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IN VITRO RECONSTITUTION OF AN ERYTHROPOIETIN GENE TRANSCRIPTION SYSTEM USING ITS 5'-FLANKING SEQUENCE AND A NUCLEAR EXTRACT FROM ANEMIC KIDNEY

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We have developed an *in vitro* transcription system for the erythropoietin (Epo) gene. This system uses a plasmid carrying 0.2 kb of 5'-flanking sequence from the human Epo gene, rNTPs and a nuclear extract from mouse kidney. The transcribed RNA was assayed by primer extension with an end-labeled primer complementary to the sequence of the plasmid, dNTPs and reverse transcriptase. The primer extension product corresponding to the transcript was detected on a sequencing gel. The *in vitro* promoter activity of the Epo 5'-flanking sequence was observed with a nuclear extract from anemic kidney but not with that from normal kidney.

Erythropoiesis is known to be induced by erythropoietin (Epo), a glycoprotein with a molecular mass of about 34 kilodalton (1). It is produced principally in adult kidney and fetal liver under the control of tissue oxygen levels (2). Epo mRNA was reported to be actively transcribed (3) and accumulate (4,5) in hypoxic kidney. It was also shown that hypoxia increased the levels of Epo mRNA in human hepatoma cell lines, Hep3B and HepG2 (6). Induction of Epo in Hep3B cells during hypoxia is controlled mainly at the transcriptional level (7,8).

The human and mouse Epo genes have been cloned and sequenced (9-13). These Epo genes are highly conserved and are 4 kilobase (kb) long, comprising five exons. The most highly homologous region in each gene consists of the 0.14 kb upstream of the capping site (12,13). Using the 4 kb complete human Epo gene, with the 0.4 kb 5'-flanking region included,

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transgenic mice were generated in which the human Epo mRNA levels increased during anemic hypoxia (14,15). In addition, hypoxia was reported to enhance transcription of the human Epo gene carrying the 0.4 kb 5'-flanking region in Hep3B cells (7). It can be assumed, therefore, that the 0.4 kb Epo 5'-flanking region contains the active promoter for inducible gene expression in response to hypoxia.

However, the physiological promoter region for Epo gene expression has not yet been defined. All previous experiments to analyze the Epo transcription mechanism were based on the long genomic fragment that included several introns, 5'- and 3'-flanking sequences (7,14), and the use of a non-physiological hepatoma cell line, Hep3B (7,8). Furthermore, inducible expression for the human Epo gene was observed in anemic liver but not in anemic kidney of the transgenic mice (14,15), even though kidney is the main organ for Epo gene expression *in vivo* in response to anemic hypoxia (4,5). To analyze the physiological mechanism of Epo gene expression in response to hypoxia, it is essential to develop an *in vitro* transcription system. In this paper, we show that 0.2 kb of 5'-flanking sequence of the human Epo gene is sufficient for transcription using a nuclear extract from anemia-hypoxic mouse kidney.

MATERIALS AND METHODS

Animal protocols. Severe anemia was induced in male BALB/c mice (8 week) by X-ray (500 R) irradiation followed by two intraperitoneal injections of 60 mg/kg phenylhydrazine sulfate (PHZ) at 72 and 120 hr after the irradiation. At 48 hr after the second PHZ administration, severely anemic mice were sacrificed. Sera and kidneys from normal and severely anemic mice were stored at -70°C until use.

Assay of Epo. The sera from normal and severely anemic mice were assayed by the sandwich-type enzyme-linked immunosorbent assay (ELISA) with monoclonal anti-Epo antibodies (16). Total RNA was prepared from the mouse kidneys and assayed by dot blot hybridization using a random-primed probe of the full-length cDNA encoding human Epo (11). All molecular biological techniques were based on the standard protocols (17).

Cloning of the 5'-flanking region of the human Epo gene and the SV40 early promoter. From the human Epo gene (11), the HindIII-Eco52I fragment (0.4 kb) of the 5'-flanking region was subcloned into pUC19. This recombinant plasmid was designated as pEpoPH. The PvuII-HindIII fragment (0.36 kb) of the SV40 early promoter was subcloned from pSV2neo (18) into pUC19 and its plasmid was designated as pSV40EP.

Preparation of nuclear extracts from mouse kidneys. The kidneys from normal and severely anemic mice were homogenized in a buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10mM KCl and 0.5 mM DTT. Crude nuclear extracts were prepared according to the protocol described (19). All nuclear extracts were stored at -70°C until the measurement of protein concentrations and *in vitro* transcription assays were performed.

In vitro transcription. Linearized templates H and A (Figure 1) were generated by digestion of pEpoPH with HindIII and ApaI, respectively. pSV40EP was linearized with EcoRI and used as a template for a positive control. *In vitro* transcription was performed in a mixture (200 µl) of 100 ng template DNA, 1mg crude nuclear extract, 10 mM HEPES (pH 7.9), 12.5 % glycerol, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM PMSF, 0.25 mM DTT, 50 mM KCl, and 0.1 mM rNTPs. To test whether RNA polymerase II is involved in this transcription, 0.1 or

100 μg/ml α-amanitin (Boehringer) was added to the mixture. The reaction mixture was incubated at 30°C for 45 min. The sample was treated with 10 U RNase-free DNase I (Takara) at 37°C for 10 min. Phenol/chloroform extraction and isopropanol precipitation were then performed.

Primer extension. The oligonucleotide primer (17mer) complementary to the sequence downstream of the cloned fragment in each template was end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase (Toyobo). The end-labeled primer was purified by phenol/chloroform extraction and gel filtration. The *in vitro* transcription product precipitated as described above was resuspended in a solution (10 μl) containing the end-labeled primer (0.1 μg). The mixture was incubated at 65°C for 10 min and hybridization was performed at 30°C for 12 hr. Primer extension was performed in a mixture (30 μl) of the RNA-primer hybrid, 0.5 mM dNTPs, 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM DTT, 5 mM $MgCl_2$, 40 U RNasin (Toyobo), and 40 U reverse transcriptase from avian myeloblastosis virus (Life Sciences). The reaction mixture was incubated at 42°C for 90 min. The reaction was stopped by incubation with 1 μl of 0.5 M EDTA (pH 8.0) and 1 μl of 1 mg/ml RNase A at 37°C for 30 min. Then phenol/chloroform extraction and isopropanol precipitation were performed. The precipitate was resuspended in a mixture of 3 μl Tris-EDTA buffer and 3 μl formamide loading buffer. The mixture was boiled for 3 min and applied on a sequencing gel with the size marker. The gel was dried and autoradiography was performed.

RESULTS

Epo concentrations in sera of severely anemic mice (hematocrits: 17 to 18 %) were determined. As shown in Table I, the Epo concentrations were much higher in the anemic mice than in normal mice. Under these conditions, Epo mRNA levels in severely anemic kidneys were at least fifteen times higher than those in normal kidneys. To test transcriptional activity of the 5'-flanking region in the human Epo gene, we prepared nuclear extracts from normal and severely anemic mouse kidneys, and constructed DNA templates H and A as shown in Figure 1. When *in vitro* transcription assays were performed using the template H and a nuclear extract from normal or severely anemic kidneys, primer extension product corresponding to the *in vitro* transcript of the template was not detected. The results suggest that the 0.4 kb HindIII-Eco52I fragment of the Epo 5'-flanking region is inactive as a promoter for *in vitro* transcription with nuclear extracts from mouse kidneys.

Table I. *Erythropoietin concentrations in sera of normal and severely anemic mice*

Condition	Epo concentration [mU/ml]
Normal	1.7 ± 0.6
Severely anemic	4875 ± 383

Sera were obtained from normal (n=5) and severely anemic mice (n=4). The Epo concentrations were determined by the ELISA. The results are expressed as the mean ± SE.

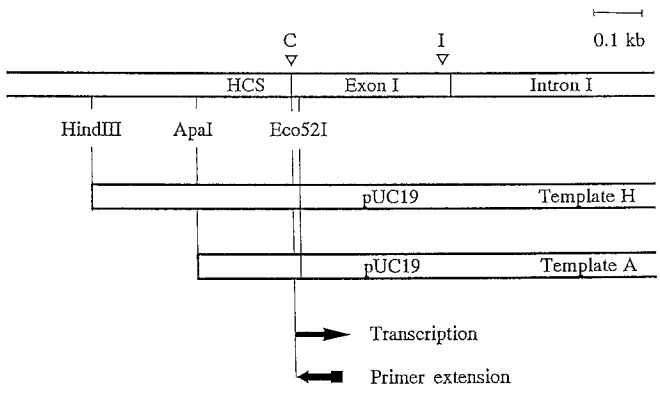

Figure 1. *Schematic representation of the 5'-structure of the erythropoietin gene, templates for in vitro transcription, and primer extension for detection of the transcript.*

The 5'-flanking region and 1st exon-intron relationship of the human Epo gene are shown. Abbreviations are as follows: HCS, highly conserved sequence; C, capping (transcription initiation) site; I, initiation codon. The HindIII-Eco52I fragment (0.4 kb) was subcloned into pUC19 and its recombinant plasmid was designated as pEpoPH. Templates H and A are generated by digestion of pEpoPH with HindIII and ApaI, respectively. The *in vitro* transcript is detected by primer extension. The primer is 17mer (5'-GTTTTCCCAGTCACGAC-3') complementary to the sequence downstream of the cloning site of pUC19. The predicted length of the primer extension of RNA which is transcribed from the native capping site is 75 nucleotides.

However, when we employed the template A and a nuclear extract from severely anemic kidneys, a significant amount of transcript was detected by primer extension as shown in lane 2 of Figure 2. The size of the primer extension coincided well with the predicted length (75 nucleotides) of RNA which is transcribed from the native capping site. In contrast, we detected no transcript using the same template and a nuclear extract from normal kidneys (lane 1), although a template to which the SV40 early promoter was linked was transcribed (upper panel). The hypoxia-inducible transcriptional activity using the template A (lane 2) was inhibited by 0.1 (lane 3) and 100 μg/ml α-amanitin (lane 4) added to the reaction mixture, thus indicating that the transcription products were not endogeneous and probably transcribed by RNA polymerase II. Therefore, the ApaI-Eco52I (0.2 kb) fragment of the Epo 5'-flanking region contains a promoter for Epo gene expression, and this region includes *cis*-regulatory element(s) that respond to hypoxia. The results also indicate that the nuclei of anemic mouse kidney contain transcription factor(s) that can activate the human Epo promoter *in vitro*.

DISCUSSION

In this paper, we have demonstrated *in vitro* transcriptional activity for the human Epo gene using its 5'-flanking sequence and a nuclear extract from mouse kidney. The activity was

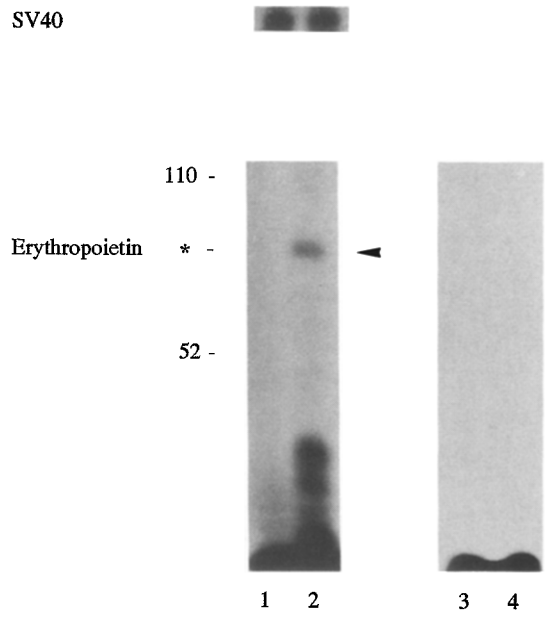

Figure 2. *Detection of the in vitro transcription product by primer extension.*

The template A including the ApaI-Eco52I fragment (0.2 kb) of the human Epo 5'-flanking region (lower panel) and the linearized pSV40EP carrying the SV40 early promoter (upper panel) were used as the DNA templates for *in vitro* transcription assays. Nuclear extracts were obtained from normal (lane 1) and severely anemic mouse kidneys (lanes 2-4). The reaction mixture was incubated in the absence (lanes 1 and 2) or presence of 0.1 (lane 3) and 100 μg/ml α-amanitin (lane 4). The *in vitro* transcript was detected by primer extension. Strong bottom bands are the end-labeled primers. The sizes in nucleotides are indicated on the left. An asterisk indicates the predicted length of RNA which is transcribed from the native capping site. An arrowhead indicates the primer extension product corresponding to the *in vitro* transcript.

induced in response to anemic hypoxia, and was highly sensitive to α-amanitin. The results indicate that anemic kidney nuclei contain factors necessary for Epo gene expression, which may include the *trans*-acting factor(s) induced in response to hypoxia and RNA polymerase II. It was previously reported that *de novo* protein synthesis is essential for Epo gene expression under hypoxic conditions (20). Furthermore, nuclear proteins that bound specifically to the 5'-flanking sequence of the human Epo gene were found to be induced in HepG2 cells (21) and mouse kidneys (22) under Epo-producing conditions such as hypoxia. These inducible binding proteins may positively regulate Epo gene transcription.

Although several experiments were previously performed to identify an essential region for physiological Epo gene expression in response to hypoxia, a distinct promoter region has not been specified. This is because the long genomic fragment of the Epo gene carrying 5'- and 3'-flanking sequences was used as a DNA template (7,14). In the present investigation, we

employed 0.2 kb of Epo 5'-flanking sequence from the human Epo gene, and found that this sequence served as an active promoter *in vitro*. Since transcriptional activation of this sequence took place in a nuclear extract from anemic kidney but not in that from normal kidney, there is strong evidence that the *cis*-regulatory element(s) for response to hypoxia are also included within this promoter region and that the extracts used in this assay reflect the physiological response to hypoxia observed *in vivo*.

It is possible, however, that total regulation of Epo gene expression *in vivo* is a more complex process, and may involve several mechanisms. For example, we could not detect the primer extension product corresponding to an *in vitro* transcript from the longer 0.4 kb fragment which included the 0.2 kb promoter region. The extra DNA may contain sequence(s) that do not function in our *in vitro* assay. In support of this is the previous observation that the promoter activity of this 0.4 kb 5'-flanking region was very weak in Hep3B cells (8). In addition, transgenic mice, containing the human Epo gene which included the 0.4 kb 5'-flanking region, showed transgene induction in liver but not in kidney under anemic conditions (14,15). Thus *cis*-acting negatively regulatory element(s), which suppress inducible Epo gene expression in kidney but not in liver during hypoxia, may be present in the sequence upstream of the 0.2 kb promoter region described here. Recently it was shown that enhancer-like element(s) which respond to hypoxia are present in the 3'-flanking region of the human Epo gene (23,24). Therefore, Epo gene expression during hypoxia is probably controlled by several *trans*-acting factors which act at regulatory elements in the 5'- and 3'-flanking regions of the Epo gene.

The results presented in this study indicate that there is a hypoxia-sensing mechanism that can induce *trans*-acting factor(s) which interact with the 0.2 kb Epo promoter sequence. Besides Epo gene expression, hypoxic stress was reported to enhance gene expression of platelet-derived growth factor-B chain (25) and aldolase (26). It would be useful to determine whether a common oxygen-sensing mechanism is involved in these different gene expression systems, and whether the same transcrption factors and *cis*-elements are employed.

Finally, since *in vitro* Epo gene transcription observed by this system resembles, in part, physiological Epo gene expression in response to anemic hypoxia, this transcription system will be useful to elucidate a molecular basis of oxygen homeostasis.

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