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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, M_1, M_2, and M_3 (as well as for the supernatant proteins) for the translation of exogenous mRNA. Liver microsomal wash will partially replace reticulocyte factors M_1 and M_2, but will not replace the requirement for reticulocyte factor M_3. 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 α and β 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 KCl-wash fraction from liver microsomes.

METHODS

The preparation of 0.5 M KCl-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 A_{260} units of salt-washed ribosomes were made 0.5% in sodium dodecyl sulfate 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 H_2O, 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 A_{260} units/ml in liquid nitrogen.

Reticulocyte Factors M_1 and M_2. Initiation factors M_1 and M_2 (containing both M_{2A} and M_{2B}) were prepared by DEAE-cellulose chromatography of the ribosomal wash fraction (12, 15).

Reticulocyte Factor M_3. A general outline for M_3 purification is given in Table 1. Initiation factor M_3 was prepared from a 35–70% ammonium sulfate fraction of the ribosomal wash fraction that was chromatographed on DEAE-cellulose with either a KCl gradient or a stepwise elution with KCl. M_3, which elutes from DEAE-cellulose at 0.18 M KCl, was further purified from elongation factors T_1 and T_2 by phosphocellulose chromatography. After application of the M_3 fraction onto a 1.5 × 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 KCl [in 50 mM Tris·HCl (pH 7.9)–1 mM dithiothreitol–0.1 mM EDTA]. Fractions were assayed for their ability to stimulate [^{14}C]-valine incorporation into protein on 0.5 M KCl-washed ribosomes in the presence of M_1, M_2, 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. M_3 prepared and stored in this manner is stable for at least 9 months.

Reticulocyte Ribosomal Subunits. Ribosomal subunits were prepared as described (16). 3000 A_{260} units of rabbit reticulocyte polysomes were made 0.5 M in KCl and 2 mM in $MgCl_2$. 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 $MgCl_2$–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 × g for 4 hr at 2°C; the resulting pellets were suspended in 0.25 M sucrose, 20 mM KCl, 2 mM $MgCl_2$, 1.0 mM dithiothreitol, 0.1 mM

TABLE 1. *Purification of M_3*

	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Fold purification	Yield (%)
0.5 M KCl wash of ribosomes	340	5474	13,685	2.5	1	100
$(NH_4)_2SO_4$ fractionation (35–70%)	75	880	—	—	—	—
DEAE-cellulose chromatography	8	72	1,920	26.7	10.7	14
Phosphocellulose chromatography	4	5	508	102	40.8	3.7

1 unit of M_3 activity is defined as the amount required to stimulate the incorporation of 50 pmol of [^{14}C]valine into protein in 20 min at 37°C.

EDTA. The subunit fractions were stored in small aliquots at concentrations of 50–200 A_{260} units/ml in liquid nitrogen.

Wash Fraction from Liver Microsomes. Livers from exsanguinated rabbits were minced and homogenized in a Waring blendor in Solution A [0.25 M sucrose–50 mM Tris·HCl (pH 7.5)–35 mM KCl–1 mM dithiothreitol–0.5 mM EDTA] made 10 mM in $MgCl_2$. The postmitochondrial supernatant obtained by low-speed centrifugation of this homogenate was centrifuged at 113,000 × *g* for 2 hr at 2°C. The postmicrosomal supernatant was discarded, and the pellets were washed three times in Solution A made 0.1 mM in $MgCl_2$, then suspended in the same buffer. This suspension was made 0.5 M in KCl by the addition of 4 M KCl, and was stirred slowly for 1 hr at 2°C. The 0.5 M KCl suspension was then centrifuged at 176,000 × *g* for 90 min. The upper 80% of the resulting supernatant was carefully removed and diluted 1:5 with 20 mM Tris·HCl (pH 7.5)–1 mM dithiothreitol–0.1 mM EDTA, concentrated 5-fold by ultrafiltration with a UM-10 membrane, and stored in small aliquots at a concentration of 9 mg of protein per ml in liquid nitrogen.

Liver Ribosomes. Liver ribosomes low in endogenous activity were prepared from post mitochondrial supernatant obtained as described above. 25 ml of postmitochondrial supernatant was layered over 5 ml of a 1.6 M sucrose cushion prepared by mixing three parts of Solution B [2.5 M sucrose–50 mM Tris·HCl (pH 7.5)–35 mM KCl–10 mM $MgCl_2$–1 mM dithiothreitol–0.5 mM EDTA] with two parts of postmicrosomal supernatant. This mixture was centrifuged in a type 60-Ti rotor at 176,000 × *g* for 4.5 hr at 2°C. The supernatant and cushion were discarded; the pellets were washed once with Solution A made 10 mM in $MgCl_2$, and then dissolved in a small volume of postmicrosomal supernatant. This solution was made 1 M in NH_4Cl, stirred slowly for 90 min at 2°C, and centrifuged at 229,000 × *g* for 90 min at 2°C. The supernatant was discarded, and the pellets were washed and then suspended in Solution A made 10 mM in $MgCl_2$. This suspension was made 0.5% in deoxycholate; 7.5 ml was layered over 2.5 ml of a 1.6 M sucrose cushion, prepared by mixing two parts of Solution A with three parts of Solution B, and centrifuged at 229,000 × *g* for 2.5 hr at 2°C. The supernatant and cushion were discarded; the pellets were washed and then dissolved in Buffer A made 10 mM in $MgCl_2$. These liver ribosomes were stored in small aliquots at a concentration of 100 A_{260} units/ml in liquid nitrogen.

Hemoglobin Synthesis Assay. The assays for hemoglobin synthesis with endogenous mRNA (17) and exogenous mRNA (10) were described. Incubations were for 20 min at 37°C in the presence of 4.5 mM Mg^{++}. Protein synthesis was measured by the incorporation of [^{14}C]valine into hot-trichloroaceticacid-precipitable material.

RESULTS

Translation of hemoglobin mRNA on reticulocyte ribosomes

Previous studies have shown that, whereas the artificial template Poly(U) requires the initiation factors M_1 and M_2 for translation at low Mg^{++}, a natural template, i.e., endogenous hemoglobin mRNA, also requires initiation factor M_3 (17). Fig. 1 indicates a near absolute requirement for M_3 in order to translate exogenous hemoglobin mRNA. In the presence of 0.2 A_{260} units of reticulocyte ribosomes, and saturating amounts of supernatant proteins and initiation factors M_1 and M_2, the amount of mRNA translation increases linearly with increasing amounts of M_3 (Fig. 1*A*). If mRNA is added in the absence of additional M_3, there is actually a decrease in total activity (Fig. 1*A* and *B*). At a half-sat-

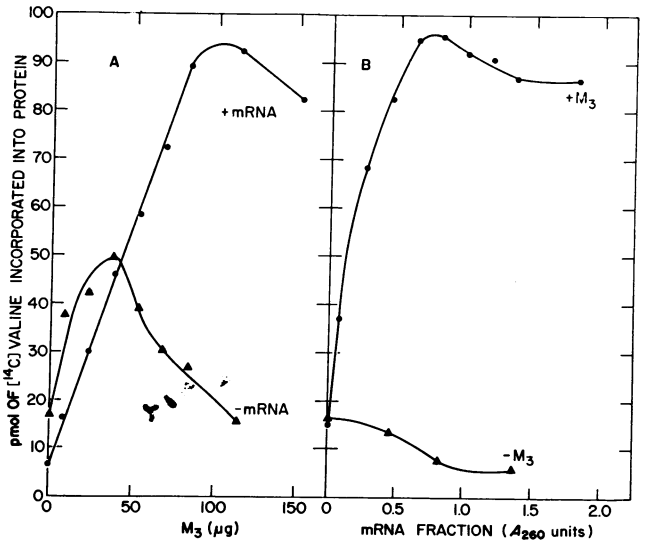

FIG. 1. Translation of hemoglobin mRNA on salt-washed ribosomes from rabbit reticulocytes. (*A*) As a function of M_3. Each 100-μl reaction mixture contained 0.2 A_{260} units of reticulocyte ribosomes, 700 μg of reticulocyte supernatant protein (containing a saturating level of the elongation factor T_2, but inadequate T_1), 56 μg of T_1, 34 μg of M_1, and 28 μg of M_2. *Upper curve,* plus 0.8 A_{260} 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 M_3 protein. *Lower curve,* no M_3 added.

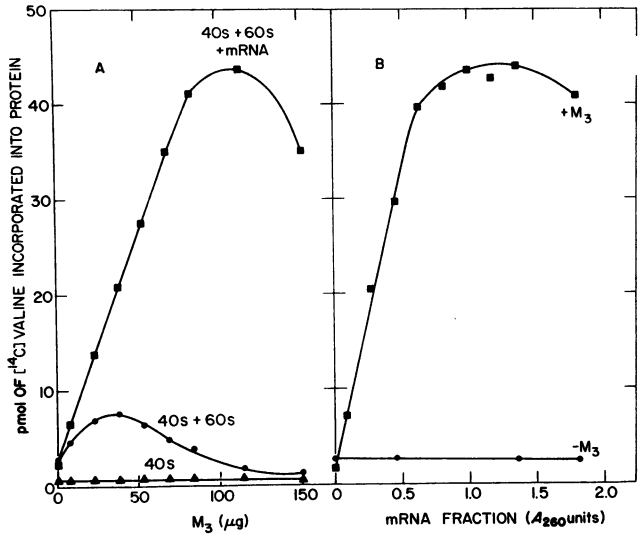

FIG. 2. Translation of hemoglobin mRNA on rabbit-reticulocyte ribosomal subunits. (*A*) As a function of M_3. Each 100-μl reaction mixture contained 700 μg of reticulocyte supernatant protein, 56 μg of T_1, 34 μg of M_1, 28 μg of M_2, and 0.12 A_{260} units of 40S or 0.12 A_{260} units of 40S plus 0.22 A_{260} units of 60S subunits, where indicated. *Upper curve*, 0.8 A_{260} units of exogenous hemoglobin mRNA added; *lower curve*, no mRNA added. (*B*) As a function of the amount of exogenous hemoglobin mRNA added. Each reaction mixture contained the same amount of reactants as in *A*, including 40S and 60S subunits, as well as the amount of mRNA fraction indicated. *Upper curve*, plus 115 μg of M_3 protein; *lower curve*, no M_3 added.

urating concentration of mRNA (0.38 A_{260} unit) 77 pmol of [^{14}C]valine is incorporated with the optimal concentration of M_3, 75 μg of protein (Table 2). Thus, addition of exogenous mRNA requires a concomitant increase in the amount of M_3 added for optimum stimulation. There is also a near absolute requirement for factors M_1 and M_2 in the translation of exogenous mRNA (data not shown). The amounts of M_1 and M_2 used in Fig. 1 were saturating for 0.8 A_{260} units of exogenous mRNA, but did not inhibit the translation of endogenous mRNA.

Translation of hemoglobin mRNA on reticulocyte ribosomal subunits

Exogenous mRNA can also be translated on reticulocyte ribosomal subunits, as shown in Fig. 2. 40S subunits alone are inactive in protein synthesis in the presence or absence of exogenous mRNA. When 60S subunits are added to the reaction mixture containing factors, energy sources, and other components, a low endogenous activity of 7 pmol of [^{14}C]-valine incorporation is obtained. Addition of exogenous hemoglobin mRNA markedly stimulates protein synthesis. Product analysis demonstrates that the products are rabbit α and β globin chains. In the absence of M_3, exogenous mRNA is not translated (Fig. 2).

TABLE 2. *M_3 requirement for optimal translation of hemoglobin mRNA*

mRNA added (A_{260} units)	Saturating amount of M_3 (μg of protein)	[^{14}C]Valine incorporated (pmol)
0	47.5	49
0.38	75	77
0.76	115	94
0.98	127	99

Incubations were performed as described in the legend to Fig. 1.

Translation of hemoglobin mRNA on liver ribosomes

In order to examine the tissue specificity of the initiation factor and ribosome requirements for translation of exogenous hemoglobin mRNA, a heterologous system was developed with rabbit liver ribosomes that were low in endogenous activity (see *Methods*). As shown in Fig. 3, liver ribosomes appear to be equally as effective as reticulocyte ribosomes in translating hemoglobin mRNA. Although an exact comparison between liver and reticulocyte ribosomes cannot be made because of their difference in endogenous protein-synthesis activity, 0.8 A_{260} units of crude mRNA, in the presence of 0.2 A_{260} units of either type of ribosome and 90μg of M_3 protein, appears to stimulate about 70 pmol of [^{14}C]valine incorporation above the endogenous activity. Analysis of the products by carboxymethyl-cellulose chromatography (18) indicates that 75–90% of the proteins synthesized are rabbit α and β globin chains (data not shown).

Hemoglobin mRNA is not translated on liver ribosomes if the reticulocyte initiation factors are replaced by the microsomal wash fraction from liver (Table 3). Hemoglobin mRNA is translated when reticulocyte factor M_3 is added to the reaction mixture containing the microsomal wash fraction from liver, while the addition of factor M_1 or M_2 does not significantly increase activity (Table 3). The ability of liver ribosomes and microsomal wash fraction to translate different amounts of hemoglobin mRNA, in the presence

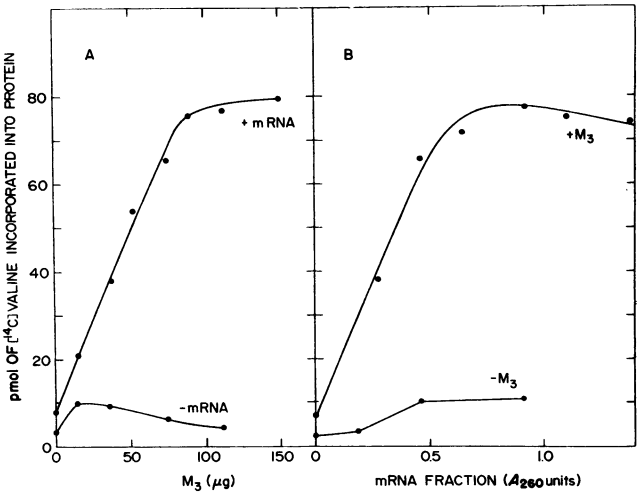

FIG. 3. Translation of exogenous hemoglobin mRNA on rabbit liver ribosomes. (*A*) As a function of M_3. Each 100-μl reaction mixture contained 0.2 A_{260} units of liver ribosomes, 700 μg of reticulocyte supernatant protein, 56 μg of T_1, 34 μg of M_1, and 28 μg of M_2. *Upper curve*, plus 0.8 A_{260} units of hemoglobin mRNA fraction added. *Lower curve*, no mRNA added. (*B*) As a function of exogenous hemoglobin mRNA added. 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 M_3 protein. *Lower curve*, no M_3 added.

of reticulocyte M_3, is shown in Fig. 4. The microsomal wash preparation from liver is not as efficient as are reticulocyte M_1 and M_2 in the translation of exogenous hemoglobin mRNA (compare Figs. 3*B* 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, M_1, M_2, and M_3, for the translation of exogenous mRNA on reticulocyte ribosomes.

M_1 and M_2 are known to be required to place the initiator tRNA, Met-$tRNA_F$, into the mRNA–tRNA–ribosome initiation complex (19–21). The function of initiation factor M_3 remains unclear. It has been demonstrated that, whereas factors M_1 and M_2 are sufficient to stimulate the synthesis of poly(U)-directed polyphenylalanine synthesis at low Mg^{++} (22), M_3 is required for the translation of a natural mRNA template (17). Crystal, *et al.* (21) showed that M_3 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-$tRNA_F$ to an endogenous mRNA template. This observation does not rule out the possibility that M_3 may have a dual function; it might also be required in the initial association of (exogenous) mRNA to the ribosome. Indeed, Heywood (8) has shown that a factor in chick reticulocytes, EF_3, which has similar chromatographic properties on DEAE-cellulose to rabbit reticulocyte M_3, appears to stimulate the binding of chick hemoglobin [^{32}P]mRNA to chick ribosomes. This factor appears to be tissue-specific, at least between reticulocyte and muscle cells (8). Stavnezer and Huang 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).

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

Additions to supernatant and liver ribosomes*	pmol of [^{14}C]Valine incorporated into protein
None	1.8
Liver microsomal wash	3.6
M_3	3.6
Liver microsomal wash + M_3	4.2
Hb mRNA + M_3	4.8
Hb mRNA + liver microsomal wash	5.3
Hb mRNA + liver microsomal wash + M_1	6.5
Hb mRNA + liver microsomal wash + M_2	6.0
Hb mRNA + liver microsomal wash + M_3	34.4

Incubations, in a total volume of 100 μl, were performed for 20 min at 37°C. Each reaction mixture contained 0.2 A_{260} 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 M_3, and 0.9 A_{260} units of hemoglobin mRNA raction.

* All M factors are from rabbit reticulocytes.

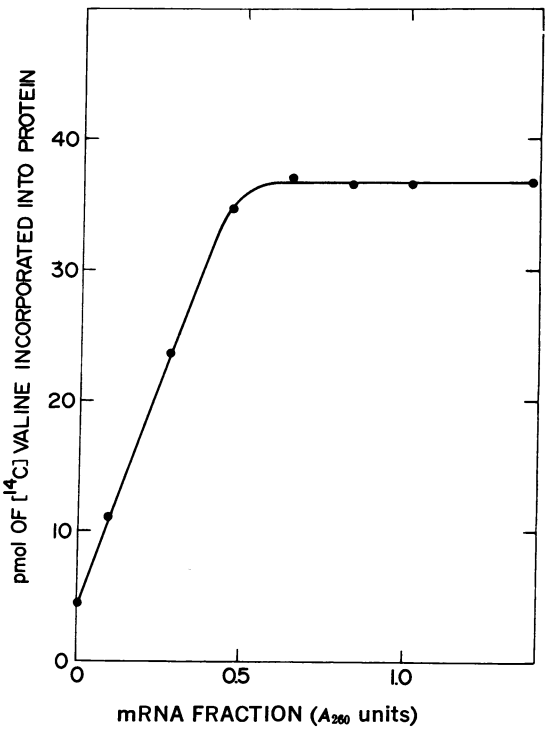

FIG. 4. Translation of exogenous hemoglobin mRNA by liver ribosomes with microsomal wash fraction from liver and rabbit reticulocyte M_3 added. Each 100-μl reaction mixture contained 0.2 A_{260} units of liver ribosomes, 700 μg of reticulocyte supernatant protein, 56 μg of T_1, 90 μg of microsomal wash fraction from liver, and 115 μg of reticulocyte M_3.

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 M_3 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 M_3 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 M_1 and M_2, but a liver factor possessing the mRNA specificity of reticulocyte M_3 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 M_3, 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 M_3, 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

mRNA. It is not yet possible to choose between these alternatives.

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