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AN S1 NUCLEASE-SENSITIVE HOMOPURINE/HOMOPYRIMIDINE DOMAIN IN THE PDGF A-CHAIN PROMOTER CONTAINS A NOVEL BINDING SITE FOR THE GROWTH FACTOR-INDUCIBLE PROTEIN EGR-1

Zhao-Yi Wang and Thomas F. Deuel

Departments of Medicine and Biochemistry & Molecular Biophysics
Jewish Hospital at Washington University Medical Center, St. Louis, MO 63110

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SUMMARY: Transcription of the platelet-derived growth factor (PDGF) A-chain gene is activated in cells exposed to growth factors. We now have identified a homopurine/homopyrimidine domain in the promoter region of the PDGF A-chain gene that exhibits S1 nuclease sensitivity in vitro and that contains a novel binding site (5'-TCCTCCTCCTCCTC-3') for the growth factor inducible transcription factor EGR-1 as demonstrated in gel mobility shift assays. Sequences similar to this novel EGR-1 binding site were observed also in five growth-related genes and shown to bind to EGR-1 in competition assays, suggesting that EGR-1 may influence the transcriptional regulation of these growth-related genes.

The homodimer of the platelet-derived growth factor (PDGF) A-chain is a potent mitogen and chemoattractant for cells of mesenchymal origin (1). Expression of the PDGF A-chain gene is observed in normal endothelial cells, fibroblasts, smooth muscle cells, and various transformed cell lines (2). Particularly high levels are found in glioblastoma, osteosarcoma, and rhabdomyosarcoma cell lines (2). Expression of PDGF A-chain gene has also been found at high levels in developing mouse central nervous system (3) and in early stage xenopus embryos (4). Levels of transcription also are found to significantly increase after cells are exposed to growth factors or other signalling agents (reviewed in 1). However, the mechanisms underlying the transcriptional regulation of this growth-related gene is largely unknown.

In order to identify control mechanisms governing levels of transcription of the PDGF A-chain gene, we characterized the structure of the human PDGF A-chain gene, identified its 5' promoter region (5), and characterized regulatory elements in the 5' flanking sequences (6,7). In the present study, we have identified a homopurine/homopyrimidine-rich region that exhibits S1 nuclease sensitivity in vitro and acts as a novel binding site for the transcription factors EGR-1 (reviewed in 8). Competition binding experiments

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have provided evidence that EGR-1 also binds to similar sequences in the promoter regions of other growth-related genes, including those genes encoding the epidermal growth factor receptor (EGF-R), the insulin receptor (I-R), c-Ki-ras, c-myc, and TGF-ß3, suggesting that EGR-1 may have a role in the transcriptional regulation of many genes involved in cell proliferation.

MATERIALS AND METHODS

S1 Nuclease Sensitivity Assays: Restriction endonucleases and S1 nuclease were obtained from Bethesda Research Laboratories. The plasmid containing the PDGF A-chain promoter (-36 to +388) was constructed as described (6) and purified by alkaline lysis and centrifugation twice through a $CsCl_2$-Ethdium bromide gradient. The supercoiled plasmid was treated with 0.05 units per µg DNA of S1 nuclease at 37°C for 5 min and the reaction was stopped by extraction with phenol and chloroform - isoamyl alcohol. The precipitated DNA was then digested with Hind III and end-labeled with either T4 polynucleotide kinase and [γ-^{32}P] ATP or with avian myeloblastosis virus (AMV) reverse transcriptase and [α-^{32}P] dATP on the 5' end of the coding strand or the 3' end of the non-coding strand, respectively. The end-labeled DNA was digested with XbaI, and the DNA fragments released were purified from agarose gels and fractionated on 6% sequencing gels in parallel with a Maxam-Gilbert sequencing ladder as marker.

Gel Mobility Shift Assay: The oligonucleotides (for sequences see Fig. 4) were synthesized with an automatic DNA synthesizer, annealed, and purified. Oligonucleotides were end-labeled with [α-^{32}P] dATP and AMV reverse transcriptase. The purified Sp1 protein was purchased from Promega. The purified EGR-1 protein was a kind gift of Dr. Frank Rauscher III (Wistar Institute of Anatomy and Biology, Philadelphia, PA). Gel mobility shift assays were performed as described (6).

RESULTS

Based upon the assumption that non-ß-form DNA may be important in the transcriptional regulation of genes, previously, we treated supercoiled plasmids containing the PDGF A-chain promoter with S1 nuclease and identified five cis-acting regulatory elements in the 5' flanking region that are sensitive to S1 nuclease *in vitro* (6). In this report, we used sensitivity to S1 nuclease to determine if a structural transition may occur within the 5' untranslated region of the PDGF A-chain gene. Plasmid DNA containing the PDGF A-chain promoter (-36 to +388, relative to the major transcription start site, Figure 1) in the native supercoiled form was isolated from the host bacteria and treated with S1 nuclease. The fragment was then end-labeled, purified, and analyzed on sequencing gels. A prominent series of intense bands that maps precisely to the sequence 5'-TACTCCTCCTCCTCCTCT-3' (+50 to +67), a homopurine/homopyrimidine motif, observed downstream of 5' cap site (Fig. 2). This sensitivity to S1 was not observed when plasmid was linearized (data not shown), suggesting that this homopur/homopyr motif is sensitive to S1 nuclease when the plasma DNA is in the superhelical structure.

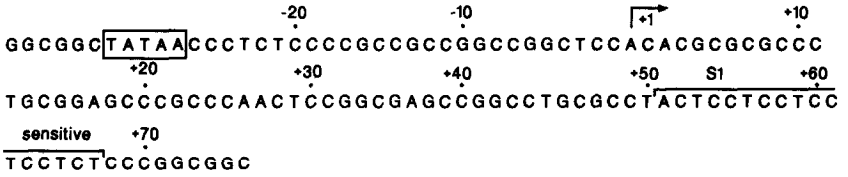


Figure 1. Partial sequence of the PDGF A-chain promoter region. Sequences are numbered according to their positions relative to the major transcription start site (+1), indicated by the bent arrow. The general region sensitive to S1 nuclease is also shown.

The human EGF-R promoter also contains an S1 nuclease-sensitive "TCC" repeat motif (9) that is similar to this repeat motif in the PDGF A-chain gene promoter. DNAse I protection studies suggested that at least two proteins,

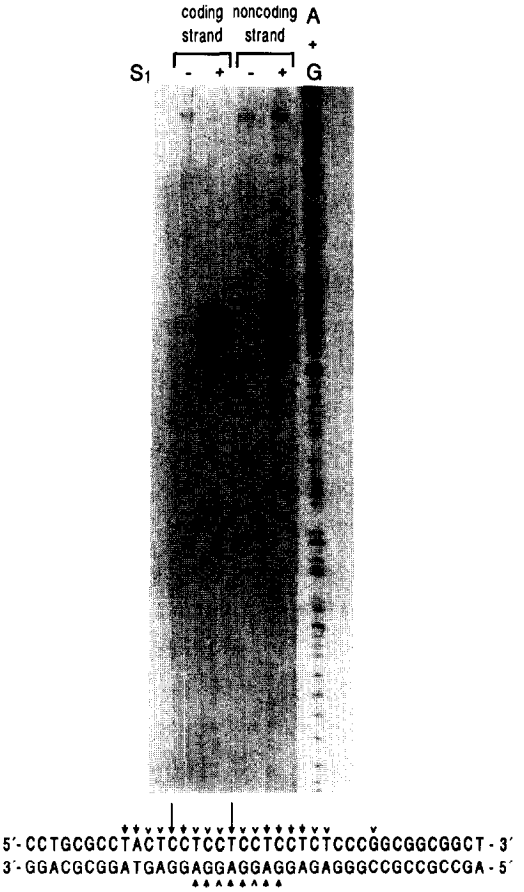


Figure 2. Fine mapping of S1 nuclease-sensitive sites in the pur/pyr-rich domain. (Upper): Reactions on the left show DNA 5' end-labeled on the coding strand. Reactions on the right show DNA 5' end-labeled on the non-coding strand. Lanes (-); controls not treated with S1. Lanes (+); S1-treated DNA. A+G: Maxam-Gilbert sequencing reactions. (Lower): Summary of S1 nuclease mapping experiments. The degree of S1 sensitivity as determined by densitometric scanning is indicated by arrow length.

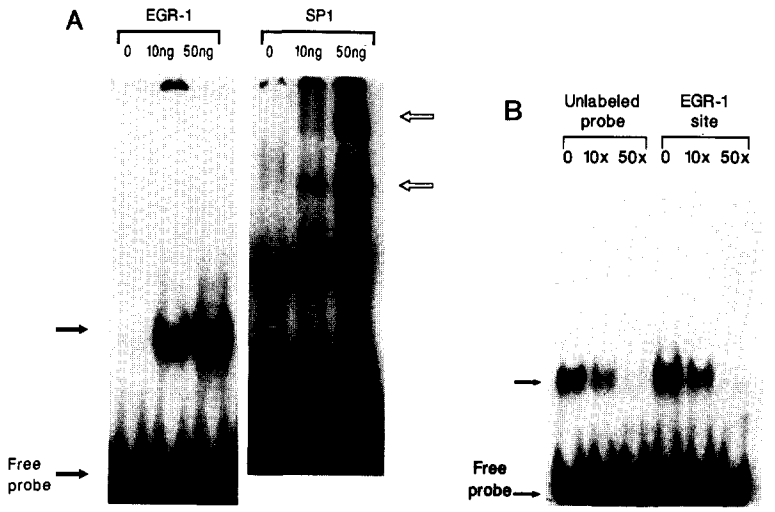
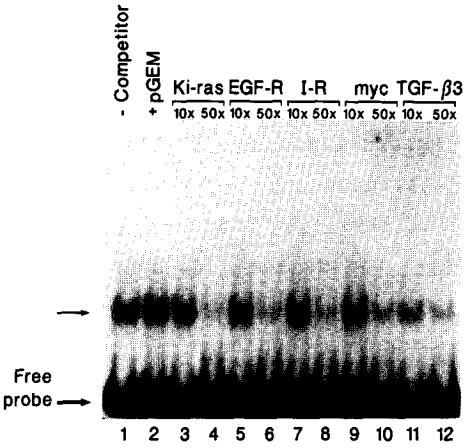


Figure 3. Gel mobility shift assays using purified EGR-1 and Sp1 proteins. A. labeled probes were incubated with 0, 10, or 50 ng purified EGR-1 or Sp1, respectively. The filled arrow marks the EGR-1/DNA complex. The open arrows indicate the Sp1/DNA complexes. Free probe also is indicated. B. Competition gel mobility shift assays. Approximately 0, 10, or 50 fold molar excess of unlabeled probe or double-stranded oligonucleotide competitor containing the typical EGR-1 binding site: 5'-CGCCCTCGCCCCCGCGCCGGG-3' was added.

Sp1 and another protein referred to as TCF, are able to bind this region (9). Sp1 shares a similar binding sequence known as a "G:C box" with another zinc finger protein, EGR-1, whose mRNA is rapidly increased in cells exposed to growth factors or other signalling agents (8). Because we suspected that EGR-1 may recognize this "TCC" repeat motif, we incubated a ^{32}P-end-labeled synthetic oligonucleotide (sequence see Fig. 4) containing the "TCC" repeat motif of the PDGF A-chain gene with purified EGR-1. The formation of DNA-protein complex was assessed by gel electrophoresis and autoradiography. A discrete complex was detected (Fig. 3A). As a control, the purified Sp1 binds to the probe as well (Fig. 3A). Binding of the ^{32}P-labeled probe to EGR-1 was inhibited by excess unlabeled probe or oligonucleotide that contains the typical EGR-1 binding site (Fig. 3B), suggesting that the S1 nuclease sensitive "TCC" repeat motif contains a novel site that specifically interacts with EGR-1.

As noted previously, the "TCC" repeat motif represents a sequence element shared to varying degrees by the promoter regions of a number of genes, in particular, certain growth-related genes that possess G+C rich promoters. These include the EGF-R (9), c-myc (10), c-Ki-ras (11), TGF-ß3 (12), and I-R (13) (Fig. 4). To determine whether these sequence similarities reflect an ability to bind EGR-1, competition gel mobility-shift assays were performed



Probe: TACTCCTCCTCCTCCTCTCCCG
Competitors:
Ki-ras : GCTCCCTCCCTCCCTCCTTCCCTCCCTCCC
EGF-R : CCTCCCTCCTCCTCGCATTCTCCTCCTCCTC
I-R : CCTCCCTCCCCTGCAAGCTTTCCCTCCCTCTC
myc : TGGTCCCTCCCTCCCCCACCCCAGG
TGF-β3: CCTTCCCCACCCTCCCCACCCTCCCCA

Figure 4. Competition gel mobility shift assays. (Upper): EGR-1/DNA complexes were competed with different double-stranded DNA oligonucleotides as indicated below. (Lower): The DNA sequences of probe and competitors.

using oligonucleotides representing the "TCC" repeat motifs in these genes as competitor. The oligonucleotide probe is composed of sequences that encompass the intact PDGF A-chain "TCC" repeat motif (Fig. 4). The competitor oligonucleotides comprising unrelated DNA sequences, including a pGEM-3 30-mer, failed to compete with probe for protein binding (Fig. 4). Competition studies were then extended to include oligonucleotides representative of the similar "TCC" repeat motifs within these genes (Fig. 4), each of which reduced to varying degrees the formation of protein-DNA complexes, indicating that the EGR-1 protein also recognizes these sequences.

DISCUSSION

The regions that regulate transcription of several eukaryotic genes contain sites either in active chromatin or in supercoiled DNA that are hypersensitive to the single-strand specific nuclease S1 (9,12). Fine mapping has demonstrated that these S1 hypersensitive sites are commonly located in pur/pyr-rich domains, regions composed of one strand containing primarily purine residues and the complementary strand containing primarily pyrimidine residues. Here we report an S1 nuclease sensitive region located in a pur/pyr-rich domain within the PDGF A-chain promoter. This "TCC" repeat motif has been shown to be sensitive to S1 in the EGF-R gene (9). Recently, a similar sequence was found in the c-Ki-ras and human δ-globin gene promoters

that also are sensitive to S1 nuclease (11,14). However, the DNA conformations that render the DNA sensitive to S1 are not clear. A similar sequence in the c-myc promoter has been reported to assume an H-DNA conformation when in a supercoiled plasmid (10). All of these regions have previously been shown to influence promoter activity of these genes. However, the mechanisms by which S1 sensitive DNA structures are involved in regulating gene transcription in vivo are unclear. Evidence in support of the idea that local changes in DNA structure alter the interctions between promoter DNA and specific DNA-binding protein has been reported (15).

As noted previously, the "TCC" repeat motif of the EGF-R gene interacts with Sp1 and with another factor referred to as TCF (9). We demonstrate here that the growth factor-inducible gene product EGR-1 recognizes these "TCC" repeat motifs. EGR-1 is thought to play a role in regulating genetic programs induced by extracellular ligands. Our findings suggest that these growth-related genes may be possible target genes whose expression may be influenced by EGR-1.

At present, we can only speculate on the role that such EGR-1 binding sequence may play in determining the transcriptional activity of the PDGF A-chain gene. It is possible that EGR-1 modulates transcription by binding to this sequence. It may be of interest that PDGF A-chain gene mRNA is increased shortly after the EGR-1 mRNA is increase in growth factor stimulated cells, suggesting that EGR may initiate other growth factor induced genetic programs. The identification of these potential target genes for EGR-1 may lead to new understanding of the role of EGR-1 in the signal transduction pathway initiating the cell proliferation.

ACKNOWLEDGMENTS

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