Structure and Phylogeny of the Venom Group I Phospholipase A_2 Gene

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Phospholipases A_2 (PLA_2s) catalyzing the hydrolysis of phospholipids form a family of proteins with diverse physiological and pharmacological properties. While there have been several reports on the cloning of PLA_2 cDNAs, very few studies have been carried out on the PLA_2 genes and, most importantly, no information has been available on the gene structure and function of group I venom PLA_2. This study, on the PLA_2 gene from a spitting cobra, besides being the very first report on any venom group I PLA_2 gene, constitutes the missing link in the biology and evolution of phospholipases. The 4-kb gene consists of four exons and three introns and resembles the human pancreatic PLA_2 gene. However, the size of intron 3 in particular is much smaller than that in the pancreatic gene. Interestingly, the information for the toxic and most of the pharmacological properties of the venom PLA_2 can be attributed to the end of exon 3 and the whole of exon 4 of the gene. This functional delineation fits in well with the theory of adaptive evolution exhibited by the venom PLA_2s. We also show that the mammalian pancreatic and elapid PLA_2s have similar paths of evolution (probably following gene duplication) from a common ancestral gene. Venom group II phospholipases, although evolved from the same ancestor, diverged early in evolution from the group I PLA_2 genes. Intriguingly, CAT reporter gene assays and DNase 1 footprinting studies on the promoter and its deletion constructs using CHO and HepG2 cell lines identified the possible involvement of *cis* elements such as Sp1, AP2, γ-IRE, and $(TG)_{12}$ repeats in the expression of the gene in a tissue-specific manner.

Introduction

Phospholipase A_2 (PLA_2; EC 3.1.1.4) catalyzes the hydrolysis of the sn-2 fatty acyl bond of phospholipids to liberate free fatty acids and lysophospholipids (Dennis 1983, 1994). PLA_2s form a diverse class of enzymes with regard to function, localization, regulation, and structure. They also play a central role in many cellular processes, including phospholipid digestion and metabolism, host defense, and signal transduction (Dennis 1994). In addition, PLA_2s provide precursors for the generation of eicosanoid and the formation of platelet-activating factor (Dennis et al. 1991; Dennis, Deems, and Yu 1992). Hence, they participate in several important human disorders, including rheumatoid arthritis, autoimmune uveitis, respiratory distress syndrome, myocardial infarction, and septic and endotoxic shock (Mukherjee, Miele, and Pattabiraman 1994).

The venom PLA_2s, on the contrary, while possessing a digestive function, exhibit a wide variety of pharmacological properties such as antiplatelet, anticoagulant, hemolytic, neurotoxic (presynaptic), myotoxic, edema-inducing, hemorrhagic, cytolytic, cardiotoxic, and muscarinic inhibitor activities (Rosenberg 1990; Harris 1991; Hawgood and Bon 1991; Yang 1994). The PLA_2s that have been extensively characterized at protein level both structurally and functionally are the extracellular forms, such as the pancreatic PLA_2 from mammals (human [Verheij et al. 1983], bovine [Fleer, Verheij, and De Haas 1978], rat [O'Hara et al. 1986], and canine [Kerfelec et al. 1986]) and the venom phospholipases from a bee (*Apis mellifera*; Kuchler et al. 1989) and snakes (viperids [Ritonja and Gubensek 1985; Wang et al. 1992], crotalids [Fukagawa et al. 1993; Faure et al. 1991], elapids [Tan and Armugam 1989; Rowan, Harvey, and Menez 1991]).

Traditionally, the venom secretory PLA_2s are classified into three main groups (groups I–III). They are cysteine-rich (five to seven disulphide bonds) proteins with low molecular mass (13–18 kDa) which require Ca^{2+} for catalysis (Heinrikson, Kruger, and Keim 1977). Many (over 100) such PLA_2s have been sequenced and found to be more than 40% identical to each other. The amino acid residues involved in catalysis have always been found to be conserved.

The authors of a phylogenetic analysis using the amino acid sequences of PLA_2s (Davidson and Dennis 1990) proposed that at least two PLA_2 genes existed at the time the ancestral lines of reptiles and mammals diverged, long before snakes came into existence. The two major groups of poisonous snakes, the Proteroglypha (Elapidae, including cobras and kraits) and the Solenoglypha (Viperidae, containing rattlesnakes and vipers), each have been considered to have acquired one of the two ancestral PLA_2 genes for the production of their venoms. Heinrikson, Kruger, and Keim (1977) also classified the PLA_2s into two groups, based on their Cys residues, as group I PLA_2s, having a disulphide bridge between the half cysteines at positions 11 and 69 (found in the venom of Elapidae snakes and in the pancreatic juices of mammals), and group II PLA_2s, characterized by a C-terminal extension containing a half cysteine linked to a half cysteine at position 50 (found in Viperidae snake venoms and in mammalian platelets, liver, and spleen).

While there have been a number of studies on the cloning of cDNAs encoding PLA_2s (Smith 1990; Pan, Chang, and Chiou 1994; Moura-da Silva et al. 1995; Armugam et al. 1997) there are only a few reports available on the cloning and characterization of PLA_2 genes (Seilhamer et al. 1986; Kordis and Gubensek 1996;

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Ohno et al. 1998). The structural organizations of the mammalian group I and group II PLA_2 genes have been found to be different from each other. The former consists of four exons and three introns (Seilhamer et al. 1986; Kerfelec et al. 1990) and the latter of five exons and four introns (Seilhamer et al. 1989; Komada, Kudo, and Inoue 1990). The genes of Crotalinae venom PLA_2 (Ohno et al. 1998), however, showed a structure similar to that of group I PLA_2 genes, although the protein structure conformed with group II enzymes. The genes of Viperidae snake venom PLA_2s (Kordis and Gubensek 1996) have been found to posses the group II PLA_2 gene structure. Kordis and Gubensek later explained that this irregular structure of the Crotalinae group II PLA_2 gene is due to a 40-bp deletion in exon 1 of the gene which resulted in abolishment of the splicing of the first intron. This apparent structural irregularity and the absence of information on the group I venom PLA_2 genes from elapids prompted us to examine the PLA_2 genes in the spitting cobra *Naja sputatrix* (synonyms: 1827 *Naja sputatrix* Boie, 1907 *Naja naja sputatrix* Stejneger, and 1989 *Naja sputatrix* Wuster and Thorpe; Wuster and Thorpe 1991). In this report, we present the structure, organization, and promoter analysis of genes encoding two isoforms (acidic and neutral) of PLA_2s in the venom of *N. sputatrix* (Armugam et al. 1997). Both of the genes contain four exons and three introns and constitute the missing link in the biology of PLA_2s and form the first report on the nonpancreatic group I PLA_2 from a venomous snake. Besides identifying some of the regulatory elements and proteins that are involved in the control of expression of the elapid PLA_2 gene, we also analyzed for the first time the phylogenetic relationship of both group I and group II PLA_2s based on the information of whole genes. Group I and group II PLA_2s appear to have evolved in separate ways from a common ancestor, whereas the human and elapid group I PLA_2s showed a common path of evolution.

Materials and Methods

Isolation of Liver DNA

Naja sputatrix was provided by N.-H.T. The snake was identified as a spitting cobra and then sacrificed for liver samples. Frozen snake liver was pulverized into a fine powder in liquid nitrogen (Sambrook, Fritsch, and Maniatis 1989). The genomic DNA was extracted in the presence of SDS and Proteinase K (Boehringer Mannheim) and purified using phenol/chloroform. DNA was precipitated with 2.5 volumes of 95% ethanol. The DNA pellet was then washed with 70% ethanol and resuspended in TE buffer.

Amplification of Genomic DNA

Custom-designed oligonucleotide primers, based on the sequence of the highly conserved regions in the PLA_2 cDNA of *N. sputatrix,* were supplied by Oswell DNA Service (United Kingdom). PCR was performed using the Advantage Genomic PCR kit (Clontech). Amplification of PLA_2 genes was carried out on 10 ng of genomic DNA of the snake liver in a 50-μl reaction mixture. The thermal profile involved a hot start at 94°C for 1 min, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 3 min at 68°C, followed by a final extension at 68°C for 10 min using a Perkin Elmer Cetus thermal cycler (model 480). The following primers were used in the amplification of the PLA_2 gene.

Sense primers were X224F (5′-ATgAATCCTgCTCACCTTCTg-3′), X347F (5′-CTTggTggCATTTTgCggACTACggTTgCTAC-3′), and X223F (5′-TTCAAgACCTATTCATACgAgTgT-3′), and antisense primers were X230R (5′-CgTCCgCAgTAgCAACCgTAgT-3′), X346R (5′-gAATAggTCTTgAAgTAgggCCAgCATCTggA-3′), and X225R (5′-gCCTTgAggTCgATATTgTAgTTg-3′). Primers were designed based on cDNA sequences of *N. sputatrix* PLA_2 (Armugam et al. 1997) using the PrimerSelect program of DNASTAR Inc. (Madison, Wis.). A fourth set of primers, PGF (sense) (5′-ggCACTgAggATgggATTg-3′) and PGR (antisense) (5′-AAggTCCCTgTTgggTCCCTggTgC-3′), were used to amplify the complete PLA_2 gene(s).

Amplification of 5′- and 3′-flanking Regions

The 5′ and 3′ ends of the PLA_2 gene were amplified using the Universal GenomeWalker kit (Clontech). Briefly, the procedure involved the construction of the adaptor-ligated libraries (GenomeWalker uncloned libraries) made by separate restriction digestion of genomic DNA with *Dra*I, *Eco*RV, *Pvu*II, *Sca*I, and *Stu*I, followed by ligation to a special adaptor provided in the kit. Two sets of primers were used for the amplification of the 5′ region: the adaptor primer 1 (AP1) (5′gTAATACgACTCACTATAgggC-3′), provided in the kit, and the gene-specific reverse primer GSP1 (X267) (5′-AATCTCCACTTACCTgCTgCCAggAT-3′) for primary PCR and the nested adaptor primer 2 (AP2) (5′-ACTATAgggCACgCgTggT-3′) and the nested gene-specific reverse primer GSP2 (X268) (5′-gCTGCCAggATCAgAAggTgAgCAgg-3′) for secondary PCR.

Primers used for mapping the 3′ end of PLA_2 gene(s) were again AP1 and the gene-specific forward primers GSP3 (X276) (5′-gACCgCTTggCAgCCATCTgCTTCg-3′) for primary PCR, followed by AP2 and the nested gene-specific forward primer GSP4 (X277) (5′-ATCTgCTTCgCCggAgCCCCTTACAA-3′) for secondary PCR. "Touchdown" PCR (Roux 1995) was conducted for the amplification of the 3′ and 5′ regions.

Subcloning and DNA Sequencing

The PCR products were analyzed on agarose gel. The appropriate bands were purified (Qiaquick gel extraction kit, Qiagen) and subcloned into pT-Adv vector (Clontech Advantage cloning kit). The ligated products were then transformed into *Escherichia coli* strain TOP10F′, and the recombinants were selected on LB-Amp (50 μg/ml) plates supplemented with IPTG and X-Gal. Putative recombinant clones were then subjected to Sanger dideoxy DNA sequencing (Sanger, Nicklen, and Coulson 1977) using M13/pUC universal primers as well as gene-specific primers where appropriate. All

double-stranded sequencing was performed with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) using an Automated DNA sequencer (Applied Biosystems, model 373).

Sequence Analysis

Nucleotide sequence homology searches of nonredundant databases in GenBank (National Centre for Biotechnology Information) were performed using the BLAST program. DNA sequence alignments were carried out using the DNASIS software package from Hitachi Software Engineering. The molecular evolutionary relationships among the phospholipase A_2 genes were examined using the MegAlign program from DNASTAR software. Acidic and neutral PLA_2s reported in this paper have been assigned GenBank accession numbers AF101235 and AF101236, respectively.

Primer Extension Analysis

Primer extension analysis was performed to identify the transcription initiation site(s) of the PLA_2 gene(s). Total cellular RNA was extracted from the *N. sputatrix* venom glands using the guanidine isothiocyanate method (Chomczynski and Sacchi 1987), and the integrity of total RNA was analyzed by denaturing formaldehyde agarose electrophoresis (Sambrook, Fritsch, and Maniatis 1989). The primer extension was carried out according to Lachumanan et al. (1998). Ten picomoles of 5′-end ^{33}P-labeled reverse primer GSP1 (5′-AATCTCCACTTACCTgCTgCCAggAT-3′) was annealed to RNA (10 μg) at 65°C, and a primer extension reaction was carried out at 42°C for 1 h in the presence of 20 U of MuMLV reverse transcriptase. The reverse-transcribed products were electrophoresed on 6% denaturing polyacrylamide DNA sequencing gel. A template harboring the 5′-flanking region of the PLA_2 gene was used as a size marker.

Transfection of Mammalian Cells and Promoter Analysis

Human hepatoma (HepG2) and Chinese hamster ovary (CHO) cells were grown in alpha-MEM media and transfected using the calcium phosphate precipitation method, enhanced by glycerol shock (Promega Corp., Madison, Wis.). Ten micrograms of $pMAM_{neo}$ CAT containing the 5′ PLA_2 promoter region its deletion constructs were cotransfected separately with 6 μg pSV-β-Gal for checking the transfection efficiency. Cells were harvested 48 h after transfection and freeze-thawed in 250 mM Tris-HCl (pH 8.0). The CAT enzyme reaction was performed in the presence of ^{14}C-chloramphenicol (0.05 mCi/ml), n-Butyryl CoA in 250 mM Tris-Cl (pH 8.0). The reaction product was separated on silica-coated plates by thin-layer chromatography (TLC), using chloroform/methanol (97:3) as the mobile phase. The TLC plate was then autoradiographed to identify the product. The CAT activity was calculated based on the counts per minute of monoacetylated species counted in the COBRA AutoGamma counter (Packard Instruments Co. Inc.).

DNase I Footprinting

The DNase 1 footprinting analysis was done using the Sure Track footprinting kit (Pharmacia Biotech, Sweden) as described by Garabedian et al. (1993). Briefly, probes were prepared by digesting the deletion and the full promoter constructs in pT7 Blue(R) vector (Novagen, Madison, Wis.) with *Hin*dIII and *Sac*I restriction enzymes and labeling the *Hin*dIII end using Klenow DNA polymerase and [α-^{32}P]dATP and [α-^{32}P]dGTP. The radiolabeled probes were incubated separately with CHO, snake venom gland, snake liver, and HepG2 nuclear extracts for 30 min on ice in binding buffer containing 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 2.5 mM $MgCl_2$, 0.5 mM DTT, and 4% glycerol. The protein-bound DNA fragment was treated with 0.3 U of DNase 1 for 1 min at room temperature and extracted with phenol/chloroform. The protected DNA was electrophoresed on an 8% polyacrylamide gel under denaturing conditions. The gel was autoradiographed on Kodak X-Omat AR5 film at −80°C.

Results

Gene Structure and Organization

PCR amplification of the PLA_2 gene was carried out using three pairs of overlapping primers. All of the primers were designed based on the highly conversed regions of the PLA_2 cDNA. Primers X224 and X230 amplified exon 1 (with signal peptide), intron 1, and a portion of exon 2 (∼1.7-kb fragment) of the PLA_2 gene (nucleotides 30–1656).

Primers X346 and X347, which overlap the reverse primer X230, amplified the regions of exon 2, intron II, and a part of exon 3 of the gene (nucleotides 1632–2740). The remainder of exon 3, intron III, and exon 4 (∼800-bp fragment) were obtained using primers X233 and X225 (nucleotides 2727–3396). From the nucleotide sequences of the above fragments, carried out on both strands, a complete sequence of the group I PLA_2 gene from *N. sputatrix* was obtained. The gene comprised four exons, exon 1 (34 bp), exon 2 (172 bp), exon 3 (113 bp), and exon 4 (122 bp), and three introns, intron I (1.45 kb), intron II (973 bp), and intron III (508 bp), for a total length of 3.379 kb from the ATG initiation codon to the TAG termination codon (nucleotides 30–3409).

The 5′-flanking region of the gene was amplified by PCR using an adaptor primer (AP1) and a reverse primer of the signal peptide. Similarly, the 3′ end of the gene was obtained by PCR using a forward primer selected from exon 4 and the adaptor primer. PCR products of 395 and 2.08 kb were obtained for the 5′ region and the 3′ region, respectively, from the *Eco*RV uncloned library. Amplification of the complete gene was achieved using a primer set at the 5′ and 3′ regions of the gene. The 4.1-kb fragment obtained was sequenced and confirmed to be the PLA_2 gene. Analysis of the gene sequences showed the presence of two PLA_2 genes, one

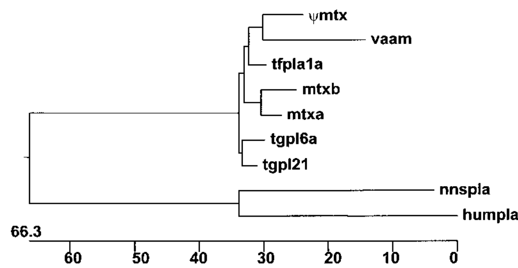

FIG. 1.—Phylogenetic analysis of the phospholipase A_2 (PLA_2) genes. A cladogram obtained from phylogenetic analysis using MegAlign from DNASTAR in shown. The length of each pair of branches represents the distance between sequence pairs. The scale under the tree measures the distance between sequences. The abbreviations of PLA_2 genes are the ψmtx gene (*Crotalus scutulatus scutulatus*; John, Smith, and Kaiser 1996), mtxa and mtxb (*C. s. scutulatus,* mtx A and B genes; John, Smith, and Kaiser 1994), tfpla1a (*Trimeresurus flavoviridis,* D10722), tgpl6a and tgpl21 (*Trimeresurus gramineus* PLA_2-VII [D31779] and $PLA_2$1 [D31780]), vaam (*Vipera ammodytes,* ammodytoxin; X76731), humpla (human PLA_2; Seilhamer et al. 1986), and nnspla (*Naja sputatrix*; this study).

of them encoding for an acidic PLA_2 (APLA) and the other for a neutral PLA_2 (NPLA). Both genes showed identical introns, as well as 5′ and 3′ regions (GenBank accession numbers AF101235 and AF101236). The variant amino acids (and the exons encoding them) $Asp^{20} \rightarrow His^{20}$ and $Asp^{39} \rightarrow Gln^{39}$ (exon 2); $Val^{46} \rightarrow Ileu^{46}$ and $Gly^{52} \rightarrow Asn^{52}$ (exon 3), and $Asp^{83} \rightarrow Asn^{83}$ (exon 4) for acidic and neutral PLA_2s, respectively (Armugam et al. 1997), can be observed on the deduced amino acid sequences. Except for these regions, both APLA and NPLA genes exhibited identical sequences on all of the introns and exons, as well as the 5′ and 3′ regions of the gene.

Phylogenetic Analysis of the PLA_2 Genes

Nucleotide sequences of the *Trimeresurus flavoviridis, Trimeresurus gramineus, Vipera ammodytes, Crotalus scutulatus scutulatus,* human, and *N. sputatrix* PLA_2 genes were aligned using the MegAlign DNASTAR software package. The noncoding regions appeared to be highly conserved among the families, while the coding regions showed significant variations. Our phylogenetic analysis (fig. 1) using the whole gene sequences showed that the two groups of snakes and mammals originated from a common ancestor and that the Elapidae and Viperidae diverged later from each other.

Putative Regulatory Sequence Elements of the PLA_2 Gene

The putative regulatory sequences in PLA_2 genes were identified using the TRANSFAC and TFD databases. The 395-bp 5′ region contained the putative enhancers AP-1, AP-2, NF-IL6 (two sites), activator SpI (four sites), a C/EBP element, and three γ-IRE sites (fig. 2*A*). Phorbol ester and protein kinase response elements, PEA1, a polyoma enhancer element, PEA3, and other sites, such as MRE CS2 associated factor, MyoD, Myogenin, E2A, and c-Myb factor, were also found. A eukaryotic consensus TATA box and a retroviral TATA box were found 60 and 186 bp upstream of the ATG codon, respectively. A ubiquitous calcium-regulated CCAAT motif–binding factor (CF1) was also observed. However, the classical CAAT element was absent. The 5′ region consisted of a significant purine-pyrimidine-rich region $(TG)_{12}$.

Functional Activity of Group 1 PLA_2 Promoter

Six constructs (5′PLA-302, 5′PLA-266, 5′PLA-233, 5′PLA-116, 5′PLA-90, and 5′PLA-51; fig. 2*B*), containing fragments of deleted promoter regions coupled to the CAT gene, were transfected into HepG2 and CHO cells to determine the activity of the PLA_2 gene promoter. The promoter in reverse orientation (r 5′PLA) and the 395-bp PLA_2 promoter fragment (5′PLA) were used as negative and positive controls, respectively. Figure 2*C* shows the results of CAT assay of the promoter constructs in both CHO and HepG2 cells. In CHO cells, the highest and the lowest promoter activities were observed for 5′PLA-233 and 5′PLA-90 constructs, respectively. On the other hand, for HepG2 cells, the 5′PLA-90 clone showed the highest activity, while 5′PLA-233 showed the lowest activity. It is interesting to note that the deletion constructs 5′PLA-233 and 5′PLA-90 show opposite effects in CHO and HepG2 cells. DNase I footprinting analysis using the whole promoter (5′PLA) and the deletion construct (5′PLA-233) demonstrated that the region −233/−147 is protected from DNase 1 digestion in all cases (with HepG2, CHO, snake venom gland, and snake liver nuclear proteins) apart from the TATA elements (fig. 3*A* and *B*). This region contains the two γ-IRE sites and one Sp I element. On the other hand, the 5′ region spanning nucleotides −220 to −233 has also been protected by proteins from CHO and snake venom gland and snake liver nuclear extracts (fig. 3*A*).

Determination of the Transcription Initiation Site of the PLA_2 Gene

The transcription initiation site (TIS) was determined by primer extension using primer GSPL1 (AATCTCCACTTACCTGCTGCCAGGAT), which is reverse-complementary to the signal peptide of the PLA_2 cDNA. The TIS was located as the adenosine nucleotide in the sequence $ATTC\mathbf{A}^{-29}$ upstream of the ATG codon (fig. 3*C*).

Discussion

The snake venom PLA_2 family of proteins is divided into two main groups, group I and group II, based on their primary structures (Dennis 1983). The group I PLA_2s are abundant in the venoms of elapids and hydrophids, while group II PLA_2s are common in crotalids and viperids. To date, only group II venom PLA_2 genes have been studied extensively (Ohno et al. 1998). This report marks the first gene structure and analysis ever reported for group I venom PLA_2. The 4-kb genes of both acidic and neutral isoforms consist of four exons and three introns. The intron/exon splice regions show-

(2A)

MyoD

atctaatccaac**cacctg**ctccagca**gggtta**gaaagga***gtgtgtgtgtg*** -317

AP-1

tgtgtgtgtgtgtgc[gcacgcgcgtgcatgg**tgag**]**tca**taacttagaaca -267
5'PLA-302

C/EBP/γ-IRE CS/NF-IL6

[cttactaagcaaccggc**c**]**attaagcaag**gacca[cttatatttggtttgcc] -217
5'PLA-266 5'PLA-233

CF1 γ-IRE CS

agtc**acatgg**ccacttggccacacccagccagtca**catgaccc**accaagc -167

SP-I γ-IRE CS SP-I cMyb NF-IL6

c**acgcccatgaatt**aagcc**acgccc**acataaa**cagttgttaggaaat**ttg -117

AP-2 SP-I

[aat**cccacccc**ttgatggg]gggcact[gaggatgggattgtagcg]ccaa**gg** -67
5'PLA-116 5'PLA-90

SP-I TATA

gggcggtggactgaa[gaagtttggggaccactg]c[**tataa**]aaggctgaact -17
5'PLA-50

TIS +1 MET

cgccaggtggaa**attcA**TCTTGCTTGCAGCTTCACCACGGACAGA**ATG**AA 34

(2B)

(2C)

ing the typical GT (donor) and AG (acceptor) splice sites (Breathnach and Chambon 1981) were assigned based on the cDNA sequences described previously (Armugam et al. 1997). The gene structure (fig. 4) is similar to the mammalian group 1 PLA_2 gene (Seilhamer et al. 1986; Kerfelec et al. 1990) and the snake venom group II PLA_2 gene from *T. flavoviridis* (Nakashima et al. 1993), *T. gramineus* (Nakashima et al. 1995), and *C. s. scutulatus* (John, Smith, and Kaiser 1994).

Exon 1 of the *N. sputatrix* PLA_2 gene encodes a 5′ untranslated region (UTR) initiated from the (+1) adenosine nucleotide and a highly conserved hydrophobic core of the signal peptide (Scheele and Jacoby 1983), as in exon I of human pancreatic (Seilhamer et al. 1986) and crotalid (Ohno et al. 1998) PLA_2 genes. The 5′ UTR of the *N. sputatrix* PLA_2 gene also shows 90%–96% similarity to the 5′ UTR in PLA_2 cDNAs of *Aipysurus laevis, Laticauda laticaudata,* and *Naja atra* (GenBank, BLASTN).

The codons for the conserved residues, important for function, are located in exon 2 of the *N. sputatrix* gene. The highly conserved substrate recognition amino terminal segment of lipophilic residues Leu^{2}, Phe^{5}, and Ile^{9} and the overlapping Ca^{2+}-binding loop, containing the glycine-rich sequence Tyr^{24}-Gly^{25}-Cys^{26}-Tyr^{27}-Cys^{28}-Gly^{29}-Arg^{30}-Gly^{31}-Gly^{32}-Ser^{33}-Gly^{34} (Kramer et al. 1989), are found in exon 2. Exon 3 contains the coding regions for the characteristic cobra loop (Glu^{53}, Ala^{54}, Glu^{55}) found in cobra PLA_2 (Heinrikson, Kruger, and Keim 1977), as well as the active-site residues His^{48}, Asp^{49}, Tyr^{52}, and Tyr^{64}, important for the formation of the catalytic network at its proximal end. The anticoagulant region, corresponding to residues 54–77 (Kini and Evans 1989), is located at the end of exon 3. The codons for the presynaptic cluster residues 55–65 and 80–89 (Arriagada and Cid 1989) are separated by intron III. The codons for both neurotoxicity (residues 80–100; Kini and Iwanaga 1986*a*) and myotoxicity (residues 80–88; Kini and Iwanaga 1986*b*) and the region contributing to lethality (Phe^{100}–Tyr^{105}; Gubensek et al. 1994) are located in exon 4. Hence, exons 3 and 4 contain the residues which are important for the pathophysiological activities described for snake venom PLA_2.

The nucleotide substitution in the gene was found to be restricted to the protein-coding region (exons 2, 3, and 4). Exon 1 and the introns, as well as the 5′ and 3′ ends of the gene, have been found to be highly conserved among the two PLA_2 genes of *N. sputatrix.* Exons 2, 3, and 4 exhibited only nonsynonymous nucleotide substitutions leading to amino acid changes in the PLA_2 protein. This observation has also been reported for PLA_2 genes from Viperidae (Ogawa et al. 1996). Thus, the elapid group I PLA_2 genes could have evolved via positive Darwinian selection in a manner similar to that of Viperidae PLA_2 genes.

The total intervening sequences (introns) in *T. flavoviridis, V. ammodytes, C. s. scutulatus, N. sputatrix,* human, and canine pancreatic PLA_2 genes have been found to be 1.28, 1.34, 1.84, 2.93, 5.00, and 8.19 kb, respectively (fig. 4). The disparity in sizes of the genes among crotalids, elapids, and mammals were found to be mainly due to the differences in sizes of introns 1 and 3. The introns of viperid PLA_2 gene are comparatively smaller (163–693 bp) than the others. Intron 1 of the *N. sputatrix* (1.45 kb) PLA_2 gene is of approximately the same size as its mammalian counterpart (1.6 kb) and is six times as large as that of crotalids. On the other hand, introns 3 of *N. sputatrix* (508 bp) and *C. s. scutulatus* (884 bp) PLA_2 genes are significantly smaller (five- and threefold, respectively) than that of the human pancreatic PLA_2 gene (fig. 4). The smaller intron 3 may have provided an added advantage in adaptive evolution of the PLA_2 genes in snakes, as illustrated by the additional pharmacological properties possibly encoded by exons 3 and 4 in the venom PLA_2 proteins. We speculate that the PLA_2 gene acquired longer introns in its sequence during the evolution of mammals from reptiles in order to minimize the rate of mutation and to protect its fundamental role as a catalytic protein. The evolution of multiple copies of cardiotoxin (Lachumanan et al. 1998) and neurotoxin (Afifiyan et al. 1999) genes from *N. sputatrix* containing shorter introns, as well as ion channel toxins encoded by intronless genes found in lower forms of animals such as sea anemone (Gendeh, Chung, and Jeyaseelan 1997) and scorpions (Delabre et al. 1995; Becerril, Marangoni, and Possani 1997), provides support for our hypothesis.

Based on the BLAST analysis, the similarities between human pancreatic and *N. sputatrix* PLA_2 intron I and II were 29% and 31% respectively in the opposite (plus and minus) strands of the DNA, while the Viperidae PLA_2 showed 24%–37% homology (in the plus strand). ART2 retrotransposons (Kordis and Gubensek 1995) and CRI-like long terminal repeats (Nobuhisa et al. 1998) reported to be present in intron 4 and the 3′ region of Crotalinae PLA_2 genes and postulated to have been acquired by homologous recombination during

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FIG. 2.—Promoter region of the phospholipase A_2 (PLA_2) gene. *A,* Nucleotide sequence of the 5′ region of the *Naja naja sputatrix* PLA_2 gene. The start codon (ATG) is indicated. The TATA box and the transcription initiation sites (TISs) are also marked in bold. The putative regulatory elements, the $(TG)_{12}$ repeats, and the gggtta motifs are also shown. Primer locations for the respective deletion constructs are boxed. *B* Map of the promoter deletion constructs. The deletion constructs, generated from the 5′ region, are shown with their respective putative *cis*-acting elements. All of the constructs contain the TATA box and the TIS. The fragments were fused to the upstream of the CAT gene in the pMAM-CAT promoterless vector. *C,* Chloramphenicol acetyl transferase activity. The promoter deletion constructs were transfected into HepG2 and CHO cells. The relative CAT activity was assayed after 48 h of growth using ^{14}C-chloramphenicol as substrate. The radioactivity of the monoacetylated product was counted (cpm) in a gamma counter, and the histogram was constructed. The whole promoter (5′PLA) and the reverse orientation promoter (r 5′PLA) were used as controls. Transfection efficiency was determined by using β-gal gene constructs and assaying for β-galactosidase. The error bars indicate an average of three independent readings (SD). Filled bars represent HepG2 cells, and open bars represent CHO cells.

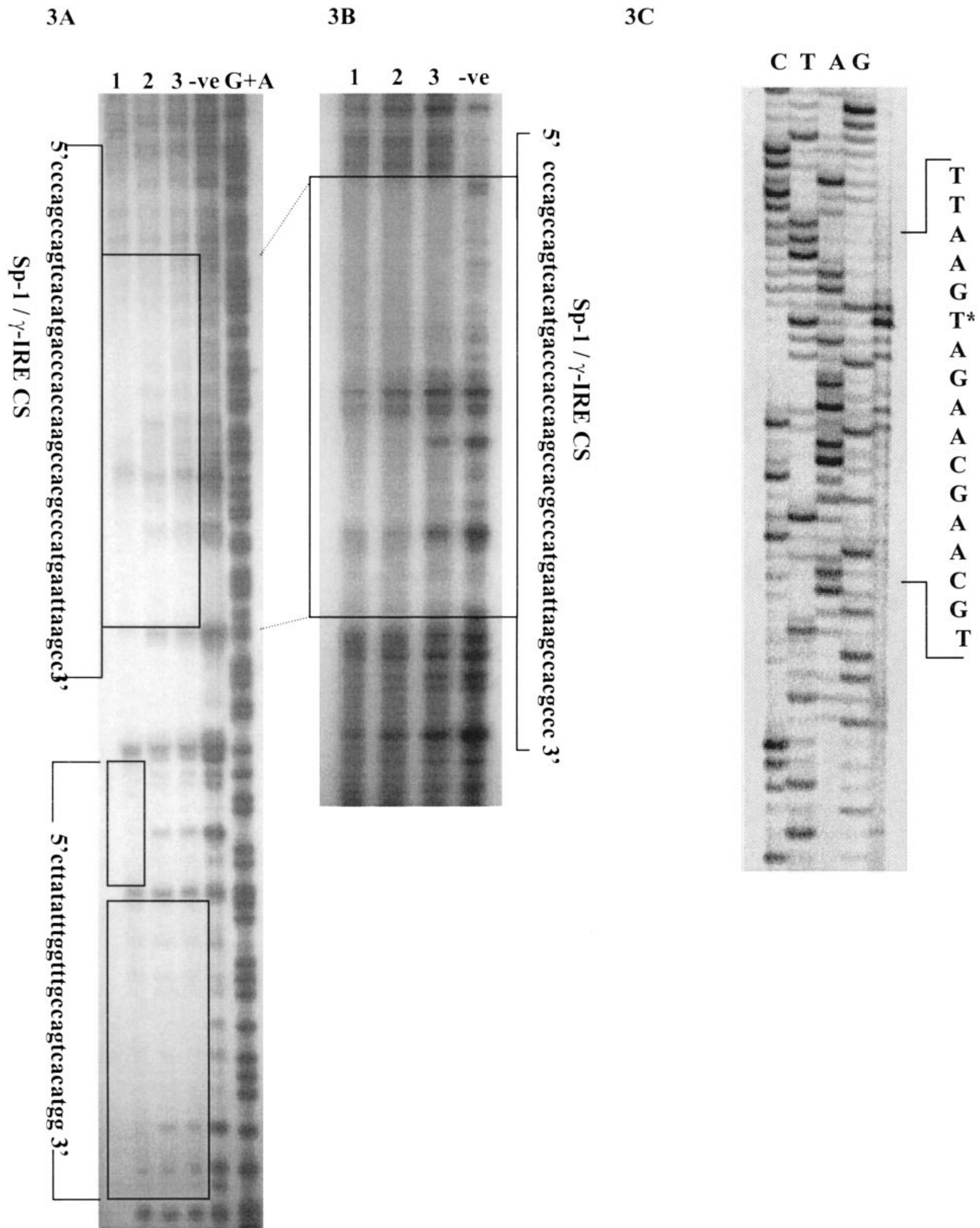

FIG. 3.—DNase 1 footprinting and primer extension analysis. For DNase 1 footprinting, the 5′PLA-233 fragment was labeled at the 5′ end of the noncoding strand using the [α-^{32}P]dATP/dGTP by end-filling and incubated with the respective nuclear proteins. The protein bound DNA fragment was digested with DNase I, electrophoresed on a 6% polyacrylamide gel under denaturing conditions, and autoradiographed. The protected regions are shown. The G+A ladder serves as a marker and -ve represents a control experiment with 40 μg of BSA. *A,* DNase I protection assay using nuclear proteins CHO (10 μg) (lane 1); snake liver (5 μg) (lane 2), and snake venom gland (5 μg) (lane 3). *B,* Footprinting using variable concentrations (15, 10, and 5 μg; lanes 1, 2, and 3, respectively) of HepG2 nuclear proteins. *C,* Primer extension analysis. The transcription initiation site of the PLA_2 gene was determined using primer extension analysis as described in *Materials and Methods.* The

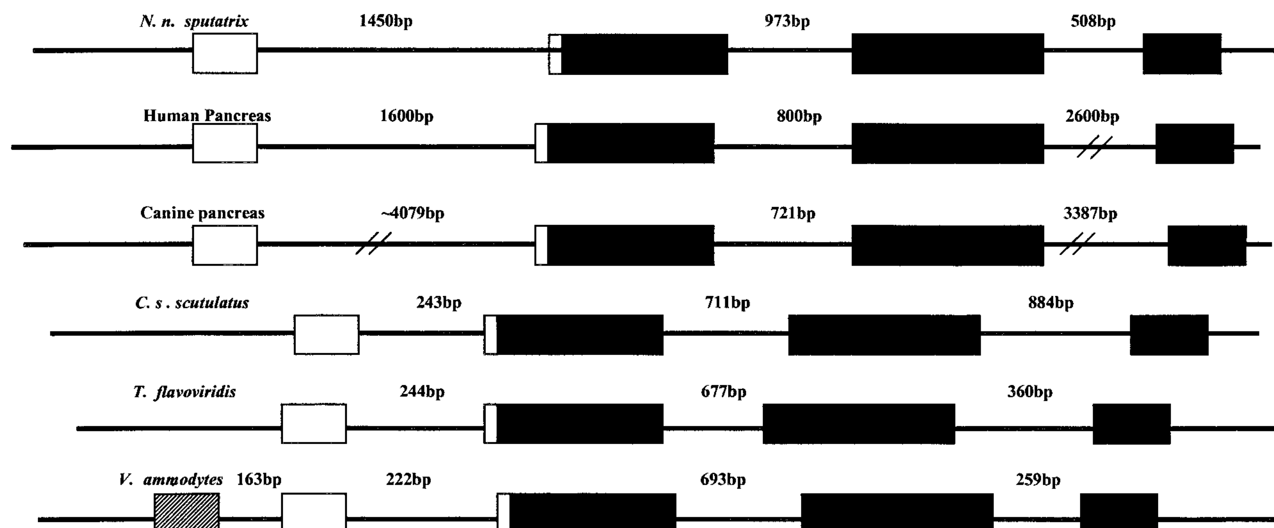

FIG. 4.—Structural organization of group I and group II PLA_2 genes. The intron/exon organization of PLA_2 genes from *Naja naja sputatrix,* human pancreas (Seilhamer et al. 1986), canine pancreas (Kerfelec et al. 1990), *Crotalus scutulatus scutulatus* (John, Smith, and Kaiser 1994), *Trimeresurus flavoviridis* (Nakashima et al. 1993), and *Vipera ammodytes* (Kordis and Gubensek 1996) are compared. The exons are boxed, and the introns are indicated as single lines. Gene regions encoding the signal peptides are represented by open boxes, while those encoding the mature protein are represented by filled boxes. The hatch box represents the 5′ untranslated region of the gene.

evolution could not be found in the PLA_2 gene of *N. sputatrix.* Nevertheless, the first 200 bp (minus strand) of intron II of the *N. sputatrix* PLA_2 gene showed 90%–100% similarity to intron II of the PLA_2 inhibitor gene (AB003473), the PLA_2 gene (D31779), and the TATA box–binding protein gene (D31782, D31777) of *Trimeresurus* sp. The 3′ end of intron II showed 80% homology to the 3′ UTR of the serine protease type toxins from the habu snake (D67080), KN-BJ2 from *Bothrops jararaca* (AB004067), plasminogen activator from *T. stejnegeri* (U21903), calobin from *Agkistrodon ussuriensis* (U32937), and salmobin from *Gloydius halys* (AF056033). This observation shows that while the exons code for the mature protein of PLA_2, the intron, particularly intron II, shows some features of ‘‘cross-talk’’ among the introns of related and unrelated genes. The significance of this has yet to be determined. The intron II also shows an 11-bp (GTCTTCTAGTC) direct repeat sequence flanking a 123-bp nucleotide sequence (nucleotides 1767–1889). Insertion elements such as γ-actin-processed pseudogene and endogenous retroviral-like elements (Ting et al. 1992) have been found to be involved in the tissue-specific expression of the salivary amylase gene. Similarly, insertion of HERV-H family of retroviral elements (Kowalski et al. 1997) in an intron of the PLA_2-like gene (PLA2L) has been shown to alter the expression of the gene in a tissue-specific manner. Although the 123-bp insertion element in intron II of the *N. sputatrix* PLA_2 gene could not be identified, it is possible that it may have a functional role in the expression of the PLA_2 gene in the venom gland of this snake.

Ohno et al. (1998), using the combined sequences of the 5′ and 3′ UTRs and the signal peptide region of the Viperidae (group II PLA_2), showed that Viperidae which evolved from a common ancestor later gave rise to the Crotalinae and Viperinae groups of PLA_2s. Our phylogenetic analysis using the secretory PLA_2 whole-gene sequences from humans, Proteroglypha (Elapidae; *N. sputatrix*), and Solenoglypha (Viperidae; *V. ammodytes, T. flavoviridis,* and *C. s. scutulatus*), while confirming the origin of Viperidae genes (Ohno et al. 1998), provided concrete evidence that the same ancestral gene was responsible for the evolution of both the group I and the group II PLA_2s, as postulated by Davidson and Dennis (1990). However, Davidson and Dennis (1990) proposed that at least two PLA_2 genes existed at the time the ancestral lines of reptiles and mammals diverged. In our analysis, based on gene sequences, the mammalian and elapid PLA_2 genes appear to have diverged from the same ancestral gene. Further evidence for the possible evolution of mammalian and elapid PLA_2 can be seen by examining the distribution of TGT/C codons encoding cysteine amino acid residues that are important for the maintenance of the tertiary structure of PLA_2. The TGT/C nucleotides are distributed as 3, 6, and 5 codons among exons 2, 3, and 4, respectively, in both *N. sputatrix* and mammalian PLA_2 genes, unlike the 2, 6, and 6 distribution of these codons in the corresponding exons of viperids and crotalids.

The presence of an extra loop is characteristic of the pancreatic PLA_2 enzymes in mammals. This loop, referred to as the ‘‘pancreatic loop,’’ has been thought to be present only in mammalian PLA_2. In recent years,

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transcription initiation site (TIS) residue is marked by an asterisk. The sequence ladder of the corresponding genomic sequence of the noncoding strand is indicated.

there have been reports on the presence of a pancreatic loop in venom PLA_2s of the elapids such as the king cobra (Huang et al. 1996), Brazilian coral snakes (Francis et al. 1997), and Australian snakes (Jeyaseelan et al. 1998). The pancreatic loop in mammalian group I PLA_2s is encoded by exon 3 of the gene. However, this loop is absent in the *N. sputatrix* PLA_2s. The loss of the pancreatic loop was suggested as an added adaptive advantage for the development of the toxic properties among venom PLA_2s (Davidson and Dennis 1990). Hence, it is possible that snake PLA_2, especially that of *N. sputatrix,* via a positive Darwinian type of accelerated evolution (Ogawa et al. 1996), could have lost its pancreatic loop from its exon 3 in order to acquire additional toxic properties, while the mammalian PLA_2s which have been considered to have evolved under neutrality retained the pancreatic loop to minimize the rate of mutation of the gene. It is interesting to note that the PLA_2s of Australian snakes and the king cobra, when compared with those of the modern elapids *N. sputatrix* and *N. atra* (Jeyaseelan et al. 1998), appear at the lower end of the evolutionary tree, indicating that the presence of the pancreatic loop in PLA_2 is an ancestral property. Thus, it appears that exons 3 and 4, which are separated by a short intron III in *N. sputatrix,* form the hot spots for mutations leading to diverse pathophysiological properties while retaining the other fundamental features of the PLA_2 enzymes, such as the catalytic property of the exon 2 of the gene.

Promoter activity studies of the CHO and HepG2 cell lines showed that the expression of the PLA_2 gene is tissue-specific, as the construct 5′PLA-233 exhibited the highest promoter activity in CHO cells and the lowest activity in HepG2 cells. This deletion construct contains, apart from the TATA and the TIS, four Sp1, two γ-IRE, one NF-IL6, and one AP-2 *cis*-acting element. The promoter region between nucleotides −116 and −233 appears to contain crucial *cis*-acting elements, which are involved in the up/down regulation of the gene expression. This region appears to recognize and differentiate between a normal cell and a hepatoma cell. DNase 1 footprinting analysis using the nuclear extracts from snake venom gland, snake liver, CHO, and HepG2 cells showed that an 87-bp region (nucleotides −146 to −233) containing one proximal Sp1 (positions −153 and −171) and two γ-IRE sites (positions −165 and −187) has been protected (fig. 3*A*). Footprinting using CHO cell nuclear proteins shows protection at nucleotides −220 to −233, containing CF1 elements, as well as nucleotides −147 to −206 (two γ-IRE sites and an Sp1 site) and an 8-bp region upstream of the CF1 site. However, only the −147 to −206 region is protected by the HepG2 nuclear protein. It appears that both the snake venom gland and snake liver nuclear proteins show interactions similar to that of CHO cell nuclear extract. Whether the proteins binding to these regions are identical or not remains to be elucidated. The binding of HepG2 nuclear proteins to this region (Sp1 and flanking γ-IREs) appears to repress the promoter activity, as demonstrated by the CAT assay of 5′PLA-233 (fig. 2*C*). The γ-IRE elements which are known to activate the human cytoplasmic PLA_2 promoter activity (Wu et al. 1994) were shown to inhibit the transcription activity of the venom secretory PLA_2. Thus, the Sp1 site and its two flanking γ-IRE elements at the upstream region of AP2 (position −113) behave as silencer elements in HepG2 cells. While only a slight increase has been observed by the removal of IRE elements (5′PLA-266 to 5′PLA-233), the removal of the 2Sp1 sites (5′PLA-266 to 5′PLA-116) showed a significant increase in CAT activity in HepG2 cells. A similar observation has been reported by Hayashi et al. (1998) for γ-IRE and Sp1 elements on PCI gene expression. Our promoter analysis using CHO cells demonstrates that these γ-IRE and Sp1 elements, in combination with the CF1 element and an unknown factor (nucleotides −220 to −233) have an effect (enhancement) opposite that observed in HepG2 cells (fig. 2*C*), thus confirming the tissue specificity of the PLA_2 promoter. Hayashi et al. (1998) observed that the AP-2 element in the 5′-flanking region is capable of exhibiting an enhancer/repressor activity depending on its position with respect to Sp1 sites. In our study, an AP2 element situated at position −113 on the promoter plays a suppressive role in HepG2 cells (fig. 2*C*). A basal promoter activity can be observed with TATA and the TIS (5′PLA-51 clone). Inclusion of two Sp1 sites at positions −65 and −68 in 5′PLA-90 yielded an increase in promoter activity in HepG2 cells and a decrease in activity in CHO cells. Similarly, Park, Plummer, and Krystal (1998) reported that selective binding of Sp1 at the promoter region is critical for maximal activity of the human c-kit promoter. Addition of an AP2 element to the 5′PLA-90 construct (5′PLA-116) lowered the level of activity by 30% in a tissue-specific manner in HepG2 cells. Thus, the AP2 site, the two SpI sites (at positions −153 and −171) and the two γ-IRE elements (at positions −165 and −187) function as suppressors in HepG2 cells and possibly as enhancers in CHO cells. The downstream Sp1 sites (at positions −65 and −68) have a suppressive role in HepG2 and an enhancing role in CHO cells. Thus, it appears that the Sp1 elements could function in combination with the nearby enhancer/repressor-binding proteins as promoter-specific factors to regulate gene expression in a tissue-specific manner, as in the case of the snake PLA_2 gene. The functions of the third γ-IRE element (at position −246) and the NF-IL6 elements (at positions −246 and −134) are still unknown.

Alternating purine-pyrimidine dinucleotide repeats are known to form Z-DNA conformations in vitro; hence, it has been speculated that their existence in the 5′-flanking regions of genes may confer a regulatory effect on gene transcription (Brahmachari et al. 1995). The CA-repeats in the 5′-flanking region have been reported to cause an inhibitory effect on the gene expression of the rat prolactin gene (Naylor and Clark 1990) and the human cytoplasmic PLA_2 gene (Hayashi et al. 1998). Singer and Gottschling (1994) also reported that telomeres (TG repeats) binding to telomerase while maintaining genomic integrity could function as silencer elements. Seeding of telomeres has been shown to require a telomeric sequence motif, GGGTTA, in verte-

brates (Konig, Fairall, and Rhodes 1998). The telomeric repeat–binding proteins are also known to contain a DNA-binding motif similar to that of the Myb-like domain. Interestingly, a (TG_{12}) repeat, a cMyb-binding site, and a GGGTTA site can be observed in the 5′ region of the PLA_2 gene of *N. sputatrix* (fig. 2*A*). Removal of this $(TG)_{12}$ repeat from the promoter region brought about an increase in promoter activity in both CHO and HepG2 cells (fig. 2*C*), thus confirming its role as a silencer in gene regulation. A second $(TG)_{12}$ repeat can be found at the 3′ end of the *N. sputatrix* PLA_2 gene. Its function is still unknown. It is possible that this $(TG)_{12}$ repeat could also be involved in the regulation of the gene activity.

The 3′ region of *N. sputatrix* PLA_2 contains two functional poly-A signal sites. Studies of 3′ rapid amplification of cDNA ends (results not shown) showed that the *N. sputatrix* PLA_2 mRNA could be terminated at either one of these two sites, which are about 50 bp apart from each other. An AT-rich region known to destabilize mRNA transcripts (Higgins 1991) is located in the 3′ region of the gene. This AT-rich region, together with the $(TG)_{12}$ repeats found in both the 5′ and the 3′ ends of the gene, could possibly explain the lower turnover of PLA_2 message in the venom gland cells in comparison with the cardiotoxin messages that we observed previously in a separate study (Lachumanan et al. 1999). Thus, the regulation of PLA_2 gene expression (in a tissue-specific manner) appears to be controlled by concerted activities of the 5′ regulatory elements, as well as the 3′ AT- and TG-rich regions.

Conclusions

The genes encoding the neutral and acidic group I PLA_2s have been cloned and characterized from an elapid, *N. sputatrix.* This study provides the information on the missing link in the biology and evolution of phospholipases. A common ancestral gene for PLA_2 enzymes could have given rise to both the mammalian and the reptilian forms, possibly by gene duplication. This eventually could have allowed the venom PLA_2 to undergo adaptive evolution in an accelerated manner to offer multiple functions from the same ancestral scaffold.

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