Erythropoietin mRNA Levels Are Governed by Both the Rate of Gene Transcription and Posttranscriptional Events

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The human hepatoma cell line, Hep3B, synthesizes large quantities of erythropoietin (Epo) mRNA and protein in a regulated manner in response to hypoxia and cobaltous chloride (CoCl₂). To further understand the regulation of Epo gene expression, we studied the effects of hypoxia and CoCl₂ on the rate of Epo gene transcription. While Northern blot analyses showed that steady-state Epo mRNA levels increase more than 50-fold in response to hypoxia or CoCl₂, nuclear run-off experiments demonstrated only a 10-fold increase in Epo gene transcription in response to these stimuli. In the presence of either actinomycin D (Act D) or cycloheximide, the stability of biologically functional Epo mRNA was much greater than that observed in the absence of these agents and much greater than that which has been reported in vivo. These findings suggest that the stability of Epo mRNA is modulated by the transcription and translation of rapidly turning over gene product(s). Thus, Epo mRNA levels are determined by both the rate of transcription and posttranscriptional events. These experiments demonstrate a potential pitfall in estimating mRNA half-lives based on Act D chase experiments alone.

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REGULATION OF the expression of highly inducible genes, such as growth factors and oncogenes, has been the subject of much investigation. The protein products of these genes are frequently quite labile and their in vivo levels may fluctuate by several orders of magnitude depending on particular physiologic conditions. The production of these proteins is dependent on precise regulation of mRNA levels. In turn, the steady-state levels of mRNA are determined by both the rate of gene transcription and the rate of mRNA decay.

Erythropoietin (Epo) is a glycoprotein hormone essential for the regulation of red blood cell production. It is produced in the fetal liver and the adult kidney in response to hypoxia and circulates in the plasma with a half-life of 4 to 7 hours. Serum levels may increase as much as 1,000-fold in severe anemia. Conversely, inappropriate overproduction of Epo may cause life-threatening polycythemia due to markedly increased blood viscosity and decreased blood flow. Thus, the Epo-synthesizing cells must possess the ability to regulate the rate of Epo production over a wide range. One of the most puzzling aspects of erythropoiesis is the mechanism by which hypoxia triggers increased expression of the Epo gene.

We have previously demonstrated that the human hepatoma cell line, Hep3B, synthesizes large quantities of Epo in a regulated manner in response to hypoxia and cobaltous chloride (CoCl₂), and that this regulation occurs at the Epo mRNA level. In addition, we have previously shown that ongoing protein synthesis is required for both the hypoxia and cobalt induction of increased levels of Epo mRNA in Hep3B cells. Northern blot analyses of RNA from murine kidneys show greater than a 100-fold increase in the level of Epo mRNA in the presence of hypoxia or cobaltous chloride. To assess changes in the rate of Epo gene transcription that might account for this marked increase in steady-state Epo mRNA levels, Schuster et al. performed nuclear run-off experiments on nuclei isolated from the whole kidneys of hypoxic and nonhypoxic mice. They could detect Epo gene transcription only in nuclei from hypoxic kidneys. A very small percentage of this mixed population of cells were potential Epo-producing cells. To gain a better understanding of the level of regulation of the Epo gene, we have examined the influence of hypoxia and CoCl₂ on Epo gene transcription and Epo mRNA stability in the Hep3B cell line, a homogeneous population of Epo-producing cells.

MATERIALS AND METHODS

Cell culture. Hep3B cells were obtained through American Type Culture Collection. They were cultured in MEM Alpha medium (GIBCO, Grand Island, NY) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% defined supplemented bovine calf serum (Hyclone, Logan, UT) and were maintained in a humidified 5% CO₂ incubator at 37°C. The cells were grown hypoxically either as described previously or in a controlled atmosphere chamber (Plas Labs, Lansing, MI) supplied with a constant flow of a hydrated 1% O₂, 5% CO₂, balance N₂ gas mixture. All experiments were begun when the Hep3B cells approached confluence.

Radioimmunoassay (RIA). The RIA for Epo was performed using a high-titer polyclonal rabbit antiserum raised in our laboratory against human recombinant Epo. Recombinant human Epo was obtained from Amersham, Inc (Arlington Heights, IL). Standards were prepared using recombinant human Epo from Amgen, Inc (Thousand Oaks, CA) diluted in MEM Alpha medium containing 10% defined supplemented calf serum (Hyclone) and 0.05% sodium azide, pH 7.4. Aliquots of 0.2 mL of standard or sample were placed in 5 mL conical polypropylene tubes. To this was added 0.1 mL of rabbit antiserum diluted 1:15,000 in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin. The mixture was diluted to 0.7 mL using the same diluent used for the recombinant Epo standards and was incubated at room temperature for 2 hours.

To Epo, 0.1 mL (approximately 10,000 cpm), prepared in the same diluent was then added, the mixture was briefly vortexed and incubated overnight at 4°C. One milliliter of Amerlex-M (Donkey antirabbit globulin, Amersham, UK) was added 0.1 mL of rabbit antiserum diluted 1:15,000 in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin. The mixture was diluted to 0.7 mL using the same diluent used for the recombinant Epo standards and was incubated at room temperature for 2 hours. The Epo-recombinant Epo standards was used for the recombinant Epo standards and was incubated at room temperature for 2 hours. The Epo-recombinant Epo standards was used for the recombinant Epo standards and was incubated at room temperature for 2 hours.

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then added to each tube and the tubes were placed at 4°C with constant shaking for 2 hours. The Amax was pelleted by centrifugation for 15 minutes at 1,500g at 4°C, washed once with 1.0 mL PBS, and counted in an LKB model 1282 gamma counter.

Northern blot analysis. Total RNA was prepared from cultured cells as described by Chirgwin et al. The RNA was denatured in formaldehyde, electrophoresed on a 1% agarose gel containing 2.2 mol/L formaldehyde and a trace amount of ethidium bromide, and transferred to a GeneScreen Plus filter (New England Nuclear, Boston, MA) using 10X standard saline citrate (1.5 mol/L NaCl, 0.15 mol/L sodium citrate). Epo cDNA in an SP65 plasmid was digested with the restriction enzyme EcoRI, the Epo insert isolated by agarose gel electrophoresis, followed by electrophoresis and transduced to a specific activity of between 3 x 10⁶ and 1.2 x 10⁷ cpm/µg of cDNA. The radiolabeled cDNA was then mixed with carrier salmon sperm DNA, denatured by boiling for 10 minutes, and hybridized to the filter at 5 x 10⁶ cpm/mL of hybridization solution (50% formamide, 1 mol/L NaCl, 1% sodium dodecyl sulfate, 10% dextran). Hybridization was performed at 42°C for 20 hours. The final washing was done in 0.5X standard saline citrate (0.075 mol/L NaCl, 0.0075 mol/L sodium citrate) at 65°C. Autoradiography was performed with intensifying screens at ~80°C using Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY).

Mouse β-actin cDNA was also radiolabeled and hybridized to the same filters to provide an internal control for the efficiency of RNA transfer to the filters.

Nuclear run-off experiments. The transcriptional component of Epo gene regulation was assessed by nuclear run-off experiments, as described by Greenberg. Hep3B cells were exposed to 75 µmol/L CoCl₂ or 1% O₂ for varying lengths of time. After isolation of nuclei by lysis of cells in NP-40 buffer, in vitro transcription was completed in the presence of α³²P-UTP. Radiolabeled RNA was then isolated and the amount of trichloroacetic acid precipitable radioactivity was quantitated. At each time point an equal amount of ³²P-RNA was hybridized to nitrocellulose filters containing a cDNA clone or a full-length, human Epo genomic clone (4.0 kb plasmid containing a linearized mouse β-actin cDNA fragment site to 0.7 kb downstream from the polyadenylation signal). A plasmid containing a linearized mouse β-actin cDNA fragment served as a positive control while a linearized plasmid containing a human β-globin genomic clone provided a negative control. Densitometric scanning was performed using a Molecular Dynamics Model 300A computing densitometer. Relative levels of Epo mRNA transcription represent arbitrary units normalized to β-actin mRNA transcription. Normalization to β-actin mRNA was used because this is an abundant mRNA and its transcriptional rate has been shown to be minimally influenced by hypoxia.6

Epo mRNA stability. Hep3B cells were placed in either a 1% or 21% O₂ containing atmosphere for 18 to 24 hours at 37°C. At “0 time” the O₂ content of the atmosphere was manipulated as described below and either actinomycin D (Act D) or cycloheximide was added to final concentrations of 5 µg/mL or 20 µg/mL, respectively. After varying periods of incubation, total cellular RNA was isolated from the cells and Northern blot analyses were performed. Densitometric scanning was performed with a Molecular Dynamics Model 300A computing densitometer. Relative Epo mRNA levels represent arbitrary units normalized relative to β-actin mRNA levels.

RESULTS

Northern blot analyses were performed on RNA obtained from Hep3B cells grown for 18 to 24 hours in either a 1% or 21% oxygen atmosphere. The RNA blots were hybridized with Epo and actin radiolabeled probes and the levels of each of these mRNAs was quantitated by densitometry as described above. Analysis of 14 such experiments demonstrated a greater than 50-fold mean increase in steady-state Epo mRNA levels (representative blots are shown in lanes 1 and 2 of Figs 2B and 3B). This stated increase in steady-state Epo mRNA levels represents a minimal estimate because densitometry loses linearity in the very low and very high ranges. In contrast, actin mRNA levels were not significantly influenced by hypoxia as estimated by concordance with the levels of 18S and 28S ribosomal RNA levels observed on the ethidium stained gels.

To assess the relative contribution of changes in the rate of Epo gene transcription to this marked increase in Epo mRNA steady-state levels, nuclear run-off experiments were performed. Initial experiments were performed with a nearly full-length Epo cDNA probe as detailed in the Materials and Methods section. However, with this probe it was difficult to detect a hybridizing signal in experiments performed on the nuclei of either hypoxic or nonhypoxic cells. In an attempt to increase sensitivity we used a human Epo full-length genomic clone that has greater homology with the unspliced primary RNA generated in the in vitro transcription reaction. With this probe we were able to detect low-level transcription even in the absence of hypoxia. In contrast to the more than 50-fold increase in steady-state Epo mRNA levels observed in RNA blot analyses, three nuclear run-off experiments performed on Hep3B cells (Fig 1) demonstrated a 5- to 10-fold increase in Epo mRNA transcription in response to hypoxia or CoCl₂. Important to the interpretation of the experiments shown in Fig 1 is the finding that the total counts per minute of ³²P-radiolabeled RNA recovered from the in vitro transcription reactions on nuclei of cells grown for 8 hours in 21% oxygen, 1% oxygen, or 75 µmol/L CoCl₂, were within 10% of one another, suggesting that 8 hours of hypoxia or exposure to 75 µmol/L CoCl₂ had no significant change on overall gene transcription.

Assuming a zero-order rate of formation of Epo mRNA and a first-order rate of mRNA decay, a mere 10-fold increase in the rate of Epo mRNA synthesis cannot by itself explain the greater than 50-fold increase in steady-state Epo mRNA levels observed after stimulation by hypoxia or cobalt. Therefore, experiments were undertaken to investigate posttranscriptional influences on steady-state Epo mRNA levels. Initially, Hep3B cells were grown in 1% O₂ to increase Epo mRNA levels, and subsequently were switched to 21% O₂. Epo mRNA levels were then determined after varying periods of time by Northern blot analyses. As demonstrated in Figs 2 and 3, in 21% O₂, Epo mRNA has a rapid rate of decay with steady-state levels decreasing by 50% within 1.5 to 2 hours and with almost undetectable levels by 6 hours. This finding is similar to reported changes in kidney Epo mRNA levels in mice switched from a hypoxic to a nonhypoxic environment, and represents a maximal estimate of the in vivo half-life of the Epo mRNA (because new transcription was not blocked). To distinguish between transcriptional and posttranscriptional con-
tions to this observed decrease in steady-state Epo mRNA levels, Act D chase experiments were performed. Initial experiments demonstrated that Act D at a concentration of 5 μg/mL blocked greater than 98% of the incorporation of [3H]-uridine into total cellular Hep3B RNA under both hypoxic and nonhypoxic conditions. Hep3B cells were made hypoxic to allow an increase in Epo mRNA levels. After the addition of Act D the cells were either kept hypoxic or rapidly equilibrated with a 21% O₂ atmosphere for various periods of time. The stability of the Epo mRNA in the presence of Act D at both O₂ tensions was much greater than that observed in the absence of Act D (Fig 2) and much greater than that reported in vivo. Thus, the addition of Act D resulted in an increased stability of Epo mRNA (estimated T₁/₂ = 7 to 8 hours). Of note, when Hep3B cells were grown continually in 1% O₂ in the absence of Act D, Epo mRNA levels continued to remain elevated (ie, did not peak and then decline) throughout the entire period of study.

If Act D was preventing the transcription of rapidly turning over mRNA whose protein product or products participate in the degradation of Epo mRNA, one would expect cycloheximide to have a similar effect on the stability of Epo mRNA. Hence, parallel experiments were performed using 20 μg/mL cycloheximide instead of Act D. These experiments demonstrated a reproducible increase in the stability of the Epo mRNA in the presence of cycloheximide as well (Fig 3). Evidence that the cycloheximide enhances Epo mRNA stability rather than de novo transcription is provided by the fact that cycloheximide blocks hypoxia and CoCl₂-induced increases in Epo mRNA and cycloheximide by itself does not cause an increase in steady-state Epo mRNA levels. However, as seen in Fig 3, the effect of cycloheximide on increasing Epo mRNA stability is not quite as marked as the effect of Act D. This may be due to the inability of cycloheximide to block translation for a sustained period of time as completely as Act D blocks transcription.

To assess the possible biologic relevance of the prolonged stability of Epo mRNA in the presence of Act D, experiments were performed to determine if this stabilized mRNA was available for translation into Epo protein. Hep3B cells were grown hypoxically for 20 to 24 hours. The culture medium was removed and frozen for subsequent determination of Epo concentration by RIA (mean ± 1 standard deviation = 91 ± 8 mU/mL, n = 32), the cells were washed thoroughly, and fresh medium was added. The cells were then grown in 1% or 21% O₂ in the presence or absence of Act D. After 14 hours the medium was removed and assayed for the presence of Epo by RIA. As shown in Fig 4, Hep3B cells switched to 21% O₂ in the presence of Act D were fully capable of sustaining Epo production and, in fact, secreted significantly more Epo protein into the culture medium than cells switched to 21% O₂ in the absence of Act D (mean ± 1 standard deviation = 52 ± 9 mU/mL vs. 33 ± 8 mU/mL, P < .05). This finding strongly supports the conclusion that the increased Epo mRNA levels observed in the presence of Act D are not due to nonspecific cytotoxic effects. Furthermore, the observation that Hep3B cells grown continually in 1% O₂ in the absence of Act D secreted even more Epo than cells grown in 1% O₂ in the presence of Act D suggests that both the rate of Epo gene transcription and posttranscriptional events influence steady-state Epo mRNA levels.
DISCUSSION

These experiments suggest that the hypoxia and cobalt induced increase in Epo mRNA steady-state levels cannot be accounted for by increased Epo gene transcription alone. As noted previously, in the nuclear run-off experiments described here, a human Epo full-length genomic clone was used to probe the in vitro transcription products. While the genomic probe allowed for increased sensitivity and enabled detection of low-level transcription even in the absence of hypoxia, it is not without potential drawbacks. If the intervening sequences include repetitive sequences that are transcribed elsewhere as well, the run-off assay may not be totally specific for Epo mRNA. In fact, in the intervening sequence between exons III and IV is a member of the Alu family of repeated sequences. This region is 70% homologous to the consensus Alu sequence. Because the 100% homologous Epo cDNA gave a relatively weak signal, it is unlikely that under the hybridization conditions used this relatively short, imperfectly conserved, repetitive sequence would give a significant hybridization signal. Furthermore, when the Epo cDNA probe was used, a weak hybridization signal was seen even from nuclei of cells grown in 21% O₂ (data not shown). This result argues strongly that some baseline Epo gene transcription is ongoing in 21% O₂. Hence, the hybridization signal seen at 21% O₂ using the genomic Epo probe is unlikely to simply represent nonspecific, background hybridization.

Like Schuster et al., we also note an increase in Epo gene transcription in response to hypoxia, although the degree of increase appears to be less. Our nuclear run-off experiments used nuclei from a homogeneous population of Epo-producing human hepatoma cells grown in continuous culture. On the other hand, Schuster et al. used nuclei from a heterogeneous population of primary murine kidney cells, only a very small proportion of which were actually Epo-producing cells. These differences may account for the difference in the degree of increase in Epo gene transcription.

The data presented in this report suggest that Epo mRNA levels are governed by a complex mechanism
REGULATION OF ERYTHROPOIETIN mRNA LEVELS

Fig 3. Epo mRNA stability. (A) [—], 21% oxygen; [— — —], 21% oxygen + cyclohex. Each data point in (A) is the average (±1 standard deviation) of at least two experiments, with most points representing three or more experiments. (B) A representative RNA blot demonstrating the effect of cycloheximide on Epo mRNA stability.

Involving both the rate of gene transcription and posttranscriptional events. The fact that steady-state Epo mRNA levels increase more than 50-fold in response to hypoxia and CoCl₂ while Epo gene transcription increases only about 10-fold in Hep3B cells suggests that there is a significant posttranscriptional component to Epo gene regulation in this cell line. Numerous examples of posttranscriptional regulation of mRNA have been well documented, particularly at the level of mRNA stability.²⁸ Many examples of regulated mRNA stability involve genes whose protein products play a crucial role in cell growth regulation and survival.²⁷⁻²⁹⁻³⁵ Although still poorly understood, multiple mechanisms for this regulation of the rate of mRNA degradation have been demonstrated; however, many of these mechanisms seem to share certain common features. In many cases mRNA degradation requires ongoing protein synthesis because stabilization occurs in the presence of various protein synthesis inhibitors.²⁵,³⁰,³¹,³³ Two explanations for the requirement of ongoing protein synthesis have been proposed: (1) certain mRNAs may be degraded by rapidly turning over specific ribonucleases, and (2) the degradation of some mRNAs may be directly coupled to their translation. Cotranslational degradation has been

Fig 4. Biologic activity of Epo mRNA. After approaching confluence, the media was changed and the cells were grown in an atmosphere containing either 1% or 21% O₂ for 20 to 24 hours. The media was then removed, the cells were washed thoroughly with PBS, and incubated with 5 mL of fresh medium with or without Act D (5 μg/mL) in either a 1% or 21% O₂ environment, as indicated in the figure, for an additional 14 hours. The media was then removed and an Epo RIA was performed in duplicate on an aliquot. Each data bar represents the average (±1 standard deviation) of eight separate experiments.
elegantly demonstrated for tubulin and histone mRNAs. On the other hand, although indirect evidence suggests the presence of rapidly turning over specific ribonucleases, thus far none of these nucleases have been well characterized.

If the expression of these putative nucleases is regulated at the level of transcription, one would expect that inhibitors of transcription as well as inhibitors of protein synthesis would stabilize the mRNA targets of these ribonucleases. This is not the case for the protein synthesis–requiring degradation of the c-myc, c-fos, and granulocyte-macrophage colony-stimulating factor mRNAs. However, the transcription inhibitor Act D does stabilize poly(I) poly(C)-induced fibroblast interferon mRNA as well as prevent the decay of human transferrin receptor mRNA. Furthermore, recent studies by Shyu et al demonstrate two independent determinants of c-fos mRNA instability, one of which appears to be dependent on ongoing transcription. Epo is another case in which mRNA instability requires both ongoing transcription and protein synthesis. However, unlike many of the other known examples, Epo’s biogenesis and its biologic actions are extremely tissue specific. The data presented here support a mechanism in which Epo mRNA degradation requires both the transcription and translation of a rapidly turning over mRNA (or mRNAs) whose protein product(s) participates in the degradation of Epo mRNA. In addition, this phenomenon clearly demonstrates a potential pitfall in relying on Act D chase experiments alone to estimate mRNA half-lives.

The evidence presented here suggesting the existence of a rapidly turning over Epo mRNA degrading protein raises the possibility that the concentration and/or the activity of this protein may be decreased during hypoxia or CoCl2 exposure. However, direct demonstration of PO2-dependent changes in Epo mRNA stability in intact Hep3B cells is impossible using Act D because the Act D itself alters the mRNA stability by blocking synthesis of the presumptive Epo mRNA destabilizing protein. One alternative might be to perform [H]-uridine pulse-chase experiments, but this approach requires medium to high abundance mRNA species. Another more direct approach, currently underway, is to characterize this Epo mRNA destabilizing protein, and the sequences to which it binds, in an in vitro system.

In the case of Epo gene regulation, the finding that steady-state Epo mRNA levels may be significantly influenced by a rapidly turning over Epo mRNA degrading protein is particularly intriguing because it may have biologic significance. The combination of the hypoxia-induced increased Epo gene transcription coupled with increased Epo mRNA stability permits a marked amplification of Epo mRNA levels. A modest increase in the rate of Epo gene transcription and a modest increase in Epo mRNA stability acting in synergy can explain how the cell is able to regulate Epo mRNA levels such that during hypoxia, circulating levels of Epo protein can increase by three orders of magnitude.

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