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Research Article

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Ethnic Differences in Polymorphism of an Immunoglobulin V_H3 Gene

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Abstract

The VH26 germline gene occupies two different loci, due to gene duplication, and is one of the most frequently expressed human immunoglobulin V_H genes. This report identifies the alleles of each VH26 locus and describes distinct patterns of VH26 polymorphism in three ethnic groups. Oligonucleotide probes targeting VH26 were used in sequence-specific RFLP analysis of DNA from 72 Caucasians, 52 Asians, 35 American Blacks, and members of six families. The A locus, on a 7.0-kb TaqI band, was detected in 89% of Caucasians, 75% of Asians, and 26% of Blacks ($\chi^2 = 46$, $P < 0.0005$). The B locus, detected on a 5.0-kb band in nearly all subjects, was found to have additional alleles occurring at 6.8 kb in 10% of Asians and 3% of Blacks ($\chi^2 = 7.8$, $P < 0.02$) and at 3.7 kb in 1.4% of Caucasians, 21% of Asians, and 9% of Blacks ($\chi^2 = 13.8$, $P < 0.001$). In Asians only, the 3.7-kb hybridization band represented a multiple-duplication unit containing three or four gene copies. Duplications of other VH26 alleles, and null alleles of the B locus, were also seen. An exact VH26 sequence was cloned from the 5.0-kb allele and likely exists in the 7.0- and 6.8-kb alleles. A novel sequence cloned from the 3.7-kb allele differed from VH26 by nine nucleotides and appears to have evolved by gene conversion in CDR2. The total diploid gene dose of the A and B loci ranged from one to as many as six copies of VH26-containing genes, and from zero to as many as six to eight copies of the 3.7-kb allele. We conclude that ethnic differences in polymorphism exist at both VH26 loci. These differences could influence VH26 expression because they involve variations in gene copy number and coding region sequence. (*J. Clin. Invest.* 1995. 96:1591–1600.) Key words: alleles • ethnic groups • genes, immunoglobulin V_H germ line • oligonucleotide probes • polymorphism, restriction fragment length

Introduction

The variable regions of immunoglobulin H chains are encoded by V_H, D, and J_H segments that are juxtaposed from their respective multigene clusters by gene rearrangement. The V_H gene segment encodes most of the V_H protein, including framework regions 1, 2, and 3 and complementarity determining regions (CDR)[1] 1 and 2. On the basis of sequence homology, V_H genes fall into seven families, V_H1 to V_H7. About half of V_H germline genes belong to the largest family, V_H3. The haploid genome contains > 100 V_H genes, of which 86 have been mapped to loci in the J_H-proximal 1,100 kb of the V_H cluster on chromosome 14 (1, 2). The functional V_H repertoire is dominated by considerably fewer genes than this total because many V_H elements are pseudogenes and because, for unclear reasons, a number of V_H genes are expressed more often than expected from random use.

An increasing body of data shows that many V_H genes are polymorphic (3–14). The repeated cloning of the exact sequences of several V_H genes from unrelated people indicates, however, that many V_H elements are highly conserved in the population. These observations are reconciled by evidence that V_H gene loci are usually dominated by only one to four prevalent alleles (8, 11). A more complete understanding of V_H allelism has been slow to emerge because, with conventional Southern analysis, the identity of V_H elements in allelic restriction fragments is usually not known, and because, when similar V_H sequences are cloned from unrelated individuals, it is routinely not possible to know whether they are alleles or from distinct V_H loci.

We have previously studied V_H gene polymorphism by using synthetic oligonucleotide probes in a modified Southern hybridization procedure (5, 8, 9, 11, 15). This technique confers exquisite specificity upon RFLP analysis. As a result, the nucleotide sequence of target regions in detected genes can be exactly predicted, and closely homologous genes can be readily distinguished. Duplications, deletions, coding region variations, and RFLP have been detected, and allelic relationships among V_H elements have been elucidated in the three largest V_H gene families, V_H1, V_H3, and V_H4.

In the present report, the study of V_H polymorphism is extended to the V_H3 gene VH26[2] (16, 17), also known as 18/2 (18), 30p1 (19), DP-47 (20), or V3-23 (1). VH26 is of interest because it is frequently expressed by both fetal and adult B lymphocytes, and often appears to be overexpressed, relative to other V_H genes (19, 21–25). VH26 has frequently been found in autoantibodies, including anti-DNA Ab and rheumatoid factors, and has been associated with systemic lupus erythematosus via the 16/6 idiotype, which it encodes (18, 26–28). VH26 also encodes specificity for several microbial antigens, including rabies virus (29), *Haemophilus influenza* type b capsular polysaccharide (30, 31), Staphylococcal protein A (32, 33), and HIV gp120 (34). The latter two are ligands with broad reactivity for V_H3 antibodies and have been suggested to be B cell superantigens (34–36).

Exact VH26 sequences have been reported in the genomes

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1. *Abbreviations used in this paper:* CDR, complementary determining region; TMACl, tetramethylammonium chloride.

2. Throughout this paper, VH26 refers to a germline gene originally reported by Matthysens and Rabbits (16), whose corrected sequence was published by Chen et al. under the name VH26c (17).

of numerous unrelated subjects, indicating that the VH26 gene is prevalent. A previous study showed that the VH26 germline gene occupies two distinct loci, presumably the result of gene duplication (37). RFLP analysis with oligonucleotide probes identified VH26 genes on TaqI fragments of 7.0 and 5.0 kb, designated the A and B loci, respectively (37). On some chromosomes 14 the A locus is absent (12, 37). The types of polymorphism at the A and B loci, and the prevalences of their respective alleles, have not been fully described. We have now examined the germline representation of VH26 in Caucasians, Asians, and American Blacks. We found that both VH26 loci are polymorphic and that significant ethnic differences exist at each VH26 locus.

Methods

Study subjects and determination of ethnicity. Peripheral blood was obtained from 159 healthy volunteers, who comprised the study population from which gene prevalences were determined. Samples were also obtained from family members of five of these volunteers, and from a sixth family (No. 0172), none of whose members were included in the main study population. Ethnicity of all subjects was determined by direct questioning by one of the authors (E.H. Sasso). Each subject was asked to state the ethnicity and country of birth of each grandparent and to state whether any earlier ancestors were of different ethnicity. When family members of mixed ethnicity were identified, the ethnicity of preceding generations was questioned in detail to identify the generation in which all ancestors had pure ethnicity. All subjects appeared honest and forthright in answering these questions. Follow-up interviews were performed when needed, and uncertainty was recorded when appropriate. For each subject, this information was used to construct a family tree of ethnicity and to calculate percentage of ethnicity. For example, a Black subject with a White maternal grandfather and a Native American paternal great grandfather would be 62.5% Black, 25% White, and 12.5% Native American.

Of the 52 Asian subjects, 50 were 100% Asian; these included 20 Chinese, 10 Koreans, 7 Japanese, 4 Cambodians, 1 Vietnamese, and 1 Filippino. 7 Asians were of mixed Asian ethnicity, including 5 subjects who were at least half Cambodian (2 were 25% Chinese, 1 was 50% Chinese, 1 was 50% Vietnamese, and 1 was 12.5% Chinese and 12.5% Vietnamese) and 2 others who were at least half Chinese (1 was 25% Vietnamese and 1 was 50% Japanese). The remaining 2 Asians were Filippino subjects who were partly Caucasian (25 and 12.5%). All 35 Blacks in this study were American Blacks. Pedigrees of 33 Black subjects established that 9 described themselves as 100% black, 30 as at least 75% Black, and 3 as less than 75% Black (62.5, 56.25, and 56.25%, respectively). The ethnic composition of these 33 Black subjects (i.e., the population mean for each ethnicity) was 86.1% Black, 8.5% Native American (several tribes), 3.2% Caucasian, 0.4% Asian, and 1.8% unknown. The pedigrees of 2 Black subjects were unavailable.

Preparation of DNA and agarose gels. The preparation and enzymatic digestion of genomic DNA extracted from peripheral blood leukocytes, and the separation of DNA on 1% agarose gels, 12 μg DNA per lane, have been previously described (8).

Hybridization in agarose gels. All techniques of in situ hybridization of ^{32}P end-labeled oligonucleotide probes to DNA in dried-down agarose gels have been previously described (8). The hybridization temperature for each probe in this study was 57°C. The temperatures of postwashes in 3.2 M tetramethylammonium chloride (TMACl) and the duration of subsequent film exposures of hybridized gels are indicated in the figure legends. Gels were stripped of probe and prepared for reuse as previously described (8).

Analysis of hybridization band intensity. Intensity of hybridization bands was determined by visual inspection. To demonstrate intensities corresponding to one and two VH26 copies at the A and B loci, respectively, gels included a control DNA sample (Virginia Mason Research Center subject 3116) whose haplotypes of VH26 (37) and other V_H germline genes (15, 37, 38) have been precisely established by hybridization analysis, V_H gene cloning, and V_H gene mapping. With the standard as a reference, interlane and intralane comparisons were made. When uncertain results needed clarification, new gels were prepared with new restriction digests of selected DNA samples. In such cases, test samples were loaded in gel lanes adjacent to the control or some other DNA whose VH26 hybridization phenotype was already established. Possible lane-to-lane variations in DNA quantity were controlled by hybridizing each gel with a Cμ oligonucleotide probe and by examining each gel under UV light following staining with ethidium bromide. It has been found previously that in gene systems where most hybridization bands contain one versus two gene copies, as in this study, phosphorimaging or quantitative image analysis do not significantly improve data accuracy (11, 15, 37).

Statistical analysis. Statistical statements are presented as a chi-square value and a *P* value derived from chi-square analysis with two degrees of freedom, that is, by considering all three ethnic groups simultaneously in a two-by-three data set. Also given in each case are the *P* values obtained from the two-ethnic-group comparisons, where C = Caucasian, A = Asian, and B = Black.

Oligonucleotide probes. All deoxyoligonucleotide probes employed in this study were 21-mers in the sense orientation and were synthesized at the Howard Hughes Medical Institute (University of Washington, Seattle, WA). Probes M8 and M24 target CDR1 and CDR2 of the VH26 sequence and have been previously described (37). Probe M1 targets the Cμ gene and has the sequence 5′-GGGAGTGCATCCGCCCCAACC-3′. Probes S1 and S2 target overlapping regions of CDR2 in the 3.7-kb gene sequence reported herein and have the sequences S1, 5′-GGTATTTATAGCGGTGGTAGT-3′ (codons 50–56), and S2, 5′-GGTAGTAGCACATACTACGGA-3′ (codons 55–61). Probes M19 (8, 15), M41 (15), S4 (=M27; reference 11), and S5 (=M28; reference 11) have been described previously. Probes M8, M24, M1, M19, and M41, and both PCR primers (described later) were the generous gift of Dr. Eric C. B. Milner (Virginia Mason Research Center, Seattle, WA).

Cloning of selected V_H elements. 1-μg aliquots of genomic DNA prepared from peripheral blood leukocytes from two subjects were amplified by 38 cycles of PCR using TaqI polymerase in Buffer A (Promega, Madison, WI). Each cycle was denatured for 30 s at 96°C, annealed for 30 s at 50°C, and extended with TaqI polymerase for 30 s at 72°C, with a 7-min extension after the last cycle. The 5′ primer (5′-CTGAATTCCATGGAGTTTGGGCTGAG-3′) contained an EcoRI site and targeted the 5′ leader region of V_H3 genes. The 3′ primer (5′-GACTCTAGACAATGAACTTCCCCTCACT-3′) contained an XbaI site and targeted the VH3 recombination signal sequence. PCR products were digested with XbaI and EcoRI, purified on Qiaquick columns (QIAGEN, Inc., Chatsworth, CA), ligated into the XbaI/EcoI site of pUC 18, transformed into *Escherichia coli* DH5 alpha competent cells, and plated on agar containing ampicillin with isopropyl-β-D-thiogalactopyranoside and bromo-4-chloro-3-indoyl-β-D-galactoside, according to standard methods. Nylon filter lifts from the colony plates were screened by hybridization with probe M8 using previously described methods (11).

Nucleotide sequence analysis of selected VH germline genes. DNA was prepared from selected colonies, and double-stranded sequence analysis was performed with the BaseMaster Sequencing Kit (Pharmacia Diagnostics AB, Uppsala, Sweden).

Results

Ethnic differences in the VH26 A locus. The VH26 gene has been shown to exist at two different loci, A and B (37). The oligonucleotide probe M8 targets CDR1 of the VH26 sequence (Fig. 1) and specifically detects the A and B loci on TaqI fragments of 7.0 and 5.0 kb, respectively (Fig. 2). To examine polymorphism of each VH26 locus, probe M8 was hybridized to

A

Genes	30			CDR1			
VH26	AGC	AGC	TAT	GCC	ATG	AGC	TGG
3.7 kb	---	---	---	---	---	---	---
Probe							
M8	---	---	---	---	---	---	---

B

Genes	50					CDR2						60
VH26	GCT	ATT	AGT	GGT	AGT	GGT	GGT	AGC	ACA	TAC	TAC	GCA
3.7 kb	-T-	---	TA-	A-C	G--	---	A--	---	---	---	--T	---
Probes												
M24						---	---	---	---	---	---	---
S1	-T-	---	TA-	A-C	G--	---	A--					
S2						---	A--	---	---	---	--T	---

Figure 1. Sequence-specific oligonucleotide probes. Nucleotide sequences of VH26, and the VH26 allele cloned from the 3.7-kb TaqI fragment, are depicted in (*A*) for CDR1 and (*B*) for CDR2. Oligonucleotide probes (*A*) M8 and (*B*) M24 target VH26. Probes S1 and S2 (*B*) target the VH26 3.7-kb fragment allele. Nucleotides are specified only where they differ from the VH26 reference sequence. Residues comprising CDR1 and a part of CDR2 are indicated by a horizontal line over the VH26 sequence. Codon numbering is according to Kabat et al. (39).

TaqI-digested genomic DNA from healthy subjects from three different ethnic groups: 72 Caucasians, 52 Asians, and 35 American Blacks. The 7.0-kb A band was detected in 64 Caucasians (89%), 39 Asians (75%), and 9 Blacks (26%) (Table I). No other TaqI band (i.e., RFLP) was seen that bore any clear relationship to the absence of the 7.0-kb band. These results indicate that the VH26 gene at the A locus is absent more often

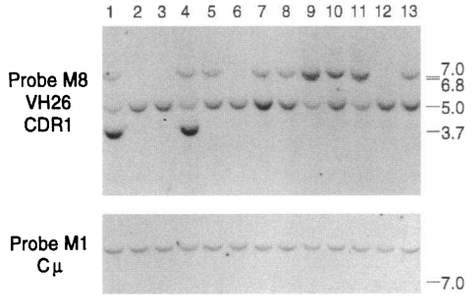


Figure 2. Detection of VH26 and related germline genes by in situ oligonucleotide probe hybridization. Aliquots of DNA extracted from peripheral blood leukocytes of 13 unrelated donors were digested with TaqI and separated on a 1% agarose gel, 12 μg DNA per lane. The gel was dried down and hybridized with [^{32}P]end-labeled probe M8 (*top*), targeting VH26 in CDR1, then stripped of probe and hybridized with probe M1 (*bottom*), targeting the Cμ gene. Probe M8 detects four VH26-related hybridization bands, on fragments of 7.0 kb (locus A) and 6.8, 5.0, and 3.7 kb (all locus B). Probe M1 detects the nonpolymorphic Cμ gene with similar band intensity in each lane. TMACl postwash temperatures and film exposure times were M8, 57°C, and 7 d, and M1, 57°C, and 14 d. Lane *13* contains DNA from a control subject with defined VH haplotypes (15, 37, 38). Restriction fragment sizes, in kb, appear to the right.

Table I. Prevalence of VH26 Hybridization Bands Detected in Three Ethnic Groups

TaqI band (kb)	White (n = 72)	Asian (n = 52)	Black (n = 35)
7.0	64 (89%)	39 (75%)	9 (26%)
6.8	0	5 (10%)	1 (3%)
5.0	72 (100%)	50 (96%)	34 (97%)
3.7	1 (1.4%)	11 (21%)	3 (9%)

Genomic DNA of subjects from three ethnic groups were digested with TaqI and hybridized with the VH26-specific CDR1 oligonucleotide probe M8. The numbers of subjects possessing M8-positive hybridization bands are indicated by ethnic group and restriction fragment size. For 7.0 kb, $\chi^2 = 45.9$, $P < 0.0005$; for 6.8 kb, $\chi^2 = 7.8$, $P < 0.02$; for 3.7 kb, $\chi^2 = 13.8$, $P < 0.001$.

in Blacks than in Asians or Caucasians ($\chi^2 = 45.9$, $P < 0.0005$; C vs. A $P < 0.043$, C vs. B $P < 0.0005$, A vs. B $P < 0.0005$).

Ethnic differences in the VH26 B locus. In the same M8 hybridizations described previously, the 5.0-kb band was detected in all Caucasians (100%), 50 Asians (96%), and 34 Blacks (97%) (Table I). The 2 Asians and 1 Black who were blank at 5.0 kb each had, in addition to a 7.0-kb band, novel bands at 6.8 and/or 3.7 kb that appear to represent locus B alleles (described later). No TaqI restriction fragments of sizes other than 7.0, 6.8, 5.0, and 3.7 kb were detected by probe M8. These data indicate that in all ethnic groups, homozygous absence of the VH26 B locus is rare, and the B locus is usually represented by a 5.0-kb band.

In this study, probe M8 detected two TaqI hybridization bands, at 6.8 kb and 3.7 kb, that were found predominantly in Asians (e.g., Fig. 2, lanes *1, 4, 9,* and *11*). One or both of these bands were seen in a total of 20 subjects: 15 Asians (29%), 1 Caucasian (1.4%), and 4 Blacks (11%) (Table I). The 6.8-kb TaqI band occurred in 5 Asians (10%), 1 Black (3%), and no Caucasians (0%) ($\chi^2 = 7.8$, $P < 0.02$; C vs. A $P < 0.007$, C vs. B $P < 0.15$, A vs. B $P < 0.223$). The Asians with a 6.8-kb+ band were Chinese (2) or of mixed Asian ancestry (1 each: Chinese/Japanese, Chinese/Cambodian, Vietnamese/Cambodian, all 50/50%). The Black with a 6.8-kb+ band was 87.5% Black and 12.5% Native American. 4 of the Asians with 6.8-kb+ bands also had the usual M8 bands at 7.0 and 5.0 kb (Table II and, e.g., Fig. 2, lanes *9* and *11;* Fig. 3, lane *10*). The 5th 6.8-kb-band–positive Asian subject had bands at 7.0 kb and 3.7 kb (Fig. 3, lane *7*). The 6.8-kb-band–positive Black had a 7.0-kb band but no 5.0-kb band (Fig. 3, lane *1*).

The other TaqI band that occurred predominantly in Asians, at 3.7 kb, was seen in a total of 11 Asians (21%), 1 Caucasian (1.4%), and 3 Blacks (9%) ($\chi^2 = 13.8$, $P < 0.001$%; C vs. A $P < 0.0005$, C vs. B $P < 0.066$, A vs. B $P < 0.001$) (Table I). Of the 3.7-kb-band–positive Asian subjects, 4 were Chinese, 4 Korean, 1 Japanese, 1 Vietnamese, and 1 of mixed Chinese (75%)/Vietnamese (25%) ancestry. The 3.7-kb-band–positive Caucasian was confirmed by a second interview to have no non-Caucasian ancestry. The ethnicities of the 3.7-kb-band–positive Blacks were 100% black, 96.9% Black, and undetermined. Of the 15 subjects possessing a 3.7-kb band, 13 also had M8 bands at 5.0 kb (e.g., Fig. 2, lanes *1* and *4;* Fig. 3, lanes *3* and *6;* and Table II) and 2 did not. In these 2 subjects, both Asians described previously, a 7.0-kb band was present (Table II). In

Table II. Phenotypic Combinations of VH26 Hybridization Bands Detected in Three Ethnic Groups

Phenotypic combination				Subjects		
Locus A	Locus B					
7.0 kb	6.8 kb	5.0 kb	3.7 kb	White (n = 72)	Asian (n = 52)	Black (n = 35)
Common phenotypes						
+	−	+	−	63 (88)	24 (46)	9 (26)
−	−	+	−	8 (11)	13 (25)	22 (63)
Uncommon phenotypes						
+	−	+	+	1 (1.4)	9 (17)	0
+	−	−	+	0	1 (2)	0
−	−	+	+	0	0	3 (9)
+	+	+	−	0	4 (8)	0
+	+	−	+	0	1 (2)	0
+	+	−	−	0	0	1 (3)

Genomic DNA of subjects from three ethnic groups were digested with TaqI and hybridized with the VH26-specific CDR1 oligonucleotide probe M8. The number of subjects possessing M8-positive hybridization bands in each of the two common and six uncommon phenotypic combinations are indicated by ethnic group. Percentages, relative to the total numbers shown at the top of each data column, appear in parentheses.

addition, 1 also had a 6.8-kb band (Fig. 3, lane *7*), and 1 had a 3.7-kb band markedly more intense than all others seen, suggesting homozygosity for the 3.7-kb gene (Fig. 3, lane *15*).

The 6.8- and 3.7-kb restriction fragments contain alleles of the VH26 B locus. The finding that the 5.0-kb band of VH26 locus B was absent in individuals who appeared to be homozygous for the 3.7-kb band, or who possessed both the 6.8- and 3.7-kb bands, suggests that the genes detected in the 6.8-kb and 3.7-kb restriction fragments are alleles of the VH26 B locus. In other words, possession of two copies of either, or one of each, precludes possession of the more common VH26 gene in the 5.0-kb band. Two lines of evidence support this conclusion. First, if the genes in the 6.8-, 5.0-, and 3.7-kb bands are alleles, heterozygotes possessing the 6.8- or 3.7-kb gene on one locus B should possess exactly one copy of the 5.0-kb gene, unless the opposite locus B happens to contain a 5.0-kb duplication (i.e., two gene copies) or be deleted (i.e., a null allele). In fact, in 82% (14 of 17) of the subjects possessing bands at 5.0 kb and either 6.8 or 3.7 kb, the 5.0-kb band had a low intensity consistent with a single gene copy (e.g., Fig. 2, lanes *1, 4, 9,* and *11;* Fig. 3, lanes *3* and *10*). In the 3 exceptions, all 3.7-kb+ subjects, the 5.0-kb band intensity suggested two gene copies (1 Asian and 1 Black subject, not shown) and greater than two gene copies (Fig. 3, lane *6,* Asian subject). These 3 subjects likely have a duplication of the 5.0-kb gene segment that does not alter the TaqI restriction fragment size (described later). In contrast, among the 139 other 5.0-kb+ subjects (Table II, Common phenotypes), low-intensity 5.0-kb bands occurred in only 6% of Caucasians (4 of 71), 13% of Blacks (4 of 31), and no Asians (0 of 37) (e.g., Fig. 3, lane *11*). The data indicate that most of the 159 subjects appeared to be homozygous for the 5.0-kb allele of locus B, as exemplified by the control DNA (reference 37; Fig. 2, lane *13*). In a significant minority of the population, heterozygosity attributable to locus B occurred as an allelic band at 6.8 or 3.7 kb, as a null allele, or as a two-gene copy locus B duplication (described in greater detail later). These locus B alleles were usually paired with the prevalent single-copy allele of the 5.0-kb gene, although other pairings sometimes occurred.

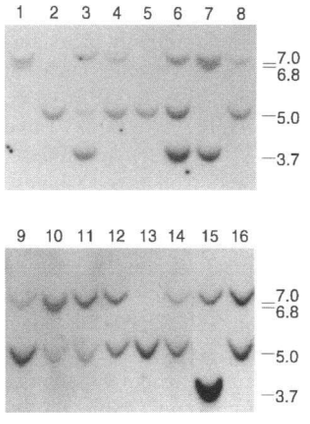
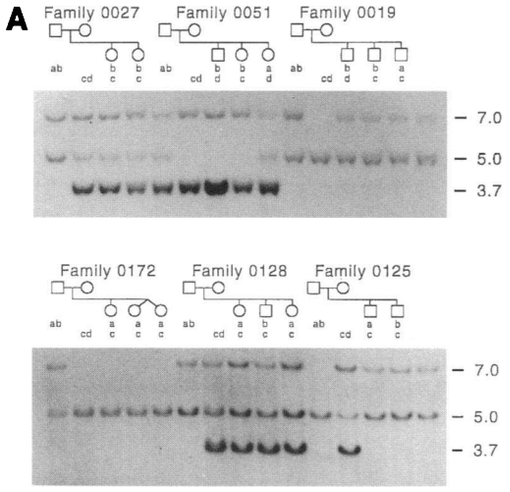


Figure 3. Selected examples of VH26 polymorphism. Aliquots of genomic DNA from 16 unrelated subjects were prepared in two gels, eight each, and hybridized with probe M8 (VH26 CDR1), as in Fig. 2. Subjects were chosen to illustrate several variations in restriction fragment size and band intensity. Subjects are all different from those in Fig. 2, except in lane *10* (equals Fig. 2, lane *9*). TMACl postwash temperature was 57°C, and film exposure times were 12 d (*top*) and 7 d (*bottom*). Lanes *4* and *14* have the same M8 hybridization result as the control DNA. Restriction fragment sizes, in kb, appear to the right.

A second line of evidence indicating that the 6.8-, 5.0-, and 3.7-kb elements are alleles of the B locus comes from segregation analysis. 6 families were studied (Fig. 4). Parental haplotypes were established with data obtained by sequential hybridization with probes to polymorphic VH genes: probes M8 (to VH26; Fig. 4 *A*), M19 (to 56p1), M41 (to hv3005), S4 (to 51p1), and S5 (to hv1263) (8, 11, 15). 4 families were Asian and had a 3.7-kb hybridization band on a total of five haplotypes. In 2 of the Asian families (0027 and 0125), the 3.7-kb band occupied a single haplotype, whereas the other haplotypes all had a 5.0-kb band (Fig. 4 *B*). In the family of Asian subject 0051, one haplotype from each parent, "b" and "d," had a 3.7-kb band (Fig. 4 *B*). Subject 0051 was shown to be homozygous for this band, as predicted (Fig. 4, *A* and *B*). The other paternal haplotype in family 0051, "a," had a 5.0-kb band. The other maternal haplotype in family 0051, "c," had no 3.7- or 5.0- (or 6.8-) kb band and is thus a locus B null allele (Fig. 4 *B*). In the family of Asian subject 0128 (who is also shown in Fig. 3, lane *6*), the paternal "a" and "b" haplotypes had a two-gene-copy and a one-gene-copy 5.0-kb band, respectively



B

		Family 0027				Family 0051				Family 0019			
		P		M		P		M		P		M	
VH 26 locus	kb	a	b	c	d	a	b	c	d	a	b	c	d
A	7.0	+	+	+	+	-	+	+	+	+	+	-	-
B	5.0	+	+	-	+	+	-	-	-	+	+	+	+
B	3.7	-	-	+	-	-	+	-	+	-	-	-	-

		Family 0172				Family 0128				Family 0125			
		P		M		P		M		P		M	
VH 26 locus	kb	a	b	c	d	a	b	c	d	a	b	c	d
A	7.0	-	+	-	-	+	-	+	-	-	-	+	+
B	5.0	+	-	+	+	+	+	+	+	+	+	+	-
B	3.7	-	-	-	-	-	-	+	-	-	-	-	+

Figure 4. Segregation in families of VH26-related germline genes. (*A*) Two gels were prepared with TaqI-digested genomic DNA from members of six families and were hybridized with probe M8, to CDR1 of VH26, postwashed in TMACl at 57°C, and exposed to film for 10 (*top*) or 15 (*bottom*) d. Families are designated by the index subject (the first sib in families 0027, 0051, and 0019; the father, last sib, and mother, respectively, in families 0172, 0128, and 0125). The last lane in each gel, unlabeled, contains DNA from the same control subject shown in the last lane of Fig. 2. DNA from subjects 0051 and 0128 also appear in Fig. 3, lanes *15* and *6,* respectively. Restriction fragment sizes, in kb, appear to the right. The VH26 haplotypes of each subject appear below each family tree. (*B*) VH26 haplotypes of six families. Paternal (P) and maternal (M) haplotypes were derived from data obtained by sequentially hybridizing the gels in (*A*) to probe M8 (shown in *A*), and to probes M19 (56p1 gene), M41 (hv3005 gene), S4 (51p1 gene), and S5 (hv1263 gene) (not shown). Boldface (+) designates a gene dose of 2 for 5.0 kb and ≥3 for 3.7 kb.

(Fig. 4 *A* and *B*). Locus B on the "a" haplotype therefore contains a duplication of the 5.0-kb gene, where both copies occupy a 5.0-kb restriction fragment. The 3.7-kb band in family 0128 occupied the maternal "c" haplotype, which also contained a 5.0-kb band—a rare linkage not seen elsewhere in the study. This finding indicates that the 5.0-kb band seen in subject 0128 contains three gene copies (Fig. 4 *A,* family 0128, third sib), two from the father and one from the mother (Fig. 4 *B*). In the 2 Caucasian families (0019 and 0172), VH26 locus B had a one-gene-copy 5.0-kb band on seven haplotypes and a null allele on one haplotype (Fig. 4, *A* and *B*). In the 6 families, 14 of the 24 haplotypes contained 7.0-kb locus A bands and 10 contained locus A null alleles (Fig. 4 *B*). Most 7.0-kb bands occurred on haplotypes shared by a 3.7-kb or a 5.0-kb band. Thus, with the exception of the unusual linkage noted in the "c" haplotype of family 0128, these data show that the 5.0- and 3.7-kb genes never occurred on the same chromosome 14, a finding consistent with their being alleles. Furthermore, these genes commonly exist on the same chromosome 14 as the 7.0-kb gene, which confirms that the 3.7- and 5.0-kb gene loci are distinct from that of the 7.0-kb gene. No families with a 6.8-kb M8+ band were available for study.

The 3.7-kb TaqI allele of VH26 B is multiply duplicated in Asians. In all Asians possessing a 3.7-kb hybridization band with probe M8, that band was unusually intense. Family segregation analysis showed that the high intensity of the 3.7-kb band in Asians represents a haploid gene dose and that the 3.7-kb band is even more intense in a homozygote (Fig. 4 *A,* family 0051, first sib). In each case, the level of intensity clearly exceeded that of other M8+ bands in the same subject (Figs. 2, lanes *1* and *4;* Fig. 3, lanes *3, 6, 7* and *15*) and that of the M8+ bands in the control DNA (Figs. 2 and 4, last lanes). On the basis of previous gene mapping, the control DNA is known to have one copy of VH26 at 7.0 kb and two copies at 5.0 kb (37). In contrast, in the 1 Caucasian and in 2 of the Blacks with a 3.7-kb band (Tables I and II, not shown on a gel), the 3.7-kb band had low intensity equal to the one-gene-copy bands seen in these and other subjects, including the control. In the third 3.7-kb+ Black, this band appeared to have a two-gene-copy intensity (Tables I and II, not shown on a gel). These findings indicate that in Asians, the 3.7-kb allele of VH26 locus B is the result of a multiple duplication that created three or four gene copies of a VH26-related gene segment, where each copy resides on a 3.7-kb restriction fragment. In the studied Blacks and Caucasians, the 3.7-kb allele was usually not duplicated and was never multiply duplicated.

Duplications of locus A and the 5.0-kb locus B allele. In 1 Caucasian subject, the 7.0- and 5.0-kb bands each had an intensity consistent with greater than two gene copies (Fig. 3, lane *16*). The result was confirmed by M8 hybridizations to three different restriction digests of this DNA sample, each in a different gel. This genome therefore contains six (or more) copies of the VH26 germline gene, presumably three at locus A and three at locus B. Similarly, the 5.0-kb locus B band had an intensity consistent with more than two gene copies in an additional 6 Caucasian, 3 Asian, and 4 Black subjects (e.g., Fig. 2, lane *7;* Fig. 3, lanes *6, 9,* and *13*). These findings indicate that there exist haplotypes containing more than one copy of the VH26 gene associated with the 5.0-kb restriction fragment (as demonstrated in family 0128, haplotype "a"; Fig. 4) and occasionally the 7.0-kb restriction fragment. These haplotypes presumably reflect local duplications of the A or B loci that created, in effect, VH26 alleles comprised of two VH26 gene segments, both on TaqI fragments of 5.0 kb (locus B) or 7.0 kb (locus A). It is possible that when the intensity of a 7.0- or 5.0-kb hybridization band indicates that it contains a total dose of two gene copies, as is common, some cases might reflect heterozygosity, that is, a duplicated allele on one chromosome 14 plus a null allele on the other. However, at either VH26 locus, such occurrences must be infrequent because in our study of 159 subjects, (1) 7.0-kb bands containing three gene copies (and therefore, a locus A two-gene-copy allele) were very rare (1 of 159), even among Caucasians (1 of 72); and (2) no homozy-

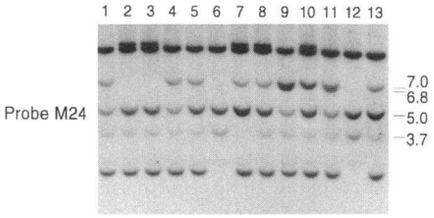


Figure 5. A VH26 CDR2 probe reveals sequence variation in the 3.7-kb locus B allele. The same gel shown in Fig. 2 was stripped of previous probe and hybridized with probe M24, which targets mid-CDR2 of the VH26 sequence. Probe M24 detected all bands previously detected by the CDR1 probe M8 at 7.0, 6.8, and 5.0 kb, but not those at 3.7 kb (see Fig. 2, lanes *1* and *4*). TMACl postwash temperature was 61°C. Film exposure time was 7 d. Restriction fragment sizes, in kb, appear to the right.

gotes for the locus B null allele were encountered, indicating that locus B null alleles were rare.

The 3.7-kb locus B allele has a distinct coding region sequence. To characterize further the coding region sequence of the VH26-related genes detected by the CDR1 probe M8, hybridization was performed with a second oligonucleotide probe, M24, which targets the middle of CDR2 in VH26 (Fig. 1). All bands detected by probe M8 at 7.0, 6.8, or 5.0 kb were also detected by M24, with the same relative intensities as previously seen with probe M8 (e.g., compare Figs. 2 and 5). In contrast, probe M24 did not detect the bands detected by M8 at 3.7 kb in 15 subjects (compare Figs. 2 and 5; the faint bands detected by probe M24 at about 3.7 kb derive from a distinct VH gene; E. H. Sasso and L. A. Suzuki, unpublished data). All M8 blanks at 7.0, 6.8, or 5.0 kb were also blank with probe M24, suggesting they represent gene absence rather than CDR sequence polymorphism. These results indicate that the VH26-related genes detected in the 7.0-kb band (locus A) and in the 6.8- and 5.0-kb bands of locus B might all contain exact VH26 sequences. The genes on the 3.7-kb TaqI fragments, which were all M8+ and M24−, are unique and must differ from the VH26 sequence in CDR2.

Cloning the 3.7- and the 5.0-kb alleles of VH26 locus B. To determine the complete nucleotide sequences of genes detected by probe M8, aliquots of genomic DNA from 2 subjects were amplified by PCR and cloned into pUC. Colony filter lifts were hybridized with probe M8. From a Caucasian subject who by genomic hybridization with probe M8 had only the 5.0-kb band (similar to Fig. 3, lane *2*), the V_H nucleotide sequence of an M8+ colony was identical to the VH26 sequence (Fig. 6 *A*, 5.0-kb sequence). This result confirms that the predominant allele of the B locus, on a 5.0-kb TaqI fragment, contains an exact VH26 gene. From Asian subject 0051, who by genomic hybridization with probe M8 had bands at 7.0 and 3.7 kb (Fig. 3, lane *15*, and Fig. 4 *A*), the V_H sequence of a M8+ colony differed from VH26 by eight nucleotides in CDR2 and one in FR3 and was identical elsewhere (Fig. 6 *A*, 3.7-kb sequence). The two most 3′ CDR2 substitutions are in the region targeted by probe M24 (Fig. 1). This cloned sequence must therefore derive from the 3.7-kb allele of VH26 locus B. The first 20 CDR2 nucleotides of the 3.7-kb gene sequence are identical to those of the V_H3 genes 8-1B (40) and 60p2 (19), suggesting that the 3.7-kb allele arose, in part, by gene conversion (Fig. 6 *A*). The translated protein sequences of the 3.7-kb allele and VH26 differ by five amino acids in CDR2 (Fig. 6 *B*).

The cloned sequence of the 3.7-kb allele exists in the germline. Confirmation that the 3.7-kb sequence cloned from subject 0051 actually resides in the germline and is unique to the M8+ 3.7-kb TaqI band was obtained by genomic hybridization with two CDR2-specific probes. Probe S1, which targets 5′ CDR2 of the 3.7-kb gene sequence (Fig. 1), detected all 3.7-kb bands previously detected by probe M8 but no other M8 bands (e.g., Fig. 7, *top*). The relative intensity of these S1-positive 3.7-kb bands paralleled that seen with probe M8. In addition, probe S1 detected polymorphic bands at > 9.0 kb and at ~ 2.5 kb, which were also detected previously by probe M24. These bands might contain the 8-1B or 60p2 germline genes but were not characterized further. The second probe, S2, targets mid-CDR2 of the 3.7-kb sequence (Fig. 1), and detected 3.7-kb bands in all the same subjects as did probes M8 and S1, but never any other bands (e.g., Fig. 7, *bottom*). These results show that the novel CDR2 sequence of the 3.7-kb allele of VH26 locus B is unique to that allele and exists in the germline of all subjects who had a 3.7-kb hybridization band detected with CDR1 probe M8. Furthermore, the data strongly suggest that the same V_H gene sequence occurs in all copies of the 3.7-kb allele.

The alleles of the A and B loci of VH26. The findings mentioned earlier identify the alleles of the VH26 A and B loci and permit estimates of their prevalences (Table III). It is possible that there exist other alleles, or linked combinations of alleles, but their prevalences are probably low in these ethnic groups. Fig. 8 depicts a proposed evolutionary relationship among the VH26 alleles.

Discussion

In this report, we have studied the germline representation of the VH26 gene in Caucasians, Asians, and American Blacks. Our data demonstrate that VH26 occupies two loci, that both VH26 loci are polymorphic, and that marked ethnic differences exist in polymorphism at each locus. We found that the A locus was absent more often among blacks than Caucasians or Asians. The B locus was infrequently absent but included two alleles that occurred in 29% of Asians, 11% of Blacks, and only 1.4% of Caucasians. The magnitude of these ethnic differences is considered to be a minimum estimate, due to intragroup heterogeneity.

Different types of polymorphism were found to predominate at each VH26 locus. The amount of polymorphism at each VH26 locus was limited, however, in keeping with a previous observation that polymorphic V_H loci are usually dominated in the population by only one to four prevalent alleles (8). At the VH26 A locus, which occurs on a 7.0-kb TaqI restriction fragment, hybridization blanks were essentially the only type of polymorphism observed. RFLP and coding region variants of the A locus were not detected, and duplications appeared to be very rare. Our hybridization data and previous reports indicated that the 7.0-kb TaqI fragment probably contains an exact VH26 gene sequence (12, 37). Hybridization blanks at 7.0 kb signify homozygous absence of the VH26 A locus, that is, two null alleles, and were seen in 74% of Blacks but only 11% of Whites and 25% of Asians. Due to this difference, most Blacks had fewer total germline copies of VH26 than did most Asians or Caucasians, that is, Blacks usually had two, rather than three, four, or sometimes more.

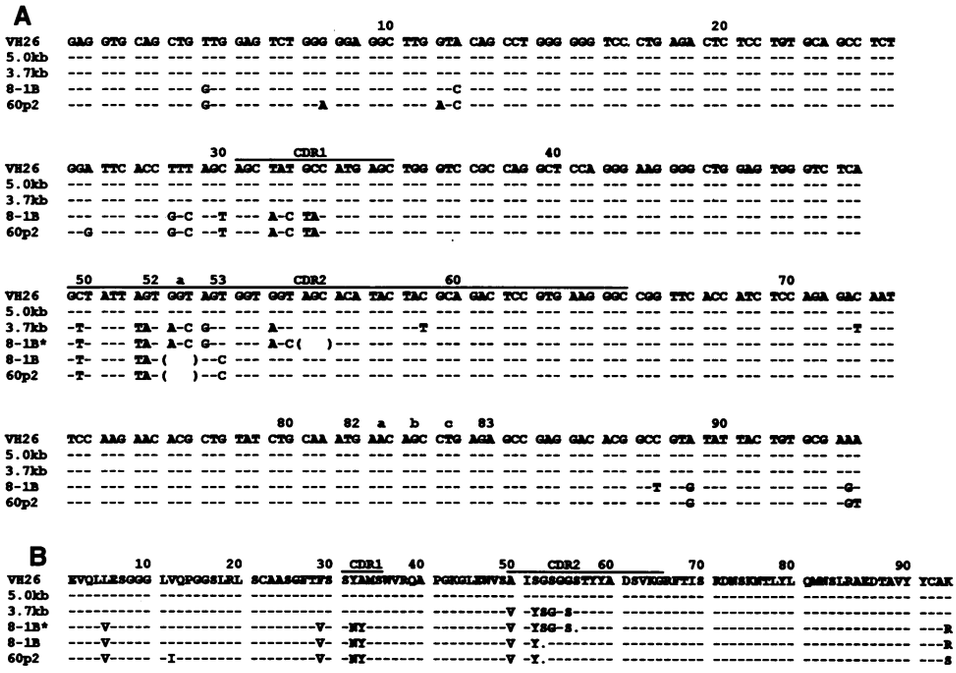


Figure 6. Sequence analysis of VH26 locus B alleles. (*A*) Nucleotide sequences cloned from the VH26 locus B alleles on 5.0- and 3.7-kb TaqI restriction fragments are shown beneath the complete VH26 reference sequence. Shown underneath for comparison are the sequences of the V_H3 germline genes of 8-1B (40) and 60p2 (19). Asterisk (*) indicates an alternative alignment of the 8-1B CDR2, to facilitate comparison with the 3.7-kb sequence. Nucleotide positions filled by a dash (−) are identical to the corresponding VH26 nucleotide. Positions enclosed by parentheses indicate nucleotide absence. CDR1 and CDR2 are indicated by horizontal lines over the VH26 sequence. (*B*) Amino acid sequences translated from the nucleotide sequences shown in (*A*). A dot (.) indicates codon absence. Codon numbering and CDR positions are according to Kabat et al. (39). GenBank accession numbers are U29481 (3.7-kb gene) and U29482 (5.0-kb gene).

In nearly all subjects studied, the B locus was present as a 5.0-kb TaqI restriction fragment, which we showed contains an exact VH26 gene. Cases of increased hybridization intensity in the 5.0-kb band indicated that its VH26 segment is sometimes duplicated without an RFLP. Other B locus alleles did involve RFLP. One occurred as a simple RFLP (the 6.8-kb band), the others as a RFLP with coding region sequence variation (the 3.7-kb bands). Hybridization data indicated that the gene on the 6.8-kb TaqI fragment matches VH26 in CDR1 and part of CDR2, and might therefore contain an exact VH26 sequence. Hybridization and gene sequence data showed that the allele on the 3.7-kb TaqI fragment differs from VH26 by nine nucleotides, including eight in CDR2 that encode five amino acid changes.

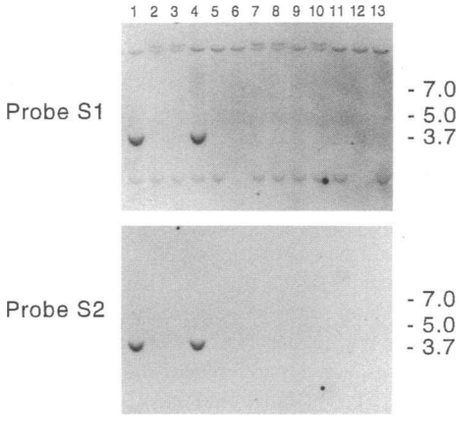


Figure 7. Specific detection of the 3.7-kb allele of VH26 by genomic hybridization. A companion gel to that shown in Fig. 2 (prepared simultaneously with DNA aliquots from the same restriction digests) was sequentially hybridized with oligo probes S1 (*above*) and S2 (*below*), which target, respectively, 5′ CDR2 and mid-CDR2 of the locus B allele cloned from a 3.7-kb TaqI restriction fragment. For each probe, TMACl postwash temperature was 57°C, and film exposure time was 7 d. Restriction fragment sizes, in kb, appear to the right.

The finding that nucleotide substitutions of V_H alleles are concentrated in the CDR has been previously seen, for example, among alleles of the V_H1 gene 51p1 (11). This tendency indicates that mutation analysis of expressed V_H genes must be performed with caution if the encoding germline allele has not been identified with certainty. Furthermore, it implies that V_H alleles can differ in their germline-encoded specificity for antigen. In fact, the close homology of the 3.7-kb gene CDR2 sequence to that of other V_H3 genes, 8-1B (V3-66) and 60p2 (V3-53), implies that its encoded V_H protein belongs to a different CDR canonical structure group than those encoded by VH26 and could on that basis have different antigen binding properties (41).

In a previous study of the same genome used here as a control, the VH26 A and B loci were mapped to a region on chromosome 14 that stretches from about 300 to 600 kb upstream of the most 3′ V_H gene, V_H6 (37). Neither the order of the A and B loci, nor the exact distance or number of V_H genes separating them has been reported. In a different genome, a VH26 gene designated V3-23 was mapped to a site 320 kb upstream of V_H6 (1). The V3-23 gene most likely represents the 5.0-kb allele of locus B, because only one VH26 locus was described in that genome. The less prevalent locus B alleles described herein, on 6.8- and 3.7-kb TaqI fragments, probably map to the same chromosomal neighborhood as the most prevalent locus B allele, on the 5.0-kb fragment, because the order of V_H genes appears largely unchanged by polymorphic varia-

Table III. Frequencies of VH26 Alleles

	Locus A			Locus B							
Allele (kb)	7.0	7.0_{II}*	Null	6.8	5.0	5.0_{II}	3.7	3.7_{II}	3.7_{multi}‡	3.7_{multi} + 5.0	Null
Caucasian	.583	.007	.410	—	.924	.042	.007	—	—	—	.028
Asian	.510	—	.490	.048	.808	.038	—	—	.106	.010	<.010§
Black	.143	—	.857	.014	.800	.071	.029	.014	—	—	.071

* II designates an allelic unit containing two gene copies; ‡ multi designates an allelic unit containing three or more gene copies; § no examples occurred in the Asian study population, but one was seen in a family member. Alleles are designated by their TaqI restriction fragment size, as detected by hybridizating probe M8 to DNA from the study population of 159 subjects. Frequencies were calculated from populations of 72 Caucasians (144 alleles), 52 Asian (104 alleles), and 35 blacks (70 alleles). For these calculations, in all cases where 7.0- or 5.0-kb bands had hybridization intensities indicating a gene dose of two, it was assumed the genome was homozygous for the single-copy allele (with the exception of 1 Asian subject with bands at 3.7, 5.0, and 7.0 kb, and 1 black subject with bands at 3.7 and 5.0 kb, in whom the respective 5.0-kb bands were assumed to be 5.0_{II} alleles).

tions (42, 43). It has been noted, however, that multigene insertions can modify the local organization of V_H elements (14, 15).

Duplication of V_H genes is considered one of the principal evolutionary mechanisms for diversity in the V_H germline repertoire (43, 44). V_H duplications differ in the distance separating the duplicated genes and the number of genes contained in the duplicated block. Several duplications that created V_H loci widely separated from each other on chromosome 14 have been described and are attributed to duplication and translocation events occurring 22–55 million years ago (44). A more localized type of duplication, proposed to have occurred ~ 9 million years ago, involved adjacent homologous blocks containing two or three genes from the V_H3 and V_H4 families (14, 15, 44). The duplication that created the A and B loci of VH26 likely resembles one of these two types, depending upon the locus-A-to-B distance. The subsequent evolutionary events leading to the ethnic differences in allelism described herein probably occurred after *Homo sapiens* evolved from its predecessors, which is generally believed to have occurred within the last million years, and perhaps as recently as the last 100,000–200,000 years (45).

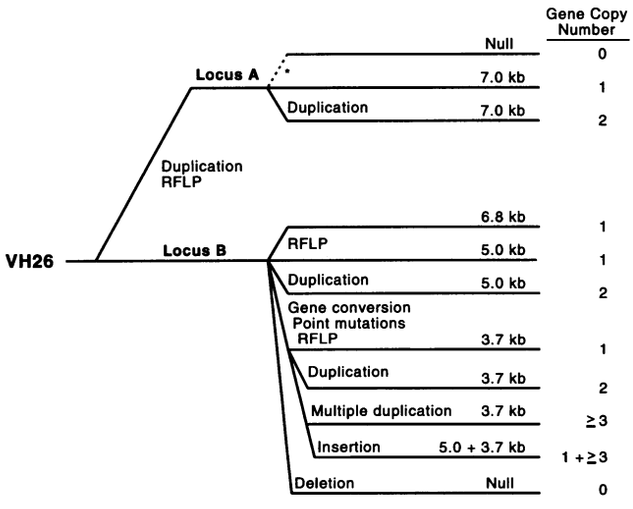


Figure 8. Phylogenetic tree of the VH26 genes. The two VH26 loci, A and B, could have arisen from a duplication of a prototype VH26 locus, followed by subsequent germline events which created the alleles identified in this study. Alleles containing more than one gene copy, as indicated to the right, presumably represent haplotypes of closely linked loci. Asterisk (*) indicates that locus A null alleles could hypothetically occur in haplotypes that lack the initial VH26 duplication or in haplotypes from which locus A was deleted.

The 3.7-kb TaqI hybridization band was a VH26 sequence variant seen in 21% of Asians, in whom it always occurred as a haplotype of at least three or four gene copies. Our data do not establish the chromosomal distance separating the multiple genes of the 3.7-kb VH26 variant, but the fact that they cosegregate and have an allelic relationship to the other VH26 locus B alleles suggests that they are in linkage disequilibrium with each other and probably comprise a closely approximated gene cluster. As such, they can be regarded as a single allele of VH26 locus B, even though in the strictest sense they represent at least three distinct gene loci. Duplications that create a closely linked pair of identical or nearly identical V_H genes have been reported (11, 14, 15). Multiple duplications have been previously described as the evolutionary basis for three V_H3 genes of unknown prevalence (none VH26) that are dispersed across a region of about 570 kb on chromosome 14 but which share only 89.7–93% nucleotide sequence identity (44). A study of the IgH chain-constant region genes identified duplicated and triplicated clusters of C genes in 2.7 and 0.2%, respectively, of an Italian study population (46). Multiply duplicated clusters of a single V_H gene sequence have not been described previously.

The novel nucleotide sequence of the 3.7-kb allele could have derived from VH26 by the combination of a gene conversion event in 5′ CDR2 and point mutations in mid-CDR2 (codon 59, TAC to TAT) and FR3 (codon 72, GAC to GAT). The putative gene conversion segment, which could have been donated by either of the V_H3 genes 60p2 (19) or 8-1B (40) (loci V3-53 and V3-66, respectively; references 1 and 2), likely included CDR2 codons 51–56, but could have extended to codon 33 (Fig. 6 *A*). These donor loci, V3-53 and V3-66, are about 390 and 470 kb upstream from the VH26 (V3-23) locus (1, 2). Because multiple duplications of the 3.7-kb gene were seen only in Asians, it can be hypothesized that the 3.7-kb VH26 allele evolved in two steps. First, before the separation of ethnic groups, it underwent germline gene conversion and point mutation; second, it underwent duplication, predominantly in Asian genomes (Fig. 8). This schema differs from previously

described V_H gene phylogenetic trees in that the gene conversion precedes rather than follows the gene duplications (47). The possibility that multiple duplications of the 3.7-kb allele occurred prior to ethnic separation but were conserved preferentially among Asians cannot be excluded.

The present data demonstrate that the locus B alleles found mostly among Asians exist in peoples from several different parts of East Asia. The 3.7-kb locus B allele was found in Chinese, Korean, Japanese, and Vietnamese subjects. The 6.8-kb locus B allele appeared in subjects of Chinese and mixed Asian ancestry (Chinese/Japanese, Chinese/Cambodian, and Vietnamese/Cambodian). The size of this study prevents us from comparing allelic prevalences among Asian subgroups or drawing conclusions regarding ethnic groups in which rare alleles were not found. Few other data concerning ethnic differences in V_H polymorphism are available. In a Southern hybridization study of Canadian Caucasian and Chinese subjects, a panel of 10 cloned probes from the V_H2, V_H3, V_H4, and V_H5 families did not detect any V_H genes unique to either group (48). In that study, a V_H3 probe derived from VH26 did detect different ethnic prevalences for a pair of allelic EcoRI bands. The V_H3 gene contained in these bands was not identified but was probably not VH26, which resides on a different EcoRI fragment (49; E. Sasso, unpublished data). In another study, an allelic RFLP of the V1-4.1 gene was found in 38% of Japanese subjects, compared with 2.4% of Caucasians from Sweden and Germany (13). These findings, combined with ours, suggest that ethnic differences in polymorphism might exist at many V_H loci.

A direct consequence of polymorphism of the VH26 loci is that individuals can differ greatly in the number of germline copies of VH26 they possess. We observed total gene copy numbers ranging from one to as many as six copies of potentially exact VH26 germline genes (i.e., 7.0 plus 6.8 plus 5.0 kb), and from zero to as many as six to eight copies of the 3.7-kb allele. Because the majority of Caucasians, and many Asians, possessed three or four copies of VH26 in the diploid genome, it seems likely that the relative overexpression of VH26 that has been reported for adult and fetal B cells is at least partly explainable by the relative abundance of the germline template (21, 23–25). This interpretation is consistent with the finding by Stewart et al., that VH26 was expressed two to three times as frequently as the V_H3 gene 56p1 (25). The 56p1 gene is not duplicated and, with a reported phenotypic prevalence of 62%, must usually be present as one copy per genome (8). Consequently, most individuals expressing VH26 and 56p1 possess two to four times as many germline copies of VH26 as 56p1. A recent study of V_H gene expression by Suzuki et al. supports this interpretation (50). However, one subject has been reported in whom no VH26 expression was detected, even though a VH26 germline gene was present (25). Thus, gene copy number probably contributes to the differential expression of V_H genes, but differences in response to intrinsic regulatory factors and to antigenic stimulation are expected to play a role as well.

In conclusion, our data show that the VH26 germline gene is polymorphic at both its A and B loci. Ethnic comparisons indicated that absence of VH26 locus A is most common among American Blacks, whereas allelic polymorphism of locus B is most common among Asians. One of the predominantly Asian alleles is a novel CDR2 sequence variant of VH26 that appears to be the product of gene conversion and multiple duplications. From these findings, we expect that distinctive patterns of V_H polymorphism will be found among other ethnic groups and at other V_H loci. Such differences could influence V_H gene expression because they can involve variations in V_H gene copy number and V_H gene coding region sequence.

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