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The Effect of Promoter-Enhancer on V(D)J Recombination of Extrachromosomal DNA Substrates

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The effect of promoter-enhancer on V(D)J recombination was analyzed by using a series of extrachromosomal DNA substrates. DNA substrates contain the polyoma replication origin and the large T gene to replicate, and recombination signal sequences of DNA substrates are transcribed in murine cell. We inserted the SV40 promoter, the enhancer-promoter region of the mouse immunoglobulin heavy chain and the SV40 poly(A) signal into the substrate, and measured the V(D)J recombination frequency using a transient assay system. The substrate containing the SV40 promoter reduced V(D)J recombination frequency 2 fold. In contrast, the enhancer region increased 2.5 fold and poly(A) signal did not have any effect. These results indicate that transcription might reduce V(D)J recombination frequency.

KEY WORDS: V(D)J Recombination / Promoter / Extrachromosomal DNA

INTRODUCTION

Complete immunoglobulin variable region genes are generated by somatic DNA rearrangement, which assembles variable (V), diversity (D), and joining (J) gene segments during the B-cell differentiation[1]. Each segments contain recombination signal sequence(RSS), which consist of heptamer-spacer (12 or 23 bases) - nonamer. A signal reaction event occurs between a 12 spacer-containing signal and a 23 spacer- containing signal. The two signal ends are joined together at their heptamers and DNA ends adjacent to their heptamers[2-5]. These pairs of RSS are sufficient to cause V(D)J recombination[6,7]. This DNA rearrangement is regulated in the B-cell differentiation. Heavy chain gene recombination initiates with the joining of D segment to J_H segment to form DJ_H complex, followed by the joining of V_H segment to DJ_H complex to form a complete V_HDJ_H gene[8]. V_L and J_L segments join together after V_HDJ_H recombination[9,10]. It is thought that the ordered recombination is regulated by transcription because RSS of segment are transcribed before V(D)J recombination and the level of transcription correlates with the frequency of V(D)J recombination. The accessibility model was proposed that transcription changes the chromatin structure to increase the accessibility of a hypothetical V(D)J recombinase to RSS[11]. To know how transcription effects on V(D)J recombination, we made a series of extrachromosomal DNA substrates and measured the V(D)J recombination frequency using a transient assay system. We show that transcription reduces the V(D)J recombination frequency.

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MATERIALS AND METHODS

Materials : Plasmid DNA MHSDRP-82[12] was provided by M. Abe (Radiation Effect Research Foundation, Nagasaki, JAPAN). Abelson murine leukemia virus (A-MuLV)-transformed PreB cell line 46-6[13] was provided by T. Takemori (NIH, Tokyo, JAPAN). Plasmid MEP203[14] and pSV2-gpt[15] were provided by Y. Kurosawa (Fujita-Gakuen, Toyoake, JAPAN). pSL1180 was purchased from Pharmacia LKB Biotechnology. HPLC column TSK-gel DEAE-NPR (TOSOH Co.LTD) used for preparation of DNA fragments was gifted by Science TANAKA Co. LTD (Sapporo, JAPAN). *E. coli* SCS-1 (F^-, *end* A1, *hsd* R17, *sup* E44, *thi*-1, *rec*A1, *gly* A96, relA1, λ^-) was used for preparation of plasmid DNA and assay of recombination.

Construction of substrates : MHSDRP-82 was digested with the restriction enzyme *Sal*I to delete the polyoma genes and the *Xho*I-*Sal*I fragment of pSL1180 which contains the multiple cloning sites (MCS) was inserted into the *Sal*I site to produce DJ63. DJ63O was constructed by insertion of the *Bam*HI fragment containing polyoma genes into the *Bgl*II site of DJ63. The 237 bp *Sau*3AI fragment containing the SV40 poly (A) signal and the 344 bp *Pvu*II-*Hin*dIII fragment containing the SV40 early promoter isolated from pSV2-gpt, were converted into blunt ends with Klenow enzyme, respectively, and inserted into the EcoT22I site of DJ63O. We named them DJ63OP and DJ63OS. The mouse heavy chain enhancer-promoter region was derived from MEP203. The 682 bp *Xba*I-*Eco*RI fragment containing the enhancer-promoter was converted into blunt ends and inserted into the *Eco*T22I site of DJ63OI containing MCS in the opposite direction to DJ63O. It was designated DJ63OEn. All fragments were purified by HPLC.

V(D)J recombination assay : A-MuLV transformed PreB cell line 46-6 was grown in 95% PRMI1640, 5% fetal calf serum, 200 units/ml penicillin and 200 units/ml streptmycin.

$2X10^7$ cells were washed with ice-cold PBS(-), suspended in 0.5 ml of ice-cold PBS(-) containing 40 μg of plasmid DNA, and incubated on ice for 10 min. After electroporation at 700V, 25 μF, using a 0.4cm electrode gap cuvette [GENE PULSER (Bio Rad;Richmond, CA], the contents of the cuvette were mixed gently, and the same pulse was applied 10 minutes later again. The cells were incubated for 10 min on ice and the complete RPMI640 medium was added. After incubation at 37℃ for 48 hr, plasmid DNA was purified from the transfected cells as follows. The transfected cells were washed with PBS(-) and the plasmid DNA was extracted by the alkali lysis method. The plasmid DNA was treated with phenol:chloroform (1:1) and precipitated with 2.5 volume of ethanol. The DNA pellet was dissolved in 100 μl of TE and an equal volume of 5M LiCl was added. After incubation on ice for 30 min, RNA was removed by centrifugation at 15,000 rpm at 4℃ and the supernatant was saved. An equal volume of isopropanol was added and DNA was precipitated by centrifugation. The pellet was dissolved in 50 μl of TE and the solution was used as the purified plasmid DNA. The DNA solution (1～3 μl) was used to transform *E. coli* SCSI. Electroporation was performed at 2350V, 25 μF using a 0.1cm electrode gap cuvette (BIO RAD). 2ml of SOC medium were added immediately and *E. coli* cells were incubated at 37℃ for 2hr with shaking. 20 μl of cultured cells were assayed on the agar plate containing Amp (100 μg/ml ,

ampicillin) and 2 ml were assayed on the agar plate containing Amp and Cm (35 μg/ml, chloramphenicol). Plates were incubated at 37℃ for 24hr and transformants were counted.

RESULTS AND DISCUSSION

Hesse *et al.* developed a system for V(D)J recombination assay using the extrachromosomal DNA as substrates[16]. Plasmid DNA can replicate and recombine in murine lymphoid cells. A series of substrates in this experiment are shown in Fig. 1.

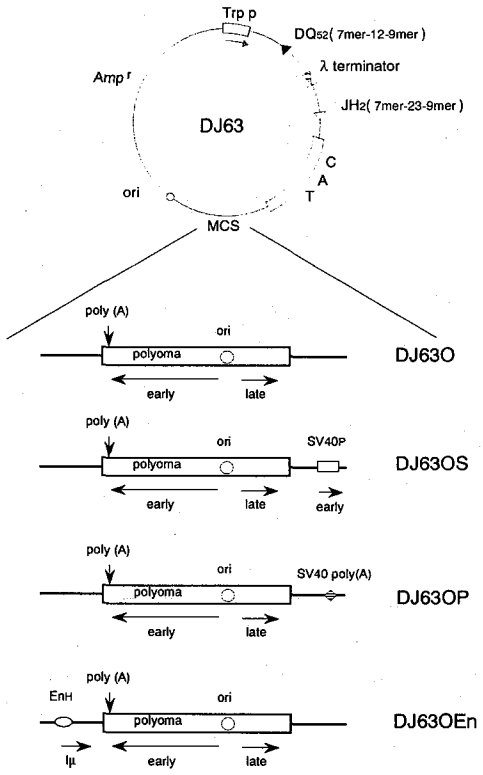

Fig. 1. Substrates for V(D)J recombination assay.
CAT is the chloramphenicol acetyl transferase gene. λ oop terminator stops the transcription from the *trp* promoter in *E. coli*. After recombination, CAT gene is transcribed by deletion of the terminator. → means the direction of transcription.

The substrates contain 12 spacer RSS (DQ_{52}) and 23 spacer RSS (J_{H2}) to detect a reciprocal joint. After the transfection of plasmids to murine PreB cell line 46-6, we recovered the plasmids and introduced them into *E. coli* cells. Bacterial cells containing the rearranged plasmid can grow on the Amp and Amp + Cm plates because of the deletion of the trascriptional terminator and the expression of Amp^r and Cm^r genes, and cells containing the non-rearranged plasmid can grow only on the Amp plate. Ampicillin-resistant (Amp^r) colonies and ampicillin chloramphenicol-double resistant ($Amp^r + Cm^r$) colonies were counted and the ratio of $Amp^r + Cm^r$ colonies to Amp^r colonies was calculated as the frequency of V (D) J recombination. We confirmed that the plasmid DNA molecules derived from $Amp^r + Cm^r$ colonies have the reciprocal joint by the restriction enzyme analysis and PCR analysis as described[12]. The substrates have the polyoma large T gene and the replication origin to replicate in the murine cell, and RSS is transcribed from the early promoter or (and) the late promoter of polyoma region. In order to examine the relationship between V (D) J recombination and trascription, we measured the recombination frequency using a series of DNA substrates (see Fig. 1).

DJ63O was used as the control substrate. We inserted the SV40 promoter between the polyoma late pormoter and the CAT gene of DJ63O and measured the recombination frequency. As shown in Table 1, the average recombination frequency of DJ63O and DJ63OS

Table 1. Frequency of V (D) J recombination

Plasmid	No. of colonies		Amp^r+Cm^r ratio
	Amp^r	Amp^r+Cm^r	Amp^r
DJ63O	18200	40	$2.2X10^{-3}$
	10900	30	$2.8X10^{-3}$
	18200	69	$3.8X10^{-3}$
	27100	72	$2.7X10^{-3}$
			$(2.9\pm0.6)X10^{-3}$
DJ63OS	7400	8	$1.1X10^{-3}$
	4600	9	$2.0X10^{-3}$
	6000	8	$1.3X10^{-3}$
			$(1.5\pm0.5)X10^{-3}$
DJ63OP	700	2	$2.9X10^{-3}$
	10700	53	$2.6X10^{-3}$
	10400	22	$2.1X10^{-3}$
			$(2.5\pm0.4)X10^{-3}$
DJ63OEn	900	7	$7.8X10^{-3}$
	11300	78	$6.9X10^{-3}$
	66400	429	$6.5X10^{-3}$
			$(7.1\pm0.7)X10^{-3}$

was $2.9X10^{-3}$ and $1.5X10^{-3}$, respectively. The SV40 promoter activity is stronger than the polyoma late promoter activity. The insertion of the strong promoter reduced V(D)J recombination frequency. As DJ63O (control plasmid) has the poly(A) signal at the end of large T gene, we inserted the SV40 poly(A) signal region at the same site (DJ63OP) to know the effect of the poly(A) signal. The recombination frequency of DJ63OP was hardly different from the frequency of DJ63O (see Table 1). The poly(A) signal does not act as the transcriptional terminator on DNA, but as the signal on RNA for cleavage and poly(A) addition[17]. These processings of the transcript do not effect on the recombination frequency. We also inserted the mouse immunoglobulin enhancer region (DJ63OEn). This enhancer region has the promoter activity in B-cell and the direction of transcription is opposite to that of polyoma early transcription. As shown in Table 1, the recombination frequency increased 2.5 fold as compared with the control substrate. The recombination frequency of DJ63OEn is 4.7 fold as high as that of DJ63OS. A factor bound to the enhancer might directly stimulate the V(D)J recombination. It is also possible that promoters in opposite directions impede the transcription from each other.

These results indicate that transcription reduces the V(D)J recombination frequency. This is not necessarily contradictory to the concept of the transcriptional activation[11]. The transcriptional activation might result from opening the chromatin structure but the presence of transcription would rather hamper the access of the V(D)J recombinase.

We could not quantify the transcripts of RSS because the efficiency of transfection is low[18] and the transcribed sequences downstream of the cleavage/polyadenylation site are extreamly unstable[19]. Hsieh et al. reported that replication is not required for V(D)J recombination[20]. It is thought that transcription or replication is not essential for V(D)J recombination.

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