Meeting report

INITIATION OF PROTEIN SYNTHESIS IN PROKARYOTIC AND EUKARYOTIC SYSTEMS

Summary of EMBO Workshop held in Noordwijkerhout, The Netherlands on 17–19 April 1974

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Received 14 August 1974

1. Prokaryotic systems

During the last few years efforts have been directed towards elucidation of the manner in which initiation factors participate in formation of the initiation complex, and of the sequence of events by which the complex is assembled. The difficulties faced by investigators in the field were evidenced by the numerous, often contradictory schemes advanced at the meeting. Some workers believe to have evidence for the existence of unstable intermediate complexes of fMet–tRNA, the 30 S subunit, and initiation factors, in the absence of messenger RNA, suggesting that initiator aminoacyl-tRNA binding may precede messenger binding (Kaempfer, Noll). A thorough analysis of the sequence of events in MS2 RNA-directed initiation led Vermeer and Bosch to accept the model in which mRNA binding precedes initiator tRNA binding. They observed that an *E. coli* 30 S subunit, MS2 RNA, and IF-3, interact stoichiometrically to form an unstable complex which, on subsequent addition of IF-1, IF-2 and fMet–tRNA, is converted to the more stable 30 S initiation complex. In further refinement of the finding (Sabol and Ochoa, Gualerzi et al., Thibault, Vidal and Gros) that 30 S-bound IF-3 is released upon formation of the 70 S initiation complex, the

Dutch workers showed that release of this factor occurs in fact as soon as the 30 S initiation complex (mRNA-30S-fMet-tRNA) is formed. Using a coupled in vitro system, in which λ or φ 80 DNA transcription occurs in the presence of washed ribosomes, with or without the various components of initiation, Crepin, Lelong and Gros (Karolinska Symposium, 1973) obtained similar results. In confirmation of Moldave's initial report they observed that 30 S ribosomes alone stimulated in vitro transcription. This effect was enhanced 4–5-fold by IF-3 but very little by IF-1 plus IF-2. Simultaneous addition of the three factors caused a 6-fold stimulation; optimal coupling was observed when initiator aminoacyl-tRNA was also present. Thus, nascent messenger can readily attach to 30 S ribosomes in an initiator-independent but factor-dependent reaction. However, IF-3 is not indespensable for ribosomal binding of mRNA because *E. coli* 30 S subunits can form an unstable complex (at 0°C) with MS2 RNA, in the absence of IF-3, provided that protein S1 (see below) is present (Szer, reported by Ochoa). On the other hand neither *C. crescentus* nor *B. subtilis* 30 S subunits form a complex with MS2 RNA in the presence or absence of *E. coli* or *C. crescentus* IF-3. Selection of a species-specific messenger is thus performed by the 30 S subunit.

During the last year or so, new approaches have been developed to study ribosomal topography and the protein subunit makeup of ribosomal binding sites. Results obtained by cross-linking neighboring ribosomal proteins or by covalent linking of labeled aminoacyl-tRNAs to ribosomes with bifunctional cross-linking reagents, were among the more important new developments presented at the Noordwijkerhout Workshop. Thus, the ribosomal protein S5 and S8, on the one hand and S13 and S19, on the other, are located in close proximity on the 30 S subunit (Kurland et al., Traut et al.), Phe–tRNA binding involves the 50 S proteins L2, L14, L15, L16, and L27 (Kuechler et al., Cantor et al.), and fMet–tRNA binding involves the 50 S proteins L15 and L27 (Kuechler et al.).

With use of radioactive initiation factors and bifunctional cross-linking reagents Hershey, Bollen and Traut found that the 30 S ribosomal proteins S1, S11, S13 and S19 participate in IF-2 binding, and S1, S11, S12, S13, S14 in IF-3 binding. The strongest cross-linking of IF-2 was with S13 and S19 and of IF-3 with S11 and S12. As shown in Nomura's laboratory, S12 is the ribosomal protein responsible for the above mentioned specific recognition of mRNA by the 30 S subunit. Involvement of S12 in the interaction of IF-3 with the 30 S subunit was also supported by other workers (Wahba et al.). Streptomycin resistant or dependent and ribosomal ambiguity mutants of *E. coli* revealed single amino acid replacements, or more drastic changes, in the 30 S proteins S4, S5 and S12 (Wittmann). Use of antibodies against 30 S ribosomal proteins (Lelong, Gros et al., Stöffler, Maschler et al.) showed that anti-S12 and S13 monovalent antibodies specifically prevent formation of an initiation complex with 30 S ribosomes but have no effect on EF-Tu-dependent Phe-tRNA binding to 70 S ribosomes. Anti-S1 and S19 inhibited initiation strongly but also interfered with binding of aminoacyl–tRNA at the aminoacyl (acceptor) site. This result is in line with Voorma's data indicating that binding sites for IF-2 and EF-Tu may overlap. Neither the cross-linking nor the immunochemical experiments can be taken as proof that IF-3 actually binds to ribosomal proteins. These proteins might act only as mediators in the binding of IF-3 to a specific region of 16 S RNA. However, Sabol et al. (1973) reported very weak binding of IF-3 to 16 S RNA. It may be noted that the ribosomal proteins implicated in initiation factor binding to the 30 S subunit are located near the 3′-end of the ribosomal RNA.

The problem of messenger recognition by the ribosome-initiation factor complex was the subject of stimulating discussions based upon Shine's and Dalgarno's recent model (PNAS, April 1974). Apparently the polypurine sequence (5′)GGAGGU(3′) is present at the same relative position in all the prokaryotic messengers analyzed to date (including the *lac* and *gal* messengers) with respect to the first translatable AUG triplet of each RNA cistron (Joan Steitz). According to Shine and Dalgarno, the 3′-proximal end of 16 S RNA has the sequence GAUC*ACCUCC*UUA (OH) so that the polypurine stretch at ribosome binding sites of mRNA can potentially base pair with the ACCUCC portion of 16 S RNA, a sequence found nowhere else in the molecule. Since the length of these regions is greater for the polypurine stretch of the A protein cistron binding site of coliphage RNAs than for the binding regions adjacent to the coat or replicase cistrons, Shine's and Dalgarno's model

would explain why A protein cistron binding site fragments are preferentially bound to ribosomes relative to fragments from other initiation sites (Steitz).

The question of how ribosomes select messenger initiation signals was the subject of much discussion. Several possibilities, including the role of base pair matching between the mRNA polypurine stretch and ribosomal RNA, the length of the region between this stretch and the AUG codon, and interference factors in the vicinity of the 3′-terminus of 16 S RNA, were considered.

As for the role of IF-3 in messenger recognition several hypotheses were discussed: (a) IF-3 might have an effect on the ribosome binding region of the messenger (Noll), perhaps acting as an unwinding factor to facilitate access of the ribosome to the initiation site. (b) IF-3, which binds to the 30 S subunit, might induce conformational changes for selective binding to the messenger initiation regions; such an effect would be mediated by ribosomal proteins. (c) IF-3 might stabilize interactions between the GGAGGU stretch on the messenger and the complementary region of the 16 S RNA (Steitz). (d) a fourth possibility places emphasis on interference factors as the actual messenger recognition agents during translation (Revel). Bij selective binding to the messenger, these proteins could conceivably prevent IF-3 from interacting with a given messenger or initiation region. Clearly the above models are purely speculative and the actual mode of mRNA selection by IF-3 remains to be elucidated. The fact that translation of chemically unfolded messengers, e.g. formaldehyde-treated, is still stimulated by IF-3 would seem to disprove hypothesis (a), but results obtained with formaldehyde-treated RNAs are difficult to interpret. A requirement for IF-3 in translation of synthetic messengers was reported (Wahba, Grunberg–Manago). However, the requirement of IF-3 for natural messenger translation is much more stringent than for translation of synthetic messengers. Moreover, most of the effect of IF-3 on synthetic messenger translation, when 70 S ribosomes are used, is referable to its DF (ribosome dissociation factor) activity.

The possible role of initiation factors in regulation of translation was extensively discussed. It will be recalled that in *E. coli* Revel's and Ochoa's groups had shown the existence of two subspecies of IF-3, differing slightly in molecular weight, with high selectivity for different messengers. Revel and collaborators discovered factors that specifically inhibit translation of different messengers. As already mentioned, this finding led to the suggestion that these factors (referred to as interference or i factors) may modulate translation by modifying ribosomal recognition of messengers. At present this attractive hypothesis is the matter of some interesting debate. Revel and collaborators isolated from high-salt ribosomal washes a protein (iα), of mol. wt. about 60 000, which inhibits translation of MS2 but not late T4 RNA. A protein with the opposite specificity (iβ) was later isolated by Lee-Huang and Ochoa. Still other proteins with different messenger specificities have been described from Revel's laboratory. Interference factor iα appears to be identical to subunit α (or I) of Qβ replicase (Revel and collaborators, Weissmann and collaborators) as well as to the 30 S ribosomal protein S1 (Pêtre et al., September 1973 Cold Spring Harbor Ribosome Symposium; Wahba et al., this meeting) and as shown by van Duin and van Knippenberg and their coworkers S1 is essential for poly(U) translation and for translation of natural messengers. S1 should not be considered as a fractional 30 S protein, but rather as a protein that is readily lost from the ribosome. Thus, the S1/30S ratio is 0.1–0.3 in monomeric ribosomes but close to 1.0 in polysomes (van Duin et al.). Under these circumstances, it is not easy to see how iα (S1) can repress messenger translation unless this protein is present in the cell in a ratio much higher than one molecule per ribosome. iα (S1) has affinity for coliphage RNA (Kaempfer) and, although lower, for poly(U) (Wahba). At high iα/mRNA ratios iα competes with the 30 S subunit for mRNA binding thus inhibiting translation (van Duin et al.).

A specific inhibitor of prokaryotic polypeptide chain initiation that can apparently exist in an active or inactive form (Lee-Huang and Ochoa) was reported to be in fact the chain elongation factor EF-G. Thus, G factor could function as a regulator of polypeptide chain initiation but there is no proof that this is so and both the mechanism of inhibition by EF-G and of the turning on and off of the inhibitory activity are unknown.

Initiation factors affect the dissociation of 70 S ribosomes and the association of the subunits. One of the novelties in this field was the use of light

scattering methods (Godefroy–Colburn and Grunberg–Manago). Dissociation by IF-3 was found to be slow (half-time about 10 min) under the conditions used. The affinity of IF-3 for the 30 S subunit was calculated to be 2.5×10^7 M^{-1} (25°C and 37°C) in agreement with that found by other groups (Ochoa, Bosch, Noll). Addition of IF-1 has very little effect on the subunits-couple equilibrium, but greatly increases both the rate and the final level of dissociation by IF-3. Contrarywise, IF-2 tends to act as an association factor, but does not cause complete association. Therefore, it binds to both the 30 S and the 70 S couples. It was also found (by the groups of Bosch, Noll and Grunberg–Manago) that ribosomes could be classified into two groups of couples: 'tight' and 'loose'. Subunits forming 'tight' couples associate (up to 80%) at 2.5–3.0 mM Mg^{2+} whereas those forming 'loose' couples require 10 mM Mg^{2+} to associate to a similar extent. IF-3 has no significant affinity for 'tight' 70 S couples but it seems to have measurable affinity for 'loose' couples.

One advantage of prokaryotic over eukaryotic systems in initiation studies could turn out to be the use of strains harbouring specific genetic defects in the formation of initiation complexes. Results from Grunberg–Manago's laboratory (M. Springer and M. Grunberg–Manago) suggest that some of the bacterial mutants that fail to grow at non-permissive temperatures could be equated with IF-3 thermosensitive mutants, but these data need further support. Another mutant of this type was found by Lupker and Bosch to harbour an altered elongation factor EF-Tu.

Piper and Clark determined the complete nucleotide sequence of a eukaryotic initiator (myeloma) tRNA. Considerable progress has also been made in our knowledge of tRNA tertiary structure through the X-ray crystallographic studies of Rich and collaborators.

2. Eukaryotic systems

Despite work in many laboratories the mechanism of initiation in eukaryotic cells is still obscure. A well established fact is that the initiator is Met–$tRNA_f$ rather than fMet-$tRNA_f$. Anderson's laboratory pioneered in the isolation of initiation factors from reticulocytes. The aim of Anderson's and more recently Staehelin's and Levin's groups was to identify, purify and characterize functionally all the factors required for the translation of natural messengers. A major difficulty lies in the definition of the ribosome structure itself and in the fact that eukaryotic messengers in vivo are associated with specific proteins. The purified systems employed in these studies used either high salt washed ribosomes or 40 S and 60 S ribosomal subunits and deproteinized messengers. Therefore, some of the rather numerous factors, although required for natural mRNA translation as opposed to e.g. poly(U) translation, may be structural in nature rather than true initiation factors.

A factor originally designated as M1 by Anderson has been isolated from rabbit reticulocytes, rabbit and rat liver and many other sources including brine shrimp embryos and ascites cells. In Anderson's and Ochoa's laboratories this factor has been obtained in homogeneous form. The preparation of virtually homogeneous M1 from rat liver was reported by Moldave. EIF-1 (for eukaryotic initiation factor 1) might be a more appropriate and general designation for this factor. It appears to be the counterpart of the prokaryotic initiation factor IF-2 but differs from it somewhat in its mode of action. EIF-1 and IF-2 are not interchangeable. EIF-1 catalyzes the GTP-independent binding of Met–(or fMet)tRNA and Phe–(or acPhe)tRNA to the 40 S ribosomal subunit; the reaction is strongly dependent on the presence of messenger (AUG, poly(U)). Upon addition of the 60 S subunit there is extensive formation of aminoacylpuromycin. In some cells, EIF-1 is found predominantly in the high-salt ribosomal wash, in others in the high-speed supernatant. Anderson's group also characterized an M3 (more properly EIF-3) which, like prokaryotic IF-3 is required for natural but not for synthetic messenger translation. Other workers (Schreier and Staehelin, Kaempfer, Heywood) have also isolated this factor from various sources. However, contrary to prokaryotic IF-3 (mol. wt. about 21 000) EIF-3 appears to consist of a number of polypeptide chains of different sizes (mol. wt. of complex 500 000 or higher). Staehelin referred to this factor as the EIF-3 organelle. The question whether there are different EIF-3's with selectivity for different messengers or whether there is only one EIF-3 whose selectivity is controlled (modulated) by additional mRNA-specific factors was discussed. This must be

considered at present an open question. Whether eukaryotic IF-3 has, like its prokaryotic counterpart, ribosome dissociation (DF) activity or this activity resides in a separate protein must also remain open. Anderson's group isolated DF from a crude M3 complex but addition of this factor was not required for globin mRNA translation. A similar factor was described by Gupta and collaborators.

Several groups (Gupta, Levin, Stanley, Schreier and Staehelin and more recently Anderson) have isolated a protein which forms in the absence of ribosomes a ternary complex with GTP and eukaryotic Met-tRNA$_i$ (initiator Met-tRNA). Met–tRNA from the ternary complex is believed to bind to the 40 S subunits prior to mRNA binding. This factor has been purified 600- to 800-fold from ribosomal wash, and it is required for initiation with natural mRNA (Schreier and Staehelin). It also restores the activity of a hemindeprived reticulocyte lysate (Anderson and London). The question whether initiator tRNA binding in eukarotes may or does precede mRNA binding, a pathway suggested by esperiments of Hunt, Staehelin, Gupta, Levin, Stanley, is not considered as settled.

An ATP requirement in eukaryotic initiation has been reported by Marcus for wheat germ systems and by Schreier and Staehelin for mammalian systems. Marcus reported that ATP is utilized for the synthesis of ppGpp. ATP is not required for the binding of initiator tRNA to ribosomes but seems to be essential for mRNA binding (Marcus, 1970). Its hydrolysis and the formation of ppGpp precede the formation of the first peptide bond (Marcus) and may be required for recycling of Met–tRNA binding factor(s).

Heywood et al. had previously reported that initiation factors from chicken myogenic systems cannot promote globin messenger binding to ribosomes, whereas reticulocyte factors do not promote myosin messenger binding. Heywood now reported that white muscles, which synthesize myosin but not myoglobin, contain an EIF-3-like factor for myosin but not for myoglobin translation whereas red muscles contain both kinds of factors. Red muscle EIF-3 could be resolved by phosphocellulose chromatography into myosin-specific and myoglobin-specific factors. Upon dialysis, total EIF-3 liberated a low mol. wt. oligoribonucleotide (mol. wt. 6000–10 000) believed to be involved in translational control (TC-RNA). Bogdanovsky and Schapira reported that a 0.5 M KCl wash from rabbit reticulocytes also liberated upon dialysis a low mol. wt. RNA which, when added back to the system, restored the initiation factor activity lost during dialysis. Heywoods functional results with TC-RNA were different, possibly because of the use of a crude reticulocyte lysate containing endogenous TC-RNA. Thus, the main effect of TC-RNA obtained from a specific source was inhibition in the reticulocyte lysate of mRNAs not related to the source of the added TC-RNA rather than specific stimulation of mRNAs obtained from the same source as the TC-RNA. Still, this lends support to the idea that a small RNA might play a key role in the function of EIF-3 factors.

Attention was also focused on changes in the level of initiation factors during ontogenic development. Cell-free extracts of *Artemia salina* at the gastrula stage can translate poly(U) but contain no endogenous messenger (Ochoa). Upon development embryo DNA is transcribed and translation of the resulting messenger(s) leads to the appearance of initiation factors of the EIF-3 type. Different factors seem to be involved in translation of bromegrass-virus RNA and globin mRNA. Appearance of new EIF-3-like factors during development was also reported by Heywood. He observed that differentiation of red muscle in chicken embryos is accompanied by a gradual rise in the myoglobin forming capacity paralleled by an increase in the cellular content of myoglobin-specific EIF-3, a factor that is undetectable at earlier stages of development.

Cell-free lens mRNA-directed protein synthesis yields α-crystallin polypeptides bearing N-terminal acetyl-methionine. Acetylation takes place when the nascent peptide chain is about 20 amino acids long (Bloemendal).

Several reports dealt with mechanisms that might control the frequency of translation. Among these one might list (a) deacylation of aminoacyl–tRNA, (b) production of an inhibitory substance under the influence of interferon, (c) lack of hemin, (d) presence of double-stranded RNA, etc. High-salt washes of reticulocyte ribosomes contain a protein inhibitor that interferes with AUG-directed Met–puromycin synthesis (Gupta). The inhibition appears to be related to a rapid deacylation of Met–tRNA. Excess of the factor that forms a ternary complex with the initiator Met–tRNA$_i$ and GTP overcomes the inhibition by protecting the initiator tRNA in the ternary complex.

Inhibition of the initiation step during viral messenger translation following interferon treatment (Revel, Lebleu and coworkers) seems to be due to the accumulation of an uncharged tRNA species, $tRNA_{Leu}$. Since amino acid starvation in eukaryotes inhibits chain initiation (Vaughan) it appears that there is a control mechanism which links initiation to elongation in a tight fashion.

As is well known, chain initiation in reticulocyte systems is inhibited in the absence of hemin (Rabinovitz and others). Lack of hemin also affects the translation of messengers other than globin mRNA, e.g. EMC-RNA. Experiments by Kaempfer suggested that in all tissues, not only in erythropoietic cell lines, hemin might be involved in regulating the conformation and activity of what he designated as factor 3. The presence of an inhibitor of eukaryotic but not prokaryotic protein synthesis in the cytosol of eukaryotic cells was mentioned (Ochoa); it seems to be a small oligonucleotide or mononucleotide.