Expression of cardiotoxin-2 gene
Cloning, characterization and deletion analysis of the promoter

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This report is the first study of the regulation of expression of a toxin gene and it also demonstrates the novel finding that the cardiotoxin (CTX)-2 gene from Naja sputatrix is expressed in the venom gland as well as in other tissues in the snake, such as liver, heart and muscle. The venom gland produces a 500-bp (spliced) CTX-2 mRNA as the final transcript. However, the liver produces two types of CTX-2 mRNA, of which the unspliced transcript (1 kb) is predominant; the 500 bp spliced transcript is the minor species. This differential expression of the CTX gene has been attributed to the usage of alternative promoter consisting of independent TATA boxes and corresponding transcription initiation sites. Among the several transcription factors that have been identified by a search of the TFIID database, the participation of two glucocorticoid elements in the expression of the CTX gene has been demonstrated by promoter deletion analysis. Putative binding sites for SP-1, C/EBP, CACCC-binding factor and at least two unknown binding factors have also been identified by DNase I footprinting of the promoter.

Keywords: alternative promoter; cardiotoxin; Naja sputatrix; tissue-specific expression.

Snake venom is a complex mixture of many different proteins. The lethal components of the venoms from cobras consist mainly of toxin molecules, such as cardiotoxins (CTX), neurotoxins (NTX) and phospholipase A2. These toxins exhibit different biological properties [1,2]. Among them, CTXs are a group of basic and highly hydrophobic small proteins found in large amounts (< 60%) in cobra venoms [3].

To date, six CTX mRNA isoforms and their corresponding genes have been cloned from Naja sputatrix [4]. The expression patterns of these CTX genes have been found to be significantly different from each other. The CTX-2 gene has been found to be expressed at a higher level than the other CTXs in N. sputatrix [4]. In N. atra [5] seven isoforms of CTX have been identified and of these, CTX-3 appears to be the most highly expressed. Recently, Lachumanan et al. [6] and Chang et al. [7] described the cloning of CTX-3 and CTX-4 genes from N. sputatrix and N. atra, respectively. The primary sequence and the gene structure of these CTX genes were found to be similar to each other. The genes consist of three exons and two introns and resemble the gene structure of the α-neurotoxin, and κ-neurotoxins [8,9]. Sequence analysis of the N. sputatrix CTX-3 gene promoter revealed the presence of two transcription initiation sites (TIS1 and TIS2), and two putative TATA box motifs and absence of a canonical upstream CCAAT element [6]. The 5′-region of the CTX-4 gene from N. atra however, has been reported to contain one (functional) TATA [7]. Other toxin genes that have been reported to contain more than one TATA box include, a K+ channel toxin from sea anemone [10], and NTX genes from N. sputatrix [8]. Moreover, genes encoding ecto-ADP-ribosyltransferase [11], and microtubule-associated protein 1B [12] are known to contain more than one TATA motif and to use them in facilitating developmental and tissue-specific expression of the proteins from alternative promoters. The importance of multiple TATA boxes and other regulatory elements within the promoter of toxin genes has not yet been examined. In this report we demonstrate for the first time that the CTX gene is under the control of different promoters in the venom gland and in other tissues of the snake, N. sputatrix. We have also characterized some putative transcriptional factor binding sites that participate in the regulation of gene expression.

MATERIALS AND METHODS

Cell culture and DNA transfection

The Chinese hamster ovary (CHO) cell line was maintained in minimal essential medium (alpha modification; α-MEM) [13] supplemented with 10% fetal bovine serum, 50 U·mL−1 penicillin and 50 μg·mL−1 streptomycin. Transfections were carried out with 6 μg reporter plasmids (pMAMneoCAT- derived [14]) by the calcium phosphate method [15]. To normalize transfection efficiencies, a plasmid containing the β-galactosidase gene (pSV-β-Gal; Promega) was cotransfected with the test plasmids in each
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Experiment. To elucidate the role of glucocorticoid receptor (GR) elements, 1 μg pcDNA-GR (a plasmid containing the mouse GR receptor) was also cotransfected with the other plasmids. Induction of gene expression was by using 1 μM dexamethasone (dex) for 48 h before processing for chlororamphenicol acetyltransferase (CAT) assay. Stable transfectants were established using complete α-MEM containing 400 μg mL⁻¹ G418.

For CAT assays, cell cytoplasmic extracts were prepared by freeze–thawing and the enzyme activity was determined by using Beckman liquid scintillation counter as described by Seed and Sheen [16]. All results were normalized by using pSV-β-gal as an internal control and are the means of determinations ± SD from six individual experiments.

RNA isolation and Northern blot analysis

Tissue obtained from a single adult snake identified as N. sputatrix by the Zoological Gardens, Singapore, were kept in liquid nitrogen until required. Samples were homogenized separately and total RNA was isolated by a single step method using the TRIZOL reagent (Life Technologies). RNA samples were separated by electrophoresis through a denaturing agarose gel and transferred to nylon membrane. Hybridization was carried out as described previously [17] by using [α-³²P]dATP-labelled CTX-2 cDNA as probe.

Rapid amplification of 5′ and 3′ ends of cDNA and the gene

5′ RACE was performed using a Marathon cDNA amplification kit according to the manufacturer’s instructions (Clontech). The 5′ RACE universal adaptor primer1 (API) 5′-GTAATACGACTCACTATAGGGC-3′ and an antisense gene-specific primer (SAND-1SPR) 5′-CCTAAGTCCAGGCCACAATTT-3′ were used for the amplification of the 5′ end of the adapted cDNA. RT/PCR was performed as described previously [4]. 3′ RACE was carried out using the gene-specific primer (SAND-1SPF) 5′-TAAGTGTCGCTGTCCTCATT-3′ and API. The partial cDNA products were analysed on a 1% agarose gel, subcloned in pT7Blue (Novagen), and their nucleotide sequences were determined by automated fluorescent DNA sequencing (Model373, Applied Biosystems).

Genome walking PCR was used to obtain the 5′ end of the CTX gene. The primers used for primary PCR were the API and a gene-specific antisense primer (GSPIR), 5′-ATTGCCACCGATGCTGTGTGTG-3′. For nested PCR, adaptor primer 2 (AP2), 5′-ACTATAGGGCAGGTCGTGTGTG-3′, and a gene-specific antisense primer (GS2PR), 5′-GGTCTTACCTAAGTCCAGGCCACAACCAAG-3′ were used. The PCR was initiated with a hot start at 94 °C for 4 min. This was followed by a touch-down PCR, consisting of 10 cycles at 94 °C for 25 s and 68 °C for 3 min, and a further 30 cycles at 94 °C for 25 s and 65 °C for 3 min. A final extension at 68 °C for 3 min was also carried out. PCR products were then subcloned and sequenced.

DNase I protection analysis

The nuclear extracts from snake venom gland and CHO cells were prepared as described [18]. CTX promoter constructs were restricted with EcoRI and labelled by using Klonev DNA polymerase and [α-³²P]dATP/dTTP. This DNA (1 μL, 20 000 c.p.m.) was incubated with 40 μg nuclear extract in binding buffer (4% glycerol, 2.5 mM MgCl₂, 10 mM Tris/HCl pH 7.5, 1.5 μg poly(dI–dC), 1 mM dithiothreitol, 50 mM NaCl) in a total volume of 20 μL. After incubation for 40 min on ice, 2 μL of Ca²⁺/Mg²⁺ buffer (10 mM MgCl₂, 5 mM CaCl₂) was added to the binding reaction and the DNA was digested by DNase I (0.5–1 U) for 1 min; the reaction was terminated with 165 μL stop solution (100 mM Tris/HCl pH 7.5, 100 mM NaCl, 1% SDS, 10 μg protease K). The reaction products were extracted with phenol/chloroform and precipitated with ethanol and 1 μL carrier yeast tRNA (6 μg μL⁻¹). The precipitate was washed with 70% ethanol, resuspended in 4 μL loading buffer (deionized formamide containing 10 mM EDTA and 0.3% each bromophenol blue and xylene cyanol), electrophoresed in a denaturing 8% polyacrylamide gel at 10 000 c.p.m. per lane [19] and subjected to autoradiography.
Primer extension analyses were performed according to Sambrook et al. [17]. Two primers were used: a 22-mer primer CAT-p(5'-AAATCTCGTCGACCCCGGGCTA-3') complementary to nucleotides 11 to 32 in the CAT gene sequence was used for RNA samples obtained from CHO, CHO cells stably transfected with pMAMneo-CAT(CTX-2), and the other promoter-reporter deletion constructs. For RNA samples from snake liver and venom gland, a 20-mer primer, SAMD-1 (5'-ATCTTGCAATCTTCTCTGGA-3') complementary to nucleotides 114 to 134 in the CTX-2 gene was used. The intensities of the bands were measured using a densitometer after autoradiography.

RESULTS

Isolation of the 5' flanking region of the N. sputatrix CTX gene

The 5' flanking region of the CTX-2 gene from the N. sputatrix genomic DraI library has been cloned by genome walking PCR. The 800 bp fragment was found to contain the promoter and exon 1 of CTX-2 genes CTX2a, CTX2b and CTX2c, which differ from each other only in the exon 1 signal peptide region. As the promoters in these three genes were identical, we chose one of them (CTX-2a) for further analysis (Fig. 1); however, it differed significantly from the sequence of the CTX-3 promoter described previously by Lachumanan et al. [6]. Only the first 100 bp upstream of the translation initiation site of CTX-2 was found to be identical to that of CTX-3. The promoter sequence of CTX-2 contained three putative TATA box motives and one canonical upstream CCAAT element between the second and the third TATA box, which has been reported to be absent from the CTX-3 promoter [6]. The transcription-factor-binding sites were identified by using Transcription Factor Search (http://bimas.dcmr.nih.gov/molbio/signal/).

Promoter activities of CTX-2 and CTX-3

The promoters of CTX-2 and CTX-3 genes were subcloned upstream of the CAT gene in the pMAMneoCAT [14] promoterless reporter vector and transfected into CHO cells. Fig. 2A shows that the promoter activity of the CTX-2 gene, is at least twofold higher than that of the CTX-3 gene. This observation is consistent with in vivo expression levels of these two genes in N. sputatrix [4]. It was also clear that the epithelial-derived CHO cells yielded comparably similar levels of expression of CTX genes and hence can be used for the investigation of CTX gene promoter activity.
CAT assays on deletion constructs

To examine the activity of the CTX promoter in detail, sequentially deleted CTX promoters were subcloned upstream of the CAT gene in the promoterless reporter plasmid, pMAMneoCAT. The vector containing the promoter of CTX-2 in opposite orientation to the CAT sequence (R-CTX) was used as a negative control. Fig. 2A shows the results of CAT activities determined for each construct. Sequential deletion of the CTX-2 promoter region generally showed a higher CAT activity except in the case of Del 6 where almost no activity was observed. Del 1 (removal of 50 bp from the 5′ end of the promoter) showed almost a 30% increase in activity over that of the whole promoter (CTX-2). Del 2, lacking 250 bp (which included the putative transactivator AP-1 and NF-1 binding sites) of the promoter showed approximately same activity as the whole promoter (CTX-2). However, when the first (Del 3) and second (Del 4) TATA boxes were removed from Del 2, an increase in activity of almost twofold to fourfold was observed. The CAT activity of Del 5 (less 630 bp from the whole promoter) decreased to about half the CAT activity for Del 4, whereas Del 6 (less 650 bp) showed no detectable CAT activity (Fig. 2A).

From the TFIIH database search and in vitro footprinting analyses, two putative GR binding sites have been located within the CTX-2 promoter region. To elucidate the possible roles of GR elements (Fig. 1), 6 μg promoter-reporter construct, 3 μg pSV-βgal and 1 μg pcDNA-GR were cotransfected into CHO cells by using the calcium phosphate method. After transfection, cells were allowed to recover for 48 h in complete α-MEM with or without 1 μM water-soluble dex. The promoter activity of CTX-2, Del 1 and Del 2 were found to decrease by 50% upon treatment with dex (Fig. 2A). However, no significant effects have been observed for other deletion constructs (Fig. 2A). A similar pattern was observed when stable transfectants of CHO cells containing the CTX-2 promoter and the deletion constructs were treated with dex (data not shown). These results show that dex might have a repressive effect on the expression of CTX genes.

**Localisation of specific protein-binding sites in the 5′ flanking region of the CTX gene**

In vitro DNase I footprinting (Fig. 3) has been used to locate the binding sites for putative regulatory proteins to the 5′ flanking region of CTX-2. Sites on the promoter protected by some of the nuclear proteins from the venom gland and CHO cells have been traced to the corresponding transcription factor binding sites using the TFIIH database search (Fig. 1). The sites that could not be identified have been named as unknown factors. The nuclear proteins from the venom gland and CHO cells were shown to have similar protection patterns, except for the region, ~66 to ~100 where protection was observed only for the nuclear proteins from the venom gland (Fig. 3A). DNase I footprinting and TFIIH database search showed that one each of Sp1 and CACCC-binding factor might be binding to this region. In addition to this, a C/EBP and two unknown transcription binding factors (Fig. 3B) and three DNase I hypersensitive sites (Fig. 3A) may be involved in the regulation of CTX gene expression. However, these data need further evaluation by promoter deletion analysis.

**Northern blot analysis and sites of transcription initiation**

Northern hybridization using the CTX-2 probe showed the presence of the CTX transcripts in the venom gland and in other tissues such as liver, heart and muscle. The predominant CTX-2 mRNAs in the liver, heart and muscle have been found to be ~1 kb in size. The liver also showed a minor transcript of 500 bp but the venom gland contained only the 500 bp product. No detectable amount of transcripts has been observed in the brain and lung (Fig. 4).

Three major transcription initiation sites situated in close proximity to each other also have been observed for the venom gland mRNAs by primer extension analysis (Fig. 5A). The major transcription initiation site for CTX-2 mRNAs in the venom gland was found to be the...
A(1) whereas the minor transcriptional initiation sites are A(+3) and C(−2). In liver tissue (Fig. 5A) the major transcription initiation site was found to be T(−25). Besides this, other minor sites such as G(−37), G(−98), C(−124) and T(−154) can be observed. The amount of liver-specific CTX-2 transcript has been found to be ~20 times lower than that of the venom specific CTX-2 transcripts.

Using mRNAs from CHO cells containing stably transfected CTX-2 promoter (CHO-CTX-2), both liver- and venom gland-specific transcription start sites could be observed (Fig. 5B). However, the major start site in this case was found to be at C(−124). The other transcription start sites at G(−37), G(−98) and G(−154) could also be observed. These data show that the CTX-2 gene promoter can function effectively in CHO cells without exhibiting any tissue specificity. On the other hand, these also imply that there could be tissue-specific factors which can help venom gland cells or liver cells to initiate tissue-specific transcription from specific initiation sites.

Roles of TATA elements on promoter activity

Three typical TATA boxes within the CTX promoter region have been identified by TFIIID database search and in vitro DNase-I footprinting. CAT assays on the CTX promoter deletion constructs showed that the highest promoter activity was obtained when the first and second TATA boxes had been removed from the CTX promoter. Primer extension analysis (Fig. 5A) showed that of CTX-2 mRNAs are initiated from different sites in the venom gland and liver and that TATA-3 could be the TATA box mainly used by the venom-specific promoter. To confirm whether both venom gland- and liver-specific TATA boxes are functional, the three TATA boxes were sequentially mutated to HinDIII sites. Fig. 2B shows that after TATA-3 had been mutated the promoter activity decreased to nearly half of the whole CTX-2 promoter activity. However, the promoter activities increased when TATA-1 was mutated. A twofold increase in promoter activity was observed when TATA-2 was mutated (Fig. 2B). Thus, all of the TATA elements are functional. Among them TATA-3 remained the strongest and might be the venom-specific TATA. The other two, TATA-1 and TATA-2 may be involved mainly in the regulation of liver-specific CTX-2 transcription.

Primer extension analysis carried out with the deletion constructs (Del 1–4; Fig. 5C) shows that the majority of the mRNA was transcribed from venom-specific TIS [A(1), A(3) and C(−2)]. In Del-1, 2 and 3, both venom- and liver-specific [T(−25)] TIS can be observed. However, in the case of Del-4, from which both TATA-1 and TATA-2 have been removed, the amount of mRNA transcribed from liver-specific TIS was decreased (Fig. 5C).

5′ and 3′ RACE analysis of liver specific cardiotoxin transcripts

The 5′ and 3′ RACE were carried out on RNase-free DNase-I-treated, total liver RNA samples. The 5′ RACE yielded a single 110-bp DNA fragment. However, the 3′ RACE gave two products of 289 bp and 408 bp; these were subcloned and sequenced (Fig. 6). Comparing these sequences with that of previously determined CTX DNA sequences [6], it was clear that one of the CTX transcripts...
from liver remained unspliced in the intron 1 region. This transcript formed the major CTX mRNA in the liver (Fig. 4, lane 3). The spliced CTX transcript (408 bp) showed the same sequence as the CTX mRNA observed in the venom gland, except that it was 26 bp longer in the 5' UTR.

**DISCUSSION**

A 706-bp 5' flanking region of the CTX-2 gene of *N. sputatrix* has been cloned and found to contain three TATA boxes and one canonical upstream CCAAT box. The CTX messages, instead of being found exclusively in the venom gland of the snake, have also been detected in its liver, heart and muscle. The mRNA transcript found in the venom gland was ∼500 bp in size. The heart and muscle were shown to contain larger transcripts of ∼1 kb whereas the liver was found to contain both the 1 kb and the 500 bp transcripts. The major TIS for liver CTX-2 was shown to be 26 bp upstream of the venom-specific TIS.

**Role of GR elements in CTX-2 gene expression**

The GR is present in almost all mammalian tissues. It is known to physiologically mediate the cellular responses to glucocorticoid. Within the nucleus GR either induces or represses gene transcription by binding to specific DNA elements in the promoter [20]. The importance of GR in salivary gland development and secretion has also been well documented. Studies on the embryonic mouse submandibular gland development indicate that the glucocorticoid/GR signal transduction pathway plays an important role in salivary gland morphogenesis [21]. Multiple GR binding sites have also been found in the 5' flanking region of a salivary gland-specific secretory protein, cystatin S, and in this case dexam treatment led to an inhibitory effect [22]. Because the mammalian salivary parotid glands and snake venom glands are believed to be analogous to each other [23], GR may also act as an important transcription factor in the CTX-2 gene regulation. Two putative GR binding sites have been identified in the CTX-2 gene promoter and experimental data demonstrated that they exhibited significant inhibitory effects on CTX-2 gene expression after treatment with 1 μM dexam. A TFIIH database search also demonstrated that these GR elements can be negative GR elements [24].

Besides GR binding sites, the consensus sequences for the binding sites of other transcription factors, such as Sp1, CACCC-binding-factor and C/EBP, have also been identified by DNase-I footprinting assay and TFIIH database search. Promoter deletion analysis showed that removal of C/EBP site (Del 4, Fig. 2A) caused an increase (twofold) in CAT activity. This indicates a suppressive role [25] for C/EBP in CTX-2 gene expression. From our deletion analysis, we also observed a suppressive effect on CAT activity by the distal end of the promoter, especially in the region up to Del 3 (Fig. 2A). This could be due either to AP1 or to unknown element(s) present in the region. Nevertheless, further investigations are needed to support our interpretation. On the other hand, Sp1, CACCC-binding-factor and the two unknown transcription factors, observed in our DNase I footprinting studies might be functioning as activators, as expected [26], as the removal of these sites resulted in a decrease in CAT activity.

**CTX-2 transcripts in snake liver**

Toxin genes have been considered to be expressed predominantly in venom gland cells and exclusively in the columnar epithelial cell [27]. From our Northern hybridization, primer extension and 5' and 3' RACE studies, it is now evident that the CTX-2 gene is also transcribed in the liver, heart and muscle tissues of the snake but not in brain and lung tissues. The function of the liver-specific CTX transcript is not clear.

Recently, it has been documented that toxins can also be expressed in tissues other than the venom gland tissue for the operation of normal cellular pathways [28]. Sarafotoxins are highly toxic components of snake venom, whereas their homologue, endothelin, is a natural compound of the mammalian vascular system [29]. Several functional homologues of three-finger toxins have been identified to participate normal cellular pathways, such as lynx1 (an endogenous neurotoxins-like modulator of nicotinic acetylcholine receptors in the mammalian central nervous system [30]), and SLURP-1 (a cytotoxin-like human secreted protein [31]). CTX is well known as a multifunctional protein, and displays a wide array of biological activities. Some of them, such as the inhibitory activity towards protein kinase C and Na+ K+ ATPase, have no relationship to its toxic activity. The CTX molecules synthesized in the liver could also be used to elicit antibody production to protect the reptile against its own venom. It may also act as a protein molecule participating in gene regulation [3].

**Tissue-specific expression of the CTX-2 gene**

Alternative promoter usage has been widely used by eukaryotic genes as a strategy to achieve developmental and tissue-specific expression [32]. Although multiple TATA boxes are common in eukaryotic genes, so far their role in tissue-specific expression has not been discussed. In this study, we found that the CTX-2 gene is under alternative promoter control in different tissues and each promoter has its own specific TATA box and transcription initiation sites. Site-directed mutagenesis on the three TATA boxes shows that they are functional and possess different promodional strength. Among them, the TATA-3 is the strongest and it may contribute to the high level expression of CTX-2 in the venom gland.

Multiple transcription initiation sites have been observed in the toxin genes [6,10]. Three prominent transcription initiation sites were observed in the mRNAs from the venom gland cells. As they are adjacent to each other it is unlikely that they elicit different promoter activity; it is more likely that they are a consequence of the different methylation status of the nucleotides of the 5' cap of the CTX gene, which may affect the efficiency of translation and/or mRNA export from the nucleus [33].

The mechanism of transcription is remarkably conserved throughout the eukaryotic kingdom; all of the basic components for eukaryotic gene transcription have been highly conserved in evolution [34,35]. It is not surprising that the CTX promoter shows high promoter activity in the CHO cell line, consistent with the in vitro CTX expression level [27]. Both venom- and liver-specific TIS could also be detected in the RNA isolated from a CHO-CTX stable cell.
clone. Hence, the CTX-2 gene promoter can function effectively in CHO cells.

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