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The Development of Cell-Free Protein Expression Systems and Their Application in the Research on Antibiotics Targeting Ribosome

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1. Introduction

There is a little doubt that increasing developments of protein synthesis are in high demand. Not only proteins are participants in all biochemical processes of the living cell, continually accelerating advances in proteomics, (i.e. the science of proteins and their reciprocal interactions in the cell) are increasingly underscoring the need to perfect techniques that facilitate the production of specified proteins at an industrial scale that meets the necessary standards of purification (Kim and Kim 2009). Investigations that have built the foundation for such protein production have largely originated from discoveries in the middle of the last century. Such advances firstly elucidated new cellular environments of protein production. Subsequent developments focused on the specificity of protein synthesis and the general efficiency of production has been developed largely by genomic analysis and genetic recombination.

Several *in vitro* systems of protein synthesis are commercially available worldwide. Many of these methods are categorized according to the derivation of their extracts, from either prokaryotic cells such as *Escherichia coli* (*E. coli)* or, alternatively, eukaryotic cells such as wheat germ or rabbit reticulocytes. While such extracts can be enriched by cofactors that enhance the efficacy of protein biosynthesis, there are obvious limitations to such systems.

An important criterion involves also simplicity of the system and its potential application: (1) simple systems, such as synthesis of phenylalanine homopolymer (*poly(U)-dependent poly(Phe) expression*) are generally applied in studies that analyze protein biosynthesis itself and on factors which block the process, i.e. antibiotics. This is in contrast to (2) the complex systems that are able to link transcription and translation into a single system.

The most advanced cell-free system based on the application of semi-permeable membrane allowing the concentration of reaction compartment during the work with ribosomes. Such membrane separates the feeding compartment where energy-rich molecules are deposed and can be moved to the reaction compartment with a simple diffusion. Moreover, such a feeding compartment is a suitable space where by-products potentially interfering with the biosynthesis can be deposed.

Recently, many of different cell-free based systems are available and the customer can select the most suitable for the specific application. Here, we described the most popular systems and we demonstrated how these systems can be utilized to study interactions between antibiotics and the ribosome.

1.1 The beginning of cell-free protein synthesis

In 1950s, several research teams independently demonstrated that protein biosynthesis can take place even after disintegration of the cell membrane (Siekievitz and Zamecnik 1951; Borsook et al. 1950; Winnick 1950; Gale and Folkes 1954). Thus, the isolated cytoplasm has been found to comprise the entire set of components necessary to conduct protein biosynthesis. As first, Zamecnik prepared fully active cell-free system based on mitochondrium-isolated ribosomes from an animal (Littlefield et al. 1955; Keller and Littlefield 1957). The team further demonstrated that the reactions were dependent on the supply of high energy molecules, such as ATP and GTP. The first *in vitro* systems of protein synthesis based on isolated bacterial ribosomes were designed independently by two teams, German (Schachtschabel and Zillig 1959) and American (Lamborg and Zamecnik 1960). However, both of them were only capable of translating endogenous mRNAs, what was their main limitation. Nevertheless, this discovery provided a proof that extracellular biosynthesis was possible at all and consequently it provided a new approach to synthesize proteins and to study molecular mechanisms of protein biosynthesis. An open nature of the *in vitro* systems was very attractive especially to the latter approach.

The discovery of protein expression systems on the template of exogenous mRNA molecules significantly extended applications of extracellular protein biosynthesis. The achievement took place in 1961 in the laboratory of Nirenberg and Matthaei (Nirenberg and Matthaei 1961). A short incubation at the physiological temperature of around 37°C proved sufficient to remove endogenous mRNA molecules from ribosomes. Free ribosomes obtained from the procedure were subsequently used for protein synthesis on the template of exogenous mRNA molecules. Of great importance, the ribosomes could be "programmed" by synthetic mRNA molecules. The technique of Nirenberg became the classical system of extracellular protein synthesis and, taking advantage of it, its originator deciphered the genetic code, for which he received the Nobel prize in 1968. In the subsequent systems, additional procedures of purifying ribosomes from endogenous mRNA molecules were applied to DEAE cellulose, permitting the separation ribosomes from free nucleic acids *via* chromatography.

Incubation of ribosomes, preceding the proper protein biosynthesis and conducted in the same manner as in the technique of Nirenberg, was later successfully applied in eukaryotic *in vitro* systems. Extracts of animal cells enriched with purified ribosomes conducted efficient protein biosynthesis. The technique was again successful using the template of exogenous mRNA molecules (Schreier and Staehelin 1973). During approximately the same timeframe, investigators applied this capacity to extracts of wheat germs and, of great interest, found that the endogenous as opposed to exogenous expression of mRNA molecules manifested naturally low levels of protein (Marcus, Efron, and Weeks 1974; Roberts and Paterson 1973; Anderson, Straus, and Dudock 1983). Other techniques of eliminating endogenous mRNA were based on application of calcium ion-dependent bacterial RNAse, used to augment the efficiency of protein expression system

in lysates of erythrocytes (Jackson and Hunt 1983; Merrick 1983; Pelham and Jackson 1976) as well as in other lysates originating from animal cells (Henshaw and Panniers 1983). In the 1980s, it was subsequently found that bypassing the expression of endogenous mRNA molecules significantly improved the efficiency of extracellular protein expression systems.

1.2 Simple systems based on synthesis of protein homopolymers

In such systems, the principal homopolymeric system involves synthesis of polyalanine on the template of poly(U) chain (*poly(U)-dependent poly(Phe) synthesis*). A buffered medium containing free ribosomes and the remaining components necessary for a translation reaction with the template of poly(U), the polyuridine homopolymer is added. The poly(U) template has the capacity to bind a ribosome without involvement of the Shine-Dalgarno sequence (sequence on mRNA which binds to the region of 16S rRNA) also has the ability to "program" the ribosome for synthesis of poly(Phe). Efficiency of the optimised systems of polyphenyloalanine synthesis may reach 300 amino acid incorporations per ribosome, which represents a significant achievement allowing for a unitemporal and complete analysis of all protein biosynthesis components (Szaflarski et al. 2008). In contrast, the first attempts of the type, performed in 1950s and 1960s resulted in merely 2-5 amino acid incorporations per ribosome.

The homopolymeric system was prepared by isolation of two cellular fractions, which were subsequently enriched in high-energy molecules, free amino acides and poly(U)-mRNA, providing the template. The fractions were obtained from bacterial extracts, which were fractionated by centrifugation (for a detailed description of ribosome isolation see (Blaha et al. 2000)). The so-called fraction S30 (obtained by centrifugation at approximately 30,000 rpm for 24 h) was rich in ribosomes and was used to purify free ribosome subunits in a sucrose gradient (centrifugation at around 45,000-60,000 rpm for 15 h). Ribosomes prepared in this manner were incubated at the temperature of 37^{o}C in a buffer containing, for instance, Mg^{2+} ions at the concentration of 4.5 mM in order to obtain complete correct 70S ribosome structure capable of performing protein synthesis.

S100 fraction was obtained from supernatant of the S30 fraction and it provides the source of protein factors indispensable to conduct translation (i.a., initiation factors: IF1, IF2, IF3, specific aminoacyl-tRNA synthetases, elongation factors: EF-Tu, EF-G, EF-Tu).

The reaction of polyphenylanaline synthesis represents a simple and widely used technique in several varieties. Instead of a poly(U) template, a poly(A) template can be used, enabling the synthesis of polylysine. Unfortunately, however, the polymer was poorly soluble in water; this property markedly restricts applicability of the system at a broader scale. Nevertheless, application of certain detergents permits its application in studies as seen in previous experiments conducted with the functional analysis of two antibiotics (pactamycin and edein), representing inhibitors of protein synthesis (Dinos et al. 2004). Here, the incorporation of near-cognate lysine instead of phenylalanine on the template of poly(U) can be precisely measured using double radioisotope labeling. If any antibiotic impacts on the translation accuracy (for example aminoglycoside paromomycin) it can be confirmed by detection of higher incorporation of lysine. Followed that technique edein was found to be

an error-prone antibiotic in contrast to pactamycin which did not induce any miscoding (Dinos et al. 2004).

1.3 Biosynthesis of protein in a couple transcription-translation system

Nirenberg and Matthaei (Matthaei and Nirenberg 1961) again were the first to describe the DNA dependence of the bacterial extracellular synthesis of protein. The dependence was corroborated by synthesis of a protein on the template of endogenous DNA molecules. Another group of investigators extended synthesis of endogenous proteins by application of exogenous DNA, which originated from a bacteriophage (Byrne et al. 1964; Wood and Berg 1962). Unfortunately, the systems manifested a relatively poor efficiency using either endogenous or viral DNA. Moreover, they were accompanied by a non-specific expression of cellular and bacteriophage proteins. However, the continuing improvements of the joint transcription and translation system resulted in its dissemination; with it ultimately becoming a significant laboratory tool (Lederman and Zubay 1967; DeVries and Zubay 1967).

In the improved system, suggested by Zubay, a preliminary bacterial extract was subjected to incubation in order to degrade mRNA and DNA molecules by cellular nucleases (Zubay 1973). The system gained popularity due to the ease of its preparation, stability of components and a relatively high efficiency. In the system designed by Gold and Schweiger ribosomes were isolated from cellular extracts to their homogenous form and so prepared ribosomes were supplemented with a cytoplasmic fraction, cleared of nucleic acids by ion-exchange chromatography (Schweiger and Gold 1969, 1969, 1970). Such a preparation of components for extracellular protein synthesis produced a remarkable reduction in the non-specific expression of protein. The troublesome procedure, however, remained the disadvantage of the system.

2. Contemporary systems of the cell-free protein expression

The 1980s and 1990s witnessed development of the *in vitro* systems in the form of optimization of cellular extracts, including the application of bacterial strains lacking the genes that code enzymes of endonuclease type (RNases) (Zaniewski, Petkaites, and Deutscher 1984). Such reaction mixes allowed researchers to keep bacteria in the reactive mix for a much longer period of time. Contemporary systems are characterized by a high mRNA level even after 24 h of the reaction (Iskakova et al. 2006). In 1990s the developing methods of genetic engineering and bioinformatics produced tremendous advances inside *in vitro* systems. One tremendous achievement was the ability to obtain data on structure of mRNA transcripts and on the effect of the structure on the efficiency of biosynthesis. The spatial structure of mRNA and primarily the sequence located at the 5′ terminus, proved to be very important (Graentzdoerfer et al. 2002) due to its ability to fold secondary structures covering Shine-Dalgarno sequence.

The several years of studies on structure and function of individual elements of mRNA sequence resulted in a design of the optimum expression vector for the *in vitro* protein expression systems. This has been exemplified by pIVEX (*In Vitro EXpression*) plasmid (Betton 2003). This plasmid is characterized by its ability to form secondary structures at the level of mRNA, particularly within the Shine-Dalgarno system and AUG initiation codon.

Due to this, both fragments of mRNA sequence are exposed and they easily bind to a ribosome. An integral part of the vector also includes the sequence of the bacteriophage promoter, T7, which permits transcription of a given gene using T7 polymerase. Two types of vectors are commercially available, including: (1) those containing His-tag sequences and (2) Strep-tag, located on the amine or carboxylic terminus of the protein, which allows for an easy and rapid purification of the biosynthesis products.

2.1 *In vitro* systems based on application of a semipermeable membrane

At present, the *in vitro* expression systems enjoy wide application due to the ease of performing the reaction without the need to apply sophisticated equipment. However, they exhibit low efficiency, not exceeding few tens of product nanograms in 50 µl reaction. The solution which markedly increased protein expression level involved application of a semipermeable membrane, used for the first time by Spirin (Baranov and Spirin 1993). This produced a significant increase volume of the so-called feeding mix, containing free amino acids, ribonucleotides and high energy molecules (mainly ATP and GTP), securing in parallel high concentration of ribosomes with the yield produced in the reaction compartment (Fig. 1, A and B). Application of a semipermeable membrane further enabled a significant extension of the biosynthetic reaction since it could be continuously supplied by inflow of indispensable reactants from the feeding mix (Fig. 1B). The maximum duration of conducting the reaction averaged at approximately 30-50 h, with *plateau* of reaction product being reached following around 30 h at 30°C. The system produced a remarkable yield. For the first time milligram quantities of protein per 1 ml of the reaction were obtained. The example involved expression of GFP (*Green Fluorescence Protein*), the synthesis of which reached the level of around 5 mg in the course of a single 24 h (Fig. 2 A and B) reaction using the RTS (*Rapid Translation System*) (Iskakova et al. 2006). RTS is manufactured by Roche company and it has been designed in the basis of A. Spirin's patent (*U.S. Pat. No. 5,478,730*).

The RTS is based not only on the ingenious application of a semipermeable membrane but also coupling the transcription and translation reactions, used also in the earlier designed systems (Fig. 1A). Such an approach markedly abbreviated duration of the process and reduced formation of nonspecific products since only the gene present in the expression vector was undergoing transcription and, then, translation.

2.2 Advantages and drawbacks of RTS

The RTS system manifests several advantages. Due to release of ribosomes from the cell and provision of appropriate conditions for the translation reaction, toxic proteins can be produced. If using *in vivo* conditions, such toxicities factors would surely block living processes in the cell. Enrichment of free amino acids with their radioactively labeled substitutes permitted the effective labeling of nascent polypeptides.

The open nature of RTS systems and other *in vitro* techniques provided a handy tool for studies on protein biosynthesis itself and on molecules such as antibiotics, which would otherwise, of course block protein production. An interesting approach within RTS involves screening analysis of known or potential antibiotics. Such an approach allows for a very rapid determination of the inhibitory concentration of a given antibiotic (determination of IC_{50}) or preliminary analysis of the mechanism of antibiotic action.

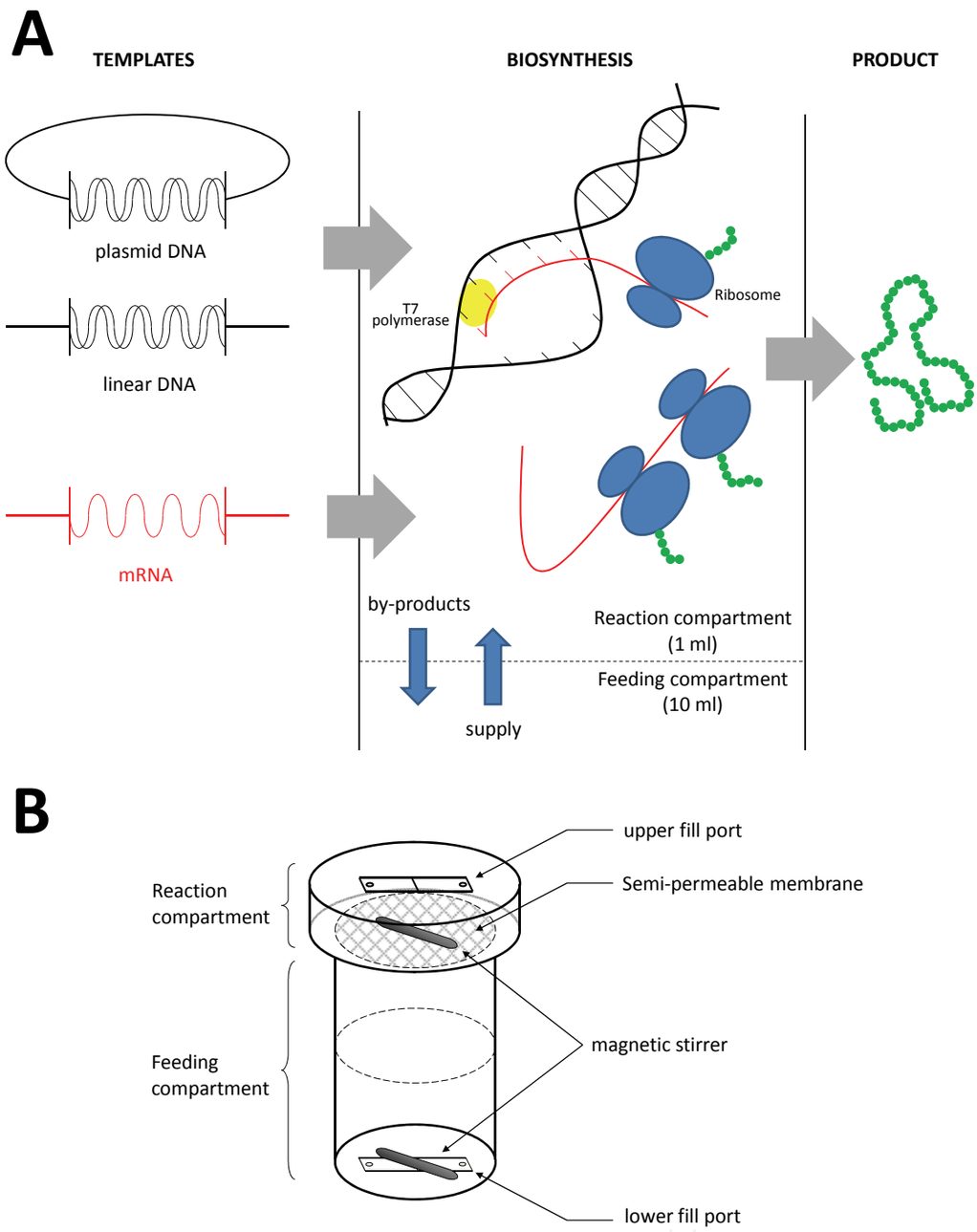

Fig. 1. Production of proteins in RTS system. (A) Principles of the process; coupled transcription-translation reaction runs on one of three templates in the reaction compartment supplied by energy rich-components and amino acids from the feeding compartment. (B) RTS reaction chamber with the semipermeable membrane separating reaction and feeding compartments.

A typical marker protein used in studies on *in vitro* systems is GFP protein, due to the ease of estimating the total product quantity and the fraction of active molecules (the phenomenon of fluorescence). The GFP protein is almost ideal for this purpose since it manifests a characteristic structure (Fig. 2A). The structure involves a barrel formed by 11 β

structures and an active centre, the chromophore, located inside the molecule. The β structures serve to protect the chromophore from the access of water molecules that could otherwise block the fluorescence of the reaction (Yang, Moss, and Phillips 1996). The appearance of inappropriate amino acids in GFP disturbs its structure and allows for penetration of its interior by water molecules.

Using this system, it was possible to demonstrate that the *in vitro* system fully confirmed the role of aminoglycosides as inducers of translation errors (Szaflarski et al. 2008). In the presence of one of the aminoglycosides (streptomycin) expression of total GFP and active fraction (i.e. GFP molecules inducing fluorescence) were followed by SDS polyacrylamide gel electrophoresis (SDS PAGE) and native PAGE, respectively. The relations between results read by these two techniques determined whether antibiotic (e.g. streptomycin) impacted on the fidelity of translation reaction. If the active fraction of GFP decreases faster than the total expression it means that full-size polypeptide chains are produced on the ribosome however their protein folding failed due to increasing number of wrong amino acids in the GFP and this protein was inactive (Fig. 2C). In view of the literature on aminoglycoside character, this provided evidence for introduction of erroneous amino acids to GFP molecule. This technique was demonstrated to be suitable to discriminate opposite effects of edeine and pactamycin acting on the ribosome (Dinos et al. 2004).

Aminoglycosides were also tested in another *in vitro* synthesis system: the poly(U)-dependent translation of polyphenylalanine (Szaflarski et al. 2008). In contrast to results obtained in the RTS system, they failed to block the ribosome. However, following addition of an additional leucine, it was found to be introduced to the polyphenylalanine chain already at low concentration of streptomycin (around 1 μM). This reflected the similarity of leucine codon and phenylalanine codon (UUC *vs.* UUU) and, thereafter, in situations inducing elevated probability of translation errors, leucine was introduced instead of phenylalanine. Thus, the observation did not allow a direct comparison between the two *in vitro* translation systems. The RTS system resembles more closely the natural conditions and, therefore, is more sensitive to action of antibiotics due to higher number of potential targets.

Nevertheless, extracellular protein biosynthesis is linked to disadvantages which for several years have been successively eliminated. In the course of studies the systems such as RTS was found to support expression of relatively high amounts of protein but around half of the proteins were found to be biologically inactive (Iskakova et al. 2006). However, application of specific translation factors as EF-4 allowed reaching 100% efficiency of RTS system (Qin et al. 2006).

The causes of lowered activity of proteins inside *in vitro* systems may be multiple but the most probable one involves application of the bacteriophage polymerase T7, which is exceedingly rapid. The transcription and translation are strictly interrelated during *in vivo* conditions with elimination of the free space on mRNA between polymerase and the ribosome. This prevents against development of spatial structures in mRNA molecule and it does not allow for a precocious termination of translation. Application of T7 polymerase disturbs the natural interrelationship between the polymerase and the ribosome, which may lead to errors at the level of translation or to incorporation of inappropriate amino acids to the growing polypeptide chain. The solution worked out by involved genetic recombination

of the polymerase T7 of such a type that the enzyme contained two point mutations that decreased the rate of activity (He et al. 1997). The results proved that despite the lowered efficiency of the total biosynthesis the content of active proteins increased to almost 100%. A similar effect was obtained decreasing temperature of the reaction. Most probably this reflected the fact that, as compared to ribosomes, polymerase is much more temperature sensitive, producing a slower rate of transcription and a similar pace of translation (Lewicki et al. 1993).

3. Energy consumption and its regeneration inside in vitro systems

Protein biosynthesis represents a process of particular energetic requirements. In biological systems the energy is obtained from hydrolysis of high energy bonds. For introduction of a single amino acid to the growing polypeptide chain, the cell sacrifices as many as 10 high energy bonds which is equivalent to hydrolysis of 10 molecules of ATP or GTP, each characterized by bonding energy of ΔG^0 = -6 kcal/mol. The extreme energetic requirement of a cell supporting protein biosynthesis explains development of sophisticated systems which control energy loss. However, the systems retain the Achilles heel of contemporary systems of protein biosynthesis *in vitro* in which their output remains seriously restricted by excessive uncontrolled leaks of energy: usually not more than 5% of energy is expended to support current protein biosynthesis while the remaining energy is wasted in uncontrolled biochemical reactions. It should be borne in mind that injuring the cell we introduce an extreme chaos to its metabolism. In contrast to *in vitro* conditions, in the *in vivo* conditions in a bacterial cell as much as 70% of energy can be directed to support protein biosynthesis (Szaflarski and Nierhaus 2007).

Therefore, in the techniques of protein biosynthesis *in vitro* a continuous replenishment of high energy compounds (i.a. ATP and GTP), necessary for efficacious transcription and translation reactions, continues to pose an enormous challenge. The earliest to be designed system of replenishing the high energy compounds involved enrichment of the cellular extract with millimolar concentrations of phosphoenolpyruvate (PEP), a derivative of pyruvic acid, and with pyruvate kinase, which catalyzes transfer of a phosphate group from phosphoenolpyruvate to AMP and ADP, yielding ADP and ATP, respectively. Also GMP and GDP represent substrates for the kinase. PEP is distinguished among all biologically active compounds by its content of the energetically most valuable bond: the phosphoester bond contained in the compound carries the energy of ΔG^0=-12 kcal/mol. Nevertheless, the PEP-based system carries also an extreme disadvantage: the by-product formed during regeneration of the high energy molecules involves orthophosphoric acid. The acid lowers pH of the reaction and, which is even more important, it binds magnesium ions (Mg^{2+}), markedly reducing their level in the reaction. Mg^{2+} stabilizes ribosome structure by its interaction with rRNA, therefore their reduced level results in a disturbed ribosome structure and a reduced efficiency of protein biosynthesis.

One of the ways in which the lowered concentration of Mg^{2+} ions can be avoided involves transformation of orthophosphoric to acetylphosphate using pyruvate oxidase and a defined prosthetic group (TPP or FAD). The reaction requires an access of molecular oxygen, the availability of which is restricted inside *in vitro* systems, particularly when the

reaction is conducted in a few milliliter volumes. Nevertheless, the application of pyruvate oxidase has significantly extend the duration of the effective protein biosynthesis reaction which has markedly increased efficiency (Kim and Swartz 2000).

However, the above approach still hardly can be considered ideal. First of all, the restricted access of molecular oxygen markedly reduces the potential for utilization of the system on a larger scale. At present, the solution widely applied involves application of a combined system, based on the traditional PEP/pyruvate kinase approach with acetyl phosphate synthesis by acetyl-CoA, which allows for an effective elimination of free phosphoric acid during synthesis of acetylphosphate from acetyl-CoA (Jewett and Swartz 2004). Application of the system provided a breakthrough and permitted milligram quantities of the produced protein in a volume of just one milliliter (Iskakova et al. 2006).

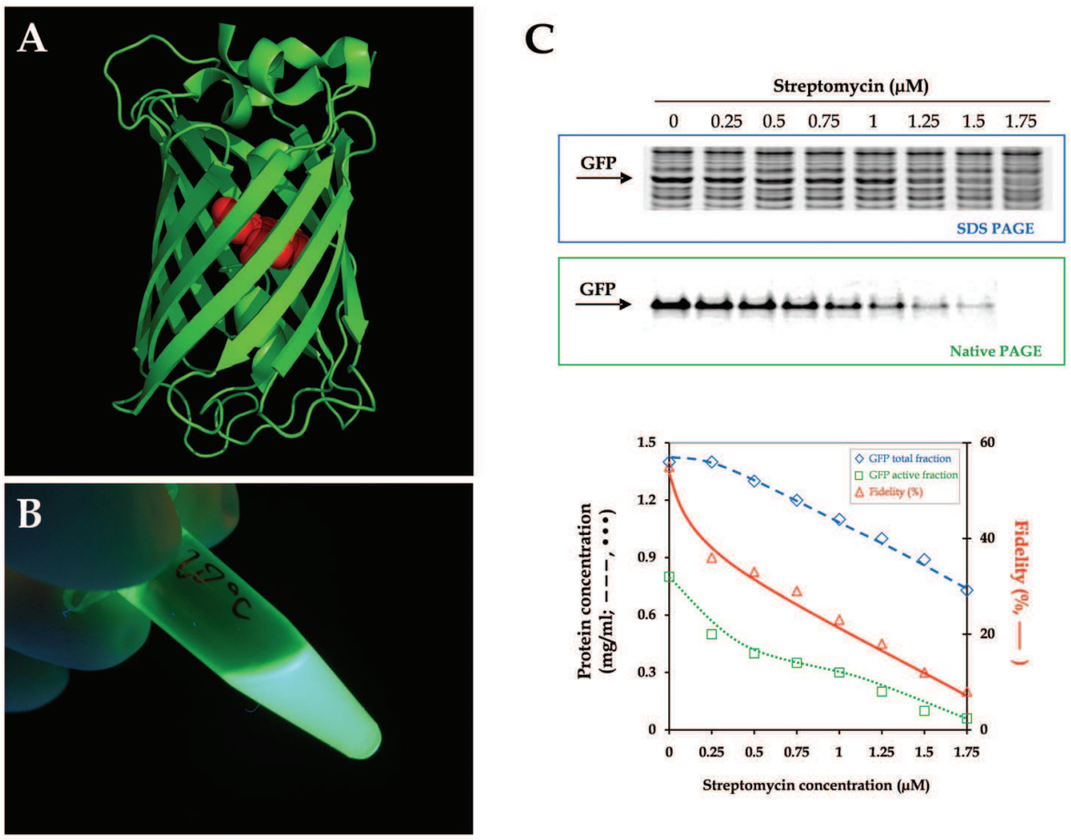

Fig. 2. Expression of GFP as a reporter protein in the presence of antibiotic streptomycin. (A) The molecular structure of GFP with the internal chromophore as red (coordinates based on PDB acc. no. 2B3Q). (B) The luminescence of GFP seen under UV lamp. (C) Parallel analysis of GFP total expression (SDS PAGE) and its activity measured as its luminescence (native PAGE). The graph demonstrates the fidelity (red line) of translation as the ratio between total expression of GFP (blue dashed line) and the active fraction of GFP (green dotted line). Increasing concentrations of streptomycin caused dramatic decrease in the ratio of the active GFP to the total protein. Techniqual and experimental details see in Dinos et al. 2004; Szaflarski et al. 2008; Qin et al. 2006.

4. Biotechnological application of extracellular protein synthesis systems

4.1 Efficient expression of several proteins and their screening analysis in parallel

The extracellular protein expression provided the base for many automated techniques of high-throughput expression and screening of several proteins in parallel (Angenendt et al. 2004; Spirin 2004). In such a system, using a single 96- or 384-well plate, multiple genes can be copied, transcribed and translated in parallel, providing substrates for subsequent high-throughput analysis of protein functions. The extensive scale of expression as well as the rate of analysis warrant that the technique deserves to be considered in proteomics and protein engineering based on screening analysis of gene and protein libraries as well as of entire genomes and proteomes (He and Taussig 2007, 2008). The *in vitro* systems of protein expression permit synthesis of protein population in a single reaction, which represents an ideal and economic solution in complete screening analysis of proteins within a given gene library (Chandra and Srivastava).

Such solutions can be proved by the technology known as *in vitro* expression cloning (IVEC) (King et al. 1997). In the technique a large genomic library is preliminarily subcloned to groups each containing 50-100 plasmids placed in the standard 96-well plate. They provide a template for expression in an *in vitro* system. The plasmid-containing genes which yield protein products are subsequently transferred by cloning to expression vectors, which allow for synthesis of milligram quantities of proteins in RTS type *in vitro* systems.

The IVEC technique can further be improved by combining it with gene cloning and amplification using the PCR reaction, thus eliminating the time-consuming cloning of the genes to plasmids (Gocke and Yu 2009). Preparation of the appropriate primers containing promoter sequences and Shine-Dalgarno sequences has facilitated protein synthesis directly from products of the PCR reaction (Rungpragayphan, Nakano, and Yamane 2003). The example includes an application of extracellular protein expression system for screening analysis of the entire *Arabidopsis thaliana* genome in order to identify new genes and products of their expression (Sawasaki et al. 2002).

Systems of *in vitro* translation have found application also in medical studies. The protein truncated test (PTT) has been worked out in order to identify open reading frames (ORF) (Roest et al. 1993). Detection of precocious translation termination within an ORF may reflect mutation at the level of DNA, which provides grounds for distinguishing another genetically-conditioned disease. Other applications are linked to production and analysis inside *in vitro* systems of potential vaccines which, even if obtained on bacterial or animal ribosomes, manifest the same immunological variables as those obtained in cultures of human cells (Kanter et al. 2007). At present, new proteins representing potential anti-malaria vaccines have been fully worked out in the systems of extracellular protein expression (Tsuboi et al. 2008). Also the expression of virus-like particles (VLP) has been characterized inside *in vitro* systems The examples include the phage protein, MS2 and C-terminal fragment of the protein core in the hepatitis B virus (HBV), the biological activity of which has been identical to the forms obtained *in vivo* (Bundy, Franciszkowicz, and Swartz 2008).

4.2 Production of proteins ”resistant” to expression

Considering the open character of *in vitro* systems due to the absence of biological membranes, the systems could have been applied with excellent results for production of

toxic or membraneous proteins, which manifest poor expression in *in vivo* systems (Jackson et al. 2004). At present the *in vitro* systems allow for expression at a large scale of membraneous proteins in modified extracts of *E. coli* (Klammt et al. 2006). Due to application in the *in vitro* systems of techniques allowing for formation of disulphide bridges, production of antibodies also has become possible. The example involves immunoglobulin G (IgG), which when obtained in the system has proven to be fully biologically active, manifesting affinity to antigen and stability identical to the natural antibody (Frey et al. 2008).

4.3 Analysis of molecular interactions

Extracellular protein expression systems markedly facilitate the molecular analysis of interactions between protein and X substance, where X may involve another protein, DNA, RNA or a ligand (Jackson et al. 2004). In order to identify the interaction, one of the reactants must be labeled (a protein, nucleotide or ligand) and placed in a system in which protein, the other reactant is synthesized. Then, the arising complex is isolated from the reaction mix using immunoprecipitation (Derbigny et al. 2000) or it may be directly analyzed in agarose or polyacrylamide gels (Lee and Chang 1995).

4.4 Protein display technologies

The essence of protein display technologies involves establishing a link between genetic information (genotype) and function of an unknown protein (phenotype) in the protein library. The principal technique involves a ribosome display (He and Taussig 1997; Hanes and Pluckthun 1997). Elimination of the STOP codon in mRNA permitted to obtain stable complexes of mRNA-ribosome-protein. Thus, a kind of a frozen structure was obtained, from the threshold of genetic world and proteomics. Subsequently, binding of the protein formed on the ribosome to a defined ligand (which may involve also DNA or RNA) resulted in development of an informational link between a given ligand and the sequence of protein mRNA. Then, a given mRNA-ribosome-protein-ligand complex can be isolated by affinity chromatography from the medium containing also other ligands while mRNA sequences are identified by reverse transcription and DNA sequencing. In order to amplify efficacy of the system, the process is conducted in a cyclic manner, i.e., the isolated mRNAs are independently amplified and added again to the mixture of ribosomes and ligands, enabling a more effective selection of an individual specific ligand. In combination with methods of genetic engineering, including mutagenesis, the protein display technologies can be applied not only in proteomics but also in molecular evolution studies. Now, the processes of interactions between DNA, RNA and protein, which took millions of years of evolution may be analyzed in the laboratory and their rate may be multiplied by selective amplification of DNA.

5. Conclusion

Cell-free systems will be optimized and improved according to their expression yield, protein specificity (“difficult proteins”) and protein folding. They will be more broadly applied in protein microarrays technology where can be utilized for the analysis of protein-protein interaction. Furthermore, protein technologies based on cell-free biosynthesis will be

applied for protein engineering in order to synthesis specific antibodies or enzymes, as well as for production of proteins for crystallisation.

The "post-genome" research requires comprehensive tool which will allow determination of structure, function and specific location of the proteins in the network of proteomes. It has to be performed effectively, quickly and on the multiple platform where large number of proteins can be analyzed in the same time. Based on cell-free systems such analysis is possible especially in the comparison to traditional cell-based systems where their miniaturization is rather impossible.

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Biotechnology is the scientific field of studying and applying the most efficient methods and techniques to get useful end-products for the human society by using viable micro-organisms, cells, and tissues of plants or animals, or even certain functional components of their organisms, that are grown in fully controlled conditions to maximize their specific metabolism inside fully automatic bioreactors. It is very important to make the specific difference between biotechnology as a distinct science of getting valuable products from molecules, cells or tissues of viable organisms, and any other applications of bioprocesses that are based on using the whole living plants or animals in different fields of human activities such as bioremediation, environmental protection, organic agriculture, or industrial exploitation of natural resources. The volume Advances in Applied Biotechnology is a scientific book containing recent advances of selected research works that are ongoing in certain biotechnological applications. Fourteen chapters divided in four sections related to the newest biotechnological achievements in environmental protection, medicine and health care, biopharmaceutical producing, molecular genetics, and tissue engineering are presented.

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