ANALYSIS OF mDRA (MOUSE DOWN-REGULATED IN ADENOMA)
-PP2A B   INTERACTION IN THE YEAST TWO-HYBRID SYSTEM

YONSEI UNIVERSITY

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-PP2A B   INTERACTION IN THE YEAST TWO-HYBRID SYSTEM

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                    is
This thesis   is not to be regarded as confidential

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Major professor

To my parents and my brother

PREFACE

These words have been my best working mate whenever I was in deep despair.

"Your life is up to you.

Life provides the canvas. ; You do the painting.

Take charge of your life, or someone else will. "

ACKNOWLEDGEMENTS

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ABSTRACT

Heesoon Chang. Ph.D., Yonsei University, August 2001. Analysis of mDRA (Mouse Down-Regulated in Adenoma)-PP2A B interaction in the yeast two-hybrid system. Major Professor : Syng-Ill Lee.

The expressed recombinant cDNA of mouse Down-Regulated in Adenoma (mDRA) originally proposed as a candidate tumor suppressor mediates DIDS-sensitive, Na^+-independent, electroneutral Cl^-/HCO_3^- exchange (34). Several potential regulatory sites of nuclear localization signals and numerous putative phosphorylation sites within the COOH-terminus of the deduced amino acid sequence of mDRA prompted us to investigate mDRA-associated regulatory proteins using yeast-based two-hybrid system. Two candidate clones containing mouse cDNA insert of PAX 3 or PP2A B regulatory subunit delta isoform (PP2A B) were verified to interact with the COOH-end of mDRA. Like rat PP2A B (46), mouse PP2A B homolog mRNAs (2.4 kb) were abundantly expressed in testis and colon, whereas PAX 3 transcripts (6.4 kb) were expressed at high levels in heart. Patterns of tissue distribution of mouse PP2A B homolog and PAX 3 were similar to those of mDRA. The functional relevance of PP2A B to mDRA as a negative regulator of tumorigenesis led us to clone the full-length of mouse PP2A B homolog cDNA. The full-length open reading frame as well as 31 nucleotides of 5'-untranslated and 565 nucleotides of 3'-untranslated were obtained using an reverse transcription-polymerase chain reaction (RT-PCR) of mouse colon cDNA. The mouse PP2A B homolog cDNA encodes a protein of 453 amino acids with a calculated Mr of 51,954 (accession number AF366393). An amino acid alignment of mouse PP2A B reveals 96% identity with rat PP2A B .

The heterologous functional expression of this full-length PP2A B   cDNA along with the core subunits of A and C in the mDRA stable transfectants will allow us to study how PP2A holoenzyme binding to mDRA can modulate mDRA function.

Taken all together, the identification and characterization of the associating proteins with mDRA, suggestive of potential regulatory proteins can be the essential study to elucidate the regulatory mechanism of mDRA function.

INTRODUCTION

Mouse DRA (mDRA) was cloned from mouse colon cDNA using an RT-PCR and 5' and 3' RACE methods based on human DRA (hDRA) sequence. Human DRA was originally considered as a candidate tumor suppressor due to a down-regulation in most adenocarcinomas, thus named as **D**own-**R**egulated in **A**denoma (DRA). However, it was reported that there was a slight increase of the incidence of intestinal cancer among people carrying mutations in the gene. Further studies related to DRA function have demonstrated that DRA protein mediates a DIDS-sensitive, Na^+-independent sulfate transport activity and Cl^-/OH^- (HCO_3^-) exchange activity. Recently, human studies of congenital chloride diarrhea (CLD) revealed that the disease is caused by null mutations in the DRA gene, probably due to a lack of Cl^-/HCO_3^- exchange activity in the ileum and colon (3, 18, 19, 20, 23). Congenital chloride diarrhea is a recessively inherited intestinal disorder affecting electrolyte transportation. This congenital chloride diarrhea (CLD) gene DRA was assigned to chromosome 7 (19).

Pattern of tissue distribution of mDRA mRNA was similar to that of hDRA transcript. mDRA transcripts were expressed abundantly in cecum and colon and at lower levels in small intestine (34). The colon is a major site for NaCl absorption which appears to be mediated by Na^+/H^+ and Cl^-/HCO_3^- exchange (28, 32, 39). Recently, it was observed that mRNA expression of mDRA was upregulated in the colon of mice deficient of the NHE3 Na^+/H^+ exchanger (34). Furthermore, like CLD incidence in mice lacking of DRA, diarrhea occurs in mice deficient of NHE3 which is distributed at high levels in the small intestine and colon (41). The expressions of DRA and NHE3 proteins are also co-localized to the apical membrane of epithelial cells in the gastrointestinal

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tracts. These observations indicate that NaCl absorption in the small intestine and colon is normally mediated by the coupled DRA Cl^-/HCO_3^- exchange and NHE3 Na^+/H^+ exchange (34). Since NHE3 Na^+/H^+ exchanger activity has been shown to be tightly regulated (24, 27), DRA that appears to act in concert with NHE3 in the colon is also suggested to be regulated. For many years, it has been demonstrated that short-term and long-term regulations of proteins are initiated by protein-protein binding or DNA-protein interaction (38). The deduced amino acid sequence of mDRA contains several potential regulatory motifs suggestive of transcription factors or of proteins that interact with transcription factors. The COOH- and NH_2-ends of the protein contain numerous charged amino acid residues. Such charged clusters have been noted in functional domains of transcription factors (5). As listed in Table 1, there are also three potential nuclear localization signals and many putative phosphorylation sites within the COOH-terminus of mDRA (42), which is suggested as a potential binding domain with regulatory proteins.

The yeast two-hybrid assay has been intensively developed to identify interacting partners of proteins (21, 48) based on the fact that many eukaryotic trans-acting transcription factors are composed of physically separable, functionally independent domains. Such regulators often contain a DNA-binding domain (DNA-BD) that binds to a specific enhancer-like sequence, which in yeast is referred to as an upstream activation site (UAS) (16). One or more activation domains (AD) direct the RNA polymerase II complex to transcribe the gene downstream of the UAS (29). Both the DNA-BD and the AD are normally required to activate a gene, as in the case of the native yeast GAL4 protein, the two domains are part of the same protein. If physically separated by recombinant DNA technology and expressed in the same host cells, the DNA-BD and AD peptides do not directly interact with each other and thus

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can not activate the responsive genes (30). However, if the DNA-BD and AD can be brought into close physical proximity in the promoter region, their function of transcription activation will be restored. In principle, any AD can be paired with any DNA-BD to activate transcription, with the DNA-BD providing the promoter specificity (6). In the MATCHMAKER GAL4 Two-Hybrid System which we employed in the present study, the DNA-BD and the AD are both derived from the yeast GAL4 protein (amino acid 1-147 and 768-881, respectively). Two different cloning vectors are used to generate fusions of these domains to genes encoding proteins that potentially interact with each other. The recombinant hybrid proteins are coexpressed in yeast and are targeted to the yeast nucleus (8, 44). An interaction between a bait protein (fused to the DNA-BD) and a library encoded protein (fused to the AD) creates a novel transcription activator with binding affinity for a GAL4-responsive UAS (Fig. 1). This factor then activates reporter genes (*lacZ* or *HIS3*) having upstream GAL4-responsive elements in their promoter, which makes the protein-protein interaction phenotypically detectable.

In the present study, we screened specific interacting proteins to identify potential regulatory proteins of mDRA using this yeast two-hybrid system. The screening system included a bait protein of the COOH-terminus of mDRA fused to GAL4-BD in pAS2-1 (Fig. 2) and mouse embryonic library cDNA fused to the GAL4-AD in pVP16 (Fig. 3). Among several real positive clones verified by the colony-lift filter -galactosidase assay, two candidate clones containing mouse PP2A B homolog and mouse PAX 3 cDNA in frame were selected for further investigation due to a functional relevance to regulation of mDRA function. Northern blot analyses showed that mouse PP2A B mRNA is expressed at high levels in testis and colon where mDRA transcript is expressed abundantly, whereas PAX 3 mRNA is highly expressed in heart

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where mDRA transcript was detectable. Since PP2A holoenzymes and DRA have been considered to regulate tumor promotion and development (49), we pursue cloning the full-length of mouse PP2A B homolog cDNA to investigate further how PP2A holoenzyme binding to mDRA modulates mDRA activity, which may lead to regulate tumor promotion and development. The mouse PP2A B homolog cDNA cloned from mouse colon consists of 31 nucleotides of 5'-untranslated and 565 nucleotides of 3'-untranslated as well as the full-length open reading frame (accession number AF366393). The cDNA encodes a protein of 453 amino acids which shows 96% identity with rat PP2A B protein.

The identification of the interacting proteins with mDRA and the cloning of the full-length mouse PP2A B cDNA can be important for the regulatory mechanism study of mDRA.

MATERIALS AND METHODS

Yeast Strain - We used the yeast strain *Saccharomyces cerevisiae* CG1945 included in the MATCHMAKER GAL4 Two-Hybrid System (Clontech). Yeast were grown in YPD medium or the transformed yeast strains were maintained in the appropriate synthetic dropout (SD) medium to keep selective pressure on the plasmid. The phenotype of the yeast CG1945 strain was verified before using them in a transformation based on the nutritional requirements of the strain. Briefly, the strain was streaked on appropriate SD selection plates, such as, SD/Trp⁻, SD/Leu⁻, SD/His⁻, SD/Ura⁻, YPD containing 1.0 /ml cycloheximide, YPD plates, and incubated at 30 for 4-6 days to allow the phenotype to appear. The strain should not grow on SD/Trp⁻, SD/Leu⁻, or SD/His⁻ plates, but should grow on SD/Ura⁻, YPD containing 1.0 /ml cycloheximide, or YPD plates. CG1945 strain is slightly leaky for HIS expression due to the presence of a minimal HIS3 promoter upstream of the gene. Therefore, we determined the optimum concentration of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of the yeast HIS3 protein (His3p) to suppress background growth on SD medium lacking His during a two-hybrid screening. The lowest concentration of 1 mM 3-AT in the medium was sufficient to suppress background growth of the untransformed CG1945 strain and the transformants.

Plasmids and Constructions - The bait plasmid DNA, the GAL4 DNA-binding domain (GAL4-BD)/mDRA in pAS2-1 (Clontech) with the selectable marker *TRP1* was constructed and named as pAS2-1/mDRA. Briefly, a pair of mDRA specific primers spanning the COOH-end cytosolic domain (amino acid 469-758) predicted as a potential binding domain were synthesized. These primers were designed to facilitate subcloning of

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GAL4-BD/mDRA fusion construct in pAS2-1 vector ; the sense primer, 5'-*ggaattccatatg*(1486)ACCCAATTTCCAAAGTG(1502)-3' and the nonsense primer, 5'-*acgcgtcgac*(2268)TTTCGTTTCAACTGGTA(2252)-3', the lowercase italic letters indicate the addition of *Nde* I and *Sal* I endonuclease sites to 5'- and 3'-ends of the PCR products, respectively. As shown in Fig. 2, the amplified PCR product of mDRA cDNA was inserted into the linearized pAS2-1 prepared by *Nde* I and *Sal* I endonuclease double digestion. The selected bait fusion construct was analyzed by restriction enzyme digestion and sequencing.

The mouse embryonic cDNAs (9.5-10.5 days) fused with the Gal4-activation domain (GAL4-AD) was subcloned into the *Not* I site of the linearized pVP16 vector to generate the two-hybrid screening cDNA library (Fig. 3), which was kindly provided by Dr. Stanley M. Hollenberg (Oregon Health Sciences University). *LEU2* is the selectable marker in pVP16 vector.

Yeast two-hybrid library screen - Protein-protein interactions in the yeast two-hybrid screening were monitored with the MATCHMAKER GAL4 Two-Hybrid system according to the company protocol.

The CG1945 cells were transformed to carry the GAL4-BD/mDRA bait plasmid using lithium acetate procedure (11).

Mouse embryonic cDNA (0.5 mg) prepared from the library was introduced into the transformants carrying the GAL4-BD/mDRA bait plasmid with the following large scale transformation procedure. The freshly grown transformants carrying the GAL4-BD/mDRA bait plasmid were transferred to 1 L of the fresh YPD for culturing until OD_{600} become 0.5. The cells were harvested, washed in 500 ml of sterile TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), and resuspended in 8 ml of 1 x TE/LiAc (TE with 100 mM lithium acetate). This 8 ml of competent cell resuspension was added to the tube containing 0.5 mg of the library cDNA and 20 mg of the denatured salmon

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sperm carrier DNA, mixed well by vortexing, and combined to 60 ml of fresh PEG/LiAc solution (50% PEG in TE/LiAc solution). Immediately after mixed at high speed vortexing, the transformation mixtures were incubated at 30 for 30 min with shaking (200 rpm). 7 ml of DMSO was added to the incubated mixtures which were then heat-shocked at 42 for 15 min with occasional swirlings. The mixtures were immediately chilled on ice for 2 min, centrifuged, and then resuspended in 10 ml of 1 x TE. These resuspended cells were then plated on SD lacking tryptophan, leucine, and histidine but containing 1 mM 3-AT, and incubated for 8 days at 30 . The rapidly growing pink colonies were isolated as positive colonies for further analyses. After the positive colonies were verified for real protein-protein interactions using the colony-lift filter -galactosidase assay as described later of this section, the yeast plasmid DNA from each positive clone was prepared. Briefly, the candidate clones were re-streaked on SD/Leu⁻ containing 1 /ml cycloheximide to eliminate the bait GAL4-BD/mDRA fusion DNAs. The GAL4-BD/mDRA fusion constructs in pAS2-1 carry the *CYH^{s}2* gene for cycloheximide sensitivity (Fig. 2), so the selected colonies grown on SD/Leu⁻ containing 1 /ml cycloheximide carried only library DNAs. The yeast DNAs from these colonies were then isolated as follows. 0.5 ml of fresh liquid culture was harvested, resuspended in 50 of the residual liquid and treated with 10 of lyticase which will weaken the tough cell wall for 60 min at 37 with shaking at 250 rpm followed by addition of 10 of 20% SDS with vigorous vortexing for 1 min. The mixture was put through one freeze/thaw cycle (at -20) and lysed completely by vortex. The volume of the lysate was brought to 200 with TE buffer (pH 7.0), and extracted with 200 of phenol:chloroform:isoamylalcohol (25:24:1). The plasmid DNA in the aqueous phase separated by centrifugation was precipitated by addition of 8 of 10 M ammonium acetate and 500 of 100% ethanol followed by placing at -70

for 1 hr and centrifugation at 14,000 rpm for 10 min. This crude yeast plasmid DNA was then transformed into the XL-1 Blue MRF' competent cells for high yield of the purified DNA. These isolated DNAs of the positive clones were analyzed by *Not* I restriction enzyme digestion that will separate the mouse embryonic cDNA insert from pVP16 vector. The DNAs were further analyzed by sequencing.

Colony-lift filter -galactosidase assay - For best results, fresh colonies (1-3 mm in diameter) grown in SD lacking tryptophan, leucine and histidine but containing 1 mM 3-AT plates were used for the assay. A sterile Whatman filter was presoaked in a plate containing Z buffer/X-gal (5-bromo-4-chloro-3-indolyl- -D-galactopyranoside) solution (0.1 M Na_2HPO_4, 45 mM NaH_2PO_4, 10 mM KCl, 2 mM $MgSO_4$, 0.3% -mercaptoethanol, and 3.3 mg/ml X-gal). A clean dry filter was placed over the surface of the plate to lift the colonies, poked to orient to the plate, and frozen in liquid nitrogen for 10 sec. The filter was allowed to thaw at room temperature (RT), placed (colony side up) on the presoaked Whatman filter, and incubated at 30   until blue colonies appeared on it. All results were reproducible in at least three independent assays.

Western blot analysis - The protein extracts from the transformed CG1945 strain carrying the bait plasmid were prepared using a freshly grown culture (OD_{600}=0.4-0.6). The pellets of the yeast cells were resuspended in the prewarmed cracking buffer (80 mM Urea, 0.05% SDS, 0.4 mM Tris-HCl, 1 μM EDTA, 40   /ml Bromophenol blue, 10   /ml -Mercaptoethanol, 70   /ml Protease inhibitor solution, 50   /ml of 100 x PMSF, 60), and transferred to a tube containing glass beads (425-600   ; Sigma #G-8772) to disrupt the yeast cell walls. The cell resuspensions with glass beads were lysed by heating at 70   for 10 min followed by vigorous vortexing for 1 min. The lysates as protein extracts were separated from unbroken cells by

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centrifugation at 14,000 rpm for 5 min. For a western blot analysis, protein extracts were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad). The blot was incubated in a blocking solution containing 4% bovine serum albumin (BSA) and 10% normal goat serum (NGS) in phosphate buffered saline plus 0.15% Tween-20 (PBS-T) at RT for 2 hrs. The blot was then probed with the primary antibody (monoclonal antibody of GAL4 DNA-BD, Clontech) for overnight at 4 , followed by detection with a horseradish peroxidase-linked goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc) and enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

GST/mDRA fusion constructs and purification of GST fusion proteins - The sense mDRA primer was synthesized to make GST/mDRA fusion construct in pGEX-6P-1 vector (Amersham Pharmacia Biotech), named as pGEX-6P-1-mDRA. The same nonsense primer synthesized for cloning of the bait plasmid construct described above was reused to generate GST/mDRA construct. The sequence of the sense primer is 5'-*cgggatcc*(1486)ACCCAATTTCCAAAGTG(1502)-3' and the lower case italic letters represent *BamH* I restriction site added to the mDRA sequence to facilitate subcloning. The clones of GST/mDRA cDNA in PGEX-6P-1 were analyzed by *BamH* I and *Sal* I double digestion followed by sequencing. The expression of GST (29 kD) or GST fusion proteins (GST/mDRA, 58 kD) in pGEX-6P-1 is under the control of the *tac* promoter which can be induced by the lactose analog, isopropyl -D-thiogalactoside (IPTG). Thus the GST or GST/mDRA fusion proteins were overexpressed by addition of 0.3 mM IPTG and purified by the Glutathione-Separose 4B as described elsewhere.

Co-sedimentation of mDRA proteins bound to the in vitro transcription/translation products from the positive clones - The proteins from

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the positive clones were produced using the TᴺT Coupled Reticulocyte Lysate Systems (Promega) according to the manufacturer's instruction. The template DNA for the TᴺT reaction was prepared by amplification of GAL4-AD/mouse embryonic cDNA insert in the positive clones using the following pair of primers. The sequences of the sense primer containing the T3 polymerase sequences and the nonsense primer are as follows ; the sense primer, 5'-TGTGCAATTAACCCTCACTAAAGGGCCGCCATGGCACCCAAGAAGAAG-3', the nonsense primer, 5'-CGAGCTAGCTTCTATCTATCTAGC-3'. The isolated PCR products (1) were added to the TᴺT reaction containing [^{35}S] methionine (1,000 Ci/mmol at 10 mCi/ml), TᴺT rabbit reticulocyte lysate, TᴺT reaction buffer, TᴺT T3 RNA polymerase, amino acid mixture minus methionine, and RNasin ribonuclease inhibitor. For the positive or the negative TᴺT reaction control, Luciferase control DNA or H$_2$O was used in the TᴺT reaction, respectively. The quality of the produced proteins in the TᴺT reactions were analyzed by SDS/PAGE followed by autoradiography. For co-precipitation, one third of the TᴺT reaction mixture was incubated with 25 of the purified GST or GST/mDRA fusion proteins bound resin in 0.5 ml of PBS plus 0.1% Triton X-100 at 4 for 2 hrs. The bound complex resin was washed four times with 0.5 ml of PBS containing 0.1% Triton X-100, analyzed by SDS-PAGE (4 to 20% Tris-glycine gradient gels), stained by Coomassie Blue, and then exposed to a film for autoradiography.

Northern blot analysis - Mouse multiple tissue northern blot containing approximately 2 of poly (A)$^+$ RNA per lane (Clontech) was probed sequentially with ^{32}P-labeled cDNA encoding mouse PP2A B homolog (500-bp of *Not* I fragment from # 3-8 positive clone), mouse PAX 3 (500-bp of *Not* I fragment from # 1-9 positive clone), and mDRA (396-bp of *EcoR* I fragment spanning codons 559-691). A cDNA for mouse ribosomal messenger RNA L32 (nucleotide 3078-3244, accession number K02060) was used to

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normalize the RNA amount loaded on each lane of the blot. The northern blot was prehybridized at 68 in ExpressHyb Hybridization solution (Clontech) for 30 min, and hybridized in ExpressHyb Hybridization solution containing the denatured ^{32}P-labeled DNA probe (1-2 x 10^6 cpm/ml) at 68 for 1 hr. The blot was washed in the following stringent way ; two times in 2 x SSC containing 0.1% SDS at RT briefly, two times in 2 x SSC containing 0.05% SDS at RT for 15 min, and one time in 0.1 x SSC containing 0.1% SDS at 55 for 10 min. The blot was then exposed to a film at -86 for autoradiography. The blot was boiled in 0.5% SDS solution for 15 min and cooled for 10 min to strip the probe.

Cloning the full-length cDNA of mouse PP2A B homolog - Total RNA was prepared from mouse colon and testis using TRIzol reagent (Life Technologies, Inc.) according to the company instruction. The isolated tissue in 1 ml of TRIzol reagent per 50 mg of tissue was homogenized two times using the Polytron homogenizer (Brinkmann) at setting 5 for 10 sec per 10 ml of homogenate. The homogenized tissue was incubated at RT for 5 min, mixed with 0.2 ml of chloroform per 1 ml of TRIzol reagent, shaked vigorously by hand, and incubated at RT for 3 min. The sample was centrifuged at 12,000 g for 15 min to separate the aqueous RNA phase (top phase) from the bottom organic phase. The aqueous RNA phase was transferred to a new tube, mixed with the same volume of isopropyl alcohol, incubated at RT for 10 min, and centrifuged at 12,000 g for 10 min to precipitate the RNA. The RNA pellet was washed in 75% ethanol, dried briefly, and dissolved in RNase-free H_2O. One microgram of the total RNA was reverse-transcribed to the 1st strand cDNA using the 1st strand cDNA synthesis kit containing oligo(dT) and random hexamer primers (Clontech). Mouse colon and testis cDNAs were then used in subsequent RT-PCR reactions. Based on the mouse sequence obtained from the positive clone containing the partial PP2A B cDNA and the rat

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sequence of the full-length PP2A B   cDNA (accession number AF180350), two pairs of primers were designed to clone the 5'- and 3'-ends of mouse PP2A B   homolog as shown in Fig. 4. The 5'-end region of mouse PP2A B   was cloned by amplification of mouse colon cDNA with the following oligonucleotides ; the sense primer from rat PP2A B   cDNA, 5'-GGCGGCGCGGTGGCGGCAGCG-3' (5'U), the nonsense primer from mouse PP2A   B   homolog   of   the   positive   clone, 5'-TTGCGGTAACCACCTGATTTTAT-3' (L1). The 3'-end region was cloned by amplification of mouse colon cDNA with the following primers ; the sense primer from mouse PP2A B   homolog   of   the   positive   clone, 5'-AAGCCTGCTAACATGGAGGAG-3' (U2), the nonsense primer from rat PP2A B   cDNA, 5'-GCACGAGAAACACTTCACAGT-3' (3'L). The RT-PCR products were subcloned into pCR2.1-TOPO vector (Invitrogen) for further sequencing   analysis   using   the   M13   reverse   primer (5'-CAGGAAACAGCTATGAC-3') and T7 promoter sequence primer (5'-CCCTATAGTGAGTCGTATTA-3'). The full-length mouse PP2A B   cDNA (accession number AF366393) was analyzed by the MacVector software (Kodak International Biotechnologies, Inc.) and the NCBI BLAST searches of nucleotide-nucleotide   or   protein-protein   alignments (http://www.ncbi.nlm.nih.gov/BLAST).

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RESULTS

Identification of the interacting proteins with mDRA using the yeast two-hybrid system - To search for binding proteins to mDRA, we employed the yeast two-hybrid screening of the mouse embryonic cDNA library using the bait plasmid GAL4-BD/mDRA in pAS2-1 (Fig. 2). The screening library carries the fusion constructs of GAL4-AD/mouse embryonic cDNAs in pVP16 (Fig. 3) and has been successfully used to identify potential regulatory proteins (37). As listed in Table 1, the COOH-terminus of mDRA containing the numerous regulatory sites was selected as a putative binding region and subcloned to make a bait GAL4-BD/mDRA fusion construct, named as pAS2-1/mDRA (Fig. 2).

As the first step of the two-hybrid screening, the bait plasmid DNA of GAL4-BD/mDRA fusion construct was transformed into the phenotype-verified CG1945 as described in the Materials and Methods. The fusion expression of GAL4-BD/mDRA bait plasmid was examined using a western blot analysis (See the Materials and Methods). As shown in Fig. 5, there was a single band detected in the lane of the lysates from the transformants carrying GAL4-BD/mDRA fusion construct and the size of the protein was 45.3 kD as expected (lane pAS2-1/mDRA). On the other hand, there was no band in the control lane of the lysates from the untransformed CG1945.

GAL4-AD/mouse embryonic cDNAs library in pVP16 was then screened for proteins that interact with the GAL4-BD/mDRA bait protein. About 0.5×10^6 transformants were plated on His⁻ plates, and rapidly growing 40 colonies as depicted with arrows in Fig. 6 were emerged as the positives library clones which may express binding proteins to mDRA. The interactions of the proteins expressed in these emerging positive clones with the GAL4-BD/mDRA bait

protein were verified by the colony-lift filter β-galactosidase assay. Particularly in CG1945 strain, the transcription of *lac*Z gene encoding β-galactosidase is activated in only real positive clones and β-galactosidase expressed in these real positives can catalyze X-gal to a indole product which will give blue color on the colonies. As shown in Fig. 7, blue color appeared on the colonies of the positive clones. In contrast, there was no color change on the control colonies.

The yeast DNAs carrying only pAS2-1/mDRA bait construct were isolated from the verified real positive clones for further analyses. To eliminate the bait GAL4-BD/mDRA fusion DNAs carrying the *CYH^{s}2* gene for cycloheximide sensitivity from the positive clones, the candidate positive clones were re-streaked on SD/Leu⁻ containing 1 μ/ml cycloheximide. The selected colonies grown on SD/Leu⁻ containing 1 μ/ml cycloheximide carried only library DNAs which were then isolated as described in the Materials and Methods. The isolated yeast DNAs of the candidate clones were digested by *Not* I restriction enzyme that will separate the mouse embryonic cDNA insert from pVP16 vector, and then analyzed by TBE/agarose gel electrophoresis. As shown in Fig. 8, each positive library clone contains similar size of about 500-bp insert which were then analyzed by sequencing. Several potential regulatory proteins were revealed from the NCBI BLAST searches of nucleotide-nucleotide or protein-protein alignments with the sequences of the cDNA inserts in the clones. The name and homology of the aligned genes with the cDNAs of the positives are as follows ; #1-9 clone, 98% identity with Mus musculus paired box gene 3 (PAX 3, accession number NM008781) ; #3-8 clone, 97% identity with Rattus norvegicus protein phosphatase 2A B regulatory subunit delta isoform (PP2A Bδ, accession number AF180350) ; #1-6 clone, 90% homology with Homo sapiens hypothetical protein (accession number AK023258) ; #1-12 clone, 89% identity with Human DNA sequence

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contained the last coding exon of the gene for p18 component of aminoacyl-tRNA synthetase complex, part of an unknown gene downstream of a putative CpG island and an STS with a CA repeat (accession number AL023694).

Our two-hybrid screening of the mouse embryonic cDNA library with the mDRA bait plasmid revealed several candidate clones expressing potential regulatory proteins of mDRA function *via* specific interaction. For further investigation, the #1-9 (PAX 3) and #3-8 (PP2A B) clones were selected because the functional roles of PP2A B and PAX 3 appear to be relevant to mDRA function and the regulatory roles of PP2A B and PAX 3 on mDRA function can be determined based on the approaches described elsewhere.

Verification of mDRA binding to the proteins produced from the candidate clones using co-precipitation - The specific strong interactions between mDRA and the candidate clones (#1-9 and #3-8) were verified using co-precipitation assay of the purified GST/mDRA proteins and *in vitro* transcription/translation products of the positive clones as depicted in Fig. 9.

First, the templates for *in vitro* transcription and translation reaction were prepared by amplification of GAL4-AD/mouse embryonic cDNA inserts of the candidate clones with the sense primer containing T3 RNA polymerase sequence and the nonsense primer containing the stop codons using *Pfu* polymerase. The PCR products of about 900-bp DNAs were separated by TBE/agarose gel electrophoresis as shown in Fig. 10.

Second, the isolated template DNAs of PCR products along with the positive and negative controls were processed by *in vitro* transcription and translation reaction (T$_N$T Coupled Reticulocyte Lysate Systems, Promega) as described in the Materials and Methods. The reaction mixtures were then resolved by the SDS/4-20% Tris-glycine gradient PAGE and visualized by autoradiography (Fig. 11). There were two major intense bands detected in the

lane of the reaction mixture from the PAX 3 template (#1-9) and the lower intense band was the expected size of 35 kD (Fig. 11, lane PAX 3). On the other hand, there was mainly one band produced in the reaction from the template of PP2A B (#3-8) as shown in Fig. 11, lane PP2A B . The positive control reaction from the Luciferase DNA template produced the major intense proteins of 61 kD as expected, whereas the negative control reaction in the absence of template DNA did not produce any proteins at all.

Third, we generated the GST/mDRA fusion construct by inserting the same COOH-terminus of mDRA cDNA into the pGEX-6P-1 vector as described in the Materials and Methods. Since the expression of GST fusion proteins (GST/mDRA) in *E. coli* is under the control of the *tac* promoter which can be induced by the lactose analog, isopropyl -D-thiogalactoside (IPTG), the GST/mDRA fusion proteins can be overexpressed by IPTG addition. This high yield of GST/mDRA fusion proteins can be easily purified by the Glutathione-Separose 4B. As shown in Fig. 12, the expression of either GST proteins (29 kD) or GST/mDRA fusion proteins (58 kD) were greatly induced by IPTG addition for 4 hrs (lanes, Unpurified GST/t=4 and Unpurified GST/mDRA/t=4) and these overexpressed proteins were purified to one homogeneous bands using the Glutathione-Separose 4B (lanes, Purified GST/t=4 and Purified GST/mDRA/t=4).

Fourth, the binding mixtures containing either the purified GST or the purified GST/mDRA fusion proteins and the i*n vitro* transcription and translation products from PP2A B or PAX 3 templates were co-sedimented and resolved by SDS-PAGE (4-20% Tris-glycine gradient gels). As shown in Fig. 13, the left panel, one major band of either 29 kD or 58 kD proteins appeared on each lane of the gel stained by Coomassie Blue, verifying that the purified GST or GST/mDRA proteins were included in the binding mixtures. However, there was no band detectable in any lanes of the gel from

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autoradiography, even though the right sizes of the major products generated by *in vitro* transcription/translation reactions of the positive clones were included in the binding mixtures (Fig. 13, the right panel).

This interesting result will be more discussed (see Discussion).

Tissue distribution of the mouse PP2A B   homolog, mouse PAX 3, and mDRA mRNA expression - We investigated the pattern of the mouse PP2A B   homolog, the mouse PAX 3, and mDRA transcripts expressions using the Mouse multiple tissue northern blot (Clontech). Our northern blot analysis showed that the mRNA expression of the mouse PP2A B   homolog was detected only in the lane of testis (Fig. 14, panel A), and the mRNA size of the PP2A B   homolog is 2.4 kb. On the other hand, mouse PAX 3 mRNA expression (Fig. 14, panel C) was widespread and particularly abundant in heart and the transcript size of the PAX 3 is 6.4 kb. The mDRA transcripts were co-expressed with either the mouse PP2A B   homolog mRNA or PAX 3 mRNA. The apparent size of mDRA mRNA in the testis is 3.2 kb as reported before (34), but the much bigger size of mDRA transcript (about 6.4 kb) was also detected in heart and testis (Fig. 14, panel B). The cDNA for mouse ribosomal messenger RNA L32 was used to normalize mRNA amount in each lane as shown in Fig. 14, panel D.

To determine co-expression of mouse PP2A B   homolog and mDRA mRNA in mouse colon where mDRA transcripts express in high levels as reported before (34), the cDNAs prepared from mouse testis and colon were amplified by the specific pair of mouse PP2A B   homolog primers (U2 and 3'L in Fig. 4) using an RT-PCR protocol. As shown in Fig. 15, the similar amount of PCR products were detected by EtBr staining of TBE/agarose gel indicating expression of mouse PP2A B   homolog in both mouse tissues.

Our northern blot analyses and RT-PCR result showed co-expression of either mouse PP2A B   homolog or mouse PAX 3 mRNA with mDRA

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transcripts, suggestive of their regulatory roles in mDRA function *via* interaction.

Cloning the full-length cDNA of mouse PP2A B homolog - To investigate how mouse PP2A B homolog binding to mDRA modulates mDRA activity, the full-length of mouse PP2A B homolog cDNA was obtained from mouse colon using an RT-PCR protocols as depicted in Fig. 4. The 5'-end cDNA of the PP2A B was amplified with the sense primer (5'U) designed from the sequences of rat PP2A B and the nonsense primer (L1) from the partial sequence of mouse PP2A B using mouse colon cDNA (Fig. 16, lane PP2A B 5'-end). The 3'-end cDNA was amplified with the sense primer (U2) from mouse PP2A B and the nonsense primer (3'L) designed from rat PP2A B (Fig. 16, lane PP2A B 3'-end). The isolated RT-PCR products of the 5'-end (430-bp) and 3'-end (1255-bp) regions were subcloned into pCR2.1-TOPO vector (Invitrogen). Three individual clones carrying the 5'-end or 3'-end region of the PP2A B in pCR2.1-TOPO vector were completely sequenced to determine the full-length cDNA of mouse PP2A B. The nucleotide and deduced amino acid sequences of mouse PP2A B are shown in Fig. 17. The full-length mouse PP2A B homolog cDNA encodes a protein of 453 amino acids with a calculated Mr of 51,954 (accession number AF366393) as well as 31 nucleotides of 5'-untranslated and 565 nucleotides of 3'-untranslated region obtained from mouse colon cDNA. The translation initiation site matches that of rat PP2A B. The 5'- and 3'-untranslated sequences of mouse PP2A B were 100% and 92% identical, respectively, to the corresponding untranslated sequences of rat PP2A B, and few gaps were required to align the sequences (data not shown). The 453 amino acid sequence of mouse PP2A B is 96% identical to rat PP2A B as aligned in Fig. 18.

The cloning of the full-length mouse PP2A B cDNA allows us to study

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further how mDRA function can be modulated by PP2A binding to mDRA.

T aken all together, identification and characterization of the interacting proteins with mDRA will be critical to elucidate the regulatory mechanism of mDRA function.

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Table 1. Analysis of potential regulatory sites in the deduced amino acid sequence of mDRA with the MacVector software of Protein subsequence (A) and Short motif (B) method.

(A) Protein subsequence method

Potential regulatory sites	Numbers of site
cAMP-dependent kinase	4
Ca^{2+}/calmodulin-dependent kinase	4
Casein kinase	1
Glycogen synthase kinase-3	14
N-glycosylation site	6
Phosphorylase kinase	1
Protein kinase C	1
Signal peptidase cleavage site	4

(B) Short motif method

Potential regulatory sites	Numbers of site
cAMP or cGMP-dependent PK phosphorylation site	7
Casein kinase site	8
Paik & Sangduck in protein methylation	9
N-myristoylation site, must be N-terminal	6
N-glycosylation sites	6
Histone kinase consensus phosphorylation sequence	9
Protein kinase C phosphorylation site	8
3' end of zinc finger motif	1
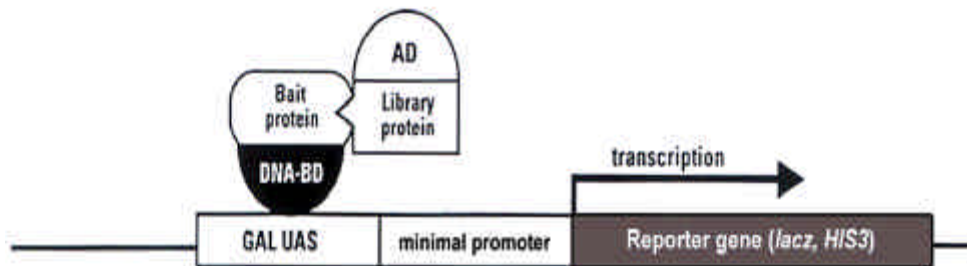
Fig. 1. Schematic diagram of the MATCHMAKER GAL4-based yeast two-hybrid system. The DNA binding domain (DNA-BD, amino acid 1-147 of the yeast GAL4 protein, GAL4-BD) binds to the GAL1 upstream activation site (UAS) in the promoter of the reporter gene, whereas the activation domain (AD, amino acid 768-881 of the GAL4 protein, GAL4-AD) has a function of transcriptional activation. The *lacZ* and *HIS3* reporter genes are separate constructs integrated in the yeast genome. GAL4-BD in the bait fusion construct is physically separated from GAL4-AD in the cDNA library fusion construct using recombinant DNA technology. When the bait protein in GAL4-BD fusion construct directly interacts with the library protein in GAL4-AD fusion construct, the GAL4-BD and GAL4-AD can be brought into close physical proximity in the promoter region (GAL UAS) of the *lacZ* and *HIS3* reporter genes, which will be then transcriptionally activated.
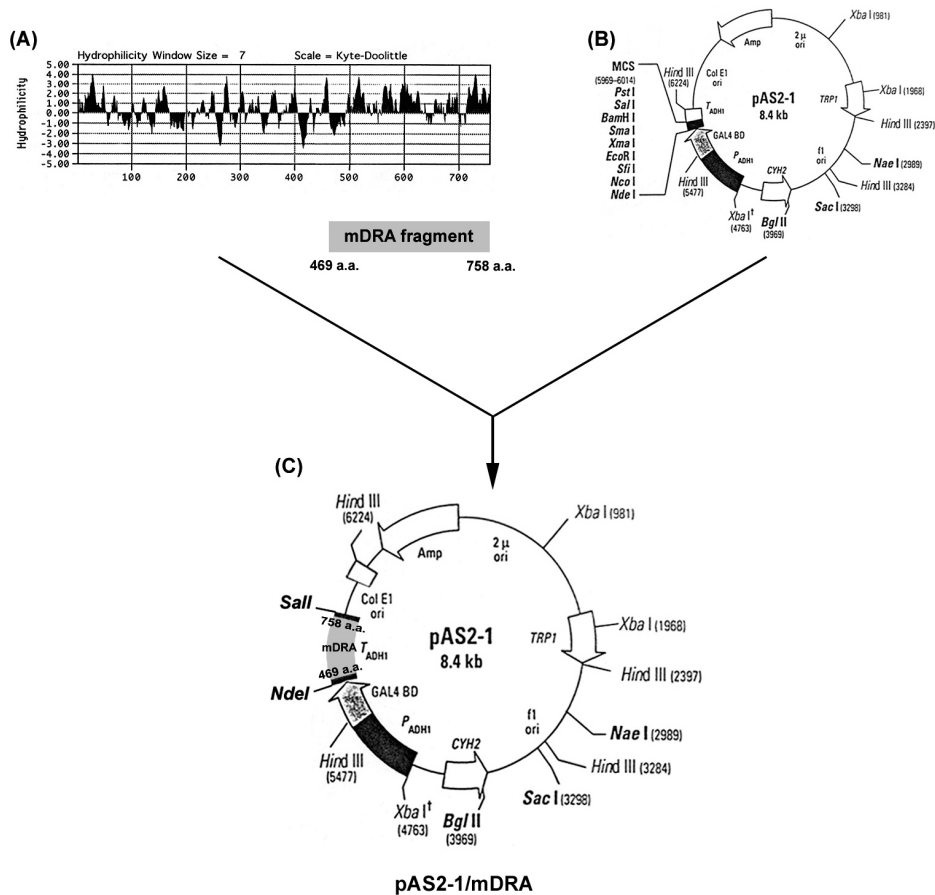
Fig. 2. Subcloning of the bait plasmid, GAL4-BD/mDRA fusion construct, pAS2-1/mDRA. (A) The hydrophilicity of the deduced amino acid sequence of mDRA cDNA and the COOH-terminus cytosolic domain (amino acid 469-758, the putative binding region) marked as the inserted fragment into pAS2-1. (B) pAS2-1 map ; pAS2-1 containing *TRP1* as a selectable marker is a cloning vector to generate a bait fusion protein. (C) Fusion construct of GAL4-BD/mDRA ; the amplified COOH-terminus of mDRA was inserted into the linearized pAS2-1 at the *Nde I* and *Sal I* sites to generate pAS2-1/mDRA.

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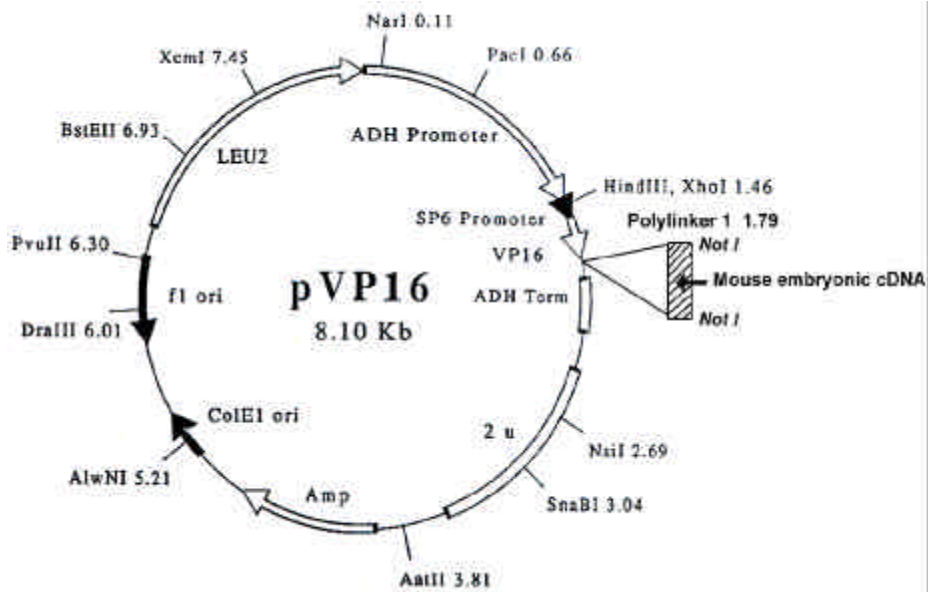
Fig. 3. The map of pVP16 library vector carrying GAL4-AD/mouse embryonic cDNAs. pVP16 is a cloning vector used to generate a library of GAL4-AD/mouse embryonic cDNA fusion constructs. *LEU2* in pVP16 is the selectable marker.

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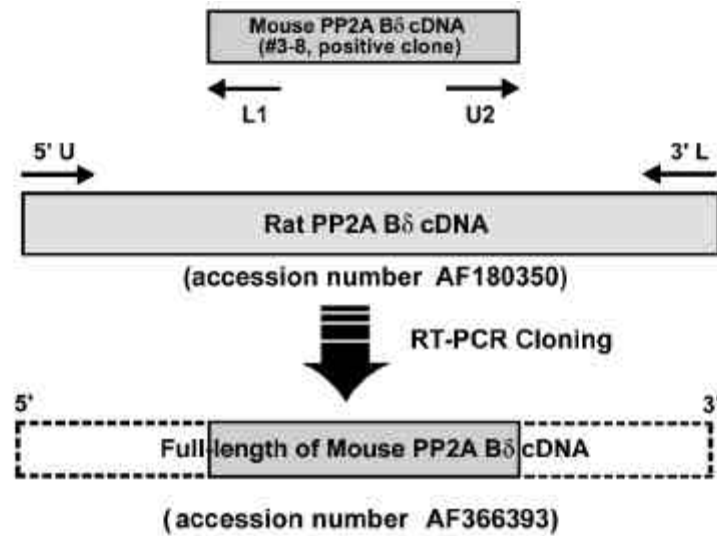
Fig. 4. Strategy for cloning the full-length of mouse PP2A B   homolog cDNA. Two pairs of primers (5'U, L1, U2, and 3'L) as indicated in the diagram were designed to clone the full-length of mouse PP2A B   cDNA based on the mouse sequence of partial PP2A B cDNA from #3-8 positive clone and the rat sequence of full-length PP2A B   cDNA (accession number AF180350). The amplified 5'- and 3'-end regions added to the partial PP2A B cDNA were depicted in the dotted line.
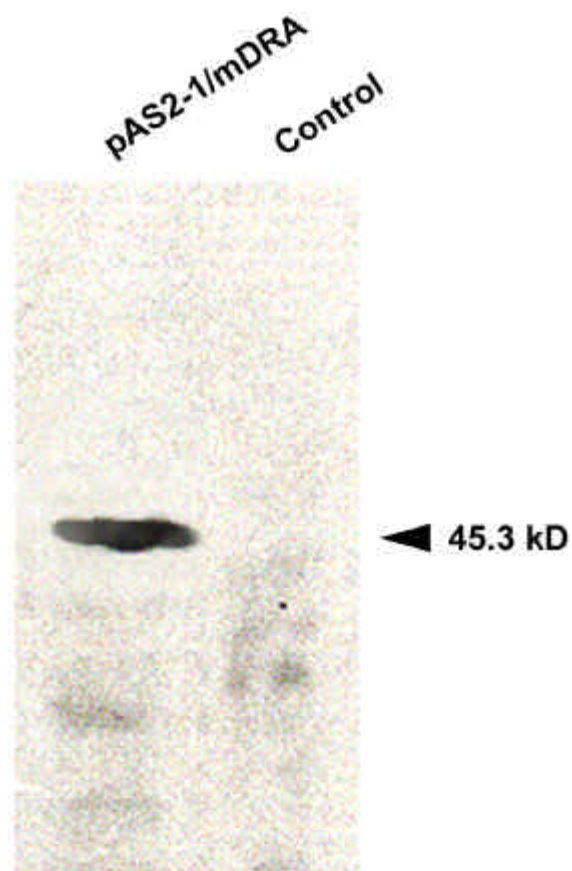
Fig. 5. The western blot analysis of the GAL4-BD/mDRA fusion protein expression in the yeast CG1945 strain. The lysates from the transformants carrying the GAL4-BD/mDRA bait fusion construct (lane pAS2-1/mDRA) or from the untransformed CG1945 (lane Control) were resolved by SDS/PAGE followed by a immuno blot analysis using GAL4-BD monoclonal antibody (Clontech).
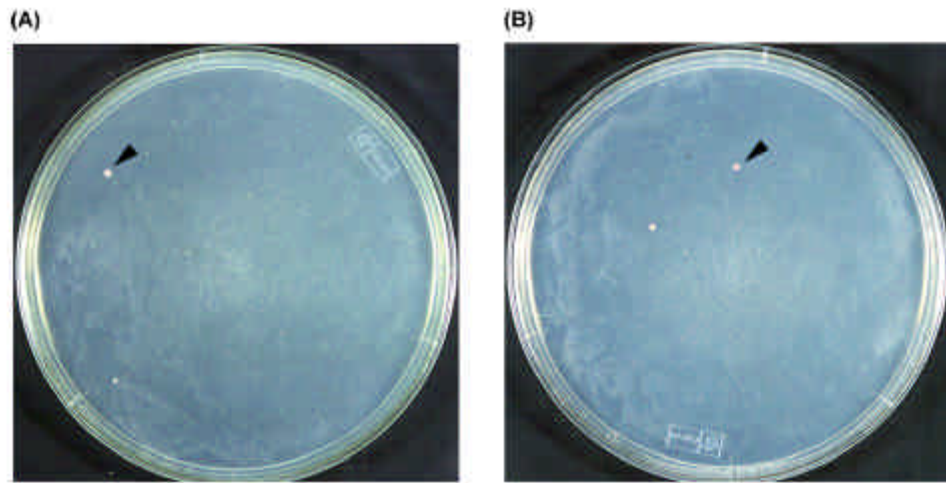
Fig. 6. Identification of the positive clones from the two-hybrid screening. After screening of 0.5×10^6 mouse embryonic cDNA library with the GAL4-BD/mDRA bait plasmid, rapidly growing colonies on His⁻ plates as pointed with arrows on (A) and (B) panels were isolated as the positive clones.

(A) Negative control
(Transformants carrying the bait plasmid only)

(B) Clone 3-8
(Rattus norvegicus protein phosphatase 2A B regulatory subunit)

(C) Clone 1-9
(Mus musculus paired box gene3)

(D) Clone 1-6
(Homo sapiens hypothetical protein)

(E) Clone 1-12
(Human DNA sequence contained the last coding exon of the gene for p18 component of aminoacyl-tRNA synthetase complex, part of an unkown gene downstream of a putative CpG island and an STS with a CA repeat))

Fig. 7. Verification of protein-protein interactions using the colony-lift filter -galactosidase assay. The binding of the proteins expressed in the positive clones to the GAL4-BD/mDRA protein was verified using transcriptional activation of *lacZ* gene which expresses -galactosidase. -galactosidase catalyzes X-gal to a indole product which will give blue color on the colonies.

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(A) Clone 3-8
(Rattus norvegicus protein phosphatase 2A B regulatory subunit delta isoform)

(B) Clone 1-9
(Mus musculus paired box gene 3)

(C) Clone 1-6
(Homo sapiens hypothetical protein)

(D) Clone 1-12
(Human DNA sequence contained the last coding exon of the gene for p18 component of aminoacyl-tRNA synthetase complex, part of an unkown gene downstream of a putative CpG island and an STS with a CA repeat)
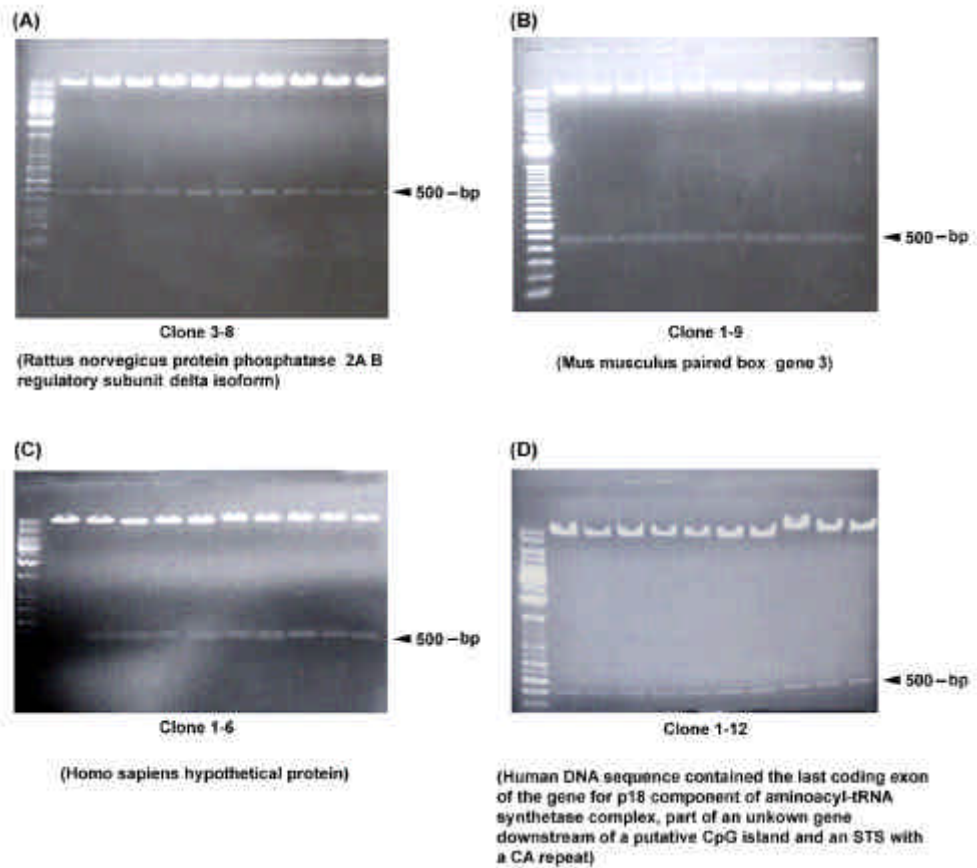
Fig. 8. Analysis of the yeast DNAs prepared from the positive clones. The isolated DNAs from the positive clones were digested by *Not I* endonuclease and resolved by TBE/agarose gel electrophoresis. The name of the best aligned gene with the sequences of each positive clone using the NCBI BLAST searches was listed in the parentheses under the panel of each positive clone.

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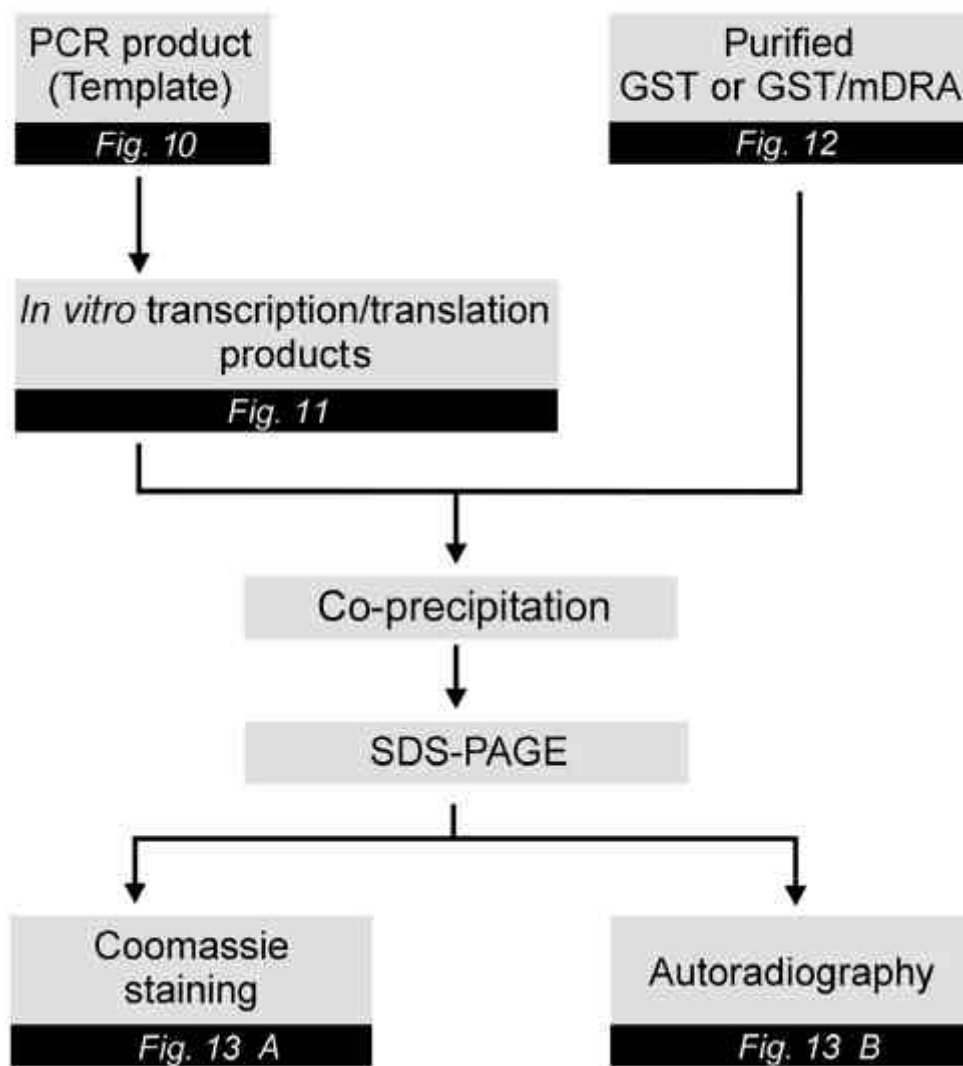
Fig. 9. Schematic diagram of verifying a specific binding of the proteins produced from the positive clones to GST/mDRA using co-precipitation (see the Materials and Methods).
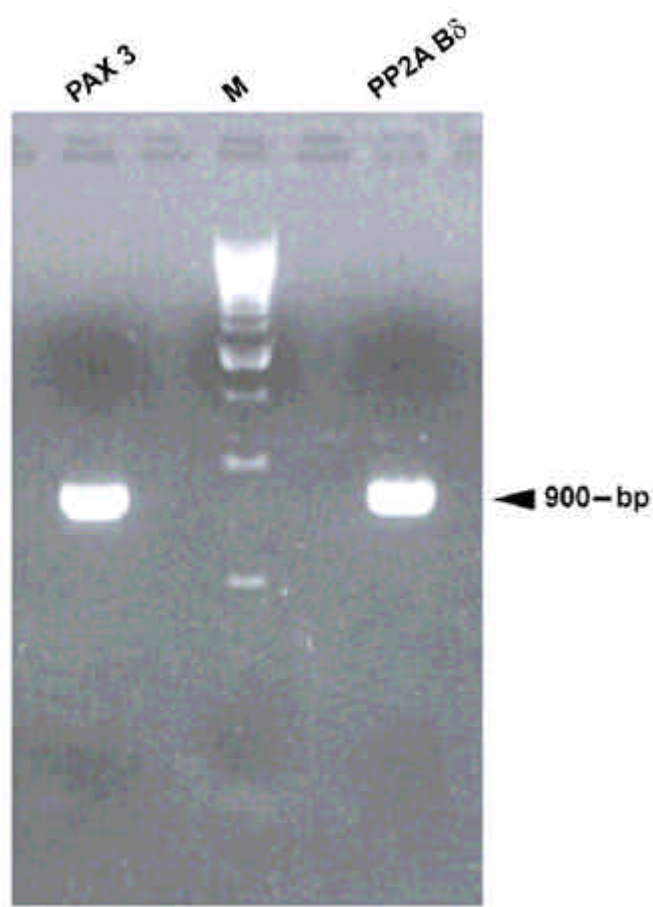
Fig. 10. Preparation of the templates used for *in vitro* transcription/translation reactions. The insert cDNAs (PAX 3 of #1-9 and PP2A B of #3-8) indicated above the panel were amplified by the sense primer containing T3 RNA polymerase sequence and the nonsense primer using *Pfu*-polymerase (Stratagene). The PCR products were analyzed by TBE/agarose gel electrophoresis. M indicates a 500-bp ladder (Life Technologies, Inc.).
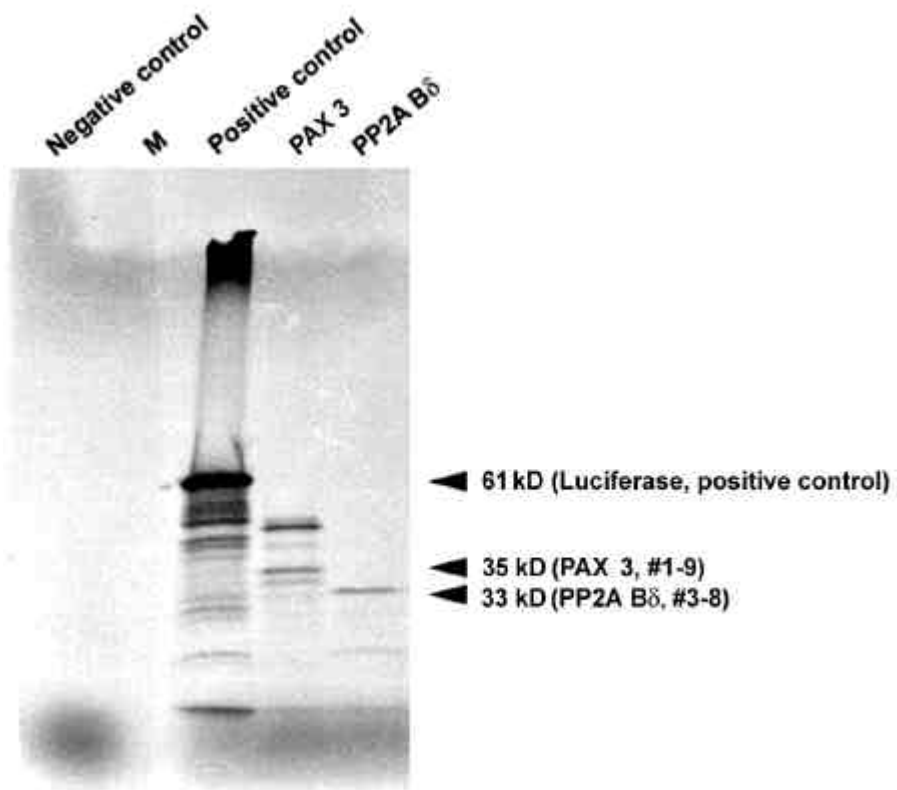
Fig. 11. Analysis of *in vitro* transcription and translation products generated by T$_N$T Coupled Reticulocyte Lysate System. The amplified templates prepared from #1-9 (PAX 3) and #3-8 (PP2A B) positive clones, the Luciferase DNA as a positive control, and H$_2$O as a negative control were processed using T$_N$T Coupled Reticulocyte Lysate System (Promega). The products from each template DNAs as indicated above the panel were resolved by SDS/PAGE and visualized by autoradiography.
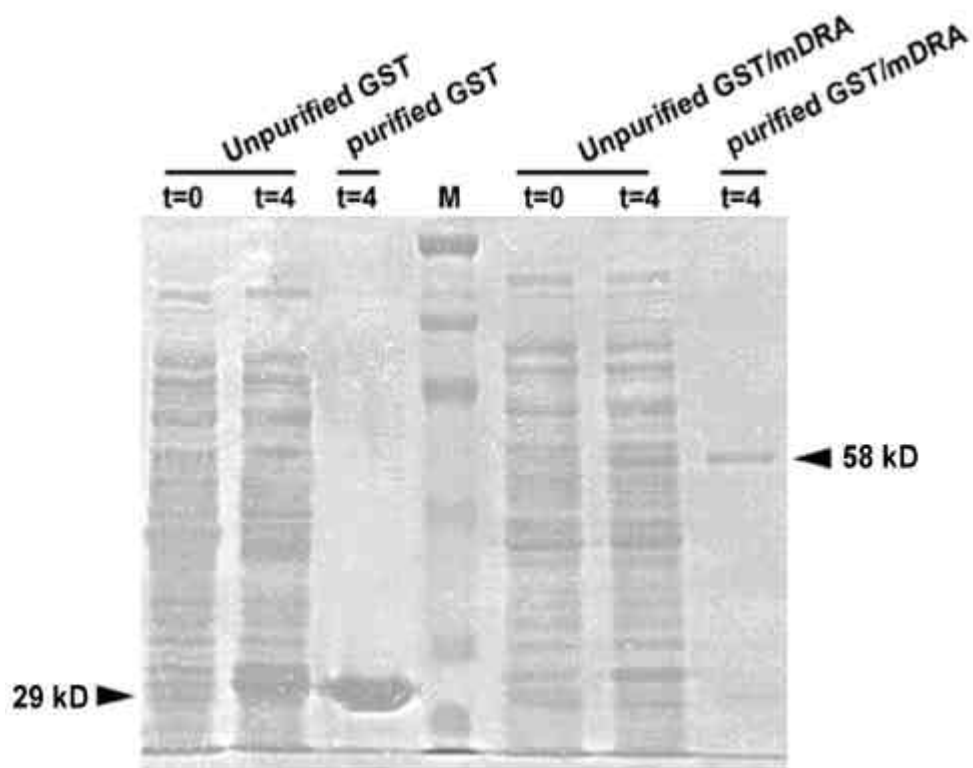
Fig. 12. Analysis of the expressed GST or GST/mDRA fusion proteins using SDS-PAGE and Coomassie staining. The lysates of *E. coli* transformants carrying pGEX-6P-1 (GST proteins) or pGEX-6P-1-mDRA (GST/mDRA fusion proteins) were analyzed by SDS/PAGE and Coomassie staining of the gel. The expressions of GST and GST/mDRA proteins were induced by addition of 0.3 mM IPTG for 4 hrs (t=4). t=0 indicates the time when IPTG was added to the culture. The GST or GST/mDRA proteins from the cultures treated with 0.3 mM IPTG for 4 hrs (t=4) were purified by Glutathione-Separose 4B. M represents the pre-stained size marker of broad range (Bio-Rad).
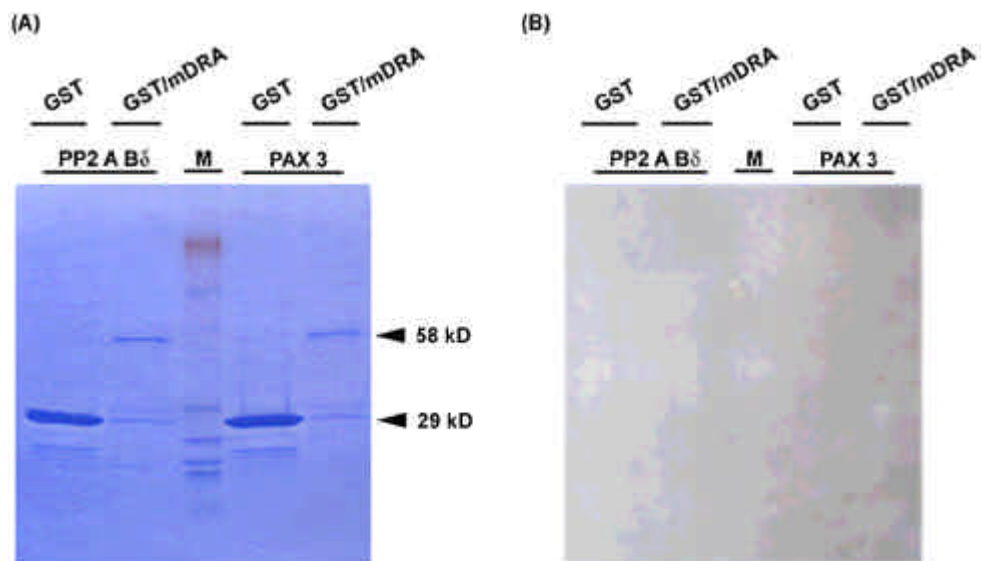
Fig. 13. Co-precipitation of PP2A B and PAX 3 with GST or GST/mDRA fusion proteins. The PP2A B or PAX 3 proteins generated by $T_N T$ Coupled Reticulocyte Lysate System were co-sedimented with the purified GST or GST/mDRA fusion proteins. The co-precipitated proteins were analyzed by SDS/PAGE followed by sequential Coomassie staining (panel A) and autoradiography (panel B).
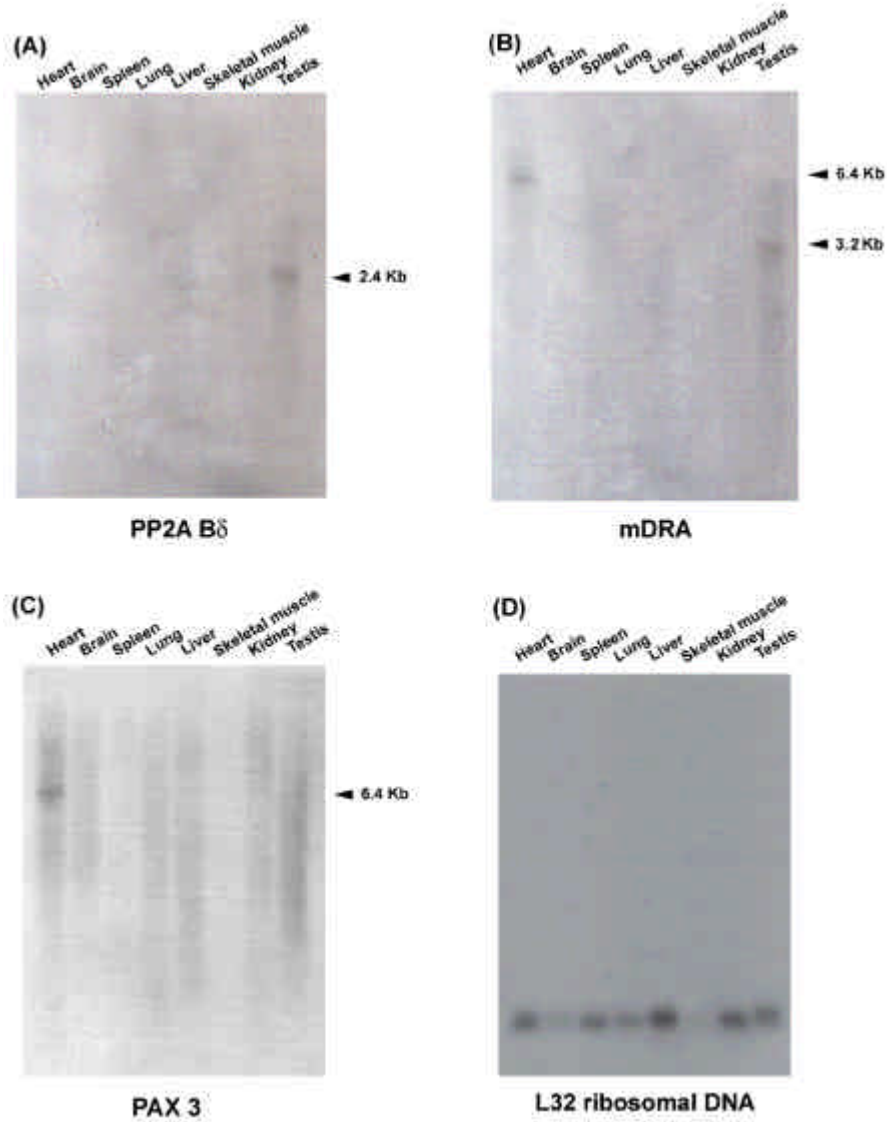
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Fig. 14. Tissue distribution of PP2A B , mDRA, and PAX 3 mRNA expression. Northern blot analysis was performed by sequential probing of the Mouse multiple tissue blot with ^{32}P-labeled cDNAs of PP2A B (panel A), mDRA (panel B), and PAX 3 (panel C). The blot was stripped and hybridized with a control mouse L32 ribosomal DNA probe (panel D).

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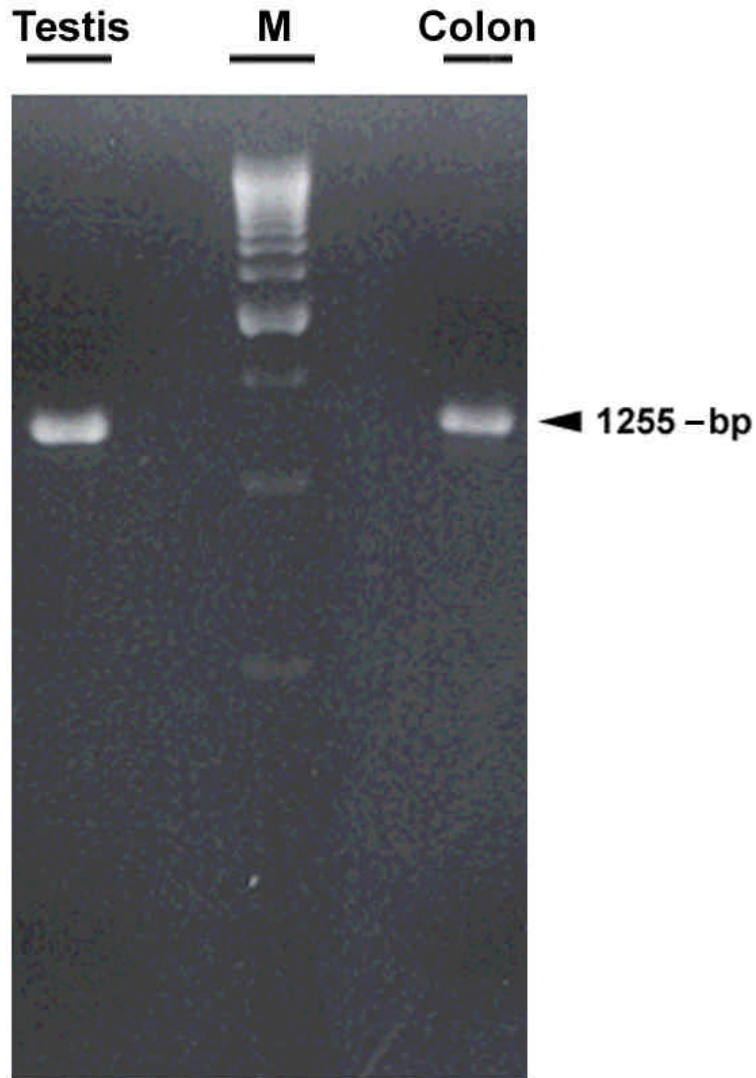
Fig. 15. Analysis of mouse PP2A B mRNA expression in mouse colon and testis using an RT-PCR protocol. The mouse PP2A B mRNA expressed in mouse colon and testis was amplified by RT-PCR using the pair of U2 and 3'L primers (Fig. 4) and cDNAs from mouse testis and colon. The PCR products were resolved by TBE/agarose gel electrophoresis. M indicates a 500-bp ladder (Life Technologies, Inc.).
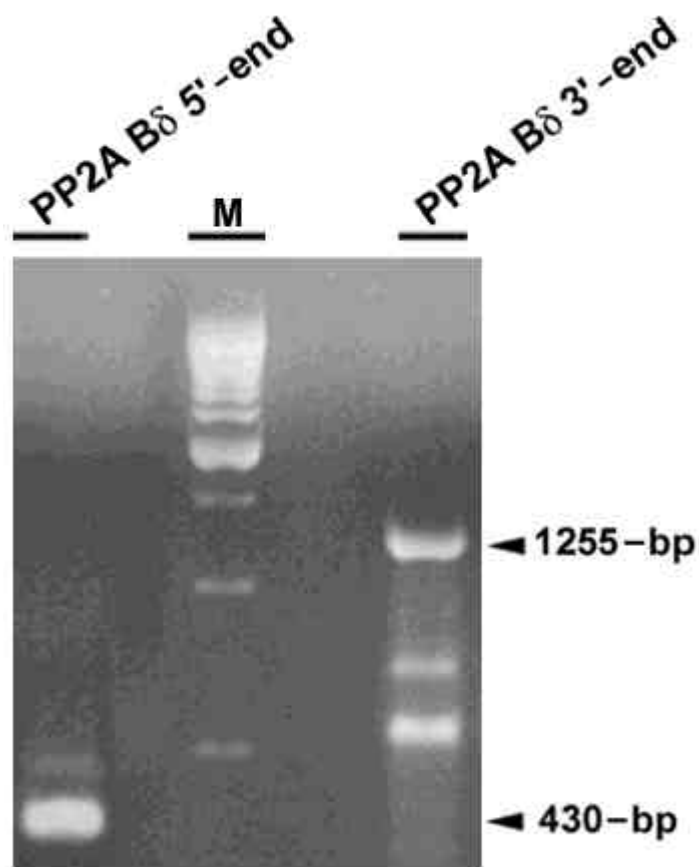
Fig. 16. Analysis of RT-PCR products to clone the full-length of mouse PP2A Bδ cDNA. The mouse colon cDNA was amplified by two pairs of primers, 5'U and L1 primers for the 5'-end (430-bp), and U2 and 3'L primers for the 3'-end region (1255-bp) of mouse PP2A Bδ cDNA. The amplified PCR products were analyzed by TBE/agarose gel electrophoresis. M represents a 500-bp ladder (Life Technologies, Inc.).

Fig. 17. Nucleotide and deduced amino acid sequence of mouse PP2A B . Nucleotides and amino acids are numbered on the *right*. The full-length of mouse PP2A B cDNA contains 1362 nucleotides encoding a protein of 453 amino acids as well as 31 nucleotides of 5'-untranslated and 565 nucleotides of 3'-untranslated region (accession number AF366393).

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Mouse PP2A Bδ    14   NDFQWCFSQVKGAVDEDVAEADIISTVEFNYSGDLLATGDKGGRVVIFQREQENKGRAHS   73
Rat  PP2A Bδ         NDFQWCFSQVKGAVDEDVAEADIISTVEFNYSGDLLATGDKGGRVVIFQREQENKGRAHS

Mouse PP2A Bδ    74   RGEYNVYSTFQSHEPEFDYLKSLEIEEKINKIRWLPQQNAAHFLLSTNDKTIKLWKISER   133
Rat  PP2A Bδ         RGEYNVYSTFQSHEPEFDYLKSLEIEEKINKIRWLPQQNAAHFLLSTNDKTIKLWKISER

Mouse PP2A Bδ    134  DKRAEGYNLKDEDGRLRDPFRITALRVPILKPMDLMVEASPRRIFANAHTYHINSISVNS   193
Rat  PP2A Bδ         DKRAEGYNLKDEDGRLRDPFRITALRVPILKPMDLMVEASPRRIFANAHTYHINSISVNS

Mouse PP2A Bδ    194  DHETYLSADDLRINLWHLEITDRSFNIVDIKPANMEELTEVITAAEFHPHQCNVFVYSSS   253
Rat  PP2A Bδ         DHETYLSADDLRINLWHLEITDRSFNIVDIKPANMEELTEVITAAEFHPHQCNVFVYSSS

Mouse PP2A Bδ    254  KGTIRLCDMRSSALCDRHAKFFEEPEDPXXXXXXXXXXXXXXXDVKFSHSGRYMMTRDYLS   313
Rat  PP2A Bδ         KGTIRLCDMRSSALCDRHAKFFEEPEDPSSRSFFSEIISSISDVKFSHSGRYMMTRDYLS

Mouse PP2A Bδ    314  VKVWDLNMEGRPVETHQVHEYLRSKLCSLYENDCIFDKFECCWNGSDSAIMTGSYNNFFR   373
Rat  PP2A Bδ         VKVWDLNMEGRPVETHHVHEYLRSKLCSLYENDCIFDKFECCWNGSDSAIMTGSYNNFFR

Mouse PP2A Bδ    374  MFDRNTRRDVTLEASRENSKPRASLKPRKVCTGGKRKKDEISVDSLDFNKKILHTAWHPM   433
Rat  PP2A Bδ         MFDRNTRRDVTLEASRENSKPRASLKPRKVCSGGKRKKDEISVDSLDFNKKILHTAWHPM

Mouse PP2A Bδ    434  ESIIAVAATNNLYIFQDKIN   453
Rat  PP2A Bδ         ESIIAVAATNNLYIFQDKIN
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Fig. 18. Amino acid sequence alignment of mouse PP2A B   and rat PP2A B . The amino acid sequence of mouse PP2A B   is compared with that of rat PP2A B . Alignment of mouse PP2A B   and rat PP2A B   reveals 96% amino acid identity.

DISCUSSION

DRA cloned from a colon cDNA subtraction library is a gene that is down-regulated in colon adenomas and adenocarcinomas in humans (42). However, mutation in human DRA gene has been shown to be responsible for congenital chloride diarrhea (CLD) (17, 18) and the functional expression studies demonstrated that DRA mediates Na^+-independent, DIDS-sensitive electroneutral exchange of sulfate, oxalate, Cl^-, and HCO_3^- (34, 35, 43). mDRA cloned from mouse colon cDNA is abundantly expressed in cecum, proximal colon and distal colon and is expressed at lower levels in each segment of the small intestine (34). Regulation of mDRA mRNA expression has suggested the functional role of mDRA in the particular tissues. CFTR induced the mRNA expression of DRA gene, which resulted in increase of the apical Cl^-/HCO_3^- exchanger activity in tracheal cells (51). Upregulation of mDRA transcript expression in the colon of mice lacking the NHE3 Na^+/H^+ exchanger (34) suggests that mDRA normally act in concert with NHE3 to absorb NaCl. The colon is a major site for NaCl absorption in the gastrointestinal tract and much of this activity appears to be mediated by coupled Na^+/H^+ and Cl^-/HCO_3^- exchange (28, 32, 39) which is considered to be tightly regulated. The deduced amino acid sequence of mDRA contains several potential regulatory sites including putative phosphorylation sites within the COOH-terminus, thus predicted as a potential binding region with the regulatory proteins.

In the present study, we have used the yeast two-hybrid screening (7, 22) to search interacting proteins which may be involved in regulating mDRA function. Among the real positive clones verified by the colony-lift filter -galactosidase assay, two candidate clones containing mouse phosphatase 2A B regulatory subunit delta isoform (PP2A B) and mouse paired box gene 3

(PAX 3) were further investigated due to a functional relevance of the clones to mDRA. However, it was interesting that GST pull-down binding assay (31, 48) of two library clones containing either mouse PP2A B homolog or mouse PAX 3 turned out to be negative, even though there were appropriate amounts of the purified GST/mDRA proteins and the right size of *in vitro* translated proteins in the assays as shown in Fig. 12 and Fig. 13. One possibility can be the weak interaction between GST/mDRA fusion protein and the *in vitro* translated products of the library clones. To co-sediment associated protein complexes with a strong interaction in the pull-down assay, we included 0.1% Triton X-100 which might have eliminated the weak binding proteins in the assay. Since we verified their specific protein-protein interactions (Fig. 7) using the colony-lift filter -galactosidase assay which is acceptable enough to confirm the protein-protein interaction, particularly in the CG1945 strain, we continued our investigation to study how these two genes may regulate mDRA function instead of pursuing more to optimize the binding condition in the pull-down assay. Patterns of tissue distribution of two clones were similar to that of mDRA transcript expression. Mouse PP2A B mRNA is highly expressed in colon and testis where mDRA transcript is also expressed abundantly (Fig. 14 : panels A and B, Fig. 15). Mouse PAX 3 is expressed at high levels in heart where mDRA mRNA expression was detectable (Fig. 14, panels B and C). Co-expression of these candidate genes and mDRA indicates the possible regulatory role of the genes on mDRA function in those particular tissues.

Protein phosphatase 2A (PP2A) is a multimeric serine/threonine phosphatase that has been highly conserved during the evolution of eukaryotes. As a heterotrimeric phosphatase, it consists of the constitutive core of A and C subunits, and one of various kinds of regulatory B subunits which determine substrate specificity, subcellular targeting and modulate the holoenzyme

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activity. PP2A has been shown to be involved in the regulation of many cellular processes including metabolism, DNA replication, transcription, RNA splicing, translation, cell-cycle progression, morphogenesis, development and viral transformation (9, 33, 36, 50). Serine/threonine protein phosphatases including PP2A are generally considered as negative regulators of cellular growth. In frog oocytes, PP2A was suggested to be a negative regulator of maturation promoting factor, a CDK complex that is essential for cell cycle progression (26). Furthermore, various protein products of small DNA tumor viruses form stable complexes with PP2A (36). This interaction inhibits phosphatase activity and leads to the activation of mitogen-activated protein kinase (MAPK) pathways in the absence of growth factor-initiated signaling (45). Similarly, other cellular proteins have been shown to interact with PP2A, thereby impinging on cellular regulation (15, 25). Moreover, a negative role of PP2A in the regulation of growth factor-regulated immediate early gene expression has been demonstrated by microinjection studies (1) and by the use of okadaic acid (2), a tumor promoter that inhibits PP2A (40). Recently, deletion of one of the regulatory subunit of PP2A has been detected in human cancers (49). Since it was reported that there was a little increase in the occurrence of intestinal cancer among individuals carrying mutations in the DRA gene, the complex of PP2A and DRA instead of DRA protein alone is likely involved in negative regulation of tumor promotion and development. mDRA mediates Na^+-independent Cl^-/OH^- (HCO_3^-) exchange and regulates the intracellular pH, which appears to be involved in cellular growth and cell division (14). The higher pH_i, for example, in the SW-620 human colon carcinoma cell line is accounted for by the absence of Cl^-/OH^- (HCO_3^-) exchange activity (4). Therefore, PP2A holoenzyme binding to mDRA may modulate the Cl^-/HCO_3^- exchange activity, which may alter the negative regulation of tumor promotion and development. To be able to examine this

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regulatory role of PP2A holoenzyme bound to mDRA, we cloned the full-length of mouse delta isoform of PP2A B regulatory subunits based on the sequences of the candidate clone and the rat PP2A B cDNA using an RT-PCR. The nucleotide and the predicted amino acid sequence alignment of mouse PP2A B to Rat PP2A B show 96% and 96% identity, respectively. Individual PP2A B regulatory subunit is distributed to many cellular compartments including cytosol, nucleus, membranes, microtubules, and neurofilaments suggesting a targeting role of the regulatory subunits. It was demonstrated that rat PP2A holoenzymes containing B regulatory subunit were fractionated into cytosol (46). Therefore, it can be predicted that mouse PP2A holoenzymes containing B targeted to cytosol may interact with the COOH-terminus cytosolic domain of mDRA to regulate the function.

Paired box gene 3 (PAX 3) is a member of a family of evolutionary conserved and developmentally regulated transcription factors (47) and also plays an important role in the formation of the neural tube and the establishment of the myogenic lineage (10, 12). Down-regulation of PAX 3 is in turn necessary to achieve muscle cell differentiation (13). In the current study, it is very difficult to predict how PAX 3 binding to mDRA regulates mDRA function even though PAX 3 protein forms the specific complex with mDRA. However, our future transcriptional regulation study of mDRA may reveal the regulatory role of PAX 3 in mDRA.

In the present study, we have identified and characterized the potential regulatory proteins which are associated with mDRA using the yeast two-hybrid screening. This study can be the essential step to elucidate the regulatory mechanism of mDRA function.

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VITA

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