Meeting Report

Third International Symposium on 'Molecular Interactions in Biological Systems' 'Eukaryotic Ribosomes and Biosynthesis of Proteins'

Summary of the Symposium held in Berlin-Buch, GDR, on 22–25 October, 1984

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1. INTRODUCTION

The 1984 International Symposium on 'Molecular Interactions in Biological Systems' of the Central Institute of Molecular Biology of the Academy of Sciences of the GDR, organized by H. Bielka, was devoted principally to selected aspects of the structural organization and regulation of eukaryotic protein synthesis.

The conference was focused mainly on the following topics: Structure of ribosomal RNAs; functional sites of ribosomal particles; messenger RNP, initiation factors and preinitiation complexes; regulation of protein synthesis; post-translational processes.

The opening talk was given by H.G. Wittmann (Berlin-West) summarizing the present state of knowledge on the structure of bacterial ribosomes. The most exciting progress, as outlined by Wittmann, is now being made in this field by the crystallization of ribosomal proteins and ribosomal particles, the three-dimensional computer reconstruction and modelling of the ribosomal structure, which support and extend data obtained by cross-linking, immune electron microscopy, neutron scattering, and reconstitution.

2. STRUCTURE OF RIBOSOMAL RNA

Several reports were closely related to biophysical studies on low molecular mass ribosomal RNAs and their protein complexes.

J. Behlke et al. (Berlin-Buch) described hydrodynamic properties of 5 S and 5.8 S rRNA from rat liver and yeast. The enhancement of sedimentation coefficients with increasing concentrations of Mg^{2+} or monovalent cations was discussed with respect to changes of conformation and the solvation shell of the molecules or salt-dependent formation of intermolecular associations.

From small- and wide-angle X-ray scattering of rat liver 5 S rRNA in Mg^{2+}-containing solutions, a flat-shape model with separate stems and a compact central branching region was proposed by J.J. Müller et al. (Berlin-Buch). The results of wide-angle X-ray scattering support evidence of conformational changes in the helical regions of 5 S and 5.8 S rRNA from the so-called A-form in the

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presence of Mg^{2+} into the A′-form upon removal of Mg^{2+}. Such polymorphism of naturally occurring RNA was described here for the first time.

From the high similarity of infrared and Raman data for 5 S rRNA of *E. coli* and rat liver H. Welfle et al. (Berlin-Buch) concluded that pro- and eukaryotic 5 S rRNAs seem to have conserved general structural features during evolution. Furthermore, from Raman data of the rat liver 5 S rRNA-protein L5 complex evidence was presented that most of the base-paired regions are unchanged in comparison to free 5 S rRNA. Specific interactions between protein residues and N(7) of guanine as well as cytosine (and/or uracil) residues in single-stranded regions were suggested.

A ribonucleoprotein complex (RNP_H) from rat liver 60 S ribosomal subunits containing 5 S rRNA and proteins L3-4, L5, L6-7, L22 and phosphoproteins P1-P2 was characterized by J.-P. Reboud et al. (Lyon). This complex shows EF-2 dependent GTPase activity and seems to be located at the subunit interface. Ultraviolet cross-linking studies with native 60 S subunits revealed that all proteins present in RNP_H were bound to the 5 S rRNA except L5.

Other presentations dealt with high molecular mass ribosomal RNAs.

A.A. Hadjiolov (Sofia) reviewed the rapidly expanding knowledge on the structure of rRNA from the large subunit of cytoplasmic ribosomes of eukaryotes (L-rRNA). He reported the complete sequence of L-rRNA from *S. cerevisiae* and rat. Together with the known sequences of *S. carlsbergensis, Physarum polycephalum, Dictyostelium discoidum, Xenopus laevis* and mouse, these data permit a detailed analysis of changes in L-rRNA primary and secondary structure in eukaryotic evolution. Along the L-rRNA chains, conserved and non-conserved segments alternate. The strongly and moderately conserved segments can be folded into double-helical loops in a way that largely corresponds to the basic structure proposed for the *E. coli* homologue. The non-conserved segments of eukaryotic L-rRNA are characterized by a very high (80–85%) GC content. These GC-rich segments can be folded in a strongly base-paired manner. This overall enrichment in G and C seems to be favoured in evolution and is typical for L-rRNA molecules in vertebrates.

A. Ladhoff (Berlin) demonstrated starfish-like secondary structures of 18 S ribosomal RNA from rabbit reticulocytes by using a novel ion-bridge technique for transfer of the molecules from aqueous solution to supporting films. The obtained data are in good agreement with the computer model as proposed by Stiegler in 1981.

3. FUNCTIONAL SITES OF RIBOSOMES

This session focused mainly on results obtained from affinity labeling experiments.

D.G. Knorre (Novosibirsk) and E. Kuechler (Vienna) presented several affinity reagents which have proved to be useful tools for identification of components involved in mRNA and tRNA binding to ribosomes.

Knorre reported on oligo(U) of defined chain length derivatized in the 5′- or 3′-position with the alkylating 4-(*N*-2-chloroethyl-*N*-methylamino)-benzylamide or -benzylidene group, respectively. These derivatives, when bound together with Phe-$tRNA^{Phe}$, EF-Tu and GTP to *E. coli* ribosomes, modify distinct ribosomal proteins and ribosomal RNA located at the mRNA binding area of the ribosome. In another approach, heptauridylate could be directly cross-linked to ribosomes by treatment with water-soluble carbodiimide. For studying components involved in tRNA binding, $tRNA^{Phe}$ bearing on average two guanine residues derivatized with photoreactive dinitroazidobenzyl groups via amide bonds was enzymatically aminoacylated and complexed with EF-Tu, GTP and ribosomes. By irradiation at 330 nm of complexes containing the reactive tRNA in the ribosomal A-site or P-site, significantly different sets of proteins were labeled.

E. Kuechler presented an interesting approach to analyse codon-anticodon interactions at the decoding site of ribosomes. Wybutine (Ywye) situated next to the 3′-side of the anticodon of $tRNA^{Phe}$ from yeast was shown to be photocross-linked to poly(U) and oligonucleotides such as pAUGUUU on *E. coli* ribosomes. In order to identify the site of reaction on the mRNA, (5′-^{32}P)-labeled pAUGUUU was used together with Phe-$tRNA^{Phe}$ from yeast bound to the acceptor site. In subsequent analyses Ywye was found to be photocross-linked to the U residue at the 5′-position of the corresponding UUU codon. The

close proximity necessary for the photoreaction may be achieved by partial unstacking between the 3′-nucleotide of the codon at the P-site and the 5′-nucleotide of the codon at the A-site. Furthermore, E. Kuechler demonstrated the importance of 23 S RNA domain V as a constituent of the peptidyltransferase site of bacterial ribosomes. 3-(4′-Benzoylphenyl)propionyl-[^{3}H]Phe-tRNA bound to the peptidyl site is photocross-linked exclusively to 23 S RNA. The cross-linked complex is still active in the transpeptidation reaction. The site of photoreaction has been identified by hybridization and primer extension as uridine-2584 and uridine-2585 which are located within the central loop of domain V of the secondary structure model of 23 S RNA.

J. Jonak reported on joint work with T.E. Petersen, K. Pokorna and I. Rychlik (Prague, Aarhus) concerning the region of EF-Tu from *E. coli* interacting with aminoacyl-tRNA. By cross-linking of [^{14}C]tosyl-PheCH_2Cl (TPCK), a specific inhibitor of aminoacyl-tRNA binding activity of EF-Tu, and by means of protection studies against photooxidation in the presence of rose bengal dye, the involvement of His-66, Cys-81, Met-91 or -112 and of His-118 of EF-Tu in the binding of the 3′-terminus of aminoacyl-tRNA was discovered.

For eukaryotic ribosomes evidence on components involved in mRNA binding was reported by J. Stahl, who summarized results obtained in joint affinity labeling experiments between groups in Berlin-Buch and the Institute of Bioorganic Chemistry in Novosibirsk. Oligo(U) derivatives with chain lengths of 7 and 12, respectively, bearing the alkylating 4-(*N*-2-chloroethyl-*N*-methylamino)benzyl group (cf. D.G. Knorre's talk) were applied to rat liver ribosomes. When the reactive group was located in the 5′-terminal position, protein S26 of the small subunit was labeled preferentially, irrespective of the chain length of oligo(U). Oligo(U) containing the reactive group in the 3′-terminal position labeled mainly ribosomal RNA of both subunits, supporting a location of parts of the mRNA binding site close to the interface. Among the ribosomal proteins which are labeled less than 10% compared with RNA by the 3′-reactive oligo(U) derivatives, proteins S3 and S3a are the main targets.

4. MESSENGER RNP, INITIATION FACTORS AND PREINITIATION COMPLEXES

The talk of H.R.V. Arnstein (London) focused on the characterization of mRNP complexes isolated from rabbit reticulocytes. He found that two proteins of globin mRNP of 50 and 78 kDa, iodinated in situ, remain attached to mRNA during initiation and elongation of protein synthesis. However, mRNA also functions in the absence of these obviously non-essential proteins. Furthermore, evidence was presented that a third protein of 66 kDa becomes bound to mRNA during 48 S pre-initiation complex formation which can be isolated in a 22 S complex together with 18 S rRNA and globin mRNA by treatment of polysomes with LiCl and SDS. After iodination this protein was reincorporated into the pre-initiation complex, but it is released before formation of 80 S ribosomes. This 66 kDa protein which obviously stabilizes the interaction between mRNA and 18 S rRNA is probably identical with the 66 kDa subunit of eIF-3 as indicated by cross-linking data obtained by P. Westermann and O. Nygård (Berlin-Buch/Stockholm). When 48 S preinitiation complexes of rat liver were treated with diepoxybutane three subunits of eIF-3 (110, 95 and 66/64 kDa) as well as the 24 kDa cap binding protein and ribosomal proteins S1, S3, S3a, S6 and S11 were covalently linked to globin mRNA, whereas only the 66 kDa subunit of eIF-3 could be cross-linked to 18 S rRNA. The results show that within the preinitiation complex mRNA simultaneously contacts the cap binding protein, large parts of eIF-3 and the same ribosomal proteins (S3, S3a and S6) which could also be cross-linked to polysomal mRNA. The results of these experiments support the idea that 'scanning' of mRNA through the pre-initiation complex does not necessarily include movement along the mRNA strand from cap to the first AUG codon, but can also be explained by subsequent binding of specific parts of the 5′-untranslated region of the mRNA to cap binding protein, eIF-3 and the binding site at the 40 S ribosomal subunit.

Electron microscopic studies of native ribosomal subunits (40 S_N), performed by the Berlin-Buch group and presented by G. Lutsch largely support and supplement the data on the location of eIF-3 on 40 S subunits obtained by the above-mentioned

cross-linking data of Westermann and Nygård. The ultrastructural analysis of 40 S_N preparations and isolated eIF-3 from rat liver and rabbit reticulocytes characterizes this factor as a flat triangular prism (17 nm side length, 7 nm height) attached with its base at the rear side of the body part of the 40 S ribosomal subunit. One side of eIF-3 was found to be arranged in parallel to the groove between the head and body of the ribosomal particle. The channel formed between eIF-3 and the 40 S ribosomal subunit is thought to be at least a part of the mRNA binding site. Ribosomal proteins S3, S3a and S6 are very likely involved in the formation of this channel because they were found cross-linked with eIF-3 and mRNA (see Westermann and Nygård). This finding is in agreement with earlier immune electron microscopic experiments in which ribosomal proteins S3, S3a and S6 were mapped between the head and the body of the 40 S ribosomal subunit (G. Lutsch et al.).

A major step forward towards the structural analysis of eIF-3 was demonstrated by J. Behlke et al. (Berlin-Buch) using hydrodynamic techniques. Working with isolated eIF-3 preparations of rat liver, prepared by Bommer et al. in Bielka's group, a frictional ratio of $f/f_0 \sim 1.6$, as obtained from sedimentation and diffusion properties, excludes a sphere, but favours a more extended shape of eIF-3. According to a molar mass of about 650000 g/mol and a partial specific volume of 0.723 cm^3/g, the volume of eIF-3 amounts to about 780 nm^3. This volume and the hydrodynamic properties match very well with the shape of a flat prism as suggested above by Lutsch et al. The volume of 780 nm^3 cannot be placed, as Behlke showed, in a sphere representing a globular protein complex as proposed before by other authors.

Further progress in the investigation of initiation factors can be expected from cloning their genes. H. Trachsel and co-workers (Basel) prepared cDNA from HeLa and mouse poly(A)$^+$ RNA and inserted it in the expression vector λgt11. Polyclonal and monoclonal antibodies were then used to isolate cDNA clones encoding eIF-2A, eIF-3 and eIF-4A sequences. A cDNA clone encoding eIF-4A was further characterized by DNA sequencing and mRNA encoding eIF-4A was identified by Northern blot analysis. In the yeast system, as a first step towards cloning the gene for eIF-4E, they purified and partially characterized the protein. Yeast eIF-4E is a 24 kDa protein which binds specifically to mRNA 5′-cap structures. It is absolutely required for translation in the yeast cell-free system, since monoclonal antibodies against this protein strongly inhibit translation. Yeast cDNA libraries in λgt11 are currently being screened to isolate clones encoding yeast eIF-4E nucleotide sequences.

In a photoaffinity labeling study, T.V. Kurzchalia, U.-A. Bommer, G.T. Babkina and G.G. Karpova (Berlin-Buch, Novosibirsk) examined the binding activity of eIF-2A for GTP by using the γ-(*p*-azidoanilide) of GTP. The data obtained clearly suggest that the γ-subunit of eIF-2A reveals the highest binding affinity for GTP.

5. REGULATION OF PROTEIN SYNTHESIS

The contributions of this session focused mainly on three aspects: (i) Compartmentation of non-ribosomal components of the translational machinery; (ii) interrelationship between cell proliferation and initiation factor activity; (iii) phosphorylation of ribosomal proteins.

A.S. Spirin (Poushchino) pointed out that the translational machinery has to be regarded as a supramolecular complex including initiation and elongation factors, mRNA-bound proteins, aminoacyl-tRNA synthetases and tRNAs attached to polysomes. The formation of large complexes can be explained by the RNA-binding properties of most components mentioned above. He furthermore discussed that the resulting compartmentation of all components necessary for protein synthesis seems to be advantageous for eukaryotic cells and any changes in the RNA-binding strength of the components may alter the efficiency of translation. In this respect it was interesting to note that both ADP-ribosylation of EF-2 and phosphorylation of EF-1 by a polysome-bound kinase abolish the functional activity and the RNA-binding properties of the elongation factors.

Using heparin-Sepharose chromatography, J. Hradec and P. Pohlreich (Prague) isolated particles containing mRNA and protein synthesis factors from post-ribosomal supernatant of rabbit liver or mouse hybridoma cells. These particles, supplemented by ribosomal subunits and an energy

source, were found to be able to synthesize albumin or $\varkappa$- and γ-chains of IgG, respectively.

The formation of a new type of messenger containing complexes was described by L. Nover and K.-D. Scharf (Halle) after reprogramming of ribosomes during heat shock in plant cells. These cytoplasmic mRNP aggregates were called 'heat shock granules' according to their appearance in electron micrographs. They contain besides mRNA RNA-binding proteins and some of the heat shock proteins and can be regarded as a storage form for untranslated mRNA.

The sequestration of eIF-2B (eRF) by phosphorylated eIF-2A was discussed by A.R. Galpine (London) from the group of M.J. Clemens who used EMAC cells, and by H.O. Voorma (Utrecht) having investigated rabbit reticulocyte factors.

eIF-2B, which is able to restore the activity of haem-deficient lysates, catalyses nucleotide exchange only with eIF-2A unphosphorylated in the α-subunit, whereas the complex of eIF-2A-αP and eIF-2B is stable and the exchange of GTP for GDP becomes inhibited. In this way the more abundant eIF-2A blocks protein synthesis initiation even if only a fraction of the molecules possesses a phosphorylated α-subunit. Reduced concentrations of active eIF-2A are also involved in the inhibition of protein synthesis during amino acid starvation of CHO cells as shown by a reduced formation of the ternary complex eIF-2A · GTP · Met-$tRNA_f$. The inhibition is not mediated by uncharged tRNA, but is observed in mutants with a temperature-sensitive lysyl-tRNA synthetase under non-permissive conditions and can be prevented by addition of lysyl-tRNA (M.J. Clemens et al.).

Also, eIF-2A was found by I. Junghahn and U.-A. Bommer (Berlin-Buch) to be involved in the regulation of protein synthesis under different growth states. In a system containing rat liver polysomes and post-ribosomal supernatant purified factor stimulates protein synthesis only in experiments with supernatant from adult and senescent animals, while no stimulation was found by addition of eIF-2A in systems containing supernatants from embryonic rat liver or from regenerating rat livers 32 h after partial hepatectomy. Other regulatory component(s) have to be presumed to be present in EMAC cells during logarithmic and stationary growth, since supernatant fractions from both states can be stimulated by eIF-2A to the same extent.

A regulatory phenomenon involving mRNA selection by eIF-4B was studied by H.O. Voorma (Utrecht) in neuroblastoma cell culture. Under serum deprivation the cells reduce their protein synthesis by 75% and change the pattern of synthesized proteins, which finally results in the formation of nerve cells making long neuronal fibres. After serum deprivation for 60 h these cells contain a reduced amount of eIF-4B as analysed by Western blotting and only the addition of isolated reticulocyte factor stimulates protein synthesis and translation of SFV mRNA remarkably.

For studying the regulation of translation of individual messages with a known N-terminal coding sequence, a simplified system was developed by T. Twardowski, N. Brot and H. Weisbach (Poznan and Nutley, NJ) which allows measurement of di- and tri-peptide formation. Using this assay, the expression of α- and β-globin mRNA, the detection of two initiation sites of S1 mRNA and the translatability of cytoplasmic mRNP complexes were described.

Several contributions referred to the phosphorylation of ribosomal proteins. I. Martin-Perez presented data on phosphorylation of S6 obtained by the group of G. Thomas (Basel). Analysis of S6 peptides of serum-stimulated 3T3 cells showed an ordered expression of 11 major phosphopeptides. The first 8 peptides can also be observed after stimulation by epidermal growth factor. At the same time S6 kinase activity which seems to be phosphorylation-dependent increases several-fold. The saturating concentration of EGF (10^{-9} M) induces S6 phosphorylation, stimulation of protein synthesis as well as DNA synthesis which underlines the functional importance of S6 phosphorylation in the regulation of cell proliferation.

In a subsequent presentation, J.A. Traugh (Riverside), also working with 3T3 cells, reported her data on S6 phosphorylation in these cells. An uptake of up to 5 mol phosphate per mol S6 was observed in a system separating 5 phosphopeptides. An identical response of 3T3 cells on insulin and EGF stimulation and of Reuber H35 cells on phorbol ester administration was observed. Evidence was presented that the complex between insulin and receptor induces (auto-) phosphoryla-

tion of the precursor of protease-activated kinase II (PAK II) which in turn permits the activation of PAK II by protease. The phosphorylation of S6 within the 40 S ribosomal subunit by PAK II involves all 5 phosphopeptides and increases poly(AUG) and globin mRNA binding as well as the translation of globin mRNA. The phosphorylation of two S6 peptides by a cAMP-activated kinase is inhibitory in the same assays. Since there was no such effect on the translation of collagen mRNA it is concluded that the phosphorylation of S6 by different kinases influences the selection of individual mRNAs.

D.P. Leader (Glasgow) summarized joint experiments with M. Katan (Belgrade) on protein phosphorylation processes in baby hamster kidney (BHK) cells upon infection by Pseudorabis virus. Maximal phosphorylation of ribosomal protein S6 was observed 2–3 h after infection. Besides the protein kinase activity present in normal BHK cells after infection a virus-induced histone kinase III operates. The latter enzyme was characterized by using protamine as phosphate acceptor. Histone kinase III is still active at KCl concentrations of 1 M, can be stimulated by spermine and by cGMP, but not cAMP, whereas NEM and $CaCl_2$ inhibit enzyme activity. The enzyme utilizes ATP but not GTP as a substrate and is able to phosphorylate two proteins in the 40 S subunit: S6 and S7.

Results presented by J.P.G. Ballesta (Madrid) concerned protein phosphorylation in yeast ribosomes. Three acidic proteins of the large ribosomal subunit (p*I* 3.0–4.0; molecular mass 11 kDa) with at least partially identical primary structures were found to be phosphorylated only in the ribosome-bound state. The large pool of these proteins in the cytoplasm is unphosphorylated. However, there is no simple correlation between the binding of these phosphoproteins to the ribosome and an activation of the translation apparatus, since ribosomes of yeast cells in the stationary phase contain a higher level of these proteins compared with ribosomes of exponentially growing cells.

Although very exciting, the experiments about the phosphorylation of ribosomal proteins are still at an early stage concerning the functional significance in terms of regulation of translation and require further work.

6. POST-TRANSLATIONAL PROCESSES

I. Rychlik (Prague) reported on recent work on the translation of a 15 S fraction of poly(A)$^+$ RNA isolated from the fourth bovine stomach. This RNA which contains information for the synthesis of chymosin and pepsinogen precursors can be translated in rabbit reticulocyte lysates or in the wheat germ system into preprochymosin and prepepsinogen. However, when translating the 15 S RNA fraction from neonatal calves only chymosin precursors and from adults only pepsin precursors are coded for as analysed by SDS gel electrophoresis and monospecific antibodies. When injecting 15 S mRNA into *Xenopus laevis* oocytes, partially processed translation products, prochymosin and pepsinogen, are formed and secreted into the medium where they could be converted into enzymatically active chymosin and pepsin under acidic conditions, as demonstrated by their milk-clotting activity.

D. Richter (Hamburg) studied the translation of mRNA encoding vasopressin from normal and diabetes insipidus rats. The nonapeptide hormone vasopressin is expressed in the hypothalamus together with its carrier protein neurophysin and an additional glycoprotein as composite precursor. Diabetes insipidus rats which lack vasopressin do contain the vasopressin gene. However, due to the absence of a single base in the coding region, the protein is not expressed. The deletion gives rise to a new reading frame predicting a precursor which is no longer terminated by a stop codon. Blot analysis of hypothalamic mRNA, transfection and microinjection experiments show that the mutant gene is correctly transcribed and spliced, but the resulting mRNA is not efficiently translated. This suggests that the lacking termination signal for protein synthesis is of significance for efficient translation of this mRNA.

T. Rapoport (Berlin-Buch) addressed the question as to whether secretory proteins in eukaryotes need a sorting signal after having crossed the rough endoplasmic reticulum membrane. He showed that *X. laevis* oocytes can secrete bacterial β-lactamase. On the assumption that the prokaryotic secretory protein cannot be adapted to the complex pathway in a eukaryotic cell, this result provides strong evidence against the requirement of signals other than the signal sequence. Soluble proteins retained in the reticular system should have a sorting signal for separation from secretory proteins.