RAPID COMMUNICATION

Characterization of Hypoxia-Responsive Enhancer in the Human Erythropoietin Gene Shows Presence of Hypoxia-Inducible 120-Kd Nuclear DNA-Binding Protein in Erythropoietin-Producing and Nonproducing Cells

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Erythropoietin (Epo) production in response to hypoxia or cobalt is primarily mediated by activation of transcription of the Epo gene. Recently an hypoxia responsive enhancer was identified in the 3' flanking region of the mouse and human Epo genes. Using functional analysis in Hep 3B cells we define here the minimal enhancer element as a 29-bp segment starting at the Aпал site in the 3' flanking region of the human Epo gene. Mutagenesis studies of the minimal element identified three different areas that are necessary for full enhancer activity. Electrophoretic mobility shift assays show the presence of hypoxia- and/or cobalt-inducible nuclear DNA-binding proteins that bind to one of the active sites of the enhancer. Induction of hypoxia-binding activity was abolished by Anisomycin, a potent protein synthesis inhibitor, suggesting that de novo protein synthesis is necessary for the activation process. Further characterization of DNA-binding proteins by use of UV light crosslinking identified a protein of molecular weight of approximately 120-Kd that was present only in hypoxic extracts. This protein was found to be present in hypoxic nuclear extracts from both Epo-producing and non-Epo-producing cells, suggesting that it may be involved in a more generalized mechanism of cellular response to hypoxia.

MATERIALS AND METHODS

Cell cultures. Hep 3B, Hep G2, and HeLa cells were obtained from the American Tissue Collection (Rockville, MD). Mouse renal mesangial, fibroblast, and tubular cell lines were obtained from Dr Eric Neilson, from the University of Pennsylvania (Philadelphia). Human endothelial cells (HUVEC) were obtained from Dr Sandor Shapiro, Cardeza Foundation. All cells were cultured in minimal essential medium (MEM) ( Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, VT), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cell lines were maintained in a well humidified incubator at 37°C in 5% CO₂, 95% air. For hypoxic stimulation the culture plates were placed for 18 hours in a modular incubator chamber (Billups-Rothenburg, Del Mar, CA) and flushed with a gas mixture containing 1% O₂, 5% CO₂, and 94% N₂.

Plasmid constructs and transfections. Our basic Epo-chloramphenicol acetyl transferase (CAT) plasmid construct has been described previously. Briefly, it consists of a 330-bp Sfα N1-XmalIII fragment from the human Epo 5' flanking region (−312 to +19) cloned through polylinker insertion upstream of the CAT coding sequences in the pSVO-CAT vector. Enhancer sequences to be tested were obtained by restriction enzyme digestion from a cloned fragment of the human Epo gene and subcloned upstream of the Epo-promoter sequences in the Epo-CAT construct. For mutant constructs double-stranded oligonucleotides were prepared by routine procedures using a DNA synthesizer. Plasmid DNA was prepared by standard procedures and purified by double banding in cesium chloride gradients. Supercoiled DNA was transfected into Hep 3B cells by electroporation as already reported. After the electrical shock, cells transfected with the same plasmid were pooled and equally distributed into two culture plates, one of which was exposed to hypoxia 48 hours later for 18 hours, while the other one was maintained at room air. For control of transfection efficiency a β-galactosidase expression vector (pSV-Gal; Promega, Madison, WI) was simultaneously transfected with the test plasmid. Cells were then harvested for further analysis using CAT and β-ga-
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Fig 1. Functional analysis of the hypoxia-responsive enhancer. (A) Determination of minimal enhancer sequences. The Apal-PstI fragment from the 3' flanking region of the human Epo gene as shown in (a) was digested with restriction enzymes and the fragments as shown in (b), (c), and (d) were subcloned upstream of the Epo promoter in pEpo-CAT. Lines (e) and (f) show end deletions of Apal-TaqI fragment. Right side panel shows results of transient transfection experiments into Hep 3B cells. Hypoxia responsiveness is expressed as the ratio of percent chloramphenicol acetylation between hypoxic and normal cells. Each value corresponds to the mean of 2 to 4 independent experiments. Numeration of Epo sequence according to Lin et al.12 (B) Mutational analysis of Apal-TaqI fragment. Sequence of the fragment starting at the Apal site as bp number 1. Site of mutations 1 to 5 (M1, M2, M3, M4, M5) are overlaid. Actual bp substitutions are shown under the sequence. Mutants were subcloned in pEpo-CAT and analyzed for enhancer activity as described above.

Electrophoretic mobility shift DNA-protein binding assays (EMSA). Nuclear extracts from normal or hypoxic Hep 3B and other cell lines were prepared essentially as described by Lee et al.22 except for the addition of 0.5% NP40 to buffer A and pepstatin (1 μg/mL), benzamidine (1 mmol/L), leupeptin (1 μg/mL), sodium fluoride (10 mmol/L), and orthovanadate (0.4 mmol/L) to buffers A and C. Usual final protein concentration of the nuclear extracts was between 5 to 10 mg/mL. Extracts were maintained at −70°C until used. For the preparation of induced extracts, cells were exposed to hypoxia (1% O2) or incubated in the presence of cobalt chloride (100 μmol/L) for 18 hours before cell harvest. Double-stranded oligonucleotides or DNA fragment probes were end-labeled by filling in 5’ overhangs with a-32P-dCTP (Amersham, Arlington Heights, IL) using the Klenow fragment of DNA polymerase I.

Electrophoretic mobility-shift assays using high ionic strength buffers were performed as described by Staudt et al.22 Binding reactions contained between 5 to 10 μg of protein extract, 1 μg of sonicated salmon sperm DNA, 16 mmol/L HEPES (pH 7.9), 70 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L DTT, and 12% glycerol in a total volume of 20 μL. After preincubation for 5 to 10 minutes at room temperature, 0.2 to 0.5 ng of radiolabeled probe were added. The reaction mixtures were incubated at 30°C for 30 minutes and then loaded onto 5% nondenaturing polyacrylamide gel (acrylamide:bisacrylamide 40:0.5 ratio). Electrophoresis was performed in 1X Tris-glycine buffer (50 mmol/L Tris-HCl, 380 mmol/L glycine, 2.1 mmol/L EDTA) at 4°C.

UV cross-linking. Direct cross-linking analysis was performed by exposing the binding reactions to UV light irradiation (305 nm) at a distance of 5 to 8 cm for 30 to 60 minutes at room temperature, after which samples were mixed with an equal volume of 2X Laemmli loading buffer and analyzed by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).23 Two-dimensional cross-linking was performed by running first the binding reactions on a 5% nondenaturing polyacrylamide gel, which was then UV-irradiated for 30 to 60 minutes on ice followed by autoradiography. Gel slices containing the bands of interest were then excised, soaked for 10 minutes in 2X Laemmli buffer, and subsequently loaded (in a second dimension) on top of a 6% SDS-polyacrylamide gel.24

RESULTS

Our previous studies had localized the hypoxia enhancer to the 146-bp, Apal-PstI fragment, in the 3’ flanking region of the human Epo gene.13 To further delineate the minimum element with enhancer activity, the Apal-PstI fragment was digested with the restriction enzymes StuI and TaqI to generate fragments of 100 and 34 bp, respectively (Fig 1A). These fragments were subcloned upstream of the Epo promoter in our Epo-CAT reporter gene construct (see Materials and Methods). The constructs were transfected into Hep 3B cells and tested for hypoxia responsiveness. As shown in Fig 1A, both the Apal-StuI and Apal-TaqI fragments gave hypoxia responses similar to the larger Apal-PstI fragment (lines b, c, d). Furthermore, minimal dele-
Fig 2. Identification of DNA-binding proteins by EMSA. (A) End-labeled probes were incubated with nuclear extracts of normoxic (N) or hypoxic (H) Hep 3B cells. Lanes 1 and 2 use mutant 5 (M5) probe, lanes 3 and 4 use mutant 1.5 (M1.5) probe, and lanes 5 and 6 use mutant 1 (M1) probe. Hypoxia inducible bands, upper (U) and lower (L), signaled by arrows. (B) DNA competition experiments. DNA-binding reactions containing labeled mutant 5 (M5) probes were incubated in the presence of no added competitor (lanes 1 and 2), or in the presence of a 100-fold molar excess of unlabeled mutant 1.5 DNA (lanes 3 and 4), or mutant 5 DNA (lanes 5 and 6). (C) Effect of cobalt on inducible activity. Labeled mutant 5 probe was incubated with nuclear extracts from normoxic (N) in lane 1, cobalt treated (Co) in lane 2, or hypoxic (H) in lane 3, Hep 3B cells.
expression, in all the cell lines tested. These comprised the
were found to completely block the induction of Epo
plexes. Similar conditions of incubation with Anisomycin
completely abolished the appearance of inducible com-
tivity was then investigated in several other cell types, both
Epo-producing and nonproducing cell lines. As shown in
Fig 4A, hypoxia-inducible binding was found, with variable
Epo-producing Hep 3B and Hep G2 cells (lanes 1 to 4), and
non-Epo-producing fibroblasts, epithelial, mesangial, and
endothelial cells (lanes 5 to 16). Furthermore, incubation
with cobalt also elicited the induction of hypoxia-enhancer
binding proteins similarly as what was observed in Hep 3B
cells (not shown). Although the rate of migration of the
inducible bands appear to differ slightly among different cell
lines, specificity of binding was confirmed by competition
experiments as shown in Fig 4B. In all cell lines tested the
hypoxic-inducible complexes were completely displaced by
an excess of unlabeled M5 DNA, but not by a similar excess
of the mutated oligo M1,5, as previously shown for Hep 3B
cells (Fig 2B).

To further characterize the protein(s) responsible for the
hypoxia-inducible binding-activity, UV cross-linking ex-
periments were performed. Labeled oligos were incubated
with nuclear extracts from Epo-producing Hep 3B and non-
producing mesangial cells, exposed to UV light irradiation
and analyzed by denaturing SDS-PAGE. As shown in Fig
5A, direct UV-cross-linking using M5-labeled oligo showed
the presence of a protein-DNA complex with an apparent
size of approximately 140 Kd in the hypoxic lanes (2 and 6)
that is not present in the nornoxic controls (lanes 1 and 5).
Furthermore, this band is not present when the mutated
M1,5 oligo is used (lanes 4 and 8). The molecular size of the
inducible band is the same in Hep 3B and mesangial cell
extracts. There are several other bands that are present with
both normal and hypoxic extracts and with the site 1 mut-
atooligo. These bands probably represent constitutive
complexes binding to other sites, as were shown in the gel-
shifting assays. To further confirm the relationship between
the 140-Kd cross-linked protein and the inducible bands
observed by gel shifting, two-dimensional gel analysis were
performed. After running a gel shift in the first dimension
(B), the gel was UV irradiated at 4°C for 30 to 60 minutes,
the areas corresponding to the inducible complexes were
excised and run in a second dimension in a denaturing SDS-
polyacrylamide gel. As shown in C, when gel slices contain-
ing the inducible DNA-binding protein complexes were run
in a second dimension, a major band of 140 to I50 Kd and a
minor one of approximately 240 Kd were found. Again,
these proteins were present in hypoxic Hep 3B and mesan-
gial cell nuclear extracts (lanes 2 and 5), but not in normoxic
extracts (lanes 1 and 4) or when the mutated M1,5 oligo was
used (lanes 3 and 6). No band was detected by crosslinking
in the area corresponding to the upper (U) inducible com-
plex of the mobility-shift gel. This is not surprising, in view
of the low abundance of that upper protein complex.

Although the cross-linking experiments showed the pres-
ence of a single major hypoxia-inducible protein-DNA
band, it still appeared possible that a more complex protein-
protein interaction could be involved in the hypoxia-induc-
ible DNA-binding reaction. To study possible protein-pro-
tein interactions, binding reactions were performed in the
presence of variable concentrations of the dissociating agent
deoxycholate (DOC) and analyzed by EMSA. As shown in
Fig 6, deoxycholate at a final concentration of 0.06% com-
pletely inhibited the hypoxia-inducible binding activity
(lanes 1 to 4). Similar concentrations of this agent had no
effect on the binding activity of the same extract when a
Sp1-labeled probe was used (lanes 5 to 7).
DISCUSSION

Recently an hypoxia-responsive enhancer was identified in the 3' flanking region of the human and mouse Epo genes. This enhancer appears to be position and orientation independent and it is able to interact with its own or with heterologous promoters in transient transfection assays. It has been suggested that specific interactions be-
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Fig 5. Characterization of DNA-binding proteins by UV cross-linking. (A) Nuclear extracts from normal (N) and hypoxic (H) Hep 3B (lanes 1 to 4) or mesangial (Mes, lanes 5 to 8) cells were incubated with labeled M5 (lanes 1, 2, 5, and 6) or M1.5 probes (lanes 3, 4, 7, and 8). UV irradiated for 45 minutes and analyzed under reducing conditions by SDS-PAGE. Inducible bands indicated by arrow heads. (B) and (C) Two dimensional cross-linking. Nuclear extract from normal (N) or hypoxic (H) Hep 3B (lanes 1 to 3) and mesangial (Mes, lanes 4 to 6) cells were incubated with labeled M5 (lanes 1, 2, 4, and 5) or M1.5 (lanes 3 and 6) probes and analyzed by EMSA (B). The gel was then UV irradiated for 45 minutes, slices containing the inducible bands were excised (shown in brackets) and analyzed in a second dimension (C) by SDS-PAGE. Large arrows indicate direction of electrophoresis. Molecular weights (Kd) of protein standards are shown.

tween the enhancer and its own promoter may facilitate the response to hypoxia. The interaction of the enhancer with other possible regulatory elements present in the 5' flanking region have not yet been investigated.

The results presented here provide further characterization of the hypoxia-responsive human Epo enhancer and shows new elements involved in its activation. Initial experiments defined the minimal enhancer element as the Apal-Taql fragment. However, base pair substitutions at the 3' end of that fragment (mutant 5) suggest that enhancer activity can be restricted to even a shorter segment of 29 bp starting at the Apal site. This segment is slightly smaller than the minimal element of 50 bp described by Semenza and Wong, and the Apal-Hpa2 fragment suggested by Blanchard et al. However, it is to note that the natural enhancer is located in the 3' flanking region of the Epo gene and not upstream of the promoter, as in our constructs, and it is thus possible that larger regions may be normally required in vivo for full enhancer activity.

Mutational analysis of the Apal-Taql fragment showed the presence of three distinct functionally significant sequences, designated as 1, 3, and 4. Because in our study we did not mutate all the possible bases, other functional sequences may exist. However, results recently reported by Semenza and Wong also suggest the presence of three functional areas in the minimal enhancer.

Gel-shifting studies showed the presence of hypoxia-inducible protein-DNA complexes that appear to bind specifically to site 1. Of the two inducible bands, the upper one was much weaker and was not consistently found in all hypoxic extracts prepared. There were also several bands that were equally present in hypoxic and normal extracts. They appear to bind to other sites of the fragment because they were also present using a mutant 1 site oligo. We have not yet characterized the binding sites of these constitutive complexes. Induction of binding activity in response to hypoxia seems to require protein synthesis. Active protein synthesis is also required for transcriptional activation and Epo mRNA accumulation, and the two phenomena are possibly linked. However, it is not clear whether the inducible DNA binding proteins are themselves synthesized de novo in response to hypoxia, or protein synthesis is required in an intermediary step, such as oxygen sensing or signal transduction.

Further characterization of the inducible proteins was obtained by UV cross-linking experiments. Direct and two-dimensional analysis after gel shifting showed the presence in hypoxic extracts of a major protein complex of molecular weight in the 140- to 150-Kd range. This complex corresponded to the lower (L) band of the gel shift and no protein corresponding to the upper (U) complex was detected. After subtraction of the molecular weight of the oligo the apparent molecular weight of the protein component is approximately 120 to 130 Kd. Another complex of about 240 Kd was also observed with hypoxic extracts in the two-dimensional gel analysis. This larger band was not found consis-
Fig 6. Effect of deoxycholate in binding activity of hypoxic nuclear extracts. End-labeled mutant 5 probe (M5) was incubated with hypoxic nuclear extracts in the presence of various concentrations of deoxycholate (DOC) and analyzed by EMSA as shown in lanes 1 to 4. Similar reactions were conducted using a labeled Sp1 probe (lanes 5 to 7).

Fig. 6: Effect of deoxycholate on binding activity of hypoxic nuclear extracts. End-labeled mutant 5 probe (M5) was incubated with hypoxic nuclear extracts in the presence of various concentrations of deoxycholate (DOC) and analyzed by EMSA as shown in lanes 1 to 4. Similar reactions were conducted using a labeled Sp1 probe (lanes 5 to 7).

Similarly, and because no equivalent band was observed in the direct cross-linking experiments, its relevance remains unclear.

Although a single major inducible band is observed in the direct cross-linking experiments (Fig. 5A), it is still possible that more complex protein-protein interactions may occur in the generation of DNA-binding activity. To further analyze this possibility, protein-DNA binding reactions were performed in the presence of deoxycholate. This agent has been extensively used to study protein-protein interactions such as those that occur in the association of NF-kB to its cytoplasmic inhibitor. In the presence of very low concentrations of deoxycholate (0.06%) there was complete suppression of hypoxia-inducible binding activity. Similar concentrations of this agent did not affect the binding of the same nuclear extracts to an Sp1 probe suggesting that deoxycholate was not affecting protein-DNA interactions, but rather protein-protein interactions. Furthermore, the same inhibition of binding activity was observed in the presence of only 0.02% of deoxycholate, a concentration that does not affect binding to DNA, if nuclear extracts had been previously preincubated with the dissociating agent. It is of note that almost ten times higher concentrations of deoxycholate did not affect the binding of NF-kB to its cognate DNA-binding site. Thus, it appears that the binding of the hypoxia-inducible activity to DNA may require initial protein-protein interactions, the nature of which, i.e., homodimerization, heterodimerization, or other protein complex formation will have to await the further purification of these factors.

Similar hypoxia-inducible DNA binding protein complexes as the ones observed in Hep 3B cells were, surprisingly, also found in non-Epo-producing cells. Cross-linking studies indicate that the hypoxia-induced DNA-binding proteins observed in Epo-producing and nonproducing cells appear to be the same or have very similar characteristics. Furthermore, cobalt was also stimulatory in these cells. Recently, Goldberg et al. have postulated that oxygen sensing in Hep 3B cells may be mediated by a heme-containing protein, such that changes in oxygen tension would result in conformational changes with subsequent activation of transcription. Cobalt would replace iron in the porphyrin ring of the heme molecule and lock the oxygen sensor in the deoxy-conformation, thus activating the Epo gene. The results presented here suggest that a common mechanism of hypoxia sensing and response is present in all the cell lines studied and possibly in almost all cells in general. Although hypoxia-regulated Epo mRNA biosynthesis is restricted to very few cells in the organism, the 29-bp element defined here allowed the detection of some more ubiquitous nuclear proteins that are responsive to changes in oxygen tension. These findings would support recent evidence published by Maxwell et al. that shows hypoxia responsiveness to the Epo enhancer transiently transfected into several cell lines that did not express the Epo gene. The fact that in our studies hypoxia-inducible proteins were also present in cells that did not express the Epo gene suggests that these proteins per se are not sufficient to induce Epo gene expression and other regulatory elements are necessary for tissue specificity, as has been shown in transgenic mice. The significance
of the presence of these hypoxia-regulated DNA-binding proteins in all cells remains speculative, and it will be important to determine whether they are involved in the regulation of expression of other genes\textsuperscript{30,31} that are also stimulated by hypoxia.

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