Erythropoietin enhancer stimulates production of a recombinant protein by Chinese hamster ovary (CHO) cells under hypoxic condition

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Abstract

Oxygen is a limiting nutrient in animal cell culture and its supply is still worthy of improvement for production of useful proteins with a high efficiency. From a different point of view, development of the system by which a high productivity can be maintained even under hypoxic condition as well as under normoxic condition may be important. A number of hypoxia-inducible genes have been found in eucaryotic cells and the induction in most cases, if not all, is due to hypoxic activation of the gene transcription. Transcription of erythropoietin gene is highly hypoxia-inducible and the induction is achieved by binding of a protein, which is widely distributed in animal cells, to a short DNA stretch (erythropoietin enhancer) in the 3' flanking region of erythropoietin gene. Using a hepatoma cell line (Hep3B) that produces the endogenous erythropoietin in an oxygen-dependent manner and Chinese hamster ovary cells that have been widely used for production of recombinant proteins, we show that, under hypoxic condition, the erythropoietin enhancer can activate not only the promoter of erythropoietin gene but also promoters of cytomegalovirus early genes and eucaryotic polypeptide chain elongation factor gene, both of which are very active in animal cells under normoxic condition.

Abbreviations: Epo – erythropoietin; bp – base pair; CHO – Chinese hamster ovary; HIF – hypoxia-inducible factor; CMV – cytomegalovirus; EF-1 α – elongation factor 1 α ; LDH – lactate dehydrogenase

Introduction

In animal cell cultures for production of recombinant proteins, oxygen is a limiting nutrient because of its low solubility in the medium. Other nutrients can be added in excess amounts at a time but oxygen has to be supplied continuously to maintain cell viability and productivity. To maintain appropriate oxygen levels in animal cell cultures, a variety of physical methods for aeration with reduced detrimental effect on cells have been developed (Kelley *et al.*, 1993). Due to fragility of animal cells to physical force, however, oxygen supply is still one of the processes worthy of improvement. Since oxygen plays a dominant role in the metabolism and viability of cells, many organisms

adapt to oxygen deficiency by activating expression of the specific genes. The use of the mechanism underlying the hypoxic-activation of the genes may provide an alternate biological method with high productivity of recombinant proteins even under low oxygen concentrations, complementing the physical methods for aeration.

In mammalian cells a number of hypoxia-inducible genes have been found (Bunn and Poyton, 1996). They include genes encoding Epo (Goldberg *et al.*, 1988; Krantz, 1991; Jelkmann, 1992), glycolytic enzymes (Semenza *et al.*, 1994; Firth *et al.*, 1995), glucose transporters (Ebert *et al.*, 1995), and vascular endothelial growth factor (Shweiki *et al.*, 1992; Stein *et al.*, 1995; Forsythe *et al.*, 1996). Epo gene has been studied most

intensively. Epo is a major stimulator of erythropoiesis (Krantz, 1991; Jelkmann, 1992; Bunn and Poyton, 1996) and more recently a novel function as a neurotrophic factor in the central nervous system has been proposed (Masuda *et al.*, 1993, 1994, 1997; Nagao *et al.*, 1995; Morishita *et al.*, 1996, 1997). Transient assays have revealed that a short DNA stretch (~40 bp) located 3' to the poly(A)-addition site of Epo gene contains the enhancer element required for the transcriptional activation in hypoxia (Beck *et al.*, 1991; Semenza *et al.*, 1991; Pugh *et al.*, 1991; Blanchard *et al.*, 1992; Semenza and Wang, 1992; Madan and Curtin, 1993). The function of this enhancer is dependent on binding of a nuclear factor termed hypoxia-inducible factor-1 (HIF-1) (Semenza and Wang, 1992) to the enhancer sequence 5'-TACGTGCT-3' (Semenza and Wang, 1992; Wang GL and Semenza GL, 1993a). The Epo 3' enhancer stimulates the transcription of reporter genes in hypoxia in many cells that do not produce Epo (Maxwell *et al.*, 1993) and most, if not all, of the hypoxic-inducible genes contain the sequences that are homologous to the human Epo enhancer and capable of binding to HIF-1 (Semenza *et al.*, 1994). Taken together with the wide distribution of HIF-1-like proteins from mammalian to fly cells (Wang and Semenza, 1993b, 1993c; Nagao *et al.*, 1996), it appears that a variety of cells carry the hypoxia-induced signal transduction pathway leading to the stimulated transcription of specific genes through HIF-1 activation.

Although the Epo promoter is extensively activated in hypoxia through the raised enhancer function, its poor basal-promoter activity in normoxia makes it undesirable to use combination of the Epo promoter and enhancer for production of recombinant proteins. In this paper we examined if the Epo enhancer can stimulate the function of strong transcriptional promoters (CMV and EF-1 α promoters) in CHO cells, widely used as a host for production of recombinant proteins. CMV promoter drives human cytomegalovirus immediate early genes and contains several enhancer elements (Sambucetti *et al.*, 1989). EF-1 α is an eucaryotic polypeptide chain elongation factor that stimulates the GTP-dependent binding of an aminoacyl-tRNA to ribosomes and a powerful expression vector containing EF-1 α promoter has been constructed (Mizushima and Nagata, 1990). Thus, both promoters are potent in driving eucaryotic gene expression.

Materials and methods

Construction of pUC-4En containing four tandem copies of Epo enhancer

A blunt-ended *ApaI*-*PvuII* fragment containing the entire Epo enhancer was cloned into the *Bam*HI and *EcoRV* sites of pBluescriptIIKS(-) (Stratagene, California, USA) to produce pB·En. A 0.2-kbp *SacI*-*ApaI* fragment of pB·En contained Epo enhancer and this fragment was subcloned into the *SacI* and *ApaI* sites of pEpoPA-Luc (see below) to prepare pEpo-P-Luc-E. The Epo enhancer excised from pEpo-P-Luc-E by *EcoRI* digestion was cloned into pUC18, yielding pUC·En. pUC·2En containing two copies of the enhancer was constructed by ligation of an *AatII*-blunted *XhoI* fragment and an *AatII*-blunted *Bam*HI fragment from pUC·En. Likewise, pUC·3En and pUC·4En were obtained by ligation of a pUC·En-derived *AatII*-blunted *XhoI* fragment with an *AatII*-blunted *Bam*HI fragment of pUC·2En and pUC·3En, respectively.

Construction of luciferase expression plasmid under control of Epo promoter

phgEpo was prepared by subcloning an *EcoRI* fragment of λ hEpo41 (Goto *et al.*, 1988), which contained 8-kbp 5' flanking region, the entire coding region and 0.7-kbp 3' flanking region of human Epo gene, into the *EcoRI* site of pUC19. The human Epo promoter region (*ApaI*-*Eco52I* fragment) was excised from phgEpo and then cloned upstream (*HindIII* site) of the luciferase cDNA of pSVOALD5' (deWett *et al.*, 1987) to prepare the reporter plasmid of Epo promoter, pEpoPA-Luc. The human Epo enhancer region (*ApaI*-*PvuII* fragment) was also cloned downstream (*Bam*HI and *ApaI* sites) of the splicing donor-acceptor and poly(A) addition sites of pEpoPA-Luc. The resulting reporter plasmid, pEpoPLE, contains Epo promoter upstream of luciferase cDNA, and Epo enhancer downstream of luciferase cDNA. p8.0k-Luc·En was generated by ligating a blunt-ended *HindIII* fragment of pEpoPLE with a blunt-ended *Eco52I*-*SalI* fragment of phgEpo. p8.0k-Luc·En consists of 8.0-kbp 5' flanking region of Epo gene, luciferase reporter gene and Epo enhancer in this order. p3.5k-Luc·En was derived by ligation of an *AatII*-blunted *NheI* fragment and an *AatII*-blunted *EcoRI* fragment from p8.0k-Luc·En. p3.5k-Luc was produced by removal of the *Bam*HI-*SseI* fragment that con-

tained Epo enhancer from p3.5k-Luc·En. p0.2k-Luc·En was constructed by ligation of two fragments, a 0.47-kbp *AatII*-blunted *EcoRI* fragment and an *AatII*-blunted *ApaI* fragment of p3.5k-Luc·En. p0.2k-Luc was prepared from p0.2k-Luc·En by removal of the *BamHI*-*SseI* fragment. pEn-0.2k-Luc, which contained Epo enhancer upstream of 200-bp promoter, was prepared by ligation of three fragments, an *AatII*-*ApaI* fragment and an *AatII*-*EcoRI* fragment of p3.5k-Luc, and an *EcoRI*-*ApaI* fragment of pEpo-P-Luc-E. p0.2k-Luc·4En was obtained by insertion of a *BamHI*-blunted *ApaI* fragment of pUC·4En into a *BamHI*-blunted *SseI* fragment of p0.2k-Luc·En. p4En-0.2k-Luc was produced by insertion of an *ApaI*-*BamHI* fragment of pUC·4En into a *BamHI*-*ApaI* fragment of pEn-0.2k-Luc. pCMV·Luc·En was prepared by inserting a *BamHI*-*ApaI* fragment of pB·En into the *ApaI* site of pCMX (Umesono *et al.*, 1991).

Construction of Epo expression plasmid under control of CMV promoter

The full length human Epo cDNA was generated by transfection of pZIP-Neo(X)1-EPO to ψ 2 cells, recovered by the plasmid rescue method and then subcloned into the *SmaI* and *BamHI* sites of M13mp8 (Matsumoto *et al.*, 1993). The plasmid containing the full length Epo cDNA was named as M13#7. pCMV·Epo was constructed by inserting a 0.8-kbp *Sau3AI* fragment of human Epo cDNA from M13#7 to the *BamHI* site of pCDNA3. Four tandemly repeated Epo enhancer was excised from an *EcoRI* fragment of pUC·4En and cloned into pBluescriptIISK(–) (Stratagene, California, USA) to prepare pB·4En. p4En·CMV·Epo and p4Enr·CMV·Epo contain four copies of Epo enhancer in the upstream of CMV promoter in forward and opposite orientation, respectively. They were prepared by insertion of a *BamHI* fragment of pB·4En into the *BglIII* site of pCMV·Epo. pCMV·Epo·4En was constructed by insertion of a *XbaI*-*ApaI* fragment of pUC·4En into the *XbaI* and *ApaI* sites of pCMV·Epo.

Construction of Epo expression plasmid under control of EF-1 α promoter

A 0.7-kbp *PstI*-*BamHI* poly(A) addition signal fragment of pCDM8 (Invitrogen Co., California, USA) was subcloned into the *PstI* and *BamHI* sites of pBluescriptIISK(–) to prepare pB·PA. pEF·Epo(-PA) contains EF-1 α promoter and human Epo cDNA but no poly(A) signal. This plasmid was produced by lig-

ating three fragments, a *HindIII*-*BamHI* fragment of pBluescriptIISK(–), a *HindIII*-*EcoRI* fragment of pEF·BOS (Mizushima S and Nagata S, 1990, a kind gift from Dr. Nagata) and a *Sau3AI* Epo cDNA fragment from M13#7. pEF·Epo was constructed by subcloning a 0.7-kbp *XbaI* fragment containing poly(A) addition signal of pB·PA into the *XbaI* site of pEF·Epo (-PA). A blunted *EcoRI* fragment containing four tandem copies of Epo enhancer was excised from pUC·4En and cloned upstream of the blunted *SalI* site of pEF·Epo, producing two plasmids, p4En·EF·Epo and p4Enr·EF·Epo which contained four tandem copies of Epo enhancer in the upstream of EF-1 α promoter in forward and opposite orientations, respectively. An *EcoRI* fragment of pUC·4En was cloned downstream of the blunted *BstXI* site of pEF·Epo to prepare pEF·Epo·4En and pEF·Epo·4Enr.

Cell culture

Hep3B cells were maintained with Dulbecco's modified Eagle's medium containing 10% fetal calf serum (growth medium in Hep3B). CHO cells were maintained with Minimum Essential Medium Alpha Medium containing ribonucleotides, deoxyribonucleotides and 10% fetal calf serum (growth medium in CHO). The cells were cultured under normoxic condition (5% CO₂, 21% O₂ and 74% N₂ atmosphere) in a Napco Model 5100 CO₂ incubator at 37 °C.

Transient reporter assay

Plasmids were transfected into Hep3B cells by LIPO-FECTIN (Life Technologies Inc., Maryland, USA) or CHO cells by LIPOFECTAMINE (Life Technologies Inc., Maryland, USA) following the protocol from the manufacturer. CHO (5×10^4 cells) or Hep3B (1×10^5 cells) were seeded in the 6 well plate (Nunc A/S, Roskilde, Denmark) and cultured for 16 h before transfection. Cells were cotransfected with 0.5 μ g of test plasmid and 0.05 μ g of p β act β gal (Seiler-Tuyns, A. *et al.*, 1984), which carried the β -galactosidase gene under the control of chicken β -actin promoter. This plasmid containing β -galactosidase gene was used to normalize transfection efficiency. The cells were cultured for 5 h, and the medium was replaced with the fresh one. After culture for 16 h under normoxia, the medium was changed to the fresh medium. For a given test plasmid, cells in two plates (2x2 wells/plasmid) were transfected at the same time. One of the plates was incubated for further 48 h under nor-

moxia, the other, for 48 h under hypoxia (1% O₂, 5% CO₂ and balance N₂) which was generated in an oxygen-regulated incubator, MODEL 9200 (WAK-ENYAKU CO., LTD, Kyoto, Japan). Then, expression of the reporter gene (luciferase and Epo) was assayed. In luciferase assay, cell extracts were prepared using PicaGene Reporter Lysis Buffer (TOYO INK MFG. CO., LTD, Tokyo, Japan), and used for measurement of transiently expressed luciferase and β -galactosidase. Luciferase activity was measured by Lumat LB9051 (EG&G Berthold, Bad Widbad, Germany) with Luciferase Assay Kit (TOYO INK MFG. CO., LTD, Tokyo, Japan), and β -galactosidase activity was measured by using chlorophenol red- β -D-galactopyranoside as a substrate (Eustice *et al.*, 1991). Luciferase activity was normalized with the β -galactosidase activity expressed by the cotransfected plasmid. In Epo assay, Epo in the spent medium was measured by EIA (Goto *et al.*, 1989), and the β -galactosidase activity in the cell extract was measured as described above.

Permanent cell lines producing Epo

CHO cells were cotransfected with two plasmids by the use of LIPOFECTAMINE; one plasmid contained Epo cDNA and the other plasmid, pcDNA3 (Invitrogen Co., California, USA), contained *neo* gene to confer G418 resistance to the transfected cells. The colonies resistant to 400 μ g ml⁻¹ G418 were picked up and examined Epo production by EIA. The CHO clones producing Epo were plated at a density of 5×10^4 cells in 24 well plate (Nunc A/S, Roskilde, Denmark) and cultured for 12 h at normoxia. The medium was changed to the fresh medium. Then, one plate was placed under normoxia (21% O₂) and the other plate, under hypoxia (1% O₂) for 48 h. Epo in the spent medium was measured by EIA, and cellular proteins were determined by protein assay kit (Bio-Rad Lab., California, USA) (Masuda *et al.*, 1994).

Results and discussion

Figure 1 depicts plasmids used in this experiment.

Transient expression

Before preparation of permanent cells, we assessed effect of Epo enhancer on expression of reporter genes (luciferase and Epo genes) by transient assay using

CMV and EF-1 α promoters.

Since it has been documented that Epo enhancer functions very effectively in Hep3B (Bunn and Poyton, 1996), we examined if Epo enhancer activates CMV promoter in hypoxia by assaying oxygen-dependency of the transient luciferase expression in Hep3B using seven plasmids as shown in Fig. 2. Hypoxic activation of the 200-bp Epo promoter without the enhancer was very small (2-fold) but the promoter with the enhancer was greatly activated by hypoxia (30~100-fold), agreeing with the results previously reported (Blanchard *et al.*, 1992). Epo enhancer linked upstream of Epo promoter was more effective for hypoxic induction than when the enhancer was linked downstream of the reporter gene (compare p0.2k-Luc·En with pEn-0.2k-Luc). The presence of four copies of the enhancer in the upstream of Epo promoter (p4En-0.2k-Luc) showed the highest expression of luciferase under 1% O₂. CMV promoter is far powerful than that of Epo promoter regardless of oxygen concentration. In the absence of Epo enhancer, the CMV promoter activity was rather reduced in hypoxia, while the promoter activity was hypoxia-inducible in the presence of the enhancer. Although the stimulation degree (1% O₂/21% O₂) is low when compared with that of Epo promoter, CMV promoter is very active and therefore its stimulation, even if it is minute, is significant with respect to production of recombinant proteins.

To examine if this hypoxic potentiation of CMV promoter by Epo enhancer in Hep3B operates for a secretory protein, we have constructed plasmids containing human Epo cDNA (Matsumoto *et al.*, 1993). The Epo enhancer containing four tandemly repeated copies of Epo enhancer element was linked, in either the forward or the opposite orientation, upstream of the promoters or downstream of the reporter Epo gene. We assayed the transient production of human Epo in culture media and its oxygen-dependency. Hep3B cells produce endogenous human Epo but its production is far lower (<1/100) than that produced by transfected plasmids. Figure 3 shows results, including those of EF-1 α promoter. The results were very similar to those obtained by using CMV promoter and luciferase (see Fig. 2). The activity of both CMV and EF-1 α promoters without Epo enhancer was slightly stimulated (in CMV promoter; pCMV·Epo) or rather reduced (in EF-1 α ; pEF·Epo) in hypoxia, while the presence of four copies of the enhancer in the downstream of Epo gene resulted in greater production of Epo in hypoxia than in normoxia (see pCMV·Epo·4En, pEF·Epo·4En and

Plasmids	Orientation	Nomenclature							
p0.2k·Luc	<table><tr><td>P</td><td>Luc</td></tr></table>	P	Luc	0.2k or <table><tr><td>P</td></tr></table> : Epo promoter	P				
P	Luc								
P									
p0.2k·Luc·En	<table><tr><td>P</td><td>Luc</td><td>E</td></tr></table>	P	Luc	E	En or <table><tr><td>E</td></tr></table> : Epo enhancer	E			
P	Luc	E							
E									
pEn·0.2k·Luc	<table><tr><td>E</td><td>P</td><td>Luc</td></tr></table>	E	P	Luc	Enr or <table><tr><td>Enr</td></tr></table> : Epo enhancer in reverse orientation	Enr			
E	P	Luc							
Enr									
p0.2k·Luc·4En	<table><tr><td>P</td><td>Luc</td><td>E</td><td>E</td><td>E</td><td>E</td></tr></table>	P	Luc	E	E	E	E	<table><tr><td>CMV</td></tr></table> : CMV promoter	CMV
P	Luc	E	E	E	E				
CMV									
p4En·0.2k·Luc	<table><tr><td>E</td><td>E</td><td>E</td><td>E</td><td>P</td><td>Luc</td></tr></table>	E	E	E	E	P	Luc	<table><tr><td>EF-1α</td></tr></table> : EF-1α promoter	EF-1α
E	E	E	E	P	Luc				
EF-1α									
pCMV·Luc	<table><tr><td>CMV</td><td>Luc</td></tr></table>	CMV	Luc	Luc or <table><tr><td>Luc</td></tr></table> : Luciferase cDNA	Luc				
CMV	Luc								
Luc									
pCMV·Luc·En	<table><tr><td>CMV</td><td>Luc</td><td>E</td></tr></table>	CMV	Luc	E	<table><tr><td>Epo</td></tr></table> : Epo cDNA	Epo			
CMV	Luc	E							
Epo									
pCMV·Epo	<table><tr><td>CMV</td><td>Epo</td></tr></table>	CMV	Epo						
CMV	Epo								
pCMV·Epo·4En	<table><tr><td>CMV</td><td>Epo</td><td>E</td><td>E</td><td>E</td><td>E</td></tr></table>	CMV	Epo	E	E	E	E		
CMV	Epo	E	E	E	E				
p4En·CMV·Epo	<table><tr><td>E</td><td>E</td><td>E</td><td>E</td><td>CMV</td><td>Epo</td></tr></table>	E	E	E	E	CMV	Epo		
E	E	E	E	CMV	Epo				
p4Enr·CMV·Epo	<table><tr><td>Enr</td><td>Enr</td><td>Enr</td><td>Enr</td><td>CMV</td><td>Epo</td></tr></table>	Enr	Enr	Enr	Enr	CMV	Epo		
Enr	Enr	Enr	Enr	CMV	Epo				
pEF·Epo	<table><tr><td>EF-1α</td><td>Epo</td></tr></table>	EF-1α	Epo						
EF-1α	Epo								
pEF·Epo·4En	<table><tr><td>EF-1α</td><td>Epo</td><td>E</td><td>E</td><td>E</td><td>E</td></tr></table>	EF-1α	Epo	E	E	E	E		
EF-1α	Epo	E	E	E	E				
pEF·Epo·4Enr	<table><tr><td>EF-1α</td><td>Epo</td><td>Enr</td><td>Enr</td><td>Enr</td><td>Enr</td></tr></table>	EF-1α	Epo	Enr	Enr	Enr	Enr		
EF-1α	Epo	Enr	Enr	Enr	Enr				
p4En·EF·Epo	<table><tr><td>E</td><td>E</td><td>E</td><td>E</td><td>EF-1α</td><td>Epo</td></tr></table>	E	E	E	E	EF-1α	Epo		
E	E	E	E	EF-1α	Epo				
p4Enr·EF·Epo	<table><tr><td>Enr</td><td>Enr</td><td>Enr</td><td>Enr</td><td>EF-1α</td><td>Epo</td></tr></table>	Enr	Enr	Enr	Enr	EF-1α	Epo		
Enr	Enr	Enr	Enr	EF-1α	Epo				

Figure 1. Structures of plasmids.

pEF·Epo·4Enr). Disposition of the enhancer upstream of the promoter is more effective for Epo production under hypoxia but the enhancer orientation does not appear to have profound effect on the production (see p4En·CMV·Epo, p4Enr·CMV·Epo, p4En·EF·Epo and p4Enr·EF·Epo). Thus, Epo enhancer activates CMV or EF-1α promoter-directed production of a secretory protein, Epo, under low oxygen concentration in Hep3B.

Next, we examined the transient expression of Epo in CHO cells being frequently used for production of recombinant proteins (Fig. 4). In both CMV and EF-1α promoters without Epo enhancer, Epo production was lower in hypoxia than in normoxia (see pCMV·Epo and pEF·Epo). The presence of four copies of Epo enhancer, however, not only prevented this hypoxic decrease but also caused higher Epo production in hypoxia than in normoxia (see p4En·CMV·Epo and

Figure 2. Transient expression of the reporter gene, luciferase, by Hep3B cells. After transfection of Hep3B cells with each plasmid, the cells were cultured for 5 h and then cultured for further 16 h in the fresh medium under normoxia. After replacement with the fresh medium, one of the two plates was incubated under normoxia (21% O₂) for 48 h and the other, under hypoxia (1% O₂). Luciferase expressed for 48 h was assayed. Transfection efficiency was normalized by the use of expression of β -galactosidase gene cotransfected. (□) normoxia, (▨) hypoxia. The cells in two wells were transfected with one plasmid and luciferase activity of two wells was averaged. Ratios of luciferase activity under hypoxia and normoxia are shown. Each column is the mean (n=2).

p4En-EF-Epo), suggesting that Epo enhancer potentiates CMV and EF-1 α promoter activity in CHO cells in hypoxia. We have no data that explain the hypoxic decrease in the activity of CMV and EF-1 α promoters without Epo enhancer, but it is conceivable that the cellular concentration of transcriptional factor(s) to activate CMV and EF-1 α is reduced in hypoxia because binding sites for a variety of the transcriptional regulator proteins have been defined in these promoters. The hypoxic induction of Epo production in the presence of Epo enhancer in Hep3B cells (Fig. 3) was always larger than that in CHO cells (Fig. 4). These results indicate that Epo enhancer potentiates the CMV and EF-1 α promoter activity in Hep3B and CHO cells under hypoxic condition but CHO cells might contain a component(s) required for the full expression of the enhancer activity at a lower level than Hep3B cells do. Indeed, the HIF-1 site is essential but not enough for the functional activity of Epo enhancer and hepatocyte nuclear factor-4 has been shown to activate Epo

enhancer activity (Galson *et al.*, 1995); Hep3B cells may contain such co-activator at a high level enough for the full expression of Epo enhancer activity, while its level in CHO cells may be very low.

Permanent CHO cell lines producing Epo

Transient expression experiments showed that Epo enhancer is effective for supporting powerful activity of CMV and EF-1 α promoters in hypoxic condition. Then we prepared permanent CHO cells in which transcription of Epo cDNA is under control of CMV or EF-1 α promoter with or without Epo enhancer. When CMV promoter was used, we established 15 clones without the enhancer and 15 clones with the enhancer. The amounts of Epo produced by the clones harboring the plasmid without Epo enhancer ranged 3–99 ng mg⁻¹ cellular protein/24 h under normoxia and the clones with the enhancer produced 0.8–55 ng. When EF-1 α promoter was used, we established total 85 clones (45

Figure 3. Transient expression of the reporter gene, Epo, by Hep3B cells. Hep3B cells were treated as described in the legend of Fig. 2. Epo produced into the medium for 48 h was assayed. Transfection efficiency was normalized by the use of expression of β -galactosidase gene cotransfected. (□) normoxia, (▨) hypoxia. The cells in two wells were transfected with one plasmid and Epo production of two wells was averaged. Ratios of Epo under hypoxia and normoxia are shown. Each column is the mean (n=2).

Figure 4. Transient expression of the reporter gene, Epo, by CHO cells. CHO cells were treated as described in the legend of Fig. 2. Epo produced into the medium for 48 h was assayed. Transfection efficiency was normalized by the use of expression of β -galactosidase gene cotransfected. (□) normoxia, (▨) hypoxia. The cells in two wells were transfected with one plasmid and Epo production of two wells was averaged. Ratios of Epo under hypoxia and normoxia are shown. Each column is the mean (n=2).

clones without and 40 clones with the enhancer). Production of Epo by the clones containing the plasmid without Epo enhancer was 0.3–103 ng mg⁻¹ cellular protein/24 h under normoxia, while Epo production in the presence of the enhancer was 0.3–93 ng. We determined Epo production by all clones under normoxic and hypoxic conditions and the production ratio

of individual clone was calculated. Figure 5 shows the number of clones with defined ratios. When CMV promoter without Epo enhancer was used, Epo production in most clones decreased to a large extent upon hypoxia (Fig. 5A). In the presence of the enhancer, however, the hypoxic decrease was small and furthermore several clones showed the hypoxic induction (Fig. 5B). When

Figure 5. Hypoxic inducibility of the recombinant human Epo production by permanent CHO clones. CHO clones harboring the plasmid shown in figure were established. Epo produced into the medium under normoxia (21% O₂) and hypoxia (1% O₂) was measured in triplicate assays and the values were averaged. The number of clones with defined ratios (hypoxia/normoxia) are shown. Transcription of Epo cDNA was driven by CMV promoter without Epo enhancer in A, CMV promoter with the enhancer in B, EF-1α promoter without the enhancer in C, and EF-1α promoter with the enhancer in D.

EF-1α promoter with Epo enhancer was used, there were some clones in which Epo production decreased upon hypoxia but most clones showed higher Epo production under hypoxia than that under normoxia; one clone showed 3-fold production in hypoxia (Fig. 5D). In the absence of the enhancer, the hypoxic decrease of Epo production was seen in 25 of total 45 clones (Fig. 5C). There were several clones with higher Epo production in hypoxia but the hypoxic stimulation in most of these clones was very low (1.2~1.8-fold). Thus, the use of promoter with Epo enhancer was very effective to obtain the clones with hypoxic induction of Epo production.

Finding several clones that harbored the plasmid without Epo enhancer showed the hypoxic induction of Epo production was rather surprising. In these clones,

the plasmid could be integrated in the vicinity of an enhancer element responsible for hypoxic induction of a gene in host cells, because a number of genes with the hypoxic inducibility have been found (Bunn and Poyton, 1996). In transient assays with CHO cells, Epo enhancer potentiated the activity of CMV and EF-1α promoters in hypoxia when compared with their promoter activity without the enhancer but the hypoxia-induced activity of EF-1α promoter did not surpass the promoter activity without the enhancer (see p4En-EF-Epo and pEF-Epo in Fig. 4). In contradiction with this result, most of the clones that harbored the plasmid with Epo enhancer showed the promoter activity higher than that in normoxia (see Fig. 5D). The results of transient experiments may involve oxygen-dependency of the cytotoxicity and intracellular stabil-

ity of the plasmids.

In summary, Epo enhancer is useful to maintain the activity of promoters in hypoxia but further improvement is necessary to develop the production system with a large hypoxic activation. In this context, preliminary experiments with the hypoxic-responsible enhancer of LDH (Firth *et al.*, 1995) shows that the promoter activity of LDH in normoxia is almost comparable with those of CMV and EF-1 α promoters and that LDH enhancer potentiates the cognate promoter activity with high efficiency (~ 10 -fold) in hypoxia. LDH is an ubiquitous house-keeping enzyme and therefore LDH enhancer may operate in a variety of cells, while the full function of Epo enhancer is restricted to a cell type such as Hep3B cells, which produce Epo inherently in an oxygen-dependent manner. Detail analyses including carbohydrate structure of Epo produced in normoxia and hypoxia by the use of LDH enhancer/CHO system are in progress.

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