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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 A_2. 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.

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Abbreviations: CTX, cardiotoxin; NTX, neurotoxin; CHO, Chinese hamster ovary cells; GR, glucocorticoid receptor; α-MEM, minimal essential medium (alpha modification); CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT/enhancer binding protein.

Note: the sequences described in this paper have been submitted to GenBank under the accession numbers AF241224, AF276222 and AF276223.

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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 $U \cdot mL^{-1}$ penicillin and 50 $\mu g \cdot mL^{-1}$ 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

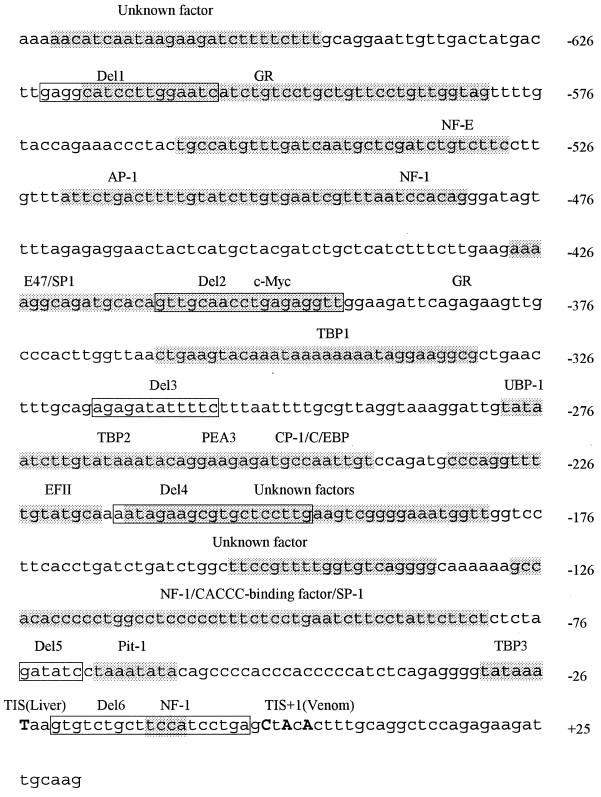

Fig. 1. Nucleotide sequence of the snake *CTX-2* promoter and transcriptional factor binding sites. A 706-bp of CTX-2 upstream of the first ATG codon is shown. The 5′ ends of the deletions (Del 1–5) of the *CTX-2* promoter are indicated as open boxes. The transcription factor binding sites identified by *in vitro* DNase I footprinting analysis are shown with a shaded background. The major transcription initiation sites (bold, capital letters) in snake venom gland and liver are also shown.

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 chloramphenicol acetyltransferase (CAT) assay. Stable transfectants were established using complete α-MEM containing 400 $\mu g \cdot mL^{-1}$ 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-^{32}P]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 (AP1) 5′-GTAATACGACTCACTATAGGGC-3′ and an antisense gene-specific primer (SAND-1SPR) 5′-CCTAAGTCCAGGCACACAATT-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′-TAAGTGTCTGCTTCCATCCTGAG-3′ and AP1. 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 AP1 and a gene-specific antisense primer (GSP1R), 5′-ATTGCCACCCGAGATCAAAGTTTGGT-3′. For nested PCR, adaptor primer 2 (AP2), 5′-ACTATAGGGCACGCGTGGT-3′, and a gene-specific antisense primer (GSP2R), 5′-GGTCTTACCTAAGTCCAGGCACACAA-3′ were used. The PCR was initiated with a hot start step 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 *Eco*RI and labelled by using Klenow DNA polymerase and [α-^{32}P]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 $MgCl_2$, 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 Ca^{2+}/Mg^{2+} buffer (10 mM $MgCl_2$, 5 mM $CaCl_2$) 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 proteinase K). The reaction products were extracted with phenol/chloroform and precipitated with ethanol and 1 μL carrier yeast tRNA (6 $\mu g \cdot \mu L^{-1}$). 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.

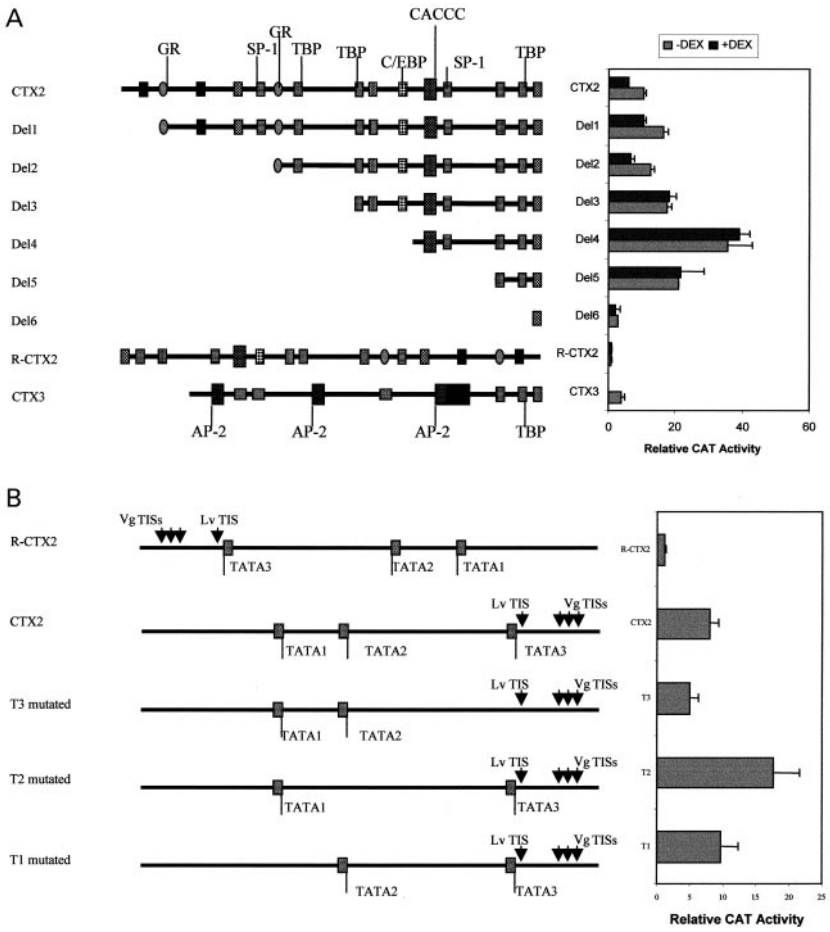

Fig. 2. Promoter activities determined by CAT assays. (A) The whole promoters of CTX-2, CTX-3 and sequentially deleted constructs. The availability of GR receptor was optimized by using mouse GR cDNAs. Dex (1 μM) was used to study the effects of GR elements on gene activity.(B) Effects of site-specific mutations of TATA elements on *CTX-2* promoter activity. In both cases, β-galactosidase activity was used for normalization of transfection efficiency. The bars represent the relative CAT activities ± SD from six individual experiments. *CTX-2* promoter in the reverse orientation was used as a negative control.

Primer extension analysis

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 +1 to +22 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′-ATCTTGCAA-TCTTCTCTGGA-3′) complementary to nucleotides +14 to +34 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 *Dra*I 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.dcrt.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 comparatively similar levels of expression of *CTX* genes and hence can be used for the investigation of *CTX* gene promoter activity.

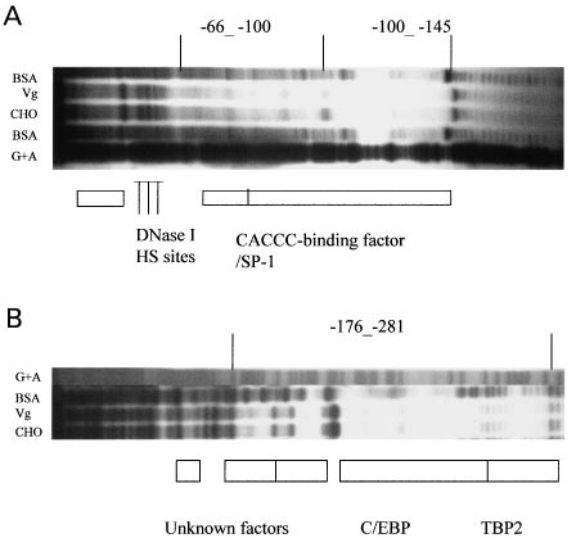

Fig. 3. DNase-I footprinting analysis. Nuclear proteins (40 μg each) extracted from CHO and venom gland were used. BSA (40 μg) was used as a negative control. Vg represents venom glands. A G + A sequencing ladder is shown in both cases. (A) DNase-I hypersensitive sites, Sp1 and CAACC binding sites. (B) Binding sites for C/EBP, TBP and unknown factors.

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 TFIID 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.

Localization 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 TFIID 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 TFIID 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.

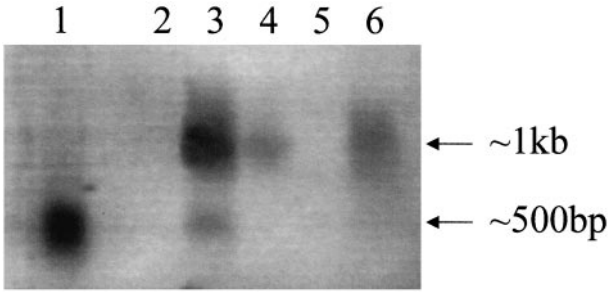

Fig. 4. Northern blot analysis. *CTX-2* cDNA was used as the probe for Northern hybridization. Lane 1, 1.5 μg of total RNA from snake venom gland; lanes 2–6, 25 μg of total RNA from snake brain, liver, muscle, lung, and heart, respectively. The autoradiograph was prepared after 18 h exposure.

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

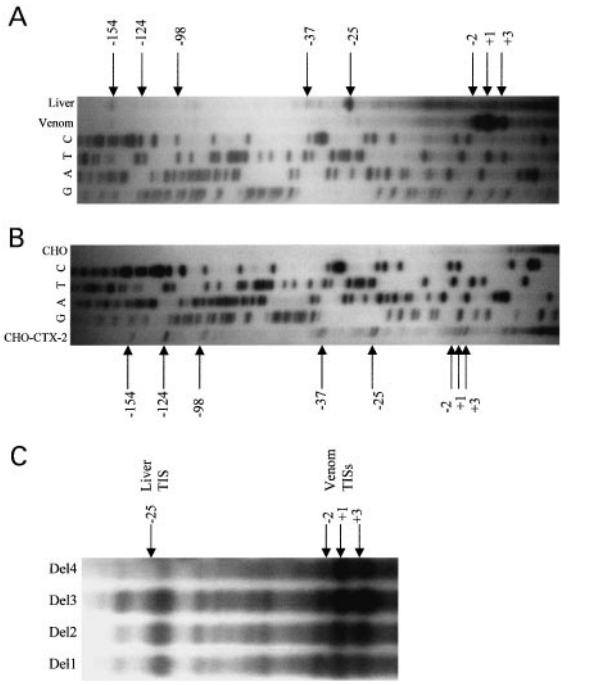

Fig. 5. Localization of transcription initiation sites. (A) Primer extension analysis using snake venom gland and liver RNAs. GATC, Nucleotide sequences of the promoter. (B) Primer extension analysis on RNAs isolated from CHO cells transfected with *CTX-2* promoter. CHO-CTX-2, total RNA from CHO stable transfectant; CHO, CHO cells; GATC, nucleotide sequences of the promoter. (C) Location of the transcription initiation sites of deletion constructs (Del 1–4).

Liver specific spliced cardiotoxin-2 transcript

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  1     TAAGTGTCTGCTTCCATCCTGAGCTACACTTTGCAGGCTCCAGAGAAGATTGCAAG

 57 ATGAAAACTCTGCTGCTGACCTTGGTGGTGGTGACAATTGTGTGCCTGGACTTAGGATAC
  1  M  K  T  L  L  L  T  L  V  V  V  T  I  V  C  L  D  L  G  Y

117 ACCTTAAAATGTAACAAACTCGTTCCTTTATTCTATAAGACTTGTCCAGCAGGGAAGAAC
 21  T  L  K  C  N  K  L  V  P  L  F  Y  K  T  C  P  A  G  K  N

177 TTATGCTATAAAATGTACATGGTGGCGACGCCAAAGGTTCCTGTCAAAAGGGGATGTATT
 41  L  C  Y  K  M  Y  M  V  A  T  P  K  V  P  V  K  R  G  C  I

237 GATGTTTGCCCTAAAAGCAGTCTCCTAGTGAAGTATGTGTGTTGCAATACAGACAGATGC
 61  D  V  C  P  K  S  S  L  L  V  K  Y  V  C  C  N  T  D  R  C

297 AACTGACAGCTCTACGAGTTCCTAATTTCCTTGAGTTTGCTCTCATCCATCATGGACATC
 81  N  *

357 CTTGAAAATTTATGCTTGTGACCTTTACCACCATGGTCCATCATCCCCCTCT
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Liver specific unspliced cardiotoxin-2 transcript (partial sequence)

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  1     TAAGTGTCTGCTTCCATCCTGAGCTACACTTTGCAGGCTCCAGAGAAGATTGCAAG

 57 ATGAAAACTCTGCTGCTGACCTTGGTGGTGGTGACAATTGTGTGCCTGGACTTAGGTAAG
  1  M  K  T  L  L  L  T  L  V  V  V  T  I  V  C  L  D  L  G  K

117 ACCACCAAACTTTGATCTTGGGTGGCAATCAAACCCAAAACAAAGGGGCAGAAGGAAAGG
 21  T  T  K  L  *

177 AGGCTATTTGAGGCAGTTTGGTCCCTTTTTTTGGTGGGACTTTGTCACTGCTCTGGTCTG

237 CATAAAGAGGGTCCATGGGAGCCTTTTGGGGAGAAAAAAGGAGGTCTCCATTTG
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Fig. 6. The cDNA nucleotide sequences of the liver-specific CTX transcripts. The asterisks indicate stop codons. The corresponding cDNA sequences have been deposited in GeneBank [accession numbers: AF276222(spliced) and AF276223(unspliced)].

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 TFIID 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 *Hin*dIII 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 dex 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 dex. A TFIID 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 TFIID 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 neurotoxin-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 bear 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 promotional 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 vivo 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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REFERENCES

1. Harvey, A.L. (1991). *Snake Toxins.* Pergamon Press, New York.
2. Tipton, K.F. & Dajas, F. (1994) *Neurotoxins in Neurobiology: Their Actions and Applications.* Ellis Horwood, New York.
3. Kumar, T.K., Jayaraman, G., Lee, C.S., Arunkumar, A.I., Sivaraman, T., Samuel, D. & Yu, C. (1997) Snake venom cardiotoxins − structure, dynamics, function and folding. *J. Biomol. Struct. Dyn.* **15**, 431–463.
4. Jeyaseelan, K., Armugam, A., Lachumanan, R., Tan, C.H. & Tan, N.H. (1998) Six isoforms of cardiotoxin in malayan spitting cobra (*Naja naja sputatrix*) venom: cloning and characterization of cDNAs. *Biochim. Biophys. Acta* **1380**, 209–222.
5. Chang, L.S., Lin, J. & Wu, P.F. (1997) cDNA sequence analysis of cardiotoxin variants from Taiwan cobra. *Biochem. Mol. Biol. Int.* **42**, 85–92.
6. Lachumanan, R., Armugam, A., Tan, C.H. & Jeyaseelan, K. (1998) Structure and organization of the cardiotoxin genes in *Naja naja sputatrix*. *FEBS Lett.* **433**, 119–124.
7. Chang, L.S., Lin, J., Chou, Y.C. & Hong, E. (1997) Genomic structures of cardiotoxin 4 and cobrotoxin from *Naja naja atra* (Taiwan cobra). *Biochem. Biophys. Res. Commun.* **239**, 756–762.
8. Afifiyan, F., Armugam, A., Tan, C.H., Gopalakrishnakone, P. & Jeyaseelan, K. (1999) Postsynaptic alpha-neurotoxin gene of the spitting cobra, *Naja naja sputatrix*: Structure, organization, and phylogenetic analysis. *Genome Res.* **9**, 259–266.
9. McLane, K.E., Weaver, W.R., Lei, S., Chiappinelli, V.A. & Conti-Tronconi, B.M. (1993) Homologous kappa-neurotoxins exhibit residue-specific interactions with the alpha 3 subunit of the nicotinic acetylcholine receptor: a comparison of the structural requirements for kappa-bungarotoxin and kappa-flavitoxin binding. *Biochemistry* **32**, 6988–6994.
10. Gendeh, G.S., Chung, M.C. & Jeyaseelan, K. (1997) Genomic structure of a potassium channel toxin from *Heteractis magnifica*. *FEBS Lett.* **418**, 183–188.
11. Haag, F.A., Kuhlenbaumer, G., Koch-Nolte, F., Wingender, E. & Thiele, H.G. (1996) Structure of the gene encoding the rat T cell ecto-ADP-ribosyltransferase RT6. *J. Immunol.* **157**, 2022–2030.
12. Liu, D. & Fischer, I. (1996) Two alternative promoters direct neuron-specific expression of the rat microtubule-associated protein 1B gene. *J. Neurosci.* **16**, 5026–5036.
13. Stanners, C.P., Eliceiri, G.L. & Green, H. (1971) Two types of ribosome in mouse-hamster hybrid cells. *Nat. New Biol.* **230**, 52–54.
14. Couette, B., Le Ricousse, S., Fortin, D., Rafestin-Oblin, M.E. & Richard-Foy, H. (1994) The establishment of the long terminal repeat of the mouse mammary tumor virus into CV-1 cells allows a functional analysis of steroid receptors. *Biochim. Biophys. Acta* **1219**, 607–612.
15. Jordan, M., Schallhorn, A. & Wurm, F.M. (1996) Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res.* **24**, 596–601.
16. Seed, B. & Sheen, J.Y. (1988) A simple phase-extraction assay for chloramphenicol acyltransferase activity. *Gene.* **67**, 271–277.
17. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.
18. Lee, K.A., Bindereif, A. & Green, M.R. (1988) A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. *Gene Analysis Techniques* **5**, 22–31.
19. Galas, D.J. & Schmitz, A. (1978) DNAse footprinting: a simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res.* **5**, 3157–3170.
20. Ito, K., Barnes, P.J. & Adcock, I.M. (2000) Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12. *Mol. Cell. Biol.* **20**, 6891–6903.
21. Jaskoll, T., Choy, H.A. & Melnick, M. (1994) Glucocorticoids, TGF-beta, and embryonic mouse salivary gland morphogenesis. *J. Craniofac. Genet. Dev. Biol.* **14**, 217–230.
22. Shaw, P.A. & Chaparro, O. (1999) The 5′ flanking sequence and regulatory elements of the cystatin S gene. *Biochem. Biophys. Res. Commun.* **261**, 705–711.
23. Kent, G.C. (1987) *Comparative Anatomy of the Vertebrates.* 6th edn. Times Mirror/Mosby College Publications, St. Louis.
24. Drouin, J., Trifiro, M.A., Plante, R.K., Nemer, M., Eriksson, P. & Wrange, O. (1989) Glucocorticoid receptor binding to a specific DNA sequence is required for hormone-dependent repression of pro-opiomelanocortin gene transcription. *Mol. Cell. Biol.* **9**, 5305–5314.
25. Lekstrom-Himes, J. & Xanthopoulos, K.G. (1998) Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J. Biol. Chem.* **273**, 28545–28548.
26. Philipsen, S. & Suske, G. (1999) A tale of three fingers: The family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res.* **27**, 2991–3000.
27. Lachumanan, R., Armugam, A., Durairaj, P., Gopalakrishnakone, P., Tan, C.H. & Jeyaseelan, K. (1999) In situ hybridization and immunohistochemical analysis of the expression of cardiotoxin and neurotoxin genes in *Naja naja sputatrix*. *J. Histochem. Cytochem.* **47**, 551–560.
28. Ohno, M., Menez, R., Ogawa, T., Danse, J.M., Shimohigashi, Y., Fromen, C., Ducancel, F., Zinn-Justin, S., Le Du, M.H., Boulain, J.C., Tamiya, T. & Menez, A. (1998) Molecular evolution of snake toxins: Is the functional diversity of snake toxins associated with a mechanism of accelerated evolution? *Prog. Nucleic Acid Res. Mol. Biol.* **59**, 307–364.
29. Kloog, Y., Ambar, I., Sokolovsky, M., Kochva, E., Wollberg, Z. & Bdolah, A. (1988) Sarafotoxin, a novel vasoconstrictor peptide: phosphoinositide hydrolysis in rat heart and brain. *Science* **242**, 268–270.
30. Miwa, J.M., Ibanez-Tallon, I., Crabtree, G.W., Sanchez, R., Sali, A., Role, L.W. & Heintz, N. (1999) Lynx1, an endogenous toxin-like modulator of nicotinic acetylcholine receptors in the mammalian CNS. *Neuron* **23**, 105–114.
31. Andermann, K., Wattler, F., Wattler, S., Heine, G., Meyer, M., Forssmann, W.G. & Nehls, M. (1999) Structural and phylogenetic characterization of human SLURP-1, the first secreted mammalian member of the Ly-6/uPAR protein superfamily. *Protein Sci.* **8**, 810–819.
32. Ayoubi, T.A. & Van De Ven, W.J. (1996) Regulation of gene expression by alternative promoters. *FASEB J.* **10**, 453–460.
33. Chiang, P.K., Gordon, R.K., Tal, J., Zeng, G.C., Doctor, B.P., Pardhasaradhi, K. & McCann, P.P. (1996) S-Adenosylmethionine and methylation. *FASEB J.* **10**, 471–480.
34. Hoey, T., Dynlacht, B.D., Peterson, M.G., Pugh, B.F. & Tjian, R. (1990) Isolation and characterization of the *Drosophila* gene encoding the TATA box binding protein, TFIID. *Cell* **61**, 1179–1186.
35. Hoffman, A., Sinn, E., Yamamoto, T., Wang, J., Roy, A., Horikoshi, M. & Roeder, R.G. (1990) Highly conserved core domain and unique N terminus with presumptive regulatory motifs in a human TATA factor (TFIID). *Nature* **346**, 387–390.