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Genetics

Complete nucleotide sequences of three V_H genes in *Caiman*, a phylogenetically ancient reptile: Evolutionary diversification in coding segments and variation in the structure and organization of recombination elements

(heterologous cross-hybridization/reptilian immunoglobulin genes/metric analysis of DNA sequences)

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ABSTRACT **Complete nucleotide sequences are described for three caiman (*Caiman crocodylus crocodylus*) immunoglobulin V_H genes (*C3*, *E1*, and *G4*) that hybridize with a murine V_H probe. The *E1* and *G4* genes are physically linked (intergenic distance, ≈6.5 kilobases) in the same transcriptional orientation but are not directly contiguous with the *C3* gene. When the coding segments, including both framework and complementarity-determining regions, of these genes and the murine probe sequences are compared by metric analysis, it is apparent that the caiman genes are only slightly more related to each other than to the mammalian sequence, consistent with significant preservation of nucleotide sequence over an extended period of phylogenetic time. Based on the presence of transcriptionally critical 5′ sequences and the absence of terminator codons, frameshift mutations, or other recognizable alterations, the genes do not appear to be pseudogenes. The *E1* gene, however, is distinguished from other V_H genes because (*i*) the spacer region within the 3′ recombination signal sequence is 12 base pairs, typical of V_κ genes but not of V_H genes, which possess 22- to 23-base-pair spacers and (*ii*) a near-perfect V_H recombination signal sequence is present within the intervening sequence that splits the segment encoding the leader. These studies establish V_H gene multiplicity in a species that arose prior to mammalian radiation and provide a description of differences in the configuration and location of recombination elements associated with an otherwise potentially functional gene.**

Based on studies in mammals, it is apparent that multiple germ line V_H genes contribute to the overall diversity of the humoral immune response that is amplified further by somatic events such as mutation and segmental joining (1–10). This latter process is mediated by relatively short DNA segments located 3′ to the coding segments of immunglobulin V_H (2, 3, 6, 7, 9) and V_L genes (9, 11, 12) and by complementary segments flanking the *D*, J_H, and J_L segments (2, 3, 9–12) located 5′ of the constant region genes. Understanding the evolution of the *V*-region gene families and their associated recombination mechanisms is essential for understanding the developmental control of antibody expression and other genetic processes involving somatic changes in DNA. Since it is likely that significant numbers of *V*-region genes are not subject to direct selection during the lifetime of an individual, the processes that govern the phylogenetic development, diversification, and stabilization of this multigenic family are important and may be unique.

To date, evolutionary studies of V_H genes largely have been restricted to comparisons between the members of structurally related families, identified in inbred mouse strains (6, 7, 13–18), as well as between murine and human sequences (19–22). In earlier reports, we described cross-hybridization between restriction enzyme-digested caiman genomic DNA and murine V_H probes (23) and demonstrated sequence similarities between caiman and prototypic mammalian V_H genes (24). In this report, we compare the coding as well as the 5′ and 3′ flanking segments of three different caiman (*Caiman crocodylus crocodylus*) V_H genes and identify unique recombination signal sequences associated with one of these genes.

MATERIALS AND METHODS

The construction, amplification, and screening of a caiman-λ47.1 library with S107V, a murine V_H probe (3), have been described (24). Standard phage purification and subcloning approaches were used. Sequences were determined using the dideoxynucleotide termination method and compared by the mathematical methods of metric analysis (25, 26).

RESULTS AND DISCUSSION

Fig. 1 *a* and *b* illustrates partial restriction maps of two V_H^+ recombinant phages, IVD and VIIIB. Comparison of the maps and additional restriction mapping data (not shown) indicates that the linked *E1* and *G4* (intergenic distance is ≈6.5 kb) are neither directly contiguous with nor most likely allelic to *C3*. Fig. 1 *c–e* identifies the functional segments of the three caiman genes and illustrates the primary strategies used in determining their nucleotide structure.

The complete nucleotide sequences of the three caiman genes and their respective noncoding 5′ and 3′ segments are presented in Fig. 2 *a* and *b*. The sequences of the gene segments adjacent to these (Fig. 1 *c–e*) will be described at a later date. All three genes encode homologous leader regions interrupted by an intervening sequence (IVS), characteristic of mammalian V_H (and V_L) genes (2, 3, 19). The putative splice donor sequence of *C3* and *E1* and acceptor sequences of *C3*, *E1*, and *G4* conform to the consensus sequence inferred from nuclear and viral genes (27) and are typical of immunoglobulin V_H genes. The *G4* donor sequence cannot be accommodated into this particular consensus sequence, but it preserves the general A·G/G·T structure and is consistent with other functional splice donor sequences (27, 28).

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Abbreviations: V_H, variable region of immunoglobulin heavy chain; V_L, light chain variable region; V_κ, κ (light) chain variable region; V_λ, λ (light) chain variable region; V, variable; D, diversity segment; J_H, heavy chain joining segment; J_L, light chain joining segment; J_κ, κ light chain joining segment; CDR, complementarity-determining region; FR, framework region; IVS, intervening sequence (segment); kb, kilobase(s); bp, base pair(s).

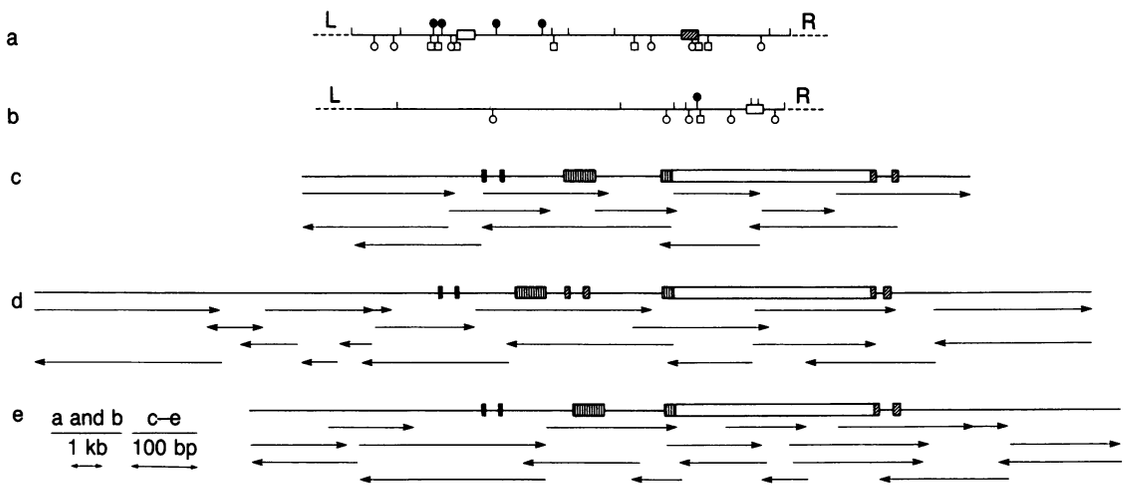
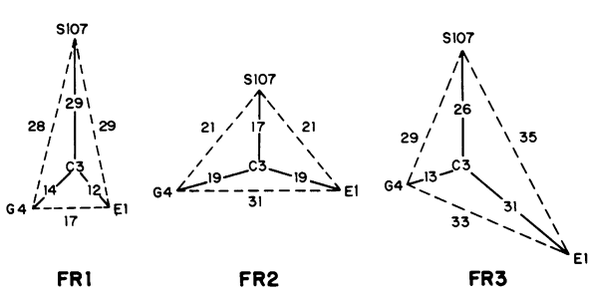


FIG. 1. Partial restriction endonuclease mapping of V_H^+ caiman genomic DNA-λ clones IVD (*a*) and VIIIB (*b*) with *Bam*HI (|), *Hin*dIII (●), *Sma* I (○), and *Sst* I (□) localizing the *G4* (□) and *E1* (▨) (*a*) and the *C3* (□) (*b*) genes. The enclosed distances correspond to the initiation codon through the recombination signal sequence of the respective genes, L and R are the left and right areas of the recombinant phage (------), and the scales for *a–e* are indicated directly as kilobases (kb) or 100 base pairs (bp). The essential organizational features of the genes, including (5′ → 3′) a hyperconserved upstream octamer and the putative promoter for RNA polymerase II transcription (▌), interrupted leader (▥), mature coding (□), and recombination signal sequences (▨) of *G4*, *E1*, and *C3* are illustrated in *c–e* along with the sequencing strategies for each of these genes. The first two rows of arrows indicate sequence determination on the (+) strand, the last two rows correspond to the (−) strand. Double-headed arrows correspond to (same direction) extension of a given sequence (typically achieved by alternative cloning strategy) overlapping the primary sequence by at least 50 bp or (opposite direction) a single clone sequenced in both directions. Dideoxynucleotide sequencing confirmed the restriction map as well as the sequence reported earlier for *C3* (24) and identified several additional *Hin*fI and *Dde* sites located within 25 bp (below the detection limits) of the map positions previously assigned for these enzymes.

The intron length is 98 bp for both *C3* and *G4* but is 171 bp for *E1*, which contains a sequence resembling an immunoglobulin recombination element (see below). The coding region of each gene is followed by a two-nucleotide spacer and a recombination signal segment. Nucleotide identity between the three genes extends 5′ and 3′ of the coding segments.

Fig. 2*b* compares the mature coding segments of the three caiman genes and *S107*, a murine V_HIII prototype (3). These can be related most efficiently by reference to the lines labeled "common acids" and "common bases" that compare the caiman gene *C3* to *E1*, *G4*, or the murine sequence. The nucleotide identities are concentrated within the framework regions FR1, FR2, and FR3 and are significantly lower in the complementarity-determining regions CDR1 and CDR2. At the level of the inferred amino acids, no residues are common to all three caiman sequences in CDR1 (residues 31–35) and the first 12 positions of CDR2 (residues 50–61). The three FR regions of all four protein sequences share 37 amino acid residues. Thus, 49% of the 76 FR residues are conserved identically in the caiman and murine prototype genes. The distances between pairs of FR gene segments are illustrated in the following spanning trees, where each distance



(edge length) is the number of base changes per 100 bases (% *bc/b*) between two segments (vertices). Since *C3* is used as the reference sequence in Fig. 2*b*, it has been used for the primary comparisons (solid line) in the spanning trees. For FR1, caiman *C3* is farthest from murine *S107*, but for FR2, *C3* is closest to *S107*. Finally for FR3, *C3* is closer to murine *S107* than to caiman *E1* but closest to caiman *G4*. Only comparison of the FR1 segments distinguishes among these particular caiman and murine V_H genes.

The three caiman sequences have also been aligned metrically with 17 additional murine (2, 6, 7, 14, 15, 17, 18) and human (19–21) V_H genes representing different subgroups and families. Overall the three caiman sequences are closest to human V_HIII prototypes—e.g., FR1, FR2, and FR3 of *G4* are 20, 12, and 20% *bc/b*, respectively, from the human *H11* gene (20) vs. 28, 21, and 29% *bc/b* for the equivalent comparisons of *G4* to the murine probe sequence illustrated above. Although it is tempting to speculate as to the various selective influences and correction processes that may have given rise to these patterns, it is important to emphasize that relaxed criteria were used in selecting the caiman genes and that members of a specific subset were not isolated. Given the apparent high degree of intraspecies variation within these families, individual gene segments from two phylogenetically divergent species may be more related to each other than are equivalent gene segments identified within the same species.

Metric analyses of sequences 5′ to the initiation codons indicate several regions of extended nucleotide identity. A potential promoter region for RNA polymerase II, $\frac{A}{C}$-T-A-A-A-T (15, 29), is located 5′ of ATG in *C3*, *E1*, and *G4*, respectively (Fig. 2*a*). All three caiman genes also possess the sequence A-T-G-C-A-A-A-T located 26–27 bp further 5′. This conserved octamer presumably is involved in transcriptional regulation (30, 31). Assuming that the start of transcription is 26–30 bp downstream of $\frac{A}{C}$-T-A-A-A-T, each of the three caiman genes possesses a C-A sequence flanked by deoxynucleotides with a pyrimidine/purine content (32) equivalent to functionally mapped transcriptional start sites of murine V_H genes (15, 29). By these criteria, it appears that all three caiman genes would support transcriptional activity. As indicated above, the presence of leader regions, typical splice sites, and uninterrupted reading frames also are consistent with functional status for these three genes. Similar consid-

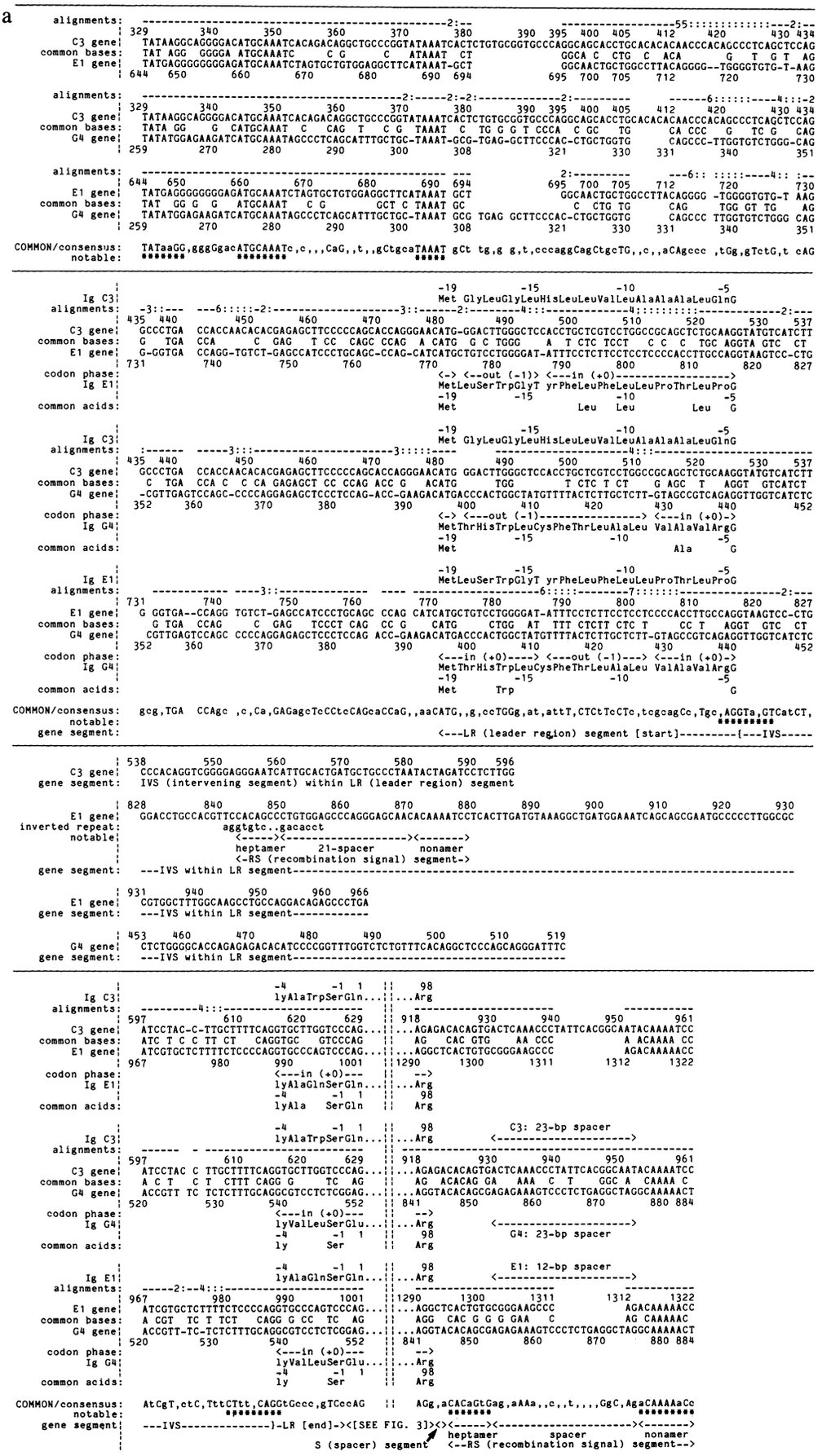
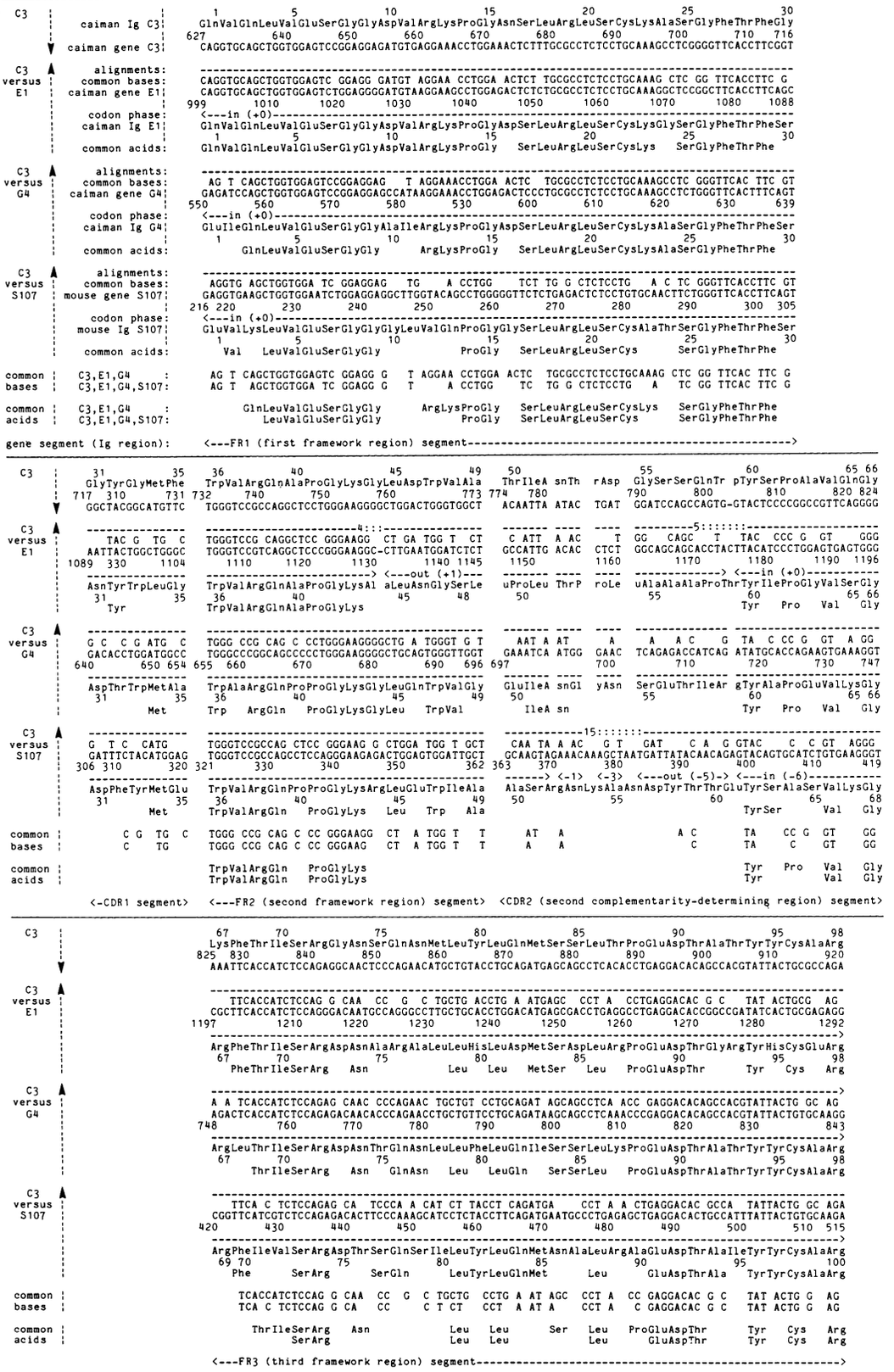


FIG. 2. DNA sequence alignments of the caiman *C3*, *E1*, and *G4* and murine *S107* (3) V_H genes. The data in *a* illustrate the sequences 5′ and 3′ of the predicted coding regions of the caiman genes and include the LR, S, and RS IVS segments. The mature coding segments are shown in *b*. Position 480 of *C3* corresponds to position 1 of the published *C3* sequence (24). Metric analysis (25, 26) was used to obtain the best pairwise

b



alignments. The symbol for each identical nucleotide pair is repeated between the two sequences. Each nonidentical nucleotide pair costs 1 base change and each nucleotide aligned with a null ("–"), corresponding to an insertion–deletion event, costs 2 base changes (26). The *alignments* line shows a dash ("–") for each position present in all metric alignments; positions having a blank space are not metrically aligned. Aligned stretches having a number of equally best alignments are indicated by that number followed by a string of colons (e.g., 4::::: denotes four alternative alignments in a stretch of six alignment positions). The *codon phase* line indicates when 2 nucleotide sequences are in or out of phase in the single metric alignment shown. In *a*, the *Common/consensus* line indicates positions having all three (*N*), two (*n*), or no (,) shared nucleotides. In *b*, *common bases* and *common acids* refer to nucleotides and amino acids shared by the caiman sequences (top row) and all four sequences (bottom row). In *a*, functionally (transcription or recombination) significant (*notable*) segments are noted by asterisks. In the IVS of *E1*, an inverted repeat and a RS segment (heptamer/spacer/nonamer) are noted. Extended deletions are present in the 5′ segments of *E1* (after 694 and 1311) and *G4* (after 330).

erations have led to classification of ≈40% of mammalian V_H sequences as pseudogenes (20, 33).

Recombination signal sequences are located 3′ to the coding segments of all mammalian V_H and V_L genes. Both *C3* and *G4* sequences match the consensus recombination 7-mer, C-A-C-A-G-$\frac{T}{C}$-G, possess a 23-bp spacer and have typical dA>dC nonamers. The *E1* 3′ 7-mer, C-A-C-T-G-T-G, is an inverse complement of the mammalian *C3*, *G4* prototype. In addition, the sequence is identical to 7-mers located at the 5′ side of *D* elements (9, 10, 34, 35) and matches the consensus for human J_H, J_κ, and J_λ recombination elements (9, 35). An identical sequence has been detected 33 bp 3′ of the prototypical recombination 7-mer of murine V_H^{441} (17) and is present at the site of an aberrant joining of a murine $J_\kappa 1$ to the nonimmunoglobulin gene segment *L10* (36). The *E1* 3′ 9-mer (A-C-A-A-A-A-A-C-C) is identical to the mammalian V_H prototypes; however, the spacer segment is only 12 bp, typical of V_κ (and *D*) but not V_H (or V_λ) recombination elements (9). A spacer segment deletion similar to the *E1* 3′ structure has been described for a pseudogene member of the *T15* family (37). As noted above (Figs. 1 and 2*a*), a V_H-like recombination element has also been detected within the IVS of *E1*. Southern blotting of restriction endonuclease-digested parent phage and plasmid subclones relative to genomic DNA isolated from several caiman genes (data not illustrated) indicates that neither 5′ nor 3′ structures arose as cloning artifacts. The close relationship of these sequences is illustrated.

```
          heptamer    21-bp spacer     nonamer
          <-----><-------------------><-------->
        843   850         862       871     879
E1 IVS  CACAGCCCTGTGGAGCCCAGGGAGCAACACAAAATCC
common  CAC G  C G G AGCCCAG         ACAAAA CC
E1 3'   CACTGTGCGG GAAGCCCAG         ACAAAAACC
     1295  1302        1313      1314    1322
          <-----><-------------------><-------->
          heptamer    12-bp spacer     nonamer
          <-RS (recombination signal) segment->
```

The two recombination elements could result in either atypical recombination (5′) or inability to undergo typical somatic reorganization (3′). The 5′ element could facilitate 5′ leader → *D* → J_H joining (assuming that these structures exist in caiman), although the abbreviated, 21 bp, spacer of the 5′ element may not be functionally active. According to the 12/23 recombination rule, the 3′ spacer segment deletion would preclude typical *V–D–J* joining; however, *E1* could participate in a direct *V–J* (light chain-like) interaction bypassing *D* joining and preserving the spacing rule.

Near-perfect direct repeats (831–839:946–954; 843–852:956–965) flanking the presumably nonfunctional 5′ recombination signal segment suggest that a recent transposition-like event (38) may have occurred within the IVS. In addition, the 7-mer is part of the 14-bp inverted repeat and contributes to one of the direct repeats (843–852) (Fig. 2*a*). The 3′ segment may have originated through homologous recombination involving caiman equivalents of *V*, *D*, or *J* segments.

Taken together these data emphasize the extended evolutionary history of this gene family and its associated reorganization mechanism. Evolution appears to preserve a core sequence and favors considerable diversification within the coding segments. The presence of atypical recombination sequences in one of the genes suggests that different alternatives to *V–D–J* joining may operate within this gene family and that these elements may be capable of recombining in previously unanticipated fashions.

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