Translation of Exogenous Messenger RNA for Hemoglobin on Reticulocyte and Liver Ribosomes

(Initiation factors/9S RNA/liver factors/reticulocyte factors/globin synthesis)

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ABSTRACT The ribosome and initiation factor requirements for translation of rabbit-reticulocyte hemoglobin mRNA on rabbit reticulocyte ribosomes, reticulocyte ribosomal subunits, and liver ribosomes have been studied. Excellent synthesis of globin chains from exogenous mRNA in the fractionated cell-free system has been achieved. There is a near absolute requirement for each of the initiation factors, M1, M2, and M3 (as well as for the supernatant proteins) for the translation of exogenous mRNA. Liver microsomal wash will partially replace reticulocyte factors M1 and M2, but will not replace the requirement for reticulocyte factor M3. Rabbit liver ribosomes and rabbit reticulocyte ribosomes are equally active in their ability to support the translation of exogenous hemoglobin mRNA.

Early attempts to investigate the ability of reticulocyte RNA to function as hemoglobin messenger RNA were hindered by the inability to separate exogenous mRNA activity from a nonspecific increase in the activity of endogenous mRNA (1-5). In an attempt to circumvent this problem, heterologous systems were developed that produced specific globin products, albeit in small amounts (6-8). In more recent studies, Lockard and Lingrel were able to obtain substantial translation of mouse hemoglobin mRNA in a rabbit-reticulocyte lysate system (9); Nienhuis et al. reported rabbit a and b globin synthesis by rabbit mRNA on human-reticulocyte ribosomes (10); and Nienhuis and Anderson demonstrated human globin synthesis by human hemoglobin mRNA (both normal and thalassemic) on mRNA-dependent ribosomes from rabbit reticulocytes (11). In the present study, we have examined the ribosome and initiation factor requirements for the translation of exogenous hemoglobin mRNA from rabbit reticulocytes in fractionated cell-free systems utilizing reticulocyte and liver ribosomes, as well as reticulocyte factors and the 0.5 M KCI-wash fraction from liver microsomes.

METHODS

The preparation of 0.5 M KCI-washed reticulocyte ribosomes, ribosomal wash fraction, supernatant fraction, and tRNA was as reported (12, 13).

Reticulocyte Hemoglobin mRNA. The preparation of rabbit-reticulocyte 9S RNA was similar to that described by Evans and Lingrel (14). 12,000 A260 units of salt-washed ribosomes were made 0.5% in sodium deoxycholate and incubated 5 min at 37°C. The mixture was diluted 1:1 with distilled water, and was then centrifuged through a 5-20% linear sucrose gradient in 10 mM Tris-HCl (pH 7.5)-10 mM KCl in a Ti-15 zonal rotor at 35,000 rpm for 16 hr at 2°C. The 9S region (which included some material from 6S to 18S) was pooled, extracted with phenol, precipitated with ethanol, dissolved in distilled H2O, and dialyzed against 10 mM KCl for 12 hr at 4°C. This “mRNA fraction” was stored in small aliquots at a concentration of 92 A260 units/ml in liquid nitrogen.

Reticulocyte Factors M1 and M3. Initiation factors M1 and M3 (containing both M24 and M28) were prepared by DEAE-cellulose chromatography of the ribosomal wash fraction (12, 15).

Reticulocyte Factor M2. A general outline for M2 purification is given in Table 1. Initiation factor M1 was prepared from a 35-70% ammonium sulfate fraction of the ribosomal wash fraction that was chromatographed on DEAE-cellulose with either a KCI gradient or a stepwise elution with KCI. M4, which elutes from DEAE-cellulose at 0.18 M KCI, was further purified from elongation factors T1 and T2 by phosphocellulose chromatography. After application of the M4 fraction onto a 1.5 x 25 cm phosphocellulose column (equilibrated with 0.1 M KCl-50 mM Tris-HCl (pH 7.9)-1 mM dithiothreitol-0.1 mM EDTA), protein was eluted by a 400-ml linear gradient of 0.1-0.4 M KCI [in 50 mM Tris-HCl (pH 7.9)-1 mM dithiothreitol-0.1 mM EDTA]. Fractions were assayed for their ability to stimulate [14C]-valine incorporation into protein on 0.5 M KCI-washed ribosomes in the presence of M1, M3, and rabbit-reticulocyte supernatant proteins. The active fractions were pooled, concentrated by ultrafiltration with a UM-10 membrane (Amicon Corp.), and stored in small aliquots at a concentration of 1.25 mg of protein per ml in liquid nitrogen. M2 prepared and stored in this manner is stable for at least 9 months.

Reticulocyte Ribosomal Subunits. Ribosomal subunits were prepared as described (16). 3000 A260 units of rabbit reticulocyte polysomes were made 0.5 M in KCl and 2 mM in MgCl2. The suspension was stirred slowly for 10 min at 4°C, and then centrifuged at 46,000 rpm for 4 hr at 2°C in a Ti-14 zonal rotor previously loaded with a 15-30% sucrose gradient in 20 mM Tris-HCl (pH 7.5)-0.5 M KCl-2 mM MgCl2-1 mM dithiothreitol-0.1 mM EDTA. The small subunit (40S) and large subunit (60S) regions were each pooled and collected by centrifugation at 176,000 x g for 4 hr at 2°C; the resulting pellets were suspended in 0.25 M sucrose, 20 mM KCl, 2 mM MgCl2, 1.0 mM dithiothreitol, 0.1 mM
Translation of Hemoglobin mRNA

**RESULTS**

**Translation of hemoglobin mRNA on reticulocyte ribosomes**

Previous studies have shown that, whereas the artificial template Poly(U) requires the initiation factors M₁ and M₂ for translation at low Mg⁺⁺, a natural template, i.e., endogenous hemoglobin mRNA, also requires initiation factor M₁ (17). Fig. 1 indicates a near absolute requirement for M₁ in order to translate exogenous hemoglobin mRNA. In the presence of 0.2 Aₙ₀₀ units of reticulocyte ribosomes, and saturating amounts of supernatant proteins and initiation factors M₁ and M₂, the amount of mRNA translation increases linearly with increasing amounts of M₁ (Fig. 1A). If mRNA is added in the absence of additional M₁, there is actually a decrease in total activity (Fig. 1A and B). At a half-sat-

![Fig. 1. Translation of hemoglobin mRNA on salt-washed ribosomes from rabbit reticulocytes. (A) As a function of M₁. Each 100-µl reaction mixture contained 0.2 Aₙ₀₀ units of reticulocyte ribosomes, 700 µg of reticulocyte supernatant protein (containing a saturating level of the elongation factor Tₚ, but inadequate T₁), 56 µg of T₁, 34 µg of M₁, and 28 µg of M₂. **Upper curve**, plus 0.8 Aₙ₀₀ units of hemoglobin mRNA fraction added. **Lower curve**, no mRNA added. (B) As a function of added exogenous hemoglobin mRNA. Each reaction mixture contained the same amount of reactants as in A, as well as the amount of mRNA fraction indicated. **Upper curve**, plus 115 µg of M₃ protein. **Lower curve**, no M₃ added.](image-url)
Table 2. M3 requirement for optimal translation of hemoglobin mRNA

<table>
<thead>
<tr>
<th>mRNA added (A260 units)</th>
<th>Saturating amount of M3 (µg of protein)</th>
<th>[14C]Valine incorporated (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>47.5</td>
<td>49</td>
</tr>
<tr>
<td>0.38</td>
<td>75</td>
<td>77</td>
</tr>
<tr>
<td>0.76</td>
<td>115</td>
<td>94</td>
</tr>
<tr>
<td>0.98</td>
<td>127</td>
<td>99</td>
</tr>
</tbody>
</table>

Incubations were performed as described in the legend to Fig. 1.
of reticulocyte M₃ is shown in Fig. 4. The microsomal wash preparation from liver is not as efficient as are reticulocyte M₁ and M₃ in the translation of exogenous hemoglobin mRNA (compare Figs. 3B and 4).

**DISCUSSION**

We have examined the ribosome and initiation factor requirements for the translation of hemoglobin mRNA in fractionated homologous and heterologous cell-free protein-synthesizing systems. The requirement for ribosomes in the translation of exogenous hemoglobin mRNA can be satisfied equally well by reticulocyte or liver ribosomes. Similar amounts of factors and crude mRNA are required to produce a given amount of globin product regardless of which type of ribosome preparation is used. There is a near absolute requirement for each of the initiation factors, M₁, M₃, and M₅, for the translation of exogenous mRNA on reticulocyte ribosomes.

M₁ and M₃ are known to be required to place the initiator tRNA, Met-tRNA, into the mRNA–tRNA–ribosome initiation complex (19–21). The function of initiation factor M₅ remains unclear. It has been demonstrated that, whereas factors M₁ and M₃ are sufficient to stimulate the synthesis of poly(U)-directed polyphenylalanine synthesis at low Mg²⁺ (22), M₅ is required for the translation of a natural mRNA template (17). Crystal, *et al.* (21) showed that M₅ is necessary for the formation of the first peptide bond in hemoglobin biosynthesis, but that it is not required, under the experimental conditions tested, for the binding of Met-tRNA to an endogenous mRNA template. This observation does not rule out the possibility that M₅ may have a dual function; it might also be required in the initial association of (exogenous) mRNA to the ribosome. Indeed, Heywood *et al.* (8) has shown that a factor in chick reticulocytes, EF₃, which has similar chromatographic properties on DEAE-cellulose to rabbit reticulocyte M₅, appears to stimulate the binding of chick hemoglobin [³⁵S]mRNA to chick ribosomes. This factor appears to be tissue-specific, at least between reticulocyte and muscle cells (8). Stavnezer and Huang (23) have demonstrated the synthesis of immunoglobulin light chains from mouse in a rabbit-reticulocyte cell-free system programmed with mouse messenger RNA, but do not rule out tissue specificity for the initiation factors since plasma cells and reticulocytes have a common stem cell (23).

We attempted to ascertain whether or not exogenous hemoglobin mRNA could be translated by the initiation factors found in liver cells. It has been determined that the microsomal wash fraction from liver cannot support the translation of hemoglobin mRNA on either liver or reticulocyte ribosomes, but can do so if reticulocyte factor M₅ is also added. No protein fraction obtained from the liver supernatant or microsomal wash fraction has yet been found that will replace the requirement for reticulocyte M₅ in hemoglobin mRNA translation. Furthermore, it has not yet been possible to obtain a completely reproducible system that uses endogenous liver mRNA with reticulocyte and liver initiation factors. In studies to be published elsewhere, the microsomal wash fraction from liver has been shown to contain factors very similar to reticulocyte M₁ and M₃, but a liver factor possessing the mRNA specificity of reticulocyte M₅ has not been found. These data can be interpreted in one of two ways: either (a) liver cells contain an initiation factor with the mRNA specificity of reticulocyte M₅, but it is present in such small amounts that it has not yet been detected in either the supernatant or microsomal wash fractions; or (b) liver cells do not contain an initiation factor with the mRNA specificity of reticulocyte M₅, but they have one or more factors that recognize their own mRNAs; these have not yet been detected since there is no specific assay for a liver

![Fig. 4. Translation of exogenous hemoglobin mRNA by liver ribosomes with microsomal wash fraction from liver and rabbit reticulocyte M₅ added. Each 100-μl reaction mixture contained 0.2 A₂₆₀ units of liver ribosomes, 700 μg of reticulocyte supernatant, 56 μg of T₁₁, 90 μg of microsomal wash fraction from liver, and 115 μg of reticulocyte M₅.](image-url)

**Table 3. Requirements for translation of exogenous hemoglobin (Hb) mRNA on liver ribosomes**

<table>
<thead>
<tr>
<th>Additions to supernatant and liver ribosomes*</th>
<th>pmol of [¹⁴C]Valine incorporated into protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.8</td>
</tr>
<tr>
<td>Liver microsomal wash</td>
<td>3.6</td>
</tr>
<tr>
<td>M₁</td>
<td>3.6</td>
</tr>
<tr>
<td>L₃ and microsomal wash + M₁</td>
<td>4.2</td>
</tr>
<tr>
<td>Hb mRNA + M₅</td>
<td>4.8</td>
</tr>
<tr>
<td>Hb mRNA + liver microsomal wash</td>
<td>5.3</td>
</tr>
<tr>
<td>Hb mRNA + liver microsomal wash + M₁</td>
<td>6.5</td>
</tr>
<tr>
<td>Hb mRNA + liver microsomal wash + M₃</td>
<td>6.0</td>
</tr>
<tr>
<td>Hb mRNA + liver microsomal wash + M₅</td>
<td>34.4</td>
</tr>
</tbody>
</table>

Incubations, in a total volume of 100 μl, were performed for 20 min at 37°C. Each reaction mixture contained 0.2 A₂₆₀ units of liver ribosomes and 700 μg of rabbit-reticulocyte supernatant protein, as well as, where indicated, 90 μg of liver microsomal wash, 115 μg of reticulocyte M₅, and 0.9 A₂₆₀ units of hemoglobin mRNA.  

* All M factors are from rabbit reticulocytes.
mRNA. It is not yet possible to choose between these alternatives.
