AN APPLICATION OF REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION IN A RELATIVE QUANTIFICATION OF GENE EXPRESSION

By

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ABSTRACT

The ability to quantify steady state levels of individual messenger-RNA (mRNA) transcripts has been the key issue for study on the control of gene expression. Although, two available techniques, Northern blot and nuclease protection assays (NPA) have been widely used for detecting mRNA, these techniques have critical limitations. The most obvious limitation of these two techniques is the required number of target mRNAs to be detected. Reverse transcription-polymerase chain reaction (RT-PCR), which has been accepted as a highly sensitive and specific method, provides a means for detecting and quantifying gene expression using, theoretically, only a single molecule of mRNA. The sensitivity and reliability of RT-PCR is dependent upon both the RT and PCR steps. The PCR step has been problematic because of the exponential nature of this reaction where small variation can lead to dramatic changes in final result. Therefore, the use of RT-PCR for quantification of gene expression requires pre-experimental planning and design. In this experiment, the procedure for pre-experimental planning, linear range determination, and subsequent relative quantification of gene expression are described in detail. A study of ornithine decarboxylase gene, a gene involved in the polyamine biosynthesis and temporally expressed, during embryogenesis of *Musca domestica* (housefly) was used as the model. The results show that during early embryogenesis (t-1 to t-4) the expression level was very low. The increase in expression profile was observed started at t-5, peaked at t-9, and followed by substantial decrease from t-10 to t-12.

Keywords: RT-PCR; Northern blot; NPA; gene expression, quantification.

ABSTRAK

Kemampuan untuk menghitung jumlah *messenger-RNA* (mRNA) pada studi ekspresi gen merupakan hal yang sangat mendasar. Walaupun teknik *Northern blot* dan *nuclease protection assay* (NPA) telah sering digunakan, kedua teknik ini masih mempunyai kelemahan. Kelemahan yang sangat mendasar pada kedua teknik ini terletak pada jumlah sampel mRNA yang dibutuhkan agar mRNA tersebut bisa dideteksi. *Reverse transcription-polymerase chain reaction* (RT-PCR) merupakan teknik yang sangat sensitif dan bahkan secara teori bisa digunakan untuk mendeteksi satu molekul mRNA yang diinginkan. Sensitifitas dan kehandalan teknik RT-PCR sangat tergantung pada dua komponen, RT dan PCR. Karena sifat dari reaksi PCR adalah eksponensial, maka kesalahan kecil pada tahap awal akan berakibat fatal pada hasil PCR yang didapat. Oleh karena itu, penggunaan RT-PCR untuk mendeteksi dan menentukan tingkat ekspresi suatu gen membutuhkan *experimental design* yang tepat. Pada artikel ini, prosedur untuk membuat dan merencanakan *experimental design*, *linear range*, dan kuantifikasi pada ekspresi suatu gen diterangkan secara mendasar dan menyeluruh. Studi pada ornithine decarboxylase, gen yang terlibat dalam proses síntesis polyamines dan diatur secara temporal, pada proses embriogenesis pada *Musca domestica* digunakan sebagai model dalam penelitian ini. Hasil yang didapat menunjukkan bahwa ekspresi ODC sangat rendah pada tahap awal embryogenesis (t-1 sampai t-4). Peningkatan ekspresi mulai terlihat pada t-5, mencapai puncak pada saat t-9, dan akhirnya menurun lagi pada t-10 sampai t-12.

Kata kunci: RT-PCR; Northern blot; NPA; ekspresi gen, kuantifikasi

INTRODUCTION

Currently, there are three widely used techniques for detecting and determining gene expression at the level of transcription. They are Northern blot, nuclease protection assays (NPA), and RT-PCR techniques. In theory, any one of these three techniques can be used to detect mRNAs and to evaluate their expression.

To obtain the size of mRNA transcript, Northern blot is the only technique available. NPA is very efficient for detection of multiple mRNA expressions. However, the obvious limitation of Northern Blot and NPA is the required number of target RNA to be detected. Northern blot takes approximately 10,000 copies of mRNA to detect the presence of mRNA transcript. While for NPA it takes approximately 4 to 5 thousands of copies, for RT-PCR theoretically it takes only a single copy to detect mRNA transcript. Hence, RT-PCR technique is widely used for cloning, complementary DNA (cDNA) library construction, probe synthesis, and signal amplification in *in situ* hybridization (Sarah and Michael, 1991; Saiki *et al.*, 1985; Margareth and Andrew, 1991; Kawasaki, 1990). Although RT-PCR is the most sensitive approach for detection and quantification of gene expression (Max *et al.*, 2001), it has drawbacks. As the PCR product amplified the target sequence errors are also amplified.

Molecular characterization of any gene usually involves a detailed analysis of temporal distribution of mRNA expression (Labuhn and Brack, 1997). Therefore, to obtain the precise profile of the band intensity throughout distributions of the messages becomes critical. Unfortunately, to obtain the actual expression profile can be extremely difficult if the expression of one sample to the other is closely expressed. Molecular study of developmentally and temporally expressed-gene(s) is one of best examples where sensitive and reproducible technique is required (Foss *et al.*, 1998; deLeeuw *et al.*, 1989; Labuhn and Brack, 1997).

A relative quantification technique for gene expression study, which is an RT-PCR based-technique, is an extremely sensitive technique for analyzing gene expression. This technique provides a way to precisely estimate changes in gene expression between samples (deLeeuw *et al.*, 1989; Margareth and Andrew, 1991; Foss *et al.*, 1998). Thus, this technique is extremely useful for a study of temporally expressed gene, where most of the time, especially in this kind of study, the expression between samples is gradual in nature. However, to perform this technique, substantial pre-experimental planning and design are required. The objective of this paper is to discuss and provide the technical background information on this approach. In this experiment, the study of ornithine decarboxylase gene, a gene involved in the polyamine biosynthesis (Pegg, 1988) and temporally expressed (Lilleberg *et al.*, 1991), during embryogenesis of *Musca domestica* (housefly) was used as the model.

MATERIALS AND METHODS

1. MATERIALS

General chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA). Radioisotopes [alpha-^{32}P]dCTP (3,000Ci/mmole) was from ICN Biochemicals (Cleveland, OH, USA). Oligonucleotides for primers were purchased from IDT, Inc. (Coralville, IA, USA). Enzymes were obtained from Perkin Elmer (Branchburg, NJ, USA).

2. TISSUE

Female *Musca domestica* were induced with 2.5 % $(NH_4)_2CO_3$ to lay their eggs (embryos) in a cup placed inside the cage at 26°C. Approximately, 6 grams of embryos (placed in a cup), were then incubated in a waterbath at 30°C. Twelve samples of embryos (each 0.5 gram) were taken at 1-hour interval (from 1 to 12-hour), from the cup

(in a waterbath) and frozen in liquid nitrogen immediately until used. In this paper, sample taken at 1-hour, 2-hour, and so on, was denoted as t-1, t-2, and so on. Since the preliminary results showed that the t-9 sample had the highest poly(A)$^+$mRNA of interest (data not shown), this sample was used for the linear range determination study.

3. ISOLATION OF poly(A)$^+$mRNA

Total RNA and poly(A)$^+$mRNA were isolated according to the method of Chirgwin *et al.* (1979), Aviv and Ledder (1972), and Davis *et al.* (1986) with several modifications. Tissue from each time point (0.5 gram each) was homogenized at 4°C in a Potter glass homogenizer in 4 ml GIT buffer (4 M guanidium isothiocyanate, 25 mM sodium acetate buffer (pH 6.0), and 120 mM β-mercaptoethanol) until no longer viscous. Following centrifugation in a Sorval HS-4 rotor (Du Pont Company, DE, USA) for 20 min at 7,000 rpm at 4°C, the clear GIT homogenates were layered onto 4 ml CsCl buffer (5.7 M CsCl and 25 mM sodium acetate). The samples were then centrifuged for 21 hr at 32,000 rpm in SW41TI rotor (Beckman Coulter, Inc. CA, USA) at 19°C. The total RNA pellets were resuspended in 200 μ diethylpyrocarbonate (DEPC)-treated water.

The poly(A)$^+$mRNAs were isolated from the total RNA. This isolation was based on base pairing between the poly(A)$^+$ residues at the 3'-end of mRNAs and the oligo(dT) residues coupled to the cellulose matrix. Oligo(dT)-cellulose column was prepared by resuspending 0.2 mg of oligo(dT)-cellulose Type 3 in 3 ml DEPC-treated water. The slurry was poured into a sterile disposable plastic column. The column was equilibrated with loading buffer (20 mM Tris-HCl (pH 7.4), 1 mM ethylenediamine tetraacetic acid (EDTA), 0.1 % sodium dodecyl sulfate (SDS), and 0.5 M NaCl) until the pH of the effluent was less than 8. Following resuspension in the loading buffer, the total RNA samples were loaded into the column. The bound poly(A)$^+$ mRNA was eluted from the column by the addition of elution buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.05 % SDS).

4. POLYMERASE CHAIN REACTION

The PCR was performed according to the method of Saiki *et al.* (1985). The final reaction conditions were 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1.0 μM of forward and reverse primers, and 2 units of *Taq* DNA polymerase. The primers for reverse transcription and PCR were the same. The sequences of the primers were as follow: forward primer (GATTATACATATGTTCAA AGAAAATGAAA), and reverse primer (CGATGAATTCCAATATTAGTCAGATT TTTGCTTTATG). For the 28S rRNA internal marker (Han *et al.*, 2002), the primers were as follow: forward primer (TCTAGTAG CTGGTTCCCTCC), reverse primer (ATATTGGTACGGCCTGTTGAG).

5. REVERSE TRANSCRIPTION REACTION AND LINEAR RANGE DETERMINATION

The reverse transcription reaction was carried out in a total volume of 20 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dNTPs, 1 unit RNase inhibitor, 2.5 unit (M-MLV) reverse transcriptase, 1 μM of reverse primer, and 1 μg of poly(A)$^+$ mRNA template (Kawasaki, 1990). The mixture was incubated for 30 min at 42°C for the synthesis of the first cDNA strand, heated at 95°C for 5 min to stop the reaction, and quick-chilled on ice. The first strand cDNA was then amplified by PCR technique in the same tube.

For linear range determination, the product of reverse transcription (20 μl) was brought to 160 μl with the final concentration was adjusted as described in PCR section (Materials and Methods 4). Since 1 μM of reverse primer had been added, only 1 μM of the forward primer was added into the PCR mixture. For the sake of detection of the PCR

product, 5 µl (alpha ^{32} P) dCTP (10 µCi/µl) was added to the mixture. The mixture was then divided into 16 aliquotes (10 µl each) and subjected to PCR amplifications. The tubes were removed from the thermal cycler at cycles 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, and 35. Based on the linear range determination , the PCR amplification consisted of 22 cycles was performed to obtain the actual gene expression profiles of ornithine decarboxylase of *Musca domestica* during embryogenesis.

6. PRECIPITATION OF DNA WITH TRICHLOROACETIC ACID

A 7 µl of RT-PCR product (from each cycle) was spotted onto a 1 cm^2 of 3MM paper, and dried completely at room temperature (Sambrook *et al,* 1992). The paper was then transferred to a beaker containing 300 ml of ice-cold 5 % trichloroacetic acid (TCA). The beaker was swirled for 2 min, and transferred to a fresh beaker containing the same volume of TCA. After soaked in 70 % ethanol for 2 min, the 3MM paper was inserted into a scintillation vial, and the radioactivity was measured in a Beckman LS5000TD scintillation counter (Beckman Coulter, Inc. CA, USA)

RESULTS

Reaction products accumulate during PCR amplification at a rate dependent on the amplification efficiency. The linear range of the reaction is defined as the period of the PCR in which the amplification efficiency is at its maximum and remains constant over a number of cycles. Consequently, a PCR reaction will remain in linear range for only a limited number of cycles. Therefore, to obtain meaningful results, the relative RT-PCR reactions must be terminated and the products quantified when all the samples are in the linear range of amplification.

In this experiment, the ornithine decarboxylase from *Musca domestica* was used as the model for relative quantification of gene expression. Figure 1 represents the steps in a relative quantitative RT-PCR technique. Isolation of total RNA samples from *Musca domestica* was performed using a guanidium-based chaotropic agent (Davis *et al.*, 1986). Oligo (dT)-cellulose was applied to isolate poly (A)$^+$mRNA from total RNA. Linear range determination was then conducted. Once the linear range was established, the highest cycle number that was still within the linear range will be used in the relative quantitative RT-PCT experiment.

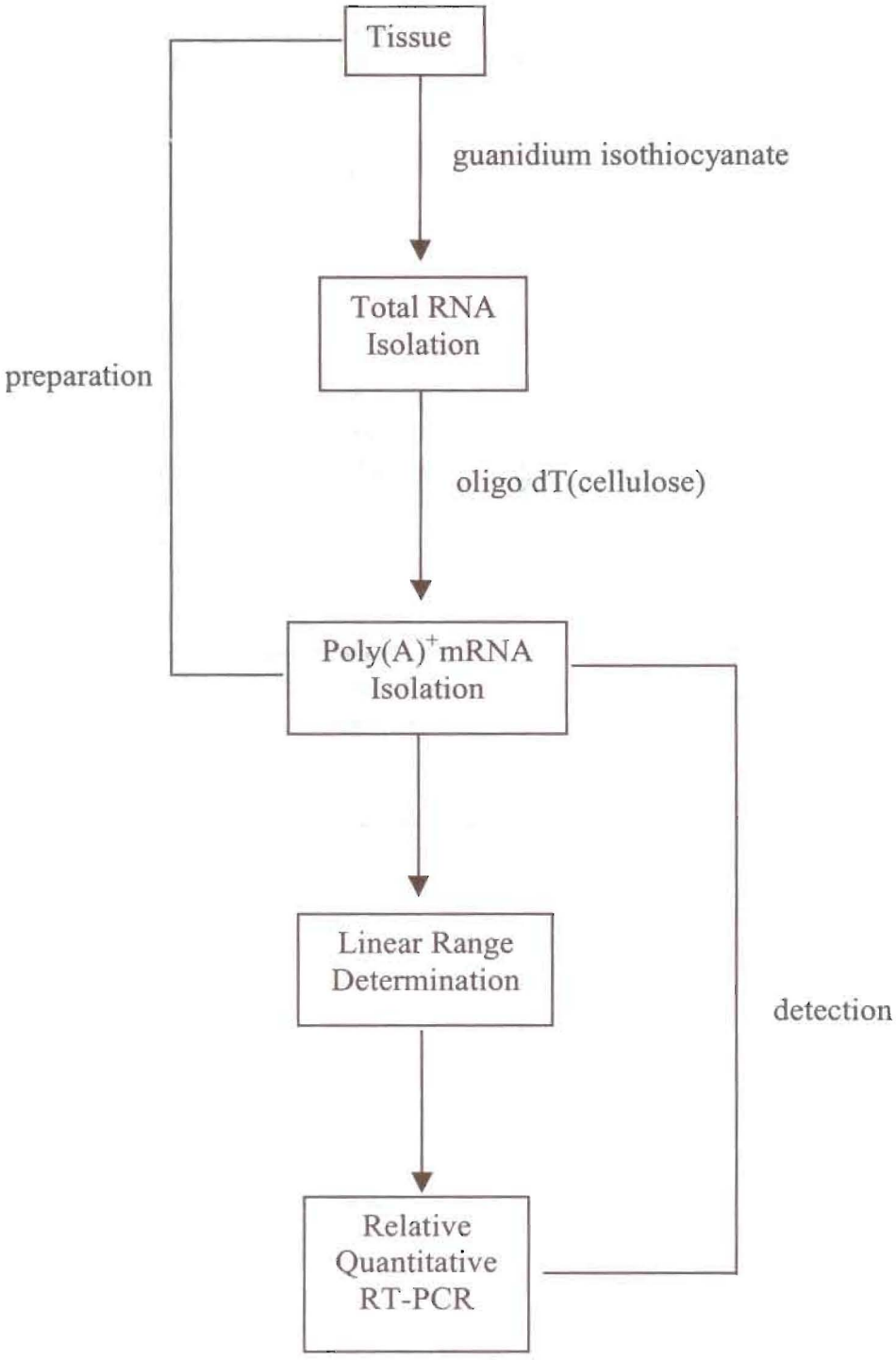

Figure 1. Flowchart of the technical steps in the relative quantification RT-PCR. The first step, preparation, total RNA isolation was accomplished by guanidine isothiocyanate method. Poly(A)$^+$mRNA was isolated from total RNA with the use of oligo (dT)-cellulose. The second step, detection, the poly(A)$^+$mRNA result was used for linear range determination followed by relative quantitative RT-PCR.

The initial step in RT-PCR is the production of a single-strand cDNA copy of the poly(A)$^+$mRNA through the action of the retroviral enzyme, reverse transcriptase (Fig. 2). An oligonucleotide reverse primer was used to initiate the cDNA synthesis from poly(A)$^+$mRNA. After the primer was annealed, the cDNA synthesis was extended toward the 5'-end of the mRNA through the RNA-dependent DNA polymerase activity of reverse transcriptase. Following the reverse transcription reaction, the cDNA was amplified by PCR.

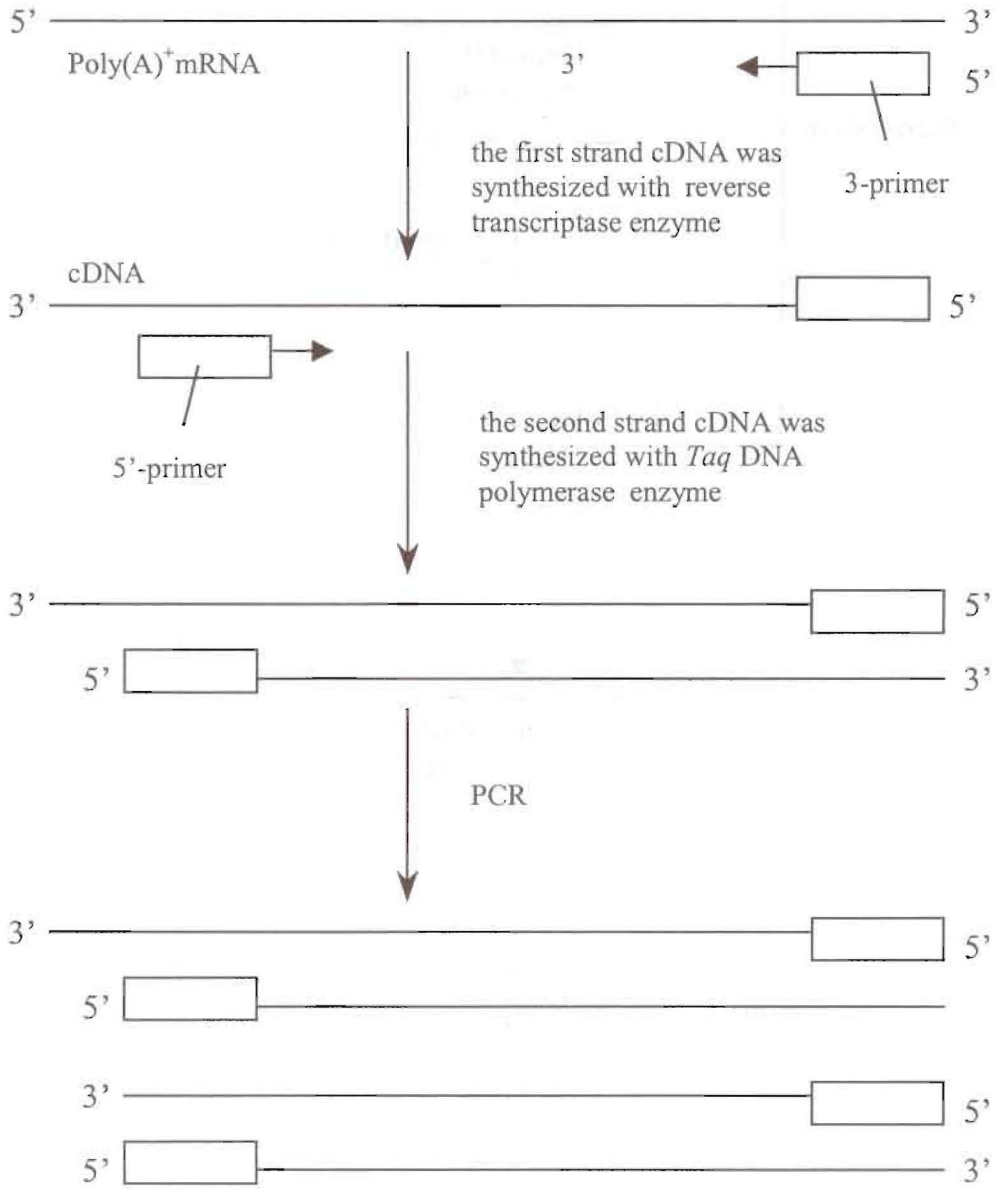

Figure 2. The schematic diagram of RT-PCR. The reverse primer was used to synthesize the first strand of cDNA from poly(A)$^+$mRNA template with reverse transcriptase enzyme. Next step, the forward primer was used to synthesize the second strand. PCR amplification was then used to amplify the target cDNA.

In theory, each amplification cycle should double the number of target molecules resulting in an exponential increase in PCR product. However, even before the substrate or enzyme becomes limiting, the efficiency of the exponential amplification is less than 100 % owing to sub-optimal DNA polymerase activity, poor primer annealing, and incomplete denaturation of the templates (Kawasaki, 1990). Thus, at some point during the reaction, the amplification efficiency falls and the rate of products accumulation slows or "plateaus" (Fig. 3).

reaction was performed with this sample. After the results were quantified and plotted, the linear range was appeared to be between cycles 5 to 22.

After the linear range determination had been established, the relative quantification study and the PCR amplification consisted of 22 cycles were performed. Figure 4 shows the real profile of the ornithine decarboxylase expression of *Musca domestica* during embryogenesis. In this experiment as seen in Figure 4A,

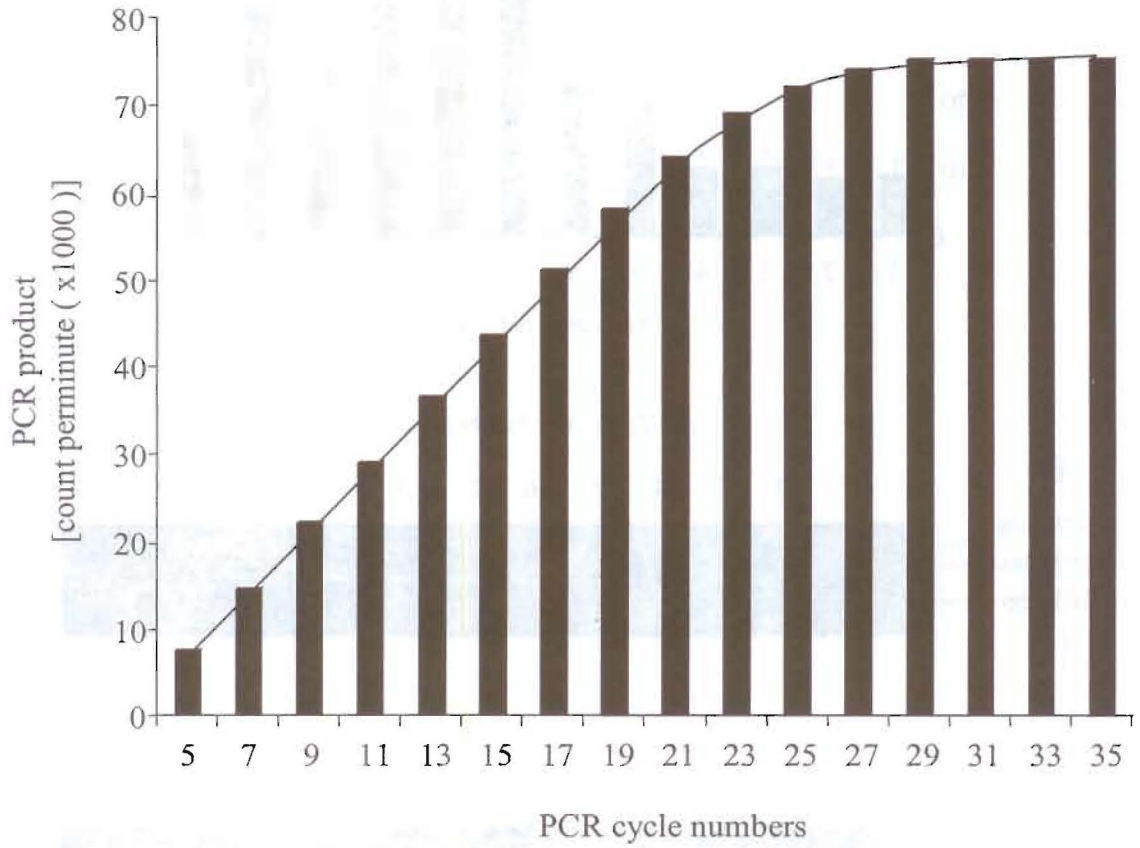

Figure 3. The graph of linear range determination. This graph shows that the linear range spans between the 5th and the 22nd cycles. After the 22nd cycle the linearity of the curve starts to diminish. The t-9 poly(A)$^+$mRNA sample was used as the template.

To determine the linearity of the "PCR cycle numbers versus PCR product", the poly(A)$^+$mRNA sample in which the target sequence is expected to be the most abundant is used for RT-PCR reaction. In this experiment, the time point t-9 hr sample was used. A single reverse transcription

radioisotope dCTP was included in the reaction so that comparison of the PCR products between samples could easily be detected. Ethidium bromide staining is a poor method for comparison of closely equal amount of DNA samples. The lower limit of ethidium bromide detection of a DNA band

is approximately 5-10 ng. Thus, radioisotope was used in this study (Ylikoski *et al.*, 2001). However, with the availability of several new DNA-detection products, such as: BrighStar Psoralen-Biotin (Ambion, Texas, USA), the use and our dependence on radioisotope for RT-PCR can be avoided.

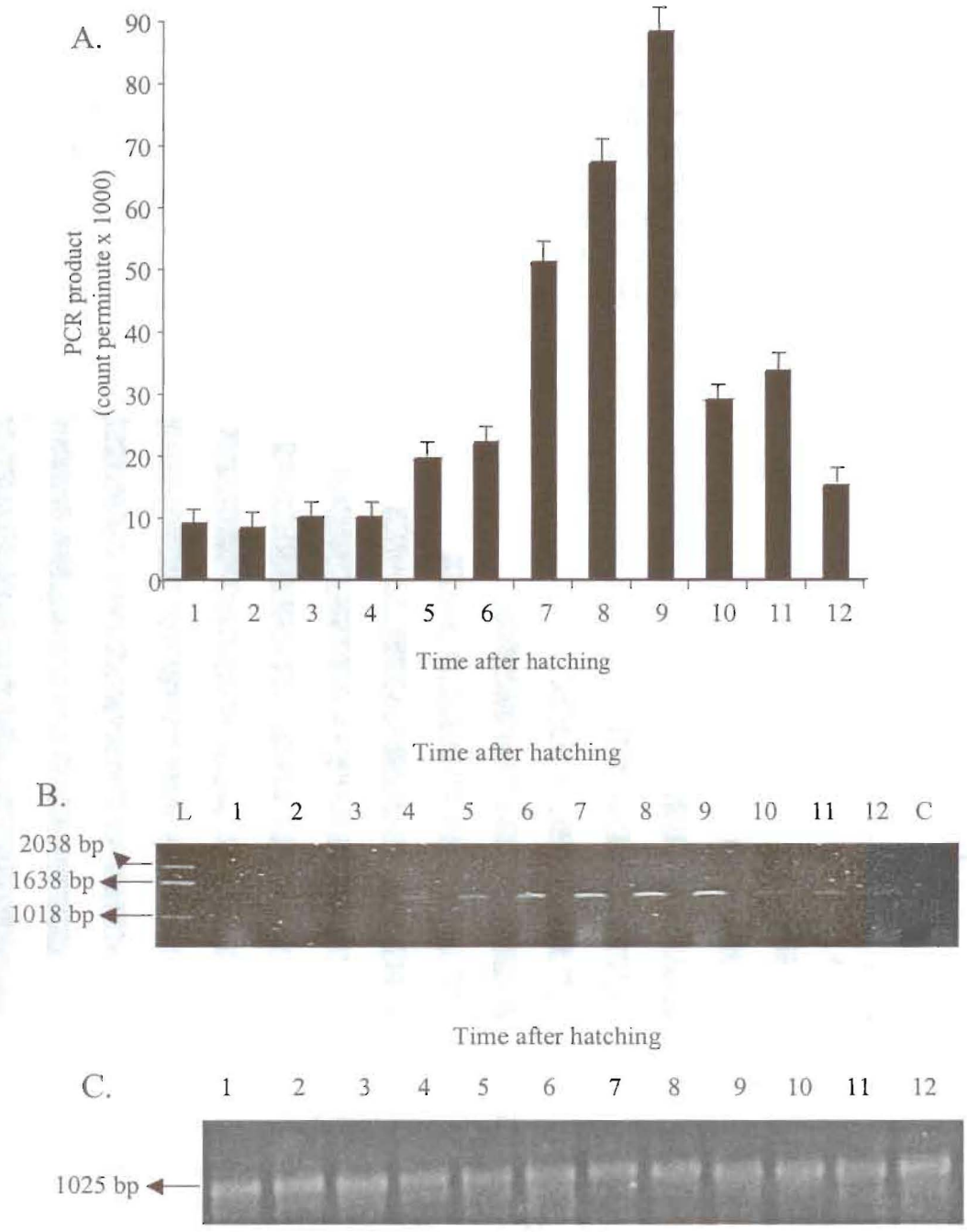

Figure 4. Relative quantification of *Musca domestica* ornithine decarboxylase gene expression during embryogenesis. This PCR consisted of 22 cycles. The conditions were the same as in Figure 3. (A) Radioisotope (alpha^{32}P)dCTP was used for detection in Beckman LS5000TD scintillation counter. Vertical lines show the mean ± SD. Each assay was repeated three times. (B) Lanes 1-12 are the RT-PCR products of ornithine decarboxylase of *Musca domestica* during embryogenesis from t-1 to t-12. Lanes L and C are ladder and negative control (no template), respectively. The size of the bands of the samples (1-12) is 1.2 kb. (C_4) 28S rRNA was used as an internal marker (Han *et al.*, 2002).

The technique employed in this experiment was able to visualize the temporal expression of ornithine decarboxylase expression. As seen in Figure 4A, at the first few hours (from t-1 to t-4) the expression level was very low. The increase in expression profile was observed started at t-5 and peaked at t-9. After t-9, the expression decreased significantly and reached very low expression at t-12. Figure 4B shows that ethidium bromide staining is not accurate enough to detect the expression profile. To ascertain that equal amount of templates were applied, RT-PCR of internal control of 28S rRNA was performed. Figure 4C shows that the intensity of the bands throughout embryogenesis was approximately similar indicating that equal amount of templates were applied.

DISCUSSION

For most organisms, the majority of development occurs during embryogenesis, and postnatal changes are primarily concerned with growth. Predictably, the molecular mechanism underlying these developmentally processes are complex and may always be driven by changes in gene expression. Since mostly gene(s) expression involved in embryogenesis is temporal in nature, to detect the pattern of gene expression at different developmental time points becomes very critical. Thus, a sensitive and reproducible technique is required to visualize developmentally and closely expressed gene(s).

Ornithine decarboxylase is the rate-limiting enzyme in the biosynthesis of the polyamines (putrescine, spermidine, and spermine) (Pegg, 1988; Pegg and McCann., 1982). This enzyme is highly regulated at transcriptional, translational, and post-translational levels (Wallon *et al,* 1995; Lovkvist *et al.*, 1993). Ornithine decarboxylase is temporally expressed during embryogenesis (Lilleberg *et al.*, 1991). Thus, to study the expression profiles of ornithine decarboxylase gene during embryogenesis at different time points in *Musca domestica*, a high-resolution technique, a relative quantitative RT-PCR was employed.

PCR is extremely sensitive method for detection of small amount of DNA or RNA (by the use of RT-PCR). However, an accurate quantification is difficult to obtain with normal PCR, as the amount of the amplified product does not necessarily reflect the amount of template initially present in the reaction. The source of inaccuracy in the PCR is due to the "plateau phase" which can be caused by several problems including deactivation of the enzyme, shortages of nucleotides and primers, inhibition by pyrophosphate, or reannealing of amplified DNAs. In this current experiment, it was observed that the "plateau phase" was begun at the 23rd cycle, and the linear range is between cycle 5 and 22. Thus, to obtain an accurate quantification of PCR product the number of PCR cycles must be between 5 and 22 cycles. Theoretically, any cycle between 5 and 22 can be used for the quantification of ornithine decarboxylase gene expression experiment. However, it is necessary to perform PCR amplification at the highest cycle in the linear range so that the maximum PCR product can be obtained and the detection will be much easier to perform. This is very important, especially, at time point t-1 where ornithine decarboxylase expression was extremely low and very difficult to detect.

Lately, the latest emerging technique for studying gene expression is microarrays. Microarrays exploit primary sequence data to measure transcript levels and detect sequence polymorphism for several genes simultaneously. Due to its complex nature and wide applications, microarrays analysis is beyond the scope of this paper. For detailed discussions, this technique has thoroughly been reviewed (Baldwin et al.- 1999; Gerhold et al., 1999; Marshall and Hodgson. 1998; Craig and David. 2000).

As explained earlier, Northern blot analysis and NPA techniques are alternatively possible to be used for gene expression studies. However RT-PCR was chosen because of its exceptional sensitivity, reliability, and the small amount of template to be used. Despite its superiority, RT-PCR is not without drawbacks, great care must be taken seriously before using RT-PCR technique, particularly if this technique is used for profiling of gene expression. As PCR is exponential in nature, errors are drastically amplified. As a result this highly sensitive technique may not serve its purpose. These data therefore suggest that with careful experimental planning RT-PCR is extremely powerful technique for quantification of different time point of closely expressed gene.

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