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V(D)J recombination frequency is affected by the sequence interposed between a pair of recombination signals: sequence comparison reveals a putative recombinational enhancer element

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ABSTRACT

The immunoglobulin heavy chain intron enhancer (Eμ) not only stimulates transcription but also V(D)J recombination of chromosomally integrated recombination substrates. We aimed at reproducing this effect in recombination competent cells by transient transfection of extrachromosomal substrates. These we prepared by interposing between the recombination signal sequences (RSS) of the plasmid pBlueRec various fragments, including Eμ, possibly affecting V(D)J recombination. Our work shows that sequences inserted between RSS 23 and RSS 12, with distances from their proximal ends of 26 and 284 bp respectively, can markedly affect the frequency of V(D)J recombination. We report that the entire Eμ, the Eμ core as well as its flanking 5′ and 3′ matrix associated regions (5′ and 3′ MARs) upregulate V(D)J recombination while the downstream section of the 3′ MAR of Eμ does not. Also, prokaryotic sequences markedly suppress V(D)J recombination. This confirms previous results obtained with chromosomally integrated substrates, except for the finding that the full length 3′ MAR of Eμ stimulates V(D)J recombination in an episomal but not in a chromosomal context. The fact that other MARs do not share this activity suggests that the effect is not mediated through attachment of the recombination substrate to a nuclear matrix-associated recombination complex but through *cis*-activation. The presence of a 26 bp A–T-rich sequence motif in the 5′ and 3′ MARs of Eμ and in all of the other upregulating fragments investigated, leads us to propose that the motif represents a novel recombinational enhancer element distinct from those constituting the Eμ core.

INTRODUCTION

Antibody diversity is generated to a large extent by recombination of DNA segments termed V, D and J (1–3). Functional immunoglobulin genes are formed during this event. With reference to the DNA segments involved the process is known as V(D)J recombination. Much progress has been made towards the elucidation of the underlying basic mechanism (4–16). In contrast, little is known about its regulation (17–30).

It has been shown that immunoglobulin κ and μ chain intron enhancers not only stimulate transcription but also V(D)J recombination (20–23,27–29). Similar observations have been made with the λ light chain enhancer located 3′ of Cλ (31) and the T cell receptor α and β chain enhancers (32–34). The stimulation of V(D)J recombination by the immunoglobulin heavy chain intron enhancer (Eμ) can be dissociated from the enhancement of transcription (28) demonstrating that recombinational enhancement is not simply the result of an elevated transcription with consequent opening of the chromatin conformation. These studies have been performed in stably transfected cells or transgenic mice with chromosomally integrated recombination substrates containing variously mutated enhancers in *cis*. These systems do not lend themselves to easy manipulation and rapid screening of potentially regulating sequences. In the present work we have attempted to circumvent this problem by using a more convenient assay based on transient transfections of variously modified plasmid recombination substrates.

MATERIALS AND METHODS

Plasmid constructs

All plasmid constructs were based on the recombination substrate pBlueRec (35). This plasmid contains a *lacZ*(α) gene interrupted by a DNA fragment flanked by consensus recombination signal sequences (RSS) on both sides (Fig. 1).

Potentially regulating elements were inserted into the unique *Eco*RV site downstream of the RSS 23 by blunt end ligation (Fig. 1). After subcloning, the presence and orientation of the inserts were checked by restriction enzyme analysis. Lambda phage inserts were restriction fragments *Nar*I (corresponding to nucleotides 45 680–48 502 of the lambda genome, EMBL accession no. V00636) and *Hin*cII (nucleotides 28 928–31 809, accession no. V00636). MII (36; accession no. L23999) and E3 (37; an earlier incomplete version is deposited in the EMBL data

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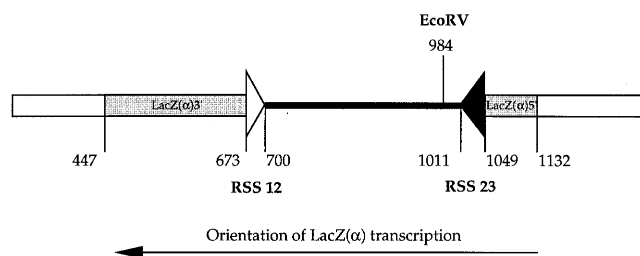

Figure 1. Map of the V(D)J recombination substrate, pBlueRec. Sequences to be tested for effects on the frequency of V(D)J recombination (Table 1) were inserted into the unique *Eco*RV site. Numbers refer to map positions as defined earlier (35). RSS, recombination signal sequence.

base under accession no. M60688, an updated version can be accessed in the data base of the National Center for Genome Resources http://www.ncgr.org/cgi-bin/ff?accloc=M60688) were cut from their vectors by *Eco*RI–*Bam*HI and *Eco*RI, respectively. BX2.95 (38) was obtained as a *Bam*HI–*Xba*I fragment (39). The *fushi tarazu* MAR insert (40) was an *Eco*RI fragment cut out of a Carnegie based construct carrying the *Drosophila fushi tarazu* gene. Eμ was cut from the corresponding OVEC construct by *Xba*I digestion (41,42; accession no. M12827). Subfragments of Eμ were obtained by digestion with *Xba*I + *Pst*I or with *Xba*I + *Hin*fI or with *Xba*I + *Eco*RI. The SV40 enhancer was cut from an OVEC construct by *Pvu*II + *Xho*I digestion (nucleotides 100–272, accession no. J02400). The CMV enhancer was cut from CMV-lacZ by *Sac*I + *Bam*HI (nucleotides 173 773–174 255, accession no. X17403). The 54 bp sequence of an established topoisomerase II cutting site originating from the human β globin gene (43) was subcloned in the same *Eco*RV site of pBlueRec by a PCR strategy with asymmetric primers.

Cell culture

Pre-B cell lines 38B9, 1-8 and 300-19 (44) were cultivated in HEPES buffered RPMI 1640 medium containing 2 mM Glutamax®, supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol (Gibco Life Technologies). Recombination-competent 31.7.12 cells (M12 lymphoma cells with stably integrated *RAG1* and *RAG2* genes driven by a heat-inducible promoter and a mutated *dhfr* gene allowing selection with the folate analogue methotrexate; 45) were grown in the above RPMI medium containing 100 mM methotrexate (Calbiochem). All cell lines were grown at 37°C in a 6% CO_2 atmosphere.

V(D)J recombination assay

The assay exploits the fact that the substrate contains an interrupted *lacZ*(α) gene, the reading frame of which is restored by V(D)J recombination (Fig. 1). Thus, upon transformation of *Escherichia coli* XL1 blue with the reaction products the recombination frequency, R, can be determined by dividing the number of blue colonies by the sum of blue and white colonies which grow on X-gal/IPTG agar plates.

Plasmid constructs were transiently transfected into recombination-competent cells by the DEAE–dextran method (46). Briefly, 3×10^6 viable cells were washed in PBS and incubated at 37°C in 0.5 ml of the following transfection mixture: 67% (v/v) RPMI 1640, 0.25 mg/ml DEAE–dextran (Sigma), 50 mM Tris–HCl pH 7.4 and 0.5–1 μg DNA. After 10 min, the cells were spun for 5 min at 300 *g* and resuspended in RPMI 1640, then spun again and finally resuspended in the above medium for a further 48 h at 37°C in a 6% CO_2 atmosphere.

Plasmid DNA was recovered from the cells by alkaline lysis, deproteinized by phenol–chloroform extraction and precipitated with isopropanol followed by centrifugation for 15 min in a refrigerated microcentrifuge. Pellets were washed in 80% ethanol, dried in open air and dissolved in 20 μl TE buffer (47). Aliquots of 10 μl were digested for 30 min at 37°C with 5 U *Dpn*I (New England Biolabs) in order to remove unreplicated plasmids that might not have entered the cells.

The digest was allowed to transform 200 μl of freshly thawed competent *E.coli* XL1 blue strain (Stratagene) for 1 h on ice. The transformation mixture was heat shocked for 40 s at 42°C, returned to ice and supplemented with 0.8 ml of cold SOC medium before incubation for 1 h at 37°C in a shaking incubator (48). The whole mixture was plated on LB-agar plates containing 100 μg/ml ampicillin (Sigma), 40 μg/ml X-gal and 50 μg/ml IPTG (Bachem Feinchemikalien) and incubated overnight at 37°C. Blue and white colonies were counted separately on a negatoscope and on a black screen, respectively.

Statistics

Duplicate transfections were done in parallel followed by transformation into XL1 blue. The resulting blue colonies from the duplicates were summed and divided by the total number of colonies to give a single r value. Independent transfection experiments (each consisting of duplicates) were performed on at least 3 separate days, each giving a separate r value. To arrive at an overall recombination frequency, R, the separate r values from independent transfection experiments were averaged and the standard deviation (SD) determined. Fold stimulation was calculated for each independent transfection experiment by dividing r experimental by r control, i.e. the r for pBlueRec obtained in parallel transfections. The individual fold stimulation values of all transfection experiments with a particular insert were then averaged and the SD determined. This normalization of R values allows comparison of different experiments (49).

In situ hybridization on colony lifts

While analyzing Eμ and its subfragments, it became apparent that the V(D)J recombination assay based on the counting of blue and white colonies could not be applied to the constructs carrying the *Xba*I–*Hin*fI and *Xba*I–*Pst*I subfragments in the genomic (+) orientation. With these subfragments (and only with these) colonies were difficult to identify as either white or blue owing to the presence of a high proportion of colonies with intermediate hue. When amplified by PCR, a significant number of them displayed a 500 bp band characteristic of non-recombined plasmids further compromising the β-galactosidase assay.

We therefore developed an alternative recombination assay based on *in situ* hybridization of colony lifts. A 29 bp oligonucleotide probe was designed which would hybridize to the coding joint of recombined plasmids but not to non-recombined substrates. The probe sequence (5′-AGAACTAGTGGAIIIIIIG-ACCTCGAGGG-3′) was complementary to the *lacZ*(α) sequence bracketing the coding joint and contained six interposed inosines allowing for hybridization even with processed joints. When checked on identified plasmids recovered from cell extracts and

Table 1. Sequences tested for effects on the frequency of V(D)J recombination in 38B9 cells

Identity of inserted sequence				Exp.	Experimental	Control	Fold stimulation [c]	
				repeats	R x 100 [b]	R x 100 [b]		
Origin	Sequence	Name [a]	Length		mean ± SD	mean ± SD	mean ± SD	p value [d]
Composite enhancer/MAR sequences								
mouse	IgH	Eμ +	992 bp	8	1.27 ± 0.69	0.34 ± 0.13	3.69 ± 1.34	p<0.01
	locus	Eμ –		9	0.61 ± 0.26	0.33 ± 0.12	1.93 ± 0.56	p<0.02
mouse	IgH	EμPXb+	610 bp	4	0.69 ± 0.37	0.30 ± 0.15	2.29 ± 0.23	p<0.05
	locus	EμPXb–		3	0.42 ± 0.18	0.35 ± 0.13	1.18 ± 0.27	
mouse	IgH	EμXbEI+	685 bp	7	0.97 ± 0.31	0.53 ± 0.14	2.09 ± 1.05	p<0.02
	locus	EμXbEI–		4	0.24 ± 0.18	0.45 ± 0.13	0.53 ± 0.35	
Enhancers								
mouse	IgH	EμHfHf+	221 bp	5	0.42 ± 0.24	0.37 ± 0.09	1.18 ± 0.72	
	core enh.	EμHfHf–		7	1.00 ± 0.58	0.40 ± 0.11	2.48 ± 1.30	p<0.05
SV40	enhancer	SV40enh+	173 bp	4	0.43 ± 0.09	0.64 ± 0.16	0.68 ± 0.17	
		SV40enh-		3	0.64 ± 0.05	0.68 ± 0.16	0.98 ± 0.15	
CMV	enhancer	CMVenh+	493 bp	4	0.24 ± 0.07	0.64 ± 0.16	0.40 ± 0.17	p<0.05
		CMVenh-		3	0.13 ± 0.04	0.68 ± 0.16	0.21 ± 0.08	p<0.05
MAR sequences								
human	topo I	MII +	2972 bp	8	2.77 ± 1.44	0.71 ± 0.39	3.96 ± 0.53	p<0.01
	gene	MII -		6	1.20 ± 0.72	0.77 ± 0.43	1.61 ± 0.30	
chicken	lysozyme	B1X1+	2950 bp	3	0.13 ± 0.04	0.23 ± 0.02	0.55 ± 0.10	p<0.05
	gene	B1X1-		3	0.03 ± 0.01	0.23 ± 0.02	0.12 ± 0.06	p<0.02
Drosophila	*fushi*	ftz3'MAR+	3200 bp	3	0.28 ± 0.36	0.44 ± 0.13	0.57 ± 0.66	
	tarazu	ftz3'MAR-		3	0.67 ± 0.44	0.44 ± 0.13	1.32 ± 0.77	
mouse	IgH	EμXbHf+	346 bp	3	2.93 ± 0.82[e]	1.05 ± 0.22[e]	2.75 ± 0.22	p<0.05
	locus	EμXbHf–		4	1.61 ± 0.68	0.48 ± 0.15	3.30 ± 1.13	p<0.05
mouse	IgH	EμXbP+	380 bp	3	2.57 ± 0.19[e]	1.24 ± 0.36[e]	2.24 ± 0.57	p<0.02
	locus	EμXbP–		4	0.38 ± 0.11	0.29 ± 0.07	1.35 ± 0.30	
mouse	IgH	EμHfXb+	424 bp	6	0.81 ± 0.36	0.48 ± 0.23	1.81 ± 0.44	p<0.02
	locus	EμHfXb–		3	0.53 ± 0.22	0.36 ± 0.10	1.45 ± 0.25	
mouse	IgH	EμEIXb+	307 bp	7	0.43 ± 0.20	0.46 ± 0.13	0.95 ± 0.45	
	locus	EμEIXb–		4	0.47 ± 0.11	0.49 ± 0.15	1.09 ± 0.22	
Non-MAR sequences								
human	topo I	E3 +	2655 bp	11	2.89 ± 1.76	0.79 ± 0.41	3.98 ± 2.36	p<0.01
	gene	E3 -		8	0.66 ± 0.49	0.90 ± 0.38	0.63 ± 0.31	
human	topo I	E3EIXh+	986 bp	3	0.35 ± 0.31	0.44 ± 0.13	0.81 ± 0.56	
	promoter	E3EIXh-		3	0.19 ± 0.09	0.44 ± 0.13	0.43 ± 0.13	
human	topo I	E3XhEI+	1669 bp	5	0.78 ± 0.38	0.62 ± 0.14	1.20 ± 0.38	
	coding	E3XhEI-		5	1.96 ± 0.76	0.62 ± 0.14	3.10 ± 0.81	p<0.02
human	topo II site	top2	60 bp	4	0.33 ± 0.11	0.62 ± 0.14	0.45 ± 0.09	p<0.01
Prokaryotic								
λ phage	NarI	λNarI +	2822 bp	7	0.41 ± 0.23	0.84 ± 0.39	0.53 ± 0.35	p<0.05
	fragment	λNarI –		5	0.13 ± 0.04	0.86 ± 0.45	0.18 ± 0.05	p<0.05
λ phage	HincII	λHincII +	2881 bp	7	0.17 ± 0.14	0.84 ± 0.39	0.23 ± 0.21	p<0.01
	fragment	λHincII –		5	0.10 ± 0.11	0.86 ± 0.45	0.13 ± 0.15	p<0.05

[a]Insert in genomic (+) or inverted (–) orientation relative to direction of *lacZ* transcription. IgH, immunoglobulin heavy chain; enh, enhancer; topo, topoisomerase; topo II site, topoisomerase II cutting site; Eμ, immunoglobulin heavy chain intron enhancer; P, *Pst*I; Xb, *Xba*I; EI, *Eco*RI; Hf, *Hin*fI; Xh, *Xho*I.
[b]Percent recombination frequency.
[c]Fold stimulation was calculated relative to control pBlueRec as described in Materials and Methods (Statistics). For statistical reasons, the ratio obtainable by dividing the mean of Experimental R × 100 by the mean of Control R × 100 would deviate from the correctly determined value of Fold stimulation as given in the table.
[d]p values identify entries where R Experimental differs significantly from R Control; calculation was by *t*-test.
[e]Values obtained by *in situ* hybridization on colony lifts.

amplified by PCR, this oligoprobe hybridized to 70–80% of the recombined plasmids (40 out of 52 analyzed).

To perform the assay, colony lifts were taken on Nylon A membranes (PALL, Biodyne), denatured for 5 min with 1.5 M NaCl/ 0.5 M NaOH, drained, and neutralized for 5 min with 1.5 M NaCl/0.5 M Tris (pH 7.4). Nucleic acids were crosslinked to the membrane by UV light, and proteins were removed by overnight digestion with 100 μg/ml proteinase K at 37°C. After prehybridization for 2 h at 60°C, membranes were hybridized overnight at 60°C with the ^{32}P-end-labelled oligonucleotide probe (6×10^6 c.p.m./ml). Washings were performed for 10 min in 2% SSC and for 25 min in 1% SSC/0.1% SDS at room temperature followed by a final wash in 0.1% SSC/0.1% SDS for 30 min at 50°C.

Computer search for sequence homology

The Wisconsin package GCG software was used. A search for homologous sequences among the upregulating fragments was performed with the programmes 'compare', 'dotplot' and 'bestfit' using standard settings. This yielded a 26 bp consensus motif, the occurrence of which was probed for in all other fragments using the command 'findpattern'. The entire EMBL and GenBank databases were then searched using the programme BLASTN.

RESULTS

The mouse heavy chain intron enhancer, Eμ, upregulates V(D)J recombination

Table 1 summarizes the recombination frequencies registered after transient transfection of the pre-B cell line 38B9 with various recombination substrates. The latter were obtained by inserting potentially regulating sequences and corresponding controls into the unique *Eco*RV site of the plasmid, pBlueRec (Fig. 1).

The composite sequence of the entire Eμ (*Xba*I–*Xba*I fragment; Fig. 2) stimulated V(D)J recombination ~4-fold when inserted in the genomic orientation and ~2-fold in the inverted orientation (Table 1). This compares well with a factor of ~5 determined in a more physiological context for a rearrangement-enhancing element upstream of the mouse immunoglobulin κ chain J cluster (50). In contrast, the SV40 enhancer had no effect, and the CMV enhancer even decreased the recombination frequency. It should be noted that in a chromosomal context the SV40 enhancer has been found to stimulate V(D)J recombination (23).

Upregulation by Eμ is conferred both by the core enhancer and by its flanking matrix associated regions

To delineate which parts of Eμ were responsible for the observed stimulation, we prepared various Eμ subfragments carrying either the core enhancer, the matrix associated regions (5′ MAR or 3′ MAR) or combinations thereof (Fig. 2). Increased recombination frequencies were observed both with the core enhancer and with fragments containing either the 5′ MAR, the intact (but not the truncated) 3′ MAR or composite sequences of either MAR with the core enhancer (Table 1 and Fig. 2). With the exception of the 5′ MAR this effect was clearly orientation dependent.

Our finding of an upregulating effect of the isolated core enhancer is in accord with the observation that a minimal version of Eμ is sufficient to target a chromosomally integrated substrate for V(D)J recombination (23). It also reflects earlier results showing that two small deletions in the Eμ core abolish V(D)J recombination in a transgenic substrate (28). Similarly, activation by the 5′ MAR agrees with a previous report showing that targeted disruption of the 5′ MAR in a chromosomal context dramatically decreased recombination of the linked J_H locus (21). Our observation that the truncated 3′ MAR (the fragment EμEIXb) does not affect the frequency of V(D)J recombination also agrees with the results of transgenic experiments (21).

In contrast, discrepant results were obtained with respect to the entire 3′ MAR, which stimulated V(D)J recombination in our system but not in a transgenic substrate (27,28).

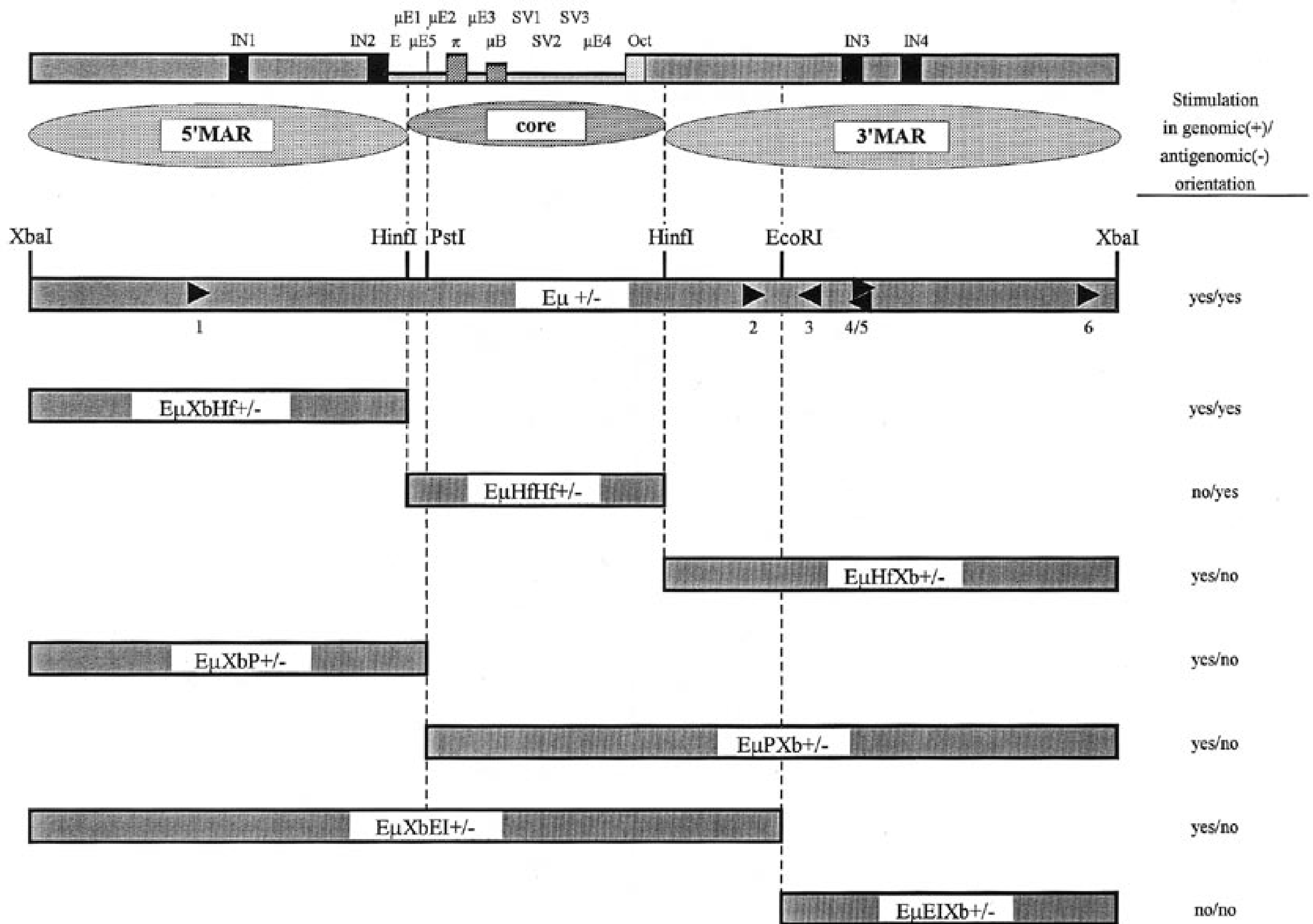

Figure 2. Eμ and its subfragments acting on recombination frequency. The top graph is a map of the known transcription elements and identifies the enhancer core with flanking 5′ and 3′ MARs (51). In Eμ, filled arrowheads with numbers indicate the positions and orientations of a 26 bp A–T-rich sequence motif (Fig. 3); orientation refers to the 5′→3′ direction in the plus and minus strand, respectively.

Matrix associated regions do not in general upregulate V(D)J recombination

We asked whether, in our system, upregulation of V(D)J recombination was a general property of MARs. To answer this question we tested for the effects of MAR sequences derived from sources other than Eμ. We chose MARs associated with the genes of human topoisomerase I, chicken lysozyme and *Drosophila fushi tarazu*. Recombination was significantly increased with the MAR (termed MII) from intron 13 of the human topoisomerase I gene, but only when it was inserted in genomic orientation (Table 1). The chicken lysozyme MAR even reduced the frequency of recombination, while the *Drosophila fushi tarazu* MAR remained without effect. Thus, MARs do not in general upregulate V(D)J recombination.

MARs often include clusters of topoisomerase II consensus sequences. One possible explanation for the discordant effects of the MARs tested might therefore be the presence or absence of such sequences particularly in view of a conceivable participation of topoisomerase II in V(D)J recombination. We therefore inserted an established human topoisomerase II cutting site (top 2) into the recombination substrate. As a result the recombination frequency was reduced to half (Table 1), thus ruling against the possibility that the presence of topoisomerase II cutting sites was responsible for the upregulation of V(D)J recombination by some MARs but not by others.

A non-MAR sequence, E3, upregulates V(D)J recombination

While investigating the influence of MARs on V(D)J recombination (see preceding paragraph) we used a control sequence (E3) of comparable length which had been proven to lack MAR activity (52). Interestingly, this sequence stimulated V(D)J recombination 4-fold when inserted in genomic orientation (Table 1). This finding further contradicted the possibility that, in our system, MARs were required for high frequency recombination.

E3 is derived from the 5′ end of the human topoisomerase I gene. It spans the promoter as well as exons 1 and 2, and extends into the adjacent intron. By digesting with *Xho*I, E3 was split into a 5′ fragment (termed topo I promoter), containing the entire promoter with the 5′ half of exon 1 attached, and a 3′ fragment (termed topo I coding) encompassing the remainder of E3. When tested in the recombination assay (Table 1) the promoter part was ineffective. In contrast, the coding part increased the recombination frequency by a factor of 3, but only when inserted in antigenomic orientation. This was surprising in light of the previous observation that the entire E3 upregulated V(D)J recombination when inserted in the opposite orientation.

Table 2. Sequences tested for effects on frequency of V(D)J recombination in 300-19, 1-8 and 31.7.12 cells

Cell line	Insert	Exp. repeats	Experimental R x 100 (mean ± SD)	Control R x 100 (mean ± SD)	Fold stimulation (mean ± SD)
1-8	Eμ +	4	0.48 ± 0.41	0.18 ± 0.15	2.72 ± 0.68
	MII +	3	0.34 ± 0.18	0.21 ± 0.17	3.78 ± 3.97
	E3 +	3	1.46 ± 0.90	0.21 ± 0.17	8.67 ± 3.17
	B1X1+	2	0.26 ± 0.33	0.21 ± 0.17	0.73 ± 0.65
300-19	Eμ +	2	0.80 ± 0.14	0.21 ± 0.10	5.39 ± 3.23
	MII +	2	1.22 ± 0.33	0.21 ± 0.10	6.61 ± 1.52
	E3 +	2	0.88 ± 0.24	0.21 ± 0.10	4.79 ± 1.09
	B1X1+	2	0.10 ± 0.03	0.21 ± 0.10	0.72 ± 0.49
31.7.12	Eμ +	3	10.07 ± 2.49	4.07 ± 0.89	2.46 ± 0.25
	MII +	3	8.51 ± 1.33	4.07 ± 0.89	2.14 ± 0.27
	E3 +	3	25.09 ± 7.21	4.07 ± 0.77	4.95 ± 2.31
	B1X1+	3	1.29 ± 1.06	4.07 ± 0.89	0.34 ± 0.24

For explanations see Table 1 footnotes.

Two unrelated λ phage fragments were tested as additional representatives of non-MAR sequences. They were selected so as to be of similar length as the MARs and the E3 non-MAR described above. These sequences strongly depressed V(D)J recombination in either orientation. A suppressive effect of prokaryotic sequences on V(D)J recombination has been described previously (27).

Other B cell lines respond similarly to a selection of test inserts

Up to this point all experiments had been performed with the Abelson murine virus-transformed pre-B cell line 38B9. It was of interest to see whether the observed responses were cell line-dependent or had a more general significance. We addressed this question by transfecting other recombination-competent cell lines with some of our recombination substrates selected for maximum efficiency (Table 2).

We used the Abelson murine virus-transformed pre-B cell lines 1-8 and 300-19 as well as the mature B cell line 31.7.12, which has been derived from M12 lymphoma cells by stable transfection with full-length *RAG1* and *RAG2*. Similar to 38B9, 1-8 cells continuously rearrange their D–J_H and V_H–DJ_H loci, whereas 300-19 cells carry fully rearranged DJ_H loci. In the more mature 31.7.12 cells endogenous VDJ loci are fully rearranged.

A comparison of the results in Table 1 (38B9 cells) and Table 2 (1-8, 300–19, 31.7.12 cells) shows that the four cell lines respond similarly to the inserts Eμ, MII, E3 and B1X1 in genomic orientation. Upregulation is consistently observed with Eμ, MII and E3, while B1X1 either downregulates V(D)J recombination or leaves it unaffected. Thus, the effects are intrinsic to the inserted sequences and are largely independent of cell stage and origin.

A 26 bp A–T-rich motif is common to all upregulating sequences other than the Eμ core

A computer search was initiated for sequences common to all of the upregulating inserts investigated. This led to the detection of a 26 bp A–T-rich sequence motif characterized by the consensus sequence shown in Figure 3 (bottom).

The motif occurred in Eμ, MII and E3 but was absent from all other inserts. It appeared in 23 variants, six of which were associated with Eμ (variants 1–6; numbering of the variants refers to their position relative to the 5′ end of the insert; Fig. 2), 14 with MII (variants 7–20) and three with E3 (variants 21–23). The positions, orientations and sequences of these variants are listed in Figure 3.

The variants were charted on their respective fragments. In Eμ, the motif was restricted to the 5′ and 3′ MARs, not being present in the core (Fig. 2). In MII, the variants were evenly distributed over the entire length of the fragment, and in E3 were confined to the topo I coding fragment.

Consistent with the possibility that the motif might represent a novel upregulating element distinct from those constituting the Eμ core, the motif was present in all fragments and subfragments found to activate V(D)J recombination except the Eμ core. In addition, the motif was absent from all inserts devoid of an upregulating activity, with the single exception of the truncated 3′ MAR of Eμ (the subfragment EμEIXb) which, despite its content of four copies of the motif, was inactive. This might be explained by assuming that certain sequence features are absolutely required for activity. In fact, closer scrutiny revealed that all upregulating fragments contained at least one variant carrying a 5′ terminal ACT triplet, while EμEIXb did not (5′ refers to the sequence motif, irrespective of whether it is located in the plus or minus strand of the insert, and does not specify its orientation relative to the direction of *lacZ* transcription). The triplet was present in variants 1 and 2 (Eμ), 9, 10, 16, 17 and 20 (MII) as well as 23 (E3) but was missing from all others (Fig. 3). It is generally recognized that functionally essential sequence characteristics are often located at the ends of recognition elements [e.g. the heptamer of the V(D)J recombination signal sequences; 53–55]. Alternatively, the activity of the motif may also vary depending on orientation (as suggested by the results presented in Table 1) and sequence context. In particular, the clustering and interdigitation (in the case of variants 4 and 5) of the four plus and minus strand variants contained in EμEIXb might abrogate the functions of the individual copies.

The computer search was extended to the entire EMBL and GenBank databases. Variants 1–6 (Eμ) were found to be unique to the mouse immunoglobulin heavy chain intron enhancer. Interestingly, a homologue of variant 6 with a T→C transition at position 3 was detected in the mouse immunoglobulin heavy chain switch region (accession no. J00478). Variants 7–20 (MII) were restricted to the human topoisomerase I gene, except for variant 12, which in addition was also associated with various other human genes but was absent from other species. Variant 21 with flanks identical to those in E3 has been described previously as part of a human CpG-containing DNA clone (accession no. Z57072). Variants 22 and 23 (E3) were not contained in the databases.

DISCUSSION

Previous studies with chromosomally integrated recombination substrates have shown that V(D)J recombination is stimulated by immunoglobulin and T cell receptor transcriptional enhancers (20–23,27–29,31,32). In the case of Eμ, upregulation was found to be associated with both the enhancer core (28) and the 5′-flanking MAR (21) and to be independent of the level of transcription (28). To accelerate the search for recombinational enhancer elements we have attempted to develop a simpler assay

Insert	Variant no.	Position from 5' end of insert (bp)	Plus or minus strand sequence	Variant sequence (5' to 3')																										Similarity (%)
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	
Eμ	1	157-182	plus	A	C	T	C	A	T	T	T	T	T	T	T	A	A	A	T	G	T	C	C	A	A	A	A	T	T	100
	2	650-675	plus	A	C	T	T	A	A	G	T	T	T	A	T	C	G	A	C	T	T	C	T	A	A	A	A	T	G	67
	3	720-695	minus	C	T	T	C	A	A	T	T	T	C	T	T	A	C	A	T	A	A	C	C	T	A	A	T	T	T	67
	4	772-747	minus	A	G	T	A	A	T	T	T	T	A	A	G	A	A	G	T	T	T	T	C	T	A	A	A	T	A	67
	5	752-777	plus	A	G	A	A	A	A	C	T	T	C	T	T	A	A	A	A	T	T	A	C	T	C	T	A	T	T	62
	6	964-988	plus	T	A	T	T	T	T	T	T	T	T	T	T	A	A	A	T	G	A	A	T	G	C	A	A	T	T	71
MII	7	188-213	plus	C	A	T	T	C	T	C	T	T	T	T	T	A	G	A	T	T	T	A	A	A	A	A	A	T	G	71
	8	217-192	minus	T	C	T	A	C	A	T	T	T	T	T	T	A	A	A	T	C	T	A	A	A	A	A	G	A	G	71
	9	431-456	plus	A	C	T	C	G	G	T	C	T	T	T	T	T	A	T	T	C	T	C	A	T	A	C	A	T	A	62
	10	503-528	plus	A	C	T	A	G	T	G	T	G	T	T	A	C	A	T	A	T	T	G	T	A	A	A	A	T	T	62
	11	821-796	minus	A	G	A	C	A	T	G	G	T	C	T	C	A	A	A	T	G	A	C	C	A	A	C	A	A	C	58
	12	959-984	plus	C	C	C	G	T	C	T	C	T	A	T	T	A	A	A	A	A	T	A	C	A	A	A	A	A	T	67
	13	1199-1174	minus	A	C	A	A	A	T	T	T	C	A	T	T	C	A	A	T	A	T	A	C	A	C	A	A	G	A	71
	14	1600-1625	plus	A	C	A	A	G	T	T	T	G	T	T	T	C	A	T	T	C	T	T	C	A	A	A	A	T	T	75
	15	1916-1891	minus	A	A	T	A	A	G	A	A	T	T	T	T	A	A	A	T	G	T	C	C	T	T	T	A	T	G	67
	16	2371-2396	plus	A	C	T	C	A	C	C	T	T	T	G	T	T	T	A	C	C	T	G	A	A	A	A	T	T	T	62
	17	2526-2551	plus	A	C	T	A	A	T	G	T	A	T	T	T	T	A	T	C	C	T	A	C	A	G	T	A	T	T	71
	18	2758-2783	plus	T	C	T	A	A	T	T	A	T	A	T	T	T	A	A	T	C	T	A	C	A	A	G	T	T	A	71
	19	2790-2815	plus	C	T	A	T	A	A	T	T	T	A	T	T	T	C	A	A	T	T	A	C	C	A	C	T	T	T	54
	20	2811-2836	plus	A	C	T	T	T	T	T	G	T	T	T	T	C	A	A	A	A	T	T	T	C	T	A	A	T	T	67
E3	21	1854-1879	plus	G	C	T	C	T	T	T	C	C	T	T	G	A	G	C	T	G	C	C	C	A	G	A	A	T	G	58
	22	2244-2219	minus	A	C	A	T	A	A	T	T	T	T	T	T	A	A	A	A	A	T	C	C	A	A	C	A	T	T	83
	23	2490-2515	plus	A	C	T	G	G	C	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	C	A	A	T	T	67
			No of A	15	3	6	9	13	7	1	2	1	5	2	1	12	16	16	6	5	3	9	4	14	15	15	18	3	4	
			No of G	1	3	0	2	4	2	4	2	2	0	1	2	0	3	1	0	5	0	2	0	1	2	1	1	1	5	
			No of T	3	2	16	6	4	12	15	16	18	15	20	19	6	2	5	14	7	19	4	4	5	2	3	4	19	13	
			No of C	4	15	1	6	2	3	3	3	2	3	0	1	5	2	1	3	6	1	8	15	3	4	4	0	0	1	
			Consensus	A	C	T	N	A	T	T	T	T	T	T	T	A	A	A	T	N	T	C/A	C	A	A	A	A	T	T	

Figure 3. A 26 bp A–T-rich consensus sequence and its variants occurring in the upregulating inserts, Eμ, MII and E3. For the positions and orientations of variants 1–6 in Eμ, see also Figure 2. Shading indicates identity with consensus sequence. A 5′ terminal ACT triplet of potential functional significance is boxed. Similarity is defined as the percentage of nucleotides corresponding to shaded positions in the consensus sequence.

based on the transient transfection of easily manipulatable extrachromosomal recombination substrates.

To evaluate the reliability of such a system we first aimed at reproducing in extra-chromosomal substrates the effects reported for chromosomally integrated constructs containing variously mutated enhancers in *cis*. For this purpose, we inserted the entire Eμ and fragments thereof into the unique *Eco*RV site of the plasmid substrate pBlueRec, and, following transfection into recombination competent cells, determined the frequency of recombination.

Consistent with previous reports we find that the entire Eμ (21,22,27,28), the Eμ core (23,28) and the 5′-flanking MAR (21) upregulate V(D)J recombination, whereas the downstream section of the 3′ MAR (the fragment EμEIXb) has no effect (21). Also, as described formerly (27), prokaryotic sequences derived from λ phage markedly suppress V(D)J recombination. Thus, with all of these constructs, our extrachromosomal assay accurately reflects the situation with chromosomally integrated substrates. It deviates from the latter only in two respects, namely that (i) the extrachromosomal SV40 enhancer does not activate V(D)J recombination (23), whereas (ii) the full-length 3′ MAR does (27,28). The lack of activity of the SV40 enhancer might be explained by the preexistence of a polyoma enhancer in the recombination substrate, which might mask the effect of additional viral enhancer elements. The same may be true for the CMV enhancer. Such interference has previously been reported with the κ enhancer (56). Alternatively, the SV40 enhancer might lack specific recombinational enhancer elements necessary for upregulating V(D)J recombination in an episomal context but, in chromosomally integrated substrates, might stimulate recombination indirectly through activation of transcription with concomitant opening of the chromatin conformation. The stimulating effect of the extrachromosomal 3′ MAR is more difficult to explain. Perhaps, in the transgenic substrates, the regulation of V(D)J recombination is not entirely physiological with parts of the enhancer being permanently silenced. In contrast, such shielding by chromosomal proteins might not exist in extrachromosomal substrates leaving the 3′ MAR enhancer sequences accessible to activating proteins. It is important to note that our extrachromosomal assay deviates from the chromosomal assays only if the latter involve ectopically integrated recombination substrates (23,27,28) but give results consistent with the more physiological assays based on the targeted deletion or disruption of endogenous enhancer sequences (21,22). Thus, the results presented in this paper indicate that our extrachromosomal system is a valuable alternative to the more elaborate assays based on chromosomally integrated substrates.

The good agreement between the results of the present work (episomal substrates) and those of previous studies (chromosomally integrated substrates) is a strong indication that the effects we see are not simply caused by methodological artefacts such as different rates of plasmid replication in the transfected cells or different efficiencies of transformation of *E.coli*. This is further substantiated by the observation that the transformation efficiencies were the same for all plasmids, and that all plasmids exhibited identical replication rates except when carrying the MII insert in

either orientation. Plasmids with this insert yielded lower numbers of total (*Dpn*I resistant) colonies than other plasmids, for an equal number of plasmid molecules transfected. Decreased replication of the substrate in comparison with the recombined product could indeed mimic an increased recombination frequency. However, if this were the explanation for the increased ratio of recombined to total colonies observed with MII, then the same result would be seen with MII in either orientation. This is not the case (Table 1) rendering this interpretation of the data unlikely. It certainly is invalid for all other inserts, none of which gave signs of an altered replication rate.

The simplest statement that our work allows us to make is that sequences interposed between a pair of RSS affect the frequency of V(D)J recombination. Previous studies have dealt with the influence of sequences immediately abutting the site of recombinational cleavage on both sides (49,53–55,57,58) but medium and long range effects of coding or non-coding elements have not been investigated. In the present study, inserts were spaced 26 bp from the RSS 23 and 284 bp from the RSS 12, respectively (Fig. 1), and had lengths of 60–3200 bp (Table 1). With a minimal distance of two and a half helix turns of DNA from the proximal end of the nearest RSS (the RSS 23), it is improbable that the effects are due to local DNA conformational changes such as unwinding or bending of the RSS. Rather, they may be attributed either to an altered flexibility of the DNA facilitating or impeding proper synapsis of the recombination sites or to the presence of *cis*-acting elements.

The activation of V(D)J recombination by the 5′ and 3′ MARs of Eμ could be due to either of two mechanisms. For instance, the MARs could bring about this effect by concentrating and immobilizing the recombination substrate on the nuclear matrix, thus bringing it into close proximity to a nuclear matrix-attached recombination machinery acting upon it. Alternatively, the MARs could stimulate recombination through *cis*-regulatory elements. Our work suggests an answer to this question. If direct attachment of recombination substrate to recombination complex were the reason for the 5′ and 3′ MARs to stimulate V(D)J recombination, then all MARs should exert this effect. This is clearly not the case (Table 1) leading us to conclude that the 5′ and 3′ MARs of Eμ harbour recombinational enhancer elements.

Enhancers and MARs are known to act largely independent of orientation. Thus, the observation that, in our assay, most of the fragments exhibited orientation dependence requires explanation. Assuming that the 5′ and 3′ MARs of Eμ mediate their effects through recombinational enhancer elements the problem is reduced to finding an explanation for enhancers. This could be related to distance. In physiological conditions, enhancers are located far remote from the elements they influence, e.g. promoters, such that the interposed stretch of DNA can easily bend back bringing enhancer and promoter complexes into contact. In contrast, our inserts were comparatively short so that functional interactions between proteins bound to asymmetrically positioned enhancer elements and sites of recombination (the RSSs) might be favoured in one of the two possible orientations and disfavoured in the other. Alternatively, proteins binding to sites close to one end of the insert might interfere with binding of the recombinational enzyme complex in only one of the orientations but not in the other (see next paragraph).

In a computer search for possible enhancer elements located in the 5′ and 3′ MARs of Eμ, a 26 bp A–T-rich sequence motif caught our attention (Fig. 3). It was present in all of the upregulating fragments investigated except the Eμ core (Table 1 and Fig. 2). This suggested that the motif might be a novel recombinational enhancer element distinct from those intrinsic to the Eμ core. In agreement with this interpretation, the motif was absent from all inactive fragments analyzed except the fragment EμEIXb. The lack of activity of EμEIXb could be due to the fact that none of the four motifs contained therein (variants 3–6) carried a 5′ terminal ACT considered to be functionally essential (see Results). Alternatively, the proximity of the RSS 23 to the nearest motif (30 bp with variant 3 in the genomic orientation and 36 bp with variant 6 in the antigenomic orientation) might impede the simultaneous binding of the recombinational enzyme complex to the RSS and of its putative activator to the nearby motif.

The consensus sequence of the motif exactly corresponded to the sequence of variant 1 located in the 5′ MAR of Eμ (Fig. 3). A computer search revealed the motif (with an intact 5′ terminal ACT triplet suggested to be essential for activity) to be restricted to the murine Eμ and the human topoisomerase I gene. An unrelated rearrangement-enhancing sequence element has been detected recently upstream of the mouse immunoglobulin κ chain J cluster (50). Its position between RSSs is comparable to the location of the 26 bp A–T-rich motif in our recombination substrates, and the enhancement of V(D)J recombination is of the same magnitude in both systems. Although further work is certainly needed to establish that the 26 bp A–T-rich motif is a constituent of a recombinational enhancer, our findings may at least provide a clue in the search for recombinational enhancer elements.

If, indeed, the motif were to define a recombinational enhancer element, what could be the reason for its occurrence in the human topoisomerase I gene? It might be argued that the motif has a function in association with RSSs only, thus lacking any physiological role in the topoisomerase I gene. Alternatively, if one accepts that the transcriptional enhancer, Eμ, also acts as a recombinational enhancer, then it does not seem unreasonable to postulate that a recombinational enhancer element in the immunoglobulin gene might also function as a transcriptional enhancer element in another gene, such as the topoisomerase I gene. One might even speculate that this reflects a functional link between V(D)J recombination and topoisomerase I transcription indicating that topoisomerase I might be a component of the recombinational complex.

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