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The class III POU factor Brn-4 interacts with other class III POU factors and the heterogeneous nuclear ribonucleoprotein U

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Abstract

The class III POU proteins are expressed throughout the central nervous system, including the hypothalamus, where they are often co-localized. Presumably, these POU proteins (Brain-1, Brain-2, Brain-4 and SCIP) serve as transcriptional transactivators. That they are co-expressed in some neurons suggests that, if they were to form homomeric and heteromeric complexes with each other, depending on the particular combination, they might have different DNA-binding specificities and, thus, activate different genes. We used purified fusion proteins of the four class III POU proteins in far-western assays to show that the proteins can interact. We confirmed their interactions using a two-hybrid system. Both techniques indicate that the interaction occurs through the POU domain. The far-western technique also allowed us to identify a 120-kDa nuclear protein that interacts with Brain-4. Subsequent affinity purification and microsequencing identified the protein as the heterogeneous nuclear ribonucleoprotein U (hnRNP U). This result suggests another mechanism by which a POU protein can influence gene expression: by facilitating the processing of pre mRNA whose transcription it has stimulated.

Keywords: Two-hybrid; Brain-4; hnRNP U; POU protein

1. Introduction

Brain-1 (Brn-1), Brain-2 (Brn-2), Brain-4 (Brn-4) and suppressed cAMP-inducible POU (SCIP; also known as Oct-6, Tst-1) are the four known class III POU factors expressed in the mammalian brain ([8]). Like all members of the POU family of transcriptional regulators, these factors are characterized by a unique bipartite DNA-binding domain consisting of a 74–82 amino acid POU-specific (POUs) region that is separated from the 60 amino acid POU homeodomain (POUhd) by a variable 15–27 amino acid linker (reviewed in [10,25,27]).

The class III POU factors have unique, but overlapping distributions during development of the central nervous system and in the adult brain [1,9,14,17,18,21]. For example, both Brn-2 and Brn-4 are expressed at high levels in the adult paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus [1,9,14,15,17,18,21]. However, while Brn-4 is expressed at relatively high levels in the striatum, Brn-2 is not. In contrast, SCIP is not expressed at high levels in either the PVN or SON but does overlap in distribution with Brn-4 within the striatum ([1]; unpublished personal observations). As another example, Brn-1, Brn-2, Brn-4 and SCIP are all robustly expressed in the medial habenula [9,14,18]. These observations raise the possibility that different combinations of class III POU factors could interact, within specific cell groups, to participate in the complex and unique transcriptional regulation that defines disparate neuronal phenotypes.

There are many junctures where class III POU factors could interact to impart unique transcriptional regulation (see examples in [27]). One possibility is that class III POU factors could form multimeric complexes prior to binding target DNA elements and in this way alter either the DNA-binding specificity and/or the protein-protein interactions of the partners involved in the complex. This scenario requires that class III POU factors interact with

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each other in a DNA-independent manner. There are few examples of POU proteins interacting with themselves in this fashion. The class IV POU factor I-POU binds to the class III POU factor Cf1-a in *Drosophila* [23,24] to inhibit the latter's ability to bind to DNA. Pit-1 and Oct-1 (classes I and II, respectively) can form a heteromeric complex through the POU domains in the absence of DNA in which the Oct-1-Pit-1 complex is favored over the Oct-1-Oct-1 complex at the prolactin 1P site [26]. Most investigations of protein-protein interactions among POU domain factors suggest that these factors are primarily monomeric in solution but can dimerize on DNA targets (reviewed in [25]). However, exhaustive evaluations of interactions amongst POU factors have not been performed. Our group is interested in class III POU factor regulation of neuropeptides and other factors within the hypothalamus. Therefore, we were motivated to investigate various aspects of class III POU factor regulation and sought to determine if class III POU factors can interact with each other. To our surprise, we found that all class III POU factors expressed in the mammalian brain can form high-affinity protein-protein interactions and that these interactions are mediated primarily by the POU domain.

Our laboratory has an additional goal of defining how class III POU factors (especially Brn-4) affect gene regulation at the level of the basal transcriptional machinery. In this paper, we also demonstrate that Brn-4 can interact with the heterogeneous nuclear ribonucleoprotein hnRNP U [13]. This interaction may be important for Brn-4 modulation of transcriptional or post-transcriptional processes.

2. Materials and methods

2.1. Expression plasmids

DNA of interest was cloned into the bacterial expression vectors pGEX-2T$^{128}/_{129}$ and/or pAR(ΔRI)$^{59}/_{60}$ ([2]; generous gift of M. Blanar, Bristol-Myers Squibb, Princeton, NJ, USA). Fusion proteins expressed from the pGEX-2T$^{128}/_{129}$ vector include a leader peptide that consists of glutathione-S-transferase (GST), a Flag epitope tag, a thrombin recognition sequence and a consensus phosphorylation site for the cAMP-dependent protein kinase. The entire leader sequence generated from the pGEX-2T$^{128}/_{129}$ vector is referred to here as GST. Fusion proteins generated from the pAR(ΔRI)$^{59}/_{60}$ vector include the Flag epitope tag, an enterokinase recognition sequence and consensus phosphorylation site. This leader sequence is referred to as Flag.

2.2. PCR amplification and subcloning of class III POU and hnRNP U DNA constructs

Synthetic 5′ and 3′ primers for amplification of Brn-4, Brn-1, Brn-2, SCIP and hnRNP U were generated containing *Eco*RI sites near the 5′ end so that amplified DNA could be cut with *Eco*RI and subcloned, in frame, into the unique *Eco*RI site within expression vectors pGEX-2T$^{128}/_{129}$ and pAR(ΔRI)$^{59}/_{60}$. Coding regions of interest, excluding the first methionine but including the TGA translation stop codon, were amplified by PCR and then subcloned into each of the expression vectors. Genomic clones of rat Brn-4 in pGEM3Z [14] or mouse Brn-1, Brn-2, and SCIP in pBluescript II SK + and KS + (gifts of Dr. Y. Hara) were used as templates for PCR amplification as class III POU factors are intronless [8]. Specific domains of the Brn-4 were also expressed as fusion proteins. These constructs included: the N-terminal transactivating region of Brn-4 (amino acids 2–188); the the entire Brn-4 POU domain (amino acids 192–361); the Brn-4 POUs domain (amino acids 192–275); and the Brn-4 POU homeodomain (amino acids 261–361). The Brn-4 POU, POUs and POU homeodomain regions were PCR-amplified from the vectors RG4pouB, RG4sB and RG4hdB (see two-hybrid section below). The N-terminal Brn-4 construct was amplified from Brn-4 in pGEM3Z. hnRNP U was amplified from the clone U21.1 ([13]; generous gift of Dr. G. Dreyfus).

DH5α-F′IQ bacteria (Life Technologies) were transformed with pGEX-2T$^{128}/_{129}$ parent vector or class III POU constructs in pGEX-2T$^{128}/_{129}$. BL21pLysS bacteria (Clonetech) were transformed with class III POU and hnRNP U constructs in the pAR(ΔRI)$^{59}/_{60}$ vector. Restriction analysis followed by DNA sequencing using the Sequenase 2.0 kit (US Biochemical) was used to select clones with inserts properly ligated into the vector.

2.3. Fusion protein expression

Overnight (10 ml) cultures were used to inoculate 90 ml LB/AMP broth and the cultures were allowed to grow at 37°C to a density of about $A_{600} = 0.60$. Cultures were next induced with 1 (GST-based) or 3 mM (Flag-based) IPTG for 4 h at 37°C, and then harvested by centrifugation at 4°C. Bacterial pellets were washed briefly with NETN buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA; [GST fusions]) or TBS (50 mM Tris [pH 7.4], 150 mM NaCl; Flag fusions) and then suspended in 10 ml NETN or TBS containing 100 μM AEBSF (Pefabloc, Boehringer-Mannheim) and lysed with mild sonication. Triton X-100 was added to 1% (v/v) and the bacterial lysate centrifuged at 4°C at 10000 × g for 5 min. The resulting crude extract was snap frozen in a dry ice/ethanol bath before storage at −70°C.

2.4. Fusion protein purification

GST and Flag fusion proteins were purified as previously described [17].

2.5. ^{32}P-labeling Flag and GST fusion proteins

Labeling of Flag and GST fusion proteins was carried out as previously described [2,12] and as outlined briefly below.

To label GST fusions, glutathione-sepharose was washed 3 times with NETN + 0.5% NP-40, incubated with crude bacterial extract for 1 h at 4°C, washed 5 times with NETN, and then washed once with heart muscle kinase (HMK) buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 12 mM $MgCl_2$). Glutathione-sepharose was then resuspended in 3 bed volumes of HMK buffer containing 1 U/μl of the catalytic subunit of cAMP-dependent kinase isolated from bovine heart (Sigma), 1 μCi/μl [γ-^{32}P]ATP (6000 Ci/mmol, 10 mCi/ml, New England Nuclear), and 1 mM dithiothreitol (DTT). The kinase reaction was allowed to progress for 30 min on ice and was stopped with 10 mM sodium phosphate [pH 8.0], 10 mM sodium pyrophosphate, 10 mM EDTA, and 1 mg/ml bovine serum albumin. The glutathione-sepharose was then washed 5 times with NETN + 0.5% NP-40 and labeled protein was eluted from the column with 10 bed volumes of 20 mM reduced glutathione/100 mM Tris (pH 8.0)/120 mM NaCl.

For labeling Flag fusions, purified aliquots of protein were thawed and approximately 0.5 μg protein was added to 50 μCi [γ-^{32}P]ATP (6000 Ci/mmol, 10 mCi/ml, New England Nuclear), 1 mM dithiothreitol, and 10 U catalytic subunit of cAMP-dependent kinase isolated from bovine heart (Sigma) in a total reaction volume of 30 μl in HMK buffer. The kinase reaction was allowed to proceed for 1 h on ice. Labeled protein was separated from unincorporated [γ-^{32}P]ATP using Sepharose-G50 chromatography.

2.6. Far-western blotting

Proteins to be screened for interactions with ^{32}P-labeled fusion protein probes were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore). Transfer buffer salts were removed from blots by washing briefly in HEPES-binding buffer (HBB; 25 mM HEPES-KOH [pH 7.7], 25 mM NaCl, 5 mM $MgCl_2$, 0.05% NP-40, 1 mM DTT). Non-specific protein interactions were blocked by incubating the blot 1 h at room temperature in 5% non-fat dry milk (NFDM) in HBB, followed by a 1-h incubation at room temperature in 1% NFDM in HBB. Blots were then incubated overnight in Hyb-75 (20 mM HEPES-KOH [pH 7.7], 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% NP-40, 1 mM DTT, 1% NFDM, 1 mM DTT) and 2.5×10^5 cpm/ml labeled fusion protein probe at 4°C with gentle rocking. Blots were washed 4 times 15 min in Hyb-75 to remove excess probe and then blown dry with warm air. Blots were apposed to BAS-III Fuji phosphorimaging plates that were then scanned and analysed with the Fuji BAS2000 image analysis system. The latent images were visually displayed and converted into cpm units.

2.7. Nuclear extracts

Adherent cells grown to confluence in 162-mm^2 flasks were resuspended in 5 volumes of buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mMDTT, and 100 μM AEBSF), incubated on ice for 10 min, and then centrifuged at $250 \times g$ for 10 min. The resulting pellet was resuspended in 3 volumes buffer A, NP-40 was added to 0.05%, and the suspension was homogenized with 20 strokes with a Dounce homogenizer. Released nuclei were pelleted by centrifugation at $250 \times g$ for 10 min and then resuspended in 1 ml buffer C (5 mM HEPES (pH 7.9), 26% glycerol (v/v), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 100 μM AEBSF). The total volume was measured, NaCl was added to a final concentration of 300 mM, and the extract was incubated on ice for 30 min. After the incubation, the extract was centrifuged at $24000 \times g$ for 20 min at 4°C and the supernatant was aliquotted, snap-frozen in a dry ice/ethanol bath and stored at −70°C. HeLa extracts were prepared according to the protocol of Dignam et al. [3].

2.8. Two-hybrid assay

To independently confirm far-weste.n observations, we developed a two-hybrid system similar to one previously described [6] with which to test protein-protein interactions in cultured mammalian cells. Expression vectors that produce fusion proteins with either the yeast GAL4 DNA-binding domain (GAL4BD) or the VP16-transactivating domain were created. If a protein-protein interaction exists, then transcription of the luciferase gene is stimulated from a promoter that contains the upstream activating sequence to which the GAL4-binding domain binds. Brn-4 or segments were inserted to test for interactions between themselves.

The specific constructs were made as follows. RSV.express was made by inserting the RSV LTR and bovine growth hormone polyadenylation signal sequences (*Nru*I and *Bbv*I, blunted) from pRC/RSV (Invitrogen) into pUC18 (*Hin*dIII/*Eco*RI, blunted). RVBp1 (without initial methionine) and RVBp2 (with initial methionine; used as a VP16-expressing control) were made by inserting the sequence encoding the VP16-transactivating domain (amino acids 413–490 + stop codon) generated by PCR from pSGVP (courtesy of M. Ptashne; see [20]) into RSV. express. RGBp1 was made by inserting the sequence encoding the GAL4BD (amino acids 1–147) generated by PCR from pSGVP into RSV.express. RPit1VB was made by inserting the entire coding sequence minus the stop codon of Pit-1 (generated by PCR from rat anterior pituitary cDNA; [11]) flanked by NotI sites at the 5′ and 3′ ends into RVBp1 cut with the same. RBrn2VBp1 was made by inserting the entire coding sequence of Brn-2 (amino acids 1–445 without stop codon generated by PCR from Brn-2; [8]) flanked

by NotI sites at the 5′ and 3′ ends into RV BpI cut with the same. Brn-4-derived plasmids were made using RHS2 PCR products with appropriate flanking restriction sites and are described in the legend to Fig. 3. The reporter plasmid, pGEL, was made by inserting the blunted the *Xho*I/*Eco*RI piece (containing 5 copies of the yeast GAL4-binding site immediately upstream from the adenovirus E1b TATA box) from 5GAL4/E1bTATA-CAT (courtesy of M.R. Green; see [16]) into pGL2.Basic (blunted *Hin*dIII/*Sma*I site; Promega). The β-galactosidase-coding region from pCH110 (Pharmacia) was subcloned into pRc/RSV (Invitrogen) and called RSV-Gal.

CV-1 cells, maintained in DMEM (BioWhittaker) with 0.45% glucose, 1 mM L-glutamine, 50 U/l penicillin, 50 μg/l streptomycin and 10% heat-inactivated fetal calf serum were plated at about 8×10^4/well in 12-well culture plates. Three days later, the media was removed, the cells washed twice with 2 ml Opti-MEM (LTI) and 1 ml of a DNA/lipofectamine (LTI) mixture in Opti-MEM was added. The DNA/lipofectamine mixture was made by adding 1.1 μg of DNA (each transfection mixture contained 0.33 μg of both POU protein expression plasmids, 0.33 μg of pGEL, and 0.1 μg of RSV-Gal to adjust for transfection/expression efficiency) to 0.1 ml Opti-MEM, and 6 μl of Lipofectamine to 0.1 ml of Opti-MEM, mixing the two Opti-MEM preparations thoroughly together, and adding 0.8 ml of Opti-MEM. Twenty-four h later, 4 ml of the DMEM media described above were added to each well and the cultures incubated for another 24 h.

48 h after the DNA/lipofectamine was added to the cells, the cells were rinsed twice with 1 ml ice-cold PBS and 100 μl of 1 × Luciferase Lysis buffer (Promega) was added to the wells. After 15 min, the lysate was transferred to 1.5-ml microfuge tubes and spun 5 min at 14 000 rpm. 1 μl of supernatant was placed in a 5-ml polyethylene tube and inserted into a Lumat LB9501 luminometer at which point 100 μl of Luciferase cocktail (Promega) was injected. After a 3-s delay, the light emissions were read for 20 s. β-galactosidase activity was measured in 5-μl aliquots from the same homogenate. The 5 μl of homogenate was incubated with reaction buffer (Tropix) for 50 min in a 5-ml polypropylene tube in the dark, exposed to light for 10 min and inserted into the Lumat luminometer. 100 μl of accelerator (Tropix) was injected and after a delay of 5 s, the light emissions were read. The legend to Fig. 3 details the quantitation and statistical analysis.

2.9. Protein microsequenceing

Bacterially expressed GST/Brn-4 was immobilized and purified on glutathione-coated beads as described above. Beads were then washed 2 times with Hyb-75 and then incubated with 1 mg of the flow-through fraction of HeLa nuclear extracts run over a phosphocellulose column in 1 ml Hyb-75 overnight at 4°C with rocking. Beads were then washed 5 times with ice-cold Hyb-75 (without milk), resuspended in 40 μl 1 × SDS loading buffer and boiled for 5 min. Proteins were separated on a 12% SDS polyacrylamide gel.

Ten lanes of protein prepared and separated as above were transferred to a PVDF membrane and stained with 0.2% Ponceau S (Sigma) in 3% trichloroacetic acid. Bands running at 120 kDa were excised and subjected to in situ proteolytic digestion with endoproteinase Lys-C [7]. The resulting peptides were purified by 2 RP-HPLC steps prior to sequencing on a Model 477A Protein Sequencer (Applied Biosystems).

3. Results

3.1. Class III POU factors can form homomeric and heteromeric complexes amongst themselves

Class III POU factors were immobilized on a PVDF membrane and ^{32}P-labeled Flag/Brn-4 was used as a probe to screen for protein-protein interactions between Brn-4 and other class III POU factors. Fig. 1A shows a Coomassie blue stain of proteins transferred to PVDF. As shown in Fig. 1B, Flag/Brn-4 interacts with full-length Flag/Brn-4 but not the C-terminal truncated Flag/Brn-$4_{2\text{-}188}$ that has the entire POU domain deleted. Flag/Brn-4 also interacts with other class III POU transcriptional regulators, including Brn-1, Brn-2, and SCIP (Fig. 1B). These interactions are independent of the Flag or GST fusions since ^{32}P-labeled Flag/Brn-4 or GST/Brn-4 interacts with Flag- or GST-fused Brn-1, Brn-2, Brn-4 and SCIP (Fig. 1, and see below).

As demonstrated in Fig. 1C and D, when GST/Brn-1 or GST/SCIP were used as ^{32}P-labeled probes these factors form homomeric and heteromeric complexes with GST/Brn-1, Flag/Brn-2, Flag/Brn-4 and GST/SCIP but do not interact with Flag/Brn-$4_{2\text{-}188}$ or GST (data not shown). These interactions were salt-dependent as KCl concentrations of less than 75 mM gave non-specific interactions with molecular weight markers and Flag/Brn-$4_{2\text{-}188}$ while specific POU-POU interactions were stable using KCl concentrations between 75 mM and 500 mM (data not shown).

3.2. The POU domain of Brn-4 is necessary for mediating protein-protein interactions between Brn-4 and other class III POU factors

Because Flag/Brn-$4_{2\text{-}188}$ did not interact with either Brn-4 or any of the other class III POU factors, we expressed GST fusions of Brn-4 POU (GST/POU), the Brn-4 POUs (GST/POUs) and the Brn-4 POUhd (GST/POUhd) as fusions with GST (Fig. 2). We then used these proteins as both probes and targets in order to

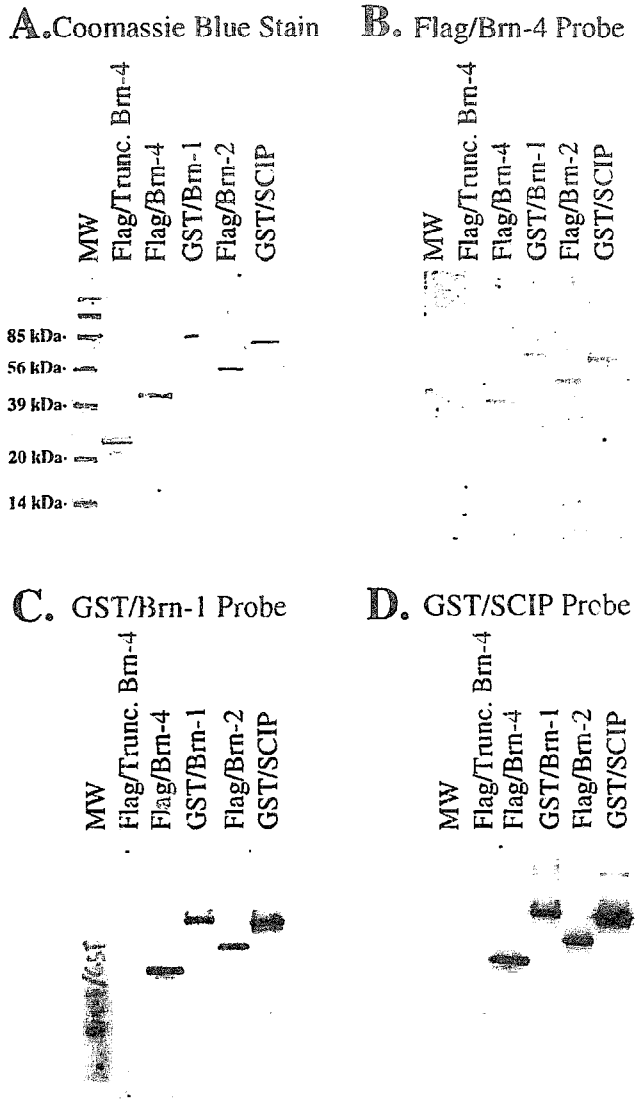

Fig. 1. Far-Western probing of triplicate blots with either ^{32}P-labeled Flag/Brn-4 (B), GST/Brn-1 (C) or GST/SCIP (D) probes shows interactions between all 4 class III POU proteins. No interaction was detected with the N-terminus of Brn-4 (lane after the molecular weight standards). Panel A is a Coomassie blue-stained replicate blot.

determine the regions of the Brn-4 protein primarily responsible for mediating class III POU factor protein-protein interactions. Fig. 2A demonstrates a Coomassie blue-stained gel of all proteins loaded into each gel and transferred to PVDF. As expected from previous experiments, GST/Brn-$4_{2\text{-}188}$ did not specifically interact with any class III POU factors including any of the truncated Brn-4 constructs (Fig. 2B). Similarly, neither the POUs nor POUhd interacted specifically with any of the truncated Brn-4 constructs or class III POU factors (Fig. 2E,F). However, as with full-length Brn-4, GST/POU interacted with full-length Brn-4, POU, and full-length Brn-1 and SCIP (Fig. 2C,D) but not with GST, GST/Brn-$4_{2\text{-}188}$, GST/POUs or GST/POUhd.

3.3. Two-hybrid analysis of POU domain regions important for mediating POU factor protein-protein interactions

We used a two-hybrid assay system to confirm and extend our findings that class III POU-POU interactions are mediated through the POU domain. The legend to Fig. 3 describes the expression constructs co-transfected into CV-1 cells in this assay. Co-transfection of the GAL4 DNA-binding domain fused to full-length Brn-4 (RGBrn4Bp1) with either full-length Brn-4 or the Brn-4 POU domain fused to the VP16 transactivation domain (RBrn4VBp1 or R4pouVBp1, respectively) resulted in activation of the luciferase reporter construct bearing the GAL4-binding site (Fig. 3, top bar on left and right graphs). Furthermore, the GAL4 DNA-binding domain

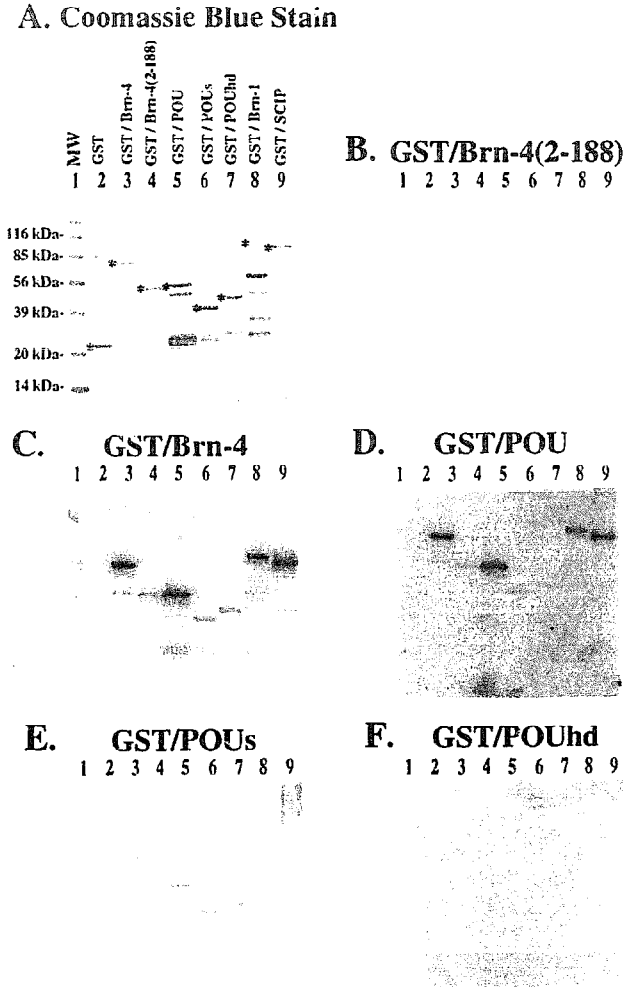

Fig. 2. Far-Western probing of quintuplicate blots with either ^{32}P-labeled GST/Brn-$4_{2\text{-}188}$ (B), GST/Brn-4 (C), GST/POU (D), or GST/POUs (E) or GST/POUhd (F) probes shows that the full-length Brn-4 (C) and the POU domain (D) interact with themselves and also with Brn-1 and SCIP (lanes 8 and 9). No interaction was detected with GST alone (lane 2), the N-terminus of Brn-4 (lane 4), POUs (lane 6) or POUhd (lane 7). Panel A is a Coomassie blue-stained replicate blot. Asterisks indicate the full-length products.

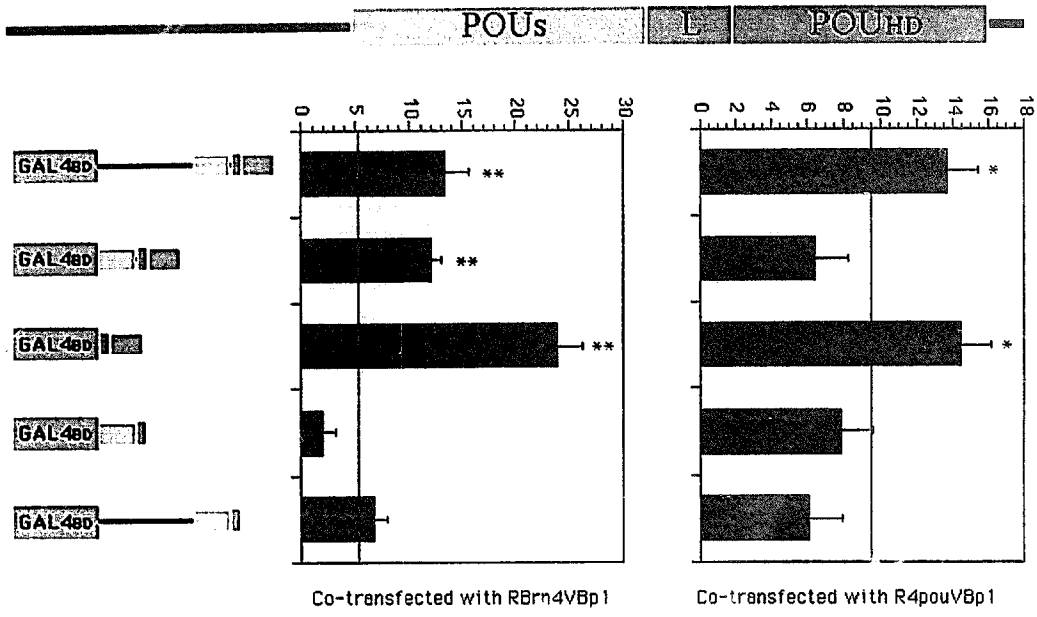

Fig. 3. The various GAL4 binding domain (GAL4BD)-Brn-4 fusion proteins were co-expressed with either RBrn4VBp1 (left graph, amino acids 1-361) or R4pouVBp1 (right graph, initiating methionine plus amino acids 192-361) in CV-1 cells along with the GAL4 binding site-containing reporter plasmid pGEL. A schematic representation of the Brn-4 cDNA is above the graphs. The co-transfected plasmids illustrated on the left are (from top to bottom): RGBrn4Bp1, amino acids 2-361 plus stop codon; RG4pouB, amino acids 192–361 plus stop codon; RG4hdB, amino acids 261–361 plus stop codon; RG4sB, amino acids 192–275 plus stop codon; and RG4ΔhdB, amino acids 2–275 plus stop codon. The backgrounds (shown as vertical lines at approximately 5.0 and 9.5 adjusted [for β-galactosidase co-expression] light units, respectively, in the two graphs) were determined by measuring and adding the adjusted light units generated by two controls. The first was the co-expression of RGBrn4Bp1 with the 'empty' plasmid RVBp2. The second was the co-expression of either RBrn4VBp1 or R4pouVBp1 with the 'empty' plasmid RGB. The backgrounds were not significantly different within the RBrn4VBp1 group (4.68 to 5.78) or within the R4pouVBp1 group (9.1 to 10.2) so one line is shown for each group. ** $P \leq 0.001$ and * $P \leq 0.05$ as determined by ANOVA with Fisher's post-hoc test.

fused to the Brain-4 POU domain (RG4pouB) also interacted with RBrn4VBp1 (Fig. 3, second bar from top on left graph); however, it did not interact with R4pouVBp1 (right graph), perhaps due to steric hinderance. These results are consistent with our far-western results above indicating that the full-length Brn-4 can interact with either

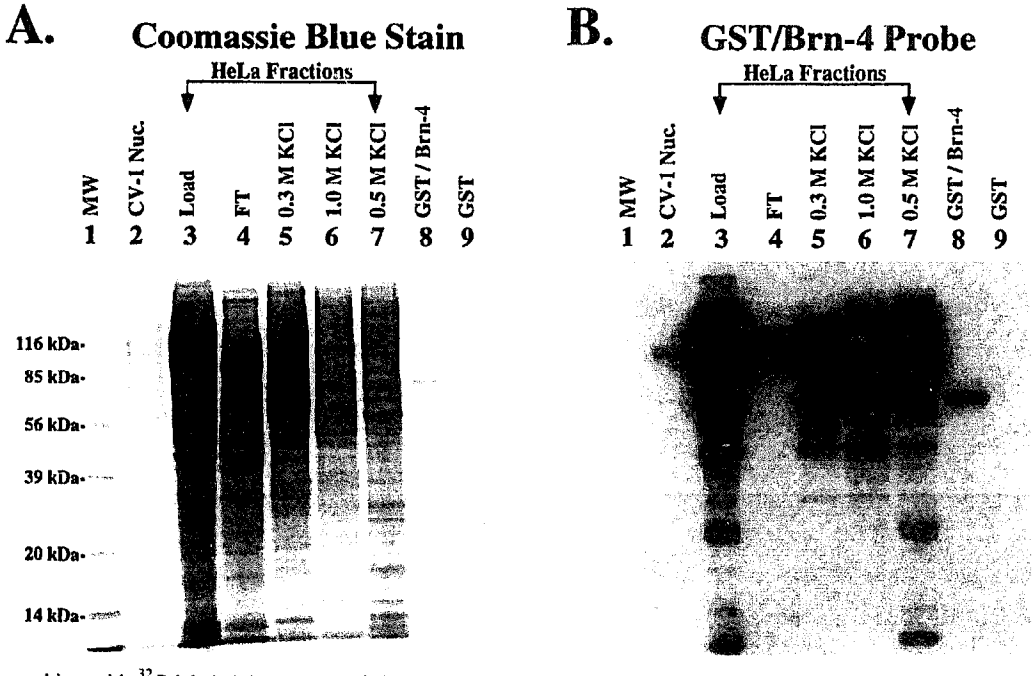

Fig. 4. Far-Western probing with ^{32}P-labeled GST/Brn-4 (B) of a blot containing various column fractions of HeLa nuclear extracts (lanes 3–7) shows that the flow-through fraction contains a substance of an apparent molecular weight of 120 kDa, similar to what we observe in the CV-1 cell line nuclear extracts (lane 2). Again, binding to GST/Brn-4 is seen (lane 8), but not to GST alone (lane 9). Panel A is a Coomassie blue-stained replicate blot.

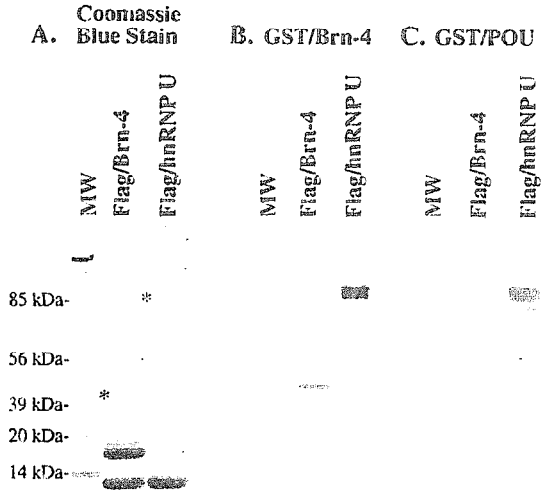

Fig. 5. Far-Western probing of duplicate blots with either ^{32}P-labeled GST/Brn-4 (B) or GST/POU (C) probes shows that they interact with Brn-4 and also with hnRNP U. Panel A is a Coomassie blue-stained replicate blot. Asterisks indicate the full-length products.

full-length Brn-4 or its POU domain. In addition, the GAL4 DNA-binding domain fused to the Brn-4 POU homeodomain (RG4hdB) also interacted with both Brn-4 and its POU domain (Fig. 3, middle bars in left and right graphs). Neither the Brn-4 POUs domain (Fig. 3, bars second from bottom) nor the N-terminus + POUs domain (Fig. 3, bottom bars) interacted with either Brn-4 or its POU domain. It should be noted that the derivation of the background values for this assay conservatively summed two different backgrounds (see legend for Fig. 3 for details) and therefore, the true background is actually less than what we graphed.

Also, in agreement with the far-western results above, RGBrn4B interacted significantly with RBrn2VB (11.4 adjusted light units, $P < 0.001$ as determined by ANOVA with Fisher's post-hoc test). Interestingly, RGBrn4B did not interact with RPit1VB (3.6 adjusted light units) which expresses a class I POU protein.

3.4. Identification of a nuclear factor that interacts with Brn-4

We used the far-western blotting technique to begin defining the interactions that Brn-4 has with other nuclear factors to influence gene expression. ^{32}P-labeled GST/Brn-4 interacts nearly exclusively with a 120-kDa factor present in nuclear extracts from CV-1 (lane 2 in Fig. 4B) and a rat neuronal precursor (line 72; generous gift of Dr. R. McKay; data not shown) cell lines. To begin to purify this substance, we used ^{32}P-labeled GST/Brn-4 to probe concentrated HeLa extracts (Fig. 4B, lane 3). Multiple interactions are detected with most fractions (over a phosphocellulose column), although the flow-through (FT) portion of the HeLa extracts contains essentially only one interacting substance that migrates at 120 kDa (Fig. 4B, lane 4). Evidence that interactions between GST/Brn-4 and the 120-kDa substance are specific include: ^{32}P-labeled Flag/Brn4 also interacts with this factor; the interaction between ^{32}P-labeled Flag/Brn4 is salt-sensitive (limited to 75–250 mM KCl); and 0.1% SDS abolishes the interaction (data not shown).

Using GST/Brn-4 immobilized on glutathione-coated beads as an affinity matrix, the 120-kDa factor was purified from the FT fraction of HeLa nuclear extracts. No residual 120-kDa factor was detected by Coomassie blue staining in that fraction after one extraction. Specifically bound proteins were eluted from the column, resolved by SDS-PAGE, transferred to PVDF membranes and then microsequenced as previously described [7]. Microsequencing determined the identity of five peptide fragments: (1) PXEDXGXGYFEYIEEN, (2) NILGTNTIM, (3) PVRHLYTK, (4) WXQHYHQGYY, and (5) SGPTSLFAVTVAPPGARQG. A GenBank database search revealed that matches for all five peptide fragments occur only for the heterogeneous nuclear ribonucleoprotein U (hnRNP U; [13]).

3.5. hnRNP U interacts with the POU domain of Brn-4

That Brn-4 interacts strongly with hnRNP U was confirmed by expressing hnRNP U as a fusion with the Flag epitope and screening for protein-protein interactions with ^{32}P-labeled GST/Brn-4 probes. Fig. 5B indicates that labeled GST/Brn-4 interacts with Flag/hnRNP U as well as the positive control, Flag/Brn-4. Similarly, labeled GST/POU interacts with Flag/hnRNP U (Fig. 5C), as well as Flag/Brn-4. The differences between the two interacting species in the doublet at about 90 kDa is unknown to us at present. However, GST/Brn-4$_{2\text{-}188}$, GST/POUs and GST/POUhd do not interact with hnRNP U (data not shown).

4. Discussion

Previous investigations have shown that various class III POU proteins are expressed within the same cells [17,21], suggesting that regulation of genes within those cells might be conferred by various combinations of the transacting factors. Different DNA-binding specificities would be determined by the formation of different homomeric and heteromeric complexes prior to DNA binding. Our results indicate that the class III POU proteins can indeed interact with each other in a DNA-independent fashion. Furthermore, as indicated in the two-hybrid assay, this interaction may be largely mediated through the POU homeodomain region. This may be similar to the interaction between the Oct-1 (a class II POU protein) homeodomain and the herpes simplex virus transactivator VP16 (24). The concentrations of the proteins used for the far-western assay are, of course, greater than those in vivo but we do not see binding to the protein markers, GST or truncated Brn-4 itself, indicating that we are not demon-

strating simple non-specific binding. Furthermore, the two-hybrid system indicates that the class IV do not exhibit promiscuous interactions with any POU protein as Brain-4 does not interact with Pit-1. It still remains to be determined whether these interactions occur in vivo and what may be the DNA-binding specificities of the various POU protein combinations.

POU proteins are known to interact with a wide variety of non-POU proteins as well. As Herr and Cleary [10] point out, the interacting proteins can be broadly categorized at this time as transcriptional activators, co-regulators and basal factors, as well as replication factors. In this report, we show that Brn-4 can interact with the heterogeneous nuclear ribonucleoprotein U, both in solution when one of them is attached to a gel matrix or to a filter. The 120-kDa protein is an abundant nucleoplasmic phosphoprotein that associates with both pre-mRNA and other hnRNP proteins ([13] and references therein). There is evidence for the role of hnRNPs in association with small nuclear ribonuclear proteins to help process pre-mRNAs [4]. For example, hnRNP A1 contains a nuclear export signal and may function to export RNA to the cytoplasm [19]. hnRNPs have been implicated in RNA splicing, as well [4]. Therefore, Brn-4 may further act to facilitate the processing of RNAs transcribed from genes that it transactivates.

There is also evidence that hnRNP K is a single-stranded DNA-binding protein that participates in the activation of human c-*myc* gene [5,22]. Whether or not Brn-4 interacts with hnRNP U to stimulate transactivation will require more detailed examination of the role of hnRNP U in the CNS, including studies of its distribution and DNA-binding preferences (if any), as well as co-transfection studies of Brn-4 and hnRNP U.

Of note is that the far-western interactions of these bacterially expressed proteins do not seem to depend on post-translational modifications of both partners, although the efficiency of interactions may be affected. This is especially evident with hnRNP U, which has an apparent molecular weight of 120 kDa when isolated from nuclear extracts, yet maintains its ability to interact with Brn-4 as a 'stripped-down' bacterial product of about 90 kDa.

In summary, our results indicate that class III POU proteins can dimerize in the absence of DNA. These homomeric and heteromeric complexes may add another level of complexity in the regulation of gene expression. Furthermore, our finding that Brn-4 can interact with hnRNP U opens a new avenue of research into the role POU proteins play in the processing of RNA.

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