mRNA Isolation and RT-PCR Using Small Amounts of Embryonic Axolotl Tissue

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Introduction

Amplification of mRNA by reverse transcription and the polymerase chain reaction (RT-PCR) has revolutionized and made more universally available molecular approaches to many questions in developmental biology. This technique can be used to analyze expression of regulatory genes that have been cloned in other organisms (Busse and Seguin 1992) and may be applicable to studies of the developmental mutant genes of the axolotl (reviewed by Malacinski, 1989). However, most methods for isolation of intact mRNA include phenol/chloroform extraction followed by oligo(dT)-cellulose affinity column chromatography (Sambrook et al. 1989). Such procedures are effective but require a relatively large amount of tissue, which for many studies can involve time-consuming dissection of embryonic tissues or organ rudiments.

In this report we present a simple, rapid method for preparation of polyA$^+$ RNA suitable for RT-PCR. The method is a modification of a method suggested to us by Dr. M. King (Medical Sciences Program, Indiana State University, Terre Haute). In addition to amplification of an mRNA of interest to us, the T-complex polypeptide 1 (TCP-1, manuscript in preparation), from various embryonic axolotl tissues, we also amplified the mRNA for elongation factor 1-α (EF-1α, Krieg et al. 1989).

Materials and Methods

PolyA$^+$ RNA isolation. PolyA$^+$ RNA was isolated with messenger affinity paper (mAP, Amersham) using the following procedure:

1. A small amount of embryonic tissue is lysed in a minimal volume of GT solution (4 M guanidium-isothiocyanate, 25 mM sodium citrate pH 7.0, and 0.5% Sarcosyl; with 2-mercaptoethanol to a final concentration of 0.1 M, and 1/10 volume of 2 M sodium acetate pH 4.0). In this experiment we lysed the following tissues from stage 35 axolotl embryos in 10 µl GT solution: 3 notochords, 3 spinal cords, 3 forebrains, one skin sample, and one gut sample.

   The tissue is pipetted in and out until it completely lyses in the solution. After tissue disruption the microtube containing the lysed sample is kept on dry ice as much as possible.

2. Small pieces of messenger affinity paper (mAP, Amersham) are prepared by cutting 1.5 mm squares and placing them on several paper towels. The mAP is then saturated with 0.5 M NaCl. Slowly and carefully the lysed sample is loaded onto the filter using 1-2 µl drops at a time so that the sample flows through the mAP, not off the sides.

3. The filter is washed with a total of 20 µl of 0.5 M NaCl using 1-2 µl drops at a time.

4. Then the filter is transferred to a 0.5 ml microtube containing 0.5 M NaCl.

5. The filter is washed 2 times for 5 minutes each time in 0.5 ml 0.5 M NaCl by inverting the tube and vortexing repeatedly .

6. Finally, it is washed 2 times in 0.5 ml 70% ethanol in the same way as in step 5. After this step, the mAP can be stored in 70% ethanol at –20°C indefinitely.

7. For use, the mAP is dried in a speed vacuum concentrator to remove the ethanol. The mAP is then transferred to a new 0.5 ml microtube.

8. Twelve ml DEPC treated H_2O are added. Then the tube is heated at 72°C for 5 minutes to elute polyA$^+$ RNA. After heating, it is snap chilled in ice. Then it is spun down. The eluted polyA$^+$ RNA can be used for reverse transcription directly.

Reverse Transcription (RT). The total volume of RT reaction mixture is 20 µl. The reaction mixture contains: 1x RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3mM $MgCl_2$), 10 mM DTT, 0.5 mM each dGTP, dATP, dTTP, and dCTP, and 25 ng/µl oligo$(dT)_{15}$, 1 unit/µl RNase Block II (Stratagene), 200 units Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, GIBCO BRL) and 9.5 µl of polyA$^+$ RNA isolated with mAP. The reaction mixture is incubated at 23°C for 10 minutes, at 37°C for 60 minutes, then heated at 99°C for 5 minutes.

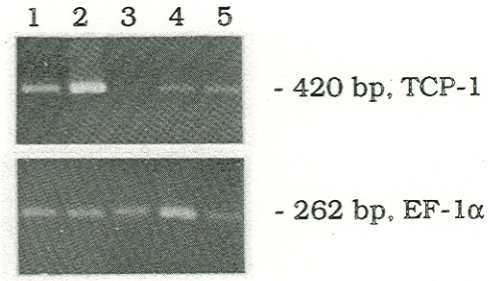
Polymerase Chain Reaction (PCR). The expression of axolotl TCP-1 in different embryonic tissues was analyzed using PCR EF-1α as the internal standard. The PCR reaction volume is 50 µl, which contains: 1x PCR buffer (500 mM KCl, 10 mM Tris-HCl), 1.5 mM $MgCl_2$, 0.2 mM each dGTP, dATP, dTTP, and dCTP, 4µl of RT reaction, 1 unit Taq polymerase, 2.5 ng/µl of each oligonucleotide primer for EF-1α mRNA (up-stream: 5'TGGGAAAGGAAAAGACTACATC3'; down-stream: 5'GTGACATAGTATTTGCTGGTCTC3') or each primer for TCP-1 mRNA. The reaction is run for 35 cycles with a "hot start." This is done by adding all components of the reaction mixture (40 µl total) except Taq polymerase, heating at 95 °C for 5 minutes, then holding at 85 °C to add 1 unit Taq polymerase, which is diluted in 10 µl 1x PCR buffer. The cycles are 95°C for 1 minute, 60°C for 30 seconds, 72°C for 30 seconds. A 5 minute "extension step," during which the reaction mixture is kept at 72°C, follows the cycles. After PCR, 10 µl of the reaction product is ethidium bromide stained and analyzed on a 2% agarose gel.

Results and Discussion

Using the polyA+ RNA isolated with mAP method, we analyzed the expression of TCP-1 in stage 35 axolotl tissues with RT-PCR. For this purpose, we needed an mRNA internal standard because the small amount of polyA+ RNA used for RT-PCR is hard to quantitate. In the axolotl, so far, there is no known gene sequence which can be used as such a standard for RT-PCR. We decided to use EF-1α as an mRNA internal standard because it is known that in *Xenopus* the EF-1α gene is expressed in all cells (Krieg et al. 1989) and a 262 bp sequence of EF-1α is a good mRNA internal standard for PCR in *Xenopus*. After optimization of the PCR conditions, the 262 bp sequence can be amplified by RT-PCR in the different tissues.

In the experiment, polyA+ RNA from each tissue was reverse transcribed to first-strand cDNA. Then the same amount of RT reaction mixture was used for the PCR amplification of TCP-1 and of EF-1α, separately. After PCR, the same amount of each reaction mixture were loaded onto the analysis gel. TCP-1 shows the highest expression level in forebrain, a lower level in spinal cord, an even lower level in skin and gut, and none at all in notochord. The expression level of EF-1α is similar in all these tissues (Figure 1).

Figure 1. RT-PCR analysis of the expression of TCP-1 in stage 35 embryonic axolotl tissues using the polyA+ RNA isolated with mAP and employing EF-1α as an mRNA internal standard.



1. spinal cord
2. forebrain
3. notochord
4. skin
5. gut

These data show that mRNA isolated with the mAP method from small amounts of embryonic tissue can be used for RT-PCR with satisfactory results. Moreover, the data suggest that EF-1α can be used as an mRNA internal standard for RT-PCR analysis of gene expression in the axolotl.

Using the mAP method to isolate mRNA has several advantages. It is simple: besides the GT solution, only 0.5 mM NaCl and 70% ethanol are needed. The procedure has few steps and is easy to do. It is rapid: one can isolate mRNA from a tissue sample in about one hour. It is sensitive: only a small amount of tissue is required, so it is easy to get "clean" tissue samples. With all of these advantages, the method make RT- PCR analysis of gene expression in a small amount of embryonic tissue more practicable.

For mAP polyA$^+$ RNA isolation, in our hands, it is important to add 1/10 by volume 1 M sodium acetate pH4.0, to the GT solution immediately before adding the tissue. Otherwise the mAP method does not work well for isolating polyA$^+$ RNA from the axolotl. After loading the sample on the mAP, it is also important to wash the membrane efficiently with 0.5 M NaCl. This step facilitates the binding of mRNA to the membrane and washes away non-specifically bound materials.

For each specific mRNA, the RT-PCR method needs to be experimentally optimized. In this report, the method has been optimized for both TCP-1 and EF-1α by changing several parameters. Optimization is achieved by the choice of primers for RT (e.g., random hexamers, oligo$(dT)_{15}$ or the down-stream primer of the specific gene), by testing concentrations of primers and $MgCl_2$, by optimizing the annealing temperature and times for annealing and elongation, and by changing the number of cycles for PCR. By optimizing the conditions of RT-PCR for EF-1α, we found that using oligo$(dT)_{15}$ for the RT reaction produced a more specific cDNA product in PCR than did the use of random hexamers. We also found that the ratio of first-strand cDNA template to primers is very important in order to get an abundant specific cDNA product with PCR, especially for the RT-PCR amplification of axolotl EF-1α mRNA. In the experiment we compared 1, 2, 3, and 4 µl of RT reaction mixture to do PCR with other parameters optimized, with 4 µl producing the most abundant specific cDNA product without non-specific product.

Acknowledgments

The authors would like to thank M. King for suggesting the original method and S. Duhon for help in preparing this manuscript. This research was supported in part by grants from the US Army Medical Research and Development Command (DAMD 17-91-2-1002) and the National Science Foundation (DIR-9013054).

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