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Deficiency of Globin Messenger RNA in Reticulocytes of the Belgrade Rat†

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ABSTRACT: The anemia of the Belgrade laboratory rat, an autosomal recessive trait (*b/b*), is associated with diminished incorporation of iron into heme and amino acids into globin by reticulocytes. We have studied the basis of decreased globin synthesis in *b/b* animals. Stimulation of protein synthesis per total RNA by *b/b* RNA was 39–46% of that by normal RNA in the wheat germ cell-free system. Hybridization of total cytoplasmic RNA to radioactive DNA complementary to rat globin mRNA showed that the number of globin mRNA molecules per total RNA in *b/b* reticulocytes was about 45% of that in normal reticulocytes. RNA fractions were also characterized by an analysis of globin products on carboxymethylcellulose chromatography after translation of RNA in the wheat germ cell-free system. The globin chain patterns synthesized by total cytoplasmic RNA and polysomal RNA isolated from *b/b* reticulocytes were indistinguishable from those of normal reticulocytes. The globin patterns synthesized by poly(A)-containing RNA derived from either total cellular or polysomal RNA were also similar when *b/b* and normal animals were compared. Our results indicate that diminished globin synthesis in *b/b* animals reflects lowered globin mRNA content in *b/b* reticulocytes, although the functional composition of globin mRNA from normal and *b/b* animals is essentially the same. The Belgrade anemia probably results from defective uptake of iron in erythroid cells, leading to heme deficiency; it is attractive to speculate that a chronic heme deficit, in turn, leads to a shortage of globin mRNA.

Belgrade laboratory rats have a hypochromic, microcytic anemia inherited as an autosomal recessive trait (*b/b*) (Sladic-Simic et al., 1963). The primary defect leading to the anemia is not yet clear. Studies on iron metabolism indicate that there is malutilization of iron in the *b/b* rats (Sladic-Simic et al., 1966). The serum iron concentration is high and the iron-binding capacity of the serum is nearly saturated (Sladic-Simic et al., 1969). Nevertheless, there is virtually no stainable iron present in the body tissue and bone marrow (Sladic-Simic et al., 1969). Although *b/b* rats respond partially to parenteral iron treatment, erythrocytes remain hypochromic and microcytic (Sladic-Simic et al., 1966). Iron-uptake studies indicate that the transport of iron from plasma into reticulocytes is markedly decreased (Edwards et al., 1978). Transferrin binding and internalization are apparently normal; however, release of iron within the cell is defective for Belgrade rats (Edwards et al., 1977).

Hemoglobin synthesis in *b/b* rats is also diminished, as measured by the incorporation of radioactive amino acids into globin by intact reticulocytes (Edwards et al., 1978). Despite the strong resemblance in red cell morphology between the *b/b* anemia and human thalassemia, starch-gel electrophoresis of hemoglobins reveals no difference between *b/b* and normal rats (Sladic-Simic et al., 1966), and incorporation into six of the seven globin chains follows the same pattern comparing *b/b* to normal rats (Edwards et al., 1978). (At the time of the cited study, the identity of a seventh protein fraction as a globin chain had not been established.) Translation of poly(U) by a cell-free system derived from *b/b* reticulocytes suggests a defect in the translational machinery, possibly at the initiation step (Cusic and Becarevic, 1976). Messenger RNA isolated from *b/b* reticulocytes is active in directing globin synthesis in cell-free systems; however, the distribution of mRNA between polysomes and the postribosomal supernatant was significantly different in *b/b* reticulocytes as compared to normal reticulocytes (Crkvenjakov et al., 1976).

We have investigated the basis for the occurrence of decreased globin synthesis in Belgrade rats despite the absence of an alteration in the pattern of incorporation into globin chains. To accomplish this goal, we studied the amount and functional composition of globin messenger RNA in *b/b* reticulocytes.

Experimental Procedure

Materials. L-[4,5-^{3}H]Leucine (62 Ci/mmol), L-[^{14}C]leucine (3.9 mCi/mmol), and [5-^{3}H]dCTP (20 Ci/mmol) were obtained from Schwarz/Mann, Orangeburg, N.Y. Oligo(dT)-cellulose (type T2) and oligo(dT)$_{12-18}$ were purchased from Collaborative Research, Inc., Waltham, Mass. Preswollen carboxymethylcellulose (Whatman CM-52) was obtained from Reeve Angel Co., Clifton, N.J. S_1 nuclease was purchased from Seikagaku Kogyo Co., Tokyo, Japan. RNA-dependent DNA polymerase of avian myeloblastosis virus, Sephadex SP-50, and Chelex 100 were generously made available by Dr. Nick Hastie of Roswell Park Memorial Institute. All other chemicals are of reagent grade.

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Animals. Anemic Belgrade laboratory rats obtained from the original Belgrade colony (Sladic-Simic et al., 1963) and the New York subcolony (Sladic-Simic et al., 1969) were bred as previously described (Edwards et al., 1978). Normal rats (Wistar strain) were made anemic by five daily injections of 1.25% (w/v) phenylhydrazine in isotonic saline. The rats were bled by cardiac puncture on the 7th day. The reticulocyte counts ranged from 45 to 80%. Because Belgrade rats are severely anemic and, thus, under severe chronic erythroid stress, their reticulocyte counts ranged from 60 to 90%. Hence, there was no need to use phenylhydrazine treatment with them.

Preparation of Stroma-Free Hemolysate. Because rat hemoglobin tends to precipitate at 4 °C and physiological pH, hemolysate was prepared at 15 °C using high-pH solutions. After removal of plasma by centrifugation, reticulocyte-rich cells were washed twice with 0.9% NaCl–15 mM Tris[1] (free base, pH 9.1). Cells were lysed with 4 volumes of 4 mM $MgCl_2$–5 mM Tris-HCl (pH 8.6). Stroma were removed by centrifugation at 30 000*g* for 30 min at 15 °C. Hemolysate thus obtained was divided into two equal aliquots. Total cytoplasmic RNA was prepared from one aliquot and polysomal RNA from the other.

Preparation of Total Cytoplasmic RNA. Hemolysates were made 50 mM in Tris-HCl (pH 9.0) (the concentration and pH were chosen to prevent hemoglobin precipitation) and 0.1% in sodium dodecyl sulfate and extracted with an equal volume of a mixture of phenol and chloroform (1:1) containing 0.1% 8-hydroxyquinoline (Lavers et al., 1974). The organic phase was back-extracted with 1 volume of 50 mM Tris-HCl (pH 9.0). The combined aqueous phase was extracted again with 1 volume of fresh phenol–chloroform mixture as just described. Total cytoplasmic RNA was precipitated from the aqueous phase by adding 0.1 volume of 3 M LiCl and 2.5 volumes of 100% ethanol and stored at −20 °C overnight. The RNA was centrifuged at 30 000*g* for 30 min, washed twice with 75% ethanol, dried, dissolved in H_2O, and stored in liquid nitrogen.

Preparation of Polysomal RNA. Hemolysates were adjusted to 10 mM Tris-HCl (pH 8.0), 100 mM KCl, and 4 mM $MgCl_2$ (TKM). The mixture (5 mL) was layered over a 4-mL cushion of 20% sucrose in TKM and then centrifuged at 145 000*g* for 4 h in a Beckman Type 40 rotor. The polysomal pellet was rinsed twice with 50 mM Tris-HCl (pH 9.0) and dissolved in the same buffer. The solution was made 0.1% in sodium dodecyl sulfate and extracted with phenol–chloroform as described above.

Oligo(dT)–cellulose Chromatography. Before preparing an RNA fraction enriched in 3′-poly(A) sequences, either total cytoplasmic or polysomal RNA was dissolved in binding buffer [10 mM Tris-HCl (pH 7.5), 0.5 M NaCl] (Hieter et al., 1976). Up to 50 A_{260} units in a volume of 2 mL was applied to a 0.9-cm diameter column containing 0.5 g of oligo(dT)-cellulose previously equilibrated with the binding buffer. After loading the sample on the column at the rate of 0.5 mL/min, the column was washed with binding buffer until no more UV-absorbing material was eluted. Poly(A)-containing RNA was then eluted with 10 mM Tris-HCl (pH 7.5) and then precipitated with ethanol, washed, dissolved, and stored as described before.

Translation of RNA in the Wheat Germ Cell-Free System. wheat germ cell-free extract was prepared by the procedure of Marcu and Dudock (1974) and stored in liquid nitrogen. The assay system contained in a final volume of 50 μL: 10 μL of wheat germ extract, 20 mM Hepes (pH 7.6), 2 mM dithiothreitol, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 40 μg/mL creatine phosphokinase, 2.5 mM magnesium acetate, 100 mM KCl, 5 μCi of L-[4,5-^{3}H]leucine, and 25 μM each of the 19 unlabeled amino acids plus the indicated amounts of RNA. Incubations were at 22 °C for 90 min. At the end of the incubation, 5 μL of the reaction mixture was spotted on a Whatman 1 paper strip (0.75 × 2.25 in.) for measuring amino acid incorporation. The paper strips were washed by the method of Mans and Novelli (1961) and counted in Omnifluor (4 g/L in toluene, New England Nuclear Corp., Boston, Mass.). The remainder of the reaction mixture was frozen until further analysis.

Preparation of [^{14}C]Leucine Hemolysate. Five milliliters of reticulocyte-rich blood from a normal rat was incubated with 250 μCi of L-[^{14}C]leucine at 37 °C for 4 h. The cells were washed and hemolysate was prepared as described above.

Carboxymethylcellulose Chromatography of the in Vitro Products. Reaction mixtures labeled with [^{3}H]leucine in the wheat germ cell-free system (1–3 × 10^5 cpm) were mixed with an appropriate amount of [^{14}C]leucine hemolysate (^{3}H/^{14}C = 10) plus 40 mg of carrier hemolysate from a normal rat. Globin was prepared from this mixture by the addition to 15–20 volumes of 2.5% (w/v) oxalic acid in acetone (Garrick et al., 1973). Globin chains were separated by a modification of the method of Clegg et al. (1966). The sample was dissolved in 2 mL of starting buffer (2.5 mM Na_2HPO_4–NaH_2PO_4 (pH 7.05), 50 mM 2-mercaptoethanol in 8 M urea) and applied to a 0.7 × 15 cm carboxymethylcellulose (CM-52) column. Globin chains were eluted with a 400-mL linear gradient of increasing Na^+ concentration (5–30 mM). Fractions of 3.2 mL were collected. Tritium and ^{14}C radioactivity were determined by mixing 0.7 mL of each fraction with 9 mL of Scintisol (Isolab, Akron, Ohio) and counting in a liquid scintillation counter.

Preliminary experiments indicated that those wheat germ endogenous proteins that bound to carboxymethylcellulose were eluted with low Na^+ concentration (before the $^0\alpha$ peak). In order to minimize errors due to this endogenous protein contamination, we analyzed only those samples in which stimulation of protein synthesis was more than 30-fold.

Synthesis of Radioactive DNA Complementary to Rat Globin mRNA. Total cytoplasmic RNA was prepared from normal rat hemolysate by the phenol–chloroform method described above. Globin mRNA was purified from total cytoplasmic RNA by collecting poly(A)-containing RNA from the oligo(dT)-cellulose column as described above and then purifying further by a second passage. Radioactive complementary DNA (cDNA) was synthesized in 200 μL containing 5 μg of globin mRNA, 50 mM Tris-HCl (pH 8.3), 60 mM NaCl, 6 mM $MgCl_2$, 20 mM dithiothreitol, 0.5 mM each of dATP, dGTP, and TTP, 150 μCi of [5-^{3}H]dCTP, 50 μg/mL actinomycin D, 1.25 μg/mL oligo$(dT)_{12-18}$ and 20 units of RNA-dependent DNA polymerase of avian myeloblastosis virus. After incubation at 37 °C for 2 h, the reaction mixture was made 0.5 N in NaOH and boiled for 5 min. Then, the mixture was acidified with acetic acid, applied to a 1.5 × 30 cm Sephadex SP 50 column with a 2-cm base of Chelex 100 and eluted with 0.3 M NaCl, 0.01 M sodium acetate (pH 5.0). The first radioactive peak that eluted from the column was precipitated with 2 volumes of 100% ethanol.

Hybridization of cDNA with Total Cytoplasmic RNA. Hybridization was performed in a sealed 5-μL micropipet. The reaction mixture contained 0.2 M NaH_2PO_4 (pH 6.8), 0.5% sodium dodecyl sulfate, 1 mM EDTA, 3000 cpm of [^{3}H]-

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

TABLE I: Translation of Normal Rat Polysomal RNA in the Wheat Germ Cell-Free System.

amount of RNA (μg)	% of total [^{3}H]Leu incorp							
	$^0\beta$	$^0\alpha$	$^I\beta$	$^I\alpha$	$^{II}\beta$	$^{II}\alpha$	$^{III}\beta$	α/β
3	5	9	8	36	22	14	6	1.44
6	4	8	10	36	22	14	6	1.38

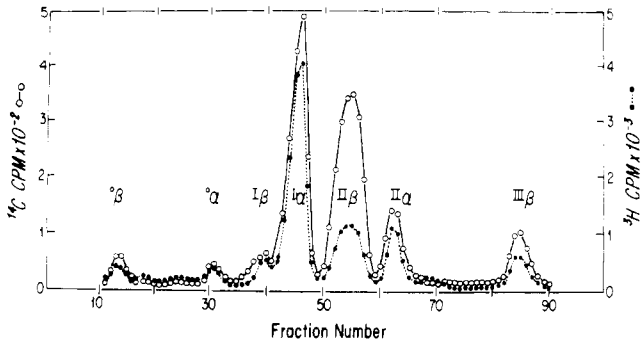

FIGURE 1: Typical CM-cellulose chromatography for analysis of in vitro translation products. Total cytoplasmic RNA isolated from normal rat reticulocytes was translated in the wheat germ cell-free system and the products were analyzed: (●) [^{3}H]leucine-labeled in vitro products; (○) [^{14}C]leucine-labeled hemolysate.

cDNA and 1-400 μg of total cytoplasmic RNA. The mixture was boiled for 5 min and incubated at 68 °C for 45 h. At the end of the incubation, the mixture was flushed into 1 mL of a solution containing 0.1 M NaCl, 1 mM $ZnSO_4$, 30 mM sodium acetate (pH 4.5), and 30 μg/mL denatured calf thymus DNA. Half of the mixture was counted in 9 mL of Scintisol to determine total radioactivity. The other half was incubated with 100 units of S_1 nuclease at 45 °C for 45 min. After incubation, 0.5 mL of 10% CCl_3COOH was added to the mixture. Acid-insoluble material was filtered on Whatman GF/C glass-fiber filters and counted in Omnifluor.

Results

To compare functional globin mRNA composition of normal vs. *b/b* animals, we translated rat RNA in the wheat germ cell-free system and analyzed the products. Optimum conditions for translation of rat reticulocyte RNA in wheat germ extract were determined with polysomal RNA from normal rats. Maximal stimulation of protein synthesis was obtained at 100 mM K^+ and 2.5 mM Mg^{2+}. Incorporation of amino acids was linear with increasing RNA concentration. The system was saturated by about 10 μg of polysomal RNA/50 μL of assay. At saturation, 100- to 120-fold stimulation over endogenous incorporation was observed. Protein synthesis was inhibited when the RNA level was above saturation.

The products synthesized in the wheat germ extract were analyzed by CM-cellulose chromatography. A typical pattern for separation of the seven globin chains is shown in Figure 1. For each separation, a standard [^{14}C]leucine hemolysate was cochromatographed with the [^{3}H]leucine-labeled products from translation of RNA. As shown by the ^{14}C radioactivity pattern, three α chains and four β chains were separated by this technique. The assignment as α or β was based on complete or partial amino acid sequence data (Garrick et al., 1975, 1978a). Approximately 80% of the [^{3}H]leucine-labeled products was recovered from the column. From the [^{3}H]leucine radioactivity pattern, it is obvious that the products eluted from the column corresponded almost entirely to the α- and β-globin peaks. At the $^I\beta$ region, the ^{3}H peak did not coincide exactly with the ^{14}C peak. Nevertheless, automated sequencing of the [^{3}H]leucine-labeled $^I\beta$ peak indicated that it represents authentic β chain rather than endogenous wheat germ protein contaminant. We have some evidence that at least part of the material in the $^I\beta$ region is a posttranslational product from $^{II}\beta$ (Garrick et al., 1978b). This may explain the observed mobility difference between these ^{3}H and ^{14}C peaks. A relative decrease in incorporation by wheat germ extracts in the $^{II}\beta$ region can also be seen. This response was typical when total cytoplasmic RNA was used and is addressed in the discussion.

The ^{14}C radioactivity pattern of each analysis gave us an indication of the reproducibility of the globin chain separation. We found the following values for the fraction of total [^{14}C]leucine incorporation in each individual chain: $^0\beta$ = 3.5 ± 0.5, $^0\alpha$ = 3.3 ± 0.3, $^I\beta$ = 5.3 ± 0.5, $^I\alpha$ = 31.8 ± 0.8, $^{II}\beta$ = 37.5 ± 0.6, $^{II}\alpha$ = 9.8 ± 0.3, and $^{III}\beta$ = 9.0 ± 0.5%. (The values are mean and SEM for ten observations.) The α/β ratio for these yielded 0.81 ± 0.01. Thus, the patterns are almost identical with variations probably attributable to uncertainties in assigning fractions between peaks to individual chains.

We compared the products made when different concentrations of rat RNA were added to the wheat germ cell-free system. At subsaturating levels of RNA (<10 μg), the globin chain pattern was independent of the RNA input (Table I). Therefore, a concentration curve was obtained for each RNA sample to ensure that subsaturating levels of RNA were used.

Functional mRNA composition in normal and *b/b* reticulocytes was compared using total cytoplasmic and polysomal RNA without fractionation on oligo(dT)-cellulose. Because various size classes of poly(A) segments are present in globin mRNA (Lingrel et al., 1974), affinity chromatography for poly(A) may result in the loss of certain types of mRNA. The percentage incorporation of [^{3}H]leucine into each globin chain translated from total cytoplasmic RNA isolated from *b/b* and normal reticulocytes is shown in Table II. The two patterns are very similar with small differences of less than 6%. Similarly, no major difference in globin pattern was observed with polysomal RNA isolated from *b/b* and normal reticulocytes (Table II). In both *b/b* and normal rats, the α/β ratio is higher for total cytoplasmic RNA as compared to polysomal RNA.

There is some evidence that poly(A) increases the structural stability of mRNA (Soreq et al., 1974; Marbaix et al., 1975; Hieter et al., 1976); thus, it is of interest to study the poly(A)-containing RNA from *b/b* and normal reticulocytes. Total cytoplasmic and polysomal RNA were passed through an oligo(dT)-cellulose column, and then the poly(A)-containing RNA was translated in the wheat germ extract. The results of product analyses are summarized in Table III. The incorporation patterns for poly(A)-containing RNA isolated from normal and *b/b* reticulocytes were generally similar, although an apparent difference was present in the $^I\alpha$ chain. We have not found any obvious explanation for this difference. In either *b/b* or normal reticulocytes, the pattern of polysomal poly(A)-containing RNA was very similar to that of total cytoplasmic poly(A)-containing RNA.

TABLE II: Translation of Total Cytoplasmic and Polysomal RNA in the Wheat Germ Cell-Free System.

source of RNA	% of total [^{3}H]Leu incorp							
	$^0\beta$	$^0\alpha$	$^I\beta$	$^I\alpha$	$^{II}\beta$	$^{II}\alpha$	$^{III}\beta$	α/β
normal cytoplasmic	4	7	6	47	14	16	6	2.33
b/b cytoplasmic	6	4	5	53	11	15	6	2.57
normal polysomal	4	5	5	41	26	11	8	1.33
b/b polysomal	6	5	8	40	21	13	7	1.40

TABLE III: Translation of Poly(A)-Containing RNA in the Wheat Germ Cell-Free System.

source of RNA	% of total [^{3}H]Leu incorp							
	$^0\beta$	$^0\alpha$	$^I\beta$	$^I\alpha$	$^{II}\beta$	$^{II}\alpha$	$^{III}\beta$	α/β
normal cytoplasmic	8	8	8	34	22	14	6	1.33
b/b cytoplasmic	5	4	6	45	19	14	7	1.70
normal polysomal	6	8	9	37	22	12	6	1.44
b/b polysomal	5	5	6	44	20	12	8	1.56

An advantage of translating unfractionated RNA in the wheat germ cell-free system is that it allows normalization of mRNA activity with respect to total cytoplasmic RNA. Comparison of the stimulation of protein synthesis by *b/b* and normal rat RNA is shown in Figure 2. Tritiated leucine incorporation per total cytoplasmic RNA was much lower for the *b/b* animals as compared to normal. This experiment was performed using RNA isolated from pooled blood. To verify that this difference does not result from an artifact such as obtaining completely inactive RNA from one or more of the *b/b* group, we prepared total cytoplasmic RNA from several individual normal or *b/b* rats and compared the mRNA activity. We found that the mRNA activity per total cytoplasmic RNA in *b/b* reticulocytes was 39–46% of that in normal reticulocytes.

The observed difference in mRNA activity may be attributed to either fewer mRNA molecules or lowered mRNA template activity in the *b/b* reticulocytes. Hybridization of radioactive complementary DNA to globin mRNA allows quantitation of mRNA sequences. Therefore, we synthesized cDNA using normal rat globin mRNA as a template. Total cytoplasmic RNA derived from normal and *b/b* reticulocytes was hybridized with excess cDNA (Figure 3). The 50% saturation point of the hybridization curve indicated that the amount of mRNA molecules in *b/b* RNA was about 45% of that in normal RNA.

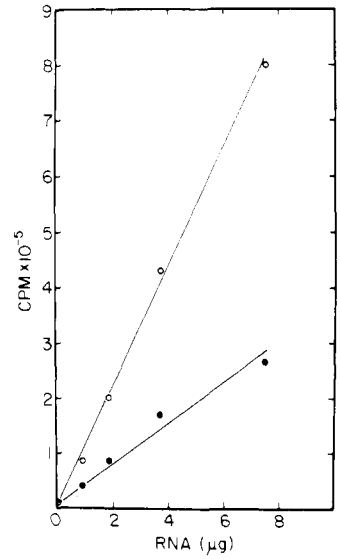

FIGURE 2: Translation of total cytoplasmic RNA isolated from normal or *b/b* reticulocytes in the wheat germ cell-free system: (○) normal rat RNA; (●) *b/b* rat RNA.

Discussion

Analysis of the products primed with mRNA in the cell-free protein-synthesizing system is a standard method to assay for mRNA composition. This method relies on the ability of the system to translate the mRNA mixture accurately. The wheat germ cell-free system has been reported to be a reliable method for the assay of human globin mRNA (Pritchard et al., 1974). Rat globin mRNA is unusually complex, containing at least seven components. Thus, it is important to determine whether such a complex mRNA mixture is accurately translated in the wheat germ extract. Our results showed that all seven globin chains were translated; however, the globin chain pattern observed after translation of polysomal RNA (Table II) was not identical to the pattern of intact reticulocytes. The α/β ratio is higher for the cell-free products as compared to those for intact cells. This alteration is probably due to a different translational control mechanism in the wheat germ as compared to reticulocytes. Since reproducible product patterns were obtained for a given RNA even at different concentrations (Table I), it is still reasonable to compare rat globin mRNA using the wheat germ cell-free system.

The data in Table II indicate that there is no difference in functional composition between *b/b* and normal rats in either total cellular or polysomal mRNA. This result is consistent with observations with intact erythroid cells where no difference has been found in the pattern of globin chain synthesis between *b/b* and normal rats (Edwards et al., 1978). Although one could object that the in vitro translation system is not reflecting faithfully the actual composition of the mRNA population, the data of Table I indicate that we are working with levels of RNA that do not influence the relative proportions of protein product. Moreover, because no difference between *b/b* and normal was detected, one would have to argue that if a difference actually existed it was coincidentally compensated by the behavior of the translational system.

More α chains were synthesized by the total cytoplasmic RNA as compared to polysomal RNA (Table II), suggesting the enrichment of α-chain mRNA in the nonpolysomal fraction. We have prepared RNA from the postribosomal supernatant and translated it in the wheat germ extract. The maximal stimulation of protein synthesis obtained was 3- and

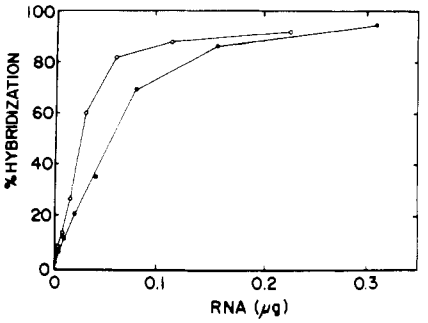

FIGURE 3: Hybridization of rat globin [^{3}H]cDNA with total cytoplasmic RNA isolated from normal or *b/b* reticulocytes. A background of S_1 nuclease resistant cDNA (5%) has been subtracted: (○) normal rat RNA; (●) *b/b* rat RNA.

0.8-fold above endogenous incorporation for normal and *b/b* nonpolysomal RNA, respectively. It appeared that an inhibitor was present in the RNA preparation because increasing RNA concentrations resulted in further inhibition of protein synthesis. This interpretation is made more likely by a report of a translational inhibitor in a nonpolysomal cytoplasmic messenger ribonucleoprotein complex (Civelli et al., 1976). The products derived from normal rat nonpolysomal RNA were analyzed. Although the minor globin chains were heavily contaminated with wheat germ endogenous proteins, the ratio for the four major chains ($^{I}\alpha + {}^{II}\alpha/{}^{II}\beta + {}^{III}\beta = 3.0$) did confirm enrichment of α-chain mRNA. Stimulation of protein synthesis by *b/b* nonpolysomal RNA was too low to allow meaningful analysis. Our results disagree with those of Crkvenjakov et al. (1976). They reported that the α/β ratio (for the four major globin chains) was similar for polysomal and nonpolysomal RNA isolated from normal rats; however, nonpolysomal RNA from *b/b* rats was enriched in α-chain mRNA. In rabbit reticulocytes, the presence of nearly pure α-chain mRNA in the postribosomal supernatant (Jacobs-Lorena and Baglioni, 1972; Olsen et al., 1972) is attributed to a translational control mechanism in which β-chain mRNA has a higher affinity for an initiation complex than α-chain mRNA (Temple and Lodish, 1975). Our results suggest that a similar mechanism may be present in the rat reticulocytes. It is interesting to note that the amount of each individual α chain is increased.

The incorporation patterns of poly(A)-containing RNA (Table III) indicate that there is no significant difference between normal and *b/b* mRNA in the degree of polyadenylation. It is remarkable that, while the patterns of total cytoplasmic and polysomal RNA are quite different, the patterns of poly(A)-containing RNA derived from cytoplasmic and polysomal RNA are almost identical. One possible explanation would be that the nonpolysomal RNA contains shorter or no poly(A) segments, but we have no additional data to support such speculation.

We found that mRNA activity per total cytoplasmic RNA in *b/b* rats is significantly lower than in normal rats. This result is in good agreement with the observation in intact reticulocytes (Edwards et al., 1978) that total protein synthesis per total RNA by *b/b* reticulocytes was about 50% of that by normal reticulocytes. Edwards et al. (1978) also noted that total RNA per reticulocyte was identical in *b/b* and normal rat reticulocytes, suggesting that the age of the reticulocyte populations was comparable. Hybridization studies indicate fewer mRNA molecules per total RNA in *b/b* reticulocytes. The data of the mRNA activity assay parallel that of the hybridization assay, suggesting that each molecule of globin mRNA of *b/b* reticulocytes is as active as that of normal reticulocytes.

Our studies of *b/b* globin mRNA indicate that the diminished globin synthesis observed in the Belgrade anemia is caused by lowered globin mRNA content in the *b/b* reticulocytes. Previous studies (Edwards et al., 1977, 1978) suggest that intraerythroid iron deficiency may be the primary defect of the *b/b* anemia. Heme deficiency in the *b/b* anemia is also supported by comparing the response to added hemin of reticulocytes and lysates from *b/b* vs. normal rats (Garrick et al., 1978c). It is well established that in the absence of iron or heme erythrocyte protein synthesis slows down and polysomes break down to smaller aggregates (Grayzel et al., 1966; Waxman and Rabinovitz, 1966). In heme deficiency, a translational inhibitor is formed which inhibits the initiation step (Maxwell et al., 1971). Lack of active translation of mRNA may result in the degradation of existing mRNA. It seems reasonable to assume that such events occur in the Belgrade anemia. The results of Crkvenjakov et al. (1976) support this hypothesis. They found that one third of the globin mRNA in *b/b* reticulocytes was in the nonpolysomal fraction, as compared to 6% for the normal reticulocytes. Correlations between hemin and globin mRNA levels were reported recently (Ross and Sautner, 1976; Dabney and Beaudet, 1977). The number of globin mRNA molecules increased when hemin was added to mouse erythroleukemia cells cultured in dimethyl sulfoxide. Heme deficiency probably exists throughout erythroid cell development in Belgrade rats. Thus, neither the data on *b/b* rats nor those with erythroleukemic cells exclude the possibilities that less mRNA is transcribed or matures properly in chronic heme deficiency.

In conclusion, we have found that, qualitatively, the globin mRNA of *b/b* rats is essentially the same as that of normal rats. Decreased globin synthesis in the *b/b* reticulocytes reflects lowered globin mRNA content in *b/b* reticulocytes.

Acknowledgments

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