Expression of the Silent Hemoglobin Gene in Sheep

STUDIES OF THE GLOBIN MESSENGER RIBONUCLEIC ACIDS

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SUMMARY

We have analyzed the messenger ribonucleic acids from reticulocytes of adult sheep carrying different globin genes and synthesizing correspondingly different hemoglobins. Hemoglobin A (αβ2) is synthesized by A/A homozygotes in the early stages of anemia, whereas these same animals switch over to hemoglobin C (αβ2) when they are severely anemic. On the contrary, only hemoglobin B (αβ2) is synthesized by normal or by anemic B/B homozygotes.

All four types of sheep globin messenger ribonucleic acids (i.e. for α, βa, βb, or βc chains) are successfully translated in a protein-synthesizing lysate from rabbit reticulocytes. The newly synthesized radioactive sheep globin chains are separated from the rabbit globin chains by chromatography on a carboxymethylcellulose column. The results suggest that a switch from βa to βc messenger ribonucleic acid accompanies the development of anemia in A/A sheep. However, the rabbit reticulocyte lysate system synthesizes sheep globin chains in different proportions than they are synthesized intracellularly; in the rabbit lysate, α and βa chains constitute a lower percentage and βc chains a larger percentage of the synthesis.

The method of Lodish (J. Biol. Chem. 246, 7131) was used to quantitate the relative levels of α, βa, and βc messenger ribonucleic acids in reticulocytes from an A/A homozygote. The proportion of βc messenger is approximately twice as high as the proportion of βc synthesis in sheep reticulocytes. On the contrary, the proportion of α messenger is lower than the proportion of α chain synthesis in sheep reticulocytes.

Domestic sheep and related species have an unusual polymorphism at the gene locus which specifies the β-chain of hemoglobin (1–5). Although sheep have only one known α chain gene, there are two common alleles, A and B, of the β chain locus. The A and B alleles are quite dissimilar since the βa and βb polypeptides differ by seven amino acid replacements (6). Furthermore, when adult sheep with the A allele are made anemic, they cease producing βa chains and they switch on the synthesis of a different type of chain, the βc chain. On the contrary, expression of the B allele is unaffected by anemia. Moreover, the βc chain differs markedly from βa and βb chains both in its amino acid sequence and in its length (6). We will refer to this switch from βa to βc synthesis in anemic sheep as “silent gene” expression. The mechanism responsible for this switch in β chain synthesis has not been elucidated.

This paper describes some of our studies of the control of sheep hemoglobin synthesis. We have tried to learn whether the βa → βc synthesis switch in anemic sheep is accompanied by a corresponding switch in types of mRNA present in reticulocytes, or whether the mRNA remains constant during the synthesis switch. Our data suggest that a switch from βa to βc mRNA does accompany anemia in A/A homozygotes. However, sheep hemoglobin synthesis is also controlled at the translational level, since the different globin mRNAs are translated intracellularly with very different efficiencies. Furthermore, silent gene expression is accompanied by changes in the proportions of several tRNAs (7).

MATERIALS AND METHODS

Preparation and Labeling of Sheep Reticulocytes—Adult Willamette sheep weighing 60 to 72 kg were made anemic and were bled according to the following schedule. Blood was always collected into heparin and was kept chilled at 0°C until used. On Day 0, 300 ml of blood were collected and the animals were injected subcutaneously with 10 mg per kg of phenylhydrazine. Additional phenylhydrazine injections were given on Days 3, 5, 9, 11, 16, and 18. On the other hand, 300-ml blood samples were collected on Days 7, 14, and 21. Hematocrits were determined and the reticulocytes were stained for microscopic examination with brilliant cresyl blue.

Blood cells were sedimented by centrifugation at 2,000 × g for 10 min and were washed three times with physiological salt solution (0.13 m NaCl, 0.005 m KCl, 0.0015 m MgCl₂). One milliliter of the packed cells was routinely resuspended at 1 × 10⁹ cells per ml in nutrient medium as described by Horii and Rabinovitz (8). The cell suspension was swirled gently in a water bath at 37°C for 10 min before the addition of 2 µCi per ml of [3H]leucine.
of a mixture of uniformly $^{14}$C-labeled 1 amino acids (New England Nuclear Corp., NEC-445). In some experiments, various concentrations of cycloheximide (Calbiochem) were added to aliquots of the cell suspension to inhibit protein synthesis. After 60 min of incorporation, the cells were chilled by dilution with 4 volumes of cold physiological salt solution. The cells were lysed with 1 volume of water and 0.4 volume of toluene. After centrifugation at 10,000 × g for 30 min, the hemolysate was dialyzed thoroughly with 0.01 M NaCl.

Separation and Analysis of Sheep Hemoglobin—The radioactive labeled sheep hemoglobin was routinely analyzed by several methods. Starch gel electrophoresis of hemoglobin at pH 8.6 was by the method of Smithies (9) and was kindly performed by Mrs. Zelma Stocklen. The gels were stained with benzidine (10). Electrophoresis of the hemoglobins in polyacrylamide gels was by the method of Smith and Evatt (11). The unstained polyacrylamide gels were scanned for absorbance at 540 nm on a gel scanner in a Gilford model 222 spectrophotometer. This method resulted in a good separation of hemoglobins A, B, and C and allowed an accurate (±5%) and rapid quantitative analysis of their proportions.

Chromatographic separation of sheep globin chains on a carboxymethylcellulose column was carried out at room temperature by a modification of the method of Adams et al. (12). Globin (75 mg) was prepared from labeled sheep hemoglobin with cold acid acetone according to the procedure of Anson and Mirsky (13). It was redissolved in Buffer A (8 M urea, 0.05 M β-mercaptoethanol, 0.00125 M NaHPO₄, adjusted to pH 7.0 with H₃PO₄) and was dialyzed overnight at 4°C against the same buffer. A stock solution of 8 M urea was deionized just before use by passage through a mixed bed ion exchange column (Bio Rad, AG 501 × 8, 20 to 50 mesh); the conductivity of the eluted urea solution was always less than 10⁻⁴ ohm⁻¹. Previously swollen carboxymethylcellulose (30 g) (Whatman, CM-52) was stirred with 150 ml of Buffer A. After setting for 30 min, the supernatant and fines were removed. This washing process was repeated twice more. The columns (1.2 × 25 cm) were then poured and were washed overnight with Buffer A at a flow rate of 12 ml per hour. The eluted globin was then placed onto the columns which were washed with 10 ml of Buffer A and then with 300 ml of a linear gradient made with 150 ml of Buffer A and 150 ml of Buffer B (8 M urea, 0.05 M β-mercaptoethanol, 0.03 M NaHPO₄, adjusted to pH 7.0 with H₃PO₄). The flow rate was maintained at 12 ml per hour and the fraction size at 5 ml. The absorbance of the eluted fractions were measured at 280 nm. As determined from the absorbance profiles, the yields of α and β chains were equal and were at least 95% of the applied globin. One-milliliter portions of each fraction were mixed with 15 ml of scintillation fluid (made by mixing 3 g of 2,5-diphenyloxazole (POPOP), 0.3 g of dimethyl 1,4 bis[2-(5-phenyloxazoyl)] benzene (POPPOP), 400 ml of toluene and 600 ml of Triton X-100) and the samples were counted in a Packard model 3125 liquid scintillation spectrometer. When the globin chains were labeled with both 3H and 14C, counting channels were counted in which the $^{3}$H window contained 14%; $^{14}$C counts and the $^{14}$C window contained essentially no $^{3}$H counts.

Preparation of Sheep Globin Messenger Ribonucleic Acids—Sheep reticulocytes which had been washed three times with cold physiological salt solution were lysed by the addition of an equal volume of lysis buffer (2 mM MgCl₂, 1 mM dithiothreitol, and 0.1 mM EDTA, pH 7.5). The lysate was centrifuged for 10 min at 30,000 × g. The supernatant was again centrifuged for 30 min at 105,000 × g for 3 hours in the Spinco 30 rotor. The pellet, which contained ribosomes and some more slowly sedimenting materials, was used for the preparation of messenger RNA. The pellets were dissolved in a buffer containing EDTA, and the ribonucleoprotein particle that contains 10 S messenger RNA was separated from ribosomal subunits on a sucrose density gradient (14). The RNA was then precipitated by adjusting the appropriate fractions (sedimentation between 10 and 30 S) to 0.15 M NaCl and to 0.5%; sodium dodecyl sulfate, followed by the addition of 3 volumes of ethanol. After 2 hours at −20°C, the precipitate was collected by centrifugation; it was dissolved in buffer containing sodium dodecyl sulfate and was extracted with phenol-chloroform, as described by Penman (15). After precipitation with ethanol, the RNA was washed at least three times with ethanol at −20°C to remove detergent, was dissolved in water at a concentration of 1 mg per ml, and was stored frozen at −70°C. This method gave biologically active messengers for all of the sheep globin chains (see below).

Although less thorough than the methods which have been used to purify rabbit and human globin mRNA (16−21), this method has the advantage that it does not require assuming that all of the sheep globin mRNAs have precisely the same sedimentation coefficient, an assumption which would have been unsuitable for this project. The present method was considered adequate because impure mRNA preparations have been successfully assayed in the rabbit reticulocyte lysate (22) and because further purification would have resulted in a lowering of yields.

Cell-free Protein Synthesis in a Lysate from Rabbit Reticulocyte—The methods used for preparing the rabbit reticulocyte lysate and for the incorporation of radioactive amino acids into hemoglobin are described elsewhere (16, 23). We employed 0.4 ml of the lysate per ml of the cell-free incorporation mixture. Incorporation of L-[4,5-3H]leucine (40 μCi per ml, 690 μCi per μm) was done in the absence of added messenger RNA, whereas a parallel incorporation of uniformly labeled L-[14C]leucine (5 μCi per ml, 259 μCi per μm) was done in the presence of 20 μg of messenger RNA. Following incorporation for 90 min at 29°C, the two incorporation tubes were mixed and 60 mg of nonradioactive carrier sheep hemoglobin were added as described for each experiment. The preparation of globin, the separation of globin chains on a carboxymethylcellulose column, and the methods for $^{2}$H and $^{14}$C radioactivity measurement are described above.

RESULTS

Development of Anemia in Sheep—Phenylhydrazine causes a lysis of circulating erythrocytes. In sheep, the resulting severe anemia is accompanied by a complete replacement of Hb A ($α_2β_2$) by Hb C ($α_2β_2$). A typical example of this replacement in an A/A homozygote is shown in the starch gel electrophoresis analysis in Fig. 1. The hemoglobins are stained with benzidine. We have observed very similar kinetics for the switch from $β^A$ to $β^C$ chains in four A/A and in one A/B sheep (e.g. see Reference 5 and 7). Following a lag of 6 to 7 days, Hb C appears in the blood. Hb A disappears very rapidly after this time because it is no longer synthesized and because the phenylhydrazine is causing very rapid destruction of circulating erythrocytes.

The abbreviation used is: Hb, hemoglobin.
The normal sheep, bled before onset of anemia, had hematocrits of 50%, contained few reticulocytes (less than 1%), and their blood was inactive in incorporation of radioactive amino acids into hemoglobin. However, by Day 7 of anemia, the hematocrits had fallen to 25 to 30% and the blood contained approximately 15% reticulocytes. By Day 14, anemia was severe, the hematocrits were approximately 20%, and the blood contained 70 to 90% reticulocytes. On Day 21, hematocrits were between 13 and 20% and the blood contained approximately 90% reticulocytes.

**Translation of Sheep Globin Messenger RNA in a Rabbit Reticulocyte Lysate**—We have assayed the sheep reticulocyte mRNA in a protein synthesizing lysate from rabbit reticulocytes (see “Materials and Methods”). One aliquot of the rabbit reticulocyte lysate system was incubated with [3H]leucine and a second aliquot was incubated with both [3H]leucine and with sheep reticulocyte mRNA. The two aliquots were then mixed with each other and with nonradioactive carrier sheep hemoglobins. The resulting mixture of materials was then converted to globin and was analyzed by chromatography on a carboxymethylcellulose column.

A typical analysis using mRNA from a B/B homozygote is shown in Fig. 2. The optical density tracing at 280 nm shows the positions of elution of the unlabeled sheep α and β chains which were added as carrier. The rabbit α and β chains co-chromatograph as a single peak which elutes slightly ahead of the sheep α chains. Generally, there is no clear separation between the rabbit globin chains and the sheep α chains. The 3H and 14C radioactivity profiles are also plotted. Clearly, there is a peak containing 14C label which co-chromatographs with the carrier PC chains, but there is no such peak which co-chromatographs with the carrier α chains. On the contrary, 3H label is absent when sheep mRNA was not added to the rabbit lysate system. We conclude that PC mRNA from the severely anemic A/A sheep was successfully translated in the rabbit reticulocyte lysate.

Fig. 3 shows the results obtained when the messenger RNA was from a severely anemic A/A sheep. The reticulocytes used as the mRNA source were synthesizing only βC and α chains, but were not producing any βA chains. The optical density tracing shows the positions of elution of the carrier βA, βC, and α chains. Clearly, there is a peak containing 14C label which co-chromatographs with the carrier βC chains, but there is no such peak which co-chromatographs with the carrier βA chains.

**Fig. 1.** Starch gel electrophoresis of sheep hemoglobins. The hemoglobins were taken from an A/A homozygote during the development of anemia. The hemoglobins are stained with benzidine and are in the following order, from right to left: 1, Day 0 of anemia; 2, Day 7; 3, Day 14; 4, Day 21; 5, a 1:1 mixture of the samples from Days 0 and 21. This latter mixture illustrates that Hb C stains more weakly with benzidine than the same amount of Hb A.

**Fig. 2.** Cell-free translation of mRNA from a B/B homozygote. The mRNA was isolated from the sheep after 14 days of anemia and was assayed in the rabbit reticulocyte lysate system. One aliquot of the cell-free lysate was incubated only with [3H]leucine, whereas the other aliquot was incubated with both [3H]leucine and with the sheep mRNA (see “Materials and Methods”). Nonradioactive carrier hemoglobin β was added after the incorporation was completed. ●—●, O.D. 280 nm; ▲—▲, 14C; ○—○, 3H.

**Fig. 3.** Cell-free translation of mRNA from a heavily anemic A/A homozygote. The mRNA was isolated from the sheep after 14 days of anemia and was assayed as described in Fig. 2; however, in this experiment, the nonradioactive carrier hemoglobin was a mixture of Hb A and Hb C. ●—●, O.D. 280 nm; ▲—▲, 14C; ○—○, 3H. The trailing portion of the last radioactivity peak has an elevated 14C/3H ratio, suggesting that they may have been some α chain synthesis.

**Fig. 4.** The results obtained when the sheep messenger RNA was from an A/A homozygote at an earlier stage of anemia; the blood was taken on Day 7 of anemia. As will be shown below, the reticulocytes which served as the mRNA source were synthesizing βA chains as well as βC and α chains. As can be seen from the optical density tracing, the separation of globin chains was especially good on this column and a resolution of the α chains from the peak of rabbit α plus β chains is clearly apparent. There are three regions of the eluate where there are
**FIG. 4.** Cell-free translation of mRNA from a mildly anemic A/A homozygote. The mRNA was isolated from the same sheep as used in Fig. 3, except that the animal had only been anemic for 7 days. The nonradioactive carrier hemoglobin was a mixture of Hb A and Hb C. •—•, 0.1 C, 280 nm; ▲—▲, 14C; ○—○, 1H. The relative levels of incorporation into the different sheep globin chains (see text) are obtained when these data are normalized to the different leucine contents of sheep α (20 leucine), βA (17 leucine), and βC (18 leucine).

**FIG. 5.** Chromatographic separation of sheep globins labeled intracellularly with a mixture of 14C-amino acids. The A/A sheep reticulocytes were the same ones used as the mRNA source in Fig. 4. They were labeled as described under "Materials and Methods." •—•, O.D. 280 nm; ▲—▲, 14C. Peaks V is often labeled in red cells from non-anemic or from only mildly anemic animals.

peaks of 14C incorporation and relatively high 14C:1 ratios, and these three regions co-chromatograph with the carrier sheep βA, βC, and α chains, respectively. We conclude that this sheep mRNA preparation contained mRNA for all three of these types of globin chains and that all of these messengers were successfully translated in the rabbit reticulocyte lysate.

However, the rabbit reticulocyte lysate system synthesized the three types of sheep globin chains (α, βA, and βC) in a different proportion than they were synthesized intracellularly. Fig. 5 shows a carboxymethylcellulose chromatographic separation of radioactive globin chains synthesized within the sheep reticulocytes. The cells were the same ones used as the mRNA source in Fig. 4. The absorbance profile shows that the cells contained mostly βA chains (94%), with only 4% βC chains; the intracellular synthesis ratio of βC:βA:α chains was 1.0:0.52:1.4 (see Fig. 5). On the other hand, the mRNAs prepared from these cells were translated in the rabbit reticulocyte lysate in the approximate ratio of βC:βA:α equal to 1.0:0.12:0.14 (see Fig. 4). Thus, the synthesis of βC and α chains were relatively inefficient in the cell-free system.

**FIG. 6.** Effect of cycloheximide on intracellular globin synthesis in reticulocytes from an A/A homozygote. The sheep had been anemic for 7 days. The cells were labeled with a mixture of 14C-amino acids and the globins were fractionated by carboxymethylcellulose chromatography (see "Materials and Methods"). The cells were labeled in the absence (a) or presence (b) of 0.5 μg per ml of cycloheximide. •—•, O.D. 280 nm; ▲ — ▲, 14C. Peak V is often labeled in red cells from non-anemic or from only mildly anemic animals.

Relative Levels of α, βA, and βC Messengers in Sheep Reticulocytes—Lodish has recently described a method for measuring the relative intracellular levels of different globin mRNA (24). Although rabbit reticulocytes contain more α mRNA than β mRNA, the β mRNA is translated more efficiently intracellularly (24). This method is based on the simple axiom that if two mRNAs are translated intracellularly with different efficiencies, then the rate-limiting rates for their translations must be unequal. However, when protein synthesis is rather severely inhibited by a drug which slows down translation in a messenger-independent fashion, then the drug-inhibited step of protein synthesis will become the rate-limiting step for translation of the intracellular mRNA. In these conditions, the efficiencies of translation of all mRNAs will become equal. It is known that cycloheximide inhibits the translational step of protein synthesis (23, 20) and that it does not selectively interfere with the translation of different messengers (24).

Fig. 6 shows a carboxymethylcellulose chromatographic fractionation of 14C-labeled globin from reticulocytes of an A/A homozygote. The globin was labeled intracellularly in the absence (a) or presence (b) of 0.5 μg per ml of cycloheximide. In this concentration of cycloheximide, protein synthesis was inhibited by 90%. Clearly, the relative levels of 14C incorporation into the different peaks are altered in the presence of cycloheximide. In the control cells (Fig. 6a), the relative incorporation ratio of βC:βA:α is 1.0:0.32:1.27, whereas in Fig. 6b the ratio is 1.0:0.16:0.77, respectively. Higher concentrations of cycloheximide did not further alter these ratios of incorporation. These data imply that the reticulocytes contained much less βA.
Fig. 7. Effect of cycloheximide on intracellular globin synthesis in reticulocytes from a heavily anemic A/A homozygote. The experiment is the same as in Fig. 6, except that the sheep had been anemic for 21 days. The reticulocytes were labeled in the absence (a) or presence (b) of 0.5 μg per ml of cycloheximide.

![Graph showing optical density and radioactivity](image)

| Fraction Number | O.D. | CPM |
|-----------------|------|-----|
| 10              | 0.0  | 100 |
| 20              | 0.0  | 200 |
| 30              | 0.0  | 300 |
| 40              | 0.0  | 400 |
| 50              | 0.0  | 500 |
| 60              | 0.0  | 600 |

mRNA than β mRNA, although the β mRNA was translated relatively efficiently in the control cells. Furthermore, the cells contained considerably less α mRNA than β mRNA.

A similar experiment using cells from a later stage of anemia is shown in Fig. 7. The relative level of incorporation into α chains is markedly reduced in the presence of cycloheximide. In the control, the incorporation ratio is β:α equal to 1.0:0.8, whereas in 0.5 μg per ml of cycloheximide the ratio is 1.0:0.5, respectively. In agreement with Fig. 6, these data suggest that sheep reticulocytes contain relatively less mRNA for α chains than for β chains, and that the α mRNA is more efficiently translated.

**DISCUSSION**

Translation of Sheep Globin Messenger RNA in a Rabbit Reticulocyte Lysate—Lockard and Lingrel (16) have shown that mouse mRNA for β chains can be translated in a protein-synthesizing lysate from rabbit reticulocytes. Furthermore, the mouse β chains were separated from the rabbit α and β chains by chromatography on a carboxymethylcellulose column. This same lysate system has more recently been used for translation of immunoglobulin mRNA (27), ovalbumin mRNA (22, 28), and lens crystallin mRNA (29). We have employed this system to translate sheep globin mRNA.

Our data indicate that the rabbit reticulocyte lysate can successfully translate all four types of sheep globin mRNA (i.e., for α, β, β', or β' chains). In all experiments, the types of globin chains made in the cell-free system were the same as were synthesized intracellularly in the sheep reticulocytes used as the mRNA source. Thus, the type of β chains programmed by mRNA from A/A sheep changed during the course of anemia. Messenger RNA taken early in anemia programed the synthesis of both β and β' chains (Fig. 4), whereas that taken later in anemia programed the synthesis of only β' chains (Fig. 3). This suggests that a switch in β chain mRNA accompanies silent gene expression in sheep. An alternative line of data which supports this conclusion is discussed below.

However, some problems with this interpretation of the cell-free synthesis data must be considered. Conceivably, non-utilized mRNA might occur in the sheep cells in an "inactive" form. If such mRNA was labile to our extraction procedure or if it was bound to cell membranes or mitochondria, it would have escaped our detection. Unfortunately, we have not succeeded in extracting active sheep globin mRNA directly from intact cells. The subcellular fraction we have utilized for mRNA extraction contained all of the cellular ribosomes and approximately 40% of the globin mRNA-protein complex which sediments in the "informosome" region at about 20 S (30-32) (see "Materials and Methods"). Our data suggest that no mRNA for β chains was present in this subcellular fraction isolated from severely anemic A/A sheep (Fig. 3). Nonetheless, it is obvious that this type of objection can never be fully excluded. In any case, we believe that our mRNA purification method makes fewer restrictive assumptions about the globin mRNA size and sedimentation properties than others (16-21) which have been previously used.

Although the rabbit reticulocyte lysate can synthesize all types of sheep globins, the relative synthesis levels differ from those made intracellularly. Several possible explanations could account for this result. (a) The various mRNAs were isolated in different yields. (b) The mRNAs were isolated in equal yields but were translated with different efficiencies in the rabbit lysate. (c) The mRNAs were translated with different efficiencies in the intact sheep reticulocytes. Although we cannot exclude any of these possibilities, the data discussed below support the conclusion that the third explanation is one contributing factor.

**Evidence for Differential Translation of Sheep Globin mRNA in Sheep Reticulocytes**—One possible explanation of silent gene expression is that the regulation occurs solely at the translational level, i.e., that both β' and β' chains are present in roughly equal amounts in normal or anemic animals and that a selective translational process is responsible for the β' → β' synthesis switch. According to this explanation, β' mRNA would be translated relatively efficiently in normal animals and relatively inefficiently in heavily anemic animals; the proportion of synthesis of either chain would be proportional to the relative efficiency of translation of its mRNA.

Our data with cycloheximide (Figs. 6 and 7) argue strongly against this interpretation. For example, β' mRNA is translated only about one-half as efficiently as β' mRNA, even when the β':β' synthesis ratio is 1.0:0.32 (Fig. 6). According to the translational regulation hypothesis, the β' mRNA should have been more efficiently translated in these cells. In addition, these data suggest that the β' and β' mRNAs occur intracellularly in grossly different amounts which change during the course of silent gene expression. We have now used this method to analyze the development of anemia in two different A/A sheep, and the results imply strongly that the proportion of β' mRNA increases during anemia. In this respect, the conclusion from these studies is the same as that arrived at from the cell-free protein synthesis data.

However, these data do suggest that the different sheep globin mRNA are translated intracellularly with very different efficiencies. Although present in relatively low levels, the α mRNA is relatively efficiently translated. When protein...
synthesis is inhibited with cycloheximide, there occurs a reduction of the \( \alpha: \beta \) incorporation ratio (Figs. 6 and 7). As can be seen from Figs. 5 to 7, we have observed that the level of incorporation into \( \alpha \) chains is frequently much less than that into \( \beta \) chains, even in the absence of cycloheximide. This disparity is heightened by cycloheximide. One possible explanation is that protein synthesis in our conditions of cell incubation may be already suboptimal, even in the absence of added inhibitors of protein synthesis. The \( \alpha: \beta \) synthesis ratio is likely to be sensitively dependent upon the conditions of the cells and upon the incubation media. Clearly, the in vivo synthesis ratio may be quite different from the ratio obtained by measuring incorporation of radioactive amino acids by cell suspensions.

Regulation of Sheep Hemoglobin Synthesis—Subject to the preceding qualification, our data suggest that silent gene expression is accompanied by a switch in types of mRNA present in reticulocytes. This implies that the regulation occurs at the level of transcription of the genes to form mRNA, at the level of processing the mRNA prior to its utilization, or at the level of mRNA degradation. On the other hand, the synthesis of sheep globins is also regulated at the translational level. The different mRNAs are translated intracellularly with different efficiencies. For example, the \( \alpha \) mRNA is present in low amounts but is relatively efficiently translated. A similar conclusion was made by Lodish with rabbit reticulocytes (24), except in that case the \( \beta \) mRNA was more efficiently translated. We have also shown that the relative proportions of different tRNAs change during silent gene expression (5). These studies are consistent with the view that the relative levels of synthesis of different proteins may be influenced at several different levels of regulation. In the case of silent gene expression our data suggest that the primary regulation occurs at the level of production, processing, or degradation of mRNA and that the translational effects occur secondarily to modulate the final result.

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