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Translational Control of Hemoglobin Synthesis by an Initiation Factor Required for Recycling of Ribosomes and for their Binding to Messenger RNA

(IF-3/mammalian ribosome cycle/R17 RNA binding)

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ABSTRACT **The continued recycling of ribosomes during protein synthesis in rabbit reticulocyte lysates at 37° requires an initiation factor whose activity is rapidly lost in the absence of added heme. Partially purified factor (*i*) fully maintains the polysomes; (*ii*) inhibits the association of 40S and 60S ribosomal subunits into single ribosomes; (*iii*) promotes the quantitative entry of added 60S subunits into polysomes; (*iv*) allows the accumulation of ribosomal subunits, instead of single ribosomes, when initiation is blocked with aurin tricarboxylate; and (*v*) is absolutely required for the binding of globin messenger RNA to ribosomes.**

These properties suggest that this mammalian initiation factor functions analogously to bacterial IF-3. In addition, the translational control of globin synthesis by heme is exerted, directly or indirectly, through this factor.

Ribosomes in growing bacteria and in the cytoplasm of yeast frequently undergo exchange of their large and small ribosomal subunits (1, 2), apparently by dissociation after each passage over messenger RNA and reformation from a pool of ribosomal subunits that continuously recycle through polysomes (3, 4). When protein synthesis slows down, synthetically inactive single ribosomes (lacking mRNA and growing polypeptide chains) accumulate at the expense of polysomes in both prokaryotic and eukaryotic cells. Bacterial 30S and 50S ribosomal subunits possess an extremely high affinity for each other and readily associate to form single ribosomes (5); this association is specifically inhibited by initiation factor IF-3 (5, 6). We have proposed that this is the mechanism by which bacterial IF-3 maintains the recycling or ribosomal subunits through polysomes and prevents their accumulation as single ribosomes (5).

We have asked if an analogous factor operates in the mammalian ribosome cycle, and, if so, whether it plays any role in exerting translational control. Rabbit reticulocytes are particularly suitable to answer these questions, because in a cell-free system prepared from them, ribosomes can recycle more than fifty times over endogenous messenger RNA (7); furthermore, when this system is incubated at 28° or higher, initiation of protein synthesis (mostly globin) decreases drastically after 5 min, and polysomes disappear, unless heme is added (7–11).

Our finding is that rabbit reticulocytes indeed contain a factor activity that regulates the flow of ribosomal subunits through the ribosome cycle in a manner strictly analogous to bacterial IF-3. This factor, tentatively named IF-3rr, is absolutely required for the binding of globin mRNA to ribosomes.

Abbreviation: rr, rabbit reticulocyte.

In the absence of added heme, reticulocyte ribosomes fail to recycle through polysomes, unless IF-3rr is added. Directly or indirectly, therefore, translational control of reticulocyte protein synthesis by heme must be exerted through this factor.

RESULTS

Control of reticulocyte ribosome cycle by the factor

The sedimentation distribution of ribosomes in a ^{32}P-labeled rabbit reticulocyte lysate is shown in Fig. 1*A*. Polysomes, which were allowed to sediment onto a dense sucrose cushion, are seen to predominate; few single ribosomes are present. Incubation of this lysate at 37° under conditions favoring rapid protein synthesis, but without added heme, results in

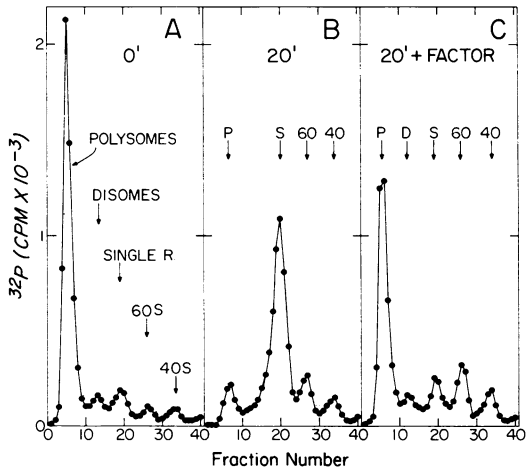

FIG. 1. Maintenance of reticulocyte polysomes during cell-free protein synthesis in the absence of added heme. A rabbit reticulocyte lysate was prepared and incubated as described by Adamson *et al.* (7). The ribosomes had been labeled *in vivo* by three intravenous injections of 10 mCi of ^{32}P at 36, 24, and 12 hr before bleeding. The reaction mixtures (225 μl) were 2 mM Mg^{2+}; they contained 30 μl of lysate (2 A_{260} units of ribosomes). After incubation at 37°, the samples were cooled to 0°, diluted twofold with 0.01 M Tris·HCl (pH 7.4)–2 mM Mg^{2+}, and centrifuged for 3.2 hr at 41,000 rpm and 5° through 13.3–19.6% exponential sucrose gradients (11 ml) supported by a 1.5-ml cushion of 75% sucrose (fractions 1–6), in 0.01 M Tris·HCl (pH 7.4)–2 mM Mg-acetate–0.05 M KCl–100 μg/ml gelatin. Incubation *A:* 0 min; *B:* 20 min; *C:* 20 min in the presence of 160 μg of DEAE-cellulose-treated factor (0.10–0.22 M KCl fraction; see Fig. 5*A*). Single R., *S* = single ribosomes.

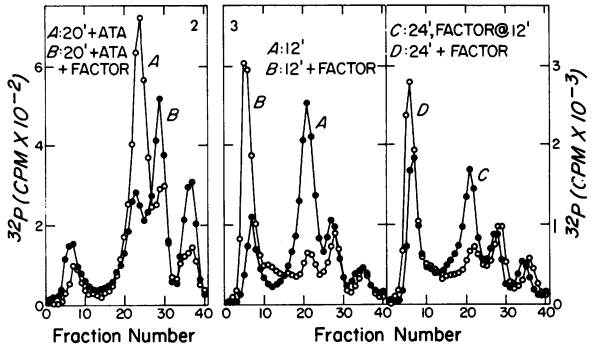

FIG. 2. (*left*) Factor-induced accumulation of ribosomal subunits during protein synthesis in the presence of aurin tricarboxylate (*ATA*). ^{32}P-labeled reticulocyte lysate was incubated as in Fig. 1*B*, (*A*) with 70 μM ATA or (*B*) with 70 μM ATA and 1 mg of crude factor (see legend of Fig. 5*A*). The two sedimentation profiles are superimposed. Peaks from right to left represent 40S, 60S, single ribosome, and polysome species.

FIG. 3. (*right*) Kinetics of polysome maintenance and single ribosome mobilization by the factor. Four samples (*A–D*) were incubated as in Fig. 1, for the times indicated. One mg of crude factor (see legend of Fig. 5*A*) was present in *B* and *D* throughout incubation, and in *C* was added after 12 min. Sedimentation profiles are superimposed. Peaks from right to left represent 40S, 60S, single ribosome, and polysome species.

the almost complete disappearance of polysomes, while large amounts of single ribosomes accumulate (Fig. 1*B*). This result, together with the observation (not shown) that under these conditions polypeptide synthesis ceases after 5 min, confirms earlier work (7–10) that has led to the conclusion that incubation in the absence of added heme causes a block in the initiation of protein synthesis.

We have detected, among the proteins removed from ribosomes with 0.4 M KCl buffer, an activity that prevents the loss of polysomes. As seen in Fig. 1*C*, in the presence of this factor polysomes are nearly fully maintained, while few, if any, single ribosomes accumulate, even after 20 min of incubation. The levels of 60S and 40S ribosomal subunits have increased somewhat relative to those in Fig. 1*A*, but are not much greater than those in Fig. 1*B*. We have found that the initial rate of protein synthesis is similarly maintained.

If this factor were to act like bacterial IF-3, it should prevent the accumulation of single ribosomes by blocking the association of ribosomal subunits generated from polysomes, while at the same time it should promote the recycling of subunits into polysomes (5, 6). Hence, in the presence of factor, we would expect ribosomal subunits to accumulate if initiation is blocked, for example, with aurin tricarboxylate (12). That this is indeed the case may be seen in Fig. 2. In the presence of aurin tricarboxylate, but no factor, single ribosomes accumulate at the expense of polysomes (*A*); when factor is also present, ribosomal subunits are the predominant product (*B*). We conclude that once they have left polysomes, ribosomal subunits are held apart by the factor.

Fig. 2*B* shows that addition of factor does not block the release of ribosomes from polysomes, confirming that the maintenance of polysomes by the factor, seen in Fig. 1*C*, is due to an active recycling of ribosomes.

The factor is much more efficient in recycling polysomal ribosomes than in recruiting single ribosomes, once they have been allowed to accumulate. As seen in Fig. 3*A*, in the absence of factor the loss of polysomes is nearly complete after incubation for 12 min; with factor present, polysomes are maintained to the same extent whether incubation is for 12 min (*B*) or for 24 min (*D*). Yet, if the same amount of factor is added at 12 min (*C*), only some of the ^{32}P label present in single ribosomes at 12 min is found in polysomes at 24 min.

Effect of factor on fate of ribosomal subunits

To analyze more directly how the factor regulates the flow of ribosomal subunits through the reticulocyte ribosome cycle, we have studied the fate of ^{32}P-labeled 60S ribosomal subunits during incubation with a nonradioactive polysome lysate, again in the absence of added heme. Fig. 4*A* shows the sedimentation distribution of ^{32}P before incubation: most label sediments at 60 S, but some single ribosomes (*S*) are present. After 15 min, however, nearly all ^{32}P label is found in single ribosomes (Fig. 4*B*); no significant amount of ^{32}P enters polysomes (*P*). Apparently, during this time the labeled 60 S subunits associated with 40 S subunits supplied by the unlabeled polysome lysate. By contrast, when factor is added (Fig. 4*C*), much less ^{32}P enters single ribosomes; most remains in the 60 S peak but some is now also found in polysomes. When a 3-fold greater amount of factor is added, moreover, the entry of 60 S subunits into polysomes is nearly quantitative (Fig. 4*D*): essentially no 60 S subunits, and only few single ribosomes, remain labeled. Because the number of unlabeled 60 S subunits in the reaction mixture exceeded labeled ones by about 15-fold, and because, as we have shown, subunits initially in polysomes are recycled very efficiently, this result means that in the presence of factor, labeled 60 S subunits enter polysomes as readily as unlabeled subunits and, unlike single ribosomes (Fig. 3*C*), rapidly attain complete equilibration.

Our conclusion is that the factor blocks formation of single ribosomes from ribosomal subunits and promotes recycling of ribosomal subunits through polysomes.

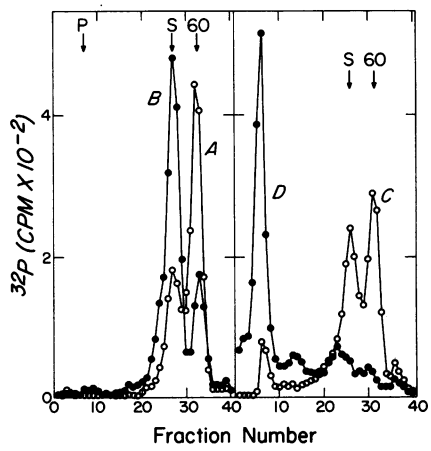

FIG. 4. Effect of factor on the fate of 60S ribosomal subunits during cell-free protein synthesis. ^{32}P-labeled 60S ribosomal subunits were prepared, from a sample made 0.25 M KCl after incubation as in Fig. 1*B*, by centrifugation through a sucrose gradient as described in Fig. 1, but containing 0.25 M KCl to dissociate the single ribosomes. Material taken from the 60S peak (about 0.08 A_{260} units) was included in a reaction mixture containing nonradioactive polysome lysate, but otherwise identical to that of Fig. 1*B*. *A:* 0 min; *B:* 15 min; *C:* 15 min in the presence of 1.4 mg of crude factor (see legend of Fig. 5*A*); *D:* 15 min in the presence of 4.2 mg of crude factor. Analysis as in Fig. 1; sedimentation profiles are superimposed.

DEAE-cellulose chromatography

The factor used to demonstrate the maintenance of polysomes in Fig. 1*C* had been partially purified by DEAE-cellulose chromatography of the crude material washed off reticulocyte ribosomes with 0.4 M KCl. A two-cycle stepwise elution technique was used to avoid dilution of the activity; a typical elution pattern is illustrated in Fig. 5*A*. The fraction of highest specific activity is eluted between 0.10 and 0.22 M KCl. The sum of the specific-activity distributions of three such stepwise elutions is depicted in Fig. 5*D;* the peak of activity elutes between 0.16 and 0.20 M KCl. The active fraction is colorless; its activity is completely lost upon trypsin digestion. Activity is expressed as polysome maintenance units; one unit is defined as the amount that maintains 1% of the total ribosomes as polysomes under standard assay conditions (Fig. 1*B* and *C*). We have found the assay to be very nearly linear with the amount of factor preparation added, up to the saturation level (about 70% polysomes), suggesting that stoichiometric amounts of factor are needed.

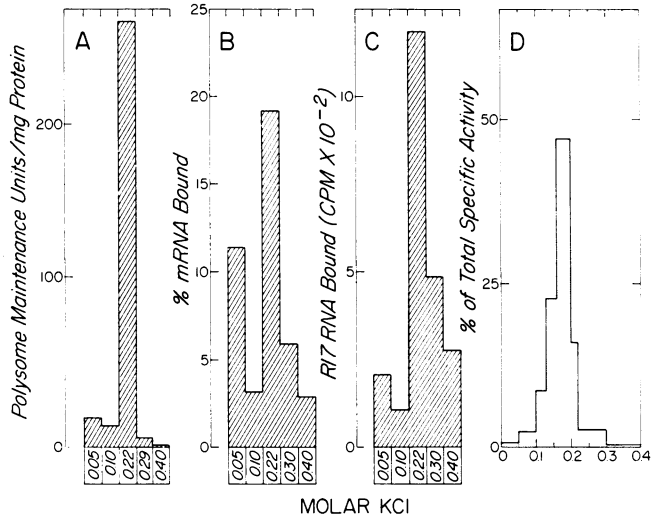

FIG. 5. DEAE-cellulose chromatography. (*A*): Elution of polysome maintenance activity. Ribosomes from nonradioactive reticulocyte lysate were washed with 0.4 M KCl buffer (13). The protein in the wash was precipitated by addition of $(NH_4)_2SO_4$ to 80% saturation at 0°, dissolved in buffer A[0.01 M Tris·HCl (pH 7.4)–0.05 M KCl–5 mM 2-mercaptoethanol] and dialyzed for 10 hr against buffer A. This preparation (crude factor) was purified on DEAE-cellulose by a micro-batch procedure: 0.5 ml of factor was agitated for 10 min at 0° with 0.25 ml of packed DEAE-cellulose equilibrated with buffer A; after centrifugation, the supernatant was removed and 0.3 ml of buffer A, containing 0.05 M KCl, was introduced. The pellet was subjected to successive cycles of extraction with 0.3 ml of buffer; to facilitate complete equilibration with DEAE-cellulose, the KCl concentration was raised after every second cycle and extracts of identical KCl concentration were pooled. Individual fractions were tested for polysome maintenance activity as in Fig. 1; the effect of the 0.10–0.22 M fraction is illustrated in Fig. 1*C*. (*B*): Elution of the activity binding 9S reticulocyte RNA to ribosomes. An equal volume of each fraction was assayed for this activity by the procedure described in Table 1. (*C*): Elution of the activity binding to R17 phage RNA. An equal volume of each fraction was assayed for its ability to bind purified ^{32}P-labeled R17 RNA (a gift from Gilbert Jay) by the filter technique of Table 1, but ribosomes were omitted. The RNA (more than 80% intact) used per assay contained 0.05 A_{260} and 4200 cpm. (*D*): Sum of three elutions of the polysome maintenance activity. Specific activity distributions of Fig. 5*A* and of two similar fractionations, performed with different increments in KCl concentration, were summed. The result is plotted as percent of total specific activity.

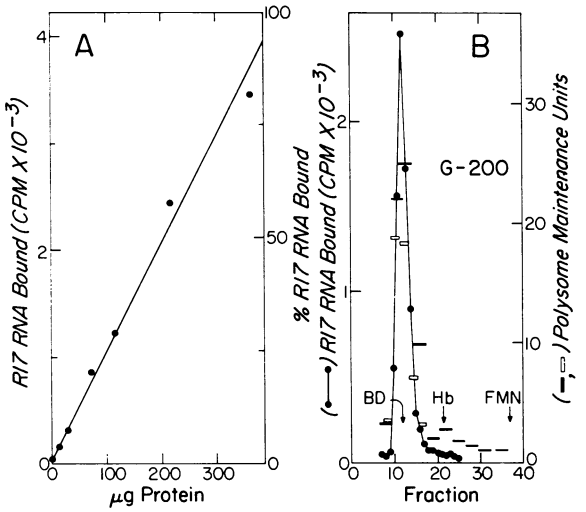

FIG. 6. (*A*): Linearity of R17 RNA-binding assay. Different amounts of DEAE-cellulose-treated factor (0.10–0.22 M KCl fraction, 2.9 mg/ml of protein) were assayed as described in the legend of Fig. 5*C*. (*B*): Sephadex G-200 chromatography of polysome maintenance and R17 RNA-binding activities. Crude factor (6.8 mg; see legend of Fig. 5*A*) was placed over a Sephadex G-200 column equilibrated with buffer A (Fig. 5) containing 0.22 M KCl. The flow rate was 2.25 ml/hr. An equal volume of each fraction was assayed for its ability to bind ^{32}P-labeled R17 RNA (5000 cpm/assay) as in Fig. 5*C*. Adjacent fractions were pooled and equal volumes were assayed for polysome maintenance activity (*bars*); *open* and *filled* symbols represent two separate analyses. *Arrows:* elution positions of markers BD (blue dextran), Hb (hemoglobin), and FMN.

Binding of globin mRNA to ribosomes requires factor

If the reticulocyte polysome-recycling factor corresponds to bacterial IF-3, one would expect the factor to stimulate binding of 9 S globin mRNA to ribosomes. Indeed, as seen in Fig.

TABLE 1. *Binding of RNA to ribosomes*

Ribosomes	Factor	% bound (counts) 9S RNA	18S RNA	28S RNA
−	−	3.4 (129)	0.2 (25)	0.5 (55)
+	−	4.5 (169)	0.2 (21)	0.5 (61)
+	+	21.4 (809)	7.9 (859)	7.4 (891)

Binding to 25-mm nitrocellulose filters was assayed as described by Heywood (14), except that the incubation buffer contained 2 mM Mg^{2+} instead of 5 mM. Per assay, 300 μg of DEAE-cellulose-treated factor (0.1–0.22 M KCl fraction), and 1 mg of 0.4 M KCl-washed ribosomes were added. ^{32}P-labeled 9S, 18S, and 28S RNA species were prepared (14) after 36 hr of *in vivo* labeling (see Fig. 1); input counts per assay were 3,772, 10,810, and 12,054, respectively.

5B, the peak of mRNA-binding activity is eluted from DEAE-cellulose in the same fraction as the polysome maintenance activity (Fig. 5A). The second peak, at 0.05 M KCl, represents unadsorbed material. Table 1 shows that the 0.10–0.22 M KCl fraction is absolutely required for binding of 9 S RNA to reticulocyte ribosomes; binding of 18 S and 28 S ribosomal RNA species also is stimulated, but to a lesser degree. The extent of binding of various RNA classes is in good agreement with that observed by Heywood for chick-embryo reticulocytes (14).

Binding of factor to R17 RNA

We have found that crude factor preparations cause bacteriophage R17 RNA to be retained on filters, even in the absence of ribosomes. The R17 RNA-binding activity is eluted from DEAE-cellulose in the same fraction (Fig. 5C) that contains both polysome maintenance and mRNA-binding activities. As shown in Fig. 6A for the 0.10–0.22 M KCl fraction, binding of ^{32}P-labeled R17 RNA is linear with the amount of factor added, over a wide range of concentration. The assay is both quantitative and sensitive.

The R17 RNA-binding activity travels with the polysome maintenance activity not only on DEAE-cellulose, but also on Sephadex G-200. When crude factor is passed through Sephadex G-200 and individual fractions are tested for their ability to bind R17 RNA to filters, a peak of activity is obtained, eluting with the excluded volume (Fig. 6B). Polysome maintenance activity, represented by bars in Fig. 6B, coincides exactly with this peak.

DISCUSSION

Mammalian ribosome cycle

We show here that the recycling of ribosomes through polysomes depends absolutely upon the presence of a factor that prevents the association of 40 S and 60 S ribosmal subunits into single ribosomes, and promotes the entry of ribosomal subunits into new polysomes.

Although in the presence of the factor, ribosomal subunits are efficiently recycled between polysomes, single ribosomes enter polysomes much more slowly. As seen in Fig. 3, if factor is added to a polysome lysate after incubation for 12 min, by which time most polysomes have disappeared and single ribosomes have accumulated (Fig. 3A), and incubation is continued for another 12 min, many single ribosomes fail to return to polysomes (Fig. 3C). Since the amount of factor added was clearly sufficient to recycle all ribosomes among polysomes between 12 and 24 min (Figs. 3B and D), and since in the sample first incubated without factor (Fig. 3A) fewer ribosomes were leaving polysomes at 12 min than could be recycled by the amount of factor added at that time, we would have expected that, if the factor actively dissociated them, all single ribosomes should have entered polysomes by 24 min. This is not the case; therefore, the rate of dissociation of single ribosomes into subunits must remain limiting, even in the presence of sufficient factor to recycle an equal amount of ribosomes in polysomes. This, and the results of Figs. 2 and 4, support the concept that the factor acts to inhibit the formation of single ribosomes from 60 S and 40 S subunits, but does not actively dissociate them. The slow entry of single ribosomes into polysomes, even in the presence of factor, can then be accounted for by the observation that at the Mg^{2+} ion concentration used in our experiments, 2 mM, reticulocyte single ribosomes exchange very slowly with 40 S and 60 S subunits (15).

We believe that the flow of ribosomal subunits through the mammalian ribosome cycle can be described in terms of the model proposed for the bacterial cycle (4, 5), in which ribosomal subunits are thought to be the only species recycling between polysomes, while single ribosomes, representing a sidetrack pool, are formed from subunits only if protein synthesis slows down. This model is in good agreement with the finding that, *in vivo*, mammalian ribosomal subunits equilibrate much more slowly with single ribosomes than with polysomes (16–19).

IF-3rr

The activities responsible for the recycling of ribosomal subunits through polysomes, for the binding of 9 S mRNA to ribosomes, and for binding of R17 RNA to filters all are eluted from DEAE-cellulose between 0.10 and 0.22 M KCl, the first activity being maximal between 0.16 and 0.20 M KCl (Fig. 5). Both ribosome recycling and R17 RNA-binding activities travel with the excluded volume on Sephadex G-200 (Fig. 6B). Although the assignment of these activities to a single protein must await their purification to homogeneity, their chromatographic properties are remarkably similar to those of IF-M_3 described by Anderson *et al.* (20, 21), and the mRNA-binding activity described by Heywood (14). The latter factors both are washed off ribosomes with 0.5 M KCl. IF-M_3, a rabbit reticulocyte factor required for initiation of translation of 9 S RNA, is eluted from DEAE-cellulose at 0.18 M KCl (20), whereas factors IF-M_1, IF-M_{2A}, and IF-M_{2B} are eluted at 0.05 M, 0.26 M, and 0.32 M, respectively (13, 22). The activity binding chick-embryo muscle mRNA to ribosomes, which elutes from DEAE-cellulose between 0.18 and 0.25 M KCl, as well as the IF-M_3 activity, are excluded from Sephadex G-200 (14, 21). These similarities in chromatographic behavior lend support to the concept that a single component is responsible for all these activities.

We propose that this factor be named IF-3rr because of its very considerable analogy to IF-3 of bacteria (5, 6, 23, 24): both are required for the binding of ribosomes to mRNA and for the initiation of polypeptide synthesis; both retain viral mRNA on filters; both control the flow of ribosomal subunits through the ribosome cycle by inhibiting their association to single ribosomes and promoting their return to polysomes.

The role of heme

Of considerable interest is the ability of IF-3rr to maintain reticulocyte polysomes in the absence of added heme, and to prevent their decay seen otherwise. The simplest explanation of this finding is that endogenous IF-3rr activity is lost in the course of cell-free protein synthesis, unless heme is present. Consequently, ribosomal subunits increasingly fail to recycle into new polysomes and instead accumulate as single ribosomes. According to this view, not only synthesis of globin, but all protein synthesis in reticulocyte lysates should cease upon the loss of IF-3rr, because the ribosomal subunits are no longer held apart and, therefore, cannot reach initiation sites on mRNA.

This consideration leads to the interesting possibility that IF-3rr, through its property of heme dependence, allows the coordinate regulation of synthesis of heme and of polypeptide chains, mostly globin, during erythroid development. The control of IF-3rr activity may be particularly relevant to the

process of terminal differentiation of reticulocytes into mature red blood cells, and may be an example of a regulatory mechanism of wider significance.

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