Immunology 1998 **95** 291–301

Co-existence of somatic hypermutation and gene conversion in hypervariable regions of single Igκ clones

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SUMMARY

In the rabbit, recent investigations have provided evidence that gene conversion leads to the generation of diversity of heavy chain rearranged V_H–D_H–J_H genes. No data have been published on a similar mechanism for rabbit light chains. In our laboratory, we initially infected rabbits with *Trypanosoma brucei*, which stimulates B-cell hyperplasia and hypergammaglobulinaemia. The heterozygous rabbits exhibited the Cκ1 b4 and b9 kappa light chain allotypes. After reverse transcription of mRNA, and cloning and sequencing of cDNA, the Vκ–Jκ–Cκ genes provided evidence for both somatic hypermutation and gene conversion. We saw that in each of the b4 and b9 kappa light chain cDNA, CDR1 and CDR3 carried both point mutation and provisional gene conversion traits. In the CDR2 region, point mutation and gene conversion inserts were observed in the b4 genes, with only gene conversion in two b9 genes. In the CDR regions, although some genes exhibited only somatic hypermutation or gene conversion, others showed linkage of both somatic hypermutation and gene conversion in the same sequence. This also marks the first time that somatic hypermutation and gene conversion in the same cloned CDR region has been observed in Vκ1 genes; however, it has been seen earlier in rabbit heavy chain VH sequences. Furthermore, the addition of several codons to the CDR3 segment by gene conversion may have provided a mechanism for length variation. In addition, we demonstrated that Jκ and framework region segments contained examples of somatic hypermutation. Confirmation of gene conversion necessitates that donor sequences be identified as providing the templated inserts. Thus after cloning two pseudogenes we found putative CDR3 donor segments for two CDR3 rearranged genes. The results offer additional mechanisms for the generation of diversity among rearranged rabbit kappa light chain genes. Whether there is a relationship or influence of gene conversion upon somatic hypermutation or vice versa is not discernable at present.

INTRODUCTION

Mechanisms responsible for the generation of diversity among complementarity determining regions (CDR) in antibody molecules are under intensive scrutiny. It is known that during antigenic stimulation, and especially during the secondary antibody response, a considerable number of nucleotides undergo change, resulting in the generation of new codons and higher affinity antibodies. Two types of mutation currently under investigation are the non-templated form called somatic hypermutation, in which single base changes result in new codons, and the templated form or gene conversion, in which blocks of codons are donated, usually from an upstream site. In gene conversion, because immunoglobulin genes in mammals show considerable complexity, it is difficult to identify specific introduction of donor sequences, in contrast to the avian system.[1–5] Nevertheless, templated insertion in mammalian immunoglobulin genes is usually deduced by inspecting databases, putative genomic donor sequences,[3] or, when more than one nucleotide insertion into one or more codons or a block of codons is demonstrated.[3,6] Thus we refer to the process here as gene conversion-like. However, as reported in this paper, we have discovered potential donor sequences for two gene converted inserts in CDR3 segments.

The rabbit is an interesting model to investigate the generation of diversity among immunoglobulins and antibodies. For example, considerable polymorphism is evident among immunoglobulin kappa light chains (IgK), in which the so-called allotypes are presumably inherited in an allelic co-dominant autosomal manner. The allotypes have been designated as Cκ1 b4, b5, b6 and b9. V_H immunoglobulin allotypes are presumably also inherited in a similar manner and are designated V_Ha1, a2 and a3. Although the rabbit has been used traditionally for the induction of antibodies even against notoriously weak antigens, there have been recent claims that it may be somewhat deficient in generating combinatorial diversity. The reasons have been ascribed to the fact that only one Jκ gene (J2) is functional for most allotypes,

Received 13 November 1997; revised 8 June 1998; accepted 8 June 1998.

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with two (J1 and J2) functioning for the Cκ1b9 allotype of the five Jκ genes found.[7–9] In contrast, all five Jκ genes are functional in the human, with four of five recombining actively in the mouse. It has also been demonstrated that a single V_H gene, V_H1, the most proximal to the D gene segment, encodes 70–90% of the entire antibody repertoire for the rabbit heavy chain.[10,11] A gene conversion mechanism in which upstream donor V_H sequences were utilized presumably led to diversification of V_Ha genes. Although the rabbit genome may contain about 100 additional V_H genes, they are thought to have limited function. Another notion to explain the rabbit's apparent deficiency to produce antibodies is the so-called 'pecking' order of allotypic expression, i.e. a1 > a2 > a3 and b4 > b5 = b6 > b9, in which, in a heterozygote, immunoglobulins are expressed unequally according to their allotype, i.e. in a b4,b9 rabbit much more antibody is synthesized by b4-expressing cells than by b9.[12,13] The reasons for this are not apparent but may be accounted for by a preponderance of b4 receptored and/or secreting cells in the b4,b9 animal, or a diminished capacity of antibody-synthesizing ability in the b9-carrying cells. However, the rabbit may invoke certain strategies to compensate for the deficit. One major mechanism is the known longer length and heterogeneity of the Vκ CDR3 region, which has been seen to vary over a range of eight amino acids, in contrast to the mouse and human in which variation of fewer amino acids has been reported. We were interested initially in exploring the mechanism(s) for the dominance of b4 over b9 in the b4,b9 animal. Therefore, after infection with *Trypanosoma brucei*, known to lead to B-cell hyperplasia and hypergammaglobulinaemia, we constructed cDNA by polymerase chain reaction (PCR) from mRNA. Although we did not discover any data to account for the abundance of b4 over b9, we demonstrated that a number of cloned Vκ sequences for both b4 and b9 contained evidence of somatic hypermutation as well as gene conversion. Furthermore, possible donor CDR3 sequences were found among two pseudogenes.

The significance of individual CDR displaying antigen-induced somatic hypermutated sites directly linked to gene conversion sites, the latter presumed to be independent of antigen induction, is discussed.

MATERIALS AND METHODS

Rabbits and infection with T.brucei

Two male rabbits from our colony, A101 and 8201A4, both typed as a1, 3/b4, 9/e15, 15, were infected with 10^6 purified live *T.brucei*, Yeager strain, by intradermal injection. Popliteal lymph nodes, spleen and liver were removed at 28 days post-infection and the tissues quick-frozen in liquid N_2 and stored at −75°.

Oligonucleotides

DNA oligomers were obtained from the DNA Synthesis Service of the Hospital of the University of Pennsylvania (Philadelphia, PA). The PCR primers and nucleic acid probes used in this study are shown in Table 1. Primers PCR01 and PCR02 included a short spacer and the recognition sequence for *Sma*I or *Hind*III at their 5′ ends, so that after PCR amplification the cDNA could be cleaved with these restriction enzymes and cloned into a plasmid vector. The PCR01 primer was taken from the 3′ end of the Cκ1 region and contained a block of identical nucleotides for b4, b5, b6 and b9.[14] PCR02 represented a partial sequence at the 5′ end of the leader region of the κ1 gene.[15] Both primers were expected to yield a fragment 766 bp long. The internal oligonucleotide probe (IPb4) used to detect b4 sequences contained a number of unique nucleotides representative of the b4 gene compared to the other b genes.[14] IPb9 was an internal oligonucleotide probe that detected only b9 sequences. The specificity of the amplification of b4 and b9 VJC sequences was confirmed by nucleotide sequence analysis.

Table 1. Oligonucleotides used as primers and probes

Designation*	Sequence (5′→3′)†
PCR01	cctactgaagctctggtccgg
PCR02	ctgggcctcctgctgctctggctccca
PCR03	ttgtatcactgtggtcatacaatcaccacc
PCR04	ttgtatcactgtggtatcactactactata
PCR05	accgacgagactcttagtgagagtcgtc
PCR06	gccgttgaaccgcgatgg
IPb4	aggtgacccagggcacgacc
IPb9	tccacaactcgggctcagcgat
IP FR1	gtggggaggcacagtcaccatca

*The primers designated PCR02 contained a short spacer and a recognition site for *Hin*dIII, whereas the primers designated PCR01, PCR03, PCR04 and PCR05 also contained a short spacer and a recognition site for *Sma*1.

†The primer PCR01 is a universal primer for the b4, b5, b6 and b9 genes. It is located at position 281–300 of the Cκ region at the 3′ end.[14] The primer PCR02 is a partial sequence at the 5′ end of the κ1 leader region.[15] Internal probes (IPb4, IPb9) were specific for b4 and b9 Cκ1 and were derived from the sequences described in our previous publication (Fig. 1)[14] PCR03 consisted of 15 nucleotides from the intron adjacent to the CDR3 region, together with 15 nucleotides from the 3′ end of CDR3 of the b4,4, A4 3 clone (Fig. 2). Primer PCR04 also consisted of 15 nucleotides from the intron adjacent to the CDR3 region but was attached to the terminal 3′ 15 nucleotides of b9,9 A4 11 clone. Primer PCR06 consisted of 18 nucleotides from codon 59 to 64.

Genomic DNA preparation

High molecular weight DNA were extracted from frozen liver of rabbit 9201 (b4,b4) and rabbit 5758 (b9,b9) by the salting-out procedure of Miller *et al.*,[16] care being taken to avoid degradation. DNA prepared in this way varied between 100 and 250 kb, as determined by pulsed field electrophoresis (J. Liu & B. Wolf, unpublished data).

Southern blot analysis of genomic DNA

We followed a strategy similar to that published by Ayadi *et al.*[15] The liver DNA were initially digested with *Bgl*II, *Bam*HI and *Hind*III, the fragments separated on 2% agarose and transferred to nylon filters. The transferred fragments of the b4 rabbit (9201) DNA were probed with a PCR product that contained the VK sequence from a cDNA b4 clone (A4-3, Fig. 1; structure shown in Fig. 2, lanes 1–3). Likewise, the transferred fragments of the b9 rabbit (5758) DNA were probed with a cloned cDNA b9 PCR product A4-11 (structure shown in Fig. 2, lanes 4–6). Figure 1 (lanes 1–6) shows the results after low stringency washing [6 × saline sodium citrate

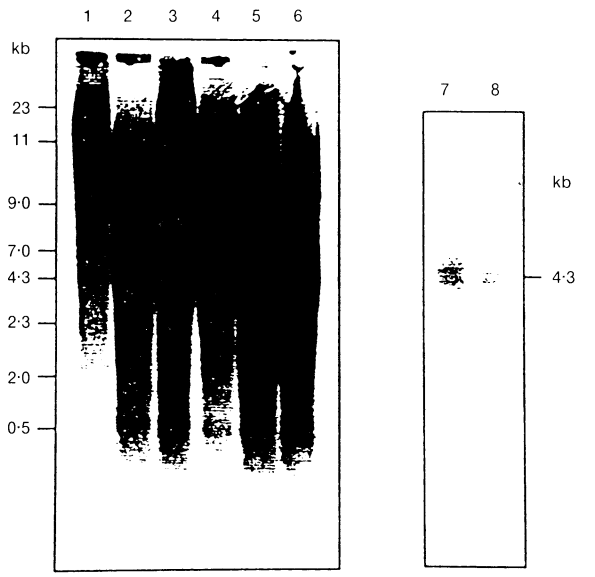

Figure 1. Genomic DNA from frozen liver of rabbit 9201 (b4,b4) were digested with *Bgl*II (lane 1), *Eco*RI (lane 2) and *Hind*III (lane 3), and probed with a PCR-derived VJC sequence from a rabbit typed as b4,b4 (see text). Similarly, genomic liver DNA from rabbit 5758 (b9,b9) were digested with *Bgl*II (lane 4), *Eco*RI (lane 5) and *Hind*III (lane 6). The fragments were visualized with the same probe after low stringency washing. Lanes 7 and 8 represent the single fragment at 4·3 kb of rabbit 9201 and 5758, respectively, after high stringency washing (see text). The single band in lane 7 contained the genomic sequences Vκ21, Vκ22, and Vκ24. The single band in lane 8 contained the genomic sequence Vκ23 and Vκ26.

(SSC), 0·1% sodium dodecyl sulphate (SDS), 42°, four washes]. The filters were then washed at high stringency (0·1 × SSC, 0·1% SDS, 65°, four washes), resulting in the disappearance of all of the bands except the one hybridizing at 4·3 kb [lane 7, rabbit 9201 (b4,b4) DNA, digested with *Hind*III; lane 8 rabbit 5758 (b9,b9) DNA also digested with *Hind*III].

Cloning and sequencing of genomic DNA

The 4·3 kb DNA fragments were isolated from agarose gels by electroelution. Using the primers PCR02 and PCR03 (Table 1) a fragment was amplified from the b4,b4 rabbit (9201) liver DNA fragment digested with *Hind*III. The primer PCR03 consisted of 15 nucleotides from the intron adjacent to the CDR3 region, together with 15 nucleotides from the 3′ end of the CDR3 of the b4 clone A4-3 (Fig. 2). Using the PCR01 and PCR04 primers, a fragment was amplified from the b9,b9 rabbit (5758) liver DNA fragment digested with *Hind*III. The primer PCR04 (Table 1) also consisted of 15 nucleotides from the 3′ intron adjacent to the CDR region, but was attached to the terminal 3′ 15 nucleotides of the CDR region of clone A4-11 (the cDNA b9 clone). The PCR products were ligated into the *Hind*III/*Sma*I site of the pUC 18 plasmid. Transformation was carried out using maximum efficiency DH5α competent cells (BRL, Gaithersburg, MD) following the manufacturer's instructions. Recombinant colonies were screened with end-labelled internal oligonucleotide probes using the same hybridizing and washing conditions as described for Southern analysis. Plasmid DNA was isolated from purified positive clones by a miniprep method using cetyltrimethylammonium bromide.[17] The sequence analysis was performed on plasmid DNA by the dideoxy chain termination technique of Sanger *et al.*[18] The genomic sequences Vκ 21, 22, 24, and 26 shown in Fig. 2 were thus derived from the cloned sequences.

Derivation of pseudogenes containing potential donor sequences

DNA was purified from livers from two rabbits, 8603A5 (b4,b4) and X-90 (b6,b6). PCR was performed using the two primers, PCR05 and PCR02 (Table 1). PCR05 was derived from the intron flanking the 3′ end of the Vκ region, as reported by Heidmann & Rougeon.[19] After cloning and sequencing, the donor segments were found in the CDR3 in single clones from each of the two rabbits (Fig. 3).

PCR of Kl mRNA

Total RNA was purified from lymph nodes and spleen by an acid guanidinium thiocyanate–phenol–chloroform extraction procedure. A total of 10 mg of total RNA was mixed with 25 pmol of PCR01 and heated at 92° for 3 min. For reverse transcription (RT) the samples were chilled in ice and mixed with 5 μl 5 × Maloney murine leukaemia virus (MMLV) reverse transcriptase buffer (BRL), 2·5 μl 10 mM dNTP, 1 μl transplacental RNase inhibitor and 200 U of MMLV reverse transcriptase in a total volume of 25 μl, and incubated at 37° for 1 hr. For PCR, RT reaction mixtures were mixed with 7·5 μl 10 × PCR buffer [200 mM Tris, pH 8·4, 500 mM KCl, 25 mM $MgCl_2$, 1 mg/ml nuclease-free bovine serum albumin (BSA)], 5 μl 10 mM dNTP, 50 pmol of PCR01 and PCR02, the volume made up to 100 μl with distilled water, heated at 94° for 8–10 min, and cooled on ice. *Thermus aquaticus* (Taq) DNA polymerase (2·5 U); Roche Molecular Systems Inc., Branchburg, NJ) was then added and subjected to 40 cycles of PCR. Each cycle consisted of denaturation at 94° for 1 min, annealing at 55° for 40 seconds and extension at 72° for 3 min. To avoid any PCR contaminations, special precautions were undertaken that included (1) a separate work area for PCR; (2) use of positive displacement pipettes; (3) aliquoting PCR premixes (containing each of the components except target DNA and Taq DNA polymerase) in sterile hoods; (4) storage of PCR premixes apart from the target DNA; and (5) running a DNA blank (a reaction that contained all the components except target DNA), as discussed in our previous paper.[14]

Analysis of PCR products

The PCR products were separated on a 2% agarose gel. The fragments were transferred to nylon membranes (ICN, Cleveland, OH) and detected by hybridization to appropriate 5′-^{32}P end-labelled internal oligonucleotide probes. The filters were prehybridized in 6 × SSC, 10 × Denhardt's solution and 100 mg/ml denatured salmon sperm DNA, at 42° for 2 hr, and hybridized in the same solution at 42° for 16–20 h. The filters were washed three times in 6 × SSC, 0·1% SDS at 42°, followed by twice in 0·5 × SSC, 0·1% SDS at 65°, and exposed to Kodak XOMAT-XAR film (Eastman Kodak, Rochester, NY) for 1–2 days at −70° using intensifying screens.

RESULTS

Following cloning of the PCR-amplified products into the pUC18 vector of 51 cloned sequences identified under low

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Genomic Controls:											FR 1													↓
Vκ20		GCG	CTT	GTG	ATG	ACC	CAG	ACT	CCA	TCC	CCT	GTG	TCT	GCA	GCT	GTG	GGA	GGC	ACA	GTC	ACC	ATC	AGT	TGC
VκAy-C		-AT	GGC	---	---	---	---	---	---	G--	--C	---	---	---	---	---	---	---	---	---	---	---	-A-	---
Vκ21	b4	-AT	GGC	---	---	---	---	---	---	---	T-C	---	---	---	---	---	---	---	---	---	---	---	-AG	---
Vκ22	b4	G-C	A-C	---	---	---	---	---	---	---	T-C	AC-	---	---	C--	---	---	---	---	---	---	---	-A-	---
Vκ24	b4	G-C	A-C	---	---	---	---	---	---	G--	T-C	AC-	---	-A-	C--	---	---	---	---	---	---	---	-AG-	---
Vκ26	b9	--C	---	---	---	---	---	---	---	--G	---	---	---	---	G--	---	---	---	---	---	---	---	-A-	---
Vκ18a		-AC	-C-	---	---	---	---	---	---	--T	T-C	AC-	---	--G	---	---	---	---	---	---	---	---	-AC	---
Vκ19a		--C	-AA	---	C--	---	---	---	---	G--	T-C	---	---	---	---	---	---	---	---	---	---	---	-A-	---
Rearranged Sequences-experimental:																								
A4-15	b9	-AC	A--	---	---	---	---	---	---	G--	T-C	---	---	-A-	C--	---	---	---	---	---	---	---	-AG	---
A4-5	b9	-AC	GGC	---	---	---	---	---	---	---	T-C	AC-	---	---	---	---	-T-	---	---	---	---	---	-A-	--T
A101-5	b9	-AC	--C	---	---	---	---	---	---	G--	T-C	---	---	---	---	---	---	---	---	---	---	---	-AG	---
A4-3	b4	--C	---	--A	---	---	---	---	---	---	T-C	---	---	---	---	---	---	---	---	---	---	---	-A-	---
A4-2	b4	--A	T-C	-AA	T--	---	---	---	---	--T	T-C	-C-	---	-A-	C--	---	---	---	---	---	---	---	-AG	---
A4-8	B4	--C	-AA	---	---	---	---	---	---	G--	T-C	---	---	---	C--	---	---	---	---	---	---	---	-A-	---
A101-1	b4	--C	A-C	-AT	---	---	---	---	---	G--	T-C	---	---	---	---	---	---	---	---	---	---	---	-AG	---
A101-2	b4	--C	A-C	-AT	---	---	---	---	---	--T	T-C	---	---	---	---	---	---	---	---	---	---	---	-AG	---

	24	25	26	27	27a	27b	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44
	CDR 1										↓				FR 2								
Genomic Controls:																							
Vκ20	CAG	TCC	AGT	GAG	AGC	GTT	TAT	AGT	AAC	AAC	CGC	TTA	TCC	TGG	TTT	CAG	CAG	AAA	CCA	GGG	CAG	CCT	CCC
	Gln	Ser	Ser	Glu	Ser	Val	Tyr	Ser	Asn	Asn	His	Leu	Ser										
VκAy-C	---	G--	---	C--	---	A--	GG-	--C	G--			---	---	---	-A-	---	---	---	---	---	---	---	---
	Gln	Ala	Ser	Gln	Ser	Ile	Gly	Ser	Ser			Leu	Ser										
Vκ21 b4	---	G--	---	---	---	A--	AG-	---	---	---	--G	---	G--	---	---	---	---	---	---	---	---	---	---
	Gln	Ala	Ser	Glu	Ser	Ile	Ser	Ser	Ser	Asn	Arg	Leu	Ala										
Vκ22 b4	---	G--	---	C--	---	---	---	---	G--	---	---	---	G--	---	-A-	---	---	---	---	---	---	---	---
	Gln	Ala	Ser	Gln	Ser	Val	Tyr	Ser	Ser	Asn	His	Leu	Ala										
Vκ24 b4	---	G--	---	---	---	A--	---	--C	G--	---	---	---	G--	---	-A-	---	---	---	---	---	---	---	---
	Gln	Ala	Ser	Glu	Ser	Ile	Tyr	Ser	Ser	Asn	His	Leu	Ala										
Vκ26 b9	--A	---	---	C--	A--	A--	---	---	---	---	---	---	G--	---	---	---	---	---	---	---	---	---	---
	Gln	Ser	Ser	Gln	Ser	Ile	Tyr	Ser	Asn	Asn	His	Leu	Ala										
Vκ18a	---	---	---	C--	-AT	---	---	---	---	---	TA-	---	---	---	---	---	---	---	---	---	---	---	---
	Gln	Ser	Ser	Gln	Asn	Val	Tyr	Ser	Asn	Asn	Tyr	Leu	Ala										
Vκ19a	---	G--	---	---			AGC	-T-	-GT	-G-	TA-	---	AA-	---	-A-	---	---	---	-T-	---	---	---	---
	Gln	Ala	Ser	Glu			Ser	Ile	Ser	Ser	Tyr												

Figure 2. Sequences of rearranged Vκ–Jκ–Cκ genes representing four of our experimentally derived b9 sequences and seven of our b4 sequences. Nine control genomic sequences are cited from the literature: Vκ20,[35] Vκ18a and Vκ19a,[34] AyC[15] and J1κ (b9), J2κ(b9) and J2κ(b9) and J2κ(b4).[9] Vκ21, 22 and 24 (b4) and Vκ23 and 26 (b9) were derived by us from rabbit 9201 (b4,b4) and rabbit 5758 (b9,b9) respectively. In CDR1, CDR2 and CDR3 in our experimentally derived sequences, the codons which contain somatic hypermutation replacement nucleotides are indicated with a single underline and those derived through give conversion contain a double underline. For the framework and Jκ region sequences, somatic hypermutation is depicted with a single underline.

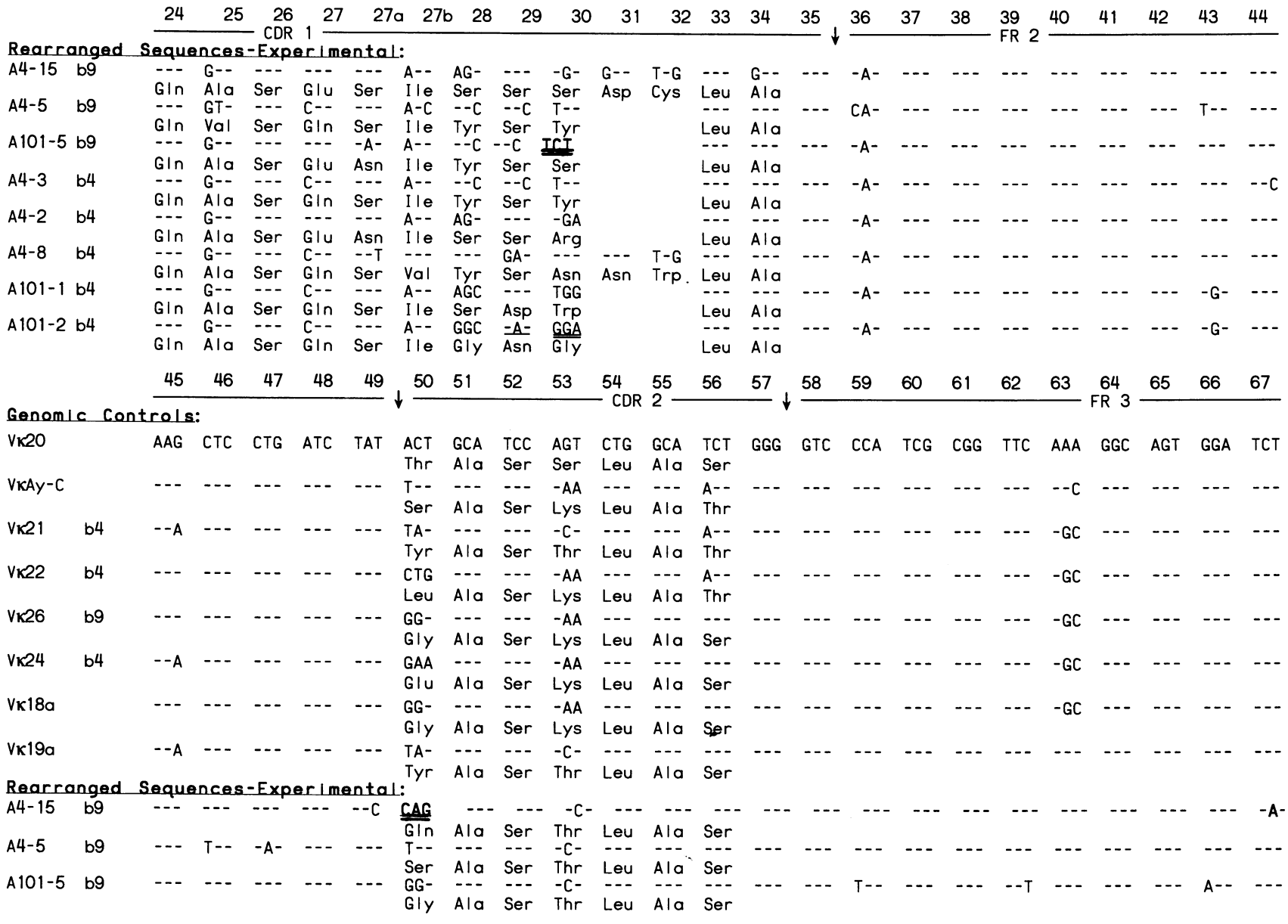

Figure 2. (*continued*).

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		45	46	47	48	49 ↓	50	51	52	53	54	55	56	57 ↓	58	59	60	61	62	63	64	65	66	67
										CDR 2												FR 3		
A4-3	b4	---	---	---	---	--C	CTG	---	---	-AA	---	---	---	---	---	---	---	---	---	---	---	---	---	---
							Leu	Ala	Ser	Lys	Leu	Ala	Ser											
A4-2	b4	---	---	---	---	---	T--	---	---	**-A-**	---	---	---	---	---	T--	---	---	---	---	---	---	---	---
							Ser	Ala	Ser	Asn	Leu	Ala	Ser											
A4-8	b4	---	---	---	---	---	GG-	---	---	-AA	---	---	---	---	---	---	---	---	---	-GC	---	---	---	---
							Gly	Ala	Ser	Lys	Leu	Ala	Ser											
A101-1	b4	---	---	---	---	---	GA-	**A--**	---	**GA-**	---	---	---	---	---	---	---	---	---	---	---	---	---	---
							Asp	Thr	Ser	Asp	Leu	Ala	Ser											
A101-2	b4	---	---	---	---	---	GA-	A--	---	-C-	---	---	---	---	---	---	---	---	---	---	---	---	---	---
							Asp	Thr	Ser	Thr	Leu	Ala	Ser											

		68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88 ↓
							FR 3															
Genomic Controls:																						
Vκ20		GGG	ACA	CAG	TTC	ACT	CTC	ACC	ATC	AGT	GGC	GTG	GAG	TGT	GAC	GAT	GCT	GCC	ACT	TAC	TAC	TGT
VκAy-C		---	---	---	--T	---	---	---	---	--C	---	---	C--	---	---	---	---	---	---	---	---	---
Vκ21	b4	---	---	G--	---	---	---	---	---	--C	---	---	C--	---	--G	---	---	---	--C	---	---	---
Vκ22	b4	---	---	---	---	---	---	---	---	---	---	A--	C--	---	---	---	---	---	---	---	---	---
Vκ24	b4	---	---	G--	-A-	---	---	---	---	--C	---	---	C--	---	---	---	---	---	---	---	---	---
Vκ26	b9	---	---	---	-A-	---	---	---	---	---	---	---	C--	C--	---	---	---	---	---	---	---	---
Vκ18a		---	-A-	---	---	---	---	---	---	---	---	---	C--	---	---	---	---	---	---	---	---	---
Vκ19a		---	-C-	G--	-A-	---	--T	---	---	--C	--T	---	C--	---	---	---	---	---	---	---	---	---
Rearranged Sequences-Experimental:																						
A4-15	b9	---	---	G--	---	---	---	---	---	--C	---	---	C--	C--	--G	---	---	---	--C	---	---	---
A4-5	b9	---	---	G--	---	---	---	---	---	--C	--T	---	C--	C-C	--G	---	---	---	--C	---	---	---
A101-5	b9	---	---	G--	--A	---	---	---	---	--C	---	---	C--	C--	--G	---	---	---	--C	---	---	---
A4-3	b4	---	---	G--	---	---	---	---	---	--C	-A-	C--	---	---	-C-	---	---	---	---	--T	--T	---
A4-2	b4	---	---	G--	--T	---	---	---	---	--C	--T	---	C--	---	---	---	---	---	---	---	---	---
A4-8	b4	---	---	G--	---	---	---	---	---	---	---	**-AI**	C--	---	---	---	---	---	---	---	---	---
A101-1	b4	---	---	---	---	---	---	---	---	--C	---	---	C--	---	---	---	---	---	---	---	---	---
A101-2	B4	---	---	---	---	---	---	---	---	--C	---	---	C--	---	---	---	---	---	---	---	---	---

Figure 2. (*continued*).

		89	90	91	92	93	94	95	95a	95b	95c	95d	96	97
							CDR 3							
Genomic Controls:														
Vκ20		GCA	GGC	TAT	AAA	AGT	TAT	AGT	AAG	TGA	TGA	TAC		
		Ala	Gly	Tyr	Lys	Ser	Tyr	Ser	Lys	*	*			
VκAy-C		CA-	T--	AC-	T-T	---	AG-	---	-CT	G-T				
		Gln	Cys	Thr	Tyr	Ser	Ser	Ser	Thr	Gly				
Vκ21	b4	CTA	--T	GG-	T-T	CA-	---	G--	-CT	G-T	A-A	A-T	GCT	
		Leu	Gly	Gly	Tyr	His	Tyr	Gly	Thr	Gly	Arg	Asn	Ala	
Vκ22	b4	---	--G	---	T-T	---	---	---	-GT	G-T	-TT	GCT		
		Ala	Gly	Tyr	Tyr	Ser	Tyr	Ser	Ser	Gly	Phe	Ala		
Vκ24	b4	CA-	---	GG-	--T	TA-	AG-	G--	---	G-T	G--	---		
		Gln	Gly	Gly	Asn	Thr	Ser	Gly	Lys	Gly	Gly	Tyr		
Vκ26	b9	CA-	---	GG-	---	TA-	A--	---	-GT	G-T	GAT	ACT		
		Gln	Gly	Gly	Lys	Thr	Asn	Ser	Ser	Gly	Asp	Thr		
Vκ18a		---	--G	---	T-T	TA-	AG-	G--	-GT	G-C	ACT	G-T		
		Ala	Gly	Tyr	Tyr	Thr	Ser	Gly	Ser	Gly	Pro	Asp		
Vκ19a		CAG	CAT	GG-	T-T	-T-	---	---	-GT	G-T	GAT	ACT		
		Gln	His	Gly	Tyr	Ile	Tyr	Ser	Ser	Gly	Asp	Thr		
Rearranged Sequences-Experimental:														
A4-15	b9	CT-	---	AG-	T-T	---	AG-	---	G-T	ACT	GCT			
		Leu	Gly	Ser	Tyr	Ser	Ser	Ser	Asp	Thr	Ala			
A4-5	b9	CT-	--T	GG-	T-T	---	ATG	-TA	--T	-AC	C--	CCT	GCT	
		Leu	Gly	Gly	Tyr	Ser	Met	Ile	Asn	Tyr	Arg	Pro	Ala	
A101-5	b9	CT-	--T	GGG	TTT	T-G	GT-	---	-TA	AAT	-AC	CGA	ACT	GCT
		Leu	Gly	Gly	Phe	Trp	Val	Ser	Ile	Asn	Tyr	Arg	Thr	Ala
A4-3	b4	CGG	CAG	GG-	T-T	GT-	AGC	---	GGT	G-T	GTT	G-T	TGT	AAT
		Arg	Gln	Gly	Tyr	Val	Ser	Ser	Gly	Gly	Val	Asp	Cys	Met
A4-2	b4	CA-	T-T	AC-	C-T	TA-	GG-	G--	--T	-AT	G--			
		Gln	Cys	Thr	His	Tyr	Gly	Gly	Asn	Tyr	GTY			
A4-8	b4	CA-	---	AG-	T-T	T--	---	-TG	TG-	--T				
		Gln	Gly	Gly	Tyr	Tyr	Tyr	Met	Trp	Cys				
A101-1	b4	CAG	CAT	GG-	T-T	-T-	---	---	-GT	G-T	GAT			
		Gln	His	Gly	Tyr	Ile	Tyr	Ser	Ser	Gly	Asp			
A101-2	b4	CAG	CAT	GG-	T-T	-T-	---	---	-GT	G-T	GAT			
		Gln	His	Gly	Tyr	Ile	Tyr	Ser	Ser	Gly	Asp			

Figure 2. (*continued*).

stringency, eight clones retained a strong hybridization signal after high stringency washing using the b4 internal probe IPb4 (Table 1). Employing the b9 internal probe IPb9 (Table 1), of 68 clones initially detected at low stringency four clones showed a strong high stringency signal. Upon sequencing, two of the b4-positive sequences were judged to be non-functional because they each contained stop codons in their CDR3 regions. Figure 2 shows five of the six b4 and three of the four b9 functional sequences. They were presumed to be functional because when compared with other published rabbit Igκ sequences, the structures were in-frame and contained unaltered framework region sequences, Jκ and Cκ regions and splice sites.

CDR variation

The CDR1, CDR2 and CDR3 regions all contained a number of hypermutated sites caused by point mutation and putative gene conversion insertions. Replacement sites representing somatic hypermutation are depicted with a single underline, and the gene conversion sites are designated with a double underline (Fig. 2). In the CDR1 region, five genomic controls for b4 and b9 cloned sequences were compared with four DNA sequences from other laboratories (Fig. 2 legend). (Heidmann *et al.*[19] have published two other genomic Vκ sequences, 18b and 19b, which were essentially similar to those shown.) In the CDR1 region amino acids 24–34 were conventionally designated as hypervariable (Wu–Kabat plot),[20,21] and in our experimental sequences for b4 the only amino acids showing nucleotide substitutions were 29 and 30. Among the b9 genes, there was a new codon in gene A101-5 at amino acid 30, a putative gene conversion insert. In the b4 genes, for sequences 101–2 and 101–4 (data not shown), amino acid 29 contained a single nucleotide replacement (G>A), and amino acid 30 in clones 101–2 and 101–4 (data not shown), respectively, contained two new codons, both glycine, which are candidates for gene conversion. The single nucleotide substitutions in both clones were adjacent to the gene conversion inserts. It is quite possible that these candidate single codon gene conversion inserts came from progenitor B cells located in the appendix, as reported by Weinstein *et al.*[3] for rabbit V_H gene conversion-like products. Locating these progenitors would, of course, put our thesis of gene conversion on firmer grounds.

In the CDR2 region for the b9 genes, the sequences were also mostly invariant, with only A4–11 (data not shown) and A4–15 showing candidate single gene conversion inserts at codon 50. For the b4-derived genes in the CDR2 region, two linked somatic hypermutation gene conversion-like products were found in sequences 101–1 and 101–4 (latter not shown).

	98	99	100	101	102	103	104	105	106	107	108	109	110	111
	———				Jκ							↓		Cκ
Genomic Controls:														
J1κb9	TTC	GGA	GCT	GGC	ACC	AAT	GTG	GAA	ATC	AAA	TGT			
	Phe	Gly	Ala	Gly	Thr	Asn	Val	Glu	Ile	Lys	Cys			
J2κb9	---	--C	-GA	--G	---	G-G	C--	--G	---	CT-	---			
	Phe	Gly	Gly	Gly	Thr	Glu	Leu	Glu	Ile	Leu	Cys			
J2κb4	---	--C	-GA	--G	---	G-G	---	-TC	G--	---	G--			
	Phe	Gly	Gly	Gly	Thr	Glu	Val	Val	Val	Lys	Gly			
Rearranged Sequences-Experimental:														
A4-15 b9	---	---	---	---	---	---	---	---	---	---	---	GAT	CCT	CCA
	Phe	Gly	Ala	Gly	Thr	Asn	Val	Glu	Ile	Lys	Cys			
A4-11 b9	---	---	---	---	---	---	---	--G	---	---	---	GAT	CCT	CCA
	Phe	Gly	Ala	Gly	Thr	Asn	Val	Glu	Ile	Lys	Cys			
A4-5 b9	---	---	---	---	---	---	---	---	---	---	---	GAT	CCT	CCA
	Phe	Gly	Ala	Gly	Thr	Asn	Val	Glu	Ile	Lys	Cys			
A101-5 b9	---	---	A-C	---	---	---	C--	---	---	---	---	GAT	CCT	CCA
	Phe	Gly	Asp	Gly	Thr	Asn	Leu	Glu	Ile	Lys	Cys			
A4-3 b4	---	--C	-GA	--G	---	G-G	---	-TC	G--	---	G--	GAT	CCA	GTT
	Phe	Gly	Gly	Gly	Thr	Glu	Val	Val	Val	Lys	Gly			
A4-2 b4	---	--C	-GA	--G	---	G-G	---	-TC	G--	---	G--	GAT	CCA	GTT
	Phe	Gly	Gly	Gly	Thr	Glu	Val	Val	Val	Lys	Gly			
A4-8 b4	---	--C	-GA	--G	---	G-G	---	-TC	G--	---	G--	GAT	CCA	GTT
	Phe	Gly	Gly	Gly	Thr	Glu	Val	Val	Val	Lys	Gly			
A101-4 b4	---	--C	-GA	--G	---	G-G	---	ATG	G--	-G-	G--	GAT	CCA	GTT
	Phe	Gly	Gly	Gly	Thr	Glu	Val	Ser	Val	Arg	Gly			
A101-1 b4	---	--C	-GA	--G	---	G-G	A--	ATG	G--	---	G--	GAT	CCA	GTT
	Phe	Gly	Gly	Gly	Thr	Glu	Val	Ser	Val	Lys	Gly			
A4-9 b4	---	--C	-GA	--G	---	G-G	---	-TC	G--	---	G--	GAT	CCA	GTT
	Phe	Gly	Gly	Gly	Thr	Glu	Val	Val	Val	Lys	Gly			
A101-2 b4	---	--C	-GA	--G	---	G-G	---	ATG	G--	---	G--	GAT	CCA	GTT
	Phe	Gly	Gly	Gly	Thr	Glu	Val	Ser	Val	Lys	Gly			

Figure 2. (*continued*).

CDR3

	89	90	91	92	93	94	95	95a	95b	95c	95d	96	97
Vκ24	CAA	GGC	GGT	AAT	TAT	AGT	GGT	AAG	GGT	GGA	TAC		
A101-5	-T-	--T	--G	TT-	-GG	GT-	A--	-TA	AA-	TAC	CGA	ACT	GCT
A4-5	-T-	--T	---	T--	AG-	-TG	ATA	--T	TAC	C--	CCT	GCT	
P1	---	---	--C	---	---	---	ATG	-TA	AA-	TAT	C--	TAG	
P2	---	---	--C	---	--G	-T-	ATA	T-T	TA-	C--	---	AGT	TAG

Figure 3. Candidate pseudogene sequences, P1 and P2, taken from liver DNA of rabbits 8603A5 (b4,4) and X-90 (b6,b6), respectively, are compared to the rearranged genes A4-5 and A101-5. The genomic sequence Vκ serves as control. The putative donor sequences as well as the gene conversion inserts are designated by a double underline.

A considerable number of clones exhibited extensive diversity in the CDR3 region for both b4 and b9. In the b9 set of genes, we obtained longer gene conversion inserts. There was a continuous six-codon insert in sequence 101–5 at amino acids 92–95b. Adjacent to these codons were other codons with single nucleotide replacements. Furthermore, sequence A4–5 had a four-codon gene conversion insert at amino acids 94–95b. This gene segment also contained a single nucleotide replacement at amino acid 89. For b4, clone A4–2 showed a three-codon gene conversion-like product at amino acids 95a, 95b, and 95c, whereas clone A4-8 contained a two-codon insert at amino acids 95 and 95a.

In the CDR3 region, both hypervariability and length variability contribute to junctional diversity. In this report, among the b4 gene segments A4-2, A4-8, and among the b9 sequences A101–5 and A4-5, the insertion of gene conversion-donated segments could have contributed to the extended length of CDR3.

Pseudogenes as candidate donors

Figure 3 depicts two pseudogene CDR3 segments (P1, P2) which could serve as donors for the four-codon A4-5 and six-codon A101–5 putative gene conversion inserts. We compared these segments (double underlined) to our Vκ 24 genomic sequence; however, other genomic sequences, as well as thousands scanned in a gene database, did not contain the candidate gene conversion inserts. The P1 and P2 'donor' segments did not completely match the inserts, having single nucleotide differences. Again, it is possible that progenitor B cells are present in the appendix, which would more yield donor

sequences directly, as described by Weinstein *et al.*[3] for V_Ha genes.

Jκ region variability

Somatic hypervariability has been reported for Jκ nucleotides at the Cκ–Jκ joint in humans,[22] as well as throughout the Jκ region in mice,[23] but not so far in rabbits. Among our clones, we saw replacement substitutions for several b4 and b9 sequences, and these are underlined in Fig. 2. The substitutions have not been reported previously for any of the κ1 and κ2 genes and may also contribute to antibody diversity in the rabbit as a limited number of Jκ genes were apparently activated, as discussed above.

Framework region variability

Somatic hypervariability has also been seen in framework regions but not to date in rabbit Vκ regions.[3,24] Many of our clones showed hypervariability and one replacement codon is underlined in Fig. 2.

To summarize these results, we demonstrated linked somatic hypermutation and candidate gene conversion sites in the CDR1 and CDR3 regions. The CDR2 region showed only gene conversion sites. There did not appear to be any bias among b4 and b9 genes in expression of linked variation. Furthermore, somatic hypermutation, but not gene conversion, was seen in the J2 gene segment as well as in the framework regions, which indicates that gene conversion targeting lies only in the area of antigen contact sites.

DISCUSSION

Although a considerable number of papers have been published on sequence alterations reflective of somatic hypermutations as well as gene conversion among immunoglobulin genes, only one report has been published for rabbit V_H genes.[3] Furthermore, the mechanism for either process remains poorly understood. Using cDNA after PCR of mRNA from lymph nodes of *T. brucei*-infected rabbits, we detected examples of linkage of the two processes in each of the CDR. In regard to somatic hypermutation, this is consistent with the findings of others in each CDR. Although induction of somatic hypermutation is often investigated using simple determinants, e.g. 4-hydroxy-3-nitrophenyl-acetic acid (NP) or 2-phenyloxazalone (phOX), a number of studies have employed multideterminant antigens.[25]

Although the variable surface glycoprotein (VSG) of *T. brucei* contributes in a major way to an antibody response, it is not known whether the VSG or other antigens contribute to somatic hypermutation. However, it is intriguing that a study was conducted in BALB/c mice in which infection with *T. brucei* led to a strong anti-phosphoryl choline antibody response that showed the same idiotype (PC) as TEPC-15 as well as antibodies to the TEPC-15 idiotype.[26] Phosphoryl choline has been routinely employed to investigate somatic hypermutation.[27]

In the mouse and human, gene conversion apparently is not responsible for CDR variation, notwithstanding certain observations attesting to it.[5,28,29] Although there are no reports of rabbit Vκ gene conversion, accumulating evidence in rabbit V_H[3,9,10,11,30] and in chicken V_H and Vλ[15] establishes templated insertion of codons in these regions. The work of Parng *et al.*[31] is especially intriguing as they reported gene conversion in bovine Vλ genes as well as identifying candidate pseudogene donors. The reasons for gene conversion to contribute to antibody diversity in rabbit, chicken and bovine and not the mouse and human systems remain unclear. As to the site of origin for somatic hypermutation as well as gene conversion, an important discovery was recently made for the rabbit V_H region.[3] It was found that both types of mutation took place in appendix germinal centres of young rabbits. The rabbit appendix may thus serve as a corollary for the chicken bursa in which gene conversion transpires.[32]

The CDR1 and CDR2 regions apparently exhibited linked templated and untemplated mutations in our studies. It is important to mention, however, that since CDR1 and CDR2 (as well as FR1) (Fig. 2) contained single codons in some clones that were interpreted to represent gene conversion as well as somatic hypermutation, the base substitution may have resulted from Taq polymerase errors. In order to confirm the validity of the sequences, we (1) sequenced two cloned PCR preparations derived at different times; (2) showed that there were no inhibitors in the PCR mix as the cloned DNA and reagents were verified to be pure; (3) analysed multiple clones; (4) performed PCR using another primer pair (internal control) taken from the leader and FR3 sites (Table 1; PCR02 and PCR06) which gave the same results as the original primer pair for CDR1, CDR2 and FR1; and (5) employed $Vent_R$ DNA polymerase, which has 3′–5′ proofreading activity. We used the conditions suggested by Mattila *et al.*[33] for optimizing fidelity. This polymerase has an approximately six-fold greater fidelity of base incorporation than Taq polymerase. Thus we feel that the clones probably exhibited putative CDR1, CDR2 and FR1 gene conversion and somatic hypermutation. Additional evidence that single codons may have been inserted by gene conversion comes from the study of Weinstein *et al.*,[3] who showed procursors in the rabbit appendix for IgH, as well as from McCormack *et al.*,[34] who presented evidence that pseudogene Vλ elements could donate sequences as short as four nucleotides in the chicken. Furthermore, Wheeler *et al.*[35] showed integration of H-2 mouse single codon gene donors into yeast.

However, the more complex alterations appear to reside in the CDR3 region. Here, we discovered two b4 allotype genes, one having undergone three-codon gene conversion alterations. Among the b9 genes, we found two clones that contained somewhat longer inserts. One was a six-codon segment in clone A101-5 in the 92–95b region. This region has not been previously reported, as judged by inspecting 10477 DNA sequences from human, mouse and other sources in a recent database. In a second clone, A4–5 (from another rabbit), a six-codon templated replacement was seen (amino acids 94–95d). Both clones contained untemplated replacement codons. It is possible that the two pseudogene CDR3 segments (Fig. 3) acted as donors for the rearranged genes, but whether they come from upstream sources is not known. However, in comparing the P1 'donor' sequence to the A101–5 acceptor we found that there is a three-codon match from codons 95a–95c plus an additional base, C, at 95d, except for a mismatch of T for C at 95c. It is possible that from the base T at 94 through to the base C at 95d (except for 95, in which

two bases are mismatched) the insert also results from the P1 donor. In respect to the P2 donor, the match for A4-5 would include five codons of the six, except for single nucleotide mismatches at 94 and 95a. We are currently searching for longer donor segments that would match the functional genes more closely. Mismatch repair may have resulted from mutations within the converted sequences or other conversion events may have led to more exact inserts. There are numerous examples in the literature in which the donor sequences do not match the functional gene. Several were found in the work of Parng *et al.*[31] in which one donor had three mismatches and another had two. A short segment was also adjudged to be a partial donor. Other papers also cite donors that have mismatches: Becker & Knight[11] found several donors that contained mismatches and, in addition to single base changes, di-nucleotide changes were reported, as was the case with us. Likewise, Weinstein *et al.*[3] found several mismatches within single donors, as did Carlson *et al.*[36] for chicken V_L1 genes and Reynaud *et al.*[37] for the chicken VλI.

The insertions in CDR3 did not come from joining variability at the Vκ–Jκ joint for the following reasons: (1) the A4–5 gene-converted clone also showed at the Vκ juncture at amino acid 96, a genomic GCT (ala) corresponding to the genomic clone Vκ21; (2) the A101-5 gene-converted clone containing a six-codon insert also exhibited at two terminal amino acid sites, 95c and 95d, the genomic codons TAC (tyr) and CGA (arg), respectively, corresponding to the genomic clones 18b[38] and our Vκ25 (both not shown); and (3) it has been well established that joining variability, i.e. N and P nucleotide additions, occur only at the V(D)J coding ends. However, N-region additions in rabbit Vκ–Jκ joints in a careful study by Ayadi *et al.*[15] were not seen, and other investigators, i.e. Davis & Bjorkman[39] and Rolink and Melchors,[40] have shown that Igκ and λ do not usually have N-region additions. As for P additions, in a study in which 138 clones were analysed, the longest of the P additions was seven nucleotides.[41] In our clones A4-2 and A4-8, in which putative inserts three codons in length were found at the terminal 3′ end, these nine nucleotide segments were non-paliindromic as well. However, without determining donor segments, it is possible, although unlikely, that N additions could have accounted for the three codons in these CDR3 sequences.

What is the role of gene conversion in somatic hypermutation if the former is independent of antigen induction? Are the cells that have undergone gene conversion more prone to somatic hypermutation and, if so, does the templated insert somehow play a primary role for hypermutation? If not, a secondary role may be postulated in which genes responsible for hypermutation use the templated insert as a signalling device, perhaps at the level of the nucleosomal apparatus.

Recently, Maizels[5] has advanced a theory that attempts to unify gene conversion and somatic hypermutation in a single model. Initially, a lesion is created that may be a single-stranded nick or a double-stranded break. The lesion could be a hot spot for hypermutation. Following this, a templated insertion or, alternatively, a targeted mutagenesis could take place. This could result in a variation of the length[42,43] at the site of the original break as well as the formation of certain structures, e.g. hairpins or cruciforms, which would lead either to repair or pairing and/or the influence of factors leading to heteroduplex formation or single base substitution. Finally repair would ensue leading to ligation. We might add that the theory does not account for the specificity of antigen-induction or whether certain cells, e.g. T-helper cells, may be involved in the mutation process.

ACKNOWLEDGMENT

We wish to thank Drs Rose Mage and Mohammad Ishaq for their careful and critical reading of the manuscript and for valuable suggestions. This work was supported by a grant from the National Science Foundation DCB 9005021.

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