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A Nuclear Factor Induced by Hypoxia via De Novo Protein Synthesis Binds to the Human Erythropoietin Gene Enhancer at a Site Required for Transcriptional Activation

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We have identified a 50-nucleotide enhancer from the human erythropoietin gene 3′-flanking sequence which can mediate a sevenfold transcriptional induction in response to hypoxia when cloned 3′ to a simian virus 40 promoter-chloramphenicol acetyltransferase reporter gene and transiently expressed in Hep3B cells. Nucleotides (nt) 1 to 33 of this sequence mediate sevenfold induction of reporter gene expression when present in two tandem copies compared with threefold induction when present in a single copy, suggesting that nt 34 to 50 bind a factor which amplifies the induction signal. DNase I footprinting demonstrated binding of a constitutive nuclear factor to nt 26 to 48. Mutagenesis studies revealed that nt 4 to 12 and 19 to 23 are essential for induction, as substitutions at either site eliminated hypoxia-induced expression. Electrophoretic mobility shift assays identified a nuclear factor which bound to a probe spanning nt 1 to 18 but not to a probe containing a mutation which eliminated enhancer function. Factor binding was induced by hypoxia, and its induction was sensitive to cycloheximide treatment. We have thus defined a functionally tripartite, 50-nt hypoxia-inducible enhancer which binds several nuclear factors, one of which is induced by hypoxia via de novo protein synthesis.

Erythropoietin (EPO) is the glycoprotein hormone which regulates mammalian erythrocyte production and, as a result, tissue oxygen delivery. EPO RNA levels increase several hundredfold in rodent liver and kidney in response to hypoxia (8, 23, 24) or anemia (3, 4, 18). Human EPO RNA levels show similar increases in transgenic mouse liver and kidney (17, 25, 26, 28). Hypoxia also induces EPO RNA expression in Hep3B human hepatoma cells (10, 12), demonstrating that the same cell type can sense hypoxia and respond by increasing EPO RNA levels. The 50-fold increase in steady-state EPO RNA in hypoxic Hep3B cells requires new protein synthesis and is accounted for by an approximately 10-fold increase in the rate of transcription, with the remaining increase due to posttranscriptional mechanisms (10, 11). Nuclear extracts prepared from hypoxic Hep3B cells support a higher level of EPO gene transcription in vitro than extracts from nonhypoxic cells (5).

We have previously demonstrated that a 256-nucleotide (nt) EPO gene 3′-flanking sequence element functions as a hypoxia-inducible enhancer when cloned 3′ to a simian virus 40 (SV40) early-region promoter-chloramphenicol acetyltransferase (CAT) reporter gene and transiently expressed in Hep3B cells (27). The functional importance of this element was underscored by finding that even when the entire EPO gene 5′-flanking sequence was deleted, the gene remained fully hypoxia inducible in Hep3B transient expression assays (1). A homologous element was identified 3′ to the mouse EPO gene and shown to impart hypoxia-inducible expression to an α-globin gene cloned 5′ to the EPO gene on the same plasmid (22). The hypoxia-inducible enhancer functions in Hep3B cells, in which the endogenous EPO gene is inducible, but not in HeLa, Chinese hamster ovary, or mouse erythroleukemia cells (1, 22), suggesting that one or more steps in the signal transduction pathway from the oxygen sensor to EPO gene transcriptional induction is cell type specific.

We have now performed functional studies and binding assays to further characterize the hypoxia-inducible enhancer. These studies indicate that hypoxia-inducible EPO gene transcription is mediated by a 50-nt element that contains at least three different transcription factor binding sites. One of these sites binds a factor in Hep3B nuclear extracts which is induced by hypoxia.

MATERIALS AND METHODS

Plasmid constructs. The pSVcat reporter plasmid (pCAT-Promoter; Promega) contained SV40 early-region promoter (excluding the 72-bp repeat enhancer), bacterial CAT coding sequence, SV40 splice site, and polyadenylation signal. EPO gene sequences were cloned into the unique *Bam*HI site 3′ to the transcriptional unit of pSVcat. In initial polymerase chain reaction (PCR) experiments, pSVcatSVE, containing the SV40 enhancer cloned 3′ to the transcriptional unit (pCAT-Control; Promega), was used as a control for high-level constitutive CAT expression. pSVβGal (Promega), utilized as a control for monitoring transfection efficiency, included bacterial *lacZ* coding sequences driven by the SV40 early promoter and enhancer.

PCR products were generated by using oligonucleotide primers A to K, which all contained the sequence 5′-TATGGATCC-3′ followed by 26 nt of EPO gene sequence (the first nucleotide of which is indicated by an arrow in Fig. 1A). PCR was performed by using 100 ng of each primer and 10 ng of pEPO4, a plasmid containing the human EPO gene within a 4-kb *Hin*dIII-*Eco*RI fragment (26), as follows: 6 min at 94°C; 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C for 30 cycles; and 10 min at 72°C. PCR products were purified on Centricon 30 columns (Amicon), digested with *Bam*HI, ligated into *Bam*HI-digested pSVcat, and transformed into DH5α cells (Bethesda Research Laboratories [BRL]). Plas-

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mid DNA was purified from alkaline lysates by Qiagen column chromatography.

Mutant EPO gene sequences were prepared by two different methods. Mutants M1 to M6 were prepared within the context of the 50-nt PCR product EJ by introducing contiguous 3-nt substitutions into forward primer E and performing PCR and cloning as described above. Mutants M7 to M9 (which contain contiguous 5-nt substitutions) were prepared within the context of a double-stranded oligonucleotide consisting of the sequence bounded by primers E and I, flanked by GATC 5′ overhangs. Complementary oligonucleotides were annealed and cloned into *Bam*HI-digested pSVcat as described above. Plasmid DNA was isolated by subjecting alkaline lysates to double cesium chloride equilibrium-density gradient centrifugation (19) and then by dialysis against 10 mM Tris HCl (pH 8.0)–1 mM EDTA. The nucleotide sequence of each cloned insert was determined by the dideoxy technique by using the Sequenase system (U.S. Biochemical).

Transient expression assays. Hep3B cells were grown in minimal essential medium with Earle's salts, supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 1 mM nonessential amino acids, 50 mU of penicillin per ml, and 50 μg of streptomycin per ml (complete medium; all components were obtained from GIBCO). The initial series of cloned PCR products (Fig. 1) was analyzed by transfection of pSVcat plasmids into Hep3B cells by using Lipofectin reagent (BRL) as previously described (27). All subsequent experiments were performed by electroporation (1). A total of 4.3×10^6 Hep3B cells in OptiMEM I (BRL GIBCO) was mixed with 450 μg of sonicated salmon sperm DNA (Sigma), 4 μg of pSVβGal (Promega), and 40 μg of a recombinant pSVcat expression plasmid in a total volume of 0.8 ml. Electroporation was performed by using a Gene-Pulser apparatus (Bio-Rad) at 260 V and 960 μF, which generated a time constant of 15.0 ± 0.5 ms. Duplicate electroporations were pooled and then split equally among two 10-cm tissue-culture dishes (Corning) containing 10 ml of complete medium. Cells were allowed to recover for 1 to 2 h in a 5% CO_2 and 37°C incubator. One set of duplicate plates was then removed to a modular incubator chamber (Billups-Rothenberg) which was flushed with a gas mixture consisting of 1% O_2, 5% CO_2, and 94% N_2 (Potomac Air Gas Inc.), sealed, and placed at 37°C. The other set of plates remained in the 5% CO_2–95% air incubator.

Cells were harvested 40 to 48 h after electroporation and resuspended in 0.25 M Tris HCl (pH 8.0), and extracts were prepared by four freeze-thaw cycles. Protein concentrations were determined by the Bradford assay (Bio-Rad) with a bovine serum albumin standard curve. CAT protein was quantitated by enzyme-linked immunosorbent assay (5 Prime → 3 Prime, Inc.) with standard curves generated with purified CAT. At least two different extract concentrations were assayed to control for linearity, and the results were corrected for values obtained by using extracts from untransfected control cells. β-Galactosidase activity was assayed by the hydrolysis of the substrate *o*-nitrophenyl-β-D-galactopyranoside (Promega) by 50 μg of extract at 37°C for 1 h, and the A_{420} was measured spectrophotometrically. The relative CAT activity was calculated as CAT (picograms of CAT per milligram of protein) divided by βGal (A_{420} per milligram of protein per hour). These values were then normalized to the expression in 20% O_2 of either pSVcatSVE or pSVcatEJ. Mean data on all plasmids were derived from at least three independent transfections. Induction values were calculated by dividing the mean CAT/βGal value for extracts of cells cultured in 1% O_2 by the value for extracts of cells cultured in 20% O_2 (1% O_2/20% O_2).

Preparation of nuclear extracts. Fresh complete medium was added to confluent Hep3B cells in 15-cm dishes, which were then incubated in 1% or 20% O_2 for 2 to 16 h. To harvest, cells were washed twice with cold Dulbecco's phosphate-buffered saline (PBS), scraped into 5 ml of PBS, and pelleted by centrifugation at 1,500 rpm for 5 min at 4°C in an IEC Centra-7R centrifuge (International). Nuclear extracts were prepared by modification of a standard protocol (7), with buffers A and C containing 0.5 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride, 2 μg of leupeptin per ml, 2 μg of pepstatin per ml, 2 μg of aprotinin per ml, and 1 mM sodium vanadate (all obtained from Sigma). The cell pellet was washed with 4 packed-cell volumes (PCV) of buffer A (10 mM Tris-HCl [pH 7.8], 1.5 mM $MgCl_2$, 10 mM KCl), resuspended in 4 PCV of buffer A, and incubated on ice for 10 min. The cell suspension was Dounce homogenized with a type-B pestle, and the nuclei were pelleted by centrifugation at 3,000 rpm for 5 min, resuspended in 3 PCV of buffer C (0.42 M KCl, 20 mM Tris-HCl [pH 7.8], 1.5 mM $MgCl_2$, 20% glycerol), and mixed on a rotator at 4°C for 30 min. Nuclear debris was pelleted by centrifugation for 30 min at 13,500 rpm. The supernatant was dialyzed against one change of buffer D (20 mM Tris-HCl [pH 7.8], 0.1 M KCl, 0.2 mM EDTA, 20% glycerol) for a total of 2 to 4 h at 4°C. The dialysate was centrifuged for 10 min at 13,500 rpm, and aliquots were frozen in liquid N_2 and stored at −80°C. Protein concentration was determined by a Bio-Rad assay with bovine serum albumin standards.

Electrophoretic mobility shift assays (EMSA). Oligonucleotide probes were generated by 5′ end labelling of the sense strand with [γ-^{32}P]ATP (NEN-Dupont) and T4 polynucleotide kinase (BRL), annealing to a 10-fold excess of antisense strand, and purification by 10% polyacrylamide gel electrophoresis. Probe was eluted from the excised gel in 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA. Binding reactions were carried out in a total volume of 20 μl containing 5 μg of nuclear extract and 0.1 μg of denatured calf thymus DNA (Sigma) in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, and 5% glycerol. After preincubation for 5 min at room temperature, probe (10^4 cpm; 0.1 to 0.2 ng) was added and the incubation was continued for an additional 15 min, after which the reaction mixtures were loaded onto 5% nondenaturing polyacrylamide gels. Electrophoresis was performed at 185 V in 0.3× TBE (1× TBE is 89 mM Tris-HCl, 89 mM boric acid, and 5 mM EDTA) at 4°C. Gels were vacuum dried and autoradiographed with intensifying screens at −80°C for 1 to 5 days. Competitor DNAs were preincubated with nuclear extract and calf thymus DNA for 5 min prior to addition of labelled probe. The USF probe (sense strand sequence: 5′-GGTAGGCCACGTGACCGGGTA-3′) was provided by C. V. Dang (The Johns Hopkins University, Baltimore, Md.).

DNase I footprint analysis. A 256-nt probe was prepared by PCR by using 5′-end-labelled forward primer A and reverse primer K (Fig. 1A) for PCR as previously described (27). Probe (10^4 cpm) was incubated with 45 μg of Hep3B nuclear extract and 1 μg of poly(dI-dC) in binding buffer consisting of 10 mM Tris HCl (pH 7.8), 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 5 mM $MgCl_2$, and 10% glycerol for 15 min on ice and then for 2 min at 25°C. A total of 0.5 μg of DNase I (Worthington) was added for 1 min at 25°C, and the reactions were terminated by the addition of an equal volume of stop

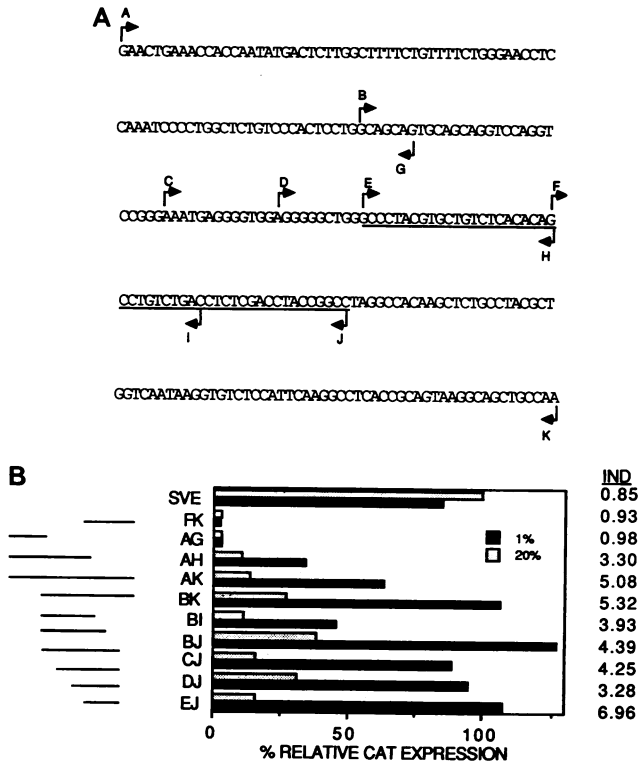

FIG. 1. Delineation of the hypoxia-inducible enhancer by functional analysis. (A) Generation of subregions by PCR. The coding-strand sequence of the 256-nt EPO gene 3'-flanking element previously demonstrated to function as a hypoxia-inducible enhancer (27) is shown. Arrows indicate the first EPO gene nucleotide included within each primer (forward primers above sequence, reverse primers below). The EPO gene polyadenylation site is at the adenine residue located 14 nt downstream of primer A (14). The 50-nt sequence EJ (underlined) is the minimal hypoxia-inducible enhancer. (B) Transient expression assays of PCR products cloned 3' to SV40 promoter-CAT reporter gene. Ten different PCR products and the SV40 enhancer (SVE) were cloned into pSVcat in the following transcriptional orientation: +, AH, AK, BK, BI, BJ, FK, and SVE; −, AG, CJ, DJ, and EJ. The relative position of each PCR product within the 256-nt sequence is indicated at left. Each plasmid was cotransfected with pSVβGal into Hep3B cells cultured in 1% versus 20% O_2. % RELATIVE CAT EXPRESSION, mean CAT/βGal ratios (normalized to results for pSVcatSVE in 20% O_2) based on three to eight independent transfection experiments. IND, inducibility (1% O_2/20% O_2).

solution (0.6 M sodium acetate [pH 7.0], 50 mM EDTA, 0.1 μg of yeast tRNA per ml). DNA was phenol-chloroform extracted, ethanol precipitated, resuspended in formamide, and analyzed by 8 M urea–8% acrylamide gel electrophoresis in 1× TBE.

RESULTS

Identification of the minimal hypoxia-inducible enhancer element. In order to determine the minimum DNA sequence able to function as a hypoxia-inducible enhancer, subregions of the 256-nt sequence were amplified by PCR and cloned 3' to an SV40 promoter-CAT reporter gene (pSVcat). By using the primers whose 5' termini are shown in Fig. 1A, 10 different regions were amplified by PCR and cloned into pSVcat. Each of these recombinant plasmids was cotransfected into Hep3B cells with pSVβGal, a plasmid in which the transcription of bacterial β-galactosidase coding sequences is driven by the SV40 early promoter and enhancer. Duplicate plates of transfected cells were cultured for 2 days in a 95% air–5% CO_2 incubator (20% O_2) or in a sealed chamber containing 1% O_2, 5% CO_2, and 94% N_2 (1% O_2). Whole-cell protein extracts were prepared from each plate, and the CAT/βGal ratio was determined as described in Materials and Methods. For each plasmid, three to eight pairs of independent transfections were performed, and CAT/βGal ratios from each experiment were normalized to the expression, in 20% O_2, of pSVcatSVE, which contains the SV40 enhancer cloned 3' to the transcription unit.

As shown in Fig. 1B, pSVcatSVE showed constitutive, high-level expression in 1% and 20% O_2, with a mean induction of 0.85. AK represents the full 256-nt sequence previously demonstrated to function as a hypoxia-inducible enhancer (27). CAT expression from pSVcatAK was induced fivefold by hypoxia. Of the other nine PCR products analyzed for enhancer activity in pSVcat, seven plasmids generated hypoxia-inducible CAT expression. The 50-nt sequence EJ enhanced CAT expression by sevenfold in hypoxic Hep34B cells compared with in nonhypoxic Hep3B cells, and this region is contained within the larger amplimers AK, BK, BJ, CJ, and DJ. The 5' end of the enhancer lies between E and F, since expression of FK is similar to that of pSVcat with no insert (30). At the 3' end, the data were less clear, since CAT expression was induced three- to fourfold from plasmids containing AH and BI compared with the fivefold induction mediated by AK and BK. There was no correlation between the transcriptional orientation of the PCR product and the level of CAT expression (Fig. 1B legend), as previously demonstrated for the 256-nt sequence (27). These experiments indicate that the 50-nt sequence EJ is the smallest fragment tested that is fully functional as a hypoxia-inducible enhancer.

Functional dissection of the 50-nt hypoxia-inducible enhancer. In order to functionally dissect the 50-nt enhancer, a series of scanning-mutagenesis experiments were performed. At the 5' end of the enhancer, six contiguous 3-nt substitutions were introduced into the sequence by synthesizing mutant versions of forward primer E for PCR in combination with reverse primer J. The six mutant PCR products (M1 to M6) were cloned 3' to the transcriptional unit of pSVcat, and their expression was compared with that of wild-type EJ (Fig. 2A). In these experiments, the CAT/βGal ratios were normalized to the results of EJ in 20% O_2. Mutants M2, M3, and M4 were uninducible by hypoxia. M5 and M6 were inducible to the same degree as EJ, and M1 functioned as a somewhat stronger enhancer in 1% O_2 than EJ. Again, there was no correlation between transcriptional orientation and level of expression (Fig. 2A legend). These studies define a 9-nt sequence (nt 4 to 12) whose alteration (mutants M2 to M4) eliminates hypoxia-inducible enhancer activity, suggesting that nt 4 to 12 encompass a binding site for a factor involved in the transcriptional induction of the EPO gene. We therefore designate this site hypoxia-inducible enhancer binding site 1 (HIE-BS1).

Analysis of the 3' end of the 50-nt enhancer was performed by first determining the effect of deleting nt 34 to 50. The resulting 33-nt sequence (bounded by primers E and I in Fig. 1A) was synthesized as a double-stranded oligonucleotide and cloned into pSVcat. As shown in Fig. 2B, expression was decreased in both nonhypoxic and hypoxic cells, and inducibility was reduced by more than one-half when nt 34 to 50 were deleted, as EI was induced threefold compared with sevenfold for EJ. We had previously analyzed nt 1 to 18 in

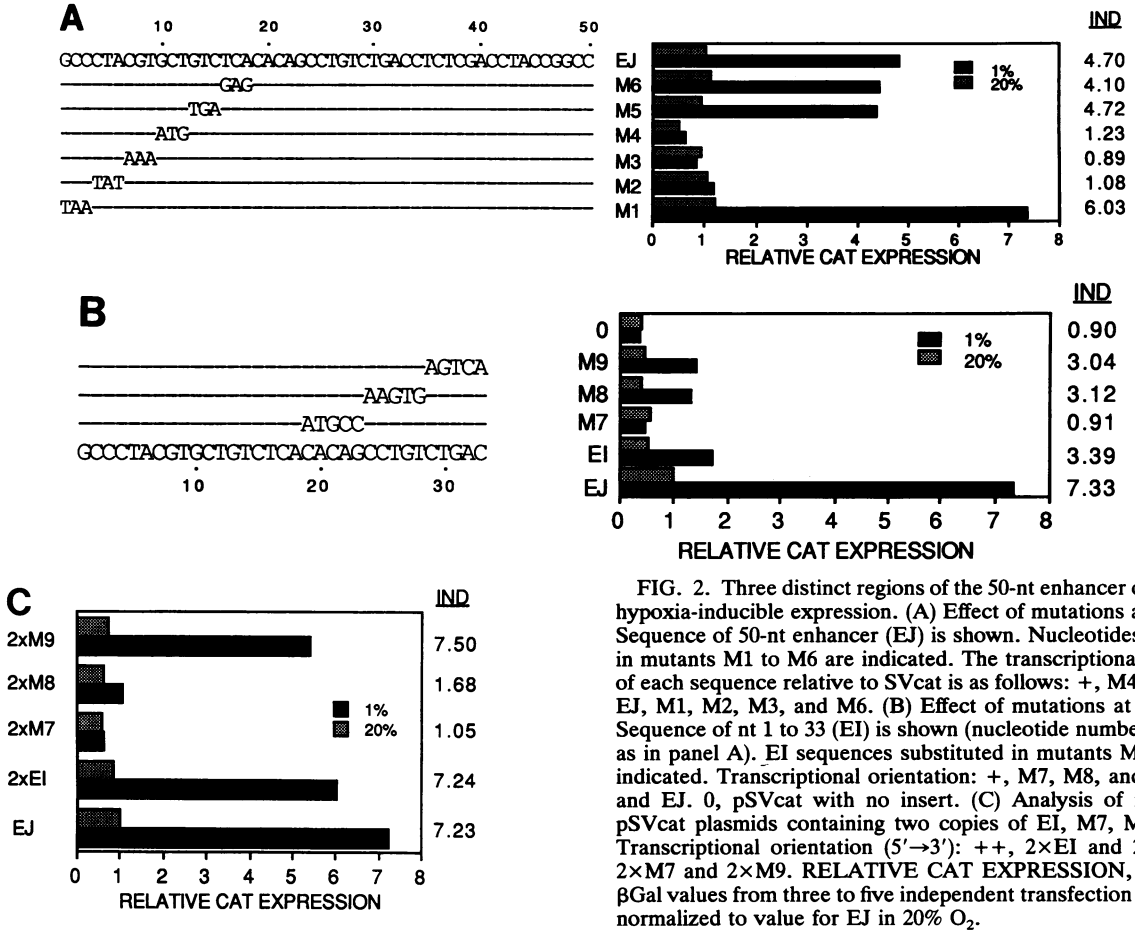

FIG. 2. Three distinct regions of the 50-nt enhancer contribute to hypoxia-inducible expression. (A) Effect of mutations at nt 1 to 18. Sequence of 50-nt enhancer (EJ) is shown. Nucleotides substituted in mutants M1 to M6 are indicated. The transcriptional orientation of each sequence relative to SVcat is as follows: +, M4 and M5; −, EJ, M1, M2, M3, and M6. (B) Effect of mutations at nt 19 to 33. Sequence of nt 1 to 33 (EI) is shown (nucleotide numbering is same as in panel A). EI sequences substituted in mutants M7 to M9 are indicated. Transcriptional orientation: +, M7, M8, and M9; −, EI and EJ. 0, pSVcat with no insert. (C) Analysis of recombinant pSVcat plasmids containing two copies of EI, M7, M8, and M9. Transcriptional orientation (5′→3′): ++, 2×EI and 2×M8; −+, 2×M7 and 2×M9. RELATIVE CAT EXPRESSION, mean CAT/βGal values from three to five independent transfection experiments normalized to value for EJ in 20% O_2.

the context of EJ. To analyze the remaining 15 nt at the 3′ end of EI, double-stranded 33-nt oligonucleotides containing contiguous 5-nt substitutions (M7 to M9 [Fig. 2B]) were synthesized and cloned into pSVcat. M8 and M9 were as inducible as EI, while M7 showed no induction by hypoxia.

Because the induction of EI was modest, we introduced two copies each of EI, M7, M8, and M9 into pSVcat (Fig. 2C). 2×EI was as inducible as EJ, suggesting that nt 34 to 50 of the enhancer were involved in binding a factor which amplifies the induction signal. 2×M9 functioned as well as 2×EI, and 2×M7 showed no induction by hypoxia, results consistent with those obtained with one copy of these oligonucleotides. The results for 2×M8 differed from the previous study, as this construct was poorly inducible. For all the plasmids, nucleotide sequence analysis and transfection studies were performed by using DNA from the same preparation, and no unexpected sequence alterations were detected. Other trivial explanations for the results were also ruled out, as identical results were obtained in multiple experiments over several weeks. Orientation also does not appear to be a factor, as the orientation of 2×M8 was identical to that of 2×EI (Fig. 2C legend). It is possible that the junctional sequence created in 2×M8 may have serendipitously generated a binding site for a transcriptional repressor. From the results shown in Fig. 2 we conclude that nt 19 to 23 (mutated in M7) are involved in the binding of a factor necessary for transcriptional induction by hypoxia. On the basis of the conflicting results obtained with M8, we cannot rule out the possibility that the binding site (HIE-BS2) may extend into nt 24 to 28.

A nuclear factor induced by hypoxia binds to the enhancer at binding site 1. Mutations at nt 4 to 12 eliminated hypoxia-induced enhancer activity, implicating this sequence as the binding site for an induced transcription factor. To confirm this hypothesis, nuclear extracts were prepared from Hep3B cells cultured in 20% versus 1% O_2 and an EMSA was performed by using a double-stranded oligonucleotide probe encompassing nt 1 to 18 (W18 [Fig. 3A]). As shown in Fig. 3B (lanes 1 and 2), EMSA revealed a binding-complex doublet (C) which was present when either induced or uninduced extracts were assayed and therefore is due to the binding of constitutively expressed factor(s). In addition, a DNA-protein complex of reduced mobility (I) was detected specifically when nuclear extracts from hypoxic Hep3B cells were assayed. As a control, an oligonucleotide containing the binding site for the ubiquitous factor USF (13) was used as a probe (lanes 5 and 6). No difference in factor binding from hypoxic versus nonhypoxic extracts was detected with the USF probe.

To demonstrate the specificity of the factors binding to W18, we synthesized a probe mutated at nt 7 to 9 (M18 [Fig. 3A]). This is the same 3-nt mutation which, in the context of the 50-nt element (M3 [Fig. 2A]), eliminated hypoxia-inducible enhancer function. When Hep3B nuclear extracts were assayed with probe M18, neither constitutive nor induced binding activities were detected (Fig. 3B, lanes 3 and 4).

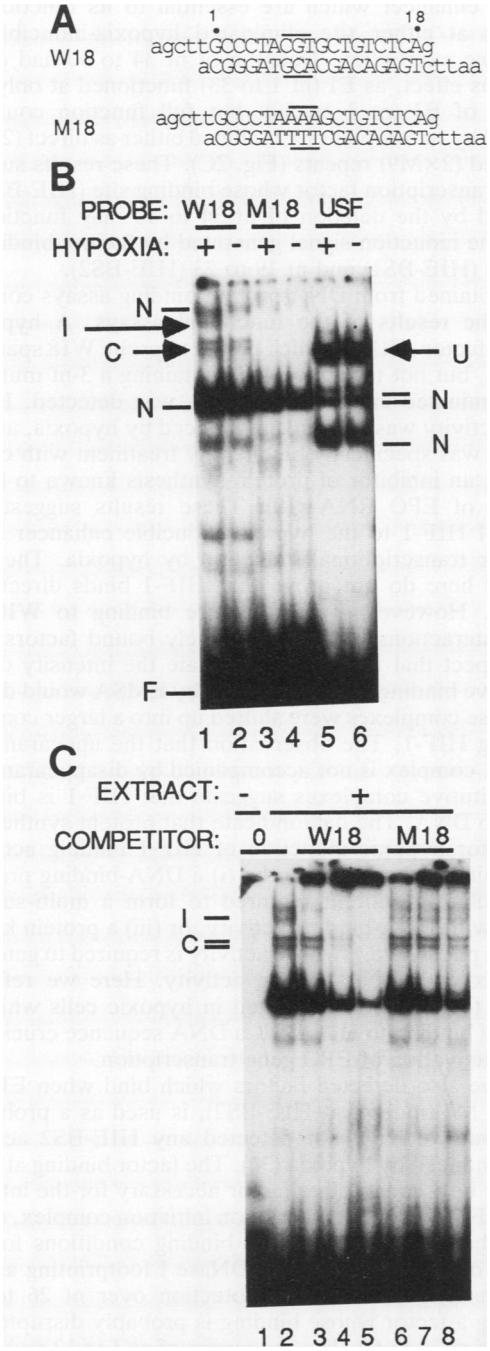

FIG. 3. Identification of a DNA-binding activity induced by hypoxia. (A) Nucleotide sequence of wild-type (W18) and mutant (M18) double-stranded oligonucleotides. Nt 1 to 18 from the hypoxia-inducible enhancer are shown in uppercase letters, with the 3-nt site of mutation in M18 overlined and underlined. (B) An induced nuclear factor binds to wild-type, but not mutant, nucleotide 1 to 18 probe. Nuclear extracts from Hep3B cells, cultured for 4 h in 1% (+) versus 20% (−) O_2, were incubated with W18 probe, M18 probe, or a probe containing a USF binding site (USF) and analyzed by EMSA. Binding activities are labelled as follows: N, nonspecific; C, constitutive; I, induced; U, USF; F, free probe. (C) Competition experiments demonstrate specificity of binding. W18 probe (lanes 1 to 8) was incubated with nuclear extract from Hep3B cells cultured for 4 h in 1% O_2 (lanes 2 to 8) in the presence of no competitor (0) or 20-, 100-, or 500-fold molar excess of unlabelled W18 (lanes 3 to 5) and M18 (lanes 6 to 8) competitor oligonucleotides and analyzed by EMSA.

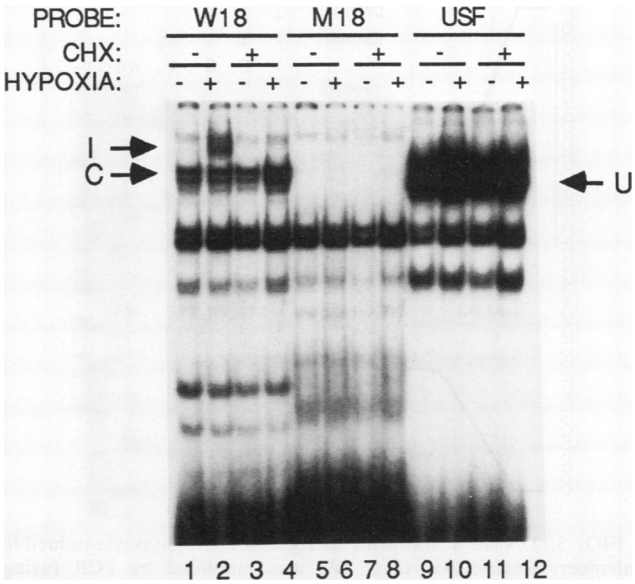

FIG. 4. Cycloheximide inhibits formation of induced binding complex. Hep3B cells were preincubated for 2 h in the presence (+) or absence (−) of 100 μM cycloheximide (CHX) and then placed in 1% (+) or 20% (−) O_2 for 4 h prior to nuclear extract preparation. Extracts were mixed with wild-type (W18; lanes 1 to 4), mutant (M18; lanes 5 to 8), and USF probes (lanes 9 to 12) and analyzed by EMSA. I, cycloheximide-sensitive binding activity induced by hypoxia; C, constitutive binding activities; U, USF.

Competition experiments (Fig. 3C) revealed that the induced binding activity, detected when the W18 probe was incubated with nuclear extract from hypoxic Hep3B cells (lane 2), was inhibited by a 20-fold molar excess of unlabelled W18 (lane 3), while a 500-fold excess of M18 did not compete for factor binding to the W18 probe (lane 8). The constitutive bands were inhibited by a 500-fold excess of W18, but a 500-fold excess of M18 again did not compete for factor binding to the W18 probe. These results identify constitutive and induced binding activities whose specificities are consistent with the results of functional assays.

A nuclear factor whose binding is induced by hypoxia via de novo protein synthesis. Hep3B cells were cultured in the presence or absence of 100 μM cycloheximide for 2 h in 20% O_2 and then cultured for an additional 4 h in 1% versus 20% O_2. As shown in Fig. 4, hypoxia-induced binding activity was eliminated by the presence of cycloheximide (compare lane 2 with lane 4). The constitutive binding activities were unaffected by cycloheximide treatment, as was binding of USF to its probe. These results indicate that a nuclear factor, designated hypoxia-inducible factor 1 (HIF-1), binds to sequences necessary for transcriptional activation of the EPO gene in response to hypoxia and that HIF-1 binding requires de novo protein synthesis. HIF-1 activity was present in nuclear extracts prepared from Hep3B cells cultured in 1% O_2 for 2, 4, and 16 h (30).

A third nuclear factor binding site is detected by DNase I footprint analysis. The 256-nt sequence AK (Fig. 1A) was amplified by PCR by using a 5′-end-labelled forward primer (27) and used to probe Hep3B nuclear extracts for binding factors which provide protection from DNase I digestion. Strong protection was seen over a region of the probe corresponding to nt 26 to 48 of the 50-nt element (Fig. 5, lane 2). This footprint was present when either hypoxic (not

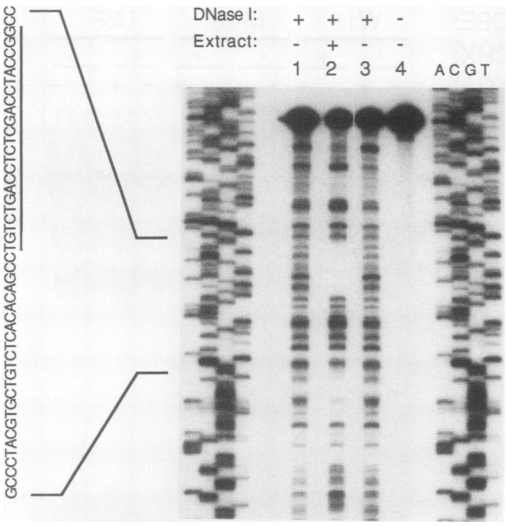

FIG. 5. DNase I footprint analysis of the hypoxia-inducible enhancer. Region AK (Fig. 1A) was amplified by PCR (using 5′-end-labelled forward primer A), incubated with 0 (lanes 1, 3, and 4) or 45 μg (lane 2) of nuclear extract from nonhypoxic Hep3B cells in the presence of 1 μg of poly(dI-dC), digested with 0 (lane 4), 0.05 (lanes 1 and 3), or 0.5 (lane 2) μg of DNase I for 1 min at 25°C, and analyzed by 8 M urea–8% polyacrylamide gel electrophoresis (only the relevant region of the 256-nt sequence is shown). Dideoxy nucleotide sequence analysis of cloned human EPO gene was also performed (ACGT) by using 5′-end-labelled primer A. Sequence of 50-nt enhancer, with footprinted region underlined, is shown at left.

shown) or nonhypoxic extracts were assayed and was specifically inhibited by a 500-fold molar excess of a double-stranded oligonucleotide spanning nt 23 to 50 but not by a 500-fold excess of an oligonucleotide sequence from outside this region (30). Binding of a factor present in both nonhypoxic and hypoxic Hep3B nuclear extracts to a nt 23 to 50 probe was also demonstrated by EMSA (30). These results are consistent with the functional data suggesting that the deletion of nt 34 to 50 removed the binding site for a constitutive factor which serves to amplify the induction signal. On the basis of the footprinting data, we designate nt 26 to 48 as HIE-BS3. No footprints were detected over HIE-BS1 or HIE-BS2. The 256-nt probe did detect other constitutive footprints, but none of these were within the 50-nt sequence and their functional significance remains to be determined.

DISCUSSION

The hypoxia-inducible enhancer is a 50-nt functionally tripartite element. We previously demonstrated that 256 nt of human EPO gene 3′-flanking sequence functioned as a hypoxia-inducible enhancer in Hep3B cells (27). In this study, we show that within this region a sequence of 50 nt, which begins 116 nt 3′ to the EPO gene polyadenylation site (Fig. 1A legend), is fully functional as a hypoxia-inducible enhancer. Compared with the expression of a reporter gene with no enhancer present, the hypoxia-inducible enhancer enhances expression 2.4-fold in Hep3B cells cultured in 20% O_2 and 20-fold in cells cultured in 1% O_2 (Fig. 2B, EJ versus 0). The eightfold transcriptional induction of CAT expression is of the same magnitude as that of the endogenous EPO gene in Hep3B cells (11). Functional studies identified two regions (nt 4 to 12 and 19 to 23) within the 50-nt hypoxia-inducible enhancer which are essential to its function, as mutations at either site eliminated hypoxia-inducible reporter gene expression. Deletion of nt 34 to 50 had a less deleterious effect, as EI (nt 1 to 33) functioned at only half the level of EJ (nt 1 to 50), but full function could be generated by two copies of EI, cloned either as direct (2×EI) or inverted (2×M9) repeats (Fig. 2C). These results suggest that the transcription factor whose binding site (HIE-BS3) is eliminated by the deletion of nt 34 to 50 may function to amplify the induction signal generated by factors binding at nt 4 to 12 (HIE-BS1) and nt 19 to 23 (HIE-BS2).

Data obtained from DNA-protein binding assays complemented the results of the functional assays. A hypoxia-inducible factor (HIF-1) which bound to probe W18 spanning nt 1 to 18, but not to probe M18 containing a 3-nt mutation which eliminated enhancer function, was detected. HIF-1 binding activity was specifically induced by hypoxia, and its induction was specifically blocked by treatment with cycloheximide, an inhibitor of protein synthesis known to block induction of EPO RNA (10). These results suggest that binding of HIF-1 to the hypoxia-inducible enhancer is required for transcriptional induction by hypoxia. The data presented here do not prove that HIF-1 binds directly to HIE-BS1. However, if HIF-1 were binding to W18 via protein interactions with constitutively bound factors, one would expect that in the induced state the intensity of the constitutive binding complexes seen by EMSA would diminish as those complexes were shifted up into a larger complex containing HIF-1. The observation that the appearance of the HIF-1 complex is not accompanied by disappearance of the constitutive complexes suggests that HIF-1 is binding directly to DNA. The data indicate that protein synthesis is required for hypoxic induction of HIF-1 binding activity. The protein synthesized may be (i) a DNA-binding protein, (ii) a regulatory subunit required to form a multi-subunit complex with DNA-binding activity, or (iii) a protein kinase or protein phosphatase whose activity is required to generate a complex with DNA-binding activity. Here we refer to HIF-1 as the complex generated in hypoxic cells which is capable of binding to HIE-BS1, a DNA sequence crucial for hypoxic activation of EPO gene transcription.

We have also detected factors which bind when EI, but not when M7 (mutant at HIE-BS2), is used as a probe for EMSA, but we have not detected any HIE-BS2 activity which is induced by hypoxia (30). The factor binding at nt 19 to 23 may be a constitutive factor necessary for the interaction of HIF-1 with the transcription initiation complex, or we may not have used appropriate binding conditions for the detection of an induced factor. DNase I footprinting assays revealed strong constitutive protection over nt 26 to 48, identifying a factor whose binding is probably disrupted by deletion of nt 34 to 50. The sequences of nt 4 to 12 and 19 to 23 were not identifiable as binding sites for known transcription factors. The nt 30 to 43 sequence on the antisense strand, 5′-AGGTCGAGAGGTCA-3′, contains perfect and imperfect direct repeats of 5′-AGGTCA-3′ (underlined). Direct repeats of this sequence separated by 1 to 5 nt have been shown to function as selective response elements for members of the thyroid-steroid hormone receptor superfamily, with binding specificity determined by the number of nucleotides separating the direct repeats (21, 29). A member of this transcription factor family may bind at HIE-BS3.

The results of DNA-protein binding assays in the present study with nuclear extracts from hypoxic versus nonhypoxic Hep3B cells are not consistent with previous results obtained by using nuclear extracts from liver and kidney of

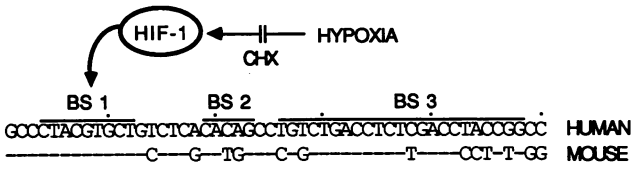

FIG. 6. Tripartite structure of the hypoxia-inducible enhancer and binding of HIF-1 to HIE-BS1. The nucleotide sequence of the 50-nt hypoxia-inducible enhancer from the human EPO gene is shown. Overscored are the three putative transcription factor binding sites identified by functional and binding assays. Below the human sequence is the homologous region of the mouse EPO gene enhancer (22), with conserved residues indicated by dashes. The hypoxia-induced, cycloheximide-sensitive binding of HIF-1 to HIE-BS1 is also depicted.

anemic versus nonanemic mice, which identified the binding of nuclear factors, whose activity appeared to be increased in anemic extracts, to sequences outside of the 50-nt enhancer (27). These differences may relate to one or more of the following considerations: (i) the whole-organ nuclear extracts contained factors from many different mouse cell types, including non-EPO-producing as well as EPO-producing cells; (ii) the mice were made anemic by phenylhydrazine treatment rather than by being subjected to hypoxia; (iii) potential activity differences between treated and untreated whole-organ extracts were not controlled by use of a probe for a ubiquitous factor like USF, as was done in the present study; and (iv) the binding factors which were detected by using whole-organ extracts may be important for in vivo expression but not for induction by hypoxia per se. Our working hypothesis is that sequences surrounding the 50-nt hypoxia-inducible enhancer play a role in EPO gene expression in liver and/or kidney in vivo. However, within Hep3B cells the hypoxia-inducible enhancer is necessary and sufficient for hypoxia-mediated transcriptional induction.

Implications for models of hypoxia-induced EPO gene transcription. Hep3B cells and EPO-producing cells in vivo are able to sense hypoxia and respond by increasing the rate of EPO gene transcription. In vivo, maximal steady-state levels of EPO RNA are attained after 2 to 4 h of hypoxia (23, 24). Hep3B nuclear run-off experiments indicate that the maximal rate of transcription is also reached at 2 to 4 h of hypoxic exposure (11). The induction of EPO RNA by hypoxia in Hep3B cells can be blocked by cycloheximide treatment (10). These results suggest that during the first 2 to 4 h of hypoxic exposure, one or more proteins required for EPO gene activation are synthesized de novo. One of these proteins appears to generate HIF-1 activity. Binding of HIF-1 to HIE-BS1 is only detected when hypoxic extracts are assayed and its induction is blocked by cycloheximide. The 9-nt sequence of HIE-BS1 is 100% conserved in the mouse EPO gene enhancer compared with a sequence conservation of 74% over the entire 50 nt (Fig. 6). If binding of HIF-1 to HIE-BS1 were indeed the crucial induced event, then the evolutionary conservation of this binding site would be necessary for the preservation of hypoxia inducibility.

The transcription of other human genes has been shown to be hypoxia inducible (15, 16), but the *cis*-acting sequences and *trans*-acting factors involved are unidentified. In other cases, the effect of hypoxia appears to be indirect. The primary cause of heat shock transcription factor activation in hypoxic myogenic cells is ATP depletion (2). The best-studied example of hypoxia-inducible gene expression is the cascade by which nitrogen fixation genes of *Rhizobium meliloti* are activated (6). In this system, the oxygen sensor is FixL, a membrane protein which binds oxygen via a heme moiety and transduces the hypoxic signal via a kinase domain (9). Under hypoxic conditions, FixL phosphorylates the transcriptional activator FixJ (20). FixJ activates transcription of *nifA*, an activator of the *nifHDK* and *fixABCX* genes (6). A hemoprotein may be involved in hypoxic induction of the EPO gene, as the induction is inhibited by carbon monoxide and desferrioxamine (10). HIF-1 may occupy a position in the EPO cascade analogous to the role of *nifA* in *Rhizobium* spp. and may be responsible for the hypoxic activation of multiple genes. Isolation of cDNA encoding HIF-1 will allow further exploration of its role in the regulation of human gene expression by hypoxia.

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