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Promoter region of the human platelet-derived growth factor A-chain gene

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ABSTRACT **The platelet-derived growth factor (PDGF) A- and B-chain genes are widely expressed in mammalian tissues and their homodimeric gene products appear to regulate the autocrine growth of both normal and transformed cells. In this study, we analyzed the 5′ flanking sequences of the human PDGF A-chain gene to seek elements important to regulating its transcription. The promoter region was exceptionally G+C-rich and contained a "TATA box" but no "CAAT box." The transcription start site was identified 845 base pairs 5′ to the translation initiation site by S1 nuclease mapping and by primer extension. Both *in vitro* transcription and transient expression of the chloramphenicol acetyltransferase gene linked to the PDGF A-chain 5′ flanking sequences established that the putative promoter region was active, and RNase H mapping established that the three characteristic mRNAs (1.9, 2.3, and 2.8 kilobases) used the same transcription start site, which was used in normal endothelial cells and in two human tumor cell lines that express high levels of A-chain transcripts. The results established an exceptionally G+C-rich promoter region and a single transcription start site active for each of the three mRNAs of the PDGF A-chain gene. DNA sites of potential importance in mediating the activation of the PDGF A-chain gene in normal cells and in transformed cell lines expressing high levels of PDGF A chain were identified.**

Platelet-derived growth factor (PDGF) is a 30-kDa heterodimeric glycoprotein composed of A and B chains linked by disulfide bonds. It is the major mitogen in serum for mesodermal tissues and is a potent chemoattractant for neutrophils, monocytes, fibroblasts, and smooth muscle cells (1). The PDGF A-chain cDNA was obtained from human glioma (2) and endothelial cell (3, 4) cDNA libraries. Its transcripts have been detected in normal human endothelial cells, smooth muscle cells, fibroblasts, placenta, and certain tumor cell lines (2); particularly high levels are found in glioblastoma, osteosarcoma, and rhabdomyosarcoma cell lines. Levels of PDGF A-chain mRNA are increased in cells that are stimulated by PDGF and epidermal growth factor (5). However, the regulation of expression of the A- and B-chain genes differs in response to ligands that induce a mitogenic response (3–8). Genomic clones of the A chain have been isolated and sequenced (9). At least seven exons and a significant number of putative regulatory elements in sequences upstream of the putative "TATA box" were observed. However, the promoter region has not been definitively identified or characterized and, importantly, it is unknown whether the three characteristic RNA transcripts of 1.9, 2.3, and 2.8 kilobases (kb) arise at different or the same start site. We have now analyzed the transcription initiation site and promoter region of the PDGF A-chain gene to seek the sites of transcriptional regulation in normal cells and in tumor cells with high levels of expression.¶

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MATERIALS AND METHODS

Cells. Human glioblastoma (A172), human embryonal rhabdomyosarcoma (RD), and human epithelioid carcinoma (HeLa) cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Human umbilical vein endothelial cells were grown in Hanks' modified medium with 10% fetal bovine serum and endothelial cell growth supplements.

Cloning and DNA Sequencing. A human leukocyte genomic library in phage EMBL3 (Clontech) was screened by plaque hybridization (10) with a synthetic oligonucleotide (A-2) corresponding to nucleotides 389–424 of the D-1 PDGF A-chain cDNA clone (2). A 3.5-kb *Bam*HI–*Bam*HI fragment of clone λ40 that hybridized with A-2 in Southern blots was isolated and subcloned into a pUC19 vector (B2). Small fragments from B2 were obtained by restriction enzyme digestion, subcloned into a pBluescript vector (Stratagene), and sequenced by the dideoxy chain-termination method.

Plasmid Construction. The *Bam*HI–*Sau*3A1 fragment (−506 to +387; Fig. 1) was subcloned into pBluescript (Sau840) and digested with *Xba* I. The free ends were filled, ligated to *Hin*dIII linkers, and digested with *Hin*dIII. The *Hin*dIII fragment was inserted in the *Hin*dIII site of pSV0cat (12) and the orientation was verified by restriction enzyme analysis.

Northern Blot Analysis. Total cellular RNA was isolated for Northern blots (10). *Xho* I–*Sau*3A1, *Bgl* I–*Taq* I, and *Pst* I–*Apa* I fragments (Fig. 1) were used as probes. The probes were labeled with [α-^{32}P]dCTP by nick-translation or random primer labeling.

S1 Nuclease Protection Assays. Total cellular RNA (20 μg) was coprecipitated in ethanol with the end-labeled *Xho* I–*Sau*3A1 fragment (10^5 cpm) (−253 to +387, Fig. 1), suspended in 15 μl of hybridization buffer (80% formamide/40 mM Pipes, pH 6.4/400 mM NaCl/1 mM EDTA), heated for 15 min at 90°C, and hybridized overnight at the designated temperatures (see Fig. 3*a*). The DNA·RNA hybrid was diluted into 300 μl of S1 nuclease buffer (280 mM NaCl/30 mM NaOAc, pH 4.4/4.5 mM $Zn(OAc)_2$ with sonicated salmon sperm DNA at 20 μg/ml) containing 200 units of S1 nuclease and was incubated at 37°C for 30 min. The digestion was terminated with 75 μl of 2.5 M NH_4OAc/50 mM EDTA, and the protected fragments were recovered by ethanol precipitation, denatured, and analyzed by electrophoresis in a 6% polyacrylamide sequencing gel.

Primer Extension Assays. Thirty micrograms of total cellular RNA was hybridized with an end-labeled oligonucleo-

Abbreviations: PDGF, platelet-derived growth factor; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M59423).

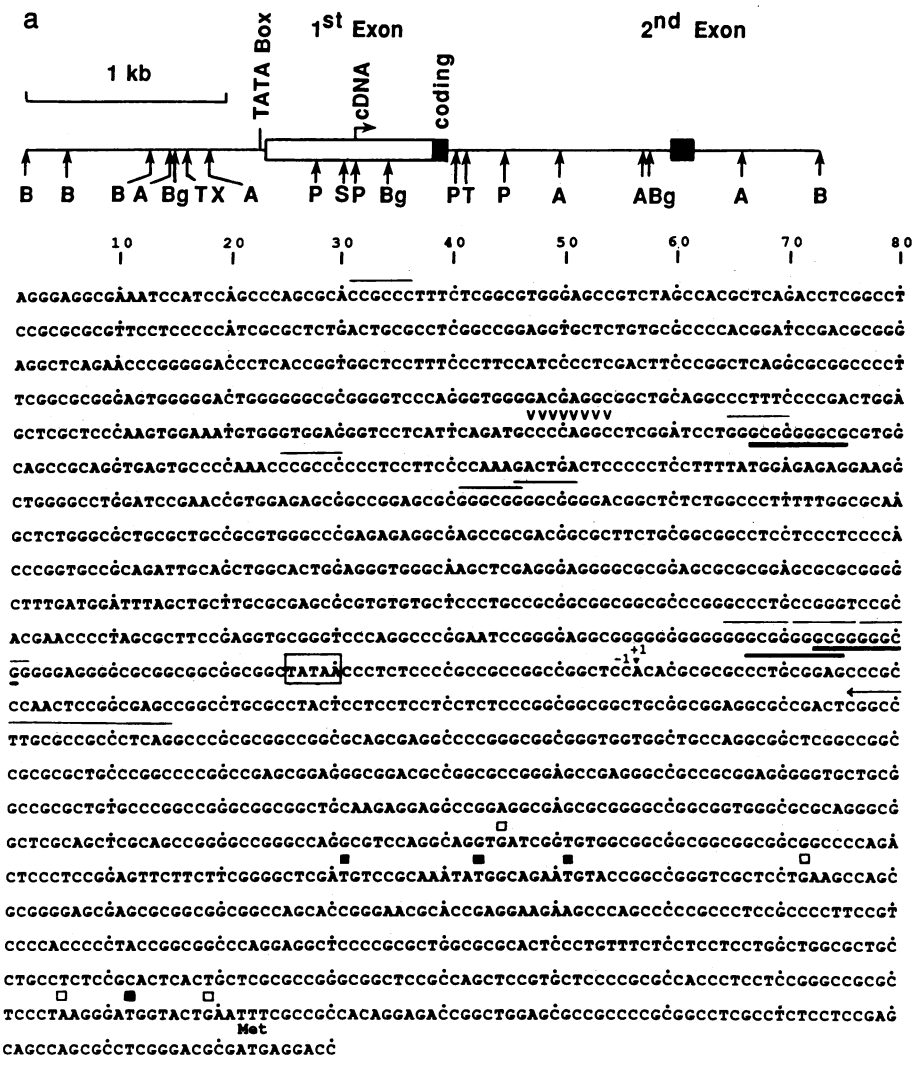

FIG. 1. Nucleotide sequence of the 5′ flanking regions of the human PDGF A-chain gene. The 3.5-kb *Bam*HI–*Bam*HI fragment was subcloned into pUC19 vector and a sequence extending upstream of PDGF A-chain cDNA was obtained by the dideoxy chain-termination method (11) after subcloning into pBluescript vector. For the purposes of analysis, a portion of previously published (9) 5′ untranslated cDNA sequence has been included. (*a*) Restriction endonuclease cleavage map of the subcloned genomic fragments of the promoter and two exon regions is also shown. A, *Apa* I; B, *Bam*HI; Bg, *Bgl* II; P, *Pst* I; S, *Sau*3A1; T, *Taq* I; X, *Xho* I. (*b*) Portion of 5′ flanking region of PDGF A-chain sequence ending at the translation initiation site. The 5′ start site of the human PDGF A-chain as determined by S1 nuclease mapping and primer extension is indicated by a triangle and designated as +1. Potential Sp1 binding sites are overlined. The consensus sequence of NGFI-A is more heavily underlined. Arrowheads indicate the binding site of AP-2. Filled squares indicate initiation codons and open squares indicate termination codons. Arrow underscores the 20-nucleotide primer used for primer extension.

tide primer (10^6 cpm) corresponding to positions +99 to +119 of the PDGF A-chain genomic DNA. The probe was precipitated with total RNA, resuspended in hybridization buffer (as in S1 nuclease protection, above), heated at 90°C for 15 min, and hybridized at 50°C overnight. The samples were precipitated in ethanol, suspended in 50 μl of 50 mM Tris·HCl, pH 8.3/75 mM KCl/3 mM $MgCl_2$/10 mM dithiothreitol/0.5 mM dNTP with bovine serum albumin (100 μg/ml), actinomycin D (50 μg/ml), RNasin (800 units/ml; Promega), and Moloney murine leukemia virus reverse transcriptase (8000 units/ml), incubated for 2 hr at 42°C, extracted with phenol/chloroform, and analyzed in 6% sequencing gels.

***In Vitro* Transcription.** Nuclear proteins were extracted from HeLa cells and *in vitro* transcription was done as described (13). The *Bam*HI–*Sau*3A1 pSV0cat 5′ → 3′ plasmid was used as template DNA. After incubation, 0.2 μg of RNase-free DNase I (BRL) was added. After 5 min more at 30°C, 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl, 0.1 M 2-mercaptoethanol, and 0.25 M NaOAc (pH 5.0) were added and the samples were incubated for 30 min at 37°C, extracted with equal volumes of ice-cold phenol saturated with distilled water and with 0.2 volume of chloroform/isoamyl alcohol (49:1, vol/vol). After ethanol precipitation, the transcripts were analyzed with the S1 protection assay.

DNA Transfection and Chloramphenicol Acetyltransferase (CAT) Assay. About 24 hr before transfection, RD cells were seeded at 5.5×10^5 per 100-mm Petri dish. Twenty micrograms of the *Bam*HI–*Sau*3A1 pSV0cat plasmid was transfected (14). After 3 hr, the cells were shocked by 15% (vol/vol) glycerol in 20 mM Hepes buffer for 3 min, washed, incubated for 48 hr, and harvested, and lysates were assayed for CAT activity (12). pA_{10}cat, which contains only the simian virus 40 (SV40) early promoter, pSV2cat, which contains both the SV40 early promoter and the SV40 enhancer region (12), and sis-CAT, which contains the PDGF B-chain promoter region (15), were used as controls.

RNase H Mapping Assay. Twenty micrograms of total cellular RNA was hybridized to 100 ng of an *Apa* I–*Apa* I fragment (see Fig. 7) in 15 μl of hybridization buffer (see S1 nuclease mapping, above). After incubation at 90°C for 15 min and at 57°C overnight, the DNA·RNA hybrids were precipitated in ethanol, suspended in 200 μl of 20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/100 mM KCl/0.1 mM dithiothreitol/5% sucrose, and digested with 6 units of RNase H (BRL) at 37°C. After 30 min, EDTA was added (10 mM) and the RNA was extracted with phenol, precipitated with ethanol, and subjected to Northern blot analysis (as described above) using a ^{32}P-labeled *Taq* I–*Taq* I fragment as the probe (see Fig. 7).

RESULTS

DNA Sequence of the Promoter Region. Four genomic clones (λ21, λ24, λ31, and λ40) that contained the 5′ end of the PDGF A-chain gene were isolated from the human leukocyte genomic DNA library as described above. Three clones were overlapping. Clones λ24, λ31, and λ40 contained the same fragment. An insert of 3.5 kb was isolated from λ40

and subcloned into pUC19 and pBluescript vectors and sequenced (11). The sequence obtained included 1392 nucleotides upstream and 2498 nucleotides downstream of the 5′ end of the human PDGF A-chain cDNA (2). The sequence obtained (Fig. 1*b*) was in complete agreement with that previously reported (9) and included an additional 630 nucleotides at the 5′ end. Several notable features were observed 5′ to the transcription initiation site (+1). The sequence TATAA beginning at −31 matches the consensus TATA-box sequence. The sequence surrounding the TATA box (residues −92 to −32 and −26 to −1) has a G+C content of 91%. Eight hexanucleotide repeats (CCGCCC or GGGCGG) that correspond to consensus binding sites for the transcription factor Sp1 (16) were found (−906, −552, −512, −416, −411, −72, −66, and −60). Consensus sequences corresponding to the binding site of NGFI-A (also known as Zif 268; ref. 17), a PDGF-inducible gene product, were found at position −550 and in overlapping sequences at −70 and −64, in close proximity to the TATA box. A consensus sequence for binding of AP-2 was identified at position −570.

Site of Transcription Initiation. The *Bgl* I–*Taq* I fragment that contained the sequence encoding the putative signal peptide and the *Xho* I–*Sau*3A1 fragment upstream of the cDNA start site (Fig. 1*a*) were used for Northern blot analyses. Hybridization (Fig. 2) to each of the three appropriate mRNAs (1.9, 2.3, and 2.8 kb) was observed. Fragments beginning 7 kb upstream of the *Xho* I–*Sau*3A1 fragment did not hybridize with the mRNAs (data not shown). The hybridization above 2.8 kb in these blots corresponded with the migration of 28S rRNA and presumably reflected the G+C-rich region of the *Xho* I–*Sau*3A1 fragment. An identical band was found when a synthetic G+C-rich oligomer was tested (data not shown), whereas the *Pst* I–*Apa* I fragment (Fig. 1*a*) that contains the first intron also failed to hybridize with mRNAs.

S1 nuclease protection and primer extension assays were used to identify the transcription initiation site(s). The 640-base-pair (bp) *Xho* I–*Sau*3A1 fragment (−253 to +387, Fig. 1) was 5′-end-labeled prior to digestion with *Xho* I, isolated from the agarose gel, hybridized to mRNA from RD, A172, and endothelial cells, and digested with S1 nuclease. A single band of about 400 bases was identified with RNA from each of the cell lines when this probe was used in Northern blot analyses (Fig. 3*a*), suggesting strongly that all of the three different transcripts (see above) were initiated from the same site in each of the three cell lines. Hybridizations for S1 mapping required very high temperatures, suggesting significant secondary structure in this region, consistent with the very high G+C-rich sequences in the region around the TATA box. Confirmation of the transcription initiation site identified by S1 nuclease analyses was obtained by primer extension analysis. An extended product of 119 nucleotides was identified (Fig. 4) when the 20-base synthetic oligonucleotide (residues +99 to +118; Fig. 1) was used as primer. The site of initiation of transcription was identified as the adenine 26 bases downstream from the last adenine of the putative TATA box, 845 nucleotides upstream of the translation start site.

Activity of the Putative A-Chain Gene Promoter. *In vitro* transcription and CAT assays were used to establish that the sequences surrounding the TATA box were sufficient to promote gene transcription. When the *Bam*HI–*Sau*3A1 fragment was linked to pSV0cat as the template for *in vitro* transcription, a 400-base band was observed that was identical to the band found *in vivo* by an S1 nuclease protection assay (Fig. 5). This same plasmid was transfected into RD cells and the *Bam*HI–*Sau*3A1 fragment was fully adequate to drive CAT transcription *in vivo* (Fig. 6).

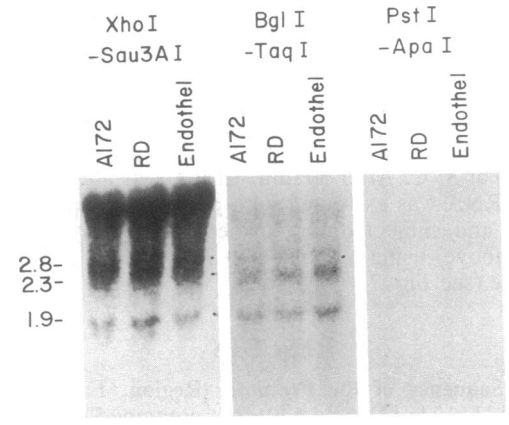

FIG. 2. Northern blot analysis of 20 μg of total RNA from A172 cells, RD cells, and endothelial cells. *Xho* I–*Sau*3A1, *Bgl* I–*Taq* I, and *Pst* I–*Apa* I fragments (see Figs. 1 and 7) were used as probes. RNA size is indicated at left in kilobases.

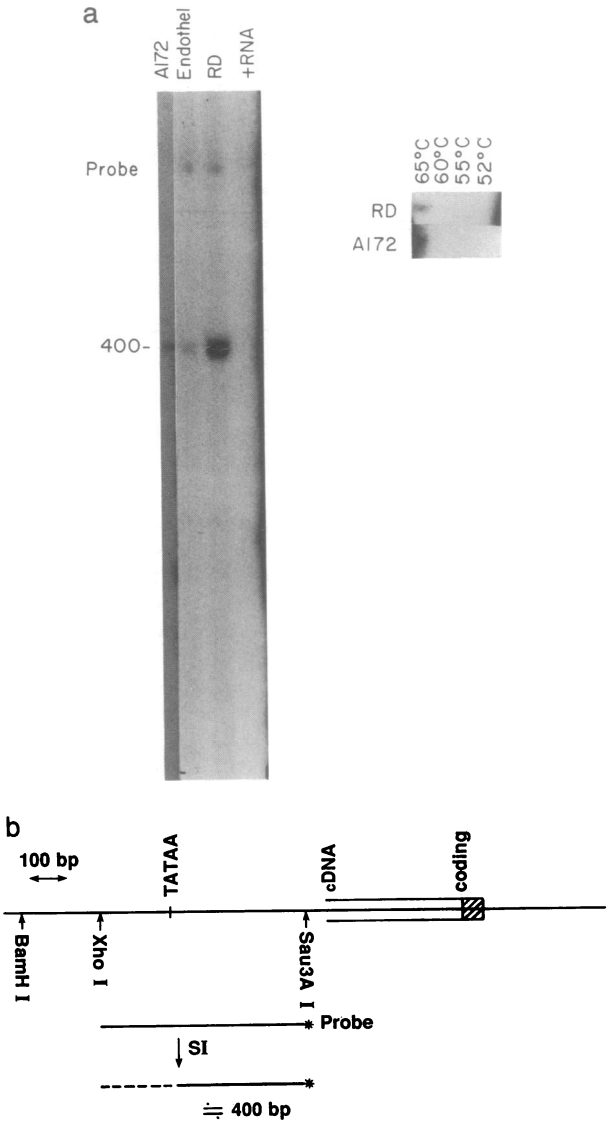

FIG. 3. (*a*) S1 nuclease mapping analysis. Twenty micrograms of total RNA from A172 cells, RD cells, or endothelial cells or 20 μg of yeast tRNA was hybridized with end-labeled *Sau*3AI–*Xho* 1 fragment. After S1 nuclease digestion, products were analyzed in a 6% polyacrylamide sequencing gel. Protected bands were detected only when hybridization temperatures were >60°C. (*b*) Probe used in S1 mapping analysis.

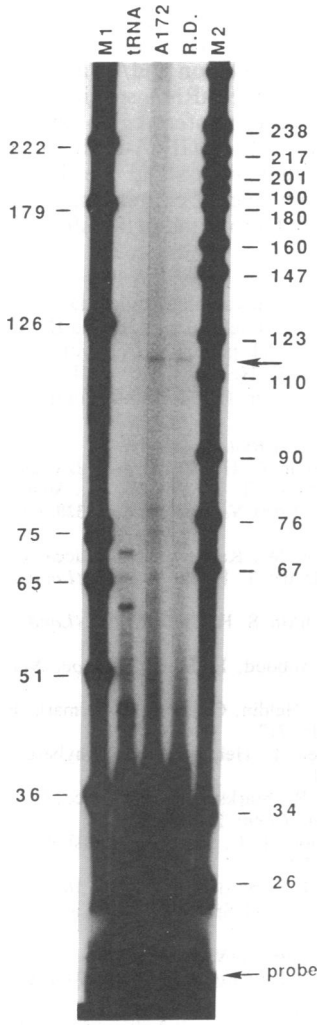

FIG. 4. Primer extension analysis. Thirty micrograms of total RNA from A172 cells or RD cells or 30 μg of yeast tRNA was hybridized with a 5'-end-labeled oligonucleotide primer. After incubation with reverse transcriptase, primer-extended products were analyzed in a 6% sequencing gel. Arrow indicates the position of the extended product. End-labeled, *Hin*fI-, *Ksa* I-, and *Sin* I-digested pGEM DNA (lane M1) and *Msp* I-digested pBR322 (lane M2) were used as size markers; lengths are indicated in base pairs.

RNase H Mapping of Initiation Site(s). RNase H mapping then was used to exclude the possibility that three different start sites were used to transcribe the three PDGF A-chain mRNA transcripts (1.9, 2.3, and 2.8 kb). After hybridization to DNA probes generated from the *Apa* I–*Apa* I fragment (Fig. 7*b*), the RNA was digested with RNase H. Northern blot analyses after RNase H digestion (Fig. 7*a*) indicated that the *Taq* I–*Taq* I fragment (Fig. 7*b*) hybridized to a 0.9-kb mRNA band that was not present before digestion. This 0.9-kb band corresponds precisely in length to the DNA sequence from the initiation site to the first splice site, indicating that each of the three mRNA species was transcribed from the same initiation site. As noted above, hybridization to the 28S rRNA also was observed above 2.8 kb.

DISCUSSION

PDGF and the homodimeric isoforms PDGF AA and PDGF BB are highly important for regulating both normal and abnormal cell growth and in the processes of inflammation and repair (1). Because the B- and A-chain genes are expressed in both normal and transformed cells and appear to be transcriptionally activated by different stimuli, we analyzed the site of initiation of transcription and the promoter region of the human PDGF A-chain gene. The major findings were the identification of the transcription start site at an adenine residue 845 bases upstream of the translation initiation site, consistent with consensus sequences for initiation of transcription of most eukaryotic mRNAs (18), and that transcription was initiated from the same site in normal

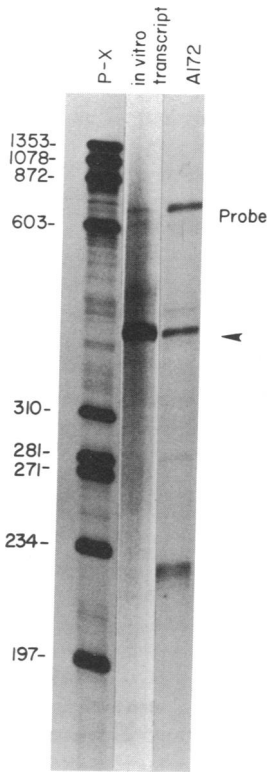

FIG. 5. S1 analysis of transcripts from *in vitro* transcription. *Bam*HI–*Sau*3A1 pSV0cat (5 → 3) was incubated with 300 μg of HeLa cell nuclear protein extract. Synthesized RNA was analyzed by S1 nuclease mapping. Twenty micrograms of total RNA from A172 cells was also analyzed by S1 nuclease mapping. End-labeled ϕX174 *Hae* III fragments (BRL) (lane P-X) were used as size markers.

human endothelial cells and in two human tumor cell lines (A172 and RD) that express very high levels of PDGF A-chain transcripts. The data also established that each of the three mRNAs uses the same promoter and initiation site. The first exon (908 bp) contained a long 5' untranslated region of 845 bp similar to that found in the PDGF B-chain gene (15, 19, 20), and each of the three PDGF A-chain mRNA species contained the first exon with the characteristic sequences encoding the signal peptide. The DNA sequences immediately

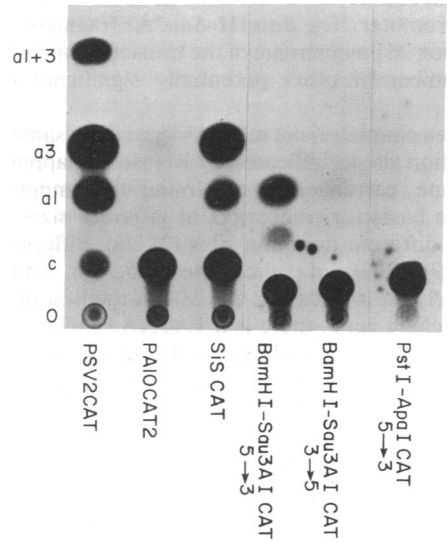

FIG. 6. CAT activity in RD cells transfected with 20 μg of various CAT plasmids. O, origin; c, chloramphenicol; a1 and a3, the two forms of monoacetylated chloramphenicol; a1+3, diacetylated chloramphenicol. After autoradiography, areas of the acetylated and nonacetylated chloramphenicol were extracted and measured in a liquid scintillation counter. The ratio of the acetylated form to the total was calculated as follows; pSV2cat, 94%; $pA_{10}cat_2$, <1%; sis-CAT, 24%; *Bam*HI–*Sau*3A1 CAT 5 → 3, 6.5%; *Bam*HI–*Sau*3A1 CAT 3 → 5, <1%; *Pst* I–*Apa* I CAT 5 → 3, <1%.

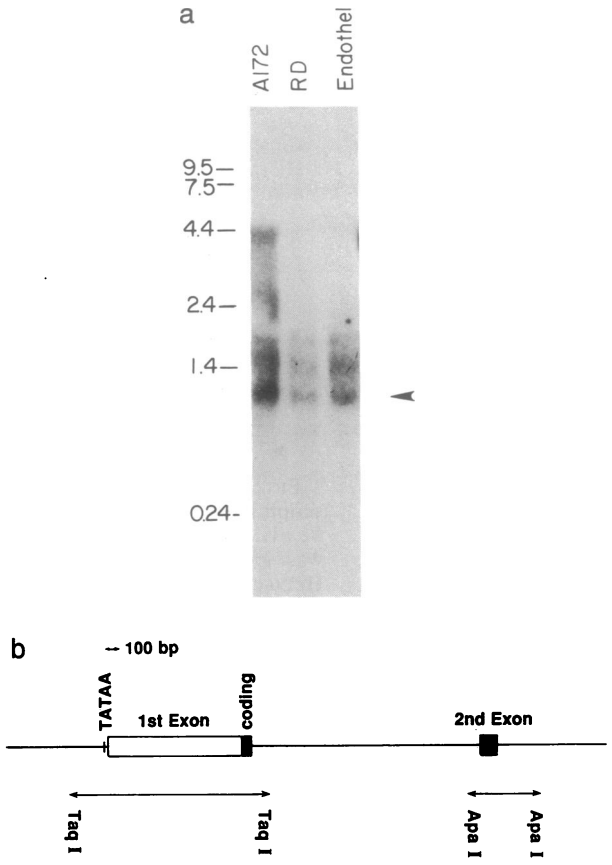

FIG. 7. (*a*) RNase H mapping. Twenty micrograms of total RNA from A172 cells, RD cells, or endothelial cells was hybridized with 100 ng of *Apa* I–*Apa* I fragment, digested with RNase H, and hybridized with ^{32}P-labeled *Taq* I–*Taq* I fragment. Arrowhead indicates the 0.9-kb band. (*b*) Probes used in RNase H mapping.

upstream of the initiation site of transcription have significant promoter activity when analyzed by *in vitro* transcription and by transient expression of the CAT gene driven by the PDGF A-chain promoter. The *Bam*HI–*Sau*3A1 fragment contains a TATAA box 26 bp upstream of the transcription initiation site and a number of other potentially significant regulatory elements.

The three characteristic mRNAs shared the same promoter and initiation site as indicated by RNase H mapping. Except for the gene rearrangements as found with immunoglobulin genes, the basis for transcripts of different sizes is varied, reflecting different initiation sites (21–26), different promoters (27–29), different splice sites (25, 28), and different polyadenylylation sites (21, 22, 28). In the first intron of the PDGF A-chain gene, there is a TATAA sequence about 250 bp upstream of the second exon and a consensus sequence for Sp1 binding (data not shown). A previously sequenced cDNA clone, 9.1 (5), begins in the first intron 150 bp upstream of the second exon and 100 bp downstream of the TATAA sequence. However, no transcript was detectable when RNA from endothelial, RD, or A172 cells was analyzed by hybridization to fragments that contained the first intron (Fig. 2), nor was any promoter activity detected in a CAT assay (Fig. 5) using the *Pst* I–*Apa* I fragment that contains the sequence from the first intron, suggesting that the previously isolated clone 9.1 represents an incompletely spliced nuclear precursor mRNA. In V-343 MGa cells (human glioblastoma), exon 6 (69 bp) was found in each of three mRNAs, whereas exon 6 was not observed in three mRNAs isolated from other cells (9). Rorsman *et al.* (9) suggested that several polyadenylylation sites are present in the 3′ flanking region of the A-chain gene and that alternative polyadenylylation and/or alternative splicing may account for the three mRNA species.

The identification of the active promoter region and 5′ upstream flanking sequences of the PDGF A-chain gene should provide the basis to further understand the tissue-specific activation and/or the high level of expression of the gene in cells stimulated by growth factors and in transformed cells.

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