ISOLATION OF FULL-LENGTH RAT AND MOUSE AMYLOID PRECURSOR PROTEIN (*APP*) CDNA FROM GLIOMA CELL LINE BY TWO-STEP PCR METHOD

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ABSTRACT: Full-length cDNA of rat and mouse beta-amyloid precursor protein gene (*App)* were isolated from glioma cell line (NG-108-15). Because of the high molecular weight of the gene (around 2.2 kb), after the first stand of *App* cDNA was acquired by RT/PCR, the two-step PCR method was subsequently applied. The first PCR was conducted to fortify and generate two separated fragments (around 1.3 kb) of 5' and 3' ends (with partial overlapping, 0.41 kb) of *App* cDNA. The second PCR was carried out to obtain the full-length of *App* cDNA, which afterward was introduced into a mammalian vector, pcDNA3.1/Zeo+. Since NG108-15 cell is hybridoma line and the two-step method was applied in this study; therefore, four chimerical *App* cDNA clones were gained. Analysis of restrictive endonuclease enzyme fingerprints was subsequently exploited to identify chimera, rat and mouse *App* cDNA positive clones. By using the RT/PCR together with the two-step PCR technique in the present study is therefore shown to be useful and applicable method for isolating complete rat and mouse *App* genes from NG108-15 cell line, and possibly further applied for isolating other genes with high molecular weight and low levels of mRNA expression. The *App* cDNA obtained in this experiment can be utilized for upcoming experiments such as studying the mechanism of beta amyloid processing, its biological and clinical significance, and screening of new potential Alzheimer's disease therapeutic agents.

Key words: Alzheimer's disease**,** amyloid precursor protein**,** two-step PCR, NG cell line

INTRODUCTION: Alzheimer's disease (AD) is a neurodegenerative disorder of the brain molecularly characterized by the neuropathologic findings of intracellular neurofibrillary tangles (from abnormal phosphorylated tau protein) and extracellular amyloid deposits (from beta-amyloid protein) that accumulate in vulnerable brain regions, especially cerebral cortex and hippocampus[1]. It has been reported that AD is the most common form of progressive dementia in the elderly. Early onset form (EOAD, patients with age less than 65) is also found and identical to the most common form of late-onset dementia (LOAD)[2]. The disorder is clinically characterized by early onset of memory deficits, decreased speed of cognitive processing and cognitive functions[3]. Later, there are progressive cognitive deficits and inability to perform the activities of daily living. On average death occurs within 6 years after onset[4]. The causes of AD are not yet fully understood. Sjogren *et al*. (1952) suggested that it has many etiologies, but only one patho-genesis[5]. However, mutations in all known pathogenic genes share in common the fact that they alter processing of beta-amyloid precursor protein (APP), thus lending strong support to the amyloid formation hypothesis[6]. Stokin *et al*. (2005) further concluded that the reduction in microtubule-dependent transport in neuronal cell possibly stimulates proteolytic processing of APP protein, resulting in the development of senile plaques and AD[7]. Furthermore, Delacourte *et al*. (2002) suggested that there is a synergistic effect of amyloid aggregation in neurofibrillary tangles from tau protein[8].

The result from the population-based study conducted by Campion *et al*. (1999) showed that

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PSEN1 and *APP* gene mutations account for 71% of autosomal dominant EOAD[9]. At least several studies have supported that the mutations of *APP* gene cause familial EOAD such as a mutation of V171I[10,11,12]. The human *APP* gene localizes to the proximal part of chromosome 21q21[13]. The *APP* gene contains 19 exons and spans more than 170 kb, and APP has several isoforms existed by alternative splicing of exons 1-13, 13a, and 14-18[14]. All of these isoforms encode multidomain proteins with a single membrane -spanning region. APP protein undergoes post-translational proteolytic processing by alpha -, beta -, and gamma - secretases. Alpha - and gamma - secretases generates soluble amyloid protein, while beta - and gamma - secretases generates APP components with amyloidogenic features[1]. The 4.2 kDa polypeptide is called the "beta-amyloid protein" because of its partial beta-pleated sheet structure[15].

Other genes may involve in AD pathogenesis as modifiers. There was a report shown that *APOE* gene plays an important role in the genetics of LOAD[16]. The presence of the butyrylcholinesterase K variant (*BCHE*) was found to be associated with an increased risk of AD[17]. The *ACE I* allele was also shown to be associated with an increased risk of LOAD[18].

There is no single diagnostic test for AD. The CSF beta-amyloid and tau protein can be used as biologic markers of AD pathophysiology, and these measures may have potential clinical utility in the future diagnosis of AD[19]. The changes in CSF biomarkers, however, occur early in the course of AD in most patients[20]. To date a variety of treatments have been reported to benefit to AD patients. None is cure, but they can temporarily relieve some of the symptoms in some patients. Cholinesterase inhibitors including donepezil, galantamine, and rivasstigmine, and NMDA receptor antagonist, namely memantine are the examples of prescribed drugs for AD patients.

The aim of this experiment was to isolate full-length cDNA of *App* gene from glioma cell line (NG-108-15). The *App* cDNA can be utilized for future experiments such as studying the mechanism of beta-amyloid processing, its biological and clinical significance, and screening of new potential Alzheimer's disease therapeutic agents.

MATERIALS AND METHODS:

Cell culture and medium

NG108-15 or NG (ECACC 88112302) cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY) with high glucose and supplemented with HAT (0.1 mM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine) and 4% fetal bovine serum (FBS). Cells were incubated at 37^{O}C in a humidified atmosphere of 90% air with 10% CO_2 and medium was changed every three days or when they reached 70-80% confluence.

Total RNA extraction

Cell cultures (70 - 80% confluence of two 100 mm culture dishes) were washed several times with phosphate buffered saline (PBS). Total RNA was extracted and cleaned by using RNA isolation kit (Isogen®, Nippon Gene, Toyama, Japan), according to the company protocol. Total RNA was precipitated with isopropanol and diluted with DEPC treated water. The concentration of total RNA was measured by spectrophotometer and kept in –80^{O}C until used for RT/PCR.

App cDNA acquisition

First strands of total cDNA were obtained from total mRNA by using reverse transcriptase system from Superscript™II kit manufactured by Invitrogen, Carlsbed, CA, USA (Superscript™II RNase H negative Reverse Transcriptase for RT/PCR kit). The amount of 2 μg of total RNA was used for 20 μl RT reactions. Two-step PCR method was applied specifically to isolate and amplify rat and mouse *App* cDNA from total cDNA (the schematic representation of two-step PCR is shown in Fig. 1). The volume of 1.0 μl from RT product was used as a template for 20 μl PCR reactions. In the first step, the 5' end of 1,320 bp of *App* cDNA was amplified using the first pair

of specific primers, named “NheI-ratAPP” containing *Nhe*I restriction site (as underlined) at 5' end (ACTG GCTAGCATGCTGCCCAGCCTGGCACTGCTCCTGCT G) and “Primer #318” (CTCTGGCCATGTGTGTCTC TA). The 3' end of 1,290 bp of *App* cDNA was amplified using the second pair of primers, “NotI-ratAPP” containing *Not*I restriction site at 3' end of *App* cDNA (ACTGCGGCCGCCTAGTTCTGCATCTG CTCAAAGAACTTGTA) and “Primer #317” (TCCCGC TGGTACTTTGATGTC). In the second step, the full-length *App* cDNA was isolated and amplified by using PCR products from the first step as templates (with 410 bp overlapping) and using the outer pair of NheI-ratAPP and NotI-ratAPP as primers. The final product contained around 2.2 kb. In both steps, Expand High Fidelity DNA polymerase enzymes (Roche Applied Science, USA) were used. In the initial step, 30 cycles of PCR (1 min 94^{O}C, 2 min 57^{O}C, and 2 min 72^{O}C) were applied, while in the later step only 10 cycles of PCR (1 min 94^{O}C, 2 min 57^{O}C, and 2 min 72^{O}C) were applied, followed by 20 cycles of 1 min 94^{O}C, 2 min 57^{O}C, and 2 min 72^{O}C plus 5 sec of each successive cycle. PCR products were isolated and excised from agarose gel, and purified by GeneMate® glass bead (ISC Kaysville, UT) binding method.

Ligation of rat and mouse *App* cDNA into pcDNA3.1/Zeo+ vector

The insert of *App* cDNA and vector pcDNA3.1/Zeo+ (Invitrogen, USA), which has around 5 kb in size, were digested with *Nhe*I and *Not*I endonuclease restriction enzymes (Roche and New England Biolabs, USA), purified by agarose gel electrophoresis and glass bead method, and later ligated together by T4 ligase (Promega, Medison, Wisconsin). The ligated products were then used to transform into *E. coli* (strain DH5α) and ampicillin was used as a selective antibiotic. Plasmids, which were harvested from *E. coli* were purified by QIAprep Spin Miniprep Kit from QIAGEN.

App-pcDNA3.1/Zeo+ positive clone identification

Colonies of *E. coli* from ampicillin-selective agar plates were marked with number, carefully picked by using pin-tip toothpick (pin-picking), and transferred into each PCR-microtube. The PCR reactions were set up with a pair of NheI-ratAPP and NotI-ratAPP primers. The *Taq* polymerase enzyme (Life Technologies, Inc., USA) was used to extend oligonucleotides. In this step, 20 cycles of PCR (1 min 94^{O}C, 2 min 57^{O}C, and 2 min 72^{O}C) were applied. Positive clones were identified by the size of PCR product, which was around 2.2 kb in size on 1% agarose electrophoresis gel. Because the NG-108-15 is a hybrid line of mouse and rat, thus four possible combinations of full-length sequence of *App* cDNA could be gained. Therefore, positive clones were then selected, propagated, and further subjected to nucleotide sequencing and endonuclease restriction sites characterization. The schematic demonstration of four possible outcomes from two-step PCR method is presented in Fig. 2.

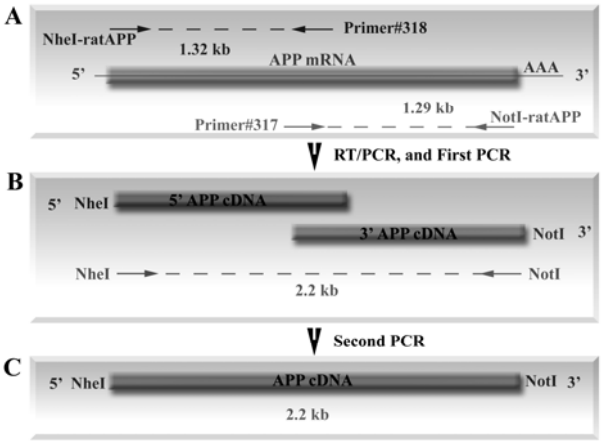


Figure 1 Schematic representation of two-step PCR method. A, in the first PCR, two pairs of specific primers were used to generate two separated 1.3 kb cDNA fragments (5' and 3' end of *App* cDNAs) with 0.41 kb overlapping. B, the second PCR was conducted by using outer specific primers and the mixture of 5' and 3' end *App* cDNA fragments as templates. C, after second step completed, the full-length sequence of *App* cDNA was obtained.

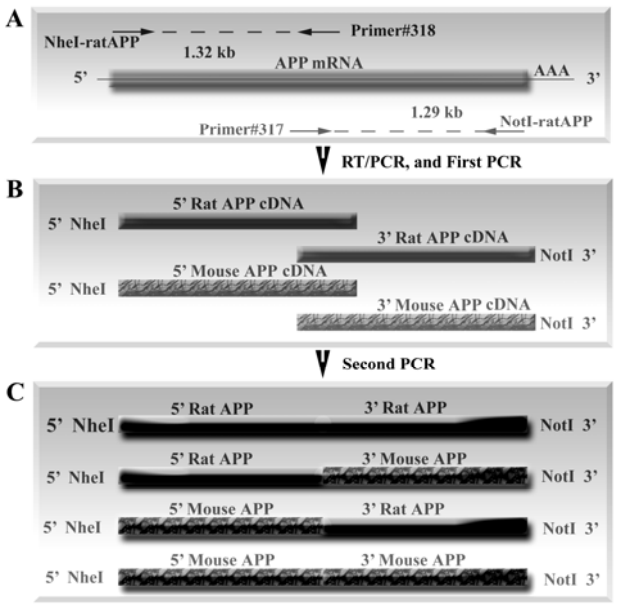
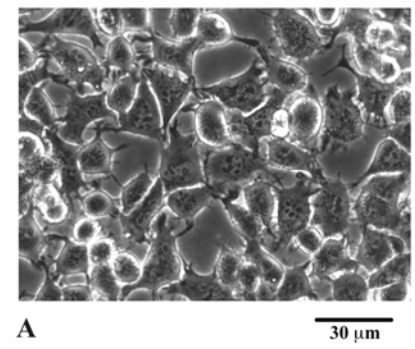


Figure 2 (above) Schematic demonstration of four possible outcomes from two-step PCR method. A, because the NG-108-15 is mouse and rat hybrid cell line, *App* cDNA generated in RT/PCR could be either from rat or mouse mRNA origin. B, four different 5' and 3' end of *App* cDNA fragments were obtained from the first step PCR. C, after the second PCR step, four possible combinations of full-length sequence of *App* cDNA (rat *App*, mouse *App* and two hybrids) could be obtained.

RESULTS: The goal of the present work was to isolate full-length *App* cDNA from NG cells by two-step RT/PCR method. The *App* gene will be further utilized for prospective studies. To achieve this goal, initially NG cells were cultured in DMEM medium with high glucose and supplemented with FBS, hypoxanthine, aminopterin and thymidine. To ensure the total RNA yields, both induced by 1 mM dibuthyl-cAMP and 0.1 μM 12-*o*-tetradecanoyl phorbol 13-acetate (TPA) and non-induced NG cells were cultured and harvested for RNA extraction. Dibuthyl-cAMP and TPA are known as agents that increase intracellular calcium ion resulting in neuronal cells differentiation, increase of neurite outgrowth, and network forming[21]. The morphologic effect of dibuthyl-cAMP and TPA on NG cells is shown in Fig. 3. However, the amount of *App* mRNA expression in induced NG cells was only around 15% more than in non-induced cells (data not shown).



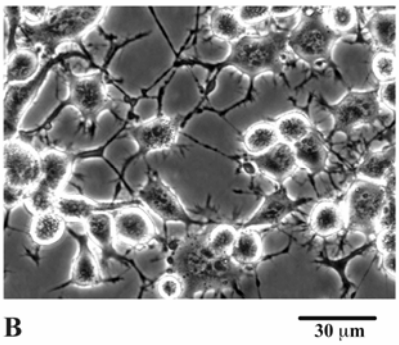


Figure 3A, 3B (right) Pictures taken from culture dishes showed the induction effect of dibuthyl-cAMP and TPA on morphology of NG-108-15 (ECACC 88112302) cells, A, without dibuthyl-cAMP and TPA induction, and B, with dibuthyl-cAMP and TPA induction. More neurite outgrowth and network forming is clearly shown in B than A.

After harvesting the cells, total RNA was isolated by RNA isolation kit, and then first strands of total cDNA were obtained from total RNA by using reverse transcriptase system (Superscript^{TM} II). Several one-step PCR with 5' and 3' ends of specific primers to full-length *App* cDNA (2,200 bp) and *Taq* polymerase were attempted; however, very low yield of full-length *App* was achieved and insufficient for further steps. Thus, two-step PCR and the Expand High Fidelity DNA polymerase were introduced (as described in materials and methods section). In the first step of PCR, two specific sets of primers were designed to generate around 1.3 kb of products, the first set covered 5' end of *App* cDNA and the second set covered 3' end of *App* cDNA. There was an overlapping of 410 base pairs between the first 5' end and 3' end PCR products. By using the outer set of specific primers for the second PCR, the final 2.2 kb of *App* cDNA was achieved (see Fig. 1).

The final PCR products were ligated into pcDNA3.1/Zeo+ vector at *Nhe*I and *Not*1 sites, and the ligated products were than used to transform into *E. coli* (DH5 α strain). Ampicillin was used as a selective antibiotic. Positive-cloned plasmids from the transformed *E. coli* were identified by PCR method. In brief, a tiny amount of positive-cloned *E. coli* in each colony was individual-picked by the tip of bamboo toothpick, and ground against the wall of PCR-microtube with certain amount of PCR reaction buffer. Then, 0.5 µl of mixture was transferred and subjected to PCR reaction, and the PCR products were loaded and electrophoresed on 1% agarose gel. The *App*-positive clones were identified by the size of the PCR product (size 2.2 kb of *App* cDNA), as shown in Fig. 4. This method used is time saving and economical, no need for *E. Coli* propagation, plasmid isolation and purification, and restriction enzyme digestion

Since the NG cell is the hybrid of mouse neuroblastoma and rat glioma, and the fact that the experiment in this study used two-step PCR method, four possible chimerical *App* cDNA products could be obtained, as illustration represented in Fig. 2. The 5' and 3' ends of the *App* cDNAs obtained from this experiment can be either from mouse or rat origin. To resolve the identity problem of obtained *App* cDNA, restriction endonuclease enzyme digestion pattern analysis was further applied. Various restriction endonuclease enzymes were introduced to digest the *App*-positive plasmids (App-pcDNA3.1/Zeo+) at various positions along the full-length *App* cDNAs. The selected

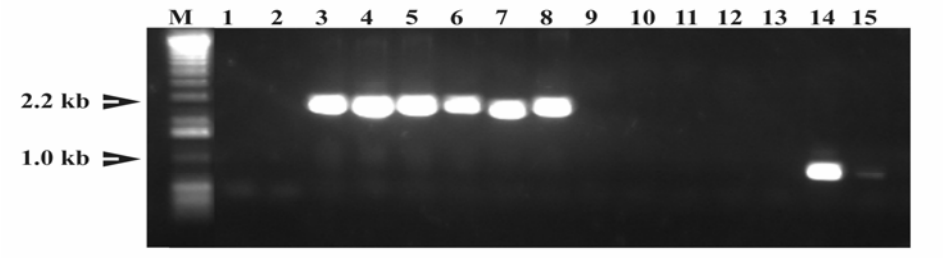


Figure 4 Agarose gel electrophoresis of PCR products of *App* cDNA (~2.2 kb) directly amplified from 15 *E. coli* colonies (8 positive and 7 negative ampicillin-resistant colonies). Positive clones with putative full-length sequence of *App* cDNA were detected in lane number 3, 4, 5, 6, 7, and 8, whereas two positive clones (lane number 14 and 15) with shorter *App* cDNA fragments (~1.0 kb) were also detected. Lane M is the DNA marker (in kb).

restriction endonuclease enzymes were those that gave informative results, leading to identification of either mouse or rat *App* cDNA. For example (see Fig. 5), *Eco*RI and *Xho*I are used to cut *App* gene at 3' end. There is only one cut-site for *Eco*RI in rat *App* cDNA but two cut-sites for *Eco*RI in mouse *App* cDNA. There are the same two cut-sites for *Xho*I in both rat and mouse *App* cDNAs. By using these two enzymes, if the positive clone at 3' end is from rat *App*, three DNA fragments can be seen on the agarose gel (~6.0 kb, 0.4 kb, ~0.4 kb); on the other hand, if the positive clone at 3' end is from mouse *App*, four DNA fragments can be seen on the agarose gel instead (~6.0 kb, 0.4 kb, 0.3 kb, and 0.2 kb). By using various combinations of restriction endonuclease enzymes (as shown in Table 1), the rat *App*, mouse *App*, or chimerical *App* containing clones were identified.

From the experiments, the rat and mouse *App* cDNA were isolated from NG cells and ligated into pcDNA3.1/Zeo+ vector. These *App* cDNAs can be utilized for future experiments.

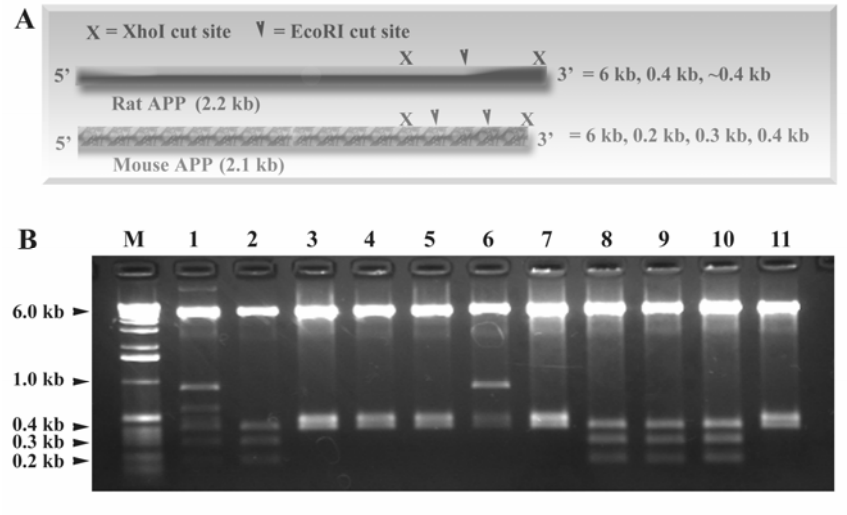


Figure 5 Example of species identification of *App* cDNA obtained from positive *E. coli* clones by analysis of the restrictive endonuclease enzyme fingerprint. A, schematic representation of the *App* cDNA fingerprint digested by *Xho*I and *Eco*RI endonuclease combination, clone containing 3' end of rat *App* generates three fragments, 6.0 kb (vector plus 5' end of *App* fragment), 0.4 kb, and ~0.4 kb; whereas clone containing 3' end of mouse *App* gives four fragments, 6.0 kb (vector plus 5' end of *App* fragment), 0.4 kb, 0.3 kb, and 0.2 kb. B, agarose gel electrophoresis showed five positive clones for 3' end of rat *App* (lane number 3, 4, 5, 7, and 11), four positive clones for 3' end of mouse *App* (lane number 2, 8, 9, and 10), and two mutants with extra ~1.0 kb band (lane number 1 and 6). Lane M is the DNA marker (in kb).

Table 1 Conclusion of the analysis of restrictive endonuclease enzyme fingerprints by using various sets of enzyme combination selected to cover the full region of *App* cDNAs.

Clone #	NheI	Size*	NheI/NotI	EcoRI	XhoI/EcoRI	NheI/XhoI	SmaI	ScaI	Conclusion
1	–	–	–	–	–	–	–	–	Negative clone
2	–	–	–	–	–	–	–	–	Negative clone
3	–	–	–	–	–	–	–	–	Negative clone
4	+	~2.2	+	Rat	Rat	+	Rat	Rat	Positive rat
5	+	~2.2	+	Mouse	Mouse	+	Mouse	Rat	Positive hybrid
6	–	–	–	–	–	–	–	–	Negative clone
7	+	~2.2	–	Rat	–	–	–	–	Positive mutant
8	+	~1.0	–	Mouse	Mouse	–	–	–	Positive mutant
9	–	–	–	–	–	–	–	–	Negative clone
10	+	~2.2	+	Mouse	Mouse	+	Mouse	Rat	Positive hybrid
11	+	~2.2	+	Rat	Rat	+	Mouse	–	Positive hybrid
12	–	–	–	–	–	–	–	–	Negative clone
13	+	~2.2	+	Mouse	Mouse	+	Mouse	Mouse	Positive mouse
14	+	~2.2	+	Rat	Rat	+	Mouse	Rat	Positive hybrid

Note * Size of putative full-length APP cDNA (~2.2 kb) by PCR
(+) Positive for both rat and mouse (–) Negative (no cut site) for that specific enzyme

DISCUSSION AND CONCLUSIONS: *APP* gene is a precursor gene for beta amyloid complex, which plays an important role in AD pathogenesis. Acquiring the full-length *App* cDNA is, therefore, important for studying of AD at its molecular level and searching for a potential substance that can be used as an AD pharmacologic therapy. At the time of the experiment, this was still no commercial full-length *App* gene or cDNA available. Thus we decided to acquire the full-length *App* cDNA by two-step RT/PCR method, and fortunately we could obtain both mouse and rat *App* cDNAs. This approach, consequently, can be applied for other gene isolation from cell lines or tissues.

NG cell line was chosen in this study to isolate *App* gene since this line is derived from neuronal cell, which is likely to constitutively express *App* mRNA, a template for *App* cDNA[22]. Furthermore its availability, easy handling and culturing, and ethical discern are also the advantages. Due to the nature of NG cell, which is a hybrid of mouse and rat cell, thus, this complicated the experiment as previous mentioned. The *App* cDNAs we obtained from this cell line contain four possible combinations. However, this issue can be dissolved easily by the restriction endonuclease enzymes fingerprints, which recognize and cut at specific sites of rat and mouse *App* genes. Subsequently, the species of the obtained *App* cDNAs can be distinguished.

Fresh neuronal tissues such as normal animal brain can be harvested for RNA isolation[23], but the amount of RNA especially full-length *App* mRNA may not be enough for further processes. Also the ethical and animal rights concerns may be unavoidable. Another source that we can get *App* gene is from mRNA or cDNA libraries[24]; nevertheless, the cost may be a major problem. Furthermore, the complete ORF of the *App* cDNA may be difficult to obtain from those libraries due to not only the high molecular weight (2.2 kb) and low abundance of the *App* mRNA itself in some tissues, but also the quality of the libraries. Genes with low molecular weight and high availability in the library normally give higher possibility of full-length cDNAs acquisition by single step PCR method such as human purine nucleoside phosphorylase (*PNP*), which is only 250 bp and presents in high

quantity in liver total RNA and cDNA libraries[24], and peanut oleosins-encoded gene, which contains 531 bp, from seed total RNA library[25]. Therefore, the RT/PCR and two-step PCR applied in this study is a better alternative method for isolating *App* cDNA from cells or tissues as clearly shown and mentioned in the results section. The first-step PCR allowed the increase of PCR yields of both 5' and 3' end of *App* cDNA fragments, especially the 5' end product, which was less availability due to inefficiency of RT/PCR system. The second step PCR resulted in higher yield of full-length product with nuclease enzyme restriction sites.

Using a newer generation of RT/PCR system such as Superscript™ III, which is claimed to be more efficient than Superscript™ II, and a high fidelity DNA polymerase in the PCR reaction[26] may improve the total full-length RNA yield, and possibly that only one-step PCR is required rather than two-step PCR. In this study, TPA and cAMP analogue could induce NG cells to increase the *App* mRNA expression by only 15% (unpublished data) when compared to non-induced condition. Previous studies by Shekarabi *et al*. (1997), Bourbonniere *et al*. (1997), and Gegelashvili (1996) nevertheless, reported that the *App* mRNA expression was increased by two to three folds after dibutyryl cAMP treatment[27,28,29] by changing agents for NG cells differentiation induction such as another cAMP analogue, higher expression of *App* mRNA is possibly expected. Interestingly, Mitani *et al*. (2004) demonstrated that by applying a *Taq* DNA polymerase -specific *Taq* aptamer (5'-GCCGGCCAATGTACAGTA TTGGCCGGC-3') or an antibody against *Tag* was shown to improve the specificity of the PCR[26].

The total cDNA obtained from the present experiment can served as an in-house cDNA library that can be benefit for further research. We applied "pick and quick PCR method" (a tiny amount of individual clones of *E. coli* was individual-picked by the tip of bamboo toothpick and then subjected to PCR) to identify positive colonies that contain and have correct size of *App* cDNA inserts. This method is much efficient in terms of time and cost.

Even though the *App* cDNA in this study has been confirmed by restriction sites analysis, nucleotide sequencing of the gene is the final confirmation. Nucleotide sequencing is also to identify potential isoform, point mutation, deletion, and addition that possibly occur on the gene of interest. From our sequencing results and the public genome database, it has shown that there is a fairly high homology (> 90%) in both nucleotide sequence of *App* cDNA and protein sequence among human (GenBank accession number NM-000484), rat (accession number BC062082), and mouse (accession number BC005490). There are several amino acids in human beta-amyloid peptide that are different from rat and mouse. The sequence of human beta-amyloid peptide is DAEFRHDSGYEVHHQKLVFFAEDVGSNK GAIIGLMVGGVVIA, whereas in rat and mouse is DAEFGHDSGFEVRHQKLVFFAEDVGSNKGAIIGLMV GGVVIA (the different amino acids are underlined). Even though the difference in those amino acids is small (they still bare the same charges) and are not essential points for well-reported protein-protein interactions or enzyme substrates, the implication of the difference might play important role in beta-amyloid aggregation cascade and/or beta-amyloid complex clearance.

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การแยกสกัดยีน "อไมลอยด์พรีเคอร์เซอร์โปรตีน" ของหนูขาวเล็กและหนูขาวใหญ่แบบเต็มส่วน จากเซลล์ไลน์ชนิดกลีโอมาโดยวิธีพีซีอาร์สองชั้น

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บทคัดย่อ: แยกสกัดยีนอไมลอยด์พรีเคอร์เซอร์โปรตีน (*App* cDNA) ของหนูขาวเล็กและหนูขาวใหญ่แบบเต็มส่วนจากเซลล์ไลน์ชนิดกลีโอมา (NG108-15 cell) เนื่องจาก *App* cDNA มีขนาดโมเลกุลค่อนข้างใหญ่ (ประมาณ 2.2 kb) การทำ RT/PCR และ PCR ตามวิธีปกติเพียงชั้นเดียวจะมีปัญหาในเรื่องการได้ยีนเต็มส่วน (full-length cDNA) ในการศึกษานี้จึงใช้วิธี RT/PCR และ PCR สองชั้น โดยการแยกทำ PCR ยีน *App* cDNA เป็นสองส่วนในชั้นแรก ซึ่งผลจะได้ชิ้นส่วนของยีน 2 ส่วน คือ ชิ้นส่วนด้าน 5′ และ 3′ของ *App* cDNA (ส่วนละ 1.3 kb) โดยทั้งสองชิ้นส่วนจะมีส่วนทับซ้อนประมาณ 0.41 kb หลังจากนั้นจะทำการ PCR ในชั้นที่ 2 โดยใช้ ไพรมเมอร์ชุดนอกที่อยู่ด้าน 5′ และ 3′ของ *App* cDNA และชิ้นส่วนด้าน 5′ และ 3′ของ *App* cDNA ที่ได้จากการทำ PCR ชั้นแรก ซึ่งผลสุดท้ายจะได้ยีน *App* cDNA แบบเต็มส่วนสมบูรณ์และมีปริมาณมากเพียงพอต่อการนำไปตัดต่อเข้ากับพลาสมิดเวคเตอร์ชนิด pcDNA3.1/Zeo+ เนื่องด้วยกลีโอมาเซลล์มีส่วนผสมของสารพันธุกรรมทั้งของหนูขาวเล็กและหนูขาวใหญ่ การแยกยีนโดยวิธีนี้ทำให้มีโอกาสเกิด cDNA ลูกผสมระหว่างหนูทั้งสองพันธุ์ได้ ดังนั้นการนำเทคนิคลายพิมพ์จุดตัดเอนไซม์ตัดเฉพาะมาใช้จึงเป็นประโยชน์ในการระบุโคลน (clone) ที่ได้ว่าเป็น *App* cDNA ของหนูขาวเล็ก หรือหนูขาวใหญ่ หรือเป็นลูกผสม และทำให้สามารถแยกสกัด *App* cDNA ของทั้งหนูขาวเล็กและหนูขาวใหญ่แบบเต็มส่วน จากเซลล์ไลน์ชนิดกลีโอมาได้อย่างมีประสิทธิภาพ วิธีการนี้อาจสามารถนำไปใช้แยกสกัด cDNA อื่นๆที่มีขนาดโมเลกุลใหญ่ หรือนำไปใช้กับเซลล์ หรืออวัยวะที่มีการแสดงออกของ mRNA ในปริมาณที่ต่ำได้ และ *App* cDNA ที่แยกสกัดได้นี้สามารถนำไปใช้ในการศึกษาและวิจัยอื่นๆต่อไป เช่น การศึกษาเกี่ยวกับขบวนการสร้างโปรตีนอไมลอยด์บีต้า หรือใช้ในการคัดกรองสารสำคัญที่สามารถยับยั้งหรือลดการเกิดโรคอัลไซเมอร์

คำสำคัญ: โรคอัลไซเมอร์ อไมลอยด์พรีเคอร์เซอร์โปรตีน พีซีอาร์สองชั้น กลีโอมา เซลล์ไลน์

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