Nuclear Factor Interleukin 6 Motifs Mediate Tissue-specific Gene Transcription in Hypoxia

(Received for publication, July 24, 1996, and in revised form, November 7, 1996)

Shi-Fang Yan‡, Yu Shan Zou, Monica Mendelsohn, Yun Gao, Yoshifumi Naka, Shi Du Yan, David Pinsky, and David Stern‡

From the Departments of Physiology, Surgery, Medicine, and Pathology, Columbia University, College of Physicians and Surgeons, New York, New York 10032

Activating transcription at the nuclear factor interleukin 6 (NF-IL-6) DNA binding motif modulates expression of multiple genes important in host adaptive and developmental mechanisms. Studies showing that hypoxia-induced transcription of IL-6 in cultured endothelial cells was due to transcriptional activation by the NF-IL-6 motif in the promoter (Yan, S.-F., Trittio, I., Pinsky, D., Liao, H., Huang, J., Fuller, G., Brett, J., May, L., and Stern, D. (1995) J. Biol. Chem. 270, 11463–11471) led us to prepare transgenic mice using 115- or 14-base pair regions of the promoter encompassing the NF-IL-6 site ligated to the lacZ reporter gene and the basal thymidine kinase promoter. On exposure to hypoxia or induction of ischemia, mice bearing either of the constructs showed prominent expression of the transgene in lung and cardiac vasculature and in the kidney but not in the liver (parenchyma or vasculature). In contrast, transgenic mice bearing a mutationally inactivated NF-IL-6 site showed no increase in transgene expression in hypoxia. Gel retardation assays revealed tissue-specific hypoxia-enhanced nuclear binding activity for the NF-IL-6 site in nuclear extracts of the heart, lung, and kidney but not in the liver; the hypoxia-enhanced band disappeared on addition of antibody to C/EBPβ-NF-IL-6. Consistent with the specificity of hypoxia-mediated activation of C/EBPβ-NF-IL-6, gel retardation assays showed no change in the intensity of the hypoxia-enhanced gel shift band in the presence of excess unlabeled oligonucleotide probes or antibodies related to other transcription factors, including NFκB, AP1, cAMP response element-binding protein, SP1, and hypoxia-inducible factor 1. These data indicate that the transcription factor NF-IL-6 is sensitive to environmental oxygen deprivation, and the tissue-specific pattern of gene expression suggests that local mechanisms have an important regulatory effect.

One of the fundamental cellular responses to environmental stress is redirection of biosynthetic mechanisms that promote adaptation and enhance survival. These mechanisms have been studied in detail following induction of heat shock and are now coming under intensive study in the setting of oxygen deprivation. Limitation of oxygen supply occurs in a range of situations, from environmental deficiency at high altitude to that resulting from diminished blood flow, or in solid tumors with growth that has outpaced the ability of neovasculature to sustain cellular activities. One of the best studied instances of gene expression modulated by hypoxia is induction of erythropoietin (1–3), which appears to be mediated largely at the transcriptional level by hypoxia-inducible factor 1 (HIF-1; Ref. 1). The DNA binding motif for this transcription factor is also present in the promoter of certain key glycolytic enzymes (4) and in a homologous form in the promoter for vascular endothelial growth factor (5–7) and non-insulin-dependent glucose transporter 1 (8), both of which demonstrate enhanced expression in response to oxygen deprivation.

In a previous study, we found that hypoxia stimulated expression of the cytokine interleukin 6 (IL-6) by cultured endothelial cells (ECs), and that this was due to enhanced transcription driven by the nuclear factor IL-6 (NF-IL-6) site in the IL-6 promoter (9). In the hypoxic cultured endothelium, activation NF-IL-6-C/EBPβ was observed, although there was no evidence for activation of C/EBPα or δ. Since NF-IL-6 binding elements are present in multiple genes with expression that might contribute importantly to the cellular response during oxygen deprivation (10), we made transgenic mice in which a 115- or 14-bp portion of the IL-6 promoter, encompassing the NF-IL-6 site, was fused to the lacZ gene as a reporter and to the basal thymidine kinase promoter. Our results indicate that transcriptional activation does occur at the NF-IL-6 site in vivo in hypoxia and ischemia, and that this is especially evident in vasculature of the lung and heart and in the kidney. In addition, nuclear extracts from the latter organs showed a prominent hypoxia-mediated increase in binding activity for an NF-IL-6 oligonucleotide probe. In contrast, hypoxic liver did not display enhanced transgene expression in vasculature or parenchyma, and there was no increase in NF-IL-6 binding activity. These results indicate the existence of tissue-specific mechanisms underlying activation of NF-IL-6, which can be modulated by the local microenvironment in response to oxygen deprivation.

MATERIALS AND METHODS

Preparation of DNA Constructs—Plasmids pTKCi 225/111 (11–13), pYSF52, and pYSF55 (9) were digested with HindIII-BamHI to remove the chloramphenicol acetyltransferase gene. The latter was replaced with the 3.7-kilobase lacZ gene from plasmid pCH110 (14) by ligation with T4 DNA ligase in one orientation to produce plasmids pYSF46, pYSF56, and pYSF57 from pTKCi 225/111, pYSF52, and pYSF55, re-

* This work was supported by United States Public Health Service Grants HL42507, HL50629, and PERC and by the Patterson Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Physiology, P&S 11-518, Columbia University, College of Physicians and Surgeons, 630 W. 168th St., New York, NY 10032. Tel.: 212-305-1615; Fax: 212-305-5337.

1 The abbreviations used are: HIF-1, hypoxia-inducible factor 1; IL-6, interleukin 6; NF, nuclear factor; EMSA, electrophoretic mobility shift assay; EC, endothelial cell; bp, base pair; ELISA, enzyme-linked immunosorbent assay; CREB, CAMP response element-binding protein; CRP, C-reactive protein.
was digested with restriction enzymes N2 (blotted, and hybridized with $^{32}$P-labeled probe (Southern analysis of founders (EcoRI constructs 1–3.

Superovulated female mice (B6CBAF1/J; Jackson Laboratory, Bar Harbor, ME) were mated with B6CBAF1/J males, and fertilized eggs were harvested and microinjected with the appropriate construct. In brief, following incubation for 18 h at 37 °C in M16 media (Specialty Media, LaVallette, NJ) in an atmosphere with carbon dioxide (9%) in air, oocytes were transferred to the oviducts of pseudopregnant foster mothers. Three independent transgenic lines for construct 1, four for construct 2, and two for the mutant construct (construct 3) were generated. To identify transgenic mice, the tails (~1 cm) were cut from 3-week-old pups, and DNA was extracted and digested with EcoRI-HindIII. Southern blotting was performed using a $^{32}$P-labeled EcoRI-HindIII fragment from pCH110. Founders were then mated with non-transgenic mice to generate new offspring (B6CBAF1/J) and to check transmission of the transgene.

Studies in Hypoxic Mice—All experiments were performed according to protocols approved by the Institutional Care and Use Committee at Columbia University, in accordance with Association for the Accreditation of Laboratory Animal Care guidelines. Mice were subjected to hypoxia using a custom designed controlled environmental chamber as described previously (15). Animals were placed in the chamber and allowed free access to food and water. During the first hour, the oxygen content of the atmosphere in the chamber was slowly reduced from ambient levels to 6% with the balance of the gas mixture made up of nitrogen. Then, 6% oxygen content was maintained for the following 6 h. Mice were then sacrificed, and tissue (lung, heart, kidney, and liver) was harvested for the assays described below. For the mouse lung ischemia model, after appropriate anesthesia, mice were placed on a Harvard ventilator (tidal volume, 0.75 ml; respiratory rate, 180/min), ventilated with 100% oxygen, and underwent bilateral thoracotomy. The left pulmonary artery was cross-clamped for a period of 60 min, and the cross-clamp was then released. After 4 h of reperfusion, lung tissue from the nonmanipulated (nonischemic) and manipulated (postischemic) mice was obtained and either placed in formalin (10%) for histologic analysis or snap frozen in liquid nitrogen.

Assays for Transgene Expression—ELISA was used to evaluate expression of β-galactosidase used tissue fragments (lung, heart, kidney, and liver) from normoxic or hypoxic transgenic mice washed three times with phosphate-buffered saline. Organs were then suspended in homogenizing buffer (NaCl, 100 mM; Tris-HCl, pH 7.4, 20 mM; EDTA, 1 mM; aprotinin, 1 μg/ml; phenylmethylsulfonyl fluoride, 1 μM) and homogenized using a Polytron. Soluble protein was isolated, and its concentration was determined using the Bio-Rad protein assay kit. Equal amounts of total protein were analyzed in each ELISA for β-galactosidase (Life Technologies, Inc.).

Immunohistological analysis of β-galactosidase expression was studied in the lung, heart, kidney, and liver. Organs were rapidly harvested from normoxic or hypoxic animals, fixed overnight in formalin (3.5%), dehydrated, and embedded in paraffin by standard procedures (16). Sections were then rehydrated, incubated in blocking buffer (phosphate-buffered saline containing bovine serum albumin, 1% and normal goat serum, 2%) for 30 min at 37 °C, washed in phosphate-buffered saline, and exposed to monospecific rabbit anti-β-galactosidase IgG (30 μg/ml; Cortex Biochem Inc., San Leandro, CA). Tissue sections were washed again in phosphate-buffered saline and incubated with biotinylated goat anti-rabbit immunoglobulin followed by peroxidase-conjugated ExtrAvidin (Sigma). Localization of peroxidase conjugates was revealed using aminomethylcarbazole as the chromogen. Nonimmune rabbit IgG replaced the anti-β-galactosidase IgG for controls. IL-6 was measured in tissue from normoxic or hypoxic transgenic mice using affinity-purified anti-mouse IL-6 IgG and the same immunostaining procedure as described previously (9). This antibody was generously

![Image](image.png)
Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from organs of nontransgenic mice (B6CBAF1/J), including lung, heart, kidney, and liver (mice had been exposed to normoxic or hypoxic conditions for 0.5, 1, 2, 3, and 4 h) using the method of Dignam et al. (17). These extracts were prepared in an environment with the same ambient oxygen tension as that in the experiment. Complementary 14-bp oligonucleotides (2158 to 2145) containing an NF-IL-6 site that included 5'-ACATTGCACAATCT-3' and 5'-AGATTGTGCAAT-GT-3' were used. Oligonucleotides were annealed and 5'-end-labeled with [32P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase according to standard procedures. Binding reactions were performed by preincubation of nuclear extract protein (1–5 μg) in HEPES (pH 7.9, 20 mM), KCl (60 mM), MgCl₂ (1 mM), EDTA (0.1 mM), glycerol (10%), dithiothreitol (0.5 mM), and poly(dI-dC) (2 μg) at room temperature for 10 min followed by addition of the double-stranded [32P]-labeled oligonucleotide (~35 fmol) and a second incubation at room temperature for 20 min. Where indicated, antiserum to C/EBPa, β, or δ, CRP1, CREB-1, CREB-2, c-Fos, c-Jun/AP1, p50 (NFκB), or p65 (NFκB) (Santa Cruz Biotechnology, Santa Cruz CA) was incubated with nuclear extract at room temperature for 1 h, and then the procedure described above was followed. Samples (5 μg of protein in each lane) were loaded directly onto nondenaturing polyacrylamide-bisacrylamide gels (4%) prepared in Trisborate/EDTA (0.5 ×; Tris, 45 mM, boric acid, 45 mM, and EDTA, 0.1 mM), and electrophoresis was performed at room temperature for 1.5–2 h at 200 V. For competition studies, a 100-fold molar excess of unlabeled NF-IL-6 probe (as above), SP1 probe (18), 18-bp probe for HIF-1 (1), CREB (this and the other the following oligonucleotides probes were purchased from Santa Cruz), AP1, and NFκB was used.

provided by Dr. Gerald Fuller (University of Alabama, Birmingham, AL).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from organs of nontransgenic mice (B6CBAF1/J), including lung, heart, kidney, and liver (mice had been exposed to normoxic or hypoxic conditions for 0.5, 1, 2, 3, and 4 h) using the method of Dignam et al. (17). These extracts were prepared in an environment with the same ambient oxygen tension as that in the experiment. Complementary 14-bp oligonucleotides (~158 to ~145) containing an NF-IL-6 site that included 5’-ACATTGCACAATCT-3’ and 5’-AGATTGTGCAAT-GT-3’ were used. Oligonucleotides were annealed and 5’-end-labeled with [32P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase according to standard procedures. Binding reactions were performed by preincubation of nuclear extract protein (1–5 μg) in HEPES (pH 7.9, 20 mM), KCl (60 mM), MgCl₂ (1 mM), EDTA (0.1 mM), glycerol (10%), dithiothreitol (0.5 mM), and poly(dI-dC) (2 μg) at room temperature for 10 min followed by addition of the double-stranded [32P]-labeled oligonucleotide (~35 fmol) and a second incubation at room temperature for 20 min. Where indicated, antiserum to C/EBPa, β, or δ, CRP1, CREB-1, CREB-2, c-Fos, c-Jun/AP1, p50 (NFκB), or p65 (NFκB) (Santa Cruz Biotechnology, Santa Cruz CA) was incubated with nuclear extract at room temperature for 1 h, and then the procedure described above was followed. Samples (5 μg of protein in each lane) were loaded directly onto nondenaturing polyacrylamide-bisacrylamide gels (4%) prepared in Trisborate/EDTA (0.5 ×; Tris, 45 mM, boric acid, 45 mM, and EDTA, 0.1 mM), and electrophoresis was performed at room temperature for 1.5–2 h at 200 V. For competition studies, a 100-fold molar excess of unlabeled NF-IL-6 probe (as above), SP1 probe (18), 18-bp probe for HIF-1 (1), CREB (this and the other the following oligonucleotides probes were purchased from Santa Cruz), AP1, and NFκB was used.

Fig. 3. Immunohistology for β-galactosidase antigen in tissues from transgenic mice bearing construct 1: comparison with IL-6. Mice were maintained in normoxia or were subjected to hypoxia as described, and then immunostaining was performed on tissue sections for β-galactosidase and IL-6 antigens. Panels shown represent β-galactosidase distribution in the lung (A, hypoxia; B, normoxia), heart (C, hypoxia; D, normoxia), and kidney (G, hypoxia; H, normoxia). A, inset, higher magnification of smooth muscle cells from hypoxic pulmonary vasculature. Immunostaining for IL-6 in nontransgenic mice in the heart (E, hypoxia; F, normoxia) and kidney (I, hypoxia; J, normoxia) shows a similar distribution to that of β-galactosidase in the transgenic mice. These experiments were repeated a minimum of three times. Bars, 61 and 20 (inset) μm.
lanes 11 and 2, respectively, each of which transmitted the transgene. Three transgenic mice were made in which the C/EBPα site was present (Fig. 1B, lane LPS). When exposed to hypoxia, these animals demonstrated enhanced expression of β-galactosidase in a similar pattern and to a degree similar to that observed with mice bearing construct 1 (data not shown). Importantly, mice bearing construct 3, in which the NF-IL-6 was mutationally inactivated, showed no increase in β-galactosidase expression compared with normoxic controls in the lung or other organs (Fig. 5, C and D, hypoxic and normoxic lung, respectively).

The effects of hypoxia on hepatic expression of construct 1 in transgenic mice (A and B) and IL-6 transcripts (C). Mice were exposed to hypoxia (as above; A) or were maintained in normoxia (B), and tissue was harvested for localization of β-galactosidase antigen. Bar, 61 μm. C, mice were exposed to hypoxia (H) or normoxia (N) for 4 h, and total RNA was harvested and subjected to polymerase chain reaction analysis using primers for murine IL-6 (upper panel) or β-actin (lower panel). The positive control was liver RNA harvested from animals treated with lipopolysaccharide (LPS; 10 μg/mouse). Lane C, positive control, the IL-6 cDNA (upper panel), or the β-actin cDNA (lower panel). These experiments were repeated a minimum of four times.

RESULTS

Generation of Transgenic Mice—Having previously demonstrated that hypoxia-mediated induction of IL-6 in cultured endothelial cells was due to activation of transcription at the C/EBPβ-NF-IL-6 site in the promoter (9), we generated transgenic mice containing this DNA binding motif. The bacterial gene lacZ and the basal thymidine kinase promoter were placed under transcriptional control of the 5'-regulatory sequence of the IL-6 gene from positions −225 to −111 and −158 to −145 (with respect to the transcription start site at +1) (Fig. 1A, constructs 1 and 2). The segment of the promoter −225 to −111 has been previously shown to function as an enhancer in response to hypoxia in cultured endothelial cells because of the presence of the C/EBPβ-NF-IL-6 motif at −158 to −145. Additional transgenic mice were made in which the C/EBPβ-NF-IL-6 motif was mutationally inactivated (Fig. 1A, construct 3). To create transgenic mice, the indicated construct was isolated, linearized, and microinjected into fertilized mouse eggs. Three and four different founders were generated with constructs 1 and 2, respectively, each of which transmitted the transgene. Southern analysis of these lines is shown in Fig. 1B (constructs 1, lanes 2–4, and 2, lanes 5–8). Two founders were identified with the mutationally inactivated C/EBPβ-NF-IL-6 construct 3 (Fig. 1B, lanes 9 and 10). Note the negative control in Fig. 1B, lanes 11 and 12, in which the same amount of DNA from nontransgenic mice was loaded and the positive control (Fig. 1B, C), in which the EcoRI-HindIII DNA fragment from pCH110 was used as the sample.

Response of Transgenic Mice to Hypoxia: Expression of β-Galactosidase and IL-6—Transgenic mice bearing either construct 1 or 2 were subjected to hypoxia in a controlled environment chamber in which the oxygen concentration was held constant at about 6%. Under these conditions, mice displayed normal activity and food consumption over the experimental period, although they were visibly tachypneic, indicative of hypoxia. First, we studied expression of the transgene in mice bearing construct 1 in the lung, heart, kidney, and liver. ELISA demonstrated 4–6-fold increased β-galactosidase antigen in the lung, heart, and kidney following exposure of animals to hypoxia versus controls maintained in normoxia (Fig. 2). The heart showed the greatest increase in β-galactosidase, about 6-fold. Immunostaining for β-galactosidase demonstrated transgene expression in hypoxic pulmonary vasculature (Fig. 3A), endothelium (Fig. 3A) and especially smooth muscle cells (the latter depicted at high magnification; Fig. 3A, inset), a distribution similar to our previously reported hypoxia-induced expression of IL-6 (9). In normoxic controls, staining for β-galactosidase antigen was virtually undetectable in the lung (Fig. 3B), analogous to the low levels of IL-6 antigen noted before (9). In the heart, immunostaining for β-galactosidase was observed in hypoxic coronary vasculature and in myocardium (Fig. 3C); there was no staining in normoxic controls (Fig. 3D). To be certain that this pattern of transgene expression indicated expression of a gene regulated by NF-IL-6/C/EBPβ, we also looked for IL-6 antigen in hypoxic cardiac tissue. Immunostaining demonstrated that IL-6 was expressed in hypoxic cardiac vasculature and myocardium (Fig. 3E) but not in normoxic controls (Fig. 3F). In the kidney, antigen was also virtually absent in normoxic controls (Fig. 3H), whereas after hypoxia there was staining in the vasculature and proximal tubules (Fig. 3G); this distribution of β-galactosidase staining was also comparable with that observed using antibody to IL-6 (Fig. 3J).

In contrast to the results in the lung, heart, and kidney, the livers of transgenic mice bearing construct 1 showed no significant induction of β-galactosidase antigen by ELISA (Fig. 2) or by immunostaining (Fig. 4, A and B show hypoxic and normoxic liver, respectively). Consistent with these findings, IL-6 transcripts (Fig. 4C) were not increased in livers of mice subjected to hypoxia (lanes N for normoxia and H for hypoxia) compared with the induction previously demonstrated in the lung (9) and also the enhancement observed in heart and kidney (data not shown). In contrast, infusion of mice with lipopolysaccharide resulted in induction of IL-6 transcripts in the liver (Fig. 4C, lane LPS for lipopolysaccharide infusion), as reported previously (19). Since the region of −225 to −111 used to prepare transgenic mice with construct 1 contains multiple possible regulatory elements, we also made mice in which only the NF-IL-6 site from the IL-6 promoter was present (−158 to −145), i.e. construct 2 (Fig. 1B, lanes 5–8). When exposed to hypoxia, these animals demonstrated enhanced expression of β-galactosidase in a similar pattern and to a degree similar to that observed with mice bearing construct 1 (data not shown). Importantly, mice bearing construct 3, in which the NF-IL-6 was mutationally inactivated, showed no increase in β-galactosidase expression compared with normoxic controls in the lung or other organs (Fig. 5, C and D, hypoxic and normoxic lung, respectively). This contrasts with increased levels of β-galactosidase antigen in lungs from hypoxic mice with the wild-type NF-IL-6
Response of Transgenic Mice to Ischemia—To extrapolate our hypoxia model to the setting of organ ischemia, a method for inducing ischemia of the left lung was developed, and activation of the transgene and expression of IL-6 were assessed. Using transgenic mice bearing constructs 1 and 2, increased \( \beta \)-galactosidase expression was observed by immunocytochemistry in ischemic lung vasculature (Fig. 6, A and C, respectively), but there was no expression of the transgene in the nonmanipulated right lung (Fig. 6, B and D). Detection of IL-6 antigen by immunostaining also showed a similar vascular pattern of enhanced expression in the ischemic left lung compared with the nonmanipulated right lung (Fig. 6, E and F, respectively).

Response of Mice to Hypoxia: Activation of C/EBP\( \beta \)-NF-IL-6—Expression of IL-6 and the two transgenes containing the NF-IL-6 site was inducible by hypoxia in a tissue-specific manner. Whereas vascular endothelium and smooth muscle in the lung demonstrated high levels of transgene expression on exposure to hypoxia, neither hepatocytes nor hepatic vasculature showed significantly enhanced \( \beta \)-galactosidase expression. To probe the nature of hypoxia-mediated transcriptional activation, EMSA was performed using nuclear extracts from the lung, heart, kidney, and liver. Nuclear binding activity for the \( ^{32} \)P-labeled NF-IL-6 probe from the IL-6 promoter (−158 to −145) (11–13) showed a strong increase in the lung, heart, and kidney (Fig. 7A). In the lung, the intensity of the gel shift band increased within 2 h of hypoxia, was sustained up to 4 h, and was due to sequence-specific binding to the NF-IL-6 oligonucleotide probe, as shown by competition with excess unlabeled probe for NF\( \kappa \)B, AP1, CREB, SP1, or HIF-1 (Fig. 7B). Supershift experiments confirmed the involvement of C/EBP\( \beta \)-NF-IL-6; addition of antibody to C/EBP\( \beta \) to the reaction mixture resulted in dose-dependent inhibition of the gel shift band (Fig. 7C). At IgG dilutions of 1:10–1:1000, appearance of the gel shift band was completely blocked, whereas at higher dilutions, 1:5000–1:10,000, the band was again observed. In contrast to these results with C/EBP\( \beta \), antibody to C/EBP\( \alpha \) or \( \delta \) or CRP1
had no effect on migration of the gel shift band (Fig. 7C). Studies with antibodies to other transcription factor components, including c-Fos, c-Jun/AP1, CREB-1, CREB-2, p50 (NFκB) and p65 (NFκB), did not change the intensity or migration of the gel shift band (data not shown). The hypoxia-enhanced gel shift band binding to the NF-IL-6 oligonucleotide probe with hypoxic lung nuclear extracts was qualitatively and quantitatively different from the band observed with extracts from normoxic lung. In addition to the gel shift band being of much lower intensity in the normoxic tissue (compared with hypoxia), there was no supershift change in intensity of the band with antibody to C/EBPβ, α, or δ, CRP1, CREB-1, CREB-2, p65 (NFκB), p50 (NFκB), c-Jun/AP1, and c-Fos (data not shown).

To further assess the nature of components binding to the NF-IL-6 probe in nuclear extracts of hypoxic kidney, competition and supershift studies were performed. The intensity of the hypoxia-enhanceable gel shift band in nuclear extracts of kidney was greatly diminished in the presence of excess unlabeled NF-IL-6 but not by excess unlabeled NFκB, AP1, CREB, SP1, or HIF-1 (data not shown). Supershift experiments with nuclear extracts from hypoxic kidney also indicated a pattern similar to what was observed with hypoxic lung; anti-C/EBPβ antibody completely blocked the appearance of the band (at a 1:10 dilution of antibody, the gel shift band is gone, but at a 1:5000 dilution, the band is again seen), whereas antibody to c-Fos, c-Jun/AP1, CREB-1, CREB-2, p65, or p50 had no effect (data not shown).

DISCUSSION

Hypoxic stress accompanies a range of pathophysiologically relevant situations. Modulation of gene expression in response to oxygen deprivation is a basic component of adaptation to such an environmental perturbation (20–23). We previously noted induction of IL-6 in cultured ECs subjected to hypoxia, which we speculated might have a protective role by suppressing the effect of potent proinflammatory mediators, such as tumor necrosis factor (9, 24–25), elicited by hypoxemia and/or ischemia. IL-6 induction in hypoxic cultured ECs was driven by increased gene transcription mediated by the NF-IL-6 site in the promoter (9). Mice subjected to hypoxia also demonstrated increased vascular expression of IL-6, corresponding with our observations in vitro. These results led us to examine whether hypoxia would trigger NF-IL-6-mediated transcription in vivo.

To address this issue, two types of transgenic mice were prepared: mice bearing a transgene spanning a 115- and 14-bp portion of the IL-6 promoter, in each case constituting the NF-IL-6 site. Expression of both transgenes was observed in lung vasculature, as expected from our previous results (9). In addition, expression of the β-galactosidase reporter was evident in the heart, in both cardiac myocytes and vasculature, and in the kidney, especially in proximal tubule cells. In contrast, the liver showed no significant hypoxia-mediated increase in transgene expression, either in hepatocytes or in the vasculature. These data suggested the existence of mechanisms for organ-specific regulation of hypoxia-inducible gene expression mediated by NF-IL-6, analogous to cell-specific expression of the erythropoietin gene in transgenic mice (although expression of the NF-IL-6 transgene follows a distinct pattern; Ref. 26). In this context, EMSA from hypoxic lung, heart, and kidney showed striking increases in nuclear binding activity for the NF-IL-6 oligonucleotide probe, whereas there was no increase in hypoxic liver. Pilot experiments with cultured hepG2 cells (data not shown) and our past studies on cultured ECs (9) confirmed these differences in the cellular response to oxygen deprivation. ECs exhibited a rapid and pronounced increase in NF-IL-6 binding activity in response to hypoxia, whereas this did not occur in hepG2 cells.
These data contrast with the well known hypoxia-mediated activation of HIF-1 in hepG2 cells (8) and suggest the existence of distinct pathways leading to expression of transcription factor-DNA binding activity.

Our findings provide a first step in understanding the contribution of NF-IL-6 motifs to the regulation of gene expression in hypoxia and ischemia. C/EBPβ-NF-IL-6-driven transcription was observed in vasculature of the heart, lung, and kidney and in cardiac myocytes. In contrast, no expression was observed in the liver, either in parenchymal cells or vasculature. This suggests the importance of local factors in the microenvironment in regulation of gene expression. Future work will be directed toward identification of such autocrine and paracrine factors produced by hypoxic and ischemic tissues.

Acknowledgments—We gratefully acknowledge the assistance of Dr. Gerald Fuller, Lester May (New York Medical College), and Dr. Gabriel Godman (Columbia University).

REFERENCES
1. Wang, G., and Semenza, G. (1995) J. Biol. Chem. 270, 1230–1237
2. Goldberg, M., Dunning, S., and Bunn, H. (1988) Science 242, 1412–1415
3. Costa-Gimi, P., Caro, J., and Weinmann, R. (1990) J. Biol. Chem. 265, 10185–10188
4. Semenza, G., Roth, P., Fang, H.-M., and Wang, G. (1994) J. Biol. Chem. 269, 23757–23763
5. Minchenko, A., Salceda, S., Bauer, T., and Caro, J. (1994) Cell. & Mol. Biol. Res. 40, 35–39
6. Goldberg, M., and Schneider T. (1994) J. Biol. Chem. 269, 4355–4359
7. Liu, Y., Coe, S., and Kourembanas, S. (1995) Circ. Res. 77, 638–643
8. Ebert, B., Firth, J., and Ratcliffe, P. (1995) J. Biol. Chem. 270, 29083–29089
9. Yan, S.-F., Tritto, I., Pinsky, D., Liao, H., Huang, J., Fuller, G., Brett, J., May, L., and Stern, D. (1995) J. Biol. Chem. 270, 11463–11471

FIG. 7. Hypoxia-mediated enhancement of nuclear binding activity for NF-IL-6 in lung, heart, liver, and kidney. A, EMSA using a 32P-labeled NF-IL-6 probe and nuclear extracts of the liver, lung, heart, and kidney harvested from mice subjected to hypoxia (H, 4 h) or maintained in normoxia (N). B, time course and competition study. Nuclear extracts were obtained from the lung at the indicated times, and EMSA was performed. As indicated, a 100-fold molar excess of unlabeled oligonucleotides for NF-IL-6, NFκB, AP1, CREB, SP1, or HIF-1 was added. C, supershift assay using antibody to C/EBP family components: EMSA using a 32P-labeled NF-IL-6 probe and nuclear extract of lungs from mice exposed to hypoxia for 4 h using antibodies to C/EBPα, β, or δ, CRP1, or nonimmune (NI) IgG. The indicated dilution of immune IgG against C/EBPβ was used (1:10–1:10,000 dilution as the final concentration in the reaction mixture) or antibody C/EBPα or δ (1:10 dilution), CRP1 (1:10 dilution), or nonimmune IgG (1:10 dilution). FP, free probe. These experiments were repeated a minimum of three times.
NF-IL-6 and Hypoxia

10. Akira, S., Ishiki, H., Sguita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990) *EMBO J.* 8, 1897–1906

11. Ray, A., Tatter, S., May, L., and Sehgal, P. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 6701–6705

12. Ray, A., Sassone-Corsi, P., and Sehgal, P. (1989) *Mol. Cell. Biol.* 9, 5537–5547

13. Ray, A., LaForge, K., and Sehgal, P. (1990) *Mol. Cell. Biol.* 10, 5736–5746

14. McKnight, S., Gavis, E., Kingsbury, R., and Axel R. (1981) *Cell* 25, 385–398

15. Shreeniwas, R., Koga, S., Karakurum, M., Pinsky, D., Kaiser, E., Brett, J., Wolitzky, B., Norton, C., Policinski, J., Benjamin W., Burns, D., Goldstein, A., and Stern, D. (1992) *J. Clin. Invest.* 90, 2333–2339

16. Brett, J., Schmidt, A.-M., Zou, Y.-S., Yan, S.-D., Weidman, E., Pinsky, D., Nepper, M., Przybyciecki, M., Shaw, A., Mighei, A., and Stern, D. (1993) *Am. J. Pathol.* 143, 1699–1712

17. Dignam, J., Lebovitz, R., and Roeder, R. (1983) *Nucleic Acids Res.* 11, 1475–1489

18. Anderson, M., and Freytag, S. (1991) *Mol. Cell. Biol.* 11, 1935–1943

19. Laye, S., Parnet, P., Goujon, E., and Dantzer, R. (1994) *Mol. Brain Res.* 27, 157–162

20. Subjeck, J., and Thung-Tai, S. (1986) *Am. J. Physiol.* 250, C1–C17

21. Anderson, G., Stoler, D., and Scarcella, L. (1989) *J. Biol. Chem.* 264, 14885–14892

22. Ogawa, S., Claus, M., Kuwahara, K., Shreeniwas, R., Butura, C., Koga, S., and Stern, D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 9897–9901

23. Ogawa, S., Gerlach, H., Esposito, C., Pasagian-Macaulay, A., Brett, J., and Stern, D. (1990) *J. Clin. Invest.* 85, 1090–1098

24. Colletti, L., Remick D., Burtch, G., Kunkel, S., Strieter, R., and Campbell, D. (1990) *J. Clin. Invest.* 85, 1936–1943

25. Maury, C., and Teppo, A.-M. (1989) *J. Intern. Med.* 225, 333–336

26. Semenza, G., Koury, S., Nejfelt, M., Gearhart, J., and Antonarakis, S. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 8725–8729