Developing a system capable of expressing heterologous proteins

Theses of the doctoral dissertation

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Introduction

Production of peptides and proteins is one of the most important field of biotechnology. The isolation of the protein of interest from its natural source is often hindered by economical, legal and/or ethical reasons. In some cases the natural source does not even exist since the protein in question is a variant or fragment of a natural protein, produced by the tools of molecular biology.

The expression systems provide solution for the above mentioned challenge. These systems using the ingenous protein expression apparatus of the choosen host are able to produce theoretically any protein based on the genetic information that is coded on the DNA fragment introduced into the host cell. The most important factor, regarding to the protein production, is the host organism. Nowdays a wide range of hosts are available: it can be prokaryotic, unicellular or higher eukaryotic organism (for example: yeast, plant or mammalian), tissue culture or *in vitro* translational system.

Among the expression hosts the yeast have several outstanding features. Their handling is as easy and cost-effective as that of the prokaryotic systems. The product produced by a yeast is socially acceptable, due to their utilization in the food industry for thousands of years.

The methylotrophic yeasts (for example the *Pichia pastoris*) have prominent importance among the yeast hosts, its industrial utilization is motivated by several advantages besides those mentioned above:

- The methanol inducible alcohol oxidase promoter results in a exceptionally high level of transcription which can be strictly controlled.
- Using secretion signals the overproduced polypeptide can be secreted into the media. This way the product recovery is more effective since the cell secrets only a few of its natural proteins, and using a media with low protein concentration is possible.
- As a result of the above features a high level of the product can be achieved inside or outside of the cell. It can be in the range ofa few gramms per liter of the media.
- In comparison to *Saccharomyces cerevisiae*, from several point of view the glycosylation pathways are more similar to the higher eukaryotes, thus the reduced/lost functionality or antigenicity of the protein, as a consequence of the improper glycosylation can be avoided.
- Thanks to the proper posttranslational modifications, several proteins that require complex modification were expressed in an active form.
- The fermentational processes can be scaled up routinely, methods for optimizing the production are available.

There is no need for using expensive and complex media for propagation, which is an advantage over the insect or mammalian cell-line based expression systems.

As for any expression systems, it is also true for the yeast-based ones that their most important element is the vector molecule. It should provide the possibility for cloning the coding DNA in a flexible way, ensure the desired level of expression and/or the secretion of the given protein, and confer the stable maintenance of the expression construct in the genome of the expression host. To fulfill these requirements different genetic signals and sequence elements should be incorporated in the vector molecule. Perhaps the most important of them is the promoter, placed 5' to the coding region of the desired protein, that is responsible for transcriptional initiation. A selectable marker element is required to distinguish cells carrying the vector from cells without it. An element for effective maintenance of the vector molecule (integration/autonomous replication) is also required. The yeast vectors in most cases are shuttle vectors, i.e. they contain bacterial elements too (origin of replication, antibiotic resistance gene), because the construction and manipulation is carried out in *E.coli.*

Specific aims

The Bay Zoltán Institute for Biotechnology is comitted to do research and development, the results of which can be directly used in the practice. During this work we frequently have the task of production of plant, animal or human proteins in large quantities and in an active form. For this purpose one may choose a generally used or a patented expression systems. The usage of a prokaryotic system for production of eukaryotic proteins is limited becasuse they lack the proper posttranslational modification pathways. This drawback can be eliminated by using other systems based on higher organisms, but the expenditures in these cases would increase significantly. For this end we initiated the development of an expression system having the advantages of the methylotrophic yeasts but could be used without legal restrictions for all the necessary elements of the system would be developed in our institute..

The development of such a system requires the selection of a yeast strain that fits to our purpose; isolation and characterization of a promoter that can be used in the expression vectors; isolation of sequence elements for plasmid maintenance and a marker gene for selection; and construction of plasmids ensuring easy insertation of the heterologous coding sequences. The thesis describes my most important results toward the above mentioned aims.

Methods

In addition to the standard methods of molecular biology and microbiology the following techniques were used:

- during the rDNA analysis, genomic fragments, coding for rRNA were isolated by touchdown PCR, and after having their sequence determined the level of similarity to related sequences in the databanks was investigated.
- degenerate and gene-specific oligonucleotides were used in standard and one-sided PCR reactions to isolate the examined genes.
- the promoter activity in the natural host was followed by the detection of the produced mRNA in qualitative and semi-quantitative RT-PCR reactions.
- in the heterologous host (*Pichia pastoris*), the activity of the promoters were followed by measuring the activity of the β-galactosidase produced by the transcriptional fusions.
- the alcohol oxidase activity was detected in a peroxidase-coupled reaction.
- genomic libraries were constructed using partially digested genomic DNA of the Pichia sp. 159 strain. The libraries were used for selection of fragments carrying ARS function.
- the rate of plasmid loss in the yeast cells was quantified.

Resuts and discussion

- For molecular characterization, genomic fragments coding for rRNA were isolated from three yeast strains, and their sequence was determined.
- The strains were classified by comparing their partial rDNA sequences to known database entries. The result confirmed the initial classifications by conventional methods: two isolates were *Candida boidinii*, and the third belonged in the *Pichia genus*.
- As the first step toward the isolation of the alcohol oxidase promoters, four genomic segments coding for alcohol oxidases were isolated using degenerate primers designed to conserved regions of the known alcohol oxidases: one from each *Candida boidinii* strain (*AOX673* and *AOX680*) and two from *Pichia sp.* 159 (*AOXA* and *AOXB*).
- Based on the analysis of the rDNA and AOX sequences the *Pichia sp*. 159 strain was chosen for further investigations.
- Having known the sequences of the alcohol oxidase coding regions gene-specific primers were designed and used in one-sided PCR reactions to isolate the 5' and 3' non-coding regions of both *Pichia sp.* 159 *AOX* genes.
- The activities of the promoters were followed in RT-PCR reactions, in the presence of various carbon sources and during starvation, by detecting the specific mRNAs transcribed from *AOXA* and *AOXB*. In parallel, the enzyme activity was also detected. We found that both promoters can be induced with methanol. Glucose strongly repressed the expressions from both promoters, but not to the same extent; a low level of *AOXA,* but not *AOXB*-specific expression was detected in the presence of glucose.
- With the exception of this last finding, the activity of the *AOXB* promoter was in all experiments higher than that of *AOXA:* for example, the level of the *AOXB*-specific mRNA was three times higher than that of the *AOXA* mRNA in cultures induced with 0.5% methanol, as measured in semi-quantitative RT-PCR.
- In a time-course experiment, the *AOXB*-specific message was detected after 1 hour of methanol induction, while the *AOXA* mRNA level reached the detection threshold 1 hour later.
- Mannitol and glycerol proved to be weak inducers. Starvation resulted in the transient induction of both promoters. Since glycerol is a poorly metabolizable carbon source, its effect on the promoters could be similar to that of starvation.
- Model expression vectors were constructed in which the expression casette contained the lacZ reporter gene under the control of the *AOXA* or *AOXB* promoters. The vectors also contained a genomic replication origin that was selected from a genomic library of the *Pichia sp.* 159 strain, and had either the dominant Zeocin resistance marker or the *HIS4* gene isolated from *Pichia sp.* 159 as a selection element.

- The model expression vectors were used to test the functionality of the *AOX* promoters in a heterologous species. In *Pichia pastoris,* the *AOXB* promoter functioned similarly as in *Pichia sp.* 159: methanol resulted in a strong induction, with a level comparable to that of the *AOX1* promoter of *P. pastoris*, while glucose repressed this activity. Starvation also induced the *AOXB* promoter in this host.
- In contrast, only a very low level of transcription was detected from the *AOXA* promoter, which did not vary after the change of the carbon source. The regulatory elements of this promoter are presumably not compatible with the transcription regulatory apparatus of the heterologous expression host.
- The experiments on heterologous expression confirmed the functionality of the replication and selection elements of the vector.
- Our results have provided data concerning the activities of the promoters under different circumstances, and furnish a solid foundation for practical applications.

Publications

Szamecz B, Urbán G, Rubiera R, Kucsera J, Dorgai L. 2005. Identification of four alcohol oxidases from methylotrophic yeasts. *Yeast* 22: 669-676

Urbán G, **Szamecz B**, Dorgai L. 2005. Identification of an autonomously replicating element from *Pichia* sp 159. manuscrript in prep.

Nielsen KH, **Szamecz B**, Valasek L, Jivotovskaya A, Shin BS, Hinnebusch AG. 2004. Functions of eIF3 downstream of 48S assembly impact AUG recognition and GCN4 translational control. *EMBO J.* 23: 1166-1177

Valasek L, Mathew AA, Shin BS, Nielsen KH, **Szamecz B**, Hinnebusch AG. 2003. The yeast eIF3 subunits TIF32/a, NIP1/c, and eIF5 make critical connections with the 40S ribosome in vivo. *Genes Dev* 17: 786-799.

Oral presentations

Béla Szamecz, Dr László Dorgai (supervisor). Experiments for developing a yeast system capable of protein expression (Hungarian)
XXIV OTDK, Section of Natural Science, Microbiology division, II. prize, Debrecen, April 1999

Klaus H. Nielsen, **Béla Szamecz**, Antonia Jitovovskaya, Leos Valasek, Alan G. Hinnebusch: In vivo evidence for critical functions of eIF3 in ribosomal scanning that impact GCN4 translational control
Translational Control Meeting Cold Spring Harbor, New York, U.S.A. September 2002

Klaus H. Nielsen, **Béla Szamecz**, Antonina Jivotovskaya, Leos Valasek, Alan G. Hinnebusch: *In vivo* evidence that translation initiation factor 3 functions in ribosomal scanning and *GCN4* translational control
XXIth International Conference on Yeast Genetics and Molecular Biology, Göteborg, Sweden July 2003

László Dorgai, **Béla Szamecz**, Gabriella Urbán: Development of an expression system based on a newly isolated methylotrophic yeast
1st Central European Forum for Microbiology (CEFORM) and the Annual Meeting of the Hungarian Society for Microbiology Keszthely, Hungary October 2005

Posters

Béla Szamecz, Gabriella Urbán, László Dorgai: Isolation and analysis of *His4*, a potential selection marker in a *Pichia* sp.
1st Central European Forum for Microbiology (CEFORM) and the Annual Meeting of the Hungarian Society for Microbiology Keszthely, Hungary October 2005

Gabriella Urbán, **Béla Szamecz**, László Dorgai: Identification and characterization of an autonomously replicating element from a *Pichia* sp.
1st Central European Forum for Microbiology (CEFORM) and the Annual Meeting of the Hungarian Society for Microbiology Keszthely, Hungary October 2005

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