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# Functional Identification of Regulatory Elements within the Promoter Region of Platelet-Derived Growth Factor 2

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**Human platelet-derived growth factor (PDGF) is composed of two polypeptide chains, PDGF-1 and PDGF-2, the human homolog of the v-*sis* oncogene. Deregulation of PDGF-2 expression can confer a growth advantage to cells possessing the cognate receptor and, thus, may contribute to the malignant phenotype. We investigated the regulation of PDGF-2 mRNA expression during megakaryocytic differentiation of K562 cells. Induction by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) led to a greater than 200-fold increase in PDGF-2 transcript levels in these cells. Induction was dependent on protein synthesis and was not enhanced by cycloheximide exposure. In our initial investigation of the PDGF-2 promoter, a minimal promoter region, which included sequences extending only 42 base pairs upstream of the TATA signal, was found to be as efficient as 4 kilobase pairs upstream of the TATA signal in driving expression of a reporter gene in uninduced K562 cells. We also functionally identified different regulatory sequence elements of the PDGF-2 promoter in TPA-induced K562 cells. One region acted as a transcriptional silencer, while another region was necessary for maximal activity of the promoter in megakaryoblasts. This region was shown to bind nuclear factors and was the target for *trans*-activation in normal and tumor cells. In one tumor cell line, which expressed high PDGF-2 mRNA levels, the presence of the positive regulatory region resulted in a 30-fold increase in promoter activity. However, the ability of the minimal PDGF-2 promoter to drive reporter gene expression in uninduced K562 cells and normal fibroblasts, which contained no detectable PDGF-2 transcripts, implies the existence of other negative control mechanisms beyond the regulation of promoter activity.**

Platelet-derived growth factor (PDGF) is one of several known growth factors involved in the regulation of cell proliferation (see reference 37 for references). This growth factor consists of two related chains, the products of different genes, PDGF-1 and PDGF-2. The PDGF-2 gene, the human homolog to the v-*sis* oncogene, confers the transformed phenotype to NIH 3T3 cells if expressed under the control of a strong eucaryotic promoter (14). PDGF-2 mRNA can normally be found in endothelial cells (5) and placental trophoblasts (17). While normal fibroblasts and glial cells do not contain detectable levels of this transcript, a large fraction of sarcoma and glioblastoma cell lines or tumor tissues express the 3.7-kilobase (kb) mRNA (13). Since fibroblasts and glial cells possess PDGF receptors and can be stimulated by PDGF (7, 19), expression of this growth factor may be important in the generation of such human malignancies. A prerequisite for understanding how PDGF-2 expression is activated in these tumors is a knowledge of the control mechanisms involved.

A system in which PDGF-2 expression can be induced makes it possible to dissect different regulatory mechanisms and determine their contribution during induction. PDGF-2 mRNA is induced in HL-60 and U-937 cells during differentiation along the monocytic-macrophage lineage (31), in activated human monocytes (28), and in mouse embryo-derived AKR-2B cells treated with transforming growth factor β (25). When the human hematopoietic stem cell line K562 is induced to differentiate into megakaryoblasts by treatment with phorbol esters, there is a concomitant appearance of PDGF-2 mRNA (3, 10). We decided to investigate PDGF-2 mRNA induction and promoter activation in this system in an effort to elucidate the control mechanisms of PDGF-2 expression.

Cloning of the PDGF-2 gene has made it possible to localize its putative promoter (34, 36). We used these sequences to control the expression of a reporter gene in determining whether the PDGF-2 promoter is a target for regulation in K562 cells and to evaluate the contribution of promoter activation to PDGF-2 mRNA induction. For this purpose we analyzed PDGF-2 mRNA expression during the early time course of K562 cell differentiation, identified PDGF-2 promoter elements, and evaluated their contribution to PDGF-2 regulation in normal and tumor cells.

## MATERIALS AND METHODS

**Cells.** The chronic myelogenous leukemia cell line K562 (26) was obtained from the American Type Culture Collection (ATCC CCL 243); normal human embryonic fibroblasts (M413 [1]), the human fibrosarcoma line 8387 (2), and the human bladder carcinoma cell line EJ (32) have been described previously. Two glioblastoma cell lines, A2781 and A1235 (S. A. Aaronson, unpublished), were also used. Exponentially growing K562 cells were treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma Chemical Co., St. Louis, Mo.) at a concentration of 2 ng/ml in RPMI 1640 (Gibco, Grand Island, N.Y.) medium (supplemented with 10% fetal calf serum, 50 U of penicillin G per ml, and 50 μg of streptomycin per ml); cycloheximide (CHX; Sigma Chemical Co.) was added at 10 or 40 μg/ml.

**Plasmid constructions.** Standard techniques were used for all recombinant DNA manipulations (27). The construction of pSRMCatPΔF.M has been described before (36). DNA templates for RNA probes were cloned into the Gemini vectors (Promega Biotec, Madison, Wis.). The PDGF-2 template was derived from pGSS2.7 (20) by deleting all 3′

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sequences between the internal PDGF-2 *Bgl*II site and the *Bam*HI site in the polylinker of pGEM4. The human β-actin template consists of a *Sal*I- *Xho*II fragment isolated from the 5′ portion of a human β-actin cDNA clone (33) inserted into pGEM1. PSV2Cat (16) was a gift from B. Howard, National Cancer Institute (NCI). The β-ActinCat plasmid contains the chicken promoter for β-actin (23) in an *Xho*I-*Hin*cII fragment which replaces the simian virus 40 promoter in pSV2Cat. It was constructed and kindly provided by B. Paterson, NCI. The eucaryotic expression vector for β-galactosidase, pCH110, was purchased from Pharmacia Inc., Piscataway, N.J.

**Deletion mutagenesis.** pSRMCatPΔF.M was linearized with *Bst*EII, incubated with exonuclease III (Bethesda Research Laboratories, Gaithersburg, Md.) at 135 U/pmol of 3′ ends in 66 mM Tris hydrochloride (pH 8.0)–5 mM $MgCl_2$ at 15°C. Samples were removed after 1 to 20 min at 1-min intervals, heat inactivated, treated with exonuclease VII, ethanol precipitated, incubated with the Klenow fragment of DNA polymerase I, and ligated with T4 DNA ligase. Plasmid 15/2-1 was generated from *Bst*EII-linearized pSRMCat PΔF.M by using nuclease Bal 31. All deletions were analyzed by sequencing.

**Site-directed mutagenesis.** Site-directed deletions were introduced into pSRMCatPΔF.M with the help of 30-mer oligonucleotides spanning the desired deletion. For this purpose, an *Eco*RI fragment of pSRMCatPΔF.M containing the promoter region was first subcloned into M13mp9. An oligonucleotide-directed in vitro mutagenesis system (Amersham, Amersham, United Kingdom), which is based on the Eckstein method (30), was used to introduce deletions into the M13mp9 inserts, which were then recloned into pSRMCatPΔF.M. The deletions were analyzed by sequencing the M13mp9 inserts as well as the respective regions in the final plasmid constructs.

**Oligonucleotide synthesis.** Oligonucleotides for in vitro mutagenesis, primer extension analysis, sequencing, and gel retardation experiments were synthesized on a Beckman System 1Plus DNA Synthesizer.

**Sequencing.** Sequencing was done by dideoxy chain termination methods (38).

**Electroporation and preparation of cell extracts.** All DNAs used for electroporation were CsCl gradient-purified, phenol-extracted plasmid DNAs which were suspended in sterile phosphate-buffered saline. Concentrations of closed circular plasmid DNAs were determined by measurement of optical densities as well as by gel electrophoresis prior to electroporation. From $2 \times 10^7$ to $4 \times 10^7$ cells were washed twice in sterile phosphate-buffered saline and suspended in a volume of 300 μl in 1.0-ml disposable cuvettes lined with aluminum foil. The cell suspensions were kept on ice, and a mixture of 20 μg of pCH110 plasmid DNA with 20 μg of test plasmid DNA was added in 100 μl of phosphate-buffered saline. Electroporations were performed at room temperature with a single electrical pulse delivered from a 494 Isco power supply at a voltage of 2 kV (K562 cells) or 1.3 kV (all other cells), limiting the current and wattage to 9 μA and 15 mW, respectively. The cells were incubated at 0°C for 15 min, resuspended in culture medium, and plated into a 175-$cm^2$ flask. For induction, the culture was divided after a 12-h incubation into two identical portions, one receiving culture medium supplemented with the appropriate amount of TPA and the other receiving medium only. The cells were harvested after 24 h. Extracts were prepared by the method of Gorman et al. (16), using three freeze-thaw cycles for cell lysis.

**CAT and β-galactosidase assays.** The amount of β-galactosidase encoded by pCH110 was used to standardize individual extracts in each electroporation experiment. The assay for β-galactosidase was performed according to Edlund et al. (12). Cell extract (20 μl) was incubated with and without *o*-nitrophenyl-β-D-galactoside (Boehringer, Mannheim, Federal Republic of Germany) for the time needed to achieve a visible conversion to the yellow chromogen; the difference in absorbance at 420 nm was used to calculate the specific activity for each extract. This activity determined the correct amount of extract to be used in the chloramphenicol acetyltransferase (CAT) assay, which was performed according to Gorman et al. (16). Standardization was restricted to each particular cell type and electroporation experiment (separately for induced and uninduced cells). To be able to compare CAT conversion values (percent conversion of chloramphenicol to its acetylated forms) between several independent experiments, we converted these values to relative CAT activity units by defining the percent conversion achieved after electroporation of pSRMCatPΔF.M into uninduced K562 cells as 1 U.

**Primer extension.** After electroporation with CAT plasmids, K562 cell suspensions were divided into two portions. One was used to prepare extracts for CAT and β-galactosidase assays, and the other was used to prepare RNA according to the method of Chirgwin et al. (4). Different amounts of RNA were hybridized to a 19-bp oligonucleotide primer specific for the 5′ end of the *cat* gene. The primer was end labeled by phosphorylation with polynucleotide kinase and [γ-$^{32}$P]ATP. Labeling and primer extension were performed as described by Geliebter et al. (15). Extension products were resolved on 8.3 M urea–8% acrylamide sequencing gels alongside sequencing reactions of pSRM CatPΔF.M performed with the same primer.

**RNase protection assays and RNA quantification.** RNase protection assays were done according to Melton et al. (29) under conditions recommended by the commercial supplier of the SP6 and T7 polymerases (Promega Biotec). The following modifications were introduced. Riboprobes were synthesized at 40°C for 1 h; the concentration of UTP in the reaction mixture was 25 μM at a specific activity of 230 Ci/mmol. If SP6 polymerase was used, 10 U of enzyme was added a second time after 30 min of incubation. Specific activities and concentrations of riboprobes were calculated based on $^{32}$P incorporation. The full-length probes were purified on 8.3 M urea–5% acrylamide sequencing gels and eluted in 1 M NaCl. For hybridizations, $10^6$ cpm of probe and 0.1 to 200 μg of total RNA were incubated at 50°C in hybridization buffer containing 80% formamide for at least 12 h. RNase digestion was performed at 37°C with 8 U of RNase A and 1,400 U of RNase $T_1$ per ml for 1 h. The protected RNA fragments were resolved on 20-cm-long 8.3 M urea–5% polyacrylamide sequencing gels alongside different amounts of untreated riboprobe. The gels were dried and autoradiographed for various amounts of time ranging from 2 to 5,760 min. Autoradiograms were scanned in a Bio-Rad model 620 videodensitometer, and RNA concentrations were calculated by comparing signal intensities with those generated by different amounts of the corresponding untreated riboprobe. By using different quantities of cellular RNA, it was verified that the hybridization time and amount of probe were sufficient to recover RNA transcripts in hybrids quantitatively.

**Gel mobility shift assays.** Nuclear extracts of K562 cells were prepared according to Dignam et al. (11). Gel mobility shift experiments were performed according to Schneider et

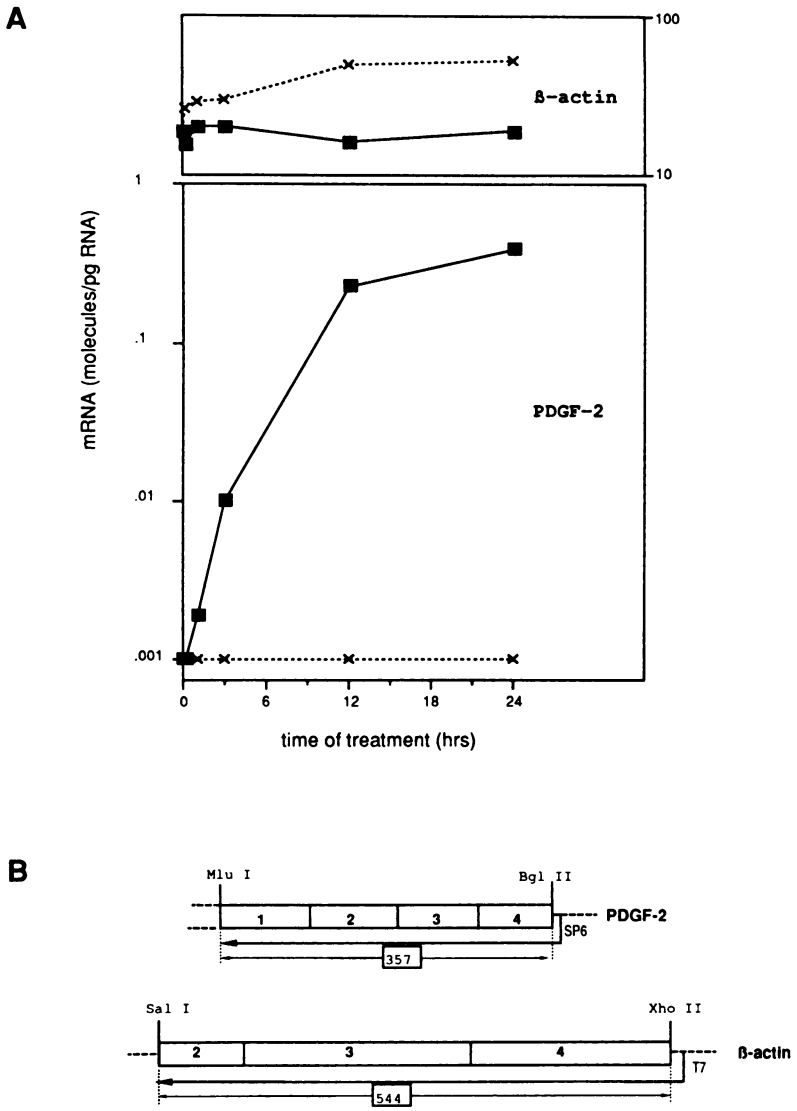


FIG. 1. TPA-mediated induction of PDGF-2 mRNA in K562 cells. The amounts of PDGF-2 (A, lower panel) and β-actin (A, upper panel, scale on right) mRNA are expressed as the number of mRNA molecules per picogram of total cellular RNA after treatment of K562 cells for 0 to 24 h with 2 ng of TPA per ml (■) or 2 ng of TPA and 40 μg of CHX per ml (×). RNAs which did not yield any detectable signal in RNase protection assays are plotted at the limit of detection (0.001 molecules/pg). (B) Structures of the riboprobes used in the RNase protection assays. Exons are boxed and numbered. The sizes (in nucleotides) of RNA fragments protected by the antisense riboprobes are boxed as well. SP6 and T7 indicate the location of the SP6 and T7 promoters used for riboprobe synthesis.

al. (40). Labeled, complementary, single-stranded oligonucleotides were annealed in equimolar amounts and purified by chromotography on DEAE-cellulose before binding. Different amounts of poly(dI-dC) · poly(dI-dC) (Sigma Chemical Co.) were used as heterologous competitor DNA (41). Protein-oligonucleotide complexes were separated on 7 or 11% Tris-glycine-polyacrylamide gels at 25 mA (40).

## RESULTS

**Time course of PDGF-2 mRNA induction in TPA-treated K562 cells.** PDGF is stored in the α-granules of platelets. Thus, megakaryocytes are thought to represent the normal site of PDGF-2 synthesis. The pluripotent K562 cell line is capable of differentiating towards the megakaryocytic lineage in the presence of TPA. This cell line might therefore be used as a relevant model system for the study of mechanisms regulating the expression of PDGF-2.

Uninduced K562 cells do not express levels of RNA detectable by Northern (RNA) blot analysis (10). In an effort to quantitate low amounts of PDGF-2 transcripts during the early time course of K562 differentiation, we used the sensitive RNase protection assay. No PDGF-2 mRNA was detectable in uninduced K562 cells even when 200 μg of total RNA was used. Thus, uninduced K562 cells contained less than 1 PDGF-2 mRNA molecule per ng of total cellular RNA. As shown in Fig. 1, PDGF-2 mRNA levels increased more than 200-fold within 24 h following TPA exposure. The β-actin mRNA levels remained unchanged at this TPA concentration. The stimulatory effect of phorbol esters on the activity of some promoters has been shown to be independent of de novo protein synthesis (21). However, when CHX (40 μg/ml) was added at the time of TPA treatment, TPA-mediated induction of PDGF-2 mRNA was completely inhibited (Fig. 1). β-Actin mRNA levels in-

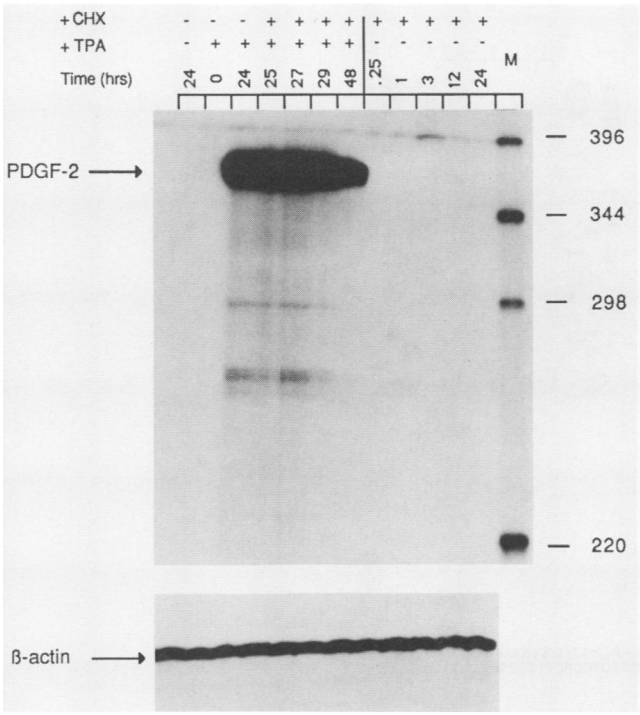


FIG. 2. RNase protection assay of PDGF-2 mRNAs in K562 cells after induction with TPA and CHX. Exponentially growing K562 cells were treated with TPA (2 ng/ml) for 24 h, followed by addition of CHX (10 μg/ml); others were treated with CHX (10 μg/ml) alone. Cells were harvested at different time points as indicated, and 20 μg of total RNA was hybridized to either the PDGF-2 or β-actin riboprobe (see Fig. 1). The protected fragments were separated on an 8.3 M urea–5% acrylamide sequencing gel. Lane M contains pBR322 *Hin*fI fragments as size markers. Sizes are shown in nucleotides.

creased about twofold under these conditions. Similar results were obtained with CHX at 10 μg/ml (data not shown).

It has been demonstrated for a number of inducible mRNAs that inhibition of protein synthesis can enhance mRNA levels (18), suggesting that a labile protein might be involved in regulating expression of these transcripts (22). When we first induced K562 cells for 24 h with TPA and then added CHX (10 or 40 μg/ml), we observed no superinduction of PDGF-2 mRNA (Fig. 2). In addition, treatment of K562 cells with CHX alone did not result in induction of PDGF-2 mRNA (Fig. 2). Together, these results demonstrated that PDGF-2 mRNA induction in K562 cells by TPA depends on protein synthesis and cannot be enhanced by CHX. Thus, in neither uninduced nor TPA-induced K562 cells did the level of PDGF-2 mRNA appear to be regulated by a labile protein.

**PDGF-2 promoter is a target for regulation in differentiating K562 cells.** In order to investigate the contribution of transcriptional control to the regulation of PDGF-2 mRNA expression, we decided to functionally characterize the PDGF-2 promoter. For this purpose, we used two plasmids, designated pSHMCatPΔF.M and pSRMCatPΔF.M, which contained the putative PDGF-2 promoter region (36). They have either 4 kbp or 447 bp of genomic PDGF-2 DNA upstream of an *Fsp*I restriction site (68 bp downstream of the TATA signal sequence) fused to the bacterial *cat* coding sequence (36), respectively. Each plasmid was introduced into K562 cells by electroporation, and cell extracts were tested for CAT activity before and after TPA induction. No significant differences in promoter activity of the two plasmids were detectable (data not shown). Thus, promoter elements required for PDGF-2 expression in the transfected cells lines seemed to be contained within the *Eco*RI-*Fsp*I restriction fragment.

We next localized the initiation of PDGF-2 and *cat* RNA synthesis in K562 cells transfected with the pSRMCat PΔF.M plasmid by primer extension analysis. Figure 3A shows the RNA initiation sites within the sequence GGTG GCAACTT. These sites were consistent with the previously determined start site of PDGF-2 RNA in human placenta or EJ cells (34) and with the consensus cap site sequence CANPyPyPy, where N is any nucleotide and Py is any pyrimidine, determined by Bucher and Trifonov (8). Thus, the PDGF-2 promoter within pSRMCatPΔF.M initiated RNA synthesis faithfully in electroporated K562 cells.

As shown in Fig. 4, induction with TPA led to a ca. 10-fold increase in CAT activity when K562 cells were electroporated with pSRMCatPΔF.M. This increase was also reflected in the amount of PDGF-2 and *cat* RNAs observed in primer extension experiments under conditions of primer excess (compare lanes 2 and 4 in Fig. 3B). Thus, the amount of CAT activity appeared to be a good measure of the transcriptional activity of the PDGF-2 promoter in K562 cells. Moreover, our results indicated that the PDGF-2 promoter was a target for regulation in these cells.

**Identification of regulatory elements within the PDGF-2 promoter by deletion analysis.** We sought to identify the promoter elements which regulate PDGF-2 transcriptional activity upon TPA-induced differentiation of K562 cells into megakaryoblasts. Thus, we analyzed the effect of various deletions in the PDGF-2 promoter region on CAT activity after electroporation into K562 cells with or without subsequent TPA induction. As shown in Fig. 4, CAT activities in uninduced cells were not affected until the deletions included sequences between 332 and 344 bp downstream of the *Eco*RI site. The variations in uninduced CAT activities observed in those experiments were judged to be not significant (Fig. 4). A minimal promoter region (Ex 15-2, Fig. 4) which included only 42 bp upstream of the TATA signal retained almost full activity.

In contrast, PDGF-2 promoter activity in K562 cells induced to differentiate into megakaryoblasts varied over more than a 20-fold range depending on the deletion mutant (Fig. 4). Deletion of the region between the *Eco*RI site and the *Nco*I site located 146 bp further downstream increased promoter activity by at least fourfold in induced K562 cells (data not shown). Mutants Ex 12-1 and Ex 13-2 had nearly identical 3′ deletion endpoints, but a region located between 31 and 71 bp downstream of the *Eco*RI site was present or not, respectively. These mutants showed a nearly fourfold difference in their induced PDGF-2 promoter activity (Fig. 4). In Ex 14-3 and Ex 13-3, the 3′ deletion endpoints were identical. However, juxtaposition of a sequence located between 1 and 61 bp downstream of the *Eco*RI site to the positive regulatory region (see below) in Ex 13-3 completely abolished inducibility. Thus, analysis of these and the other deletion mutants led to the tentative localization of a transcriptional silencer (− symbol in Fig. 4) within a stretch of 30 bp between 32 and 61 bp downstream of the *Eco*RI site.

Only one of the two potential Sp1-binding sites (34) in the PDGF-2 promoter region seemed to have an influence on the level of transcription; deletion of this site caused a reduction in inducibility (Ex 7-1 versus Ex 8-1 in Fig. 4) that could be counteracted by also deleting the transcriptional silencer. This same Sp1-binding site seemed not to be necessary for

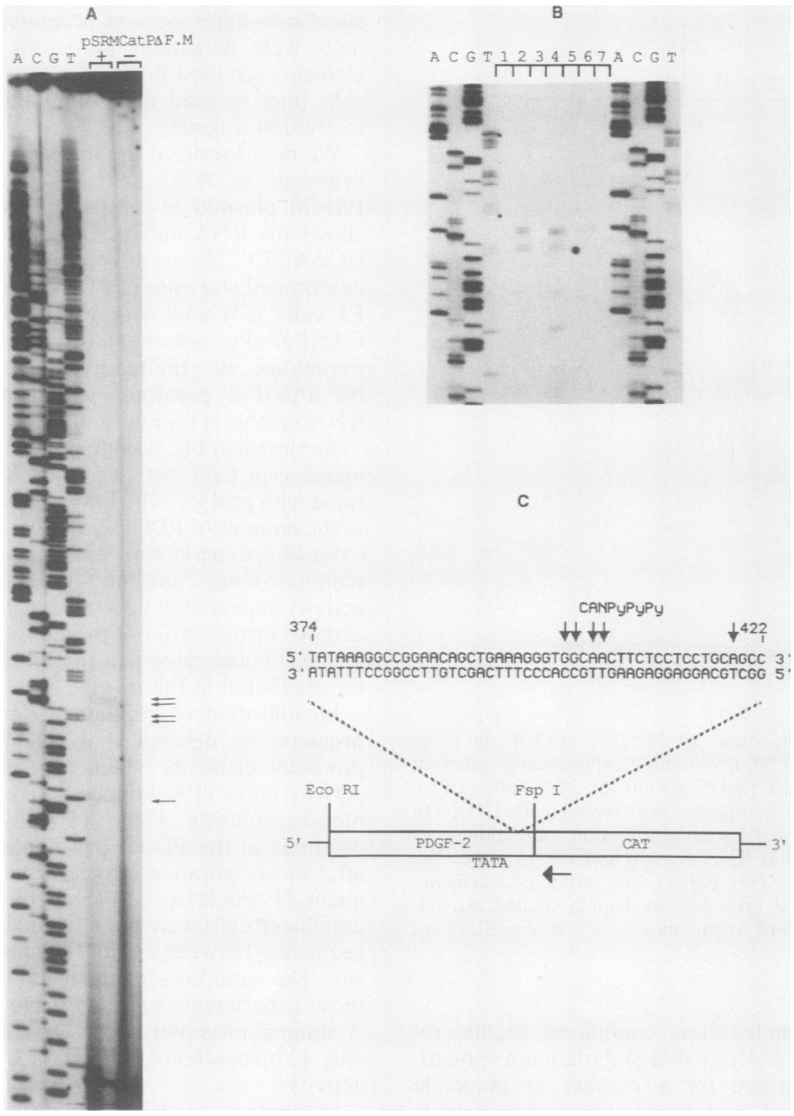


FIG. 3. Primer extension analysis of K562 cells transfected with PDGF-2/*cat* constructs. K562 cells were electroporated with 20 μg of pCH110 and 20 μg of the test plasmid. Each culture was divided into two portions, one of which was induced for 24 h with TPA (2 ng/ml). RNAs were then prepared from uninduced and induced cultures. PDGF-2/*cat* RNA initiation (A) and the amount of specific PDGF-2/*cat* RNA (B) were determined with an oligonucleotide primer (horizontal arrow in panel C) complementary to *cat* RNA (C). (A) Sequence reaction of plasmid pSRMCatPΔF.M obtained with the end-labeled primer and primer extension reactions of RNA from K562 cells with (+) or without (−) electroporated pSRMCatPΔF.M DNA after TPA induction. Primer extension products are indicated by arrows. (B) Primer extension reactions with RNA from K562 cells electroporated with pSRMCatPΔF.M (lanes 1, 2, 4, 5), Δ3 (lane 6), Δ2 (lane 7), or no DNA (lane 3). The cells were untreated (lanes 1, 3, 4, 6, 7) or induced with TPA (lanes 2, 5). Different amounts of cellular RNA were used for the primer extension reaction: 1 μg (lane 5), 10 μg (lanes 1, 2, 3), or 100 μg (lanes 4, 6, 7). The signal intensity achieved by using 100 μg of RNA from uninduced K562 cells electroporated with pSRMCatPΔF.M (lane 4) is comparable to the intensity from 10 μg of RNA isolated from the corresponding TPA-induced culture (lane 2). (C) Sequence in the pSRMCatPΔF.M construct where the RNA start sites (vertical arrows) were mapped. The consensus cap site is shown.

high levels of induction in the absence of the silencer element. Induced activity declined more than fivefold when the deletions included sequences from 301 to 306 bp downstream of the *Eco*RI site (Ex 14-2 to 15-2/1, 73 to 68 bp upstream of the TATA box; Fig. 4 and 5) and dropped to background levels when the deletions extended to position 345 (Ex 17-1, Fig. 4 and 5). Thus, by deletion mapping we were able to localize a positive regulatory region (+ symbol in Fig. 4) between 301 and 344 bp downstream of the *Eco*RI site.

**Sequence elements required for PDGF-2 promoter stimulation by TPA differ from the target sequence of the AP-1 transcriptional factor.** In order to confirm our conclusions, we created deletions mutant generated by site-directed mutagenesis with synthetic oligonucleotides. Such mutants retained all the sequences in pSRMCatPΔF.M but carried deletions in the positive regulatory region. As shown in Fig. 5, deletion of sequences from 75 to 45 bp upstream of the TATA box (Δ4 versus Δ3 in Fig. 5) led to a threefold reduction in inducibility, and further deletions downstream (Δ2) reduced both uninduced and induced activity to nearly background levels. The RNA start site was not altered in PDGF-2/*cat* RNA encoded by this deletion mutant (Δ3), as tested by primer extension analysis (Fig. 3B, lane 6).

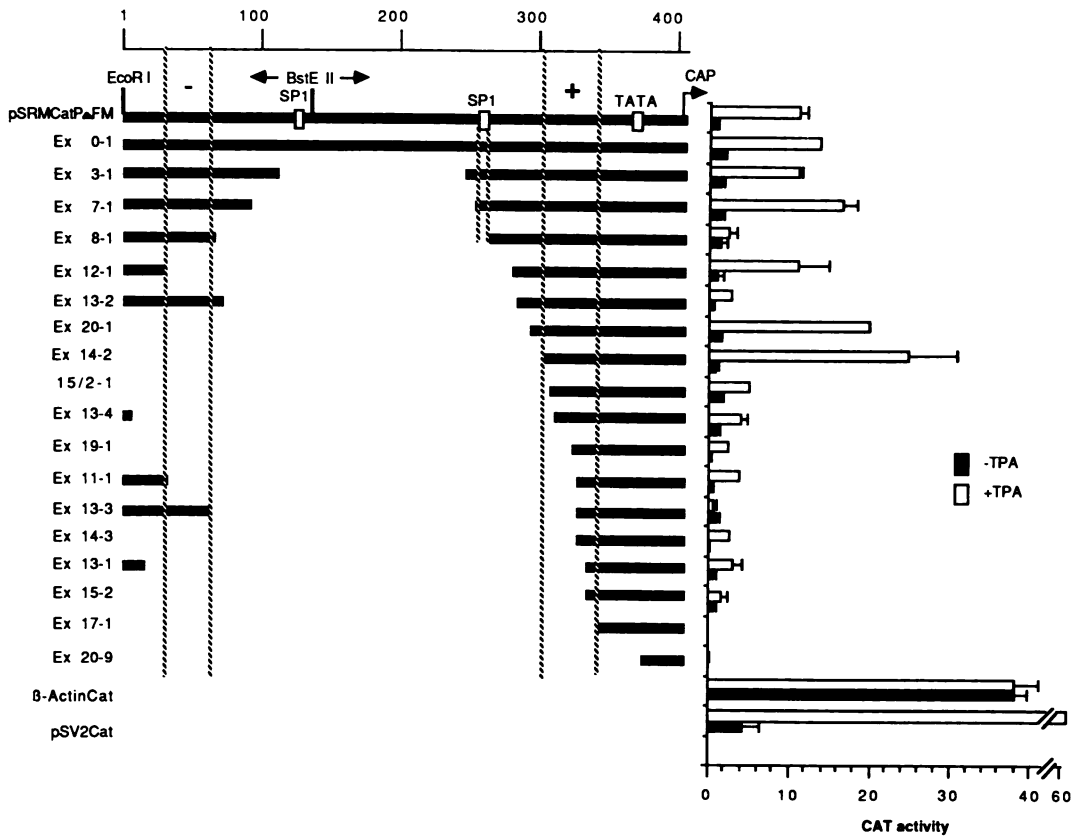


FIG. 4. Deletion mapping of PDGF-2 promoter activity. The deletion mutants generated from *Bst*EII-linearized pSRMCatPΔF.M with exonuclease III (Ex 0-1 to Ex 20-9) or Bal31 (mutant 15/2-1) were electroporated together with pCH110 DNA into K562 cells. Cultures were split into two portions, one of which was treated with TPA. The amount of extracts used for determination of CAT activity was standardized by measuring β-galactosidase activity (see Materials and Methods). Calculations of CAT activities for each deletion mutant were based on the activity determined after electroporation of pSRMCatPΔF.M into uninduced K562 cells. Thus, the CAT activity of pSRMCatPΔF.M in uninduced K562 cells was defined as 1 U. CAT activities of deletion mutants which were tested in more than two independent experiments are shown with their standard errors. Ex 14-2 and Ex 15-2 were tested in seven independent experiments for their activity in uninduced K562 cells and in four independent experiments in TPA-induced cells. The induced activity of Ex 14-2 (24.8 ± 6.9 U) is at a probability level of $P < 0.01$ (Student's $t$ test) larger than the induced activity of Ex 15-2 (1.8 ± 0.4 U), whereas the difference in uninduced activities is not statistically significant ($P = 0.05$). The *Eco*RI-*Fsp*I restriction fragment including the Sp1-binding sites, TATA signal sequence, and cap site within the parental plasmid is represented by a solid bar. The extent of the deletion within different mutants is indicated by the size of the gap. Ex 0-1 is identical in its sequence to the parent plasmid. PSV2Cat was strongly induced by TPA, whereas β-ActinCat showed identical activities in induced and uninduced cells. Regions defined by deletion analysis to exert a positive (+) or negative (−) effect on induced PDGF-2 promoter activity are demarcated by vertical lines. Deletion of only one of two hypothetical Sp1-binding sites also decreased induced promoter activity. The scale refers to fragment length in nucleotides.

Recent studies have identified a consensus sequence that is the target for activation protein 1 (AP-1), seems to regulate the activities of certain promoters in cells treated with phorbol esters (4, 24). We found a sequence homologous to this consensus sequence, TGAGTCAG (4) or TGACTCA (24), within the positive regulatory region. It was located 62 to 55 bp upstream of the TATA box (sequence TGACTCCG at positions 312 to 319, Fig. 5). Deletion of this region, however, did not change the promoter activity in uninduced or TPA-induced K562 cells (Δ5 in Fig. 5). Thus, this sequence element, which could serve as a target sequence for AP-1, is not required for PDGF-2 promoter activity.

**PDGF-2 upstream promoter region is a target for nuclear factors expressed in differentiated K562 cells.** To analyze whether nuclear factors specifically bound to the PDGF-2 upstream promoter region in uninduced and induced K562 cells, we used oligonucleotide retardation electrophoresis. The abundance and specificity of nuclear factors binding to the PDGF-2 upstream promoter region were investigated in gel retardation experiments. An oligonucleotide spanning the region from positions 301 to 344 (73 to 30 bp upstream of the TATA box) was retarded in 11% polyacrylamide gels after binding to nuclear extracts from K562 cells. The gel retardation was observed even at a $10^5$-fold excess of the nonspecific competitor poly(dI-dC) · poly(dI-dC). This activity was higher in cells induced with TPA than in untreated cells and could be competed with by the identical unlabeled oligonucleotide but not by unrelated DNA (Fig. 6A). Lowering the poly(dI-dC) · poly(dI-dC) concentration unmasked two other specific oligonucleotide-nuclear protein interactions which could be detected at lower polyacrylamide concentrations (Fig. 6B). The specificity of these interactions was confirmed by competition experiments (Fig. 6B). The abundance of these two complexes did not seem to change after TPA induction. Thus, interaction of the PDGF-2 upstream promoter region with nuclear factors from K562 cells could generate three different complexes, one of which was more abundant after TPA-mediated differentiation. That

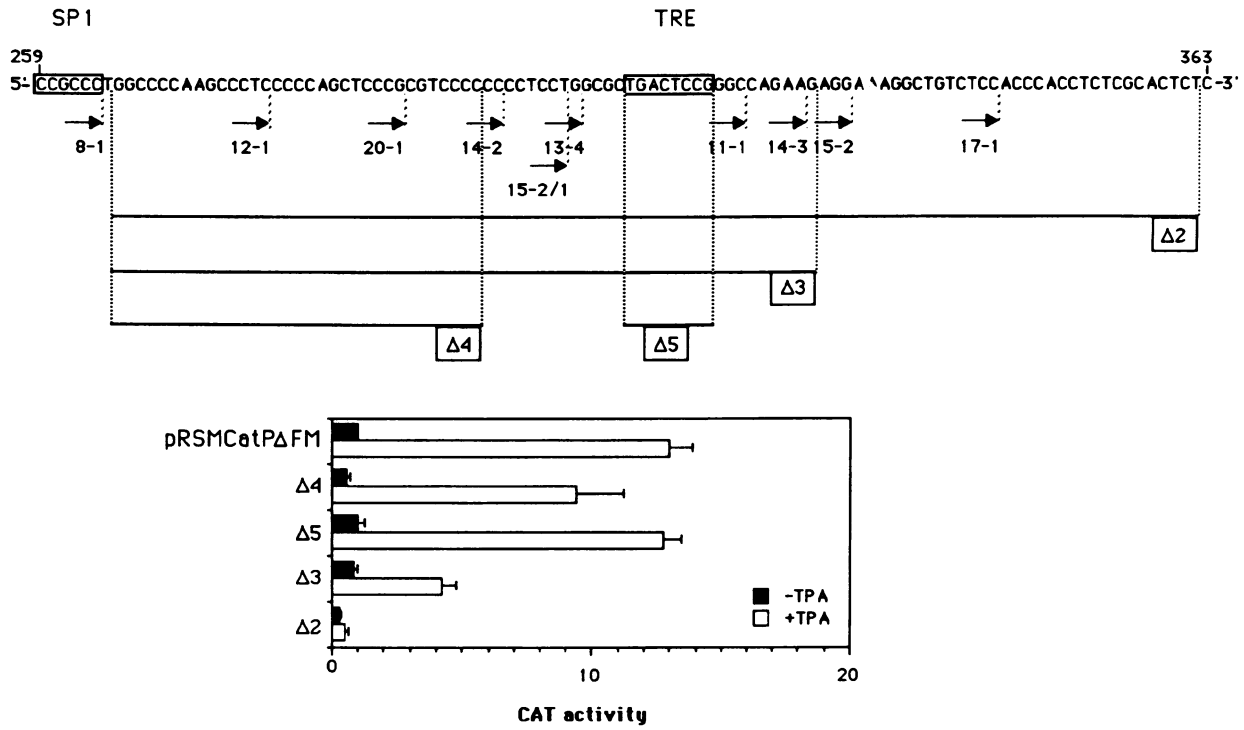


FIG. 5. CAT activities of PDGF-2 promoter mutants generated by site-directed mutagenesis. Electroporation and determination of PDGF-2 promoter activities were performed as described in the legend to Fig. 4 and Materials and Methods. Deletions generated by site-directed mutagenesis are indicated by horizontal bars below a section of the PDGF-2 promoter sequence. The 3' deletion endpoints of some of the mutants shown in Fig. 4 are also illustrated. Sequences homologous to the binding sites for the transcriptional factor Sp1 (SP1) or AP-1 (TRE) are boxed. Nucleotide positions are given relative to the *Eco*RI site.

different nuclear proteins can bind to one short promoter element is well documented (39).

**Role of the positive regulatory region in PDGF-2 promoter expression in normal and tumor cells.** We wanted to investigate the possiblity that the presence of PDGF-2 mRNA in some human tumor cells might be due to transcriptional activation involving the identified upstream promoter region. Thus, deletion mutants Ex 14-2 and Ex 15-2 (Fig. 4 and 5) were introduced by electroporation into normal fibroblasts (M413 cells) and four different human tumor cell lines which have been shown to express different levels of PDGF-2 mRNA (13, 20). As shown in Table 1, deletion of the sequence between 73 and 42 bp upstream of the TATA box had nearly identical effects on PDGF-2 promoter activity in human fibroblasts as well as in the glioblastoma and fibrosarcoma cell lines independent of whether they expressed PDGF-2 mRNA. In one line, the EJ bladder carcinoma cell line, there was a significantly reduced level of promoter activity associated with the construct in which the positive regulatory region had been deleted. Thus, the higher level of PDGF mRNA in EJ cells (20) appears to correlate with the ability of EJ cells to *trans*-activate the target sequences between 73 and 42 bp upstream of the TATA signal in the PDGF-2 promoter.

TABLE 1. Enhancement of PDGF-2 promoter activity in normal and tumor cells by an upstream promoter region

| Cell line | PDGF-2 RNA expression | CAT activity[a] (% chloramphenicol conversion) | | Relative increase in activity[b] (fold) |
|---|---|---|---|---|
| | | Ex 15-2 | Ex 14-2 | |
| M413 (fibroblasts) | − | 21 | 85 | 4 |
| A 1235 (glioblastoma) | − | 3.5 | 15 | 4 |
| 8387 (fibrosarcoma) | + | 0.5 | 3 | 7 |
| A 2781 (glioblastoma) | + | 0.4 | 1.3 | 3 |
| EJ (bladder carcinoma) | ++ | 0.7 | 21 | 30 |

[a] Ex 15-2 or Ex 14-2 was electroporated together with pCH110 (see Materials and Methods). Efficiencies of plasmid uptake and cell survival show large differences for each cell line but are standardized (with pCH110) for electroporations into the same cell line.

[b] Relative increase in promoter activity in Ex 14-2 compared with Ex 15-2.

## DISCUSSION

PDGF-2 expression appears to be tightly controlled, since only a few differentiated tissues normally express its transcript (37). In fact, there is accumulating evidence that expression of PDGF-2 in cells possessing the cognate receptor may play a role in the development of certain malignancies (35). Our present report describes the initial functional analysis of the PDGF-2 promoter region. For these studies, we used the K562 cell line, which differentiates along the megakaryocyte pathway following TPA treatment associated with activation of PDGF-2 mRNA expression (3, 10). Our studies defined the early kinetics of induction in which, over a 24-h period, the levels of PDGF-2 mRNA increased more than 200-fold above an undetectable level of less than 1 molecule per ng of total cellular RNA.

Using deletion mutagenesis and the *cat* reporter gene, we identified a minimum region of 42 bp upstream of the TATA box which was required for promoter activity in uninduced cells (Fig. 7). In addition, two upstream regions which

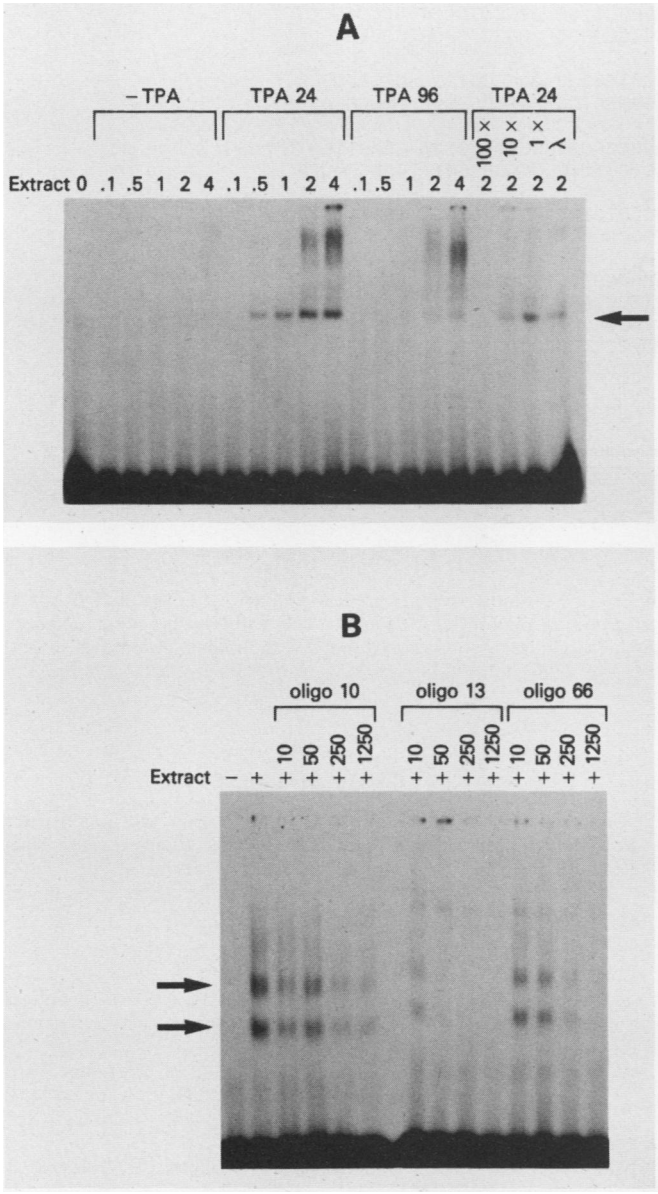


FIG. 6. Binding of nuclear proteins to PDGF-2 promoter elements. Nuclear extracts of K562 cells were prepared as described in Materials and Methods and used for gel retardation experiments. (A) Autoradiogram of an 11% acrylamide gel with the gel retardation of double-stranded oligonucleotide 13 (positions 301 to 344, Fig. 5). Poly(dI-dC) · poly(dI-dC) (2 μg) was incubated with increasing amounts of extracts prepared from uninduced K562 cells or the same cells treated for 24 or 96 h with TPA, and 2 μg of extracts from K562 cells induced for 24 h with TPA were also incubated with a 1- to 100-fold molar excess of unlabeled oligonucleotide 13 or with 1 μg of lambda DNA. Then, 1 fmol of labeled oligonucleotide 13 was added. The retardation of oligonucleotide 13 is most prominent with extracts from cells induced for 24 h and can be competed with by unlabeled oligonucleotide 13 but not by lambda DNA. (B) Autoradiogram of a 7% acrylamide gel in which two protein-oligonucleotide 13 complexes can be observed in the presence of 70 ng of poly(dI-dC) · poly(dI-dC). The amounts of oligonucleotides added for competition are given in femtomoles. The protein binding to 10 fmol of labeled oligonucleotide 13 is competed with by a fivefold excess of unlabeled oligonucleotide 13, in contrast to a more than 100-fold excess of the unrelated oligonucleotide 66 or oligonucleotide 10 necessary for a signal reduction.

affected TPA inducibility were identified by deletion analysis. A transcriptional silencer exerted a negative effect on TPA inducibility; a positive regulatory region was also identified (Fig. 7).

The silencer was further shown to exert its inhibitory effect when fused to sequences within the positive regulatory region. This silencer contains part of the sequence CTG AGTCCC which was homologous to the sequence CTGA CTCCG located within the positive regulatory region. This raises the possiblity that homologous sequences may be targets for identical or related transcriptional factors exerting positive as well as negative effects on promoter activity. The consensus sequence for the target of the transcriptional factor AP-1 is TGAGTCAG or TGACTCA (4, 24). Deletion of the sequence TGACTCCG within the positive regulatory element did not lead to a decrease in promoter activity. Thus, AP-1 is probably not responsible for the stimulation of PDGF-2 promoter activity by TPA at the phorbol ester concentrations used to induce K562 differentiation. However, if AP-1 can bind to sequences other than the consensus sequences which have been reported so far (4, 24), this transcriptional factor could still be involved in the activation of PDGF-2 expression.

We detected binding of nuclear factors to a sequence 75 to 45 bp upstream of the TATA box. Since deletion of the octamer TGACTCCG had no effect on promoter activity and extending the 5′ deletions from position 301 to 306 (Fig. 4 and 5) led to a significant decline in inducibility, one target element for *trans*-activation or an essential part of it is likely to be contained within the sequence CCTCCTGGCGC between positions 301 and 311 (sequence 1, Fig. 7). This element was part of oligonucleotide 13, used in the gel retardation assays. The sequence is located at the beginning of a stretch of 100 bp encompassing the TATA signal sequence and the cap site. This stretch shows 98% sequence identity with the 5′ sequence of the feline c-*sis*/PDGF-2 proto-oncogene (42), which points to its functional significance. While deletion of the positive regulatory region reduced the TPA inducibility of the reporter gene about eightfold when comparing mutant Ex 14-2 with Ex 15-2, it led to only a threefold reduction when comparing mutant Δ4 with Δ3. The latter were generated by site-directed mutagenesis, whereas the former had all the other upstream sequences deleted as well. Thus, the absence of the silencer region is likely to increase the effect of deleting the positive regulatory element. Deletion of another 13 bp downstream (sequence 2, Fig. 7) reduced induced and uninduced promoter activity to nearly background levels. Thus, sequences essential for PDGF-2 promoter activity must be located within those 13 bp. Although this initial analysis of the PDGF-2 promoter identifies promoter elements which are important for PDGF-2 gene regulation in megakaryocytes, we do not know whether additional elements are also required. The correlation between the different oligonucleotide-protein complexes and promoter activity in vivo has to be evaluated further. Introducing point mutations into the regulatory sequence elements and evaluating their effects on protein binding and promoter activity should aid in this characterization.

While the PDGF-2 promoter could be activated about 10-fold as measured by CAT assays in K562 cells, the increase in endogenous PDGF-2 mRNA was much greater. Moreover, while the promoter showed a readily detectable baseline activity in CAT assays in uninduced K562 cells, no endogenous PDGF-2 mRNA was detectable in those cells. That CAT expression was not due to aberrantly initiated

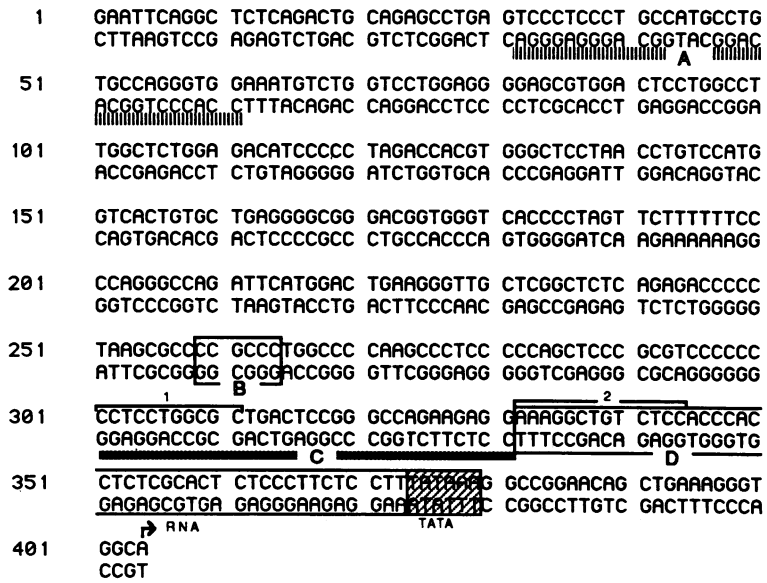


FIG. 7. PDGF-2 promoter elements. Sequences involved in regulating PDGF-2 promoter activity are the silencer region (A), the postulated Sp1-binding site (B), the positive regulatory region (C), and the minimal promoter region (D). Sequences delineated by brackets indicate a positive regulatory element (labeled 1) and the region (labeled 2) beyond which deletions cannot be extended without losing promoter activity in uninduced and induced K562 cells. The TATA box is shaded.

transcripts was demonstrated in primer extension assays. Although it is difficult to compare directly promoter activities based on CAT assays with endogenous mRNA levels, the differences observed were large enough that it seems very likely that the PDGF-2 promoter is constitutively active in uninduced K562 cells. Thus, other levels of regulation must exist. The PDGF-2 transcript might be either terminated prematurely, as has been demonstrated for c-*myc* mRNA expression (6), or degraded very rapidly. If pre-mRNA degradation were to constitute a second level of regulation, it is unlikely to be controlled by a labile protein in K562 cells, since the PDGF-2 transcript could not be superinduced by CHX. Nuclear runoff experiments with K562 nuclei should help to differentiate between these possibilities.

There is evidence of dysregulation of PDGF-2 RNA expression in a majority of human fibrosarcomas and glioblastomas (13, 20). Measurement of PDGF-2 transcripts in normal human fibroblasts indicates levels at least 200- to 1,000-fold lower than observed in TPA-induced K562 cells or under steady-state conditions in normal human endothelial cells and some human glioblastomas and fibrosarcomas (M. Pech, unpublished). Yet human fibroblasts as well as uninduced K562 cells expressed the *cat* reporter gene driven by the minimal PDGF-2 promoter. In one of several tumor cell lines examined, the positive regulatory region identified by us conferred a significant enhancement in reporter gene activity, consistent with the fact that this cell line constitutively expresses high levels of PDGF-2 mRNA. These findings support the concept that alterations in tumor cells can enhance PDGF-2 expression by means of *trans*-acting factors that interact with this element. However, our findings strongly argue that PDGF-2 gene expression in fibroblasts and uninduced K562 cells is also regulated beyond promoter control. Evaluation of this level of control will be critical in determining the mechanism which causes PDGF-2 mRNA to be expressed under most conditions in human malignancies.

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