Functional analysis of an oxygen-regulated transcriptional enhancer lying 3' to the mouse erythropoietin gene

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ABSTRACT Erythropoietin, the major hormone controlling red-cell production, is regulated in part through oxygen-dependent changes in the rate of transcription of its gene. Using transient transfection in HepG2 cells, we have defined a DNA sequence, located 120 base pairs 3' to the poly(A)-addition site of the mouse erythropoietin gene, that confers oxygen-regulated expression on a variety of heterologous promoters. The sequence has the typical features of a eukaryotic enhancer. Approximately 70 base pairs are necessary for full activity, but reiteration restores activity to shorter inactive sequences. This enhancer operates in HepG2 and Hep3B cells, but not in Chinese hamster ovary cells or mouse erythroleukemia cells, and responds to cobalt but not to cyanide or 2-deoxyglucose, thus reflecting the physiological control of erythropoietin production accurately.

Oxygen-regulated gene expression is widely observed in biological systems (1–3). Several control systems have been defined in prokaryotes (1, 2) and yeast (3) but little is understood about the mechanisms operative in higher animals. Feedback control of erythropoiesis by erythropoietin (Epo) is perturbed by changes in blood oxygen tension and hemoglobin affinity (4), indicating that the sensor controlling Epo production responds to tissue oxygenation itself. Confirmation of this, for one source of Epo, was obtained in hepatoma cell lines HepG2 and Hep3B, which were shown to regulate Epo production in culture in response to oxygen tension (5). Although the range of cell types responsible for Epo production in normal liver and kidney remains unclear (6, 7), regulated expression of Epo by hepatoma cells presumably represents at least one of the oxygen-sensing systems operating physiologically. This view is supported by the observation that in these cells, as in normal liver and kidney, Epo production is stimulated by both cobalt and hypoxia, and control is achieved primarily through modulation of mRNA levels (4, 8, 9).

To determine the cis-acting sequences responsible for regulation, we have measured inducible expression of constructs derived from the mouse Epo gene in transiently transfected HepG2 cells.

MATERIALS AND METHODS

Transient Transfection and Experimental Incubation Conditions. HepG2 and Hep3B cells were grown in minimal essential medium with Earle's salts supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (50 units/ml) and streptomycin sulfate (50 µg/ml). Chinese hamster ovary (CHO) and murine erythroleukemia (MEL) cells were grown in RPMI 1640 medium with the same supplements.

In all experiments, cells from each transfection were divided into aliquots for parallel 16-hr incubations under control (normoxic) and test conditions. For transfection,

≈10⁷ cells were mixed with molar equivalent quantities of test plasmid equal to 50–150 µg for different-sized constructs. A control plasmid containing 10 µg of either an α₁-globin gene or a ferritin-growth hormone (FGH) fusion gene, without Epo sequence, was added to permit correction for transfection efficiency. A 1-mF capacitor array charged at 375 V was discharged through the cuvette and the cell suspension was divided for parallel incubations. Normoxic incubation was in humidified air with 5% CO₂. Hypoxic incubation was in an atmosphere of 1% O₂, 5% CO₂, and 94% N₂ in a Napco 7100 incubator. In pharmacological studies, substances were added to the culture medium as follows: cobaltous chloride (50 µM), 2-deoxyglucose (5, 10, or 25 mM), or potassium cyanide (10, 100, or 1000 µM).

RNA Analysis. RNA was prepared using a modified single-step acid/guanidinium thiocyanate/phenol/chloroform extraction method (RNAzol B, Cinna/Biotech Laboratories) and was assayed by RNase protection. Continuously labeled RNA probes were produced using [α -³²P]GTP and the SP6 system. For analysis of Epo mRNA, the RNA probe was transcribed from either *Xba* I–*Sac* I or *Xba* I–*Bal* I fragments of genomic sequence that crossed the cap site and protected 214 base pairs (bp) and 67 bp, respectively, in correctly initiated transcripts. For the *Sma* I-deleted construct (see Fig. 1), this sequence was deleted, and a probe protecting a portion of exon V was used. The α₁-globin and FGH RNA probes also crossed the cap sites and protected 97 bp and 145 bp of the respective first exons. In α₁-globin and FGH mRNA assays, 3 µg of total RNA was subjected to double hybridization with the appropriate probes. In Epo mRNA assays, 30 µg of total RNA was used and the expression of the cotransfected plasmid was analyzed separately on a 3-µg aliquot of total RNA. Hybridization was performed at 60°C in 80% formamide/40 mM Pipes, pH 6.4/400 mM NaCl/1 mM EDTA and RNase digestion was performed at 20°C for 30 min. Protected fragments were subjected to denaturing PAGE and quantified by measuring radioactivity of excised portions of the dried gel in an LKB flat-bed scintillation counter (Pharmacia-Wallac, Turku, Finland). Values are related to expression of the cotransfected control plasmid.

Plasmids. Recombinant plasmids were grown in *Escherichia coli* DH5α and purified on a cesium chloride gradient. Mouse Epo DNA sequence was obtained by screening a BALB/c mouse genomic library in bacteriophage λ EMBL3 (Clontech).

Epo constructs with 5' and 3' deletions were made in pBluescript SKII (Stratagene) and pAM19 (Amersham) by using the restriction enzymes indicated in Figs. 1 and 4, respectively.

Plasmids containing linked Epo and α₁-globin genes were made by inserting a *Bgl* II–*Ppu*MI fragment containing the intact human α₁-globin gene with 1.4 kilobases (kb) of 5' sequence, in either orientation, into the *Not* I site of pBluescript SKII with appropriate linkers. Portions of mouse Epo

sequence were inserted into adjacent polylinker sites by using the restriction enzymes indicated in Fig. 2. Precise deletions through the active sequence (*Apa* I–*Pvu* II restriction fragment) were made by PCR amplification of plasmids using a specific primer in the sequence of interest and a common primer in the plasmid, placed so that the appropriate cloning sites were included in the product. Rearrangements of the sequence were generated either by concatamerization of PCR products or by digestion of the *Apa* I–*Pvu* II fragment with *Hae* III and religation.

The control FGH plasmid contained 290 bp of the promoter of the mouse ferritin heavy-subunit gene fused with 90 bp of 5' noncoding sequence of the human growth hormone gene.

Direct plasmid sequencing for determination of unknown sequence at the 3' end of the Epo gene and for verification of closely deleted and rearranged constructs was performed by the dideoxy chain-terminator method.

S1 Nuclease Mapping. S1 nuclease mapping was performed using two overlapping 3'-end-labeled probes. Hybridization was at 55°C with 250 μ g of total RNA from hypoxic mouse kidney and 30 μ g of total RNA from transfected HepG2 cells expressing mouse Epo transcripts. S1 nuclease digestion was at 30°C.

RESULTS

Oxygen-Dependent Expression of the Mouse Epo Gene in HepG2 Cells. Following transient transfection into HepG2 cells, constructs containing a 7.6-kb *Xba* I fragment that included the entire mouse Epo gene together with ≈ 0.4 kb of 5' sequence and 3.4 kb of 3' sequence were expressed in an oxygen-dependent manner with a time course similar to that of the endogenous gene. As with the endogenous gene (5), optimal hypoxic induction was observed in an atmosphere containing 1% oxygen.

RNAse mapping demonstrated two sites of initiation, one corresponding to the correct cap site in anemic mouse kidney (10) and one lying ≈ 140 bp 3' (Fig. 1). Transcripts from both sites showed oxygen-dependent regulation. The effect of 5' deletions on expression is also shown in Fig. 1. Although the total amount of transcript was increased for the deleted constructs, the ratio of hypoxic to normoxic expression ($\approx 5:1$) was similar for each deletion, even in the +198 construct, which lacked all 5' upstream sequence and almost all of the 5' untranslated region. The deletion to –9 abolished initiation from the correct cap site, and initiation occurred instead from sequences upstream in the plasmid. Thus oxygen-dependent modulation was observed in transcripts arising from the correct cap site, a second downstream initiation site, and unknown sequences within the plasmid.

Expression of cotransfected plasmids containing an intact human α_1 -globin gene or the FGH fusion gene showed no evidence of hypoxic regulation, indicating that the property was conferred by DNA sequence lying within, or 3' to, the Epo gene.

Expression of α_1 -Globin–Epo Fusion Gene Constructs. Since the poly(A)-addition site of the mouse Epo gene was unmapped and evidence of oxygen-dependent changes in mRNA stability had been obtained (11), we linked the Epo gene to the α_1 -globin gene to determine whether oxygen-dependent regulation could be conferred on that gene, distinguishing transcriptional from posttranscriptional effects. When the α_1 -globin gene was placed in either orientation 5' to the Epo gene, oxygen-dependent expression was indeed conferred on α_1 -globin (Fig. 2). A series of deletions localized this activity within a 123-bp *Apa* I–*Pvu* II fragment situated just 3' to the gene. A total of seven constructs that contained this region all showed a similar level of hypoxic induction, whereas six constructs that did not contain this element but that covered all of the other 6 kb of DNA sequence between

FIG. 1. RNase protection assay showing oxygen-dependent expression of the mouse Epo gene after transient transfection into HepG2 cells. Alternate lanes are from normoxic (20% O₂) and hypoxic (1% O₂) cells. Deletion sites are shown schematically and indicated above each lane. For the first two constructs (lanes 1–4), two sites of initiation are observed, one corresponding to the normal cap site (band A), and a second lying 140 bp 3' (band B). Deletions to –9 and beyond abolished initiation from the correct cap site, and the size of the longer protected fragment (lanes 5 and 6) indicates that the transcripts were initiated from upstream sequences in the plasmid. The initiation site was not determined in the *Sma* I-deleted construct (lanes 9 and 10) since expression was measured using a different RNA probe, which protected a portion of exon V. Oxygen-dependent expression was observed from all constructs irrespective of the initiation site.

the *Xba* I and *Nco* I sites did not show hypoxic induction (Fig. 2). The activity was independent of orientation and was manifest at distances of up to 5.5 kb from the α_1 -globin promoter. The element also conveyed oxygen-regulated expression on the ferritin gene promoter (data not shown). Using the PCR to generate successive close deletions from each end of the enhancer, we defined a minimal element of ≈ 70 nucleotides that was necessary and sufficient for full activity. Deletions within the minimal element either severely reduced or abolished activity (Fig. 3). However, evidence of functionally discrete regions within the sequence was provided by the finding that reiteration of sequence that had minimal activity restored full enhancer activity. This was observed in two constructs both of which contained two copies of the first 60 nucleotides. In these constructs, reiterated sequences were oriented differently with respect to each other, and in one, the elements were separated by 67 bp of plasmid sequence (Fig. 3).

Enhancer Action on the Epo Gene. To confirm that this sequence operated on the Epo gene itself, the 123-bp *Apa* I–*Pvu* II fragment was excised from the 3' end of the mouse Epo gene. Expression was reduced to an undetectable level. When the first 96 nucleotides of the *Apa* I–*Pvu* II fragment containing the enhancer sequence were inserted as an *Xba* I-linked PCR amplification product into the *Xba* I site lying 0.4 kb 5' to the Epo cap site, oxygen-dependent expression was completely restored (Fig. 4).

Anatomy of the 3' End of the Mouse Epo Gene. S1 nuclease mapping localized the poly(A)-addition site in both normal mouse kidney and mouse Epo transcripts from transiently transfected HepG2 cells ≈ 120 bp 5' to the *Apa* I site. Thus, the 3' untranslated region of the mouse gene is significantly

FIG. 2. Oxygen-regulated expression of α_1 -globin after transient transfection of HepG2 cells with constructs containing an intact α_1 -globin gene linked to portions of the mouse Epo gene. (A) Position of the linked human α_1 -globin and mouse Epo genes used in constructing the α_1 -globin-Epo plasmids. Restriction sites used in making the deletions are indicated. Exons are marked by stippling. The table shows the amount of human α_1 -globin mRNA accumulated in normoxic (20% O₂) and hypoxic (1% O₂) cells when each of the indicated deletion fragments of the mouse Epo gene was linked to the α_1 -globin gene. Values give the expression of α_1 -globin relative to that of the cotransfected control plasmid and refer to the mean of two to five experiments each. The ratios of hypoxic to normoxic expression are also shown (R). Oxygen-regulated expression is shown to depend on the presence of the Apo I-Pvu II fragment indicated by the solid box. (B) RNase protection assays demonstrating oxygen-regulated expression of α_1 -globin in some of the constructs used in A. Alternate lanes show normoxic and hypoxic α_1 -globin expression and expression of the control plasmid (FGH). The Apo gene fragments in the constructs are indicated above each lane. The presence of the Apo I-Pvu II fragment is indicated below the autoradiograph and its orientation with respect to the α_1 -globin gene is indicated (+ or -). This element conveys regulated expression independently of distance or orientation with respect to the α_1 -globin promoter.

longer than that of the human gene (approximately 820 bp vs. 560 bp). The DNA sequence of the Apo I-Pvu II fragment is shown in Fig. 3. This region is $\approx 80\%$ identical to a region in the human Epo gene lying 125 bp 3' to the poly(A)-addition site.

Stimulus Specificity. To determine whether the physiology of Epo regulation was reflected in the operation of the enhancer, we exposed the transiently transfected HepG2 cells to cobaltous chloride (50 μ M) immediately after transfection. Fig. 5A compares the actions of cobaltous chloride and hypoxia on α_1 -globin expression in constructs where α_1 -globin was linked to Epo constructs with and without the enhancer. Responsiveness to cobaltous chloride was conveyed to the α_1 -globin gene by the presence of the enhancer, although the stimulus was somewhat less effective than hypoxia. Further experiments defined the same minimal element of ≈ 70 bp as necessary and sufficient for this response. To determine whether other potentially damaging metabolic inhibitors, which might create a nonspecific cellular stress, could induce enhancer-mediated transcriptional changes, cells transfected with the construct containing α_1 -globin linked to the Apo I-Pvu II fragment were exposed to cyanide (10, 100, or 1000 μ M), and to 2-deoxyglucose (5, 10, or 25 mM). None of the doses of either of these agents induced enhancer-mediated transcriptional changes (Fig. 5B).

Tissue Specificity of the Enhancer. In studies of tissue specificity of the enhancer, we found activity in Hep3B cells, where the same minimal element was required, although a

somewhat greater level of inducibility was seen. However, in CHO and MEL cells, neither constitutive nor inducible enhancer activity was observed. The possibility that the α_1 -globin reporter was refractory to further increases in transcription in MEL cells was excluded by the demonstration that even higher levels of α_1 -globin expression could be produced by the operation of a 1.9-kb *HindIII* fragment containing the second hypersensitive site of the β -globin locus control region (12) (data not shown).

DISCUSSION

We have demonstrated a cis-acting regulatory element at the 3' end of the Epo gene that acts on linked heterologous promoters irrespective of distance and orientation and thus has the typical features of a eukaryotic transcriptional enhancer. The length of the minimal element, 70 bp, suggests that its function requires interaction with multiple DNA-binding proteins. For some inducible enhancers, such as that controlling expression of the β -interferon gene (13), deletion analysis within the enhancer has defined positive and negative regulatory elements. In our deletion analysis, we did not obtain evidence of such an organization. However, our analysis did not include internal mutations and it remains possible that these might delineate functionally discrete elements within the sequence. Evidence in favor of this is provided by the observation that while a single truncated copy of the enhancer (positions 1-60) was inactive, reiteration of this sequence restored full activity. In the constructs

FIG. 3. (A) Nucleotide sequence of the 123-bp *Apa* I-*Pvu* II fragment containing the enhancer. The *Hae* III sites are underlined. (B) Activity of constructs containing portions of this sequence. Nucleotides are numbered as in A and the bars give the ratios of hypoxic to normoxic expression. Construct X contains two copies of nucleotides 1-60 separated by a 67-bp polylinker spacer. Construct Y contains the nucleotide sequence 59-26, 59-26, 4-59, 25-1.

containing these reiterations, the two truncated elements were in opposite orientations, and in one case, were separated by 67 bp of polylinker sequence. Comparison of the sequence in these elements (positions 1-60) with that of the missing 20 bp (positions 61-80) did not reveal any similarity. Thus reiteration does not, in any simple way, restore the sequence lost when nucleotides 61-80 are deleted. However, any repeated DNA conformation may be cryptic. Studies of other enhancers have shown these to be built up from a

FIG. 4. RNase protection assays demonstrating enhancer function in the 6-kb *Xba* I-*Nco* I fragment containing the Epo gene transiently transfected into HepG2. Alternate lanes show normoxic (20% oxygen) and hypoxic (1% oxygen) Epo expression. Bands arise from protection of correctly initiated transcripts. In lanes N, the transfected plasmid was intact. In lanes D, the *Apa* I-*Pvu* II portion had been deleted from the construct. In lanes R, the *Apa* I-*Pvu* II fragment was also deleted but nucleotides 1-96 of that fragment were reinserted into the *Xba* I site at the 5' end of the Epo gene. Constructs are illustrated schematically above the autoradiograph. Restriction sites: X, *Xba* I; A, *Apa* I; P, *Pvu* II; Nc, *Nco* I.

FIG. 5. Action of cobalt, cyanide, and 2-deoxyglucose on the Epo enhancer. (A) RNase protection assay showing the action of hypoxia and cobaltous chloride (50 μ M) on the expression of α_1 -globin from constructs containing Epo sequence coupled to the α_1 -globin gene. As is the case for the endogenous gene, cobalt is a somewhat less effective stimulus than hypoxia. The cobalt-dependent modulation is again conveyed by the *Apa* I-*Pvu* II restriction fragment. Expression of the control plasmid is shown for comparison (FGH). (B) Action of cyanide and 2-deoxyglucose on the expression of constructs containing Epo sequence linked to the α_1 -globin gene. Numbers indicate the ratio of expression under pharmacological or hypoxic stimulation to that with no added stimulus. No action of cyanide or 2-deoxyglucose was observed.

number of discrete, sometimes dissimilar, elements (14, 15). While function is lost with the removal of one of these, it can be restored by reiteration of the remaining elements without stringent requirements for spacing between them (15).

The finding that the minimal element conveys responsiveness to both hypoxia and cobalt exposure, but not to cyanide and 2-deoxyglucose, strongly supports the physiological relevance of this element in the control of Epo production and indicates that this response is distinct from other stress responses (16). The operation of both cobalt and hypoxia on the same minimal element also supports the view of Goldberg *et al.* (5) that these stimuli operate through a common mechanism.

For constructs containing the enhancer sequence linked to the α_1 -globin gene, mRNA accumulation was consistently 5- to 10-fold greater with hypoxic incubation than with normoxic incubation. Expression was also increased 2- to 3-fold under normoxic conditions when similar α_1 -globin-containing plasmids, with and without the Epo enhancer, were compared. Thus the total increase in expression arising from enhancer function was up to 20-fold.

These changes in transcription are less than the 100- to 200-fold modulation in mRNA levels seen *in vivo* (8), but they are similar to changes in transcriptional rate observed in nuclear run-on assays from mouse kidney and Hep3B cells (9, 11). Oxygen-dependent changes in mRNA stability, which could amplify the effects of changes in transcriptional rate on Epo mRNA accumulation, have also been described in Hep3B cells (11). When we excised the enhancer element, mRNA accumulation was almost undetectable, so that we

could not confidently analyze these other influences on Epo gene expression. Comparison with the nucleotide sequence for the human Epo gene revealed $\approx 80\%$ identity between the mouse sequence and a region lying 125 bp 3' to the poly(A)-addition site in the human gene. Recent reports have described enhancer activity 3' to the human Epo gene (ref. 17; see Note).

By electrophoretic mobility-shift assays with nuclear extract from normal or cobalt-treated mouse kidneys, cobalt-dependent DNA-binding activity has been defined to nucleotides -61 to -45 relative to the start site of transcription of the mouse gene (18). In our experiments, we observed a similar ratio of hypoxic to normoxic gene expression when this region was deleted. The total level of expression was increased in our 5'-deleted constructs, but given the shift in transcriptional start from the Epo promoter to unknown sequences within the plasmid, the significance of this finding is unclear. Many explanations are possible for the apparent discrepancy between DNA binding studies in mouse kidney nuclear extract and the current functional studies in HepG2. One possibility is of differences between the mechanisms controlling gene expression in liver and kidney. This has been suggested by studies in transgenic mice in which regulated expression of human Epo transgenes containing 0.7 kb of 3' sequence and up to 6 kb of 5' sequence was observed in liver but not in kidney (19, 20). Recently, Semenza has also reported the regulated expression in kidney of a human transgene containing 10 kb of 5' sequence (21). Such a distant 5' sequence may itself operate to provide oxygen-dependent control in kidney or may operate to bring the Epo promoter under control of the 3' enhancer.

In Hep3B, enhancer-like activity has also been reported within a 255-bp restriction fragment lying in the 3' untranslated region of the human gene (22). The element we have described is not homologous with this region and we did not detect enhancer activity in either HepG2 or Hep3B using α_1 -globin-Epo constructs that contained either this region from the human cDNA or the homologous region from the mouse. Although our experiments define a minimal element that is necessary and sufficient for enhancer activity in our system, it is quite possible that under different conditions, interactions with other sequences assume rate-limiting activity.

Maneuvers designed to interfere with heme synthesis or with the liganding properties of heme affect Epo production in Hep3B cells and have led Goldberg *et al.* (5) to propose that the signaling mechanism operates through conformational change in a putative heme protein. Based on similar responses, such a mechanism has also been proposed in oxygen-dependent control of platelet-derived growth factor β -chain expression (23). A heme-protein sensor in the Fix L/J system controls oxygen-regulated gene expression in *Rhizobium meliloti* (2), and heme is involved in oxygen-regulated gene expression in yeast (3). These observations suggest that similar mechanisms of oxygen-regulated gene expression may operate widely in nature. We have not identified any homology in our sequence with DNA binding sites for oxygen-regulated transcription factors in lower organisms, but we hope that definition of this oxygen-regulated enhancer element will permit further analysis of mechanisms of oxygen-regulated gene expression that are of broad biological and medical significance.

Note. Since submission of this manuscript for review, a report has appeared (24) describing the differential binding of nuclear factors

from normal and anemic animals to a region located 3' to the human Epo gene. Functional enhancer activity in a 256-bp fragment from that region was also demonstrated. This region contains the area that is homologous to the mouse minimal functional element we have described.

Note Added in Proof. Further experiments (25) using transient transfection of Hep3B cells with Epo minigene constructs have identified an enhancer in a 150-bp *Apa* I-*Pst* I fragment from the 3' flanking region of the human Epo gene, homologous to the mouse minimal functional element we have described.

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