Altered Sensitivity to Single-strand-specific Reagents Associated with the Genomic Vascular Smooth Muscle $\alpha$-Actin Promoter during Myofibroblast Differentiation*

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Stimulation of quiescent AKR-2B mouse fibroblasts with transforming growth factor $\beta1$ results in uniform conversion to a myofibroblast-like phenotype as judged by a rapid accumulation of smooth muscle $\alpha$-actin mRNA and protein. Because transcriptional regulation of the smooth muscle $\alpha$-actin gene in these cells might be mediated by single-stranded DNA-binding proteins, we have examined the sensitivity of genomic DNA to chemical reagents with specificity for unpaired bases in a region of the promoter previously implicated in Pur, Pur/T, and MSY1 binding in vitro (Kelm, R. J., Jr., Cogan, J. G., Elder, P. K., Strauch, A. R., and Getz, M. J. (1999) J. Biol. Chem. 274, 14238–14245). Our data reveal specific differences between purified DNA treated in vitro and nucleoprotein complexes treated in living cells. Although some differences were observed in quiescent cells, treatment with transforming growth factor $\beta1$ resulted in the development of additional sensitivity within 1 h. This enhancement was most pronounced in bases immediately upstream of an MCAT enhancer element-containing polypurine-polypyrimidine tract. A TATA-proximal element of similar base distribution showed no such hyperreactivities. These results suggest that activation of the endogenous smooth muscle $\alpha$-actin gene during myofibroblast conversion is accompanied by specific structural changes in the promoter that are consistent with a decline in single-stranded DNA repressor protein binding.

Although many studies have focused on malignant cells themselves, there is an emerging appreciation of the essential role played by stromal components in regulating tumor growth and progression (1). In the stroma of invasive breast cancer for example, myofibroblasts are the most abundant cell type (2), and their persistence has been linked to stromal modifications believed to facilitate tumor cell invasion and metastasis (3, 4). Myofibroblasts, which exhibit morphological and biochemical features intermediate between fibroblasts and smooth muscle cells, were originally identified in granulation tissue (5) where they appear transiently during wound healing and are thought to provide the contractile force necessary for wound closure (6–9). Because vascular smooth muscle (VSM)$^1$ $\alpha$-actin gene expression is generally considered the hallmark of the myofibroblast phenotype (10), elucidation of the molecular mechanisms that control VSM $\alpha$-actin gene expression during fibroblast to myofibroblast conversion will likely provide greater insight into the role of this distinctive cell type in cancer progression and wound healing. Unfortunately, convenient in vitro model systems of myofibroblast cytodifferentiation are lacking due to the manifest heterogeneity of VSM $\alpha$-actin expression in many established fibroblastic cells lines (11).

Some of our recent studies have focused on identifying the operative cis-acting elements and DNA-binding proteins that regulate mouse VSM $\alpha$-actin promoter activation and repression in serum-stimulated AKR-2B fibroblasts (12–18), a non-transformed clonal cell line derived from AKR mouse embryonic tissue (19). Early transient transfection experiments in AKR-2B cells suggested that transcriptional activation and repression of the mouse VSM $\alpha$-actin promoter in myogenic and fibroblastic cell lines was mediated by promoter-proximal elements spanning the region from −224 to −150 relative to the start site of transcription (12, 13). Subsequent site-directed mutagenesis analysis of the −191 to −150 sequence revealed a purine-rich motif spanning −181 to −176 (GGAATG) that was absolutely required for serum growth factor-dependent activation (15). This motif forms the core of an inverted MCAT enhancer element (AGGAATG) common to a number of genes encoding muscle-specific proteins (20–22). Not surprisingly, band shift studies conducted with fibroblast extracts revealed that a double-stranded oligonucleotide spanning −191 to −162 served as a binding site for an activating protein related by molecular weight, DNA-binding specificity, and immunoreactivity to the MCAT enhancer-binding protein, transcription enhancer factor 1 (TEF-1 (15)). Inspection of sequence spanning −195 to −165 revealed that the MCAT enhancer was centered within a region of high puryury/polyuridyimine (Pu/Py) asymmetry. Because certain Pu/Py tracts have been frequently associated with S1 nuclease-hypersensitive sites in the promoter regions of a number of different genes (23), alterations in DNA secondary structure (especially structures with

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This paper is dedicated to the memory of Dr. Michael John Getz.

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1 The abbreviations used are: VSM, vascular smooth muscle; CAA, chloroacetaldehyde; DMS, dimethyl sulfate; EGF, epidermal growth factor; FGF, fibroblast growth factor; KMnO4, potassium permanganate; PCR, polymerase chain reaction; BMP2, bone morphogenetic protein 2; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline; bp, base pair.
single-stranded regions) were postulated as a mechanism to explain differences in promoter activity observed between VSM α-promoter deletion mutants. Although this region lacks the mirror symmetry thought to be required for H-DNA, recent in vitro studies have none the less identified three single-stranded binding proteins (SSBs) that specifically interact with opposite isolated strands of the Pu/Py tract in the region −195 to −165 (14, 17, 18).

Other groups have suggested the potential role for sequence-specific SSBs in transcriptional regulation (for review see Ref. 24). Several SSBs have been found to be sequence-specific in their DNA association and offer in vivo evidence for promoter-specific activation (25–27) and repression (28–30). Association of SSBs with the DNA target sequence requires the DNA to be unwound or unpaired. Although DNA is typically conceived to be a stiff, static macromolecule existing in vivo in standard B-form, strong in vitro evidence has shown that DNA is capable of assuming a variety of non-B DNA structures (24, 31, 32). Such in vitro evidence of non-B DNA structures with regions of unpairing suggests the potential for such structures in vivo. Whereas the prevailing intracellular temperature, pH, and ionic strength would seem to preclude stable DNA unpairing, there is growing evidence that a small subclass of sequence- and/or conformation-specific, single-stranded DNA-binding transcription factors may utilize such structures as recognition sites in modulating gene expression (25–27, 33–35).

The present study sought to determine if in vivo DNA secondary structure alterations could be detected in the VSM α-actin promoter as a function of gene stimulation. Previous studies have indicated that AKR-2B embryonic fibroblasts are highly sensitive to the mitogenic effects of transforming growth factor-β1 (TGF-β1) (36). We first sought to determine whether AKR-2B fibroblasts could simulate myofibroblast cytodifferentiation in vitro. Immunocytochemical analyses revealed a cytoskeletal composition consistent with that of an in vivo myofibroblast, whereas Northern and Western blotting analyses indicated strong induction of VSM α-actin gene expression following recombinant TGF-β1 treatment of quiescent, G0-arrested cells. To ascertain whether the effect of TGF-β1 was mediated by activation of the endogenous promoter, we employed chemical probes of DNA structure and ligation-mediated PCR (LMPCR) to monitor differences in genomic DNA reactivity in the vicinity of a 5′ Pu/Py tract containing an essential MCAT enhancer element. Single-strand selective reagents were used because previous promoter mapping and in vitro DNA binding studies had implicated several sequence-specific SSBs in repression of MCAT enhancer activity in reporter gene constructs transfected into AKR-2B fibroblasts (14–18). Interestingly, we observed development of strand-specific hyperreactivity in bases immediately flanking the endogenous genomic Pu/Py tract. This observation suggests that DNA topology, and perhaps single-stranded DNA protein binding, is modulated in response to TGF-β1 stimulation. Collectively, these results establish the utility of AKR-2B fibroblasts as a useful cell line for the study of myofibroblast conversion in vitro and support the possible involvement of SSBs in transcriptional control of the VSM α-gene.

**Experimental Procedures**

**Immunocytochemical Analyses of AKR-2B Fibroblasts—Mouse embryonic-derived AKR-2B fibroblasts were maintained and passaged as described previously (13). Cells were seeded in chamber slides (Lab-Tec) and grown to ~75% confluence in McCoy’s 5A medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (HyClone). Cells were rendered quiescent and then treated with 1 ng/ml (40 pM) TGF-β1 as described below for 11.5 h. Cells were washed with phosphate-buffered saline (PBS) and fixed for 10 min in methanol at −20 °C. All subsequent steps were performed at room temperature unless otherwise indicated. Endogenous peroxidase was blocked by incubation with 0.3% H2O2/methanol for 10 min. After washing three times with PBS, potential nonspecific antibody-binding sites were blocked by 30 min incubation with 0.1% crystallized bovine serum albumin dissolved in PBS. The blocking solution was removed by aspiration, and the primary antibody was diluted in blocking buffer and applied to the cells. Following three PBS washes, the primary antibody was detected by indirect immunoperoxidase staining using a Vectastain ABC Elite mouse IgG kit (Vector) as directed by the manufacturer. Following color development with metal-enhanced diaminobenzidine substrate (Pierce) for 1–2 min, slides were rinsed with water, and cells were counterstained by incubation in methyl green (Vector) for 5 min at 65 °C. Slides were dehydrated and mounted with Cytoseal 60 (Stephens Scientific). The monoclonal anti-cytoskeletal antibodies used were anti-smooth muscle α-actin, clone 1A4 (Sigma) at 1:400, anti-desmin, clone DE-U-10 (Sigma) at 1:25, anti-cytokeratin AE1/AE3 (Roche Molecular Biochemicals) at 1:400, anti-pan actin, clone C4 (Roche Molecular Biochemicals) at 1:100, and anti-vimentin, clone Vim 3B4 (Roche Molecular Biochemicals) at 1:5.

**Treatment of AKR-2B Fibroblasts with TGF-β1 and Other Growth Factors—**AKR-2B fibroblasts were plated in 100-mm dishes and grown to 75% confluence as described above. Cells were rendered quiescent by incubation in serum-free MCD 402 medium (JRH Biosciences) for 48 h and then fed 5 ml of fresh medium supplemented with recombinant TGF-β1 (10–400 pM) for 5 min at 65 °C. Slides were dehydrated and mounted with Cytoseal 60 (Stephens Scientific). The monoclonal anti-cytoskeletal antibodies used were anti-smooth muscle α-actin, clone 1A4 (Sigma) at 1:400, anti-desmin, clone DE-U-10 (Sigma) at 1:25, anti-cytokeratin AE1/AE3 (Roche Molecular Biochemicals) at 1:400, anti-pan actin, clone C4 (Roche Molecular Biochemicals) at 1:100, and anti-vimentin, clone Vim 3B4 (Roche Molecular Biochemicals) at 1:5.

**Northern and Western Blot Analyses—**Duplicate 100-mm dishes containing quiescent or TGF-β1/growth factor-treated cells were washed twice with cold PBS. Total cellular RNA was harvested and pooled using TRIzol reagent (Life Technologies, Inc.) as directed by the manufacturer. RNA was quantified spectrophotometrically, and integrity was confirmed by electrophoresis (3 μg/lane) on a 1.2% non-denaturing agarose gel. For Northern blotting, RNA (10 μg/lane) was electrophoresed on 1% agarose gels containing formaldehyde/formamide and transferred to a GeneScreen Plus membrane (NEN Life Science Products). The efficiency of RNA transfer was monitored by staining the membrane with 0.2% methylene blue in 0.02 M NaOAc as described previously (37). The membrane was probed with a mouse smooth muscle α-actin 3′-untranslated region probe (obtained from D. Foster, University of Minnesota) labeled with 32P by random priming and diluted in Quick Hybridization solution (Stratagene) as directed by the manufacturer. Blots were exposed to Kodak XAR film. For Western blotting, whole cell extracts of washed cells were prepared by hypotonic lysis in 1× radiolysis buffers, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and visualized using chemiluminescence. Following Western blot analyses, the membrane was probed with antibodies against smooth muscle α-actin, desmin, and α-actin 3′-untranslated region. The efficiency of RNA transfer was monitored by staining the membrane with 0.2% methylene blue in 0.02 M NaOAc as described previously (37). The membrane was probed with a mouse smooth muscle α-actin 3′-untranslated region probe (obtained from D. Foster, University of Minnesota) labeled with 32P by random priming and diluted in Quick Hybridization solution (Stratagene) as directed by the manufacturer. Blots were exposed to Kodak XAR film. For Western blotting, whole cell extracts of washed cells were prepared by hypotonic lysis in 1× radiolysis buffers, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and visualized using chemiluminescence. Following Western blot analyses, the membrane was probed with antibodies against smooth muscle α-actin, desmin, and α-actin 3′-untranslated region. The efficiency of RNA transfer was monitored by staining the membrane with 0.2% methylene blue in 0.02 M NaOAc as described previously (37). The membrane was probed with a mouse smooth muscle α-actin 3′-untranslated region probe (obtained from D. Foster, University of Minnesota) labeled with 32P by random priming and diluted in Quick Hybridization solution (Stratagene) as directed by the manufacturer. Blots were exposed to Kodak XAR film. For Western blotting, whole cell extracts of washed cells were prepared by hypotonic lysis in 1× radiolysis buffers, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and visualized using chemiluminescence. Following Western blot analyses, the membrane was probed with antibodies against smooth muscle α-actin, desmin, and α-actin 3′-untranslated region.

**Oligonucleotides—** Oligonucleotides were synthesized by standard phosphoramidite chemistry on an Applied Biosystems Inc. 394 synthesizer. The oligonucleotides were purified on 20% denaturing polyacrylamide gels, eluted from gel slices, and desalted by Sep-Pak C18 cartridges. Oligonucleotide photographs were prepared by standard techniques. A 32P-labeled probe for the TGF-β1 coding region was prepared by standard methods. The probe was annealed to a poly-dG/dC oligomer (Marligen Bioproducts) and purified on a polyacrylamide gel. The probe was used to prepare a 32P-labeled probe for the TGF-β1 coding region.

**In Vitro Chemical Treatment of Plasmid and Genomic Samples—** Anhydrous hydrazine, dimethyl sulfate (DMS), formic acid, potassium permanganate (KMnO4), and piperidine were obtained from Aldrich. Chloroacetaldehyde (CAA), 45% wt solution in H2O, was obtained from Fluka. All chemical reagents were used without further purification. The chemical reactivities of naked genomic DNA were analyzed using modifications of published procedures (38). Briefly, 40 μg of purified...
AKR-2B genomic DNA was dissolved in 300 μl of buffer containing 25 mM MOPS, pH 7.1, 4 mM MgCl₂, and 100 mM NaCl. To monitor KMnO₄ reaction, samples were treated with 5 mM (final concentration) KMnO₄ for 2 min at 24 °C. KMnO₄ reactions were terminated by adding 0.1 volume of sodium acetate, pH 5.2, 1 μM-mercaptoethanol, followed by two precipitations from ethanol. To monitor DMS reactivity, samples were treated in a solution of 0.5% aqueous DMS in 50 mM sodium cacodylate, 1 mM EDTA for 2 min at 24 °C. DMS reactions were terminated by adding 0.1 volume of sodium acetate, pH 5.2, 1 μM-mercaptoethanol, followed by two precipitations from ethanol. To monitor CAA reactivity, samples were treated with 2% (final concentration) CAA for 30 min at 37 °C. CAA reactions were terminated by adding 50 mM (final concentration) NaCl followed by two precipitations from ethanol. CAA-treated samples were resuspended in H₂O and then incubated with either formic acid or hydrazine in high salt followed by two precipitations from ethanol to superimpose the CAA data on Maxam and Gilbert reference ladders (39). Dried DNA samples were then resuspended in 1 M piperidine, and base modifications were cleaved by incubation at 90 °C for 30 min. The samples were lyophilized extensively and resuspended in H₂O. DNA samples were then ready for analysis by LMPCR.

In Vivo Chemical Treatment—AKR-2B fibroblasts were subjected to the following chemical treatments after stimulation with 1.0 ng/ml (40 pM) TGF-β1 for 1, 5, and 10 h. As a control, quiescent G₀-arrested cells were similarly treated following 48 h serum deprivation (time 0). For KMnO₄ treatment, cells were exposed to 10 mM KMnO₄ in PBS for 2 min at 24 °C. For DMS treatment, cells were exposed to 0.1% DMS in PBS for 2 min at 24 °C. For CAA treatment, cells were exposed to 3% CAA in PBS for 2 min at 37 °C. All reactions were terminated by washing cells twice with ice-cold PBS. Cell lysis and DNA purification were by standard protocols (40). CAA-treated DNA was then processed as described above for in vitro treated DNA to superimpose either G:A (CAA-R) or C:T (CAA-Y) chemistry. Dried genomic DNA was cleaved with piperidine and prepared for LMPCR as described for in vitro genomic samples. The genomic DNA concentration was then determined spectrophotometrically at 260 nm.

LMPCR Analysis—For analysis of the VSM α-actin promoter by LMPCR, four gene-specific primer sets were designed with the assistance of Sequenase 2.0 software (41). Primer set A was used to analyze the purine strand of the VSM α-actin MCT enhancer element and included gene-specific primers A1 (5'-AGCAAGACGGGCTGAGAAGC), A2 (5'-GCTGAAAGTGCGCCTCCTACTAAC), and A3 (5'-ACTTAAACAACCATATAAGGACACTCAG). Primer set B was used to analyze the pyrimidine strand of the VSM α-actin MCT enhancer element and included gene-specific primers B1 (5'-TCCCCATGACACTAGCCGATG), B2 (5'-AGCTGCTGGCATTTAAGCCGATGCT), and B3 (5'-AGGTGTTCTGAGGCTTGAAGT). Primer set C was used to analyze the top purine strand of the TATA-proximal promoter and included gene-specific primers C1 (5'-TGGCTACATTGGATGATAAAAG), C2 (5'-TGGATGATAAAGAGAGCCGTTGAAAATC), and C3 (5'-GAAACTCGAAGTCATATCCCTGG). Primer set D was used to analyze the bottom purine strand of the TATA-proximal promoter and included gene-specific primers D1 (5'-GGAAATGGCGAACGAGAGACC), D2 (5'-AGAGAACCACGGCTCTGGCCAACCC), and D3 (5'-GCCACCCCAGTTAGGAGGATTGTGTC). The chemical reactivities of the genomic DNA samples were analyzed using standard LMPCR methods (42, 43). Briefly, 2 μg of chemically treated and purified genomic DNA samples were used at the start of LMPCR. Primer extension was performed using Sequenase 2.0 (Amersham Pharmacia Biotech) with the first gene-specific primer. After ligation of a blunt unidirectional linker duplex (42, 43), PCR was performed in the presence of Taq polymerase (Fisher), the second gene-specific primer, and the unidirectional linker primer (5'-CGCGTGACCAGAGGAGATCTG-AATTC) for 20 cycles. The PCR products were separated on 6% or 8% denaturing polyacrylamide sequencing gels (19:1 acrylamide:bisacrylamide). Gels were then electrophoresed to nylon membranes (NEN Life Science Products), prehybridized, and hybridized with a radioactive probe created by single-sided PCR using the third gene-specific primer. Sequencing markers were created by standard Maxam and Gilbert chemical modifications of genomic DNA in vitro (39), together with standard LMPCR detection. Radioactive signals were analyzed using a Molecular Dynamics Storm 840 PhosphorImager.

RESULTS

AKR-2B Fibroblasts as a Model System for the Generation of the Myofibroblast (VSM α-Actin Expressing) Phenotype—Immunocytochemical analyses were initially conducted to ascertain the cytoskeletal composition of AKR-2B fibroblasts following treatment with recombinant TGF-β1. Serum-starved quiescent cells at roughly 75% confluence were exposed to 40 pg TGF-β1 for 11.5 h. Panactin and VSM α-actin-specific antibodies (44) displayed identical patterns of immunoreactivity (compare top right and bottom right panels). Importantly, VSM α-actin staining of cytoskeletal stress fibers was observed in all cells indicating that AKR-2B fibroblasts are homogeneous with respect to their ability to express the VSM α-actin isoform. Moreover, whereas vimentin staining was apparent in all cells (Fig. 1, middle right panel), staining for desmin (middle left panel) and keratin (bottom left panel) was completely negative. Since desmin, keratin, and vimentin are cytoskeletal markers for cell types of myogenic, epithelial, and mesenchymal origin, respectively, these data indicate that AKR-2B fibroblasts exhibit a uniform pattern of cytoskeletal protein expression characteristic of myofibroblasts in vivo. This conclusion was confirmed by Western blot analyses of whole cell extracts from AKR-2B fibroblasts using the same antibodies applied in immunohistochemical detection (data not shown).

To ascertain the relative effect of purified recombinant TGF-β1 on endogenous VSM α-actin gene transcription and translation in AKR-2B fibroblasts, Northern and Western blot analyses were performed following treatment of quiescent cells with various growth factors. As shown in Fig. 2A, TGF-β1 proved to be by far the most potent inducer of VSM α-actin mRNA accumulation (lanes 6–8), relative to serum (lane 3), insulin (lane 4), or PDGF-BB (lane 5). The stimulatory effect of TGF-β1 appeared to be regulated in a temporal manner in that elevated mRNA levels were detectable at 2 h, peaked between 6 and 12 h, and declined at 24 h (Fig. 2B). Moreover, accumulation of mRNA coincided with a progressive increase in VSM α-actin protein from 2 to 24 h following addition of TGF-β1 (Fig. 2C). Other growth factors/cytokines such as acidic FGF, basic FGF, and tumor necrosis factor α were also tested, but all failed to effect VSM α-actin gene expression in quiescent AKR-2B fibroblasts by themselves. Importantly, however, basic FGF was found to inhibit induction of VSM
Altered DNA Structure Induced by TGF-β1

**FIG. 2.** TGF-β1 specifically induces endogenous VSM α-actin gene expression in AKR-2B fibroblasts. A, quiescent AKR-2B fibroblasts were treated for 4 h with a variety of peptide growth factors including EGF (10 ng/ml), insulin (500 ng/ml), PDGF-BB (2.5 ng/ml), and TGF-β1 (1 ng/ml). Total cellular RNA was harvested and subjected to Northern blot analysis (10 μg/lane) using CDNA probe that hybridizes to the 3’-untranslated region of mouse VSM α-actin. Data shown are from an overnight exposure. B, quiescent AKR-2B fibroblasts were stimulated with 1 ng/ml (40 pM) TGF-β1, and total RNA was harvested at various time points. Northern blot analysis was performed to detect VSM α-actin mRNA as above. Data shown are from a 4-h exposure. C, quiescent AKR-2B fibroblasts were stimulated with 1 ng/ml (40 pM) TGF-β1, and whole cell protein extracts were prepared at various time points. Denatured protein (5 μg/lane) was subjected to Western blot analysis using a monoclonal anti-smooth muscle α-actin-specific antibody (44). D, quiescent AKR-2B fibroblasts (lane 1) were stimulated with TGF-β1 alone (lane 2) or with TGF-β1 and basic FGF (lanes 3–5). Whole cell protein extracts were prepared 8 h after the addition of growth factors, and 2 μg of denatured protein was subjected to Western blot analysis as above.

α-actin protein expression by TGF-β1 in a dose-dependent manner (Fig. 2D). This result is reminiscent of the inhibitory effect of basic FGF on TGF-β1-induced myofibroblast conversion in primary breast fibroblasts (2) and provides further credence to the notion that AKR-2B fibroblasts are a useful model system for studying the generation of the myofibroblast phenotype.

**Strategy for in Vivo Analysis by LMPCR—**LMPCR has been shown to be an excellent tool for in vivo footprinting of protein-DNA interactions and methylation patterns (42, 43). LMPCR permits the analysis of specific gene sequences at nucleotide resolution using as little as 2 μg of total eukaryotic genomic DNA. We employed LMPCR to analyze features of the in vivo DNA structure in two regions in the VSM α-actin promoter (Fig. 3). The upstream Pu/Py tract (−195 to −165) containing an inverted, consensus MCAT enhancer element (AGGAATG) has been previously implicated as an important region for transcriptional regulation in serum-stimulated fibroblasts (12–15). Moreover, the opposing strands of the Pu/Py sequence have been shown to serve as binding sites for three distinct SSBs (Purα, Purβ, and Mst1) in vitro (17, 18). Within the TATA-proximal promoter, a distinct, purine-rich element is found that has been implicated in controlling the TGF-β1 responsiveness of a transfected rat promoter:reporter gene construct in smooth muscle cells. This element, designated TGF-β1 control element (TCE) by Hautmann and co-workers (45), spans −48 to −57 of the rat promoter and is completely conserved in the mouse promoter.

To survey completely the DNA regions of interest, multiple chemical probes were chosen for use in combination with LMPCR. Chemical reagents that react preferentially with unpaired or unmatched DNA structures (CAA and KMnO4) or serve as footprinting agents (DMS) were employed to characterize differences in reactivities within the VSM α-actin promoter DNA sequences as a function of TGF-β1 stimulation. These chemical probes have been previously shown to be valuable for detecting unusual non-B DNA structures and indicating regions of protection resulting from the binding of proteins (46).

To validate the use of these chemical probes and to explore the reproducibility of sample preparation, we first analyzed two independent purified genomic DNA samples and two independent DNA samples prepared from quiescent G0-arrested AKR-2B cells chemically treated in vivo (Fig. 4). Extremely consistent patterns of reactivity were seen for both the purified genomic DNA samples (Fig. 4, compare lanes 7 and 8) and the quiescent G0-arrested in vivo DNA samples (Fig. 4, compare lanes 13 and 14) between the two independently prepared sample sets. We also noted differences in the pattern of chemical reactivity between the isolated genomic DNA samples and samples from intact cells (Fig. 4, circles). CAA-R, CAA-Y, DMS, and KMnO4 treatments yielded interpretable patterns of reactivity for both in vitro and in vivo genomic samples (Fig. 4, lanes 7–22). CAA alone did not provide useful data for analysis of hyperreactivities or protections within this region (Fig. 4, lanes 3–6). Together, these data demonstrate the remarkable reproducibility of LMPCR data and validate the use of these chemical probes in combination with LMPCR for our in vivo structural analysis.

**LMPCR Analysis of VSM α-Actin Promoter Sequences—**Chemical probes of DNA structure were used in combination...
with LMPCR to analyze the upstream MCAT-containing Pu/Py element and flanking sequence as a function of TGF-β1 stimulation (Fig. 5). Initial experiments compared the differences between chemically treated genomic samples versus quiescent G₀-arrested in vivo samples (Fig. 5, A and B, open symbols). A small number of differences were observed, distributed on both strands, and were scattered over the analyzed sequences (Fig. 5, A and B). An area of DMS protection was indicated (bases −209 to −205) upstream from the Pu/Py element on the pyrimidine strand (Fig. 5B, compare lanes 14 and 15, open arrowheads). The guanine residues that are faint in the in vitro CAA-Y lane (Fig. 5A, lane 4) become more reactive in vivo (Fig. 5A, lane 5). An adenine residue at position −164 in the CAA-Y sample becomes reactive indicating that the base is unpaired in vivo (Fig. 5A, compare lanes 9 and 10). Examples of increased thymine reactivity within in vivo quiescent samples (versus in vitro samples) are noted on both strands indicating an unpairing or unstacking of these bases (Fig. 5, A and B, compare lanes 19 and 20, open circles).

AKR-2B cells were monitored at 1, 5, or 10 h following TGF-β1 treatment to determine what structural changes occurred as a function of stimulation (Fig. 5). Differences in reactivity patterns were seen within 1 h following TGF-β1 stimulation (Fig. 5, closed symbols). Only two examples of DNA protections were reproducibly observed indicating protein binding as a function of TGF-β1 stimulation (Fig. 5, closed arrowheads). Most of the observed changes following stimulation were hyperreactivities to single-strand-specific chemicals, indicating regions of enhanced base flexibility or unpairing. Hyperreactivities were seen immediately downstream of the Pu/Py element on both strands (Fig. 5, A and B, bases −162 to −160). Hyperreactivities on the purine strand are localized to the downstream Pu/Py boundary and flanking sequence (Fig. 5A, closed circles). Interestingly, most of the hyperreactivities on the pyrimidine strand occurred in the upstream or downstream flanking regions of the Pu/Py element (Fig. 5B, closed circles). The CAA-Y hyperreactivity was localized to a region between bases −196 and −209 (Fig. 5B, compare lane 10 with lanes 11–13, closed circles). Together, these data indicate that a structural transition is occurring within this region as a function of TGF-β1 stimulation and is localized to the region immediately downstream of the MCAT enhancer. Importantly, transient transfection studies conducted with mouse VSM α-actin reporter gene constructs have also implicated nucleotides spanning −192 to −224 in mediating transcriptional repression in fibroblasts (12, 13). Thus, there appears to be some commonality between the bases that become hyperreactive upon TGF-β1 induction of the genomic promoter and those required to maintain full repression of a transfected promoter, albeit in rapidly growing or serum-stimulated fibroblasts. Fig. 7A summarizes chemical reactivities for the Pu/Py sequence and flanking bases.

By using a different set of LMPCR primers, we then analyzed the DNA samples studied in Fig. 5 to observe the pattern of reactivity within the TATA-proximal promoter that, like the Pu/Py element, is purine-rich and also contains a putative TCE of unknown relevance in fibroblasts. We wanted to determine if a reactivity pattern similar to that in the Pu/Py element would be observed as a function of TGF-β1 stimulation. When we compared chemically treated genomic samples versus quiescent G₀-arrested in vivo samples, several differences were detected in the analyzed region (Fig. 6, A and B, open symbols). A region immediately upstream of the TATA box (bases −27 to −36) revealed modest protections from DMS, CAA, or KMnO₄ in vivo (versus in vitro) indicating possible protein association (Fig. 6, A and B, open arrowheads). An adenine residue immediately downstream of the +1 site was hyperreactive in the in vivo samples, indicating a site of unpairing near the transcription start site (Fig. 6A, compare lane 9 with lanes 10–13, open symbols). When AKR-2B cells were stimulated with TGF-β1, only modest changes were noted (Fig. 6, closed symbols). Hyperreactivities comparable to those detected in the Pu/Py element and flanking sequence were not observed in the TATA-proximal promoter. These data indicate that this region does not undergo a dramatic change in structure to accommodate changes in protein binding as a function of TGF-β1 stimulation. Fig. 7B summarizes chemical reactivities for the TATA-proximal promoter that includes the putative TCE sequence.

**DISCUSSION**

Historically, cis-acting regulatory elements responsible for activation or repression of particular genes have been analyzed using transient transfections and plasmid-based reporter gene expression systems in which the promoter sequence of interest is linked to a reporter gene not commonly expressed in the cell under investigation. The assumption with this type of analysis is that the transfected promoter in plasmid DNA is an accurate and reliable model of the native promoter in genomic DNA. Although this is obviously an oversimplifica-
tion in both practical and theoretical terms (e.g. nucleosome structure, matrix attachment sites, genomic insulator/boundary elements, etc.), reporter gene assays remain the most commonly used and accepted tool for studying transcriptional regulation in cultured cells.

Another approach to examine the validity of conclusions drawn from promoter-reporter experiments is to monitor genomic DNA in vivo for changes in base hypersensitivity or footprinting patterns as a function of endogenous gene activation or repression. This approach also offers the opportunity for further insights. In this paper, we present such an analysis of the VSM α-actin promoter in TGF-β1-stimulated AKR-2B fi-

**FIG. 5.** TGF-β1 stimulation induces base hyperreactivities in sequences flanking the MCAT enhancer element. DNA samples were analyzed using different primer sets to examine either the purine (A) or pyrimidine (B) strands. The sequence analyzed is indicated (left of each panel) with the MCAT enhancer-containing Pu/Py element highlighted (gray box). The positions relative to the major VSM α-actin tsp are labeled (right of each panel). Lanes 4–8 and 9–13 indicate relative reactivities to CAA superimposed on Maxam and Gilbert G + A (CAA-R) and C > T (CAA-Y) ladders, respectively. Lanes 14–18 indicate relative reactivities to DMS. Lanes 19–23 display the results of in vivo treatment of purified genomic DNA with the indicated chemicals. Lanes 5, 10, 15, and 20 display the results of in vitro treatment of quiescent AKR-2B cells with the indicated chemicals. Lanes 6–8, 11–13, 16–18, and 21–23 display the results of in vivo treatment on AKR-2B cells with the indicated chemicals following TGF-β1 stimulation (1, 5, or 10 h). Reference markers for both panels are Maxam and Gilbert G + A (lane 1) and C > T (lane 2) ladders. Lane 3 in both panels indicates results for purified genomic DNA samples treated with piperidine alone. Hyperreactive bases (circles) and protected bases (arrowheads) are indicated to the left of in vitro treated samples. Differences detected between in vitro genomic DNA versus quiescent G0-arrested in vivo samples (open symbols) and between quiescent G0-arrested in vivo versus in vivo TGF-β1 stimulation (closed symbols) are indicated. Samples were analyzed on a 8% denaturing polyacrylamide sequencing gel.

**FIG. 6.** LMPCR analysis of the VSM α-actin proximal-TATA region. The same DNA samples as in Fig. 5 were analyzed using different primer sets to examine either the top, purine-rich (A), or bottom, pyrimidine-rich (B), strands of the proximal TATA region. The sequence analyzed is indicated (left of each panel) with the purine-rich TCE sequence highlighted (gray box). The positions relative to the major VSM α-actin tsp are labeled (right of each panel). The sample lanes and symbol notations are labeled as in Fig. 5. Samples were analyzed on a 6% denaturing polyacrylamide sequencing gel.
broblasts. This promoter and cell type were selected as a model system for two main reasons. First, from the perspective of pathology and disease, the molecular mechanisms that promote fibroblast to myofibroblast conversion are of interest in delineating the role of the myofibroblast in wound healing and tumor cell metastasis. Second, from a basic molecular biology perspective, alteration of DNA secondary structure is of functional interest in the study of mechanisms of gene regulation.

The implied involvement of non-B-DNA structures in VSM α-actin gene transcriptional control was inferred from previous promoter mapping and in vitro DNA binding studies that identified an asymmetric Pu/Py sequence spanning a region from 195 to 165 (13, 14, 17, 18). The crede of this hypothesis was further supported by computer modeling that predicted two possible alternative, intrastrand hairpin (cruciform) structures that might arise within this region of the promoter if DNA unpairing was somehow induced. Because both structures placed a core MCAT enhancer motif contained within the Pu/Py tract in a partially single-stranded conformation, the possibility existed that transcriptional repression required the formation of single-stranded DNA in the vicinity of the MCAT enhancer. Biochemical analysis identified three single-strand-specific DNA-binding proteins (MSY1, Purα, and Purβ (14, 17, 18)). Purα, a retinoblastoma-binding protein initially identified in HeLa cells (47, 48), and Purβ, a related protein of unknown function, interact with each other and with the purine-rich strand of the Pu/Py element as homo- and heterodimers (18). MSY1, a member of the evolutionarily conserved Y box family of nucleic acid-binding proteins (49), binds to the pyrimidine-rich, non-coding strand of the Pu/Py element but also interacts with Purα and Purβ in the absence of single-stranded DNA (18). Collectively, these results have led to the formulation of a working model whereby transcriptional repression of the VSM α-actin gene in fibroblasts is mediated by competitive interaction of Pur proteins and MSY1 with opposing strands of the MCAT enhancer-containing Pu/Py sequence (14). In such a hypothetical model, disruption of enhancer base pairing would necessarily preclude TEF-1 enhancer protein binding to double-stranded DNA. One seemingly testable prediction arising from this model is that changes in MCAT enhancer topology, as evidenced by the detection of unpaired bases, should accompany changes in the state of endogenous VSM α-actin gene activation in living cells (i.e. AKR-2B fibroblasts).

To test this prediction, we wished to confirm 1) that AKR-2B fibroblasts uniformly express VSM α-actin and 2) that transcription of the endogenous VSM α-actin gene could be strongly induced by serum or some other growth factor. Results of immunocytochemistry clearly showed that AKR-2B cells were homogeneous with respect to VSM α-actin expression, and moreover, the overall cytoskeletal protein composition appeared to be consistent with a myofibroblast phenotype (Fig. 1). Northern blotting suggested that recombinant TGF-β1 was more potent than serum or any other tested growth factor with respect to induction of endogenous VSM α-actin gene expression in AKR-2B fibroblasts (Fig. 2A). Importantly, this effect appeared to be mediated by activation of gene transcription and not stabilization of pre-existing mRNA (Fig. 2, A and B). Furthermore, Western blotting revealed that accumulation of VSM α-actin protein coincided with accumulation of mRNA in TGF-β1-stimulated fibroblasts (Fig. 2, C and D). Together, these initial experiments established the utility of AKR-2B fibroblasts as a model system to search for alterations in genomic VSM α-actin promoter structure during fibroblast to myofibroblast conversion.

Admittedly, our in vitro footprinting data indicate only subtle changes in promoter architecture as a function of TGF-β1 induction. Nevertheless, these changes were not only reproducible (e.g. Fig. 4) but were mapped to regions of the promoter implicated by previous reporter gene studies to be involved in mediating transcriptional activation and repression (Fig. 5). For example, stimulation by TGF-β1 tended to induce accumulation of CAA hyperreactivities rather than DNA protection both within and flanking the Pu/Py tract containing the core MCAT enhancer element (195 to 165). The appearance of such hyperreactivities is indicative of base unpairing and is consistent with reduced binding by SSBS in this region of the promoter. Curiously, most of the hyperreactivities induced by TGF-β1 stimulation map to sequences immediately flanking the Pu/Py tract (196 to 209 and 160 to 162), although several bases were also identified within the 195 to 165 element. Interestingly, only a few bases were protected from methylation by DMS. Such bases were included in a region between 209 to 205 that appeared to be refractory to TGF-β1 induction, and a base at position 185 on the pyrimidine strand that appeared to become more protected as a consequence of TGF-β1 induction. This latter result should not be over-interpreted. However, because of its proximity to the core MCAT enhancer element (182 to 176), increased DMS protection at 185 might reflect increased TEF-1 activator protein binding to double-stranded DNA.

It is important to note that there is precedent for the involvement of a MCAT element in mediating TGF-β1 induction. McLellan and co-workers (50) previously reported that a TEF-1-binding site (i.e. MCAT element) derived from the chicken skeletal α-actin promoter functioned as a TGF-β1 response element in transfected rat cardiac myocytes. Although two copies of the TEF-1-binding site were required to mediate TGF-β1 inducibility of a heterologous promoter, the native MCAT element in concert with an upstream serum response element (i.e. serum response factor-binding site) were neces-
sary and sufficient for conferring TGF-β1 responsiveness upon the skeletal $\alpha$-actin promoter. Such a result is reminiscent of the cooperative interactions between TEF-1 and serum response factor that are thought to contribute to activation of the mouse VSM $\alpha$-actin promoter in fibroblasts stimulated with serum growth factors (13, 15). Thus, we do not consider it implausible that VSM $\alpha$-actin genomic DNA might undergo a structural transition in the vicinity of an MCAT enhancer element in response to TGF-β1 stimulation.

In addition to the 5' Pu/Py region, we also performed LMPCR analysis of the TATA-proximal region of the VSM $\alpha$-actin promoter using our fibroblast model system. This was done, in part, in deference to Hautmann and co-workers (45) who utilized reporter constructs transfected into rat aortic smooth muscle cells to identify a TGF-β control element (TCE, −48 to −57) in the rat smooth muscle $\alpha$-actin promoter. In short, we found little evidence for an overt structural change in this region using either single- or double-strand selective chemistries, nor did we observe any discernible footprint over this region (Fig. 6). These data, however, should not be viewed as contradictory to those of Hautmann et al. (45) since there is substantial experimental evidence indicating that the VSM $\alpha$-actin promoter may be differentially regulated in smooth muscle versus non-smooth muscle cells (33, 34). Reactivity differences were noted near the +1 site of transcription and near the TATA box when we compared in vitro genomic DNA with in vivo quiescent samples (Fig. 6). Curiously, when compared with the MCAT-containing Pu/Py element and its flanking sequence, the TATA proximal sequences showed little or no clustering of either hyperreactive or protected bases (Fig. 7). Within the TATA-proximal promoter there is little evidence of structural transitions as a function of TGF-β1 stimulation, reaffirming that the differences seen in the MCAT region are reliable and not an artifact of chemical treatment or LMPCR (Fig. 7, compare A and B).

DNA is a structurally dynamic macromolecule that is capable of assuming a variety of non-B DNA structures in vitro (32). There is growing evidence that a small subclass of sequence- and/or conformation-specific single-stranded DNA-binding transcription factors may utilize such structures as recognition sites to regulate gene expression (25–27, 33–35). Moreover, in the specific case of the human c-MYC gene, DNA in the vicinity of cis-element binding sites for the SSB transactivators, FUSE-binding protein and hnRNP K has been shown to exist in a configuration that is hypersensitive to cleavage by single-strand-selective agents (51). Our similar analysis of the endogenous VSM $\alpha$-actin promoter supports (but does not prove) the concept, advanced by previous reporter gene and nucleic acid binding studies, that control of VSM $\alpha$-actin gene expression in fibroblasts is regulated, in part, by SSBS. The growth of base hypersensitivity in the vicinity of a Pu/Py tract containing an essential MCAT enhancer element as a function of time and TGF-β1 stimulation is indicative of a structural transition that may give rise to a decline in single-stranded DNA represor protein binding. In this regard, it will be interesting to evaluate whether TGF-β1 induces any change in subcellular localization and/or post-translational modification of Purc, Purβ, and/or MSY1 since inhibition of single-stranded DNA binding by these factors could, in principle, result in derepression of the promoter in fibroblasts. In future studies, it will also be important to determine whether similar changes in genomic structure are observed in smooth muscle cells where activation of VSM $\alpha$-actin gene transcription appears to be regulated by a fundamentally different mechanism (33, 34, 45).
Altered Sensitivity to Single-strand-specific Reagents Associated with the Genomic Vascular Smooth Muscle $\alpha$-Actin Promoter during Myofibroblast Differentiation
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