Abundance and Stability of Erythropoietin Receptor mRNA in Mouse Erythroid Progenitor Cells

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The abundance and stability of erythropoietin (EPO) receptor mRNA were measured in Friend virus-infected mouse splenic erythroblast (FVA) cells. FVA cells correspond to the colony-forming unit-erythroid (CFU-E) and cluster-forming unit-erythroid stages of differentiation and they mature in vitro into reticulocytes during 45 to 60 hours of culture. After 20 hours, there was a rapid decline in the EPO receptor mRNA level through the 45-hour culture period. Levels of actin and β-globin mRNAs were monitored as two representative proteins that are actively synthesized in early versus late stages of terminal erythroid differentiation. A general decrease of actin mRNA level was apparent, whereas globin mRNA levels increased throughout the culture period. The greatest decrease in both EPO receptor and actin mRNA was observed when the cell population was at the stage of basophilic and polychromatophilic erythroblasts. The half-life for EPO receptor mRNA was approximately 75 minutes, as determined using the transcriptional inhibitor actinomycin D. The half-life measured at several times during the 48-hour culture period remained constant. These results indicate that EPO receptor mRNA must be transcribed continuously until late in the maturation process of FVA cells in order to maintain the levels seen by Northern analysis. The number of copies of EPO receptor mRNA at 0 hours was determined to be 25 copies per cell. This low number and the decline of EPO receptor mRNA correlate with the low receptor numbers on FVA cells, as well as the decline of binding sites with cell maturation.

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ERYTHROPOIETIN (EPO) is a glycoprotein hormone (30,400 d) that acts as the principle regulator of erythroid progenitor cell development into red blood cells. EPO controls terminal differentiation by acting on colony-forming units-erythroid (CFU-E) and cluster-forming units-erythroid stages, as well as earlier stage progenitor cells. The cloned EPO receptor gene codes for a protein that is 55,000 d as predicted by the cDNA sequence.
no receptors can be detected once these cells have reached the stage of reticulocytes.11,19,20 The only exception to this is the observation that in murine erythroleukemia cell cultures, the number of receptors increases when induced by dimethyl sulfoxide.21,22 Recent cloning of the mouse erythropoietin receptor cDNA has provided a specific probe to study the synthesis and abundance of the receptor mRNA in erythroid progenitor cells. Because of their relative homogeneity of developmental stage and their uniform differentiation to reticulocytes in a well-defined time period in vitro, the FVA cells have allowed us to follow precisely the fate of EPO receptor mRNA during terminal differentiation of these cells in vitro. In addition, the half-life and the mRNA copy number for FVA cells have been determined.

MATERIALS AND METHODS

Materials. CD/F1 mice were obtained from the National Institutes of Health, Bethesda, MD. RQD Nase and pGEM TZ plasmid were purchased from Promega, Madison, WI. Human recombinant EPO was a gift from Ortho Pharmaceutical, Raritan, NJ. RNAzol solution was purchased from Cinna/Biotex Laboratories, Friendswood, TX. The EPO receptor cDNA clone 1904 was provided by Genetics Institute, Cambridge, MA. Mouse α-actin clone, PAM91,23 was a gift from Michael J. Getz, Mayo Foundation, Rochester, MN. Plasmids containing mouse β-major globin gene and its flanking regions24 were provided by James Darnell, Rockefeller University, New York, NY.

Cell culture. Erythroid progenitor cells were obtained from spleens of mice infected with the anemia strain of Friend leukemia virus (FVA). A highly pure population of these cells was isolated by velocity sedimentation in a continuous gradient of bovine serum albumin. A highly pure population of these cells was isolated by velocity sedimentation in a continuous gradient of bovine serum albumin. The actin probe was a PstIIPstII fragment containing the entire a-actin coding region obtained from mouse actin clone PAM91. This probe recognizes one or both of the actin mRNAs (β and γ) present in FVA cells.25 The mouse β-globin probe was the HindIII/HindIII fragment containing the first two exons from the β-major globin gene.

RNA isolation and Northern analysis. Total RNA was extracted from mouse erythroid progenitor cells by a single-step guanidium thiocyanate-phenol method using RNAzol.25 RNA was electrophoresed in 1.9% agarose gels containing 0.66 mol/L formaldehyde according to the method described by Davis et al.26 Each lane contained 20 μg of RNA. Before electrophoresis, 10 μg of ethidium bromide was added to each sample. After electrophoresis, the gels were photographed and blotted onto nitrocellulose. Prehybridizations and hybridizations were done in 50% formamide, 6× SSC (1× SSC = 0.15 mol/L NaCl, 0.015 mol/L sodium citrate), 1× Denhardt's solution (0.02% Ficol, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), and 0.5% sodium dodecyl sulfate (SDS). DNA probes were labeled by the random primer labeling method28 using [α-32P]dCTP (800 Ci/mmol). Hybridizations were performed at 42°C for 16 to 20 hours using 1× 106 cpm of the labeled probes per milliliter of hybridization fluid. Blots were washed with 2× SSC, 0.5% SDS at room temperature for 40 minutes, followed by 0.1× SSC, 0.1% SDS at 55°C for 1.5 hours. Quantitation of the autoradiographic signal was performed by a laser scanning densitometer (Bio-Rad video densitometer model 620, Richmond, VA). The relative amounts of total RNA present in each lane were estimated by scanning a photographic negative of the ethidium bromide-stained gel over the 18S RNA band. Then for each lane, the autoradiographic hybridization signal was normalized to the amount of total RNA present in each lane by comparing it with the peak area of 18S RNA in the scan of the stained gel. The signals on the autoradiograms and the photographic negatives of the ethidium bromide-stained gels were within the linear range of the densitometer. Control experiments also showed that the estimates of RNA amounts based on the photography and scanning of stained gels were linear with respect to actual amounts of RNA loaded onto the gels.

EPO receptor mRNA copy number determination. The 1.42-kb XhoI/Cla I fragment of the EPO receptor gene was subcloned into a pGEM 7Z vector. The plasmid containing the insert was then linearized with Clal restriction enzyme to construct a template for in vitro transcription. High-yield transcription reactions using unlabeled nucleotides were performed according to the protocol suggested in the Promega technical bulletin. DNA template was removed by addition of DNase (1 U/μg DNA) to the RNA synthesis reaction and incubating at 37°C for 15 minutes, followed by phenol/chloroform extraction and ethanol precipitation. Quantitation of the synthesized RNA was performed by taking OD260 readings. This was also confirmed by running a formaldehyde/agarose gel of the transcribed RNA in a parallel lane to a known amount of RNA molecular weight marker and visualizing the ethidium bromide-stained gel. All of the visible stained product RNA migrated as a single band of 1.4 kb in size. Immediately following the quantitation, mouse kidney RNA (15 μg) was added to 10 μg of the transcribed EPO receptor RNA before performing serial dilutions. Serial dilutions of the in vitro synthesized RNA were done in deionized H2O containing 10 μg/mL kidney RNA. Also prior to electrophoresis of the transcribed RNA, 5 μg of mouse kidney RNA was mixed with each sample. These additions of mouse kidney RNA assured the presence of sufficient carrier RNA to prevent loss of the in vitro transcript during dilution, electrophoresis, or blotting. Mouse kidney RNA yielded no hybridization signal with the EPO receptor probe (see Results). Electrophoresis and Northern blot hybridizations were performed as described previously using the 1.42-kb XhoI/ClaI DNA fragment as the probe. Quantitation of the autoradiograms was made by densitometry. The number of pico moles of EPO receptor mRNA present in FVA cell RNA samples was determined by comparison with a standard curve constructed using known amounts of in vitro transcribed RNA. The standards of in vitro transcript and the erythroid progenitor cell RNA were analyzed on the same Northern blot. The number of copies of EPO receptor mRNA was calculated using a value of 1.5 × 106 of total RNA per 1× 106 FVA cells.9

RESULTS

FVA cells cultured with EPO expressed EPO receptor mRNA at levels that were easily detectable by the Northern
ERYTHROPOIETIN RECEPTOR mRNA

A 0
28s
18s

mRNA decreases with time of culture as the erythroid cells mature.

Normalization of the autoradiographic signal to the amount of RNA (18S) present on each lane allowed us to directly analyze the changes occurring in the level of EPO receptor mRNA in the erythroblasts during their maturation (0- to 45-hour culture period). The experiment represented by Fig 1 was repeated three times (four times for points up to 20 hours). Although ribosomal RNAs provided standards for any loading differences among lanes, actin and globin transcript levels were monitored as two representative proteins that decrease and increase respectively during the 0- to 45-hour time period. These were analyzed as controls to assure that the cells of the particular experiments were undergoing the differentiation program.

Figures 2 and 3 show the results of analyzing such timed RNA samples for actin and globin mRNA in addition to EPO receptor mRNA on the same blot.

During 0 to 6 hours in culture, there is a 30% decrease in the EPO receptor mRNA level. Between the times 6 hours and 20 hours, the EPO receptor mRNA level stays un-

Fig 1. Levels of EPO receptor mRNA during maturation of mouse erythroid cells. (A) Ethidium bromide staining of 1% formaldehyde-agarose gel containing 20 μg of total RNA isolated from FVA cells at times 0 hours (lane 1), 6 hours (lane 2), 20 hours (lane 3), 26 hours (lane 4), 32 hours (lane 5), and 45 hours (lane 6). Mouse kidney total RNA (lane 7) was used as a control. (B) Autoradiogram of the Northern blot showing a band corresponding to EPO receptor RNA.

hybridization technique using 15 to 20 μg of total cellular RNA per gel lane (Fig 1). The cDNA probe detected a broad band of mRNA in FVA cells corresponding to 1.8 kb in size, which was visible as a doublet in some experiments (a clear doublet is seen in Fig 4B and the original autoradiogram of Fig 6, lane 7). Mouse kidney RNA (Fig 1, lane 7) exhibited no signal, even at long exposure times. In the experiment shown in Fig 1, RNA samples were analyzed from FVA cells that had been cultured for 0 to 45 hours in the presence of EPO. The RNAs were thus from cells at progressively later stages of maturation between CFU-E or cluster-forming units–erythroid and reticulocyte stages. Results of this experiment showed that EPO receptor

Fig 2. Levels of EPO receptor mRNA during maturation of mouse erythroid progenitor cells. Northern blot containing 20 μg of total RNA isolated from FVA cells at times 0 hours (lane 1), 6 hours (lane 2), 20 hours (lane 3), 26 hours (lane 4), 32 hours (lane 5), and 45 hours (lane 6). Mouse kidney total RNA was used as a control (lane 7). (A) Ethidium bromide-stained formaldehyde gel corresponding to the Northern blot. (B) The level of EPO receptor message during the time course. (C) Levels of actin mRNA, and (D) β-globin mRNA during the same time period. Hybridization for EPO receptor was performed on the same blot after stripping the actin and globin probes.
Changes in the level of EPO receptor (■), actin (X), and globin (○) mRNAs during maturation of mouse erythroid progenitor cells. Data are ± SD of quadruplicate determinations for EPO receptor mRNA time points 0 to 20 hours and triplicate determinations for time points 26 to 45 hours. The data for actin mRNA are ± SD of duplicate determinations.

changed. After the 20-hour time period, there is a further decline in the receptor mRNA levels. By 45 hours, only 15% of the initial amount is present in these late cells. The decline between 0 and 6 hours, as well as that after 20 hours, is statistically significant (P < .005 and P < .025, respectively) by the criterion of the paired t test applied to the percentage. Actin and β-globin are major erythrocyte proteins whose synthesis kinetics are known in FVA cells through protein gel analyses and specific transcription rate analyses. Actin synthesis follows the pattern of total protein synthesis in these cells; its synthesis rate remains approximately constant for about 24 hours of culture, then decreases to less than 25% of this rate by 48 hours. Globin protein chains are first detected at about 12 hours of culture of FVA cells, then the synthesis rate increases steadily to a peak rate between 40 and 48 hours of culture. Both actin and globin mRNA levels followed the expected patterns in these experiments, as shown quantitatively in Figs 2 and 3.

The half-life of EPO receptor mRNA was determined by Northern analysis following the addition of actinomycin D at three separate time periods beginning at 0 hours (Fig 4A), 10 hours (Fig 4B), or 20 hours (Fig 4C) of culture. The isolation and analysis of equivalent RNA samples were done after 15 minutes to 3 hours of subsequent incubation. The reduction of the EPO receptor mRNA signal during the course of incubation with actinomycin D is shown in Fig 4. The actin probe used on the same blots shows approximately a 10% decrease in its signal during the same time period (Fig 4). The decay kinetics of both EPO receptor mRNA and actin mRNA are shown in Fig 5. There is an initial 15-minute lag period during which actinomycin D enters the cells and takes effect. This lag period was determined by [3H]uridine incorporation into RNA (data not shown). The half-life for EPO receptor message was approximately 75 minutes at 0 hours, 68 minutes at 10 hours, and 83 minutes at 20 hours after taking into account the initial 15-minute lag period. Actinomycin D had no effect on cell viability during the experiments as determined by trypan blue exclusion.

Experiments were performed to determine the number of copies of EPO receptor mRNA per FVA cell at the time of culture (Fig 6). The number of receptor mRNA copies per cell, calculated by comparison to the standard curve, was 25. This experiment was repeated three times with two
Fig 5. Measurement of half-life of EPO receptor mRNA. Level of EPO receptor mRNA after addition of actinomycin D at time 0 hours (○), 10 hours (■), and 20 hours (▲). Level of actin mRNA beginning at 0 hours (X), 10 hours (■), and 20 hours (▲).

independently synthesized in vitro transcripts as standards, and yielded the same results in each experiment. The same result was also obtained with a dot blot, as well as the Northern blot (data not shown).

DISCUSSION

The decrease in the level of EPO receptor mRNA seen in FVA cells during maturation is similar to the decrease observed in EPO binding. Studies performed by Sawyer and Koury with FVA cells, by Landschultz et al with mouse spleen cells, and by Fraser et al with human bone marrow cells showed that as erythroid cells mature from proerythroblasts into late-stage erythroblasts or reticulocytes, the EPO binding activity disappears from their cell surfaces. In FVA cells, the total number of receptors decreases by 50% after the cells have been in culture for 24 hours. During the next 24 hours, the receptor number decreases to approximately 17% of the original number that was present at the initiation of cell culture. When regenerating mouse CFU-E were used after the mice had been treated with thiampenicol, the number of receptors decreased at a faster rate than the rate observed with FVA cells. In fact, in the regenerating mouse CFU-E study, there were only approximately 25% of the original number of receptors left by 24 hours of culture and almost all the receptors were lost by 36 hours of culture. In the study by Fraser et al, using normal human bone marrow cells, a loss of 70% of the receptors was seen between the pronormoblast and orthochromatic erythroblast stages. The data from our study in FVA cells showing a loss of 50% of the EPO receptor mRNA during the first 24 hours of cell culture, followed by a 33% loss of the message during the next 24 hours, agrees with the EPO binding study by Sawyer and Koury for the disappearance of the EPO receptor during the maturation of FVA cells. The initial decrease in the EPO receptor mRNA levels seen between the times 0.75 and 6 hours is due to reasons other than adding EPO to these cultures. An experiment performed to determine whether EPO causes this apparent
downregulation showed that there was no difference in the EPO receptor mRNA level during this time frame with or without EPO (results not shown). One possible explanation is that the shift from in vivo to in vitro conditions is creating the downregulation.

Koury and Bondurant have shown that an increased synthesis of total cellular RNA was observed in FVA cells during the first 24 hours in culture, followed by decreased synthesis from 24 to 48 hours. The decrease in the level of actin mRNA observed is similar but not identical to the decline that is observed with the EPO receptor mRNA. Thus, both EPO receptor mRNA and actin mRNA levels decrease before there is any indication of a general decrease in total cellular RNA synthesis. The dramatic increase of globin mRNA that is seen here is consistent with the earlier study on globin transcription in FVA cells by Bondurant et al. Therefore, the data suggest that EPO receptor, actin, globin, and ribosomal RNAs are regulated by multiple mechanisms during the late CFU-E and cluster-forming unit–erythroid stages of the FVA cells.

The half-life of EPO receptor mRNA as determined by addition of the actinomycin D to the cells was approximately 75 minutes. Since there is no significant change in the half-life of the EPO receptor message between 0 and 23 hours, the decrease in the EPO receptor mRNA level observed during the course of cell culture probably reflects a decrease in rate of transcription over time. The short half-life for EPO receptor mRNA indicates that in order for this message to remain in these cells, continuous transcription must take place. The relatively short half-life of EPO receptor is similar to the half-lives of other receptor mRNAs, ie, insulin receptor, epidermal growth factor receptor, and thyrotropin-releasing hormone receptor. During the 2- to 3-hour period of the experiment, actin mRNA showed only a 10% decay. Based on the 10% decay rate, the estimated half-life for actin was 12 hours. The long half-life of actin mRNA in these cells is enigmatic, because the mRNA level decreases greatly at later times of culture. One possibility is that the actin transcription rate is extremely slow, and therefore what is being measured in the time course (Fig 3) primarily reflects the decay of actin mRNA.

The number of copies of EPO receptor mRNA is 25 per FVA cell at the time of their isolation from mice. Since these cells resemble normal mouse CFU-E by many criteria, including number of EPO binding sites, we propose that this receptor mRNA copy number is probably similar in normal mouse CFU-E. Previous studies to measure concentrations of mRNAs using rates of hybridization of total mouse liver cDNA with its mRNA conclude that there are approximately 100 species of RNA that have between 5,000 and 50,000 copies per cell, followed by several hundred species that are present in the range of 100 to 1,000 copies per cell and over 10,000 species of RNA that have as few as 0.1 to 10 copies per cell. Based on these studies, EPO receptor mRNA falls between low and intermediate abundance categories. The relatively low mRNA copy number is consistent with the low (800 to 1,000) receptor numbers that have been determined in FVA cells and other types of erythroid cells. The broad bands seen for EPO receptor mRNA that can be visualized as a doublet in some Northern blots might well be two transcripts arising due to alternate splicing or having two transcriptional initiation sites. Although the study by Youssoufian et al showed the presence of three transcriptional start sites (a 4-base separation between the shortest and the longest transcript), this cannot account for the doublet, since the 1% agarose gel is not capable of resolving such small differences in transcript length.

This study demonstrates that the time course of disappearance and the number of mRNA copies for the cloned EPO receptor gene in FVA cells are consistent both with the number of EPO binding sites and the timing of their disappearance in these cells. Thus, whatever the quaternary structure of the total EPO receptor may be, our data are consistent with the idea that the receptor molecule identified by D'Andrea et al correlates with binding activity in late erythroid precursor cells.

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