The Single-stranded DNA-binding Proteins, Purα, Purβ, and MSY1 Specifically Interact with an Exon 3-derived Mouse Vascular Smooth Muscle α-Actin Messenger RNA Sequence*

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Amino acids 44–53 of mouse vascular smooth muscle α-actin are encoded by a region of exon 3 that bears structural similarity to an essential MCAT enhancer element in the 5′ promoter of the gene. The single-stranded DNA-binding proteins, Purα, Purβ, and MSY1, interact with each other and with opposite strands of the enhancer to repress transcription in fibroblasts (Sun, S., Stoflet, E. S., Cogan, J. G., Strauch, A. R., and Getz, M. J. (1995) Mol. Cell. Biol. 15, 2429–2436; Kelm, R. J., Jr., Cogan, J. G., Elder, P. K., Strauch, A. R., and Getz, M. J. (1999) J. Biol. Chem. 274, 14238–14245). In this study, we employed both recombinant and fibroblast-derived proteins to demonstrate that all three proteins specifically interact with the mRNA counterpart of the exon 3 sequence in cell-free binding assays. When placed in the 5′-untranslated region of a reporter mRNA, the exon 3-derived sequence suppressed mRNA translation in transfected fibroblasts. Translational efficiency was restored by mutations that impaired mRNA binding of Purα, Purβ, and MSY1, implying that these proteins can also participate in messenger ribonucleoprotein formation in living cells. Additionally, primary structure determinants required for interaction of Purβ with single-stranded DNA, mRNA, and protein ligands were mapped by deletion mutagenesis. These experiments reveal highly specific protein-mRNA interactions that are potentially important in regulating expression of the vascular smooth muscle α-actin gene in fibroblasts.

In eukaryotes, protein synthesis is the end result of an integrated program of gene transcription, pre-mRNA processing, and mRNA transport, translation, and metabolism. The molecular mechanisms that function to integrate these diverse processes are largely unknown, but in some cases appear to involve multifunctional proteins capable of associating with regulatory sequences in both DNA and RNA. In Xenopus for example, FRGY2 was originally identified as an oocyte-specific transcription factor that associates with a DNA regulatory element termed the Y-box (1). However, recent studies have shown that FRGY2 also functions to repress mRNA translation, in part, by binding to a specific mRNA sequence motif (2). Thus, expression of FRGY2 leads to both increased transcription from promoters containing a Y-box and translational silencing of the mRNA transcripts (3, 4). Similarly, MSY1, the mouse homologue of FRGY2, has been proposed to have dual roles in activating germ cell-specific transcription in the testis and translational repression of the resulting mRNA (5). Both situations are reminiscent of the dual role of transcription factor IIIA in the synthesis and storage of 5 S rRNA in the Xenopus oocyte (6).

Evidence also exists to suggest that pre-mRNA splicing may be coupled to transcription, in part, through common regulatory proteins. In yeast, the group I intron splicing stimulatory protein, Cbp2, has been reported to enhance transcription of the mitochondrial COB gene (7), while in higher organisms, an apparent pre-mRNA splicing factor, heterogeneous nuclear ribonucleoprotein K, has been shown to stimulate RNA polymerase II transcription in vitro and to activate and repress transcription in vivo (8, 9). Thus, cotranscriptional splicing, which has been observed in a variety of different systems (10–12), may involve the participation of proteins that function as both transcription factors and pre-mRNA splicing factors.

Studies in our laboratory have centered on the participation of a group of single-stranded DNA (ssDNA)1-binding proteins in transcriptional regulation of the mouse vascular smooth muscle (VSM) α-actin gene in fibroblasts. These proteins, recently identified as the mouse Y-box protein, MSY1 (13), and the purine-rich ssDNA-binding proteins, Purα and Purβ (14), interact with opposite strands of a polypurine-polypyrimidine tract conserved in VSM α-actin gene promoters to negatively regulate transcription by a mechanism not yet fully understood (13, 15). This tract, designated the promoter element (PE), resides between −165 and −195 relative to the start site of transcription, contains an inverted core MCAT enhancer element (AGGAATG), and exhibits a high degree of purine-pyrimidine asymmetry (15). Interestingly, an inspection of the complete VSM α-actin cDNA and genomic sequences revealed an exon 3-derived sequence with strong similarity to the 5′ PE (16). This sequence, termed the coding element (CE), encoded amino acids 44–53 of the mature protein, exhibited a similar

1 The abbreviations used are: ssDNA, single-stranded DNA; VSM, vascular smooth muscle; VACesBF, vascular actin single-stranded DNA binding factor; PE, promoter element; CE, coding element; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; β-gal, β-galactosidase; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; ELISA, enzyme-linked immunosorbent assay; HRP, horse-radish peroxidase; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); UTR, untranslated region.

‡ Deceased.

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† Deceased.

This paper is dedicated to the memory of Dr. Michael John Getz, who died on October 10, 1999 as a result of complications of cancer. Mike will be remembered by students, faculty, and co-workers alike as an accomplished scientist, dedicated teacher, inspiring mentor, and loyal friend. We of the Getz laboratory will honor him by continuing the research that brought so much joy to his life.

*This paper is available on line at http://www.jbc.org

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were synthesized using a 2 Biosystems model 394 DNA/RNA synthesizer. RNA oligonucleotides ethylene glycol spacer (BioTEG CPG, Glen Research) on an Applied thesis using a biotin phosphoramidite containing a mixed polarity tri-gel purification.

denote transversion mutations. Underlined letters

transcript and encode amino acids 43–53 (caG-GGA-GUA-AUG-GUU-GGA-AUG-GGC-CAA-AAA-GAc, QGVMVGMGQKD). Therefore, 1, 17–19) as well as by a recent report that Pur may be capable of associating with a 7 SL-like RNA (20). Our results indicate that RNA binding of all three proteins is sequence-specific and does not appear to require protein-protein interaction. Moreover, mutational analyses of mouse Pur indicate that the determinants of ssDNA, mRNA, and protein binding differ from each other and from those previously reported for human Pur. These findings suggest that MSY1, Puro, and Purβ may be multifunctional regulators of VSM α-actin gene expression by virtue of specifically interacting with both DNA and RNA regulatory elements.

**EXPERIMENTAL PROCEDURES**

*Cell Culture, Transient Transfection, and Reporter Gene Assays—*

Cell culture and transfection of mouse embryo-derived AKR-2B fibroblasts were performed as described previously (16). Total protein in lysed transfectants was determined by BCA dye-binding assay (Pierce) using bovine serum albumin (BSA) as a standard. Chloramphenicol acetyltransferase (CAT) and β-galactosidase (β-gal) reporter proteins were measured by ELISA (Roche Molecular Biochemicals). Correctly initiated CAT mRNA transcripts were detected by primer extension analysis as described previously (16).

**Synthesis of DNA and RNA Oligonucleotide Probes—**Oligonucleotides possessing a 3′ biotin moiety were prepared by chemical synthesis using a biotin phosphoramidite containing a mixed polarity tri-ethyleneglycol spacer (BioTEG CPG, Glen Research) on an Applied Biosystems model 394 DNA/RNA synthesizer. RNA oligonucleotides were synthesized using 2′-OMe-RNA CE (β-Cyanoethyl) phosphoramidites (Glen Research). Following synthesis, all oligonucleotides were purified by gel filtration over a NAP-25 column in sterile water. A 120-base RNA oligonucleotide corresponding to a tetramer of VSM α-actin exon 3 coding element sequence, (GGGAGTAATGGTTGGAATGGGCCAAAAAGA), used in cDNA expression library screening and Northwestern blotting was subjected to additional polyacrylamide gel purification.

**Construction of Mouse Purβ Deletion Mutants—**Complementary DNAs encoding amino acids 2–263, 2–236, 2–186, 2–135, and 2–87 were amplified by PCR using primers that generated 5′ BamHI and 3′ Kpn1 cloning sites. The PCR products were agarose gel-purified, digested with restriction enzymes, and subcloned into pQE30 (Qiagen) to create fusion constructs encoding an N-terminal 6 histidine tag. Plasmids were transformed into Escherichia coli strain JM109 and the fidelity of the mutant Purβ cDNAs was verified by DNA sequencing. Mutant proteins were expressed and purified as described previously (13).

*Analytical Methods—*Western blotting, ELISA, and cDNA library screening were performed as described previously (13, 14, 16). For Northwestern and Southwestern blotting, PVDF transblots were blocked overnight in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20 (TBST) buffer with 5% (w/v) nonfat dry milk. Blots were probed with either CE-F, (Southwestern) or CE-RNA (Northwestern) oligonucleotide tetramers end-labeled with [%32P]ATP and T4 polynucleotide kinase. The RNA probe was diluted to ~100 ng/ml (~400,000 cpm/ml) in binding buffer consisting of 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA (TBSE), 2% nonfat dry milk supplemented with 4.0 μg/ml CE-RNA mutant 2 (160-fold molar excess), 10 μg/ml poly(dI-dC), and 10 μg/ml total ARK-2B RNA. The DNA probe was diluted to ~30 ng/ml (~100 cpm/ml) in the same binding buffer minus the RNAs but supplemented with a 100-fold molar excess of CE-Fmu2. Blots were incubated for 2 h at room temperature with gentle agitation. Blots were washed four times (10–12 ml/wash) with TBSE buffer containing 0.1% Tween 20 for 40–45 min total and then one final time with TBSE. Blots were air-dried for 20 min and then exposed to Kodak XAR-5 film for 10–90 min.

**Discontinuous Colorimetric Nucleic Acid Binding Assay—**Purified His-tagged Purβ (100-μl application of a 50 nM solution) was passively absorbed to polystyrene microtiter wells (Costar EIA strips) as described previously for ELISA (13). After an overnight incubation at 4 °C, the coating solution was aspirated and the wells washed twice with 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20 (HBSET). A blocking solution of 0.2% (w/v) ultrapure BSA (Roche Molecular Biochemicals) dissolved in HBSET was then applied (250 μl/well) for 2 h at room temperature. Blocking solution was removed, and a 50-μl solution of ssDNA or RNA oligonucleotide competitor diluted from 1000 to 0.064 nM in assay buffer (0.1% BSA-HBSET with 10 μg/ml poly(dI-dC)) was applied to selected wells in quadruplicate. This was followed immediately by the addition of a 50-μl solution of 1.0 nM 3′-biotinylated DNA probe diluted in assay buffer. After a 1-h incubation at 37 °C, the wells were washed three times with HBSET and a 100-μl solution of ExtrAvidin-HRP (Sigma) diluted 1:2000 in HBSET was applied. Following a 30-min incubation at 37 °C, wells were washed as above and 100 μl of the HRP chromogenic substrate, ABTS (Roche Molecular Biochemicals), was applied. Absorbance readings at 405 nm were determined after 15 min using a 96-well microplate spectrophotometer.

**RESULTS**

Puro, Purβ, and MSY1 Specifically Bind to an Exon 3-derived VSM α-Actin mRNA Sequence—To assess whether Puro, Purβ, or MSY1 could interact with the RNA sequence encoding amino acids 44–53 of VSM α-actin, we utilized a synthetic, biotinylated RNA oligonucleotide (CE-RNA, Table I) coupled to

**TABLE I Synthetic ssDNA and RNA probes used in this study**

| Designation | Type     | Sequencea                           |
|-------------|----------|-------------------------------------|
| PE-F        | ssDNA    | 5′-GGGAGTAATGGTTGGAATGGGCCAAAAAGA-3′ |
| PE-R        | ssDNA    | 5′-GGGAGTAATGGTTGGAATGGGCCAAAAAGA-3′ |
| CE-F        | ssDNA    | 5′-GGGAGTAATGGTTGGAATGGGCCAAAAAGA-3′ |
| CE-Fmu2     | ssDNA    | 5′-GGGAGTAATGGTTGGAATGGGCCAAAAAGA-3′ |
| CE-RNA      | RNA      | 5′-GGGAGTAATGGTTGGAATGGGCCAAAAAGA-3′ |
| CE-RNAmu2   | RNA      | 5′-GGGAGTAATGGTTGGAATGGGCCAAAAAGA-3′ |

a The location of the sequence element within the mouse VSM α-actin gene and its corresponding designation are indicated within parentheses. Nucleotides are numbered relative to the start site of transcription (+1). For simplicity, intron sequences have been omitted from the numbering scheme. Therefore, +201 to +299 refers to the location of the sequence within the mouse VSM α-actin cDNA while G/+201 denotes the intron 2/exon 3 splice site that would exist in pre-mRNA. The coding element RNA sequence (CE-RNA) as written would also be present in the mature transcript and encode amino acids 43–53 (caG-GGA-GUA-AUG-GUU-GGA-AUG-GGC-CAA-AAA-GAc, QGVMVGMGQKD). Underlined letters denote transition mutations.

were measured by ELISA (Roche Molecular Biochemicals). Correctly
paramagnetic particles in an attempt to capture MSY1 and/or Purα/Purβ from an AKR-2B fibroblast nuclear extract. As shown in the left panel of Fig. 1, MSY1 was selectively captured using either the pyrimidine-rich, reverse DNA strand of the promoter element (PE-R, lane 4) or the purine-rich, RNA counterpart of the coding element (CE-RNA, lane 5). MSY1 was not captured by the purine-rich, forward DNA strand of the promoter element (PE-F, lane 3), the forward DNA strand of the coding element (CE-F, lane 7), or a mutant RNA counterpart of the CE in which transversions were introduced into positions held in common with the PE (CE-RNAmu2, lane 6). Moreover, when a parallel blot was probed with a Pur protein specific antibody, both Purα and Purβ were detected using the CE-RNA-coupled particles (Fig. 1, right panel, lane 5). In contrast to MSY1 and consistent with previous results, both Pur proteins were also captured using the forward, purine-rich DNA strands of both the promoter (PE-F, lane 3) and coding (CE-F, lane 7) elements. Purα and Purβ were not captured, however, using the mutant RNA oligonucleotide (lane 6). These data indicate that all three proteins can specifically associate the same purine-rich, exon 3-derived CE-RNA sequence. This result was somewhat unexpected, given that MSY1 and the Pur proteins specifically bind to opposing pyrimidine-rich and purine-rich strands of the PE sequence, respectively (13, 14). Thus, while the ssDNA and mRNA sequences that bind the Pur proteins are related, the ssDNA and mRNA sequences that bind MSY1 are completely unrelated (compare PE-R with CE-RNA in Table I).

The Exon 3-derived RNA Sequence Suppresses Translation of a Heterologous Reporter Gene in a Manner Dependent on Purα, Purβ, and MSY1 Binding—Analysis of translational repression conferred by a protein-binding site located within the 5′-UTR of a reporter mRNA has been shown to be useful as a basic strategy for detecting RNA-protein interactions in living cells (21). We previously constructed several chimeric promoters in which the double-stranded CE sequence was incorporated both 3′ and 5′ of the transcriptional start site of a deletionally active VSM α-actin promoter-CAT reporter construct (VSMP4) to create P4/CE(3′) and P4/CE(5′), respectively (Fig. 2A) (16). When analyzed for CAT mRNA expression, the P4/CE(3′) construct was found to be transcriptionally identical to VSMP4 while the P4/CE(5′) construct was markedly repressed (16). However, the P4/CE(3′) was not tested for CAT protein output (16). Because care was taken to ensure that translational initiation at an internal VSM α-actin ATG codon within the 5′-UTR of the CAT reporter would not alter the CAT reading frame, we decided to evaluate whether or not this construct would exhibit impaired CAT protein expression as a function of Purα/Purβ/MSY1 binding to CE-RNA. Thus, P4/CE(3′) and P4/CEEmu2(3′) were tested in parallel transfections with their parent construct, VSMP4, and two transcriptionally repressed promoters, VSMP3 (22) and P4/CE(5′) (16), for both CAT protein and CAT mRNA expression in AKR-2B fibroblasts. As a measure of the relative abundance of CAT mRNA, correctly initiated CAT transcripts were assayed by primer extension analysis of poly(A)+ RNA harvested from mock transfected cells (mock) served as a control for the fidelity of the CAT mRNA primer.

**FIG. 1.** Pur proteins and MSY1 bind to opposing strands of the VSM α-actin PE and simultaneously to an exon 3-derived RNA sequence (CE-RNA). Parallel reaction mixtures containing 200 μg of AKR-2B fibroblast nuclear protein (14) and 100 pmol of biotinylated DNA or RNA oligonucleotide (lanes 3–7) were incubated as described previously (14). Nucleic acid-bound proteins were captured on streptavidin-coupled paramagnetic particles, washed three times with 20 mM HEPES, pH 8.0, 500 mM NaCl, and eluted with 2× Laemmli sample preparation buffer. Eluates were assayed by Western blotting using rabbit anti-PurB42–69 (13) for detection of both Purα and Purβ, or rabbit anti-MSY276–302 (13) for detection of MSY1 (left panel), and rabbit anti-PurB42–69 (13) for detection of both Purα and Purβ (right panel). Each lane represents the amount of Purα, Purβ, or MSY1 captured from 100 μg nuclear protein (i.e., 50% sample load). Lane 1 shows the immunoreactivity present in 10 μg of nuclear extract. Lane 2 is a control for nonspecific binding by paramagnetic particles.

**FIG. 2.** The CE sequence does not effect CAT mRNA transcription when positioned in the 5′-UTR of the CAT reporter gene. A, schematic representations of chimeric reporter gene constructs in which the CE sequence is positioned both 3′ and 5′ of transcriptional start site in the deletionally activated VSM α-actin promoter (VSMP4) are shown (16). B, VSM α-actin promoter constructs (10 μg) were tested in parallel transfections for CAT mRNA expression in AKR-2B fibroblasts. Cotransfection with 5 μg of a β-actin promoter construct (p99Ac-CAT; Ref. 22) served as a control for transfectional efficiency. To enhance mRNA accumulation, quiescent transfecants were co-stimulated for 6 h with 20% fetal bovine serum and 10 μg/ml cycloheximide (16). Cycloheximide inhibits normal transcriptional attenuation that occurs after serum stimulation (50). Correctly initiated CAT mRNA transcripts were assayed by primer extension analysis of poly(A)+ RNA isolated and pooled from four independent transfecants. Arrows indicate the positions of p99Ac-CAT (pAc, 129 nucleotides), VSMP4 (pAc, 168 nucleotides), P4/CE(3′) and P4/CEEmu2(3′) (pAc, 207 nucleotides) extension products. 32P markers (M, pBR322 MspI digest) are shown for reference. Primer extension of RNA harvested from mock transfected cells (mock) served as a control for the fidelity of the CAT mRNA primer.
Cotransfection with pCMV-promoter constructs (5 μg) shown in Fig. 2A were tested in parallel transfections for CAT protein expression in G0 arrested AKR-2B fibroblasts. Cotransfection with pCMV-βGal (0.1 μg) served as an internal control for transfection efficiency. Cellular extracts were assayed for total protein by BCA dye binding assay and for CAT and β-gal proteins by immunoblot, as shown in Fig. 3. CAT protein data were normalized for transfection efficiency. Cellular extracts were assayed for total protein. Values shown are the mean ± S.D. of three independent transfections.

Consistent with our previous data (16), the two repressed promoters, P4/CE(5') and VSMP3, produced clearly less CAT mRNA relative to VSMP4 while P4/CE(3') produced a comparable amount (Fig. 2B, lanes 1–4). Importantly, an export product of identical size (207 bases) and intensity was obtained from P4/CE(3') and P4/CEmu2(3'), indicating that insertion of the wild type CE, or a mutant thereof, into the 5'-UTR of VSMP4 had no substantive effect on the ability of the promoter to drive transcription of the CAT reporter gene (Fig. 2B, lanes 4 and 5).

Because quiescent, G0-arrested AKR-2B fibroblasts exhibit relatively low rates of protein synthesis (23), lysates of quiescent transfectants were assayed for CAT protein by quantitative immunoblotting. While VSMP3, VSMP4, and P4/CE(5') produced CAT protein at levels that were consistent with CAT mRNA expression, P4/CE(3') produced only 10% of the CAT protein of its parent construct, VSMP4 (Fig. 3). Importantly, mutation of the MSY1/Pur binding site in the 5'-UTR of VSMP4 had no substantive effect on the ability of the promoter to drive transcription of the CAT reporter gene (Fig. 2B, lanes 4 and 5).

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MSY1, Pur, and Purβ Can Independently Bind the Exon 3-derived CE-RNA Sequence, as Evidenced by RNA Binding Site-dependent cDNA Cloning and Northwestern Blotting—Because of the mutual interactions between MSY1 and the Pur proteins (13), these initial data did not allow us to determine whether all three proteins directly interacted with RNA, or whether one or more proteins interacted indirectly via protein-protein interaction. To approach this problem, a mouse lung cDNA expression library was screened with a tetramer of the exon 3-derived CE-RNA sequence. Such a strategy was successfully used to clone cDNAs encoding Purα and Purβ with the ssDNA counterpart of the CE-RNA sequence (CE-F) (14) and cDNAs encoding MSY1 with the pyrimidine-rich strand of the promoter element (PE-R) (13). Using a 32P-end-labeled tetramer of the CE-RNA sequence as a probe in the presence of a 160-fold molar excess of the CE-RNAμ2, seven individual clones were purified through tertiary screens. Fig. 4 shows a representative filter-binding assay for one of these clones in which ssDNA and RNA binding capacity was compared. All seven clones demonstrated the same binding specificity for CE-RNA but not CE-F (i.e. the ssDNA counterpart of the CE-RNA sequence) suggesting that these clones did not encode the Pur proteins. Indeed, subsequent DNA sequencing revealed that all seven clones encoded overlapping sequences corresponding to MSY1. These cloning data validated the results of Fig. 1 and again indicated that MSY1 can specifically interact with both the pyrimidine-rich strand of the promoter element sequence and an unrelated, purine-rich, exon 3-derived mRNA sequence. Moreover, interaction of MSY1 with CE-RNA does not require simultaneous Purα or Purβ binding. It is also noteworthy that this mRNA sequence is unrelated to a previously identified Y-box protein consensus RNA binding motif (5'-AACAU-3') (19).

The fact that no clones corresponding to Pur α or Purβ were obtained from 300,000 plaques initially screened suggested to us that the Pur cDNAs were either of low abundance relative to MSY1, or perhaps, the Purα/β-CE-RNA interaction observed in Fig. 1 occurred only indirectly via association with MSY1. To test for direct interaction between mouse Purα and Purβ with CE-RNA, we analyzed recombinant His-tagged proteins purified from E. coli (13) by Northwestern blotting using [32P](CE-RNA)₄ as a probe. As a control for loading equivalence, a control...
Ligand Binding Properties of Purβ Deletion Mutants Reveal Primary Structure Determinants Necessary for Interaction of Purβ with ssDNA, mRNA, Purα, and MSY1—Because of the unusual pattern of RNA binding displayed by the Purβ deletion mutants, we also asked whether the same was true for ssDNA binding. As shown in Fig. 6, Southwestern blotting of the Purβ mutants using the ssDNA counterpart of the CE-RNA tetramer (CE-F$_4$) as a probe revealed a markedly different profile. For example, the β186 mutant, which bound CE-RNA quite efficiently (Fig. 5, lane 6), showed no evidence of ssDNA-binding capacity (Fig. 6, lane 3). In fact, only the full-length and β263 proteins were capable of binding ssDNA efficiently (Fig. 6, lanes 1 and 6). Importantly, since the β236 mutant was impaired in its ability to bind both ssDNA (Fig. 6, lane 2) and RNA (Fig. 5, lane 5), the third basic-aromatic (class I) repeat is likely required for both ssDNA and RNA binding. However, because mutants such as β186 and, to a lesser extent, β135 also bind to RNA, additional sequences, such as the second class II repeat, or perhaps the N-terminal glycine-rich region, appear to assist in the Purβ-RNA interaction.

The affinity and specificity of the interaction between recombinant Purβ and ssDNA or RNA were also evaluated quantitatively using a discontinuous, colorimetric nucleic acid binding assay. Full-length, His-tagged Purβ was passively immobilized on microtiter wells and tested for its ability to bind fluid-phase biotinyl-CE-F (ssDNA) in the presence of varying amounts of unlabeled wild type and mutant ssDNA and RNA oligonucleotides. Complex formation between biotinyl-CE-F and Purβ was detected following subsequent incubations with HRP-coupled streptavidin and a chromogenic substrate. As shown in Fig. 7, the competition curves generated with CE-F and CE-RNA oligonucleotides do not overlap. This is likely a reflection of the non-identity of the ssDNA and RNA-binding regions within Purβ. However, despite the difference in apparent affinity between the CE-F (IC$_{50}$~25 nM) and CE-RNA (IC$_{50}$ >500 nM) oligonucleotides, Purβ binding to these sequences is nevertheless specific, as evidenced by the right-shifted curves obtained with the CE-Fmu2 (IC$_{50}$~150 nM) and CE-RNAmu2 (IC$_{50}$ >500 nM) oligonucleotides.

To address whether there is any overlap between sequences required for ssDNA/RNA binding and those required for protein-protein interaction, we utilized an ELISA method to test the protein-binding capacity of the Purβ deletion mutants. This assay system was previously used to identify interactions between Purα and Purβ and between Purα/Purβ and MSY1 in fibroblast nuclear extracts (13). As a control, the coating efficiency of each mutant was first assessed with respect to the

![Fig. 5. Binding of recombinant MSY1, Purα, Purβ, and Purβ C-terminal mutants to the CE-RNA sequence.](image-url)

![Fig. 6. Binding of Purβ C-terminal deletion mutants to the DNA counterpart of the CE-RNA sequence.](image-url)
full-length protein by ELISA using an antibody directed against an N-terminal peptide epitope present in all the mutants. As shown in Fig. 8, the coating efficiencies of β263, β186, and β135 did not differ substantially from the full-length protein. The β87 mutant displayed a somewhat higher coating efficiency, while the β236 mutant demonstrated a slightly lower coating efficiency. When tested for MSY1 and Purβ binding, only the full-length protein demonstrated high affinity binding capacity (Fig. 9). Importantly, deletion of amino acids 264–324 substantially impaired the ability of the β263 mutant to complex with either fibroblast-derived MSY1 or Purβ. This result is in stark contrast to the data obtained from analyses of nucleic acid binding, where this mutation appeared to have no adverse effect on the ability of the protein to bind either ssDNA (Fig. 6) and RNA (Fig. 5). However, because further deletion of amino acids 237–263 completely eliminates Pur and MSY1 binding (Fig. 9), we cannot exclude the possibility that there may be some partial overlap in amino acid sequences, which contribute to nucleic acid and protein binding capacity.

**DISCUSSION**

Earlier analyses of mouse vascular smooth muscle α-actin gene transcription in fibroblasts and undifferentiated myoblasts led to the formulation of a molecular model in which the activity of an essential, transcription enhancer factor 1-dependent, MCAT enhancer element located within a polypurine-polypyrimidine tract (~165 to ~185) was subject to negative regulation by sequence-specific, ssDNA-binding proteins (15, 25). It was later discovered that the purine-rich strand binding activity, then termed vascular actin single-stranded DNA-binding factor 2 (VACsaBP2), also interacted specifically with the sense strand of a protein-coding element (CE) sequence bearing similarity to the 5’ promoter element (PE) sequence (16). This CE sequence was located at the 5’ end of exon 3 and encoded amino acids 44–53 of VSM α-actin. The sense strand of the CE sequence was subsequently used to screen a mouse lung cDNA library and to isolate clones encoding the purine-rich strand-binding proteins, Puro and Purβ (14). DNA-binding analyses of Puro and Purβ expressed in mouse fibroblasts confirmed that these proteins were identical to the p46 and p44 components of VACsaBP2 (14). More recently, we employed the same binding site screening strategy to identify the mouse Y-box protein, MSY1, as VACsaBP1 (13). Biochemical studies conducted with peptide-specific polyclonal antibodies confirmed that fibroblast-derived Purα/Purβ and MSY1 specifically bind to opposite strands of the MCAT containing PE sequence and revealed that Puro and Purβ associate with each other and with MSY1 via DNA-independent protein-protein interactions (13). Armed with specific immune reagents (13) and knowledge that human Purα (20) and several Y-box proteins can also bind RNA (5, 17–19), we decided to test the mRNA counterpart of the aforementioned CE sequence as a target for Puro, Purβ, and MSY1 binding.

In this study, we present several lines of evidence that support the conclusion that mouse Puro, Purβ, and MSY1 can each specifically interact with a purine-rich mRNA sequence encoding amino acids 44–53 of VSM α-actin. First, all three proteins were efficiently captured from a fibroblast nuclear extract using a biotinylated synthetic CE-RNA oligonucleotide coupled to paramagnetic particles (Fig. 1). Second, multiple cDNAs encoding MSY1 were cloned from a mouse lung cDNA expression library using CE-RNA as a probe (Fig. 4). Third, purified re-
combinant Purα, Purβ, and MSY1 each independently bound a CE-RNA probe by Northwestern blotting (Fig. 5). Fourth, the wild-type CE sequence but not a mutant deficient in Purα, Purβ, and MSY1 binding, impaired translation of a CAT reporter mRNA when placed in the 5′-UTR of the CAT gene (Figs. 2 and 3). These new findings, together with our previous results, suggest that there are at least two potential regulatory binding sites for Purα, Purβ, and MSY1 in the mouse VSM α-actin gene. Although human and avian Purα (20, 26) as well as several Y-box protein homologues (5, 17–19) have been previously implicated in functionally relevant RNA binding, this is the first study, to our knowledge, to document the interaction of Purα, Purβ, and MSY1 with distinct ssDNA and mRNA-binding sites within the promoter and protein-coding regions of the same gene.

Because RNA binding by Purβ had not been previously reported, we also felt it important to identify amino acid sequences that contributed to RNA binding affinity in relation to those which mediated ssDNA and protein-protein interaction. Mouse Purβ is ~70% identical to mouse Purα at the amino acid level (14). Like Purα, Purβ is a modular protein characterized by a N-terminal domain (amino acids 1–39) rich in glycine residues, a central domain (amino acids 40–263) consisting of three basic-aromatic (class I) and two acidic-leucine rich (class II) repeats, a C-terminal domain (amino acids 264–324) containing a region of limited similarity to T-antigen (termed the psycho motif in Purα; Ref. 27), and a glutamate-rich tail (Ref. 14; see Fig. 5). Comparative ssDNA and RNA binding analyses of C-terminal deletion mutants revealed that amino acids 264–324 were dispensable for ssDNA and RNA binding (Figs. 5 and 6), while ELISA data suggested that this region was critical for high affinity binding to Purα and MSY1 (Fig. 9). Additional deletion of amino acids 237–263, which includes the third basic-aromatic repeat, eliminated ssDNA, RNA, and protein-binding capacity, implying that there is some partial overlap in amino acid sequences that mediate nucleic acid binding and protein-protein interaction. In contrast to these results, the third basic-aromatic repeat does not seem to be required for Purα binding to either ssDNA (27, 28) or RNA (20). Curiously, further deletion of amino acids 187–236 restored the ability of mutant Purβ to bind RNA (Fig. 5) but failed to restore ssDNA binding (Fig. 6). Amino acids 187–236 encompass an acidic-leucine repeat that is interrupted by a glycine-rich stretch in Purβ but not in Purα (Ref. 14; see Fig. 5). This structural difference could explain why this region appears to function as an inhibitory domain in Purβ-RNA binding. In Purα, sequences within this region appear to be required for binding to the purine-rich strand of c-myc PUR element (27) and to 7 SL-like RNA (20) but not to a single-stranded target sequence in the JC virus lytic control element (29). Thus, one cannot exclude the possibility that the relative importance of the second acidic-leucine repeat module to single-stranded nucleic acid binding by the Pur proteins may be dictated by the target sequence.

What is the functional consequence of Purα, Purβ, and MSY1 binding to VSM α-actin CE-RNA? Incorporation of this sequence into the 5′-UTR of a CAT reporter gene substantially reduced the efficiency of CAT protein translation in a manner that was dependent on nucleotides that contribute to high affinity Pur protein/MSY1 mRNA-binding (Figs. 1, 2, 3, and 7). Interestingly, this same CE sequence suppressed CAT mRNA transcription when positioned 5′ and directly flanking an enhancer-protein binding site in chimeric promoter-reporter gene constructs in a manner that was again dependent on Purα/Purβ (then termed VACssBF2) binding, but in this instance, to the CE-F DNA strand (16). While these results do not directly address the issue of the functional significance of Pur protein and/or MSY1 binding to the CE in its natural exonic position, they do, however, suggest that these proteins possess the capacity to influence gene expression at the level of transcription and translation. In this regard, it is noteworthy that Y-box protein homologues have been implicated in transcriptional activation (3, 30, 31), transcriptional repression (32, 33), and translational repression (4, 18). Similarly, Pur protein homologues have been implicated in both transcriptional activation (34–40) and repression (14, 41), and in several notable cases, appear to functionally cooperate with Y-box proteins (42, 43).

Clearly, one of the most striking and unexpected findings to emerge from these studies is that MSY1 exhibits specific binding to a pyrimidine-rich ssDNA sequence and to a completely unrelated purine-rich mRNA sequence within the same gene. This mRNA sequence is also unrelated to a previously characterized Xenopus Y-box protein (FRGY2) consensus RNA binding motif identified through in vitro selection (19) and implicated in translational repression (4). Although MSY1 has been proposed to similarly participate in translational masking and mRNA packaging (5), the distinctiveness of its VSM α-actin CE-RNA binding site suggests an alternative functional role. It is tempting to speculate that expression of the VSM α-actin gene in fibroblasts could be regulated through the coordinated interaction of Purα, Purβ, and MSY1 with promoter- and mRNA-derived sequences. For example, sequestration of Purα,
Purβ and MSY1 by virtue of binding to newly transcribed mRNA might establish an autoregulatory loop to augment VSM α-actin transcription. An analogous mechanism involving β-casein mRNA-dependent sequestration of a ssDNA-binding repressor has been proposed to explain the lactogenic hormone-induced expression of the β-casein gene in mammary epithelial cell (44). It is also possible that Purβ, Purγ, and MSY1 may function in regulating some other aspect of mRNA metabolism, such as pre-mRNA splicing. This is suggested by the similarity between the exon-derived CE sequence and a class of purine-rich sequences located within the exons of several different cellular and viral genes, which affect splice site selection and kinetics of pre-mRNA processing (45–49). The position of the CE precisely at the intron 2/exon 3 boundary lends further credence to this notion. Clearly, the significance of Purβ function in regulating some other aspect of mRNA metabolism, and MSY1 binding to VSM α-actin CE-RNA will only be resolved through further experimentation.

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