主論文

Construction of Chimeric Processed Immunoglobulin Genes that Contains Mouse Variable and Human Constant Sequences

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The exquisite specificity of monoclonal antibodies has provided powerful diagnostic and therapeutic tools for human neoplasia. Radiological scanning and immune therapy with mouse tumor-specific monoclonal antibodies have been applied to patients with preliminary success[1-3]. However, a major problem of these trials is neutralization of a mouse monoclonal antibody by patients' antibodies induced by repeated administration of heterologous antibodies. To avoid or reduce such immune reaction it is reasonable to synthesize chimeric immunoglobulins consisting of mouse V and human C regions. To construct the chimeric immunoglobulin cDNA we employed the retrovirus vector with a unique life cycle that includes two different forms of the genome in host cells, RNA and DNA. We took advantage of the fact that the retroviral genome including inserted sequences is transcribed into RNA which is then spliced to remove the intron sequence[4]. In addition, a splicing donor site of SV40 and an acceptor site of the mouse β-globin gene were properly spliced in monkey cells[5], suggesting that the donor and acceptor sites are separate entities. We therfore constructed recombinant retrovirus DNA carrying a genomic V_HDJ_H and $C_{\gamma 1}$ genes from different species and showed the chimeric intervening sequences were spliced out precisely. This procedure provides a useful method to construct the chimeric mouse-human immunoglobulin gene to be expressed in E.coli, yeast and animal cells. During the course of this study we found unexpectedly that a hidden splice donor site in the 5' flanking region of a human V_H gene was utilized in place of the donor site of the leader sequence exon, resulting in the formation of the V region without the leader sequence. The removal of the leader sequence, which is essential for secretion in eukaryotic cells but

often toxic in prokaryotic cells, is also potentially useful for efficient expression in microorganisms.

We have chosen two pairs of immunoglobulin V_H and C_H genes of different species origins as models for the chimeric recombinant immunoglobulin heavy chain gene. One pair consists of the mouse $C_{\gamma 1}$ gene[6] and a human V_H gene encoding a V_H region against the surface antigen of hepatitis B virus (HBV). These two genes were inserted into a retrovirus vector plasmid, pTK-2$\Delta\Delta$ ter(R)[7]. The recombinant virus plasmid was abbreviated as $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$ (Fig. 1A). The other pair of the human $C_{\gamma 1}$ gene[8] and the mouse V_H gene of TEPC15 myeloma[9] was inserted into pSNVΔTK[4] and the recombinant virus plasmid was abbreviated as $pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$ (Fig. 1B). These two vectors are derived from spleen necrosis virus (SNV). Since the removal of the poly A addition signal AATAAA of an inserted foreign gene increases the recovery of the SNV vector[7], we removed the sequence 3' to the HinfI site located 39 bp downstream of the mouse $C_{\gamma 1}$ coding sequence and the sequence 3' to the HhaI site located 88bp downstream of the human $C_{\gamma 1}$ coding sequence.

To recover infectious viruses carrying the immunoglobulin gene, $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$ and $pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$ DNAs were used to transfect chicken embryo fibroblasts with or without one tenth as much DNA of a provirus clone of a non-defective helper virus, reticuloendotheliosis virus strain A (REV-A)[10]. Before transfection $pSNV_1 \cdot hV_{HBS}\text{-}mC_{\gamma 1}$ DNA was digested with SalI to remove pBR322 sequence electrophoretically. The virus production was assayed by its reverse transcriptase activity[11]. The enzyme activity of the culture supernatants of the cells transfected with recombinant and helper

virus DNAs arose about 10 folds higher than that of the background on day 5-7 after transfection. Viruses in the culture supernatants recovered 7-10 days after transfection were used to infect fresh chiken embryo fibroblasts. Three days later, unintegrated linear viral DNA was isolated and examined for processing of the inserted DNA by Southern blot hybridization.

The parental $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$ DNA contains the 3.6-kb BglII fragment that hybridizes to both human V_{HBV} and mouse $C_{\gamma 1}$ probes (probes A and B in Fig. 1A, respectively). On the other hand BglII digestion of the processed $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$ DNA should yield a 2.6-kb fragment hybridizing to both probes (Fig. 1). As shown in Fig 2A, the viral DNA obtained from chicken embryonic fibroblasts transfected with $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$ and helper virus DNA contained the 3.6- and 2.1-kb BglII fragments hybridizing to both human V_{HBV} and mouse $C_{\gamma 1}$ probes, suggesting that the viral DNA contains the parental and processed forms. No significant viral DNA was recovered from transfectants without the helper virus DNA (Fig. 2A lanes 2,3, 7 and 8).

The parental $pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$ DNA contains the 1.3-kb BamHI fragment hybridizing to the mouse V_{T15} probe (probe C) and a 3.6-kb BamHI fragment hybridizing to the human $C_{\gamma 1}$ probe (probe D). BamHI digestion of the processed $pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$ should yield a 1.7-kb fragment hybridizing to both probes C and D. Viral DNA isolated from transfectants by $pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$ and helper viruses contained the 1.7-kb and 1.3-kb BamHI fragments hybridizing to probe C (Fig. 2B lanes 1-5) and the 1.7-kb and 3.6-kb BamHI fragments hybridizing to probe D (Fig. 2B lanes 6-10). There are three other potential donor splice sites (J_{H2}, J_{H3} and J_{H4}) paired with the acceptor splice site of the CH1 exon. If the donor sites of the J_{H2},

J_{H3} and J_{H4} segments were used, we should see the 2.2-, 1.5- and 2.0-kb BamHI bands, respectively, hybridizing to the human $C_{\gamma 1}$ probe. There were no additional faint bands between 3.6- and 1.7-kb BamHI bands, excluding the above possibilities. These results suggest that the chimeric immunoglobulin genes were processed at the expected intron-exon splice sites.

To analyse the structure of the progeny viruses in more detail, the inserts of the progeny viruses were cloned into Charon 28H vector a derivative of Charon 28 (see legend of Fig. 3). The progeny viral DNAs of $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$ and $pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$ were digested with BglII and BamHI, respectively, and fractionated by agarose gel electrophoresis. DNA fractions containing the 2.1-kb BglII and 1.7-kb BamHI fragments were ligated into the BamHI site of phage Charon 28H DNA. Recombinant phages were screened by appropriate immunoglobulin gene probes. We obtained 8 clones containing $hV_{HBV}\text{-}mC_{\gamma 1}$ and 2 clones containing $mV_{T15}\text{-}hC_{\gamma 1}$. Restriction enzyme cleavage analysis indicated that eight $hV_{HBV}\text{-}mC_{\gamma 1}$ clones were indistinguishable from each other. Similarly two $mV_{T15}\text{-}hC_{\gamma 1}$ clones were identical with respect to restriction site mapping.

The restriction map of the cloned $hV_{HBV}\text{-}mC_{\gamma 1}$ DNA is consistent with that of the processed chimeric gene of the human V_{HBV} and mouse $C_{\gamma 1}$ gene except that the left most TaqI-XbaI is shortened by 500 bp (Fig. 1A). Disappearance of the BamHI site in the 5' flanking region of the processed $hV_{HBV}\text{-}mC_{\gamma 1}$ agrees with the reduction of the TaqI-XbaI fragment size, suggesting that segmental deletion occurred in the 5' flanking region of the V_{HBV} gene. The total length of the cloned $hV_{HBV}\text{-}mC_{\gamma 1}$ insert is 2.1-kb, 500 bp shorter than predicted, in agreement with the size of the BglII fragment

(2.1 kb) observed by Southern blot hybridization (Fig. 2A). The 500-bp deletion in TaqI-XbaI fragment is, therefore, not due to the cloning artifact but to genetic events during virus replication. The results of restriction site mapping of the progeny mV_{T15}-$hC_{\gamma 1}$ clones are consistent with loss of all introns from the parental mV_{T15}-$hC_{\gamma 1}$ insert.

The DNA sequences at the junctions of exons were determined according to the strategy shown in Fig. 1. Nucleotide sequences surrounding the junctions of the V and C gene sequences of the progeny clones are aligned with those around the splice sites of the parental V and C gene exons as shown in Fig. 3. It is clear that the V-C junctions of $pSNV_1 \cdot hV_{HBV}$-$mC_{\gamma 1}$ and $pSNV_2 \cdot mV_{T15}$-$hC_{\gamma 1}$ are precisely processed according to the GT-AG rule[12], indicating that interspecies pairs of exons have little trouble for precise removal of introns. Nucleotide sequences of other exon junctions also demonstrated that all the introns were spliced out exactly as expected (data not shown).

We also read the sequence of the 5' flanking region of the genomic V_{HBV} gene and found that there was a potential donor splice site CCGGTGAGA at 268-276 bp downstream from the TaqI site. Comparison of the nucleotide sequences of the parental and progeny V_{HBV} genes and their upstream regions revealed that the region extending from this potential donor site to the acceptor site of the V_{HBV} exon was removed (Fig. 3). Therefore, there is preference of the 5' potential splice site to the normal donor site of the signal peptide exon. The nucelotide sequences of both the 5' potential and normal donor sites mismatch at two bases with the consensus 9-base sequence surrounding the donor splice site[13]. Except for GGTGA sequence, there is

little sequence homology between the nucleotide sequences surrounding the potential and normal donor sites. There are several examples that mutations in normal splice donor sites awoke potential donor splice sites in exons[14-17]. The presence of a donor splice site more potent than the normal donor site in the flanking region has not been reported.

We have shown that the strategy using the retrovirus vector is useful for construction of hybrid immunoglobuin genes and eventually for expression of the hybrid protein in yeast, E.coli and animal cells. Our results demonstrate that the genomic V and C exons derived from different species is properly fuzed. We have employed successfully the same strategy to construct the hybrid light chain genes composed of a mouse V_K and the human C_K genes. It is needless to say that antibodies against human cells and proteins are easier to produce in mouse than in man. Most clinically useful antibodies are thus made in mouse and difficult to be administered to man. If the C region of the mouse monoclonal antibody against human cancer cells could be replaced by a human immunoglobulin C region, there would be several advantages of such a hybrid immunoglobulin to cure patients suffered from cancer. First, the hybrid immunoglobulin is less antigenic to human. Secondly, we can choose the most useful C_H region that has the specific effector functions required for the treatment.

Legends to Figures

Fig. 1. Structure of parental and progeny recombinant immunoglobulin genes. Parental recombinant plasmids containing retroviral and immunoglobulin sequences are shown at top. Immunoglobulin gene sequences in progeny retroviruses are shown at bottom. Retrovirus, mouse and human immunoglobulin sequences are indicated by open, oblique and solid rectangles, respectively. Long terminal repeat (LTR) and exons are shown by wider rectangles. pBR322 sequence is shown by solid line. Corresponding DNA segments in parental and progeny viruses are aligned by dotted lines. Solid bars indicate probes used. Directions and ranges of sequences read are shown by horizontal arrows. L, leader exon; V, VDJ exon; H, hinge exon; C_{H1}, C_{H2} and C_{H3}, exons encoding each domain. Restriction sites are indicated as follows: A, AvaII; B, BamHI; Bg, BglII; E, EcoRI; H, HinfI; Ha, HaeIII; Hh, HhaI; S, SalI; Sc, SacII; Sm, SmaI; T, TaqI; X, XbaI. A)$pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$. Fragments A and B indicate Sau3A-HhaI fragment of human V_{HBV} gene and BamHI-BglII fragment of mouse $C_{\gamma 1}$ gene, respectively. Closed triangle indicates the hidden donor splice site in the TaqI-BamHI fragment. B)$pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$. Fragments C and D indicate BamHI fragment of mouse V_{T15} gene and SacII fragment of human $C_{\gamma 1}$ gene, respectively.

Method: Construction of $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$. We cloned the V_H gene encoding the V region against the surface antigen of hepatitis B virus (HBV) from the Epstein-Barr-virus-transformed cell and determined the nucleotide sequence of the coding region (unpublished data). The 2.2 kb (SacII/SmaI) fragment of the human V_{HBV} gene was prepared. The SacI fragment (2.5 kb) including

the mouse $C_{\gamma 1}$ gene sequence was electrophoretically isolated from λgtWES·IgH-2 (ref. 6). The SacI fragment was digested separately with XbaI and SmaI, and with SmaI and HinfI. HinfI digests the $C_{\gamma 1}$ gene at 30 bp upstream from the poly(A) addition signal AATAAA. The 0.88-kb XbaI-SmaI and 0.64-kb SmaI-HinfI fragments were isolated. We inserted the 2.2-kb SacII-SmaI fragment of the human V_{HBV} gene and the 0.88-kb XbaI-SmaI and the 0.64-kb SmaI-HinfI fragments of the mouse $C_{\gamma 1}$ gene into the BglII site of pTK-2△△Ter (R) (ref. 7). The XbaI site of the mouse $C_{\gamma 1}$ gene was treated with Klenow fragment of DNA polymerase I to make the blunt end and ligated with the SmaI site of the human V_{HBV} gene. The SacII site of human V_{HBV} gene and the HinfI site of mouse $C_{\gamma 1}$ gene were filled with Klenow fragment and ligated with BglII linker (Takara Co. Ltd.). The intron between the V and C exons consists of 460 bp of 3' noncoding sequence of the $V_{HBV}DJ_{H4}$ gene and 50 bp of the 5' noncoding sequence of the mouse $C_{\gamma 1}$ gene.

Construction of $pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$. pSNV-TK (ref. 4) was digested with SacII to remove electrophoretically the 1.6 kb SacII fragment in the tk gene and then recircularized with T4 ligase. This plasmid is referred to pSNV△TK. If the tk gene is left intact, the total length of the viral DNA containing the mouse V_{T15} and human $C_{\gamma 1}$ genes exceeds the upper limit for encapsidation. The HindIII fragment of Igγ1-10 containing human $C_{\gamma 1}$ gene[8] was first subcloned into pBR322 and this plasmid was then digested separately with HindIII and PstI, and with PstI and HhaI. HhaI cleaves the $C_{\gamma 1}$ gene at 15 bp upstream from AATAAA sequence. Equimolar amounts of the HindIII-PstI fragment containing the CH1 exon and the PstI-HhaI fragment containing the CH2, hinge and CH3 exons were ligated together. The 1.9-kb

fragment corresponding to the length of the intact HindIII-HhaI fragment of the human $C_{\gamma 1}$ gene was electrophoretically purified and the ends of the fragment were filled by Klenow fragment of DNA polymeraseI. The blunt-ended DNA fragment was iserted into the SmaI site of pSNVΔTK. The XbaI fragment containing the V_{T15} sequence[9] was then inserted into the XbaI site of pSNVΔTK$\cdot hC_{\gamma 1}$. The intron between the V_{T15} and $C_{\gamma 1}$ exons consists of the 3' noncoding region of the V_{T15} (2 kb) containing the J_{H2}, J_{H3} and J_{H4} exons, the 5' noncoding region of the human $C_{\gamma 1}$ gene (200 bp) and the XbaI-SmaI fragment of pSNV-TK (200 bp).

Fig. 2. Structural analyses of unintegrated progeny viral DNA from chicken fibroblasts infected with recombinant viruses. Unintegrated viral DNA was analysed by Southern blot hybridization using nick-translated probes indicated. (A) progeny virus of $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$. DNA in each lane is as follows; lanes 1 and 6, parental $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$; lanes 2, 3, 7 and 8, progeny of $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$ recovered from chicken fibroblastic cells transfected without helper virus DNA; lanes 4, 5, 9 and 10, progeny of $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$ recovered from fibroblasts transfected with helper virus DNA. DNAs in lanes 1, 3, 5, 6, 8 and 10 were digested with BglII. Hybridization probes used are; lanes 1-5, probe A; lanes6-10, probe B indicated in Fig. 1. (B) Progeny virus of $pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$. DNA in each lane is as follows; lanes 1 and 6, parental $pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$; lanes 2, 3, 7 and 8, progeny of $pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$ recovered from fibroblasts transfected without helper virus DNA; lanes 4, 5, 9 and 10, progeny of $pSNV_2 \cdot mV_{T15}\text{-}C_{\gamma 1}$ recovered from fibroblasts transfected with helper virus

DNA. DNAs in lanes 1, 3, 5, 6, 8 and 10 were digested with BamHI. Hybridization probes used are; lanes 1-5, probe C; lanes 6-10, probe D indicated in Fig. 1. Lengths of hybridized bands are given in kb. Arrows indicate the bands derived from the parental or processed hybrid immunoglobulin gene.

Method The 8.2-kb SalI fragment of $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$ were isolated by agarose gel electrophoresis and ligated with T_4 DNA ligase at 15°C for 10 hours. Ten µg each of homopolymeric $SNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$ and circular $pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$ DNA were introduced separately into chicken fibroblastic cells in a tissue culture flask (25 cm^2) in the presence or the absence of REV-A DNA (1µg) by the calcium phosphate coprecipitation technique[18]. Virus production was assayed by the reverse transcriptase activity[11]. After 7-10 days the tissue culture medium containing the recombinant virus was collected and fresh chicken cells in a 100mm Petri dish were infected with undiluted virus. Three days after infection unintegrated viral DNA was isolated by the Hirt procedure from the infected cells[19,20]. The unintegrated viral DNAs were digested with BglII or BamHI and analyzed by electrophoresis in a 0.8% agarose gel. Each lane contained DNA from one 100-mm Petri dish. The gel was blotted onto nitrocellulose filter paper and the filter was hybridized with nick-translated ^{32}P-labelled probe indicated.

Fig. 3. Nucleotide sequences surrounding processed exon junctions in progeny viruses. (A) Sequence surrounding the V_{HBV} and mouse C_{H1} exon junction in progeny virus of $pSNV_1 \cdot hV_{HBV}\text{-}mC_{\gamma 1}$. (B) Sequence surrounding the V_{T15} and human C_{H1} exon junction in progeny virus of $pSNV_2 \cdot mV_{T15}\text{-}hC_{\gamma 1}$. (C)

Sequence surrounding the recombination site between the potential donor splice site in the 5' flanking region of the V_{HBV} gene and the V_{HBV} exon. Nucleotide sequences around the exon-intron junctions in parental virus are shown above the sequence of processed gene. The consensus sequence of the donor splice site[13] and the 5' splice site of leader sequence of V_{HBV} are also shown. GT and AG are underlined. Splice sites are designated by arrow heads. Sequencing strategies are shown in Fig. 1. Nucleotide sequences determined (about 200 bp at each junction) are identical to those predicted from parental gene sequences taken from litterature[6,22,21]. V_{HBV} sequence was determined completely and will be published elsewhere.

Method Virus-infected chicken cells in twelve 100-mm Petri dishes were lysed with sodium dodecyl sulfate 3 days after infection. Unintegrated viral DNA was isolated by Hirt's procedure[19,20]. The 1.7-kb BamHI fragment containing the processed mV_{T15}-$hC_{\gamma 1}$ gene and the 2.1 kb BglII fragment containing the processed hV_{HBV}-$mC_{\gamma 1}$ gene were isolated by agarose gel electrophoresis. These fragments were inserted separately into Charon 28H a modified Charon 28 vector (described below) with T_4 ligase and packaged in vitro. Recombinant phages containing hV_{HBV}-$mC_{\gamma 1}$ and mV_{T15}-$hC_{\gamma 1}$ were screened using probes A and C, respectively. We identified 8 phage clones that contained the hybrid hV_{HBV}-$mC_{\gamma 1}$ gene fragment and 2 phage clones that contained the hybrid mV_{T15}-$hC_{\gamma 1}$ gene fragment. There is a EcoRI site in each arm of Charon 28H vector. We electrophoretically purified the EcoRI fragment containing processed hV_{HBV}-$mC_{\gamma 1}$ gene and the BamHI fragment containing processed mV_{T15}-$hC_{\gamma 1}$ gene for restriction enzyme analysis and

sequencing. The EcoRI fragment containing hV_{HBV}-$mC_{\gamma 1}$ and the BamHI fragment containing mV_{T15}-$hC_{\gamma 1}$ were cleaved with restriction enzymes shown in Fig. 1 and their ends were labeled by polynucleotide kinase. Labeled fragments were isolated and sequenced according to Maxam and Gilbert[23]. We also sequenced the TaqI-XbaI fragment of the 5' flanking region of the V_{HBV} gene.

Construction of Charon 28H vector. 6.8-kb BamHI fragment of mouse immunoglobulin $S_{\gamma 2a}$ region gene[24] was cloned in PBR322. The recombinant plasmid was digested with BglII and inserted into BamHI site of Charon 28 (ref 5) to construct Charon 28H vector. Charon 28H DNA was digested with BamHI and SalI, and the internal fragment derived from pBR322 was removed to prepare arms. Longer and shorter arms of the modified Charon 28H are 4.1- and 2.7-kb, respectively, longer than the original Charon 28. We can use the BamHI site of Charon 28H to clone as small as 1-kb fragment.

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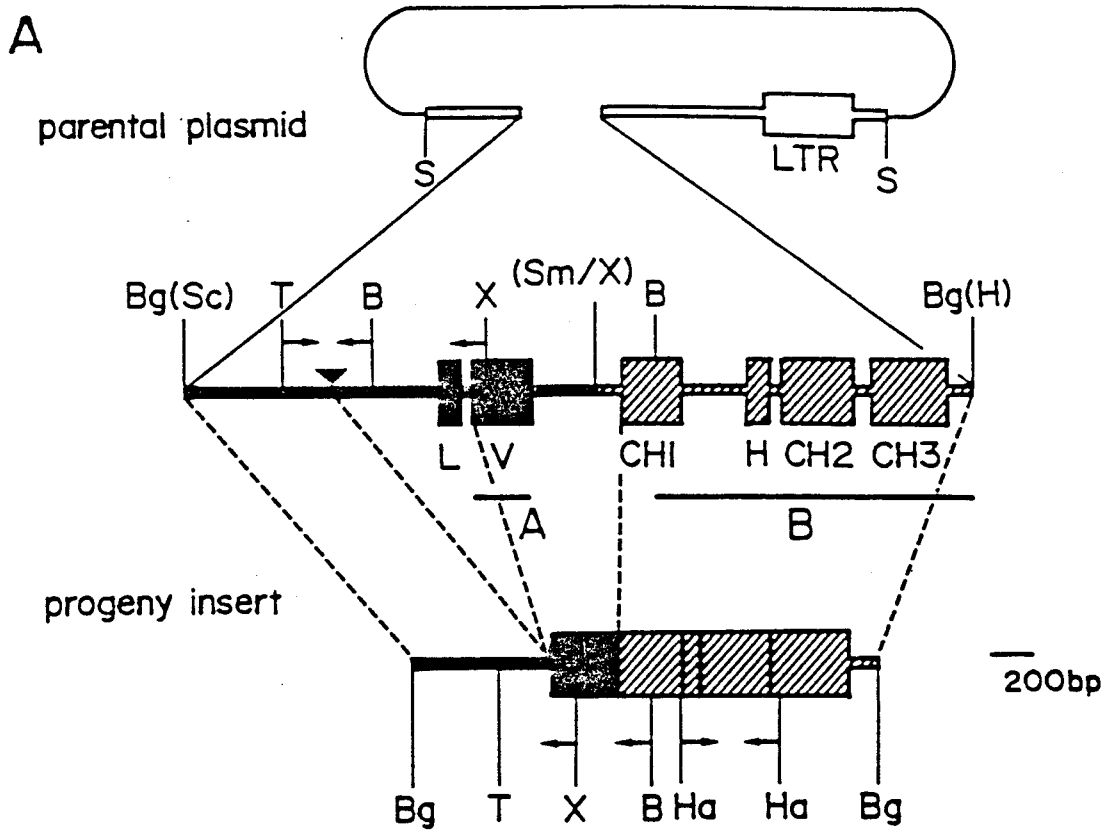
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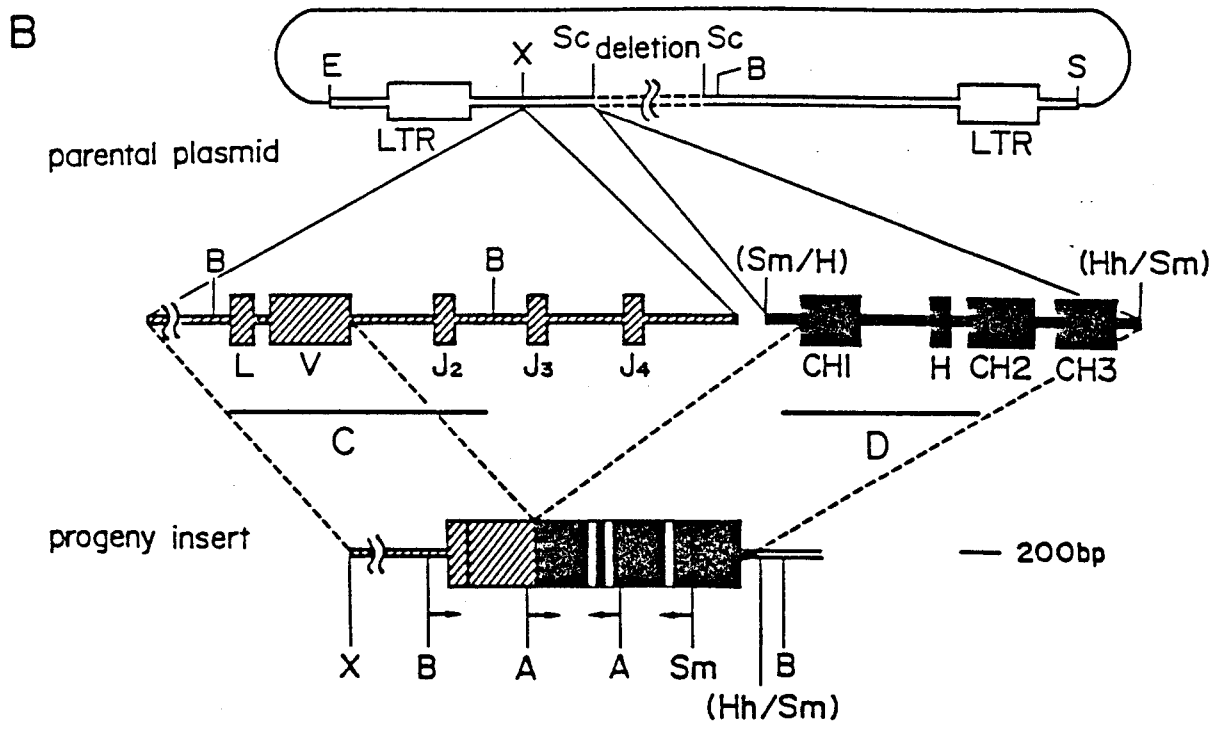
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Figure 1

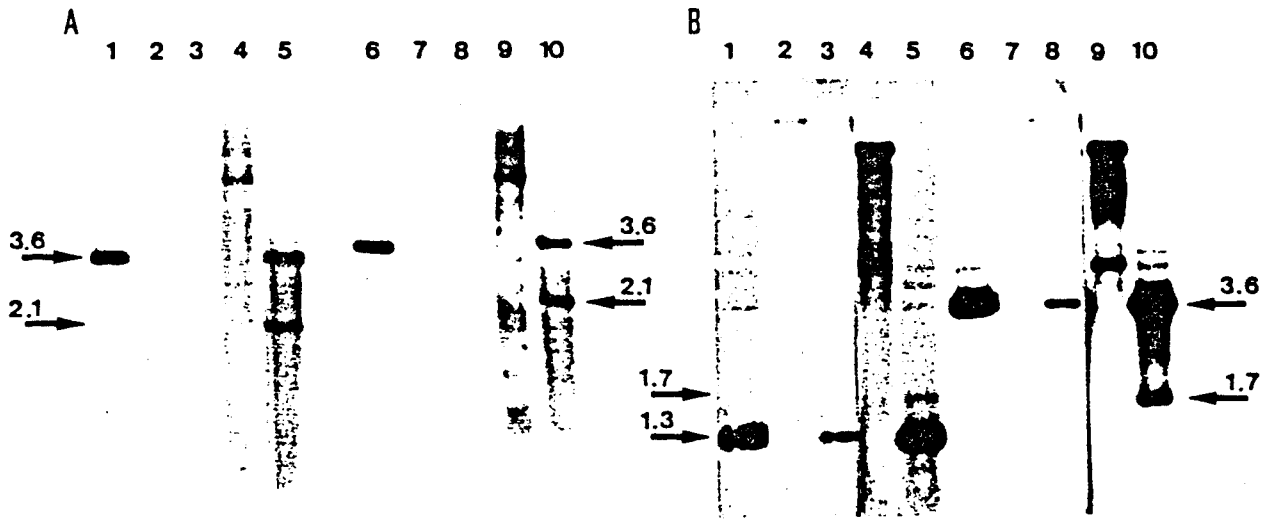
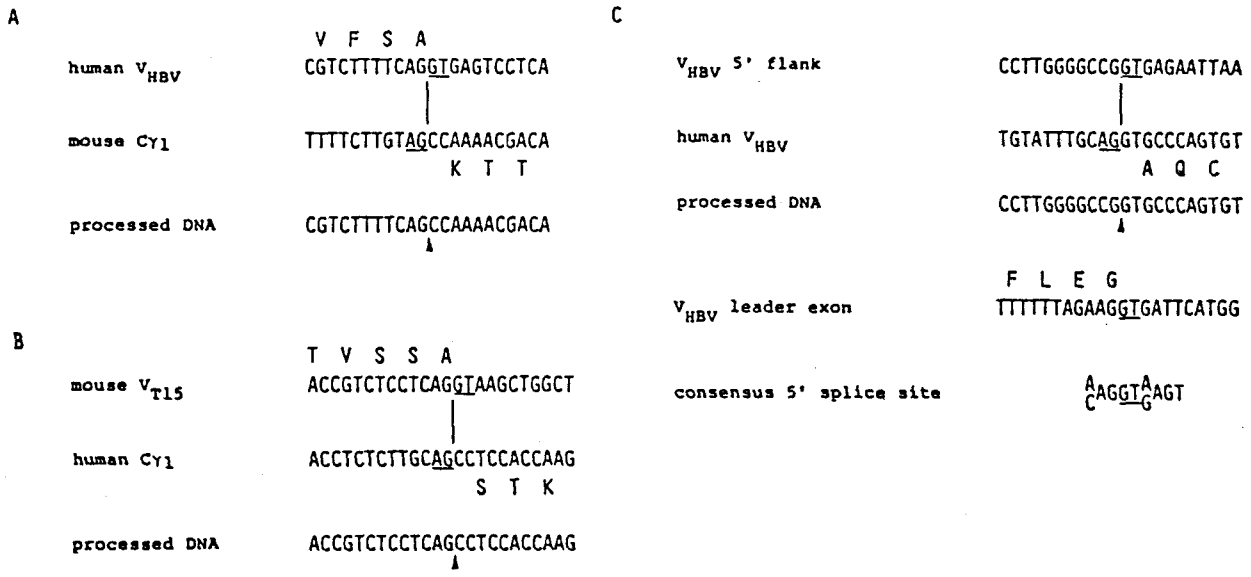


Figure 2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mouse $C\gamma_1$
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CGTCTTTTCAGCCAAAACGACA
B
T V S S A
mouse V_{T15}
ACCGTCTCCTCAGGTAAGCTGGCT
human $C\gamma_1$
ACCTCTCTTGCAGCCTCCACCAAG
S T K
processed DNA
ACCGTCTCCTCAGCCTCCACCAAG
C
V_{HBV} 5' flank
CCTTGGGGCCGGTGAGAATTAA
human V_{HBV}
TGTATTTGCAGGTGCCCAGTGT
A Q C
processed DNA
CCTTGGGGCCGGTGCCCAGTGT
F L E G
V_{HBV} leader exon
TTTTTTAGAAGGTGATTCATGG
consensus 5' splice site
A/C AGGT A/G AGT

Figure 3

From: GENETICS OF THE IMMUNE RESPONSE
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ORGANIZATION AND REORGANIZATION OF CONSTANT REGION GENES OF IMMUNOGLOBULIN HEAVY CHAINS: GENETIC BASIS FOR CLASS SWITCHING

Tasuku Honjo, Norio Ishida, Tohru Kataoka,
Sumiko Nakai, Toshio Nikaido, Yasuyoshi Nishida,
Yoshihiko Noma, Masahiro Obata, Yasuhiko Sakoyama,
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SUMMARY

We have determined the complete organization of the mouse C_H gene family, which is comprised of the 8 C_H genes in the order 5'-J_H-6.5kb-C_μ-4.5kb-C_δ-55kb-$C_{\gamma 3}$-34kb-$C_{\gamma 1}$-21kb-$C_{\gamma 2b}$-15kb-$C_{\gamma 2a}$-14kb-C_ε-12kb-C_α-3'. The S regions, which contain characteristic tandemly repeated unit sequences, are located 5' to each C_H gene except for the C_δ gene. There are at least two types of repetitive sequences dispersed in this 200 kb region. No pseudogenes are present. The arrangements of the C_H genes in BALB/c and C57BL mice are similar, but the lengths of the S regions vary. The basic structures of all the C_H genes are similar in that coding sequences are interrupted at the junctions of the domains and the hinge regions. Comparison of the nucleotide sequences of the C_H genes revealed that sequence segments have been exchanged among members of the C_H gene family. Cloning and characterization of human C_γ genes, i.e. $C_{\gamma 1}$, $C_{\gamma 2}$, $C_{\gamma 3}$, $C_{\gamma 4}$ and ψC_γ, indicate that the human C_γ gene family evolved by dynamic DNA rearrangements, including gene duplication, exon duplication, and exon reassortment by unequal crossing-over. A human pseudo-epsilon gene ($C_{\varepsilon 3}$) is a processed gene that has completely spliced out introns. The presence of movable genetic elements surrounding the $C_{\varepsilon 3}$ gene suggests that the C_ε gene evolved by a translocation mechanism. Although S-S recombination has been shown to take place in myelomas and hybridomas secreting a large amount of immunoglobulin, analyses of the C_H gene organization in normal

spleen B cells bearing immunoglobulin on their surface suggest that RNA splicing may be responsible for the first step in class switching, followed by S-S recombination. The nucleotide sequences of S regions contain short common sequences, TGGG(G) and (G)AGCT. Comparison of nucleotide sequences surrounding recombination sites revealed common sequences TGAG and TGGG. A sister chromatid exchange model was proposed to explain deletion of C_H genes accompanying S-S recombination. We have found that the S region serves as a preferred recombination site in E.coli extracts.

I. INTRODUCTION

Church bells in western countries have a high, clear ring. In contrast, bells in Buddhist temples in east Asia, especially in Japan, have a low, rumbling roar. Beautiful gardens in European palaces are usually designed with symmetry, or at least according to an obvious plan. In contrast, stone and sand gardens in Zen temples look like anything you wish. They do not provide any explanation. We love the bells and gardens in Japanese temples because they have what we call yo-in, which may be translated into the English words reverberation, aftertaste, or trailing note. None of them exactly express the meaning of yo-in. Yo-in means the state or space left unexplained or unspoken, left for imagination. To me, yo-in is also important in science. It is the problem full of yo-in that is really fascinating. If you knew everything about a problem you would be bored by it.

The genetic bases of antibody diversity and class switching have been central problems in modern immunology. These fascinating questions stimulated molecular biologists to introduce their powerful technology, molecular genetics, into immunology. This technology, using recombinant DNA, has been so powerful that the outlines of the answers to the above problems have been obtained in less than a decade. Although the organization of the V genes is not completely known, the complete organization of the C_λ, C_κ and C_H gene loci have been elucidated at least in the mouse. We know that DNA rearrangements accompanied by DNA deletion play major roles in the generation of antibody diversity as well as in class switching. The nucleotide sequences surrounding the recombination sites have already been determined.

Rapid progress in a certain area of science often causes people outside the area to think that everything has been solved in that field, which would be really disappointing because of the absence of yo-in. As was the case with many other scientific advances, however, such achievements do not necessarily complete the story. Instead, the introduction of new technology, in turn, has raised more fundamental questions such as "How is the DNA

rearrangement regulated during B cell differentiation?" and "Do T cells regulate the DNA rearrangement?", thus opening a new era in contemporary immunology.

We have been working on the genetic basis of class switching. We have proposed a model in which class switching is mediated by the deletion of the intervening C_H genes between a V_H gene and the C_H gene to be expressed. We have obtained substantial evidence for this model by analysis of the germline and expressed forms of the C_H genes. In due course we have determined the complete organization of the mouse C_H genes. The complete characterization of such a complex gene family offers a unique opportunity to study evolution of a gene family in different species, thus we are currently studying the structure and organization of human C_H genes. Although S-S recombination was shown to take place in myelomas and hybridomas, analyses of the C_H gene organization in normal spleen B cells bearing immunoglobulins on their surface suggest that RNA splicing may be the first biochemical step in class switching. We have also set out to study the molecular mechanism of S-S recombination in vitro using E.coli extracts, as a step into a new era.

II. ORGANIZATION AND STRUCTURE OF THE CONSTANT REGION GENES OF THE IMMUNOGLOBULIN HEAVY CHAINS

1) Organization of Mouse C_H Genes

Hybridization kinetic analyses using cDNA 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 immunoglobulin genes were used (Coleclough et al., 1980 ; Cory and Adams, 1980 ; Cory et al., 1980 ; Rabbitts et al., 1980 ; Yaoita and Honjo, 1980 a,b). Such studies also support the proposed order of C_H genes.

To determine directly the order of C_H genes, we and others set out to clone mouse Ig genes using recombinant DNA technology. The strategy has been successful in isolating all the eight C_H genes of mouse and in their physical linkage (Shimizu et al., 1981, 1982a ; Takahashi et al., 1981 ; Nishida et al., 1981). As summarized in Fig. 1, we have now cloned the entire C_H region gene cluster encompassing about 200kb. Portions of the cluster were also cloned in other laboratories (Liu et al., 1980 ; Moore

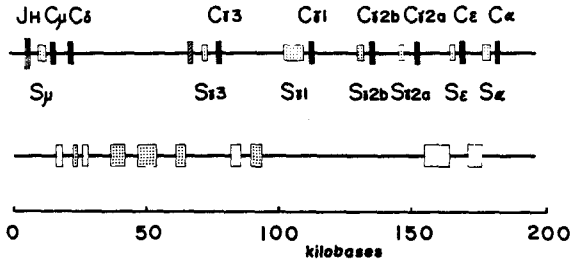

Fig. 1. Organization of the C_H gene family of BALB/c mouse. Locations of structure genes (closed boxes) and S regions (broken-lined boxes) are indicated on the top line. An oblique-lined box indicates the region which is homologous to the $C_{\gamma 2b}$ probe. The second line indicates the location of reiterated sequences. Taken from Shimizu et al. (1982a).

et al., 1981 ; Roeder et al., 1981). The organization of the entire C_H gene cluster is 5'-J_H-6.5kb-C_μ-4.5kb-C_δ-55kb-$C_{\gamma 3}$-34kb-$C_{\gamma 1}$-21kb-$C_{\gamma 2b}$-15kb-$C_{\gamma 2a}$-14kb-C_ε-12kb-C_α-3' in agreement with the originally proposed order of the C_H genes (Honjo and Kataoka, 1978).

Using these isolated overlapping DNA segments, we have characterized several stuctural features of the mouse C_H gene loci. The results are summarized below. There are no other J_H region segments except for those at the 5' side of the C_μ gene. Namely, the J_H segments are shared among all the C_H genes. This is consistent with the fact that the same V region sequence is associated with different C region sequences in the linage of a lymphocyte. The S region is present 5' to each C_H gene except for the C_δ gene, and the nucleotide sequences of the S regions share some homology as described later. There is no reasonably conserved pseudogene in the whole C_H gene cluster in contrast to the human C_H gene family which has many pseudogenes. There are at least two species of reiterated sequences scattered in these loci. 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 and S regions are schematically represented (Fig. 1).

Cloning and Southern blot hybridization analyses indicate that the arrangements 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 length of S regions (Fig. 2). In contrast, we found that the $C_{\gamma 2a}$ gene is duplicated in a Japanese wild mouse Mus musculus molossinus (Shimizu et al.,

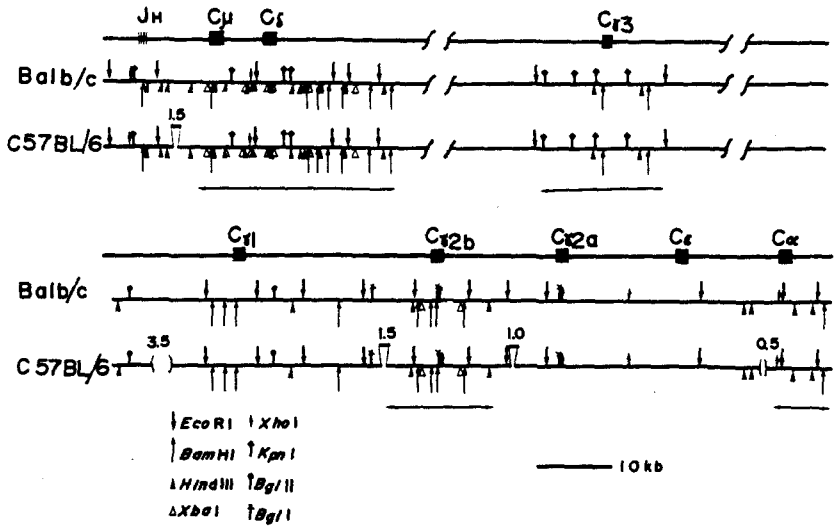

Fig. 2. Structural comparison of the C_H gene families in BALB/c and C57BL/6 mice. At the top line, structural genes are shown in closed boxes. Second and third lines show restriction maps of BALB/c and C57BL/6 mice, respectively. Only the restriction sites identified in C57BL/6 and their corresponding sites in BALB/c are shown. Numbers indicate lengths (kb) of deletions (parentheses) or insertions (bars) in C57BL/6 DNA as compaired with BALB/c DNA. Horizontal arrows below the third line indicate the regions which were cloned from C57BL/6 DNA. Taken from Shimizu et al. (1982a).

1982b). Both of these $C_{\gamma 2a}$ genes seem to be expressed because two individual mice of inbred M. m. molossinus have two allotypes of IgG2a (L. Herzenberg, personal communication).

2) Structure of Mouse C_H Genes

We and others have determined the complete nucleotide sequences of all the eight C_H genes of mouse. The exon-intron organization of each C_H gene is schematically shown in Fig. 3. The coding regions of these genes are split at the junctions of the domains and the hinge regions by intervening sequences (IVS). The results suggest that IVS was introduced into the C_μ gene before divergence of the H chain classes, and also support the hypothesis (Gilbert, 1978 ; Darnell, 1978) that the splicing mechanism has facilitated the evolution of eukaryotic genes by linking duplicated domains or exons of prototype peptides not directly adjacent to one another.

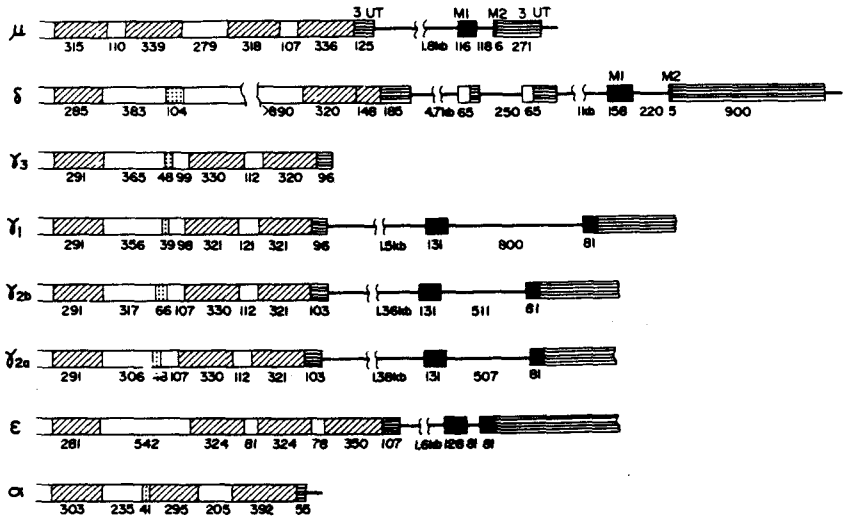

Fig. 3 Structures of mouse C_H genes. Lengths of exons, introns and 3' untraslated sequences were determined by nucleotide sequencing. Data are taken from various sources ; C_μ (Kawakami et al., 1980 ; Early et al., 1980 ; Rogers et al., 1980), C_δ (Tucker et al., 1980 ; Cheng et al., 1982), $C_{\gamma 3}$ (F. Blattner, unpublished data), $C_{\gamma 1}$ (Honjo et al., 1979), $C_{\gamma 2b}$ (Yamawaki-Kataoka et al., 1980), $C_{\gamma 2a}$ (Yamawaki-Kataoka et al., 1981), membrane exons of C_γ (Yamawaki-Kataoka et al., 1982 ; Tyler et al., 1982 ; Nakai et al., 1982 ; Roger et al., 1981) C_ε (N. Ishida and T. Honjo, unpublished data) and C_α (Tucker et al., 1981).

Similarities in structure of C_H genes indicate that all the C_H genes are derived from a common ancestral gene, probably a prototype C_μ gene because some lower vertebrates can produce only IgM.

3) IVS-mediated domain transfer

Comparison of nucleotide sequences of C_H genes, especially those of γ subclass genes has revealed an interesting conservation of nucleotide sequences at limited portions of the gene (Miyata et al., 1980 ; Yamawaki-Kataoka et al., 1981, 1982). In order to evaluate the divergence in the coding and non-coding segments of the gene at the same level, the sequence divergence of the coding region was determined by measuring two distinct types of substitutions: one leading to the amino acid change (Ka) and the other leading to the synonymous change (Ks). Obviously, the former is under the influence of selective constrains at both protein and RNA levels and the latter is under the influence of selective constraint at the RNA level alone. The Ks values and substitution rates at the non-coding region were used for comparison.

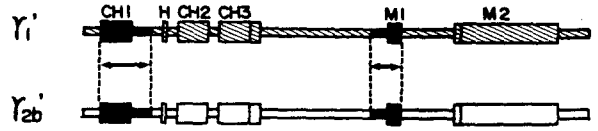

Fig. 4. Schematic representation of IVS-mediated domain exchange between ancestors of $C_\gamma 1$ and $C_\gamma 2b$ genes. Wider rectangles represent exons. Closed rectangles are homologous regions which were exchanged between the two genes. Taken from Yamawaki-Kataoka et al. (1982).

Such comparison between the γ1 and γ2b genes has shown that at least two segments, one including the CH1 domain and the 5' portion of the adjacent IVS and the other including the M1 exon and the flanking region are highly conserved as shown in Fig. 4. When the nucleotide sequences of the human γ4 and murine γ1 genes were compared, there was no particularly conserved segment in the gene. It is therefore likely that some correction mechanisms operate in the gene family. The mechanism could be either double unequal crossing-over or gene conversion (Baltimore, 1981). The exchange or transfer of genetics informations between a gene family seems to be common as growing numbers of examples were provided in immunoglobulin genes as well as in other genes (Schrier et al., 1981 ; Ollo and Rougeon, 1982 ; Slightom et al., 1980 ; Liebhaber et al., 1981 ; Lalanne et al., 1982).

4) Evolution of the Human gamma Gene Family

Cloning and characterizaion of human γ gene clones

We and others have recently cloned most of the human C_H genes including C_μ, C_δ, C_γ, C_ε and C_α genes (Takahashi et al., 1980b ; Ravetch et al., 1980 ; Ellison et al., 1981 ; Ellison and Hood, 1982 ; Rabbitts et al., 1981 ; Nishida et al., 1982 ; Takahashi et al., 1982). We have isolated five human γ gene clones from phage libraries as shown in Fig. 5 (Takahashi et al., 1982). We have cloned four γ genes γ1, γ2, γ3 and γ4. The γ2 and γ4 genes were shown to be linked in this order by overlapping the flanking sequences and they are about 19kb apart. In addition, we obtained another γ gene clone called γ-11 which we think a pseudogene because of the several reasons to be discussed below. Since nucleotide sequences of human γ genes are similar to each other, we have identified these clones by determining nucleotide sequences of the hinge regions which are most divergent.

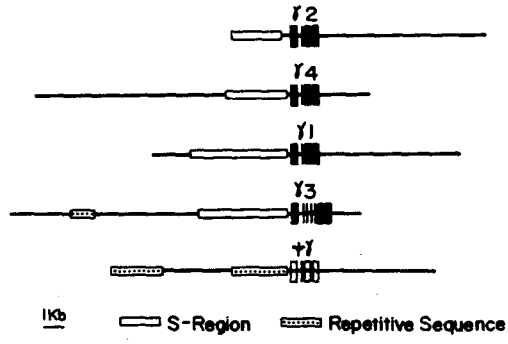

Fig. 5. Structure of human γ gene clones. Four γ genes (γ1, γ2, γ3 and γ4) and a pseudo γ gene (clone γ-11) are schematically shown. Exons are shown by closed rectangles. Taken from Takahashi et al. (1982).

As shown in Fig. 6 the amino acid sequences of the hinge regions predicted from the nucleotide sequences of the γ1, γ2, γ3 and γ4 genes agree with the published sequences except for one residue in the γ4 gene (Edelman et al., 1969 ; Pink et al., 1970 ; Michaelsen et al., 1977 ; Wang et al., 1980). However, the amino acid sequence predicted from the nucleotide sequence of clone γ-11 does not coincide with any of the know human γ sequences.

The S regions of the μ, ε and α genes have been shown to be well conserved between human and mouse (Takahashi et al., 1980b ; Ravetch et al., 1980 ; Nishida et al., 1982). Similarly, the restriction fragments containing the 5' flanking regions of the γ1, γ2, γ3 and γ4 genes hybridized with the mouse $S_{\gamma 2b}$ probe. The γ-11 clone, however, did not have any fragment hybridizing with the mouse $S_{\gamma 2b}$ region that crosshybridized with all the S_{γ} regions of mouse. As the S region is essential for the class switch recombination, the γ-11 clone may not have any chance to be expressed. Furthermore, the γ-11 clone has highly repetitive sequences in the 5' flanking region where the S region is expected to be. Four gamma genes γ1, γ2, γ3 and γ4 have S region sequences at reasonable locations whereas the γ-11 clone has only repetitive sequences (Fig. 5). These observations described above, namely the absence of a known amino acid sequence, the absence of the S region and the presence of repetitive sequences, lead us to conclude tentatively that the clone γ-11 is a pseudogene.

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γ2    GCAGAGCGCAAA------TGTTGTGTCGAG---------TGCCCACCGTGCCCAGGTAA
        GluArgLys       CysCysValGlu          CysProProCysPro

γ4    GCAGAGTCCAAA------TATGGTCCCCCA---------TGCCCATCATGCCCAGGTAA
        GluSerLys       TyrGlyProPro          CysProSerCysPro

γ1    GCAGAGCCCAAA------TCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGGTAA
        GluProLys       SerCysAspLysThrHisThrCysProProCysPro

γ3H1  GCAGAGCTCAAAACCCCACTTGGTGACACAACTCACACATGCCCACGGTGCCCAGGTAA
        GluLeuLysThrProLeuGlyAspThrThrHisThrCysProArgCysPro

γ3H2  GCAGAGCCTAAA------TCTTGTGACACACCTCCCCCCGTGCCCACGGTGCCCAGGTAA
        GluProLys       SerCysAspThrProProProCysProArgCysPro

γ3H3  GCAGAGCCTAAA------TCTTGTGACACACCTCCCCCGTGCCCACGGTGCCCAGGTAA
        GluProLys       SerCysAspThrProProProCysProArgCysPro

γ3H4  GCAGAGCCCAAA------TCTTGTGACACACCTCCCCCGTGCCCAAGGTGCCCAGGTAA
        GluProLys       SerCysAspThrProProProCysProArgCysPro

ψ     GCAGAGCCCAAAACCCCATGTTGTGACACAACTCACACATGCCCACCATGTGCAAGTAA
        GluProLysThrProCysCysAspThrThrHisThrCysProProCysAla
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Fig. 6. Nucleotide sequences of the hinge regions of human γ genes. The nucleotide sequences of the hinge regions are aligned to maximize homology. Amino acids predicted by the nucleotide sequences are shown below. Amino acid residue and nucleotide sequences which are inconsistent with the published sequences are underlined. Taken from Takahashi et al. (1982).

The γ3 gene may have been created by the exon reassortment between the γ1 and pseudo γ genes. The structure of the γ3 gene is interesting because there are four exons for the hinge region. Apparently, such structure indicates that the hinge exon was quadruplicated in the γ3 gene as proposed from the amino acid sequence (Michaelsen et al., 1977). When we compared the nucleotide sequences of the hinge exons of the γ3 gene with those of the other γ genes, we realized that the fact is more interesting than anticipated (Fig. 6). The first hinge exon (H1) of the γ3 gene is very similar to that of the pseudogene which is quite distinct from those of the other γ genes. However, the other hinge exons H2, H3 and H4 of the γ3 gene are most homologous to that of the γ1 gene. Consequently, it is most likely that the γ3 gene was created by unequal crossing-over in the intervening sequences (IVS) between the ancestors of the pseudo γ and γ1 genes, followed by two successive duplications of the γ1-type hinge exon as shown in Fig. 7. Such unequal crossing-over reassorted the exons of the ancestors of the γ1 and pseudo γ genes, creating a new gene.

This assumption inevitably leads us to propose the order 5'-γ1-γ3-ψγ-3' in the human genome. Otherwise, the parental genes would have been lost. The γ2 and γ4 genes are already physically

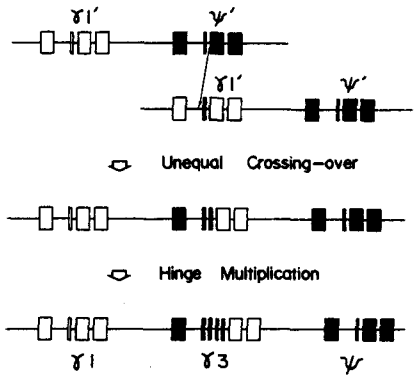

Fig. 7. An evolutionary pathway to create the γ3 gene. Open and closed rectangles indicate exons of the γ1 and pseudogene linages, respectively. Taken from Takahashi et al. (1982).

linked and their hinge region sequences are homologous to each other but different from those of the other γ genes. Two alternative γ gene orders can be proposed ; 5'-γ2-γ4-γ1-γ3-ψγ-3' and 5'-γ1-γ3-ψγ-γ2-γ4-3'. Neither of them agree with the order previously proposed on the basis of genetic studies (Natvig et al., 1967 ; Lefranc et al., 1977).

Phylogenetic trees of human gamma genes From these studies we can estimate the phylogenetic tree of the human γ genes as shown in Fig. 8. There are two possibilities. In the first case (A), we assume the prototype γ gene is either γ1 or γ2 (γ4) type, both of which have deletions in the hinge exon as compared with the pseudogene. The prototype γ gene underwent duplication and segregated into the anscestors of the γ1 and γ2 genes. The γ2 gene ancestor again duplicated and evolved the γ2 and γ4 genes which are about 19kb apart. The γ1 gene anscestor also duplicated and evolved the anscestors of the γ1 and pseudo γ genes. Then, unequal crossing-over took place between these two genes and the γ3 gene anscestor was created as proposed above. In the other case (B), the prototype γ gene segregated into the ancestors of the pseudogene and all the other γ genes. Then, the γ1 gene segregated from the γ2 and γ4 gene ancestors. Like the first case, unequal crossing-over took place between the γ1 and pseudo γ genes and the γ2 and γ4 genes evolved by duplication. Had no recombination taken place among gamma genes, we could distinguish between the two models by determining whether the γ1 gene is more homologous to the pseudogene than the γ2 (or γ4) gene. Comparison of the nucleotide sequences so far determined of the pseudogene, γ2 and γ4 genes with that of the γ1 gene did not allow us to distinguish these models.

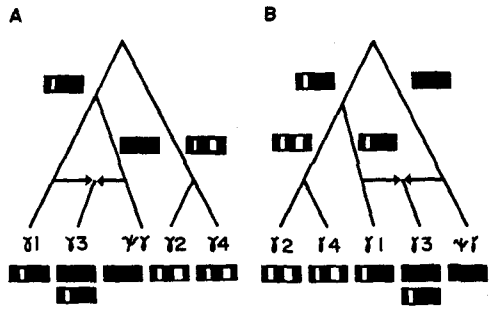

Fig. 8. Phylogenetic trees of human γ genes. The γ genes are best distinguished by the structures of the hinge exons which are schematically shown by closed boxes with deletions (see Fig. 6). Horizontal arrows indicate unequal crossing-over. Taken from Takahashi et al. (1982).

In any case, these results clearly demonstrate that human gamma genes underwent dynamic rearrangements during evolution. To create a human γ gene family there have been several types of gene rearrangement, which include at least three duplications of a complete γ gene, two duplications of the hinge exon and the exon reassortment by unequal crossing-over between two adjacent genes. Certainly many point mutations have accumulated in the γ subclass genes. Nonetheless, DNA rearrangements seem to have played a more important role for the evolution of the γ subclass genes. This might be the best example of how a gene family evolved by exon reassortments.

5) Human Pseudo Epsilon Gene Family

We have also cloned the human epsilon gene (Nishida et. al., 1982). Since the amino acid sequence homology between the human and mouse ε chains is only 43%, it is impossible to use the mouse ε gene (Nishida et al., 1981) as a probe for cloning the human ε gene. Instead, we have used the J_H probe and cloned a rearranged ε chain gene from DNA of a human ε-producing myeloma 266B1 (Nilsson, 1971).

The ε chain gene clone was identified by the complete nucleotide sequence determination. The sequence matched almost perfectly with the published amino acid sequence except for 15 residues out of 427 residues. The human ε gene has four exons, each encoding one domain just like the mouse ε gene. BamHI digestion of human DNA produced three C_ε fragments of 3.0, 6.5 and

9.2kb, which were named $C_{\varepsilon 1}$, $C_{\varepsilon 2}$ and $C_{\varepsilon 3}$ genes, respectively (Fig. 9). We found the three C_{ε} gene fragments in all of the human DNA preparations from 11 individuals, excluding the possibility of polymorphysm. The C_{ε} gene expressed in the myeloma was identified as the $C_{\varepsilon 1}$ gene from the restriction map. Since the $C_{\varepsilon 2}$ gene is deleted from the myeloma DNA, the $C_{\varepsilon 2}$ is located 5' to the $C_{\varepsilon 1}$. The nonrearranged $C_{\varepsilon 3}$ gene was also cloned from the myeloma DNA.

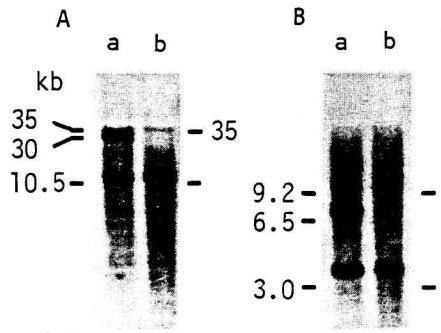

Fig. 9. Southern blot hybridization of human DNA with the C_{ε} probe. Human placenta and 266Bl DNAs were digested with BamHI(A) or EcoRI(B). Southern blots were hybridized with the C_{ε} probe. Lanes a and b contain placenta and 266Bl DNAs, respectively. Numbers indicate sizes of hybridized bands in kb. Taken from Nishida et al. (1982).

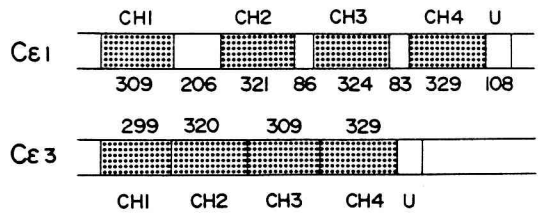

Fig. 10. Comparison of structure of the $C_{\varepsilon 1}$ and $C_{\varepsilon 3}$ genes. Dotted and open boxes indicate exons and introns, respectively. Numbers show lengths (bp) of exons and introns. U, untranslated region.

The heteroduplexes formed between the $C_{\varepsilon 1}$ and $C_{\varepsilon 3}$ genes have revealed that the $C_{\varepsilon 3}$ gene might have deleted three introns. The complete nucleotide sequence of the $C_{\varepsilon 3}$ gene was determined and compared with that of the $C_{\varepsilon 1}$ gene. Although the nucleotide sequence of the $C_{\varepsilon 3}$ gene is 83% homologous to that of the $C_{\varepsilon 1}$ gene in the exons, the introns are precisely spliced out of the $C_{\varepsilon 3}$ gene. The $C_{\varepsilon 3}$ gene does not have any J or V-like sequence at the 5' side but does have poly(A)-like sequence at the 3' side. The structures of the $C_{\varepsilon 1}$ and $C_{\varepsilon 3}$ genes are schematically shown in Fig. 10. This type of the gene was called a processed gene as previously found in the mouse α globin gene family, the human immunolobulin gene family and the human β-tubulin gene family (Nishioka et al., 1980 ; Hollis et al., 1982 ; Wilde et al., 1982).

The most exciting was the finding that movable genetic elements including LTR-like sequences flank both the 5' and 3' sides of the $C_{\varepsilon 3}$ gene as shown in Fig. 11. We can assign two sets of LTR-like sequences, both of which have an inverted repeat, a TATA box and a poly(A) signal (AATAAA). The inverted repeat of one LTR is $(T)_6$-$(A)_6$ and that of the other (TGAA)-(TTCA). In addition, there are short direct repeats at the 5' and 3' ends of each set. However, the 5' and 3' LTR-like sequences in each set do not constitute a direct repeat (Fig. 11A). There are multiple copies of the 3' LTR-like sequence in the human genome. The evolutional origin of the spliced gene is a fascinating question. The presence of the LTR-like structure suggests that the $C_{\varepsilon 3}$ was transcribed into RNA and that the spliced RNA might have been integrated back to the genome by way of the reverse transcription

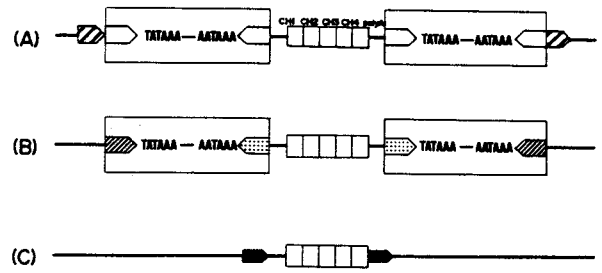

Fig. 11. The possible arrangements of movable genetic elements flanking to human immunolgobulin epsilon pseudogene $C_{\varepsilon 3}$. The sequences of direct and inverted repeats in each model are ; (A) GCT or ACC, TTTTTT or TGAA, (B) TGGCANGAG, TGGNCAAGG, (C) CCTAGAG, respectively.

as proposed previously (Hollis et al., 1982). There are several other possibilities such as the gene conversion model (Nishioka et al., 1980) and two step model (S. Ueda and T. Honjo, in preparation). Note that other types of movable genetic elements are also found in the vicinity of the $C_{\varepsilon 3}$ gene. We can identify two sets of large inverted repeats surrounding the $C_{\varepsilon 3}$ gene (Fig. 11B). It is also possible to locate the direct repeat on both sides of the $C_{\varepsilon 3}$ gene (Fig. 11C).

The $C_{\varepsilon 2}$ gene is also a pseudogene because the two exons of the C_{H1} and C_{H2} domains are deleted.

III. MOLECULAR GENETIC BASIS FOR CLASS SWITCHING

1) The S-S Recombination

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 (Davis et al., 1980a ; Kataoka et al., 1980 ; Sakano et al., 1980). According to this model (Fig. 12) rearrangement called S-S recombination brings a

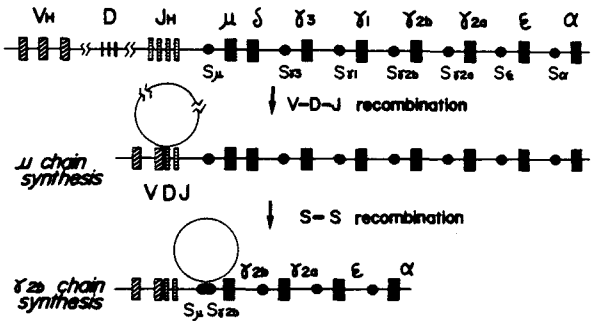

Fig. 12. Rearrangements during differentiation of B lymphocytes. Two successive recombinations V-D-J and S-S complete the expressed form of the γ2b chain gene. Both events are accompanied by deletion of intervening DNA segments which might be accomplished through looping-out or sister chromatid exchange (Obata et al., 1981). Reproduced from Honjo et al. (1981b)

completed V_H gene, which is located originally 5' to the C_μ gene, close to another C_H gene by deletion of an intervening DNA segment (Honjo and Kataoka, 1978 ; Cory et al., 1980 ; Coleclough et al., 1980 ; Rabbitts et al., 1980 ; Yaoita and Honjo, 1980). The S-S recombination takes place between S regions located in the 5' flanking region of each C_H gene. The nucleotide sequences of S regions are shown to comprise tandem repetitive sequences sharing short common sequences (Kataoka et al., 1981 ; Davis et al., 1980b ; Dunnick et al., 1980 ; Nikaido et al., 1981, 1982).

2) C_H Genes Are Not Rearranged in $\mu^+\varepsilon^+$ B Cells

It is known that resting B cells often bear two different isotypes on their surface. The most common are those bearing IgM and IgD. Some carry IgG, IgE or IgA in addition to IgM. It has been difficult to explain this type of class switching, namely from $\mu^+\delta^+$ to $\mu^+\gamma^+$ or $\mu^+\varepsilon^+$ by the S-S recombination that is accompanied by deletion of C_H genes including the C_μ gene. We have, therefore, analyzed the organization of C_H genes in sorted $\mu^+\varepsilon^+$ B-lymphocytes and found that they retain the C_μ, C_δ, C_γ and C_ε genes in the germline configulation, suggesting that the simultaneous expression of the C_μ and C_ε genes is mediated by an RNA splicing mechanism (Yaoita et al., 1982).

Borges et al (1981) found that Igh congenic strain SJA/9 (Igh^a) having SJL background cannot produce a detectable amount of IgE in the sera even after the infection of Nippostrongylus brasiliensis, which is known to stimulate polyclonal IgE production. Furthermore, Okumura and his associates (unpublished data) also found that the increase of IgE-bearing B cells after N. brasiliensis infection occurs equally in SJA/9 as well as SJL mice. Approximately 10% of the spleen cells of the N. brasiliensis-infected mice carry IgE on their surface. The advantage of SJA/9 mice is that a low IgE level in sera minimizes binding of IgE to Fc receptors of ε-negative lymphocytes, thus avoiding the contamination of ε-negative cells into sorted ε^+ B cells.

The IgE-bearing B cells were isolated from spleen cells of N. brasiliensis-infected SJA/9 mice by the fluorescence-activated cell sorter. Only brightest top 9% of the stained cells were collected. The purity of the isolated cells was examined under the fluorescent microscope, which is less sensitive and gives a lower limit value of staining. 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. However, they were not stained with either anti-δ, anti-γ2a or anti-γ1 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 (2.5mg/ml) for 30 min at 37°C to strip off all cell surface immunoglobulins, then after culturing, newly synthesized immunoglobulins on the surface were reexamined by fluorescence staining. As expected, 2hr and 5hr 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 secret IgE when T cells of SJL were provided (K. Okumura, unpublished data).

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

Table 1 C_H Genes in $\mu^+\varepsilon^+$ B Cells

C_H genes examined	length (kb) of fragments identified*	origin of DNA	
		liver	$\mu^+\varepsilon^+$ B cell
C_μ	13 (EcoRI)	+	+
C_δ	11.5 (BamH1)	+	+
$C_{\gamma 3}$	14 (EcoRI)	+	+
	6.6 (Hind III)	+	+
$C_{\gamma 1}$	23 (Hind III)	+	+
	6.6 (EcoRI)	+	+
$C_{\gamma 2b}$	6.6 (EcoRI)	+	+
	9 (Hind III)	+	+
$C_{\gamma 2a}$	21.3 (EcoRI)	+	+
	6.2 (Hind III)	+	+
C_ε	21.3 (EcoRI)	+	+
	4.8 (Bam1)	+	+
J_H	6.4 (EcoRI)	+	- (faint)

* Restriction enzymes used are indicated in parentheses.

EcoRI, blotted and hybridized with the C_ε gene probe, it produced a 21.3 kb fragment which is identical to that produced in SJA/9 liver DNA (Table 1). Since the EcoRI fragment (21.3kb) 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 (Nishida et al., 1981, 1982).

EcoRI digestion of the ε^+ B cell DNA produced the germline form of the C_μ gene fragment (13 kb) as shown in Fig. 13. 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. *BamHI digestion of the ε^+ B cell and SJA/9 liver* DNAs yielded the 11.5 kb fragment hybridizing with the C_μ probe. Since the 11.5 kb BamHI fragment encompasses the C_δ as well as C_μ gene, the results indicate that the C_δ gene is not rearranged in the ε^+ B cells. Similarly, the $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\gamma 2b}$ and $C_{\gamma 2a}$ genes are not rearranged in the $\mu^+\varepsilon^+$ B cells. In contrast, 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 EcoRI fragments of different lengths in polyclonal B cells (Nottenberg and Weisman, 1981). From these results it is likely that the organization of the C_H gene in the IgE-bearing cells is the same with the germline 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.

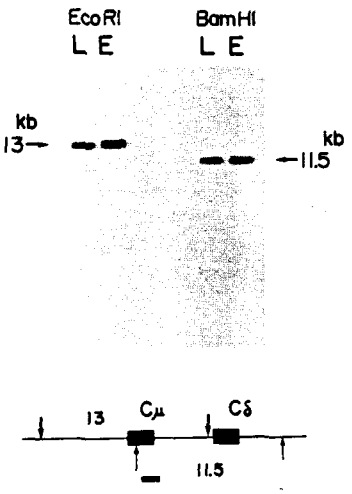

Fig. 13. Analysis of EcoRI and BamHI fragments of ε-bearing cell and SJA/9 liver DNAs using cloned mouse C_μ gene as probe. The fragments produced by EcoRI and BamHI digestion of ε-bearing cell DNA (E) and SJA/9 liver DNA (L) were electrophoresed and blotted to nitro cellulose filters. The restriction map surrounding probe used is shown below. Each lane contains about 1μg DNA. ↓, EcoRI; ↑, BamH1.

3) Class Switching Proceeds by Two Biochemical Steps

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. 14. 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 (Lerner et al., 1980 ; Roger and Wall, 1980). The size of the primary transcript is estimated to be about 180 kb from the C_H gene organization (Shimizu et al., 1982a). Naturally, the μ and ε mRNAs share an identical V_H region sequence. Since we handled a mixed population of $\mu^+\varepsilon^+$ lymphocytes, we were unable to obtain the evidence that 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 (Maki et al., 1981). We presume that the step I does not involve any major DNA rearrangement.

IgM-IgE-bearing B lymphocytes differentiate into IgE-secreting B cells or plasma cells by the step II which involves the S_μ-S_ε recombination and the simultaneous DNA deletion as established before (Nishida et al., 1981). Needless to say, similar mechanism should apply to the switch from IgM-bearing B

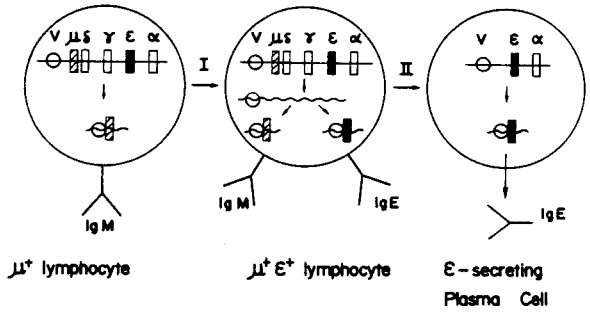

Fig. 14. Two steps of differentiation from μ^+ lymphocytes to ε-secreting plasma cells. Step I involves the activation of differential splicing. Alternate splicing of a long transcript containing V_H, C_μ, C_γ and C_ε sequences will produce mRNA encoding either μ or ε chain with the same V region sequence. Step II involves DNA deletion. Taken from Yaoita et al., (1982).

cells to IgD-, IgG-or IgA-secreting plasma cells. It is not cle whether DNA rearrangement accompanies the differentiation from IgM-bearing B cells to IgM-secreting plasma cells. It is worth noting that most of the IgM-secreting myelomas and hybridomas seem to have deletion in the S_μ region (Coleclough et al., 1980; Hurwitz et al., 1980 ; Yaoita and Honjo, 1980). We think it reasonable that deletion of the S_μ region facilitates the transcription of the C_μ gene and promotes the secretion of IgM since the extremely GC-rich S_μ region (Nikaido et al., 1981) may hinder the efficient transcription.

There are no data as to how long the primary transcript of μ mRNA is in IgM-bearing B cells. They may transcribe the whole C_H gene locus from the beginning. If so, the 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 the step I requires at least two new biochemical events, i.e. 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 biochemichal changes in the C_H gene loci such as demethylation (Razin and Riggs, 1980). In fact the C_δ gene is demethylated in $\mu^+\delta^+$ hybridoma but not in μ^+ lymphoma (Rogers and Wall, 1981).

This model favors 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) untill 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 (Sher et al., 1975 ; Kishimoto et al., 1979). A lymphoma cell line I.29 was shown to switch always from μ to α (Sitia et al., 1981). Such results appear to indicate that the S-S recombination is catalyzed by the class-specific enzyme(s).

IgM-IgE-bearing lymphocytes accumulated in spleens of N. brasiliens-infected SJA/9 mice are capable of differentiating into IgE-secreting plasma cells when T cells of SJL are provided (K. Okumura, unpublished data). Since 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 (Kishimoto, 1982). Furthermore, the step II is a likely step at which the T cells affect the B cell differentiation.

Perlmutter and Gilbert (1982) found the existence of the

C_μ gene in γ_1-bearing B cells purified from normal spleen using antibody-coated petri dishes. Alt et al. (1982) reported that the C_μ and $C_{\gamma 2b}$ genes remain intact in a γ2b chain-producing variant of the Abelson virus-transformed cell line 18-81.

IV. MOLECULAR MECHANISM OF THE S-S RECOMBINATION

1) Nucleotide Sequences of S Regions

In order to elucidate molecular mechanism for the S-S recombination we have determined the nucleotide sequences of the S regions (Takahashi et al., 1980a ; Kataoka et al., 1981 ; Nikaido et al., 1981, 1982). The results indicate that the 5' flanking regions of all the C_H genes except for the C_δ gene contain the S regions which comprise tandem repetition of short unit sequences. The nucleotide sequences of the repeat units of the S reigons are summerized in Fig. 15. Comparison of the nucleotide sequences of all the S regions revealed that length as well as nucleotide sequences of the S region sequences vary among different classes of the C_H gene but share short common sequences, (G)AGCT and TGGG(G). The nucleotide sequence of the S_μ region is homologous to those of the other S regions in the decreasing order of the S_ε, S_α, $S_{\gamma 3}$, and ($S_{\gamma 1}$, $S_{\gamma 2b}$, $S_{\gamma 2a}$) regions.

S_μ*	GAGCTGAGCTGGGGTGAGCT
$S_{\gamma 3}$	PGGACCAGGCTGGGACAGCTCTGGGGAGCTGGGGTGGGTGGGAGTGTGG
$S_{\gamma 1}$	GPPTCCAGGCTGAGCAGCTACAGGGGAGCTGGGGYAPPTGGGAPTPTPG
$S_{\gamma 2b}$	GGGACCAG(T/A)CCTAGCAGCTPTGGGGGAGCTGGGGA(A/T)GGTPGGAPTPTGA
$S_{\gamma 2a}$	GGGACCAGGCAGTACAGCTCTGGGTPGGGPNCAGGCAGTACAGCTCTGNGTG
$S\varepsilon$*	GGGCTGGGCTGAGCTGPGCTGAGCTGPGCTGAGCTGPPNT
$S\alpha$	ATGAGCTGGGATGAGCTGAGCTAGGCTGGAATAGGCTGGGCTGGGCTGGT GTGAGCTGGGTTAGGCTGAGCTGAGCTGGA
common sequences	(G)AGCT(G), TGGG(G)

*, variable unit length

Fig. 15. Nucleotide sequences of repeat units of S regions. The consensus sequence of the repeat unit of each S region is shown. Common short sequences are underlined. P, purine ; Y, pyrimidine. Taken from Nikaido et al. (1982).

The order of the S region homology mentioned above does not seem to correlate with the relative contents of the immunoglobulin classes in mouse serum. In contrast, the order of the S region lengths appears to bear some correlation with the relative contents of the immunoglobulin classes in the mouse serum. In BALB/c IgG1 is the most abundant immunoglobulin class, followed by IgG2a, IgG2b and IgA in decreasing order (Kalpaktsoglou et al., 1973). This observation is consistent with the fact that the $S_{\gamma 1}$ region is the longest among the S reigons of BALB/c (Fig. 16). In C57BL/6 serum IgG2b is the highest in the concentration, followed by IgG1, IgG2a and IgA in that order (Barth et al., 1965), which is in general agreemnt with the order of the S reigon length in this strain, namely the $S_{\gamma 2b}$, $S_{\gamma 1}$, $S_{\gamma 2a}$, $S_{\gamma 3}$ and S_{α} regions (Fig. 2). In both strains the S_{ε} is the shortest and IgE is the least abundant.

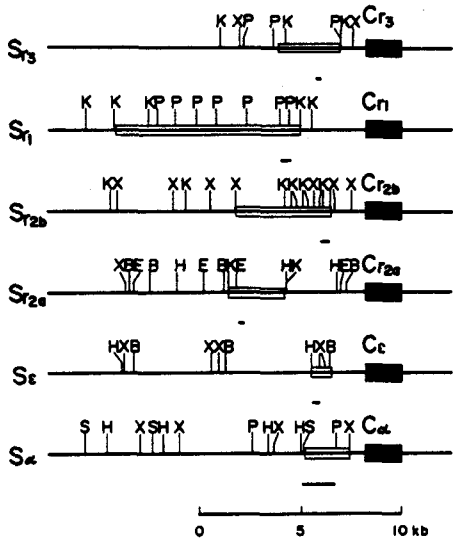

Fig. 16. Locations and ranges of the S regions. Schematic representation of the locations and sizes of the $S_{\gamma 3}$, $S_{\gamma 1}$, $S_{\gamma 2b}$, $S_{\gamma 2a}$, S_{ε} and S_{μ} regions is shown. Open boxes indicate the location of restriction DNA fragment containing the S region. Structural genes are shown as closed boxes with direction of transcription from left to right. Taken from Nikaido et al. (1982).

It is hard to believe that the length of the S reigon and the S reigon homology with the S_{μ} region directly determine the relative concentration of the immunoglobulin class as the latter depends on many other regulatory steps such as the B-lymphocyte proliferation and the half life of immunoglobulins. However, it is conceivable that the C_H gene organization may affect the relative abundance of the immunoglobulin class to some extent because the longer the S region, the higher the chance of the class switch recombination.

We have compared the nucleotide sequences immediately adjacent to the recombination sites of seven rearranged genes as shown in Fig. 17. Note that tetranucleotides TGAG and/or TGGG are always found except for one case. Such tetranucleotides may constitute a part of the recognition sequence of a putative recombinase. These results provide a support to our previous proposal that the S-S recombination may be facilitated by short common sequences dispersed in all the S regions.

Clones		S regions
Ig_{ϵ}-1	GA<u>TGGGTGGG</u>CTTCTC<u>TGAG</u>	S_{μ}
	TGTAGGGGAGC AGGGATAGG	$S_{\gamma 2b}$
Ig_{ϵ}-1	AGGGAGC<u>TGGG</u>GCAGG<u>TGGG</u>	$S_{\gamma 2b}$
	CTAAGCTTAGTTTAGC<u>TGAG</u>	S_{ϵ}
J606	C<u>TGAGC</u><u>TGGGG</u><u>TGAGC</u><u>TGAG</u>	S_{μ}
	GGGAGTG<u>TGGG</u>GACGGGTTG	$S_{\gamma 3}$
MOPC141	TGTTAAAGAATGGTATCAAA	S_{μ}
	GCCAGGAGAGTTGTCCGATT	$S_{\gamma 2b}$
MC101	<u>TGAG</u>GTGATTACTC<u>TGAG</u>GT	S_{μ}
	C<u>TGAG</u>CTGGAA<u>TGAG</u>C<u>TGGG</u>	S_{α}
MC101	GCTAGGTTGGTC<u>TGAG</u>CTGA	S_{α}
	CTGGAGCTGA<u>TGGG</u>TATAAA	$S_{\gamma 1}$
TEPC15	TGGTATCAAAGGACAGTGCT	S_{μ}
	TGGAA<u>TGAG</u>C<u>TGGG</u>T<u>TGAG</u>C	S_{α}
M603	<u>TGGG</u>C<u>TGAG</u>C<u>TGAG</u>C<u>TGAG</u>	S_{μ}(deleted)
	ATAGGT<u>TGGG</u>C<u>TGGG</u>CTGGT	S_{α}
MPC11	TAGAGCTGAC	$S_{\gamma 3}$
	GCGGGGATAGG<u>TGGG</u>AGTAT	$S_{\gamma 2b}$

Fig. 17. Nucleotide sequences around S-S recombination sites. TGGG and TGAG are underlined. Dots indicate putative recombination sites. Nucleotide sequences of MOPC141, TEPC15 and M603 sequences are taken from Davis et al., (1980).

2) Sister Chromatid Exchange Model

The basic mechanism for the S-S recombination may be a sort of a homologous recombination mediated by short reiterated common sequences. It is now established that the S-S recombination is accompanied by deletion of C_H genes. Two alternative models can be proposed to explain the mechanism of the C_H gene deletion in B-lymphocytes as shown in Fig. 18. The first model postulates that the S-S recombination takes place on a single chromosome by mutual

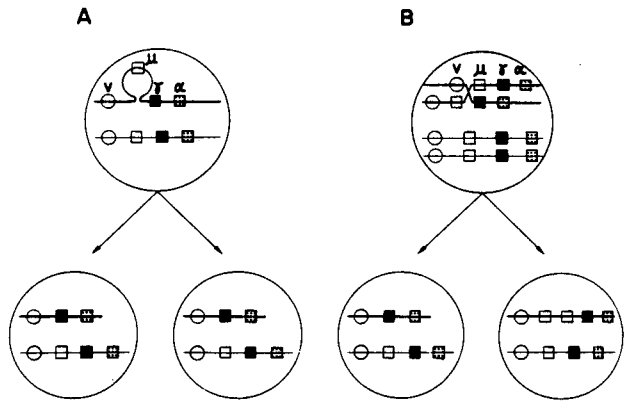

Fig. 18 Possible models for deletion of C_H genes in class switch recombination. A, looping-out model. B, sister chromatid exchange model. Taken from Obata et al. (1981).

recognition of two S regions. The intervening DNA segment is looped out and lost from the chromosome. This model is referred to as a looping-out model. Such recombination can occur at any stage of the cell cycle in principle. The other model, called a sister chromatid exchange model, explains the deletion of DNA segment by an unequal crossing-over event between sister chromatids (Honjo et al., 1981a ; Obata et al., 1981). 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.

I will describe observations which lead us to conclude that the sister chromatid exchange model is more favorable than the looping-out model. First, the expressed $\gamma 1$ gene from MC101 myeloma contains the S_α segment between the S_μ and $S_{\gamma 1}$ regions (Obata et al., 1981). It appears as if the presence of the S_α segment between the S_μ and $S_{\gamma 1}$ segments contradicted the linear arrangement of C_H genes ($5'$-C_μ-$C_{\gamma 3}$-$C_{\gamma 1}$-$C_{\gamma 2b}$-$C_{\gamma 2a}$-C_ε-C_α-$3'$) and the deletion mechanism for the class switch. It is too complicated to explain such rearrangement by recombination events within a chromosome. The generation of such $\gamma 1$ gene, however, can be explained by two or three successive unequal crossing-over events. There are various possible pathways to create the MC101 $\gamma 1$ gene.

Secondly, inhibition of the cell division leads to an increase in the frequency of binucleated cells able to direct synthesis of both IgM and IgG under the conditions that a single lymphocyte can give rise to progeny cells synthesizing IgM, IgG or IgA (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 only IgG was rather high (10-20%) and increased 2 to 3 fold by inhibition of cell division, switching process appears to take place during cell division, probably during or after replication of DNA.

3) S-S Recombination in E. coli Extracts

Assay system The molecular mechanism of the S-S recombination including the possibility of the sister chromatid exchange can be directly tested by the in vitro recombination system. For this purpose it is important to set up an assay system that allows us to detect an extremely small number of recombinants like one recombinant out of 10^7 molecules (Kataoka et al., 1982). The basic idea of this assay system is illustrated in Fig. 19. We made two kinds of λ phage derivatives, each carrying an immunoglobulin S region as well as a coding sequence. In this case μ and α phages are shown. The two phages have different

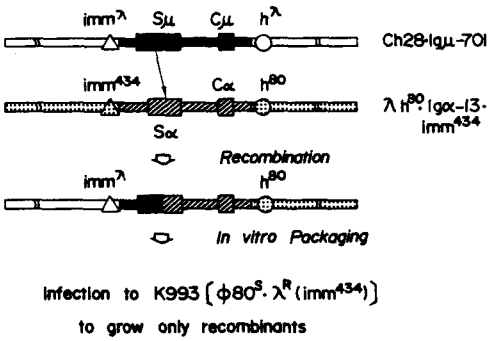

Fig. 19. In vitro assay system for the S-S recombination. Ch28·Igμ-701 was constructed by a simple transfer of the insert EcoRI fragment of λgtWES·IgH 701 (Kawakami et al., 1980). λh$^{\phi 80}$·Igα-13·imm^{434} was constructed by recombination between Ch28·Igα-13 and a recombinant phage λh$^{\phi 80}$·Imm^{434}·pgal, followed by the second recombination between the recombinant obtained (Ch28·Igα-13·h$^{\phi 80}$) and a λ phage (h$^{\lambda}$·imm^{434}). Two phages were incubated with lymphocyte extracts and DNAs were extracted. DNAs were packaged in vitro into coat proteins and the recombinants produced by recombination within two genetic markers were recovered by infection to E.coli K993 [φ80^{S}·λ^{R} (imm^{434})].

genetic markers on each arms. The μ phage has immunity to λ and the tail protein (host restriction) of λ phage whereas the α phage has immunity to λ434 and the tail protein of φ80. After in vitro reaction with lymphocyte extracts, we can extract phage DNAs, package them into coat proteins and have them infect E.coli to recover recombinant phages. If we choose a proper host bacteria like K993 which is φ80-sensitive, λ-resistant and immune to λ434, we can detect a recombinant very efficiently.

Recombination in vitro To our surprise, however, we soon found that this assay system has very high back ground. Many recombinants were produced without lymphocyte extracts. So we decided to characterize the recombinational system in E.coli or λ phage which seem to catalize the S-S recombination of the immunoglobulin gene. To exclude the recombination which took place

Table 2 Recombination of Immunoglobulin Genes in E.coli and in vito Packaging System

Experimental System	Recombination frequency ($\times 10^{-4}$)	Recombination site[a] (%)	
		insert	vector
Expt I (in vitro packaging)			
Complete	3.5	30	70
With inversely inserted μ gene	1.6	1.6	98
Mixed after separate packaging	2.0	2	98
Expt II (double infection)			
Complete	43	0	100
With inversely inserted μ gene	37	0	100

a, recombinat phages were screened by in situ hybridization using the DNA fragment 5' to the S_μ region and that 3' to the C_α gene as probes. Recombinants that hybridized to both probes were classified into those which recombined within the inserts.

within phage arms between two genetic markers, putative recombinants grown on K993 were screened by hybridization with μ and α probes. We determined the number of clones which hybridized to both μ and α probes. As shown in Table 2 the recombination frequency is in the order of 10^{-4}. The reaction requires that two S regions are in the same orientation because the S-S recombination was not detected when one of the insert is inverted. The recombination reaction seems to take place or initiate during the in vitro packaging reaction because the S-S recombination was not observed when two phages were packaged separately. Also double infection at 100 times higher moi did not induce the S-S recombination although the recombination in the vector arms took place at 100 times higher frequency.

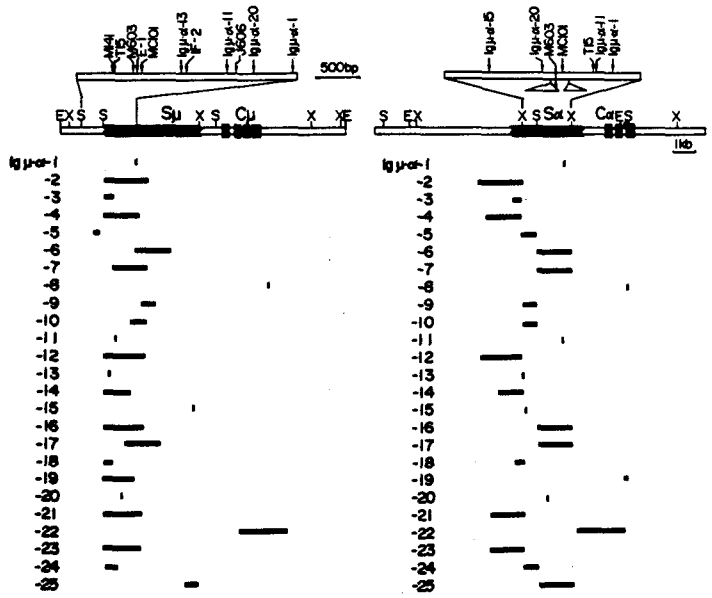

Fig. 20. Distribution of the recombination sites on μ and α chain genes. The left and right horizontal rectangles represent the restriction cleavage sites of the Igμ-701 and Igα-13 inserts, respectively. Horizontal bars below the restriction maps of the parental clones indicate the estimated ranges of the recombination sites. In the clones, the recombination sites of which are definitely assigned, the sites are shown as the vertical lines. Top rectangles above Igμ-701 and Igα-13 represent enlargements of portions of the S_μ and S_α regions which include the class switch recombination sites of various myelomas or hybridomas as well as those of the several in vitro recombinant clones. Triangles below the top bar of Igα-13 indicate the locations of deletions introduced upon cloning of $\lambda h^{\phi 80}$·Igα-13·imm^{434}. S, SacI; X, XbaI; E, EcoRI.

Nucleotide sequences surrounding recombination sites We have determined locations of recombination sites of randomly-chosen 25 recombinant phages by restriction site mapping. The majority of recombination (about 90%) occurred within the S_μ and S_α regions. Some took place in the coding regions (Fig. 20). Then, we have arbitrarily chosen 4 recombinants and determined the nucleotide sequences surrounding recombination sites. As shown in Fig. 21 the recombinations must have taken place somewhere within the boxed regions. These regions always contain TGAG or TGGG which is also found around the class switch recombination site in mouse myelomas. These results taken together, the λ phage-E.coli system may have the recombinational system that can recognize short sequences similar to those used in the class switch recombination of the immunoglobulin gene.

Note that the Chi sequence (TCTGGTGG), which enhances the generalized recombination in E.coli (Smith et al., 1981), bears striking homology with the S_μ sequence and short sequences found around the recombination sites in E.coli as well as in myelomas. We have recently shown that the nucleotide sequences almost identical to the mouse S_μ region are represented in variety of organisms such as yeast, sea urchin and Drosophila (Sakoyama et al., 1982). Although the biological function of these sequences is not known, one may speculate that these sequences have been conserved in variety of organisms because they serve as recognition signals for DNA recombination of other genes.

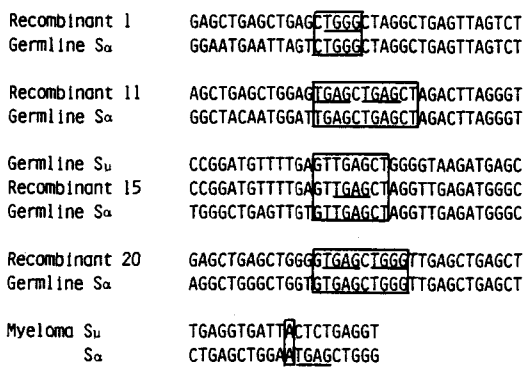

Fig. 21. Nucleotide sequences of recombination sites in S-S recombinants isolated from E.coli. Nucleotide sequences of recombination sites in four independent S-S recombinants were determined. The germline S_μ sequence was not available except for the recombinant 15 but the nucleotide sequence of the S_μ region where these recombination took place is shown to be represented by $[(GAGCT)_3GGGGT]_n$ (Nikaido et al., 1981). Myeloma sequence (MC101) is taken from Obata et al. (1981). Possible recombination sites are boxed. TGGG or TGAG closest to the recombination sites is underlined.

ACKNOWLEGMENT

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DISCUSSION

Leder: Indirect evidence from a human IgE-producing cell line suggests that the E pseudogene (called E2 by Honjo) is 5' to the active gene - that is, the pseudogene is deleted in this line.

Milstein: Dr. Honjo, did you say most of the C_H has been left intact, but that some may be deleted?

Honjo: No, it is identical to the germ line sequence.

Vitetta: Have you tried activating the epsilon positive cells with LPS to see if they will be activated to secretion? That is, can you induce a DNA rearrangement?

Honjo: This has not been done.

Jones: What is the role of antigen in expanding the number of cells with a particular combination of C_H on the surface? Do you think that the decision of which second C_H will be expressed (through long transcript) along with C_μ is random, with a role for antigen in selecting B cells expressing a certain C_H?

Honjo: T cells may recognize the surface Ig and may prefer a certain V region associated with a particular C_H region. If T cells give a special signal to begin the class-specific S-S recombination, this will result in a tight coupling of RNA splicing (step I) and DNA recombination (step II).

Leder: Are these epsilon positive cells normal B cells?

Honjo: Yes.

Leder: How do you know that you are not looking at both chromosomes?

Honjo: Because the analysis is quantitative.

Cohn: What happens in E. coli that lack Rec. A?

Honjo: Switch recombination in the E.coli system does not depend on Rec. A.

Hood: Expression of immunoglobulin IgA by plasmacytomas occurs as a result of DNA rearrangement that brings the variable gene, (V_H), a few kilobases 5' to the constant region gene, C_{alpha}. We have demonstrated that the allelic or nonexpressed C_{alpha} gene also is rearranged in virtually all plasmacytomas. Cloning, restriction mapping, heteroduplex analyses, and sequence analyses of the nonproductively

rearranged C_{alpha} genes from two plasmacytomas, M603 and M167, have demonstrated that the nonproductive rearrangement occurs within the alpha switch region, S_{alpha}. In each case, the same DNA sequence has been joined to the 5' side of the C gene and we have termed this DNA NIRD (for nonimmunoglobulin rearranged DNA). Southern blotting analysis of genomic DNAs from a variety of IgG-, IgM-, or IgA-producing plasmacytomas suggests that NIRD is rearranged in almost all plasmacytomas. However, NIRD rearranges to the S_{alpha} regions only in IgA-producing cells and not in IgM or IgG producers.

Cytogenetic evidence has shown than translocations between chromosones 12 and 15 are common in murine plasmacytomas. Immunoglobulin heavy genes are located on chromosome 12 and the translocation breakpoint in plasmacytomas occurs near the immunoglobulin genes. NIRD has been mapped to chromosome 15 by Southern blotting analysis of mouse or hamster cell lines, suggesting that the nonproductively rearranged C_{alpha} gene clones represent the 12-15 chromosomal translocations identified cytogenetically. Therefore, we have identified a region of DNA on chromosome 15 that is commonly rearranged in transformed mouse lymphocytes. Obviously one can speculate on the possible significance of the NIRD sequence in the neoplastic transformation of mouse lymphocytes, particularly with regard to the possibility that it is a cellular oncogene that has been activated through chromosomal rearrangement.

Leder: We have also seen this rearrangement (Kirsch et al., Nature, 1981) and find that it has occurred in all the non-u myelomas we have looked at.

Cohn: What happens in the abberrantly rearranged chromosome when the expressed chromosome switches to another class?

Weigert: In what B cells do you find the rearranged x sequence?

Hood: We find that it is rearranged in every IgA producer and in some B cells expressing other classes of immunoglobulins. In these latter case we do not know where x rearranges to.

Cohn: The switch from IgM to other Ig classes is driven by the effective level of T helper activity

There are two views possible on the regulation of this class switch. Either the switch is random in which case any disproportionate expression of an isotype must involve an isotype recognizing mechanism, or the switch is directed (non-random), in which case any disproportionate expression of an isotype must involve a set of isotype inducing hormones.

The argument used by Lee Hood that the switch is non-random is based on studies with C_{alpha}. It is not strong because C_{alpha} is the

3' ultimate C-gene and successive random switches in both chromosomes would have to end up at C alpha, deleting everything in between. Besides, I believe that there exist data showing that the chromosome pair does not switch in parallel to the same C-gene.

In any case, the randomness or non-randomness of the switch is the key question to settle before discussions of mechanism can become meaningful. It is the reason I asked the question "What happens to the aberrantly rearranged chromosome during class switching of the functionally rearranged chromosome?"

Leder: I simply wish to point out that this is one of two poorly understood genetic rearrangements that occur in immunoglobulin producing cells. The other is the disappearance or inevitable rearrangement of kappa genes in lambda producing B-cells.

Nabholz: Carlo Croce has claimed that the Burkitt lymphoma specific rearrangements involve the "non-expressing" Ig-chromosome. Is this true?

Leder: I think both models are valid even if the translocation is to the "inactive" chromosome. For example, both kappa alleles are transcribed, though only one makes a "normal" transcript.

Mäkelä: How low is actually the production of circulating IgE in the SJA mouse? It is probably increased by the nematode infection, is it still low enough to exclude the problem of cytophilic antibodies?

Hood: Dr. Honjo has demonstrated that one may strip IgE from these B cells and in five hours they regain (presumably resynthesize) surface IgE.

Vitetta: One could answer this more definitely by looking at the size of the H chains on the cell surface IgE. Cell surface IgE should be larger than secreted IgE.

Uhr: I want to clarify Dr. Honjo's speculation concerning the coordination of RNA splicing and DNA rearrangements, which is necessary for a B cell to differentiate into a plasma cell that secretes the same isotype as was expressed by the B cell. Your last figure suggested that a T cell was responsible. The implication, therefore, is that the non-IgM isotype is a receptor for an isotype specific T helper cell or T cell derived lymphokine that signals the B cell to undergo the DNA rearrangements resulting in synthesis of the isotype in question. Is this your viewpoint?

Honjo: T cells may recognize the surface Ig and may prefer a certain V region associated with a particular C_H region. If T cells give a special signal to begin the class-specific S-S combination, this will result in a tight coupling of RNA splicing (step I) and DNA

recombination (step II).

Coutinho: If we come back to Dr. Honjo's model, I would like to ask him about the "rigidity" of the relationship he postulates between the specificities of the splicing and recombination steps. The model postulates that such specificities are related, in order to "use" surface expression in the regulation of the secreted isotypes (e.g. by isotype-specific helper cells or factors). In contrast, however, we have evidence pointing out that the relationship between membrane and secreted isotypes is not absolute in the case of IgG subclasses. Together with Luciana Forni at the Basel Institute for Immunology we have analysed the production of membrane and secreted forms of all isotypes (except IgD) in single clones of normal B cells and at single cell level. We detected a sizeable fraction of all cells bearing any given IgG which also express a second IgG isotype on the membrane. Furthermore, and here the data have direct implications to the model, a proportion of all cells carrying a given membrane IgG are secreting a different IgG subclass, indicating that the specificity of splicing (membrane expression) is often different from the specificity of recombination (secreted forms).

Honjo: It is possible that the IgM-IgE-bearing lymphocytes are in a transient stage and they can shift to express the different isotypes. In that case there is no solid linkage between the steps I and II.

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参考論文

Escherichia coli extract-catalyzed recombination in switch regions of mouse immunoglobulin genes

(phage vector/*in vitro* packaging/nucleotide sequence)

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ABSTRACT **We have shown that *Eschericha coli* extracts catalyze recombination between mouse immunoglobulin μ and α genes inserted separately in λ phage vectors carrying different genetic markers. Most of the recombination sites in the inserts are located in the switch regions of the heavy chain genes, as previously found in the expressed genes of myeloma cells. The recombination took place at relatively high frequency (10^{-4}). The recombinational system in *E. coli* or λ phage seems to prefer short nucleotide sequences similar to those used in the class switch recombination.**

During differentiation of B lymphocytes, immunoglobulin heavy (H) chain genes undergo two steps of DNA rearrangement called the variable–diversity–joining (V-D-J) and switch–switch (S-S) recombinations (1–3). The latter provides the genetic basis for the class switch phenomenon of H chains, in which a single B lymphocyte can associate a given V-region sequence with several constant (C) region sequences, first with the μ chain and subsequently with the γ, ε, or α chain. The S-S recombination takes place between the S regions located in the 5′ flanking region to each C_H gene except for the C_δ gene (4–10). We and others have shown that the S regions are comprised of tandem repetition of unit sequences. Although the lengths, as well as the sequences, of the repeat units are varied among different S regions, all the S regions share the short common sequences G-A-G-C-T-G, G-A-G-C-T, and T-G-G-G (5–8). These sequences themselves are the constituents of the S_μ region, which pairs with anyone of the other S regions. These results led us to propose that the S-S recombination may be mediated by recognition of the common repeated sequences.

Inasmuch as the immunoglobulin gene recombination is a developmentally regulated process and a key to understand the molecular mechanism for lymphocyte differentiation, we are interested in analyzing the enzymatic mechanism for the S-S recombination. For this purpose, we have set out to construct an *in vitro* system for assay of the S-S recombination. In this report, we will present evidence that, in *Escherichia coli* extracts, recombination takes place between mouse immunoglobulin μ and α genes inserted separately in λ phage vectors.

MATERIALS AND METHODS

Bacteria and Phages. *E. coli* LE392 is identical to *K803suII/suIII*, m_k^+, r_k^+, *gal*$^-$ (11). *E. coli* km993 (C600[$\phi 80^S \cdot \lambda^R \cdot (imm^{434})$]), km738 (C600[$\phi 80^S \cdot \lambda^R \cdot (imm^{\lambda})$]), and B12 [C600($\phi 80^S \cdot \lambda^S$·Sup0)] and a phage, $\lambda h^{\phi 80} \cdot imm^{434} \cdot gal^+$, were constructed and donated by K. Matsubara of Osaka University (K. Matsubara, personal communication). The basic structure of $\lambda h^{\phi 80} \cdot imm^{434} \cdot gal^+$ was similar to that of $h^{\phi 80} \cdot imm^{434} \cdot C$ (12), except that the *gal* gene of *E. coli* was inserted by recombination. *E. coli* strains used for *in vitro* packaging reactions were NS428 [N205 *recA*$^-$(λ*Aamb2 red3 Sam7*)], λdg805 [W3350 (λ*dgal 805 cI857 Sam7*)], and NS433 [N205 (λ*Eam4b2 red3 cIts857 Sam7*)] obtained from F. Blattner, University of Wisconsin, and HI501 [HI225 *recA1* (λ*cI857 Dam15FIam96B Sam7 int6 red3*)] and HI507 [HI225 *recA1* (λ*cI857 Eam4 Sam7 int6 red3*)] obtained from H. Ikeda, Tokyo University.

***In Vitro* Recombination.** Initially, 1 μg each of Ch28·Igμ-701 and $\lambda h^{\phi 80}$Igα-13·imm^{434} DNA were incubated with extracts from B lymphocytes, but this incubation was later omitted as described in *Results and Discussion*. The DNA mixture was packaged *in vitro* into phage coats and the recombinant phages were selected by infection to *E. coli* km993. The *in vitro* packaging system was composed of the extracts from *E. coli* NS428 and purified protein A from λdg805 (13). Other systems using combined extracts of different *E. coli* strains, NS433 and NS428 (14) or HI501 and HI507 (15) were also examined and gave similar results. Although these packaging systems have a tail protein of λ phage origin, the *in vitro*-constructed phages were shown to infect λ-resistant *E. coli* with about one-third of the efficiency as compared with λ-sensitive cells. The same phenomenon occurred when *in vitro*-constructed $\lambda h^{\phi 80}$·Igα-13·imm^{434} phages infected km738. We suspect that this may be due to the altered structures of the coat proteins constructed *in vitro*.

Determination of the Location of the Recombination Sites. Phage DNAs in the plaques on km993 were transferred *in situ* onto two sheets of nitrocellulose filters (16). The two sheets were separately hybridized with nick-translated probes A and B (see Fig. 1). Phages that hybridized with both of the probes were considered to be recombined between the inserts of the parental phages. The recombination sites were mapped by comparison of the *Sac* I, *Xba* I, and *Eco*RI restriction cleavage maps of the phage DNAs with those of the parental phage DNAs.

RESULTS AND DISCUSSION

Construction of the Assay System for *in Vitro* Recombination. To detect a small number of recombinants among millions of the parental DNA, we have used λ phage genetics. We constructed recombinant phages that carry immunoglobulin S regions as well as C_H genes as inserts and selection markers of the host restriction (*h*) and the immunity (*imm*) genes in phage arms.

Ch28·Igμ-701 was constructed by ligation of Charon 28 arms (17) with a 13-kilobase (kb) *Eco*RI fragment of λgt·*WES*·Igμ-701 (1) carrying the mouse C_μ gene and the whole S_μ region as shown in Fig. 1. $\lambda h^{\phi 80}$·Igα-13·imm^{434} was constructed from Ch28·Igα-13 (18), a recombinant phage of Charon 28 that carries the mouse

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Abbreviations: V, D, J, S, and C, variable, diversity, joining, switch, and constant regions, respectively, of the immunoglobulin heavy (H) chain; moi, multiplicity of infection; kb, kilobase(s).

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C_α gene together with the whole S_α region. Ch28·Igα-13 and $\lambda h^{\phi 80}$·imm^{434}·gal^+ phages were permitted to coinfect LE392 ($\phi 80^S$·λ^S) and the resultant recombinants carrying the markers of $h^{\phi 80}$ and λ immunity were selected by infection of *E. coli* km993. The recombinant phages obtained were hybridized with the C_α probe to test for the presence of the Igα-13 insert. One of the phages thus isolated was then crossed with λ·imm^{434} and recombinants bearing $h^{\phi 80}$ and imm^{434} were selected by infection to *E. coli* km738. Unfortunately, all 10 clones hybridizing to the C_α probe examined have small deletions in the S_α region. Since the majority of S_α sequence was maintained, we decided to use one recombinant, $\lambda h^{\phi 80}$·Igα-13·imm^{434}, for the further experiments. The location of the deletion in this clone is shown in Fig. 2.

Recombination reactions were designed to take place between $\lambda h^{\phi 80}$·Igα-13·imm^{434} and Ch28·Igμ-701 DNAs when mixtures of them were incubated with extracts of various eukaryotic or prokaryotic cells. If the recombination occurs between the mouse DNA inserts of both clones, the recombinants produced have either $h^{\phi 80}$·imm^λ or h^λ·imm^{434} markers, the former being selectively isolated by infection of an appropriate *E. coli* strain (e.g., km993). After incubation with cellular extracts, DNAs were extracted and packaged into coat proteins *in vitro* using extracts from the *recA*$^-$ mutant of *E. coli* carrying lysogenized λ phage with the *red*$^-$ mutation. Hence, the extracts were free from generalized homologous recombination systems in *E. coli* and λ phages (13, 14). The recombinants that infected km993 were further tested for whether they had two immunoglobulin gene sequences by hybridization with the 5′ flanking region of the S_μ region and the 3′ segment of the C_α gene (probes A and B; see Fig. 1).

Recombination in *E. coli* Extracts. To our surprise, however, we soon found that many recombinants were formed without any cellular extract. When $\lambda h^{\phi 80}$·Igα-13·imm^{434} and Ch28·Igμ-701 DNAs were simply mixed, packaged *in vitro*, and permitted to infect km993, recombinants with $h^{\phi 80}$ and imm^λ markers were detected at the relatively high efficiency of 3×10^{-4} (Table 1). Approximately 30% of recombinants were shown to hybridize with both probes A and B, indicating that recombination took place between the inserts of both phages. The results also suggest that about 70% of the recombination took place in the phage arms between the genetic markers and inserts. The recombinations within the phage arms may be due to homologous recombination similar to those observed on the double infection by the separately packaged phages of km993 (*recA*$^+$) or on double infection by the two phages (see below).

Since the recombination took place only outside the inserts when separately packaged $\lambda h^{\phi 80}$·Igα-13·imm^{434} and Ch28·Igμ-701 DNAs coinfected km993, the recombination within the insert DNAs seems to take place or at least to initiate during the *in vitro* packaging reaction. Although comparable numbers of recombinants were formed when phage DNAs without mouse DNA inserts or those with inversely orientated inserts were used, most of the recombination took place within the phage arms.

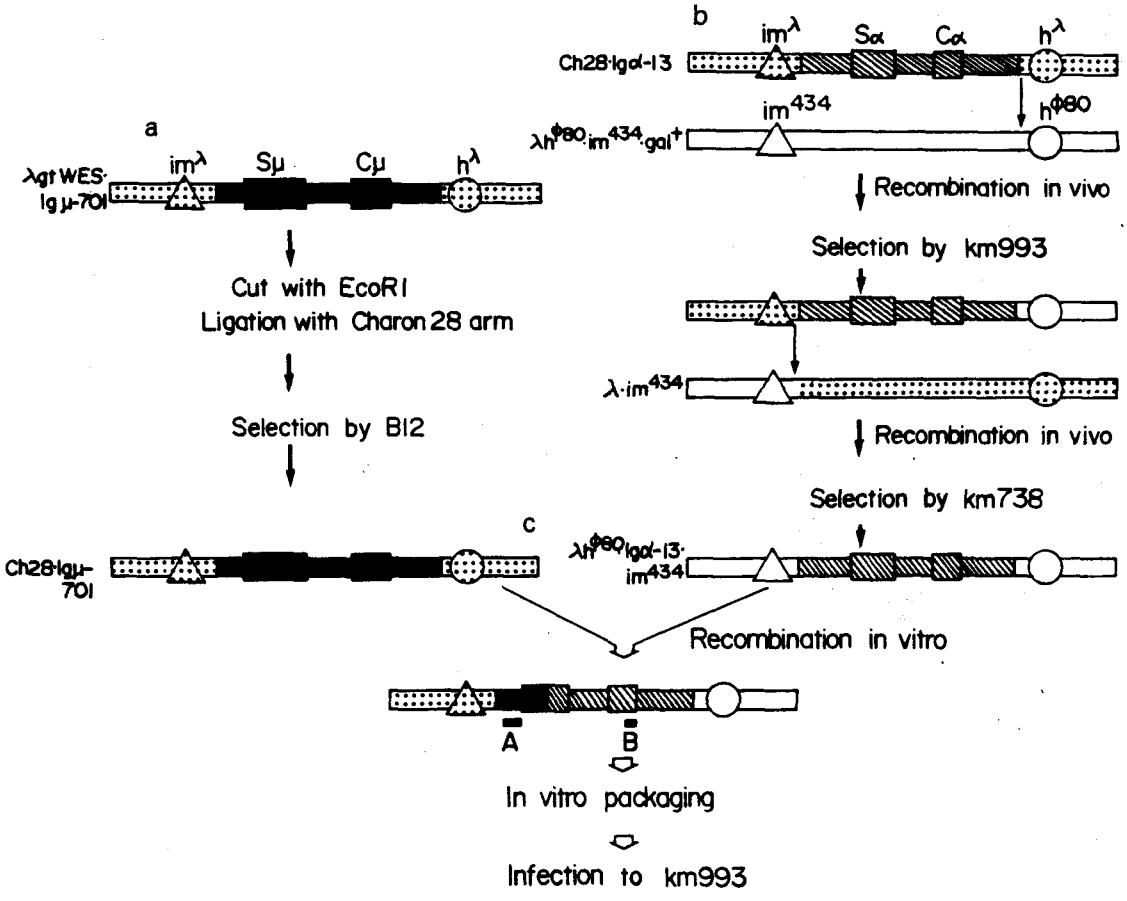

FIG. 1. *In vitro* assay system for S-S recombination. (*a*) Formation of Ch28·Igμ-701. λgt·*WES*·Igμ-701 (1) was cleaved with *Eco*RI and ligated in the presence of Charon 28 arms. The resultant phage DNAs were packaged *in vitro* and permitted to infect B12(*sup* 0). One of the phages grown in B12 was isolated and tested for the presence of the intact 13-kb *Eco*RI fragment by restriction cleavage. (*b*) Formation of $\lambda h^{\phi 80}$·Igα-13·imm^{434}. Genetic markers $h^{\phi 80}$ and imm^{434} were introduced into the phage arms of Ch28·Igα-13 by two steps of crosses. (*c*) *In vitro* recombination assay. Ch28·Igμ-701 and $\lambda h^{\phi 80}$·Igα-13·imm^{434} DNAs are mixed, treated with extracts from B lymphocytes, and packaged *in vitro* into phage coats. Recombinants bearing the $h^{\phi 80}$ and imm^λ markers are then selected. Fragments A and B are the 0.8-kb *Hin*dIII fragment of Igμ-701 and the cloned α-chain cDNA (pABα-1), respectively. Im^λ and im^{434}, immunity to wild-type λ phage and to lambdoid phage 434, respectively.

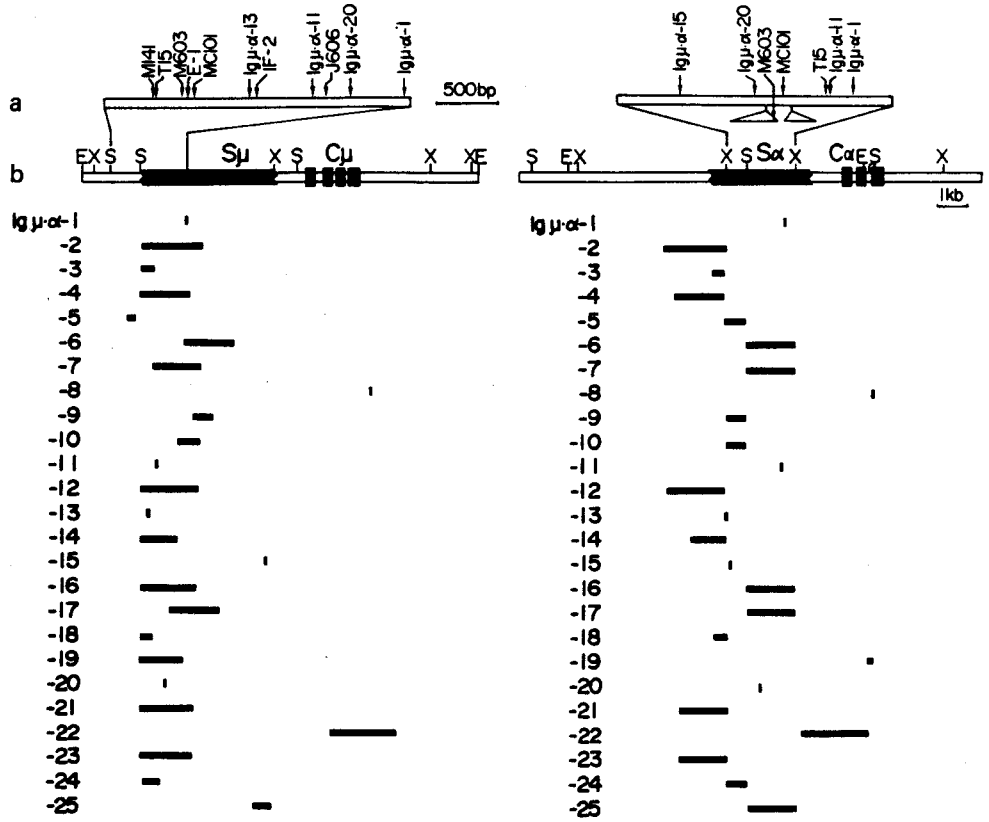

FIG. 2. Distribution of the recombination sites on the μ and α chain genes. Portions of the S_μ and S_α regions (*a*) and approximate locations of recombination sites of 25 clones (*b*) are shown. (*b*) Restriction cleavage sites of the Igμ-701 (*Left*) and Igα-13 (*Right*) inserts. The approximate locations of the recombination sites of the 25 arbitrarily selected recombinant clones were determined by comparison of restriction cleavage maps of the clones with those of Igμ-701 and Igα-13. The horizontal bars below the restriction maps of the parental clones indicate the estimated ranges of the recombination sites. In those clones for which the recombination sites are definitely assigned, the sites are shown as vertical lines. (*a*) Enlargement of portions of the S_μ and S_α regions that include the class switch recombination sites of various myelomas; MOPC141 (M141) (19, 20), MCPC603 (M603) (4), TEPC15 (T15) (4), IgE-1 (E1) (7), MC101 (4, 9), IF-1 (10), and J606 (7, 8), as well as those of the several *in vitro* recombinant clones. Triangles below the bar (Igα-13) indicate locations of deletions introduced. S, *Sac* I; X, *Xba* I; E, *Eco*RI. bp, base pair(s).

Table 1. Frequency of recombination *in vivo* and *in vitro*

System	Plaques, no. × 10^{-5}	Recombination		Location of recombination site			
				Insert		Vector arms	
		Total no.	Frequency × 10^4	No.	%	No.	%
		In vitro recombination					
Exp. 1							
Ch28·Igμ-701/$\lambda h^{\phi 80}$·Igα-13·imm^{434}	21/7.9	246	3.5	74	30	172	70
Ch28·Igμ-701*/$\lambda h^{\phi 80}$·Igα-13imm^{434}	21/7.8	128	1.6	2	1.6	126	98
Charon 28/$\lambda h^{\phi 80}$·imm^{434}·gal^+	5.0/1.6	23	1.4				
Ch28·Igμ-701† and $\lambda h^{\phi 80}$·Igα-13·imm^{434}	18 and 2.0	105	2.0	2	2	103	98
Exp. 2‡							
Ch28·Igμ-701/$\lambda h^{\phi 80}$·Igα-13·imm^{434}	5.5/2.0	97	4.8		42		50
Ch28·Igμ-701/$\lambda h^{\phi 80}$·Igα-13·imm^{434}/ oxolinic acid	5.3/2.1	68	3.3		41		59
Ch28·Igμ-701/$\lambda h^{\phi 80}$·Igα-13·imm^{434}/ oxolinic acid/coumermycin	5.0/2.1	54	2.7		39		61
		In vivo recombination					
Ch28·Igμ-701/$\lambda h^{\phi 80}$·Igα-13imm^{434}	1.8/2.1	907	43	0	0	907	100
Ch28·Igμ-701/$\lambda h^{\phi 80}$·imm^{434}·gal^+	1.9/2.4	925	39				
Ch28·Igμ-701*/$\lambda h^{\phi 80}$·Igα-13·imm^{434}	1.9/2.2	821	37	0	0	821	100
Ch28·Igμ-701 alone	790	0	0				
$\lambda h^{\phi 80}$·Ig-13·imm^{434}	590	0	0				

In vitro recombination: Phage DNAs were packaged *in vitro* and permitted to infect km993 at a moi of 0.001–0.008. Recombination frequency was calculated as the ratio of the number of phages grown on km993 to that of phages grown on km738. Determination of the locations of the recombination sites was by plaque hybridization. *In vivo* recombination: Phages were doubly infected at a moi of 5 on LE392 cells and grown for 1 hr at 37°C, and progeny were examined as above. Oxolinic acid and coumermycin were used at 45 and 5 μg/ml, respectively. Ch28·Igμ-701*, as Ch28·Igμ-701 except that the insert (Igμ-701) is inversely oriented relative to phage arms.

† Mixture of phages packaged separately *in vitro*.

‡ Only a portion of the recombinant phages in this experiment were tested by hybridization.

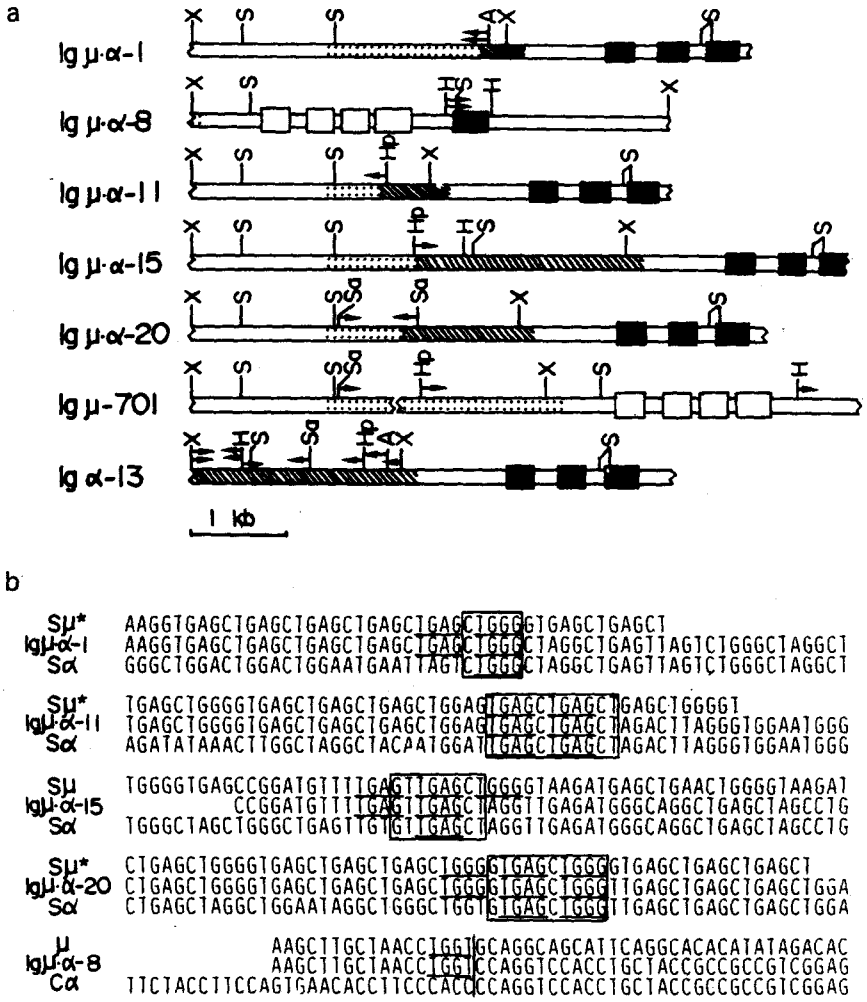

FIG. 3. Sequence analysis strategy and nucleotide sequences surrounding the recombination sites. (*a*) Analysis strategy. The rectangles represent portions of the inserts of the recombinant phages containing the recombination sites. Only those cleavage sites of *Hin*dIII, *Sau*3A, *Hpa* II, and *Ava* II used for the sequence determination are shown. ▭ and ▨, S_μ and S_α regions, respectively; ■ and □, exons of the C_α and C_μ genes, respectively. The nucleotide sequences surrounding the recombination sites and corresponding regions of the germ line α gene Igα-13 were determined by the method of Maxam and Gilbert (21). The nucleotide sequence of such a region of the germ line μ gene Igμ-701 that corresponds to the neighbor of the recombination site of Igμ·α-15 was determined. Horizontal arrows represent directions and ranges of analysis. (*b*) Nucleotide sequences. Nucleotide sequences surrounding the recombination sites are represented with the direction of transcription of exons from left to right. The boxed areas indicate the regions in which the recombination sites are located. T-G-G-G, T-G-A-G, and T-G-G-T sequences around the recombination sites are underlined. S_μ, S_α, μ, and C_α indicate the corresponding germ line nucleotide sequences. S_μ* indicates the possible nucleotide sequences of the germ line S_μ region estimated from the repetitive pattern of the S_μ region. X, *Xba* I; S, *Sac* I; A *Ava* II; H, *Hin*dIII; Hp, *Hpa* II; Sa, *Sau*3A.

The recombination, therefore, requires that the S_μ and S_α region sequences be presented in the same orientation. As control experiments, we allowed the two phages to coinfect LE392. Although recombination took place 10 times more frequently, no recombination took place within the inserts. These recombinations in phage arms may be due to the recA-dependent homologous recombination in bacterial cells.

Nucleotide Sequences Around the Recombination Sites. To determine the locations of the recombination sites within the inserts, 25 recombinant phages having nucleotide sequences of both S_μ and C_α genes were randomly chosen and their DNAs were analyzed by digestion with various restriction endonucleases. The results are summerized in Fig. 2. Of 25 recombinants, 23 phages recombined in the S_μ region and two phages combined around the C_μ coding region. Similarly, of 25 recombinants, 22 recombined in the S_α region and three phages combined around the C_α coding region. The results thus show the preference of the S regions as the recombination site in the *in vitro* packaging system.

Five recombinant phages were arbitrarily selected to determine nucleotide sequences surrounding the recombination sites. Four of them have recombination sites in the S regions and the other has recombined within the structural genes. Restriction fragments containing recombination sites were isolated and the nucleotide sequences were determined (Fig. 3). Nucleotide sequences of the parental germ line S_α region were also determined. We were unable to determine all the nucleotide sequences of the parental germ line S_μ region because appropriate restriction sites were not available except for that of Igμ·α-15. However, nucleotide sequences derived from the S_μ region were easily identified by the characteristic tandem repetition of G-A-G-C-T and G-G-G-G-T (7). Recombinants Igμ·α-1, Igμ·α-11, Igμ·α-15, and Igμ·α-20 were shown to be formed by direct joining of the S_μ and S_α regions. Igμ·α-8 was created by recombination between the 3′ flanking region of the C_μ gene and the third domain of the C_α gene.

The recombination sites were located by the comparison of nucleotide sequences with those of parental genes. These regions always contained abundant T-G-A-G or T-G-G-G sequences (or both), which have also been found near the class switch recombination sites in mouse myelomas (8). These results indicate that the *in vitro* packaging system, which consists

solely of extracts from *E. coli* and λ phages, might have a recombination system that prefers these short common sequences similar, albeit not identical, to those used in the class switch recombination of the immunoglobulin genes. The nucleotide sequence of Igμ·α-8 indicated that a short sequence (T-G-G-T) similar to those in the other recombinants was also found around the recombination site, supporting the above assumption.

Mechanism for *in Vitro* Recombination. Since the *in vitro* packaging system used is free from both *recA* and *red* functions, the *in vitro* recombination may be carried out by other minor recombination pathways of *E. coli* or λ phages, although we cannot exclude contamination by a tiny amount of recA protein. We have tested whether DNA gyrase is involved in this recombination by using the specific inhibitors of the enzyme oxolinic acid and coumermycin (22). Since these agents had virtually no effect on the recombination frequency (Table 1), the DNA gyrase-dependent recombination, which was previously shown to occur in the *in vitro* packaging system (15, 23), is not likely to be responsible for the recombination of the S regions. Neither does the int protein seem to be involved in this recombination as the nucleotide sequences surrounding the recombination sites were quite different from that of the *att* region.

Farabaugh and Miller (24) have reported that recombinations between tandemly repeated short sequences generate deletion or insertion mutations at a high frequency. The most frequent mutation site has involved tandem repetition of the sequence C-T-G-G, which is reminiscent of a building block of the short common sequence of the S regions. They have suggested that this recombination is also independent of the recA function. It is not known whether their recombination system is related to that described here.

The nucleotide sequences of the switch regions bear considerable homology to the χ sequence (25, 26). Although χ-mediated recombination *in vivo* is dependent on the products of the *recA* and *recBC* loci (27), it is not clear whether, under the conditions of *in vitro* packaging, there would be a strict requirement for the *recA* product. The present reaction may be mediated by χ-like elements, as suggested (26).

Significance of S-S Recombination in *E. coli* Extracts. Recently, site-specific recombination systems of prokaryotic cells have been shown to share some properties similar to those of eukaryotic cells. The nucleotide sequence involved in the flip-flop inversion of the flagellar genes of *Salmonella* was shown to be quite homologous to the sequences possibly involved in the V-J or V-D-J recombination of the immunoglobulin genes (28–31). Furthermore, Sakoyama *et al.* (32) have shown that the nucleotide sequences homologous to the mouse S_μ region are represented in variety of organisms such as yeast, sea urchin, and *Drosophila*. Such sequences of *Drosophila* are almost identical to the mouse S_μ region. These findings suggest that prototypes of the eukaryotic recombination system might be found in lower eukaryotes and possibly in prokaryotes, although the S_μ-like sequence is not found in *E. coli*. As a further speculation, the *in vitro* recombination observed in this study might be related to a prototype of the eukaryotic recombinational system.

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