Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 278–282, January 1990
Biochemistry

Sequence analysis and DNA–protein interactions within the 5′ flanking region of the Ca^{2+}/calmodulin-dependent protein kinase II α-subunit gene

(protein phosphorylation/transcription regulation/neuronal differentiation)

TERESA SUNYER AND NAJI SAHYOUN

Cell Biology Division, Wellcome Research Laboratories, 3030 Cornwallis Road, Research Triangle Park, NC 27709

Communicated by George H. Hitchings, September 18, 1989

ABSTRACT **The 5′ flanking region of the brain Ca^{2+}/calmodulin-dependent protein kinase II α-subunit gene was identified and characterized. A total of 430 bases was sequenced upstream from the translation initiation codon, and the site of transcription initiation was located at −149 or −147 bases as determined by primer extension and S1 nuclease protection analysis, respectively. TATA and CAAT boxes were absent from their standard positions; however, the 5′ flanking region was rich in G+C and contained a GGGCG and a TATATAA sequence 76 and 160 bases upstream from the transcription initiation site, respectively. Moreover, the sequence CAACGG was found 85 and 146 bases upstream from this site, indicating presumptive binding sites for the Myb protein. Gel-mobility shift assays revealed that a 120-base-pair fragment, which included the G+C-rich, TATA, and CAACGG sequences bound nuclear proteins specifically. DNA–protein complexes with similar gel mobilities were obtained with nuclear extracts from rat forebrain or cerebellum and from neonatal or adult brains. Extracts from rat liver, kidney, and spleen generated specific DNA–protein complexes with different electrophoretic mobilities, suggesting the occurrence of different nuclear proteins that bind to 5′ regulatory elements of the Ca^{2+}/calmodulin-dependent kinase II α-subunit gene.**

Neuronal multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) represents a major Ca^{2+}-operated molecular switch subject to regulation by Ca^{2+}/calmodulin and by autophosphorylation (1, 2). This abundant enzyme is located within different subcellular compartments including cytosol, cytoskeleton, nuclei, presynaptic terminals, and postsynaptic densities (3–13) and may regulate important neuronal functions such as neurotransmitter release and long-term potentiation (14–17).

Soluble CaM kinase II was initially purified from brain as a 10- to 12-subunit oligomer with an apparent M_r of ≈600,000, composed primarily of two related α and β subunits. The α-to-β ratio varies from 3 or 4:1 in forebrain to 1:4 in cerebellum (3–5, 18). A third relatively minor subunit, β′, has also been identified (3). α and β subunits are encoded by distinct genes (19–21), whereas β′ is generated by alternative splicing of the β-gene transcript (22).

Enzyme levels and subunit composition were dependent on the stage of neuronal development; the levels increased dramatically between the second and fourth postnatal weeks in rat brain (23–27). The use of cDNA probes revealed that the α and β subunits of CaM kinase II are encoded by 5- to 5.4-kilobase (kb) and 3.9- to 4.8-kb mRNA species, respectively (19–21), and that the level of the 5-kb mRNA species increased 10-fold during the first 3 postnatal weeks in rat forebrain; a further 2.5-fold increase occurred by the end of 90 days (19).

CaM Kinase II isoenzymes from skeletal muscle (28), liver (29, 30), lung (31), and heart (32) have also been isolated, and the wide distribution of CaM kinase II immunoreactive proteins was demonstrated (33, 34). Moreover, α/β-subunit DNA or RNA probes reacted with mRNA species ranging in size from 2.7 kb to 5.7 kb in various peripheral tissues and cultured cells (19–22).

Because of the varying subunit composition, cellular distribution, and developmental regulation of CaM kinase II, we investigated possible regulatory mechanisms that may govern the expression of the α-subunit gene in rat brain. We sought to isolate and define genomic fragments* that extend upstream from the transcription initiation site and to study their interaction(s) with nuclear proteins. We hope that these studies will constitute a first step in our understanding of the transcriptional regulation of the expression of a major neuronal protein.

MATERIALS AND METHODS

Radioisotopes were obtained from DuPont/New England Nuclear. Restriction enzymes, DNA modification enzymes, and sequencing reagents were purchased from Bethesda Research Laboratories; avian myeloblastosis virus reverse transcriptase was obtained from Life Sciences; nitrocellulose was purchased from Schleicher & Schuell; the pGEM single-strand system was obtained from Pharmacia. Oligonucleotides were prepared in a Biosearch model 8600 DNA synthesizer and purified on a Nensorb Prep column from DuPont/New England Nuclear.

A rat genomic partial *Eco*RI library in Charon 4A was obtained from Clontech, and replica filters were screened with synthetic oligonucleotides α-I and α-II following standard procedures (35, 36). DNA fragments containing sequences complementary to our probes were shot-gun subcloned into pGEM-3Zf(+), and single-stranded DNA was prepared following the manufacturer's instructions. DNA sequencing was performed with the Sequenase system (United States Biochemical) as suggested by the manufacturer (37). DNA analysis and subcloning of small fragments of DNA into pUC18 followed standard techniques (38–40). RNA was obtained from rat brain (41), and poly(A)$^+$ RNA was isolated as described (42). Primer extension analysis and S1 nuclease protection assays were performed as detailed (40–43) with some modifications.

For electrophoretic mobility assays, the vector was digested with the indicated restriction enzymes and separated

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M29699).

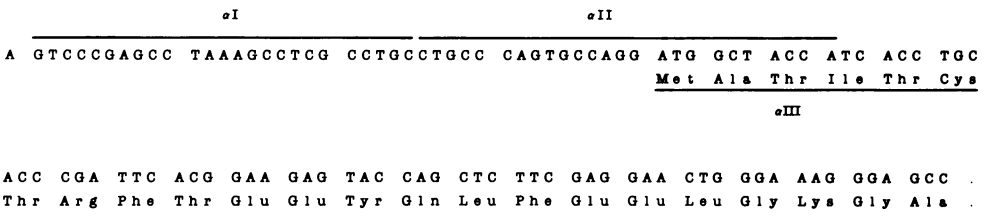

FIG. 1. Synthetic oligonucleotides used for screening a rat genomic library and studying the CaM kinase II α-subunit gene. The nucleotide sequence of the 5′ region of the CaM kinase II α-subunit cDNA was determined by Lin *et al.* (20). The position of the synthetic oligonucleotides α-I (25-mer), α-II (25-mer), and α-III (18-mer) are indicated. α-I and α-II derive from the coding strand, while α-III represents the noncoding complementary strand that hybridizes with mRNA.

in a 2% agarose gel. The fragments of interest were recovered with DE-81 ion-exchange paper (Whatman), end-labeled, and digested again with a second restriction enzyme, and the final DNA products were separated in a 12% acrylamide gel in 1 × TBE (50 mM Tris borate, pH 8.3/1 mM EDTA). The DNA was retrieved by electroelution and kept at −20°C until used.

Nuclei from various rat tissues were prepared (44), and nuclear proteins were extracted (45) and dialyzed against 40 mM KCl/1 mM dithiothreitol/10% (vol/vol) glycerol/0.1 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/25 mM Tris·HCl, pH 7.5. Protein concentrations were determined as described by Bradford (46).

Electrophoretic mobility assays were performed (47) at room temperature. Nuclear extracts (1–10 μg) or dialysis buffer was preincubated for 20 min with 2 μg of carrier DNA [poly(dI-dC)] in a final volume of 20 μl containing 75 mM KCl, 0.2 mM dithiothreitol, 25 mM Tris·HCl (pH 7.5), and 13% glycerol. When indicated, unlabeled competitor DNA was added to the preincubation mixture for an additional 5 min. End-labeled DNA (3–10,000 cpm, 0.5–5 ng) was then introduced in 0.5- to 1.5-μl samples and incubated with the reaction mixture for 20 min. Unbound (free) DNA was separated from protein-bound DNA in a nondenaturing 5% polyacrylamide gel at low ionic strength (48).

RESULTS AND DISCUSSION

Isolation of a Genomic Fragment Containing the 5′ Flanking Region of the CaM Kinase II α-Subunit Gene. A rat genomic library in Charon 4A was screened with two oligonucleotides derived from the 5′ region of the CaM kinase II α-subunit cDNA (ref. 20; Fig. 1). Five clones were isolated that hybridized to both probes, and restriction enzyme analysis showed that all five contained very similar 18- to 20-kilobase-pair (kbp) inserts. One clone (c4) was chosen for further investigation; digestion of c4 with *Eco*RI and *Bam*HI produced 10 fragments, including one that contained 7 kbp that hybridized to both oligonucleotide probes. The 7-kbp fragment was subcloned into the pGEM-3Zf plasmid for characterization and sequencing.

Analysis of the 5′ Flanking Sequence of the CaM Kinase II α-Subunit Gene and Determination of the Transcription Initiation Site. A sequence of ≈300 nucleotides upstream from the initiation codon was obtained by using oligonucleotide α-III (Fig. 1) as a primer for the dideoxy chain termination reaction. Based on this information, the oligonucleotide, designated α-IV, encoding 20 nucleotides 265 base pairs (bp) upstream from oligonucleotide α-III was synthesized and used as a primer for additional nucleotide sequencing. Together, the sequencing data yielded ≈500 nucleotides. The first 45 bp upstream from the initiation codon corresponded to the untranslated cDNA sequence reported (20) and confirmed that the genomic fragment cloned with oligonucleotides α-I and α-II contained at least part of the CaM kinase II α-subunit gene. In spite of the high degree of homology between the amino-terminal end of the α and β subunits of brain CaM kinase II, the 5′ untranslated sequences of the α- and β-subunit cDNAs (20, 21) diverge considerably. These observations further indicate that the cloned fragment does not correspond to the β-subunit gene but to the α-subunit gene.

Primer extension and S1 nuclease protection analyses were used for the determination of the transcription initiation site. The most abundant primer extension product covered 149 bases from the initiation codon in three independent determinations (Fig. 2*A*); the S1 nuclease protection analysis

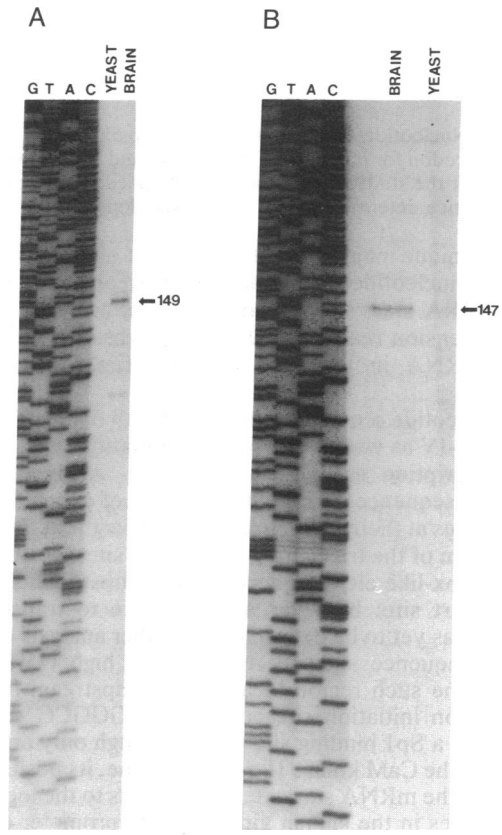

FIG. 2. Determination of the transcription initiation site by primer extension and S1 nuclease protection analysis. (*A*) Primer extension analyses were performed with adult rat brain poly(A)$^+$ RNA (2.5 μg) or yeast tRNA (5 μg) and end-labeled oligonucleotide α-III as a primer. A major product (arrow) was observed only with brain mRNA and corresponded to a fragment extending 149 nucleotides upstream from the initiation codon. As a standard, the pGEM-3Zf construct was sequenced with α-III as primer in a parallel dideoxy-sequencing reaction (lanes G, T, A, and C). (*B*) S1 nuclease protection analysis was carried out with adult rat brain poly(A)$^+$ RNA (2 μg) or yeast tRNA (5 μg) and a continuously labeled DNA fragment that extended 2 kb upstream from the initiation codon obtained by primer extension with single-stranded DNA pGem as template and oligonucleotide α-III as primer. A major protected fragment that extended 147 bases from the initiation codon (arrow) was observed only with brain mRNA. Fragment size was determined as in *A* (lanes G, T, A, and C).

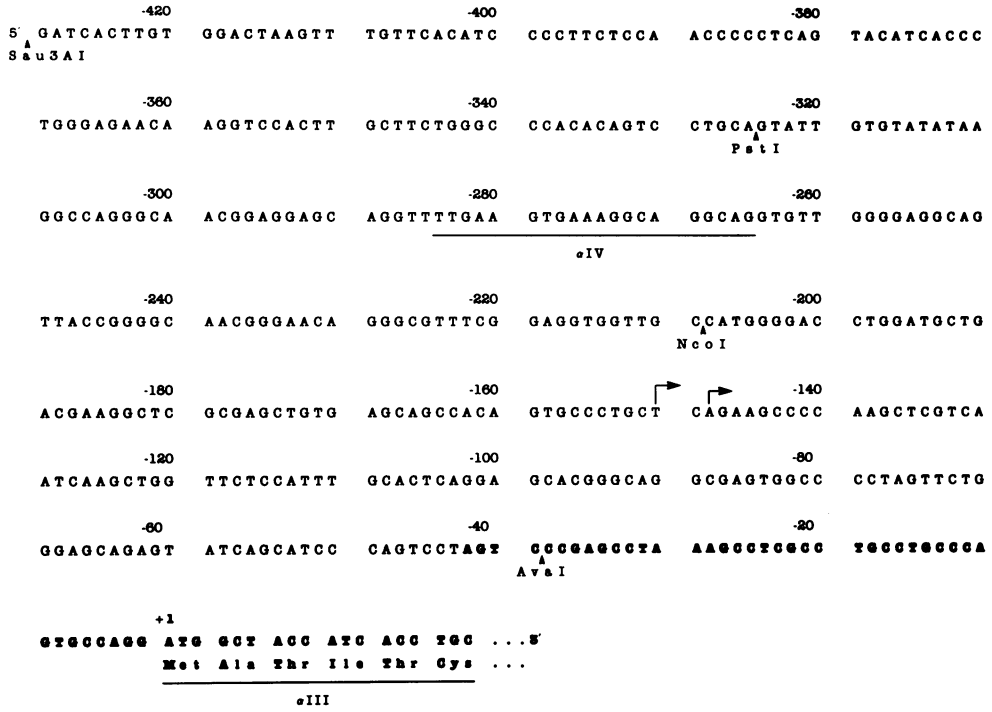

FIG. 3. Nucleotide sequence of the 0.4-kbp 5' flanking region of the CaM kinase II α-subunit gene. Nucleotide residue +1 denotes the A of the ATG codon for initiation of translation, and the arrows indicate the deduced site for initiation of transcription. Oligonucleotides α-III and α-IV used for the study of the gene are underlined, and relevant restriction sites are indicated. The nucleotide sequence in boldface is identical to the sequence determined from the cDNA clone (ref. 20; Fig. 1).

yielded a unique major protected fragment of 147 bases (Fig. 2*B*). Oligonucleotide α-IV, located upstream from the deduced mRNA start site, did not serve as a primer for the primer extension reaction and did not hybridize with brain poly(A)$^+$ RNA in Northern blot experiments (data not shown).

The nucleotide sequence determined with oligonucleotides α-III and α-IV as well as the sites for initiation of translation and transcription are shown in Fig. 3. Analysis of the nucleotide sequence revealed the absence of either TATA or CAAT boxes at their respective standard positions, 30 and 70 bp upstream of the transcription initiation site. Interestingly, a TATA box-like element occurs 160 bp upstream from the mRNA start site; however, its relevance to transcription initiation has yet to be determined. Further analysis of the 5' flanking sequence showed regions with high (75%) G+C content; one such region located 76 bp upstream from the transcription initiation site contained a GGGCG sequence resembling a Sp1 binding site (49). Although only one site is present in the CaM kinase II α-subunit gene, its position with respect to the mRNA start site corresponds to the location of the Sp1 sites in the simian virus 40 early promoter (49, 50). A number of eukaryotic genes that have promoters high in G+C content lack the characteristic TATA and CAAT boxes. In most cases, these genes are constitutively expressed and code for enzymes with a housekeeping function or participate in growth regulation (cited in ref. 51). In addition, two CAACGG presumptive binding sites for the v-*myb*-encoded protein (52) have been located 85 and 146 bp upstream from the mRNA start site. This observation and the presence of c-*myb*-encoded mRNA in the hippocampus of 3-day-old rats (53) are consistent with putative Myb regulation of CaM kinase II gene expression in brain.

Study of DNA Sites for Binding Nuclear Proteins in the 5' Flanking Region of the CaM Kinase II α-Subunit Gene. A 290-bp *Ava* I/*Pst* I fragment (Fig. 3) that includes at least 170 bp upstream from the transcription initiation site exhibited an apparent shift in electrophoretic mobility following incubation with protein extracts obtained from rat brain nuclei (Fig. 4*A*). A 391-bp *Ava* I/*Sau*3A1 fragment (Fig. 3) that included the 0.3-kbp *Ava* I/*Pst* I fragment mentioned above was isolated from the pGEM-3Zf plasmid and was subcloned into pUC18. Treatment of the construct with only two or three restriction enzymes led to the isolation of three different DNA fragments designated A, B, and C (Fig. 4*B*). Fragment A (*Ava* I/*Nco* I) comprises 170 bp and contains the transcription initiation site; fragment B (*Nco* I/*Pst* I) and fragment C (*Pst* I/*Ava* I) comprise 120 and 106 bp, respectively (diagram in Fig. 4*B*). When the three fragments were individually tested for DNA–protein interactions by gel-mobility assays, only fragment B exhibited a single, discrete shift (data not shown). Interestingly, fragment B is high in G+C content and contains the TATA box-like element and the CAACGG binding site for the *myb* gene product.

To study the specificity of this shift, labeled fragment B was incubated with rat brain nuclear extract in the absence or presence of different competitor DNA fragments (Fig. 4*C*). A 4-fold excess of the 0.3-kbp *Pst* I/*Ava* I fragment that contained fragment B was an effective competitor for the DNA-protein binding, while a different 1.0-kbp fragment starting 70 bp downstream from the initiation codon was unable to compete for the binding at an 8-fold molar excess. Trypsin pretreatment of the nuclear extract abolished the mobility shift of fragment B (Fig. 4*D*). These data are consistent with a specific, high-affinity interaction between fragment B and nuclear proteins.

Developmental Profile and Tissue Specificity of the DNA–Protein Interaction(s) with Fragment B. The presence of DNA-binding protein(s) that may modulate the transcription of CaM kinase II α-subunit gene was studied at different stages of development. Gel-mobility assays of fragment B were performed in the presence of brain nuclear extracts prepared from rat embryonic day 16 fetuses as well as newborn, 2-week-old, and adult rats. A similar mobility shift was observed with 1 and 3 μg (Fig. 5*A*) or 2 and 4 μg (data not shown) of nuclear protein independent of animal age.

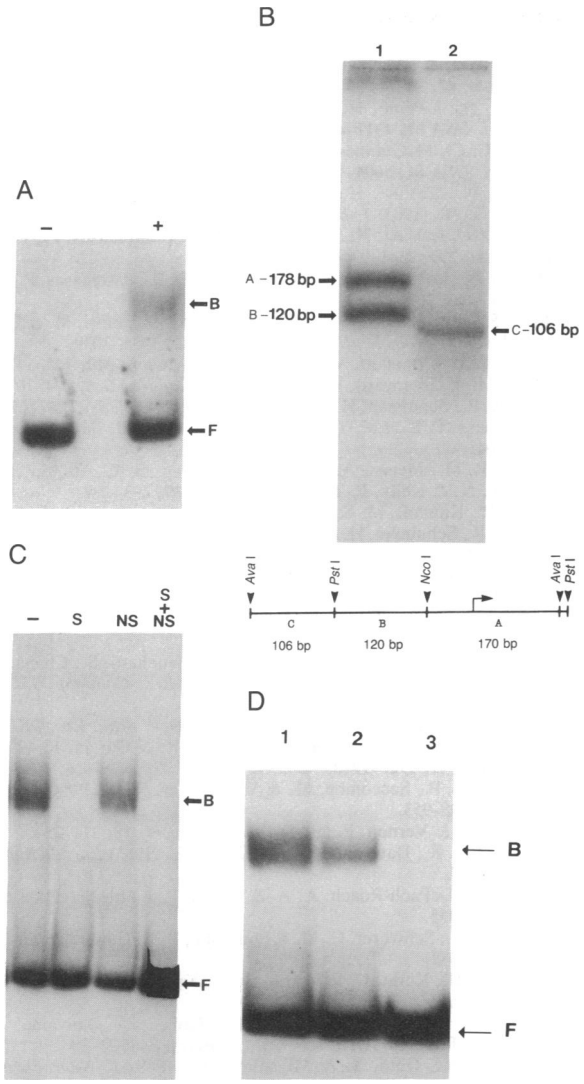

FIG. 4. Analysis of DNA–protein interaction(s) in the 5′ flanking region of the CaM kinase II α-subunit gene. (*A*) Electrophoretic mobility assay for the 0.3-kbp fragment. End-labeled 0.3-kbp *Ava* I/*Pst* I DNA fragment (3000 cpm) was tested for protein binding by incubation in the absence (−) or presence (+) of 7 μg of rat brain nuclear extract. Unbound DNA (F) was separated from protein-bound DNA (B) in a 5% polyacrylamide gel and was detected by autoradiography. (*B*) Isolation of DNA fragments A, B, and C. The pUC18 construct containing the 0.4-kbp insert was digested with *Nco* I (lane 1) or *Ava* I and *Pst* I (lane 2), the fragments obtained were isolated from a 2% agarose gel, end-labeled, and digested again with *Pst* I (lane 1) or *Nco* I (lane 2). Resultant fragments A, B, and C were isolated from a 12% polyacrylamide gel as shown; fragment sizes are indicated. A schematic representation of the restriction map for the 0.4-kbp pUC18 insert is shown below the gel; fragments A, B, and C are indicated. The arrow denotes the site for transcription initiation. (*C*) Electrophoretic mobility assay for isolated fragment B. Labeled fragment B (4000 cpm) was incubated with 7.5 μg of nuclear extract in the absence (−) or presence of a 4-fold molar excess of a specific competitor DNA fragment (S), in the presence of an 8-fold molar excess of a nonspecific DNA fragment (NS) or in the presence of both specific and nonspecific DNA fragments (S+NS). Protein-bound (B) and free (F) fragment B are indicated. (*D*) Effect of trypsin treatment of nuclear extract on the mobility shift of fragment B. Nuclear extracts (3.5 μg) were incubated for 1 hr in the absence (lanes 1 and 2) or presence (lane 3) of 1 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin at 4°C (lane 1) or at room temperature (lanes 2 and 3). Trypsinization was stopped by the addition of 4 μg of soybean trypsin inhibitor. Electrophoretic mobility assays were then performed with fragment B (4000 cpm).

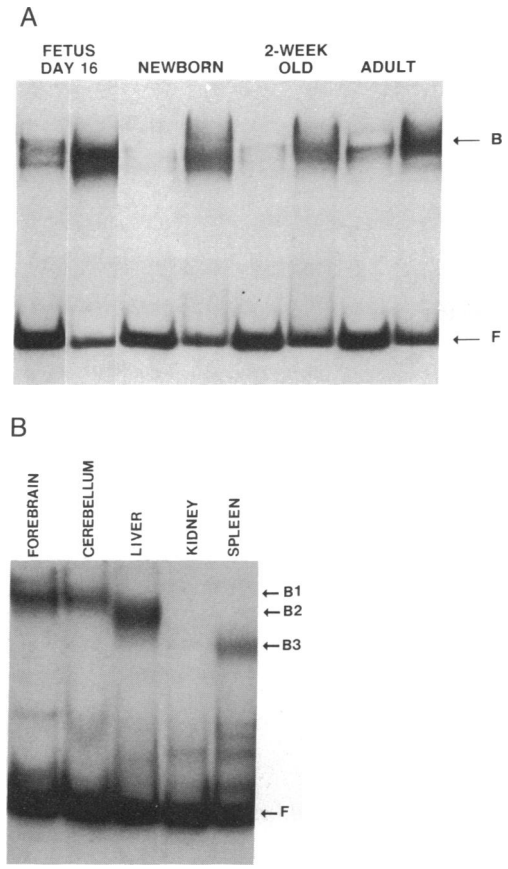

FIG. 5. Developmental profile and tissue specificity for the DNA–protein interaction(s) with fragment B. (*A*) For the developmental study, electrophoretic mobility assays of fragment B (4000 cpm) were performed in the presence of 1 μg (left lane) or 3 μg (right lane) of brain nuclear extracts from rat embryonic day 16 fetuses, from newborn rats, from 2-week-old rats, and from adult rats. All the extracts shifted fragment B from free (F) to bound (B) irrespective of animal age. (*B*) For the tissue distribution analysis, electrophoretic mobility assays for fragment B (4000 cpm) were performed in the presence of 4 μg of nuclear protein extract obtained from adult rat forebrain, cerebellum, liver, kidney, or spleen. The five tissue extracts shifted fragment B from free (F) to different bound states designated B1, B2, and B3.

This suggests that fragment B-binding proteins are present, at least in some brain cell types, from early developmental stages. Thus, the increased postnatal level of mRNA for the α subunit of CaM kinase II cannot be merely ascribed to altered levels of the nuclear proteins that bind to fragment B. Other factors such as protein–protein interactions and protein phosphorylation or protein interaction with other DNA sequences may be involved in the developmental regulation of CaM kinase II α-subunit gene expression.

We also examined the presence of fragment B-binding proteins in nuclear extracts from adult rat forebrain, cerebellum, liver, kidney, and spleen (Fig. 5*B*). An electrophoretic mobility shift was effected by all five protein extracts tested. However, the nature and abundance of the protein(s) may vary from tissue to tissue, as suggested by the differences in the extent and the amount of DNA shifted in each case. Whereas forebrain and cerebellar extracts altered the mobility of fragment B to the same extent, liver extract seemed to produce a consistently smaller gel shift. The mobility changes observed with kidney and spleen extracts were smaller than those produced by brain and liver. The DNA–protein interactions observed in all the tissues were specific and dependent on protein concentration (Fig. 6).

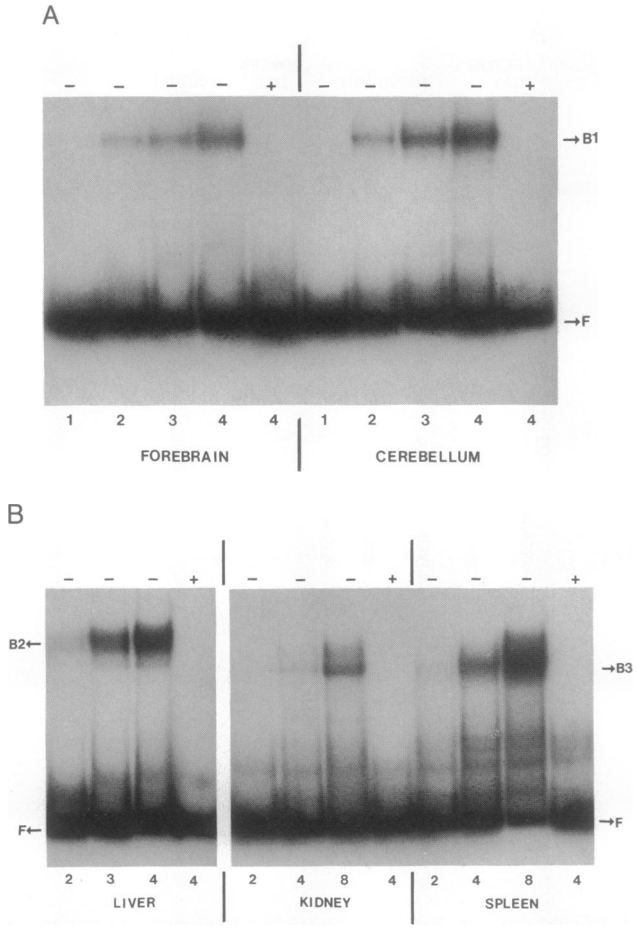

FIG. 6. DNA–protein interaction(s) for fragment B as a function of extract concentration and DNA specificity. Electrophoretic mobility shifts of fragment B were produced by increasing concentrations of nuclear extracts from forebrain and cerebellum (*A*) as well as liver, kidney, and spleen (*B*) in the absence (−) or presence (+) of a 5-fold molar excess of fragment B. The amount (in μg) and the source of the nuclear extract used are indicated. Fragment B shifted specifically from free (F) to bound states B1, B2, and B3 as a function of protein concentration.

The differences observed in the electrophoretic mobility of DNA–protein complexes implies that fragment B binds to different nuclear proteins from different tissue sources. Therefore, fragment B may contain one or more sites for the binding of nuclear proteins that regulate the transcription of CaM kinase II α-subunit gene in brain. The same or similar sequences to those in fragment B may regulate the transcription of the CaM kinase II isoenzymes in different tissues.

We thank Dr. Carol A. Ohmstede for her expert assistance and advice.

1. Schulman, H. (1988) in *Advances in Second Messenger and Phosphoprotein Research*, eds. Greengard, P. & Robison, G. A. (Raven, New York), Vol. 22, pp. 39–112.
2. Colbran, R. J., Schworer, C. M., Hashimoto, Y., Fong, Y.-L., Rich, D. P., Smith, M. K. & Soderling, T. R. (1989) *Biochem. J.* **258,** 313–325.
3. Bennett, M. K., Erondu, N. E. & Kennedy, M. B. (1983) *J. Biol. Chem.* **258,** 12735–12744.
4. Yamauchi, T. & Fujisawa, H. (1983) *Eur. J. Biochem.* **132,** 15–21.
5. Goldenring, J. R., Gonzalez, B., McGuire, J. S., Jr., & DeLorenzo, R. J. (1983) *J. Biol. Chem.* **258,** 12632–12640.
6. Kennedy, M. B., Bennett, M. K. & Erondu, N. E. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 7357–7361.
7. Kelly, P. T., McGuinness, T. L. & Greengard, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 945–949.
8. Goldenring, J. R., McGuire, J. S., Jr., & DeLorenzo, R. J. (1984) *J. Neurochem.* **42,** 1077–1084.
9. Sahyoun, N., LeVine, H., III, & Cuatrecasas, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 4311–4315.
10. Ouimet, C. C., McGuinness, T. L. & Greengard, P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5604–5608.
11. Sahyoun, N., LeVine, H., III, Bronson, D., Siegel-Greenstein, F. & Cuatrecasas, P. (1985) *J. Biol. Chem.* **260,** 1230–1237.
12. Sahyoun, N., LeVine, H., III, Bronson, D. & Cuatrecasas, P. (1984) *J. Biol. Chem.* **259,** 9341–9344.
13. Sahyoun, N., LeVine, H., III, McDonald, O. B. & Cuatrecasas, P. (1986) *J. Biol. Chem.* **261,** 12339–12344.
14. Llinás, R., McGuinness, T. L., Leonard, C. S., Sugimori, M. & Greengard, P. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3035–3039.
15. Baines, A. J. & Bennett, V. (1986) *Nature (London)* **319,** 145–147.
16. Bähler, M. & Greengard, P. (1987) *Nature (London)* **326,** 704–707.
17. Malinow, R., Schulman, H. & Tsien, R. W. (1989) *Science* **245,** 862–866.
18. McGuinness, T. L., Lai, Y. & Greengard, P. (1985) *J. Biol. Chem.* **260,** 1696–1704.
19. Hanley, R. M., Means, A. R., Ono, T., Kemp, B. E., Burgin, K. E., Waxham, N. & Kelly, P. T. (1987) *Science* **237,** 293–297.
20. Lin, C. R., Kapiloff, M. S., Durgerian, S., Tatemoto, K., Russo, A. F., Hanson, P., Schulman, H. & Rosenfeld, M. G. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 5962–5966.
21. Bennett, M. K. & Kennedy, M. B. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1794–1798.
22. Bulleit, R. F., Bennett, M. K., Molloy, S. S., Hurley, J. M. & Kennedy, M. B. (1988) *Neuron* **1,** 63–72.
23. Sahyoun, N., LeVine, H., Burgess, S. K., Blanchard, S., Chang, K.-J. & Cuatrecasas, P. (1985) *Biochem. Biophys. Res. Commun.* **132,** 878–884.
24. Weinberger, R. P. & Rostas, J. A. P. (1986) *Dev. Brain Res.* **29,** 37–50.
25. Kelly, P. T., Shields, S., Conway, K., Yip, R. & Burgin, K. (1987) *J. Neurochem.* **49,** 1927–1940.
26. Rostas, J. A. P., Seccombe, M. & Weinberger, R. P. (1988) *J. Neurochem.* **50,** 945–953.
27. Kelly, P. T. & Vernon, P. (1985) *Dev. Brain Res.* **18,** 211–224.
28. Woodgett, J. R., Davison, M. T. & Cohen, P. (1983) *Eur. J. Biochem.* **136,** 481–487.
29. Ahmad, Z., DePaoli-Roach, A. A. & Roach, P. J. (1982) *J. Biol. Chem.* **257,** 8348–8355.
30. Payne, M. E., Schworer, C. M. & Soderling, T. R. (1983) *J. Biol. Chem.* **258,** 2376–2382.
31. Schulman, H., Kuret, J., Jefferson, A. B., Nose, P. S. & Spitzer, K. H. (1985) *Biochemistry* **24,** 5320–5327.
32. Iwasa, T., Inoue, N., Fukunaga, K., Isobe, T., Okuyama, T. & Miyamoto, E. (1986) *Arch. Biochem. Biophys.* **248,** 21–29.
33. Fukunaga, K., Goto, S. & Miyamoto, E. (1988) *J. Neurochem.* **51,** 1070–1078.
34. Shenolikar, S., Lickteig, R., Hardie, D. G., Soderling, T. R., Hanley, R. M. & Kelly, P. T. (1986) *Eur. J. Biochem.* **161,** 739–747.
35. Benton, W. D. & Davis, R. W. (1977) *Science* **196,** 180–182.
36. Ullrich, A., Berman, C. H., Dull, T. J., Gray, A. & Lee, J. M. (1984) *EMBO J.* **3,** 361–364.
37. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
38. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517.
39. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 363–402.
40. Davis, L. G., Dibner, M. D. & Battey, J. F. (1986) *Basic Methods in Molecular Biology* (Elsevier, New York), pp. 222–226 and 276–284.
41. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
42. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1408–1412.
43. Reeder, R. H., Roan, J. G. & Dunaway, M. (1983) *Cell* **35,** 449–456.
44. Thompson, R. J. (1973) *J. Neurochem.* **21,** 19–40.
45. Gorski, K., Carneiro, M. & Schibler, U. (1986) *Cell* **47,** 767–776.
46. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
47. Weinberger, J., Baltimore, D. & Sharp, P. A. (1986) *Nature (London)* **322,** 846–848.
48. Sen, R. & Baltimore, D. (1986) *Cell* **46,** 705–716.
49. Dynan, W. S., Sazer, S., Tjian, R. & Schimke, R. T. (1986) *Nature (London)* **319,** 246–248.
50. Dynan, W. S. & Tjian, R. (1983) *Cell* **35,** 79–87.
51. Sehgal, A., Patil, N. & Chao, M. (1988) *Mol. Cell. Biol.* **8,** 3160–3167.
52. Biedenkapp, H., Borgmeyer, U., Sippel, A. E. & Klempnauer, K.-H. (1988) *Nature (London)* **335,** 835–837.
53. Gozes, I., Nakai, H., Byers, M., Avidor, R., Weinstein, Y., Shani, Y. & Shows, T. B. (1987) *Somatic Cell Mol. Genet.* **13,** 305–313.