THE MOLECULAR MECHANISM OF HEMOGLOBIN SYNTHESIS AND ITS REGULATION IN THE RETICULOCYTE

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I. INTRODUCTION

The synthesis of hemoglobin involves the processes of polypeptide chain initiation, elongation, and termination. Initiation is the assembly of ribosomes, messenger RNA (mRNA), and initiator Met-tRNA$_f$ by protein factors which regulate the temporal sequence of the assembly process and the spatial arrangement of components required for formation of the first peptide bond. Elongation is the addition of amino acids one at a time to the growing polypeptide chain, while termination is the release of the completed polypeptide from the 80S ribosome. We will first examine the steps involved in 80S initiation complex formation and the specific function of each of the eukaryotic initiation factors. Next, we will briefly review the processes of elongation and termination, and then will discuss in detail the nature of hemin-deficient translational inhibition of hemoglobin synthesis.

II. INITIATION

A. Steps in the Pathway

During the past 10 years, the basic sequence of events required for the initiation of protein synthesis has been established. An overall view of the four major steps involved is shown in Figure 1. First, native 40S$_N$ ribosomal subunits (40S$_N$) are formed by the binding of specific proteins to free ribosomal particles. The initiator aminoacyl-tRNA species Met-tRNA$_f$ then binds to these 40S. Formation of the Met-tRNA$_f$:40S complex is followed by mRNA insertion at the correct initiation sequence. Joining of the 60S ribosomal subunit then completes formation of the 80S initiation complex. Fractionation of the reticulocyte lysate has resulted in the purification of seven polypeptide initiation factors, all of which are required for optimal assembly of the initiation complex with natural mRNA. Five of these factors are absolutely required, while two have only stimulatory effects. Two additional factors have also been purified, whose activity can only be shown in model assay systems with artificial templates and whose physiological function is not clear. The physical and biochemical properties of these nine factors are summarized in Table 1. At the present time, all major components required for in vitro 80S initiation complex formation appear to have been purified. An alter-

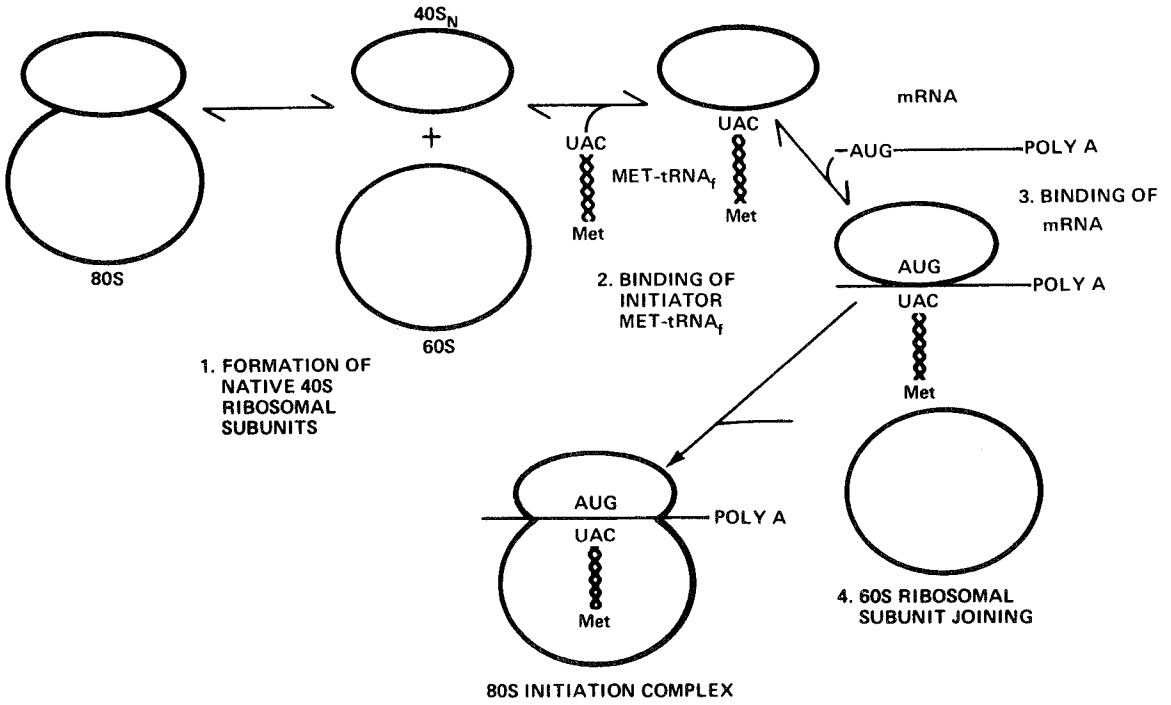

FIGURE 1. The four basic steps in the pathway of 80S initiation complex formation. Figures 2 through 5 expand on each of the steps.

TABLE 1

Physical and Biochemical Properties of Eukaryotic Initiation Factors

Nomenclature[a]		Molecular weight		
New	Old	SDS Gels	Native	Primary function
eIF-1	—	15,000	15,000	Preinitiation complex stabilization; 80S ribosome dissociation
eIF-2	IF-MP	55,000		Met-tRNA$_f$ binding to 40S$_N$
		52,000	125,000	
		35,000		
eIF-2A	IF-M1	65,000	65,000	[Regulation of Met-tRNA$_f$ binding][b]
eIF-3	IF-M5	130,000		Formation of 40S$_N$; mRNA binding
		110,000		
		69,000		
		49,000	700,000	
		43,000		
		39,000		
		35,000		
eIF-4A	IF-M4	50,000	50,000	mRNA binding
eIF-4B	IF-M3	80,000	80,000	mRNA binding
eIF-4C	IF-M2Bβ	19,000	17,000	Preinitiation complex stabilization subunit joining
eIF-4D	IF-M2Bα	17,000	15,000	[Preinitiation complex stabilization][b]
eIF-5	IF-M2A	150,000	125,000	Initiation factor release; subunit joining

[a] See Reference 232.

[b] Exact function uncertain.

native approach used to establish the sequence of events during initiation has been to perturb the unfractonated reticulocyte lysate using specific inhibitors of protein synthesis and radiolabeled RNA components. At the present time, however, it appears that while both have been successful in establishing many basic aspects of eukaryotic protein synthesis, an overall understanding will only result from an integration of the major findings from both experimental approaches. An attempt to accomplish this is the basic philosophy on which the following is based.

1. Formation of Native 40S Ribosomal Subunits ($40S_N$)

The first step of 80S initiation complex formation appears to be the binding of the initiation factor eIF-3 to newly released 40S ribosomal subunits (see Figure 2) to form native 40S ribosomal subunits, designated $40S_N$. During polypeptide chain termination, 80S ribosomal couples transiently dissociate into free subunits upon release from mRNA. Under in vivo conditions, however, the dissociation-reassociation equilibrium strongly favors nonenzymatic reassociation to nonfunctional 80S couples. Dissociation of these monosomes to 40S and 60S subunits by nonribosomal proteins is thought to be an obligatory step for all subsequent stages of functional 80S initiation complex formation.[1-5] The specific initiation factor responsible for formation of an active $40S_N$ subunit pool is eIF-3.

The initiation factor eIF-3 is a large multisubunit complex composed of seven to ten polypeptide chains having molecular weights ranging from 35 to 130×10^3 (see Table 1). The mass of native eIF-3 is ca. 700,000 daltons, corresponding to a sedimentation coefficient of 15 to 17S.[6-10] This initiation factor has been purified and characterized by several groups as: IF-E_3,[6,9] IF-M5,[8] IF-3,[10] and EIF-3.[11] It was also contained as a major activity in cruder factor preparations: IF-M3,[12] IF-3,[13] DF,[14] and EIF-3.[15]

Although the functions of eIF-3 appear to be pleiotropic commensurate with its complex structure, one major effect is to prevent the spontaneous reassociation of 40S and 60S ribosomal subunits outside the 80S initiation complex pathway. In contrast to all other initiation factors, mRNA or aminoacyl-tRNA species, eIF-3 binds to 40S ribosomal subunits independent of energy or other factors.[6,9,10,11] As a result, the 40S:eIF-3 complex does not spontaneously associate with 60S ribosomal subunits until a specific sequence of events during

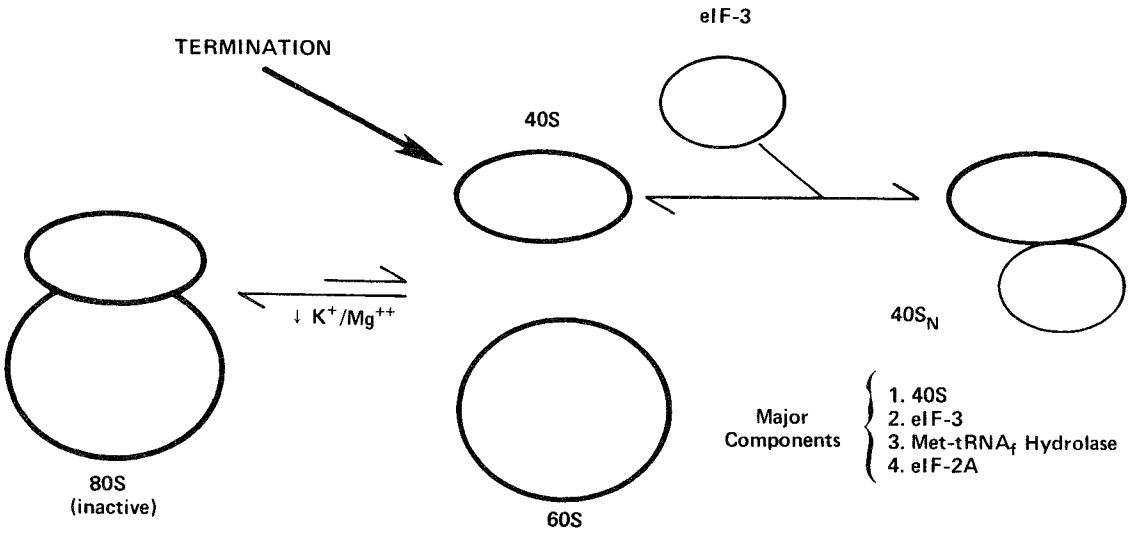

FIGURE 2. Formation of native 40S ribosomal subunits. The first step of 80S initiation complex formation is the binding of eIF-3 to the 40S ribosomal subunits which are released upon termination of polypeptide synthesis. No other initiation factors or energy source are required. Binding of eIF-3 to form $40S_N$ prevents the spontaneous reassociation of 40S and 60S ribosomal subunits that would occur under in vivo ionic conditions. In vitro, formation of inactive 80S ribosomal couples is promoted by decreased K^+/Mg^{++}. Other major components associated with $40S_N$ are Met-$tRNA_f$ hydrolase and eIF-2A.

initiation complex formation has occurred. Although the exact mechanism of this effect is unclear, a stoichiometric association of one eIF-3 per native 40S subunit,[7,9,10,16,17] but not to 60S subunits or 80S ribosomes, suggests that such binding is highly specific and stable. Evidence has also been obtained in support of eIF-3 preventing reassociation of free subunits rather than actively dissociating 80S ribosomal couples.[10]

Cesium chloride (CsCl) buoyant density gradient analysis can be used to measure the relative amounts of protein and RNA in ribosomal particles. By this technique, essentially all free 40S ribosomal subunits in vivo are found to be associated with specific nonribosomal protein components.[18] Five distinct species of native 40S ribosomal subunits ($40S_N$) have been identified, both in vivo[19,20] and following in vitro reconstitution of derived 40S subunits with ribosomal wash proteins.[21] In vitro, Met-tRNA$_f$ is only found associated with $40S_N$ having a buoyant density of 1.42 g $\cdot cm^{-1}$.[22] This is roughly equivalent to an association of 7.5×10^5 daltons of nonribosomal protein. In vivo, Met-tRNA$_f$ is only associated with $40S_N$ having a buoyant density of 1.40 g $\cdot cm^{-3}$.[23]

A specific role for the proteins associated with $40S_N$ is indicated by observations that the extent of such binding changes under a variety of physiologic conditions, in concert with the rate of protein synthesis.[24,25] Identification of these nonribosomal proteins in $40S_N$ as eIF-3 (700,000 daltons) and eIF-2 (130,000 daltons) agrees with the demonstration that in vitro Met-tRNA$_f$ binding to 40S subunits is mediated by eIF-2 and stabilized by eIF-3.[7,10,16] (see Section II.A.2). Four other $40S_N$ of buoyant density 1.39, 1.45, 1.49, and 1.51, respectively, have been identified.[19,21] These do not bind Met-tRNA$_f$ and may arise from the variable association of a specific Met-tRNA$_f$ deacylase (30,000 daltons) or eIF-2A with $40S_N$ containing eIF-2 and/or eIF-3. As will be discussed subsequently (see Section V.H), the presence of a Met-tRNA$_f$ deacylase specific for Met-tRNA$_f$ bound to $40S_N$ may be functionally important to both the economy of translation and translational control. Although other initiation factors may associate with $40S_N$ in fractional amounts,[16,26-29] only eIF-3 and Met-tRNA$_f$ hydrolase are associated with 40S particles in near stoichiometric amounts.[7,16,30] Another important observation relevant to the sequence of 80S initiation complex formation is that less than 5% of $40S_N$ in reticulocytes are associated with globin mRNA.[17]

2. Met-tRNA$_f$ Binding to $40S_N$

The second step of 80S initiation complex formation is the binding of initiator Met-tRNA$_f$ to $40S_N$ (see Figure 3). This is accomplished through the initiation factor eIF-2, which first forms a stable ternary complex with Met-tRNA$_f$ and GTP. Many laboratories have now reported the isolation of a similar or identical factor designated: IFL3,[31] IF1,[32] 1F-1,[33] IF-E2,[34] IF-MP,[35] EIF3,[36] and EIF2[37] from mammalian tissues.

Homogeneous eIF-2 is composed of three nonidentical polypeptide subunits with approximate molecular weights of 55,000, 52,000 and 35,000[38,39,56] (see Table 1). The native molecular weight of eIF-2, however, has been estimated to be 125,000 to 180,000, depending on the analytical techniques used.[16,35,39] Subunit separation by isoelectric focusing has suggested that the intermediate molecular weight subunit has Met-tRNA$_f$ and mRNA binding activities, while the small subunit binds guanine nucleotides.[38] Phosphorylation of two of these subunits by highly specific protein kinases, accompanied by changes in their pI, may be related to altered eIF-2 function.[40-44] The function(s) of the 55,000 dalton subunit (possibly regulatory[38]) is uncertain, and active preparations of eIF-2 not containing this subunit have been isolated.[45,46] The recent isolation of a protein cofactor which stimulates ternary complex formation might be related to these observations.[47]

a. Ternary Complex Formation

Under physiological conditions, eIF-2 exclusively binds the initiator aa-tRNA species Met-tRNA$_f$.[48-51] Although a stable binary eIF-2:Met-tRNA$_f$ complex can be observed, formation of a ternary eIF-2:Met-tRNA$_f$:GTP complex is kinetically favored.[50] Binding proceeds as an ordered sequential reaction, with GTP binding preceding Met-tRNA$_f$.[50] Formation of a transient eIF-2:GTP complex appears to transform eIF-2 into a metastable state which can bind Met-tRNA$_f$ more efficiently, but which leads to the rapid inactivation of eIF-2 if

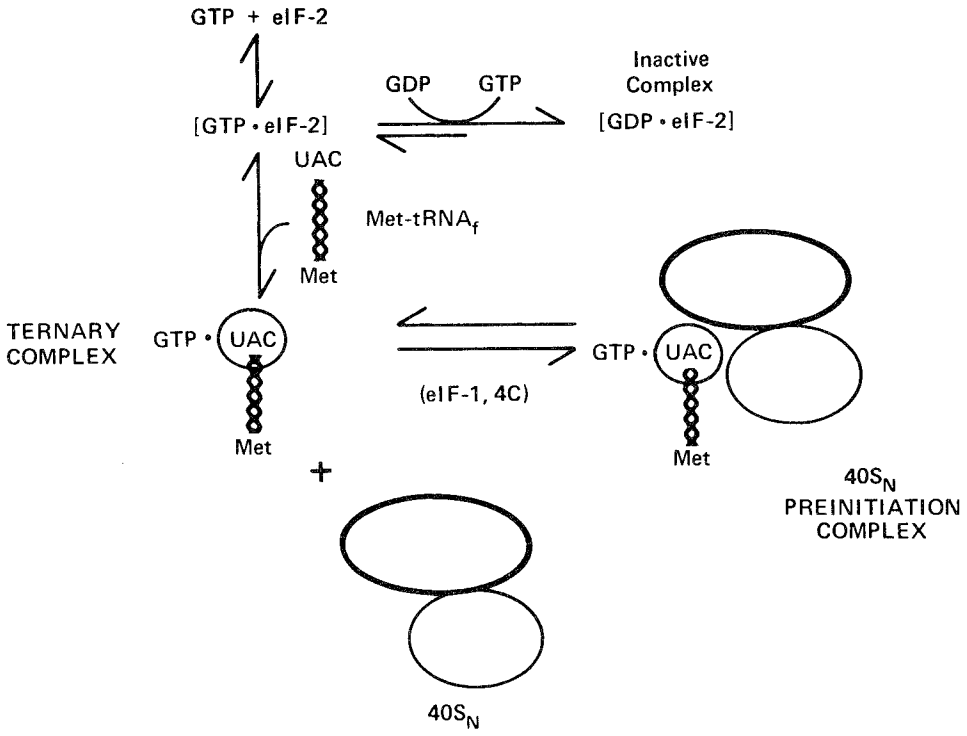

FIGURE 3. Met-tRNA$_f$ binding to 40S$_N$. The second step of 80S initiation complex formation is the binding of Met-tRNA$_f$ to 40S$_N$. This is accomplished through the formation of a ternary complex composed of Met-tRNA$_f$, eIF-2, and GTP. eIF-1 and eIF-4C appear to stabilize the [40S:eIF-3:eIF-2:Met-tRNA$_f$:GTP] preinitiation complex. Physiological regulation of this early step of protein synthesis may be mediated through the greater affinity of eIF-2 for GDP than GTP. This results in the formation of an inactive eIF-2:GDP binary complex when the energy charge of the cell is low. Binding of the ternary complex occurs independent of the initiation codon AUG. In contrast, mRNA binding is absolutely dependent on Met-tRNA$_f$ binding to 40S$_N$.

Met-tRNA$_f$ is not available.[50] Nonhydrolyzable GTP analogs appear to substitute for GTP, with GMP-P(NH)P being relatively more effective than GMP-P(CH$_2$)P. Binding of 5.5 pmol Met-tRNA$_f$ per 8 pmol of homogeneous eIF-2 has been reported.[50]

Ternary complex formation appears to be a primary site for the rapid adjustment of the rate of protein synthesis to the energy state of the cell.[52] GDP is a potent competitive inhibitor of ternary complex formation with a $K_i = 3.4 \times 10^7$ *M*.[53] Since GTP and Met-tRNA$_f$ are readily exchangeable in the ternary complex and GDP will not support ternary complex formation, binding of Met-tRNA$_f$ to the 40S$_N$ is inhibited by GDP. By linking the guanine nucleotide pool with the larger adenine nucleotide pool, nucleoside diphosphate kinase rapidly coordinates ternary complex formation with the overall adenylate energy charge of the cell.[53]

b. Ternary Complex Binding to 40S$_N$

Binding of the [eIF-2:Met-tRNA$_f$:GTP] ternary complex to 40S$_N$ occurs independent of mRNA. In the reticulocyte lysate, Met-tRNA$_f$ is only found associated with 40S$_N$ having a buoyant density of 1.40 to 1.42, in which no mRNA can be detected.[17,19,21,54] In reconstituted fractionated systems where it is possible to selectively omit components, it has been directly demonstrated that Met-tRNA$_f$ binding does not require either AUG initiation codon or mRNA.[6,55-58] In contrast, in the presence of all components required for 80S initiation complex formation, mRNA binding is totally dependent on Met-tRNA$_f$.[56,58] Observations that AUG codon is required for Met-tRNA$_f$ binding may reflect increased stability of the complex or are based on Millipore® filter assays dependent on dissociation of nonbound ternary complex by Mg^{++}.[21,60] Highly purified prepara-

tions of mammalian eIF-2 are not affected by Mg^{++},[35,36,39,46] although sensitivity of eIF-2 from *Artemia salina* and wheat germ has been reported.[61,62] Partially purified preparations of eIF-2 may contain an as yet unidentified factor conferring sensitivity to Mg^{++},[47,61] but caution is warrented in view of possible GTPase activation by Mg^{++}.

At this stage, all components of the 40S preinitiation complex are in rapid equilibrium with their free counterparts.[63,64] It is not known whether such exchange occurs at the level of single components with the 40S preinitiation complex or if dissociation of ternary complex precedes such exchange.

Binding of Met-tRNA$_f$ to 40S$_N$ involves the participation of all major polypeptide subunits of eIF-2 and eIF-3.[16,39] In the presence of eIF-3, the rate of methionyl-puromycin synthesis at nonsaturating levels of eIF-2 is enhanced.[16] This corresponds to a direct stabilization of ternary complex binding to 40S$_N$ by the large complex eIF-3 observed on sucrose density gradient analysis.[16,64] A possible conformational change of the 40S subunit mediated by eIF-3 resulting in the increased stability of Met-tRNA$_f$ binding may be indicated.[63] Observations of a requirement for AUG or mRNA[59,60,65] may be related to an additional stabilization of Met-tRNA$_f$ binding to the 40S preinitiation complex by the subsequent binding of mRNA;[58] however, additional factors are required for this (see next section).

3. Binding of mRNA to the 40S Preinitiation Complex

The third step of initiation complex formation is mRNA binding (see Figure 4). When elongation is blocked in the unfractionated reticulocyte lysate, 40S ribosomal preinitiation complexes containing Met-tRNA$_f$, eIF-2, eIF-3, and GTP can be converted to 80S initiation complexes by the addition of mRNA.[66] In reconstituted fractionated systems, mRNA binding is totally dependent on bound Met-tRNA$_f$, whereas the binding of initiator tRNA does not require mRNA. Such observations suggest that the sequence of initiation complex formation is the initial binding of Met-tRNA$_f$ followed by mRNA.[55,56]

a. Initiation Factor Requirements

Three distinct initiation factors are necessary for mRNA binding. One of these, eIF-3, which first binds to 40S ribosomal subunits to form 40S$_N$, was discussed previously (Section II.A.1). Two additional factors (eIF-4A and eIF-4B) and ATP are also required[8,56,58,67] (see Table 1). eIF-4A is a single polypeptide chain with a molecular weight of 48,000 to 50,000, which has previously been identified as IF-EMC,[68] IF-M4,[8] and IF-E4.[46,67] eIF-4B is also a single polypeptide of 80,000 mol wt, corresponding to IF-M3[69] and IF-E6.[46,67] Omission of any of these components results in a 10- to 20-fold decrease in either the rate of protein synthesis or in the extent of mRNA binding to 40S$_N$. As previously mentioned, this and all other subsequent steps of initiation complex formation are absolutely dependent on bound Met-tRNA$_f$.

b. Energy Requirements

The requrement for ATP during mRNA binding to the 40S preinitiation complex was first observed in wheat germ[70] and later confirmed in a fractionated mammalian system.[34,46,56] During mRNA binding, ATP is hydrolyzed; while such hydrolysis is nonstoichiometric in vitro,[63] this may not be true in vivo. The function of ATP hydrolysis during mRNA binding is unknown. One possibility advanced is that energy-dependent translocation might be involved during the correct alignment of the 40S bound Met-tRNA$_f$ anticodon with the correct initiation codon AUG. Although it was initially thought that eIF-4B had ATPase activity,[56] this was later shown not to be the case.[63] Substitution of nonhydrolyable analogs of ATP and GTP during 80S initiation complex formation shows that while Met-tRNA$_f$ and mRNA binding to 40S$_N$ is unaffected or increased, mRNA binding and 60S ribosomal subunit joining to form 80S complexes is strongly inhibited.[16,58]

c. Do Initiation Factors Required for mRNA Binding Exist as a Complex?

The initiation factors eIF-3 and eIF-4B were probably first isolated as a complex designated IF-M3.[71] Separation by several laboratories has subsequently been achieved, but this appears to require high salt dissociation followed by sucrose or glycerol density gradient fractionation.[9,46] Because of the difficulty in separating these activities and their similar functions (mRNA binding) during initiation complex for-

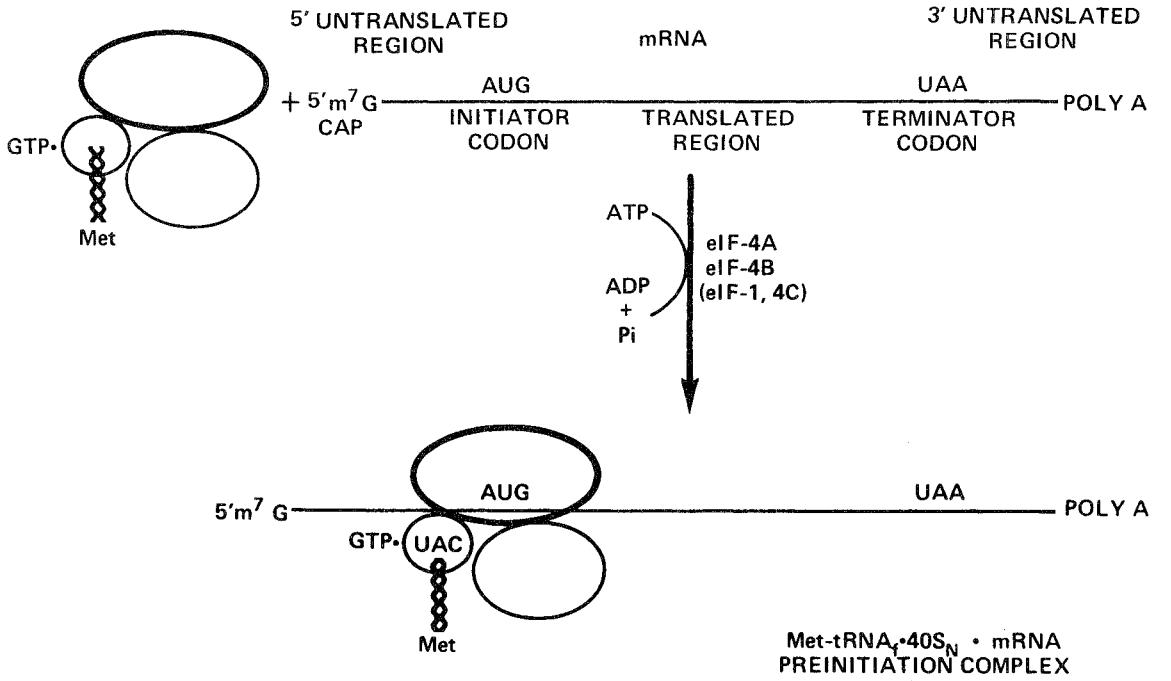

FIGURE 4. Binding of mRNA to the 40S preinitiation complex. The third step of initiation complex formation is mRNA binding to the [40S:eIF-3:eIF-2:Met-tRNA$_f$:GTP] complex. Three initiation factors are required for translation of mRNA: eIF-3, eIF-4A and eIF-4B. It is not known at the present time whether the eIF-3 already bound to 40S$_N$ can serve this function. Actively translated messenger RNA is associated with characteristic proteins which appear to be tightly and specifically bound to the mRNA. The relationship of these proteins to initiation factors is unknown, but no convincing evidence has been obtained for the general existence of message-specificity factors (see text). Binding of these ribonucleoprotein particles (mRNP) requires ATP hydrolysis.

mation, it has been speculated that under in vivo conditions eIF-3 and eIF-4B may constitute a single complex.[8] Similarly, the third initiation factor required for mRNA binding, eIF-4A, also appears to be present in small amounts in this complex.[8,9] A possible argument against such a functional complex has been the report that such minor polypeptide components appear to be lost upon binding of the eIF-3 complex to the 40S$_N$.[9] However, it is not known at the present time if the eIF-3 which is responsible for 40S$_N$ formation is identical to that required for subsequent mRNA binding. In this regard, it has recently been observed that eIF-3 and eIF-4B can bind to mRNA independent of 40S$_N$.[72] A possible function of such binding may be to "melt" locally the extensive secondary structure of globin mRNA to facilitate translation.[73,74] It is therefore possible that discrete eIF-3 complexes may exist corresponding to different functions during initiation complex formation, analogous to the various subpopulations of 40S$_N$. The availability of radiolabeled factors and mRNA and the use of crosslinking reagents to stabilize weak interactions should permit direct evaluation of this concept.

d. Message Specificity Factors

In the rabbit reticulocyte, β-globin mRNA is translated more efficiently than α-globin mRNA.[75,76] Since balanced synthesis of α and β globin is required, the cell must have some mechanism to achieve this. The purification of initiation factors specific for natural mRNA seemed to offer such a possibility. The initiation factor eIF-4B (IF-M3)[77] (and more recently I_{Hb}[78])was thought to stimulate preferentially the translation of α-globin mRNA. A second factor, eIF-4A (IF_{EMC}),[68] was also thought to increase translation of β-globin mRNA and EMC viral mRNA. However, the apparent specificity of these factors for α or β globin mRNAs can also be explained in terms of rate-limiting levels of these initiation factors in various translation systems.[79,80] In very basic terms, more efficient mRNAs will outcompete intrinsically less effi-

cient mRNAs for rate-limiting amounts of an initiation factor. Since the reticulocyte lysate system can be used to translate a wide variety of eukaryotic and viral mRNAs, the most likely explanation for an apparent message specificity appears to reside in a differential affinity of various mRNAs for a rate-limiting factor. Although various eukaryotic initiation factors may recognize specific features of mRNA sequence or secondary structure, the message specificity of various factors probably reflects preference for broad classes of mRNA structure (message discrimination) rather than distinct mRNAs (message specificity).[80] Exceptions to this general rule may exist, however.[11,81]

4. 60S Ribosomal Joining and Initiation Factor Release

The fourth step during 80S initiation complex formation is the joining of the 60S ribosomal subunit to the 40S preinitiation complex (see Figure 5). A major uncertainty at this time is whether initiation factor release precedes or follows this step. It is generally agreed, however, that functional 80S initiation complex formation is mediated by eIF-5.

The initiation factor eIF-5 is a large single polypeptide chain of 125,000 daltons (see Table 1). The factors which appear to be similar or identical are IF-M2A,[82] IF-L2,[55] IF-E5,[46] IF-3,[32] IF-II,[86] F-0.25,[87] and IF-S2.[88]

Immediately before joining of the 60S ribosomal subunit, the $40S_N$ preinitiation complex has been demonstrated to contain equimolar amounts of the following components: 40S ribosomal subunits, eIF-2, eIF-3, Met-tRNA$_f$, GTP, and mRNA. Other components required or facilitating formation of this complex but which are not yet shown to be bound in a stable manner are ATP, eIF-4A, eIF-4B, and eIF-1. Although hydrolysis of ATP is apparently required for functional MRNA binding,[63] the GTP originally bound as part of the eIF-2:Met-

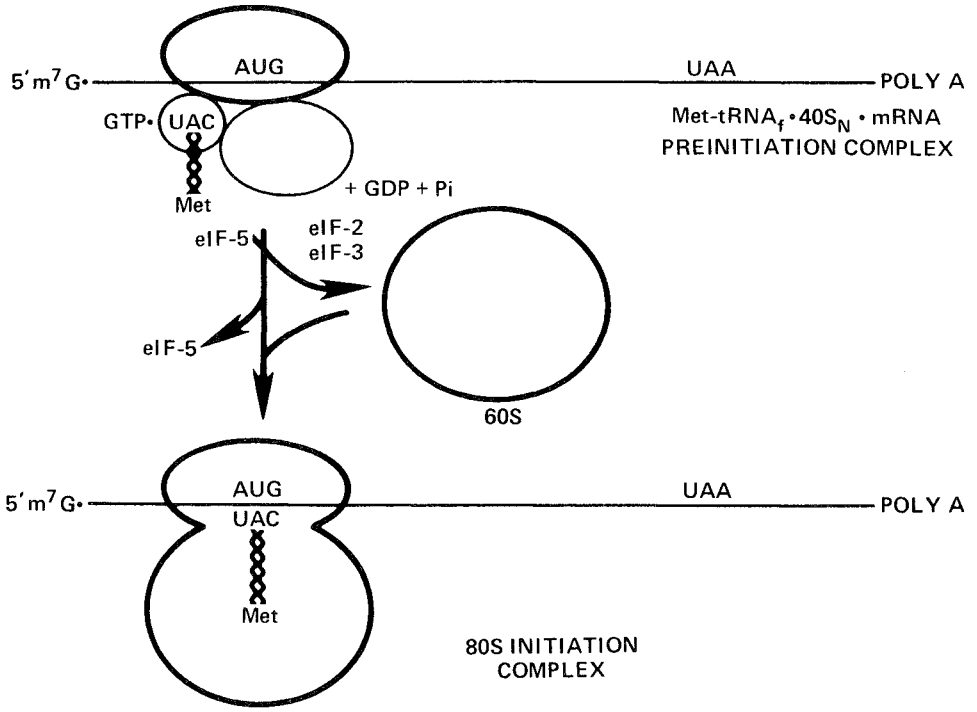

FIGURE 5. 60S ribosomal subunit joining. Joining of the 60S ribosomal subunit to the $40S_N$-mRNA complex is associated with release of bound eIF-2 and eIF-3. eIF-5-dependent GTPase activity appears to mediate factor release prior to 80S initiation complex formation. A stable association of eIF-5 with the Met-tRNA$_f$:$40S_N$:mRNA complex has not been observed. Indirect evidence for an eIF-2 recycling mechanism before reformation of the ternary complex by released eIF-2 has been obtained (see Section V.F.3).

$tRNA_f$ ternary complex has not been utilized yet. This is shown by the ability of nonhydrolyzable analogs of GTP to substitute fully for GTP.[16,50,56,58,83,86,89] It appears that the primary function of bound GTP is to allow an energy-dependent, eIF-5-mediated release of eIF-2 and eIF-3 prior to subunit joining. Substitution of nonhydrolyzable analogs of GTP work equally well to stimulate Met-$tRNA_f$ (and mRNA) binding; however, joining of the 60S ribosomal subunit is inhibited.[16,50,56,58,83,86] In the presence of GTP, prevention of 60S ribosomal subunit joining by the single omission of eIF-5 does not alter the stoichiometry of components bound to the 40S preinitiation complex. In the presence of GTP and eIF-5, when 80S initiation complex formation is prevented by omission of 60S ribosomal subunits, Met-$tRNA_f$, eIF-2, and eIF-3 binding to 40S preinitiation complexes is destabilized. Possibly for this reason, 40S ribosomal subunit binding studies have been more successful when performed with GDPCP or in the absence of eIF-5.[58]

Rather than being a nonspecific disruption of the 40S preinitiation complex, it appears that eIF-5-dependent GTPase activity mediates the release of bound eIF-2, eIF-3, and possibly other factors prior to 60S ribosomal subunit joining.[16,90] Following such factor release, the 40S:Met-$tRNA_f$ complex is left in a metastable state. If 60S ribosomal subunits are available, formation of functional 80S initiation complexes will occur; if for any reason 60S ribosomal joining does not occur, Met-$tRNA_f$ (and mRNA) bound to 40S are destabilized by loss of eIF-2 and eIF-3. It is reasonable to assume that initiation factor release precedes 60S ribosomal joining, since the presence of two factors whose total mass is greater than 50% of the 40S ribosomal subunit may form the basis for their antiassociation activity.

It is experimentally difficult to determine if initiation factor release precedes or is the result of 60S ribosomal subunit joining. However, experiments with the inhibitor pactamycin are informative. At low concentrations, this antibiotic leads to the formation of 48S preinitiation complexes that either remain free or bind to polysomes to form "half-mers," but which cannot bind 60S ribosomal subunits.[87,91] Although not directly examined in this study, observations from several other laboratories would suggest that 48S preinitiation complexes accumulating during pactamycin inhibition contain eIF-2 and eIF-3 utilized during their formation. Since pactamycin does not directly inhibit ribosomal subunit joining, but prevents catalytic activity of eIF-5, inhibition of factor release by pactamycin (or GDPCP) may be the immediate effect which then blocks 60S ribosomal subunit joining.

B. Other Initiation Factors

The specific function(s) of two other initiation factors which stimulate globin synthesis has not been established. eIF-4C is a single 17,000 dalton polypeptide chain (see Table 1), formerly designated IF-M2Bβ[92] and IF-E7.[63] A similar protein eIF-4D, a single 15,000 dalton polypeptide chain, while required for model assay systems, does not stimulate translation of natural mRNAs.[92] Both proteins, however, appear to stabilize all intermediate complexes made during 80S initiation complex formation.

eIF-1, a 15,000 dalton polypeptide, was formerly IF-E1.[56] Although similar in molecular weight to eIF-4C and eIF-4D, it is functionally distinct.[92] It had been reported previously that eIF-1 was primarily involved in stabilization of the 40S:Met-$tRNA_f$:mRNA complex,[56] whereas eIF-4C and D were primarily involved in 60S ribosomal subunit joining. More recently, however, an emphasis has been placed on its role in preinitiation complex stabilization, specifically to ensure that coordinate Met-$tRNA_f$ and mRNA binding occur before 60S subunit joining.[58]

A third initiation factor characterized by model assay systems, but apparently not required for translation of natural mRNAs, is eIF-2A. This factor is active as a single polypeptide chain of 65,000 daltons which promotes the template-dependent, GTP-independent binding of Met-$tRNA_f$ to 40S ribosomal subunits.[93] With artificial polynucleotide templates, both eIF-2A and eIF-2 appear to bind Met-$tRNA_f$ to an identical binding site on the 40S subunit, which subsequently can be made puromycin-reactive. However, a puromycin-sensitive 80S initiation complex containing globin mRNA cannot be formed with eIF-2A,[57] although this factor has been identified in $40S_N$.[30] Similar factors have been isolated and characterized: EIF-1,[94] IF-M1[93] and IF-1.[95,96] Because

of the similarity between mammalian eIF-2A and the equivalent factor in *Artemia salina* and because of the similarity of the *Artemia* factor with E. coli IF-2, it is possible that eIF-2A is the direct evolutionary descendant of prokaryotic IF-2.[94]

C. Supportive Evidence for this Pathway from Unfractionated Systems

If this pathway of 80S initiation complex formation is correct, it should be possible to demonstrate, in an unfractionated system or in vivo, complexes corresponding to the postulated intermediate stages. In addition, tissue levels of such complexes should reflect the rate of protein synthesis and the metabolic state of the cell. The availability of highly purified, active, radiolabeled components, their use as probes in unfractionated systems, and the refinement of analytical techniques such as CsCl density gradient and polyacrylamide gel electrophoresis (PAGE) have allowed direct confirmatory experiments to be carried out. It should be noted, that with respect to RNA and ribosomal components, several of the early pathways proposed without benefit of such techniques were essentially correct.[6,55,66]

In vitro, binding of Met-tRNA$_f$ to the 40S ribosomal subunit by eIF-2 can occur with or without eIF-3.[6,16,39] Several observations suggest, however, that the ternary complex preferentially binds to native 40S subunits (40S$_N$) which contain bound eIF-3. In unfractionated systems, radiolabeled Met-tRNA$_f$ is primarily found in 40S$_N$ of buoyant density 1.40 to 1.42. The density shift from 1.51, characteristic of salt-washed 40S ribosomal subunits, to 1.40 indicates that the amount of nonribosomal protein bound is ca. 900,000 daltons.[22] In view of the presence of near stoichiometric amounts of eIF-3[7,10,17] in the total 40S$_N$ population and the mutual and absolute interdependence of Met-tRNA$_f$ and eIF-2 binding,[16,39,50] the 1.40 to 1.42 g·cm^{-3} 40S$_N$ population probably contains equimolar amounts of eIF-2, eIF-3, Met-tRNA$_f$, and 40S subunits. The formation of this complex from an initial 40S:eIF-3 complex, rather than subsequent binding of eIF-3 to a [40S:eIF-2:Met-tRNA$_f$:GTP] complex, is indicated by failure to observe Met-tRNA$_f$ binding to 40S$_N$ of any other buoyant density (i.e., Met-tRNA$_f$ binding to 1.48 to 1.50 40S$_N$ should be seen, implying an associated nonribosomal protein mass equivalent to eIF-2).[22] Evidence for changes in the amount of Met-tRNA$_f$ bound to 1.40 g·cm^{-3} 40S$_N$ in parallel with changes in the rate of protein synthesis produced by glucose or amino acid starvation implies a possible physiological regulation at this step.[25] As expected, the extent of the initial, energy-independent binding of eIF-3 to 1.51 g·cm^{-3} 40S subunits is not greatly affected.

Although the 40S preinitiation complex containing both Met-tRNA$_f$ and mRNA can be readily formed in vitro, it is extremely difficult to detect in vivo (or in vitro) when excess 60S ribosomal subunits allow 80S initiation complex formation. This suggests that mRNA binding to 40S preinitiation complexes probably constitutes the primary rate-limiting step of protein synthesis under normal conditions. As expected, then, inhibition of protein synthesis after this step has allowed demonstration of a [40S:Met-tRNA$_f$:mRNA:eIF-2, -3, -4A, 4B] complex in unfractionated systems.[97a] Such complexes were initially suggested by the appearance of "half-mers" in polyribosomal populations analyzed by sucrose density gradient analysis following fluoride[98] or pactamycin inhibition.[87,91] Direct evidence was subsequently obtained in the unfractionated lysate by using [^{125}I]-labeled globin mRNA[99] in the sparsomycin shift assay.[66] Although 40S complexes containing both Met-tRNA$_f$ and mRNA ± GTP, eIF-2, and eIF-3 can be demonstrated in highly fractionated systems,[9,58,90] the initiation factor composition of [40S:Met-tRNA$_f$:mRNA] complexes in unfractionated systems in the absence of inhibitors is not known. It is not possible, therefore, to know if eIF-5-mediated initiation factor release[90] occurs prior to 60S ribosomal joining in vivo. However, edeine, a drug which prevents 60S ribosomal joining in vivo and in vitro,[10] does not inhibit initiation factor release in a highly fractionated system.[90,100] A possible physiological regulation of protein synthesis at this step is suggested by the detection of presumptive [40S:Met-tRNA$_f$:eIF-2:eIF-3:GTP:mRNA] complexes upon refeeding of lysine-deprived cells.[25]

D. The Possible Relationship of Initiation Factors and mRNP Proteins

It has been postulated that association of mRNA with nonribosomal proteins, some of which may be initiation factors, prior to initia-

tion complex formation may affect control of protein synthesis at the translational level.[102-104] Globin mRNA exists in two distinct forms of ribonucleoprotein particles (mRNP) in the reticulocyte. Globin mRNP isolated from polysomes has a sedimentation value of 15S and, similar to mRNA isolated from a wide variety of tissues, contains major proteins with respective molecular weights of ca. 52,000, 78,000, and 130,000.[105-112] In contrast, globin mRNP existing as free cytoplasmic particles has a sedimentation value of 20S and is associated with major proteins of 51,000 and several in the range 15,000 to 24,000 mol wt.[108,113] Binding of these proteins appears to be highly specific and stable since such mRNA-protein complexes will survive high salt-washing as well as Cs_2SO_4 density gradient analysis without prior fixation.[114] Since both types of particles contain translatable 9S globin mRNA as their major RNA species, one possible interpretation is that 20S mRNP-associated proteins regulate and are exchanged during entry of globin mRNA into the translation mechanism.[108,113] In contrast, proteins associated with globin mRNA in polysomes (15S mRNP) appear to have neither an inhibitory nor stimulatory effect on translation.[63,115-117] A similar concept involving oligonucleotide RNA sequences has been postulated.[118,118a] So-called translational control RNA (tcRNA), which is thought to be produced during mRNA processing, may exert negative control by binding to both the 3′ and 5′ ends of its mRNA of origin. On the other hand, tcRNA associated with polysomal mRNP might be activated through partial hydrolysis of the inhibitor form which would open the free, inactive, circular mRNP-tcRNA complex.[118]

A possible identity of certain initiation factors with 15S RNP proteins has been postulated. The molecular weights of three initiation factors/subunits are very close or identical to mRNP proteins: eIF-5 = 125,000, eIF-4B = 80,000, and a subunit of eIF-2 = 52,000. Coelectrophoresis of the 52,000 dalton eIF-2 subunit and mRNP also suggest a physical, if not functional, identity. All three of these initiation factors bind tightly to mRNA and synthetic polyribonucleotides.[109,119,120] However, it has not been possible to demonstrate any alteration of initiation factor dependence in a highly fractionated reticulocyte system using mRNP in place of naked mRNA. It is possible, however, that functional properties of the bound initiation factors could be altered. In view of these conflicting results, peptide mapping of the mRNP and initiation factor polypeptide chains following denaturation may reveal unambiguous relationships.[121] A relationship of the 78,000 dalton mRNP protein with eIF-4B appears unlikely, since binding of this mRNP protein to the poly(A) tail of mRNA appears to be related to mRNA transport from the nucleus to the cytoplasm.[122]

Most viral and eukaryotic mRNAs have a 5′ $m^7G(5')pppN$ "cap" which is essential for binding and translation.[123-125] Cap analogs inhibit translation of these mRNAs by competing with the message for ribosomal binding.[72,126-129] Recently, a cap-binding protein has been identified which may promote binding of eukaryotic mRNA to 40S ribosomal subunits.[130] The relationship of this protein to other known translational components is unknown.

III. ELONGATION

Two factors are required for polypeptide chain elongation in eukaryotic cells (see Figure 6). Elongation factor 1 (EF-1) binds noninitiator aminoacyl tRNA to the A site of 80S ribosomal couples. Elongation factor 2 (EF-2) is required for translocation of peptidyl tRNA and the associated mRNA codon from the ribosomal A site to the P site and for the release of deacylated tRNA from the A site. As a result of one round of activity of these factors, the nascent polypeptide chain is extended by one amino acid, and the ribosomal A site is able to bind the next aminoacyl tRNA specified by the adjacent triplet sequence.

EF-1 has been isolated from a variety of eukaryotic cells.[131-137] The monomeric active species appears to be a single polypeptide chain of 47,000 daltons, which has been called EF-1α [135-138] or EF-1_L.[139] Much confusion in the literature has resulted from the tendency of this factor to aggregate both with itself and with other polypeptide complexes that may mediate EF-1 recycling.

A model which has recently been proposed for the mechanism of EF-1_L is shown in Figure 6.[139] In this scheme, EF-1_L enters the ribosomal A site upon completion of the initiation com-

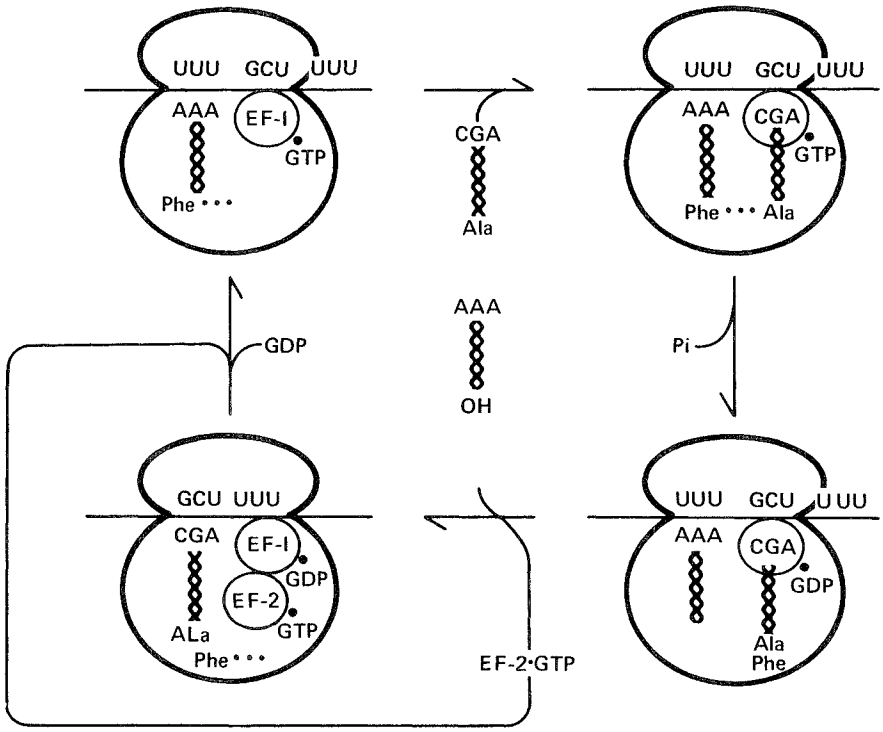

FIGURE 6. Polypeptide elongation. Following initiation factor release and 80S initiation complex formation, EF-1 binds to the 80S ribisomal couple and in the presence of GTP binds the aminoacyl-tRNA specified by the triplet codon of mRNA. Upon hydrolysis of GTP, transpeptidation occurs, and the growing nascent chain is elongated by one amino acid. Binding of a stable EF-2:GTP complex mediates release of the uncharged tRNA from the P site, translocation of the peptidyl tRNA from the A site to the P site, and exchange of EF-1-bound GDP with GTP to complete the elongation cycle.

In this scheme, EF-1 remains associated with the 80S ribosomal couple until termination.[139] In an alternate mechanism, binding of an EF-1:aminoacyl-tRNA:GTP ternary complex, release, and reactivation analogous to the prokaryotic EF-Tu-Ts cycle[140] is thought to occur. See text for details.

plex where it remains bound until termination of polypeptide chain elongation. In the presence of GTP, ribosomes carrying EF-1 can bind aminoacyl-tRNA as specified by the triplet codon of mRNA. Upon hydrolysis of GTP, bound aminoacyl-tRNA is modified to permit transpeptidation and translocation. GDP is then released through subsequent interaction with EF-2. In this scheme, therefore, the free [EF-1α:aa-tRNA:GDP] ternary complex plays no physiological role, and during the process of elongation, EF-1_L functionally becomes a ribosomal protein. Direct support for a stable association of EF-1_L with actively translating polysomes has recently been reported.[145-147] It is not known at the present time if other factors participate in this mechanism.

While several laboratories have purified high-molecular-weight forms of EF-1 corresponding to aggregates of the light species,[131,132,134] others have presented evidence that EF-1α is associated with a complementary factor designated EF-1β.[135-138,140] This factor is composed of two polypeptide chains, of 55,000 and 30,000 daltons, respectively. EF-1α, analogous to EF-Tu, has been reported to form a stable binary and ternary complexes with aminoacyl-tRNA ± GTP;[141] EF-1β may be analogous to EF-Ts in that it appears to be required for regeneration of EF-1α·GDP, which is released from the ribosome following aa-tRNA binding.[137,140,142] In this model, therefore, EF-1α binds aa-tRNA to the 80S A site in a template-specific manner as part of a stable ternary complex EF-2:aa-tRNA:GTP. Upon hydrolysis of GTP, EF-1α release is promoted as an EF-

1α·GDP binary complex by EF-1β.[140] In contrast to prokaryotic EF-Tu, which has an affinity for GDP 100 times greater than GTP, EF-1α appears to have a greater affinity for GTP than GDP.[141,144] The factor EF-1β, however, appears to facilitate the exchange of bound GDP for GTP. The EF-1α·GTP complex can then bind aa-tRNA to participate in subsequent rounds of polypeptide chain elongation. Since the subunit composition of the EF-1α, β complex, and eIF-2 are similar and their functions analogous to each other, it is possible, if not probable, that a similar mechanism for recycling eIF-2 might be found.

In both of the above elongation mechanisms, translocation of peptidyl tRNA and mRNA from the ribosomal A to the P site is mediated by EF-2. This process is accompanied by release of deacylated tRNA from the P site as well as GTP hydrolysis.[148,149] The molecular weight of EF-2 is ca. 100,000,[150-152] and it has 80S ribosome-dependent GTPase activity.[151,153,154] A highly specific ADP ribosylation of EF-2 catalyzed by diphtheria toxin inhibits both GTP hydrolysis and translocation activity.[151-155]

EF-2 can form a stable binary complex with GTP, which can subsequently bind to 80S ribosomes with hydrolysis of GTP; hydrolysis is not required for EF-2 binding,[151,153,154] but is a prerequisite for EF-2 release.[155a] In contrast to previous results, radiolabeled preparations of EF-1 and EF-2 have been shown not to interfere mutually with binding of the other;[146,147] previous interference reported was found to result from competition between aminoacyl tRNA and EF-2 for binding at the A site. While EF-2 permits catalytic utilization of EF-1, radiolabeled EF-1 does not freely exchange with cold, unbound EF-1 during resumption of elongation following harvest of EF-1 polysome complexes. Many theories have been proposed to explain EF-2-mediated ribosomal translocation along mRNA;[156-159] however, definitive proof is still lacking.

IV. TERMINATION

The termination of polypeptide synthesis is considered to occur as two distinct processes: terminator codon recognition and hydrolysis of peptidyl tRNA on the P site of the ribosome. In eukaryotes, the terminator codon UAA has been identified in normal as well as mutant hemoglobins,[160-163] while yeast suppressor tRNAs which recognize UAA and UAG have been purified and shown to suppress premature chain termination in vitro.[164,165] Recognition of these terminator codons requires a protein release factor (RF) which has recently been purified to greater than 75% homogeneity.[166] Reticulocyte RF has a subunit molecular weight of 56,000 and a native molecular weight of 105,000. RF activity, detected by release of bound f[^{3}H]Met-tRNA$_f$ from reticulocyte ribosomes, requires the presence of terminator oligonucleotide and GTP. Purified RF can form stable [RF:f[^{3}H]Met-tRNA$_m$:UAAA:ribosome] complexes which bind to Millipore filters.[167] The mechanism by which RF participates in terminator codon recognition is unclear. While RF may interact with the terminator oligonucleotide independent of ribosomes, interaction with the 3′ end of 18S ribosomal RNA may also be involved.[166,168] An additional stimulatory factor for reticulocyte RF which is neither EF-1 nor EF-2 has also been reported.[166]

The following model has been proposed:[167] Following the addition of the carboxy terminal aminoacyl tRNA, translocation from the A site to the P site results in the alignment of the terminator codon in the A site. In the absence of an aminoacyl tRNA having a complementary recognition codon, RF and GTP bind to the terminator codon-A site region. RF and peptidyl transferase then interact to hydrolyze the ester bond of peptidyl tRNA, resulting in release of the completed protein chain. The concomitant hydrolysis of GTP then leads to dissociation of release factor from the ribosome. Detailed studies of each of these steps, however, still remain to be done.

V. REGULATION OF GLOBIN mRNA TRANSLATION BY HEMIN

A. Introduction

Protein synthesis in the intact reticulocyte is tightly coupled to the availability of hemin. Under conditions of hemin or iron deficiency, polyribosomes disaggregate to 80S ribosomal couples with the release of completed globin chains. Addition of hemin leads to the rapid restoration of translation to normal levels. This suggests that hemin is able to modulate the rate

of protein synthesis at the translational level and that such regulation occurs during initiation complex formation rather than elongation or termination.[42,169-172]

B. Kinetics of Hemin-deficiency Translational Inhibition

Similar observations to those obtained in the intact reticulocyte have been made in the cell free reticulocyte lysate. The kinetics of globin synthesis in the lysate are summarized in Figure 7.[173-176] In the presence or absence of hemin, maximal rates of globin synthesis are maintained for 4 to 6 min. In the presence of hemin, a linear role of translation continues for up to 120 min. In the absence of hemin, after 4 to 6 min, protein synthesis abruptly slows by 90 to 95%. The onset of translational inhibition, ac-

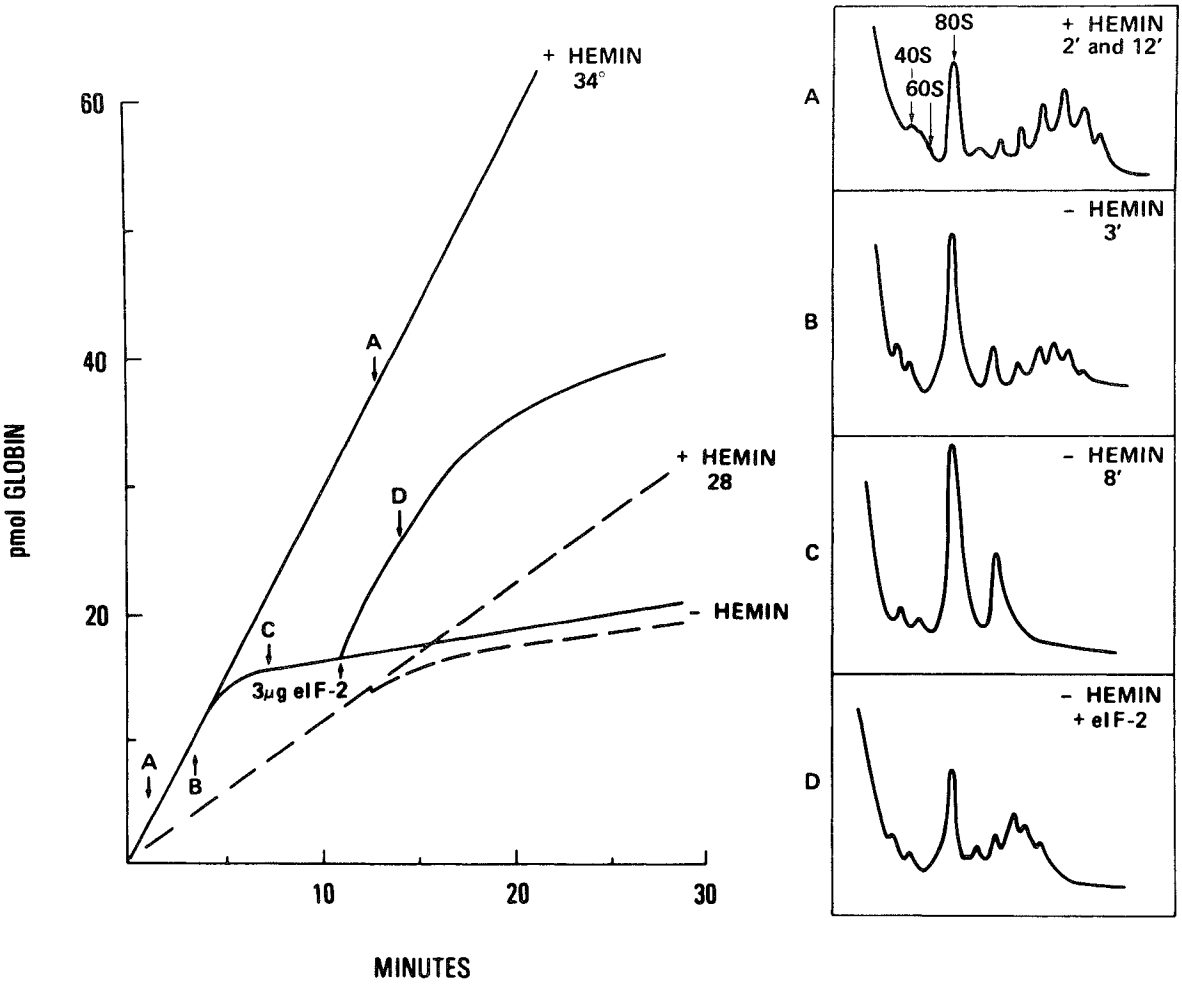

FIGURE 7. The kinetics of hemin-deficiency translational inhibition in rabbit reticulocyte lysate. In the presence of hemin, globin synthesis at 34° remains linear for up to 2 hr, and sucrose density gradient analysis of incubated lysates sampled at A shows a normal distribution of 40S and 60S ribosomal subunits, 80S monosomes, and polysomes. By 3 min of incubation in the absence of hemin (B), sucrose density gradient analysis shows partial disaggregation of the heavy polysomes and an increase in the number of inactive monosomes. In the absence of hemin, translation is severely impaired after 5 min of apparently normal protein synthesis. After 8 min, no polysomes can be seen (C). Addition of eIF-2 at 10 min, however, results in an immediate resumption of control rates of protein synthesis and, as shown by lysate sampled at D, a relatively normal ribosomal profile. Nonphosphorylated or prephosphorylated (2 pmol phosphate per pmol eIF-2) preparations give identical results. Note that recovery of globin synthesis is transient and stoichiometric with the amount of eIF-2 used (ca. 24 pmol). In lysate incubated at 28°, a slower but longer period of synthesis at control rates is seen in the absence of hemin. Note that the extent of globin synthesis in the absence of hemin is the same as that obtained at 34°.

companied by disaggregation of polysomes (shown in Figure 7C), is immediately preceded by a depletion of initiator Met-tRNA$_f$ in native 40S ribosomal subunits.[42,177] This inhibition appears to be caused by a high-molecular- weight protein inhibitor, formed in the lysate during incubation in the absence of hemin.[174,177,181] Addition of the purified inhibitor to lysate, even in the presence of hemin, yields similar kinetics of inhibition as obtained with endogenously generated inhibitor.[43,178] Addition of large amounts of purified inhibitor does not reduce the time required for shutoff of protein synthesis below a minimum value of 1.5 to 2 min.[174] Since the transit time required for completion of a globin chain in this system has been estimated to be 0.5 to 0.75 min,[173,179] several rounds of initiation occur during this period. Both elongation and termination appear to be unaffected. The time required for shutoff of protein synthesis is inversely proportional to the rate of translation before shutoff, and the extent of protein synthesis before the onset of inhibition appears to be constant over a wide range.[175] These observations have been interpreted to reflect the time required for expenditure of a fixed pool of a factor required for active translation, a factor not directly inactivated by the inhibitor, but whose reutilization is prevented.[42,175,179] This factor is thought to be eIF-2 (see Section V.F.3). Various mechanisms which have been proposed to explain aspects of hemin-deficiency translational control are summarized in Figure 8. These will be discussed below in Section V.E,F, and H.

C. Three Stages of Inhibitor Formation

The hemin-regulated translational inhibitor (HRI), which has also been called hemin-controlled repressor (HCR), appears to be formed in three stages by prolonged incubation of reticulocyte postribosomal supernatant at 34°.[170,180-183] These can be distinguished by the effects of hemin on the kinetics of inhibition obtained in reticulocyte lysates incubated at various temperatures. Incubation of the inactive proinhibitor at 34° for 10 min in the absence of hemin results in the formation of an active inhibitor which can be rapidly inactivated by hemin. Upon continued incubation, an intermediate form of inhibitor can be detected which cannot be directly inactivated by hemin, but whose inhibitory effects can be reversed under conditions of protein synthesis in the presence of hemin. Prolonged incubation of lysate in the absence of hemin forms an irreversible translational inhibitor which cannot be inactivated by hemin or translation under any conditions. A scheme summarizing the effects of various agents on this conversion is shown in Table 2. It should be stressed at this time that the molecular basis for the conversion of inactive proinhibitor to these inhibitory forms is not known.

Although evidence is available for the existence of distinct forms of HRI, it is possible to interpret the data in terms of a combination of a) proinhibitor activation and b) inactivation of a component capable of converting activated forms of the inhibitor to the inactive proinhibitor state. For example, the inability of hemin to inactivate the intermediate form of the inhibitor (in contrast to the reversible form) except in the presence of other components of the translational apparatus may result from inactivation of a reversal factor in the postribosomal supernatant present in the actively translating lysate.

D. Purification and Characterization of HRI

Irreversible HRI has been purified ca. 4800-fold and appears to be identical to the cAMP-independent, ATP-specific protein kinase which phosphorylates the 35,000 dalton subunit of eIF-2[42,43] (see Section V.G). One to two pmol phosphate per pmol eIF-2[16,40] are incorporated; other substrates are unknown.[40,43,184-187] By gel filtration, the molecular weight has been estimated to be 140,000 or 300,000. A sedimentation value of 6S has been obtained. The subunit structure, if any, is unknown. On isoelectric focusing, HRI activity is found in two fractions with pI values of 5.56 and 6.36. The amount of purified inhibitor required to decrease protein synthesis in 25 μl of control lysate to the level obtained in the absence of hemin is 0.2×10^{-7} g. Based on a content of 200 pmol eIF-2 or ribosomes per ml of lysate, each molecule of inhibitor is capable of inactivating up to 1000 ribosomes or molecules of eIF-2.[42,43]

Although the exact relationships are unknown, translational inhibitors similar in physiological effect or identical in mechanism to HRI have also been isolated. In this regard, it has been suggested that activation of such inhibitors may be the final pathway for transla-

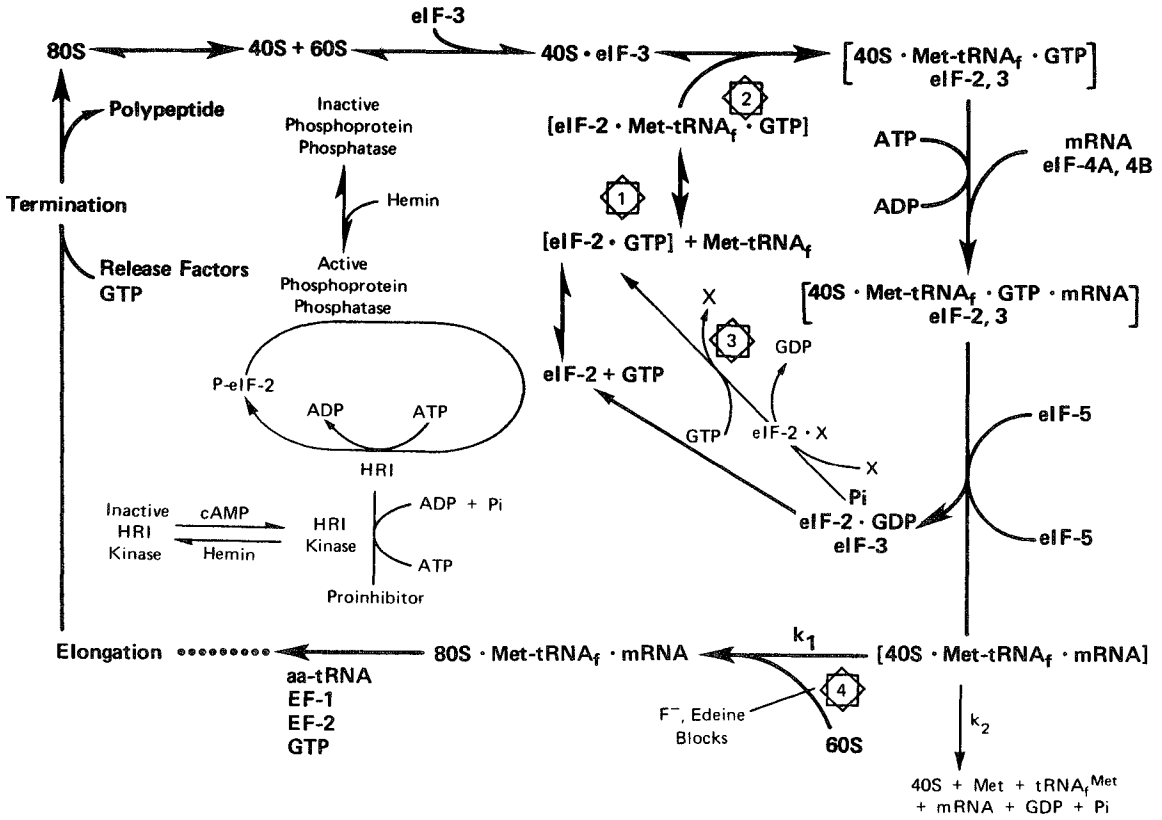

FIGURE 8. Summary of the various mechanisms proposed for hemin regulation of hemoglobin synthesis. The initiation, elongation, and termination pathways are shown in bold type. Hypothetical sites of inhibition resulting from hemin deficiency are indicated by □. In the mechanisms responsible for □ and □ hemin deficiency results in an increase in the proportion of eIF-2 in the phosphorylated state. This can occur either through a relative inactivation of phosphoprotein phosphatase or activation of HRI by a cyclic AMP-dependent HRI kinase. Ternary complex formation or binding of the ternary complex to $40S_N$ is then directly inhibited by phosphorylated eIF-2. In mechanism □ phosphorylated eIF-2 can be used normally for one round of initiation, but reactivation through an as yet unidentified pathway is inhibited, either directly or as a result of eIF-2 phosphorylation. In □, 80S initiation complex formation is inhibited at the site of 60S ribosomal subunit joining. All previous steps in the initiation sequence are normal. Although $k_1 >> k_2$ normally allows 80S initiation complex formation, Met-tRNA$_f$ in the metastable [40S:Met-tRNA$_f$:mRNA] complex is subject to deacylation by an AUG- and 40S-dependent Met-tRNA$_f$ hydrolase. Such a mechanism of inhibition would be very similar to that seen during fluoride inhibition of protein synthesis. Edeine, which blocks mRNA binding (and therefore the AUG initiation codon), would prevent deacylation of the $40S_N$-bound Met-tRNA$_f$ during inhibition of protein synthesis.

tional inhibition produced by double-stranded RNA[42,188,189] and oxidized glutathione.[189-191] Similar translational inhibitors have been found and/or partially purified in cells other than the reticulocyte: Ehrlich ascites tumor cells,[192-194] HeLa cells,[195] mature erythrocytes,[196] liver,[197] and Friend erythroleukemia cells.[198]

E. Specific Modifications Resulting in the Activation of Proinhibitor

It has recently been proposed that activation of proinhibitor is mediated by a cAMP-dependent protein kinase in analogy to a similar mechanism modulating the activity of phosphorylase kinase (see Figure 8).[199,199a] However, the activity of protein synthesis in reticulocyte lysate was not inhibited by cAMP unless exogenous cAMP beef heart protein kinase or its catalytic subunit was added. Also, experiments actually showing that such inhibition was mediated through activation of proinhibitor were not done. It has not been possible so far to determine the specific type of modification involved during activation of the proinhibitor. One reason for this has been the extremely low amounts of inhibitor, in any form, that appears to be present in the reticulocyte lysate. Although the

TABLE 2

Stages During Formation of the Hemin-regulated Translational Inhibitor

	↑temp cAMP GTP	block	
	Proinhibitor ⇄ Reversible HRI ⇄ Intermediate HRI → Irreversible HRI		
—	Formed within 10 min at 34°	Detected by 45 min at 34°	Not formed until after 150 min at 34°
—	Hemin prevents formation; hemin and/or translation inactivates	Hemin has no effect, but can be inactivated during translation	Hemin and translation have no effect
Temperature of lysate incubation	Effect of HRI addition		
25°	No inhibition	Transient inhibition	Progressive inhibition
27°	Transient inhibition ± hemin	Transient inhibition ± hemin	Progressive inhibition
30°	—	—	Immediate inhibition

irreversible form has recently been purified to a high degree in several laboratories, it is extremely difficult to isolate highly purified proinhibitor, since activation occurs readily if it is exposed to $(NH_4)_2SO_4$ or oxidized partners of reducing couples.[200] Evidence suggesting that HRI is a specific protein kinase which phosphorylates eIF-2 will be presented later[42,43,201,202] (see Section V.G). While the mechanism of hemin control of HRI activity is unknown, stabilization of the postulated regulatory subunit: catalytic subunit complex, direct allosteric control of catalytic subunit activity, and modulation of HRI phosphorylation and dephosphorylation by hemin have all been proposed.

Mixing experiments have shown that while incubation of the postribosomal supernatant at 55° prevents activation of proinhibitor, this is not the result of direct inactivation of proinhibitor, since addition of unincubated supernatant generates the amount of inhibitor expected from the sum of control and heat-inactivated components.[174] Therefore, it is possible that hemin regulation of translation may involve a cascade system for activation of proinhibitor.

F. Reversal of HRI-mediated Inhibition

1. Hemin

The effect of hemin addition to lysate inhibited by endogenously generated, reversible HRI at different times following the start of incubation has been studied.[172,181,203] Although hemin is able to reverse translational inhibition, its effectiveness is diminished with increasing incubation time in the absence of hemin. While this observation could be correlated with an increasing extent of conversion of the hemin-reversible form of HRI to the hemin-irreversible intermediate form (see Figure 7), an additional set of experiments complicate this interpretation. In the absence of hemin, protein synthesis at 25° does not become inhibited for up to 2 hr.[180] Lysate previously inhibited at 34° resumed translation immediately after cooling to 24°. A satisfactory explanation for this experiment is not immediately obvious. A rapid conversion of active inhibitor to inactive proinhibitor should still require a lag period before the control rate of protein synthesis is observed. If inhibition is the result of modification of a rate-limiting pool of translational component (i.e., eIF-2), it should still require time to reactivate this pool. Recently, a direct activation by hemin on the activity of a phosphoprotein phosphatase specific for phosphorylated eIF-2 was observed and it was speculated that this might be the basis for hemin-mediated reversal.[204,205] (However, see Section V.H).

2. Purine Nucleotide Analogs

In 1973, reversal of translational inhibition

of energy-depleted cells by cAMP and purine derivatives was reported.[203] It has subsequently been found that high concentrations of purine derivatives (including cAMP and 2-amino-purine) could also replace hemin.[189,206,207,208] Based on the idea that these compounds acted as analogs of naturally occurring purine nucleotides which participate in the action of HRI, the effects of ATP or GTP were investigated. As previously noted, GTP was found to inhibit formation of the inhibitor directly. Other simple purines, however, blocked expression of inhibitor activity. ATP significantly enhanced inhibition and blocked reversal by cAMP.[208] These observations were the basis of the postulate that the inhibitor functions as a protein kinase. Although the antagonistic effects of GTP and ATP on inhibitor activation was thought to occur by virtue of the inhibitor being a protein kinase containing both catalytic and regulatory subunits,[187a] this is now thought unlikely.[42,43] These observations in the hemin-deficient reticulocyte lysate have been extended to similar inhibitions produced by purified translational inhibitor, low levels of double stranded RNA, and by oxidized glutathione.[42,189,207]

More recently, it has been shown that high concentrations of caffein, theophylin, 2-aminopurine, and cAMP, all of which antagonize the inhibitory activity of HRI, also inhibit phosphorylation of the 35,000 dalton subunit of eIF-2.[42,207,208] This does not prove, however, that the cAMP-independent protein kinase activity associated with HRI is the basis for its inhibitory activity (see Section V.H).

3. eIF-2

As early as 1972, it was observed that inhibition of protein synthesis by crude preparations of HRI (Q fraction) could be reversed by the addition of 0.5 *M* KCl wash proteins (I fraction) of reticulocyte polysomes.[209,210] Partially purified fractions of initiation factors could also reverse inhibition by HRI.[11,13,211] An example is the crude factor M3,[11] which upon further fractionation resulted in the purification of four distinct initiation factors: eIF-2 (IF-MP), eIF-3 (IF-M5), eIF-4A (IF-M4), and eIF-4B (IF-M3).[8,35,69] It was then demonstrated that eIF-2 was the factor responsible for reversal of protein synthesis produced by double-stranded RNA, oxidized glutathione, or the absence of hemin.[119,191,202,212] This work has since been confirmed and extended by several other laboratories.[42,214] Recently, two additional fractions capable of reversing hemin-deficiency translational inhibition have been reported.[215-217]

G. Does Phosphorylation of eIF-2 Mediate Hemin-regulated Translational Control?

Several observations have suggested the hypothesis that phosphorylation of eIF-2 is the mechanism by which the hemin-controlled repressor exerts translational control: 1. An eIF-2-specific protein kinase activity co-purifies with HRI.[42,43,185] 2. Hemin-deficiency translational inhibition can be reversed by eIF-2[16,42,212,214] (see Figure 7). 3. Other inhibitors of translation in the reticulocyte lysate, such as double-stranded RNA and oxidized glutathione, appear to act through phosphorylation of eIF-2.[42] 4. The extent of translational inhibition appears to correlate with an increase in eIF-2-specific kinase activity and phosphorylation of ribosomal bound eIF-2.[42,43,185,216a] 5. Compounds such as caffein, theophylin, 2-aminopurine, and 3′-5′ cyclic AMP, which antagonize the inhibitory activity of HRI, also inhibit phosphorylation of eIF-2.[16,42] 6. The time required for shutoff of protein synthesis in the absence of hemin is reported to reflect endogenous levels of eIF-2 in the reticulocyte lysate.[179] While it is tempting to accept phosphorylation of eIF-2 as the basis for hemin-deficient translational inhibition, several observations caution against this at the present time. In the HRI-inhibited reticulocyte lysate, addition of phosphorylated eIF-2 (prephosphorylated by the identical kinase preparation used to produce translational inhibition) transiently restores control rates of protein synthesis to the same extent as nonphosphorylated eIF-2.[16] None of the partial reactions of eIF-2 in highly fractionated translation systems have been convincingly shown to be affected by *de novo* phosphorylation.[16,42,44,58,218,218a] Ternary complex formation is not affected by phosphorylation of homogeneous eIF-2; when such inhibition has been reported, either nonmammalian or partially purified factor preparations[199] have been used. It is possible that other proteins or factors may affect the formation or stability of the [eIF-2:Met-tRNA$_f$:GTP] ternary complex, but no definite conclusions can be drawn at this time.[47] Similarly, binding of the ternary com-

plex to native 40S ribosomal subunits is also unaffected by the phosphorylation state of eIF-2. Again, inhibition has only been seen in the unfractionated lysate or in the presence of other crude fractions of the ribosomal salt-wash proteins.[184,199,212,219] Release of eIF-2 following transfer of Met-tRNA$_f$ to the 80S initiation complex also is identical, whether phosphorylated or nonphosphorylated preparations of eIF-2 are used;[16] in addition, the phosphorylation state of eIF-2 in reticulocyte lysate does not have an exact relationship to the onset, the extent, and timing of translational inhibition.[219a]

Such observations have led to the hypothesis that phosphorylation does not directly inactivate eIF-2, but rather interferes with its catalytic recycling during initiation complex formation.[16,42,179] This is supported by experiments showing a 1:1 stoichiometric relationship between the amount of globin synthesized and the amount of eIF-2 utilized to reverse transiently an HRI-inhibited lysate.[16] The amount of globin synthesized before shutoff of translation in the absence of hemin also appears to correlate with the estimated amount of eIF-2 present in reticulocyte lysate.[179] It is extremely important, therefore, to know if eIF-2 recovered from inhibited reticulocyte lysates is functionally and/or physically modified from standard preparations.[202] The relative importance of eIF-2 phosphorylation to the mechanism of hemin-deficiency translational inhibition cannot be established until normal mechanisms involved in its recycling have been defined.

H. Alternatives to Direct Inhibition of eIF-2 Utilization by HRI

In the absence of hemin,Met-tRNA$_f$ bound to 40S ribosomal subunits declines from the 2nd to the 5th min of incubation; at this time, disaggregation of polysomes is complete, and inhibition of protein synthesis is maximal (see Figure 7). Since the level of Met-tRNA$_f$ in the lysate remains unaltered, binding of Met-tRNA$_f$ to the 40S subunit was thought to be inhibited.[177] Subsequent studies of the effects of various antibiotics which inhibit [Met-tRNA$_f$:40S] complex formation suggested, however, that inactivation of eIF-2 or mechanisms which allow eIF-2 recycling, may not be the pathway by which inhibition is initially produced.[201,202] The proposed sites of HRI-mediated inhibition and the locus of action of various agents affecting HRI activity are shown in Figure 8. Addition of cycloheximide to lysates inhibited by exogenous HRI or hemin deficiency results in an accumulation of Met-tRNA$_f$ bound to 40S$_N$. Increased binding is probably the result of an inhibition of [40S:Met-tRNA$_f$] complex utilization coupled with a slow rate of complex formation; however, it is important to realize that only steady-state levels of this complex rather than rate of formation have been measured. Whereas the addition of the initiation codon AUG or mRNA promotes an almost quantitative shift of bound Met-tRNA$_f$ from the 40S to the 80S region in control lysates,[66] in inhibited lysate (added HRI or hemin-deficient), only deacylation of Met-tRNA$_f$ bound to 40S$_N$ is seen. Deacylation appears to be dependent on the presence of both Met-tRNA$_f$ *and* AUG on the 40S preinitiation complex. Edeine, an inhibitor of 60S ribosomal subunit subjoining to the 40S preinitiation complex,[101] blocks the AUG-dependent shift reaction, but in addition, Met-tRNA$_f$ deacylation is prevented.[201]

Recent experiments from this laboratory have shown that interruption of 80S initiation complex formation at the level of mRNA binding or 60S joining results in the destabilization of the [40S:Met-tRNA$_f$:GTP:eIF-2:eIF-3] preinitiation complex. This effect appears to be mediated by the factor, eIF-5, required at this stage.[90] Based on this observation, it is important to reevaluate the mechanism of hemin-deficiency inhibition of protein synthesis proposed several years ago.[169,201,202] This hypothesis postulated that the observed inhibition of Met-tRNA$_f$ binding to native 40S ribosomal subunits and the accompanying disaggregation of polysomes did not result from any direct inactivation of the factor(s) responsible for Met-tRNA$_f$ binding, but occurred secondary to deacylation of the Met-tRNA$_f$ when joining of ribosomal subunits was blocked by HRI. The presence of a Met-tRNA$_f$ deacylase bound to native 40S ribosomal subunits has been reported by several laboratories.[95,220-223] Met-tRNA$_f$ present in the ternary complex [eIF-2Met-tRNA$_f$:GTP], in 40S$_N$ or 80S ribosomes is protected against deacylation.[220-223] Met-tRNA$_f$ bound to 40S by eIF-2A is readily hydrolyzed.[95] Activity of the deacylase is dependent on the

presence of the AUG initiation codon,[95,220,222] but appears to be inhibited by GTP.[223]

It has been proposed that Met-tRNA$_f$ deacylase may be responsible for the accumulation of ribosomal-bound deacylated tRNA$_f^{Met}$ seen during sodium fluoride inhibition of protein synthesis.[99,224-226] Such inhibition leads to the formation of polysomes with an unpaired 40S ribosomal subunit at the initiation site[98] or to 80S ribosomes containing mRNA.[227] The point at which fluoride inhibits initiation seems to be at the joining of 60S ribosomal subunits, since reincubation of fluoride-inhibited polyribosomes in uninhibited lysate results in completion of nascent polypeptide chains. This is completely insensitive to edeine, an inhibitor of 60S joining to the 40S preinitiation complex and to aurintricarboxylic acid, which inhibits eIF-2-dependent Met-tRNA$_f$ binding to 40S ribosomal subunits[224] and, as a direct result, subsequent mRNA binding.[228] Although the extent of deacylation of Met-tRNA$_f$ in native 40S not bound to mRNA has not been examined, it appears that Met-tRNA$_f$ bound to mRNA as a "half-mer" during fluoride inhibition is deacylated. In addition, during fluoride inhibition in the intact reticulocyte, total cellular mRNA activity is quantitatively associated with monoribosomes.[227]

A likely interpretation of these observations is that fluoride inhibits joining of the 60S ribosomal subunit to the [40S:Met-tRNA$_f$:mRNA] preinitiation complex, which then allows hydrolysis of bound Met-tRNA$_f$. It is important to know if such deacylation occurs in the presence of bound eIF-2 and eIF-3 (which appear to be released following mRNA binding, but preceding 60S ribosomal subunit joining). It is possible that the presence of these factors inhibits deacylase activity. Since mRNA binding is not prevented by nonhydrolyzable analogs of GTP,[58] but factor release and 60S subunit joining are,[16,90] this hypothesis can be directly tested.

A similar mechanism could easily account for many of the observations made on hemin-regulated translational control. Specifically, either an inhibition of initiation factor release prior to 60S ribosomal subunit joining or a direct inhibition of 60S ribosomal subunit joining would result in deacylation of bound Met-tRNA$_f$. This would then be followed by disaggregation of the 40S preinitiation complex. It has been reported that formylation of Met-tRNA$_f$ prevents deacylase activity and partially reverses inhibition in hemin-deficient lysates.[228a] Teleologically, such a mechanism could be useful to the cell to allow reutilization of active components required for initiation complex formation, if for any reason defective 80S initiation complex occurs. Such altered release of initiation factors might not allow normal recycling of factors. This would agree with the stoichiometric utilization of eIF-2 and the absence of initiation factor recycling seen during rescue of the hemin-deficient or HRI-inhibited lysate by added eIF-2.[16,179] It is quite likely, however, that inhibition caused by HRI produces both inhibition of eIF-2 recycling as well as inhibition of 60S ribosomal subunit joining. Although it is difficult to eliminate possible artifacts, rescue of the hemin-deficient lysate by eIF-2 preparations (ranging from crude to homogeneous) does not result in the resumption of control rates of protein synthesis maintained during the entire recovery phase.[16,179,218,229] This would be expected if the eIF-2-dependent binding of Met-tRNA to 40S$_N$ were the only step affected in the absence of hemin.[42,179,230]

At the present time, it is difficult to decide the validity and/or relative importance of both postulated mechanisms to the phenomenon of hemin-mediated translational control. Many basic observations required to do this have not yet been made, or conflicting results have been obtained. A minimal list of necessary information might include:

1. Do the kinetics of eIF-2 phosphorylation agree with the onset of translational inhibition?
2. Does deacylation of bound Met-tRNA$_f$ occur? At what stage during initiation complex formation does it occur?
3. What is the fate and distribution of globin mRNA during hemin-deficiency inhibition?
4. Is eIF-2 in the inhibited hemin-deficient lysate inactive?

VI. CONCLUSIONS

Initiation of protein synthesis in the eukaryotic cell appears to require a minimum of five

distinct initiation factors to achieve the proper temporal sequence for assembly of the 80S initiation complex. The polypeptide complexes eIF-3 and eIF-2 are required for $40S_N$ formation and Met-$tRNA_f$ binding. In addition to eIF-3, two other factors, eIF-4A and eIF-4B, are necessary for mRNA binding. Joining of the 60S ribosomal subunit to the 40S:Met-$tRNA_f$:mRNA complex is mediated by eIF-5. Two other initiation factors, eIF-1 and eIF-4C, also participate in this sequence through regulation and/or stabilitization of intermediary complexes formed during initiation complex formation. Based on in vitro kinetic arguments and the pool sizes of the intermediary complexes in vivo, it would appear that initiation complex formation proceeds primarily along an ordered sequential pathway with respect to both RNA and initiation factor components. Indirect evidence also exists for the action of certain initiation factors as part of large macromolecular complexes ([eIF-3:eIF-4A:eIF-4B]; [eIF-2:mRNA]).

Initiation factor release may occur prior to 60S ribosomal subunit joining. Similar to prokaryotic recycling of EF-Tu, both EF-1 and eIF-2 may undergo modification which permits their catalytic utilization. In the case of eIF-2, interference with recycling may be part of the translational control mechanism by which hemin and globin synthesis are coordinated. The exact sequence of events required to achieve this is unknown, however.

At the present time, it is possible in vitro to radiolabel (and maintain the functional activity of) highly purified components required for protein synthesis. Utilization of these labeled components as probes in fractionated and unfractionated systems to follow initiation under normal and experimentally altered states should lead to a better understanding of the mechanisms that mediate the control of protein synthesis at the translational level.

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