Characterization of Hypoxia-inducible Factor 1 and Regulation of DNA Binding Activity by Hypoxia*

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Hypoxia-inducible factor 1 (HIF-1) is a DNA binding activity detected in nuclear extracts from Hep3B cells cultured in 1% O₂ but not in extracts from cells cultured in 20% O₂. HIF-1 binds to a sequence within the human erythropoietin gene enhancer that is required for hypoxic activation of transcription. Induction of HIF-1 is inhibited by cycloheximide, which also inhibits induction of erythropoietin RNA. We now demonstrate that induction of both HIF-1 and erythropoietin RNA is inhibited by the protein kinase inhibitor 2-aminopurine. HIF-1 binding to DNA was eliminated by phosphatase treatment of nuclear extracts. Actinomycin D also inhibited HIF-1 induction, suggesting that de novo transcription is required. The kinetics of HIF-1 induction by hypoxia paralleled the kinetics of erythropoietin gene transcriptional induction. HIF-1 DNA binding activity decayed rapidly when hypoxic cells were exposed to increased oxygen tension. In vitro, the kinetics of HIF-1 association with, and dissociation from, its binding site were extremely rapid, with a t½ for both processes of <1 min. These findings are consistent with the proposed function of HIF-1 as a physiologic regulator of gene expression that responds to changes in cellular oxygen tension. Methylation interference analysis indicated that HIF-1 makes specific contacts with DNA in the major groove.

Cells respond to environmental stimuli by altering their patterns of gene expression. One of the most fundamental physiologic stimuli is oxygen. In the bacterium Rhizobium meliloti, hypoxia is sensed by a hemoprotein, resulting in the activation of its kinase domain and subsequent transcription of a gene encoding a transcriptional activator (1–3). In the yeast Saccharomyces cerevisiae, heme appears to function as an internal barometer of O₂ tension by interacting with transcription factors to regulate gene expression (4). A major physiologic regulator of O₂ supply in mammals is erythropoietin (Epo), the primary growth factor for erythroid progenitor cells (5, 6). In mice, expression of the endogenous Epo gene (7–9) and of human EPO transgenes (10–12) is induced by anemia or hypoxia in fetal and adult liver and kidney. Tissue hypoxia, whether due to altered O₂ tension, O₂-carrying capacity, or O₂ affinity of the blood, is the primary stimulus for Epo production (5, 6). The kidney is the major site of Epo synthesis in adults, but there are no kidney-derived cell lines in which EPO expression is regulated by O₂ tension (13). The human hepatoblastoma cell line Hep3B has been utilized extensively to study the regulation of EPO transcription by O₂ tension, as the rate of EPO transcription increases at least 10-fold within 4 h of changing Hep3B cells from 20% to 1% O₂ (14). Induction of EPO expression in Hep3B cells may require a hemoprotein (15) and protein kinase activity (16, 17), suggesting that aspects of the hypoxia signal transduction pathway may be conserved between bacteria and mammals.

A cis-acting hypoxia-inducible enhancer element has been localized to 3'-flanking sequences of the human EPO gene by nuclelease sensitivity studies in transgenic mice (18) and transient expression studies in Hep3B cells (18–20). A homologous enhancer element has also been identified 3' to the mouse Epo gene (21). The enhancer is functional when positioned in either orientation 3' or 5' to a reporter gene (18–21). EPO gene 5'-flanking sequences may also contribute to transcriptional regulation by O₂ tension (20, 22, 23). However, deletion of the 3' enhancer, but not 5'-flanking sequences, eliminates hypoxia-inducible transcription (19, 21). We recently defined a functionally tripartite 50-nucleotide (nt) EPO gene hypoxia-inducible enhancer, and our data indicated that site 1 (nt 4–12) and site 2 (nt 19–23) are absolutely required for hypoxic induction while site 3 (nt 26–48), a putative thyroid-steroid hormone receptor superfamily binding site, functions to amplify the induction signal (24). We identified a DNA binding activity that is induced by hypoxia and binds specifically to site 1 of the enhancer, which we have designated hypoxia-inducible factor 1 (HIF-1). HIF-1 binds to the wild-type site 1 sequence but not to a sequence containing a mutation at nt 7–9 that eliminated enhancer function in transient expression assays. HIF-1 induction is dependent upon de novo protein synthesis (24), as is Epo RNA induction (15), suggesting a causal relationship between induction of HIF-1 and of Epo RNA. In this paper we provide further evidence for the importance of HIF-1 in the hypoxic activation of EPO transcription and present data that provide insight into the regulation of HIF-1 DNA binding activity by hypoxia.

MATERIALS AND METHODS

Cell Culture and Nuclear Extract Preparation—Hep3B cells were maintained as described (24). Cells were subjected to hypoxia in a sealed chamber flushed with 1% O₂, 5% CO₂, and 94% N₂ (1%O₂) or placed directly in a 5% CO₂ and 95% air incubator (20% O₂) and cultured at 37 °C. For inhibitor experiments, actinomycin D (Sigma) was dissolved in ethanol and 2-aminopurine (nitrate salt; Sigma) was dissolved in tissue culture medium and neutralized with sodium bicarbonate. Confluent cells were given fresh medium and pretreated with inhibitors, or solvent alone, for 2 h before being placed in 1% or 20% O₂ for 4 h. Nuclear extracts were prepared as described (24).
Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed in binding buffer consisting of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 50 mM KCl, 1 mM MgCl₂, 5 mM diethiothreitol, 1 mM EDTA, and 5% glycerol to which 0.1 µg of denatured calf thymus DNA, 5 µg of Hep3B nuclear extract, and 10⁴ cpm of probe were added per 20-µl reaction (24). The binding-site sequence (coding strand) of the wild-type probe (W18) was 5'-GCCCTACGTGCTGTCTCA-3', corresponding to nt 1-18 of the 50-nt enhancer (24) or nt 3454-3471 within the 3'-flanking region of the published EPO gene sequence (25). The sequence of the mutant probe (M18) was 5'-GCCCTAAAAAGGTGTCTCA-3' (nt 7-9 mutation underlined). The USF probe (5'-GGTAGGCCACGTGACCGGGTA-3') was provided by C. V. Dang (The Johns Hopkins University, Baltimore, MD).

For estimation of binding kinetics, reactions were incubated at 22 or 0 °C for 1-80 min before gel loading. For dissociation kinetics, 100- or 1000-fold molar excess of unlabeled oligonucleotide was added after the binding reaction was incubated at 22 °C for 15 min. Aliquots were withdrawn at different time points and loaded onto a running gel. For in vitro dephosphorylation, 5-µg aliquots of nuclear extract from hypoxic cells were incubated with 0.05-1 unit of calf intestinal alkaline phosphatase (New England Biolabs) for 15 min at 22 °C prior to addition of probe and analysis by EMSA as described above. DNA binding activities were quantitated by scanning of autoradiographs with an LKB Ultrascan XL laser densitometer with computerized peak integration.

RNA Extraction and Analysis—Total cellular RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (26). A 32P-labeled, 1.4-kilobase anti-sense RNA riboprobe was hybridized to 75 µg of total RNA for 3 h at 65 °C, ribonuclease-digested, and analyzed by 8 M urea, 8% PAGE as described (11).

Methylation Interference Analysis—The W18-coding or noncoding strand was 5'-end-labeled with [γ-32P]ATP and T4 polynucleotide kinase and annealed with unlabeled complementary oligonucleotide (24). Labeled probes were gel-purified, ethanol-purified, and partially methylated with dimethyl sulfate for 5 min at 22 °C (27). EMSA was performed as described above, except that binding reactions were scaled up 20-fold in a total volume of 60 µl. After autoradiography overnight at 4 °C, bands corresponding to free and complexed probe were eluted from the gel. Probe DNA was purified by elution from NA-45 DEAE paper (Schleicher & Schuell). The eluate was extracted with phenol-chloroform and ethanol-purified. DNA was resuspended in 100 µl of 1 M piperidine and cleaved by incubation at 90 °C for 30 min. Equal counts per minute were loaded in each lane and resolved by 8 M urea, 15% polyacrylamide gel electrophoresis. Autoradiographs were scanned for densitometric analysis.

RESULTS

Actinomycin D Inhibits Induction of HIF-1 DNA Binding Activity—We have shown previously that induction of HIF-1 DNA binding activity can be inhibited by treatment of hypoxic Hep3B cells with cycloheximide, a protein synthesis inhibitor known to block induction of Epo RNA (15), suggesting that de novo protein synthesis is required for induction of HIF-1 binding activity and EPO transcriptional activation (24). To investigate whether HIF-1 induction requires de novo transcription, Hep3B cells were treated with 15 µg/ml actinomycin D or solvent (ethanol) alone for 2 h in 20% O₂ followed by 4 h of incubation in 1% or 20% O₂. Nuclear extracts were prepared, 5-µg aliquots were incubated with a 32P-labeled double-stranded oligonucleotide probe (W18), and analyzed by EMSA. From the autoradiograph shown in Fig. 1 (and those in all subsequent figures), DNA binding activities were quantitated by scanning laser densitometry.

As previously demonstrated (24), the W18 probe specifically detected constitutive (Fig. 1, lanes 1 and 2, C) and hypoxia-induced (HIF-1; lane 2) DNA binding activities in Hep3B nuclear extracts. Both sequence-specific DNA binding activities, HIF-1 and constitutive, migrated as doublet bands on the non-denaturing polyacrylamide gels used in this assay (24). HIF-1 DNA binding activity was markedly (>78%) reduced in nuclear extract prepared from actinomycin D-treated hypoxic cells (lane 4) compared to mock-treated hypoxic cells (lane 2), while constitutive and non-specific DNA binding activities were unchanged. An oligonucleotide probe (M18) with a 3'-nt substitution that eliminated hypoxia-inducible enhancer function (24) detected only non-specific DNA binding activity (lanes 5-8). A probe containing a binding site for the ubiquitous transcription factor USF (28) was used to further demonstrate that HIF-1 was specifically induced by hypoxia and that actinomycin D specifically inhibited induction of HIF-1 activity in hypoxic cells (lanes 9-12). These results and our previous studies (24) suggest that induction of HIF-1 activity in hypoxic cells requires de novo transcription and translation. Definitive proof of transcriptional induction will require nuclear run-off experiments for which cDNA sequences encoding HIF-1 must first be isolated.

2-Aminopurine Inhibits Induction of HIF-1 Activity and Epo RNA—Many signal transduction pathways involve protein phosphorylation (29). To investigate whether hypoxic activation of EPO transcription via HIF-1 induction is dependent upon phosphorylation events, Hep3B cells were treated with 10 mM 2-aminopurine (2-AP), a protein kinase inhibitor. 2-AP caused a marked (>70%) reduction in HIF-1 binding activity in nuclear extracts from hypoxic cells (Fig. 2A, compare lane 4 with lane 2). 2-AP had no effect on binding of constitutive and nonspecific factors to the W18 probe (Fig. 2A), nor did it affect USF binding activity in these extracts (data not shown). To confirm that the effect of 2-AP on HIF-1 was also manifested at the level of Epo RNA, total RNA was isolated from Hep3B cells incubated in 1 or 20% O₂ in the presence or absence of 10 mM 2-AP, and analyzed by ribonuclease protection assay. The increase in steady-state Epo RNA in hypoxic cells was markedly reduced by treatment with 2-AP (Fig. 2B, compare lane 4 with lane 2). The metabolic inhibitors cycloheximide and 2-AP therefore both reduce the level of HIF-1 DNA binding activity and the steady-state level of Epo RNA induced by hypoxia, underscoring the importance of HIF-1 induction for EPO transcriptional activation.

Kinetics of HIF-1 Hypoxic Induction and Post-hypoxic Decay—In Hep3B nuclear run-off experiments, the maximal rate of EPO transcription was reached by 4 h of exposure to 1% O₂ (14). To compare the induction kinetics of EPO transcription
and HIF-1 activity, Hep3B cells were subjected to 1% O₂ for 0–4 h prior to nuclear extract preparation. HIF-1 activity was first detectable at 15 min (data not shown) and was maximal at 4 h. In the experiment shown in Fig. 3A, HIF-1 activity was 8% at 30 min (lane 2) and 50% at 1 and 2 h (lanes 3 and 4) relative to maximal levels (100%) at 4 h (lane 5). HIF-1 activity at 16 h was no greater than at 4 h (not shown). The induction kinetics of HIF-1 therefore paralleled the previously reported (14) kinetics of EPO transcription in Hep3B cells exposed to 1% O₂.

The kinetics of post-hypoxic decay were also determined by exposing Hep3B cells to 1% O₂ for 4 h at 37 °C. The cells were then returned to 20% O₂ and incubated at 37 °C for 5 min to 2 h prior to extract preparation. The post-hypoxic decay of HIF-1 DNA binding activity was remarkably rapid (Fig. 3B). After only 5 min at 20% O₂ (lane 3), HIF-1 activity had decayed to 34% of the level in cells harvested directly from 1% O₂ (lane 2) and HIF-1 activity was completely eliminated by a 15-min exposure of hypoxic cells to 20% O₂ (lane 4). USF binding activity in the same extracts showed no change (lanes 8–14). HIF-1 DNA binding activity is thus tightly regulated by O₂ tension.

HIF-1 DNA Binding and Dissociation Kinetics—To study the rate of HIF-1 binding to DNA in vitro, hypoxic Hep3B nuclear extract was incubated with W18 probe for 1–80 min prior to analysis by EMSA (Fig. 4A). There was substantial (77% of maximal) HIF-1 binding after only 1 min of incubation (lanes 2 and 9) and maximal binding at 5 min (lanes 3 and 10). At 22 °C, HIF-1 binding decreased progressively after 5 min (lanes 3–7) to 15% of maximal binding by 80 min, while at 0 °C the level of binding remained constant through 80 min (lanes 10–14). Constitutive DNA binding activity was affected by incubation at 22 °C to a lesser degree, with 42% of maximal activity present after 80 min (lane 7). These results suggest the presence of a factor (e.g. protease or phosphatase) in the nuclear extract whose activity over time (at 22 °C) markedly reduced the DNA binding activity of HIF-1.

In addition to the rapid on-rate, HIF-1 displayed an equally rapid off-rate (Fig. 4B). Hypoxic Hep3B nuclear extract was incubated with W18 probe for 15 min, and then either a 100-fold (lanes 2–7) or a 1000-fold (lanes 9–14) molar excess of unlabeled W18 oligonucleotide was added for 0–60 min prior to gel loading for EMSA. Probe binding to HIF-1 was decreased by 41% after 1 min (compare lane 3 with lane 2) and was com-

**Fig. 2.** Effect of 2-aminopurine on hypoxic induction of HIF-1 DNA binding activity and Epo RNA. Hep3B cells were cultured in the presence (+) or absence (−) of 2-AP in 20% O₂ (lanes 1 and 3) or 1% O₂ (lanes 2 and 4). A, HIF-1. Nuclear extracts were incubated with W18 probe and analyzed by EMSA. B, Epo RNA. Total RNA was analyzed by ribonuclease protection assay. The protected fragment corresponds to exon 2 of the EPO gene.

**Fig. 3.** Kinetics of HIF-1 induction and post-hypoxic decay. A, hypoxic induction of HIF-1 DNA binding activity. Nuclear extracts, prepared from cells cultured in 1% O₂ for 0–4 h, were incubated with W18 probe and analyzed by EMSA. B, post-hypoxic decay of HIF-1 DNA binding activity. Nuclear extract, prepared from Hep3B cells cultured in 20% O₂ (N) or cells cultured in 1% O₂ for 4 h at 37 °C followed by incubation in 20% O₂ for 0 to 2 h 37 °C, were mixed with W18 (lanes 1–7) or USF probe (lanes 8–14) and analyzed by EMSA.
**Induction of HIF-1 by Hypoxia**

**A**

| TEMP: | 22°C | 0°C |
|------|------|-----|
| TIME: | N 1 5 10 20 40 80 | N 1 5 10 20 40 80 |

- HIF-1 → C
- NS →

**B**

| COMPETITOR: | 100-FOLD | 1000-FOLD |
|-------------|----------|-----------|
| TIME:       | P 0 1 5 15 30 60 0 | 0 1 5 15 30 60 0 |

- HIF-1 → C
- NS →

**Fig. 4. HIF-1 DNA binding and dissociation kinetics.** A, binding kinetics. Aliquots (5 μg) of nuclear extract from non-hypoxic (N, lanes 1 and 8) or hypoxic (lanes 2–7) Hep3B cells were incubated with W18 probe for 1–80 min at either 22°C (lanes 1–7) or 0°C (lanes 8–14) prior to EMSA. B, dissociation kinetics. Aliquots (5 μg) of nuclear extract from hypoxic cells were incubated with W18 probe for 15 min at 22°C followed by addition of a 100-fold (lanes 2–7) or 1000-fold (lanes 8–14) molar excess of unlabeled W18. Reactions were incubated for an additional 0–60 min (lanes 2–7 and 9–14) at 22°C, and each sample was immediately loaded onto a running gel. Lane 1 contains probe (P) only (no extract); lanes 8 and 15 contain probe and extract only (no competitor). Free probe is seen only for samples that were loaded on to the gel last.

completion of the 5-min incubation with unlabeled oligonucleotide (lane 4). The use of 100-fold excess competitor revealed that the constitutive binding activity appeared to be more stable, as detectable binding remained after 60 min (lane 7). The rapidity of HIF-1 dissociation is illustrated by the increased intensity of HIF-1 in lanes 8 and 15, which contain no competitor, compared to lanes 2 and 9, in which 100- and 1000-fold excess competitor, respectively, were added to samples, which were then immediately loaded onto the gel. The binding studies shown in Fig. 4B were performed at 22°C. However, similar results were obtained at 0°C (data not shown) as expected since the competition was complete by 5 min (Fig. 4B), whereas the effects of temperature were not apparent until 10 min (Fig. 4A). The loss of binding activity over time demonstrated in Fig. 4A does not significantly alter the interpretation of the data presented in Fig. 4B, although it is clear that binding studies performed at 0°C provide the most accurate estimates of the dissociation constant. Overall, the data suggest that the t₁/2 for HIF-1 binding and for HIF-1 dissociation is <1 min.

**Elimination of HIF-1 DNA Binding Activity by Phosphatase Treatment**—The inhibitory effect of 2-AP (Fig. 2) and the loss of HIF-1 activity at 22°C (Fig. 4A) suggested that phosphorylation of HIF-1 may be required for DNA binding. To test this hypothesis, nuclear extract from hypoxic Hep3B cells was incubated in the presence of varying amounts of calf intestinal alkaline phosphatase (CIP) prior to HIF-1 binding assays with the W18 probe (Fig. 5). Compared to untreated extract from hypoxic cells (lane 2), HIF-1 activity was reduced by 53% after treatment with 0.1 unit (lane 6) and completely eliminated by 1 unit (lane 3) of CIP. Constitutive DNA binding activity was reduced by 36% and 98% by incubation with 0.1 and 1 unit of CIP, respectively. Nonspecific DNA binding activity was unaffected even in the presence of 1 unit of CIP (lane 3) and thus served as an internal control for loss of DNA binding activity due to proteolysis. The sensitivity of HIF-1 and the constitutive factor to CIP thus paralleled their sensitivity to incubation at 22°C suggesting, but by no means proving, that the loss of DNA binding activity at 22°C was associated with dephosphorylation events. The results of CIP treatment are consistent with the hypothesis that phosphorylation of HIF-1 is required for DNA binding.

**Characterization of the HIF-1 DNA Binding Site by Methylation Interference Analysis**—We previously demonstrated that, within the context of the 50-nt EPO gene enhancer, three contiguous 3-nt substitutions (nt 4–6, 7–9, and 10–12) each eliminated hypoxia-inducible enhancer function in Hep3B transient expression assays (24). We also showed that, within the context of the W18 probe (comprising nt 1–18 of the enhancer), the nt 7–9 substitution completely eliminated binding of HIF-1 and the constitutive factor (Fig. 1). Since 3-nt substitutions on either side of nt 4–12 did not eliminate enhancer function (24), these results suggested that nt 4–12 encompassed the residues contacting HIF-1.

To provide more direct data on the DNA-protein contacts involved in HIF-1 binding, we performed methylation interference analysis using the W18 oligonucleotide, end-labeled on the coding or noncoding strand and partially methylated by treatment with dimethyl sulfate. Methylation of guanine at the N-7 position interferes with binding in the major groove, while methylation of adenine at the N-3 position interferes with binding in the minor groove of the DNA double helix (27). The
partially methylated probe was incubated with nuclear extract from hypoxic Hep3B cells and a preparative EMSA was performed. Individual radioactive bands were excised from the gel representing free probe (F), the doublet bands of HIF-1 (I1 and I2), the doublet bands of the constitutive binding activity (C1 and C2), and nonspecific DNA binding activity (N). DNA was extracted, cleaved with piperidine, and analyzed by denaturing polyacrylamide gel electrophoresis.

Both bands of the HIF-1 doublet (I1 and I2) gave similar patterns of methylation interference (Fig. 6A). On the coding strand, methylation of the guanine residue at nt 8 (G-8; see Fig. 6B for numbering scheme) almost completely (98%) eliminated HIF-1 DNA binding. Methylation of G-10 also markedly (89%) reduced HIF-1 binding. In contrast, methylation of G-1 or G-13 had no effect on HIF-1 binding. On the noncoding strand, methylation of G-7 completely (99%) eliminated binding. Methylation of G-11 also greatly (86–90%) reduced binding. In contrast, methylation of guanine residues 2, 3, 4, 15, and 17 had no effect on HIF-1 binding to the probe. Methylation of adenine residues on either strand also had no effect on HIF-1 binding.

The constitutive DNA binding activity (C1 and C2) showed reproducible (n = 3) differences in the methylation interference pattern compared to HIF-1. On the coding strand, methylation of G-8 and G-10 had a less pronounced effect on the constitutive binding activity. The C1 and C2 bands were reduced by 94 and 97%, respectively, at G-8 and by only 88 and 79%, respectively, at G-10. In contrast, methylation of G-1 reduced the formation of the C1 and C2 complexes by 16 and 54%, respectively, but had no effect on HIF-1 binding. On the noncoding strand, methylation of G-7 reduced C1 and C2 binding by 94 and 97%, respectively. Most striking was the fact that methylation of G-11 reduced C1 and C2 binding by only 1 and 31%, respectively. Methylation of adenine residues on either strand had no effect on binding of the constitutive factor. As expected, nonspecific DNA binding activity was unaffected by methylation at any guanine or adenine residue on either strand.

These results demonstrate that, within the sequence 5'-CTACGTGCT-3' at nt 4–12 of the EPO enhancer, which we had previously established as a putative HIF-1 binding site (24), HIF-1 makes specific contacts with guanine residues at nt 8 and 10 on the coding strand as well as nt 7 and 11 on the noncoding strand (Fig. 6B). The effect of these guanine methylations and the lack of effect of adenine methylations indicate that HIF-1 primarily interacts with DNA in the major groove, with no detectable contacts being made in the minor groove. Finally, HIF-1 clearly does not make contact with G-4 on the noncoding strand, thus limiting the HIF-1 binding site to the sequence 5'-TACGTGCT-3'.

**DISCUSSION**

**Involvement of HIF-1 in Transcriptional Activation of the EPO Gene by Hypoxia**—In this paper, we have presented additional evidence supporting the hypothesis, advanced on the basis of previous studies (24), that HIF-1 plays a key role in the hypoxic induction of EPO transcription. (i) Hypoxic induction of Epo RNA and HIF-1 activity were inhibited by 2-AP, as they were by cycloheximide (24). (ii) The kinetics of HIF-1 induction by hypoxia paralleled those of EPO transcriptional activation. (iii) HIF-1 DNA binding activity decayed rapidly when hypoxic Hep3B cells were exposed to increased O2 tension. (iv) HIF-1 binding to DNA in vitro reached equilibrium conditions in <5 min, as might be expected of a physiologic transcriptional regulator. The half-life of the DNA-protein complex was <1 min, compared to a half-life of 6–10 min for DNA binding of the developmental regulator GATA-1 (30).

We have recently demonstrated that cobalt chloride, the other known inducer of EPO transcription (14), also induced HIF-1 DNA binding activity in Hep3B cells (31). Hypoxia and cobalt chloride induced HIF-1 and Epo RNA with similar kinetics, and induction was sensitive to cycloheximide treatment in both cases (24, 31). Thus, inducers of EPO expression (1% O2, CoCl2) induced HIF-1 activity and inhibitors of EPO induction (cycloheximide, 2-aminopurine) also inhibited HIF-1 activity. Taken together, these observations strengthen our conclusion that induction of HIF-1 binding to the EPO enhancer is a critical event in the hypoxic activation of EPO transcription.

**Metabolic Inhibitors Block Activation of EPO Gene Transcription Mediated by HIF-1**—We have now evaluated the effect of inhibitors of protein synthesis (cycloheximide), transcription (actinomycin D), and protein phosphorylation (2-AP) on induction of HIF-1 activity and Epo RNA by hypoxia. In our experimental protocol, Hep3B cells were pretreated with inhibitor for 2 h in 20% O2 and then exposed to 20 or 1% O2 for 4 h. A 4-h hypoxic exposure was chosen because of maximal HIF-1 activity at this time point (Fig. 3A). The 6-h exposure to inhibitors might cause multiple metabolic alterations, which could affect HIF-1 binding activity. Nevertheless, one interpretation of these results is that hypoxia induces transcription and translation, resulting in de novo synthesis of a protein required for HIF-1 induction. Candidates for the induced protein(s) include one or more HIF-1 subunits, or perhaps an activating protein kinase (see below). Addressing this issue in a definitive fashion will require the isolation of cDNA sequences encoding.
HIF-1. Thus, the results reported here have generated hypotheses that can be tested when additional reagents become available.

The role of protein phosphorylation in the activation of EPO transcription mediated by HIF-1 is uncertain. The mechanism by which 2-AP inhibits phosphorylation reactions catalyzed by protein kinases has not been established. A known target for inhibition by 2-AP is the heme-regulated eukaryotic initiation factor-2 (eIF-2) kinase (32, 33). At present, there are no data addressing the possibility that 2-AP inhibition of HIF-1 DNA binding activity and EPO transcription occurs at the level of translation via inhibition of eIF-2 kinase activity. Transcriptional activation of the mouse cytochrome P450 gene Cyp1a-1 is mediated by induction of aryl hydrocarbon receptor DNA binding activity (34, 35). Aryl hydrocarbon induction of Cyp1a-1 RNA is blocked by 2-AP and also by inhibitors of protein kinase C (36), as is the case for induction of Epo RNA by hypoxia (16, 17). In addition, DNA binding activity of both HIF-1 (Fig. 5) and the aryl hydrocarbon receptor (36) is eliminated by treatment with CIP. Activation of a protein kinase could thus represent an early step in the hypoxia signal transduction pathway or phosphorylation could directly regulate HIF-1 DNA binding activity or both mechanisms could be operative. Our data suggest that HIF-1 phosphorylation may be necessary for DNA binding, but it is important to acknowledge that we are presently limited by our ability to only assay HIF-1 DNA binding activity, rather than HIF-1 proteins (and phosphoproteins) directly.

**HIF-1 Binds DNA via Specific Base Contacts in the Major Groove**—Methylation interference analysis revealed that HIF-1 makes close contacts with 4 guanine residues (G-8 and G-10) on the coding strand and 2 (G-7 and G-11) on the non-coding strand of the binding site probe. These results further refine the HIF-1 binding site in the EPO enhancer to the sequence 5'-TACGTGCT-3' and provide evidence that HIF-1 binds in the major groove of the DNA double helix. Methylation interference patterns differed between HIF-1 and the constitutive factor that binds to the same site. The less pronounced effect of guanine methylations at positions 7, 8, 10, and 11 on the constitutive binding activity is consistent with the observation that DNA-binding proteins of the constitutive factor are UV-cross-linked to W18 much less efficiently than are HIF-1 proteins (31). These results suggest that (i) HIF-1 may make closer contacts with its binding site than does the constitutive factor and (ii) the two factors do not share a common DNA-binding subunit, especially in light of the differences in molecular weight of the UV-cross-linked proteins (31).

**General Involvement of HIF-1 in Hypoxia-inducible Transcription**—We recently demonstrated that HIF-1 activity and transcription of reporter genes containing HIF-1 binding sites are induced by hypoxia in non-Epo-producing cells (31). The mouse Epo enhancer also mediates hypoxia-inducible transcription of reporter genes in non-Epo-producing cells (37). The generalized induction of HIF-1 activity stands in contrast to restricted expression of the EPO gene, which is regulated by multiple positive and negative cis-acting elements (11, 12, 20, 22). The induction of HIF-1 activity in non-Epo-producing cells suggests that HIF-1 may mediate transcriptional activation of many genes in response to hypoxia. Candidate genes should contain HIF-1 binding sites within enhancer or promoter elements and transcription of these genes might be expected to respond appropriately to inhibitors and inducers of HIF-1 de-