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Hypoxia Response Elements in the Aldolase A, Enolase 1, and Lactate Dehydrogenase A Gene Promoters Contain Essential Binding Sites for Hypoxia-inducible Factor 1*

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Hypoxia-inducible factor 1 (HIF-1) is a basic helix-loop-helix transcription factor which is expressed when mammalian cells are subjected to hypoxia and which activates transcription of genes encoding erythropoietin, vascular endothelial growth factor, and other proteins that are important for maintaining oxygen homeostasis. Previous studies have provided indirect evidence that HIF-1 also regulates transcription of genes encoding glycolytic enzymes. In this paper we characterize hypoxia response elements in the promoters of the *ALDA*, *ENO1*, and *Ldha* genes. We demonstrate that HIF-1 plays an essential role in activating transcription via these elements and show that although absolutely necessary, the presence of a HIF-1 binding site alone is not sufficient to mediate transcriptional responses to hypoxia. Analysis of hypoxia response elements in the *ENO1* and *Ldha* gene promoters revealed that each contains two functionally-essential HIF-1 sites arranged as direct and inverted repeats, respectively. Our data establish that functional hypoxia-response elements consist of a pair of contiguous transcription factor binding sites at least one of which contains the core sequence 5′-RCGTG-3′ and is recognized by HIF-1. These results provide further evidence that the coordinate transcriptional activation of genes encoding glycolytic enzymes which occurs in hypoxic cells is mediated by HIF-1.

Multiple homeostatic mechanisms are employed by mammals to respond to chronic hypoxia. In the case of systemic hypoxia due to decreased environmental O_2 (hypobaric hypoxia) or decreased blood O_2-carrying capacity (anemia), erythropoiesis is stimulated by the production of erythropoietin (EPO).[1] In the case of local hypoxia due to inadequate perfusion (ischemia), angiogenesis is stimulated by the production of vascular endothelial growth factor (VEGF). The production of EPO and VEGF ultimately increases O_2 delivery, thus reducing the discrepancy between O_2 supply and demand in affected tissues. Regardless of etiology, hypoxia is sensed by individual cells which undergo metabolic adaptations to compensate for an inadequate O_2 supply. A major intracellular adaptation to severe hypoxia is the transition from oxidative phosphorylation to glycolysis as the principal means of generating ATP (1, 2). When tissue culture cells were subjected to hypoxia, expression of genes encoding respiratory chain components decreased and expression of genes encoding glycolytic enzymes increased (2). As in the case of the *EPO* (3, 4) and *VEGF* (5–7) genes, increased expression of genes encoding glycolytic enzymes in hypoxic cells is due at least in part to increased gene transcription (8).

Transcription of genes encoding EPO, VEGF, and glycolytic enzymes is activated in hypoxic cells by a common molecular mechanism (reviewed in Ref. 9). For both *EPO* (10–14) and *VEGF* (5, 6, 15, 16), hypoxia response elements (HREs) of 50 bp or less mediate transcriptional activation of reporters in hypoxic cells. In recent studies (15, 17), the human *EPO* and *VEGF* HREs have been shown to contain functionally essential binding sites for hypoxia-inducible factor 1 (HIF-1), a basic helix-loop-helix transcription factor composed of HIF-1α and HIF-1β subunits, the expression of which is induced in hypoxic cells (18). The HIF-1 binding site sequences in the human *EPO* and *VEGF* HREs are 5′-TACGTGCT-3′ and 5′-TACGTGGG-3′, respectively (14–17). Mutations within these elements that eliminated HIF-1 binding also eliminated transcriptional activation of reporters in hypoxic cells and forced expression of recombinant HIF-1 in non-hypoxic cells activated expression of reporters containing HREs with wild-type, but not with mutant, HIF-1 sites (15, 17). Mutagenesis analysis of the *EPO* and *VEGF* HREs identified sequences just 3′ to the HIF-1 binding site that were also required for function (10, 14, 16).

The expression of genes encoding the glycolytic enzymes aldolase A (ALDA), lactate dehydrogenase A (Ldha), phosphoglycerate kinase 1 (PGK1), and pyruvate kinase M was induced in cells exposed to 1% O_2, cobalt chloride, or desferrioxamine, which each induce HIF-1 DNA binding activity as well as *EPO* and *VEGF* expression (19–25). Treatment of hypoxic cells with cycloheximide blocked induction of glycolytic mRNAs and HIF-1 activity (14, 22). HIF-1 binding sites were identified in genes encoding ALDA, enolase 1 (ENO1), Ldha, phosphofructokinase L, and PGK1 and transcription of reporters containing

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[1] The abbreviations used are: EPO, erythropoietin; HIF-1, hypoxia-inducible factor 1; ALDA, aldolase A; ENO1, enolase 1; Ldha, lactate dehydrogenase A; VEGF, vascular endothelial growth factor; HRE, hypoxia response element; PGK1, phosphoglycerate kinase 1; β-Gal, β-galactosidase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; bp, base pair(s); nt, nucleotide(s).

ALDA, Ldha, PFKL, and *PGK1* promoter sequences was activated in hypoxic cells (19, 20, 22). However, definitive evidence supporting the role of HIF-1 in activating transcription of these genes has not been presented. We have now analyzed in greater detail the regulation of *ALDA* and *ENO1* transcription in hypoxic cells, identified novel HIF-1 binding sites in the *ALDA, ENO1*, and *Ldha* genes, correlated HIF-1 binding with transcriptional activation, and demonstrated transcriptional activation in non-hypoxic cells by forced expression of HIF-1α. These studies provide further evidence for the coordinate regulation of genes encoding glycolytic enzymes by HIF-1 and demonstrate that the presence of a HIF-1 binding site is necessary but not sufficient to direct hypoxia inducible transcription.

MATERIALS AND METHODS

Construction of ALDA Reporter Plasmids—Construction of pHcat, pHcatSacII, pHcatAvaI, and pHcatAvaIHphI was described previously (26). Plasmid pHcatSacIImut was generated from pHcatSacII using the Transformer Site-directed Mutagenesis kit (Clontech) and the mutagenic primer 5′-GTGGTCCGAGTGAATTCCGAGGGGG-3′ (mutations underlined).

Construction of ENO1 Reporter Plasmids— ENO1 reporter plasmid pAcat1 was constructed by inserting a 2.0-kilobase *Hin*dIII/*Nae*I fragment from the human *ENO1* gene (27) between the *Hin*dIII and blunt-ended *Bam*HI sites of pBLCAT3 (28). Plasmids pAcat2 and pAcat3 were derived from pAcat1 by removal of a *Hin*dIII/*Bst*XI or *Hin*dIII/*Pst*I fragment, respectively, followed by treatment with T4 DNA polymerase and T4 DNA ligase (Life Technologies, Inc.). pAcat4 was derived from pAcat3 by excision of a *Bgl*I/*Xho*I fragment which was inserted between the *Bam*HI and *Xho*I sites of pBLCAT3. pAcat2T and pAcat2X were derived from pAcat1 by removal of *Hin*dIII/*Tth*111I and *Hin*dIII/*Xcm*I fragments, respectively, followed by treatment with T4 DNA polymerase and T4 DNA ligase. pAcat2S was derived from pAcat2 by excision of a *Sac*I/*Xho*I fragment which was inserted between the *Bam*HI and *Xho*I sites of pBLCAT3. To generate plasmids p2.1, p2.2, p2.3, p2.4, and p2.5, *ENO1* sequences between −416 and −349 were amplified by polymerase chain reaction using the following oligonucleotides: forward primer 3 (F3), 5′-aaaggtaccAGGGCCGGACGTGGGGCCCC-3′ (nucleotides introduced for cloning are in lower case and *ENO1* sequences are in upper case); F4, 5′-aaaggtaccAGGGCCGGAAAAGGGGCCCC-3′ (mutation underlined); reverse primer 5 (R5), 5′-aaactcgagGGGGCTCCGTCACGTACTCC-3′; R6, 5′-aaactcgagGGGGCTCCGTCTTTTACTCC-3′; and R7, 5′-aaactcgagAAAAATCCGTCACGTACTCC-3′. Polymerase chain reaction products amplified by the indicated primer pairs were digested with *Kpn*I and *Xho*I and ligated to *Kpn*I/XhoI-digested p0 (pGL2-Promoter (Promega)): p2.1, F3 and R5; p2.2, F4 and R5; p2.3, F3 and R6; p2.4, F5 and R6; and p2.5, F3 and R7. To generate p2.6 and p2.7, the primers used included F8, 5′-aaaggtaccACGCTGAGTGCGTGCGGGAC-3′ and F9, 5′-aaaggtaccACGCTGAGTGAAAGCGGAC-3′ as follows: p2.6, F8 and R5; and p2.7, F9 and R5. The identity of cloned polymerase chain reaction products was confirmed by nucleotide sequence analysis.

Transient Expression Assay—Hep3B cells were transfected with 20 μg of a CAT or 10 μg of a luciferase reporter plasmid and 5 μg of control plasmid pSVβgal (Promega) by electroporation (22) and split onto six plates which were incubated at 20% O_2 for 24 h. The medium was changed and three plates were incubated at 1 or 20% O_2 for 24–48 h prior to preparation of cell lysates. β-Galactosidase (β-Gal), chloramphenicol acetyltransferase (CAT), and luciferase assays were performed as described previously (14, 15, 25). For co-transfection assays, cells also received 1 μg each of pCEP4/HIF-1α3.2–3T7 (15, 17) and pBM5neo/M1–1 (29), which contain human HIF-1α and HIF-1β (ARNT) cDNA sequences, respectively, downstream of a cytomegalovirus promoter. For co-transfection experiments with pCEP4/HIF-1αDN (15), cells were transfected with various amounts of pCEP4 (Invitrogen) and pCEP4/HIF-1αDN such that all cells received a total of 40 μg of expression plasmid. pEBB/HIF-1α was constructed by cloning the HIF-1α coding sequence into pEBB (30) downstream of the human EF-1α promoter.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from Hep3B cells and EMSA was performed as described (14, 15, 31), using the following ^{32}P-end-labeled double-stranded oligonucleotide probes (sequence of sense strand is shown): Aw, 5′-GTGGTCCGAGTCACGTCCGAGGGGG-3′; Cw, 5′-AGGGCCGGACGTGGGGCCCC-3′; Dw, 5′-GGAGTACGTGACGGAGCCCC-3′; Ew, 5′-GCCCTACGTGCTGTCTCA-3′; A1, 5′-TTCCCGCACGTCCGCTGGGCTCCCAC-3′; A2, 5′-CCCAGCCTACACGTGGGGTTC-3′; and Uw, 5′-ACGCTGAGTGCGTGCGGGAC-3′. For competition assays 0–50 ng of unlabeled oligonucleotide were added to the binding reaction. The following mutant oligonucleotides were used (mutation underlined): Am, 5′-GTGGTCCGAGTGAATTCCGAGGGGG-3′; Em, 5′-GCCCTAAAAGCTGTCTCA-3′; Cm, 5′-AGGGCCGGAAAAGGGGCCCC-3′; and Dm, 5′-GGAGTTTTTGACGGAGCCCC-3′. For supershift assays, 1 μl of polyclonal antiserum raised in rabbits against recombinant HIF-1α or HIF-1β at 1:3 or 1:6 dilution, respectively, was added to the EMSA reaction as described (18).

RNA Blot Hybridization—Total RNA was isolated from Hep3B cells by acid-guanidinium-phenol-chloroform extraction (32). Fifteen-μg aliquots of RNA were fractionated by 1.4% agarose, 2.2 M formaldehyde gel electrophoresis and transferred to a nitrocellulose filter. A human ENO1 cDNA (American Type Culture Collection, Rockville MD) was ^{32}P-labeled using the Random Primers DNA Labeling System (Life Technologies, Inc.) and blot hybridization was performed at 68 °C in Quik-Hyb (Stratagene), followed by washes at 55 °C in 0.1 × SSC, 0.1% SDS. Following autoradiography, the blot was stripped of radioactivity and hybridized to a ^{32}P-end-labeled oligonucleotide complementary to 18 S rRNA, 5′-ACGGTATCTGATCGTCTTCGAACC-3′. Autoradiographic signals were quantitated by scanning with a laser densitometer (Molecular Dynamics, Sunnyvale CA).

RESULTS

Identification of Sequences Required for Hypoxia-inducible Transcription from the Aldolase A Promoter—We previously demonstrated that mRNA initiated from exon H of the human *ALDA* gene was induced 3-fold in hypoxic Hep3B and HeLa cells (incubated in 1% O_2 for 16 h), whereas levels of mRNA initiated from upstream exon N were unaffected by hypoxia (22). Reporter plasmid pHcat, containing the 172-bp exon H, 420 bp of 5′-flanking DNA, and 168 bp of intronic DNA downstream of exon H fused to CAT coding sequences, was transfected into Hep3B cells with plasmid pSVβgal (to correct for variability in transfection efficiency). Transfected cells were split onto plates which were incubated for 24 h in 20% O_2 and then for 48 h in either 20 or 1% O_2. CAT and β-Gal assays were performed on cell lysates and the CAT/β-Gal ratio was calculated (relative CAT activity). Expression of pHcat was induced 4.1-fold in hypoxic cells (Fig. 1). We previously identified a HIF-1 binding site, 5′-CACGTGCG-3′, within intron sequences downstream of exon H (22) that are included within pHcat (indicated by *open circle* on map in Fig. 1). To determine whether intron and/or promoter sequences were required for activation of the *ALDA* promoter in hypoxic cells, we analyzed the expression of constructs pHcatSacII, pHcatAvaI, and pHcatAvaIHphI. Deletion of promoter sequences from nt −420 to −212 resulted in a decrease in the level of expression at both 20 and 1% O_2 but pHcatSacII expression was induced 8.6-fold in hypoxic cells. When promoter sequences from −212 to −118 were deleted the level of expression declined further and there was no induction in hypoxic cells. Deletion of sequences between +92 and +341 further decreased expression at 20 and 1% O_2 to a similar degree. These results indicate that (i) sequences located between −212 and −118 were required for activation of the *ALDA* promoter in hypoxic cells and (ii) the HIF-1 binding site downstream of exon H played no role in the response to hypoxia. Disruption of the intronic HIF-1 binding site by insertion of two different oligonucleotides into the 5′-CAC ↓ GTGCG-3′ sequence (arrow indicates insertion site) also had no effect on the expression of pHcat (data not shown).

A candidate HIF-1 site, 5′-GACGTGAC-3′, was identified on the antisense strand between −188 and −195 (*closed square* on map in Fig. 1), which was identical to a putative HIF-1 site located within an HRE in the *PGK1* promoter (19). A 3-bp substitution within this sequence was introduced into pHcatSacII (Fig. 2*A*). Expression of pHcatSacIImut was similar to

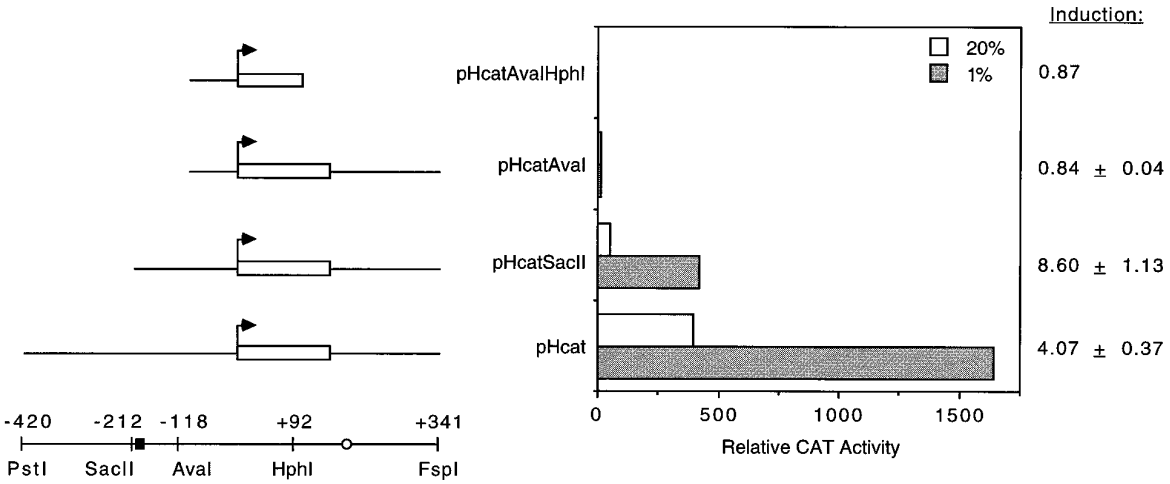

FIG. 1. **Functional analysis of the aldolase A gene promoter.** *Left, ALDA* sequences were cloned 5′ to CAT coding sequences. *Open box*, exon H; *arrow*, 5′-most transcription initiation site. HIF-1 binding sites are indicated on the *map below* as *closed square* and *open circle. Right, ALDA*-CAT reporters were transfected into Hep3B cells with pSVβgal, split onto duplicate plates, incubated at 20% O_2 for 24 h, and then incubated at 1 or 20% O_2 for 48 h. The CAT/β-Gal ratio was determined for each plate and normalized to the result for pHcatAvaIHphI in cells at 20% O_2 (*Relative CAT Activity*). The ratio of relative CAT activity in cells at 1% O_2 compared to 20% O_2 was also calculated to determine induction by hypoxia (mean ± S.E.; n = three independent experiments, except for pHcatAvaIHphI where n = 1).

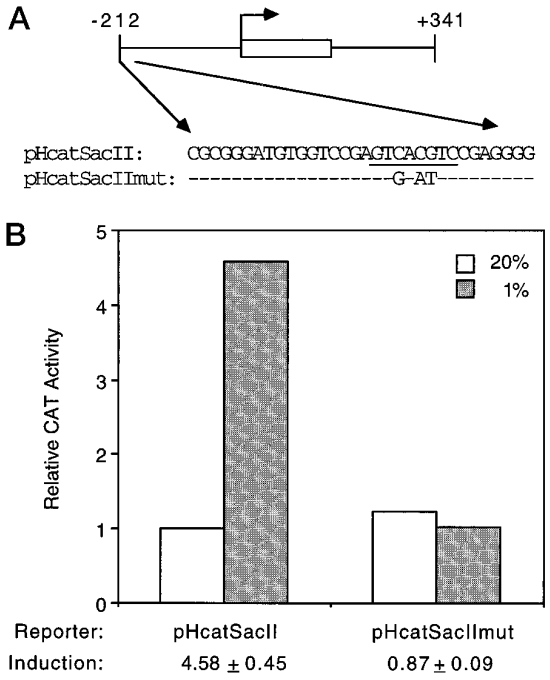

FIG. 2. **Effect of site-directed mutagenesis on aldolase A promoter function.** *A*, sequence of wild-type and mutant *ALDA*-CAT reporter plasmids. pHcatSacII and pHcatSacIImut sequences were identical (*hyphens*) except for a 3-bp substitution within the HIF-1 binding site (*underlined*). *B*, transient expression assay. Hep3B cells were transfected with pSVβgal and pHcatSacII or pHcatSacIImut. Transfected cells were incubated at 20% O_2 for 24 h and then duplicate plates were incubated at 20 or 1% O_2 for 24 h. The CAT/β-Gal ratio was determined for each plate and normalized to the result for pHcatSacII in cells at 20% O_2 (*Relative CAT Activity*). The ratio of relative CAT activity in cells at 1% O_2 compared to 20% O_2 was also calculated to determine induction by hypoxia (mean ± S.E.; n = three independent experiments).

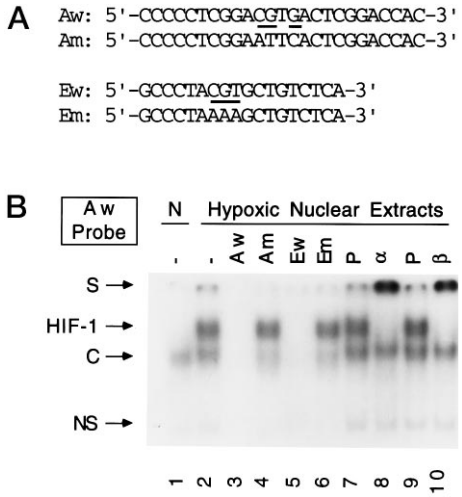

FIG. 3. **Binding of HIF-1 to aldolase A promoter sequences.** *A*, oligonucleotide sequences. Aw and Am, wild-type and mutant *ALDA* sequences (antisense strand is shown to demonstrate canonical HIF-1 site). Ew and Em, wild-type and mutant *EPO* sequences. *B*, EMSA using probe Aw. Nuclear extracts were prepared from Hep3B cells exposed to 20 or 1% O_2 for 4 h. Aliquots (5 μg) were incubated with probe Aw and DNA binding activities were analyzed by EMSA. For competition assays (*lanes 3–6*), binding reactions included 50 ng of the indicated oligonucleotides. For supershift assays (*lanes 7–10*), binding reactions contained antiserum raised against HIF-1α (*lane 8*) or HIF-1β (*lane 10*), or the respective preimmune serum (*lanes 7* and *9*). The following probe complexes are indicated by an *arrow*: *S*, HIF-1 supershifted by antibody; *C*, constitutive DNA binding activity; *NS*, nonspecific DNA binding activity. The *bottom* of the gel containing free probe is not shown.

that of pHcatSacII at 20% O_2 but was not induced in hypoxic cells (Fig. 2*B*).

To demonstrate that these sequences represented a HIF-1 binding site, oligonucleotide Aw spanning nt −204 to −181 was used as a probe in an EMSA with Hep3B nuclear extracts (Fig. 3*A*). As previously demonstrated for probes from the *EPO* and *VEGF* HREs (14, 15), the Aw probe bound constitutive and nonspecific DNA binding activities that were present in nuclear extracts of hypoxic and non-hypoxic cells and also bound a hypoxia-induced factor (Fig. 3*B, lanes 1* and *2*). Competition with 50 ng (500-fold molar excess) of unlabeled Aw (*lane 3*) or Ew containing the HIF-1 site from the *EPO* HRE (*lane 5*) prevented detection of all three DNA binding activities. In contrast, Am, containing the 3-bp substitution present in pHcatSacIImut, did not compete with the probe for binding of the constitutive and hypoxia-induced factors (*lane 4*). Oligonucleotide Em, containing a 3-bp substitution in the HIF-1 binding site of the *EPO* enhancer that was previously shown to eliminate enhancer function (14), also failed to compete with the

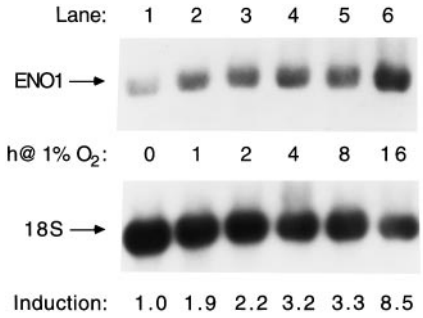

FIG. 4. **Induction of enolase 1 mRNA in hypoxic cells.** Hep3B cells were incubated in 20% O_2 (*lane 1*) or in 1% O_2 for 1–16 h (*lanes 2–6*). Total RNA was isolated and 15-μg aliquots were analyzed by blot hybridization using probes complementary to *ENO1* mRNA (*top panel*) or 18 S rRNA (*bottom panel*). Autoradiographic signals were quantitated by laser densitometry and normalized to the results obtained from cells incubated in 20% O_2. The *ENO1* mRNA/18 S rRNA ratios are indicated. Results are representative of two independent experiments.

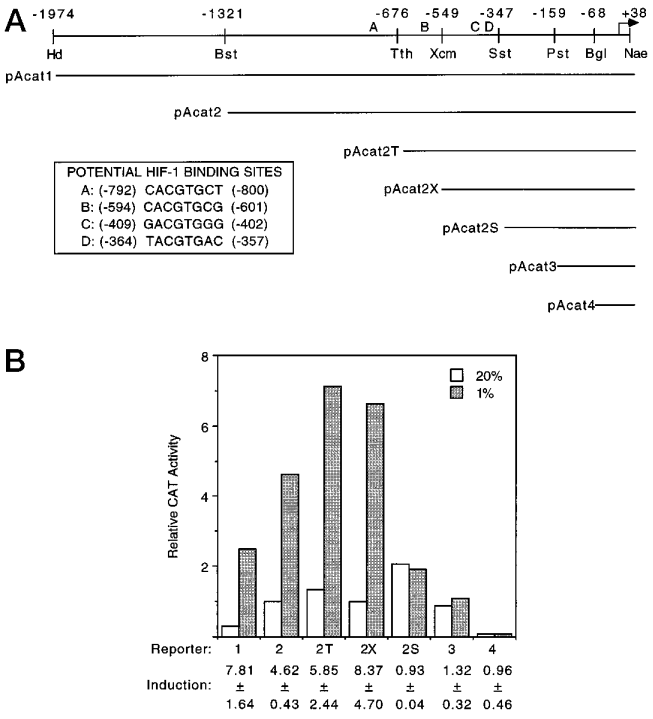

FIG. 5. **Enolase 1 promoter sequences mediate transcriptional responses to hypoxia.** *A*, structure of *ENO1*-CAT reporters. *ENO1* sequences were cloned 5′ to CAT coding sequences. The location of restriction sites relative to the transcription start site (*arrow*) is shown: *Hd*, *Hin*dIII; *Bst*, *Bst*XI; *Tth*, *Tth*111I; *Xcm*, *Xcm*I; *Sst*, *Sst*I; *Pst*, *Pst*I; *Bgl*, *Bgl*I; *Nae*, *Nae*I. The nucleotide sequence (*box*) and location of potential HIF-1 binding sites is indicated. *B*, transient expression of reporters. Hep3B cells were transfected with pSVβgal and *ENO1*-CAT reporter, duplicate plates were incubated at 20% O_2 for 24 h and then incubated at 20 or 1% O_2 for 48 h. The ratio of relative CAT activity in cells at 1% O_2 compared to 20% O_2 was also calculated to determine induction by hypoxia (mean ± S.E.; n = two to five independent experiments).

probe for binding of these factors (*lane 6*). To prove that the hypoxia-induced factor that bound to probe Aw was HIF-1, supershift assays were performed. Addition of polyclonal antiserum raised in rabbits against recombinant HIF-1α (*lane 8*) or HIF-1β (*lane 10*) resulted in the formation of a supershift complex of reduced mobility, whereas the respective preimmune sera had no effect (*lanes 7* and *9*).

Induction of Enolase 1 mRNA Expression in Hypoxic Cells—Hep3B cells were exposed to 1% O_2 for 0–16 h, total RNA was isolated, and the expression of ENO1 mRNA was quantitated by blot hybridization relative to the expression of 18 S rRNA (as a control for variation in RNA loading and transfer). Compared to cells incubated at 20% O_2 (Fig. 4, *lane 1*), the ENO1 mRNA/18 S rRNA ratio was increased 8.5-fold in cells exposed to 1% O_2 for 16 h (*lane 6*). These results indicate that *ENO1* expression in Hep3B cells is induced by hypoxia.

Identification of Sequences Required for Hypoxia-inducible Transcription from the ENO1 Promoter—A 2.0-kilobase genomic DNA fragment from the 5′-flanking region of the human *ENO1* gene that extended through the transcription initiation site was cloned 5′ to CAT coding sequences to generate pAcat1. Four potential HIF-1 binding sites were noted within the sequence of this region (Fig. 5*A*). In Hep3B cells transfected with pAcat1, CAT expression was induced 7.8-fold by hypoxia (Fig. 5*B*), indicating that increased ENO1 mRNA levels in hypoxic cells resulted at least in part from increased transcription directed by these 5′-flanking sequences. Deletion of 5′-flanking DNA from nt −1974 (pAcat1) to −1321 (pAcat2), −676 (pAcat2T), or −549 (pAcat2X) did not affect the response to hypoxia, as pAcat2X was induced 8.4-fold. These results indicated that HIF-1 binding sites A and B were not required for transcriptional activation of *ENO1* in hypoxic cells. Deletion of sequences from nt −549 to −347 resulted in a reporter (pAcat2S) that was unresponsive to hypoxia. Further deletion to nt −159 (pAcat3) or −68 (pAcat4) did not restore the response. These results indicate that sequences between nt −549 and −347, which include putative HIF-1 binding sites C and D, were necessary for the transcriptional response to hypoxia.

Identification of a Hypoxia Response Element in the ENO1 Promoter—In order to identify sequences which were sufficient to direct hypoxia-inducible transcription from a heterologous promoter, a 68-bp *ENO1* promoter fragment, extending from nt −416 to −349 and encompassing potential HIF-1 binding sites C and D (Fig. 6*A, bottom*), was cloned 5′ to an SV40 promoter-luciferase transcription unit. The reporter plasmid was co-transfected into Hep3B cells with pSVβgal and duplicate plates were incubated in 20% O_2 for 24 h and then in 20 or 1% O_2 for 24 h. In cells incubated at 20% O_2, expression of p2.1 (the reporter containing the wild-type 68-bp fragment) was increased 2-fold compared to the expression of p0, the parental SV40-luciferase plasmid (Fig. 6*A, top*). Expression of p2.1 was induced 34-fold by hypoxia, whereas p0 was induced only 1.3-fold. Thus, the 68-bp *ENO1* sequence functions as an HRE. To determine whether putative HIF-1 sites C and D were required for this induction, 3-bp (CGT to AAA) substitutions were introduced at one or both of these sites (Fig. 6*A, bottom*). For all three mutant reporters (p2.2, p2.3, and p2.4) expression at 20% O_2 remained increased 2-fold over that of p0. Reporter p2.2, mutated at site C, was induced 29-fold by hypoxia, similar to the response of p2.1. In contrast, reporter p2.3, mutated at site D, showed a minimal response to hypoxia that was similar to that of p0. The double mutant p2.4 also showed a response that was similar to p0.

HIF-1 Mediates Sequence-specific Transactivation via the ENO1 Hypoxia Response Element—The wild-type and mutated reporter plasmids were next co-transfected into Hep3B cells with pSVβgal in the absence or presence of expression vectors encoding HIF-1α and HIF-1β (Fig. 6*B*). At 20% O_2, reporter expression was increased 2-fold over that of p0. In this set of experiments, expression of p2.1, p2.2, p2.3, and p2.4 in the absence of HIF-1 expression vectors was increased 99-, 35-, 2.3-, and 1.5-fold, respectively, in cells incubated in 1% O_2 relative to cells incubated in 20% O_2, whereas p0 was induced 1.6-fold. When co-transfected with recombinant HIF-1 expression vectors (rHIF-1), transcription of p2.1, p2.2, p2.3, and p2.4 in cells at 20% O_2 was increased 90-, 58-, 11-, and 7.7-fold

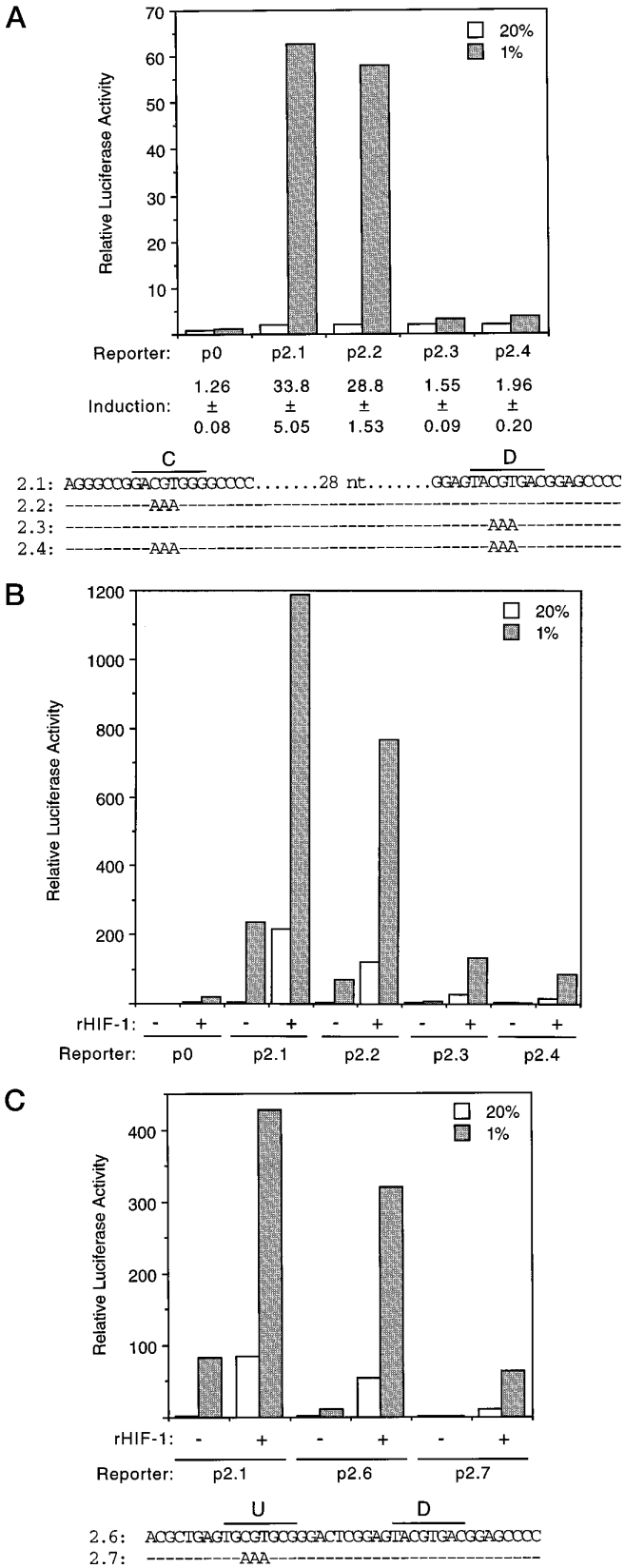

FIG. 6. **Functional analysis of the *ENO1* hypoxia-response element in the context of a heterologous promoter.** *A*, identification of sequences required for hypoxia inducible transcription. A 68-bp fragment from the *ENO1* promoter which was either wild-type (2.1) or mutated (2.2, 2.3, and 2.4) (*bottom*) was cloned 5′ to an SV40 promoter-luciferase transcription unit in the reporter p0. Reporters were transfected into Hep3B cells with pSVβgal and exposed to 20 or 1% O_2 for 24 h (*top*). The ratio of luciferase/β-Gal activity was normalized to the result obtained for cells transfected with pO and exposed to 20% O_2 (*Relative Luciferase Activity*). Expression at 1% relative to 20% O_2 was calculated (*Induction*) and mean ± S.E. (n = 3) is shown. *B*, co-transfection of reporters with HIF-1 expression vectors. Hep3B cells were transfected with pSVβgal and one of the reporters in the absence (−) or presence (+) of 1 μg of HIF-1α and HIF-1β expression vectors (*rHIF-1*), exposed to 20 or 1% O_2 for 24 h, and analyzed as described above. Mean data from three transfections are shown. *C*, functional analysis of sequences upstream of site D. Expression of p2.1 (wild-type 68-mer), p2.6 (wild-type 42-mer), and p2.7 (mutant 42-mer) was compared as described above.

relative to transcription in the absence of rHIF-1, whereas p0 transcription was increased 5.1-fold. Thus, the relative response of the reporters to rHIF-1 paralleled the response to hypoxia. The effect of hypoxia and rHIF-1 was synergistic: expression of p2.1, p2.2, p2.3, and p2.4 in the presence of rHIF-1 at 1% O_2 was increased 495-, 366-, 52-, and 39-fold relative to expression in cells incubated at 20% O_2 in the absence of rHIF-1, whereas p0 expression was increased 20-fold under these conditions. Transactivation of p0 by rHIF-1, which was 20-fold less than that of p2.1, may be due to the presence of a cryptic HIF-1 site in p0 (data not shown). These results indicate that responses to hypoxia and rHIF-1 are mediated by the same cis-acting sequences within the 68-bp HRE. The mutagenesis experiments suggest that site D plays a greater role than site C in mediating these responses.

Since the ability of the *ENO1* HRE to respond to hypoxia and rHIF-1 was more dependent upon site D than site C, we next mutated the 5 bp immediately downstream of site D (GCCCC to TTTTT) to generate p2.5, expression of which was not significantly different from that of p2.1 (data not shown), indicating that these downstream sequences are not essential for HRE function. To further delineate the minimal HRE, we generated reporter p2.6 which included 42 bp from the 3′ end of p2.1, containing site D but not site C (Fig. 6C). In this experiment, expression of p2.1 was induced 54-, 85-, and 427-fold by hypoxia, rHIF-1, and hypoxia + rHIF-1, respectively, whereas expression of p2.6 was induced 9-, 44-, and 260-fold. Thus, compared to p2.1, p2.6 showed a diminished but still significant response to hypoxia and/or rHIF-1. We identified another potential HIF-1 binding site U, 5′-TGCGTGCG-3′, immediately upstream of site D, which differed from known HIF-1 binding sites by the presence of a G, rather than A, residue at the second position. Expression of p2.7, containing a 3-bp substitution in site U, was only induced 2-, 11-, and 57-fold by hypoxia, rHIF-1, and hypoxia + rHIF-1, respectively. These results suggest that site U also plays an important role in transcriptional responses to hypoxia.

Binding of HIF-1 to Sites in the ENO1 Promoter—The binding of nuclear proteins to oligonucleotide probes spanning putative HIF-1 sites C, D, and U was examined by EMSA. When the site C oligonucleotide Cw (Fig. 7*A*) was used as probe to assay nuclear extracts constitutive, nonspecific, and hypoxia-induced DNA binding activities were detected (Fig. 7*B, lanes 1* and *2*). Excess unlabeled Cw competed with the probe for binding of all three activities (*lane 3*), whereas Cm, containing the same 3-bp substitution as in p2.2, competed with probe Cw for binding of the nonspecific and, to a lesser extent, the constitutive DNA binding activities, but did not compete with the probe for binding of the hypoxia-induced factor (*lane 4*). Wild-type *EPO* oligonucleotide Ew successfully competed with probe Cw for binding of all three activities (*lane 5*), whereas mutant Em only competed for binding of the nonspecific and, to a lesser extent, constitutive DNA binding activities (*lane 6*). When antiserum raised against HIF-1α (*lane 8*) or HIF-1β (*lane 10*) was included in the binding reaction, the HIF-1-probe complex was replaced by a HIF-1-probe-antibody supershift complex. In contrast, when the respective preimmune serum was included in

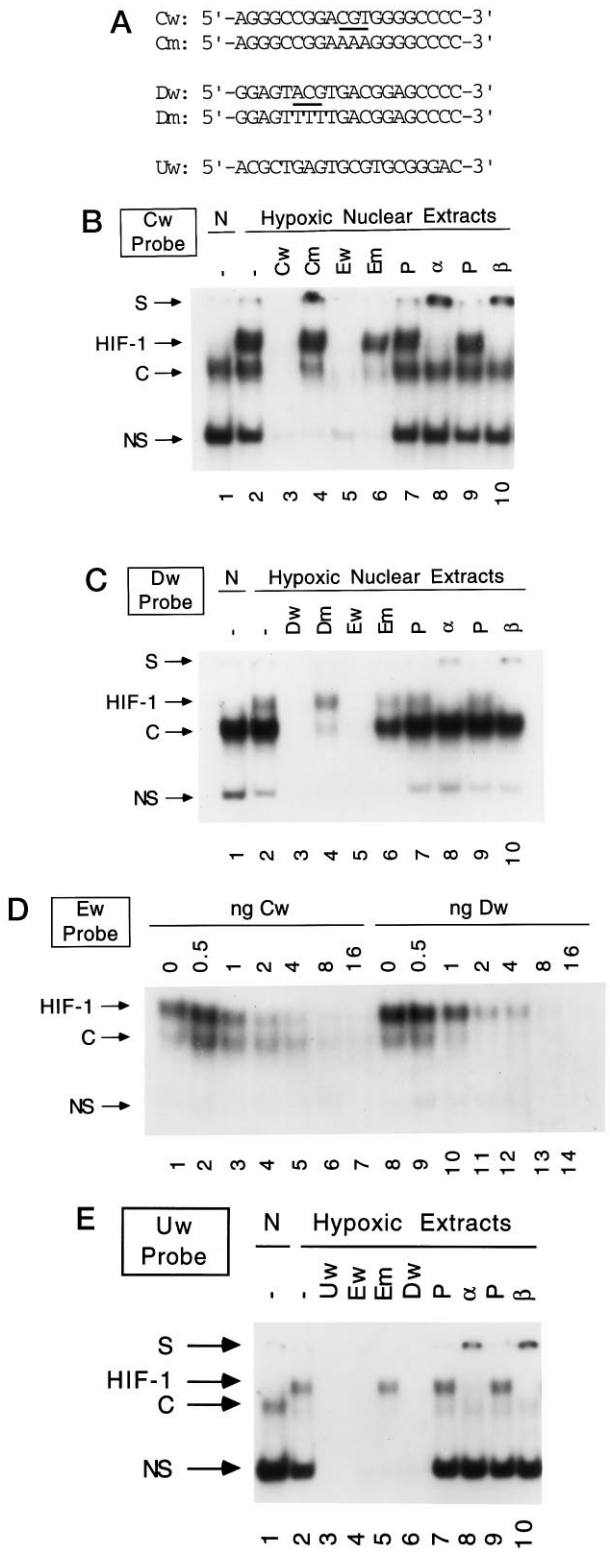

FIG. 7. **Binding of HIF-1 to *ENO1* promoter sequences.** *A*, oligonucleotide sequences. *Cw* and *Cm*, wild-type and mutant *ENO1* site C sequences (*bar* indicates site of mutation). *Dw* and *Dm*, wild-type and mutant site D sequences. *Uw*, wild-type site U sequence. *B*, EMSA using probe Cw. *C*, EMSA using probe Dw. Aliquots (5 μg) of nuclear extracts prepared from Hep3B cells incubated in 20% O_2 (*lane 1*) or 1% O_2 for 4 h (*lanes 2–10*) were analyzed by EMSA. For competition assays (*lanes 3–6*), binding reactions included 50 ng of the indicated unlabeled oligonucleotides. For supershift assays (*lanes 7–10*), binding reactions contained 1 μl of antiserum raised against HIF-1α (*lane 8*) or HIF-1β (*lane 10*), or the respective preimmune serum (*lanes 7* and *9*). Probe complexes are indicated by *arrow: S*, HIF-1 supershifted by antibody; *C*, constitutive DNA binding activity; *NS*, nonspecific DNA binding activity. *D*, analysis of relative binding affinities. Aliquots of hypoxic nuclear extract were incubated with probe Ew in the presence of the indicated amount (in ng) of unlabeled Cw or Dw. *E*, EMSA using probe Uw. Competition assays were performed with Uw (*lane 3*), Ew (*lane 4*), Em (*lane 5*), and Dw (*lane 6*). Supershift assays (*lanes 7–10*) were performed as described above.

the binding reaction, the mobility of the HIF-1-probe complex was unaffected (*lanes 7* and *9*). These results demonstrate that HIF-1 can bind to the *ENO1* site C sequence *in vitro*.

Analagous experiments were performed with probe Dw that spans *ENO1* site D (Fig. 7*A*). The probe again detected constitutive, nonspecific, and hypoxia-induced DNA binding activities (Fig. 7*C, lanes 1* and *2*) but, compared to probe Cw, there appeared to be greater binding of constitutive factors to probe Dw. Excess unlabeled Dw competed for binding to all three factors (*lane 3*), whereas unlabeled mutant Dm competed for binding to the nonspecific and constitutive factors, but not the hypoxia-induced factor (*lane 4*). Ew also competed for binding to all three factors (*lane 5*), whereas Em only competed for binding to the nonspecific factors (*lane 6*). The hypoxia-induced DNA binding activity was supershifted by antiserum raised against HIF-1α (*lane 8*) or HIF-1β (*lane 10*) but not by preimmune serum (*lanes 7* and *9*). These results demonstrate that HIF-1 can bind specifically to the *ENO1* site D sequence *in vitro*. Taken together, the EMSA data indicate that (i) any wild-type or mutant oligonucleotide tested was bound by the nonspecific activity; (ii) the same 3-bp substitution (CGT to AAA) had different effects on binding of constitutive factors depending upon the surrounding sequence context in different oligonucleotides; (iii) HIF-1 bound to Cw, Dw, and Ew with similar sequence specificity.

To determine the relative binding affinity of HIF-1 for Cw and Dw, a more extensive competition assay was performed. Aliquots of nuclear extract from hypoxic cells were incubated with probe Ew in the presence of increasing amounts of unlabeled Cw or Dw (Fig. 7*D*). Whereas Dw was a more effective competitor for binding to constitutive factors, Cw and Dw were similar in their ability to compete with probe Ew for binding of HIF-1. These results indicate that the greater functional importance of *ENO1* site D compared to site C is not a reflection of an increased binding affinity of HIF-1 for the former compared to the latter site.

Oligonucleotide Uw spanning site U (Fig. 7*A*) was also tested by EMSA. Probe Uw bound constitutive and nonspecific DNA binding activities present in nuclear extracts of non-hypoxic and hypoxic Hep3B cells (Fig. 7*E, lanes 1* and *2*). Sequence specific binding of Uw to HIF-1 present in hypoxic extracts was demonstrated by competition (*lanes 3–6*) and supershift (*lanes 7–10*) assays. Thus, HIF-1 also recognizes the sequence 5′-TGCGTGCG-3′.

Forced Expression of HIF-1α Is Sufficient to Activate Transcription via the ENO1 Hypoxia Response Element—Whereas the co-transfection experiments described above (Fig. 6*B*) were performed with both HIF-1α and HIF-1β expression plasmids, we next co-transfected p2.1 either with 1 μg of HIF-1α or 1 μg of HIF-1β expression plasmid or both (Fig. 8*A*). Co-transfection of HIF-1α was necessary and sufficient for activation of reporter gene transcription in non-hypoxic cells and superactivation in hypoxic cells. HIF-1β had no effect either alone or in combination with HIF-1α. Since HIF-1α cannot bind to DNA in the absence of HIF-1β (17), these results suggest that sufficient endogenous HIF-1β protein is present in non-hypoxic and hypoxic cells to form heterodimers with recombinant HIF-1α. Reporter transcription increased in a dose-dependent manner with increasing amounts of transfected HIF-1α expression vector. At 20% O_2, reporter transcription was increased 1385-fold

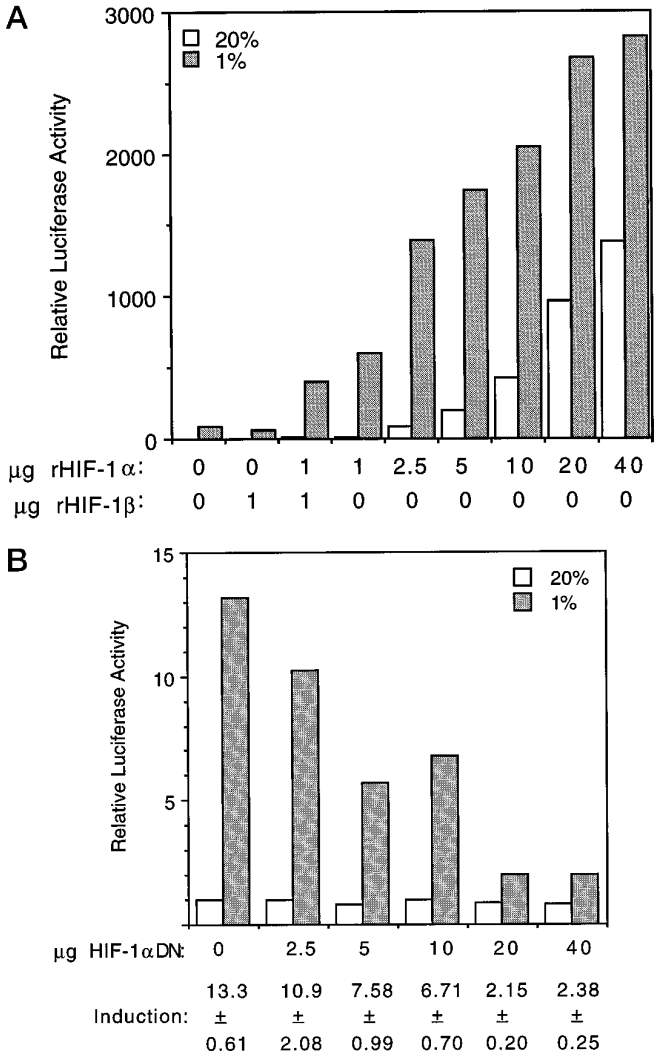

FIG. 8. **Effect of wild-type and mutant forms of HIF-1α on transcription directed by the *ENO1* hypoxia-response element.** *A*, forced expression of HIF-1α is sufficient to activate transcription in non-hypoxic cells. Hep3B cells were co-transfected with pSVβgal, p2.1, and HIF-1α and/or HIF-1β expression plasmids, and pEBB to a total of 40 μg, and exposed to 20 or 1% O_2 for 24 h. Luciferase/β-Gal ratios (mean of six transfections) were normalized to the results obtained from non-hypoxic cells transfected with p2.1 and 40 μg of pEBB. *B*, repression of hypoxia-induced transcription mediated by a dominant-negative form of HIF-1α. Hep3B cells were co-transfected with pSVβgal, p2.1, and various amounts of pCEP4/HIF-1αDN and pCEP4 to a total of 40 μg (amount of pCEP4/HIF-1αDN is shown) and exposed to 20 or 1% O_2 for 24 h. Luciferase/β-Gal ratios were normalized to the results obtained from non-hypoxic cells transfected with 40 μg of pCEP4 (*Relative Luciferase Activity*). Mean data for relative luciferase activities at 20 and 1% O_2 and hypoxic induction (± S.E.) are based upon three independent transfections.

in cells transfected with 40 μg of HIF-1α expression vector relative to cells transfected with 40 μg of empty vector. In cells incubated in 1% O_2, reporter transcription appeared to plateau at higher levels of transfected HIF-1α expression vector, suggesting that HIF-1β or some other component of the system may have become limiting.

A Dominant Negative Form of HIF-1α Inhibits Transcriptional Activation Mediated by the ENO1 Hypoxia Response Element—A dominant negative form of HIF-1α was utilized to provide further evidence that transcription of p2.1 in hypoxic cells is activated by HIF-1. We constructed pCEP4/HIF-1αDN by deletion of sequences encoding the amino-terminal basic domain and carboxyl-terminal transactivation domain. HIF-1αDN can dimerize with HIF-1β, but the resulting heterodimer cannot bind to DNA or activate transcription (15, 17). Overexpression of HIF-1αDN thus prevents the formation of biologically-active HIF-1 in hypoxic cells. Hep3B cells were co-transfected with pSVβgal, p2.1, and various amounts of pCEP4/HIF-1αDN and the parental vector pCEP4 such that all cells received a total of 40 μg of vector. Transfection of pCEP4/HIF-1αDN had no greater effect on p2.1 expression in cells at 20% O_2 than did pCEP4 (Fig. 8*B*). However, transfection of pCEP4/HIF-1αDN resulted in a dose-dependent inhibition of hypoxia-induced expression. In cells transfected with 40 μg of pCEP4/HIF-1αDN, hypoxic induction was decreased by 82% relative to cells transfected with 40 μg of pCEP4. Thus, mutations either in cis (p2.3; see Fig. 6) or trans (pCEP4/HIF-1αDN) that affected HIF-1 binding to the *ENO1* HRE also affected transcriptional activation.

HIF-1 Binding Sites in the Hypoxia Response Element of the Lactate Dehydrogenase A Gene Promoter—We identified a HIF-1 binding site in the mouse *Ldha* promoter consisting of the sequence (on the antisense strand) 5′-GACGTGCG-3′ (22). A 52-bp sequence that encompassed this HIF-1 site was shown to function as an HRE and mutations within the HIF-1 site resulted in a complete loss of hypoxia-induced transcription (20). Mutations in sequences 5′ to the HIF-1 site also decreased hypoxia-induced transcription 4-fold (20). The mutations affected a second potential HIF-1 binding site (on the sense strand) 5′-CACGTGGG-3′. When oligonucleotide A1 spanning the 3′ site (Fig. 9*A*) was used as a probe for EMSA, a hypoxia-induced DNA binding activity was detected (Fig. 9*B, lane 2*). Binding of probe A1 to the hypoxia-induced factor was competed by excess unlabeled A1 or Ew but not by Em (*lanes 3–5*). The hypoxia-induced DNA binding activity was supershifted by antibodies against HIF-1α or HIF-1β but not by preimmune serum (*lanes 6–9*), indicating that HIF-1 can bind to the *Ldha* sequence 5′-GACGTGCG-3′.

Probe A2, spanning the 5′ putative HIF-1 site, also detected a hypoxia-induced factor as well as a novel DNA binding activity not previously detected by other probes (Fig. 9*C, lane 2*). Probe binding to the hypoxia-induced factor was competed by excess unlabeled A2 or Ew but not by Em (*lanes 3–5*) and was supershifted by HIF-1 antibodies but not by preimmune serum (*lanes 6–9*). These results indicate that there are two functionally-important HIF-1 binding sites in the *Ldha* HRE. To determine the relative binding affinity of HIF-1 for oligonucleotides A1 and A2, a competition assay was performed using probe Ew (Fig. 9*D*). Whereas A2 was a slightly more effective competitor for the constitutive factors, A1 was a more effective competitor for binding of HIF-1, as equivalent effects were seen with 0.5 ng of A1 and 4 ng of A2. Mutations involving site A1 resulted in complete loss of hypoxia-induced transcription whereas mutations involving site A2 decreased induction approximately 4-fold (20).

DISCUSSION

Previous studies have provided evidence for the induction of glycolytic enzyme gene expression via cis-acting DNA sequences containing putative HIF-1 binding sites (19, 20, 22). In this study we have utilized HIF-1 antibodies and expression vectors to perform definitive experiments which: (i) establish that the same cis-acting DNA sequences (HREs) are required for HIF-1 binding and transcriptional responses to hypoxia; and (ii) establish the effect of trans-acting factors (full-length and dominant negative forms of HIF-1α) on the transcriptional activation of reporters containing these HREs. The analyses of both cis-acting sequences and trans-acting factors, by both DNA binding and transcription assays, provide complementary evidence demonstrating the essential role of HIF-1 in the activation of *ALDA* and *ENO1* transcription in hypoxic cells.

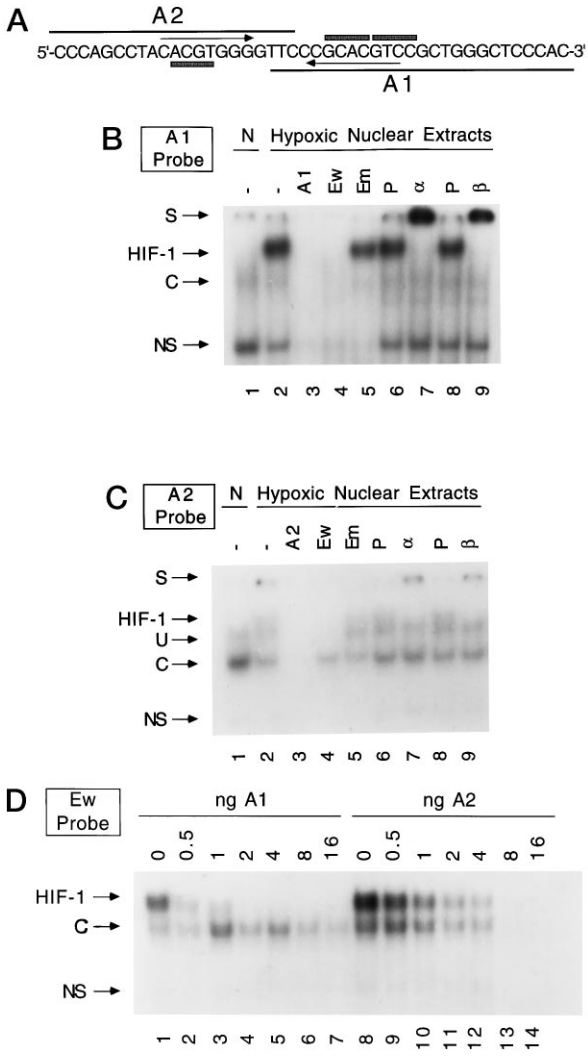

FIG. 9. **Binding of HIF-1 to lactate dehydrogenase A promoter sequences.** *A*, oligonucleotide sequences. The nucleotide sequence of the mouse/rat *Ldha* promoters from −93 to −50 relative to the transcription initiation site is shown with the sequence of the A1 and A2 oligonucleotides indicated by *solid line* and HIF-1 binding site indicated by the *arrow*. Sequences which when mutated resulted in a loss of hypoxia-induced transcription (20) are indicated by the *bars*. *B*, EMSA using A1 probe. *C*, EMSA using A2 probe. Aliquots (5 μg) of nuclear extracts prepared from Hep3B cells exposed to 20 O_2 (*lane 1*) or 1% O_2 for 4 h (*lanes 2–9*) were analyzed by EMSA. For competition assays (*lanes 3–5*), binding reactions included 50 ng of the indicated unlabeled oligonucleotide. For supershift assays (*lanes 6–9*), binding reactions contained antiserum raised against HIF-1α (*lane 7*) or HIF-1β (*lane 9*), or the respective preimmune serum (*lanes 6* and *8*). Probe complexes are indicated by *arrows* as described in legend to Fig. 7. *D*, analysis of relative binding affinities. Aliquots of hypoxic nuclear extract were incubated with probe Ew in the presence of the indicated amount (in ng) of unlabeled A1 (*lanes 1–7*) or A2 (*lanes 8–14*).

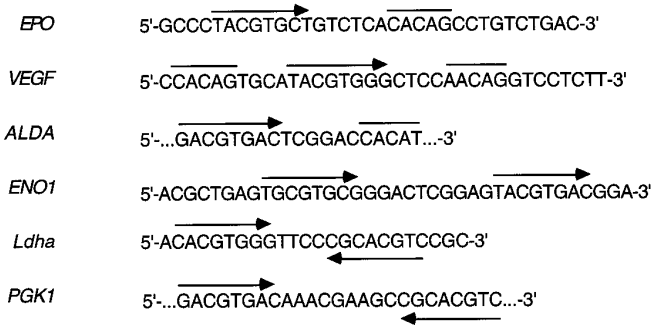

FIG. 10. **Compilation of functional HIF-1 binding sites.** HREs from the *EPO, VEGF, ALDA, ENO1, Ldha,* and *PGK1* genes are shown. *Arrow*, HIF-1 binding site. *Overline*, sequence with ≥4/5 match to the functionally-essential *EPO* sequence 5′-CACAG-3′. *Ellipses* indicate that the minimal functional HRE has not been determined.

Whereas HREs in the *ALDA* and *ENO1* promoters contain functionally-essential HIF-1 binding sites, other HIF-1 sites in the same genes make no contribution to the hypoxic response, indicating that the presence of a HIF-1 binding site is necessary but not sufficient for HRE function. Finally, we have shown that both the *ENO1* and *Ldha* HREs contain multiple HIF-1 sites that are required for maximal function. The 68-bp *ENO1* sequence, which contains three HIF-1 binding sites, is the most powerful HRE identified thus far, as it mediated greater than 50-fold increased transcription in cells exposed to hypoxia or rHIF-1.

Several mechanisms could discriminate between functional and non-functional HIF-1 sites. (i) Location of site: the functional HIF-1 sites in the *ENO1* promoter were located most proximal to the transcription start site. However, the *EPO* HRE is located in the 3′-flanking region more than 3 kilobases from the transcription start site (10, 14) and can function when placed 5′ or 3′ to a reporter gene, as is also the case for the *VEGF* HRE (16) which is located distal to a non-functional HIF-1 site (15). Thus, distance from the transcription initiation site is unlikely to determine whether a HIF-1 site is functional. (ii) Relative binding affinity: HIF-1 showed similar relative binding affinities for sites C and D in the *ENO1* promoter, as determined by an oligonucleotide competition EMSA, suggesting that differences in the affinity of HIF-1 for isolated binding-site sequences do not determine function, although the possibility of cooperative binding cannot be excluded (see below). (iii) Binding of constitutive factors: the basic-leucine-zipper transcription factors ATF-1 and CREB have recently been identified as components of the constitutive binding activity (33). Although a marked variability in the binding of constitutive factors to different oligonucleotides was noted, there was no correlation with HRE function. It will be necessary to identify wild-type or mutant HREs that are recognized by HIF-1 but not by constitutive factors in order to conclusively rule out an essential role for the latter in transcriptional responses to hypoxia. (iv) Bipartite structure of HREs: for both *EPO* and *VEGF*, mutation of sequences 3′ to the HIF-1 site resulted in loss of HRE function (10, 12, 14, 16). Comparison of the *EPO, VEGF,* and *ALDA* HREs (Fig. 10) reveals the presence of a DNA sequence located 4–6 nt 3′ to the HIF-1 site with similarity to the *EPO* sequence 5′-CACAG-3′ which when mutated results in loss of HRE function (14). In contrast, the *ENO1, Ldha,* and *PGK1* HREs contain two HIF-1 binding sites arranged as either direct or inverted repeats separated by 4–10 bp.

These data suggest that a functional HRE consists of a pair of contiguous transcription factor binding sites, at least one of which is bound by HIF-1. Including the novel HIF-1 sites identified in this study, the consensus HIF-1 binding site sequence can now be represented as 5′-RCGTG-3′. Factors that recognize the two sites may function cooperatively either at the level of DNA binding or transactivation. Response elements containing dual binding sites have been identified in many genes that are inducible by stimuli other than hypoxia (34–40). For several of these elements, cooperative DNA binding has been demonstrated (35, 40). HIF-1 has been shown to have a rapid dissociation rate from oligonucleotide probes containing an isolated HIF-1 binding site *in vitro*, with a half-time of less than 1 min (24). HIF-1 binding *in vivo* may therefore require stabilization via protein-protein interactions with another factor bound immediately downstream.

Taken together with previous studies (19, 20, 22), the data presented in this paper provide compelling evidence that HIF-1 activates transcription of the *ALDA, ENO1,* and *Ldha* genes in response to hypoxia. More indirect evidence also implicates HIF-1 in the regulation of *PFKL, PGK1,* and *PKM* (19, 22). There is thus a growing body of data which is consistent with the hypothesis that the coordinate induction of glycolytic enzyme activity which occurs in hypoxic cells (2) is mediated at the transcriptional level by HIF-1. The coordinate induction of multiple glycolytic enzymes in response to hypoxia appears inconsistent with theories of rate-limiting enzymatics. However, recent studies indicate that cardiac glucose metabolism is controlled by several enzymes depending upon cellular conditions (41) and suggest that complex metabolic patterns of regulation exist *in vivo* (42). Under chronic hypoxic conditions, coordinate induction of the glycolytic enzymes may increase flux through the pathway such that ATP generation is maximized under conditions that prevent the more efficient process of oxidative phosphorylation from being utilized. In support of this hypothesis, expression of the glucose transporter-1 gene (*GLUT-1*) is also induced by hypoxia and an HRE containing a putative HIF-1 binding site has been identified in the *GLUT-1* promoter (43). Our demonstration that HIF-1β is present in excess and that HIF-1α protein levels increase exponentially as cellular O_2 tension decreases (44), suggest that regulation of HIF-1α protein levels plays an important role in determining the rate of glycolytic gene transcription and other aspects of cellular O_2 homeostasis.

The ability of cells to maintain ATP production under conditions of limited O_2 availability is of obvious adaptive significance. The induction of glycolytic gene expression in response to hypoxia may be relevant to the phenomenon of myocardial hibernation, a local contractile defect observed in patients with chronic myocardial ischemia in which affected tissue is akinetic but viable (45). Since hibernating myocardium can recover following revascularization, hibernation may represent an adaptive response that prevents infarction by reducing myocardial O_2 requirements through decreased contractility and increased glycolysis. Isolated heart preparations subjected to ischemia showed metabolic recovery in the presence of glucose and pyruvate but not pyruvate alone, suggesting that glycolytic generation of ATP may be essential for recovery (46).

Whereas metabolic adaptation to hypoxia may promote survival in the context of coronary artery disease, the adaptation of tumors to hypoxic conditions correlates inversely with patient survival. The probability of developing metastatic disease was 2-fold higher for soft tissue sarcomas with $pO_2 < 10$ mm Hg (1.5% O_2) compared to tumors with $pO_2 > 10$ mm Hg (47). Tumor levels of lactate were signficantly higher in cervical carcinoma biopsies from patients who developed metastases compared to patients who remained free of metastatic disease (48). Given that tumor metastases are initially avascular and therefore poorly supplied with O_2 their survival may be dependent upon glycolytic generation of ATP. These observations suggest that pharmacological manipulation of the glycolytic pathway *in vivo* by targeting HIF-1 may provide a novel approach to the treatment of cancer and ischemic heart disease.

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Hypoxia Response Elements in the Aldolase A, Enolase 1, and Lactate Dehydrogenase A Gene Promoters Contain Essential Binding Sites for Hypoxia-inducible Factor 1

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