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Characterization of the transcript for a depressant insect selective neurotoxin gene with an isolated cDNA clone from the scorpion *Buthotus judaicus*

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The poly(A)$^+$ mRNA isolated from the venomous terminal segments of the scorpion *Buthotus judaicus* was reversed transcribed into cDNA. PCR amplification of the cDNA in presence of oligonucleotide primers prepared on basis of the known amino acid sequence of the depressant insect toxin II yielded a 125 bp long product. This fragment was cloned and its sequence determined. The deduced amino acid sequence has revealed a complete homology with the amino acid sequence of the toxin. This clone was used to probe a Northern blot resolving the poly(A)$^+$ and poly(A)$^-$ fractions derived from the scorpion. An organ specific 360 nucleotide transcript which might be the processed product of a ~4.0 kb precursor was elucidated. This cDNA clone may pave the way for a molecular genetic approach to study the structure-function relationship of scorpion selective insect toxins.

Scorpion neurotoxin; cDNA; Polymerase chain reaction; Organ-specific transcription

1. INTRODUCTION

The depressant insect toxins which are small polypeptides (7 kDa) derived from Buthinae scorpion venoms were shown to paralyze exclusively insects through a modification and blockage of their neuronal sodium conductance [1,2] and to possess a characteristic primary structure [3]. The pharmacological uniqueness of the depressant toxins strongly suggest the elucidation of their structure-function relationship to enable the construction of specific models for the future design of selective insecticides. Genetic modification of the related genes may provide information in regard to specific amino acid residues involved in the unique selectivity of the depressant toxins.

As the first step in a genetic approach to this study, we isolated a partial cDNA clone encoding the depressant insect toxin of the scorpion *Buthotus judaicus* (BjIT2). This clone was used to demonstrate the organ specific transcription of the BjIT2 gene.

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Abbreviations: BjIT2, *Buthotus judaicus* insect toxin II; bp, base pairs; kb, kilobase pairs; kDa, kilodalton; PCR, polymerase chain reaction; DEPC, diethylpyrocarbonate; *dit*, 'depressant insect toxin' gene

2. MATERIALS AND METHODS

2.1. *Biological material*

Black scorpions, *Buthotus judaicus (Buthidae)*, were collected in the Judaen and lower Galilee mountains. 52 h prior to the RNA extraction the scorpions were 'electrically milked' as previously described [4]. Propagation of plasmid DNA was carried out in DH5α *E. coli* cells [5]. *E. coli* JM107 was used to produce single stranded DNA [6] for sequence determination. The rat β-actin gene in plasmid pAC18.1 was kindly provided by Dr. R. Stein at Tel-Aviv University.

2.2. *RNA purification*

52 h after 'milking' the scorpions, the various abdominal segments were cut out into an RNA extraction solution containing half volume of 4 M guanidium thiocyanate, 0.5% Na-lauryl-sarcosinate and 1% β-mercapto ethanol, and half volume of TE-saturated phenol, chloroform and isoamylalcohol (25:24:1 respectively, pH 7.4). This mixture was immediately frozen in dry ice-ethanol and homogenized with a Polytron apparatus (Kinematica, PT 10-35, Switzerland) in 5 bursts 30 s each interrupted by 30 s intervals in dry ice-ethanol. Guanidinium thiocyanate and guanidinium hydrochloride were utilized in the extraction as described [7]. Poly(A)$^+$ RNA was purified by using oligo(dT)-cellulose columns (Pharmacia) and the remaining RNA was considered as the poly (A)$^-$ fraction. All procedures were performed under RNase-free conditions.

2.3. *DNA synthesis*

cDNA was synthesized by using the poly(A)$^+$ RNA with a poly(dT) primer supplied in a 'cDNA synthesis plus' kit from Amersham, and according to the instructions of the manufacturer. The oligonucleotide mixtures were synthesized at the Biochemistry Service Unit of Michigan State University. The polymerase chain reaction was carried out in a Perkin Elmer 'Cetus' thermocycler programmed for 30 cycles as described [8]. 0.5 μl of Taq polymerase were used to

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initiate the reaction containing: 50 ng of cDNA and 5 μg of each of the primers in the presence of 1 × TBE buffer and dNTPs (100 μl final volume). Each cycle was composed of 60 s at 95°C, 60 s at 45°C and 90 s at 72°C. Oil was removed with chloroform.

2.4. *Northern blot analysis*

Dried 10 μg of poly(A)$^-$ RNA and 0.25 μg of poly(A)$^+$ RNA were dissolved in 5 μl of sample buffer composed of: 50% formamide, 2.2 M formaldehyde in 1× MEN buffer (20 mM MOPS, 5 mM Na-acetate and 1 mM EDTA pH 7.0). The samples were heated for 6 min at 68°C and immediately cooled on ice for 5 min. 1.3 μl of 5 × loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol) were added prior to loading onto a 1.5% agarose gel prepared with 2.2 M formaldehyde in 1× MEN buffer. Electrophoresis was carried out for 2.5 h at 200 V in 1× MEN buffer. Blotting to a nylon BioTrace™ RP membrane (Gelman Sci. Inc.) was as described [5]. Prehybridization was at 42°C for 2 h with 43% formamide, 43 mM Tris pH 7.5, 8.5% Dextran sulfate, 2.1× Denhardt's, 0.43% SDS, 100 μg/ml sheared salmon sperm ssDNA and 5.1× SSC. Hybridization was performed by adding the ^{32}P-radiolabeled probe and continued for 14 h at 42°C. Prior to exposure, the membranes were washed 3 times (20 min each) at 42°C with 2× SSC plus 0.1% SDS.

3. RESULTS

3.1. *Cloning of BjIT2 cDNA*

The cDNA synthesis was performed in the presence of ^{32}P-dCTP to enable 'follow up' of the product. Electrophoresis of 25 ng of the cDNA on 1.4% agarose gel followed by its drying and exposure to X-ray film visualized its electrophoretic profile which was scanned by densitometry. As illustrated in Fig. 1 the majority of the molecules migrated at the 375 bp-long region but a few larger size bands were obtained as well. Fig. 2A illustrates the sequence of the two peptidic regions used for the synthesis of the corresponding oligonucleotide primers. The PCR amplification yielded a 125 bp product (Fig. 2B), which was blunt-end ligated into the *Sma*I site at the polylinker of Bluescript phagemid. Recombinant ampicillin resistant colonies were selected and analysed for content. One of the recombinants designated as pBjITb61, was analysed by sequencing of the single stranded insert. The resulting deduced amino acid sequence revealed a complete homology to the sequence determined by chemical procedures [3]. This cDNA clone of the BjIT2 gene (designated as *dit*2 for 'depressant insect toxin 2'), was further used as a homologous probe to examine the BjIT2 transcripts produced by the scorpion.

3.2. *Northern blot analysis of RNA*

The RNA containing blots were probed with radiolabeled inserts purified from plasmids, pBjITb61 for BjIT2 sequence, and pAC18.1 for the β-actin sequence [9]. As illustrated in Fig. 3A, a clear signal, averaging at ~360 nucleotides long, lit up in lane 1 [poly(A)$^+$ RNA kept for nine months under ethanol at −70°C] and in lane 3 [fresh preparation of poly(A)$^+$

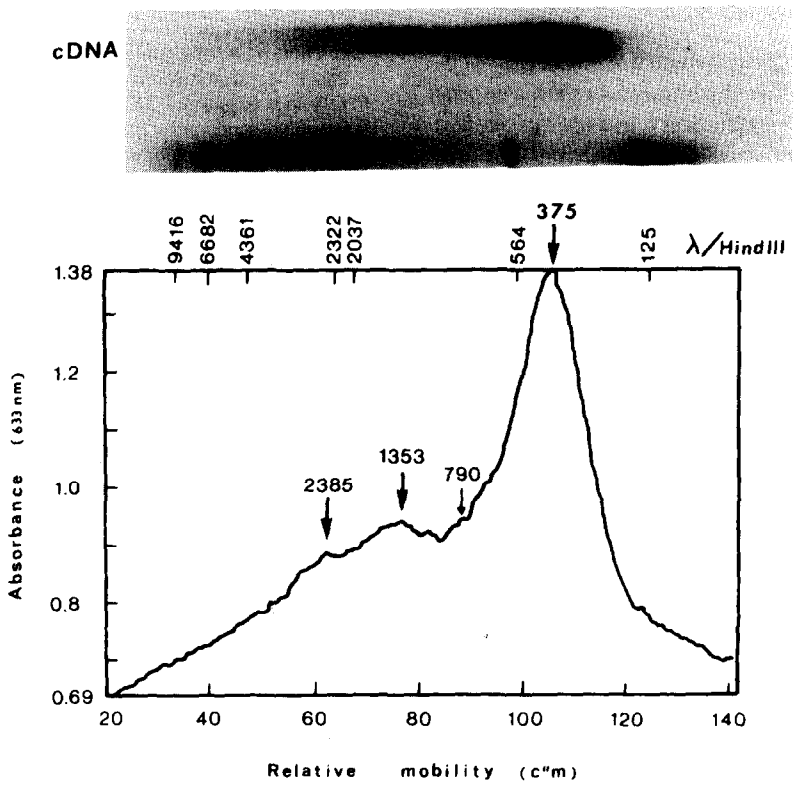

Fig. 1. Electrophoretic profile of the ^{32}P-cDNA derived from the venom-producing segment (telson) of the scorpion, *Buthotus judaicus*. Densitometry of the resulting X-ray film was carried out in a 2222-020 Laser densitometer of LKB. The size markers were the end labeled fragments of lambda digested with *Hind*III. The estimated size of the major bands is indicated.

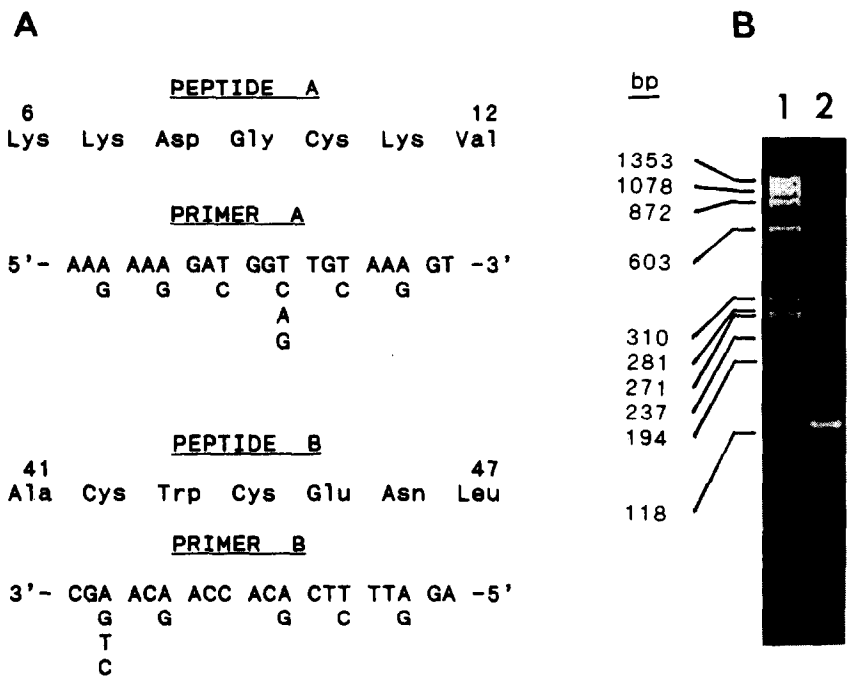

Fig. 2. Polymerase chain reaction (PCR). (A) Two 20-mer DNA mixtures (degeneracy = 128) were designed as opposing primers for the PCR. (B) Lane 1, *Hae*III digest of phage X174RF for sizing; lane 2, 10 μl aliquot from the reaction run on 3% Nusieve-1% agarose, in 1× TBE buffer at 110 V for 60 min. The gel was stained with ethidium bromide and photographed.

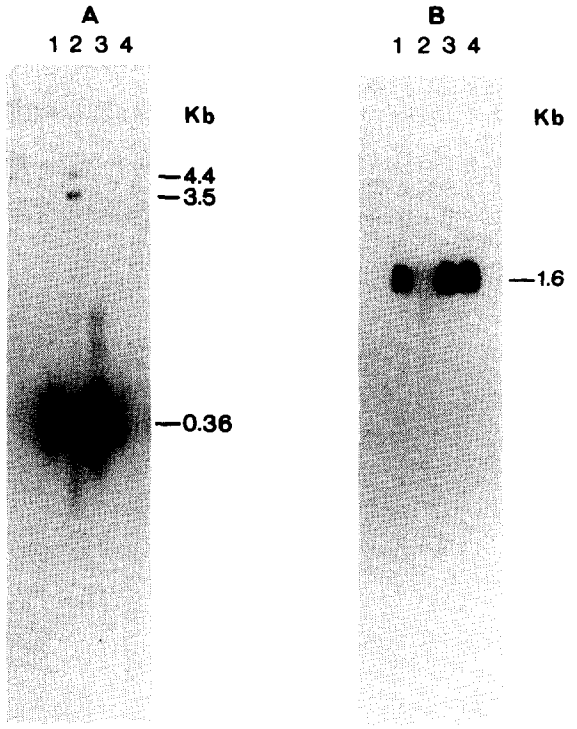

Fig. 3. Northern blot analysis of mRNA derived from various abdominal segments of *Buthotus judaicus*. (A) Blot probed with the radiolabeled insert from plasmid pBjITb61. (B) Blot probed with the ratiolabeled rat β-actin gene. 1-poly(A)$^+$ RNA from telsons after nine months incubation at −70°C; 2-poly(A)$^-$ and 3-poly(A)$^+$ RNAs, were freshly prepared from telsons; 4-poly(A)$^+$ RNA from abdominal segments which do not produce venom.

RNA] resolving RNA derived from the venom-containing segment (telson) of *B. judaicus*. In lane 4 which contained the poly(A)$^+$ RNA from the rest of the abdonimal segments of the scorpion, no such signal was obtained. The probe was then removed by 4 washes in boiling water, and the blot was probed again with the rat β-actin radiolabeled fragment. Lanes 1, 2 and 4 in Fig. 3B have revealed a signal of ~1.6 kb long for the scorpion β-actin transcript. Lane 2, which contained the poly(A)$^-$ RNA, did not show any transcript in the ~360-nucleotide region when the first probe was applied, or at the 1.6 kb region when using the second probe. However, in lane 2, two bands appeared at the 4.4 and 3.5 kb region when probed with the *dit*2 gene. No high molecular size bands were detected in the blot probed with the β-actin gene.

4. DISCUSSION

As illustrated in Fig. 1, the average size of the major cDNA product, was rather small (~375 bp). This could perhaps be attributed to the fact that the poly(A)$^+$ RNA used for its synthesis was derived from the telson in which a majority of the transcripts might be related to the short toxic products. This assumption was supported by the fact that the size of the transcript for BjIT2 was approximately 360 nucleotides long (Fig. 3A lanes 1,3). Recently, it was demonstrated in the scorpion *Androctonus australis* [10], that the majority of the telson-derived cDNA was similar in size to the

cDNA obtained by us from *B. judaicus*. However, this 360 nucleotides transcript could also be a processed molecule originating from a larger precursor like that appearing in the poly(A)$^-$ containing lane (Fig. 3A, lane 2). In fact, two bands were detected in the poly(A)$^-$ lane. Since both hybridized with the same probe, it was possible that these were the transcripts of different genes possessing homology to the *dit* probe. Other possibilities could be: The two transcripts were produced by one gene; Two genes were involved in coding for BjIT2; One of the bands was a processed product derived from the other. Actually, two closely related bands seemed to hybridize in the lane containing poly(A)$^+$ RNA. This result was repeatedly demonstrated in a few separate experiments and most interestingly, obtained as well with the β-actin poly(A)$^+$ mRNA. This observation might be related to the presence of more than one transcript for both types of genes in the telson of *B. judaicus* (see lane 2 in Fig. 3A).

The organ specific transcription of the *dit2* gene was examined by utilizing two different DNA probes. The isolated cDNA of the BjIT2 gene (in plasmid pBjITb61) and the rat β-actin gene (in plasmid pAC18.1) were applied to a Northern blot containing purified RNAs from various segments of *B. judaicus*. From comparison between lanes 1, 3 and 4 in Fig. 3A, and lanes 1, 3 and 4 in Fig. 3, panel B, it was clear that while the transcript for the β-actin gene has been synthesized in the telson as well as in the other abdominal segments of *B. judaicus*, the transcript of the *dit2* gene has been synthesized exclusively in the telson.

These results demonstrate our ability to study the transcriptional control of the *dit2* gene by utilizing the isolated cDNA clone. This partial cDNA can be used for the isolation of the genomic *dit* sequence in order to elucidate the mechanism controlling its expression. Concomitantly, the isolation of a full length BjIT2 cDNA clone and corresponding homologous sequences from other scorpions, may be facilitated. The various clones will be employed in a comparative genetic study focused on the structure-function relationship of scorpion derived neurotoxic polypeptides.

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