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Genetics

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 *K803sulI/sulIII*, 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.

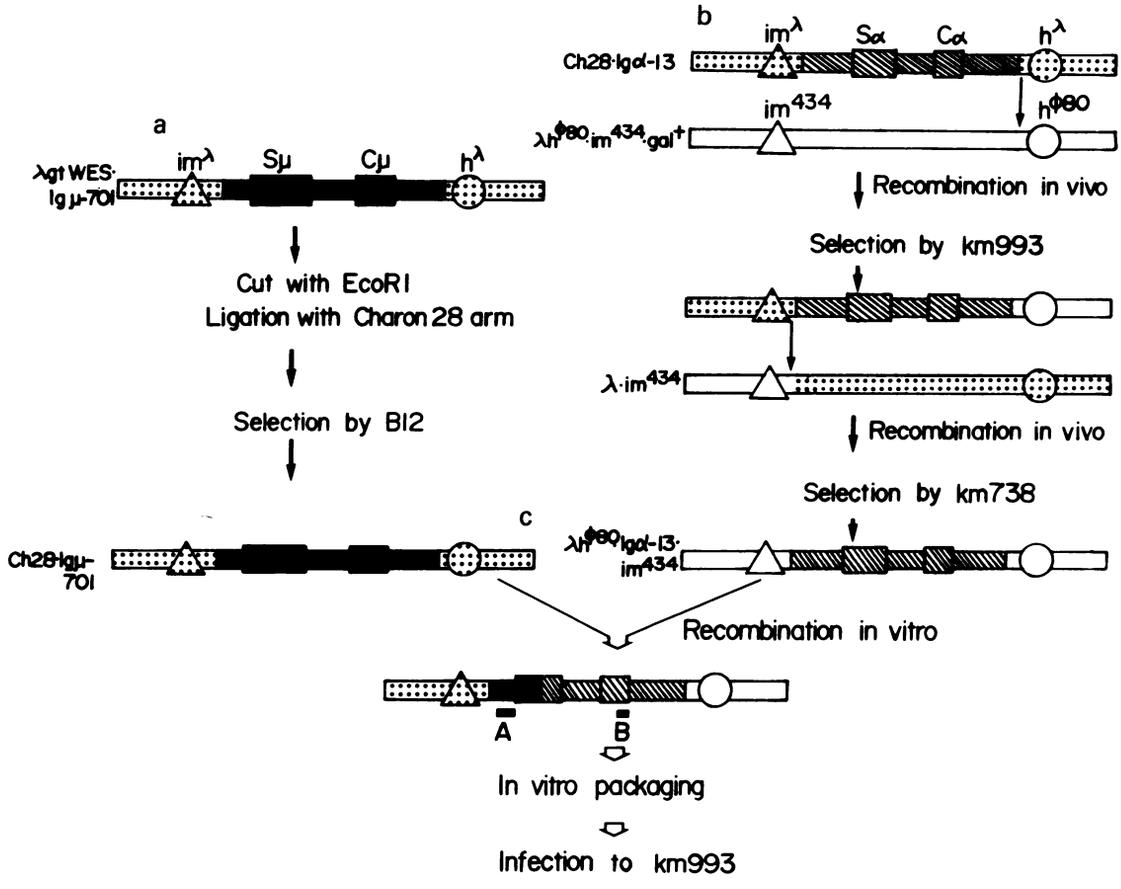

FIG. 1. *In vitro* assay system for S-S recombination. (*a*) Formation of Ch28·Igμ-701. λgt·*WES*·Igμ-701 (1) was cleaved with *Eco*RI and ligated in the presence of Charon 28 arms. The resultant phage DNAs were packaged *in vitro* and permitted to infect B12(*sup* 0). One of the phages grown in B12 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$^\lambda$ and im^{434}, immunity to wild-type λ phage and to lambdoid phage 434, respectively.

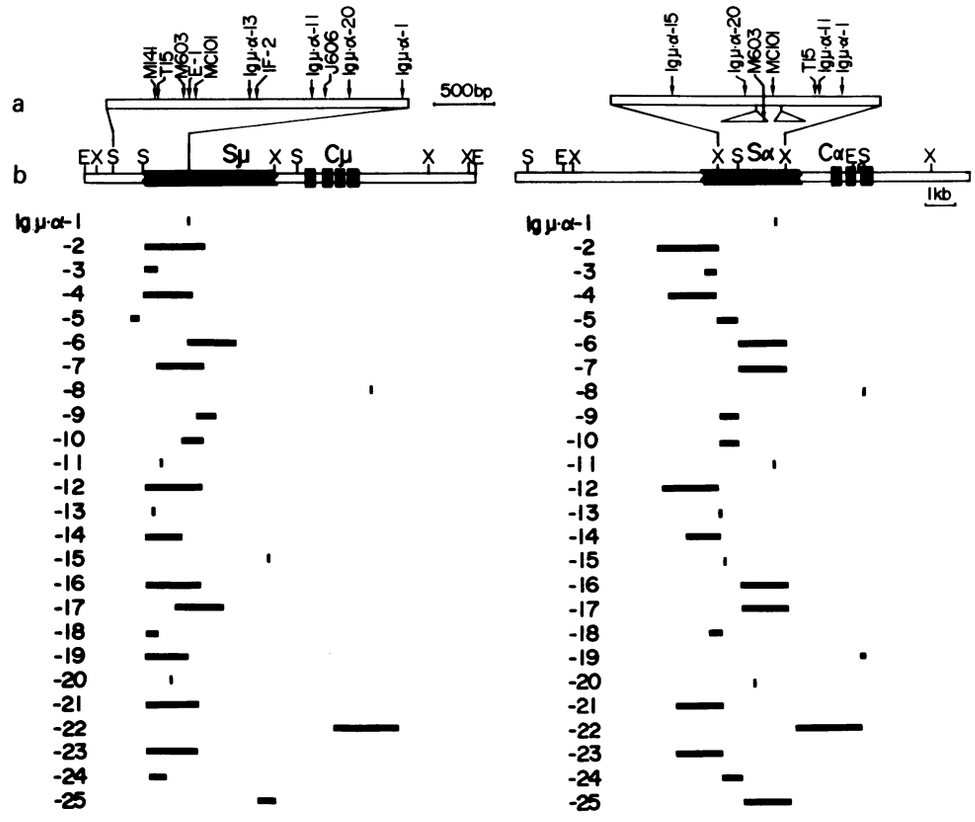

FIG. 2. Distribution of the recombination sites on the μ and α chain genes. Portions of the S_μ and S_α regions (*a*) and approximate locations of recombination sites of 25 clones (*b*) are shown. (*b*) Restriction cleavage sites of the Igμ-701 (*Left*) and Igα-13 (*Right*) inserts. The approximate locations 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. $\times 10^{-5}$	Recombination		Location of recombination site			
				Insert		Vector arms	
		Total no.	Frequency $\times 10^4$	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-13*imm*^{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-13*imm*^{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.

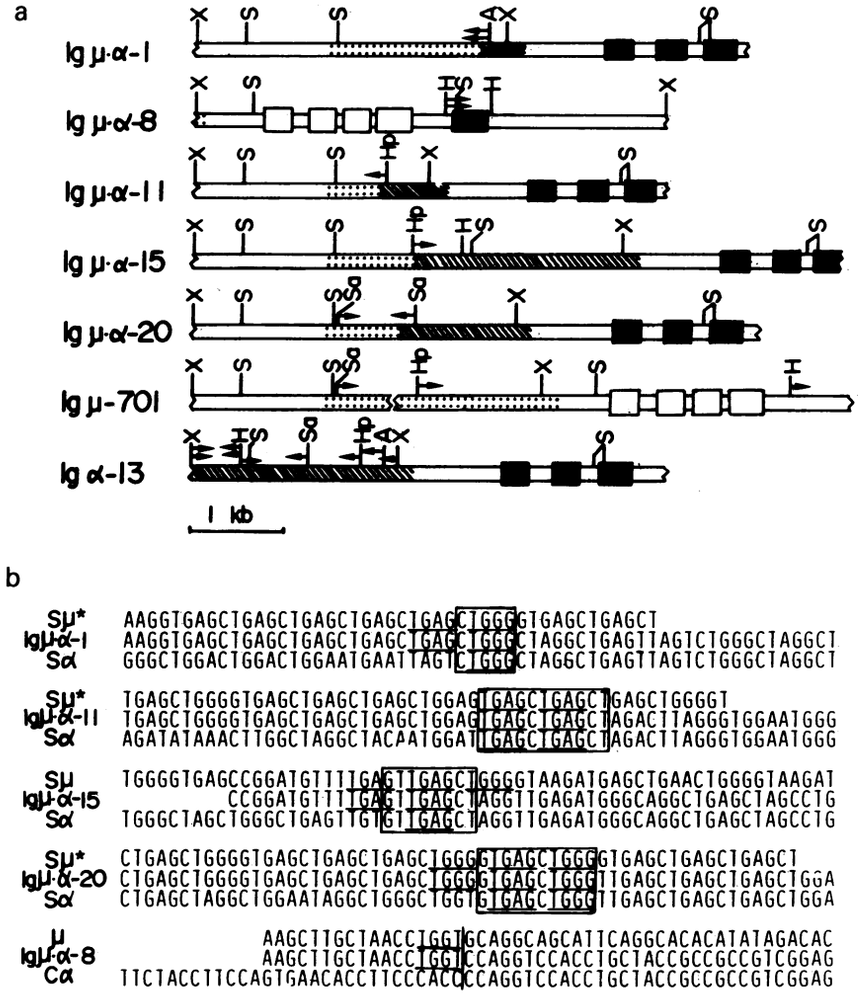

FIG. 3. Sequence analysis strategy and nucleotide sequences surrounding the recombination sites. (*a*) Analysis strategy. The rectangles represent portions of the inserts of the recombinant phages containing the recombination sites. Only those cleavage sites of *Hin*dIII, *Sau*3A, *Hpa* II, and *Ava* 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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