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A Constitutively Active Hypoxia-Inducible Factor-1α/VP16 Hybrid Factor Activates Expression of the Human B-Type Natriuretic Peptide Gene

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ABSTRACT

Hypoxia-inducible factor-1 (HIF-1) is a primary regulator of the physiological response to hypoxia. A recombinant adenovirus expressing a constitutively active hybrid form of the HIF-1α subunit (Ad2/HIF-1α/VP16) is being evaluated as a gene therapy for the treatment of peripheral vascular disease. Ad2/HIF-1α/VP16 up-regulates known HIF-1-responsive genes, including those involved in angiogenesis. Expression profile analysis revealed that the brain natriuretic peptide (BNP) gene was significantly up-regulated in response to HIF-1α/VP16 in human fetal cardiac cells. Real-time reverse transcription-polymerase chain reaction analyses confirmed transcriptional activation of the BNP gene by HIF-1α/VP16 in human but not rat cardiac cells. Because hypoxia itself did not increase human BNP gene expression in these analyses, the mechanism of the HIF-1α/VP16 effect was determined. Analyses of promoter deletion mutants suggested that the *cis*-acting sequence in the human BNP promoter mediating activation by HIF-1α/VP16 was a putative HIF-1 responsive element (HRE) located at −466. An SV40 basal promoter-luciferase plasmid containing a minimal BNP HRE was up-regulated by HIF-1α/VP16, whereas a similar construct carrying a mutation within the HIF-1 binding site was not. Mutation of an E-box motif within the BNP HRE reduced HIF-1α/VP16-mediated transcriptional activation by 50%. Gel-shift analyses showed that both the native HIF-1α and HIF-1α/VP16 are able to bind to a probe containing the HIF-1 binding site. These experiments demonstrate the existence of a functional HRE in the BNP promoter and further define the scope and mechanism of action of Ad2/HIF-1α/VP16.

Hypoxia-inducible factor-1 (HIF-1) is triggered by low oxygen to initiate a coordinated physiological response to hypoxia via the regulation of gene expression. This role as a "master switch" has made HIF-1 an attractive candidate for pharmacological manipulation (Giaccia et al., 2003). Genes regulated by HIF-1 are involved in a variety of processes, including glucose metabolism, erythropoiesis, regulation of vascular tone, and cell proliferation and survival (Wenger and Gassmann, 1997). Because HIF-1 functions to up-regulate the expression of several genes involved in angiogenesis (Kelly et al., 2003; Yamakawa et al., 2003), HIF-1 has been a focus of strategies to promote therapeutic angiogenesis in patients with coronary or peripheral ischemia. Because neovascularization results from the complex interplay of a variety of factors, an upstream regulatory protein such as HIF-1 could potentially be more effective as a therapeutic than any single proangiogenic factor.

HIF-1 is a heterodimeric transcription factor composed of an α and β subunit; both are bHLH-Per-ARNT-Sim domain proteins. Intracellular oxygen concentration regulates HIF-1 activity by influencing both stability and transcriptional activity of the HIF-1α subunit (Poellinger and Johnson, 2004). Under normoxic conditions, HIF-1α protein stability is regulated by prolyl hydroxylases via the modification of conserved prolines within the oxygen-dependent degradation domain of HIF-1α. These are subsequently recognized by the von Hip-

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ABBREVIATIONS: HIF-1, hypoxia-inducible factor-1; BNP, brain natriuretic peptide; ANP, atrial natriuretic peptide; HRE, hypoxia-inducible factor-1-responsive element; ARNT, aryl hydrocarbon nuclear receptor translocator; bHLH, basic helix-loop-helix; VEGF, vascular endothelial growth factor; SAGE, serial analysis of gene expression; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase polymerase chain reaction; EMSA, electrophoretic mobility shift assay; HAS, hypoxia-inducible factor-1 ancillary sequence; ROS, reactive oxygen species; nt, nucleotide; ANOVA, analysis of variance; HNF, hepatic nuclear factor; bp, base pair; AP-1, activator protein-1; NF-κB, nuclear factor κB; ELISA, enzyme-linked immunosorbent assay; m.o.i., multiplicity of infection; mHRE, minimal hypoxia response element; mmHRE, mutant minimal hypoxia response element; 2xmHRE, duplex minimal hypoxia response element; 2mmHRE, CACAG mutant minimal hypoxia response element; SV40, simian virus 40; MI, myocardial infarction.

pel-Lindau tumor suppressor protein, which targets HIF-1α for ubiquitination and proteosomal degradation. Likewise, transcriptional activity of HIF-1α is regulated by hydroxylation at an asparagine residue in the C-terminal activation domain. This modification regulates the interaction of HIF-1 and p300/CRB, a coactivator protein required for HIF-1-mediated transactivation (Arany et al., 1996; Ebert and Bunn, 1998).

A constitutively active version of HIF-1α was generated by replacing the C terminus of the protein, including the oxygen-dependent degradation domain and endogenous transactivation domains, with the transactivation domain from the herpesvirus VP16 protein. Administration of plasmid DNA encoding HIF-1α/VP16 has been shown to increase blood flow in animal models of both cardiac and peripheral ischemic disease (Vincent et al., 2000; Shyu et al., 2002). In a similar approach, constitutively active, modified versions of the native HIF-1α protein have been reported to promote angiogenesis in the hind limbs of mice and rabbits (Pajusola et al., 2005; Patel et al., 2005). A phase I clinical trial evaluating an adenovirus vector expressing HIF-1α/VP16 (Ad2/HIF-1α/VP16) for the treatment of peripheral arterial disease has been completed (S. Rajagopalan, J. Olin, S. Deitcher, A. Pieczek, J. Laird, P. Grossman, C. Goldman, K. McEllin, R. Kelly, and N. Chronos, manuscript in preparation).

To further characterize the effect of the potential therapeutic gene HIF-1α/VP16 on target-cell gene expression, a gene expression profile of human cardiac cells infected with Ad/2HIF-1α/VP16 was determined using SAGE analysis (Jiang et al., 2002). A gene found to be significantly up-regulated by HIF-1α/VP16 was brain natriuretic peptide (BNP). However, in this analysis, hypoxia had no effect on BNP gene expression. BNP is a member of the natriuretic peptide family that also includes the structurally similar atrial natriuretic peptide (ANP) and the C-type natriuretic peptide. Human BNP is a 32-amino acid cardiac hormone, primarily synthesized in the ventricular myocardium, that possesses natriuretic, diuretic, vasorelaxant, and antigrowth activity (de Lemos et al., 2003).

BNP gene expression and release into the circulation are enhanced in association with myocardial infarction, heart failure, and hypertrophy. A primary stimulus for increased production of BNP by the ventricular myocyte is believed to be mechanical strain due to wall stress (Liang and Gardner, 1999). However, there is growing evidence that plasma BNP levels are elevated in patients with coronary artery disease in the absence of ventricular dysfunction, suggesting that BNP production might also be enhanced by myocardial ischemia (Yeo et al., 2005). Evidence suggesting a possible role for hypoxia in influencing BNP gene expression came initially from studies in which rats were exposed to long-term hypoxia (Hill et al., 1994; Perhonen et al., 1997). Recent experiments performed in a porcine model have shown that ventricular BNP mRNA levels are increased by exposure to short-term hypoxia (Goetze et al., 2004). Although it has been demonstrated that transcription of the ANP gene is enhanced by hypoxia and that this effect is mediated by HIF-1 (Chen et al., 1997; Chun et al., 2003), a direct role for HIF-1 in the regulation of BNP gene expression has not yet been reported. In light of the SAGE results indicating activation of BNP transcription by HIF-1α/VP16 but not by hypoxia, the experiments described here were designed to investigate the mechanism of HIF-1α/VP16-mediated up-regulation of BNP gene expression. The results document the existence of an HIF-1 response element in the BNP promoter and serve to further define the scope and mechanism of action of the HIF-1α/VP16 hybrid transcription factor.

Materials and Methods

Cells and Viruses. Human fetal cardiac cells, passaged twice and supplied frozen, were obtained from Cambrex Bioscience (Walkersville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum and 1% chick embryo extract. Neonatal rat ventricular cardiomyocytes were isolated as described previously (Lee et al., 1988). After isolation, cells were seeded on rat collagen type 1-coated dishes (Becton Dickinson and Co., Bedford, MA) and maintained in DMEM/F-12 medium containing 10% fetal bovine serum and insulin-transferrin-sodium selenite (Sigma-Aldrich, St. Louis, MO). HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U of penicillin, and 100 μg of streptomycin (Invitrogen/Life Technologies, Carlsbad, CA) per milliliter. Hep3B cells were cultured in minimum essential medium containing 10% fetal bovine serum, 2 mM glutamine, 1 mM minimal essential medium sodium pyruvate, 100 U of penicillin, and 100 μg of streptomycin.

Recombinant adenoviral vectors were generated as described previously (Armentano et al., 1995). Ad2/HIF-1α, Ad2/HIF-1α/VP16, and Ad2/HIF-1α/NF-κB adenoviruses are in a backbone that is $E1^-$, $E2^+$, $E3^+$, and $E4^-$ (except for ORF6) and in which the protein IX coding sequence is moved into the deleted E4 region (Hehir et al., 1996). Adenoviral infections (m.o.i. = 100; except for Ad2/HIF-1α, where the m.o.i.= 2000) of primary human fetal cardiac cells were performed on 2.5×10^6 cells in a 100-mm dish for 4 h in 5 ml of media, and then an additional 5 ml of fresh media was added, and infections were allowed to proceed overnight. The media were then changed, and the cells were treated (maintained under normoxic conditions, treated with 100 μM desferrioxamine, or subjected to hypoxia). Twenty-four hours later, either the supernatants were harvested (for ELISA and Western analysis) or RNA was prepared from the cells (for real-time RT-PCR).

Plasmid Constructions. The human BNP promoter (from −1578 to +37) was amplified by PCR from HeLa cell cDNA (Clontech Laboratories Inc., Palo Alto, CA). To create a luciferase reporter plasmid, the PCR product was cloned into the pGL3-Basic plasmid (Promega, Madison, WI). 5′-Deletion mutants were generated by cleaving at convenient restriction enzyme sites (BsaBI, PflMI, AflIII, StyI) within the promoter. Further deletions between the AflIII and StyI sites were generated by PCR amplification of the appropriate promoter sequences and cloning into pGL3-Basic. A luciferase reporter plasmid containing the minimal hypoxia response element (mHRE) from the BNP promoter was constructed by cloning a double-stranded oligonucleotide (sense strand: 5′-CAGGCAGGGTGCACAGCGGCGAGCAGGTGCTGCGCTA**CGT**GCGGGCCAGG-3′) corresponding to the sequences between −503 and −454 into the pGL3-promoter plasmid (Promega). In the mutant mHRE (mmHRE), the three nucleotides in boldface type are changed to AAA. The duplex mHRE (2xmHRE) contains two tandem copies of the mHRE, whereas in the CACAG mutant (2mmHRE), the underlined nucleotides CACAG are mutated to ATGCC. In the E-box mutant, the underlined nucleotides AGCAG are changed to TTACC. The VEGF HRE sequence (Forsythe et al., 1996) was similarly cloned into pGL3-promoter plasmid and consisted of sequences between −985 and −939 in the VEGF promoter (sense strand, 5′- CCACAGTGCATACGTGGGCTCCAACAGGTCCTCTTCCCTCCCATGCA-3′).

The HIF-1αΔ/VP16 construct, which contains a deletion within the HIF-1α DNA binding and dimerization domains, was constructed by cleaving pcDNA3/HIF-1α/VP16 (Vincent et al., 2000) with XhoI and BsgI. Oligonucleotides 1 (5′-CTCGAATGAAGTGTACACTAAC-

TAGCCGAGGAAGAACATGAACATAAAGTCTGCAACATGG-3′) and 2 (5′-CCATGTTGCAGACTTTATGTTCGGCTAGTTAGTGTAC-ACTTCATTCGAG-3′) were annealed and ligated between the XhoI and BsgI sites to delete amino acids 24 to 170 and recreate the restriction sites. The nucleotides underlined in the oligonucleotides represent an alanine/threonine to cysteine/glycine mutation to create a BsrGI site for screening.

A second constitutively active version of HIF-1α contains amino acids 1 to 390 of HIF-1α fused to amino acids 350 to 550 of the human NF-κB p65 subunit. This region of p65 contains two activation domains, TA_1 and TA_2 (Schmitz and Baeuerle, 1991). The hybrid construct was generated by first amplifying this region of p65 via PCR (Advantage cDNA PCR kit; Clontech, Mountain View, CA) from a HeLa cell cDNA library (Clontech). The PCR fragment was then cloned between the AflII and XbaI sites of pcDNA3/HIF-1α/VP16, replacing the VP16 activation domain with that of p65.

RNA Preparation and Real-Time RT-PCR. Total RNA was extracted with RNAzolB (Tel-Test, Inc., Friendswood, TX). Real-time RT-PCR analysis (TaqMan) was performed using an ABI-Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers and probes were designed according to software provided by Applied Biosystems and were synthesized by either Operon (Alameda, CA) or Integrated DNA Technologies (Coralville, IA). Human BNP primer sequences are as follows: forward primer, 5′-AGCCTCGGACTTG-GAAACG-3′; reverse primer, 5′-CGACAGTTTGCCCTGCAAAT-3′; probe, CGGGTTACAGGAGCAGCGCAAC. Rat BNP primer sequences are as follows: forward primer, 5′-TGGGCAGAAGATAGAC-CGGA-3′; reverse primer, 5′-ACAACCTCAGCCCGTCACAG-3′; probe, 5′-CCAAGCGACTGACTGCGCCG-3′. RT-PCR reactions were performed in a one-step format with the reverse-transcriptase reactions (using the reverse PCR primer) and PCR reactions taking place sequentially. Comparison to 18S RNA values was carried out for normalization. Standard curves consisting of serially diluted RNAs were run in duplicate for both BNP and 18S. Relative expression levels were determined in duplicate for each sample by comparison with the standard using the ABI Sequence Detector version 1.6.3 software for analysis.

Transfections and Luciferase Assays. Transfections of rat neonatal cardiac myocytes were performed using Lipofectamine 2000 (Invitrogen/Life Technologies). Cells (seeded at 4×10^5 cells/cm^2 in a six-well plate) were treated with 5 μg of total DNA (2.5 μg of each plasmid) and 16 μl of Lipofectamine 2000 and infected with an empty adenoviral vector (Ad2/CMVEV) at an m.o.i. of 200. All transfections also included pCMVβgal (1 μg) (Clontech) to control for transfection efficiency. The DNA/Lipofectamine mix and Ad/CMVEV were added simultaneously and incubated on the cells for 3 to 4 h followed by a media change. On the following day, fresh media were added again, and the cells were then either maintained under normoxic conditions (21% O_2, 5% CO_2, 37°C), placed in a hypoxia chamber (1% O_2, 5% CO_2, 37°C), or treated with 100 μM desferrioxamine (Sigma-Aldrich). Cells were harvested for the luciferase activity assay 24 h later. Transfection of the cardiomyocytes (10–20% efficiency) by this method was confirmed by transfection with a green fluorescent protein-expressing plasmid and immunofluorescence analysis [using an antitropomyosin (sarcomeric) antibody (mouse IgG from Sigma-Aldrich) to identify the cardiomyocytes]. HeLa cells (seeded at 4×10^5 cells/well of a six-well plate) were transfected with Lipofectamine 2000 (10 μl) and 4 μg of DNA (1.5 μg of each plasmid and 1 μg of pCMVβgal). Luciferase assays were performed using the Dual-Light kit (Tropix Inc., Bedford, MA).

BNP ELISA and Western Analyses. The BNP ELISA (for human BNP-32) was performed using commercially available reagents (Peninsula Laboratories, Inc., San Carlos, CA). Samples were prepared, and the assay was performed according to manufacturer's instructions. For Western analysis, samples were prepared as for the ELISA and were run on a 16% Tricine gel (Invitrogen). The primary antibody was rabbit anti-BNP-32 (human) IgG (Peninsula Laboratories) used at 1:500 dilution. The secondary antibody was donkey anti-rabbit IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK) used at a dilution of 1:2000. Chemiluminescent detection was carried out using the ECL kit (GE Healthcare).

Electrophoretic Mobility Shift Assays. Probes were prepared, and gel-shift assays were performed according to published protocols (Wang and Semenza, 1996). Nuclear extracts were isolated from uninfected Hep3B cells or cells infected with either Ad2/HIF-1α/VP16 (m.o.i. = 200), Ad2/HIF-1αFL (m.o.i. = 200) (which expresses the native HIF-1α protein), or Ad2/HIF-1α/VP16 (m.o.i. = 1×10^4). Cells infected with Ad2/HIF-1αFL were exposed to hypoxia for 24 h before harvest. Reactions were performed as described but with 5 μg of nuclear extract, 0.1 μg of denatured calf thymus DNA (GE Healthcare), and 10^4 cpm of probe in a reaction volume of 20 μl. For supershift experiments, reactions also included 2 μg of either anti-VP16 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-HIF-1α (Novus Biologicals, Inc., Littleton, CO) antibody. Nuclear extract, calf thymus DNA, and antibody were incubated on ice for 15 min before adding probe. Competition experiments were performed with 50 or 500 M excess of unlabeled double-stranded oligonucleotide. Reaction products were resolved by electrophoresis in a 6% retardation gel (Invitrogen). Probe sequences are as follows: BNP: sense, 5′-TGCTGCGCTACGTGCGGGCCAGGG-3′, antisense, 5′-CCCTGGCCCGCACGTAGCGCAGCA-3′; erythropoietin: sense, 5′-GCCCTACGTGCTGTCTCA-3′; antisense, 5′-TGAGACAGCACG-TAGGGC 3′. To create mutant versions of the probes, the underlined nucleotides were changed from CGT to AAA (sense strand) or ACG to TTT (antisense strand).

Statistical Analysis. Data (mean ± S.E.M.) were analyzed either by Student's t test or by one-way ANOVA, followed by a Newman-Keuls test. A value of $P < 0.05$ was considered statistically significant.

Results

Confirmation of SAGE Results. Gene-expression profiling was used to identify the spectrum of genes regulated by a constitutively active HIF-1α hybrid, HIF-1α/VP16, and to compare these with the genes regulated by hypoxia (or native HIF-1α). A SAGE experiment performed on human fetal cardiac cells (Jiang et al., 2002) revealed that of all the genes in the analysis, BNP was up-regulated to the greatest extent in response to HIF-1α/VP16. To confirm the SAGE results, primary human fetal cardiac cells and primary rat neonatal cardiomyocytes were infected with either an empty adenovirus or adenoviruses expressing the native HIF-1α or HIF-1α/VP16 and then maintained under normoxic conditions or exposed to hypoxia (Fig. 1A). Real-time RT-PCR analysis of RNA isolated from the human cells confirmed the SAGE results (i.e., dramatic up-regulation of BNP expression in response to HIF-1α/VP16 but no effect with either hypoxia or overexpression of the native HIF-1α). Direct analysis of the SAGE RNAs via real-time RT-PCR also confirmed these results (data not shown). Increases in BNP peptide were also observed in human cells as assayed either by ELISA or Western analysis (Fig. 1, B and C). In rat cardiomyocytes, there was no up-regulation with hypoxia, whereas HIF-1α/VP16 seems to somewhat down-regulate BNP expression.

To further investigate whether hypoxia up-regulates BNP in human cardiac cells, cells were exposed to hypoxia for up to 72 h (Fig. 2). In parallel, cells were infected with an empty adenoviral vector or vectors expressing the native HIF-1α or HIF-1α/VP16. BNP expression was analyzed via real-time RT-PCR. Even with a 72-h exposure to hypoxia, there was no observed effect on BNP expression. A measurable effect was observed with overexpressed HIF-1α at 72 h. As observed in

previous experiments, BNP gene expression was up-regulated in response to HIF-1α/VP16, and this effect increased with time. In light of this apparent difference in activity between the native HIF-1α and HIF-1α/VP16, experiments were performed to characterize the mechanism of action of the hybrid factor.

Analysis of the HIF-1αΔ/VP16 Deletion Mutant. To rule out the possibility that the VP16 activation domain alone was mediating the observed effect on BNP gene expression, the HIF-1αΔ/VP16 deletion mutant was constructed. A deletion was engineered within the DNA binding and dimerization domain of HIF-1α/VP16 (between amino acids 24 and 170 in the bHLH and Per-ARNT-Sim domains), and this construct was tested to determine its effect on BNP gene expression. Recombinant adenoviruses expressing HIF-1α/VP16 and HIF-1αΔ/VP16 were used to infect human fetal cardiac cells. Protein expression, expression of the VEGF and BNP genes, and DNA binding were tested (Fig. 3). Real-time RT-PCR analysis showed that HIF-1αΔ/VP16 is unable to up-regulate either VEGF or BNP expression (Fig. 3C). The inability of the HIF-1αΔ/VP16 protein to bind DNA was confirmed via electrophoretic mobility shift assays (EMSAs) (Fig. 3D). Western analysis (Fig. 3B) demonstrated that in these experiments, HIF-1αΔ/VP16 was expressed at a level comparable with HIF-1α/VP16 and confirmed that lack of activity was not due to insufficient transgene expression. These results confirm that up-regulation of BNP gene expression by HIF-1α/VP16 is not dependent on the VP16 activation domain alone and suggest that this effect is dependent on DNA binding via the HIF-1α portion of the protein.

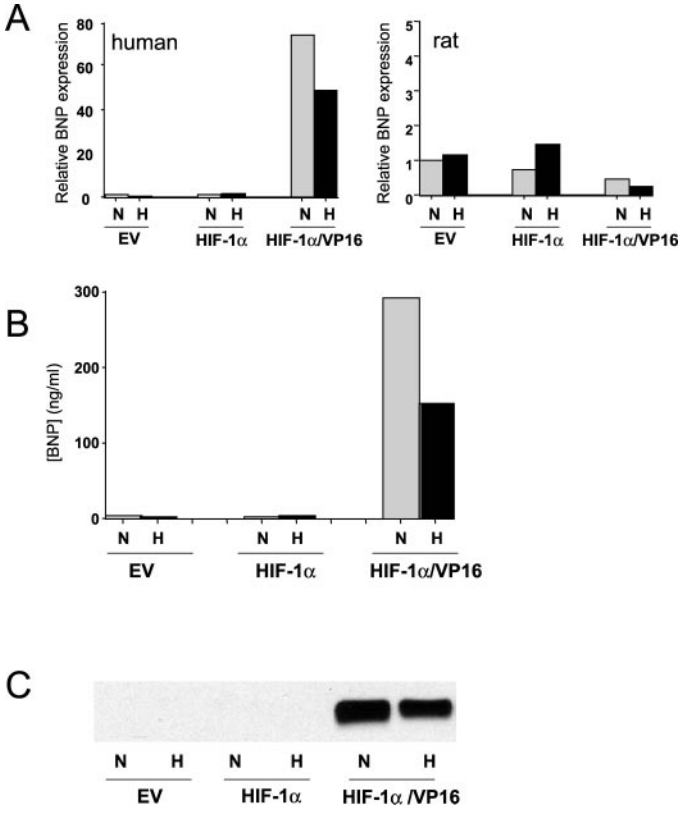

Fig. 1. Analysis of BNP expression induced by hypoxia or infection with Ad2/HIF-1α/VP16. A, primary human fetal cardiomyocytes (Cambrex) or primary rat neonatal cardiomyocytes were infected with the viruses indicated (CMVEV = Ad2/CMVEV; 100 m.o.i.; HIF-1α = Ad2/HIF-1α, a vector expressing the full-length wild-type HIF-1α cDNA; 2000 m.o.i.; HIF-1α/VP16 = Ad2/HIF-1α/VP16; 100 m.o.i.). The cells were either maintained under normoxic conditions (N) or exposed to hypoxia (H) for 24 h. Expression of the BNP gene was assayed via semiquantitative RT-PCR (TaqMan) and normalized to 18S RNA levels to control for RNA concentration. Relative BNP expression is the ratio of that value to the value obtained from uninfected cells under normoxic conditions. B, primary human fetal cardiomyocytes were infected with the viruses indicated at an m.o.i. of 100 (Ad2CMVEV and Ad2/HIF-1α/VP16) or 2000 (Ad2/HIF-1α) and either maintained under normoxic conditions (N) or exposed to hypoxia (H) for 24 h. The higher m.o.i. (2000) for the latter virus was used because at that m.o.i., expression levels of HIF-1α were equivalent to that of HIF-1α/VP16 in cells infected with Ad2/HIF-1α/VP16 at an m.o.i. of 100. BNP peptide was concentrated from the media according to manufacturer's instructions and assayed using an ELISA kit (Peninsula Laboratories). C, the concentrated BNP peptide sample was also analyzed via Western analysis using an antibody specific for human BNP (Peninsula Laboratories).

Deletion Analysis of the BNP Promoter. The sequence of the human BNP promoter was examined to identify putative HIF-1 binding sites. Shown in Fig. 4 is a schematic of the BNP promoter. The promoter contains binding sites for known transcription factors, such as AP-1, and three GATA sites. There are seven possible HIF-1 binding sites within the promoter (Fig. 4B). It is noteworthy that the site at −466 is identical with the consensus sequence (Wenger and Gassmann, 1997). To determine the location of the *cis*-acting sequence conferring activation by HIF-1α/VP16, the promoter was isolated from human genomic DNA via PCR, and four deletion mutants were generated by cleavage at convenient restriction sites. The mutants were cloned into a luciferase reporter plasmid and analyzed as shown in Fig. 4C.

Primary rat neonatal cardiac myocytes were used for the analysis of the human BNP promoter deletion mutants because of the relative ease of isolating and manipulating these cells relative to human cardiac myocytes. However, potential limitations of this approach are differences in signaling pathways and *trans*-acting factors between species. Each luciferase reporter construct was transfected into rat cardiomyocytes in the presence and absence of an HIF-1α/VP16 expression plasmid. The BglII, BsaBI, PflMI, and AflIII constructs all up-regulate luciferase activity from 3- to 6-fold, whereas the StyI construct fails to elevate luciferase expres-

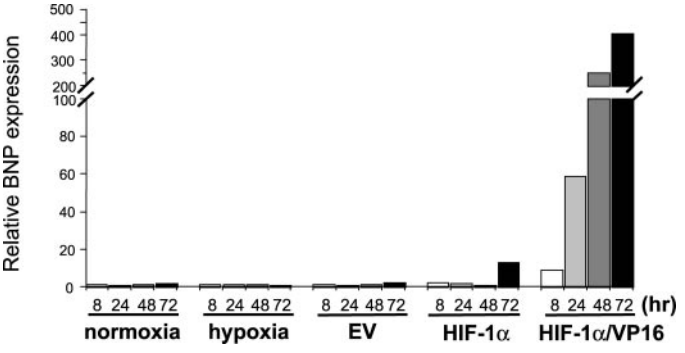

Fig. 2. Time course of BNP expression after exposure to hypoxia or infection with Ad2/HIF-1α/VP16. Primary human fetal cardiomyocytes were infected with the viruses indicated. Cells were either maintained under normoxic conditions [normoxia group, Ad2/CMVEV (EV; m.o.i. = 200) and Ad2/HIF-1α/VP16 (HIF-1α/VP16; m.o.i.= 200)] or exposed to hypoxia [hypoxia group, Ad2/HIF-1α (HIF-1α; m.o.i. = 2000)[for the times indicated. Virus infections were for 6 h (i.e., 8-h time point is 2 h after the removal of virus). Total RNA was isolated, and BNP expression was assayed via a semiquantitative RT-PCR method (TaqMan); values were normalized to 18S levels. Relative BNP expression is the ratio of that value to the value obtained from cells maintained under normoxic conditions for 8 h.

sion. These results suggest that a *cis*-acting sequence-mediating activation by HIF-1α/VP16 might be located between the AflIII site at −880 and the StyI site at −283. An increase in activity in the absence of HIF-1α/VP16 observed with the StyI deletion relative to the other deletion constructs may be due to the removal of an inhibitory sequence that affects basal transcription activity. A similar result was observed previously after deletion of the sequences between −904 and −500 (La Pointe et al., 1996).

Because there are three putative HIF-1 binding sites in the region between the AflIII and StyI sites, more finely tuned deletions were constructed to distinguish among them. These deletions were designed to remove each of the putative HIF-1 binding sites between the AflIII and StyI sites one at a time (Fig. 5A). The results show that activation of gene expression by HIF-1α/VP16 is decreased sharply with the deletion of the site at −466 (Fig. 5B).

Analysis of the BNP HRE. The putative HRE was cloned upstream of the SV40 promoter in a luciferase promoter plasmid. This 50-base pair mHRE extending from −503 to −454 contains the putative HIF binding site at −466 and a CACAG motif; this sequence has been shown to function as

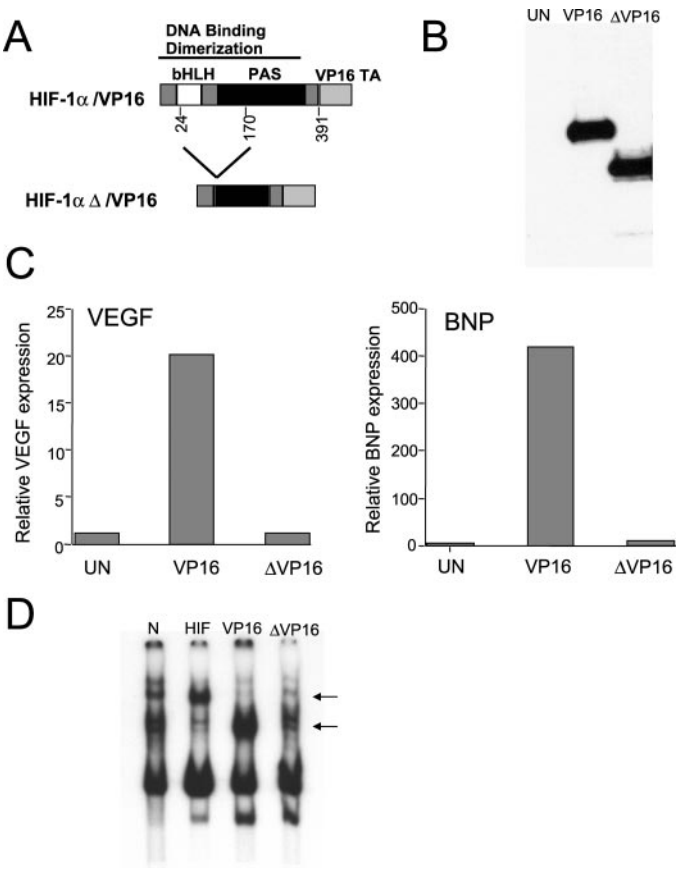

Fig. 3. Analysis of the HIF-1αΔ/VP16 mutant. A, HIF-1α/VP16 was modified to inactivate the DNA binding and dimerization activity of HIF-1α by deleting amino acids 24 to 170 (portions of the bHLH and Per-ARNT-Sim domains of HIF-1α, known to be required for these activities). A recombinant adenovirus expressing this mutant (Ad2/HIF-1 αΔ/VP16) was used in the experiments described here. B, primary human fetal cardiomyocytes were infected with Ad2/HIF-1α/VP16 (VP16; m.o.i. = 100) or Ad2/HIF-1 αΔ/VP16 (ΔVP16; m.o.i. = 10^5); control cells were uninfected (UN). Total protein was isolated 48 h after infection; expressions of HIF-1α/VP16 and HIF-1αΔ/VP16 were analyzed via Western analysis using an antibody specific for the C terminus of VP16 (Santa Cruz Biotechnology). C, from the same experiment, RNA was isolated 48 h after infection; BNP and VEGF mRNA were assayed via a semiquantitative RT-PCR method (TaqMan). Values were normalized to 18S levels to control for RNA concentration. Relative BNP and VEGF expression is the ratio of the normalized value to that obtained from uninfected cells. D, EMSA analysis of the HIF-1 αΔ/VP16 mutant. Hep3B cells were infected with Ad2/HIF-1α/VP16 (VP16) and Ad2/HIF-1α (HIF) at an m.o.i. of 200; infection with Ad2/HIF-1 αΔ/VP16 (ΔVP16) was carried out at an m.o.i. of 10^4. In addition, Ad2/HIF-1α-infected cells were exposed to hypoxic conditions for 24 h. Nuclear extracts were prepared as described under *Materials and Methods*. Gel-shift experiments were performed with a BNP HRE oligonucleotide probe. The top arrow indicates the position of the shifted HIF-1α complex; the bottom arrow indicates the position of the shifted HIF-1α/VP16 complex.

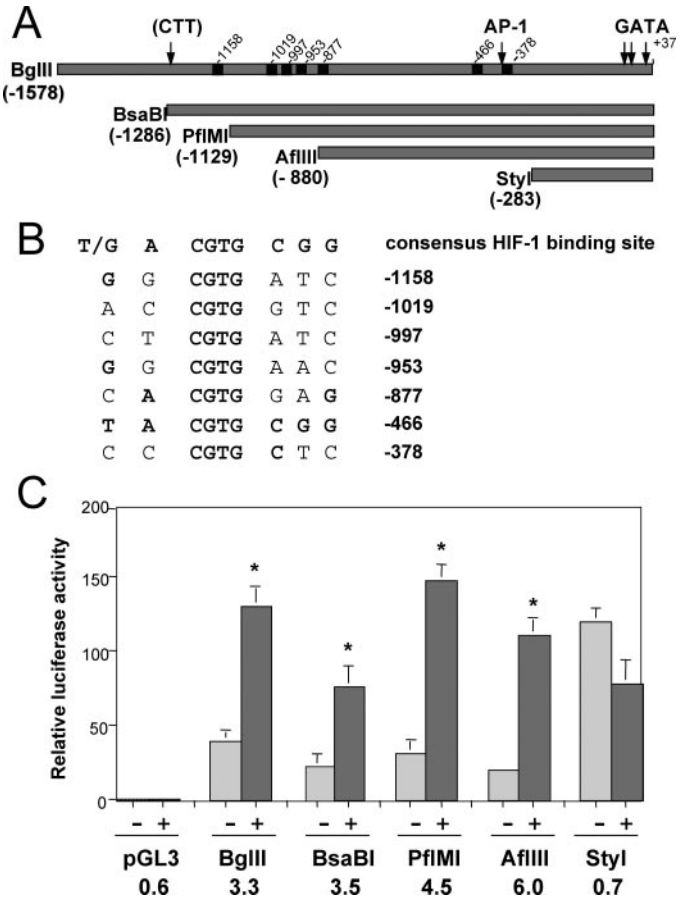

Fig. 4. Deletion analysis to locate functionally significant *cis*-acting sequences within the BNP promoter. A, a diagramatic representation of the BNP promoter. Known transcription factor binding sites (AP-1 and GATA) and proposed functional regions (CTT) are highlighted (Ogawa et al., 1995). Filled boxes indicate the positions of possible HIF-1 binding sites. The full-length BNP promoter (BglII; −1578 to +37) was isolated by PCR from human genomic DNA and cloned into a luciferase expression vector (pGL3; Promega). Four deletions with endpoints at convenient restriction sites within the promoter (BsaBI, PflMI, AflIII, and StyI) were also generated. B, shown are the consensus HIF-1 binding site and the sequences of the seven possible HIF-1 binding sites within the BNP promoter. Individual nucleotides are highlighted in boldface type where they match the consensus. C, the BNP promoter deletion constructs were transfected into rat neonatal cardiomyocytes either in the presence (+) or absence (−) of a plasmid expressing HIF-1α/VP16. All transfections also included pCMVβgal to control for transfection efficiency. Luciferase activity in each of the samples was normalized to β-galactosidase activity; relative luciferase activity is the ratio of that value to the value obtained from cells transfected with pGL3 in the absence of HIF-1α/VP16. Results are averaged from four independent experiments; each transfection was performed in duplicate. Bars represent S.E. Numbers below the graph are the fold increase in luciferase activity in the presence of HIF-1α/VP16. *, $P < 0.01$, compared with the absence of a plasmid expressing HIF-1α/VP16 (Student's *t* test).

part of the erythropoietin HRE (Semenza and Wang, 1992). A similar motif, termed the HIF-1 ancillary sequence, has been identified in other HIF-1 target genes as well (Kimura et al., 2001). In this experiment, the negative control is the plasmid pGL3-promoter (the parental SV40 promoter-luciferase reporter plasmid). The positive control was a pGL3-based plasmid containing the VEGF HRE (Forsythe et al., 1996). Each of these reporter plasmids was cotransfected into HeLa cells with either pcDNA3 (the empty expression vector) or a vector expressing native HIF-1α, HIF-1α/VP16, or HIF-1α/NF-κB; the latter is an HIF-1α hybrid containing the activation domain from the p65 subunit of human NF-κB. Cells were either maintained under normoxic conditions, subjected to hypoxia, or treated with desferrioxamine, an iron-chelator that is known to mimic the effect of hypoxia. The results (Fig. 6) show that the 50-bp sequence comprising the mHRE is able to mediate activation by HIF-1α/VP16. The HIF-1α/NF-κB hybrid also up-regulated the BNP mHRE, demonstrating that a construct with a different heterologous activation domain can exert an effect similar to that of HIF-1α/VP16. The HIF-1α/NF-κB hybrid is also able to up-regulate expression of the endogenous BNP gene in human fetal cardiac cells (data not shown). Overexpressed HIF-1α activated the mHRE construct 6-fold under hypoxic conditions and in the presence of desferrioxamine ($P < 0.001$), suggesting that the BNP HRE does indeed contain an authentic HIF-1 binding site. Although not statistically significant, there was also a trend toward enhanced expression with hypoxia alone and over-expressed HIF-1α under normoxic conditions. The levels of activation observed with the BNP mHRE are similar to those observed with the VEGF HRE construct.

These results were further confirmed in a second experiment, which included a construct that contained point mutations within the HIF-1 binding site consensus sequence (mmHRE), one carrying two tandem copies of the BNP mHRE (2xHRE), and one which bears substitutions within the CACAG motif (2mmHRE) (Fig. 7). Each of these reporters was cotransfected into HeLa cells with either the empty pcDNA3 vector or a vector overexpressing HIF-1α/VP16 or the native HIF-1α. Cells were maintained under normoxic conditions. Similar to what has been observed with the erythropoietin HRE (Pugh et al., 1994), tandem BNP HREs result in more potent induction of transcription than a single copy. This is the case with both HIF-1α/VP16 and overexpressed HIF-1α. In the latter case, up-regulation of approximately 30-fold is observed, further confirming the ability of native HIF-1α to act on the BNP HRE. Unlike the erythropoietin HRE, however, mutation of the CACAG motif in the BNP HRE had no effect on up-regulation by either HIF-1α/VP16 or native HIF-1α.

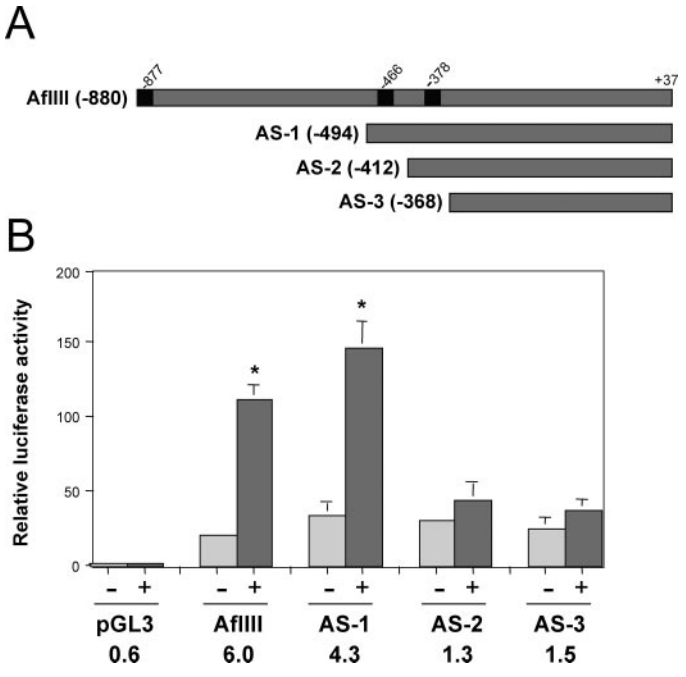

Fig. 5. Deletion analysis within a defined region of the BNP promoter to identify the HRE. A, shown in the diagram are the endpoints of the three 5′ truncation constructs (AS-1, AS-2, and AS-3) engineered between the AflIII and StyI sites within the BNP promoter. Each sequentially removes one possible HIF-1 binding site (shown in filled boxes). B, the deletion constructs were transfected into rat neonatal cardiac myocytes either in the presence (+) or absence (−) of a plasmid expressing HIF-1α/VP16. Luciferase activity in each of the samples was normalized to β-galactosidase activity; relative luciferase activity is the ratio of that value to the value obtained from cells transfected with pGL3 in the absence of HIF-1α/VP16. Results are averaged from four independent experiments; each transfection was performed in duplicate. Error bars represent S.E. Numbers below the graph are the fold increase in luciferase activity in the presence of HIF-1α/VP16. $*$, $P < 0.001$ compared with the absence of a plasmid expressing HIF-1α/VP16 (Student's t test).

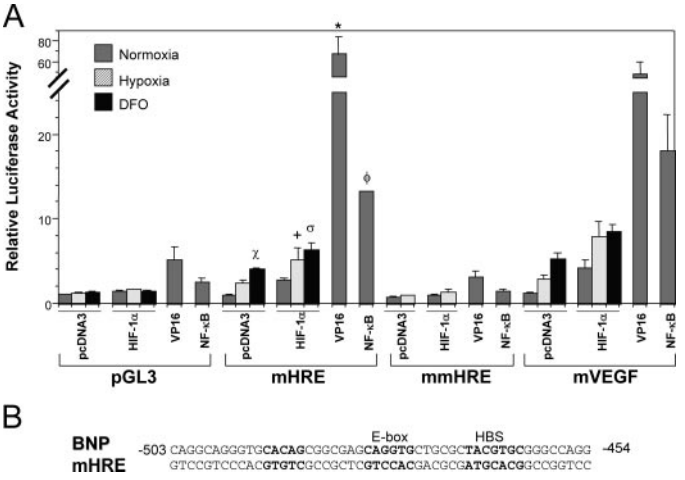

Fig. 6. Functional analysis of the BNP HRE in the context of a heterologous promoter. A, a luciferase reporter plasmid containing a minimal SV40 promoter (pGL3-promoter) was engineered to include either the minimal HRE (mHRE) from the BNP promoter (nt −503 to −454), a mutated version (mmHRE), or as a positive control, the VEGF HRE (mVEGF). These plasmids were cotransfected into HeLa cells with either pcDNA3 (negative control), a plasmid expressing the full-length HIF-1α cDNA (HIF-1α), a plasmid expressing HIF-1α/VP16 (VP16), or a plasmid expressing another HIF-1α hybrid, HIF-1α/NF-κB (NF-κB), which contains the activation domain from the NF-κB subunit p65. Cells were then either maintained under normoxic conditions, subjected to hypoxia, or treated with 100 μM desferrioxamine (DFO). Luciferase activity was normalized to β-galactosidase activity; relative luciferase activity is the ratio of that value to the value obtained from cells cotransfected with pGL3-promoter and pcDNA3 under normoxic conditions. Results are averaged from five separate experiments (except for the mVEGF control data, which are from three experiments); each transfection was performed in duplicate. Error bars represent S.E. B, the sequence of the BNP mHRE. In boldface type are the HIF-1 binding site (HBS), the E-box motif, and the CACAG sequence. $*$, $P < 0.001$, compared with pGL3/VP16 or mmHRE/VP16; +, $P < 0.001$, compared with mHRE/pcDNA3/N, mHRE/pcDNA3/H, or mHRE/HIF-1α/N; ϕ, $P < 0.001$, compared with pGL3/NF-κB and mmHRE/NF-κB; σ, $P < 0.0.001$, compared with mHRE/pcDNA3/N, mHRE/pcDNA3/DFO, and mHRE/HIF-1α/N; χ, $P < 0.01$, compared with mHRE/pcDNA3/N (one-way ANOVA followed by Newman-Keuls test).

A search for the possible additional transcription factor binding sites within the BNP HRE identified an E-box motif (CANNTG) just upstream of the HIF-1 binding site (at −480 to −475). A luciferase reporter construct containing a mutation within this site showed a trend toward reduced expression when cotransfected with an HIF-1α/VP16 expression plasmid (Fig. 8). This result suggests that an E-box-binding transcription factor may be contributing to the induction of BNP gene expression by HIF-1α/VP16.

EMSA Analysis of HIF-1α/VP16 Binding to the BNP HRE. To determine whether the HBS in the BNP promoter is an authentic one, gel-shift analyses were performed using nuclear extracts derived from either uninfected Hep3B cells or cells infected with Ad2/HIF-1α/VP16 or Ad2/HIF-1αFL. As shown in Fig. 9, A and B, both overexpressed native HIF-1α and HIF-1α/VP16 proteins are able to form a complex with a 24-bp oligonucleotide that contains the HIF-1 binding site. With both extracts, a nonspecific complex is detected as well as a doublet band corresponding to a constitutive binding activity, similar to that observed previously with erythropoietin and VEGF HRE probes (Semenza and Wang, 1992; Forsythe et al., 1996). The HIF-1α/VP16-containing complex comigrates with the constitutive complex (Fig. 9B). To further confirm the specific binding activity of both HIF-1α and HIF-1α/VP16, a probe was synthesized to contain mutations within the HIF-1 binding site consensus sequence. These mutations, when introduced into the minimal BNP HRE, had prevented the activation of expression within the context of the luciferase reporter plasmid (Fig. 7). Likewise, both HIF-1α and HIF-1α/VP16 are unable to form a complex with the mutant sequence (Fig. 9, A and B, lanes 3 and 4). Furthermore, the presence of HIF-1α and HIF-1α/VP16 proteins in the specific complex was confirmed by supershift analysis (Fig. 9, A and B, lanes 5 and 6). Competition experiments demonstrated that the specific binding activity could be inhibited by a 50-fold molar excess of unlabeled wild-type probe but not by a 500-fold molar excess of mutant probe. Figure 9C shows a similar panel of reactions using an HIF-1α/VP16-containing extract and an erythropoietin probe as a positive control, demonstrating that this protein is also capable of recognizing and binding to an HRE characterized previously. The results of the EMSA experiments, in conjunction with the HIF-1αΔ/VP16 mutant results, demonstrate that up-regulation of BNP gene expression by HIF-1α/VP16 is dependent on binding to the HBS via the HIF-1α portion of the protein.

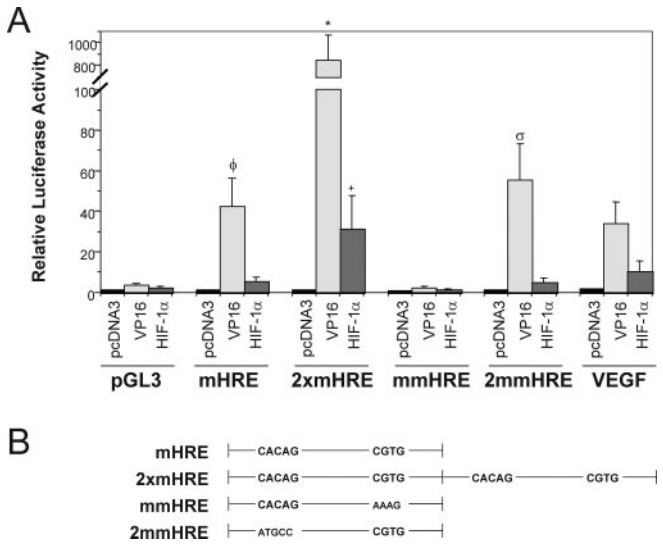

Fig. 7. Functional analysis of modified versions of the BNP HRE. A, the experiment was carried out as described in the legend to Fig. 6; only normoxic conditions were analyzed. Results are averaged from four separate experiments; each transfection was performed in duplicate. Error bars represent S.E. B, a schematic of the constructs analyzed in this experiment. CGTG represents the canonical core of the HIF-1 binding site; CACAG is a possible secondary site that has been shown to function as part of the erythropoietin HRE (Semenza and Wang, 1992). The mHRE is composed of nt −503 to −454 from the BNP promoter; the 2xmHRE contains two copies of the mHRE; the mmHRE is mutated within the CGTG sequence of the HIF-1 binding site; the 2mmHRE bears mutations within the CACAG motif. +, $P < 0.05$ compared with pGL3/HIF-1α, mHRE/HIF-1α, and mmHRE/HIF-1α. ∗, $P < 0.001$ compared with pGL3/VP16, mHRE/VP16, and mmHRE/VP16; ϕ, $P < 0.05$ compared with pGL3/VP16 and mmHRE/VP16; σ, $P < 0.05$ compared with pGL3/VP16 and mmHRE/VP16 (one-way ANOVA followed by Newman-Keuls test).

Discussion

A gene expression profile analysis aimed at characterizing the effect of infection with Ad2/HIF-1α/VP16 revealed that expression of the BNP gene in human fetal cardiac cells was significantly up-regulated (Jiang et al., 2002). A similar approach taken with a recombinant adenovirus expressing a constitutively active mutant of native HIF-1α did not identify BNP as an induced gene; however, that analysis was performed in human pulmonary arterial endothelial cells (Manalo et al., 2005). As reported here, the original SAGE findings were confirmed via real-time RT-PCR analysis of independently derived RNA samples. Subsequent experiments were therefore focused on determining the mechanism of Ad2/HIF-1α/VP16-mediated up-regulation of BNP gene expression. The results confirmed the existence of an HRE in the BNP promoter and demonstrated that binding of HIF-1α/VP16 to this sequence is via the HIF-1α portion of the protein.

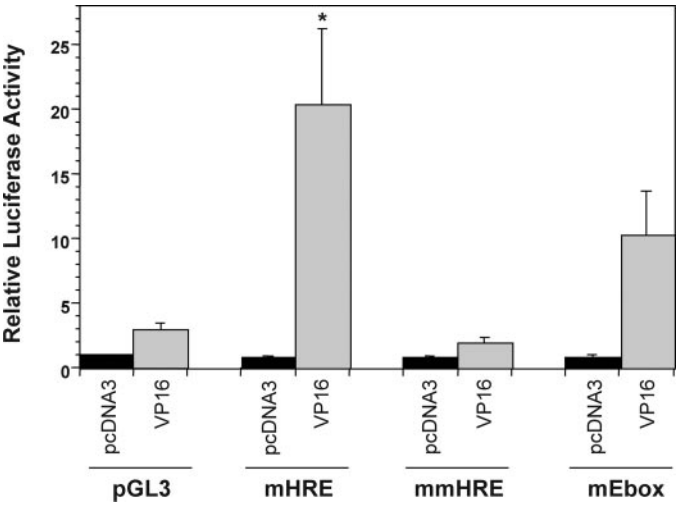

Fig. 8. Analysis of an E-box motif in the BNP HRE. The experiment was carried out as described in the legend to Fig. 6; only normoxic conditions were analyzed. Results are averaged from three separate experiments; each transfection was performed in duplicate. Error bars represent S.E. mEbox is a luciferase reporter plasmid containing a mutation in the E-box motif within the minimal BNP HRE. ∗, $P < 0.05$ compared with pGL3/VP16 and mmHRE/VP16 (one-way ANOVA followed by Newman-Keuls test).

Deletion analysis of the human BNP promoter indicated that the HRE was located between 494 and 412 bp upstream of the transcription initiation site. Within this region is an HIF-1 binding site, TACGTGCGG, that exactly matches a consensus sequence, (T/G)ACGTGCGG (Wenger and Gassmann, 1997). Mutation of the HIF-1 binding site resulted in inhibition of both DNA binding and transcriptional activation by either HIF-1α/VP16 or HIF-1α. It is noteworthy that deletion of the DNA binding domain in the HIF-1αΔ/VP16 mutant abrogated binding to the HRE and transcriptional activation of the BNP gene, demonstrating that the HIF-1α/VP16 effect is dependent on the HIF-1α domain. Although HIF-1α/VP16 strongly activates transcription of the human BNP gene in vitro, this effect was not observed when rat cardiac myocytes were infected with Ad2/HIF-1α/VP16. One possible explanation might be the absence of a strong HRE in the rat BNP promoter. There are three potential HIF-1 binding sites within the rat BNP promoter (at −873, −614, and −543). The latter, CACGTGTGG, matches the consensus at seven of nine positions; however, a threonine at the seventh position does not appear in 20 functional HBSs (Wenger and Gassmann, 1997).

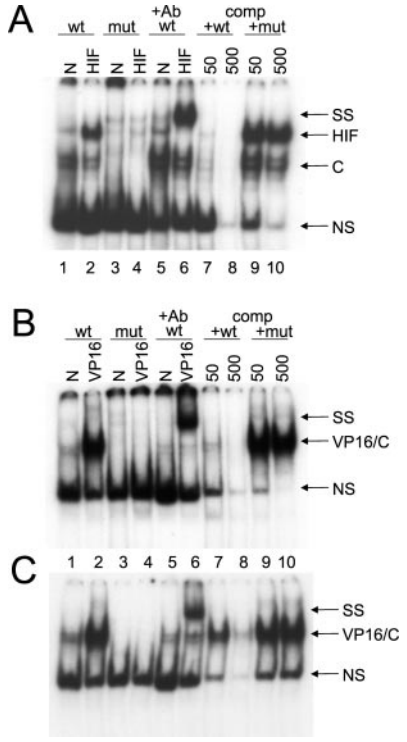

Fig. 9. EMSA analysis of HIF-1α/VP16 binding to the BNP HRE. A, the results of gel-shift assays using extracts from uninfected Hep3B cells maintained under normoxic conditions (N) or cells infected with Ad2/HIF-1α m.o.i. = 200) and subjected to hypoxia for 24h (HIF). Probes were derived from the BNP HRE (nt −476 to −μ453) and were either wild-type (wt, lanes 1 and 2) or mutated (mut; lanes 3 and 4) versions. Reactions in lanes 5 and 6 were supershifted with an antibody specific to HIF-1α. Lanes 7 to 10 were competition experiments which included either 50- or 500-fold excess of unlabeled wt (lanes 7 and 8) or mutant (lanes 9 and 10) oligonucleotides. Arrows show the positions of a nonspecific complex (NS), a constitutive complex (C), the complex containing HIF-1α (HIF), and the supershifted complex (SS). The free probe was run off the gel and is not shown here. B, a similar experiment performed with an extract from cells infected with Ad2/HIF-1α/VP16 (VP16). The HIF-1α/VP16-containing complex comigrates with the constitutive complex. The supershifts were performed with an antibody specific to the C terminus of VP16. C, similar to panel B but using an oligonucleotide probe derived from the erythropoietin HRE.

It has been demonstrated for several hypoxia-responsive genes that transcriptional regulation by HIF-1 is mediated by interactions among multiple factors. For example, the human erythropoietin HRE includes the HIF-1 binding site, an adjacent CACAG (termed the HIF-1 ancillary sequence or HAS), and a binding site for hepatic nuclear factor 4 (HNF; Ebert and Bunn, 1999; Kimura et al., 2001). Three functional domains also exist within the mouse lactate dehydrogenase A HRE, including the HIF-1 binding site, an adjacent HAS, and a cAMP response element (Ebert and Bunn, 1998). In general, each of these sites within the HRE is necessary but not sufficient for hypoxic induction; interactions between the factors binding these sites are believed to be required for high-affinity binding of the transcriptional coactivator protein p300/CBP (Ebert and Bunn, 1998). The human BNP HRE, defined as nucleotides −494 to −454, includes a candidate HAS motif; however, it appears 24 bp upstream of the HBS. Mutation of this sequence does not interfere with activation by either HIF-1α or HIF-1α/VP16. This result is not surprising, because the position of the HAS (8–9 bp downstream of the HBS) was shown to be crucial for activity (Kimura et al., 2001). The human BNP HRE also contains an E-box motif (CAGGTG) just upstream of the HIF-1 binding site (−484 to −475). Similar to the HNF site and the cAMP response element in the erythropoietin and lactate dehydrogenase A and VEGF HREs, respectively, mutation of the E-box motif within the BNP HRE reduces but does not completely abolish transcriptional activation. This site may be bound by muscle-specific, basic helix-loop-helix proteins such as MyoD, myogenin, myf-5, and MRF4. The E-box motif might therefore be functionally homologous to the HNF site in the erythropoietin HRE, acting to recruit, for example, MyoD, which is known to bind p300 (Yuan et al., 1996).

Although both HIF-1α/VP16 and native HIF-1α bind to the BNP HRE in vitro, the two factors differ with respect to their ability to activate BNP gene expression in primary human fetal cardiac cells. Results of experiments presented in Figs. 1 and 2 confirm the original SAGE analysis and suggest that under hypoxic conditions, there is little increase in expression of the endogenous human BNP gene. This result may be explained by differences in the potency of the VP16 and endogenous HIF-1α transcriptional activation domains. Three pieces of data suggest that native HIF-1α is capable of binding to the BNP HRE but that sensitivity of the BNP promoter to HIF-1α is low. First, the EMSA data show that native HIF-1α binds to the BNP HRE. Second, experiments performed with the minimal HRE in HeLa cells show that there is an effect of native HIF-1α when overexpressed (6-fold; Fig. 6); the latter effect is increased when the HRE is duplicated (to 30-fold; Fig. 7). Finally, a small yet detectable effect was observed at 72 h with overexpressed, native HIF-1α in the time course experiment in primary human fetal cardiac cells. Thus, although up-regulation of BNP gene expression by the HIF-1α/VP16 hybrid protein depends on binding to the HRE via the HIF-1α domain, the magnitude of the observed effect compared with native HIF-1α may be attributable to the strong VP16 activation domain. This possibility may have implications regarding the performance of Ad2/HIF-1α/VP16 in vivo. For example, negative consequences might arise from the up-regulation of genes not normally regulated by native HIF-1α and/or the activation of transcription of HIF-1α target genes to a greater than normal

extent. However, to date, no transgene-related toxicity has been observed either in experiments performed in animals or in the phase I clinical trial (Rajagopalan et al., 2006).

A potential limitation of the studies presented here is that cells were exposed to hypoxic conditions of 1% oxygen. It has been suggested that mitochondrial-derived reactive oxygen species (ROS) might play a role in oxygen-sensing under these conditions (e.g., 1–3% O_2). The ROS are proposed to act through signaling pathways to inactivate the prolyl hydroxylases and lead to stabilization of HIF-1α (Schroedl et al., 2002; Brunelle et al., 2005; Guzy et al., 2005; Mansfield et al., 2005). In contrast, it has been suggested that ROS do not play a role under anoxic conditions (e.g., 0.1–0.5% O_2) when limiting oxygen would directly inhibit the prolyl hydroxylases. It is a formal possibility that under 1% O_2, generation of ROS by the mitochondria might activate a signaling pathway that would repress BNP promoter activity and that this might explain why BNP gene expression is not up-regulated by hypoxia. The role of ROS in hypoxia is not necessarily relevant to the HIF-1α/VP16 effect, because HIF-1α/VP16 up-regulates BNP under normoxic conditions.

An additional hypothesis to explain the ability of the wild-type HIF-1α to activate transcription of the minimal HRE constructs but not the endogenous BNP promoter in human cardiac cells is that a repressor could interact with a regulatory element present in the full-length, chromosomal BNP promoter. A general limitation of studies using minimal HRE constructs is that most of the endogenous promoter and associated *cis*-acting transcriptional elements are absent. A repressor might act by inhibiting transcriptional activation by the native HIF-1α activation domain specifically or by influencing HIF-1α binding at the HRE. Such a role has been proposed for the AP-1 and GATA-2 factors in the function of the endothelin-1 HRE (Yamashita et al., 2001) and for USF-2a in modulating plasminogen activator inhibitor-1 expression (Samoylenko et al., 2001). The influence of an inhibitory factor that affects DNA binding may be mitigated by the strong VP16 activation domain in HIF-1α/VP16.

The finding that the endogenous human BNP promoter in primary human fetal cardiac cells is not up-regulated by hypoxia seems to conflict with recent clinical data suggesting that the BNP gene is up-regulated by ischemia in patients with coronary artery disease (Yeo et al., 2005). Likewise, neither overexpressed HIF-1α nor HIF-1α/VP16 activated the expression of the BNP gene in primary neonatal rat cardiomyocytes, whereas ventricular BNP mRNA levels are increased in rats after myocardial infarction (MI) (Hama et al., 1995; Shimoike et al., 1997; Hystad et al., 2001) and after exposure to chronic hypoxia (Hill et al., 1994; Perhonen et al., 1997). These inconsistencies might be explained by differences in the properties of primary cells in vitro and cells in situ and/or between adult and fetal or neonatal cells. Another possibility is that the observed increase in BNP gene expression is not a direct effect of hypoxia but might be due to regional wall motion abnormalities elicited by ischemia (Yeo et al., 2005). It is well established that BNP expression is enhanced in cardiac myocytes in response to mechanical strain (Liang and Gardner, 1999; de Lemos et al. 2003). Transgenic mice carrying a luciferase gene expressed from a minimal human BNP promoter (−408 to +100) show up-regulation of BNP promoter activity after MI. Because this region of the promoter does not contain the putative HRE, activation of promoter activity after infarction may be attributed to pathophysiological stimuli other than hypoxia (He et al., 2001).

The ability of Ad2/HIF-1α/VP16 to promote neovascularization (Vincent et al., 2000; Shyu et al., 2002) is probably attributable to the multifaceted effects of HIF-1, including the enhancement of expression of angiogenic growth factors such as VEGF, Ang-2, Ang-4, and placental growth factor (Vincent et al., 2000; Jiang et al., 2002; Yamakawa et al., 2003). In addition, HIF-1 has been shown to up-regulate the chemokine stromal cell derived factor-1, which promotes the recruitment of endothelial progenitor cells to ischemic tissue (Ceradini et al., 2004). Up-regulation of the BNP gene is potentially relevant to the proangiogenic effect of HIF-1α/VP16, because it has been reported that angiogenesis was accelerated in ischemic hind limbs of transgenic mice overexpressing BNP (Yamahara et al., 2003).

The possibility that BNP might be regulated by HIF-1 supports the hypothesis that BNP serves an adaptive role in ischemic myocardium. D'Souza et al. (2003) found that administration of exogenous BNP to isolated perfused rat hearts was protective against ischemia/reperfusion injury. Possible beneficial effects of enhancing BNP expression extend also to heart failure. Production of BNP is enhanced in the diseased heart; plasma BNP levels are elevated early in acute MI, during congestive heart failure, and in conjunction with other conditions characterized by ventricular hypertrophy. Indeed, BNP concentration is considered a diagnostic marker of congestive heart failure (de Lemos et al., 2003). By virtue of its systemic effects on the kidney and vasculature, BNP may play a compensatory role in this setting. However, the role of BNP may not be limited to fluid homeostasis. By selectively knocking out the ANP/BNP receptor guanylyl cyclase-A in cardiomyocytes and thereby circumventing a systemic effect, Holtwick et al. (2003) provided evidence that guanylyl cyclase-A functions within the myocardium to moderate cardiac hypertrophy. BNP may influence cardiac remodeling after MI by inhibiting fibrosis (Tamura et al., 2000), possibly either via the inhibition of collagen synthesis and induction of metalloproteinases (Tsuruda et al., 2002; Kawakami et al., 2004) or by opposition of transforming growth factor-β (Kapoun et al., 2004). Therefore, through the activation of BNP gene expression, Ad2/HIF-1α/VP16 may not only enhance angiogenesis but may also promote additional therapeutic benefits within ischemic tissues.

Acknowledgments

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