

# Involvement of an Upstream Stimulatory Factor as Well as cAMP-responsive Element-binding Protein in the Activation of Brain-derived Neurotrophic Factor Gene Promoter I\*

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**The use of different brain-derived neurotrophic factor (BDNF) gene promoters results in the differential production of 5'-alternative transcripts, suggesting versatile functions of BDNF in neurons. Among four BDNF promoters I, II, III, and IV (BDNF-PI, -PII, -PIII, and -PIV), BDNF-PI was markedly activated, as well as BDNF-PIII, by Ca<sup>2+</sup> signals evoked via neuronal activity. However, little is known about the mechanisms for the transcriptional activation of BDNF-PI. Using rat cortical neurons in culture, we assigned the promoter sequences responsible for the Ca<sup>2+</sup> signal-mediated activation of BDNF-PI and found that the Ca<sup>2+</sup>-responsive elements were located in two separate (distal and proximal) regions and that the DNA sequences in the proximal region containing cAMP-responsive element (CRE), which is overlapped by the upstream stimulatory factor (USF)-binding element, were largely responsible for the activation of BDNF-PI. CRE-binding protein (CREB) family transcription factors and USF1/USF2 bind to this overlapping site, depending upon their preferred sequences which also control the magnitude of the activation. Overexpression of dominant negative CREB or USF reduced the BDNF-PI activation. These findings support that not only CREB but also USF1/USF2 contributes to Ca<sup>2+</sup> signal-mediated activation of BDNF-PI through the recognition of an overlapping CRE and USF-binding element.**

Brain-derived neurotrophic factor (BDNF),<sup>1</sup> a member of the neurotrophin family, plays an important role in promoting

neuronal survival, neuronal differentiation, and synaptic plasticity (1). BDNF is predominantly expressed in the central nervous system, and its mRNA expression is up-regulated by neuronal activity accompanying the influx of Ca<sup>2+</sup> into neurons (2, 3). The BDNF gene consists of four short 5'-exons (exons I, II, III, and IV) and a common 3'-exon V encoding a prepro-BDNF protein (4). Four promoters, BDNF-PI, II, III, and IV, were mapped upstream of the 5'-exons, respectively, which are differentially regulated by kainic acid-induced seizure in distinct regions of the rat brain (5), suggesting versatile BDNF functions in the brain (6). Therefore, elucidating the regulatory mechanisms of BDNF gene transcription will provide a better understanding of BDNF functions in the brain.

We previously reported that BDNF-PI and -PIII were differentially activated by Ca<sup>2+</sup> signals evoked via two distinct Ca<sup>2+</sup> entry sites, the L-type voltage-dependent Ca<sup>2+</sup> channel (L-VDCC) and the N-methyl-D-aspartate glutamate receptor (NMDA-R) (7). BDNF-PI mainly responds to Ca<sup>2+</sup> signals evoked via L-VDCC, whereas BDNF-PIII reacts to those evoked via either NMDA-R or L-VDCC but less to those via NMDA-R, suggesting that the transcriptional mechanisms initiated in response to Ca<sup>2+</sup> signals evoked via Ca<sup>2+</sup> channels, at least in part, differ between BDNF-PI and -PIII. It has already been reported that the binding of cAMP-responsive element (CRE)-binding protein (CREB) to CRE on BDNF-PIII is required for the Ca<sup>2+</sup> responsiveness of BDNF-PIII (8, 9). Quite recently, however, novel Ca<sup>2+</sup>-responsive elements (CaREs) of BDNF-PIII have been characterized and three CaREs, called CaRE1, CaRE2, and CaRE3 (or CRE), identified within a stretch of 170 bp upstream of exon III (10, 11). In addition to CREB, which specifically binds to CaRE3 (or CRE), a novel calcium-responsive transcription factor, which specifically binds to CaRE1, has been found to drive the neuronal specific activation of BDNF-PIII in response to the Ca<sup>2+</sup> signals (11). However, it is still unknown whether the CREB or another transcription factor is involved in the activation of BDNF-PI.

In the present study, we assigned the Ca<sup>2+</sup>-responsive DNA elements of BDNF-PI and identified the transcription factors binding to these responsive elements. As a result, we found that not only the CREB family transcription factors but also the upstream stimulatory factor (USF) bound to the responsive site on which the CRE and the USF-binding element overlap each other. It is well established that CREB is a transcription factor, which conveys intracellular Ca<sup>2+</sup> signals to the gene

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<sup>1</sup> The abbreviations used are: BDNF, brain-derived neurotrophic factor; CRE, cAMP-responsive element; CREB, cAMP-responsive element-binding protein; USF, upstream stimulatory factor; BDNF-P, brain-derived neurotrophic factor promoter; L-VDCC, L-type voltage-dependent Ca<sup>2+</sup> channel; RT, reverse transcription; NMDA-R, N-methyl-D-aspartate glutamate receptor; CaRE, Ca<sup>2+</sup>-responsive element; DIV, days in culture; APV, DL-amino-5-phosphonovalerate; TRE,

tetradecanoylphorbol-13-acetate -responsive element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CBP, cAMP-responsive element-binding protein-binding protein; EF1 $\alpha$ , human elongation factor 1 $\alpha$ .

**DNA Transfection, Luciferase, and  $\beta$ -Galactosidase Assays**—DNA transfection was carried out over 3 days in culture and using calcium phosphate/DNA precipitation as already described (18). Briefly, the calcium phosphate/DNA precipitates were prepared by mixing one volume (100  $\mu$ l) of plasmid DNA (6  $\mu$ g, pBDNFpL:pRL-EF1 $\alpha$  = 10:1) in a 250 mM CaCl<sub>2</sub> solution with an equal volume of 2 $\times$  HEPES-buffered saline (50 mM HEPES-NaOH (pH7.05), 280 mM NaCl, 1.5 mM



Na<sub>2</sub>HPO<sub>4</sub>), and added to each 60-mm dish. For overexpression experiments, we used plasmid DNA (6 µg, pBDNFpI:EF1α-β-gal:expression vector = 5:1:30). The dish was washed three times with phosphate-buffered saline and fresh TIS medium added. After 40 h, the transfected cells were stimulated with 25 mM KCl (25 mM KCl stimulation) or vehicle for 6 h and cell lysates were prepared.

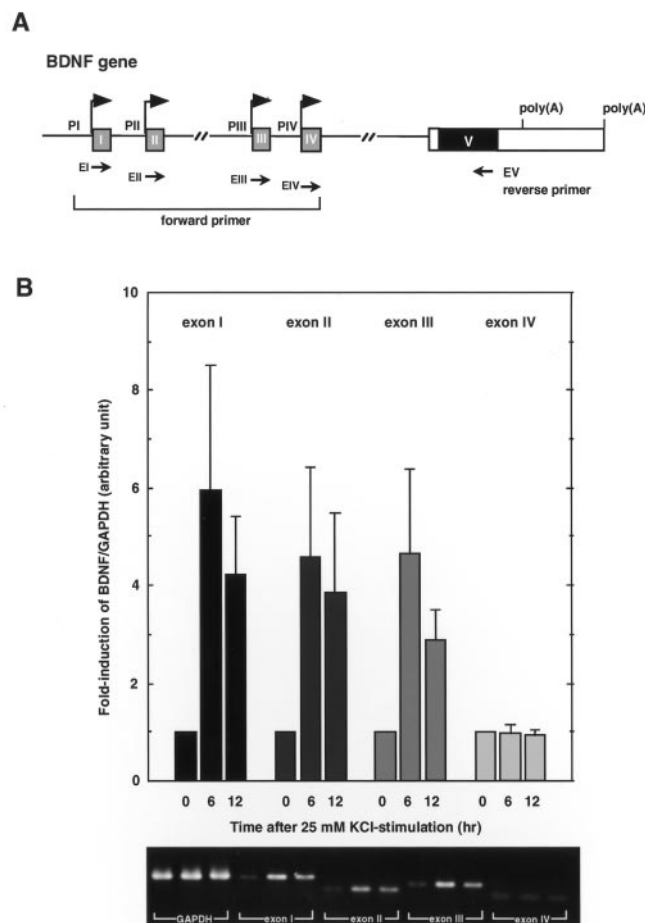
For the dual (firefly and *Renilla*) luciferase assay, cell lysates were extracted with passive lysis buffer (Promega) and used as described previously (7). For the measurement of β-galactosidase activity as an internal control, cell lysates were extracted with 250 µl of cell lysis buffer containing 1 mM potassium phosphate (pH 7.8), 1 mM dithiothreitol, and 0.5% Triton X-100; 30 µl of the lysate was used for the chemiluminescence-based β-galactosidase assay (CLONTECH), and 20 µl for the firefly luciferase assay.

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)**—Forty hours after the medium exchange at 3 DIV, cells were stimulated with 25 mM KCl (25 mM KCl stimulation) or vehicle and incubated for 6 h. Then, nuclear extracts were prepared as described with minor modifications (20). EMSAs were carried out using probes corresponding to the BDNF-PI sequence (these are indicated in Fig. 4) as reported previously (21). Briefly, end-labeling of DNA probes was performed at 37 °C for 20 min in 10 µl of reaction mixture (6.6 mM Tris-HCl, pH 7.4, 50 mM NaCl, 6.6 mM MgCl<sub>2</sub>, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 1 mM dithiothreitol) containing 200 ng of DNA, 2 µl of [<sup>32</sup>P]dCTP, and 1 unit of Klenow fragments. Then, DNA probes were recovered with a Sephadex G-50 column. The DNA-protein binding reaction was carried out at 25 °C for 15 min in 20 µl of binding buffer (20 mM HEPES-NaOH, pH 7.9, 80 mM NaCl, 0.3 mM EDTA, 0.2 mM EGTA, 0.2 mM phenylmethanesulfonyl fluoride, 10% glycerol, 2 µg of poly(dI-dC), 0.2–0.4 ng of <sup>32</sup>P-labeled DNA probes, and 5 µg of nuclear extract). Then, DNA-protein complexes were separated on 4% polyacrylamide gel at 132 V for 2.5 h. The protein concentration was determined by the method of Lowry. For supershift EMSA, a series of antibodies (2 mg/ml) was used at a dilution of 1:20.

## RESULTS

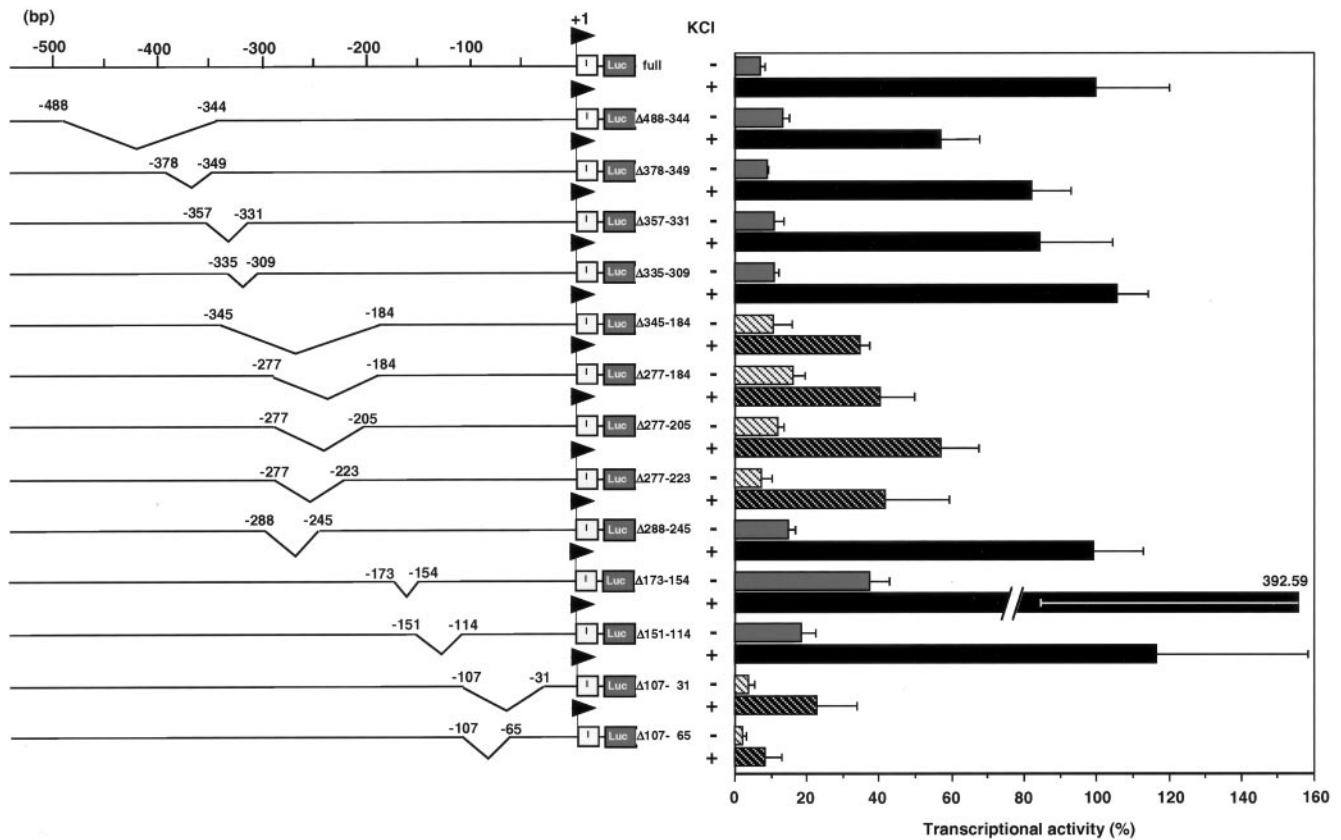
**Production of BDNF Transcripts Containing Exons I, II, III, and IV Induced via Membrane Depolarization**—Although the complicated structure of the BDNF gene is suggested to give rise to at least eight species of BDNF transcript (4), it has already been reported that the exon III-containing transcript is the major form, the expression of which is controlled by BDNF-PIII in response to the Ca<sup>2+</sup> signals evoked via membrane depolarization in rat cortical neurons (8, 9). In addition, we have also reported that BDNF-PI is activated by membrane depolarization, the basal promoter activity being ~8-fold less than that of BDNF-PIII (7, 18). To confirm whether endogenous 5'-exon specific BDNF transcripts, particularly those containing exon I, are induced by membrane depolarization or not, we examined the increase in each transcript by RT-PCR. To distinguish each transcript, we designed a forward primer corresponding to exon I, II, III, or IV and a reverse primer corresponding to the common exon V (Fig. 1A). As shown in Fig. 1B, the expression of three transcripts containing exons I, II, and III was up-regulated by the exposure of cells to 25 mM KCl in medium (25 mM KCl stimulation), whereas the expression of the exon IV-containing transcript remained constant even after the stimulation. The minimum cycle numbers required for detecting transcripts by RT-PCR were 32 and 26 cycles for exon I and III, respectively (see the legend of Fig. 1), indicating that the expression level of the exon I-containing transcript is lower than that of the exon III-containing transcript. These findings indicate that BDNF-PI is activated by membrane depolarization in neurons although the expression level of the exon I-containing transcript is quite low, compared with that of the exon III-containing transcript.

**Calcium-responsive Elements Located in Two Distinct Regions of BDNF Promoter I**—To assign the calcium (Ca<sup>2+</sup>)-responsive elements in BDNF-PI, we constructed a series of firefly luciferase reporter vectors containing various lengths of the promoter I region with internal deletions (see Fig. 2) for the measurement of promoter activity. Each vector was transfected



**FIG. 1. Expression of 5'-exon-specific BDNF transcripts stimulated by membrane depolarization.** A, structure of the BDNF gene and location of primers for RT-PCR. The BDNF gene accommodates four alternative promoters, BDNF-PI, -PII, -PIII, and -PIV, which reside upstream of exons I, II, III, and IV, respectively (4). Gray boxes indicate 5'-noncoding exons I, II, III, and IV; the closed box the region coding prepro-BDNF mRNA in exon V; and the open box the 3'-noncoding region. Arrows indicate the positions of primers used for RT-PCR. EI, EII, EIII, and EIV are specific forward primers corresponding to exons I, II, III, and IV, respectively, and the reverse primer (EV) is located in the coding region. B, rat cortical neurons (5 DIV) were stimulated with 25 mM KCl (25 mM KCl stimulation), and total RNA was isolated at the times indicated (0, 6, and 12 h) and subjected to RT-PCR. The relative intensities of amplified DNA fragment containing each exon were analyzed with an Imaging scanner and plotted. The minimum cycle numbers required for detecting the amplified DNA were 32, 31, 26, and 29 for exons I, II, III and IV, respectively. The data represent the mean ± S.E. from the experiment performed in triplicate, and the same tendency was obtained from at least two separate experiments.

with *Renilla* luciferase control vector driven by EF1α promoter (pRL-EF1α) into rat cortical neurons at 3 DIV. We previously reported that the luciferase activity derived from the vector carrying the region from -528 to +138 increased in response to the Ca<sup>2+</sup> influx through L-VDCC, which was caused by 25 mM KCl stimulation, suggesting the presence of Ca<sup>2+</sup>-responsive elements within the region up to nucleotide position -528 on BDNF-PI (18). As shown in Fig. 2, the luciferase activities of plasmid vectors pBDNFpI(Δ378–349), -(Δ357–331), -(Δ335–309), -(Δ288–245), and -(Δ151–114) were nearly as inducible as those of pBDNFpI(full). Although the use of pBDNFpI(Δ488–344) decreased the luciferase activity, the deletion of the 5'-flanking region up to -310 (pBDNFpI(-311 to +138)) did not decrease the luciferase activity, compared with that of pBDNFpI(full) (data not shown), suggesting that the upstream region



**FIG. 2. Effect of internal deletion on transcriptional activation of BDNF promoter I.** Firefly luciferase reporter vectors carrying various lengths of BDNF promoter I with an internal deletion were transfected into rat cortical neurons with pRL-EF1 $\alpha$  control vector at 3 DIV. Forty hours after DNA transfection, cells were stimulated with 25 mM KCl (+) or vehicle (–), and incubated for 6 h. Then, cell lysates were extracted and used for luciferase reporter assay. The structures of a series of deletion constructs used here are illustrated on the left. An arrow indicates the transcriptional initiation site (+1). The open box indicates the region of exon I (+1 ~ +138). Luciferase in the gray box indicates the firefly luciferase gene. The deleted regions in the BDNF promoter I are indicated by a bent line with the nucleotide positions. pBDNFpI(full) means the wild-type construct without any deletion. The value of KCl-stimulated transcriptional activation of pBDNFpI(full) was indicated as 100%. The data represent the mean  $\pm$  S.E. from at least four independent experiments.

up to –310 is not so largely involved in the Ca<sup>2+</sup> signal-mediated activation of BDNF-PI. In contrast, the use of plasmid vectors pBDNFpI(Δ345–184), (–Δ377–184), (–Δ377–205), and (–Δ377–223) resulted in a decrease in Ca<sup>2+</sup> responsiveness of ~40–60%, suggesting that this region includes the Ca<sup>2+</sup>-responsive elements. In addition, more marked decreases in Ca<sup>2+</sup> responsiveness were observed with the deletion mutants pBDNFpI(Δ107–31) and (–Δ107–65), also suggesting a localization of elements responsible for a strong Ca<sup>2+</sup> response in this region. We called these two regions the distal and proximal region, respectively. On the other hand, deletion of DNA sequences from –173 to –154 (pBDNFpI(Δ173–154)) rather increased the luciferase activity, indicating that DNA elements which function as silencers could be located within this region.

**Critical Role of DNA Sequences Including the CRE-like Element and a Few Upstream Sequences in the Activation of BDNF-PI**—As shown in Fig. 2, the proximal region from –107 to –65 was extremely effective in the activation of BDNF-PI. Therefore, we focused on the proximal region and investigated which DNA sequences within this region contribute to the activation of BDNF-PI. Using the TFSEARCH program ([www.rwcp.or.jp/papia/](http://www.rwcp.or.jp/papia/)) (22), we found that this region contained several candidate DNA-binding sites for transcription factors, such as CRE- and 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (TRE)-like sequences (Fig. 3A). To investigate the role of these sites in the activation of BDNF-PI, we constructed a series of plasmid DNAs having internal mutations and deletions at the sites and transiently transfected

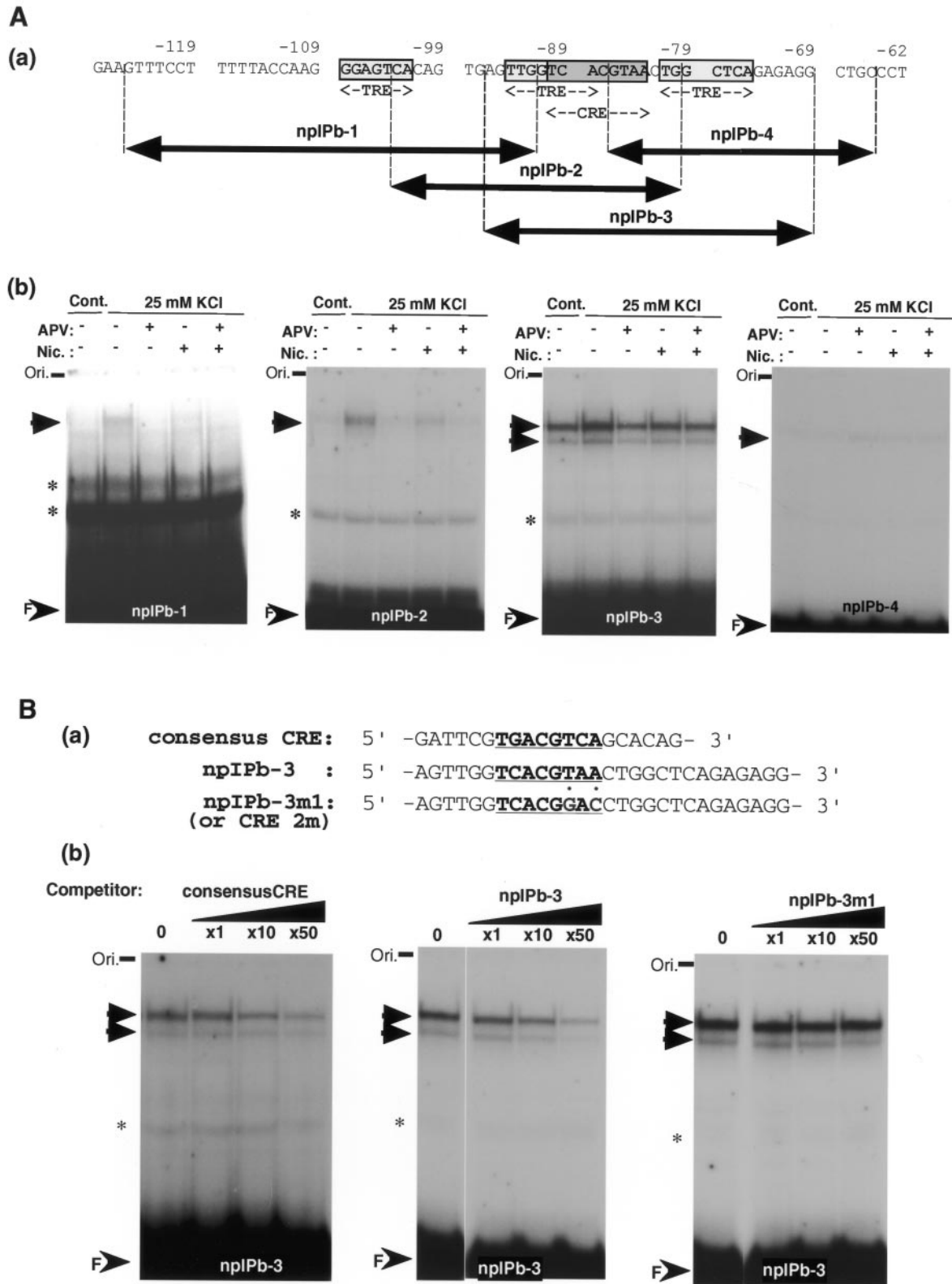
them into rat cortical neurons. As shown in Fig. 3B, mutation and deletion of the downstream TRE-like sequence (TRE3m and TRE3Δ) did not affect the luciferase activity, whereas a series of mutants of the CRE-like sequence (CRE2m, CRE2Δ) showed a marked decrease in luciferase activity. Deletion of the middle TRE-like sequence which partially overlapped with CRE (TRE2Δ) resulted in the same decrease as with the CRE2 mutants (CRE2m, CRE2Δ). On the other hand, mutants of the upstream TRE-like sequence (TRE1m, TRE1Δ) were also effective in decreasing the transcriptional activity. However, the mutation combined with the deletion of CRE (TRE1/CRE2Δ) decreased the BDNF-PI activation to almost the same level as CRE2Δ, suggesting a minor contribution of upstream TRE to the activation of BDNF-PI. Among the mutant plasmid vectors constructed, plasmid TRE2/CRE2Δ with an 11-base pair deletion containing middle TRE- and CRE-like sequences was most effective in reducing the level of BDNF-PI activity, indicating that the DNA containing the CRE-like sequence and a few bases upstream of this sequence are mainly responsible for the activation of BDNF-PI.

Additionally, we constructed a plasmid vector, pBDNFpI(Δdistal/CRE2m), with both a deletion of the distal region (Δ277–223) and mutation of CRE2 (Fig. 3C). Compared with the luciferase activity of wild-type pBDNFpI(full), the activity of Δdistal was low, which was consistent with the data shown in Fig. 2. On the other hand, Δdistal/CRE2m showed a greater decrease in luciferase activity than Δdistal, the level of which was almost the same as that of CRE2m (Fig. 3C). These results

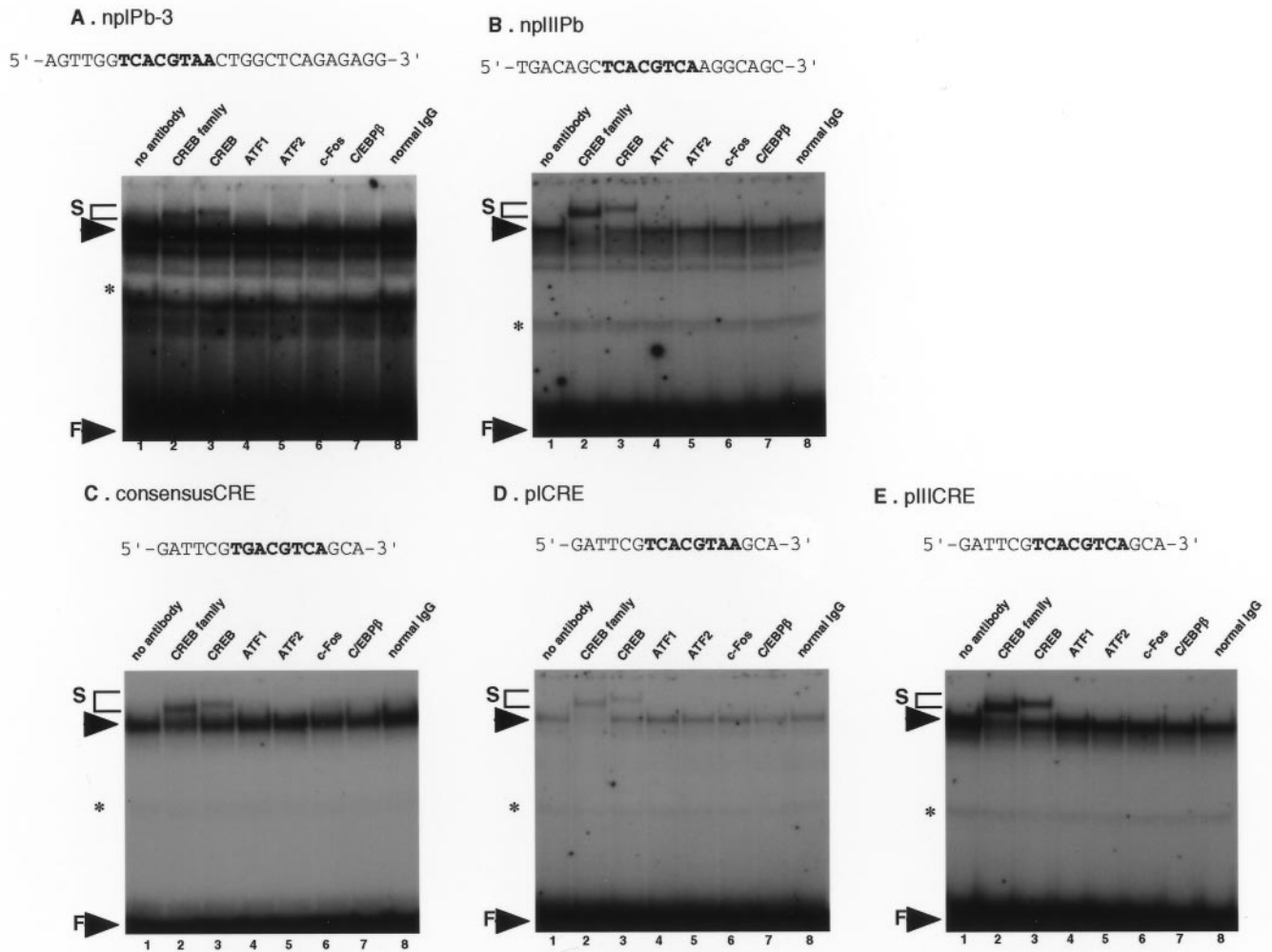


To investigate whether the binding to npIPb-3 probe involves the CRE-like sequence, we examined the competitive effect of a consensus CRE. As shown in Fig. 4B (b), both the upper and lower bands were gradually erased when an excess of not only the cold npIPb-3 but also consensus CRE competitors was added. The mutation of two bases introduced into the CRE-like sequence (npIPb-3m1) failed to affect the DNA binding (Fig. 4B, a and b). These results indicate that the CRE-like sequence in the proximal region functions as the CRE. The competitors





**FIG. 4. DNA binding activities in the proximal region and their sensitivities to CRE.** *A*, DNA binding activities using four kinds of DNA probes covering the proximal region. *a*, nucleotide sequences covering the proximal region and position of DNA probes for EMSA used here. Four native promoter I DNA probes (npIPb-1, -2, -3, and -4) were designed to cover the proximal region from -109 to -62 of BDNF promoter I. *b*, EMSA for detecting transcription factors which bind to the proximal region of BDNF promoter I. Rat cortical neurons were stimulated with 25 mM KCl and incubated for 6 h. Nuclear extracts were then prepared and subjected to EMSA after DNA-protein binding reaction. Inhibitors APV (200  $\mu$ M) and nicardipine (Nic.; 5  $\mu$ M) were added 10 min before the 25 mM KCl stimulation. *B*, competitive effect of consensus CRE (*consCRE*), npIPb-3, and its mutant (*npIPb-3m1*) on the DNA binding activity to npIPb-3. *a*, nucleotide sequences of competitors. Bold letters indicate the position of consensus CRE, CRE-PI, and mutated CRE-PI. Dotted gray letters indicate the substituted bases. *b*, using a labeled npIPb-3, a competitive binding reaction was performed with a 1-, 10-, and 50-fold molar excess of cold competitors. Arrows indicate the doublet of bands, the upper and the lower band, and asterisks nonspecific bands. F means free probes. The experiments were done twice, and the same results were obtained.

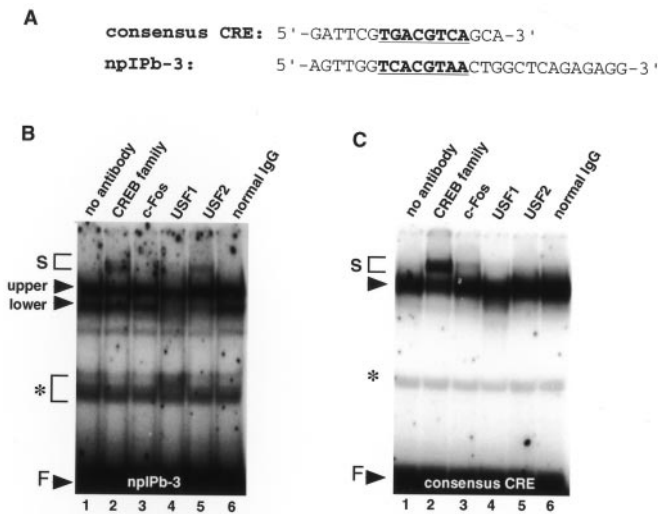


**FIG. 5. Analysis of transcription factors binding to the proximal region using supershift EMSA.** Nuclear extracts were prepared from the cells stimulated with 25 mM KCl and were subjected to supershift EMSA after DNA-protein binding reaction. Antibodies (0.08  $\mu$ g/ml) against the CREB family, CREB, ATF1, ATF2, c-Fos, C/EBP $\beta$ , and normal IgG were added to the reaction mixture before the DNA-protein binding reaction was started at 25  $^{\circ}$ C (see "Experimental Procedures"). S indicates the positions of supershifted bands. The experiments shown in A and B were performed using the DNA probes derived from BDNF promoter I (npIPb-3) and from BDNF promoter III including the CRE of BDNF-PIII (from bp -36 to -29) (npIIPb), respectively. Experiments in C-E were performed using the DNA probes containing the consensus CRE (C), and the CRE-like sequence of BDNF-PI (CRE-PI) (D) and of BDNF-PIII (CRE-PIII) (E), surrounded by DNA sequences that were randomly chosen from the nucleotide sequences of pBR322. The **bold letters** in the DNA probes indicate the position of the consensus CRE, CRE-PI, and CRE-PIII. The experiments were done twice, and the same results were obtained.

containing a consensus TRE failed to compete with the binding to npIPb-3 probe, indicating that the TRE-like sequence on npIPb-3 was unable to function as the TRE. However, the binding to npIPb-2 probe was reduced by an excess of consensus TRE (data not shown), indicating that the TRE-like sequence on npIPb-2 probe functions as the TRE. These findings indicate that the CRE-like sequence in the proximal region works as the CRE and the proteins binding to npIPb-3 probe were different from those binding to the npIPb-2 probe.

**Binding of CREB Family Transcription Factors to the CRE of BDNF Promoter I**—To identify the DNA-binding proteins that can bind to the CRE-like sequence of BDNF-PI (CRE-PI), we performed EMSA using npIPb-3 probe, in which antibodies against transcription factors were added prior to the start of the DNA-protein binding reaction. When the anti-CREB family or anti-CREB specific antibody was added to the mixture containing npIPb-3 probe with nuclear extracts prepared from 25 mM KCl-stimulated cortical neurons, supershifted bands were observed but faintly (Fig. 5A, lanes 2 and 3). When anti-ATF1, anti-ATF2, anti-c-Fos, or anti-C/EBP $\beta$  specific antibody was added to the mixture, no shifted band was observed (Fig. 5A,

lanes 4–7). When the native promoter III DNA probe (npIIPb) containing CRE-PIII that was previously identified as a calcium-responsive element (CaRE3) (8, 9) was used, stronger shifted bands than those with npIPb-3 probe were observed in the presence of anti-CREB family or anti-CREB specific antibody (Fig. 5B, lanes 2 and 3). Anti-phosphorylated CREB specific antibody was also effective in shifting the bands upon EMSA (data not shown). This supershift of bands caused by anti-CREB family or anti-CREB specific antibody was clearly observed with consensus CRE probe (Fig. 5C, lanes 2 and 3). To test further whether the CRE-PI in the proximal region could solely function as the CRE, we used DNA probes, pICRE and pIIICRE, in which the CRE-like sequence of BDNF-PI or -PIII was surrounded by the same sequences as surrounded the consensus CRE. As shown in Fig. 5, D and E (lanes 2 and 3), supershifted bands were observed in the presence of anti-CREB family or anti-CREB specific antibody, in which the supershift of the bands was observed at almost the same level as that of pIIICRE probe. Thus, the CRE-PI in the proximal region of BDNF-PI functions as the CRE by itself, which is able to introduce CREB or CREB family proteins for binding, and its

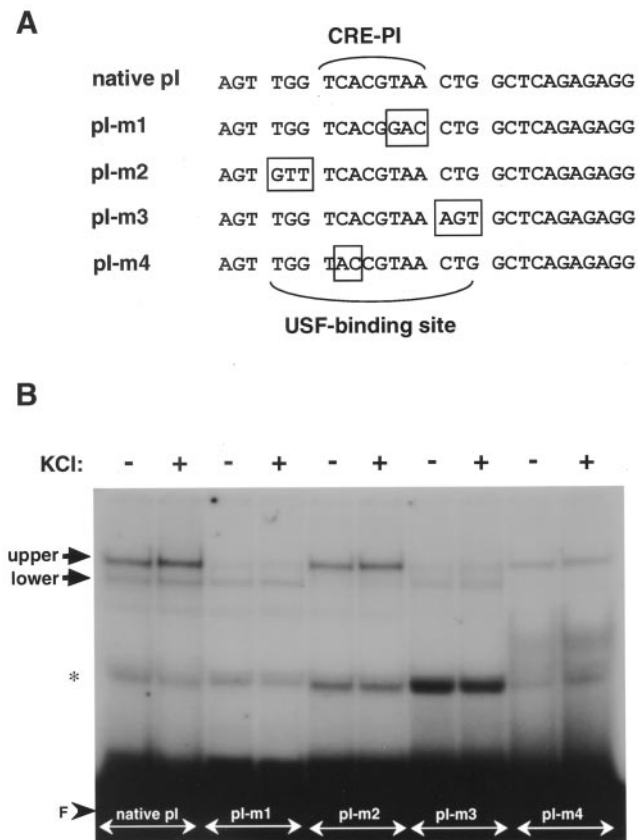


**FIG. 6. Binding of USFs to the proximal region.** Supershift EMSA was performed according to the same experimental schedule as described in the legend of Fig. 5. **A**, the DNA sequences of the probes used here, consensus CRE and native promoter I DNA probe (npIPb-3), are shown and the underlined bold letters indicate the consensus CRE and CRE-PI. **B** and **C**, USF1 and USF2 indicate antibodies acting specifically against USF1 and USF2, respectively. Antibodies against the CREB family and c-Fos were also added. The experiments were done three times, and the same results were obtained.

effectiveness seems to be equal to that of BDNF-PIII. As described above, however, the level of supershift of the bands formed on npIPb-3 probe in the presence of anti-CREB family or anti-CREB antibody appeared to be lower than that formed on native pIII DNA probe (Fig. 5, **A** and **B**).

**Binding of USF to a Site Overlapping CRE**—Searching other binding sites for known transcription factors in the proximal region of BDNF-PI using the data base TFSEARCH, we found that the USF-binding site (5'-TGGTCACTGAACTG-3') was located and overlapped the CRE-PI (Fig. 7A). Therefore, we next investigated whether USF could be included in the DNA-protein complex formed on the npIPb-3 probe. As shown in Fig. 6B, addition of anti-USF1 or -2 specific antibody prior to the DNA-protein binding reaction resulted in a complete disappearance of the lower band of doublet (lanes 4 and 5). Furthermore, addition of anti-USF1 but not anti-USF2 antibody partially erased the upper band and slightly shifted its position downward (lane 4). The partial disappearance and downward shift of the upper band were also observed with the consensus CRE probe (Fig. 6C, lane 4), although only the upper band was obtained with this probe. When anti-CREB family antibody was added, supershifted bands appeared faint with npIPb-3 probe but were clear with consensus CRE probe (Fig. 6, **B** and **C**, lanes 2), which was consistent with the results shown in Fig. 5 (**A** and **C**). Furthermore, using the npIPb-3 probe or consensus CRE, anti-c-Fos antibody did not give rise to the shifted band (Fig. 6, **B** and **C**, lane 3). Thus, the upper band of the doublet formed by npIPb-3 probe, at least in part, consists of CREB family transcription proteins or, at least, CREB and the lower band of the doublet consists of USF1/USF2 only.

**Changes in DNA Binding Activities Induced by Mutations of CRE-PI and/or the USF-binding site**—Because we found that the CRE-PI and USF-binding site overlapped in the proximal region, we next investigated which DNA sequences are preferentially or commonly used for the binding of CREB family proteins or USF. For this purpose, we introduced several substitutions of nucleotide bases in the CRE-PI and USF-binding site by constructing four mutant DNA probes for EMSA (Fig. 7A). The npIPb-3m1 and -3m4 probes were designed to destroy

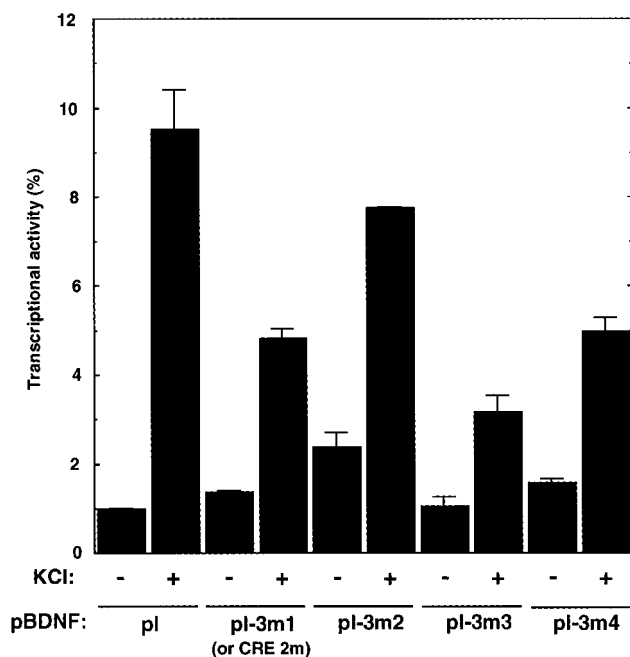


**FIG. 7. Effects of mutations in CRE-PI and the USF-binding site on DNA binding activity.** EMSA was performed with the same experimental schedule as described in the legend of Fig. 4. **A**, nucleotide sequences of five DNA probes. Nucleotide bases in open boxes are substituted. In npIPb-3m1 and -3m4 probes, both CRE-PI and USF-binding sequences were destroyed by mutations. In npIPb-3m2 and -3m3, the USF-binding site but not CRE-PI was lost. **B**, the cells were treated with 25 mM KCl (+) or vehicle (-), and the nuclear extracts were prepared for EMSA after DNA-protein binding reaction. Arrows indicate the upper and the lower bands and asterisks nonspecific bands. F indicates free probes. The specific radioactivity of every mutant DNA probe was almost the same (data not shown). The experiments were done twice, and the same results were obtained.

both CRE-PI and the USF-binding site. On the other hand, the npIPb-3m2 and -3m3 were designed for destruction of the USF-binding site only. Comparing the intensities of the upper and lower bands formed on the npIPb-3 probe, as shown in Fig. 7B, the upper band was found to disappear with npIPb-3m1 and -3m3 but remain with npIPb-3m2 and partially so with -3m4, whereas the lower band tended to remain with npIPb-3m1 and -3m3 but disappear with npIPb-3m2 and -3m4. Mutation of npIPb-3m3 probe, even though it is located outside the CRE-PI, markedly decreased the upper band, the DNA-protein complex of which should include the CREB family. Mutation of npIPb-3m2 probe, introduced at the 5' outside of the CRE-PI, selectively removed the lower band but not the upper band. Taken together, the formation of the upper band tended to be dependent upon the 3' side sequences, and that of the lower band upon the 5' side sequences within this region.

**Transcriptional Activity of BDNF Promoter I Carrying the Mutated CRE/USF-binding site**—Because substitutions of CRE-PI and/or the USF-binding site affected the binding activity of CREB family proteins and USF (Fig. 7), we constructed plasmid vectors in which the same substitutions were introduced into the proximal region of BDNF-PI and, then, measured transcriptional activity using transient DNA transfection. The luciferase activity of vector pBDNFpI-3m1 (also termed





**FIG. 8. Effect of mutation within CRE-PI and/or the USF-binding site on the activation of BDNF promoter I.** We constructed reporter vectors carrying the same substitutions in CRE-PI and the USF-binding site as were introduced into the DNA probes for EMSA (Fig. 7) and transfected them into rat cortical neurons for measuring transcriptional activation. Cells were stimulated with 25 mM KCl (+) or vehicle (–). Transcriptional activation was indicated as a -fold increase of the control in which pBDNFpl(full) was transfected and the 25 mM KCl stimulation was omitted. The nucleotide sequences of pBDNFpl, pI-3m1, pI-3m2, pI-3m3, and pI-3m4 were shown in Fig. 7A. The data represent the mean  $\pm$  S.E. from experiments performed in triplicate. The same tendency was obtained from two independent experiments.

pBDNFpl-CRE2m in Fig. 3, A and B) decreased by approximately 50% in terms of  $\text{Ca}^{2+}$  responsiveness (Fig. 8), which was consistent with the data shown in Fig. 3. Mutation of npIPb-3m2, which preferentially removed the lower band but not the upper band (Fig. 7B), resulted in a decrease of  $\sim 20\%$  in  $\text{Ca}^{2+}$  responsiveness. In contrast, mutation of npIPb-3m3, by which both the upper and the lower bands were erased (Fig. 7B), resulted in the greatest decrease in  $\text{Ca}^{2+}$  responsiveness. The luciferase activity of pBDNFpl-3m4 was almost the same as that of pBDNFpl-3m1, whose mutation resulted in the disappearance of both the upper and lower bands but the upper band tended to be stronger than that for mutation of npIPb-3m3. Thus, the  $\text{Ca}^{2+}$  responsiveness of four reporter mutant vectors showed a good correspondence with the preferential binding of DNA probes into which the same mutation was introduced (Figs. 7 and 8), indicating an involvement of the transcription factors that preferentially bind to CRE-PI and the USF-binding site in the activation of BDNF-PI.

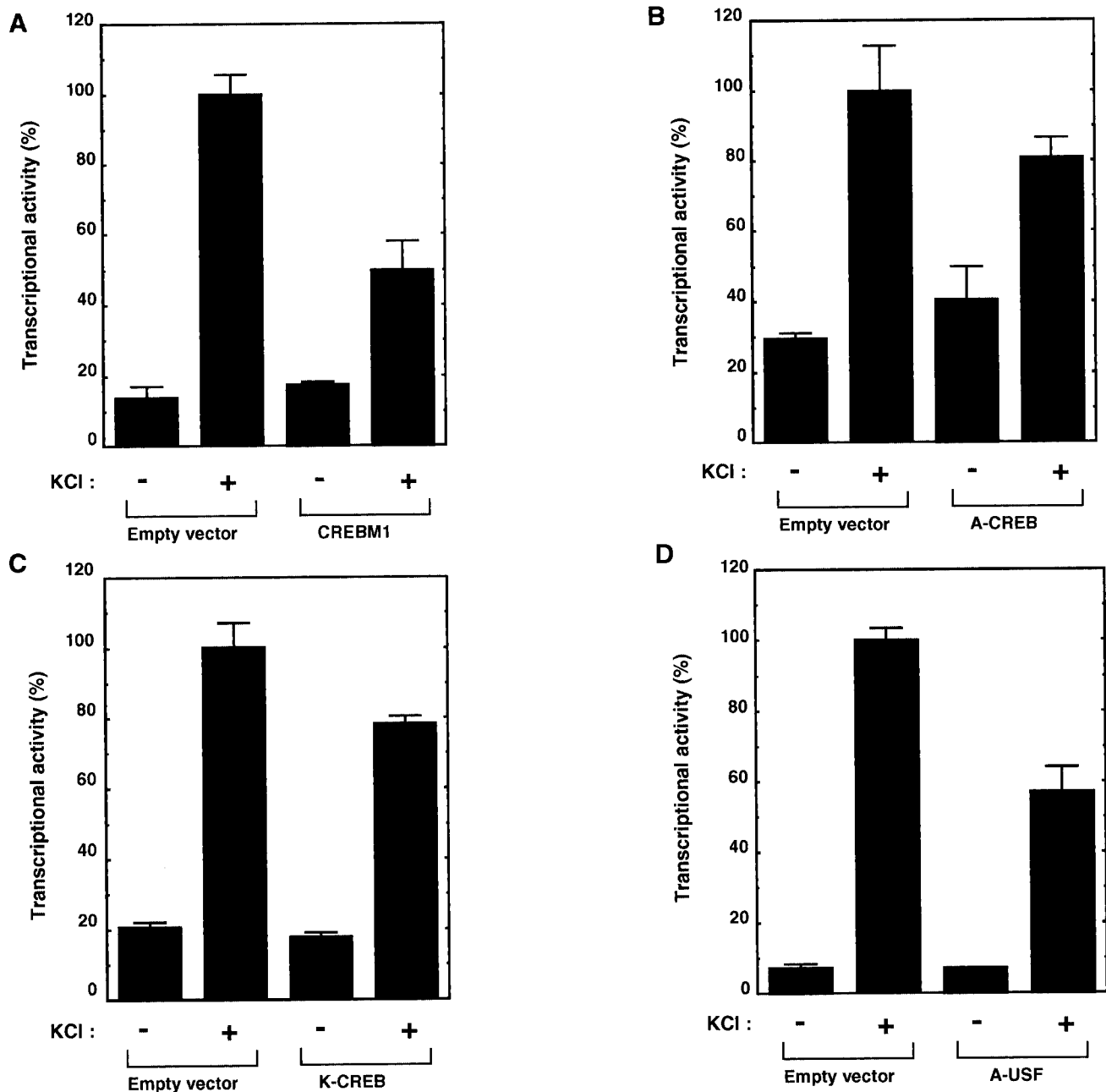
**Effect of Dominant Negative CREB or USF on the  $\text{Ca}^{2+}$  Responsiveness of BDNF-PI**—To address further the crucial role of CREB family proteins and USF in the  $\text{Ca}^{2+}$  signal-mediated activation of BDNF-PI, mutant plasmid vectors of dominant negative CREB and USF were introduced into rat cortical neurons with a reporter vector, pBDNFpl(full). Instead of the *Renilla* luciferase vector, we used another control vector carrying an EF1 $\alpha$  promoter-driven *Escherichia coli*  $\beta$ -galactosidase gene. Overexpression of CREB M1, a mutant in which the serine-133 residue was converted to alanine (23), inhibited the activation of BDNF promoter I up to  $\sim 50\%$  of the control transfected with empty vector (Fig. 9A). Overexpression of A-CREB, a mutant with an acidic extension at the N terminus of the CREB leucine zipper domain that could interfere with

the binding of DNA and transcriptional activation of wild-type CREB (24), or K-CREB, a mutant with a substitution in the DNA-binding domain that could inhibit the binding of wild-type CREB (25), decreased the activation of BDNF-PI by  $\sim 20\%$  (Fig. 9, B and C). On the other hand, overexpression of A-USF, a mutant with an acidic extension at the N terminus of USF (26), resulted in a marked reduction of BDNF-PI activity up to  $\sim 60\%$  of the control (Fig. 9D). Combined DNA transfection of CREB M1 and A-USF, however, tended to induce the death of cortical neurons transfected (data not shown).

#### DISCUSSION

Prior to the characterization of the molecular mechanisms for the  $\text{Ca}^{2+}$  responsiveness of BDNF gene promoter I (BDNF-PI), we first demonstrated using RT-PCR that not only the expression of exon III-containing transcript but also that of the exon I-containing transcript was induced by membrane depolarization, although the basal expression level of the latter seems to be quite low, compared with that of the former (Fig. 1). This observation indicates that endogenous BDNF-PI in genome DNA responds to  $\text{Ca}^{2+}$  signals to increase the expression of exon I-containing transcript in rat cortical neurons. By promoter assay using transient DNA transfection in cultures of rat cortical neurons, in addition, we demonstrated that  $\text{Ca}^{2+}$ -responsive elements of BDNF-PI were located in two regions, distal and proximal (Fig. 2). Detailed analysis of the proximal region with deletions or mutations revealed that the CRE-like sequence in the proximal region of BDNF-PI (CRE-PI) functioned as the CRE (Figs. 4 and 5) and was potentially required for the activation of BDNF-PI (Figs. 2 and 3). Besides this CRE-PI, the TRE-like sequence can work as the TRE to bind the AP1 transcription factor because the DNA-protein complexes formed on native promoter I DNA probe-2 (npIPb-2) for EMSA, in which the TRE-like sequence was centered (Fig. 4A), included c-Fos (data not shown). Because this TRE-like sequence partially overlapped not only the CRE-PI but also the USF-binding site, however, the TRE-like sequence in native promoter I DNA probe-3 (npIPb-3) does not seem to work as a TRE but rather as a part of the CRE-PI and USF-binding site. On the other hand, the upstream TRE-like sequence seems to be responsible for the activation of BDNF-PI (Fig. 3B) but would play a minor role because the mutant of the upstream TRE-like sequence combined with CRE-PI (TRE1/CRE2 $\Delta$ ) had the same effect as the mutant of CRE-PI alone (CRE2 $\Delta$ ) (Fig. 3C). The downstream TRE-like sequence was ineffective in the activation (Fig. 3B). With respect to the distal region, the elements involved in the  $\text{Ca}^{2+}$  responsiveness of BDNF-PI have not been determined. A search for these elements using a series of plasmid vectors with a deletion or mutation in the distal region failed to specify their location because the  $\text{Ca}^{2+}$  responsiveness resulting from the distal region was dispersed by every mutation and deletion (data not shown). As shown in Fig. 2, in addition, the mutant plasmid with both the deletion of the distal region and the mutation of CRE-PI ( $\Delta$ distal/CRE2m) showed the same level of activation as that having the mutation in CRE-PI (CRE2m) alone (Fig. 3C). These findings indicate that the proximal region could play a major role in activating BDNF-PI, whereas the distal region has a minor role.

Focusing on the proximal region, we have identified the transcription factors that could bind to CRE-PI. As shown in Figs. 4–6, a doublet of shifted bands, upper and lower, were detected by EMSA using npIPb-3 probe, whereas only a single band corresponding to the upper band of the doublet was detected with the consensus CRE, pICRE, and pIIICRE probes (Figs. 5 and 6). The DNA binding activity responsible for the upper band was susceptible to consensus CRE (Fig. 4B), and



**FIG. 9. Effects of overexpression of dominant negative CREB or USF on the activation of BDNF promoter I.** The dominant negative expression vectors of CREB M1 (A), K-CREB (B), A-CREB (C), and A-USF (D) were co-transfected with firefly luciferase reporter vectors carrying BDNF promoter I and *E. coli*  $\beta$ -galactosidase internal control vectors carrying EF1 $\alpha$  promoter into rat cortical neurons. Empty vectors were co-transfected as a control. Forty hours after transfection, cells were stimulated with 25 mM KCl (+) or vehicle (–) and incubated for another 6 h. Cell lysates were then extracted for measuring luciferase activity. The value for the transcriptional activation of pBDNFpI(full) obtained with 25 mM KCl was standardized to 100%. The variability in the basal transcriptional activities is probably caused by an experimental flexibility, which could be caused mostly by culturing conditions. The data represent the mean  $\pm$  S.E. from experiments performed in triplicate. The same result was obtained from three independent experiments.

the supershift of the upper band was caused by anti-CREB family or anti-CREB specific antibody, which was faintly observed (Figs. 5A and 6B). In addition, mutations introduced into CRE-PI in the proximal region (Fig. 8) and the overexpression of dominant negative CREB M1 decreased the level of activation of BDNF-PI (Fig. 9). Thus, it is evident that the binding of CREB to CRE-PI, at least in part, contributes to the activation of BDNF-PI. In addition, the fact that the three adjacent downstream bases outside the CRE-PI in the proximal region were required for the upper band to form on the npIPb-3 probe (Fig. 7) but not on the consensus CRE, pICRE, or pII-ICRE probe (Figs. 5 and 6), which were effective in the tran-

scriptional activation of BDNF-PI (Fig. 8), may suggest that different transcription factors as well as CREB bind to npIPb-3 probe and the bases adjacent to the CRE-PI are required for their binding. Thus, the DNA sequences surrounding CRE-PI seem to affect the DNA binding of CREB and other transcription factors, which may confer some differences in the CREB binding to CRE between BDNF-PI and -PIII.

On the other hand, the lower band of the doublet was completely diminished by anti-USF1 or -USF2 antibody (Fig. 6B), indicating that it was formed only by the binding of USF1 and USF2 to the npIPb-3 probe. In support of this, the USF-binding site (5'-TGGTCACTAACTG-3') covered the CRE-PI (5'-

TCACGTAA-3') (Fig. 7A). These overlapping sequences are conserved in the rat, human, and mouse BDNF promoters I (data not shown). Some mutations within the USF-binding site preferentially diminished the lower band (Fig. 7), which also decreased the level of activation of BDNF-PI (Fig. 8). In addition, the overexpression of dominant negative A-USF was effective in reducing the transcriptional activation, the level of which was lower than that of CREB M1 (Fig. 9, A and D). These findings indicate that USF1 and/or USF2 are involved in the transcriptional activation of BDNF-PI through their bindings to the USF-binding site.

USF1 and USF2 are members of the basic helix-loop-helix/leucine zipper family of transcription factors and bind to DNA as homodimers or heterodimers (16). In addition, differential splicing has been shown to give rise to at least two isoforms, the 44-kDa USF2a and the 38-kDa USF2b (27, 28). Therefore, the complicated formations of homodimer or heterodimer among these transcription factors are involved in the complex formation via the USF-binding site. USF1 has been reported to interact with other transcription factors, such as Fos-related antigen 1 (Fra1) (17) and transcription factor II-I (29). On the other hand, CREB is a member of the leucine zipper family that is also able to form a homodimer or heterodimer with ATF/CREB family members or other factors at the leucine zipper motif (30, 31). Although there are several cases in which the CRE and the USF-binding site (or E-box) neighbor each other (32, 33), BDNF-PI is unique because the CRE and the USF-binding site overlap. Nevertheless, a tendency was observed for the 5'-side DNA sequences within the USF-binding site to be preferentially used for the formation of the lower band upon EMSA, whereas the 3'-side sequences were used for that of the upper band, *i.e.* a preferential usage of the DNA sequences within the USF-binding site for USF1/USF2 and CREB. These observations may also support the binding of a heterodimer between CREB and USF to this overlapping site, which should be investigated.

Overexpression of the dominant negative CREB and USF mutants, CREB M1 and A-USF, resulted in a marked reduction of BDNF-PI activation, indicating an involvement of CREB and USF in the activation of BDNF-PI (Fig. 9, A and D). Because CREB M1 binds to CRE but is devoid of phosphorylation at Ser-133, the dominant negative effect of CREB M1 seems to be caused by an interference in the interaction between CREB and coactivators like CREB-binding protein (CBP) on the promoter. All three types of dominant negative CREB mutants also reduced the activation of BDNF-PIII (data not shown).

As  $\text{Ca}^{2+}$  signal-stimulated transcription factors other than CREB, MEF2 and NF-AT have already been found in neuronal as well as non-neuronal cells (34, 35), and, quite recently, calcium-responsive transcription factor has been detected in neuronal cells as a calcium- and neuron-selective transcription factor (11). As described in the present study, it is highly possible that USF1/USF2 also functions as a  $\text{Ca}^{2+}$  signal-stimulated transcription factor in neurons. It has been reported that the phosphorylation of USF1 enhanced its DNA binding activity (36); quite recently, Galibert *et al.* (37) reported that phosphorylation of USF1 at Thr-153 increased its transcriptional activity. In addition, the activation of gene transcription by USF1/USF2 could be mediated by CBP/p300 (38). Thus, it is very likely that USF1/USF2 is phosphorylated by  $\text{Ca}^{2+}$  signaling pathways, such as extracellular signal-regulated kinase/mitogen-activated protein kinase, CaM (calmodulin) kinase IV and adenylyl cyclase/cAMP-dependent protein kinase (39), and involved in the transcriptional activation through mediation of CBP. For full activation of the transcription of the mouse renin

gene, co-operation between CREB/CREM and USF1/USF2 is required, in which the CRE-like element and E-box, for the binding of CREB and USF1/USF2, respectively, neighbor each other with an interval of ~10 bases (33). In BDNF-PI, however, the CRE-like sequence and the USF-binding site overlapped, suggesting a different mechanism for transcriptional activation of BDNF-PI. Rather than co-operation by these transcription factors for promoter activation, a competitive binding of CREB and USF1/USF2 to this overlapping site could be considered for controlling the BDNF-PI activation in accordance with the  $\text{Ca}^{2+}$  signalings evoked in neurons. Differential usage of CREB or USF1/USF2 for promoter activation in response to  $\text{Ca}^{2+}$  signals might be controlled by  $\text{Ca}^{2+}$  signaling pathways among which extracellular signal-regulated kinase/mitogen-activated protein kinase, CaM kinase IV, and adenylyl cyclase/cAMP-dependent protein kinase are activated by the influx of  $\text{Ca}^{2+}$  into neurons via the NMDA-R or L-VDCC. It is important to elucidate the molecular mechanisms of the CREB- and USF-mediated activation of BDNF-PI evoked via  $\text{Ca}^{2+}$  signals in neurons for a better understanding of the intracellular mechanisms of neuronal activity-dependent gene expression, which might contribute to neuronal survival and plasticity.

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**Involvement of an Upstream Stimulatory Factor as Well as cAMP-responsive Element-binding Protein in the Activation of Brain-derived Neurotrophic Factor Gene Promoter I**

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