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CCAGCGCTGGAGG-3', R: 5'-CCGCGGCTGTTC-CCGG-3'; exon 2 (168 bp), F: 5'-AGTAACGTACT-TCTTAACTTTGGC-3', R: 5'-AGAGGAAGATACC-TATCACGTG-3'; exon 3 (227 bp), F: 5'-AAAATG-GAAGCATTTGGTAATCA-3', R: 5'-AGTGAACTAA-AATTCTTAGAGGG-3'; exon 4 (250 bp), F: 5'-AAG-CAATGATGACAAAGTGCTAAC-3', R: 5'-TGGTC-CACAATGTCACATTTCGG-3'; exon 5a (223 bp), F: 5'-CTGAAGGGCTGTGCTGTGGA-3', R: 5'-TGTC-CTTACAAACGGGGCT-3'; and exon 5b (224 bp), F: 5'-CCCATGCTCAAGACATACTCC-3', R: 5'-ACA-GTAAGGAAAAAACAAACAGCC-3'. Amplifications for exons 2, 3, 4, 5a, and 5b consisted of 30 cycles with the following parameters: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. To amplify the highly GC-rich exon 1, we raised the annealing temperature to 68°C and 10% dimethyl sulfoxide was added to the reaction. The search for mutations was conducted with single-strand conformation polymorphism analysis [M. Orita, Y. Suzuki, T. Sekiya, K. Hayashi, *Genomics* **5**, 874 (1989)] in 168 FRDA patients, and chemical cleavage [J. A. Saleeba, S. J. Ramus, R. J. H. Cotton, *Hum. Mutat.* **1**, 63 (1992)] in 16 patients.
26. Assuming a FRDA carrier frequency in Italy of 1 out of 120 individuals (2) and a frequency of I154F of 1 out of 40 FRDA chromosomes in Southern Italians, one individual in 3300 in that population is expected to be a carrier of I154F. Finding such a person in a random sample of 210 individuals can occur with >6% probability.
27. The Perkin-Elmer XL long-PCR reagent kit was used to set up the reactions, and standard conditions were used as suggested by the manufacturer with primers 5200Eco (5'-GGGCTGGCAGATTCCTCCA-G-3') and 5200Not (5'-GTAAGTATCCGCGCCGG-GAAC-3'). Amplifications were performed in a Perkin-Elmer 9600 machine and consisted of 20 cycles of the following steps: 94°C for 20 s, 68°C for 8 min, followed by 17 cycles in which the length of the 68°C-step was increased by 15 s per cycle. The generated amplification product is 5 kb from normal chromosomes, and about 7.5 kb from FRDA chromosomes.
28. The primers GAA-F (5'-GGGATTGGTTGCCAGT-GCTTAAAAGTTAG-3') and GAA-R (5'-GATCTAAG-GACCATCATGGCCACACTTGCC-3') flank the GAA repeat and generate a PCR product of 457 + 3*n* bp (*n* = number of GAA triplets). With these primers, efficient amplification of normal alleles could be obtained by using the traditional PCR procedure with Taq polymerase after 30 cycles consisting of the following steps: 94°C for 45 s, 68°C for 30 s, and 72°C for 2 min. Enlarged alleles were much less efficiently amplified, particularly when present together with a normal allele; therefore, use of these primers is not indicated for FRDA carrier detection. A more efficient amplification of expanded alleles, also in FRDA carriers, could be obtained by using the primers Bam (5'-GGAGGGATC-CGTCTGGGCAAAGG-3') and 2500F (5'-CAATCCAG-GACAGTCAGGGCTTT-3'). These primers generated a 1.5-kb normal fragment. Amplification was conducted with the long PCR protocol, in 20 cycles composed of the following steps: 94°C for 20 s, 68°C for 2 min and 30 s, followed by 17 cycles in which the length of the 68°C step was increased by 15 s per cycle.
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32. See G. Imbert *et al.*, *Nature Genet.* **4**, 72 (1993), where the absolute linkage disequilibrium in myotonic dystrophy is explained by recurrent mutations on such an at-risk allele.
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37. This work is dedicated to the memory of Dr. A. E. Harding. Supported by grants from NIH (NS34192, M.P. and P.I.P.); from the Muscular Dystrophy Association (M.P.); from the Association Francaise contre les Myopathies, the Groupement de Recherche et d'Etudes sur les Genomes, CNRS, and INSERM (M.K.); from the Italian Telethon (S.C.); from the Comision Interministerial de Ciencia y Tecnologia; and from the Generalitat Valenciana (F.P.). V.C. is a recipient of a fellowship from Ministerio de Educacion y Ciencia, Spain, M.D.M. is a recipient of a fellowship from the Generalitat Valenciana and from the University of Valencia, L.M. is a recipient of a fellowship from the Italian Telethon, M.C. is a recipient of a fellowship from the Fondation pour la Recherche Médicale, F.D. is a recipient of a fellowship from the Association Française contre l'Ataxie de Friedreich (AFAF), E.M. and J.C. are recipients of fellowships from the Generalitat Valenciana, and S.B. is a recipient of a fellowship from the Muscular Dystrophy Association. We wish to thank S. Vicaire (IGBMC) and P. Milasseau and C. Crueau (Généthon, Evry) for large-scale sequencing. We thank the Baylor College of Medicine Human Genome Center for access to DNA analysis resources and databases. We are grateful to the many clinicians who referred their patients to us, and to all patients and their families for their willingness to participate in this research and for their continuous encouragement.

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Egr-1–Induced Endothelial Gene Expression: A Common Theme in Vascular Injury

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A number of pathophysiologically relevant genes, including platelet-derived growth factor B-chain (PDGF-B), are induced in the vasculature after acute mechanical injury. In rat aorta, the activated expression of these genes was preceded by a marked increase in the amount of the early-growth-response gene product Egr-1 at the endothelial wound edge. Egr-1 interacts with a novel element in the proximal PDGF-B promoter, as well as with consensus elements in the promoters of other genes induced by endothelial injury. This interaction is crucial for injury-induced PDGF-B promoter–dependent expression. Sp1, whose binding site in the PDGF-B promoter overlaps that of Egr-1, occupies this element in unstimulated cells and is displaced by increasing amounts of Egr-1. These findings implicate Egr-1 in the up-regulated expression of PDGF-B and other potent mediators in mechanically injured arterial endothelial cells.

Vascular endothelium constitutes a nonthrombogenic surface of normally quiescent cells that line blood vessels and regulate molecular and cellular movement across the vessel wall. In response to denuding injury, endothelial cells at the wound edge spread and migrate into the vacant area, undergo proliferation, and secrete factors that stimulate endothelial and smooth muscle cell growth. These responses provide an important homeostatic mechanism for maintaining normal vascular function. PDGF has been implicated in the regenerative events that follow vascular injury. The induction of PDGF expression in vascular endothelium may have profound chemotactic and mitogenic effects on the underlying smooth muscle cells and may contribute to the structural remodeling that typically occurs in experimental arterial repair, in restenosis, and in the pathogenesis of atherosclerotic vascular disease (*1*). Despite a wealth of descriptive studies that correlate the formation of vascular occlusive lesions with the inappropriate expression of PDGF and other growth-regulatory molecules (*2, 3*), a direct link between a transcription factor and the induced expression of a pathophysiologically relevant gene has not yet been demonstrated in the context of arterial injury.

In a survey of immediate-early genes that could be induced by acute vascular injury in rat aorta (*4, 5*), we examined the expression of Egr-1, a serum-inducible zinc-finger nuclear phosphoprotein and member of a family of related transcription factors (*6*). In situ hybridization techniques that visualize the endothelium of the vessel wall en face revealed that Egr-1 expression was markedly induced exclusively at the endothelial wound edge within 30 min of partial denudation (Fig. 1B). Egr-1 expression was undetectable in endothelium from unmanipulated arteries (Fig. 1A). Induced Egr-1 mRNA remained apparent after 2 hours (Fig. 1D), and the time-dependent decrease in the specific hybridization signal (Fig. 1, B to D) demonstrates the transient induction of endothelial Egr-1 expression by injury. In contrast, the

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sense Egr-1 riboprobe failed to hybridize with mRNA from normal or injured tissue (Fig. 1, E and F). Amounts of PDGF-B transcript were also low in unmanipulated vessels (Fig. 1G), consistent with the results of studies that used other techniques (*3, 4, 7*). Partial denudation did not induce PDGF-B expression at the endothelial wound edge until 4 hours after injury (Fig. 1H); this induction continued for several weeks during endothelial regeneration (*4*). The colocalization of the spatial patterns of Egr-1 and PDGF-B expression, and the potential kinetic association between these two genes in injured arterial endothelium, led us to investigate whether Egr-1 could inducibly regulate the expression of PDGF-B.

In response to mechanical injury in vitro, confluent endothelial cells initiate movement into the open "wounded" area by actively responding to locally derived signals or autocoids from injured cells. This standard in vitro model of vascular injury (*8*) was used to address the possible link between Egr-1 and injury-induced PDGF-B expression. Nuclear runoff analysis revealed that Egr-1 gene transcription was induced in cultured bovine aortic endothelial cells (BAECs) within 1 hour of injury (*9*). In a previous study (*10*), 5′ deletion analysis of the PDGF-B promoter in endothelial cells defined a region necessary for core promoter activity (d77) that contained a binding site for the ubiquitous transcription factor, Sp1.

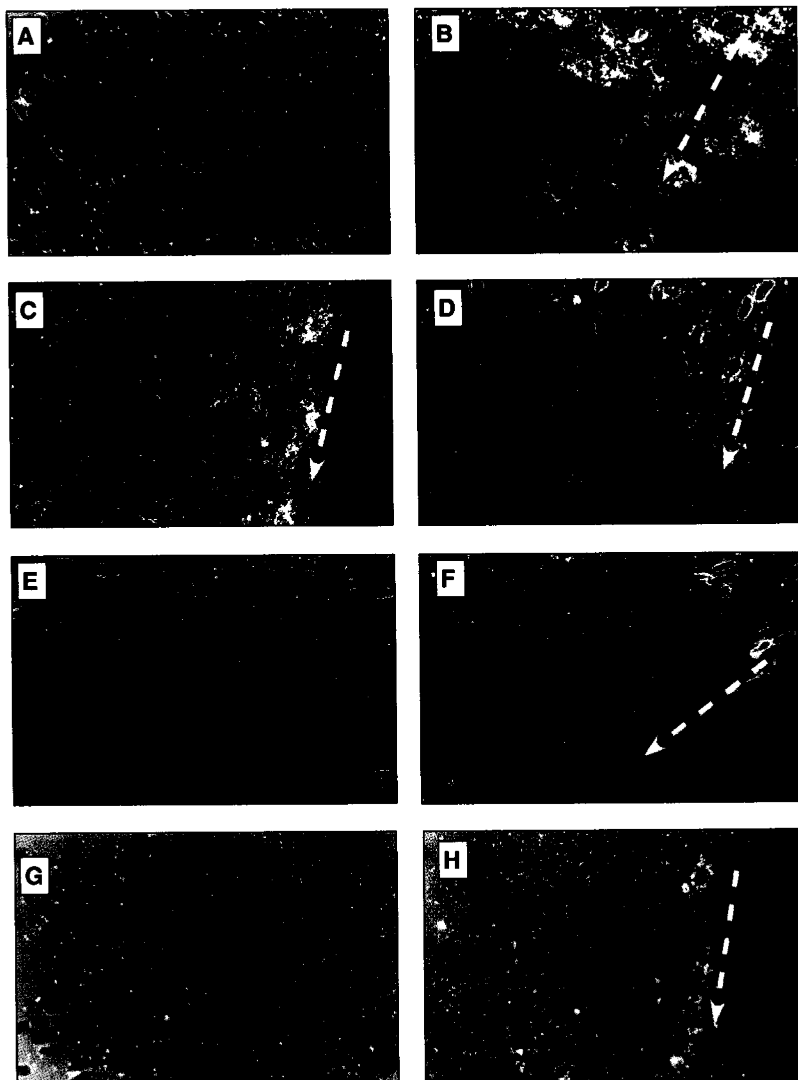

Fig. 1. Endothelial gene expression in rat aorta after partial denudation with a balloon catheter. Photomicrographs of en face preparations of rat aortic endothelium hybridized with ^{35}S-UTP–labeled antisense riboprobes (*22*) are shown. (**A**) Endothelium of normal aorta showed no expression of Egr-1 mRNA. Egr-1 mRNA was detectable at the leading edge (**B**) 30 min, (**C**) 1 hour, and (**D**) 2 hours after wounding. Sense ^{35}S-UTP–labeled Egr-1 riboprobe failed to hybridize with unmanipulated endothelium (**E**) or endothelium 1 hour after wounding (**F**). Endothelium of normal aorta showed no expression of PDGF-B mRNA (**G**). However, PDGF-B mRNA was detectable at the leading edge 4 hours after injury (**H**). The area to the right of the dashed arrow indicates the denuded zone; intact endothelium is to the left of the arrow. Magnifications, ×300.

Recent in vivo footprint analysis of the promoter demonstrates that the Sp1 element is indeed occupied in intact cells (*11*). In vitro deoxyribonuclease I (DNase I) footprinting revealed that recombinant Egr-1 protected a region overlapping this site from partial DNase I digestion (Fig. 2A). When nuclear extracts from endothelial cells 1 hour after injury were incubated with a ^{32}P-labeled oligonucleotide spanning this region [^{32}P–Oligo B (*12*)], a distinct nucleoprotein complex formed (Fig. 2B, band B2). The injury-induced complex was eliminated by antibodies to Egr-1 (Fig. 2B). Nuclear Sp1 also bound to the PDGF-B promoter fragment; however, its concentration was unaltered by injury (Fig. 2B). Thus, injury-induced endothelial Egr-1 expression precedes the induction of PDGF-B (Fig. 1), and Egr-1 binds to a distinct region in the PDGF-B promoter also bound by Sp1 (Fig. 2, A and B).

We next investigated the functional importance of this interaction for PDGF-B promoter–dependent gene expression. Northern (RNA) blot and transient transfection analysis using PDGF-B promoter-reporter constructs revealed that this gene is basally expressed in vascular endothelial cells (*10*). Chloramphenicol acetyltransferase (CAT) expression driven by the PDGF-B promoter (d77-CAT) was induced by injury within 36 hours (Fig. 2C). Reporter activity also increased in cells exposed to phorbol 12-myristate 13-acetate (PMA) or by cytomegalovirus-mediated overexpression of Egr-1 (CMV–Egr-1) (Fig. 2C). When a mutation that abolished the ability of Egr-1 to bind to the PDGF-B promoter (Fig. 2D) was introduced into the d77-CAT construct, basal expression driven by the promoter was attenuated, and expression inducible by injury was abolished (Fig. 2C). The mutant construct also failed to mediate increased reporter activity when Egr-1 was overexpressed or when the cells were exposed to PMA (Fig. 2C). The Egr-1 binding site in the proximal PDGF-B promoter is thus required for inducible promoter-dependent expression in vascular endothelial cells.

The interaction of Egr-1 and Sp1 with overlapping binding elements in the proximal PDGF-B promoter suggests that Sp1, resident on the promoter in unstimulated cells, may be displaced by increasing amounts of Egr-1. Running gel shifts (*13*) indicated that recombinant Egr-1 bound to the PDGF-B promoter in a stable and reversible manner (Fig. 3A). The relative efficiency with which Egr-1 was displaced from ^{32}P–Oligo B by its unlabeled counterpart (Fig. 3A) indicates that Egr-1 interacts with the PDGF-B promoter with a faster dissociation rate than its comparable site in the proximal PDGF-A promoter (*13*). Sp1

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was displaced from the promoter by Egr-1 in a dose-dependent manner (Fig. 3B). Decreasing amounts of Egr-1 in the presence of a fixed concentration of Sp1 allowed reoccupation of the promoter by Sp1 (Fig. 3B). The absence of a higher order complex when both factors were present indicates that Egr-1 and Sp1 do not bind the promoter simultaneously (Fig. 3B). These findings with recombinant proteins indicate that an interplay involving Egr-1 and Sp1 can occur on the PDGF-B promoter.

The localized induction of Egr-1 at the endothelial wound edge precluded a direct determination of whether a displacement mechanism was involved in the induction of PDGF-B expression by injury. PMA is a model agonist of Egr-1 expression in vascular endothelial cells (*13*). The marked induction of Egr-1 mRNA and protein that preceded the increase in the amount of PDGF-B in endothelial cells exposed to PMA (Fig. 3C) is like the temporal pattern with which these genes are expressed at the endothelial wound edge after arterial balloon injury (Fig. 1). The amounts of Sp1 transcript and protein were also unaffected by PMA (Fig. 3C). Nuclear proteins from PMA-treated endothelial cells bound to the PDGF-B promoter with a pattern similar to that observed with injury-induced extracts (compare Figs. 2B and 3D). Immunobinding studies determined that complexes B1 and B2 contained Sp1 and Egr-1, respectively (Fig. 3D). The extreme Egr-1 induction by PMA demonstrates the ability of this transcription factor to displace Sp1 from the PDGF-B promoter in the context of nuclear extracts (Fig. 3D). Accordingly, the PMA-inducible endothelial expression of PDGF-B, like that of PDGF-A (*13*), involves an interplay between Egr-1 and Sp1 at overlapping binding sites in the proximal promoter. This result contrasts with a previous report suggesting that Egr-1 may serve as a negative regulator of gene transcription by blocking the binding of Sp1 to its own recognition sequence (*14*). These findings suggest that the localized induction of PDGF-B expression at the endothelial wound edge may also involve displacement of promoter-bound Sp1 by elevated amounts of nuclear Egr-1. Egr-1 may be involved in interactions with other transcriptional activators and the basal complex to mediate increased gene expression in response to injury.

Transcriptional activation mediated by Egr-1 may be an important common theme that integrates vascular injury with specific patterns of induced gene expression. Putative nucleotide recognition elements for Egr-1 appear in the promoters of a number of pathophysiologically important genes, including human transforming growth factor (TGF)–β1 (*15*), tissue factor (TF) (*16*),

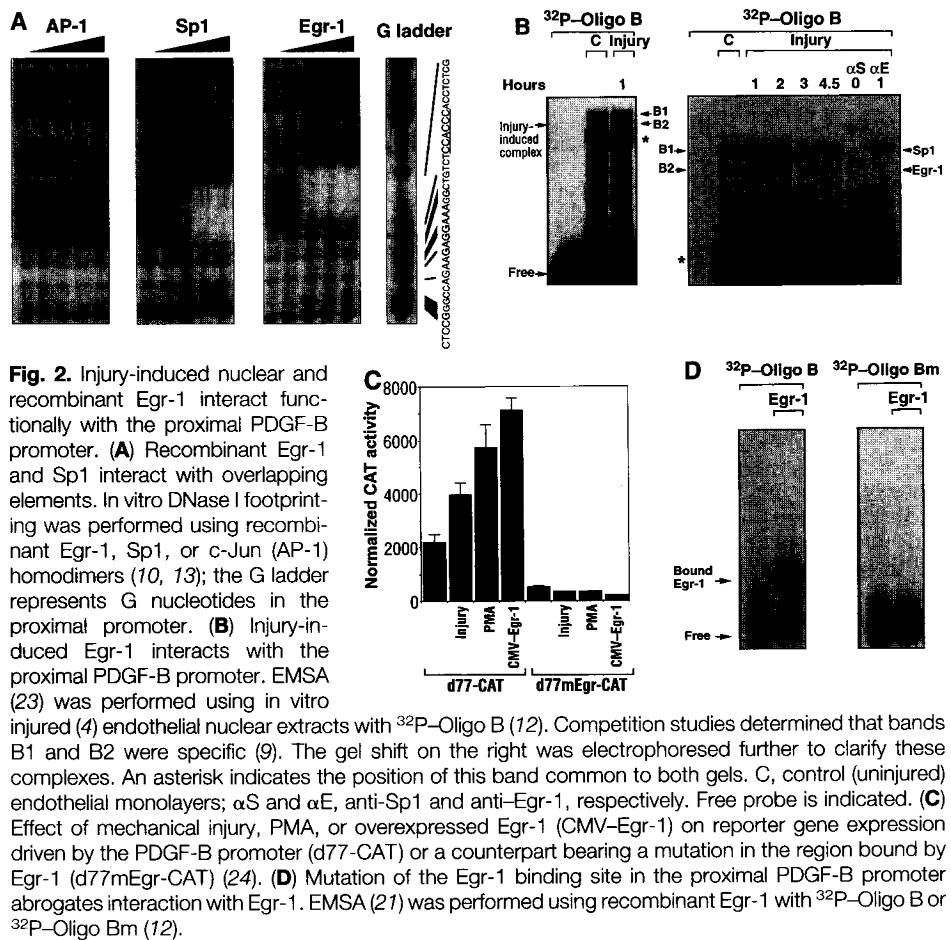

Fig. 2. Injury-induced nuclear and recombinant Egr-1 interact functionally with the proximal PDGF-B promoter. (**A**) Recombinant Egr-1 and Sp1 interact with overlapping elements. In vitro DNase I footprinting was performed using recombinant Egr-1, Sp1, or c-Jun (AP-1) homodimers (*10, 13*); the G ladder represents G nucleotides in the proximal promoter. (**B**) Injury-induced Egr-1 interacts with the proximal PDGF-B promoter. EMSA (*23*) was performed using in vitro injured (*4*) endothelial nuclear extracts with ^{32}P–Oligo B (*12*). Competition studies determined that bands B1 and B2 were specific (*9*). The gel shift on the right was electrophoresed further to clarify these complexes. An asterisk indicates the position of this band common to both gels. C, control (uninjured) endothelial monolayers; αS and αE, anti-Sp1 and anti–Egr-1, respectively. Free probe is indicated. (**C**) Effect of mechanical injury, PMA, or overexpressed Egr-1 (CMV–Egr-1) on reporter gene expression driven by the PDGF-B promoter (d77-CAT) or a counterpart bearing a mutation in the region bound by Egr-1 (d77mEgr-CAT) (*24*). (**D**) Mutation of the Egr-1 binding site in the proximal PDGF-B promoter abrogates interaction with Egr-1. EMSA (*21*) was performed using recombinant Egr-1 with ^{32}P–Oligo B or ^{32}P–Oligo Bm (*12*).

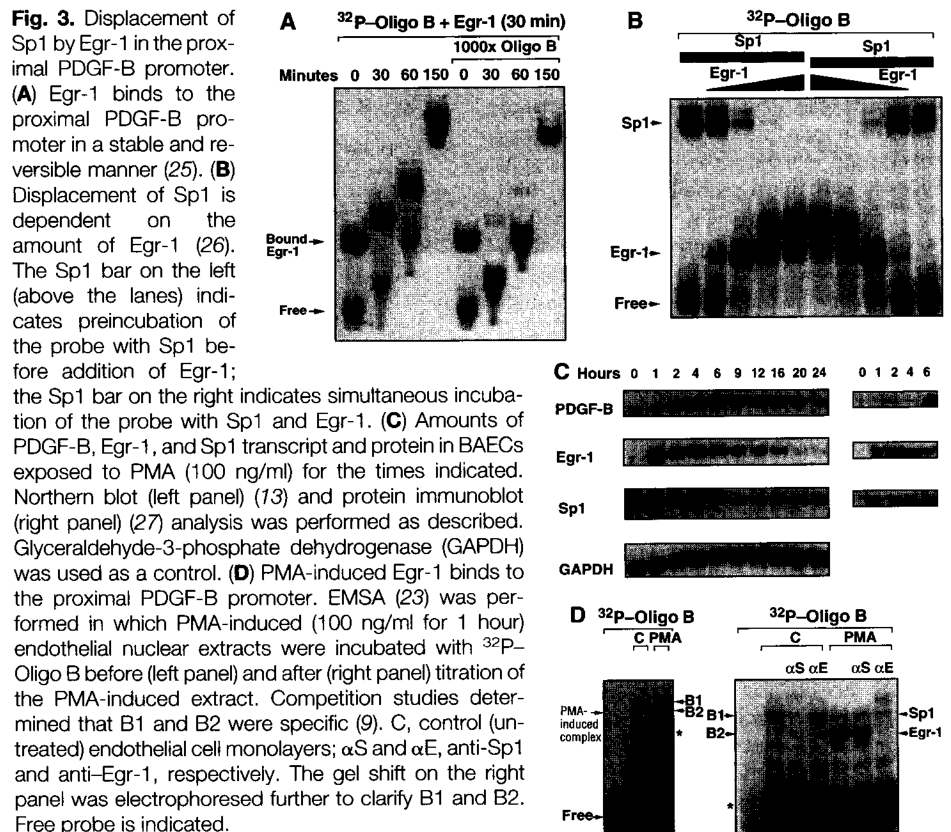

Fig. 3. Displacement of Sp1 by Egr-1 in the proximal PDGF-B promoter. (**A**) Egr-1 binds to the proximal PDGF-B promoter in a stable and reversible manner (*25*). (**B**) Displacement of Sp1 is dependent on the amount of Egr-1 (*26*). The Sp1 bar on the left (above the lanes) indicates preincubation of the probe with Sp1 before addition of Egr-1; the Sp1 bar on the right indicates simultaneous incubation of the probe with Sp1 and Egr-1. (**C**) Amounts of PDGF-B, Egr-1, and Sp1 transcript and protein in BAECs exposed to PMA (100 ng/ml) for the times indicated. Northern blot (left panel) (*13*) and protein immunoblot (right panel) (*27*) analysis was performed as described. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. (**D**) PMA-induced Egr-1 binds to the proximal PDGF-B promoter. EMSA (*23*) was performed in which PMA-induced (100 ng/ml for 1 hour) endothelial nuclear extracts were incubated with ^{32}P–Oligo B before (left panel) and after (right panel) titration of the PMA-induced extract. Competition studies determined that B1 and B2 were specific (*9*). C, control (untreated) endothelial cell monolayers; αS and αE, anti-Sp1 and anti–Egr-1, respectively. The gel shift on the right panel was electrophoresed further to clarify B1 and B2. Free probe is indicated.

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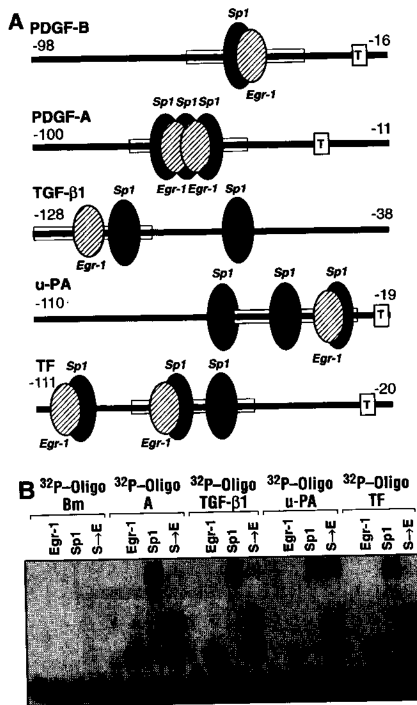

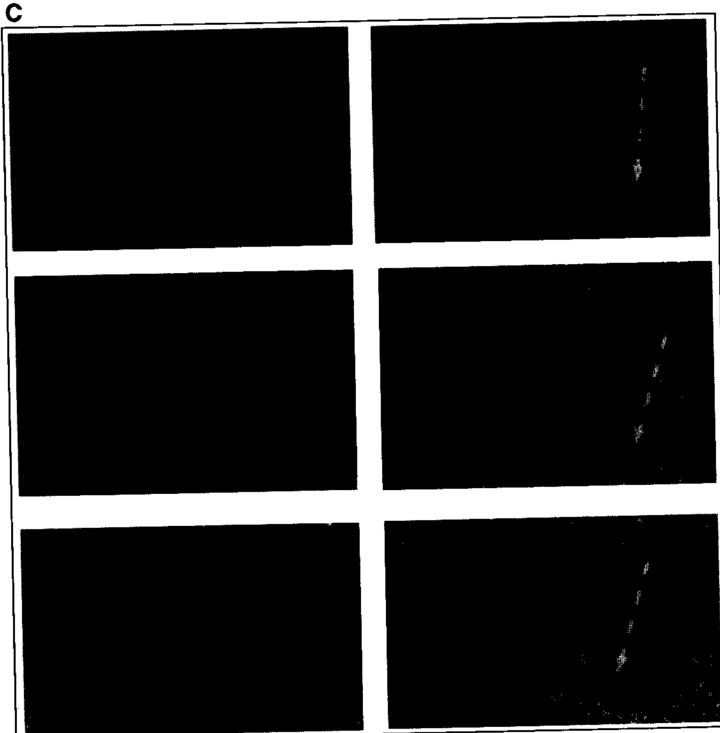

Fig. 4. A common theme in injury-induced endothelial gene expression. (**A**) Comparison of the organization of the human PDGF-B, PDGF-A, u-PA, TF, and TGF-β1 promoters, illustrating positions of consensus Sp1 (*28*) and Egr-1 (*29*) elements. The putative Egr-1 binding site in the u-PA promoter is one base pair mismatch from the consensus. The position of the TATA element is indicated by the letter T. Regions of the promoters used as oligonucleotides in EMSA are boxed. (**B**) Interaction of Egr-1 and Sp1 with the promoters of several pathophysiologically relevant genes. EMSA (*21*) was performed using ^{32}P-labeled oligonucleotides from the PDGF-A (5′-GGGGGG-GGCGGGGGCGGGG-GCGGGGGAGGG-3′), TGF-β1 (5′-CCTGGGGGCCGCCCCCGCTCCCGCCCCGTG-3′), u-PA (5′-GGAGAGGGAGGGGCGGCGCCGGGGCGGGCC-3′), and TF (5′-GCG-GCGGGGGCGGGCGCCGGGGGCGGGCAG-3′) promoters as well as recombinant Egr-1 and Sp1. In lanes marked S→E, Sp1 was preincubated with the probe [indicated in (A)] for 15 min, and a molar excess of Egr-1 was incubated with the binding mixture for 30 min. (**C**) Endothelial gene expression in rat aorta after partial denudation (*22*). Photomicrographs of en face preparations of aortic endothelium hybridized with ^{35}S-UTP–labeled antisense riboprobes for PDGF-A (a and b, unmanipulated and 4 hours after injury, respectively), TGF-β1 (c and d, unmanipulated and 24 hours after injury, respectively), and u-PA (e and f, unmanipulated and 24 hours after injury, respectively). Area to the right of the dashed arrow indicates the denuded zone; intact endothelium is to the left of the arrow. Magnifications, ×300.

urokinase-type plasminogen activator (u-PA) (*17*), PDGF-A (*13*), and PDGF-B (Fig. 2A), as well as in the Egr-1 promoter itself (*18*) (Fig. 4A). The PMA- and serum-response regions in several of these promoters contain elements that can interact specifically with both recombinant Egr-1 and Sp1 (Fig. 4B). Moreover, elevated amounts of Egr-1 can displace prebound Sp1 from these promoters (Fig. 4B). Egr-1 is able to transactivate inducible expression through several of these binding sites (*13*, *19*). Arterial balloon injury induced the expression of these growth-regulatory molecules in vascular endothelial cells specifically at the wound edge (Fig. 4C) or in smooth muscle cells (*20*), and injury-induced endothelial Egr-1 can interact with the promoters of these genes (*9*). Thus, injury-induced Egr-1 expression is both spatially and temporally consistent with a possible role for this pleiotropic mediator in the regulation of multiple genes involved in vascular remodeling (Figs. 1 and 4C). The coordinated expression of sets of growth factors and components of the coagulation system mediated by Egr-1 may contribute to the complex series of cellular and thrombogenic events in the development of vascular occlusive lesions.

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21. An electrophoretic mobility-shift assay (EMSA) was done in buffer containing 50 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), 5% glycerol, 0.5% Nonidet-P40 (Sigma), 1 mM dithiothreitol, and 20 μg of bovine serum albumin (BSA) for 30 min at 22°C.
22. Aortic endothelium of male Sprague-Dawley rats (400 g) was partially denuded with an uninflated 2 French balloon catheter. Deendothelialized regions were identified by intravenous injection of Evans blue (0.3 ml of 5% solution in phosphate-buffered saline) 10 min before killing. The rats were perfusion-fixed with phosphate-buffered 4% paraformaldehyde. Vessel segments were treated with proteinase K (1 μg/ml) at 37°C, prehybridized for 2 hours at 55°C in 0.3 M NaCl, 20 mM Tris (pH 7.5), 5 mM EDTA, 1× Denhardt's solution, 10% dithiothreitol, and 50% formamide, and incubated with the appropriate ^{35}S-uridine 5′-triphosphate (UTP)–labeled riboprobe for 16 hours. After washing, the slides were coated with autoradiographic emulsion and exposed for 3

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weeks. The images were photographed and digitized. The hybridization signal of the radiolabeled probe appears as white grains. All specimens were observed under dark field illumination after nuclear counterstain with hematoxylin. Immunostaining for factor VIII–related antigen (4) confirmed that injury was limited to endothelium.
23. Binding was carried out in buffer containing 50 mM NaCl, 5 mM $MgCl_2$, 5% glycerol, 2.5 mM Hepes (pH 7.9), 1 μg of polydeoxyinosinic-deoxycytidylic acid, and 20 μg of BSA for 30 min at 22°C. Polyclonal antipeptide antibodies to Sp1 and Egr-1 (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with nuclear extracts 15 min before the addition of the probe.
24. d77mEgr-CAT was constructed with the use of an oligonucleotide bearing the Oligo Bm (12) sequence as the 5′ primer for the polymerase chain reaction. Transfections in BAECs were performed with 10 μg of reporter plasmid and the calcium phosphate protocol (10). The cells were incubated with PMA (100 ng/ml), cotransfected with CMV–Egr-1, or injured with a sterile comb (8), and then incubated for 36 hours at 37°C. CAT activity was assessed by the two-phase fluor diffusion technique (13) and was normalized to the amounts of protein in the cell lysate.
25. Recombinant Egr-1 was incubated with ^{32}P–Oligo B (12) for 30 min at 22°C and applied to a running nondenaturing 5% polyacrylamide gel at the times indicated. Alternatively, a 1000-fold molar excess of the unlabeled cognate was added after the 30-min incubation and applied to the gel (21).
26. Increasing amounts of recombinant Egr-1 were applied to a solution in which Sp1 was preincubated with ^{32}P–Oligo B (12) for 30 min at 22°C (Fig. 3B, left side). Alternatively, ^{32}P–Oligo B was incubated with a fixed concentration of Sp1 and decreasing amounts of Egr-1 (Fig. 3B, right side) (21).
27. Protein immunoblots were analyzed with polyclonal antibodies to Egr-1 and Sp1 (1:2500; Santa Cruz Biotechnology) and to PDGF-B (1:200; Genzyme). Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham) with horseradish peroxidase–linked donkey secondary antiserum to rabbit immunoglobulin at 1:10,000 dilution.
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TECHNICAL COMMENTS

Sperm-Egg Binding Protein or Proto-Oncogene?

Recently, D. J. Burks *et al.* (1) identified and characterized a human sperm receptor protein tyrosine kinase (RPTK), called Hu9, as a receptor for ZP3, a glycoprotein of the egg-surrounding matrix, zona pelucida. The finding is a step forward in our understanding of the initiation of the acrosome reaction. However, the sequence presented bears some features that are problematic:

1) The RPTK Hu9 is virtually identical over large parts with the human putative proto-oncogene c-*mer* (2); in these regions, Hu9 is more similar to human c-*mer* than is the mouse orthologue of c-*mer* (3) (Fig. 1). c-*mer* is a member of the growing *axl* subfamily of RPTK genes that is characterized by high similarity and conservation of special features among the tyrosine kinase domains and by the presence of two immunoglobulin (Ig) and two fibronectin type III (FNIII) domains in its extracellular parts (3) (features that are missing in Hu9). This family includes c-*eyk* (4), which has [as reported by Burks *et al.* (1)] 55% identity to Hu9 in the catalytic domain.

2) Although Burks *et al.* (1) did not find any similarity to other proteins in the extracellular part of Hu9, the first 70 amino acids are almost identical with an extracellular region of human c-*mer* and still 48% identical to the corresponding region in c-*eyk* (Fig. 1A).

3) The proposed signal peptide of Hu9 is unusual as it is almost entirely composed of hydrophilic rather than hydrophobic residues. This putative signal peptide is identical with a region within the second FNIII domain in the much larger extracellular part of c-*mer* and similar to other *axl*-like RPTKs. FNIII modules are globular domains common to many extracellular proteins (5); it is extremely unlikely that a part of a globular domain with a known β-sandwich fold is able to function as a membrane-spanning helical segment. The presence of a signal sequence upstream from the stated (1) NH_2-terminus is also supported by 73 bases of the *hu9* complementary DNA (cDNA) (GenBank database accession no. L08961) upstream of the predicted protein that are identical to human c-*mer*.

4) Two regions of Hu9 seem to be frameshifted as compared with c-*mer* and the other *axl*-like RPTKs; one is located proximal to the transmembrane region and the other within the kinase domain (Fig. 1A). In both cases, the cDNA is 100% identical to human c-*mer*, but the reported (1) amino acid sequence of Hu9 is totally different.

5) Hu9 contains two inserts compared to the *axl*-like RPTKs. The insert in the extracellular part contains a segment of 22 residues that is identical (except for two small gaps) to a part of an Ig-like domain in rat PDGF receptor β (Fig. 1B). The two frameshifted regions are located at the end of these inserts. In the case of alternative splicing this could be explained by difficulties in finding the exact termination of the introns.

How can one make sense out of these observations? I offer two extreme interpretations:

1) Hu9 could contain an extremely high sequencing error rate and could be identical to with human c-*mer*: (i) the cDNA is far from being complete; it can be extended in both directions, by about 400 amino acids upstream from the NH_2-terminus as well as by 30 amino acids or so downstream from the COOH-terminus. (ii) As the Hu9 RNA was apparently extracted from a testis library that includes cell nuclei (1), the *hu9* cDNA could correspond with a nuclear pre-mRNA transcript or a defectively spliced transcript, implying that the two inserts would be spliced out of the mature RNA. (iii) The putative (double) frameshifted regions could be corrected by insertion or deletion of four bases in the appropriate positions (Fig. 1) leading to 100% c-*mer* identity. (iv) Numerous small errors such as multiple omission of bases could be corrected. Finally, a protein with an estimated mass of about 95 kD could result, very similar in size to a ZP3 receptor characterised earlier in mouse and human by the same group (6). The interaction of the Ig domains with ZP3 would make sense, as ZP3 also contains a module (ZP) that is common to other zona pelucida proteins (7). Thus, a fine-tuned network of Ig-ZP interactions could initiate the signaling processes required for the complex acrosome reaction.

2) Although unlikely, one could argue that the sequence presented shows very recent evolution at work. (i) Hu9 would then be one of the first traceable cases where evolution incorporates frameshifts to create variety in proteins. (ii) The inserts in Hu9 compared to c-Mer and other *axl*-like RPTKs could be natural and would be explained by alternative splicing or exon shuffling. The high similarity to a part of a globular Ig-like domain in PDGF receptor β supports the latter; reports of an alternative splicing site in human c-*mer* proximal of the membrane (2) point to the former. (iii) Alternative splicing might also lead to a truncated c-*mer*–like receptor that misses a large fraction of the NH_2-terminus and does not contain a signal sequence (that is, a nearly full-length cDNA has been presented). (iv) As Hu9 is closer to human c-*mer* in the regions of similarity than the putative mouse c-*mer* ortholog, one conclusion would be that divergence of Hu9 occurred after the descent of humans and rodents. This would, however, exclude a mouse or-

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