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Genetics

Conservation of an immunoglobulin variable-region gene family indicates a specific, noncoding function

(heavy-chain variable region gene/molecular evolution/gene rearrangement/conserved DNA sequences)

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ABSTRACT **Blot-hybridization and DNA sequence analyses reveal the particular evolutionary conservation of a group of immunoglobulin heavy-chain variable-region (V_H) genes in all mammalian species examined. These particular genes are group III genes—the V_H7183 family in the mouse and the homologous V_H III family in human. This conservation is localized to sequences encoding framework regions 1 and 3 of the antibody variable region and is exerted at the nucleotide level. Because selection acting at the amino acid level alone cannot explain the conservation of these sequences, these sequences must have a noncoding function. The preferential rearrangement of V_H7183 and V_H III genes, together with the similarity of the conserved sequences to elements implicated in recombination in other systems, suggest that these sequences function to target the series of rearrangements that assemble complete immunoglobulin genes.**

There are three major groups of immunoglobulin heavy-chain variable-region (V_H) genes (I, II, and III) in both mouse and human (1). In both species, these groups have been further divided into families on the basis of cross-hybridization and sequence similarity (2–6). Homology between several human and mouse V_H families in each group has been inferred from sequence similarity (Table 1). Thus, the divergence of the three major groups of V_H families (and certain V_H families therein) preceded the mammalian radiation 60–80 million years (Myr) ago, when contemporary orders of placental mammals last shared a common ancestor. The recent isolation of V_H genes homologous to groups I and III from *Xenopus* (7, 8) indicates that the divergence of the three major groups is considerably more ancient, over 300 Myr ago.

If the three groups of V_H genes were subjected to similar evolutionary pressures, then they would be expected to be similarly conserved. However, when cloned mouse V_H genes were used as probes to isolate their human counterparts, mouse group III V_H genes identified their human homologues more easily than did mouse group I and II V_H genes (4, 5). Mouse group III probes could also identify V_H genes in several other mammalian species (9, 10), the reptile *Caiman* (9, 11), the amphibian *Xenopus* (7), and the elasmobranch *Heterodontus* (12). Moreover, V_H genes in the rabbit and chicken, which may have only one V_H gene family, belong to group III (13, 14). Collectively, these observations suggest that while other V_H groups may evolve more freely (and in some cases even be lost), group III V_H genes, in particular, are a conserved and obligatory component of heavy-chain loci in widely divergent mammalian and nonmammalian lineages.

Here, we use Southern blot hybridization to show that discrete segments of group III V_H genes have been conserved throughout mammalian evolution. Examination of published sequences identifies specific candidate sequences in mouse V_H7183 and human V_H III gene families; these sequences have been conserved at the level of nucleotide sequence rather than coded protein sequence. Our results indicate that these V_H genes have a specific, noncoding function that, rather than their antigenic binding specificity, is responsible for their conservation.

MATERIALS AND METHODS

Animals, DNA, and Blot Hybridization. Frozen tissues from cat, dog, goat, opossum, raccoon, and spotted and striped skunks were the gift of H. Johnstone (San Diego County veterinarian). Rabbit spleens, human cells, and squirrel and woodchuck spleens were provided by W. Mason, D. Mosier, and T. London (Fox Chase Cancer Center). Fresh or frozen tissue from cow, pig, and sheep were purchased from local vendors. Deer mouse and shrew samples were provided by domestic house cats. Rat DNA was provided by D. Gold and C. Gritzmacher (Medical Biology Institute). Dolphin and horse DNAs were the gifts of A. Dizon (Southwest Fisheries Center) and J. Woodward (University of Kentucky), respectively. Species are indicated in Table 2. Preparation of genomic DNA, all Southern blot hybridization techniques, and V_H family probes were as described (15), except for the V_HVGAM3-8 family probe, provided by P. Brodeur (16). Final stringency of all Southern blot washes was 0.2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate)/0.1% $NaDodSO_4$, at 65°C.

V_H7183 Subregion-Specific Hybridization Probes. Probes spanning framework (FW) and complementarity-determining regions (CDR) of the cloned V_H7183 member 81X (gift of G. Yancopolous; ref. 17) were prepared by subcloning or purifying restriction fragments.

Published V_H Gene Nucleotide Sequences. Sequences from V_H families representing groups I, II, and III in mouse (M), human (H), and *Xenopus* (X) were as follows: M III—V_H7183 members E4.15 (17), 37.1, 50.1 (18), and 62 (M. Solazzo, personal communication); H III—V_H III members H11 (19), 26 (4), 30P1, 56P1, 38P1, and 60P2 (20); M II—V_HJ558 members ID11 (21), J558 (D. Livant and L. Hood, personal communication), DX11 (P. Early and L. Hood, personal communication), and 28 (22); H II—V_H I members HA2, HG3 (4), 20P3, and 51P1 (20); M I—V_H36–60 members 1210.7 (24), LB8 (25), V_HQ52N members PJ14 (26), MC101 (27), and V_H3609P member 23.9 (28); H I—V_H II member CE1 (29), V_H IV members 71.2, 58 (3), 15P1, and 58P2 (20); X III—1 (7); X I—8 and 14 (8). Crocodile V_H sequences were C3 (11) and G4

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Abbreviations: V_H, D_H, and J_H, immunoglobulin heavy-chain variable, diversity, and joining regions, respectively; FW, framework regions; CDR, complementarity-determining regions; Myr, million years.

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(30). Shark was represented by HXIA (12). All V_H genes are full length (mature peptide-coding sequence) and are germ line or were expressed in early development and likely germ line, except for LB8 and CE1, derived from a hybridoma and myeloma, respectively. No pseudogenes were analyzed.

Sequence Alignment and Construction of Phylogenetic Trees. Sequences were aligned for comparison by visual inspection and with the aid of the NUCALN program of Wilbur and Lipman (ref. 31, as implemented for VAX computers in the SEQ package from the Institute for Cancer Research, Philadelphia) using recommended default parameter values (ktup = 3, window = 20, gap = 7). To obtain biologically meaningful alignments of distantly related sequences, gaps consistent in size and placement with gaps used to align more closely related sequences were often maintained (32). This alignment was sometimes at the expense of a few percent greater nucleotide identity obtained by the placement of multiple small gaps. Evolutionary distances between aligned sequences were measured as amounts of nucleotide substitution at replacement and silent sites, estimated using the MYNEID program provided by M. Nei and T. Gojobori (33). Following suggestions of M. Nei, method I was used, with simple averaging and including evolutionary pathways involving intermediate terminators (tables of these estimates are available from the authors). Unrooted phylogenetic trees of V_H sequences were constructed from the estimates of substitutions between sequences using the tree-building algorithm of Fitch and Margoliash (34), implemented as the FITCH program in the PHYLIP package written by J. Felsenstein (35). The IBM-PC version of this package was obtained from G. D. F. Wilson (Scripps Institute of Oceanography). For estimates of substitutions between mouse and human groups I, II, or III, only the most homologous mouse and human families within each group were compared (Table 1).

RESULTS

Group III V_H Genes Are Conserved in Different Mammalian Species. To determine whether any V_H families are conserved in mammalian species, cloned DNA probes for nine mouse V_H gene families were hybridized under stringent conditions to *Eco*RI-digested, Southern-blotted genomic DNA from species representing most contemporary mammalian orders. Fig. 1 shows the cross-hybridization of V_H families representing groups I, II, and III in *Peromyscus* (deer mouse), *Marmota* (woodchuck), *Oryctolagus* (rabbit), and *Sorex* (shrew). These results were obtained with probes for the mouse V_H families having the closest identified human homologues (Table 1). Results obtained with all nine V_H families in these and all other analyzed species are summarized in Table 2. Probes for the group III V_H7183 and V_HS107 families hybridized to DNA

Table 1. Human and mouse V_H gene family homologues

Group I		Group II		Group III	
Human	Mouse	Human	Mouse	Human	Mouse
V_H II	V_H3609P	V_H I	V_HJ558	V_H III	V_H7183
V_H IV	V_H36–60	V_H V	V_HVGam3–8		V_HS107
V_H VI	V_HQ52N				V_HJ606
					V_HX24

V_H gene families in mouse and humans are divided according to nucleotide similarity into three major homologous groups, which correspond to Kabat's classification of mouse V_H protein subgroups (ref. 1; but note that Kabat's assignments of human subgroups I and II are reversed). The V_H families most similar between species are boxed; these mouse–human pairs are between 70 and 83% identical (refs. 3–5; additional analysis not shown). The mouse V_H7183 family is most like human V_H III and a consensus mammalian group III sequence; other mouse group III families have diverged relatively recently and are still similar (36).

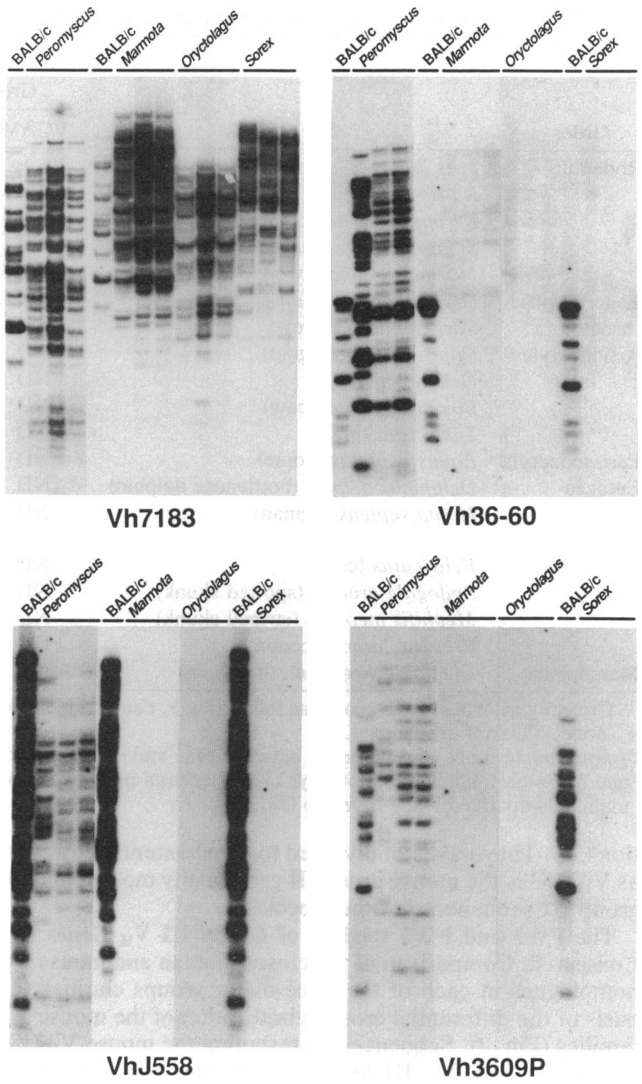

FIG. 1. Cross-hybridization of V_H families representing groups I, II, and III in *Peromyscus*, *Marmota*, *Oryctolagus*, and *Sorex*. Genomic DNA from each species was digested with *Eco*RI, Southern blotted, and probed with V_H7183 (group III), V_HJ558 (group II), and V_H36–60 and V_H3609P (group I). Lanes of mouse (BALB/c) DNA were included in each blot as internal positive controls.

from all species examined with an intensity comparable to that of control BALB/c mouse DNA. In contrast, probes for group I families hybridized strongly only to DNA from the muroid rodents *Rattus* and *Peromyscus* and weakly or not at all with other species; more dramatically, group II families hybridized weakly to *Rattus* and *Peromyscus* DNA and failed entirely to hybridize to DNA from species further removed from *Mus*. Moreover, in several cases—e.g., rabbit—the faint hybridization of group I families actually reflects cross-hybridization with a set of restriction fragments detected more intensely by group III probes (data not shown). These results are consistent with reports of hybridization of the V_HS107 probe with DNA from several different mammals (9, 10), but not with the results of Berman *et al.* (5), who describe hybridization of a mouse V_HJ558 probe with human DNA; this difference is likely explained by the less stringent conditions used in the latter study.

Probes for each of the mouse group III families detect the same set of restriction fragments in species that diverged from *Mus* >50 Myr ago, indicating that these families became distinct (diverged beyond the point of cross-hybridization at high stringency, ≈80% identity) after the mammalian radia-

Table 2. Differential cross-hybridization of murine V_H gene families in mammalian species

		V_H gene family								
		Group II		Group I			Group III			
Order	Species*	VGAM	J558	3609P	Q52N	36–60	J606	X24	S107	7183
Rodentia	*Mus domesticus* (mouse)	++	++	++	++	++	++	++	++	++
	Rattus norvegicus (rat)	+	+	++	++	++	++	++	++	++
	Peromyscus maniculatis (deer mouse)	+	+	++	++	++	++	++	++	++
	Sciurus carolinensis (grey squirrel)	−	−	−	+/−	+/−	++	++	++	++
	Marmota monax (woodchuck)	−	−	−	+/−	+/−	+	++	++	++
Lagomorpha	*Oryctolagus cuniculus* (rabbit)	−	−	−	+	+/−	+	++	++	++
Insectivora	*Sorex fumeus* (shrew)	−	−	−	+	−	+	++	++	++
Artiodactyla	*Capra aegagrus* (goat)	ND	−	ND	+/−	+	ND	ND	++	++
	Ovis aries (sheep)	ND	−	ND	+/−	−	ND	ND	++	++
	Bos primigenius (cow)	ND	−	ND	+/−	+	ND	ND	++	++
	Sus scrofa (pig)	ND	−	ND	−	−	ND	ND	++	++
Perissodactyla	*Equus caballus* (horse)	ND	−	ND	+/−	+/−	ND	ND	++	++
Cetacea	*Delphinus delphis* (bottlenose dolphin)	ND	−	ND	−	+/−	ND	ND	++	++
Primate	*Homo sapiens* (human)	ND	−	ND	+/−	+/−	ND	ND	++	++
Carnivora	*Canis familiaris* (dog)	ND	−	ND	−	−	ND	ND	++	++
	Felis catus (cat)	ND	−	ND	−	−	ND	ND	++	++
	Spilogale gracilis (spotted skunk)	ND	−	ND	−	−	ND	ND	++	++
	Mephitis mephitis (striped skunk)	ND	−	ND	−	−	ND	ND	++	++
	Procyon lotor (raccoon)	ND	−	ND	−	−	ND	ND	++	++
Marsupialia	*Didelphis virginiana* (opossum)	ND	−	ND	−	−	ND	ND	++	++

Cross-hybridization was scored as follows: ++, comparable to BALB/c control; +, less than BALB/c control; +/−, faint and inconsistent; −, none; ND, not determined.

*Approximate times of divergence between *Mus* and other rodent species are as follows: *Rattus*, 20 Myr (37, 38); *Peromyscus*, 40 Myr (37); and *Marmota* and *Sciurus*, 50 Myr (39). Placental mammalian orders diverged 60–80 Myr ago, and placental and nonplacental (opossum) mammals diverged ≈120 Myr ago (38).

tion (36). They have not diverged to equal extents, however, as V_H7183 is the mouse group III gene family most similar to group III sequences in other species.

The FW1 and FW3 Regions of Group III V_H Genes Are Conserved. Comparison of the closest human and mouse V_H homologues in each of the three major groups clarifies the basis of the differential cross-hybridization of the mouse V_H families (Fig. 2). Sequences representing the mouse V_H7183 and the human V_H III families share the greatest overall similarity, as much as 83% nucleotide identity, as compared with 77% between the closest group I or group II homologues. Probably equally important is the presence of continuous stretches of nucleotide identity between the V_H7183/V_H III homologues, both longer [22, 27, and up to 35 base pairs (bp) in comparisons of different genes] and more frequent than the longest identical tracts (up to 20 bp) in group I and II homologues. These stretches of nucleotide identity are found in FW1 and FW3 regions.

To test whether these regions are conserved in other mammalian species, we repeated the species surveys with probes spanning subregions of a V_H7183 sequence. As shown in Fig. 3, a probe spanning almost all of FW1 hybridized to DNA from all species. Other experiments showed that this probe detects most or all of the restriction fragments detected by the entire V_H1783 probe. A probe containing all of FW3 and the 3′ heptamer and nonamer involved in V-D-J rearrangement hybridized to all species except the opossum, the only nonplacental mammal examined, detecting a subset of the restriction fragments detected by the FW1 probe (Fig. 3). Removal of the 3′ noncoding region did not affect these results (data not shown). In contrast, probes spanning the region between FW1 and FW3 (including CDR1, FW2, and CDR2) or 500 bp of 5′ flanking sequence (including transcription signals, leader exon, and intron) failed to hybridize with species further removed from *Mus* than *Peromyscus* (data not shown). These results demonstrate that FW1 and FW3 regions of V_H7183 homologues are conserved in diverse mammalian lineages, whereas FW2 and both CDRs are no more conserved than their counterparts in group I and II V_H genes. The lack of conservation of group III CDRs, which form the antibody

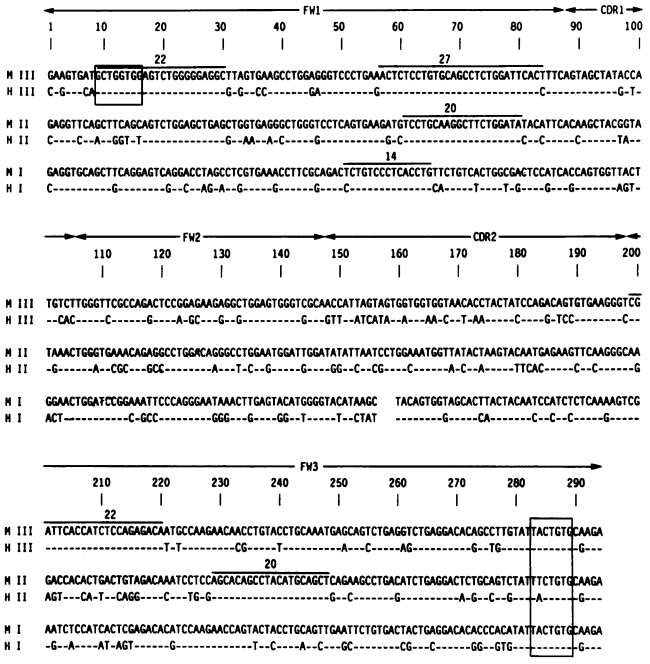

FIG. 2. Comparison of the closest published human (H) and mouse (M) group I, II, and III V_H homologues. M III, E4.15 (V_H7183); H III, 56P1 (family III); M II, ID11 (V_HJ558 family); H II, HA2 (family I); M I, 1210.7 (V_H36–60 family); H I, 71.2 (family IV). See *Materials and Methods* for references. FW1, -2, -3: framework regions 1, 2, and 3; CDR1, 2: complementarity-determining regions 1 and 2 (1). Bars indicate stretches longer than 19 bp, lengths of which are indicated above the bars [except for the group I homologues, in which the longest string (14 bp) is noted]. Boxes enclose the Chi octamer (FW1) and the embedded recombination signal heptamer (FW3).

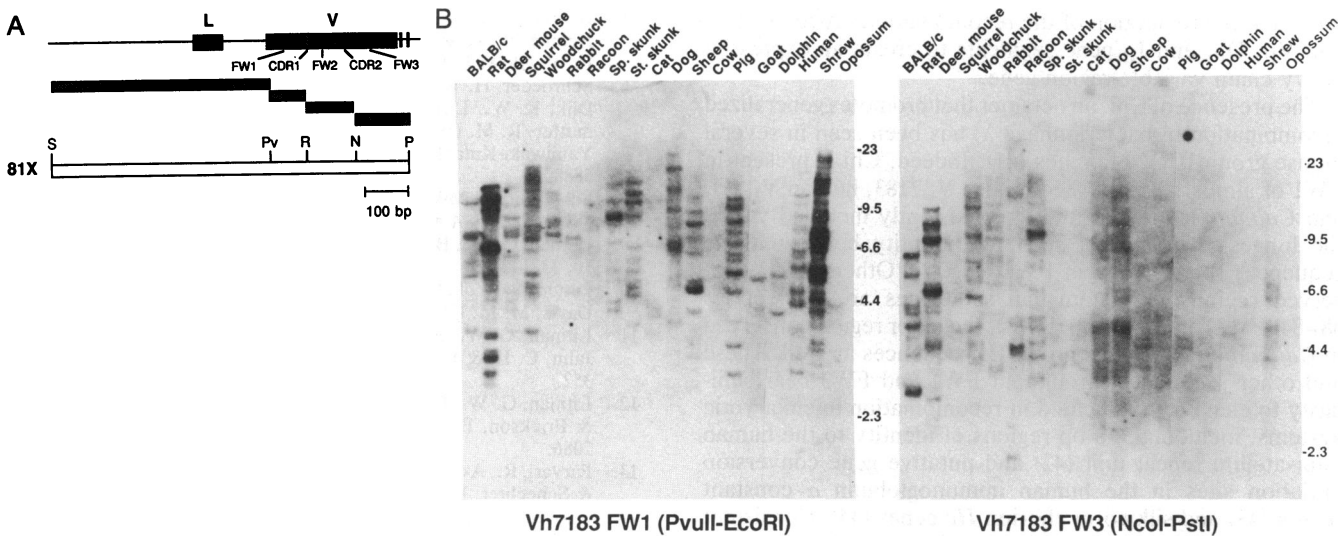

FIG. 3. Conservation of group III FW1 and FW3 regions in different mammalian species. (*A*) Probes specific for subregions of the V_H7183 member 81X (17); R, *Eco*RI; P, *Pst* I; Pv, *Pvu* II; S, *Sac* I. Solid vertical lines represent the heptamer and nonamer recombination signals. L, leader (*B*) *Eco*RI Southern blots of different mammalian species hybridized to probes specific for V_H7183 FW1 and FW3 subregions.

combining site, indicates that the antigenic specificities of these V_H genes are not the basis for their conservation.

Group III V_H Genes Are Conserved at Both Silent and Replacement Sites. If evolutionary conservation of a gene is effected by selection on protein function, the rate of replacement substitutions will be reduced relative to that of silent substitutions. If conservation is exerted at the nucleotide level, the rate of silent substitutions will also be reduced. The long continuous stretches of identical sequence in group III genes indicate that these sequences are conserved at the nucleotide level. To test in another way whether group III V_H genes are conserved at the nucleotide rather than the amino acid level, we compared evolutionary rates of silent and replacement substitution in the three groups of V_H genes by constructing phylogenetic trees of V_H sequences from widely divergent lineages.

We generated a matrix of the estimated number of nucleotide substitutions per replacement and silent site that have accumulated between the three groups of V_H genes in human and mouse, the two groups in *Xenopus*, and V_H sequences from *Caiman* and *Heterodontus*. Phylogenetic trees were constructed from these estimates (Fig. 4) using the algorithm of Fitch and Margoliash (34). This method was chosen because it generates a "realized distance tree," in which branch lengths are proportional to the number of nucleotide substitutions experienced, rather than elapsed evolutionary time (40).

Although the trees in Fig. 4 are unrooted, we can assume that the divergence of groups I, II, and III occurred before that of Amphibia and Mammalia and that the root (i.e., the earliest point in evolutionary time) is therefore located in the central region of each tree. In trees constructed from substitution at replacement or silent sites branches leading from the central region to group III termini are shorter than those to group I or II termini. Thus, group III V_H genes have accumulated fewer nucleotide substitutions at both replacement and silent sites, approximately half the amounts incurred in group I or II. These results indicate that the conservation of group III V_H genes is exerted at the nucleotide, rather than the amino acid level.

DISCUSSION

Blot-hybridization experiments and sequence analysis demonstrate that the V_H7183/V_H III family is particularly conserved in mammalian lineages. This conservation is localized to FW1 and FW3 and is exerted at the nucleotide level, indicating an important noncoding function for these sequences. A role in transcription is one possibility, but no family-specific differences in transcriptional efficiency of rearranged V_H genes have been reported. Instead, several observations suggest that the conserved sequences are in-

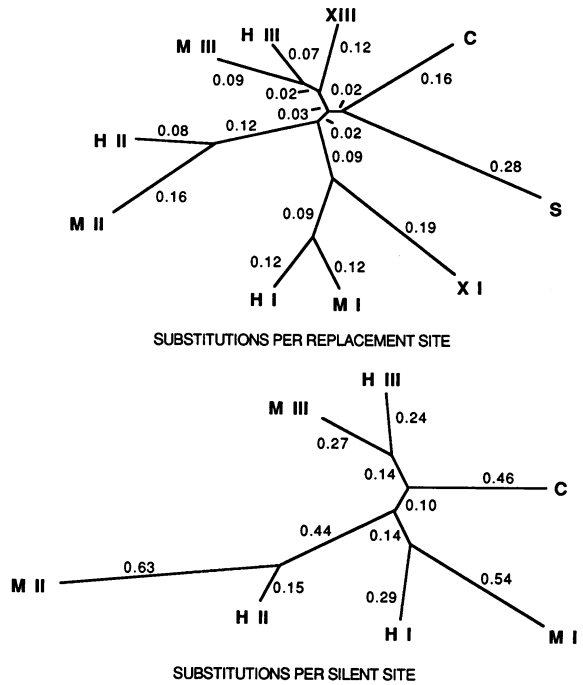

FIG. 4. Phylogenetic trees of V_H sequences from human (H), mouse (M), *Xenopus* (X), crocodile (*Caiman*, C), and shark (*Heterodontus*, S). Unrooted trees were constructed from estimates of substitutions per replacement or silent site. Numbers alongside branches indicate the estimated nucleotide substitutions accumulated along each branch. Mammalia diverged from reptilia (*Caiman*) ≈300 Myr ago, from Amphibia (*Xenopus*) ≈325 Myr ago, and from Elasmobranch (*Heterodontus*) ≈400 Myr. The trees constructed from substitutions per replacement site (*Upper*) and per silent site (*Lower*) were chosen from a total of 216 and 75 examined trees, respectively, and have average percent SDs of 5.05 and 8.62, respectively. Topology and branch lengths were optimized by global rearrangement (35) and were insensitive to input order of sequences.

volved in the regulation of the recombination processes that join V_H, D_H, and J_H gene segments to create an expressed heavy-chain variable-region gene.

The presence of Chi, an octamer that promotes generalized recombination in bacteriophage λ, has been seen in several mouse group III V_H families (41). Indeed, Chi is present in FW1 of almost all published mouse V_H7183, human V_H III, and *Caiman* sequences and is consistently included within the longest stretches of sequence identity between these sequences (boxed area in FW1, Fig. 2). Other highly conserved regions in mammalian sequences are nucleotides 58–84 (FW1) and 199–220 (FW3), the latter region conserved in *Caiman* and *Xenopus* group III sequences as well. These and other sequences throughout FW1 and FW3 bear similarity to elements implicated in recombination in eukaryotic systems, including 5–8 bp regions of identity to the human minisatellite repeat unit (42) and putative gene conversion initiation sites in the human immunoglobulin α constant region (43) and silkworm chorion *Hc* genes (44). Consistent with the idea that these genes contain recombinogenic elements is the finding that many meiotic recombination events seen in the mouse *Igh-V* locus are in the vicinity of V_H7183 genes (unpublished data).

Members of the V_H7183 family, which maps to the 3′, D_H-proximal end of the mouse V_H locus (16), are preferentially rearranged in early mouse development (45). A model involving the proximity of these and other frequently rearranging V_H genes to the D_H locus together with a one-dimensional tracking recombinase has been postulated to explain this phenomenon (17). In light of the striking conservation of V_H7183/V_H III genes and their seeming recombinogenic sequence content, we suggest an alternative hypothesis—that conserved group III sequences serve to activate or target local chromosomal regions for rearrangement, so that V_H7183/V_H III genes and V_H genes in the immediate vicinity are the first to be rearranged. In support of this model, V_H III genes in humans are preferentially rearranged even though they are not located closest to D_H (5, 6, 20, 45). The conserved V_H7183/V_H III sequences may be inherently recombinogenic or may serve as a recognition/binding site for a regulatory factor or component of the recombinational machinery.

Because the V_H7183/V_H III family and its homologues in other species are evolving more slowly than other V_H genes, they are the closest descendants of the primordial V_H genes from which the contemporary V_H groups diverged. Thus, the conserved features in V_H7183/V_H III were likely present and functional in those ancestral V_H genes. Studies of immunoglobulin genes in the chicken (23) have revealed that in different vertebrate lineages, alternative recombinational strategies were selected to generate antibody diversity from the same genetic elements. We speculate that the presence of recombinogenic sequences in primordial variable-region genes facilitated the evolution of different mechanisms for the generation of antibody diversity.

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