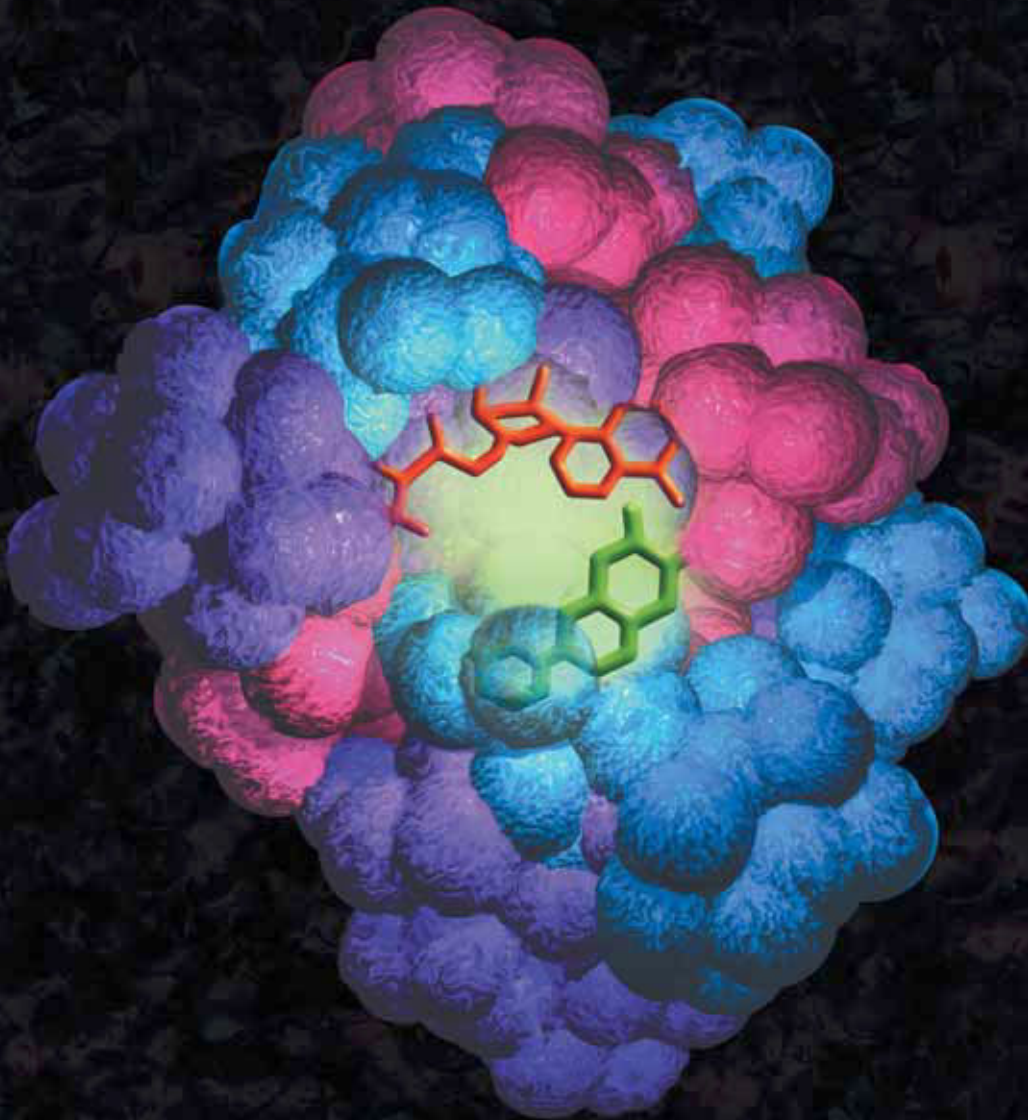


CHAPTER

9

About the Image:

Proteins produced in vitro in cell-free systems exhibit varying degrees of protein function or activity, depending on the factors necessary for correct synthesis, folding and cofactor incorporation. In this illustration the protein demonstrates luminescence once the essential cofactor ATP is present.



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Chapter Nine: Protein Function

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Introduction

Various expression systems for the in vitro production of exogenous proteins are currently in use. The proteins expressed in the coupled and uncoupled rabbit reticulocyte lysate (RRL), wheat germ extract (WGE), and *E. coli* S30 Systems include both prokaryotic and eukaryotic templates, and exhibit varying degrees of activity upon completion of translation. The level of activity of a particular protein when expressed in an in vitro expression system will be dependent on the factors necessary for correct synthesis, folding, and cofactor incorporation if necessary, and whether those factors are present in the in vitro expression system being used.

Many different types of proteins have been expressed in vitro, and the activity of these proteins has been determined using many different enzymatic and functional assays. Most commonly, RNA- or DNA-binding proteins are expressed in vitro, and the activity of such proteins determined using mobility (gel) shift assays with the appropriate RNA or DNA probe. For such studies, the choice of in vitro expression system can be critical, as background RNA or DNA binding activities can vary dramatically between rabbit reticulocyte lysate, wheat germ extract and *E. coli* S30. For more detailed information concerning the in vitro expression and functional determination of nucleic acid-binding proteins, see Chapter 3 of this guide.

Another common function of in vitro expressed proteins is the determination of protein-protein interactions. Proteins expressed by in vitro systems may be assayed for the ability to bind to other proteins endogenous to the expression system or to exogenous proteins either supplied as pure or semi-pure proteins. Alternately, the target protein may also be expressed in vitro, in either the same reaction or in a separate reaction as the bait protein. For a more detailed discussion concerning the determination of protein-protein interactions with in vitro expressed templates, see Chapter 2 of this guide.

The functionality of other in vitro expressed proteins has been assayed using a number of different techniques, and the remainder of this chapter will provide some examples of proteins and activities detected following in vitro expression.

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Enzymatic Activity

The expression and enzymatic activity of firefly luciferase are commonly used as both positive controls for in vitro expression system functions (1–6), and for studies investigating protein refolding in vitro (7,8) or protein characterization through mutagenesis studies (9).

Other enzymatic functions for in vitro expressed proteins have also been measured in addition to luciferase, and include protease, transferase, phosphatase, kinase, reductase, synthase and oxidase activities. Functional human lysozyme has been expressed in RRL (10), while a number of active viral proteases, including those from tomato black ring virus (TBRV), hepatitis C (HCV), and herpes simplex virus (HSV-1), have been expressed in either RRL or WGE (11–13). Active tissue-type plasminogen activator (TPA) has been expressed in RRL in the presence of canine microsomal membranes and oxidized glutathione (14), while active 6-hydroxy-D-nicotine oxidase was expressed in RRL supplemented with the cofactor FAD (15). A variety of active transferase enzymes have been successfully expressed and detected in either RRL or WGE, and include aspartate aminotransferase (16), carnitine palmitoyltransferase (17), and thiopurine S-methyltransferase (18). Expression of B59 ERK phosphatase (19) and murine thymidine kinase (20) in RRL produced functional proteins with detectable phosphatase and kinase activities, respectively. A continuous-flow cell-free WGE translation system was used to synthesize active dihydrofolate reductase (21), while RRL has been utilized to express functional 2'–5'-oligoadenylate synthase (22). Human

hepatitis B virus (HBV) polymerase was expressed by RRL-coupled transcription/translation. The in vitro expressed polymerase possessed protein priming activity demonstrated by [³²P]-dGTP labeling. In addition, polymerization activity was evident by synthesis of HBV-specific DNA products between 100 and 500 nucleotides. Polymerization activity was also detected in in vitro polymerase assay by incorporation of radionucleotides into acid precipitable polynucleotides (23).

Proteins with incorporated biotinylated lysines can also be assayed for enzymatic activity. For example, pectin methylesterase was assayed enzymatically from TnT[®] and Transcend[™] Systems (24).

Other Functions

Biologically active proteins have been successfully expressed in vitro. Active human interleukin-6 (IL-6) has been synthesized using a continuous-flow cell-free WGE translation system (26), as has interleukin-2 (IL-2) (27).

The functional assembly of protein subunits into a mature, active complex has been demonstrated for fibrinogen (28), connexins (29) and steroid hormone receptors (30). The expression of functionally active complexes requires the presence of canine microsomal membranes for both fibrinogen and connexins, and the activity of functional fibrinogen also requires the presence of oxidized glutathione during translation. The assembly of connexin proteins into functional gap junction channels was demonstrated using single channel conductance for connex-

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ins that were integrated into microsomal vesicles. Rabbit reticulocyte lysate contains a multiprotein system that assembles steroid hormone receptors and, in particular, the glucocorticoid receptor, into an active heterocomplex with heat shock protein 90 (hsp90).

The addition of oxidized glutathione to in vitro expression systems to allow for the synthesis of active proteins is not uncommon and is necessary for the expression of active mannose-6-phosphate-specific receptor (30). In this instance, protein activity was determined using phosphomannan affinity chromatography.

PFK1 and PFK2 coding for the subunits of 6-phosphofructokinase were cloned into plasmids. In vitro translation products resulted, using RRL as the synthesis and folding system. Folding and assembly of both the α - and β -subunits of 6-phosphofructokinase occurred, resulting in an enzymatically active protein. The in vitro-generated enzyme exhibited a folding state similar to that of the heterooctameric 6-phosphofructokinase, as demonstrated by size exclusion followed by ELISA (32).

The assembly of intact viral capsids in vitro has been demonstrated for a number of viral coat proteins, including human immunodeficiency virus type 1 (HIV-1; 33) and human papillomavirus type 16 (HPV-16; 34). Rabbit reticulocyte lysate in vitro translation was used to create HIV capsids in vitro, and these in vitro capsids appeared essentially identical to immature capsids produced in vivo as demonstrated by electron microscopy. Expression of the HPV L1 protein in RRL resulted in the assembly of virus-

like particles that closely resembled papillomavirus virions and that retained various conformational epitopes.

More recently, the use of in vitro transcription/translation systems for in vitro expression cloning (IVEC[®]) has become more widely used as an alternative to library screening with either nucleic acid or antibody probes. This method of cloning is discussed in more detail in Chapter 6.

Finally, the use of coupled in vitro transcription/translation systems may be used in the future for the in vitro evolution of catalytic function (35). Laboratory evolution has been carried out successfully with RNA molecules, and studies by Joyce and coworkers were able to operate isothermal RNA amplification and in vitro translation simultaneously with the TNT[®] RRL System^(a,b,c,e). The problem of colocalization of gene and protein product remains for this type of in vitro evolution system.

Thus a wide variety of proteins can be expressed in their active states in vitro, and these activities can be measured using many different types of assays and techniques. The success of expressing and detecting a specific protein will depend highly on the efficiency with which the particular protein of interest is translated and folded in the in vitro expression system used, as well as the potential background activities that may be present in the expression system itself. For many proteins, this information can only be obtained empirically by experimentation and optimization of both protein expression and functional detection.

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