Heparanase is an endoglycosidase that degrades heparan sulfate chains of heparan sulfate proteoglycans, a key component of extra-cellular matrix and basement membranes. Studies using heparanase inhibitors and gene silencing have provided evidence to support an important role for heparanase in tumor metastasis and angiogenesis. The expression of heparanase is normally very tightly controlled, however, it is commonly deregulated in tumor cells, which express elevated heparanase activity that correlates with high levels of heparanase mRNA. We recently identified the transcription factor early growth response gene 1, EGR1, as a key regulator of inducible heparanase transcription in T cells. In this study using chromatin immunoprecipitation, we demonstrate for the first time that EGR1 binds to the heparanase gene promoter in vivo. The important question of the role of EGR1 in regulating heparanase transcription in tumor cells was then assessed. Studies were carried out in four epithelial tumor lines of different tissue origin. Functional dissection of the heparanase promoter identified a 280-bp region that was critical for transcription of the heparanase gene. Transactivation studies using an EGR1 expression vector co-transfected with a reporter construct containing the 280-bp region showed EGR1-activated heparanase promoter activity in a dose-dependent manner in prostate or breast adenocarcinoma and colon carcinoma cell lines. In contrast, overexpression of EGR1 resulted in a dose-dependent repression of promoter activity in melanoma cells. Using site-directed mutagenesis the 280-bp region was found to contain two functional EGR1 sites and electrophoretic mobility shift assays showed binding of EGR1 to both of these sites upon activation of tumor cells. Furthermore, the heparanase promoter region containing the EGR1 sites was also inducible in tumor cells and induction corresponded to HPSE expression levels. These studies show that EGR1 regulates heparanase transcription in tumor cells and importantly, can have a repressive or activating role depending on the tumor type.

Understanding the molecular basis of tumor metastasis and angiogenesis remains a key focus in cancer research and is critical for the development of novel interventional approaches in the treatment of neoplastic pathologies. The extracellular matrix (ECM) is an important structure in these processes, in particular the specialized form known as basement membranes that surround vessels and provides a physical barrier to the migration of cells. An essential structural component of the ECM and also cell surfaces are heparan sulfate proteoglycans. These are composed of a protein core covalently linked to complex sulfated glycosaminoglycan, heparan sulfate side chains (1–3), that interact with other components of the ECM, such as fibronectin, collagen, and laminin, to provide matrix assembly and stability. The main mechanism of heparan sulfate cleavage is by the β-D-endoglucuronidase, heparanase (HPSE) (4, 5). The cleavage of heparan sulfate chains by heparanase expressing cells such as metastatic tumor cells, proliferating endothelial cells, and activated leukocytes facilitates the degradation of the ECM promoting cell invasion associated with tumor metastasis, angiogenesis, and inflammation (6, 7). In addition, heparan sulfate chains in the ECM specifically bind many proteins such as growth factors and cytokines (2) that upon cleavage by HPSE, modulate the environment of the ECM to facilitate angiogenic responses required for wound healing and tumor angiogenesis (8). The cloning and characterization of HPSE (6, 9–11) has suggested that this gene encodes for the dominant heparan sulfate-degrading enzyme in mammalian tissues. Consequently, HPSE has become a very attractive drug target for new anti-metastatic and antiangiogenic therapies.

Human HPSE is expressed as a 543-amino acid, 65-kDa proenzyme (6, 10) that is proteolytically processed to produce an active enzyme composed of 8- and 50-kDa polypeptide chains that form a heterodimer (12–14). Northern blot analysis has identified two major transcripts in human, a 1.7- and 5-kb mRNA (10, 15), which encode for the same protein product. HPSE expression under normal physiological conditions is restricted to activated leukocytes, endothelial cells, and smooth muscle cells (16, 17), as well as cytrophoblasts, keratinocytes, and platelets (11, 16, 18, 19). However, HPSE expression is also up-regulated by many tumor cells. Numerous studies of clinical tumor samples have established that HPSE is highly expressed in tumors at both the protein and mRNA levels (20–23) and its expression is correlated with disease (22, 24). Furthermore, studies using HPSE inhibitors (25, 26) or HPSE gene silencing approaches (27–29) have confirmed the important in vivo role of HPSE in the processes of tumor metastasis, angiogenesis, and growth.

Recent studies are attempting to identify the mechanisms by which HPSE switches from being a normally tightly controlled gene to one that is deregulated in tumor cells. Whereas advances have been made into understanding the processing of the proenzyme (14, 30–32) and the

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**Early Growth Response Gene 1 (EGR1) Regulates Heparanase Gene Transcription in Tumor Cells**

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environmental factors that influence its activity (33–35), our knowledge on the mechanisms of the complex regulatory processes of HPSE gene transcription remains limited. Two studies have highlighted the importance of several ETS response elements in the promoter, although the emphasis in each study was on different sites and the studies were limited to a breast carcinoma line (36) and thyroid carcinoma lines (37). Jiang et al. (37) also showed there were three functional Sp1 sites in the promoter that acted cooperatively with the ETS family member, GABP.

At the endogenous gene level, it has been shown that methylation of a CpG region in the HPSE promoter can contribute to the silencing of the gene (21, 38, 39) more recently, the expression of a dominant negative form of cAMP-response element-binding protein was shown to decrease HPSE mRNA levels in melanoma cells (40).

We recently reported the identification of the serum inducible zinc finger transcription factor human early growth response gene 1 (EGR1) (41), as a key regulator of inducible HPSE transcription in T lymphocytes (42). These current studies address the important question as to whether EGR1 may also be playing a role in the deregulated expression of HPSE in human tumor cells. EGR1 is a member of the early growth response family that also includes, EGR2, EGR3, EGR4, and WT-1. This family of transcription factors share the ability to bind GC-rich recognition motifs in DNA (41). EGR1 is a nuclear phosphoprotein that is rapidly induced in response to a variety of extracellular and environmental signals (including growth factors, cytokines, vascular injury, and hypoxia) (41, 43, 44) where it binds to the promoters of a range of genes (45) to mediate responses such as wound healing and neo-vascularization, and has been strongly associated with vascular proliferative disorders (46, 47). EGR1 expression has been shown to be variable between tumor cells of different tissue origin (48–50). The well characterized neo-vascularization promoting function of EGR1 in tissue injury suggests it would play an important role in tumor growth and tumor angiogenesis.

Indeed, studies using knockdown strategies have confirmed a central in vivo role for EGR1 in tumor angiogenesis, growth, and metastasis in breast adenocarcinoma (47, 51) and tumor progression in prostate adenocarcinomas (52, 53). A recent study of human prostate cancer samples has shown a correlation between EGR1 expression and HPSE mRNA expression (39), further supporting a role for EGR1 in regulating HPSE transcription in tumor cells. In this article we have functionally dissected the human HPSE promoter and identified EGR1 as critical in binding to two elements in the HPSE promoter and regulating transcription in tumor cells. Interestingly, EGR1 was found to have differential regulatory effects on HPSE transcription depending on the tumor type. These results represent an important advance into understanding the mechanisms that control transcription of the HPSE gene in tumor cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfections, and Luciferase Assays**—MCF7 human breast carcinoma cells were from American Type Culture Collection (ATCC, Manassas, VA), PC-3 human prostate adenocarcinoma cells (ATCC) MM170 human melanoma cells, COLO397 human colon carcinoma cells, and B16-F1 mouse melanoma cells were cultured in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum in a humidified atmosphere of 5% CO2 and 37 °C. Transient transfections of MCF7, PC-3, COLO397, and B16-F10 cells were performed using Lipofectin (Invitrogen) as per the manufacturer’s instructions. Briefly for the luciferase reporter experiments, cells at 70–80% confluence were transfected with luciferase reporter constructs and a Renilla luciferase construct, pRLTK (Promega), as an internal control. Transient transfection of MM170 melanoma cells were performed using Bio-Rad Gene Pulser II (Bio-Rad) at 270 volts and 975 capacitance with 5 μg of reporter construct and 1 μg of pRLTK being transfected per 4–5 × 106 cells. For the EGR1 overexpression experiments, each of the tumor cell lines were transfected as described above, with a constant amount of total DNA used, i.e. when decreasing amounts of pCR3.1-EGR1 or pCB6-Egr1 were transfected, the balance was made up by co-transfection with the backbone constructs, pCR3.1 or pCB6+. Each of the tumor cell lines were transfected at similar efficiencies. After transfection, cells were rested for 24 h and then assayed for luciferase activity using a Dual-Glo™ luciferase assay system (Promega). Plates were read on a Reporter Microplate Luminometer (Turner Biosystems, Sunnyvale, CA). In activation assays, cells were rested for 24 h and then stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) for 16 h then assayed for luciferase activity as described above. Overexpression studies and mutagenesis effects were analyzed for statistical significance by using a two-tailed Student’s t test.

**Plasmid Constructs**—Luciferase reporter constructs pXP-1/1300bp, pXP-1/520bp, pXP-1/280bp, pXP-1/120bp, pXP-1/280bpMUT, and pCB6-Egr1 have been previously described (42). The pXP-1/280bp-MUT2 construct was generated by splice overlap extension PCR by mutating the core sequence of the second putative EGR1 binding site, TGGG, to GTTT using the pXP-1/280bp construct as a template. Briefly two PCR were performed to generate overlapping 5’ and 3’ fragments encoding each mutation, which were then spliced together in a third PCR. The oligonucleotide primers used to generate these fragments were MWT-1 and HH1P1 or MWT-2 and HH3P. The sequences of oligonucleotide primers MWT-1, MWT-2, HH1P1, and HH3P were 5’-AACAGAGGAGTTAGGGATGGAGGGC-3’, 5’-TC- CATCCCTAAACCTCTCTTCTG-3’, 5’-TTGAGGCCGACGAC- CATC-3’ and 5’-GTAAGTGAAACGTGACC-3’, respectively. A 478-bp fragment of the mouse HPSE gene promoter was amplified by PCR from genomic DNA isolated from C57BL/6 mice, using oligonucleotide primers, MHP1 and MHP6. The amplified fragment was cloned into the BglII/KpnI site in pXP-1, to generate the vector pXP-1/0.5kb. The sequences of MHP1 and MHP6 were 5’-CAGCATCCCCACTGGTGCT-3’ and 5’-TAAAGGCGAGAGGAGCTC-3’, respectively. The pXP-1/0.5kbMUT1 construct was generated using the QuikChange® XI XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) as described by the manufacturer, using pXP-1/0.5kb as a template. The core binding motif (underlined) of the putative Egr1 site, AGGGGGAGGA, was mutated to TGTT. The sequences of the oligonucleotide primers used to generate this mutation were: MEGRM1F, 5’-GG- ACTTCCGGGAGGTTGGAGGGATGGAGCGCTG-3’ and MEGRM1R, 5’-CAGGGAGGTTGAGGGATGGAGCGCTG-3’, respectively. This fragment was cloned into pCR3.1 (Invitrogen) as per the manufacturer’s instructions. The nucleotide sequence integrity of all clones was confirmed by automated sequencing (Biomolecular Resource Facility, The John Curtin School of Medical Research, Canberra, Australia) using an ABI 3730 Analyzer (Applied Biosystems, Foster City, CA) following the manufacturer’s protocol.

**RNA Extraction and Real Time Quantitative RT-PCR**—Total RNA was extracted from tumor cells at 80% confluence, using TriReagent® (Molecular Research Center Inc., Cincinnati, OH) as described by the manufacturer. cDNA synthesis was performed on 1 μg of total RNA using Superscript™ II Reverse Transcriptase as per the manufacturer’s instructions (Invitrogen). SYBR Green (Qiagen GmbH, Hilden, Germany) was used as the fluorophore in a real-time PCR analysis using the ABI 7000 System (ThermoFisher, CA) as previously described (56). The results were normalized to the expression of HPSE, using HPSE as a housekeeping gene as previously described (37). The primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA) and are shown in Table I. The reactions were performed using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer’s guidelines. The amplifications conditions were as follows: 50 °C for 2 min; 95 °C for 10 min; 50 cycles of 95 °C for 15 s, and 60 °C for 1 min.

**Plasmid Constructs**—Luciferase reporter constructs pXP-1/1300bp, pXP-1/520bp, pXP-1/280bp, pXP-1/120bp, pXP-1/280bpMUT, and pCB6-Egr1 have been previously described (42). The pXP-1/280bp-MUT2 construct was generated by splice overlap extension PCR by mutating the core sequence of the second putative EGR1 binding site, TGGG, to GTTT using the pXP-1/280bp construct as a template. Briefly two PCR were performed to generate overlapping 5’ and 3’ fragments encoding each mutation, which were then spliced together in a third PCR. The oligonucleotide primers used to generate these fragments were MWT-1 and HH1P1 or MWT-2 and HH3P. The sequences of oligonucleotide primers MWT-1, MWT-2, HH1P1, and HH3P were 5’-AACAGAGGAGTTAGGGATGGAGGGC-3’, 5’-TC- CATCCCTAAACCTCTCTTCTG-3’, 5’-TTGAGGCCGACGAC- CATC-3’ and 5’-GTAAGTGAAACGTGACC-3’, respectively. A 478-bp fragment of the mouse HPSE gene promoter was amplified by PCR from genomic DNA isolated from C57BL/6 mice, using oligonucleotide primers, MHP1 and MHP6. The amplified fragment was cloned into the BglII/KpnI site in pXP-1, to generate the vector pXP-1/0.5kb. The sequences of MHP1 and MHP6 were 5’-CAGCATCCCCACTGGTGCT-3’ and 5’-TAAAGGCGAGAGGAGCTC-3’, respectively. The pXP-1/0.5kbMUT1 construct was generated using the QuikChange® XI XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) as described by the manufacturer, using pXP-1/0.5kb as a template. The core binding motif (underlined) of the putative Egr1 site, AGGGGGAGGA, was mutated to TGTT. The sequences of the oligonucleotide primers used to generate this mutation were: MEGRM1F, 5’-GG- ACTTCCGGGAGGTTGGAGGGATGGAGCGCTG-3’ and MEGRM1R, 5’-CAGGGAGGTTGAGGGATGGAGCGCTG-3’, respectively. This fragment was cloned into pCR3.1 (Invitrogen) as per the manufacturer’s instructions. The nucleotide sequence integrity of all clones was confirmed by automated sequencing (Biomolecular Resource Facility, The John Curtin School of Medical Research, Canberra, Australia) using an ABI 3730 Analyzer (Applied Biosystems, Foster City, CA) following the manufacturer’s protocol.
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Germany) real time PCR for amplification of heparanase, EGR1, or the housekeeper gene ubiquitin-conjugating enzyme, E2D 2, were performed using an ABI PRISM 7700 sequence detector (PerkinElmer Life Sciences) as previously described (42). A dissociation curve was performed after each experiment to confirm a single product was amplified. A standard curve was generated for each gene using known copy numbers of a plasmid containing the cdNA specific to the gene.

**Chromatin Immunoprecipitation (ChIP) Assay—**ChIP analysis was performed on 5 × 10^6 Jurkat T cells either non-stimulated or following stimulation with PMA (20 ng/ml) and calcium ionophore (Sigma) (1 μM) for 4 or 12 h following a protocol previously described with some modifications (54, 55). In brief, cells were harvested and cross-linked with 1% formaldehyde for 10 min, and the reaction was terminated by the addition of 0.25 M glycine. Cells were washed four times in ice-cold phosphate-buffered saline, resuspended in ChIP SDS lysis buffer (Upstate Biotechnology, Charlottesville, VA) in the presence of Complete protease inhibitors (Roche Diagnostics), and sonicated to shear chromatin using a Cole Palmer Ultrasonic processor (Cole Palmer, Vernon Hills, IL). The sonicated DNA fragments were in the range of 100 to 1000 bp. The samples were pre-cleared with 60 μl of salmon sperm DNA-protein A-agarose (Upstate Biotechnology) and subsequently incubated with either 4 μg of anti-EGR1 antibody Egr1X-588 (Santa Cruz Biotechnology) or without antibody as a control. overnight with 4°C. Immunocomplexes were recovered with salmon sperm DNA-protein A-agarose (Upstate Biotechnology), washed extensively as described previously (55), and eluted with ChIP elution buffer (Upstate Biotechnology). Following the reversal of cross-links at 65 °C overnight, samples were extracted with phenol/chloroform and resuspended in Milli Q water for real time PCR analysis. ChIP-purified DNA was screened for HPSE and CD69 proximal promoter fragments or –1 kb HPSE promoter fragments by real time PCR analysis as described previously (54, 55). The sequence for the oligonucleotide primers were: HPSE set A (proximal HPSE promoter) sense, 5′-TTCTGTAAGTGAAGCTCAGCCGGCC-3′ and antisense, 5′-CCCTCAGATCTCCCTCCACT-3′; HPSE set B (–1 kb HPSE promoter) sense, 5′-TTCTGACACTTCACATGCCGGC-3′ and antisense, 5′-AACCTGCCGAGTGTGACTTCGATCT-3′; and CD69 (proximal CD69 promoter) sense, 5′-AACCTGCAGCCTTCTGCTCT-3′ and antisense, 5′-GCCGCCATCTTGCTGACTA-3′. The amount of precipitated target sequence was calculated by normalization with the total input DNA after subtraction of the no antibody background as described previously (54, 55).

**Nuclear Extraction and Electrophoretic Mobility Shift Assay—**PC-3 or MCF7 cells at 80% confluence were harvested and washed in 10 ml of ice-cold phosphate-buffered saline either non-stimulated or after activation with PMA for 1.5 h. Nuclear extraction was then performed as previously described (56). Nuclear extracts were incubated for 30 min at 4°C for 1.5 h. Nuclear extraction was then performed as described previously (54, 55). In brief, cells were harvested and cross-linked with 1% formaldehyde for 10 min, and the reaction was terminated by the addition of 0.25 M glycine. Cells were washed four times in ice-cold phosphate-buffered saline, resuspended in ChIP SDS lysis buffer (Upstate Biotechnology, Charlottesville, VA) in the presence of Complete protease inhibitors (Roche Diagnostics), and sonicated to shear chromatin using a Cole Palmer Ultrasonic processor (Cole Palmer, Vernon Hills, IL). The sonicated DNA fragments were in the range of 100 to 1000 bp. The samples were pre-cleared with 60 μl of salmon sperm DNA-protein A-agarose (Upstate Biotechnology) and subsequently incubated with either 4 μg of anti-EGR1 antibody Egr1X-588 (Santa Cruz Biotechnology) or without antibody as a control. overnight with 4°C. Immunocomplexes were recovered with salmon sperm DNA-protein A-agarose (Upstate Biotechnology), washed extensively as described previously (55), and eluted with ChIP elution buffer (Upstate Biotechnology). Following the reversal of cross-links at 65 °C overnight, samples were extracted with phenol/chloroform and resuspended in Milli Q water for real time PCR analysis. ChIP-purified DNA was screened for HPSE and CD69 proximal promoter fragments or –1 kb HPSE promoter fragments by real time PCR analysis as described previously (54, 55). The sequence for the oligonucleotide primers were: HPSE set A (proximal HPSE promoter) sense, 5′-TTCTGTAAGTGAAGCTCAGCCGGCC-3′ and antisense, 5′-CCCTCAGATCTCCCTCCACT-3′; HPSE set B (–1 kb HPSE promoter) sense, 5′-TTCTGACACTTCACATGCCGGC-3′ and antisense, 5′-AACCTGCCGAGTGTGACTTCGATCT-3′; and CD69 (proximal CD69 promoter) sense, 5′-AACCTGCAGCCTTCTGCTCT-3′ and antisense, 5′-GCCGCCATCTTGCTGACTA-3′. The amount of precipitated target sequence was calculated by normalization with the total input DNA after subtraction of the no antibody background as described previously (54, 55).

**RESULTS**

**EGR1 Binds the HPSE Promoter in Vivo—**We have previously shown that EGR1 binds to the proximal HPSE promoter in vitro and regulates inducible HPSE expression in the human leukemic T cell line, Jurkat (42). Initially ChIP analysis was carried out to confirm EGR1 binding to the HPSE gene promoter in vivo. Jurkat T cells that were either non-stimulated or stimulated for 4 or 12 h with the pharmacological agonists PMA and calcium ionophore were treated with formaldehyde to cross-link protein to binding sites in DNA. As a positive control, the precipitated EGR1-DNA complexes were initially analyzed by real time RT-PCR using primers specific to the proximal CD69 gene promoter. EGR1 sites have been previously characterized in this region of the CD69 gene promoter that regulate inducible CD69 expression (57). As shown in Fig 1A, activation of Jurkat T cells resulted in a 185-fold increase in the binding of EGR1 to the proximal CD69 promoter. Samples were then analyzed using primers specific to the proximal HPSE gene promoter containing the previously characterized EGR1 binding site (HPSE set A). Activation of Jurkat T cells for 12 h resulted in an increase in EGR1 binding to the proximal HPSE promoter of 40.5-fold when compared with EGR1 binding in resting cells (Fig 1B). No PCR amplification could be detected in the no antibody control precipitated DNA (TABLE ONE). Interestingly, EGR1 binding could be detected at low levels in anti-EGR1-treated chromatin from non-stimulated Jurkat T cells (TABLE ONE). To determine whether this binding was specific to the proximal promoter, DNA samples were also analyzed by real time RT-PCR using primers specific to the –1 kb HPSE promoter region predicted to contain no EGR1 sites (HPSE set B). Activation of cells for 4 and 12 h resulted in a 5-fold increase in EGR1 binding to the –1 kb HPSE promoter when compared with non-stimulated cells (Fig 1B). This small increase in EGR1 binding is consistent with the detection of low numbers of longer than 1000 bp DNA sonication fragments that would encompass the proximal promoter. These in vivo data confirm previous observations using in vitro assays (42) that EGR1 binds to the proximal HPSE gene promoter.

**Identification of a cis-Responsive Element within the HPSE Promoter in Tumor Cell Lines—**To investigate the potential regulation of HPSE transcription in tumor cells by the transcription factor, EGR1, four tumor cells lines of different epithelial and tissue origins were selected for study, human colon carcinoma (COLO397), human prostate adenocarcinoma (PC-3), human breast adenocarcinoma (MCF7), and human melanoma (MM170). Clinical tumor samples from each of these tissues have been previously described to overexpress heparanase (23, 39, 58, 59). Real time RT-PCR was used to confirm HPSE mRNA expression in each of the cell lines described above. Expression was high although variable in each of the cell lines ranging from 43,000 copies mRNA/μg of RNA for MCF7 cells to 276,000 copies per μg of RNA for MM170 melanoma cells (data not shown).

To identify the region of the HPSE promoter responsible for regulating transcription of the HPSE gene in tumor cells, transient transfection
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In contrast, the longer 1300-bp fragment showed variable activity, with the highest activity noted in COLO397 cells. These data indicate that the −164 to +2 region of the HPSE promoter is most active in all four tumor cell types. It should be noted that the longer promoter fragments suggest the existence of an upstream repressor element that is active in all four tumor cell lines. Interestingly, in contrast to that observed for the PC-3, MCF7, and MM170 cell lines, the repressor activity was not apparent in the 1300-bp promoter fragment of the COLO397 cell line.

EGR1 Can Activate or Repress the HPSE Promoter Depending on Tumor Cell Type—The −164 and +2 region in the HPSE promoter identified above, contains two putative EGR1 binding sites (Fig. 3A). The site designated EGR1/A, i.e. GTGAGGAGGCGT, was previously characterized to be critical to inducible expression of HPSE in T cells (42). A second site designated EGR1/B, i.e. AGGAGTGGAGG, was predicted by bioinformatics (Match™) as a potential binding site for all members of the EGR family, including EGR1, EGR2, EGR3, and EGR4. To determine the functionality of these EGR1 sites in tumor cells, we co-transfected an EGR1 expression construct (pCR3.1-EGR1) in increasing amounts along with the reporter construct pXP-1/280bp that contained the two putative EGR1 binding sites and performed luciferase assays. EGR1 trans-activated HPSE promoter activity in a dose-dependent manner when compared with cells co-transfected with pXP-1/280bp and backbone pCR3.1 alone, in PC-3, COLO397, and MCF7 cells (Fig. 3B). In contrast, co-transfection of EGR1 resulted in a dose-dependent repression of promoter activity in the melanoma cell line, MM170. These results suggested that the 280-bp human promoter fragment contains an EGR1 responsive element in the four tumor cell lines; and it is likely that at least one of the above described EGR1 sites was functional.

Identification of Two EGR1 Binding Sites—To confirm that the EGR1 sites described above were functional and to differentiate between the potential activity of the two sites, transversion mutations of the predicted core binding motif of either EGR1/A or EGR1/B were carried out on the reporter construct, pXP-1/280bp (resulting in reporter constructs, pXP-1/280bpMUT and pXP-1/280bpMUT2 respectively), and transient transfection analysis was performed. Mutation of the EGR1/A site previously characterized in T cells resulted in a 74–86% reduction in promoter activity in PC-3, COLO397, MCF7, and MM170 cells when compared with wild-type activity (Fig. 4A). Interestingly, mutation of the EGR1/B site also resulted in a 64–72% decrease in promoter activity when compared with wild-type activity in PC-3, COLO397, and MCF7 cell lines; indicating that this site is also active and therefore identifies a second functional EGR1 site in the HPSE promoter. Mutagenesis of the EGR1/B site resulted in a 38% decrease in promoter activity in MM170 cells (Fig. 4A).

To further characterize the contribution of each putative EGR1 site in mediating activation of the HPSE promoter observed in Fig. 3B, we performed EGR1 trans-activation studies using the mutant reporter constructs described in the legend to Fig. 4A. We co-transfected the EGR1 expression construct (pCR3.1-EGR1) in increasing amounts along with either pXP-1/280-bpMUT or pXP-1/280-bpMUT2 and performed transient transfection analysis in PC-3, MCF7, and MM170 cells. EGR1 retained the ability to trans-activate the HPSE promoter despite mutation of either EGR1/A or EGR1/B sites in PC-3, MCF7, and MM170 cells. EGR1 retained the ability to trans-activate the HPSE promoter even when compared with wild-type trans-activation observed in Fig. 2B. In MM170 cells, EGR1 mediated only a small repression effect with the EGR1/A site mutated that was not significant. In contrast, EGR1 mediated a significant repression effect with the EGR1/B site mutated, although this was less marked when compared with the effects on the wild-type construct,
TABLE ONE

| HPSE set A (No Ab) | HPSE set A | HPSE set B (No Ab) | HPSE set B | Primer CD69 (No Ab) | Primer CD69 |
|-------------------|------------|--------------------|------------|--------------------|------------|
| Relative copy enrichment (IP/total input) × 100% |
| NS                 | 0.000%     | 0.000%             | 0.000%     | 0.000%             | 0.000%     | 0.000%     |
| 4 h                | 0.000%     | 0.410%             | 0.000%     | 0.070%             | 0.000%     | 0.170%     |
| 12 h               | 0.000%     | 1.500%             | 0.000%     | 0.070%             | 0.000%     | 5.600%     |

FIGURE 2. Identification of a cis-responsive element within the HPSE promoter in tumor cell lines. A, schematic of the varying lengths of the HPSE promoter cloned into the luciferase reporter construct, pXP-1. The arrow indicates the transcription start site. The numbering describes the promoter fragments relative to the transcription start site (+1). B, luciferase reporter analysis of the HPSE promoter. HPSE promoter activity was assessed using the constructs shown in A. Each construct along with a Renilla control construct were transfected into PC-3, MCF7, COLO397, or MM170 cells. At 24 h after transfection, cells were harvested and the luciferase activity was measured for firefly and Renilla luciferase using the Dual-Glo luciferase assay system (Promega) and a luminometer. Luciferase ratio was calculated as firefly expression relative to Renilla expression. The data represents two independent experiments each with duplicate transfections. The error bars represent the mean ± S.E.

pXP-1/280bp shown in Fig. 3B. These results, together with those observed in Fig. 4A, confirm that both EGR1 sites are active in PC-3, MCF7, and MM170 cells. Furthermore, results obtained in MM170 cells suggest that both EGR1/A and EGR1/B sites contribute to the repression effect of EGR1 in this cell type.

EGR1 Represses the Mouse HPSE Promoter in Melanoma Cells—To explore the repression effect of EGR1 on the HPSE promoter in MM170 melanoma cells, we carried out trans-activation experiments in a mouse melanoma cell line, B16-F1. Co-transfection of Egr1 along with a 478-bp mouse Hpse promoter construct (−494 to +84 bp relative to transcription start site) in the B16-F1 mouse melanoma cells, resulted in a dose-dependent repression of HPSE promoter activity (Fig. 5A) similar to that observed in human MM170 cells. This is in contrast to our findings in mouse mammary tumor cells where EGR1 positively regulates mouse Hpse promoter activity.4 The conservation across species of this repressive activity in melanoma cells by EGR1 suggests that the effect is melanoma cell-specific and not simply a unique property of the human cell line, MM170. Sequence analysis of the mouse Hpse gene promoter using bioinformatics analyses (Match™) revealed that the EGR1/B site but not the EGR1/A site is conserved across both the mouse and human promoter sequences. To investigate whether this putative Egr1 site is functional in the mouse Hpse promoter, transversion mutation of the core binding motif, GTGG, of the putative Egr1 site was carried out on the reporter construct pXP-1/0.5kb, and transient analysis performed in B16-F1 cells. Mutation of the GTGG motif results in a 73% decrease in promoter activity when compared with wild-type activity (Fig. 5B).

4 A. M. de Mestre, T. Soe-HTwe, and M. D. Hulett, manuscript in preparation.
Regulation of HPSE Transcription in Tumor Cells by EGR1

EGR1 Protein from Activated Tumor Cells Binds to Both the EGR1/A and EGR1/B Sites in the HPSE Promoter—Electrophoretic mobility shift assays were performed to determine whether EGR1 protein from tumor cells could bind to both EGR1/A and EGR1/B sites. Initially nuclear extracts from resting PC-3 and MCF7 cells were incubated with a HPSE promoter oligonucleotide (\(^{32}\)P-hep1) 5'-GAGGGGTAGGGAGGCGGJAAACGGGGCGGAGG-3', which encompassed the EGR1/A binding site (underlined). Two nucleoprotein complexes were formed, however, pre-clearing with an anti-EGR1 antibody confirmed that neither complex involved EGR1 (Fig. 6A). Consistent with previous observations in thyroid tumor cells (37), one of these complexes involved Sp1, as shown by a supershift in lanes 4 and 10 and partial loss of binding to a mutant probe (lanes 6 and 12). Recent in vivo studies indicate that EGR1 does play an important role in tumor growth and angiogenesis or tumor progression in MCF7 and MDAMB231 breast adenocarcinoma cells and prostate adenocarcinoma cells (47, 51, 52). In the case of MCF7 and MDAMB231 cells, this key function occurs despite low EGR1 expression as measured in vitro (48). EGR1 has been shown to be induced by numerous bioactive proteins and environmental conditions such as hypoxia (41, 44), which are present in the context of the tumor environment. Therefore, these studies were extended to determine the interaction between EGR1 induced in tumor cells and the HPSE promoter oligonucleotide \(^{32}\)P-hep1. Nuclear extracts harvested from PC-3 cells stimulated with PMA for 1.5 h and incubated with the \(^{32}\)P-hep1 probe formed two nucleoprotein complexes (C1 and C2) that were also present in resting PC-3 cells (Fig. 6B), as well as a third complex that was confirmed to contain EGR1 by supershift using an anti-EGR1 antibody (Fig. 5B, lane 4). The complex labeled C1 was determined to be Sp1 by supershift (data not shown). Competition studies using 100 times excess of an unlabeled probe hep1 confirmed the specificity of the complexes. To determine the requirement of the EGR1 core binding motif for EGR1 binding, an additional probe, \(^{32}\)P-mhep1 (EGR1/A), was then designed with the same 4-bp transversion mutation of the core EGR1 binding site as that performed to create the mutant reporter construct used in transient transfection analysis. No EGR1 nucleotide complex formed with either \(^{32}\)P-mhep1 (Fig. 6C, lane 3), indicating that the EGR1 complex observed in Fig. 6B is dependent on the core motifs GGCG. Interestingly, there is also partial loss of binding of C1 (Sp1). The same nucleoprotein complexes were observed using nuclear extracts from activated MCF7 cells (data not shown).

The studies were then repeated using a second HPSE promoter oligonucleotide, (\(^{32}\)P-hep2) 5'-TGGAGAGGGAGGAGGGATGGAGGGCCGAGC-3', which encompassed the EGR1/B site (underlined). Nuclear extracts from resting PC-3 cells incubated with \(^{32}\)P-hep2 resulted in three nucleoprotein complexes, C3, C4, and C5 (Fig. 6B). The complex labeled C3 is Sp1 (data not shown). Similarly to that observed with the \(^{32}\)P-hep1 probe, an EGR1 containing nucleoprotein complex forms upon PMA activation of cells as shown by a supershift in lane 9. Competition studies using 100 times excess of an unlabeled probe hep2 confirmed the specificity of complexes C3, C4, and EGR1. To determine the requirement of the EGR1 core binding motif for EGR1 binding to the EGR1/B site, an additional probe, \(^{32}\)P-mhep2, was then designed with the same 4-bp transversion mutation of the core EGR1 binding site as that performed to create the mutant reporter construct pXP-1/280bpMUT2 used in transient transfection analysis. No EGR1 nucleotide complex formed with \(^{32}\)P-mhep2 (Fig. 6C, lane 7), indicating
that the EGR1 complex observed in Fig. 6B is dependent on the core motif TGGG. Interestingly, there is also loss of binding of C3 (Sp1) and partial loss of C4 and C5 on the 32P-mhep2 probe.

HPSE has also been shown to be induced in tumor cells in response to bioactive proteins such as growth factors (60) and estrogen (61). To confirm that the HPSE promoter fragment that contains the EGR1 sites has inducible transcriptional activity, transient transfection analysis was performed on PC-3 and MM170 cells either non-stimulated or stimulated with PMA for 16 h. As shown in Fig. 7A, the pXP-1/280bp construct shows significant inducible promoter activity in PC-3 cells. Only minimal inducible activity is observed by the constructs that contain mutations of either the EGR1/A or EGR1/B sites in PC-3 cells. In contrast, there is a small decrease in promoter activity of the pXP-1/280bp construct upon activation of MM170 cells, consistent with the differential regulation of the HPSE promoter in melanoma cells (Fig. 7A). The promoter activity of the construct with a mutation of the EGR1/B site also shows a small decrease in promoter activity upon activation of MM170 cells, whereas no change in promoter activity is observed by the construct with a mutation in the EGR1/A site (Fig. 7A).

**DISCUSSION**

HPSE function has been implicated in tumorigenesis for over two decades, and the recent cloning of the HPSE gene has enabled experimental confirmation that it does indeed play a key role in the metastasis of solid tumors as well as mediating tumor angiogenesis both directly by promoting endothelial cell migration and indirectly via release of pro-angiogenic molecules from the ECM (27–29). Numerous studies in...
tumor cells have correlated the metastatic potential of tumor cells with HPSE activity and protein expression (4, 5). Furthermore, extensive studies of clinical tumor samples have correlated high HPSE mRNA expression with tumor progression and in some cases poor patient post-operative survival (22, 24). This strongly suggests that deregulated HPSE gene function and protein expression in tumor cells is likely to be linked to dysfunctional HPSE transcription. Clearly, there is significant interest in identifying the molecular mechanisms that control HPSE transcription in normal and pathological settings. We have previously investigated regulatory mechanisms responsible for the normal induction of HPSE in T lymphocytes, and found that HPSE inducible expression in a Jurkat T cell model was regulated by the transcription factor EGR1 (42). Based on these studies, we asked the important question of whether deregulation of this physiological mechanism of HPSE gene expression in lymphocytes was contributing to the high levels of HPSE transcription observed in tumor cells. These current studies have shown that EGR1 can act as a regulator of HPSE transcription in four different tumor cells, but this role is cell specific as EGR1 can have differential effects, functioning as an activator in the case of prostate or breast adenocarcinoma cells and colorectal carcinoma cells, whereas it acts as a repressor in the case of melanoma cells. Furthermore, this study provides the first evidence that EGR1 binds to the HPSE gene promoter in vivo.

EGR1 is an early growth response gene that is induced by a variety of stimuli, including hypoxia (44), growth factors (41), estrogen (62), and neurotrophins (63), all of which are well known to act as agonists promoting tumor angiogenesis and metastasis. Studies in Egr1 knock-out mice have defined a key role for Egr1 in tumor progression of prostate carcinomas (52) and fibroblast growth factor-dependent tumor growth of breast carcinoma cells (47). Interestingly, growth of B16 melanoma cells progressed normally in these mice (47). These data correlate with our observations using mutagenesis and trans-activation studies presented herein that EGR1 up-regulates HPSE transcription in breast, prostate, and colorectal tumor cell lines, but has repressive activity in melanoma cells. Furthermore, a recent study in human prostate cancer cells has found that EGR1 expression correlates with HPSE mRNA expression (39). Our findings provide the first evidence as to the mechanism that EGR1 mediates regulation of HPSE expression in tumor cells. Electrophoretic mobility shift assay studies showed that EGR1 could bind to two distinct sites in the HPSE promoter after activation of the cells with PMA, a general pharmacological agonist that we used to mimic extracellular stimuli. Furthermore, trans-activation studies carried out using reporter constructs with mutations in the EGR1 sites confirmed that both sites contributed to the trans-activation effect observed by EGR1. The EGR1 site located at the 3′ end of the 280-bp fragment has been previously characterized in T cells and our results confirm that this site is also active in tumor cells. These studies are the first to identify the functionality of the 5′ EGR1 site in any cell type.

HPSE is induced in tumor cells by growth factors (hepatocyte growth factor, basic fibroblast growth factor, and platelet-derived growth factor) (60), estrogen (61), and neurotrophins (64), agonists that also result in rapid induction of EGR1 expression. Under the experimental conditions described herein we found that the 280-bp HPSE promoter fragment had significant inducible activity that was dependent on the EGR1 sites in the HPSE promoter, and furthermore, this corresponded to an induction of HPSE protein expression in prostate carcinoma cells. Interestingly, Elkin et al. (61) also identified an inducible region in the HPSE promoter, and speculated that four putative estrogen response regions located in this region may be mediating the induction of HPSE expression in response to estrogen treatment. The previously characterized induction of EGR1 in MCF7 cells upon stimulation by estrogen (62) lends weight to the notion that the induction of HPSE mRNA observed by Elkin et al. (61) could be contributed to by EGR1. Our findings,
together with these observations, have led us to hypothesize that EGR1 may mediate growth factor, estrogen, and neurotrophin induction of HPSE expression. Two previous studies (36, 37) have reported deletion analysis of the HPSE promoter in resting tumor cells. These differ slightly in their definitions of the minimal promoter to a region 300- or 700-bp upstream of the translation start codon (36, 37). Our findings in four different tumor cell lines define the minimal HPSE promoter to the 280-bp region (specifically /H11002 to /H11001 compared with the transcription start site), which is consistent with findings in three different thyroid tumor lines (37) and other cell types, including endothelial cells and T cells (42, 65). These data suggest that the longer minimal promoter noted only in MDAMB231 cells may be unique to this cell line (36). Three Sp1 sites and four ETS response elements have been defined in the minimal promoter region (37). Two of these Sp1 sites and all four of the ETS sites are located in the 280-bp promoter fragment used in our studies. As the consensus, sequences of EGR1 and Sp1 are very similar, it was not surprising to find that Sp1 bound to both EGR1 promoter probes in the electrophoretic mobility shift assays studies. Furthermore, previous studies have shown Sp1 binding to the EGR1/A site (37, 42). It is likely that EGR1 and Sp1 cooperate in promoting HPSE transcription, as described on several gene promoters in vascular systems (66), although additional studies are required to determine whether this interaction is occurring on the HPSE promoter. Two further ETS sites that are trans-activated by Ets-1 and to a lesser extent, Ets-2, PEA3, and ER81, have been defined in MDAMB231 cells (36). One of these sites is located in the 520-bp promoter fragment used in our studies, the other situated in the 1300-bp fragment. Both the 520- and 1300-bp fragments

![Figure 6](http://www.jbc.org)
FIGURE 7. Identification of an inducible promoter element. A, HPSE promoter constructs along with Renilla control constructs were co-transfected into PC-3 or MM170 melanoma cells and rested for 24 h. Cells were then either stimulated with PMA, 50 ng/ml, or non-stimulated (NS). At 16 h after stimulation, cells were harvested and the luciferase activity assayed as described in the legend to Fig. 2B. The data are representative of two independent experiments each with duplicate transfections. The error bars represent the mean ± S.E. The asterisk indicates a p value of <0.03 when compared with resting cells. B, Western blot analysis using a human HPSE antibody was carried out on 1.25 × 10^6 PC-3 cells either resting (NS) or activated with PMA for 8, 12, 24, 48, or 72 h. An ~50-kDa HPSE-specific band is indicated. The positions of the molecular mass markers in kDa are shown. A Coomassie Blue-stained gel identical to the above represents the protein loading control.
showed low promoter activity in three of the four tumor cell lines used in our studies, suggesting that these sites play a less important role in regulating HPSE transcription in these cell lines. Of note is the previous identification of EGR1 as a Ets-1 target gene (67). Again, it would be interesting to investigate if the trans-activating response elicited by Ets-1 on the HPSE promoter also has an indirect component mediated through Ets-1 activation of EGR1.

It is interesting to note that studies of cis-acting mechanisms of HPSE gene regulation have defined a 483-bp CpG island (38), which encompasses the 280-bp fragment used in our studies, and methylation of this region confers low gene expression that can be reversed by treatment with demethylating agents (21, 38). This effect was shown to be independent of changes in chromatin structure, and transacting mechanisms were not excluded. Significantly, Simizu et al. (21), showed the effect of demethylating agents on HPSE transcription was absent when de novo protein synthesis was inhibited, suggesting that transacting factors may be mediating this methylation response. A recent study in prostate tumor cells and benign prostatic hyperplasia samples suggests that methylation at the EGR1/A site may contribute HPSE expression (39). Interestingly, this study also found that EGR1 expression was more restricted to MM170 cells, but is rather melanoma cell-specific. Induction of EGR1 may repress HPSE promoter activity by displacement of another transcription factor previously shown to have a repression activity. These are currently being investigated to define the repression activity in this region of the HPSE promoter.

In contrast to the observed trans-activation role of EGR1 in PC-3, MCF7, and COLO397 cells, this transcription factor was noted to play a repressive role in regulating HPSE expression in MM170 melanoma cells. Interestingly, this repressive role of EGR1 on HPSE promoter activity was conserved across species, as shown by the repressive activity of EGR1 on mouse HPSE promoter activity in B16 melanoma cells. Together, this provides significant evidence that this effect is not restricted to MM170 cells, but is rather melanoma cell-specific. Sequencing of the 280-bp human HPSE promoter region in the four tumor lines did not reveal any nucleic acid changes that could be responsible for this effect (data not shown). Mutagenesis experiments in melanoma cells indicate that mutation of the EGR1 sites in the human and mouse HPSE promoters results in loss of HPSE promoter activity. Whereas the mechanism by which EGR1 mediates this repressive role in melanoma cells remains unclear, this mutagenesis data suggests that EGR1 may repress HPSE promoter activity by displacement of another transcription factor whose activity is both specific and activatory in melanoma cells.

In conclusion, we have demonstrated that EGR1 regulates HPSE transcription in tumor cells, interestingly, in an activatory manner in PC-3 human prostate adenocarcinoma cells, MCF7 human breast carcinoma cells, and COLO-397 human colon carcinoma cells, or a repressive manner in human and mouse melanoma cells. The basis of this differential regulatory function awaits further investigation. Furthermore, we have identified a second functional EGR1 site in the HPSE promoter that is conserved between human and mouse. In addition, we have demonstrated that EGR1 binds the HPSE promoter in vivo providing the first evidence of in vivo binding of any protein to the HPSE gene promoter. In conjunction with the characterized key regulatory role of EGR1 in inducible HPSE gene transcription in T cells, this provides strong evidence that EGR1 is a major player in controlling HPSE expression across different cell types. The targeting of EGR1 to inhibit HPSE gene expression may well represent a novel approach for inhibiting heparanase function in tumorigenesis. Indeed, gene silencing of EGR1 in vascular proliferative disorders and tumorigenesis has proven a promising interventional approach in animal models of these diseases.

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