Molecular Interactions between Single-stranded DNA-binding Proteins Associated with an Essential MCAT Element in the Mouse Smooth Muscle α-Actin Promoter*

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Transcriptional activity of the mouse vascular smooth muscle α-actin gene in fibroblasts is regulated, in part, by a 30-base pair asymmetric polyuridine-polypyrimidine tract containing an essential MCAT enhancer motif. The double-stranded form of this sequence serves as a binding site for a transcription enhancer factor 1-related protein while the separated single strands interact with two distinct DNA binding activities termed VACssBF1 and 2 (Cogan, J. G., Sun, S., Stoflet, E. S., Schmidt, L. J., Getz, M. J., and Strauch, A. R. (1995) J. Biol. Chem. 270, 11310–11321; Sun, S., Stoflet, E. S., Cogan, J. G., Strauch, A. R., and Getz, M. J. (1995) Mol. Cell. Biol. 15, 2429–2936). VACssBF2 has been recently cloned and shown to consist of two closely related proteins, Purα and Purβ (Kelm, R. J., Elder, P. K., Strauch, A. R., and Getz, M. J. (1997) J. Biol. Chem. 272, 26727–26733). In this study, we demonstrate that Purα and Purβ interact with each other via highly specific protein-protein interactions and bind to the purine-rich strand of the MCAT enhancer in the form of both homo- and heteromeric complexes. Moreover, both Pur proteins interact with MSY1, a VACssBF1-like protein cloned by virtue of its affinity for the pyrimidine-rich strand of the enhancer. Interactions between Purα, Purβ, and MSY1 do not require the participation of DNA. Combinatorial interactions between these three single-stranded DNA-binding proteins may be important in regulating activity of the smooth muscle α-actin MCAT enhancer in fibroblasts.

Eukaryotic gene transcription requires the coordinated assembly of upstream cis-element binding proteins, intermediary cofactors, and components of the basal transcription machinery into a multicomponent complex competent to initiate transcription. During this process, sequence-specific DNA-binding transcriptional activators and/or repressors play a pivotal role in modulating the cell-type specific expression of genes. While most such proteins bind to double-stranded DNA target sequences, a small but intriguing subclass has been identified that show enhanced affinity and specificity for either the sense or antisense strands of certain cis-regulatory elements required for promoter-specific activation (1–4) or repression (5–9). We have recently cloned and identified two single-stranded DNA (ssDNA)1-binding proteins, Purα and Purβ, that interact with the purine-rich strand of an essential transcription control sequence upstream of the mouse vascular smooth muscle (VSM) α-actin gene promoter (10).

The involvement of ssDNA-binding proteins in VSM α-actin gene transcription was discovered as a consequence of promoter mapping studies that led to the identification of a conserved 5′-flanking sequence required for both activation and repression of promoter activity in fibroblasts and undifferentiated myoblasts (11, 12). This proximal promoter element (PE) sequence (–195 to –165) exhibited polyuridine-polypyrimidine asymmetry, an inverted muscle-specific MCAT (AGGAATG) enhancer element, and bound at least three distinct DNA binding activities in a sequence and strand-specific manner. The two ssDNA binding activities, formerly designated vascular actin single-strand binding factors, VACssBF 1 and 2, appeared to play a role in repression (11) while a transcription enhancer factor 1-related protein was implicated in activation (12, 13). Although the mechanism of repression remains to be formally established, a hypothetical model involving VACssBF-mediated disruption of MCAT element base pairing and competition for transcription enhancer factor 1 binding was proposed (11). Interestingly, an additional binding site for VACssBF2 was later identified on the purine-rich coding strand of a GGAATG-containing sequence element located in a downstream VSM α-actin exon (14). This coding element sequence functioned as a VACssBF2-dependent repressing element when positioned 5′ and adjacent to a transcription enhancer factor 1- or activator protein 1-dependent enhancer element in chimeric promoter constructs (14). Because the noncoding strand of the coding element sequence lacked detectable VACssBF1 binding affinity (14), these data suggested that VACssBF2 binding was necessary and sufficient for repression. Screening of a mouse lung cDNA expression library with the exonic VACssBF2-binding site ultimately resulted in the isolation of two clones encoding the purine-rich ssDNA-binding proteins, Purα and Purβ (10). Biochemical analyses of the cloned mouse Pur proteins expressed in fibroblasts confirmed that Purα and Purβ corresponded to the p46 and p44 components of VACssBF2 that bind to the purine-rich strand of the PE and presumably down-regulate VSM α-actin gene expression (10).

In the present study, we used a similar binding site screen to confirm the identity between VACssBF1 and the mouse Y-box

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The abbreviations used are: ssDNA, single-stranded DNA; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); KLH, keyhole limpet hemocyanin; HRP, horseradish peroxidase; PVDF, polyvinylidene difluoride; DHFR, dihydrofolate reductase; ELISA, enzyme-linked immunosorbent assay; VSM, vascular smooth muscle; PE, promoter element.
protein, MSY1. By utilizing recombinant proteins and isoform-specific immune reagents, we demonstrate highly specific protein-protein interactions between Purα, Purβ, and MSY1 which seem likely to have functional significance. These include the binding of Pur proteins to ssDNA as both homo- and heterodimeric complexes and the formation of heterotrimeric complexes between all three proteins in the absence of DNA. These data suggest that protein-protein interactions between Purα, Purβ, and MSY1 play an important role in regulating transcriptional activity of the VSM α-actin gene in fibroblasts.

**EXPERIMENTAL PROCEDURES**

Cloning of a cDNA Encoding a PE-MCAT Strand Binding Protein, MSY1—A mouse lung cDNA expression library (Stratagene) was screened for cDNA-encoded proteins that interact with the pyrimidine-rich strand of the VSM α-actin MCAT enhancer element using a binding site cloning methodology described previously (10). Eight independent clones were isolated from 250,000 plaques initially screened. DNA sequencing by semi-automated dideoxy termination indicated that all eight clones encoded the mouse Y-box protein, MSY1 (15).

**Construction of Bacterial Expression Vectors and Purification of 6xHis-tagged Purα, Purβ, and MSY1**—The cDNAs encoding the open reading frames were amplified by PCR using the start and stop nucleotides of the murine β-actin (14239-11421) and MSY1 (clone 7-1, this study) were amplified by polymerase chain reaction using primers which generated 5′ BamHI and 3′ KpnI cloning sites. The polymerase chain reaction products were gel purified, cut with restriction enzymes, and subcloned into pQE-30 (Qiagen) to generate fusion constructs encoding a N-terminal 6xHis tag. The resultant plasmids were transformed into Escherichia coli strain JM109 and the orientation and fidelity of the polymerase chain reaction-amplified cDNA inserts were determined by DNA sequencing. For protein preparation, 1 liter of terrific broth containing 100 mg/ml ampicillin was inoculated (1:50) with an overnight bacterial culture and incubated for 5–7 h at 37 °C. Recombinant protein synthesis was induced by the addition of isopropyl-β-D-thiogalactopyranoside to 2.0 mM and an additional 4-h growth period. E. coli were collected by centrifugation at 5000 × g for 10 min and resuspended in 14 ml of cold 50 mM sodium phosphate, pH 8.0, 300 mM NaCl (lysis buffer) supplemented with 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 μg/ml each pepstatin, leupeptin, and aprotinin. Cells were lysed by sequential 30-min incubations on ice with lysozyme added to 1.0 mg/ml followed by Triton X-100 added to 0.13% (v/v). Lysates were centrifuged at 100,000 g for 1 h and collected, centrifuged, and packed Ni-NTA agarose resin (Qiagen) equilibrated in lysis buffer, and mixed overnight at 4 °C. The resin was washed sequentially at room temperature with 50 mM sodium phosphate, pH 8.0, buffer containing 0.3, 1.0, and 2.0 mM NaCl. The resin was then packed into a column and washed with 50 mM sodium phosphate, 2.0 mM NaCl, pH 8.0, until the A260 of the flow-through was <0.02. His-tagged protein was eluted with a step gradient of buffer, 2.0 mM NaCl, pH 8.0, 200 mM NaCl, pH 8.0, 300 mM sodium imidazole. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue R-250 staining. Pur protein or MSY1-enriched fractions were pooled, dialyzed versus 50 mM sodium phosphate, 1.0 mM NaCl, pH 8.0, and chromatographed a second time on Ni-NTA agarose to ensure optimal purity. His-tagged protein eluates were dialyzed against 25 mM HEPES, 0.5 mM NaCl, pH 8.0, aliquoted, and stored at −20 °C. Recombinant proteins were also purified as above but in buffers supplemented with 8 μM urea. Purification under such conditions enhanced the yield of His-tagged protein. No differences were observed in the in vitro protein-binding properties between His-tagged proteins purified under nondenaturing or denaturing conditions after dilution and/or dialysis into aqueous buffer. Recombinant protein concentration was estimated by optical density measurement using molar extinction coefficients and molecular weights of 18,610 and 35,000 for Purα, 18,610 and 34,000 for Purβ, and 26,170 and 36,000 for MSY1. In some cases, proteins were quantified by dye-binding assay (Bio-Rad) using bovine serum albumin as a standard.

**Preparation of Peptide-specific Polyclonal Antibodies against Mouse Purα, Purβ, and MSY1**—Peptides corresponding to mouse α-amidated acids 42−69, 210−229, and 302−324 of mouse Purα (10), 149−175 amide 291−313 of mouse Purα (10), and 85−110, 139−165, 242−267, and 276−302 of MSY1 (this study) were synthesized using modified Merrifield solid-phase chemistry and purified by reverse-phase high performance liquid chromatography by the Mayo Protein Core Facility. The composition of each peptide was confirmed by amino acid analysis. Each peptide was synthesized with a cysteine residue at either the N or C terminus to facilitate coupling to maleimide-activated KLH (Pierce) and iodoacetyl-agarose (SulfoLink, Pierce). KLH-coupled peptides were used as immunogens and rabbit polyclonal antisera production was carried out by a commercial vendor (Cocalico) using a 60-day standard protocol. Polyclonal rabbit IgGs were affinity purified using peptide-coupled agarose (20). Briefly, whole IgGs from rabbits were absorbed at 4 °C with 40% ammonium sulfate precipitation. Following centrifugation for 10 min at 5000 × g, the IgG-rich pellet was reprecipitated, dissolved in phosphate-buffered saline, and dialyzed. Rabbit IgG was then applied to the appropriate 2-ml peptide-agarose (0.5−1.0 mg of peptide/ml) column equilibrated with phosphate-buffered saline. The flow-through fraction was retained and roister washing the column with phosphate-buffered saline, peptide-bound IgG was eluted with 0.1 M glycine, pH 2.5, and immediately neutralized with 1 M Tris, pH 9.5. Affinity purified IgG was precipitated by the addition of solid ammonium sulfate to 75% saturation. The pellet was collected by centrifugation, dissolved in 50% (v/v) glycerol/phosphate-buffered saline, and stored at −20 °C. Rabbit IgG from pooled preimmune serum was used on Protein A/G-agarose (Calbiochem). IgG concentration was estimated by optical density measurement based upon a molar extinction coefficient and molecular weight of 210,000 and 150,000, respectively.

**Screening of Peptide Affinity-purified Antibodies by ELISA**—His-tagged mouse Purα, Purβ, or MSY1 diluted to 50 ng in 25 mM HEPES, 150 mM NaCl, pH 7.5 (HBS), containing 5.0 μg/ml crystalline grade bovine serum albumin (BSA), was applied to polystyrene microtiter wells (100 μl/well) (Corning ELISA plate number 25805) and incubated 16−20 h at 4 °C. The resultant Purα-, Purβ-, or MSY1-coated wells were washed once with HBS containing 0.05% (v/v) Tween 20 (HBST), and blocked for 1 h with 0.2% (w/v) bovine serum albumin in HBS (250 μl/well). Wells were washed once and rabbit anti-mouse Pur or MSY1 peptide antibody (1.0−0.016 μg/ml, 100 μl/ well) diluted in HBST containing 0.1% bovine serum albumin was applied for 2 h at room temperature. Primary antibody solution was aspirated and wells were washed three times with HBST. Goat anti-rabbit IgG-HRP (Santa Cruz) diluted 1:2000 in HBST was then applied for 1 h. Wells were washed as above and 100 μl of ABTS chromogenic substrate (Roche Molecular Biochemicals) was added. Absorbance readings at 405 nm were determined after 5−6 min using a 96-well microplate spectrophotometer.

**Screening of Peptide Affinity-purified Antibodies by Western (Immuno) blotting**—Cellular Purα, Purβ, or MSY1 were enriched from 1 mg of AKR-2B fibroblast nuclear protein by selective capture on biotinylated ssDNA (PE-F for Pur proteins and PE-R for MSY1) coupled streptavi- din-paramagnetic particles as described previously (10). DNA-bound proteins were resuspended in 4% (w/v) glutaraldehyde mini-curtain gel, and electrotransferred to a polycrylamide diffusion membrane (Immobilon-P) for 90 min (300 mA) in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol at 4 °C. After blocking overnight in 25 mM Tris, 150 mM NaCl, pH 7.5 (TBS), with 5% (w/v) Carnation nonfat dry milk at 4 °C, the membrane was fitted into a multiscan apparatus (Mini-PROTEAN II, Bio-Rad) and selected channels were loaded with either anti-Pur or MSY1 peptide antibody diluted to 2.0, 0.5, and 0.1 μg/ml in 2% nonfat dry milk/TBS. Following a 1-h incubation at ambient temperature with gentle mixing, the antibody solutions were aspirated, and each channel was washed once with TBS, containing 0.05% Tween 20 (TBST). The initial wash solution was aspirated, the apparatus disassembled, and the entire blot washed three more times (5 min/25 ml wash). Goat anti-rabbit IgG-HRP (Santa Cruz) diluted 1:2000 in TBST was then applied for 1 h. The blot was washed four times (30 min total) and chemiluminescence reagent (ECL, Amersham) was applied for 1 min. Immune complexes were visualized on x-ray film (KAR-5, Kodak) following a 5−10 x exposure.

**Electrophoretic Mobility Shift Assay for Protein-DNA Binding**—Band shift assays were performed as described previously (11, 14). For antibody supershift experiments, rabbit IgGs (0.25−1.0 μg) were preincubated for 20 min with AKR-2B nuclear protein (10) in binding buffer containing poly(dI-dC) (11). A 22P-ssDNA probe corresponding to the purine-rich coding strand of the PE was then added (≈1 nM final) and mixtures were incubated for an additional 20 min prior to electrophore- sis on a 6% non-denaturing polyacrylamide gel.

**ELISA for Protein-Protein Interactions**—His-tagged Purα, Purβ, MSY1, or dithiothreitol reductase (DHR)-coupled microtiter wells (50 nm application as described above) were incubated with varying amounts of AKR-2B nuclear protein (10) (100 μl/well) diluted in binding buffer (HBST with 0.1% bovine serum albumin) for 16−18 h at 4 °C. Wells were aspirated and washed 3 times with HBST and 100 μl of anti-Pur or anti-MSY1 peptide IgG diluted to 1.0 μg/ml in binding buffer was applied for 1 h at room temperature. Primary antibody solution was
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RESULTS

Development and Characterization of Isoform-Specific Immune Reagents to Purα and Purβ—A repertoire of immune reagents based upon cDNA-deduced amino acid sequences of Purα and Purβ were produced to assist in defining determinants of protein-DNA and putative protein-protein interactions. Synthetic peptides, corresponding to both conserved and unique sequences within the Pur proteins, were used as immunogens. Polyclonal IgGs were enriched from rabbit antisera by ammonium sulfate fractionation and then subjected to affinity purification on peptide-coupled agarose columns.

Common Peptides: B42-69, A149-175

Isoform Specific Peptides: B210-229, B302-324, A291-313

Fig. 1. Peptides used in the preparation of polyclonal antibodies to Purα and Purβ. Five peptides corresponding to selected amino acid sequences (underlined) were synthesized, coupled to KLH, and used as immunogens. Polyclonal IgGs were enriched from rabbit antisera by ammonium sulfate fractionation and then subjected to affinity purification on peptide-coupled agarose columns.

Aspirated and wells were washed three times with HBST. Goat anti-rabbit IgG-HRP (Santa Cruz) diluted 1:1000 in HBST was then applied for 1 h. Secondary antibody solution was aspirated and wells were washed four times with HBST. Immune complexes were detected using 100 μl of ABTS chromogenic substrate. Absorbance readings at 405 nm were determined after 45 min.

Immunoprecipitation Assay for Protein-Protein Interaction—Mouse AKR-2B fibroblasts were transiently transfected with mouse Purα and Purβ expression vectors as described previously (10). All subsequent steps including cell synchronization, serum-stimulation, harvest, extraction, and protein assay have been detailed previously (10, 14). Whole cell protein extract (100 μg) from transfected cells or nuclear extract from nontransfected rapidly growing AKR-2B fibroblasts (10) was combined with 2.5 μg of selected rabbit anti-Pur or MSY1 peptide IgGs in a final volume of 250 μl. After a 1-h incubation at room temperature, ~10⁷ sheep anti-rabbit IgG-coupled magnetic dynabeads (Dynal) were added and the mixtures incubated for an additional 90 min. In some experiments, goat anti-rabbit IgG-biotin (Santa Cruz) coupled to streptavidin-coated paramagnetic beads (Promega) were used. The beads were then captured with a magnet and washed three times with HBS. Rabbit IgG-bound protein was specifically eluted by adding a vast excess of free peptide (20 μl at 50 μM) and incubating for 30 min at room temperature. Eluates were supplemented with Laemmli SDS sample preparation buffer and 5% (v/v) β-mercaptoethanol, and subjected to electrophoresis on a 10% (29:1) polyacrylamide mini-gel. Immunoprecipitates were evaluated for the presence Pur proteins and MSY1 via immunoblotting as described above.

Development and Characterization of Isoform-Specific Immune Reagents to Purα and Purβ—A repertoire of immune reagents based upon cDNA-deduced amino acid sequences of Purα and Purβ were produced to assist in defining determinants of protein-DNA and putative protein-protein interactions. Synthetic peptides corresponding to both conserved and unique sequences within the Pur proteins were used as immunogens in rabbits (Fig. 1). IgGs were enriched from rabbit antisera and then subjected to affinity purification using peptide-coupled agarose columns. The resultant affinity purified IgGs were tested for reactivity using both recombinant (Histagged) and cellular Purα and Purβ (Figs. 2 and 3). Assessment of antibody binding to immobilized recombinant Purα and Purβ by ELISA (Fig. 2) indicated that several antibodies possessed remarkable specificity for either Purα (anti-A291–313) or Purβ (anti-B210–229 and anti-B302–324) while another antibody directed against a conserved region (anti-B42–69) cross-reacted with Purα and Purβ (Fig. 2). The specificity of these antibodies was also evaluated by Western blotting of cellular Pur proteins enriched from an AKR-2B fibroblast nuclear extract by selective capture on paramagnetic particles coupled with the purine-rich strand of the VSM α-actin MCAT element, PE-PrMss, or PE-F (10). Consistent with ELISA data, anti-B42–69 demonstrated similar reactivity toward fibroblast-derived Purα (p46, Mr = 46,000) and Purβ (p44, Mr = 44,000) which migrate as a closely spaced doublet (Fig. 3, lanes 4–6). Anti-A291–313 recognized only the slower migrating Purα (p46, Mr = 46,000) and Purβ (p44, Mr = 44,000) isoform (Fig. 3, lanes 16–18) while both anti-B210–229 and anti-B302–324 preferentially detected the faster migrating Purβ (p44) isoform (Fig. 3, lanes 10–12 and 13–15). As expected, preimmune rabbit IgG failed to detect the Pur proteins in both screening assays.

Effect of Anti-Pur Antibodies on Protein-DNA Complex Formation—Band shift assays were conducted in the presence of anti-Pur peptide antibodies to confirm the identity of Pur protein-DNA complexes previously suggested by overexpression studies (10). Initial experiments utilized nuclear protein from AKR-2B fibroblasts as a source of cellular Purα and Purβ and...
a \(^{32}\)P-oligonucleotide probe corresponding to the purine-rich coding strand of the PE (PE-PrMas or PE-F) (11). As shown in earlier studies, Pur and Pur\(\beta\)-ssDNA complexes migrate as a closely spaced doublet (Fig. 4A, lane 2). A slower migrating complex (NS) that is also detected is composed of an unrelated, nonspecific DNA-binding protein (11, 14). As illustrated in Fig. 4A, three out of the four anti-Pur antibodies tested (lanes 4–6) were found to selectively supershift the two major Pur protein-ssDNA complexes into two slower migrating complexes, designated SS1 and SS2. These supershifted complexes were not formed when preimmune IgG or anti-B42–69 were included in the reaction mixtures (lanes 2 and 3). The inability of anti-B42–69 to supershift suggests that this antibody is unable to bind Pur proteins in their native (i.e., nondenatured) state since immobilization on polystyrene (Fig. 2) or denaturation by SDS (Fig. 3) did not interfere with epitope recognition.

Closer inspection of the band shift patterns obtained using isoform-specific antibodies provided unexpected results. While anti-A291–313 appeared to only partially supershift the major Pur protein-ssDNA complex (Fig. 4A, lane 6, arrow), a minor and slower migrating complex (denoted by a \(^*\)) was completely supershifted by this antibody. Moreover, anti-B210–229 and anti-B302–324 clearly supershifted the most rapidly migrating Pur\(\beta\) complex and, surprisingly, the major (middle) Pur protein-containing complex as well (Fig. 4A, lanes 4 and 5). These data suggested that the major (middle) Pur protein complex is heterogeneous and likely contains Pur\(\beta\) while the minor, slowest migrating complex is composed exclusively of Pur. Owing to the low abundance of this minor Pur-containing complex, we also performed antibody supershift analyses using extracts from AKR-2B fibroblasts transfected with mouse Pur and/or Pur\(\beta\) expression vectors (10). As shown in Fig. 4B, overexpression of Pur enhanced formation of the minor (slowest migrating) Pur protein-ssDNA complex (lane 2, \(^*\)) which was supershifted by anti-A291 (lane 5) but not by anti-B302 (lane 8). Overexpression of Pur\(\beta\) enhanced formation of the major (most rapidly migrating) Pur\(\beta\)-ssDNA complex (Fig. 4B, lane 3) which was supershifted by anti-B302 (lane 9) but not by anti-A291 (lane 6). Co-expression of both proteins enhanced formation of the two major (middle and most rapidly migrating) Pur protein complexes but not the minor (slowest migrating) Pur protein complex (Fig. 4B, lane 4). Both major complexes were efficiently supershifted by anti-B302 (compare lanes 4 and 10) while only the middle Pur\(\beta\)-ssDNA complex was affected by anti-A291 (compare lanes 4 and 7). Importantly, formation of all three Pur protein-ssDNA complexes was abolished by low concentrations of sodium deoxycholate, a mild ionic detergent known to disrupt protein-protein interactions (16) (Fig. 4C, lanes 2–5). In contrast, the nonspecific ssDNA-binding complex (NS) was largely unaffected by deoxycholate while the Pur protein-ssDNA complexes were unaffected by Triton X-100 (lanes 6–9). It is important to note that no faster migrating ssDNA complex was detected in samples treated with deoxycholate implying that monomeric binding of the Pur proteins to ssDNA does not occur under these conditions. Together, these data provide strong evidence that mouse Pur and Pur\(\beta\) bind to the purine-rich strand of the MCAT enhancer in the form of homo- and heterodimers. No evidence was obtained for the existence of monomeric ssDNA-binding complexes. These data are consistent with a recent finding that human Pur binds to a recognition element in the myelin basic protein gene promoter as a homodimer (17) and suggest that dimerization between Pur proteins may be a necessary prerequisite for functional activity.

**Puro and Pur\(\beta\) Associate in the Absence of ssDNA**—To determine whether Pur protein dimerization requires coincident interaction with a ssDNA recognition element, we evaluated the ability of the Pur proteins to associate in the absence of ssDNA. Initially, we performed immunoprecipitation experi-
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Fig. 5. Detection of a Puro-Purβ complex by immunoprecipitation and ELISA. Top, AKR-2B fibroblasts were transiently transfected with 2 μg each of pCI-Pura and pCI-Purβ expression vectors (10). Transfectants were rendered quiescent and serum-stimulated for 6 h. Cell extracts were prepared and 100 μg of lysed cell protein was supplemented with 2.5 μg of the indicated rabbit IgG and incubated for 1 h. Following an additional incubation with sheep anti-rabbit-coupled magnetic beads, immune complexes were captured on a magnet, washed, and rabbit IgG bound protein eluted with free peptide. Eluates were analyzed by immunoblotting using anti-B42–69 to detect Purα and Purβ. Lane 1 shows Pur protein immunoreactivity observed in 2 μg of whole cell extract prior to immunoprecipitation. Bottom, His-tagged Purβ and DHFR-coated microtiter wells (50 nM application) were incubated with varying amounts of AKR-2B nuclear protein for 16 h at 4°C. Wells were aspirated, washed 3 times, and solid-phase Puro-Purβ complexes were detected by ELISA using anti-Pura 291–313 as the primary antibody. Absorbance readings at each point were corrected by subtracting a background A405 nm reading generated with His-tagged protein-coated wells and binding buffer.

Fig. 6. Cloning of a cDNA encoding a PE-MCAT strand binding protein. Screening of a mouse lung cDNA expression library (250,000 plaques) with a 32P-end labeled tetramer of the pyrimidine-rich strand of the PE yielded eight independent PE-MCATss-binding clones. Each clone was tested for its ssDNA-binding specificity in a filter binding assay. All eight clones produced identical results. The data for one of the clones, 7-1, is shown. Excess (150-fold molar) wild-type oligonucleotide (right side) completely inhibited the binding of the 32P-tetramer while a mutant oligonucleotide deficient in VACssBF1 binding did not (left side).

Identification of MSY1 as a PE-MCAT Strand-binding Protein—Screening of a mouse lung cDNA expression library with a 32P-end-labeled tetramer of the pyrimidine-rich strand of the PE yielded eight independent PE-MCATss-binding clones. Each clone was tested for its ssDNA binding specificity using wild-type and mutant oligonucleotide competitors in a tertiary filter binding assay. All eight clones produced identical results. The data for one of the clones, 7-1, where excess wild-type oligonucleotide (PE-MCATss) completely inhibited the binding of the 32P-tetramer in comparison to a mutant oligonucleotide (PE-MCATmu2) lacking VACssBF1 binding affinity (11). DNA sequencing revealed that each clone contained overlapping nucleotide sequences that were virtually identical to the cDNA sequence encoding MSY1 previously reported by Tafuri and co-workers (15). The full-length cDNA sequence of clone 7-1 and the published MSY1 cDNA sequence differ by only a single nucleotide within the open reading frame. Alignment of the deduced amino acid sequences illustrates that clone 7-1 encodes a glycine residue rather than an alanine residue at codon 29 (Fig. 7). The reason for this discrepancy is probably due to a polymorphism although the glycine codon is conserved in the rat and human Y-box homologues, EF1α (18), dpbB (19), and YB-1 (20) (Fig. 7).

Synthetic peptides corresponding to several sequences of predicted antigenicity were used as immunogens to derive MSY1-specific antibodies. Affinity purified rabbit IgGs were tested for specificity as described for the panel of anti-Pur antibodies (data not shown), and two, anti-MSY242–267 and anti-MSY276–302, were selected for use in further experiments. Importantly, neither antibody exhibited detectable cross-reactivity with either of the Pur proteins. These antibodies were used to test whether cellular, as opposed to recombinant, MSY1 would bind to the pyrimidine-rich strand of the MCAT enhancer. As shown in Fig. 8, an MSY1 immunoreactive...
species was captured from a crude AKR-2B fibroblast nuclear extract by paramagnetic particles coupled with the pyrimidine-rich, reverse strand of the enhancer (PE-F, lane 3) but not by particles coupled with the opposing, purine-rich forward strand (PE-F, lane 2). In contrast, the purine-rich forward strand, but not the pyrimidine-rich reverse strand, effectively captured Purα and Purβ (compare lanes 5 and 6). These data validate the expression cloning results and provide strong evidence that the ssDNA-binding complex previously termed VACssBF1 (11, 12) is identical, or closely related to the mouse Y-box protein, MSY1.

**Interaction of Purα and Purβ with MSY1**—The identification of MSY1 as a pyrimidine-rich strand, VSM α-actin MCAT enhancer-binding protein was particularly intriguing given a previous report implicating the human homolog, YB-1, as a transient Purα-binding protein in the context of a different promoter element (22). As an independent evaluation of the potential for protein-protein interaction between mouse Purα and/or Purβ and MSY1, quantitative binding studies were conducted with purified, recombinant proteins. The binding of cellular Purα and Purβ to His-tagged MSY1 passively immobilized on polystyrene microtiter wells was evaluated by ELISA. Fluid-phase AKR-2B nuclear protein was incubated with both MSY1 and DHFR-coated wells. After removal of unbound nuclear protein, solid-phase Purα and Purβ-MSY1 complexes were detected using antibodies that specifically recognize the C-terminal region of either Purα or Purβ. While virtually no signal was obtained from wells coated with DHFR, the colorimetric signal generated with MSY1-coated wells was dose-dependent and saturable (Fig. 9A). Similar results were obtained when the assay was performed using His-tagged Purα or Purβ as the solid-phase ligands and an MSY1-specific antibody to detect complex formation (Fig. 9B).

Although MSY1 binding was not completely saturable in this orientation (owing to decreased accessibility of binding sites, lower affinity of the detecting antibody, and/or limiting fluid-phase MSY1), the immobilized Pur proteins were nonetheless indistinguishable in terms of their ability to specifically partner with cellular MSY1. These data demonstrate that both Purα and Purβ can form a stable, heterotrimeric complex with MSY1 in the absence of ssDNA.

To test whether or not such a heterotrimeric complex could be detected in a nuclear extract without disturbing the equilibrium by exposure to immobilized recombinant ligand, immunoprecipitation experiments were performed with anti-Pur and anti-MSY1 antibodies and a nuclear extract from nontransfected AKR-2B fibroblasts. The Pur protein and MSY1 composition of each immunoprecipitate was analyzed by Western blotting using anti-PurB42–69 to detect both Pur isoforms, blotting with a mixture of anti-MSY1 peptide antibodies, and with both MSY1 and DHFR-coated wells. After removal of unbound nuclear protein, solid-phase Purα and Purβ-MSY1 complexes were detected using antibodies that specifically recognize the C-terminal region of either Purα or Purβ. While virtually no signal was obtained from wells coated with DHFR, the colorimetric signal generated with MSY1-coated wells was dose-dependent and saturable (Fig. 9A). Similar results were obtained when the assay was performed using His-tagged Purα or Purβ as the solid-phase ligands and an MSY1-specific antibody to detect complex formation (Fig. 9B). Although MSY1 binding was not completely saturable in this orientation (owing to decreased accessibility of binding sites, lower affinity of the detecting antibody, and/or limiting fluid-phase MSY1), the immobilized Pur proteins were nonetheless indistinguishable in terms of their ability to specifically partner with cellular MSY1. These data demonstrate that both Purα and Purβ can form a stable, heterotrimeric complex with MSY1 in the absence of ssDNA.

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FIG. 9. Detection of cellular Pur and Purβ binding to recombinant MSY1 by ELISA. A, His-tagged MSY1 and DHFR-coated microtiter wells (50 nM application) were incubated with varying amounts of AKR-2B nuclear protein for 16 h at 4°C. Wells were aspirated, washed 3 times, and solid-phase Purα-MSY1 or Purβ-MSY1 complexes were detected by ELISA using anti-PurA 291–313 or anti-PurB 302–324, respectively, as the primary antibodies. B, His-tagged Purα, Purβ, or DHFR-coated microtiter wells (50 nM application) were incubated with varying amounts of AKR-2B nuclear protein for 16 h at 4°C. Wells were aspirated, washed 3 times, and solid-phase MSY1-Puro or MSY1-Purβ complexes were detected by ELISA using anti-MSY1 242–267 as the primary antibody. Absorbance readings at each point were corrected by subtracting a background A₄05 nm reading generated with His-tagged protein-coated wells and binding buffer.

**DISCUSSION**

Because Purα and/or Purβ appear to function as repressors of VSM α-actin MCAT enhancer activity (11, 14) and because Purβ has not previously been characterized, we felt it important to study potential molecular interactions between these two ssDNA-binding proteins. Recombinant mouse Pur proteins and rabbit polyclonal antibodies directed against specific domains both common and unique to Purα and Purβ were used in these studies (Figs. 1–3). Surprisingly, we found that several antibodies specific for the Purβ isoform supershifted protein-ssDNA complexes composed of both Purα and Purβ, implying that the Pur proteins can transiently associate via protein-protein interaction (Fig. 4). This conclusion was validated by both immunoprecipitation and ELISA-based, protein-protein binding experiments which indicated that a specific and stable Purα-Purβ complex can form in the absence of a ssDNA recognition element (Fig. 5). This interaction likely underlies the previously unrecognized ability of Purα and Purβ to form heterodimeric ssDNA-binding complexes and is likely of consequence given the diverse functional roles attributed to Purα. For example, Purα has been previously implicated in DNA replication of several viral genomes (23–25) and transcriptional activation of both viral (22, 26) and mammalian promoters (27–32). In contrast, our results suggest that mouse Purα and/or Purβ are able to repress the activity of a nearby enhancer in the context of both natural and chimeric promoters (11, 14). While it is not uncommon for a transcription factor to function as either an activator or repressor depending on promoter context, it is equally likely that the properties of a homodimeric Purα ssDNA-binding complex differ substantially from a homodimeric Purβ complex, or from a Purα/Purβ heterodimer. This could easily explain why the VSM α-actin MCAT enhancer is negatively regulated despite the presence of a transcriptional activator like Purα. This possibility is lent additional credence by the structural differences between Purα and Purβ, most notably the absence of a C-terminal polyglutamine sequence, a potential transactivation domain, in Purβ (Fig. 1).

In an earlier study, a binding site screen of a human astrogloma cell cDNA expression library was used to tentatively identify a member of the Y-box family of nucleic acid-binding proteins as the pyrimidine-rich ssDNA binding activity previously termed VACssBF1 (33). In the present study, we confirmed this result using a cDNA library from a mouse tissue enriched in smooth muscle. This screen yielded eight independent clones, all encoding the mouse Y-box protein MSY1. Moreover, MSY1 was selectively captured from fibroblast nuclear extracts by the pyrimidine-rich strand of the MCAT enhancer coupled to paramagnetic beads (Fig. 8). Together, these studies provide convincing evidence that MSY1 does indeed represent the ssDNA binding activity which interacts with the strand of the MCAT enhancer opposing the Pur protein recognition site. Thus, it may be highly significant that MSY1 specifically interacts with both Purα and Purβ in vitro (Fig. 9), and indeed, can be co-immunoprecipitated from fibroblast nuclear extracts in the form of an MSY1-Pur protein complex (Fig. 10).

While the functional significance of these interactions to transcriptional regulation of the VSM α-actin gene remains to be established, it is noteworthy that the human Y-box protein homologue, YB-1, has been shown to similarly interact with Purα to reciprocally modulate each others binding to the JC polyomavirus lytic control element (22). Similar cooperative interactions seem likely to occur within the context of the
MCAT enhancer element. Because Purα and/or Purβ appear capable of repressing MCAT enhancer activity independently of MSY1 (14), protein-protein interactions with MSY1 might serve to antagonize this effect by virtue of sequestering one or both of the Pur proteins into an inactive complex. Alternatively, MSY1 may potentiate the effect(s) of the Pur proteins owing to the fact that vertebrate Y-box proteins have been functionally implicated in both transcriptional activation (18, 34–36) and repression (37, 38). Other possibilities can easily be envisioned but can only be resolved through experiment.

While potential combinatorial interactions between Puro, Purβ, MSY1, and their respective ssDNA recognition motifs are numerous, the binding of Puro to a ssDNA recognition element has also been shown to be modulated by specific protein-protein interaction with the retinoblastoma tumor suppressor protein, Rb (39). Whether Rb similarly interacts with Purβ is not known. However, a Puro sequence implicated in Rb binding, termed the “psycho” motif (39, 40), is largely conserved in Purβ, albeit with modification (10). We are currently exploring the potential of Rb in regulating VSM α-actin gene transcription. Irrespective of the outcome of these experiments, it seems quite clear that complex combinations of protein-protein and protein-ssDNA interactions are likely to be important to the ability of Puro, Purβ, and MSY1 to modulate the activity of the VSM α-actin MCAT enhancer element. Delineation of the effects of such interactions on both the topology and activity of this element is an important priority.

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