Correspondence

Evidence that BmTXKβ–BmKCT cDNA from Chinese scorpion *Buthus martensii* Karsch is an artifact generated in the reverse transcription process

Xian-Chun Zeng*, San-Xia Wang

First published online 15 May 2002

cDNA is a man-made copy of mRNA. There are risks of producing artifactual copies of natural mRNAs in the process of in vitro cDNA synthesis. For example, cDNA synthesis can be primed either from a poly(A) tail of mRNA or from an A-rich internal region. Some artifacts could arise from a GC-stem skipping mechanism in the reverse transcription reaction. Reverse transcription is also known to be an error-prone process and thus might generate mutations in the cDNA. Moreover, it has been shown that the two forms of commercially available transcriptase, AMV and MMLV reverse transcriptase, can switch from one template to another in a homology-dependent manner and result in a recombinant DNA artifact [1]. Therefore, no cDNA can be completely free from suspicion of cloning errors.

BmTXKβ is a putative K^+-channel specific toxin [2], and BmKCT is a Cl^--channel toxin [3], both of which were identified from the Chinese scorpion *Buthus martensii* Karsch. Recently, a novel cDNA clone (BmTXKβ–BmKCT), which is a hybrid of the 5′-terminal region of BmTXKβ cDNA and the 3′-terminal part of BmKCT cDNA, has been isolated from *B. martensii* Karsch by Zhu et al. [4], and our group, independently. The junction site is a common region of 11 bp shared by both BmTXKβ and BmKCT cDNAs. Three hypotheses might explain the generation of the hybrid cDNA: a site-specific recombination at the genomic level, a trans-splicing mechanism of two different transcripts [5] or a homology-dependent template switching during in vitro cDNA synthesis [1]. Zhu et al. proposed that the hybrid cDNA was produced from BmTXKβ and BmKCT transcripts by a naturally occurring trans-splicing mechanism, and inferred that it might be implicated in the regulation of scorpion toxin gene expression [4]. However, our investigation argued against this hypothesis. Based on our experiment, we proposed that BmTXKβ–BmKCT cDNA is an artifact produced by the homology-dependent template switching mechanism in the process of reverse transcription. The mechanism involves three steps: (1) BmKCT first-strand cDNA synthesis is primed by a base-pairing hybrid of BmKCT mRNA and oligo(dT) primer; (2) when the reverse transcriptase reaches the 5′-end of the '11-bp common region' in the BmKCT mRNA, the nascent cDNA tail anneals to the '11-bp common region' of BmTXKβ mRNA (template switching); (3) the 5′-terminal part of BmTXKβ mRNA serves as a new template for reverse transcription.

We obtained BmKCT, BmTXKβ and BmTXKβ–BmKCT (referred to as BmKβ-CT by our group; GenBank accession number AY055475) cDNAs by oligonucleotide probe hybridization, selective sequencing or subtractive screening strategy from a venom gland cDNA library of *Buthus martensii* Karsch. Genomic amplification excluded the possibility that BmTXKβ–BmKCT was generated from a site-specific recombination event between BmTXKβ and BmKCT genes. In order to determine whether the hybrid cDNA resulted from template switching during in vitro cDNA synthesis or is a naturally occurring transcript resulting from a trans-splicing event, the following experiments were performed.

The mMESSAGE mMACHINE™ kits (Ambion) were used to synthesize in vitro both BmKCT and BmTXKβ sense RNA according to the Kit user's instruction. The templates for transcription, which contained SP6 or T7 RNA Polymerase promoter upstream of BmKCT or BmTXKβ cDNA, were prepared by PCR amplification using a proofreading polymerase (Pfu, Stratagene). The primers for BmKCT are 5′-ATTTAGGTGACACTATAGAAGTGCAAAACTCTATTAAAAATG-3′ (sense), and 5′-$T_{(20)}$AATGCTTATTAATATTTATTATTC-3′ (antisense), and BmTXKβ primers used are 5′-TAATACGACTCACTATAGGGAGAATTTTACATAGCCCCGAA (sense), and 5′-$T_{(20)}$AGCTATAAATGTCTTTTAATATA-3′ (antisense). The PCR reaction was carried out as follows: denature the template for 2 min at 94°C, perform 35 cycles of denaturation at 94°C for 15 s, and extension at 68°C for 30 s. After the transcription reaction, DNase I was added to remove template DNA. Ambion's Spin Column was used to remove unincorporated nucleotides and small premature transcripts. The quantity of RNA samples was monitored by ethidium bromide spot assay. About 0.1 μg of BmKCT and BmTXKβ RNA mixture (each 0.05 μg) was converted into double-strand cDNA and generated a cDNA 'library' using SuperScript™ cDNA library construction kit (Gibco BRL) as described elsewhere [6]. The recombinant plasmid pSPORT1 was introduced into competent *Escherichia coli* DH5α by transformation. From 0.1 μg cDNA, approximately 5.0×10^4 *E. coli* DH5α transformants were produced. A PCR strategy was used to screen BmTXKβ–BmKCT cDNA. The primers used are 5′-GGTAAATGATGAAACAACAGTTC-3′ (sense) corresponding to the 5′-terminal partial sequence of BmTXKβ cDNA, and 5′-TCATATACGGTTACACAGAC-3′(antisense) corresponding to the 3′-terminal sequence of BmKCT encoding region. The screening protocol was as follows: 1000 transformants from the cDNA 'library' were plated on LB/Ampicillin plates and cultured overnight. Each bacterial colony was inoculated into individual Eppendorf tubes containing LB/Ampicillin medium and incubated overnight. As a first screening, 10 PCR reactions were run by dividing 1000 clones into 10 groups (100 clones/group). Each positive group, chosen from the first screening, was divided into 10 groups (10 clones/group), and 10 PCR reactions for a second screening were performed. The positive group was then divided into 10 groups (1 clone/group), and 10 PCR reactions for a third screening were performed. Positive clones containing an insert longer than 200 bp were chosen for sequencing. The result indicated that from 5000 clones we identified 5 positive clones which were consistent with

0014-5793/02/$22.00
PII: S0014-5793(02)02812-0

BmTXKβ–BmKCT cDNA except for the 5′-terminal 1–6 nucleotides that came from the 3′-terminus of T7 promoter incorporated during transcription (T7 promoter: TAATACGACTCACTATA**G**GGAGA, the base in bold being the first base incorporated into RNA during transcription). The existence of the incorporated nucleotides excluded the possibility that these positive clones resulted from contamination.

This investigation strongly suggested that BmTXKβ–BmKCT cDNA is an artifact generated in the cDNA synthesis process by a homology-dependent template switching. The low frequency of recombination is due to the low reaction temperature we used, and the short length of the homologous region.

Reverse transcriptase template switching has been shown to be responsible for many homologous recombination events of retroviruses in vivo, and has been reported in vitro for the AMV and MMLV reverse transcriptase. During in vitro cDNA synthesis, they can switch from one template to another in a homology-dependent and temperature-dependent manner, and result in artificial cDNA recombination.

Acknowledgements: This work was partially supported by the National Natural Science Foundation of China (no. 39970897), by the National Medicine Creation Doctor-Foundation of China (no. 96-901-033), as well as by the Zhiqiang Foundation of Wuhan University to Dr. Xian-Chun Zeng.

References

[1] Ouhammouch, M. and Brody, E.N. (1992) Nucleic Acids Res. 20, 5443–5450.
[2] Zhu, S.Y., Li, W.X., Zeng, X.C., Jiang, D.H., Mao, X. and Liu, H. (1999) FEBS Lett. 457, 509–514.
[3] Zeng, X.C., Li, W.X., Zhu, S.Y., Peng, F., Zhu, Z.H., Wu, K.L. and Yang, F.H. (2000) Toxicon 38, 1009–1014.
[4] Zhu, S., Li, W. and Cao, Z. (2001) FEBS Lett. 508, 241–244.
[5] Finta, C. and Zaphiropoulos, P.G. (2002) J. Biol. Chem. 277, 5882–5890.
[6] Zeng, X.C., Peng, F., Luo, F., Zhu, S., Liu, H. and Li, W.X. (2001) Biochimie 83, 883–889.

*Corresponding author. Present address: Laboratory of Cell Biology, National Heart, Lung and Blood Institute, National Institutes of Health, Building 50, Room 2529, 9000 Rockville Pike, Bethesda, MD 20892, USA. Fax: (1)-301-402 15 19.
E-mail address: zengx@nhlbi.nih.gov (X.-C. Zeng).

Department of Biotechnology, Institute of Virology, College of Life Sciences, Wuhan University, Wuhan 430072, PR China