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Negative Regulation of the Vascular Smooth Muscle α-Actin Gene in Fibroblasts and Myoblasts: Disruption of Enhancer Function by Sequence-Specific Single-Stranded-DNA-Binding Proteins

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Transcriptional activation and repression of the vascular smooth muscle (VSM) α-actin gene in myoblasts and fibroblasts is mediated, in part, by positive and negative elements contained within an approximately 30-bp polypurine-polypyrimidine tract. This region contains binding sites for an essential transcription-activating protein, identified as transcriptional enhancer factor 1 (TEF-1), and two tissue-restrictive, sequence-specific, single-stranded-DNA-binding activities termed VACssBF1 and VACssBF2. TEF-1 has no detectable single-stranded-DNA-binding activity, while VACssBF1 and VACssBF2 have little, if any, affinity for double-stranded DNA. Site-specific mutagenesis experiments demonstrate that the determinants of VACssBF1 and VACssBF2 binding lie on opposite strands of the DNA helix and include the TEF-1 recognition sequence. Functional analysis of this region reveals that the CCAAT box-binding protein nuclear factor Y (NF-Y) can substitute for TEF-1 in activating VSM α-actin transcription but that the TEF-1-binding site is essential for the maintenance of full transcriptional repression. Importantly, replacement of the TEF-1-binding site with that for NF-Y diminishes the ability of VACssBF1 and VACssBF2 to bind to separated single strands. Additional activating mutations have been identified which lie outside of the TEF-1-binding site but which also impair single-stranded-DNA-binding activity. These data support a model in which VACssBF1 and VACssBF2 function as repressors of VSM α-actin transcription by stabilizing a local single-stranded-DNA conformation, thus precluding double-stranded-DNA binding by the essential transcriptional activator TEF-1.

DNA is a structurally dynamic macromolecule which is capable of assuming a number of alternative conformations containing single-stranded regions. These topologically distinct conformations include localized single-stranded "bubbles," cruciform structures, the junctions between B- and Z-DNA, H-DNA, and several other non-B-DNA structures (reviewed in reference 37). Since the intracellular norms of temperature, pH, and ionic strength are hostile to DNA melting, it is generally assumed that the formation and maintenance of single-stranded DNA (ssDNA) within the nucleus require the action of effector molecules such as proteins or cRNA, whose role would be to initiate and maintain localized single-stranded conformations.

The sensitivity of chromatin-associated DNA to single-strand-specific nucleases or chemical reagents that specifically modify ssDNA has suggested the presence of ssDNA within the promoter regions of numerous genes (reviewed in reference 37). Indeed, in several cases, the formation of S1 nuclease-sensitive sites in the 5′-flanking regions of genes correlates with the onset of cell type-specific gene expression (16, 22), and a number of ssDNA-binding proteins have been identified which interact with specific promoter sequences (1, 8, 14, 23, 34, 35). Such findings are generally interpreted to indicate that the generation of ssDNA within promoter regions might play a key role in the control of transcription. To date, however, the actual mechanisms by which ssDNA contributes to specific gene regulation remain obscure.

The vascular smooth muscle (VSM) α-actin gene encodes the predominant isoform of the contractile protein actin found in blood vessel walls. However, expression of this gene is not restricted to differentiated smooth muscle cells but occurs in certain other cells and tissues, including a specialized cell type termed a myofibroblast (27, 30). Myofibroblasts display properties intermediate between those of smooth muscle cells and fibroblasts and typically appear during the granulation phase of wound healing, where they are believed to provide the major contractile force for wound closure (6, 30). While myofibroblasts are present only transiently during normal wound healing, they persist in fibrotic lesions of many organs (27, 30) including stromal reactions to tumors of epithelial origin (3, 17, 28, 29). Although the cellular precursor of myofibroblasts has been a controversial issue, recent evidence suggests that myofibroblasts arise from normal cutaneous fibroblasts under the influence of transforming growth factor β1 (TGF-β1) (9, 25). During wound healing, TGF-β1 is released by platelets and other cells associated with the inflammatory response, while there is evidence to suggest that in tumor stroma, the source of TGF-β1 is the tumor cells themselves (25). The expression of the VSM α-actin gene in both differentiating smooth muscle cells and myofibroblasts suggests a degree of plasticity in the mechanisms which regulate VSM α-actin transcription. Thus, we have been interested in delineating the mechanisms which govern VSM α-actin transcription in both cell types.

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We have previously shown that expression of the mouse VSM α-actin gene in both cultured AKR-2B fibroblasts and cytodifferentiating BC3H1 myoblasts is regulated by the concerted action of a number of positive and negative *cis*-acting promoter elements (11, 31). One of these was shown to represent an essential binding site for an activating protein closely related to, or perhaps identical to, the enhancer-binding protein TEF-1 (4), previously known as M-CAT binding factor (20). Interestingly, the TEF-1-binding site was found to be in close proximity to a strong negative control region and to be centered within a polypurine-polypyrimidine tract, a sequence motif frequently associated with S1 nuclease-sensitive sites in the promoter regions of a variety of genes (37). Deletion of a 5′ portion of the polypurine-polypyrimidine tract resulted in a strong transcriptional activation in both fibroblasts and undifferentiated myoblasts (11, 31), suggesting the possibility that transcriptional repression requires the formation of ssDNA in the vicinity of the TEF-1-binding site. Most recently, we identified two strand-specific ssDNA-binding proteins in both myoblast and fibroblast cellular extracts which bound to single-stranded oligonucleotide probes encompassing the polypurine-polypyrimidine tract (4). In vivo, both ssDNA-binding activities appeared highly restricted to tissues enriched in smooth muscle, suggesting a role in regulating the smooth muscle phenotype.

In the present study, we further characterized these ssDNA-binding factors and found that nucleotides critical to their binding include, but are not limited to, the TEF-1-binding site. Functional analyses of these sequences by enhancer substitution and other site-directed mutagenesis strategies suggest that the ssDNA-binding factors repress VSM α-actin transcription by disrupting base pairing within the TEF-1 enhancer element. These findings add to the growing body of evidence that ssDNA-binding proteins play an important role in regulating specific gene transcription in higher eukaryotes.

MATERIALS AND METHODS

Cell culture and transfection. Cell culture and transfection of mouse embryo-derived AKR-2B fibroblasts and BC3H1 myogenic cells were performed as previously described (11, 31). Chloramphenicol acetyltransferase (CAT) activity was determined as described previously (12) and quantitated in an AMBIS radioanalytic scanner.

Construction of VSM α-actin transversion mutants and chimeric VSM α-actin and β-actin constructs. Transversion mutants of VSM α-actin promoter construct pC3VSMP3 (VSMP3) (31) and chimeric VSM α-actin and β-actin promoters were made by PCR amplification. Oligonucleotide primers with a 5′ *Sal*I restriction site, which create transversion mutations (G↔T, A↔C) or anchor sequence substitutes, were made on an Applied Biosystems 394 DNA/RNA synthesizer and desalted over a Sephadex G-25 NAP column (Pharmacia) in distilled water. In PCR amplifications, each of these primers was paired with *Bam*27, the 3′ primer with a *Bam*HI restriction site. Briefly, primers were annealed to 0.1 to 1 μg of template DNA (VSMP3) and amplified for 30 to 35 cycles in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, Conn.) with standard PCR kit (Perkin-Elmer-Cetus) reaction mixes and times (94°C for 1 min, 50°C for 2 min, and 72°C for 3 min). PCR products were gel purified from a 1 to 2% agarose gel on spin-X columns (Costar, Cambridge, Mass.). VSM or chimeric fragments were then *Sal*I-*Bam*HI digested and ligated into the promoterless reporter plasmid pBLCAT3 (18) that was similarly digested and treated with calf intestinal alkaline phosphatase. Transformation into *Escherichia coli* HB101 cells was done by standard $CaCl_2$ techniques. All cloned sequences described in this paper were confirmed by double-stranded dideoxy sequencing with a Sequenase kit (United States Biochemical, Cleveland, Ohio). DNA used in transfection experiments was purified by double cesium chloride gradient centrifugation.

EMSA. Electrophoretic mobility shift assays (EMSAs) were performed as previously described (4). Briefly, single-stranded oligonucleotides were labeled with T4 kinase and [γ-^{32}P]ATP and purified over G-50 Quick-spin columns (Boringers). Annealed double-stranded probes were labeled with α-^{32}P-deoxynucleoside triphosphates and Klenow fragment to ensure incorporation of radioactivity only into the double-stranded DNA. Each reaction mixture contained 1.5 to 4.0 μg of whole-cell protein, prepared as previously described (4), 0.6 μg of poly(dI-dC), 5 mM Tris · HCl (pH 7.5), 1 to 3 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 0.6 to 0.9 mM dithiothreitol, 3.5 to 5.5% glycerol, and 20,000 cpm (~0.1 to 1 ng) of ^{32}P-labeled probe in a volume of 20 μl. Reaction mixtures were incubated for 30 min at room temperature and resolved either in 0.25× TBE (25 mM Tris base, 25 mM boric acid, 0.5 mM EDTA [pH 8.3]) on a 1.5-mm-thick 4% nondenaturing polyacrylamide gel (see Fig. 3) or in 1× TGE (25 mM Tris base, 190 mM glycine, 1 mM EDTA) on a 6% nondenaturing polyacrylamide gel (see Fig. 2, 4, 5, and 7). In competitive EMSA reactions, molar fold excesses of cold DNAs were added to the mixture at the same time as the labeled probes were added.

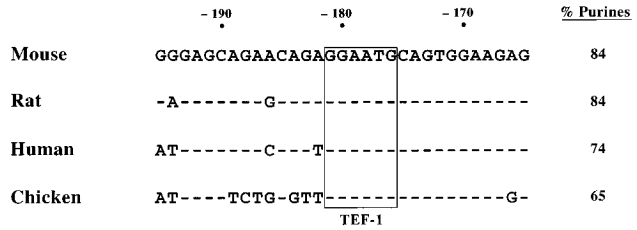

FIG. 1. Conserved polypurine-polypyrimidine tract found in VSM α-actin gene promoters. The boxed sequence represents an essential binding site for the transcription-activating protein TEF-1.

RESULTS

Fibroblast ssDNA-binding factors bind to opposite strands of a polypurine-polypyrimidine tract containing an essential TEF-1-binding site. Previous deletion-mapping studies established that a VSM α-actin promoter truncated to position −224 (VSMP3) is inefficiently expressed in AKR-2B fibroblasts and in undifferentiated BC3H1 myoblasts (11, 31). Further truncation to position −191 resulted in the inactivation of a strong negative control element and yielded a promoter (VSMP4) which exhibited a high degree of inducibility by serum growth factors in both cell types (11, 31).

Figure 1 illustrates the nucleotide sequence from −165 to −195 of the coding strand of the mouse VSM α-actin gene promoter. This sequence is heavily purine rich (84%) and contains a 6-bp GGAATG sequence (boxed), which is 100% conserved at the identical coordinates in the rat, human, and chicken homologs (2, 21). We designated this sequence the purine-rich motif (PrM) and showed that double-stranded oligonucleotides containing this sequence specifically bind a protein closely related to TEF-1 (4). Importantly, mutations within the PrM impair TEF-1 binding and completely abolish promoter activity in AKR-2B fibroblasts (4). Thus, TEF-1 appears essential for transcriptional activation of the VSM α-actin promoter in fibroblasts.

In the experiment whose results are shown in Fig. 2, AKR-2B whole-cell extracts were applied to a heparin-agarose column and eluted stepwise with increasing salt concentrations. An aliquot of each fraction was then tested in an EMSA for DNA-binding activity with a double-stranded oligonucleotide probe corresponding to the sequence shown in Fig. 1 (PrM30, Fig. 2C), or the individual single strands (PrMss, Fig. 2B; MCATss, Fig. 2A). We designated the pyrimidine-rich complementary strand to the sequence shown in Fig. 1 the MCAT strand, since it contains an inverted MCAT motif (4, 19). As shown in Fig. 2C, TEF-1 eluted in the 0.4 M NaCl fraction as detected with the double-stranded PrM30 probe. Two distinct ssDNA-binding activities were detected with the separated single strands. An activity denoted VACssBF1 (for vascular actin single-stranded binding factor 1) also eluted in the 0.4 M NaCl fraction and preferentially bound to the pyrimidine-rich MCAT strand (Fig. 2A), while a second activity, denoted VACssBF2, eluted in both the 0.4 M and 0.6 M NaCl fractions and preferentially bound to the purine-rich PrM strand (Fig. 2B). As noted previously (4), VACssBF2 consists

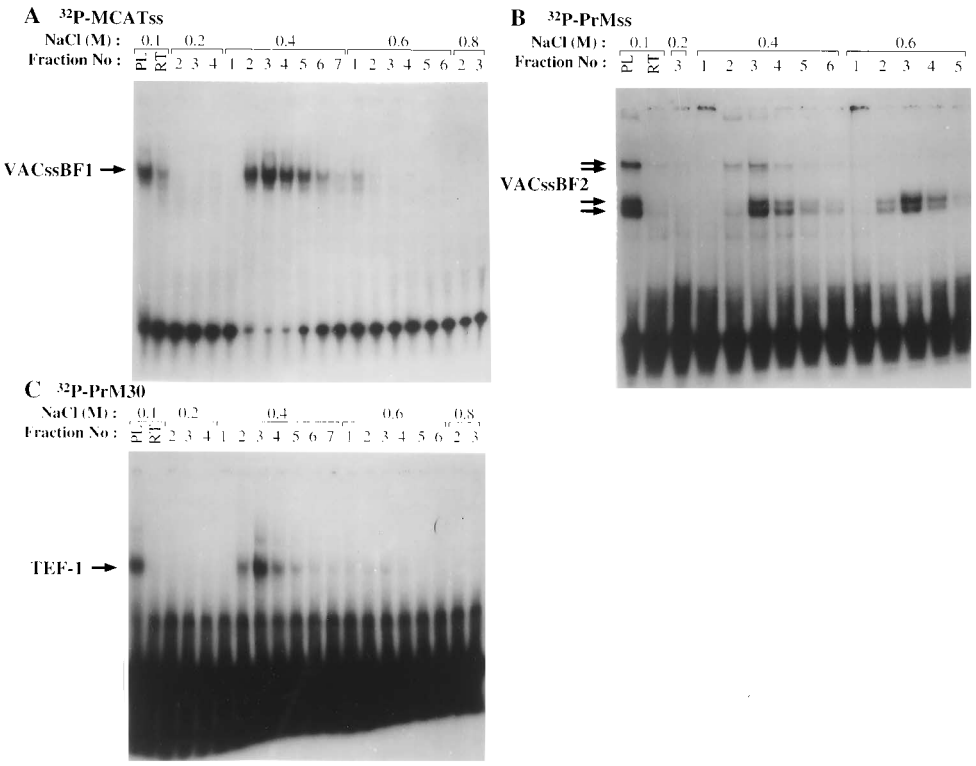

FIG. 2. Heparin-agarose chromatography of dsDNA- and ssDNA-binding factors which interact with the VSM α-actin polypurine-polypyrimidine tract. AKR-2B whole-cell extract was fractionated on heparin-agarose as described previously (4). Aliquots of each column fraction were tested in EMSAs with ^{32}P-labeled probes corresponding to the MCAT strand (A), the PrM strand (B), or an annealed double-stranded probe (C) corresponding to positions −164 to −195 of the VSM α-actin promoter. PL, preload; RT, run-through.

of multiple electrophoretically distinct species, a fast-migrating doublet, and a more slowly migrating band which is frequently resolved as a doublet as well (see Fig. 4 and 5).

TEF-1 lacks detectable ssDNA-binding activity. Some muscle-regulatory factors such as MyoD have been reported to possess both ssDNA- and double-stranded-DNA (dsDNA)-binding activity (26). However, TEF-1 binding is not detectable with either PrM or MCAT single-stranded oligonucleotide probes in gel shift assays (4) (Fig. 2; also see Fig. 4). To further determine whether TEF-1 possessed ssDNA-binding activity, the ability of unlabeled single-stranded oligonucleotides to compete for TEF-1 binding to a labeled double-stranded probe was investigated. As shown in Fig. 3A, TEF-1 binding to the PrM30 probe was abolished by a 200-fold molar excess of unlabeled double-stranded oligonucleotide (Fig. 3A, lanes 5, 6, 11, and 12) but was unaffected by even a 2,000-fold molar excess of either the PrM strand or the MCAT strand (lanes 4 and 10). Figure 3A also includes the results of an EMSA performed under identical conditions with a single-stranded DNA probe corresponding to the MCAT strand (lane 13). The intense binding of VACssBF1 relative to TEF-1 indicates that the ssDNA-binding factors are present in much higher abundance than TEF-1 and/or bind to their respective probes with much higher affinity than TEF-1.

VACssBF1 and VACssBF2 lack detectable dsDNA-binding activity. The absence of detectable VACssBF1 or VACssBF2 binding activity when the double-stranded PrM30 probe was used in the experiment in Fig. 3A indicated that the ssDNA-binding factors lacked the ability to bind to the double-stranded form of the TEF-1 enhancer. This was further confirmed by the experiments whose results are shown in Fig. 3B. Here, the ability of VACssBF1 and VACssBF2 to bind to their respective single-stranded probes was unaffected by the presence of unlabeled double-stranded oligonucleotide under conditions where binding was readily inhibited by homologous unlabeled single-stranded DNA. Together, the data of Fig. 3A and B further reinforce the notion that TEF-1 binds exclusively to the double-stranded form of the TEF-1 enhancer element while VACssBF1 and VACssBF2 exhibit a strong preference for the separated single strands.

Determinants of VACssBF1 and VACssBF2 binding are broadly distributed throughout the polypurine-polypyrimidine tract and include the TEF-1-binding site. Many ssDNA-binding activities which have been reported do not appear to interact with a single narrowly defined sequence motif but, rather, exhibit a somewhat relaxed binding specificity (23, 26, 37). To analyze the sequence specificity of VACssBF1 and VACssBF2 binding, EMSAs were performed with fibroblast cellular extracts and a series of single-stranded oligonucleotide probes containing transversion mutations of 1 to 4 bp distributed throughout the entire polypurine-polypyrimidine tract. The resulting EMSA gels are shown in Fig. 4A. To correct for minor differences in probe loading, an AMBIS radioanalytic imaging scanner was used to quantitate the percent radioactivity incorporated into free and bound forms for each of the wild-type and mutant probes. The values obtained for the two wild-type probes were arbitrarily set at 1.0, and the resulting data were plotted in the form of a histogram (Fig. 4B). These data demonstrated that while no single mutation eliminated binding entirely, destabilizing effects were produced by several mutations distributed throughout the entire 30-bp polypurine-polypyrimidine tract including the PrM-MCAT sequence itself.

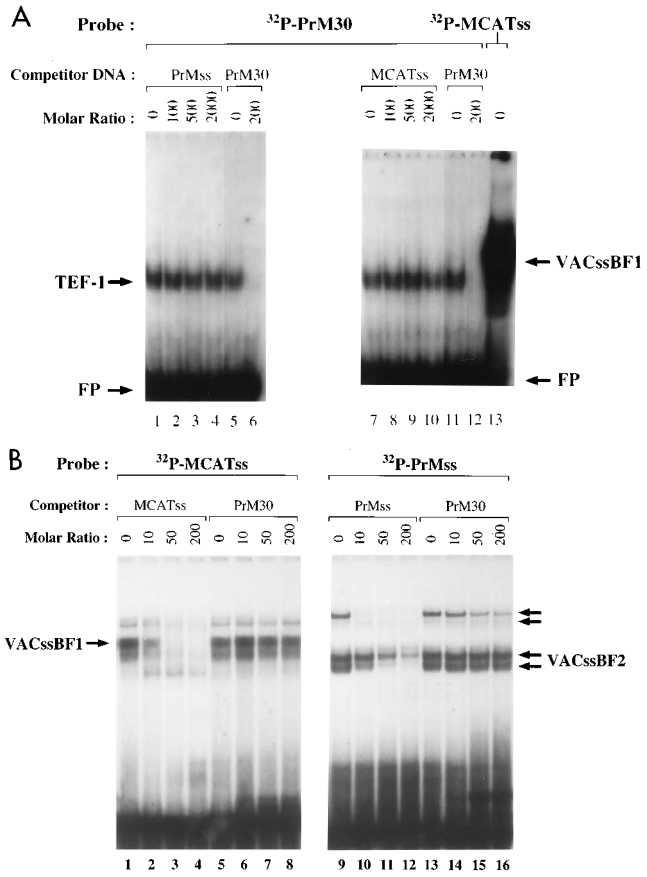

FIG. 3. dsDNA- and ssDNA-binding specificity of TEF-1, VACssBF1, and VACssBF2. (A) Effect of single-stranded oligonucleotides on TEF-1 binding to dsDNA. A ^{32}P-labeled double-stranded oligonucleotide (PrM30) was incubated with AKR-2B whole-cell extract in the presence of the indicated molar excess of either unlabeled double-stranded PrM30 oligonucleotide (lanes 5, 6, 11, and 12) or unlabeled oligonucleotides corresponding to the respective single strands (PrMss, MCATss). VACssBF1 binding to a labeled MCATss probe was also included for comparison with TEF-1 (lane 13). (B) Effect of double-stranded oligonucleotide on VACssBF1 and VACssBF2 binding to ssDNA. ^{32}P-labeled single-stranded oligonucleotides corresponding to the MCAT and PrM strands of the TEF-1 enhancer were incubated with AKR-2B whole-cell extract in the presence of the indicated molar excess of either homologous unlabeled single-stranded oligonucleotides (lanes 1 to 4 and 9 to 12) or the double-stranded PrM30 oligonucleotide (lanes 5 to 8 and 13 to 16).

Both binding activities appeared particularly sensitive to transversions introduced toward the 5′ ends of their respective strands. The binding of VACssBF1 to the pyrimidine-rich strand was particularly affected by mutations within a repeated TCT motif, which occurs three times and which was altered by five mutations (TV167, TV171, TV182, TV184, and TV188). Of these five, only TV184 appeared to have little effect on VACssBF1 binding. This is notable, since TCT motifs have recently been shown to be important binding determinants for a rat pyrimidine tract-binding protein (14). This protein differs from VACssBF1, however, in apparent molecular weight and tissue specificity (4).

An inspection of the actual EMSA gel profiles shown in Fig. 4A also revealed an important characteristic of VACssBF2 binding. In the top gel, VACssBF2 is clearly resolved into two pairs of differently migrating doublets labeled AB and CD. Importantly, the effect of the tested mutations was restricted almost entirely to the more rapidly migrating CD doublet, while the more slowly migrating doublet AB was virtually unaffected. This suggested that the two pairs of doublets possessed different DNA-binding specificities, a possibility which was confirmed by the experiment whose results are shown in Fig. 5.

The effects of mutations on VACssBF1 and VACssBF2 binding are cumulative. Since no tested mutation was found to eliminate ssDNA-binding activity, we tested the effect of several additional combinations. As shown in Fig. 5, at least five of these severely impaired binding, including three combinations which retained an unaltered TEF-1 binding site (PrM strand mutant 2 and MCAT mutants 1 and 2). An additional PrM strand mutant (mut 1) reduced but did not eliminate binding of the CD doublet. As was observed in the experiment whose results are shown in Fig. 4A, the binding of VACssBF2 doublet AB to the PrM strand was not affected, even by mutations which almost completely abolished binding of the CD doublet (compare lanes 2 to 4). To date, we have not identified specific mutations which impair binding of the VACssBF2 AB doublet, although it does not bind to the complementary MCAT strand or to several other unrelated oligonucleotides (results not shown). Thus, while the binding specificity of this component remains to be determined, the fact that it is clearly different from that of the CD doublet makes it unlikely that the latter is simply a proteolytic degradation product of the former.

Mutations which impair ssDNA factor-binding activity result in transcriptional activation of a repressed VSM α-actin promoter. Since the determinants of VACssBF1 and VACssBF2 binding to ssDNA overlap the determinants of TEF-1 binding to dsDNA, we reasoned that these ssDNA-binding factors might repress transcription by disrupting base pairing of the TEF-1 recognition sequence. One prediction arising from this hypothesis is that an enhancer element unrelated to that of TEF-1 might functionally substitute for the TEF-1 site in supporting activated transcription but not in maintaining full transcriptional repression, since it would lack determinants important to VACssBF1 and VACssBF2 binding. To test this prediction, we constructed two chimeric promoters in which we substituted an essential 9-bp CCAAT box element (AGCC AATCA) from the cytoskeletal β-actin promoter for the 6-bp GGAATG TEF-1 recognition sequence. The β-actin CCAAT box element has been shown to specifically bind nuclear factor Y (NF-Y) (5), and we have determined that these two elements are interchangeable within the context of the β-actin promoter (unpublished data). The activities of the CAAT box-substituted promoters were compared with those of their wild-type counterparts by transfection into both AKR-2B fibroblasts and BC3H1 myoblasts.

In accordance with previous results (11, 31), the data in Fig. 6 illustrate that a VSM α-actin promoter truncated to position −224 (VSMP3) is repressed approximately 10-fold in both fibroblasts and myoblasts relative to a construct deleted to position −191 (VSMP4). However, when the two CAAT box-substituted promoters were compared, VSMP3caat was repressed only twofold relative to its VSMP4caat counterpart in both cell types. Thus, substitution of the NF-Y-binding site for that of TEF-1 significantly relieved transcriptional repression in both AKR-2B fibroblasts and BC3H1 myoblasts. EMSAs performed with AKR-2B cellular extracts confirmed that both VACssBF1 and VACssBF2 CD doublet binding activities were reduced by the CAAT box substitution (results not shown). However, some residual binding activity was retained, as would be expected given the fact that other binding determinants in the polypurine-polypyrimidine tract were unaltered.

To further investigate the relationship between ssDNA-binding activity and transcriptional repression, we analyzed the effects of a 10-bp transversion between −186 and −195. This region contains nucleotides which stabilize the binding of both

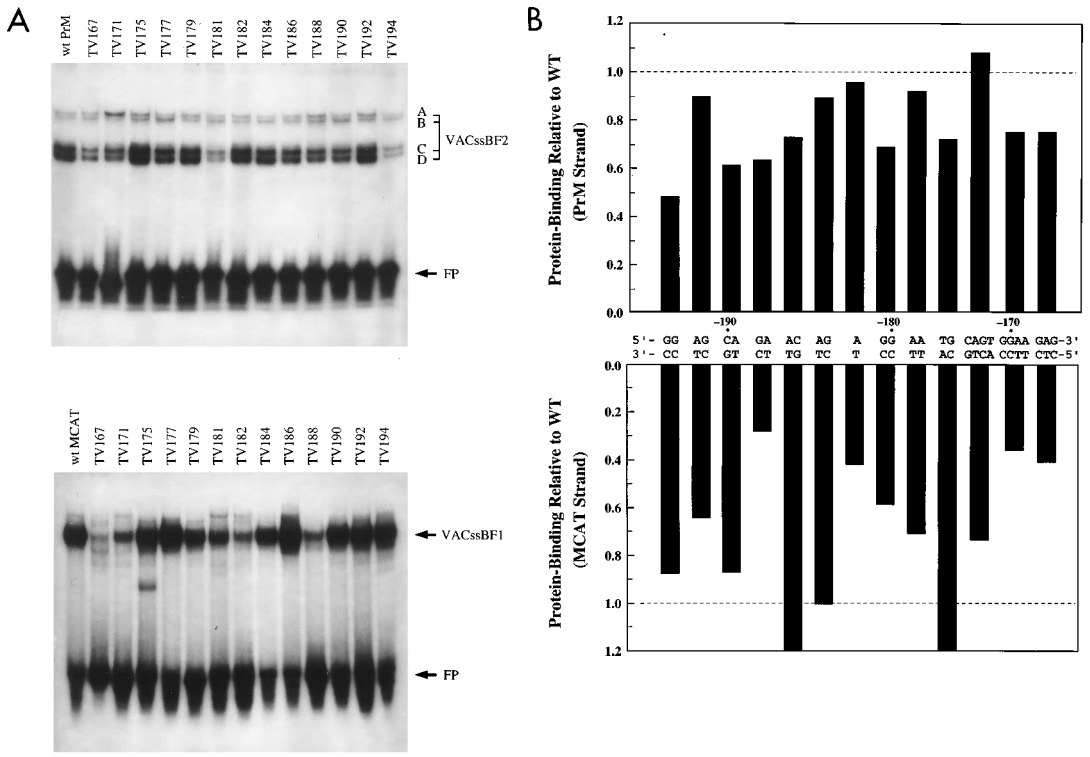

FIG. 4. ssDNA-binding specificity of VACssBFs. Single-stranded PrM- and MCAT-strand oligonucleotides containing transversion mutations of 1 to 4 nucleotides at the indicated positions were synthesized and tested for their ability to bind VACssBF1 and VACssBF2 in EMSAs with AKR-2B whole-cell extract. An AMBIS radioanalytic imaging scanner was then used to directly measure the percent radioactivity incorporated into free and bound forms for each of the wild-type and mutant probes. The value obtained for each wild-type probe was arbitrarily set at 1.0. (A) EMSA gels. (B) Binding relative to wild-type (WT) probes. The bars represent the relative binding activity which resulted from transversion of the bases shown directly under or over the bars.

VACssBF1 and VACssBF2 (Fig. 4) and spans the −191 deletion point, which results in strong transcriptional activation of the wild-type promoter. The EMSA data shown in Fig. 7A confirm that single-stranded oligonucleotides containing this mutation (designated TV195) are impaired in their ability to bind both VACssBF1 and the CD doublet of VACssBF2. This reduction in binding activity was greater than that observed with the CCAAT box-substituted probes. Importantly, Fig. 7B illustrates that introduction of the same mutation into the transcriptionally repressed VSMP3 promoter results in transcriptional activation to about 70% of the level exhibited by VSMP4 in quiescent fibroblasts. Identical results were obtained with cells stimulated with serum or serum plus cycloheximide (not shown), again implicating the ssDNA-binding factors in the maintenance of transcriptional repression in fibroblasts. However, in contrast to the similar results obtained with the CCAAT box-substituted promoter, transfection of the TV195 mutant into BC3H1 myoblasts yielded only a small (10%) increase in CAT activity when compared with the wild-type VSMP3 promoter (data not shown). However, this increase was observed in three independent experiments and was statistically significant ($P = 0.025$). Whether these results reflect the existence of additional forms of negative regulation in myoblasts (see Discussion), developmental stage variability (11), or other factors is currently unknown.

DISCUSSION

In a recent study, we identified two ssDNA-binding factors in BC3H1 myoblasts and AKR-2B fibroblasts which bound to opposite strands of the VSM α-actin promoter in a region

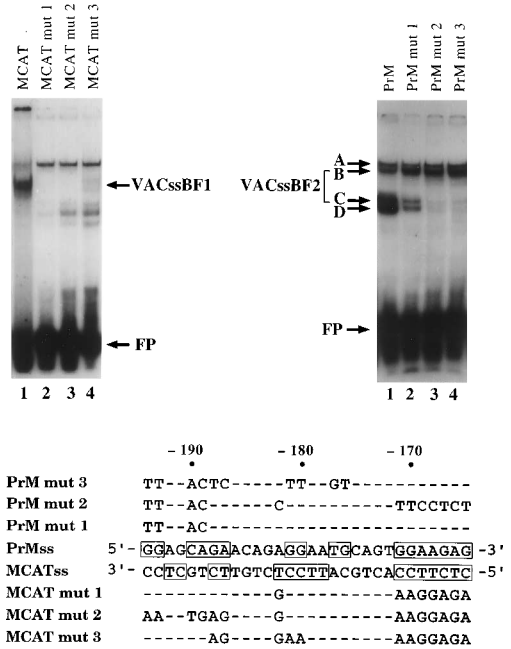

FIG. 5. Effect of multiple mutations within the polypurine-polypyrimidine tract on VACssBF binding. Single-stranded oligonucleotides corresponding to the PrM (PrMss) or the MCAT (MCATss) strand or the indicated mutants were ^{32}P labeled and used as probes in EMSAs with AKR-2B whole-cell extracts. The boxed nucleotides are those which influence complex formation with VACssBF1 and VACssBF2 as determined in Fig. 4A and B.

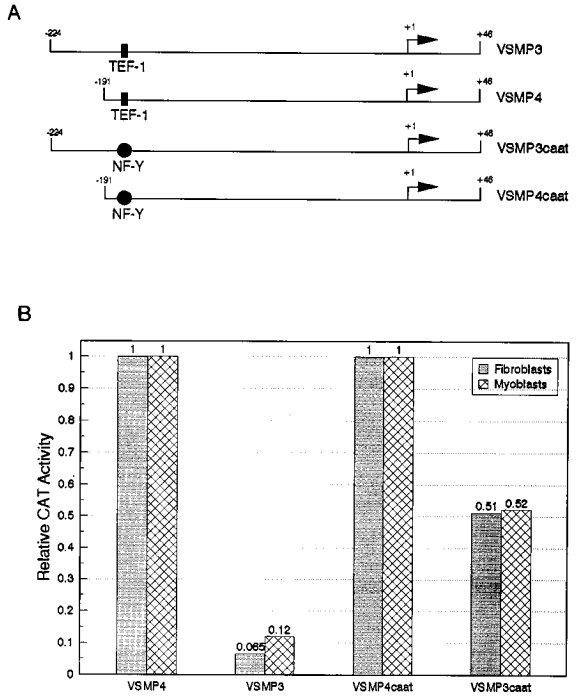

FIG. 6. Effect of enhancer substitution on transcriptional repression of the VSM α-actin promoter. (A) Structure of wild-type (TEF-1) and enhancer-substituted (NF-Y) promoters. (B) Relative expression in AKR-2B fibroblasts and BC3H1 myoblasts. Following transfection, AKR-2B cells were rendered quiescent, stimulated for 6 h with 20% fetal calf serum in the presence of cycloheximide, and subjected to a 2-h washout with the same medium lacking cycloheximide, while BC3H1 myoblasts were grown in serum-supplemented medium as described previously (11) and harvested at a subconfluent stage. To facilitate comparison between the two cell types, the percent conversion of chloramphenicol to its acetylated derivatives for VSMP4 (70.6% ± 0.8%, AKR-2B cells; 76.0% ± 4.0%, BC3H1 cells) and VSMP4caat (51.4% ± 4.5%, AKR-2B cells; 31.0% ± 3.0%, BC3H1 cells) was assigned a value of 1.0. All values represent the mean of two (AKR-2B) or three (BC3H1) independent transfection experiments.

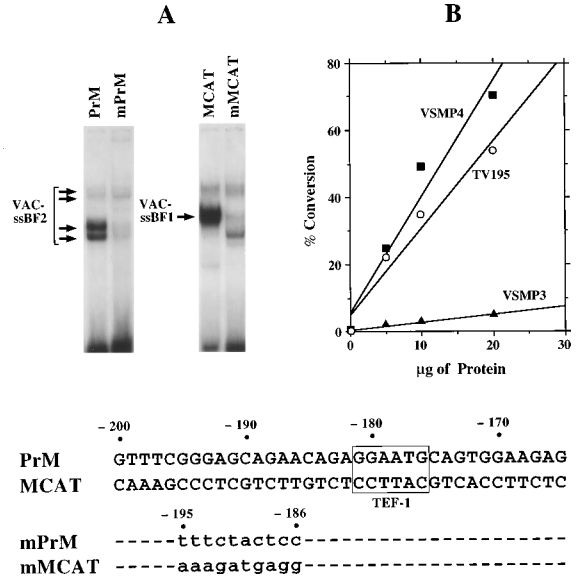

FIG. 7. Effect of polypurine-polypyrimidine tract transversion mutation on VACssBF binding and expression properties in fibroblasts. (A) ^{32}P-labeled single-stranded oligonucleotides corresponding to either the PrM or MCAT strands or the indicated mutants (mPrM and mMCAT) were tested in EMSAs with AKR-2B whole-cell extracts. (B) The indicated base sequence changes were introduced into the transcriptionally repressed promoter VSMP3 as described in Materials and Methods. The resulting promoter (TV195) was tested in parallel transfections with VSMP3 and the transcriptionally activated promoter VSMP4 for expression in quiescent AKR-2B fibroblasts. Increasing amounts of cellular extract were analyzed for CAT activity, and the resulting slopes were determined. Values shown are the average of two independent transfection experiments.

containing an essential TEF-1 enhancer site (4). Since the enhancer was closely linked to a strong transcriptional silencing element (11, 31), we postulated that the ssDNA-binding factors might function as repressors of VSM α-actin transcription by disrupting base pairing within the enhancer. This model is supported by the following observations. First, we have found that nucleotides which are important to specific binding of VACssBF1 and VACssBF2 include components of the TEF-1-binding site itself, as well as other specific nucleotides distributed throughout the polypurine-polypyrimidine tract. Second, and more importantly, mutations which impaired binding of VACssBF1 and VACssBF2 in EMSAs also resulted in the relief of transcriptional repression when incorporated into a repressed VSM α-actin promoter. These activating mutations included both an enhancer substitution in which the TEF-1-binding site itself was replaced by an unrelated CCAAT box element and a transversion mutation in which nucleotides outside of the TEF-1 binding site were altered. These results establish a relationship between the ability of VACssBF1 and VACssBF2 to bind to the individual single strands of the TEF-1 enhancer and the maintenance of transcriptional repression. Although correlative, the establishment of similar relationships for double-stranded enhancer-binding proteins is generally regarded as strong evidence for an essential function in transcriptional regulation.

Although both of the tested site-directed mutants clearly exhibited an increase in promoter activity relative to the parental VSMP3 promoter, neither was fully activated to the degree displayed by the deletion mutant VSMP4. CAT activity directed by the TV195 mutant in both quiescent and serum-induced fibroblasts approximated 60 to 70% of that directed by the VSMP4 construct, while the CCAAT enhancer-substituted VSMP3caat was about 50% as active as VSMP4caat in both fibroblasts and myoblasts. Because we have previously shown that CAT activity directed by transcriptionally activated and repressed VSM α-actin promoter constructs is an accurate reflection of the level of correctly initiated RNA transcripts (31), these measurements indicate that the mutated promoters retain a significant degree of transcriptional repression. Because none of the tested mutations significantly impaired binding of the slowly migrating (AB) component of VACssBF2, the continued interaction of this component may, in itself, be responsible for a degree of repression. Alternatively, the VSM α-actin promoter may be additionally repressed by mechanisms which are independent of ssDNA-binding activity. For example, we have recently identified a differentiation stage-specific dsDNA-binding activity in BC3H1 myoblasts, which interacts with a "CArG-like" motif previously shown to be required for repression in myoblasts but not in AKR-2B fibroblasts (4, 11, 31). This sequence lies immediately upstream of the polypurine-polypyrimidine tract and may account, in part, for the diminished effect of the TV195 mutation on transcriptional repression in BC3H1 myoblasts relative to AKR-2B fibroblasts. Indeed, the available mutagenesis data are consistent with a model in which the mechanisms of repression differ in these two cell types, perhaps reflecting a multifactorial involvement of both cell-type-specific and non-cell-type-specific factors.

As noted previously, VACssBF1 and VACssBF2 exhibit a highly tissue-restrictive pattern of expression in mice (4). In particular, they are abundant in tissues enriched in smooth muscle but are virtually absent in all other tested tissues, including skeletal muscle and liver. Since the latter tissues do not express VSM α-actin in the adult, the mechanism of repression in these tissues may differ from the model described here. A corollary to this hypothesis is that VACssBF1 and VACssBF2 may function to repress VSM α-actin transcription only in tissues and cell types capable of activation in response to external or internal stimuli. Consistent with this interpretation are recent studies demonstrating that fibroblasts can be stimulated to differentiate into VSM α-actin-expressing myofibroblasts, both in vivo and in vitro, by TGF-β1 (9, 25). Indeed, recent studies in one of our laboratories have shown that TGF-β1 treatment of AKR-2B fibroblasts, the cell type used in these experiments, results in the rapid accumulation of VSM α-actin mRNA and protein and in the appearance of VSM α-actin-positive stress fibers (33). Thus, it will be important to determine the role of VACssBF1 and VACssBF2 in repressing TGF-β1-inducible transcription of the VSM α-actin gene in fibroblasts.

Since TEF-1 has now been shown to regulate a number of viral and cellular promoters (4, 7, 10, 13, 15), we performed additional experiments to determine whether other TEF-1-binding enhancer elements might also interact with VACssBF1 and VACssBF2. EMSAs with opposite-strand probes corresponding to the chicken cardiac troponin T MCAT motif (19) and the simian virus 40 GT IIC and *Sph*-I and *Sph*II enhanson motifs (7) revealed that VACssBF1 bound very weakly to the cardiac troponin T MCAT motif and not at all to the simian virus 40 enhanson sequences (data not shown). Notably, none of these sequences exhibit the high degree of polypurine-polypyrimidine asymmetry displayed by the VSM α-actin TEF-1-binding region. In contrast to the restricted binding displayed by VACssBF1, VACssBF2 bound avidly to all of the motifs tested, with the cardiac troponin T probe giving the strongest signal. While this is not an exhaustive survey, these results suggest that VACssBF1 may be relatively promoter specific while VACssBF2 has the ability to interact with a broader spectrum of sequences. The interaction of these two different types of factors with the VSM α-actin promoter is reminiscent of combinatorial strategies of gene regulation involving dsDNA-binding proteins (32).

An important question is whether either of the two VSM α-actin ssDNA-binding factors also interacts with RNA. RNA is the most prevalent single-stranded nucleic acid within the cell, and other ssDNA-binding factors have been shown to exhibit a dual specificity for both ssDNA and RNA. Notably, members of the highly evolutionarily conserved Y-box family of nucleic acid-binding proteins have been implicated in both transcriptional regulation and the binding and sequestering of mRNA during gametogenesis (reviewed in reference 36). Similarly, the rat pyrimidine tract-binding protein interacts with both a liver-specific enhancer element (14) and nuclear pre-mRNA (24). In preliminary studies (data not shown), we observed that VACssBF1 binding to the MCAT strand of the TEF-1 enhancer element can be effectively inhibited by AKR-2B fibroblast whole-cell RNA. However, VACssBF2 binding to the PrM strand was unaffected by the same molar excess of whole-cell RNA. Thus, VACssBF1 may have dual specificity for both ssDNA and RNA, while VACssBF2 may preferentially interact with ssDNA. Experiments are in progress to determine whether VACssBF1 recognizes specific RNA sequences or binds in a sequence-independent manner.

An ssDNA binding activity which interacts with a muscle-specific TEF-1/MCAT sequence has been reported previously (26). This factor, termed muscle factor 3 (MF3), appears to differ from either of the two ssDNA-binding factors described here in that MF3 readily binds to both strands of the TEF-1/MCAT sequence or to dsDNA. Moreover, MF3 appears to be expressed in a variety of tissues, including skeletal muscle, while VACssBF1 and VACssBF2 are smooth muscle specific (4). The existence of multiple ssDNA-binding factors which interact with muscle-specific genes may indicate a generalized role for such factors in muscle cell differentiation.

ssDNA-binding activities have also been detected in association with promoter sequences of a variety of nonmuscle genes (1, 8, 14, 23, 34, 35, 37) and, in the case of several genes, appear positioned to function as transcriptional repressors by a mechanism similar to that proposed here. These include the rat growth hormone gene (23), the mouse adipsin gene (35), and, most recently, the rat and mouse β-casein gene (1). ssDNA-binding activity in the β-casein gene was shown to be under hormonal control in mammary epithelial cells and to disappear during lactation in mice. In each case, the recognition sequence(s) for ssDNA-binding activity exhibited purine-pyrimidine asymmetry and was closely associated with a positive element required for transcription. To our knowledge, however, activating mutations similar to those described here have not yet been delineated and the ssDNA-binding activities detected appear distinct from VACssBF1 and VACssBF2 by at least one of several criteria. These include cell or tissue specificity, DNA-binding specificity, and apparent molecular weight. In aggregate, the data suggest that VACssBF1 and VACssBF2 are members of a growing class of ssDNA-binding proteins which play a key role in regulating cell-type-specific gene expression by modulating enhancer topology and function. The details of their interaction with DNA and whether either has dual roles in processes such as RNA metabolism or DNA replication are important issues for future study.

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