-chain gene probe. Myeloma BKC F_1 # 15, (kindly supplied by Dr M. Potter) was propagated in F_1 mice between female BALB/c and male C57BL/6. BKC F_1 # 15 was induced in F_1 mice between C57BL and BALB/c and shown to produce IgG2b (ref. 27). The allotype of the BKC F_1 # 15 protein was determined to be BALB/c type by Drs K. Hayakawa and K. Okumura in Prof. T. Tada's laboratory (University of Tokyo). Mouse livers or myeloma tumours were homogenized in 50 mM Tris·HCl (*p*H 8.0), 10 mM EDTA, 0.15 M NaCl with a Potter–Elvehejm homogenizer at 0 °C. Sodium dodecylsulphate to 0.3% and $NaClO_4$ to 0.5 M were added to a whole homogenate. The homogenate was extracted with 0.5 vol. each of water-saturated phenol and a chloroform–isoamyl alcohol mixture (24 : 1). The water phase was separated by centrifugation, dialysed against 0.1 × SSC (0.015 M NaCl, 0.0015 M Na citrate) for 24 h digested with RNase (100 μg ml^{-1}) in a dialysis bag for 24 h at room temperature and then extracted with phenol. After dialysis against 0.1 × SSC for 48 h, 1.27 g of CsCl was added to 1 ml of the water phase. After centrifugation at 30,000 r.p.m. for 48 h, fractions with high viscosity were collected and dialysed against 0.1 × SSC for 48 h. DNAs (5 μg each) were digested with *a*, *Xba*I or *b*, *Eco*RI, electrophoresed in 0.5% agarose gels (Sigma type I) and transferred to nitrocellulose filters (Schleicher and Schuell) according to the method of Southern[32]. DNA fixed on filters was hybridized with the μ-chain gene probe as described previously[8]. The probe DNA was labelled with [α-^{32}P]TTP (Amersham) by nick translation[33]. The specific activity of the probe was 200 c.p.m. pg^{-1}. After hybridization, filters were rinsed with 2 × SSC and then washed in 0.1 × SSC plus 0.1% SDS (four times) for 40 min each at 67 °C. Filters were finally rinsed with 2 × SSC, dried and exposed to X-ray film using a DuPont Cronex lightening plus intensifier screen at −80 °C. Origins of DNA used are: lanes 1, BALB/c liver; lanes 2, C57BL/6 liver; lanes 3, F_1 mouse liver; lanes 4, BKC F_1 # 15 myeloma.

and 2). BKC F_1 # 15 DNA, when digested with *Xba*I, yielded only the μ-chain gene fragment of the C57BL allele while F_1 mouse DNA contained the μ-chain gene fragments of both BALB/c and C57BL alleles (Fig. 2*a*, lanes 3 and 4). Faint bands larger than 6 kilobase pairs are due to contamination of other *Hin*dIII fragments of the μ-chain gene clone to the 1.2-kilobase pair *Hin*dIII fragment used as a probe (see Fig. 1) because these faint bands disappeared when the recloned 1.2-kilobase pair *Hin*dIII fragment was used as a probe. It is worth noting that a faint band (at 6.1 kilobase pairs) of the BALB/c chromosome is also absent from the F_1 myeloma DNA. The μ-chain gene of the C57BL allele in BKC F_1 # 15 appeared to be unchanged at its 3′ distal region since DNAs of C57BL and BKC F_1 # 15 produced identical μ-chain gene fragments of 12- and 8.5-kilobase pairs when digested with *Bam*HI and *Bgl* II, respectively (ref. 28 and see Fig. 1). However, the 5′ region flanking the μ-chain gene of the C57BL allele in BKC F_1 # 15 is rearranged because the μ-chain gene fragment (9.8-kilobase pairs) in *Eco*RI-digested BKC F_1 # 15 DNA was smaller than the band (14-kilobase pairs) observed in *Eco*RI-digested C57BL DNA (Fig. 2*b*, lanes 2 and 4).

BALB/c and C57BL DNAs, when digested with *Bgl*II, yielded the γ1-chain gene fragments of 18- and 14.5-kilobase pairs, respectively (Fig. 3, lanes 1 and 2). Naturally, F_1-mouse DNA contained the γ1-chain gene fragments derived from both BALB/c and C57BL alleles. It is evident that BKC F_1 # 15 DNA has lost the γ1-chain gene of the BALB/c allele (the 18-kilobase pair band), whereas the gene on the C57BL allele (the 14.5-kilobase pair band) seems to remain intact (Fig. 3, lane 4).

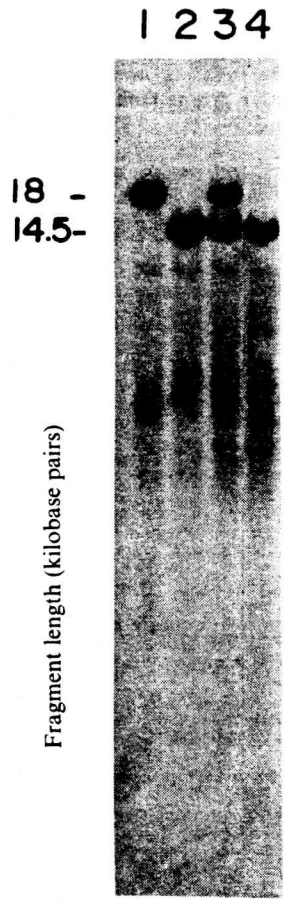

Fig. 3 Hybridization of mouse liver and F_1 myeloma DNAs with the γ1-chain gene probe. DNAs (30 μg each) were digested with *Bgl*II and hybridized as described in the legend to Fig. 2 with the [α-^{32}P]-labelled γ1-chain gene probe. Origins of DNA used are: lane 1, BALB/c liver; lane 2, C57BL/6 liver; lane 3, F_1 mouse liver; lane 4, BKC F_1 # 15 myeloma.

Table 1 Restriction fragments of immunoglobulin H-chain genes in mouse liver and myeloma

Origin of DNA	Size of restriction DNA fragments (kilobase pairs)			
	μ (*Xba*I)	γ1 (*Bgl*II)	γ2b (*Bgl*I)	γ2a (*Eco*RI)
BALB/c liver	4.8	18.0	7.5	23.0
C57BL liver	2.8	14.5	9.5	23.0
F_1 liver	4.8 + 2.8	18 + 14.5	–	–
BKC F_1 # 15	2.8	14.5	9.5 and 6.5	23.0

The results of Figs 2–4 are summarized. Restriction enzymes used are shown in parentheses.

Rearrangement of the γ2b-chain gene of the expressed allele

The γ2b-chain gene in BKC F_1 # 15 DNA was analysed by digestion with *Bgl* I and Southern blot hybridization using the 5′ fragment of the cloned γ2b-chain gene (γ2b probe a) as a probe. As shown in Fig. 4 (lanes 1 and 2), *Bgl* I digestion of BALB/c and C57BL DNAs produced the fragments of 7.5- and 9.5-kilobase pairs respectively, which hybridized with the γ2b chain probe a. BKC F_1 # 15 DNA, when digested with *Bgl* I, yielded the γ2b-chain gene fragments of 6.5- and 9.5-kilobase pairs (Fig. 4*a*, lane 4). The results indicate that the 5′ region of the γ2b-chain gene on the BALB/c allele was rearranged, while that gene on the C57BL allele seems to remain unchanged in BKC F_1 # 15 DNA. However, we do not exclude the possibility that the γ2b-chain gene on the C57BL allele may have undergone some rearrangements which we did not detect. We also found that the 3′ region flanking to the γ2b-chain gene of the BALB/c allele is rearranged in BKC F_1 # 15 DNA when the 3′-portion probe of the γ2b-chain gene was used[28].

Presence of the γ2a-chain genes

Both BALB/c and C57BL DNAs, when digested with *Eco*RI, produced the 6.6- and 23-kilobase pair fragments hybridizing to the γ2b-gene probe b which cross-hybridizes with the γ2a-chain gene (Fig. 4*b*, lanes 1 and 2). The 6.6-kilobase pair fragment corresponds to the cloned γ2b-chain gene and the 23-kilobase pair fragment was identified as the γ2a-chain gene fragment[21]. F_1-mouse and BKC F_1 # 15 DNA contained the γ2a-chain gene fragment (23-kilobase pairs) (Fig. 4*b*, lanes 3 and 4). The γ2a-chain gene seems to be intact in BKC F_1 # 15 although we cannot exclude the possibility that the γ2a-chain gene contains an undetected rearrangement.

Organization of immunoglobulin H-chain genes in BKC F_1 # 15 myeloma

Restriction DNA fragments of the μ-, γ1-, γ2b- and γ2a-chain genes in BALB/c mice, C57BL mice, F_1 mice and BKC F_1 # 15 myeloma are listed in Table 1. It is evident that BKC F_1 # 15 has lost the μ- and γ1-chain genes of the BALB/c chromosome and contains the rearranged γ2b-chain gene of the BALB/c chromosome. This myeloma contains the μ-, γ1- and γ2b-chain genes of the C57BL chromosome. BKC F_1 # 15 contains the γ2a-chain gene although we did not distinguish two alleles. These results are in agreement with the prediction of the allelic deletion model. Nevertheless, we found an unexpected rearrangement at the 5′ region flanking the μ-chain gene of the C57BL chromosome that is unexpressed in BKC F_1 # 15. The two chromosomes of BKC F_1 # 15 are schematically represented in Fig. 5.

Unexpected rearrangements in BKC F_1 # 15

Although the deletion profile of C_H genes in BKC F_1 # 15 DNA is as predicted by the allelic deletion model, we found two unexpected rearrangements in BKC F_1 # DNA; one in the 5′

region flanking the μ-chain gene of the unexpressed chromosome (Fig. 2*b*, lane 4) and the other in the 3′ region flanking to the γ2b-chain gene of the expressed chromosome[28].

It is possible that rearrangement in the 5′ region flanking the μ-chain gene of the C57BL allele is related to the mechanism to suppress the H chain genes on the C57BL chromosome. It may be relevant to this assumption that the 5′ region flanking the μ-chain gene is proposed to contain the putative J region and the *S* region that is important to class-switch recombination[22].

It is difficult to understand why the rearrangement in the 3′ region flanking the γ2b-chain gene of the expressed chromosome is necessary to express the γ2b-chain gene. It may be possible that the 3′ rearrangement of the C_H gene takes place so that the class switch stops at this particular gene. Otherwise, a V_H gene together with a J region moves farther down to a C_H gene remaining at the 3′ side of the expressed gene.

Deletion of C_H genes in other myelomas

Rabbitts *et al.*[21] reported that C_H genes, which are located 5′ to the expressed C_H gene, are absent from both chromosomes in several myelomas induced in BALB/c mice. We also observed similar deletion from both chromosomes and found that the expressed C_H genes are rearranged in different manners between homologous chromosomes in these myelomas[28]. The results may suggest that C_H gene deletion, which accompanies the rearrangement of the expressed C_H genes, takes place successively, but not simultaneously, in the allelic chromosomes. Several forms of rearranged κ-type L-chain genes were isolated from κ-chain-producing myelomas[29,30].

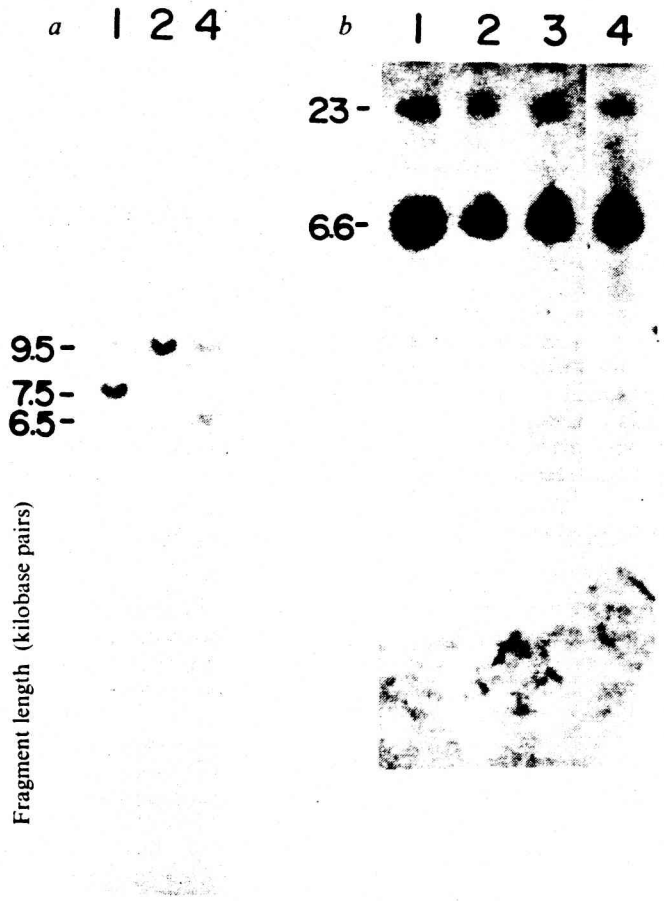

Fig. 4 Hybridization of mouse liver and F_1 myeloma DNAs with the γ2b-chain gene probes. DNAs were digested with *a*, *Bgl*I or *b*, *Eco*RI, and hybridized as described in the legend to Fig. 2 with [α-^{32}P]-labelled probes indicated. The γ2b probes a and b were used in *a* and *b* respectively. Origins of DNA used are: lanes 1, BALB/c liver; lanes 2, C57BL/6 liver; lanes 3, F_1 mouse liver; lanes 4, BKC $F_1 \neq 15$ myeloma.

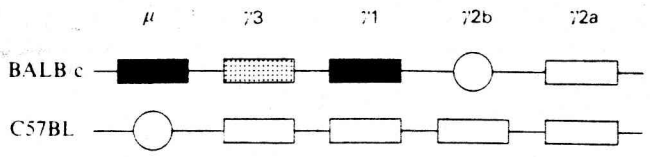

Fig. 5 Schematic representation of C_H genes in BKC $F_1 \neq 15$. Each line shows one chromosome derived from each parent. The order of C_H genes is as proposed before[20]. Open and closed rectangles indicate 'intact' and deleted genes, respectively. (The word 'intact' simply means that the change is not detected in the present study.) Circles indicate rearranged genes. The γ3-chain gene, which was not determined in the present study, is assumed to be deleted from the BALB/c chromosome and indicated by a dotted rectangle.

However, we cannot exclude the possibility that such rearrangements could be secondary mutations in myelomas. We are quite aware of the fact that chromosomal anomalies are rather frequent in myeloma cells[29]. Nonetheless, the perfect agreement of the C_H-gene deletion profile (the order and allelic chromosome) with the prediction of the allelic deletion model is something more than accidental coincidence. Seidman and Leder[5] reported that only one member of an allelic pair of L-chain genes is rearranged in κ-chain-producing myelomas, MOPC 149 and MOPC 41. Studies on differentiated B lymphocytes are necessary to determine whether or not the case of BKC $F_1 \neq 15$ is universal and directly applicable to normal lymphocytes.

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Immunoglobulin switch region-like sequences in *Drosophila melanogaster*

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ABSTRACT

We found immunoglobulin switch (S) region-like sequences in DNAs of wide variety of organisms including sea urchin, yeast and Drosophila that do not produce immunoglobulins. DNA fragments carrying S_μ-like seqences were cloned from Drosophila and the nucleotide sequence of a clone is almost identical to that of the mouse S_μ region. Restriction fragments of Drosophila S_μ-like sequences and their flanking regions seem to vary among Drosophila species. Possible evolutionary significance of the S_μ-like sequences in invertebrates was discussed.

Recent structural studies on immunoglobulin heavy chain genes demonstrate that two distinct types of DNA rearrangement take place during the differentiation of B lymphocytes(1-3). One type, termed V-D-J recombination, generates the complete V_H gene by joining the V_H-, D- and J_H-gene segments. A second type of recombination mediates the H chain class switch that associates a particular antigen binding specificity, a completed V region with a series of different heavy-chain constant (C_H) regions such as C_μ, C_γ, C_ε and C_α. 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. To switch from μ to γ chain, the S_μ region recombines with the S_γ region, resulting in the replacement of the C_μ gene with the C_γ gene without affecting the V region sequence.

It has been shown that the S region comprises tandem repetition of short unit sequences(4-9). The simplest S region, S_μ consists of tandem repetition of two kinds of pentanucleotides, GAGCT and GGGGT which disperse in repeat units of all the other S region sequences. We have proposed that the S-S recombination is mediated by recognition of short common sequences(4-9). During evolutionary studies of immunoglobulin genes we and others found that the S_μ, S_γ and S_ε region sequences as well as the coding sequences are conserved between mouse and human(10-14). The results suggest that the S region sequence per se has some important biological functions although it does not code for a protein.

In this paper we report that the S_μ-like sequences are present in variety of organisms such as yeast, sea urchin, Drosophila and calf. We have cloned

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several DNA fragments containing the S_μ-like sequence from Drosophila and shown that the nucleotide sequence of one clone is almost identical to the repeating unit (GAGCT, GGGGT) of the mouse S_μ region. We have discussed evolutionary origin and implication of the presence of the S_μ-like sequences in invertebrates which do not produce immunoglobulins.

MATERIALS AND METHODS

Isolation of High Molecular Weight DNA

High molecular weight DNA of adult Drosophila flies was prepared as described previously (15). D. virilis, D. simulans and D. melanogaster (three stocks; Oregon R, Hikone R and T(Y;2) CyO, DTS-513) were used for preparation of DNA. DNA was also isolated from several developmental stages of D. melanogaster (T(Y;2) CyO, DTS-513), namely, 0-12hr embryos, larvae and pupae. Embryonic DNA was prepared by a simplified method described by Yaoita et al. (34). The virginator stock, T(Y;2) CyO, DTS-513, was obtained from Bowling Green, Stock Center (Ohio).

Recombinant DNA and DNA Sequence Determination

Drosophila genomic DNA library (sheared DNA of Canton S stock in the Charon 4A vector) was provided by T. Maniatis (16). The library was screened by in situ plaque hybridization technique (17) using the nick-translated Hind III fragment of the mouse S_μ region as a probe (Fig. 1). Phage DNA was prepared as described (18). The Hind III fragment of Dm S_μ-6 (6.8 kb) and Eco RI-Hind III fragment of Dm S_μ-9 (6.0 kb) were recloned into pBR322 (19). The two subclones were referred to as pDm S_μ-6 and pDm S_μ-9. pDm S_μ-9 was used for DNA sequencing analysis. Plasmid DNA was prepared as described (20). Recombinant DNA experiments were done under the P2-EK2 containment. DNA sequence determination was carried out by the method of Maxam and Gilbert (21) with minor modifications (22).

Filter Hybridization

DNA fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose filters (23). The filters were hybridized with appropriate ^{32}P-labeled probes as described (24). ^{32}P-labeled nick-translated probes were synthesized as described (25). Hybridization was done at 65°C for 12-18 hr in the hybridization buffer (1M NaCl, 50mM Tris-HCl pH7.4, 10mM EDTA, 0.1% SDS, 1xmodified Denhardt's solution, 20µg/ml denatured E. coli DNA) containing labeled probes. After hybridization, the filters were washed four times for 40 minutes intervals in 1xSSC-0.1% SDS at 65°C.

Materials

α-^{32}P-dCTP (spec. act. 2000-3000Ci/mmole) was obtained from the Radiochemical Center (Amersham, England). Sources of restriction endnucleases and other enzymes were described previously (15,22,26).

RESULTS

Mouse S_μ-like Sequences in Varaiety of Organisms

To search for the distribution of S_μ-related DNA sequences in different species, we analysed DNAs from various organisms by Southern blot hybridization. We used a 3.7kb Hind III fragment (S_μ probe) of IgH 701(6,27) which contains a major portion of the mouse S_μ region as a probe (Fig. 1). DNAs of various species such as sea urchin, yeast, Drosophila, calf and mouse were digested with Eco RI. DNA digests were electrophoresed in a 0.7% agarose gel, blotted to nitrocellulose filters and hybridized with the nick-translated S_μ probe. Eco RI digestion produced several fragments hybridizing to the S_μ probe in each DNA. For example, a dark 12-14kb fragment in sea urchin DNA ; 19, 9.8, 6.0, 4.3, 2.6 and 1.5 kb fragments in yeast DNA ; 17, 12, 10, 8, 5.8, 3.6 and 3.0kb fragments in Drosophila DNA, a dark 6.4kb band above background smear in calf DNA (Fig. 1). In addition to the prominent C_μ gene fragment (13kb) numerous fainter bands were produced in mouse DNA as described before(7,28,29). Similarly many bands were seen in chicken, rabbit, rat and human DNAs. Most abundant S_μ-like sequences were found in lampery, rainbow traut and frog DNAs. We found only a faint 3.0kb band in Stytonychia DNA (lane 1). These results demonstrate that the S_μ-like sequence is distributed among the genomes of wide variety of organisms.

Isolation of Recombinant Phages Containing S_μ-like Sequences of *Drosophila melanogaster*

To characterize the S_μ-like sequence in invertebrates we cloned DNA fragments containing S_μ-like sequences from Drosophila DNA. We have screened a phage library of D. melanogaster (Canton S strain) DNA(16) using the mouse S_μ probe. Out of approximately 1.8×10^5 plaques, 36 positive plaques were identified upon autoradiography. Eleven of these positive phages were isolated and their DNAs were prepared. Southern blot hybridization of their restriction DNA fragments allowed us to classify them into nine independent phages. These phages are referred to as Ch·Dm·S_μ-4, Ch·Dm·S_μ-5, Ch·Dm·S_μ-6, Ch·Dm·S_μ-9, Ch·Dm·S_μ-10, Ch·Dm·S_μ-17, Ch·Dm·S_μ-18, Ch·Dm·S_μ-19 and Ch·Dm·S_μ-24. Restriction endonuclease cleavage maps of the inserts were constructed by conventional procedures. Comparison of these maps and Southern blot hybridization revealed that Dm·S_μ-6, Dm·S_μ-9 and Dm·S_μ-10 DNAs overlap partially with each other (Fig. 2). The

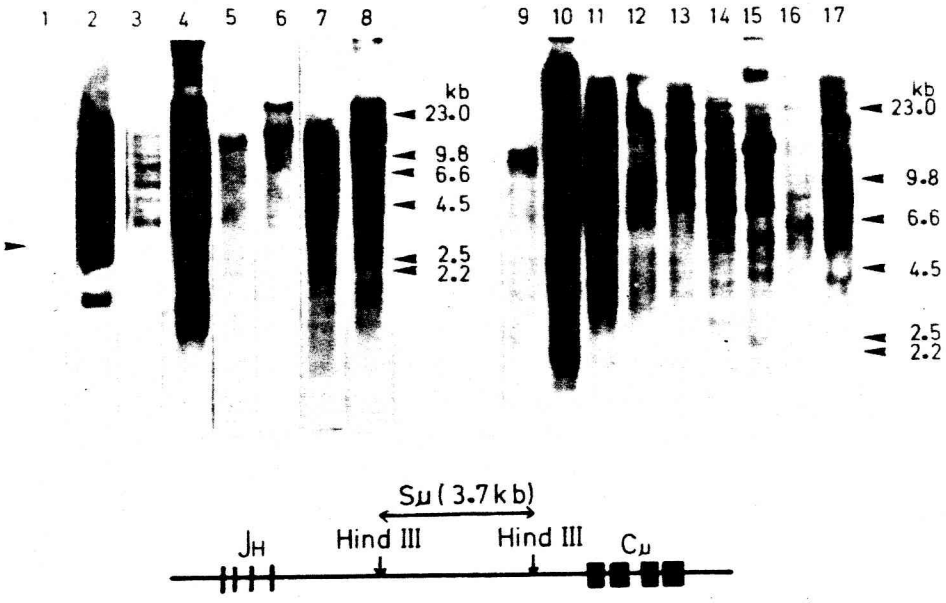

Fig. 1. S_μ-like Sequences in Various Organisms.

DNA (2_μg) was digested with a 5-fold excess of Eco RI, separated by agarose gel electrophoresis, blotted onto nitrocellulose filters, and hybridized with radioactive S_μ probe (3.7 kb-Hind III fragment shown at the bottom). DNA used in each lane is as follows; 1, Stylonychia pustlata (macronuclei); 2, yeast; 3, D. melanogaster; 4, lamprey; 5, sea urchin; 6, mouse (BALB/c); 7, sea urchin; 8, mouse (BALB/c); 9, sea urchin; 10, rainbow traut; 11, frog (Rana catesbeiana); 12, chicken; 13, rabbit; 14, rat (Rattus norvegicus); 15, mouse (BALB/c); 16, calf thymus; 17, human placenta. Exposure times were 12 hrs (lanes 5 and 6), 3 days (lane 1-4, 7 and 8) and 4 days (lanes 9-17). Specific activities of probes were 800 cpm/pg (lanes 1-8) and 600 cpm/pg.

overlapped regions contain the DNA segment that is homologous to the mouse S_μ sequence. The inserts of other clones are shown in Fig. 3.

The 6.8kb Hind III fragment (fragment B) of Dm·S_μ-6 and the 6.0kb Eco RI-Hind III fragment of Dm·S_μ-9 were subcloned into the plasmid pBR 322 and the subclones were referred to as pDm·S_μ-6 and pDm·S_μ-9, respectively. Comparison of detailed restriction maps of these clones confirmed that both inserts contain segments homologous to the S_μ region and that the 6.0kb Eco RI-Hind III fragment of Dm·S_μ-9 contains the same restriction enzyme cleavage sites as the fragment B except that the left most portion (900bp) of the fragment B is absent from the 6.0kb Eco RI-Hind III fragment of Dm·S_μ-9. Similarly, the 3.6kb Eco RI-Hind III fragment of Dm·S_μ-10 hybridized when the fragment A of Dm·S_μ-6 (Fig. 2) is used as a probe. These results demonstrate

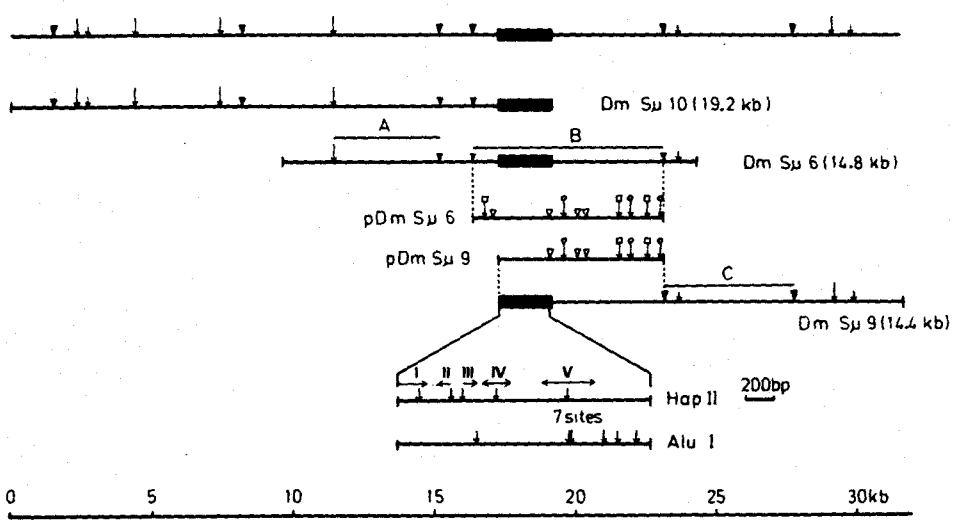

Fig. 2. Restriciton maps and sequence strategy of the cloned S_μ-like sequences of Drosophila .

The restriction maps of Dm S_μ-6, Dm S_μ-9 and Dm S_μ-10 are shown. The upper diagram shows the composite map of the chromosomal region containing the S_μ-like sequence. Subclones used in the detailed mapping and sequencing of Dm S_μ segments are indicated by pDm S_μ-6 and pDm S_μ-9. Sequencing strategy is shown by an enlarged segment of pDmS_μ-9. Horizontal arrows indicate the directions and ranges of sequencing. The S_μ-like sequences hybridizing to the mouse S_μ probe are represented by closed rectangles. Each insert of S_μ-like clone terminates with Eco RI linker sites. Restriction sites are ; EcoRI (↓), Hind III (▼), Bam HI (↓), Hinc II (▽), Pst I (↑) and Pvu II (↑).

that three clones, Dm·S_μ-6, Dm·S_μ-9 and Dm·S_μ-10 share overlapping segments and encompass an about 32kb region of the Drosophila genome.

Since there are multiple bands of the S_μ-like sequence in Drosophila DNA, we further confirmed that the restricton fragments expected from the constructed chromosomal map was identified in the genomic DNA. When the fragment A was used as probe, the 17kb Eco RI and 21kb Bam HI fagments of the Drosophila DNA were hybridized in consistence with the restriction map of the clones. Similarly, the 17kb Eco RI fragment expected from the chromosomal map was identified in Drosophila DNA by hybridization with the fragment C as probe. The rest of the clones have quite distinct restriction maps and do not overlap with any part of Dm·S_μ-6, Dm·S_μ-9 and Dm·S_μ-10 DNA. Dm·S_μ-17 and Dm·S_μ-18 share considerable portions in common (Fig. 3).

Nucleotide Sequence of *Drosophila* S_μ-like Sequence

We have determined partial nucleotide sequences of the S_μ-related DNA

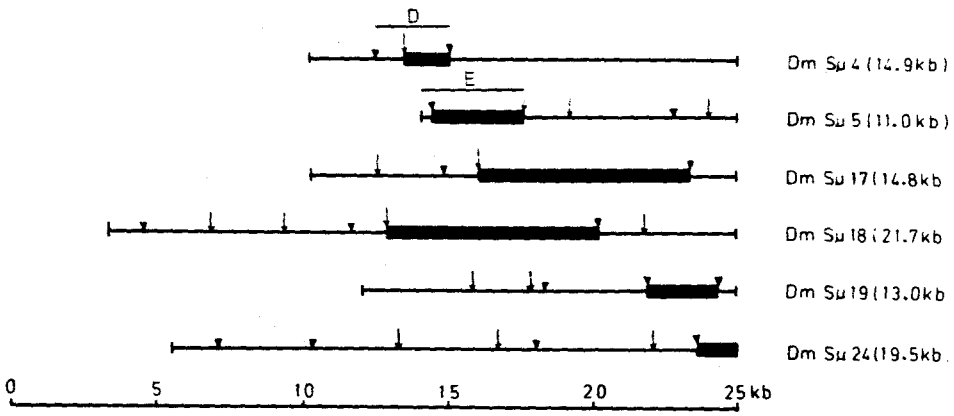

Fig. 3. Restriction maps of the cloned Drosophila DNA containing S_μ-like sequences.
The S_μ-like sequences are represented by closed squares. Restriction sites are ; Eco RI (↓) and Hind III (▼).

segment and its flanking regions of Dm·S_μ-9. The S_μ-like sequence was localized by Southern blot hybridization and sequenced by the strategy shown (Fig. 2). The nucleotide sequences I, II, III, IV and V consist of rather simple sequences as shown in Fig. 4. There are abundant homo-trimers and homo-pentamers. In the sequence V we found a simple tandem repetition of two kinds of 9 nucleotides, GAGCTGGGT and GAGCTGGGG which repeated seven times without interspaced sequences. The unit sequences determined are almost identical to that of the mouse S_μ, namely GAGCT and GGGGT. However, repetition of the S_μ-like sequence in Drosophila is very short in length (63bp) as compared to the mouse S_μ region (about 3kb). When the Hap II fragment (600bp) containing the right half of the sequence V was digested with Alu I, four large fragments (240, 140, 120 and 100bp) were produced, indicating that the Hap II fragment does not have more Alu I sites (AGCT) other than found in the sequence V. Similarly there is only one other Hph I site (GGTGA) than shown in the sequence V. We conclude, therefore, that there is no other S_μ-like sequences in the visinity of the sequence V. The presence of AAAAAA and TTTTTT at the left boundery of the S_μ-like segment is interesting. Such streches of T (or A) base also have been found in the boudaries of repeated DNA elements (30,31)

We have asked whether or not the cloned S_μ-like sequences are conserved among Drosophila species. Fragments B, D and E (Fig. 2 and 3), which carried S_μ-like sequences, were isolated and hybridized to restricted DNAs of D.

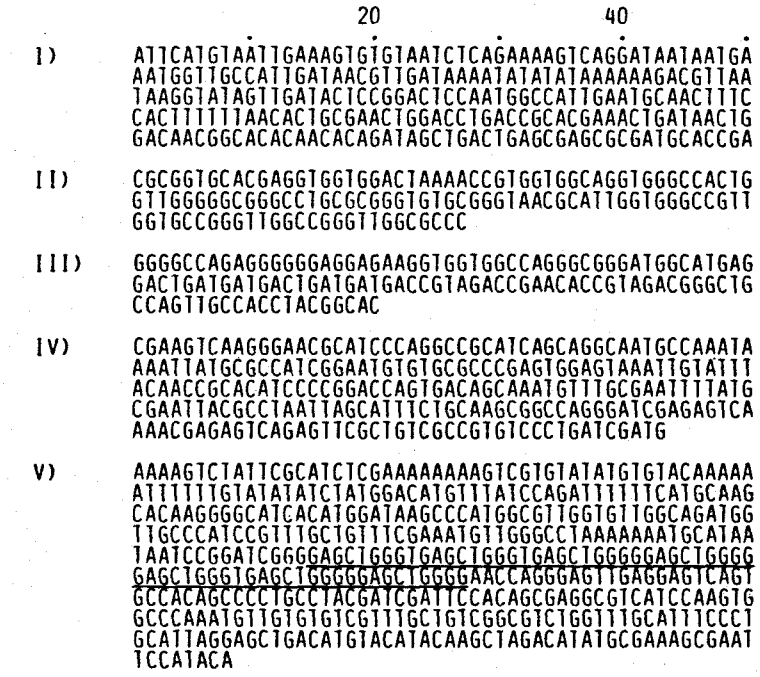

Fig. 4. Nucleotide sequences surrounding the S_μ-like sequence of Dm S_μ-9. Eco RI/Hinc II fragment (1.8 kb) of pDm S_μ-9 was sequenced. Sequences I, II, III, IV and V are indicated in Fig. 2. Underlined sequence in the sequence V comprises 7 repeat of 9bp units.

melanogastor, D. simulans and D. virilis. As shown in Fig. 5, DNAs of three stocks of D. melanogastor contained the single identical restriction fragment which hybridized to the fragment B. However, D. virilis did not have any sequences which hybridized with the fragment B except for a faint band similar in size to that of D. melanogaster. Similar results were obtained using the fragment E as a probe. In addition, DNA of D. simulans contained the identical restriction fragment hybridizing to the fragment E with that of D. melanogastor. D. virilis did not have any bands other than two very faint ones.

On the other hand, the fragment D hybridized to many minor bands in addition to the major band expected from the restriction map of the clone Dm·S_μ-4 in DNAs of D. melanogastor and D. simulans. Again, DNA of D. virilis did not contain the major band hybridizing to the fragment D although a number of minor bands were observed. These results indicate that the sequences flanking to the S_μ-like sequences in Dm·S_μ-6 and Dm S_μ-5 are unique, giving a single major

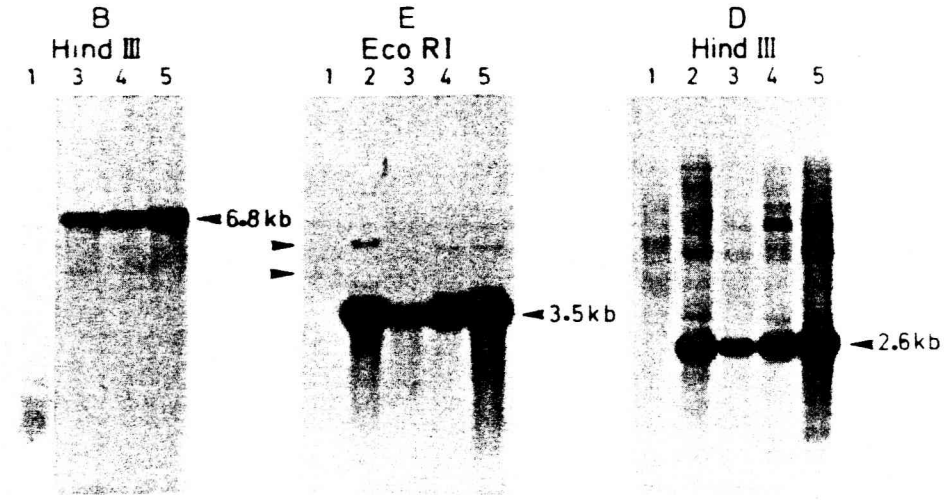

Fig. 5. Southern blots of Drosophila DNA hybridized with cloned Drosophila S_μ-like sequences and their flanking sequences.

Drosophila genomic DNA (2µg) were digested with a 5-fold excess of restriction endonucleases (Eco RI and Hind III), separated by agrose gel electrophoresis, blotted onto nitrocellulose filters, and hybridized with radioactive probes derived from restriction fragments B, D and E shown in Fig. 2 and 3. The radioactive probes were B fragment of Dm S_μ-6, D fragment of Dm S_μ-4 and E fragment of Dm S_μ-5. DNA used in each lane is as follows; 1, D. virilis; 2, D. simulans; 3, D. melanogester, (Oregon R), 4, D. melanogaster (Hikone R) 5. D. melanogaster (T(Y;2) CyO, DTS-513). Arrows indicate the faint bands in lane 1.

hybridizing band for each probe. The sequences are not necessarily conserved among Drosophila species but rather likely to be deleted in D. virilis. Furthermore, the sequences in the fragment D seem to contain diverged sequences which cross-hybridize variety of restriction fragments.

We have further confirmed the divergence of the S_μ-like sequences in Drosophila using mouse S_μ probe. Even among DNAs of three different stocks of D. melanogaster, there are differences in relative intensities of several S_μ-hybridizing bands although a pattern of bands is similar as shown in Fig. 6. The profile of the S_μ-like bands in D. melanogaster is clearly different from those in D. simulans and D. virilis. These results taken together, the S_μ-like sequences and their flanking sequences move rapidly during evolution. The repetitive nature of the S_μ-like sequences may be responsible at least in part for such evolutionary rearrangements.

Do S_μ-like Sequences Rearrange during Development?

The next obvious question is whether or not the S_μ-like sequences rearrange

during differentiation of Drosophila. DNAs were extracted from 0-12hr embryos, larvae, early pupae, late pupae, adult heads, adult bodies and whole adult corpuses, and their S_μ-like sequences were compared by Southern blot hybridization using cloned S_μ-like DNAs and mouse S_μ DNA as probes. We were unable to detect any significant changes by these analyses which had obvious limitations such that we could detect only changes taking place in a significant proportion of the cells. It is possible that a S_μ-like sequence rearranges only in a few percent of cells of a larva, which we will never see by this analysis.

DISCUSSION

What is the Significance of the S_μ-like Sequence in Invertebrates?

The S sequence has been shown to mediate the class switch recombination of immunoglobulin genes. The nucleotide sequence of the S_μ region seems to be important for the organism since it is conserved more than the coding sequences between mouse and human (10,11), although the S region does not code for any protein. In fact we have recently isolated a pseudo gamma (14) and pseudo epsi-

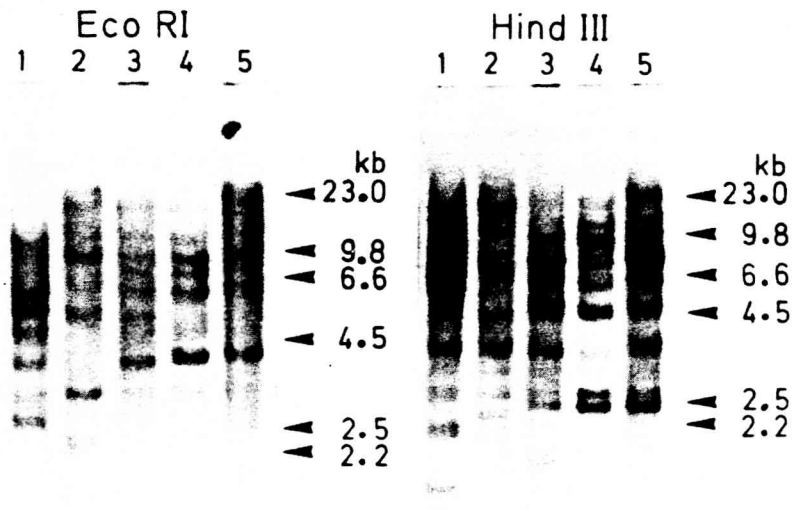

Fig. 6. Southern blot of Drosophila DNA hybridized with mouse S_μ probe. Drosophila DNA (2µg) was digested with a 5-fold excess of Eco RI and Hind III, separated in agarose gel electrophoresis, blotted onto nitrocellulose filters, and hybridized with radioactive S_μ (3.7 kb fragment in Fig. 1). DNA used in each lane is; 1, D. virilis; 2, D. simulans; 3, D. melanogaster (Oregon R); 4, D. melanogaster (Hikone R); 5, D. melanogaster (T(Y,2) CyO, DTS-513).

lon (13) genes which seem to have lost the S region. The S region seems to be essential to immunoglobulin heavy chain genes which undergo class switch recombination.

It is well known that the primitive vertebrates like hagfish and lamprey are able to produce only an IgM-like immunoglobulin (32). In these organisms it is unnecessary or, more precisely, impossible to switch the class because they have only the mu gene. Then, a simple question arises. Does the mu gene of hagfish and lamprey carry the S_μ region? Nobody knows the answer yet but there are several reasons which allow us to presume that the mu genes of hagfish and lamprey may not have the S_μ region. The S_μ-like sequence is not found in the kappa or lambda gene which may be an ancestral gene of the mu gene. Since there is no use of the S_μ-like sequence in these organisms there is no selection pressure to maintain it even if they had the S_μ sequence at the beginning.

The important question is what is the origin of the S_μ region in higher vertebrates which have multiple C_H genes. It is easier to imagine that the S_μ region was introduced into the C_μ-flanking region from another gene probably immediately prior to the duplication of the C_μ gene than to assume that the S_μ sequence was created together with the C_μ gene and conserved in lower vertebrates which cannot make use of the sequence (Fig. 7). It is also possible, albeit more difficult, that the S region sequences were introduced after duplication of the C_H gene.

We speculate that the S_μ sequence was transferred from some other gene which has the S_μ-like sequence but nothing to do with the immunoglobulin gene just like one we cloned from Drosophila. Then, what is the function of such S_μ-like sequences in invertebrates which do not make the immunoglobulin? One attractive speculation might be that S_μ-like sequences are responsible for DNA

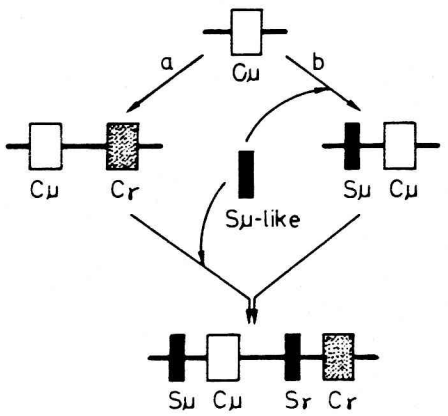

Fig. 7. Possible Evolutionary Origin of S Sequence in the Immunoglobulin Gene. Two pathways to create the C_H gene cluster comprising S_μ, C_μ, S_γ and C_γ were schematically represented. In (a) the S_μ-like sequence was incorporated into the C_H genes after duplication of the C_μ gene. In (b) the S_μ-like sequence was transferred to the visinity of the C_μ gene before the duplication.

rearrangements in invertebrates as recently reported about the Alu family in human (33). Immunoglobulin genes might have borrowed the DNA-rearranging machinery that had been already established in other gene systems yet to be exploired.

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参考論文

ORGANIZATION OF IMMUNOGLOBULIN HEAVY CHAIN GENES AND GENETIC MECHANISM OF CLASS SWITCH[1]

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INTRODUCTION

During differentiation of a given B lymphocyte, a single variable (V) gene is first expressed as a part of the μ chain and at a later stage, the constant (C) region of the expressed heavy (H) chain switches from μ to γ or to α without alteration of the V region sequence. Genetic and molecular bases for such a phenomenon called H chain class switch is one of the most fascinating biological questions.

Comparison of rearranged and germline H chain genes lead us to propose a molecular mechanism to explain H chain class switch (1-3). A complete H chain gene is formed by two types of recombinational events as shown in Figure 1. The first type of recombination takes place between a given V_H, D and a J_H segments, which completes a V region sequence. This recombination is referred to as V-D-J recombination. After such recombination the V region sequence is expressed as a part of the μ chain. The second type of recombination takes place between S_μ and S_γ (or S_α) regions. The S region was defined as a functional region responsible for the class switch and assumed to be located in the 5' flanking region of each C_H gene. The S_μ region is located between J_H and the μ gene and the S_γ (or S_α) is present at the 5' side of each γ gene (or α gene). The second type of recombination, which is called S-S recombination, can replace the C part of the H chain without affecting the V region sequence. Other groups (4,5) also reached similar conclusion.

Both types of recombination result in the deletion of the intervening DNA segment from the chromosome (6-12). The order of C_H genes was previously proposed to be 5'-μ-γ3-γ1-γ2b-γ2a-α-3' (6). This is based on the deletion

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profile of C_H genes in myelomas which produce different classes of the H chain proteins.

In this paper we will present the direct evidence for the proposed organization of the C_H gene cluster as described in Figure 1. We will also describe the structural basis of the S region which was originally defined as a functional region and discuss the molecular mechanism of the deletion. Finally, we will present evidence that there are conserved segments at the 3' side of each C_γ gene.

Linkage of Mouse C_H Genes

Most of the mouse C_H gene fragments including C_μ, C_δ, $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, $C_{\gamma 3}$ and C_α have been cloned. We have already reported complete nucleotide sequences of the C_μ, $C_{\gamma 1}$, $C_{\gamma 2a}$ and $C_{\gamma 2b}$ genes (13-16). Recently, we have suceeded in cloning the ε gene from an IgE-producing hybridoma (17). Partial nucleotide sequences of the cloned ε gene were determined and the amino acid sequences deduced from the nucleotide sequences were similar to that of the human ε chain (Figure 2). Using these cloned DNA segments as probes, we have isolated overlapping chromosomal segments of the C_H gene cluster from phage libraries containting embryonic mouse DNA (17-19). We have isolated a number of clones containing C_H genes and their flanking regions and aligned them by characterization with restriction enzyme cleavage and

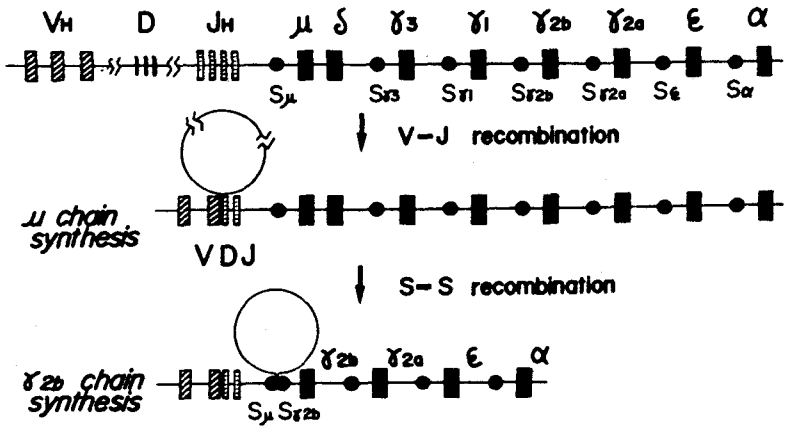

FIGURE 1. A genetic mechamism of heavy chain class switch. Recombination events, which take place to form a complete $\gamma 2b$ gene during differentiation of a B lymphocyte, are schematically represented.

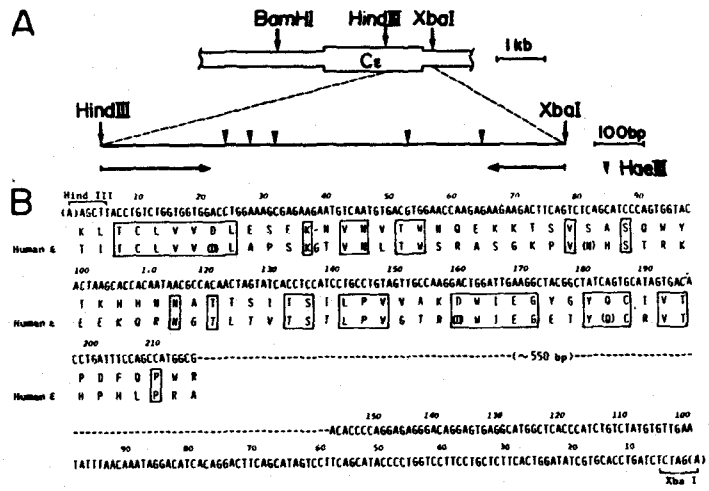

FIGURE 2. Partial nucleotide sequence of the ε gene. (A) Strategy for nucleotide sequence determination. The 0.95 kb Hind III-Xba I fragment was isolated and sequenced according to Maxam and Gilbert (20). Ranges and direction of sequences read are shown by horizontal arrows. (▼), Hae III sites. (B) Nucleotide sequences at both termini. The amino acids predicted by the nucleotide sequence are shown under the coding sequences. The amino acid sequence corresponding to the C_{H3} domain (residue 345-418) of human ε chain (21) is presented at the bottom row and the homologous amino acids are boxed. Amino acids are expressed by one letter code. Reproduced from Nishida et al. (17).

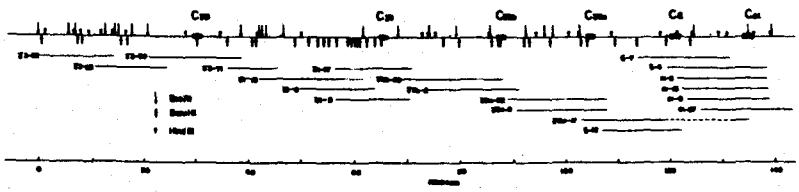

FIGURE 3. Restriction endonuclease cleavage maps of overlapping cloned fragments in the C_H gene cluster. The chromosomal segment containing γ3, γ1, γ2b, γ2a, ε and α genes is shown schematically. Mouse C_H gene clones were isolated from phage libraries of embryonic mouse (Balb/c) DNA and characterized by restriction enzyme cleavage, Southern blot hybridizaton and R-loop formation. All the clones were aligned with use of overlapping regions (3,17-19). Closed boxes indicate structural genes.

Southern blot hybridization experiments. Figure 3 summarizes all the clones thus far characterized.

For example, when we screened a partial Sau 3A fragment library with the ε gene probe, we isolated two clones which were designated as clones 6 and 7. Fortunately, clone 6 was shown to hybridize with the α gene probe and the linkage of ε and α was easily determined.

The most difficult part was the linkage between the γ3 and γ1 genes. We started from both directions and two steps from both directions were required to join two genes. In each step we isolated the 5' or 3' terminal fragment of the newly isolated clone and used as a probe. So far, we have isolated clones covering more than 150 kp in the C_H gene cluster. It is clear that the C_H gene is ordered γ3, γ1, γ2b, γ2a, ε and α, which is in agreement with the order proposed previously (6) and with the order of γ subclass genes reported by Tonegawa and his associates (5,22). We have confirmed that these cloned DNA segments are found in germline DNA by Southern blot to exclude the possibility of cloning artefact.

Several groups reported that J segments are present about 6.5 kb 5' of the μ gene. (23-25). Liu et al. (26) reported that the δ gene is only 4.5 kb 3' to the μ gene. These results taken together, the general organization of the immunoglobulin C_H gene is now elucidated except that we do not know the distance between the δ and γ3 gene (Figure 4). It is worth noting that all the C_H genes ordered have the same orientation and thus are transcribed from the same strand of DNA.

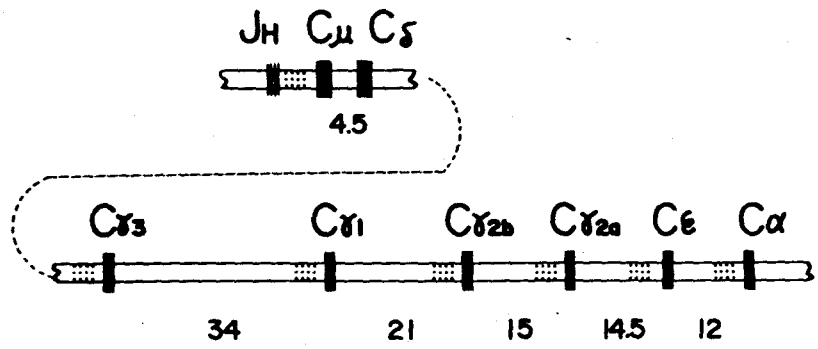

FIGURE. 4 Summary of the C_H gene organization. Closed boxes indicate structural genes. Dotted areas show regions containing repetitive sequences. Numbers in kb.

Structure of the S Region

Originally we have defined the S region as such that is functionally responsible for the class switch recombination. Recent nucleotide sequence determination indicated that the structural basis of the S region is the beautiful tandem repetition of related nucleotide sequences. The class switch recombination sites of 7 rearranged clones were thus far determined and they cluster in the region located about 5 kb upstream of the C_μ gene. This region was previously sequenced and characteristic of a simple repetitive sequence (2,25). Downstream to this region there is a region which is often deleted during cloning. The μ gene fragments cloned by other groups seem to have such deletion (23,25). Fortunately, we have isolated and characterized the whole S_μ region, probably because we used λgtWES as vector in stead of Charon 4A which was used by others.

Nucleotide sequences of the S_μ region can be represented by (GAGCT)n(GGGGT) where n ranges from 1 to 17 with the highest frequency at 3 (27). The core part of the S_μ region was very difficult to be sequenced because there were not appropriate restriction sites. When we digested it with Alu I and analyze the digests by polyacrylamide gel

Table I Nucleotide Sequences of Repeat Units of S Regions

S Region	Unit Sequences
S_μ	GAGCTGAGCTGGGGTGAGCT
$S_{\gamma 1}$	GPPTCCAGGCTGAGCAGCTACAGGGGAGCTGGGGYAPPTGGGAPTPTPG
$S_{\gamma 2b}$	GGGACCAG$^{T}_{A}$CCTAGCAGCTPTGGGGGAGCTGGGGA$^{A}_{T}$GGTPGGAPTPTGA
$S_{\gamma 3}$	PGNACC$^{A}_{T}$GPNTPAGCAPYYACAGGGGAGC$^{T}_{A}$GGGG$^{A}_{T}$PGGTGGGAGTATPP
S_α	ATGAGCTGGGATGAGCTGAGCTAGGCTGGAATAGGCTGGGCTGGGCTGGT
	GTGAGCTGGGTTAGGCTGAGCTGAGCTGGA
Common Sequences	GAGCTG, TGGGG

Common sequences are underlined. Sources of sequences are S_μ (27), S_γ (28) and S_α (29).

electrophoresis, major bands of 5 and 10 bp and minor bands of 20 and 15 bp were found. The results indicate that the core sequence also contains a tremendous number of Alu I sites (AGCT) probably one site every five or ten bases. This is consistent the sequence determined at the region adjacent to the core fragment. There are regions which contain precise tandem repeat of 20 bp unit exactly where deletion occurrs.

We have also determined partial nucleotide sequences surrounding the class switch recombination site in the $S_{\gamma 1}$, $S_{\gamma 2b}$ and $S_{\gamma 3}$ regions (28). All of them comprise tandem repetition of 49 base-pair units. The nucleotide sequence of the $S_{\gamma 2b}$ region shows that each repeating unit is similar to each other. Such tandem repetitive region lies between 1.8 to 5.2 kb 5' to the structural gene. We have also determined a partial nucleotide sequence of the S_{α} region, which comprises tandem repetition of 80 bp unit (29,30). Table 1 summarizes nuclotide sequences of the repeat units of S regions. The S_{μ} region shares short common sequences (GAGCTG and TGGGG) with other S regions. GAGCTG and TGGGG appear 50 to 100 times in all the S regions.

A combined structure of tandem repetition and short common sequences provides a large number of the possible recombination sites in the S region and increase the chance of S-S recombination. The S-S recombination does not have to be highly specific to the nucleotide joined together because it takes place in the intervening sequence. Instead, the class switch recombination is expected to be efficient since it takes place during relatively short period of time after stimulation with an antigen. The above structure satisfies these biological features required for the class switch recombination. We think that these repetitive sequence is the structural basis for the S region. We find the repetitive sequence in the 5' flanking region of each C_H gene except for the δ gene which we have not tested as shown in Figure 4.

We have cloned human μ gene from human DNA library and compared its structure with mouse μ gene (31). Studies using heteroduplex analysis and Southern blot hybridization clearly show that not only the coding region but also the S_{μ} region is homologous between human and mouse μ genes. The results suggest that the nucleotide sequence in the S_{μ} region plays an essential biological function and thereby it has been conserved in these organisms for decades of million years (31,32).

In addition, we found that the S_{μ}-related sequences are found among a wide variety of organisms. It is remarkable that DNA of sea urchin, which obviously does not have immunoglobulin genes, also contains clear bands hybridizing with mouse S_{μ} sequence. The results indicate that the immunoglobulin gene seems to have used, as S region sequences, some pre-

existing sequence which may or may not have other biological functions. We have cloned these S_μ-related sequence from sea urchin, Drosophila and Xenopus (33). We are going to test that these sequences are linked to a gene expressed in these organisms or not.

Sister Chromatid Exchange Model

It is established that deletion of C_H genes accompanies the S-S recombination. Two alternative models can be proposed to explain the mechanism of the C_H gene deletion in B-lymphocytes as shown in Figure 5 (3,29). The first model postulates that the S-S recombination takes place on a single chromosome by mutual recognition of two S regions. The intervening DNA segment if 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 DNA segment by an unequal crossing-over event between sister chromatids. 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.

In either case we think the basic mechanism of the S-S recombination is mediated by repeated short common sequences. It is likely that a putative recombinase or a recombinase complex catalyze excision and ligation of two DNAs. But actual recombination sites do not seem to be highly specific to the nucleotide joined together.

Structural analyses of an expressed $\gamma 1$ gene clone lead us to conclude that the sister chromatid exchange model may be more favorable. The structure of the expressed $\gamma 1$ gene of myeloma MC101 can be represented as follows; $5'\text{-}V\text{-}D\text{-}J\text{-}S_\mu\text{-}S_\alpha\text{-}S_{\gamma 1}\text{-}C_{\gamma 1}\text{-}3'$ (29,34). This $\gamma 1$ gene contains a short S_α segment(490 bp) between S_μ and $S_{\gamma 1}$ regions. This fact appears to contradict the linear arrangement of C_H gene ($5'\text{-}\mu\text{-}\gamma 3\text{-}\gamma 1\text{-}\gamma 2b\text{-}\gamma 2a\text{-}\varepsilon\text{-}\alpha\text{-}3'$) and the stepwise looping-out mechanism. Such $\gamma 1$ gene, however, can be created by two or three successive unequal crossing-over events.

There are various possible pathways to create MC101 $\gamma 1$ gene, several examples of which are illustrated in Figure 6. In one pathway, 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

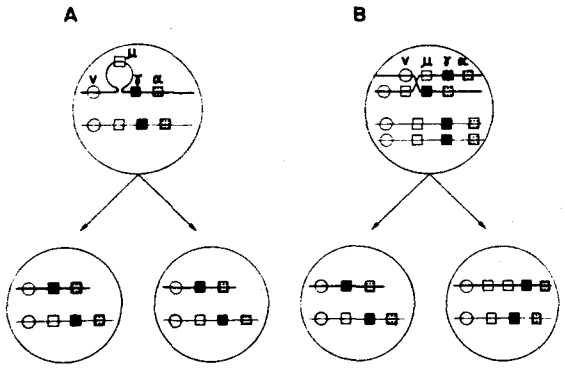

FIGURE 5. Possible models for deletion of C_H genes in class switch. A. looping-out model. B. sister chromatid exchange model. Reproduced from Obata et al. (29).

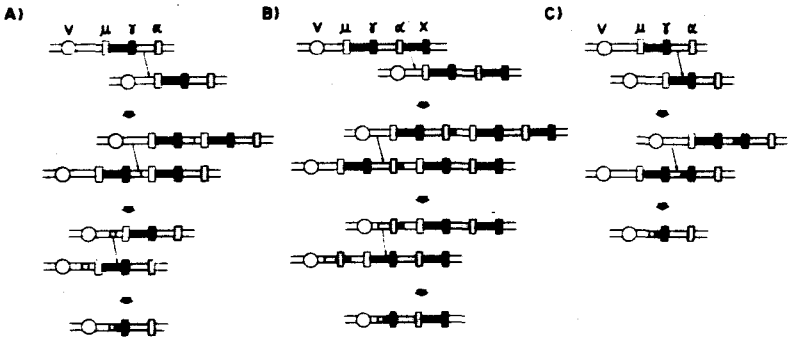

FIGURE 6. Possible pathways to create the expressed γ1 gene of MC101 myeloma. Reproduced form Obata et al. (29).

giving rise to a γ gene that is linked to a V gene, the S_μ and the S_α region at its 5' side. In a second pathway we postulated another C_H gene at the 3' side to the α gene.

In a third we allowed a recombination event that does not result in the class switch. 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. The number of clones switching in the reverse direc-

tion may be lower than that switching in the forward direction because the number of the recombination 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. (35) recently reported that a variant of myeloma X63 can switch from γ2b to γ1. Reverse switch can be easily explained by sister chromatid exchange as shown Figure 6 B.

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 innevitably produces progeny clones with duplicated as well as deleted C_H genes.

Membrane Domain Exons in γ Genes

We have compared the flanking regions of different γ genes by heteroduplex analyses. Such study revealed interesting homology regions in the 3' flanking region of all the γ genes (36). Heteroduplex molecules formed between γ2a and γ3 genes showed four separate homology regions as shown in Figure 7. Comparison of these pictures with the restric-

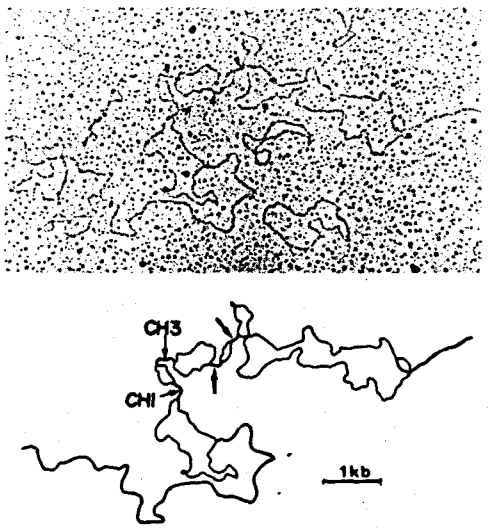

FIGURE 7. Heteroduplex formed between γ2a and γ3 genes. Upper picture shows electronmicrograph of the heteroduplex formed between Igγ2a-11 and Igγ3-31 (clone of J606) and its interpretation is illustrated below. The homology regions in the 3' flanking region are indicated by arrows.

tion maps of the γ2a and γ3 genes indicated that two homology regions are located at the 5' side of the clones correspond correspond to the CH1 and CH3 domains. In the 3' flanking region there are two homology regions of 270 and 250 bp in length which are separated by a 550 to 600 bp long intervening sequence. These 3' homology regions are located about 1.4 to 1.7 kb 3' to the CH3 domain. Similar analyses carried out with all the γ genes showed that all the γ genes have two homology regions of similar size at similar location. It is likely that these conserved segments are membrane domains similar to those found in the μ gene (37,38). We have tested this possibility by hybridizing a DNA segment of the γ2a-gene homology region to 2PK3 mRNA (prepared by Drs. V. Oi and L. Herzenberg) which contains mRNA encoding membrane-form γ2a chain (39). The homology segment of the 3' flanking region of the γ2a gene hybridized to 4kb mRNA while the DNA segment of the CH1 and CH2 domains hybridized to both 1.8 and 4 kb mRNAs. We have also demonstrated R-loop formation between 2PK3 mRNA and γ2a gene with the size and location similar to the homology regions in the 3' flanking region.

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Immunology

A T15-idiotype-positive T suppressor hybridoma does not use the T15 V_H gene segment

(phosphocholine-specific hybridoma/T suppressor factor/Southern blot analysis)

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ABSTRACT The T suppressive factor (TsF) released from a T15-idiotype-positive phosphocholine (*P*Cho)-specific T hybridoma, F18-3-4, which was formed by fusion between BALB/c T cells and BW5147 thymoma, was immunochemically characterized. TsF inhibited the *in vitro* induction of both IgE and IgG1 antibody responses of 2,4-dinitrophenyl keyhole limpet hemocyanin (DNP-KLH)-primed spleen cells in the presence of *P*Cho-KLH-DNP. TsF had the ability to bind to *P*Cho determinants and possessed T15 idiotype determinants as well as Ia^d products. However, we were unable to detect either the rearrangement of the T15 V_H gene or the presence of T15 V_H gene transcripts in hybridomas by DNA and RNA blot hybridization analyses with the T15 V_H DNA probe.

Both B and T lymphocytes show antigen specificity. Antigen-binding receptors of B lymphocytes are known to be membrane forms of conventional immunoglobulin molecules. In spite of numerous serological studies on T cell receptors, however, the molecular properties of antigen-binding receptors of T lymphocytes are almost entirely unknown. Binz and Wigzell (1) and Rajewsky and Eichmann (2) have documented that T and B lymphocytes specific for the same antigenic determinant express the same idiotype and that the idiotypes of T cell receptors are controlled by genes in the immunoglobulin (Ig) heavy (H) chain linkage group. Since then, numerous other investigators have obtained similar evidence supporting the same conclusion (3, 4). Thus, those results suggested that antigen receptors of T lymphocytes possessed antigen-combining sites encoded by genes for the immunoglobulin H chain variable region (V_H), although their constant portions were not identical to those of immunoglobulin molecules.

Recently, we have established phosphocholine (*P*Cho)-specific suppressor T hybridomas by somatic cell hybridization of the BW5147 thymoma cell line with T cells primed with *P*Cho-conjugated mycobacteria (*P*Cho-Myc) (5). A *P*Cho-specific T hybridoma, F18-3-4, was stained with anti-T15 idiotype (T15 Id) antibody and showed rosette formation with *P*Cho-conjugated sheep erythrocytes (SRBC) that was specifically inhibited by the addition of anti-T15 Id antibody. Hybridoma F18-3-4 secreted a T suppressive factor (TsF) that showed *P*Cho-specific suppression on both the IgE and IgG1 antibody responses. In this study, we immunochemically characterized the TsF molecules and analyzed genes coding for *P*Cho-specific TsF by employing cloned T15 V_H genes. The results show that TsF has *P*Cho-binding sites with T15 Id but the T15 V_H gene is not rearranged in the T hybridoma.

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MATERIALS AND METHODS

Animals. BALB/c mice (8–10 weeks old) were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Japan).

Antigens. *p*-Aminophenylphosphocholine was prepared from *p*-nitrophenylphosphocholine (Sigma) and diazotized as described (6). (*P*Cho)-derivatized *Mycobacterium tuberculosis*, Aoyama B strain (*P*Cho-Myc), *P*Cho-conjugated keyhole limpet hemocyanin (*P*Cho-KLH), *P*Cho-conjugated bovine serum albumin (*P*Cho-albumin), and 2,4-dinitrophenyl (DNP) derivatives of KLH or *P*Cho-KLH were prepared as described (5).

Myeloma Proteins and Anti-T15 Id Antibody. *P*Cho-binding myeloma proteins (TEPC15, MOPC511, and MOPC167) were purified with *P*Cho-tyramine-Sepharose CL-4B as described (6). Anti-T15 Id antiserum was prepared in rabbits. The antiserum was extensively adsorbed with a MOPC511-coupled Sepharose CL-4B column. Then, anti-T15 Id was specifically purified by passage through a TEPC15-coupled Sepharose CL-4B column. The specificity of anti-T15 Id was examined by inhibition solid-phase radioimmunoassay as described (6).

Generation of T15 Id-Positive T Hybridomas. Serologically defined T15 Id^+ T hybridoma F18-3-4 was selected from clones that had been established by hybridization of the BW5147 thymoma cell line with the T cell fraction (BALB/c) enriched from *P*Cho-Myc-primed T cells by anti-T15 Id-coated plastic Petri dishes (5). Cell surface phenotypes of F18-3-4 were $H\text{-}2^{k+}$, $H\text{-}2^{d-}$, $Thy\text{-}1.2^+$, $Lyt\text{-}1.2^-$, $Lyt\text{-}2.2^+$, Ig^-, and T15 Id^+ as determined by immunofluorescence and microcytotoxicity tests. About 30–40% of cells consistently formed rosettes with *P*Cho-coupled SRBC (*P*Cho-SRBC); formation was specifically inhibited by preincubation of cells with *P*Cho-albumin or anti-T15 Id. The details were described in a previous article (5).

***In Vitro* Antibody Formation for Testing the Suppressive Activity.** The culture system has been described in detail (5). Briefly, DNP-KLH-primed spleen cells (25×10^6) were suspended in 1 ml of either fresh culture medium (Eagle's minimal essential medium supplemented with 10% fetal calf serum, penicillin at 100 units/ml, and streptomycin at 100 μg/ml) or the supernates from T hybridomas that had been extensively dialyzed against fresh culture medium and stimulated with *P*Cho-KLH-DNP or KLH-DNP at 1 μg/ml. Four days later, a reverse plaque assay was carried out to enumerate immunoglobulins (IgE or IgG1)-secreting cells as described (7). All cultures were set up in duplicate.

Abbreviations: *P*Cho, phosphocholine; ABA, *p*-azobenzene arsonate; DNP, 2,4-dinitrophenyl; Mlg, mouse immunoglobulin; T15 Id, idiotype of TEPC15 myeloma protein; V_H, variable region of immunoglobulin heavy chain; TsF, T suppressive factor; Myc, mycobacteria; KLH, keyhole limpet hemocyanin; SRBC, sheep erythrocytes; kb, kilobases; PFC, plaque-forming cells.

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Affinity Chromatography. Immunoadsorbents were prepared by conjugation of protein-ligands to Sepharose CL-4B activated with cyanogen bromide as described (6). The immunoadsorbents used in this study were Sepharose coupled to anti-IgG1 (1.5 mg of goat anti-mouse IgG1 per ml of beads), anti-IgA (3 mg of goat anti-mouse IgA per ml of beads), anti-IgM (2.3 mg of goat anti-IgM per ml of beads), anti-Mlg (2 mg of rabbit anti-mouse Ig per ml of beads), anti-T15 Id (2.4 mg of rabbit anti-T15 Id per ml of beads), anti-Ia^d ($I\text{-}J^d$, E^d, C^d) [2 ml of anti-B10BDR, from (B10 × D2GD) F_1 mice per ml of beads, kindly provided by D. C. Shreffler (Washington Univ.)], IgE (2 mg of monoclonal mouse anti-DNP-IgE per ml of beads), *P*Cho-tyramine, and *p*-azobenzene arsonate coupled to bovine serum albumin (ABA-albumin). Affinity chromatography was carried out by the incubation of cell-free supernatants with beads at 20:1 volume ratio for 1 hr at 4°C. After the beads had been washed with 20 vol of 0.025 M sodium borate-buffered saline (pH 8.0), the binding materials were eluted with 0.05 M sodium acetate buffer (pH 4.0, containing 0.085 M NaCl, 0.005 M KCl, and 0.1% fetal calf serum), immediately neutralized with 1 M sodium bicarbonate buffer (pH 9.0), and concentrated by ultrafiltration with Diaflo YM10 membranes (Amicon).

External Radioiodination of the *P*Cho-Binding Molecules from F18-3-4 Supernates. The acid eluates of F18-3-4 supernates from anti-T15 Id-Sepharose columns were obtained as described above. The concentrated eluate was radiolabeled with 0.5 mCi (1 Ci = 3.7×10^{10} becquerels) of carrier-free $Na^{125}I$ (Amersham) by the chloramine-T method (8), then applied onto a *P*Cho-tyramine-Sepharose column in order to purify the *P*Cho-binding materials. When parallel experiments in which only ^{125}I labeling procedures were omitted were performed, the TsF activity of chromatographed material was 100-fold greater than the activities of original F18-3-4 supernates in limiting dilution assays.

Molecular Sieve Chromatography. The eluates of F18-3-4 supernates from *P*Cho-tyramine-Sepharose were concentrated by ultrafiltration with a YM10 membrane and applied to a Sephadex G-200 column (96 × 2 cm) that had been equilibrated with sodium phosphate-buffered saline (pH 7.4) and calibrated with IgG2a, bovine serum albumin, ovalbumin, and chymotrypsinogen. Two or 3 fractions (2.6 ml per fraction) were pooled and dialyzed against fresh culture medium to test for their suppressive activity.

Preparation of T15 V_H DNA Fragment. The total cellular DNA of TEPC15 myeloma was extracted (9) and digested to completion with *Eco*RI. The DNA fragment from 7.8 to 9.3 kilobases (kb) was purified by preparative agarose gel electrophoresis and ligated to outer fragments of DNA from phage λgtWES (10). The recombinant phage DNA was packaged *in vitro* into coat proteins (11). About 2×10^6 phages were screened with the ^{32}P-labeled J_{H4} gene segment, the 1.5-kb *Hin*dIII/*Eco*RI fragment containing the J_{H4} gene and its 3′ flanking sequence. Twenty-three positive clones were obtained. The T15 V_H clones were identified by comparison of their restriction map with the published one (12, 13). The 1.1-kb *Bam*HI fragment of the T15 V_H clone was inserted into the *Bam*HI site of pBR322 and subcloned. The 280-base-pair *Hha* I/*Hin*fI fragment consisting of only the coding sequence of the T15 V_H gene was isolated by polyacrylamide gel electrophoresis. This fragment was designated as the T15 V_H fragment.

Preparation of DNA and Southern Blot Hybridization. High molecular weight DNA was prepared according to the method described previously (9). DNA (3 μg) digested with restriction enzymes was electrophoresed in 0.5% agarose gels, transferred to nitrocellulose filters (14), hybridized with nick-translated probes, and autoradiographed as described (9).

Preparation of RNA and Blot Hybridization. Cytoplasmic RNA was extracted from myelomas and T hybridomas as described previously (15) except that the homogenizing buffer was replaced by 0.1 M Tris·HCl (pH 9.0) containing 0.25 M sucrose, 0.1 M NaCl, and RNase inhibitor from rat liver at 5 units/ml (16). Poly(A)-containing RNA was enriched by two successive applications onto oligo(dT)-cellulose columns and transferred to nitrocellulose filters as described (17). Hybridization was performed under the conditions of Southern blot hybridization (9). After hybridization, filters were rinsed twice with 2× NaCl/Cit (1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate)/0.1% $NaDodSO_4$ at room temperature and then washed twice in 0.1× NaCl/Cit/0.1% $NaDodSO_4$ for 15 min at 50°C.

RESULTS

Antigen (*P*Cho)-Specific Suppressive Effect of Culture Supernatants from T15 Id^+ T Hybridoma F18-3-4. DNP-KLH-primed spleen cells (25×10^6) were suspended in 1 ml of fresh culture medium, supernates of BW5147, or supernates of F18-3-4 and stimulated with *P*Cho-KLH-DNP or KLH-DNP at 1 μg/ml. Four days later, IgE- or IgG1-secreting cells were enumerated by a reverse plaque assay. As shown in Table 1, supernates of F18-3-4 suppressed both the IgE and IgG1 responses when DNP-KLH-primed spleen cells were stimulated with *P*Cho-KLH-DNP but not with KLH-DNP. Supernates of BW5147 were employed for control experiments because either supernates of BW5147 or fresh medium showed the same response (5). The results showed that F18-3-4 cells secreted TsF that inhibited the induction of antibody responses in *P*Cho-specific and immunoglobulin class-nonrestricted fashion.

Immunochemical Characterization of TsF from F18-3-4. For the immunochemical characterization of the TsF from F18-3-4 cells, the supernates of F18-3-4 were adsorbed with various immunoadsorbents and tested for their suppressive activity. As shown in Table 2 (Exp. I), the suppressive activity was specifically removed by the adsorption with *P*Cho-tyramine-Sepharose beads but not with ABA-albumin-Sepharose beads. The TsF activity was also completely removed by the incubation with anti-T15 Id Sepharose, but not with anti-Mlg-, anti-IgM-, anti-IgG1, or anti-IgA-coupled Sepharose beads, showing that the TsF had no determinants of immunoglobulin constant portions. It was also found that anti-Ia^d ($I\text{-}J^d$, E^d, C^d)-Sepharose beads removed the TsF activity from the supernates.

In the next series of experiments, we attempted to elute the materials with TsF activity from these adsorbents. Among var-

Table 1. *P*Cho-specific suppressive effect of culture supernates from T15 Id^+ T hybridoma F18-3-4

Culture medium*	Antigen *in vitro*	PFC per culture† IgE	PFC per culture† IgG1
Fresh medium	None	62 ± 10	198 ± 17
BW5147	*P*Cho-KLH-DNP	239 ± 8	1,620 ± 127
BW5147	KLH-DNP	189 ± 8	1,452 ± 27
F18-3-4	*P*Cho-KLH-DNP	95 ± 8‡	275 ± 42‡
F18-3-4	KLH-DNP	189 ± 19	1,328 ± 34

DNP-KLH-primed spleen cells (25×10^6) were suspended in 1 ml of culture medium and stimulated with *P*Cho-KLH-DNP or KLH-DNP at 1 μg/ml for 4 days. All cultures were set up in duplicate.

* Cell-free supernate derived from T hybridomas or BW5147 had been dialyzed against fresh culture medium before use.

† Immunoglobulin-secreting cells were enumerated by a reverse plaque assay and the mean (±SEM) numbers of plaque-forming cells (PFC) were determined.

‡ Significantly suppressed response compared with that of corresponding control ($P < 0.01$).

Table 2. Immunochemical characterization of TsF in cell-free supernates from F18-3-4

Exp	Sepharose affinity column	Fraction of TsF*	% suppression of PFC response per culture† IgE	IgG1
I	None	Unfractionated	82 ± 3	81 ± 10
	ABA-albumin	Effluent	77 ± 2	85 ± 6
	*P*Cho-tyramine	Effluent	18 ± 7	<0
	Anti-T15 Id	Effluent	2 ± 13	10 ± 2
	Anti-Ia^d	Effluent	<0	27 ± 12
	NMS	Effluent	69 ± 2	86 ± 4
	Anti-MIg	Effluent	75 ± 5	71 ± 1
	Anti-IgM	Effluent	98 ± 8	>100
	Anti-IgG1	Effluent	>100	84 ± 9
	Anti-IgA	Effluent	100 ± 4	96 ± 8
II	None	Unfractionated	85 ± 2	71 ± 1
	*P*Cho-tyramine	Effluent	<0	28 ± 12
		Eluate	89 ± 12	85 ± 8
	Anti-T15 Id	Effluent	<0	9 ± 14
		Eluate	102 ± 2	56 ± 8
III	None	Unfractionated	66 ± 1	80 ± 3
	ABA-albumin	Effluent	69 ± 5	82 ± 5
	Anti-Ia^d	Effluent	<0	14 ± 4
		Eluate	52 ± 5	64 ± 2

See legend of Table 1. The antigen for *in vitro* stimulation was *P*Cho-KLH-DNP (1 μg/ml). NMS, normal mouse serum.

* Cell-free supernatants from F18-3-4 were adsorbed with 1/20th vol of an affinity matrix for 1 hr at 4°C. The binding materials were eluted with 0.05 M sodium acetate buffer (pH 4.0).

† Percent suppression = [1 − (experimental group − background)/(positive control − background)] × 100. Exp. I, background responses (*B*) (all responses expressed in PFC per culture) without an antigen were 47 ± 1 for IgE and 1,313 ± 61 for IgG1. Positive control responses (*P*) with *P*Cho-KLH-DNP were 170 ± 7 for IgE and 6,900 ± 707 for IgG1. Exp. II, *B* was 33 ± 2 for IgE and 171 ± 21 for IgG1; *P* was 80 ± 3 for IgE and 1,260 ± 59 for IgG1. Exp. III, *B* was 34 ± 6 for IgE and 378 ± 12 for IgG1; *P* was 127 ± 6 for IgE and 2,856 ± 60 for IgG1. All values are given ± SEM.

ious elution buffers employed in these experiments (0.1 M sodium citrate buffer, pH 3.5; 0.1 M sodium citrate buffer, pH 4.5; 0.175 M glycine·HCl, pH 3.2; 0.05 M sodium acetate buffer, pH 4.0; or 0.1 M NH_4OH, pH 10.7), 0.05 M sodium acetate buffer (pH 4.0) consistently eluted the TsF with intact suppressive activity from *P*Cho-tyramine- and anti-T15 Id-Sepharose beads. The other buffer systems, which are generally employed for the purification of antibodies, gave remarkable loss of TsF activity in the eluates from the beads (data not shown). The finding also suggested that the TsF molecules in the supernates were not conventional immunoglobulin molecules.

As shown in Table 2 (Exps. II and III), the TsF activity was detected in the eluates from *P*Cho-tyramine-, anti-T15 Id-, or anti-Ia^d-Sepharose beads. None of TsF activity was shown in effluents from these beads. The experiment outlined in Fig. 1 was carried out to confirm these results. Supernates of F18-3-4 were successively passed through *P*Cho-tyramine columns and either anti-Ia^d columns or anti-T15 Id columns, then tested for their suppressive activity. The suppressive activity was found in the acid eluates from *P*Cho-tyramine-Sepharose (fraction B). When fraction B was subsequently applied to an anti-Ia^d column, the effluents (fraction C) showed little suppressive activity, whereas the acid eluates (fraction D) from this column retained the activity. Similarly, in the case of the successive passage of fraction B through anti-T15 Id-Sepharose column, the suppressive activity was detected in the acid eluates (fraction E), but not in the effluents (fraction F). The acid eluates of BW5147 supernatants from a *P*Cho-tyramine column did not show any suppressive activity. When these results are taken together, it appears that *P*Cho-binding sites, T15 Id determinants, and Ia^d product are expressed on the same TsF molecule, although it is not known whether TsF is composed of a single polypeptide chain or more than one.

Estimation of Molecular Weight of TsF from F18-3-4. The acid eluates of supernates of F18-3-4 from *P*Cho-tyramine-Sepharose beads were concentrated 22-fold relative to the original volume by ultrafiltration and applied to a Sephadex G-200 column together with externally ^{125}I-labeled "TsF fraction" as described in *Materials and Methods*. As shown in Fig. 2, suppressive activity was detected in the fractions with the approximate molecular weight of 138,000. Radiolabeled "TsF fraction" as a tracer showed two distinct radioactive peaks. One fraction had a molecular weight of 138,000 and completely overlapped with the peak of TsF activity, whereas the other, smaller, fraction had a molecular weight of 54,000 and no suppressive activity.

Southern Blot Analysis. Serological and functional analyses of the suppressive factor from the *P*Cho-specific T hybridoma F18-3-4 suggested that the antigen-binding sites of the TsF might be encoded by the T15 V_H gene. To examine this pos-

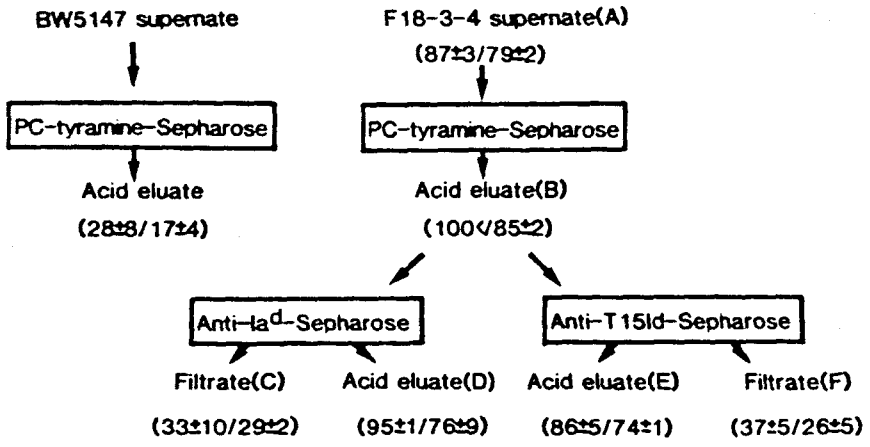

FIG. 1. Coexistence of antigen-binding sites, T15 Id determinants, and Ia^d products on a single TsF molecule. See footnotes of Table 2. The numbers in parentheses are percent suppression (±SEM) of IgE or IgG1 response (i.e., % suppression of IgE response/% suppression of IgG1 response). Positive control responses were 116 ± 13 PFC per culture for IgE and 4,675 ± 124 PFC per culture for IgG1. Background responses without antigen were 40 ± 2 PFC per culture for IgE and 668 ± 26 PFC per culture for IgG1.

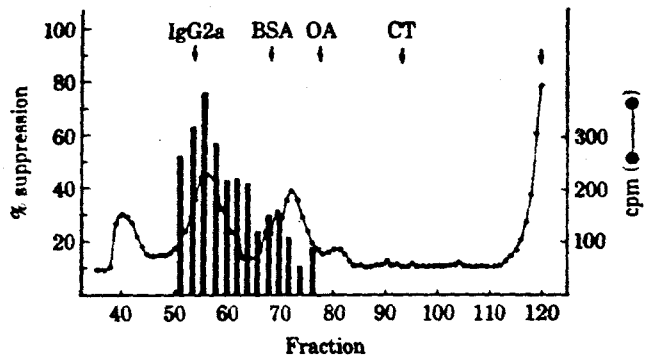

FIG. 2. Sephadex G-200 chromatography of *P*Cho-binding molecules from cell-free supernate of F18-3-4. After pools of two or three fractions (2.6 ml per fraction) had been dialyzed against fresh culture medium, they were tested for the suppressive activity (IgG1 response is presented by vertical bars as percent suppression; see Table 2 for definition of percent suppression). ●——●, cpm of ^{125}I-labeled TsF fraction sequentially purified with anti-T15 Id and *P*Cho-Sepharose columns from supernate of F18-3-4 cells. Marker proteins were IgG2a (M_r 155,000), bovine serum albumin (BSA; M_r 69,000), ovalbumin (OA; M_r 43,000), and chymotrypsinogen (CT; M_r 26,000).

sibility, we cloned the T15 V_H DNA probe from a myeloma cell line, TEPC15, and studied whether the T15 V_H gene of F18-3-4 cells was rearranged or not. Southern blot analysis of restriction fragments of F18-3-4 DNA with the T15 V_H DNA probe is presented in Fig. 3. We have employed hybridization conditions under which only the T15 V_H gene is detected and cross-hybridizable pseudogenes (13) are faintly visible. Although the bands derived from BALB/c DNA of F18-3-4 cells were consistently fainter than those derived from DNA of BW5147, no rearrangements of the T15 V_H gene segment of BALB/c DNA were observed in F18-3-4 cells. Also, none of the cross-hybridizable V_H fragments rearranged under different hybridization conditions.

Blot Analysis of RNA. Although gene rearrangements are closely associated with immunoglobulin gene expression in B cells, it may be that T cells express the Ig V_H gene without rearrangements. Therefore, an attempt was made to detect the T15 V_H gene sequence in the mRNA fraction extracted from F18-3-4 cells. F18-3-4 cells were transplanted subcutaneously in

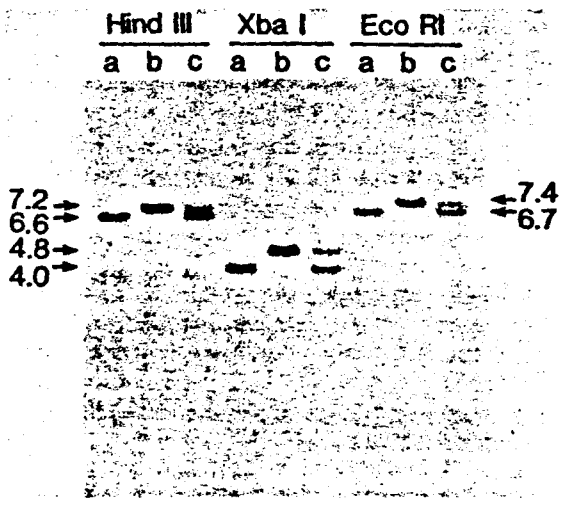

FIG. 3. Southern blot analysis with the T15 V_H DNA probe. DNA was digested with *Hin*dIII, *Xba* I, or *Eco*RI. The origins of the DNA in each lane are a, BW5147; b, BALB/c liver cells; c, *P*Cho-specific T hybridoma F18-3-4. Size markers, in kb, are indicated.

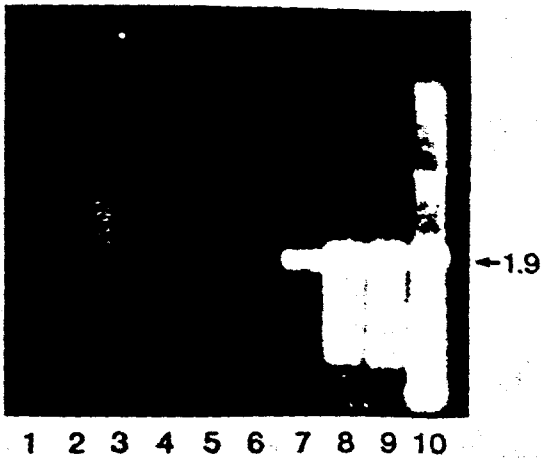

FIG. 4. Blot analysis with a T15 V_H DNA probe. Lane 1, 3 μg of F18-3-4 poly(A)$^+$ RNA; lane 2, 7.5 μg of F18-3-4 poly(A)$^+$ RNA; lane 3, 7.5 μg of BW5147 poly(A)$^+$ RNA; lane 4, 7.5 μg of IgE-53-569 (anti-DNP IgE-producing B hybridomas) poly(A)$^+$ RNA; lanes 5–8, TEPC15 poly(A)$^+$ RNA (0.5 ng in lane 5, 5 ng in lane 6, 50 ng in lane 7, and 500 ng in lane 8); lane 9, 500 ng of MOPC511 poly(A)$^+$ RNA; lane 10, 5 μg of TEPC15 whole RNA. RNA molecular weight standards included murine 18S and 28S rRNA. A size marker, in kb, is indicated.

BALB/c *nude* mice. More than 90% of cells recovered from *nude* mice expressed the T15 Id and the suppressive activity of their sera was 40-fold stronger than that of the culture supernates. Poly(A)$^+$ RNA was obtained from the F18-3-4 cells transplanted in *nude* mice. Size-separated poly(A)$^+$ RNAs from various T and B cell lines were hybridized with ^{32}P-labeled T15 V_H probes. Poly(A)$^+$ RNA from TEPC15 myeloma was run on the same gels to estimate our detection limit; we found that we could detect less than 3 copies per cell of T15 $V_H$$^+$ RNA on this autoradiography. However, as shown in Fig. 4, we were unable to detect any T15 V_H sequence in poly(A)$^+$ RNA of F18-3-4 cells.

DISCUSSION

In this study, immunochemical characterization of the *P*Cho-specific TsF released from a *P*Cho-specific, T15 Id$^+$ T hybridoma, F18-3-4, was carried out, and the result showed that the TsF had the ability to bind to *P*Cho-determinants and possessed T15 Id determinants as well as Ia^d products. Previously, we showed that F18-3-4 cells were stained with anti-T15 Id antibody and formed rosettes with *P*Cho-SRBC, and formation of rosettes was inhibited by the addition of anti-T15 Id antibody (5). When these results are taken collectively, it appears that the *P*Cho-specific T hybridoma F18-3-4 expresses T15 Id determinants in their antigen receptors similar to those of *P*Cho-specific B cells.

In the recent study by Crews *et al.* (13), four distinct germ-line V_H gene segments belonging to the T15 V_H gene family have been cloned. A comparison of the four different germ-line V_H gene sequences with 19 V_H regions derived from *P*Cho-binding myeloma and hybridoma proteins showed that all of the V_H regions were derived from the T15 germ-line V_H gene segment. It appears that virtually the entire immune response to *P*Cho is derived from a single V_H-coding sequence in B cells. Thus, it seems reasonable to study whether the *P*Cho-specific T hybridomas utilize a T15 V_H gene identical to that of B cells for the construction of the antigen-binding sites of their receptor molecules. In this paper, no rearrangements of the T15 V_H gene segments in the *P*Cho-specific T15 Id$^+$ hybridoma were detected by Southern blot analysis with the T15 V_H DNA probe.

Furthermore, RNA blot analysis did not detect any T15 V_H sequence-positive mRNA in the hybridoma. The results strongly suggest that T cells with specificity for *P*Cho did not utilize a V_H gene identical to that expressed in B cells.

Kronenberg *et al.* (18) have shown that joining (*J*) segments for immunoglobulin light and heavy chains are not rearranged in helper and killer T cell lines. Kurosawa *et al.* (19) also have demonstrated in the analysis of various T cell lines that the J_H gene may not be involved for the construction of combining sites of T cell receptors.

In this study, we showed clear evidence that T cells do not use the same V_H gene as that expressed in B cells with the same specificity. However, we can still speculate that another member of the V_H gene family was involved in the antigen binding of T cells by assuming that *P*Cho-specific T cells might use a V_H gene distinct from the T15 V_H gene family; i.e., *P*Cho-specific T cells might use an unknown germ-line V_H gene for the *P*Cho specificity, as a result of which B cells express totally different antigen specificity in combination with diversity (*D*) and *J* segments. Another possibility, that a set of V genes for T cell receptors V_T may be distinct from V_H genes of B cells and may be located elsewhere either in or out of chromosome 12, is also conceivable.

Under the conditions employed in the RNA blot experiment the probe could hybridize to RNA in the limit of 80% DNA sequence homology. The V gene for the *P*Cho-specific T hybridoma therefore has sequence homology below 80%. Thus, the amino acid sequence of the V region of *P*Cho-specific receptor molecules of T cells should be different from those of *P*Cho-specific T15 Id^- B cells. The T15 idiotype specificity of the antibody employed in this experiment was confirmed by several pieces of data from the *P*Cho-specific delayed-type hypersensitivity system (6) and the *P*Cho-specific, IgE class-restricted suppression system (5). Then, the question to be asked is why *P*Cho-specific T cells that did not use the T15 V_H gene expressed T15 Id-like determinants similar to those of *P*Cho-specific B cells. Recently, Wysocki and Sato (20) have shown that the anti-ABA major crossreactive idiotype (CRI) family of A/J mice included members with no reactivity toward ABA. Recent work of Miller *et al.* (21) has also shown the similar result that administration of anti-idiotypic antibody against anti-nuclease antibody induced the appearance of idiotype-bearing immunoglobulins in the serum without any detectable antigen (nuclease)-binding activity. These results strongly suggest that serologically defined idiotypes of immunoglobulins and possibly T cell receptors do not reflect their antigen specificity. Nor does their coincidence necessarily mean the expression of the identical *V* gene.

Taking these findings collectively, we suggest that idiotype study of T cell receptors should be reconsidered and another approach, such as analyzing constant determinants of T cell receptors as markers (22–24), would be more reasonable to solve the puzzle of T cell receptors and genes coding for them.

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Organization and Reorganization of Immunoglobulin Heavy-chain Genes

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Each of the immunoglobulin polypeptides, light (L) and heavy (H) chains, comprises an aminoterminal variable (V) region, which determines antigen-binding specificity, and a carboxyterminal constant (C) region, which mediates biological effector functions. Immunoglobulins can be divided into five major classes, IgM, IgG, IgA, IgD, and IgE, which are defined by their heavy-chain constituents, μ, γ, α, δ, and ϵ, respectively.

Dreyer and Bennett (1965) proposed a hypothesis that the V and C regions of immunoglobulin proteins are encoded by separate genes and that these two genes, namely, V and C genes, are brought together during differentiation of lymphocytes. This hypothesis was supported by a number of genetic studies using allotype markers and by hybridization kinetic studies using cDNA (see Leder et al. 1977). Finally, Tonegawa et al. (1977a) obtained direct evidence for the Dreyer-Bennett hypothesis. They have cloned both V and C genes of a light chain in separate DNA segments from embryonic DNA (Brack and Tonegawa 1977; Brack et al. 1978). They have also shown that the V and C genes are brought into close proximity in a myeloma expressing V and C genes.

The next question was, What is the mechanism that brings V and C genes close to each other? Several possible models were proposed. In one, a V gene undergoes transposition to be associated with a C gene (Gally and Edelman 1970). In a second model, a copy of a particular V gene is inserted next to a C gene (Dreyer et al. 1968; Sledge et al 1976). In a third model, V genes and a C gene are arranged in a chromosome in opposite orientations, and a segment of DNA is inverted to associate a V gene with a C gene (Tonegawa et al. 1977b). In a fourth model, a V gene and a C gene are joined together following deletion of the DNA segment between the two joining genes (Kabat 1972). Finally, in a splicing model (Rabbitts 1978; Tonegawa et al. 1978), splicing of a putative large precursor RNA, containing a large spacer sequence in addition to a V gene sequence and a C gene sequence, generates a mature mRNA in which a V gene sequence is directly adjacent to a C gene sequence.

To answer this question, we have taken advantage of an immunobiological phenomenon called heavy-chain class switch (Kincade et al. 1970; Nossal et al. 1971; Fu et al. 1975; Gearhart et al. 1975; Goding and Layton 1976; Sledge et al. 1976; Bleux et al. 1977; Wang et al. 1977; Abney et al. 1978). During differentiation, a given lymphocyte appears to be able to associate sequentially a single V region with two or more different classes of the heavy-chain C region. The sequence of the class switch is always from μ to γ or to α. In such a system, we can study the mechanism of the immunoglobulin gene rearrangement by comparing DNAs of lymphocytes that produce different classes of the heavy-chain protein.

In 1978, we presented the first experimental data to support the deletion model, using hybridization kinetic studies with cDNA probes (Honjo and Kataoka 1978). Our data suggested that a V gene is linked to a C gene to be expressed following deletion of the intervening DNA segment, including other C genes that are located 5′ of the expressed C gene. The original version of our model consisted of three major points, as shown in Table 1.

More recently, we have cloned, from mouse myelomas, γ genes that have accomplished the class-switch rearrangement. Comparison between embryonic and differentiated forms of γ genes led us to expand the original model and to propose a detailed molecular mechanism for the heavy-chain class switch (Kataoka et al. 1980; Takahashi et al. 1980). The complete heavy-chain gene appears to be formed by at least two recombination events, the first one (V-J recombination) completing a V gene and the second one (S-S recombination) replacing the C gene from μ to γ or to α. Both types of recombinations result in deletion of the intervening DNA segment from the chromosome (Coleclough et al. 1980; Cory and Adams 1980; Cory et al. 1980; Rabbits et al. 1980; Yaoita and Honjo 1980a,b). A similar conclusion was drawn from studies on rearranged α and γ genes (Davis et al. 1980; Maki et al. 1980).

In this paper we briefly describe further evidence for the deletion model. We have also analyzed the structure and organization of the heavy-chain genes by cloning these genes and determining their nucleotide sequences. Such analyses provide more direct evidence for the proposed order of the heavy-chain genes and the molecular mechanism for the heavy-chain class switch. Finally, we propose a model in which the S-S (class-switch) recombination may be mediated by unequal crossing-over events between sister chromatids.

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Table 1. Original Deletion Model

1. V and C genes are joined following deletion of the intervening DNA segment
2. Order of C_H genes is 5′-μ-γ3-γ1-γ2b-γ2a-α-3′
3. Deletion takes place on only one of the homologous chromosomes

Deletion of the γ Gene from the Expressed Allelic Chromosome

To prove the deletion of the C_H gene from one chromosome, it is essential to distinguish the C_H genes in an allelic pair of chromosomes. Several inbred strains of mice such as BALB/c and C57BL have different serological markers (allotypes) in heavy-chain C regions (Lieberman 1978), suggesting that their C_H genes may have different nucleotide sequences. We searched for restriction endonucleases that produce different C_H gene fragments between BALB/c and C57BL alleles. Among the restriction endonucleases tested, we found that *Xba*I, *Bgl*II, and *Bgl*I can distinguish the μ, γ1, and γ2b genes, respectively, of BALB/c and C57BL alleles (Yaoita and Honjo 1980b).

Our strategy is as follows: BALB/c and C57BL DNAs are digested with appropriate restriction enzymes, electrophoresed in an agarose gel, and blotted to a nitrocellulose filter according to the method of Southern (1975). When the filter is hybridized with a ^{32}P-labeled immunoglobulin gene probe, the autoradiograph is expected to show immunoglobulin gene bands of different sizes in BALB/c and C57BL DNAs. In the liver DNA of an F_1 mouse from these two strains of mouse, two bands, one from BALB/c and one from C57BL, will be observed. A single lymphocyte of an F_1 animal can produce only one of the parental types of immunoglobulin protein, which is called allelic exclusion (Pernis et al. 1965; Cebra et al. 1966). If we use the DNA of a myeloma induced in an F_1 mouse producing a certain class of heavy chain with the BALB/c allotype, the allelic deletion model predicts the presence of all the immunoglobulin genes in the C57BL chromosome and the absence of certain immunoglobulin genes from the BALB/c chromosome, according to the linear order of the C_H-chain genes proposed. We can also expect rearrangement of the particular C_H gene of the BALB/c chromosome that is expressed in the myeloma. Only a few F_1 myelomas are available. BKC F_1 #15 was induced in a BALB/c-C57BL F_1 mouse and produces the γ2b-chain protein with the BALB/c allotype. This myeloma is suitable for our purpose, since the allelic deletion model predicts the deletion of the γ1, γ3, and μ genes from the BALB/c chromosome that is expressed in this myeloma.

When digested with *Bgl*II, BALB/c DNA produced an 18-kb γ1 gene fragment and C57BL DNA produced a 14.5-kb γ1 gene fragment, as shown in Table 2. Naturally, F_1 mouse liver DNA contained the γ1 gene fragments derived from both BALB/c and C57BL alleles. In contrast, the F_1 myeloma DNA has lost the γ1 gene of the BALB/c allele, whereas the γ1 gene on the C57BL allele remains intact in the F_1 myeloma DNA. Similarly, we have analyzed the μ, γ2b, and γ2a genes of the F_1 myeloma (Table 2). The F_1 myeloma has lost the μ gene of the BALB/c chromosome but retains the μ gene of the C57BL chromosome. The γ2b gene of the C57BL chromosome remains intact. On the other hand, the γ2b gene of the BALB/c chromosome is rearranged, consistent with the expression of the γ2b gene of the BALB/c chromosome in this myeloma. Although we did not distinguish the γ2a genes of two alleles, it is clear that this myeloma contains the γ2a gene. We have not examined the γ3 gene. The chromosomes of BKC F_1 #15 are schematically represented in Figure 1. These results are in agreement with the prediction of the allelic deletion model.

After digestion with *Eco*RI, however, we found unexpected rearrangement at the 5′ flanking region of the μ gene of the C57BL chromosome that is unexpressed in this myeloma (Yaoita and Honjo 1980b). It is possible that this rearrangement of the C57BL allele is related to the mechanism that suppresses the heavy-chain genes in the C57BL chromosome. It may be relevant to this point that the region flanking the 5′ end of the μ gene contains the J (joining) region and the S (switch) region, which is important to class-switch recombination, as will be discussed later.

Deletion of C_H Genes in Other Myelomas

Several groups (Coleclough et al. 1980; Cory and Adams 1980; Cory et al. 1980; Rabbitts et al. 1980) have reported that some C_H genes are deleted from both chromosomes in several myelomas induced in BALB/c or NZB mice. We have also observed similar deletions from both chromosomes (Yaoita and Honjo 1980a). In

Table 2. Restriction Fragments of Immunoglobulin Heavy-chain Genes in Mouse Liver and Myeloma

	Sizes of restriction DNA fragments of immunoglobulin genes (kb)			
Origin of DNA	μ (*Xba*I)	γ1 (*Bgl*II)	γ2b (*Bgl*I)	γ2a (*Eco*RI)
BALB/c liver	4.8	18	7.5	23
C57BL liver	2.8	14.5	9.5	23
F_1 liver	4.8 + 2.8	18 + 14.5	n.d.	n.d.
F_1 myeloma (BKC F_1=15)	2.8	14.5	9.5 + 6.5	23

Restriction enzymes used are shown in parentheses; n.d. indicates not done. Probes used were restriction DNA fragments of cloned immunoglobulin genes (Yaoita and Honjo 1980b).

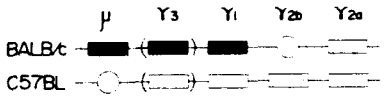

Figure 1. Schematic representation of allelic chromosomal segments containing C_H genes in BKC F_1=15 myeloma. Each line shows one chromosome derived from each parent. (□) Intact genes; (■) deleted genes; (○) rearranged genes. The γ3 gene was not studied, and its estimated state is shown in parentheses. Data were taken from Yaoita and Honjo (1980b).

addition, we found that the expressed C_H genes are rearranged at different locations between an allelic pair of chromosomes of these myelomas. The results can be most easily explained if C_H gene deletion takes place in one chromosome at a time. Later, during proliferation of myeloma cells, it is possible that the successive deletion of C_H genes takes place in the other chromosome. Our original data (Honjo and Kataoka 1978), indicating the presence of a half copy of γ gene per haploid in these myelomas, seem to be due to a combined background by cross-hybridization of γ genes, by impurity of the probe, and by the presence of host cells among tumor cells (Yaoita and Honjo 1980a).

The question is which is the case in normal B lymphocytes; whether C_H gene deletion is confined to one homologous chromosome containing the expressed C_H gene or whether C_H gene deletion occurs in both chromosomes. Since chromosomal anomalies are rather frequent in myeloma cells (Yosida et al. 1968; Ohno et al. 1979), we hesitate to settle this issue by majority vote of the number of myelomas studied so far. In fact, Joho and Weissman (1980) recently reported that only one of the homologous chromosomes undergoes rearrangement of the light-chain gene in normal spleen lymphocytes. It is shown that inhibition of cell division increases the population of binucleated B lymphocytes that have both IgM and IgG in the cytoplasm (Lawton et al. 1977; van der Loo et al. 1979). Since the results suggest that the heavy-chain class switch may involve asymmetric cell division, it is likely that at least the initial DNA rearrangement of the heavy-chain class switch may be confined to one of the homologous chromosomes.

Order of C_H Genes

We have proposed that the order of C_H genes is μ, γ3, γ1, γ2b, γ2a, and α in the 5′→3′ direction. To prove the proposed order of C_H genes, we have cloned the γ1 and γ2b genes as well as the μ gene from *Eco*RI digests of newborn-mouse DNA (Honjo et al. 1979; Kataoka et al. 1979; Kawakami et al. 1980; Yamawaki-Kataoka et al. 1980). Nucleotide sequence determination of these genes has unequivocally demonstrated that intervening sequences split immunoglobulin genes into segments encoding each domain and the hinge region. More recently, we have also cloned the γ2a and γ3 genes, which also appear to be separated by intervening sequences at the junction of the domains (Y. Yamawaki-Kataoka et al., in prep.).

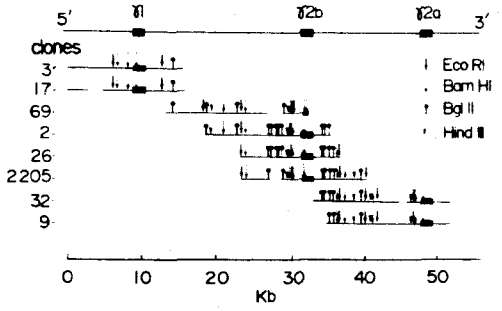

Figure 2. Order of γ-chain genes. Mouse myeloma (MPC-11) DNA was partially digested with *Eco*RI. and DNA fragments of 21–14 kb in length were isolated by agarose gel electrophoresis. The fragments were ligated with Charon-4A arms, packaged in vitro, and amplified (Blattner et al. 1978). Libraries were screened by γ gene probes according to the method of Benton and Davis (1977). Alternatively, DNA fragments hybridizing with a γ gene probe were purified from partial *Eco*RI digests of newborn-mouse DNA by agarose gel electrophoresis. The fragments were ligated with λgtWES arms, and the recombinant phages were screened as above. Cloning experiments were done under EK2-P2 conditions. The clones obtained were analyzed by R-loop formation, Southern blot hybridization, and restriction mapping and were aligned along the chromosome in the direction of transcription. The orientation of the structural gene was assigned by nucleotide sequence determination. Not all the restriction sites are shown. Horizontal lines show the inserts of the clones. Closed rectangles indicate structural genes.

Using the cloned γ genes as probe, we then screened a partial *Eco*RI digestion library of mouse myeloma DNAs and a partial *Hae*III digestion library of mouse embryonic DNAs. We have obtained a number of clones that hybridized with these probes. We have characterized these clones by restriction endonuclease cleavage, R-loop analysis, and Southern blot hybridization. On the basis of these characterizations, we have aligned these clones along the chromosome, as shown in Figure 2. The results have demonstrated that the order of γ genes is γ1, γ2b, and γ2a in the 5′→3′ direction. This order is in perfect agreement with that proposed by our model (Honjo and Kataoka 1978). The γ1 and γ2b genes are 21 kb apart, and the γ2b and γ2a genes are about 15 kb apart. Our preliminary data suggest that the γ3 gene is located 5′ of the γ1 gene and that they are about 34 kb apart. The distance (21 kb) between the γ1 and γ2b genes agrees with a preliminary report by Maki et al. (1980).

Rearranged γ1 Gene

To elucidate the molecular mechanism of the heavy-chain class switch, we cloned a rearranged γ1 gene from a γ1-chain-producing myeloma, MC101. The structure of this clone was analyzed by R-loop formation, Southern blot hybridization, and nucleotide sequence determination. As shown in Figures 3 and 4, this clone consists of five DNA segments. The 5′ segment of 4.7 kb is derived from the V-region gene fragment. A germ-line V_H gene finishes at amino acid residue 97, and a

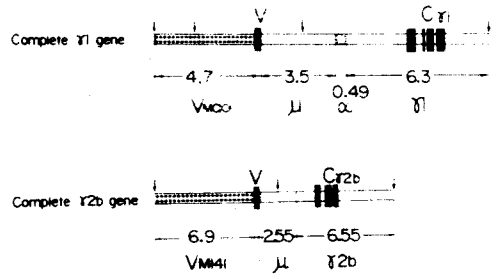

Figure 3. Structure of rearranged γ genes. The complete γ1 gene of MC101 (upper bar) and the complete γ2b gene of MOPC-141 (lower bar) are shown schematically. Cloning and characterization of the 3′ *Eco*RI fragments of these DNAs were described previously (Kataoka et al. 1980; Takahashi et al. 1980). The complete γ1 gene of MC101 was cloned from a partial *Eco*RI digestion library of MC101 DNA. The complete γ2b gene of MOPC-141 was cloned by Maki et al. (1980). The origin of each DNA segment was determined by Southern blot hybridization and nucleotide sequence determination.

complete V_H gene extends to amino acid residue 120 (Fig. 4). The segment corresponding to residues 107–120 appears to be derived from a J segment (Sakano et al. 1980). The DNA segment corresponding to residues 98–106 is the D (diversity) gene segment, whose germ-line location is not known. The presence of the D segment also has been reported by other investigators (Early et al. 1980). At the 3′ side of the germ-line V gene, we found a characteristic nucleotide sequence capable of forming an inverted-repeat stem structure, as shown in other germ-line V_H genes (Early et al. 1980; Sakano et al. 1980). The third fragment contains J regions and is derived from the 5′ flanking region of the μ gene clone, whose nucleotide sequence has already been determined (Kawakami et al. 1980). The fourth fragment of 490 bases seems to be derived from the 5′ flanking region of the α gene. The nucleotide sequence of this segment is almost identical to a portion of the 5′ flanking region (about 3.1–3.6 kb 5′ of the structural gene) of the α gene, but we cannot exclude the possibility that this segment is derived from another gene that has a 5′ flanking region similar to that of the α gene. The nucleotide sequence of a similar portion of the 5′ flanking region of the α gene also has been determined by other investigators (Hood et al., this volume). The 3′ segment of 6.3 kb is derived from the embryonic γ1 gene fragment.

These results indicate that a complete heavy-chain gene is formed by at least two types of recombination events. The first one is called the V-J (or V[D]J) recombination, which takes place between V and D and between D and J to form a complete V-region gene sequence, in a manner similar to the V-J recombination in the light-chain V genes (Max et al. 1979; Sakano et al. 1979). The second type of recombination, which joins the 5′ flanking region of the μ gene and that of the γ1 gene, is different from the V-J recombination. We have determined the nucleotide sequence surrounding the

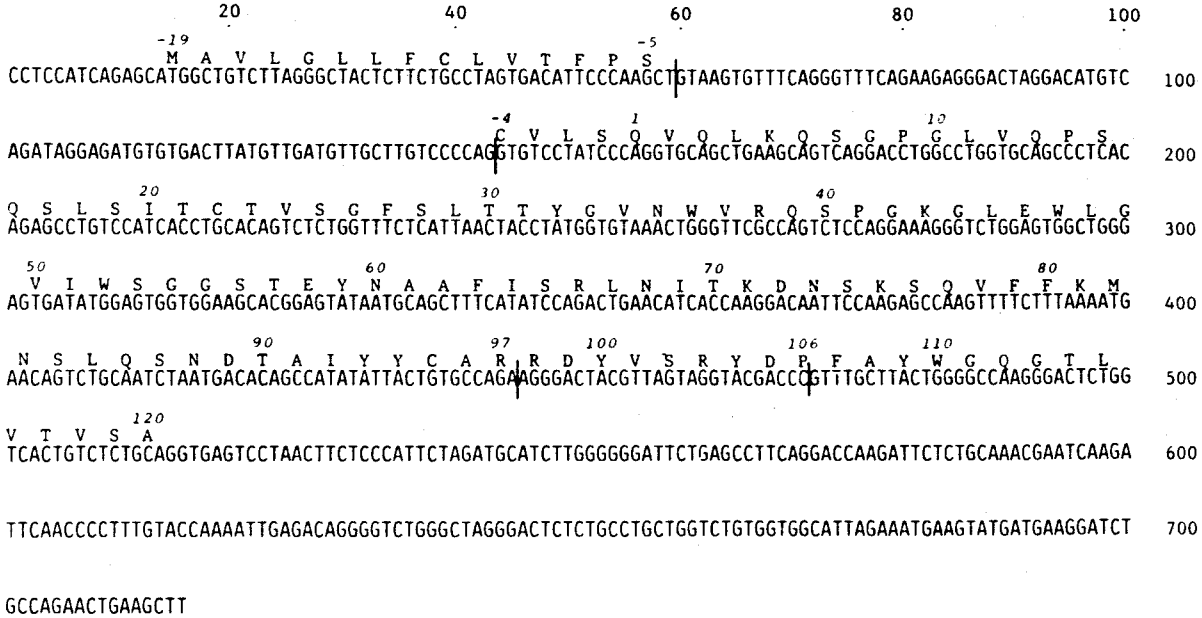

Figure 4. The nucleotide sequence of the V region of the MC101 γ1 gene. Nucleotide sequence was determined according to the method of Maxam and Gilbert (1977), with slight modifications (Honjo et al. 1979). Amino acids predicted from the nucleotide sequence are shown above the base sequence by one-letter code (see Honjo et al. 1979). Vertical lines at positions 59 and 143 indicate possible splicing sites within the hydrophobic leader sequence. Five germ-line V_{MC101} genes were cloned from newborn-mouse DNA. We have determined the nucleotide sequence of the gene that has the identical restriction pattern in its 5′ flanking region with that of MC101 γ1 gene (T. Kataoka and T. Nikaido, in prep.). The nucleotide sequences of the germ-line V_{MC101} gene and the complete γ1 gene of MC101 are identical before position 445 (amino acid residue 97), but completely disagree after position 446. The nucleotide sequence after position 471 (amino acid residue 107) agrees with J_3, determined by Sakano et al. (1980). Thus, we conclude that the segment between position 446 and position 471 is derived from the D-segment gene.

second type of recombination site (Kataoka et al. 1980). We did not find a J region or V region in the immediate vicinity of the recombination sites. We call the portion surrounding the recombination site the S region, since this region is thought to be responsible for class switch. The second type of recombination is called S-S recombination.

Rearranged γ2b Gene

We have cloned another rearranged γ gene from a γ2b-chain-producing myeloma, MOPC-141 (Takahashi et al. 1980). Our clone contained a 7.8-kb *Eco*RI fragment that corresponds to the 3′ fragment of the complete γ2b gene fragment (16 kb) cloned by Maki et al. (1980). The complete rearranged γ2b gene consists of four DNA segments. The 5′ part of 6.9 kb is derived from the V gene of MOPC-141. The second segment contains a D gene segment (Sakano et al. 1980). The third segment is derived from the 5′ flanking region of the μ gene fragment and contains the J region. The 3′ fragment is derived from the embryonic γ2b gene. The complete γ2b gene of MOPC-141 seems also to have undergone two types of recombination events. The first type is the V-J recombination and the second type is the S-S recombination between the 5′ flanking region of the μ gene and that of the γ2b gene. We have determined the nucleotide sequence around the S-S recombination site, as shown in Figure 5. It is clear from the sequence that the rearranged γ2b gene was formed by the recombination of the embryonic μ and γ2b genes. We looked for nucleotide sequences similar to the J region or V region around the recombination site and here again there were no such sequences.

Model for Heavy-chain Class Switch

On the basis of the above results, we have expanded our previous model and have proposed a more detailed mechanism for heavy-chain class switch, as shown in Figure 6 (Kataoka et al. 1980). The first recombination event during differentiation of a lymphocyte brings a V gene directly adjacent to the J region in a manner similar to that shown for light-chain genes (Max et al. 1979; Sakano et al. 1979). This event, which we call V-J recombination, also involves joining with a D segment whose location in a germ line is unknown. After such recombination, the V-region gene sequence is now completed and can be expressed as a μ chain.

At a later stage of differentiation, probably after encountering an antigen, the lymphocyte changes the C part of the heavy chain from μ to α. This requires the second recombination event, called S-S recombination. This recombination occurs between the S_μ and S_γ regions. The S_μ region is present between the J-region gene and the μ gene, and the S_γ region is present at the 5′ side of each γ gene. By this recombination, the completed V gene can change the C partner without affecting the V-region sequence. Consequently, the same V gene is now expressed as a γ chain. In both types of recombinations, the intervening DNA segments are deleted from the chromosome. It is also possible that the successive S-S recombination can switch the C region from γ to α. This model postulates two important structural features in the heavy-chain gene organization: (1) the presence of only one set of J-region genes at the 5′ side of the μ gene and (2) the presence of the S region before each C-region gene.

Figure 5. Nucleotide sequences around recombination sites of the embryonic μ gene, embryonic γ2b, and rearranged γ2b gene of MOPC-141. Nucleotide sequences are displayed from left to right in the direction of transcription of the structural gene sequence. Homologous nucleotide sequences are underlined. Arrows indicate the recombination sites. The sequences were taken from Takahashi et al. (1980).

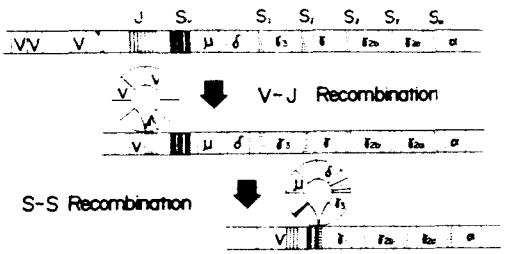

Figure 6. Model for heavy-chain class switch. Recombination events producing a complete γ1 gene are schematically represented. D-segment genes are not shown because their original location in the germ-line gene is not known. We postulate the S region for each C_H gene, except for δ gene (see text).

Recently, Blattner et al. (this volume) have reported that the δ gene is located only 3 kb downstream from the 3′ end of the μ gene. It is also known that the μ and δ chains are usually expressed simultaneously (Knapp et al. 1973; Rowe et al. 1973). Taken together, the results indicate that the switch from μ to δ may be done by an RNA-splicing mechanism.

Structure of S Regions

Figure 7 shows a schematic representation of a chromosomal segment containing C_H genes. We and others (Early et al. 1980; Sakano et al. 1980) have identified several J regions in the 5′ flanking region of the μ gene. Using this segment as probe, we have tested whether or not each embryonic γ gene clone contains the sequence homologous to the J region. The answer was no. There was no J or J-like sequence anywhere from the 5′ flanking region of the γ3 gene to the 3′ flanking region of the γ2a gene. The class-switch-recombination sites so far determined by comparison of the embryonic and rearranged γ gene clones are indicated by arrowheads in Figure 7. This includes two recombination sites in S_μ,

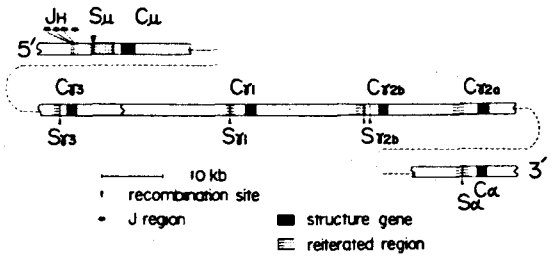

Figure 7. Schematic representation of a chromosomal segment containing C_H genes. The location of the structural gene was determined by nucleotide sequence determination (Honjo et al. 1979; Kawakami et al. 1980; Yamawaki-Kataoka et al. 1980) or by R-loop formation (S. Nakai et al., unpubl.). The range of the reiterated region was estimated from Southern blot hybridization data and from the deletion during cloning. Recombination sites were determined by cloning rearranged genes from myelomas J606 (γ3), MC101 (γ1), MOPC-141 (γ2b), and MPC-11 (γ2b) (Kataoka et al. 1980; Takahashi et al. 1980; Y. Yamawaki et al., unpubl.). A recombination site in the S_α region was determined by Davis et al. (1980).

one in $S_{\gamma 3}$, one in $S_{\gamma 1}$, and two in $S_{\gamma 2b}$. One site in S_α was determined by Davis et al. (1980).

We have determined the partial nucleotide sequences of the $S_{\gamma 1}$, $S_{\gamma 2b}$, and $S_{\gamma 3}$ regions, all of which comprise tandem repetition of 49-bp units, as shown in Figures 8 and 9. In the $S_{\gamma 2b}$ region, five or six repeating units form a large group, and this large group again repeats at least six times (Fig. 8). All repeating units are similar to one another. Restriction enzyme cleavage analysis indicates that the tandem repetitive region lies between 1.8 kb and 5.2 kb 5′ of the structural gene. In the $S_{\gamma 1}$ region, two units form a group structure. In the $S_{\gamma 3}$ region, such a set structure is not clear. The nucleotide sequences of the repeating units of the $S_{\gamma 1}$ and $S_{\gamma 3}$ regions are more divergent from one another than from those of the $S_{\gamma 2b}$ region (Fig. 9).

Comparison of Different S Regions

The S regions of the γ genes have some homology with one another, as judged by comparison of nucleotide sequences and also as shown by Southern blot hybridization. The prevalent sequences of the repeating units of the $S_{\gamma 1}$, $S_{\gamma 2b}$, and $S_{\gamma 3}$ regions are shown in Figure 10. The homologous nucleotides are boxed. These results suggest that $S_{\gamma 1}$, $S_{\gamma 2b}$, and $S_{\gamma 3}$ regions have a common ancestral sequence and may have been derived from tandem duplication of the 49-bp unit. It is worth noting that the recombination takes place at the identical position within the 49-bp repeating unit of the $S_{\gamma 1}$ and $S_{\gamma 2b}$ regions.

Southern blot hybridization experiments (data not shown) indicate that the S_μ region has good homology with the S_α region and less, but significant, homology with the $S_{\gamma 1}$, $S_{\gamma 2a}$, $S_{\gamma 2b}$, and $S_{\gamma 3}$ regions. A partial nucleotide sequence determination of the S_μ region (Sakano et al. 1980; Takahashi et al. 1980; T. Nikaido et al., unpubl.) indicates that the S_μ region shares short common sequences, such as AGCT, TGGG, and AGCTGGGG, with the $S_{\gamma 1}$, $S_{\gamma 2b}$, and $S_{\gamma 3}$ regions. Such common sequences are extremely abundant in the S_μ region so far sequenced.

We have cloned a human μ gene from the human DNA library and have compared its structure with that of a mouse μ gene. Studies using heteroduplex analysis and Southern blot hybridization clearly show that not only the coding region, but also the S_μ region, is homologous between human and mouse μ genes. The results suggest that the nucleotide sequence in the S_μ region plays an essential biological function and therefore has been conserved in these organisms for millions of years.

Nucleotide Sequences Adjacent to the S-S Recombination Sites

We have compared the nucleotide sequences immediately adjacent to the S-S recombination sites of various rearranged genes. Table 3 summarizes such sequences around three sets of the S-S recombination sites. We found trinucleotides TGG, TGA, TAG, and TTG close to these recombination sites. Such trinucleotides also

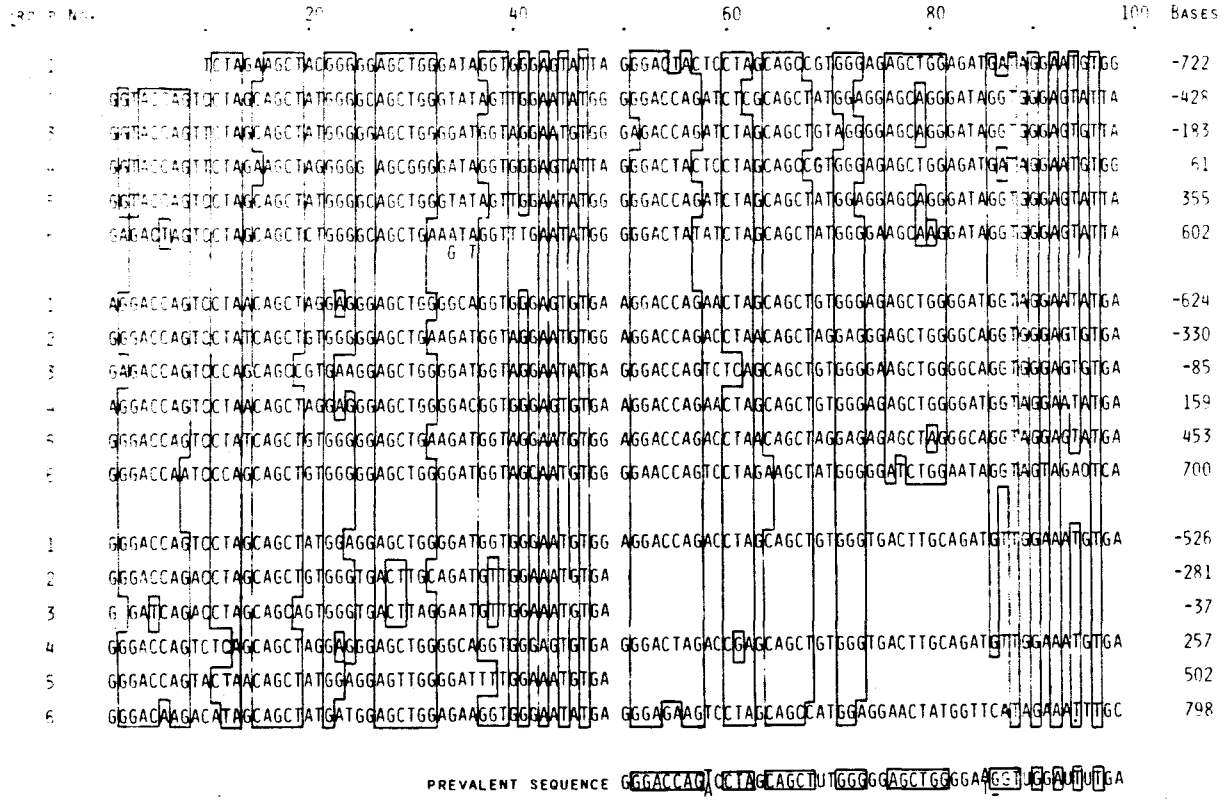

Figure 8. Nucleotide sequence of the $S_{\gamma 2b}$ region. The region sequenced encompasses the DNA segment located 2–3.6 kb 5′ of the γ2b structural gene. The continuous sequence is divided into six groups by homology, each group containing 5 or 6 units of 49 bp. Homologous bases are boxed. The prevalent sequence for the 49-bp unit is shown at the bottom. The arrowhead indicates the recombination site in the rearranged γ2b gene of the MPC-11 myeloma. Data taken from Kataoka et al. (1981).

appear very frequently in the S_μ region. It is worth noting that the recombination sites of two rearranged γ2b genes cloned from MOPC-141 and MPC-11 myelomas share an identical tetranucleotide, AGNTG, the middle nucleotide being the recombination site.

Since the class-switch–recombination site is located in the region intervening a V_H gene and a C_H gene, the S-S recombination does not have to be highly specific to the nucleotides joined together. Instead, the S-mediated recombination is expected to be efficient, because in a small B lymphocyte, the heavy-chain class is switched from μ to γ or to α within a short period of time after an antigen is encountered. In this respect, the S-S recombination is in sharp contrast to the V-J recombination, which requires extremely specific excision and ligation. Otherwise, the V-region sequence may be modified and the immunoglobulin protein may lose the antigen-binding activity. From these points of view, it is possible to imagine that a combined structure of tandem repetitive sequences and relatively short common sequences may serve as a recognition signal for the S-S recombination.

Sister-chromatid Exchange May Explain the S-S Recombination

It has been established that the S-S recombination results in the deletion of a DNA segment between two joining sequences (Honjo and Kataoka 1978; Coleclough et al. 1980; Cory et al. 1980; Rabbitts et al. 1980; Yaoita and Honjo 1980a,b). 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 (Kindt et al. 1970; Landucci-Tosi et al. 1970; Knight and Hanly 1975). Two alternative models can be proposed to explain the mechanism of the C_H gene deletion in B lymphocytes, as illustrated in Figure 11. The first model postulates that the S-S recombination takes place in a single chromosome by mutual recognition of two S regions (Fig. 11A). The intervening DNA segment is looped out and lost from the chromosomes. This model is referred to as a looping-out model. In principle, such recombination can occur at any stage of the cell cycle. The other model, called a sister-chromatid-exchange model, explains the deletion of a DNA segment by an unequal crossing-over event between sister chromatids (Fig. 11B). 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 (Gearhart et al. 1975, 1980). Inhibition of cell division

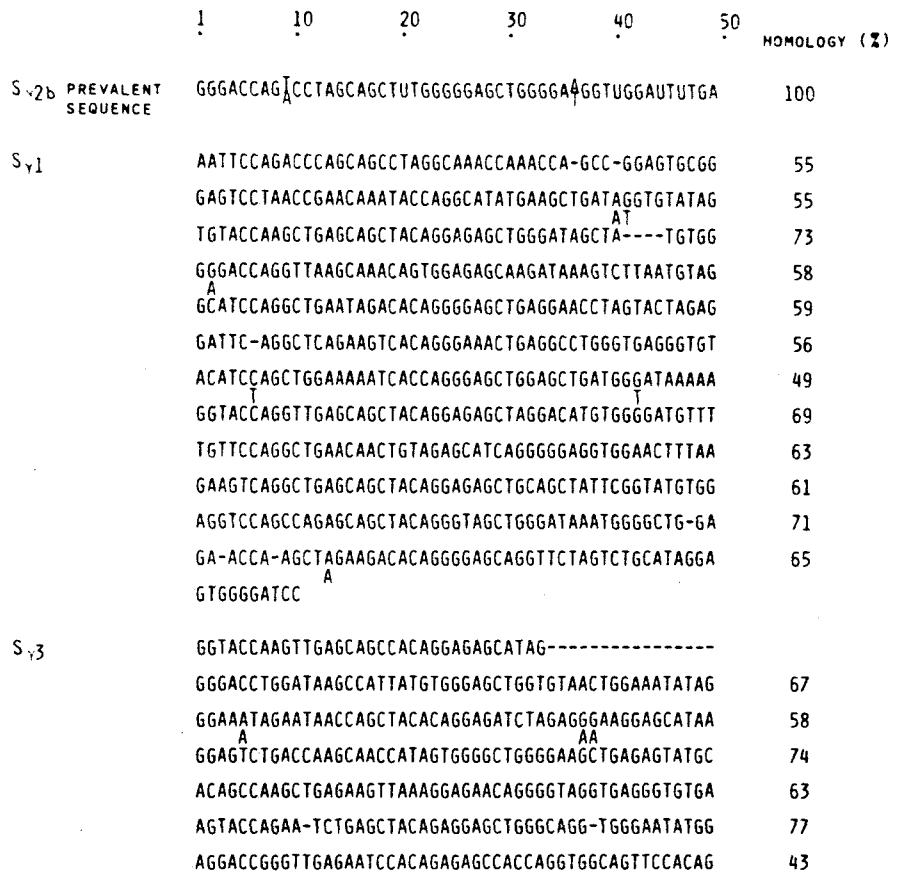

Figure 9. Nucleotide sequences of the $S_{\gamma 1}$ and $S_{\gamma 3}$ regions. The nucleotide sequence of $S_{\gamma 1}$ was taken from Kataoka et al. (1980). The region shown corresponds to the DNA segment located 2.4–3.0 kb 5′ of the γ1 structural gene. The nucleotide sequence of the $S_{\gamma 3}$ region corresponds to the DNA segment located around 3.6 kb 5′ of the γ3 structural gene. Each 49-bp unit was compared with the prevalent sequence of the $S_{\gamma 2b}$ region (Fig. 8), and homology is shown at the right. Data taken from Kataoka et al. (1981).

leads to an increase in the frequency of binucleated cells able to direct synthesis of both IgM and IgG (Lawton et al. 1977; van der Loo et al. 1979). The results suggest that the class switch from IgM to IgG may involve an asymmetric cell division, which is consistent with the sister-chromatid-exchange model, although they do not necessarily exclude the looping-out model. Since the percentage of cells containing both IgM and IgG relative to cells containing IgG was rather high (10–20%) and increased two- to threefold by inhibition of cell division (van der Loo et al. 1979), the switching process appears to take place during cell division, probably during or after replication of DNA.

It appears as if the presence of an S_α segment between S_μ and $S_{\gamma 1}$ segments of a rearranged γ1 gene of MC101 myeloma (Fig. 3) contradicted the linear arrangement of C_H genes and the deletion mechanism for the class switch. The generation of this γ1 gene, however, can be explained by two or three successive unequal crossing-over events. There are various possible pathways to create the MC101 γ1 gene, and several examples are illustrated in Figure 12. In one pathway (Fig. 12A), the

Table 3. Nucleotide Sequence around Class-switch-Recombination Site

	5′ Side		3′ Side	
Myeloma	S region	sequence	sequence	S region
M141	S_μ	AAAGAAT*GGTATC	GGAGAGTTGTCCGATTGA	$S_{\gamma 2b}$
MC101	S_μ	AACTGAC*TCTGAGGTA	T*GAGCTGGGA	S_α
MC101	S_α	GGCTGG*GCTGATT	AGCTGAT*GGGTATAAA	$S_{\gamma 1}$
MPC-11	$S_{\gamma 3}$	AGCTGAC*	GTATAGTTGGAATATGG	$S_{\gamma 2b}$

Asterisk indicates recombination site.

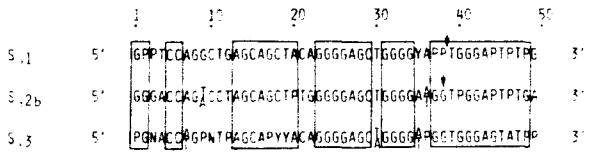

Figure 10. Comparison of the prevalent sequences of the $S_{\gamma 1}$, $S_{\gamma 2b}$, and $S_{\gamma 3}$ regions. The prevalent sequences of the $S_{\gamma 1}$, $S_{\gamma 2b}$, and $S_{\gamma 3}$ were deduced from the sequences shown in Figs. 8 and 9. Similar nucleotides are boxed. Arrows indicate the actual recombination sites used in the rearranged γ1 gene of the MC101 myeloma and the rearranged γ2b gene of the MPC-11 myeloma. (P) Purine; (Y) pyrimidine. Data taken from Kataoka et al. (1981).

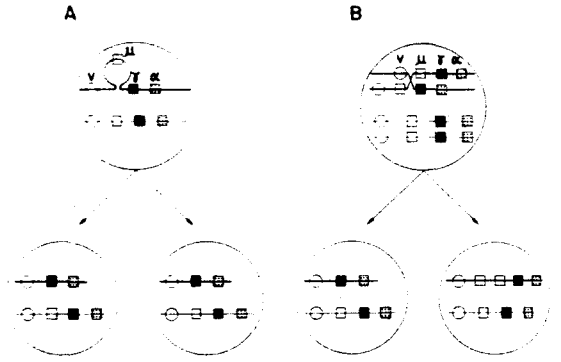

Figure 11. Models for the mechanism of the C_H gene deletion accompanied by the heavy-chain class switch. (—, —) Two parental chromosomes; (○) V gene; (□) μ gene; (■) γ gene; (▨) α gene. The γ subclass genes are not shown. (*A*) Looping-out model; (*B*) sister-chromatid-exchange model. Data taken from Obata et al. (1981).

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 a μ gene that is linked to a V gene and 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 and the S_μ and S_α regions at its 5′ side. In the second pathway (Fig. 12B), we postulated another C_H gene at the 3′ side of the α gene. In the third pathway (Fig. 12C), we allowed a recombination event that does not result in the class switch. Although other explanations by rather rare events such as somatic recombination between homologous chromosomes, recombination at two sites by the formation of double loops of a chromosome, translocation of chromosomal segments, and so on, are also possible, the C_H gene organization in the MC101 myeloma may imply that the sister-chromatid-exchange mechanism operates for switching the heavy-chain class. There are several myelomas whose C_H gene context appears exceptional to the deletion model. For example, MPC-11 (γ2b producer), MOPC-141 (γ2b producer), and HOPC-1 (γ2a producer) contain the μ gene (Cory et al. 1980; Coleclough et al. 1980; Yaoita and Honjo 1980a). These apparent exceptions may also be explained without difficulty by the sister-chromatid-exchange model, although other mechanisms (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 to the C_H gene order proposed (Honjo and Kataoka 1978), an example of which (α → γ switch) is shown in Figure 12B. 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 progeny. Nonetheless, Radbruck et al. (1980) recently reported that a variant of myeloma X63 can switch from γ2b to γ1.

The sister-chromatid-exchange model can be tested directly 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, as well as deleted, C_H genes.

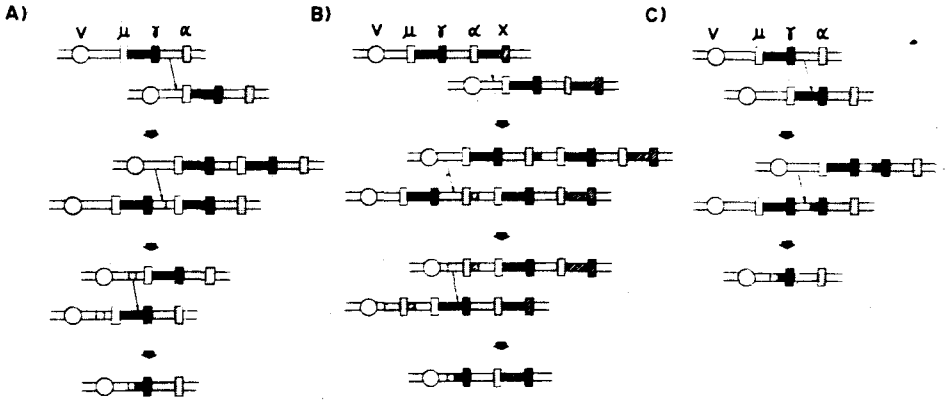

Figure 12. Possible explanation for the presence of the S_α sequence between the S_μ and $S_{\gamma 1}$ sequences in the cor..plete γ1 gene of the MC101 myeloma. (*A, B, C*) Three pathways of unequal crossing-over events between sister chromatids. Symbols for structural genes are given in the legend to Fig. 11, and we have postulated an unknown gene (X) at the 3′ side of the α gene (*B*). Unknown genes are shown by hatched rectangles. The S region for each C_H gene is shown by a narrower rectangle with the same marking as for the structural gene. Data taken from Obata et al. (1981).

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Organization of the Constant-Region Gene Family of the Mouse Immunoglobulin Heavy Chain

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Summary

We cloned overlapping DNA segments that encompass the region from the immunoglobulin J_H segments to the $C_{\gamma 3}$ gene of BALB/c mouse. We have now cloned the entire region (about 200 kilobases) of the constant-region gene family of the immunoglobulin heavy chain, the organization of which is 5′–J_H–6.5 kb–C_μ–4.5 kb–C_δ–55 kb–$C_{\gamma 3}$–34 kb–$C_{\gamma 1}$–21 kb–$C_{\gamma 2b}$–15 kb–$C_{\gamma 2a}$–14 kb–C_ϵ–12 kb–C_α–3′. Using these cloned DNAs, we have characterized several structural features of the constant-region gene loci. There are no other J region segments except for those at the 5′ side of the C_μ gene. The S region is 5′ to each C_H gene except for the C_δ gene, and the nucleotide sequences of the S region share some homology. There is no reasonably conserved pseudogene. There are at least two species of reiterated sequences scattered in these loci. Cloning and Southern blot hybridization analyses indicate that the general organizations of the heavy-chain gene loci of BALB/c and C57BL/6 mice, which have many different serological markers, are fundamentally similar but different in the lengths of S regions. Restriction enzyme cleavage maps of the whole constant-region gene loci were constructed with respect to eight restriction endonucleases.

Introduction

Immunoglobulin (Ig) heavy (H) chain genes comprise a family of variable (V) region genes and a set of the constant (C) region genes, which, in mouse, consist of eight classes and subclasses; C_μ, C_δ, $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, C_ϵ and C_α. These C_H genes, which are genetically termed *Igh* loci, are so tightly linked that thousands of genetic crosses did not give rise to a recombinant within these loci (Herzenberg, 1964; Potter and Lieberman, 1967).

During differentiation of a single B-lymphocyte, a given V_H gene is first expressed in combination with the C_μ gene of the same allelic chromosome, and later in the lineage of the B-lymphocyte, the same V_H gene is expressed in combination with a different C_H gene. This phenomenon is called H chain class switch.

Hybridization kinetic analyses in which cDNA is used have shown that specific C_H genes are deleted in mouse myelomas, depending on the C_H genes expressed (Honjo and Kataoka, 1978). The order of the C_H genes, 5′–V_H gene family–spacer–C_μ–$C_{\gamma 3}$–$C_{\gamma 1}$–$C_{\gamma 2b}$–$C_{\gamma 2a}$–C_α–3′, was consistent with the assumption that the DNA segment between a V_H and the C_H gene to be expressed is deleted, bringing these genes close to each other. Deletion of the intervening DNA segment during H chain class switch was confirmed by Southern blot hybridization analyses of myeloma DNAs in which cloned Ig genes were used (Coleclough et al., 1980; Cory and Adams, 1980; Cory et al., 1980; Rabbitts et al., 1980; Yaoita and Honjo, 1980a, 1980b). Such studies also support the proposed order of C_H genes.

Recent cloning experiments comparing expressed (rearranged) and germline C_H genes demonstrated that at least two types of recombination are necessary for the extensive production of the Ig H chain gene (Davis et al., 1980a; Kataoka et al., 1980; Maki et al., 1980). First, V–D–J recombination creates a complete V_H gene by joining a germline V_H gene, a D segment and a J_H segment. The completed V_H gene can be expressed as μ chain because the C_μ gene is located immediately 3′ to the J_H segment. Second, S–S recombination takes place between the S_μ region and another S region (S_γ, S_ϵ or S_α) located in the 5′ flanking region of each C_H gene, the complete H chain gene replacing its paired C_H gene from C_μ to C_γ, C_ϵ or C_α gene without alteration of the V_H region sequence. This model, which is an elaborate version of the deletion model, postulates several important characteristics of the C_H gene structure and organization: the order of the C_H genes is as proposed previously (Honjo and Kataoka, 1978); there is only one set of J_H segments located at the 5′ side of the C_μ gene, and there is an S region at the 5′ side of each C_H gene.

To test the model of the class switch, we set out to clone the entire region of the C_H gene family, and partially succeeded in linking the $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, C_ϵ and C_α genes in this order (Nishida et al., 1981; Shimizu et al., 1981; Takahashi et al., 1981). Other investigators also reported the linkage of the J_H, C_μ and C_δ genes (Liu et al., 1980; Moore et al., 1981) and of the $C_{\gamma 3}$ and $C_{\gamma 2a}$ genes (Roeder et al., 1981).

We report the isolation of clones that link the J_H, C_μ, C_δ and $C_{\gamma 3}$ genes. We have now completed cloning the entire region of the C_H gene family and have characterized the whole C_H gene cluster. The results are in agreement with the predictions of the deletion model described above.

Results and Discussion

Isolation of Clones That Encompass the Region Between the J_H Segments and the $C_{\gamma 3}$ Gene

To link the C_μ and $C_{\gamma 3}$ genes, we cloned overlapping DNA fragments, using either the 5′ or the 3′ DNA fragment of hitherto unlinked C_H genes as probe. In the 5′ flanking region of the $C_{\gamma 3}$ gene, we have already reached the region 30 kb 5′ to the $C_{\gamma 3}$ gene (Takahashi et al., 1981). We cut out the 5′ Eco RI fragment (5.7 kb) of γ3–32 and used it as probe (Figure 1,

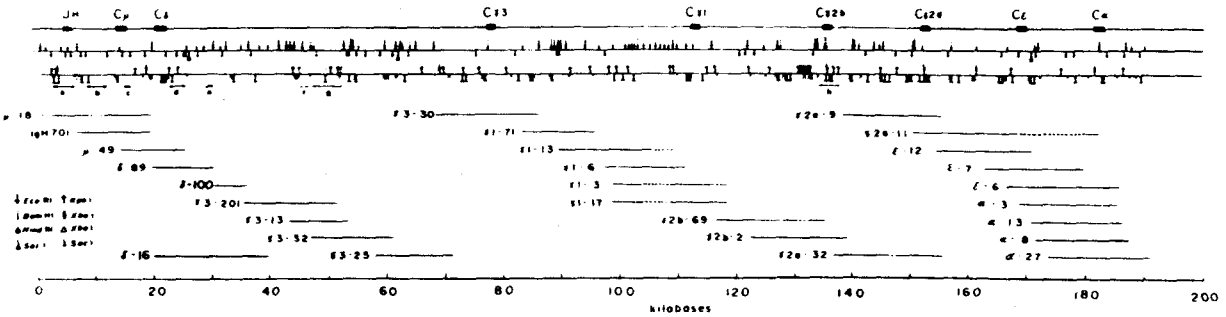

Figure 1. Organization and Restriction Enzyme Cleavage Map of the Entire Region of BALB/c Mouse C_H Gene Cluster

Structural genes are shown by closed boxes at the top line. Second and third lines show restriction sites with symbols indicated. Horizontal arrows under the third line indicate fragments used for probes in this study. Ranges covered with isolated clones are shown in horizontal bars with abbreviated names. Dashed lines indicate deletions. Locations of J_H and C_δ genes were from Liu et al., 1980; Moore et al., 1981; Newell et al., 1980, and Sakano et al., 1980. Clones which are located 3′ to γ3–32 were described previously (Nishida et al., 1981; Shimizu et al., 1981; Takahashi et al., 1981).

probe g) to screen a Charon 4A library containing partial Eco RI* digests of newborn BALB/c mouse DNA. After screening 2×10^5 plaques, we obtained one positive phage, which we designated γ3–13. To extend further toward the 5′ direction, we cut out the 5′ Xba I fragment of γ3–13 and used it as probe (Figure 1, probe f) for screening another library containing partial Eco RI* digests of BALB/c embryo DNA. After screening 1.4×10^6 plaques, we isolated five identical copies of a phage, which we designated γ3–201.

We have already cloned a 13 kb Eco RI fragment (IgH701) that contains the C_α gene (Kataoka et al., 1980; Kawakami et al., 1980). Using a Hind III fragment of IgH701 (Figure 1, probe c) as probe, we have isolated from a newborn mouse DNA library a clone μ–18 that contains both the J_H and the C_μ genes 6.5 kb apart as described by other investigators (Newell et al., 1980; Sakano et al., 1980).

To extend from the C_μ gene toward the 3′ direction, we cloned restriction DNA fragments that were partially purified by agarose gel electrophoresis. First, we isolated an 11.5 kb Bam HI fragment (μ–49), using a Hind III fragment (Figure 1, probe c) of IgH701 (Kawakami et al., 1980) as probe. Then we isolated a 10.5 kb Eco RI fragment (δ–89) using an Xba I–Bam HI fragment (Figure 1, probe d) of μ–49 as probe. Finally, we cut out the 3′ Bam HI–Eco RI fragment of δ–89 (Figure 1, probe e) and used it as probe to screen a Charon 4A library containing partial Hae III digests of C57BL/6 liver DNA. After screening 10^6 plaques, we obtained a clone called δ–16. This clone has regions overlapping both δ–89 and γ3–201. We have confirmed that the region linked by the DNA fragment of C57BL/6 DNA is identical to that of BALB/c DNA by cloning δ–100 from partial Eco RI digests of BALB/c DNA. DNA fragments isolated by other investigators (Moore et al., 1981; Maki et al., 1981) also indicate that δ–89 and δ–100 are adjacent Eco RI fragments in BALB/c DNA. The results demonstrate unequivocally that the J_H, C_μ, C_δ and $C_{\gamma 3}$ genes are organized in BALB/c DNA as follows: 5′–J_H–6.5 kb–C_μ–4.5 kb–C_δ–55 kb–$C_{\gamma 3}$–3′ (Figure 1).

Organization of the Entire C_H Region Gene Family of BALB/c Mouse

As summarized in Figure 1, we have now cloned the entire C_H region gene cluster, encompassing about 200 kb. The origins and some properties of these clones are listed in Table 1. Organization of the entire C_H gene cluster is 5′–J_H–6.5 kb–C_μ–4.5 kb–C_δ–55 kb–$C_{\gamma 3}$–34 kb–$C_{\gamma 1}$–21 kb–$C_{\gamma 2b}$–15 kb–$C_{\gamma 2a}$–14 kb–C_ϵ–12 kb–C_α–3′, in agreement with the originally proposed order of C_H genes (Honjo and Kataoka, 1978).

Using these isolated overlapping DNA segments, we determined maps of several restriction endonuclease cleavage sites of the entire C_H gene cluster, which can serve as a basis of comparison with those of other haplotypes and species. As shown in Figure 1, a Bam HI site cluster is 5′ to the $C_{\gamma 1}$ gene and a Kpn I site cluster is 5′ to the $C_{\gamma 2b}$ gene, indicating that these regions contain tandem repetitious sequences characteristic of S regions (Takahashi et al., 1980; Kataoka et al., 1981; Nikaido et al., 1981).There are only seven Xho I and three Sal I sites throughout the whole 200 kb region. Since these enzymes have a dinucleotide CG in their recognition sequences, the scantness of Xho I and Sal I sites could be related to the fact that the dinucleotide CG is relatively rare in the eucaryotic genome.

There Are No Other J Region Segments Except for Those 5′ to the C_μ Gene

It is well established that there are four J_H segments 6.5 kb 5′ to the C_μ gene (Newell et al., 1980; Sakano et al., 1980). To test whether there are other J_H segments, we used as probe the Xba I fragment (Figure 1, probe a) containing all of four J_H segments and hybridized it to cloned DNAs encompassing the entire C_H gene family with some overlaps. As shown in Figure 2, there are no hybridizing bands other than a positive control, even under very mild washing con-

Table 1. List of Recombinant Clones

Clones[a]	Abbreviations	Sizes of Inserts (kb)	Structure Genes	Direction of Inserts[b]	Sources of DNA[c]	Restriction[d]	Libraries[e]	References
Ch·M·Ig·μ-18	μ-18	16.8	J_H, μ	B	NB	Eco RI-P	S	This paper
λgtWES·IgH701	IgH701	13.0	μ	B	NB	Eco RI-C	R, A	Kataoka et al. 1980
Ch28·M·Ig·μ-49	μ-49	11.5	μ, δ	B	NB	Bam HI-C	A	This paper
λgtWES·M·Ig·δ-89	δ-89	10.5	δ	B	L	Eco RI-C	A	This paper
Ch·M·Ig·δ-16	δ-16	19.2	δ	L	BL	Hae III-P	S	This paper
λgtWES·M·Ig·δ-100	δ-100	6.2	—	B	L	Eco RI-P	A	This paper
Ch·M·Ig·γ3-201	γ3-201	16.3	—	R	Em	Eco RI*-P	S	This paper
Ch·M·Ig·γ3-13	γ3-13	10.3	—	R	NB	Eco RI*-P	S	This paper
Ch·M·Ig·γ3-32	γ3-32	14.5	—	L	NB	Eco RI-P	S	Takahashi et al. 1981
Ch30·M·Ig·γ3-25	γ3-25	14.0	—	R	NB	Sau 3A-P	S	Takahashi et al. 1981
Ch·M·Ig·γ3-30	γ3-30	18.0	γ3	R	NB	Eco RI-C	R, A	Kataoka et al. 1981
Ch30·M·Ig·γ3-71	γ3-71	12.5	—	L	NB	Sau 3A-P	S	Takahashi et al. 1981
Ch·M·Ig·γ1-13	γ1-13	17.4	—	L	NB	Eco RI-P	S	Takahashi et al. 1981
Ch30·M·Ig·γ1-6	γ1-6	14.8	—	R	NB	Sau 3A-P	S	Takahashi et al. 1981
Ch·M·Ig·γ1-3	γ1-3	14.4	γ1	R	Em	Hae III-P	S	Shimizu et al. 1981
Ch·M·Ig·γ1-17	γ1-17	12.4	γ1	R	Em	Hae III-P	S	Shimizu et al. 1981
Ch·M·Ig·γ2b-69	γ2b-69	18.9	γ2b	R	Em	Hae III-P	S	Shimizu et al. 1981
Ch·M·Ig·γ2b-2	γ2b-2	17.3	γ2b	R	Em	Hae III-P	S	Shimizu et al. 1981
Ch·M·Ig·γ2a-32	γ2a-32	18.7	γ2a	R	Em	Hae III-P	S	Shimizu et al. 1981
Ch·M·Ig·γ2a-9	γ2a-9	17.5	γ2a	L	Em	Hae III-P	S	Shimizu et al. 1981
Ch·M·Ig·γ2a-11	γ2a-11	20.0	γ2a	L	RPC5	Eco RI-C	R, A	Shimizu et al. 1981
Ch·M·Ig·ε-12	ε-12	16.4	ε	L	Em	Hae III-P	S	Shimizu et al. 1981
Ch30·M·Ig·ε-7	ε-7	18.2	ε	L	NB	Sau 3A-P	S	Nishida et al. 1981
Ch30·M·Ig·ε-6	ε-6	19.4	ε, α	L	NB	Sau 3A-P	S	Nishida et al. 1981
Ch30·M·Ig·α-3	α-3	17.1	ε, α	R	NB	Sau 3A-P	S	Nishida et al. 1981
Ch30·M·Ig·α-13	α-14	16.4	α	L	NB	Sau 3A-P	S	Nishida et al. 1981
Ch·M·Ig·α-8	α-8	15.6	α	L	Em	Hae III-P	S	Nishida et al. 1981
Ch30·M·Ig·α-27	α-27	17.4	α	L	NB	Sau 3A-P	S	Nishida et al. 1981

[a] Clone names indicate vectors used. Ch, Charon 4 A; λgtWES, λgtWES; Ch28, Charon 28; Ch30, Charon 30.
[b] R, direction of insert is the same as that of structural genes of phage arms; L, direction of insert is reverse to that of structural genes of phage arms; B, identical inserts in both directions.
[c] NB, BALB/c newborn mice; L, BALB/c liver; Em, BALB/c embryo; BL, C57BL/6 liver; RPC5, RPC5 myeloma.
[d] P, partial digests; C, complete digests.
[e] R, DNA fragments were purified by RPC 5 column chromatography; A, DNA fragments were purified by agarose gel electrophoresis; S, shotgun library.

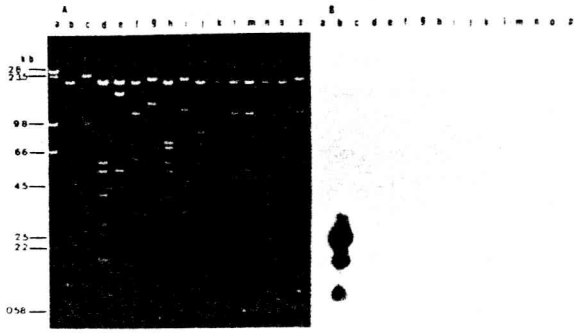

Figure 2. Southern Blot Hybridization Analysis with J Region Probe

About 0.1 μg each of cloned DNAs that cover the entire C_H gene family was digested with Eco RI (lanes e, f, g, i, k, l, m and n) or with Eco RI + Hind III (lanes b, c, d, h, j, o and p) and subjected to electrophoresis in a 0.5% agarose gel. These DNAs were transferred to a nitrocellulose filter, hybridized with probe a in Figure 1 and washed under the mild conditions described in Experimental Procedures. Ethidium bromide stain (A) and autoradiogram (B) are shown. DNA used in each lane is as follows: a, λCI857S7 DNA digested with Hind III as size marker; b, clone μ–18; c, μ–49; d, δ–16; e, γ3–201; f, γ3–32; g, γ3–25; h, γ3–30; i, γ3–71; j, γ1–13; k, γ1–3; l, γ2b–69; m, γ2b–2; n, γ2a–9; o, γ2a–11; p, ϵ–6.

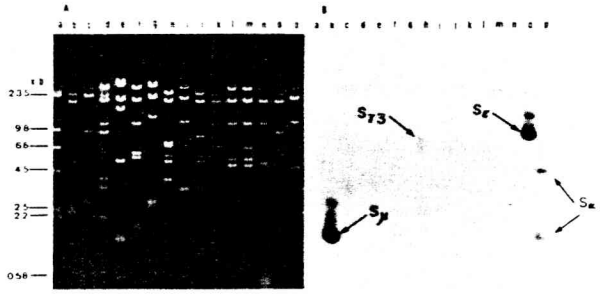

Figure 3. Cross-hybridization between S_α and Other S Regions

Experiments were performed as described in the legend to Figure 2 except that the Hind III fragment which contains the S_α region (probe b in Figure 1) was used as probe. DNA used in each lane is the same as in Figure 2 except that lane d contains δ–16 DNA digested with Eco RI.

ditions. A prolonged exposure, which completely darkened the positive control lane, did not yield any other band (data not shown), indicating that there are neither J_H nor J_H-like sequences throughout the 200 kb region. Since the same V–D–J sets are used in IgM and IgG (Gearhart et al., 1981), it is most unlikely that completely different J_H gene segments are present for different C_H genes.

There Is an S Region for Each C_H Gene Except C_δ

The nucleotide sequences of the S_μ, $S_{\gamma 1}$, $S_{\gamma 2b}$, $S_{\gamma 3}$ and S_α regions comprise tandem repetitions of short unit sequences that contain common sequences GAGCT and GGGGT (Kataoka et al., 1981; Takahashi et al., 1980; Davis et al., 1980b; Nikaido et al., 1981; Dunnick et al., 1980; Obata et al., 1981).

To determine whether there are other similar sequences elsewhere in the C_H gene loci and also to assess the degree of homology among S regions, we hybridized the Hind III fragment (Figure 1, probe b), which contains the S_μ region, to cloned DNAs encompassing the entire C_H region gene family under mild washing conditions. The S_μ probe cross-hybridized most strongly to the S_ϵ region, followed by the S_α region, and most weakly to the $S_{\gamma 3}$ region (Figure 3). After a longer exposure one can barely see hybridized bands of the $S_{\gamma 1}$, $S_{\gamma 2b}$ and $S_{\gamma 2a}$ regions (data not shown). Thus the degree of homology among the S_μ region and other S regions is in the order $S_\epsilon > S_\alpha > S_{\gamma 3} > S_{\gamma 1} > S_{\gamma 2b} \simeq S_{\gamma 2a}$. These results are in agreement with comparison of the nucleotide sequences of the S regions thus far determined (Nikaido et al., 1981; Kataoka et al., 1981; T. Nikaido and T. Honjo, submitted manuscript). The results also indicate that there are no regions other than the S regions which are homologous to the S_μ region in the C_H gene cluster. However, there are many other sequences that cross-hybridize, albeit to a lesser extent, with the S_μ region elsewhere in the mouse genome (Y. Yaoita and T. Honjo, unpublished observations; Dunnick et al., 1980; Kirsch et al., 1981). The above order of the S region homology and the absence of other regions homologous to S regions are also supported by similar experiments in which other S regions are used as probes. We were unable to detect any sequences homologous to the S_μ region in the 5′ flanking region to the C_δ gene, although two δ-producing cells have deleted the C_μ gene (Maki et al., 1981; Moore et al., 1981).

There Is No Well Conserved Pseudogene in the C_H Gene Loci

Many gene families such as mouse V_H, human V_L, α globin, β globin and mouse histocompatibility antigen genes contain several pseudogenes that have well conserved structure similar to active genes, but variable degrees of mutation fatal to their expression (Bentley and Rabbitts, 1980; Givol et al., 1981; Jahn et al., 1980; Lacy and Maniatis, 1980; Leder et al., 1980; Nishioka et al., 1980; Proudfoot and Maniatis, 1980; Steinmetz et al., 1981; Vanin et al., 1980).

We tested the existence of such pseudogenes or of hidden genes that have not been found but are actually expressed. We hybridized restriction DNA fragments containing the C_μ, C_ϵ or C_α gene to cloned DNAs encompassing the entire C_H region but failed to find such genes. When we used the Xba I–Hha I fragment of the $C_{\gamma 2b}$ gene (Figure 1, probe h) as probe, it hybridized to the fragments (12.8 and 3.0 kb) located around 10 kb 5′ to the $C_{\gamma 3}$ gene under conditions in which the probe strongly hybridized to the $C_{\gamma 3}$ gene (lane b) least homologous to the $C_{\gamma 2b}$ gene of the C_γ subclass genes (Yamawaki-Kataoka et al., 1978) (Figure 4A). However, the homology is not in the coding region but in the 3′ flanking region because the γ3–25 clone DNA hybridized to the 3′ flanking region (3.4

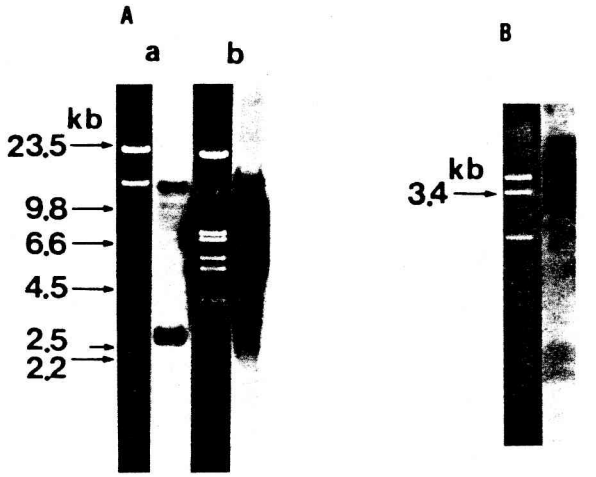

Figure 4. Cross-hybridization of the $C_{\gamma 2b}$ Gene to the Region 5′ to the $C_{\gamma 3}$ Gene

(A) Cross-hybridization between $C_{\gamma 2b}$ probe and clone γ3-25. About 0.2 μg each of γ3-25 DNA (lane a) and γ3-30 DNA (lane b) that contains the $C_{\gamma 3}$ gene as positive control was digested with Eco RI (a) and Eco RI + Hind III (b), respectively, and subjected to electrophoresis on a 0.5% agarose gel. The Southern blot of the gel was hybridized with the nick-translated Xba I–Hha I fragment containing the $C_{\gamma 2b}$ gene (probe c in Figure 1). The $C_{\gamma 3}$ gene is located in the 6.8 kb Hind III fragment of γ3-30 DNA. Ethidium bromide stain (left) and autoradiogram (right) are shown side by side. (B) Cross-hybridization of γ3-25 probe to $C_{\gamma 2b}$ gene. About 0.3 μg of pIgH22 (Yamawaki-Kataoka et al., 1979), which is a recombinant of pBR322 carrying the 6.6 kb Eco RI fragment of the $C_{\gamma 2b}$ gene, was digested with Eco RI + Sac I and run in a 1.0% agarose gel. The Southern blot of the gel was hybridized with nick-translated total DNA of the γ3-25 clone. Ethidium bromide stain (left) and autoradiogram (right) are shown side by side.

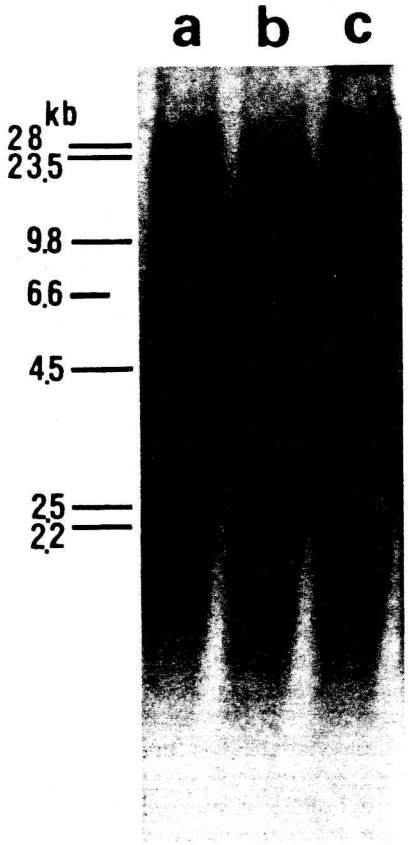

Figure 5. Reiterated Sequences in the C_H Gene Family

BALB/c liver DNA was digested with Bam HI (lane a), Hind III (lane b) or Eco RI (lane c) and subjected to electrophoresis in a 0.5% agarose gel. The Southern blot of the gel was hybridized with the nick-translated Xba I fragment 31 kb 5′ to the $C_{\gamma 3}$ gene (probe f in Figure 1).

kb Sac I fragment and some partially digested bands) of the $C_{\gamma 2b}$ gene (Figure 4B). Although another region 14 kb 5′ to the $C_{\gamma 1}$ gene barely cross-hybridized with the $C_{\gamma 2b}$ gene probe and the γ1 cDNA clone (pG1-6) (Obata et al., 1980), the intensity of hybridization was much less than that found 5′ to the $C_{\gamma 3}$ gene, and so we did not characterize this region further. We tentatively conclude that there is no pseudogene of any Ig class that has reasonably conserved structure like those in other gene families such as globin, V_H, V_L and histocompatibility antigen genes, though the above two regions remain possible candidates for a pseudo C_γ gene until we sequence them.

There Are at Least Two Species of Reiterated Sequence in the C_H Region Gene Family

During isolation of clones that link the C_μ and $C_{\gamma 3}$ genes, we found that some restriction DNA fragments gave very high background when used as screening probes. When we screened libraries with these fragments as probes, one of three to five randomly chosen plaques hybridized, albeit with much less intensity than true positive plaques. This made it difficult to isolate true positive clones and to decide whether a library contains necessary clones. Nucleotide sequences similar to these fragments seem to be dispersed throughout the mouse genome. One such experiment, in which the Xba I fragment 31 kb 5′ to the $C_{\gamma 3}$ gene (Figure 1, probe f) is used, is shown in Figure 5. All the lanes containing restriction enzyme digests of mouse DNA were strongly darkened with several intense bands that did not coincide with the restriction fragment of the cloned DNA. We presume that these fragments may contain highly reiterated sequences such as Alu I family (Jelinek et al., 1980).

To determine precisely which regions in the C_H gene loci contain reiterated sequences, we hybridized ^{32}P-labeled total mouse liver DNA or the ^{32}P-labeled Xba I fragment (Figure 1, probe f) to restricted cloned DNAs of the C_H region gene family. The region around the $C_{\gamma 3}$ gene hybridized strongly with both probes (Figure 6). Most hybridizing bands are common to these two probes, indicating that these probes contain homologous reiterated sequences. In addition, a few bands hybridized only with total mouse DNA probe, as indicated by arrows in Figure 6B. No other regions hybridized to either probe except that weakly hybrid-

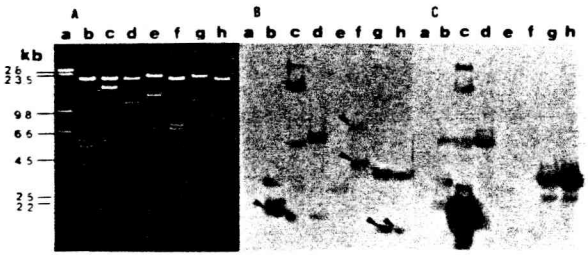

Figure 6. Location of Reiterated Sequences in the C_H Gene Family

About 0.2 μg each of δ–16 (lane b), γ3–201 (lane c), γ3–32 (lane d), γ3–25 (lane e), γ3–30 (lane f), γ3–71 (lane g) and γ1–13 (lane h) DNAs were digested with Eco RI (lanes c, d, e and g) or with Eco RI + Hind III (lanes b, f and h) and run in 0.5% agarose gels. Two identical Southern blot filters were made. One (B) was hybridized with total BALB/c liver DNA probe (10 ng per milliliter of hybridization solution for 12 hr) and the other (C) was hybridized with the Xba I fragment (probe f in Figure 1). Ethidium bromide stain of the gel is shown in A. Lane a contains λCI857S7 DNA digested with Hind III as size marker.

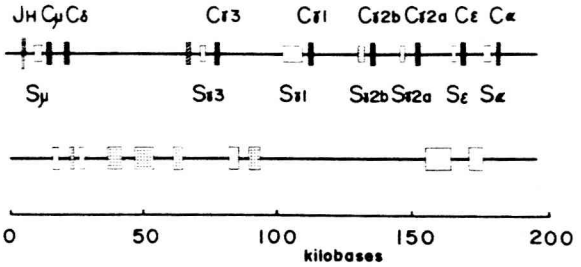

Figure 7. Organization of the C_H Gene Family of BALB/c Mouse

Locations of structure genes (closed boxes) and S regions (broken-lined boxes) are indicated on the top line. Lengths of S regions were determined by hybridization analysis (T. Nikaido and T. Honjo, submitted manuscript) and maximum ranges of deletions which occurred during propagation of phages. These two criteria coincided. An oblique-lined box indicates the region that is homologous to the $C_{\gamma 2b}$ probe. The second line indicates the location of reiterated sequences. Boxes indicate reiterated sequences, and dotted boxes indicate reiterated sequences homologous to probe f in Figure 1.

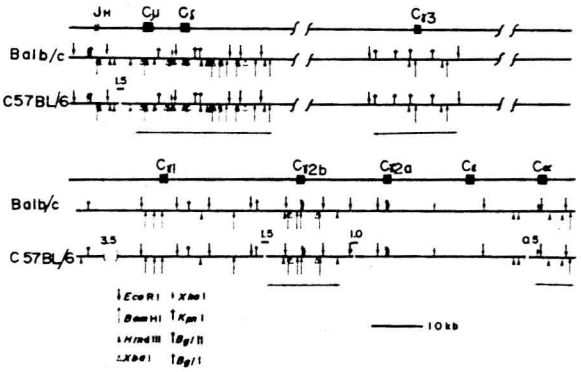

Figure 8. Structural Comparison of the C_H Gene Families in BALB/c and C57BL/6 Mice

At the top line, structural genes are shown in closed boxes. The second line shows a restriction map of BALB/c mice, and the third line of C57BL/6 mice. Only the restriction sites identified in C57BL/6 and their corresponding sites in BALB/c are shown. Numbers indicate lengths (kb) of deletions (parentheses) or insertions (bars) in C57BL/6 DNA as compared with BALB/c DNA. Horizontal arrows below the third line indicate the regions cloned from C57BL/6 DNA.

izing bands specific to total DNA probe were seen around the C_ϵ gene (data not shown) and at the 3′ flanking region of the C_μ gene that was previously reported to hybridize with the reiterated sequence of the nontranscribed spacer of the ribosomal RNA gene (Arnheim et al., 1980).

These results demonstrate that there are at least two kinds of reiterated sequence, one homologous to probe f and the other different from probe f, in the C_H region gene family. The distribution of such reiterated sequences in the C_H gene family is not random, but their functional significance is not known. Locations of reiterated sequences, S regions and the region homologous to the 3′ flanking region of the $C_{\gamma 2b}$ gene are schematically represented in Figure 7.

Comparison of Structure of the C_H Gene Family in BALB/c and C57BL/6 Mice

BALB/c and C57BL/6 mice have different sets of serological markers in many heavy chains, which are called haplotypes. We have shown that C57BL/6 DNA also contains one copy each of eight C_H genes by Southern blot hybridization (Honjo et al., 1981; Y. Yaoita and T. Honjo unpublished data). We have isolated several C_H gene clones from a library of C57BL/6 mouse liver DNA and compared their restriction endonuclease maps with those of BALB/c clones shown in Figure 1. For the regions not covered by isolated clones, we compared restriction sites determined by Southern blot hybridization experiments of BALB/c and C57BL/6 DNAs, using cloned BALB/c C_H genes as probes.

As summarized in Figure 8, the C_H genes of the two haplotypes are very similar. Major differences between BALB/c and C57BL/6 DNAs reside in lengths of S regions such as S_μ, $S_{\gamma 1}$, $S_{\gamma 2b}$, $S_{\gamma 2a}$ and S_α. As S regions contain tandem repetitions of short sequences, mismatching and unequal crossing-over between units of repetition might occur with rather high frequency, resulting in deletion or insertion of unit sequences. The variation in the lengths of the S_μ and S_α regions was also reported in other strains of experimental mice and wild mice as well (Marcu et al., 1980; Honjo et al., 1981). Although the $S_{\gamma 3}$ region of the C57BL DNA clone is 0.8 kb shorter than that of the BALB/c DNA clone, we could not confirm the difference in the Southern blot analyses. The difference might be due to deletion during propagation of the phages. We tentatively conclude that the $S_{\gamma 3}$ region is identical in C57BL and BALB/c DNAs.

We find a new Xba I site 3′ to the C_μ gene of C57BL/6. In addition, if one chooses restriction fragments encompassing S regions of different lengths, one can distinguish alleles of the C_H gene in F1 mice or hybridomas made between different strains (Yaoita and Honjo, 1980b). Structure of the C_H genes seems to

be more conserved among mouse strains than V_H genes, which show considerable difference in restriction fragments (Kataoka et al., 1982; Honjo et al., 1981).

Experimental Procedures

Materials

We obtained α-^{32}P-dCTP (spec. act. 2–3000 Ci/mmole) from Radiochemical Center. Eco RI, Bam HI and T4 ligase were prepared as described previously (Honjo et al. 1979). Other enzymes were purchased from New England BioLabs, Bethesda Research Laboratories and Takara Shuzo Ltd. and used according to their recommendations. Bacterial strains for in vitro packaging were obtained from F. Blattner.

Libraries and Cloning

We used several libraries of BALB/c embryonic, newborn or liver DNA that were constructed in different ways, because none of these libraries was really complete even if it satisfied the statistical calculations. Libraries of partial Hae III digests of BALB/c embryo DNA in Charon 4A (Blattner et al., 1977), partial Eco RI* digests of BALB/c embryo DNA in Charon 4A and partial Hae III digests of C57BL/6 liver DNA in Charon 4A were gifts from P. Leder, J. Adams and J. G. Seidman, respectively. Numbers of independent phages are 5×10^5, 1×10^6 and 6×10^6, respectively. Libraries of partial Eco RI and partial Eco RI* digests of newborn BALB/c mouse DNA were prepared as described (Maniatis et al., 1978; Kataoka et al., 1982; N. Takahashi, unpublished results). Partial Sau 3A digests of newborn BALB/c mouse DNA ranging from 12 kb to 19 kb were prepared by agarose gel electrophoresis, ligated to outer fragments of Charon 30 (Liu et al., 1980), packaged in vitro (Blattner et al., 1978) and screened directly without propagation because sizes of plaques are so different from each other that we were afraid of selective proliferation of unexpected phages; 2 to 6×10^5 plaques were screened each time. Screening was performed as described (Shimizu et al., 1981), by the method of Benton and Davis (1977).

Clones μ–49, δ–89 and δ–100 were obtained as follows. Complete Bam HI and Eco RI digests and partial Eco RI digests of BALB/c liver DNA were separated by agarose gel electrophoresis, and fragments of appropriate sizes were ligated to outer fragments of Charon 28 or λgtWES (Leder et al., 1977). Recombinant phage DNA was packaged in vitro and screened as described above. We screened 3×10^5 to 10^6 plaques and usually isolated more than three independent but identical clones containing inserts in both directions relative to phage arms. Cloning experiments were performed according to Japanese guidelines for recombinant DNA research.

Southern Blot Hybridization

High molecular weight DNAs of BALB/c and C57BL/6 livers were obtained as described (Yaoita and Honjo 1980a, 1980b). When total DNA was used, 1–3 μg of DNAs were digested by appropriate restriction endonucleases, subjected to electrophoresis in 0.5% agarose gels and transferred to nitrocellulose filters as described (Southern, 1975). When cloned DNAs were used, amounts of DNAs were decreased to 0.1–0.3 μg per lane.

DNA fragments used as probes were labeled with α-^{32}P-dCTP by nick translation to specific activity 200–1000 cpm/pg (Maniatis et al., 1975). Hybridization was carried out in 1 M NaCl at 65°C as described previously (Honjo et al., 1979). Stringent washing conditions indicate that filters were washed four times (40 min each) in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS at 65°C, and mild conditions indicate filters were washed three times (15 min each) in the same buffer at 50°C.

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apanese wild mouse, *Mus musculus nolossinus*, has duplicated mmunoglobulin γ2a genes

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he immunoglobulin heavy chain loci of the mouse contain eight ghtly linked genes that encode the constant region (C) of the mmunoglobulin heavy chains (H). As no recombination curred within the immunoglobulin C_H gene loci among more an 3,000 crosses of mice[1-3], the mouse C_H genes were believed be inherited as a set, designated the *Igh* haplotype. Recent olecular cloning experiments[4-9] have demonstrated that the ganization of the mouse *Igh* loci of BALB/c (*Igh*^{a} haplotype) 5′-Cμ-(4.5 kilobases [kb])-C_δ-(55 kb)-$C_{\gamma 3}$-(34 kb)-$C_{\gamma 1}$-1 kb)-$C_{\gamma 2b}$-(15 kb)-$C_{\gamma 2a}$-(14 kb)-C_ε-(12 kb)-C_α-3′. The uctural analyses of four C_γ subclass genes have shown that ey are essentially identical in terms of lengths and locations each of the structural and intervening sequences[6,10-12]. Comrison of the nucleotide sequences of the $C_{\gamma 1}$, $C_{\gamma 2b}$ and $C_{\gamma 2a}$ nes has shown that limited portions of the C_γ gene are nserved between compared pairs of the C_γ genes[12]. The sults imply that the nucleotide sequences of the C_γ gene gments have been exchanged by recombination among ated clustered genes. Similar studies on the $C_{\gamma 2a}$ genes of ferent haplotypes, *Igh*^{a} and *Igh*^{b}, have also suggested that se two types of $C_{\gamma 2a}$ gene have undergone recombinational change of a part of the gene sequence[13]. These results imply, contrast to the previous belief, that frequent recombinational ents have taken place within the *Igh* loci and that their cleotide sequences have been rearranged during evolution. ere we present direct evidence that a Japanese wild mouse, *us musculus molossinus*, contains duplicated $C_{\gamma 2a}$ genes while e copy numbers of the neighbouring C_H genes, that is, the 1, $C_{\gamma 2b}$ and C_ε genes, remain constant. The results indicate at the duplicated $C_{\gamma 2a}$ genes of *M. m. molossinus* arose from equal crossing-over between homologous chromosomes.

During our evolutionary study of immunoglobulin C_H genes .Y. and T.H., in preparation) we found that DNAs of the panese wild mouse *M. m. molossinus* had several extra restricn DNA fragments which hybridized with a $C_{\gamma 2b}$–$C_{\gamma 2a}$ probe, ereas DNAs of many laboratory strain mice had a single gment for each C_γ gene (ref. 6 and Y.Y. and H.T., in eparation). DNA of *M. m. molossinus* had four *Eco*RI fragnts (12, 9.3, 5.6 and 1.85 kb) hybridizing with a $C_{\gamma 2b}$–$C_{\gamma 2a}$ obe in addition to the 23-kb $C_{\gamma 2a}$ and 6.6-kb $C_{\gamma 2b}$ fragments ich are shared by BALB/c, C57BL and other inbred strain ce[6]. We used the $C_{\gamma 2b}$ gene fragment (probe b in Fig. 1) as robe for both $C_{\gamma 2a}$ and $C_{\gamma 2b}$ genes. The $C_{\gamma 2a}$ gene fragments re usually less intense than the $C_{\gamma 2b}$ gene fragment because $C_{\gamma 2a}$ gene sequence was detected by cross-hybridization to art of the probe. As the 12- and 5.6-kb fragments were less ense than the 9.3- and 1.85-kb fragments, we presumed that former fragments are the $C_{\gamma 2a}$ gene.

To determine unequivocally which of the above *Eco*RI fragnts are the $C_{\gamma 2a}$ gene, we used as a probe the restriction NA fragment that contains the 3′ half of the intervening luence between the coding sequences for the C_H1 domain d the hinge region of the γ2a chain (probe c in Fig. 1). This obe has little cross-hybridization with the $C_{\gamma 2b}$ gene[12]. As own in Fig. 1*a*, DNA of an individual *M. m. molossinus* ouse (which was trapped at Mishima, in Honshu Island) has

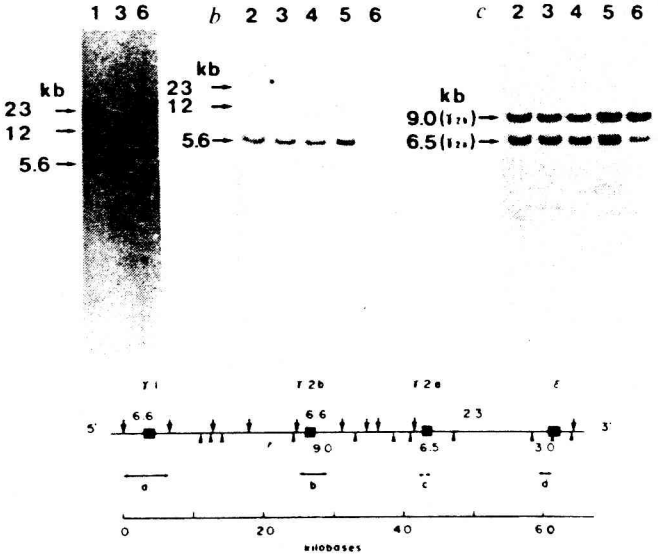

Fig. 1 Southern blot hybridization of *M. m. molossinus* liver DNA with $C_{\gamma 2a}$ or $C_{\gamma 2b}$–$C_{\gamma 2a}$ gene probe. High molecular weight DNA was extracted from each individual liver of a wild heterozygous *M. m. molossinus* or inbred *M. m. molossinus* mice, or from livers of BALB/c mice as described previously[20]. We used three individual mice of inbred *M. m. molossinus* which are derived from wild mice trapped at Teine, Hokkaido Island. These mice are designated as Mol. Ten (individual numbers 101, 102 and 103). The other individual mouse of inbred *M. m. molossinus*, designated as MOL. A, is the progeny of wild mice trapped at Anjo, Aichi, Honshu Island. DNAs (1.5 μg each) were digested with *Eco*RI (*a*, *b*) or *Hin*dIII (*c*), electrophoresed in a 0.5% agarose gel and transferred to nitrocellulose filters as previously described[20,21]. Probes used are $C_{\gamma 2a}$ (probe c) for *a* and *b* and $C_{\gamma 2b}$–$C_{\gamma 2a}$ (probe b) for *c*. Probes were labelled by nick-translation and hybridized as described elsewhere[20,22]. Origins of DNAs used are as follows: lane 1, a wild *M. m. molossinus*; lane 2, Mol. Ten 103; lane 3, Mol. Ten 102; lane 4, Mol. Ten 101; lane 5, MOL. A; lane 6, BALB/c. A restriction endonuclease cleavage map around the $C_{\gamma 2a}$ gene of the BALB/c mouse and fragments used for probes are shown at the bottom. Data for the restriction map are taken from ref. 4. Structural genes are shown in closed boxes. Numbers above and below the top line indicate the sizes (kilobases) of *Eco*RI and *Hin*dIII fragments, respectively, which are described in the present study. Horizontal arrows under the top line indicate the fragments used as probes: a, 6.6-kb *Eco*RI fragment of γ1 gene[10]; b, 4-kb *Xba*I–*Hha*I fragment of $C_{\gamma 2b}$ gene[11] which hybridizes to not only the $C_{\gamma 2b}$ gene but also the $C_{\gamma 2a}$ gene[4,20]; c, 217-base pair *Hae*III fragment of $C_{\gamma 2a}$ gene which contains the 5′ 12 base pairs of the hinge region exon and the 3′ half of the intervening sequence between the C_H1 domain and the hinge region exons[12]; d, 2-kb *Bam*HI-*Hin*dIII fragment of C_γ gene[5]. ↓ and ▲ indicate *Eco*RI and *Hin*dIII sites, respectively.

three *Eco*RI fragments (23, 12 and 5.6 kb) that hybridize with the $C_{\gamma 2a}$ probe. The results seem to indicate that the $C_{\gamma 2a}$ gene is duplicated in this wild mouse, although the presence of heterozygous $C_{\gamma 2a}$ gene cannot be excluded.

We next examined DNAs of four individual mice of *M. m. molossinus* which have been maintained in laboratories by full-sibmating for 11 generations in the Mol. Ten strain and for 23 generations in the MOL. A strain. The Mol. Ten strain was derived from wild mice caught at Teine in Hokkaido Island and the MOL. A strain from those caught at Anjo in Honshu Island. *Eco*RI digestion of DNAs of these *M. m. molossinus* strains gave two DNA fragments (12 and 5.6 kb) which were identical for the two strains, whereas DNA of BALB/c mice had a single *Eco*RI fragment of 23 kb (Fig. 1*b*). The results strongly support the idea that *M. m. molossinus* has the duplicated $C_{\gamma 2a}$ genes, one of which is located in the 12-kb *Eco*RI fragment and the other in the 5.6-kb fragment. One can still argue the possibility, however, that *M. m. molossinus* has introduced point mutations which have created new *Eco*RI sites, thus converting the 23-kb fragment into the 12- and 5.6-kb fragments.

To exclude this possibility, we used another restriction

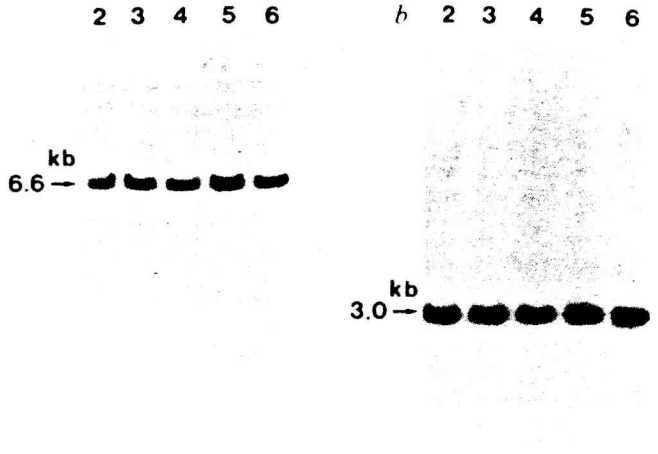

Fig. 2 Southern blot hybridization of *M. m. molossinus* liver DNA with $C_{\gamma 1}$ or C_ε probe. DNAs (1.5 μg each) were digested with *Eco*RI (*a*) or *Hin*dIII (*b*) and electrophoresed, blotted and hybridized as described in Fig. 1 legend except that probes used are $C_{\gamma 1}$ (probe a in Fig. 1) for *a* and C_ε (probe d in Fig. 1) for *b*. Origins of DNAs used in lanes indicated were the same as described in Fig. 1 legend.

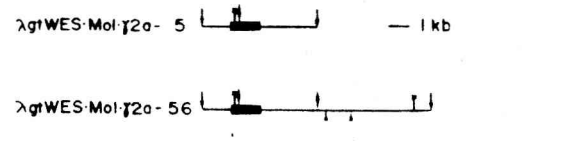

Fig. 3 Restriction map of two cloned $C_{\gamma 2a}$ genes of *M. m. molosssinus*. High molecular weight DNA of Mol. Ten 103 was digested with *Eco*RI to completion and the digests were fractionated by electrophoresis in a 0.5% agarose gel. Fragments of 5.6 and 12 kb were collected and ligated to λgtWES arms as described previously[9]. Ligated DNAs were packaged *in vitro* and screened with BALB/c $C_{\gamma 2b}$–$C_{\gamma 2a}$ probe (probe b in Fig. 1) as described elsewhere[4]. Two and three identical clones were isolated from 5.6- and 12-kb fragments, respectively. Inserts of λgtWES·Mol·γ2a-5 (5.6 kb) and λgtWES·Mol·γ2a-56 (12 kb) are shown by horizontal bars. Closed boxes indicate structure genes. Restriction enzyme cleavage sites are indicated as follows: ▼, *Eco*RI; ▲, *Hin*dIII; ◆, *Xho*I; ⚷, *Bgl*I.

enzyme, *Hin*dIII. *Hin*dIII digestion of *M. m. molossinus* produced the 9.0-kb $C_{\gamma 2b}$ fragment and the 6.5-kb $C_{\gamma 2a}$ fragment, both of which are identical in size to those of BALB/c DNA (Fig. 1*c*). However, 6.5-kb bands of the $C_{\gamma 2a}$ gene of *M. m. molossinus* DNAs are about twice as dark as that of the BALB/c DNA. In contrast, the intensity of the $C_{\gamma 2b}$ gene bands (9.0 kb) remains the same among DNAs of *M. m. molossinus* and BALB/c mice.

To examine whether neighbouring C_H genes are duplicated, we analysed the $C_{\gamma 1}$ and C_ε genes. As Fig. 2 clearly shows, the intensity and size of the $C_{\gamma 1}$ and C_ε genes in these wild mice are indistinguishable from those of BALB/c mice. We have also found that the $C_{\gamma 3}$ gene is not duplicated (data not shown). Previously we showed that the number of the C_μ and C_α gene fragments of *M. m. molossinus* are identical to those of many laboratory inbred strain mice although the sizes of the fragments vary slightly[6].

We have further cloned two DNA fragments, each containing a complete $C_{\gamma 2a}$ gene, from DNA of an individual *M. m. molossinus*. One $C_{\gamma 2a}$ gene is located in the 12-kb *Eco*RI fragment and other in the 5.6 kb *Eco*RI fragment (Fig. 3). The restriction sites in the cloned DNA fragments are consistent with those determined by Southern blot analyses.

These results demonstrate that *M. m. molossinus* has a new haplotype which contains the duplicated $C_{\gamma 2a}$ genes. In spite of the fact that these four mice are derived from two independent populations in different islands of Japan, the mice seem to have a similar haplotype, indicating that this haplotype is widely distributed among various local colonies of *M. m. molossinus*.

Lieberman and Potter[14] analysed allotype markers of serum immunoglobulins of homozygous *M. m. molossinus* (Kyushu) and found that this wild mouse had $IgG2a^a$ markers in addition to the $IgG1^b$, $IgG2b^b$, $IgG2a^b$ and IgA^b markers. They proposed that this haplotype is created by unequal crossing-over between the Igh^a and Igh^b haplotypes. Using monoclonal antibodies which can distinguish *a* and *b* allotypes, Herzenberg and his associates (personal communication) also found that two individual mice of inbred *M. m. molossinus* (Mol. Ten strain) have two allotypes of IgG2a, namely $IgG2a^a$ and $IgG2a^b$. Because the restriction sites around the coding regions of the $C_{\gamma 2b}$ and $C_{\gamma 2a}$ genes are well conserved between C57BL (Igh^b) and BALB/c (Igh^a)[6,9], we are unable to correlate the $C_{\gamma 2a}$ gene fragments with serological markers. The restriction map of the cloned $C_{\gamma 2b}$ gene of *M. m. molossinus* is different from those of BALB/c and C57BL (A.S. *et al.*, in preparation). As Fig. 4 shows, it is therefore likely that the duplicated $C_{\gamma 2a}$ genes of *M. m. molossinus* arose from unequal crossing-over between two different haplotypes which must be characterized and identified by nucleotide sequence determination.

Since immunoglobulin allotypes are distributed among various subspecies of *M. musculus*, the allotype seems to have evolved before the divergence of the subspecies of *M. musculus*. The duplication of the $C_{\gamma 2a}$ gene must have taken place after the divergence of *M. m. molossinus* and *M. m. domesticus*, an estimated one million years ago[15–17]. Duplication of immunoglobulin genes has also been suggested in humans by serological studies[18,19].

*Eco*RI digestion of DNA of the first heterozygous *M. m. molossinus* mouse produced three $C_{\gamma 2a}$ gene fragments (23, 12 and 5.6 kb) and three $C_{\gamma 2b}$ gene fragments (9.3, 6.6 and 1.85 kb). It is now clear that the 12- and 5.6-kb $C_{\gamma 2a}$ gene fragments are located on one chromosome and the 23-kb $C_{\gamma 2a}$ gene fragment on the other. We also know that the 6.6-kb $C_{\gamma 2b}$ gene fragment is derived from the chromosome containing the duplicated $C_{\gamma 2a}$ gene. Consequently, the other chromosome should contain two $C_{\gamma 2b}$ gene fragments (9.3 and 1.85 kb) and one $C_{\gamma 2a}$ gene (23 kb). It is not clear whether these two *Eco*RI fragments of the $C_{\gamma 2b}$ gene are due to the gene duplication or

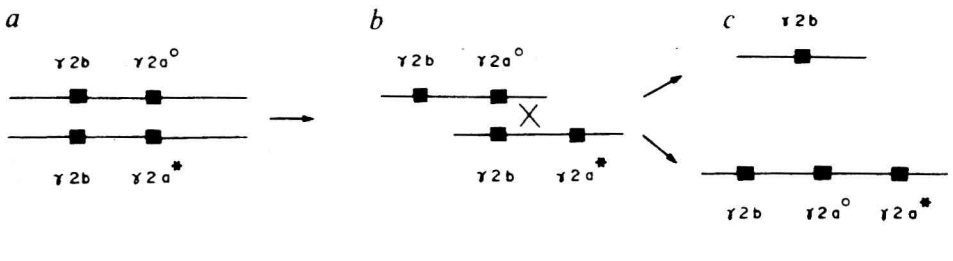

Fig. 4 A possible recombination that created duplicated $C_{\gamma 2a}$ genes with different allotypes. *a*, schematic representation of heterozygous mouse chromosomes containing two different $C_{\gamma 2a}$ genes. $\gamma_{2a}{}^*$ and $\gamma_{2a}{}^\circ$ indicate $C_{\gamma 2a}$ genes with different allotypes. *b*, Unequal pairing between homologues at meiosis and unequal crossing-over. As $C_{\gamma 2b}$ and $C_{\gamma 2a}$ genes have extensive homology[8,12], such a misalignment may occur at meiosis. *c*, Resultant chromosomes. In one chromosome (upper) the $C_{\gamma 2a}$ gene has been deleted, while the other chromosome contains the duplicated $C_{\gamma 2a}$ genes which have different allotypes. The latter chromosome may be an ancestor of the present chromosome in *M. m. molossinus*.

to the introduction of new *Eco*RI sites by point mutation.

The number of C_H genes has been thought to be invariant among inbred strains, although definitive proof is lacking. Previous studies (ref. 6 and Y.Y. and H.T., in preparation) using Southern blot analyses indicate the presence of a single gene for each of the C_μ . $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, C_ε and C_α genes in many inbred strains including BALB/c, C57BL, AKR, AJ, NZB and SJL. It may be safe to conclude that inbred strains mentioned above have similar sets of C_H genes although they have considerable divergence in the nucleotide sequence.

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