Cryptic MCAT Enhancer Regulation in Fibroblasts and Smooth Muscle Cells

SUPPRESSION OF TEF-1 MEDIATED ACTIVATION BY THE SINGLE-STRANDED DNA-BINDING PROTEINS, Purα, Purβ, and MSY1

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An asymmetric polyurine-polypyrimidine cis-element located in the 5′ region of the mouse vascular smooth muscle α-actin gene serves as a binding site for multiple proteins with specific affinity for either single- or double-stranded DNA. Here, we test the hypothesis that single-stranded DNA-binding proteins are responsible for preventing a cryptic MCAT enhancer centered within this element from cooperating with a nearby serum response factor-interacting CArG motif to trans-activate the minimal promoter in fibroblasts and smooth muscle cells. DNA binding studies revealed that the core MCAT sequence mediates binding of transcription enhancer factor-1 to the double-stranded polyurine-polypyrimidine element while flanking nucleotides account for interaction of Purα and Purβ with the purine-rich strand and MSY1 with the complementary pyrimidine-rich strand. Mutations that selectively impaired high affinity single-stranded DNA binding by fibroblast or smooth muscle cell-derived Purα, Purβ, and MSY1 in vitro, released the cryptic MCAT enhancer from repression in transfected cells. Additional experiments indicated that Purα, Purβ, and MSY1 also interact specifically, albeit weakly, with double-stranded DNA and with transcription enhancer factor-1. These results are consistent with two plausible models of cryptic MCAT enhancer regulation by Purα, Purβ, and MSY1 involving either competitive single-stranded DNA binding or masking of MCAT-bound transcription enhancer factor-1.

Current models of transcriptional repression take into account the ability of negative-acting factors to function in multiple capacities with or without DNA binding specificity (1, 2). For example, evidence exists that certain activator proteins can simply be masked or sequestered by protein binding partners that do not interact with DNA directly, as in the case of retinoblastoma tumor suppressor protein-mediated repression of the E2F family of trans-activators (3). Alternatively, a repressor protein may bind and displace components of the basal transcription machinery as in the case of Drosophila even-skipped-mediated repression of the Adh proximal promoter (4).

Recent work suggests that some repressors can participate, indirectly, in modifying chromatin structure by binding activator sites and recruiting specific histone deacetylases to silence genes through histone modification. This scenario is implicated in repression of E-box motifs through Mad/Max-mediated recruitment of histone deacetylases 1 and 2 (2). Still other models of repression propose that activator protein access to a DNA target sequence can be blocked by repressor protein binding to either the same site or an overlapping site. Such a mechanism appears to be operative in the competitive DNA binding of YY1 and serum response factor (SRF)1 to serum response elements (SREs) of the c-fos promoter (5).

An interesting variation on the theme of multiple proteins competing for overlapping binding sites arises from our attempts to elucidate the mechanism of vascular smooth muscle (VSM) α-actin promoter regulation in fibroblasts where plasticity of actin expression is necessary to accommodate switching to a contractile myofibroblast phenotype (6–8). In early studies, deletion mapping of the 5′-flanking region of the mouse VSM α-actin gene revealed several positive and negative cis-regulatory elements important for cell type-specific expression of promoter activity in skeletal myoblast-like cells versus embryonic fibroblasts (9, 10). While the region between –191 and –150 functioned as a positive transcriptional control element in transfected myogenic and fibroblastic cell lines, inclusion of the sequence spanning –224 to –191 restricted expression of the promoter to fully differentiated BC3H1 myogenic cells (9). Although the full –224 to –191 sequence was required to function as a negative regulatory element in undifferentiated myoblasts, retention of a relatively small GGGA motif located on the 3′ end (–195 to –192) was sufficient to confer complete repression in fibroblasts. This implied that the se-

1 The abbreviations used are: SRF, serum response factor; SRE, serum response element; VSM, vascular smooth muscle; TEF-1, transcription enhancer factor-1; Pu/Py, polyurine-polypyrimidine; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; VACSSBF, vascular actin single-stranded DNA-binding factor; SMC, smooth muscle cell; PE, promoter element; PBS, fetal bovine serum; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus; TV, transversion; pRb, retinoblastoma tumor suppressor protein.

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sequence spanning −195 to −150 contained closely aligned cis-elements responsible for both activation and repression of promoter activity in fibroblasts. In a later study, mutational analysis of several non-canonical SREs of the general form, CC(A/T)_{n}GG, located 3′ of the −191 to −150 positive control element revealed that a CArG box spanning −120 to −111 was essential for serum growth factor induction of a deletionally activated reporter in fibroblasts and for DNA binding by in vitro synthesized SRF (10). Similarly, mutation of an inverted muscle CAT (MCAT)-like motif (GGATAG) residing between −181 to −176 was also found to eliminate transcriptional activity of the same truncated reporter and to impair DNA binding by a fibroblast-derived factor provisionally identified as transcription enhancer factor-1 (TEF-1) (11). Collectively, these data suggested that functional cooperation between CArG-bound SRF and a MCAT-interacting factor related to TEF-1 was necessary for activation of the VSM α-actin promoter in fibroblasts once the MCAT enhancer was released from repression by proximate negative-acting factors.

Biochemical support for the existence of MCAT-suppressing factors came about when it was fortuitously noted that nucleotides spanning −195 to −165, which include the MCAT element, demonstrated a high degree of polyurine-polypyrimidine (Pu/Py) asymmetry (12), a characteristic common to many promoter-associated S1 nuclease-hypersensitive sites (13). Computer modeling of the sequence spanning −224 to −162 also indicated that this region possessed the theoretical potential to form a non-B-DNA cruciform structure with placement of the MCAT motif in a partially unpaired configuration. These observations prompted an assessment of the region encompassing the MCAT site as a target for single-stranded DNA (ssDNA)-binding proteins in vitro. Several sequence-specific vascular actin ssDNA-binding factors (VACsBFs) were identified in cell and tissue extracts that selectively bound to opposing strands of the Pu/Py element in manner that was consistent with suppression of MCAT enhancer activity (11, 12). These results led to the formulation of a hypothetical model whereby interaction of VACsBFs with opposing strands of the Pu/Py element was proposed to mediate transcriptional repression of the VSM α-actin promoter in fibroblasts (12). In this model, disruption or stabilization of MCAT enhancer base pairing by VACsBFs was envisioned to exclude binding of a TEF-1-like activator to double-stranded DNA (dsDNA) (14). Screening a mouse lung expression library with VACsBF target sequences subsequently resulted in the cloning of cDNAs encoding these putative repressor proteins and identification of VACsBF2 as Purα and Purβ (15), and VACsBF1 as MSY1 (16).

Purα and Purβ are highly homologous proteins that interact with the purine-rich strand of the Pu/Py element as homo- or heterodimers (16). They represent a newly delineated family of proteins whose founding member, Purα, appears to play a role in both cell growth and differentiation by modulating cell cycle progression as well as the replication and transcription of certain viral and cellular genes via interaction with specific single-stranded purine-rich elements of the general form, (GGN)\_n (17). MSY1 is a member of the evolutionarily conserved Y-box family of nucleic acid-binding proteins implicated in transcriptional and translational regulation (18). MSY1 binds to the pyrimidine-rich strand of the VSM α-actin Pu/Py element and can be detected in association with both Purα and Purβ in fibroblast cell extracts (16). Precedence exists for the human Y-box protein, YB-1, and human Purα to both physically and functionally associate to enhance the transcriptional activity of the JC virus promoter in glial cells (19). However, our data suggest that mouse Purα and Purβ together with MSY1 cooperate to repress transcriptional activity of the VSM α-actin promoter in fibroblasts. Owing to the high degree of sequence conservation between rodent and human Pur proteins (17) and Y-box proteins (16), it is unlikely that such functional differences are due to a species-specific phenomenon. Rather, promoter context and cellular environment are likely to be critical factors in determining the functional activity of Pur and Y-box proteins both individually and collectively (20). This contention is supported by this study in which a variety of experimental approaches are used to biochemically define the scope of protein-ssDNA, protein-dsDNA, and protein-protein interactions that may contribute to modulating the activity of a cryptic VSM α-actin MCAT enhancer element in rodent fibroblasts and smooth muscle cells (SMCs).

**EXPERIMENTAL PROCEDURES**

**VSM α-Actin Promoter-Reporter Constructs and Oligonucleotides**—Chloramphenicol acetyltransferase (CAT) reporter gene constructs containing the minimal mouse VSM α-actin promoter (VSMP7, −59/+48) or two additional promoter proximal 5′ flanking sequence (VSMP1, −1063/+48; VSMP2, −724/+48; VSMP3, −224/+48; VSMP4, −191/+46; VSMP5, −150/+46; VSMP6, −90/+46) were described previously (10). CarG box mutants of the VSMP4 reporter, VSMP4+31 and VSMP4Stu2 (10) and other transcriptionally repressed reporters including Δ195 (−195/+46) and P4/TV177 (VSMP4 with a 2 bp transversion mutation within the MCAT element) were also detailed in earlier reports (9, 11). The mutant reporter, Δ195-PvM, was constructed as follows. A Sull/BamHI (restriction sites underlined) fragment was generated by PCR amplification using 115S as a template with a 5′ primer (mutated bases in lowercase letters) (5′-TGGAGGTTGGAGTACG-GAACAGCAGCAGAATGCTTCCCACTAAAGCTTCTC-3′) and the 3′ primer (5′-AGATCTGTTGAGTCGAGCAGAGATGAGAATGCGAGTCTC-3′). The mutant promoter fragment was gel purified, cut with restriction enzymes, subcloned into pBlCAT3, and sequenced. All reporter plasmids used for transfection were purified by double cesium chloride gradient centrifugation.

Unlabeled and 3′-biotinylated oligonucleotides were synthesized on an Applied Biosystems model 394 DNA/RNA synthesizer. The double-stranded form of the mouse VSM α-actin Pu/Py promoter element (dsPE) containing a core MCAT motif (underlined) was generated by annealing synthetic oligonucleotides corresponding to the forward (PE-F) and reverse (PE-R) strands of the sequence spanning −194 to −165 relative to the transcription start site (5′-ggagcGAAAGACAGAGAATGAGAATGCGAGTCTC-3′). Mouse VSM α-actin CarG box (underlined) containing dsDNA probes were similarly generated by annealing complementary strands of sequences located −80 to −51 nucleotides (CarG1, 5′-gctttTTCGACTCCAGTGGACAGGACGcgtcTTTGCTCCTTGTTTGGGAGGCGagtg-3′) and −130 to −100 nucleotides (CarG2, 5′-gctttTTCGACTCCAGTGGACAGGACGcgtcTTTGCTCCTTGTTTGGGAGGCGagtg-3′-ctgcAGTGGACAGGACGcgtcTTTGCTCCTTGTTTGGGAGGCGagtg-3′) upstream of the transcription start site. To assist in 32P labeling, all dsDNA probes used for band shift assays were engineered with 4 base overhangs (lowercase letters) on each end to allow for filling-in by Klenow fragment. Blunt-ended biotinylated dsDNA probes used for pull-down of nucleic acid-binding proteins from cell extracts (see below) were prepared by annealing complementary oligonucleotides in which only one strand was biotinylated on the 3′ end.

**Cell Culture, Transient Transfection, and Reporter Gene Assays**—Rat A7r5 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS) and transiently transfected using GenePORTER™ reagent as directed by the manufacturer (Gene Therapy Systems). Mouse embryo-derived AKR-2B cells were cultured and transiently transfected as described previously (10). In experiments designed to compare the activity of various VSM α-actin promoter constructs in asynchronous cultures, 4 × 10^6 AKR-2B cells and 2 × 10^6 A7r5 cells were transfected with 4.8 μg of VSM α-actin promoter-CAT reporter and 0.2 μg of pCMVβ (CLONTECH, control β-galactosidase reporter) and harvested after 48 h incubation in complete growth medium. In experiments designed to compare the activity of selected VSM α-actin promoter constructs in synchronized cells, 5 × 10^6 AKR-2B or 2 × 10^6 A7r5 were transfected, serum-starved, and restimulated as previously described (10, 21). To harvest cell lysates, transfected cells were washed three times with phosphate-buffered saline and 0.5 ml of 1 × hypotonic CAT lysis buffer (Roche Molecular Biochemicals) was applied for 30 min. Whole cell lysates were collected after centrifugation at 14,000 × g for 10 min at 4°C and total protein content was...
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determined by BCA protein assay (Pierce) using bovine serum albumin as a standard. CAT and β-galactosidase reporter proteins were measured using colorimetric enzyme immunoassay kits as directed by the manufacturer (Roche Molecular Biochemicals). β-Galactosidase served as reference to monitor transfection efficiency between replicate transfectants.

**Rabbit Anti-mouse TEF and Other Antibodies**—Peptides corresponding to the first 15 amino acids of mouse TEF-1 (EPSSWSGSESPAE), TEF-3 (ITSNEWSSPDSPSPEG), TEF-4 (MGDPTRAPLDDGGG), and TEF-5 (IA5SNNWANSPPGEO) were synthesized, purified, and coupled to carrier protein as previously described (16). Immunization of rabbits and collection of antisera were carried out by a commercial vendor (Cocalico). Polyclonal antibodies were affinity purified from an immunoglobulin G (IgG)-enriched ammonium sulfate fraction of rabbit antiserum using peptide-coupled agarose columns. Details regarding the production and specificity of rabbit antibodies against mouse PurA, PurB, and MSY1 were described previously (16). Anti-human pBR (G3-245) was from PharMingen. Rabbit anti-RGS(H)4 was from Qiagen. Rabbit anti-TATA-binding protein (SI-1), rabbit anti-Sp3 (D-20), and rabbit anti-human pRb (G3-245) was from PharMingen. Rabbit anti-SRF (G-20), rabbit anti-TATA-binding protein (SI-1), rabbit anti-Sp3 (D-20), and mouse anti-c-Myc (9E10) were from Santa Cruz Biotechnology, Inc. Mouse anti-rGS(H)4 was from Qiagen.

**Biotinyl-DNA Pull-down Assay**—Nuclear protein (100–200 μg) extracted from AKR-2B fibroblasts or SMCs as previously described (15) was combined with 50–100 pmol of either wild type or mutant biotinylated DNA probe in binding buffer consisting of 10 mM HEPES, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 25 μg/ml 2-mercaptoethanol and heated at 95 °C for 5 min in preparation for SDS-PAGE and immunoblotting with either mouse anti-RGS(H)4, at 250 ng/ml or mouse anti-c-Myc at 40 ng/ml.

**RESULTS**

An MCAT Element in the 5’-Flanking Region of the Mouse VSM α-Actin Gene Functions as a Cryptic Enhancer Element in Both Fibroblasts and SMCs—To quantitatively compare the contribution of several consensus cis-regulatory elements to transcriptional activity of the minimal mouse VSM α-actin promoter in fibroblasts and SMCs, a variety of CAT reporter constructs containing deletion or point mutations of the 5’-flanking region (Fig. 1A) were transiently transfected into mouse AKR-2B or rat A7r5 cells. Unlike previous studies (9–11), all constructs were: 1) co-transfected with a pCMVβ (β-galactosidase) reporter to control for potential differences in transfection efficiency; 2) tested under identical experimental conditions; and 3) quantitatively compared relative to the minimal VSM α-actin promoter in both fibroblasts and SMCs. In AKR-2B fibroblasts, ~1 kb of 5’-flanking region failed to substantially enhance the transcriptional activity of the minimal promoter, defined empirically as TATA box-containing sequence spanning −59 to +46 relative to the start site of transcription (Fig. 1B, compare VSMP1 to VSMP7). However, deletion of nucleotide sequence between −1063 and −192 generated a reporter (−191 to +46:CAT or VSMP4) that produced ~10-fold more CAT protein than the minimal, TATA-driven promoter in fibroblasts (Fig. 1B, compare VSMP4 to VSMP7). Deletion or point mutation of a conserved MCAT motif spanning −181 to −176 was sufficient to reduce the activity of VSMP4 to basal levels (Fig. 1B, compare VSMP5 and P4/T177 to VSMP4 and VSMP7). Similarly, mutation of the second of two CArG elements located upstream of the TATA box (CarG2) also decreased VSMP4 activity by ~5-fold (Fig. 1B, compare P4/Stu2 to VSMP4). Alteration of the CarG element closest to the TATA box (CarG1) lowered activity by only ~2-fold (Fig. 1B, compare P4/Stu1 to VSMP4) suggesting that CarG2 is the more critical of the two CarG sites in terms of co-regulating promoter activation in concert with the MCAT element in VSMP4. Importantly, the enhanced transcriptional activity of VSMP4 was also eliminated by the addition of just 4 bp (GGGA) of 5’ VSM α-actin promoter sequence (Fig. 1B, compare Δ195 to VSMP4). These data confirmed that the MCAT element, while capable of conferring robust enhancer activity in cooperation with CarG2 in the deletionally activated VSMP4 reporter, is normally cryptic in the context of longer promoter constructs (e.g. Δ195, VSMP3, VSMP2, and VSMP1). Furthermore, although additional negative regulatory elements may reside between −196 to −224 (Fig. 1B, compare VSMP3 and Δ195), −195 to −192 constitutes the minimal enhancer sequence required to inactivate the MCAT enhancer and to reestablish basal promoter activity in AKR-2B fibroblasts (compare Δ195 to VSMP7).

To test whether the cryptic behavior of the MCAT enhancer was unique to AKR-2B fibroblasts, the same set of reporters were also evaluated for transcriptional activity in A7r5 cells, a rat aortic SMC line that exhibits a differentiated adult SMC phenotype (24). As shown in Fig. 1C, although the total activity of each reporter was about an order of magnitude greater in A7r5 cells relative to AKR-2B fibroblasts (compare y axes in B and C), the pattern of activity displayed among the reporters was remarkably similar in both cell lines. In particular, the Δ195 reporter was repressed relative to VSMP4, while mutation of the MCAT and CarG2 elements in the context of VSMP4 dramatically reduced promoter activity in both cell types (Fig. 1, compare P4/T177 and P4/Stu2 in B and C). One notable
Fig. 1. Truncation of the 5′-flanking region of mouse VSM α-actin exposes a cryptic MCAT enhancer which operates with a downstream CArG element to activate the minimal promoter in rodent fibroblasts and SMCs. Mouse AKR-2B fibroblasts (B) or rat A7r5 SMCs (C) were transiently transfected with 4.8 μg of the indicated VSM α-actin promoter:CAT reporter plasmids (A) along with 0.2 μg of pCMVβ. In VSMP4 mutant reporters, P4/Stu1 and P4/Stu2, a Stu1 restriction site was substituted for CArG1 and CArG2, respectively. P4/TV177 contains a 2-bp transversion mutation within the core MCAT element (see Fig. 3). Mutagenized elements are designated with an X. After a 48-h growth period in serum-containing medium, cell lysates were prepared and assayed for total protein, CAT, and β-galactosidase reporter expression. CAT values were normalized for total protein content and β-galactosidase values were used to correct for any differences in transfection efficiency between the various VSM α-actin reporters. Bars show the mean corrected CAT value (n = 4) and standard deviation for each construct.

The Double-stranded Form of the Mouse VSM α-Actin Pu/Py-rich Element Is a Target of TEF-1 Interaction in AKR-2B Fibroblasts—We next sought to define the molecular mechanism responsible for keeping the MCAT enhancer in a cryptic state by biochemically characterizing the protein-binding properties of wild type and mutant versions of the Pu/Py tract surrounding the core MCAT motif. To assist in identifying the
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presumptive MCAT-interacting trans-activator, rabbit polyclonal antibodies were raised against the divergent N termini of four known murine TEF gene products (26, 27). To evaluate the MCAT dependence and to definitively identify the TEF isoform(s) present in nucleoprotein complexes with the double-stranded Pu/Py element, EMSAs were conducted with wild type or mutant DNA probes, AKR-2B nuclear protein extracted selectively capture these proteins from an AKR-2B nuclear extract. As has been documented previously using this pull-down assay (16, 28), the purine-rich strand of the MCAT-containing Pu/Py element (PE) bound Pur (−46 kDa band) and Purβ (−44-kDa band) (Fig. 4, lane 2) whereas the complementary pyrimidine-rich strand (PE-R) specifically bound MSY1 (lane 10). Consistent with EMSA data, TEF-1 (−57-kDa band) interacted with the double-stranded Pu/Py element (dsPE) (Fig. 4, lanes 16 and 20) but failed to bind ssDNA (lanes 21 and 22) using the pull-down approach. The 2-bp transversion mutation within the core MCAT element (TV177) did not affect the binding of the Pur proteins and MSY1 to ssDNA under the experimental conditions employed (Fig. 4, lanes 6 and 12, respectively). However, this same mutation completely eliminated the binding of TEF-1 to dsDNA (Fig. 4, compare lanes 16 and 17). Importantly, mutation of specific nucleotides flanking the MCAT motif (PrM) did not prevent TEF-1 from binding to dsDNA (Fig. 4, compare lanes 16 and 18). Conversely, Purα, Purβ, and MSY1 binding to ssDNA was either abolished or weakened to a much greater extent by the flanking PrM mutations than by the MCAT transversion mutation (Fig. 4, compare lanes 6 and 14 to lanes 4 and 12). The dramatically reduced affinity of Pur proteins and MSY1 for opposing strands of the PrM mutant probe was independently confirmed by EMSA (Fig. 5A, compare lanes 2−4 with lanes 6−8 and B, compare lanes 10−12 with lanes 14−16). All three Pur-containing nucleoprotein complexes detected by EMSA (αα, ββ, and αβ) were similarly affected by this mutation. The composition of each nucleoprotein complex was established previously by

Effects of Mutations within or Flanking the MCAT Motif on Pu/Py Element Binding by Purα, Purβ, MSY1, and TEF-1—To ascertain the effects of MCAT mutations on Purα, Purβ, and MSY1 ssDNA-binding relative to TEF-1 dsDNA binding, we compared the ability of wild type and mutant biotinylated DNA probes in both single- and double-stranded configurations to

FIG. 2. The CArG1 and CArG2 elements of the mouse VSM α-actin promoter demonstrate differential SRF binding capacity. Varying amounts of AKR-2B nuclear protein (0, 1, 2, or 4 µg) were incubated with 32P-labeled probes containing mouse VSM α-actin CArG1 (lanes 1−4), CArG2 (lanes 5−10), or mutated CArG2 (lanes 11−16) possessing 2-bp transversions (lowercase letters) within the core CArG element (underlined). In some reactions, either nonimmune rabbit IgG (lanes 9 and 15) or a commercial SRF-specific antibody (lanes 10 and 16) was included. Nucleoprotein complexes were resolved by EMSA. SRF denotes the major complex detected with the CArG2 probe. Probe indicates unbound DNA, Ab, antibody; NE, nuclear extract; NS, nonspecific band; ss, supershifted complex.

FIG. 3. TEF-1 binds to the double-stranded form of the MCAT-containing Pu/Py element. Varying amounts of AKR-2B nuclear protein (0, 1, 2, or 4 µg) were incubated with 32P-labeled probes corresponding to the double-stranded VSM α-actin Pu/Py promoter element, dsPE (lanes 1−4), or to the MCAT mutant, dsTV177 (lanes 5−8). Nucleoprotein complexes were resolved by EMSA. The major nucleoprotein complex formed with the wild type dsPE probe is labeled TEF. In a separate experiment, the indicated N-terminal specific rabbit antimouse TEF IgGs (1.0 µg) were preincubated with AKR-2B nuclear protein (4 µg) prior to addition of 32P-dsPE probe (lanes 10−13). The supershifted complex formed with rabbit anti-mouse TEF-1 (lane 10) is designated ss. Probe denotes unbound DNA. NE, nuclear extract; NS, nonspecific band.
double-stranded configuration, flanking nucleotides are essential for high affinity ssDNA binding by Pur, Purβ, and MSY1.

Functional Implications of Differential Binding by Pur, Purβ, and MSY1 to Wild Type and Mutant Versions of the Pu/Py Element—In an attempt to define the molecular basis for the difference in activity between the VSMP4 (active) and Δ195 (repressed) reporters, a VSMP4-like version of the Pu/Py element (P4, −191 to −162) was evaluated for its protein-binding properties relative to the PE sequence spanning −194 to −165. As shown in Fig. 6A, analysis by EMSA revealed a striking reduction in total Pur protein binding to ssDNA when a 5′-GGA trinucleotide is removed from the PE forward strand (compare lanes 2−4 with lanes 6−8). Importantly, deletion of the 5′ GGA motif was nearly as effective as the PrM mutations at eliminating ssDNA binding by Pur and Purβ (compare Figs. 5A and 6A). Similar results were obtained when MSY1 binding to the reverse strands of the PE and P4 probes was tested by EMSA (Fig. 6B). In contrast, TEF-1 binding to dsDNA was unaffected by altering the 5′ end of the Pu/Py element (Fig. 6C). Hence, the marked reduction in Pur protein and MSY1 binding to opposite strands of the truncated Pu/Py sequence in vitro might explain why the promoter activities of Δ195 and VSMP4 differ in transfected fibroblasts and SMCs.

An experimentally testable prediction that follows from the results presented above is that mutations within Δ195 that preclude Pur, Purβ, and MSY1 ssDNA binding in vitro, should de-repress the promoter in living cells. To directly assess the functional significance of nucleotides flanking the MCAT element in the context of a repressed reporter, a Δ195-PrM mutant reporter (Fig. 7A) was constructed and tested for CAT expression in transiently transfected AKR-2B fibroblasts. As shown in Fig. 7B, Δ195-PrM was markedly more efficient than its parent construct, Δ195, in terms of driving CAT reporter expression in AKR-2B fibroblasts irrespective of the stimulation condition. Relative to Δ195, Δ195-PrM promoter activity was enhanced by ~6-fold in quiescent cells, ~7-fold in serum-stimulated cells, and ~15-fold in cells superinduced with serum and cycloheximide. In short, Δ195-PrM exhibited the same high level of transcriptional activity demonstrated by VSMP4, the deletionally activated reporter containing a truncated Pu/Py element and a functional MCAT enhancer (Fig. 7B). Hence, the flanking PrM point mutations were just as effective as the −195 to −192 5′ deletion in terms of freeing the cryptic

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FIG. 4. Mutation of the core MCAT element eliminates TEF-1 binding to dsDNA but does not prevent ssDNA-binding by Pur, Purβ, and MSY1. Parallel reaction mixtures containing 200 μg of AKR-2B nuclear protein and 100 pmol of biotinylated single- or double-stranded probes corresponding to wild type (PE) or mutant (PrM) versions of VSM α-actin Pu/Py element were incubated for 30 min. Biotinylated DNA and associated proteins were captured with streptavidin-coated paramagnetic particles and washed with HEPES-buffered NaCl as indicated. Proteins bound to DNA were eluted with SDS and analyzed by immunoblotting with affinity purified rabbit antibodies specific for mouse Pur, Purβ, and MSY1.

FIG. 5. Mutations flanking the MCAT element prevent ssDNA binding by Pur, Purβ, and MSY1. Varying amounts of AKR-2B nuclear protein (0, 1, 2, or 4 μg) were incubated with 32P-labeled probes corresponding to wild type (PE) or mutant (PrM) versions of the forward (F, coding) or reverse (R, non-coding) strands of the VSM α-actin Pu/Py element. Nucleoprotein complexes were resolved by EMSA. A, the migration pattern and composition of the heteromeric (αβ) and homomeric (αα, ββ) Pur-PE-F complexes were documented previously (16). B, identification of putative MSY1-containing nucleoprotein complexes is based upon results of pull-down experiments (see Fig. 4). Probe denotes unbound DNA. Oligonucleotide sequences are shown in the 5′ → 3′ direction. NS, nonspecific band.
These data are completely consistent with results obtained by Purα, Purβ, and MSY1 while leaving the MCAT element competent to bind TEF-1 (see Figs. 4–6), the transfection data strongly implicate Purα, Purβ, and MSY1 as negative regulators of MCAT enhancer activity in the AKR-2B fibroblast model.

To ascertain whether Purα, Purβ, and MSY1 function similarly in the A7r5 SMC model, the mutant Δ195-PrM construct was also evaluated for promoter activity in transiently transfected A7r5 cells. As shown in Fig. 7C, this mutant reporter demonstrated ~4-fold more promoter activity in A7r5 cells relative to its transcriptionally repressed parent construct, Δ195, under all treatment conditions. The absence of promoter induction by serum in A7r5 cells is consistent with the constitutive nature of the VSM α-actin gene expression in highly differentiated adult SMCs versus embryonic fibroblasts. To biochemically confirm that de-repression of the MCAT enhancer in SMCs could be correlated with loss of repressor protein binding to ssDNA comprising the Pu/Py element, biotinyl-DNA pull-down assays were performed using nuclear protein extracted from A7r5 cells. As shown in Fig. 8, A and B, rat Pur proteins were selectively captured with wild type Pu strand probe (PE-F, lane 2) and probe containing the 2-bp MCAT mutation (TV177-F, lane 5) while leaving the MCAT motif failed to bind Pur proteins (PrM-F, lane 7). Likewise, when the Pu strand binding properties of rat MSY1 (known as EF1α or rYB-1) were analyzed (Fig. 8C), A7r5-derived rYB-1 bound to the wild type probe (PE-R, lane 3) and the MCAT mutant (TV177-R, lane 6) but failed to interact with the PrM mutant probe (PrM-R, lane 8). In its double-stranded configuration, the Pu/Py element (dsPE) did not bind rat Pur, Purβ, or rYB-1 under high ionic strength wash conditions (see lane 4 in Fig. 8, A–C). On the other hand, TEF-1 binding to the wild type dsPE probe was clearly detected using this assay system (Fig. 8D, lane 1). Moreover, dsDNA binding by A7r5-derived TEF-1 was markedly reduced by the core MCAT mutation (dsTV177, lane 2, and dsP4TV177, lane 5) but not by the flanking PrM mutations (dsPrM, lane 3) or by the 5’ VSMP4-like deletion (dsP4, lane 4). These data are completely consistent with results obtained using mouse AKR-2B nuclear protein as a source of Purα, Purβ, MSY1, and TEF-1 (see Fig. 4).

Weak Binding by Purα, Purβ, and MSY1 to dsDNA—The preceding biochemical data did not allow us to strictly exclude the possibility that Purα, Purβ, and MSY1 may also bind to opposing strands of the Pu/Py when in its B-DNA conformation with or without simultaneous binding of TEF-1. To address this issue, we performed pull-down assays with wild type and mutant forms of the Pu/Py element under low and high stringency wash conditions (i.e. 500 mM NaCl) where sequence-specific ssDNA binding is clearly observed (Fig. 4). AKR-2B-derived Purα, Purβ, and MSY1 did not interact with the double-stranded Pu/Py element (data not shown). This is consistent with previous findings made on the basis of EMSAs (12) and pull-down assays using A7r5 nuclear protein (Fig. 8, A-C, lane 4). However, using low stringency washes (i.e. 75 mM NaCl), Purα, Purβ, and MSY1 interaction with dsDNA was clearly detectable and inhibited by the same PrM mutations that impaired higher affinity ssDNA binding (Fig. 9, compare lanes 2 and 6 to lanes 4 and 8).
Evidence for Protein-Protein Interaction among Repressors (Pura, Purβ, and MSY1) and Activators (TEF-1 and SRF)—We have previously established that Pur a and Purβ can associate with each other and with MSY1 in the context of a cell extract and independent of DNA binding (16). To ascertain whether interaction of Pura and/or Purβ with TEF-1 could be similarly detected, we performed co-immunoprecipitation assays using whole cell extracts of AKR-2B fibroblasts expressing either His-tagged Purα or His-tagged Purβ. As shown in Fig. 10, A and B, anti-TEF-1 IgG purified from two different lots of rabbit antisera (lanes 4 and 5) co-precipitated His-Pura (A) and His-Purβ (B) while negative control IgGs including nonimmune rabbit IgG (lane 1) and mouse IgG (lane 9) did not. Consistent with previous findings using non-tagged proteins (16), positive control antibodies, anti-Pura 291–313 (lane 2), and anti-Purβ 302–324 (lane 3), precipitated both His-Pura (A) and His-Purβ

Curiously, unlike the pattern observed with ssDNA probes (Fig. 4), the TV177 mutation, which disrupts the core MCAT element, also eliminated interaction of Purα, Purβ, and MSY1 with dsDNA (Fig. 9, compare lanes 2 and 6 to lanes 3 and 7). Because this mutation also impairs dsDNA binding by TEF-1 (Fig. 4, lane 17, and Fig. 8D, lane 2), these results suggest that co-association of Pura, Purβ, and MSY1 with dsDNA may require simultaneous TEF-1-MCAT interaction. It is important to note that these data do not allow us to discount the possibility that Purα, Purβ, MSY1 (repressors), and TEF-1 (activator) bind to the double-stranded Pu/Py element independently. But unlike TEF-1, the determinants required to facilitate weak interaction of Pura, Purβ, and MSY1 with the double-stranded Pu/Py element include not only the core MCAT element but also flanking nucleotides implicated in mediating high affinity binding to ssDNA.
Whole cell protein (100 μg) for TEF-1 and SRF. MSY1 expressed in AKR-2B fibroblasts with antibodies specific to His-Pur/β-H9251 and MSY1/H9252 combined with the indicated rabbit polyclonal antibodies (2.5 μg). Sheep anti-rabbit IgG-coupled magnetic beads were used to isolate and wash IgG complexes. After elution with SDS, precipitated proteins were subjected to SDS-PAGE and immunoblotting with mouse anti-RGS(H)4, anti-pRb, anti-TEF-1, or Myc-tagged MSY1 as well. As shown in Fig. 11, using extracts of AKR-2B cells expressing either Myc-tagged MSY1 or Myc-TEF-1 (C and D). The peptide epitopes recognized by the antibodies used for precipitation are designated in parentheses. Markers are indicated in kDa.

In addition, commercial antibodies against other known Pur-interacting proteins including Sp3 (lane 7) and retinoblastoma tumor suppressor protein, pRb (lane 8), also co-precipitated His-Pur/β (A) and His-Pur/β (B) using this assay system. To evaluate whether TEF-1 could also be co-precipitated using anti-Pur antibodies and to test for interaction between TEF-1 and MSY1, immunoprecipitation experiments were repeated using extracts of AKR-2B cells expressing either Myc-tagged TEF-1 or Myc-tagged MSY1 as well. As shown in Fig. 11, antibody specific to the N terminus of TEF-1 (lane 5) precipitated His-Pur/β (A), His-Pur/β (B), Myc-MSY1 (C), and Myc-TEF-1 (D) while antibodies against Pur/β (lanes 2, Pur/β lane 3), His-Pur/β (lane 4), precipitated His-Pur/β (A), His-Pur/β (B), Myc-MSY1 (C), and Myc-TEF-1 (D). These interactions were highly specific and unlikely due to fortuitous antibody cross-reactivity since the His- or Myc-tagged proteins were not precipitated by nonimmune rabbit IgG or anti-TATA-binding protein (Fig. 11, lanes 1 and 7). As an additional positive control for TEF-1 interaction, a commercial SRF antibody was also tested. As expected, Myc-TEF-1 was pulled-down by the SRF antibody (Fig. 11D, lane 6). However, co-precipitation of His-Pur/β (A), His-Pur/β (B), and Myc-MSY1 (C) by anti-SRF was also observed (Fig. 11, lane 6). These results expand the potential repertoire of Pur/β, His-Pur/β, and MSY1 protein-binding partners to include not only TEF-1, but also SRF. Such interactions could conceivably effect the ability of TEF-1, and perhaps SRF, to trans-activate the VSM α-actin promoter in fibroblasts and SMCs (see “Discussion”).

**DISCUSSION**

Using mouse AKR-2B and A7r5 cell lines as models for myofibroblast and SMC differentiation, respectively, in vivo cis-element mapping studies and in vitro protein-DNA and protein-protein binding analyses suggest that VSM α-actin gene expression is regulated by a complex set of interactions involving ssDNA-binding repressors and dsDNA-binding activators. One of the activators, TEF-1, is the prototype of the TEA/ATTS family of DNA-binding proteins. This family includes TEF-1, which was originally identified in HeLa cells by virtue of its interaction with the simian virus 40 GT-IIC and SpH++II enhancers (29), and other TEA domain-containing and/or TEF-1-related homologues from human, mouse, chicken, yeast, *Drosophila melanogaster*, and *Aspergillus nidulans* (30). Although family members encoded by the vertebrate genome (TEF-1, TEF-3, TEF-4, and TEF-5) demonstrate a high degree of amino acid sequence similarity, clear differences in cooperative DNA binding potential among TEF-1, TEF-3, and TEF-4 proteins have been noted (26). Moreover, the transcripts encoded by the four related TEF genes appear to be differentially expressed in both cultured cell lines (26, 31) and in developing tissues during mouse embryogenesis (27, 26). In particular, while widely expressed at early gestational stages, TEF-1 shows preferential expression in mitotic neuroblasts, the developing myocardium, and muscle anlagen at mid-gestational stages and persistent expression at late stages in developing skeletal muscle and differentiating myocardium (26).

Hence, it is not surprising that TEF-1 has been implicated in the transcriptional control of a variety of cardiac, skeletal, and smooth muscle-associated genes via interaction with MCAT-containing cis-regulatory elements (30, 32). Here, we provide compelling physical evidence (Figs. 3, 4, 6, and 8) for the participation of TEF-1 in modulating mouse VSM α-actin gene transcription in rodent fibroblasts and SMCs via a cryptic MCAT enhancer element (Figs. 1 and 7).

Although TEF-1 is apparently required for enhanced promoter activity in the deletionally activated VSMP4 reporter, it clearly cannot function autonomously. In addition to the cryptic MCAT enhancer, two downstream CArG elements (CArG1, −70 to −61; and CArG2, −120 to −111) are also implicated in VSM α-actin promoter activity in AKR-2B fibroblasts and A7r5 SMCs (Fig. 1). In a previous study, dissection of these elements from their native context by linkage to the minimal thymidine kinase promoter, revealed that both elements functioned as relatively weak SREs in the context of a heterologous promoter when compared with a canonical β-actin SRE (10). However, when CArG2 is mutated in the context of the VSMP4 reporter, promoter activity is reduced to near basal levels. That CArG2 functions as a strong, promoter context-dependent SRE in VSMP4 is consistent with our biochemical studies demonstrating that markedly greater SRF binding occurs to a VSM α-actin CArG2 probe rather than a CArG1 probe in vitro, when AKR-2B nuclear protein is used as a source of SRF (Fig. 2). The sequence difference between the individual CArG elements may underlie this difference in SRF binding capacity with perhaps the TATA-like sequence of CArG2 mediating enhanced SRF binding affinity. Alternatively, the differential SRF binding affinity and transcriptional activity of the mouse VSM α-actin CArG elements may reflect their noncanonical sequences in which a single G or C is substituted in the 6-bp A/T-rich core. It is also possible that cooperative interactions with other factors, such as TEF-1, may enhance binding or recruitment of SRF to specific VSM α-actin CArG elements.

The mutual dependence of TEF-1 and SRF in up-regulating VSM α-actin promoter activity in fibroblasts and SMCs is substantially by the loss of enhancer activity that occurs upon mutation of either the MCAT or CArG2 elements in VSMP4 (Fig. 1). Importantly, such cooperativity has also been independently observed in the skeletal α-actin promoter where SRF and TEF-1 appear to function in concert to mediate TGF-β1
and α1-adrenergic stimulation in cardiac myocytes (33, 34). However, it is important to note that such a cooperative mechanism may be cell-type and/or promoter-context specific. For example, in cultured primary rat SMCs, there is no evidence for CArG and MCAT (i.e. SRF and TEF-1) collaboration in activating the rat VSM α-actin promoter (35). In fact, Owens and co-workers (25) have reported that the two proximal SRF-interacting CArG elements (CArG 1 and 2 or CArG A and B in Ref. 25) are necessary and sufficient for activation of the rat VSM α-actin promoter in primary rat SMCs (25, 36, 37). Our results would suggest that CArG2 is the more critical of the two proximal CArG elements in terms of MCAT enhancer cooperation and mouse VSM α-actin promoter activation in both mouse AKR-2B fibroblasts and clonal rat A7r5 SMCs. While it is evident that the core MCAT element spanning −181 to −176 is required for mediating high level transcriptional activation of the mouse VSM α-actin promoter in these model cell lines, it is equally apparent that flanking sequences can modulate the activity of the enhancer in a negative fashion. The addition of the 4-bp sequence (GGGA) to the 5′ end of VSMp4 is sufficient to convert VSMp4 to a transcriptionally repressed construct (Δ195) (Figs. 1 and 7). Importantly, at least three of these bases are required for creating a high affinity binding site for Purα and Purβ, the ssDNA-binding proteins that interact with the purine-rich strand of the MCAT-containing Pu/Py-element in vitro (Figs. 4, 6, and 8 and Refs. 15, 16, and 28). In this study, we now provide evidence suggesting that the molecular basis for the difference in activity between VSMp4 and Δ195 is directly related to the relative binding capacity of Pur proteins for full-length and truncated versions of the Pu/Py sequence (Fig. 6). Moreover, we also show that mutation of nucleotides flanking the MCAT element which eliminate ssDNA binding of Purα, Purβ, and MSY1 to opposite strands of Pu/Py element in vitro (Figs. 4, 5, and 8), de-repress the Δ195 promoter in cultured fibroblasts and SMCs (Fig. 7). It is noteworthy that, in the context of the Δ195 reporter, de-repressing mutations involved located both 5′ (−194/−193, −190/−189) and 3′ (−171 to −165) of the core MCAT motif. Collectively, these data reinforce the idea that these three ssDNA-binding proteins are intimately involved in negatively regulating MCAT enhancer activity and VSM α-actin gene transcription in fibroblasts and SMCs as proposed in earlier studies before their identity was known (11, 12). Although the involvement of ssDNA-binding proteins is somewhat novel, the relative importance of flanking sequences and associated DNA-binding factors in modulating the cell type-specific activity of MCAT elements is a concept that has been acknowledged by others (38).

What is the molecular mechanism by which Purα, Purβ, and MSY1 mediate transcriptional repression in the context of the VSM α-actin gene promoter? The model which was initially proposed was a simple competitive one, in which simultaneous binding of Purα, Purβ, and MSY1 to opposing strands of the DNA helix comprising the Pu/Py element was conceived to promote or stabilize disruption of MCAT enhancer base pairing (Fig. 12A and Refs. 12 and 14). Such a nucleoprotein complex would necessarily preclude the binding of TEF-1 to dsDNA, and as a consequence, prevent promoter activation. While conceptually consistent with the high affinity ssDNA-binding properties of Purα, Purβ, and MSY1 in vitro, this model rests on a thermodynamically unfavorable assumption that the MCAT enhancer is capable of existing in a sustained, presumably ssDNA-binding protein-stabilized, non-B-DNA conformation in the cell (Fig. 12A). Curiously, nucleoprotein pull-down assays conducted with dsDNA clearly indicate that these ssDNA-binding proteins can also be detected in association with the Pu/Py element in its double-stranded configuration (Fig. 9). Taking into account that the experimental conditions under which these data were acquired were less stringent than the conditions used to detect interaction with ssDNA, dsDNA binding by Purα, Purβ, and MSY1 appears to be intrinsically weaker at least in vitro. Nevertheless, we cannot ignore the fact that the same mutations that block co-association of Purα, Purβ, and MSY1 with dsDNA also result in complete de-repression of the MCAT enhancer when functionally assayed in the context of plasmid-based reporter gene constructs transfected into fibroblasts and SMCs (Fig. 7). Hence, we are compelled to also offer an alternative model in which simultaneous binding of Purα, Purβ, and MSY1 to dsDNA is proposed to create a cryptic MCAT enhancer by masking TEF-1 (Fig. 12B). In this model, strand-specific contact by Purα, Purβ, and MSY1 with nucleotides flanking the MCAT motif would, in theory, stabilize the formation of a macromolecular complex containing TEF-1 that is transcriptionally inactive. In this scenario, one does not need to invoke bizarre alterations in DNA secondary structure to explain the contribution of Purα, Purβ, and MSY1 to masking MCAT enhancer activity in cultured fibroblasts and SMCs.

Credence for the masking model is enhanced by our additional co-immunoprecipitation data that expand the repertoire of protein interaction partners of Purα, Purβ, and MSY1 to include not only TEF-1, but also apparently, SRF (Figs. 10 and 11). Such an observation is quite interesting given the precedent that human Purα can bind to other transcriptionally relevant proteins such as pRb (26, 39), MyEF-2 (40), JC virus T-antigen (41), HIV-1 Tat protein (42, 43), YB-1 (19), E2F-1 (44), Sp1 (45), and hmRNP K (46). Detection of Purα, Purβ, and MSY1 co-immunoprecipitating with TEF-1 (and SRF) provides a strong biochemical basis for future studies aimed at evaluating whether such protein-protein associations might interfere with the ability of TEF-1 and SRF to interact with each other, with coactivating factors, or with components of the general transcription factor complex. In this regard, a recent report showing direct protein-protein binding between TEF-1 and SRF (47), leads one to question whether this interaction is subject to regulation by Purα, Purβ, MSY1, or some combination thereof. Furthermore, in light of the changes in DNA structure surrounding the genomic Pu/Py element that accompanies myofibroblast differentiation (48), analysis of nucleoprotein complex assembly/disassembly on the genomic promoter will likely provide further insight into the mechanism of MCAT enhancer regulation by Purα, Purβ, MSY1 vis-à-vis the proposed models.
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