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Hypoxic Induction of the Human Erythropoietin Gene: Cooperation between the Promoter and Enhancer, Each of Which Contains Steroid Receptor Response Elements

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Transcription of the human erythropoietin (Epo) gene is stimulated by exposure to hypoxia and/or cobalt in whole animals and in Hep3B cells. We have systematically investigated the promoter and 3′ enhancer elements necessary for this induction by transient transfection of Hep3B cells. We define a promoter region of 53 bp and an enhancer region of 43 bp that confer hypoxia and cobalt inducibility. Each element gives rise to a 6- to 10-fold induction alone. In combination they produce a 50-fold induction after stimulation, similar to the 50- to 100-fold induction of the endogenous Epo gene. Two areas of DNA sequence homology are present in these regions. We demonstrate specific DNA-protein interactions in the enhancer and the ability of the promoter element to compete with these interactions in electrophoretic mobility shift assays. DNase I footprinting and methylation interference data further refine the *cis*-acting element in the 43-bp enhancer to a short region containing a direct repeat of a steroid/thyroid hormone receptor response element half-site separated by a 2-bp gap. Two half-site consensus sequences are also present in the 53-bp promoter. Site-specific mutation of the half-site sequences in the enhancer destroys the functional activity of the enhancer.

Since oxygen plays such a dominant role in the metabolism and viability of cells, it is not surprising that hypoxia can result in adaptationally appropriate alterations in gene expression. For example, when bacteria such as *Vitreoscilla* spp. (30) and *Rhizobium meliloti* (18) are exposed to low oxygen tension, there is a marked increase in the expression of heme protein that serves as a storage depot for oxygen. Such induction of hemoglobin synthesis extends to water fleas of the genus *Daphnia*, which turn red in anaerobic water and revert to their colorless state upon aeration (17). In the yeast *Saccharomyces cerevisiae*, varying oxygen tension affects the expression of a number of metabolically relevant genes, including genes for certain cytochromes (38–40).

In higher eukaryotes, the gene that responds most dramatically to hypoxic stress encodes erythropoietin (Epo), a 30.4-kDa glycoprotein hormone that regulates erythrocyte production by supporting the proliferation and terminal differentiation of erythroid precursor cells in bone marrow (21, 28, 33). In humans and in many other mammals, Epo is produced in the kidneys (26) and fetal liver (57) in response to hypoxia. The human fetal hepatoma cell line Hep3B has proven to be useful for investigating the regulation of the Epo gene (23). These cells produce a small amount of Epo when incubated in a standard 20% oxygen atmosphere. When the cells are challenged with hypoxia, production of Epo protein and Epo mRNA increases 50- to 100-fold (16). Treatment with cobaltous chloride results in a similar induction in Epo expression (23), mimicking that observed in vivo (6). The marked increase in Epo mRNA levels following exposure to hypoxia or cobalt is primarily transcriptional (22). In Hep3B cells, the rate of induction of Epo mRNA upon switching to low partial O_2 pressure and the rate of decay following an abrupt return to a normoxic atmosphere (16, 22) are very close to changes in the levels of kidney Epo mRNA observed in intact animals undergoing similar challenge (49). Thus, Hep3B cells are a physiologically appropriate model system to study oxygen responsive elements within the Epo gene.

A comparison of the sequences of mouse and human Epo genes provides insight into candidate regulatory *cis* elements. Regions of homology in the noncoding portions of the gene include 150 bp upstream of the transcription initiation site, two 40-bp segments within the first intron, a 30-bp segment in the untranslated region of the fifth exon (53), and a 70-bp segment 130 bp downstream from the polyadenylation site (45). Transfection experiments with Hep3B cells utilizing these elements in conjunction with reporter genes have proven useful in testing their contributions to hypoxic induction. Several groups (4, 45, 51) have shown that the 3′ element serves as an oxygen-responsive enhancer but confers only a 4- to 14-fold induction of reporter gene expression, compared with the 50- to 100-fold induction of the endogenous Epo gene. The conserved sequences within the Epo promoter may contain stimulus-specific binding sites for protein and RNA (7), but thus far it has been difficult to demonstrate oxygen responsiveness of the Epo promoter in a reporter gene assay.

In this study, we have identified the minimal portion of the Epo promoter that confers hypoxic induction and demonstrate that it cooperates with the downstream element to cause at least 50-fold induction in luciferase reporter gene analysis. We also demonstrate that these oxygen-responsive elements in the enhancer and promoter include tandem repeats of consensus steroid hormone receptor half-sites.

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MATERIALS AND METHODS

Cell lines. All lines were maintained in α minimal Eagle's medium supplemented with iron-enriched, defined calf serum (HyClone, Inc.) at a concentration of 10%. Penicillin and streptomycin were added to final concentrations of 100 U/ml. Serum was stripped of steroid hormones by treatment with sterile Dowex AG1-X8 for 24 h and activated charcoal for 4 h.

Plasmid DNA. Plasmid DNA was prepared as described previously (47). The promoterless luciferase reporter vectors pXP1 and pXP2 were obtained from S. Nordeen (44), and TK-LUC, a luciferase reporter driven by the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) promoter (*Pvu*II to *Hin*cII), was provided by A. Brasier (8). pCH110, which contains the β-galactosidase gene driven by the simian virus 40 early promoter, was purchased from Pharmacia, and pBluescript KS(+) was purchased from Stratagene.

Constructs. All sequence coordinates of the human Epo gene are based on the GenBank file HUMERPA (accession number M11319 [37]). A 10.5-kb, *Bam*HI-to-*Bam*HI genomic clone of the human erythropoietin gene was obtained from R. Young and S. Orkin. A 5.6-kb fragment from the upstream *Bam*HI site to the sole *Ecl*XI site was subcloned into pBluescript KS(+). This insert was removed by digestion with *Sal*I and *Sac*I, which cleave in the polylinker of the vector. This DNA fragment was subcloned into the *Sal*I and *Sac*I sites of the promoterless luciferase reporter vector pXP2. The Epo promoter in this construct was forward in orientation and immediately 5′ of the luciferase gene. Multiple transcriptional start sites may exist for the human Epo gene (11, 50, 53). For this study, the position of the Epo transcriptional start site was arbitrarily defined as bp 385, 23 bp 5′ of the *Ecl*XI site. A 5′ Epo deletion series, starting at the *Asp* 718 site (a single site 100 bp 3′ of the natural BamHI site in the Epo clone), was created with exonuclease III and mung bean nuclease. The 65-bp Epo promoter (bp 321 to 385) was produced synthetically from four oligonucleotides and is identical to the region from bp 321 to 394. This promoter fragment was subcloned into the *Bam*HI and *Xho*I sites of pXP2.

A 259-bp fragment of the 3′ untranscribed enhancer region of the human Epo gene (E-259) was produced by the polymerase chain reaction (PCR) with the following synthetic oligonucleotide primers: 5′, bp 3324 to 3347, and 3′, bp 3579 to 3556. The wild-type 126-bp 3′ enhancer (E-126) was generated by digesting the E-259 fragment with *Apa*I and subcloning the blunted distal fragment into the *Sma*I site of pBluescript KS(+). The mutant 126-bp 3′ enhancer (mE-126) was made by site-specific mutagenesis (34) with a single mutated primer. The mutations were verified by dideoxy sequencing. The mutated enhancer was cleaved from pBluescript KS(+) by digestion with *Bam*HI and *Eco*RI, blunted, and ligated into the blunted *Bam*HI site immediately upstream of the thymidine kinase promoter in TK-LUC.

DNA sequence analysis. The 5′ Epo deletion constructs described above were used as templates for dideoxy DNA sequencing of the 5-kb 5′ flank of the Epo gene. DNA sequencing reactions were performed with 7-deaza-dGTP and Sequenase (U.S. Biochemical) as described previously (48). Initially, a primer based on the pXP2 polylinker (5′-CCAAGCTCAGATCCAAGC-3′) was used to sequence the 5′ end of each deletion construct. Subsequent primers for further sequencing were derived from new DNA sequence information. The DNA sequence from the original 10.5-kb Epo *Bam*HI clone in pUC19 was obtained with the same primers.

Transfections. One day before transfection, the cells were harvested and plated at a density of 10^6 cells per 10-cm-diameter plate. Ten micrograms of test DNA and 2 μg of pCH110 were prepared as calcium phosphate precipitates and transfected as described previously (31). All transfections were done in triplicate. Cells were incubated with the precipitate for 14 to 16 h at 37°C in an atmosphere of 5% carbon dioxide and 20% oxygen. In appropriate plates, cobaltous chloride was added to a final concentration of 100 μM. Hypoxic stimulation was achieved by exposure to a 1% oxygen–5% carbon dioxide–94% nitrogen atmosphere. The percent oxygen (±0.1%) was maintained by nitrogen washout in an ESPEC triple-gas incubator. The duration of stimulation with hypoxia and cobalt was 48 to 54 h. The cells were harvested, lysed by freeze-thawing, and assayed for luciferase activity as described by de Wet et al. (13). The cell lysates were assayed for β-galactosidase activity in 500-μl reaction mixtures containing 50 mM Tris HCl (pH 7.6), 100 mM NaCl, 10 mM $MgCl_2$, and 2.5 mM ONPG (*ortho*-nitrophenyl-β-D-galactopyranoside) at 37°C. The reactions were terminated by adding 500 μl of 1 M Na_2CO_3. In all transfection experiments, relative light units were calculated by dividing 10^4 light units by the β-galactosidase activity.

Electrophoretic mobility shift assays. Nuclear extracts were prepared by the method described by Shapiro et al. (52). In some cases, the cells were lysed by sonication. K562 and MOLT-4 extracts were prepared according to the method of Dignam et al. (14). The protein concentration of the nuclear extracts was 5 to 10 mg/ml. Unless otherwise indicated, cells were induced with hypoxia for 6 h. In some experiments, cells were incubated in 100 μM cobaltous chloride for 24 h before cell harvest.

The single-stranded oligonucleotides composing the double-stranded DNAs, E-A (bp 3448 to 3480), E-B (bp 3480 to 3512), mE-B (same as E-B, except the cytosines at bp 3486, 3487, 3494, and 3495 are deleted), E-C (bp 3513 to 3547), and P-1 (bp 261 to 320) (see Figure 3), and the DR series were synthesized in a biopolymer facility. DNA probes were single end labeled with α-^{32}P-deoxynucleoside triphosphates by filling in restriction enzyme-created 5′ overhangs with the Klenow fragment of *Escherichia coli* DNA polymerase I. Probe DNA was isolated from nondenaturing polyacrylamide gels as described previously (10). The estimated recovery was 80%, and the specific activity of the probes was >1 × 10^8 cpm/μg.

The DR-series sequences were as follows:

DR-0 5′-AGCTCGTAACTTGACCTTGACCTCAATAAGTAACT-3′
DR-1 5′-AGCTCGTAACTTGACCTcTGACCTCAATAAGTAACT-3′
DR-2 5′-AGCTCGTAACTTGACCTcaTGACCTCAATAAGTAACT-3′
DR-3 5′-AGCTCGTAACTTGACCTcagTGACCTCAATAAGTAACT-3′
DR-4 5′-AGCTCGTAACTTGACCTcatgTGACCTCAATAAGTAACT-3′
DR-5 5′-AGCTCGTAACTTGACCTcagtgTGACCTCAATAAGTAACT-3′
DR-6 5′-AGCTCGTAACTTGACCTcagctgTGACCTCAATAAGTAACT-3′

The lowercase letters indicate the gap sequence inserted between the direct repeats of the steroid receptor element half-sites.

Electrophoretic mobility shift assays were done in 20-μl reaction mixtures containing 10 fmol of radiolabeled probe, 2 μg of poly(dI-dC), and 5 to 10 μg of protein from nuclear extracts. Binding buffer contained final concentrations of 10 mM Tris HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM EDTA, 5%

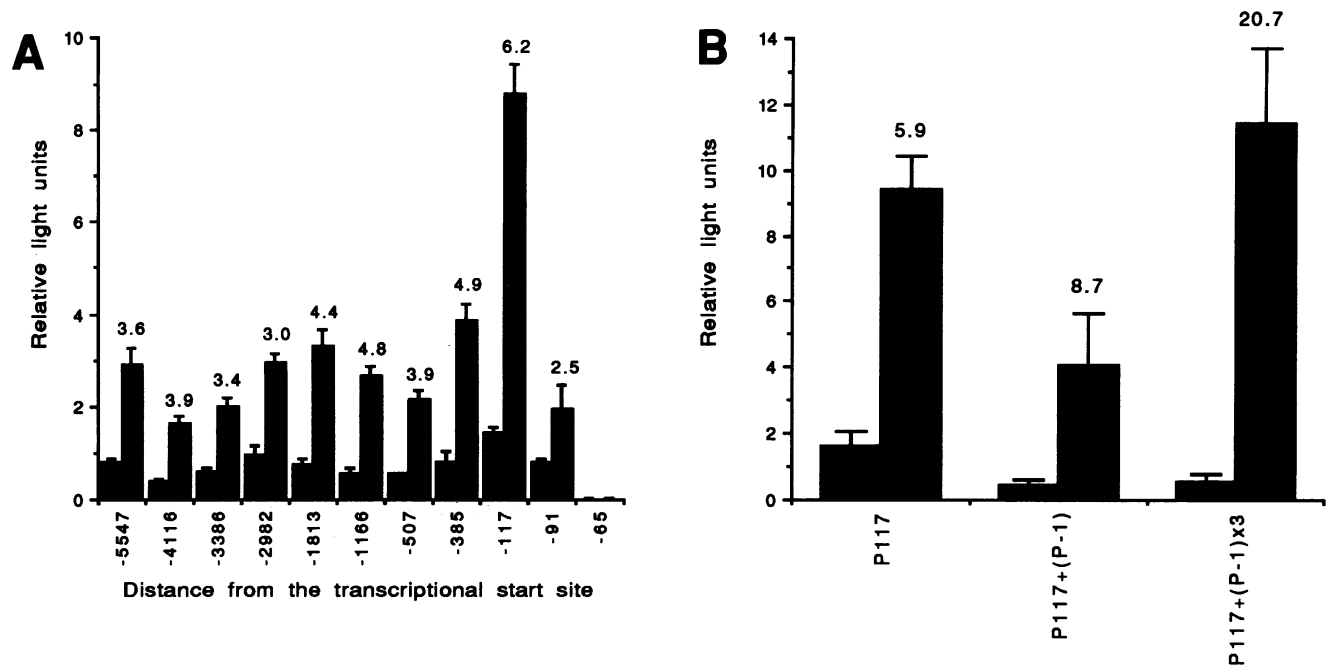

FIG. 1. Dissection of the Epo promoter. (A) 5′ deletion series of the human Epo promoter. Epo-luciferase deletion constructs were transfected into Hep3B cells. (B) Hypoxia- and cobalt-sensitive promoter (P) elements. Constructs containing one [P117+(P−1)] or three [P117+(P−1)×3] copies of the Epo sequence extending from bp −125 to −65 inserted upstream of the minimal inducible promoter (−117) were transfected into Hep3B cells. All samples were measured in triplicate. Solid columns represent normoxic incubations, and hatched columns represent hypoxic and cobalt stimulation for 48 h. Error bars represent one standard deviation. The fold induction (induced activity divided by basal activity) is shown above each column. Transfection efficiency was estimated by cotransfection with pCH110.

(vol/vol) glycerol, and 40 mM NaCl. The binding reaction mixtures were incubated at room temperature for 30 min. Electrophoresis was done in 4% polyacrylamide gels (acrylamide/bisacrylamide ratio, 29:1) containing 2.5% glycerol and 0.5× Tris-borate-EDTA (TBE) (47) at 250 V and 35 mA at room temperature.

DNase I footprinting. Preparative electrophoretic mobility shift reactions were scaled up 8- to 10-fold. DNase I and $CaCl_2$ (final concentration, 0.5 mM) were added after the completion of standard binding reactions. The sample was incubated for 2 min at room temperature, and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid] was added to a final concentration of 10 mM. After electrophoresis on a preparative gel, the complexes were identified by autoradiography, and individual complexes and free probe were excised from the gels. DNA from the polyacrylamide slices was deposited on an NA-45 membrane by electrophoresis in an agarose gel, isolated (9), and analyzed by electrophoresis, on denaturing polyacrylamide gels.

Methylation interference. Ten nanograms of DNA was methylated with dimethyl sulfate without a carrier according to the method of Maxam and Gilbert (41). Methylated DNA was subjected to preparative electrophoretic mobility shift assays as described above. Isolated DNAs were treated with 1 M piperidine for 30 min at 90°C, purified by three cycles of lyophilization in water, and subjected to electrophoresis on denaturing polyacrylamide gels.

Nucleotide sequence accession number. The nucleotide sequence of the 5′-flanking sequence of the Epo gene from *Bam*HI to *Hin*dIII has been assigned GenBank accession number M97764.

RESULTS

DNA sequence analysis. Sequence analysis of the 5-kb region of the Epo 5′ flank responsible for the negative regulation of constitutive expression in non-Epo-producing tissues (50) reveals 5 copies of *Alu* repetitive elements (300 bp each), a frequency of 1 *Alu* element every 1,000 bp, or 29% of the region. The Epo gene contains an *Alu* element within IVS3 (37) and at least two more in the 3′ flank of the Epo gene contained in the 10.5-kb *Bam*HI fragment (17a). In total, there are at least eight *Alu* elements in the 10.5-kb *Bam*HI fragment (23%). The expected distribution of *Alu* elements is 5% (27, 55); hence, *Alu* elements are overrepresented in the region of the human Epo gene. The murine Epo gene has a similar clustering of B1 repeat elements (5, 42, 53), the murine homologs of the human *Alu* type I family.

Minimal basal promoter. To define the minimal basal promoter of the human erythropoietin gene, a deletion series of the 5.6-kb *Bam*HI-to-*Ecl*XI Epo promoter upstream of the luciferase gene was analyzed by transient transfection. As shown in Fig. 1A, the minimum DNA sequence required for the basal transcription of the human Epo promoter in Hep3B cells extends from 91 bp upstream of the transcriptional start site to the transcriptional start site (bp 295 to 385). In contrast, an Epo promoter consisting of 65 bp upstream of the transcriptional start site (the −65-bp promoter) produced less than 1% of the luciferase activity observed with the most active Epo promoter. The transcriptional activity of the −65-bp Epo promoter was similar to the activity of the promoterless luciferase vector pXP2 (data not shown). In these constructs, the Epo promoter was relatively weak; the TK promoter of HSV-1 was approximately 50 times stronger

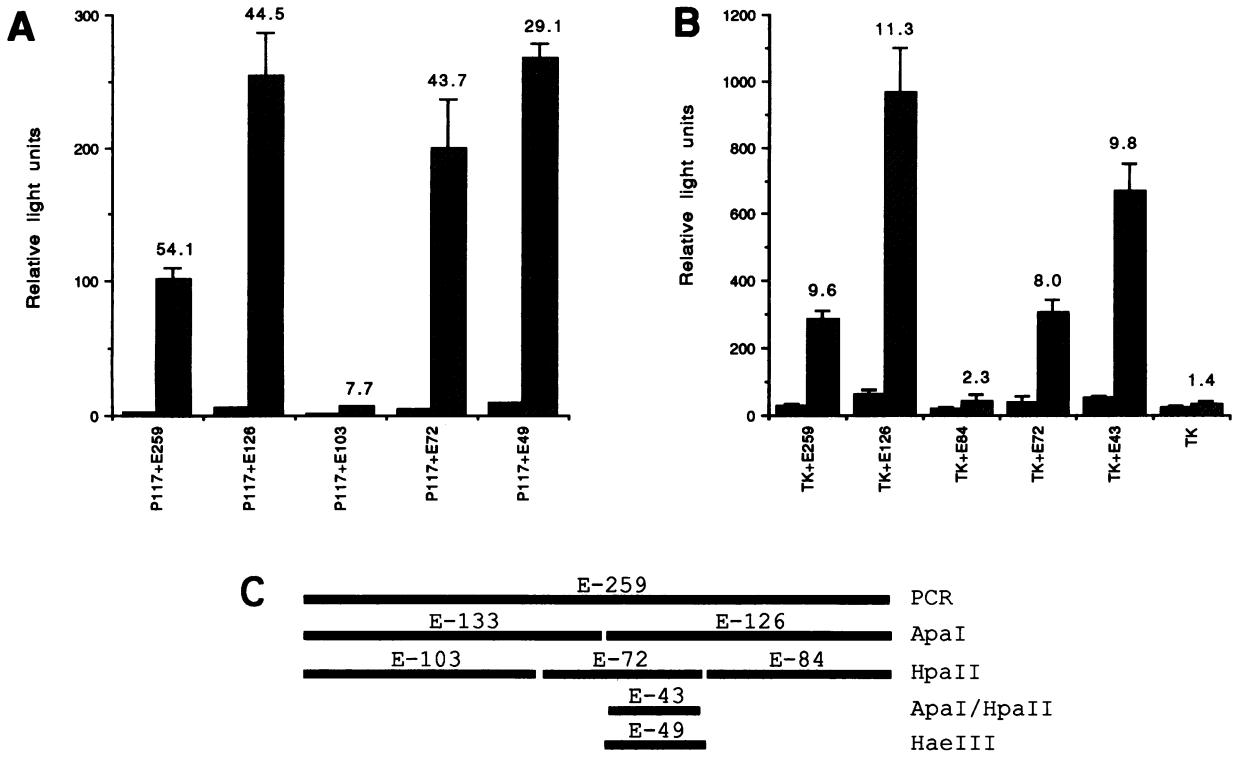

FIG. 2. Dissection of the 3' enhancer. Restriction fragments of the 3' enhancer were subcloned 5' of the minimal inducible promoter of Epo (A) and of the HSV-1 TK promoter (B). Solid columns represent basal activity, while hatched columns represent activity after induction with hypoxia and cobalt. Error bars represent one standard deviation. (C) The enhancer (E) fragments are designated by size in base pairs: E-259, PCR product; E-126, *Apa*I terminal fragment; E-103, *Hpa*II proximal fragment; E-72, *Hpa*II middle fragment; E-84, *Hpa*II terminal fragment; E-49, *Hae*III fragment; E-43, *Apa*I-to-*Hpa*II fragment.

than the basal Epo promoter. The promoter deletion constructs from the −5.6-kb promoter to the −91-bp promoter varied in strength by no more than 2.5-fold. Thus, this 5.6-kb region does not appear to have any strong positive or negative regulatory elements.

Transfection of the 5' deletion series into 293 cells, an embryonal human kidney cell line, demonstrated basal regulation similar to that seen in Hep3B cells. The −65-bp promoter was more active in this cell line (about 20% of the activity of the most robust Epo promoter and two times that of the promoterless reporter vector pXP2). The most active Epo promoter was at least 50-fold less active than the HSV-1 TK promoter in 293 cells (data not shown).

Minimal inducible promoter. Transfections with the 5' deletion series also provided information on the minimal promoter sequence of the Epo gene required for hypoxia and cobalt stimulation of transcription in Hep3B cells. After transfection of these constructs, the cells were subjected to a 1% oxygen (7 torr [ca. 933 Pa])–5% carbon dioxide (38 torr [ca. 5,070 Pa]) atmosphere in medium containing 100 μM cobaltous chloride. Coincident exposure to hypoxia and cobalt was chosen because hypoxic conditions could not be maintained during cell lysate preparation. The response to cobalt was directly proportional to the response to hypoxia for all of the Epo constructs, and the induction with coincident exposure was additive compared with individual exposures, as we have seen with native Epo production (data not shown). Each of the 5' promoter deletion constructs conferred hypoxia and cobalt inducibility upon the reporter gene, with the exception of the −65-bp promoter. The most inducible promoter construct contained the 117-bp region (bp 269 to 385) upstream of the transcriptional start site of the Epo gene. Hypoxia and cobalt reproducibly resulted in a 6- to 10-fold increase in luciferase activity. The −91-bp promoter construct showed basal activity similar to that of the −117-bp promoter but led to only a 2.5-fold augmentation of activity during stimulation. These results define the −117-bp promoter as the minimal inducible promoter of the human erythropoietin gene and suggest that the region of the promoter between bp −117 and −65 contains *cis*-acting elements essential for maximal inducible expression of Epo gene in Hep3B cells (Fig. 1A).

To further investigate the importance of this region in directing inducible expression of the Epo gene, the region from −125 to −65 bp relative to the promoter (bp 261 to 320), which we designated P-1, was subcloned immediately upstream of the −117-bp Epo promoter in single and triple copies in the forward configuration. Figure 1B demonstrates the basal activity of these constructs and their ability to direct inducible expression of luciferase in Hep3B cells. The introduction of multiple copies of this element into the −117-bp Epo promoter led to decreased basal transcription and augmented transcription under incubation conditions that induce endogenous Epo production. The minimal inducible promoter showed a 5.9-fold stimulation with induction, while the upstream addition of a single element or triple elements gave rise to an 8.7- and a 20.7-fold induction, respectively. Despite the effect of this element on the minimal inducible Epo promoter, when it was placed up-

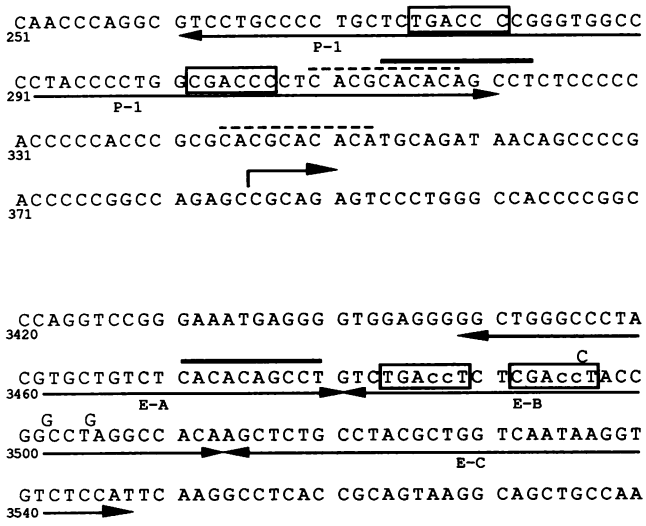

FIG. 3. Sequence of the human Epo promoter and 3′ enhancer region. Sequence coordinates are according to the GenBank HUMERPA file. Arrows under the sequence represent synthetic oligonucleotides used in this study. P indicates an oligonucleotide derived from the promoter, E indicates an oligonucleotide derived from the enhancer. Steroid/thyroid hormone receptor response element half-sites within the inducible elements are boxed. The lowercase c's in the E-B sequence indicate the two pairs of cytosine residues that are deleted in mutant mE-B. The dashed lines identify a repeated sequence in the promoter, while the solid lines show a sequence present in the promoter and enhancer. The bent arrow over the promoter sequence shows the transcriptional start site (11, 50, 53). The three nucleotides placed over the sequence of the enhancer fragment indicate the additional nucleotides present in the independent human clone.

stream of the TK promoter no hypoxic induction was seen (data not shown).

Minimal 3′ enhancer. We then tested the effect of the 3′ Epo enhancer on the minimal inducible Epo promoter. The enhancer was synthesized by PCR with a human genomic Epo clone as a template. The PCR product should be a 256-bp fragment. However, DNA sequence analysis (see Fig. 3) demonstrated three single-base-pair insertions in the PCR product compared with the published human Epo sequence (25, 37), thereby resulting in a 259-bp product (E-259). Identical insertions were present in the DNA sequence of the genomic clone; therefore, these insertions presumably represent DNA polymorphisms.

We prepared reporter constructs that contain E-259 or fragments thereof inserted immediately upstream of the minimal inducible (−117-bp) Epo promoter. The combination of the minimal inducible promoter and the 3′ enhancer led to a 40- to 50-fold induction of luciferase activity in response to the combination of hypoxia and cobalt in Hep3B cells (Fig. 2A). The magnitude of induction depended to a small degree on several factors, such as the subline of Hep3B cells, the lot of calf serum, and the cobalt concentration (data not shown). Fragmentation of the enhancer localized the active region of the enhancer to a 43-bp area extending from an *Apa*I site to the second *Hpa*II site. The proximal *Hpa*II fragment (E-103) and the distal *Hpa*II fragment (E-84) did not act as inducible enhancers when juxtaposed onto the minimal inducible Epo promoter or the TK promoter, respectively. In the context of a heterologous promoter (HSV-1 TK), active enhancer fragments generated a 9- to 11-fold induction with the combination of hypoxia and cobalt (Fig. 2B). E-259, E-126, and E-72 were subcloned into the luciferase reporter vector in both forward and reverse orientations. In no case did the orientation of the enhancer fragment affect the level of induction produced by hypoxia and cobalt exposure. None of the enhancer or promoter constructs was able to direct inducible activity in 293 cells (data not shown).

Our functional studies identify two short regions in the Epo gene that, when combined, are able to confer hypoxia and cobalt induction to a reporter gene that is similar in magnitude to the induction of endogenous Epo mRNA. These regions consist of a 53-bp element in the 5′ promoter and a 43-bp fragment of the 3′ enhancer. DNA sequence comparison (Fig. 3) of the two areas reveals sequence similarities. Both regions contain the 10-bp sequence CAC ACAGCCT, and both contain two copies of a sequence, YGACCY, which is fully homologous to the consensus steroid/thyroid hormone receptor response element (3, 15). In addition, the promoter contains two copies of a 10-bp sequence (CACGCACACA). The upstream copy overlaps the 10-bp repeat sequence mentioned above.

DNA-protein interactions. (i) Electrophoretic mobility shifts. Nuclear extracts were prepared from Hep3B cells grown under normoxic and hypoxic atmospheres as well as in the presence of 100 μM cobaltous chloride. The presence of DNA binding proteins in the nuclear extracts was assayed by electrophoretic mobility shift assays. Multiple DNA-protein interactions were identified as retarded complexes by mobility shift gel electrophoresis using E-259 as a probe (Fig. 4A). Addition of nonradiolabeled E-259 demonstrated a single specific DNA-protein complex and multiple nonspecific complexes (Fig. 4A). The patterns of specific and nonspecific complexes were similar in extracts prepared from normoxic, hypoxic, and cobalt-treated Hep3B cells. Reaction mixtures containing nonradioactive double-stranded oligonucleotide P-1 or E-B showed displacement of the specific complex with a 25-fold molar excess of these competitors. The displacement by E-B appears to be stronger than that of P-1. Oligonucleotides E-A and E-C, synthetic fragments that flank E-B in the enhancer, failed to compete for the formation of the specific complex observed with probe E-259. mE-B, a site-specific mutant of E-B, failed to compete (for sequence, see Fig. 3).

E-126 contains the DNA elements necessary and sufficient for hypoxic and cobalt induction as determined by transient transfection (see above). Incubating probe E-126 with Hep3B nuclear extract gave rise to one major and two minor specific complexes that were inhibited by E-259, P-1, and E-B (Fig. 4B). The specific complexes were not inhibited by a 50-fold excess of mE-B or E-C. E-C competes with a complex that overlies the predominant nonspecific complex in the electrophoretic mobility shift pattern. Several complexes with rapid mobility were present in scant amounts in the cobalt and hypoxic extracts, but were displaced by E-259 and E-C only in cobalt extract.

Electrophoretic mobility shift assays with labeled E-B (Fig. 4C) revealed three major nonspecific complexes, three major specific complexes, and a diffuse specific complex overlying a nonspecific complex. No difference in complex formation was seen with nuclear extracts prepared from induced or uninduced Hep3B cells (data not shown). The major DNA-protein complex migrates as a doublet. Competitor titrations demonstrated that unlabeled E-B competes approximately 8-fold better than P-1 and that mE-B is an ineffective competitor despite a 200-fold molar excess.

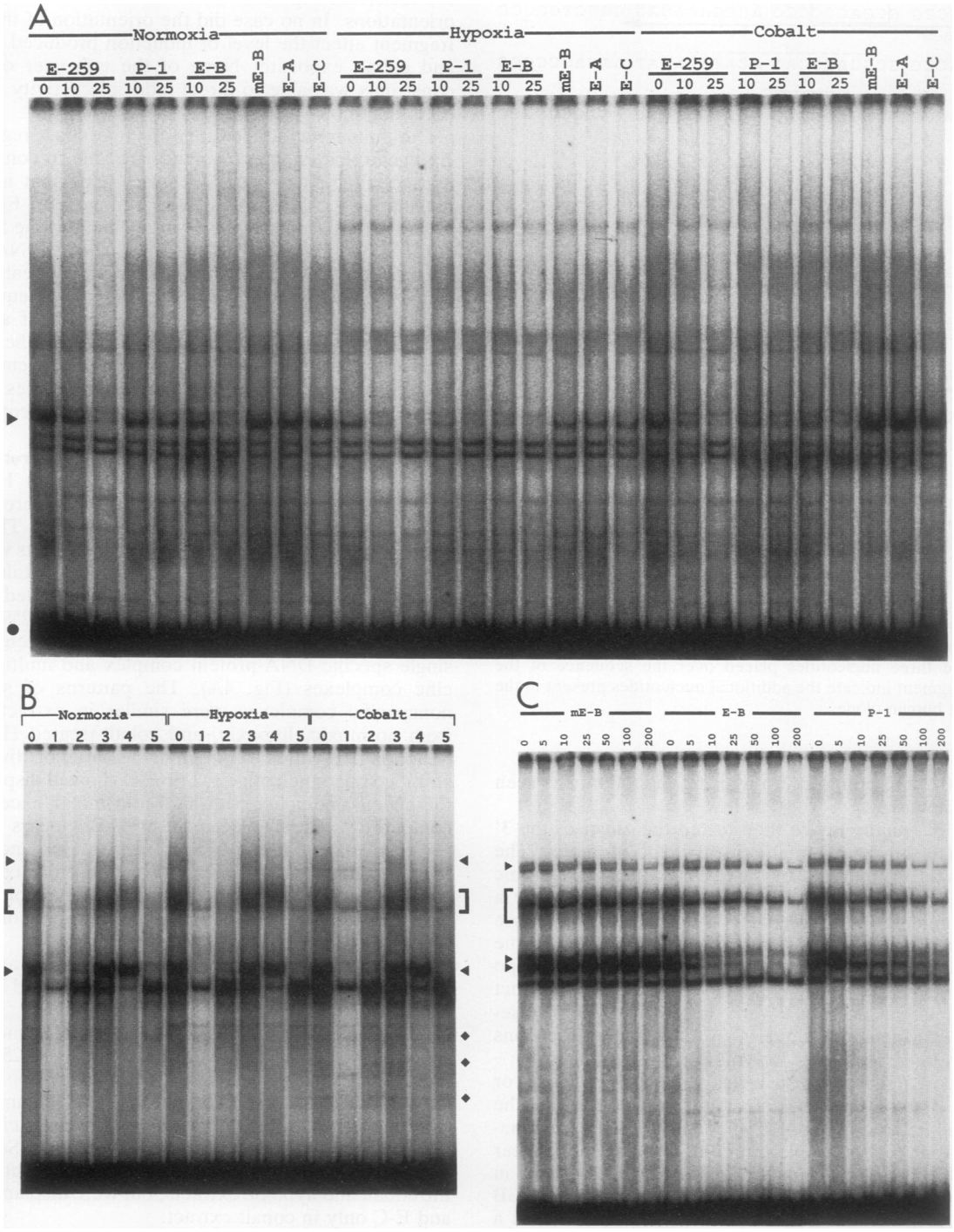

FIG. 4. Electrophoretic mobility shift analysis of E-259, E-126, and E-B (35 bp). End-labeled probes were incubated with nuclear extract from Hep3B cells. In panels A and C, competitor DNA was added at the molar ratio noted above each lane, before extract was added. The triangles in each panel indicate complexes that migrate as a discrete band and are specifically inhibited by E-259, P-1, and E-B. The brackets indicate in panels B and C a diffuse complex(es) that is specifically inhibited by E-259, P-1, and E-B. (A) E-259 with normoxic, hypoxic, and cobalt nuclear extract. mE-B, E-A, and E-C were added at a 25-fold molar excess. The dot on the left corresponds to the position of free probe. (B) E-126 with normoxic, hypoxic, and cobaltous nuclear extract. The lane numbers correspond to the following competitors (added at a 50-fold molar excess): 0, no competitor; 1, E-259; 2, P-1; 3, mE-B; 4, E-C; and 5, E-B. The diamonds indicate minor complexes found in extract from cobalt-treated Hep3B cells that are displaced by E-259 and E-C. (C) E-B with hypoxic nuclear extract from Hep3B cells.

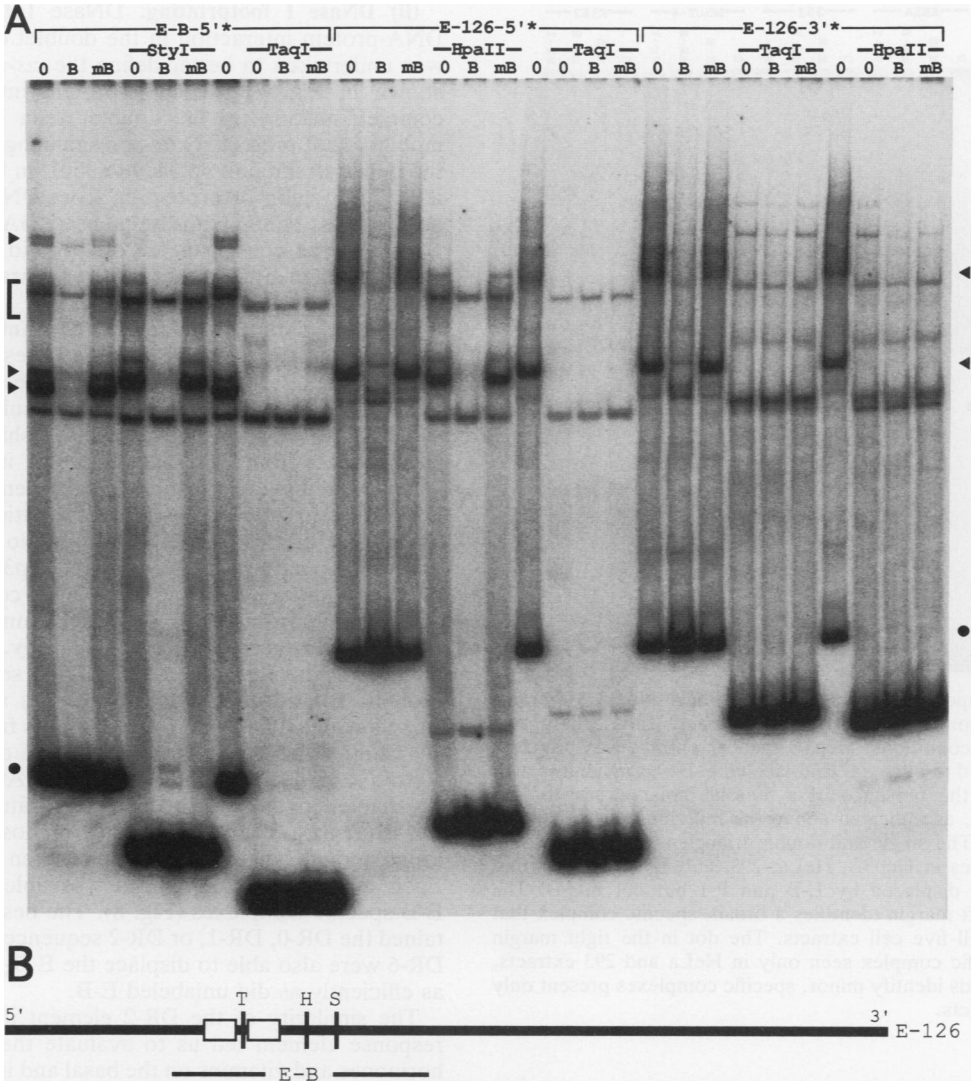

FIG. 5. Definition of the binding site in the 3′ enhancer by electrophoretic mobility shift. (A) Nuclear extract from Hep3B cells exposed to hypoxia was incubated with E-B and E-126 probes. The 35-bp E-B probe was radiolabeled on the 5′ end of the fragment (E-B-5′*), and the E-126 probe was radiolabeled independently on the 5′ (E-126-5′*) or 3′ (E-126-3′*) end. As indicated, in some cases the probes were treated with *Sty*I, *Hpa*II, or *Taq*I before incubation with nuclear extract. Competitor DNAs are indicated above the individual lanes: 0, no competitor; B, E-B; mB, mE-B. Competitor DNA was added at a 50-fold molar excess. The triangles and bracket on the left identify the specific complexes formed on E-B, and the dot identifies the position of the full-length E-B probe. The triangle on the right indicates the specific complexes formed on E-126, and the dot indicates the position of the full-length E-126 probe. (B) Schematic map showing the positions of the restriction enzyme sites in the fragments. T, *Taq*I; H, *Hpa*II; S, *Sty*I. The boxes represent the direct repeat sequences.

The region of E-126 responsible for DNA-protein complex formation was evaluated by sequential shortening of the probe. Probe E-126 was radiolabeled at one end or the other and then digested with *Hpa*II or *Taq*I. These probes were incubated with nuclear extract and subjected to electrophoretic mobility shift assays (Fig. 5). When the probe was labeled at the 5′ end, digestion with *Hpa*II (generating a 43-bp probe) permitted the formation of a specific complex that was displaced by E-B but not by mE-B. Digestion with *Taq*I (generating a 34-bp probe) ablated the formation of a specific radiolabeled complex. With the label at the 3′ end of E-126, digestion with either *Hpa*II or *Taq*I also prevented formation of a radiolabeled complex. These experiments limit the region of specific protein binding to the 43-bp sequence between the *Apa*I site and the *Hpa*II site.

Similar experiments were carried out with E-B labeled at the 5′ end. Specific complex formation was observed after digestion with *Sty*I (which cleaves 4 bp 3′ of *Hpa*II), but digestion with *Taq*I interfered with formation of specific radiolabeled complexes. The E-B sequence begins 20 bp 5′ of the *Hpa*II site (Fig. 5B). Thus, these data limit the DNA sequence required for complex formation to this 20-bp region. No significant differences in complex formation were observed between the *Sty*I-digested E-B probe and the larger *Apa*I-to-*Hpa*II probe. Minor variations in complex migration may relate to the position of the DNA binding site relative to the end of the probe (29). Digestion with *Taq*I destroyed the specific, radiolabeled DNA-protein complexes on all three DNA probes.

The tissue specificity of the E-B DNA-protein interactions

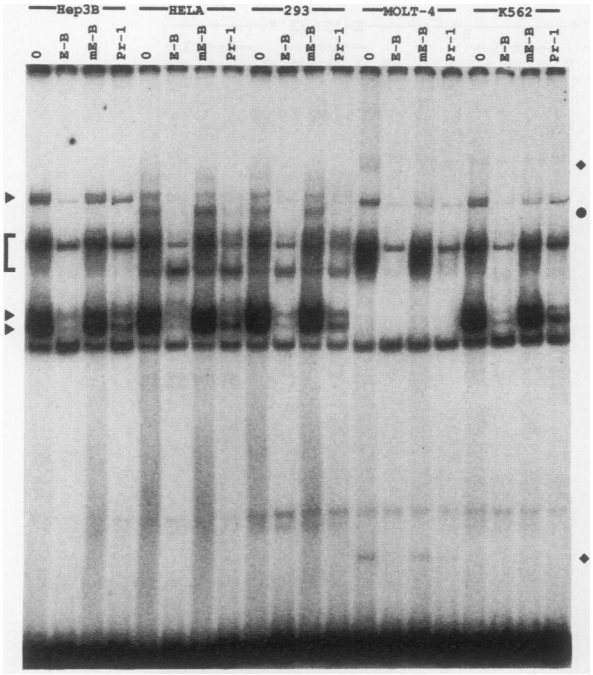

FIG. 6. Cell specificity of E-B interactions. Nuclear extracts were prepared from HeLa, 293, MOLT-4, and K562 cells grown under normoxic conditions. Hep3B nuclear extract was prepared from cells exposed to hypoxia. End-labeled E-B was incubated with each extract in the presence of a 50-fold molar excess of the competitor DNA, as indicated above the individual lanes. Pr-1 is identical to P-1. The single and double triangles in the left margin indicate complexes in Hep3B, HeLa, 293, and K562 extracts that were specifically displaced by E-B and P-1 but not mE-B. The bracket in the left margin identifies a broad, specific complex that was present in all five cell extracts. The dot in the right margin indicates a specific complex seen only in HeLa and 293 extracts, while the diamonds identify minor, specific complexes present only in MOLT-4 extracts.

was examined by electrophoretic mobility shift assays with nuclear extracts made from other cell lines (Fig. 6). The major specific complex observed as a doublet in Hep3B cells was detected in nuclear extracts prepared from HeLa, 293, and K562 cells but not from MOLT-4 cells (a T-lymphocyte line). However, the other specific complexes were observed in all of the nuclear extracts. This observation suggests that the upper complexes are not dependent on the presence of the protein(s) involved in the formation of the lower doublet. A specific complex forms in the presence of nuclear extracts from 293 and HeLa cells but is not present in Hep3B nuclear extracts. This complex is relatively minor compared with the major specific doublet. In addition, there are two specific complexes observed with MOLT-4 nuclear extract that are not present in any of the other extracts tested.

The sensitivity of the mobility shift complexes to protease digestion, RNase digestion, or thermal denaturation was tested by using Hep3B nuclear extracts and radiolabeled E-43. The specific complexes were destroyed by proteinase K digestion, and preincubation of the nuclear extract for 10 min at 70°C prevented complex formation. RNase A digestion for 30 min at 37°C diminished the resolution of specific and nonspecific complexes, but the general pattern of mobility shift complexes was preserved (data not shown) (cf. reference 7).

(ii) DNase I footprinting. DNase I footprinting of the DNA-protein interaction in the doublet observed with E-B was undertaken to better define the *cis*-acting element. As shown in Fig. 7, the DNA-protein interactions in each complex making up the doublet seen by electrophoretic mobility shift protected a region spanning bp 3482 to 3504 on the upper strand and bp 3479 to 3501 on the lower strand of E-B. The region of protection from DNase I digestion on both strands contains the sequence TGACCTCTCGACCT, a form of the consensus steroid/thyroid hormone receptor response element, a direct repeat separated by a 2-bp gap (DR-2 element) (19).

(iii) Methylation interference footprinting. DNA-protein contact points in both of these complexes were evaluated by methylation interference. The E-B probe, labeled on either strand, was methylated by dimethyl sulfate exposure and subjected to electrophoretic mobility shift assays with nuclear extract from Hep3B cells grown in a hypoxic atmosphere. Methylated guanines and adenosines within the DR-2 element interfered with the formation of DNA-protein complexes in this region (Fig. 7). No difference in the interference pattern was seen with Hep3B nuclear extracts prepared during normoxic, hypoxic or cobalt incubation or with extracts from 293 or HeLa cells (unpublished data).

(iv) Steroid receptor element homology. The DNA-protein interactions between the putative DR-2 sequence in E-B and proteins from Hep3B nuclear extracts were evaluated by using unlabeled DNA competitors (gifts from P. Yen and W. W. Chin). These competitors contain a direct repeat of the steroid hormone response element (TGACCT) separated by a variable-length gap sequence (see Materials and Methods for sequence). The sequences were chosen to match those found in the F2 element from the chicken lysozyme gene (2). Each of the DR competitors was able to compete with E-B-specific complexes (Fig. 8). The best competitors contained the DR-0, DR-1, or DR-2 sequence, but DR-3 through DR-6 were also able to displace the E-B-specific complexes as efficiently as did unlabeled E-B.

The similarity of the DR-2 element in E-B to a steroid response element led us to evaluate the effects of steroid hormones and vitamins on the basal and inducible activity of the Epo enhancer. Hep3B cells were transfected with a construct consisting of E-126 placed upstream of the TK promoter. After transfection, the cells were exposed to medium containing stripped calf serum with or without steroid hormones and vitamins. The cells were subjected to hypoxia and cobalt stimulation for 48 h and assayed for luciferase activity. As demonstrated in Fig. 9, none of the ligands had an impact on the basal activity of the construct or the inducibility of the Epo enhancer by hypoxia and cobalt.

Functional characteristics of the 3′ enhancer. In order to test the functional characteristics of E-B and mE-B, they were subcloned immediately upstream of the minimal inducible Epo promoter (−117 bp) in a luciferase reporter vector. Analysis of these constructs by transient transfection revealed that E-B alone is not sufficient for hypoxia or cobalt induction, nor does mE-B inhibit basal or inducible activity of the Epo promoter (data not shown). Irrespective of these results, the inability of mE-B to compete with E-B in electrophoretic mobility shift analysis and the methylation interference pattern on E-B provide evidence that the DR-2 element is an important region in E-B. Therefore, we synthesized a mutated form of the E-126 3′ enhancer containing the two 2-bp deletions as in mE-B. The mutated enhancer was subcloned upstream of the TK promoter and was

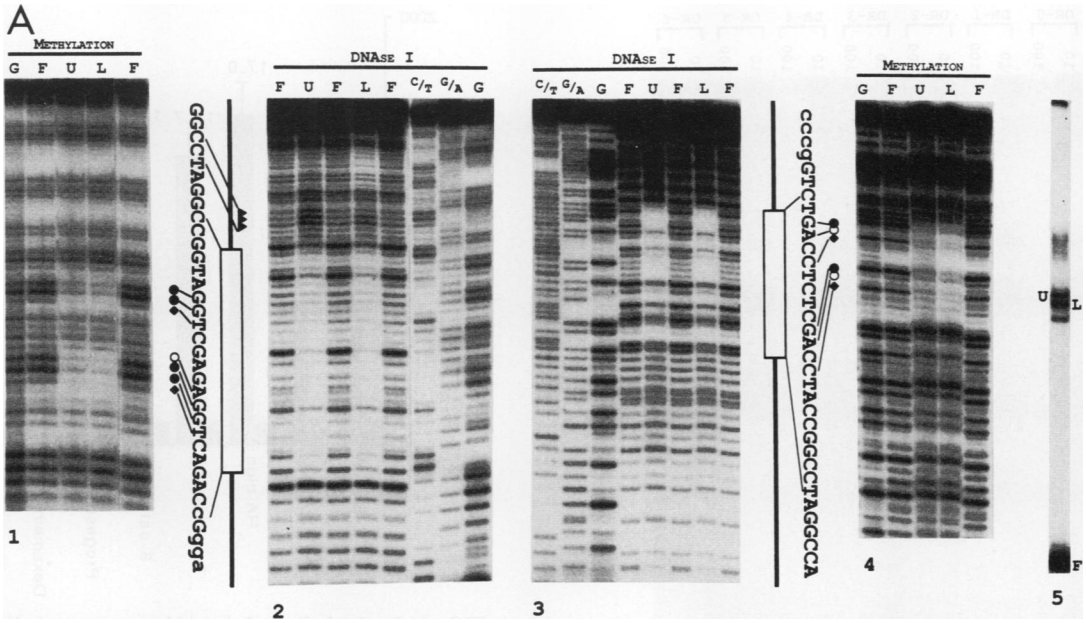

FIG. 7. (A) DNase I and methylation interference footprinting of E-B. E-B was independently radiolabeled on the 3′ end of each strand. Panels 1 and 2 correspond to E-B radiolabeled on the lower strand, while panels 3 and 4 correspond to E-B radiolabeled on the upper strand. Panel 5 is a analytical electrophoretic mobility shift gel with E-B. U and L indicate the positions of the upper and lower specific complexes in the major doublet, and F indicates the position of the free probe. In all cases, the nuclear extract was prepared from hypoxic Hep3B cells. G, C/T, and G/A indicate chemical sequencing ladders (41). The open boxes between panels 1 and 2 and between panels 3 and 4 indicate the extent of the DNase I footprint on each strand. Symbols: ▲▲▲, an area of DNase I hypersensitivity on the lower strand; ●, methylated guanines that interfere with protein binding; ○, methylated adenosines that interfere with protein binding; ◆, fragments ending in pyrimidines that are overrepresented in the free probe. (B) DNase I and methylation interference footprinting composite. The DNA sequence encompasses bp 3474 to 3509 in the 3′ enhancer. The horizontal arrows represent the DNase I footprint. Symbols: ▲▲▲, an area of DNase I hypersensitivity induced by protein binding; ● ○, guanines and adenines, respectively, that strongly interfere with DNA-protein interactions after methylation; ◆, pyrimidines that are underrepresented in the bound complexes (the type of base modification at these sites is unknown).

analyzed by transient transfection, as shown in Fig. 10. In the context of the TK promoter, mutations in the DR-2 element abolished the hypoxic and cobalt responsiveness of the E-126 enhancer.

DISCUSSION

The regulation of erythropoietin production is a complex process that requires the transduction of a change in intracellular oxygen tension into a transcriptional response. We have identified minimal elements in the promoter that regulate basal and inducible transcription of human erythropoietin in Hep3B cells. The minimal basal promoter is no greater than 91 bp in length, while maximal induction requires no more than 117 bp of Epo sequence upstream of the transcriptional start site. Data presented in Fig. 1 suggest that the region between positions −117 and −65 upstream of the promoter (bp 268 to 320) contains DNA sequences necessary for hypoxia and cobalt sensitivity. When P-1 (bp 261 to 320) is placed in multiple copies upstream of the Epo minimal inducible promoter, additive effects on transcriptional induction are observed. In contrast, in the context of the heterologous TK promoter, P-1 does not confer hypoxia sensitivity. The Epo basal promoter may contain elements not present in the TK promoter, with which P-1 interacts. It is notable that the TK promoter has both TATA and CAAT boxes, elements that are absent from the Epo promoter.

We have functionally dissected the 3′ region of the human Epo gene to define the smallest region necessary and sufficient for hypoxia and cobalt induction of Epo transcription. These fragments have been analyzed in constructs containing the minimal inducible Epo promoter as well as in constructs containing the HSV-1 TK promoter. The vector we chose to evaluate Epo promoter and enhancer combinations contains two strong polyadenylation signals upstream of the promoter inserted to minimize cryptic promoter interference (13). Previous experiments done by others (4) have been complicated by the presence of a strong cryptic promoter in the vector used for transfection, which permitted high-level production of reporter RNA despite the absence of the Epo promoter or any other known promoter. As shown in Fig. 2, the minimal sequence retaining full functional activity is a 43-bp *Apa*I-*Hpa*II fragment. Only the 3′ fragments containing this 43-bp sequence demonstrated inducible activity. The induction conferred by the minimal inducible (−117-bp) Epo promoter (6-fold) and by the 3′ enhancer (10-fold) as individual elements increased in a multiplicative manner (40- to 50-fold) when the elements were juxtaposed, demonstrating cooperativity between these transcriptional elements.

We have demonstrated the presence of a protein(s) in Hep3B nuclear extracts that binds specifically to functionally important elements. The major specific complexes

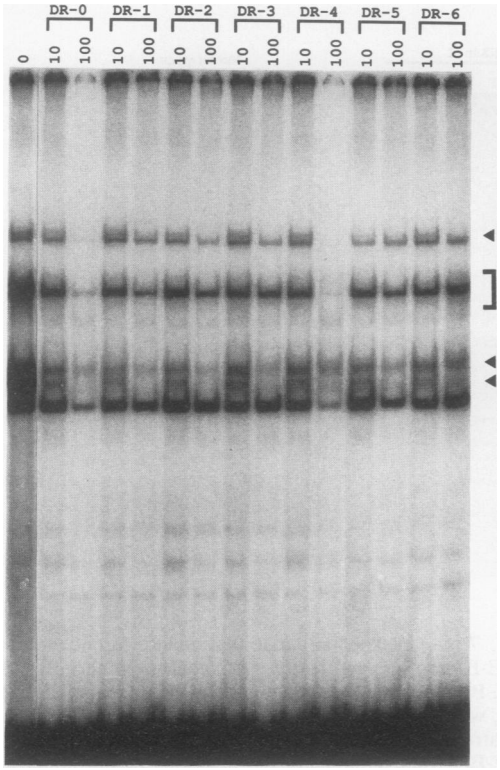

FIG. 8. Competition between complexes formed on E-B with direct repeats of thyroid hormone response element (TRE) half-sites. Nuclear extract prepared from hypoxic Hep3B cells was incubated with the E-B probe. Competitor DNA was added at the molar ratio indicated above each lane. The competitor DNA contains direct repeats of TGACCT separated by the number of base pairs after the DR above each set of lanes (see Materials and Methods for sequences). The triangles and bracket on the right indicate the specific complexes formed on E-B.

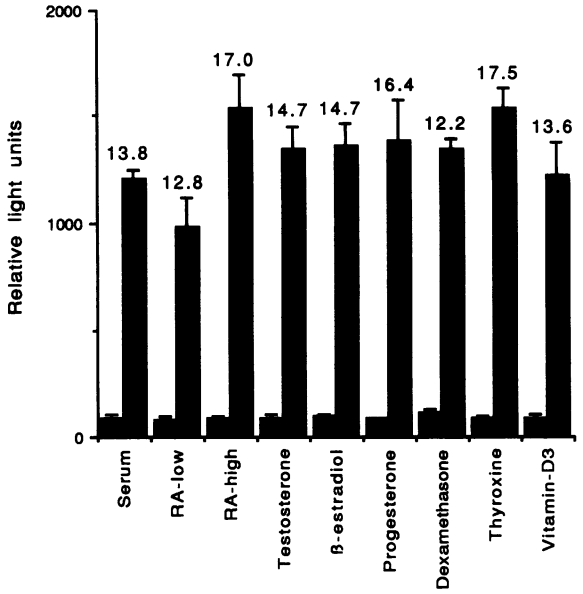

FIG. 9. Lack of effect of steroid hormones and vitamins on basal and inducible activity of the Epo enhancer. A construct containing the 126-bp wild-type 3′ enhancer placed upstream of the TK promoter was transfected into Hep3B cells. After transfection, the cells were fed with medium containing stripped serum (see Materials and Methods). Hormones and vitamins were added at physiologic concentrations. Cells were exposed to hypoxia and cobalt or to normoxia for 48 h in the presence of hormones and vitamins as indicated. All samples were tested in triplicate. Symbols: ■, normoxia; ▨, hypoxia and cobalt. Error bars indicate one standard deviation. Numbers above bars are ratios of relative light units under hypoxia and cobalt induction to relative light units under normoxia.

formed by using the enhancer as a probe can be displaced by a 60-bp element in the promoter that confers responsiveness to hypoxia (Fig. 4). Conversely, specific complexes formed between nuclear extract from Hep3B cells and P-1 can be displaced by 3′ enhancer competitors (17a). DNase I footprinting confines the binding site in the enhancer to a 26-bp region containing a nearly perfect direct repeat of the thyroid hormone receptor half-site, TGACCT, separated by a 2-bp gap (DR-2). We have shown by methylation interference analysis that DNA-protein contact points reside in the GACC portions of the footprint. P-1 contains two putative half-sites that may be responsible for the competition between the promoter and the enhancer fragments in electrophoretic mobility shift assays. Regardless of its physical interaction with factors in Hep3B nuclear extract, a 32-bp region of the enhancer that includes DR-2 is not sufficient for cooperative levels of induction by hypoxia and cobalt when placed upstream of the minimal inducible Epo promoter. The DR-2 site in E-B is necessary for the activity of the enhancer, however, since mutation of DR-2 abolishes induction by E-126. We posit that yet another element in E-43 is involved in cooperative interactions between the Epo promoter and enhancer.

Comparison of the mouse (bp 22 to 55) (45) and human (bp 3473 to 3505 + 3) (25, 37) Epo sequences reveals that the DR-2 site is present in both species in a portion of the enhancer that is necessary and sufficient for induction:

ACAGCCTGTCTGACCTCTCGACCCTACCGGGCCT (human)
AtgGCCcGgCTGACCTCTtGACCCctCtGGGCtT (mouse)

The sequence of the DR-2 site (boldface type) is highly conserved (93%), while the sequence around the DR-2 site diverges significantly (60% conserved). The stringent conservation of the DR-2 site between species suggests an important role for this site in gene regulation.

Despite the similarity of the DR-2 site to a steroid response element, the addition of steroid hormones, thyroxine, vitamin D_3, or retinoic acid to Hep3B cells had no effect on basal or inducible expression. Thyroxine stimulated basal activity of the Epo promoter/enhancer combination two- to threefold (7a) but had no effect on the TK promoter/Epo enhancer combination. Thus, the minor effect of thyroxine on basal Epo transcription appears to be at the level of the promoter, not the 3′ enhancer. Many steroid receptor gene family members have no known ligand and are classified as "orphan receptors" (36, 54, 56). It seems plausible that the DR-2 site of the Epo enhancer interacts with such an orphan receptor.

DNA-protein binding to the functionally active DR-2 region of the Epo enhancer under basal and induced conditions suggests that the regulation conferred by this region is due to a modification of the protein bound to the site. Such a modification could be phosphorylation or some other posttranslational modification of a protein. The progesterone receptor has been shown to undergo DNA-dependent phos-

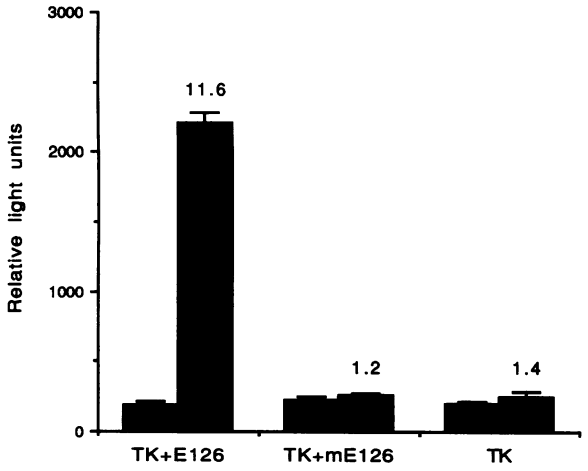

FIG. 10. Site-specific mutation of the 126-bp enhancer 5' of the TK promoter. The wild type (E-126) and mutant (mE-126) forms of the 126-bp enhancer were subcloned 5' of the TK promoter. The constructs were transfected into Hep3B cells and subjected to stimulation with hypoxia and cobalt for 48 h. All samples were tested in triplicate. Error bars represent one standard deviation. For explanation of symbols and numbers above bars, see the legend to Fig. 9.

phorylation (1a), and this phosphorylation of the receptor stimulates transcription in the absence of ligand (12). The similarity of the DR-2 site to a steroid/thyroid hormone receptor binding site (19, 35) suggests two other explanations. If a steroid receptor family member binds the DR-2 site, transcriptional activity could depend upon the presence or absence of a specific ligand. For example, at some sites thyroid hormone receptor acts as a activator in the absence of thyroxine but as a repressor in the presence of thyroxine (43). A ligand that is metabolized differently under hypoxic and under normoxic conditions might bind the receptor with differential affinity and repress or induce transcription depending on the oxidative state of the ligand. Another mode of regulation recognized in the steroid receptor gene family is the formation of heterodimers of receptor molecules that differ in relative activity (24, 32, 46) and could be altered by hypoxia.

Our electrophoretic mobility shift assays with the enhancer region of the Epo gene and nuclear extract from Hep3B cells did not show hypoxia- or cobalt-specific shifts. Semenza et al. (51) demonstrated hypoxia-specific interactions between extracts from the kidneys and liver of anemic mice and small oligonucleotide probes from the enhancer region of the human Epo gene. Three of their probes that generated hypoxia-specific complexes fall outside the region of the enhancer that we have determined is responsible for inducible activity in Hep3B cells. Since Hep3B cells produce Epo at a low level at 20% oxygen tension, it is possible that factors that bind the enhancer and promoter are present in low levels even under basal conditions. The extreme sensitivity of electrophoretic mobility shift assays may reveal these factors in Hep3B cell nuclear extracts, whereas in the extracts made from whole organs, the basal binding activity may be overwhelmed by the predominance of non-Epo-producing cells. Furthermore, the heterologous admixture of human Epo gene and murine organ extracts might lead to interactions that are not present in a system homogeneous as to both species and cell type.

We have shown that nuclear extracts from cells of liver (Hep3B), epithelial (HeLa), kidney (293), and myeloid (K562) origin contain factors that bind specifically to the DR-2 element and comigrate in electrophoretic mobility shift assays. The methylation interference and DNase I footprint patterns of these interactions are identical (1). A nuclear extract from cells of T-lymphocyte origin (MOLT-4 cells) did not form this specific complex with the DR-2 element. Of these cells, only Hep3B cells produce Epo. These findings suggest that factors that can recognize the DR-2 element are present in many types of cells, but functional interactions with this element leading to transcription of the Epo gene occur only in Hep3B cells. Cells may contain different members of the steroid hormone receptor family, and transcriptional activation at a DNA binding site might depend on the relative concentrations and presence of specific family members (20). For a productive interaction, the steroid hormone receptor family member may require a ligand that is cell specific or condition specific. Even in the presence of the appropriate receptor and ligand, a cell-type-specific adaptor or coactivator may be required to mediate a response.

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