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Strand specificity in the transcriptional targeting of recombination at immunoglobulin switch sequences

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ABSTRACT **B-lymphocyte-specific class switch recombination is known to occur between pairs of 2- to 10-kb switch regions located immediately upstream of the immunoglobulin constant heavy-chain genes. Others have shown that the recombination is temporally correlated with the induction of transcription at the targeted switch regions. To determine whether this temporal correlation is due to a mechanistic linkage, we have developed an extrachromosomal recombination assay that closely recapitulates DNA deletional class switch recombination. In this assay, the rate of recombination is measured between 24 and 48 hr posttransfection. We find that recombinants are generated in a switch sequence-dependent manner. Recombination occurs with a predominance within B-cell lines representative of the mature B-cell stage and within a subset of pre-B-cell lines. Transcription stimulates the switch sequence-dependent recombination. Importantly, transcription activates recombination only when directed in the physiologic orientation but has no effect when directed in the nonphysiologic orientation.**

Immunoglobulin class switch recombination takes place in the peripheral lymphoid tissues and replaces the initial heavy-chain constant regions μ and δ with any of several downstream constant regions (1, 2). Immediately upstream of the constant domain exons are 2- to 10-kb switch (S) regions, each of which has a different repeated sequence within it. For the immunoglobulin S_μ region (called the donor S region), the repeat length is 20 bp, whereas for the downstream S regions (acceptor S regions), the repeat length is 40–80 bp. A given rearrangement reaction appears to be directed by two S regions (S_μ and any one of the acceptor S regions). Only 50–60% of the recombination points at the donor sequences (μ) are actually within the tandemly repetitive S_μ region (3). Of the remainder, about half are within a few kilobases upstream, and the remainder are downstream. Recombination points are usually within the tandemly repetitive acceptor S regions.

It is unclear how S regions are differentially targeted for recombination (4, 5). Prior to recombination to a given S region, intron promoters located immediately upstream of the S region sequences (called the upstream activator region) are activated, generating sterile transcripts (designated I_μ transcripts in the case of μ). The temporal correlation between transcriptional and recombinational activation raises the possibility that transcription of S regions either modulates DNA accessibility or is an indicator of locus accessibility (6, 7).

Several studies have examined the importance of the transcriptional control region and the sterile transcript promoter upstream of the S regions (8–10). In one of these (9), the heavy-chain gene enhancer (E_μ)/heavy-chain variable region promoter was inserted in place of the endogenous control region for S_ε. These cells were found to undergo constitutive switch recombination, although at a somewhat lower efficiency. Similar results were obtained with the same promoter at I_ε in a gene replacement system (11). Jung *et al.* (8) did the reciprocal experiment by completely removing the upstream control region but leaving $S_\gamma 1$ intact. The absence of this region greatly diminished recombination. Similar results were obtained for $S_\gamma 2b$ (10). These studies support a positive regulatory role for the upstream control regions but do not dissect out the mechanistic role of transcription from the I exon or other functions of these regulatory regions. In all three cases, enhancers or other implied control elements were also manipulated. It remains a possibility that transcription either has a causal role in recombination or is a temporally related, but causally unrelated, consequence of locus opening.

Previous research has utilized exogenous substrates to study class switch recombination (12–14). Using both endogenous control regions and heterologous promoter/enhancers (12), the data were consistent with a causal link between transcriptional regulatory elements and recombination, although without directly linking recombination to transcriptional initiation and elongation. In fact, subsequent use of these recombination substrates reported that an enhancer component of the upstream activating region is important for recombination and not transcription itself (13). To ask specific mechanistic questions, we have recapitulated switch recombination on minichromosome substrates. Our studies have found a causal link between transcription and recombination and suggest that the transcript targets recombination to S region sequences.

MATERIALS AND METHODS

Cell Lines, Transfection, and Plasmids. Cells were grown as described and transfected by the hypotonic DEAE-dextran method (15), except for M12 and S194, which were transfected by electroporation. Electroporation was performed with a Bio-Rad gene pulser set at 0.25 V, 960 μF, 200 Ω, and a 0.4-cm cuvette containing 1×10^7 cells plus 10 μg of DNA in 0.3 ml of complete medium at room temperature.

A 1.2-kb *Hin*dIII fragment originating from pJ14 (16) and containing $\approx$0.9 kb of repetitive DNA was used as the source of S_μ in the cellular assay system. $S_\gamma 3$ was subcloned from the vector pγ3BgH2.5 (17) as a 2.2-kb *Sac* I/*Hin*dIII fragment. The cellular assay vector backbone was constructed by using the mouse polyoma large T and origin on a 3.6-kb *Bam*HI/*Hin*cII fragment inserted into pBR322 (18). An oligonucleotide fragment containing sequences of the gastrin transcription terminator (19) was cloned into the *Eco*RV site of pBR322 at position 185. A 1.5-kb *Mlu* I fragment from pBGS8 (20) conferring kanamycin resistance was inserted into an engineered *Bgl* II site downstream of the gastrin terminator. A *Sma* I fragment containing the simian virus 40/thymidine kinase (SV/*tk*) promoter (21) was placed into the *Nhe* I/*Nde* I site to create pGD117. The *supF* tRNA gene from pS189 (22) was inserted downstream of the SV/*tk* promoter into a *Bgl* II site. The major immediate-early human cytomegalovirus (hCMV)

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Abbreviations: S region, switch region; hCMV, human cytomegalovirus.

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promoter was inserted into a *Bam*HI site to create the backbone for the switch sequence and control plasmids. A partial *Eco*RI digest to remove most of the SV/*tk* promoter was done to create pGD255. The 300-bp λ phage transcriptional terminator *oop* was positioned upstream of the CMV promoter in pGD275.

Other DNA fragments include a 1.1-kb (positions 3128–4206) *Hae* III fragment used as prokaryotic control fragment P1 and a 1.0-kb (positions 4948–434) *Hae* III fragment used as prokaryotic control fragment P2 from the bacteriophage φX174. A 1.0-kb *Dra* I fragment from the RAG1 cDNA and a 1.4-kb *Bst*XI/*Eco*RV fragment from the RAG2 cDNA were used as eukaryotic control fragments E1 and E2 (23, 24).

Recombination Assay. Recombination substrates (Fig. 1) were transfected into eukaryotic cells using either hypotonic DEAE-dextran (15) or electroporation. The transfected cells were plated into 100-mm dishes at a density of 3×10^5 cells per ml in complete medium. Covalently closed circular DNA molecules were recovered by alkaline lysis at the indicated times (25) and 10-fold digested with *Dpn* I (New England Biolabs) to recover only those molecules that have entered the nucleus and undergone at least one round of replication (15). The digested DNA was electroporated into strain MLB7070 (22) containing an amber mutation in the β-galactosidase gene. The transformed bacteria were plated onto 5-bromo-4-chloro-3-indolyl β-D-galactoside/isopropyl β-D-thiogalactopyranoside (United States Biochemical) LB plates containing either ampicillin (100 μg/ml) or ampicillin and kanamycin (25 μg/ml). The substrate molecules give rise to blue colonies while any recombinant molecules that have deleted *supF* are white. The ratio of white colonies to total colonies gives percentage recombination for that substrate. Recombination values (*R*) are reported as the percentage of recombination per hr from 24 to 48 hr posttransfection. *R* values are given for recombinants selected on ampicillin- or ampicillin/kanamycin-containing plates. The number of blue colonies arising from transformation of *Escherichia coli* by substrate is the same when either ampicillin or ampicillin/kanamycin plates were used. Each plasmid was transfected in at least three independent experiments with multiple replicates for each time point in each experiment, and the average *R* value is given ± SE.

RESULTS

Substrate and Assay Design. Because transcriptional activation has been associated with class switch recombination, we have bypassed tissue-specific regulation of these promoters by the use of constitutive promoters in a minichromosome substrate assay. These promoters are positioned immediately upstream of positions A and B, the sites where test sequences are inserted (Fig. 1). The SV/*tk* and hCMV promoters function well in a wide variety of cells and are active in each of the cell lines used in this study (data not shown). To facilitate the analysis of recombinants, a second prokaryotic selection marker was inserted upstream of the promoter for region A. Therefore, by selecting for recombinants that preserve both the β-lactamase- and kanamycin-resistance genes, we can readily observe a more restricted group of recombinants. The majority of these recombinants will have junctions lying within or close to regions A and B. Because class switch recombination in the genome also includes larger deletions that extend outside of the S regions, we were interested in using this assay system to examine a broader target region. Positive selection of recombinants with ampicillin alone allows identification of recombinants with junctions further upstream of S_μ. Transformation of switch sequence-bearing substrates, such as pGD244, directly into *E. coli* without passing the plasmid through eukaryotic cells results in a 0.01% (10^{-4}) or lower background of white colonies.

Recombination Is Switch Sequence Dependent and Time Dependent. S regions have been implicated as the cis-directing

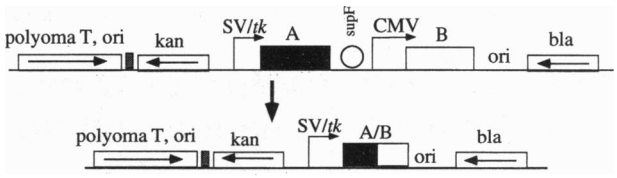

FIG. 1. Switch sequence minichromosome substrates. In the substrate (top line), the tRNA gene *supF* complements an amber mutation in the *E. coli* strain MLB7070 giving rise to blue colonies on 5-bromo-4-chloro-3-indolyl β-D-galactoside plates. Switch or control sequences were placed in regions A and B, which flank *supF*. The substrates were transfected into murine hematopoietic cell lines and incubated for specified times, and the plasmid DNA was harvested and transformed into *E. coli* for genetic assay of product (bottom line). Recombination is scored by the deletion of *supF* and is reported as the change in ratio of replicated recombinant product molecules (which give rise to white colonies) to total replicated molecules (blue and white colonies) from 24 to 48 hr posttransfection. All values are reported as the average slope (±SE) of at least three independent transfections. The *kan* and *bla* segments are the kanamycin- and ampicillin-resistance genes, respectively. The polyoma large T antigen and polyoma origin (polyoma T, ori) allow replication of the substrate in murine cells. Between the polyoma large T and the kanamycin gene is the gastrin transcriptional terminator (solid rectangle). SV/*tk* and hCMV are constitutive eukaryotic promoters oriented. Ori is the prokaryotic origin of pBR322. Arrows represent direction of transcription.

sequences responsible for class switching. We compared the recombination frequency of switch DNA with nonswitch DNA in Abelson murine leukemia virus immortalized pre-B-cell line 18-81 and in mature B-cell line Bal17. Although class switch recombination is thought to be restricted to mature B cells *in vivo*, previous work has shown that some pre-B cells actively recombine their endogenous S regions (5, 26–28). Recombination of the minichromosome substrates is >50-fold greater for switch DNA than for segments of either prokaryotic or eukaryotic DNA in both cell lines (Fig. 2 and Fig. 3, lines 1–3). The slope is linear from 24 to 48 hr and represents the rate of recombination (calculated as the change in percentage of recombined molecules per hr). The recombinants selected with ampicillin/kanamycin are a subset of recombinants selected on ampicillin alone. The former have junctions that fall either within or up to 1 kb upstream of S_μ. Comparing the levels of recombination under the two selections gives an

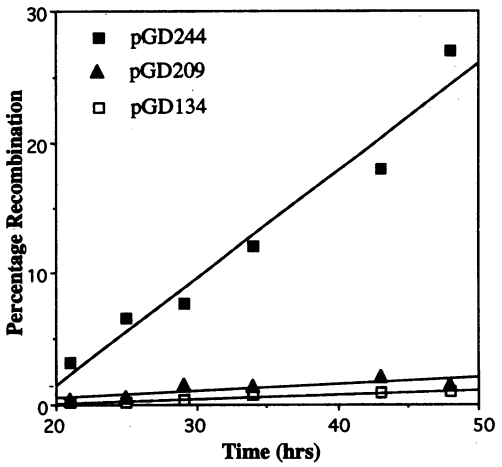

FIG. 2. Time course of recombination. Duplicate transfection points for three recombination substrates are plotted for pre-B-cell line 1-8 21–48 hr after introduction into the cells. pGD134 has two different pieces of prokaryotic DNA inserted into regions A and B, and pGD209 contains two different pieces of eukaryotic DNA fragments. pGD244 contains S_μ in region A and $S_\gamma 3$ in region B. Only the restricted target zone is shown; time course for the broad target zone is similar but absolute levels of recombination are higher at each point.

Table 1. Recombination of a substrate bearing class switch recombination sequences

Substrate	ApKn^r [white/(blue + white)], % 24 hr	ApKn^r [white/(blue + white)], % 48 hr	R-ApKn, % change per hr
pGD134	0/85,500 = 0	3000/675,000 = 0.4	0.02
pGD209	150/93,150 = 0.2	1200/571,200 = 0.2	0.002
pGD244	1950/36,375 =5.4	20,400/48,150 = 42.4	1.54

Specified substrates were transfected into murine mature B-cell line Bal17 and then analyzed by recombination assay. Numerator of each ratio is the total number (pooled replicates) of white ampicillin/kanamycin double-resistant transformant colonies. Denominator is total of white and blue ampicillin/kanamycin double-resistant transformant colonies. R-ApKn is calculated as percentage corresponding to this ratio at 48 hr minus the same percentage at 24 hr, and this quantity is divided by 24 hr to yield percentage change per hr.

indication of the propensity for recombination to occur near S_μ or other sequences in region A on the substrate. A representative comparison of switch sequence substrates with control substrates at two time points illustrates that there is not only an increase in recombinants relative to substrate but also an increase in the absolute number of recombinants over time (<2000 at 24 hr increasing to >20,000 at 48 hr) (Table 1). Recombinant products do not undergo a greater number of rounds of DNA replication than switch recombination substrates (data not shown) as determined by monitoring the prokaryotic *dam* methylation pattern on the plasmids (29, 30).

Switch Recombination Is Enhanced in the B-Cell Lineage. It is not known which components of class switch recombination make this process cell lineage specific and developmentally regulated. To address this issue, the recombination activity of the S_μ- and $S_\gamma 3$-containing substrates was analyzed in a variety of hematopoietic cell lines (Table 2). We find that cell lines representing the mature B stage of development generally show the highest activity for recombination. This is the case for both the narrow and the broad recombination target zone (only the former is shown in Table 2). In addition, two pre-B-cell lines, but not three others, have relatively high activity. One of the pre-B-cell lines (18-81) has been found to be positive for recombination in a retroviral substrate assay (14). All other cell types had relatively low recombination values. Most notably, six mature T-cell lines had values lower than the lowest mature B-cell line (Table 2). The low level of recombination present in most nonmature B-cell lines is either an inherent background level of the switch substrates in the assay or illustrates a low level of switch recombination activity, perhaps used for other physiologic DNA metabolic processes.

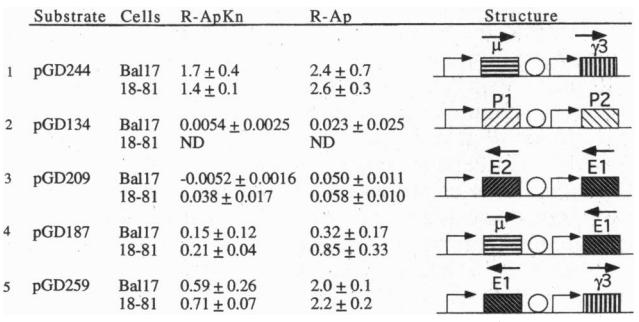

FIG. 3. Dependence of recombination on switch sequences. Plasmid pGD187 contains a single S region, S_μ, together with eukaryotic fragment E1. Likewise, pGD259 contains only $S_\gamma 3$ together with the same eukaryotic fragment E1. Arrows above DNA inserts illustrate direction of transcription for these regions in the genome. Circles represent *supF*. Arrows in front of each box show orientation of the eukaryotic promoters SV/*tk* and hCMV (see Fig. 1, Fig. 2, Table 2, and *Materials and Methods*). R-Ap and R-ApKn, *R* values for recombinants selected on ampicillin- and ampicillin/kanamycin-containing plates; ND, not determined.

Table 2. Cell line survey

Lineage and stage	Cell line	R-ApKn, % substrate converted to product per hr: Switch substrate pGD244	Control substrate pGD209
B-lymphoid			
Pre-B	18-81	**1.4 ± 0.1**	0.038 ± 0.017
	1-8	**0.88 ± 0.11**	0.019 ± 0.020
	PD31	0.052 ± 0.040	0.011 ± 0.018
	PD0	0.024 ± 0.018	0.012 ± 0.024
	S41	0.053 ± 0.10	0.020 ± 0.017
Mature B	Bal17	**1.7 ± 0.44**	−0.0052 ± 0.0016
	X16C	**1.2 ± 0.4**	0.053 ± 0.012
	2PK-3	**0.95 ± 0.28**	0.028 ± 0.014
	L10A6	**0.57 ± 0.17**	−0.0039 ± 0.0052
	M12	**0.32 ± 0.12**	0.0041 ± 0.0067
Plasma cell	S194	0.39 ± 0.19	0.0052 ± 0.0037
	MPC11	0.072 ± 0.032	0.031 ± 0.059
T-lymphoid			
Pre-T	2017	0.025 ± 0.13	0.0058 ± 0.0045
Mature T	EL4	0.11 ± 0.05	−0.023 ± 0.018
	WR19L	0.048 ± 0.025	−0.0012 ± 0.0080
	WEHI 7.1	0.017 ± 0.013	−0.016 ± 0.0056
	S49.1	0.018 ± 0.016	−0.0031 ± 0.0031
	R1.1	0.019 ± 0.20	0.0046 ± 0.0028
	TIMI.4	−0.014 ± 0.023	−0.014 ± 0.0010
Nonlymphoid			
Myeloblast	M1	0.0074 ± 0.21	0.14 ± 0.17
Monocyte	RAW309	−0.020 ± 0.043	0.048 ± 0.073
Mastocytoma	P815	0.056 ± 0.016	0.0042 ± 0.0017
Erythroid	F-MEL	0.079 ± 0.071	0.00029 ± 0.0073

Hematopoietic cell lines were assayed by using the episomal class switch recombination substrate pGD244 and the control substrate pGD209. For all cell lines, recombination for the period from 24 to 48 hr was found to be linear. At least three independent transfections were performed. Recombination values (% recombinant product per hr) are given ± SE for the narrow target zone (R-ApKn); the broad target zone gave values that were higher in absolute value but were similar when comparing lines relative to each other (data not shown). Measurements in boldface are mean values that are >2 SD above 0.05% recombinant product per hr. Cell lines were obtained from the American Type Culture Collection with the following exceptions: sources and features of cell lines 18-81, 1-8, 2017, F-MEL PD31, and PD0 are described in ref. 15; cell line S41 is described in ref. 31; and cell lines Bal17, X16C, L10A6, and M12 are described in ref. 32.

S Regions Can Act Independently in Recombination. Evidence consistent with activation of single S regions (independent of a pairwise interaction with a second S region) has been reported (33–35). To examine this issue in this system, substrates were constructed containing either S_μ (pGD187) or $S_\gamma 3$ alone (pGD259) combined with a segment of nonswitch DNA (Fig. 3, lines 4 and 5). Recombination was measured in pre-B-cell line 18-81 and in mature B-cell line Bal17. Both S regions independently show significant levels of recombination compared to the nonswitch sequence-containing substrates pGD134 and pGD209. However, in both mature B- and pre-B-cell lines, the level of recombination when both S_μ and $S_\gamma 3$ are present (pGD244) is greater than simply adding the rates of recombination for the individual regions. In addition, the spectrum of recombination is altered to favor deletions more limited to the S_μ region (recombination rates with ampicillin/kanamycin vs. ampicillin selections).

Upstream Promoter Elements Regulate Recombination of Switch Substrates. The heterologous promoters upstream of each S region were removed in order to study their effects on recombination. pGD255 is similar to pGD244 except for the

removal of the SV/*tk* promoter in front of S_μ (Fig. 4, line 4). Removal of this promoter had no effect on the level of recombination. Similarly, only a small effect (2-fold) is observed on recombination in the broad target zone (ampicillin) when the hCMV promoter upstream of $S_\gamma 3$ is removed (pGD247 in Fig. 4, line 3). In contrast, recombination within the restricted target zone decreases 12-fold (compared with pGD244) when the $S_\gamma 3$ promoter is removed. This drop is due to the effect of the hCMV promoter on $S_\gamma 3$ and not to the promoter itself being a hotspot recombination, pGD187 (Fig. 3, line 4). Using the substrate pGD189 as a baseline for recombination of S_μ, both the hCMV promoter (pGD187 in Fig. 3, line 4) and the $S_\gamma 3$ segment (pGD247 in Fig. 4, line 3) increase recombination individually; however, together the promoter and the correctly oriented S region generate the highest rate of recombination. Both $S_\gamma 3$ and the hCMV promoter individually have $\approx 1/10$th the values of when they are combined. The lack of effect seen upon removal of the SV/*tk* promoter upstream of S_μ may be due to either a lower dependence of S_μ on transcriptional activation or read-through transcription from the polyoma large T gene. Although these results suggest an association between transcription and recombination, we were interested in probing this association further.

Transcription Activates Recombination in a Strand-Specific Manner. The previous data indicate that recombination is dependent on S region sequences and stimulated by transcriptional promoters. We tested a series of substrates to address how the promoters activate recombination. If transcription is required simply for increased chromatin accessibility to the recombinase, we would expect pGD275 (Fig. 4, line 5) to recombine at rates comparable to pGD244 (line 1). Both substrates contain the same switch sequences and promoters but differ in positioning of the fragments. The substrate pGD275 retains the original physiologic orientation of the S regions to each other but changes transcription to the opposite strand. The results are quite clear. The rates of recombination are substantially lower for pGD275 compared to pGD244 (Fig. 4). Because pGD275 retains the same promoters and orientation of the S regions to one another, the low level of recombination eliminates "accessibility" as a possible role of transcription on our extrachromosomal substrates. The difference between pGD244 and pGD275 also points out that simple transcription of repetitive switch DNA is not sufficiently recombinagenic to account for the high rate of recombination seen in pGD244. Thus, S regions have a defined strand specificity for transcriptional activation of recombination.

	Substrate	R-ApKn	R-Ap	Structure
1	pGD244	1.7 ± 0.4	2.4 ± 0.7	μ γ3
2	pGD189	-0.0035 ± 0.013	0.048 ± 0.020	μ E1
3	pGD247	0.14 ± 0.010	1.1 ± 0.59	μ γ3
4	pGD255	1.8 ± 0.7	2.1 ± 0.41	μ γ3
5	pGD275	-0.020 ± 0.009	0.037 ± 0.032	γ3 μ
6	pGD262	0.46 ± 0.040	0.58 ± 0.050	E1 E1
7	pGD263	0.034 ± 0.035	0.087 ± 0.035	E1 E1
8	pGD264	0.52 ± 0.15	0.56 ± 0.19	E1 E1

FIG. 4. Effect of promoters and orientation on switch sequence recombination. Rate of recombination for substrates lacking promoters upstream of the S regions was determined for mature B-cell line Bal17. Fragments μ and γ3 are from S regions, while fragment E1 is a similarly sized nonswitch DNA fragment. Substrates lacking promoters (pGD189, pGD247, and pGD255) are indicated by the corresponding promoter arrow being left off the figure. Lines 6–8, rate of recombination was determined for mature B-cell line Bal17. For both pGD262 and pGD264, restriction analysis of recombinants showed that >90% were the result of homologous recombination between the two copies of E1 (data not shown).

Sequence inspection of the more usual S region-dependent recombination events shows no evidence for homologous recombination (3, 34, 36, 37). Nevertheless, the system here provides an opportunity for a direct test of this issue. Substrates pGD262 through pGD264 contain the same nonswitch DNA fragment in both regions A and B but in different orientations (Fig. 4, lines 6–8). Homologous recombination is the basis for the high deletion rate between directly repeated sequences in substrates pGD262 and pGD264. However, it is important to note that there is no transcriptional strand bias for the recombination reaction. Substrates pGD262 and pGD264 are inverted with respect to transcription but both recombine well in either orientation. This is in striking contrast to the switch DNA substrate pair pGD244 and pGD275. Thus, transcription in the physiologic orientation activates switch sequence-dependent recombination and uniquely distinguishes it from homologous recombination as well as other previously identified recombination pathways.

DISCUSSION

The temporal correlation between activation of transcription and recombination is strong. The same exogenous factors that stimulate transcription from the upstream activating regions also direct class switching to that isotype (38). Sterile S region transcription appears to be regulated at the level of both specific transcription factor production and chromatin accessibility around the sterile transcript promoter (39). However, whether this transcription is required for switch recombination has remained uncertain.

The recombination on the minichromosome substrates here correlates in many ways to class switch recombination in the genome. First, as in the genome, recombination on our substrates is switch sequence dependent. Although the exactly analogous experiment has never been performed in the genome, recombination on our substrates is >300-fold greater for switch DNA than for nonhomologous, nonswitch DNA. Second, endogenous class switch recombination is regulated in a lineage and developmental stage-specific manner. Recombination in the genome occurs predominantly at the mature B-cell stage, but it has also been found to occur earlier in some pre-B-cell lines (14). Recombination in this minichromosomal substrate assay is similarly restricted, showing a strong predominance at the mature B-cell stage but also in some pre-B-cell lines (Table 2). Third, isotype switching in the genome is strongly associated with transcriptional activation of a targeted S region. We find a dependence of recombination on transcription of at least one of the S regions. In addition, the transcriptional dependence is orientation specific. Transcriptional regulation of the mechanism of switch recombination would provide an additional level of lineage and developmental stage specificity because the sterile transcript promoters are active only in a lineage and developmental stage-specific fashion (6, 7).

Finally, in the genome, junctional endpoints involving S_μ are scattered over the entire S_μ region and occur to a significant extent both upstream and downstream of S_μ (3). In contrast, junctional endpoints for the endogenous γ3 acceptor S region fall almost exclusively in the repetitive region. As in the genome, recombinants characterized by this minichromosomal substrate assay do not exclusively map to S_μ. We have characterized 34 recombination junctions generated by this assay from pre-B-cell line 18-81 (data not shown). Approximately half of the recombinants have their 5′ endpoints in or within

1 kb upstream of S_μ. The fraction of recombinant junctions observed to occur upstream of S_μ in our substrates may be a consequence of using a fragment of S_μ that is 2.0 kb smaller than S_μ in the genome and of not having the I region protein binding sites present (40–43). On the other side of the junction, the majority of the sequenced recombinants (seven of eight) mapped to repetitive $S_\gamma 3$ sequences, as in the genomic recombination events. Interestingly, we find nucleotide substitutions at some recombinant junctions (data not shown). This feature has been observed for switch recombination events in the genome (3).

Due to the nature of the assay, nonswitch DNA will always have a background level of recombination that is not present in the genome. Events leading to white colonies potentially can occur at four steps in this type of assay: (*i*) the propagation of substrates in *E. coli*; (*ii*) the transfection of mammalian cells; (*iii*) propagation of substrates in mammalian cells; and (*iv*) the isolation and measurement of recombinants in *E. coli*. We can eliminate background problems at steps 1, 2, and 4 in our data by measuring the slope of recombination. Repair of damaged transfected molecules after 24 hr to generate white colonies does not significantly contribute to the rate of recombination because essentially all of the repair processes appear to be complete by 15 hr as measured by linear DNA transfection (unpublished data). To address background associated with step 3, segments of prokaryotic and eukaryotic DNA were studied as negative control substrates (Fig. 3 and Table 2). In addition, we inverted the S regions themselves, providing the ideal control (Fig. 4). Comparing the rate of recombination with these substrates to the level of recombination of the switch substrates gives the rate of correctly oriented switch sequence-dependent recombination. As shown in the results, the background level is very small compared to the recombination rate (Figs. 3 and 4 and Table 2).

Recombination on our substrates differs from recombination of the endogenous locus in an interesting way. Recombination in the genome is regulated by upstream control regions. These upstream control regions are not included in our substrates. Instead, we have used heterologous promoter elements upstream of each S region. These promoters appear to contain the necessary features of the upstream control regions. In addition, the lack of higher-order chromatin conformation of the substrates may eliminate the need for certain functions of the control region necessary for chromatin accessibility.

The unique orientation dependence seen in our assay suggests a role for the promoter and transcription rather than enhancer elements or changes in supercoiling. Both the effects of an enhancer and changes in supercoiling would be expected to be orientation independent. Precisely how transcription directs recombination is unclear. It is intriguing to speculate that an RNA·DNA structure similar to that observed upon cell-free transcription of S_α (44, 45) also directs the action of the recombinase. The orientation of transcription that leads to a stable cell-free RNA·DNA association in S_α (44, 45) is the physiologic one leading to recombination. The evidence for a direct role for the sterile RNA transcript has remained tentative for lack of a functional assay system and the unique nature of the sequence of S_α for which the model was proposed. Extrapolating from the data presented here, we propose that the RNA molecule itself is necessary but not sufficient to direct recombination. Upon stimulation of a B cell to undergo class switch recombination, transcriptional induction may induce structural changes via the RNA transcript that target particular S regions for recombination. Thus, locus targeting may, in large part, be the direct result of transcriptional activation, which provides a level of tissue and stage specificity due to the developmental regulation of sterile transcription of S regions. Therefore, transcriptional targeting combined with lineage and developmental stage predominance of the recombinase may dictate the relatively tight regulation of S region recombination observed in B lymphocytes.

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