A Novel Member of the BTB/POZ Family, PATZ, Associates with the RNF4 RING Finger Protein and Acts as a Transcriptional Repressor*

Monica Fedele‡, Giovanna Benvenuto‡, Raffaella Pero‡, Barbara Majello‡, Sabrina Battista‡, Francesca Lembo†, Erminia Vollono†, Patricia M. Day§, Massimo Santoro‡, Luigi Lania§, Carmelo Bruno Bruni‡, Alfredo Fusco‡, and Lorenzo Chiariotti‡**

From the ‡Centro di Endocrinologia ed Oncologia Sperimentale del CNR “G. Salvatore” Dipartimento di Biologia e Patologia Cellulare e Molecolare L. Califano Università degli Studi di Napoli “Federico II” via S. Pansini, 5, 80131 Napoli, Italy, the §Dipartimento di Genetica, Biologia Molecolare e Generale, Università di Napoli “Federico II,” 80134 Napoli, Italy, the ¶Laboratory of Cellular Oncology, NCI, National Institutes of Health, Bethesda, Maryland 20892; and the †Dipartimento di Medicina Sperimentale e Clinica “G. Salvatore,” Università degli Studi di Catanzaro “Magna Graecia,” via Tommaso Campanella 115, 88100 Catanzaro, Italy

We have identified a novel human gene encoding a 59-kDa POZ-AT hook-zinc finger protein (PATZ) that interacts with RNF4, a mediator of androgen receptor activity, and acts as a transcriptional repressor. PATZ cDNA was isolated through a two-hybrid interaction screening using the RING finger protein RNF4 as a bait. In vitro and in vivo interaction between RNF4 and PATZ was demonstrated by protein-protein affinity chromatography and coimmunoprecipitation experiments. Such interaction occurred through a small region of PATZ containing an AT-hook DNA binding domain. Immunofluorescence staining and confocal microscopy showed that PATZ localizes in distinct punctate nuclear regions and colocalizes with RNF4. Functional analysis was performed by cotransfection assays: PATZ acted as a transcriptional repressor, whereas its partner RNF4 behaved as a transcriptional activator. When both proteins were overexpressed a strong repression of the basal transcription was observed, indicating that the association of PATZ with RNF4 switches activation to repression. In addition, RNF4 was also found to associate with HMGI(Y), a chromatin-modeling factor containing AT-hook domains.

Several RING finger proteins play a crucial role in the control of transcription. mel-18, RING1, and KRIP-1 proteins act as transcriptional repressors and/or interact with transcriptional repressors (1–4). The RING finger protein PML interacts with Sp1 and inhibits the Sp1-mediated transcriptional activity (5). Most of the human RING finger proteins are localized in the nucleus or both in the cytoplasm and the nucleus and are often involved in oncogenesis by interfering with the transcriptional machinery (1). Chimeric proteins containing RING finger domains such as RET/Trf (6), TIF1/B-Raf (T18) (7), and PML/RARα (8) are generated by chromosomal translocations occurring in different human neoplasias. Other transcription-related RING finger proteins have oncogenic (c-Cbl and Bmi-1) or tumor suppressor (BRCA-1 and mel-18) activity (1). It is believed that in most cases RING finger proteins participate in the formation of multimeric complexes.

We have recently isolated a human RING finger gene (RNF4) that encodes a protein of 190 amino acids (9). RNF4 is expressed at low levels in several tissues with the exception of a very high expression in the testis. The mouse homolog of RNF4 is abundantly expressed in embryonic tissues from the earliest days after gestation and exhibits a ubiquitous pattern of expression. The human RNF4 gene is located at 4p16.3, a chromosome region associated with several genetic and neoplastic diseases, between the huntingtin (HD) and the fibroblast growth factor receptor 3 (FGFR3) genes. Recently, the rat homolog of RNF4 has been shown to associate with the DNA binding domain of the androgen receptor (10) and to enhance both steroid receptor-dependent and basal transcription, suggesting that RNF4 may act as a bridging factor between nuclear receptors and other transcriptional factors.

To identify molecular partners of RNF4 we have performed a two-hybrid screening. We report here the isolation and the characterization of a novel human gene encoding a POZ-AT hook-zinc finger protein (PATZ)1 that associates with RNF4. PATZ was localized in specific nuclear domains. We also show that PATZ is a transcriptional repressor that acts in a selective manner on different promoters and that RNF4, a transcriptional activator, may act as a corepressor in association with PATZ.

EXPERIMENTAL PROCEDURES

Strains and Media—The genotype of the Saccharomyces cerevisiae reporter strain LA0 is MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ (11). Yeast strains were grown at 30 °C in rich medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose) or in synthetic minimal medium with appropriate supplements.

Yeast Two-hybrid Screen—The pLexA-RNF4 plasmid was constructed by inserting the entire RNF4 coding sequence, amplified with oligonucleotides containing EcoRI and BamHI restriction sites (9), into

1 The abbreviations used are: PATZ, POZ-AT hook-zinc finger protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; ORF, open reading frame; NLS, nuclear localization signal; CAT, chloramphenicol acetyltransferase; bp, base pairs; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride.
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the pBTM116 plasmid (11). The yeast reporter strain L40, which contains the reporter genes LacZ and HIS3 downstream from the binding sequences for LexA, was sequentially transformed with the pLexA-RNF4 plasmid and with a mouse embryo cDNA library (CLONTECH) in vector plasmid pVP16, using the lithium acetate method and subsequence selection (11). Plasmid DNA was prepared from colonies lysine. The plates were incubated at 30 °C for 3 days. His+ colonies were patched on selective plates and assayed for β-galactosidase activity using a filter assay (11). Plasmid DNA was prepared from colonies displaying a His+/LacZ phenotype and used to retransform the L40 strain containing the selected L40-1 hybrids. pVP16 library plasmids were then rescued from His+/LacZ colonies and tested for specificity by cotransformation into L40 with pLexA-RNF4, pLexA-galectin, pLexA-Rab7, and pLexA-ras. The cDNA inserts from specific clones were sequenced using the dye deoxy termination method (12). Nucleotide and protein sequence analysis and comparisons were carried out with the BLAST (13) and PROSITE (14) programs.

Screening of cDNA Library—A human breast carcinoma cDNA library in Agt11 was purchased from CLONTECH. A total of 2 × 106 recombinant clones was screened by an in situ plaque hybridization technique (15). The 489-bp insert of pVP16-A23 was labeled by the random priming procedure (16) and used as a probe. Hybridizations were carried out at 60 °C, and filters were washed under low stringency (0.6× SSC, 0.1% SDS at 40 °C). Positive clones were isolated by limited proteolysis and scale purification procedure (15). The 3038-bp insert of one positive phage was subcloned in plasmid pGem3Z (Promega Biotec, Madison, WI) (plasmid pGEM-PATZ). Sequence analysis was performed using the dye deoxy chain termination procedure (12).

Construction of Fusion Genes and Expression Plasmids—For construction of the glutathione S-transferase (GST) fusion genes, different fragments were amplified by PCR with pairs of primers linked to restriction sites and cloned in pGEX-2T plasmids (Promega); pGST-RNF4 was obtained by cloning the entire RNF4 coding sequence in the pGEX-2T plasmid; pGST-PATZ and pGST-PATZΔIR were obtained by cloning a 1092-bp and a 777-bp fragment of the human PATZ coding sequence coding amino acids 1 to 366 and 1 to 259 of the PATZ protein, respectively. Downstream primers used for PATZ were 5′-ATCGGAATTCGTTCCCTTCCACT-3′ and 5′-ATCGGAATTCATGGAGCGGGTGGAACGACGCT-3′, respectively. Upstream primers used for PATZ were 5′-GGAAGGGCATGGA-3′ and different portions of the untranslated sequence and different portions of the PATZ coding sequence were amplified and inserted in the pDNA3 expression vector: 5′-GAGTACAAGAAAGCGTCGTG-3′ (for PATZ); and 5′-ATCGGAATTCGTTCCCTTCCACT-3′ and 5′-GGAAGGGCATGGA-3′ (for PATZΔIR); and 5′-ATCGGAATTCGTTCCCTTCCACT-3′ and 5′-GGAAGGGCATGGA-3′ (for PATZΔIR). For RNF4 expression plasmid (pRFN4), a 1400-bp SflI fragment was inserted in the pDNA3 expression vector (Invitrogen, San Diego, CA). pHa-tagged RNF4 was made by cloning a PCR-generated full-length RNF4 fragment into the EcoRI site of pCEFL-HA (gift of S. Gutkind, NIDCR, National Institutes of Health, Bethesda, MD) expression vector. Primers used were AA1Eco (5′-ATCGGAATTCATGGAGCGGGTGGAACGACGCT-3′) and GREco (5′-GGCACTATATAATAGGGGTTG-3′); for PATZ (1–366), PBam (5′-ATCGGAATTCATGGAGCGGGTGGAACGACGCT-3′) and GREco (5′-GACTATACCATGAGCATGGAACC-3′); and for PATZIR (1–259), PBam and P2Eco (5′-ATCGGAATTCATGGAGCGGGTGGAACGACGCT-3′).

For RNF4 expression plasmids (pGST-RNF4, pGST-PATZ, or pGST-PATZΔIR), were diluted from 5 to 400 ml in LB with ampicillin (100 μg/ml), grown at 30 °C to A600 = 0.6, and induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. After an additional 2 h at 30 °C, the cultures were harvested and resuspended in 10 ml of cold phosphate-buffered saline (PBS; 140 mM NaCl, 5 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4). Protease inhibitors (Roche Molecular Biochemicals). The cells were broken using the French pressure cell. The lysate was rocked at 4 °C for 20 min with Triton X-100 to 1% and clarified by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatant was then incubated at 4 °C for 1 h with 250 μl of glutathione-Sepharose beads (Amersham Pharmacia Biotech). GST fusion proteins were captured on the beads with 10 μl of PBS, 1 mM PMSF, and protease inhibitors. The recombinant proteins were eluted with a buffer containing PBS, 10 mM reduced glutathione, and 10% (v/v) glycerol. Polyclonal antibodies were raised against purified GST-RNF4 and GST-PATZ in rabbits by using 100 μg of protein at each immunization.

In Vivo Translation and Protein-Protein Binding—The plasmid pGEM-PATZ and the plasmid pHRNF4 (1) containing the entire human PATZ or RNF4 cDNA, respectively, were used in in vitro transcription and translation assays using the TNT SP6 polymerase-coupled reticulocyte lysate system (Promega), by adding 40 μCi of [35S]methionine (Amersham Pharmacia Biotech) in a total volume of 50 μl. The translated products were either analyzed directly by electrophoresis on SDS-12% polyacrylamide gel or subjected to in vitro protein-protein binding. GST-RNF4 or GST-PATZ expression plasmids (pRFN4, and luciferase) were incubated with recombinant 4–5 μg of each of the following: GST, GST-RNF4, GST-PATZ, GST-PATZΔIR, or GST-HMG1(Y). Reactions were carried out in binding buffer (150 mM NaCl, 0.1% Nonidet P-40, 50 mM Hepes (pH 7.5), 1 mM PMSF, and protease inhibitors at 4 °C for 4 h of gentle rocking. The protein-protein complexes formed on the resin were centrifuged at 14,000 rpm for 1 min at 4 °C. The resin was washed five times at 4 °C with 1 ml of cold binding buffer. The PATZ and the RNF4 proteins, which remained attached to the resin-bound GST or GST fusion proteins, were resolved on SDS-12% polyacrylamide gel, the gel was dried, and autoradiography was performed.

Cell Culture, Transient Transfections, Transcription Assays, and Immunoprecipitation—Human C33A and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (GIBCO/BRL, Gaithersburg, MD). Cells were plated at a density of about 250,000 per 60-mm Petri dish 16 h before transfection.

DNA transfections were carried out by calcium phosphate precipitation using Calphos (CLONTECH). Cultures were cotransfected with 5 μg of test plasmids and different amounts of effector plasmids as indicated in the figures. Normalization of transfection efficiencies, a β-galactosidase expression plasmid pSVβ-gal (Promega) (1 μg) was included in the cotransfections as the internal standard for transfection efficiency. CAT assays were performed with different amounts of extracts to ensure linear correlation of the chloramphenicol with each extract. The results are presented as the means of at least three independent transfection experiments. The CAT activity was quantified using the PhosphorImager System (Molecular Dynamics, Sunnyvale, CA). The top panel of the autoradiogram was cropped and the densities were measured by scanning the autoradiogram. The activity was determined using the luminometer Monolight 1010 (Analytical Luminescence Laboratory, San Diego, CA). Lysates (20 μl) were automatically mixed with 100 μl of the luciferase substrate solution (20 mM Tris (pH 8), 4 mM MgSO4, 0.1 mM EDTA, 30 mM dithiothreitol, 0.5 mM ATP, 0.5 mM d-luciferin, and 0.25 mM coenzyme A), and the emitted fluorescence was measured for 15 s. 20 μg of total protein extract was assayed for β-galactosidase activity as described previously (18).

For immunoprecipitation experiments, transfected human 293T cells were lysed with Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl) with proteinase inhibitors on ice for 15 min. Total proteins were incubated with mouse monoclonal anti-HA antibody (CA125; Roche Molecular Biochemicals) at 4 °C for 1 h and further incubated with protein A-Sepharose (Amersham Pharmacia Biotech) at 4 °C for 2 h with continuous mixing. The mixture was centrifuged at 7500 × g for 40 min at 4 °C. The supernatant was added to 40 μl of 50 μg/ml protein A-Sepharose, washed, and the protein A-Sepharose was incubated with 4 μg of a rabbit anti-PATZ antibody followed by horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin antibody (Amersham Pharmacia Biotech) for 1 h for each step at room temperature. The antibody detection reaction was performed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Immunocytofluorescence—For microscopic analyses the cells were...
seeded onto acid-washed no. 1 coverslips in 24-well plates at a density of 10^5 cells/well and cultured overnight. The cells were washed three times with PBS (pH 7.4), fixed by a 10-min incubation at room temperature with 2.0% paraformaldehyde diluted in PBS, and washed three times with PBS-200 mM glycine. They were then incubated with primary antibody diluted in PBS-0.1% polyethylene glycol 20 cetyl ether (Brj 58; Sigma) and incubated at 4 °C. Anti-HA monoclonal antibodies were from Babco (clone HA1.1) and anti-Myc monoclonal antibodies from Sigma (clone 9E10). Polyclonal antibodies raised against RNF4 and PATZ protein were obtained by injection of bacterial recombinant proteins into rabbits, as described above. The working dilutions were 5 μg/ml for monoclonal antibodies and 1:2000 for polyclonal sera. For double immunofluorescent staining, the primary antibodies were incubated in unison. After incubation, the cells were washed thoroughly in PBS-0.1% Brj and inverted onto Fluoromount-G mounting solution (Southern Biotechnology Associates, Birmingham, AL) on a glass slide. Fluorescence was examined with a Bio-Rad MRC 1024 laser scanning confocal system attached to a Zeiss Axioplan microscope. All the images were acquired with a Zeiss 63× N.A. 1.4 Planapo objective. The use of control coverslips established that fluorescence in the green and red channels was not overlapping and that antibody binding was specific for the intended antigen. Images were merged and subjected to scale adjustment using image software (Photoshop; Adobe Systems, Inc., San Diego, CA). For matrix extraction experiments, cells were grown on coverslips as described above. Prior to immunofluorescent staining the cells were subjected to a sequential extraction procedure as described previously (19).

RESULTS

Interaction Screening of a Mouse Embryo cDNA Library with RNF4.—To identify partners of the RNF4 protein, we performed interaction screening using the two-hybrid system in yeast (11). Four groups of interacting clones were identified on the basis of sequence identity. The first group, comprising five clones, was further characterized. The specificity of interaction was assessed as described in the “Experimental Procedures.” These clones contained DNA inserts between 350 and 489 bp, which differed only in length but contained the same cDNAs. Analysis of the sequence (GenBank™ accession number AF119255) revealed the presence of an entirely open reading frame (ORF) of 163 amino acids with no identity with known gene products. Using the PROSITE program we identified a conserved AT-hook DNA binding domain and a zinc finger domain. The region containing the AT-hook domain, but not the entire zinc finger motif, was sufficient for the interaction with RNF4 (Fig. 1B).

Isolation and Characterization of a Human Full-length PATZ cDNA Clone.—The incomplete mouse cDNA clone was used to screen a full-length human breast carcinoma cDNA library. One positive clone (B23) contained an ORF of 1611 nucleotides, encoding a 537-amino acid polypeptide, leaving 660 and 606 nucleotides of 5’ and 3’-untranslated sequences, respectively (GenBank™ accession number AF119256). The amino acid sequence of the putative translation product is shown in Fig. 1A. The protein displayed three main features from the N to C terminus (Fig. 1, A and B): i) a region located at the extreme N terminus (amino acids 1–149) with high homology to a conserved domain called BTB/POZ. An alignment of the N-terminal regions of some POZ-containing proteins is shown in Fig. 1C; ii) a canonical AT-hook DNA binding domain (amino acids 282–272) typical of HMGI proteins and involved in the binding to the minor groove in correspondence of AT-rich regions (20); iii) four zinc finger motifs belonging to the C2H2 type, the last of which, confirmed to be consensus FYYX2C...CX3FX5LX3HX5...H (21), whereas the remaining three contained a substitution in one of the hydrophobic residues thought to stabilize the formation of zinc finger. Interestingly, a large region from amino acids 356 to 444, including three zinc fingers, shows a striking homology (82% identity) with three of the six zinc fingers (amino acids 280–368) of the myc-associated zinc finger protein MAZ (GenBank™ accession number P56270). Further examination of the amino acid sequence revealed two nuclear localization signals (NLSs) (22), one within the AT-hook domain (amino acids 268–271) and the other between the first and the second zinc finger motif (amino acids 344–347). A comparison of the available mouse and human PATZ-predicted amino acid sequences showed that the two proteins were very similar with an overall identity of 95% (data not shown).

In Vitro and in Vivo Association Analysis between PATZ and RNF4.—Physical interaction between RNF4 and PATZ was assessed by GST pull-down experiments. [35S] Methionine-labeled PATZ produced by translation in vitro was allowed to bind to RNF4 fused to GST and immobilized onto a glutathione-Sepharose matrix, after which the bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis. PATZ associated with RNF4 in vitro because more than 20% of the input protein was recovered as complexes with RNF4 (Fig. 2A). The specificity of the interaction was confirmed by the observation that PATZ did not adhere to GST resin devoid of RNF4 (Fig. 2A) and no binding of a control protein, 35S-labeled luciferase, was observed under identical conditions (data not shown). The same experiments were performed using 35S-labeled RNF4 and PATZ fused to GST (Fig. 2B). These experiments gave similar results that further confirmed the physical interaction between PATZ and RNF4. As a further control, we showed that 35S-labeled RNF4 was not able to bind the N-terminal region of PATZ (amino acids 1–259) (Fig. 2B), which lacks the region found to interact with RNF4 in yeast.

To investigate whether RNF4 and PATZ are physically associated in intact cells, 293T cells were transfected with pcDNA-PATZ and HA-tagged RNF4 (pHA-RNF4) expression vectors. Protein complexes associated with RNF4 were first immunoprecipitated with the anti-HA monoclonal antibody, and the bound proteins were subsequently analyzed by immunoblotting with the PATZ-specific antibody. PATZ protein was present in immunoprecipitates from cells transfected with both RNF4 and PATZ expression plasmids but not in those expressing only PATZ in the absence of RNF4, confirming that PATZ and RNF4 were found as complexes in vivo (Fig. 2C).

Subcellular localization of PATZ and RNF4 was determined by immunofluorescence analysis. In single-transfected NIH-3T3 cells, PATZ protein was localized exclusively in cell nuclei and showed a speckled nuclear distribution in the majority of transfected cells (Fig. 3A). In cells transfected with both RNF4 and PATZ, a speckled distribution of PATZ protein was again observed, whereas RNF4 showed a more diffuse nuclear distribution possibly due to overexpression (data not shown). In order to unmask a possible colocalization of PATZ and RNF4 in nuclear domains, we also examined the protein staining after in vitro matrix extraction. By this method, double staining of coexpressing cells showed a distinct colocalization of the PATZ and RNF4 proteins on the nuclear matrix (Fig. 3, B–D).

PATZ Functions as a Transcriptional Repressor.—Several zinc finger DNA-binding proteins with the BTB/POZ domain such as ZF5 (23), PLZF (24), BCL6 (25), and BAZF (26) have the ability to repress gene transcription. The transcriptional functions of PATZ were tested by transient transfection assays into human C33 cells, using the G5-myc- XDN and the G5-SV40 promoter-reporter constructs, bearing five GAL4 DNA binding sites upstream of the c-myc P2 and SV40 minimal promoters, respectively (27). The various expression constructs used in this study are schematically represented in Fig. 4A. The PATZ protein repressed the c-myc and the SV40 promoter activities in a dose-dependent manner (Fig. 4B). Such activity was mainly associated with the BTB/POZ domain, because repression activity on the c-myc promoter was retained by the
GAL4-PATZ fusion devoid of both the zinc finger region and the AT-hook regions (pGAL4-PATZDD, amino acids 1–259), whereas it was abolished by the deletion of the POZ domain (pGAL4-DPOZ) (Fig. 4B).

To determine whether PATZ could repress the expression of natural promoter-reporter constructs, an expression vector containing the full-length PATZ cDNA was cotransfected with a series of chimeric genes. Expression driven by the c-myc, CDC6, and galectin-1 promoters was significantly inhibited in a concentration-dependent manner when PATZ was coexpressed (Fig. 5A). In contrast, expression driven by RSV and TK promoters was unaffected by PATZ. These findings demonstrate that PATZ is able to repress authentic promoter-reporter constructs in a selective manner and that the artificial recruit-

**Fig. 1. Characteristics of the PATZ protein.** A, complete predicted amino acid sequence of the human PATZ protein as deduced from the nucleotide sequence of the full-length cDNA clone B23. The complete cDNA sequence is available under DDBJ/EMBL/GenBank™ accession number AF119256. The initiator ATG was determined as the first ATG codon downstream of an in-frame stop codon. The N-terminal POZ domain is indicated by _underlining_; the AT-hook domain is _boxed_; and the four C2H2 zinc finger motifs are indicated by _heavy underlining_.

B, schematic structure of human PATZ (top) and relative position of overlapping murine PATZ cDNAs representing regions of interaction with RNF4. POZ, BTB/POZ domain; AT, AT-hook domain; Zn, zinc finger motif.

C, comparison of the N-terminal 149 amino acid residues of PATZ with the N-terminal BTB/POZ domains of other zinc finger proteins of human origin indicated on the left. Black columns denote the positions of amino acid residues that are identical in all four proteins; shaded columns represent residues conserved in three of the four proteins. For the other human proteins the sequence GenBank™ accession numbers and positions shown are: O05516, positions 1–115 (PLZF); P41182, positions 1–118 (BCL-6); P24278, positions 1–108 (KUP).
FIG. 2. Characterization of RNF4-PATZ interactions in vitro and in mammalian cells. Interaction between PATZ and RNF4 was assessed by a pull-down assay. A, [35S]methionine-labeled PATZ was left untreated (INPUT) or incubated with either immobilized GST alone or GST-RNF4. B, [35S]methionine-labeled RNF4 was left untreated (INPUT) or incubated with each of the following: GST alone, GST-PATZ (amino acids 1–259), GST-PATZ (amino acids 1–366), or GST-HMGI(Y). C, interaction between PATZ and RNF4 in vivo. pHA-RNF4 was cotransfected with pcDNA3-PATZ in 293T cells. After 48 h in culture, whole-cell extracts were prepared. Lysates of 293T cells transfected with pcDNA3-PATZ (−) or with pcDNA3-PATZ and pH-A-RNF4 (RNF4) were immunoprecipitated with anti-HA antibody. Interaction of PATZ with HA-RNF4 was detected into immunoprecipitates by Western blotting with anti-PATZ antibody. Total lysate of 293T cells cotransfected with pcDNA3-PATZ and pHA-RNF4 (INPUT) was loaded as a positive control. The arrow indicates the PATZ protein. Molecular masses are indicated on the right.

The expression of the PATZ protein on the promoter by fusion to the GAL4 DNA binding domain was not strictly required for transcriptional repression. PATZ, devoid of the putative DNA binding region (pcDNA-PATZ1), was still able to repress transcription from the myc promoter in a dose-dependent manner albeit at a lower level (Fig. 5B). Therefore, we concluded that PATZ repression ability is likely a result of a functional interaction between PATZ and components of the transcriptional apparatus and such interaction does not require sequence-specific DNA binding of PATZ. Deletion of the N-terminal region completely abolished the PATZ repression activity from the natural myc promoter (Fig. 5B), which again shows that such activity is associated with the BTB/POZ domain.

As the PATZ protein associates both in vitro and in vivo with RNF4, it was of interest to examine the possible functional cooperation of both proteins in transcriptional regulation. First, we assayed the effect of RNF4 protein in transcription, and we found that RNF4 enhanced c-myc promoter activity (Fig. 5C) and that such activation was concentration-dependent (data not shown). However, coexpression of PATZ and RNF4 resulted in a strong repression of RNF4-mediated activation. The extent of transcriptional repression achieved when both RNF4 and PATZ proteins were coexpressed was substantially higher than that observed with PATZ alone, suggesting that coexpression of both proteins enhances the PATZ repression ability. On the contrary, when RNF4 was cotransfected with PATZ without the N-terminal region containing the POZ domain (pcDNA-APOZ), the RNF4 activation potential prevailed (Fig. 5C). These data indicate that the presence of the POZ domain is essential for both the repression activity of basal transcription (Fig. 5B) and for repression of RNF4-mediated activation (Fig. 5C). Because the RNF4 interacting domain is conserved in the pcDNA-APOZ construct, we suggest that the simple interaction between RNF4 and PATZ is not sufficient to switch the activation ability of RNF4 to repression if PATZ is devoid of the repression domain.

Collectively, these results indicate that PATZ is a transcriptional repressor that acts in a selective manner on different promoters and that RNF4, which alone functions as a transcriptional activator, may act as a corepressor in association with PATZ.

RNF4 Associates with HMGI(Y)—Because the physical interaction between RNF4 and PATZ occurs through a small region of PATZ containing an AT-hook domain (Fig. 1B), we questioned whether RNF4 could also associate with other proteins containing AT-hook domains. The human HMGI(Y) is a small protein of 107 amino acids and displays three AT-hook domains. To further confirm the interaction between RNF4 and HMGI(Y), an in vitro pull-down assay was performed. Immobilized GST-HMGI(Y) was incubated with the in vitro translated product of pGEM-RNF4 (encoding the full-length RNF4). RNF4 associated with HMGI(Y) in vitro, because more than 15% of the input protein was recovered as complexes with HMGI(Y) (Fig. 2). These findings suggest that RNF4 can form complexes with
different transcriptional regulators containing AT-hook DNA binding domains.

**DISCUSSION**

A human gene encoding a small nuclear RING finger protein, RNF4, has been isolated and characterized in our laboratory (9). Recently, the rat homolog of RNF4, SNURF, has been demonstrated to associate with and to mediate the activity of steroid receptors (10). In this study, we have identified a novel transcriptional regulatory factor gene, PATZ, whose product associates with RNF4 and displays significant repression potential in cotransfection experiments.

PATZ is predicted to encode a 59-kDa protein containing an N-terminal POZ domain, an AT-hook domain in the central region, and four C2H2 zinc finger motifs. Although such motifs are common in factors involved in transcriptional regulation, the presence of all these domains in the same protein appears to be an unique feature of PATZ.

The BTB (for Broad complex, tramtrack, and bric-a-brac) or POZ domain (for poxvirus and zinc finger) (28) mediates homomic and heteromic protein-protein interactions (29), targets the protein to distinct nuclear substructures (30), and is involved in transcriptional repression or chromatin modeling (31–34). Our results indicate that PATZ can function as a transcriptional repressor on selected promoters. Although it remains possible that the transcriptional activity of POZ/ZF proteins may be dependent on the cellular environment and may include the ability to transactivate, all POZ/ZF proteins studied so far have displayed a consistent transrepressive activity in a variety of cell types and on various promoters (34). Similarly to the other ZF/POZ proteins, the repressor activity of PATZ associates with the POZ domain (Fig. 5). The mechanisms by which transrepression by PATZ occurs remain to be elucidated. The POZ domain of BCL6 and PLZF, as well as other POZ domains, may associate with the SMRT-mSin3A-HDAC-1 complex and form a multimeric repressor complex involving histone deacetylation activity (34, 35). PATZ may also be involved in the formation of such a complex. Moreover, it is likely that the four zinc finger motifs could target the PATZ protein to specific DNA sequences.

In addition, PATZ displays an AT-hook motif in the central region. The AT-hook motif is a small AT-rich DNA binding domain that was first described in the high mobility group nonhistone chromosomal protein HMGI(Y) and then identified in a few other proteins such as HMGI-C and ALL-1, which are both frequently found fused to other genes in different human tumors (36–39). Based on the observation that all the PATZ partial clones showing ability to interact with RNF4 in yeasts contained the AT-hook motif (Fig. 1C), we found that RNF4 represses transcriptional activation by the c-myc and SV40 promoters. A, outline of the expression constructs used in the transfections. B, the pG5-myc-XΔN or the pG5-SV40 reporter plasmids (5 μg) were transfected into C33A cells in the presence of the indicated effectors at increasing concentrations (0, 1, 3, and 9 μg). Shown is the level of transcription, given as a percentage of CAT activity relative to the level seen in the absence of expression plasmids (set at 100%). GAL4, GAL4 binding domain; POZ, POZ domain containing region (amino acids 1–259); AT, AT-hook domain containing region (amino acids 260–366); Zn3, region containing the zinc fingers (amino acids 367–537); CAT activity was quantitated as described under “Experimental Procedures.” The results are presented as an average of multiple experiments ± SD. Each histogram bar represents the mean of three independent transfections.
Fig. 5. Effect of PATZ and RNF4 expression. A, effects of PATZ on the expression of various promoter-reporter constructs. 5 μg of the various promoter-reporter plasmid DNAs was transiently cotransfected into C33 cells with increasing amounts (0, 1, 3, and 9 μg) of pcDNA-PATZ expression vector. Promoters tested were c-myc P2 (myc-CAT) (27), RSV (RSV-CAT) (45), mouse galectin-1 (gal-1-CAT) (45), thymidine kinase (TK-CAT), and the human CDC6 (CDC6-Luc) (46). B, effect of different PATZ deletion constructs on the expression of the c-myc P2 promoter. 5 μg of the c-myc promoter was cotransfected with increasing amounts of each indicated expression vector (0, 1, 3, and 9 μg). C, effects of PATZ and RNF4 coexpression. 3 μg of each expression plasmid were cotransfected into C33 cells together with 5 μg of pmyc-XΔN. CAT and luciferase activity were assessed as described under “Experimental Procedures.” Shown is the level of transcription, given as a percentage of CAT or luciferase activity relative to the level seen in the absence of expression plasmids (set at 100%). The histograms represent the mean of three replicative assays. The results are presented as an average of multiple experiments ± SD.
may physically interact with a chromatin architectural factor, HMGB(Y) (20, 40). HMGB(Y) can have positive (41) or negative (42) effects on transcription. Whether HMGB(Y) and PATZ are competitors in the binding to RNF4 and whether their activity is synergistic or antagonist remain to be determined. Our data raise the intriguing possibility that the AT-hook DNA binding motif might also be involved in protein-protein interaction and that RNF4 could also modulate the multiple activities of other AT-hook-containing proteins.

The finding that PATZ localizes in the nucleus is consistent with the ability of PATZ to modulate transcription and with the presence of typical features of nuclear proteins, including two NLSs. RNFI which interacts with PATZ, has been shown to localize in particular nuclear substructures (10). PATZ colocalizes with RNF4 and is distributed in distinct nuclear domains. Consistently, the POZ domain has been shown to target the proteins to nuclear domains that have a potential role in transcriptional regulation (30, 43). Interestingly, an example of proteins that interact and colocalize in nuclear substructures, i.e., the PML oncogenic domains, are the RING finger protein PML and the POZ/ZF protein PLZF (44), both of which are leukemia-associated retinoic acid receptor α fusion partners.

Finally, our data show that RNF4 enhances basal transcription from minimal promoters but also show that the simultaneous overexpression of RNF4 and PATZ leads to a transcriptional repression stronger than that obtained by the overexpression of PATZ alone. Thus, we conclude that RNF4 may act both as an activator and as a corepressor depending on the presence of overexpressed PATZ. Because RNF4 enhances the androgen receptor activity, it is possible that PATZ and HMGB(Y) can also modulate the androgen response either in agonist or in antagonist manner. The precise role of these molecules remains to be defined, but it is likely that they participate in the formation of higher macromolecular complexes and their final activity may depend on the relative abundance of the different components of such complexes.

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A Novel Member of the BTB/POZ Family, PATZ, Associates with the RNF4 RING Finger Protein and Acts as a Transcriptional Repressor

Monica Fedele, Giovanna Benvenuto, Raffaela Pero, Barbara Majello, Sabrina Battista, Francesca Lembo, Erminia Vollono, Patricia M. Day, Massimo Santoro, Luigi Lania, Carmelo Bruno Bruni, Alfredo Fusco and Lorenzo Chiariotti

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