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A Novel RING Finger Protein, BFP, Predominantly Expressed in the Brain

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RING finger is a variant zinc finger motif present in a new family of proteins including transcription regulators. A genomic DNA fragment containing RING finger motifs was identified by the polymerase chain reaction using degenerate primers. Using this fragment as a probe, we have isolated a novel cDNA from rat brain library. The predicted open reading frame contains a RING finger domain at its N-terminal portion. The corresponding transcript was detected predominantly in the brain and therefore was designated brain finger protein (bfp). An antibody raised against a recombinant bfp reveals the presence of the bfp in the brain. Interestingly, the bfp is induced during retinoic acid-mediated differentiation of P19 embryonal carcinoma cells into neural cells. These findings suggest the possible involvement of bfp in some aspects of neural cell regulation.

RING finger is a variant type of zinc finger motif (C3HC4) that emerged through sequence comparison (1). Members of the RING finger family are mostly nuclear proteins, some of which are implicated in transcriptional regulation. Typical examples include PML that is frequently fused to the retinoic acid receptor α in acute promyelocytic leukemia translocations (2-5), Rfp (6) which is a possible regulator of spermatogenesis, and XNF7 (7) and rpt-1 (8) that are potential transcription regulators.

Several RING-finger-containing proteins have been implicated in cell transformation. PML, rfp and T18 (9), acquire transformation capabilities upon chromosomal translocation. In each of these translocations, the RING finger domain is retained in the fusion protein, suggesting an important role for this domain in the transformation process. BRCA1, a tumor suppressor gene whose mutations are implicated in early-onset breast and ovarian cancer (10), also harbors a RING finger motif. Still, the normal functions of RING finger proteins, as well as their relation to carcinogenesis are poorly understood at the present time.

Recently, using genomic binding-site cloning method (11) to pick up estrogen responsive genes, we have identified several estrogen responsive elements in human genomic DNA and cloned a novel estrogen responsive gene, efp (12) that encodes a RING finger protein. By in situ hybridization histochemistry, the transcripts of efp were detected in uterus, mammary gland, ovary and brain, and the co-localized expression patterns of efp and estrogen receptor (ER) mRNA were especially demonstrated in these female organs (13). Moreover, the level of efp mRNA in uterus and brain, which are known as target organs for estrogen, was up-regulated by 17β-estradiol suggesting some roles of efp in the estrogen action.

To gain better understanding of RING finger functions we set out to isolate additional RING finger containing genes en route to functional characterization. By performing polymerase chain reaction (PCR) using degenerate primers corresponding to conserved domains in the RING finger motif, we identified and cloned a novel RING finger gene that was predominantly expressed in the brain, and named it bfp (brain finger protein). Interestingly, the bfp protein is up-regulated during neural differentiation of P19 embryonal carcinoma cells.

EXPERIMENTAL PROCEDURE

PCR with degenerate primers. Two degenerate primers PRIMER1; 5′-TCNTG(CT)TCN(GA)TNTG(CT)CT-3′ and PRIMER2; 5′-(GT)(CT)-

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Abbreviations: bfp, brain finger protein; PCR, polymerase chain reaction; NLS, nuclear localization sequence; RA, retinoic acid; PBS, phosphate buffered saline; FBS, fetal bovine serum; SMS, Smith-Magenis syndrome.

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NC(GT)(AG)CA(CT)TGNGG(AG)CA-3′ were used to obtain DNA fragments encoding the RING finger motif. PCR reactions were performed in a final volume 20 μl containing 100 ng of rat genomic DNA, 10μM of each primer, 200 μM dGTP, dATP, dTTP and dCTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 2 U Taq DNA polymerase. After thirty temperature cycles (each for 1 min at 94 °C, for 2 min at 46 °C and for 3 min at 72 °C), PCR products were cloned into pCRII vector (Invitrogen). One of them, 7R8, encodes the RING finger motif.

Screening of rat cDNA libraries and DNA sequence analysis. A λZAPII (Stratagene) cDNA library prepared from poly (A)+ RNA of the rat brain was screened as described previously (10). 600,000 plaques were screened by hybridization with the ^{32}P-labeled 0.2 kb Eco RI fragment of 7R8. Both strands of the cDNA insert of clone RCA having the longest insert were sequenced completely by dideoxy-method according to the manufacturer's instruction (Sequenase, US Biochemical).

Northern blot analysis. Rat multiple tissue Northern blot containing 2 μg of poly(A)+ RNA per tissue was purchased from Clontech. The membrane was hybridized with ^{32}P-labeled 1.2 kb Pst I fragment of rat bfp cDNA and then β-actin cDNA. Washing conditions were 0.2XSSC, 0.1% SDS at 65° for 30 min and the autoradiograph was taken by 8 hour exposure at −80°C with an intensifying screen.

Cell culture. COS-7 cells and P19 embryonal carcinoma cells (14) were were maintained in alpha modification of Eagle's minimum essential medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) (Cell Culture Laboratories) at 37°C in a humidified atmosphere of 5% CO_2. Retinoic acid treatment (0.3 μM) of P19 cells was performed as described (14). Briefly, cells were cultured in bacterial dishes in the presence of 0.3 μM retinoic acid (RA) for the first 4 days with a medium change at day 2 and then transferred to tissue culture dishes.

Nuclear extract preparation. Mouse and rat brain was minced and nuclear extracts were prepared as described (15). The cDNA insert of clone RCA was recloned into EcoRI site of pSSRα expression vector (16) having SRα promoter in sense orientation to construct pSSRαBFP. Either pSSRα vector (10 μg) or pSSRαBFP (10 μg) was transfected into COS-7 cells by calcium-phosphate precipitation method (17). Nuclear extracts from these transfected COS-7 cells and P19 cells were prepared as described (18).

Antibody preparation, Western blot analysis. Partial rat bfp cDNA containing amino acids 22-631 was ligated in EcoRI site of pGEX-1T (Pharmacia) and an in-frame fusion was constructed. The fusion protein expressed in *Escherichia coli* and rabbit polyclonal anti-bfp antibody (IgG fraction) was generated by courtesy of Medical and Biological Laboratories, Ina, Japan. Nuclear extracts prepared from the rat and mouse brain, COS-7 cells and P19 cells were separated in 10% SDS-polyacrylamide gel, electroblotted to PVDF membrane (Millipore) and analyzed by Western blot analysis as described (19). The membrane was probed with the anti-rat bfp antibody (1:1000) and then anti-rabbit IgG (Fc) conjugated with alkaline phosphatase (AP) (1:7500). For extracts from P19 cells, the ECL system (Amersham) was utilized according to the manufacturer's instruction. The experiments were carried out three times and representative patterns are shown.

RESULTS

Isolation and structure of a novel member of the RING finger protein family. To isolate genomic DNA fragments containing the RING finger motif, PCR was performed on rat genomic DNA using degenerate primers corresponding to consensus amino acid sequences

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          PRIMER1                                        PRIMER2
            ---->                                         <----
7R8       CSICLERLREPISLDCGHDFCIRCFDTHRIPG    CELPCCPECR

efp     ELSCSICLEPFKEPVTTPCGHNFCGSCLNETWAV    QGSPYLCPQCRAV
mefp    ELSCSVCLELFKEPVTTPCGHNFCTSCLDETWVV    QGPPYRCPQCRKV
rpt-1   EVTCPICLELLKEPVSADCNHSFCRACITLNYESNRNTDGKGNCPVCRVP
XNF7    ELTCPLCVELFKDPVMVACGHNFCRSCIDKAWEG    NSSFACPECCES
BRCA1   ILECPICLELIKEPVSTKCDHIFCKFCMLKLLNQKK    GPSQCPLCKND
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FIG. 1. Isolation of a genomic DNA fragment containing the RING finger motif. Amino acid alignment of proteins with the RING finger motif. A deduced amino acid sequence of rat genomic DNA fragment (7R8) that encodes a RING finger motif obtained by PCR using degenerate primers is shown. The portions of amino acids corresponding to the degenerate primers are shown by arrows. Conserved amino acids, including Cysteines/Histidines, are denoted in bold type.

of the motif (Fig. 1). One of these fragments, designated 7R8, harbored a potential novel RING finger sequence which showed 49 % identity with the human efp, mouse efp and human rpt-1 RING finger domains and 42 % identity with each of *Xenopus* XNF7 and human BRCA1 (Fig. 1). Using this genomic DNA fragment as a probe, two cDNA clones were isolated from a rat brain cDNA library. Both cDNA clones were found to derive from the same RNA as reflected by restriction mapping and partial sequencing. The clone that had the longest insert was completely sequenced and the longest open reading frame derived from it contained 631 codons (Fig. 2). It harbors two putative initiator 'ATG's preceded by an upstream in-frame stop codon. Usually the upstream initiation codon is preferentially used when there are two 'ATG's. Neither of the sequences around the 'ATG's resembles the translation initiation consensus sequence proposed by Kozak (20). The predicted sequence contains a RING finger motif at the N-terminal portion. The calculated relative molecular mass (Mr) of the predicted protein was 68,643. There is no signal peptide sequence or a hydrophbic transmembrane-like sequence, suggesting that the protein is probably intracellular. The coding sequence also features a potential nuclear localization sequence (NLS), KRAR (Fig. 2), similar to those of polyoma large-T antigen (KKAR and RKRPR) (21). The C-terminal portion of the protein is rich in glycine and alanine, while no acidic or serine rich domains are found. We named this gene brain finger protein (bfp), as it is predominantly expressed in the brain (see below).

Expression of bfp in the brain. Northern blot analysis showed that the transcript of rat bfp is predominantly expressed in the brain (Fig. 3A). Two positive bands of 3.3 kb and 3.7 kb were detected and the faster migrating band corresponded to the size of the cDNA isolated here. The 3.7 kb band may corresponded to a high molecular mRNA precursor, a splicing variant or an mRNA with an alternative end.

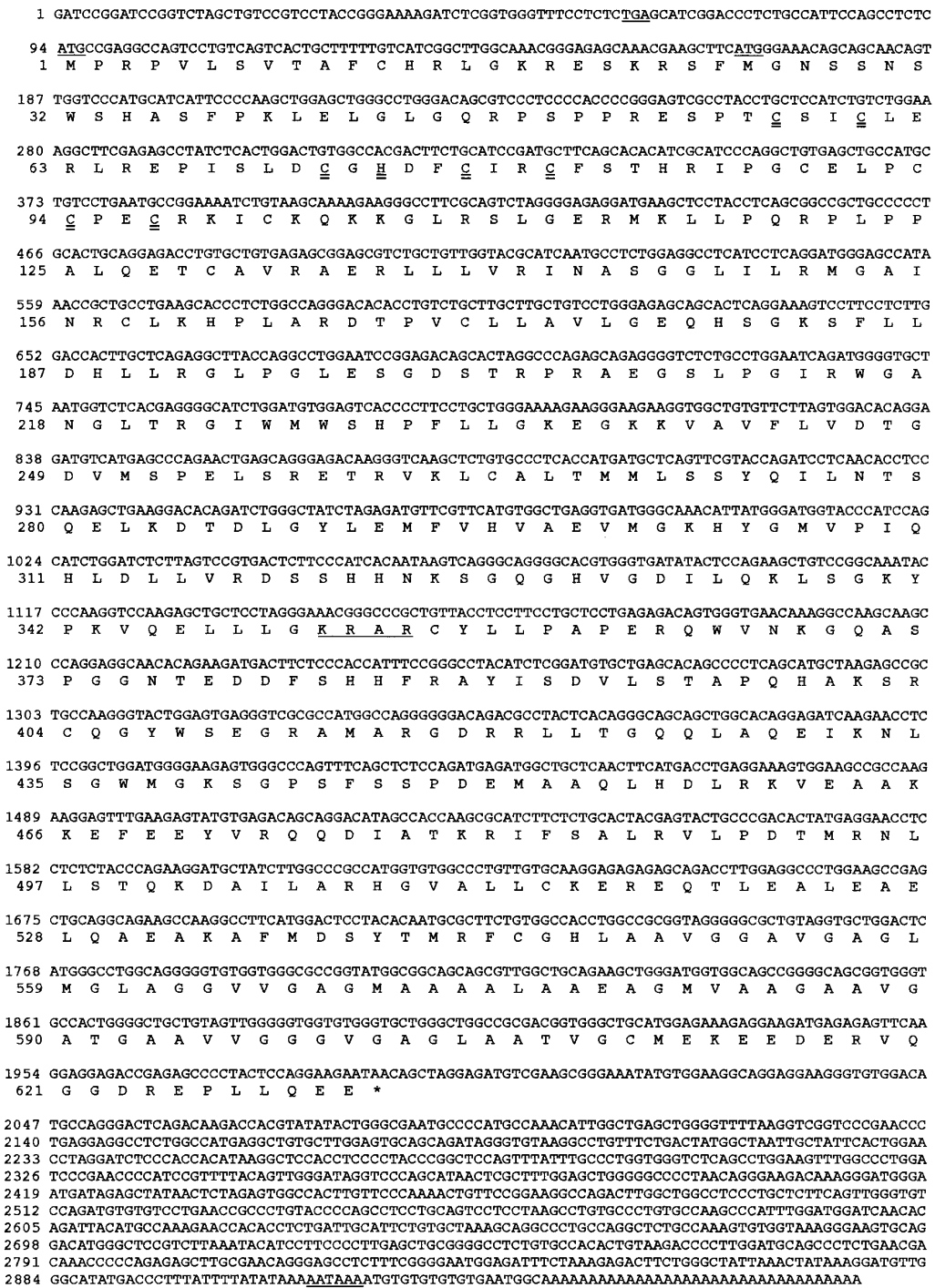

FIG. 2. Molecular structure of the rat bfp cDNA. Nucleotide and deduced amino acid sequences of the rat bfp cDNA. The deduced amino acids are shown below their respective codons. Two putative initiation codon 'ATG's, an upstream stop codon 'TGA,' and the poly adenylation signal are underlined. The putative nuclear localization sequence is also underlined. Conserved Cysteines/Histidines residues in the RING finger domain that may be involved in zinc finger-like structure are double underlined.

Utilizing GST fusion protein of the rat bfp expressed in *Escherichia coli* as antigen, a rabbit polyclonal anti-bfp antibody was generated. The specificity of this anti-body was examined by the COS-7 expression system. A 70 kDa band was detected by Western blot analysis in COS-7 cells transfected with the bfp expression con-

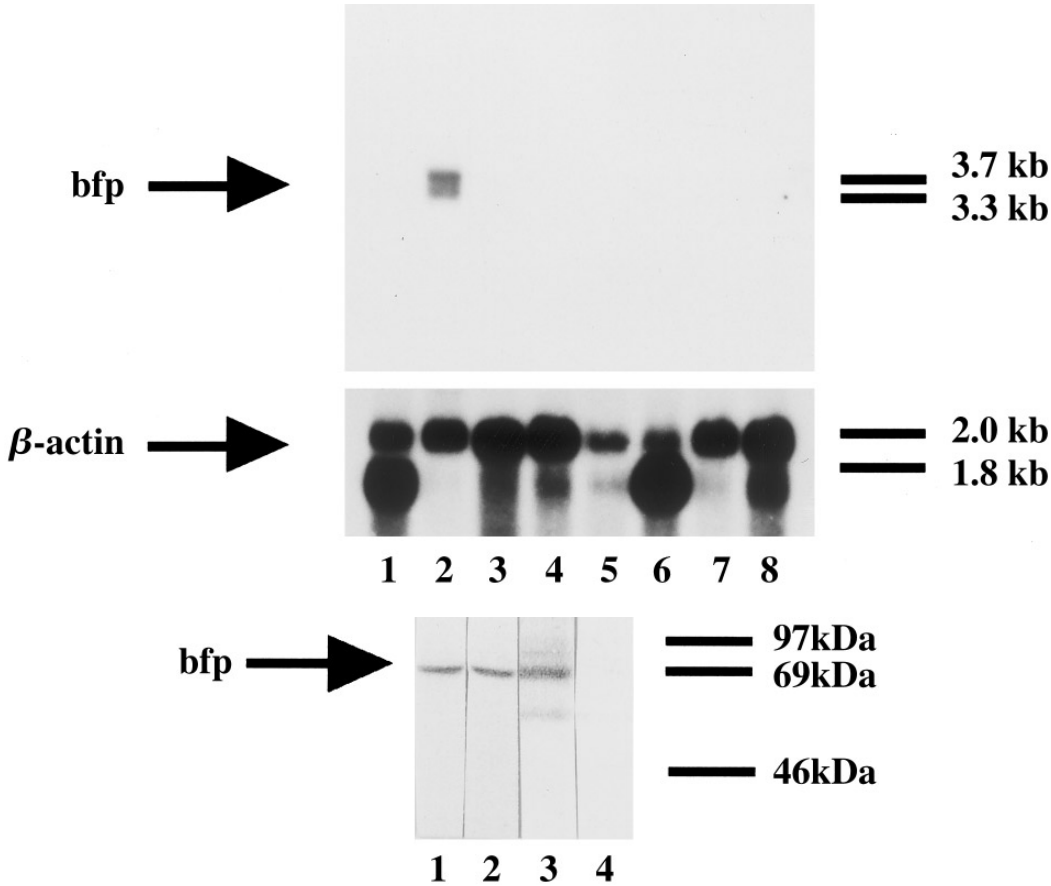

FIG. 3. Expression of bfp in the brain. (A) Tissue distribution of the rat bfp mRNA was analyzed by Northern blotting. Two μg poly(A)+ RNA of each tissue (lane 1: heart; lane 2: brain; lane 3: spleen; lane 4: lung, lane 5: liver, lane 6: skeletal muscle, lane 7: kidney, and lane 8: testis) was separated on the membrane. The ^{32}P-labeled 1.2 kb Pst I fragment of rat bfp cDNA or β-actin cDNA fragment was used as the probe. The 3.3 kb and 3.7 kb bands were detected predominantly in RNA from the brain. (B) Western blot analysis of the bfp protein in the brain. Nuclear extracts (20 μg) prepared from the rat (lane 1) and the mouse (lane 2) brain were analyzed by Western blotting. The 70 kDa band was detected with the anti-bfp antibody (1:1000) and the size agreed with the Mr predicted from the bfp cDNA. Nuclear extracts (20 μg) prepared from COS-7 cells transfected with the bfp expression plasmid (pSSRαBFP) (lane 3) or with the control expression vector (pSSRα) (lane 4) were also analyzed. The 70 kDa band, the size of which agreed with the natural products, was detected only in COS-7 cells transfected with the bfp expression plasmid.

struct (Fig. 3B; lane 3), whereas this band was not detected in COS-7 cells transfected with the control expression vector (Fig. 3B; lane 4). Western blot analysis using this anti-bfp antibody detected a 70 kDa band in nuclear extracts of the rat brain (Fig. 3B; lane 1). This antibody also recognized a band of the same size in the mouse brain extracts (Fig. 3B; lane 2). Thus, the size of the natural product agreed well with the predicted Mr and with that of the product derived from the transfected bfp expression construct.

Expression of bfp protein in retinoic acid treated P19 cells. Mouse embryonal carcinoma P19 cells were treated with retinoic acid. After 7 days, the P19 cells had neural dendrite-like structures as shown in Fig. 4A. Interestingly, the expression of bfp, as detected by anti-bfp antibody, appeared only after retinoic acid treatment (Fig. 4B).

DISCUSSION

In the present study, a novel RING finger protein, bfp has been identified. A number of proteins having the RING finger motif are involved in regulating gene expression. For example, rpt-1 is a transcription factor that down-regulates the IL-2 receptor and human immunodeficiency virus type 1 genes (8). XNF-7 is a putative transcription regulator expressed maternally in *Xenopus laevis* (7). PWA33 is associated with the nascent transcripts on the lampbrush chromosome loops and may function as a regulatory protein during early development (22). Posterior Sex Comb (Psc) and Suppressor two of zesta (Su(z)2) are *Drosophila* Polycomb group (Pc-G) genes, the members of which are involved in maintaining homeotic genes in the suppressed state after their local down regulation at a specific time dur-

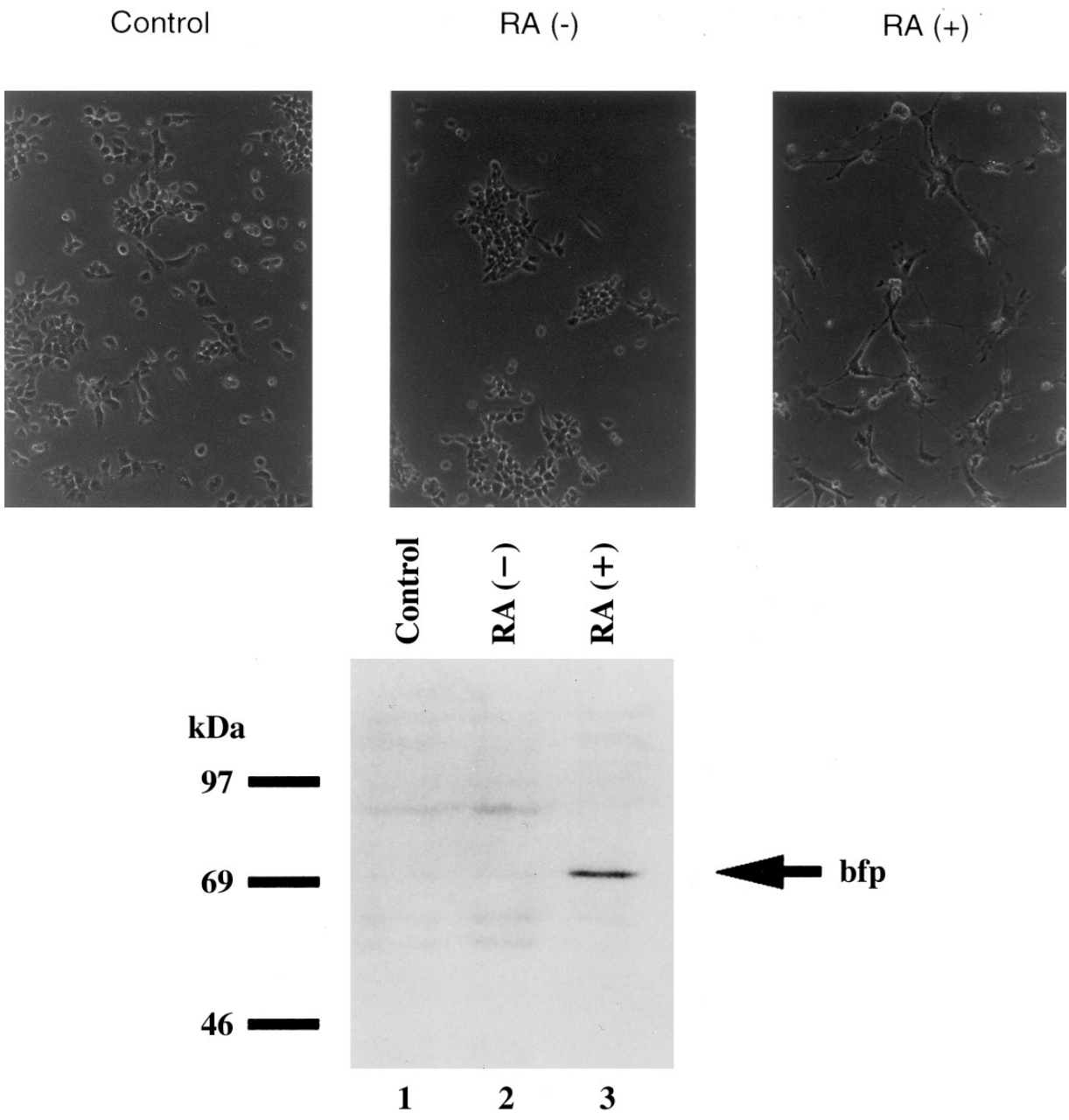

FIG. 4. Expression and localization of bfp protein in P19 cells treated with retinoic acid. (A) Mouse embryonal carcinoma P19 cells were differentiated toward neural cells by treatment with retinoic acid. Microphotographs of P19 cells before treatment (Control) and 7 days after treatment without (RA(−)) or with retinoic acid (RA(+)) are shown (original magnification, ×100). (B) Western blot analysis was performed with nuclear extracts (5 μg) from P19 cells before treatment (Control) and after treatment without (RA(−)) or with retinoic acid (RA(+)). The signal detected by anti-bfp antibody (1:5000) is increased after retinoic acid treatment.

ing development (23, 24). Bmi-1 (25, 26) was shown to be the mouse homolog of Psc. Its disruption causes posterior transformation (27), whereas its overexpression causes anterior transformation of the axial skeleton (28). These observations suggest that bmi-1 is a regulator of homeotic genes in mice. Male-specific lethal-2 (msl-2) required for X chromosome dosage compensation in *Drosophila* males also possesses the RING finger motif (29, 30). The msl-2 protein localizes on the male X chromosome and may mediate the 2-fold increase in transcription that is characteristic of dosage compensation. TIF1 was suggested to be a putative mediator of the ligand-dependent activation of nuclear receptors, including retinoic acid receptor, RXR, vita-

min D_3 receptor, progesterone receptor and estrogen receptor (31). Thus, RING finger proteins, of which the bfp is a member, appear to be involved in transcription regulation as well as other diverse functions.

Some members of the RING finger family have been implicated in carcinogenesis and cell transformation. For example, PML is a fusion oncoprotein with the retinoic acid receptor α in acute promyelocytic leukemia (APL) (2-5). T18, the N-terminal portion of TIF1 including the RING finger domain, is found fused with the B-*Raf* proto-oncoprotein in mouse liver tumor as a transforming fusion protein (9, 31). Human rfp is also found fused with the *c-ret* tyrosin kinase as ret oncogene (6). Mouse *bmi-1* cooperates with *myc* in lymphoma development in Eμ-myc transgenic mice (25, 26). BRCA1 gene, a putative tumor suppressor gene, was found to be mutated in families of early-onset breast and ovarian cancers (10). Interestingly, in three families, when one of the conserved cysteine residues in the RING finger motif was changed into a glycine residue, these one-base missense mutations cause the diseases in the families (32, 33). This data strongly suggests the importance of the RING finger motif in BRCA1. To investigate the possible involvement of bfp in carcinogenesis, molecular cloning of the human and mouse bfp gene are now underway.

Chromosome mapping showed that human bfp is located at 17p11.2 (34). Interestingly, Smith-Magenis syndrome (SMS) is linked to interstitial microdeletions of band 17p11.2 (35,36). SMS patients show several clinical signs associated with the central nervous system. These include mental retardation, speech delay, prepsychotic behavior and sleep problems. These features are variable among patients probably due to varying extent of deletions. The bfp may be a candidate gene that is responsible for some features of SMS associated with the central nervous system.

The expression of bfp mRNA was restricted in the brain as far as we examined by Northern blot analysis. Western blot analysis also showed the presence of bfp protein in both rat and mouse brain. To investigate the regulation of bfp in the course of neural differentiation, we utilized a P19 embryonal carcinoma cell model. P19 cells are known to differentiate toward neural cells when treated with retinoic acid (14). The P19 cells treated with retinoic acid change their shape, sprouting neural dendrite-like structures. We previously confirmed that they express 160 kDa neurofilament and 45 kDa fibrillary acidic glial protein under these conditions (37). Using this model, the bfp protein was probed by Western blot analysis. Indeed, the bfp protein was not detected in untreated P19 cells but was clearly shown after treatment with retinoic acid when the cells differentiated to neural cell-like cells. This up-regulation of the bfp protein was also confirmed using P19 cell extracts (data not shown). The genes that are developmentally regulated in the course of neural differentiation should be important to understand the development and function of the central nervous system. If the bfp is a putative transcription regulator, it may play a crucial role in neural differentiation. Further studies are required to reveal the roles of the bfp in the brain.

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