Repression of Platelet-derived Growth Factor A-chain Gene Transcription by an Upstream Silencer Element

PARTICIPATION BY SEQUENCE-SPECIFIC SINGLE-STRANDED DNA-BINDING PROTEINS*

(Received for publication, May 30, 1996, and in revised form, July 25, 1996)

Bin Liu, Raymond S. Maul, and David M. Kaetzel, Jr.‡
From the Department of Pharmacology, University of Kentucky Medical Center, Lexington, Kentucky 40536

Platelet-derived growth factor A-chain is a potent mitogen expressed in a restricted number of normal and transformed cells. Transient transfection and deletion analysis in BSC-1 (African green monkey, renal epithelial) cells revealed that the −1680 to −1374 region of the A-chain gene repressed homologous and heterologous promoter activities by 60–80%. An S1 nuclease-hypersensitive region (5′SHS) was identified within this region (−1418 to −1388) that exhibited transcriptional silencer activity in BSC-1 and a variety of human tumor cell lines (U87, HepG2, and HeLa). Electrophoretic mobility shift assays conducted with 5′SHS oligodeoxynucleotide probes revealed several binding protein complexes that displayed unique preferences for binding to sense, antisense, and double-stranded forms of the element. Southwestern blot analysis revealed that the antisense strand of 5′SHS binds to nuclear proteins of molecular mass 97, 87, 44, and 17 kDa, whereas the double-stranded form of 5′SHS is recognized by a 70-kDa factor. Mutations within 5′SHS element indicated the necessity of a central 5′-GGGGAGGGGG-3′ motif for protein binding and silencer function, while nucleotides flanking both sides of the motif were also critical for repression. These results support a model in which silencer function of 5′SHS is mediated by antisense strand binding proteins, possibly for stabilizing single-stranded DNA conformations required for interaction with enhancer sequences in the proximal promoter region of the A-chain gene.

Platelet-derived growth factor (PDGF)1 is a potent mitogen and chemoattractant for cultured cells of mesenchymal origin, such as fibroblasts, smooth muscle cells, and glial cells (1). PDGF is a family of cationic glycoproteins composed of two chains, A and B, that combine via disulfide linkages to yield three isoforms (AA, BB, and AB). PDGF B-chain is the product of the c-sis proto-oncogene and is located on human chromosome 22 (2, 3), whereas the homologous A-chain gene is found on human chromosome 7 (4, 5). Expression of the A-chain has been detected in normal endothelial, epithelial, smooth muscle, neuronal, and glial cells, suggesting various functions in cell differentiation, embryogenesis, and wound healing (4, 6, 7). PDGF is also strongly implicated as an etiologic factor in such disparate fibrillar-degenerative diseases as atherosclerosis, glomerulonephritis, pulmonary fibrosis, and neoplasia (8–10).

Transcription of the PDGF A-chain gene can be stimulated or inhibited by a variety of growth factors and cytokines (reviewed in 11). The transcriptional mechanisms mediating these effects and cell-specific patterns of A-chain expression are not fully understood, although accumulating evidence indicates that the transcriptional regulation of the A-chain gene is governed by a complex interaction of positive and negative regulatory elements. A highly GC-rich region in the proximal 5′-flanking sequence of the human A-chain promoter (−80 to −50) has been shown to contribute over 80% promoter activity (12–14), probably mediated through the binding of Sp1-like proteins. Deletion mutagenesis and transient transfection analyses (12, 13) have indicated that the gene is also subject to transcriptional repression by two discrete negative regulatory elements located in more distal areas of the 5′-flanking DNA (−1853 to −1032 and −1031 to −883). In addition, an S1 nuclease-hypersensitive (SHS) region has been localized within the first intron (+1605 to +1630; intron SHS or int-SHS) and demonstrated to exhibit transcriptional silencer activity in HeLa, but not human glioblastoma (A172) cells, suggesting a cell-specific function (15).

In this study, we have employed deletion mutagenesis, S1 nuclease hypersensitivity assay, and transient transfection to identify a 31-bp silencer element in the distal 5′ flanking region (−1418 to −1388; 5′SHS) that represses transcription of the A-chain promoter in a variety of cell lines. Mutational analysis of the 5′SHS element suggests that a centrally located GGGAGGGGG motif, found in both 5′SHS and int-SHS elements, is required both for binding of antisense strand-specific proteins and for full silencer function. Furthermore, we have determined that 5′SHS and int-SHS bind to the same antisense strand-specific binding proteins (p97, p87 and p17), strongly suggesting a common mechanism of transcriptional silencing by these topologically distant DNA elements. These results highlight the role of transcriptional repression in the regulation of A-chain gene expression and indicate the importance of single strand DNA-specific binding proteins in eukaryotic gene transcription.

MATERIALS AND METHODS

DNA Sequencing and Plasmid Constructs—Construction of the plasmids pAC261 and pUTKAT3 has been described (12, 16). The plasmid pGL261 was constructed by inserting an XhoI-HindIII fragment of the

* This work was supported by Grant DK45518 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Pharmacology, University of Kentucky, Chandler Medical Center, MS 305, Lexington, KY 40536. Tel.: 606-257-6558; Fax: 606-323-1981; E-mail: dmkaetzel@pop.uky.edu.

‡ The abbreviations used are: PDGF, platelet-derived growth factor; th, thymidine kinase; HSV, herpes simplex virus; cat, chloramphenicol acetyltransferase; β-gal, β-galactosidase; EMSA, electrophoretic mobility shift assay; SHS, S1 nuclease-hypersensitive; bp, base pairs; ds, double-stranded; as, antisense; s, sense; DTT, dithiothreitol; hnRNP, heterogeneous nuclear ribonucleoprotein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Bicine, N,N-bis[2-hydroxyethyl]glycine; DMEM, Dulbecco’s modified Eagle’s medium.
PDGF A-chain Silencer Element

PDGF A-chain promoter fragment (−261 to +8) into pGL2-basic (Promega) was subcloned into a plasmid containing the CAT reporter gene. To localize DNA elements in the upstream negative regulatory region involved in repression of PDGF A-chain transcription, the −1853 to −883 region was digested with restriction endonucleases AccI and BssHIII into four fragments, designated A (−1853 to −1716), B (−1680 to −1374), C (−1373 to −1032), and D (−1031 to −883). These fragments were inserted in both orientations into the XhoI site of pUTKAT3, which is a derivative of pGL261, DNA sequence (−1853 to −883) region of the PDGF A-chain promoter was obtained by double-stranded DNA sequencing of plasmids containing fragments A–D by the Sequenase procedure (Amersham Corp.). Wild type and mutated 5’ SHS oligodeoxynucleotides and their complementary strands were synthesized (Integrated DNA Technologies), annealed, and inserted in both orientations into the XhoI site of pUTKAT3. The wild type 5’ SHS was also inserted in both orientations 3’ to the cat gene into the BamHI site of pAC261, and 5’ to the cat sequence at the XhoI site of pUTKAT3.

Cell Culture and Transient Transfection—BSC-1, A172 (human glioblastoma), U87 (human astrocytoma), and HepG2 (human hepatocellular carcinoma) cells were obtained from American Type Culture Collection (Manassas, VA). Human cervical carcinoma (HeLa) cells were kindly provided by Dr. Anuradha Ray (Rockefeller University). All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

All plasmids used in transient transfection were purified by an ion-exchange chromatographic procedure (Qiagen). BSC-1 and HepG2 cells were transfected by calcium phosphate/DNA co-precipitation as described previously (12, 17). Cells were harvested 48 h after transfection and were lysed for cat assays by three freeze-thaw cycles in 100 μl of 0.25 mM Tris, pH 7.6, per dish. For luciferase assays, cells were lysed in 200 μl of 1 × reporter lysis buffer (25 mM Bicine, pH 7.6, 0.05% Tween 20, 0.05% Triton X-100). U87 and HeLa cells were transfected with Lipofectin reagent (Life Technologies, Inc.) as described (18). U87 cells were plated 24 h prior to transfection on fibronectin-coated (1 μg/cm2 for 1 h, Sigma) 60-mm diameter tissue culture dishes at a density of 4 × 104 cells per dish. HeLa cells were plated at a density of 1.4 × 105 cells per 60-mm dish 24 h prior to transfection. Lipofectin was added to DMEM at a concentration of 16 μl/ml, mixed with an equal volume of DMEM containing 1.5 μg of pRSV-bgal, and 3.5 μg of test plasmid, and the DNA-Lipofectin mixture was incubated at room temperature for 10 min. Cells were rinsed twice with 1 × PBS and incubated with the Lipofectin-DNA mixture (1.6 ml per dish) at 37 °C for 5 h. An equal volume of DMEM containing 10% FBS was then added to the dishes, and the cells were harvested 48 h after transfection.

CAT, β-Galactosidase and Luciferase Assays—Assays of cat and β-gal activity were performed on cell lysates as described (12, 19, 20). Relative cat activity represents the percent conversion of [14C]chloramphenicol to acetylated products after correction for transcription efficiency by β-gal activity. Luciferase assays were conducted as described by the manufacturer (Promega). Twenty-μl aliquots were assayed by injection of 100 μl of reconstructed luciferase assay reagent (20 mM Tricine, 1.07 mM MgCl₂, 0.1 mM EDTA, 33.3 mM DMF, 270 μM coenzymes A, 470 μM Luciferin, 530 μM ATP, pH 7.8) and measurement of light output for 15 s with a LB9850 Lumet luminometer (Wallac).

Nuclear Extract and Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from HepG2, U87, HeLa, BSC-1, and A172 cells by the method of Dignam et al. (22). Electrophoretic mobility shift assays (EMSAs) were conducted as described (23). A 36-bp irrelevant DNA sequence (5′-GGGCCGGCGAAGGTCGCTGTTAAACC-3′) was used as a nonspecific competitor. Competitive EMSAs with wild type and mutated 5’SHS were scanned with an M2 densitometer (Imaging Research Inc.) to quantify radiolabeling in protein-DNA and free probe bands.

Southwestern Blotting—Southwestern blotting was performed as described (24). Crude nuclear extract (50 μg) was mixed with DIG-labeled buffer D and 6 μl of 4 × sample loading buffer (8% SDS, 28% glycerol, 0.32% Tris, pH 6.8, 0.2% bromophenol blue, 10 μM DTT) to a final volume of 24 μl, heated at 95 °C for 5 min, and then loaded onto a denaturing SDS-polyacrylamide gel (3% stacking, 10% or 15% separating) in a buffer of 25 mM Tris, 192 mM glycine, 0.1% SDS. After electrophoresis, gels were incubated for 30 min in gel transfer buffer (25 mM Tris, 192 mM glycine, 0.025% methanol) and blotted to nitrocellulose membranes with a Bio-Rad transfer cell at 20 V for 1 h. Membranes were blocked for 1 h at room temperature in TNE (10 mM Tris, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT) containing 5% Carnation nonfat dry milk, washed in TNE for 5 min, and placed in a sealed plastic bag containing 5 ml TNED, 5 × 10⁶ cpm/ml oligodeoxynucleotide probe, with or without unlabeled competitor DNA. Membranes were incubated with the probe mixture for 1 h at room temperature, washed twice with TNE for 10 min, and air-dried. Bands were visualized by autoradiography.

RESULTS

Localisation of Multiple Negative Regulatory Regions in the PDGF A-chain Promoter—We previously employed 5’-end point deletion analysis to identify two regions in the 5’-flanking sequence of the A-chain promoter that exhibited negative regulatory activity, designated NRE1 (−1853 to −1032) and NRE2 (−1031 to −883) (12). The DNA sequence of the −1853 to −883 region was obtained (Fig. 1), and the region was digested into four fragments, A, B, C, and D, with restriction endonucleases AccI and BssHIII (Fig. 2A). These fragments were subcloned into pGL261, which contains the −261 to +8 segment of the PDGF A-chain promoter and the luciferase reporter gene. BSC-1, cells, which exhibit considerable A-chain expression when maintained in basal serum-containing culture medium conditions (12), were transiently transfected with these constructs. Fragment A had no effect on luciferase activity when oriented correctly with respect to the A-chain promoter (sense) but modestly inhibited transcription in the opposite orientation (Fig. 2B). Fragment B displayed the most inhibitory activity of the four fragments, reducing luciferase activity to 35% of pGL261 when oriented correctly, although it failed to inhibit transcription in the opposite orientation. Fragment C did not affect transcriptional activity, whereas fragment D displayed a 50% inhibition in either orientation.

To test the ability of fragments A–D to repress transcription of a heterologous promoter, they were inserted into the plasmid pUTKAT3, which contains a promoter/effector segment of the thymidine kinase gene from herpes simplex virus (HSV-tk) and the cat reporter gene (16). Fragment A modestly inhibited tk promoter activity in both orientations (Fig. 2C). Fragments B and C were much more active than fragment A in their sense orientations, decreasing cat activity to 20 and 35% of the pUTKAT3 parent vector, respectively. In their negative orientations, fragments B and D also inhibited tk activity, albeit to a lesser extent, decreasing cat activity to 55 and 75% of the parent construct. Fragment D also significantly inhibited tk promoter activity but only in the opposite orientation. These results indicate the presence of multiple subregions in the −1853 to −883 fragment of the PDGF A-chain gene that repress transcription. Fragment B exerted the strongest inhibitory activity upon both promoters, and its ability to inhibit transcription in a distance-independent manner suggested that it could be liberally characterized as a classical silencer element.
Localization of a Predominant SHS Site (−1416 to −1399) in the Upstream Negative Regulatory Region—Hypersensitivity to S1 nuclease has proven useful in mapping cis-acting DNA transcriptional elements, since single-stranded, non-B form DNA structures often serve as recognition sites for binding of trans-acting factors. When a supercoiled plasmid containing the upstream negative regulatory region of the A-chain promoter (−1836 to −814) was treated with S1 nuclease and then digested with Bam HI, a single, predominant S1 nuclease-hypersensitive (SHS) site was revealed within the fragment B region, at approximately −1400 (data not shown). To localize this SHS site, high resolution mapping was performed as described under “Materials and Methods” with the plasmid pGL261−B, which contains the fragment B region (see Fig. 2B). S1 nuclease hypersensitivity was observed on both DNA strands over an 18-bp region (−1416 to −1399), consistent with the location identified by crude mapping. The antisense strand was particularly accessible to S1 nuclease, with two adjacent nucleotides T and C showing a lesser degree (Fig. 3, lane 1). Cleaveage induced at the site was directly correlated with nearby nucleotides A and C displaying the most sensitivity and two adjacent nucleotides T and C showing a lesser degree (Fig. 3, lane 1). Restriction enzyme sites used in subcloning are relative to the transcriptional start site (−1418 to −1388) denotes the 5′ SHS nuclease-hypersensitive (5′ SHS) element.

To assess 5′ SHS silencer activity across a panel of cell lines that express varying amounts of A-chain, HeLa (low expression), HepG2 (intermediate), and U87 (high) cells were transiently transfected with plasmid constructs 5′SHS/pAC261 and 5′SHS/pAC261. As previously observed in BSC-1 cells, the 5′ SHS element reduced cat activity to 40–50% of control in all three cell lines and both orientations (Fig. 4C). The ability to repress transcription in multiple, unrelated cell lines indicates a ubiquitous role of 5′ SHS as a silencer.

5′SHS and int-SHS Bind the Same Single-stranded and Double-stranded DNA-binding Proteins from BSC-1 Cells—To identify nuclear factors that bind to 5′ SHS, EMSAs were performed with BSC-1 cell nuclear extract and oligodeoxynucleotide probes derived from the 5′ SHS. To address whether 5′ SHS and int-SHS bind to the same proteins, an oligodeoxynucleotide containing the int-SHS sequence was also included. Double-stranded 5′ SHS probe (ds-5′SHS) formed two distinct DNA-protein complexes in the absence of unlabeled competitor (Fig. 5A, lane 1). The intensities of these two bands were both reduced by more than 80% in the presence of unlabeled ds-5′SHS competitor at a 10-fold molar excess (lane 2), with complete displacement at a 100-fold molar excess (lane 3). Unlabeled, double-stranded int-SHS oligodeoxynucleotide (ds-int-SHS) also competed at a 100-fold molar excess with ds-5′SHS probe for binding to these two complexes (lane 10). The double-stranded form of the irrelevant oligodeoxynucleotide competed inefficiently at a 100-fold molar excess for binding to complex 1 (lane 11), at a level less than that seen for binding to complex 2 (lane 12).
PDGF A-chain Silencer Element

Fig. 2. Localization of negative regulatory activity in the 5’ A-chain promoter by transient transfection of BSC-1 cells. A, relative positions of fragments A, B, C, and D in the A-chain promoter, with the transcriptional start site (+1) noted with an arrow. Shown are the results of transient transfection with plasmids containing fragments A–D, inserted 5′ of the A-chain promoter (pGL261, B) or the heterologous HSV-tk promoter (pTK, C). The activities of the four fragments in both sense (black) and antisense (shaded) orientations are corrected for co-transfected β-gal activity and expressed as a relative percent of the activities of pGL261 or pTK alone, which are set respectively to 100%. The results represent the mean of four independent transfections (± S.E.).

with a 10-fold excess of homologous ds-5’ SHS competitor. The irrelevant oligodeoxynucleotide competed somewhat more efficiently for binding to complex 2, suggesting this binding interaction was less sequence-specific.

The S1 nuclease hypersensitivity of 5’SHS suggested that proteins with specificity for single-stranded structures might bind to this region. Thus, EMSAs were conducted with single-stranded oligodeoxynucleotides probes derived from both strands of the 5’SHS. Antisense 5’SHS probe (as-5’SHS) formed two distinct DNA-protein complexes (Fig. 5B, lane 1) with significantly greater mobility than those formed with ds-5’SHS probe. Efficient dose-dependent competition of binding was observed with unlabeled homologous competitor (lanes 2 and 3), indicating a high affinity interaction between these two protein complexes and as-5’SHS. Unlabeled ds-5’SHS competed to a lesser extent (lanes 4 and 5), indicating the affinity of these complexes was higher for the antisense strand form. The as-int-SHS sequence competed effectively with as-5’SHS probe for protein binding (lanes 6 and 7), strongly suggesting that the antisense strands of both 5’SHS and int-SHS are recognized as high affinity binding sites for the same nuclear proteins. Double-stranded int-SHS competed less efficiently for binding of these proteins to the as-5’SHS probe at a 100-fold molar excess (lanes 8 and 9). Neither the sense strand of int-SHS (lane 10) nor nonspecific competitor (lanes 11 and 12) significantly displaced protein binding to the antisense 5’SHS probe, indicating the sequence specificity of these complexes. EMSAs conducted with 5’SHS sense probe (s-5’SHS) revealed the formation of numerous DNA-protein complexes, only one of which was specifically displaced by a 100-fold molar excess of unlabeled a-5’SHS competitor (Fig. 5C, lanes 1–3).

The ability of unlabeled int-SHS oligodeoxynucleotides to compete with radiolabeled 5’SHS probes for protein binding suggested that the 5’SHS and int-SHS sequences bind the same set of proteins. This conclusion was reinforced by EMSAs conducted with radiolabeled as-int-SHS probes, in which a doublet of bands identical in mobility to that formed with as-5’SHS probes was observed (data not shown). Taken together, the EMSA analyses suggest that the antisense strands of 5’SHS and int-SHS are recognized as high affinity binding sites for the same set of nuclear proteins, whereas sense and double-stranded forms of 5’SHS are recognized by their own cognate DNA-binding proteins.

Mutations within the 5’SHS Reveal Different Nucleotide Sequence Requirements for Binding of Double-strand, Antisense Strand, and Sense Strand DNA-binding Proteins—To identify individual nucleotides within the 5’SHS sequence required for protein binding, the ability of a panel of mutant 5’SHS oligodeoxynucleotides to compete for binding to radioactive 5’SHS probes was evaluated (Fig. 6). Disruption of the GGGGAGGGG core (m2) resulted in a complete loss in ability to compete for binding to both ds-5’SHS and as-5’SHS probes (Fig. 7, A and B), whereas mutation of the 5′- and 3′-portions of the 5’SHS (m1 and m3, respectively) had little effect. This indicates that the GGGGAGGGG core comprises the recognition site for the 5’SHS binding proteins, whereas the nucleotides flanking the core are not critical for binding.

Smaller mutations revealed subtle differences in nucleotide sequence requirements for binding to double-stranded, sense, and antisense forms of the 5’SHS (Fig. 7, C–E). While a 2-bp mutation in the region 5′ to the core sequence (m4) did not affect binding to any 5’SHS binding proteins, a 2-bp mutation (m5) just 3′ to m4 significantly diminished binding to anti-
sense-strand binding proteins (D) and abolished binding to the sense-strand binding protein (E). The ability of the m5 mutation to affect binding is somewhat unexpected, since these two nucleotides were altered in the m1 mutant without effect (A and B). The apparent disparity could be related to the different nucleotide substitutions employed in these two mutants. Mutation of the 5' portion of the core sequence (m6: GGGGA to CTCTT) inhibited binding to all three forms of 5'SHS probe, whereas mutation of the 3'-portion of the core sequence (m7: GGGGG to CTCTC) or the centrally located A residue of the core (m8) decreased binding to ds-5'SHS and as-5'SHS but not s-5'SHS. A 5-bp mutation in the region flanking the 3'-end of the core sequence (m9) did not affect binding of any 5'SHS binding proteins. Although oligodeoxynucleotides m5–m8 all exhibited partial reductions in affinity for the antisense binding protein complexes, none exhibited a complete loss, suggesting that the antisense strand DNA recognition site is multivalent over the GTGGGGAGGGGG core sequence.

The different binding specificities of these proteins can be summarized as follows. Binding of complexes 1 and 2 to ds-5'SHS (Fig. 5A) appears to require the entire core sequence as well as the central A residue. Similarly, complexes 1 and 2, which bind to the antisense strand of the 5'SHS (Fig. 5B), require both halves and central A of the core sequence but also appear to require the presence of a GT pair (nucleotides –1411 and –1412) just 5' to the core. Finally, binding of the single complex to the sense strand (Fig. 5C) requires the 5'-portion of the core and the adjacent GT pair but neither the central A residue nor the 3'-half of the core.

Both the Core GGGGAGGGGG Sequence and Nucleotides Flanking the Core Are Required for 5'SHS Silencer Function—To explore DNA sequence requirements for silencer function of 5'SHS, mutant oligodeoxynucleotides m1 through m9 were inserted into pAC261 in both orientations and analyzed by transient transfection of BSC-1 cells. Mutant oligodeoxynucleotides m1, m2, and m3, in which nucleotide sequence

![Fig. 4.](http://www.jbc.org/)

**Fig. 4.** The 5'SHS sequence functions as a silencer element in multiple cell lines. A, DNA sequence homology between SHS sites in the first intron (+1605 to +1630, int-SHS) and 5' promoter region (–1418 to –1388, 5'SHS) of the PDGF A-chain gene. Lowercase letters indicate overhanging ends of restriction enzyme sites. B, transient transfection of BSC-1 cells with plasmids containing 5'SHS inserted in both orientations in either the A-chain promoter (pAC261, black) or tk promoter (pTK, shaded). C, transient transfection with plasmids containing 5'SHS inserted in both orientations upstream of the A-chain promoter (pAC261) in HepG2 (unshaded), U87 (shaded), and HeLa (black) cells. Relative cat activity is expressed as a percent of the activity of the parent vectors pAC261 or pTK. The results are the average of three independent experiments, with error bars representing the S.E.

![Fig. 5.](http://www.jbc.org/)

**Fig. 5.** EMSAs with single-stranded and double-stranded probes derived from 5'SHS reveal sequence- and strand-specific binding by nuclear factors. Oligodeoxynucleotides were 5'-end-labeled by [γ-32P]ATP and T4 polynucleotide kinase. Nuclear extract from BSC-1 cells in the amount of 5 μg/lanefor single-stranded probes or 10 μg/lanefor double-stranded probe was incubated with radiolabeled double-stranded 5'SHS (A), antisense 5'SHS (B), or sense 5'SHS (C), in the absence or presence of unlabeled competitors. Non-sp, nonspecific competitor; ds, double-stranded; as, antisense strand; s, sense strand. Bands corresponding to DNA-protein complexes and the free probe are indicated by arrows.
of the 5′SHS was disrupted in three large discrete blocks (Fig. 6), all displayed significant decreases in silencer activity in either orientation (Fig. 8A). m1 and m3, which contained mutations of the sequences flanking the 5′ and 3′ sides of the GGGAGGGGG core, respectively, exhibited almost total loss of silencer activity, whereas mutation of the core sequence (m2) resulted in nearly a 50% decrease (Fig. 8A). A nearly total loss in silencer function was observed when two adjacent nucleotides 5′ to the core (m4) were mutated (Fig. 8A). Taken together, these observations indicate that nucleotides required for 5′SHS function are dispersed over the entire DNA element. A second panel of oligodeoxynucleotides containing smaller mutations (m5–m9) failed to display any alterations in silencer activity, with the exception of a partial loss observed when m5 was inserted in the opposite orientation relative to the promoter (Fig. 8B). The loss in 5′SHS silencer activity seen when the core sequence was mutated in toto (m2) correlated with loss in binding of nuclear proteins to the antisense strand, suggesting that binding of these factors is required for full function of the element. In contrast, mutations that ablated binding of double-stranded (m6 and m7) and sense strand (m5) binding proteins had no appreciable effect on silencer activity, suggesting these proteins were not required. Two mutations that completely disrupted silencer function, m1 and m3, had no effect on binding of double-stranded or antisense forms (Fig. 7).

Southwestern Blot Analysis of Nuclear Proteins Binding to 5′SHS—To further characterize the proteins that bind the 5′SHS element, Southwestern blot analysis was conducted with the single-stranded and double-stranded probes employed in EMSA analyses. Four cell lines exhibiting a range of A-chain expression, HepG2 (intermediate A-chain expression), U87 (high), HeLa (low), and BSC-1 (high), were examined. When equal concentrations of nuclear extract from these cells were subjected to denaturing SDS-polyacrylamide gel electrophoresis, blotted to nitrocellulose membranes, and probed with radiolabeled as-5′SHS, four protein species of molecular mass 97,000, 87,000, 44,000, and 17,000 (p97, p87, p44, and p17) were identified (Fig. 9A). These bands represented specific DNA-protein complexes, as demonstrated by displacement with a 100-fold molar excess of unlabeled homologous competitor DNA (data not shown). Whereas nearly equivalent concentrations of p97 were observed across these cell lines, BSC-1 cells contained significantly less p44 (Fig. 9A). p87 was ob-

**FIG. 6.** Panel of wild type (wt) and mutant (m1–m9) 5′SHS oligodeoxynucleotides employed in EMSA and transient transfection analysis. Mutated nucleotides are highlighted.

**FIG. 7.** Mutations within the 5′SHS localize nucleotides critical for interaction of nuclear proteins with double-stranded, antisense, or sense strand 5′SHS. EMSAs were conducted with BSC-1 cell nuclear extract and radiolabeled, double-stranded (A and C), antisense (B and D), or sense strand 5′SHS (E), in the absence or presence of a 100-fold molar excess of unlabeled competitors. Radioactivity incorporated into bound and free bands was quantified with an M2 densitometer. Results were expressed as relative binding percent ([bound/(free + bound)] × 100%), with binding percent in the absence of unlabeled competitor DNA assigned a value of 100%. The data are representative of several experiments with similar results.
served in HeLa nuclear extracts and to a lesser extent in BSC-1, but was not observed in HepG2, U87 (Fig. 9A), or A172 cells (data not shown). Hybridization of blots with as-int-SHS oligodeoxynucleotide probes revealed three proteins (p97, p87, and p17), identical in molecular weight to those seen with the as-5′SHS probe (data not shown), suggesting that these elements are recognized by the same set of three antisense strand-specific binding proteins. An additional as-int-SHS binding protein species (p37) was observed that was not found in 5′SHS-probed blots.

When ds-5′SHS was employed as probe, binding to a 70-kDa protein was seen at approximately equal concentrations in all cell lines examined (Fig. 9B). Two additional species were observed in HeLa cells (p48 and p44), with a small amount of p48 also seen in HepG2 cells. p97, p87, and p17 were not detected, however, indicating these proteins bind preferentially to the antisense strand of 5′SHS. Conversely, absence of p70 from the as-5′SHS-probed blot demonstrates specificity of this protein for the double-stranded form of 5′SHS (Fig. 9A). No specific DNA-protein complexes were detected with s-5′SHS probe (data not shown), suggesting that the sense strand binding protein detected by EMSA is sensitive to denaturing SDS-polyacrylamide gel electrophoresis.

**DISCUSSION**

In this report, deletion analysis and S1 nuclease hypersensitivity assay have been employed to localize a silencer element, designated 5′SHS, to nucleotides −1418 to −1388 in the upstream promoter region of the PDGF A-chain gene. The 5′SHS bears homology to a recently described silencer in the first intron of the A-chain gene (int-SHS, 15). Our study extends previous observations by demonstrating the contribution of single strand-specific DNA-binding proteins in the function of the A-chain silencers and by identifying the molecular weight species involved in binding to both elements. We have also employed site-directed mutagenesis to define DNA sequences required for binding of nuclear factors and silencer function. Multiple negative regulatory elements have been identified in the promoters of genes encoding human PDGF B-chain (25–27), chicken (28), hamster (29), and human (30) vimentin, human collagen II (31), interleukin-4 (32), and e-globin (33), and rat glutathione P-transferase (34). The 5′SHS and int-SHS appear to be unique in their relatively large distance from the transcription start site (>1 kilobase) and locations both 5′ (5′SHS) and 3′ (int-SHS) to the start site. The appear-
ance of the GGGAGGGGG motif in these two topologically distant elements and the sharing of cognate binding proteins suggest possible interactions in repression of A-chain gene transcription.

Probing of several promoter regions by single strand-specific nucleases such as S1 has revealed the existence of non-B-form DNA, which often correlates with important transcriptional functions. However, the molecular mechanisms through which single-stranded DNA structures mediate the regulation of gene transcription remain obscure. Many transcription factors exhibit high affinity binding to single-stranded DNA, including myoD (35) and estrogen receptor (36, 37). In addition, specific DNA sequences found in a number of promoters, such as β-casein (38), c-Myc (39), adipin (40), and the serum response element of the PDGF A-chain (41) have been shown to exhibit considerable S1 nuclease sensitivity, suggesting the presence of single-stranded structure. More specifically, repressor/silencer function has been attributed to S1 nuclease-sensitive sequences and/or cognate binding proteins that exhibit preferential binding to single-stranded DNA, such as the intron 1 silencer (int-SHS) of the PDGF A-chain (15, 42), and silencers of genes encoding lipoprotein lipase (43), mouse androgen receptor (44), vascular smooth muscle α-actin (45), and mouse myelin basic protein (46). A number of molecular structures have been proposed for unwound DNA, including simple single strands, junctions between B- and Z-DNA, and triplex forms such as H-DNA (reviewed in Ref. 47). In vivo formation and maintenance of these structures are presumed to require assistance in the form of superhelical DNA winding and/or the binding of effector molecules (i.e. protein, cRNA), since DNA melting is unlikely under normal conditions of intracellular pH, temperature, and ionic strength. Thus, it is noteworthy that the antisense strand of 5′SHS is S1 nuclease-hypersensitive when presented to the enzyme in supercoiled form but is not recognized by single-stranded DNA binding proteins in a double-stranded oligodeoxynucleotide. This suggests that the element may indeed require chromosomal superhelicity and/or binding of accessory factors, such as the 5′SHS antisense strand binding proteins for generation of single-stranded structure in vivo.

Mutation of the GGGAGGGGG core sequence resulted in loss of both silencer activity and binding to antisense strand-specific binding proteins, suggesting an important role of these proteins in silencer function. Also implicating these proteins is the observation that the same antisense-specific 5′SHS binding proteins bound with high affinity to the int-SHS silencer element. Southwestern blot analysis has provided the first insight into the identity of proteins that bind to the 5′SHS and int-SHS silencers. The 5′SHS antisense binding protein p97 (Fig. 9A) is close in size to a 95-kDa species shown to bind a homologous silencer element (5′-CCCCCTCC-3′) in the vimentin promoter (28, 30), raising the interesting possibility that p97 may represent a transcription factor or family of related factors involved in silencing expression of growth-related genes. Homologues of the 5′SHS core sequence (5′-CCCTCCCTCC-3′) in the rat prolactin and human urokinase-type plasminogen activator promoters have been proposed as binding sites for AP-2, although no AP-2 binding has been demonstrated (48–50). Members of the heterogenous nuclear ribonucleoprotein (hnRNP) family of proteins also exhibit affinity for DNA sequences similar to 5′SHS and int-SHS. hnRNP K is relevant since it binds DNA with higher affinity than RNA, exhibits preferred binding to C-rich, single-stranded DNA sequences such as the four direct repeats of the sequence CCCTCCCTCA (CT element) of the c-myc gene (51), and has been recently implicated in c-myc transcriptional activation (51, 52). hnRNP K is close in size (p68) to that of the 5′SHS binding protein p70 (Fig. 9, but its preference for single-stranded DNA is inconsistent with the double-stranded DNA specificity of p70. No hnRNPs have been identified in the molecular weight range of p97, p87, or p17. NSEP-1 also interacts with the C-rich strand of the c-myc CT element and is close in size (322 amino acids; Ref. 53) to the int-SHS binding protein p37. Detailed analyses will be required to conclusively identify the complex of transcription factors that interact with the 5′SHS and int-SHS silencers and to better understand the molecular mechanism mediating silencer function.

Mutation of regions flanking the core sequence (m1, m3, m4) inhibited silencer activity but did not affect binding to proteins specific for the 5′SHS, suggesting a role for these nucleotides in conferring a silencer-competent DNA structure and/or binding to proteins not detected by our EMSA analyses. Two inverted repeats (5′-GACGT-3′ and 5′-TGCGA-3′) are found in the core-flanking sequence and contribute to a mirror symmetry over the whole region (Fig. 3), suggesting a potential for stabilizing a DNA triplex structure such as H-DNA. The prominent S1 hypersensitivity on the 5′SHS antisense strand in the region of the downstream inverted repeat is consistent with the H-DNA structure shown in Fig. 10, with a sharp turn of the DNA strand and relatively short length of single-stranded DNA on the antisense strand. Presumed contact points between DNA and binding proteins that were mapped by mutagenesis and EMSA studies are also represented in the model. Since many transcription factors bind to bent DNA, or may generate DNA bending upon binding (54–58), it is tempting to speculate that formation of H-DNA or other single-stranded DNA structures in the 5′SHS region may serve to bring silencer element binding proteins into correct orientation with downstream positive regulatory elements.

The 5′SHS was equally efficient in repressing A-chain promoter activity in either orientation when placed 5′- to the promoter, conforming to a classical definition of a silencer element. The ability to function in upstream and downstream locations relative to the A-chain promoter in pMC261 further suggests position independence, although 5′SHS failed to repress transcription in the opposite orientation at a downstream location. The lack of strict adherence to the criteria of orienta-
tion-, distance-, and promoter-independent function is prece-
dented in other DNA response elements (15, 59–63).

The 5′SHS repressed transcription from promoter/enhancer
fragments of the A-chain and TK genes, both of which contain
GC-rich enhancers and TATAA boxes (64). This suggests an
inhibitory interaction between 5′SHS binding proteins and en-
hancer transcription factors bound to the promoter-proximal
GC-rich region (--82 to --42) and/or the general transcription
factors of the RNA polymerase initiation complex. We
previously showed that Sp1-like proteins bind with high affinity to
the GC-rich region (12), suggesting this family of transcription
factors may be the target of inhibitory action by 5′SHS binding
proteins.

The 5′SHS silencer was active both in cell lines that express
considerable amounts of A-chain (BSC-1, HepG2, U87) and in
HeLa cells that express very little (15). This is consistent with
our previous observation that the upstream negative regula-
tory region of the A-chain gene (--1853 to --883), which con-
tains the 5′SHS, appears to function in cells regardless of their
level of basal transcription (12). The ubiquitous silencer activ-
ity of the 5′SHS and its cognate binding proteins suggests a
fundamental role in repressing A-chain expression, thus pre-
venting inappropriate expression of this potent mitogen. In
cells where A-chain expression is elevated, transcriptional
silencing continues to oppose enhancement mediated by the
proximal GC-rich region. Wang et al. (15) reported that the
int-SHS functioning as a negative regulatory element in HeLa
but not in human glioblastoma (A172) cells and ascribed a
function to the sequence in the maintenance of cell-specific
expression of the A-chain. Although the study compared si-
lence activity in only two transformed cell lines, it raises the
possibility that tissue- and developmental-specific patterns of
A-chain expression may be partially determined by transcrip-
tional silencing. The Southwestern blot analysis has provided
preliminary identification of proteins that may participate in
these aspects of A-chain expression. Although p97 was
expressed at similar levels in all cell lines examined, concentra-
tions of p87, p44, and p17 and the as-int-SHS-binding protein p37
varied considerably, suggesting cell-specific possible roles in
transcriptional repression. Taken together, our observations of
structural and functional similarity between the 5′SHS and
int-SHS suggest that the 5′SHS and int-SHS and their cognate
trans-acting factors may cooperate to affect cell-specific expres-
sion of the A-chain gene.

PDGF is developed as a mediator of malignant growth in
astrocytoma and sarcoma (65, 66), with elevated expression
of PDGF A- and B-chains often seen in transformed cells. Loss of
silencer function and ensuing A-chain overexpression could be
envisioned, therefore, to constitute an important step in tumor
progression. Sharing of protein components (i.e. p97, p87, and
p17) between two separate silencer elements (5′SHS and int-
SHS) would appear to make the A-chain gene vulnerable to
such a loss of transcriptional control. The recent observation
that the 95-kDa silencer element binding protein of the vimen-
tin gene is absent from the metastatic breast cancer cell, MD-
MB-231, indeed suggests that such a mechanism may mediate
progression to the metastatic phenotype (67). Also relevant is
a recent observation that overexpression of PDGF B-chain con-
fers a metastatic phenotype to human T98G glioblastoma cells
(68). Characterization of nuclear factors binding to the A-chain
silencers and their roles in expression of A-chain in malignancy
and tumor metastasis will be of interest in future studies.

Acknowledgments—We thank James Reid and Dewey Morgan, III
for their technical contributions.
54. Gustafson, T., Taylor, A., and Kedes, L. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 2162–2166
55. Horikoshi, M., Bertuccioli, C., Takada, R., Wang, J., Yamamoto, T., and Roeder, R. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 1060–1064
56. Ryder, K., Silver, S., DeLucia, A., Fanning, E., and Tegtmeyer, P. (1986) Cell 44, 719–725
57. Spana, C., and Corces, V. (1990) Genes Dev. 4, 1505–1515
58. Verrijzer, C., van Oosterhout, J., van Weperen, W., and van der Vliet, P. (1991) EMBO J. 10, 3007–3014
59. Hata, A., Ohno, S., Akita, Y., and Suzuki, K. (1989) J. Biol. Chem. 264, 6404–6411
60. Kiss, I., Bosze, Z., Szabo, P., Altanchimeg, R., Barta, E., and Deak, F. (1990) Mol. Cell. Biol. 10, 2432–2436
61. Mizuno, K., Goto, M., Masamune, Y., and Nakanishi, Y. (1992) Gene (Amst.) 119, 293–297
62. Pierce, J. W., Gifford, A. M., and Baltimore, D. (1991) Mol. Cell. Biol. 11, 1431–1437
63. Smith, K. P., Liu, B., Scott, C., and Sharp, Z. D. (1995) J. Biol. Chem. 270, 4484–4491
64. Jones, K. A., Yamamoto, K., and Tjian, R. (1985) Cell 42, 559–572
65. Hermansson, M., Funa, K., Hartman, M., Claesson-Welsh, L., Heldin, C.-H., Westmark, B., and Nister, M. (1992) Cancer Res. 52, 3213–3219
66. Leveen, P., Claesson-Welsh, L., Heldin, C.-H., Westmark, B., and Betsholtz, C. (1990) Int. J. Sci. 46, 1066–1076
67. Stover, D. M., Carey, I., Garzon, R. J., and Zehner, Z. (1994) Cancer Res. 54, 3092–3095
68. Potapova, O., Fakhrai, H., Baird, S., and Mercola, D. (1996) Cancer Res. 56, 280–286
