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Regulation and Targeting of Recombination in Extrachromosomal Substrates Carrying Immunoglobulin Switch Region Sequences

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We have used extrachromosomal substrates carrying immunoglobulin heavy-chain Sμ and Sγ3 switch region sequences to study activation and targeting of recombination by a transcriptional enhancer element. Substrates are transiently introduced into activated primary murine B cells, in which recombination involving S-region sequences deletes a conditionally lethal marker, and recombination is measured by transformation of *Escherichia coli* in the second step of the assay. Previously we found that as many as 25% of replicated substrates recombined during 40-h transfection of lipopolysaccharide (LPS)-stimulated primary cells and that efficient recombination was dependent on the presence of S-region sequences as well as a transcriptional activator region in the constructs (H. Leung and N. Maizels, Proc. Natl. Acad. Sci. USA 89:4154–4158, 1992). Here we show that recombination of the switch substrates is threefold more efficient in LPS-cultured primary B cells than in the T-cell line EL4; the activities responsible for switch substrate recombination thus appear to be more abundant or more active in cells which can carry out chromosomal switch recombination. We test the role of the transcriptional activator region and show that the immunoglobulin heavy-chain intron enhancer (Eμ) alone stimulates recombination as well as Eμ combined with a heavy-chain promoter and that mutations that diminish enhancer-dependent transcription 500-fold diminish recombinational activation less than 2-fold. These observations suggest that the enhancer stimulates recombination by a mechanism that does not depend on transcript production or that is insensitive to the level of transcript production over a very broad range. Furthermore, we find that Eμ stimulates recombination when located either upstream or downstream of Sμ but that the position of the recombinational activator does affect the targeting of recombination junctions, suggesting that the relatively imprecise targeting of switch junctions in vivo may reflect the availability of many potential activator sites within each switch region.

Immunoglobulin isotype switch recombination is a regulated recombination event which joins an expressed heavy-chain variable [V(D)J] region to a new downstream constant (C) region, deleting the DNA between (for reviews, see references 5, 6, and 8). Isotype switching occurs in B cells which have accomplished V(D)J joining and have been activated by antigen. Recombination involves G-rich switch or S regions, from 1 to 8 kb in length, which are found in the intron upstream of each C region that undergoes switching—Cμ, Cγ, Cα, and Cε; expression of Cδ is regulated by alternative splicing, not recombination, and there is no G-rich S region upstream of Cδ. In contrast to V(D)J joining, in which heptamer-nonamer recognition elements precisely target recombination (13), switch recombination is notably imprecise. No conserved sequences comparable to the heptamer-nonamer elements are apparent within S regions or at switch recombination sites, and chromosomal switch junctions produced in vivo are heterogeneous with regard to both the site of recombination within each S region and the sequence at the recombination junction itself. Switch recombination is therefore region specific but not site specific, and the absence of site specificity suggests that switch recombination might depend at least in part on components involved in general cellular processes of recombination and/or repair.

The mechanisms which regulate and effect switch recombination are not well understood. We have developed a transient recombination assay that uses extrachromosomal substrates carrying immunoglobulin heavy-chain switch region sequences to study sequences and regulatory elements that may be important to isotype switching (19). The substrates consist of a shuttle vector carrying Sμ and Sγ3 sequences flanking a conditionally lethal marker, the leftward promoter of phage λ (λP_L). Substrates are transfected into primary murine spleen cells cultured with lipopolysaccharide (LPS) to induce switch recombination, low-molecular-weight DNA is recovered 40 h later, and recombination is assayed as deletion of the λP_L region. When we first described this assay, we demonstrated that efficient recombination required the presence of S-region sequences and that an enhancer-promoter upstream of Sμ stimulated recombination 12-fold, so that about 25% of recovered, replicated molecules had recombined during transfection of the eukaryotic cells. Both the immunoglobulin heavy-chain enhancer-promoter ($E\mu P_H$) and the cytomegalovirus IE1 enhancer-promoter (EP_{CMV}) stimulated recombination in the substrates.

Here we show that substrate recombination is threefold more efficient in LPS-cultured primary B cells than in the murine T-cell thymoma line EL4. The activities that carry out substrate recombination therefore appear to be more

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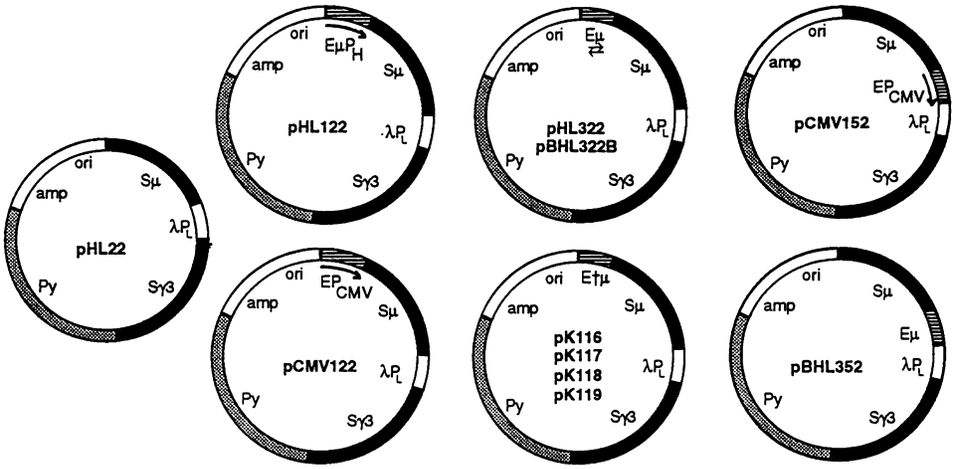

FIG. 1. Extrachromosomal switch substrates. The constructs carry 2.2 kb of Sμ sequence and 2.7 kb of Sγ3 sequence flanking the leftward promoter of λP_L, cloned into a shuttle vector consisting of the polyomavirus early region (Py) and the pBR322 origin of replication (ori) and Amp^r gene (amp). pHL122 carries Eμ and P_H upstream of the Sμ region, and pCMV122 carries EP_{CMV} in the same position; in both constructs, transcription from P_H would proceed clockwise, as shown by the arrow, and G-rich sequences in the Sμ and Sγ3 sequences are on the top strand. pHL322 and pBHL322B carry Eμ with no promoter in forward and reverse orientations, respectively. pK116, pK117, pK118, and pK119 carry Eμ regions mutated at specific protein binding sites (E†μ) (mutants 16 to 19 described in Fig. 5 of reference 18; see text for further description). pBHL352 carries Eμ between Sμ and λP_L in the forward orientation, and pCMV152 carries EP_{CMV} between Sμ and λP_L.

abundant or more active in cells capable of carrying out chromosomal switch recombination. We examined recombinational activation by the transcriptional regulatory region in detail and found that $E\mu P_H$ and Eμ alone, with no promoter, stimulated recombination to comparable levels, although these elements differed by at least 50-fold in the ability to drive expression of a reporter construct in activated primary B cells. Furthermore, mutations in Eμ that diminish transcriptional enhancement 10-fold (18) diminished recombination less than 2-fold. A 500-fold decrease in enhancer-dependent transcription therefore has only a 2-fold effect on recombination. This suggests that transcript production is not critical to the recombination process or that if transcript production is critical, recombination is insensitive to the level of transcript production over a very broad range. Recombination does depend on factor binding to the enhancer, because an Eμ region mutant at the core, E1, E2, E3, E4, and octamer (oct) sites was unable to activate recombination. Stimulation of recombination by Eμ is orientation independent and occurred when Eμ was upstream or downstream of Sμ, but the position of the recombinational activator did affect the targeting of recombination junctions in the switch substrates. The relatively imprecise targeting of switch junctions in vivo may reflect the availability of many potential activator sites within each switch region.

MATERIALS AND METHODS

Recombination substrates. pHL22 and pHL122 have been described previously (19) and are diagrammed in Fig. 1. Briefly, pHL22 carries 2.2 kb of Sμ sequence and 2.7 kb of Sγ3 sequence flanking λP_L, cloned into a shuttle vector consisting of the polyomavirus early region (for replication in murine B cells) and the origin of replication and ampicillin resistance (Amp^r) gene of pBR322 (for replication and selection in *Escherichia coli*). In pHL122, the immunoglobulin heavy-chain intron enhancer (Eμ) and a minimal heavy-chain promoter (P_H) are just upstream of the Sμ region. pHL322 and pBHL322B, carrying Eμ in forward and reverse orientations, respectively, were constructed by insertion of the 682-bp Eμ *Xba*I-*Eco*RI fragment into *Xho*I and *Not*I sites in a short polylinker upstream of Sμ in pHL22. (To simplify terminology, we will use "upstream" and "downstream" to describe positions with respect to direction of transcription in the genome, without implying that a sequence is itself transcribed, and "forward" and "reverse" to describe orientation with genomic orientation as the reference.) pK116, pK117, pK118, and pK119 carry Eμ regions mutated at specific protein binding sites inserted in forward orientation upstream of Sμ. Mutant enhancers 16 to 19 (18) were used to generate pK116 to pK119. Enhancers were PCR amplified from constructs generously supplied by T. Kadesch, using primers which annealed at the *Eco*RI and *Xba*I sites which define the boundaries of the enhancer; *Xho*I and *Not*I recognition sequences had been incorporated into the 5′ and 3′ primers, respectively, to permit directional cloning into the *Xho*I and *Not*I sites just upstream of Sμ in pHL22. pBHL352, carrying Eμ in forward orientation downstream of Sμ, was generated by cloning the Eμ fragment into the *Bsa*BI site at position −67 of λP_L; this destroys one of the three λ repressor binding sites (the most promoter distal) but does not interfere with the genetic selection. pCMV152 was generated by inserting EP_{CMV} at this same *Bsa*BI site.

Transfection. Transfection of primary murine splenocytes has been described in detail previously (19). Briefly, cells were cultured at 10^6 cells per ml in RPMI 1640 medium supplemented with 10% fetal bovine serum and 40 μg of LPS per ml. After 3 days in culture, 5×10^7 washed cells were transfected with 10 to 20 μg of DNA in 100 μg of DEAE-dextran per ml. For transfection of EL4, a T-cell line derived from a murine thymoma, 10^7 washed cells were incubated at room temperature for 20 min with 10 μg of DNA in RPMI 1640 containing 500 μg of DEAE-dextran per ml, washed twice, and cultured at 2×10^5 cells per ml. Transfection protocols for both cell types were optimized by using a

chloramphenicol acetyltransferase (CAT) expression construct.

Reporter constructs and expression assays. To construct pIgEP1-CAT, $E\mu P_H$ was subcloned from pUC-IgEPV (19) into the *Eco*RV site of the Bluescribe KS+ polylinker (Stratagene) to generate pBP-IgEP, excised by *Xba*I-*Hin*dIII digestion, and inserted (by using an *Xba*I linker) into the *Acc*I-*Hin*dIII sites of pSV2CAT (12), so that $E\mu P_H$ replaced the simian virus 40 early enhancer-promoter upstream of the CAT gene in the reporter construct. To construct pIgE-CAT, P_H was deleted from pBP-IgEP by *Bam*HI digestion and recircularization, and Eμ was excised and inserted into the *Acc*I-*Hin*dIII sites of pSV2CAT as described above for pIgEP1-CAT. DEAE-dextran transfection was carried out as described previously (19). Whole cell extracts were prepared 48 h after transfection and assayed for CAT activity (12).

Extrachromosomal switch recombination assay. The switch recombination assay has been described in detail previously (19). Briefly, low-molecular-weight DNA was prepared from transfected cells 40 h after transfection, mixed with a small amount of the compatible plasmid pACYC184, which carries a chloramphenicol resistance (Cm^r) marker and served as an internal transformation control, and digested with *Dpn*I to destroy molecules that had not replicated in the eukaryotic cells. Equal aliquots of DNA were then electroporated into *E. coli* DH10B and the isogenic λ lysogen DH10B(λ). The recombination frequency, R, was calculated as the fraction of ampicillin-resistant colonies produced by transformation of the nonlysogen compared with the lysogen, normalized for any slight differences in transformation efficiencies of the two strains: R = Amp^r [DH10B/DH10B(λ)] × Cm^r [DH10B(λ)/DH10B].

Control experiments have addressed the following critical points. (i) The S-region sequences in these constructs are not preferential sites for deletion in *E. coli*. We have assayed mutation of λP_L following extensive propagation of a variety of constructs in *E. coli* and have found this mutation frequency to be $\leq 2 \times 10^{-5}$ in all constructs examined. Furthermore, in contrast to the deletions that occur during transfection of activated primary B cells, essentially all mutations that occur during propagation in *E. coli* consist of point mutations or deletions so small that they do not affect the restriction map. (ii) The genetic selection does not bias the assay. We assayed recombination frequencies by hybridization of a λP_L probe to transformants of DH10B(λ) and found these measurements to be comparable to those produced in the genetic assay. (iii) Deletion of λP_L does not enhance replication of the shuttle vector in the mammalian cells. Constructs carrying and lacking λP_L display comparable levels of replication during transfection. (iv) Deletion endpoints are heterogeneous and distinct from bacterial deletion endpoints. Both mapping and sequencing (see below) showed that sites in the constructs are not functioning as hotspots for deletion in the mammalian cells. Furthermore, regions of homology of 4 to 6 nucleotides (nt) or more characterize deletion junctions in multicopy plasmids in bacteria (15, 41), and such regions are not found in recombinants of the switch substrates.

Mapping and sequencing of recombinants. Minipreps of recombinant DNA were prepared by a standard alkaline lysis protocol. Deleted regions in the recombinant plasmids were mapped by digestion with *Hin*dIII, *Bam*HI, or *Eco*RI. Recombinants were sequenced in chain termination reactions by using the following primers complementary to sequences within Sμ: μ85 (5′-CCCGGATCCACATGCTC TGTGTGA), μ105 (5′-TCCCTCTGGCCCTGCTTATTGTT GAA), μ414 (5′-TTAGGGGGAGAAAGAGAC), and μ1072 (5′-ACTAACTCTCCAGCCACA).

RESULTS

Figure 1 shows some of the extrachromosomal substrates used in the transient recombination assay. A shuttle vector carries Sμ and Sγ3 switch region sequences flanking a conditionally lethal marker, λP_L. These plasmids can be propagated in strains of *E. coli* lysogenic for bacteriophage λ, in which the endogenous *c*I repressor shuts off λP_L transcription, but the high level of transcription driven by λP_L interferes with plasmid replication in nonlysogenic strains of *E. coli*. The frequency of recombination events that remove λP_L can therefore be assayed by comparing transformation of isogenic nonlysogenic and lysogenic strains of *E. coli*.

Previously we observed that following transfection of primary murine B cells, 2.1% of replicated pHL22 substrates had undergone recombination that deleted λP_L and that an active enhancer-promoter placed upstream of Sμ stimulated recombination 10- to 12-fold (19). Two different transcriptional regulators known to function in primary murine B cells were shown to stimulate recombination to comparable levels: the recombination frequency for pHL122, which carries $E\mu P_H$ upstream of Sμ, was 25.6%, and that for pCMV122, which carries EP_{CMV} upstream of Sμ, was 21.1%.

Cell type specificity of switch substrate recombination. Only B cells carry out switch recombination in vivo, and only LPS-stimulated primary cells carry out efficient chromosomal switch recombination in culture. To test whether recombination of the extrachromosomal switch substrates displays cell type specificity, we assayed recombination following transfection of EL4, a murine T-cell line. This T-cell line was chosen because the polyomavirus shuttle vector constructs replicate at comparable levels during transfection of T-cell and B-cell lines; while the polyomavirus shuttle vector also replicates in fibroblasts, replication in these cells is 50- to 100-fold more efficient than in lymphocytes, and in the absence of any appropriate internal standard for recombination, it is therefore impossible to compare recombination frequencies in the two cell types. Furthermore, like LPS-cultured primary B cells, EL4 cells are nonadherent cells that can be efficiently transfected by using DEAE-dextran. Preliminary experiments established that (i) EP_{CMV} drives reporter gene expression efficiently in EL4 cells, (ii) deletion of λP_L and switch region sequences does not confer a measurable replication advantage in EL4 cells; and (iii) recovery of replicated DNA from transfections of this T-cell line is only about fivefold higher than from primary B cells cultured with LPS and comparable to recovery from the B-cell line J558L. The increased yield of DNA from EL4 cells is probably partly due to the fact that primary B cells do not transfect well, even in the optimized conditions that we are using, and also to somewhat enhanced replication of the constructs in the T-cell line. If recombination is coupled to replication, then enhanced replication would lead to an overestimate of the recombination frequency.

We assayed recombination in EL4 cells of two substrates, pHL22 and pCMV122, which differ in that pCMV122 carries EP_{CMV} upstream of the Sμ region. As shown in Table 1, the recombination frequency of pCMV122 is 7.1%, about threefold below the recombination frequency of this construct in LPS-stimulated primary B cells. The recombination frequency of pHL22 is 2.2%, showing that the transcriptional

TABLE 1. Recombination of switch substrates in the T-cell line EL4[a]

Plasmid	No. of transformed colonies				R (%)
	Amp^r		Cm^r		
	DH10B	DH10B(λ)	DH10B	DH10B(λ)	
pCMV122	775	13,500	82	124	8.7
	1,150	25,750	478	440	4.1
	750	22,750	66	100	5.0
	1,650	25,875	170	278	10.4
					7.1 ± 2.6[b]
pHL22	975	71,250	94	208	3.0
	250	16,000	42	34	1.3
					2.2 ± 0.85[b]

[a] Plasmids pCMV122 and pHL22 were assayed for recombination following transfection into the murine T-cell line EL4. Shown are the numbers of Amp^r and Cm^r colonies recovered after transformation of DH10B and the isogenic lysogen DH10B(λ). Each line represents data from a single transfection experiment. *R* was calculated as the ratio of Amp^r transformants in DH10B to those in DH10B(λ), normalized by the control Cm^r colonies: *R* = Amp^r [DH10B/DH10B(λ)] × Cm^r [DH10B(λ)/DH10B].

[b] Average *R* ± standard error.

TABLE 2. Stimulation of recombination in constructs carrying a heavy-chain enhancer but no promoter[a]

Plasmid	No. of transformed colonies				R (%)
	Amp^r		Cm^r		
	DH10B	DH10B(λ)	DH10B	DH10B(λ)	
pHL322	282	481	445	281	37.0
	68	200	290	302	35.4
	66	318	193	208	22.4
	100	728	35	43	16.9
	72	560	146	164	14.4
	123	556	226	214	20.9
	60	271	162	153	20.8
	117	469	584	581	24.9
					24.1 ± 2.9[b]
pBHL322B	383	3,750	118	146	12.6
	299	814	274	194	26.0
	382	2,323	310	426	22.6
	125	538	492	290	13.7
	146	1263	159	183	13.4
					17.7 ± 2.8[b]

[a] Plasmids pHL322 and pBHL322B were transfected into LPS-cultured primary murine B cells and then assayed for recombination as described in Materials and Methods. Shown are the numbers of Amp^r and Cm^r colonies recovered after transformation of DH10B and the isogenic lysogen DH10B(λ). Each line represents data from a single transfection experiment. *R* was calculated as the ratio of Amp^r transformants in DH10B to those in DH10B(λ), normalized by the control Cm^r colonies: *R* = Amp^r [DH10B/DH10B(λ)] × Cm^r [DH10B(λ)/DH10B].

[b] Average *R* ± standard error.

activator sequences contribute to the observed recombination. Restriction mapping of recombination products showed that they carried a heterogeneous array of deletions that span λP_L (data not shown), as do substrates that have recombined in activated primary B cells.

These data show that substrate recombination is about threefold more efficient in activated primary B cells than in the T-cell line. Most of the recombination that we observe is therefore likely to be due to activities that are unique to or enhanced in activated primary B lymphocytes. Is it surprising to find any recombination of the substrates during transfection of T cells? Only B cells have been shown to carry out regulated chromosomal switch recombination in vivo, but this does not mean that the enzymatic apparatus responsible for switch recombination is restricted to B cells. In fact, as considered in greater detail in Discussion, much of the control on chromosomal switch recombination may well depend on locus-specific regulation rather than a cell-type-restricted enzymatic apparatus.

Eμ alone can stimulate recombination. Our previous experiments showed that the recombination frequency of pHL122, which carries $E\mu P_H$ upstream of Sμ, is 25.6%. This is dramatically higher than the recombination frequency of 2.1% observed with pHL22, which lacks these transcriptional regulatory sequences, or the recombination frequency of 1.2% observed with pHL422, which carries P_H alone upstream of Sμ (19). To determine whether the high level of recombination observed with pHL122 correlates with transcription, we measured recombination of two constructs which lacked the promoter and carried Eμ alone upstream of Sμ, in either orientation. As shown in Table 2, substrate pHL322, with Eμ in its natural orientation, recombined at a frequency of 24.1%, and pBHL322B, with Eμ in the reverse orientation, recombined at a frequency of 17.7%. Eμ alone can therefore stimulate recombination as well as $E\mu P_H$ can.

To verify that removal of P_H did in fact depress transcription in primary LPS-stimulated B cells, we compared expression of a reporter gene in constructs carrying Eμ alone or $E\mu P_H$ as a transcriptional regulatory element. Figure 2 shows that expression of the CAT reporter gene was at least 50-fold lower in the construct carrying Eμ alone than in the construct carrying $E\mu P_H$. We also attempted to assay transcription from transfected switch substrates by RNase protection, using a λP_L antisense probe and comparing protection of RNA isolated from primary B cells transfected with substrate pHL122 or pHL322. Neither RNA preparation showed any protection of the λP_L probe (data not shown), but we do not believe that any conclusions can be drawn about transcript production from these data because an Sμ-λP_L-Sγ3 transcript would not be polyadenylated and

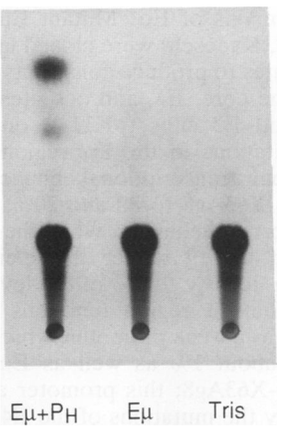

FIG. 2. $E\mu P_H$- and Eμ-driven expression of a CAT reporter gene in primary splenocytes cultured with LPS. LPS-stimulated primary murine B cells were transfected with pIgEP1-CAT, which carries a CAT reporter gene under control of Eμ combined with P_H ($E\mu+P_H$), and pIgE-CAT, which carries Eμ but no promoter (Eμ). A control reaction in which cell extract was replaced with 0.25 M Tris (pH 7.8) is also shown (Tris). Data are from a typical assay. Conversion in extracts of cells transfected with pIgEP1-CAT ranged between 1 and 3%, while extracts of cells transfected with pIgE-CAT never showed conversion above background (no extract) levels.

TABLE 3. Stimulation of recombination in constructs carrying mutations in Eμ[a]

Plasmid	No. of transformed colonies				R (%)
	Amp^r		Cm^r		
	DH10B	DH10B(λ)	DH10B	DH10B(λ)	
pK116	234	2,125	217	177	9.0
	28	115	431	402	22.7
	543	5,150	345	353	10.8
	486	1,646	158	120	22.4
					16.2 ± 3.7[b]
pK117	344	5,213	194	195	6.6
	557	4,413	156	147	11.9
	381	4,612	188	194	8.5
					9.0 ± 1.6[b]
pK118	171	2,500	162	128	5.4
	146	910	204	174	13.7
	197	788	195	141	18.1
					12.4 ± 3.7[b]
pK119	32	3,700	180	238	1.1
	23	794	307	279	2.6
	102	2,525	449	298	2.7
	48	3,063	155	125	1.3
					1.9 ± 0.4[b]

[a] Plasmids pK116, pK117, pK118, and pK119 were transfected into LPS-cultured primary murine B cells and then assayed for recombination as described in Materials and Methods. The reference construct was pK116. Test constructs carried mutations at the E4 and oct sites (pK117), at the E1, E2, and E3 sites (pK118), or at all five sites (pK119). Shown are the numbers of Amp^r and Cm^r colonies recovered after transformation of DH10B and the isogenic lysogen DH10B(λ). Each line represents data from a single transfection experiment. *R* is the ratio of Amp^r transformants in DH10B to those in DH10B(λ), normalized by the control Cm^r colonies: *R* = Amp^r [DH10B/DH10B(λ)] × Cm^r [DH10B(λ)/DH10B].

[b] Average *R* ± standard error.

TABLE 4. Stimulation of recombination in constructs carrying transcriptional regulatory elements downstream of Sμ[a]

Plasmid	No. of transformed colonies				R (%)
	Amp^r		Cm^r		
	DH10B	DH10B(λ)	DH10B	DH10B(λ)	
pBHL352	146	1,718	179	184	8.7
	104	588	147	157	18.9
	19	298	132	192	9.6
	17	395	125	294	10.1
					11.8 ± 2.4[b]
pCMV152	68	455	415	450	16.2
	73	615	401	294	8.7
	92	1,165	124	157	10.0
	45	215	237	177	15.7
	104	1,423	264	286	10.6
					12.2 ± 1.5[b]

[a] Plasmids pBHL352 and pCMV152 were tested in a recombination assay as described in Materials and Methods. Shown are the numbers of Amp^r and Cm^r colonies recovered after transformation of DH10B and the isogenic lysogen DH10B(λ). Each line represents data from a single transfection experiment. *R* was calculated as the ratio of Amp^r transformants in DH10B to those in DH10B(λ), normalized by the control Cm^r colonies: *R* = Amp^r [DH10B/DH10B(λ)] × Cm^r [DH10B(λ)/DH10B].

[b] Average *R* ± standard error.

would therefore probably be extremely short-lived in the cell.

Effect of mutation in Eμ on recombinational activation. To determine which elements within Eμ can stimulate recombination of the extrachromosomal substrates, we constructed substrates carrying mutations in either or both of the functional domains of Eμ. Mutant Eμ regions (18) (a generous gift of T. Kadesch) were cloned upstream of Sμ in the switch substrates to produce constructs that carry linker substitutions at the core, E4, and oct sites (pK117), at the core, E1, E2, and E3 sites (pK118), or at all six sites (pK119). The mutations in the Eμ regions of pK117 and pK118 diminish their transcriptional enhancer activity in the plasmacytoma P3-X63Ag8 to 80 and 20%, respectively, of the level of the normal enhancer, while the six mutations in the Eμ region of pK119 render this element essentially inactive, reducing activity to 2% of the level of the normal enhancer (18). The Eμ region functions not only as an enhancer but also as a weak promoter, which drives reporter gene expression about 1% as well as EμP$_H$ does in the plasmacytoma P3-X63Ag8; this promoter activity of Eμ is reduced 10-fold by the mutations of the E4 and oct binding sites in pK117 (29).

Each of the enhancer derivatives in pK117-119 carries a linker insertion at a core site; the three reiterated core sites, C1, C2, and C3, interact with a general factor, and core site mutation does not affect transcription in B cells (17). As a reference construct, we therefore generated a substrate, pK116, which carries a linker insertion at a core site but is not mutated at the E1, E2, E3, E4, or oct binding site. Recombination of pK116 was compared with recombination of pHL322, which carries an unmutated Eμ region. As shown in Table 3, the core mutation in the Eμ region of pK116 appears to diminish recombinational activation slightly: the recombination frequency of pK116, the reference construct, was 16.2%, or about 65% of the recombination frequency of pHL322, which carries an intact core region.

We compared recombination of constructs pK117, pK118, and pK119 with that of the reference construct, pK116. As shown in Table 3, the recombination frequency of pK117, mutated at the core, E4, and oct sites, was 9.0%, and the recombination frequency of pK118, mutated at the core, E1, E2, and E3 sites, was 12.4%. The recombination frequency of pK119, in which the Eμ region is mutated at the core, E1, E2, E3, E4, and oct sites, was 1.9%, comparable to that of pHL22, which has no Eμ or P$_H$ region. Eμ-dependent stimulation of recombination therefore does require protein binding to the regulatory element. In the immunoglobulin heavy-chain locus, Eμ is located just upstream of Sμ in the J-C intron, and it is possible that Eμ functions as a recombinational enhancer for isotype switching in vivo.

The ability of the regulatory element to stimulate recombination in the switch substrates appears to be essentially independent of its ability to stimulate transcription. Removal of the promoter from EμP$_H$ decreased transcription (as measured by expression) 50-fold or more but did not affect recombination (compare pHL122 and pHL322), and a further 10-fold decrease in the promoter activity of Eμ by mutation of the E4 and oct binding sites diminished recombination only about 2-fold (pK117). A 500-fold decrease in transcription thus had only a 2-fold effect on recombination. This result suggests that the mechanism by which Eμ stimulates recombination either does not depend on transcription or is insensitive to the actual level of transcription over a broad range.

Transcriptional regulatory elements enhance recombination whether upstream or downstream of Sμ. Eμ and EP$_{CMV}$ were tested for recombinational activation when inserted downstream rather than upstream of Sμ. Table 4 shows that the

TABLE 5. Summary of deletion endpoints in switch substrate recombinants[a]

Substrate	Elements	R (%)[b]	% Containing: Sμ endpoint 5′Sμ[d]	Sμ endpoint Sμ	Sγ3 endpoint Sγ3	Sγ3 endpoint 3′Sγ3[d]	n[c]
pBHL352	Sμ-Eμ-λP_L-Sγ3	11.8	11	89	45	55	44
pCMV152	Sμ-EP_{CMV}-λP_L-Sγ3	12.2	12	88	82	18	101
pHL122	EμP_H-Sμ-λP_L-Sγ3	25.6	78	22	86	14	47
pHL322	Eμ-Sμ-λP_L-Sγ3	24.1	62	38	50	50	26
pCMV122	EP_{CMV}-Sμ-λP_L-Sγ3	21.1	100	0	48	52	23
pTK119	E†μ-Sμ-λP_L-Sγ3	1.9	33	67	85	15	46

[a] Maps of substrates are shown in Fig. 1. Substrates that had undergone recombination during transfection of LPS-cultured primary murine B cells were isolated from Amp^r transformants of DH10B. Regions in which junctions mapped were determined by *Hin*dIII, *Bam*HI, or *Eco*RI digestion of recombinant plasmid DNA.

[b] From Tables 2 to 4 of reference 19.

[c] Number of recombinants of each substrate mapped.

[d] Refers to position with respect to direction of transcription of Sμ or Sγ3 sequence in the genome and does not imply that these sequences are transcribed in the extrachromosomal substrates.

recombination frequency of pBHL352, which carries Eμ with no promoter between Sμ and λP_L, was 11.8%, or about half of that of pHL322, in which Eμ is upstream of Sμ (Table 2). The recombination frequency of pCMV152, which carries EP_{CMV} between Sμ and λP_L, was 12.2%, or about half of that of pCMV122. While the position of the recombinational activator had a relatively small effect on the recombination frequency, as shown below, it had a dramatic effect on targeting of recombination junctions.

Transcriptional regulatory elements appear to target recombination endpoints. To determine whether the position or activity of the activator element might affect junction targeting, we mapped deletion endpoints in recombinants recovered from transfection of LPS-cultured primary murine B cells. Table 5 shows that in recombinants of substrates in which the regulatory element is between Sμ and λP_L, the majority of upstream endpoints mapped within Sμ: 89% of pBHL352 recombinants and 88% of pCMV152 recombinants. Furthermore, we found that in constructs in which the enhancer is located downstream of Sμ, not only did the majority of junctions map within Sμ, but about three times as many junctions were evident in the enhancer-proximal restriction fragment as would be predicted from size considerations alone (data not shown). In contrast, in constructs which carry a functional regulatory element upstream of Sμ (pHL122, pHL322, and pCMV122), the regulatory element was frequently deleted during recombination, and in only a minority of these recombinants did endpoints map within Sμ: 22% of pHL122 recombinants, 38% of pHL322 recombinants, and none of the pCMV122 recombinants. Deletion of the regulatory element correlated with factor binding. In most recombinants of pK119, which carries upstream of Sμ an Eμ region mutated at the C2, E1, E2, E3, E4, and oct sites and undergoes recombination at very low levels, upstream junctions mapped within the Sμ region (67%). These results suggest that binding of regulatory factors may define a boundary of the recombination zone.

The results in Table 5 show that targeting of recombination in the switch substrates is strikingly dependent on the position and activity of the regulatory element. If so, the regulatory factors that bind the polyomavirus sequences downstream of the Sγ3 region to direct shuttle vector replication may also affect junction targeting, and this may explain why in some cases (pCMV152, pHL122, and pTK119), over 80% of downstream junctions map in the Sγ3 region, while in others (pBHL352, pHL322, and pCMV122), only about half the downstream junctions are within Sγ3 and the rest are 3′ of Sγ3, within the polyomavirus sequences. However, it is also important to note that chromosomal switch junctions frequently map outside of S-region sequences. A recent compilation of chromosomal switch junctions shows that while essentially all downstream junctions map within the G-rich repetitive S-region sequences, about 40% of upstream junctions map not within but upstream of the Sμ region (7). The significance of these upstream junctions in chromosomal switch recombination is currently unresolved: junctions outside Sμ occur frequently and are stably maintained during cell passage, suggesting that they are primary products of switch recombination, but they are not observed in certain data sets, particularly the circles that are produced as apparently reciprocal products of switch recombination in primary cells cultured with lymphokines and LPS (see reference 7 for a discussion). The junctions in the switch substrates that map outside of S-region sequences may therefore be analogous to some of the chromosomal recombinants observed in vivo, or they may reflect the design of these particular substrates.

Recombination junction sequences. Recombination junctions in two classes of recombinants were sequenced to determine whether recombinants were simple deletions and whether there were obvious hotspots for recombination, particularly when recombination involved sequences outside of the S regions. In a major class of pCMV152 recombinants, upstream endpoints mapped within the first few hundred bases of Sμ; Fig. 3A shows sequences of seven such pCMV152 recombinant junctions, three of which joined Sμ and Sγ3 sequences and four of which joined Sμ to sequences downstream of Sγ3. There appears to be no sequence preference or targeting at the recombination site. Four share no common sequences between the upstream and downstream recombination partners at the junction, and three share 1 to 4 nt of sequence identity. One recombinant, pRCMV152.25, has a 4-nt untemplated addition at the junction; additions of 1 to 33 nt are also observed at switch junctions, where they are typically composed of G-rich DNA that is similar but not identical to S-region sequence.

Sequences of four recombinants derived from pHL122 are also shown in Fig. 3; all of these sequences display limited (2 and 4 nt) identity between upstream and downstream sequences involved in recombination. The Sγ3 region consists of about 40 reiterations of a consensus repeat 49 nt in length (30), and two of the Sγ3 recombination junctions mapped to repeat 33 in the germ line Sγ3 region (pRHL122.3 and pRBHL122.15), but neither mapping nor sequence analysis thus far shows any consistent recombinational targeting in the substrates.

Short (1- to 4-nt) regions of sequence identity were apparent at the recombination junction in 6 of 11 extrachromosomal substrates. Switch recombination is region specific, not site specific, and while chromosomal junction sequences do not bear any unique hallmark of the switch recombination apparatus, about half of the chromosomal products of chromosomal switch recombination do carry identities of 1 to 4 nt at the junctions (7). Although the sequences do not prove that enzymes involved in switching have produced these junctions, they are certainly most consistent with this possibility. Short regions of identity have also been observed at junctions produced by nonhomologous recombination in

pCMV152 Recombinants

```
pRCMV152.19     TTGAATGGGCCAAAGGTCTG GGGGTGTGGGGACCAGGCTG
Sμ              -------------------- A-ACCAG-CT-CTGCT-GGT
Sγ3             -G-GGGATCTGGGG-TAGGT --------------------

pRCMV152.25     GCCAGCCTCGGTGGCTTTGA AGGA TAAGTGAGGATGTGGGGACC
Sμ              -------------------- ---- AC-A-TCCACACAAAGACT-
Sγ3             -GACAG---T-GA-GGAGCT ---- --------------------

pRCMV152.24     CCACACAAAGACTCTGGACC AGGC GGGACCAGGCTGGGCAGCCT
Sμ              --------------------      TCTCCG-AA-CA----C-GC
Sγ3             A-GTGGGT-TGTGGG-TGTG      --------------------

pRCMV152.37     AATGTATGGTTGTGGCTTCT CAAAACAAACTGTAGGAGCA
Sμ              -------------------- GCC-CCC-TCCACTT-GCTG
Polyoma         GCACATTACAAAGTATA--A --------------------

pRCMV152.9      TCCACTTGGCTGCTCATGGA C GTTTATGCGGAAGAGGTAAA
Sμ              -------------------- - CAGCCA--CTCG-T--CTTT
3' λP_L         A-T-TAAACGCTGATGGAAG - --------------------

pRCMV152.34     TCTTCAGTCATTGCTTTAGG GG CTCAATGGAAAGCAGCAAAT
Sμ              -------------------- -- GAG--A-AG-CATTTGTGTG
λP_L            GAACGTCGCGCA-AGAA-CA -- --------------------

pRCMV152.8      GTGAACTCCCTCTGGCCCTG GTCCGGCGTAGAGGATCTAG
Sμ              -------------------- C-TATTGT-GA-T-GG-C-A
λP_L-Sγ3        -GATC-C--GG-CACGATGC --------------------
```

pHL122 Recombinants

```
pRHL122.1       AAACGCGCGAGGC AGCC CAGGAGAGGT
5' of IgEP      ------------- ---- GGATCATAAT
Sγ3             GTAGCTCTGGGGG ---- ----------

pRHL122.3       TGACGGGCTT GT TCAAGTATGGGG
5' of IgEP      ---------- -- CTGCTCCCGGCA
Sγ3             GCTGGGGTAG -- ------------

pRBHL122.15     CATCCACTT GGC AGCTCTGGGGCA
Sμ              --------- --- TGCTCATGGACC
Sγ3             ACTAACCTG --- ------------

pRBHL122.5      CTGCTCATGG AC AACTACATG
Sμ              ---------- -- CAGCCAGCC
Polyoma         GGCATAATAT -- ---------
```

FIG. 3. Sequences of recombination junctions. For each recombinant, the first line shows the top-strand sequence at the recombination junction, and the second and third lines show the sequences of the regions of the construct that participated in recombination. Dashes indicate identity; gaps indicate sites of recombination or define boundaries of recombination in cases where homology is evident at the junction.

mammalian cells (25) and at junctions in *Saccharomyces cerevisiae* produced by recombination between similar but not identical DNA sequences, for example, divergent alleles of a single gene (homeologous sequences) (22). This similarity is consistent with the possibility that some of the same or very similar enzymes carry out switch recombination and nonhomologous recombination.

Switch substrate junction sequences contrast with the pattern of spontaneous deletions in bacteria, in which sequence identity appears to be essential to the recombination mechanism. Studies of spontaneous deletions on the *E. coli* chromosome (2) and multicopy plasmids (15, 41) showed that the majority of deletions occur at sites with identity of 4 to 6 nt or longer, and deletions between sequences with no identity were rarely recovered. This finding further establishes that recombination of the substrates occurs during transfection of the eukaryotic cells, not during transformation of *E. coli*.

DISCUSSION

We have studied recombination of extrachromosomal substrates carrying immunoglobulin switch region sequences during transient transfection of primary B cells stimulated in culture to undergo chromosomal switch recombination. Previously we found that a functional promoter-enhancer upstream of the Sμ region in these constructs could stimulate recombination 12-fold (19). Here we show that the ability of the regulatory element to stimulate recombination does not correlate with its ability to stimulate transcript production. The immunoglobulin heavy-chain intron enhancer, Eμ, stimulated recombination to the same high level alone or when combined with a minimal heavy-chain promoter: the 50-fold reduction in transcriptional activity associated with removal of the promoter had no effect on recombination. Furthermore, additional mutations at the E4 and oct sites in Eμ that reduce the promoter activity of the isolated enhancer element 10-fold (29) resulted in only a 2-fold decrease in recombinational enhancement. Thus, a 500-fold reduction in transcription diminished recombination only 2-fold. Recombinational enhancement does depend on the presence of functional factor binding sites in Eμ, however, since an enhancer mutated at six separate binding sites cannot stimulate recombination.

Do the extrachromosomal substrates assay the same activities that carry out chromosomal switch recombination? To test cell type specificity, we assayed recombination of the switch substrates in the T-cell line EL4 and found that recombination is about threefold more efficient in activated primary B cells than in the T-cell line. Most of the recombination that we observe is therefore likely to be due to activities that are unique to or enhanced in activated primary B lymphocytes. Nonetheless, we do observe a significant level of recombination in EL4 (R = 7.1% for pCMV122). While this may be particular to the cell type or construct that we are using, it is also possible that the same enzymes that carry out switch region recombination in activated B cells participate in other recombination processes in other cell types.

Switch recombination in vivo is confined to a limited stage in B-cell development and could in principle require a recombination apparatus restricted to that cell type. However, no experimental evidence thus far has shown that this is the case. The cell type specificity of S-region-mediated recombination has been addressed in experiments that genetically assay the loss of a selectable marker from integrated retroviral constructs; in those experiments, recombination appeared to occur at a higher frequency in pre-B-cell lines than in a fibroblast line (23). However, the validity of these experiments depends largely on the untested assumption that only a single copy of the retroviral construct was integrated in the derivative cell lines assayed. Moreover, recent genetic knockout experiments show that regulatory sequences must function in *cis* to stimulate switch recombination in activated B lymphocytes (16, 42). Removal of the *cis*-activator sequences in homozygous knockouts essentially abolishes switching to a given isotype, despite the presence of *trans*-acting factors that carry out recombination. This finding suggests that the specificity of switching may depend on activation of locus-specific regulatory factors

rather than the presence or absence of recombination enzymes. The dramatic stimulation of switch substrate recombination by an adjacent enhancer element may be analogous to targeting of chromosomal switching by a locus-specific *cis*-activator sequence.

Our data show that recombinational stimulation in the switch substrates does require protein binding to the regulatory element, but recombination appears to be independent of transcript production. A temporal correlation between recombination and transcriptional activation has been documented both for isotypes switching and for V(D)J joining in mammalian lymphocytes, and Alt and coworkers have suggested that transcription could regulate recombination by rendering a region of DNA accessible to factors or lesions that promote recombination (3, 5, 21, 39). However, there is considerable recent data suggesting that transcript production and recombination may not be mechanistically linked in mammalian cells. While culture with interleukin 4 induces sterile γ1 and ε transcription in B lymphocytes, a second signal is necessary for induction of switch recombination (24). Conversely, overproduction of the transcription factor E47 in a pre-T-cell line stimulates endogenous immunoglobulin D-J joining but not sterile transcription of the D-J region (26); and μ heavy-chain expression in a pre-B-cell line stimulates κ light-chain rearrangement independent of increased germ line κ transcription (28). These results are consistent with observations that V(D)J recombination in extrachromosomal substrates is independent of transcription through the recombination zone (14). Homologous recombination between extrachromosomal substrates in mouse L cells similarly appears to be independent of transcription through the recombining regions (40). In *S. cerevisiae*, in which recombinational activation has been studied in the greatest genetic detail, the HOT1 element has been shown to be identical with the rRNA promoter (31), but while both the *ARG4* and *HIS4* recombinational hotspots in *S. cerevisiae* map very near promoters, there appears to be no correlation between transcription and recombination at either of these loci (27, 33).

In the extrachromosomal switch substrates, factor binding but not transcription stimulates recombination. If there is no mechanistic link between recombination and transcription, why might transcription frequently correlate with recombinational activation at the chromosomal immunoglobulin loci? One possibility is that since transcription and recombination both involve alterations in chromatin structure and DNA architecture, transcriptional activation can occur as a by-product of recombinational activation. The prokaryotic integration host factor has been shown to act as either a recombinational or transcriptional activator (reviewed in reference 9), and both the prokaryotic transcription factor catabolite gene activator protein and the eukaryotic factor LEF-1 can substitute for integration host factor in λ Int-promoted recombination in vitro (10, 11). This functional interchangeability appears to also characterize some of the transcription factors that regulate the yeast *HIS4* recombination hotspot (33), and it may be a widespread property of proteins that regulate transcription or recombination.

The position of the regulatory element in the switch substrates affected the selection of recombination endpoints, and binding of protein factors therefore appears to target as well as stimulate recombination. This may have implications for targeting of chromosomal switch recombination. Our laboratory has identified an inducible DNA-binding and -bending protein, LR1, with multiple binding sites in each of the S regions (34–36). Others (20, 32, 37, 38) have identified sites within the S regions for other factors, including another B-cell-specific factor, BSAP (1, 4), distinct from LR1 in both molecular weight and recognition sequence (34, 35). This finding raises the possibility that binding site selection by regulatory factors could explain the apparently random distribution of switch junctions.

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