Repression of Transcriptional Enhancer Factor-1 and Activator Protein-1-dependent Enhancer Activity by Vascular Actin Single-stranded DNA Binding Factor 2*

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Transcriptional repression of the murine vascular smooth muscle α-actin gene in fibroblasts results from the interaction of two sequence-specific single-stranded DNA binding activities (VACssBF1 and VACssBF2) with opposite strands of an essential transcriptional enhancer factor-1 (TEF-1) element (Sun, S., Stoflet, E. S., Cogan, J. G., Strauch, A. R., and Getz, M. J. (1995) Mol. Cell. Biol. 15, 2429–2436). Here, we identify a sequence element located within a protein-coding exon of the gene that bears structural similarity with the TEF-1 enhancer. This includes a 30-base pair region of purine-pyrimidine asymmetry encompassing a perfect 6-base pair GGAAATG TEF-1 recognition motif. Unlike the enhancer, however, the exon sequence exhibits no TEF-1 binding activity nor does the pyrimidine-rich strand bind VACssBF1. However, VACssBF2 interacts equally well with the purine-rich strand of both the enhancer and the exon sequence. To test the ability of VACssBF2 to independently repress transcription, the exon sequence was placed upstream of a deletional activated promoter containing an intact TEF-1 binding site. The exon sequence repressed promoter activity, whereas a mutant deficient in VACssBF2 binding did not. Moreover, VACssBF2 similarly repressed activator protein-1-dependent transcription of a heterologous tissue factor promoter. These results suggest that VACssBF2 possesses an intrinsic ability to disrupt enhancer function independently of the enhancer-binding proteins involved.

The cell- and tissue-specific transcription of eukaryotic genes is regulated by cis-acting elements that are generally localized outside of the RNA/protein coding region of the gene. These regulatory elements, known commonly as enhancers or silencers, are DNA sequences that influence gene transcription in either a positive or negative fashion by virtue of their ability to bind specific proteins that, in turn, regulate the assembly of a basal transcriptional complex. Although these DNA elements are commonly found upstream of the transcription start site, cis-acting silencing elements have also been localized within introns and the protein-coding sequences of a number of genes (1–3). In most cases, the fidelity of enhancer or silencer function is strongly correlated with the sequence-specific binding of a protein(s) to a region of double-stranded DNA (dsDNA) encompassing the enhancer or silencer motif (4, 5). However, recent studies suggest that proteins that bind to single-stranded DNA (ssDNA) in a sequence-specific manner may also participate in the regulation of gene transcription. Although the molecular mechanism(s) by which ssDNA-binding proteins affect transcription remains largely speculative, these proteins are often associated with transcriptional silencing elements (6–11).

Recent studies have shown that repression of vascular smooth muscle (VSM) α-actin promoter activity in both BC3H1 myoblasts and AKR-2B fibroblasts can be linked to the interaction of two sequence-specific ssDNA-binding activities within a 30-bp polypurine-polypyrimidine tract residing in the 5′ promoter (12, 13). These activities, designated VSM α-actin single-strand binding factor 1 and 2 (VACssBF1 and VACssBF2) bind to opposite strands of the DNA helix within a region of the promoter (−194 to −165) spanning the binding site for a dsDNA-binding protein closely related with, and possibly identical to, transcriptional enhancer factor 1 (14–20). Promoter activation, site-directed mutagenesis, and DNA-binding studies have led to the hypothesis that VACssBFs function by stabilizing a local single-stranded DNA conformation within the 30-bp promoter element (PE) which precludes TEF-1 binding and hence transcriptional activation (12). Interestingly, amino acids 44–53 of VSM α-actin are encoded by a DNA sequence that is structurally similar to the 5′ PE. This intragenic sequence element or coding element (CE) lies immediately adjacent to the 3′ end of intron 2 and is positionally conserved in all vertebrate actin genes (21). The coding element (CE) and promoter element (PE) each possess a core GGAATG consensus TEF-1 binding motif centered within an asymmetric polypurine-polypyrimidine tract.

The present study illustrates that VACssBF2, but not VACssBF1, interacts with the CE in a sequence- and single strand-specific manner. The identification of this promoter-independent VACssBF2 binding site permitted a more rigorous

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1 The abbreviations used are: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; VSM, vascular smooth muscle; VACssBF, VSM α-actin single-strand binding factor; TEF-1, transcriptional enhancer factor-1; PE, promoter element; CE, coding element; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; mu, mutant; wt, wild type; AP-1, activator protein-1; PVDF, polyvinylidene difluoride; bp, base pair.
evaluation of the ssDNA-binding properties, subunit composition, and functional activity of VACssBF2. VACssBF2 is shown to consist of multiple distinct polypeptide species of differing apparent molecular weights and ssDNA binding specificity. Functional analysis of the VACssBF2-CE interaction conducted within the context of homologous and heterologous promoters suggests that VACssBF2 can repress enhancer-dependent transcription independently of both VACssBF1 and the dsDNA-binding protein governing enhancer activity.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Reporter Gene Assay—Mouse embryo-derived AKR-2B fibroblasts were maintained in culture and transfected as described previously (22, 23). Transfected cells were allowed to recover in McCoy’s 5 A medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) for 18–24 h. The cells were then washed twice and rendered quiescent by incubating an additional 48 h in serum-free IMDM (JRH Biosciences, Lenexa, KS). Quiescent cells were stimulated for 6 h with MCD402 medium and 20% fetal bovine serum or for 4 h with the same medium supplemented with 10 μg/ml cycloheximide (Sigma). Serum- and cycloheximide-stimulated cells were washed twice and subjected to a 2-h washout in medium lacking cycloheximide to allow for the recovery of CAT protein synthesis (24). Cells were harvested, and cellular extracts were prepared by repeated freeze-thawing. Protein concentration was determined by BCA dye-binding assay (Pierce) using bovine serum albumin as a standard. Chloramphenicol acetyltransferase (CAT) reporter protein was measured in cell lysates using an immunonassay kit (Boehringer Mannheim).

Construction of VSM α-Actin Promoter/ Coding Element Reporter Plasmids—The deletionally activated VSM α-actin promoter construct pCSVSMP4 (VSMP4) (25) was modified by insertion of a synthetic copy of a 33-base pair segment of DNA encoding the intron 2/exon 3 splice site and amino acids 44–53 of VSM α-actin (Table I) both 3′ and 5′ of the transcription start site. Complementary oligonucleotides possessing the appropriate splice ends as well as an internal Bcl I restriction site (underlined) of the form 5′-AGCTTTGATCATGgofVSM-A GCTTTGATCATGAACgofVSMP-4 were synthesized on an Applied Biosystems model 394 DNA/ Oligonucleotide Synthesizer and subsequently end-labeled with T4 polynucleotide kinase (Boehringer Mannheim) and [γ-32P]ATP (Amersham Corp.) and purified using S-200 HR microspin columns (Pharmacia). Annealed double-stranded 200 bp DNA probes were labeled with [α-32P]dATP (A and C) (Amersham Corp.) and Klenow fragment (Boehringer Mannheim) and purified by electrophoresis on a 6% polyacrylamide gel (Pharmacia Biotech, Inc.) in distilled water. Oligonucleotides were phosphorylated, annealed, and ligated into either HindIII and Sal I (construct) or BamHI and XhoI (3′ construct) cut, and phosphatase-treated VSMP4 to create P4/CE(5′-actin coding sequence element and the CAT coding sequence are in noncoding strand) or coding element (CE, G/nucleotide corresponding to either the promoter element (PE, nuc- leotide −194 to −165) or coding element (CE, G/nucleotide +201 to +229) (Fig. 1, Table I) were synthesized and purified as described above.

Preparation of DNA-binding Protein Extracts—Whole cell protein extract of rapidly growing AKR-2B fibroblasts was prepared by hyper- tonic lysis as described previously (13). Cytosolic and nuclear protein extracts were prepared from rapidly growing AKR-2B and NIH-3T3 fibroblasts according to the mini-extraction method of deGroot and co-workers (26). Protein concentration was determined by dye-binding assay (Bio-Rad) using bovine serum albumin as a standard.

Electrophoretic Mobility Shift Assay (EMSA)—Protein-DNA interactions were analyzed by band shift assay using AKR-2B fibroblast pro- tein extracts and 32P-labeled oligonucleotide probes. Single-stranded oligonucleotides were end-labeled with T4 polynucleotide kinase (Boe- hringer Mannheim) and [γ-32P]ATP (Amersham Corp.) and purified using S-200 HR microspin columns (Pharmacia). Annealed double-stranded 300 bp DNA probes were labeled with [α-32P]dATP (A and C) (Amersham Corp.) and Klenow fragment (Boehringer Mannheim) and purified by electrophoresis on a 6% polyacrylamide gel (Pharmacia Biotech, Inc.) in distilled water. Oligonucleotides were phosphorylated, annealed, and ligated into either HindIII and Sal I (construct) or BamHI and XhoI (3′ construct) cut, and phosphatase-treated VSMP4 to create P4/CE(5′) and P4/CE(3′) constructs, respectively. The CE(3′) plasmids were constructed such that the VSM α-actin coding sequence element and the CAT coding sequence are in reading frame. The CE was similarly cloned into a heterologous tissue factor promoter construct (AP1TFlf60CAT) upstream of two AP-1 transcription independent of both VACssBF1 and the dsDNA-binding protein governing enhancer activity.

| Designation | Sequence* |
|-------------|-----------|
| PE-PrMas (coding strand) | 5′-GGGCGAAAGAGATGATGAAAGAGG−3′ |
| PE-PrMas (coding strand) | 5′-GGGATGTGAGGTGAGAAGGCAA−3′ |
| PE-PrMmu2 (coding strand) | 5′-TGATGACTGTTGTTCCGGAGCACAG−3′ |
| PE-MCATes (nondocking strand) | 5′-CTCTCTACGTCATCCCTGCTGCTC−3′ |
| CE-MCATes (nondocking strand) | 5′-CTCTTCGTGCACCACCAATGCATCCC−3′ |

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poly(dI-dC), was then added, and the membranes were incubated for 3 h with continuous agitation. The membranes were washed three times, 10 min each with TNE containing 0.05% Tween 20 and then one final time with TNE alone. After air drying, the membranes were wrapped in plastic, placed in a x-ray cassette, and autoradiographed at –70 °C for 1–3 days as described above. Apparent molecular weights were calculated from a plot of log molecular weight versus relative mobility of pre-stained molecular weight standards (Bio-Rad) using a linear least squares regression fit.

**RESULTS**

**Promoter Element and Coding Sequence Element Homology**—In previous studies transcriptional regulation of the murine VSM α-actin gene in fibroblasts and myoblasts was shown to be mediated, in part, by both positive and negative elements residing in a 30-bp asymmetric polypurine-polypyrimidine tract within the 5′ promoter (12, 13, 22, 25). Promoter activation and DNA-binding experiments suggested a model in which repression of this TEF-1-dependent enhancer element is maintained by disruption of base pairing via the interaction of two sequence-specific ssDNA binding proteins, VACssBF1 and VACssBF2, that bind opposite strands of the DNA helix encompassing the TEF-1 recognition sequence (12, 13). Interestingly, a search of the cDNA sequence (28) revealed that a protein coding exon of the gene contains another, perfect, consensus TEF-1 recognition motif (GGAATG) centered within a purine-rich tract. As shown in Fig. 1, this protein-coding element or PE and coding element (CE) are in bold type.

**Interaction of a Subset of Single-stranded PE-binding Proteins with the CE**—Direct and competitive electrophoretic mobility shift assays (EMSA) with wild-type and mutant 30 base oligonucleotides (Table I) and AKR-2B cellular extract were performed to delineate the protein-binding properties of the CE in comparison to the PE. The double-stranded form of the PE has been previously shown to interact with a protein that is related with, and likely identical to, TEF-1 (13). Despite the presence of a GGAATG motif in both elements, only the double-stranded form of the PE exhibited detectable affinity for TEF-1 (data not shown). Similar results were obtained on examination of VACssBF1 binding affinity. As shown in Fig. 2, the pyrimidine-rich, noncoding strand of the CE (CE-MCATs) did not compete for binding of VACssBF1 to 32P-PE-MCATs (lanes 6–10), whereas competition was readily observed by the homologous PE-MCATs sequence (lanes 1–5). Direct binding EMSAs also indicated that VACssBF1 had little or no detectable affinity for 32P-CE-MCATs (data not shown).

In marked contrast to both TEF-1 and VACssBF1, the previously described, electrophoretically distinct band-shifted components of VACssBF2 (12) were clearly resolved when either the PE (PE-PrMss) or CE (CE-PrMss) sense strands were used as probes (Fig. 3). The ability of excess, unlabeled CE-PrMss and PE-PrMss to independently compete for binding of VACssBF2 to 32P-CE-PrMss (Fig. 4, lanes 2–6 and 14–18) confirmed that these ssDNA probes likely interact with the identical protein(s). Importantly, as illustrated by the competitive properties of CE-PrMm2 (Fig. 4, lanes 8–12), mutation of nucleotides that are positionally conserved in both the PE and CE (Fig. 1, bold letters) significantly reduces the affinity of the CE for the more rapidly migrating VACssBF2 doublet. The binding of the more slowly migrating VACssBF2 component was not affected by these mutations implying that this species has a weaker affinity for ssDNA or, alternatively, a different sequence specificity (see “Discussion”) than that of the VACssBF2 doublet. These data are consistent with previous mutagenesis experiments conducted with the homologous PE sense strand which indicated that binding activity of the VACssBF2 doublet is impaired by select purine to pyrimidine transversions introduced either within the GGAATG motif or toward the 5′ and 3′ ends of the ssDNA (12).

**Subunit Composition and Molecular Weight Estimation of Polypeptide Components of VACssBF2**—Binding site mutagenesis studies presented above (Fig. 4) as well as those reported previously (12) indicate that the effect of tested mutations within either the PE or CE is exclusively restricted to the more rapidly migrating VACssBF2 doublet, implying that VACssBF2 is not a homogeneous ssDNA-binding entity. Therefore, an analysis of the individual VACssBF2 band shift species was undertaken. Single-stranded DNA-protein complexes were first resolved in nondenaturing EMSA gels following incubation of AKR-2B whole cell protein extract with a coding strand-specific oligonucleotide probe (32P-PE-PrM). The protein-DNA complexes were irreversibly cross-linked by exposure of the wet gels to 254 nm UV light and then excised and subjected to denaturing electrophoresis in SDS-polyacrylamide gels. Fig. 5 illustrates that VACssBF2 consists of multiple distinct polypeptide species. Relative apparent molecular weights of the individual species were calculated after correcting for the contribution of the DNA component (35). The two bands of the rapidly migrating doublet were independently excised following UV cross-linking and analyzed separately. As shown in Fig.

**Fig. 1. DNA sequence elements within the promoter and coding region of the VSM α-actin gene containing a TEF-1 recognition motif (GGAATG).** A simplified model of the VSM α-gene is shown. Nucleotides encoding the promoter, 5′-untranslated (5′-UTR), and protein coding regions are numbered with respect to the start site of transcription (+1). Arrows indicate the predicted positions of introns 1–8 that are not included in the nucleotide numbering. Amino acids are numbered with respect to the start methionine (+1). Nucleotides shared between the promoter element (PE) and coding element (CE) are in bold type.
Since these bands were absent or diminished in the parallel
ence in intensity of the two bands, the slowly migrating species (\( \text{M}_{\text{slowly migrating species}} \)) was incubated with AKR-2B whole cell extract protein (3.6 \( \mu \text{g} \)). Anumber of other bands signal generated in a parallel blot probed with a mutant CE ssDNA-protein complexes was indicated by the lack of denotesthe intron2/exon3 splicesite. Underlined bases denoted differences with respect to the VSM \( \alpha \)-actin isoform. Bold nucleotides are required for high affinity VACssBF2 binding.

Nucleotide sequences encoding amino acids 44–53 (GVMVGMGQKD) of the nascent murine actin isoforms are shown (28, 30–34). CAG/G denotes the intron 2/exon 3 splice site. Underlined bases denote differences with respect to the VSM \( \alpha \)-actin isoform. Bold nucleotides are required for high affinity VACssBF2 binding.

| Isoform     | Base sequence                  |
|-------------|--------------------------------|
| \( \alpha \) (VSM) | CAG/GGA-GTA-ATG-GTT-GGA-ATG-GGC-CAA-AAA-GAC |
| \( \alpha \) (cardiac) | CAG/GGA-GTA-ATG-GTT-GGA-ATG-GGC-CAA-AAA-GAC |
| \( \alpha \) (skeletal) | CAG/GGA-GTA-ATG-GTT-GGA-ATG-GGC-CAA-AAA-GAC |
| \( \beta \) (cytokkeletal) | CAG/GGA-GTA-ATG-GTT-GGA-ATG-GGC-CAA-AAA-GAC |
| \( \gamma \) (enteric) | CAG/GGA-GTA-ATG-GTT-GGA-ATG-GGC-CAA-AAA-GAC |

5 (lanes 2 and 3), the individually cross-linked components of the VACssBF2 doublet migrated somewhat differently with corrected apparent molecular weights of 52,000 and 49,000, respectively. The more slowly migrating component (Fig. 5, lane 1) resolved as two components, a major \( M_r \sim 84,000 \) species and a faint \( M_r \sim 30,000 \) species. Given the relative difference in intensity of the two bands, the \( M_r \sim 30,000 \) species is likely only a minor component of the more slowly migrating VACssBF2 complex. Southwestern blotting experiments were also performed with 30 base CE ssDNA probes to validate the conclusion that VACssBF2 consists of multiple distinct polypeptide species. As shown in Fig. 6, the profile of the Southwestern blot probed with the wild-type CE oligonucleotide (CE-PrMss) is similar in appearance to previously described EMSAs. In particular, the CE ssDNA-binding reactivity of AKR-2B-derived cellular protein resolved into at least two distinct components, a more rapidly migrating doublet (\( M_r \sim 51,000/48,000 \)) and a more slowly migrating species (\( M_r \sim 121,000 \)).

The specificity of these ssDNA-protein complexes was indicated by the lack of signal generated in a parallel blot probed with a mutant CE oligonucleotide (Fig. 6, CE-PrMmu2). A number of other bands (\( M_r \sim 133,000, 39,000, 33,000 / 32,000 \) doublet, 25,000) were also discernible in the CE-PrMss blot (Fig. 6, left panel), most notably in the lane containing AKR-2B nuclear extract (lane 3). Since these bands were absent or diminished in the parallel CE-PrMss blot, they may represent different post-translationally modified forms and/or degradation products of VACssBF2 that are not detected by band shift analysis. Nuclear extracts from cultured NIH-3T3 fibroblasts also exhibited VACssBF2 ssDNA binding activity (Fig. 6, lane 4). It is also noteworthy that the overall intensity of signal relative to whole cell extracts (Fig. 6, lane 1) was enhanced in nuclear extracts (lane 3) and diminished in cytosolic extracts (lane 2) of AKR-2B fibroblasts. Because an equivalent amount of protein from each extract was applied to the gel, these data suggest that VACssBF2 is enriched in the nucleus. Results of comparative band shift assays conducted with whole cell, nuclear, and cytosolic extracts of AKR-2B fibroblasts were consistent with this conclusion (data not shown).

Repression of Enhancer-dependent Transcription by VACssBF2—Previous deletion-mapping studies demonstrated that a mouse VSM \( \alpha \)-actin promoter truncated to position –224 (VSMP, Fig. 7) is transcriptionally silent in both AKR-2B fibroblasts and undifferentiated BC3H1 myoblasts (22, 25). Deletion to position –195 (the 5’ end of the PE) yielded a promoter that was still fully repressed in AKR-2B fibroblasts but only partially repressed in subconfluent myoblasts (25). Further truncation of the PE to position –191 produced a serum-inducible promoter (VSMP4, Fig. 7) that was transcriptionally active in both cell types (22, 25). Subsequent studies revealed that a purine-rich, TEF-1-binding element located between –181 and –176 is essential for the activity of VSMP4 (13) and that ssDNA binding activities (VACssBF1 and
VACssBF2) that interact with opposite strands of the TEF-1 enhancer (i.e. the PE) are, at least, partially responsible for the repression of VSMP3 in both fibroblasts and myoblasts (12, 13).

The discovery that the CE element binds VACssBF2 but not VACssBF1 nor TEF-1 allowed us to examine the functional activity of VACssBF2 independently by assessing the effect of the CE on promoter function. A synthetic copy of the CE was inserted 5' of the TEF-1 enhancer binding site in VSMP4 (13) to create P4/CE(5') (Fig. 7). This chimeric construct was tested in parallel transfections with VSMP4 and VSMP3 for CAT protein expression in quiescent and serum-stimulated AKR-2B fibroblasts. As shown in Fig. 7, the activity of P4/CE(5') was repressed approximately 5–10-fold relative to its parent construct, VSMP4. This level of repression is similar, although not identical, to that of VSMP3, which was repressed 9–16-fold relative to VSMP4 in quiescent and serum-stimulated transfectants. These data suggest that the CE can partially substitute for the intact PE in suppressing the activity of the promoter when positioned 5' of the TEF-1 binding site.

Because promoter activity was also suppressed in P4/CE(5') transfectants superinduced with serum and cycloheximide (Fig. 7), we next tested whether or not the effect of the CE was mediated at the transcriptional level. The above constructs were transiently transfected into AKR-2B fibroblasts together with a β-actin promoter construct (p99βAc-CAT, (22)) as a transfection efficiency control. Following superinduction with serum and cycloheximide, poly(A)+ mRNA was isolated. Promoter activity was evaluated by assaying the levels of correctly initiated CAT RNA transcripts by primer extension analyses using a CAT coding sequence-specific primer. As shown in Fig. 8, while positive control transcripts from the β-actin promoter were equally expressed in all cotransfectants, the levels of VSM α-actin promoter-driven transcripts varied. This pattern was also evident using shorter autoradiographic exposure times. In particular, VSMP3 was transcriptionally silent while VSMP4 was transcriptionally active (Fig. 8, compare lanes 2 and 3). Incorporation of the CE into the 5' position of VSMP4 significantly impaired the ability of the promoter to drive transcription of the CAT reporter gene (Fig. 8, compare lanes 3 and 4). However, the CE had no substantive effect when positioned 3' of the transcription start site at a distance from the TEF-1 binding site (Fig. 8, lane 6) suggesting that the repressive function of the CE is position-dependent. Importantly, a promoter construct possessing a 5' mutant CE defective in VACssBF2 binding, P4/CEmu2(5'), exhibited transcriptional activity comparable with that of VSMP4 (Fig. 8, compare lanes 3 and 5, and Fig. 9). These data suggest that VACssBF2 can function in the absence of VACssBF1 to transcriptionally re-
press a nearby TEF-1 enhancer in fibroblasts.

To test whether VACssBF2-induced repression was unique to the VSM α-actin TEF-1-dependent enhancer element, the CE was also incorporated into a heterologous tissue factor promoter construct immediately upstream of two activator protein-1 (AP-1) dsDNA-binding elements (Fig. 9). This construct was chosen because these two AP-1 elements had been previously shown to be necessary and sufficient for serum inducibility of the basal tissue factor promoter in AKR-2B fibroblasts (23). The heterologous tissue factor/VSM α-actin CE construct, TF/CE(59), was tested in parallel transfections with its parent construct, TFAP1, as well as VSMP3, VSMP4, P4/CEwt(59), and P4/CEmu2(59) for CAT protein expression in serum and cycloheximide-stimulated fibroblasts as described under “Experimental Procedures.” Cellular extracts from duplicate transfectants were assayed for CAT protein by immunoassay.

DISCUSSION

A recent study suggested a model in which two ssDNA-binding factors, designated VACssBF1 and VACssBF2, function to repress transcription of the mouse VSM α-actin gene by disrupting base pairing within an essential TEF-1 enhancer element (12). Although these activities bind to opposite strands of the DNA helix encompassing the TEF-1 recognition motif, we could not determine whether both or only one of these factors were required to repress transcription. In this study we provide evidence that VACssBF2 can function autonomously (i.e. in the absence of an opposing VACssBF1 binding site) to transcriptionally repress the activity of two distinct promoters in an enhancer-independent manner. These results were based upon the initial identification of a DNA element within the protein-coding region of the gene that bears intriguing sequence similarity to the negatively regulated enhancer element.
located in the proximal promoter (Fig. 1). This sequence element, termed the coding element (CE), contains a 6-bp GGAAATG TEF-1 recognition motif positioned within a purine-rich sequence and is thus structurally analogous to the promoter element (PE). Comparative DNA binding studies revealed that while TEF-1 and VACssBF1 binding are restricted to the PE (Fig. 2), VACssBF2 interacts with the purine-rich coding strand of the CE (Fig. 3) in a sequence-dependent manner (Fig. 4). Because the noncoding stra of both the PE and CE exhibit a high pyrimidine content (83 and 77%, respectively), the determinants of VACssBF1 binding must also include specifically ordered sets of bases rather than a simple high ratio of pyrimidines. Our results also demonstrate that TEF-1 binding is strongly influenced by sequences that flank the central GGAAATG motif.

Several recent reports identifying a silencing element within the protein-coding sequence of the osteocalcin gene (1, 2) prompted us to investigate the possibility that the CE may function as an intragenic, transcriptional silencer. Although the CE mimicked the PE when placed 5' of a TEF-1 binding site in a deletionally activated promoter (P4/CE(5'), Fig. 3) in a sequence-dependent manner (Fig. 4). Because the noncoding stra of both the PE and CE exhibit a high pyrimidine content (83 and 77%, respectively), the determinants of VACssBF1 binding must also include specifically ordered sets of bases rather than a simple high ratio of pyrimidines. Our results also demonstrate that TEF-1 binding is strongly influenced by sequences that flank the central GGAAATG motif.

While we cannot formally preclude some residual VACssBF1 binding to the truncated PE in P4/CE(5'), VACssBF1 binding to either strand of the two AP-1 elements in TFAP1 is highly unlikely given the lack of sequence homology between AP-1 and TEF-1 enhancer elements (13, 23). Therefore, the ability of the CE to also repress the activity of a heterologous, AP-1-dependent tissue factor promoter (Fig. 9) suggests that VACssBF2 can disrupt enhancer function by a mechanism that is independent of both VACssBF1 and the double-stranded enhancer-binding protein affected.

In a previous survey of selected TEF-1-dependent enhancers, VACssBF1 binding was found to be largely restricted to the VSM α-actin PE, but VACssBF2 readily interacted with a broader spectrum of ssDNA sequences including those of the cardiac troponin T MCAT motif and the SV40 GT IIC, SpI and SpII enhancer motifs (12). The ability of VACssBF2 to interact with enhancer elements present in diverse promoters, coupled with an intrinsic ability to disrupt enhancer function, greatly expands the regulatory potential of this ssDNA-binding factor. Thus, there is a strong likelihood that VACssBF2 participates in the regulation of a larger number of genes than is currently appreciated.

The chimeric promoter activation studies presented here provide important clues as to the functional role of VACssBF2. Given the ssDNA binding properties of VACssBF2 and its ability to function in the absence of VACssBF1, we postulate that VACssBF2 may interfere with enhancer activity by inducing a localized ssDNA conformation that diminishes proximal enhancer protein binding affinity. Precedence for a mechanism of this type exists in the literature. Several other ssDNA-
binding proteins, including YB-1 (36) and the far-upstream element binding protein of c-myc (37–39), appear to influence transcription, in part, by altering DNA structure within specific promoter elements. However, because VACssBF2 does not appear to be enhancer type-selective, we cannot exclude the possibility that VACssBF2 does not disrupt enhancer protein dsDNA binding directly but rather functions to disrupt interactions between such proteins and components of the basal transcription complex. VACssBF1 binding within the context of the native promoter may be required to augment the function of VACssBF2 under some conditions or to increase specificity for the VSM α-actin TEF-1 enhancer element.

The issue of whether these ssDNA-binding proteins induce a localized dsDNA conformation or stabilize a preexisting, non-B-DNA structure will ultimately require the purification, cloning, and characterization of these factors. A review of the literature reveals that VACssBF1 and VACssBF2 are not unique in their affinity for ssDNA encompassing a TEF-1 motif. Another ssDNA binding factor, designated muscle factor 3 (MF-3), has been previously reported to interact with the non-coding strand of the muscle-specific TEF-1/M-CAT sequence element from the chicken skeletal α-actin gene (40). However, MF3 differs from either VACssBF1 or VACssBF2 in that it also interacts with dsDNA comprising the TEF-1/M-CAT motif (41). VACssBF1 and VACssBF2 have no detectable binding affinity for the double-stranded form of the VSM α-actin TEF-1-binding sequence (12). Moreover, while VACssBF1 and VACssBF2 ssDNA binding activity is restricted to tissues that are enriched in smooth muscle (13), MF3 is expressed in both skeletal muscle and liver (40).

Structural studies have revealed that while VACssBF1 is a single, M, = 52,000, polypeptide,2 VACssBF2 is more complex. UV cross-linking and SDS-polyacrylamide gel electrophoresis analyses indicated that VACssBF2 consists of multiple polypeptide components depending upon the analytic technique employed (Fig. 5). Results of Southwestern blots confirmed that VACssBF2 consists of at least three distinct electrophoretic species, a M, = 51,000/48,000 doublet and a larger molecular weight component (Fig. 6). The estimated apparent molecular weight of the more slowly migrating component varied somewhat depending upon the analytic technique employed (M, 84,000 by UV cross-linking and M, 121,000 by Southwestern blotting). The inherent nonideality in electrophoretic mobility of a ssDNA-protein complex (UV cross-linking) versus protein alone (Southwestern blot) may account for this difference. Results of preliminary VACssBF2-RNA binding studies suggest that the more slowly migrating VACssBF2 species preferentially interacts with the mRNA counterpart of the CE, whereas the VACssBF2 doublet binds exclusively to ssDNA.3 These data reinforce the conclusion that the M, 121,000 component is distinct from the VACssBF2 doublet and also suggest that this species may also play a role in post-transcriptional regulation.

Using a DNA-binding site screening methodology, VACssBF1 has been tentatively identified on the basis of its molecular size, binding site specificity, and antigenic determinants as the murine homolog of YB-1, a member of the Y-box family of proteins.5 Owing to the recent study that reported that YB-1 promotes the formation of single-stranded regions within the major histocompatibility class II major histocompatibility

2 J. G. Cogan, E. S. Stoﬀet, M. J. Getz, and A. R. Strauch, manuscript in preparation.
3 R. J. Kelm, S. Sun, and M. J. Getz, unpublished observations.
