INITIATION OF GLOBIN SYNTHESIS

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1. Introduction

Initiation of globin synthesis requires the interaction of a small and large ribosomal subunit, the initiator tRNA, methionyl-$tRNA_F$, protein initiation factors, globin mRNA, and GTP to form a stable configuration termed the "initiation complex". Different aspects of this process have been studied in our laboratory with components isolated from rabbit reticulocytes. Specific partial reactions of globin initiation include:

1) binding of Met-$tRNA_F$ to the triplet ApUpG or to endogenous globin mRNA,

2) binding of artificially formylated eukaryotic initiator tRNA, f-Met-$tRNA_F$, to ApUpG,

3) synthesis of the naturally occurring initial dipeptide of rabbit α and β globin, methionyl–valine, on endogenous globin mRNA,

4) synthesis of an initial dipeptide analog, methionyl–puromycin, on ApUpG, or on endogenous globin mRNA,

5) synthesis of the natural initial tripeptides, methionyl–valyl–leucine (rabbit α chain), and methionyl–valyl–histidine (rabbit β chain), on endogenous globin mRNA,

6) synthesis of polyphenylalanine with poly (U) as messenger, and

7) synthesis of complete globin chains on globin mRNA.

Abbreviations: IF-M_1, IF-M_{2A}, IF-M_{2B}, IF-M_3: initiation factors M_1, M_{2A}, M_{2B}, M_3; EF-1, EF-2: elongation factors 1 and 2; Met_f: methionine donated from methionyl-$tRNA_f$.

The individual rabbit reticulocyte initiation and elongation factor requirements for each of these assays are summarized in table 1. In the present study, we have extended our investigations to include:

1) the synthesis of the initial dipeptide of rabbit and sheep globin on exogenous mRNA,

2) the expression of genetic information contained in erythroid cells by the translation on rabbit ribosomes of human, sheep and goat mRNA derived from reticulocytes and bone marrow cells, and

3) the isolation of initiation factors IF-M_1, IF-M_{2A}, IF-M_{2B}, and IF-M_3 from nucleated (rabbit liver) cells.

2. Materials and methods

The preparation of the fractionated cell-free globin synthesizing system from rabbit reticulocytes has been described [1] as have the details of the specific assays [2, 3]. The components and assays used in the experiments described here are in the figure legends.

3. Results and discussion

Four initiation factors, IF-M_1, IF-M_{2A}, IF-M_{2B}, and IF-M_3 have been isolated from the 0.5 M KCl wash of reticulocyte lysate ribosomes [3, 4]. Studies with the partial reactions of globin initiation listed above (table 1) have suggested the following roles for each of the initiation factors. IF-M_1, IF-M_{2A},

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Table 1
Factor requirements for globin initiation.

	Artificial templates			
	Binding of Met-tRNA$_F$ (ApUpG)	Binding of f-Met-tRNA$_F$ (ApUpG)	Met$_f$-puromycin synthesis (ApUpG)	Polyphenylalanine synthesis (poly U)
IF-M$_1$	+	+	+	+
IF-M$_{2A}$	+	–	+	+
IF-M$_{2B}$	+	+	+	+
IF-M$_3$	–	–	–	–
EF-1	–	–	+	+
EF-2	–	–	–	+

	Natural globin mRNA				
	Binding of Met-tRNA$_F$	Met$_F$-puromycin synthesis	Initial dipeptide synthesis	Initial tripeptide synthesis	Globin synthesis
IF-M$_1$	+	+	+	+	+
IF-M$_{2A}$	+	+	+	+	+
IF-M$_{2B}$	+	+	+	+	+
IF-M$_3$	–	+	+	+	+
EF-1	–	+	+	+	+
EF-2	–	–	–	+*	+

The initiation and elongation factor requirements for the individual steps during the initiation of rabbit globin synthesis are shown; (+) factor is required, (–) factor is not required. The preparation of individual factors and the techniques of each assay have been previously described [1–6].

* The requirement for EF-2 during the synthesis of the initial tripeptides of α and β globin is inferred from the inhibition of tripeptide synthesis by fusidic acid, a known inhibitor of EF-2 function. Fusidic acid does not inhibit Met-tRNA$_F$ binding nor initial dipeptide synthesis [2].

and IF-M$_{2B}$ bind the initiator tRNA, Met-tRNA$_F$, to the initiator codon ApUpG contained within the globin α and β mRNA. This interaction specifically requires GTP [2, 6, 7]. When the prokaryotic initiator, f-Met-tRNA$_F$ is substituted for Met-tRNA$_F$, IF-M$_{2A}$ and GTP are not required for the binding reaction. This suggests that the requirement for M$_{2A}$ in the binding of naturally occurring initiator, Met-tRNA$_F$, may involve GTP hydrolysis.

Artificial mRNA (poly U) can be translated with fidelity at a low Mg^{2+} concentration with IF-M$_1$, IF-M$_{2A}$ and IF-M$_{2B}$, but natural globin mRNA requires the addition of IF-M$_3$ before initiation is complete and the first peptide bond can be formed. In addition to all of the initiation factors, the synthesis of the initial dipeptide requires EF-1 and the synthesis of the initial tripeptide requires EF-1 and EF-2.

The binding of the initiator tRNA to either the template ApUpG [6, 7] or to endogenous globin mRNA [5] takes place on the small ribosomal subunit. The synthesis of the initial dipeptide however, requires the presence of both the small and the large ribosomal subunits (fig. 1a).

The initiation factor requirements for the synthesis of the initial dipeptide on *exogenous* rabbit globin mRNA (fig. 1b) are the same as for the synthesis of the initial dipeptide on *endogenous* globin mRNA [2, 5]. This finding suggests that if one or more of the initiation factors are necessary to "bind" the mRNA [8, 9], this must be a dual role of IF-M$_1$, IF-M$_{2A}$, IF-M$_{2B}$ and/or IF-M$_3$.

Met-tRNA$_F$ has been demonstrated to be the initiator tRNA [10] for rabbit globin α and β chains [2, 11] and the duck α chain [12]. Since valine is the N-terminal amino acid of sheep α chains, Met-tRNA$_F$

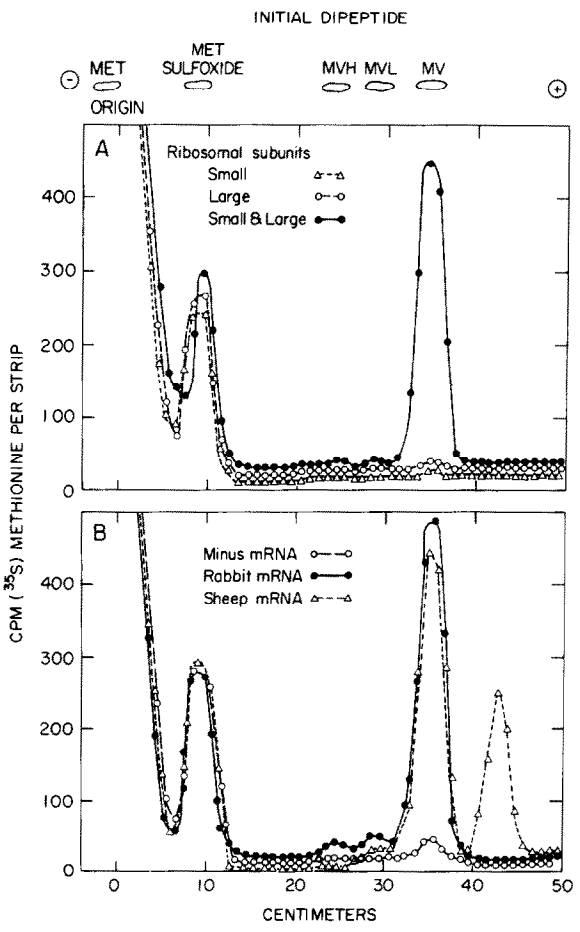


Fig. 1. (A) Ribosomal subunit dependence for the synthesis of the initial dipeptide of rabbit globin α and β chains (Met_f-valine). Monosomes isolated from salt-washed ribosomes were separated into small and large ribosomal subunits by the method of Blobel [20]. The assay conditions were as previously described except 0.5 A_{260} units of 40 S and 0.4 A_{260} units of 60 S substituted for the salt-washed ribosomes usually used in this assay [5]. Significant quantities of endogenous globin mRNA are associated with small subunits prepared in this manner. (B) mRNA dependence for the synthesis of the initial dipeptide of rabbit globin α and β chains (Met_f-valine) and the sheep α globin chain (Met_f-valine). mRNA was prepared from rabbit and sheep lysate as previously described [21]. The sheep were made anemic and were synthesizing α and β^C globin chains. The initial dipeptide Met_f-valine corresponds to that expected from the sheep α chain; the second peak of [^{35}S]methionine containing oligopeptide directed by sheep mRNA has not yet been identified. The details of the mRNA dependent initial dipeptide assay have been described elsewhere [2]. In both (A) and (B) [^{35}S]methionyl-$tRNA_f$ and [^{12}C]valyl-tRNA were the only aminoacyl-tRNA's added to the reaction mixture, hence there was no synthesis of tripeptides or larger oligopeptides [2]. Oligopeptide markers run as standards in the reaction mixtures are: MV: methionyl–valine, MVL: methionyl–valyl–leucine, MVH: methionyl–valyl–histidine.

is probably the initiator tRNA for this globin (fig. 1B). $Met\text{-}tRNA_M$ will not donate methionine to form this initial dipeptide. In studies to be published elsewhere, we have also demonstrated that $Met\text{-}tRNA_F$ is the initiator tRNA for human α, β and γ chains and for goat α chains.

Globin mRNA isolated from human reticulocytes can be translated with fidelity in the rabbit cell-free system. The ratio of α/β chains synthesized in such a system is approximately 1 when the globin mRNA is derived from normal human (fig. 2a). When the mRNA is isolated from reticulocytes of patients with β-thalassemia, the ratio is abnormally high (fig. 2b). Thus the defect in this genetic disease is carried by the β-globin mRNA and is either a decrease in the amount of β globin mRNA or an abnormality in the sequence of this mRNA [13].

Sheep with HbA ($\alpha_2\beta_2^A$), when made anemic, "switch" to the production of HbC ($\alpha_2\beta_2^C$). This is believed to be a model system for examining gene expression. mRNA isolated from reticulocytes of anemic sheep that have partially switched, direct the translation of α, β^A, and β^C chains, (fig. 2c) so the information for the "switch" is carried by the mRNA [14].

mRNA isolated from goat bone marrow directs the synthesis of goat Iα, IIα, and β^A globin in the rabbit reticulocyte cell-free system. These are the same globin chains synthesized by the intact cells from which the mRNA was isolated. mRNA has also been isolated from sheep and rabbit marrow cells [14] as well as human marrow cells (unpublished observations A.W.N.).

The fidelity with which human, sheep and goat reticulocyte and marrow mRNA's are translated in the rabbit reticulocyte cell-free system suggests that the mechanisms of translation (initiation, elongation, and release) are probably similar in these cell types. Initiation factors $IF\text{-}M_1$, $IF\text{-}M_{2A}$, $IF\text{-}M_{2B}$, and $IF\text{-}M_3$ have been isolated from rabbit liver cells by D. Picciano in our laboratory and can substitute for the corresponding reticulocyte factors in the

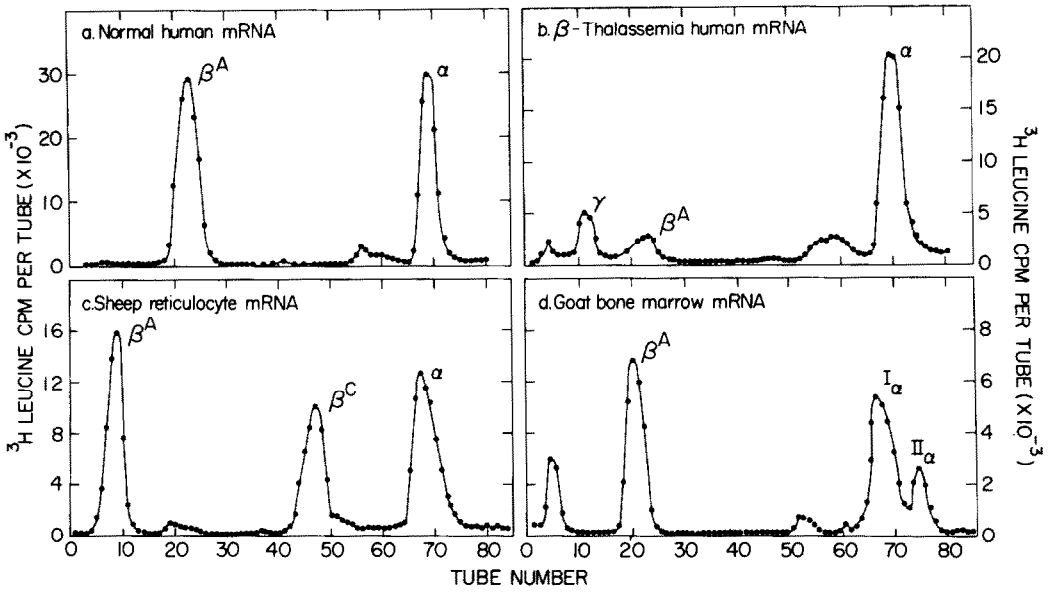
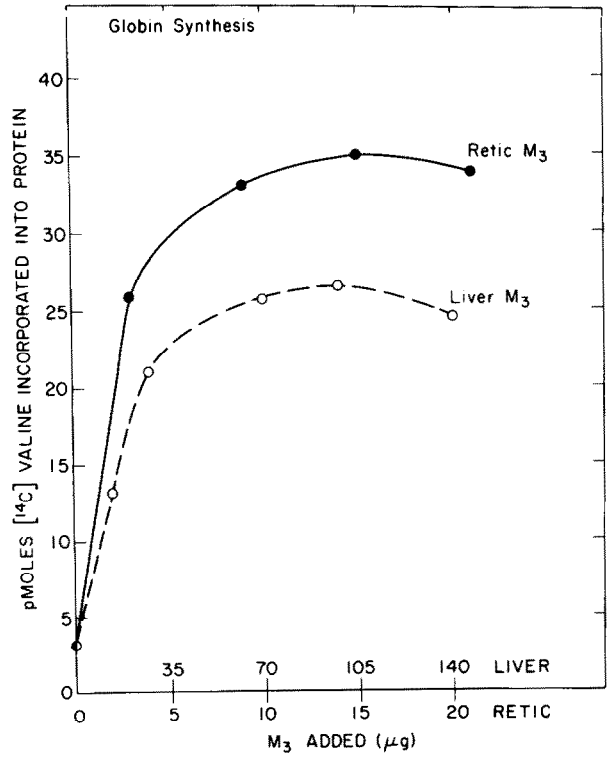


Fig. 2. Translation of globin mRNA in the rabbit reticulocyte cell-free system. Separation of globin chains was done by carboxymethyl cellulose chromatography in 8 M urea–phosphate [22]; isolation of mRNA and assay conditions have been previously described [13, 14, 21]. (a) mRNA isolated from normal human reticulocytes, (b) mRNA isolated from the reticulocytes of patients with homozygous β-thalassemia, (c) mRNA isolated from reticulocytes of anemic sheep and (d) mRNA isolated from the bone marrow of non-anemic goats.



translation of globin mRNA. Globin synthesis on rabbit reticulocyte ribosomes when liver IF-M_3 is substituted for reticulocyte IF-M_3 is shown in fig. 3. The liver initiation factors IF-M_1, IF-M_{2A}, and IF-M_{2B} can also substitute for the corresponding reticulocyte factors in the translation of artificial mRNA and in the binding of the initiator tRNA. These data further suggest the probable universality of the eukaryotic initiation mechanism [15–19].

Fig. 3. Requirement for eukaryotic initiation factor M_3 in tl synthesis of globin on reticulocyte ribosomes. Conditions fo the initiation factor dependent globin synthesizing assay fro rabbit reticulocytes have been described [1]. Salt-washed rabbit reticulocyte ribosomes containing endogenous globin mRNA were used in the presence of reticulocyte IF-M_1, IF-M_{2A}, and IF-M_{2B}. Similar results can be obtained using liver ribosomes and exogenous rabbit globin mRNA. Reticulocyte IF-M_3 was prepared as described [2]. Liver IF-M_3 was isolated from the 0.5 M KCl wash fraction of liver micr somes followed by DEAE-cellulose chromatography; IF-M_3 activity elutes at 180 mM KCl.

4. Conclusion

The fully fractionated rabbit reticulocyte cell-free globin synthesizing system is proving useful in elucidating the mechanism of eukaryotic initiation. Studies with mRNA isolated from a variety of sources have demonstrated that this mechanism, at least for globin initiation, is probably universal. Initiation factors from liver cells can substitute for reticulocyte initiation factors suggesting that, in general, the processes described for globin initiation are probably applicable to other mammalian cells. In addition, the rabbit reticulocyte cell-free system can be used to study human genetic defects and the control of gene expression.

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