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AN S1 NUCLEASE SENSITIVE REGION IN THE PDGF A-CHAIN GENE PROMOTER CONTAINS A POSITIVE TRANSCRIPTIONAL REGULATORY ELEMENT

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SUMMARY: Homodimers of the platelet-derived growth factor (PDGF) A-chain are strong mitogens for cells of mesenchymal origin and appear to be functionally important during development and perhaps in phenotypic transformation. In order to understand mechanisms of the developmental regulation of the PDGF A-chain gene and its dysregulation in transformation, we used S1 nuclease to identify and map an S1 hypersensitive region that is located 482 to 513 base pairs upstream of the transcription initiation site of the PDGF A-chain gene. A single nuclear protein binds to this site in gel mobility shift assays. This site confers a 2-3 fold increase in transcriptional activity when inserted into a heterologous promoter and analysed in transient transfection assays. The results suggest that this region of DNA under torsional stress locally assumes a single stranded character and functions to upregulate promoter activity.

Transcriptional regulation is influenced by a number of mechanisms that depend upon interactions of proteins and DNA (1). Many of these proteins have been identified through their binding to specific DNA sequences; however, DNA is a structurally dynamic macromolecule that adopts a number of conformations that may have important regulatory roles in biological processes, including transcription (2). Recently, evidence that poly(dG)-poly(dC) sequences function as negative regulators through their ability to adopt intramolecular triplex structures *in vivo* (3), supporting strongly the idea that alternating DNA conformations may contribute to transcriptional regulation.

Previously, we described several cis-acting transcriptional regulatory elements in the platelet-derived growth factor (PDGF) A-chain gene promoter that assume a conformation sensitive to S1 nuclease under

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torsional stress *in vitro* and demonstrated that these regions of DNA contribute to the transcriptional regulation of the PDGF A-chain gene (4-7). Here, we report the identification of a novel S1 hypersensitive region within the PDGF A-chain promoter that is located -482 to -513 base pairs relative to the transcription start site (+1). This region is specifically recognized by a nuclear protein and functions as a positive regulatory element.

MATERIALS AND METHODS

Plasmid DNA Manipulations. Plasmid pB2 was constructed by fusing the BamH I fragment of the PDGF A-chain gene (-442 to -553, Fig. 1) into the BamH I site of Bluescript (+KS) (Stratagene, San Diego, CA). The appropriate orientation was confirmed by DNA sequencing. Supercoiled plasmid DNA was isolated by alkaline lysis and purified twice by ultra-centrifugation through cesium chloride-ethidium bromide gradients (8).

The oligonucleotide used in gel mobility shift assay was cloned in both orientations into the Hind III site upstream or downstream Sal I site of pBLCAT2 in which the CAT gene was regulated by the herpes simplex virus (HSV) thymidine kinase (tk) promoter (9).

S1 Nuclease-Sensitive Assay. The supercoiled plasmid was digested with 0.05 unit of S1 nuclease/μg of DNA for 5 min at 37°C in low salt buffer as described (5). The nicked plasmid DNA was digested with Xba I, end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase or [α-^{32}P]dCTP and avian myeloblastosis virus reverse transcriptase at the 5' and 3' ends of both strands, secondarily digested with Hind III, and purified from 1% agarose gels. Control plasmid DNA was similarly prepared but not treated with S1 nuclease. Samples were analyzed in 6% sequencing gels parallel with a chemical sequencing ladder of the 3' end-labeled fragments as marker.

Preparation of Nuclear Extracts, Gel Mobility Shift Assays. Nuclear extracts were made from HeLa and A172 cells by methods of Dignam et al (10). Particulate materials were removed by centrifugation and the supernatants were applied to a heparin sepharose column in 0.1 M KCl HEMG buffer (25 mM HEPES (K^+), pH 7.5, 25 mM $MgCl_2$, 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol). The column was washed with loading buffer until the elutes contained less than 0.05 μg/ml protein (estimated by A_{280}) and the bound proteins were eluted with a linear gradient from 0.1 to 1 M KCl in HEMG buffer (pH 7.5). Fractions were pooled, dialyzed against 0.04 M KCl/HEMG, and frozen at -70°C for subsequent use.

Oligonucleotides were synthesized on an Applied Biosystem 380 B DNA synthesizer and end-labeled as previously described (5). The oligonucleotide used in this work is as follows: 5'-TCCTTCCCCAAA GACTGACTCC-3'.

DNA binding assays were carried out in 25-µl mixtures containing 2 or 4 µg of the 0.4 M KC1 heparin-agarose fraction of nuclear extracts from HeLa and A172 cells, 1-2 fmol of end-labeled oligonucleotide, and 2 µg of poly(dI-dC)•poly(dI-dC) in 25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 15 mM $MgCl_2$, for 30 min at room temperature. For competition assays, unlabeled oligonucleotides were mixed with the binding mixtures before addition of probes. Samples were fractionated on 7.5% polyacrylamide gels in 50 mM Tris-HCl, pH 8.0, 0.38 mM glycine buffer at 5-8 V/cm for 5-6 h and gels were dried and exposed to Kodak XAR-5 film at -70°C overnight for analysis.

DNA Transfection and CAT Assays. Plasmid DNAs were transfected into cells by calcium phosphate coprecipitation (11); 10 µg of plasmid DNA was precipitated with calcium phosphate and added to cultured cells that had been plated 24 h previously at $1x10^6$ cells/100-mm dish. After 48 h, extracts were prepared from the transfected cells, and CAT assays were performed by using acetyl-CoA and [^{14}C]chloramphenicol (Du Pont-New England Nuclear), as described by Gorman et al (12). Acetylated and nonacetylated chloramphenicol were separated by thin layer chromatography. After autoradiography, regions corresponding to acetylated and nonacetylated chloramphenicol were excised and quantitated by scintillation counting. The efficiency of transfections was normalized in cells co-transfected with an SV40 early promoter β-galactosidase plasmid (pCH110, Clonetech). β-Galactosidase activity in cell lysates was assayed as described (13).

RESULTS

We previously used S1 nuclease sensitivity to analyse a promoter fragment of the PDGF A-chain gene and reported that several transcription factor binding sites, including those for Sp1, NF-κB and the Serum Response Factor (SRF), assume a conformation that is sensitive to S1 nuclease when supercoiled in plasmids (4-7). Because evidence suggested that these S1 sensitive regions of DNA may be important in the transcriptional regulation of the PDGF A-chain gene, we tested an additional plasmid (pB2) containing a fragment of the PDGF A-chain promoter from -442 to -553 (Fig. 1). The supercoiled plasmid was treated with S1 nuclease, digested with restriction enzymes, end-labled with ^{32}P, and analyzed on a DNA sequencing gel. A prominent series of intense bands was mapped to a region that joins two polypyrimidine motifs in the non-coding strand within the PDGF A-chain fragment. Only several bands of lower intensity were observed from this region of the coding strand (Fig. 2) and thus, the non-coding strand is preferentially cleaved by S1. Sensitivity of the fragment is highly dependent on supercoiled stress;

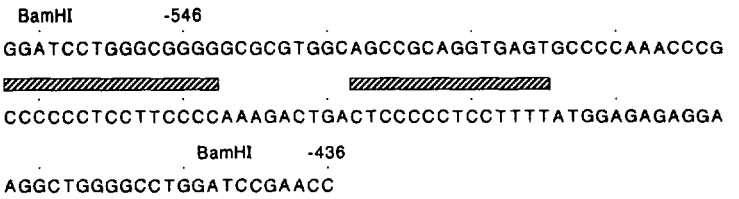

Figure 1. Nucleotide sequence of the BamH I-BamH I DNA fragment of the PDGF A-chain gene promoter. The bars indicate the homopurine-homopyrimidine motifs. The BamH I sites are also shown.

linearized or relaxed plasmid DNA is not recognized by S1 (data not shown), indicating that this region of the PDGF A-chain gene promoter adopts a supercoiling-dependent structural transition that preferentially establishes a single stranded-like conformation to the non-coding strand.

Previously, sites that assumed a single-stranded conformation under supercoiling stress within the PDGF A-chain gene were found to be recognized by nuclear proteins such as Sp1 and NF-κB (5). We therefore tested an end-labeled double-stranded oligonucleotide (5'-TCCTTCCCCAAAGACTGACTCCC-3') encompassing the S1 sensitive region with nuclear extracts from HeLa cells and from A172 (neuroblastoma) cells and analyzed complex formation on native polyacrylamide gels. A distinct protein-DNA complex was observed (Fig. 3A) with both HeLa and A172 nuclear extracts. To assess the specificity of the interaction of this protein with the end-labelled fragment, we performed competition assays with unrelated oligonucleotides from the pUC19 polylinker region and with the unlabled probe itself. The unlabled probe was strongly competitive with the labeled probe for the nuclear protein identified, while the pUC oligonucleotide had no effect. This result indicated that a nuclear protein from two different cell lines appears to bind directly and specifically to the region identified as an S1 sensitive element within the promoter of the PDGF A-chain gene.

The S1 sensitive regions previously identified within the PDGF A-chain gene have not only recognized nuclear proteins but also were associated with regulatory function (4,5). We therefore wished to determine if this element also had regulatory function. Previously, we observed that deletion of the region of the PDGF A-chain gene promoter containing this element decreased promoter activity ~50% (14), suggesting that this region may contain a positive regulatory element. To test this function further, we fused the oligonucleotide used in gel mobility shift assays in an upstream position in both the 5'→3' and 3'→5' orientation and downstream in the 5'→3' orientation in the thymidine kinase (TK) promoter driven CAT reporter gene (pBLCAT2) and transfected

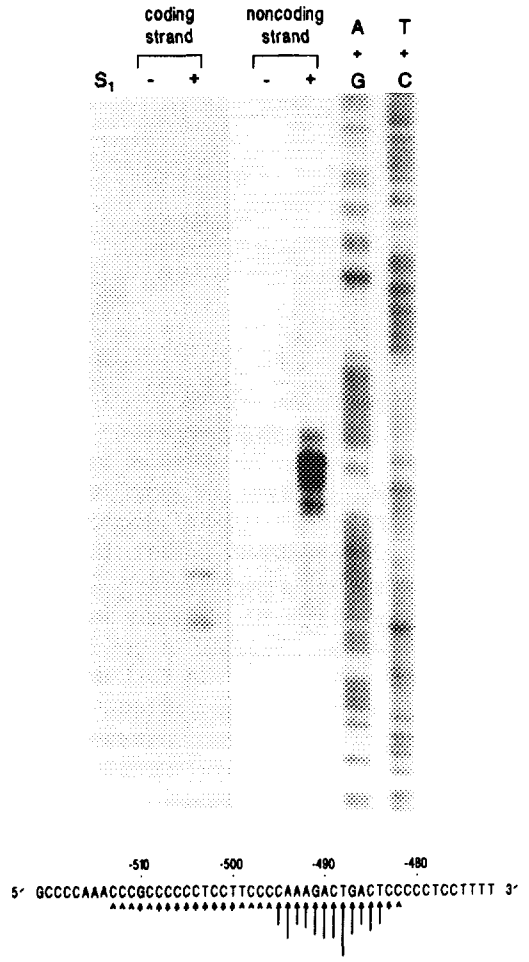

Figure 2. S1 nuclease sensitivity assay of the supercoiled plasmid, pB2. The supercoiled plasmid was treated with S1 nuclease and digested with Xba I. The plasmid was then end-labeled on the coding or noncoding strand. The end-labeled DNA fragments were electrophoresed on a sequencing gel along with a chemical sequencing ladder as a marker. Lane -, no S1 added. Lane +, S1 added. The S1 sensitive sequence is shown and numbered at the bottom. The length of the arrows correlate with the frequency of S1 digestion as determined by densitometric scanning.

these constructs into HeLa and A172 cells. Analysis of the CAT activities of extracts of transfected cells demonstrated a 2-3 fold increase of the TK promoter activity that was both position and orientation independent (Fig. 4). Thus, these data suggest that this S1 sensitive region of the PDGF A-chain gene promoter contains a positive regulatory element that functions as an enhancer and binds to a single nuclear protein in gel retardation assays.

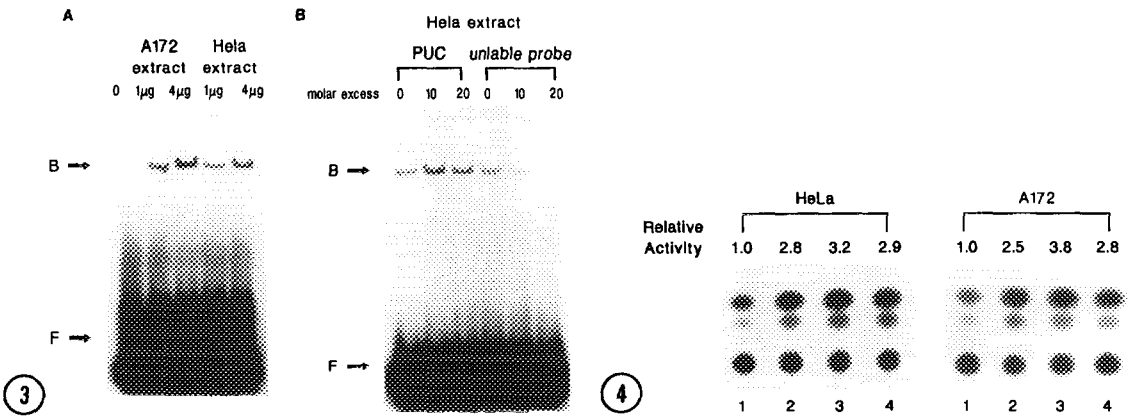

Figure 3. Gel mobility shift assays and competition assays. A. The probe was end-labeled, incubated with nuclear extracts from HeLa and A172 cells and separated on native polacrylamide gels. B. The protein/DNA complex was incubated with different molar excesses of non-specific competitor (PUC) and with the unlabeled probe. B indicates the protein-DNA complex. F indicates the free probe.

Figure 4. Functional activity of the S1 nuclease sensitive site. The pBLCAT2 carrying the oligonucleotide used in gel shift assays was transfected into the HeLa and A172 cells for CAT analysis. Lane 1: pBLCAT2. Lane 2: oligonucleotide cloned upstream of pBLCAT2 in a 5'→3' orientation. Lane 3: oligonucleotide cloned upstream of pBLCAT2 in a 3'→5' orientation. Lane 4: oligonucleotide cloned downstream of pBLCAT2. Relative CAT activities are shown.

DISCUSSION

Sequences in purified supercoiled plasmid DNA or in chromatin residing in regulatory regions of active genes have been identified with chemical probes and/or endonucleases such as S1 that preferentially recognize single-stranded DNA (15-17). Although different sequences confer different DNA conformations under superhelical strain, many of the sequences that are recognized by S1 nuclease have been identified in purine:pyrimidine rich regions. Depending on the conditions of analysis, homopurine-homopyrimidine motifs may assume a variety of conformations, such as an A-like conformation (18). Homopurine-homopyrimidine sequences have been mapped in the regulatory regions of the human c-myc gene (19) and the chicken β-globin gene (15,16) and appear to be important in the regulation of transcription of these genes. Although the mechanisms by which the local topological features of promoter DNA regulate gene transcription are unclear, two possibilities have been suggested; two or more transacting factors may differentially recognize different conformations of the same DNA element or,

alternatively, DNA binding proteins may differentially recognize sequences with single- and double-stranded-like conformations with different apparent affinities for each.

In this work, we have identified an element within the PDGF A-chain promoter that adopts an S1-sensitive conformation dependent on supercoiling. Because this element is located at the junction of two homopurine-homopyrimidine sequences, it is possible that the conformational changes observed may be induced by these homopurine-homopyrimidine motifs, as previously suggested (18). However, apart from its single stranded character, the structural configuration within this S1-sensitive region that we described is not clear. Interestingly, however, this element functions as an enhancer by activating transcription in a position and orientation independent manner, and thus this enhancer element of PDGF A-chain gene may be an important regulator of PDGF A-chain gene transcription *in vivo*. Recently, we identified a single-stranded DNA binding protein that specifically recognizes another S1 nuclease sensitive region in the PDGF A-chain gene promoter region (6) and suggested that the single-stranded DNA binding protein may stabilize the altered DNA conformation and thereby reduce the potential for a double-stranded DNA binding protein to recognize this sequence. As noted, other S1 sensitive sites also have been identified in this promoter and thus it may be that growth related genes in general may rely on DNA conformations that differ from the usual Watson-Crick double-stranded DNA conformation as a means to further the potential for the fine regulation of transcription.

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