EXPRESSION PATTERN OF GLYCOGEN SYNTHASE, GLYCOGEN PHOSPHORYLASE-2, PHOSPHODIESTERASE INHIBITOR, AND SP60 IN *Dictyostelium discoideum*

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ABSTRACT

In *Dictyostelium discoideum* the transition from growth to differentiation depends on the availability of food. Various gene expressions are involved at each time point throughout the 24 h life cycle. The 0 h (amoeba), 8 h (mound of aggregating cells), and 12 h (a structure of discrete tip containing prestalk cells) stages were selected because of their distinctive morphologies. Total RNA containing the visible 17s and 26s rRNA bands (1,900 and 4,100 bp, respectively) was used. Primers for RT-PCR of glycogen synthase (*GS*: 5'-GAAGC ATCTA AACGT GGTAT C–3' and 5'-CAGAG TGGAT CAAAG ATGAC AGTTG-3'), glycogen phosphorylase-2 (*GP-2*: 5'-GCAGG TTTAG GTAAT GGTGG-3' and 5'-TCACC ATGGG AAAGT GAACG-3'), phosphodiesterase inhibitor (*PDI*: 5'-GATAA ATGCA CTAGC CCAGA-3' and 5'-GGTGG TTGGC CATGT ATTAA-3'), and *SP60* (5'-GTAGG TGCCT TATGT ATGGG-3' and 5'-ACTTG GGTTG TCCAA AGGGT-3') were designed from the conserved regions of those genes in other organisms recorded in GenBank. The difference of products from mRNA/cDNA and genomic DNA amplification can be differentiated because those primers were designed to surround an intron (100 bp) of the genes. The 400 bp RT-PCR product of *GS* was detected from 0-8 h. The 200 bp RT-PCR product of *GP-2* was detected after 8 h while the expression of *PDI* (529 bp) could be detected from 0-8 h. The 418 bp RT-PCR product of *SP60* was expressed at 12 h. The above results show that RT-PCR can indicate the expression of developmentally regulated genes in *D. discoideum* instead of Northern analysis and protein purification.

KEYWORDS: *Dictyostelium discoideum*, slime mold, development, differential expression, slime mold, RT-PCR, RNA

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1. INTRODUCTION

Dictyostelium discoideum is a simple eucaryotic cellular slime mold which is rarely found in nature. Normally, they live together with bacteria, especially *Escherichia coli* (*E. coli*) which they use a source of food. It is hard to grow *D. discoideum* in pure culture except for mutant strains. The morphology depends on the availability of nutrients. When food is abundant, a unicellular amoeba will be found. If nutrients become limited, cells start to differentiate [1]. During this time, the individual amoeba can switch its genetic program from growth to cellular differentiation. Developmentally regulated genes and factors have been studied for many years in order to understand a mechanism of development which may apply to similar mechanisms in other organisms, especially human. For example, the enzyme activity of glycogen phosphorylase (gp-2) increases after 12 h of starvation during *D. discoideum* development. Later, it is known that the synthesis of gp-2 is required to catalyze the breakdown of glycogen into glucose monomers that are then used to generate the carbohydrate structural components of the final differentiated state [2, 3]. The *gp-2* has also been shown to be responsive to various developmental signals, i.e. cAMP, Differentiation Initiation Factor (DIF), NH_3, and adenosine [4]. Another enzyme is 5' nucleotidase (5nu) which has its highest activity at 20 h of development. This enzyme can degrade 5' Adenosine Monophosphate (5'AMP) which is the product of the degradation of cAMP by cAMP-phosphodiesterase. The products from the activity of 5nu are adenosine and inorganic phosphate. If 5nu is inactivated at this time, future development of *D. discoideum* can not occur [5, 6, 7]. Based on the temporal regulation of developmentally regulated genes and their responsiveness to developmental cues, it is likely that analysis of the control of these genes may uncover basic transcriptional determinants involved in the regulation of genes required for the transition from growth to differentiation [8].

2. MATERIALS AND METHODS

Cell culture and harvesting

Dictyostelium discoideum strain AX3K was used to inoculate HL5 media (60 mM glucose, 1% (w/v) Oxoid peptone, 0.5% (w/v) Oxoid yeast extract, 2 mM Na_2HPO_4 and 3 mM KH_2PO_4). Stock culture of amoebae was diluted to a density of 10^5 cells/ml. The medium was supplemented with 25 μg/ml dihydrostreptomycin or streptomycin sulfate to prevent bacterial growth. Cells were shaken 130 rpm at 20°C until the density reached log phase ($5x10^6$ cells/ml). For differentiated cells, 10^8 cells were washed free of nutrient media, then plated on filter saturated with 1x MES LPS (7 mM MES, 20 mM KCl and 2.5 mM $MgSO_4$, pH 6.5). Plated cells were incubated at 20°C until the desired stage of development was reached.

Genomic DNA isolation

Genomic DNA was isolated by a column based method from Qiagen and a procedure for small amounts of *D. discoideum* cells as described in [9]. For the Qiagen method, $1x10^8$ amoeba were used as the starting material. The method followed is described in the QIAamp Blood kit, catalog number 29104, from Qiagen.

The method in [9] required AX3K amoeba (200 μl of $5x10^6$ cells/ml). The cells were pelleted at 2,200xg for 4 min, washed twice with cold (4°C) milli Q H_2O, then resuspended in 100 μl PCR buffer. The sample was mixed, then incubated at 56°C for 45 min, 95 °C for 10 min and chilled on ice. After centrifugation at 14,000xg for 2 min, the supernatant was transfered into a new tube and was used for PCR analysis.

RNA and mRNA isolation

Total RNA was isolated from *D. discoideum* cells as described in [10]. Briefly, the frozen cell pellet was resuspended in 1 ml denaturing solution per 10^7 cells (2 M guanidium thiocyanate, 45 mM sodium citrate, pH 7, 1% Sarkosyl and 0.7% (v/v) 2-mercaptoethanol) and mRNA was isolated from total RNA using either the Poly A Tract mRNA isolation kit (Promega) or the Poly A+ mRNA isolation kit (Oligotex). mRNA was also directly isolated from *D. discoideum* cells by the Quick prep micro mRNA purification kit (Pharmacia Biotech). The concentration of RNA and mRNA were assayed spectrometrically at 260 nm. Agarose gel electrophoresis was performed using 5 μg of total RNA or 1 μg of mRNA to verify the quality of the RNA.

Primers for Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and PCR

Primers directed against actin 8
5' GGACG GTGAA GATGT TCAAG 3' and
5' CTCCC ACACT GTACC AATCT 3'
Primers directed against *gs*
5' GAAGC ATCTA AACGT GGTAT C 3' and
5' CAGAG TGGAT CAAAG ATGAC AGTTG 3'
Primers directed against *gp-2*
5' GCAGG TTTAG GTAAT GGTGG 3'
5' TCACC ATGGG AAAGT GAACG 3'
Primers directed against *SP60*
5' GTAGG TGCCT TATGT ATGGG 3' and
5' ACTTG CGTTG TCCAA AGGGT 3'
Primers directed against *PDI*
5' GATAA ATGCA CTAGC CCAGA 3' and
5' GGTGG TTGGC CATGT ATTAA 3'

Optimal conditions of RT-PCR and PCR

RNA (150 ng) in DEPC-H_2O were transfered into two PCR tubes. One tube received a reaction mixture containing RT while the other reaction mixture contained no RT (control). PCR buffer containing 2 μM of 3' PCR primer, 20 units of RNasin, and 2.5 units of AMV RT was added to the RNA sample and mixed. The reaction was incubated at 42°C for 1 h and 100°C for 10 min. After it was cooled on ice for 5 min, 0.5 μM 3' PCR primer, 1 μM 5' PCR primer, and 2.5 units of *Taq* DNA polymerase were added to the template reaction. The PCR program started with the denaturing temperature at 94°C for 2.5 min, followed by 34 cycles of 94°C for 30 sec, annealing temperatures of 58°C for 30 sec and 72°C for 3 min, and followed by 72°C of extension for 10 min. After amplification, agarose gel electrophoresis was performed.

3. RESULTS AND DISCUSSION

Three time points (0 h, 8 h and 12 h of development) from the 24 h life cycle of *D. discoideum* were chosen because of distinguished morphology (**Figure 1**). When nutrients are abundant, this organism exists as a unicellular organism which is defined as 0 h of development. Mound of several thousands begin to form after 8 h of starvation, but the cells retain their individual identities and do not fuse. At 12 h, the "first finger" stage is visible. The structure posseses a

discrete tip containing prestalk cells that will develop into stalk cells at maturation. The finger-like structure will form a slug which is a motile, worm-like creature that migrates toward light or warmth until the second finger forms at 20 h of development. Finally, true stalk and spore cells develop.

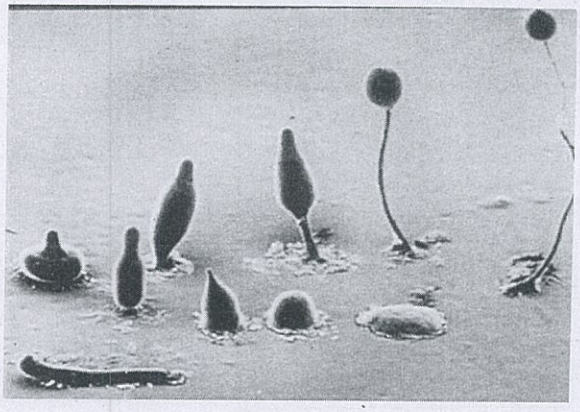

Figure 1. The life cycle of *Dictyostelium discoideum.*

In order to observe the developmentally regulated expression of interesting genes, it is very important to obtain a high yield of undegraded mRNA. The condition of the extracted RNA was assessed by agarose gel electrophoresis as described in Materials and Methods. Total RNA from the amoeba developmental stage is a representative. The 17S and 26S ribosomal RNA bands (1,900 and 4,100 bp, respectively) are clearly visible, indicating that the RNA sample was not degraded (**Figure 2**).

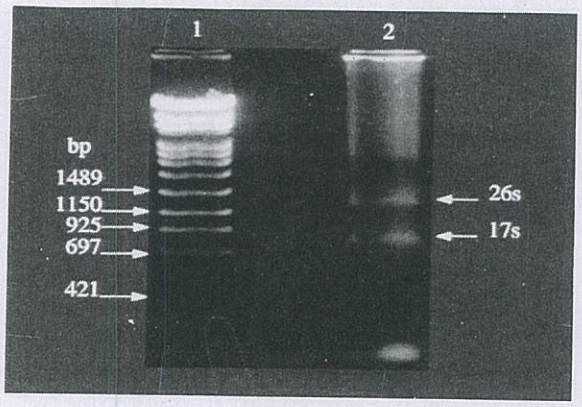

Figure 2. Examination of total RNA. Lane 1 contains DNA marker IV . Lane 2 contains total RNA from the amoeba developmental stage separated on a 1% agarose gel.

Then, RT-PCR was carried out using extracted total RNA or mRNA as template to detect developmentally regulated genes (*gp-2* and *gs*, **Figure 3**). Both of these genes are expressed at a low level. Detection of these genes from genomic DNA serves as a positive control for the PCR portion of the reaction. For both genes, PCR primers were designed to surround an intron, so that DNA and RNA could be distinguished. Additionally, identical reactions lacking reverse transcriptase (RT) were performed in order to show that the RNA-

derived band is RT-dependent. Thus, based on this RT-dependence and on the size of the band, it can be established that the band is the result of amplifying mRNA.

Figure 3 shows the result of the RT-PCR analysis for amoeba, 8 h, and 12 h time points. When using primers for *gs* and *gp-2*, smaller PCR products (400 bp and 200 bp, respectively) can be seen in the lanes containing AMV RT, but the larger DNA-derived PCR products (500 and 300 bp, respectively) are seen in the lanes lacking RT. The lack of a 200 bp product for *gp-2* at 0 h was visible. It shows that this gene is not expressed in amoeba. The larger PCR products in the lanes containing RT are the result of contaminating genomic DNA. Although the 300 bp genomic product can be seen faintly in the +RT lane for amoeba RNA (See **Figure 3b**, lane 1), it is far fainter than the -RT control (**Figure 3b**, lane 2) for this sample. It is possible that this result can be attributed to promiscuous binding of the *gp-2* 3' primer to non-*gp-2* sequence. Several smaller PCR products can be seen faintly in the +RT lane and these smaller products may be substracting primer, nucleotides, etc. from the reaction amplifying the genomic contaminant, thus reducing the intensity of the 300 bp genomic band. The *gs* 400 bp PCR product can be detected, even though this gene is known to be expressed only in low quantities. In addition, the RNA-derived PCR product for *gp-2* demonstrates the expression pattern of the gene, there is no expression during amoeba stage and detectable expression at 8 h of development.

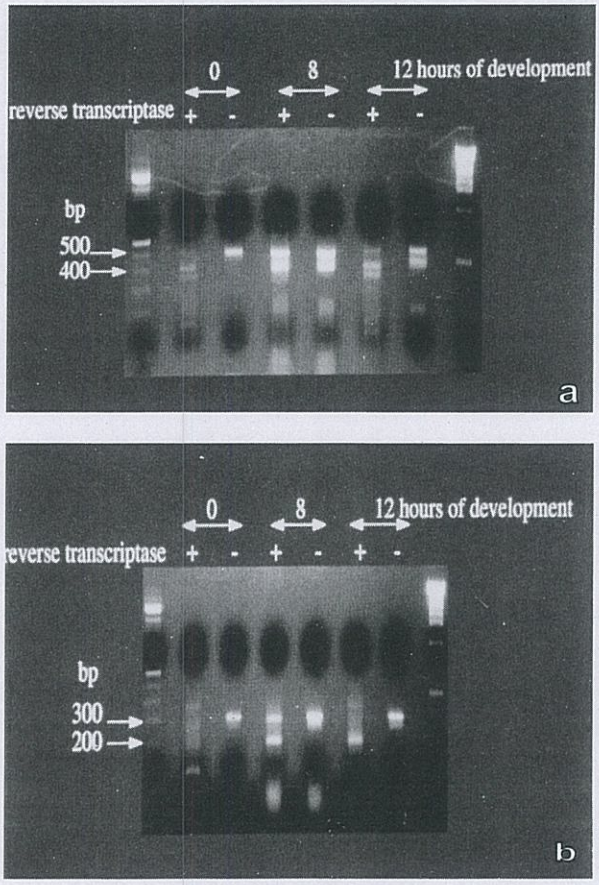

Figure 3. Analysis of mRNA by RT-PCR. mRNA from amoeba (0), 8, and 12 h developmental stages were subjected to RT-PCR using primers for *gs* (3a) and *gp-2* (3b). + and - refer to the presence or absence of RT, respectively. For *gs,* the 500 bp band is DNA-derived, while 400 bp band is RNA-derived. For *gp-2,* the 300 bp band is DNA-derived, while the 200 bp band is RNA-derived.

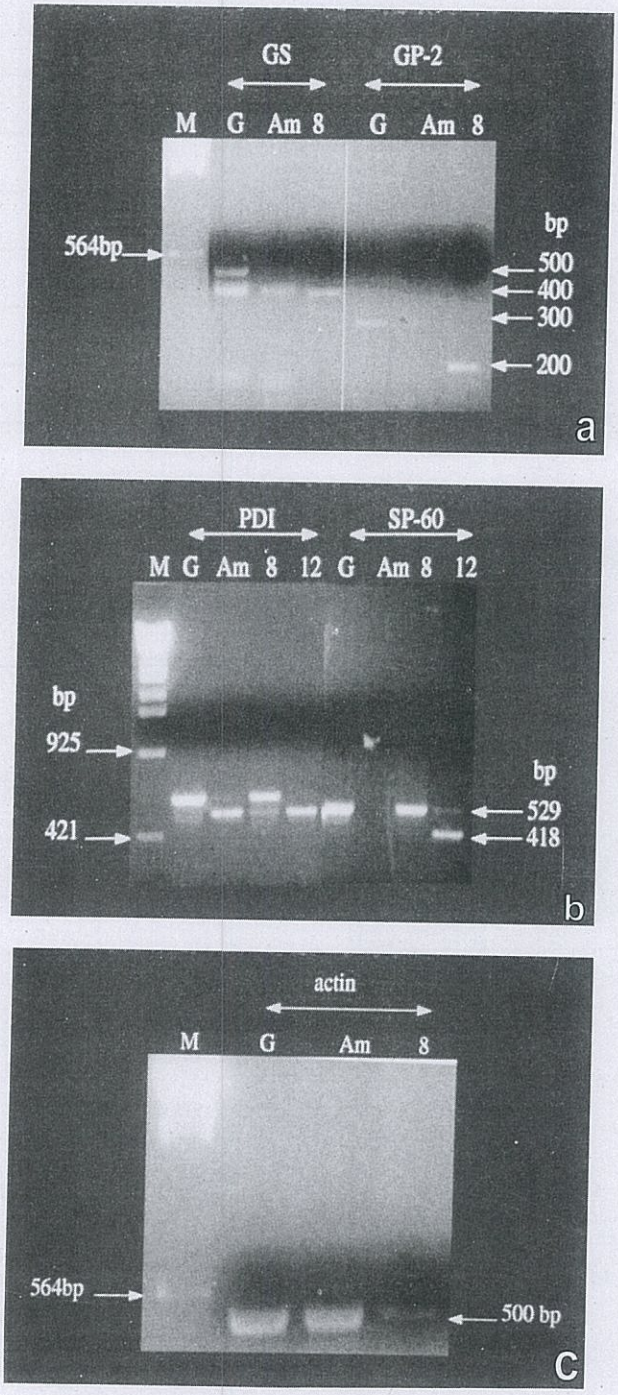

Figure 4. Comparison of PCR product and RT-PCR product. RNA from amoeba (Am), 8 h and 12 h developmental stages were amplified by using primers directed against *gs, gp-2, PDI, SP60,* and actin 8. G: genomic DNA, M: λ *Hind*III marker (**Figures 4a** and **4c**), λ *Sty* I marker (**Figure 4b**).

Furthermore, RT-PCR was performed by using other primers designed to surround introns and against *PDI* and *SP60*. Identical RT-PCR reactions were performed as described in Materials and Methods. The product of 529 bp from *PDI* and 418 bp from *SP60* primers were present in the mRNA. Furthermore, the product of 629 bp and 518 bp from *PDI* and *SP60* primers were present in the -RT reactions as expected (**Figure 4**). Also, *PDI* expresses from 0-8 h while *SP60* expresses at 12 h of development. In addition, amplification using actin 8 primers was used as control to indicate that the entire coding sequence of the message was likely to be found in the RT-PCR experiments. The actin primers were designed to direct against the extreme 5' end of this gene and included the start codon. The product of 500 bp from actin 8 was present in the experiment (**Figure 4**)

From the above data, actin 8 is constitutively expressed at constant, high levels throughout the life cycle of *D. discoideum*. *gs* is included as an additional constitutively expressed gene; however, it is expressed in low levels throughout the life cycle. *gp-2* is inactive during vegetative growth, but becomes transcriptionally active very early in development during aggregation. *SP60* is expressed only late in development during the first finger stage. *PDI* is expressed from the onset of starvation to about 8 h of development.

4. CONCLUSION

During the development of *D. discoideum,* the expression of developmentally regulated genes can be detected by RT-PCR technique, as an alternative to Northern analysis and protein purification. RNA at 0, 8, and 12 h of development were collected. Good quality RNA was determined by the following methods; 1) the ratio of OD260/OD280 should be 2.0 spectrometrically. 2) the 17s and 26s (1,900 and 4,100 bp, respectively) rRNA bands should be visible on 1% agarose gel. RT-PCR was performed by using the presence and absence of AMV Reverse Transcriptase with various primers from *gp-2, SP-60*, and *PDI*. Accurate time points of the expression of some developmentally regulated genes were obtained. *gp-2* was expressed after 8 h of development and *SP60* after 12 h of development. The expression of *PDI* was detected from 0-8 h of development. The full length of mRNA could be determined by using actin 8 primers which were designed to encompass the start codon of the gene. Primers from rare gene (*gs)* was also used. If such a rare gene could be obtained from the isolation, it is possible that other rare genes may be similarly identified.

5. ACKNOWLEDGMENTS

The author would like to thank Professor Dr. Charles L. Rutherford and Dr. Reyna L. Favis for their help and advice. Also, the author would like to thank the Development Grants for New Faculty Researchers of Chulalongkorn University and the Asahi Glass Foundation for financial support.

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