Biol. Proced. Online 2002;3(1):79-90.

Investigation of Neuronal Cell Type-Specific Gene Expression of Ca^{2+}/Calmodulin-dependent Protein Kinase II.

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Submitted: February 22, 2002; Revised: March 20, 2002; Accepted: March 26, 2002; Published: April 12, 2002

Indexing terms: Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II, CaMKII), gene expression, promoter activity, silencer element, neuronal cell, gel shift analysis

Abbreviations: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; CPRG, chlorophenol red β-D-galactopyranoside; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EGTA, ethylene glycol bis (β-aminoethylether)-N,N,N',N'-tetra acetic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethyl sulfonyl fluoride.

ABSTRACT

The promoter activity of the rat Ca^{2+}/calmodulin-dependent protein kinase II gene was analyzed using the luciferase reporter gene in neuronal and non-neuronal cell lines. Neuronal cell type-specific promoter activity was found in the 5'-flanking region of α and β isoform genes of the kinase. Silencer elements were also found further upstream of promoter regions. A brain-specific protein bound to the DNA sequence of the 5'-flanking region of the gene was found by gel mobility shift analysis in the nuclear extract of the rat brain, including the cerebellum, forebrain, and brainstem, but not in that of non-neuronal tissues, including liver, kidney and spleen. The luciferase expression system and gel shift analysis can be used as an additional and better index by which to monitor gene expression in most cell types.

INTRODUCTION

Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) is one of the most abundant protein kinases in mammalian brain and a major mediator of calcium signaling in neurons. CaM kinase II is involved in diverse functions in neurons, including neurotransmitter synthesis and neurotransmitter release, modulation of ion channel activity, synaptic plasticity, learning and memory, and gene expression (1-7). The α and β isoforms of CaM kinase II are expressed almost exclusively in the nervous system, but the expression of both isoforms is not uniform throughout the brain, and is regulated developmentally. In the adult rat, the α isoform is abundant in the forebrain, whereas the β isoform is abundant in the cerebellum (8,9). The enzyme level is increased most rapidly in postnatal brain during the most active phase of synapse formation (10-12). Thus, the space-temporal gene expression of CaM kinase II indicates that the enzyme is carefully regulated at the level of transcription. The transcriptional regulation of many eukaryotic genes has been extensively studied and tissue-specific enhancer and silencer elements have been identified. However, little is known about the neuronal cell type-specific expression of the CaM kinase II gene. Therefore, it is imperative that there is a method to analyze the gene expression. We have analyzed the promoter activity and a protein bound to the DNA sequence of the gene (13,14). Some of our procedures may be modified, although major procedures are known to provide good results.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (specific activity of 110 TBq/mmol) was purchased from NEN™ Life Science Products, Inc. (Boston, MA.). Double strand poly(dI-dC) and Sephadex G-50 were obtained from Amersham Pharmacia Japan (Tokyo, Japan). Oligonucleotide primers were synthesized by Biologica Co. (Nagoya, Japan), or by Invitrogen Life Tech. Japan (Tokyo,

Japan). Luciferase assay system, luciferase reporter pGL2- and pGL3-control vectors, pGL2- and pGL3-basic vectors, and Wizard™ Plus Minipreps DNA purification system were from Promega Corp. (Madison, WI). Geneclean II DNA purification kit was from BIO 101 Inc. (Vista, CA). FuGENE™6 transfection reagent and chlorophenol red β-D-galactopyranoside (CPRG) were from Roche Diagnostics Japan Corp. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), Ham F12 medium, and DMEM/Ham F12 (1:1) medium were from Invitrogen Life Tech. Japan (Tokyo, Japan). Eagle's MEM amino acids and vitamins medium was from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was from JRH Bioscience (Lenexa, KS). X-OMAT film was from Eastman Kodak Company (Rochester, NY).

Construction of α and β CaM Kinase II Promoter-Luciferase Reporters

α CaM kinase II promoter-luciferase reporters were created by polymerase chain reaction (PCR) with cloned genomic DNA (CL-1 clone) (15) as a template. PCR fragments possessing 5' flanking sequences with various 5' sense primers and a common 3' end at +112 were inserted into a promoterless pGL3-basic vector. The 5' sense and 3' antisense primers were designed according to the sequence of the gene as shown in Table 1 (see Protocols section). To the 5' sense and 3' antisense primers were added BglII and HindIII restriction sites, respectively. PCR products were separated by agarose gel electrophoresis and excised, and then purified using the Geneclean II kit. PCR products were also separated by polyacrylamide gel electrophoresis (PAGE) and excised, and then purified as described (16). Purified PCR products were digested with BglII and HindIII, and ligated into a promoterless pGL3-basic vector. The 5' flanking sequence containing 8.5 kbp of the mouse α CaM kinase II gene (17) was digested with SalI and inserted into pGL3-basic vector to make pGLmα8.5k.

β CaM kinase II promoter-luciferase reporters were prepared in the same way as the α CaM kinase II luciferase reporters. Genomic DNA clone (BCL-1) or the subcloned plasmid DNA of the β CaM kinase II gene (14) was used as a template. To the 5' sense and 3' antisense primers were added KpnI and XhoI restriction sites, respectively. PCR fragments were cloned directly into a promoterless pGL2-basic vector. A construct devoid of the transcription initiation site (pGLΔ0.57) was prepared from the fragment of NcoI (nucleotide -577) and EheI (nucleotide -45) of the gene.

Cell Culture and Transfection

The following cell lines were used in the experiment; neuronal NG108-15, N18TG2, and CAD cells and non-neuronal BALB/c3T3, CHO-K1, and HT1080 cells. For transient transfection, cultured cells were plated on 35-mm tissue culture dishes at approximately 1-3 x 10^5 cells/dish, and cultured 24 h. Luciferase reporter DNA (1.2 μg of pGL2- or pGL3-control vector or the molar equivalent of luciferase constructs) with β-galactosidase expression vector (pcDNA3/β-gal) DNA (0.8 μg) were introduced into the cells via FuGENE™6 reagent treatment (3 μl of FuGENE™6 reagent/dish) as instructed by the manufacturer. After 24 h, cells were harvested and extracted for assay. All transfections were performed in duplicate.

Luciferase Expression

Plated cells were washed with phosphate-buffered saline (PBS), and then lysed in 200 μl of lysis buffer. Luciferase activity was assayed in a reaction mixture containing 20 mM tricine, pH 7.8, 470 μM luciferin, 530 μM ATP, 270 μM Coenzyme A, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\bullet}5H_20$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, and nuclear extract. Luciferase activity was measured for 20 s after the addition of reagents. The efficiency of transfection was corrected by the β-galactosidase activity. Promoter activity was compared by measuring the luciferase activity of the pGL2- or pGL3-control vector with the SV40 early gene promoter, and expressed as normalized luciferase activity. β-Galactosidase activity was assayed by a modification of the method described by Herbmel *et al*. (18) The enzymatic activity of individual samples was measured in triplicate.

Electrophoretic Mobility Shift Analysis

Nuclear extracts were prepared as described (19). Sense and antisense oligonucleotide probes were annealed, and end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The binding reaction was carried out in a reaction mixture containing 25 mM Tris buffer (pH 7.5), 75 mM KCl, 1 mM EGTA, 1 μg of poly(dI-dC), 13% glycerol, 10 μg of the nuclear extract, and 20-50 fmol ^{32}P-labeled probe (about 10,000 cpm) with or without non-labeled probe or competitor DNA. After incubation for 30 min at 0 °C, the reaction mixture was separated by 6.4% polyacrylamide gel electrophoresis at 200 V for 30 min in 0.5x TBE, fixed, dried, and autoradiographed.

RESULTS AND DISCUSSION

Analysis of Promoter Activity of α CaM Kinase II Gene

We examined the promoter activity in neuronal and non-neuronal cell lines. Transient transfection was used to compare the promoter activity of the luciferase reporter gene. The structure of the 5'-flanking region of the α CaM kinase II gene and one of the luciferase reporters (pGLα2281) is shown in Fig. 1A. Fragments possessing a common 3' end (+112 bp) containing a sequence of 2281, 1622, 757, 275, 199, 145, and 100 bp 5' upstream from the transcription initiation site were

cloned upstream of the luciferase reporter gene in a promoterless pGL3-basic vector to make respective pGLα constructs. α CaM kinase II promoter activity was compared with that of pGL3-control vector by measuring luciferase activity, and expressed as normalized luciferase activity.

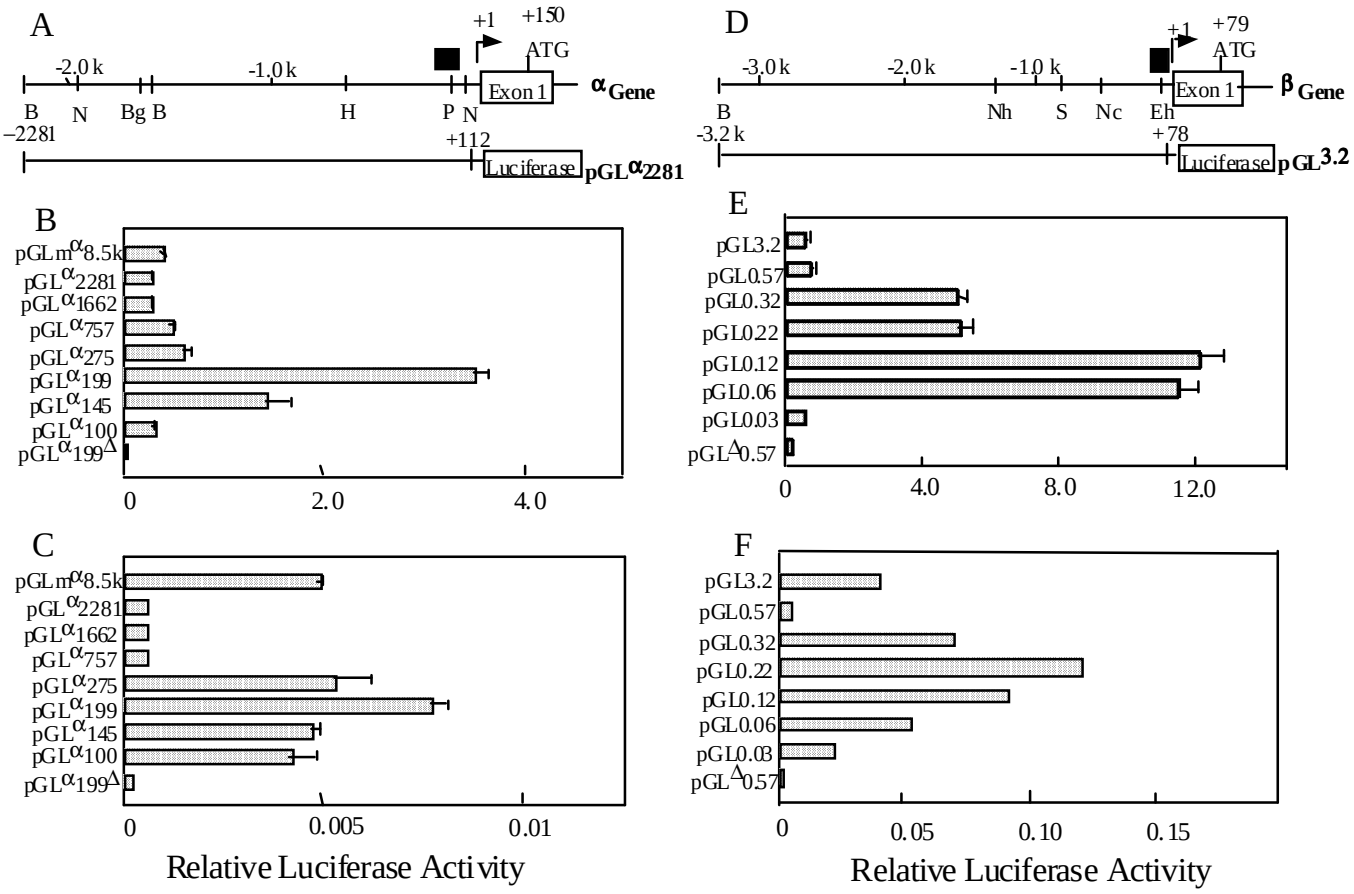

Fig. 1: Promoter-luciferase reporter and deletion analysis of CaM kinase II promoter activity in transiently transfected neuronal and non-neuronal cells. A. A schematic representation of the 5' flanking region of the α CaM kinase II gene (Upper) and one of the α CaM kinase II promoter-luciferase reporters (Lower). α CaM kinase II promoter-luciferase reporters were created by PCR amplification using the 5' flanking sequence of the gene. The sequence is numbered with respect to the transcription initiation site (indicated as +1) shown by an arrow. The box represents exon 1 and the translation initiation site is shown by ATG and +150. Black box shows neuronal cell type-specific promoter region found in this study. Restriction enzyme sites and their positions are shown; B, BamHI; Bg, BglII; H, HindIII; N, NcoI; P, PstI. **B** and **C.** Deletion analysis of α CaM kinase II promoter activity in transiently transfected neuronal and non-neuronal cells. Luciferase reporters were co-transfected with the internal control of pcDNA3/β-gal into neuronal cells, NG108-15 (B), and into non-neuronal cells, BALB/c3T3 (C). Luciferase activity was measured 24 hr later, and is expressed relative to that obtained with the control vector after correcting for the transfection efficiency based on co-transfection with pcDNA3/β-gal. The activity observed with pGL3-control vector was used as a positive control and expressed as 1.00. All values are presented as the mean of three assays. The data are representative of three independent experiments. **D**. A schematic representation of the 5' flanking region of the β CaM kinase II gene (Upper) and one of the β CaM kinase II promoter-luciferase reporters (Lower). β CaM kinase II promoter-luciferase reporters were created by PCR. The sequence is numbered as in A. Black box shows neuronal cell type-specific promoter region found in this study. Restriction enzyme sites and their positions are shown; B, BamHI; Eh, EheI; Nc, NcoI; Nh, NheI; S, Sac I. **E** and **F**. Deletion analysis of β CaM kinase II promoter activity in transiently transfected cells. Luciferase reporters were co-transfected with the internal control of pcDNA3/β-gal to neuronal cells, NG108-15 (E), and non-neuronal cells, BALB/c3T3 (F). Activity observed with pGL2-control vector was used as a positive control and expressed as 1.00. Luciferase activity was measured after 24 h. Data are representative of three experiments and similar results were obtained.

In NG108-15 cells, the strongest promoter activity was obtained with pGLα199, which yielded a 3.68-fold increase in luciferase activity relative to pGL3-control vector (Fig. 1B). pGLα145 also showed high promoter activity at about 40% of that of pGLα199, but pGLα100 showed very little promoter activity. An internal deletion mutant (pGLα199Δ) that had no transcription initiation site showed no significant promoter activity in NG108-15 cells, indicating that the transcription initiation site was important to the transcription of the α CaM kinase II gene. Similar results were obtained in neuronal cell lines, N18TG2 and CAD (data not shown), and normalized luciferase activity of pGLα199 in NG108-15, N18TG2, and CAD cells was 3.68, 3.08, and 1.87, respectively. However, luciferase activity was extremely weak in non-neuronal cells - about 0.007 in BALB/c3T3 (Fig. 1C). In other non-neuronal cell lines, HT1080, and CHO-K1, the activity was also low and less than 0.05 (data not shown). Promoter activity in non-neuronal cells was 100- to 500-fold lower than that in neuronal cells. These results indicated that the 5' upstream region -199 to -100 bases from the transcription initiation site showed neuronal cell type-specific strong promoter activity.

The inclusion of an additional 5' flanking sequence of 275 bases (pGLα275) decreased luciferase expression only 0.57-fold relative to the pGL3-control vector in NG108-15 cells (Fig. 1B). The luciferase activity of pGLα275 was almost the same as that of pGLα757, pGLα1662, and pGLα2281. Since the sequence of the 8.5 kbp 5' flanking region is known to show brain-specific expression of α CaM kinase II (17), the promoter activity of pGLmα8.5k was compared with that of the rat promoter. pGLmα8.5k showed weak promoter activity, as did pGLα275 in NG108-15 cells. Similar results were obtained in N18TG2 and CAD cells (data not shown). These results indicate that there is a strong silencer element within the 5' upstream region -275 to -199 bp from the transcription initiation site.

The promoter activity of all regions of the 5'-flanking sequence was much weaker in non-neuronal cells including BALB/c3T3, HT1080 and CHO-K1 than in neuronal cells (Fig. 1C, and data not shown).

Analysis of Promoter Activity of β CaM kinase II Gene

Since the β isoform was expressed almost exclusively in the brain, the promoter activity of the β CaM kinase II gene was also examined. The structure of the 5' flanking region of the β CaM kinase II gene and one of the luciferase-reporters (pGL3.2) is shown in Fig. 1D. Fragments possessing a common 3' end (+78 bp) containing sequences of 3.2, 0.57, 0.32, 0.22, 0.12, 0.06, and 0.03 kbp 5' upstream from the transcription initiation site were cloned upstream of the luciferase reporter gene in a promoterless pGL2-basic vector to make respective pGL constructs. The luciferase activity of these reporters was analyzed in neuronal cells, NG108-15, N18TG2, and CAD cells, and in non-neuronal cells, BALB/c3T3, HT1080, and CHO-K1 cells. The results indicate that the 5' upstream region -66 to -35 bp from the transcription initiation site contained neuronal cell type specific strong promoter activity, and that there are two silencing elements within the regions -222 to -123 bp and -576 to -323 bp. Representative results are shown in Fig. 1E and 1F.

The relative luciferase activity of the α isoform gene was lower than that of the β isoform, because the luciferase activity of the pGL3-control vector was about 5 to 10-fold that of the pGL2-control vector. pGL3-control vector contains more SV40 early gene promoter than pGL2-control vector as described by the manufacturer.

Investigation of Nuclear Protein Bound to DNA of the Promoter Region of β CaM kinase II

Since neuronal cell type-specific promoter activity was found in the region from -66 to -35 bp of the β CaM kinase II gene, a gel mobility shift analysis was performed using nuclear extract from various rat tissues. The 32P-labeled probe was incubated with nuclear extract of the brain, including forebrain, cerebellum, and brainstem, and non-neuronal tissues, including liver, kidney and spleen. A band specific to the brain was observed as indicated by an arrow in Fig. 2A, although some other bands were expressed ubiquitously. Its density was highest in the cerebellum, consistent with the expression of β CaM kinase II (8,9).

The radioactivity of this band detected by incubation of the extract of cerebellum was reduced almost completely in the presence of a 5-fold excess of non-radioactive probe (Fig. 2B). These results indicate that this band was formed by specific binding of the nuclear protein in the brain.

A protein bound to the promoter regions (nucleotides -145 to -100, and -199 to -146) of α CaM kinase II was also found in the nuclear extract of rat brain, including forebrain, brainstem and cerebellum, but not in non-neuronal tissues, including liver, kidney and spleen (data not shown). There is no homology in the sequence in the promoter region of both α and β genes. The sequence of the promoter region is 5'- CTT CTG GGC CCA CAC AGT CCT GCA GTA TTG TGT ATA TAA GGC CAG GGC AAC GGA GGA GCA GGT TTT GAA GTG AAA GGC AGG CAG GTG TTG GGG AGG CAG T -3' (-199 to -100) for the α gene and 5'- CAG CCA GCC CGG CCC CCG CCC GGC GCC GCG AG -3' (-66 to -35) for the β gene. Known transcription factor binding sites were underlined which corresponded to TATA box, v-myb, and MyoD in the α gene and Sp1 in the β gene, respectively. These sites were not specific to neuronal cells. Thus, it is suggested that there are unidentified promoter elements in these regions.

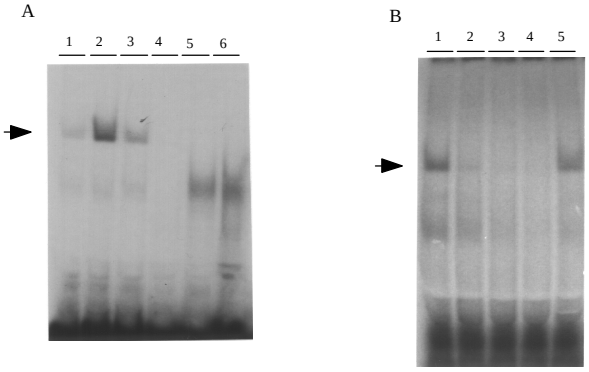

Fig. 2: Gel mobility shift analysis using the -66 to -35 region of β CaM kinase II. Ten μg of nuclear extract and 0.2 ng of ^{32}P-labeled probe (about 1 x 10^4 cpm) were used in the experiments. **A**, tissue specificity of DNA binding protein, Lanes 1 - 6, nuclear extract from forebrain, cerebellum, brainstem, liver, kidney and spleen of rat, respectively. **B**, specificity of probe. Nuclear extract of cerebellum was incubated with ^{32}P-labeled probe in the presence of non-radioactive probe or competitor DNA. Lane 1, without non-radioactive probe; lanes 2 - 4, in the presence of a 1-, 2.5-, and 5-fold mole excess of non-radioactive probe, respectively; lane 5, in the presence of a 5-fold mole excess of the scrambled sequence.

Conclusion

We have established a method for luciferase reporter analysis by which the number of cells expressing a specific gene can be monitored through the assay of luciferase and β-galactosidase activities. The method provides an alternative for analyzing the promoter activity of different genes. The main observations we have made with this technique are 1) neuronal cell type-specific expression of the α and β gene occurs in neuronal cell lines (silencer elements were also found in further upstream promoter regions) and 2) neuronal cell type-specific proteins bound to the promoter region are found in nuclear extracts of brain cells.

ACKNOWLEDGEMENTS

This study was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

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PROTOCOLS

Construction and purification of α and β CaM Kinase II Promoter-Luciferase Reporter

α and β CaM kinase II promoter-luciferase reporters were created by PCR with genomic DNA as a template.

1. PCR primers of α CaM kinase II are shown in Table 1. To the 5' sense and 3' antisense primers were added 2 base plus BglII (5'-GAAGATCT-3') and HindIII (5'-CCAAGCTT-3') restriction sites, respectively.
2. PCR primers of β CaM kinase II are shown in Table 1. To the 5' sense and 3' antisense primers were added 2 base plus KpnI (5'-GGGGTACC-3') and XhoI (5'-CCCTCGAG-3') restriction sites, respectively.
3. The PCR reaction mixture contains the following in a final volume of 25 µl:
   - 20 mM Tris/HCl, pH 8.8, 2 mM dNTP,10 mM KCl, 2 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 1 unit of pfu DNA polymerase, 0.4 µM 5' sense- and 0.4 µM 3' antisense-oligonucleotide primers, and 100 pg of genomic DNA clone containing the 5' flanking region of α or β CaM kinase II
4. The amplification profile involved initial denaturation at 94 °C for 2 min, then denaturation at 98 °C for 20 s, annealing at 56 °C for 1 min, and extension at 72°C for 1 min except for the extension of pGLα2281, pGLα1162, and pGL3.2 (3 min). The reaction was repeated for 30 cycles. An automated DNA thermal cycler was used.
5. Separate the PCR products by agarose gel or polyacrylamide gel electrophoresis.
6. Detect the DNA with ethidium bromide under a UV lamp, and cut out the segment of the gel containing the band of interest.
7. Extract and purify the DNA from the gel as described below.
8. Digest the PCR product of the α CaM kinase II gene with 1 unit each of BglII and HindIII or that of the β CaM kinase II gene with 1 unit each of KpnI and XhoI for 1 hr at 37 °C.
9. Heat the reaction mixture at 70 °C for 5 min to inactivate the restriction enzymes.
10. Withdraw a 1 µl aliquot and add to 20 µl of ligation reaction mixture.
11. Add 20 ng of appropriately digested pGL3- and pGL2-basic vectors and 1 unit of T4 ligase to the mixture, and incubate for 1 hr at 16 °C.
12. Transform the ligation product to *E. coli* DH5α.
13. Purify the luciferase reporter plasmid using the Wizard™ Plus Minipreps DNA purification system.
14. Check the purity of the reporter plasmid DNA by 1% agarose gel electrophoresis. The yield of purified reporter plasmid DNA was usually 50-100 µg from 10 ml of culture of *E. coli*. under these conditions.

Purification of PCR Product by Agarose Gel Electrophoresis

The PCR product was purified from agarose gel using Geneclean II DNA purification kit according to the manufacturer's instructions.

1. Prepare the following solutions:
   - TAE: 40 mM Tris-acetate, pH 8.0, and 1 mM EDTA
   - TE7.5: 10 mM Tris/HCl, pH 7.5, and 1 mM EDTA
2. Separate the PCR products by 1% agarose gel electrophoresis in TAE at 100 V for 30 min. Cut out the segment of the gel containing the band of interest.
3. Transfer the gel slice (about 100 µg) to a 1.5-ml microfuge tube. Add 3 volumes of NaI solution to the agarose gel. Heat at 50 °C for 5 min to dissolve the agarose gel, then cool at 0 °C.
4. Add 3 µl of glassmilk to the solution, mix well, and centrifuge for 5 s at 15,000 rpm. Remove the supernatant.
5. Wash the precipitate with 500 µl of new wash solution. Centrifuge for 5 s at 15,000 rpm. Remove the supernatant. Repeat these steps twice.
6. Add 10 µl of 0.1x TE7.5 to the precipitate and mix well. Centrifuge at 15,000 x for 40 s. Transfer the supernatant to a new tube and obtain the purified PCR product.

Table 1: Oligonucleotide primer sequences used for PCR

Construct	5' Sense primer (nucleotide number)		3' Antisense primer (nucleotide number)	
α Gene				
pGLα2281	5'-CTTAGAGCAGTGGTTCTCAA-3'	(-2281 to -2262)	5'-GACTAGGACTGGGATGCTGA-3'	(+93 TO +112)
PGLα1622	5'-CTTGAGCCTCAGTTGGGTC-3'	(-1622 to -1604)		
pGLα757	5'-CTTCAGAGTTCTGGGTTTGC-3'	(-757 to -738)		
pGLα275	5'-CTTGTGGACTAAGTTTGTTCA-3'	(-275 to -255)		
pGLα199	5'-CTTCTGGGCCCACACAGTC-3'	(-199 to -181)		
pGLα145	5'-GGAGCAGGTTTTGAAGTGAAA-3'	(-145 to -125)		
pGLα100	5'-TTACCGGGGCAACGGGAAC-3'	(100 to -82)		
pGLα199δ	5'-CTTCTGGGCCCACACAGTC-3'	(-199 to -181)		
β Gene				
pGL3.2	5'-CTCCTGGCGGGGACAGAG-3'	(PUC vector sequence)	5'-GGCGGTGGCGATTGGGCTC-3'	(+60 to +78)
pGL0.57	5'-CTCCTGGCGGGGACAGAG-3'	(PUC vector sequence)		
pGL0.32	5'-CGACATGTGTGCGAAGGGAG-3'	(-320 to -301)		
pGL0.22	5'-CGAGTGTAGGGTGCGAGAGAG-3'	(-218 to 198)		
pGL0.12	5'-CTCCTGGCGGGGACAGAG-3'	(-116 to -99)		
pGL0.06	5'-CAGCCAGCCCGGCCCC-3'	(-66 to -51)		
pGL0.03	5'-CCGAGGTGTCTCCCGCG-3'	(-34 to -18)		

Table 1: Oligonucleotide sequences were derived as previously described (13, 14).

Purification of PCR Product by Polyacrylamide Gel Electrophoresis

The PCR product was purified from polyacrylamide as previously described (16).

1. Prepare the following solutions:
   - TBE: 9 mM Tris-borate, pH 8.0, and 2 mM EDTA.
   - Elution buffer: 0.5 M CH_3COONH_4, 10 mM $Mg(CH_3C00)_2$, 1 mM EDTA, and 0.1% SDS
2. Separate the PCR products by 5.6% polyacrylamide gel electrophoresis in TBE at 75 V for 1 hr.
3. Transfer the gel slice (about 100 µg) to a 1.5-ml microfuge tube. Add 2 volumes of elution buffer and crush the gel with the plastic tip.
4. Incubate the mixture at 37 °C for 4 hr with shaking. Centrifuge at 12,000 rpm for 1 min, then transfer the supernatant to a new tube.
5. Add 2 volumes of ethanol. Let stand for 30 min at 4 °C. Centrifuge at 15,000 rpm for 15 min at 4 °C. Remove the supernatant.
6. Dissolve the precipitate in 100 µl of TE7.5.
7. Add 8 µl of 5 M NaCl and 250 µl of ethanol to the solution and mix well. Stand for 30 min at 4 °C and then centrifuge at 15,000 rpm for 15 min at 4 °C. Remove the supernatant and dry the precipitate under reduced pressure.
8. Dissolve the purified PCR product in 10 µl of TE7.5.

Transformation and Purification of Luciferase Reporter Plasmid DNA

Luciferase reporter plasmid DNA was purified using the Wizard™ Plus Minipreps DNA purification system according to the manufacturer's instructions with some modifications.

The solution contains the following:

- SOC medium: 2% trypton, 0.5% yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose (adjust the pH to 7.0 with 5 N NaOH)
- LB (Luria-Bertani)-ampicillin medium: 1% trypton, 0.5% yeast extract, 1% NaCl, and 50 µg/ml ampicillin (adjust the pH to 7.0 with 5 N NaOH)

- TB (Terrific Broth)-ampicillin medium: 17 mM KH_2PO_4, 72 mM K_2HPO_4, 1.2% trypton, 2.4% yeast extract, 0.4% glycerol, and 50 µg/ml ampicillin
- Cell resuspension solution: 50 mM Tris/HCl, pH 7.5, 10 mM EDTA, and 100 µg/ml RNase A
- Cell lysis solution: 0.2 N NaOH, and 1% SDS
- Neutralization solution: 1.32 M K-acetate, pH 4.8
- Column wash solution: 20 mM Tris/HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, and 55% ethanol

1. Transfer 4 µl of the ligation mixture into 96 µl of *E. coli* DH5α competent cell suspension. Incubate successively at 0 °C for 1 hr at 42 °C for 45 s, and then at 0 °C for 2 min.
2. Add 900 µl of SOC medium. Incubate at 37 °C for 1 hr with shaking.
3. Withdraw a 200-µl aliquot of *E. coli* suspension, and plate onto LB-ampicillin. Incubate at 37 °C for 15 hr.
4. Remove a single colony and culture in 10 ml of TB-ampicillin medium at 37 °C for 15 hr.
5. Harvest cells by centrifugation at 5,500 rpm for 5 min.
6. Completely resuspend the cell pellet in 1 ml of cell resuspension solution.
7. Add 1 ml of cell lysis solution and mix by inverting the tube 4 times. The cell suspension should clear up immediately.
8. Add 0.5 ml of 2x neutralization solution and mix by inverting the tube 4 times.
9. Centrifuge the lysate at 17,000 rpm for 15 min at 4 °C. Transfer the supernatant to a 15-ml plastic tube.
10. Add 4 ml of 7 M guanidine/HCl and 2 ml of resuspended DNA purification resin into the cleared lysate. Mix by inverting the tube for 5 min.
11. Transfer the mixture to a 10-ml syringe attached to a minicolumn. Carefully insert the syringe plunger and gently push out the slurry into the column.
12. Wash the column with 5 ml of column wash solution.
13. Remove the syringe and transfer the minicolumn to a 1.5-ml microfuge tube. Centrifuge the column at 10,000 rpm for 20 s to dry the resin.
14. Transfer the minicolumn to a new 1.5-ml microfuge tube. Apply 50 µl of TE7.5 prewarmed at 65-70 °C and wait 1 min. Centrifuge at 10,000 rpm for 20 s to elute DNA. Remove the minicolumn. Store the purified plasmid DNA in the microfuge tube at -20 °C.

Transfection of Luciferase Reporter and Preparation of Cell Extract

The culture medium for each cell line is the following. Note that all culture media contained 0.007% penicillin and 0.01% streptomycin:

- Mouse neuroblastoma-rat glioma hybridoma NG108-15 cells; DMEM supplemented with 10% FBS and 0.1 mM hypoxanthine, 1 mM aminopterin and 16 mM thymidine (HAT)
- Mouse neuroblastoma N18TG2 cells; DMEM supplemented with 10% FBS
- Mouse central nervous cell line CAD cells; DMEM/F12 medium supplemented with 8% FBS
- Mouse fibroblast BALB/c3T3 cells; DMEM supplemented with 10% FBS
- Human fibroblast HT1080 cells; DMEM supplemented with 10% FBS
- Chinese hamster ovary CHO-K1 cells; Ham F12 medium supplemented with 10% FBS

The lysis buffer contains the following:

- 25 mM Tris buffer, pH 7.8, 2 mM EDTA, 2 mM dithiothreitol, 10% glycerol, and 1% Triton X-100

Ca^{2+}/ Mg^{2+}-free-DMEM is prepared as follows:

- Dissolve the following reagents in a total volume of 200 ml of water; 176 mg of Eagle's MEM amino acids and vitamins medium, 50 mg of KCl, 1280 mg of NaCl, 25 mg of $NaH_2PO_4 \cdot 12H_2O$, 200 mg of D-glucose, 22 mg of sodium pyruvate, 6 mg of glycine, 8.4 mg of L-serine, and 440 mg of $NaHCO_3$. Add 14 mg of penicillin and 20 mg of streptomycine. Introduce CO_2 gas, and sterilize by passage through a 0.22-µm filter

1. Seed cells at approximately 1-3 x 10^5 /35 mm dish in 1 ml of culture medium, and culture at 37 °C in a 7 % CO_2 humidified incubator for 24 hr.
2. Mix luciferase reporter cDNAs (1.2 µg) and β-galactosidase expression vector (0.8 µg) with the FuGENE™6 transfection reagent (3 µl) in 100 µl of serum-free DMEM medium, and stand for 15 min at room temperature.
3. Add DNA-FuGENE™6 reagent mixture directly to the cells, and ensure even dispersal by swirling the wells of dishes.
4. Withdraw the medium after 24 h, and wash cells once with 1 ml of Ca^{2+}/ Mg^{2+}-free-DMEM.

5. Add 200 µl of lysis buffer to the cells. Incubate for 15 min at room temperature. Scrape the lysed cells from the dish using a rubber policeman. Transfer the cell lysate to a 1.5-ml microfuge tube.
6. Centrifuge the cell lysate at 18,500 x g for 2 min at 4 °C. Save the supernatant as cell extract.
7. Divide an aliquot, and store frozen at -80 °C until use. Usually, about 20 µg of protein (0.1 mg/ml, 200 µl) was obtained under our conditions.

Assay of Luciferase Activity

Luciferase activity was measured using Promega's luciferase assay system according to the manufacturer's instruction. The luciferase assay reagent contains the following in a final volume of 50 µl:

- 20 mM tricine, pH 7.8, 470 µM luciferin, 530 µM ATP, 270 µM Coenzyme A, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, and 33.3 mM dithiothreitol

1. Initiate the reaction by adding 10 µl of cell extract.
2. Measure the light output for a period of 20-40 s at room temperature in a luminometer.

Assay of β-Galactosidase

β-Galactosidase activity is assayed using CPRG as substrate. The reaction mixture contains the following in a final volume of 250 µl:

- 0.1 M Na-phosphate buffer, pH 7.5, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, 2.5 mM CPRG, and cell extract

1. Initiate the reaction by adding 2-5 µl of cell extract.
2. Incubate at 37 °C for 5 - 15 min.
3. Withdraw a 90-µl aliquot, and add 37.5 µl of 1 M Na_2CO_3
4. Measure the absorbance at 574 nm (A_{574}) in a spectrophotometer.

Preparation of Nuclear Extract

The buffer contains the following:

- Buffer A: 10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptine, 10 µg/ml antipain, and 10 µg/ml pepstatin A
- Buffer B: 20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptine, 10 µg/ml antipain, and 10 µg/ml pepstatin A

1. Dissect the tissue quickly after decapitation. Wash the tissue with PBS. Pulverize it gently with a Teflon pestle in 4 volumes of buffer A. Transfer to a centrifuge tube. The weight of tissues of one adult rat was as follows; forebrain, about 0.9 g; brainstem, about 0.9 g; cerebellum, about 0.2 g; liver, about 5 g; kidney, about 3 g; spleen, about 0.8 g.
2. Incubate the homogenate for 15 min at 0 °C.
3. Add 40% Nonidet P-40 to a final concentration of 1%. Vortex the tube vigorously for 10 s.
4. Centrifuge the mixture at 5,000 rpm for 5 min.
5. Remove the supernatant, and resuspend the nuclear pellet in buffer B.
6. Incubate for 5 min at 4 °C with shaking.
7. Centrifuge at 15,000 rpm for 15 min at 4 °C. Save the supernatant as nuclear extract. Divide the fraction into aliquots and store at -80 °C. Usually about 5-7 mg/ml protein was obtained under the above conditions.

Preparation of End-labeled Probe DNA

The buffer contains the following:

- STE solution: 0.1 M NaCl, 10 mM Tris/HCl, pH 8.0, and 1 mM EDTA
- Labeling buffer: 50 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 5 mM spermidine, and 1 mM EDTA

The sequence of the oligonucleotide corresponding to bases -66 to -35 of the 5'-flanking region of the β CaM kinase II gene is as follows:

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  -66
5'-CAG CCA GCC CGG CCC CCG CCC GGC GCC GCG A-3'
3'-  TC GGT CGG GCC GGG GGC GGG CCG CGG CGC TC-5'
                                                -35
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1. Mix the sense and antisense oligonucleotide probes (10 ng each) in 5 µl of STE solution (annealing buffer). Heat the mixture at 90 °C for 3 min. Cool it gradually to room temperature for about 1 hr.
2. Add 20 µl of labeling buffer to the solution, including 10 units of T4 nucleotide kinase and [γ-^{32}P]ATP (0.37 MBq).
3. Incubate at 37 °C for 60 min.
4. Heat at 70 °C for 5 min to terminate the reaction.
5. Add 75 µl of STE solution (total volume about 100 µl).
6. Apply to a spun column of Sephadex G-50 equilibrated with STE solution.
7. Centrifuge the column at 2000 rpm for 50 s.
8. Obtain the effluent (about 100 µl) as labeled probe. Usually about 2 - 3 x 10^6 cpm was incorporated into the probe under the conditions.

Isolation of Labeled Probe by Spun-column

The spun-column procedure was adapted from a method described previously (20).

1. Plug the 1-ml disposable syringe with a small amount of sterile glass wool.
2. Prepare a column of Sephadex G-50 (0.9-ml bed volume) equilibrated with STE solution in the syringe.
3. Insert the column into a plastic centrifuge tube and centrifuge at 2000 rpm for 50 s. Since usually the Sephadex G-50 packs down during centrifugation, Sephadex G-50 is added until the packed column volume is 0.9 ml.
4. Add 100 µl of STE to the column, and centrifuge at 2000 rpm for 50 s. Repeat this step once.
5. Apply the labeled DNA probe to the column in a total volume of 100 µl. Centrifuge at exactly the same speed and time as before.
6. Collect about 100 µl of effluent from the syringe into a plastic tube.
7. Prepacked columns for the isolation of labeled probe are commercially available (such as MicroSpinTM S-300 HR column, Amersham Pharmacia Biotec, etc).

Gel Mobility Shift Analysis

The solution contains the following:

- Binding buffer: 25 mM Tris/HCl, pH 7.5, 75 mM KCl, 1 mM EGTA, 1 µg poly (dI-dC), and 13% glycerol
- Polyacrylamide gel mixture: 6.4% polyacrylamide/bisacrylamide (29:1), 5% glycerol, and TBE
- Dye solution: 25% Ficol, 0.05% bromophenol blue, and 0.05% xylene cyanol

1. Preincubate nuclear extract (10 µg protein) in 20 µl of binding buffer for 20 min at 0 °C.
2. Initiate the reaction by adding ^{32}P-labeled probe DNA (20-50 fmol DNA, about 1 x 10^4 cpm).
3. Incubate at 0 °C for 30 min.
4. Add 2 µl of dye solution to the reaction mixture.
5. Withdraw a 15-µl aliquot and subject to 6.4% polyacrylamide gel electrophoresis with 0.5x TBE at 10 V/cm (25-30 mA) for 30 min.
6. Fix the gel in 10% acetic acid and 10% methanol solution for 10 min, and then wash the gel with distilled water.
7. Dry the gel at 70 °C for 2 hr.
8. Expose to X-ray film with an enhancing screen for 4 - 24 h at -80 °C.
9. Develop the film and detect the radioactive band. Alternatively, the radioactive bands can be analyzed in an imaging analyzer BAS 1500 Mac (Fuji Film Co., Tokyo) according to the manufacturer's instructions.