Cloning and characterization of an apolipoprotein C2 promoter in the mouse central nervous system

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Abstract
Apolipoprotein C2 is an important member of the apolipoprotein C family, and is a potent activator of lipoprotein lipase. In the central nervous system, apolipoprotein C2 plays an important role in the catabolism of triglyceride-rich lipoproteins. Studies into the exact regulatory mechanism of mouse apolipoprotein C2 expression have not been reported. In this study, seven luciferase expression vectors, which contained potential mouse apolipoprotein C2 gene promoters, were constructed and co-transfected with pRL-TK into HEK293T cells to investigate apolipoprotein C2 promoter activity. Luciferase assays indicated that the apolipoprotein C2 promoter region was mainly located in the +104 bp to +470 bp region. The activity of the different lengths of apolipoprotein C2 promoter region varied. This staggered negative-positive-negative arrangement indicates the complex regulation of apolipoprotein C2 expression and provides important clues for elucidating the regulatory mechanism of apolipoprotein C2 gene transcription.

Key Words
neural regeneration; basic research; apolipoprotein C2; promoter; dual-luciferase reporter assay; transcriptional activity; regulatory elements; grants-supported paper; neuroregeneration

Research Highlights
(1) The present study analyzed the transcriptional activity of the mouse apolipoprotein C2 promoter using the dual-luciferase assay.
(2) This study analyzed transcriptional activities of different regions of the upstream apolipoprotein C2 gene sequence and the binding site of the transcription factor. It also identified the promoter region of apolipoprotein C2, and primarily revealed the structure and functional characteristics of the apolipoprotein C2 upstream sequence.

Abbreviations
GATA1, GATA binding protein 1; TFIID, TATA binding protein of transcription factor IID

INTRODUCTION
The brain is the most lipid-enriched organ in the body, containing 25% of the body’s total unesterified cholesterol[1]. Due to the blood-brain barrier, lipid and lipoprotein metabolism in the brain is different from the rest of the body. Apolipoprotein C2 is a necessary cofactor for the activation of lipoprotein lipase and plays an important role in lipid metabolism through hydrolysis.
of triglycerides\textsuperscript{[2-4]}. Apolipoprotein C2 also plays an important role in the transport and metabolism of lipids in the central nervous system. Apolipoprotein C2 polymorphism has been identified as a risk factor for several neurological diseases. Furthermore, cerebral expression of apolipoprotein C2 is significantly altered in several brain disorders. The apolipoprotein C2 locus has also been tested for linkage to familial Alzheimer's disease. In fact, an abnormality in apolipoprotein C2 has been demonstrated to increase the incidence of Alzheimer's disease\textsuperscript{[5-8]}. Apolipoprotein C2 deficiency is related to a lipid encephalopathy and spinocerebellar ataxia type 12\textsuperscript{[9-10]}.

Mouse apolipoprotein C2 localized on chromosome 7 includes four exons and three introns. Nucleotide sequence analysis has indicated that apolipoprotein C2 is synthesized with a 22-residue signal peptide that is cleaved cotranslationally in the rough endoplasmic reticulum. The remaining single-chain polypeptide of 75 amino acid residues has a molecular mass of 8.5 kDa\textsuperscript{[11]}. Given the above observations, studies into apolipoprotein C2 expression and regulation mechanisms are important for research into neurological diseases.

To the best of our knowledge, studies concerning the exact regulatory mechanisms of mouse apolipoprotein C2 expression have not been reported. In this study, seven luciferase expression vectors which contained potential mouse apolipoprotein C2 promoter were constructed, and co-transfected with pRL-TK into 293T cells. Apolipoprotein C2 promoter activity was investigated, including analysis of the promoter structure and cis-acting elements. This study analyzed the transcription factor binding site in the apolipoprotein C2 promoter region (from the Bioinformatics Database) in efforts to provide a better understanding of the transcription regulation of apolipoprotein C2.

**RESULTS**

**Cloning of DNA fragments from the apolipoprotein C2 promoter region**

Electrophoresis revealed bands for PCR products of the apolipoprotein C2 gene promoter region. The positive bands appeared to contain 2 430 bp, 1 821 bp, 1 365 bp, 968 bp, 685 bp, 425 bp, and 367 bp (Figure 1).

**Construction and verification of the recombinant luciferase reporter plasmid**

The recombinant luciferase plasmids containing apolipoprotein C2 promoter were double digested by \textit{KpnI} and \textit{XhoI}, and the electrophoresis result is shown in Figure 2. The lengths of PCR production were consistent with expectations. DNA sequencing results further confirmed that the recombinant plasmid pGL3-Basic-apolipoprotein C2-X was successfully constructed.

![Figure 1](https://example.com/figure1.png)

*Figure 1  PCR products of different fragments of apolipoprotein C2 (ApoC2) gene promoter region.*

M1: DL15000 DNA marker; M2: DL2000 DNA marker; 1: ApoC2-A; 2: ApoC2-B; 3: ApoC2-C; 4: ApoC2-D; 5: ApoC2-E; 6: ApoC2-F; 7: ApoC2-G. A–G: 7 promoter segments with different lengths.

![Figure 2](https://example.com/figure2.png)

*Figure 2  Identification of pGL3-ApoC2-X by Kpn I and Xho I double digestion.*

M1: DL15000 DNA marker; M2: DL2000 DNA marker; 1: pGL3-basic-ApoC2-A; 2: pGL3-basic-ApoC2-B; 3: pGL3-basic-ApoC2-C; 4: pGL3-basic-ApoC2-D; 5: pGL3-basic-ApoC2-E; 6: pGL3-basic-ApoC2-F; 7: pGL3-basic-ApoC2-G. A–G: Recombinant luciferase plasmids containing 7 ApoC2 promoter segments with different lengths. ApoC2: Apolipoprotein C2.

**Transient co-transfection and luciferase assays**

The seven recombinant luciferase reporter plasmids containing different lengths of mouse apolipoprotein C2 promoter region (pGL3-Basic-apolipoprotein C2-A–G) were co-transfected with internal control plasmid pRL-TK into 293T cells, and the luciferase activity was then detected. The wells with pGL3-Basic and pRL-TK were designated the control group. Transcriptional activity analysis of the apolipoprotein C2 promoters showed that...
the activity of pGL3-Basic-apolipoprotein C2-F was highest, whilst the activity of pGL3-Basic-apolipoprotein C2-E, G and C was intermediate. The activity of pGL3-Basic-apolipoprotein C2-A, B and D was lowest (Figure 3). These results suggested that the apolipoprotein C2 core promoter was located in the region of +104 bp to +470 bp. There were also negative regulatory elements in the region at −1 350 bp to −894 bp and −497 bp to −214 bp, and positive regulatory elements at +45 bp to +104 bp.

Analysis of transcription factor binding sites of the apolipoprotein C2 promoter

Analysis for transcription factor binding sites in the apolipoprotein C2 promoter (−1 350 bp to +470 bp) using http://www.cbil.upenn.edu/cgi-bin/tess/tess revealed many transcription factor binding sites in this region. Analysis of the apolipoprotein C2 promoter (−1 350 bp to −894 bp) showed that there were six AP1 binding sites (Figure 4A). The region of the promoter, −497 bp to −214 bp, contained several consensus sites for factors including GATA binding protein 1 (GATA1), YY1 transcription factor (YY1) and TATA binding protein of transcription factor IID (TFIID; Figure 4B). There were GATA-1, trans-acting transcription factor 1 (Sp1), CAC-binding protein binding sites and TATA, CAAC conserved sequences in the region of +104 bp to +470 bp, where the core promoter is located (Figure 4C). A schematic map of mouse apolipoprotein C2 upstream regions is displayed in Figure 5.

DISCUSSION

The apolipoprotein C family includes apolipoprotein C1, C2 and C3. These molecules play important roles in lipoprotein metabolism and pathogenesis of various human diseases[12]. Apolipoprotein C2, the most important member of the family, is conserved in mammals, and maintains the stability of lipid metabolism mainly through its c-terminal mediated activation of lipoprotein lipase[13]. Based on these background findings, it has been suggested that apolipoprotein C2 may be critically involved in the development of human evolution characterized by lipid profile[14].

There are dramatic quantitative changes in the expression of apolipoprotein C1 and apolipoprotein C2 in the human brain. It has been reported that apolipoprotein C1 mRNA level is variable, with the level being higher in children younger than 5 years of age, and then gradually dropping to adult levels[15-17].

Figure 3  Activity analysis of different DNA fragments of apolipoprotein C2 (ApoC2) promoter in HEK293T cells. □: pGL3-Basic+TK; ■: pGL3-ApoC2-X+TK. TK is the pRL-TK vector, which contains the herpes simplex virus thymidine kinase promoter to provide low to moderate levels of Renilla luciferase expression in co-transfected mammalian cells. *P < 0.05, vs. pGL3-Basic+TK group.

Figure 4  Transcriptional factor binding site analysis of the regions of apolipoprotein C2 promoter –1 350 bp → –894 bp (A), −497 bp → −214 bp (B), and +104 bp → +470 bp (C). Underlined sequences represent binding sites for transcriptional factors.
By contrast, apolipoprotein C2 expression is at minimum levels in most babies, and increases during the adolescent period. This pattern indicates that while apolipoprotein C1 is being down-regulated, apolipoprotein C2 may be up-regulated\[15-17\]. Twenty-two known apolipoproteins exist in the central nervous system, nine have been able to be detected at the protein level, and eight at mRNA level. Apolipoprotein C2 has been detected both at the protein and mRNA level\[15-17\]. However, the regulation and possible role of apolipoprotein C2 in the central nervous system are poorly understood.

To the best of our knowledge, the present study is the first report characterizing transcriptional activity of the mouse apolipoprotein C2 promoter. Results show that the transcriptional activities of seven recombinant luciferase reporter plasmids containing different lengths of mouse apolipoprotein C2 promoter region were greatly different in HEK293T cells. The results suggested that the apolipoprotein C2 core promoter was located between +104 bp to +470 bp. Negative regulatory elements between –1 350 bp to –894 bp and –497 bp to –214 bp were found. Positive regulatory elements between +45 bp to +104 bp were also seen. Bioinformatics analyses were used to analyze the transcriptional factor binding sites of the apolipoprotein C2 promoter –497 bp to –214 bp. The promoter region of apolipoprotein C2 –497 bp to –214 bp contains several consensus sites for factors including GATA-1, YY1 and TFIID. YY1 is a ubiquitously distributed transcriptional factor belonging to zinc finger proteins. Studies in mouse and Xenopus have shown that YY1 plays an important role in the developing nervous system\[23-25\]. YY1, as an activator of the beta-site amyloid precursor protein-cleaving enzyme 1 promoter in neurons and astrocytes, plays a role in Alzheimer’s disease\[26\]. Transcriptional factor binding sites for TATA, CAAC conserved sequence, GATA-1, Sp1, and CAC-binding protein were also seen in the region of the apolipoprotein C2 promoter +104 bp to +470 bp, where the core promoter was located. Sp1, known as Specificity Protein 1, is a transcriptional factor involved in gene expression in early development\[27-28\]. Sp1 is expressed in the central nervous system, with expression being much higher in glia than neurons\[29\]. AP1, YY1 and SP1 may directly bind to cis-sites in the promoter region of the apolipoprotein C2 gene and regulate the expression of apolipoprotein C2 in the central nervous system. However, this hypothesis requires further investigation.

Research into the genetic structure and the transcriptional regulation of apolipoprotein C2 in the brain is important to explore the underlying mechanisms of neurological diseases, and so reveal novel therapeutic targets. More and more evidence has shown that apolipoprotein C2 plays a beneficial role in several neurological diseases. Thus, it is tempting to speculate that agents aimed at altering apolipoprotein C2 expression level may have therapeutic applications.

**MATERIALS AND METHODS**

**Design**

An engineered cytological controlled experiment.

**Time and setting**

Experiments were performed at the Department of Laboratory Animals of China Medical University, China from 2009 to 2011.

**Materials**

Human embryonic kidney cells (HEK293T) and C57BL/6J mice were supplied by the Department of
Laboratory Animals of China Medical University, China.

**Methods**

**Cell culture**

HEK293T were grown in RPMI-1640 containing 10% heat inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA), at 37°C in 5% CO₂. When the cells were in the logarithmic phase, they were cultured on 96-well plates. All cell culture reagents other than fetal bovine serum were purchased from Invitrogen, Carlsbad, CA, USA.

**Bioinformatics analysis and PCR primer design**

We analyzed the region of apolipoprotein C2 (GenBank Gene ID: 11813) –2 000 bp to +700 bp using http://www.fruitfly.org/seq_tools/promoter.html, and designed seven pairs of PCR primers based on the results:

| Primer        | Sequence (5’–3’ Position (bp)) |
|---------------|---------------------------------|
| Apolipoprotein | CCGGGTACCGTCTAGGT –1 959 to –1 940 |
| C2-A (sense)  | CCGGGTACCGCACCACCC –1 350 to –1 332 |
| Apolipoprotein | CCGGGTACCTCTGCCT –214 to –197 |
| C2-C (sense)  | CCGGGTACCTCTGCCT –214 to –197 |
| Apolipoprotein | CCGGGTACCTCTGCCT –214 to –197 |
| C2-D (sense)  | CCGGGTACCTCTGCCT –214 to –197 |
| Right-         | CCGGGTACCGGAGGTGA –894 to –877 |
| apolipoprotein | CCGGGTACCGGAGGTGA –894 to –877 |
| C2 (antisense) | CCGGGTACCGGAGGTGA –894 to –877 |

Right-apolipoprotein C2 (antisense) is the common downstream primer. KpnI, XhoI sites and their protection bases were added to the 5’-termini of the upstream and downstream primers (underlined).

**Construction of mouse apolipoprotein C2 promoter-luciferase reporter**

C57BL/6J mouse tail genomic DNA was used as a PCR template, and seven promoter segments with different lengths were amplified using PCR. Plasmid pGL3-basic and the seven promoter DNAs were double digested with restriction enzymes KpnI and XhoI. Several colonies were obtained after ligation and transformation. The colonies were identified by PCR, restriction enzyme and sequence analysis. The successfully constructed plasmids were named pGL3-Basic-apolipoprotein C2-A (2 430 bp), pGL3-Basic-apolipoprotein C2-B (1 821 bp), pGL3-Basic-apolipoprotein C2-C (1 365 bp), pGL3-Basic-apolipoprotein C2-D (968 bp), pGL3-Basic-apolipoprotein C2-E (685 bp), pGL3-Basic-apolipoprotein C2-F (425 bp), and pGL3-Basic-apolipoprotein C2-G (367 bp).

**Transient transfection**

pGL3-Basic-apolipoprotein C2-X and pRL-TK were co-transfected into 293T cells under the mediation of Lipofectamine™2000 (Invitrogen). pGL3-Basic-apolipoprotein C2-X plasmid was 200 ng/well, and pRL-TK was 20 ng/well, and 10% fetal bovine serum was added 4 hours after co-transfection.

**Luciferase assays**

Transfected cells were incubated for 48 hours at 37°C and washed three times with PBS. Cells were lysed and the dual-luciferase assay was performed according to the manufacturer’s instructions (Promega, Madison, WI, USA). Each transfection was performed in triplicate and experiments were carried out a total of three times.

**Transcriptional factor binding sites of the apolipoprotein C2 promoter**

Transcription factor binding sites of the apolipoprotein C2 promoter –1 350 bp to +470 bp were analyzed through http://www.cbil.upenn.edu/cgi-bin/tess/tess.

**Statistical analysis**

All experimental data were analyzed using the SPSS 17.0 software (SPSS, Chicago, IL, USA). The differences in the mean value between groups were compared using one-way analysis of variance. Paired comparisons of the mean value between groups were performed using a two-sample t-test. A value of $P < 0.05$ was considered statistically significant.

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**Author contributions:** Zhihong Zheng was in charge of funds, provided and integrated the data, designed the study and approved the final version of the manuscript. Zhaoyang Li provided technical support, collected and analyzed data, and wrote the manuscript. Bing Du and Wei Wang cultured cells. Zhaoyang Li and Shengyang Li analyzed the data. Xiangchuan Lv, Shenglai Zhou and Yang Yu supplied the C57BL/6J mice (specific pathogen free). All authors approved the final version of the manuscript.

**Conflicts of interest:** None declared.

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