The Molecular Biology of Mammalian Hemoglobin Synthesis*

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ABSTRACT

The protein subunits of hemoglobin are made by the usual reactions of eukaryotic protein biosynthesis. Control of the rate, amount and kind of hemoglobin synthesis occurs at several levels. These include the transcription of globin messenger ribonucleic acid (mRNA), and the synthesis of other elements of the protein biosynthetic components. The translation of globin is restricted by mRNA abundance and possibly by mRNA structure, and by the availability and activity of the macromolecules required in protein synthesis. The process of red cell development provides a finite time during which the complement of hemoglobin can be synthesized. The events of red cell maturation include the enucleation of precursors, following which the biosynthetic components cannot be renewed, and the lability of the components imposes limitations on the duration of synthesis. The final result is mature erythrocytes which, in the healthy individual, are quite uniform and contain an average of 300 million hemoglobin molecules per cell with little excess of any of the hemoglobin moieties.

Introduction

Several human red blood cell diseases involve abnormal hemoglobin synthesis, and a knowledge of the molecular biology of hemoglobin synthesis is essential to their understanding. During the past 20 years, this topic has been studied intensively. Much of what is now known about the biochemical of protein synthesis in eukaryotic cells in general was first investigated as hemoglobin synthesis in reticulocytes. In addition much is now known about the regulation of the kind, rate and amount of hemoglobin synthesis. Both of these subjects are herewith reviewed.

Protein Biosynthesis

The reactions of hemoglobin biosynthesis, which are like the reactions in the synthesis of other proteins in eukaryotic cells, are summarized in figure 1. Hemoglobin synthesis can be considered as commencing with the synthesis of the globin mRNA's by transcription of the globin subunit genes. It is now known that the genes for the alpha subunit are dupli-

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Figure 1. Outline of hemoglobin synthesis. The reactions of protein synthesis in eukaryotic cells are outlined briefly from the point of view of globin mRNA synthesis, translation and cycling. It must be recognized that some of the processes indicated are understood in great detail, and references should be consulted.
located in most human populations and are located on chromosome number 16. The human non-alpha (beta, gamma and delta) subunits are located in close proximity on the 11th chromosome. The gamma genes are also duplicated, and there are two forms of gamma subunit differing by only a single amino acid. The globin genes contain segments whose base sequences are not translated although these so-called intervening sequences are transcribed and are present in the precursors of globin mRNA.

The mRNA is processed through several steps in which the intervening sequences are excised, a polyadenylic acid sequence is added enzymatically at the 3'-end of the molecule and nucleotides at the 5'-end are methylated to form a structure known as the cap. These features are characteristic of most eukaryotic mRNA's. Globin mRNA's from reticulocytes can be isolated readily, and the base sequences of several have been determined including both the portion that is translated and corresponds to the amino acid sequences of the globin subunits and the untranslated portions at the ends.

Desoxyribonucleic acid (DNA) with a base sequence complementary to globin mRNA (cDNA) can be synthesized in vitro using the mRNA as a template and viral reverse transcriptase for the incorporation of the appropriate bases. This cDNA molecule can be used as a probe to determine the presence of globin genes and to measure levels of globin mRNA in cells by DNA-RNA hybridization. Alternatively, DNA fragments obtained by the site specific scission of DNA can be used as probes to detect the globin genes in cells of interest by DNA-DNA hybridization. Evidence that some kinds of thalassemia are based on gene deletions and that others have reduced globin mRNA was obtained by these hybridization procedures.

The various elements of the protein biosynthetic apparatus, including globin mRNA, are cycled so that they can be re-used. Amino acids are incorporated into the nascent globin subunits. Energy provided by guanosine triphosphate (GTP) is consumed both in the formation of the initiation complexes for protein synthesis and during the elongation of nascent protein chains. Additional energy required for peptide bond formation is derived from adenosine triphosphate (ATP) and is used in the enzymatic formation of ester bonds between amino acids and the 3'-hydroxyl groups of tRNA by the aminoacyl-tRNA synthetases.

The process by which protein synthesis is initiated involves several steps in which initiation complexes are formed. At least eight protein factors, some of them very well defined biochemically, are required for the reactions of initiation. Initiator tRNA (met-tRNA<sub>m</sub>), GTP, and the two ribosomal subunits are associated with the beginning of the translated poly-nucleotide sequence near the 5'-end of globin mRNA to form the protein biosynthetic unit. The process of initiation, which will be discussed further, is important in the regulation of hemoglobin synthesis.

In the process of elongation, the various aminoacyl-tRNA's as determined by the genetic code in the base sequence of globin mRNA are bound to ribosomes, thus bringing the amino acids into proper relationship for incorporation into the nascent polypeptide chain. Peptide bonds are formed, tRNA is cycled through two ribosomal positions and then released, and the mRNA is advanced or translocated in its relationship to the ribosome so that the process of nascent chain elongation proceeds. The nascent globin chain is transferred from one tRNA to the next on the ribosomes. Initiation occurs frequently as mRNA is being translated, so that there are characteristically several ribosomes (polysomes) associated with each mRNA molecule being translated. The polysomes involved in globin mRNA translation normally contain four or five ribosomal monomers.
The tRNA of red cell precursors is of special interest because this family of molecules actually translates the genetic code. These molecules alone possess the triple specificity needed for recognition by the cognate aminoacyl-tRNA synthetase, ribosome binding and codon-anticodon interaction. There is evidence that the content of tRNA is specialized in red cell precursors for the synthesis of the globin subunits which have an unusual amino acid composition compared to most proteins.\(^2\)

About 30 seconds are required for the synthesis of an alpha or beta globin subunit at 37° in rabbit or human reticulocytes.\(^12\) Therefore, on the average the nascent hemoglobin subunit chain is elongated by five amino acids per second. Completed subunits are released from ribosomes, and it is only after release that the full hemoglobin molecule, which is a tetramer of two alpha and two non-alpha subunits plus heme moieties, is assembled. The reticulocytes of rabbits subjected to phlebotomy or to phenylhydrazine treatment synthesize 20,000 to 30,000 hemoglobin molecules per minute.\(^15\) It must be recognized that these reticulocytes, produced under the stress of anemia, are more active in globin synthesis than those which normally circulate and are analogous to the so-called “stress reticulocytes” seen in human hemolytic and hemorrhagic disease.\(^2\)

**Regulation of Hemoglobin Synthesis**

The processes of transcription and translation, as described previously, are regulated so that the mature red cell is provided a characteristic endowment of hemoglobin. Three aspects of regulation have been thoroughly investigated and are described.

**COORDINATION OF ALPHA AND BETA SUBUNIT SYNTHESIS**

One characteristic of hemoglobin synthesis is that approximately equal numbers of alpha and beta subunits are synthesized. Failure of this coordination occurs in the thalassemias, and its consequence is the accumulation of uncombined subunits, their early denaturation and precipitation, and the premature destruction of the red cells containing them.

There are several factors which combine to provide for the coordination of alpha and beta subunit synthesis. In the case of rabbit and human reticulocytes, there is about 40 percent more mRNA for the alpha subunit than for the beta subunit. Initiation of beta subunit synthesis is, however, more efficient and frequent. The polysomes on which beta subunits are synthesized are, therefore, larger than those for alpha subunits and there are more nascent beta than alpha subunit chains per globin mRNA molecule.\(^16\)

A question still not answered conclusively is whether or not the time required for translation of the two kinds of subunits is equal. There is some evidence favoring each of these possibilities.\(^9,12,17\) The availability of the aminoacylated tRNA species required for translation and the structure of the mRNA molecules could provide restrictive conditions limiting rates of amino acid addition and thereby necessitating differences in the time of translation of the two subunits. In the case of human hemoglobin A2, there is evidence both for and against a much prolonged translation time for the delta subunit compared to the alpha and beta subunits. The major factor in the low level of synthesis of the delta subunit is, however, mRNA abundance. It appears, moreover, that delta subunit synthesis ceases earlier during red cell maturation than the synthesis of other subunits.\(^11,25\)

Although most of the thalassemic conditions are associated with gene deletions or low or absent mRNA for the affected subunit, there is evidence that one form of the disease found in the region of Ferrara, Italy results from a deficiency in some protein biosynthetic component of the red cell precursors. It can be demonstrated
that the beta subunit mRNA is present in the cells, but it is not translated.21

In many of the human qualitative hemoglobinopathies the synthesis of the mutant subunit in the heterozygote is less than the corresponding normal subunit despite equal gene dosage for each subunit.8 This phenomenon is well exemplified by hemoglobin S which accounts for only 25 to 45 percent of the total hemoglobin of sickle cell heterozygotes. Beta8 subunit synthesis is reduced, corresponding to the lower level of the abnormal hemoglobin.6 The mechanisms causing the deficient synthesis of mutant subunits remain obscure.

COORDINATION OF GLOBIN SYNTHESIS WITH HEME AVAILABILITY

Some years ago it was observed that the cell-free synthesis of hemoglobin in rabbit reticulocyte lysates ceases after a few minutes of incubation but that it can be prolonged manyfold at approximately the initial rate if the lysate is supplemented by hemin. It is now known that in the absence of hemin the process of initiation is soon inhibited coincident with the accumulation of a phosphorylated form of the initiation factor eIF2. Phosphorylation is performed by a protein kinase which appears in the absence of hemin, which is quite specific for the small subunit of the factor and which is cyclic-AMP independent. A phosphatase is recognized which can reverse the phosphorylation.22 The role of the phosphorylation was enigmatic until recently because it could not be demonstrated that the phosphorylated eIF2 was inactive. It now appears that there is an interaction between eIF2 and a second protein that is necessary for full factor activity, and the interaction is affected by the phosphorylation of eIF2.3

While the coordination of hemin availability with the synthesis of globin is a reasonable mechanism for the regulation of hemoglobin synthesis, the process of initiation is universal to all eukaryotic cells as is the occurrence of eIF2. The specificity of the control mechanism to hemoglobin synthesis seems to lie in the levels of the specific protein kinase in red cell precursors. While similar kinase activity is present in other kinds of cells, it is much lower, and the extent to which hemin can maintain protein synthesis in extracts of these cells is less impressive.

Interestingly, the protein kinase mediated inactivation of initiation through the phosphorylation of eIF2 as seen in reticulocyte lysates is closely paralleled by the inhibition of translation in other kinds of cells which have been treated with interferon or with double stranded RNA or both. It appears, however, that with these agents different protein kinases are involved.14

REGULATION OF HEMOGLOBIN SYNTHESIS DURING RED CELL DEVELOPMENT AND DURING ONTOGENY

The process of red cell development includes provision for the expression of the globin genes. Red cell differentiation, the process by which red cell precursors become committed to erythrocyte formation, and red cell maturation occur in the bone marrow. Approximately 72 hours are required as proerythroblasts and basophilic erythroblasts undergo several divisions and are converted to reticulocytes, at which point they are discharged into the peripheral circulation. Among the early features of maturation is the synthesis of the protein biosynthetic machinery required for globin synthesis. This includes the mRNA's for the globin subunits in characteristic amounts. It also includes the enzymes of prophyrin synthesis so that heme, required both in the structure of hemoglobin and for the activity of initiation factor eIF2 as discussed before, can be formed.7 It includes the synthesis of a specialized population of tRNA molecules as discussed previously.52
DNA synthesis ceases prior to the last division of red cell precursors and, subsequent to the division, the nuclei are discharged from the daughter cells. After this, RNA synthesis can no longer occur. The reticulocyte is, therefore, a closed system containing the information and protein biosynthetic components for a limited amount of hemoglobin synthesis. The lability of the components limits the amount of hemoglobin synthesis in reticulocytes, and hemoglobin synthesis terminates with the disappearance of the first essential component. The loss of crystal violet staining, the tinctorial property defining reticulocytes, is histochemical evidence of the loss of RNA. It appears, at least in rabbit reticulocytes, that the half life of globin mRNA is about 24 hours, but it is not known if this is the first required component in hemoglobin synthesis to disappear. Thus the events of macromolecular synthesis and turnover provide constraints on the amount of hemoglobin finally synthesized.

An important event in both red cell differentiation and the regulation of hemoglobin synthesis is the commitment of red cell precursors to the synthesis of a particular kind of hemoglobin at a very early step in their development. During fetal life most red cell precursors synthesize a preponderance of fetal hemoglobin, which has gamma subunits rather than the beta subunits of adult hemoglobin. The switching of the principal hemoglobin synthesized from a fetal to an adult form is universal among vertebrates, and it involves the transcription of mRNA for the adult rather than the fetal subunit. The switching process may be based on altered determinants of the gene transcription or on the selection of a population of early red cell precursors which is already committed to the synthesis of one of the alternative subunits.

The process of hemoglobin switching is perhaps best observed in sheep, some strains of which synthesize a hemoglobin called hemoglobin C under the stress of anemia which has unique beta subunits (beta^c). It appears that a population of early red cell precursors appears in the bone marrow shortly after the onset of anemia which is committed to the synthesis of beta^c subunits. Hemoglobin C does not appear in the peripheral blood cells, however, until several days after the onset of anemia when there has been time for the maturation of the precursors. With the reversal of the anemia, the synthesis of hemoglobin C ceases quickly as the population of precursor cells reverts to one committed to synthesizing normal adult hemoglobin.

Although hemoglobin C of sheep is not a fetal hemoglobin, the switching process observed is similar to the compensatory synthesis of hemoglobin F in the various beta-thalassemias, including the beta-delta thalassemia of southern Europe which is based on the deletion of the genes for the beta and delta subunits. In this form of the disease, the only hemoglobin which can be synthesized is hemoglobin F. The emergence of an increased population of red cell precursors committed to gamma chain synthesis under increased erythropoietin levels, however, explains only part of the compensatory effect which also includes a higher level of gamma chain synthesis than is usual in the small population of cells in which some hemoglobin F synthesis occurs (F cells) in the postnatal human. The increased gamma subunit synthesis per cell is an effect that cannot be reproduced in committed cultured red cell precursors by increasing the level of erythropoietin. The program of development, transcription and translation by which globin synthesis and red cell maturation are regulated is subject, therefore, to modification by external factors such as anemia with its associated pathophysiological consequences.

References

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