Platelet-derived growth factor A-chain gene transcription is mediated by positive and negative regulatory regions in the promoter

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Platelet-derived growth factor (PDGF) is a disulphide-linked heterodimer of two polypeptide chains, the A and B chains, which are encoded by genes on separate chromosomes. The A-chain gene is transcribed in a number of transformed and non-transformed cell lines and is inducible by a wide variety of growth factors, cytokines and other mitogenic agonists. To localize DNA elements that mediate basal transcription in the promoter regulatory region of the A-chain gene, we have employed 5'-endpoint deletion mutagenesis and transient expression analysis in the renal epithelial cell line BSC-1 (African green monkey). Studies conducted in this cell line, which expresses high concentrations of PDGF A-chain mRNA, reveal a positive regulatory element (PRE) in a GC-rich stretch of the A-chain promoter between –82 and –40, relative to the transcription start site. Two discrete regions of the promoter were identified as negative regulatory elements (NREs), located between –1029 and –880 (NRE1) and between –1800 and –1029 (NRE2). The –1800 to –812 region, which contains both NREs, functions as a potent NRE when relocated in either orientation

adjacent to the herpes simplex virus thymidine kinase promoter, reducing transcription activity by 60 % in the positive orientation and 85 % in the negative orientation. Comparison of BSC-1 cells and Saos-2 cells (human osteogenic sarcoma), which do not express significant quantities of PDGF A-chain mRNA or protein, indicates that basal transcription of the gene is determined by enhancer activity mediated by the GC-rich region rather than through de-repression of the upstream NREs. Electrophoretic gel mobility shift assays reveal a complex pattern of nuclear protein binding to the GC-rich PRE (–73 to –46). Competition studies conducted with mutant oligonucleotides that alternately disrupt consensus binding sites for Sp-1 or Egr-1 demonstrate a requirement for the presence of an Sp-1-like core sequence (GGCGGG) but not Egr-1/Krox-24 [GCG(G/T)-GGGCG] for the formation of specific DNA–protein complexes. Our observations suggest that basal transcription of the A-chain gene in renal epithelial cells is achieved through active enhancement, mediated by a GC-rich PRE and nuclear proteins that bind to Sp-1-like consensus DNA sequences.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a family of three distinct but structurally related polypeptide dimers derived from the disulphide linkage of two subunits that share high amino acid sequence identity, designated the A and B chains. Depending on a number of factors, PDGF homodimers (PDGF-AA, PDGF-BB and PDGF-AB) are produced in varying amounts. The A chain, as a component of either PDGF-AA or PDGF-AB, is a potent mitogen and chemoattractant for cells that harbour the PDGF α -receptor (Bowen-Pope et al., 1991). This property of the molecule is responsible for important physiological functions in wound healing, cellular differentiation, embryogenesis and haemodynamics. The PDGF A chain has also been implicated as a contributor to such pathologies as atherosclerosis (Ross, 1986), fibroproliferative disease in liver (Pinzani et al., 1991), lung and kidney (Abboud et al., 1987), and malignancy (Betsholtz et al., 1986). Cultures of Wilms' tumour cells, both of epithelial and fibroblast origin, express PDGF (Fraizer et al., 1987), suggesting a role in the aetiology of this renal cancer.

Synthesis of the PDGF A and B chains is regulated in large part by the transcriptional rate of their cognate genes, and both genes are expressed in a restricted group of normal and transformed cell types (for a review, see Silver, 1992). Transcription of PDGF A- and B-chain genes is subject to positive and negative influence through the actions of various growth factors and cytokines (for a review, see Kaetzel et al., 1993).

In this study, we have examined the molecular mechanisms that mediate basal transcription of the PDGF A-chain gene in the renal epithelial cell line BSC-1. These cells secrete a PDGF-like activity and have been shown previously to express the PDGF B-chain gene (Mendley and Toback, 1988). We demonstrate that these cells express significant amounts of the A-chain mRNA during unstimulated conditions and that these mRNA concentrations can be increased by treatment with a phorbol ester. Transient transfection and promoter deletion analyses reveal that A-chain transcription is determined through the interplay of positive and negative regulatory elements (PREs and NREs respectively) in the proximal 5'-flanking sequence. Most of the basal transcription activity is mediated by a GC-rich

Abbreviations used: AchBS, A-chain binding sequence; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; β gal, β -galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSV, herpes simplex virus; MCS, multiple cloning site; NRE, negative regulatory element; AchP, A-chain promoter; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; PRE, positive regulatory element; TGF, transforming growth factor; TK, thymidine kinase.

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region located between nucleotides -82 and -40 relative to the transcription start site. A complex of nuclear proteins binds with high affinity to this region, as measured by electrophoretic mobility shift analysis (EMSA), suggesting a possible role for these proteins in the activation of A-chain gene transcription in BSC-1 cells.

EXPERIMENTAL

Expression vectors

A panel of AchP-CAT (AchP, A-chain promoter; CAT, chloramphenicol acetyltransferase) fusion reporter constructs bearing different 5'-endpoints was prepared by deletion mutagenesis. The numbering system employed in this paper to describe 5'-deletion endpoints for the promoter fragments is that described by Bonthron et al. (1988). The DNA sequence has been determined for the AchP from the start site of transcription to -936 (Bonthron et al., 1988; Takimoto et al., 1991). The plasmid pAC-CAT12 was constructed by insertion of an 87 bp *Bss*HII fragment of the A-chain gene (-883 to +8) in a sense orientation into the *Hind*III site of the promoterless CAT vector, pSPCAT3 (Ratner et al., 1987; Bonthron et al., 1988; Gashler et al., 1992). This vector was designed to fuse only 5'-flanking sequence to the reporter CAT gene, omitting 5'-untranslated DNA which could possibly confer stability and/or translation efficiency effects onto transiently expressed CAT mRNA. pAC-CAT12 served as the parent vector in the subsequent construction of pAC-CATX1 (-18 kb to +8 kb), pAC-CATX11 (-5 kb to +8 kb), pAC-CATX-20 (-1800 to +8 kb). These vectors were constructed by (1) excision of an A-chain genomic fragment from pAC-CAT12, extending from a 5' *Sall* site in the pSP65-derived multiple cloning site (MCS) to a 3' *Xho*I site located 261 bp upstream of the transcription start site; and (2) insertion of A-chain genomic DNA fragments that contained *Sall* in their respective 5'-endpoints but shared the *Xho*I termination point. pAC-CAT(-1029) was obtained from pAC-CATX-20 by excision of promoter sequence between -1800 and -1029 by digestion with *Sall* and *Kpn*I, filling in of 5'-overhanging ends with Klenow fragment of DNA polymerase I, and blunt-end ligation with DNA ligase. pAC-CAT(-638) (or pAC-CATΔ*Sac*I), pAC-CAT(-261) and pAC-CATF27(-60) have been described previously (Gashler et al., 1992). The remaining promoter constructs, pAC-CATe36 (-120 to +8), pAC-CATF11 (-82 to +8), and pAC-CATF47 (-40 to +8), were obtained by linearization of pAC-CAT(-261) with *Pst*I (MCS) and *Xho*I, digestion with either exonuclease III (e36) or *Bal*31 (F11, F27 and F47), and ligation.

The NRE of the AchP (-1800 to -812) was obtained as a *Bam*HI fragment from pAC-CATX20 and was relocated adjacent to the minimal promoter element of the thymidine kinase (TK) gene from herpes simplex virus (HSV) within the parent vector, pUTKAT3 (Prost and Moore, 1986). Plasmids were derived that contained the element in the correct orientation with respect to the promoter [pAC(-1800/-812)TK-CAT], in the opposite orientation [pAC(-812/-1800)TK-CAT] and a double copy insert with both copies in the positive orientation {[pAC(-1800/-812)₂]TK-CAT}.

Cell culture and transient expression assays

BSC-1 (African green monkey, renal epithelial), U2-OS and Saos-2 (human osteogenic sarcoma) cells were obtained from the American Type Culture Collection. BSC-1 cells (passage 47-52)

were maintained in Dulbecco's modified Eagle's medium (DMEM, containing 4.5 g of glucose/l) supplemented with 5% (v/v) fetal bovine serum, 10 mM nonessential amino acids, 2 mM glutamine, 50 i.u./ml penicillin, and 50 µg/ml streptomycin. Saos-2 cells (passage 30-35) were maintained in the same medium, with the exception that fetal bovine serum was included at a concentration of 15%.

All plasmids used in transient transfection experiments were purified by an ion-exchange chromatographic procedure (Qiagen, Chatsworth, CA, U.S.A.). Cells were plated at a density of 10⁶ cells per 100 mm diameter plastic dish (Costar, Cambridge, MA, U.S.A.). After incubation for 24 h, cells were transfected by calcium phosphate/DNA co-precipitation, as previously described (Graham and Van der Eb, 1973). A portion (10 µg) of test plasmid and 5 µg of the vector pRSV-βgal (βgal, β-galactosidase) (provided by J. Nilson, Case Western Reserve University) were included in each transfection plate. pRSV-βgal contains a constitutively expressed promoter/enhancer (3'-long terminal repeat) from the Rous sarcoma virus linked to the β-galactosidase gene and was included to correct for possible differences in transfection efficiency between experimental replicates. At a fixed period (24 h) after transfection, cell lysates (100 µl/plate) were harvested and analysed by CAT (Gorman et al., 1982) and β-galactosidase (Herbomel et al., 1984) assays, as described. CAT assays were conducted with 0.2 µCi of [¹⁴C]chloramphenicol substrate and 60 µl of cell lysate. β-Galactosidase activity was measured in duplicate on 15 µl lysate samples. Both assays were incubated at 37 °C for 4-6 h, and measurements of enzyme activity were made in the linear ranges of the respective assays. Conversion of [¹⁴C]chloramphenicol to acetylated products was 30% and *A*₄₁₀ readings for the β-galactosidase assay were in the range 0.05-1.0.

EMSA

EMSAs were conducted as described (Bokar et al., 1988). Radioactive probes for the assays were prepared by 5'-end labelling of oligodeoxyribonucleotides with [γ-³²P]ATP (3000 Ci/mmol) and T₄ DNA polynucleotide kinase. Nuclear extracts were prepared from BSC-1 cells by the method of Dignam et al. (1983). Binding assays were conducted in the presence of 20-22 fmol of probe, 5 µg of nuclear extract protein, 2 µg of poly[d(I-C)-d(I-C)] and a suitable amount of competitor DNA at 20-22 °C for 20 min. Binding reactions were applied to 6% (w/v) polyacrylamide gels with a recirculating electrophoresis buffer system of 25 mM Tris/HCl, 0.19 M glycine, pH 7.9.

Isolation and measurement of PDGF A-chain mRNA by blot hybridization

Total cellular RNA was extracted from confluent cultures of cells with acid guanidinium thiocyanate, phenol and chloroform (Chomczynski and Sacchi, 1987). RNA samples were subjected to electrophoresis through 1% (w/v) agarose gels and transferred to a nylon membrane by capillary blotting. PDGF A-chain transcripts were detected by hybridization with digoxigenin-UTP labelled antisense RNA probe, using a 633 nt A-chain cDNA in pBluescript as template (Fenstermaker et al., 1993). Bound probe was visualized by incubation with alkaline phosphatase-conjugated anti-digoxigenin and detection of chemiluminescence with Lumi-Phos 530, as described by the manufacturer (Boehringer-Mannheim). To verify equivalent loading and blot transfer of samples, blots were re-hybridized with a probe encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Bound A-chain probe was removed by incubation of blots

in stripping buffer [50% (v/v) formamide, 50 mM Tris/HCl, 1% (w/v) SDS, pH 8.0] for 1 h at 68°C, rinsing them in water, and then incubating them in 2×SSC at ambient room temperature. GAPDH mRNA was detected with an antisense RNA probe derived from a 780 bp *Xba*I/*Pst*I human GAPDH cDNA fragment described by Tso et al. (1985). Digoxigenin-UTP-labelled probe was prepared from a vector bearing the GAPDH cDNA fragment (pGEM-3Z; Promega) by linearization at the 3' end of the insert with *Hind*III and *in vitro* transcription with T7 RNA polymerase.

RESULTS

The renal epithelial cell line BSC-1 expresses significant quantities of PDGF A-chain mRNA

BSC-1 cells exhibited significant steady-state concentrations of A-chain mRNA, as determined by Northern blot hybridization (Figure 1; lane 1). Transcripts of three different sizes, 2.8, 2.3 and 1.9 kb, were observed, similar to those reported for a number of human transformed and non-transformed cell lines (Betsholtz et al., 1986; Peres et al., 1987; Silver et al., 1989). The mRNA concentration in BSC-1 cells was approximately equivalent to that seen in U2-OS cells (results not shown), a human osteogenic sarcoma line previously shown to express A-chain mRNA at high levels (Betsholtz et al., 1986). When BSC-1 cells were treated with the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), concentrations of the three A-chain transcripts were increased 4-fold within 2 h (Figure 1; lane 2). In addition to the three predominant mRNAs, another transcript of 4 kb was observed under PMA-stimulated conditions. All four mRNA species returned to steady-state concentrations within 8 h of treatment. Treatment with epidermal growth factor (EGF) led to an increase in the A-chain mRNAs of a magnitude and time course similar to that observed with PMA (results not shown).

Basal transcription of the PDGF A-chain gene is mediated through an interplay of PREs and NREs in the promoter region

To localize the regions in the PDGF AchP that determine its high basal transcriptional activity in BSC-1 cells, transient transfection

experiments were conducted with a panel of expression vectors containing pAC fragments linked to the CAT gene. All AchP fragments were terminated at a site just downstream from the transcription start site (+8) to prevent any possible interference by mRNA stability and/or translation efficiency conferred by 5'-untranslated sequence. Correct initiation of transcription has been demonstrated for several of the reporter construct deletions by primer extension analysis in U2-OS cells (Gashler et al., 1992). CAT activity was corrected for transfection efficiency with respect to β -galactosidase activity in cell lysates.

Maximum promoter activity was exhibited by pAC-CAT(−120), indicating that the 120 bp immediately upstream of the transcription start site contained the essential DNA sequence for basal transcription of the A-chain gene (Figure 2a). Further 5'-deletion of DNA sequence from −120 to −82 resulted in a modest (40%) reduction in CAT activity. However, deletion as far as −60 led to an 83% reduction, and further deletion to −40 resulted in a 94% loss. These data indicate the importance of the −82 to −40 region in determining basal transcription of the A-chain gene in BSC-1 cells. These observations are in agreement with those of others who have demonstrated the importance of the GC-rich enhancer for basal transcription activity in a variety of cell lines that produce PDGF A-chain (Takimoto et al., 1991; Gashler et al., 1992; Lin et al., 1992; Wang et al., 1992a).

Inclusion of longer segments of the AchP to −880 had no significant effect on expression. When the promoter sequences were increased to −1029 and −1800, reductions to 34 and 17% of maximal CAT activity respectively were observed (Figure 2a). This indicates the existence of two NREs, between −1029 and −880 (NRE1) and between −1029 and −1800 (NRE2). These results are also consistent with a *cis*-dominant effect of the upstream NREs upon the PRE of the A-chain gene.

To further characterize the transcriptional silencing function of this region, a −1800 to −812 *Bam*HI fragment bearing both NREs was inserted upstream of the minimal promoter/enhancer of the TK gene from HSV. When the region was oriented correctly with respect to the HSV-TK promoter [pAC(−1800/−812)TK-CAT], CAT activity was reduced to 43% of the control vector, pUTKAT3 (Figure 2b). Two positively oriented copies of the element {[pAC(−1800/−812)₂]TK-CAT} were more inhibitory than a single copy, reducing CAT activity to 27% of the control. Promoter activity was inhibited to 15% of control when a single copy of the element was inserted in the negative orientation [pAC(−812/−1800)TK-CAT]. These data characterize the NREs of the AchP as prototypical silencer elements that function in either orientation and can inhibit transcription of a heterologous promoter.

Basal transcription of the A-chain gene is mediated by the GC-rich region and not by de-repression of the upstream NRE

Deletional analysis of the PDGF AchP in BSC-1 cells indicated the presence of an important PRE between −82 and −40, as well as potent negative regulatory activity (NRE1 and NRE2). The high basal promoter activity in BSC-1 cells could be caused by high constitutive activation mediated by the GC-rich PRE, or a diminished repressor activity of the upstream NREs. To distinguish between these possibilities, we compared the transcriptional activity in BSC-1 cells of CAT plasmids bearing pAC deletions to that observed in Saos-2 cells that do not express A-chain mRNA.

In both cell lines, a vector containing the PRE [pACCAT(−261)] exhibited significantly higher CAT activity than a construct bearing only the minimal AchP [pAC-CAT(−40)] (Table 1). As expected, inclusion of upstream DNA sequence

Figure 1 BSC-1 cells contain high steady-state concentrations of PDGF A-chain mRNA that are inducible with phorbol ester treatment

Northern blot analysis of total cellular RNA (10 μ g) from BSC-1 cells either made quiescent by a 24 h treatment with serum-free culture medium (Con), or starved of serum and then incubated with PMA (10 nM) for the times (h) indicated. The relative mobilities of RNA molecular mass standards (kb) are indicated on the left, and probes employed are indicated to the right of each panel.

Figure 2 Localization of PREs and NREs in the PDGF AChP by deletional analysis

(a) The organization of the PDGF AChP, with the transcription start site (+1), pertinent restriction endonuclease (B, *Bss*HI; K, *Kpn*I; Sc, *Sac*I; S, *Sal*I; X, *Xho*I) sites and 5'-deletion endpoints indicated. Beneath the A-chain map are represented the various constructs employed in this study, consisting of the indicated 5'-flanking sequence fragments fused to the CAT gene. On the right, the relative CAT activities obtained in the analysis are summarized as means ± S.D., with the number in parentheses indicating the number of independent transfection experiments performed. The CAT activities are expressed relative to that of pAC-CAT(-120). (b) The left-hand portion of the panel displays the plasmid constructs containing the -1800 to -812 *Bam*HI fragment relocated adjacent to the HSV-TK promoter and the CAT gene. On the right, the relative CAT activities observed in triplicate transfection experiments are displayed as means ± S.D. Relative CAT activities in both (a) and (b) represent values that have been corrected for differences in transfection efficiency by measurement of co-transfected β -galactosidase.

containing the NREs [pAC-CAT(-1800)] resulted in reduced CAT activity, with a similar extent of inhibition in both BSC-1 and Saos-2 cells. CAT activity was consistently higher in BSC-1 cells with all promoter constructs, which may reflect a small but significantly higher rate of A-chain gene transcription in BSC-1

cells. These results reinforce the observation that A-chain gene transcription is maintained through enhancement mediated by the PRE and strongly suggest that the lack of transcriptional activity in Saos-2 cells is not caused by increased NRE activity. Other negative regulatory mechanisms not mediated by the

Table 1 Basal transcription of the A-chain gene in BSC-1 cells is maintained through active enhancement mediated by the GC-rich region and not by de-repression of the upstream NRE

The table contains CAT assay results from a representative transient transfection experiment with the indicated promoter constructs and cell lines. Three separate transfection experiments were conducted, and similar results were obtained in each. Relative CAT activity represents the CAT activity as a percentage of β -galactosidase activity (means $\pm$ S.D., conducted in triplicate transfection plates).

Construct	Cell line ...	Relative CAT activity	
		BSC-1	SaOS-2
pAC-CAT(-1800)		5 $\pm$ 3	2.1 $\pm$ 0.2
pAC-CAT(-261)		30 $\pm$ 11	11 $\pm$ 2
pAC-CAT(-42)		6 $\pm$ 1	3 $\pm$ 1

promoter sequence between -1800 and +8, such as other NREs or decreased mRNA stability, may contribute to the absence of A-chain mRNA in Saos-2 cells.

The PRE of the AchP is a site for high-affinity binding of nuclear proteins

EMSA was employed to examine the nuclear factors in BSC-1 cells that bind to the PRE. A double-stranded oligonucleotide probe corresponding to the GC-rich -73 to -46 region (known as the A-chain binding sequence, AchBS) was 5'-end labelled with 32 P, incubated with BSC-1 nuclear extract and subjected to non-denaturing PAGE. Figure 3 displays the results of an EMSA experiment in which the unaltered AchBS and two different mutant oligonucleotides were evaluated for their relative abilities to compete for binding of nuclear extract proteins to the radiolabelled probe. As indicated in Figure 3(a), the oligonucleotide 3T mutant consisted of the same -73 to -46 region but contained single nucleotide substitutions (T for G to give GGCGTG) that inactivated the three consensus Sp-1 sites, while maintaining the consensus Egr-1 binding sequences [GCG(G/T)GGGCG] contained in three overlapping Egr-1 sites. Thus, a loss in binding activity with this mutant sequence would be consistent with an interaction of nuclear factors with Sp-1-like sequence requirements, but not Egr-1. The oligonucleotide +3G mutant contained additions of single G residues to each of the three Egr-1 binding sites [GCGGGGGGCG], which disrupts the 9 base symmetry of these sites (Christy and Nathans, 1989) while keeping the three Sp-1 binding sites intact (Figure 3a).

Binding reactions conducted with BSC-1 nuclear extract and radiolabelled probe in the absence of unlabelled competitor DNA revealed the presence of three distinct protein-DNA complexes (Figure 3b, lane 1). The wild-type version of the DNA sequence, AchBS, competed effectively for binding of the probe, with half-maximal displacement achieved with a 5-fold molar excess of unlabelled DNA over the probe concentration (Figure 3b, lanes 2-4). In contrast, the 3T mutant oligonucleotide failed to compete for binding to the wild-type DNA probe at concentrations up to 100-fold molar excess (Figure 3b, lanes 5-8). The +3G mutant oligonucleotide, however, displaced the probe from all three bands at concentrations equal to that achieved with the wild-type sequence (Figure 3b, lanes 9-12). These results indicated that the -73 to -46 PRE of the AchP is a site for high-affinity binding of nuclear proteins, and that the three protein-DNA complexes possess nucleotide sequence requirements for

Figure 3 Analysis of nuclear protein binding to the PRE of the PDGF A-chain gene (-82 to -51) by EMSA

EMSA was conducted as described in the Materials and methods section, using a double-stranded oligonucleotide probe corresponding to the GC-rich PRE of the A-chain gene. The DNA probe was end-labelled with [γ - 32 P]ATP and polynucleotide kinase. (a) DNA sequences of deoxynucleotides employed in the EMSA experiments. Endpoints of the AchBS are displayed (-73 and -46), along with the consensus DNA-binding sites for Egr-1 and Sp-1. Asterisks mark the positions of multiples of 10 with respect to the transcription start site (i.e. -70, -60, -50). For comparison, the mutant deoxynucleotides, the +3G mutant and the 3T mutant, have been aligned with the wild-type sequence. Guanine insertions within the +3G mutant sequence are denoted by g, and GT substitutions in the 3T mutant sequence are underlined. (b) Nuclear extract (5 μ g/lane) obtained from BSC-1 cells was incubated with radiolabelled AchBS probe, 500 ng of poly(dI-dC), and the indicated unlabelled DNA competitors, and subjected to non-denaturing PAGE (6%, w/v) as described (Bokar et al., 1988). Dried gels were subjected to autoradiography for 24-48 h. Unbound probe and three major DNA-protein complexes (labelled 1, 2 and 3) are displayed.

DNA binding that are consistent with those of Sp-1, but not Egr-1.

To confirm that the +3G mutant oligonucleotide retained the binding characteristics of the native sequence, as would be predicted by its retention of the Sp-1 motif, the pattern of nuclear protein binding to a radiolabelled +3G mutant probe was analysed by EMSA. Protein binding to the +3G mutant probe displayed a banding pattern virtually identical to that seen with the AchBS probe (Figure 4a, lanes 1-3). A fourth band of higher mobility (arrow closest to the unbound probe) evident in this EMSA was seen intermittently in other assays with the AchBS and +3G mutant probes. Each band could be displaced with a 50-fold molar excess of AchBS (Figure 4, lane 2), but not by the 3T mutant oligonucleotide. The competing activity of homologous +3G mutant competitor was identical to that of AchBS in repeated EMSAs (results not shown). These data are consistent with the hypothesis that proteins binding to this region of the AchP display nucleotide sequence requirements similar to Sp-1, but not Egr-1.

In contrast, when the 3T mutant oligonucleotide is employed as a probe, a significantly different banding pattern is observed (Figure 4b, lanes 1-3). Although two bands displayed mobilities similar to the AchBS probe, (compare bands 4 and 5 in lane 1 to

Figure 4 Formation of sequence-specific DNA–protein complexes with the PRE of the A-chain gene is dependent upon conservation of the Sp-1 motif, but not the Egr-1 sequences

(a) EMSA conducted with either the +3G mutant or AchBS as probes and the indicated unlabelled competitor DNAs (A, AchBS; T, 3T mut). Unbound probe, the three major DNA–protein complexes identified in Figure 3(b) (bands 1–3) and an additional band of more rapid mobility (band 4) are identified in the Figure. (b) Radioactive probes, competitor DNAs and the major DNA–protein complexes are identified.

bands 2 and 4 in lane 4), their relative intensities were different from those seen with the AchBS probe, and both were displaced by unlabelled 3T mutant competitor (lane 3). As observed in Figure 3(b) and lanes 4–6 of Figure 4(b), the 3T mutant does not displace the proteins that bind to the AchBS. Three new bands also appear when 3T mutant is used as the probe (bands 1–3 in lanes 1–3; Figure 4), although these appear to be of lower affinity and sequence specificity than those DNA–protein complexes observed with the AchBS and +3G mutant probes. Disruption of the Sp-1 consensus binding sites within the AchBS, therefore, interferes with specific, high-affinity binding of the normal complement of nuclear factors, reinforcing the hypothesis that Sp-1, but not Egr-1, binds to the GC-rich PRE of the A-chain gene.

DISCUSSION

These data indicate that BSC-1 cells express significant quantities of PDGF A-chain mRNA under basal culture conditions and suggest that renal epithelial cells may be a physiologically

significant source of PDGF production in the kidney. PDGF B-chain mRNA and protein is also expressed in these cells (Karthan et al., 1988; D. M. Kaetzel, Jr., R. S. Maul, B. Liu, D. Bonthron, R. A. Fenstermaker and D. W. Coyne, unpublished work). As with other epithelial cells, BSC-1 do not express PDGF receptors but may stimulate adjacent connective or vascular tissues by a paracrine mechanism. Furthermore, expression of A-chain mRNA is highly inducible in BSC-1 cells by the protein kinase C activator PMA and by EGF (results not shown), suggesting a greater potential for renal epithelial cell production of A-chain *in vivo* under stimulated conditions. Increased epithelial production of PDGF A-chain, in turn, could contribute to pathological cellular proliferation in adjacent PDGF-responsive cell types such as tubular and mesangial cells, fibroblasts, and infiltrating macrophages.

Agonist-induced [i.e. PMA, EGF and transforming growth factor- β (TGF β)] increases in A-chain mRNA are mediated in other cell types by increased rates of PDGF gene transcription (Kavanaugh et al., 1988; Daniel and Fen, 1988; Press et al., 1989). No evidence could be obtained in BSC-1 cells, however, for the existence of DNA response elements for these agents in the proximal 18 kbp of AchP sequence (results not shown). Lin et al. (1992) identified a functional serum response element between –477 and –468 that mediates induction of A-chain transcription by PDGF in HeLa cells. This apparent discrepancy with our results could be explained by (1) participation of 5'-untranslated DNA sequence in the response, as the +1 to +392 bp region of the gene was present in their promoter constructs, (2) a cell-specific difference in the responsiveness of the gene, or (3) activation by PDGF of second messenger systems independent of those activated by PMA, EGF or TGF- β .

The PRE of the PDGF AchP is GC-rich and has been hypothesized to be a potential site of protein–DNA interaction and transcriptional regulatory activity (Bonthron et al., 1988). This region contains a total of 11 overlapping consensus binding sites for the transcription factor AP-2, four sites for Sp-1, and three overlapping consensus binding sites for the transcription factor Egr-1 [Krox-24, NGFI-A, zif268 and TIS8; for a review, see Kaetzel et al. (1993)]. In addition, the Wilms' tumour gene product (WT1) recognizes a core consensus DNA sequence similar to the Egr-1 binding sequence (5'-GCGGGGGCG-3') and has been shown to bind to this region of the A-chain gene (Gashler et al., 1992; Wang et al., 1992b, 1993). Furthermore, overexpression of the WT1 protein was shown to repress expression *in trans* from CAT vectors bearing the GC-rich region of the A-chain gene, suggesting that loss of this repressor gene product could result in overexpression of the PDGF A-chain and growth stimulation in Wilms' tumours.

Our data indicate that the GC-rich PRE from –73 to –46 is an area of complex DNA–protein interaction. The observation that DNA-binding proteins found in BSC-1 cells recognize the Sp-1-like binding sites and not the Egr-1 sites from this region suggests that Sp-1 may play an important role in the maintenance of basal transcription of the A-chain gene in BSC-1 cells, and perhaps, renal epithelial cells in general. In contrast, Egr-1 does not bind to the PRE, even though the cells do express an Egr-1-like protein which binds to a probe containing a single Egr-1 motif (results not shown). Cumulatively, these observations are consistent with the hypothesis that the Sp-1-like protein complexes effectively compete with Egr-1 for binding to the –73 to –46 region of the A-chain gene, either with higher affinity, by increased numbers of the Sp-1-like complexes, or a combination of both factors. Further detailed studies will be required, however, to identify and characterize conclusively the proteins that bind to the PREs in BSC-1 and other cell types.

Our studies indicate that the AchP contains a region that exerts a potent transcriptional silencing activity, both on the pAC and the heterologous HSV-TK promoter. We demonstrated that the silencer activity could be localized to two discrete regions, the first between -1029 and -880 (NRE1) and the other between -1800 and -1029 (NRE2). Our results extend those of Lin et al. (1992), who localized negative regulatory activity in the AchP to a region between -1900 and -900. Surprisingly, the -1800 to -812 fragment exhibited stronger silencer activity when re-located in the opposite orientation with respect to the HSV-TK promoter. The lack of strict orientation independence could be explained by a spatial requirement for optimal function of the A-chain silencers. Nevertheless, the region did exhibit significant silencer activity in the correct orientation and that activity was magnified when two correctly orientated copies of the element were placed adjacent to the HSV-TK promoter.

Further characterization of the A-chain silencer elements could prove valuable in identifying possible mechanisms of oncogenesis and other diseases of inappropriate cellular proliferation. For example, overexpression of the A-chain could be caused by impaired NRE function, either through somatic mutation of the NRE or by loss of cognate transcription factors which interact with the element. The DNA sequence within NRE1 contains a 27 bp segment (-1004 to -978) bearing considerable dyad symmetry and an 8 bp core with limited sequence identity to the AP-1/PMA and cyclic AMP response elements (TRE and CRE respectively). We have observed high-affinity binding between this element and a nuclear factor found in numerous cell types (HeLa, A172 astrocytoma, renal mesangial). The sequence confers neither silencer activity nor PMA/cyclic AMP responsiveness onto the heterologous HSV promoter/enhancer (results not shown), although its function has not been tested in the context of the A-chain PRE. In this regard, the immunoglobulin κ gene is repressed in non-B cells through the interaction of a silencer element with the adjacent κ B enhancer, but does not function with heterologous enhancers (Pierce et al., 1991). Promoter deletion and DNA-protein binding studies should be useful to localize further the minimal silencer elements located within the NRE1 and NRE2 DNA segments.

Regulation of PDGF A-chain gene transcription in response to various developmental, cell-specific and environmental cues is complex, involving the participation of multiple DNA elements and cognate transcription factors. Our studies demonstrate clearly that transcriptional tone of this gene is set by the net activities of multiple NREs and PREs within the promoter region. Precise identification and characterization of these molecular participants should facilitate understanding of the mechanisms which mediate the regulated expression of the A-chain gene in both physiological and pathophysiological conditions.

The assistance of Sherry Beecher, Dianne Kube, Susan Miljkovic, James Reid, Amy Wandstradt and Andrea Hujer is gratefully acknowledged. This work was supported by funds from the Department of Veterans Affairs (to D.K., R.F. and D.C.), National

Institutes of Health DK45518 (to D.K.), Community Dialysis Center of Cleveland (to D.K.), Case Western Reserve University Ireland Cancer Center Grant (CA43703), and the Capt. David M. Kaetzel Memorial Cancer Research Fund. D.T.B. was supported by the Leukaemia Research Fund, U.K. (grant 90/30).

REFERENCES

- Abboud, H. E., Poptic, E. and DiCorleto, P. (1987) *J. Clin. Invest.* **80**, 675-683
- Betsholtz, C., Johnsson, A., Heldin, C.-H., Westermark, B., Lind, P., Urdea, M. S., Eddy, R., Shows, T. B., Philpott, K., Mellor, A. L., Knott, T. J. and Scott, J. (1986) *Nature* (London) **320**, 695-699
- Bokar, J. A., Roesler, W. J., Vandenbark, G. R., Kaetzel, D. M., Hanson, R. W. and Nilson, J. H. (1988) *J. Biol. Chem.* **263**, 19740-19747
- Bonthron, D. T., Morton, C. C., Orkin, S. H. and Collins, T. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1492-1496
- Bowen-Pope, D. F., van Koppen, A. and Schatteman, G. (1991) *TIG Rev.* **7**, 413-418
- Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156-159
- Christy, B. A. and Nathans, D. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8737-8741
- Daniel, T. O. and Fen, Z. (1988) *J. Biol. Chem.* **263**, 19815-19820
- Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475-1489
- Fenstermaker, R. A., Poptic, E., Bonfield, T. L., Knauss, T. C., Corsillo, L., Piskurich, J., Kaetzel, C. S., Jentoft, J., Gelfand, C., DiCorleto, P. E. and Kaetzel, D. M., Jr. (1993) *J. Biol. Chem.* **268**, 10482-10489
- Fraizer, G. E., Bowen-Pope, D. F. and Vogel, A. M. (1987) *J. Cell. Physiol.* **133**, 169-174
- Gashler, A. L., Bonthron, D. T., Madden, S. L., Rauscher, F. J., III, Collins, T. and Sukhatme, V. P. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10984-10988
- Graham, F. L. and Van der Eb, A. J. (1973) *Virology* **52**, 465-467
- Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044-1055
- Herbomel, P., Bourachot, B. and Yaniv, M. (1984) *Cell* **39**, 653-662
- Kaetzel, D. M., Coyne, D. W. and Fenstermaker, R. A. (1993) *BioFactors* **4**, 71-81
- Kartha, S., Bradham, D. M., Grotendorst, G. R. and Toback, F. G. (1988) *Am. J. Physiol.* **255**, F800-F806
- Kavanaugh, W. M., Harsh, G. R., Starksen, N. F., Rocco, C. M. and Williams, L. T. (1988) *J. Biol. Chem.* **263**, 8470-8472
- Lin, X., Wang Z., Gu, L. and Deuel, T. F. (1992) *J. Biol. Chem.* **267**, 25614-25619
- Nilson, J. H., Nejedlik, M. T., Virgin, J. B., Crowder, M. E. and Nett, T. M. (1983) *J. Biol. Chem.* **258**, 12087-12090
- Mendley, S. R. and Toback, F. G. (1988) *Annu. Rev. Physiol.* **51**, 33-50
- Peres, R., Betsholtz, C., Westermark, B. and Heldin, C.-H. (1987) *Cancer Res.* **47**, 3425-3429
- Pierce, J. W., Gifford, A. M. and Baltimore, D. (1991) *Mol. Cell. Biol.* **11**, 1431-1437
- Pinzani, M., Knauss, T. C., Pierce, G. F., Hsieh, P., Kenney, W., Dubyak, G. R. and Abboud, H. E. (1991) *Am. J. Physiol.* **251**, C485-C491
- Press, R. D., Misra, A., Gillasp, G., Samols, D. and Goldthwait, D. A. (1989) *Cancer Res.* **49**, 2914-2920
- Prost, E. and Moore, D. D. (1986) *Gene* **45**, 101-111
- Ratner, L., Thielan, B. and Collins, T. (1987) *Nucleic Acids Res.* **15**, 6017-6036
- Ross, R. (1986) *Acta Med. Scand. Suppl.* **715**, 33-38
- Silver, B. J., Jaffer, F. E. and Abboud, H. E. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1056-1060
- Silver, B. J. (1992) *BioFactors* **3**, 217-227
- Takimoto, Y., Wang, Z. Y., Kobler, K. and Deuel, T. F. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1686-1690
- Tso, J. Y., Sun, X.-H., Kao, T., Reece, K. S. and Wu, R. (1985) *Nucleic Acids Res.* **13**, 2485-2502
- Wang, Z.-Y., Lin, X. H., Nobuyoshi, M., Qiu, Q.-Q. and Deuel, T. F. (1992a) *J. Biol. Chem.* **267**, 13669-13674
- Wang, Z.-Y., Madden, S. L., Deuel, T. F. and Rauscher, F. J., III (1992b) *J. Biol. Chem.* **267**, 21999-22002
- Wang, Z.-Y., Qiu, Q.-Q. and Deuel, T. F. (1993) *J. Biol. Chem.* **268**, 9172-9175