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Egr-1 Mediates Transcriptional Activation of IGF-II Gene in Response to Hypoxia[1]

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ABSTRACT

We have previously reported that the exposure of human HepG2 cells to hypoxic conditions results in the overexpression of human insulin-like growth factor II (IGF-II) mRNA whose size is 6.0 kb. This particular size of IGF-II mRNA is transcribed under the control of the IGF-II P3 promoter. In the present study, to delineate the molecular mechanism for the activation of the IGF-II gene, we examined the induction of P3 promoter activity in HepG2 cells by hypoxia in the transient expression system. In this system, hypoxia induced a linear increase within 24 h in the expression of luciferase that was driven by the IGF-II P3 promoter. To further delineate which factors mediate this response, the expression pattern of regulators of the P3 promoter, *Egr-1*, *Sp1*, and *WT1*, were analyzed by reverse transcription-PCR and Northern blot analysis. We found that hypoxia increased the expression of *Egr-1* but not of *Sp1*. In contrast, the level of *WT1*, a repressor of IGF-II expression, was markedly decreased during hypoxia. The mRNA stability assay revealed that the induction of transcription is the mechanism of underlying *Egr-1* mRNA elevation. We then investigated the effects of hypoxia on the DNA binding activity of Egr-1. Both electrophoretic mobility shift assay and supershift assay demonstrated that the DNA binding activity of the Egr-1 protein was increased by hypoxia. In addition, the level of Egr-1 protein was also increased under the hypoxia as determined by Western blot analysis. Cotransfection of HepG2 cells with an Egr-1 expression vector and an IGF-II P3 promoter-luciferase reporter plasmid showed that the transcription of IGF-II was activated by Egr-1 in a dose-dependent manner. Moreover, the elevation of IGF-II P3 promoter activity was induced synergistically by the cotreatment of hypoxia with Egr-1 overexpression. Deletion of sequences in the IGF-II P3 promoter containing Egr-1 binding sites did not respond to hypoxic stress. Taken together, these data strongly indicate that hypoxia-induced IGF-II expression in HepG2 cells is due to the enhanced activity of Egr-1 on the IGF-II P3 promoter and that the Egr-1 binding site in the IGF-II P3 promoter is essential for the transcriptional regulation of IGF-II under hypoxic conditions.

INTRODUCTION

Low cellular oxygen tension, or hypoxia, is a feature of physiological conditions, such as adaptations to high altitude and physical endurance exercise (1, 2), and pathophysiological conditions, including ischemia, fibrosis (3), and neoplasia (4). Human solid tumors, even those <1 cm in diameter (*i.e.*, at the limits of clinical detection), may have substantial hypoxic fractions (5).

Recent studies have shown that hypoxia detected in the central region of a tumor can be the leading cause of angiogenesis by activating the expression of angiogenic factors (2, 6, 7). Of the angiogenic factors, expression of the vascular endothelial growth factor (2, 8), platelet-derived growth factor- β (7), interleukin-6 (9), interleukin-8 (10), and acidic/basic fibroblast growth factor (11) genes are known to be hypoxia-inducible.

Much interest in IGF-II[4] has been generated by recent reports on the role of IGF-II protein as a potent mitogen (12) and inhibitor of apoptosis (13, 14) in several normal and neoplastic cell types. IGF-II is also known to exhibit an angiogenic activity in rat cornea assay (15) and quantitative chorioallantoic membrane assay (16). The human IGF-II gene is located on chromosome 11p15.5, downstream of the insulin gene, and spans 30 kb, which includes nine exons and four promoters (17). Transcription of the human IGF-II gene yields six kinds of mRNA species, which are expressed on tissue-specific manners during the development phase (18).

A dynamic control of the four promoters (P1-P4) of the IGF-II gene has been shown in hepatic development (19). The P1 promoter is active exclusively in human adult liver, whereas the activity of the P2 promoter has been detected only in certain human tumor cell lines. In contrast, P3 and P4 are highly active in fetal hepatic tissues but down-regulated shortly after birth (20). Enhanced levels of P3- and P4-driven IGF-II mRNA have been detected in many human tumors of different origins including hepatocellular carcinoma (21). These data suggest a role for P3- and P4-derived transcripts in autocrine or paracrine growth stimulation during tumorigenesis. However, the molecular mechanisms underpinning the activation of P3 and P4 promoters during the hepatocarcinogenesis are still unclear.

In this study, we show that the expression of the IGF-II gene is induced by hypoxia-mediated P3 promoter activation. Furthermore, we present the first evidence that Egr-1, a zinc finger-containing transcription factor, is involved in the activation of IGF-II transcription by hypoxia.

MATERIALS AND METHODS

Materials. Chemical reagents were obtained from Sigma (St. Louis, MO), except as noted. HepG2 cells were obtained from American Type Culture Collection (ATCC HB-8065). The cDNA probe of human *Egr-1* (3.2 kb) and *Egr-1* expression vector pEgr-1 were kindly provided by Dr. S. Murakami (Kanazawa University, Kanazawa, Japan). The IGF-II P3 promoter, HUP3, was a generous gift of Dr. J. S. Sussenbach (State University of Utrecht, Utrecht, the Netherlands).

Primers. Oligonucleotide primers for PCR were designed as follows: *β-actin*, 5′-GACTACCTCATGAAGATC-3′ and 5′-GATCCACATCTGCTGGAA-3′; *Egr-1*, 5′-GAGCCGAGCGAACAACCCTACGAGCACCTG-3′ and 5′-GCGCTGAGGATGAAGAGGTTGGAGGGTTGG-3′; *Sp1*, 5′-AATTTGCCTGCCCTGAGTGC-3′ and 5′-TTGGACCCATGCTACCTTGC-3′; *WT-1*, 5′-ACGCCCTCGCACCATGCGGCGCAGTTCCCC-3′ and 5′-CCTTTGGTGTCTTTTGAGCTGGTCTGAACG-3′. A double-stranded oligonucleotide (Ew) containing two *Egr-1* consensus sequences (CGCCCCCGC) separated by one nucleotide and an oligonucleotide (Em) containing two mutated *Egr-1* consensus sequences (CGCCCTAGC), and an oligonucleotide containing consensus sequences for transcriptional factor Sp1 (CCGCCC) were from Santa Cruz Biotechnology (Santa Cruz., CA).

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[4] The abbreviations used are: IGF-II, insulin-like growth factor II; WT1, Wilms' tumor suppressor gene; MEM, minimal essential medium; RT-PCR, reverse transcription-PCR; RLU, relative light units; EMSA, electrophoretic mobility shift assay; EBS, Egr-1 binding site; TBS, Tris-buffered saline.

Cell Culture and Hypoxic Condition. The human hepatoma cell line, HepG2, was cultured in MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin, and 100 mg/ml of streptomycin. After 3 days, the cells were washed with serum-free MEM, replaced with complete medium, and incubated under normoxic (5% CO_2, balanced with air) or hypoxic (1% O_2, 5% CO_2, balanced with N_2) conditions in a humidified incubator with an interior temperature of 37°C.

RT-PCR. Total RNA was isolated from cell cultures by a single-step procedure with TRI REAGENT (Life Technologies, Inc.). First-strand cDNAs were synthesized from 5 μ g of total RNA by incubating in 20 μ l of aliquots containing 200 units of superscriptase (Life Technologies, Inc.) and 500 ng of oligo-dT primer at 42°C for 1 h. PCR reaction was performed on the first-strand cDNA using the PCR reaction kit (Perkin-Elmer) and primer sets. Amplification by PCR was performed using an automated thermal cycler (Perkin-Elmer). A thermal cycler was set to the following cycle parameters: *β-actin* , at 94°C for 4 min (1 cycle), 94°C for 30 s, 55°C for 30 s, 72°C for 1 min (35 cycles), 72°C for 5 min (1 cycle); *Egr-1* , at 94°C for 3 min (1 cycle), 94°C for 30 s, 55°C for 30 s, 72°C for 1 min (30 cycles), 72°C for 5 min (1 cycle); *Sp1* , at 94°C for 3 min (1 cycle), 94°C for 1 min, 60°C for 1 min, 72°C for 1 min (30 cycles), 72°C for 5 min (1 cycle); *WT-1* , at 94°C for 3 min (1 cycle), 94°C for 1 min, 55°C for 2 min, 72°C for 2 min (35 cycles), 72°C for 5 min (1 cycle).

Northern Blot Analysis. Total RNA was prepared as described above. RNAs (20 μg) were fractionated on 1.2% agarose-6.6% formaldehyde gels, transferred to Zeta-Probe nylon membrane (Bio-Rad, Hercules, CA), and were covalently linked by UV irradiation (UV Stratalinker 1800; Stratagene, La Jolla, CA). The membrane was prehybridized at 42°C for 1 h in 50% formamide, 0.12 M Na_2HPO_4 (pH 7.2), 0.25 M NaCl, 7% SDS, and 1 mM of EDTA. Hybridization was then performed with the addition of 20 ng of α ^{32}P-labeled DNA probes at 42°C for 16 h. The membrane was washed twice with washing solution (0.5 × SSC/0.1% SDS) at 50°C for 30 min and exposed to X-ray film with intensifying screens at −70°C for 24 h. α -^{32}P-labeled DNA probes were prepared by using random primer oligonucleotides (*redi* prime DNA Labeling System, Amersham, United Kingdom).

mRNA Stability Assay. The mRNA stability of *Egr-1* was determined by treating HepG2 cells with the transcription inhibitor, actinomycin D, as follows. Actinomycin D was added into the growth medium (25 μg/ml) to block transcription. After 1 h, the cells were returned either to normoxic or to hypoxic conditions and incubated for 2 h. Total RNA was isolated at 3 h after treatment of actinomycin D, and RT-PCR was performed as described.

Plasmid Construction and Serial Deletions of IGF-II P3 Promoter. The human IGF-II P3 construct, HUP3, was cloned into the pSLA3 vector, which contains the luciferase gene as a reporter, by the procedure reported previously (22). HUP3 was cut with *Eco* RI and *Sal* I, agarose gel-purified, and cloned between the *Eco* RI and *Sal* I sites in the multiple cloning site of the vector pBluescript (Stratagene, Heidelberg, Germany). This construct was then digested with *Bam* HI and *Xho* I and subcloned into pXP-2 luciferase vectors to construct N-terminal deletion mutants of the IGF-II P3 promoter, pXP-2-P3. p Δ125 (from −169 to +110) and pΔ255 (from −39 to +110) were made in pXP-2 luciferase vectors using the *Sma* I and *Alu* I site in the IGF-II P3 promoter sequence.

Transient Transfection. The calcium phosphate-mediated cotransfection procedure (23) for adherent cells was used with the following modifications. Briefly, 5 × 10^5 HepG2 cells were plated in 60-mm dishes and transfected after changing the medium the next day with the calcium phosphate method. The medium was removed after 24 h, and the cells were supplied with fresh medium and incubated under hypoxic conditions. Control cells were transfected in parallel and cultivated under normal oxygen pressure. After 6, 16, and 24 h, cells were harvested, lysed, and then assayed for luciferase activity. For studying involvement of Egr-1 in the induction of IGF-II P3 promoter activity under hypoxic conditions, HepG2 cells were cotransfected with the IGF-II P3 promoter-luciferase reporter plasmid and increasing amounts of Egr-1 expression vector pEgr-1. After the 24-h transfection, cells were washed with serum-free medium and supplied with fresh medium. After another 48 h, cells were harvested, lysed, and then assayed for luciferase activity.

Luciferase Assay. Cells were harvested after transfection, and extracts were prepared with reporter lysis buffer (Promega, Madison, WI). Cell extracts were assayed for luciferase activity with the luciferase assay kit (Promega) and a luminometer (Turner Designs, Sunnyvale, CA). Extracts were also assayed for β-galactosidase activity with the β-galactosidase enzyme assay system (Promega) and assayed for protein concentration with the protein assay kit (Bio-Rad). Each extract was assayed three times, and the mean RLU was corrected by values obtained from an extract prepared from nontransfected cells. The relative luciferase activity was calculated as RLU/β-galactosidase.

Preparation of Nuclear Extracts. Cells were washed once in PBS followed by preparation of nuclear extracts using modifications of the procedure of Dignam *et al.* (24). Briefly, the cells were lysed in buffer A [10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM NaCl, 0.25% NP40 (pH 7.5)] for 5 min at 4°C, followed by centrifugation at 4000 rpm for 2 min. The supernatant containing cytosol was removed, and the nuclei were extracted with buffer C [20 mm of HEPES, 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.25% NP40 (pH 7.5)]. The nuclei were vortexed vigorously several times over 20 min, followed by centrifugation at 14,000 rpm for 5 min. The supernatant (nuclear extract) was removed, diluted 1:2 with buffer D [20 mm of HEPES, 50 mM of KCl, 0.2 mM of EDTA, 20% glycerol (pH 7.5)], and frozen at −80°C until use.

EMSAs and Supershift Assays. EMSAs were performed as previously described methods (25). Nuclear proteins from HepG2 cells were used in binding reactions. The double-stranded oligonucleotides for the *Egr-1* were 5′-end-labeled with [γ-^{32}P]ATP (10 mCi/ml; Amersham, United Kingdom) using T4 polynucleotide kinase and purified by ethanol precipitation. The DNA binding reaction was performed by preincubating nuclear extract proteins (10 μg) in 20 mM of HEPES (pH 7.5), 50 mM of KCl, 1 mM of DTT, 1 mm of EDTA, 5% glycerol, 2 μg of poly(dI·dC) at 4°C for 15 min, followed by the addition of the double-strand 32P-labeled oligonucleotides (~0.2 ng) and a second incubation at 25°C for 30 min. Oligonucleotide competition experiments were performed with unlabeled wild-type or mutant *Egr-1* or unlabeled *Oct-1* double-stranded oligonucleotide. After preincubation for 15 min on ice, labeled wild-type *Egr-1* probe was added and incubated at 25°C for 30 min.

In the supershift assays, the antibodies (Santa Cruz Biotechnology, Santa Cruz., CA) were added to the reaction mixture subsequent to the addition of the ^{32}P-labeled oligonucleotide probe and incubated at 4°C for 1 h. Samples were subjected to electrophoresis on a native 5% polyacrylamide gel in 0.25 × Tris-borate EDTA for 2 h at 200 V. Gels were vacuum-dried before autoradiography.

Western Blot Analysis. Nuclear proteins (10 μg) were separated on 12% nonreducing PAGE (26). After transfer, nitrocellulose membranes were washed in TBS-T solution [20 mM Tris-HCl buffer (pH 7.6) containing 137 mM NaCl, 1% Tween 20] and incubated in a blocking buffer (5% skim milk in TBS-T) at room temperature for 2 h. After washing with TBS-T three times, the filters were probed with first polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz., CA) against Egr-1 at room temperature for 5 h and then washed three times with TBS-T. The primary antibodies were detected by horseradish peroxidase-conjugated secondary antibody and visualized by the Amersham ECL system after intensive washing of the membranes.

RESULTS

Hypoxia Increases the Activity of IGF-II P3 Promoter. As we have shown previously, hypoxia significantly enhances the expression of 6.0-kb mRNA of the human IGF-II gene (16). The IGF-II gene is a complex transcription unit, and the 6.0-kb human IGF-II mRNA is under the control of the P3 promoter. To investigate whether the P3 promoter region of the IGF-II gene could mediate transcriptional responses to cellular hypoxia, the activity of the P3 promoter was first examined under hypoxic conditions with a reporter plasmid. In this case, the IGF-II P3 promoter sequence from −1229 to +140 relative to the cap site of IGF-II exon 5 was fused to luciferase coding sequences. This reporter plasmid was transfected into HepG2 cells. To correct the variable transfection efficiency, cells were cotransfected with a pSV-β-gal plasmid constitutively expressing β-galactosidase under the control of the SV40 promoter and enhancer. The transfected cells were exposed to hypoxia, harvested, and assayed for luciferase and β-galactosidase activities. As shown in Fig. 1, reporter gene expression by hypoxia was increased 1.4-fold at 6 h, 2.9-fold at 16 h,

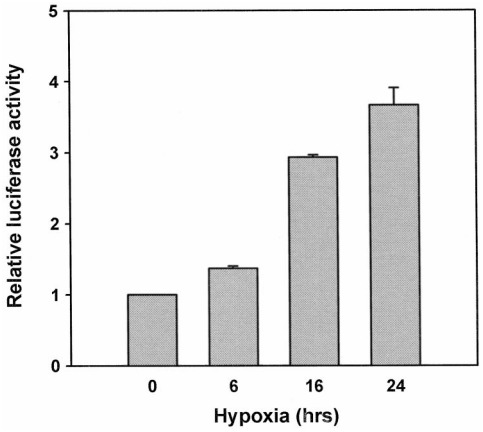

Fig. 1. Effect of hypoxia on the transcriptional activity of the IGF-II P3 promoter. HepG2 cells were cotransfected with 9 μg of the IGF-II P3 promoter-luciferase reporter plasmid and 1 μg of the pSV-β-galactosidase plasmid and then allowed to recover for 24 h after transfection. Transfected cells were incubated for 24 h before exposing them to hypoxia or maintaining them in normoxia for the indicated times and assayed for luciferase and β-galactosidase activities. The relative luciferase activity refers to the ratio of RLU/β-galactosidase measured in hypoxia-treated cells compared to normoxic cells. Values represent the means of at least three independent transfections. The results shown are the mean $\pm$ SE of three to five experiments.

and 3.7-fold at 24 h. This result demonstrates that the activation of the P3 promoter was responsible for the induction of the IGF-II gene by hypoxia.

Transcriptional Regulation of Egr-1 and WT1 by Hypoxia. The IGF-II P3 promoter contains a TATA and a CCAAT box and is highly G/C rich. Transcription factors Egr-1, Sp1, and WT1 are known to play a role in P3 promoter regulation (27). To determine which factors confer responsiveness to hypoxia, the transcriptional regulation of *Egr-1*, *Sp1*, and *WT1* was examined under hypoxic conditions. As shown in Fig. 2*A*, RT-PCR analysis demonstrated that the strong induction of *Egr-1* transcripts was detected at 2 h of hypoxia, with declined expression up to 8 h. In contrast to *Egr-1* mRNA, neither a significant increase nor decrease of *Sp1* and β-actin mRNA by hypoxia was observed at up to 8 h of hypoxia. Interestingly, the level of *WT1* mRNA was dramatically decreased after 8 h of hypoxia. The induction of *Egr-1* transcripts under hypoxia was confirmed by Northern blot analysis (Fig. 2*B*). Hypoxia could increase *Egr-1* mRNA levels either by increasing its rate of transcription or by decreasing its destruction. To evaluate the effect of hypoxia on the steady-state level of *Egr-1* mRNA, HepG2 cells were pretreated with actinomycin D for 1 h before hypoxic or normoxic treatment for 2 h. Total RNA was harvested, and the amount of *Egr-1* mRNA was measured by RT-PCR. Hypoxia did not affect the stability of *Egr-1* mRNA (Fig. 2*C*). Thus, the increase of *Egr-1* mRNA levels during hypoxia was not due to the increased stability but to the transcriptional activation.

Regulation of DNA Binding Activity of Egr-1 by Hypoxia. Next, we examined whether hypoxia also regulates the DNA-binding activity of the Egr-1 protein. As shown in Fig. 3, EMSAs of nuclear extracts from hypoxia-exposed HepG2 cells showed enhanced binding activity of Egr-1 to its binding sequence (*Lanes 3* and *4*), compared with their counterparts in normoxia (*Lane 2*). Competition experiments showed that an excess of unlabeled *Egr-1* oligonucleotides (*Lanes 6* and *7*) completely blocked the appearance of the hypoxia-induced gel-shift band. In contrast, excess unlabeled mutant *Egr-1* (*Lane 8*) or *Oct-1* oligonucleotides (*Lane 9*) had no effect, indicating that sequence-specific DNA binding activity of Egr-1 was involved. Moreover, the protein-DNA complex was supershifted by the anti-Egr-1 antibody (*Lanes 11–13*). But the DNA-binding activity of Sp1 was not changed by hypoxia (data not shown). Taken together, the results in Fig. 3 indicate that induction of the P3 promoter activity of the human IGF-II gene by hypoxia is, in part, mediated through the EBSs in the P3 promoter region and that hypoxia enhances the DNA binding activity of the Egr-1 protein.

Abundance of Egr-1 Protein in Hypoxia-Treated Cells. Because the DNA binding activity and the level of protein may not be necessarily correlated, we performed Western blot analysis to confirm that Egr-1 is present in a greater level in nuclear extracts from hypoxia-treated HepG2 cells than in control cells. Findings from immunoblot analysis indicate that Egr-1 levels were indeed greater in hypoxia-treated cells at 4 h than in control cells (Fig. 4), which is consistent with observations from EMSA and supershift studies (Fig. 3). These results demonstrate that Egr-1 was constitutively induced in hypoxia-

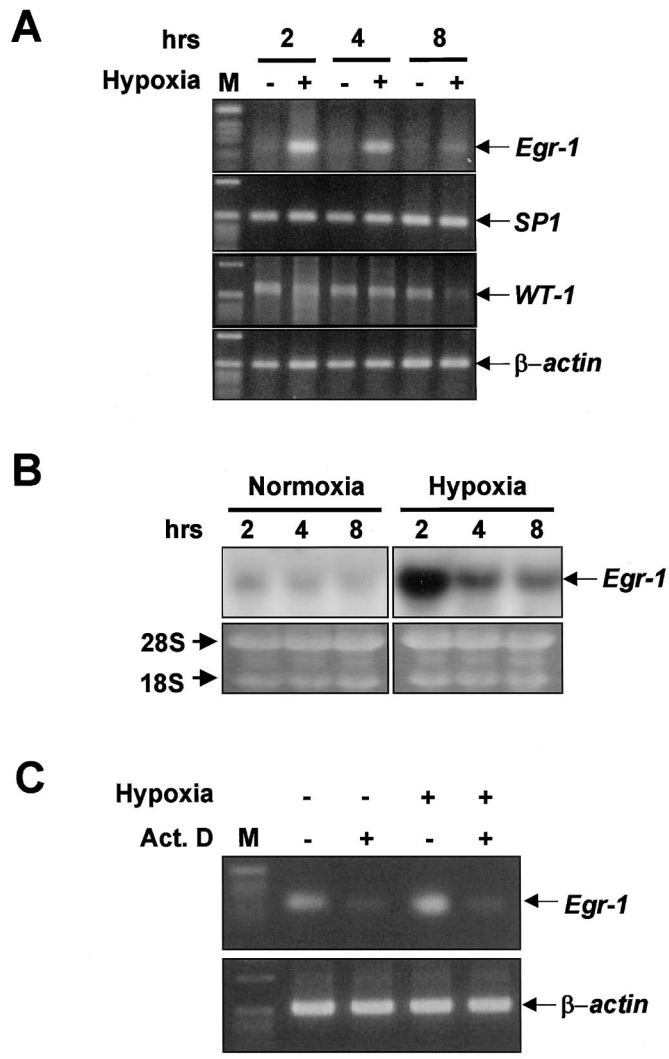

Fig. 2. Hypoxic regulation of *Egr-1*, *Sp1*, and *WT1* gene expression in HepG2 cells. *A*, RT-PCR analysis. HepG2 cells were exposed to hypoxia or maintained in normoxia for the indicated times. Total RNA was harvested and RT-PCR analysis was performed as described in "Materials and Methods." First-strand cDNA was prepared from total RNA and served as a template for PCR in combination with primers specific for *Egr-1*, *Sp1*, *WT1*, or *β-actin*. *B*, Northern blot analysis of *Egr-1* mRNA during hypoxia. HepG2 cells were incubated under hypoxic or normoxic conditions for the indicated times. Total RNA was extracted and analyzed for *Egr-1* mRNA by Northern blot hybridization. The *top panel* is an autoradiograph with an *Egr-1* cDNA probe; the *bottom panel* shows the ethidium bromide-stained photograph of the same gel before transfer to demonstrate equal loading of RNA. Migration of rRNA is indicated by *arrows* (28S and 18S). *C*, *Egr-1* mRNA stability under hypoxia. HepG2 cells were pretreated with vehicle or actinomycin D (Act. D; 25 μg/ml) for 1 h before incubation under hypoxic or normoxic conditions for 2 h. Total RNA was harvested, and RT-PCR analysis was performed with primers specific for *Egr-1*. M, molecular size markers.

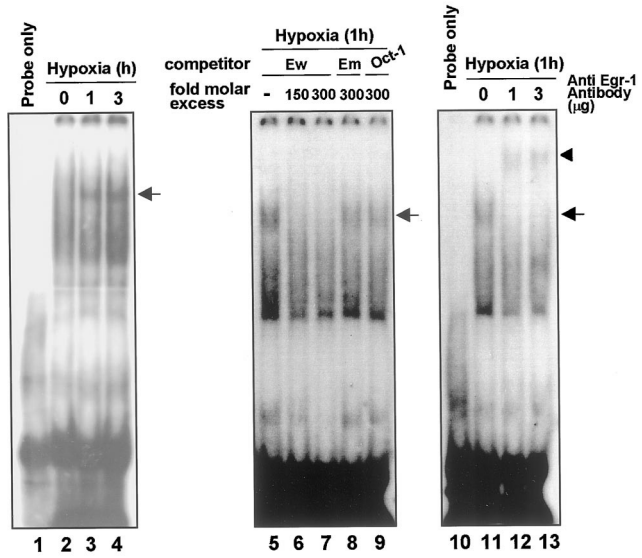

Fig. 3. Effect of hypoxia on the DNA binding activity of nuclear factor Egr-1. Binding of nuclear factors from HepG2 cells to the *Egr-1* consensus fragment (EMSA). Ten-microgram aliquots of nuclear extract prepared from HepG2 cells exposed to normoxic (*Lane 2*) and hypoxic (*Lanes 3* and *4*) conditions for the indicated times were incubated with the ^{32}P-labeled *Egr-1* probe (oligonucleotide competition assay). Nuclear extracts from HepG2 cells exposed to hypoxic conditions for 1 h were analyzed for the DNA-binding activity of Egr-1 using the ^{32}P-labeled Egr-1 probe in the absence (*Lane 5*) or presence of the indicated amount (in fold molar excess) of unlabeled wild-type *Egr-1* (*Ew*; *Lanes 6* and *7*), mutated *Egr-1* (*Em*; *Lane 8*), or Oct-1 (*Lane 9*) oligonucleotides (supershift assay). Nuclear extracts from HepG2 cells exposed to hypoxic conditions for 1 h were incubated with the labeled *Egr-1* probe in the absence (*Lane 11*) or presence of an antibody against the Egr-1 protein (*Lanes 12* and *13*). The *arrows* and *lines* mark the positions of the Egr-1-DNA complex whose migration is further retarded by interaction with the anti-Egr-1 antibody (marked by an *arrowhead*).

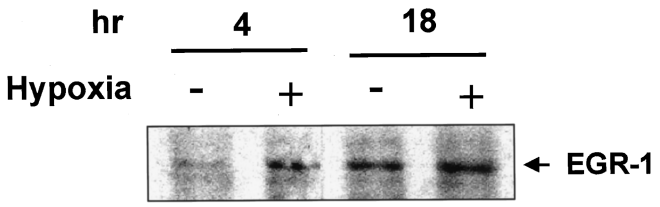

Fig. 4. Western blot analysis of nuclear extracts from HepG2 cells. Nuclear extracts (10 μg) were prepared from HepG2 cells incubated under hypoxia or normoxia for 4 h and 18 h. The nuclear extracts were fractionated by 12% nonreducing PAGE and transferred to a nitrocellulose membrane. The filter was exposed to the polyclonal antibody directed toward Egr-1 and detected by ECL .

exposed HepG2 cells and that the enhanced level of Egr-1 by hypoxia contributes in part to the DNA binding activity.

Effects of Egr-1 Protein on the Activity of IGF-II P3 Promoter. To establish that IGF-II P3 promoter-region-mediated hypoxia-inducible transcription was in fact activated by the Egr-1 protein, we cotransfected HepG2 cells with an expression vector encoding Egr-1 protein along with a reporter vector containing the IGF-II P3 promoter and a pSV-β-gal control plasmid. As shown in Fig. 5*A*, increasing Egr-1 expression vector relative to the reporter plasmid resulted in increasing activity of the P3 promoter, causing maximally 2.5-fold higher luciferase values than those when only the reporter vector is present. Furthermore, hypoxia synergistically induced the promoter activities observed in the Egr-1 cotransfectant (Fig. 5*B*). All these data support the possibility of Egr-1 as a critical factor in the activation of P3-directed IGF-II expression by hypoxia.

Deletion Analysis of the IGF-II P3 Promoter Region. We generated a set of deletion constructs encompassing the IGF-II P3 promoter region and ligated them to a luciferase reporter gene in plasmids pXP-2 (Fig. 6*A*). A construct containing the promoter sequence of the three EBSs, HUP3, was included as a positive control of the hypoxia-induced promoter. These constructs were transiently transfected in the HepG2 cells to assess a putative effect of EBSs on the transcription of IGF-II with the IGF-II P3 promoter. Luciferase activities were measured 24 h later and normalized to β-galactosidase activities. The activity of the hypoxia-treated IGF-II P3 promoter compared to that of the control promoter was reproducibly 3.3-fold higher in the HepG2 cells (Fig. 6). Activities of the deletion constructs are shown as a relative activity of the longest HUP3 construct (Fig. 6*A*). The pXP-2-P3 construct (from -294 to $+110$ bp), which contains three EBSs, retains most of the promoter activity responding to hypoxic stress (Fig. 6*B*). In contrast, deletion of the region from -294 to -169 bp (pΔ125) and -294 to -39 bp (pΔ255) resulted in an almost complete loss of the promoter activity (Fig. 6*B*). Therefore, we propose that the region of -294 to -169 bp, the first EBS, is likely to contain the important regulatory elements in response to hypoxia.

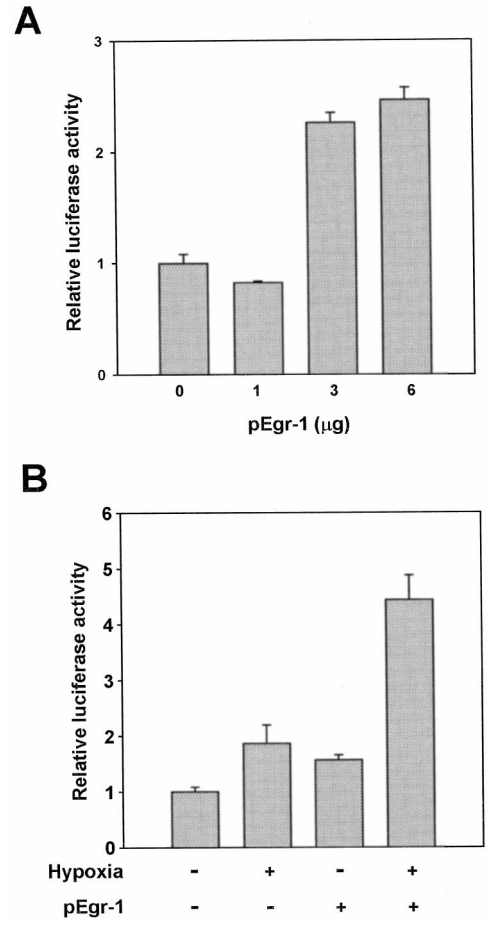

Fig. 5. Cotransfection of IGF-II P3 promoter-luciferase reporter and Egr-1 expression vectors. *A*, effect of the Egr-1 protein on the transcriptional activity of the IGF-II P3 promoter. HepG2 cells were cotransfected with 3 μg of the IGF-II P3 promoter-luciferase reporter plasmid and increasing amounts of Egr-1 expression vector pEgr-1. To maintain equal amounts of total DNA (10 μg), the appropriate amount of empty vector was added. Transfected cells were incubated for 48 h before harvesting for luciferase and β-galactosidase assays. *B*, synergistic effect of hypoxia and Egr-1 protein on the transcriptional activity of the IGF-II P3 promoter. Six micrograms of the IGF-II P3 promoter-luciferase reporter plasmid and 1 μg of the pSV-β-gal plasmid were cotransfected into HepG2 cells with or without 3 μg of the Egr-1 expression vector, incubated for 24 h at 21% O_2, and then incubated at 21% or 1% O_2 for an additional 24 h. The mean RLU/ β-galactosidase ratios from three transfection experiments were normalized to the result for the IGF-II P3 promoter-luciferase reporter plasmid alone. The results shown are the mean $\pm$ SE of three to five experiments.

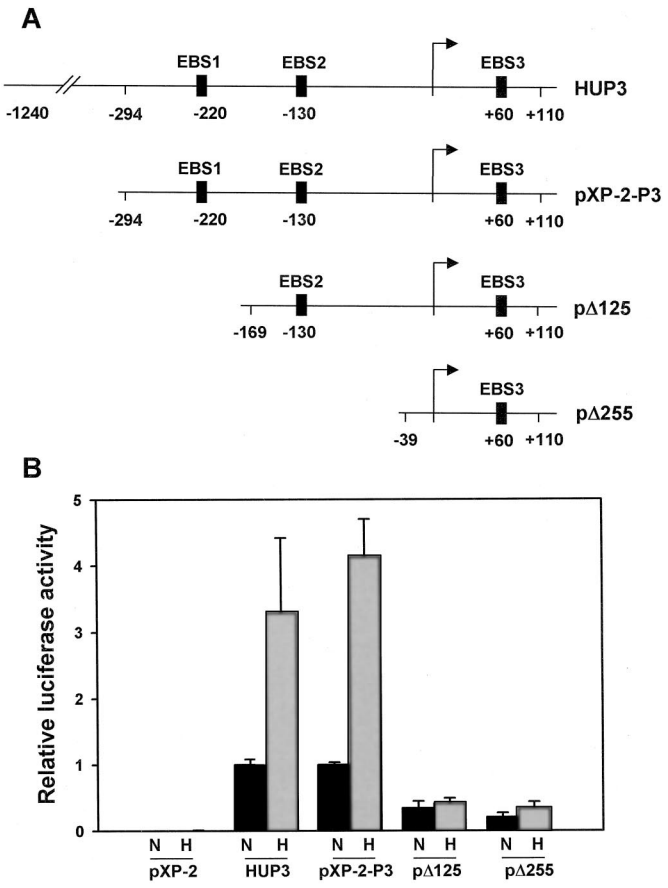

Fig. 6. Activation of luciferase reporter gene constructs containing deletions of the −1240 to +110 human IGF-II P3 promoter. *A*, deletion constructs of the human IGF-II P3 promoter were used in transient transfection experiments. Sequences are numbered relative to the transcription start site (*arrow*). ■, indicates *EBS* s (GCGGGGGCG), and each *EBS* is numbered. *B*, the plasmids (9 μg) were transfected into HepG2 cells in the presence of 1 μg of the pSV-β-gal plasmid. Twenty hours after transfection, the medium was changed to MEM (10% fetal bovine serum), and cells were exposed to either normoxia or to hypoxia for 24 h. Cells were lysed, and extracts were prepared. The level of luciferase activity was measured and normalized for β-galactosidase activity. Luciferase activity is expressed relative to the activity of the −1240 to +110 promoter from cells not exposed to hypoxia. Luciferase activity from static control cells is represented by *black bars* (*N*), whereas luciferase activity from cells exposed to hypoxia for 24 h is represented by *shaded bars* (*H*). The results shown are the mean ± SE of three to five experiments.

DISCUSSION

Our previous results indicated that exposure of HepG2 cells to hypoxia results in the overexpression of the IGF-II transcript with 6.0 kb in size (16). Among the six kinds of mRNA species transcribed from the four promoters of IGF-II genes, 6.0 kb-transcripts are under the control of P3 promoter. The P3 promoter is active at the fetal stage in liver cells but is repressed at parturition and then reappears in most adult human primary liver cancer.

Reactivation of the P3 promoter of the IGF-II gene, resulting in continued expression of IGF-II, was found to be an important driving force of cell proliferation during hepatocarcinogenesis (21, 28). However, little is known about the molecular mechanism of P3 promoter reactivation during tumorigenesis. In the present studies, we have demonstrated that hypoxia in the central region of the tumor cells may act as a triggering signal for reactivating the human IGF-II P3 promoter (Fig. 1). Our sequence analysis of the human IGF-II P3 promoter failed to find hypoxia-responsive element sequences that exist in the promoters of vascular endothelial growth factor and erythropoietin genes. This failure strongly implies the existence of other regulatory mechanisms for the IGF-II P3 promoter activation.

Transcription factors Egr-1 and Sp1 are known to play a role in the activation of the P3 promoter (27). Interestingly, WT1 that binds the same DNA sequences as Egr-1 displays opposite effects on the transcription of target genes of EBSs (29). In fact, it was previously reported that the human IGF-II P3 promoter was repressed by the WT1 product and a continued synthesis of large amounts of IGF-II was observed by functional loss of WT1 activity in Wilms' tumor (30). Our data demonstrated for the first time that hypoxia up- and down-regulates the expression of the *Egr-1* (Fig. 2, *A* and *B*) and *WT1* genes (Fig. 2*A*), respectively, and hypoxia also activates the DNA binding activity of the Egr-1 protein as detected by EMSA (Fig. 3). Moreover, our Western blot analysis reveals that the amount of Egr-1 is markedly elevated in the nuclei of HepG2 cells by hypoxia (Fig. 4). The increase of *Egr-1* mRNA was not due to its increased stability because actinomycin D treatment did not change the Egr-1 mRNA level under hypoxic conditions (Fig. 2*C*). By deletion analysis of the IGF-II P3 promoter sequences, we showed that the sequences containing the upstream EBS1 is primarily responsible for transcriptional activation by hypoxia (Fig. 6*B*). From these results, we conclude that hypoxia increases the level and/or the activity of Egr-1, thus enhancing the transcription of the IGF-II gene through Egr-1-mediated P3 promoter activation. In addition, we further defined a critical role for the EBS1 region of the IGF-II P3 promoter in the elevated expression of the *Egr-1* gene of HepG2 cells and identified that Egr-1 nuclear proteins bind to the IGF-II P3 element in a specific and a functional manner under hypoxic conditions.

However, it remains unclear by what precise mechanisms hypoxia induces the expression of *Egr-1*. Recently, it was shown that a posttranslational modification of the transacting factors, such as a phosphorylation event (31) or redox shift (32), might play a role in the hypoxia signal transduction pathway. Previous reports (33) indicate that the possible phosphorylation event is required for the activation of the Egr-1 protein and also that the induction of *Egr-1* gene expression mediates through its own binding site because the *Egr-1* promoter contains a high-affinity binding site for the Egr-1 protein. From these reports, it seems that hypoxia causes at first the phosphorylation of the Egr-1 protein or other proteins that regulate the activity of Egr-1. Then the active form of the Egr-1 protein increases the expression of the *Egr-1* gene itself and other target genes containing EBSs in their promoter regions such as the IGF-II gene. As shown in Fig. 4*B*, a synergistic response was observed when cells were cotransfected with Egr-1 and exposed to hypoxia. If the effect of hypoxia *per se* was to increase the synthesis of Egr-1 and such synthesis was sufficient for a maximal transcriptional response to hypoxia, the effects of Egr-1 and hypoxia should be additive at best.

Therefore, the observed synergism indicates that additional events, including the posttranslational modification (*e.g.*, phosphorylation) of Egr-1, should occur under hypoxia. Another possible explanation for this synergism relates to the level of other proteins affecting the activity of Egr-1. This possibility is supported by our present result that WT1, functioning as a repressor of Egr-1, is reduced in its expression under hypoxia. This decreased level may be necessary for a maximal activation of the IGF-II P3 promoter in hypoxic cells.

It has been previously reported that *Egr-1* and *c-fos* have a similar signaling pathway (34). Therefore, it might be that the mitogen-activated protein kinase-dependent pathway is involved in the induction of *Egr-1* by hypoxia. In fact, a recent report (35) shows that p38 and Jun kinase 1, not extracellular signal-regulated kinase 2 that is activated by various stresses such as heat shock, sodium arsenite, UV radiation, and anisomycin are involved in the induction of *Egr-1* in NIH 3T3 cells. However, our data do not exclude these possibilities or any other unknown pathways of signal transduction in hypoxia-

induced *Egr-1* expression. Further delineations are needed to define underlying mechanisms.

In conclusion, our studies demonstrate that Egr-1 activated by hypoxia can positively regulate the activity of the human IGF-II P3 promoter. *Egr-1* induction and the role of IGF-II as a autocrine/paracrine growth factor as well as an angiogenic factor can be significant in pathological conditions such as hepatoma where cells are exposed to hypoxic environments. Thus, it is likely that the Egr-1 protein is an important nuclear intermediate in signal transduction processes under hypoxia and one of the crucial targets in hepatocellular carcinogenesis.

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