Leishmania tarentolae for the Production of Multi-subunit Complexes

10

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Abstract

Multi-subunit protein complexes are involved in a wide variety of cellular processes including DNA replication, transcriptional regulation, signal transduction, protein folding and degradation. A better understanding of the function of these protein complexes requires structural insights into the molecular arrangement and interactions of their constituent subunits. However, biochemical and structural analysis of multi-subunit protein complexes is still limited because of technical difficulties with their recombinant expression and reconstitution. This chapter presents an overview of a novel protein expression system based on *Leishmania tarentolae*, a unicellular protozoan parasite of lizards, and practical considerations for the production of multi-subunit protein complexes. The *Leishmania tarentolae* expression system offers fully eukaryotic protein expression with post-translational modifications but with ease of handling similar to bacteria. This chapter also summarizes studies on the production of laminins, large heterotrimeric glycoproteins of the extracellular matrix, using this expression system. In addition, a recently developed *Leishmania tarentolae*-based cell-free translation system is briefly described.

Keywords

Cell-free translation • Heterologous protein expression • Laminin chain assembly • Protozoan parasite • Trypanosomatids

10.1 Introduction

Protozoan parasites of the genus *Leishmania* belong to the family Trypanosomatidae, which comprises unicellular organisms characterized by the presence of a single flagellum and a unique mitochondrial DNA-containing organelle, the

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kinetoplast [1–4]. *Leishmania* parasites are transmitted to their vertebrate hosts by the bite of infected female phlebotomine sandflies, and alternates between two life-forms: an extracellular promastigote form living in the digestive tract of female sandflies and an intracellular amastigote form residing in vertebrate macrophages. More than 20 *Leishmania* species have been reported to cause a wide spectrum of tropical diseases, collectively termed leishmaniasis [5–7]. According to a recent report from the World Health Organization, there are an estimated 1.3 million new cases of, and 20,000–30,000 deaths from leishmaniasis annually in 98 countries worldwide [8].

Not all members of genus *Leishmania* are parasites of mammals. *Leishmania tarentolae* (*L. tarentolae*) is a lizard-infecting species, which was first isolated from the Moorish gecko, *Tarentola mauritanica* (Fig. 10.1) [9]. *L. tarentolae* does not cause pathology in humans nor in severe combined immunodeficient (SCID) mice [10]. This lack of pathogenicity has been addressed in several studies comparing *L. tarentolae* with pathogenic *Leishmania* species [11–13]. One study showed that several of the genes expressed preferentially in the intracellular amastigote form of pathogenic *Leishmania* species are lacking from *L. tarentolae*, providing a possible explanation for why *L. tarentolae* is unable to replicate efficiently in mammalian macrophages [13].

As *L. tarentolae* grows rapidly in simple nutrient media and is not pathogenic to humans, it has been used as a model organism for studying unique features of Trypanosomatids, such as RNA editing and polycistronic transcription [14–16]. In Trypanosomatids, messenger RNAs (mRNAs) are transcribed as polycistronic precursors that are post-transcriptionally processed into individual mRNAs by trans-splicing and polyadenylation (Fig. 10.2) [17, 18]. Trans-splicing adds a capped 39-nucleotide spliced leader sequence to the 5′ end of the mRNA, which is necessary for RNA transport, stability, and translation efficiency. In these organisms, regulation of gene expression occurs predominantly post-transcriptionally through the structure of the intergenic untranslated regions (UTRs) [19].

Using the unique features of protozoan parasites, Breitling et al. developed a novel eukaryotic expression system based on *L. tarentolae* for the production of recombinant proteins [20]. Constitutive or inducible expression of target proteins for the cytosolic or secretory pathway is possible, and the expression vector can be either stably integrated into the genome or maintained episomally [21, 22]. Now that the *L. tarentolae* protein expression system has been commercialized by Jena Bioscience GmbH (http://www.jenabioscience.com/), it is being used more widely. There are increasing reports of heterologous protein expression in *L. tarentolae*; however, few examples of the expression of multi-subunit

Fig. 10.1 ***L. tarentolae*** **cells expressing green fluorescent protein (GFP) and their natural host**, ***Tarentola mauritanica*** **.** Scale bar = 5 μm

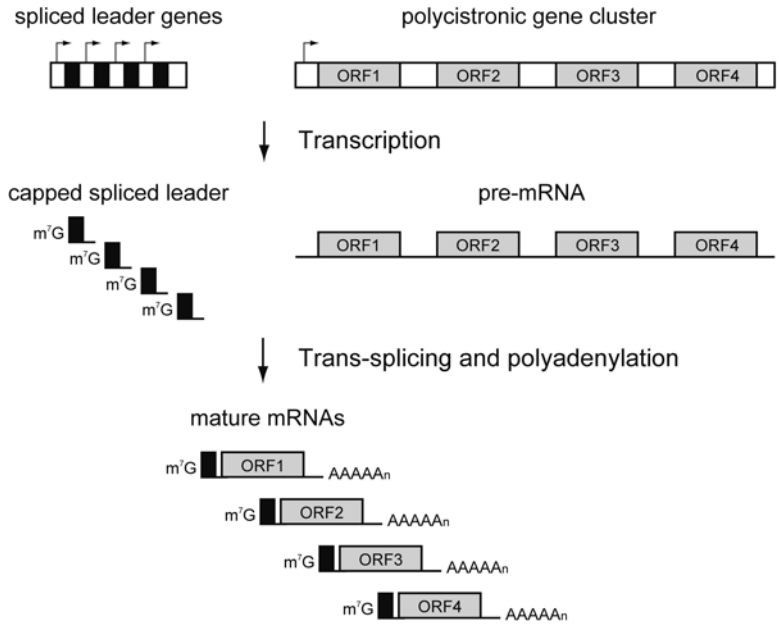

Fig. 10.2 The general process of mRNA synthesis in Trypanosomatids. Most *Leishmania* genes are organized in tandem repeats as indicated by the *grey boxes*. At another chromosomal location there are several hundred tandem direct repeats encoding spliced leader RNAs (*black boxes*). In some cases, a single promoter is present upstream of the first gene in the cluster, whereas in the spliced leader gene cluster each repeat is presumed to bear a transcriptional promoter. After transcription, the polycistronic pre-mRNA is processed by trans-splicing and polyadenylation to produce mature mRNAs

proteins have been reported [23–30]. This chapter outlines the *L. tarentolae* expression system and our research on multi-subunit protein expression in this system.

10.2 Maintenance of *L. tarentolae* Cells

The culture of *L. tarentolae* is much easier than that of mammalian cells. Because it is not pathogenic to mammals, it requires only biosafety level 1 facilities. *L. tarentolae* cells can be grown in brain–heart infusion broth without serum but supplemented with hemin, which is essential for growth of heme-deficient organisms such as Trypanosomatids [31, 32]. *L. tarentolae* cells require aerobic conditions, so the cells are maintained in a suspension culture in ventilated tissue culture flasks. A CO_2 incubator is not necessary. Conventional static cultures are incubated in the dark at 26 °C. Agitated cultures for protein expression are incubated on an orbital shaker at 140 rpm using Erlenmeyer flasks. *L. tarentolae* cells can be grown indefinitely *in vitro*, with a doubling time of around 5 h and to high cell densities in shaking culture (approximately 5×10^8 cells/mL).

10.3 Expression Vectors for the *L. tarentolae* Expression System

In most eukaryotes, ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) are transcribed by RNA polymerase I and III, respectively, and the protein-coding genes are transcribed by RNA polymerase II to yield mRNAs [33]. In Trypanosomatids, however, translation of RNA polymerase I-generated transcripts is possible because of trans-splicing of polycistronic pre-mRNAs [17]. In a *L. tarentolae* expression system, integration of an expression cassette into the chromosomal small-subunit (ssu) rRNA locus enables the generation of large numbers of transcripts for constitutive expression of target proteins [20]. Thus, the expression cassette is flanked by two fragments of the ssu rRNA locus for homologous recombination, and contains three optimized UTRs, flanking the target and marker gene insertion sites, which provide the trans-splicing signal (Fig. 10.3).

In this system, alternative cloning strategies allow heterologous proteins to be expressed cytosolically or secreted into the medium. To promote secretion, a signal sequence derived from the secreted acid phosphatase of *L. mexicana* is uti-

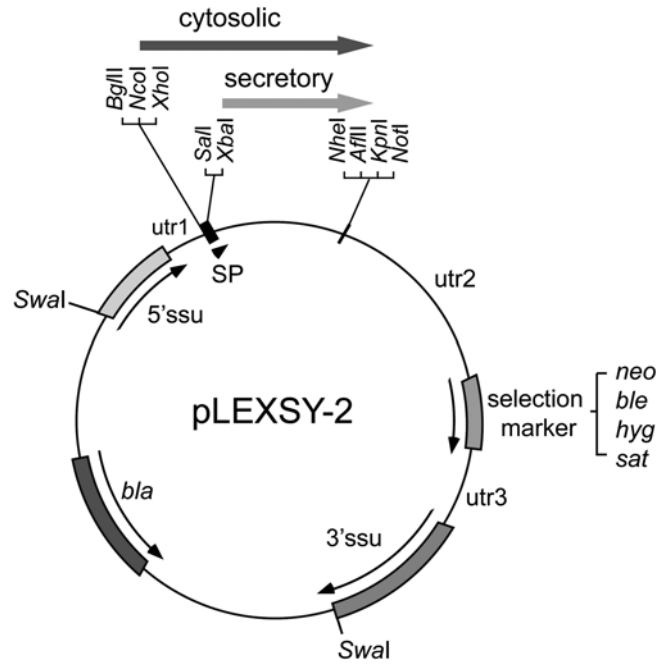

Fig. 10.3 Map of the *L. tarentolae* expression vector. 5′ssu and 3′ssu are regions for homologous recombination into the host chromosome following linearization of the expression plasmid with *Swa*I. The vector contains three optimized intergenic untranslated regions (UTRs) for post-transcriptional mRNA processing: utr1 derived from the 0.4 kb intergenic region (IR) of the *L. tarentolae* adenine phosphoribosyl transferase gene; utr2 from the 1.4 kb IR of the calmodulin cluster containing three tandemly arranged calmodulin genes; and utr3 from the 1.7 kb IR of the *L. major* dihydrofolate reductase–thymidylate synthase gene. SP indicates the signal peptide of *L. mexicana* secreted acid phosphatase. Alternative cloning strategies result in cytosolic or secretory expression of the target protein. As selection markers, four genes are available: *neo*, *ble*, *hyg*, and *sat* genes

lized [34]; however, the native signal sequence has also been used successfully for secretion of several proteins [20, 35]. Use of the native signal sequence may enable native processing of proteins at the N terminus.

After construction, the expression plasmid is linearized and integrated into the genome of *L. tarentolae* by homologous recombination. *L. tarentolae* cells can be routinely transfected with plasmid DNA by electroporation and the transfected cells can be selected with antibiotics. Currently, four selectable marker genes are available in this system: neomycin phosphotransferase (*neo*), hygromycin phosphotransferase (*hyg*), bleomycin resistance protein (*ble*), and streptothricin acetyltransferase (*sat*) that confer resistance to G418, hygromycin, bleomycin, and nourseothricin, respectively. Therefore, up to four genes can be simultaneously expressed to produce multi-subunit proteins. If additional selection marker genes, for example, puromycin acetyltransferase (*pac*), are incorporated into the expression vector, more than four genes could be simultaneously expressed. The construction of markerless *L. tarentolae* strains carrying multiple expression cassettes may be possible, but is known to be difficult [36]. Platforms for inducible expression of target proteins are available; however, only one, or at most two, selectable marker genes are offered.

10.4 Post-translational Modifications (PTMs) in *L. tarentolae*

In contrast to prokaryotic systems, expression of recombinant proteins in eukaryotic expression systems allows PTMs. Protein glycosylation is one of the most common PTMs in eukaryotes, and it plays essential roles in many biological processes, such as cell recognition, cell-cell communication, signaling, embryo development, and immunity [37, 38]. The pattern of N-linked glycosylation of glycoproteins is important because the number and position of N-linked oligosaccharides often have significant effects on protein function [39, 40]. *L. tarentolae* has been reported to produce higher eukaryote-like biantennary N-glycans with terminal galactose and core fucose but lacking sialic acid residues, indicating that the N-glycosylation pathway of *L. tarentolae*

is more similar to that in mammals than in yeast and insect cells [20]. It has been recently demonstrated that human soluble amyloid precursor protein α (sAPPα) produced in *L. tarentolae* was both N- and O-glycosylated on similar sites as described for mammalian-expressed sAPPα [41]. However, more complex O-glycan structures commonly found in mammalian expression systems were not observed. This insufficient glycosylation is probably due to the lack of certain glycosyltransferase activities in *L. tarentolae* [20]. Genetic engineering of the *L. tarentolae* host or *in vitro* glycosylation using specific glycosyltransferases may provide a method for producing glycoproteins with more complex glycan structures.

L. tarentolae has the potential to perform other eukaryotic PTMs, including processing of signal sequences, proper protein folding, and disulfide bond formation. Previously, we successfully produced the disulfide-linked heterotrimeric glycoprotein, laminin-332, in the *L. tarentolae* expression system [42]. In the following section, the production of multi-subunit proteins in the *L. tarentolae* expression system is described.

10.5 Production of Recombinant Laminin-332 Using the *L. tarentolae* Expression System

Laminins are a family of extracellular matrix glycoproteins localized in the basement membrane, and bind to cell surface receptors such as integrins, supporting various cellular functions including adhesion, migration, proliferation, and differentiation [43–45]. They consist of three subunits, α, β, and γ chains, which bind to each other via disulfide bonds between laminin coiled-coil (LCC) domains to form a cross-shaped structure with three short arms and one long arm (Fig. 10.4a). To date, five α, three β, and three γ chains have been identified to combine into at least 16 heterotrimeric isoforms [46, 47]. They are named according to their chain composition; for example, laminin-111 consists of α1, β1, and γ1 chains.

There is a great need to develop a method for the efficient and mass production of recombinant laminins because some laminin isoforms (laminin-511 and -332) are able to support the stable culture of human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) [48–51]. Chemically defined, xeno-free, and feeder-free culture systems are required for future use of hESCs and hiPSCs in regenerative medicine, and recombinant laminins can be used to replace feeder cells. Because they are large heterotrimeric proteins (400–900 kDa), it is difficult to express correctly folded laminins in bacterial and yeast expression systems. Thus, mammalian cells have been used to prepare recombinant laminins; however, mass production of recombinant laminins remains laborious.

The *L. tarentolae* expression system combines the ease of handling found with bacteria and yeast, with eukaryotic protein folding and mammalian-type PTMs of target proteins. These advantages of the *L. tarentolae* system prompted us to examine whether recombinant laminins produced in *L. tarentolae* acquire proper conformation and bioactivity. As a model for the production of laminins in *L. tarentolae*, laminin-332, which consists of α3, β3, and γ2 chains, was selected because it is the smallest laminin isoform, with truncated short arms in all three chains (Fig. 10.4a). The full-length cDNAs of laminin β3 and γ2 chains, without signal sequences, were cloned into the *L. tarentolae* expression vector (Fig. 10.3) behind the signal sequence of *L. mexicana* secreted acid phosphatase. The α3 chain undergoes extracellular proteolytic processing of both ends in mammals [52–54], so the cDNA of the α3 chain containing the fully processed form was cloned into the expression vector in the same way as β3 and γ2 chains. These three plasmids were sequentially transfected into *L. tarentolae* cells by electroporation, and stable transfectants were selected by culturing cells on solid media with three antibiotics.

For the assembly of the laminin chains, the β and γ chains first assemble to form heterodimers in the endoplasmic reticulum after translation of individual chains [55]. Subsequently, one α chain joins the β–γ heterodimers to form α–β–γ heterotrimers, which are transported through the

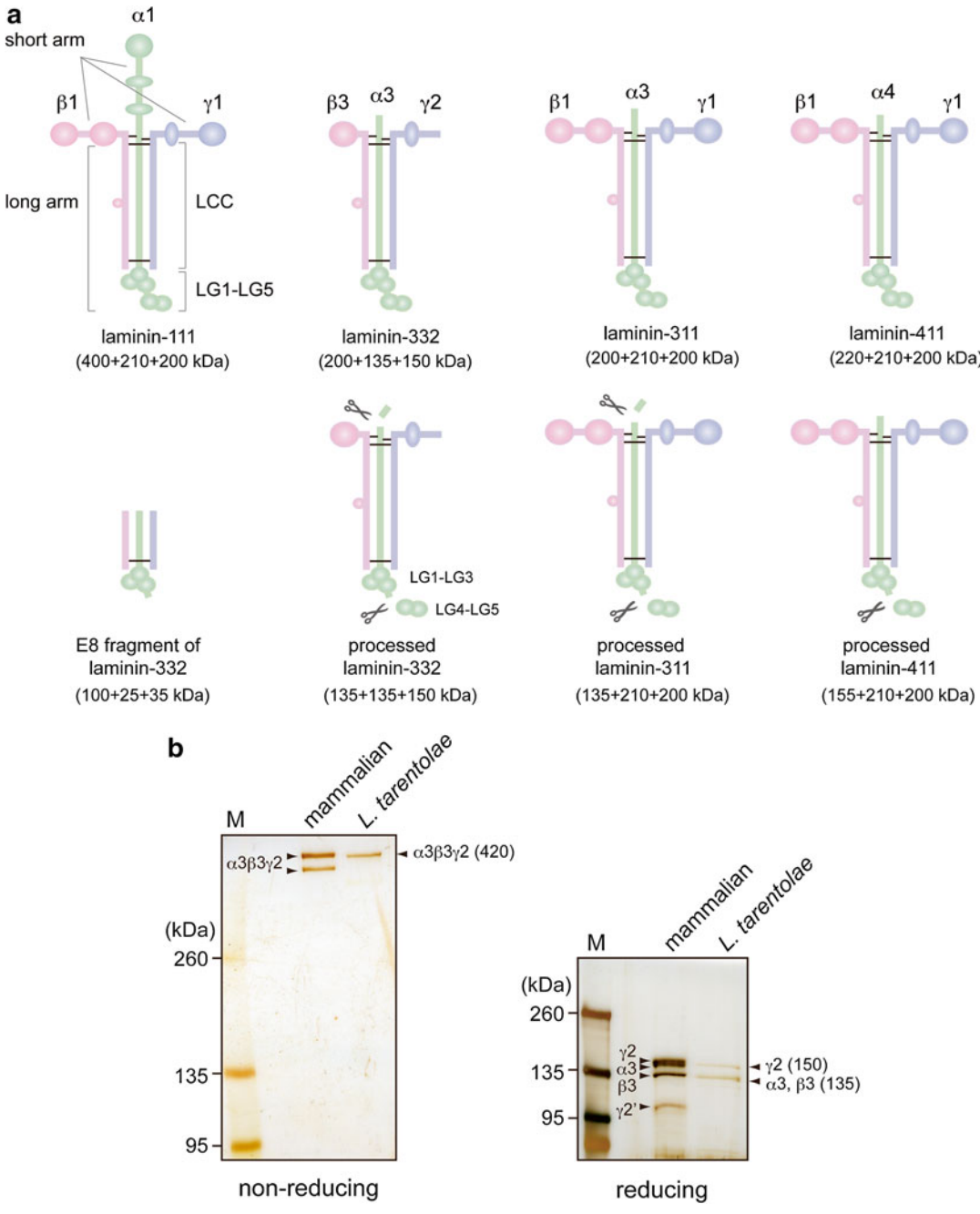

Fig. 10.4 Production of recombinant laminins using the *L. tarentolae* expression system. (**a**) Schematic structure of laminin isoforms. The α, β, and γ chains assemble to form a triple-stranded α-helical coiled-coil structure in the laminin coiled-coil (LCC) domain. The laminin globular (LG) domains are typically involved in cellular interactions. The size of each chain is shown below the laminins. Physiological cleavage by enzymes known to occur for the α3 and α4 chains is indicated by scissors. The laminin isoforms produced successfully in *L. tarentolae* expression system are shown in the lower panel. (**b**) Purified laminin-332 from mammalian 293-F cells (*left lane*) and *L. tarentolae* cells (*right lane*) were separated by SDS-PAGE under non-reducing or reducing conditions and analyzed by silver staining

secretory pathway. Single cysteine residues at the C termini of the β and γ chains form an interchain disulfide bond. At the N termini of the LCC domains, all three chains have two cysteine residues and are disulfide linked to each other before secretion. Accordingly, the heterotrimer can be viewed by SDS-PAGE under reducing and nonreducing conditions. The recombinant *L. tarentolae* strain, harboring the three subunits of laminin-332, efficiently formed α3–β3–γ2 heterotrimers (~420 kDa) with disulfide bonds and secreted it into the medium, demonstrating for the first time that the three chains of semi-intact laminin can form heterotrimers in a unicellular eukaryote (Fig. 10.4b) [42]. Hydrophobic interactions within the α-helical coiled-coils are the main driving force for laminin chain assembly, so synthetic peptides or small fragments of the LCC domains of the three subunits can assemble themselves *in vitro* [55]. However, assembly of the whole unprocessed laminin chains is difficult to achieve *in vitro* or in bacterial and yeast expression systems, probably because the individual chains need to fold correctly before assembly. Correct folding is also required to facilitate the proper positioning of cysteine residues, which allows the correct formation of intra-chain disulfide bonds in the short arm region of all three chains and in the laminin globular (LG) domain at the N-terminal end of the α chain. As we were able to efficiently form laminin heterotrimers, *L. tarentolae* cells may provide the appropriate molecular chaperones to aid proper protein folding as well as a transport system for large proteins. When analyzing the recombinant *L. tarentolae* strains harboring only β3 or β3/γ2 subunits, the β3 monomers and the β3–γ2 heterodimers were detected in the cells but not secreted into the medium, suggesting that the monitoring system that allows only heterotrimers to be transported through the secretory pathway is also present in *L. tarentolae* cells [42]. The purified laminin-332 showed similar cell adhesion activity to laminin-332 purified from mammalian cells [42]. The production yield (about 0.5 mg per liter of culture medium) was also similar to that of mammalian cells [42].

10.6 Production of Other Laminins Using the *L. tarentolae* Expression System

The successful production of laminin-332 led us to investigate whether other isoforms of the laminin family could be produced in *L. tarentolae* (Fig. 10.4a). When the β1 and γ1 chains were swapped with the β3 and γ2 chains in the recombinant *L. tarentolae* strain, laminin-311 (~545 kDa) was formed and secreted into the culture medium [42]. In addition, α4 chain without the LG4–LG5 domains could assemble with β1–γ1 heterodimers to form laminin-411 (~565 kDa) (unpublished observation). However, it was difficult to express other α chains with or without LG4–LG5 domains in *L. tarentolae.* In fact, intact α chains can be often expressed but not folded correctly, and then are unable to assemble with β–γ heterodimers. These results suggest that larger and more complex laminin chains could not be expressed in *L. tarentolae* cells. Thus far, laminin-411 (~565 kDa) is the largest recombinant protein with multiple subunits produced in the *L. tarentolae* expression system.

The expression level of laminin-332 in *L. tarentolae* was insufficient for mass production despite it being the smallest laminin. Therefore, the laminin E8 fragment, which is a truncated laminin composed of the C-terminal regions of all three chains, was expressed in *L. tarentolae* (Fig. 10.4a). It contains the active integrin-binding site but lacks other activities of whole laminins such as heparin-binding activity; therefore, it serves as a functionally minimal form that efficiently maintains hESCs and hiPSCs [50, 51]. When the three chains corresponding to the E8 fragment of laminin-332 were expressed in *L. tarentolae*, these chains successfully formed heterotrimers (~160 kDa) and were secreted into the culture medium (unpublished observation). The expression level was at most twice that of the processed form of laminin-332. For high-level expression of laminin heterotrimers in the *L. tarentolae* expression system, careful optimization of culture conditions might be required.

10.7 An *L. tarentolae*-Based Cell-Free Translation System

Cell-free translation systems offer several advantages over cell-based expression systems, including the synthesis of difficult targets, such as toxins and membrane proteins, the easy modification of reaction conditions, suitability for high-throughput strategies, and rapid production [56–58]. Although any organisms could potentially be used as sources for the preparation of a cell-free translation systems, the most popular are those based on *Escherichia coli*, wheat germ, and rabbit reticulocytes. The choice of the system should be determined by the biochemical nature of the target protein and the downstream application. In general, *Escherichia coli*-based systems provide higher yields and more homogeneous proteins suitable for structural studies. Eukaryotic cell-free systems, although less productive, provide a better platform for functional studies, particularly for proteins with PTMs. We have previously expressed laminin-332 subunits in a cell-free translation system based on insect cell extract [59]. β3–γ2 LCC domain heterodimers (~130 kDa) and α3–β3–γ2 LCC domain heterotrimers (~200 kDa) were successfully formed with disulfide bonds following co-translation of each chain, however, intact β3 and γ2 chains were unable to form β3–γ2 heterodimers, indicating that the proper folding of laminin-332 subunits that included the short arm region was deficient in this system.

Recently, Alexandrov's group developed a eukaryotic cell-free translation system based on extracts of *L. tarentolae* cells [60–62]. They discovered species-independent translational sequences that mediate efficient cell-free protein synthesis in any prokaryotic and eukaryotic systems, and applied them to express proteins in *L. tarentolae* cell extract. Moreover, addition of an anti-spliced leader oligonucleotide to *L. tarentolae* cell extract suppressed the translation of endogenous *L. tarentolae* mRNAs. Using this system, Guo et al. could produce *in vitro* all six subunits of the 600 kDa HOPS and CORVET multi-subunit membrane tethering complexes [63]. This cell-free translation system is also available from Jena Bioscience GmbH. Although a limited number of proteins have been tested because of the recent development of this product, this system may be suitable for high-throughput analysis of expression of multi-subunit proteins.

10.8 Conclusions

In this chapter, the production of multi-subunit proteins using the *L. tarentolae* expression system was discussed. Laminin-332, a large heterotrimeric glycoprotein, could be produced using the *L. tarentolae* expression system, however, it was not in intact form but in processed form, suggesting that *L. tarentolae* cells do not have the same protein folding machinery as mammalian cells for expression of large proteins with complex structures like laminin α chains. Using the *L. tarentolae* expression system, laminin-332 subunits could assemble to form heterotrimers with disulfide bonds and were secreted into the culture medium, whereas it is difficult in bacterial and yeast expression systems. Thus, the *L. tarentolae* system provides an alternative platform to mammalian cells for the production of multi-subunit proteins. Up to four genes can be introduced into *L. tarentolae* cells to produce a stable cell line that can be scaled up to larger volumes. The drawbacks of this system include the limited number of expressible genes and the long experimental time line. One round of transfection and clonal selection can take up to 2 weeks (Fig. 10.5). Cell-free translation systems, where an unlimited number of genes can be co-expressed simultaneously, overcome these drawbacks. Although cell-free translation systems are relatively high cost and low yield, a recently developed cell-free system based on *L. tarentolae* enabled rapid production and reconstitution of six subunits of the multimeric membrane tethering complexes. With the range of expression systems now available, it is important for researchers to understand their advantages and disadvantages so the optimal expression systems can be selected, depending on the purpose of the target proteins [64, 65]. Both expression systems based on *L. tarentolae*

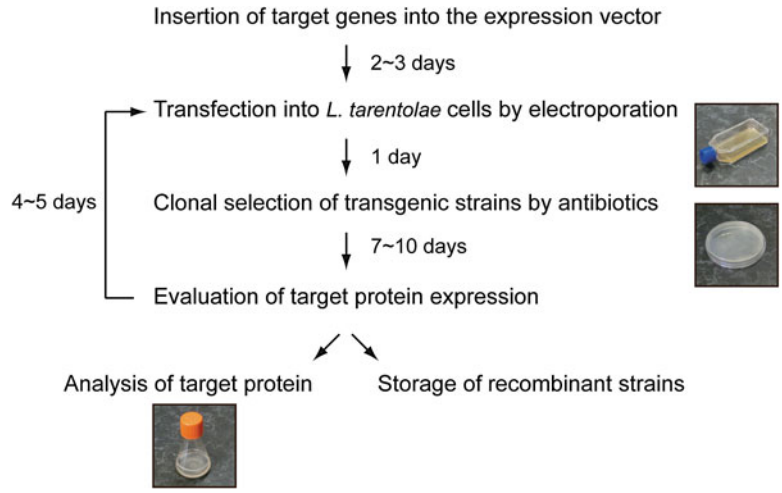

Fig. 10.5 The workflow for the production of multi-subunit proteins using the *L. tarentolae* expression system

are relatively new, so there are few examples of multi-subunit protein expression using them. The structural and biochemical analysis of many other multi-subunit proteins will benefit from the use of these expression systems, and will lead to future improvements in the technology.

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