The role of the near upstream sequence in hypoxia-induced expression of the erythropoietin gene

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ABSTRACT

Transcription of the erythropoietin (epo) gene is regulated in response to tissue hypoxia. In this study we show that constructs containing 117 bp of the epo promoter sequence cloned upstream of a luciferase reporter, respond to hypoxia when transfected into the human hepatoma cell line, Hep3B. The sequence –61 to –45 (EP17) relative to the transcription start of the murine epo gene imparted a ~4-fold induction of reporter gene expression due to hypoxia. Internal deletion of EP17 resulted in loss of induction by hypoxia without altering basal expression of the 117 bp epo promoter reporter construct. Mutagenesis studies showed that the bases at positions –53, –59, from –49 to –51 and from –55 to –57 are essential for hypoxic induction. The EP17 sequence is required for the 3′ enhancer element of the epo gene to be maximally functional. Gel shift and UV cross-linking experiments showed the presence in Hep3B nuclear extracts, of two protein factors with approximate molecular weights of 52 kDa and 25 kDa that bind to EP17. Introduction of specific mutations in the EP17 region that abolish induction by hypoxia, also eliminated the binding of one or both of these factors. These experiments demonstrate a role for the proximal region of the epo promoter in hypoxic induction of the epo gene.

INTRODUCTION

The glycoprotein, erythropoietin (epo) is the primary regulator of blood cell formation in mammals (1). It stimulates the proliferation and differentiation of erythroid progenitor cells (2,3). In adult animals it is mainly produced in kidneys in response to reduced tissue oxygenation while in fetal life the liver is the major site of production (4,5). In spite of active investigation, the mechanisms by which specific cells can recognize relative hypoxia and respond by increased transcription of the epo gene are still not clarified.

Transcriptional regulation of gene expression is, in part, at the level of initiation and is due to interaction of sequence-specific DNA binding proteins with the regulatory elements located in the promoter, the 5′ and 3′ untranslated and intronic regions of the eukaryotic genes. Transcriptional activation involves complex and often cooperative interactions of various factors and gene expression is probably determined at least in part by the presence or activity of specific factors in a particular cell type (6–8). The binding activity or abundance of these factors may be regulated by extracellular signals. Since transcription of the epo gene is affected by tissue oxygenation, it offers an excellent model for the study of the factors that are sensitive to extracellular cues.

The human hepatoma cell line, Hep3B, which can synthesize and secrete epo in a regulated manner in vitro (9), has proven to be a useful system for investigating the transcriptional regulation of the epo gene (10). During the last few years, using these cells, gene transfer experiments with deletions of the promoter and 3′ untranslated regions of the epo gene have identified the presence of a cis-acting element in the promoter region and an enhancer element in the 3′ untranslated region (11–17).

Deletion and mutation experiments have established a 117 bp promoter sequence and a 50 bp 3′ enhancer sequence as minimal sequences necessary for the epo gene to respond to hypoxia (11,12,15). Hypoxic induction of the epo gene was shown to involve a cooperative interaction of the promoter and enhancer binding factors (11). In vitro DNA binding studies using nuclear extracts from a number of different cell lines have shown the presence of a 120 kDa hypoxia-inducible factor (HIF-1) that binds to the 5′ portion of the enhancer (15). The induction of HIF-1 was shown to be dependent on protein phosphorylation (16) and the recent cloning of the HIF-1 cDNA showed that it is a basic helix-loop-helix-PAS heterodimer (18). The possible roles of hepatic nuclear factor 4 and of the COUP family of proteins in tissue-specific and hypoxia-inducible expression of the epo gene have also been demonstrated (19).

We have previously shown that the region from –61 to –45 (EP17) relative to the transcription start site of the mouse epo gene binds to both a protein and a ribonucleoprotein in kidney nuclear extracts. The RNA component of the latter is down-regulated in response to hypoxia, suggesting a functional (possibly repressor) role for this component in epo transcription in kidneys (20). In the present study we demonstrate the functional importance of EP17 in hypoxic induction of epo gene transcription in Hep3B cells. Our data also suggest that this sequence of the promoter region cooperates with the previously identified 3′ enhancer element. In addition, we present evidence for two factors binding to EP17 in nuclear extracts of Hep3B cells exposed to normal O2 level and to hypoxia.

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Preparation of nuclear extracts

Hep3B cells were grown to confluence and exposed to normoxic and hypoxic conditions for 48 h, as described above. Nuclear extracts were prepared by the method of Abnoury and Workman (26), with the following modifications: the high salt buffer contained 25% glycerol and 50 mM KCl and the dialysis buffer contained 25% glycerol and 20 mM KCl. The extracts were dialyzed for 6 h at 4°C. The dialysate was quick frozen in liquid N2 and stored in aliquots at −70°C. The nuclear extract from differentiated Sol8 cells was prepared essentially as described above for Hep3B cells.

Probe synthesis for DNA binding assay

Synthetic oligonucleotides were purified by electrophoresis on 15% denaturing polyacrylamide gels and then subjected to chromatography on Sep-Pak C18 columns (Waters Associates, Milford, MA) (27). For use as competitors or probes in gel mobility shift assays, complementary strands of each oligonucleotide were denatured in 10 mM Tris–HCl (pH 7.5), 1 mM EDTA at 85°C for 3 min, annealed by slow cooling at room temperature and purified on a 15% polyacrylamide gel. Double stranded oligonucleotide probes were 5’ end labelled with [γ32P]ATP using T4 polynucleotide kinase (United States Biochemical Corp., Cleveland, OH). The following double stranded oligonucleotides (oligos) were used in this study, Oligo EP17 (CCCCCAACCCCTACCCGCCG) corresponds to the region from −61 to −45 of the murine epo gene. The mutant oligos were: mut 1 (CCGACGCCGACCCGGG), mut 2 (CCCCCCGCCGCCCGC), and mut 3 (CCCCCTTTTTTTTTCCCGG). Oligo SP1 (CTAAAAGGGGCGGCTGCTGCA) corresponds to the SP1 binding site in the human γ-globin gene (28).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed using nuclear extracts of Hep3B (normoxic and hypoxic) and Sol8 cells. Binding reactions were carried out in a total volume of 25 µl containing 50 000 c.p.m. (0.1–1 ng) of the radiolabelled probe, 5–10 µg of total protein of the nuclear extract and 2 µg of double stranded poly (dl-dc) as non-specific competitor. The binding buffer consisted of 10 mM Tris–HCl (pH 7.4), 100 mM NaCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.3 mM MgCl2, 8% glycerol and 0.5 mM phenylmethylsulfonyl fluoride. Binding was performed at 25°C for 20 min. Putative, unlabelled competitor oligos were added at 25–100-fold molar excess over the labelled probe for 3–5 min prior to addition of the probe. Binding reaction mixtures with purified SP1 (3 ng) from HeLa cells contained 5 µg of bovine serum albumin (BSA) and 0.5 µg of the double stranded poly (dl-dc) nonspecific competitor. The reaction mixtures were loaded on 5% polyacrylamide gels (44:1, acrylamide/bis-acrylamide) containing 0.5× Tris-borate-EDTA buffer and electrophoresed with 0.5× Tris-borate-EDTA electrode buffer (0.045 M Tris base, 0.045 M boric acid, 0.001 M EDTA). Electrophoresis was carried out at 150 V at 4°C (27), the gels were fixed in 10% acetic acid, dried and exposed to Kodak XAR-5 film.

UV cross-linking molecular weight determination

Double stranded probes were radiolabelled as described above. Binding reactions were replicated and multiple identical reactions were run on the same polyacrylamide gel. Following electrophoresis...
the wet gels were exposed to UV irradiation (254 nm) for 20 min and the gel exposed to X-ray film overnight. The regions corresponding to protein–DNA complexes were cut from the gel and placed in a 1.5 ml Eppendorf tube. The cross-linked complexes were eluted from the polyacrylamide by crushing the gel in 460 mM Tris (pH 6.8), 2.5% sodium dodecyl sulfate (SDS), and 0.025% (wt/vol) bromophenol blue, supplemented with 0.2 M NaCl. The slurry was incubated at 37°C for 2 h and at 95°C for 2 min and spun through a Schleicher and Schuell Centrex spin filter, packed with glass wool. The filtrate was run on an SDS 12.5% polyacrylamide electrophoresis gel with Rainbow molecular weight markers (Amersham, Arlington Heights, IL).

RESULTS

Hypoxia-induced epo gene expression in Hep3B cells is regulated in part by an upstream element

The role of the EP17 sequence in the 5′ untranslated region of the epo promoter in the regulation of epo gene expression was studied in Hep3B cells transiently transfected with luciferase reporter constructs containing different sub-fragments of the human epo upstream sequence and compared with the promoterless control plasmid pX2. As shown in Figure 1, following hypoxic stimulation, cells transfected with the plasmid ΔH [385 bp of epo 5′ flanking sequence linked to the luciferase reporter] showed 4–6-fold greater activation of luciferase than those with pX2. When the plasmid, Δ18, containing 117 bp of the epo 5′ flanking sequence was transfected into Hep3B cells there was a similar increase in luciferase activity in response to hypoxia. To determine whether EP17 plays a functional role in the response to hypoxia, an internal deletion of the EP17 sequence in the Δ18 plasmid was constructed (ΔEP18). With Hep3B containing this construct hypoxia did not induce increased reporter expression. The loss of response to hypoxia is not due to a distance effect caused by the deletion in the epo promoter because, as described later, introduction of specific mutations in the EP17 region resulted in a similar loss of hypoxia-induced luciferase activity. These data clearly indicate that the EP17 region of the epo promoter participates to some extent in hypoxia-inducible expression of the epo gene. When the EP17 sequence was cloned upstream of the TK promoter driving luciferase expression, no hypoxic induction was seen (Table 1).

Table 1. Luciferase reporter activity in normoxic and hypoxic Hep3B cells following transient expression of various DNA constructs

<table>
<thead>
<tr>
<th>DNA</th>
<th>Luciferase (RLU/µg protein)/β-gal (A420)</th>
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<tbody>
<tr>
<td>pX2</td>
<td>18% O₂ 1 ± 0.4</td>
</tr>
<tr>
<td>1. Δ18</td>
<td>2% O₂ 2% 1 ± 0.6</td>
</tr>
<tr>
<td>2. Δ18</td>
<td>9 ± 1.4</td>
</tr>
<tr>
<td>3. ΔEP18</td>
<td>38 ± 3.8</td>
</tr>
<tr>
<td>4. Δ18Enh</td>
<td>11 ± 2.9</td>
</tr>
<tr>
<td>5. ΔEP18Enh</td>
<td>8 ± 1.6</td>
</tr>
<tr>
<td>6. TK</td>
<td>20 ± 2.6</td>
</tr>
<tr>
<td>7. TKenh</td>
<td>370 ± 8.5</td>
</tr>
<tr>
<td></td>
<td>15 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>60 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>120 ± 9.5</td>
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<td>150 ± 8.8</td>
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<td></td>
<td>110 ± 6.6</td>
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<td>460 ± 8.1</td>
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Values represent mean ± SD of four experiments (triplicate assays) and are expressed as relative light units (RLU/µg of protein normalized for cotransfected β-galactosidase activity). TK is a DNA construct where luciferase expression is under the control of thymidine kinase promoter, TKenh, the enhancer element (fragment from 3′ untranslated region of the epo gene) cloned upstream of the TK promoter, pX2 the promoterless luciferase reporter vector. For details of other DNA constructs, numbers 2 through 5, refer to Figure 1.

EP17 is required for the epo enhancer element to be maximally functional

In agreement with others (11), we have found that the enhancer element located in the 3′ untranslated region of the epo gene is 5-fold more active in conjunction with the epo promoter than when cloned upstream of a heterologous promoter. It therefore suggests that factors binding to the enhancer region must, in some way, interact with those binding to the promoter region.
determine whether this interaction is mediated via factors binding to the EP17 region, three additional constructs were made and the response with respect to induction by hypoxia was compared with Δ18. These three were Δ18Enh where the 101 bp enhancer element was cloned upstream of Δ18, ΔEPA18Enh where the enhancer was cloned upstream of the EP17 deletion mutant and TKenh where the same enhancer element was cloned upstream of the thymidine kinase promoter driving luciferase expression. Each of these DNAs in combination with pCMV β-galactosidase was transfected into Hep3B cells which were then exposed to normal levels of O2 or to low O2 and luciferase activity, normalized to β-galactosidase activity, determined. As shown in Figure 1B, Δ18Enh showed ∼18-fold induction of luciferase activity in response to hypoxia. In contrast the magnitude of enhancer activity was drastically reduced (4-fold induction) when the EP17 sequence was deleted in the same construct (ΔEPA18Enh) (Fig. 1B). There was only a 5-fold induction in luciferase activity with the heterologous promoter, TKenh (Table 1).

These data suggest that the C rich EP17 region is necessary but not sufficient for the contribution of the promoter to the response to hypoxia.

Characterization of nuclear factors that bind to the EP17 region

These transfection experiments indicate that the EP17 region of the epo gene is functionally important for the hypoxia-inducible expression of the epo gene. Electrophoretic mobility shift assays were performed to identify nuclear proteins that may bind to this region. Extracts were prepared from Hep3B cells grown under normoxic and hypoxic conditions. A synthetic double stranded oligonucleotide corresponding to the EP17 sequence of the epo promoter was radiolabelled and used as a probe. As shown in Figure 2, this sequence binds to two nuclear factors (C1 and C2) in extracts from both normoxic and hypoxic Hep3B nuclei (lanes 2 and 8). Each of the EP17 binding factors was sequence specific, because the binding was competed for by excess unlabeled specific oligonucleotides EP17 (Fig. 4, lane 4) as well as by EP31, a DNA sequence from –31 to –61 in the epo gene which includes EP17 (Fig. 2, lanes 3 and 11) but not by the nonspecific oligonucleotides EP22, corresponding to –80 to –101 region of the epo gene (Fig. 2, lanes 4 and 9), or the polylinker region of the pX2 vector (Fig. 2, lanes 6 and 12). Rough quantitation showed that the C1 and C2 components were not significantly induced by hypoxia.

Since the EP17 sequence has a close homology to that recognized by SP1 or the CACCC binding factor, we performed gel shift assays with the SP1 oligomer and various others where the CACCC sites were mutated. As seen in Figure 3A, when the unlabelled SP1 binding site oligomer was used as a potential competitor, it did not displace the C1 complex although a slight competition with C2 complex was seen when a 100-fold molar excess of the SP1 oligo over the labeled EP17 was used (lanes 8, 9, 10). The specific competitor (EP31) competed for both complexes at 25-fold molar excess (lane 5). This observation was confirmed further when, in place of nuclear extracts, we used purified SP1 in the binding reaction. In this experiment, a faint band with slower mobility than the C1 complex was seen (Fig. 3B), signal was not detected in the vicinity of the C2 complex (data not shown). Using a similar amount of purified SP1 and similar binding conditions, strong binding of SP1 was shown with another sequence element (29), suggesting that despite a close homology between EP17 and the SP1 binding site, the EP17 region of the epo gene has only a weak affinity for SP1. The specificity of the Hep3B nuclear extracts was tested by experiments using a non-epo producing cell line Sol 8 which is derived from mouse skeletal muscle (30). Non-epo producing cells do not show epo promoter activity (14). Nuclear extracts from these cells in the gel shift assay showed a trace amount of only the C1 complex (Fig. 3B).
Figure 3. Characterization of factor(s) binding to the EP17 element. (A) End-labelled EP17 was incubated with 5 µg of Hep3B nuclear proteins. The molar excess of putative competitors over the labelled probe is given above each lane. The sequences of mut 1, mut 2 oligos are shown in Figure 4 and are mutant versions of the EP17 sequence. (B) The end-labelled EP17 oligo was incubated with 5 µg of Hep3B, HepG2, or Sol8 nuclear proteins or with 3 ng of purified SP1. Overnight exposure (upper panel) and 10 day exposure (lower panel) of the gel to X-ray film are shown. Positions of the C1, C2 complexes and the free probe (F) are indicated.

The properties of the C1 and C2 complexes were studied further using a mutant oligo where the polypyrimidine nature of the EP17 region was disrupted by introducing A and G in place of C at positions –53 and –59 (mut 1). When a double stranded mut 1 oligo was used as a competitor in the gel shift assay it largely competed for the faster mobility complex, C2 (Fig. 3, lanes 11, 12, 13). In some experiments we found competition for the C1 complex with mut 1 (Fig. 2, lanes 5 and 10; Fig. 4B, lane 3). To verify further the effect of this mutation on complex formation, the mut 1 oligo was radiolabeled, and used as a probe, gel shift assay showed only one complex which had the same mobility as C2 (Fig. 4B, lane 8). This binding was competed effectively by the specific competitor EP31 but not by the non-specific oligo EP22 (Fig. 4B, lanes 9, 10). Thus, by introducing two point mutations in the EP17 region, it was possible to eliminate the formation of the C1 complex. When CACCC boxes were disrupted by introduction of G in place of A at positions –50 and –56 (Fig. 4A, mut 2), the unlabeled double stranded mut 2 oligo effectively competed for both the C1 and C2 complexes (Fig. 3, lanes 14, 15, 16). When the CAC sequence from positions –49 to –51 and from –55 to –57 was replaced by TTT (mut 3), and the double stranded 17 mer used as a probe, neither the C1 nor C2 complex was formed (Fig. 4B, lane 11).

To determine the functional significance of the C1 and C2 complexes in hypoxic induction of the epo gene we introduced specific mutations corresponding to mut 1, mut 2 and mut 3, into the Δ18 plasmid by PCR. The wild type (Δ18) and its mutant variants were transfected into Hep3B cells, which were then grown under normoxic or hypoxic conditions and the extracts analysed for luciferase activity. As shown in Figure 4A, activation of luciferase transcription was substantially reduced in mut 1 and mut 3 transfectants but not in the mut 2 transfectant, suggesting that the factor that binds to EP17 to yield the C1 and/or C2 complex may play a crucial role in EP17-mediated hypoxic induction of the epo gene.

Further characterization of the factors binding to the EP17 region was performed by UV cross-linking analysis. Complexes were first run on a native gel, exposed to UV radiation and the bound complexes eluted and resolved on an SDS gel. The C1 complex had an approximate molecular weight of 52 kDa and the C2 complex 25 kDa (Fig. 5).

DISCUSSION

We have identified a DNA element, EP17, at –61 to –45 relative to the transcription start site of the mouse epo gene, that is involved in hypoxia-regulated epo gene expression in Hep3B cells. Transfections of Hep3B cells with a human 117 bp epo promoter/luciferase construct conferred 4-fold induction in reporter activity due to hypoxia. Internal deletion or point mutations in EP17 resulted in loss of response to hypoxia but the basal expression remained unaltered, suggesting that factors binding to the EP17 element are activators of epo gene transcription in hypoxic cells. These data are in agreement with earlier reports documenting the role of the promoter region in hypoxia-induced regulation of the epo gene (11,12,31). Two earlier reports (13,32) in contrast failed to show the presence of an hypoxia-responsive element in the 5′ flanking region.

In the present study, when the EP17 region was placed upstream of a heterologous promoter, there was no induction by hypoxia, suggesting that it must interact with other elements in the epo promoter region. It is interesting to note that a DNA element (P-1) located adjacent to the EP17 element (from –65 to –117) was shown to bind to factors in Hep3B nuclear extracts and to regulate the hypoxia-induced increase in epo gene transcription (11). Neither the P-1 (11) nor the EP17 (the present study)
Figure 4. Effect of various mutations in EP17 sequence on reporter gene activity and factor binding. (A) In Δ18 (WT) plasmid, containing 117 bp upstream sequence and luciferase reporter gene, nucleotides at position –53 and –59 were replaced by G and A (mut 1), at position –50 and –56 by G (mut 2), –49 to –51 and –55 to –57 by T (mut 3). Both wild type and mutant plasmids were transfected into Hep3B cells, exposed to 20% or 2% oxygen. The activation of luciferase activity due to hypoxia is shown as fold increase. (B) End-labelled EP17 (WT), mut 1 or mut 3 oligos were incubated with 5 µg of Hep3B nuclear proteins and subjected to gel shift analysis. The specificity of DNA–protein interaction was examined by addition of 100-fold molar excess of various oligonucleotides as possible competitors. Competitors ‘a’ and ‘b’ are defined in the legend to Figure 2.

Elements were found active on a heterologous promoter. Further when EP17 was either deleted or point mutated in the 117 bp epo promoter/reporter construct, any induction contributed by the P-1 element was lost. These data suggest a possible interaction of the P-1 and EP17 binding factors. Our data, in addition, suggest that EP17 acts cooperatively with the enhancer element located ∼120 bp 3′ to polyA addition site of the human epo gene, because when EP17 is deleted, the hypoxia inducibility conferred by the enhancer element is not different from that found with a heterologous promoter.

Gel shift and UV cross-linking experiments with Hep3B nuclear extracts show that two proteins of apparent molecular weights of 52 kDa and 25 kDa interact with EP17. The factors C1 and C2 that bind to EP17 are not induced by hypoxia. Further, we have no evidence to suggest that these factors are modified by hypoxia because in gel-shift assay the mobility pattern of the complexes are not altered by exposure of cells to low oxygen. The increased transactivation of the epo promoter/reporter constructs seen in the transfection experiments is possibly achieved via factor–factor interaction with the epo promoter and enhancer element, that binds to a hypoxia-inducible factor, HIF-1 (15). Such an interaction may be responsible for the EP17 role in hypoxia-induced reporter expression shown in Figure 1. If so, the interaction may be transient since we see no evidence for it in mobility shift experiments possibly because of dissociation of the protein–protein complexes during electrophoresis. Unstable protein–protein interaction was demonstrated for a homeodomain protein, Phox, and the serum response factor (SRF), for target sequence element, SRE. In this study Phox-1 could modulate the functional response of SRF and the rate of SRF–SRE association/dissociation in the absence of its own target binding sequence and without altering the mobility of the SRF–SRE complex in mobility shift assay (33). With the recent cloning of HIF-1 cDNA (18), it may now be possible to study the interaction of the EP17 DNA–protein complex with HIF-1.

Gel shift analysis with mutated EP17 indicated that in each of the two CAC sequences the flanking Cs are essential for binding to C1 and C2 but that the A can be replaced by G without affecting binding. Cross-linking studies, however, show that the apparent molecular weights of C1 (52 kDa) and C2 (25 kDa) are different from those of SP1 (130 kDa) or of an SP1 related transcription factor (82 kDa) that bind to the CAC sequence (34,35).

The mechanisms by which hypoxia affects epo gene transcription are unclear. Reports from various laboratories indicate that there are several points in the epo gene where trans-acting factors could regulate epo gene transcription. For example, the enhancer element is shown to bind to at least two transcription factors, one of which (HIF-1) is induced in response to hypoxia (15). The protein factor binding to the P-1 element is shared by the enhancer element and is not induced by hypoxia (11). The two protein factors binding to EP17 described in this study are not induced by hypoxia and their molecular weights do not correspond to the
previously described factors involved in regulation of epo gene transcription. Based on these studies we propose that the induction of HIF-1 due to hypoxia may allow a cooperative interaction of various constitutively expressed factors interacting with the 3′ region of the enhancer element, the P-1 element and the EPI7 region. These interactions may lead to the strong induction of epo gene transcription following exposure of Hep3B cells to hypoxia.

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