Half-Lives of Beta and Gamma Globin Messenger RNAs and of Protein Synthetic Capacity in Cultured Human Reticulocytes

By Jeffrey Ross and Thomas D. Sullivan

The turnover rates of beta and gamma globin messenger RNAs and of beta and gamma globin protein synthesis in human reticulocytes have been measured. Our goal was to determine whether beta globin mRNA is significantly more stable than gamma globin mRNA during the final stages of erythroid cell maturation. Such a result could explain the reported increase in the beta-gamma protein synthetic ratio during erythroid maturation. As determined by molecular hybridization and cell-free translation assays, the half-lives of both mRNAs are 20 to 29 hours in adult and neonatal reticulocytes. Protein synthetic capacity in intact cells decays with a half-life of six to eight hours, but beta protein synthesis declines at the same rate as gamma.

Although the expression of most specialized genes is probably controlled at the transcriptional level, post-transcriptional control mechanisms modulate the quantities of some proteins. Amphibian and echinoderm oocytes contain a reservoir of untranslated mRNAs that remain stable for months ("maternal" or "masked" mRNAs) (reviewed in reference 1). After fertilization, masked mRNAs begin to be translated and are then rapidly degraded before gastrulation. Production of embryonic proteins is thus regulated by factors that affect translation and mRNA turnover. In a similar fashion, the control of histone mRNA stability during and after DNA synthesis may ensure that histone protein production is restricted to the S-phase.1,2

The experiments described here investigated the possibility that hemoglobin (Hb) A and F production in human erythroid cells is modulated by changes in beta and gamma globin mRNA turnover. The onset of Hb production in erythroblasts appears to depend on the induction of transcription of the globin genes.3 However, two observations suggest that posttranscriptional controls may play a role in determining the final levels of HbA and F in erythroblasts: (1) During differentiation of cultured erythroid progenitors (BFU-E) to reticulocytes and erythroblasts, the gamma-beta globin protein synthetic ratio decreases.3-4 That is, the gamma-beta synthetic ratio is lower in older, more mature (red) burst colonies than in younger, less mature (pink or white) colonies. This decrease occurs even in cells that make predominantly HbF.5 (2) HbF is synthesized in bone marrow normoblasts but not in reticulocytes, while HbA continues to accumulate in reticulocytes.6 Thus, the chain synthesis is reduced, relative to beta, in late-stage erythroid cells.

Three mechanisms could account for asynchronous synthesis of these globins: (1) Transcription of the gamma and beta genes is asynchronous, i.e., gamma globin gene transcription ceases before beta globin transcription. (2) Translational efficiencies change. Beta globin mRNA may be translated more efficiently than gamma globin mRNA in late-stage cells. There is some precedent for this hypothesis. For example, alpha and beta globin mRNAs are translated with different efficiencies in rabbit reticulocytes, and the concentrations of various reaction components affect alpha-beta translation ratios in vitro.6-12 (3) Transcription of both genes is synchronous, but gamma mRNA turns over faster than beta mRNA. This mechanism would affect protein synthetic ratios, especially in late-stage cells that no longer produce globin mRNAs. The level of each mRNA in normoblasts and reticulocytes would then depend solely on mRNA turnover rate.

In view of the importance of mRNA turnover during embryonic development, and because of recent evidence that human globin mRNAs with similar structures (beta and delta) are degraded at different rates,13 we tested the third hypothesis by measuring the stability of gamma and beta globin mRNAs. We find that the turnover rates are approximately equivalent in cultured reticulocytes from an adult patient with mild sickle cell anemia and from neonatal umbilical cord blood. Furthermore, although reticulocyte protein synthetic capacity decays twofold to threefold more rapidly than mRNA, the rates of synthesis of beta and gamma globins decay in parallel. These observations exclude the hypothesis that the change in the gamma-beta ratio results from differences in mRNA stability in late-stage erythroid cells. They support the hypothesis that the ratio changes because the gamma and beta globin genes are transcribed asynchronously.

Materials and Methods

Cell culture and RNA isolation. Umbilical cord blood from three unrelated neonates and peripheral blood from one transfused patient with mild sickle cell anemia were used as sources of reticulocytes. Blood cells were collected in heparinized tubes and washed twice at room temperature with a fivefold to tenfold-volume excess of Iscove's medium containing 5% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cell pellet was resuspended at 1 to 4 x 10⁸ cells (reticulocytes plus erythrocytes) per milliliter in warm Iscove's medium containing 20% FCS.
and the same antibiotics (growth medium). Cells were cultured at 37 °C in a humidified 5% CO₂ atmosphere. In each experiment, total cells were counted at different culture intervals, and no loss (hemolysis) was observed. The percentage of reticulocytes assessed by staining with new methylene blue remained constant throughout the culture period.

To prepare total RNA, cells were harvested and centrifuged at 4 °C (2,400 rpm, eight minutes, PRJ centrifugation). Pellets were stored at −70 °C without further washing. They were subsequently thawed but not allowed to warm up above 4 °C. An approximately tenfold-volume excess of ura lysis buffer (7 mol/L urea, 2% [wt/vol] sodium dodecyl sulfate, 0.35 mol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Tris-Cl, pH 8.0) plus, in some experiments, 40 μg/mL of Escherichia coli tRNA (as carrier) was added. The mixture was vortexed vigorously at room temperature and was extracted two times with 1.0 vol of phenol and 0.5 vol of chloroform-isooamyl alcohol (CIAA, 24:1, vol/vol) and then two times with CIAA alone. The RNA was then precipitated twice in ethanol and then twice in ethanol containing 0.1 mol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Tris-Cl, pH 7.4. For cell-free translation and Northern blot analysis, the RNA was further purified by pelleting through a CsCl cushion. For each experiment, each RNA sample was resuspended in the same volume of buffer, ie, a given volume of RNA was added. A given volume of RNA represents the same number of total cells (reticulocytes plus erythrocytes) harvested for each time point.

Hybridization analyses. Solution hybridizations to measure mRNA levels were performed with 5'-32P-labeled probes specific for either β or γ globin mRNA. This method has been described in detail. The percentage of hybridization was determined by measuring the number of counts resistant to S, nuclease digestion. The β globin probe is a 285 bp HinfI fragment of clone H91S,15 32P-5'-end labeled at +595 from the mRNA cap site, as previously described.16 The γ globin probe is an approximately 900 nucleotide fragment prepared from the cDNA clone JW151.14 After incubating JW151 DNA with Sau 3A, which cuts at codons 80-81, the mRNA-complementary strand was purified by affinity chromatography on oligo dT-cellulose. This probe does not hybridize to α, δ, or β globin sequences (data not shown). The γ and β probes were single and double stranded, respectively. Because only one labeled strand of the β probe will hybridize with mRNA, the theoretical maximum hybridization to β mRNA is 50% (see Fig 1A). See Ross and Donaldson14 for detailed discussion of why hybridization frequently fails to achieve the theoretical maximum. Data for all solution hybridizations were analyzed by a modification of the standard Cot curve format.19 In this case, the percentage of hybridization (S, resistant cpm with reticulocyte RNA minus S, resistant cpm with RNA divided by input cpm × 100) was plotted vs volume of RNA added. A given volume of RNA represents the same number of total cells (reticulocytes plus erythrocytes) harvested for each time point in a given experiment. Therefore, the Cot curves actually plot the amount of globin mRNA on a per cell basis, rather than on a per microgram basis. The relative amounts of mRNA at each time point were determined by comparing the amounts of RNA necessary to give 50% of maximum hybridization and were plotted as a percentage of time zero (Figs 1 and 2). Straight lines, determined by linear regression analysis, were drawn through the points, taking time zero as equivalent to 100%.

Fig 1. Solution hybridization analysis of beta and gamma globin mRNAs in cultured reticulocytes. Umbilical cord blood was cultured at 10⁶ total cells per milliliter, as described in Materials and Methods. Aliquots (3 mL) were harvested at the times noted below, and total RNA was extracted without added carrier. Each sample was dissolved in 300 μL so that each microliter contained RNA from approximately 3.3 × 10⁶ cells (reticulocytes plus erythrocytes). RNA was hybridized in solution with 5'-32P-labeled DNAs specific for β or γ globin mRNA, each at a specific activity of approximately 10⁶ cpm/μg, and S, resistant cpm were determined. (A) Hybridization with the double-stranded 32P-β-globin probe: 285 bp, HinfI fragment of H91S, 5' end-labeled at nucleotide +595 from the mRNA cap site. (B) Hybridization with the single-stranded 32P-γ-globin probe: 900 nt single-stranded Sau3A fragment of JW151, 5' end-labeled at +195 from the mRNA cap site. Closed circles, 0 hour; open circles, 6½ hours; closed squares, 16 hours; open squares, 23 hours; triangles, 41½ hours.

Fig 2. Half-lives of beta and gamma globin mRNAs and of total reticulocyte RNA. Points for β (top panel) and γ (middle panel) globin mRNA decay were derived from panels A and B of Fig 1, respectively, by determining the quantity of RNA required to protect a given percentage of the labeled mRNA probe (see Materials and Methods). Total RNA decay data (bottom panel) were derived from Orcinol assays of RNA recovered at each time point. Lines were determined by linear regression analysis, including the time zero point as 100%. The half-lives of β and γ mRNAs and of total RNA are 31, 29, and 43 hours, respectively. These data are experiment 5 of Table 1.
Table 1. Summary of Molecular Hybridization Experiments

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Source of Reticulocytes</th>
<th>Half-life of Globin mRNA (h)</th>
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<tbody>
<tr>
<td>1</td>
<td>Sickle cell anemia</td>
<td>Beta: 17, Gamma: 15</td>
</tr>
<tr>
<td>2</td>
<td>Sickle cell anemia</td>
<td>Beta: 32, Gamma: 34</td>
</tr>
<tr>
<td>3</td>
<td>Neonatal umbilical cord</td>
<td>Beta: 30, Gamma: 22</td>
</tr>
<tr>
<td>4</td>
<td>Neonatal umbilical cord</td>
<td>Beta: 34, Gamma: 34</td>
</tr>
<tr>
<td>5</td>
<td>Neonatal umbilical cord</td>
<td>Beta: 31, Gamma: 29</td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td></td>
<td>Beta: 29 (± 6), Gamma: 27 (± 8)</td>
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The half-life values are derived from the slopes of lines such as those in Fig 2. Experiments 1 and 2 used samples collected at different times from the same adult patient. Experiments 3 through 5 used different samples from unrelated newborns.

RESULTS

Turnover rates of beta and gamma globin mRNAs. Reticulocytes were cultured in enriched medium containing 20% FCS. No decrease in total cell number or percentage of reticulocytes was observed during the 40- to 48-hour culture periods (data not shown). Total RNA was extracted and annealed in solution with 5'-32P-labeled DNA probes, to quantitate β or γ globin mRNA levels. Although these RNAs possess similar primary structures, each probe detects one, but not the other, mRNA because the 32P-labeled terminus of each probe is located in a region of nonhomology. For example, a large unlabeled segment of the β probe might anneal with γ mRNA, but the labeled terminus does not anneal, remains single-stranded, and is digested by S1 nuclease.

The quantity per cell of each mRNA decreases as the reticulocyte matures (Figs 1 and 2). The relative amount of mRNA in each sample (Fig 2) was determined by measuring the volume of RNA required to saturate a given percentage of DNA (20% hybridization for β; 50% hybridization for γ). (Fig 1). The greater the volume of RNA needed to hybridize a given percentage of probe, the lower the amount of mRNA in that fraction. Summary data from five experiments, three with umbilical cord blood from unrelated neonates and two with different samples from an adult with mild sickle cell anemia, indicate that the turnover rates of both mRNAs are

Fig 3. Polyacrylamide gel electrophoresis of reticulocyte protein. Reticulocyte culture, protein labeling, and cell lysis were performed as in Table 2A legend and Materials and Methods. Duplicate samples from different culture time points were electrophoresed in a polyacrylamide gel containing urea and Triton X-100. (Panel A) Protein stain. After electrophoresis the gel was stained with Coomassie brilliant blue, destained by diffusion, and photographed. (Panel B) Fluorography. The gel was treated with ENHANCE (New England Nuclear, Boston) and dried according to the manufacturer's instructions. Preflashed film (Kodak XAR-5) was exposed to the gel at -70 °C for 12 days. Globin proteins are indicated on the left. These data are experiment 3 of Table 2A. Lane 1, 13 μg; 0 hour, 4,000 dpm; lane 2, 39 μg, 0 hour, 12,000 dpm; lanes 3 and 4, 130 μg, 0 hour, 40,000 dpm; lanes 5 and 6, 130 μg, 6½ hours, 44,000 dpm; lanes 7 and 8, 130 μg, 16 hours, 20,600 dpm; lanes 9 and 10, 130 μg, 23 hours, 8,400 dpm; lanes 11 and 12, 130 μg, 41½ hours, 6,250 dpm.

EOSINOPHIL PEROXIDASE
similar (Table 1). We do not know why the values were different between experiments 1 and 2.

**Turnover rates of beta and gamma globin protein synthesis in intact reticulocytes.** Synthesis of γ and β globin proteins was monitored in cultured reticulocytes for several reasons. First, this assay measures the functional, as opposed to the structural, half-lives of the mRNAs. Second, it should detect changes, if any, in the relative translational efficiencies of the mRNAs during culture.

Intact cells were incubated in medium containing $^3$H-leucine for one hour. Globin chains in crude cell lysates were separated by electrophoresis in acrylamide–Triton polyacrylamide gels (see Materials and Methods) (Fig 3). The gels were stained, and globin bands were excised and counted. The results of three such experiments indicate that the half-life of β and γ globin protein synthesis in intact cells is similar, approximately seven to nine hours for each (Table 2A).

To summarize, β and γ mRNAs decay at similar rates. However, the rate depends on how the mRNA is assayed. The structural half-lives are 27 to 29 hours, as measured by molecular hybridization with deproteinized RNA (Table 1). The functional half-lives are seven to nine hours, as determined by translation in intact cells (Table 2A). There are at least two possible explanations for this discrepancy: (1) The hybridization assay overestimates mRNA half-life. This assay could detect mRNA fragments that are large enough to anneal to the probe but cannot be translated. For example, putative β globin mRNA degradation products in rabbit reticulocytes are detectable by hybridization. (2) The functional assay in whole reticulocytes underestimates mRNA half-life because something else limits translation. That is, this assay measures the half-life of a limiting translation factor(s), not mRNA.

**Size and translational capacity of mRNAs.** To address these two possibilities, additional experiments were performed. First, Northern blot hybridization analysis was exploited to determine whether major mRNA breakdown products accumulate with time. Second, RNA isolated from cultured reticulocytes was translated in a cell-free protein synthesis system, to determine the levels of functional mRNA during culture.

Umbilical cord blood cells were cultured for 0, 20, or 44 hours. At each time, total cell RNA was purified by pelleting through a CsCl cushion, electrophoresed in an agarose gel containing formaldehyde, and transferred to a nylon support. The transfer was hybridized with a nick-translated human β globin cDNA clone and autoradiographed. The probe should hybridize to β and γ globin mRNAs under these conditions. A single band of approximately 650 nucleotides is observed at each time point (Fig 4). The band is broad, probably because the probe hybridizes to two mRNAs and because the poly A size is not uniform. More important, no discrete, lower mol wt bands are observed. Bands containing 20% or less of the input can easily be detected (lane 1). Therefore, there is no evidence for the accumulation of

<table>
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<th>Table 2. Summary of Experiments on the Translation of Beta and Gamma Globin mRNAs in Intact Reticulocytes v Cell-free Extracts</th>
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<tr>
<td>Experiment No.</td>
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<tr>
<td>A. Globin synthesis in cultured reticulocytes*</td>
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<tr>
<td>1</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
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<tr>
<td>Mean (± SD)</td>
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<tr>
<td>B. In vitro translation†</td>
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<tr>
<td>4</td>
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<tr>
<td>5</td>
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<tr>
<td>Mean (± SD)</td>
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*Neonate umbilical cord blood cells were cultured and pulse-labeled for one hour with $^3$H-leucine at different times (Materials and Methods). Lysates were prepared, samples were electrophoresed in polyacrylamide gels to separate globin chains (see Fig 3), and the amount of radioactivity in each globin band was determined. Half-lives were measured by plotting cpm (as the percentage of time zero) v culture time, as described for Fig 2 and Table 1. The slopes of the lines were used to determine decay rates.

†Neonate umbilical cord blood cells were cultured, and total RNA was deproteinized and isolated after different culture times (Materials and Methods). In vitro translation of the RNA was performed in hemin-supplemented, nuclease-treated rabbit reticulocyte lysates containing methionine. Aliquots of the translation mixture were analyzed in acid-urea–Triton gels as in A.

**Fig 4. Blot transfer analysis of reticulocyte globin RNAs.** Umbilical cord blood cells were cultured for 0, 20, or 44 hours. Total RNA was prepared by phenol extraction without added carrier and was pelleted through a CsCl cushion (see Materials and Methods). RNA samples were electrophoresed in a 1.5% agarose gel containing formaldehyde and transferred to a nylon support (Biodyne A paper, Pall). The transfer was hybridized with nick-translated β globin cDNA clone JW102, and the autoradiogram was exposed for six days at –70 °C with Kodak XAR film and a Lightning Plus intensifying screen. Size marker fragments in nucleotides are noted on the right side of the figure (pBR322 DNA treated with HaeII and kinased). Lane 1: tγ, 0.2 nM; lane 2: tγ, 1.0 nM; lane 3: tβ, 1.0 nM; lane 4: tβ, 1.0 nM.
discrete, stable mRNA degradation products. Of course, small RNA fragments that transfer or anneal poorly and large fragments that migrate immediately below the intact mRNA might go undetected in this assay. For this reason we have also performed S1 mapping experiments with a DNA probe that anneals to the 3' one third of β globin mRNA.25 Low levels of shortened protected fragments were observed with long exposures (data not shown). These fragments could result from stable degradation products, but they did not accumulate with time in culture and were not investigated further.

The functional state of each mRNA was analyzed by cell-free translation in nuclease-treated rabbit reticulocyte lysates.26 Subtle structural changes that escape detection with the Northern blot or S1 mapping assays but that alter mRNA function might be detected by in vitro translation. The results of this assay parallel the molecular hybridization results: β and γ globin mRNAs decay with half-lives in the range of 20 to 30 hours (Table 2B). Thus, the turnover rates of reticulocyte β and γ globin mRNAs are similar, as measured by both structural and in vitro functional assays. This conclusion highlights the observation that the protein synthetic capacity of a reticulocyte disappears faster than does the globin mRNA.

DISCUSSION

We draw two conclusions from this work: (1) β and γ globin mRNAs decay at the same rate in cultured reticulocytes. (2) The protein synthetic capacity of reticulocytes disappears faster than does either globin mRNA.

Work from other laboratories had already demonstrated that globin mRNA levels rise in parallel with globin chain synthesis between the BFU-E and late erythroblast stages of development.27 These data, along with those reported here, indicate that the shift in γ-β synthetic ratios or in HbF-HbA accumulation ratios in late-stage erythroid cells does not result from differential stability of γ and β globin mRNAs. This conclusion makes it probable that transcription of these genes is repressed asynchronously. Thus, expression of these genes during erythroid cell maturation is determined by controls that affect both the rate of transcription and the time period during which each gene is transcribed.

The second conclusion, that protein synthesis decays faster than mRNA levels in reticulocytes, extends the original observation of Temple and Lodish that translation in rabbit reticulocyte lysates is not limited by the amount of endogenous mRNA.11 Analogous results have been observed in amphibian oocytes28 and in the lens of the developing chick eye.29 Additional experiments are required to characterize which protein synthesis factor(s) become rate limiting in maturing reticulocytes.

ACKNOWLEDGMENT

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