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Biased reading frames of pre-existing D_H-J_H coding joints and preferential nucleotide insertions at V_H-DJ_H signal joints of excision products of immunoglobulin heavy chain gene rearrangements

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During B cell differentiation immunoglobulin (Ig) D_H segments join to J_H segments, followed by joining of V_H to DJ_H. Although circular excision products of D_H-J_H rearrangements have been characterized, excision products of V_H to DJ_H joining have never been isolated. We selectively denatured chromosomal DNA of mouse splenocytes and enriched circular DNA spanning the long distance between V_H and D_H. Subsequent PCR amplifications allowed the identification of signal joints of V_H to DJ_H. Sequence analysis indicated that pre-existing D_H-J_H coding joints of excision products showed a strong bias for reading frame 1, and the absence of reading frame 2, which would allow the expression of a truncated μ chain called D_μ protein. When comparing the joints of the V_H-DJ_H and D_H-J_H rearrangements we observed N-nucleotide insertions to be abundant at the V_H-D_H signal joint, but very sparse at the D_H-J_H signal joint, while the coding joints of both contained abundant N-insertions. These differences in N region insertions at the signal joints suggest a differential control of the D-J and V-DJ rearrangements.

Key words: alkaline lysis/circular DNA/D-J and V-DJ joining/DJ_H reading frame/N sequences

Introduction

The initial diversity in Ig and TCR is afforded by combinatorial diversity generated by the combination of different V, (D) and J gene segments. In addition, considerable diversity is generated at the junction of these segments, namely junctional site diversity (deletion) by exonuclease activity, and junctional insertion diversity (addition of N nucleotides, thus 'N region diversity'), presumably by the enzyme terminal deoxynucleotidyl transferase (TdT).

Although it is common in adult lymphocyte repertoires, N region diversity at the junctions is rare in prenatal and neonatal repertoires of B cells (Gu *et al.*, 1990; Meek, 1990; Feeney, 1990; Bangs *et al.*, 1991), $\gamma\delta$ T cells (Asarnow *et al.*, 1988; Elliott *et al.*, 1988; Lafaille *et al.*, 1989) and $\alpha\beta$ T cells (Bogue *et al.*, 1991). The lack of N region diversity correlates with low levels of the enzyme TdT in fetal liver and fetal thymus (Gregoire *et al.*, 1979; Rothenberg and Triglia, 1983; Opstelten *et al.*, 1986).

The V(D)J joining results in two distinct classes of recombination products: a coding joint and a signal joint created by the fusion of two joining signal sequences flanking the coding elements. According to recombination analysis performed with extrachromosomal recombination substrates, the incidence of N-insertions in a signal joint correlates well with the level of TdT activity in the cell line, but the incidence of coding joint insertions does not (Lieber *et al.*, 1988). Differential N-insertions at the signal joint in the presence of TdT activity were obvious in TCR gene rearrangements of adult mouse thymocytes because N-insertions in coding joints were common for every TCR gene γ, δ, β and α, but those in signal joints were mostly restricted to TCR γ and δ rearrangements (Takeshita *et al.*, 1989; Iwasato and Yamagishi, 1992). This may correlate with the overall temporal order of TCR gene rearrangement as suggested by Pardoll *et al.* (1987).

During B cell differentiation, the Ig genes encoding H and L chain are assembled by sequential rearrangements (Tonegawa, 1983). First, D_H segments are joined to J_H segments, followed by V_H to DJ_H joining. After successful IgH rearrangement with possible N region addition at the coding joint, V_L to J_L rearrangement takes place at the IgL loci. No N region diversity is introduced at the V_L-J_L junctions, possibly because TdT activity is turned off at this later stage of B cell development. For the IgH rearrangement, two separate controls seem to operate in D-J and V to DJ rearrangements as shown for the TCR β locus (Ferrier *et al.*, 1990; Rolink and Melchers, 1991). These stage-specific rearrangements led us to investigate whether N-insertions at signal joints might be differentially regulated in B cell precursors.

To answer this question, we isolated the circular excision products containing signal joints of D_H to J_H and V_H to DJ_H rearrangements. Excision products of D_H-J_H rearrangements were prepared from the 'cleared lysate' (Stanfield and Helinski, 1976) of splenocytes and the linear DNA was digested by ATP-dependent DNase (Yamagishi *et al.*, 1983). We introduced alkaline lysis methods (Carroll *et al.*, 1987) for the enrichment of large circular molecules spanning the long distance of murine V_H locus containing 500-1000 or more members (Livant *et al.*, 1986; Rathbun *et al.*, 1989). V_H-DJ_H signal joints were sequenced following PCR amplification from the large circular DNA-enriched fraction. These results show that N region nucleotides were lacking in almost all D_H-J_H signal joints, but were present abundantly at V_H to DJ_H signal joints. Therefore, the addition of N regions at the signal joint suggests the accessibility of TdT to recombination complexes, or the higher TdT activity at the stage of V_H to D_HJ_H joining.

We also examined the reading frame of the D_H-J_H coding joints obtained from circular excision products and discussed the role of a short μ protein consisting of DJ_H and C_μ sequence (D_μ protein) in the early B cell development.

Results

Circular DNA preparation and DNA clones homologous to D_H or V_H

We have enriched small circular DNAs in the 'cleared lysate' (Stanfield and Helinski, 1976) from mouse splenocytes and purified them with the use of ATP-dependent DNase as previously described (Yamagishi *et al.*, 1983). We prepared a phage DNA library of *Eco*RI or *Bam*HI fragments of circular DNA and screened circular DNA clones by plaque hybridization with a D_{SP2} probe and a mixture of V_H probe composed of V_{HJ558}, V_{H36-60}, V_{HS107}, V_{HQ52} and V_{H7183} (Table I). Many clones reacted with D_{SP2} but no clones were positive with V_H probes. The high frequency of mitochondrial DNA clones showed the purity of circular DNA. Since the total length of the human V_H region is estimated to be ~3000 kb (Matsuda *et al.*, 1988), circular DNA spanning the long distance between V_H and DJ_H may have been lost during the high-speed centrifugation of 'cleared lysate' which was standardly used for the preparation of 150–200 kb circular DNAs (Fujimoto and Yamagishi, 1987; Iwasato *et al.*, 1990, 1992). In an attempt to isolate larger DNA circles we used the alkaline lysate method, involving alkaline lysis to denature selectively chromosomal linear DNA ('alkaline lysate') and then performed phase separation of circular DNA (Carroll *et al.*, 1987). Now, we obtained several V_H-positive clones even in a small scale screening. In this library, mitochondrial DNA clones were enriched 500-fold and V_H-positive clones are enriched several 10-fold as compared with the chromosomal genomic library. To purify the circular DNA further we used CsCl–ethidium bromide (EtBr) gradients to band it. As measured by the frequency of mitochondrial DNA clones, circular DNAs were enriched to 3000-fold in the alkaline lysate method (15–16.5% of the clones consist of mitochondrial DNA), but not to the high levels of purity obtained with the cleared lysate method (65% mitochondrial DNA clones). Many V_H-positive clones as well as D_{SP2}-positive clones were obtained from these adult splenocytes. Nevertheless, circular DNA libraries prepared from day 18 fetal liver by these two methods did not show any V_H-positive clones. V–DJ rearrangements may be rare if not totally absent in fetal liver.

DJ_H recombinants in circular DNA

The D_{SP2} probe (pHC100) used in this study contained the germline sequence of $D_{SP2.9}$ (Lawler *et al.*, 1987) followed by a new pseudo D element [93% homologous to ψD_1 (Lennon and Perry, 1989) and termed ψD_3] (EMBL, GenBank and DDBJ nucleotide sequence database under the accession number D13199). $D_{SP2.9}$ has an unique sequence in the coding region but a high homology to other D_{SP2} genes in the flanking sequence and is virtually identical with $D_{SP2.y}$ shown by Gu *et al.* (1991).

Circular DNAs were prepared from the 'cleared lysate' of splenocytes, digested by *Eco*RI and cloned into λgt11 phage. Out of 5×10^5 phage, 32 D_{SP2}-positive clones were obtained (Table I). We selected 28 clones and recloned into plasmid pHSG399 (Table II). Although a linkage map of D_{SP2}-positive *Eco*RI fragments is known (Kurosawa and Tonegawa, 1982), recombinant structures were not predictable from the insert length, possibly due to a strain-specific polymorphism. We performed preliminary nucleotide sequencing with respect to the specified base using specific sequence primers from the upstream sense strand or downstream antisense strand of D_{SP2} as shown in Figure 1. D_{SP2}-sequence primers reacted to 23 clones but did not react to the other five clones (pHC6, 13, 17, 21 and 31). Out of 23 D_{SP2}-sequence reactive clones, 13 clones carried the germline configuration of D_{SP2} and 10 clones showed a rearrangement of D_{SP2} sequence, namely four clones contained a single signal joint and six clones contained both coding joint and signal joint in the same molecule. In Figure 1, the rearranged sequences of these 10 clones are shown with the previous three sequences (Toda *et al.*, 1989). The five clones negative for D_{SP2} primers are 1.2 kb (pHC6, 13, 21 and 31) and 1.1 kb (pHC17) in length. We subcloned pHC6 and pHC17, and completed the sequences (1190 bases for pHC6; 1085 bases for pHC17) as registered in the databases under the accession number D13197 and D13198, respectively. pHC6 contained the region upstream of the pseudo D element (ψD_1) possessing a functional 3′ recombination signal but no 5′ signal (Lennon and Perry, 1989). pHC17 contained two recombination sites; one was a coding joint of another new pseudo D element [94% homologous to ψD_1 (Figure 1a) and thus termed ψD_2] and J_{H3}, and the other a signal joint of $D_{SP2.9}$ with J_{H4} (Figure 1b). The sequence downstream of signal joint was identical with the D_{SP2} probe containing ψD_3 element. Negative reaction of pHC17 to the antisense primer downstream of D_{SP2} may be due to a single base change in the $D_{SP2.9}$ spacer sequence.

Table I. Preparation of circular DNA clones and plaque hybridization

Mouse strain	Source	DNA preparation	Vector (cloning site)	No. of clones screened	Probes		mtDNA clones (%)
					D_{SP2}	V_H	
BALB/c	7WS	CL/CsCl–EtBr	λgt11 (*Eco*RI)	2.4×10^5	22	0	65.0
				2.7×10^5	10	ND	ND
BALB/c	7WS	CL/CsCl–EtBr	Charon 27 (*Bam*HI)	1.2×10^6	404	0	57.5
BALB/c	4WS	AL	λgt10 (*Eco*RI)	4.4×10^4	0	10	2.5
BALB/c	4WS	AL/CsCl–EtBr	λgt10 (*Eco*RI)	1.5×10^5	25	13	15.0
BALB/c	4WS	AL/CsCl–EtBr	λgt10 (*Eco*RI)	2.6×10^5	17	41	16.5
C57BL/6	fetal liver	CL/CsCl–EtBr	λgt10 (*Eco*RI)	2.7×10^5	1	0	87.0
C57BL/6	fetal liver	AL/CsCl–EtBr	λgt10 (*Eco*RI)	1.7×10^6	22	0	15.5
BALB/c	4WS	total	λgt10 (*Eco*RI)	1.9×10^6	15	38	0.005

Probe-positive clones were scored. V_H, coding regions of V_{HJ558}, V_{H36-60}, V_{HS107}, V_{HQ52} and V_{H7183}. Circular DNA was prepared from 'cleared lysate' (CL/CsCl–EtBr) or 'alkaline lysate' (AL) followed by CsCl–EtBr branding (AL/CsCl–EtBr). 4WS, 4 week old mouse splenocytes; 7WS, 7 week old splenocytes.

A partially rearranged DJ_H allele including ψD_1 segment has the capacity to initiate a D_μ transcript starting at the 5′ flanking region of the D segment (Nelson *et al.*, 1983; Reth and Alt, 1984; Lennon and Perry, 1989). However, the D_μ protein is produced only when the D segment rearranges to the J_H segment in the appropriate reading frame (RF), RF2 according to the numbering system of Ichihara *et al.* (1989). We thus examined the RF of DJ_H coding joints obtained from circular excision products produced by secondary DJ_H rearrangements (Figure 1a). We detected six RF1 and two RF3 while no RF2 was found.

N-insertions were noticed at eight of nine coding joints. However, only two N-insertions were found in 14 DJ_H signal joints (Figure 1b).

V_H-DJ_H recombinants in circular DNA clones

In order to analyze V_H-positive clones in circular DNA libraries prepared by the alkaline lysis method (Table I) in more detail, we selected four clones at random for recloning, restriction site mapping and sequencing. Three clones, pHC102 (1.5 kb), pHC103 (3.5 kb) and pHC104 (5.0 kb) contained different members of V_{HJ558} family in the germline configuration and another clone pHC105 (1.2 kb) contained the germline sequence of V_{H36-60} family (database accession numbers D13200–D13203). We prepared 3′V_H probes from these germline V_H sequence clones; 0.2 kb *Dde*I fragment (from pHC102), 0.15 kb *Ban*I–*Eco*RV fragment (from pHC103) and 0.1 kb *Ban*II–*Eco*RI fragment (from pHC105). However, no double-positive clones for 3′V_H and D_{SP2} probes were obtained in the circular DNA libraries. Since the excision products of V_H-DJ_H rearrangement may harbor several thousand varieties of V_H in germline configuration, a single V_HD_H signal joint may be too rare to be cloned into phage vectors. Alternatively, the murine V_H orientation may be completely inverted with respect to the DJ_H loci and the V_H-DJ_H rearrangements could generate both coding and signal joints in the same chromosomes. To distinguish between those two possibilities we tried to clone the V_HD_H signal joint following PCR amplification from either circular DNAs or chromosomal DNAs digested by *Eco*RI using primers specific to 3′V_H and 5′D_{SP2} as shown in Figure 2. For 3′V_H-specific primers, we used the most J_H-proximal V_H gene, V_{H81X} of the V_{H7183} family which is preferentially utilized in neonatal spleen (Yancopoulos *et al.*, 1984; Malynn *et al.*, 1990) and the most upstream and largest gene family, V_{HJ558} (Brodeur *et al.*, 1988). We amplified circular DNAs from 4 week old mouse spleen and fetal liver prepared by alkaline lysis and purified by banding in CsCl–EtBr gradients as well as chromosomal DNA from 4 week old mouse spleen and liver. PCR amplification of DNA using 3′V_H and 5′D_{SP2} primers having *Eco*RI or *Bam*HI cloning sites was attained exclusively in the circular DNAs from adult splenocytes. No PCR products were

Table II. D_{SP2}-positive circular DNA clones analyzed

Clones	Size (kb)	Sequences determined		
		Coding joint	Germline	Signal joint
pHC1	7.0		D_{SP2}	
pHC2	4.6		D_{SP2}	
pHC3	4.6		D_{SP2}	
pHC5	4.6		D_{SP2}	
pHC6	1.2		ψD_1	
pHC7	4.2	$D_{SP2.2}-J_{H1}$		$D_{FL16.1}-J_{H2}$
pHC8	4.1			$D_{SP2}-J_{H1}$
pHC9	5.0		D_{SP2}	
pHC11	4.0	$D_{SP2.6.7}-J_{H2}$		$D_{SP2.9}-J_{H3}$
pHC13	1.2		ψD_1	
pHC14	4.1			$D_{SP2}-J_{H1}$
pHC16	4.6	$D_{SP2.1.5}-J_{H1}$		$D_{FL16.1}-J_{H3}$
pHC17	1.1	ψD_2-J_{H3}		$D_{SP2.9}-J_{H4}$
pHC18	4.6		D_{SP2}	
pHC19	4.6		D_{SP2}	
pHC20	4.6	$D_{SP2.1.5}-J_{H3}$		$D_{FL16.1}-J_{H4}$
pHC21	1.2		ψD_1	
pHC22	4.6		D_{SP2}	
pHC23	5.2		D_{SP2}	
pHC24	5.1	$D_{SP2.8}-J_{H2}$		$D_{SP2}-J_{H3}$
pHC25	5.2		D_{SP2}	
pHC26	5.4			$D_{SP2}-J_{H4}$
pHC27	4.1			$D_{SP2}-J_{H1}$
pHC28	4.6		D_{SP2}	
pHC29	5.0		D_{SP2}	
pHC30	4.6		D_{SP2}	
pHC31	1.2		ψD_1	
pHC32	4.3	$D_{SP2.8}-J_{H1}$		$D_{FL16.1}-J_{H2}$

D_{SP2} probe-positive phage clones were recloned into plasmid vector pUC19 or pHSG399 and the sequences were determined using the specific primers of D_{SP2} and J_{H2-4} or the universal M13 primers for ψD_1 clones. Fragment lengths were measured using the references of *Sty*I digests of λ DNA. Fragment lengths 7.0, 5.2, 5.0 and 4.6 kb may correspond to the germline *Eco*RI fragments, 6.7, 5.4, 5.2 and 5.0 kb (Kurosawa and Tonegawa, 1982) respectively.

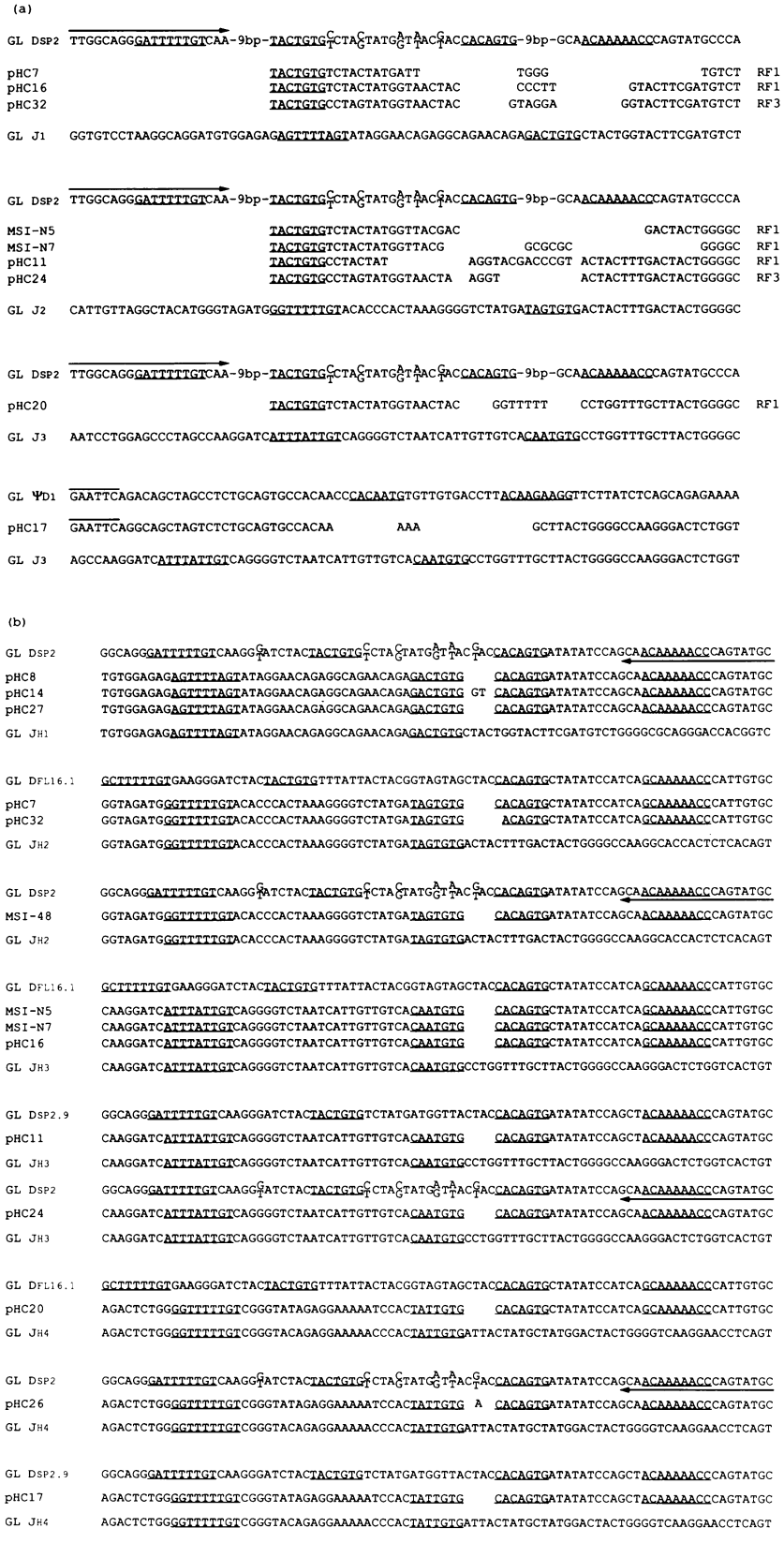

Fig. 1. Nucleotide sequences of D−J coding joint (**a**) and signal joint (**b**) of circular DNA clones. The recombinant sequences including the previous sequence, MSI-48, N5 and N7 (Toda *et al.*, 1989) are compared with the corresponding germline sequences of D_{SP2}, $D_{FL16.1}$ (Kurosawa and Tonegawa, 1982), $D_{SP2.9}$ (this work, database accession number D13199), J_H (Sakano *et al.*, 1980) and ψD_1 (Lennon and Perry, 1989). Two strain-specific polymorphic base changes are observed in the J_{H4} spacer sequence. Each reading frame is shown at the right. The signal sequences are underlined and the terminal *Eco*RI site is overlined. The sense and antisense sequence primers for D_{SP2} are overlined and underlined with an arrow, respectively.

recovered from chromosomal DNA of adult spleen and liver cells. These results demonstrated the presence of circular DNA excised by V_H to DJ_H joining and disproved the inverted orientation of all V_H loci. The amplified DNAs were digested by *Eco*RI and *Bam*HI, and cloned into the plasmid vector pHSG399. Southern blot analysis of undigested PCR products amplified from the circular DNA of adult splenocytes and hybridized with the mixed DNAs of V_{H81X}/D_{SP2} PCR clones revealed a fragment close to 93 bp which is expected from a precise signal joint of V_H-D_H joining (data not shown).

For sequence determination, we selected 10 clones amplified by $3'V_{H81X}$ and $5'D_{SP2}$ primers (Figure 2a) and eight clones by $3'V_{HJ558}$ and $5'D_{SP2}$ primers (Figure 2b). Every clone carried the V_H to DJ_H signal joint. Unexpectedly, N-insertions were found at every $V_{H81X}-D_{SP2}$ signal joint of 10 clones and at seven of eight $V_{HJ558}-D_{SP2}$ signal joints. In clone pHC122, one base was lost from the 3′ signal heptamer of V_{HJ558}. To confirm the V gene replacement circles identified in the cell line (Usuda *et al.*, 1992), circular DNAs from adult splenocytes were amplified using PCR primers, $5'V_{H81X}$ and $3'V_{HJ558}$. However, no PCR products were recovered. The specified V_H sequence to be replaced may be rare if not totally absent in the mixed cell population.

Discussion

Circular DNA preparations

Since extrachromosomal circular DNAs in eukaryotic cells form only a small fraction of total DNA (Rush and Misra, 1985), various preparation procedures have been developed. Stanfield and Helinski (1976) prepared a cleared lysate with detergent, partially purified extrachromosomal DNAs by banding in CsCl−EtBr gradients and then finally purified by a combination of denaturation−renaturation and nitrocellulose chromatography. To minimize the loss of nicked open circles, we introduced the treatment of ATP-dependent DNase, which acts only upon linear DNA molecules, in place of denaturation−renaturation (Yamagishi *et al.*, 1983). We successfully purified circular DNAs of a large size up to 150−200 kb as measured by electron microscopy (Fujimoto and Yamagishi, 1987; Iwasato *et al.*, 1990, 1992). This method allowed the isolation of the relatively small sized excision products of D_H-J_H rearrangement in high purity but failed to isolate larger circular DNAs of V_H-DJ_H rearrangement products. However, isolation of circular DNA by alkaline lysis and phase separation without pelleting the chromosomal DNA (Griffin *et al.*, 1981; Carroll *et al.*, 1987) in combination with PCR technology was useful to identify the presence of excision products of V_H to DJ_H rearrangements. This isolation method is indispensable for analyzing the large circular DNA of several thousand kb in length as estimated from the human V_H region locus (~3000 kb) (Matsuda *et al.*, 1988). However, the purity obtained by this method is limited compared with the cleared lysate and ATP-dependent DNase method.

Defective D elements

The D_{SP2} probe we used also contained the sequence homologous to ψD_1 gene (Lennon and Perry, 1989). Accordingly, we found new pseudo D elements, ψD_2 and ψD_3 among circular DNA clones. Although these entire germline sequences are truncated by an *Eco*RI cloning site, the ψD_2 element is 94% homologous to the 33 bp sequence downstream of the ψD_1 *Eco*RI site (Figure 1a) and ψD_3 is 93% homologous to the 394 bp sequence upstream of the ψD_1 *Eco*RI site (database accession number D13198). While ψD_1 is located upstream of $D_{SP2.3}$, another pseudogene ψD_{ST4} maps downstream of $D_{SP2.8}$ (Gerondakis *et al.*, 1988; Lennon and Perry, 1989). New pseudogenes ψD_2 and ψD_3 located between ψD_1 and ψD_{ST4} suggest that many other ψD elements are also scattered in D locus. Since

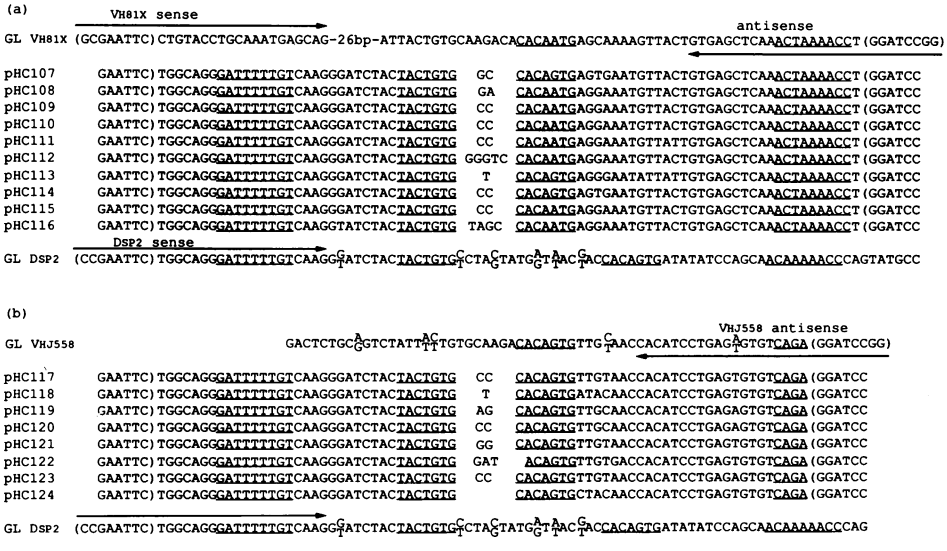

Fig. 2. Nucleotide sequences of V_H-DJ_H signal joints of DNA clones isolated by PCR amplification of circular DNA preparations. The sense and antisense PCR primers are overlined and underlined with an arrow, respectively. Cloning site sequences of *Eco*RI and *Bam*HI at the 5′ end of primers are shown in brackets. A pair of the V_{H81X} (V_{H7183} family) sense primer and the V_{HJ558} antisense primer was used to amplify the possible V gene replacement products.

it is suggested that D_H gene families are diverged from a primordial gene by repeated duplication (Ichihara *et al.*, 1989), the primordial D gene may have contained a set of D_{SP2} and ψD which multiplicated during evolution. Both pseudogenes ψD_1 and ψD_{ST4} possess a functional 3′ recombination signal, but no 5′ signal. However, any rearranged ψDJ_H allele may not produce the D_μ protein because every reading frame for the pseudo D elements contains a stop codon.

Reading frames of the D_H-J_H complex of circular DNA

The first step of rearrangements during the development of B-lineage cells is the joining of D and J_H. This is followed by either the replacement of existing DJ_H rearrangement by joining an upstream D segment to a downstream J_H segment, or the attachment of a V_H segment to the DJ_H complex. These two types of rearrangement occur at approximately equal frequencies (Reth *et al.*, 1986). Although $D-J_H$ joining generates all three reading frames of $D-J_H$ complex, a strong bias for the expression of one particular reading frame (RF1) has been noticed in murine antibodies (Kaartinen and Mäkelä, 1985; Ichihara *et al.*, 1989). Gu *et al.* (1991) explained the selection of D_H reading frames by the molecular properties of the D_H elements in the germline and the joining process itself. According to this model, RF1 usage is promoted by short sequence homologies between D and J_H elements (Gu *et al.*, 1990), RF3 is counterselected on the basis of stop codons, and D_H-J_H joints in RF2 are expressed as a membrane protein (D_μ protein) (Gu *et al.*, 1991; Tsubata *et al.*, 1991) which may prevent V_H-DJ_H recombination through a mechanism analogous to allelic exclusion so that the majority of cells die. Thus, biased reading frames found in the excision products of DJ_H rearrangements (Figure 1a) support the current model of D_H RF selection. As shown in Figure 1a, the absence of RF2 in D_H-J_H coding joints replaced by secondary D_H-J_H joining indicates that the membrane-bound D_μ protein may inhibit further D_H-J_H replacements as well as V_H-DJ_H rearrangements. Both D_H-J_H and V_H-DJ_H joining processes may be subject to allelic exclusion mediated by the D_μ protein.

Differential N-insertion at signal joints

Two separate controls operate in D−J and V to DJ rearrangements of the TCR β locus and probably also in the IgH locus (Ferrier *et al.*, 1990). A dominant *cis*-acting enhancer element activated in both pre-T and pre-B cells initiates D−J rearrangements, while a second cell type specific element controls V to DJ rearrangements. In adult B cells, N region diversity is generally greater at the V_H to D_H coding joint than at the D_H to J_H coding joint (Gu *et al.*, 1990). This could be accounted for by the level of TdT expression at the two types of rearrangement. However, the striking difference in N-insertions at signal joints between the D−J and V−DJ joints was unexpected. This indicates that N-insertions in the signal joint may not only be correlated with the TdT activity in lymphocytes but also with the local differences in chromatin structure or in 'accessibility' of TdT to chromatin. Since most D_H segments carry their own 5′ transcriptional promoter element and also produce a D_μ messenger RNA (Reth and Alt, 1984), the chromatin structure of the D_H to J_H region may be opened for recombinase 'accessibility' by transcriptional activation as previously suggested (Yancopoulos and Alt, 1986). Such full recombinase accessibility may compete with the TdT activity for the signal ends and generate no N-insertions at the D_H-J_H signal joint. However, signal ends in V_H-DJ_H recombination may be long-lived enough to interact with the TdT. In this way N-insertions may depend on the unequal access of the recombinase and TdT to signal and coding ends.

Materials and methods

Preparation of circular DNA

Lymphocytes were obtained by teasing the tissues on a wire-mesh from spleens of 4 week old and 7 week old BALB/c mice or from fetal liver of C57BL/6 mice at 18 days gestation. Circular DNAs were enriched from the 'cleared lysate' (Stanfield and Helinski, 1976) or from the 'alkaline lysate' (Carroll *et al.*, 1987) of lymphocytes. Circular DNAs of the 'cleared lysate' were purified by banding in a CsCl−EtBr gradient and ATP-dependent DNase treatment as described (Yamagishi *et al.*, 1983; Harada and Yamagishi, 1991). Circular DNAs of the 'alkaline lysate' were purified by phase separation after extraction with phenol and chloroform, followed by ATP-dependent DNase treatment. However, digestion of linear DNA fragments was incomplete, due to the presence of single-stranded DNA fragments that are inhibitory to the enzyme action on double-stranded DNA. Residual linear DNA fragments were separated by banding in a CsCl−EtBr gradient.

Circular DNA clone libraries

Circular DNAs were digested by restriction enzymes and cloned in the *Eco*RI site of λgt10 or λgt11 phage vector, or in the *Bam*HI site of Charon 27 phage vector. The recombinant DNA was packaged *in vitro*. Phage titers per μg of vector DNA were 10^5-10^6. Plaque hybridization was performed according to the method of Maniatis *et al.* (1982). The D_{SP2} probe (pHC100) was a 0.8 kb *Hin*dIII−*Eco*RI fragment subcloned into plasmid pSP65, originally derived from a phage DNA clone provided by Dr Y.Kurosawa (Kurosawa and Tonegawa, 1982). V_H probes were a mixture of 260 bp *Pst*I−*Hin*fI fragment of V_{HNP} (V_{HJ558}), 180 bp *Bam*HI−*Hae*III fragment of V_{HA} (V_{H36-60}), 285 bp *Hin*fI−*Hha*I fragment of V_{HT15} (V_{HS107}), 250 bp *Sin*I−*Bst*EII fragment of V_{HS9} (V_{HQ52}) and 240 bp *Eco*RI−*Hae*III fragment of V_{HC5} (V_{H7183}) (Nakanishi *et al.*, 1982; Neuberger, 1983; Yaoita *et al.*, 1983, 1988). All V_H probes contained only the coding region of the respective V_H gene. The mitochondrial DNA probe was a 0.2 kb and a 2.0 kb *Eco*RI fragment of BALB/c mouse mitochondrial DNA.

PCR amplification

Polymerase chain reaction (PCR) amplification was performed using *Tth* DNA polymerase (Toyobo Co. Ltd) and the *Eco*RI digested circular DNA or chromosomal DNA as described by Saiki *et al.* (1988). After 4 min preincubation at 94°C, 30 cycles of PCR amplification were run for 1 min at 94°C, 2 min at 56°C and 3 min at 72°C, and followed by 7 min incubation at 72°C. The 20mer sequences of the sense primer for V_{H81X} (V_{H7183} family, Yancopoulos *et al.*, 1984) and for D_{SP2} (Kurosawa and Tonegawa, 1982), and those of the antisense primer for V_{H81X} and for V_{HJ558} [consensus germline sequence of 23 members registered in the database, MUSIGHV(A,B,I1,J2,K2,L,M2,R), MUSIGHW(A,C,D,E,F,G,H,O,T,U,VX,W) and MUSIGHX(M,N,O)] are shown in Figure 2. *Eco*RI and *Bam*HI cloning sites were joined to the 5′ end of PCR primers so as to facilitate cloning of PCR products. PCR products were confirmed by Southern blot hybridizations (Maniatis *et al.*, 1982).

DNA sequence analysis

For sequence determinations, recombinant phage clones were recloned into plasmid vectors, pUC19 or pHSG399. Nucleotide sequences were determined by the dideoxy chain termination method (Sanger, 1981) using the universal M13 primer M4, reverse primer RV, the sense primers upstream of J_{H2}, GAGGCAGTCAGAGGCTAGCT; J_{H3}, GGCCTCCATCTGAGATAATC; J_{H4}, CTGCACAGGCAGGGAACAGA (Sakano *et al.*, 1980), or appropriate specific primers for D_{SP2} as shown in Figure 1.

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