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Neurotrophin-3 increases the DNA-binding activities of several transcription factors in a mouse osteoblastic cell line

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Abstract

In the mouse osteoblastic cell line MC3T3-E1, the signaling responses of several DNA-binding proteins induced by the treatment of neurotrophin-3 were examined using electrophoretic mobility shift assay. Neurotrophin-3 increased binding activities in nuclear extracts of MC3T3-E1 cells to TPA-responsive element (TRE), cyclic AMP-responsive element (CRE) and serum-responsive element (SRE), but not binding activity in the nuclear extracts to c-Myc binding DNA element. Competition experiments revealed that the binding activity to TRE in the nuclear extracts of neurotrophin-3-treated MC3T3-E1 cells was entirely inhibited by the both unlabeled TRE and CRE probes. On the other hand, the binding activity to CRE was abolished by the unlabeled CRE probe but not by the same amount of unlabeled TRE probe. Moreover, immunodepletion/supershift assay using antibodies directed to Fos, Jun and CREB proteins, showed that the binding activities to TRE and CRE in the nuclear extracts were derived in part from these proteins.

Keywords: Neurotrophin-3; DNA binding activity; Transcriptional factor; Electrophoretic mobility-shift assay

1. Introduction

Neurotrophic factors, especially neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), play an important role in the normal development of the central and peripheral nervous systems [1–4]. These factors promote the survival and differentiation of various types of neurons [5–10]. Recently, the *trk* family of proto-oncogene products (TRK), which are members of the family of tyrosine kinase receptors, have been identified as the neurotrophin receptors [11–13]. NGF and BDNF bind to TRKA and TRKB, respectively [14,15] and NT-3 binds mainly to TRKC [16].

Neurotrophic factors are also produced by non-neural target cells of peripheral neurons [17]. We previously demonstrated that osteoblastic cell line MC3T3-E1 expressed mRNA of neurotrophins and also produced these proteins [18]. These findings suggest that neurotrophins produced by osteoblastic cells have developmental and survival effects on bone-associated neurons. On the other hand, we recently reported that osteoblastic cells expressed receptors for neurotrophins, especially TRKC, and NT-3 stimulated the proliferation of osteoblastic cells via TRKC [19]. This non-neural, novel function of NT-3 suggests the involvement of neurotrophic factors in the regulation of bone metabolism. It is known that NGF induces the expression of immediate-early genes, such as NGFI-A [20,21], c-*fos* [22–25], c-*myc* and c-*jun* [26] in PC12 cells. These immediate-early genes encode transcription factors which regulate the induction of late genes, including ornithine decarboxylase [27,28], tyrosine hydroxylase [29], GAP-43

Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; TRK, the trk family of proto-oncogene products; TRE, TPA-responsive element; CRE, cyclic AMP-responsive element; SRE, serum-responsive element; EMSA, electrophoretic mobility shift assay.

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[30], SCG10 [31] and VGF [32,33] genes. These late gene-related products play important roles in cellular functions. Although many studies have revealed the molecular mechanism of NGF action, little is known about the intracellular response to NT-3. In the present study, we examined NT-3-dependent changes in DNA-binding activities which bound to several DNA-binding motifs, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE), cyclic AMP-responsive element (CRE), serum-responsive element (SRE) and c-MYC-binding element in the nuclear extracts of MC3T3-E1 cells using electrophoretic mobility shift assay (EMSA).

2. Materials and methods

2.1. Cell culture and reagents

MC3T3-E1 osteoblastic cells derived from C57BL/6 newborn mouse calvaria [34,35] were kindly provided by Dr. Hiroaki Kodama, Ohu University Dental School. The cells were grown in α-minimum essential medium (ICN Biomedicals Inc., Costa Mesa, CA, USA) supplemented with 2% fetal bovine serum (Bioserum, Victoria, Australia) under 5% CO_2 in air at 37°C. The cells were inoculated at a density of 5×10^4 cells/ml. Three days after the inoculation of cells, 20 ng/ml of NT-3 (Pepro Tech Inc., Rocky Hill, NJ, USA) was added to each culture. The cells were harvested at the appropriate times for preparation of nuclear extracts.

2.2. Preparation of nuclear extracts

Nuclear extracts were prepared from the cultured MC3T3-E1 cells at the appropriate times after NT-3 stimulation according to the method of Schreiber et al. [36] with minor modifications. In brief, the cultured cells collected in 1 ml of Tris-buffered saline (25 mM Tris-HCl (pH 7.4), 130 mM NaCl and 5 mM KCl) were centrifuged at 7000 × *g* for 30 s. The pellet was suspended in 400 μl of buffer A (10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride). The suspension was placed on ice for 15 min. Then 30 μl of 10% Nonident P-40 was added, and vigorously mixed for 10 s. The nuclear fraction was precipitated by centrifugation at 15000 × *g* for 5 min and suspended in 50 μl of ice-cold buffer B (20 mM Hepes (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride). The mixture was placed on ice for 15 min with frequent agitation. Then the supernatants of the nuclear extracts were prepared by centrifugation at 15000 × *g* for 5 min and stored in aliquots at −80°C. The protein concentration was determined using a Bio-Rad protein assay kit with bovine serum albumin as a standard.

2.3. Electrophoretic mobility shift assay (EMSA)

Double-stranded oligonucleotides indicated in Fig. 1 were labeled with [α-^{32}P]dCTP (Amersham International, Amersham, UK) using DNA polymerase I (Klenow fragment) and purified by Sephadex G-50 column chromatography.

The DNA-protein binding reaction was performed for 15 min at 20°C in a reaction mixture (20 μl) containing 20 mM Hepes-NaOH (pH 7.9), 2 mM Tris-HCl, 1 mM dithiothreitol, 0.4 mM EDTA, 0.2 mM EGTA, 80 mM NaCl, 2 μg of poly(dI-dC), 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 200 pg of ^{32}P-labeled double-stranded oligonucleotide probes, and nuclear extract (2 μg). In the case of competition assays, the reaction was performed in the presence of excess volume of non-radiolabeled competitor DNAs.

For immunodepletion/supershift assay, 4 μl of anti-c-Fos antibody (c-fos (K-25), Santa Cruz Biotechnology, Santa Cruz, CA), anti-c-Jun antibody (c-jun/AP-1 (D), Santa Cruz Biotechnology, Inc.) or anti-CREB antibody (CREB-1 (C-21), Santa Cruz Biotechnology) were incubated with nuclear extract for 1 h at 4°C before EMSA. The DNA-protein complexes were resolved on 4% polyacrylamide gel (30:1 cross-linking ratio) containing 6.7 mM Tris-HCl (pH 7.6), 3.3 mM sodium acetate, 2.5% glycerol, 1 mM EDTA and 0.06% ammonium persulfate. Electrophoresis was carried out at 11 V/cm for 2.5 h at 4°C. After gel electrophoresis, the gel was dried and exposed to X-ray film (Kodak X-OMAT, Eastman Kodak, Rochester, NY) with intensifying screens for 1 day at −80°C. The reactive density of detected bands on the autoradiograms was measured and analyzed with an image scanner (Epson GT-6500, Seiko-Epson, Tokyo) and computerized image analysis software (Image 1.44, NIH).

2.4. Northern blot analysis

Subconfluent MC3T3-E1 cells were cultured with or without NT-3 (20 ng/ml) and harvested at the appropriate times after the stimulation with NT-3. Total RNA was

TRE : 5'-GATTCG**TGACTCA**GCACAGG-3'
3'-CTAAGC**ACTGAGT**CGTGTCC-5'

CRE : 5'-GATTCG**TGACGTCA**GCACAG-3'
3'-CTAAGC**ACGTAAGT**CGTGTC-5'

SRE : 5'-TATGGATGT**CCATATTAGG**ACATCTACA-3'
3'-ATACCTACA**GGTATAATCC**TGTAGATGT-5'

c-Myc : 5'-AAGCAGAC**CACGTG**GTCAGGCTA-3'
3'-TT CGTCTG**GTGCAC**CAGTCCGAT-5'

Fig. 1. Oligonucleotide sequences used as probes or competitors for EMSA. Consensus sequences of TRE [42], CRE, SRE and c-MYC [43] are indicated in bold letters.

isolated from the cells by the method of Wilkinson [37] and denatured with 6.7% formaldehyde-50% formamide solution. The denatured RNA (5 μg/ml) was electrophoresed on 1% agarose/formaldehyde gels and then the RNA was transblotted directly onto a nylon membrane (Hybond-N, Amersham). Murine osteosarcoma virus

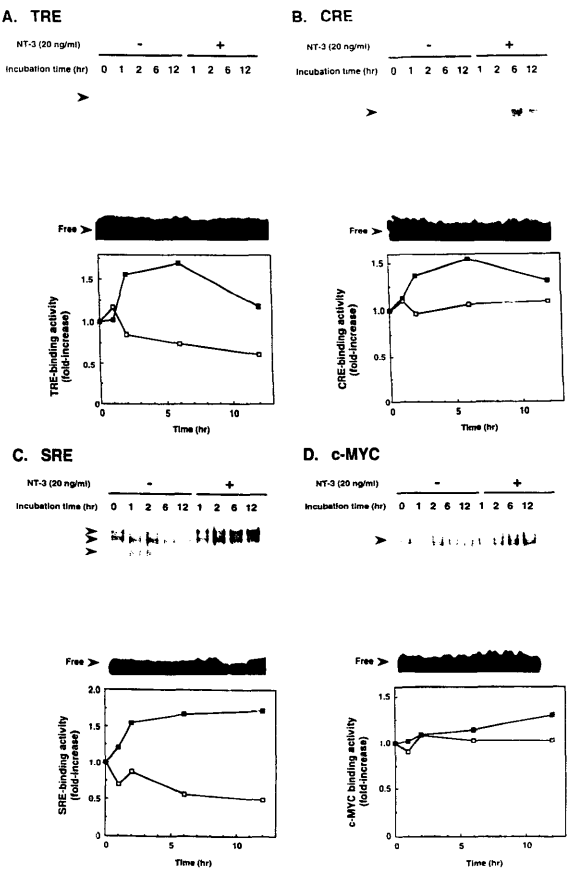

Fig. 2. Effects of NT-3 on TRE-, CRE-, SRE- and c-MYC-binding activities in nuclear extracts of MC3T3-E1 cells. Nuclear extracts were prepared from MC3T3-E1 cells at the indicated times after incubation with 20 ng/ml of NT-3. Two micrograms of proteins from nuclear extracts were incubated with ^{32}P-labeled double-strand oligonucleotide probes and subjected to electrophoresis. Gels were exposed to X-ray film (top) and analyzed with an image scanner (bottom). Arrowheads point to the shifted bands. (A) TRE, (B) CRE, (C) SRE and (D) c-MYC. Changes in DNA-binding activity are also plotted. (■) NT-3, (□) PBS-treated.

(MSV)-derived v-*fos* DNA probe (1 kb *Pst*I-*Pvu*II fragments) (Takara Shuzo, Otsu, Japan) was radiolabeled with [α-^{32}P]dCTP (Amersham Int. plc) using a random prime labeling kit (Amersham). Blots were pre-hybridized for 3 h at 42°C in hybridization buffer (5 × SSC, 5 × Denhardt's solution, 50% formamide, 0.5% SDS and 20 μg/ml denatured salmon sperm DNA). Hybridization was performed for 18 h at 42°C in the same buffer containing about 10^5 Bq/ml ^{32}P-labelled DNA probes. After the hybridization, membranes were washed twice with 1 × SSC containing 0.1% SDS for 15 min at room temperature, then washed for 30 min at 50°C and exposed to X-ray film (Hyperfilm MP, Amersham Int. plc) for 5 days at −80°C. The relative density of the exposed bands on the film was measured with an image scanner (Epson GT-6500) and image analysis software (Image 1.44, NIH).

3. Results

3.1. Increase of DNA-binding activities of several transcription factors in nuclear extracts of MC3T3-E1 cells by NT-3

Binding activities of nuclear proteins to probes of TRE, CRE, SRE and c-MYC were analyzed by EMSA. MC3T3-E1 cells were incubated with NT-3 (20 ng/ml) and harvested at 0, 1, 2, 6 and 12 h after the stimulation to prepare nuclear extracts for EMSA. As shown in Fig. 2, binding activities to TRE, CRE and SRE in the nuclear extracts increased 2 h after the addition of NT-3, and this increased level persisted for more than 12 h after the stimulation. The increased levels of TRE- and CRE-binding activities in the nuclear extracts of NT-3- treated MC3T3-E1 cells were 1.8- to 2.3-fold and 1.2- to 1.45-fold, respectively, compared with the activities of non-treated cells (Fig. 2A,B). Three bands were detected for nuclear proteins bound to SRE. Especially, the induction of the upper band was remarkable (Fig. 2C); NT-3-induced level of radiolabel of the upper band was 3.0–3.5 times that of non-treated control cells. On the other hand, the level of the lower band was not increased by treatment with NT-3. No significant difference was observed in the binding activities to probes for c-MYC (Fig. 2D).

3.2. Competition assays of the TRE- and CRE-binding activities of nuclear proteins

Because of the similarity of the nucleotide sequences of TRE and CRE, we compared the binding abilities of TRE and CRE by using competition assays between labeled and unlabeled probes to analyze the binding specificity of TRE- or CRE-binding activity. We used the nuclear extracts collected 6 h after NT-3 stimulation and changed the molar ratios of competitor unlabeled probes to labeled probes (3-, 10-, 30-, 100- and 200-fold). The addition of unlabeled CRE probes dose-dependently inhibited TRE-binding activity in the nuclear protein to the same extent as when unlabeled TRE probes were used as competitors (Fig. 3A). TRE-binding activity was entirely inhibited by both TRE and CRE unlabeled probes at a 200-fold excess of unlabeled versus labeled probes. Conversely, unlabeled TRE probes inhibited CRE-binding activity (Fig. 3B). Although the binding activities to labeled CRE probe were abolished by the addition of 200-fold of excess unlabeled CRE probe, the addition of 200-fold excess unlabeled TRE

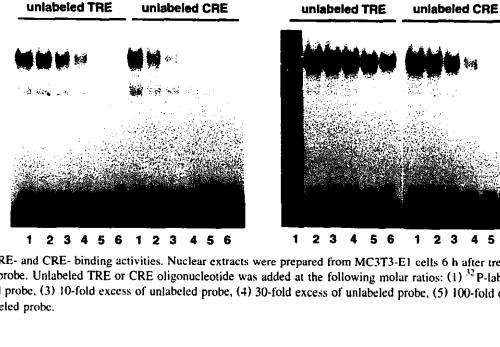

Fig. 3. Competition of TRE- and CRE- binding activities. Nuclear extracts were prepared from MC3T3-E1 cells 6 h after treatment of 20 ng/ml of NT-3. A: TRE probe. B: CRE probe. Unlabeled TRE or CRE oligonucleotide was added at the following molar ratios: (1) ^{32}P-labeled probe only (control), (2) 3-fold excess of unlabeled probe, (3) 10-fold excess of unlabeled probe, (4) 30-fold excess of unlabeled probe, (5) 100-fold excess of unlabeled probe, (6) 200-fold excess of unlabeled probe.

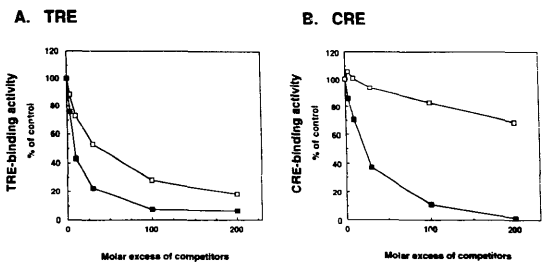

Fig. 4. Results of competitor experiments. Each band shown in Fig. 3 was measured and analyzed with an image scanner (Epson GT-6500) and computerized image analysis software (Image 1.44 NIH). The extent of competition was calculated as a percentage of the control. (□) unlabeled TRE probe, (■) unlabeled CRE probe.

probe only reduced the CRE-binding activities to approximately 70% of the total binding activities (Fig. 4).

3.3. Immunodepletion / supershift of the band formed by TRE- and CRE-binding activity

To quantitate the amount of c-Fos, c-Jun and CREB proteins contained in the DNA-protein complexes formed with TRE and CRE probes, we performed an immunodepletion/supershift assay using antisera raised against c-Fos, c-Jun and CREB proteins. Anti-c-Fos antibody was reactive with Fos-family proteins as well as the c-Fos protein. Likewise, anti-c-Jun antibody was cross-reactive with Jun-family proteins.

When the nuclear extracts of MC3T3-E1 cells harvested 6 h after the NT-3 stimulation were preincubated with anti-c-Jun antibody and then incubated with radiolabeled probes, a new band of higher molecular weight appeared, indicating the presence of a DNA-protein-antibody complex (Fig. 5). About 40% of total binding activity to TRE- or CRE-probe was shifted to a higher molecular weight. On the other hand, normal rabbit antiserum had no significant effect on the banding pattern (Fig. 5). Pretreatment of anti-CREB antibody reduced the signals of DNA-binding proteins bound to TRE and CRE sequences to 56% and 48%, respectively (Fig. 5). On the other hand, the addition of anti-c-Fos antibody reduced the TRE-binding activity to 62% of the total binding activity. CRE-binding activity was slightly reduced by pretreatment with anti-c-Fos antibody.

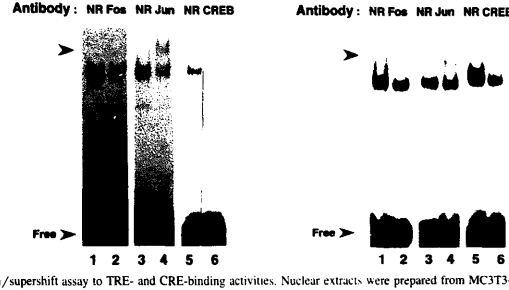

Fig. 5. Immunodepletion/supershift assay to TRE- and CRE-binding activities. Nuclear extracts were prepared from MC3T3-E1 cells 6 h after treatment of 20 ng/ml of NT-3. A: TRE probe. B: CRE probe. (1,3,5) nuclear extracts were preincubated with normal rabbit antiserum, (2) preincubated with anti-c-Fos antibody, (4) preincubated with anti-c-Jun antibody, and (6) preincubated with anti-CREB antibody. Arrowheads point to the shifted bands.

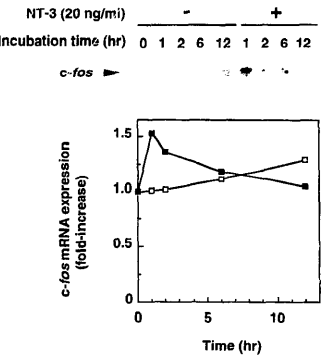

Fig. 6. The expression level of c-*fos* mRNA in NT-3-stimulated MC3T3-E1 cells. MC3T3-E1 cells were harvested at the indicated times after the addition of 20 ng/ml of NT-3 for preparation of RNA. Total RNA (5 μg/lane) was subjected to Northern blot analysis using ^{32}P-labeled c-*fos* cDNA as a probe. The membrane was exposed to X-ray film after the hybridization (A). The intensity of the bands corresponding to the mRNA size of c-*fos* was analyzed by an image scanner (B). (■) NT-3, (□) PBS-treated.

3.4. Induction of de novo synthesis of c-fos mRNA in MC3T3-E1 cells

To clarify whether the enhancement of DNA-binding activity shown in Fig. 2 was caused by enhancement of DNA-binding activity of pre-existed proteins or induction of de novo synthesis of DNA-binding proteins, we performed Northern blot analysis of c-*fos* mRNA because supershift analysis showed that c-Fos was contained in the DNA-binding activities to TRE and CRE. As shown in Fig. 6, the level of c-*fos* mRNA was rapidly increased in MC3T3-E1 cells by the addition of NT-3, indicating that NT-3-dependent enhancement of the DNA-binding activity was caused mainly by the induction of de novo synthesis of the DNA-binding proteins.

4. Discussion

In this study, we found that NT-3 increased DNA binding activities to TRE, CRE and SRE probes in the nuclear extracts of MC3T3-E1 cells. We also detected c-Fos, c-Jun and CREB proteins in the DNA binding activities to TRE and CRE. This is the first report on changes in DNA binding activities of these transcription factors in response to NT-3.

Among many immediate-early genes, overexpression of c-*fos*, c-*jun* and c-*myc* genes led to cellular transformation, and it is considered that these genes promote cellular proliferation. These immediate-early genes encode transcription factors which are thought to play an important role in regulating the transcription of the target genes which contribute to generating long-lasting or flexible alterations of particular cellular responses such as survival, proliferation and differentiation.

In the case of osteoblastic cells, we have already reported that NT-3 stimulated the proliferation of MC3T3-E1 cells [19] and in the present study, we showed that there was an increase in TRE, CRE and SRE binding activities in the nuclear extracts of the cells at 2 h and still an appreciable increase at 12 h following treatment with 20 ng/ml of NT-3 (Fig. 2A,B,C). These changes were also observed in the cells treated with 1 ng/ml of NT-3, although the levels were lower than that observed in the cells treated with 20 ng/ml of NT-3 (data not shown). As a result, the concentration of NT-3 required to stimulate the proliferation of MC3T3-E1 cells was almost the same as those needed to increase the DNA binding activities to TRE, CRE and SRE probes. These data suggest that NT-3 treatment could lead to an increase in DNA-binding activities of particular transcription factors, for example c-Fos, c-Jun and CREB, and of other transcription factors which are associated with the TRE, CRE and/or SRE DNA binding motifs, and these factors may regulate the transcription of the target genes with these motifs in their upstream regions which, in turn, promote the proliferation of MC3T3-E1 cells. On the contrary, no significant elevation of c-MYC binding activity was noted in NT-3-treated MC3T3-E1 cells (Fig. 2D). In the osteoblastic cell line MC3T3-E1, c-Myc may not contribute to the NT-3-induced proliferation.

In the competition assays of nuclear extracts obtained from the MC3T3-E1 cells 6 h after the NT-3 treatment using labeled and unlabeled DNA probes, the addition of not only unlabeled TRE probe but also unlabeled CRE probe inhibited the TRE-binding activity of nuclear extracts dose-dependently (Fig. 3A). Conversely, not only unlabeled CRE probe but also unlabeled TRE probe inhibited the CRE-binding activity of nuclear extracts (Fig. 3B). These results suggest that TRE- and CRE-binding activities were induced in part via activation or synthesis of common DNA-binding factors that recognized both TRE and CRE motifs. Recently, it has been reported that, in cultured cerebellar granule cells, activation of NMDA or kainate receptors induced TRE- and CRE-binding activities mediated by common DNA-binding complexes [38]. However, the inhibition of CRE binding activity by TRE probe was only 30% of the total binding activities even when the 200-fold excess unlabeled TRE probe was used (Fig. 4B). These data suggest that there are other DNA binding factors induced by NT-3 which selectively recognize the CRE probe. It is known that AP-1 has a high affinity for both the TRE and CRE sequences. On the other hand, CREB/ATF protein has higher DNA-binding affin-

ity for CRE than for TRE. So, in MC3T3-E1 cells, CREB/ATF protein might recognize CRE selectively and bind weakly to TRE because of the high homology of nucleotide sequences between CRE and TRE.

Furthermore, we examined immunodepletion/supershift assays. The protein composition of the shifted bands were investigated using antibody against components of the AP-1 and CREB/ATF complexes. Anti-c-Fos and anti-c-Jun antibodies affected not only the signals of TRE-binding activity but also the signals of CRE-binding activity (Fig. 5). These antibodies might recognize the AP-1 complex that cross-reacts with the CRE sequence. AP-1 transcription factor consists of either Jun homodimers or Fos/Jun heterodimers, which bind the TRE sequence and homologous CRE sequence. Furthermore, it has been well documented that certain CREB/ATF proteins can form leucine zipper dimers with members of the AP-1 transcription factor family [39]. The CREB/ATF family binds to the CRE sequence selectively. Thus, these antibodies might also recognize the AP-1 component which were included in CRE-binding proteins.

Anti-CREB-antibody also reduced the signals of DNA-binding proteins bound to both TRE and CRE (Fig. 5), suggesting that CREB/ATF transcription factors cross-bind to the TRE sequence or bind to TRE via the AP-1 complex. Another possibility is that the anti-CREB-antibody recognized the c-Jun protein included in AP-1 because the anti-CREB-antibody used in this experiment reacts with the DNA-binding domain of CREB and it is known that CREB proteins are about 50% similar to Jun proteins in this basic DNA-binding domain [40].

In addition, high molecular weight-shifted extra bands which contain DNA-protein-antibody complexes were observed only in the case of anti-c-Jun antibody (Fig. 5). It is assumed that anti-c-Fos and anti-CREB antibodies recognize DNA binding sites on the c-Fos and CREB molecules, respectively, and then inhibit the binding of protein-antibody complexes to DNA probes.

Northern blot analysis revealed that NT-3 induced the expression of c-*fos* mRNA in MC3T3-E1 cells (Fig. 6). It has been reported that serum growth factors induce phosphorylation of transcription factor $p62^{TCF}$ by MAP kinase and stimulates ternary complex formation at c-*fos* promoter which leads induction of c-*fos* mRNA expression [41]. We have already shown that NT-3 stimulates the proliferation of MC3T3-E1 cells [19], so the induction of c-*fos* mRNA expression by NT-3 may be caused in a similar manner. The induction of c-*fos* mRNA expression in the NT-3-stimulated MC3T3-E1 cells strongly suggests that the enhancement of DNA-binding activity in the cells resulted from stimulation of de novo synthesis of c-Fos protein.

In the previous study, we found that NT-3 and its receptor TRKC were co-expressed in the proliferating phase of osteoblastic MC3T3-E1 cells and that NT-3 stimulates the proliferation of MC3T3-E1 cells via TRKC. In this study, we showed that exogenous NT-3 induced DNA-binding activities of particular transcription factors in the cells. These transcription factors may modulate transcription of certain genes, which play an important role in the proliferation of osteoblastic cells. Future investigations searching for the target genes of these transcription factors in osteoblastic cells are now under way.

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