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Development of an insect cell factory for the production of complex biopharmaceuticals using a synthetic biology approach

João Miguel Nunes Vidigal

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Index

Abbreviation list

AcIE1 promoter – Immediate-early promoter from *Autographa californica* (Ac)

BEVS - Baculovirus Expression Vector Systems

CHO - Chinese Hamster Ovary

DIG – Digoxigenin

DNA - Deoxyribonucleic acid

Dox - Doxycycline

G418 – Geneticin Antibiotic

dsRED – Red fluorescent protein

E. coli - *Escherichia coli*

ECL - Enhanced chemiluminescence

eGFP – Enhanced green fluorescent protein

ES - Embryonic stem

FLP - Flippase

Flp-RMCE - Flp-based recombinase-medicated cassette exchange

Fn - FRT variants

FRT - FLP recognition target sites

GOI - Gene of interest

HeLa cells - Cell line derived from the cervical cancer cells of patient Henrietta Lacks.

hESCs - Human Embryonic stem cells

iPS - Induced pluripotent stem cells

LB – Lysogeny broth

Mtn - Metallothionein

OpMNPV -*Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus

Ori - Origin of replication

PBS - Phosphate-buffered saline

PCR – Polymerase Chain Reaction

PhCMV - human Cytomegalovirus minimal promoter

RMCE - Recombinase mediated cassette exchange systems

RT - Recognition-target

rtTA – Tetracycline transactivator

SSR - Site-specific Recombination

Sf9 – Cell line derived from *Spodoptera frugiperda ovarian cells.*

TAFII40 – TATA binding protein-associated factor II 40

TBP – Tata Binding Protein

Tet - Tetracycline

TetO - Tet operator

Tet-Off system – Inducible system which needs tetracycline to shut off.

TetR - Tet repressor

TFIIB – Trascription Factor II B

TIRs - Translation initiation regions

tTA - Tetracycline transactivator

TRE – Tetracycline response element

1.Preface

This master thesis is within the scope of the project PTDC/EBB-EBI/102266/2008, entitled "Novel Cell Factories for the production of secreted complex bioproducts: a syntetic biology approach for improved product stoichiometries" funded by the Portuguese Fundação para a Ciência e Tecnologia (FCT). The global aim of this project is to develop a flexible and re-usable insect cell line able to produce different correctly assembled multi-component biopharmaceuticals at relevant yields. The transcription rate of the different components will be stoichiometrically tuned by bridging synthetic biology and molecular biology approaches, namely by combining transcriptional regulatory circuits and the recombinase mediated cassette exchange (RMCE) technology.

The majority of the results described in this thesis were presented at several international scientific meetings:

Fernandes F, **Vidigal J**, Teixeira AP, Coroadinha AS, Alves PM *"Implementation of Recombinase Mediated cassette Exchange Systems in Sf9 cells for expression of multiple recombinant proteins"*, *22nd ESACT Meeting 2011,* Viena, Austria.

Fernandes F, **Vidigal J**, Coroadinha AS, Teixeira AP, Alves PM "*Implementation of Synthetic Circuits in Sf9 Insect Cells at Tagged loci generated by Recombinase Mediated Cassette Exchange Systems"*, *5th Annual Workshop on the Business-Government Interface: Systems and Synthetic Biology*, June 2011, Braga, Portugal.

Fernandes F, **Vidigal J**, Tomás H, Teixeira AP, Coroadinha AS, Alves PM *"Fast insect cell line development for production of complex biopharmaceuticals"*, *11thCHEMPOR 2011, Sept 2011,* Caparica, Portugal.

Fernandes F, **Vidigal J**, Tomás H, Teixeira AP, Coroadinha AS, Alves PM *"Development of an insect cell factory for production of complex biopharmaceuticals using flipase-mediated cassette exchange"*, *10th PEACe, Sept 2011,* Cascais, Portugal.

Fernandes F, **Vidigal J**, Tomás H, Carrondo MJT, Coroadinha AS, Teixeira AP, Alves PM *"Exploring novel approaches to produce virus-like particles"*, *2nd ENVVP,* Oct 2011, Frankfurt, Germany.

2.Abstract

Insect cells, in particular the *Spodoptera frugiperda* Sf9 cell line, are a popular system for the production of biologically active recombinant proteins. However, the current technology uses baculovirus infection which has two main disadvantages: firstly, the recombinant gene is only expressed transiently during the infection cycle, after which cells die; secondly, due to the lytic nature of this system, the cellular protein processing machinery is severely compromised at the end of the infection cycle, affecting the correct formation of recombinant proteins whose expression is usually controlled by very-late baculovirus promoters.

Stably transformed insect cell lines represent an alternative system for continuous protein production. However, their establishment is laborious, requiring the identification of cell clones that display the right expression properties due to random integration of the gene-of-interest. Furthermore, independently of the expression system, multimeric biopharmaceuticals continue to have very low yields, mainly because the stoichiometry between components is not properly reached in the producer cells.

To overcome these issues, this report shows work towards the development of a novel Sf9 cell factory combining (i) recombinase mediated cassette exchange (RMCE) for targeted gene integration and (ii) a tetracycline (Tet) inducible system for the modulation of gene expression levels.

We started by evaluating the strength of different promoters to drive GFP expression in Sf9 cells as well as different transfection protocols. The baculovirus promoter OpIE2 allowed the strongest expression when compared to others, and the efficiency of transfection was significantly higher using the lipotransfection method. From the different transfection protocols, 4 cell populations with distinct fluorescence distributions were obtained after 3 weeks under hygromycin selection. These populations were independently subject to limiting dilution and isolated cell clones were analyzed by southern blot to screen for single-copy integration of the tagging GFP construct. While the majority of clones were single copy, cassette exchange was performed in a few clones by co-transfection with flippase and the target plasmid containing dsRed. Cells were selected in the presence of neomycin and cassette exchange was confirmed by PCR of genomic DNA, revealing the absence of tagging cassette and the presence of the target cassette in the some locus.

In parallel, the functionality of a tetracycline inducible system in Sf9 cells was evaluated by substituting mammalian promoters with insect cell specific promoters. However, transactivation was suboptiomal, requiring additional changes to the original system.

In summary, this work reports for the first time the implementation of a RMCE system in Sf9 cells and a preliminary assessment of Tet transactivation in these cells. This new cell line will be a major breakthrough since it will combine the advantages inherent to Sf9 cell growth, continuous protein production, the ability of re-using a well characterized locus for targeted DNA integration and inducible gene expression.

Keywords: Sf9 cells, cell line development, multimeric biopharmaceuticals, RMCE systems, Tetracycline inducible systems.

3.Resumo

A cultura de células de insecto conjugada com a infecção por baculovirus representa um dos sistemas biológicos de eleição para a produção de proteínas recombinantes biologicamente activas. Há vários biofármacos em ensaios clínicos e alguns já no mercado, como é o caso da vacina contra o vírus do papiloma humano (Cervarix, GSK), que utilizam este tipo tipo de sistema de expressão. No entanto, o uso de baculovirus representa um sistema de expressão transiente / lítico inerente ao processo de infecção.

Em contraste, quando estavelmente transformadas, as células de insecto podem ser usadas como um sistema de expressão contínuo e não-lítico. Contudo, o desenvolvimento de linhas celulares estáveis é significativamente demorado e laborioso, sendo necessário identificar e isolar clones que exibam elevadas taxas de expressão. A grande variabilidade existente nos níveis de expressão entre clones deve-se ao chamado efeito de posição, ou seja a aleatoriedade de integração no genoma da célula.

Além disso, e independentemente do sistema de expressão, quando se pretende expressar biofármacos multiméricos, como as particulas semelhantes a vírus, estes continuam a ter rendimentos muito baixos. A estequiometria entre os componentes é um factor chave e, quando não é alcançada adequadamente nas células produtoras, resulta na formação de uma grande percentagem de partículas incorrectamente formadas.

De forma a superar estas limitações, neste trabalho é iniciado o desenvolvimento de uma linha celular derivada de *Spodoptera frugiperda*, usando uma combinação de duas tecnologias: (i) sistema de troca de cassete mediada por recombinase (RMCE) e (ii) circuito transcricional indutível. Os sistemas RMCE utilizam um eficiente e avançado processo de troca génica localizada por intermédio de uma reacção de recombinação. Anteriormente implementada em células de mamífero, este tipo de tecnologia foi agora aplicada num cenário de expressão estável em células de insecto com o objectivo de permitir o uso repetido de locais genómicos pré-caracterizados com elevada taxa de expressão. Já os circuitos indutíveis sintéticos, como o sistema de indução por tetraciclina, permitem ajustar a expressão de diferentes genes de interesse por intermédio de um indutor, facilitando a produção de produtos multiméricos correctamente formados.

O trabalho desenvolvido nesta dissertação de mestrado começou por centrar-se na análise de diferentes promotores no controlo da expressão de genes repórter na linha celular Sf9. Foram estudados promotores com diferentes origens mas com uma base de funcionamento comum em

células de insecto, como os promotores de baculovirus OpIE2 e OpIE1, o promotor da metalotioneína e o das proteínas de choque térmico (hsp70) de *Drosophila*. Em resultado, verificou-se que o promotor OpIE2 superou consideravelmente os restantes na expressão de eGFP. Por outro lado, o promotor da metalotioneína induzido por cobre revelou ser um sistema desadequado para expressão de proteínas de interesse em células Sf9, uma vez que apenas para doses citotóxicas se verificou uma expressão de eGFP mensurável.

Para o funcionamento do sistema RMCE são necessários dois vectores distintos e complementares (*Tagging* / *Target*). Em ambos os vectores foi utilizado o promotor OpIE2 para a expressão das proteínas repórter (diferentes nos dois vectores: *Tagging* – dsRed; *Target* - eGFP), e o promotor OpIE1foi usado para a expressão do agente de selecção, necessário para garantir a expressão estável dos dois vectores no genoma da célula (*Tagging* – higromicina; *Target* -neomicina). Enquanto a integração do plasmídeo *Tagging* no genoma se dá através de um processo aleatório, a integração do *Target* é o resultado da troca bem-sucedida por recombinação enzimática. A selecção pelo agente neomicina assegura o sucesso da troca génica uma vez que o gene de resistência é activado pela inserção de um códão de iniciação (ATG) presente apenas no *Target*.

Antes do processo de troca, é importante que apenas uma cópia da cassete *Tagging* seja integrada no genoma da célula para que o sistema RMCE seja funcional. Utilizaram-se dois métodos em paralelo para transfectar as células Sf9 com o plasmídeo *Tagging*: lipotransfecção usando *cellfectin* e eletroporação, donde resultaram quatro populações celulares com distribuições de fluorescência distintas. Para cada população de células foi posteriormente realizada uma diluição progressiva para a obtenção de clones produtores, os quais foram analisados por citometria de fluxo para comparar o nível de expressão de eGFP. Posteriomente, foi implementado um protocolo de *Southern blot* para analisar o número de cópias integradas da cassete *Tagging* em cada clone., Dos nove clones analisados, apenas um revelou ter mais do que uma cópia. Em alguns dos clones com apenas uma cópia procedeu-se à co-transfecção da construção *Target* com a enzima recombinase (*flippase*). Foi possível seleccionar células resistentes à neomicina sugerindo o sucesso da troca de cassetes, tendo sido mais tarde confirmado por meio de PCR do DNA genómico e de RT-PCR do RNA dos genes repórteres.

No que toca o circuito indutível de transcrição, o trabalho conduzido teve como objectivo fazer uma avaliação preliminar da sua funcionalidade em células Sf9 antes da implementação directa na cassete de recombinação. No entanto, os resultados revelaram uma fraca indução por parte do agente tetraciclina, justificando a necessidade de uma optimização do sistema original.

Em conclusão, com este trabalho reportamos pela primeira vez a implementação bem sucedida de um sistema de troca de cassete por intermédio de recombinação enzimática em células Sf9. Esta

tecnologia representa um grande avanço na produção de novas linhas celulares de insecto, combinando as vantagens inerentes à cultura deste tipo de células com a possibilidade de atingir elevados níveis de expressão contínua de diferentes proteínas recombinantes através da fácil integração de genes de interesse no genoma celular.

Palavras-chave: cultura de células de insecto, biofármacos complexos, sistemas de troca de cassetes mediada por recombinase, sistemas transcricionais indutiveis.

4.Introduction

4.1 Animal Cell technology and Biopharmaceutical Production

Biopharmaceutical compounds produced in living systems are continuously gaining importance in medicine and are expected to help cure diseases not yet treatable today (Schmidt, 2004; Butler, 2005)**.** Currently, these products represent approximately one in every four genuinely new pharmaceuticals coming on the market and this ratio is expected to increase as seen by the increasing number of new products enrolling in clinical trials (Hunt, 2005; Walsh, 2005)**.** To this class of compounds belong the nucleic acid-based medicinal products and recombinant therapeutic proteins, including monoclonal antibodies. The majority of animal cell-derived biopharmaceuticals on the market are monoclonal antibodies targeting mainly cancer therapy (Walsh, 2005).

Animal cell lines have been the preferred biological system to produce complex therapeutic proteins due to their innate capacity to perform post-translational modifications. In particular, glycosylation is of special interest since it influences functionality, serum half-life and immunogenicity of some proteins. The ability to perform correct post-translational modifications has justified a rapid increase in the number of approved therapeutics produced from animal cells over the last twenty years, which surpassed the number of biopharmaceuticals produced in *E. coli* or yeast around 1996. Table 1 compares different expression systems in terms of their ability for recombinant protein production. Animal cells have lower growth rates and reach lower maximum cell densities when compared to bacteria or yeast systems, resulting in lower volumetric productivities. However, during the last years these discrepancies have been progressively shortened through successive improvements in expression technology, media composition and process operation.

Characterization of cellular metabolism and physiology allowed the design of efficient fed-batch and perfusion bioreactor processes. In some monoclonal antibody processes, product yield is now over 5 g/L (Wurm 2004). However, some analysts indicate that this still may not be enough, especially due to the large doses of mAB required; future manufacturing demand may exceed production capacity as the number of approved biotherapeutics increases (Butler, 2005). Therefore, if in the past, the concept of ‘‘time-to-market’’ dominated the industrial arena, nowadays, companies are willing to focus on process optimization and cost reduction (Hunt, 2005).

Table 1- Main characteristics of the most important expression systems in terms of quality and yield, two major criteria for recombinant pharmaceuticals (adapted from www.invitrogen.com and Beljelarskaya, 2002).

Host System	Advantages	Challenges	Protein yield, % dry weight
Bacterial expression	• Low cost • Simple culture conditions • Higher growth rates and maximum cell densities	• Protein solubility • Minimal posttranslational modifications • Difficult to express functional mammalian proteins	1-5%
Yeast Expression	• Eukaryotic protein processing • Simple media requirements	• Fermentation required for very high yields	1%
Insect Expression	• Posttranslational modifications similar to mammalian systems • Higher yields than mammalian systems	• More demanding culture conditions • Transient/lytic system (BEVS)	20%
Mammalian Expression	• Improved levels of correct posttranslational modifications • Increased probability of obtaining fully functional human proteins	• More demanding culture conditions	<1%

4.2 Insect Cell Expression: Baculovirus vs Stable expression

Insect cell culture is a mature technology and is being applied in the routine production of recombinant proteins. This technology have major advantages compared with mammalian systems: insect cells are more stress-resistant, easier to culture, more productive and they have the capability to grow in suspension in defined serum-free culture media (Ikonomou *et al,* 2003; Schmidt, 2004; Weber and Fussenegger, 2009). Their efficiency to perform post-translational modifications is comparable to mammalian cells. Furthermore, these cells tolerate higher levels of free amino acids and glucose without switching to overflow metabolism, which enables the design of more nutrient-rich culture media. Unlike mammalian cells, they support lower pH (6.2–6.9) which is commonly maintained by a phosphate buffer, removing the need for carbon dioxide in the culture, as required for the open bicarbonate buffer system in mammalian cell culture media. Growing at lower temperatures (27°C) is also an advantage in terms of process (Ikonomou *et al*, 2003; Weber and Fussenegger, 2009).

Two of the most used insect cell lines (Sf21 and Sf9) were isolated from the ovarian tissue of *Spodoptera frugiperda* pupae. The Sf21 cell line was established at the USDA Insect Pathology Laboratory in 1970 (Vaughn *et al*, 1977) and Sf9 cells constitute a sub-clone of Sf21 cells, selected for faster growth rate and higher cell densities than the Sf21 cells. Both cell lines are 1) robust and easy

to culture in mono-layer or suspension, 2) able to grow rapidly to high cell densities, 3) highly susceptible to Baculovirus (BV), 4) able to produce high virus titers, and 5) readily adaptable to serum-free medium and scale up culture (Granados *et al*, 2007). The Baculovirus expression vector systems (BEVS) is the most used insect cell expression system, mainly because of the successful expression of a large variety of proteins, either intracellularly expressed, surface bound, or secreted into the culture medium (Geisse *et al*, 1996). For application in biotechnology, baculoviruses have been engineered to express recombinant target genes under the control of the very late promoters p10 or polyhedrin, while eliminating the genes that form occlusion bodies.

The baculovirus-insect cell technology is currently being used by numerous small to midsize startup biotechnology companies in North America and Europe to produce custom recombinant proteins for research and commercial applications (Ikonomou *et al,* 2003; Granados *et al,* 2007). Furthermore, since it is an accepted technology for the production of viral antigens, several vaccine candidates are now in clinical trials, such as FluBlock (a flu vaccine from Protein Sciences Corporation) or already in the market, such as Cervarix (vaccine against the human papilloma virus, from Glaxo Smith Kline Biologicals).

While existing insect cell lines provide good results in baculovirus expression vector systems, they can still be engineered for high quantity and quality of recombinant protein. In this respect, (Jarvis et al 1997) engineered a *Spodoptera frugiperda* cell line to perform human-like glycosylation patterns in recombinant therapeutic proteins. In addition, insect cell lines can be engineered for stable protein expression (Granados *et al*, 2007). Indeed, the expression of proteins from an uninfected engineered cell line can provide certain advantages in protein quality, when compared with protein expression from a baculovirus expression vector. Major drawbacks due to baculovirus infection are: (1) an impairment of the protein folding and secretion capacity of the cell (McCarroll and King, 1997), (2) a high, in part baculovirus-encoded, protease activity implying the use of protease inhibitors in the culture media or protease-deficient vectors (Ikonomou *et al,* 2003), (3) deviations of the posttranslational modification pattern, which could act immunogenically and (4) probably the major disadvantage of the system, is the impossibility of continuous protein production due to the lytic nature of the viral infection process (Schmidt, 2004; Yamaji *et al*, 2008).

To overcome these problems, stable plasmid-based expression systems, capable of continuous protein production in *Spodoptera frugiperda* cells started to be developed. There is a small number of promoters normally used for stable protein expression in insect cells, including baculovirus-derived immediate-early (IE) promoters, insect cells constitutive promoters, such as actin and Hsp70 promoters, and inducible promoters such as the *Drosophila* metallothionein promoter (McCarroll and King, 1997) reported similar expression levels for the tissue–plasminogen activator expressed under

the control of the AcIE1 promoter (an IE promoter from *Autographa californica* (Ac) *baculovirus*) in stably transfected Sf9 cells or in baculovirus-infected Sf9 cells. There are studies showing even better protein yields in stable engineered insect cell lines compared with BEVS (Jarvis and Summers, 1989; French and Roy, 1990; Park *et al*, 2004). Despite potential advantages, two major botlenecks still needed to be adressed i) the long development timelines of stable cell lines and ii) the random integration of the transgene into the host chromosome.

4.3 Traditional Cell Line Development

The first step in the development of a recombinant stable cell line is the transport of the foreign DNA into the cells by physical, chemical or biological methods (e.g. microinjection, liposomes, electroporation, gene-gun or viral vectors) (Wurm, 2004). Stable integration of the foreign DNA will only occur in a small portion of the cells that have taken up the DNA. The integration is mediated by cellular DNA repair enzymes and occurs at random sites of the host genome. The genome is packaged into a unique pattern of heterochromatin and euchromatin, which is maintained after cell division. This structure plays a major role in regulating gene transcription by allowing or not the access of the transcriptional apparatus to genes (Narlikar et al. 2002), thus it determines which genes will be active when integrated (Orphanides and Reinberg, 2002). A stringent selection for the generation of stable transgenic cell lines proceeds by means of a cytotoxic antibiotic or depriving cells from an essential metabolic enzyme (Wurm, 2004). The bacterial neomycin phosphotransferase enzyme encoded by the *neo* gene is one of the most used antibiotic resistance marker in eukaryotic cells (Trill *et al,* 1995; Li *et al,* 2007). In the presence of the drug G418 it confers selected ability to the cells that have integrated the plasmid. A number of alternative antibiotic selection markers are available (e.g., *hygromycin, zeocin)*.

The expression level of transfected cells surviving from selection is variable and unstable due to different and unpredictable locations of the integrated copies. Furthermore, having in mind that high-producers are only a minor portion of successfully transfected cells, and that non- and low-producers tend to overgrow, an intensive screening is needed to find highproducing clones which have to divert their metabolic resources to the expression of the gene-of-interest (GOI) (Kromenaker and Srienc, 1994). To isolate clonal cell lines stably expressing the recombinant protein at high levels, the most commonly used method is limiting dilution. Although simple, it is a very laborious and time-consuming process (Fig. 1). Finally, even if a transcriptional hotspot is hit by incidence, a random screening process cannot guarantee the recurrence of such an event.

In this respect, reuse of an identified hotspot would radically change the time needed for the development of a production cell line for various recombinant proteins. Site-specific recombination, which enables the reuse of *loci* with the desired expression characteristics, is nowadays being exploited to this end.

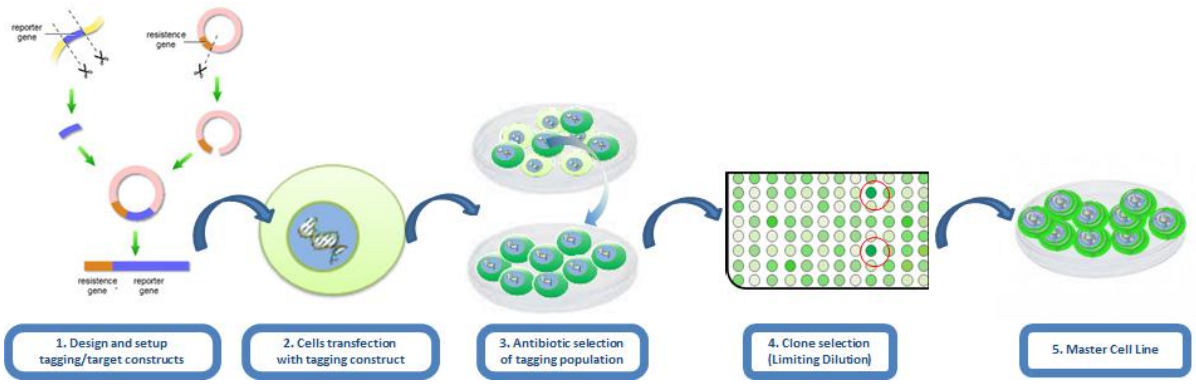


Figure 1 – Traditional cell line development step-by-step, being the transgene one fluorescent reporter protein.

4.4 Site-specific Recombination (SSR)

To integrate a transgene into a pre-tagged genomic site, a number of site-specific recombinases have been of great help. The most widely used site-specific recombinases are the *E. coli* P1 phage-derived Cre (Sternberg *et al.* 1986), the *Saccharomyces cerevisiae*-derived Flp (Buchholz *et al,* 1996; Schaft *et al,* 2001) and the bacteriophage ΦC31-derived integrase (Thorpe and Smith 1998). These enzymes can only mediate recombination between two copies of their target sequence, i.e. loxP site (for Cre), Flp-recognition target site (FRT; for Flp) or a combination of attP/attB (for ΦC31), which enable to excise (Cre, Flp), insert (Cre, Flp, ΦC31), or invert (Cre, Flp) DNA molecules. When two copies of the recognition-target (RT) site are arranged as direct repeats, the corresponding enzyme excises the DNA segment within the RTs and releases it as circular DNA, whereas the reverse reaction leads to an insertion of a circular DNA.

4.4.1 Flippase Site-specific Recombination (SSR)

In this work we will focus on the flippase system. Flippase was originally isolated by Broach and Hicks (1980) from the *Saccharomyces cerevisiae* 2-μm circle; it is a member of the integrase family of site-specific recombinases. The FLP protein targets two FRT sites that are within the 599 bp inverted repeats separating a small and a larger unique region in the 2-μm plasmid. A wild type FRT site consists of 48 bp, composed of two inverted 13 bp repeats and an 8 bp spacer together with a third 13

bp direct repeat and a single additional base pair. FLP protein binds in a site-specific manner to the 13-bp symmetry elements. The 8 bp spacer is involved in DNA-DNA pairing during strand exchange. Together with the extra repeat, its asymmetry determines the direction of site alignment in the recombination event, which will consequently lead to either inversion or excision (Zhu & Sadowski, 1995; Turan *et al.,* 2011). Mutations in the 8 bp spacer result in functional FRT variants (Fn), which will recombine with a second site of the same composition but not with a wild type one (Schlake and Bode, 1994). If F and Fn sites are in a strategically favorable position, flippase can mediate the exchange of expression cassettes in a double reciprocal crossover event, which is called Flp-based recombinase-medicated cassette exchange (Flp-RMCE).

4.4.2 Flippase RMCE, Recombinant mediated cassette exchange systems

The RMCE concept was first introduced by Schlake and Bode (1994) to enable multiple uses of pre-characterized genomic sites; it is a process in which a tagging cassette (inserted in host genome) flanked by a set of incompatible sites (for non-cross-interactions), is exchanged for a target cassette (targets a tagged place) that is flanked by identical sites by means of site-specific recombinases as illustrated by the Fig. 2 (Qiao *et al*, 2009; Turan *et al*., 2011). In the particular case of flippase-mediated cassette exchange, two different FRT sites, for instance the wild type F and the mutant F3 (Schlake and Bode 1994), flank an expression cassette anchored in the genome which is then site-specific recombined by an analogous cassette which is provided on an exchange plasmid.

RMCE technology has been successfully used for different purposes in cultured mammalian and silkworm cells and in living organisms such as *Drosophila* and mice (Coroadinha *et al*. 2006; Nakayama *et al*, 2006; and Cobellis *et al*, 2005). For instance, introduction of RMCE in mouse embryonic stem (ES) cells by homologous recombination could be used repeatedly to generate different knock-ins in the same locus in order to study gene function or towards the development of models for human diseases (Roebroek *et al*, 2003). Also studies in human cell cultures, such as embryonic stem cells (hESCs) and induced pluripotent stem (iPS) cells, with great interest in transplant therapies for genetic diseases, have benefited from the application of RMCE. Indeed, much of these medical applications benefit from continuous expression of therapeutic transgenes in this case without random integration, preventing the possibility of mutagenesis and subsequent tumor formation (Ramachandra *et al*, 2011).

Concerning cell line development for biopharmaceuticals production, Coroadinha *et al* (2006) reported RMCE for the production of retroviral vectors for gene therapy, in which the target integration was used for eventual exchange of the therapeutic gene. Targeted integration strategies have been

adopted also to express relevant therapeutic proteins, such as monoclonal antibodies and other proteins in Chinese Hamster Ovary (CHO) cells (Groth *et al,* 2000; Thyagarajan *et al,* 2001; Kito *et al,* 2002; Wiberg *et al,* 2006; Huang *et al,* 2007). Recently, a flippase-mediated cassette exchange system was exploited to generate 25 CHO cell lines to produce a human polyclonal anti-RhD antibody mixture composed by equal amounts of 25 monoclonal antibodies (Frandsen *et al*, 2011). The engineered polyclonal cell pool displayed highly consistent manufacturing yields of each antibody in the final composition. This strategy was important to minimize genomic position effects allowing the development of cell lines with similar growth and production characteristics (Frandsen *et al*, 2011). Despite the required single copy integration of the gene of interest, the identification of a potent integration site can allow to reach competitive productivity levels in RMCE systems (Wiberg *et al*, 2006).

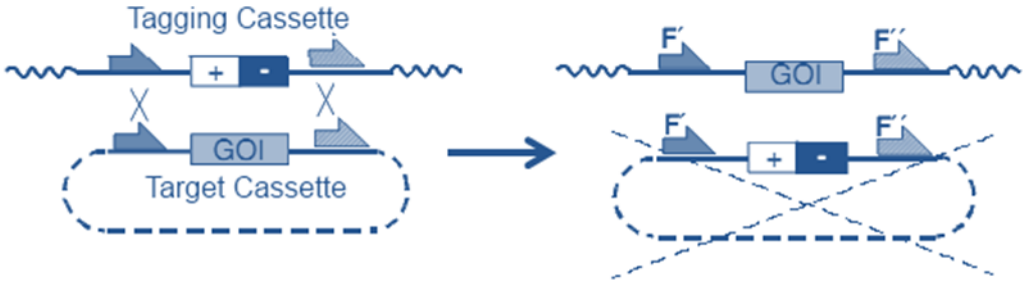


Figure 2 – Recombinase mediated cassette exchange in tagged genomic loci. The tagging cassette is exchanged by a target cassette encoding the gene of interest. (Adapted from Turan *et al*, 2011)

4.5. Inducible Gene Networks

Indeed the integration site is very important but for complex multimeric proteins not enough to guarantee their production at commercially relevant levels and costs. Genes must be expressed at appropriately balanced levels to avoid the accumulation of intermediate products that result in suboptimal yields of correctly assembled products (Pfleger *et al*, 2006). This is the case of the production of a rotavirus-like particle, composed by three structural proteins of the virus, in which the percentage of correctly assembled particles represent only about 15% of the total viral protein mass produced (Vieira *et al,* 2005; Roldão *et al*, 2006).

Reversibility and tuning of expression rate can be achieved by drug inducible/repressible systems that can work by two means: 1) molding activity pattern of the protein (post-transcriptional regulation) or by 2) directly control of the transgene's transcription (transcriptional regulation) (Fussenegger 2001).

The main system now being used for regulating recombinant protein expression is based on transcriptional regulation. Drug inducible transcriptional networks are composed by two major components: 1) a transactivator/transrepressor which activates/blocks transcription in the presence or

presence of a specific molecule (e.g. an antibiotic) and 2) a responsive promoter driving expression of the gene of interest (GOI) (May *et al*., 2006). This control can be done by dimerisation of the transactivator homodimers or other conformational change determining the transactivator's capacity to bind to its target DNA sequence. Examples for this kind of regulation mechanism are the streptogramin system (Pip/pristinamycin; Fussenegger *et al*., 2000), the macrolide based system (E/erythromycin, clarithromycin, and roxithromycin; Weber *et al*, 2002) and the most common the tetracycline (tet) regulated system (Gossen and Bujard, 1992) addressed in more detail below.

These systems have been combined into artificial regulatory networks allowing for sensitive tuning of expression (Kramer *et al*, 2003). For bioprocessing applications, the ideal regulation system should fulfill the following criteria (Fussenegger, 2001):

*i) Specificity***:** In early attempts to regulate transgene expression endogenous regulatory elements were utilized that were activated by exogenous or stress signals (e.g. heat, hypoxia, metal ions). However, this strategy interfered with (stress) response mechanisms from the host cell leading to unpredictable effects. Thus, the regulation system should respond to heterologous or modified endogenous inducing/repressing molecules that are not toxic and do not crossreact with host regulatory networks avoiding pleiotropic side effects.

*ii) Inducibility***:** Low basal activity and high expression levels upon induction are favoured. The extent of inducibility is measured by the ratio *expression level after induction/basal expression level*.

iii) Reversibility: The system should allow repeated induction and repression of transgene expression by addition or depletion of the regulating drug. The time that is required to reach basal/maximal expression levels after aministration/removal of the respective drug is dependent on the degradation rate of the chemical.

iv) Dose-dependence: Expression levels of the regulated transgene should proportionally correlate to concentrations of the regulating agent.

4.5.1. Tetracycline (Tet) regulated gene expression.

Gossen and Bujard (1992) developed a bacterial promoter-based tetracycline-controlled gene expression system that later became the most frequently used inducible expression system for mammalian cells. Regulation in this system involves a highly specific interaction between the transcription factor Tet repressor (TetR) and a Tet operator (TetO) DNA sequence (Tet response element or TRE). In the original Tet-Off system, the DNA binding domain of TetR was fused with the

potent herpes simplex virus VP16 transactivation domain to form the tetracycline responsive transactivator (tTA) (Kaern *et al*, 2003). Homodimers of this fusion protein bind to the TRE which consists of 7 TetO repeats placed close to the minimal human Cytomegalovirus promoter (PhCMV) (Boshart *et al*, 1985). Thereby, the VP16 domain is capable of initiating transcription of the downstream transgene by recruitment of endogenous transcription factors like TFIIB (Lin *et al*, 1991), TBP (Ingles *et al*., 1991), or TAFII40 (Goodrich *et al*., 1993).

In the Tet-Off system (Fig. 3), the transactivator tTA is constitutively expressed and in the absence of tetracycline (Tet) or its analogous most used doxycycline (dox), it binds to the TetO to initiate transcription; in the presence of dox, tTA cannot bind to the Tet operators in the hybrid promoter, and thus transcription is inhibited. To control gene expression in cultured cells, dox concentrations of 1 to 5 ng/ml are required, which are far below the toxicity threshold reported for HeLa cells (5 to 10 g/ml) (Gossen and Bujard, 2002).

Random mutagenesis of TetR generated a new transactivator (rtTA), which binds and transactivates gene expression in the presence of dox – this is called the Tet-On system. Improved versions of rtTA have been developed to give tighter gene expression, increased sensitivity towards the inducer, enhanced stability and expression in mammalian cells, and more uniform transgene expression in the induced cells (Gossen and Bujard, 2002; Kaern *et al*, 2003; Alexander *et al*, 2007).

Tetracycline systems have proven to be particularly valuable for inducible expression as the inducer doxycycline is inexpensive, ii) they have a high dynamic range, iii) low background in un-induced state and iv) can be used for in vivo and in vitro applications (Gossen and Bujard, 2002).

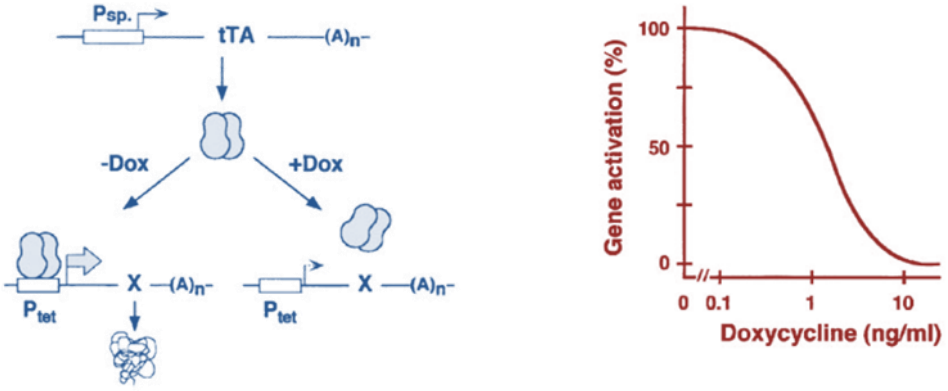


Figure 3 - Tetracycline inducible system, in this case the Tet-Off, in which the presence of doxycycline inhibits gene expression in a dose-dependent manner. Left part shows the mode of action of the Tc-controlled transactivator (tTA). In the absence of the effector molecule Dox, tTA binds to the *tet*O sequence within Ptet and activates transcription of gene x. Addition of Dox prevents tTA from binding and, thus, abolishes the initiation of transcription. Right part depicts the dose response curve for the effects of Dox on tTA-dependent gene expression. Gene activity is maximal in the absence of the antibiotic, but as effector concentrations increase, transcription gradually decreases to background levels at Dox concentration of 5 ng/ml (addapted from Gossen and Bujard, 2002).

As for RMCE, gene inducible networks have become essential tools for studies of gene function in vivo, expression of protein or RNA molecules, and for the development of different approaches for cancer and genetic disease, like gene therapy (Tigges *et al*, 2009). Indeed in gene therapy these transcriptional regulatory systems have been encoded within several viral vectors to facilitate gene expression and further improve the kinetics of gene regulation (Chtarto *et al*, 2003). The application of inducible expression systems to achieve stoichiometric expression of the different proteins composing a multimeric product, as intended in this work, has not been reported so far.

5.Aim of the thesis

The work developed in this master thesis is in the scope of a project which aims at developing a flexible Sf9 insect cell platform to produce different complex products that require co-expression of 2 or 3 genes, such as triple-layered rotavirus like-particles, monoclonal antibodies or viral vectors for gene therapy. The final goal is to implement in the same cell line two or three Recombinase Mediated Cassette Exchange (RMCE) systems, each enconding one of the sub-units composing the target product, and to be able control the expression rate of the subunit in relation to other sub-units by implementing synthetic gene circuits inside those cassettes.

The work developed in this thesis will cover two main tasks of the global project: i) the implementation of the first cassette system in Sf9 cells and also ii) the first steps in the development of an inducible system for Sf9 cells. To accomplish these main objectives several sub-tasks were addressed, namely the selection of the most suitable promoters to be used in this Sf9 cell line, the optimization of the transfection protocols, the design and construction of the expression vectors, and the implementation of several analytical techniques crucial to support the development of this cell line.

6.Materials and Methods

6.1 Molecular Biology

6.1.1. Vector Design and Construction

Stable expression Study with Different Promoters

pIZT/OpIE2 vector: The pIZT/OpIE2 vector was derived from two vectors: pIZT/V5-His (Invitrogen, Carlsbad, USA) and pMDISGFP (provided by Dr. Dagmar Wirth, HZI Braunschweig, Germany). eGFP from pMDISGFP was amplified by PCR (primers in Table 2, Appendix) and cloned into an EcoRI/NotI excised pIZT/V5-His.

pIZT/hsp70 vector: The pIZT/hsp70 vector was derived from pIZT/OpIE2 and pUAST (provided by Dr. Pedro Domingos, ITQB, Portugal) vectors. Hsp70 *Drosophila* promoter was amplified by PCR (primers in Table 2, Appendix) and cloned into an BspHI/AgeI excised pIZT/OpIE2.

pIZT/mtn vector: The pIZT/hsp70 vector was derived from pIZT/OpIE2 and pRmHa (provided by Dr.Pedro Domingos) vectors. Mtn *Drosophila* promoter was amplified by PCR (primers in Table 2, Appendix) and cloned into an BspHI/AgeI excised pIZT/OpIE2.

RMCE related vectors

Tagging vector (Flp-tagging): The vectors pIZT/V5-His (Invitrogen), pCAG-dsRed (Addgene) and pTagFwF5 were used to construct the tagging cassette. pTagFwF5 was designed by Dr. Ana Coroadinha and synthetized by Geneart (Carlsbad, USA). OpIE2 and OpIE1 promoters were cloned into pCAG-dsRed by excision with EcoRI/AgeI and HindIII/AvrII, respectively. From this construct a fragment containing the OpIE2 promoter, dsRed, poly A terminator and OpIE1 was amplified by PCR (primers in Table 2, Appendix). This amplicon was ligated by digestion of SacI/EcoRI into pTagFwF5 that contains a wild-type FRT site (FW) and a spacer mutant FRT site (F5) with and hygromycin resistance gene followed by an ATG-deleted neomycin resistance gene.

Flippase vector (pOpIE2FLP): this vector results from pSVFLP (provided by Dr. Dagmar Wirth) which was modified by cloning: SV40 promoter was replaced by OpIE2 promoter.

Target vector: Vectors pIZT/V5-His (Invitrogen), pMDISGFP (provided by Dr. Dagmar Wirth) and pTAR-MCS (provided by Dr. Ana Coroadinha, ITQB, Portugal) were used for designing the target cassette. eGFP from pMDISGFP was amplified by PCR and cloned into an EcoRI/NotI excised pIZT/V5-His. OpIE2 promoter and eGFP which resulted from the previous ligation were amplified and cloned into pTAR-MCS in SphI/XhoI unique recognition sites. Subsequently, using ECORI/ECORV recognition sites OpIE1 promoter was placed upstream to an ATG start codon followed by FRT mutant site to complement the inactive neomycin resistance gene after targeting.

Tet–Off Inducible Circuit vectors

Transactivator vector: The vector pIZT/V5-His (Invitrogen) was used to construct the transactivator vector. ptTa was designed by Dr. Ana Coroadinha and Fabiana Fernandes (ITQB, Portugal) and synthesized by Geneart. The zeocin resistence marker was amplified by PCR from pIZT/V5-His (primers in Table 2, Appendix). This amplicon was ligated by digestion of SacI/EcoRI into ptTa.

TetOminhsp70 vector: The three plasmids pIZT/V5-His (Invitrogen), Flp-tagging and pTetO (Dr. Pedro Domingues) were used to construct the TetOminhsp70 vector. The TetOminHsp70 was amplified by PCR from pTetO and the GFP from pIZT/V5-His (primers in Table 2, Appendix). The two amplicon were ligated by digestion of SacI and AvrII.

6.1.2. Techniques supporting vector construction

The herein studied genes and DNA-fragments were constructed with the methods described below. The composition of the utilized primer pairs are found in Table 2 in the Appendix.

General PCR-protocol

PCR enables the rapid and specific amplification of DNA-sequences. The oligonucleotides used for PCR were custom-made by Sigma Aldrich (St.Louis, USA). A typical PCR-reaction contained 10 µl of 10 x polymerase buffer, 1µl of 10 mM dNTPs from Promega (Madison, USA), 1 µM of the 5'- and 3'-primers, 5 to 100 ng of template, 1 to 5 U of Promega's polymerase. Distilled water was also added to the final volume of 50 µl. The PCR-amplification program started with a 1 min denaturation step at 94 to 98 °C, followed by 10 to 30 sec primer annealing, which was performed 5 to 10 °C below the melting point of the primer. The next step in the cycle was primer extension at 72 to 75 °C for 1 to 10 min. After 30 cycles, a single primer extension of DNA-fragments was performed for 10 min at 72 to 75 °C. The quality of the amplified DNA-fragments was analyzed by agarose gel electrophoresis and purified using the Qiagen (Venlo, Netherlands) PCR-purification kit.

Colony PCR-screening

PCR-screening was used to screen transformed bacterial colonies to evaluate if they contained the vector with the desired insert by using vector- or gene-specific 5'- and 3'-primers. Single transformed bacterial colonies were selected from the LB-agar-plate and transferred into a PCR-tube containing 20 µl of the pre-pipetted PCR-reaction mixture. PCR was performed immediately and checked by agarose gel electrophoresis.

Digestion of DNA

DNA-digestion of both PCR-fragments and the vector-DNA was performed with appropriated restriction endonucleases using the buffer system, with time and temperature as recommended by the manufacturer, NEB (Ipswich, USA). To prevent religation of the digested linearized vector-DNA, the 5'-phosphate groups were removed by treatment with Antarctic phosphatase (NEB). Antarctic

phosphatase is compatible with the NEB buffer 2 to 4 used by the restriction enzymes. One unit of it is directly added to 2.5 µg linearized vector-DNA digestion mix and incubated for 1 h at 37 °C. The digested DNA fragments were excised and purified with the GE healthcare DNA-extraction kit (Little Chalfont, United Kingdom) were analysed by agarose gel electrophoresis.

Ligation with T4 ligase

For the ligation of digested DNA-fragments the molar ratio of insert-DNA to linearized vector-DNA was varied in a ratio of 1 to 1 up to 5 to 1, typically using 100 µg of vector. The DNA-fragments were ligated using 1 µl of T4 ligase in a total reaction volume of 20 µl following the instructions of the manufacturer (NEB). The ligated vector-DNA mix was used for transforming bacterial cells without further purification.

Preparation of competent E. coli cells

A small amount ~10 µl of frozen competent *E. coli* cells were added to a flask containing 200 ml of LB-Medium with no antibiotic and incubated at 37 °C and 250 rpm. When the bacterial culture reached an OD600 value of approximately 0.5, the flask was removed from the shaker and cooled on ice. After 10 min, the bacteria culture was transferred to a pre-chilled 250 ml centrifuge tube and the cells were harvested by centrifugation at 3.5 K, 4 °C for 10 min. The cell pellet was re-suspended in 80 ml ice cold TFB1-buffer and incubated on ice for 5 min. The cells were once again harvested by centrifugation at 3.5 K, 4 °C for 10 min. The new cell pellet was resuspended in 8 ml ice cold TFB2-buffer, aliquoted into micro-centrifugation tubes (75 µl), shock-frozen in liquid nitrogen, and stored at -80 °C until further use.

TFB1-Buffer: 100 mM RbCl, 50 mM MnCl2, 30 mM Potassium acetate, 10 mM CaCl2, 15% glycerol, pH 5.8, filter sterilize

TFB2-Buffer: 10 mM MOPS, 10 mM RbCl, 75 mM CaCl2, 15% glycerol, adjust to pH 6.8 with KOH, filter sterilize

Transformation of E. coli cells

Competent *E. coli* cells were thawed and mixed with 20 µl of the ligated vector-DNA mix or 100 ng vector-DNA. The mixtures were kept on ice for 30 min, followed by a heat shock for 1 min at 42 °C and then immediately 2 min incubation on ice. Transformed *E. coli* cells were incubated for 1 h at 37 °C, with 0.3 ml LB-media containing no antibiotics. After this, the *E. coli* cells were spread on LB-Agar plates containing Amp. Subsequent to over-night incubation at 37 °C, *E. coli* transformants were visible as colonies. To identify whether transformants contained the gene of interest, PCR screening and vector digestion were performed from selected bacterial colonies. Both PCR-fragments and vector digestion were analyzed using agarose gel electrophoresis

Isolation of vector-DNA

Transformed *E. coli* cultures were grown over-night at 37 °C and 240 rpm. The next day, 5 ml (200 ml) cell culture was harvested by centrifugation at 3.5 K for 10 min and DNA was purified with the DNA-Miniprep kit (Qiagen) or for higher concentrations with the DNA-Maxiprep kit (Roche, Basel, Switzerland).

Agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate DNA-fragments according to their size. The agarose concentration of the gels varied from 0.8 to 2.0% (w/v) depending on the size of DNA-fragments to be analyzed. The agarose was melted in 1 x TAE buffer and 0.5 µg/ml Gel red was added before pouring the gel. Gel red is an organic dye with a plane structure that intercalates into DNA. It gets excited with UVlight (254 to 366 nm) and emits light of the orange-red spectrum (590 nm) that visualizes the DNA-fragments. Samples were mixed with 1/5 vol of agarose gel loading buffer before loading. Gels were run at 5 V/cm and photographed. A 100 bp- or 1 kbp ladder from Promega was applied as a size standard.

Determination of DNA-concentration

DNA has an UV-light absorption maximum at 260 nm due to the aromatic rings of its bases. Therefore, DNA-concentrations of the samples were determined by measuring the OD at 260 nm using a spectrophotometer. An OD260 of one equals a concentration of 50 µg/ml DNA.

6.2. Insect Cell Culture

6.2.1. Culture maintenance

Sf9 insect cell lines were grown in serum-free Sf900II medium (Gibco, Carlsbad, U.S.A.) at 27 ºC. For suspension cultures, shake-flasks of 125 ml or 500 ml containing 20 ml or 50 ml were kept in orbital shakers at 130 or 90 rpm, respectively. Suspension cultures were maintained by splitting at a cell density of 3-4 x 10^6 cells/ml back to a density of 4.5-5 x 10^5 cells/ml in fresh culture medium normally every 3-4 days. Sf9 monolayer cultures were maintained in well plates (Nunc, Roskilde, Denmark) and sub-cultured when confluent.

6.2.2. Freezing and thawing of insect cells

The cell culture vial was thaw in hand and cells were washed with 10 ml of culture media. Centrifugation was then performed at 1200 rpm for 10 min to eliminate cryo preservation medium. After this, the cell pellet was re-suspended in 50 ml suspension culture media and cells were cultivated at 27 °C and 90 rpm.

Cells from mid-exponential growth phase of a concentration of about 3.5 x 10^6 cells/ml were centrifuged at 1200 rpm for 10 min and the cell pellet re-suspended in cryo preservation media

(Cryostor, San Diego, U.S.A.) into a concentration of 2 x 10^7 cells/ml. Aliquots of the cell culture were slowly frozen in the blue-topped isopropanol container (Mr.Frosty) and cell culture vials were kept at –80 °C or transferred after a day into the liquid nitrogen tank for long-term storage.

6.2.3. Transfection of insect cells

For the transfection of insect cells two methods were used.

Electroporation

Tagging tranfections:

For the electroporation (NeonTM transfection system, Invitrogen, Carlsbad, U.S.A) procedure 1 µg or 0.5 µg of tagging DNA were used to transfect 4 million cells at 250V or 500V, respectively, with two sequential pulses of 5 ms duration.

Lipotranfection

Promotor Strenght Studies transfections:

One unit of Cellfectin II (Invitrogen) reagent was transfected to 0.5 million cells 0.3 µg of the DNA. Fluorescence was observed using a Zeica microscope and analysed by FACS after a period of 72 h post transfection.

Tagging tranfections:

One unit of Cellfectin II (Invitrogen) reagent was transfected to 0.5 million cells with 0.03 or 0.3 µg of tagging DNA. Fluorescence was observed using a Zeica microscope and analysed by FACS after a period of 72 h post transfection.

Transactivator and tetOminhsp70 transfections:

One unit of Cellfectin II (Invitrogen) reagent was transfected to 0.5 million cells with 0.05 or 0.3 µg of tranactivator DNA . After selected the two populations , 0.3 µg of tetOminhsp70 was transfected into the more expressing one, 0*,3* µg . Fluorescence was observed using a Zeica microscope and analysed by FACS after a period of 72 h post transfection.

6.2.4. Killing curves

The killing curves for the different antibiotics were done in shake flasks with 20 ml cellular suspension at a concentration of 2.5x10^6 cell/ml. Several concentrations were tested for Zeocin (InvivoGen), hygromycin (Invivogen) and G418. Every 24 hours cells were counted to determine cellular concentration (Fig. 20, Appendix).

6.2.5. Cloning by Limiting dilution

Cloning by limiting dilution is a procedure for separating cells based on the assumption that if a suspension of cells is diluted with enough culture medium, a concentration of cells will be produced such that an accurately measured volume of the diluted suspension will contain 1 cell. A series of dilutions were preformed until the amount of 1 cell in 100 µl in the final dilution was reached. In this dilution, the medium is composed by 50% conditioned and 50% fresh medium. Then, 100 µl of this

mixture was transferred into separate wells of a 96-well plate, so each well will receive one cell. The conditioned medium is usually needed because of the obviously low cell density of 1 cell/well. After this cell proliferates, a new cell clone has been established. When the 96 well is confluent, each clone is transferred to a 12 well, then to a 6 well and when the 6 well is confluent cells are detached with the pippete and and put in 5ml suspension culture in a 50 ml falcon placed in an orbital shaker. From one cell per well in the 96 well plates to clones in shake flask suspension cultures, it took about 3 months.

6.2.6. Doxycycline Assay

Sf9 cells stably harboring both tTA and TetOminhsp70 were seeded in 6 well plates at a low cell density with different doxycycline doses (0, 0.01, 0.1, 1, 10 and 100 ng/ml). After 7 days, cells were harvested and analysed by flow cytometry.

6.3. Analytical techniques

6.3.1. Cells counting

Cell concentration and viability were assessed by haemocytometer (Brand, Wertheim, Germany) using the trypan blue exclusion dye (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS).

6.3.2. Flow cytometry

To determine eGFP and dsRED expression cells were analysed by flow cytometry using a CyFlow® space (Partec) instrument. Ten thousand events were registered per sample using the appropriate scatter gates to avoid cellular debris and aggregates. When the measured fluorescence intensity exceeds the signal obtained by untransfected cells, the cell is considered fluorescent.

6.3.3. Western Blot to the Transactivactor

Samples were separated under reducing electrophoresis on a 1-mm NuPAGE Novex BisTris gel (Invitrogen) and electrically transferred to a nitrocellulose membrane (HybondTMC extra, Amersham Biosciences, Little Chalfont, United Kingdom). The membrane was then stained with TetR Monoclonal Antibody (Clonetech 9G9, Otsu, Japan), at a 1:1000 dilution during 2h at room temperature. Blots were developed using the ECL (enhanced chemiluminescence) detection system after incubation with horseradish peroxidase-labeled anti mouse IgG antibody (Amersham Biosciences) at 1:5000 dilution during incubation at 1h at room temperature.

6.3.4. Southern Blot

Genomic DNA was extracted from each cell clone using the Wizard® Genomic DNA Purification Kit (Promega). A DIG labeled probe was obtained according to the instructions of the PCR DIG Probe Synthesis Kit (Roche) using specific primers for the neomycin resistance gene. Genomic DNA was digested with both SacI and EcoRI and loaded into a 0.7% agarose gel. Gel content was transferred to a filter by alkaline transfer. DIG filter hybridization protocol was applied (hybridization

overnight at 60ºC) and DIG Nucleic Acid Detection Kit (Roche) was used for detection. Alkaline transfer harms the DIG labeled DNA from the marker it requires longer exposition times to detect it.

6.3.5. RNA extraction and RT-PCR

RNA extraction was done using the Quiagen RNAeasy kit and the synthesis of cDNA by First Strand cDNA Synthesis Kit for RT-PCR (AMV), as explained in kit's protocol. One tenth to one-twentieth of this reaction was used as a template for PCR amplification with Taq polymerase (Promega) for 25–35 cycles at 94 8C for 1 min, 55 8C for 1 min, and 72 8C for 1 min.

6.3.6. Statistical analysis

The results were expressed as the mean ± standard deviation. The statistical test used, One-way ANOVA, was performed in Graph Pad 5.1 for Windows for a level of confidence of 95% ($\alpha = 0.05$) followed by the Scheffé multiple comparison test.

7.Results

7.1 Comparison of promoters strength

For any cell system, a number of central elements are essential in the design of recombinant expression systems. Expression is normally induced from a plasmid harboring a set of compatible genetic elements, including an origin of replication (ori), an antibiotic resistance marker, transcriptional promoters, translation initiation regions (TIRs) as well as transcriptional and translational terminators (Baneyx, 1999). Among these genetic elements a strong transcriptional promoter is one of the main determinants to achieve high-level gene expression.

The first step in the development of the Sf9 cell line incorporating a flippase-mediated cassette exchange system was the selection of the promoter to drive the expression of the gene of interest. Different plasmids with the same backbone were constructed, as illustrated in Fig. 4, by changing the transcriptional promoter in order to compare expression strengths. Three insect cell promoters were tested: i) one constitutive promoter, OpIE2, which is an immediate-early promoter from the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) and ii) two promoters from *Drosophila melanogaster*, the heat shock protein 70 promoter, induced by several stresses but in particular the heat shock stress and the metallothionein (Mtn) promoter which is induced by high intracellular concentrations of heavy metals.

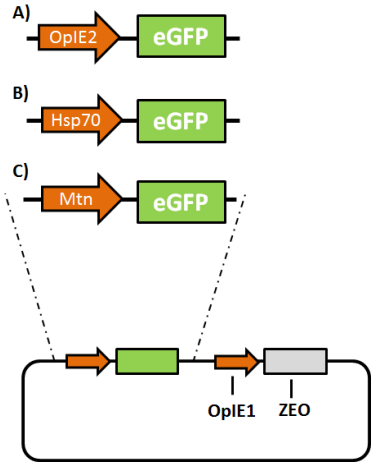


Figure 4 – Scheme of the vectors used to compare the strength of the different promoters. The same backbone vector was used changing only the different promoters. A) OpIE2, B) HSP70 and C) Metallothionin.

Once the vectors were constructed, Sf9 cells were transfected individually with each of them. These transfections were based on Cellfectin and the same conditions were used for all constructs (see

materials and methods). The promoter driving the expression of the resistance gene, OpIE1, is also an immediate-early promoter from the baculovirus OpMNPV, but from preliminary results we observed that it is weaker than OpIE2 promoter (data not shown).

7.1.1 Metallothionein Promoter

Concerning the use of the metallothionein promoter in Sf9 cells, the gene product expression is dependent on the inducer levels. Thus, first it is important to determine the impact of copper (one of the possible inducers), on cellular growth and physiology. Sf9 cells were cultured in the presence of different concentrations of copper(II) sulfate pentahydrated ($CuSO_4*5H_2O$). When adding this salt to the culture medium, the availability of cooper ions (Cu^{2+}) to enter the cell is assured.

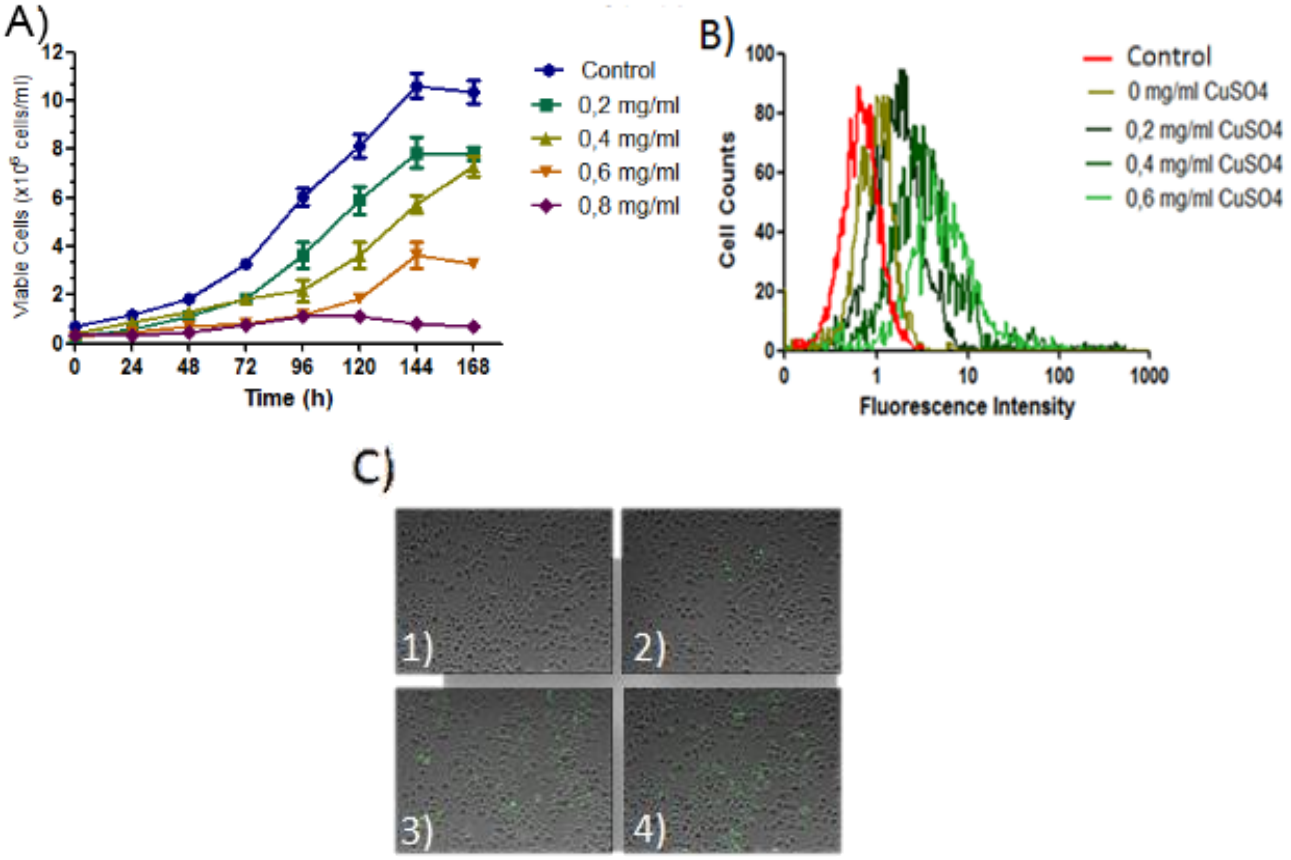


Figure 5- A) Sf9 cell density profiles when subjected to different concentrations of $CuSO_4$, B) Flow cytometry analysis of GFP expression in the Mtn cell population, 96h after copper addition and the control with a non-transfected population, and C) Fluorescence microscopy images of cells subjected to different levels of inducer, 1- 0 mg/ml; 2- 0,2 mg/ml; 3- 0,4 mg/ml; 4- 0,6mg/ml of $CuSO_4$.

In Fig. 5, the cell growth profiles of Sf9 cells when cultured in the presence of the indicated concentrations of copper sulfate are shown. The data indicate that copper affects substantially the cellular growth, promoting longer lag phases, lower growth rates and lower maximum cell densities when comparing to cultures without the heavy metal. Cells grown in concentrations up to 0.6 mg/ml of copper sulfate continue to double, but the replicate time increases as the concentrations of $CuSO_4$ increases. For concentrations higher than 0.6 mg/ml, cells can no longer enter the exponential phase.
Taking the above results, we assessed the GFP expression levels only for the three lower concentrations of copper (0.2, 0.4 and 0.6 mg/ml). Only 96h after copper sulphate addition to the culture medium it was detected GFP by fluorescence microscopy examination (Fig. 5C). Even at high induction conditions, GFP expression is low when compared to the fluorescence detected for the other two promoters (see results below). The flow cytometry analysis for the same concentration range (Fig. 5B), shows how the addition of $CuSO_4$ increases the expression of GFP controlled by the Mtn promoter. Indeed, a clear increase in fluorescence intensity is observed with increasing $CuSO_4$ concentrations, confirming the inducible pattern which characterizes this promoter. The fluorescence intensity of the Mtn cell population cultured without inducer is similar to the negative control (untransfected Sf9 cells), marked in red (Fig. 5B).

7.1.2 OpIE2 and Hsp70 promoters

The cell populations transfected with the constructs harboring GFP expression under OpIE2 or Hsp70 promoters were also analyzed by flow cytometry and fluorescence microscopy (Figure 6). Although Hsp70 is an inducible promoter, in stable transfection apparently loses the need for induction and acts like a constitutive promoter allowing continuous expression of the transgene. Nevertheless, the OpIE2 promoter revealed to be much stronger than Hsp70 and Mtn promoters (Fig. 7).

Comparing the growth curves of the transfected populations with the non-transfected we see that the main difference is in maximum cell densities reached, with a slight decrease for both transfected populations.

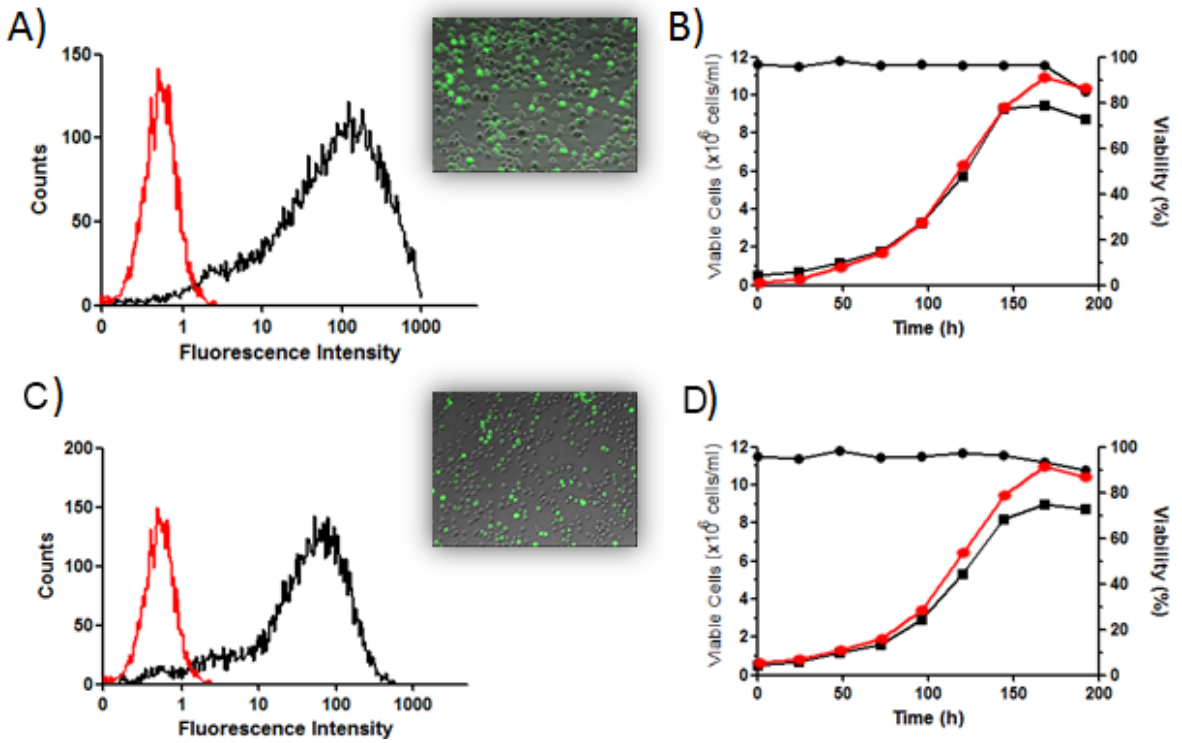


Figure 6- Distribution of fluorescence intensity in the transfected cell populations after 3 weeks of antibiotic selection, each with a different promoter driving eGFP expression: a) OpIE2, c) Hsp70 and corresponding growth curves with the transfected and selected cells b) OpIE2 and d) Hsp70.

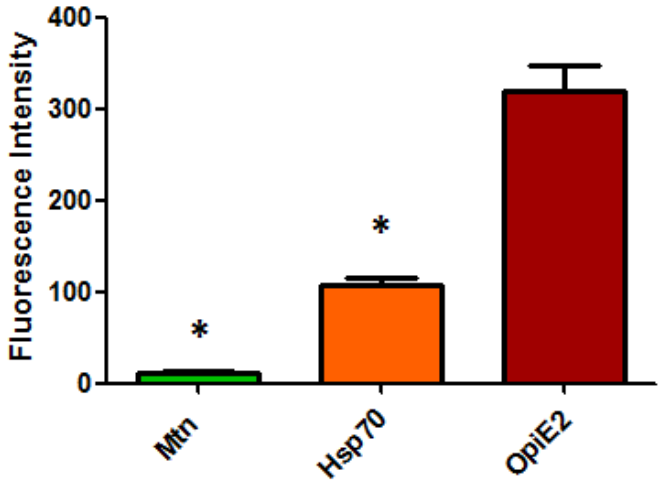


Figure 7 – Maximum GFP fluorescence Intensity of three cell populations, each having a different promoter driving GFP expression. Errors bars correspond to standard desviation (n = 3). *p<0.05 compared to OpIE1.

7.2 Tag and target cassettes

To generate cell clones which are suitable for targeted integration it was adopted a Flp recombinase mediated cassette exchange strategy, as outlined in Fig. 8. This technology comprises two main

steps. The first is the tagging of chromosomal integration sites within the genome of the sf9 cell line with a red fluorescence reporter gene (dsRED) and a hygromycin resistance marker (Fig. 8A). At this stage we create a cassette acceptor allele with heterotypic and incompatible recognition sites that by means of the dsRED reporter enables the choice and characterization of the best integration locus. The second step is based on a targeting cassette containing eGFP flanked by the same set of heterotypic recognition target sites. This cassette encodes the ATG starting codon which is lacking in the ATG defective neomycin resistance gene in the tagging construct (Fig. 8B).

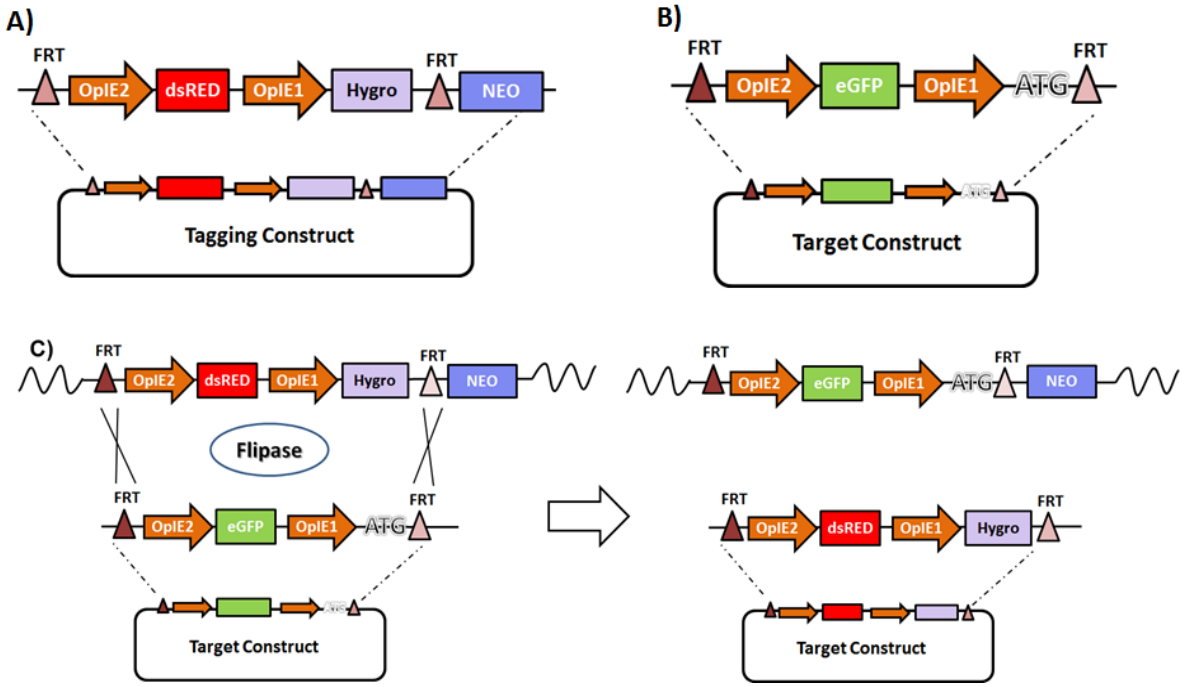


Figure 8 – Schematic representation of the (A) tagging and (B) target cassettes and (C) how the recombination between the two cassettes occurs in the cells in the presence of FLP.

Both cassettes have the same promoters, OpIE2 and OpIE1, driving the expression of the reporter genes and the resistance markers, respectively (Fig. 8). The choice for these promoters was based on the expression levels obtained in the previous presented data (Section 7.1) with the different insect cell specific promoters. For cassette exchange, the tagged cells will be co-transfected with the targeting cassette and the plasmid that encodes the Flp recombinase. This enzyme which will catalyze the exchange reaction is expressed also under the control of the OpIE2 promoter. The ATG entrapment present in the target construct is then triggered by the cassette exchange, leading to the transcription of the neomycin gene (Fig. 8C).

7.3 Transfection of Sf9 cells with tagging cassette

The tagging and Target cassettes were constructed (see details in material and methods) and the tagging cassette was transfected into Sf9 cells. It is important to highlight that RMCE technology was never performed in Sf9 cells and single copy integration of the tagging cassette must be ensured for the system to work properly. Due to the lack of knowledge regarding which transfection method would allow preferentially single copy integration of the tagging cassette in Sf9 cells, random integration was performed by two transfection methods in parallel: i) lipotransfection using Cellfectin II, a commercial cationic lipid mixture that ensures the transport of DNA by means of endocitose within liposomes carrying DNA and ii) electroporation, in which an electric field is created that causes permeability of the cell plasma membrane enabling the entrance of DNA. The Cellfectin reagent allows high transfection efficiencies of Sf9 cells, but one concern is the number of copies associated to such high transfection activities that can compromise the objective of having clones with single copy integration. Bearing this in mind, two quantities of tagging DNA were used (0.3 µg and 0.03 µg of DNA). The transfection efficiency was analysed by flow cytometry 72 h post-transfection and significant different values were obtained for each condition (Fig. 9).

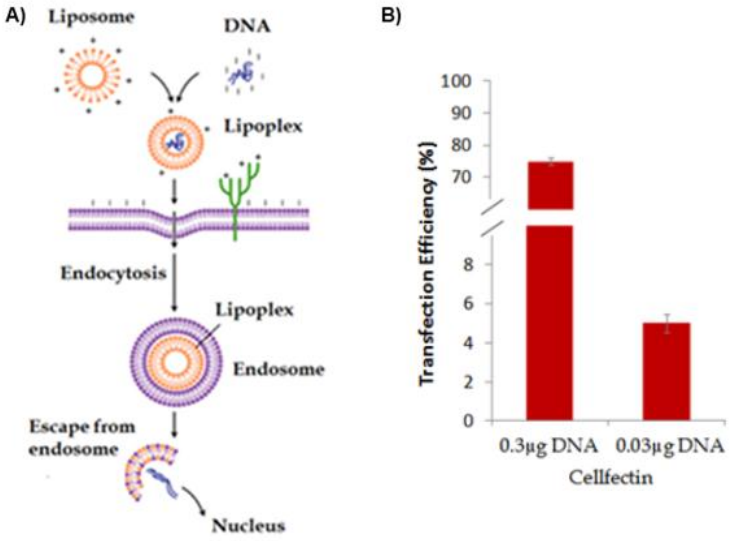
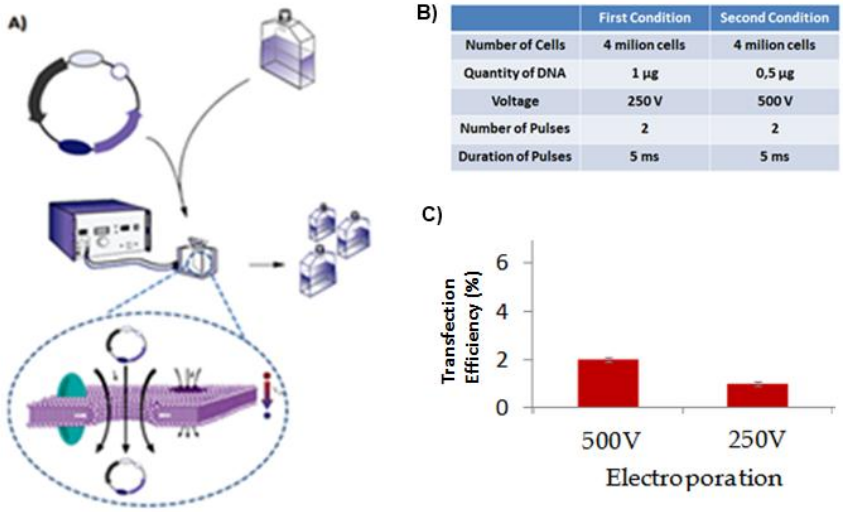


Figure 9 – A) Schematic representation of the lipotransfection method; B) Transfection efficiencies obtained for each amount of tagging cassette used. Error bars correspond to standard desviation (n=3).

Electroporation-based transfection (Figure 10), on the other hand is not a common transfection method used in insect cells but with the experience brought from transfecting mammalian cells in our lab, we know that electroporation allows lower transfection efficiencies than methods based on

cationic lipids. This could be associated to less DNA entrance and consequently more clones with single copy integration. We applied this method to Sf9 cells, testing several parameters, namely the voltage, number of pulses, duration of pulses, quantity of cells and DNA (data not shown).



	First Condition	Second Condition
Number of Cells	4 milion cells	4 milion cells
Quantity of DNA	1 µg	0,5 µg
Voltage	250 V	500 V
Number of Pulses	2	2
Duration of Pulses	5 ms	5 ms

Figure 10 – A) Schematic representation of the electroporation method B) the two conditions used and C) the transfections efficiencies with both protocols. Error bars correspond to standard desviation (n =3).

From the different protocols employed, two populations could be selected (Fig. 10B), which resulted from electroporations using 4×10^6 cells and two pulses of 5 ms length each, but different quantities of the tagging construct were added to the cells (either 1 µg or 0.5 µg), and different voltages applied (250 V or 500 V, respectively).

All four populations of tagging, two from lipotransfection (0.3 and 0.03 µg DNA) and two from electroporation (250 V and 500 V) were analysed by flow cytometer for transfection efficiency 72 h post-transfection and put in hygromycin selection. The 0.3 µg population has clearly a higher percentage of transfection efficiency with values of 75 % contrasting with the other three populations with percentages only up to 5 %. After three weeks of selection the four populations were analyzed by flow cytometer and fluorescence microscope. The results are shown in Fig.11.

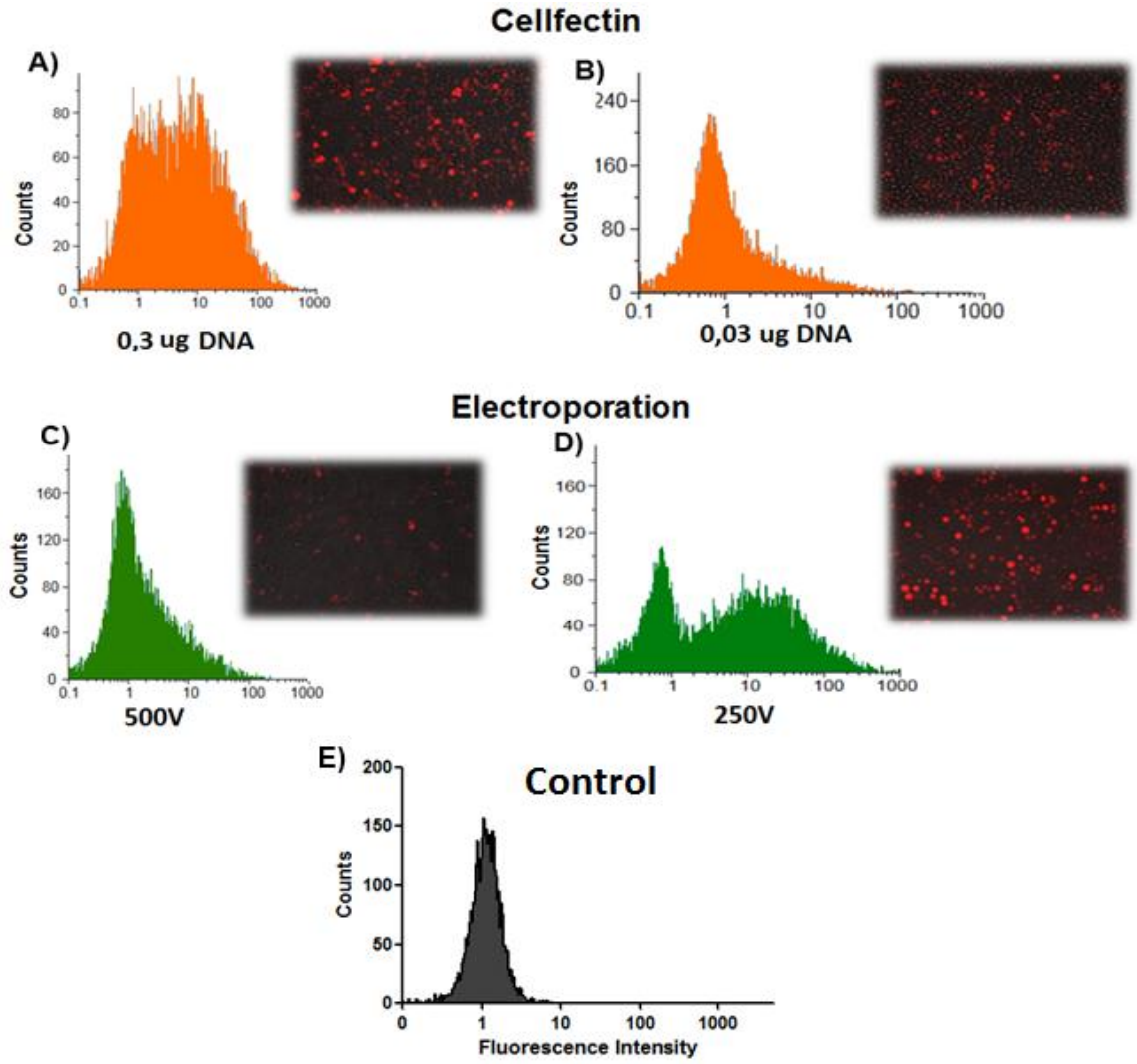


Figure 11- Distribution of fluorescence intensity in the four cell populations obtained with different transfection methods using the tagging plasmid: lipotransfection using A) 0.3 μg and B) 0.03 μg of tagging and electroporation using voltages of C) 500V and D) 250V. E) Is the control with non-transfected cells.

The different transfection methods resulted in substantially different populations in terms of fluorescence distribution. Cellfectin 0.3 μg DNA and Electroporation 250V population have higher number of dsRED positive cells which could mean undesirable integration of multiple copies. To identify high producer cell clones, a limiting dilution approach was established for tagging chromosomal loci evaluation.

7.4 Limiting dilution and clone screening

After having a selected population of tagging cells, clonal cells need to be obtained. The limiting dilution constitutes a long and laborious process. We have obtained on average 15 clones per 96 well plate. Due to the lack of time and resources to continue with all clones, we selected the most fluorescent by fluorescence microscopy. For the clones to grow from a single cell to a confluent well from a 96 well-plate a whole month was needed. At confluence, clones were successively sub-cultured to higher surface growing areas until having a 6 well plate confluent was obtained. At this stage, cell clones were transformed to 5ml suspension culture flasks. There is an adaptation period when cells pass from adherent to suspension and the viability decreases from detaching from the plate. After two to three passages in suspension, clones were cryopreserved. To go from a single cell to a small cell bank of each clone it took on average 3 months.

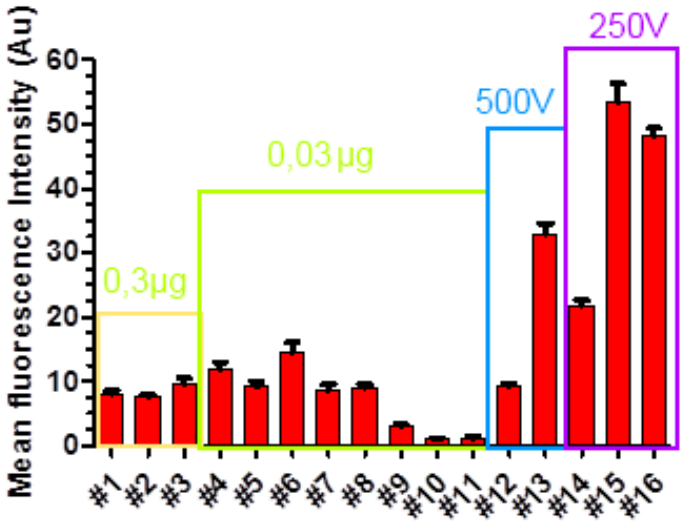


Figure 12- Variability of fluorescence in the screening tagged clones from the four different poulations, as indicated.

After intensive screening of hygromycin-resistant tagged clones, 16 clones were analyzed by flow cytometer in terms of dsRed expression, 11 clones from lipotransfection and 5 clones from electroporation (Fig. 12). Clones with higher expression levels of the reporter protein were, in general, obtained from the cell populations obtained by electroporation. These differences can be due to multiple copies or can be related to influence of the chromosomal surroundings.

7.5 Southern blot analysis of tagged clones

To access copy number of the tagging construct in the genome of the different clones, southern blot analysis was performed. For single copy integrations, the neo probe will only detect a single fragment with size dependent on the genomic integration site of the tagging construct (as illustrated in Fig. 8). Five clones from lipotransfection populations were analysed so far and all of them proved to have single copy integration. Three out of four clones from electroporation also have single copy integration (Fig. 13). As expected from fluorescence analysis, electroporation provided lower efficiencies of single copy integration events, 75% against 100% obtained with lipotransfection. Anyway, clones with single copy integration and better expressing levels of reporter proteins were obtained from electroporation; therefore, both transfection protocols turned out to be suitable for efficient tagging according to the RMCE strategy.

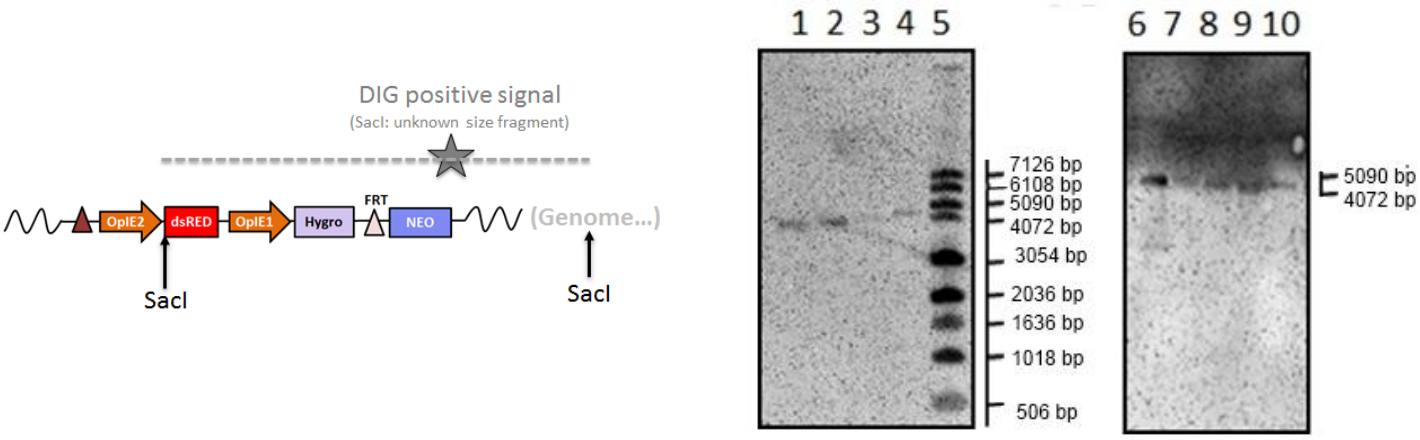


Figure 13 - Southern blot analysis of tagging clones. Clones 5, 8, 9 and 10 (0,03 µg population) are represented in lanes 1 to 4. In lane 5 is the ladder. In Lane 6 is represented clone 16 (250 V) which has two copies of the tagging cassette. In lane 7 is represented the clone 14 (250V). Lane 8: clone 12 (500 V); Lane 9: clone 13 (500 V); Lane 10: clone 1 (0,3 µg).

7.6 Flippase-mediated cassette exchange

We evaluated the efficiency and accuracy of RMCE upon targeting with the eGFP expressing cassette. Targeting and flippase plasmids were co-transfected into one of the clones which had proven to have stable expression from single copy integration (clone 8, from the 0.03 µg population). By fluorescence microscopy we saw a decrease in the number of red cells and an increase in the number of green cells over time (Fig. 14). In agreement with this, PCR analysis showed a decrease in the mRNA levels of dsRed and an increase in the mRNA levels of GFP, 15 days after cassette exchange when comparing to the constitutive18S gene expression (Fig. 15A). These results suggested correct flippase-mediated cassette exchange.

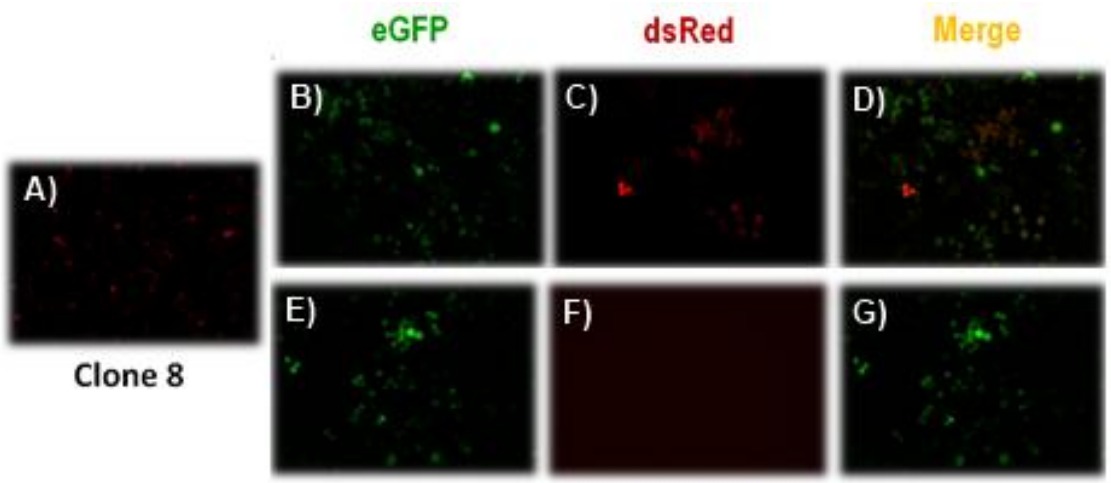


Figure 14 – Fluorescence microscopy images of clone 8 (A) before targeting, (B - D) 72 h after targeting and (E – G) 2 weeks after targeting, using either GFP, dsRED or merging both channels.

After 20 days in neomycin selection, there were practically no red cells and some green colonies had reached a reasonable size, so they could be transferred to 6 well-plates for expansion and further analysis.

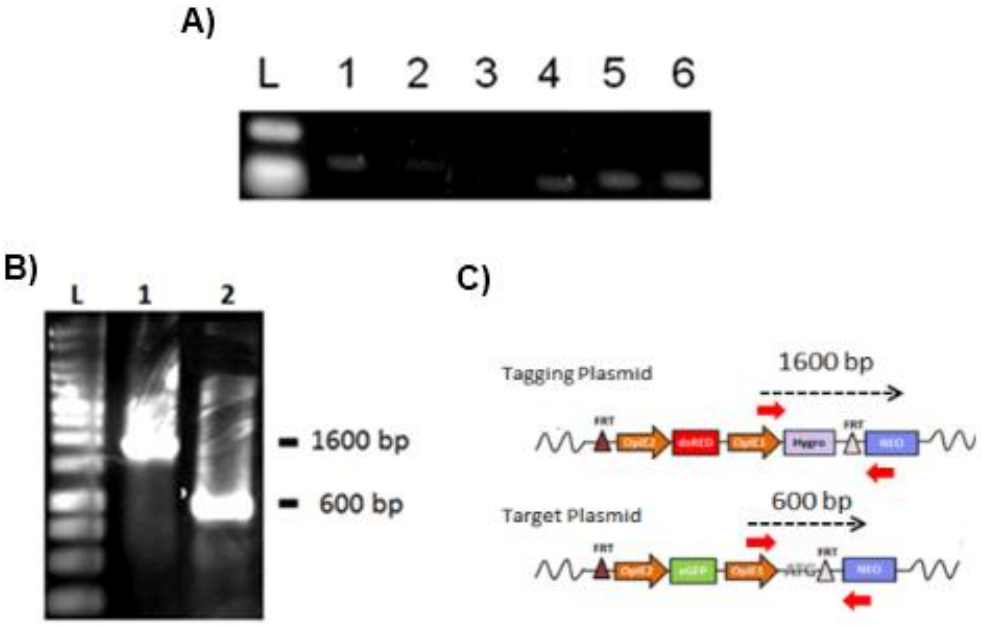


Figure 15 – A) mRNA levels of dsRED and eGFP from clone 8 cells. L – Ladder; 1- red expression of tagging cells; 2- red expression 15 days after targeting; 3- GFP expression of tagging cells; 4- GFP expression 15 days after targeting and G418 selection; 5- 18S expression of tagging cells (housekeeping gene); 6 – 18S expression 15 days after targeting. B) PCR to genomic DNA from clone 8 cells. L – Ladder; 1- Tagging cells; 2- Targeted cells after 15 days in G418 selection. C) Schemetic representation of the result of PCR to genomic DNA, in order to confirm cassette exchange. The same primers were used (pOpIE1 and neo marker).

The cassette exchange was also confirmed by PCR of genomic DNA. The same primers were used in the tagging clone and in the cells after cassette exchange: the primers used were in OpIE1 promoter and in neo marker. A difference of 1000 bp in size confirmed the exchange, since the target cassette lacks the hygromycin gene between OpIE1 promoter and the neo resistance marker. This is the first proof of flippase-mediated cassette exchange in Sf9 cells (Figs. 15B-C). Subclones of the targeting of clone 8 can be seen in figure 16. These targeted cell clones still need to be screened for absence of additional random integration events of the targeting cassette by real-time PCR or Southern Blot analysis. The remaining clones which have proven to have single copy integration of the tagging cassette were already targeted and placed in neomycin selection. From fluorescence microscopy analysis, these clones show an increase in GFP expression and a decrease in dsRed expression which is a good indication that cassette exchange has occurred well in these tagging clones too.

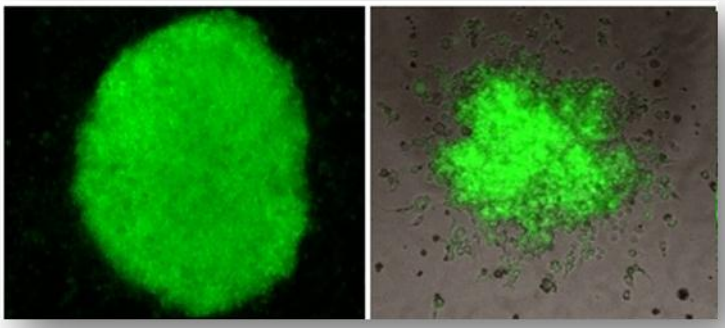


Figure 16 – Fluorescence microscopy images of the colonies obtained after targeting clone 8 cells when they were picked for growth as sub-clones.

7.7 Tetracycline Inducible Circuits

In order to control and tune recombinant gene expression in Sf9 cells, a tetracycline-based transcriptional regulatory circuit, developed originally for mammalian cells, was modified to work in insect cells. This system is composed by two elements: i) a transactivator being continuously expressed under the control of OpIE2 promoter, that will bind to ii) a sequence of seven Tet-operators placed upstream of the Hsp70 minimal promoter and activate the expression of the gene of interest. The hybrid promoter (Tet-operators and minimal hsp70 promoter) were obtained from a plasmid that was being used in S2 *Drosophila* cells. The scheme of the two constructs is illustrated in Fig. 17.

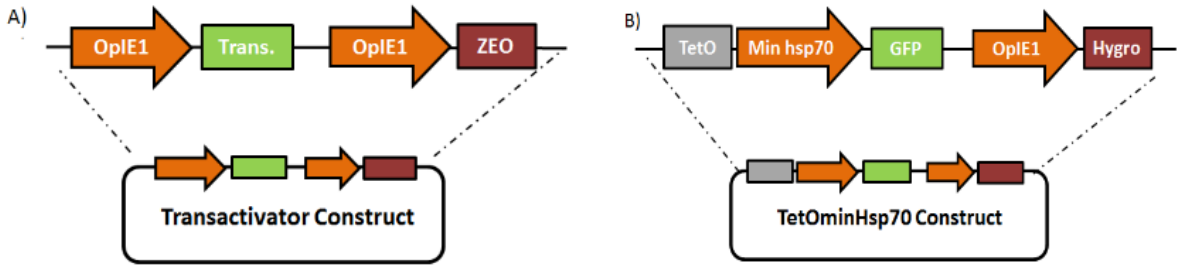


Figure 17- Schematic representation of (A) transactivator (ptTA) and (B) TetOmin Hsp70 constructs.

Once the vectors were constructed, Sf9 cells were transfected with the transactivactor construct. The transfection conditions were the same as in the first study for promoter strength (see materials and methods). The population of Trans (transactivator) was then selected with zeocin for two weeks and then analysed for transactivator expression by western blot (Fig. 18A). After confirming that cells were expressing the transactivator, they were transfected with the second construct, the hybrid promoter (TetOminHsp70) driving the expression of eGFP. We have also transfected parental Sf9 cells with TetOminHsp70 to have a control for the basal expression (without transactivator). After two weeks in hygromycin selection, both populations (with and without transactivator expression) seem to have similar GFP expression levels (by fluorescence microscopy analysis).

A preliminary study was conducted by adding different doxycycline doses to evaluate how the system responds. As explained in the Introduction section, when doxycycline is added to cells harboring the Tet-Off system, it will bind to the transactivator, subsequently preventing the binding of the transactivator to the promoter and thus reducing expression of the transgene (Fig. 19). Cells were subjected to different levels of Dox during 7 days and then analysed by flow cytometry. GFP expression decreased slightly (mean fluorescence intensity decreased from 22.0 to 16.9 as Dox was increased from 0.01 to 100 ng/ml (Fig. 18C). In Fig. 18B, it is a comparison of GFP expression in three cell populations: 1) the population with the full Hsp70 promoter, 2) the population of cells not expressing no legends for the above figure, transactivator but harboring the TetOminhsp70 construct and 3) the population transfected with both transactivator and TetOminhsp70 constructs. We can see that there is a huge difference in GFP expression driven by the full and minimal Hsp70 promoter, which was supposed to be rescued in the transactivator expressing cells. However, only a slight increase in mean fluorescence intensity was observed suggesting that somehow the transactivator is not reaching the Tet-operators sequence in the hybrid promoter as expected.

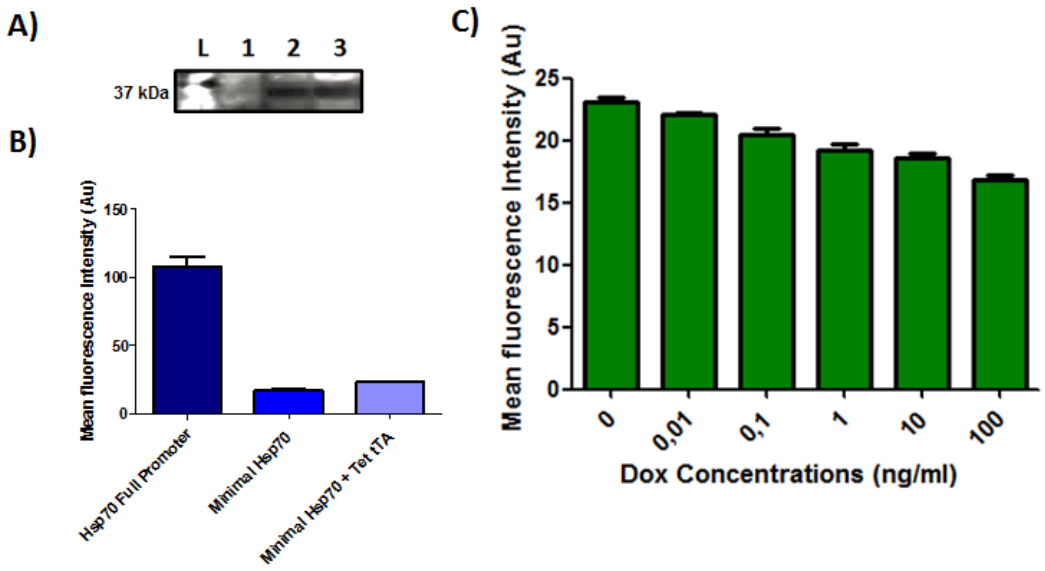


Figure 18 – A) Western blot analys of the transactivator (37 kDa). L - Ladder, 1- Non transfected cells, 2- Transfected cells with 0,05µg of transactivator plasmid and 3- Transfected cells with 0,3µg of transactivator plasmid. B) comparison of GFP expression in three cell populations: i) the population with the full Hsp70 promoter, ii) the population of cells not expressing transactivator but harboring the tetOminhsp70 construct and iii) the population transfected with both transactivator and tetOminhsp70 constructs. C) Cells harboring both constructs were subjected to different levels of Dox during 7 days and then analysed by flow cytometry.

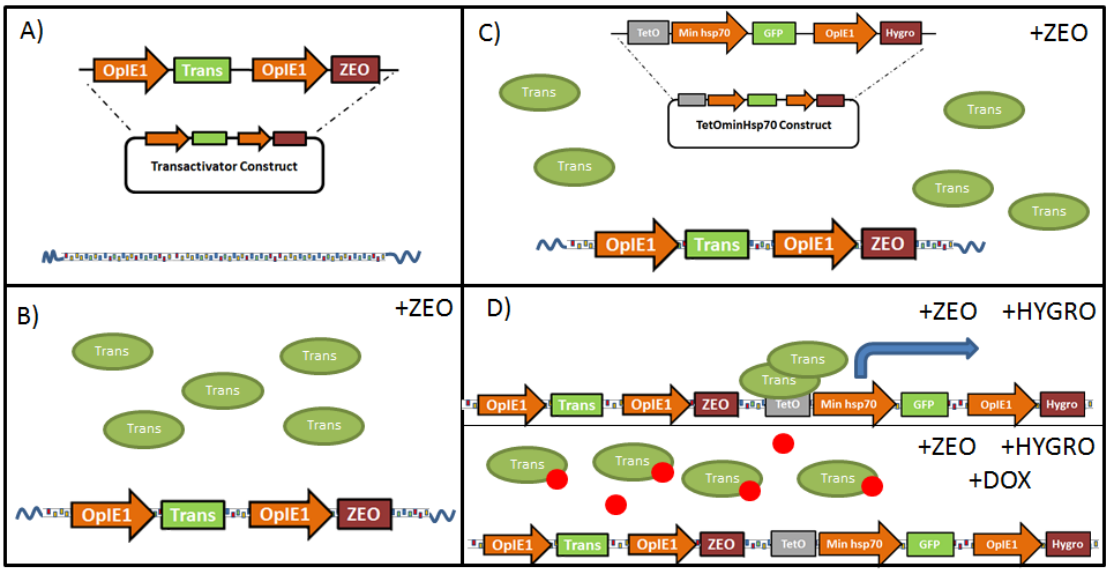


Figure 19 – Scheme of the way how the system should work inside the cells. A) First, cells were transfected with the transactivator B) integrated and selected in order to have a population expressing transactivator C) This population was then transfected with tetominhsp70 and selected. D) If no DOX is on the system there is a maximum of expression of GFP. When dox is added, expression decreases in a dose-dependent manner.

8. Discussion

The first steps in the development of a reusable insect cell factory were undertaken in this thesis. This cell line is expected to be advantageous over the baculovirus-insect cell system, the current gold standard to produce multimeric proteins. Three major tasks were performed during this thesis: 1) different transcriptional promoters were first compared in terms of reporter protein expression and genetic stability in Sf9 cells; then 2) a Flippase RMCE system was implemented in Sf9 cells using the best performing promoter and finally 3) some preliminary work concerning the implementation of an inducible system for proper stoichiometric expression of multicomponent complex proteins was performed.

8.1 Comparison of different promoters strength

For expression of recombinant proteins in biological systems, an initial task is the selection of suitable promoters. Strong promoters with little or almost no tissue specificity must be considered. In what concerns Sf9 cells, constitutive or inducible promoters have not yet been extensively studied, much because these cells have been used essentially in the context of the baculovirus expression system and also because the genome of these cells is not sequenced yet. Thus, constitutive and inducible promoters from closer species, such as *Drosophila,* and immediate early promoters from *baculovirus* these cells were evaluated. When these promoters were cloned into the same vector systems, their expression activities could be compared. Two inducible promoters were tested: *Drosophila* hsp70 and metallothionein promoters (Steller and Pirrotta, 1986; Hegedus et al., 1998). The Hsp70 for a cellular promoter is rather strong; it gives rise to high background activity without heat shock treatment, acting just like a constitutive promoter (Clem and Miller, 1994; Prikhod'ko and Miller, 1996). The metallothionein promoter revealed to be the worst alternative for stable protein expression in Sf9 cells due to the high concentration of metals required to have measurable protein expression, leading to high cytotoxicity. Hegedus et al. (1998) have shown that the *Drosophila* metallothionein promoter allows higher level expression in dipteran cells than in lepidopteran cells such as Sf9 cells used in this work which corresponds to our results. The baculovirus early promoters are stronger, allowing higher protein levels than the *Drosophila*-derived promoters. Their capability of inducing high translational rates in most lepidopteran cell lines have been described previously (Pfeifer et al, 1997). From our results, OpIE2 promoter allowed a four-fold increase in gene expression comparing to hsp70

promoter, but there are some studies reporting up to ten fold increase (Mclachlin et al, 1997). Moreover, the comparison between OpIE1 and OpIE2 promoters was performed previously in our lab, with the OpIE2 promoter allowing about five-fold more reporter protein, in agreement with Pfeifer et al, (1997).

8.2 Flippase-Mediated Cassette Exchange

Our approach to establish a reusable high producer recombinant insect cell line relies on a flippase site-specific recombination system. This strategy was already successfully implemented in several mammalian cell lines for the production of various proteins, including secreted monoclonal antibodies and even to produce highly complex biological entities like retroviral vectors for gene therapy purposes (Schucht et al. 2006; Coroadinha et al. 2006; Wirth et al. 2007). The success of the establishment of this producer cell line depends on some critical aspects, such as an efficient tagging of a hotspot supporting higher expression levels, single copy integration of the tagging cassette, genetic stability and the ability of targeting the tagged site.

The tagging cassette was randomly integrated in Sf9 cells by two transfection methods, cellfectin-based lipotransfection and electroporation, as we did not know which one would allow preferentially single copy integration. Although cellfectin-based transfection allowed higher transfection efficiencies, so far all seven cellfectin clones analysed by Southern blot revealed to have single copy integration. From electroporation, only one from the four analyzed clones has two copies of the tagging cassette. Therefore both transfection methods seem to be adequate for single copy integration purposes.

A remarkable advantage of this tagging/targeting strategy in relation to traditional cell line development is that the cells are marked by fluorescence and so high producers can be pre-selected for dsRED expression to ensure only active transcriptional sites to be tested for their single copy integration and exchangeability (Qiao et al., 2009). The broad expression pattern of the selected clones indicates variable integration sites.

Correct cassette exchange was confirmed in clone 8, one of the clones of lipotransfection with 0,03 µg of tagging cassette. Our system uses for selection an ATG-deficient *neomycin* gene positioned downstream to the FRT site. This system assures correct targeting of recombinants by ATG complementation of neomycin resistance gene, allowing for neomycin selection after exchange. If only excision would occur without introduction of the targeting cassette, cells would not be G418 resistant (Seibler, 1999). In the absence of this positive selection with NEO complementation, random integration would be favored over RMCE since this reaction can only take place at a single site whereas random integration can be widespread in the whole genome (Oumard et al, 2006; Qiao et al,

2009). Nevertheless, high expressing clones can overpass the ATG deficiency and resist to the G418 without exchanging cassettes favoring in this case random integration (Coroadinha et al 2006). To exclude this scenario, we performed a PCR analysis directly to genomic DNA from clone 8 before and after co-transfection with flippase and target cassette. The difference in size between the two bands confirmed undoubtedly cassette exchange. Therefore, we have shown for the first time that with the right modifications flippase-RMCE systems can be applied also to insect cells. This approach opens the way to a highly flexible method for protein expression in Sf9 cells.

Once we generate a master cell line, it will be an ideal platform for the integration of various genes of interest. In contrast to random approaches, screening efforts are minor. Therefore, compared to the traditional integration and selection that takes up to 12 months (Wurm et al, 2004), the tag-and-targeting RMCE strategy only takes about 4 – 6 months to have a novel expression clone with predictable properties (Schucht *et al.* 2006).

8.3 Transcriptional regulatory circuits

Tightly controlled expression of transgenes is a challenge in the establishment of sophisticated cellular systems. Two different patterns of control are normally attempted: (1) the restriction of gene expression to a certain time period while shutting off transgene expression when not needed and (2) to express adjustable levels of a protein in order to individually modulate the concentration of the transgene (Schucht *et al*, 2010). Our aim fits into the second pattern as we intend to control expression of one protein in relation to other protein, both composing the same particle, to obtain stoichiometric balanced expression and improve in this way the percentage of correctly assembled particles. A tetracycline transcriptional control system was implemented in Sf9 cells; the expression of the mammalian transactivator originally conceived by Gossen and Bujard (1992), was driven by the strong Opie2 promoter, and the reporter plasmid was modified from one applied to *Drosophila* (Bello et al. 1998) in which the seven tandem repeated TetO sequences were placed upstream to a minimal Hsp70 promoter, and this hybrid promoter drives the expression of GFP.

The first result we obtained was that, comparing to other systems like *Drosophila* and mammalian cells (Gossen and Bujard, 2002), this Tet-Off system could not induce GFP expression with the same strength. Sf9 cells transfected with just the reporter TetOminHsp70 plasmid exhibit only a slight difference in terms of GFP fluorescence intensity when comparing to cells harboring both parts of the Tet-system: the transactivator and the reporter TetOminHsp70 construct. Moreover, the presence of Dox just decreased only slightly GFP expression, even using high doses of Dox (100 ng/ml). This

means that there is a problem with one of the parts concerning this system, most likely with the transactivator as it has been recently reported in other insect cell line (Karasaki *et al*, 2009) .

One major concern is that the transactivator has to function in the nucleus, thus a nuclear import of this protein should occur. The recognition is in general well conserved for nuclear protein import mechanisms but in some pathways is different among species (Riolobos *et al*, 2010). Indeed some studies have shown that this type of import can be mediated by recognition of the pattern of phosphorylation in the proteins and it was observed that recombinant proteins from mammalian virus when produced in insect cell are not correctly imported (Karasaki *et al,* 2009, Riolobos *et al*, 2010). The transactivator is composed by two proteins fused: the tetracycline repressor protein from *E. coli* and VP16 which is an abundant phosphoprotein present in the tegument of the herpes simplex virus. VP16 enhances gene expression in the tet transactivator (Schmelter *et al.,* 1996). An incorrect phosphorylation of this mammalian virus protein could lead to an incorrect nuclear importation or incorrect action as transactivator (Riolobos *et al*, 2010).In *Bombyx mori* cells Karasaki *et al.* (2009) observed a 100 fold increase in luciferase expression in a Tet-On system when a nuclear locazation signal (NLS) sequence was added to the transactivator.
This might suggest that nuclear protein import mechanisms are different between the two species. Despite the benefits of NLS in the closely related *Bombyx mori cells,* we cannot exclude the possibility of the transactivator being expressed in limiting amounts in Sf9 cells.

9. Conclusion and Future Work Perspectives

In this work it was approached for the first time site-directed engineering of defined chromosomal site in Sf9 cells for the development of a flexible insect cell factory for the production of multiple complex biopharmaceuticals, bypassing the need for baculovirus infection. Although the work is far from being finished we have proved that the use of RMCE can be implemented into these cells with extremely success. The RMCE technology, which allows the introduction of heterologous genes at a predefined chromosomal locus, makes production more predictive and, importantly, allows to reduce the time necessary to define the best producer clone for a particular product each time new complex biopharmaceuticals are intended to be expressed in this cell line.

In parallel, the firsts steps, for the implementation of a tetracycline regulatory network in sf9 cells were conducted. From the results, it is clear that additional changes to the original system must be performed to obtain a Tet-Off system working properly, as it seems that too few if any transactivator is reaching the Tet-operators and so the transactivator is not exerting any control in GFP expression.

Therefore, as future work, we intend to optimize the Tetracycline inducible system. We will focus on the transactivator, in which we can test the effect of adding a nuclear localization signal or even substitute the VP16 mammalian protein to a transcription factor form of virus infecting Sf9 cells, such as baculovirus. Also, instead of minimal Hsp70 promoter we can test minimal OpIE2 promoter. We can anticipate a higher basal expression using this stronger promoter in a Tet-Off system, but this is not really a disadvantage for the application in question. Ideally, the expression without inducer should be as higher as possible and then we should be able to decrease it with some precision by adding a certain amount of inducer.

In addition, we will construct a second cassette system, which will be based on the cre-recombinase system. The best flippase-tagging clone will be transfected with the second cassette and we will screen again for clones with high and stable expression of this cassette and obviously with single copy integration. Then, to be able to control expression of both proteins in relation to each other, the transcriptional circuit will be implemented in one of the cassettes. Finally, we will exchange cassettes with the genes of interest, for instance VP2 and VP6 which are structural proteins from rotavirus that self-assemble to produce a rotavirus-like particle vaccine, and evaluate how this master cell line performs in terms of yields and quality comparing to the gold standard baculovirus expression system.

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Appendix

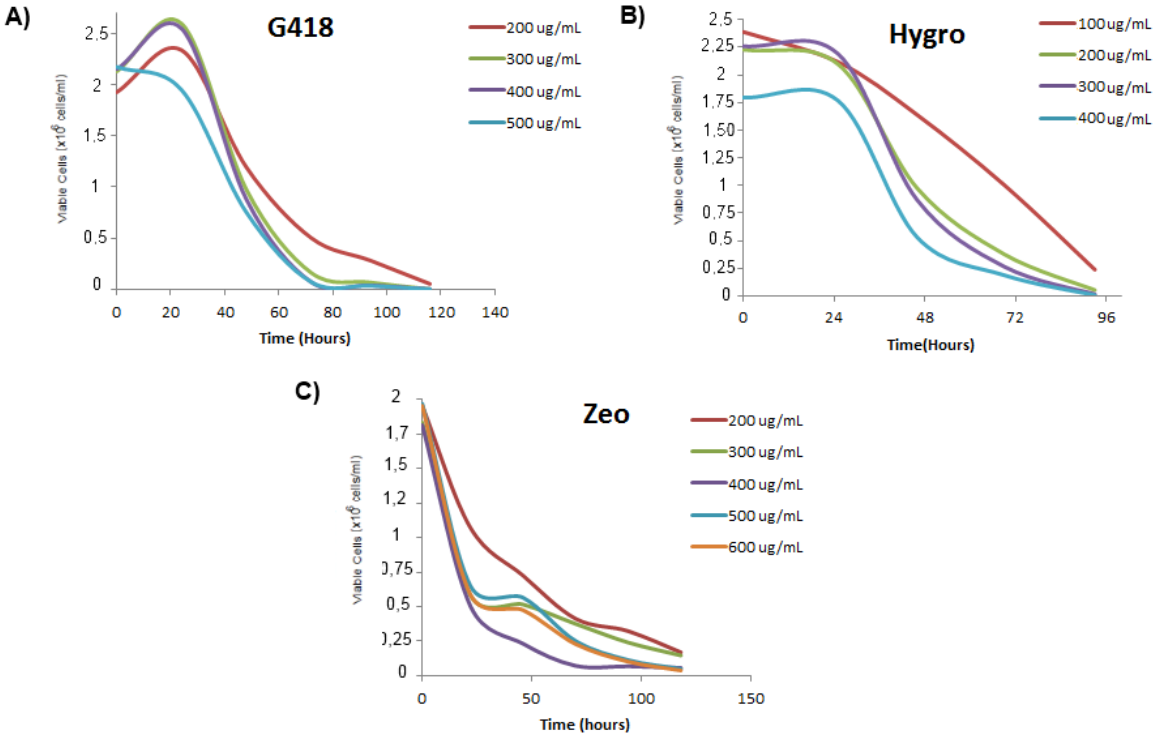

Figure 20 - Killing Curves for the three antibiotics used for selection of the cells. A) G418, B) Hygro and C) Zeo.

Table 2 – Primers used in the construction of vectors need in this work.

Amplified element	Original Vector	Fw Primer	Rv Primer	Receving Vector
eGFP	pMDISGFP	AAACGAATTCATGGTGAGCAAGGGCGAGGA	TATAGAGCTCGCCGCTTTACTTGTACAGCT	pIZT V5
hsp70 promoter	pUAST	AAATCATGAGACGCCGGAGTATAAATAGAGG	TATGAGCTCTTGTATTCAATAATTACTTC	pIZT/OpIE2
mtn promoter	pRmha	AAATCATGACCTCTGGTTCCGATAAGAGA	TATGAGCTCTACCCTTTAGTTGCACTGAGAT	pIZT/OpIE2
OpIE2 promoter	pIZT/OpIE2	AAAACGAATTCTATCAGTCGACTCATGATGATAAACAATGTATGG	AAACACCGGTAAACGGATCCTAAATTCGAACAGATGCTGT	pCAG-dsRed
OpIE1 promoter	pIZT/OpIE2	AAATAAGCTTTTTTGGTCATGCGAAACACG	AATCCTAGGAATAGATCCCGGCAAAGGTGCTGCGCG	pCAG-dsRed
OpIE2+dsRED+OpIE1	pCAG-dsRED	AAAGAGCTCGAAGCTTGCATGCCTCATGA	TATAGAATTCCTACAGGAACAGGTGGTGGC	pTagFwF5
OpIE2 + eGFP	pIZT/OpIE2	AATGCATGCGGATCATGATGATAAACAATG	TATCTCGAGGCTTTACTTGTACAGCTCGT	pFem46(mcs)
OpIE1 promoter	pIZT/OpIE2	AATGAATTCTCATGATGATAAACAATGTA	AATGATATCCCCGGCAAAGGTGCTGCGCG	pFem46(mcs)
Zeo Rest. Marker	pIZT/OpIE2	AATCTGCAGAGAAAAAATGGCTAGCAAAG	TATTTAATTAAGTCCTGCTCCTCGGCCACGAAGTGC	ptTa
TetO+minhsp70	ptetO	AATCCTAGGTAACTTACATACATACTAGA	AATACCGGTGGGGAAAGTATTCAGAGTTCTC	pFlptagging