On the Mechanism of Erythropoietin-induced Differentiation

IX. INDUCED SYNTHESIS OF 9 S RIBONUCLEIC ACID AND OF HEMOGLOBIN*

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SUMMARY

Erythropoietin induces the synthesis of 9 S RNA and hemoglobin by cultured bone marrow cells derived from polycythemic rats and mice in which hemoglobin synthesis has been suppressed. Synthesis of 9 S RNA starts within 1 hour (rat cells) and 4 hours (mouse cells) of addition of erythropoietin to the medium, while hemoglobin synthesis is not initiated until 8 to 10 hours later. These findings indicate that there is another regulatory step between transcription, initiated by erythropoietin, and translation. Under conditions in which no hemoglobin synthesis occurs, bone marrow cells still synthesize a very small amount of 9 S RNA which may be a messenger for a protein other than globin. Comparison of stimulated hemoglobin synthesis by cells from normal animals with cells from polycythemic animals indicates that erythropoietin has an effect on some differentiated cells in the former population as well as on the undifferentiated cells in the latter population.

The pivotal role of erythropoietin in the initiation of erythroid cell development is, by now, well established. We have shown in previous papers that primary cultures of rat bone marrow cells respond to the addition of erythropoietin by increasing the rate of hemoglobin synthesis (1). This effect is preceded by an increase in uptake of iron (2) and by an increase in the synthesis of a variety of RNA species, one of which has a sedimentation coefficient of about 9 S (3). Similar RNA isolated from rabbit reticulocyte polysomes proved to contain messengers for the synthesis of α and β chains of globin when tested in cell-free systems (4-6). Bone marrow cells in culture provide a system in which cell development is, by now, well established. We have shown that erythropoietin has an effect on some differentiated cells in the former population as well as on the undifferentiated cells in the latter population.

Preparation of Polycythemic Animals—Male, Sprague-Dawley rats (250- to 300-g) were made polycythemic by the intraperitoneal injection of 10 ml of packed, washed red cells from homologous donors on Days 1, 2, and 6. Hematocrits and reticulocyte counts measured on Day 7 were 65 to 80 and 0.02 to 0.10% (normal 1.5 to 3.5%), respectively. Bone marrow cells were harvested on Day 8. Female CF 1 mice (15 to 20 g) were made polycythemic by the intraperitoneal injection of 1 ml of packed, washed red cells from homologous donors on Days 1 and 3. Hematocrits were 65 to 75 on Day 8, when the bone marrow cells were isolated. The mice were reticulocyte-free and had no cells in their marrow that were recognizable erythroid (7).

Preparation of Hyperplastic Rats—Erythroid hyperplasia was induced in 250- to 300-g, male, Sprague-Dawley rats by subcutaneous injection of 50 mg per kg of neutralized phenylhydrazine hydrochloride on 2 successive days. Bone marrow cells were isolated on the 4th day, when the hematocrit was about 30 and the peripheral blood showed 10 to 15% reticulocytosis.

Cell Culture—Rat marrow cells were cultured as previously described (3) unless otherwise indicated. Bone marrow was obtained from the mice by flushing culture medium through the marrow cavity of the femora. These cells were cultured in the same manner as the rat marrow cells, with mouse serum replacing rat serum, unless otherwise indicated. We have found that it is important to maximize the yield of cells from the mouse femur by vigorous flushing of medium through the bone. If flushing is gentle and only the loosely attached cells are removed, the yield is about 6 to 8 million nucleated cells per femur, and little or no response to erythropoietin is seen. If the flushing is vigorous, the yield is about twice as great and the cells respond to erythropoietin. It is of interest that the concentration of colony-forming cells in the mouse femur is 2 to 3 times greater in the firmly

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Fig. 1. Effect of erythropoietin on marrow cells from polycythemic rats. Quadruplicate cultures (1.0 ml) containing $10 \times 10^6$ cells per ml were incubated for increasing periods and pulsed with 1.0 $\mu$Ci of $^{59}$Fe for 4 hours at the indicated times. The points are placed at the midpoints of the pulse periods. $\bullet-\bullet$, control cells; $\bigcirc-\bigcirc$, erythropoietin-treated cells (0.10 unit per ml); and $\Delta-\Delta$, the difference between control and stimulated. The upper portion of the figure shows iron uptake and the lower portion shows hemoglobin synthesis. Heme was isolated by the Fox and Thomson method (9). In comparison, parallel cultures with cells from normal rats under identical conditions at the first pulse time showed an iron uptake of 7250 cpm and heme synthesis equivalent to 2350 cpm. The vertical bars indicate the standard deviation of the means.

attached cells than in those removed by gentle treatment. All cell numbers are expressed as nucleated cells.

Measurement of RNA Synthesis—RNA synthesis was determined by a 15-min pulse with 4 $\mu$Ci per ml each of uridine-$^3$H and cytidine-$^3$H followed by RNA isolation with Method II previously described (3). Where indicated, cells were chased with actinomycin D ($8 \mu$g per ml) and cold cytidine and uridine (5 mM final concentration) as described previously. In the experiment in which rats were labeled in vivo, they were given an intravenous injection of 50 $\mu$Ci each of uridine-$^3$H and cytidine-$^3$H. Marrow cells were collected and RNA was extracted by Method II 15 min later. The RNA was fractionated on sucrose density gradients with the SW-50 rotor in a Beckman model L-2 ultracentrifuge as previously described. The conditions of centrifugation are indicated in the figure legends. Absorbance and radioactivity were determined as described previously (3). Quantitative evaluation of gradient profiles was done by planimetric integration.

1 B. I. Lord, personal communication.

Determination of Iron Uptake and Hemoglobin Synthesis—Iron uptake was measured as the radioactivity in the marrow cell pellet after three washes with 5 ml of PBS$^*$ (2). Radioiron ($^{59}$Fe$^{3+}$) was added bound to rat serum for rat cells or to mouse serum for mouse cells. The amount of iron was enough for 50 to 80% saturation of the transferrin in the serum. Radioiron was counted in a Nuclear-Chicago model 4227 counter.

Hemoglobin synthesis was determined by one of three methods after labeling with $^{59}$Fe, valine-$^{14}$C, or glycine-$^{14}$C. The first method was the heme isolation procedure of Fox and Thomson (8). The washed marrow cell pellet was mixed with 0.25 ml of PBS containing 10 $\mu$Ci of packed rat red cells as carrier and then lysed by the addition of 0.03 ml of 2% Nonidet-P-40. After hemoglobin was precipitated by the addition of 4 $\mu$M of rabbit antiserum against twice crystallized rat hemoglobin, any precipitable radioactivity was measured as described earlier (3). Quantitative evaluation of gradient profiles was done by planimetric integration.

2 The abbreviation used is: PBS, phosphate-buffered NaCl solution (0.145 M NaCl, 0.013 M phosphate, pH 7.3).
To the stroma-free lysate, 0.10 ml of antiserum was added, and the mixture was incubated at 37°C for 30 min and then kept at 3°C overnight. The precipitates were collected by centrifugation (5 min at 1500 x g), washed three times with 0.50 ml of PBS, and then suspended in 0.50 ml of acetone. Heme was solubilized according to the method of Fox and Thomson, and the heme in the acid acetone supernatant was counted as an infinitely thin layer on aluminum planchets. If less than 0.10 ml of antiserum was added, the amount of heme remaining in the supernatant was inversely proportional to the volume of antiserum added. If, after precipitation of all of the hemoglobin, an additional amount of unlabeled hemoglobin and antiserum was added to the supernatant, no significant radioactivity was precipitated. Methemoglobin was not precipitated by the antiserum.

A third method was the procedure developed by Hrinda (9) which depends upon the specificity of the binding of hemoglobin to haptoglobin. DEAE-cellulose was equilibrated with 0.5 M sodium acetate (pH 5.5) and then washed thoroughly with water. The stroma-free lysates from the marrow cells and 5 µl of packed rat red cells were dialyzed overnight against 0.005 M sodium acetate, pH 5.5 (acetate buffer), and run through a DEAE-cellulose column (8 x 0.2 cm²). Hemoglobin was washed through with the effluent with the same acetate buffer. Sufficient haptoglobin was added to the effluent to form a complex with all the hemoglobin present. These samples were then applied to separate DEAE-cellulose columns (8 x 0.2 cm²) to which the hemoglobin-haptoglobin binds at this pH and ionic strength. After the second column was washed with 10 1-ml volumes of acetate buffer, the hemoglobin-haptoglobin complex was eluted with 1 M sodium chloride, 0.10 M sodium phosphate (pH 7.2). The hemoglobin in the eluate was treated and counted as described for the antisi-

### Table I

**Early stimulation of hemoglobin synthesis in normal marrow cells**

Rat marrow cell cultures contained 15 X 10⁶ cells per ml, and mouse cultures had 5 X 10⁶ cells per ml. One microcurie of ⁶⁶Fe was added to each plate at the indicated times. Rat cell cultures studied at 4 hours were in quintuplicate, others were quadruplicate, and mouse cell cultures were in triplicate. The difference between control and erythropoietin groups is significant (0.001 < p < 0.01) by t test.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>⁶⁶Fe incorporated into hemoglobin</th>
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<td>Rat cells</td>
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<td>Control</td>
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<td>All additions at zero, stopped at 4 hr</td>
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<td>Erythropoietin and ⁶⁶Fe added at 24 hr, stopped at 28 hr</td>
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<td>Erythropoietin added at 24 hr, ⁶⁶Fe added at 39 hr, stopped at 43 hr</td>
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<td>430</td>
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**Fig. 3.** Presence of 9 S RNA in rat marrow cells. RNA from hyperplastic rat marrow (O--O), normal marrow (●—●), and polycythemic rat marrow (△—△) was isolated by Method II (3). Sucrose gradients were run for 14 hours at 48,000 rpm.

**Fig. 4.** Synthesis of 9 S RNA by cultured cells from polycythemic rats. Triplicate cultures (2.5-ml) containing 20 X 10⁶ cells per ml were incubated for 1 hour and then pulsed for 15 min with 4 µCi each of uridine-³H and cytidine-³H. Cells were pooled, and RNA was isolated and run on sucrose gradients for 11 hours at 48,000 rpm. ●—●, controls; ○—○, erythropoietin-treated cells.
body precipitation method, except that the heme was extracted with chloroform to remove the salt in the elution buffer.

We have found that all three of these methods agree in the determination of heme synthesis, and the antibody and haptoglobin methods agree in the determination of globin synthesis in rat marrow cell cultures maintained for as long as 42 days (10). In this system, measurement of heme synthesis is equivalent to measurement of hemoglobin synthesis (11).

Materials—NCTC-109 was purchased from Microbiological Associates, Bethesda, Maryland, newborn calf serum was from Colorado Serum Company, Denver, Colorado, and culture dishes were from Falcon Plastics, Baltimore, Maryland. Uridine-5-$^3$H (27.6 Ci per mmole), cytidine-5-$^3$H (26.4 Ci per mmole), valine-1-$^{14}$C (34.2 mCi per mmole), and $^{55}$FeCl$_3$ (10 Ci per g) were bought from New England Nuclear. Glycine-2-$^{14}$C (57 mCi per mmole) was bought from Nuclear-Chicago. Actinomycin D was a commercial sample from Merck Sharpe, and Dohme. Nonidet-P40 was a gift from Shell Chemicals. The antihemoglobin antisem was generously provided by Dr. B. Hurn of the Wellcome Research Laboratories, Kent, England.

RESULTS

The time course of control and erythropoietin-induced iron uptake and hemoglobin synthesis by marrow cells from polycythemic rats is shown in Fig. 1. In comparison with cells from normal rats, iron uptake was suppressed by about 75% and hemoglobin synthesis was suppressed by about 85%. Stimulation of iron uptake by erythropoietin was evident about 7 hours after incubation was started, while stimulation of hemoglobin synthesis was not detected before 12 hours. Induced iron uptake (as shown by the difference curve) went through two maxima, at 12 and 48 hours, while induced hemoglobin synthesis rose steadily after the initial lag period, reaching a maximum at about 36 hours. The control rate of hemoglobin synthesis declined steadily from the start of the incubation, but the control rate of iron uptake showed an appreciable increase after about 18 hours of decline. We have shown previously (12) that this secondary increase in rate of iron accumulation in the absence of erythropoietin is due to the carrier iron added to the medium.

A similar study of the rate of hemoglobin synthesis by marrow cells from polycythemic mice showed that the control incorporation of iron into heme in these cultures was virtually zero throughout the 58 hours of incubation, as would be expected from observations on living mice (Fig. 2). Erythropoietin caused an increase that was apparent between the 10th and 24th hours of incubation, and reached a maximum at about 33 hours. The stimulation of the rate of hemoglobin synthesis fell almost to zero by 58 hours. Nakao, Miura, and Takaku (13) have reported similar findings in organ cultures of spleen from polycythemic mice.
Cells from normal rats and mice, in marked contrast to the suppressed cells, showed a small but significant effect of erythropoietin on incorporation of iron into hemoglobin as early as 4 hours. If the normal cells were previously incubated for 24 hours before erythropoietin was added, there was no stimulation of hemoglobin synthesis until about 15 hours later (Table I).

The data in Fig. 3 show the effects of polycythemia and erythropoietic hyperplasia (induced by anemia due to phenylhydrazine) on the amount of 9 S RNA from equivalent amounts of total marrow cell RNA. The suppression of red cell formation by polycythemia resulted in the disappearance of 9 S RNA, while the acceleration of erythropoiesis in the anemic rats resulted in a doubling of the 9 S RNA present.

Marrow cells from polycythemic rats in culture still synthesized some 9 S RNA even though none could be detected in the absorbance profile (Fig. 4). When erythropoietin was added to the cultures 1 hour before pulsing for 15 min, there was an increase of about 40% in the amount of labeled 9 S RNA but still none was detectable by absorbance measurement. With this short pulse the only difference between the meniscus and the 15 S region of the gradient due to erythropoietin was in the 9 S peak.

A chase experiment studying the fate of pulse-labeled 9 S RNA indicated that all of it, in both control and stimulated cells, persisted for 3 hours (Fig. 5). If the half-life of 9 S RNA was 24 hours or greater, we would not have been able to detect any loss in 3 hours. Chasing for longer periods, however, is not possible since the cells do not survive long treatment with actinomycin. It is interesting to note that a considerable amount of RNA in the 4 S region accumulated during the chase, possibly because of terminal labeling of tRNA.

The unexpected synthesis of 9 S RNA by cells from polycythemic rats might have been due to a small amount of erythropoietin in the calf serum used in the culture medium. In order to determine whether this was so, or whether that 9 S RNA synthesis seen in cells not making hemoglobin might be unrelated to erythropoietin action, we did two experiments. In one of these (Fig. 6), polycythemic, normal, and hyperplastic rats were pulsed in vivo with labeled uridine and cytidine and the RNA from marrow cells was examined on a sucrose gradient. Even under the in vivo condition of suppressed hemoglobin synthesis there was a small, but detectable, synthesis of 9 S RNA amounting to about 60% of that seen in normal rats. In the hyperplastic rats, as expected, 9 S RNA synthesis was about 2.5 times as great as in normal animals. In the second experiment, we cultured marrow cells from polycythemic rats in media containing serum either from polycythemic animals (Fig. 7A) or from normal rats (Fig. 7B). Unstimulated cells in the medium containing "polycythemic" serum synthesized 9 S RNA at a rate very similar to that of

![Graph A](image1)

**Fig. 7.** 9 S RNA synthesis by cells from polycythemic rats incubated in serum from polycythemic rats. Triplicate cultures (2.5-ml) containing 30 x 10⁶ cells per ml were incubated in a medium containing 70% NCTC-109 and 30% serum from polycythemic rats (A) or serum from normal rats (B). The cultures were pulsed for 15 min at 4 hours and the pooled RNA samples were run on sucrose gradients at 49,000 rpm for 12 hours (A) and 13 hours (B). •••, controls; ○○○, erythropoietin-treated cells.

![Graph B](image2)

**Fig. 8.** 9 S RNA synthesis by cells from polycythemic mice. Duplicate cultures (2.5-ml) containing 15 x 10⁶ cells per ml were pulsed at 4 hours for 15 min, and the pooled RNA samples were run on sucrose gradients for 16 hours at 49,000 rpm. •••, control cultures, ○○○, erythropoietin-treated cells.
control cells in normal serum, indicating that at most 5 to 10% of the 9 S RNA synthesized by cells in normal serum could have been due to the presence of endogenous erythropoietin. In both cases, erythropoietin caused the same increase in 9 S RNA synthesis. These data indicate that there is an erythropoietin-independent synthesis of 9 S RNA in rat marrow cells.

Because the suppression of hemoglobin synthesis by artificial polycythemia in rats is not complete, we examined the synthesis of 9 S RNA in cells from polycythemic mice, where red cell production is nil. The results (Fig. 8) show that, as with rat cells, no 9 S RNA is detectable by absorbance measurement, and there is a similar synthesis of 9 S RNA in controls, amounting to about 20% of that seen in normal mouse cells. This level of synthesis was about the same when serum from polycythemic mice was substituted for calf serum in the medium. Addition of erythropoietin caused an increase of about 2-fold in 9 S RNA synthesis when measured by a 15-min pulse after 4 hours of incubation.

The effect of erythropoietin on 9 S RNA synthesis is limited to a relatively short period of time (Fig. 9). When cells from polycythemic rats were pulsed with uridine and cytidine after 3 hours of incubation, the erythropoietin stimulation was about 2-fold, but when the cells were pulsed after 24 hours of incubation there was no erythropoietin effect; control 9 S RNA synthesis was reduced by about 65%. During the 24-hour incubation in the presence of erythropoietin, however, the cells did accumulate a significant amount of 9 S RNA as determined from the absorbance profile. Cells that have been previously incubated 24 hours in the absence of erythropoietin can respond to its addition at that time with an increase of about 40% in total RNA synthesis. These data suggest that the lack of response to erythropoietin with regard to 9 S RNA synthesis at 24 hours is not a generalized consequence of culture conditions, but represents a selective loss of a particular function.

**DISCUSSION**

Our results indicate that at least two types of 9 S RNA, one of which is the erythropoietin-dependent globin messenger, may be found in marrow cells. The synthesis of 9 S RNA that occurs in marrow cells from polycythemic mice, however, clearly takes place in cells that do not form hemoglobin and, since this synthesis is independent of erythropoietin, it may represent an RNA having no role in globin synthesis. The same may be true for the 9 S RNA synthesized by the cells 24 hours after incubation in the presence of erythropoietin but after its action has stopped. It is quite possible that marrow cells not concerned with red cell formation make 9 S RNA that are messengers for proteins (other than globin) with a molecular weight of about 16,000. Another interpretation of these data is that undifferentiated cells synthesize 9 S RNA globin messengers that do not accumulate until some other aspect of erythroid differentiation (e.g., heme synthesis) is initiated.

The identification of that part of marrow cell 9 S RNA formed in response to erythropoietin as globin messenger must await the demonstration that it can serve as a template for globin synthesis in a cell-free system. Since the unfractionated, normal marrow cell population contains reticulocytes, it is certain to have some 9 S RNA that is known to be globin messenger. The use of marrow from polycythemic animals would entail a massive experiment since so little 9 S RNA is present. Another interpretation is that undifferentiated cells synthesize 9 S RNA that are messengers for globin synthesis can be found in marrow cells. The synthesis of 9 S RNA that occurs in marrow cells from polycythemic animals, and that globin synthesis can persist in the absence of heme synthesis.

![Graph](image-url)

**Fig. 9.** 9 S RNA synthesis by rat marrow cells incubated for 24 hours in the presence of erythropoietin. Triplicate cultures (2.5 ml) containing 25 x 10^6 cells per ml were incubated for 3 hours with (O—O) and without (●—●) erythropoietin and for 24 hours with (□—□) and without (■—■) erythropoietin. All cultures were pulsed for 15 min at the end of the incubations. The pooled RNA samples were run on sucrose gradients for 13 hours at 49,000 rpm. In the lower part of the figure the triangles (Δ—Δ) indicate marker RNA isolated from hyperplastic rat marrow cells.

Initiation of 9 S RNA synthesis by erythropoietin occurs in 1 hour in cells from polycythemic rats and in 4 hours in cells from polycythemic mice. Induced hemoglobin synthesis, on the other hand, does not begin until about 12 hours. The delay in initiation of hemoglobin synthesis, both in vitro and in vivo, clearly must be due to a post-transcriptional regulatory process since the presumptive messenger (9 S RNA) is synthesized several hours before its function is expressed. One aspect of this post-transcriptional regulation may be the transposition of RNA synthesized in the nucleus to the cytoplasm. We have found that the bulk of the RNA newly synthesized in erythropoietin-treated marrow cells is retained in the nucleus for at least 3 hours (10).

The remaining interval between transcription of globin messenger RNA and hemoglobin synthesis may be required for those transcriptional and translational processes needed for the enzymes used for heme synthesis. We will present data elsewhere showing that there is a discrepancy between heme and globin synthesis in cells from polycythemic animals, and that globin synthesis can persist in the absence of heme synthesis.
Marrow cells that have been previously incubated for 24 hours before erythropoietin is added behave as cells from polycythemic animals, in that erythropoietin has no effect on hemoglobin synthesis in the first 4 hours, as there is in normal cells. These findings suggest that erythropoietin can act on cells that are already at some stage in erythroid differentiation, since the difference between the populations is essentially the lack of recognizable erythroid cells in the polycythemic and previously incubated cultures. We have shown elsewhere (14) that the erythropoietin stimulation of hemoglobin synthesis by marrow cells from normal rats, occurring within the first 4 hours of incubation, is much less sensitive to inhibitors of DNA synthesis than is the response at 24 and 48 hours. Ortega and Dukes (15) have made similar observations. In addition, Barcos and Goldwasser (16) have found that the erythropoietin response of marrow cells from polycythemic rats is more radiosensitive than is the response of normal cells and that previously incubated cells are more similar to cells from polycythemic animals than to normal cells in their response to ionizing radiation. These findings all suggest that the early erythropoietin stimulation of hemoglobin synthesis represents an action on cells already started on the pathway toward erythroid differentiation. There is evidence from studies in vivo which also suggests this type of action (16, 17).

While many aspects of this problem remain to be clarified, it is quite clear that marrow cells can synthesize 9 S RNA and retain it in an unexpressed form for several hours, and that superimposed on the regulation of gene expression at the transcriptional level there are other regulatory mechanisms.

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