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Evolution

Accelerated evolution in the protein-coding regions is universal in crotalinae snake venom gland phospholipase A_2 isozyme genes

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ABSTRACT **The nucleotide sequences of four genes encoding *Trimeresurus gramineus* (green habu snake, crotalinae) venom gland phospholipase A_2 (PLA2; phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) isozymes were compared internally and externally with those of six genes encoding *Trimeresurus flavoviridis* (habu snake, crotalinae) venom gland PLA2 isozymes. The numbers of nucleotide substitutions per site (K_N) for the noncoding regions including introns were one-third to one-eighth of the numbers of nucleotide substitutions per synonymous site (K_S) for the protein-coding regions of exons, indicating that the noncoding regions are much more conserved than the protein-coding regions. The K_N values for the introns were found to be nearly equivalent to those of introns of *T. gramineus* and *T. flavoviridis* TATA box-binding protein genes, which are assumed to be a general (nonvenomous) gene. Thus, it is evident that the introns of venom gland PLA2 isozyme genes have evolved at a similar rate to those of nonvenomous genes. The numbers of nucleotide substitutions per nonsynonymous site (K_A) were close to or larger than the K_S values for the protein-coding regions in venom gland PLA2 isozyme genes. All of the data combined reveal that Darwinian-type accelerated evolution has universally occurred only in the protein-coding regions of crotalinae snake venom PLA2 isozyme genes.**

Phospholipase A_2 (PLA2; phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) catalyzes the hydrolysis of the 2-acyl ester bond of 3-*sn*-phosphoglycerides with the requirement of Ca^{2+}. Aspartate-49 (numbered according to the aligned numbering of PLA2 enzymes from various sources) constitutes a part of the Ca^{2+}-binding site. The PLA2s are classified into two groups, [Asp^{49}]PLA2s with high lipolytic activity and [Lys^{49}]PLA2s with extremely low lipolytic activity (1–3). Analysis of the nucleotide sequences of six genes encoding *Trimeresurus flavoviridis* (habu snake, crotalinae; Tokunoshima Island, Japan) venom gland [Asp^{49}]PLA2s and [Lys^{49}]PLA2s showed that the protein-coding regions of exons are less conserved than introns by a factor of 4 and that Darwinian-type accelerated substitutions have occurred in the protein-coding regions except for the signal peptide domain (4, 5). Such evolutionary findings are compatible with the fact that *T. flavoviridis* venom contains PLA2 species with diverse physiological activities (6). These observations suggest that *T. flavoviridis* venom gland PLA2 isozyme genes have evolved via Darwinian-type accelerated substitutions to gain new physiological activities.

Since such accelerated evolution as found in *T. flavoviridis* venom gland PLA2 isozyme genes has not so far been known, establishment of universal occurrence of such evolution in the genes encoding venom gland PLA2s of crotalinae snake species is of great importance from the viewpoint of molecular evolution. Therefore, an evolutionary study was conducted for the genes encoding *Trimeresurus gramineus* (green habu snake, crotalinae; Taiwan) venom gland PLA2 isozymes, since we have determined the amino acid sequences of five PLA2 isozymes—four [Asp^{49}]PLA2s named PLA2-I (7), PLA2-II (8), PLA2-III, and PLA2-IV (9) and one [Lys^{49}]PLA2 named PLA2-V (unpublished results). Furthermore, to gain insight into the evolutionary feature of venom gland PLA2 isozyme genes, the evolutionary rates of introns in *T. gramineus* and *T. flavoviridis* venom gland PLA2 isozyme genes were compared with those of introns of general (nonvenomous) genes to investigate whether the former is at the same level as the latter. For this purpose, *T. gramineus* and *T. flavoviridis* PLA2 isozyme genes were compared, in terms of nucleotide divergence in introns, with *T. gramineus* and *T. flavoviridis* TATA box-binding protein (TBP) genes (10), which are assumed to be a general gene commonly present in all cell types.‖ Based on the data obtained in the present study, it became evident that accelerated evolution in the protein-coding regions is universal in crotalinae snake venom gland PLA2 isozyme genes.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases and other enzymes were obtained from Takara Shuzo (Kyoto). [α-^{32}P]dCTP ($\approx$3000 Ci/mmol; 1 Ci = 37 GBq) and deoxyadenosine [α-(^{35}S)thio]triphosphate ($\approx$1000 Ci/mmol) were from Amersham. Other reagents were of reagent grade.

Construction of cDNA and Genomic DNA Libraries. Total RNAs were extracted from *T. gramineus* venom gland (11). The mRNAs were purified by using Daynabeads mRNA purification kit (Daynal, Lake Success, NY) according to the manufacturer's protocol and were used to construct the λgt10 cDNA library with the standard method (12). *T. gramineus* and *T. flavoviridis* genomic DNA libraries were constructed by using EMBL3 as a vector in the same manner as described (5).

Screening of cDNA and Genomic DNA Libraries. *T. gramineus* venom gland cDNA library was screened by the plaque hybridization method as described (12) with full-length cDNA encoding *T. flavoviridis* [Lys^{49}]PLA2, called "basic protein I" as a probe (4). *T. gramineus* genomic DNA library was screened by the plaque hybridization method (12) with the

Abbreviations: PLA2, phospholipase A_2; UTR, untranslated region; K_N, number of nucleotide substitutions per site in the noncoding regions; K_S, number of nucleotide substitutions per synonymous site; K_A, number of nucleotide substitutions per nonsynonymous site; TBP, TATA box-binding protein.

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‖The nucleotide sequences reported in this paper have been deposited in the GenBank data base (accession nos. D01235–D01238, D13383, D13384, and D31774–D31782).

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*Bam*HI/*Sma* I fragment of *T. flavoviridis* [Asp^{49}]PLA2 isozyme gene called pgPLA 1b (5) or full-length *T. gramineus* [Lys^{49}]PLA2 (PLA2-V) cDNA (cPLA2-V) as probes. The DNAs employed for screening were labeled with [α-^{32}P]dCTP by the random priming method (13).

Subcloning and DNA Sequencing Analysis. The cloned cDNAs encoding [Asp^{49}]PLA2 (PLA2-I) and [Lys^{49}]PLA2 (PLA2-V) in phage vectors were digested with *Eco*RI and then subcloned into pUC 118 at the *Eco*RI site.

The genomic DNA clones encoding [Asp^{49}]PLA2 isozymes (PLA2-I and PLA2-VI) in phage vectors were digested with *Eco*RI and then subcloned into pUC118 at the *Eco*RI site. The genomic DNA clone encoding two [Lys^{49}]PLA2s (PLA2-V and PLA2-VII) in tandem in phage vector was digested with *Bam*HI and *Bam*HI/*Sal* I, respectively, and the inserts were then subcloned into pUC118 at the corresponding sites. The nucleotide sequences were determined by the dideoxy chain-termination method (14) using denatured plasmid as template (15).

Data Analysis. The DNASIS package developed by Hitachi Software Engineering was used for analysis of DNA sequences, and the GENAS system (16) at Kyushu University Computer Center was used to search the GenBank genetic sequence data bank for sequence homology.

For each of the pairwise comparisons of the nucleotide sequences of four *T. gramineus* and six *T. flavoviridis* PLA2 isozyme genes, the numbers of nucleotide substitutions per site (K_N) for the noncoding regions including introns and the numbers of nucleotide substitutions per synonymous site (K_S) and per nonsynonymous site (K_A) for the protein-coding regions were computed by the method of Miyata and Yasunaga (17) with corrections for multiple substitutions (18). The same computation was made for *T. gramineus* and *T. flavoviridis* TBP genes, which consist of eight exons and seven introns (10). Synonymous site is a site of a codon at which base substitution causes no amino acid change. Nonsynonymous site is a site of a codon at which base substitution causes an amino acid change.

RESULTS AND DISCUSSION

Isolation and Characterization of cDNAs Encoding *T. gramineus* PLA2 Isozymes. The *T. gramineus* venom gland cDNA library was screened with full-length *T. flavoviridis* basic protein I cDNA, which contains the 5′ and 3′ untranslated regions (UTRs), that are highly conserved throughout *T. flavoviridis* venom gland PLA2 isozyme cDNAs (4). Twenty-two positive clones were selected from 8 × 10^5 plaques. The inserts of the clones were subcloned into pUC118 at the *Eco*RI site and sequenced. Four clones encoded [Asp^{49}]PLA2 (PLA2-I), and the remainder encoded [Lys^{49}]PLA2 (PLA2-V). The longest clone encoding PLA2-I, termed cPLA2-I, was 701 bp long and that of PLA2-V, termed cPLA2-V, was 697 bp long, respectively. The sectional homologies between cPLA2-I and cPLA2-V were 97.1% for the 5′ UTR, 95.2% for the 3′ UTR, and 76.6% for the protein-coding region, indicating that the protein-coding region is much more diversified than the UTRs as in the case of *T. flavoviridis* venom gland PLA2 isozyme cDNAs (4). Moreover, the homology in the amino acid sequences was 51.6% and was much lower than the homology in the nucleotide sequences of the protein-coding regions, indicating that nonsynonymous base substitutions have occurred more frequently than synonymous base substitutions.

Isolation and Characterization of Genes Encoding *T. gramineus* PLA2 Isozymes. The *T. gramineus* genomic DNA library was screened with a *Bam*HI/*Sma* I fragment of pgPLA1b, a *T. flavoviridis* [Asp^{49}]PLA2 isozyme gene, and two positive clones with the same restriction enzyme site were obtained from 8 × 10^5 plaques. One clone termed TgPLA2-VI was 13.8 kb long (Fig. 1) and contained a 2.4-kb *Eco*RI fragment that hybridized with the probe (data not shown). This 2.4-kb fragment was designated gPLA2-VI. To obtain other clones of PLA2 isozyme genes, the genomic DNA library was screened with full-length cPLA2-V, and 29 positive clones were obtained. One clone designated TgPLA2-I was 12.3 kb long, contained a 2.4-kb *Eco*RI fragment (Fig. 1) that hybridized with the protein-coding region of cPLA2-I, and was designated gPLA2-I. Another clone termed TgPLA2-V-VII was 14.2 kb long and contained 2.8-kb *Bam*HI and 3.6-kb *Bam*HI/*Sal* I fragments, both of which hybridized with the protein-coding region of cPLA2-V and were designated gPLA2-V and gPLA2-VII, respectively. These two fragments are arranged in tandem as shown in Fig. 1. Because numerous PLA2 isozyme genes have been isolated from the genomic DNA library, *T. gramineus* PLA2 isozyme genes form a

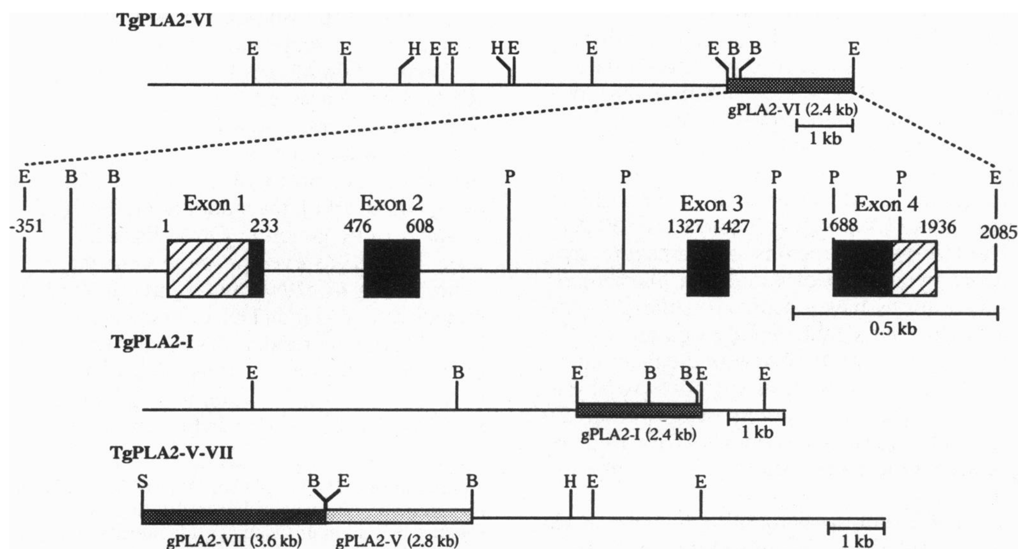

FIG. 1. Structures of phage clones TgPLA2-VI, TgPLA2-I, and TgPLA2-V-VII, the former two encoding *T. gramineus* [Asp^{49}]PLA2 isozymes and the latter encoding two *T. gramineus* [Lys^{49}]PLA2 isozymes. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I; S, *Sal* I. The fragments that hybridized with the *Bam*HI–*Sma* I fragment of pgPLA1b gene encoding *T. flavoviridis* [Asp^{49}]PLA2 isozyme or with the coding region of cPLA2-I or cPLA2-V encoding *T. gramineus* [Asp^{49}]PLA2 or [Lys^{49}]PLA2 isozyme, respectively, are shown by shaded boxes. These fragments were sequenced. The exon–intron structure of gPLA2-VI with the nucleotide position numbers is shown below the structure of TgPLA2-VI as a representative. This structure is common for all venom gland PLA2 isozyme genes. Four exons are indicated by boxes, and the UTRs are indicated by hatching.

multigene family as in the case of *T. flavoviridis* PLA2 isozyme genes (5).

Nucleotide Sequences of Four *T. gramineus* PLA2 Isozyme Genes. The nucleotide sequences of gPLA2-I, gPLA2-V, gPLA2-VI, and gPLA2-VII were determined by the dideoxy chain-termination method (14, 15) after being subcloned into pUC118. The protein-coding regions of four genes were determined by matching their sequences with those of *T. gramineus* [Asp[49]]PLA2 cDNA (cPLA2-I) and [Lys[49]]PLA2 cDNA (cPLA2-V).

The transcription initiation site of *T. gramineus* PLA2 isozyme genes was assigned by comparison with *T. flavoviridis* PLA2 isozyme genes. Because of their highly homologous sequences, the transcription initiation site of *T. gramineus* PLA2 isozyme genes could be assumed to be identical to that of *T. flavoviridis* PLA2 isozyme genes. The site was assigned to

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gPLA2-I     229 gtgagtgaagcaaaactgtaaaatgggcagctaattc-tgctccctttgcagaaggtgaaacggaggg
gPLA2-V     229 ..a........................................t........................
gPLA2-VI    234 ...........................................t............c........a..
gPLA2-VII   223 .....................................c.....t........................
pgPLA 1a    245 ...........................................t........................
pgPLA 2a    245 ...........................................t........................
pgPLA 1b    245 ...........................................t.................g....t.
pgPLA 2b    245 ...........................................t.................g......
BP-I        245 ........................................-..t.................g......
BP-II       245 ........................................-..t.................g......

298 gttacaggttggctttggctttagtggccgacatcgg-acagg-aaattcaccgttaagcgaggcatcgtgtcaccacat
298 .................a........a..........g.....g.........a..............c...........
301 .................c........a..........g.....g........................c.......g-..
291 .................a........a..........g.....g.........a..............c...........
312 .................a........a..........g.....g....................................
312 .................a........a..........g.....g....................................
312 .................t........t..........g....aa....................................
312 .................t........t..........g....aa....................................
311 .................a.g......a........t.g.....t....................................
311 .................a.g......a........t.g.....t........g...........................

376 cttctttgctgtggtcgttaag-cgaggactgccagcatctgccattaaccctacagagaagccgagggaggctgacac
378 .............................................................................a..
380 ...............c........g.......t...............................................
371 .......................g.....................................................a..
392 .a.g.........a.........................................a.....................a..
392 .a.g.........a.........................................a.....................a..
392 .acg............t......................................a.....................a..
392 .acg............t......................................a.....................a..
391 ...g....c.......t............g........................t.........................
391 ...g....c.......t............g........................t.........................

455 tttctgtcttttccagTCGAGGGGCACCTGATGCAATTTGAGACACTGATCATGAAAGTGGCGGGGAGAAGCGGTGTTT
                     alGluGlyHisLueMetGlnPheGluThrLeuIleMetLysValAlaGlyArgSerGlyValT
457 ............................................ATA......A.......A.......G......A...
                     *  *  *  *  *  *  *  *  * AsnMet * Lys *  * Thr *  *  *  * Ile
459 ..................T......GG.G....CG....GAC..AGA.....G.CC...A.AT.....A..A..C.C..A
                     *  *  * GlyValIle * LeuThrLysMet * ValGlnGluMet * LysAsnAlaLeuT
451 ....----..........T......AG.G....CG....G.G..AGA.....T.CC...A.A......A..A.CC..C.A
                     *  *  * SerValIle * LeuGlyLysMet * PheGlnGluThe * LysAsnProAlaT
471 .............a...........GG....TG......C....ATA.......T.......T.AA..A.......A.AC
                     *  *  * Gly * Trp *  *  * AsnMet * Ile *  * ValLysLys *  * IleL
471 .............a...........GG....TG......C....ATA.......T.......T.AA..A.......A.AC
                     * Asp * Gly * Trp *  *  * AsnMet * Ile *  * ValLysLys *  * IleL
471 ...................A...................C....ATA......A.......A.......G..T...A...
                     * Lys *  *  *  *  *  *  * AsnMet * Lys *  * Thr *  *  *  * Ile
471 ...................A...................C....ATA......A.......A.......G..T...A...
                     * Lys *  *  *  *  *  *  * AsnMet * Lys *  * Thr *  *  *  * Ile
470 .....c...............T...AG....G.C.....GTG..AGA.....T.CC...A.A......A.GAA.C..C A
                     * Asp * Ser * Val * LeuTrpLysMet * PheGlnGluThr * LysGluAlaAlaL
470 .....c...............T...AG....G.C.....GTG..AGA.....T.CC...A.A......A.GAA.C..C A
                     * Asp * Ser * Val * LeuTrpLysMet * PheGlnGluThr * LysGluAlaAlaL

535 GGTACTACGGCTCTTACGGATGCTTCTGCGGCGCGGGGGGCCAAGGCCGGCCACAGGACGCCAGTGACCG
    rpTyrTyrGlySerTyrGlyCysPheCysGlyAlaGlyGlyGlnGlyArgProGlnAspAlaSerAspAr
537 ...GG.....T..C..........A.......AA..............T...C..............T..
    * Trp *  *  *  *  *  * Tyr *  * Lys *  *  *  * Leu *  *  *  *  *  *  *
539 CA.C....A. CT...T......AA...T...C.........G.C.TAA....AT........CC...A.
    hrSer * SerLeu *  *  * Asn *  * Pro *  * ArgArgLys * Lys *  * Thr * Se
527 CA.C.......CT...T......AA...T...C.........G.C.TAA....A.........CC.....
    hrSer *  * Leu *  *  * Asn *  * Pro *  * ArgArgLys * Lys *  * Thr *  *
551 TT.CG...A.TG............A.......TG.........G...AA....A.........CC.....
    euSer * SerAla *  *  * Tyr *  * Trp *  * Arg * Lys * Lys *  * Thr *  *
551 TT.CG...A.TG............A.......TG.........G...AA....A.........CC.....
    euSer * SerAla *  *  * Tyr *  * Trp *  * Arg * Lys * Lys *  * Thr *  *
551 ...GG...................A.......AA...A..TG.................C..........
    * Trp *  *  *  *  *  * Tyr *  * Lys *  * Glu *  *  *  *  * Pro *  *  *
551 ...GG...................A.......AA...A..TG.................C..........
    * Trp *  *  *  *  *  * Tyr *  * Lys *  * Glu *  *  *  *  * Pro *  *  *
550 AAA.........TA.........AA........T....A.G.G....AA....A.......T.C....A.
    ysAsn *  * Leu *  *  * Asn *  * Val * ArgArg * Lys * Lys *  * Thr * Se
550 AAA.........TA.........AA........T....A.G.G....AA....A.......T.C....A.
    ysAsn *  * Leu *  *  * Asn *  * Val * ArgArg * Lys * Lys *  * Thr * Se
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FIG. 2. The nucleotide sequences of introns 1 and exons 2 of *T. gramineus* (gPLA2-I, gPLA2-V, gPLA2-VI, and gPLA2-VII) and *T. flavoviridis* PLA2 isozyme genes (pgPLA 1a, pgPLA 2a, pgPLA 1b, pgPLA 2b, BP-I, and BP-II) with the predicted amino acid sequences for exons 2. The nucleotide sequences of exons are capitalized.

the adenosine residue located at about 200 nucleotides upstream from the translation initiation codon (ATG) (19). The TATA-like sequence (20) (CATAAA) was found 34 nucleotides upstream from the transcription initiation site. All *T. gramineus* PLA2 isozyme genes, like *T. flavoviridis* PLA2 isozyme genes (5), spanned about 1.9 kb, contained four exons and three introns, and encoded proteins of 138 amino acid residues, including the highly conserved signal sequence of 16 amino acid residues. The exon–intron structure of gPLA2-VI is shown in Fig. 1.

The genes gPLA2-I and gPLA2-VI encode PLA2-I (7) and PLA2-VI, respectively, both of which are [Asp^{49}]PLA2 isozymes. The genes gPLA2-V and gPLA2-VII encode PLA2-V and PLA2-VII, respectively, both of which are [Lys^{49}]PLA2 isozymes.

Analysis of Nucleotide Substitutions in PLA2 Isozyme Genes Suggests Accelerated Evolution. The nucleotide sequences for intron 1 and exon 2 of four *T. gramineus* PLA2 isozyme genes are shown in Fig. 2 together with those of six *T. flavoviridis* PLA2 isozyme genes. Exon 2 is evidently much more diversified than intron 1. This is also true of other introns and the protein-coding regions of other exons except for the signal sequence-coding domain of exon 1.

The nucleotide sequences of *T. gramineus* and *T. flavoviridis* PLA2 isozyme genes were analyzed by computing K_N, K_S, and K_A values for pairs of genes within and between species (17, 18). Table 1 shows the values when compared within species (*T. gramineus* vs. *T. gramineus*) and between species (*T. gramineus* vs. *T. flavoviridis*). The K_N values for the noncoding regions including introns are approximately one-third to one-eighth of the K_S values for all of the pairs of genes, irrespective of comparison within species or between species. This indicates that the noncoding regions including introns are unusually conserved compared with the protein-coding regions. The K_A/K_S values for the protein-coding regions are much greater than those reported for other isoprotein genes (18, 21). Although synonymous sites are known to be much more variable than nonsynonymous sites because of much less functional constraint in the former (18, 21), this is not the case in the protein-coding regions of *T. gramineus* and *T. flavoviridis* PLA2 isozyme genes. In terms of *T. gramineus* PLA2 isozyme genes, K_A/K_S values of the protein-coding regions for pairs of gPLA2-I and gPLA2-VI and of gPLA2-VI and gPLA2-VII and of gPLA2-VI and gPLA2-V are close to 1, those for pairs of gPLA2-I and gPLA2-V and of gPLA2-I and gPLA2-VII are about 1.1, and those for the pair of gPLA2-V and gPLA2-VII is 2.63 (Table 1). The exceptionally low K_A/K_S values (0.253) obtained for a pair of gPLA2-VI and pgPLA2 1b remains to be solved.

The fact that the nucleotide sequences of *T. gramineus* venom gland PLA2 isozyme genes showed the same characteristics as found for those of *T. flavoviridis* venom gland PLA2 isozyme genes reveals that accelerated evolution is rationally assumed to be universal for crotalinae snake venom gland PLA2 isozyme genes. Recently, it was reported that in the genes encoding mojave toxins from *Crotalus scutulatus scutulatus* (crotalinae), which are heterodimeric neurotoxic PLA2s, the noncoding regions are much more conserved than the protein-coding regions (22). This further supports the conclusion that has been drawn from the present study.

Accelerated Substitutions in the Protein-Coding Regions Are Responsible for Darwinian Evolution of Venom Gland PLA2 Isozyme Genes. In *T. gramineus* and *T. flavoviridis* venom gland PLA2 isozyme genes, the protein-coding regions have been evolving at much higher rate than the noncoding regions including introns. On the other hand, it was previously noted that in *T. flavoviridis* venom gland PLA2 isozyme genes, the evolutionary rates for all introns are almost equivalent to those for all the noncoding regions (5). This is also the case for *T. gramineus* venom gland PLA2 isozyme genes. For example, when gPLA2-I and gPLA2-VI were compared, K_N values for all introns and for all the noncoding regions were computed to be 0.0716 and 0.0635, respectively. This holds for all of the pairs of genes compared between species, indicating that the evolutionary rates are almost equal between introns and other noncoding regions in venom gland PLA2 isozyme genes.

To gain a further insight into the evolutionary feature of venom gland PLA2 isozyme genes, the evolutionary rates of introns of venom gland PLA2 isozyme genes were compared with those of introns of general (nonvenomous) genes. For this purpose, we determined the nucleotide sequences of *T. gramineus* and *T. flavoviridis* TBP genes (17 kb) (10), which contain eight exons and seven introns and which are assumed to be a general gene ubiquitously present in all cell types. The K_N, K_S, and K_A values for a pair of gPLA2-VI and BP-I, as an example, were compared with those for *T. gramineus* and *T. flavoviridis* TBP genes (Fig. 3). It is noted that the K_N values for venom gland PLA2 isozyme genes are comparable to those of TBP genes. This indicates that introns of PLA2 isozyme genes have evolved at similar rates to introns of TBP genes,

Table 1. K_N/K_S and K_A/K_S values for pairs of PLA2 isozyme genes within species (*T. gramineus* vs. *T. gramineus*) and between species (*T. gramineus* vs. *T. flavoviridis*)

Pair of genes		K_N	K_S	K_N/K_S	K_A	K_A/K_S
T. gramineus vs. *T. gramineus*						
gPLA2-I	gPLA2-VI	0.0635	0.174	0.365	0.103	0.594
gPLA2-I	gPLA2-VII	0.0657	0.301	0.218	0.331	1.10
gPLA2-I	gPLA2-V	0.0550	0.263	0.209	0.296	1.13
gPLA2-VI	gPLA2-VII	0.0620	0.371	0.167	0.324	0.873
gPLA2-VI	gPLA2-V	0.0539	0.325	0.166	0.290	0.902
gPLA2-VII	gPLA2-V	0.0317	0.024	0.133	0.063	2.63
T. gramineus vs. *T. flavoviridis*						
gPLA2-I	pgPLA 1a	0.0697	0.272	0.257	0.173	0.635
gPLA2-I	pgPLA 1b	0.0634	0.184	0.345	0.101	0.551
gPLA2-I	BP-I	0.0640	0.238	0.269	0.309	1.30
gPLA2-V	pgPLA 1a	0.0639	0.324	0.197	0.292	0.903
gPLA2-V	pgPLA 1b	0.0567	0.285	0.199	0.312	1.10
gPLA2-V	BP-I	0.0477	0.156	0.306	0.138	0.885
gPLA2-VI	pgPLA 1a	0.0743	0.203	0.367	0.178	0.877
gPLA2-VI	pgPLA 1b	0.0565	0.169	0.253	0.0427	0.253
gPLA2-VI	BP-I	0.0644	0.231	0.278	0.301	1.30
gPLA2-VII	pgPLA 1a	0.0747	0.357	0.209	0.312	0.872
gPLA2-VII	pgPLA 1b	0.0693	0.329	0.210	0.345	1.04
gPLA2-VII	BP-I	0.0618	0.166	0.372	0.165	0.995

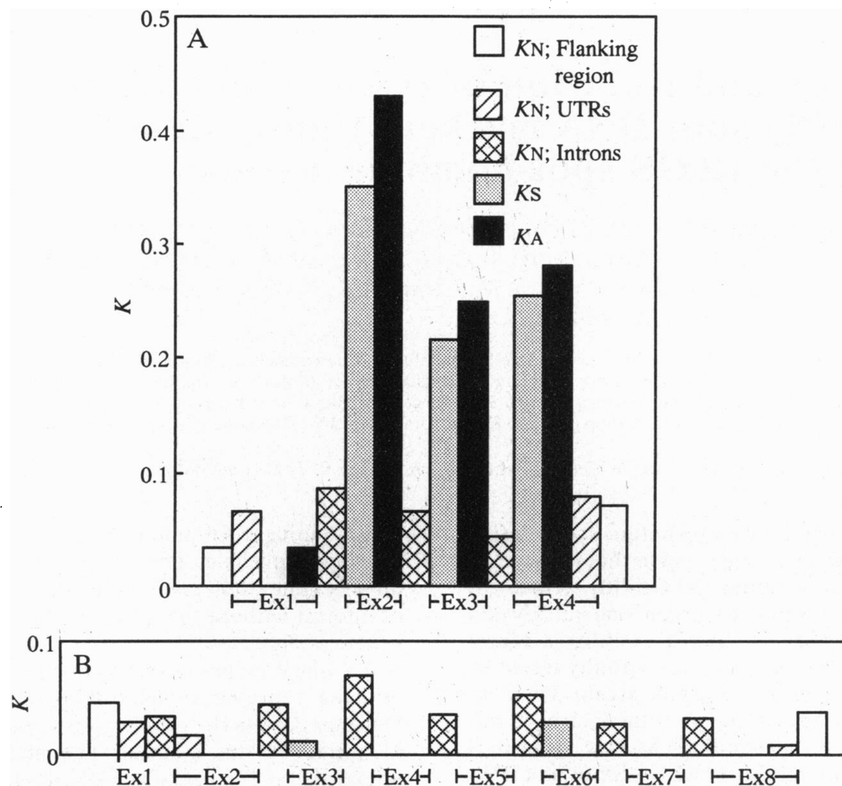

FIG. 3. Schematic representation of sectional K_N, K_S, and K_A values for a pair of gPLA2-VI and BP-I (*A*) and those for *T. gramineus* and *T. flavoviridis* TBP genes (*B*). Ex designates exons, and introns are portions sandwiched between exons. Each K is expressed by different patterns, as indicated in the key in *A*. In PLA2 isozyme genes (*A*), K_S for exon 1 is zero. In TBP genes (*B*), exon 1 contains the UTR only. In TBP genes, K_A for exons 3 and 6 and K_S and K_A for exons 2, 4, 5, 7, and 8 are all zero. It is also evident from *A* that in PLA2 isozyme genes, K_S and K_A values are much greater than K_N values.

which could be assumed to have evolved under neutrality (18, 21). Some functional constraints in the introns of PLA2 isozyme genes that had once been considered because of high homology of introns compared with the protein-coding regions could be excluded. Thus, it is emphasized that the highly accelerated substitutions having occurred only in the protein-coding regions except for the signal sequence domain are responsible for Darwinian evolution of venom gland PLA2 isozyme genes to produce new physiological activities. Indeed, distinct physiological functions have been found in *T. gramineus* venom gland PLA2 isozymes in terms of lipolysis (or hemolysis), myolysis, edema-inducing (unpublished results), and muscle contraction (8). The acquisition of such diverse PLA2 isozymes in the venom must have a strong selective advantage to disrupt the physiological integrity of animals for catching prey or for defense against predators.

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1. Tanaka, S., Mohri, H., Kihara, H. & Ohno, M. (1986) *J. Biochem. (Tokyo)* **99,** 281–289.
2. Yoshizumi, K., Liu, S.-Y., Miyata, T., Saita, S., Ohno, M., Iwanaga, S. & Kihara, H. (1990) *Toxicon* **28,** 43–54.
3. Liu, S.-Y., Yoshizumi, K., Oda, N., Ohno, M., Tokunaga, F., Iwanaga, S. & Kihara, H. (1990) *J. Biochem. (Tokyo)* **107,** 400–408.
4. Ogawa, T., Oda, N., Nakashima, K., Sasaki, H., Hattori, M., Sakaki, Y., Kihara, H. & Ohno, M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8557–8561.
5. Nakashima, K., Ogawa, T., Oda, N., Hattori, M., Sakaki, Y., Kihara, H. & Ohno, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5964–5968.
6. Kihara, H., Uchikawa, R., Hattori, S. & Ohno, M. (1992) *Biochem. Int.* **28,** 895–903.
7. Oda, N., Nakamura, H., Sakamoto, S., Liu, S.-Y., Kihara, H., Chang, C.-C. & Ohno, M. (1991) *Toxicon* **29,** 157–166.
8. Fukagawa, T., Matsumoto, H., Shimohigashi, Y., Ogawa, T., Oda, N., Chang, C.-C. & M. Ohno. (1992) *Toxicon* **30,** 1331–1341.
9. Fukagawa, T., Nose, T., Shimohigashi, Y., Ogawa, T., Oda, N., Nakashima, K., Chang, C.-C. & M. Ohno. (1993) *Toxicon* **31,** 957–967.
10. Nakashima, K., Nobuhisa, I., Deshimaru, N., Ogawa, T., Fukumaki, Y., Shimohigashi, Y., Hattori, M., Sakaki, Y., Hattori, S. & Ohno, M. (1995) *Gene* **152,** 209–213.
11. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **15,** 5294–5299.
12. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
13. Feinberg, A. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
14. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
15. Hattori, M. & Sakaki, Y. (1986) *Anal. Biochem.* **152,** 232–238.
16. Kuhara, S., Matsumoto, F., Futamura, S., Fujita, A., Shimohara, T., Takagi, T. & Sakaki, Y. (1984) *Nucleic Acids Res.* **12,** 88–99.
17. Miyata, T. & Yasunaga, T. (1980) *J. Mol. Evol.* **16,** 23–36.
18. Kimura, M. (1983) *The Neutral Theory of Molecular Evolution* (Cambridge Univ. Press, Cambridge).
19. Kozak, M. (1984) *Nucleic Acids Res.* **12,** 857–872.
20. Coden, J., Wasylyk, B., Buchwalder, A., Sassone-Corsi, P., Kedinger, C. & Chambon, P. (1980) *Science* **209,** 1406–1414.
21. Nei, M. (1987) *Molecular Evolutionary Genetics* (Columbia Univ. Press, New York).
22. John, T. R., Smith, L. A. & Kaiser, I. I. (1994) *Gene* **139,** 229–234.