In vivo footprinting and functional analysis of the human c-sis/PDGF B gene promoter provides evidence for two binding sites for transcriptional activators

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ABSTRACT

By in vivo DMS footprint and reporter gene analyses we identified two transcription factor binding sites in the human c-sis/PDGF B gene promoter. The low basal activity of the PDGF B promoter in HeLa and undifferentiated K562 cells, which express low PDGF B mRNA levels, and in PC3 cells, which express a high PDGF B mRNA level, results from binding of a weak transcriptional activator between positions −64 and −61 relative to the transcription start site. Cytotrophoblast-like JEG-3 cells, which do not express the 3.5 kb PDGF B mRNA, contain a transcriptional activator directed at the −64/−61 sequence, but DNA methylation may render the endogenous promoter inaccessible to this activator. A CCACCCAC element at position −61/−54 was identified as the in vivo binding site for a strong transcriptional activator in phorbol ester-treated megakaryocytic K562 cells, which express a high PDGF B mRNA level. Primary human fibroblasts, which do not transcribe the PDGF B gene, contain a transcriptional activator that recognizes an element between positions −60 and −45 but does not bind to the endogenous unmethylated promoter. Our results show that the complex expression pattern of the human PDGF B gene involves the cell type-specific expression of weak and strong transcriptional activators and regulation of promoter accessibility to these factors.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a collective term for the three dimeric proteins that can be constituted out of type A and/or type B polypeptide chains. The human PDGF A and B chain precursors are encoded by two homologous genes located on chromosomes 7 (1) and 22 (2,3), respectively. The B chain gene is identical to the c-sis proto-oncogene (4–6). PDGF is a potent growth factor and chemo-attractant for cultured cells of mesenchymal origin, such as fibroblasts (7,8) and smooth muscle cells (9,10). Expression of PDGF is tightly regulated in a cell type- and developmental stage-specific manner and is thought to play a role in wound healing and early development (reviewed in 11). PDGF is synthesized in bone marrow megakaryocytes (12,13) and stored within α-granules of platelets, from which it is released during the clotting process (14). Other examples of cell types that express PDGF are vascular endothelial (15) and smooth muscle cells (16), placental cytrophoblasts (17), macrophages (18) and activated monocytes (19). Depending on the cell type, expression of PDGF can be affected by very diverse extracellular stimuli, such as transforming growth factor β (20), hypoxia (21) and shear stress (22).

Via autocrine and/or paracrine pathways aberrant expression of PDGF may play a role in the etiology of atherosclerosis, fibrosis and certain types of cancer (reviewed in 11). Whereas the PDGF A chain has only weak transforming activity, over-expression of normal PDGF B chains induces transformation of NIH 3T3 cells with high efficiency (23–26). The long and GC-rich leader of the PDGF B transcript can act as a potent translational inhibitor (27,28). However, if transcription of the PDGF B gene is driven by a sufficiently strong promoter, such as the SV40 early promoter/enhancer, the leader sequence does not prevent development of fibrosarcomas in mice (29). Upon transfection of PDGF B cDNA into NIH 3T3 cells the acquisition of features of transformation was shown to correlate directly with the expression level of PDGF B mRNA (30). Modulation of the transcription rate is an obvious mechanism by which the expression level of the PDGF B chain under normal and pathological conditions could be regulated.

Since localization of the transcription start site of the human PDGF B gene (31), only little progress has been made in understanding regulation of transcription of the gene. An attractive model system in which to study regulation of the PDGF B promoter is the human erythroblastic leukemia cell line K562. Upon treatment with the phorbol ester 12-O-tetradecanoylphorhbol 13-acetate (TPA) K562 cells differentiate to megakaryocytes (32), which is accompanied by a 200-fold increase in PDGF B mRNA content (33). The induction of PDGF B mRNA is at the transcriptional level (32) and is dependent on de novo protein synthesis (33). In previous studies, DNA elements that may regulate activity of the PDGF B gene promoter during TPA-mediated differentiation of K562 cells were mapped by reporter gene analyses and gel retardation assays (33,34). The first 72 bp upstream of the transcription start site were found to be sufficient.

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for minimal promoter activity in untreated K562 cells (33). Regions between positions −101 and −58 bp (33) and −64 and −39 bp (34) were reported to be essential for TPA inducibility of the PDGF B promoter. In gel retardation assays, both regions were shown to interact specifically with nuclear proteins from K562 cells and some complexes correlated with TPA-mediated megakaryocytic differentiation (33,34). A recent reporter gene analysis revealed that an AP-1-like sequence between −92 and −85 and an ETS-like sequence between −80 and −70 relative to the major transcription start site are required for maximal activity of the PDGF B promoter in cultured vascular endothelial cells. An Sp1-like sequence between −61 and −56 appeared to be required for basal PDGF B promoter activity in endothelial cells and was shown to interact with purified Sp1 in a DNase I footprint experiment (35). Reporter gene analysis of the PDGF B promoter in human osteosarcoma cell line U2-OS indicated that regions between −244 and −203, −102 and −95 and −63 and −44 relative to the transcription start site contain positive regulatory elements. By gel retardation analysis the −63−44 sequence was shown to interact in vitro with an Sp1-like factor present in nuclear extracts derived from U2-OS cells (36). In the studies that have been described thus far, interactions between PDGF B promoter sequences and nuclear proteins have always been examined by reporter gene analysis and in vitro DNA binding experiments. However, in living cells the PDGF B promoter is part of a specific chromatin structure, which is not maintained in the in vitro experiments. Therefore, DNA–protein interactions that were mapped in vitro may not always reflect the interactions that actually occur at the endogenous gene. We decided to try to map the actual protein binding sites at the PDGF B promoter in a diverse panel of human cell types by in vitro dimethyl sulfate (DMS) footprinting, which has become more readily applicable by the development of the ligation-mediated polymerase chain reaction (LMPCR) (37). To complement the in vivo footprint results with functional data, we also mapped transcription regulatory elements by reporter gene analysis of the PDGF B promoter. The combined results enable us to accurately map two transcription regulatory elements within the PDGF B gene promoter that are recognized by activating transcription factors in vivo in a cell type-specific manner.

Previous studies provided evidence for negative regulation of the PDGF B promoter: in cultured dermal fibroblasts and cytotrophoblast-like JEG-3 cells the PDGF B gene promoter is active in a reporter gene assay, whereas these cells do not detectably express the corresponding PDGF B transcript (33, 38, 39). Recently, we showed that the PDGF B promoter is not hypersensitive to DNase I in these cells and that nucleosome phasing may play a negative transcription regulatory role in fibroblasts. In addition, we mapped silencer elements within intron 1 and downstream of the gene that may suppress activity of the promoter in JEG-3 cells (39). To examine a potential role for DNA methylation in regulating promoter activity we analyzed the chromosomal methylation status at the PDGF B promoter by genomic sequencing (40,41) and restriction enzyme analysis.

**MATERIALS AND METHODS**

**Cell culture**

Cell lines K562, JEG-3, HeLa and PC3 were all from the American Type Culture Collection (Rockville, MD). Primary human dermal fibroblasts (HuFi) were derived from fresh foreskin biopsies, which were a kind gift of the Department of Surgery (University Hospital, Nijmegen). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 125 U/ml streptomycin and 125 U/ml penicillin. Megakaryocytic differentiation of K562 cells was induced by addition of TPA (Sigma) to the culture medium (final concentration 2 nM).

**Reporter gene constructs**

All recombinant DNA constructs were made according to standard protocols (42). psis-112/+43CAT and psis-1758/+43CAT have been described earlier (39). psis-425/+43CAT was made by Aval digestion and subsequent recircularization of psis-1758/+43CAT. Unidirectional deletion mutants of Aval/SstI-linearized psis-425/+43CAT (Fig. 1A) were made with exonuclease III according to the technical manual of the Erase-a-Base System (Promega): mutants that were to be tested in a reporter gene assay were characterized by dideoxy sequence analysis (43). psis-112/+18CAT was made by religation of the 130 bp PstI fragment of psis-112/+43CAT into PstI-linearized psis-112/+43CAT (this results in the deletion of an internal 28 bp PstI fragment). psis-112/+18mutaCAT was made as follows. The 130 bp PstI fragment of psis-112/+18CAT was cloned into the PstI site of pGem3-Zf(-) (Promega) and saDNA was isolated. A 24 bp oligonucleotide primer [5'−dGAG GTG GAT TCC−3'] was used for site-directed-mutagenesis according to the Amersham protocol of the Oligonucleotide-Directed In Vitro Mutagenesis System Version 2 (Amersham). Dideoxy sequence analysis revealed that mutagenesis had resulted in a C→A change at position −63 and the insertion of an A residue between positions −64 and −65 (data not shown). The mutated 130 bp PstI fragment was recloned into the vector part of PstI-digested psis-112/+43CAT. pSuperCAT is a promoterless chloramphenicol acetyltransferase (CAT) gene vector. pCH110 (Pharmacia LKB) contains the β-galactosidase gene driven by the SV40 promoter.

**Electroporations and CAT assays**

Superoiled plasmid DNA was purified by CsCl gradient centrifugation (42) and quantitated by means of both ethidium bromide staining and spectrophotometric measurement. Cells (3–5 × 10⁷) were electroporated with 10 μg pCH110 and 10 pmol CAT construct as previously described (39). All cells except K562 were harvested 24–48 h after electroporation. Immediately after electroporation, K562 cells were divided equally over two dishes. TPA was added to one dish and 24 h later both undifferentiated and TPA-treated cells were harvested. Cell lysates were prepared (42) and the protein content of each lysate was determined in a protein assay (BioRad). Fixed amounts of protein were tested for β-galactosidase activity (44) and, subsequently, amounts of protein corresponding to equal β-galactosidase activity were assayed for CAT activity (45). First, pilot assays were carried out to determine what time of incubation and amount of protein derived from reference sample psis-112/+43CAT would yield 20% conversion of [14C]chloramphenicol after subtraction of the background CAT activity of pSuperCAT. These parameters were then used to determine the relative CAT activities of all other constructs. Radioactive spots were quantitated by measuring in a liquid scintillation counter, from which the percentage acetylated chloramphenicol could be determined. CAT values were corrected for background activity by subtracting the mean values of pSuperCAT.
In vivo footprinting

Monolayers of PC3, HeLa and Hufi cells were cultured to subconfluence on 15 cm dishes. The tissue culture medium was replaced by medium containing 0.1% dimethyl sulfate (DMS). After an incubation of 2 min at 37°C, the DMS-containing medium was quickly aspirated and cells were washed four times with phosphate-buffered saline (PBS) at 37°C. Cells were harvested in lysis solution [300 mM NaCl, 50 mM Tris–HCl, pH 8.0, 25 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 200 μg/ml proteinase K] at 1.5 ml/15 cm dish and incubated overnight at 37°C. To induce megakaryocytic differentiation, K562 cells were cultured in the presence of 2 ng/ml TPA for 5 days before DMS treatment. Suspensions of untreated and TPA-treated K562 cells (3–5 × 10^5 cells/ml) were centrifuged (5 min, 500 g, room temperature) and the cell pellet was resuspended in culture medium at 5 × 10^7 cells/ml. DMS was added to 0.1% and after a 1 min incubation at 37°C, the DMS-containing medium was diluted with 50 vol ice-cold PBS. Cells were quickly centrifuged (5 min, 500 g, 4°C) and the cell pellet was washed three times with ice-cold PBS. Cells were resuspended in lysis solution at 2 × 10^7 cells/ml and incubated overnight at 37°C.

Chromosomal DNA from all DMS-treated cells was further purified by repeated extractions with phenol, chloroform and diethyl ether. Subsequently, the DNA was precipitated with ethanol and dissolved in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA (TE). DMS methylation of control protein-free chromosomal DNA and pipiderine cleavage of in vivo and in vitro methylated DNA at sites of modified guanine residues were performed according to Maxam and Gilbert (46). Pipiderine was removed by lyophilization and the DNA dissolved in 0.3 M sodium acetate, pH 7.0. The DNA was ethanol precipitated repeatedly and finally dissolved in water. Trace amounts of pipiderine were removed by lyophilization and the DNA was redissolved in TE at 0.4 μg/ml.

Genomic footprinting was done by means of LMPCR as described by Mueller and Wold (37) and modified by Garrity and Wold (47). The PDGF B promoter-specific primers 1(s), 2(s) and 3(s), respectively 5'-d(CAT GGA CTG AAG GGT TGC TC)-3', 5'-d(CTC TCA GAG ACC CCC TAA GCG CCC C)-3' and 5'-d(AGA CCC CCT AAG CGG CCC GGT G)-3' were used for analysis of the lower strand. Primers 1(a), 2(a) and 3(a), respectively 5'-d(CGC AAA GTA TCT CTA TCT AGG GAA)-3', 5'-d(TAG GGA ATG AAA ATG GCC GCT GGC)-3' and 5'-d(GGA ATG AAA ATG GCC CGC TGG CGG C)-3' were used for the upper strand. For first strand synthesis, 2 μg DMS/pipiderine-treated DNA was annealed to primer 1(s/a) at 60°C. During the PCR amplification (18 cycles) annealing of primer 2(s/a) was at 72°C. Annealing of the labeling primer 3(s/a) was at 74°C. First strand synthesis, PCR amplifications and labeling reactions were done using Thermococcus litoralis DNA polymerase (Vent: New England Biolabs). Labeling reaction mixtures were electrophoresed on 6% sequencing gels. Gels were dried and exposed to X-ray film with two intensifying screens. The footprinting data were quantified by scanning the autoradiograms with a densitometer (Kipp & Zonen, Delft, The Netherlands). Nucleotide sequence markers were generated by dideoxy sequence analysis (43) of human c-sis subclone pAO121 (48).

DNA methylation analysis

Chromosomal DNA was isolated according to standard protocols (42). Chromosomal DNA (–20 μg) was digested with EcoRI and BamHI and subsequently with either MspI or HpaII. MspI and HpaII digestions were monitored by adding small aliquots of the digestion mixtures to undigested phage λ DNA. Upon complete digestion of the λ DNA, the chromosomal DNA samples were electrophoresed in a 1% agarose gel and transferred to a Hybond-N+ filter (Amersham, UK). DNA was hybridized overnight in 0.9 M NaCl, 50 mM sodium phosphate, 5 mM EDTA, pH 7.7, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.5% SDS, 20 μg/ml herring sperm DNA at 68°C with 32P-labeled probe PR16 (39,49). Filters were washed at 68°C in 18 mM NaCl, 1 mM sodium phosphate, 0.1 mM EDTA, pH 7.7, 0.1% SDS and then exposed to X-ray film with two intensifying screens.

For genomic sequencing, chromosomal DNA was treated with hydrazine in the presence of 1.5 M NaCl (46). Pipiderine cleavage, DNA purification and LMPCR were done as described above for in vivo DMS footprinting. Nucleotide sequence markers were generated by dideoxy sequence analysis (43) of human c-sis subclone pAO121 (48).

RESULTS

Functional analysis of the human PDGF B promoter reveals cell type-specific transcription regulatory elements

Earlier we showed that in a panel of human cell lines (listed in Fig. 5) the activity of the first 112 bp upstream of the PDGF B transcription start site does not differ significantly from the activity of the first 1758 bp upstream of that site (39). To map the transcription regulatory elements within the 112 bp region in more detail, we prepared a set of unidirectional deletion mutants fused to the CAT reporter gene (depicted in Fig. 1A). The deletion mutants were tested for promoter activity in the same panel of human cell lines that differ in level of PDGF B mRNA expression (see also Fig. 5), namely untreated and TPA-treated (i.e. megakaryocytic) K562 cells, Hufi, cytrophoblast-like JEG-3 cells and carcinoma-derived HeLa and PC3 cells. Culturing K562 cells in the presence of TPA has already been shown to result in 15–30-fold increased activity of the 112 bp fragment after 24 h (Fig. 5; 39). Therefore, data for untreated and TPA-treated K562 cells are presented in separate diagrams. Figure 1B shows that deletion up to position –64 does not significantly lower promoter activity, except for a slight decrease in JEG-3 cells. In TPA-treated K562 cells the 64 bp fragment displays >4-fold increased activity compared with the 112 bp fragment. Further deletion up to position –60 results in >5-fold decreased activity in PC3, HeLa, JEG-3 and untreated K562 cells compared with the activity of the 64 bp fragment, but does not significantly affect activity in TPA-treated K562 cells and in fibroblasts. Deletion up to position –44 leads to 10- and 3-fold decreased promoter activity in TPA-treated K562 cells and fibroblasts, respectively compared with the activity of the 60 bp fragment. A 36 bp promoter fragment, which extends to 6 bp upstream of the TATA box, displays near background activity in all cell lines tested. The results suggest that sequences located between positions –64 and –60 are required for activity of the PDGF B promoter in PC3, HeLa, JEG-3 and untreated K562 cells, but not in fibroblasts and TPA-treated K562 cells. In these last two cell types sequences located between positions –60 and –44 appear to be indispensable for promoter activity.
Figure 1. Reporter gene analysis of unidirectional deletion mutants of the human PDGF B gene promoter. (A) Schematic overview of PDGF B promoter deletion mutants. Unidirectional deletion mutants were generated as described in Materials and Methods. Numbers indicate the positions of PDGF B sequences relative to the transcription start site. The transcription start site is marked by an arrow. (B) Relative CAT activities of lysates from cells electropermeated with the indicated PDGF B gene promoter deletion mutants. Cells were electropermeated with a mixture of pCH110 and a deletion mutant. Aliquots of lysate normalized to mean $\beta$-galactosidase activity were tested for CAT activity. CAT activity was determined as percentage acetylated $[14\text{C}]$chloramphenicol/time. For background correction, mean CAT activity of the promoterless construct pSuperCAT was subtracted from each value. For each cell type, the activity of psi-112/+43CAT was defined as 100%.

To provide unequivocal proof for the cell type-specific activity of the -64/-61 region site-directed mutagenesis was performed, which resulted in a C→A change at position -63 and the insertion of an A residue between positions -64 and -65. The necessary cloning steps inevitably resulted in deletion of a small downstream fragment located between positions +19 and +43 (see Materials and Methods). Therefore, we also tested whether the +19/+43 region might affect reporter gene activity. As can be seen in Table 1, the minor change in the -64/-61 region reduces activity of the PDGF B promoter to background levels in untreated K562 cells and PC3 cells and by >5-fold in JEG-3 cells. However, it does not affect promoter activity in TPA-treated K562 cells, which is in agreement with the deletion mutant analysis. In addition, the results show that the +19/+43 region is dispensable for activity of the PDGF B promoter. The combined results from the reporter gene assays enable us to map two transcription activating elements. The first element harbors (part of) the -64/-61 region and is essential for promoter activity in
PC3, HeLa, untreated K562 and JEG-3 cells, whereas the second element is located between positions \(-60\) and \(-44\) and is required for promoter activity in TPA-treated K562 cells and fibroblasts.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>CAT activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>PC3</td>
</tr>
<tr>
<td>(-112/+43)</td>
<td>169.3 ± 9.5</td>
</tr>
<tr>
<td>(-112/+18)</td>
<td>100 ± 29.4</td>
</tr>
<tr>
<td>(-112/+18)mut</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

See legend to Figure 1B for details, except that CAT activity of psis-112/+18CAT was defined as 100%. All data are presented as means ± SD of two to four independent electroporation events. Only the PDGF B promoter sequences present in the CAT constructs are indicated.

**In vivo DMS footprinting reveals protein binding sites in the human PDGF B promoter that co-map with the transcription activating elements**

Transient reporter gene assays are a powerful means to reveal the presence of transcription factors and to map transcription regulatory DNA elements. However, as the methylation status and chromatin structure at the promoter in the reporter gene construct may differ significantly from the situation at the endogenous promoter, the assays may provide an artifactual view of the promoter in situ. To study the PDGF B promoter directly in the living cell, we performed in vivo DMS footprint analyses in fibroblasts, untreated and TPA-treated K562 cells, HeLa cells and PC3 cells according to the method of Mueller and Wold (37). In HeLa, PC3 and both untreated and TPA-treated K562 cells methylation at G-61 in the lower strand was increased by 2-3-fold compared with the naked DNA lane (Fig. 2A). Enhanced methylation was sometimes observed at G-63 in PC3 and untreated K562 cells, but was never as pronounced as at G-61. In the same strand methylation at G-1 was increased by 300% in PC3 and TPA-treated K562 cells and by nearly 200% in HeLa cells. In addition, methylation of all G residues from position \(-60\) to \(-54\) in the lower strand was decreased by almost 70% in TPA-treated K562 cells. In the upper strand, we detected 2-3-fold increased methylation at G-2 in PC3 and TPA-treated K562 cells (Fig. 2B). The hypermethylation at G-61 in untreated K562, HeLa and PC3 cells co-maps with the \(-64/-61\) element that was identified in the reporter gene assays. Similarly, the footprint at \(-61/-54\) bp in TPA-treated K562 cells corresponds with the \(-60/-45\) element found in the reporter gene assays. No DMS footprint was found in fibroblasts, suggesting that the PDGF B promoter does not interact with transcription factors in vivo in these cells. The enhanced methylation close to the transcription start site in PC3, HeLa and TPA-treated K562 cells could not be directly linked to a functional element and may result from the binding of basal transcription factors.

**DNA methylation may inhibit accessibility of the human PDGF B promoter in JEG-3 cells, but not in fibroblasts, HeLa and K562 cells**

Fibroblasts and JEG-3 cells express transcriptional activators of the PDGF B promoter, despite the absence of corresponding PDGF B mRNA in these cells (33,38,39; this paper). To examine whether DNA methylation could play a negative regulatory role in these cells or in HeLa and untreated K562 cells, which express very low levels of PDGF B mRNA, we analyzed the chromosomal methylation status at the PDGF B promoter. Genomic sequencing analysis reveals that the CpG dinucleotides at positions \(-20\), \(-48\), \(-85\) and \(-94\) are heavily methylated in JEG-3 cells, whereas they are unmethylated in fibroblasts and in untreated and TPA-treated K562 cells (Fig. 3). These results are in full accordance with an isoschizomer experiment focusing on the chromosomal methylation status of a representative MspI site at position \(-85\) of the PDGF B promoter (Fig. 4A). Chromosomal DNA was pre-digested with EcoRI and BamHI and subsequently with either MspI or its methylation-sensitive isoschizomer HpaII. As can be seen in Figure 4B, the MspI site at \(-85\) is unmethylated in fibroblasts,
HeLa and untreated K562 cells, as well as in PC3 and TPA-treated K562 cells, which express high levels of PDGF B mRNA. Only in JEG-3 cells are most of the MspI sites within the PDGF B promoter and the 5'-part of exon 1 heavily methylated. In conclusion, these results do not support a role for CpG methylation in regulating activity of the PDGF B promoter in fibroblasts, HeLa and untreated K562 cells. However, DNA methylation may inhibit activity of the PDGF B promoter in JEG-3 cells.

DISCUSSION

By in vivo DMS footprint analyses and transient reporter gene assays (summarized in Fig. 5) we have demonstrated that the first 64 bp upstream of the human c-sis/PDGF B gene transcription start site contain two specific binding sites for transcriptional activators. A transcriptional activator expressed in HeLa, PC3 and untreated K562 cells recognizes an element containing at least part of the TCTC sequence at positions −64/−61. This is supported by the observation that unidirectional deletion of the −64/−61 region reduces promoter activity in a reporter gene assay to near background levels in all three cell lines. In addition, a site-directed mutation in the −64/−61 element abolishes promoter activity in PC3 and untreated K562 cells. Finally, genomic footprinting reveals enhanced methylation at G−61 in all three cell lines, which indicates that the element is bound by a trans-acting factor in vivo. As the activity of the PDGF B gene promoter is 100−300-fold lower than that of the SV40 promoter/enhancer in untreated K562, HeLa and PC3 cells (Fig. 5; 39), we conclude that the factor that binds to the −64/−61 element is a weak transcriptional activator. Although our previous results showed that the high PDGF B mRNA level in PC3 cells compared with HeLa cells probably results from a higher transcription rate in PC3 cells (39), both cell types use the same weak −64/−61 element for basal promoter activity. Presumably, the promoter activity in PC3 cells is increased by an enhancer element located downstream of the gene, while a silencer located within intron 1 may down-regulate promoter activity in HeLa cells (39,50). Despite contrary observations by others (32,33), we reproducibly detected a low expression level of PDGF B mRNA in untreated K562 cells (39,50). A low percentage of untreated K562 cells may differentiate spontaneously to megakaryocytes (reviewed in 51), which could explain the expression of PDGF B mRNA. However, our reporter gene assays demonstrate that activity of the PDGF B promoter in untreated K562 cells is abolished by mutagenesis of the −64/−61 element, whereas activity in TPA-treated megakaryocytes is not affected. In addition, hypermethylation of G−61 would not be expected if only a low percentage of untreated K562 cells had that site occupied by a transcription factor. Thus, our current data indicate that the PDGF B promoter is constitutively active in untreated K562 cells due to binding of a weak transcriptional activator to the −64/−61 element. The observation that the PDGF B promoter is fully demethylated in untreated K562 cells is consistent with this hypothesis. Whether the −64/−61 element interacts with a known transcription factor is unclear, as a TCTC sequence or its complement was not found as a specific binding site in a recent compilation of transcription factors (52). PDGF B promoter
complexes result from interaction with extracts that are directed by the PDGF B promoter. The transcriptional activity of the PDGF B promoter is dependent on the GATA-1 gene, which encodes a transcriptional activator directed at the PDGF B promoter. The footprint of the PDGF B promoter in vivo is shown in bold. Expression levels of 3.5 kb PDGF B mRNA are summarized from Northern blot analysis (39): -- , no detectable footprint; **+, +++, +++++, increasing levels of expression. Activity of the PDGF B promoter (39) is indicated as the activity of contract psis+112/+43CAT relative to the activity of the SV40 promoter/enhancer construct pSV2CAT (100%). The methylation status of the CpG dinucleotides (m) was determined by restriction enzyme analysis (all cell types) and by genomic sequencing (all cell types except HeLa and PC3): no, unmethylated; yes, heavily methylated.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>3.5 kb PDGF B mRNA</th>
<th>Promoter activity</th>
<th>Activating element</th>
<th>In vivo DMS footprint</th>
<th>CpG methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>++</td>
<td>0.39 ± 0.01</td>
<td>-64/-61</td>
<td>I, III (G-1)</td>
<td>no</td>
</tr>
<tr>
<td>PC3</td>
<td>+++</td>
<td>0.31 ± 0.01</td>
<td>-64/-61</td>
<td>I, III</td>
<td>no</td>
</tr>
<tr>
<td>Hufi</td>
<td>-</td>
<td>1.4 ± 0.07</td>
<td>-60/-45</td>
<td>--</td>
<td>no</td>
</tr>
<tr>
<td>JEG-3</td>
<td>-</td>
<td>5.9 ± 0.14</td>
<td>-64/-61</td>
<td>N.D.</td>
<td>yes</td>
</tr>
<tr>
<td>K562</td>
<td>+</td>
<td>0.95 ± 0.3</td>
<td>-64/-61</td>
<td>I</td>
<td>no</td>
</tr>
<tr>
<td>K562 + TPA</td>
<td>+++++</td>
<td>24.4 ± 6.3</td>
<td>-60/-45</td>
<td>I, II, III</td>
<td>no</td>
</tr>
</tbody>
</table>

Figure 5. Summary of structural and functional analysis of the human PDGF B gene promoter. The nucleotide sequence of the PDGF B promoter (31) is numbered relative to the transcription start site. Sites of DNA–protein interactions that were mapped by in vivo DMS footprint analysis are boxed and indicated by Roman numerals I, II and III; -- , no detectable footprint; N.D., not determined. Activity of the -64/-61 (underlined) and the -60/-45 (double underlined) transcription activating element in a transient reporter gene assay is indicated for each cell type. The TATA box is shown in bold. Expression levels of 3.5 kb PDGF B mRNA are summarized from Northern blot analysis (39): -- , undetectable; **+, +++, +++++, increasing levels of expression. Activity of the PDGF B promoter (39) is indicated as the activity of contract psis+112/+43CAT relative to the activity of the SV40 promoter/enhancer construct pSV2CAT (100%). The methylation status of the CpG dinucleotides (m) was determined by restriction enzyme analysis (all cell types) and by genomic sequencing (all cell types except HeLa and PC3): no, unmethylated; yes, heavily methylated.

fragments previously used in gel retardation assays with nuclear extracts from K562 cells also contained the -64/-61 element, suggesting that at least part of the in vitro DNA–protein complexes result from interaction with the weak transcriptional activator (33,34). JEG-3 cells, which do not contain the 3.5 kb PDGF B mRNA, also express a transcriptional activator directed at the -64/-61 element. Earlier we showed that the PDGF B promoter is not hypersensitive to DNase I in JEG-3 cells (39), suggesting that the transcriptional activator does not bind to the endogenous promoter. We now show that the PDGF B promoter and a large part of exon 1 is heavily methylated in JEG-3 cells, which might cause inhibition of PDGF B promoter activity.

A second transcription activating element was localized by deletion mutant analysis between positions -60 and -44 and co-mapped with an in vivo DMS footprint extending from -61 to -54 bp in TPA-treated K562 cells. TPA-mediated megakaryocytic differentiation of K562 cells is accompanied by a >200-fold increase in PDGF B mRNA level and a 10–30-fold increase in activity of the PDGF B promoter in a reporter gene assay (Fig. 5; 33,39). In addition, the TPA-mediated increase in PDGF B mRNA is dependent on de novo protein synthesis (33). Therefore, we conclude that the increased activity of the PDGF B promoter in TPA-treated K562 cells is the result of binding of a potent transcriptional activator to the -60/-54 element. Enhancer elements located downstream and far upstream of the PDGF B gene may further increase the transcription rate in TPA-treated K562 cells (39,50). Genomic footprinting revealed hypermethylation at G–61 in both untreated and TPA-treated K562 cells, which may result from binding of the same factor. This is supported by previous observations that complexes between PDGF B promoter fragments and nuclear proteins from either untreated or TPA-treated K562 cells migrate at similar positions in gel retardation assays (33,34). Our observation that mutagenesis or deletion of the -64/-61 element does not affect PDGF B promoter activity in TPA-treated cells could be explained by assuming that the weak activity of this element is obscured by the potent transcription activating element located between -60 and -45 bp. Alternatively, hypermethylation at G–61 may result from binding of different factors in untreated and TPA-treated cells. The footprint at -61/-54 in TPA-treated K562 cells closely resembles an in vivo footprint at an identical sequence element shown to be required for full promoter activity of the GATA-1 gene in mouse erythroleukemic MEL cells. The GATA-1 gene encodes an erythroid transcription factor that is specifically expressed in erythroid precursors, mast cells and megakaryocytes (53). In addition, the GATA-1 product itself may interact with CACC binding transcription factors (54). This raises the interesting possibility that GATA-1 and PDGF B promoter elements are regulated by related transcription factors. CACC element binding transcription factors were cloned from a murine erythroleukemia cell line (55) and isolated from rat liver (56). Recent in vitro binding studies indicated that the -61/-54 region of the PDGF B promoter interacts with purified Sp1 (35) and with Sp1-like proteins present in nuclear extracts from a human osteosarcoma cell line (36). Whether known CACC binding transcription factors are involved in regulation of the PDGF B promoter in megakaryocytic K562 cells remains to be determined.

Our present results indicate that primary dermal fibroblasts express a transcriptional activator that recognizes DNA sequences between positions -60 and -45 bp of the PDGF B promoter. Inhibition of PDGF B gene expression in fibroblasts seems important, as over-expression of normal PDGF B chains in fibroblasts may cause the development of fibrosarcomas via autocrine stimulation of cell proliferation (11,23–26,29). Since we could not detect transcription of the PDGF B gene in a nuclear run-on assay (39) and the transcriptional activator does not detectably bind to the endogenous promoter in vivo (this paper),
the gene is probably repressed at the transcriptional level. This is further supported by our previous observation that the PDGF B promoter is not hypersensitive to DNase I in fibroblasts (39). Thus far we have not found a transcriptional silencer within a region stretching out from -12 to +25 kb relative to the transcription start site of the PDGF B gene in fibroblasts (39,50). Now that we show that the PDGF B promoter is unmethylated in fibroblasts, a negative regulatory role for DNA methylation is also unlikely. Possibly, a DNase I-hypersensitive site located immediately downstream of the transcription start site in fibroblasts (39) contains an element involved in specific nucleosome phasing, which may render the PDGF B promoter inaccessible to trans-acting factors. A similar regulatory mechanism was suggested to explain the observation that the cAMP-responsive element (CRE) located upstream of the tyrosine aminotransferase (TAT) gene is inaccessible to the CRE binding protein (CREB) in certain cell lines that express nuclear CREB, even after 5-azacytidine-mediated demethylation of CpG residues in the TAT CRE (57).

In conclusion, we provide direct in vivo evidence for cell type-specific binding of a weak and a strong transcriptional activator to the PDGF B gene promoter in a panel of human cell lines. In addition, we have discussed the putative role of chromatin structure in negative and positive regulation of the gene. These studies illustrate the complexity of regulatory mechanisms used by this gene to achieve its broad, but highly specific expression pattern.

REFERENCES