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An element in the endogenous IgH locus stimulates gene targeting in hybridoma cells

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ABSTRACT

Gene targeting of the immunoglobulin (Ig) heavy chain locus is the basis of improved methods of investigating gene expression and of antibody engineering. The VH-Cμ intron is a convenient region for mediating homologous recombination events which result in production of Ig bearing an altered heavy chain. Also, this segment includes several elements which are important for gene expression, replication and isotype switching: in some cases it will be advantageous to alter these processes by modifying this intron. Considering that multiple targeting steps might be needed to accomplish all the requisite changes, it is important to know whether any of the anticipated modifications also alter the recombinogenicity of the IgH locus. To test this possibility we have measured the frequency at which a mutation in the Cμ3 exon of the endogenous μ gene is corrected by homologous recombination with a transfected segment of Cμ DNA. Comparison of recombination frequencies in several engineered hybridomas indicates that deletion of a 7.1 kb segment from the VH-Cμ intron depresses recombination by ~10-fold.

INTRODUCTION

It has long been observed that genetic recombination does not occur uniformly in the genome. Numerous elements of different types and from widely divergent organisms have been found to stimulate homologous recombination in nearby genetic intervals. In some cases the molecular basis of such differences in recombinogenicity are understood. Thus in *Escherichia coli* the *chi* sequence (GCTGGTGG) alters the activity of the RecBC(D) protein to reduce exonuclease and enhance helicase activities, leading to an increased frequency of homologous recombination in nearby intervals (1). The M26 site in *Schizosaccharomyces pombe* (ATGACGT) binds a specific heteromeric protein (2) and appears to be a preferred site of DNA cleavage (3). Homologous recombination is also stimulated in regions which are near an origin of replication, perhaps because the nicks or single-stranded segments which are associated with replication initiate recombination (4). Also, transcription has been seen to increase homologous recombination in yeast (5), in mammalian cells (6,7) and inconsistently in bacteria (reviewed in 8). The mechanism linking transcription and recombination is unknown, but it has been proposed that transcription might render the DNA more accessible to recombinases or that topoisomerase cleavages entrained by the unwinding associated with transcription might create structures which promote recombination.

Gene targeting of the immunoglobulin loci has been studied intensely, both because this technique permits highly controlled analysis of gene function and because modified immunoglobulins have diverse practical and medical applications. The immunoglobulin heavy chain (IgH) locus contains several elements which function in transcription, replication and DNA rearrangement. In constructing recombinant hybridoma cells to analyse the role in gene expression of elements lying in the intron between the VH and Cμ exons it became important to know whether these elements also affect the efficiency of gene targeting of the IgH locus. Here we report that deletion of a major part of this intron depresses recombination in an adjoining interval by ~10-fold.

MATERIALS AND METHODS

Tissue culture

The Sp6 hybridoma cell line and its normal (Sp6/HL) and mutant (igm482 and igm692) subclones have been described (9), as have the derivation of the recombinants 692R1 from igm 692 and 482R1 from igm482 (10).

Construction of plasmids and targeted recombinants

Figure 1 shows construction of the vector pIΔCμ482, which bears the frameshift mutation in Cμ3 and the 7.1 kb intron deletion and was used to generate the I^+ and IΔ recombinant hybridomas. An analogous vector with a normal Cμ3 exon was used to construct the ER50 cell line (A.E.Oancea and M.J.Shulman, manuscript in preparation). The pCμ(*Acc*I) plasmid was prepared by inserting the 2.4 kb *Acc*I fragment bearing the Cμ exons into pTZ18. To control for efficiency of transfection we measured the frequency of puromycin-resistant transformants conferred by pPur/sqs, a vector derived from pBABEDpCMVpuro by A.Cochrane.

Recipient cell lines were grown to a density of 2×10^5 cells/ml. A sample of 3.6×10^7 cells were electroporated as described (11). After DNA transfection cells were grown in normal medium for

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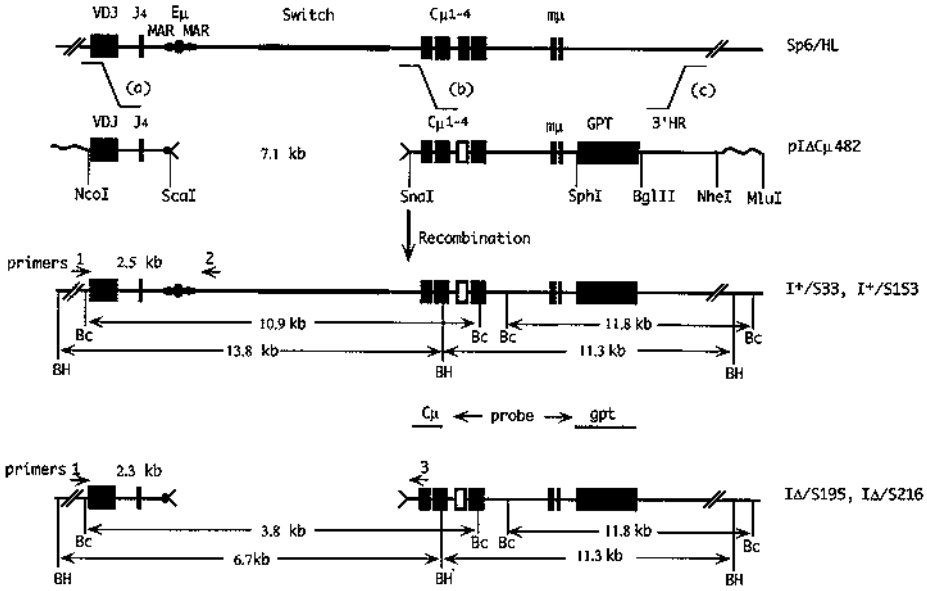

Figure 1. Construction of cell lines with the altered μ gene for measuring recombination frequency. The top line shows the structure of the μ gene of the hybridoma cell line Sp6/HL. The second line indicates the the segments in the vector, IΔCμ482, shown after linearization at the unique *Mlu*I site. Cross-overs in intervals (b) and (c) yield *gpt*$^+$ recombinants with a normal intron and the 2 bp deletion in the Cμ3 exon. Cross-overs in intervals (a) and (c) yield *gpt*$^+$ recombinants with a truncated intron and the 2 bp deletion in the Cμ3 exon. The sizes of the expected DNA fragments after digestion of genomic DNA with *Bam*HI (BH) and *Bcl*I (Bc) enzymes are indicated. The location of the 5′-most *Bam*HI site lies outside the sequenced region of the hybridoma IgH locus. The 6.7 kb value indicated for the IΔ recombinants corresponds to the actual measurement (see Fig. 3). Considering that the deletion in these recombinants extends for 7.1 kb, the results for the IΔ cell lines then predict 13.8 kb for the I$^+$ recombinants. The probes were the 870 bp *Xba*I–*Bam*HI Cμ1-2 fragment and the 1960 bp *Sph*I–*Hpa*I *gpt* fragment. The arrows show the positions of primers 1–3.

2 days in bulk culture and then distributed in 96 well plates in MHX medium for *gpt*$^+$ selection (12) and in medium containing 10 μg/ml puromycin for *pur*$^+$ selection at the cell concentrations indicated in Results. ELISAs specific for the Cμ1 and Cμ4 domains (13) were used to analyze supernatants from *gpt*$^+$-positive clones for selection of targeted recombinants.

Measurement of recombination frequency

To measure the frequency of recombination a mixture of 70 μg plasmid pCμ(*Acc*I) DNA and 2 μg pPur/sqs was transfected into 4×10^7 cells, grown as indicated for construction of targeted recombinants. Cells were returned to normal medium for 2 days, after which they were plated at limiting dilution in selective medium containing 10 μg/ml puromycin to assess transfection efficiency. On day 7, as well as several weeks thereafter, the transfected cells were plated to measure plaque-forming cells (PFC), and thus recombinants, as described (14).

Analysis of DNA structure

PCR analysis was performed using *Taq* DNA polymerase (Boehringer Mannheim) according to the following protocol for 30 cycles: denaturation, 1 min at 94°C; re-annealing, 2 min at 65°C; extension, 3 min at 72°C, which was increased by 3 s/cycle. Oligonucleotide primers: 1, 5′-TTACCTGGGTCTATGGCAGT-3′; 2, 5′-GTCACTGTAAATGCTTCGGG-3′; 3, 5′-GGGCACATGCAGATCTCTGTTTTTGC-3′ (3).

Genomic DNA for Southern blot analyses was prepared by the SDS/proteinase K method (15). DNA (7 μg) digested with *Bam*HI or *Bcl*I was electrophoresed in 0.8% agarose, transferred to Hybond N membrane (Amersham) and hybridized with the ^{32}P-probes radiolabeled by random priming.

RESULTS

The system which we used to measure recombination frequency is based on the hybridoma Sp6, which secretes IgM(κ) specific for trinitrophenyl (TNP) and forms plaques on TNP-coupled erythrocytes (14). This cell line bears a single copy of the μ heavy chain gene (16). To prepare cell lines with a convenient genetic marker for measuring recombination frequency we introduced a 2 bp deletion into the Cμ3 exon of the endogenous μ gene of the hybridoma cell line, thus causing production of a truncated μ heavy chain (Fig. 1). Because the resulting IgM is non-cytolytic, the recombinant hybridomas do not form plaques. Recombination between a transfected normal Cμ segment and the mutant endogenous μ gene can restore normal IgM production (Fig. 2). Our previous analyses indicate that most if not all PFC arise by homologous recombination (11,14), so recombination frequency can be measured by assaying the number of PFC. To assess the importance of the VH-Cμ intron segment we constructed cell lines in which the 2 bp deletion was *in cis* with either a normal or a truncated intron and then measured the frequency of PFC which arise after transfection of the normal Cμ segment, as described below. By using a Cμ segment which lies entirely outside the intron deletion, each cell line will present the same target for homologous recombination and any differences in recombination can then be ascribed to differences in recombination efficiency.

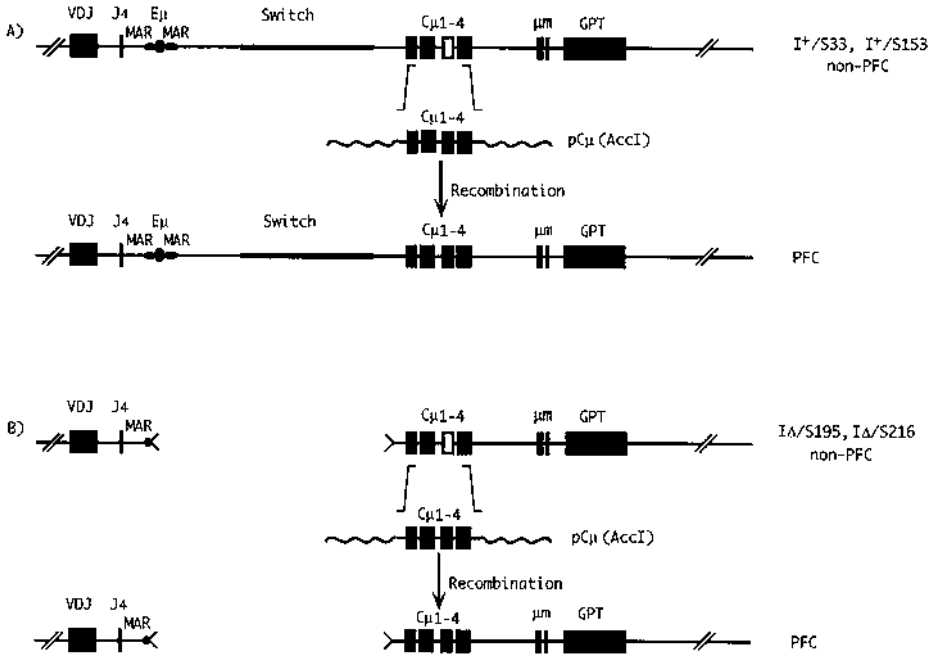

Figure 2. Assay for recombination frequency. This diagram depicts recombination between the transferred Cμ fragment with hybridomas bearing the normal and truncated introns.

Construction of materials for measuring recombination

To construct the modified hybridomas for measuring recombination frequency we used the vector pIΔCμ482, which lacks a 7.1 kb segment of the VH-Cμ intron (Fig. 1). Transfection of this vector into Sp6/HL or related hybridomas is expected to generate two types of homologous recombinants. Thus cross-overs at (a) and (c) will yield a recombinant with the 7.1 kb deletion (designated IΔ recombinants); cross-overs at (b) and (c) will generate recombinants with a normal intron (designated I^+ recombinants). The vector pIΔCμ482 was digested with *Mlu*I and transfected into Sp6/HL cells and into the Sp6-derived mutant cell line igm692, which lacks the Cμ1 and Cμ2 exons and part of Sμ. After incubating the cells for 2 days in normal medium we selected gpt^+ transformants by plating the cells at limiting dilution in MHX medium. Using ELISAs specific for either the Cμ1 or Cμ4 domain we then distinguished transformants bearing randomly inserted vectors ($C\mu4^+$ transformants) from those which had targeted the IgH locus ($C\mu1^+$ and $C\mu4^-$ transformants). In summary, 35 of 164 gpt^+ transformants derived from Sp6/HL and eight of 240 gpt^+ transformants of igm692 produced IgM lacking the Cμ4 domain. To establish whether the cross-over in the VH-Cμ intron of the Sp6/HL transformants occurred 5′ or 3′ of the deletion we measured the size of the fragments amplified using the indicated primers. Thirteen of the gpt^+, $C\mu4^-$ transformants yielded a 2.5 kb segment with primers 1 and 2, implying that the cross-over occurred 3′ of the 7.1 kb deletion in interval (b); 15 transformants yielded a 2.3 kb segment with primers 1 and 3, indicating a cross-over 5′ of the deletion in interval (a). We further tested that the vector had inserted by homologous recombination by measuring the size of the indicated junction fragments. Figure 3 shows results for several transformants: I^+/S33, I^+/S153, IΔ/S195 and IΔ/S216 derived from Sp6/HL and IΔ/6-42 derived from igm692. Thus using the Cμ1-2 probe we found that transformants I^+/S33 and I^+/S153 yielded the ~13.8 kb *Bam*HI and ~10.8 kb *Bcl*I bands expected for recombinants with an intact intron, while transformants IΔ/S195 and IΔ/S216 and IΔ/6-42 yielded the corresponding ~6.7 kb and ~3.8 kb bands expected for recombinants with the truncated intron. Probing with *gpt* to test the 3′ junction indicated that all the selected cell lines had the 11.3 kb *Bam*HI and 11.8 kb *Bcl*I bands expected for properly targeted recombinants.

To prepare DNA for transfection into these cell lines the 2.4 kb *Acc*I Cμ segment was inserted in pTZ18. As illustrated in Figure 2, the intron deletions do not overlap this segment, so the target for homologous recombination is the same in all cell lines.

Preliminary experiments: measurement of plaquing and transfection efficiency

There are two potential problems which could confound the relationship between the frequency of PFC and frequency of recombination. First, the intron deletion might affect the efficiency of plaque formation. To test this possibility we measured the plaquing efficiency of various $C\mu3^+$ recombinant cell lines which were constructed as described above so that the VH-Cμ intron was either normal or lacked the same 7.1 kb segment deleted from the IΔ cell lines (Fig. 4). Thus 482R1, derived from the igm482 mutant hybridoma, has an intact intron (10) and ER50, derived from igm692, lacks the 7.1 kb segment of the VH-Cμ intron (A.Oancea and M.J.Shulman, manuscript in preparation). We also analyzed the recombinant 692R1, which is derived from the igm692 hybridoma and bears a 2.8 kb deletion of part of the Sμ region, a deletion which does not affect μ expression (17). Table 1 presents the results of plaquing these cell lines on four occasions using independently prepared assay

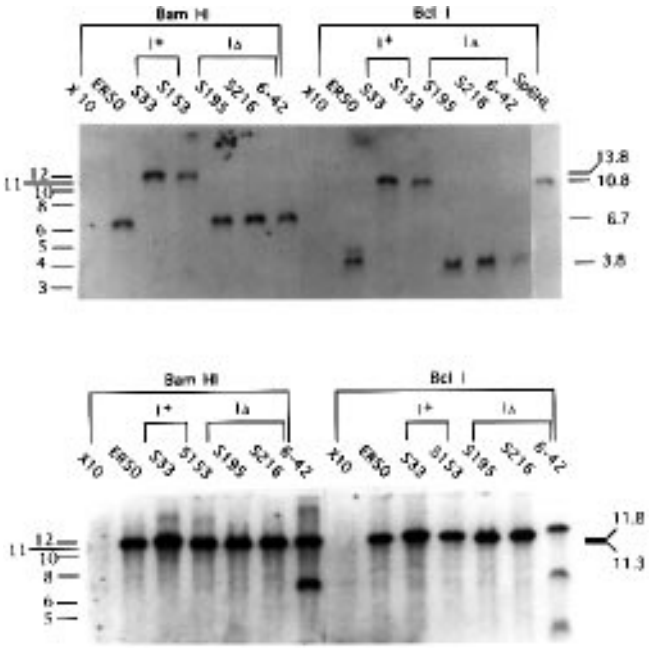

Figure 3. Analysis of DNA structure. DNA (7 µg) from the recombinant cell lines was digested with *Bcl*I or *Bam*HI, as indicated, fractionated by electrophoresis and probed with Cµ1-2 (upper panel) and *gpt* (lower panel) probes (described in Fig. 1). The cell line X10, which lacks the µ gene as well as the *gpt* gene, was included as a negative control. The position of marker bands is indicated to the left of the blots. The size of the predicted bands (Fig. 1) is shown on the right. The extra band seen in the *gpt* probing of the IΔ/6-42 transformant reflects the fact that the subclone of igm692 used as a recipient in these experiments had been previously transformed with pSV2neo, i.e. the *gpt* probe detects the pSV2neo DNA, presumably because of common sequences in the probe preparation. The extra band in ER50 represents partial digestion.

materials. These results indicate a moderate day-to-day variation and that the plaquing efficiencies of the intron-deleted (ER50) and other cell lines are in the ratio ~0.8 ± 0.2.

A second potential problem is that the efficiency of transfection, which depends on unknown aspects of the recipient cells, might vary and thus influence the frequency of recombination. To monitor transfection efficiency we included a small amount of the pPur/sqs plasmid, which confers resistance to puromycin. As indicated in Table 2, transformation frequency was approximately proportional to added DNA when between 0 and 9 µg pPur/sqs was included during electroporation under our normal assay conditions, i.e. in the presence of 70 µg pCµ(*Acc*I).

Table 1. Plaque-forming efficiency of I^+ and IΔ cell lines

Cell line	Plaque-forming efficiency (%)			
Sp6/HL	48	92	58	37
482R1	55	73	50	35
692R1	66	57	49	38
ER50	36	77	42	31

Two hundred cells of the indicated cell line were mixed with 3×10^6 cells of the non-cytolytic hybridoma igm482, as noted and tested for PFC, as described in Materials and Methods. This table presents results for PFC measurements on four typical occasions.

Measurement of PFC and recombination frequency

To measure whether the intron deletion affects recombination frequency two recombinant cell lines with a normal intron (I^+/S33 and I^+/S153) and three with a truncated intron (IΔ/S195, IΔ/S216 and IΔ/6-42) were transfected on one or more occasions with a normal Cµ segment [linearized vector pCµ(*Acc*I)] and pPur/sqs to monitor transfection efficiency. We then measured the frequency of PFC and of puromycin-resistant transformants (Table 3). As indicated in the table legend, the frequency of PFC was comparable whether measured 7 days or several weeks post-transfection. The frequencies of puromycin-resistant transformants imply that on any particular occasion the transfection efficiency for the I^+ and IΔ cell lines was approximately the same. However, the cell lines bearing the intact intron yielded on average 12-fold more PFC than did the cell lines with the truncated intron. When corrected for the lower (0.8) plating efficiency of hybridomas with the truncated intron, these results indicate that the intron deletion depresses the frequency of recombination in the adjoining Cµ interval by ~10-fold.

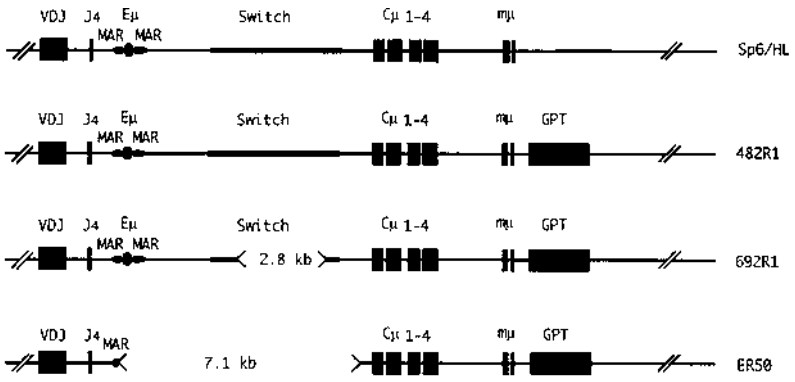

Figure 4. The structure of the µ genes in the recombinant hybridoma cell lines which were used to assay plaque-forming efficiency.

Table 2. Dependence of transformation (drug resistance) frequency on amount of added DNA

Cell lines	Amount (μg/ml) pPur/sqs vector	Frequency ($\times 10^{-3}$) of *pur*^{r} transformants
I^+/S33	0	<0.001
	0.36	0.03
	2.0	0.17
	9.0	0.42
I^+/S153	0	<0.001
	0.36	0.01
	2.0	0.06
	9.0	0.24

As described in Materials and Methods, 4×10^7 of the indicated recombinant hybridomas were transfected with 70μg pCμ (Acc1) DNA plus the indicated amount of pPur/sqs DNA. After incubation for 2 days in normal medium the transfected cells were plated at various cell concentrations in medium containing 10 μg/ml puromycin. The number of growth-positive wells was determined and the frequency of puromycin-resistant cells was then calculated according to the Poisson distribution.

Table 3. (A) Frequency of drug resistant transformants homologous recombination in I^+ and **I**Δ cell lines

Cell line	Frequency ($\times 10^{-3}$) of *pur*^{r} transformants	
	Experiment 1	Experiment 2
I^+/S33		0.3
I^+/S153	1.0	0.26
IΔ/S195		0.4
IΔ/S216	0.9	0.35
IΔ/6-42	1.7	0.28

(B) Frequency of plaque-forming cells

Cell line	Plaque-forming cells ($\times 10^{-7}$)				
	Experiment 1	Average 1	Experiment 2	Average 2	Average all
I^+/S33			(30) (28) (25)	27.7	21.0
I^+/S153	(13,12,11) (10,9) (13,15)	11.9	(25) (24) (21)	23.3	
IΔ/S195			(1) (1) (1)	1	1.8
IΔ/S216	(0, 0, 2) (2,1,1) (1,2,1)	1.3	(1) (1) (1)	1	
IΔ/6-42	(1,0,0,4) (4,5,4) (3,3)	3.0	(4) (2) (2)	2.7	

As described in the legend to Table 2, the indicated cell lines were transfected with a mixture of 70 μg pCμ(*Acc*I) DNA and 2 μg pPur/sqs DNA. The transfected cells were then incubated in normal medium for 2 days. (A) At this time an aliquot of the culture was plated at various cell concentrations in 10 μg/ml puromycin and the frequency of drug-resistant transformants was determined as indicated in Table 1. (B) On day 7 and several weeks thereafter aliquots of 3×10^6 cells from the transfected cultures were assayed for PFC, as described (14). This table lists the number of PFC observed on each plate and the results for each occasion that the cultures were assayed are grouped within parentheses. No PFC were observed in the absence of transfected DNA.

DISCUSSION

Genetic recombination depends on both enzymatic factors and *cis*-acting elements. Because the cell lines used to measure recombination bear only a single copy of the μ gene, the increased recombination which we observe to be associated with the intact VH-Cμ intron could in principle reflect an IgH-derived *trans*-acting factor, RNA or protein, as well as the action of a *cis*-acting element. However, only two transcripts have been detected for this region, that which yields mRNA for the μ heavy chain and the apparently non-coding Iμ transcript, which intiates in the vicinity of the Eμ enhancer (18). It is therefore unlikely that this region encodes a protein which promotes recombination.

As summarized in the Introduction, homologous recombination in other systems has been found to be stimulated by transcription and replication, as well as by seemingly recombination-specific elements. Neither the *chi* nor M26 sequences occur in the sequenced part of the deleted region. However, the 7.1 kb deletion examined here has removed other elements of interest. First, the deletion removed a segment which appears to function as a replication origin in B cell lines (19). Second, the deletion removed the switch region (Sμ), which is the preferred site for the rearrangements which cause isotype switching (20). The switch region also promotes rearrangements involving nearby non-switch DNA (21). Third, the deleted interval contains several elements which greatly stimulate transgene expression: the Eμ enhancer (22), the flanking matrix attachment regions (MARs) (23), Sμ and two other switch-associated elements, RegA and RegS (24,25).

It is still unclear whether these elements function in the recipient hybridoma cell line and, if so, whether their identified

activities contribute to the higher recombination frequency associated with the intact intron. Inasmuch as the Eμ-associated replication origin was observed in B cells but not in fibroblasts, we suppose that it functions in the I^+ recipient cell lines. Switch recombination in these hybridomas occurs very rarely, if at all (26,27), and studies with artificial switch substrates suggest that similar cell lines lack the switch recombinases (28). Nevertheless, the switch region in a closely related hybridoma cell line is a preferred site for insertion of transfected DNA (29). Inasmuch as normal switch recombination occurs in adjoining non-switch DNA, we consider it possible that the switch region might also stimulate homologous recombination in an adjoining interval.

The functional importance of the 'transcriptional' elements in the endogenous IgH locus is uncertain. Previous studies on myeloma and hybridoma cells imply that deletion of Eμ, the MARs and the switch-associated elements does not depress expression of the natural IgH locus in mature or pre-B cell lines (17,29–33). In the case of the particular hybridoma used in our present study the deletion depressed transcription ~4-fold, an effect which correlates with the loss of MAR content (A.E.Oancea and M.J.Shulman, manuscript in preparation). Further experiments will be needed to ascertain which, if any, of these defined elements contributes to recombination and, if so, whether their importance relates to a role in transcription or in some other process. It is also possible that the deletion did not remove a specific element which contributes to recombination. For example, the deletion might have changed the interval which separates the Cμ exons from an important stimulatory or inhibitory recombination element.

Our results also have practical implications for genetic engineering. In some cases it will be advantageous to construct recombinant loci in multiple steps. For example, it might be important that the modified loci lack the drug resistance genes commonly used to enrich for targeted recombinants. In principle, several methods using either homologous (hit-and-run or bait-and-switch) or site-specific (lox/Cre or frt/Flp) recombination can be used for this purpose in constructing recombinant immunoglobulin loci (10,16,34). Because recombination frequency can be affected by the changes introduced in a previous recombination step, the order of operations, as well as the structure of the intermediate recombinants, can be important for success.

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