Expression of Antibodies and Retroviral Vectors from Defined Chromosomal Sites

Strategies towards Reliable Production Systems

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Foreword

The present thesis dissertation is the result of four years of research at the Department of Gene Regulation and Differentiation at the Helmholtz Centre for Infection Research, Braunschweig, Germany, under supervision of Dr. Hansjörg Hauser and in close collaboration with the Animal Cell Technology Unit of ITQB-UNL/IBET, Oeiras, Portugal, under the supervision of Dr. Paula M. Alves.

It gave me the opportunity to be introduced to the challenging field of cell line development for antibody and retroviral vector production, in particular to the demanding task of constitutive lentiviral vector production. All over the years, I had the opportunity to participate actively in other non-related scientific projects.

Altogether, this research experience supported my growth as a person and as a student.

This thesis work intends to explore strategies based on site-directed engineering of defined chromosomal sites towards improvement of cell line development for reliable production of biotechnological relevant products such as antibodies, gamma-retroviral and lentiviral vectors.

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FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Clinigene

Abstract

Technologies enabling expression of recombinant genes in mammalian cells belong to the tools of the trade in molecular biology. These technologies can serve a wide variety of purposes, ranging from basic research to elucidate gene functions *e.g.*, in transgenic mouse models, to biotechnological applications such as manufacturing antibodies or producing viral vectors.

In cell line development, the integration of transgenes into the chromosomal DNA of the host cell is a crucial step, since chromosomal surroundings have a major impact on the expression of the transgene. With regard to safety, product consistency and operational transparency, expression of biotechnological relevant products from single copy DNA integrants is preferable over a scenario of multi-gene integrants.

Currently, most strategies rely on random integration of the transgene and are accompanied by large scale screening efforts to identify suitable clones. The re-use of a favorable locus would be a breakthrough towards production of biotechnological relevant molecules.

Site-directed engineering of defined chromosomal sites has gained considerable relevance in the cell line development field upon establishment of Recombinase Mediated Cassette Exchange (RMCE) technology. This technology permits the rapid exchange of expression cassettes of choice in defined genomic surroundings. In this way, RMCE allows the manipulation of a single chromosomal locus that supports the desired expression level of the relevant protein and renders its repeated re-use feasible.

In this thesis, strategies have been followed that employ targeted integration for antibody expression as well as for viral vector design and production. In particular, the development and exploitation of cell lines with a single copy DNA integrant that sustain high and stable levels of antibody, gamma-retroviral and lentiviral vector expression was addressed.

In **Chapter I**, a review on the current strategies towards site-directed engineering of defined chromosomal loci and the importance and application of locus specific integration towards optimal expression of recombinant proteins is presented. In particular, the first part of the chapter focuses on the creation and exploitation of genetic platforms that sustain optimal levels for transgene expression, highlighting the advantages of targeted integration of transgenes over random insertion-based strategies. In the second part of this chapter, the state-of-the-art on the development of stable producer cell clones for antibody, gamma-retroviral and lentiviral vector production is given. The advantage of locus specific integration towards optimal expression of these bio-molecules is specifically addressed.

In **Chapters II** and **III**, the applicability of chromosomal targeted integration of transgenes is particularly demonstrated. The results presented reinforce the importance of implementation of RMCE technology in the development of consistent and predictable recombinant protein production systems, namely antibodies and gamma-retroviral vectors. Finally, in **Chapter IV**, the extension of chromosomal targeted integration based strategies towards lentiviral vector expression is investigated. This allows the definition of cellular restrictions and requirements that are relevant for the establishment of stable and safe lentiviral vector producer cell lines.

In particular,

Chapter II reports on a comparative approach to identify potent integration sites that support predictable and consistent antibody production levels, based on implementation of RMCE technology. In order to identify chromosomal loci in HEK293 and CHO-K1 cells that support high levels of transgene expression, genomic tagging was performed using various strategies. Cell clones producing > 20 pg/cell in 24 hours were able to be identified. Since the tagged sites had been flanked with heterologous recombinase target sites, selected high expressing clones harboring single integration of the tagging cassette were further systematically exploited by RMCE towards the production of antibodies. A panel of different antibody expressing targeting cassettes was evaluated, differing in the nature of the promoter, order and orientation of the transgenes. Antibody production was found to be highly consistent within the individual cell clones as expected from their isogenic character. However, the nature and orientation of expression control elements were revealed to be critical. It is shown that the composition of the initial tagging vector defines the capacity of the integration site with respect to supporting promoters, indicating that *cis*-effects exerted by surrounding sequences on integrated heterologous regulatory elements play a detrimental role in the expression performance of the integrated construct. This work highlights the significance of chromosomal site tailored-design of expressing cassettes, in order to obtain the maximum benefit of a defined chromosomal site for recombinant protein production by means of RMCE-based technology.

The specific requirements of a particular integration site relating to the maximum level of production is also the main focus of attention in

Chapter III, here specifically related to production of gamma-retroviral vectors.

Chapter III reports on the potential of targeted integration for the evaluation of efficiency and safety of different gamma-retroviral vectors designs. In Part A, two different, previously established HEK293-derived modular packaging cell lines are exploited for the production of a panel of distinct gamma-retroviral vectors upon targeted integration into the defined loci. In Part B, the use of the RMCE-based tagging/targeting procedure is described to generate a murine-based modular packaging cell line, for GALV-pseudotyped gamma-retroviral vectors production. The murine-based production system is exploited for the production of a panel of distinct gamma-retroviral vectors, including clinically relevant therapeutic vectors. Vectors that are distinguished by different components, such as LTR promoters, are introduced in either sense or reverse orientation in a defined locus of the three distinct cell lines. The results show that a combination of promoter, viral vector orientation and integration site are the main determinants of the titer. The modular packaging cell lines are further exploited to evaluate the safety of each viral production system, by assessment of viral vector read-through activity which results in the illegitimate transduction of sequences flanking the targeted retroviral vectors.

Overall, from the work presented in this Chapter it is concluded that the level of viral vector production capacity associated with a producer cell line, is strictly dependent on trans-effects related to the different nature of producer cell lines tested (*i.e.*, human and murine nature). Moreover, the work presented shows that the expression of an integrated viral vector and concomitantly the production of infectious viral particles is strongly dependent on positive/ negative *cis*- effects that

are mediated by the chromosomal sequences present in the vicinity of the integration site(s) of the retroviral construct. These *cis* –mediated effects have different outcomes depending on the molecular composition of the integrated vector(s).

Altogether, this knowledge is believed to be crucial in contributing to a rational design of retroviral vectors, by exploitation of the intrinsic properties of a defined integration site. The definition of optimal combinations of vector elements and integration sites allows the maximization of vector production while providing the high level of safety needed for the implementation of these vectors into clinics.

In **Chapter IV** attention is given to production of lentiviral-based vectors upon integration in biotechnological relevant cell lines such as HEK293. The establishment of cellular systems able to produce lentiviral vectors in a predictable and controlled manner, *i.e.* from a single chromosomal locus, is a milestone that has not been achieved so far. This is in contrast to the most recent strategies for gamma-retroviral vector production. The advantages of exploitation of single copy lentiviral genome integration are obvious. Similar to modular packaging cell lines for gamma-retroviral vector production, the identification of suitable chromosomal loci that sustain high levels of lentiviral vector expression would allow the generation of a flexible lentiviral producer cell line by taking advantage of RMCE technology. Once established, the production of safer infectious particles is envisaged from lentiviral vectors that have been modulated towards their maximal performance from that specific integration site.

In the work presented in this Chapter it is shown that the identification of loci supporting high levels of lentiviral vector expression is not straight-forward. None of the more than 100 individual clones

tested is able to sustain lentiviral vector production at satisfactory levels. Importantly, it is shown that the requirements for producing viral vectors from single copy integration in HEK293 cells are different for gamma-retroviral and lentiviral derived vectors. Intriguingly, the impairment of infectious particle production from single copy integrated lentiviral vector is partially circumvented upon prolonged expression of SV40 T-Ag. The results suggest that pleiotropic effects of T-Ag contribute to the conversion of a non productive to a productive cellular system, acting *a posteriori* of lentiviral genome transcription. A detailed study of the putative restriction mechanisms that are behind the impairment of lentiviral vector production from a defined site is also presented.

This work represents a systematic study of specific cellular requirements for lentiviral genome expression from single copy in a perspective that has not been addressed so far. It is envisioned that GMP-based genetic manipulation of HEK293 cell towards functional replacement of T-Ag will render HEK293-derived cell line capable of producing lentiviral infectious particles from one copy integrated vector.

Long-term stability of genetic modifications in mammalian cells is a prerequisite for the reliable production of recombinant proteins, such as antibodies and viral vectors. This thesis presents important contributions towards the consolidation of methodologies and strategies for cell line development for the stable and predictable production of recombinant molecules with high biotechnological significance. The development and exploitation of novel antibody and retroviral producing cell lines based on targeted integration strategies is presented as paradigms of the outreach of site-directed chromosomal engineering technologies.

Sumário

Tecnologias que permitem a expressão controlada de genes recombinantes em células de mamífero são de particular importância em biologia molecular. Estas tecnologias podem servir uma série de propósitos, que vão desde a investigação fundamental, de modo a elucidar funções de genes- por exemplo em modelos de ratos transgénicos, a aplicações biotecnológicas, como seja a produção de anticorpos e vectores virais.

No desenvolvimento de linhas celulares, a integração de transgenes no DNA da célula hospedeira (produtora) é um passo crucial, uma vez que as zonas cromossómicas adjacentes ao local de integração têm um impacto relevante nos níveis de expressão do transgene. No que respeita à segurança, produção consistente e transparência operacional, é preferível que a expressão de moléculas com relevância biotecnológica seja feita a partir de uma única cópia de DNA transgénico em detrimento de um cenário no qual o transgene está presente em múltiplas cópias.

Actualmente, a maioria das estratégias de produção baseia-se na integração ao acaso do transgene no genoma celular, implicando enormes esforços de rastreio, de modo a identificar clones celulares que apresentem características de expressão desejáveis. Assim, a re-utilização de um *locus* cromossómico favorável à expressão de determinado transgene é visto como uma importante mais-valia para a produção de moléculas de interesse biotecnológico.

Na área do desenvolvimento de linhas celulares, a engenharia cromossómica que visa a manipulação de *loci* específicos tem ganho considerável relevância com o estabelecimento da tecnologia de troca de

cassette mediada por recombinase (do ingles *Recombinase Mediated Cassette Exchange*, RMCE).

Esta tecnologia possibilita a troca rápida de *cassettes* de expressão em *loci* genómicos pré-definidos. Desta forma, RMCE permite a manipulação de uma região cromossómica de interesse, que suporta um nível de expressão desejável de uma determinada proteína, possibilitando a sua repetida reutilização.

Nesta tese, foram seguidas estratégias que empregam integração sítio-específica para expressão de anticorpos, assim como para o refinamento do design e produção de vectores virais. Em particular, foi especialmente abordado o desenvolvimento e exploração de linhas celulares que suportam níveis elevados e constantes de expressão de anticorpos, gamma-retrovírus e lentivírus, onde o transgene de interesse está presente apenas numa única cópia.

No **Capítulo I**, é apresentada uma revisão das estratégias actuais que visam a engenharia cromossómica sítio-específica para a máxima expressão de proteínas recombinantes. Na primeira parte do Capítulo é abordada a criação e exploração de plataformas genéticas que suportam níveis óptimos de expressão de transgenes, dando especial ênfase às vantagens associadas a integração sítio-específica em relação a estratégias que implicam uma integração não controlada desse mesmo transgene. Na segunda parte do Capítulo, é descrito o estado da arte em desenvolvimento de linhas celulares estáveis produtoras de anticorpos, gamma-retrovírus e lentivírus. A vantagem de integração sítio-específica para expressão dessas bio-moleculas é especialmente abordada.

Nos **Capítulos II** e **III**, a aplicabilidade de integração sítio-específica é demonstrada. Os resultados apresentados reforçam a importância da implementação da tecnologia RMCE no desenvolvimento

de sistemas celulares produtores de proteínas recombinantes, nomeadamente anticorpos e gamma-retrovírus de um modo estável e previsível. No **Capítulo IV** é abordada a produção de vectores lentivirais com base na aplicação de estratégias que visam a integração sítio-específica do genoma viral. A definição de restrições e requisitos celulares que são relevantes para o estabelecimento de linhas celulares produtoras de vectores lentivirais com elevados níveis de segurança e consistência é reportada.

Em particular,

O **Capítulo II** descreve uma abordagem comparativa que visa a identificação de *loci* cromossómicos com capacidade de suportar níveis elevados de produção de anticorpos, baseada na implementação da tecnologia RMCE. Foi efectuada, em linhas celulares HEK293 e CHO-K1, uma marcação genómica de um *locus* por célula, de modo a identificar *loci* cromossómicos de interesse para a expressão de anticorpos. Linhas celulares com capacidades de produção superiores a 20pg/célula em 24 horas foram identificadas. Uma vez que estes *loci* estão marcados geneticamente com sequências específicas que são reconhecidas por recombinases, a troca de cassette residente por outra é um processo exequível. Assim, linhas celulares seleccionadas e associadas a elevada capacidade de expressão do transgene a partir de um determinado sítio de integração podem ser sujeitas a uma sistemática exploração pela aplicação da tecnologia de engenharia molecular RMCE, pela simples troca sítio-específica de *cassettes* genéticas. Um painel de diferentes *cassettes* que expressam anticorpos mas que diferem na composição de promotores, ordem e orientação do transgene foi avaliado após integração sítio-específica mediada por RMCE. A produção de anticorpos

por linhas celulares clonais é altamente consistente, o que é expectável devido ao carácter isogénico das diferentes linhas individuais. Contudo, a natureza e orientação dos elementos que regulam a expressão génica revelou-se como sendo crítica. É referido que a composição genética da *cassette* marcadora inicial (residente) define a capacidade daquele local de integração no que respeita a suportar sequências promotoras, o que indica que efeitos mediados em *cis* e exercidos pelas sequências genéticas adjacentes ao local de integração exercem um papel fundamental na capacidade de expressão da sequência integrada. Este trabalho enfatiza o significado e mais-valia do design de *cassettes* desenvolvido especificamente para expressão do transgene a partir de um local de integração específico. Deste modo, pretende-se, aplicando tecnologia RMCE, obter o máximo proveito do *locus* definido para a produção de proteínas recombinantes.

Os requisitos específicos de um local de integração particular relacionados com o máximo nível de produção são também abordados no Capítulo III, aqui especialmente relacionado com a produção de vectores gamma- retrovirais.

Capítulo III descreve o potencial da integração sítio-específica para a avaliação da eficiência e segurança associada a diferentes designs de vectores gamma-retrovirais. Na Parte A, duas linhas celulares de empacotamento derivadas de HEK293 e previamente estabelecidas são exploradas para a produção de um painel de diferentes vectores gamma-retrovirais após integração sítio-específica. Na Parte B, é descrito o uso da tecnologia RMCE para gerar uma nova linha celular de empacotamento derivada de células de murino, capaz de produzir vectores gamma-retrovirais pseudotipados com o envelope GaLV (do Inglês *gibbon ape leukemia virus*). Este sistema de produção é explorado

para a produção de distintos vectores gamma-retrovirais, incluindo vectores terapêuticos. Vectores diferindo em vários componentes, como sejam, promotores, são integrados nos *loci* pre-definidos das três células de empacotamento quer em orientação directa ou reversa. Os resultados demostram que a combinação de sequências promotoras de transcrição, a orientação da integração do vector viral bem como o local de integração, são os maiores determinantes da capacidade de produção dos diferentes sistemas celulares. Estas células modulares de empacotamento são ainda exploradas de modo a avaliar o grau de segurança associada a cada sistema de produção de vectores virais, medindo a actividade de *read-through* da qual resulta a transdução ilegítima de sequências cromossómicas que ladeiam o local de integração dos vectores virais.

Em suma, do trabalho apresentado neste Capítulo conclui-se que o nível de capacidade de produção de vectores virais associada a uma linha celular é estritamente dependente de efeitos exercidos em *trans* e relacionados com a diferente natureza das linhas celulares testadas (isto é, linha celulares humanas e murinas). Além do mais, o trabalho apresentado demostra que a expressão de um determinado vector viral integrado no genoma da célula produtora e concomitantemente a produção de partículas virais infecciosas é fortemente dependente de efeitos positivos/negativos exercidos em *cis* e que são mediados pelas sequências cromossómicas adjacentes ao local de integração do transgene retroviral. Estes efeitos exercidos em *cis* têm diferentes resultados, dependendo na composição molecular do vector integrado.

Este conhecimento é tido como crucial para o desenvolvimento de uma estratégia racional de design de vectores retrovirais, com base na exploração das propriedades intrínsecas de um local específico de

integração genómica. A definição de combinações ideais de elementos vectoriais e do respectivo local de integração permite a maximização da produção de vectores, com o grau de segurança necessário para a implementação destes moléculas em prática clínica.

No **Capítulo IV**, é prestada atenção à produção de vectores lentivirais após integração sítio-específica em células de interesse relevante para biotecnologia, como sendo HEK293. O estabelecimento de sistemas celulares capazes de produzir vectores lentivirais de modo previsível e controlado, isto é, a partir de um *locus* definido, é um objectivo ainda não alcançado. Isto está em contraste com as mais recentes estratégias para a produção de vectores gamma-retrovirais, atrás descritas. As vantagens da exploração de um *locus* definido para a produção de vectores lentivirais são óbvias. De modo semelhante ao que é descrito para gamma-retrovírus, a identificação de *loci* cromossómicos capazes de suster níveis elevados de expressão de vectores lentivirais abre a possibilidade da geração de linhas celulares flexívies para a produção de vectores, aplicando a tecnologia RMCE. Uma vez estabelecida, a produção de partículas infecciosas a partir de vectores lentivirais optimizados para um determinado *locus* é previsível.

Pelo trabalho apresentado neste Capítulo demonstra-se que a identificação de *loci* que suportam níveis elevados de expressão de vectores lentivirais não é trivial. Em mais de 100 clones celulares, nenhum foi identificado como estando associado à produção de lentivírus a níveis de produção satisfatórios. É demonstrado que os requisitos de linhas celulares derivadas de HEK293 para a produção de lentivírus a partir de integração em cópia única do vector viral são diferentes dos associados à produção de vectores gamma-retrovirais nas mesmas condições. No entanto, a incapacidade de produção de

partículas infecciosas a partir de vectores lentivirais presentes no genoma de células produtoras em cópia única é parcialmente aliviado após prolongada expressão de Antigénio-T do Vírus Símio 40. Os resultados sugerem que os efeitos pleiotrópicos de Antigénio-T contribuem para a conversão de um sistema celular não produtivo para um sistema produtivo actuando *a posteriori* da transcrição do genoma lentiviral. Um estudo detalhado sobre os possíveis mecanismos que restringem a produção de vectores lentivirais a partir de integração sítio-específica do transgene viral são também apresentados.

Este trabalho descreve um estudo sistemático dos requerimentos para a expressão do genoma lentiviral a partir de uma única cópia integrada na célula produtora, numa perspectiva que até agora não tinha sido abordada. É expectável que manipulação genética de linhas celulares em condições de *Boas Práticas de Produção* (do inglês *Good Manufacturing Practice*) que visem a substituição do Antigénio –T, renda o estabelecimento de uma linha celular capaz de produzir vectores lentivirais a partir de uma única cópia do transgene viral.

A estabilidade a longo-termo de modificações genéticas em células de mamífero é um pré-requisito para a produção de proteínas recombinantes, como sejam anticorpos e vectores virais. Esta tese apresenta importantes contribuições para a consolidação de metodologias e estratégias que visam o desenvolvimento de linhas celulares para a produção consistente e previsível de moléculas recombinantes de alto valor biotecnológico.

O desenvolvimento e exploração de novas linhas celulares produtoras de anticorpos e retrovírus baseados em estratégias de integração sítio-específica são apresentados como paradigmas do alcance das tecnologias baseadas em engenharia cromossómica.

Thesis publications

- Wirth D., Gama-Norton L., Riemer P., Sandhu U., Schucht R., Hauser H.(2007).Road to precision: Recombinase-based targeting technologies for genome engineering. *Curr Opin Biotechnol* 18(5): 411-9.

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Brief Contents

CHAPTER I

INTRODUCTION

This Chapter is adapted from the paper

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and book chapter

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CONTENTS

OVERVIEW

This chapter constitutes a detailed revision on the current approaches towards the establishment of biotechnologically relevant producer cells. It comprises both the state-of-the-art strategies for stable production of antibodies and retroviral vectors and also highlights the recently evolved recombinase-based targeting technologies for genome engineering.

In Part A, the impact of the chromosomal surroundings on transgene expression is emphasized – which was basically the motivation to develop methods for site specific integration, as detailed in this chapter. The advantages of targeted integration over random integration for expression of transgenes are discussed. Furthermore, this part focusses on strategies towards exploitation of favorable loci for production of different molecules.

In Part B, the current status on production of antibodies, gamma-retroviral and lentiviral vectors is specially addressed. The biotechnological benefit of targeted integration for the predictable expression of antibodies is discussed. Attention is given to gamma-retroviral vector production upon locus specific integration and to strategies to exploit this approach for the production of safer gamma-retroviral and lentiviral vectors for application in the field of gene therapy.

PART A

Standard and advanced strategies towards optimal transgene expression in mammalian cells.

1. Limitations of the classical random gene integration strategy.

Efficient production of proteins and virus particles using mammalian cell lines often relies on random and stable integration of an expression construct into the cell's chromosomal DNA. Once a construct is incorporated its expression levels will be determined by neighboring genetic elements (Festenstein *et al.*, 1996; Bell and Felsenfeld, 1999). For instance, enhancers will support expression while silencers and formation of heterochromatin will suppress it. A so called "position effect" constitutes a major drawback of the random approach of integration; expression from many integration sites is silenced or severely reduced and stability of long term expression is often constricted. This results in an unpredictable expression pattern. Expression can also differ due to variable gene copy number. Importantly, the intuition that a higher copy number supports stronger expression is not necessarily valid. Conversely, single copy integration can as well provide the required expression strength and stability (Yarranton, 1990; Coroadinha *et al.*, 2006; Schucht *et al.*, 2006). If a toxic protein is to be produced, regulated transgene expression is required. In this case, genomic loci have to meet even higher demands. Here, they need not simply support efficiently high expression but should display low basal expression levels as well as strong inducibility. Consequently, extensive screenings are necessary to identify cell clones (*i.e.*, integration sites) with suitable expression characteristics. This selection step usually requires several weeks and it has to be repeated for each new expression construct, slowing down the generation of production cell

lines to a significant degree. Thus, the ability to repeatedly reuse a single chromosomal locus that supports the desired expression characteristics is highly advantageous. To enable exploitation of a favorable chromosomal integration site, a primary genomic modification to first mark (tag) the site needs to be performed. This in turn, would create a genomic platform that eventually supports subsequent modifications of that particular site.

2. Primary genomic modification to integrate a tag.

Tagging a genomic site that possesses the desired expression properties is a prerequisite to generate a platform for further engineering. To utilize a particular chromosomal site, two approaches can be considered for the primary integration of a tag: (a) functional screening of an unknown locus using random integration, (b) exploiting a characterized genomic site using homologous recombination. The second approach requires knowledge about the properties of chromosomal sites. This information is usually not available; in particularly not in cell lines that are used for protein or viral vector production. On the other hand, random integration provides the possibility to screen for the desired properties amongst thousands of loci. This is why currently most endeavors follow the random approach. The random integration of recombinant genes into a host cell line is characterized by unpredictable *cis* -effects which might either positively or negatively regulate gene expression. Productivity as well as clonal stability consequently depends on the site(s) of integration. Exhaustive screening to identify loci with

desirable (*e.g.*, high and/or regulated) and stable expression patterns is hence mandatory. Current transfection methods such as lipofection, electroporation, calcium phosphate precipitation and even viral transduction lead to unpredictable integration patterns, although a specific bias has been shown for individual methods (Schroder *et al.*, 2002; Wu *et al.*, 2003; Mitchell *et al.*, 2004; Bushman *et al.*, 2005). Thus, any of the above mentioned methods are suitable tools to integrate the tags randomly as long as single copy integrates are achieved. Alternatively, homologous recombination permits the integration of tags to use predefined chromosomal loci with known expression characteristics. This strategy exploits the cells' own recombination apparatus to exchange a certain DNA sequence for the construct of interest which is flanked by ends homologous to the endogenous locus. Homologous recombination has been widely applied in murine embryonic stem cells to establish *e.g.*, knockout mouse models. However, the frequency of homologous recombination in differentiated mammalian cells is very low (one in a million or even less) requiring a large number of clones to be screened for correct integration. In differentiated cells the high ratio of illegitimate recombination masks homologous recombination showing that this strategy is not suitable for routine use (Glaser *et al.*, 2005). For circumventing these drawbacks, zinc finger nucleases (ZFNs) have emerged for site-specific genome modifications (Porteus and Carroll, 2005). ZFNs are composed of DNA recognizing zinc finger domains fused to an endonuclease (Kim *et al.*, 1996; Cathomen and Joung, 2008). They can be designed to generate sequence-specific double-strand DNA breaks which are then repaired exploiting the natural

homology-directed DNA repair machinery. Originally developed to repair point mutations, these ZFNs have been recently applied for gene addition into a pre-determined endogenous locus in human cells (Moehle *et al.*, 2007). Initial limitations of their specificity (Bibikova *et al.*, 2002; Porteus and Baltimore, 2003) can now be overcome by newly engineered variants (Miller *et al.*, 2007). While these ZFN-based approaches are a promising tool, they have not yet been applied in mammalian biotechnology.

3. Exploiting the tagged loci by site specific recombination.

Once a good chromosomal integration site has been identified and tagged, repeated extensive screenings can be circumvented by the ability to constantly reuse the desired locus. These loci can hence be used to specifically integrate any DNA sequence of interest providing predictable expression levels. Homologous recombination in principle can permit this, but the above mentioned drawbacks limit the feasibility of this approach. Efficient and flexible ZF-nuclease based technologies are still being developed and have not yet found routine use for biotechnological applications. Currently, the technology fulfilling at best the requirements that allow the recognition of a specific genomic site are based on the use of site-specific recombinases (SSR).

3.1. Site-specific recombinases as tools for targeting a previously tagged locus.

In the past 10 years, SSRs have been widely exploited for targeted integration of transgenes into the mammalian genome. The best-studied and most commonly used SSRs in mammalian cell cultures are the bacteriophage P1-derived Cre, the *Saccharomyces cerevisiae*-derived Flp and the bacteriophage □C31-derived integrase. These SSRs recognize distinct sequence-specific motifs termed as recombination target sites-RTs (LoxP, FRT and attB/ attP, respectively) catalyzing efficient conservative DNA rearrangements (reviewed in Branda and Dymecki, 2004). The basic principle of their use involves a tagging and screening step, in which suitable chromosomal loci are identified and marked by the integration of specific recognition sequences for the recombinase enzyme. These sites can hence later be used for specifically integrating any expression construct of interest into the predefined locus providing predictable expression levels. On the whole, screening for well expressing chromosomal loci has to be performed just once, reducing both time and effort to establish producer cell lines.

3.2. Flip-In and Recombinase Mediated Cassette Exchange (RMCE) as versatile applications of site-specific recombinases.

First generation of SSR-based chromosomal targeting relies on the integration of a single LoxP or FRT site into the genome of the chosen cell line. This is followed by the transfection of a plasmid carrying the

matching RT in the presence of the recombinase and subsequent trapping of the rare integration event (O'Gorman *et al.*, 1991). These pioneering approaches have three limitations: firstly, due to the reversibility of the reaction catalyzed by the recombinase, excision is favored over insertion as a result of which extensive screenings and temporal expression of the recombinase are required; secondly, prokaryotic sequences are co-integrated and thirdly, a positive selection marker is left behind in the chromosome after the integration.

A second-generation chromosomal targeting system arose with recombinase-mediated cassette exchange (RMCE), which is based on the replacement of gene cassettes flanked by two non-interacting RTs (Karreman *et al.*, 1996). Apart from the naturally occurring heterotypic RTs (*e.g.*, □C31), several mutants have been developed for Cre and Flp (Branda and Dymecki, 2004) providing the required heterospecificity crucial for RMCE.

The technique of RMCE involves two steps:

1. *To tag the genomic locus of interest*: In this step, the heterotypic and incompatible recognition targets are introduced into a genomic locus (please refer section 2). This creates a cassette acceptor allele, *i.e.*, the tagged locus can now be used to integrate and exchange different DNA cassettes of choice.

2. *To target the genomic locus of interest*: A targeting vector containing the desired transgene flanked by the same set of heterotypic recognition target sites can now replace the DNA region flanked by the recognition target sites in the tagged locus. This reaction is catalyzed by the recombinase via a double reciprocal

crossover recombination event. The overall strategy can hence be termed as the "Tag and Target" strategy (Baer and Bode, 2001).

RMCE thus permits the reuse and rapid modification of a pre-defined chromosomal locus through targeted integration of transgenes. Meanwhile, this technique has proven to be highly efficient enabling routine integration of gene cassettes in any given locus supporting the desired expression level (Seibler *et al.*, 1998; Verhoeyen *et al.*, 2001; Coroadinha *et al.*, 2005b; Schucht *et al.*, 2006; Nehlsen *et al.*, 2009).

PART B

Production of Antibodies and Retroviral Vectors.

Importance and application of locus specific integration towards optimal expression of bio-molecules.

The successful production of any recombinant protein for diagnostic or therapeutic purposes is dependent on the generation of stable producing cell clones. The state-of-the-art of the development of stable cell lines for antibodies and retroviral vector production will be reviewed in the following section. The potential of recently established RMCE technology towards production of these bio-products will be addressed.

4. Antibody production. *The importance of gene targeting approaches on the optimization of antibody expression technologies.*

Since recombinant DNA technologies evolved 30 years ago, developments in protein expression systems and cell culture methods have been of central interest for the biopharmaceutical industry. Important factors for the development of a protein production cell line include the ability of a cell type to serve as a good producer cell line in terms of growth conditions and the yield and quality of the resulting recombinant molecule. The majority of clinical biopharmaceuticals are currently being produced in CHO cells and their DHFR-deficient derivates, as well as in NS0 and HEK293 cells and the human retina-derived PER.C6® (Crucell, N.V., Netherlands). Much effort has been made in recent decades towards the development of cell lines with excellent safety profiles, scalability and productivity under serum-free culture conditions. The bottleneck of stable protein expression from such cell lines seems to be with the optimization of antibody expression technologies. There is an urgent need for

systematic genetic approaches that allow simple screening and result in desired expression of any therapeutically relevant protein. One classical procedure for the establishment of a stable production cell line is to transfect a host cell (*e.g.*, DHFR-deficient CHO cell lines) with plasmids containing the recombinant gene and its necessary regulatory elements along with a selectable marker gene. Commonly used selection genes are dihydrofolate reductase (DHFR) and the glutamine synthetase (Kingston *et al.*, 2002). Following application of increasing amounts of methotrexate or methionine sulfoximine the production levels can increase due to amplification of the inserted genes. Although these selection methods are applicable to increase expression levels of antibody expression cassettes, their limitations have been described (Kim *et al.*, 1998; Kim *et al.*, 2001; Jun *et al.*, 2006). High clone to clone variations as well as instability in expression levels after amplification have been shown to be due to severe genetic rearrangements during gene amplification as well as the emergence of drug-resistance. Hence, the procedure of gene amplification needs intense screening to identify a clone with the potential to express adequate amounts of protein after gene amplification, which remains stable over time. In contrast to the drawbacks of gene-amplification such as genomic instability, followed by ambiguous expression levels, a single copy integration can yield levels of production that are competitive with state of the art industrial productivity levels (Yarranton, 1990). Still, productivity as well as clonal stability can be influenced by the site of integration. Productivity will vary among clones and the identification of high producers will require intensive screening for clones with high and

stable expression patterns. As a consequence, for every new production clone the process development must be re-established, which is time consuming and expensive. It is therefore desirable to reduce the production cost and the time needed for cell line development. To this end, gene targeting strategies can be applied to expression of biopharmaceutical relevant proteins. Most efforts reported until today rely on the first generation targeting systems with the above-mentioned limitations (please refer to section 3). The potential of this technology was exploited for the production of a human polyclonal anti- RhD antibody (Wiberg *et al.*, 2006) by integrating 25 individual antibody expression cassettes into a defined FRT tagged integration site in CHO cells (Flp-In TM cell line; Invitrogen). An oligoclonal cell pool derived thereof provided a highly reproducible relative distribution of each antibody. Furthermore, comparable antibody expression levels could be achieved upon targeting at defined integration sites in CHO cells, thereby realizing the concept for antibody production using Cre- (Kito *et al.*, 2002) or Flp mediated integration (Huang *et al.*, 2007). Nevertheless, only marginal numbers of applicable clones could be obtained by both groups following sustained screenings for appropriate integration loci. These fulfilled the desired properties such as expression stability and the ability for gene amplification, which could be applied for transgene targeting and would express satisfactory levels of a desired antibody. So far, the identification of potent integration sites that allow efficient expression of variable recombinant genes without the use of additional gene-amplification steps has not yet been achieved. It is envisioned that once these favourable sites are identified, RMCE

provides a unique tool towards their exploitation for predictable expression of biotechnologically relevant proteins such as antibodies.

In **Chapter II**, one strategy focusing at this point is described. It comprises a comparative study evaluating different approaches for identification of potent chromosomal sites for antibody production. Moreover, the flexibility of these chromosomal sites to support predictable and consistent antibody production levels, based on implementation of RMCE technology it is addressed.

5. Retroviral vector production. *State-of-the-art of production strategies and the importance of predictable production systems towards generation of safer vectors.*

Gamma-retroviral and lentiviral- vectors (generally referred as retroviral vectors) are important and well characterized tools widely used for stable gene transfer into mammalian cells (*e.g.*, as recently shown for somatic cell reprogramming, Takahashi and Yamanaka, 2006; Takahashi *et al.*, 2007; Yu *et al.*, 2007; Carey *et al.*, 2009; Chang *et al.*, 2009) .

The consistent progress of retroviral-based gene therapy approaches (Aiuti *et al.*, 2002; Hacein-Bey-Abina *et al.*, 2002; Ott *et al.*, 2006; Aiuti *et al.*, 2009) and the most recent successful clinical trials employing the use of lentiviral vectors (Cartier *et al.*, 2009) have contributed to strengthen gamma-retroviral and lentiviral vectors as tools of excellence in biological sciences. Their high relevance in basic research and more importantly their consolidation as vehicles for gene delivery in pre- and clinical settings contributes to the

mandatory improvement of their safe and standardized production on a large-scale.

5.1. Production of retroviral vectors upon transient transfection and from stable producing cell lines.

The production of viruses, or viral vectors, should be regarded as a special case of recombinant protein production. In contrast to single protein expression, viruses and viral vectors consist of a number of molecules that must be assembled into a functional unit – primarily to incorporate and protect genetic information and deliver it into target cells. Accordingly to the most recent protocols for retroviral vector generation, expression of the following packaging genes is necessary:

- the gag gene: encoding the core proteins capsid, matrix, and nucleocapsid;
- the pol gene: encoding for the viral enzymes protease, reverse transcriptase, and integrase;
- a gene encoding an envelope protein: which will determine the tropism of the viral vector (one of the most widely used heterologous proteins for psedotyping retroviral vectors is the G protein from vesicular stomatitis virus, VSV-G).

Beside the components described above, in case of the generation of lentiviral vectors, the expression of the accessory protein rev, responsible for nuclear export of unspliced viral RNA is also required.

In order to minimize the risk of generating replication-competent viruses by recombination, all these components must be physically separated on different transcriptional units. Since, all the components

are provided to the producer cell in *trans*, generation of infectious viral particles at high titers is dependent on balanced stoichiometric expression of all viral components (Yap *et al.*, 2000; Carrondo *et al.*, 2008).

Two different approaches towards production of retroviral vectors can be undertaken:

- transient transfection of a suitable cell line with plasmids encoding the retroviral genome as well as the different components necessary for the assembly of an infectious viral particle;
- the generation of stable cell lines constitutively expressing these elements from chromosomally integrated plasmids.

Transient production of retroviral vectors and the associated drawbacks

Transient transfection as a method to generate pre-clinical and clinical grade retroviral vectors has a number of non negligible drawbacks. The most relevant are listed below:

a) The purification of plasmid DNA and viral vector batch: The plasmid DNA to be transfected into the cell line must be produced in cGMP conditions. The plasmid DNA must have very low or non-existing traces of bacterial endotoxins, bacterial host cell DNA and RNA, residual solvents and proteins while being highly enriched in supercoiled DNA. Accordingly, the manufacture of these molecules can be extremely costly. Concerning the purification of the viral

vector supernatant, the transient production method requires an elaborate downstream treatment of the biological product that ensures the total elimination of residual plasmid DNA in the stock that is to be administered *in vivo*;

b) The characterization of the viral batch: Large quantities of transfected plasmid DNA may exacerbate the risk of replication competent lentiviral particle (RCL) generation by increasing the potential for recombination between cotransfected plasmids. Thus, exorbitant costs may be incurred to continuously produce the reagents and execute the tests required for a detailed characterization of each viral batch produced;

c) The reproducibility: The production of a viral stock by a transient transfection process can result in a very high level of batch to batch variation requiring standardization of the plasmid production, quality and yield of the vector stock that is difficult to achieve;

d) The up-scaling: The large scale production of retroviral vectors based in transient transfection techniques is not straight forward. Some of the difficulties are related to the large quantities of reagents required for each production and technical limitation.

In conclusion, the production of vector using a transient transfection method is strongly discouraged and a producer cell line that constitutively expresses all of the components necessary for the

production of infectious particles arises as the most preferred scenario for clinical grade production of retroviral vectors.

The particular case of transient lentiviral vector production: the so far uncharacterized benefit of T-Ag expression on vector production

Current protocols for lentiviral vector production are still based on transient transfection of 293-T cells, a SV40 large T-antigen (T-Ag) transfected HEK293 cell line. These cells are highly transfectable with low-cost calcium phosphate based methods and generally associated to viral titers in the range of $1x10^6$ to $1x10^7$ i.p./ml prior concentration.

Over-expression of the large T-Ag allows enhanced episomal replication of packaging and lentiviral expression plasmids containing the SV-40 origin of replication and generally enhances lentiviral production through additional unknown mechanisms. SV40 derived T-Ag has been reported as an oncoprotein that elicits cellular transformation. It has been extensively shown the role of T-Ag in regulating cellular gene expression through its actions on tumour suppressor genes pRb and p53, being these proteins two key targets during cellular transformation exerted by this viral molecule (Ahuja *et al.*, 2005). But, in addition to control expression of cellular genes involved in the progression of cell cycle, it has been reported that T -Ag is a promiscuous activator of many viral and other cellular promoters (Gilinger and Alwine, 1993; Rice and Cole, 1993; Moens *et al.*, 1997). The pleiotropic effects of T-Ag expression are believed to account for the dramatic and not yet fully characterized alterations in

the cellular transcriptome of 293-T, rendering it as the cell line of choice for high lentiviral titers.

As the transition of lentiviral-derived vectors from bench to bed-side has been consolidated (Levine *et al.*, 2006; Cartier *et al.*, 2009), the transient production in 293-T of such infectious viral particles is gaining particular importance. Nevertheless, relatively little progress has been made towards optimization of the transient transfection parameters and only minor modifications of the protocol originally published by Naldini and collaborators (1996) have been reported (Mitta *et al.*, 2005; Segura *et al.*, 2007).

As already referred, SV40 large T-Ag is a multifunctional protein with oncogenic proprieties since affects both checkpoint control in G1 of the cell cycle and p53 activity. Therefore, the putative occurrence of co-transduction of T-Ag gene it is of major concern and raises limitations to the use of 293-T cell line for production of vectors with application in human medicine.

Stable production of retroviral vectors - advantages of single copy viral genome integration.

Much effort has been laid on the development of gamma-retroviral and lentiviral stable producing cell lines. Typically, satisfactory titers are achieved upon chromosomal integration of viral genome at high copies number into the producer cell. Consequently, this renders a cell line that is hard to characterize, since, the number of viral integrants and their chromosomal localization are difficult, or impossible to be evaluated.

In depth molecular characterization of the stable producer cell line ensures a higher level of safety (minimizing the possibility of generation of RCL), and less complex vector production reduces batch-to-batch variation, balancing the initial investment of cost and time to generate a therapeutically useful producing cell line.

Conceptually, stable production of retroviral vectors should be achieved from a low copy number or, most beneficial, from single vector copy integrated into the producer cell genome. Since inefficient termination of vector transcription is an inherent characteristic of the nature of retroviral vectors, read-through transcription and packaging of sequences in the vicinity of the integration site is a matter of major concern (Zaiss *et al.*, 2002; Schambach *et al.*, 2007). In the scenario of known viral vector chromosomal integration site, the sequence of produced viral mRNA can be predicted and the putative co-packaging of harmful cellular sequences fully anticipated.

Moreover, retroviral producer cell lines containing a single copy of the viral genome can gain considerable importance if their further exploitation towards production of other related viral vectors is considered. Application of RMCE technology for targeted integration of the retroviral vector genome is therefore of major relevance towards establishment of flexible viral vector producer cell lines in a expedite way (for details on site-directed integration by RMCE, please refer to section 3.2 of this chapter). In this respect, highly expressed and stable chromosomal integration site that particularly supports retroviral vector transcription must be first identified and tagged (*i.e.*, *FRT* –flanked). Upon site-specific targeting by RMCE, a precise

exchange of the resident vector with other retroviral vector of interest would result in the generation of a different producer cell line with reproducible and predictable expressing proprieties.

5.2. Stable gamma-retroviral vector production.

The present section gives an overview of the landmarks achieved in the field of stable gamma-retroviral vector production. Special attention will be paid to the last generation of modular packaging cell lines. In these systems, defined chromosomal loci are successfully exploited towards production of gamma-retroviral vector particles with high safety standards. Moreover the further potential of targeted integration towards the systematic design and evaluation of efficiency and safety of gamma-retroviral vectors optimized for a given modular producer cell line will be addressed.

5.2.1. Conventional and new modular producing cell lines for generation of gamma-retroviral vectors.

Gamma-retroviral vectors constitute a powerful tool for stable gene transfer into mammalian cells. They can be used to efficiently infect dividing cells of diverse origin of various species. Vectors derived from murine leukaemia virus (MLV) are used as gene delivery systems in clinical gene therapy trials. They have been the vectors of choice in *ex vivo* hematopoietic stem cell gene therapy and have proven to be useful in correcting several inherited diseases by integrating an expression unit for the therapeutic transgene(s) into

the cellular genome (Cavazzana-Calvo *et al.*, 2000; Aiuti *et al.*, 2002; Hacein-Bey-Abina *et al.*, 2002; Ott *et al.*, 2006).

A number of retroviral vector packaging cell lines has been developed. These are based on mouse or human cell lines, which stably express the retroviral helper genes gag, pol and env and support high titer virus production (Miller and Buttimore, 1986; Danos and Mulligan, 1988; Markowitz *et al.*, 1988; Miller *et al.*, 1991; Cosset *et al.*, 1995a; Cosset *et al.*, 1995b; Miller and Chen, 1996; Davis *et al.*, 1997). Conventionally, the three components are consecutively integrated into the producer cells via co-transduction of a selectable marker, leading to random integration of unpredictable copy numbers and yielding variable expression levels. Packaging cell lines are thus developed to achieve high, stoichiometric and stable expression of these three genes (Carrondo *et al.*, 2008). Hence, the development of a high titer production cell line is time consuming and connected with tremendous screening efforts – a process that can take several months when conventional retrovirus packaging cell lines are used (Miller and Miller, 1993). The titer to be achieved from a given helper cell line strongly depends on the strength of expression of the viral vector. Since in classical settings this is achieved by random integration, the selection of a producer cell clone would ideally be based on a functional principle delivered by the transgene itself, *e.g.*, conferring drug resistance. Alternatively, surface expression of markers supports the direct or indirect detection of a transgene product by antibodies and could serve as a method for isolation of highly expressing cells. If the transgene is undetectable or located intracellularly, the amount of work during cloning procedures

increases dramatically, even more so if there is no protein product, *e.g.*, for vector-encoded therapeutic RNA. Co-expression of a therapeutic and a selectable marker gene has been achieved by employing methods such as: differential splicing (Becker *et al.*, 1998); fusion of two open reading frames using a self-digesting protease recognition site, *e.g.* FMDV-2A (de Felipe *et al.*, 1999; Klump *et al.*, 2001; Szymczak *et al.*, 2004); or by the much more widely used strategy based on construction of bicistronic units in which the expression of the two genes is translationally separated by an IRES element (Pfutzner, 2008). However, co-expression of a marker gene and the transgene in the target cells may not be desirable in gene therapy approaches, because of possible side effects of the marker gene, *e.g.* possible immunogenicity potential. Loew and collaborators (2004) have developed a strategy that relies on the reversible introduction of a marker gene flanked by recombinase recognition sites (*LoxP*) into the gamma- retroviral genome. After selection and titration of the "best" producer clone, the marker gene can be removed from the provirus by Cre recombinase – mediated excision. Although this strategy clearly facilitates the isolation of virus producing cells coding for transgenes which are difficult or impossible to detect, it has some limitations, such as the size of therapeutic gene that can be packaged.

With the application of RMCE to a selected locus for gamma-retroviral vector insertion in packaging cells, flexible gamma-retrovirus producer cell lines were established, that simplify the isolation of highly productive producer clones (Coroadinha *et al.*, 2005b; Schucht *et al.*, 2006). This approach is less laborious and

increases safety. It is based on an advanced site-specific cassette replacement strategy, that combines retroviral tagging and a positive selection trap with the Flp/FRT recombination (Hauser *et al.*, 2000; Verhoeyen *et al.*, 2001). To meet the above mentioned balanced expression of gag/pol, env and the retroviral vector for high titer production, the following strategy was applied: Firstly, an extensive screening for a single highly expressed and stable chromosomal integration site that particularly supports retroviral vector transcription was identified and tagged. Secondly, cells expressing balanced levels of gag/pol and env were created. The vector genome is integrated into the tagged (FRT -flanked) locus by RMCE. In order to exclusively select cells that underwent the site-specific recombination and to avoid contamination of viruses released by the master cell line, the tagging cassette contains a transcriptionally inactive selection marker that is only activated by correct site-specific integration. Thus, the resulting producer cell clones are genetically identical and yield reproducibly high levels of viruses. Virus titers up to 2×10^7 i.p./10^6 cells.24h were achieved with high reproducibility (Coroadinha *et al.*, 2005b; Schucht *et al.*, 2006). Also, high-titer producer cells for a therapeutic vector that encodes the 8.9 kb collagen VII cDNA in a marker-free cassette were obtained within three weeks without screening (Schucht *et al.*, 2006). Since the master cell line is fully characterized with respect to retroviral vector production conditions, the establishment of a producer cell line is reduced to the replacement step of integrating the vector of interest.

As the translation of retroviral vector for gene therapy demands high titer preparations and stringent quality standards of the vectors

produced from stable producer cell lines, intensive research in the field of vector production has been conducted, such as on the development of serum free and suspension cultures (Gerin *et al.*, 1999a; Gerin *et al.*, 1999b; Chan *et al.*, 2001; Pizzato *et al.*, 2001; Rodrigues *et al.*, 2009), or improving downstream processes that contribute to the quality of retroviral vector preparations (Rodrigues *et al.*, 2008; reviewed in Rodrigues *et al.*, 2007).

5.2.2. Factors affecting retroviral vector performance upon chromosomal targeted integration.

Much effort has been made towards the optimization and fine-tuning of recombinant gamma-retroviral vectors (Yu *et al.*, 1986; Zufferey *et al.*, 1999; Kraunus *et al.*, 2004; Schambach *et al.*, 2006; Schambach *et al.*, 2007), but the impact of such modification on both vector titers and safety has never been systematically evaluated from integrated copies. The vector titer associated to a producer cell line is strongly dependent on positive/ negative *cis*- effects that are mediated by the chromosomal sequences present in the vicinity of the integration site(s) of the retroviral construct. These *cis* –mediated effects have different outcomes depending on the molecular composition of the integrated vector(s).In this respect, modulation of vector design would take maximal benefit if it is done according to the requirements of a defined chromosomal locus.

Besides the advantage of modular cell lines for production of clinically relevant gamma-retroviral vectors, they constitute, as shown in the **Chapter III**, a platform to systematically evaluate and directly

compare different vector compositions in a specific chromosomal locus. Modular gamma-retroviral producing cell lines arise as essential tools for the development of vectors with increased efficacy and safety levels by permitting the rational design of vectors that meet the requirements of the respective integration site.

5.3. Stable lentiviral vector production.

The stable production of lentiviral vectors meets another level of complexity when compared to constitutive production of gamma-retroviral vectors. It has been cumbersome to establish clinical grade lentiviral packaging cell lines, in part due to the toxicity associated to packaging proteins such as rev (Miyazaki *et al.*, 1995), pol encoded protease (Konvalinka *et al.*, 1995) and VSV-G (Burns *et al.*, 1993) but also due to gene silencing that occurs during the long culture period necessary for sequential addition of packaging constructs (Kafri *et al.*, 1999; Ni *et al.*, 2005). The need to establish regulatory networks that support transient levels of protein expression below their cytotoxic/cytostatic level has become one of the major obstacles to continuous- lentiviral vector production. Typically, the coordinated expression of the packaging elements has been, over the years, achieved by implementation of drug-dependent regulatory systems such as the tetracycline-depended regulatory system (Gossen and Bujard, 1992). The major landmarks achieved in the field of developing safer and large-scalable lentiviral packaging cells will be addressed.

5.3.1. The first generations of lentiviral packaging cell lines.

The first relevant reports on the generation of inducible lentiviral packaging cell lines were published more than 10 years ago. An initial report by Kafri and collaborators (Kafri *et al.*, 1999) described the production of the first-generation of packaging systems, in which the expression of both the HIV-1 packaging genome and the *trans*-given VSV-G coding plasmid were under a tetracycline responsive promoter. Further on, the next step towards safer producing systems was achieved with the development of cell lines based on the second generation of packaging elements (Farson *et al.*, 2001; Pacchia *et al.*, 2001). In this systems, the virulent genes (vif, vpr, vpu and nef) had been removed while gag-pol and regulatory genes (such as Tat or/and Rev) were still present in the same packagable construct. The simultaneous expression of these genes was driven by a tetracycline (Farson *et al.*, 2001) or ecdysone (Pacchia *et al.*, 2001) regulatable promoter present 5' of the HIV-1 genome. With the creation of the so-called third-generation of lentiviral packaging elements (Dull *et al.*, 1998), new prospects for safer inducible packaging cell systems arose, by drastically reducing the chances of RCL generation (Klages *et al.*, 2000; Sparacio *et al.*, 2001). In such packaging systems, rev acquired its independent expression due to the segregation of its sequence from the structural genes gag-pol and its controlled production was achieved by the tet-off system. In order to overcome the constitutive expression of the transactivator (tTA is a cytotoxic protein and its constitutive expression leads to leaky expression of regulatable packaging genes), Ni and collaborators (2005) developed a

three-level cascade gene regulation system in which the expression of the transactivator was regulated by a positive feedback loop mechanism. This report was considered to be the first showing HIV-1-based lentiviral vector production on a large scale using a packaging cell line. Although the authors claimed stable vector production over a period of greater than 11 days reaching very satisfactory titers (higher than $1x10^7$ i.p./ml), they also refer to long term genetic silencing of VSV-G, Rev and gag-pol. In an attempt to avoid the silencing of these components, insulator sequences flanking each one of the intervening expressing cassettes were used. Nevertheless, insulators were demonstrated to be of no benefit in preventing gene silencing over long-term culture (Ni *et al.*, 2005).

In all of the previously described packaging systems, the induction of regulated genes was achieved upon withdrawal of a regulator drug, such as tetracycline or its analogue doxycycline (Tet-off system). As above referred, it has been suggested that Tet-off system is related to leaky expression of regulatable genes, such as VSV-G (Farson *et al.*, 2001), contributing to the poor stability of inducible packaging cell lines.

A major improvement in the field was described recently, referring to the establishment of a 293-derived third-generation packaging cell line, in which the induction of gene expression is triggered by the presence of the inducer, with the capacity to stably produce high-titers (above $1x10^7$ i.p./ml) of lentiviral vectors (Broussau *et al.*, 2008). In this work is reported the establishment of a lentiviral packaging cell line based on the implementation of optimized Tet-On switch (Urlinger *et al.*, 2000) and the newly developed cumate-inducible

transcription system (Mullick *et al.*, 2006) to tightly and independent control different cassettes expressing the proteins necessary for the production of the viral particle. Moreover, the authors described inducible production of lentiviral vectors in suspension culture and in serum-free medium, important requirements of a packaging cell line for stable large-scale production of viral vectors in a clinical setting.

The implementation of the third-generation of packaging lentiviral systems dramatically increased the levels of safety and RCL production has no longer been a matter for major concern. Nevertheless, the issue of genomic instability associated with most of the packaging cells described so far is still a major limitation. An important achievement in the field, although not entirely understood, came with the demonstration that stable expression and continuous production of lentiviral vectors was obtained upon gamma-retroviral transduction of the lentiviral packaging functions (Ikeda *et al.*, 2003). Through the delivery of codon optimized HIV gag-pol gene by MLV-based vector to the lentiviral packaging cell line, the expression of HIV-1 gag protein could be sustained for a certain period of time in a constitutive fashion. However, the molecular reason why gag-pol is stably expressed after retroviral integration without apparently compromising the viability of the cell remains unclear. The authors claimed that due to the gamma-retroviral profile of insertion, favorable sites for stable expression of HIV gag-pol are targeted. However, this can only explicate the high levels of expression of an integrated cassette but hardly explains the apparent overcoming of the need of regulatable expression systems. This HIV-1 vector production system is unlikely to be of value for clinical applications,

due to expression of T-Ag oncogene and the significant instability reflected in loss of titer after a short time in culture, making large scale viral production from these cells a cumbersome task.

In summary, work on development of third-generation of packaging cell lines has been done essentially regarding the need to circumvent the cytotoxicity associated to the elements that constitute the lentiviral vector virion. In this respect, implementation of different gene switcher systems (Ni *et al.*, 2005; Broussau *et al.*, 2008), or strategies that circumvent or reduce the need for gene regulation (Ikeda *et al.*, 2003) have been described as major landmarks towards cell line development for lentiviral vector production. Nevertheless, little attention has been paid to strategies that would contribute to decrease the levels of lentiviral genome silencing, undoubtedly contributing to the reduction of viral titers upon short term culture of the producer cells.

5.3.2. From lentiviral packaging to lentiviral producing cell lines.

Once the packaging cell line is obtained, it is constituted as a master cell line and can be exploited for the production of different vectors simply by transfer of the lentiviral vector genome. Two approaches lead to the expression of the lentiviral genome: either the transient transfection or the genomic integration of the viral vector into the packaging cell line. The latter is the method of choice, since is the only one that makes possible the scaling-up of lentiviral vector production in a clinical grade process (as already mentioned in section

5.1). The genomic integration of the DNA vector into the cells can be accomplished either upon a classical DNA transfection followed by random integration of the vector sequences (Ikeda *et al.*, 2003) or by viral transduction of a replication competent vector (Kafri *et al.*, 1999; Klages *et al.*, 2000; Ikeda *et al.*, 2003; Ni *et al.*, 2005; Cockrell *et al.*, 2006). The viral transduction has been the typical method of choice towards establishment of lentiviral producer cell lines. Remarkably, satisfactory titers are achieved upon infection of the packaging cell at high multiplicity of infection (m.o.i.) and upon successive infection rounds. This suggests that high numbers of viral vector integrants are required and/or only few integration sites allow for high titer expression.

With the advent of the self-inactivating (SIN) lentiviral vectors (Miyoshi *et al.*, 1998; Zufferey *et al.*, 1998), improvements in vector safety were achieved. Nevertheless, promoter inactivation associated to the deletion in the U3 sequence in 3' LTR of SIN vectors is accompanied with the expense of limitation in their stable production: the development of lentiviral producer cell lines upon classical transduction of viral vector became no longer possible, due to the formation of replication-deficient proviral vector. Due to this limitation, stable SIN vector-producer cell lines started to be generated by co-transfecting vector DNA with a selection marker gene into packaging cells followed by exhaustive screening for stable cell clones yielding the highest vector titers. There are many shortcomings related to the generation of packaging cell lines upon transfection of the lentiviral genome. Since stable transfection results in tandem integration of multiple copies of the vector construct, most of the

positively screened clones are genetically unstable and often subjected to transcription shut-off after a few passages. To make possible the generation of stable SIN lentiviral vector- producer cell lines by transduction of the viral vector, Xu and co-workers (2001) have developed a lentiviral vector in which the SIN nature is given through the conditional transcription of the full length genome. In this so-called conditional SIN (cSIN) vector, expression of lentiviral genome is achieved upon replacement of wild-type U3 sequence by a tet-regulatable promoter, allowing vector production exclusively in cells expressing the synthetic Tet-regulated transactivator (tTA). They demonstrate production of cSIN lentiviral vector from a first generation packaging cell line (Kafri *et al.*, 1999) with titers above $1x10^6$i.p./ml. Later on, the production of cSIN vectors from the third-generation of lentiviral packaging systems was reported, achieving titers in the range of $1x10^7$ i.p./ml, in the absence of the inducer (Cockrell *et al.*, 2006). One of the major limitations for the acceptance of cSIN lentiviral vectors (which contain regulatable enhancers and promoters in the LTR) is related to the lack of rigorous tests in newly developed insertional mutagenesis assays (Modlich *et al.*, 2006; Modlich *et al.*, 2009). Due to the inducible expression of conditional lentiviral vector production systems (Xu *et al.*, 2001; Cockrell *et al.*, 2006) it is difficult to foresee their application in a human clinical setting. Therefore, other strategies have been developed in order to achieve producing cells associated with high and stable titers of safer SIN lentiviral vectors with clinical repercussion, as the one described by Thorm and collaborators (2009). In order to improve transfection methods that lead to the generation of high titer SIN- lentiviral

producing cell lines, the referred authors report on an interesting concatemeric array-based technique (Throm *et al.*, 2009). The novelty of the work presented is related to *in vitro* manipulation of the structure of the viral vector DNA to be transfected into the packaging cells. By promoting a directed tandem ligation of multiple copies of the viral vectors (the so-called "concatemeric array"), the authors claimed a new method towards an increase of delivery efficiency, integration and stable expression of the vector genome, resulting in the generation of SIN lentiviral producing cell lines. It was reported the generation of clones contained approximately 200 copies of the viral genome sequence and reaching titers of about $1x10^7$ i.p./ml for 3 months. The applicability in a clinical setting was demonstrated with the generation of concatemeric insulated SIN lentiviral vector encoding the human IL2 receptor common gamma-chain (IL2RG) gene in a 293-T derived producer cell line. To this end, the regulatable packaging functions were first introduced upon retroviral transduction (in a similar approach described by Ikeda *et al.*, 2003), followed by transfection of SIN lentiviral DNA concatemers. The generation of a high titer producer cell line for SCID-X1 gene therapy setting was achieved, revealing the value of the approach presented.

5.3.3. Factors affecting lentiviral vector production upon chromosomal targeted integration.

The tandem arrangement of several copies of a DNA sequence in a concatemeric molecule has as a consequence the integration of several viral DNA copies in a single locus of the genome of the

producing cell line. In this respect, it is expected that the internal segments of the integrated molecule are shielded from silencing by the flanking sequences that act as insulators, protecting those internal units from the positive and negative effects that the genomic chromosomal surroundings could exert. In the work reported by Throm and collaborators (2009), it is not completely clear if the benefit of the concatameric assay for establishment of high titer lentiviral producing cell lines relies on the augmentation of DNA integrants in a single genomic locus or if it is related to the decrease in silencing that integrated vectors are prone to. Nevertheless, it is suggested that it is due to the concatameric structure of integrated DNA that viral production can be achieved for a longer period when compared to what is observed for the counterpart STAR cell lines (Ikeda *et al.*, 2003). Despite the advantages of integration of concatameric viral DNA molecules on viral titers, the establishment of lentiviral producer cell lines based in a multitude of inserted copies is not the most desired scenario for a consensual cell system for production of therapeutic lentiviral vectors. The high copy number of the vector DNA cassette curtails one's ability to genetically characterize the integrated vector DNA and to exclude rearrangements that may pose a danger.

As already mentioned (please refer to section 5.1.), production of retroviral vectors should be achieved from low or most beneficial single copy integrated into the producer cell genome. In this respect, production of gamma-retroviral vectors from single copy integration in modular packaging cell lines has been already described (Coroadinha *et al.*, 2005b; Schucht *et al.*, 2006) but up until now,

lentiviral vector production from single copy integration and the translation of modular packaging cells towards lentiviral production has never been under the scope of research.

Chapter IV discusses the capacity of biotechnological relevant cell lines such as HEK293 to support lentiviral production upon chromosomal targeted integration. The work presented gives an important contribution to the definition of cellular restrictions and requirements that are relevant for establishment of novel stable and safe lentiviral vector producer cell lines.

6. SCOPE OF THE THESIS

Besides pioneering work conducted, site-directed engineering of defined chromosomal sites has not yet been systematically investigated towards production of highly valuable bio-products.

Accordingly, up to now, no viable production process is known to be based on this highly potent technology. This thesis has the aim to exploit and validate targeted integration-based approaches and to specify its advantages and eventual limitations for production of biotechnological relevant products such as antibodies and retroviral vectors.

By means of exploitation of a defined chromosomal locus, it is intended to evaluate if expression of integrated transgenes can be maximized according to the specific characteristics conceded by the chromosomal surroundings. In this respect, expression of a given transgene from single chromosomal locus is the main focus of attention.

Evaluation of the impact of cassette design towards identification of suitable chromosomal sites supporting high levels of recombinant protein is addressed taking the case of antibody production as a paradigm. As a second application, the defined loci are evaluated as platforms for systematic design and evaluation of efficiency and safety of gamma-retroviral vectors, thereby allowing to optimize vectors for a given producer cell line. Furthermore, targeting approaches are assessed as a method of choice for production of lentiviral infectious particles, a strategy that has not yet been considered so far.

Together, the present thesis intends to give a significant contribution to the next generation of producing systems for reliable expression of molecules for broad applications ranging from basic research to pharmaceutical and gene therapy field. It is envisioned that cell line development will gain considerable impetus with the consolidation and systematization of site-directed engineering based strategies as the ones presented in this work.

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CHAPTER II

ANTIBODY PRODUCTION FROM DEFINED CHROMOSOMAL LOCI

This Chapter is adapted from the paper

Nehlsen K., Schucht R., **Gama-Norton L.**, Krömer W., Baer A., Cayli A., Hauser H. and Wirth D. (2009). Recombinant protein expression by targeting pre-selected chromosomal loci. *BMC Biotechnol*, 9: 100.

ABSTRACT

Background: Recombinant protein expression in mammalian cells is mostly achieved by stable integration of transgenes into the chromosomal DNA of established cell lines. The chromosomal surroundings have strong influences on the expression of transgenes. The exploitation of defined loci by targeting expression constructs with different regulatory elements is an approach to design high level expression systems. Further, this allows to evaluate the impact of chromosomal surroundings on distinct vector constructs.

Results: We explored antibody expression upon targeting diverse expression constructs into previously tagged loci in CHO-K1 and HEK293 cells that exhibit high reporter gene expression. These loci were selected by random transfer of reporter cassettes and subsequent screening. Both, retroviral infection and plasmid transfection with eGFP or antibody expression cassettes were employed for tagging. The tagged cell clones were screened for expression and single copy integration. Cell clones producing > 20 pg/cell in 24 hours could be identified. Selected integration sites that had been flanked with heterologous recombinase target sites (FRTs) were targeted by Flp recombinase mediated cassette exchange (RMCE). The results give proof of principle for consistent protein expression upon RMCE. Upon targeting antibody expression cassettes 90-100% of all resulting cell clones showed correct integration. Antibody production was found to be highly consistent within the individual cell clones as expected from their isogenic nature. However, the nature and orientation of expression control elements

revealed to be critical. The impact of different promoters was examined with the tag-and-targeting approach. For each of the chosen promoters high expression sites were identified. However, each site supported the chosen promoters to a different extent, indicating that the strength of a particular promoter is dominantly defined by its chromosomal context.

Conclusion: RMCE provides a powerful method to specifically design vectors for optimized gene expression with high accuracy. Upon considering the specific requirements of chromosomal sites this method provides a unique tool to exploit such sites for predictable expression of biotechnologically relevant proteins such as antibodies.

CONTENTS

1. INTRODUCTION

High level expression of proteins from mammalian cells is crucial for diverse questions in basic research such as structure analysis and is a key issue for biopharmaceutical production. The current state of the art for establishment of recombinant protein production cell lines relies on transfection of producer cells with a plasmid that encodes the gene of interest driven by a potent promoter. Upon uptake into the nucleus, the incoming DNA, in particular through double-strand breaks, is sensed by the cellular repair machinery. These enzymes stably integrate the incoming recombinant DNA into the cellular DNA by illegitimate recombination. This procedure is largely random and accordingly, the sites of integration are mostly spread all over the genome (Wurtele *et al.*, 2003). Once integrated into the cellular DNA, the transgene cassette is affected by neighboring chromosomal elements that modulate the promoter to a high extent (West and Fraser, 2005). Enhancers and silencers directly affect promoters in *cis* and may be shielded by insulators. Beside this, chromatin modeling elements such as locus control regions and S/MARs significantly influence the transgene expression level (Bode *et al.*, 2000; Li *et al.*, 2002; West and Fraser, 2005). Finally, evidence has been provided that also nearby/close promoter elements interact with incoming promoters (promoter crosstalk) and can result in their downregulation (so called promoter occlusion) or potentiation (Hampf and Gossen, 2007). Thus, upon random integration, individual cell clones display a highly

heterogenous expression pattern and have to be screened for appropriate expression.

Homologous recombination is used in stem cells for targeting transgene to specific loci. In differentiated cells homologous recombination is very infrequent. Recently, Zn-finger nuclease based approaches have been designed for targeting transgene cassettes in mammalian cells to defined loci (Porteus and Carroll, 2005; Cathomen and Joung, 2008). Thus, tools are available to target individual chromosomal sites for various applications in order to overcome the limitations of random integrations. While such methods are useful for gene therapies and basic research, their value for protein expression is limited since chromosomal sites in production cell lines that support high level recombinant protein expression are usually not known.

Indeed, in order to meet the requirements for high and stable protein expression extensive screenings are performed to identify those cell lines that provide optimal protein production. For industrial purposes devices for robotic cell and sample propagation were developed that support high throughput screenings of millions of individual cell clones, thereby allowing the identification of those cell clones with favorable expression of the transgene. Beside this, procedures for enhancing the copy number of transgene integrations by gene amplification have been employed (Kingston *et al.*, 2002; Chusainow *et al.*, 2009). However, high clone to clone variations as well as instability in expression levels have been found. The latter is due to genetic rearrangements during gene amplification as well as to the emergence of drug-resistance (Kim *et al.*, 1998; Kim *et al.*, 2001; Jun *et al.*, 2006). Although these procedures have provided potent

producer clones, the pitfalls are obvious: for any and every new protein and/or expression construct the screening process has to be re-established which is both time consuming and expensive. Alternative methods to decrease the production cost and the time needed for cell line development are thus essential.

In the last two decades specific genetic engineering of mammalian cells via site specific recombinases such as Cre and Flp has been explored. These recombinases specifically bind and recombine short recombination target sites, the 34 bp loxP site and 48 bp FRT site, respectively. Once a chromosomal site in the host genome is tagged with a single recombination target sequence, the recombinases mediate site specific targeting of plasmids carrying the same recombination target site. Thereby, targeted integration of whole plasmids into pre-tagged integration sites in mammalian cells is feasible (O'Gorman *et al.*, 1991; Kim *et al.*, 1998). This first generation gene targeting strategy suffers from low efficiency due to possible excision of the integrated cassette and also from integration of extended plasmid backbone sequences. Accordingly, only few cell lines have been provided so far that allow for successful targeted integration of a transgene cassette. Although some tagged cell lines are commercially available, they are not screened for high production and do not meet the requirements for many applications.

The method of recombinase mediated targeting was significantly improved by flanking an initial tagging cassette with a set of non interacting recombinase recognition sites. Upon integration, such cassettes can be precisely exchanged for an incoming vector that is flanked with the same set of recombinase recognition sites

(O'Gorman *et al.*, 1991; Schlake and Bode, 1994; Karreman *et al.*, 1996). Hence, the term recombinase mediated cassette exchange (RMCE) was coined (Bouhassira *et al.*, 1997). Basically, RMCE relies on two heterologous recombinase target sites (spacer mutants) that resist site specific recombination between each other but still undergo recombination with their respective homologous counterparts. Mutants that can be exploited in this respect have been identified both for the Flp (Schlake and Bode, 1994) and the Cre system (Wirth *et al.*, 2007). The main advantage of RMCE is the lack of excision which reduces the targeting efficiency in simple first generation targeted integration approaches. Upon implementation of stringent selection strategies the frequency of targeting can be increased (Seibler and Bode, 1997) even up to 100% (Verhoeyen *et al.*, 2001; Schucht *et al.*, 2006). Further, RMCE overcomes the integration of bacterial vector sequences which potentially limits mammalian gene expression. Meanwhile, this technology has been exploited in various cell systems and for various applications including the establishment of viral producer cells (Coroadinha *et al.*, 2005; Schucht *et al.*, 2006), erythropoietin production (Kim and Lee, 2008), and for the evaluation of vector design (Verhoeyen and Cosset, 2004).

A systematic exploitation of defined chromosomal sites for expression of proteins has not yet been followed. The present study focuses on the evaluation of different approaches for tagging and the evaluation of the flexibility of incoming cassettes concerning the predictability of expression upon targeting. Moreover, we give evidence that the strength of a given promoter is strongly linked to the properties of the respective chromosomal integration site.

Together, the study gives new insights in the interactions between promoters and chromosomal elements. Thereby, it contributes to a deeper understanding of these mechanisms which is a prerequisite for a systematic exploitation of chromosomal integration sites for various applications including protein production.

2. MATERIAL AND METHODS

2.1. Plasmids.

The retroviral tagging cassette RV-GFP has been described elsewhere (Schucht *et al.*, 2006). It contains in its 3'LTR a wild-type FRT site and a F5 spacer mutant FRT site, followed by an ATG-deleted neomycin phosphotransferase gene. Plasmidic tagging cassettes P-HTG and P-GFP harbor the respective reporter gene(s) flanked by a wild-type FRT site and F5 spacer mutant FRT site, and are also followed by an ATG-deficient neomycin phosphotransferase gene. In P-GFP, eGFP as fluorescence marker is expressed from the PGK promoter, in P-HTG the SV40 promoter drives the HTG fusion protein comprising hygromycin phosphotransferase, thymidine kinase and eGFP. The antibody coding tagging cassette harbors the SV40 driven HTG fusion and the heavy and the light chain of an IgG molecule each controlled independently by an SV40 promoter/ enhancer.

Targeting cassettes contain the FRT wild-type and the F5 FRT mutant site flanking an antibody expression unit. The design of the cassettes is depicted in the individual Fig.s. The cassettes carry the SV40 promoter, the CMV promoter, a hybrid promoter comprising the MPSV enhancer elements and the CMV promoter (Mielke *et al.*, 2000)

or a bidirectional promoter composed of the Adenovirus major late gene promoter and the elongation factor 1 promoter (Wiberg *et al.*, 2006). Targeting vectors encode RFP, eGFP or antibody expression cassettes. In all targeting vectors, the PGK promoter or an IRES element and an ATG start codon is positioned upstream of the FRT mutant site to complement the inactive neoR gene after targeting. Maps or sequences are available upon request.

2.2. Mammalian cell culture and transfection.

CHO-K1 cells (ATCC CCL 61) were cultivated at 37°C in a humidified atmosphere with 7.5% CO_2 in CD Hybridoma medium (Gibco) with 2% fetal calf serum (Biowest), 8 mM L-glutamine and 4 ml of 250× Cholesterol lipid concentrate (Gibco) *per* litre medium. Selection was performed in medium supplemented with hygromycin B (150 U/ml), G418 (500 µg/ml) or ganciclovir (10 µg/ml). HEK293 cells (BioReliance) were cultivated at 37°C in a humidified atmosphere with 5% CO_2 in DMEM (Gibco) with 10% fetal calf serum (Cytogen), 2 mM L-glutamine, penicillin (10 U/ml) and streptomycin sulfate (100 µg/ ml). Selection was performed in medium supplemented with hygromycin B (200 U/ml), G418 (1500 µg/ml) or ganciclovir (10 µg/ml).

For plasmidic transfer CHO-K1 cells were transfected with 4 µg of the tagging vector using the nucleofection standard protocol (amaxa AG; Cologne; Germany; Nucleofector™ Kit V) and selected with hygromycin B for optimal generation of single copy clones. HEK293 cells were plasmid transfected using the GenePulser

electroporator (BioRad). For this purpose 1 × 10^6 cells were transfected with 2.6 µg of the tagging vector carrying an eGFP cassette. eGFP positive cells were sorted using flow cytometry and individual clones were expanded. Retroviral tagging was performed as described earlier (Verhoeyen *et al.*, 2001). In brief, tagging viral vectors were generated upon transfection of PG13 packaging cells. The supernatant was used to infect HEK293 and CHO-K1 cells at an m.o.i. of 0.1 and the cells were subjected to selection with hygromycin B.

2.3. Targeted cassette exchange.

For site-specific cassette exchange 4 × 10^5 of tagged HEK293 cells were co-transfected with 2 µg Flp recombinase- expressing vector (Schucht *et al.*, 2006) and 2 µg of targeting plasmid using lipofection (GenePORTER™ 2 Transfection Reagent, Peqlab). Targeting of tagged CHO-K1 cells was performed using nucleofection (amaxa) by cotransfer of 4 µg Flp recombinase-expressing vector and 1 µg of the targeting plasmid. For both, the medium was replaced 24 h post transfection and the cells were cultivated for 4 days to allow cassette exchange. On the fifth day the cells were transferred to a 60-mm culture plate and G418- and ganciclovir- containing medium to select for targeted daughter clones.

2.4. PCR analysis.

Neo-resistant clones were checked for correct integration of the targeting vector by PCR. The use of a set of primers where the 5'

primer is located in the newly integrated targeting cassette (e.g. the IRES element or PGK promoter) and the 3' primer located in the tagging backbone (e.g. the neomycin phosphotransferase gene) leads to the amplification of a targeting-specific product. The PCR is performed using the Mango-Taq Polymerase Kit (Bioline). The annealing temperature of the used primers (e.g. primer pair: PGKfwd 5' TCTCGCACATTCTTCACGTCC 3' and Neorev2 5' GTCATAGCCGAATAGCCTCTCC-3') was 58°C with an elongation time of 30 sec.

2.5. Flow cytometry.

FACSCalibur and FACSVantage SE (Becton Dickinson) were used for evaluation and isolation of eGFP positive cells. The cells were washed, trypsinized and stained with propidium iodide (50 µg/ml) to exclude dead cells from the analysis.

2.6. ELISA.

The specific productivity of the clones was analysed by sandwich enzyme linked immunosorbent assay (ELISA). The cells were seeded on a 6-well plate with a density of 5×10^5 cells and incubated for 24 h with 2 ml of medium. The next day the cell number was determined, the supernatant harvested and centrifuged (5 min for 1000 rpm) and added to a 96-well plate covered with an Fc-specific anti-human IgG (SIGMA). The photometric measurement was done based on a substrate conversion by peroxidase (HRPO) labelled goat anti human IgG (H+L), (CALTAG™ Laboratories). Levels significantly

above background and below 0.1 pg per cell in 24 h (pcd) are indicated as 0.01- 0.1.

3. RESULTS

3.1. Strategies for generation of targetable clones.

To generate cell clones which are suitable for targeted integration we followed a Flp recombinase based strategy as outlined in Fig. 1A. It comprises the tagging of chromosomal integration sites within the host genome of a given cell line with a reporter gene cassette. Catalyzed by the Flp recombinase that is encoded by a plasmid and is co-transferred together with the targeting vector cassette exchange will occur (Flp recombinase mediated cassette exchange, RMCE, Fig. 1A). To generate high producer cell clones, different approaches for tagging chromosomal loci were evaluated (Fig. 1A).

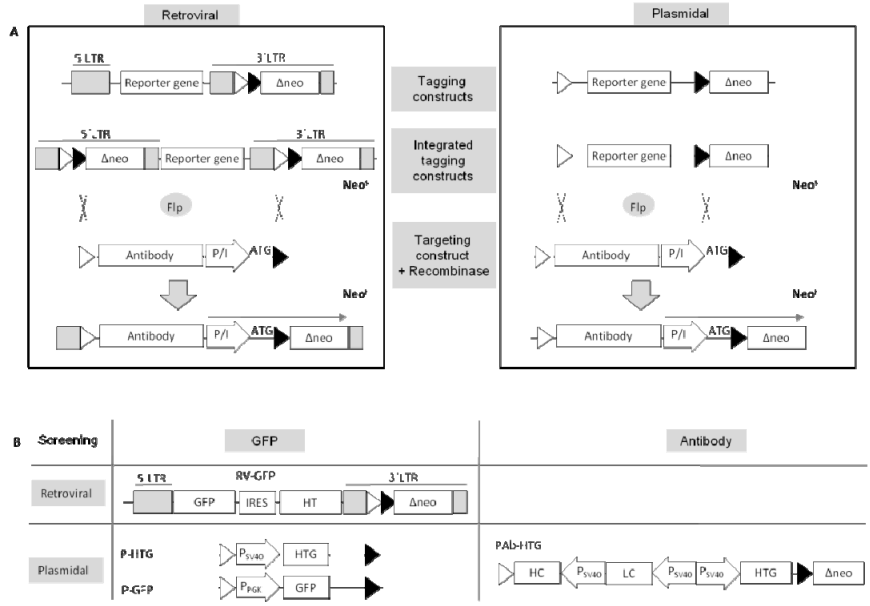


Figure 1. Strategy for the identification of single copy tagged high expressing cell clones for cassette exchange. (A) High expression chromosomal loci were tagged with either retrovirally or plasmid mediated transduced expression cassettes. These cassettes were flanked by a set of heterospecific recombinase target sites. Tagged cells lines were analyzed for the stable integration of one single copy of the respective tagging vector and screened for high expressing integration loci. The transfer of a targeting vector carrying the same heterospecific recombinase target sites as the tagging vector in presence of the Flp-recombinase leads to a site-specific cassette exchange. The selection for successfully targeted clones was performed by complementing a silent, ATG-defective neomycin resistance gene pre-integrated upon tagging. This renders successfully targeted cells resistant to G418. For this purpose, the incoming targeting vector carried next to its gene of interest (antibody expression unit) a specific sequence (P/I) that facilitates expression of the neomycin resistance gene. (B) The vectors used for tagging are depicted. All tagging cassettes contain a promoter or internal ribosomal entry site for activation of the neomycin resistance gene and are flanked by heterospecific (Fwt-F5) FRT sites. Retroviral tagging was performed as described in (Schucht *et al.*, 2006) with a vector that transduces a bicistronic cassette of eGFP and a hygromycin phosphotransferase/thymidine kinase fusion protein. For plasmidic tagging vectors with different reporter genes (eGFP and/or antibody

expression unit) as well as varying promoter elements (SV40/PGK) were employed. All tagging vectors express eGFP, either as a fusion protein with the hygromycin phosphotransferase/thymidine kinase or as a single protein, allowing fluorescence-based screening for the expression of the tagging cassette.

We employed plasmid transduction, in particular a classical electroporation protocol for HEK293 cells and nucleofection which has been the method of choice for many cell lines including CHO (Cho *et al.*, 2003). Further, retroviral transduction was followed for both cell lines for two reasons: first, this method allows to statistically adjust the copy number by using a low, defined ratio of recombinant virus particles to the number of infected cells (multiplicity of infection). Second, this method has been reported to favor high expression integration sites (Mielke *et al.*, 1996; Johnson and Levy, 2005). As recipients we used HEK293 and CHO-K1, both cell lines used in basic research and industrial biotechnology.

All tagging vectors include an expression unit encoding eGFP, either as a fusion protein with the hygromycin phosphotransferase/thymidine kinase (HTG) or as a single protein unit (GFP), allowing a fluorescence-based screening for the expression of the tagging cassette (see Fig. 1B). Identification of high expression cell clones (screening) was either carried out by multiple rounds of FACS sorting or limited dilution cloning steps.

3.2. Expression strength, stability and copy number of tagged cell clones.

We comparatively evaluated the different methods for their capability to identify high expression cell clones from both cell lines. CHO-K1 and HEK293 cells were tagged with an eGFP reporter cassette according to Fig. 1. GFP expression of representative CHO-K1 and HEK293 master cell clones for each gene transfer method is depicted in Fig. 2A. In all these cell clones, eGFP expression was found to be stable for more than 6 months. In an independent approach, CHO-K1 cells were tagged with an antibody expressing construct. Cells tagged with antibody expression constructs were cloned into 96 well plates after selection of hygromycin resistance and then tested for antibody expression by ELISA and for GFP by FACS. After intensive screening of >800 hygromycin-resistant clones, about twenty clones were identified producing more than 2 pg per cell in 24 h (pcd), which was considered an arbitrary threshold for high expression. High expressing cell clones were also analyzed for their expression stability over time. This revealed that the highest expressing clones #6 and #8, producing 12.0 and 22.5 pcd, respectively, showed long term instability. Although not being considered for RMCE, clone #8 gives evidence that high level expression can be obtained from single copy integrations.

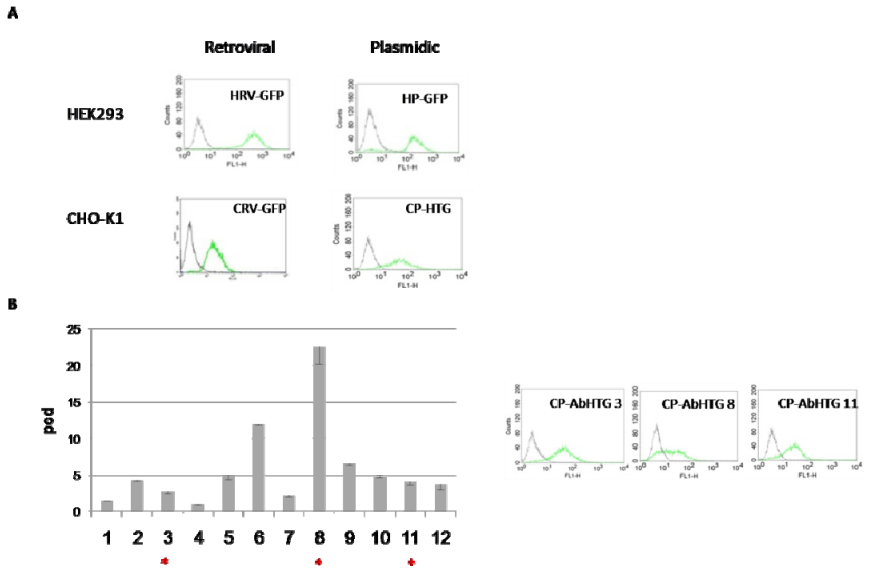


Figure 2. Characterization of the tagged integration sites. (A) GFP expression pattern of single copy HEK293 and CHOK1 clones obtained upon retroviral and plasmidic transfer of the tagging cassette. Representative clones are shown. Green lines: tagged clones. Gray lines represent non-transfected HEK293 and CHOK1 cells, respectively. (B) CHO cells were tagged with an antibody expression construct. The antibody expression level production was evaluated. Only cell clones that produced >2 pcd are shown; the data were obtained from 3-5 replicates. Clones showing single copy integration events (data not shown) are marked by asterisks. Their GFP expression profile is given presented on the right. Green lines: tagged clones; gray lines: nontransfected CHOK1 cells. Nomenclature: HRV-GFP: HEK293 cells retrovirally tagged with eGFP (RV-GFP according to Fig. 1B); HP-GFP: HEK293 cells plasmid-tagged with eGFP (P-GFP); CRV-GFP: CHO cells retrovirally tagged with eGFP (RV-GFP); CP-AbHTG: CHO cells plasmid- tagged with antibody cassette (PAb-HTG).

The eGFP and antibody expressing cell clones were analyzed by Southern Blot for the copy numbers of the respective tagging construct (supplementary data, Fig. S1). This proved single copy integrations in clones #3, #8 and #11. We further evaluated the

frequency of single copy integrations with the various tagging methods. In agreement with previous studies classical electroporation protocols (Baer *et al.*, 2000), but also nucleofection provided high efficiencies of single copy integration events (Table 1). The single copy integration rates obtained were about 30% for CHO and 56% for HEK293 cells after plasmidic transfer. As expected, a very high frequency (> 90%) of single copy integration events was achieved for retrovirally tagged cell lines.

Table 1. Single copy integration rate of tagged high-expressing cells.

	Retroviral		Plasmidal (transfer method)	
HEK293	44/48	92%	5/9 (EP Gene Pulser, BioRad)	56%
CHO-K1	2/2	100%	6/20 (Nucleofection, Amaxa)	30%

Thus, all chosen transduction protocols turned out to be suitable for efficient tagging according to the RMCE strategy. Successfully tagged single copy cell clones from the various strategies providing long term expression stability are called "master cell clones" henceforth. They were used for further evaluation of the targeting accuracy as well as the homogeneity of expression upon targeting.

3.3. Targeting efficiency and specificity.

We evaluated the efficiency and accuracy of RMCE upon targeting various eGFP and antibody expressing vectors specified in more detail below. All vectors carried an IRES element or a promoter

as illustrated in Fig. 1A and were co-transfected into the tagged master cell lines that had proven to stably express their reporter gene from a single copy locus. The selection of successfully targeted cells was accomplished as shown in Fig. 1A. The efficiency of integrating targeting vectors into the preselected chromosomal sites was evaluated. The complementation of the defective neomycin resistance gene resulting in G418 resistant cell clones (Fig. 1) was taken as a measure for the targeting efficiency. Resistant daughter cell clones were expanded for further analysis. Successfully targeted master cell clones should have lost the parental construct and show correct site-specific integration of the targeting cassette. Fig. 3 demonstrates the loss of eGFP expression after exchange with an antibody encoding cassette in representative HEK293 and CHO-K1 daughter cell clones. Specific integration into the previously tagged locus was confirmed by PCR analysis as exemplified in Fig. 3B. We also proved the specifically targeted cell clones for absence of additional random integration events of the targeting construct by PCR or Southern Blot analysis (supplementary data, Fig. S1). In Table 2, the overall targeting efficiency is given as the percentage of correctly targeted within the obtained G418-resistant clones.

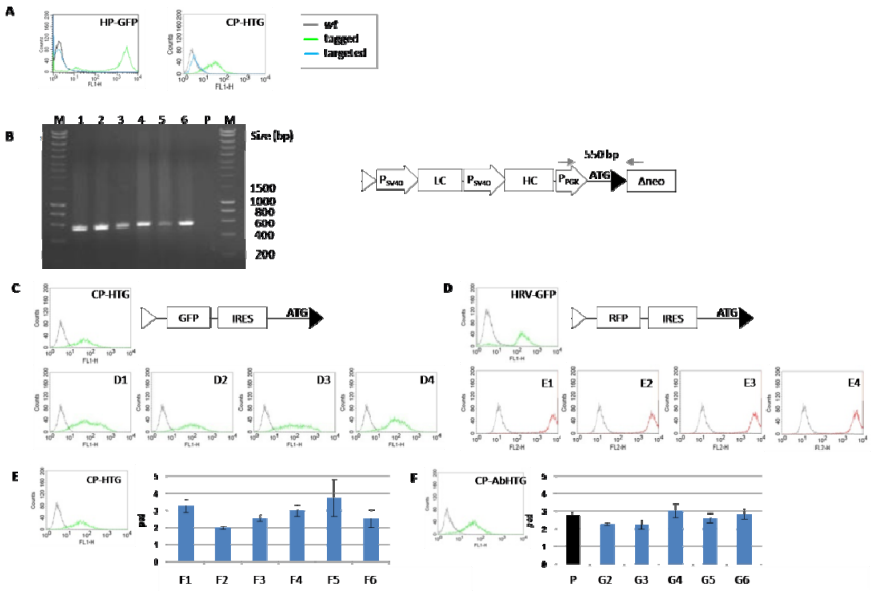


Figure 3. Gene expression of cell clones after targeting by cassette exchange. Characterization of HP-GFP and CP-GFP upon targeting. The cell clones show homogeneous loss of GFP expression after targeting with an antibody expression vector as depicted in (B). (B) Molecular characterization of the targeting event by PCR. Amplification of a 550 bp fragment from the daughter cell clones (D1-D6) using primers located at the indicated positions (arrows) is specific for integration of the targeting construct (P: parental tagged cell clone). (C-F) Homogeneity of the targeted daughter clones. The expression level of daughter clones after cassette exchange is compared with the expression level of the parental clones. Upper left: CP-HTG tagged clone targeted with a SV40 promoter driven GFP cassette. Upper right: HRV-GFP tagged clone targeted with a CMV-RFP cassette. Antibody targeting: CP-HTG tagged clone (lower left) and CP-AbHTG tagged clone (lower right) were targeted with an antibody expression construct as depicted in (B). The expression of the targeted daughter clones is shown.

All HEK293 G418-resistant derived clones (daughter clones) analyzed showed the specific integration of the targeting construct and lack unspecific random integration. In CHO-K1 cells the targeting efficiency was slightly lower but still above 85%. In some of the

successfully targeted CHO-K1 cell clones we could find additional randomly integrated copies of the targeting construct.

Table2. Targeting efficiency and additional random integration.

	Targeting		Random Integration	
HRV-GFP	30/30*	100%	0/30	<3%
CP-HTG	48/56*	85,7%	8/56	14,3%

*targeted/neo^r clones

3.4. Homogeneity of expression strength from the targeted cell clones.

To evaluate the homogeneity of expression level of the daughter cell clones, three master cell clones were targeted with expression vectors encoding eGFP, RFP or heavy and light chains of an antibody. Plasmid tagged as well as retrovirally tagged clones were chosen and individual daughter clones were characterized for expression. As illustrated in Fig. 3, CHO-K1 based CP-HTG cells plasmid-tagged with the eGFP-fusion reporter construct were targeted with an eGFP (Fig. 3C) and an antibody targeting vector (Fig. 3D), respectively. From the analyzed targeted daughter clones, all showed a pronounced homogeneity in expression of both reporters which would be expected from isogenic clones. Accordingly, retrovirally tagged HEK293 cells (HRV-GFP) were targeted using an RFP coding vector (Fig. 3E) and the RFP expression in the isogenic daughter clones again proved to be homogenous. Finally, an antibody expressing master cell line (CPAbHTG) was targeted with an antibody

cassette harboring an expression unit with the same antibody. Again, a uniform expression pattern within the daughter clones (lower right). This setting also allowed to compare the antibody titer of the master cell clone and upon targeting. Notably, the antibody expression within the daughter clones (gray) was consistent with that of the parental cell line (black).

3.5. Evaluation of cassette design for antibody expression.

To assess the capacity of the tagged integration sites of the individual master clones to support different cassette designs and promoters, we designed a set of antibody targeting vectors differing in the architecture. In these targeting vectors, the heavy and the light chain genes are either individually transcribed from SV40 promoter elements or they are encoded in a bicistronic expression unit driven by SV40 or MPSV/CMV (Mielke *et al.*, 2000) promoter elements and employing the NRF (Oumard *et al.*, 2000) or the Poliovirus IRES element (Pelletier and Sonenberg, 1988; Oumard *et al.*, 2000). In addition, tricistronic expression constructs were created in which a second IRES element combines the antibody cassette with the ATG start codon that complements the defective neomycin resistance gene in the tagging locus upon targeting. The mean antibody expression level of the daughter clones after targeting the indicated vectors in plasmid tagged (P) or retrovirally (RV) tagged HEK293 master cells is shown in Fig. 4. Interestingly, in these cells the targeting constructs with antibody chains individually controlled by SV40 promoter elements resulted only in basal expression levels (0.1-

0.1 pcd). This was observed upon targeting in either of the two transcriptional orientations. However, upon targeting with the MPSV/CMV driven tricistronic expression cassette the retrovirally tagged clone yielded 3.4 pcd in the same locus.

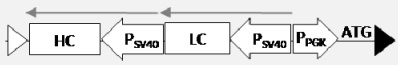
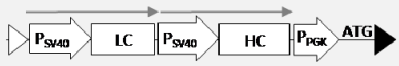
Tagging	Reporter	Targeting construct	Mean Expression (pcd)
RV	GFP		0.01-0.1
P	GFP		0.01-0.1
RV	GFP		0.01-0.1
P	GFP		0.01-0.1
RV	GFP		3.38 ±1.4
P	GFP		n.t.
RV	GFP		1.69 ±0.2
P	GFP		n.t.

Figure 4. Antibody expression after targeting in plasmid-mediated and retrovirally tagged HEK293. The retrovirally (RV) or plasmid-mediated (P) tagged GFP expressing HEK293 cells were targeted with the indicated antibody expression cassettes. Antibody expression levels are given as mean expression of 5 targeted daughter clones (pcd). n.t.: not tested.

We evaluated the same set of antibody expression cassettes in two different plasmid-tagged loci in CHO-K1 cells, clones CP-HTG and CP-AbHTG. Fig. 5 depicts the mean antibody expression level of the daughter clones. In opposite to the tagged HEK293 cells discussed before, all antibody vectors harbouring two SV40 promoters showed high expression levels (2.6 - 4.4 pcd). In contrast, in these integration sites the MPSV/CMV driven expression setup performed poorly (0.3 pcd). Interestingly, a targeting construct that harbors an SV40 driven

bicistronic antibody expression cassette performed well in CP-AbHTG but failed in the CP-HTG. Together, these data indicate that specific regulatory elements are strongly modulated by flanking chromosomal elements and thus their performance is critically dependent on the nature of the specific chromosomal site.

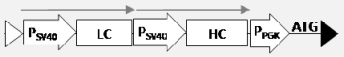
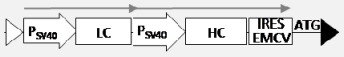
Tagging	Reporter	Expression (pcd)	Targeting construct	Expression (pcd)
P	HTG	-		2.59 ±0.27
P	AbHTG	2.79 ±0.15		4.44 ±0.31
P	HTG	-		3.87 ±0.21
P	AbHTG	2.79 ±0.15		2.58 ±0.35
P	HTG	-		2.84 ±0.44
P	AbHTG	2.79 ±0.15		2.64 ±0.41
P	HTG	-		n.t.
P	AbHTG	2.79 ±0.15		0.30 ±0.07
P	HTG	-		0,01-0,1
P	AbHTG	2.79 ±0.15		2.48 ±0.25
P	HTG	-		n.t.
RV	GFP	-		0.57 ±0.06

Figure 5. Antibody expression after targeting of plasmid-mediated tagged CHO-K1. CHO-K1 derived clones plasmid-mediated tagged with either a HTG or AbHTG cassette as indicated were targeted with different antibody expression cassettes. The mean expression of 5 daughter clones after targeting with the depicted constructs as well as the antibody expression of the parental cell in pcd is presented. n.t: not tested.

4. DISCUSSION

High level recombinant protein expression in mammalian cells not only relies on potent transcription promoting elements and optimal design of the expression cassette but also crucially depends on appropriate chromosomal sites that support the incoming expression cassette upon integration. Sophisticated chromosomal engineering approaches based on site specific recombinases allow the precise/controlled integration of expression cassettes into tagged chromosomal sites (Kolb, 2002; Akopian and Marshall Stark, 2005; Wirth *et al.*, 2007). A first-generation targeting system with a single recombinase target site has recently been evaluated for protein production. Comparable (consistent) antibody expression levels could be achieved upon targeting at defined integration sites in CHO cells using Cre-(Kito *et al.*, 2002) or Flp-mediated integration (Huang *et al.*, 2007). The potential of this technology was further exploited for the production of a human polyclonal anti-RhD antibody (Wiberg *et al.*, 2006) by integrating 25 individual antibody expression cassettes into a defined FRT tagged integration site in CHO cells (Flp-In™ cell line; Invitrogen). Irrespective of this success first generation targeting systems are limited by fact that the tagging sequences cannot be eliminated. Usually, complete vectors including bacterial sequences are co-integrated which have been shown to decrease transgene expression from neighboring promoters (Riu *et al.*, 2007; Chen *et al.*, 2008). Also, since excision of the targeted cassette is favored, the targeting efficiency can be unsatisfactory. In this respect, exchange of tagging cassettes via RMCE seems to be the method of choice. While

proof of principle has been given for production of retroviral vectors (Coroadinha *et al.*, 2005; Schucht *et al.*, 2006) so far, a systematic approach evaluating RMCE for protein production has been missing.

This report describes a comparative approach to identify potent integration sites that support stable protein production and to exploit these sites by Flp RMCE to target expression cassettes of choice. For this purpose we used CHO-K1 and HEK293 cells - the most relevant cell lines for protein production. Motivated by the notion that any tagging procedure has an intrinsic bias for specific patterns types of integration sites we employed random tagging strategies based on retroviral or plasmidic transfer of screening vectors. For monitoring expression we employed eGFP (either in a single expression unit or in a fusion to a selection marker) or an IgG molecule. With these screening approaches we established a set of tagged master cell lines that stably express the respective reporter gene(s). Importantly, both plasmidic and retroviral tag-ging proved to be appropriate for RMCE since they lead to a high-percentage of single copy integrants ranging from 30 to > 90% - a prerequisite for targeted integration.

Evaluation of the performance of cassette exchange in the different master cell clones derived from HEK293 and CHO-K1 cells using various targeting vectors proved to be highly efficient with ≥85% correctly targeted daughter clones. In certain applications, this high efficiency might overcome the need for subsequent sub-cloning. Further, analysis of the production levels could confirm that site directed integration significantly reduces the variations of clonal expression levels. This is expected from isogenic clones and is in

accordance to previous reports evaluating this method for production of retroviral vectors (Schucht *et al.*, 2006).

Further, we investigated the flexibility of the tagged integration sites with respect to supporting other promoters. Unexpectedly, in the master clones HP-GFP and HRV-GFP targeting of SV40 based antibody cassettes failed to provide significant levels of antibody expression (Fig. 4). In contrast, high level antibody expression was obtained in these integration sites upon targeting either an MPSV/CMV chimeric promoter or a bidirectional composite promoter, resulting in 3.38 and 1.69 pcd, respectively. Interestingly, the opposite situation was observed in CHO-K1 cell clones: while targeting of the SV40 promoter cassettes resulted in high level antibody expression in the range of 2.5-4.4 pcd, performance of promoters such as the composite MPSV/CMV and bidirectional AdMLP/pEF promoters was significantly impaired (Fig. 5). Several reports and also results from our lab (Lin *et al.*, 1994); data not shown) give evidence that the SV40 promoter is a potent, although not the most favorable promoter in both CHO and HEK293 cells. This rules out that this differential performance obtained upon targeting is a consequence of a diverse set of transcription factors differentially supporting in these two cell lines. Rather, it seems to be that the nature/composition of the initial tagging vector would define the capacity of the integration site with respect to supporting promoters: the master cell clones HP-GFP and HRV-GFP which were incompatible with SV40 based targeting vectors were tagged and screened for high level GFP expression from a PGK promoter and an MSCV promoter, respectively. In contrast, the master cell clones CP-HTG and CP-ABHTG

which showed high level of SV40 based expression upon targeting were initially screened to support an SV40 promoter driven tagging vector.

Together, these data indicate that specific promoters show preferential performances in certain integration sites. Our data from the screening for high expression clones show that chromosomal sites that support high level expression can be identified with both promoters. However, it seems that the nature of the integration site specifically defines the final strength of a given promoter. This interpretation is not immediately compatible with the general believe that certain promoters are particularly strong in certain cell lines. It is important to note that the data that led to this conclusion are derived from transient expression experiments or from experiments in which pools of transfectants were analyzed. For transient expression the composition of soluble (transcription) factors might indeed constitute the dominant level of promoter strength (Backliwal *et al.*, 2008). However, upon stable integration into the host genome the influence of the surrounding chromatin might be dominant over the influence of the soluble factors, given that the composition of the promoter allows expression at all. This suggests that the strength/potential of a specific integration site is linked to a certain promoter - and is not necessarily supporting any integrated expression cassette. For application of the tag and targeting approach it indicates that the molecular composition of targeting vectors and chromosomal integration site go hand-in-hand. Thus, it will be important to consider the specific requirements of a particular integration site relating to the maximum level of recombinant protein production that can be

achieved. Various types of chromosomal elements have been identified that contribute and modulate individual expression cassettes upon integration (Bode *et al.*, 2000; Li *et al.*, 2002; West and Fraser, 2005; Lindahl Allen and Antoniou, 2007). In the last years increasing evidence has been provided showing that not only specific genetic elements but also complex epigenetic mechanisms can be involved. We employed RMCE to test if expression from a weak integration site can be increased by chromosomal engineering of the integration site. However, neither the cHS4 element nor a potent S/MAR could significantly (more than 2 fold) increase the level of expression (data not shown). This indicates that the mere integration of chromosomal elements into specific loci is not of benefit *per se* but would require certain prerequisites. At the same time this gives evidence that our knowledge about the chromosomal elements and their influence on transgene expression is still rudimental. This might explain why the rational construction of synthetic expression domains providing *per se* all the needs for position independent and high expression is not straight forward and is still in its infancy. With the technologies now available for targeting transgenes to pre-defined loci our understanding of mechanism governing the crosstalk of chromosomal elements should be broadened.

5. CONCLUSION

RMCE provides a powerful strategy to specifically adapt vector designs for optimized gene expression to the specific requirements of chromosomal sites. Thereby, this method provides a unique tool to exploit such sites for predictable expression of biotechnologically relevant proteins such as antibodies.

6. AUTHORS' CONTRIBUTIONS

KN, RS, HH and DW conceived and designed the experiments. KN, RS and LGN performed the experiments and analyzed the data. LGN, AB, AC and WK helped drafting the manuscript. KN, DW and HH wrote the manuscript. All authors edited, read and approved the final manuscript.

7. ACKNOWLEDGMENTS

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8. SUPPLEMENTARY DATA

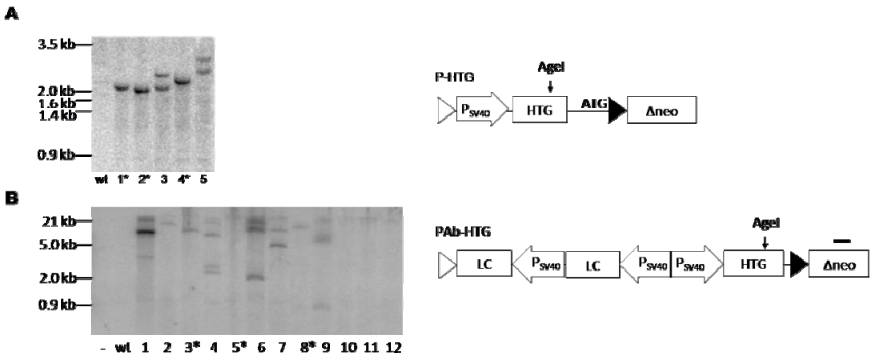

Figure S1. Evaluation of the number of integrated copies. (A) Analysis of bordering fragments from P-HTG tagged HEK293 cells. lanes 1-5 individual clones obtained upon tagging. (B) Analysis of bordering fragments of P-Ab-HTG antibody tagged CHO cells. Lane wt, untransfected CHO-K1 cells, lanes 1-12 correspond to clones #1-12 in Fig. 2B. Clones with single bands are marked with an asterisk. wt: untransfected cells; the neo probe used for hybridization is indicated. For analysis, high molecular DNA of individual clones was extracted according to Ramirez-Solis et al., 1992. The DNA was cut with AgeI and subjected to Southern Blotting. For detection of bordering fragments, the blot was hybridized to the Neo sequence. Each band corresponds to an individual integration site. Single bands indicate single integration sites.

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CHAPTER III

RETROVIRAL VECTOR PRODUCTION FROM DEFINED CHROMOSOMAL LOCI

PART A

Retroviral vector performance upon integration into defined chromosomal loci of modular packaging cell lines.

This Chapter is adapted from the paper

Gama-Norton L., Herrmann S., Schucht R., Coroadinha A.S., Löw R., Alves P., Bartholomae C., Schmidt M., Baum C., Schambach A., Hauser H., Wirth D. Retroviral vector performance upon integration in to defined chromosomal loci of modular packaging cell lines. *Hum Gene Ther*. *In Press*.

ABSTRACT

The improvement of safety and titer of retroviral vectors using standard retroviral packaging cell lines is hampered since they rely on uncontrollable vector integration events. The influences of chromosomal surroundings make it difficult to dissect the performance of a specific vector from the chromosomal surroundings of the respective integration site. Taking advantage of a technology that relies on the use of packaging cell lines with pre-defined integration sites we have systematically evaluated the performance of several retroviral vectors. In two previously established modular packaging cell lines (Flp293A and 293 FLEX) with single, defined chromosomal integration sites, retroviral vectors were integrated by means of Flp-mediated site specific recombination. Vectors that are distinguished by different LTR promoters were introduced in either sense or reverse orientation. The results show that a combination of promoter, viral vector orientation and integration site are the main determinants of the titer. Furthermore, we exploited the viral production systems to evaluate the read-through activity. Read-through is thought to be caused by inefficient termination of vector transcription and is inherent to the nature of retroviral vectors. We assessed the frequency of transduction of sequences flanking the retroviral vectors from both integration sites. The approach presented here provides a platform for systematic design and evaluation of efficiency and safety of retroviral vectors optimized for a given producer cell line.

CONTENTS

1. INTRODUCTION

Gamma-retroviral vectors are one of the most promising tools for gene therapy (Edelstein *et al.*, 2007). Much effort has been laid on the optimization and fine-tuning of recombinant retroviral vectors in order to increase both vector titers and safety (Yu *et al.*, 1986; Zufferey *et al.*, 1999; Kraunus *et al.*, 2004; Schambach *et al.*, 2006a; Schambach *et al.*, 2007), thereby rendering them as reliable tools for gene therapy applications.

A number of distinct packaging cell lines have been developed based on either murine or human cell lines. Upon stable integration of the retroviral vector into the host genome they are used for the production of infectious particles (Danos and Mulligan, 1988; Markowitz *et al.*, 1988; Miller *et al.*, 1991; Cosset *et al.*, 1995; Miller and Chen, 1996; Ory *et al.*, 1996; Ward *et al.*, 2003). The titer of retroviral vectors produced from these packaging cell lines depends not only on the vector design but also on the copy number and the position effects mediated upon its chromosomal integration. Genetic modification of these cells relies on unpredictable integration site distribution of the viral vector making it impossible to investigate the properties of retroviral vectors independently from the variable influences of the integration site. Thus, a rational design of vectors that takes advantage of beneficial features of a given integration site on virus production is not possible. Accordingly, screening of appropriate integration sites that support optimal vector expression is still the state-of-the-art for establishment of producer cell lines – a highly time consuming and laborious procedure.

The chromosomal site of vector integration is further of major importance due to the leakiness of termination of the retroviral vector transcription (polyadenylation). Indeed, the suboptimal polyadenylation signal in the R region of the retroviral LTRs results in the formation of read-through transcripts (Furger *et al.*, 2001; Zaiss *et al.*, 2002; Schambach *et al.*, 2007). As much as 10% of viral transcripts are estimated to be a consequence of illegitimate read-through in retroviruses (Zaiss *et al.*, 2002). This read-through activity is considered to be a mechanism that can lead to the transduction/activation of cellular oncogenes flanking viral genome integration site (Swain and Coffin, 1992; Uren *et al.*, 2005). For production of recombinant retroviral vectors the adventitious transduction of cellular genes by read-through activity is thus a safety concern. To exclude this, the specific vector integration site(s) in the chromosome of packaging cells would have to be characterized.

Previously, two new modular retroviral packaging cell lines have been developed, Flp293A with an amphotropic murine leukemia virus (MLV) host envelope (Schucht *et al.*, 2006) and 293 FLEX expressing the gibbon ape leukemia virus (GALV) envelope (Coroadinha *et al.*, 2006). In each of these HEK293 based cell lines a single retroviral vector integration site was identified for its capacity to provide high retroviral vector production levels. Titers up to $2.5x10^7$ infectious particles (i.p.)/10^6cells in 24hours were achieved from a single retroviral vector copy integrated into these highly active chromosomal sites.

A specific feature of these cells is the fact that the respective retroviral vector integration site is tagged with Flp recombinase target

sites (FRTs) and moreover linked to a sensitive selection system (Verhoeyen *et al.*, 2001). This enables the efficient excision of integrated retroviral screening (tagging) vector and concomitant targeting by any (retroviral) vector of interest (Flp mediated cassette exchange, RMCE). For both cell lines, cassette exchange is highly efficient and allows the rapid generation of recombinant virus with high and predictable titers. The schematic representation of the targeting reaction mediated by Flp in Flp293A and 293 FLEX cell lines is depicted in Fig. 1 A-C.

Apart from obvious advantages for highly controlled production of a given vector, these systems constitute an exceptional platform to systematically evaluate different vector compositions in defined chromosomal loci, allowing a direct comparison of vector performance and thus contributing for the development of a rational strategy for retroviral vector design. Further, since the 3' elements flanking the retroviral vector integration site are known, a detailed investigation of vector-related read-through activities is feasible.

The aim of this study was to evaluate retroviral vector design within the unique and defined chromosomal sites of modular cell lines upon Flp-mediated integration. In particular, we tested the impact on retroviral production level of both, 5' promoter composition and vector orientation of the integrated retroviral genome. Further, we utilized the defined retroviral integration sites to assess the risk of the formation and transduction of specific read-through transcripts generated from retroviral vectors. Together, this knowledge will contribute to a rational design of retroviral vectors, thereby exploiting the properties of defined vector integration site. We envision that the

definition of optimal combinations of vector elements and integration sites will allow maximizing the production of vectors without compromising the biosafety needed for the implementation of these vectors into clinics.

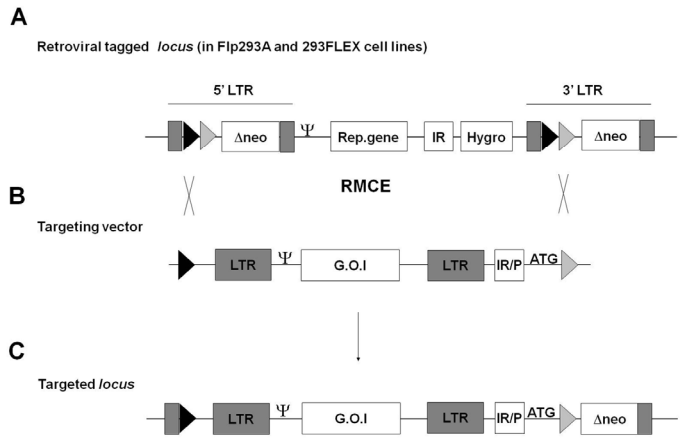


Figure 1. Vector targeting mediated by RMCE in Flp293A and 293 FLEX. The tagged retroviral locus in Flp293A and 293 FLEX cell is represented (A). In each cell line the locus is characterized by a single copy of retroviral integrated cassette harbouring GFP or LacZ as reporter gene in Flp293A (Schucht *et al.*, 2006) and 293 FLEX (Coroadinha *et al.*, 2006), respectively. Due to the ATG deficiency of the neomycin phosphotransferase (neo) gene, the tagged cell lines are G418 sensitive. For targeting via Recombinase Mediated Cassette Exchange (RMCE), the incoming targeting vectors (B) are flanked by two heterospecific and incompatible FRT sites (F-WT and the spacer mutant F5, as depicted by the black and grey triangles, respectively) and contain a sequence that complements and drives expression of neo gene (IR/P). Upon cassette exchange reaction mediated by Flp-recombinase, correctly targeted clones are G418 resistant and hygromycin sensitive (C). Ψ-retroviral packaging signal; black triangles- FRT wild type site (F-WT); light gray triangles- mutant FRT site (F-5); Rep.gene- reporter gene; IR- IRES element; IR/P- IRES/Promoter; G.O.I- gene of interest; Hygro – hygromycin phosphotransferase gene.

2. MATERIAL AND METHODS

2.1. Plasmids.

Targeting GFP-expressing SIN gamma-retroviral vectors: Retroviral vectors were integrated in a precursor targeting vector (pEMTAR-derived) containing FRT wild-type (F-WT) and FRT mutant (F-5) and an EMCV-IRES element (sense vectors) or a human PGK promoter (reverse vectors). In all vectors, an ATG start codon is positioned upstream to the F-5 site to complement the ATG-deficient neomycin phosphotransferase gene upon targeting. Sense and reverse targeting vectors differ in the orientation of the transcription of the retroviral cassette (convergent or divergent) in respect to the transcription of the ATG-complemented neo gene upon targeting event.

The sense targeting vectors:

• pRSV, pCMV, pSVe-RSV and pMPSV are derived from pSRS11.SF GFPpre, pSCS11.SF GFPpre, pSERS11.SFGFPpre and pSIN11.SFGFPpre, respectively, all previously described elsewhere (Schambach *et al.*, 2006b). Briefly, the precursor vectors were cloned in pEMTAR-derived vector in order to create targetable retroviral vectors. The vector names are modified from the original names in order to create more intuitive designations. The original names of the targeting vectors are: pEMTARSRS11.SF GFPpre (pRSV), pEMTARSCS11.SF GFPpre (pCMV), pEMTARSERS11.SFGFPpre (pSVe-RSV) and pEMTARSIN11.SFGFPpre (pMPSV).

• pCMVe-MoMLV is derived from pE336 (Loew *et al.*, 2009). The vector was modified into a targetable vector by cloning it into the pEMTAR-derived vector pTAR (Loew *et al.*, 2009).

• pCMV-CPCol is derived from the targeting vector pBulletSintec 4 (Schucht *et al.*, 2006) (referred as pSINColVII). The EF1α internal promoter of pBulletSintec 4 was replaced by the human collagen promoter from the pCPColVII vector (Kindly provided by Merten, O.W., Genethon, France).

The reverse targeting vectors:

• pPGK-MPSV and pPGK-SVe-RSV are derived from pSIN11.SFGFPpre and pSERS11.SFGFPpre, respectively, further modified by cloning the retroviral cassette into a pEMTAR-derived vector containing the PGK promoter as the regulatory element. The original designations of these vectors are pPGK- pEMTARSIN11.SFGFPpre (pPGK-MPSV) and pPGK- SERS11.SFGFPpre (pPGK-SVe-RSV).

• In order to generate pPGK-CMV-CPCol, the cassette containing the human collagen gene driven by the human collagen promoter was cloned into an EMTAR-derived vector containing the PGK promoter as the regulatory element driving expression of the neo gene in targeted cells.

Further details of all the vectors included in this study can be provided upon request.

2.2. Mammalian Cell Culture.

The modular packaging cell lines Flp293A (Schucht *et al.*, 2006), 293 FLEX (Coroadinha *et al.*, 2005), were used for retroviral vector production upon targeting via RMCE with the different targeting vectors described above. These cell lines are based on HEK293 cells and provide a single chromosomal site tagged with two heterospecific and non compatible FRT sites to allow Flp mediated cassette exchange. Further, they carry constructs encoding the respective envelope and the gag-pol gene randomly integrated in the genome at an unknown copy number. NIH3T3 cells (ATCC CRL-1658) and TE671 cells (ATCC CRL-1537) were used as target cells to titer the infectious retroviral vector particles produced from Flp293A and 293 FLEX, respectively. All cell lines were cultivated at 37ºC in an humidified atmosphere with 5%CO_2 in Dulbecco's modified Eagles medium (D-MEM, Sigma-Aldrich, Germany) supplemented with 10% of fetal bovine serum (BioWest, France), 2mM L-glutamine, penicillin (10U/ml) and streptomycin sulphate (100µg/ml). Selection of targeted cells was performed with medium supplemented with G418 (1500µg/ml) and ganciclovir (10µg/ml). Neo-transducing virus titration was performed with medium supplemented with G418 (1500µg/ml).

2.3. Recombinase mediated cassette exchange (RMCE).

For site-specific cassette exchange, Flp293A and 293 FLEX cells were seeded in a six-well plate ($3x10^4$ cells/cm^2) at the day 0. At day 1, the cells were co-transfected with 2µg of Flp recombinase

expressing vector (pFlpe; K. Maass, unpublished) and 2 µg of targeting vector using GenePORTER 2 transfection reagent (Genlantis, USA). Cells only transfected with the Flp coding vector and non transfected cells were included as negative controls. 24 hours posttransfection, the medium was replaced. On the 5^{th} day, the cells were transferred to a 60-mm culture plate and cultivated in G418 and ganciclovir containing medium to select for targeted clones. The selection was carried out for 14 days during which it was ascertained that the cells in a negative control were dead. Putative RMCE targeted G418/ganciclovir resistant clones were then picked and cultured.

2.4. Characterization of correctly targeted clones.

G418 and ganciclovir resistant single clones were evaluated for correct targeting by PCR. Genomic DNA was extracted accordingly to Ramirez-Solis *et al.*, (1992) and used as template for PCR with the primer pair 5EMCV 5'-GGCAGCCAGTCGACGTTATTTTCCACCATATTGCCG-3' and neorev2 5'-GTCATAGCCGAATAGCCTCTCC-3' in case of sense targeting vectors and the primer pair PGKFwd1 5'- TCTCGCACATTCTTCACGTCC-3' and neorev2 in case of antisense targeting vectors.

2.5. Viral supernatant production and titration.

Flp293A and 293 FLEX derived target clones were seeded at $4x10^4$ cells/cm^2 on 25 cm^2 T-flask with 3ml of medium, assuring a cell confluence of about 70% in 24h. The next day, supernatant was harvested, filtered (45µm) and used for infection of target cells upon

supplementation with 8µg/ml of polybrene (Sigma, Germany). The cell number of the producer clones was determined. Viral titration of GFP containing vectors generated upon targeting of Flp293A and 293 FLEX was determined by flow cytometry. At day 0, NIH3T3 and TE671 cells were seeded at $5x10^3$ cells/cm^2 in a 12-well plate. At day 1, NIH3T3 and TE671 cells were infected with 300µl of serial dilutions of viral supernatant generated from Flp293A or 293 FLEX derived clones, respectively. At day 2, the viral supernatant was replaced by fresh medium. At day 3, the infected cells were analyzed by flow cytometry. The titer was calculated based on the percentage of GFP positive cells. Infections that rendered 2-20% of infected cells were considered for titer calculations.

Viral titration of collagen VII containing vectors upon targeting of Flp293A and 293 FLEX was done by immune staining of infected cells. Upon staining, cells were analyzed by flow cytometry and the titer determined. At day 0, NIH3T3 and TE671 cells were seeded at $1x10^5$cells/cm^2 in a six-well plate. At day 1, NIH3T3 cells and TE671 cells were infected with 1ml of dilutions 1:2 and 1:10 of viral supernatant generated from Flp293A or 293 FLEX derived clones, respectively. At day 2, the viral supernatant was replaced by fresh medium. At day 3, the cells were stained and the titer determined. Briefly, the cells were washed twice with phosphate buffered saline (PBS), fixed with 100µl of 2% PFA (paraformaldehyde) solution and again washed with PBS. The cells were stained with 100µl of 1:300 primary antibody solution (anti-human collagen type VII; Sigma; C6805) in 0.5% triton/PBS and incubated for 30 minutes on ice. After incubation the cells were washed three times with 0.5% Triton/PBS.

The secondary goat anti-mouse IgG antibody (FITC labeled; Dianova GmbH, Germany) was applied (1:400) and the cells incubated 20 minutes on ice. After incubation, the cells are washed twice with 0.5% Triton/PBS and finally resuspended in PBS/2%FBS FACS buffer.

2.6. Flow cytometry.

Flow cytometric analysis was performed on a FACSCalibur (Becton Dickinson). For titration of GFP expressing virus, the cells were washed, trypsinized and stained with propidium iodide (50µg/ml) to exclude dead cells.

2.7. Neo- transducing virus titration.

Viral supernatants were harvested from targeted 293 FLEX and Flp293A cells and used to infect TE671 or NIH3T3 cells, respectively. Briefly, TE671 and NIH3T3 cells were seeded at $1.0x10^4$cells/cm^2 in 6 a well-plate, day 0. At day 1, the cells were infected with 1ml of serial 10- fold dilutions of viral supernatant. At day 2, the cells were transferred to a 60-mm culture dish in presence of medium supplemented with G418. Fourteen days after, the number of G418 resistant cells was determined and the neo titer calculated.

3. RESULTS

3.1. Re-use of a defined chromosomal locus of a modular packaging cell line for evaluation of retroviral vector performance.

Efficiency of viral RNA transcription is dependent on the repertoire of transcription factors provided by the packaging cell line (so-called *trans* factors) and on *cis* effects. The latter comprise regulatory elements within the vector sequence, genetic elements as well as epigenetic organization *status* of the chromosomal integration site of the vector. In order to compare the performance of different retroviral vectors and their dependence on the chromosomal surroundings of the integration site, a panel of different gamma-retroviral vectors (Fig. 2) was included in this study. These vectors were targeted into the tagged single copy chromosomal loci of the modular packaging cell lines 293 FLEX and Flp293A (Coroadinha *et al.*, 2006; Schucht *et al.*, 2006).

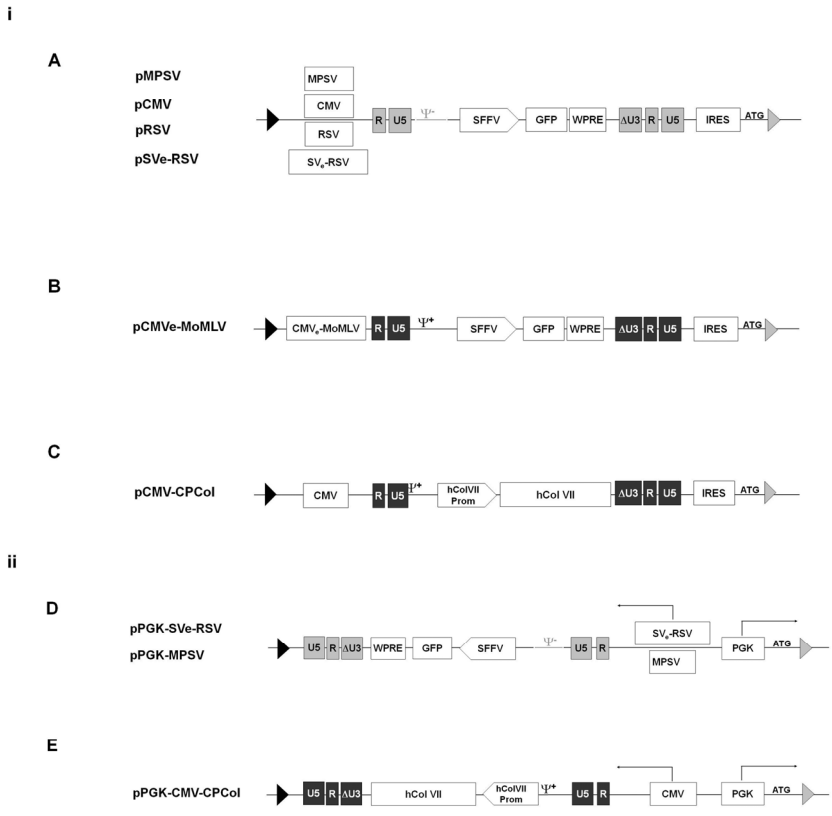


Figure 2. Schematic representation of targeting SIN gamma-retroviral vectors used in this study. The retroviral vectors are flanked by two non-interacting FRT sites, F-WT and F-5, depicted in the figure as black and grey triangles, respectively. The selection of the correctly targeted clones is assured by the presence of the EMCV IRES element, in the case of the sense vectors (i) or by the PGK promoter, in case of the reverse vectors (ii). The regulatory element is followed by the ATG codon, promoting translation of the complemented neo gene (present in the tagged loci) upon targeting events. Light gray boxes and dark gray boxes represent the LTR regulatory sequences from the MPSV or MoMLV, respectively. Thin black arrows represent the direction of transcription driven by the promoters represented below the arrows. i) *Sense targeting vectors.* The transcription of the gamma-retroviral genome is convergent to the transcription of the neo gene upon targeting. A) *GFP targeting vectors comprising various 5'LTR promoter compositions in a defined retroviral backbone.* In all of these targeting vectors, the gamma-retroviral backbone contains GFP as a reporter gene. Due to the self-inactivating

(SIN) nature of these vectors, the reporter gene expression, in infected cells is assured by the U3 sequence of the spleen focus forming virus (SFFV), present as an internal promoter. The vectors contain a post-regulatory element from Woodchuck hepatitis virus (WPRE). The vectors pMPSV, pCMV, pRSV and pSVe-RSV constitute a set of vectors based on the same retroviral backbone, solely differing on the promoter composition at the 5' LTR. The residual vector sequence is identical among these four vectors and is composed by the untranslated leader region of the MESV (represented as a gray line below *psi*) and the LTR regulatory sequences from the MPSV (represented as gray boxes). The packaging region is partially deleted (no gag/vector overlap) (Ψ-). B) *eGFP targeting vector based on alternative viral backbone.* The vector pCMVe-MoMLV (Loew *et al.*, 2009) is SIN vector and as the vectors described above is composed by the GFP as a reporter gene driven by the U3 sequence of the SFFV and followed by the post-regulatory element from Woodchuck hepatitis virus (WPRE). The 5'LTR is composed by the CMV enhancer and the U3 promoter sequence of the MoMLV. The viral backbone is derived from MoMLV and contains the enlarged packaging region (Ψ+). C) *human collagen VII targeting vector providing a large packagable viral RNA.* Targeting vector expressing the human collagen VII gene, driven by the internal human collagen VII promoter. The CMV promoter is present in the 5'LTR, driving expression of the viral genome. The viral backbone is derived from MoMLV and contains the enlarged packaging region (Ψ+). ii) *Reverse targeting vectors.* The gamma-retroviral backbone is cloned in antisense in respect to the transcription of the neo gene upon targeting. D) *GFP expressing vectors.* The vectors pPGK-SVe-RSV and pPGK-MPSV are the reverse counterparts of the vectors pSVe-RSV and pMPSV, respectively. E*) human collagen VII expressing vector.* The vector pPGK-CMVCPCol is the reverse counterpart of the vector pCMV-CPCol. Please note that the schemes in the figure are not represented to scale. For the original nomenclature of vectors and references, please consult Material and Methods section.

The retroviral vector integration site of these two cell lines was mapped using a non-restrictive LAM-PCR protocol (Gabriel *et al*., 2009). For Flp293A the tagged locus is located on the short arm of the chromosome 12, either in the first intron of DDX11 (p11.21, 31227164) or DDX12 gene (p13.31, 9600360), reversely orientated in

relation to the endogenous gene transcription. Since these genes are highly homologous, both positions are possible. For 293 FLEX cell line, the tagged locus is positioned in an intergenic region 100Kb upstream the gene ARRDC3 on chromosome 5 (q14.3, position 90781745). After Flp-mediated targeting of the retroviral vectors in Flp293A and 293 FLEX cells, G418 resistant clones were isolated and targeting was confirmed by PCR (data not shown). Virus production from the targeted cells was determined. In agreement with previous findings (Coroadinha *et al.*, 2005; Schucht *et al.*, 2006), the virus production levels of clones generated upon cassette exchange with a given vector were highly homogeneous. This is exemplified in Fig. 3 for the vectors pSVe-RSV and pCMV after targeting.

a) Impact of regulatory elements

Among other elements, the nature of the promoter that drives retroviral vector transcription is important for the titer. To compare various commonly used promoter elements in the defined chromosomal context of the modular cell lines Flp293A and 293 FLEX, we used self-inactivating (SIN) gamma-retroviral vectors as displayed in Fig. 2A and 2B (for sake of simplicity, the name of each vector is deduced from the promoter within the 5'LTR). Four of these SIN vectors only differ in 5' promoter/enhancer elements used for driving the viral genomic mRNA (Schambach *et al.*, 2006b). Both native promoter sequences (Myeloproliferative Sarcoma Virus-MPSV, Cytomegalovirus-CMV, Rous Sarcoma Virus-RSV) and a combination of Simian Virus 40 enhancer and RSV promoter element (SVe-RSV)

were employed (Fig. 2A). Further, we included a Moloney Murine Leukemia Virus (MoMLV) based SIN vector in which the endogenous U3 sequence in the 5'LTR was replaced by a CMV enhancer MoMLV composite promoter (CMVe-MoMLV) (Fig. 2B). Common to the vectors included in this study is the internal cassette composed by the Spleen Focus Forming Virus (SFFV) U3 sequence that drives the GFP reporter gene.

High clonal homogeneity was observed for all cell clones generated upon targeting of the same retroviral vector (Fig. 3).

However, we found up to 15-fold differences in retroviral titers when the five promoter/enhancers LTRs were compared in the same locus. Fig. 4 depicts a summary of the titers obtained from clones generated upon targeting of Flp293A and 293 FLEX with the five vectors. Interestingly, the hierarchy of performance of the 5'LTR-modified retroviral vectors was the same in both packaging cell lines. The composite promoter vectors pSVe-RSV and pCMVe- MoMLV provided the highest titers: about $1x10^7$ i.p./$1x10^6$ cells in 24 hours in Flp293A and about $7x10^5$ i.p./$1x10^6$ cells in 24 hours in 293 FLEX. pRSV and the pCMV gave rise to significantly lower titers in both cell lines.

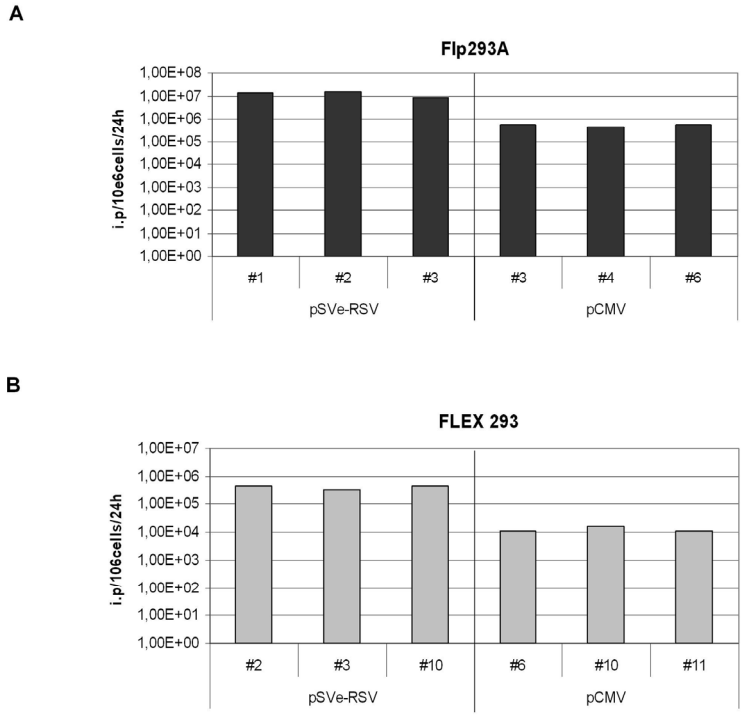


Figure 3. Analysis of virus production from independent cell clones obtained upon targeting of Flp293A (A) and 293 FLEX (B) with the vectors pSVe-RSV and pCMV. Flp293A and 293 FLEX cells were targeted with the retroviral vectors pSVe-RSV and pCMV. G418 resistant clones were confirmed for correct targeting by PCR. The supernatant from three independent cell clones from each targeting in the Flp293A and 293 FLEX lines was used to infect NIH3T3 and TE671cells, respectively. Virus titrations were done by assessing the number of GFP expressing cells by FACS. The individual clones were analyzed at least twice, with a titer deviation less than 25%. Each bar represents one independent cell clone.

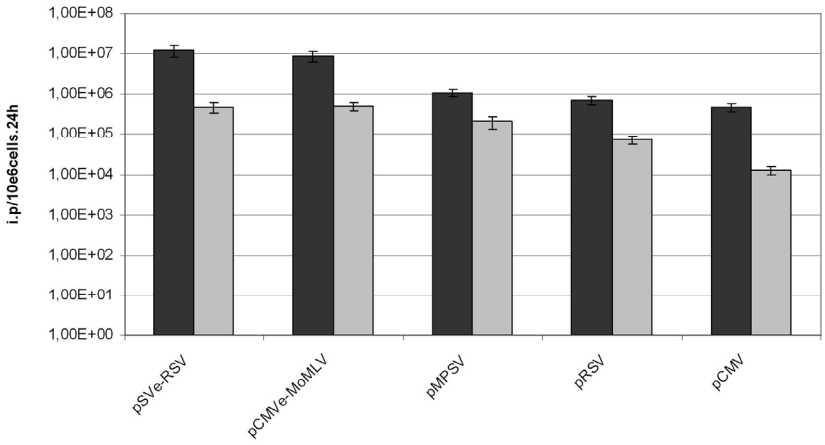


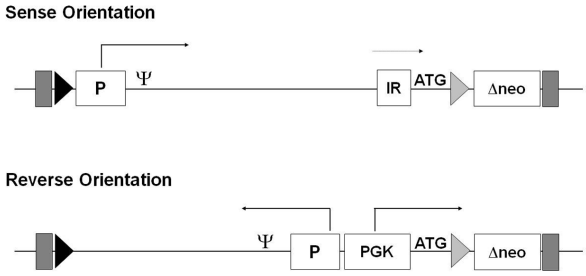
Figure 4. Influence of the 5'LTR promoter on virus titer from targeted retroviral modular cells. The gamma-retroviral SIN vectors as depicted in Fig. 2 (A, B) were targeted in Flp293A (dark gray bars) and 293 FLEX (light gray bars) cell lines. The supernatant from three different cell clones from each targeting in the Flp293A and 293 FLEX lines was used to infect NIH3T3 and TE671 cells, respectively. Virus titrations were done by assessing the GFP expressing cells by FACS. The average titres were calculated based on at least three measurements of GFP expression upon infection using independent cell clones obtained after RMCE.

b) Impact of the orientation of vector integration

We further asked if the integration sites in Flp293A and 293 FLEX cells support efficient vector production for both orientations of the retroviral vector according to Fig. 5A. For this study, the two GFP containing SIN gamma-retroviral vectors pMPSV and pSVe-RSV (Fig. 2A) were chosen and integrated in a targeting vector in reverse orientation. To activate the neo gene upon integration, the human PGK promoter was used. This provided the targeting plasmids pPGK MPSV and pPGK-SVe-RSV (Fig. 2D). In this study we further included a MoMLV-derived collagen VII gene transducing vector, pCMV-CPCol

(Fig. 2C). In its reverse counterpart, pPGK-CMV-CPCol (Fig. 2E), the genomic viral RNA transcription is driven by a CMV promoter. Interestingly, the vector orientation had different effects in the two integration sites. In the 293 FLEX site, both orientations resulted in either similar titers or even in an increase in titer from the reversely orientated collagen VII containing vector when compared to its sense counterpart (Fig. 5B). In contrast, in the Flp293A site, a significant lower titer was observed in all reverse targeting vectors (Fig. 5C). For the vector pPGK-SVe-RSV a difference of more than 7-fold was observed when compared with pSVe-RSV, while for the pPGK-MPSV a reduction of about 13-fold was observed when compared to the sense counterpart pMPSV. An even more severe reduction in titer was observed when comparing the sense and the reverse collagen expressing vectors in Flp293A. This shows that while the integration site of 293 FLEX cells supports equally both orientations, in Flp293A cells the sense orientation is highly favored.

A



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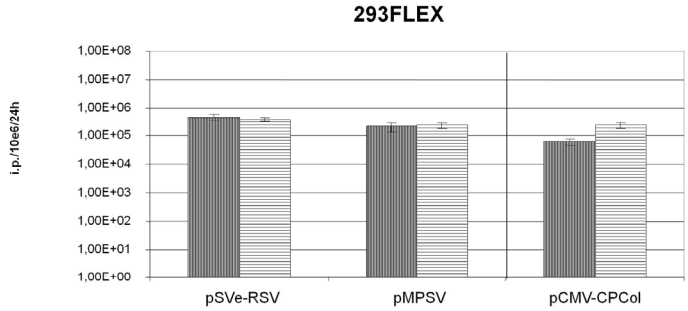

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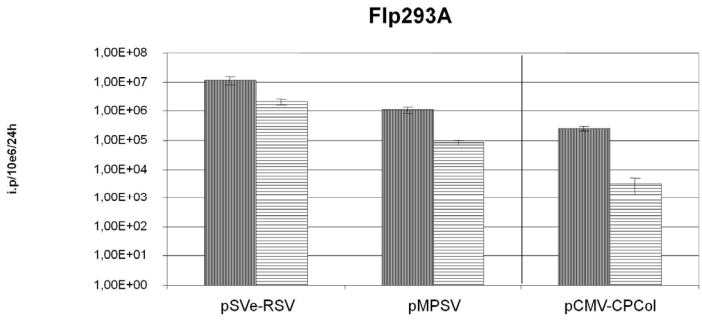

Figure 5. The impact of orientation of the retroviral transcription on infectious viral production from Flp293A and 293 FLEX cell lines. (A) The impact of retroviral transcript orientation was evaluated upon targeting of three different constructs in sense and in reverse orientation. In the sense orientation, the expression of the complemented neo gene is assured by an EMCV IRES element (IR) in the targeting vector while in the reverse orientation, the transcription of the neo gene is driven by the PGK promoter. "P" represents the heterologous promoter in the 5' LTR of

the retroviral vector. The vectors implemented in this study are the sense vectors pSVe-RSV, pMPSV and pCMV-CPCol as well their reverse counterparts pPGK-SVe RSV, pPGK-MPSV and pPGK-CMVCPCol, respectively. The sense and reverse retroviral constructs were titrated upon targeting in 293 FLEX (B) and Flp293A (C) cell lines (vertical and horizontal lines represent the sense and the reverse vectors, respectively). The vectors pSVeRSV and pMPSV as well as their reverse counterparts harbor the GFP gene while in the pCMV-CPCol and its reverse counterpart the transduced gene is human collagen VII. Indicated for each set of bars are the names of each sense vector.

3.2. Evaluation of packaging of read-through transcripts in modular packaging cell lines.

In the modular producer cell lines Flp293A and 293 FLEX, the downstream region of the 3' LTR of the targeted vectors is defined. It is composed of the FRT F5 site followed by the neomycin phosphotransferase gene (neo) and the residual 3'LTR sequences. These elements were used to identify correctly targeted retroviral vectors. Upon targeting of a retroviral vector in sense orientation into the tagged sites, the vector is flanked at its 3' end by the EMCV-IRES element followed by the neomycin resistance gene and an inverted repeat-deleted viral LTR, as depicted in Fig. 1C. Because the termination of retroviral vector transcripts is provided by a weak polyadenylation signal present in the R region of the viral LTR, we asked if this constellation would result in transduction of the downstream sequences. As these events are expected to be comparably rare, we exploited the unique situation in the modular packaging cell lines and determined the ability of the above described producer clones to transduce the neo gene ("read-through titer"). Packaging of these read-through transcripts upon infection and

integration should render neomycin resistance. In particular, we intended to investigate if the read-through events are strictly dependent and directly correlated with the strength of a certain promoter or with certain promoter composition.

In order to evaluate the read-through frequency, viral supernatants from targeted 293 FLEX and Flp293A cells were used to infect TE671 and NIH3T3 cells, respectively. These read-through titers associated with the tested vectors in 293 FLEX and Flp293A are summarized in table 1 and 2.The number of the G418 resistant cells reflects the frequency of read through and successful transduction of the neo gene during virus production. The read-through titers were compared with the number of GFP expressing infected cells, which were taken as a measure for total infectious viral particle production (table 1 and 2). The ratio between the read-through titer and the total infectious particle titer gives a quantitative measure for read-through occurrence frequency.

Table 1. Neo titers from 293FLEX targeted with sense and reverse orientated vectors.

Retroviral vector		Titer		
		Neo Titer[a]	Infectious Particles[b]	Neo Titer/Infectious Particles[c]
Sense	pSVe-RSV	370,5 ± 131,4	$3,0x10^5$	1 : 810
	pCMVe-MoMLV	12,3 ± 3,6	$1,8x10^5$	1 : 15000
	pMPSV	790,4 ± 277,7	$1,7x10^5$	1 : 215
	pRSV	922,0 ± 78,1	$7,1x10^4$	1 : 77
	pCMV	15,6 ± 4,8	$1,3x10^4$	1 : 831
	pCMV-CPCol	12,5 ± 3,7	$6,1x10^4$	1 : 5000
Reverse	pPGK-SVe-RSV	< 1	$3,9x10^5$	< 1 : $3,9x10^5$
	pPGK-MPSV	< 3	$2,5x10^5$	<1 : $8,3x10^4$
	pPGK-CMVCPCol	< 1	$2,5x10^5$	< 1 : $2,5x10^5$

Table 2. Neo titers from Flp293A targeted with sense and reverse orientated vectors.

Retroviral vector		Titre		
		Neo Titer[a]	Infectious Particles[b]	Neo Titer/Infections Particles[c]
Sense	pSVe-RSV	791,1 ± 293,6	$1,2 \times 10^7$	1 : 15548
	pCMVe-MoMLV	50,6 ± 19,9	$8,8 \times 10^6$	1 : 174337
	pMPSV	1340,2 ± 694,0	$1,1 \times 10^6$	1 : 821
	pRSV	1640,8 ± 579,3	$7,1 \times 10^5$	1 : 435
	pCMV	16,9 ± 9,4	$4,8 \times 10^5$	1 : 28143
	pCMV-CPCol	40,8 ± 18,2	$2,6 \times 10^5$	1 : 6406
Reverse	pPGK-SVe-RSV	n.d		
	pPGK-MPSV	n.d		
	pPGK-CMVCPCol	< 3	$3,2 \times 10^3$	<1 : 1067

[a]Neo Titer: G418 resistant particles/$1x10^6$ producer cells in 24h. Between 3 to 8 independent measurements for each targeting group were considered. For the sense vectors, neo titer reflects the read-through activity. [b]Infectious Particles Titer: GFP transducing particles/$1x10^6$ producer cells in 24h. [c]Neo Titers/Infectious Particles: The ratio represents the frequency of neo transduction events *per* total of infectious particles produced. n.d.: not determined.

The read-through titers produced by the clones targeted with the different sense vectors are considerably heterogeneous, with two orders of magnitude of difference in the two cell lines tested. Namely, the 293 FLEX cell line targeted with pCMVe-MoMLV and pCMV produced about 12 to 16 neo-transducing i.p./10^6 cells in 24 hours, while the group composed by 293 FLEX cell line targeted with pSVe RSV, pMPSV and pRSV produced 370 to 922 neotransducing i.p./10^6 cells in 24 hours. Similarly, Flp293A-derived cells targeted with pCMVe- MoMLV and pCMV were associated with a read-through frequency 30-100 fold lower than the read-through frequency observed for pSVe-RSV, pMPSV and pRSV targeted cell lines. Of note is that efficiency of read-through transduction was not correlated to the GFP-transducing infectious viral particles produced by the constituent vectors in both of the cell lines tested: among the vectors with low neo-titers, pCMVe-MoMLV was one of the two vectors with the highest infectious particle production. In contrast, pCMV also showing low neo-titers was the vector that showed the lowest infectious particle production capacity. At the same time, for the vectors that gave rise to the highest infectious particles titers, pSVe RSV and pCMVe-MoMLV, the efficiency of the read-through transcript transduction was found to be low. In 293 FLEX one neo transducing particle was generated for every 810 or 15000 infectious particles produced from cells targeted with pSVe-RSV and pCMVe-MoMLV, respectively. Interestingly, the frequency of read-through transcripts generation was maximally 2 to 4-fold increased in Flp293A cells although this cell line provided an up to 40 to 50-fold increased GFP transduction capacity.

In general, the maximum size of naturally occurring retroviruses is around 9.5 Kb. Viral RNAs that exceed this length are found to be compromised for packaging and transduction. Hence, theoretically, long read-through transcripts should be less efficiently packaged than the shorter, correctly poly-adenylated transcripts. We evaluated the efficiency of neo transduction from cells targeted with the vector pCMV-CPCol (Fig. 2C). This cDNA comprises about 9 Kb; upon correct poly-adenylation at the 3' R region a transcript of 10.5 Kb is expected, while illegitimate readthrough transcripts would result in a 12.5 kb RNA. As expected, 293 FLEX as well Flp293A cells targeted with pCMV-CPCol are related to low levels of read-through transcript generation, producing in average, 12.5 and 40.8 neo transducing particles per 10^6 cells in 24 hours, respectively and thereby ranging at the lower level. These results also show that the increase of RNA length *per se* cannot further reduce the neo titer, indicating that additional mechanisms are involved.

3.3. Overcoming the formation of neo transducing read-through transcripts by targeting the retroviral cassette in reverse orientation.

Accidental transduction of the neo gene does not represent a critical issue *per se* and indeed many therapeutic vectors use neo gene as a co-transduced selection marker. However, apart from potential antigenicity, the neo transduction from modular cell lines is accompanied by an unwanted transfer of the 3´ LTR elements (remaining sequences from the tagging construct after a targeting

event). This illegitimate transduction thus represents the transfer of sequences with promoter/enhancer properties with the ability to restore LTR activity upon infection. Indeed, it has been shown that these promoter/enhancer elements have the potential to deregulate genes in the vicinity of the vector integration site (Hacein-Bey-Abina *et al.*, 2003).

Although the frequency of putative insertional events triggered upon infection with the read-through derived particles is rather low, we intended to improve the safety of these cellular systems for retroviral vector production. Thus, we evaluated if a reverse orientation of retroviral vectors in the previously tagged integration sites would efficiently overcome transduction of the residual sequences (*i.e.* the neo and LTR) provided by the modular cell lines. For this purpose, the vectors pSVe-RSV, pMPSV and pCMV CPCol were chosen. Readthrough titers from targeted cells with reverse orientated vectors were determined and compared with the read through titers produced by the sense counterparts (table 1 and 2).

As expected, a significant reduction of neo transducing particles was observed for the reverse oriented vectors. In the case of the reverse GFP containing vectors targeted into the 293 FLEX cell line, the ratio between neo transducing particles to total infectious particles was only 1 in $1x10^5$ and less than 1 in $4x10^5$ for pPGK-MPSV and pPGK-SVe-RSV, respectively. In case of the reverse oriented vector pPGK-CMV-CPCol no G418 resistant colonies were detected, indicating that less than one in $2x10^5$ of infectious particles transduces the neo containing transcript. A significant 15-fold reduction was observed in the case of Flp293A targeted with the collagen encoding

reverse vector. Thus, we show that in modular packaging cells 293 FLEX and Flp293A, the expression of the reversely oriented retroviral vector is efficiently supported and significantly reduces transduction of the neo gene and 3' LTR containing transcripts.

4. DISCUSSION

The recently described modular packaging cell lines Flp293A and 293 FLEX represent universal tools for production of any gamma-retroviral vector by application of recombinase mediated cassette exchange technique. Due to the controlled genetic modification mediated by targeting of a characterized integration site, a predictable, fast and safe production of viral vectors is possible, thereby overcoming limitations of strategies that rely on random integration of the viral genome. Both modular packaging cell lines are derived from the same cell line (HEK293), but are distinguished by the integration sites of the retroviral construct. The absolute titer cannot be compared between both of the cell lines since Flp293A is producing the amphotropic envelope and 293 FLEX packages the virus with the GALV envelope. Nevertheless, the relative amount of viral production can be compared. This allowed the performance evaluation of different retroviral vectors in each of the two integration sites. Among the variables affecting the viral titer in a given cell, the cell's repertoire of transcription factors (*trans* effects) defines the overall activity of specific promoters. These *trans* effects should act independently of the chromosomal integration. In addition to these 'global' effects, any integration site in the genome may be

affected by positive and/or negative effects mediated by genetic and epigenetic elements. These *cis* effects are site specific and may significantly modulate the transcription efficiency of cassettes that are integrated into specific site.

The consistent graduation obtained after targeting various vectors differing in the 5' promoters into the chromosomal sites of Flp293A and 293 FLEX (Fig. 1A) suggests that a corresponding set of cellular factors determines the titer in both cell lines. This is expected for typical *trans* effects like the availability of certain transcription factors required for promoting transcription of the individual promoter/enhancer combinations. Also, *cis* factors acting on both integration sites in Flp293A and 293 FLEX cells would give rise to a consistent performance of vectors. In this respect, the tagging procedure applied for identification of the integration sites and in particular the common flanking 3' LTR sequences of the tagging virus might contribute to this consistency. Interestingly, a totally different hierarchy was met if the vectors were evaluated in a mouse derived modular packaging cell line that was established using the same screening procedure (Loew *et al.*, 2009). This suggests that the *trans* effects have a strong influence in determining viral vector production levels from stable helper cell line.

Besides this consistent behavior of vector performance in the two chromosomal loci, an integration site-specific modulation is thought to result in a locus specific 'fine-tuning' of the transcription regulatory elements. An example is the vector pCMV in comparison to the pRSV vector: while these vectors are showing comparable performances in Flp293A cell line the CMV vector is 5.5-fold less

active in 293 FLEX cell line. Conversely, pSVe-RSV and pMPSV vectors behave similarly in 293 FLEX ($5x10^5$ and $2x10^5$ i.p./$1x10^6$ cells in 24 hours, respectively) but display more than 10-fold difference in titer in Flp293A. We conclude that these different performances are a consequence of locus specific *cis*-effects on the respective promoter elements. In this respect, the CMV promoter is either negatively influenced by the chromosomal surroundings of the 293 FLEX locus, or positively affected by the surroundings in Flp293A cell line. This gives evidence that indeed, optimal combinations of specific integration sites and promoter content of a retroviral vector have to be defined in order to maximize the level of recombinant virus produced by those systems.

Notably, the significant difference on the performance of the vectors with the highest and the lowest viral titers (pSVe-RSV and pCMV, respectively) of 25-33 fold in Flp293A and 293 FLEX was not obtained in transient production experiments (Schambach *et al.*, 2006b). Nevertheless, in both production systems (transient and stable production), the vector pSVe-RSV performed best and the vector pCMV performed worst. This supports the view that *trans*-acting factors in HEK293 derived cells (*e.g.* transcription factors) govern this hierarchy. In addition, this gives evidence that in transient production systems, the simultaneous expression of multiple copies of the retroviral genome can mask the real impact of the different heterologous 5'LTR promoter on the full length transcript production and concomitantly on the infectious viral titers.

Further, our study clearly shows that the performance of transcription of a targeted vector in either one or the other

orientation is integration site-dependent. While in Flp293A the sense orientation is highly favored (over the reversed vector), in 293 FLEX cells, efficient production is supported upon reversion of the vector at the same extent as for the sense vector.

We assume that crosstalk between cellular and vector elements (*cis*-effects) plays a major role in determining the expression level of a certain integrated cassette. The tagging vector integration site of Flp293A has been mapped to chromosome 12 in the first intron of DDX11 or DDX12 gene, with reverse orientation to the DDX transcript. Despite potential antisense transcripts in Flp293A, a high expression of targeted retroviral vector requires its orientation in antisense to the endogenous transcript. A possible explanation concerns the promoters of the endogenous gene (DDX11/12) and the vector. In antisense orientation, the endogenous DDX11/DDX12 gene and the retroviral vector are divergent and the promoters are separated by 10 kb of intervening sequence. A different scenario is met for the 293 FLEX cell line. As referred, the tagged site is positioned in an intergenic region of chromosome 5, 100 Kb upstream the ARRDC3 gene. In this integration site, we determined no preference for the sense vs. the reverse orientation. We hypothesize this might be due to the absence of interfering promoters or endogenous transcriptional activity in the vicinity of the tagged site.

Together, our study shows that retroviral titer can be maximized by acknowledging the specific requirements of the nature of the retroviral vector integration site in the producer cell line, suggesting that the chromosomal locus can modulate the performance of specific regulatory elements presented in integrated

viral vector. Thus, to achieve an optimal titer, the demands of the respective integration site have to be considered. Accordingly, only certain vectors/promoters can provide satisfactory high titers in a given integration site.

Retroviruses have been shown to acquire and transduce cellular genes. This can be accomplished by accidental packaging of cellular RNAs devoid of any retroviral sequences. This process is designated as retrofection (Linial, 1987; Levine *et al.*, 1990; Lum and Linial, 1998) and has been shown to occur with frequencies of 10^{-6}. The frequency of retrofection has to be considered as the basal transduction level inherent of any retroviral transfer. More relevant for accidental transduction are cellular genes that are located next to retroviral integration sites. This is the initial event that can lead to the capture of proto-oncogenes (Swanstrom *et al.*, 1983; Herman and Coffin, 1987; Swain and Coffin, 1992; Schwartz *et al.*, 1995; Muriaux and Rein, 2003). Illegitimate read-through of viral RNA is supposed to be one mechanism followed by copackaging and subsequent recombination with a correctly processed RNA. To exclude transduction of proto-oncogenes by this mechanism, the proximal 3' region of a retroviral vector integration site in the packaging cell line has to be confirmed to be devoid of critical sequences. For the retroviral integration sites of Flp293A cells and 293 FLEX cells the proximal 3' region is constituted by elements used for the establishment of the integration site including an additional poly adenylation signal provided by the residual 3' LTR. In this respect, upon transcription of a sense vector transduction of cellular neighboring sequences can be largely excluded for both of the cell

lines. Further, due to the chromosomal localization of 293 FLEX tagged sites, putative read-through in either direction would not lead to expression of a functional protein in the infected cells.

Despite the high level of safety of modular packaging cells with respect to the adventitious transduction of cellular sequences, an inherent property of these cellular systems is the potential read-through transcription and packaging of the downstream sequences to the targeted retroviral vector (composed by the neomycin phosphotransferase gene and retroviral LTR) that constitute the remaining sequences of the tagging vector upon a targeting event. This specific feature could be used to determine the extent of read-through capacity of different retroviral vectors targeted in these specific loci simply by the quantification of G418 resistant cells that arise upon infection. A comparison of the neo transduction frequency of the used vectors, we observed that the frequency of neo transduction did not correlate with the GFP titer. The reason for this is not obvious. One explanation for this might be that the transcription termination is affected by specific DNA configurations promoted by the different regulatory sequences presented in a certain targeted vector. Such regulatory sequences might positively or negatively affect the recognition of transcription termination signals presented at 3' end of the retroviral vector. Alternatively, unique genetic or structural elements inherent to both integration sites might provoke this consistency.

As suppression of transcriptional read-through into downstream sequences can be regarded as an improvement, vectors like the pCMVe-MoMLV targeted into the modular cell lines constitute

a better choice in terms of biosafety (table 1 and 2). Moreover, incorporation of sequences that improve transcript termination such as upstream sequence elements (Schambach *et al.*, 2007) can be regarded as a benefit for the safer vector systems production for therapeutics. It has been shown that promoters derived from human genes such as EF1alpha or PGK promoter, showed a lower potential to activate neighboring promoters than virally derived promoters (Zychlinski *et al.*, 2008).

The safety of the production system presented in this work has also been shown to increase upon targeting of retroviral vectors in reverse orientation. This strategy is of great value for the minimization of the production of viral transcripts that are formed upon read-through events of the neo gene and LTR sequences in the targeted cells.

In the reverse orientation, read through of the viral transcript cannot contribute to neo gene transduction. The low frequency of neo transduction is in the same range as has been reported for unspecific retrofection and in this respect has to be considered as the natural basal level of transduction of cellular sequences independent of the orientation of the retroviral vector. While the integration of reversely orientated vectors represents a beneficial feature for increasing the safety of the retroviral vectors (concerning to the specific characteristics inherent to these modular cell systems), it is of note that the performance of these reverse retroviral vectors is strictly dependent on the chromosomal locus. A similar conclusion is also supported by results obtained in another cellular background (Loew *et al.*, 2009).

In order to achieve the production of vector particles with the indispensable safety levels for the translation of those cellular systems into the clinics, the incorporation of sequences that improve transcription termination of the retroviral tagging sequence is an alternative strategy (Schambach *et al.*, 2007). We anticipate that the read-through titer of sense vectors targeted in modular cell lines can be reduced by implementing additional poly-adenylation sequences downstream the viral genome, without significant decrease of viral titers.

The data show that a systematic comparison of vector constructions is feasible in modular producer cell lines. Moreover, the data also suggest that a next generation of helper cell lines should have additional features: in particular, they should provide maximal flexibility with respect to supporting vector sequences and also chromosomal surroundings that do not lead to unpredictable effects like oncogenic transfer or antigenic effects. As RMCE implies that the site of interest is tagged a priori by specific sequence motifs, we envisioned that technologies for site-directed modifications of mammalian genome *e.g.* Zinc-Finger recombinase-based technologies could be used to direct FRT docking sites to specific loci of interest. The tagging of a well characterized defined locus and its exploitation by means of RMCE provides the next step towards the establishment of safer cell lines suitable for clinical grade production of therapeutic vectors, representing a particular case of production of proteins with biological/therapeutical significance.

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PART B

A new PG13-based packaging cell line for stable production of clinical-grade self-inactivating gamma-retroviral vectors using targeted integration.

This Chapter is adapted from the paper

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ABSTRACT

The clinical application of self-inactivating (SIN) retroviral vectors has been hampered by the lack of reliable and efficient vector production technologies. To enable production of SIN gamma-retroviral vectors from stable producer clones, a new PG13-based packaging cell, known as PG368, was developed. Viral vector expression constructs can be reliably inserted at a predefined genomic locus of PG368 packaging cells by an Flp-recombinase mediated targeted cassette exchange (RMCE) reaction. A new, carefully designed vector-targeting construct, pEMTAR-1, eliminated the co-packaging of the selectable marker gene used for the identification of successful recombination at the predefined genomic locus and thus, improved the safety of the production system. Selected clones produced vector supernatants at consistent titers.

The targeted insertion of therapeutically relevant SIN vectors for chronic granulomatous disease and X-linked severe combined immunodeficiency into PG368 cells results in stable titers within the range necessary for clinical application. The production of retroviral SIN vectors from stable clinical-grade producer cells is feasible and will contribute to the safe production and application of SIN gamma-retroviral vectors for clinical trials.

CONTENTS

1. INTRODUCTION

Retroviral-mediated gene therapy has been shown to be an efficient treatment option for a number of inherited blood disorders (Cavazzana-Calvo *et al.*, 2004). Patients with X-linked severe combined immunodeficiency (SCID-X1) (Hacein-Bey-Abina *et al.*, 2002), adenosine deaminase deficient SCID (ADA-SCID) (Aiuti *et al.*, 2002; Aiuti *et al.*, 2009) and chronic granulomatous disease (CGD) (Ott *et al.*, 2006) have clearly benefited from gene therapy treatment, and almost all treated patients have shown phenotypic correction of the disease for at least several months. However, the occurrence of severe adverse events such as leukemias in SCID-X1 patients (Hacein-Bey-Abina *et al.*, 2003) and myelodysplastic- like syndromes in CGD patients (M. Grez, personal communication) has shown the limitations of the current generation of vectors. Vector-related side effects can be significantly reduced by relatively modest changes in vector architecture (Montini *et al.*, 2006; Zychlinski *et al.*, 2008; Montini *et al.*, 2009). In this respect, several studies have shown that self-inactivating (SIN) vectors, which harbor a deletion of the promoter/enhancer elements in the LTR, do have a more favorable biosafety profile compared with conventional LTR-driven retroviral vectors (Modlich *et al.*, 2006; Montini *et al.*, 2006; Montini *et al.*, 2009). Self-inactivating retroviral vectors have been constructed on the basis of lentiviral (for example, human immunodeficiency virus-1, simian immunodeficiency virus) (Dull *et al.*, 1998; Negre and Cosset, 2002) and gamma-retroviral (MLV) backbones (Yu *et al.*, 1986; Schambach *et al.*, 2000; Schambach *et al.*, 2006a). Production of SIN

vectors is routinely accomplished by transient cotransfection of packaging and transfer vector plasmids into HEK293T cells (Dull *et al.*, 1998; Schambach *et al.*, 2009). Although transient production is more flexible and avoids the longer time required to generate stable vector-producing cell lines, stable producer clones still have theoretical advantages, such as higher reproducibility, predictability of viral vector output and the possibility of cell banking. Stable packaging cell lines are usually generated by transducing packaging cells with LTR-driven retroviral vectors, followed by a selection of the best producers. However, this procedure is not feasible for SIN vectors, as the transduction of a SIN vector would result in the inactivation of the 5'LTR promoter, which is necessary to generate vector genomic RNA used for packaging. Therefore, the SIN vector, including an intact 5'LTR with active promoter/enhancer elements, has to be transfected as a DNA plasmid into the packaging cells. However, this procedure results in unpredictable and variable titers.

Site-specific recombinases such as Cre and Flp have the capability to integrate foreign DNA sequences into the predefined loci. The RMCE (recombinase-mediated cassette exchange technology) (reviewed in Bode *et al.*, 2000 and Wirth *et al.*, 2007) has the potential to replace an integrated cassette, flanked by two hetero-specific/ noninteracting recognition sites (FRT) with a second cassette by homologous recombination at the FRT sites. RMCE-mediated targeted integration allows for the rapid generation of retroviral producer cells with high, reproducible and predictable titers. This technology was used previously to generate two packaging cell lines, Flp293A and 293FLEX, providing either amphotropic or

GALV-pseudotyped envelopes for the production of gamma-retroviral vectors (Coroadinha *et al.*, 2006; Schucht *et al.*, 2006). In this study, we describe the use of the RMCE-based tagging/targeting procedure (Verhoeyen *et al.*, 2001; Schucht *et al.*, 2006) to generate a PG13-based packaging cell line known as PG368 (Miller *et al.*, 1991), for the production of GALV-pseudotyped SIN gamma-retroviral vectors. We chose PG13 as the starting material as this line has successfully been used to establish clinical-grade retroviral vector producer clones for recent clinical trials (Gaspar *et al.*, 2004; Ott *et al.*, 2006). We established a tagged locus in PG13 cells under fully controlled conditions. This will allow the transfer of the PG368 packaging cells into a GMP process for production of gamma-retroviral vectors. Optimization of SIN vector architecture led to the selection of the most suitable design for SIN-vector production in PG368 cells after targeting the tagged locus. We used this system for generating stable producer clones for therapeutic vectors aimed at correcting CGD and X-linked severe combined immunodeficiency (SCID-X1).

2. MATERIAL AND METHODS

2.1. Cell culture.

293T (ATCC no. CRL-11268), HT1080 (ATCC no. CCL-121), PG13 (ATCC no. CRL10686), T-Fly A33 and PG368 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal calf serum (PAA, Pasching, Austria) at 37ºC in a humified atmosphere with 5% CO_2.

Cultures were propagated at logarithmic growth and were split at 70–80% confluency.

2.2. Plasmid constructs.

The retroviral SIN-vectors SIN11-SFgfp, SCS11-SFgfp, SRS11-SFgfp and SERS11-SFgfp have been described previously (Schambach *et al.*, 2006b). The retroviral SIN-vectors of the pES.1 series were similar to the previously described SFG-ECT2-luc vector (Bueler and Mulligan, 1996; Lindemann *et al.*, 1997) with the following modifications: (1) deletion of the ATG codon within the psi+ region, (2) an altered 5' promoter (CMVenhancer (-673/-53) with minimal MLV promoter (50/+1) hereafter known as cm-hybrid promoter) driving genomic RNA expression and (3) modification of the 3'LTR in which the DNA region around the TATA-box was modified to avoid initiation of transcription (details provided on request). pEMTAR-1 was derived from pEMTAR and contains the vector genome in reverse orientation relative to the neomycin gene. Furthermore, the ECMV IRES present in pEMTAR was replaced by the human PGK promoter (Freas-Lutz *et al.*, 1994). The pEMTAR-1.PGK.HS4.SIN11 contains a small insulator fragment) (Chung *et al.*, 1997) of the chicken β-globin gene, inserted between P2 and P3 promoters (Fig. 4B). The modification in pEMTAR-1.PGK.Tet11 was the replacement of the PGK promoter by a tet-regulatable promoter (Gossen and Bujard, 1992), and in pEMTAR-1.bi-SIN11, the PGK promoter was replaced by a CMV minimal promoter (-53/+75). The SIN gamma-retroviral vectors, SIN11(fes-gp91) and SIN11(EFS-gp91), were derived from

SERS11.SF.gp91.W31 by replacing the SFFV LTR by a 500-bp DNA fragment obtained from the human c-fes promoter and the intronless version of the elongation factor 1a promoter (REF), respectively. The SERS11(EFS-γc) viral vector has been described previously (Thornhill *et al.*, 2008) and was used for construction of the SIN11(EFS-γc) retroviral vector transferred by pEMTAR- 1 (introduction of vectors into pEMTAR or pEMTAR- 1 was done using standard molecular biology techniques).

2.3. Transient vector production.

TAGeGFP vector supernatants were generated by transient transfection on 293T cells with a DNA mixture consisting of 5 µg pHIT60 (Soneoka *et al.*, 1995), 5 µg pczVSV-G (Pietschmann *et al.*, 1999) and 5 µg of the tagging vector (Schucht *et al.*, 2006) using lipofection (TransIt 293 reagent, Mirus, Madison, WI, USA) as recommended by the supplier. Supernatants were titrated on HT1080 cells after one freeze/thaw cycle. Three to five serial dilutions were used to determine viral vector titer on the basis of eGFP-positive cells.

2.4. Generation of tagged packaging cells.

Transiently produced TAGeGFP vector supernatants were used to transduce PG3C11 cells at an MOI of 0.02. Transduced cells were selected in hygromycin-containing media (75µg/ml, Invitrogen) for 5–7 days. Approximately 100 clones were tested for vector production and the 20 best clones were selected for detailed analysis. Finally,

four clones (PG368, no. 21, no. 23, no. 69 and no. 86) were selected and tested for vector production by targeted exchange reaction.

2.5. Generation stable producer clones for SIN gamma-retroviral vectors by targeted exchange reaction.

PG368 producer clones were established by Flp-recombinase-mediated targeted exchange reaction after transient cotransfection of the targeting constructs, pEMTAR or pEMTAR-1. Briefly, $5x10^4$ PG368 cells were transfected with a DNA mixture consisting of 4 µg Flp-recombinase expression plasmid (Schucht *et al.*, 2006) and 1 µg of the pEMTAR- or pEMTAR-1 exchange plasmid. Twenty-four hours later transfection cells selection with G418 (400µg/ml) was started and individual clones were expanded under continued selection.

2.6. Titration of viral vectors.

All titrations of vector supernatants were performed on HT1080 cells by limiting dilution. Briefly, $2x10^5$ HT1080 cells were transferred into 6-well dishes the day before transduction and transduced using standard methods. Cells were harvested for fluorescence-activated cell sorting analysis on day 5. Titration of the therapeutic SIN-vectors, SIN11(fes-gp91) and SIN11(EFS-γc), was performed in a similar manner, but titers were determined by Q-PCR using WPRE (woodchuck hepatitis virus posttranscriptional regulatory element) sequences. Genomic DNA was prepared using the QIAmp DNA Blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primer/probe sets were designed by the

Primer Express software program (Applied Biosystems, Darmstadt, Germany). The 5'-oligo (PRE-1f) was 5'-AGCACACCACGCCACGTT, the 3'-oligo (PRE-1r) was 5'-CCTTGTATAAATCCTGGTTGCTGTCT and the probe (PRE-1p) was 5'-FAM-ACGGACAACGGGCCACAACTCCTCT-TAMRA. For reference, the primer/probe set for detection of human erythropoietin (Hepo) was used. The 5'-oligo (Hepo-f) was 5'-CTGCTGCCAGCTTTGGAGTACACTA, the 3-oligo (Hepo-r) was 5'-GAGATGCCAGAGTCAGATACCACAA and the probe (Hepo-p) was VIC-5'-ACCCCAGCTCCCAGCTCTTGCGT-3'-TAMRA. The DCT values were determined by comparing Hepo with WPRE signals in the transduced Ht1080 cells. Titers were calculated by normalizing the DCT values in the transduced cells to those of a HT1080 control clone containing a single copy of the WPRE element.

2.7. Northern analysis of total RNA.

Total RNA was isolated using the acidic phenol method (Chomczynski and Sacchi, 1987) and subjected to non-radioactive northern analysis according to a previously published method (Low and Rausch, 1994), except that CPD-Star (Tropix, Bedford, MA, USA) was used as substrate for chemiluminescence detection. Rat GAPDH (glyceraldehyde 3-phosphate dehydrogenase) served as internal standard. Probes were biotin labeled during PCR amplification. Detection of the relative mRNA steady-state levels was achieved after exposure of the gel to X-Ray film (Kodak Bio-Max light, Sigma, Munich, Germany). RNA size markers were from Promega (Mannheim, Germany). Oligonucleotides used for hybridization were: for GAPDH,

sense: 5'-AATGCATCCTGCACCACCAAC, antisense: 5'-GCCATATTCATTGTCATACCAGG; for eGFP, sense: 5'-TTCAGCGTGTCCGGCGAG, antisense: 5'-TCCTCGATGTTGTGGCGG; for WPRE, sense: 5'-GGGCTGCAGGATcCGAGCATCTTACG, antisense: 5'-GATATCTCCGCAGGAAGATCTCGACGTCAGCT; for MLV gag/pol, sense: 5'-AGACGGCACCTTTAACCG, antisense: 5'-GGTGATGAGAACAGACTC. The densitometric analysis was carried out using the NIH-1.57 software (public domain). Signal intensity is expressed as mean density of identical blot areas.

3. RESULTS AND DISCUSSION

3.1. Establishment of a targetable PG13-based packaging cell line.

The experimental setup for establishing a targetable packaging cell line is shown schematically in Fig. 1A. In essence, PG13 packaging cells were transduced with the retroviral 'tagging' vector, TAGeGFP (Schucht *et al.*, 2006) at an MOI of 0.02 to ensure single copy integration. About 100 clones were obtained after hygromycin B selection. Transduced clones, termed 'PG368' hereafter, were tested for production of the tagging vector (Fig. 1B). Among the PG368 clones, four generated infectious particles in the range of $\approx 1x10^6$ i.p./ml, namely clone nos 21, 23, 69 and 86. All four clones did not survive G418 selection, indicating that at the 'tagged' loci no activation of the neomycin resistance gene had occurred by transcriptional readthrough.

These four PG368 clones were subsequently targeted with pEMTAR.MFGeGFP (Schucht *et al.*, 2006), a plasmid containing the Fn and F5 recombination sites (Schlake and Bode, 1994) and an LTR driven MFG-based retroviral vector (Riviere *et al.*, 1995) with enhanced green fluorescent protein (eGFP) as a marker gene and an IRES-coupled 'atg' start codon downstream of the 3'LTR (Fig. 1A). Cells were cotransfected with a plasmid encoding for the Flp-recombinase to allow site-specific recombination at the FRT sites. Flp-mediated recombination resulted in the loss of hygromycin resistance and activation of the NeoR gene. Five targeted clones for each of the four selected PG368 clones were isolated after G418 selection and tested for vector production (Fig. 1C). The most consistent and robust titers were obtained from the PG368#23 packaging cell clone, with highly homogeneous titers differing only by twofold among the different clones. The mean titer for the PG368#23-derived producer clones was $2.3x10^6 \pm 7.6x10^5$ i.p./ml. A primary and secondary seed bank of the PG368#23 producer clone was generated and is referred to as the PG368 cell line hereafter.

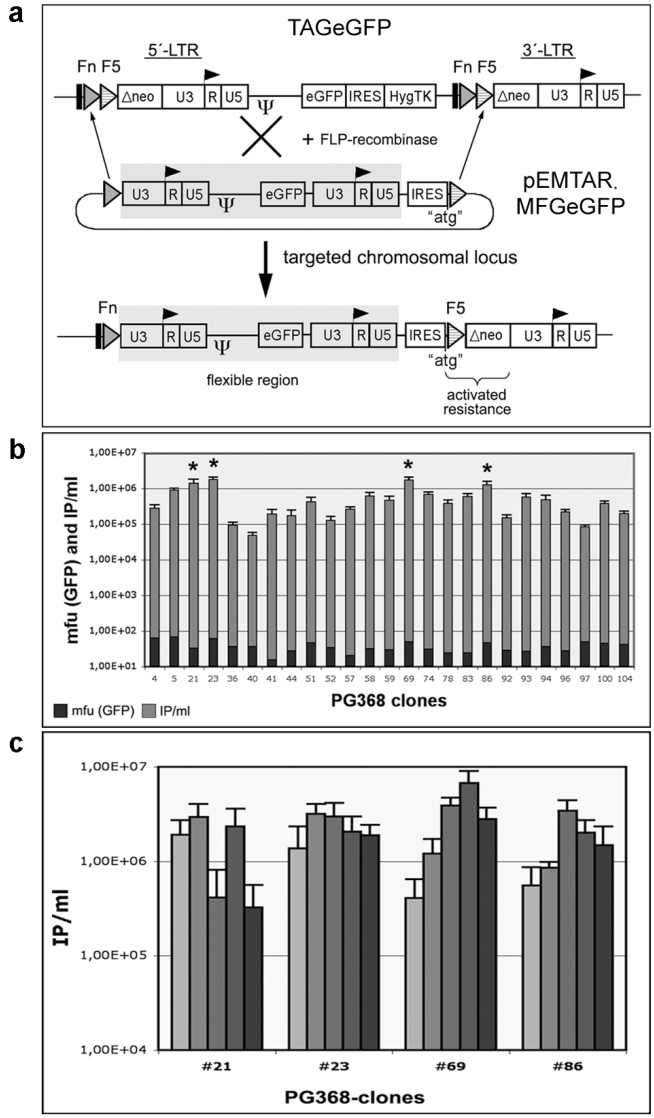


Figure 1. Generation and titers of selected PG368 packaging clones. (A) Schematic outline of the targeted exchange reaction. The TAGeGFP vector was transduced into PG13 packaging cells to 'mark' defined integration sites in the genome of the packaging cells. Clones were subsequently selected in hygromycin-containing media. These producer clones were named PG368. Thereafter, the plasmid pEMTAR.MFGeGFP was transfected into individual PG368 clones together with a plasmid coding for the FLP-recombinase. After targeted recombination at the FRT sites, the neomycin gene is activated, whereas the Hygromycin-TK hybrid

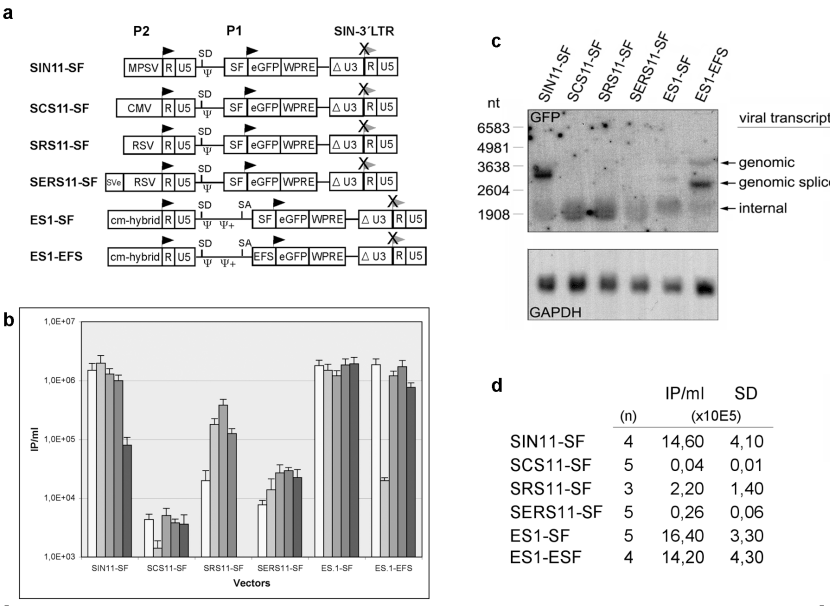
gene is eliminated from the tagged site. 'Flexible region' (gray boxed) indicates the variable sequence within pEMTAR into which viral vectors can be inserted. Fn/F5 represents the recombination sites (FRT sites) for the FLP-recombinase. Δneo represents an ATG-deleted neomycin gene. (B) Hygromycin-selected PG368 clones were tested for the expression of enhanced green fluorescent protein (eGFP) (mean fluorescence units, mfu, dark gray bars) and TAGeGFP vector production. The four clones with highest titers (*, i.p./ml) were used for further analysis. (C) Titers of selected PG368 clones after the introduction of the pEMTAR-MFGeGFP vector into the tagged site of selected PG368 clones by targeted exchange reaction are shown. The titers of five selected clones for each of the four previously selected PG368 clones are also shown. Error bars indicate s.d.; Neo: neomycin resistance gene; HygTK: hygromycin resistance gene/thymidine kinase fusion.

3.2. High-titer SIN-vector production in PG368 producer cells depends on promoter activity and vector cargo.

Next, we constructed a series of pEMTAR-based exchange plasmid vectors containing safety-improved SIN retroviral vector backbones as described by Schambach *et al*., (2006a, 2006b). These vectors termed 'SIN11-SF,' 'SCS11-SF,' 'SRS11-SF' and 'SERS11-SF' differ only in the enhancer/ promoter combination (P2) driving the expression of the viral genomic mRNA. All these vectors contained an internal SFFV-derived promoter (P1) driving the eGFP expression. In addition, we tested a second line of retroviral backbones, termed 'ES.1-SF' and 'ES.1–EFS,' which contained a CMV/MLV-hybrid promoter at the 5'LTR in addition to an enlarged packaging region (Armentano *et al.*, 1987; Adam and Miller, 1988) and either an internal SFFV promoter or the elongation factor short promoter driving the eGFP expression (Fig. 2A). Stable PG368 cell clones were generated as described above and titers were determined on HT1080 cells (Fig. 2B and D). Best vector producer clones were obtained with

SIN11-SF, ES1-SF and ES1-EFS (Fig. 2B). Analysis of genomic transcripts produced by selected packaging clones for each SIN-vector revealed abundant transcripts in SIN11-SF clones (Figure 2C). Interestingly, titers obtained from the SIN11-SF producer clones were similar to those of the ES1-SF and ES1-EFS clones, despite much lower amounts of genomic RNA in the latter two. This observation is suggestive of packaging constraints related to vector architecture and promoter interference as described previously (Schambach *et al.*, 2006b). The introduction of the same vectors into the two independent loci in HEK293-based modular Flp293A cells (Schucht *et al.*, 2006) and 293Flex cells (Coroadinha *et al.*, 2006) revealed highest titers for the SERS11-SF and the ES.1-SF constructs (Gama-Norton *et al.*, 2010). This may indicate that the generation of high-vector titers depends on P2-driven viral genome expression and on the influence of P1 on P2 (Schambach *et al.*, 2006b). In addition, the strength of P1 and P2 may depend on cellular context (for example, human vs mouse cells) adding another level of complexity to the system. Besides the genomic transcripts, transcripts initiated at P1 were evident in all producer cells and were most abundant in SCS11-SF and SRS11-SF producer cells (Fig. 2C).

In fact, this may indicate a strong negative interference for genome synthesis with particular promoter configurations and/or still unknown structural constraints and cellular context may influence SIN gamma-retroviral titers. In addition, a splice transcript was detected in ES1-SF and ES1-EFS producer cells, which was attributed to the presence of splice donor and acceptor site in the enlarged leader region used in these vectors.



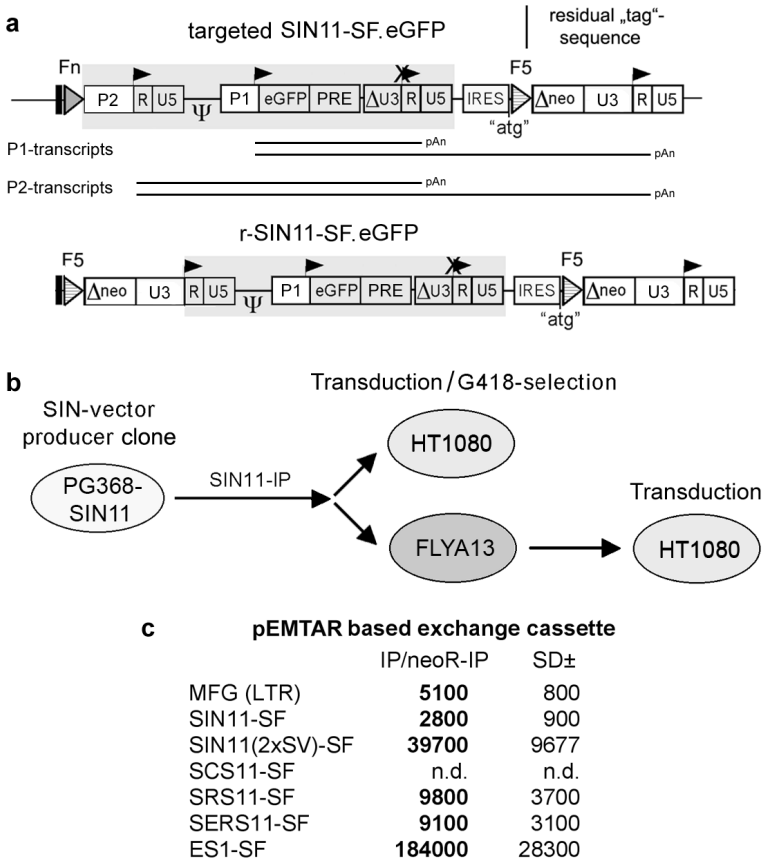
	(n)	IP/ml (x10E5)	SD
SIN11-SF	4	14,60	4,10
SCS11-SF	5	0,04	0,01
SRS11-SF	3	2,20	1,40
SERS11-SF	5	0,26	0,06
ES1-SF	5	16,40	3,30
ES1-ESF	4	14,20	4,30

Figure 2. Stable production of self-inactivating (SIN) vectors in PG368 packaging cells. (A) Schematic overview of the SIN vectors introduced by targeted recombination into the PG368#23 cell clone. (B) Titration of vector supernatants obtained from PG368#23 clones after targeted exchange reaction. (C) Northern blot analysis of one individual PG368 producer clone for each of the viral vectors. The blot was probed against eGFP. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (D) Vector titers were obtained from stable PG368 producer clones. HT1080 cells were used for titration by limiting dilution. (n) indicates the numbers of independent clones used for mean titer calculations. P2, 5' promoter driving the expression of the viral genomic RNA; P1, internal promoter driving transgene expression; SD/SA, splice donor /acceptor; SF, viral SFFV-U3 promoter; cm-hybrid, CMV-enhancer fused to the minimal MLV core promoter; EFS, elongation factor 1a promoter short; eGFP, enhanced green fluorescent protein; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

3.3. pEMTAR-mediated exchange leads to generation of NeoR-transfer vectors containing the U3-region of the tagging vector.

As *bona fide* insertions into the TAGeGFP-tagged chromosomal loci by pEMTAR-derived plasmids results in the activation of the neomycin resistant gene by readthrough mechanisms from transcripts starting at P1 and/ or P2 (Fig. 3A), we asked whether transcripts containing the neomycin resistance gene could be co-packaged with the transfer vector. To test this possibility, we used vector supernatants produced by different PG368 producer clones after FLP-mediated recombination for the transduction of HT1080 cells (Fig. 3B). Transduced cells were cultured in the presence of G418, and neomycin-resistant colonies were counted 10–14 days thereafter. As shown in Fig. 3C, all producer clones generated infectious vector particles containing the neomycin resistance gene. The number of neomycin resistant colonies varied among the different vector supernatants and was the highest in HT1080 cells transduced with vector supernatants obtained from PG368/SIN11-SF producer cells (1 Neo-transfer vector particle per 2800 infectious particles). Interestingly, the inclusion of a termination enhancer (2xSV40 USE, 2xSV) (Schambach *et al.*, 2007) within the viral 3'LTR reduced the number of Neo-transfer vector particles (NTVP). An even greater reduction in the NTVP numbers was seen in supernatants obtained from PG368/ES1-SF producers, although the reason for this remains unclear. The generation of neomycin-resistant colonies from readthrough transcripts starting at P2 potentially could lead to the

restoration of the LTR U3 function (Fig. 3A). Therefore, we used supernatants from PG368/SIN11-SF producers to transduce the packaging cell line FLYA13. After G418 selection, the supernatants from NeoR FLYA13 cells were used to transduce HT1080 cells. We found that two out of three neomycin-resistant FLYA13 clones generated neomycin-resistant colonies in transduced HT1080 cells proving that the configuration of the pEMTAR target exchange plasmid leads with high frequency to the inclusion of a fully functional LTR in the reverse transcribed product (data not shown). In conclusion, these findings exclude the use of pEMTAR-based target exchange plasmids for the production of retroviral SIN-vectors suitable for clinical use.



c **pEMTAR based exchange cassette**

	IP/neoR-IP	SD±
MFG (LTR)	**5100**	800
SIN11-SF	**2800**	900
SIN11(2xSV)-SF	**39700**	9677
SCS11-SF	n.d.	n.d.
SRS11-SF	**9800**	3700
SERS11-SF	**9100**	3100
ES1-SF	**184000**	28300

Figure 3. pEMTAR-based targeted exchange reaction leads to the coproduction of vector particles containing the neomycin gene. (A) Schematic view of an integrated pEMTAR.SIN11-SF.eGFP construct into the tagged site of PG368#23. Transcripts generated from P1 and P2 promoters are shown below. In addition, a possible structure for the recombinant provirus (r-SIN11-SF.eGFP), containing a full-length, functional 5'LTR and the neomycin resistance gene is indicated. (B) Diagram of the procedure used to test for the transmission of neo containing vector particles after pEMTAR-mediated targeted exchange reaction. Viral supernatant (SIN11-IP) was harvested from a self-inactivating (SIN)-vector producer cell (for example, PG368/SIN11 (SF-eGFP) and used for the transduction of HT1080 cells. After transduction, HT1080 cells were cultured in the presence of 200 mg G418 *per* ml for 14 days. The emergence of G418-resistant colonies indicates the transmission of the NeoR gene into the target cells. The same viral supernatant was used to transduce the packaging cell line, FLYA13. The supernatant of G418- resistant FLYA13 clones was subsequently used to

transduce HT1080 cells. The appearance of NeoR-HT1080 cells indicates the repair of the SIN-LTR. (C) Frequency of NeoR-transfer vectors generated after pEMTAR-mediated targeted exchange recombination. Numbers represent the number of total infectious particles (IPs) *per* neo-resistant colony detected in HT1080 cells after 14 days culture in the presence of G418. Each vector supernatant was tested in duplicate.

3.4. Redesign of the pEMTAR-targeting construct overcomes transfer of the neomycin resistance gene and restoration of U3 function.

To circumvent the problems associated with pEMTAR, we developed a bidirectional design for the targeting construct in which the viral vector was placed in antisense orientation to the neocassette at the tagged locus. Furthermore, the IRES element in pEMTAR was replaced by the constitutive human PGK promoter (P3) rendering activation of the neomycin resistance independent from the expression of the viral genome. This new targeting construct was named pEMTAR-1 (Fig. 4A). We used pEMTAR-1 to introduce the SIN11-SF vector into PG368 cells. Vector containing supernatants from NeoR cells were tested for the cotransfer of NTVP. No neomycin-resistant colonies were obtained after transduction of HT1080 cells with $1.2x10^7$ SIN11-SF vector particles. We believe that the number of infectious particles tested is sufficiently high to exclude the generation of NTVP also in large-scale productions, as we saw more than a four-log reduction in the transmission of neomycin resistance by pEMTAR-1.SIN11-SF supernatants in comparison with pEMTAR.SIN11-SF supernatants. Taken together, redesign of the targeting construct improved safety; however, at the expense of a 2–

3-fold reduction in the virus titer (Fig. 4C). Next, we asked whether further modifications in pEMTAR-1 would lead to increased vector titers, as the cluster of the LTR and housekeeping promoters in pEMTAR-1 might result in negative promoter interference at P2 and consequently in low titers (Fig. 4A).

To approach this, we considered the promoter interaction between the internal P1 and the external P2 promoter of the viral vector not to be relevant, as type, spacing and orientation of the two promoters were identical in pEMTAR and pEMTAR-1. Therefore, we focused our attention on the interaction between P2 and P3, as P2 is relevant for the production of packageable genomic RNA. Several strategies were tested to avoid promoter interference between P2 and P3. First, a chicken HS4 insulator sequence element was included between P2 and P3 as the HS4 insulator has been shown to have enhancer blocking activity and to shield promoters from adjacent enhancers (Chung *et al.*, 1997). Second, we replaced P2 by a tet-inducible promoter, and finally, we replaced P3 by a CMV-minimal promoter thereby transforming P2 into a bidirectional promoter (Fig.4B). All producer clones derived from modified pEMTAR-1 backbones produced virus titers in the range of 5–8x10^5 i.p./ml (except the construct containing the HS4 insulator element), and thus slightly lower than the ones obtained after pEMTAR-mediated recombination (Fig. 4C). Again, northern blot of total RNA from these clones showed lower genomic RNA abundance for pEMTAR-1 constructs compared with pEMTAR, in agreement with the observed titer differences (Fig. 4D).

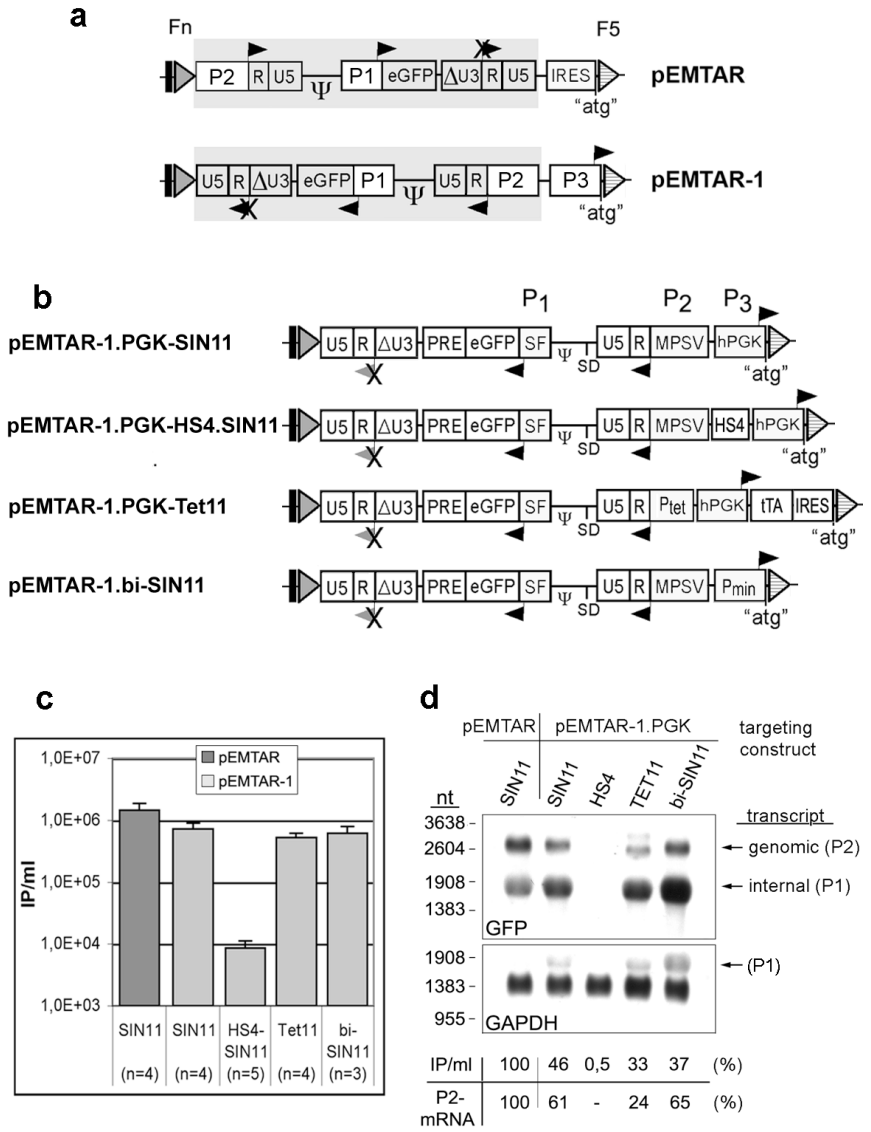


Figure 4. Design of pEMTAR-1 and titers of stable producer cells. (A) Schematic comparison between pEMTAR and the new redesigned targeting construct, pEMTAR-1. In pEMTAR-1, the orientation of viral vector genome was inverted, and the IRES element was replaced by the constitutive PGK promoter (P3). The transcriptional start sites are indicated by arrows. (B) Diagram outlining the modifications done on the pEMTAR-1-targeting construct carrying the SIN11-SF gamma-retroviral vector to overcome putative negative interference between P2 and P3

promoters. For structural details, see text and the 'Materials and Methods' section. (C) Titers generated from PG368 producer cells into which the SIN11-SF SIN gamma-retroviral vector was introduced either by pEMTAR or pEMTAR-1 after targeted exchange reaction are shown. (D) Northern blot analysis of PG368 producer clones, that have been targeted with SIN11-SF vector by either the pEMTAR- or pEMTAR-1-targeting construct. The table below compares the relative abundance of the P2- expressed genomic transcript and the titers observed for each producer clone analyzed. The titer and the genomic transcript level of pEMTAR-SIN11 are set to 100%. For detection of genomic and internal transcripts, the blot was probed with enhanced green fluorescent protein (eGFP). For normalization of RNA input, the blot was reprobed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

3.5. Generation of stable PG368 packaging cells for clinically relevant gamma-retroviral vectors.

Having defined an improved targeting system, we evaluated this system in the context of clinically relevant therapeutic vectors. For this purpose, two therapeutic vectors aimed at the correction of CGD (Moreno-Carranza *et al.*, 2009) and X-linked severe combined immunodeficiency (Thornhill *et al.*, 2008) were inserted into pEMTAR-1 and targeted into PG368 packaging cells. The internal P1 promoter in the SIN11- SF vector was replaced by either the EFS promoter or the c-fes promoter (M. Grez, unpublished data), and eGFP was replaced by either a codon-optimized gp91phox cDNA (Moreno-Carranza *et al.*, 2009) or the wt IL2Rγc-chain cDNA (Thornhill *et al.*, 2008) (Fig. 5A). The titer of the best PG368 producer clones for SIN11(fes-gp91) and SIN11(EFS-γc) was similar and ranged between $1.0x10^5$ and $4.1x10^5$ i.p./ml, which is ≈ 5- to 20-fold lower than the titer detected for SIN11-SF ($2x10^6$ i.p./ml) after pEMTAR-mediated targeted exchange reaction (Fig. 5B). Titer reduction was not caused

by low levels of packageable genomic RNA, as no correlation was found between the amount of vector genomic RNA found in the producer cells and the estimated titers (Fig. 5C). For example, the amount of genomic RNA found in PG368/SIN11(fes-gp91) was two-fold higher than that of PG368/SIN11(SF-eGFP), whereas the titer generated by PG368/SIN11(fes-gp91) was at least fivefold lower than that of PG368/SIN11(SF-eGFP). Clearly, the promoter configuration or other vector cargo features seem to influence titer, for example, by promoter interference, mRNA stability or accessibility of the packaging signals to the retroviral nucleocapsid proteins. Interestingly, the highest amount of vector genomic RNA was found in the PG368-PGK.SIN11(fes-gp91) producer clone. The vector backbone in this construct includes a myeloid-specific promoter at P1 (c-fes), which is not active in murine fibroblasts. One may speculate that the presence of a weak promoter at P1 may allow for maximal expression from P2 at the targeted locus, although this did not lead to a concomitant increase in viral titers. In summary, both therapeutic vectors could be produced at reasonable titers (1–4x10^5 i.p./ml) and after concentration, pre-loading these supernatants should be useful for the transduction of hematopoietic stem cells in the context of clinical trials.

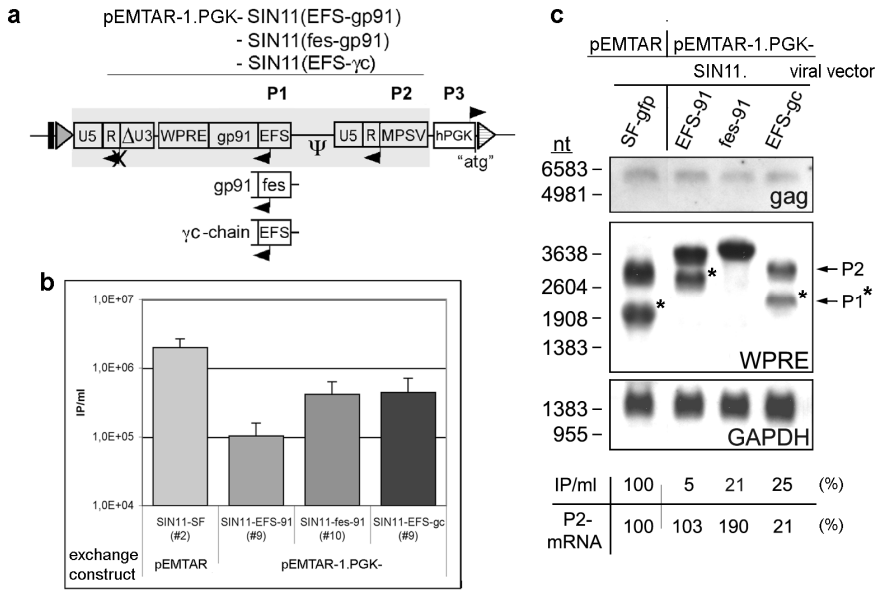


Figure 5. pEMTAR-1 enables vector production in a clinically relevant setting. (A) Outline of the therapeutic viral vectors inserted into pEMTAR 1. In all pEMTAR-1 vectors, P2 was derived from the MPSV LTR and P3 contains the human PGK promoter. The self-inactivating (SIN) gamma-retroviral vector contained in pEMTAR-1.PGK.SIN11(EFS-gp91) includes the human elongation factor 1α short promoter driving the expression of a codon optimized gp91phox cDNA, whereas in pEMTAR-1.PGK.SIN11(fes-gp91), the EFS promoter was exchanged with a 500-bp DNA fragment derived from the promoter region of the human c-fes gene. In pEMTAR 1.PGK.SIN11(EFS-γc), the elongation factor short (EFS) promoter was used to drive the expression of the IL2R- γc cDNA. (B) Titers of the best PG368 producer clones after pEMTAR-1- mediated targeted exchange reaction. Titers (infectious particles -i.p. *per* ml) were determined on HT1080 cells by fluorescence-activated cell sorting (FACS) and/or quantitative PCR (qPCR). (C) Northern blot analysis of PG368 producer clones that have been targeted with different SIN11-vectors by either the pEMTAR- or pEMTAR-1-targeting construct. The vector variants, as shown in panel a differ by the P1 promoters introduced for transgene expression. As a hybridization probe, a DNA fragment derived from woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was used to detect specific transcripts, originating from P1 (asterisk) and P2 of the vectors. For normalization of the mRNA steady state, the blot was reprobed against MLV gag/pol and glyceraldehyde 3-

phosphate dehydrogenase (GAPDH). Numbers at the left indicate molecular weight markers. The table below compares the relative abundance of genomic RNA started at P2 and the titers observed for each producer clone analyzed. The titer and the genomic transcript level of pEMTAR-SIN11-SF are set to 100%.

4. CONCLUSION

The RMCE technology, which allows the introduction of a vector genome at a predefined chromosomal locus, makes vector production more predictive and noteworthy and greatly reduces the time necessary to define the best producer clone for a particular vector. In this study, we designed a bidirectional exchange construct (pEMTAR-1), which greatly improved the safety of vector production by elimination of the readthrough- based selection of targeted producer clones and now allows the use of the targeted exchange technology together with the tagged PG368 packaging cells to establish stable producer clones for clinically relevant vectors. Moreover, as the tagging and selection process of the PG368 cells was carried out under highly controlled conditions, this cell line can be readily transferred into a GMP process for the production of such vectors.

Our results identified two major constraints for efficient SIN-vector production: (1) The external P2 promoter, which provides the genomic transcript of the vector. This problem was solved by the use of a highly active P2 promoter (MPSV-U3 promoter), providing large quantities of vector genomes in PG368. (2) Effects of the vector cargo also affect viral titers, possibly through promoter interference, mRNA stability and/or accessibility of packaging signals. In retroviral SIN

vectors, concerns related to the presence of an extended packaging region (Riviere *et al.*, 1995; Schambach *et al.*, 2006b) are less eminent than in LTR vectors, because the SIN design reduces the risk of the formation of replication-competent retrovirus and the extended packaging region is not contained in the therapeutic transcript initiated by the internal promoter. Thus, further vector developments should address the issue of efficient packaging of vector genomes regardless of their promoter and cargo configurations.

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CHAPTER IV

LENTIVIRAL VECTOR PRODUCTION FROM DEFINED CHROMOSOMAL LOCI

PART A

Lentiviral production is influenced by chromosomal integration of the vector and SV40 large T-Antigen in HEK293 cells.

This Chapter is adapted from the paper

Gama-Norton L., Herrmann S., Alves P.M., Hauser H., Wirth D. Lentiviral production is influenced by chromosomal integration of the vector and SV40 large T-Antigen in HEK293 cells. *Submitted to Molecular Therapy*.

ABSTRACT

Currently, lentiviral vectors for research and gene therapy are produced from 293-T cells that are loaded with vector and helper functions. However, transiently transfected vectors as well as the presence of SV40 virus large T-antigen (T-Ag) cause serious technical and safety considerations. We aimed at the exploitation of single chromosomal locus of HEK293 cells supporting lentiviral vector production. We found that lentiviral vectors commonly used in transient production protocols result in only little infectious particle production from single copy integrants in HEK293. Moreover, once this cell line harbours an integrated single copy of lentiviral genome, it becomes impaired to transiently produce lentiviral vectors. T-Ag has a dramatic effect on virus production. Low levels of constitutive T-Ag expression can overcome the production restriction imposed by integrated lentiviral vectors copies. Interestingly, T-Ag does not exert its role at the level of transcriptional activity of the vector, rather it seems to impose an indirect effect on the cell, thereby rendering it capable for lentiviral vector production. Together, our studies highlight the restrictions and requirements for integrated lentiviral vectors that are relevant for the establishment of stable and safe producer cell lines.

CONTENTS

1. INTRODUCTION

The capacity of lentiviral vectors to efficiently deliver genetic cargo to proliferating and non-proliferating cells rendered HIV-based vectors the tool of choice for stable genetic modification of many cell types including terminally differentiated cells (Naldini *et al.*, 1996a; Naldini *et al.*, 1996b) and HSC (Uchida *et al.*, 1998; Case *et al.*, 1999; Miyoshi *et al.*, 1999). Meanwhile, they have been also employed in clinical applications (Levine *et al.*, 2006; Cartier *et al.*, 2009; Sommer *et al.*, 2009). The state –of-art technology for lentiviral vector production is still based on transient transfection of 293-T cells, a SV40 T-antigen transfected HEK293 cell line. In these production systems both the helper plasmids (encoding the structural and enzymatic components Gag-Pol, accessory protein Rev and an envelope gene, commonly VSV-G) as well as the lentiviral vector are transfected by means of $CaPO_4$ precipitation. Virus production takes place upon transcription of DNA molecules present in the cell in an episomal *i.e.*, non integrated state (Sinn *et al.*, 2005; Cockrell and Kafri, 2007). Despite the high lentiviral titres achieved (in the range of $1x10^7$ infectious particles-i.p./ml), transient production systems hardly fulfil the requirements of safety and reproducibility, indispensable for their consolidation in clinical settings. For more than 20 years, development of stable production systems has been subject of intense research. Due to the cytotoxic/cytostatic properties of helper proteins such as protease, Rev and VSV-G envelope, one of the major obstacles to the successful generation of a stable packaging cell line is the need for establishment regulatory networks that allow to

accurately adjust protein expression. Exploitation of novel gene switcher systems (Broussau *et al.*, 2008) or strategies that circumvent or reduce the need for gene regulation (Ikeda *et al.*, 2003) resulted in the generation of helper cell lines with reasonable stability and titer. Still, a stable cellular system from which the production of the lentiviral vectors can be totally predicted and controlled is a milestone to be attained. Despite advances achieved towards establishment of lentiviral packaging cell lines, only little attention has been paid to the requirements of stable vector integration and strategies that would overcome lentiviral genome silencing which would result in reduction of viral titers upon cultivation of producer cells.

Typically, for the generation of lentiviral producer cell lines, the vector containing the transgene is introduced in an already established packaging cell line. Accordingly, the helper cells are considered as master cell lines and can be exploited for the production of different vectors simply by transfer the lentiviral vector genome. For establishment of producer cells, vector DNA was either introduced upon a classical DNA transfection (Ikeda *et al.*, 2003) or by viral transduction (Kafri *et al.*, 1999; Klages *et al.*, 2000; Ikeda *et al.*, 2003; Ni *et al.*, 2005; Cockrell *et al.*, 2006). The last referred strategy is the method of choice for the generation of the majority of cell lines described to date. Viral transduction allows to integrate increased numbers of vector copies by employing very high titres and even successive infections rounds. However, this is only conceivable for replication competent vectors. Due to their U3 sequence deletion at the 3'LTR, stable production of safer self-inactivating (SIN)-derived vectors can only be performed by transfection of vector DNA in the

selected packaging cell line. Introducing a vector by transfection has been observed to be accompanied by silencing of full-length vector mRNA expression upon a certain number of passages. One reason for this observation might be the tandem pattern of genomic insertion (Garrick *et al.*, 1998). This so-called repeated-induced gene silencing phenomenon has been related to increase of chromatin condensation and methylation and can frequently result in variegated pattern of expression in a population of transfectants. A novel strategy for DNA transfection has been described leading to the introduction of hundreds of concatamerized vector copies in the cellular genome resulting in satisfactory titers for a certain period of time (Throm *et al.*, 2009). There is no information about the minimal number of vector integrants needed to achieve high titres upon DNA transfection or transduced viral genomes. It is well known that a critical parameter is the nature of the vector integration site which considerably contributes to the overall titer (Coroadinha *et al.*, 2006; Schucht *et al.*, 2006; Gama-Norton *et al*., 2010). Higher copy numbers might increase the probability for beneficial integration sites, however, with the expense of losing genetic stability.

Conceptually, safe *i.e.*, defined and genomically stable production systems for retroviral vectors (gamma- and lenti-) require a low copy number or, most beneficial, a single vector copy integrated into the producer cell genome. Only in this scenario the sequence of produced viral mRNA including deleterious readthrough transcripts can be predicted and the putative co-packaging of harmful cellular sequences fully anticipated. In this respect, high titer production from a single copy gamma-retroviral vector in modular packaging cell lines

gave proof of concept (Coroadinha *et al.*, 2005; Schucht *et al.*, 2006; Loew *et al.*, 2009).

In the work presented here, we intended to evaluate the feasibility of lentiviral vectors commonly used in transient production protocols to contribute to the generation of infectious viral particles upon integration in HEK293 cells. We give evidences that HEK293 cells as such do not support efficient lentiviral vector production when the vector is integrated at low copy number in the cellular genome. Intriguingly, this impairment is partially circumvented only upon prolonged expression of T-Ag, exerting its effects at a post-transcriptional level. This work represents a systematic study on lentiviral genome expression in HEK293 upon chromosomal integration - a perspective that has not been addressed so far.

2. MATERIAL AND METHODS

2.1. Lentiviral vectors.

All the vectors represented are derived from HIV-1 genome. Vector I represented in Fig.1A is derived from pRLL.cPPT.PGK.eGFP.PRE (kindly provided by L. Naldini, Milano, Italy). The vector II is a derivative of vector I, harboring a GFP gene driven by the 5'LTR and a RFP cassette downstream the PGK promoter. The GFP was cloned into the *EcoR*V site upstream of the cPPT sequence. Vector III is a Tat-dependent non-SIN lentiviral vector derived from pHR'-CMVLacZ plasmid (kindly provided by Trono D., Lausanne, Switzerland). The vector IV is a targeting lentiviral vector used for RMCE, based on pRLL.cPPT.PGK.eGFP.PRE. Briefly, the lentiviral

cassette was cloned in pEMTAR vector (Verhoeyen *et al.*, 2001) in order to create a targetable lentiviral vector. It contains F-WT site and an F-5 site flanking the viral genome. An EMCV-IRES elements and an ATG start codon are positioned upstream to the F-5 site to complement the ATG-deficient neomycin-phosphotransferase expression upon targeting.

Sequences or maps as well as additional features and cloning details of the vectors are available upon request.

2.2. Mammalian cell culture.

HEK293-derived 1B2 and 293-3 clones were previously described elsewhere (Schucht *et al*., 2006; Coroadinha *et al*., 2006, respectively). Briefly they contain a single targetable site flanked by two heterospecific FRT sites and an ATG-deficient neo gene at 3' end. The chromosomal location of each tagging locus is described elsewhere (Gama-Norton *et al*., 2010).

HEK293 cells (BioReliance), 293-T (ATCC # CRL-11268) and all the derived lentiviral tagged clones were cultivated at 37°C in a humidified atmosphere with 5% CO_2 in DMEM (Sigma, Germany) with 10% fetal calf serum (Cytogen, Germany), 2 mM L-glutamine, penicillin (10U/ml) and streptomycin sulfate (100 µg/ ml). Selection was performed in medium supplemented with hygromycin B (300 U/ml) or G418 (1500 µg/ml). In all the HEK293-derived clones harboring SV40 T-Ag gene, cultivation was performed with DMEM supplemented with Dox (2µg/ml). NIH3T3 cells (ATCC CRL-1658) were

used to titer the lentiviral vector particles with cultivation conditions similar to the ones described above.

2.3. Lentiviral tagging.

DNA transfection

Plasmidic transfer was performed by conventional electroporation protocol or a nucleofection-based method as described in Nehlsen *et al*. (2009). HEK293 cells were electroporated using the GenePulser® electroporator (BioRad, Germany). For this purpose $1x10^6$ cells were transfected with 2.6 µg of the tagging lentiviral vector. For nucleofection based method, 2 µg of lentiviral plasmid DNA were used, using Nucleofector® Kit V (Amaxa®,Lonza, Germany). Individual cell clones expressing eGFP were isolated and expanded.

Viral transduction

Lentiviral tagging vectors were generated upon transient transfection of 293-T following a standard protocol. Briefly, 293T cells were transfected with vector III, together with packaging plasmids pLP1, pLP2, pLP/VSV-G (all from Invitrogen) and pCMV-TAT, using the calcium phosphate precipitation method. Upon filtration (45µm) and supplementation with polybrene (8µg/ml, Sigma, Germany), the supernatant was used to infect HEK293 and 293-T cells at m.o.i. of 0,01. Forty-eight hours after infection, single transduced clones expressing GFP were isolated by flow cytometry.

2.4. Viral vector production.

HEK293 and 293-T derived cell clones with integrated lentiviral genome were transiently transfected with plasmids encoding the packaging functions, pLP1, pLP2 and pLP/VSV-G (Invitrogen) using calcium phosphate precipitation standard method. In order to monitor the overall capacity of these clones to produce lentiviral vectors from episomal copies, the packaging helper functions were co-transfected with a lentiviral genome coding vector in an independent experiment.

2.5. Flow cytometry.

Flow cytometric analysis was performed on a FACSCalibur® (Becton Dickinson). For titration of GFP expressing virus, the cells were washed, trypsinized and stained with propidium iodide (50 µg/ml) to exclude dead cells.

Single cell sorting was performed on a FACSVantage SE® (Becton Dickinson).

2.6. Recombinase mediated cassette exchange.

For site-specific cassette exchange, HEK293-derived tagged clones 1B2 and 293-3 were co-transfected with Flp recombinase expressing vector (pFlpe) and the targeting vector, using GenePORTER® 2 transfection reagent (Genlantis, USA), following a protocol described elsewhere (Gama-Norton *et al*., 2010). Correctly targeted clones were isolated upon selection in G418 and ganciclovir

containing medium for 14 days. G418 and ganciclovir resistant single clones were evaluated for correct targeting by PCR or Southern blot.

2.7. Random Integration of T-Ag encoding plasmid.

pRITA (May *et al*., 2004) encoding the SV40 T-Ag was stably integrated into the genome of HEK293-derived cells upon co-transfection with a plasmid encoding hygromycin as a selectable marker (ratio 10:1) using calcium phosphate precipitation method. Selection started forty-eight hours after transfection and was carried out for about 14 days, after which individual colonies were picked, expanded and confirmed for SV40 T-Ag integration by PCR.

2.8. Real-time PCR.

For the relative quantification of SV40 T-Ag and lentiviral packaging sequence (psi) containing transcripts, human β-actin mRNA was used for normalization and to allow a comparison between experiments.

Total mRNA was isolated using the RNeasy Kit® (Qiagen, Germany) with a DNAse digestion to remove genomic DNA from the sample, accordingly to the instructions from the supplier. Synthesis of first-strand cDNA templates from total RNA was generated using the Ready-To-Go® You-Prime First-Strand Beads (GE Healthcare, Germany) with oligo dT primers, following the manufacturer's instructions.

Relative real time (RT)-PCR was performed using QuantiTect SYBR Green® PCR Kit (Qiagen, Germany). Briefly, for each sample, 10 µl of SYBR Green master mix was added to 1µl of a 10mM forward

and reverse primer. Diluted cDNA sample and RNase-free water were combined to a final volume of 20µl. For each sample duplicates or triplicates were performed and a no-template reaction was included as negative control. Standard conditions were used for the PCR reaction (15 min at 95ºC, then 60 cycles of 95ºC 15 sec, 58ºC 20 sec and 72ºC 30 sec, followed by a cooling step). RT-PCR was performed using the 96-well LightCycler® 480 Real-Time PCR System (Roche, Germany). For detection of T-Ag gene, the primers used were as followed: T-Ag1 5'TAGTGGCTGGGCTGTTCTTT3' and T-Ag2 5'GGTGGGTTAAAGGAGCATGA3'; for detection of psi sequence in lentiviral genome, the primers used were as followed: LentiPsiFwd1 5'ATCTCTAGCAGTGGCG3' and LentiPsiRev1 5'CTCCCCCGCTTAATAC3'; for detection of the housekeeping gene human β-actin, the primers used were as followed: hActβ Fwd 5'TCTTCCCCTCCATCGTG3' and hActβ rev 5'TTCAGGGTGAGGATGCC3'.

All the bars in graphs of Fig. 1-8 represent average values obtained from at least two independent experiments. Error bars represent standard deviation (SD) associated to at least three measurements.

3. RESULTS

3.1. HEK293-derived clones tagged with lentiviral genome are impaired to produce infectious viral particles.

In order to tag a HEK293 chromosomal locus that supports high levels of lentiviral genome expression, we used three different strategies applying the lentiviral vectors described in Fig.1A-I, II and III. In standard production protocols, these vectors gave rise to high titers, above $1x10^6$ i.p./ml (Fig.1B). In the first approach, a conventional SIN-lentiviral vector encoding GFP (Fig.1A, vector I) was transfected into the cells and stable clones expressing high levels of GFP were identified. The expression of the reporter gene was supposed to identify chromosomal loci that support high level transcription, reflected by the activity of the internal promoter. Thus, in this strategy, high GFP expressing clones do not necessarily correspond to clones with high performance of the 5' LTR promoter which would be required for generation of high levels of lentiviral mRNA. Hence, we also employed a dual reporter gene screening vector, in which GFP was cloned downstream of the LTR followed by the internal PGK promoter driving RFP as a second reporter (Fig.1A, vector II). We anticipated that upon integration, the expression of GFP would allow to identify a chromosomal locus that supports high levels of 5'LTR promoter activity and concomitantly, sustain high levels of lentiviral genome expression.

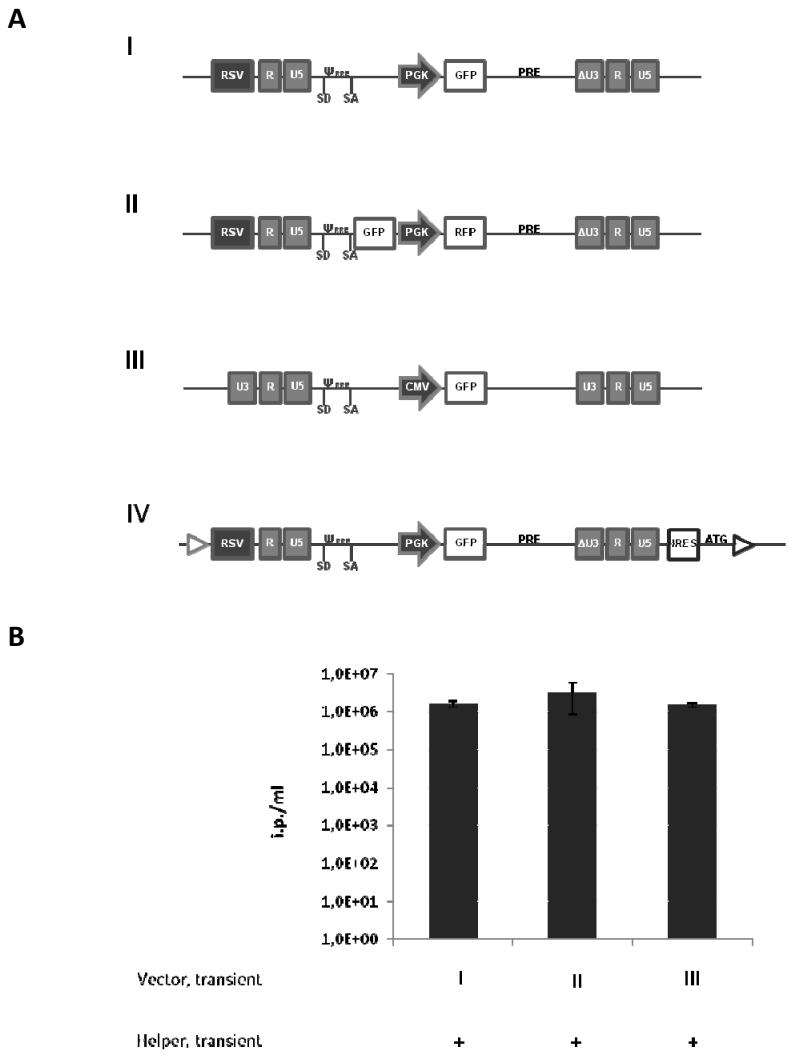

Figure 1. (A) Schematic representation of the lentiviral vectors used in this study. The vectors I and II are self-inactivating (SIN) lentiviral vectors. In I, the internal PGK promoter drives the expression of GFP reporter gene. In II the RSV promoter within the 5'LTR drives expression of GFP and an internal PGK promoter drives the RFP gene. Vector III is a Tat-dependent non-SIN lentiviral vector encoding GFP driven by an internal CMV promoter. Vector IV is a targeting vector, based on vector I. FRT-WT and FRT-5 sites are indicated by light and dark gray opened triangle, respectively. It contains the EMVC IRES element followed by ATG codon immediately located downstream the viral sequences. Dark gray filled

objects-Heterologous promoter sequences; light gray filled objects-HIV-1 derived LTR sequences; SD-major splice donor site; SA-splice acceptor site; ψ-packaging signal; RRE- rev responsive element; PRE- Hepatitis B virus post-transcriptional regulatory element. (B) Lentiviral vector production upon transient transfection of 293-T with indicated vectors. "Vector, transient", refers to transient transfection of lentiviral vectors; "Helper, transient", refers to transient transfection of HIV-1 gag-pol, HIV-1 rev and VSV-G envelope coding plasmids.

Both SIN-vectors were randomly integrated into HEK293 cells, following either a conventional electroporation protocol or a nucleofection-based method. These methods have been previously shown to provide a high percentage of single or low copy integration of different DNA cassettes (Nehlsen *et al.*, 2009). After isolation of clones on the basis of GFP expression strength (data not shown), their capacity to produce infectious particles was evaluated (Fig.2).

To this end, selected clones were transiently transfected with plasmids encoding HIV-1 derived gag-pol, rev and VSV-G envelope (in this figure and further figures as "Helper, transient"). Under these conditions, infectious viral particles are produced upon packaging of the full length lentiviral mRNA expressed from the lentiviral vector integrated into the genome of HEK293 cells (designated in the following figures as "Vector, integrated"). More than 40 independent clones were analysed.

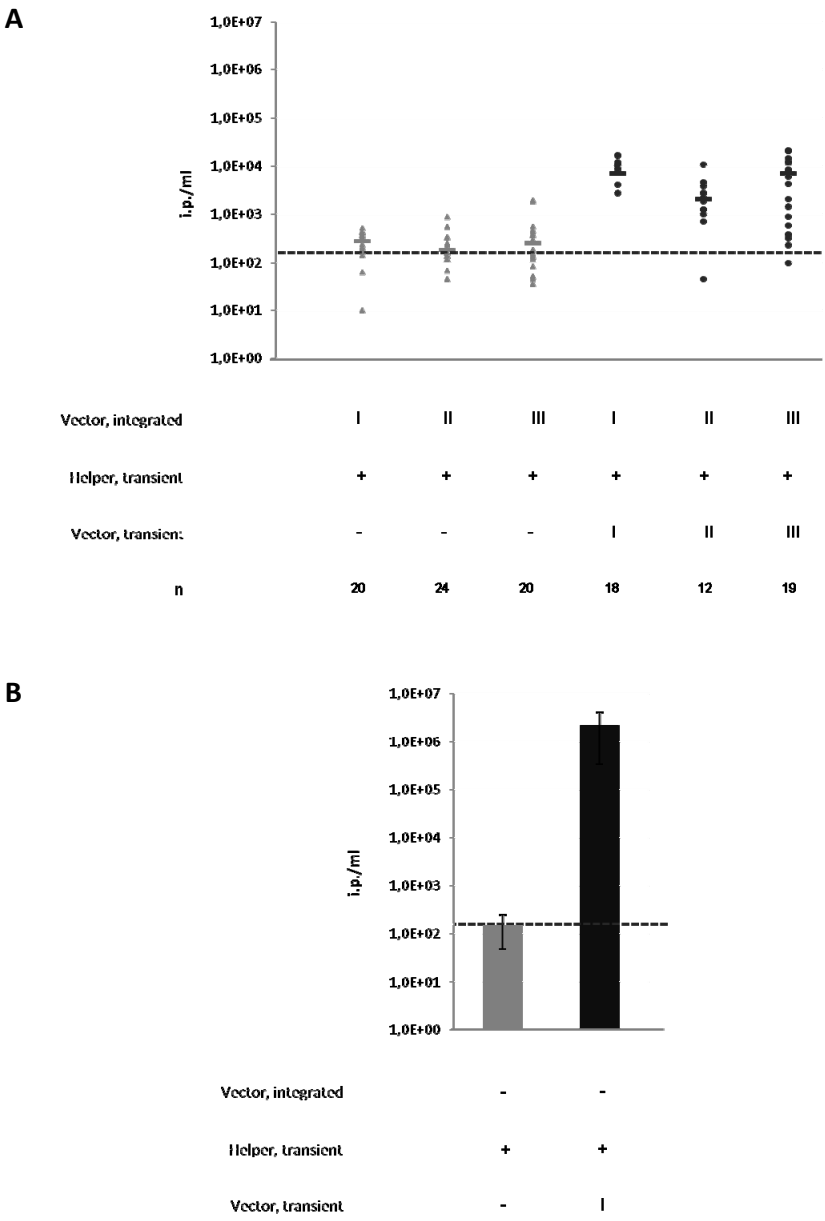

Figure 2. (A) HEK293-derived clones tagged with the indicated lentiviral vectors were analysed for their capacity to produce lentiviral infectious particles. Each filled light gray triangles refers to measurements from an independent cell clone. Filled dark gray circles refer to measurements associated to the same set of cell clones when additionally transfected with a lentiviral vector. The horizontal bars indicate the average of titer obtained from the different clones for each transfection group. "n"-

number of independent clones. Dotted horizontal line represents the confidence value of measurements which was defined to indicate infectious viral production. See also Fig. 1 for further explanations. (B) Transient lentiviral vector production from HEK293 cells. Vector I was co-transfected together with the helper functions as indicated. "Vector, integrated" refers to genomic integration of the indicated lentiviral vector in HEK293-derived cell clones; "Vector, transient" refers to transient transfection of the designated lentiviral vector.

Unexpectedly, none of the clones examined was able to produce lentiviral vectors above $1x10^3$ i.p./ml (Fig.2A, light gray triangles). Since plasmid transfection might impose a bias towards specific integration sites that are unfavourable with respect to viral vector production, we employed lentiviral transduction of screening vectors to tag 'natural' lentiviral integration sites. For this purpose, we utilized the Tat-dependent non-SIN lentiviral vector depicted in Fig. 1A, vector III. Upon infection with a moi of 0.01, GFP expressing clones were isolated with different levels of reporter gene expression (data not shown). Upon expansion, stable GFP expressing clones were analysed for lentiviral vector production. Again, among 20 clones with an integrated vector, none was able to produce relevant amounts of infectious lentiviral particles (Fig.2A).

Since none of the 64 clones tagged with the various vectors was able to produce lentiviral vectors above the background levels, we presumed that the potential of the cell to produce virus was compromised once the vector was integrated into the genome. We confirmed this assumption by transient co-transfection of a lentiviral vector in 49 of the clones (in this figure and followings as "Vector, transient"). We found that all these clones showed only low levels (< $1x10^4$ i.p./ml) of lentiviral vector production when the lentiviral

genome was additionally provided as an episome (Fig.2A, dark gray circles). This is about 100-fold less than the titer of wild type HEK293 cells.

3.2. Exploitation of well defined HEK293 genomic loci towards lentiviral vector expression.

We decided to evaluate the capacity of two well described HEK293 loci, which previously allowed to establish production systems for very high titers of gamma-retroviral vectors, to support lentiviral expression (1B2 and 293-3 in Schucht *et al.*, 2006; Coroadinha *et al.*, 2006, respectively). Of particular importance is the fact that these two tagged loci harbour two flanking heterospecific Flp recombinase target sites (FRTs) linked to a sensitive selection system (Verhoeyen *et al.*, 2001). This feature makes these two loci targetable with any cassette of interest that is flanked by the two respective FRT sites by Recombinase Mediated Cassette Exchange (RMCE). Taking advantage of this feature, we integrated the lentiviral vector represented in Fig.1A-IV into 1B2 and 293-3 loci of HEK293 cells as described in Fig.3A. Upon selection and molecular characterization of correctly targeted clones (data not shown), we evaluated their capacity to support infectious vector particle production. Lentiviral vector production was severely impaired both from the 1B2 locus (TAR293LV cell line, Fig.3B) and the 293-3 locus (not shown). Moreover the capacity of these clones to transiently produce lentiviral vectors was impaired if compared to HEK293 cells. This observation is in line with the results described above.

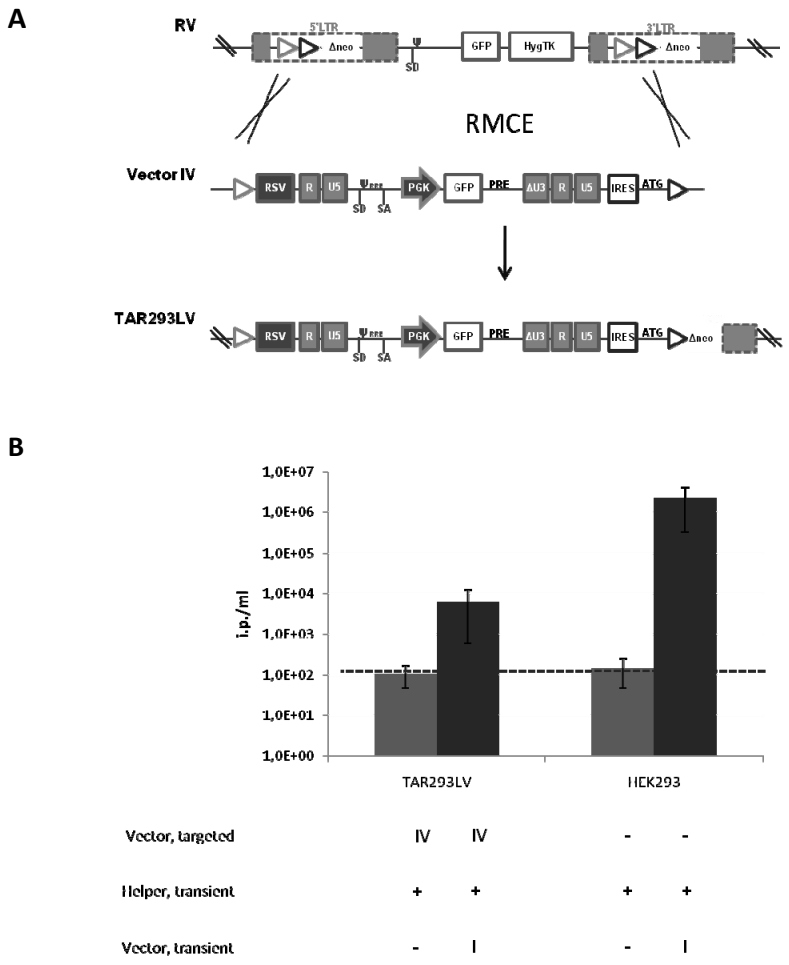

Figure 3. (A) To direct the lentiviral vector to a chromosomal site with high transcriptional activity, vector IV was targeted into tagged 1B2 cells (Schucht *et al*., 2006) via RMCE (for further details, please refer to Schucht *et al*., 2006; Gama-Norton *et al*., 2010). Upon cassette exchange reaction mediated by Flp-recombinase (stretched crosses), correctly targeted clones are G418 resistant. As a result, the lentiviral vector is integrated and the retroviral tagging cassette "RV" is excised as indicated (TAR293LV cell line). For further details, please consult legend of Fig. 1 and 2. (B) HEK293-derived clone with a targeted integration of the lentiviral vector represented in Fig.3A (TAR293LV) was analysed for its capacity to produce infectious particles. Production from integrated lentiviral copy and upon transfection of a viral vector is represented by light and dark gray bars, respectively. Transient lentiviral production in HEK293 is given as a reference.

3.3. In 293-T cells the cellular ability to produce lentiviral vectors upon chromosomal integration is improved.

The good performance of 293-T for transient vector production motivated us to evaluate the generation of lentiviral infectious particles upon stable vector integration in this cell system. In this respect, we tagged 293-T upon infection with replication competent HIV-1 derived vector (Fig.1A, vector III), according to the protocol described above. Transduced clones were identified on the basis of GFP reporter gene expression. Independent clones were analysed for vector production upon transient transfection of HIV-1 derived helper function and VSV-G envelope. The results obtained for 41 representative clones are shown in Fig.4. Overall, production of lentiviral vectors upon chromosomal integration in 293-T was increased about 10-fold when compared to corresponding HEK293 clones, as depicted in Fig. 2A and Fig. 3B. Importantly, 293-T derived clones were not impaired to produce lentiviral vectors when the viral genome was additionally provided as an episome, achieving titres up to $1x10^7$ i.p./ml which is comparable to virus production capacity of the parental cell line.

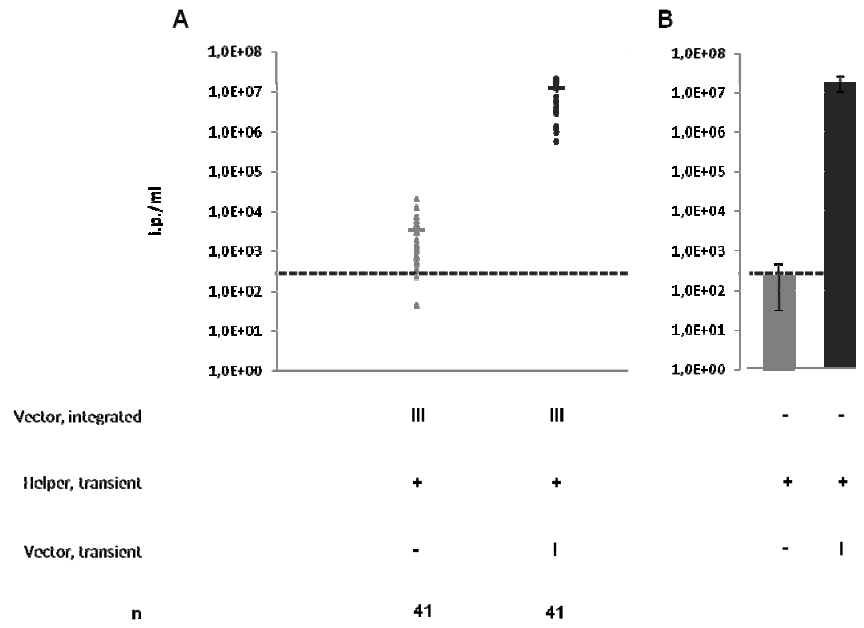

Figure 4. (A) Forty one independent cell clones generated upon infection of 293-T cells with the non-SIN vector III at m.o.i. 0,01 were evaluated for virus production. Filled light gray triangles refer to measurements of titer from integrated vector copies in independent clones. Filled dark gray circles refer to measurements associated to the same set of cell clones when additionally transfected with a lentiviral vector. The horizontal bars indicate the average of titer generated from the 41 clones for each transfection group. (B) As a control, lentiviral vector production from 293-T was assessed. For further details see Fig. 1 and 2.

3.4. Stable integration of SV40 large T-Ag improves lentiviral vector production from HEK293-derived cell lines.

To evaluate if T-Ag expression exerts a positive effect on the production of infectious lentiviral particles from single integrated HEK293 cell line, we complemented TAR293LV cells with SV40 T-Ag. The effect of T-Ag expression on lentiviral producing capacity of TAR293LV cells was firstly evaluated upon transient expression. For

this purpose, the plasmid encoding T-Ag was co-transfected with helper functions and production of viral particles was evaluated either from single copy integrated genome or from non-integrated episomal lentiviral genome (Fig.5).

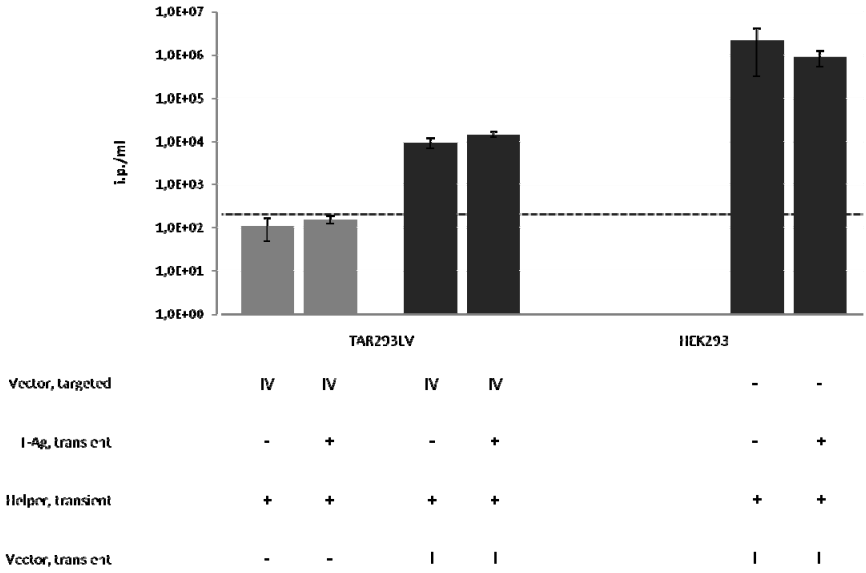

Figure 5. Effect of transient T-Ag expression on lentiviral vector production capacity from TAR293LV cell clone. TAR293LV cells were transiently transfected with SV40 T-Ag with and without additional lentiviral vector. The virus production capacity was assessed. For further details see Fig. 1 and 2.

The results showed that transient expression of T-Ag did not affect virus production in TAR293LV cells in both experimental settings. We thus hypothesised that long-term expression of T-Ag would be required to promote lentiviral vector production from a single copy at satisfactory levels. In this respect, stable transfection and continuous expression of SV40 large T-Ag was pursued. Upon

calcium phosphate precipitation of the T-Ag plasmid into TAR293LV cells, four single clones were arbitrarily selected and confirmed for integration of T-Ag gene by PCR (data not shown). These clones were designated as TAR293LV-TAg#1-4 and they were considered to be representative cell clones from the pool of T-Ag transfectants. We monitored the capacity of these clones to produce infectious particles (Fig.6).

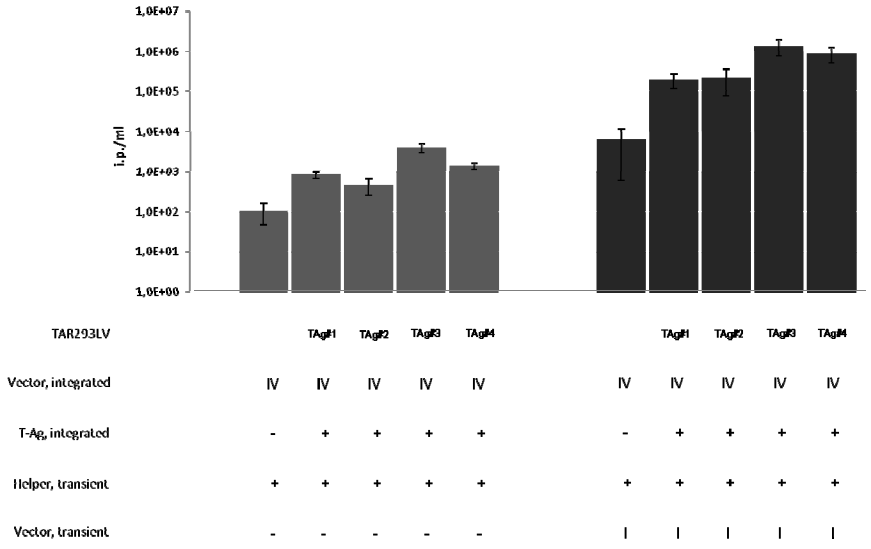

Figure 6. Effect of stable T-Ag expression on lentiviral vector production capacity from TAR293LV- derived cell clones. Four independent cells clones derived upon stable transfection of T-Ag in TAR293LV cells (TAR293LV-TAg#1-4) were analyzed for virus production. Additionally, the cells were transfected with a lentiviral vector as indicated. For further details see Fig. 1 and 2.

Importantly, upon integration of T-Ag gene in the genome of TAR293LV cell line, transient production of lentiviral vectors increased up to 200-fold when compared to the same experimental setting in absence of T-Ag integration. Noteworthy, even a 4 to 35-fold increase

of infectious particle production was monitored from integrated lentiviral genome in TAR293LV harboring T-Ag when compared to the parental cell line TAR293LV. This suggests that the impairment for lentiviral vector production from TAR293LV cell clone was partially overcome by integration of T-Ag gene.

3.5. Rescue of lentiviral vector production capacity is associated to low levels of T-Ag expression.

In order to attribute the observed phenomenon to expression of T-Ag gene and to evaluate the levels of expression that trigger such increment, a quantitative Real Time (qRT)-PCR was performed for two selected clones, TAR293LV-TAg#1 and TAR293LV-TAg#2. The results are shown in the Fig.7. Both clones expressed significantly lower levels of T-Ag if compared to 293-T cells. In clone TAR293LV-TAg#1, 4.2% of T-Ag expression was monitored, while TAR293LV-TAg#2 was related to a 40-fold lower level of T-Ag, corresponding to 0,1% of T-Ag in 293-T. Interestingly, despite the different levels of T-Ag expression, these two clones displayed similar and significantly increased capacity to produce lentiviral vectors from episomal templates (Fig.6). Moreover, upon expression of low levels of T-Ag in the clones TAR293LV-TAg#1 and #2 an up to 10-fold increase of lentiviral vector production from a single copy integrated vector was monitored (Fig.6).

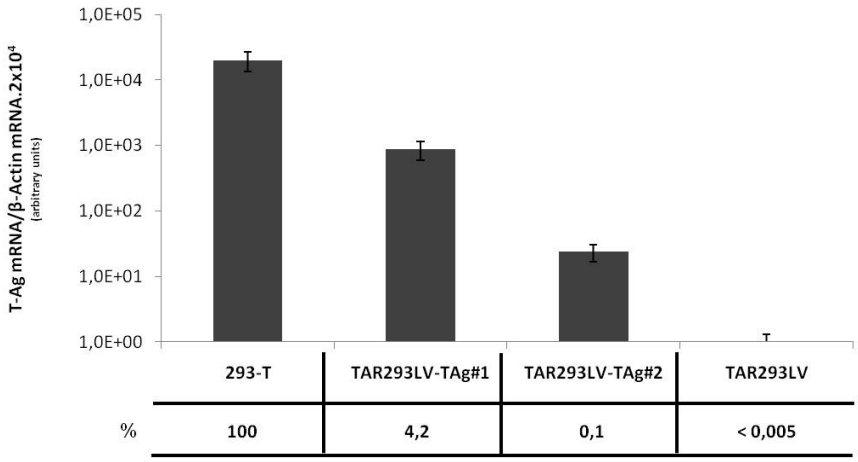

Figure 7. Relative quantification of stable T-Ag expression levels in two selected TAR293LV-derived clones. The level of T-Ag expression in clones TAR293LV-TAg#1 and #2 was analyzed by RT-PCR. The expression of T-Ag in these two clones was normalized against β-actin transcription. 293-T was included as a control for expression of T-Ag in a cell line with high transient lentiviral production capacity (set as 100%). TAR293LV was included as negative control and the value associated to it is given as the background value for T-Ag detection.

3.6. Restoration of infectious particle production in TAR293LV by T-Ag expression is not due to increased levels of lentiviral mRNA.

We evaluated if T-Ag has a direct effect on the amount of lentiviral mRNA produced in transformed cells. For this purpose, we relatively quantified the level of full length mRNA in TAR293LV cells expressing and not expressing T-Ag by qRT-PCR, using primers that specifically bind in the packaging signal (psi) present in the leader region of the lentiviral genome. The levels of psi encoding mRNAs in the different clones were compared to the mRNAs detected in control 293-T cells upon transient expression of the lentiviral vector. The

results are described in Fig.8. 293-T cells expressed the highest levels of psi containing mRNA. In contrast, in HEK293-derived cells with single copy vector integration, expression of full length mRNA was decreased about 2-3 orders of magnitude. This was observed also for the episomal state of the vectors in these cells. Interestingly, upon stable integration of T-Ag, psi-containing mRNA level was not significantly affected, despite the increased vector production capacity from these clones. These results suggest that the restriction of cell clones with single copy integration of lentiviral genome to produce infectious particles is not related to impairment of full length mRNA transcription.

These results indicate that T-Ag exerts a positive effect which acts downstream of the transcription of the viral mRNA. Our data suggest that this effect does not depend on high levels of T-Ag expression and is not directly related with an increase of packagable mRNA transcription.

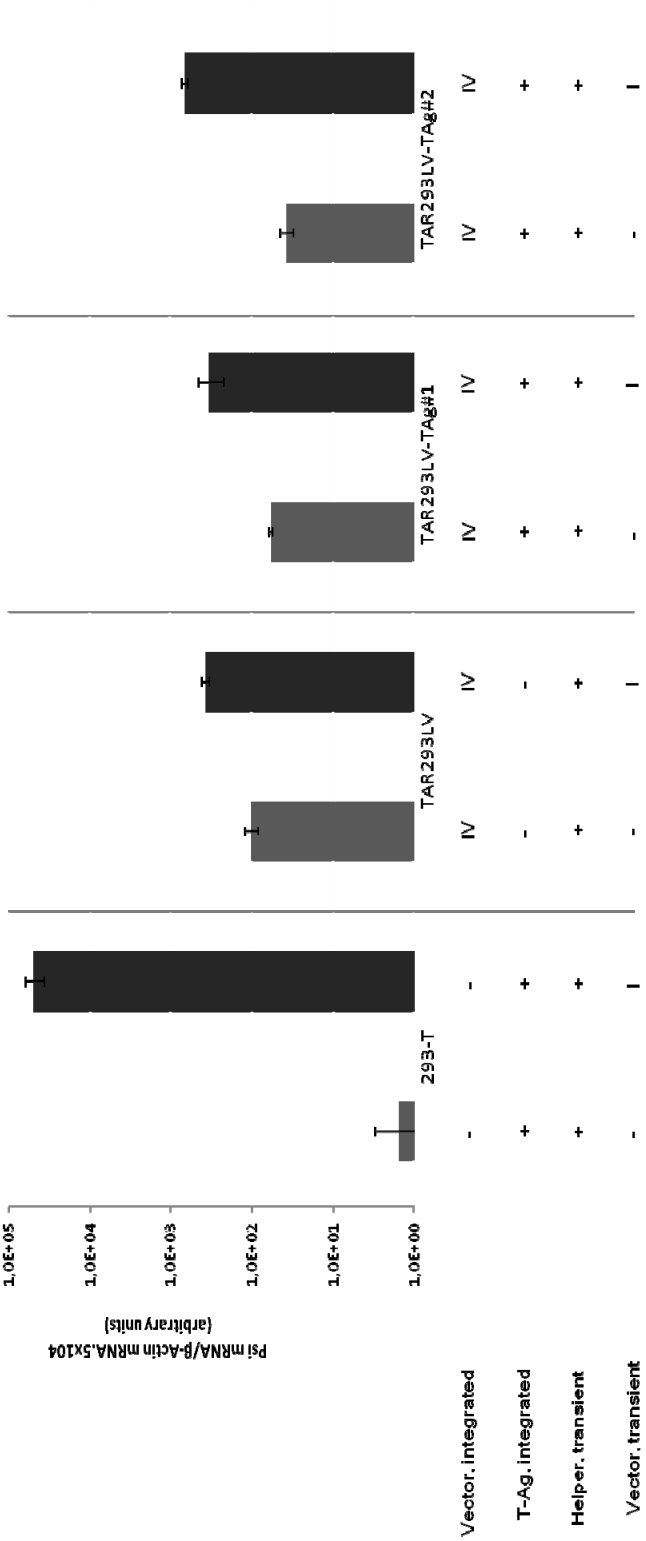

Figure 8. Effect of stable T-Ag expression on full length lentiviral transcript production in TAR293LV-derived clones. The relative production of full length lentiviral mRNA in TAR293LV-Tag#1-2 cells was determined by RT-PCR detecting the psi region. This level was normalized against β-actin transcription. As a control, the level of psi containing transcripts in 293-T transient production system was assessed.

4. DISCUSSION

Recently, important achievements have been accomplished towards generation of reliable lentiviral packaging cell lines. These systems pave the way to establish clinical relevant high titer producer cells upon integration of a therapeutic vector of interest into the cellular genome. In this respect, the integration of multiple vector copies is usually accompanied by a genetic instability and loss of titer. To meet the requirements for gene therapy applications, lentiviral production from a well characterized stable producer cell line would be mandatory. Integration of viral vectors into single, defined chromosomal locus has been previously exploited for generation of high titer gamma-retroviral producer cells (Coroadinha *et al.*, 2006; Schucht *et al.*, 2006). The modular nature of these cells allowed to evaluate and optimize vector designs in the specific loci (Gama-Norton *et al.,* 2010). We intended to identify chromosomal loci in HEK293 that support high levels of lentiviral vector genome transcription. This would be the prerequisite to the establishment of a stable, modular packaging cell line for lentiviral vector production.

In order to identify genomic loci in HEK293 cells supporting lentiviral vector production, we screened more than 100 independent clones, tagged with a HIV-1 derived lentiviral vector. Different DNA transducing methods were used (plasmid transfection as well transduction of lentiviral sequence) to exclude any bias with respect to integration sites favoured by individual methods. Although the reporter gene was readily expressed, none of the individual chromosomal sites in these clones was capable to support expression

of lentiviral packagable genome. In addition, we exploited two defined integration sites in HEK293 cells that have been previously shown to provide high transcriptional levels of integrated cassettes. These sites have been used for integration of retroviral SIN and non-SIN vectors, resulting in high virus titers upon complementation with the helper functions (Coroadinha *et al.*, 2006; Schucht *et al.*, 2006; Gama-Norton *et al*., 2010). These loci also support expression of non-viral cassettes, such as antibody and tet-regulated expressing cassettes (data not shown). Unexpectedly, upon integration of the lentiviral vector by RMCE we could not restore a reasonable titer although reporter gene expression was very high (data not shown). Together, the results clearly show that none of the loci tested was able to support production of lentiviral vectors from integrated viral genomes. Intriguingly, once a lentiviral vector is stably integrated, cells were severely impaired to produce infectious particles from episomal DNA molecules. It is not clear how the presence of an integrated lentiviral vector unable the production of lentiviral particles from episomal copies. However, the fact that all the clones tested showed comparable results excludes that the vector integration knocked out a crucial cellular factor. This suggests a general phenomenon behind the impairment for lentiviral production from a single copy integration site in HEK293 cells.

We evaluated if the above described phenomena is a result of uncharacterized feature of the vectors applied in this study. Thus, we tested an unrelated Tat dependent SIN- vector which was previously shown to give high titers upon transient transfection in 293-T cell line (Ikeda *et al.*, 2003). Also in this case, upon targeted integration of this

vector, the production of lentiviral vectors was impaired (data not shown). Moreover, we tested non HIV-1 derived vectors, namely a Tat-independent SIN simian immunodeficiency virus (SIV)-derived vector without rescuing viral infectious particles form single stable integrated copy (data not shown). This suggests that the impairment is not a specific feature of the HIV-1 based lentiviral vectors used but that it reflects a more general phenomenon.

Current protocols for lentiviral vector production are based on transient transfection of 293-T cells, a SV40 T-Ag transfected HEK293 cell line. The titres of lentiviral vectors associated to this method are high, typically ranging at $1x10^7$i.p./ml of a not concentrated supernatant. If compared to HEK293 cells, this corresponds to a 10-fold increase. The molecular reason for this difference has not been yet elucidated. SV40 derived T-Ag has been characterized as an oncoprotein that elicits cellular transformation. The role of T-Ag in regulating cellular gene expression through its actions on tumour suppressor genes pRb and p53 has been matter of intense research (reviewed in Ahuja *et al.*, 2005). Furthermore, T-Ag has been reported to be a promiscuous activator of many viral and other cellular promoters (Gilinger and Alwine, 1993; Rice and Cole, 1993). The pleiotropic effects of T-Ag expression account for the dramatic alterations in the cellular transcriptome. This is reflected by the deregulation of around 5% of cellular genes upon expression of T-Ag (May *et al.*, 2004). In addition, T-Ag is known to exert non-reversible effects as a result of increased genome instability, a consequence of impaired p53 function.

Here, we show that expression of T-Ag is associated with higher lentiviral vector titres both for single copy integrated vectors and for episomal state. The molecular reason for the differences in titers observed in HEK293 and 293-T cells is not clear. Recently, differential lentiviral mRNA processing has been described (McLaren and Cochrane, 2009).

It has also been suggested that one of the possible mechanisms of transcription activation by T-Ag could involve the chromosomal status of the DNA molecule (Rice and Cole, 1993), being T- antigen's activities in transcriptional activation directed, in some manner, towards episomal DNA. This hypothesis could explain, at least in part, the restitution of transient lentiviral vector production capacity upon T-Ag integration. Nevertheless, the moderate restoration of lentiviral vector production from a single copy indicates that other mechanisms then the above described are involved in the conversion of an impaired cellular system towards a productive cellular system. Moreover, the fact that in our system, transient expression of SV40 T-Ag did not provide any benefit suggests indirect effects associated to long term changes within the cell rather than mere expression of T-Ag as such.

The cause of a low-vector titre can occur at any step from the vector production/packaging of the RNA genome into the virus particle to the integration of the vector into the cellular genome of the target cell. We evaluated if sub-optimal transcriptional level of lentiviral genome is the limiting step that is behind the impairment of lentiviral vector production from a single copy in HEK293. Real-time PCR analysis showed that 293-T cells transiently transfected express a

higher relative value of psi containing transcripts than all the other cell clones analyzed. In contrast, TAR293LV clones displayed only low levels of psi containing transcripts (0,7 to 1,3% compared to 293-T). Importantly, this level was not significantly affected upon stable integration of T-Ag. This shows that the increase in titer as observed in T-Ag expressing TAR293LV cells is not due to an increase of full length mRNA transcripts. It would be interesting to evaluate if higher levels of T-Ag expression in these clones are related to an augmentation of lentiviral vector production capacity close to the levels observed for 293-T transient production and how would be this capacity related to the amounts of available psi containing transcripts in these cells.

The transient production of lentiviral vectors in cell lines impaired for virus particle production is alleviated upon expression of T-Ag, but T-Ag only has marginal effects on production of lentiviral vectors from single copy. The capacity of HEK293 expressing T-Ag or 293-T single integrated with a lentiviral genome is never higher then $1x10^4$ i.p./ml. This indicates that lentiviral vector production from single copy and the restriction of single integrated HEK293-derived cells to transiently produce viral vectors are uncoupled.

In summary, the reason why HEK293-derived cell clone with an integrated lentiviral cassette is compromised to produce infectious particles remains obscure. We anticipate that global effects are triggered by small amounts of T-Ag expression and are necessary to convert a non productive cell system towards a cell with capacity for lentiviral production. We hypothesize that pleiotropic effects are long term achieved and thus, 293-T cell line must have intrinsic proprieties

that are not present (or down regulated) in HEK293-derived cell line with an integrated lentiviral genome.

In order to improve lentiviral packaging systems, it would be crucial to reveal the mechanism of effect of T-Ag in overcoming the impairment of infectious particle production in HEK293-derived cell clones. This should allow to suggest non-oncogenic alternative strategies. We envision that GMP-based genetic manipulation of HEK293 cell towards functional replacement of T-Ag will render HEK293-derived cell line capable of producing lentiviral infectious particles from one copy integrated vector.

5. ACKNOWLEDGMENTS

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PART B

SUPPLEMENTARY DATA

In Part B, supplementary data on the putative restriction mechanisms for lentiviral vector production from single copy integrated vector in a HEK293-derived cell line is given. For this purpose, the TAR293LV cell line was chosen (for further details, please refer to Part A). Since the restrictions observed for virus production are not restricted to a specific clone, *i.e.*, integration site or related to a specific lentiviral vector, a general mechanism is anticipated that is independent from the vector integration site. Hence, it is assumed that the results obtained can be extrapolated for all the HEK293-derived clones with a single copy integration from which production of lentiviral vectors was shown to be impaired (Part A).

As Part B aims to the complementation of data presented and discussed in Part A, it is only composed by Results and Discussion sections, supplemented with additional Material and Methods and References.

CONTENTS

1. MATERIAL AND METHODS

This section refers to only material and methods that have not yet been described in Part A. For further information, please consult Part A of this Chapter.

1.1. Lentiviral vectors.

The dual reporter gene lentiviral vector (represented in Fig.3) is a targeting vector for Flp mediated RMCE, based on vector II of Fig.1A, Part A.

The lentiviral targetable vectors pRSV-SFFV, pMSCV-SFFV and pMSCV-PGK (represented in Fig.7) are derivatives of the vector IV, Fig.1, Part A.

1.2. Real-time PCR.

For detection of R sequence, the primers used were as followed: RT-LV-R-Fwd1 5'GTCTCTCTGGTTAGACC3' and RT-LV-R-Rev1 5'AAGCACTCAAGGCAAGC3'.

1.3. Northern Blot.

The Northern Blot was performed as described in standard protocols. Briefly: Total mRNA was isolated using the RNeasy Kit® (Qiagen, Germany) with a DNAse digestion to remove genomic DNA from the sample, accordingly to the instructions from the supplier. 5µg of each RNA sample were loaded on the denaturing 1.2% agarose gel. RNA size marker used was 0.5-10kb RNA ladder from Invitrogen (Germany). Probe against eGFP of 700bp length was generated by

restricting vector I, Fig.1 Part A with *Xma*I/*Age*I*I*. Generation of radioactively labeled DNA probe was performed using the RediPrime® kit (GE Healthcare, Germany). Phospho-screens were imaged after more than 6h exposition with a Storm 860 Phosphoimager (Molecular Dynamics).

1.4. Transfection of RSS encoding plasmids.

The standard protocol for generation of lentiviral vectors has been already described in Part A. For the analysis of the effect of Tat overexpression on viral production 1µg of pCMV-TAT was co-transfected along with the lentiviral helper functions (set as 1x). The amount of Tat encoding plasmid cotransfected was adjusted accordingly to the different concentrations tested (e.g., 0,1x corresponding to 0, 1 µg of DNA, *etc*…).

For the analysis of the effect of VP35 overexpression on viral production, 8µg of pHY16 (Kindly provided by Peter de Haan, Viruvation B.V., Leiden, The Netherlands) were co-transfected along with the lentiviral helper functions.

2. RESULTS

2.1. TAR293LV supports very high levels of reporter gene expression from targeted lentiviral vector.

As previously referred (Part A), the cell line TAR293LV is a well-characterized derivative of the HEK293, harboring a single copy of lentiviral vector, integrated in the previously tagged 1B2 locus (Schucht *et al*., 2006). In Part A, was shown that this clone is impaired to produce lentiviral vector particles at satisfactory levels. In order to give insights on the reasons for this observed phenomenon, molecular characterization of the integrated construct as well as evaluation of the expression level of the reporter gene present in the targeted lentiviral vector were performed and the results are described in this section.

The clone was confirmed to contain a single copy integration of the lentiviral vector in the defined locus by Southern blot and PCR (data not shown). Moreover, the integrity of the targeted sequence was confirmed upon sequencing and mutations and/or deletions could be excluded (data not shown).

The expression of the reporter gene from single copy integrated lentiviral vector was analyzed by flow cytometry. As illustrated by Fig. 1, the expression profile of GFP gene in the context of lentiviral targeted cells was extremely high.

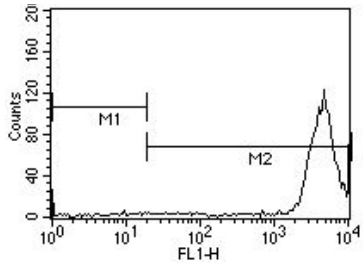


Figure 1. Reporter gene expression from TAR293LV cell clone analyzed by flow cytometry.

2.2. TAR293LV supports expression of a reporter gene driven by the 5'LTR promoter.

Once an expression cassette is integrated into the genome of a cell line, the performance of its regulatory elements (*i.e.*, promoters), is strongly dependent on positive/negative *cis*- effects that are mediated by the chromosomal sequences present in the vicinity of the integration site(s) of the heterologous construct. In this section we intended to evaluate how the impairment of lentiviral production of TAR293LV was related to the performance of the promoter sequence present in the 5'LTR. In this respect, the transcriptional activity of the 5'LTR was specifically considered.

The transcription of the SIN lentiviral vector targeted in TAR293LV cell line can render three different mRNA molecules, as represented in Fig.2. The full length packagable mRNA (in the figure as mRNA I) is transcribed from the heterologous promoter present in 5'LTR. Because the targeting vector retains the HIV-1 major splice donor signal and a splice acceptor signal (at 3' of the RRE sequence), it is expected that a proportion of full length mRNA is spliced (in the

figure as mRNA II). The internal promoter drives expression of a smaller transcript (mRNAIII). As only the unspliced full length mRNA contains the packaging signal as well as the 5' and 3'LTRs, is the only RNA molecule contributing to the generation of infectious viral particles.

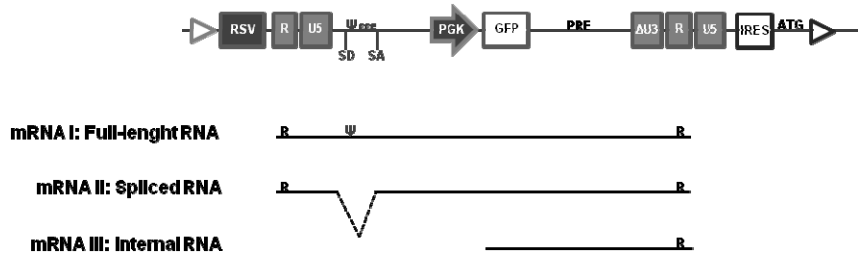


Figure 2. Different mRNA molecules transcribed from targeted SIN lentiviral vector in TAR293LV cell line. For further details of the vector, please consult legends of Fig. 3A, Part A.

Nevertheless, and as represented in Fig.2, all the different transcripts produced can contribute for the expression of GFP. In this respect, the expression profile of the targeted lentiviral cassette does not directly indicate activity of the 5'LTR promoter, which drives expression of the packagable mRNA, since GFP expression can be uncoupled from transcription of packagable lentiviral genome.

We have previously shown that 5' LTR promoter in the context of targeted lentiviral genome is active, since we were able to identify psi-containing transcripts by RT-PCR (please refer to relative quantification of psi containing transcripts described in Fig.8, Part A).

Nevertheless, the production of a reporter protein which expression is solely dependent on the activity of 5' LTR promoter was

not previously shown. In this respect, we modulated the previously targeted lentiviral vector towards a dual reporter gene targetable vector, as represented in Fig. 3A.

This vector contains a GFP gene driven by the 5'LTR and a RFP gene which expression is controlled by the PGK internal promoter (being this vector the targetable version of the dual reporter vector represented in Fig.1A vector II, Part A). The vector has a high capacity to produce lentiviral particles upon transient transfection in 293-T (data not shown), confirming a titer associated in the range of $1x10^6$ i.p./ml.

The dual reporter vector was targeted in the HEK293-derived 1B2 tagged clone, by means of RMCE. Molecular characterization of resistant clones was performed in order to confirm correct targeted integration of the lentiviral vector (data not shown). A correctly targeted clone was selected and subjected to further analysis. The activity of the 5' promoter was evaluated upon monitoring the expression of GFP. The heterologous promoter at the integrated 5'LTR was confirmed to be active and its transcriptional level high enough to produce the reporter protein (Fig.3B). These results reinforce the conclusion that impairment of lentiviral production is not related to an inactivity of the 5'LTR.

A

B

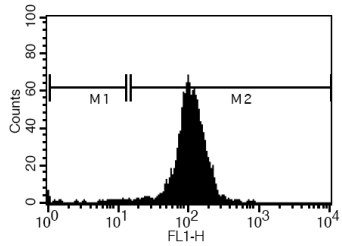


Figure 3. (A) Schematic representation of the targetable dual reporter lentiviral vector. For further details of the vector please consult Fig.1A vector II, Part A. (B) GFP expression from targeted dual reporter lentiviral vector represented in (A) analyzed by flow cytometry. GFP expression is driven by the RSV promoter presented in 5'LTR.

2.3. Unbalanced production of full length and internal lentiviral transcripts is observed in TAR293LV.

The results above described indicate that the promoter that drives expression of packable mRNA transcript is active. Nevertheless, the fact that infectious viral particles could not be produced from targeted cell lines might suggest that activity of 5'LTR promoter is sub-optimal. As SIN vectors contain an internal promoter (assuring expression of the transgene in infected cells), we hypothesized that reciprocal interference between the regulatory sequences present in the lentiviral targeted vector could account to sub-optimal levels of full length genome transcription. In order to evaluate the relative

activity of the two promoters, we next quantified the abundance of full length transcript in relation to the internal transcript of the targeted lentiviral vector by performing a quantitative RT-PCR.

To this end, the packagable mRNA was detected by using primers that bind specifically to the lentiviral packaging signal (psi). The exclusive detection of the internal transcript was not possible to be performed due to the inexistence of sequences that are only presented in this transcript (Fig.2). Therefore, we opted to use primers directed to the R region, localized in the lentiviral 5' and 3' LTR sequences. The relative abundance of R-containing transcripts and full length transcripts in TAR293LV was compared to 293-T. Lentiviral vector production in 293-T was defined as being related to equal production of R and psi transcripts, *i.e.*, a ratio of 1:1. The results obtained, presented in Fig.4, indicate an unbalanced ratio between R containing transcripts and full length transcripts in TAR293LV when compared to what is observed for the 293-T control. This suggests that in TAR293LV, the production of internal transcript is favoured over the transcription of the full length packagable transcript.

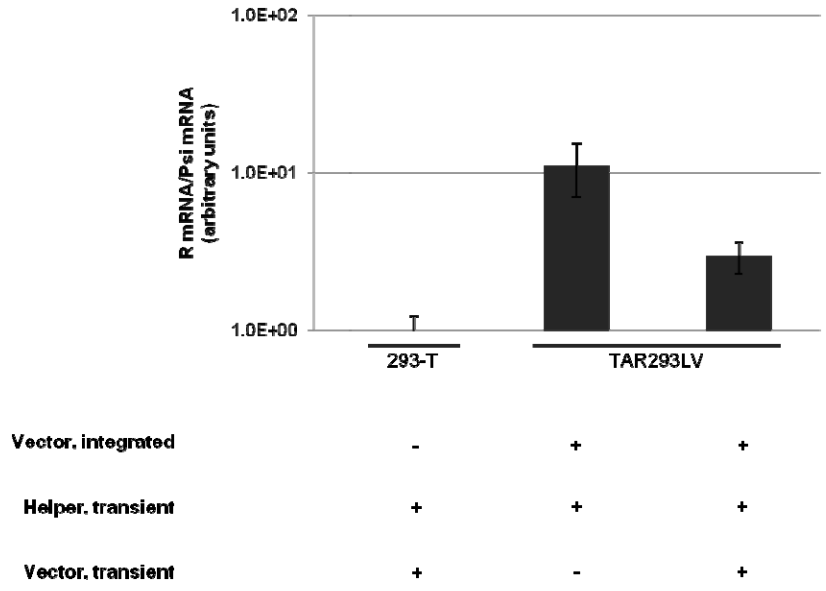


Figure 4. Relative quantification of R- and psi-containing lentiviral transcripts in TAR293LV cell clone. The expression of R-mRNA was normalized against transcription levels of packagable lentiviral mRNA (psi-mRNA). 293-T was included as a control, as a cell line with high transient lentiviral production capacity (ratio between the R and Psi-containing mRNA set as 1:1).

2.4. Lentiviral transcript splicing is not the major cause for TAR293LV vector production incapacity.

The results from the RT-PCR indicated that the amount of psi-containing transcripts is decreased in relation to R containing transcripts in HEK293-derived cell lines with a single lentiviral vector integrated. In this respect, it could not be excluded that un-characterized splicing events account for the reduction of packagable constructs in single targeted cell line. In order to qualitatively characterize the nature of the transcripts produced by TAR293LV cell line, a Northern blot analysis was performed (Fig.5).

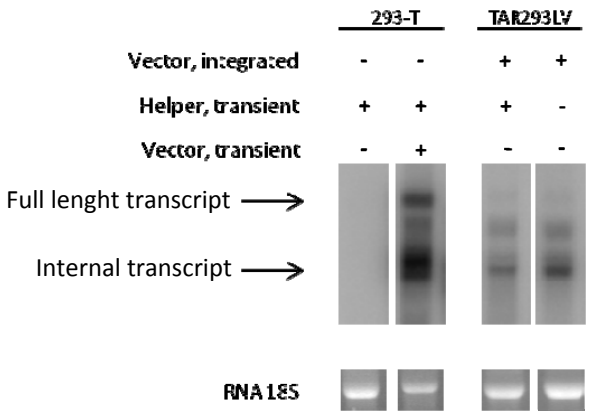


Figure 5. Northern blot analysis of lentiviral transcripts produced in TAR293LV. RNA detection from 293-T transiently transfected with lentiviral genome was included as control. For detection of the different species of mRNA transcribed from lentiviral genome, the blot was probed for GFP. Ribosomal RNA 18S is presented as loading control in ethidium bromide-stained gel. Upper and lower arrows indicate packagable and internal transcripts, respectively.

The Northern blot analysis confirmed the results obtained by RT-PCR. In TAR293LV, there is a marked upregulation of the internal transcript over the full length transcript. Importantly, the same pattern of transcripts is observed for 293-T and TAR293LV, indicating that the major cause of impairment of lentiviral vector production from TAR293LV is not related to uncharacterized and unexpected splicing events specifically occurring in this cell line.

Although uncharacterized splicing events do not occur in lentiviral targeted cell line, the Northern blot indicates the presence of a transcript smaller than the packagable lentiviral mRNA, most possibly related to a spliced transcript. We speculated that this transcript must be the result of the expected splicing event that occurs between the major splice donor and splice acceptor site as

represented in Fig.2. We next hypothesized that the amount of full-length transcripts in TAR293LV cell line would augment if splicing was impaired. In this respect, the HIV-1 major splice donor site was mutated by replacing the core sequence TGGT for TGCA in the lentiviral targetable vector depicted in Fig.2. After confirming that the mutated vector was not impaired for transient vector production in 293-T (data not shown), it was targeted in 1B2 locus following the above described protocol. A representative HEK293-derived clone targeted with a splice donor-mutated sequence was selected and further analysed in respect to its capacity to sustain lentiviral vector production. The results presented in Fig.6 indicate that the mutation of splice donor site did not contribute to an increase of lentiviral vector production from single copy integrated cell line.

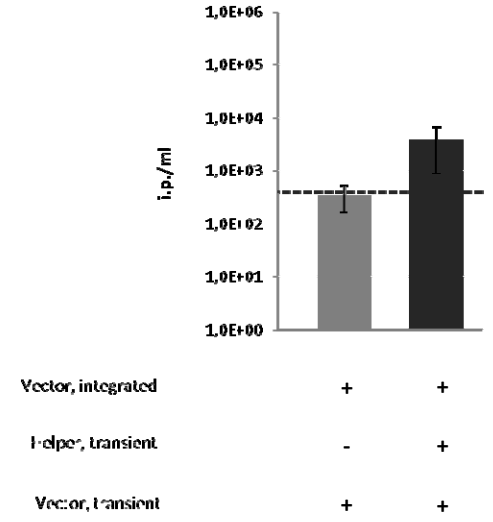


Figure 6. HEK293-derived clone with a targeted integration of the lentiviral vector containing a mutation in SD site was analysed for its capacity to produce infectious particles. Production from integrated lentiviral copy and upon transfection of a viral vector is represented by light and dark gray bars, respectively.

2.5. Altering promoter composition of the viral vector does not significantly improve production from single copy.

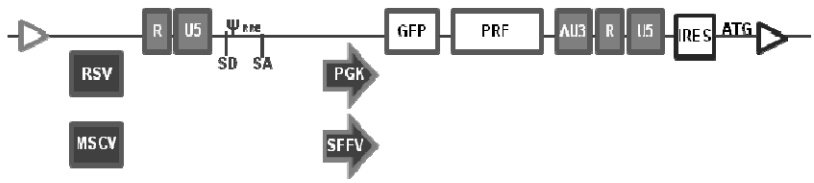
The RT-PCR and the Northern Blot results presented above (Fig.4 and 5, respectively), along with the quantification of psi-containing transcripts by RT-PCR in Part A (Fig.8), indicate that although expressed, the production of full length transcript is unfavoured in relation to the expression of the internal promoter. This fact suggests, as already referred, that promoter interference occurring between the two regulatory sequences present in the targeted lentiviral vector could account to the observed phenomenon.

We next evaluated if such inefficient full length transcription rate could be overcome by a more favourable combination of promoter content present in the targetable lentiviral vector. To this end, we designed a panel of different targetable lentiviral vectors with different promoter combinations (Fig. 7A). As internal regulatory elements we used the human PGK promoter and the spleen focus forming virus (SFFV) promoter, the latter being described as being related to high SIN gamma-retroviral titers when present as internal promoter in single targeted vector in 1B2 locus (Gama-Norton L. *et al*., 2010). For the promoter present in 5' LTR and driving the full length mRNA transcription, we evaluated, beside the RSV promoter, the Murine Stem Cell Virus (MSCV) U3 sequence. Previous studies showing that the 1B2 locus supports high levels of gamma-retroviral titers, when the viral genome is transcribed from this gamma-

retroviral promoter (unpublished data) motivated us to evaluate its performance in the context of lentiviral vector.

In order the address the capacity of these vectors to generate infectious lentiviral particles, 293-T cells were transiently co-transfected with each of these vectors and the packaging functions. The results shown in Fig. 7B indicate that not all the promoter combinations created are related to high transient production of lentiviral particles.

A



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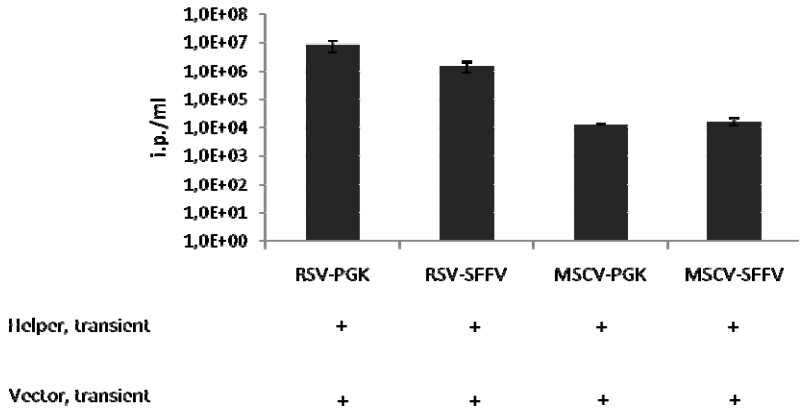

Figure 7. (A) Schematic representation of the different targetable lentiviral vectors differing at 5'LTR and internal promoter composition.(B) The capacity of the vectors to generate lentiviral infectious particles was evaluated upon transient transfection in 293-T. Bars indicate the results obtained for at least three independent measurements.

We postulated that the chromosomal surroundings in the vicinity of the targetable site of TAR293LV could modulate the performance of each vector, once integrated in the cellular genome. Therefore, we targeted the newly designed lentiviral vectors in HEK293-derived 1B2 clone, to generate (TAR293LV)-RSV-SFFV; (TAR293LV)-MSCV-SFFV and (TAR293LV)-MSCV-PGK. Upon identification and molecular characterization of correctly targeted clones (data not shown), their potential to express the reporter gene was evaluated. The FACS profile represented in Fig.8A shows that expression of GFP was intrinsically dependent on the vector content of the analysed targeted vectors, evidencing that promoter interference plays indeed a role in the transcription activity of the regulatory elements present in the targeted lentiviral vector. In this respect, the vector composed by the PGK as internal promoter showed higher expression levels of GFP, when compared to vectors composed by SFFV as internal promoter. Next, we evaluated the capacity of these clones to produce lentiviral infectious particles from single copy (results are summarized in Fig.8B). The vector composed by RSV-SFFV combination is the one related to higher lentiviral production once integrated as single copy, being the titer more then 10-fold higher to what was observed for the promoter combination RSV-PGK (present in TAR293LV). Interestingly, expression level of GFP from the vector with combination RSV-SFFV ranges at a lower level when compared to vector containing the PGK as internal promoter (Fig. 7A). This clearly points out that selection of clones with single copy integration by the extension of reporter gene expression is not indicative for the capacity of that clone to produce infectious viral

particles from single copy integration. Although an increase in virus titer was observed, the modulation of promoter content did not significantly rescue production of lentiviral vectors from single copy integrated genome.

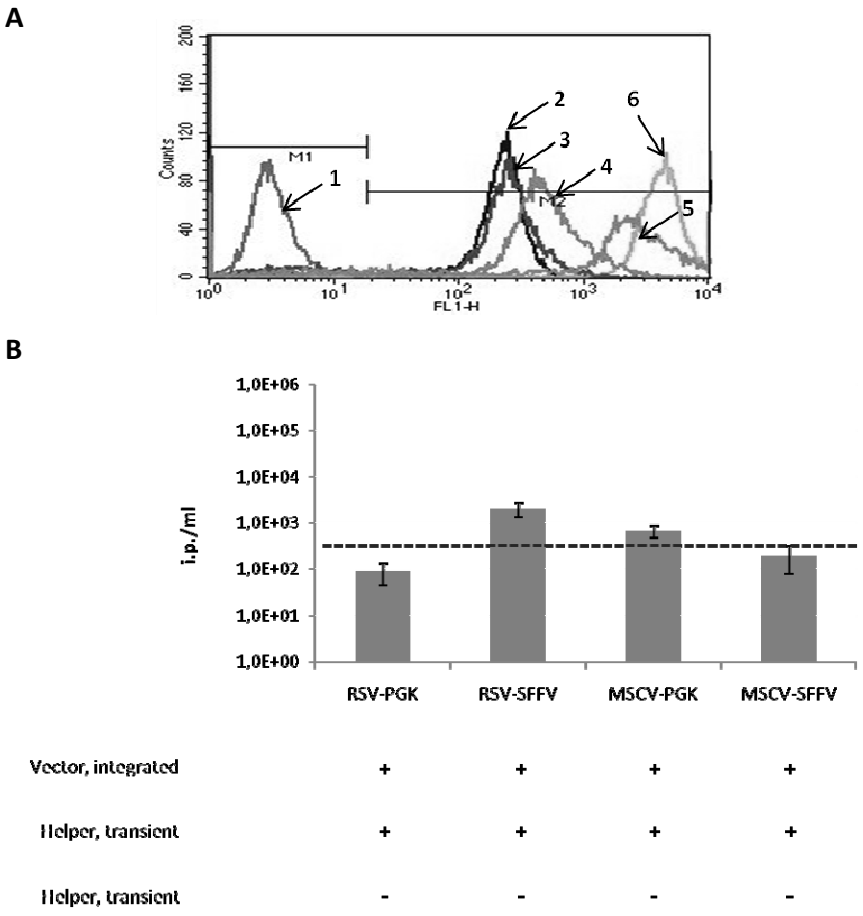

Figure 8. (A) Reporter gene expression from HEK293-derived cell clones targeted with the lentiviral vectors represented in Fig.7A. (1) HEK293 given as control; (2) reporter gene expression from 1B2 cell line, for further details please refer to Schucht *et al*., 2006 and Fig.3A, Part A; (3-6) reporter gene expression from HEK293-derived cell line targeted with MCSV-SFFV, RSV-SFFV, MCSV-PGK and RSV-PGK (aka TAR293LV), respectively. (B) HEK293-derived clones targeted integrated with the lentiviral vectors Fig.7A were analysed for their capacity to produce infectious particles from lentiviral integrated copy.

In summary, the results described above (and in part A of this Chapter) indicate that although transcriptional levels of full length mRNA from single copy integration in TAR293LV are rather low, the restriction for lentiviral vector production from HEK293-derived cell lines with single copy integrated lentiviral vector is not mainly exerted at a transcriptional level. Therefore, we evaluated if by manipulating post-transcriptional mechanisms, production of lentiviral vectors from single integrated HEK-derived cell line could be rescued.

2.6. Production of lentiviral vectors in TAR293LV is not rescued by RNA silencing suppressor proteins.

RNA interference (RNAi) is a gene regulation mechanism in eukaryotes that controls cell differentiation and developmental processes via expression of microRNAs. RNAi also serves as an innate antiviral defense response in plants, nematodes, insects and was recently shown to serve as an innate defense response also in mammals (Haasnoot *et al*., 2007). This antiviral response is triggered by virus-specific double-stranded RNA molecules that are produced during infection. To overcome antiviral RNAi responses, many viruses encode RNA silencing suppressors (RSSs) proteins that enable them to replicate at higher titers. It has been demonstrated that RNAi-suppressing activity of RSSs is correlated with their ability to bind dsRNA, thus influencing its further downstream processing and consequently affecting virus replication (Haasnoot *et al.,* 2007). Moreover, it has been demonstrated that expression of RSSs derived from human viruses and a plant virus in human cells can improve

production of viruses and viral gene delivery vectors in producer cells (de Vries *et al*., 2008).

We hypothesized that one of the causes for restriction of lentiviral production from single copy could be an effect of active RNAi response triggered by the lentiviral targeted cell. Therefore, we next evaluated if this putative restriction to viral vector production could be bypassed by overexpression of certain viral proteins described as having RSS activity. It has been proposed that Tat protein, besides its well-described activity as transcriptional activator of HIV-1 wild-type genome, also has an important RSS activity in HIV-1 infected cells (Bennasser *et al*., 2005). The effect of Tat overexpression was evaluated upon transient co-transfection of plasmid encoding the HIV-1 accessory protein at different amounts and the packaging functions necessary to produce infectious lentiviral particles. The results are summarized in Fig.9. No increase in lentiviral vector production upon transient transfection of lentiviral Tat encoding plasmid at different amounts was observed. Moreover it is shown that overexpression of Tat has a deleterious effect in the lentiviral vector production from 293-T cell line, being related to a decrease of more than 100-fold lentiviral vector production.

A

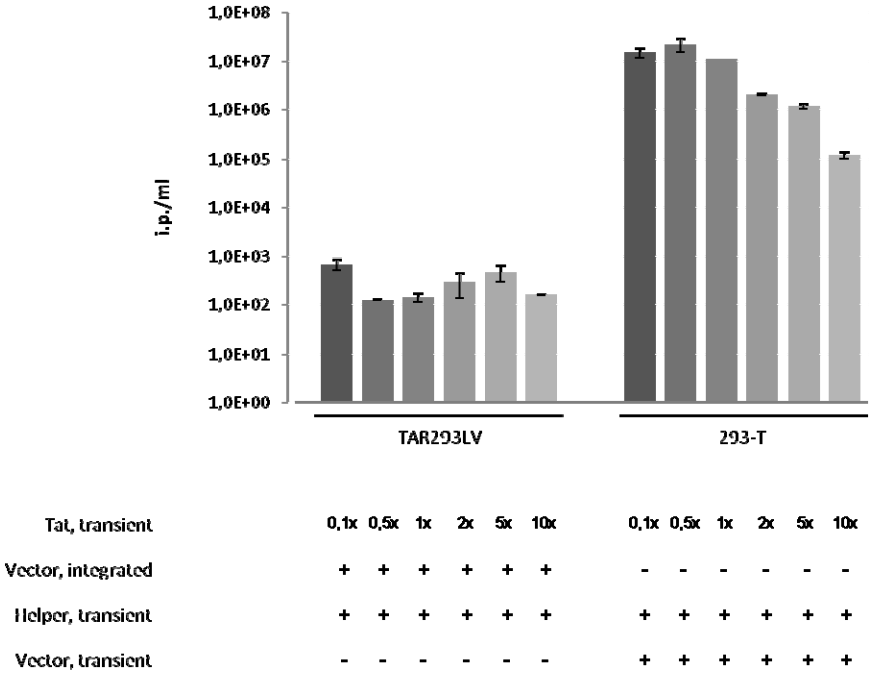

Figure 9. Effect of Tat overexpression on production of infectious particles from single copy integrated lentiviral genome in TAR293LV. As control, transient lentiviral vector production in 293-T with different amounts of Tat encoding plasmid was included. 1x is the equivalent of the amount of Tat-encoding DNA to be transfected in standard protocol for Tat-depended virus production. For detailed information, please consult Material and Methods section.

Besides Tat as a RSS protein encoded by HIV-1, it has been shown that other human virus encode proteins also with the capacity to suppress RNAi pathways in mammalian cells, such as the VP35 protein encoded by the Ebola virus. deVries and collaborators (2008) showed that HIV-1-based vector production can be upregulated in HEK293 producer cell lines by expression of RSS proteins, such as VP35 coding protein. We next evaluated the effect of expression of

VP35 in production of lentiviral vector form single copy integrated in TAR293LV. The results shown in Fig.10 indicate that expression of VP35 does not rescue production of lentiviral vectors from a cell line harboring an integrated lentiviral genome.

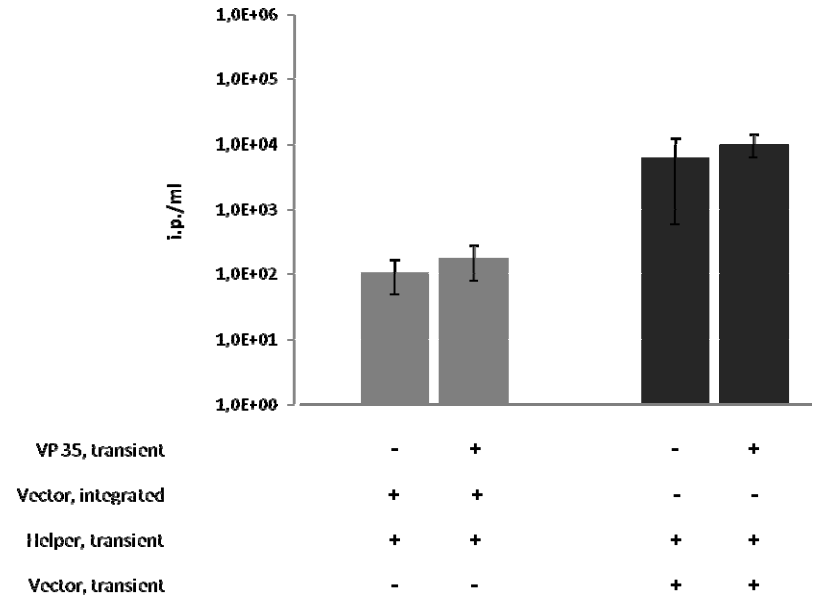

Figure 10. Effect of VP35 overexpression on production of infectious lentiviral particles from TAR293LV. Production from integrated lentiviral copy and upon transfection of a viral vector is represented by light and dark gray bars, respectively.

3. DISCUSSION

The results presented in Part B described additional approaches performed in order to elucidate the restriction of production of viral vectors upon chromosomal integration. As referred, Part B, aimed the complementation of the results described in Part A and therefore, should be regarded as supplementary data.

From the results presented in this Chapter, it was shown that TAR293LV targeted chromosomal locus does not support lentiviral vector production although expression of the reporter gene in the context of lentiviral targeted sequence was extremely high. In Part B, this observation was taken as a starting point to evaluate mechanisms that could account for limitations in viral production and strategies in order to circumvent such restriction. In this respect, we evaluated if such restrictive phenomenon could be exerted either at lentiviral genome transcriptional or at post-transcriptional level.

At transcriptional level, attention was paid to strategies that could account to an increment of full length viral mRNA production, either by manipulating the epigenetic status of the producer cell (data not shown) or by altering splicing frequency of viral genome or finally by modulating the promoter content of the targeted vector. None of these strategies revealed to be of significant value in order to render infectious viral particles production at satisfactory levels. Altogether, the results presented in Part A and B, indicate that transcription of lentiviral packagable genome occurs at levels that must be sufficient to render vector production at least at moderate levels.

The other level of analysis was performed addressing the hypothesis that post-transcriptional mechanisms could contribute to the incapacity of lentiviral vector production from single copy in HEK293-derived cell line. In this respect, it was anticipated that long-term expression of T-Ag down-regulates such restrictive mechanism, partially circumventing the restriction observed in HEK293 (please refer to Part A). Among post-transcriptional mechanisms that could play a role in the impairment of HEK293 derived cell line to produce

infectious particles from single copy, we focused on RNA interference (RNAi) pathway. It has been reported that RNAi serves as a potent antiviral mechanism in mammalian cells (Bennasser *et al*., 2005; Haasnoot *et al*., 2007; Triboulet *et al*., 2007) including HIV-1 infection, acting as a posttranscriptional gene regulation mechanism as part of an innate immune response exerted by the infected cells. The virus in turn evolved strategies/mechanisms to counteract these innate-immune defenses from the host cells. The putative effect of blocking RNAi pathway in production of viral particles from TAR293LV cell line was evaluated. This question was addressed by overexpression of two RSS proteins, namely HIV-1 Tat accessory protein and VP35 protein from Ebola Virus. Lentiviral production from single copy in TAR293LV was not rescued upon expression of either of these proteins, suggesting that RNAi mechanisms, at least at the level addressed in this study, are not the cause of impairment to produce infectious virus from single lentiviral integrated cell lines.

Together, the results presented in this study suggest that restriction of viral production is much likely exerted primarily at a post-transcriptional level and is partially circumvented by expression of T-Ag. The nature of such restrictive phenomenon as well its *modus operandi* still needs to be defined.

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CHAPTER V

DISCUSSION AND CONCLUSIONS

CONTENTS

1. DISCUSSION

Site-directed engineering of defined chromosomal sites for recombinant protein expression was evaluated to rationally develop highly valuable producing cellular systems in a expedite way. As paradigm, production of antibodies and retroviral vectors was particularly addressed. Together, the results obtained show that this approach is indeed exceptional in its ability to establish reliable production systems.

In Chapter II, RMCE-based targeted integration approaches were systematically exploited for antibody production. Chromosomal loci supporting production of antibodies in biotechnologically relevant cell lines were identified following a rational screening strategy based on the molecular composition of the tagging cassette. It was shown that the identified loci were able to be engineered in order to support expression of (other) antibody targeting cassettes. Nevertheless, the capacity for the expression of different cassettes was found to be intrinsically related to their molecular composition, namely promoter content. The work presented shows that high level of antibody expression in mammalian cells not only relies on potent transcription-promoting elements and optimal design of the expression cassette, but also crucially depends on appropriate chromosomal sites from which that cassette is expressed.

In conclusion, it is important to consider the specific requirements of a particular integration site to achieve maximum level of recombinant protein– a relevant finding that is also corroborated by the results present in Chapter III.

In Chapter III, defined chromosomal loci were exploited by means of RMCE towards production of well characterized gamma-retroviral vectors. It was shown that besides the outstanding advantage

of modular cell lines for production of clinically relevant retroviral vectors, they constitute an exceptional platform to systematically evaluate and directly compare different vector compositions in a specific chromosomal locus, an issue that had not been addressed so far. Targeted exchange technology allowed the evaluation of viral vector design in two orders, namely, viral titers and safety associated to the producing system. It was shown that a given chromosomal locus only supports efficient expression of certain retroviral cassettes. This gives evidence that, indeed, optimal combinations of specific integration sites and promoter content of a targeting vector have to be defined in order to maximize the level of expression (viral vector production) by those systems. The work present in this Chapter contributes to the development of a rational strategy for vector design that takes into consideration not only the achievement of high viral titers but importantly, that assures maximal safety levels, necessary for the translation of these cellular systems into therapy.

The exploitation of defined chromosomal sites for production of other viral vectors was also under the scope of this thesis.

In Chapter IV the exploitation of site specific integration for lentiviral vector production was addressed. The production of lentiviral vectors from single copy integration revealed to be challenging. In fact, production of lentiviral vectors at satisfactory titers could not be achieved. Interestingly, it was revealed that long term expression of heterologous proteins such as T-Ag plays an important role in circumventing non characterized cellular restrictions for production of lentiviral vectors. The results indicate that the requirements for a cell line to support high levels of lentiviral vectors from single copy integration are different from those necessary to produce gamma-retroviral vectors.

It is envisioned that GMP-based genetic manipulation of HEK293 cell towards functional replacement of T-Ag could render HEK293-derived cell line capable of producing lentiviral infectious particles from one copy integrated vector. Finally, this cell line can be exploited towards the establishment of modular packaging cell lines for lentiviral vector production, in conjugation with lentiviral vectors optimized for constitutive expression upon single integration in cell genome. It is foreseen that this modular packaging cell line will constitute a platform to produce lentiviral vectors in a flexible, efficient and safe manner, representing a new dimension of engineered cell lines for constitutive virus production with clinical relevance that has been not achieved so far.

1.1. Holistic perspective of the results presented in this thesis.

In cell line development, the integration of transgenes into the chromosomal DNA of the host cell is a crucial step and thus is of main consideration. Once integrated into the cellular DNA, the transgene cassette is affected by neighboring chromosomal elements that modulate their expression to a high extent (Bell *et al*., 2001). Thus, expression of recombinant proteins would be ideally from *a priori* defined chromosomal site, known to sustain optimal expression of that particular transgene. But so far it is not feasible to bring this idea to light. In one hand chromosomal sites that provide the required properties for recombinant protein expression are not identified and in another hand, genomic tools for site specific engineering are not yet available (Cathomen *et al*., 2008). Therefore raises in importance the targeted integration of transgenes through the re-use of already defined and characterized loci.

Concerning the exploitation of a defined locus by RMCE for production of molecules of biotechnological interest the data provided in this thesis allow to draw some general conclusions. Since the interaction of promoter elements with the flanking chromosomal sites cannot be foreseen, a conservative exchange of expression cassettes seems to be a reasonable approach. Thus, the tagging cassette should be as similar as possible to the cassette to be targeted in order to guarantee the maximal positive *cis* -effects that this locus could exert on expression. A defined locus is not necessarily universal, which means that the production of different biological products is not guaranteed even if the vector composition (*i.e.*, regulatory sequences) is constant among the different vectors. If these requirements are fulfilled, the exceptional advantages of the RMCE technique could be vastly applicable to the generation of cell lines suitable for production of a wide variety of molecules with biological significance.

RMCE technology is not only of major benefit towards establishment of flexible cell lines for production levels. It reveals to be an essential tool towards development of optimized targeting cassettes designed to take maximal advantages of the specific positive effects exerted by the defined chromosomal site from which they are expressed. Thereby, the approach allows to optimize an expression cassette to the requirements of a given locus – a strategy that may also exceed the expression level with respect to the initial tagging cassette.

It is important to consider the specific requirements of a particular integration site relating to the maximum level of recombinant protein production that can be achieved. In other words, molecular composition of the targeting vectors and the chromosomal integration site go hand-in-hand. As a paradigm, this concept was shown for the case

of antibody and retroviral vector production but, in theory can be translated to any protein production system in general.

1.2. Further perspectives for the next generation of producer cell lines.

The potential of RMCE for re-engineering a targeted genomic locus.

Expression cassettes use strong viral or cellular promoters and enhancers to confer stable and high expression of a transgene. However, the performance of these regulatory elements strongly depends on the chromosomal surroundings, *i.e.*, sequence and epigenetic status of the integration site. The incorporation of protective *cis* –regulatory elements has been used to avoid this position effect. Such DNA elements should exhibit specific characteristics like being small in size, conferring stable and high expression levels and universal applicability so that they can be inserted into various cell lines and combined with many different promoters. Different genetic elements have been used in order to protect the integrated transgene from silencing or increase transcriptional rate of a transgene by uncouple its expression from the influence of other genetic elements present in the chromosomal surroundings of the integration site. Regulatory elements such as insulators (Chung *et al*., 1993; Emery *et al*., 2000), locus control regions (LCR) (Grosveld *et al*., 1987), scaffold/matrix attachment regions (S/MAR) (Bode *et al*., 1995; Zahn-Zabal *et al*., 2001; Girod *et al*., 2007), ubiquitous chromatin opening elements (UCOEs) (Williams *et al*., 2005; Zhang *et al*., 2007) and STAR- (stabilizing and antirepressor-,Kwaks *et al*., 2003; van Blokland *et al*., 2007) have been used in order to increase the expression of a certain integrated transgene. The results achieved are dependent on

the regulatory element applied, transgene and integration site considered. Nevertheless, a systematic approach in order to define the performance of different regulatory elements in a give integration site has not yet been performed. In this respect, RMCE can be a technology of excellence in order to re-engineer a genomic locus towards its optimal expression. Different genetic elements can be introduced / excised or exchanged to modulate the expression in a given locus for maximal production of a given expressing cassette. The combination of different non-interacting recombinase sites or the combination of recombinase systems (*e.g.*, Cre/ *LoxP* and FLP/ *FRT)* allows a flexible introduction of two and more expression cassettes within independent chromosomal integration sites.

The overall result could be the creation of a genomic locus that supports expression of a transgene independent of its chromosomal surrounding (as a result of neutralizing the *cis*-effects that the integration site surrounding could exert over the integrated transgene). Another scenario would be the capitalization of a given integration site upon its modulation in order to take maximal benefit of *cis*-regulatory elements present in the vicinity of the integration site.

Boosting the production capacity of a cell line by multiplication of a defined locus.

It is envisioned that strategies that allow the "multiplication" of a certain locus region in a producer cell line could improve exponentially the expression of a certain recombinant protein.

Bacterial artificial chromosomes are engineered DNA constructs that have the propriety to accommodate very large genomic sequences (usually >150kb) without the risk of rearrangement. Due to their high

sequence capacity, they can incorporate an entire chromosomal region, including regulatory elements upstream and downstream the encoding sequence, including even enhancer sequences that govern a gene's expression level usually several kb of distance from the transcriptional starting site (reviewed in Copeland *et al*., 2001). In this respect, it is expected that the expression of a given gene from a BAC containing its cognate chromosomal environment would recapitulate the expression of that gene in its natural chromosomal environment.

It is anticipated that development of a producer cell line by integration of several copies of engineered BAC containing the transgene targeted integrated in a highly potent locus region of interest would allow the increase of production rates of that particular recombinant protein. Moreover, the application of BAC-based technology can open widows for the establishment of "hybrid" producer cell systems, in which a highly potent locus region from a heterologous cell line is transferred to any cell line of interest. In this respect, transgene expression would benefit from the positive proprieties exerted by the transferred heterologous locus, circumventing the need for additional screening procedures in order to identify highly potent loci in other producing system.

Cell line development considering the exploitation of molecular tools such as BACs in order to artificially create multiple copies of a given locus supporting high levels of certain transgene expression has not yet been performed. A new generation of producer cell lines for biotechnological relevant products is thus expected to arise contributing to another dimension of the current state-of-the-art of cell engineering technologies.

2. CONCLUSION

In this thesis, site-directed engineering of defined chromosomal sites is shown to be a valuable approach towards establishment and exploitation of reliable production systems for antibodies and viral vectors. Although significant contributions for rational cell line development for biotechnological relevant molecules production are presented, the systematic exploitation of such technology is still in its infancy. It is envisioned that further refinements of targeted integration based-technologies will lead to the successful establishment of the first commercial processes in the near future.

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