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REAL-TIME QUANTITATIVE RT-PCR METHOD FOR ESTIMATION OF mRNA LEVEL OF CCAAT/ENHANCER BINDING PROTEIN IN THE CENTRAL NERVOUS SYSTEM OF *LYMNAEA STAGNALIS**

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The fluorescence-based real-time reverse transcription polymerase chain reaction (RT-PCR) is becoming widely used to quantify mRNA level in cells and tissues and is now a crucial tool for basic biological researches and biotechnology. In the present study, on the basis of the real-time quantitative RT-RCR, we detected and quantified mRNA copies of the transcription factor, CCAAT/enhancer binding protein (C/EBP; an immediate-early gene that is involved in synaptic plasticity and learning and memory) in the central nervous system of the pond snail *Lymnaea stagnalis.* We designed the primer set and the probe in the specific insert for the detection of *Lymnaea* C/EBP (LymC/EBP) clone 1. This insert is not contained in LymC/EBP clone 2 by alternative splicing. The copy number of LymC/EBP clone 1 was linearly decreased relative to the dilution of cDNA, and it was estimated 30 copies/µl in test sample. The availability of the present study showed that the real-time quantitative RT-PCR technique is more accurate and more specific for the detection and quantification of the mRNA level of genes in *L. stagnalis* than the other PCR methods.

Keywords: C/EBP – mRNA – qRT-PCR – CNS – *Lymnaea*

INTRODUCTION

The real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) is useful for simplicity, rapidity and a higher degree of reproducibility for quantification of mRNA [7]. This method takes advantage of the 5' to 3'-exonuclease activity of TaqDNA polymerase and utilizes the PRISM™ 7700 sequence detection system of Applied Biosystems (AB) for direct monitoring of PCR product accumulation through a dual-labeled fluorogenic probe (Fig. 1A–D) [1]. When the probe is intact, the proximity of the reporter dye (shown "R" in Fig. 1) to the quencher dye (shown "Q" in Fig. 1) results in suppression of the reporter fluorescence (Fig. 1A). During the strand displacement of PCR cycle (Fig. 1B), the 5' to 3' exonuclease

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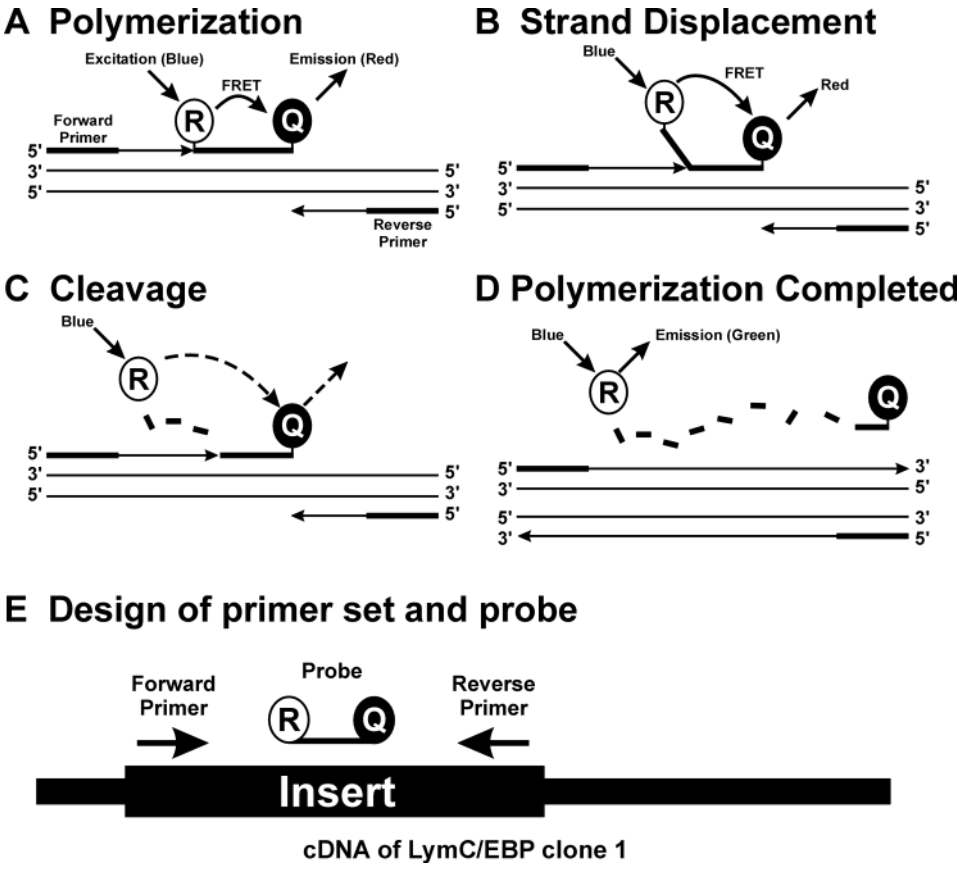

Fig. 1. (A–D) Schematic representation of the 5' to 3' exonuclease activity-based nucleic acid quantification. (E) Design of the primer set and the probe for detection of LymC/EBP clone 1

activity of the AmpliTaq Gold DNA polymerase (AB) cleaves the probe. This cleavage releases the reporter dye from the probe, resulting in an increase of the reporter dye fluorescence (Fig. 1C). After cleavage, the shortened probe dissociates from the target and polymerization of the strand continues. This process occurs in every PCR cycle and does not interfere with the exponential accumulation of the product. The exonuclease activity of the AmpliTaq Gold DNA polymerase acts only if the probe hybridizes to the target; it does not cleave free probe in solution (Fig. 1D). As a result, the increase of the reporter dye fluorescence is directly proportional to the amount of PCR product accumulated. In the present study, we detected and quantified mRNA copies of C/EBP in the central nervous system of the pond snail *Lymnaea stagnalis*, which can acquire conditioned taste aversion (CTA) [2, 3, 4], by the real-time qRT-PCR method.

MATERIALS AND METHODS

The total RNA of *Lymnaea* CNS for test sample was purified with TRIzol Reagent (Invitrogen). The insert region of LymC/EBP clone 1 was amplified by RT-PCR and inserted into pGEM™-T Easy vector (Promega). This plasmid purified with Plasmid Midi Kit (QIAGEN) was digested with restriction enzyme *Sal* I. RNA synthesis was performed according to the documents of manufacturer (ABI Prism™ 7700 system, AB) with MAXIscript™ (Ambion). The synthesized RNA was quantified and used as the standard RNA. Serially diluted standard RNA (5×10^1– 5×10^6 copies/µl) were reverse-transcribed in 10 µl of reaction mixture to prepare the first strand cDNA with 2.5 µM specific primer, diluted to 1 : 5 and used as the standard cDNA. The RT solution of samples and standards were added to PCR-reaction solution containing AmpliTaq Gold DNA polymerase (AB). The primer set and the probe were designed in the specific insert region of LymC/EBP clone 1 (Fig. 1E). Reaction was carried out

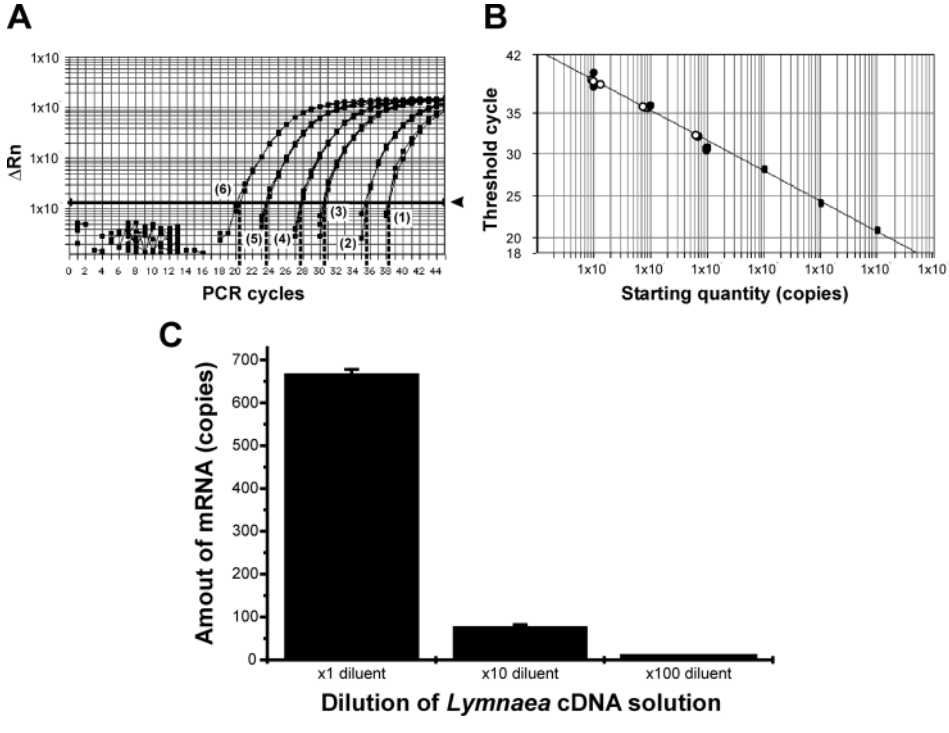

Fig. 2. (A) Amplification plot of serially diluted standard RNA. (1) 1×10^1, (2) 1×10^2, (3) 1×10^3, (4) 1×10^4, (5) 1×10^5 and (6) 1×10^6 copies/µl. Arrowhead shows the threshold value for definition of standard curve. Dotted line indicates the C_T value of each standard amplification plot. (B) Standard curve for the determination of nucleic acid quantity in unknown samples. Filled circles and open circles showed the plots of C_T values of standards and test samples, respectively. (C) Copy numbers of LymC/EBP clone 1 in test samples

at 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. In the assay, several doses of standard cDNA ($1 \times 10^1 - 1 \times 10^6$ copies) were applied in triplicate to estimate inter assay coefficients of variation between runs. The entire process of calculating C_T (threshold cycle), preparing a standard curve, and determining starting copy number for unknowns was performed by the software of the 5700 and 7700 systems (AB).

RESULTS AND DISCUSSION

The computer software constructed the amplification plots (Fig. 2A) from the fluorescence emission data that are collected during the PCR amplification. These plots marked fluorescence signal intensity versus cycle number. In the initial cycles of PCR (from the 1st cycle to the 17th cycle in Fig. 2A), we did not observe the change in fluorescence signals, and so we defined them as "baseline". An increase in fluorescence above the baseline indicated the detection of accumulated PCR product. A fixed fluorescence "threshold" (arrowhead in Fig. 2A) represented the PCR cycle at which an increase in the reporter fluorescence above the baseline signal can first be detected. The parameter "C_T" value of each concentration of standard was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Plots of the logs of initial target copy numbers for a set of the standards versus C_T values were arranged in a straight line, which is referred to as "standard curve" (Fig. 2). The C_T values of test samples were plotted versus the log of initial amount of cDNA to give the standard curve (Fig. 2C).

The copy number of LymC/EBP clone 1 was linearly decreased relative to the dilution of cDNA (Fig. 2B, C). The volume of 1× dilution of test sample was 23 µl and the concentration of cDNA of LymC/EBP clone 1 was estimated 30 copies/µl (Fig. 2C). The real-time qRT-PCR method can provide precise and reproducible quantification of genomic samples over a 1,000,000-fold dynamic range with a minimum input of 10 copies [6]. Actually, the amount of cDNA of LymC/EBP clone 1 in the test sample diluted to 100 fold was quantified about 10 copies. We previously demonstrated that the cerebral giant cell (CGC) plays a crucial role in the regulation of CTA in *L. stagnalis* [5]. The real-time qRT-PCR method can quantify mRNA levels more sensitively than the other RT-PCR methods, and it can be applied to analyze the change in mRNA level of LymC/EBP clone 1 in single CGCs during CTA. If multiple probes are labeled with other reporter dyes, the mRNA levels of many genes in a single cell can be monitored simultaneously. The real-time qRT-PCR method will be a very powerful tool for facilitating the comprehensive understanding of the molecular mechanisms underlying learning and memory of *L. stagnalis*.

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