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Detection of living cells that express AP1 using a fluorolabeled DNA probe

Noriaki Shimokawa*, Mitsuhiko Miura

Department of Physiology 1st Division, Gunma University School of Medicine, 3-39-22 Showa-machi, Maebashi City 371, Japan

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Abstract Activator protein 1 (AP1) is a complex of Fos and Jun, and it regulates the transcription of genes possessing the AP1-binding sequence. The purpose of this study was to detect living cells that express AP1 after stimulation with a tumor promoter. The Fos and Jun components of AP1 were induced rapidly and transiently in PC12 cells following the addition of phorbol ester (phorbol 12-myristate 13-acetate, PMA). The DNA fragment containing the AP1-binding sequence was combined with ethidium bromide, which was used as a fluorescent probe. The probe was transfected into the cells using cationic liposomes. Fluorescence in the transfected cells was observed using a fluorescence microscope. The nuclei of transfected cells emitted strong fluorescence in the presence of PMA, whereas weak fluorescence was retained in the cytoplasm in its absence. The former phenomenon is evidence that AP1 combined with the fluorescent probe was transported into the nuclei. This study suggests that such a fluorolabeling method is feasible to detect living AP1-expressed neurons.

Key words: Activator protein 1; AP1-binding sequence; Fluorescent probe; Transfection; Living cell; Detection method

1. Introduction

Activator protein 1 (AP1), a nuclear transcription factor, is a complex formed through leucine zipper motifs [1]. The major components of the complex are products of the *c-fos* and *c-jun* protooncogenes, i.e. the c-Fos and c-Jun proteins. The complex binds to the palindromic AP1-binding sequence TGAC/GTCA [2,3] and regulates the transcription of genes that have the AP1-binding sequence [4,5]. Recently, several investigators showed that the components of AP1 were induced rapidly and transiently within neurons following synaptic activation [6–8] and pharmacological stimulation [9].

Since the AP1-expressed neurons always lost viability during immunohistochemical and biochemical procedures such as fixation and homogenization of cells, the isolation and culture of living AP1-expressed neurons have yet to be studied. This urged us to establish an appropriate marking method for living AP1-expressed neurons. Since PC12 cells readily differentiate into sympathetic neurons by induction of nerve growth factor (NGF), PC12 cells are frequently used as a model system for the culture of neurons [10]. In this study, we tried to detect living PC12 cells that expressed AP1 following stimulation of a tumor promoter. The results showed that our newly devised method using a fluorescent probe is feasible for the isolation of living AP1-expressed neurons.

*Corresponding author. Fax: (81) (272) 20-7926.

Abbreviations: DOSPA, 2,3-dioleyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate; DOPE, dioleoylphosphatidylethanolamine

2. Materials and methods

2.1. Chemicals

Lipofectamine reagent (DOSPA/DOPE 3:1, mol/mol), a polycationic liposome, for DNA transfection was purchased from Life Technologies (Gaithersburg, MD). Phorbol 12-myristate 13-acetate (PMA) as a tumor promoter was obtained from Wako Pure Chemical Co. (Osaka, Japan). The fluorescent dye ethidium bromide was obtained from Sigma Chemical Co. (St. Louis, MO). Other reagents were purchased from the indicated sources: Dulbecco's Modified Eagle's Medium (DMEM) and L-glutamine from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan); deoxycytidine 5′-[α-^{32}P]triphosphate ([^{32}P]dCTP; 110 TBq/mmol) and nylon membranes (Hybond N$^+$) for Northern hybridization from Amersham (Bucks, UK); anti-c-Fos and c-Jun antisera from Cambridge Research Biochemicals (Cheshire, UK); polyvinylidene difluoride (PVDF) membranes from Millipore (Bedford, MA); ABC reagents from Vector Labs. (Burlingame, CA); restriction enzymes (*ApaI* and *SacI*) and DNA polymerase from Takara Shuzo Co. Ltd. (Kyoto, Japan).

2.2. Cell culture and PMA treatment

PC12 rat pheochromocytoma cells, from a line initiated by Greene and Tischler [10], were kindly supplied by the Riken Cell Bank (Tsukuba, Japan). PC12 cells were grown in DMEM supplemented with 10% heat-inactivated horse serum (HS), 5% fetal bovine serum (FBS), 4 mM L-glutamine and 60 μg/ml kanamycin in tissue culture dishes. The tissue culture dishes had previously been treated with rat-tail collagen and washed with serum-free DMEM. For experiments, the cells were plated at a density of 2×10^5 cells per 35 mm dish and incubated at 37°C in an atmosphere of 95% air/5% CO_2. 24 h after plating, cells were stimulated with PMA for various times. PMA (5 mM) was kept as a stock solution in dimethyl sulfoxide (DMSO) and added directly to the culture dish to a final concentration of 100 nM. Control dishes contained appropriate concentrations of DMSO. After mixing by gently swirling, cells were immediately returned to the culture incubator.

2.3. Northern blot analysis

Total cellular RNA from cultured PC12 cells was prepared by the guanidinium thiocyanate method [11]. Northern hybridization was performed as described previously [12]. RNA (10 μg/lane) was electrophoresed in 1.2% agarose denaturing gels containing 2.2 M formaldehyde. Separated RNAs on the gels were transferred to nylon membranes by alkaline blotting. Complementary DNA (cDNA) of *c-fos* (obtained from the Japanese Cancer Research Resources Bank) and *c-jun* (obtained from the Riken Gene Bank) were labeled with [^{32}P]dCTP using random primers with DNA polymerase Klenow fragment [13]. These radioactive probes were used for the detection of RNAs on blots. The membrane was hybridized in buffer solution with labeled cDNA at 42°C for 16 h. After hybridization, the membrane was washed, and the blot was used for the detection of radioactivity using a Fujix BAS-2000 Bio-Imaging Analyzer (Fuji Photo Film Co. Ltd., Japan).

2.4. Western blotting

Cells were harvested at 2 h after the addition of PMA. Nuclear extracts for Western blotting were prepared according to the method of Dignam et al. [14]. Extracts from the PC12 cells were subjected to

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SDS-PAGE [15], and the proteins were transferred to a PVDF membrane by electroblotting. Membranes were blocked with 5% bovine serum albumin and incubated with appropriate dilutions of primary antibody (anti-c-Fos or c-Jun antiserum) at 4°C for 12 h. After incubation, membranes were incubated with biotinylated secondary antibody, incubated in ABC solution, and then treated with diaminobenzidine plus nickel chloride (DAB-Ni) solution containing 0.003% H_2O_2.

2.5. DNA-protein binding test

The binding of PC12 nuclear extracts to the consensus AP1-binding sequence was examined as follows [16]. A 96 bp *ApaI-SacI* fragment containing the AP1-binding site was excised from the *c-jun*/CAT reporter gene (*c-jun*-730CAT; obtained from Riken Gene Bank) [17]. The fragment (150 μg) was conjugated with 100 μg of ethidium bromide, a fluorescent dye. The unincorporated ethidium bromide was removed from fluorolabeled DNA fragments by precipitation with ethanol. Binding reaction mixtures (10 μl) contained 25 μg PC12 nuclear protein, 25 mM HEPES-KOH, 0.5 mM EDTA-NaOH, 50 mM KCl, 0.75 mM phenylmethylsulfonyl fluoride (PMSF), 0.75 mM dithiothreitol (DTT), 10% glycerol, and 150 ng of fluorolabeled AP1 probe, incubation being performed at room temperature for 20 min. The reaction mixture was subjected to electrophoresis through 7% polyacrylamide gels containing 50 mM Tris, 50 mM boric acid and 1.25 mM EDTA. DNA-protein complexes in the gel were detected by illumination with UV light (wavelength, 302 nm). The specificity of the binding complex was demonstrated by competition with a 20-fold excess of non-fluorolabeled probe.

2.6. Transfection

The fluorolabeled probes were introduced into cells using cationic lipids, lipofectamine reagent, following the manufacturer's recommended protocol with minor modifications. Fluorolabeled DNA probes (50 μg) and lipofectamine (50 μg) were separately diluted in 0.1 ml 4% HS, 2% FBS, and antibiotic-free DMEM (low-serum medium), then mixed and incubated for 1 h at room temperature. Low-serum medium (0.8 ml) was added, and the entire mixture was added to the cells. Subsequently, cells were treated with PMA as described in Section 2.2. The cells were then transferred to a 37°C incubator for 8 h. Fluorescence in the transfected cells was observed under a fluorescence microscope (excitation wavelength, 488 nm). Viability of the transfected cells was estimated by trypan blue exclusion.

2.7. Image processing

Transfected cells were photographed using Fujichrome color slide film. The slides and results of the blotting and binding test were scanned into digital memory using scanners, and arranged in composites using Photoshop software (Adobe Inc., Mountain View, CA).

3. Results

To determine whether the expression of *c-fos* and *c-jun* is induced by PMA stimulation, we carried out Northern blot analysis of the expression of *c-fos* and *c-jun* mRNAs in PC12 cells following stimulation with PMA. We prepared RNA blots that contained cytoplasmic RNA obtained from the PMA-treated PC12 cells. The blots were hybridized with a labeled fragment of *c-fos* or *c-jun* cDNA under conditions of high stringency. Fig. 1A shows that the level of *c-fos* mRNA (2.2 kb) increased markedly 1 h after the addition of PMA, but returned to a low level 2 h after PMA addition. These observations were consistent with the previous report [18]. Fig. 1B shows that two *c-jun* transcripts (approx. 3 and 3.5 kb) increased 1 h after the addition of PMA and gradually decreased, maintaining a high level until 4 h. This suggests that the *c-jun* gene be autoregulated by the binding of its own gene product to an AP1 sequence in the *c-jun* promoter [19].

Moreover, the expression of c-Fos and c-Jun proteins in the nuclei of PMA-treated PC12 cells was monitored by Western blot analysis (Fig. 2). c-Fos (62 kDa) and c-Jun (39 kDa) were

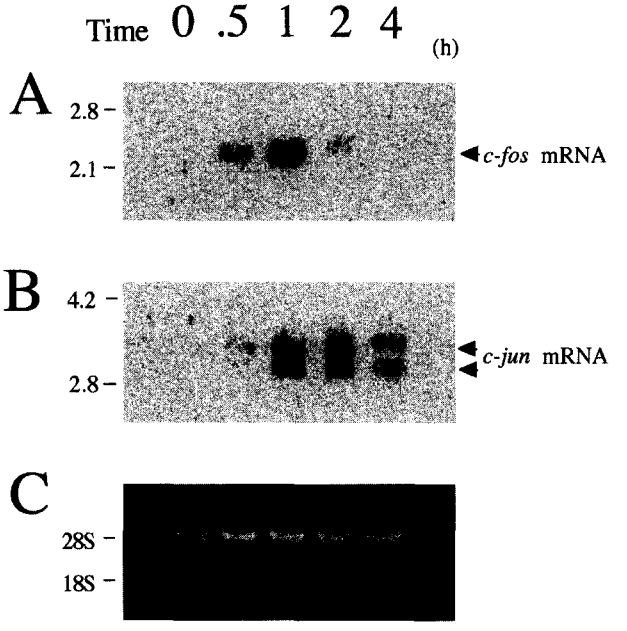

Fig. 1. Time course of *c-fos* (A) and *c-jun* (B) mRNA expression in PMA-treated PC12 cells. Cells were treated with PMA (100 nM) for the indicated times. Northern blotting was performed as described in Section 2. The arrowhead indicates hybridizing bands corresponding to mRNA encoding c-Fos or c-Jun. The two ribosomal RNAs stained with ethidium bromide are shown as control (C), and positions of standard molecular mass (kb) are indicated on the left.

detected by screening using each antibody, whereas neither c-Fos nor c-Jun was detected in untreated cells. This result reflected not only the elevation of *c-fos* and *c-jun* mRNAs but also the accumulation of Fos and Jun in the nuclei.

To determine whether AP1 proteins bind to the fluorolabeled AP1-binding sequence and whether the complexes are detectable by fluorescence, we undertook a DNA-protein binding test, the results of which are illustrated in Fig. 3. Lane 1 shows a fluorescent band of free probes (96 bp) to which nuclear extracts from the untreated PC12 cells did not bind. Lane 2 demonstrates the appearance of a high molecular weight fluorescent band, a complex of fluorolabeled probes and AP1 in nuclear extracts from PMA-treated PC12 cells. Lane 3 shows that a high molecular weight fluorescent band disappeared when non-fluorolabeled cDNA was added to the nuclear extracts from PMA-treated PC12 cells.

To visualize the living cells that expressed AP1 protein, we introduced the fluorolabeled probes into the PC12 cells. When PMA was added to the cell culture, it was observed that the nuclei of PC12 cells emitted strong fluorescence (Fig. 4C). The average percentage of the cell population that emitted fluorescence was about 40% (8×10^4 emitting cells per 2×10^5). Since the fluorescent cells were not stained with trypan blue, they may be living cells. When an organic solvent for PMA (DMSO) was added, weak fluorescence was found only in the cytoplasm as shown in Fig. 4A.

4. Discussion

To detect cells that express Fos, Jun and AP1, several methods have been used. These include immunohistochemical techniques [8,20], Western blotting [20,21], immunoprecipitation

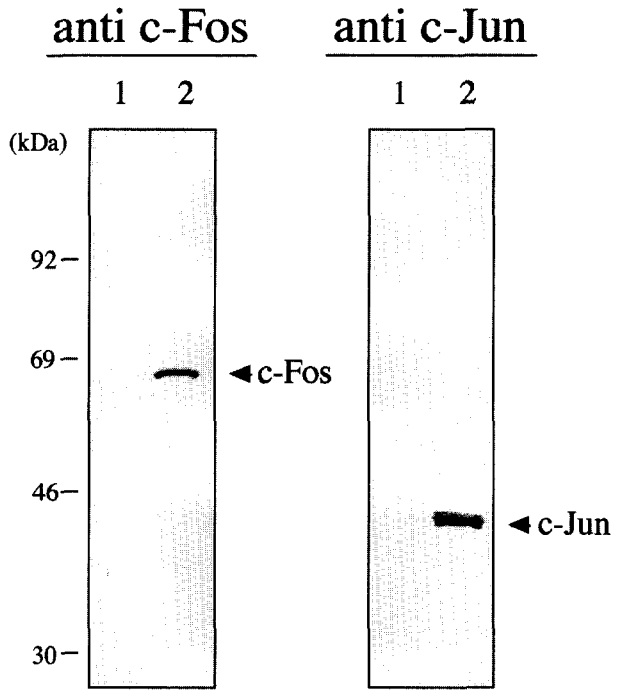

Fig. 2. Expression of c-Fos and c-Jun in PMA-treated PC12 cells. Cells were treated with PMA (100 nM) for 2 h. Nuclear proteins were analyzed by SDS-PAGE followed by immunoblotting with anti-c-Fos or c-Jun antibody. The antigenic signals were amplified with an avidin-biotin complex and visualized by DAB-Ni treatment. Lanes: 1, nuclear extracts from untreated cells; 2, nuclear extracts from PMA-treated cells. The molecular masses (kDa) of protein markers (Rainbow marker, Amersham) are indicated on the left.

[22,23] and gel mobility shift assays [17,21]. These techniques involve the procedures of sectioning and/or homogenizing cells which do not allow cells to survive. Our newly devised method, however, allows AP1-expressed cells to survive. This success is possibly due to two merits. First, we used ethidium bromide for labeling DNA. Ethidium bromide is a basic fluorescent dye suitable for staining nucleic acids in living cells [24]. Ethidium bromide intercalates between the stacked bases of nucleic acids, and fluoresces with a wavelength of 590 nm. Using the ethidium bromide-labeled probe, we succeeded in detecting AP1 in non-denaturing polyacrylamide gels. Second, we used liposomes to transfect cells on account of their high efficiency and low toxicity to cells. With the help of liposomes, the fluorescent probes containing the AP1-binding sequence were successfully introduced into cells and bound to AP1 formed in the cytoplasm [25]. AP1 possesses nuclear localization signals (NLS) that attract proteins into the nuclei [26]. This is the reason why the fluorescent probes containing the AP1-binding sequence accumulate in the nuclei.

Several investigators have reported that the expression of AP1 is depressed in most neurons including PC12 cells under unstimulated conditions [8,20,27]. Acute stimulation, however, elevates mRNAs of *c-fos* and *c-jun* to a peak 30 min after the onset of stimulation [28,29], and ultimately the production of Fos and Jun proteins reaches a maximum 1–1.5 h after the onset of stimulation and persists for 2–6 h [28,29]. These observations are consistent with the results of this study, since the fluorescence in cellular nuclei began 8 h after PMA stimulation.

Most of the PC12 cells expressed AP1 with PMA (100 nM, data not shown), while the average percentage of the cell population emitting fluorescence was as low as 40%. Since the instruction manual for the cationic liposomes shows that nucleic acids are introduced into 30–40% of PC12 cells with the help of the liposomes, it is reasonable to suppose that the emitting cells make up 40% of the population.

Montminy et al. [30] reported that a DNA element subserving induction by adenosine 3′,5′-monophosphate (cAMP) to some mammalian gene promoters (cAMP-responsive element; CRE) is different from the AP1-binding sequence, because a single nucleotide, guanylic acid, is inserted into a sequence TGACGTCA of the latter [30]. Curran and Franza [31] reported that a CRE-binding protein (CREB) binds to the AP1 sequence but the binding affinity of the CREB and AP1 is 5–10-fold less than that of the CREB and CRE. These data suggest that our newly devised method may be applicable to the detection of living cells that express various DNA-binding proteins by replacing the DNA-binding sequence.

In conclusion, this study has shown that the combination of a fluorescent probe with the transfection procedure is a useful technique to detect living cells that express AP1.

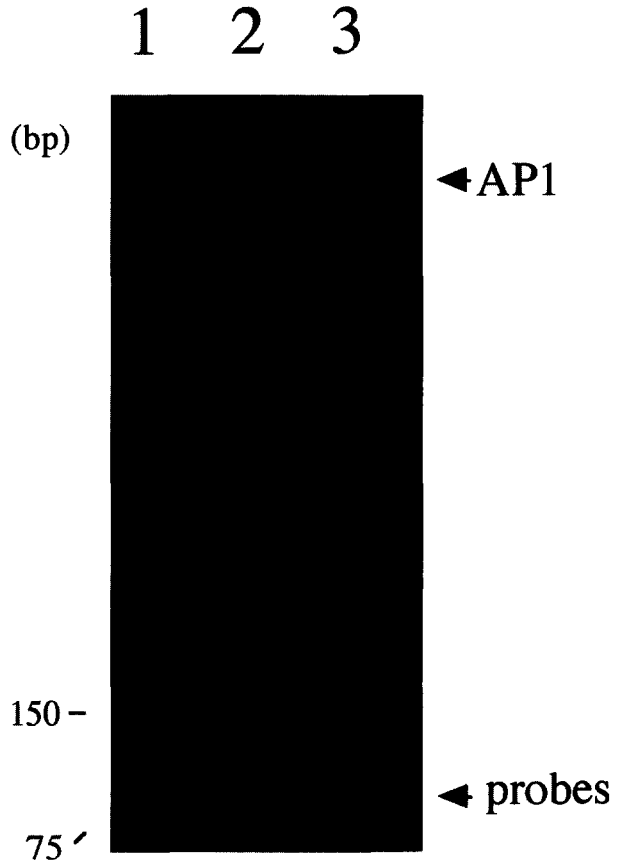

Fig. 3. Detection of complex of fluorolabeled DNA probe and AP1 protein. PC12 cells were treated with PMA (100 nM) for 2 h. The reaction mixture containing the nuclear proteins and fluorolabeled DNA probe was incubated, electrophoresed and then exposed to UV. Lanes: 1, mixture of nuclear extract from untreated cells and fluorolabeled probe; 2, mixture of nuclear extract from PMA-treated cells and fluorolabeled probe; 3, mixture of nuclear extract from PMA-treated cells and non-fluorolabeled probe. The molecular masses of nucleic acids (bp) are indicated on the left.

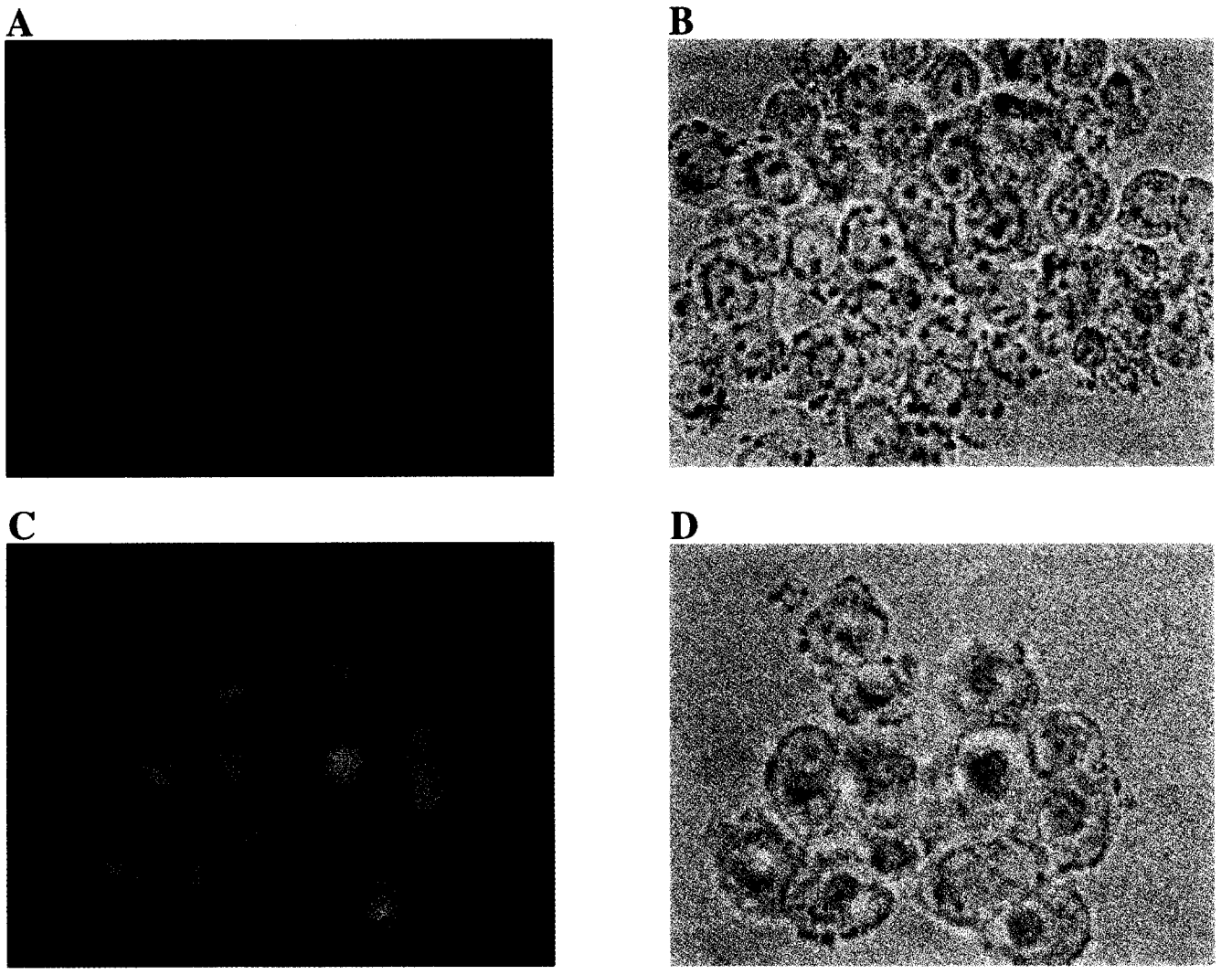

Fig. 4. Fluorescence observation of PC12 transfected with the AP1-binding sequence. PC12 cells were treated with PMA (100 nM) for 8 h after transfection of fluorolabeled probes. (A) Cells without PMA treatment. (B) Bright field of (A). (C) Cells with PMA treatment. (D) Bright field of (C).

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