Table 1
Oligonucleotide primers used for PCR amplification

Gene	Accession No.[a]	Direction	Sequence
CYP1A4	X99453	Sense	5′-ATCGCCGCAATGCTTCAC-3′
		Antisense	5′-CAGACCCTTCCTTTTATTAGCC-3′
CYP1A5	X99454	Sense	5′-AGGGACCGAAGTGAACAAAG-3′
		Antisense	5′-GGTTCCGTGAGGTTATGCC-3′
GAPDH	K01458	Sense	5′-ACGCCATCACTATCTTCCAG-3′
		Antisense	5′-CAGCCTTCACTACCCTCTTG-3′
AhR	AF260832	Sense	5′-GCGCTCTTTCAAGATAAC-3′
		Antisense	5′-AGCATAACCGACTCAGAA-3′
ANF	X57702	Sense	5′-GACCCGTGCTCTGAAG-3′
		Antisense	5′-AGAGGTCCAGCATAGA-3′

[a] Accession numbers of sequences from GenBank database.

Production of anti-AhR antibody

cDNA fragment (1269 bp) was obtained by RT-PCR with primers for chicken AhR (summarized in Table 1). The fragment was ligated to pGEM-T Easy vector (Promega). The insert containing the partial coding region for AhR was obtained by digestion with *Eco*RI and further subcloned to the *Eco*RI site of the pGEX-4T-3 expression vector (Amersham–Pharmacia Biotech). The constructed vector was used for transformation of *Escherichia coli* BL21 (DE3), and recombinant AhR was purified with glutathione agarose beads according to the manufacturer's instructions (Amersham–Pharmacia Biotech). The fusion protein was digested with thrombin protease and was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [26] followed by electrophoretic elution of AhR peptide from gels. AhR peptide was dialyzed in PBS and used as antigen. Rabbits were immunized with the antigen emulsified in Freund's complete adjuvant and boosted twice with the same antigen emulsified in Freund's incomplete adjuvant. Serum containing anti-AhR antibody was purified by incubating the serum with nitrocellulose-bound antigen and then eluting it at low pH [27].

Western blot analysis

Hearts and livers removed from chick embryos at each stage were frozen immediately in liquid nitrogen, crushed into fine powder, and dissolved in PBS containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, and 5 μg/ml pepstatin). After centrifugation at 10,000*g* for 10 min at 4 °C, supernatants were collected, and the protein concentration was determined by the method described by Bradford [28]. Proteins (15 μg/lane) were resolved by SDS–PAGE and transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The membranes were blocked with 5% non-fat dried milk in TPBS (0.05% Tween 20 in PBS) for 30 min at room temperature, followed by incubation with anti-AhR primary antibody overnight at 4 °C. After incubation with the primary antibody, the membranes were washed five times in TPBS and incubated for 2 h at room temperature with a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated secondary antibody. Enhanced chemiluminescence (ECL) detection reagents (Amersham–Pharmacia Biotech) were used to enable detection of antibody reactivity on XAR film (Kodak).

Preparation and staining of tissue sections

Tissues were dissected, fixed with 4% paraformaldehyde in PBS overnight at 4 °C, and frozen in Tissue-Tek OCT compound (Miles). To detect lipid vesicles, sections (20 μm thickness) were washed twice with PBS for 5 min and immersed in 50% ethanol for 30 min. Visualization was achieved by immersing sections in 0.2% (w/v) Sudan IV in 70% ethanol for 1 h at 37 °C, followed by two washings with 50% ethanol for 30 s and staining with hematoxylin solution. In situ hybridization [29] was performed as previously described [30]. In brief, sections (20 μm thickness) were treated with 20 μg/ml proteinase K for 3 min at room temperature. After post-fixation with 4% paraformaldehyde in PBS for 10 min at room temperature, samples were rinsed with PBS, preincubated with the hybridization mixture for 30 min at 65 °C, and reacted overnight at 65 °C with 0.5–1 μg/ml digoxigenin (DIG)-labeled RNA probes. To obtain sense and antisense riboprobes for CYP1A4, plasmid clone prepared as described above was linearized and used as a template for the generation of riboprobes with a MAXI script in vitro transcription kit (Ambion, Austin, TX, USA). After 60 °C washes and blocking, the samples were incubated overnight at 4 °C with alkaline phosphatase-conjugated anti-DIG antibody (1:2000 dilution, Boehringer, Mannheim, Germany). After a washing to remove unbound antibodies, samples were stained with

the NBT/BCIP mixture at room temperature until color development.

Results

Effects of TCDD exposure on chick embryo heart and liver

We injected TCDD or corn oil vehicle control into white leghorn eggs prior to incubation. After incubation for 12 (E12) or 18 days (E18), chick embryos were dissected. Control embryos exhibited 20% mortality (4/20), whereas TCDD-exposed embryos exhibited 65% mortality (39/60) at E12. At E12, hearts from TCDD-exposed embryos were larger than control hearts, and histological examination revealed that most instances of enlargement occurred in left ventricle (Fig. 1A). To establish morphological change in the heart, we measured the wet weight of hearts. Heart wet weight relative to body wet weight increased when hearts were exposed to TCDD for 12 or 18 days (Fig. 1B). An increase in ANF, one of the hallmarks of the heart disease [31], was observed in hearts from E12 embryos (Fig. 2). Thus, morphological and functional alterations were evident in TCDD-exposed chick embryo hearts. Morphological abnormality was also observed in livers of TCDD-exposed chick embryos. TCDD exposure partially altered the color of the liver from pale pink to yellowish white (Fig. 3A). These changes in color were detected in both E12 and E18 livers (data not shown). However, no significant change in liver wet weight was detected when we compared the wet weights of TCDD-exposed and control livers, regardless of incubation periods (Fig. 3B). High-magnification images of TCDD-exposed liver sections showed that clearly extensive vesicle formation occurred in hepatocytes and that the abnormal vesicle formation was restricted to the periphery of TCDD-exposed liver, where color change was observed macroscopically (Figs. 4A–C). Accumulation of fatty vesicles was confirmed by Sudan IV staining (Fig. 4D). Staining of fatty vesicles was obvious in the region where abnormal vesicular formation was observed, suggesting that extensive vesicular fatty metamorphosis was induced in the periphery of TCDD-exposed liver.

Contribution of AhR-CYP1A pathway to TCDD signaling

Transcriptions of CYP1A family genes are initiated by binding of the complex to the DRE region [16,17]. To assess AhR expression at the protein level, an anti-AhR polyclonal antibody was generated. Affinity-purified anti-AhR antibody was specifically reacted with approximately 100-kDa peptide in both the heart and liver (Fig. 5A). In chick embryo, expression of AhR is detected from E2 to E10 in the heart and liver [23]. Consistent with the findings of Walker et al., Western blot analysis in the present study revealed constant AhR expression in the heart throughout embryogenesis. Expression of AhR was observed at approximately the same level in the liver as in the heart throughout embryogenesis (Fig. 5B).

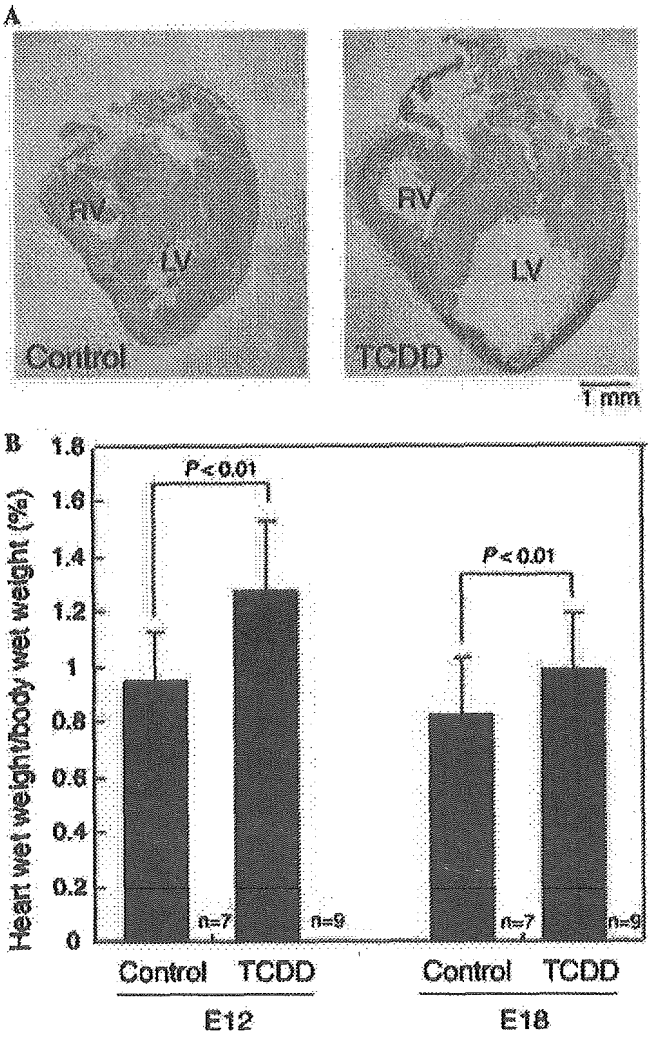

Fig. 1. (A) Representative images of frontal sections of chick embryo hearts 12 days after TCDD (1.0 pmol/g egg) or vehicle (corn oil for control) was injected into egg yolks. Sections are stained with hematoxylin and eosin. The TCDD-exposed heart (right panel) has an enlarged ventricular cavity compared to that of the control heart (left panel). RV, right ventricle; LV, left ventricle. (B) Heart wet weights after 12 (E12) and 18 days (E18) of incubation in the presence or absence (for control) of TCDD (1 pmol/g egg). Weights are relative to body wet weight. The *P* values refer to comparisons between TCDD-exposed and control hearts. The number of embryos in each group is specified.

To determine whether CYP1A4 and CYP1A5 gene expression occurred under our experimental condition, Northern blot analysis for these enzymes was done. In both analyses, an increase in the mRNAs was observed only in the liver when embryos were exposed to TCDD for 12 days, and no signals were observed in the heart

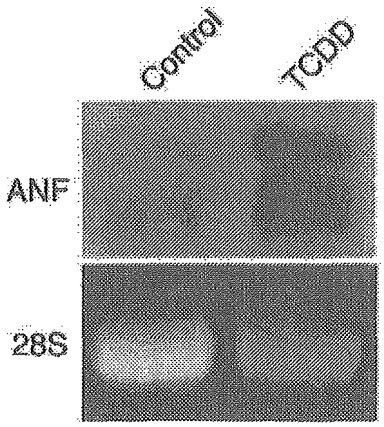

Fig. 2. Northern blot analysis of ANF expression levels in chicken embryo hearts. Approximately 20 μg total RNA was dissolved by agarose gel electrophoresis. Induction of ANF expression in the E12 heart was examined in TCDD-injected chick embryos. 28S, 28S ribosomal RNA. Control, vehicle (corn oil) was injected into egg yolks.

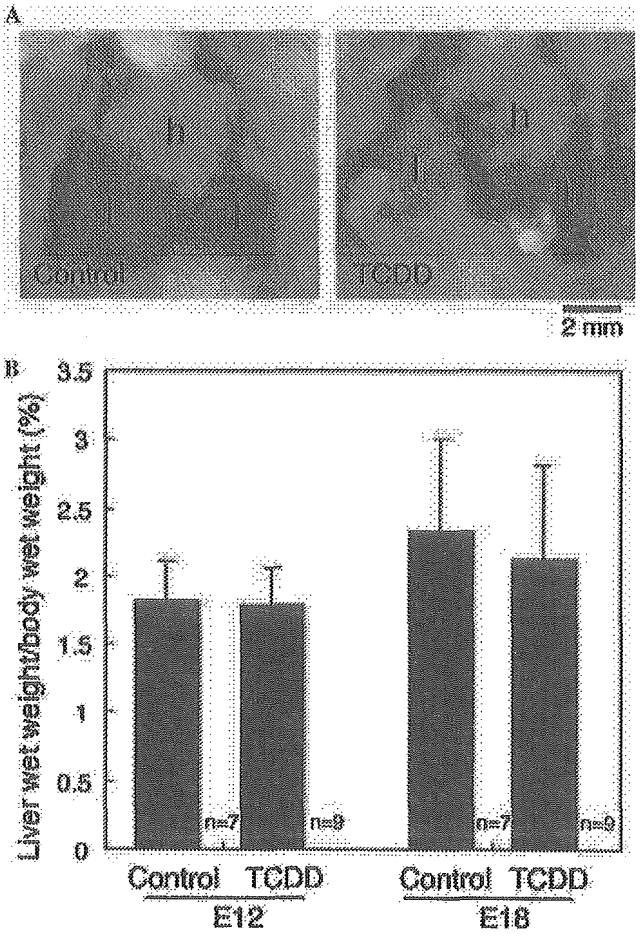

Fig. 3. (A) Representative image of abnormal liver in embryos collected after 12 days of incubation in the presence of TCDD (right panel). Control embryos (left panel) were exposed to vehicle only. An obvious change in color is seen in the TCDD-exposed liver. Arrowhead indicates the areas of color change: (h) heart, (l) liver. (B) Liver wet weights after 12 (E12) and 18 days (E18) of incubation in the presence or absence of TCDD. Weights are relative to body wet weight. The number of embryos in each group is specified.

(Fig. 6). No induction of CYP1A family genes in the heart was observed in E6, E8, and E10 TCDD-exposed chick embryos (data not shown). We further analyzed expression of the CYP1A gene by in situ hybridization. Signals for CYP1A4 were evident in TCDD-exposed liver, although the signal was confined to the normal liver tissue in which no vascular fatty metamorphosis was observed (Fig. 7). No significant difference was observed in the heart when the CYP1A gene was detected with antisense or sense probes (data not shown). Our data show clearly that AhR-mediated induction of CYP1A family gene expression occurred only in the liver after eggs were exposed to TCDD, although nearly the same amount of AhR protein was present in the heart.

Accumulation of TCDD in the liver was reported after in ovo injection [32–34]. This simply raises the question whether the different responses of the heart and liver to TCDD are caused by differences in TCDD concentration or by the nature of each cell type.

EROD assay

We prepared primary cultures of cardiac myocytes and hepatocytes from E12 embryos, and the same number of each type of cell was seeded into 96-well plates. EROD activity in wells was measured as an index of CYP1A family enzyme induction. Weak EROD activity induced by TCDD exposure was detected after 24 h of culture of cardiac myocytes, whereas EROD activity of TCDD-exposed hepatocytes was markedly increased (Fig. 8). Exposure to a high concentration (10 nM) of TCDD resulted in a decrease of EROD activity in hepatocytes. These data show that the different responses to TCDD of the heart and liver were due to the nature of each cell type.

Discussion

We found that morphological and functional alterations induced by TCDD were marked in the heart and liver of the chick embryo. The ventricular cavity of the heart was enlarged, and an increase in heart wet weight was observed. These alterations are implicated in the functional changes induced by TCDD exposure. Previous studies showed dilated cardiomyopathy with an increased heart weight induced by TCDD in E10 and E12 chick embryos [12,23]. In our experiment, induction of ANF, a hallmark of heart disease, was detected in the heart exposed to TCDD. This result supports the idea that the observed morphological alterations in heart were the result of functional defects induced by TCDD. We injected TCDD into egg yolks prior to incubation at a concentration of 1 pmol/g egg, a concentration approximately twice as high as that of LD_{50} for white leghorn chickens (*Gallus domesticus*) [7]. This might

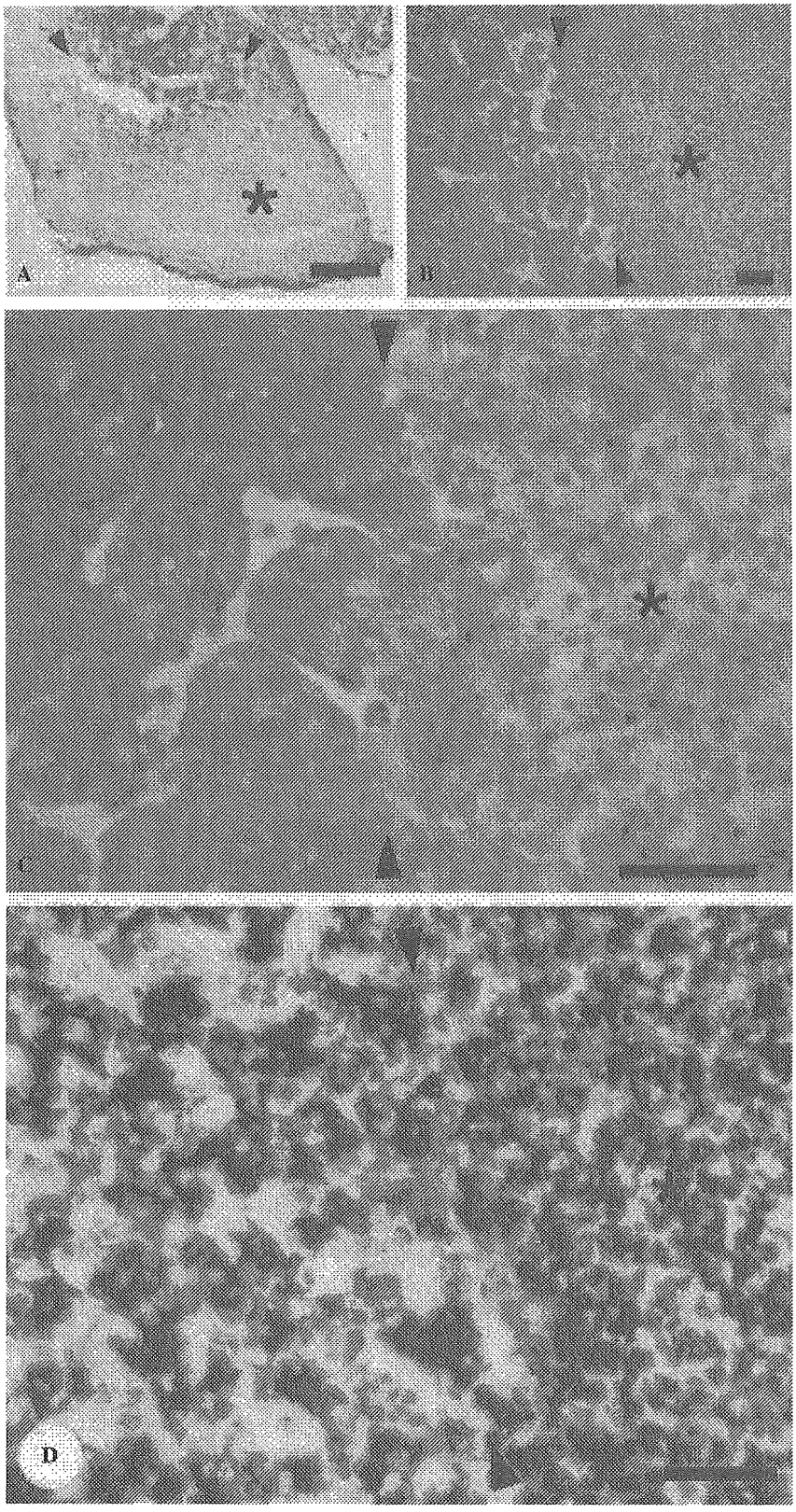

Fig. 4. Histological analysis of TCDD-exposed liver. TCDD (1 pmol/g egg) was injected into egg yolks prior to incubation. The liver dissected from chick embryos after 12 days of exposure to TCDD was fixed, sectioned, and stained with hematoxylin and eosin. Images of different magnifications from lower (A) to higher (C). Fatty vesicles are stained with Sudan IV (D). Arrowheads indicate boundaries between normal and abnormal liver tissue where color change is seen macroscopically (asterisk). Accumulation of fatty vesicles was observed in hepatocytes and was restricted to the abnormal liver. Bars indicated in (A)–(D) are 1 mm and 50 μm, respectively.

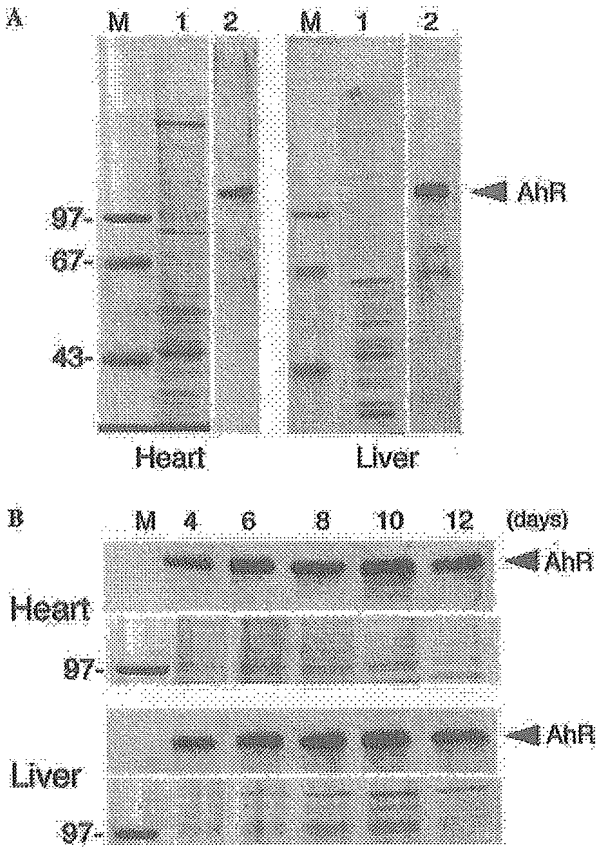

Fig. 5. (A) Western blot analysis of AhR with the purified anti-AhR antibody. Proteins (15 μg) from E12 embryo heart and liver were resolved by SDS–PAGE and stained with Coomassie brilliant blue (lane 1). The purified anti-AhR antibody reacted specifically with an approximately 100-kDa peptide both in heart and liver (lane 2). Lane M shows molecular weight markers, which are specified by numbers to the left of the panels. (B) Change in expression levels of AhR in heart and liver was examined by Western blot analysis. Heart (upper two panels) and liver (lower two panels) extracts (15 μg) from embryos at 4, 6, 8, 10, and 12 days were resolved by SDS–PAGE. Coomassie brilliant blue stained gels are shown on the lower panel of each pair. Western blots with the purified anti-AhR antibody are shown on upper panel of each pair. Levels of AhR expression are constant in the heart and liver throughout embryogenesis. Arrowheads indicate the expected molecular weight for AhR. Lane M shows molecular weight markers; numbers to the left of the panels indicate the 97-kDa marker peptide.

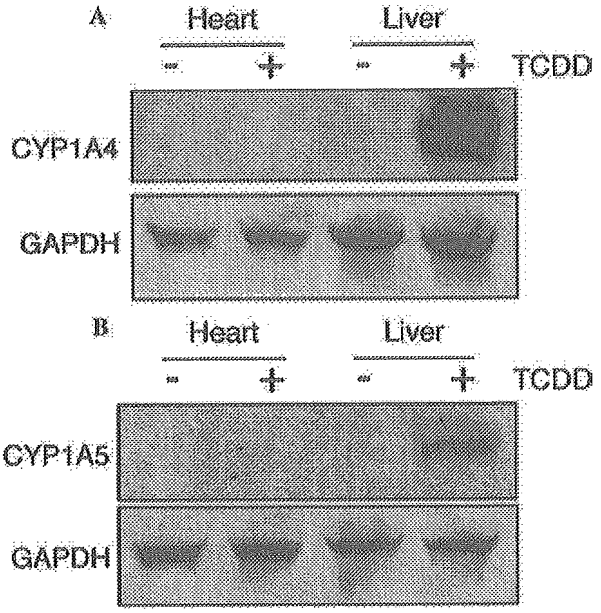

Fig. 6. Induction of CYP1A4 and CYP1A5 12 days after injection of TCDD or vehicle into egg yolks. Northern blots include mRNA of GAPDH as an internal standard. Induction of CYP1A4 (A) and CYP1A5 (B) by TCDD is evident only in the liver. Control (−), 1 pmol TCDD/g egg (+).

explain the high mortality (65% on E12) in our experiment. Even with this high concentration of TCDD, no significant change in wet weight of embryonic liver was observed. There are a number of studies that showed a statistically significant increase in heart weight [7,12,23]; however, inconsistent changes in liver weight were observed [7,32].

TCDD-induced toxicity is mediated through binding to AhR. Therefore, we examined expression of AhR at the protein level. Commercially available antibody raised against human AhR was less sensitive to chicken AhR (data not shown); therefore, expressed chicken AhR was used as antigen to obtain a specific antibody. Western blot analysis with the specific antibody showed that approximately the same levels of AhR were expressed in the heart and liver throughout embryogenesis. However, our results showed that a principal AhR response, induction of the CYP1A family genes, occurred in the liver but not in the heart in response to TCDD. Gannon et al. [20] reported that CYP1A4 was induced in chick embryo hearts when TCDD was injected into eggs. However, they injected TCDD at late stages of embryogenesis (E15 or E16) and used a 20-fold higher concentration of TCDD (1 nmol/egg) than we did in our experiments (1 pmol/g egg; approximately 0.05 nmol/egg) for a shorter exposure time (24 h). Walker et al. [23] reported that, under conditions similar to ours, AhR was expressed continuously in cardiac myocytes during cardiogenesis. They also reported that no induction of CYP1A family genes by TCDD was detectable at protein or mRNA levels except at the distal outflow tract of the embryonic heart. Our EROD assays confirmed that induction of CYP1A4 and CYP1A5 genes occurred only in the hepatocytes of E12 chick embryos, although weak activity indistinguishable from that in control hearts was observed. The same effects were observed when cells were exposed to TCDD for 48 h (data not shown), indicating that the CYP1A family genes were not inducible in cardiac myocytes in embryos or monocultures.

TCDD exposure also altered the function of the liver. A change in color of the liver surface from pale pink to yellowish white was observed. Histochemical analysis revealed a fatty vascular metamorphosis at the liver

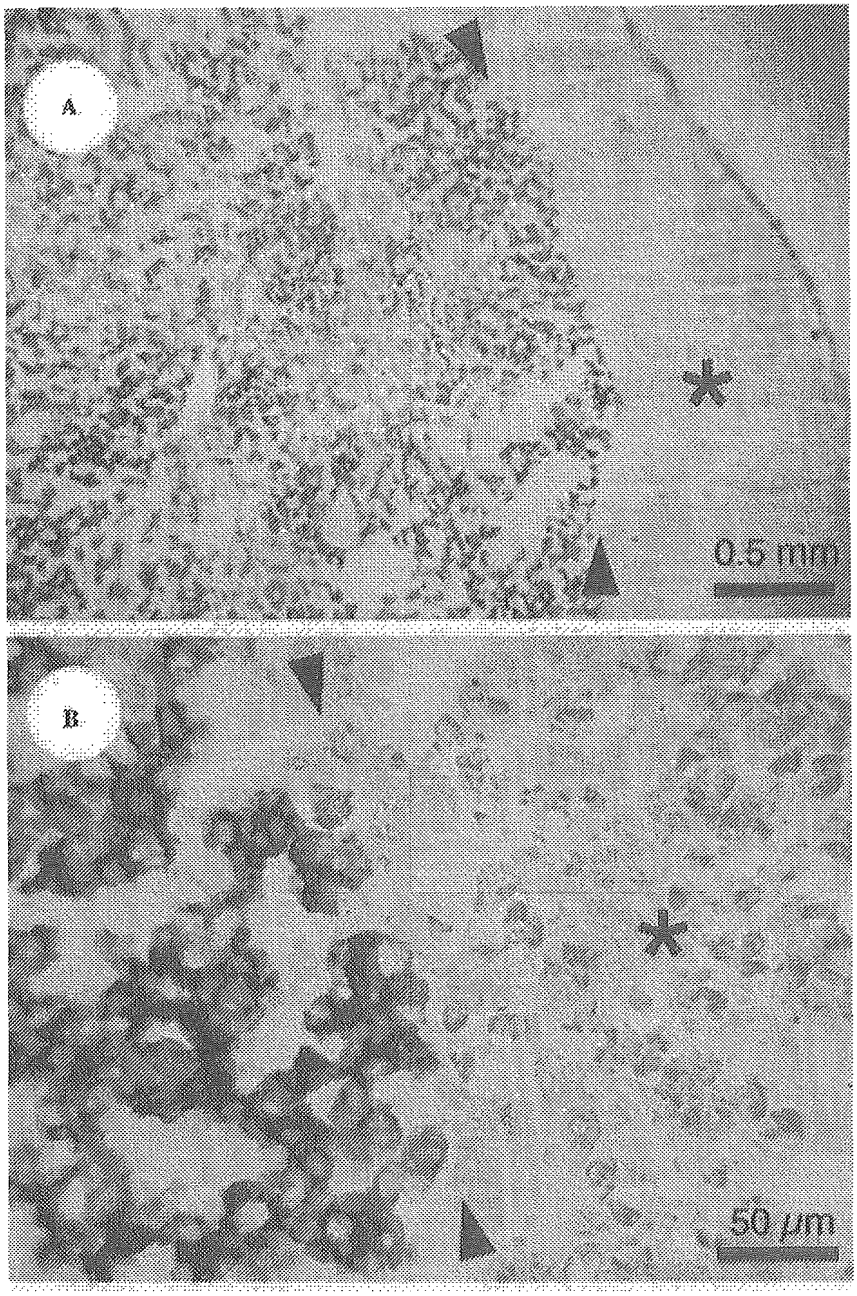

Fig. 7. In situ hybridization study of liver section with probes for CYP1A4. The liver dissected from chick embryos after 12 days of exposure to TCDD was fixed and sectioned, and the signals for CYP1A4 were detected with DIG-labeled antisense probes. Low (A) and high (B) magnification images show restricted expression of CYP1A4 in normal liver tissue but not in abnormal liver tissue (asterisk). Arrowheads indicate the boundary line between normal and abnormal liver tissue.

periphery, where the color change was observed. Similarly, alteration in hepatocytes with transient microvesicular formation was observed in the liver of AhR null mice. And the vesicular formation was due to a metabolic deficit in hepatocyte function [35]. Studies of TCDD toxicity in the liver have been done with 7- to 9-week-old immature chicks. And it is reported that lipoprotein lipase and glucose transporting activities were decreased by TCDD, which resulted in reduced fatty acid synthesis, wasting syndrome, profound loss of adipose tissue and lean body mass, and increased liver wet weight [36,37]. We consistently observed an accumulation of fatty vesicles in the liver. In situ hybridization analysis showed that the CYP1A gene was not expressed in the liver periphery of TCDD-exposed embryos. This novel finding suggests alteration of hepatocytes induced by TCDD toward a loss of function for metabolizing xenobiotics. The CYP1A gene can produce reactive oxygen species during its catalytic cycle, and CYP1A-based production of H_2O_2 suppresses expression of the CYP1A gene itself in HepG2 cells [38]. Decrease of EROD activity in hepatocytes as shown in Fig. 8 may due to the same suppressive effects of

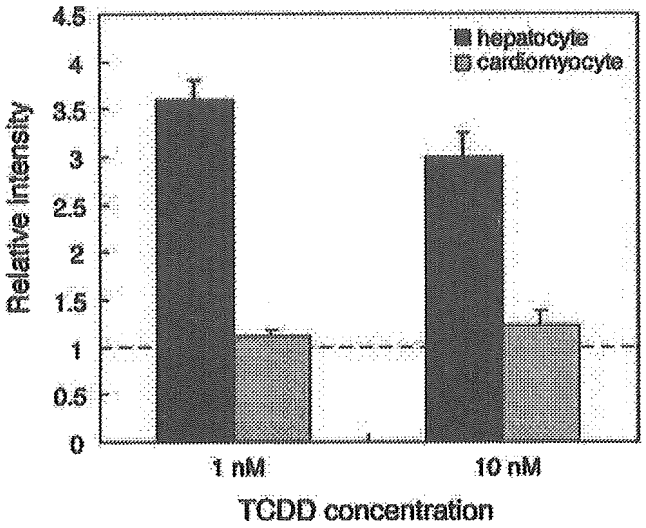

Fig. 8. Induction of EROD activity in hepatocytes and cardiac myocytes from chick embryos after 12 days of exposure to TCDD. Average EROD intensity relative to control intensity is given for two TCDD concentrations. A relative intensity of 1.0 means there is no TCDD effect. Error bars represent standard deviations.

the reactive oxygen. Thus, absence of CYP1A gene expression in the liver periphery of TCDD-exposed embryos can be explained by a similar autoregulation mechanism.

Possible mechanisms for TCDD-induced toxicity have been postulated. Toxicity might result from transcriptional activation or repression of genes by binding the AhR–Arnt complex to the regulatory sequence upstream of the genes. TCDD toxicity might also result from the regulatory alteration of unrelated genes by sequestering bHLH-PAS proteins [35]. Transcriptional activation of toxicity-implicated genes by TCDD has been postulated because immunohistochemical analysis revealed that AhR and Arnt are present in the heart [23].

We found that expression of the CYP1A family genes was induced in the liver but not in the heart. Our findings suggest that the signaling cascade through AhR in response to TCDD is different in the heart from that in the liver. Paracrine factors, for example, may be implicated in the pathway leading to heart defects. Defects in the liver or other organs cause an increased circulatory load. The increased load induces expression of paracrine factors such as endothelin that can induce cardiac hypertrophy [39]. Further analysis is needed to elucidate the mechanism underlying the heart defects induced by TCDD. The physiological function of AhR in the heart should also be determined.

Acknowledgments

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Comparative analysis of gene expression mechanisms between group IA and IB phospholipase A_2 genes from sea snake *Laticauda semifasciata*☆

Takahiko J. Fujimi, Sakiko Yasuoka, Eri Ogura, Takahide Tsuchiya, Toru Tamiya*

Department of Chemistry, Faculty of Science and Technology, Sophia University, 7-1, Kioi-cho, Chiyoda-ku, Tokyo 102-8554, Japan

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Abstract

Phospholipase A_2 (PLA_2) genes expressed in the venom glands of the sea snake, *Laticauda semifasciata*, were investigated. Both mRNAs, encoding group IA (without a pancreatic loop) and group IB (with pancreatic loop), were detected from venom glands by Northern blot hybridization analysis and RT-PCR. The results of quantitative PCR analysis indicated that the expression amount of group IA genes was around 100–300 times greater than that of group IB genes. Sequence analysis of 5′-upstream regions and a reporter gene assay of the genes (groups IA and IB) previously cloned showed that the functional sequence (411 bp) was inserted in the 5′-flanking region of the group IA PLA_2 genes. It seemed that the contribution of the inserted sequence to the amount of transcribed mRNAs was greater than that of number of genes present in the genome. Comparative analysis of the 5′-flanking sequences from several snake genes encoding toxic PLA_2s revealed that this sequence was probably inserted into an ancestral gene of PLA_2 with a pancreatic loop. After the duplication of the gene, which contained the inserted sequence, the PLA_2 gene without a pancreatic loop evolved from one of the duplicate genes. This inserted sequence might determine the future of the genes expressed in the venom glands.

Keywords: Snake toxin; Phospholipase A_2; Molecular evolution; Promoter

1. Introduction

Phospholipase A_2 (PLA_2, EC 3.1.1.4) catalyzes the hydrolysis of the acyl ester bond at the *sn*-2 position of phosphoglycerides (Tischfield, 1997). Phospholipase A_2s are commonly classified into twelve groups and many subgroups (Six and Dennis, 2000). Snake venoms are well known as good sources of low molecular weight secreted type PLA_2s. Venoms of the Viperidae and Colubridae families contain group II PLA_2s, whereas venoms of the Elapidae family contain group I PLA_2s. Phospholipase A_2s in group I are further divided into two subgroups, IA and IB. The difference between these two subgroups is the existence (IB) or the absence (IA) of a pancreatic loop in their amino acid sequences. The digestive PLA_2s secreted as zymogens in the pancreas of mammals are classified into group IB PLA_2. In mammals, group IB PLA_2 is well known as an enzyme contributed to the some pharmacological responses in the cells mediated through the arachidonic acid releasing or the specific receptor binding (Tohkin et al., 1993; Fonteh et al., 1998; Yokota et al., 2000).

Most of toxic PLA_2s from the venoms of the Elapidae family are classified into group IA PLA_2s (*Naja*, Bhat et al., 1991; *Notechis*, Halpert and Eaker, 1976; *Laticauda*, Yoshida et al., 1979). Snake venomous PLA_2s with a pancreatic loop (IB) have been isolated from the venom of *Oxyuranus*

Abbreviations: GADPH, glyceraldehyde-3-phosphate dehydrogenase; GL, genomic library; LsGADPH, *Laticauda semifasciata* glyceraldehyde-3-phosphate dehydrogenase; LsPLA2, *Laticauda semifasciata* phospholipase A_2; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction.

☆ The nucleotide sequence data reported here have been submitted to the DDBJ sequence data bank. The 5′-upstream regions of group IA and IB PLA_2 genes from *L. semifasciata*, IA: AB111958, IB: AB111959; Group IB PLA_2 mRNA partial sequences from *L. semifasciata* venom glands, AB120375 (VG11) and AB120376 (VG8); GAPDH mRNA partial sequence from, *L. semifasciata* AB111960.

* Corresponding author. Tel.: +81-3-3238-3363; fax: +81-3-3238-3361.

E-mail address: t_tamiya@sophia.ac.jp (T. Tamiya).

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scutellatus scutellatus (Fohlman et al., 1976; Lambeau et al., 1990), *Pseudonaja textilis* (Pearson et al., 1991), *Micropechis ikaheka* (Gao et al., 1999) and *Notechis scutatus scutatus* (Francis et al., 1995). Both types of PLA_2s (groups IA and IB) have been purified as one of the toxic components from the venoms of the snake species, mentioned above, *P. textilis*, *O. scutellatus scutellatus* and *N. scutatus scutatus* (Danse et al., 1997).

Although toxin genes expressed in the snake venom glands, almost nothing is known about the mechanism of the expression regulation system. Jeyaseelan et al. (2000, 2001) and Ma et al. (2001) assessed the promoter activity of several snake toxin genes, including the group IA PLA_2 gene, using a reporter gene assay. They have found that the promoter activities of the snake toxin genes in the CHO cells are proportional to their in vivo expression levels (Jeyaseelan et al., 2001; Ma et al., 2001).

We have reported the nucleotide sequences of genes encoding groups IA and IB PLA_2s from the genomic library and cDNAs cloned from the venom gland (IA) and pancreas (IB) cDNA pools of *Laticauda semifasciata* (Table 1) (Fujimi et al., 2002a,b). Analyses of the structural genes of both group IA and IB PLA_2 revealed that they evolved from the same ancestral gene, and the expression products acquired the character of digestive enzymes or toxins. However, the regulation mechanisms of transcription involved in the diversification of these PLA_2 genes were still not understood. It is very important to clarify the regulation mechanisms of transcription in the venom glands, because the gene expression in the appropriate tissue is necessary for the expression product to act as a toxin.

In this report, the expression of group IB PLA_2 genes in the venom gland of *L. semifasciata* was proven by Northern blot hybridization and RT-PCR. The result of Northern blot hybridization and quantitative PCR analyses also showed that the expression levels of group IB genes in venom glands were quite low compared with those of the group IA genes. To elucidate the cause of the vast difference about the expression levels of both groups of genes, a series of reporter gene assay and gene quantitative analysis were performed. The reporter gene assay of group IA and IB genes from *L. semifasciata* using CHO-K1 cells, showed that the specific sequence was present in the promoter regions of the group IA gene and suggested the group IA specific sequence played an important role in the gene expression.

Table 1
Description regarding *L. semifasciata* phospholipase A_2 clones used in this study

Clone name	Accession no.	Clone type	Group	Origin
LsPLA2cPm09	AB037415	cDNA	IA	venom gland
LsPLA2pkP5	AB078348	cDNA	IB	pancreas
VG11[a]	AB120375	cDNA	IB	venom gland
VG8[a]	AB120376	cDNA	IB	venom gland
LsPLA2GL1-1	AB062439	gene	IA	genom library
LsPLA2GL5-1	AB062440	gene	IA	genom library
LsPLA2GL16-1	AB078346	gene	IB	genom library
1-1up1[a]	AB111958	upst ream of LsPLA2GL1-1	–	genom library
16-1up1[a]	AB111959	upstream of LsPLA2GL16-1	–	genom library

[a] These clones were newly reported in this report. The other clones had been reported in our previous reports (Fujimi et al., 2002a,b).

2. Materials and methods

2.1. Synthesis of single-stranded cDNA pool

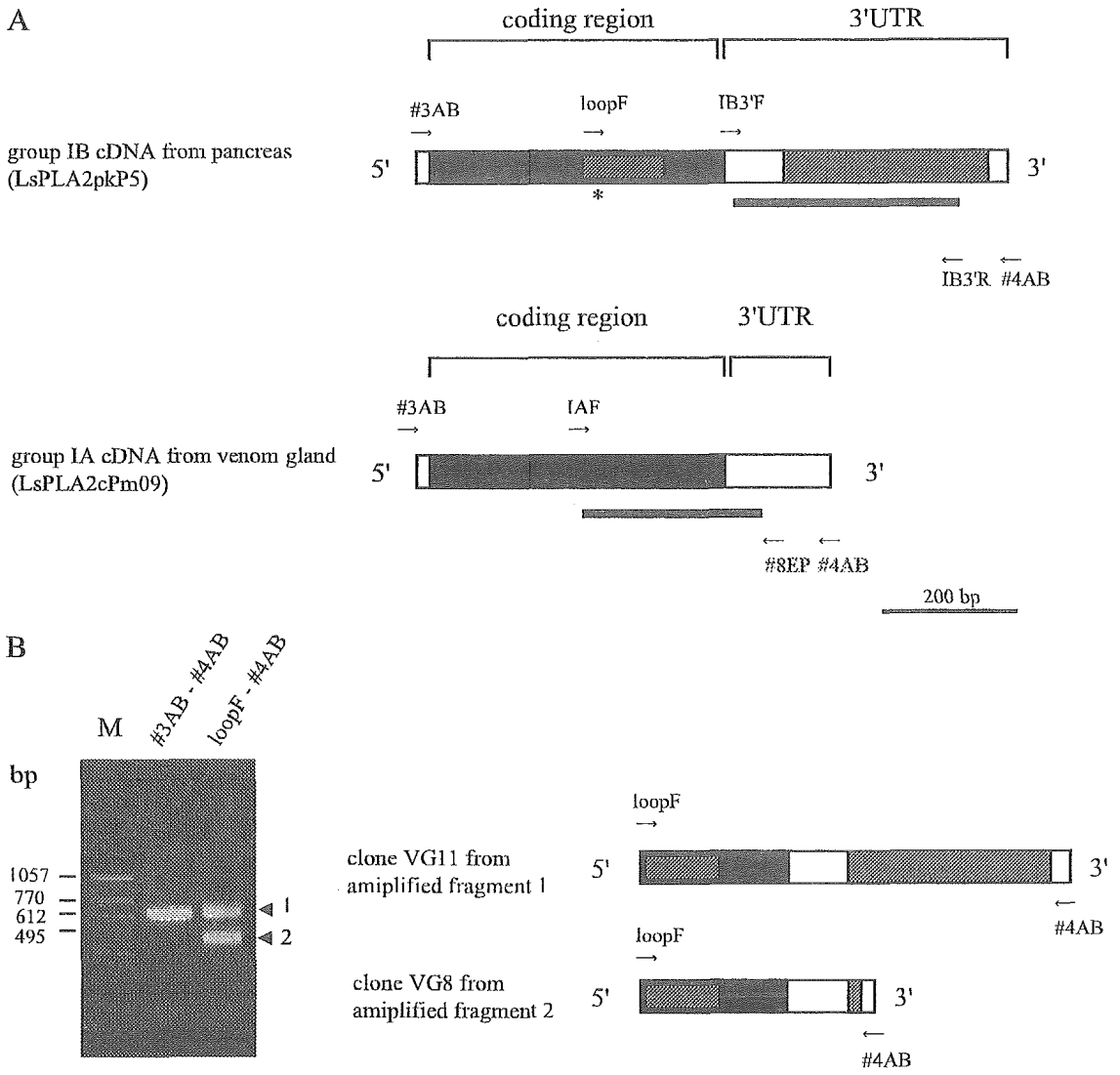
Total RNA was extracted from 1 g of frozen venom glands using TRIZOL, according to the manufacturer's protocol (Invitrogen). Poly(A)$^+$ RNA was purified from total RNA with Dynabeads Oligo $(dT)_{25}$ (Dynal). Single-stranded cDNA (cDNA pool) was synthesized by reverse transcriptase M-MLV (Takara) with an anchored oligo (dT) primer (5′-CTGATCTAGAGGTACCGGATCCT$_{20}$-3′) according to the manufacturer's protocol.

2.2. PCR amplification, cloning and sequence determination of PLA_2 cDNAs

Three primers were used (#3AB: 5′-CATYKWGCTTGCAGITTCACCAC-3′, #4AB: 5′-ATYGGCACCTCACTTTATTGTTCA-3′ and loopF: 5′-ACATCCTGCATGTAAGTCCC-3′) for amplification of PLA_2 cDNAs from the cDNA pool (Fig. 1). The primers #3AB and #4AB were designed to anneal with cDNAs that encoded both groups of PLA_2s (IA and IB). The sequence of loop F primer is complimentary to the pancreatic loop coding sequence of LsPLA2pkP5 (DDBJ accession number AB078348). Amplified fragments were cloned using pGEM-T Easy vector systems (Promega) and plasmid DNAs obtained were purified by Wizard plus Minipreps DNA Purification Systems (Promega).

2.3. Probes for hybridization analysis

Partial sequences of cDNA clones LsPLA2cPm09 (group IA PLA_2: AB037415) from venom glands and LsPLA2pkP5 (group IB PLA_2: AB078348) from the pancreas (Fujimi et al., 2002a,b) were amplified by PCR using IBF (5′-ACAGGCATCGCCCACCCACCTG-3′), IBR (5′-ATTGCACTCAGCCCCGTTTG-3′), IAF (5′-TAGGTGCTCCAAAATACATG-3′) and #8EP (5′-TTGAATTCTGCAGAGGCCCATCCAGAGAATTGCCAT-3′) as primers. Origins and accession numbers of the template cDNA clones are described in Table 1. The positions and directions of the primers are indicated in Fig. 1A. A cDNA clone encoding the partial sequence of *L. semifasciata* glyceraldehyde 3-phosphate dehydrogenase (LsGAPDH: AB111960), previously cloned, was used for the probe for detection of LsGAPDH mRNA. The fragments obtained were then purified by agarose gel electrophoresis and labeled by the ECL direct nucleic acid



C

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pkP5        -27  MNPAHLLVLLAVCVSLLGASTIPPLPLNLVQFSNMIKCTIPGSRPLLDYADYGCYCGAGG  33

pkP5         34  SGTPVDELDRCCQTHDNCYSQAKKHPACKSPLDSPYIKIYSYTCSGGSLTCRDDNDECGA  93
VG11                                     LDSPYIKIYSYTCSGGSLTCRDDNDECGA
VG8                                      LDSPYIKIYSYTCSGGSLTCRDDNDECGA

pkP5         94  FICNCDRTAAICFAGAPYNKENYNIDTKKHCK  125
VG11             FICNCDRTAAICFAGAPYNKENYNIDTKKHCK
VG8              FICNCDRTAAICFAGAPYNKENYNIDTKKHCK
```

Fig. 1. Cloning of group IA and IB PLA_2 cDNAs from venom glands. (A) Schematic comparison of typical cDNA clones encoding group IA (LsPLA2cPm09) and IB (LsPLA2pkP5) PLA_2s. Arrows indicate the annealing positions and directions of the primers. The regions with black or white color are highly homologous to each other. The regions in gray indicate the low homology between the two groups. An asterisk shows the position of the pancreatic loop coding sequence. Bold underlines indicate the annealing region of probes for Northern blot hybridization analysis. (B) Amplification of partial group IB PLA_2 cDNA from the cDNA pool of venom glands by PCR. Left: Agarose gel (2%) electrophoresis of amplified fragments. M indicates the DNA size marker. The primers used for amplification are represented above the each lane. Arrow heads with numbers (1 and 2) in the right side of the bands indicate the two amplified fragments of different sizes obtained by PCR using loop F and #4AB as primers. Schematic drawings of the clones from each amplified fragment (VG8 and VG11) are indicated on the right. (C) Comparisons of partial amino acid sequences of VG8, VG 11 and pancreas group IB PLA_2 deduced from the clone LsPLA2pkP5 (pkP5).

labeling and detection system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Specificity of the probes was checked by Southern blot hybridization analysis using the same detection system as Northern blot analysis.

2.4. Northern blot hybridization analysis

Poly(A)$^+$ RNAs purified from the 100 μg total RNAs (venom glands or pancreas) by Dynabeads Oligo $(dT)_{25}$ (Dynal), and electrophoresed on a 1% formaldehyde agarose gel. RNAs in the agarose gel were transferred on Hybond-N+ (Amersham Pharmacia Biotech) by aspiration, and then fixed by UV. Phospholipase A_2 mRNAs on the blotted membrane were detected by the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech) and X-ray film (X-OMAT AR film, KODAK) according to the manufacturer's protocols. Exposure time was an important factor to detect the probe specific signal, especially when the quantity of target mRNAs was low, because the intensity of the detected bands on the X-ray film was the integrated value of chemiluminescence. To obtain the sufficient or moderate signals, three X-ray films were exposed with time variance (10 min, 2 h and 12 h) per an assay using a probe. The same membrane was reacted sequentially with three kinds of probes to detect mRNA encoding GAPDH, group IB PLA_2 and group IA PLA_2.

2.5. Cloning and sequence analysis of 5′-upstream regions of PLA_2 genes

Lambda FIXII (Stratagene) genomic clones, LsPLA-2GL1-1 (group IA) and LsPLA2GL16-1 (group IB) were digested with *Hin*dIII (for group IA), and *Cla*I and *Bam*HI (for group IB). The DNA fragments corresponding to −3742 to +1571 of LsPLA2GL1-1, and −6514 to +129 of LsPLA2GL16-1 were inserted into pBluescript II SK(+) vectors (Stratagene). The subclones obtained were named 1-1up1 (group IA) and 16-1up1 (group IB). Origins and accession numbers of the clones are summarized in Table 1.

2.6. Construction of the vectors for reporter gene assay

The 5′-upstream region containing the conserved sequence for the gene of each group (IA and IB) was amplified by PCR using 1-1up1 as a template and the following primers: F2AMlu (5′-ACGCGTAAATGAGAG-CAGATTTCCGT-3′) and RAXho (5′-CTCGAGTTTGT-CAGTGGTGAAGCTGC-3′). The amplified fragment was digested with *Mlu*I and *Xho*I, and ligated into the pGL3-Basic vector (Promega). A Kilo-Sequence deletion Kit (Takara) was used to generate the 5′-progressive deletion mutant series of the 5′-upstream region of the group IA PLA_2 gene. Clones containing a suitable length of the 5′-upstream region were selected and sequenced. Finally, the reporter gene series of the group IA PLA_2 gene 5′-upstream region (IA-633, -545, -410, -382, -232, -162, -83 and -33) was obtained. For the group IB PLA_2 gene 5′-upstream region, three sense primers [for IB-220: F2Bmlu (5′-ACGCGTAAATCAGAGCAGATTTCCGT-3′), for IB-220: F150Mlu (5′-ACGCGTACAGCTCTGGTTTTATCTCC-3′) and for IB-33: F33Mlu (5′-ACGCGTGCTATAAAAGGTT-GAACTCG-3′)] and one antisense primer [RBXho (5′-CTCGAGTTTGTCTGTGGTGAATCTGC-3′)] were used for amplification of appropriate regions. The amplified fragments were digested with *Mlu*I and *Xho*I, and ligated into the pGL3-Basic vector (Promega). Finally, the reporter gene series of the group IB PLA_2 gene 5′-upstream region (IB-220, -132 and -33) was obtained.

2.7. Cell culture and DNA transfection

The Chinese hamster ovary cell K1 line (CHO-K1) was used for a luciferase assay of *L. semifasciata* PLA_2 genes. The cell line was maintained in an α-MEM medium supplement with 10% fetal bovine serum. The day before transfection, cells were trypsinized and subcultured into a 24-well plate at a density of 5.0×10^4 cells per well. To normalize for transcriptional efficiency, a pRL-TK vector (Promega) was cotransfected with reporter vector construct. Tranfections were carried out with 1 μg of total plasmid DNAs by Tfx-20 Reagent (Promega), according to the manufacturer's protocol. The DNA amount ratio of reporter and normalizing vectors for transfection was 10:1.

2.8. Reporter gene assay

Luciferase reporter gene assay (Gould and Subramani, 1988) was performed with the Dual-Luciferase Assay System (Promega) according to the manufacturer's protocol and the luciferase activity was determined by Turner Designs Luminometer, Model TD-20/20 (Promega). Six replicate measurements were performed for each reporter clone.

2.9. Gene quantitative analysis by slot blot Southern hybridization

Total genomic DNA was prepared from the livers of four individual snakes (*L. semifasciata*), as described previously (Fuse et al., 1990) and digested with *Spe*I. The plasmid DNAs containing cDNA encoding group IA PLA_2 (LsPLA2cPm09: AB037415), group IB PLA_2 (LsPLA2pkP5: AB078348) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (LsGAPDH: AB111960) were used as the standard to calibrate the relationship between the chemiluminescent intensity and quantity of DNA molecules. The genome-digested and standard DNA samples were transferred and fixed on Hybond-N+ (Amersham Pharmacia Biotech) by aspiration under alkaline conditions with a slot blotter (Sanplatec). The blotted membranes were rinsed with 2× SSC and allowed to air dry. The sequences of the specific probes for each gene were 5′-CATCGTCAACTTTGGGTAGC-3′(for the group IA

PLA_2 gene), 5′-GCCTCCAGAACAGGTGTACG-3′ (for the group IB PLA_2 gene) and 5′-ACCCTTCATTGGGCCTTCAG-3′ (for the GAPDH gene). Labeling and detection of probe DNAs were performed with Gene Images 3′-Oligolabelling Module and ECF signal amplification module (Amersham Pharmacia Biotech), respectively, according to the manufacturer's protocol. Membranes were incubated with a detection reagent for 24 h and scanned by the FluorImager 595 (Molecular Dynamics). The images obtained were saved as TIFF files by the Image Quant ver.1.0 (Molecular Dynamics) and analyzed by KODAK 1D Image Analysis Software EDAS 292 (Eastman Kodak, version 3.5). The amounts of DNAs on the blots were determined by triplicate runs.

2.10. Quantitative real-time PCR analysis

The ABI PRISM Primer Express ver. 2.1 software (Perkin-Elmer Applied Biosystems) was used to design all PCR primers and probes (Table 2). To determine the quantity of cDNAs encoding group IA and IBPLA_2s, two sets of probes and primers were designed for each group of PLA_2 cDNA. The protein coding sequences were used to design the primers and probes. The regions used were highly conserved among the PLA_2 cDNA clones in each group.

The cDNA pools prepared from three independent snakes were used for the template of quantitative PCR. At the quantification assay for one group of PLA_2 cDNA, 0.1–10 ng cDNA pool was used for the template. The iQ Supermix (Bio-Rad) was used for the amplification of real-time PCR. TaqMan primers and probe concentrations in the final volume of 25 μl were 300 and 200 nM, respectively. The thermal cycling condition were: 3 min at 95 °C, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C 30 s. The quantitative PCR reaction was performed by iCycler iQ real-time PCR detection system (Bio-Rad). Quantity of the target cDNA in the pool were estimated from the standard curves obtained from the known amounts of target cDNA by software (ver. 3.0a) associated with the system. The quantities of the three independent reactions were averaged.

2.11. Nucleotide sequence determination

Nucleotide sequences were determined by the dideoxynucleotide chain-termination method using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction FS kits and ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems).

3. Results

3.1. PCR analysis of PLA_2 genes expressed in the venom glands of L. semifasciata

To investigate the expression of the group IB PLA_2 genes in the venom glands, cDNA pool from venom glands was used as a template of PCR. When loopF and #4AB primers (Fig. 1A) were used, two amplified fragments (650 and 450 bp) were detected by agarose gel electrophoresis (Fig. 1B). The size of the longer one was the same as the amplified fragment of the pancreas group IB PLA_2 cDNA clone (LsPLA2pkP5). Both fragments were cloned and sequenced (VG11: AB120375; VG8: AB120376). Both nucleotide sequences were similar to that of group IB PLA_2 clone from pancreas (Fig. 1C). Existence of two different types of genes was checked by the PCR using loopF and #4AB as primers and *L. semifasciata* genome as template (data not shown). Two types (with long or short 3′UTR) of the genes encoding PLA_2 with a pancreatic loop (group IB) exist in the genome of *L. semifasciata* and they expressed in the venom glands. The group IB PLA_2 gene, previously cloned (LsPLA2GL16-1: AB078346), had the same structure as the group IB PLA_2 mRNA with long 3′UTR.

3.2. Expression of PLA_2 genes in the venom glands of L. semifasciata

To confirm the expression of group IB PLA_2 genes in the venom glands, Northern blot hybridization analysis was performed. Before the Northern blot hybridization analysis, the specificity of the probes used for the analysis (Fig. 1A) was checked by Southern blot hybridization analysis. The probe for group IA PLA_2 weekly cross-reacted with group IB PLA_2 cDNA (Fig. 2A, middle). On the other hand, the probe for group IB PLA_2 specifically reacted with the group IB PLA_2 cDNA (Fig. 2A, bottom). As shown in Fig. 2B, group IA, the strongest signal was detected in the venom gland mRNAs with the probe for group IA in just 10 min. Because the size of the band was slightly smaller than that of pancreas mRNA, the signal detected here should be derived from group IA PLA_2 mRNA. In the case of using group IB probe, week signals were obtained when the film exposed only after 12-h exposure (Fig. 2, group IB) though no signals were obtained after 10-min and 2-h exposure (data not shown). Two bands were detected by the probe for group IB PLA_2 in the lane of venom gland mRNAs, though only one band was detected

Table 2
Primers and probes used for quantitative PCR analysis

Target	Primer 1 (5′-3′)	Reporter–5′-Probe-3′–Quencher	Primer 2 (5′-3′)
Group IA PLA_2	AATGGGATGCTACCCAAAGTT	FAM–TGGCACAGAGGGACCCTACTGCAATACA–TAMURA	CACAAGCACACACATAACGTTGA
Group IB PLA_2	GCCCCTACATCAAGATCTATTCG	FAM–ACACCTGTTCTGGAGGCAGCCTCAC–TAMURA	CCACACTCATCGTTGTCATCTC

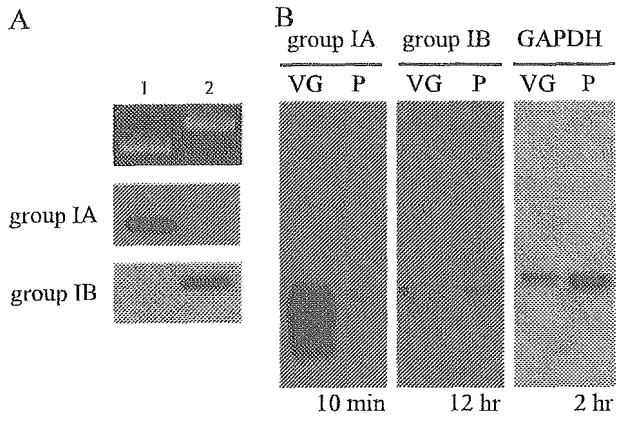


Fig. 2. Expression of PLA_2 genes in the venom glands of *L. semifasciata*. (A) Specificity of the probes was checked by Southern blot hybridization analysis. Lane 1: group IA PLA_2 cDNA (LsPLA2cPm09); lane 2: group IB PLA_2 cDNA (LsPLA2pkP5); Top panel: EtdBr staining of 100 ng cDNAs fragments; Middle and bottom panels: Southern blot hybridization analysis of 10 ng cDNA fragments. Probes were indicated in the left side of panels. The X-ray films were exposed for 1 min. (B) Northern blot hybridization analysis to the mRNAs purified from the 100 μg of total RNA of venom glands (VG) and pancreas (P). The probes using the hybridization indicated on the top of each panel. GAPDH: glyceraldehyde 3-phosphate dehydrogenase. The exposure time of the X-ray films indicated at the bottom of each panel. The asterisk mark indicates the weak signal of group IB PLA_2 with long size 3UTR in the lane of venom gland mRNA.

in the lane of pancreas mRNAs (Fig. 2, group IB). This result confirmed the presence of two types of group IB PLA_2 genes, and they expressed in the venom glands of *L. semifasciata*. These two bands probably corresponded to the cDNA clones of VG11 and VG8 (Fig. 1B). The signal intensity of the band with the longer size (Fig. 2B, asterisk) was very weak compared with the shorter one in the same lane. It suggested that the expression levels of the longer size of group IB genes were very low compared with that of the shorter size gene. The difference in the signal intensity and the exposure time required to obtain the sufficient signal suggested that the expression level of the group IB PLA_2 gene might be very low compared with those of group IA genes.

Quantitative PCR analysis also showed the substantially different amounts of mRNAs between group IA and IB PLA_2 in venom glands (Table 3). In all snakes, the amount of expressed group IA genes was around 100–300 times greater than that of group IB genes. The primers and probe for group IB PLA_2 in this assay could detect the group IB PLA_2 cDNAs regardless of the size of 3′UTR. The primers and probes for group IB PLA_2 targeted the 3′UTR could not be designed because sequence of the region did not have sufficient homology to design for all clones of group IB PLA_2 with long UTR. If such a probe were designed, the difference of expression levels would be greater than the results in Table 3. The expression levels of toxin genes in venom glands are affected with the time after the venom extraction (Lachumanan et al., 1999). The expression levels of PLA_2 genes in venom glands were slightly different among the snakes in this study, because the times after the venom extraction were not controlled on each snake.

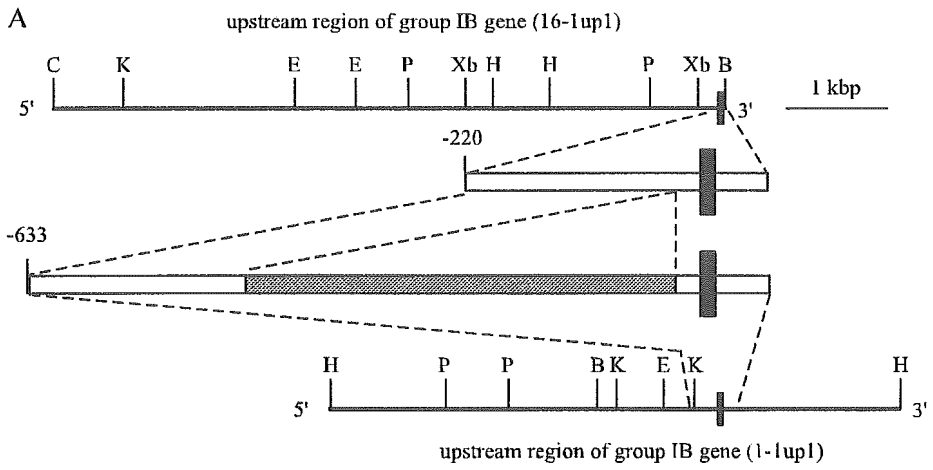
3.3. Nucleotide sequence comparison of 5′-upstream regions of PLA_2 genes

The difference in the amount of mRNAs encoding group IA and IB PLA_2s may be caused by the regulatory elements present in the 5′-upstream region of the genes. To define the difference in the regulatory sequences, the nucleotide sequences of the 5′-upstream regions of both groups of genes, LsPLA2GL1-1 (AB062439 encoding group IA PLA_2, Fujimi et al., 2002b) and LsPLA2GL16-1 (AB078346 encoding group IB PLA_2, Fujimi et al., 2002a), were determined and analyzed. The nucleotide sequences of upstream clones (1-1up1: AB111958 and 16-1up1: AB111959) were less homologous, except for the 5′ flanking regions of the genes. As shown in Fig. 3A, even the restriction enzyme maps of the genes confirmed this observation. An inserted sequence (−444 to −34, Fig. 3B) was found in the putative promoter region of group IA PLA_2 gene. The homologous region was interrupted by this inserted sequence. The presence of the inserted sequence was probably general for the genes encoding group IA PLA_2 in *L. semifasciata*, because this sequence was also present in the another clone of group IA PLA_2 gene, which is previously isolated (LsPLA2GL5-1: AB062440) (Fujimi et al., 2002b). The promoter structure was dramatically changed by this inserted sequence in the 5′-flanking region of the group IA PLA_2 genes. In general, the minimum promoter region of a gene is present in the 5′-upstream region up to −200 from the transcription initiation site in eukaryotes. The CCAAT box, a *cis* element found in the promoter region of group IB gene (LsPLA2GL16-1), was shifted to more than 400-bp upstream in group IA gene (LsPLA2GL1-1) by the insertion of this sequence. In addition, there were one GC box and two E boxes, which were not found in the putative promoter region of group IB gene (LsPLA2GL16-1), in the 5′-upstream of group IA gene (LsPLA2GL1-1) (Fig. 3B). This observation suggested that the promoter activities

Table 3
Quantitative PCR analysis of PLA_2 genes expressed in the *L. semifasciata* venom glands

Sample	Group IA		Group IB		Ratio of average (IA/IB)
	Ave.	S.D.	Ave.	S.D.	
Snake A	3.40×10^{-7}	$\pm1.13\times10^{-8}$	1.08×10^{-9}	$\pm3.09\times10^{-10}$	315
Snake B	3.33×10^{-7}	$\pm6.71\times10^{-9}$	3.30×10^{-9}	$\pm3.86\times10^{-10}$	101
Snake C	1.47×10^{-7}	$\pm3.16\times10^{-8}$	1.51×10^{-9}	$\pm1.48\times10^{-10}$	97

The results were evaluated based on the standard curves predetermined with known amounts of target sequence. The results were derived from three independent reactions. Indicated numbers show the mole number of PLA_2 cDNA in the 1 ng venom gland cDNA pool. Ave. indicate the average of thee independent reactions. S.D. is standard deviation.



B

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 1-1up   -633 AAATGAGAGCAGATTTCCGTTTCTTCACAAAAAAACAGAACATCTTGTTCCAACTCTTGG
16-1up   -220 AAATCAGAGCAGATTTCCGTCTCTGCACAAGAAAACAAAACATCTAGATCCAACTCTTGG

 1-1up   -573 AGGAAAACTGGCAGATTTTTGCAAGCAAACAGCTCTGGTTTTATCTCCTTCCTGGAAGGA
16-1up   -160 AGGAAAACTGGCAGATTTTTGCAAGCAAACAGCTCTGGTTTTATCTCCTTCCT--ACATA

 1-1up   -513 GTGCTGATAGGCCAATAAAACCATCCATCTCTTATCTCCCATCTTAGCTGGGTCCAAGGT
16-1up   -102 GTGCTGATAAGCCAATAAAACTATCCATCTCTTATCTCCCATCTCAGCTAAGGCCAAGGT
                       CCAAT box
 1-1up   -453 TACGGCAAGgctataagacagtggttcccaaagtgggtggtaccacccccctgaggggcag
16-1up    -42 TATGACAAG----------------------------------------------------

 1-1up   -393 tgggatgctttaggggggcactaagaggcaagggggtgccggaggtgggcctctcgcag
16-1up        ----------------------------------------------------------

 1-1up   -333 ggaacaattcaggagtgggattctaccagttcattaccgttcccagtagtttggagaagt
16-1up        ----------------------------------------------------------

 1-1up   -273 ggtaaaatgggcccctggctggccctgctcctctccacacactcttccattttcctgctc
16-1up        ----------------------------------------------------------

 1-1up   -213 agtccgtccttattgcgcatctgtaacatcgcacgcatgacccccaagccatgcccacaa
16-1up        ----------------------E box---------------------------------

 1-1up   -153 attaggccacgcccacagaaacatttgttcacaaatttgaatcccacccccttggtggggg
16-1up        ----------------------------------------------------------
                                     E box
 1-1up    -93 cactgaggatgggattatagcaccaaggggcggggactgaaaaagtttgggaaccact
16-1up        ----------------------------------------------------------
                                                GC box
 1-1up    -33 GCTATAAAAGGCTGAACTCTCCAGGTACTGAAATTCATCTTGCTTGCAGCTTCACCACTG
16-1up    -33 GCTATAAAAGGTTGAACTCGCCTGGTATCGAAGTTCATTGAGCTTGCAGATTCACCACAG
              TATA box
 1-1up     28 ACAAAATG
16-1up     28 ACAAAATG
```

Fig. 3. Comparison of 5′-upstream region sequences of group IA and IB PLA_2 genes. (A) Restriction maps of 5′-upstream regions of group IA (1-1up1) and IB (16-1up1) PLA_2 genes are indicated. The sites of enzymes are indicated as C: *Cla*I, K: *Kpn*I, E: *Eco*RI, P: *Pst*I, Xb: *Xba*I, H: *Hin*dIII and B: *Bam*HI. Black boxes in the maps indicate exon I. Schematic representations of the homologous regions around exon I are shown between the maps. The gray region in 1-1up1 indicates the inserted sequence. (B) A nucleotide sequence comparison of 5′-flanking regions of exon I. Boxed sequences indicate the putative promoter motifs. The bar represents a gap (-). The inserted sequence is written in lowercases.

and/or tissue specific expression of these genes were/was affected by the inserted sequence in their promoter region.

3.4. Reporter gene assay

As a practicable experiment to confirm the function of the inserted sequence, reporter gene assay was performed. Ma et al. (2001) showed that the CHO cells yielded comparatively similar levels of expression to that of toxin genes in vivo and hence can be used as a cell line for the investigation of the promoter activity of toxin gene. Jeyaseelan et al. (2001) also showed that the results of reporter gene assay of a snake toxin gene using the nuclear extracts from both venom glands and CHO cells were similar.

According to their reports, we also performed the reporter gene assay with CHO-K1 cells.

A deletion series of the 5′-upstream region-luciferase gene fusion constructs was generated. The regions used for this assay were the ranges of −633 to +32 of group IA gene (LsPLA2GL1-1) and −220 to +32 of group IB gene (LsPLA2GL16-1). These regions contained both homologous and specific sequences (Fig. 3B). The transcription activity of these constructs in the CHO-K1 cell line was detected as luciferase activity (Fig. 4). Comparison of luciferase activities among the deletion series containing the 5′-upstream region of the group IA PLA_2 gene revealed that the inserted sequence significantly affected the expression ability (Fig. 4A). Two regions (−232 to −162) and (−410 to −382) especially affected the expression of group IA PLA_2 gene in CHO-K1 cell were found out in the specific inserted sequence. The former region showed the highest luciferase activity. The latter region showed the strong suppression of reporter gene transcription.

The constructs from group IA gene showed higher luciferase activity than those from group IB gene containing the corresponding regions (Fig. 4B). A construct named Δ411 was a mutant that lacked the specific inserted sequence (411 bp) from the construct IA−633. The luciferase activity of Δ411 was about one third that of IA−633 (Fig. 4B). These observations suggested that the transcription activities and/or tissue specific expression of group IA and IB PLA_2 genes were/was different from each other, and that the difference depended on the existence of a group IA PLA_2 specific inserted sequence.

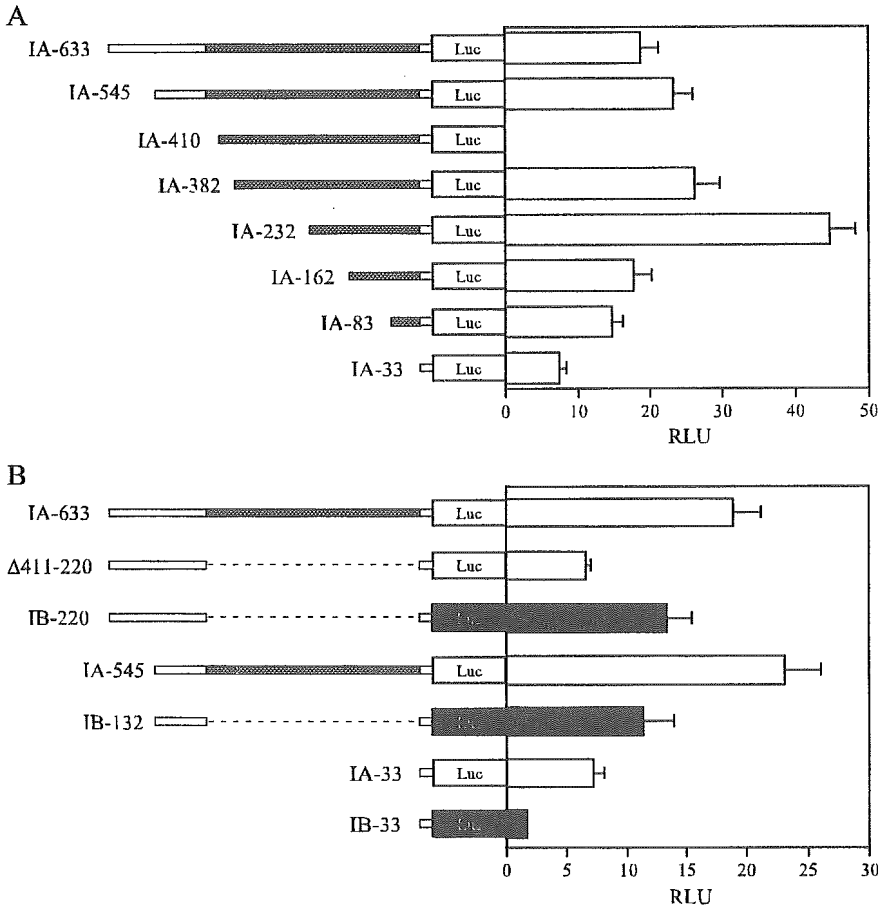

Fig. 4. Luciferase assay analysis of PLA_2 promoters. (A) Luciferase activity of the constructions from the 5′-upstream region sequence of group IA gene in CHO-K1 cell. Left: a diagrammatic representations of the 5′-upstream regions (−633 to 31) of group IA PLA_2 gene (LsPLA2GL1-1) containing the reporter gene system. Each deletion mutant was ligated to a promoter-less firefly luciferase gene (Luc) in an expression vector (pGL3B). Numbers in the names of each construction (left side of schema) indicate the distances in bp from the transcription start site. The region colored in gray is the group IA specific sequence. (B) Comparison of luciferase activities of the constructions between the derivatives from 5′-upstream regions of group IA and IB PLA_2 genes. The clones constructed from the 5′-upstream region of the group IA (LsPLA2GL1-1) gene are indicated as IA at the left side of the schema. Δ411-220 is the mutant lacking the group IA specific sequence (411 bp) from IA-633. The clones constructed from the 5′-upstream region of the group IB (LsPLA2GL16-1) gene are indicated as IB at the left side of the schema and black out the scheme of the luciferase gene. Dashed lines indicate gaps. Each activity is normalized by *Renilla* luciferase activity from a cotransfected vector (pRL-TK). RLU indicates the Relative Luciferase Unit (Firefly/*Renilla*). Data are the means of six determinations. Bar: standard deviation.

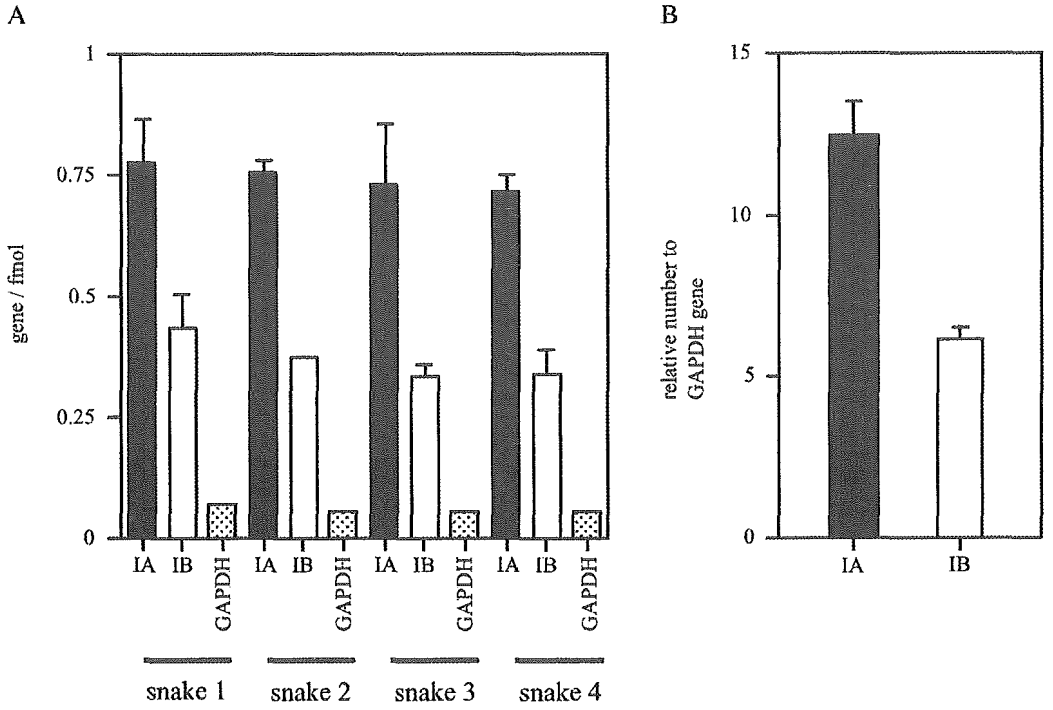

Fig. 5. Quantitative analysis of PLA_2 genes by slot blot hybridization. (A) Results for the determination of the mole numbers of three genes per 1 μg of genome DNA from four individual snakes. IA and IB correspond to the group IA and IB PLA_2 genes, respectively. GAPDH indicates the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Data are the means of three determinations. Bar: standard deviation. (B) The relative numbers of each PLA_2 gene to GAPDH gene. The mole numbers of each PLA_2 gene was normalized by that of GAPDH gene. Bar: standard deviation.

3.5. Quantitative analysis of PLA_2 genes by slot blot hybridization

One of the assumable factors causing the difference in the expression level between the group IA and IB genes was their difference in the number of gene copies. To estimate the relative abundance for each PLA_2 gene (groups IA and IB) to the GAPDH gene in genomic DNA from *L. semifasciata*, quantitative analysis by slot blot hybridization was performed. From the determined chemiluminescent intensities of the fractions-blotted genomic DNA, mole number of the gene per unit mass of genomic DNA was calculated from the calibration curves. The results showed that the number of group IA PLA_2 gene copies was around 0.72–0.77 fmol/μg of genomic DNA (Fig. 5A). Using the value of the GAPDH gene as the internal standard, the abundance of each gene from four individual snakes was normalized. As a result, the abundance of the group IA PLA_2 gene was twice as much as that of the group IB PLA_2 gene (Fig. 5B). The difference in the amount of each group PLA_2 gene indicated that the contribution of the transcription mechanisms to the gene expression level in venom glands was higher than that of the gene copy numbers.

4. Discussion

In our previous paper, we described a model for the evolution of snake group I PLA_2 genes from the analysis of the structural gene sequences (Fujimi et al., 2002a). In the model, toxic PLA_2 genes arose from PLA_2 genes with a pancreatic loop coding sequence in the genome of *L. semifasciata*. However, we did not address an important question: How did the group IA genes evolve to express as toxin genes? In this report, we proposed to address this question from the analysis of the 5′-upstream regions of these genes.

Analysis of Northern blot hybridization revealed the difference of the expression level between group IA and IB genes in venom glands. In the preliminary experiment of mass spectrometry (MALDI-TOF MS) analysis, group IB PLA_2 was not detected in the venom of *L. semifasciata* (data not shown). Quantitative PCR analysis confirmed the low level of expression on group IB genes in venom glands. Expression level of group IA genes was more than 100 times higher than that of group IB genes. It seems that the group IB PLA_2 did not act as the toxin in *L. semifasciata* venom glands.

The difference of the expression level detected by the Northern blot hybridization and quantitative PCR analysis was greater than that of the copy numbers of the gene and may be explained by the difference in the transcription activity of these two genes. Analysis of the 5′-upstream sequences of group IA and IB genes from *L. semifasciata* revealed a specific inserted sequence in the 5′-flanking region of the group IA PLA_2 gene. Comparison of the 5′-flanking regions of several snake toxic group I PLA_2 genes showed that the specific inserted sequence was present before the divergence of Elapidae family (Fig. 6). It is also

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LsGL1-1    -633 AAATGAGAGCAGATTTCCGTTTCTTCACAAAAAAACAGAACATCTTGTTCCAACTCTTGG
LsGL16-1   -220 AAATCAGAGCAGATTTCCGTCTCTGCACAAGAAAACAAAACATCTAGATCCAACTCTTGG

LsGL1-1    -573 AGGAAAACTGGCAGATTTTTGCAAGCAAACAGCTCTGGTTTTATCTCCTTCCTGGAAGGA
LsGL16-1   -160 AGGAAAACTGGCAGATTTTTGCAAGCAAACAGCTCTGGTTTTATCTCCTTCCT--ACATA

LsGL1-1    -513 GTGCTGATAGGCCAATAAAACCATCCATCTCTTATCTCCCATCTTAGCTGGGTCCAAGGT
LsGL16-1   -102 GTGCTGATAAGCCAATAAAACTATCCATCTCTTATCTCCCATCTCAGCTAAGGCCAAGGT

LsGL1-1    -453 TACGGCAAGGCTATAAGACAGTGGTTCCCAAAGTGGGTGGTACCACCCCCTGAGGGGCAG
LsGL16-1    -42 TATGACAAG----------------------------------------------------

LsGL1-1    -393 TGGGATGCTTTAGGGGGGCACTAAGAGGCAAGGGGGGTGCCGGAGGTGGGCCTCTCGCAG
LsGL16-1        ----------------------------------------------------------
PtIB       -380  CTGGTGCCTTACGGGGGCACTGAAAAGCAAGGGGGGCGCTGGAGGTGGGCCCCTCACAG
NsA        -366                              ATCTAATCCAACCACCTGCTCCAGCAGGGTTAGAA
                                             *   **       * *     *     *   *

LsGL1-1    -333 GGAACAATTCAGGAGTGGGATTCTACCAGTTCATTACCGTTCCCAGTAGTTTGGAGAAGT
LsGL16-1        ----------------------------------------------------------
PtIB       -321 GGAACAATTCAGGGGTGGGATTCTACCGGTTCATTACCGTTCCCAGTAGTTTGGAGAACT
NsA        -331 AGGAGTGTGTGTGTGTGTGTGTGTGCGCACGCGCGTGCATGGTGAGTCATA-ACTT
                 * *   *    * *** *  * *    *  **    *** *   **   *   * *  *

LsGL1-1    -273 GGTAAAATGGGCCCCTGGCTGGCCCTGCTCCTCTCCACACACTCTTCCATTTTCCTGCTC
LsGL16-1        ----------------------------------------------------------
PtIB       -261 GGTAATATGGATCCCTGGCTGGCCCTGCTCCTCTCCACGCGCTCCTCCATTTTCCTGCTC
NsA        -272 AGAACACTTACTAAGCAACCGGCCATTAAGCAAGGACCACTTATATTTGGTTTGCCAGTC
                 * *   *          * **** *    *      * *     *    *** *   **

LsGL1-1    -213 AGTCCGTCCTTATTGCGCATCTGTAACATCGCACGCATGACCC-CCAAGCCATGCCCACA
LsGL16-1        ----------------------------------------------------------
PtIB       -201 ACTCAGTCCTTATTGCGCACCTGCAACATCGCACGCATGACCC-C-AA-CCACGCCCACA
NsA        -212 ACAT-GGCC-ACTTGGCCACACCCAGC--CAGTCACATGACCCACCAAGCCACGCCCATG
                *    * **   **** **     * *  *   * ******** * ** *** *****

LsGL1-1    -154 AATTAGGCCACGCCCACAGAAACATTTGTTCACAAATTTGAATCCCACCCCTTGGTGGGG
LsGL16-1        ----------------------------------------------------------
PtIB       -144 AATTAG-CCAC-CCCACAGAAACAGTTGTT-AAAAATTTGAATCCCACCCCTCCGTGGGG
NsA        -156 AATTAAGCCACGCCCACATAAACAGTTGTTAGGAAATTTGAATCCCACCCCTTGATGGGG
                ***** **** ****** ***** *****   ******************   *****

LsGL1-1     -94 G-CACTGAGGATGGGATTATAGCACCAAGGGGGCGGGGGACTGAAAAAGTTTGGGAACCA
LsGL16-1        ----------------------------------------------------------
PtIB        -87 C-CACTGAG-ATTGGATTATA-CACCAA-GGGGCGGTGGACTGAAAAA--TTTGGAACCA
NsA         -96 GGCACTGAGGATGGGATTGTAGCGCCAAGGGGGCGGTGGACTGAAGAAGTTTGGGGACCA
                  ******* ** ***** ** * **** *** *** ******** **  ** ** ****

LsGL1-1     -35 CTGCTATAAAAGGCTGAACTCTCCAGGTACTGAAA
LsGL16-1    -33 --GCTATAAAAGGTTGAACTCGCCTGGTATCGAAG
PtIB        -33 CTGCTATAAAAGGCTGAACTCACCAG-TAC-GAAA
NsA         -36 CTGCTATAAAAGGCTGAACTCGCCAGGT--GGAAATTC
                ********************* **** *    *
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Fig. 6. Sequence comparison of the 5′-flanking regions of the group I PLA_2 genes from various snakes. The 5′-flanking regions of group IA and IB PLA_2 genes are compared. LsGL1-1 and LsGL16-1 indicate the 5′-flanking region sequences of the *L. semifasciata* group IA (AB062439) and IB (AB078346) genes, respectively. PtIB and NsA indicate the 5′-flanking region sequence of the *P. textilis* group IB PLA_2 gene (AY027495) and *N. naja sputatrix* group IA acidic PLA_2 gene (AF101235), respectively. The asterisks under the aligned sequences indicate the conserved nucleotide. The bar represents a gap (-).

significant that the functional inserted sequence in the 5′-flanking region of *L. semifasciata* group IA PLA_2 gene was also found in the *P. textilis* group IB PLA_2 gene (AY027495) expressed abundantly in venom glands. These observations indicate that the expression levels of PLA_2 genes in venom glands depend on the presence or absence of the functional inserted sequence. Ma et al. (2001) reported that the reporter gene assay of snake toxin genes in the CHO cells is proportional to the in vivo expression level. Thus, we also performed the assay using the CHO-K1 cell line. Upstream region sequences of the genes expressed in the snake venom glands were highly conserved up to around −200. The luciferase assay showed that the −232 to −162 region is important for the transcription of LsPLA2GL1-1 (group IA). Jeyaseelan et al. (2000) reported that −233 to −116 region activates the

transcription of the *Naja naja sputatrix* toxic PLA_2 (group IA) gene. The conservation of both sequences and function suggest that the mechanism of the regulation of the gene encoding toxic PLA_2 is highly conserved and under the strong selective pressure during the evolution of toxin genes.

Since group IB PLA_2s are found in many different organisms, it is appropriate to predict the presence of the sequence encoding pancreatic loop in the ancestral PLA_2 gene. We have previously presented a model for the evolution of snake toxic PLA_2 genes based on the analysis of nucleotide sequences of the structural genes (Fujimi et al., 2002a).

Following is our hypothesis related to the functional divergence of PLA_2 in snakes based the evidences in this report. During the evolution of the group IB PLA_2 genes, the functional sequence (411 bp) was inserted into the 5′-flanking region of an ancestral gene. The gene might have started to evolve as a toxin gene by gaining the expression mechanism in venom glands. To evolve as a toxic component, it is thought that a dynamic change in the transcription mechanism might have occurred before the mutation of the coding sequence. Actually, the 5′-upstream region sequences of group IA (1-1up1) and IB (16-1up1) genes were not conserved except for the 5′-flanking region of exon I (Fig. 3A). Promoter insertion is known as a dynamic alteration event of the 5′-upstream sequence associated with changes in promoter activity (Adler et al., 1988). This phenomenon occurs by the insertion of a viral sequence in the transcription regulatory region. However, such a viral sequence in the 5′-upstream regions of the genes (1-1up1 and 16-1up1) was not found by the DNA database search. Defining the origin of the functional inserted sequence found in the 5′-flanking region of *L. semifasciata* PLA_2 gene will resolve where the toxins come from.

In this study, the presence of two types of group IB genes was defined (with long or short 3′UTR). One of them, with short 3′UTR, has not been cloned yet. To define the structure of this clone will also helps us to understand the evolutionary process of snake PLA_2 genes.

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