Systematic Study on Generation of Mammalian Production Cell Lines by Targeted Integration (RMCE)

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Summary

In this study, the targeted integration of a gene-of-interest (GOI) into pre-characterized loci with high and stable expression properties is further elaborated to overcome variable and unstable transgene expression caused by position effects that are typical for random integration. The Flp-based recombinase-mediated cassette exchange (RMCE) strategy permits the exchange of a target cassette for a cassette with the GOI, introduced as part of an exchange vector. According to this protocol RMCE enables a “clean” process and it overcomes silencing by not co-introducing prokaryotic vector parts nor leaving behind a selection marker (“pFAR” principle). Contrary to certain single-site recombination strategies (such as the “Flp-in” technique) RMCE is a virtually irreversible process that can be driven to completion by a molar excess of the exchange cassette and it does not suffer from the presence of genomic pseudo-sites as reported for the Cre- and ΦC31-recombinases.

The present work has extended our RMCE technology and made it applicable for the generation of permanent (production-) cell lines that are characterized by an unstable genotype due to continuous chromosomal rearrangements. Using a two-step RMCE pre-screen, this protocol

- guarantees the selection of loci with high-level and long-term stable expression of a transgene under a given promoter
- enables a highly efficient “trap-principle” without introducing unwanted sequences/elements. This has been achieved via a promoter residing on the target in combination with a promoter-free targeting cassette
- provides persistent exchangeability (i.e. high RMCE potential)
- promotes the introduction of a single transgene at the target site in the absence of any (additional) random integration events
- selects for sites with properties that are amenable to the transcription-augmenting potential of histone deacetylases inhibitors (HDACi).

The generation of suitable master cell lines is feasible within 8 weeks. These lines permit further RMCE cycles within 4 weeks in the absence of any drug selection and with a considerable efficiency (~10%). The particular advantages of Flp-RMCE will therefore grant its use for Biotechnology, Gene Therapy, Stem Cell/transgenic animal research.
1. Introduction

1.1. Current status in cell line development and engineering for recombinant protein production

1.1.1. Selection of stable cell lines

During recent years the market for biopharmaceuticals has widely expanded and is expected to reach US$ 70 billion by 2010 (Walsh 2006). The bulk of items are recombinant protein-based products, which are mostly produced in cultivated mammalian cell lines, among which the Chinese hamster ovary (CHO) line is the most prominent one (Trill et al. 1995; de la Cruz Edmonds et al. 2006). Traditionally, a transgene under the control of potent transcription regulatory elements and a selectable marker gene are randomly integrated into the host chromosomes. A stringent selection procedure in media containing a cytotoxic antibiotic or devoid of an essential metabolic enzyme is used for the generation of stable transgenic cell lines (Wurm 2004). The most common antibiotic resistance marker used is the bacterial neomycin phosphotransferase enzyme encoded by the neo gene (Trill et al. 1995; Li et al. 2007). It confers selectability by the drug G418 in eukaryotic cells. There are a number of alternative antibiotic selection markers available (e.g., puromycin, zeocin), but the information on their use in mammalian expression systems is poorly reported. The metabolic selection markers such as dihydrofolate reductase (DHFR, Trill et al. 1995; Kim et al. 1998) or glutamine synthetase (GS, de la Cruz Edmonds et al. 2006) have provided effective platforms for high level expression systems. In both cases, cell lines defective in the respective enzyme are available. In these systems, selection pressure is applied to the cell culture with an inhibitor of the DHFR (methotrexate, MTX) or GS enzymes (methionine sulfoximine, MSX) that cause an increase in the number of copies of the selection gene and the transfected genes. With these systems, a significant improvement in product yield could reach 5 g/l in bioreactor processes (Wurm 2004; Butler 2005). However, since the targeted gene and the selection marker gene are transfected and randomly integrated into the host chromosomes, the expression level of transfected cells surviving from selection is variable and unstable due to different and unpredictable locations of the integrated copies, a phenomenon known as
“position effect”. The co-integrated selection marker might cause repressive effects on targeted expression (Artelt et al. 1991) and/or disrupt the expression of neighboring gene within a locus (Pham et al. 1996) since the target gene and selection marker gene are mostly aligned on a (bicistronic) construct. A further complication arises from the fact that the presence of multiple integrated gene copies at different loci tends to cause recombination over time accompanied by chromosomal aberration (Derouazi et al. 2006) and/or repeat-induced silencing (Garrick et al. 1998) in case of tandem-array integration patterns. Furthermore, high-producers only account for a minor portion of successfully transfected cells. This fact calls for immediate extensive screening since non- and low-producers tend to overgrow the desired producing clones that have to divert their metabolic resources to the expression of the gene-of-interest (GOI) (Kromenaker and Srienc 1994). Therefore a screening process is necessary to isolate clonal lines stably expressing recombinant products at high levels in a timeline-efficient, cost-effective and high-throughput manner.

1.1.2. Screening of suitable clones for production

In the existing studies, isolating single clones with desired expression properties usually relies on extensive screening of a huge number of individual clones, for which the most commonly used method is limited dilution (LD). Although the screening process based on LD and subsequent product analysis and characterization is simple and inexpensive, it is laborious and time-consuming. After the advent of flow cytometry applied for cell screening, subpopulations or single cells can be isolated from the mixture in a short range of time and with high throughput. Combined with a cell surface labeling technique using fluorescently tagged antibodies that bind to secreted target proteins, high producers can be directly evaluated by the fluorescence intensity at the cell surface (Brezinsky et al. 2003). Repeated sorting based on the selection of cells with the highest fluorescence intensity results in significant increases regarding specific cellular productivity. Kuafman et al. (2008) reported a similar method to establish stable cell lines only by repeated sorting steps rather than drug selection, which warrants a high degree of homogeneity and stability in gene expression. In more refined cell surface labeling strategy, based on semisolid methylcellulose growth medium, the high expressers can be visualized as a halo around the colony and even be picked up by automated colony pickers (www.genetix.com) according to the halo size.
Besides that, many other efforts have been undertaken to improve the screening process but the general principle of screening has remained unchanged except that more clones can be screened in a shorter time span thereby improving throughput. Progress can only be expected if, prior to screening, an enrichment process is applied in order to reduce the number of clones needed to be screened and characterized. Finally, even if a transcriptional hotspot is hit by incidence, a random screening process cannot guarantee the recurrence of such an event.

For this purpose, reuse of these identified hotspots could dramatically reduce the timeline in development of production cell lines for various recombinant proteins. A procedure to this end could rely on site-specific recombination, which permits the reuse of loci with the desired expression characteristics for the expression of any GOI. Repeated use of the same integration site would also simplify the regulatory approvals.

In brief, an ideal expression system should have the following characteristics. It should:

1- mediate high and stable expression level in the absence of a drug selection;
2- co-integrate no auxiliary sequences (selection marker gene, prokaryotic sequences) besides the GOI;
3- accommodate a single copy of the gene in order to reduce variable expression levels and genomic instability caused by multiple copies at the same or different integration sites;
4- enable to repeatedly target a given transcriptionally active site at a reasonable efficiency to reduce the number of clones to be screened.

1.2. Site-specific recombination (SSR)

In the past decade, a number of site-specific recombinases have been exploited for the targeted integration of a transgene into a pre-tagged genomic site, especially for introducing mouse mutations in a pre-specified temporal and spatially restricted manner (Branda and Dymecki 2004). The combined use of site-specific recombination and gene-targeting or -trapping in mouse embryonic stem (ES) cells has provided a useful platform to precisely manipulate the mouse genome.

For applications in mammalian cell culture, this combination matches some, but not all of the requirements as stated above. The most widely used site-specific recombinases in
mammalian cell culture are the *E. coli* P1 phage-derived Cre (Sternberg et al. 1986), the *Saccharomyces cerevisiae*-derived Flp (Buchholz et al. 1996; Schaft et al. 2001) and the bacteriophage ΦC31-derived integrase (Thorpe and Smith 1998). These enzymes mediate a recombination between two copies of their target sequence, i.e. loxP site (for Cre), Flp-recognition target (FRT; for Flp) site or a combination of attP/attB (for ΦC31), respectively, which enable to excise (Cre, Flp), insert (Cre, Flp, ΦC31) or invert (Cre, Flp) DNA molecules. When two copies of the recognition-target (RT) site are arranged as direct repeats, the corresponding enzyme excises the DNA segment intervening the RTs and releases it as circular DNA, whereas the reverse reaction leads to an insertion of a circular DNA in case it is present at a large molecular excess (Fig 1.1A). Due to thermodynamic reasons, the entropy-driven (monomolecular) excision step is much favored over the (bimolecular) insertion step (Baer and Bode 2001). When two copies of RT are positioned in inverse orientation, the intervening DNA becomes inverted by the action of the appropriate recombinase. Thereby, in the presence of recombinase activity, a dynamic equilibrium is established, in which half of the products will be in one orientation and half in the other (Fig 1.1B). In contrast, the ΦC31 integrase, a member of the large family of serine recombinases mediates recombination between two different RTs, attP and attB, which leads to the insertion of the whole plasmid sequence giving rise to a different product pair of RTs, attL and attR (Fig 1.1A'). Since the excision would require cofactors (such as excisionase, Xis) that cannot be expressed in a biologically active form in mammalian cells, the integration or inversion reaction becomes irreversible (Fig 1.1B'), other than for, Cre and Flp (Baer and Bode 2001). Although the irreversible integration principle may be favorable, yields are rather low due to the fact that an attP site requires negative superhelicity in order to recombine with attB. Improvement has been achieved by adding a nuclear localization signal (NLS) to the recombinase, whereby its efficiency became comparable to the widely used Cre/loxP system. Even through, the conventional ΦC31 system still cannot avoid the integration of prokaryotic DNA that is part of the integration plasmid. The consequences of such a co-integration will be detailed in the following context.
The recombination is mediated through a single reciprocal crossover between the RTs and the whole sequences of the plasmid DNA are integrated. In the persistent presence of Flp/Cre-recombinase activity, however, such an event will be immediately reversed in an entropy-driven excision reaction. This requires that the rare desired events can only be captured by a stringent selection principle (usually based on a positive selection strategy).

All of the recombinases introduced before have found applications to construct engineered CHO cell lines for high-level production of recombinant proteins (Groth et al. 2000; Thyagarajan et al. 2001; Kito et al. 2002; Wiberg et al. 2006; Huang et al. 2007). Although, in principle, the SSR systems guarantee the reproducible and stable transcription of various GOIs at the same genomic site, certain drawbacks still cannot be avoided. For example, a
selection marker is required in order to enrich the targeted events, which will cause un-expected repressive effects as encountered in the conventional cell line development processes. Even worse, transcriptional levels of the GOI may be affected by the co-expressed selection marker and the negative effect may be fortified by host-defense mechanisms against prokaryotic vector parts (Riu et al. 2005). These circumstances should be considered when designing novel expression systems.

1.3. Recombinase-Mediated Cassette Exchange (RMCE)

An advanced generation of targeted integration strategies relies on Recombinase-Mediated Cassette Exchange (RMCE) techniques (Schlake and Bode 1994; Feng et al. 1999; Bode et al. 2000b; Baer et al. 2000; Belteki et al. 2003). Since genomic pseudo-sties have been reported for Cre and ΦC31, which cause genomic instability and, as a consequence, apoptosis (Thyagarajan et al. 2000; Thyagarajan and Calos 2005), we have focused on Flp-based RMCE application.

A wild type FRT site consists of 48 bp, composed of two inverted 13 bp repeats and an 8 bp spacer together with a third 13 bp direct repeat and a single additional base pair.

![Figure 1.2: Composition of a wild type FRT site (cf. Table 1 in Schlake and Bode 1994)](image)

The 8 bp spacer is involved in DNA-DNA pairing during strand exchange. Together with the extra repeat, its asymmetry determines the direction of site alignment in the recombination event, which will consequently lead to either inversion or excision. Foot-printing techniques have defined two 12 bp Flp recombinase binding sites that include 11 bp of each of the inverted repeats but only the outermost bases of the spacer, suggesting that most of the spacer can be mutagenized without a loss of function. These mutations in the 8 bp spacer result in functional FRT variants (Fn), which will recombine with a second site of the same composition but not with a wild type one (Schlake and Bode 1994). If sites F and Fn are in a strategically favorable position, they can mediate the exchange of expression cassettes in a
double reciprocal crossover event, which is called Flp-based recombinase-mediated cassette exchange (Flp-RMCE). In this case, Flp-RMCE utilizes a set of two 48 bp FRT sites, for instance wild type F and mutant F3 (Schlake and Bode 1994). Under these conditions, Flp-RMCE enables the site specific exchange of an expression cassette anchored in the genome which is flanked by an F3 site on one end and an F site on the other for an analogous cassette which is provided on an “exchange plasmid” (Fig 1.3).

![Diagram of Flp-RMCE concept](image)

**Figure 1.3: Principle of the Flp-RMCE concept.**
First, a +/- selection marker flanked by wild type F and mutant F3 sites is integrated into host cell genome as a recombination target. This is done either by random integration or by homologous recombination. Only single copy integration cells are recovered by selecting against the presence of negative marker or by positive selection or by a combination of both. By the action of Flp, exchange between the genomically anchored target cassette and exchange cassette is initiated. After this step, non-exchanged parental clones can be eliminated by negative selection.

Compared with the simple site-specific strategy, Flp-based RMCE, combined with a negative selection principle (Seibler et al. 1998), enables the “clean” exchange of a selection marker for the GOI in the sense that no extra sequences besides the GOI are left behind in the genome. These advantages have motivated applications of the RMCE principle for the generation of various production cell lines (Feng et al. 1999; Schucht et al. 2006; Kim and Lee 2008; Malchin et al. 2008), although additional (positive) selection markers had to be introduced as a part of the exchange cassette. Since the documented unstable karyotype of
CHO cell line lowers the efficiency with which suitable targets can be recovered, major efforts have to be undertaken to overcome the main bottleneck, i.e. the identification of transcriptional hotspots amenable to the RMCE principle. These hotspots should be consistently target-able and mediate high and persistent transcription levels. Ideally, the targeted integration events are recovered in the absence of any drug selection to avoid cellular stress-related phenomena (Rodolosse et al. 1998), to prevent the repressive and disruptive effects caused by the co-expression of a selection marker (Artelt et al. 1991; Pham et al. 1996), and to reduce the risk of antibiotic contaminants in industrial fermentation processes (Sommerfeld and Strube 2005).

1.4. Transgene expression in eukaryotic cells

Recombinant gene expression in mammalian cells is a complicated process with multiple-steps, from the activation of transcriptional regulators to the synthesis of a functional protein. It starts from transcription, and continues with posttranscriptional processing, translation and posttranslational modification. Subsequent steps are protein folding under the control of chaperones and final secretion. The level of protein expression is determined by each of these individual control points, although, traditionally, transcription is considered to be the dominant factor controlling expression rate (Barnes et al. 2003).

After a transgene has been transfected into a cell and stably integrated into the host genome, it is wrapped by histones to form nucleosomes, which are the basic building block of chromatin. Both, the genomic locus and the surrounding environment determine its expression properties. A transgene inserted into or close to a heterochromatin (highly condensed regions of chromatin) is more prone to be silenced than a gene in a euchromatic (more open and active) environment. Taking a close look at the molecular level, histone modifications including acetylation, methylation, phosphorylation, poly(ADP-ribosyl)ation and ubiquitination (collectively known as the “histone code”) determine whether a gene is active or inactive. Transcriptional repression can occur by histone-deacetylation (Jeppesen et al. 1992) and -methylation (Peters et al. 2001; Lorincz et al. 2004) as well as by methylation of the transfected DNA’s promoter sequence, whereas an open and active chromatin domain is generally associated with histone acetylation (Schlake et al. 1994). Therefore, two
approaches have been adopted to create chromatin environments around the transgene that will favor active transcription: the surrounding chromosomal environment is physically prevented from depressing transcription or the overall epigenetic environment of the surrounding chromosomal DNA is altered by making use of the histone code (Barnes and Dickson 2006). These two approaches will be addressed in the following chapters.

1.5. Introduction of boundary elements

Eukaryotic genomes are organized into functionally and structurally distinct domains in which gene expression is either repressed or facilitated. Individual domains can be maintained independent of their surroundings through the establishment of insulators or boundary elements (West et al. 2002). Insulator or bordering functions protect genes within a chromatin domain from repressive effects arising from the surrounding genomic environment. In order to convey stable and high expression levels, incorporating cis-acting DNA elements such as the scaffold/matrix attachment regions (S/MARs) and/or genomic insulators into the transgene construct is the usual way to organize an artificial mini-domain around the transgene. Recent halo fluorescence in situ hybridization (halo-FISH) studies confirm that S/MARs act by organizing eukaryotic chromatin into separate loops (Heng et al. 2004). Following histone extraction, these loops can be visualized as part of a DNA halo anchored to the densely stained nuclear matrix or chromosomal scaffold (Goetze et al. 2003).

S/MARs and their associated sequences have a number of properties, which make them powerful tools for designing predictable gene expression systems as parts of integrating transgenes. They increase transcriptional initiation rates in the integrated but not in the pre-integrative state. This phenomenon has been called “augmentation” as it is different from enhancement. Association of loop DNA with the nuclear matrix via S/MAR elements present at the flanks is observed which consequently leads to an open access of the transcriptional factors to start the transcription machinery (Iarovaia et al. 2005). Moreover, S/MARs can function as insulators to establish a physically autonomous domain to block the negative regulatory influences of the surrounding heterochromatin (Goetze et al. 2005). When the transgene expression cassette was flanked by S/MARs, multi-copy integration with tandem transgene arrays was also observed, which augments the transgene expression in a site-independent manner (Klehr et al. 1991; Bode et al. 1996).
but not least, due to their recombinogenic propensity, S/MARs might also direct the transgene to
the nuclear matrix and cause integration into regions of the genome with S/MAR-like sequences
(Bode et al. 2000a). For this purpose, different S/MAR elements were integrated into transgene
cassette to generate high producer cell lines. The chicken lysozyme 5’ matrix attachment region
almost fully abolishes the occurrence of CHO cell clones with low transgene expression levels that
may result from integration into unfavorable chromosomal environments. At the same time it
increases the overall expression of the transgene, allowing the recovery of producer strains with
significantly lower screening efforts (Zahn-Zabal et al. 2001; Girod et al. 2005). Using the human β
S/MAR elements, systems were also developed for expressing high levels of recombinant proteins
in CHO cells (Kim et al. 2005).

The 2.2 kb S/MAR element E (http://smartdb.bioinf.med.uni-goettingen.de/cgi-bin/SMARtDB/
getSMAR.cgi?SM0000002) used in this study is an EcoRI restriction fragment from the 14 kb
domain of the human interferon gene, where it is located upstream of the well-characterized
β-interferon gene (Mielke et al. 1990). The 1.3 kb S/MAR element W
(http://smartdb.bioinf.med.uni-goettingen.de/) goes back to the first intron of the potato leaf
stem-specific protein ST-LS1. As element E, S/MAR W has been used to confer
position-independent expression to a transgene in potato and tobacco plants (Stockhaus et al.
1987) as well as in mammalian cells.

Besides S/MARs, there are many other cis-acting DNA elements reported to improve transgene
expression. Placing the human β-globin locus control region (LCR) in front of a gene construct
leads to high and stable transgene expression in erythroid tissue of transgenic mice (Grosveld et
al. 1987). Insulators block the action of the enhancer on the promoter when placed in between.
Insulators also work as barrier to stop the spread of surrounding heterochromatin. Incorporation of
the well characterized cHS4 insulator from the chicken β-globin LCR confers long-term stability of
transgene expression (Pikaart et al. 1998). Integration of ubiquitous chromatin opening elements
(UCOE) upstream of the transgene also permits rapid expression of a protein in an integration
independent manner (Benton et al. 2002). Stabilizing anti-repressor (STAR-) elements flanking the
reporter gene are also able to secure higher levels of stable expression in CHO cells, indicating
anti-silencing effects (Kwaks et al. 2003).
1.6. Synergistic function of S/MARs and HDACi on transgene expression

In addition to the transcription augmentation function introduced in chapter 1.5, S/MARs are involved in chromatin remodeling and subsequent transcriptional activation events (Bode et al. 2000a). When S/MARs are used to flank the transgene cassette, their strand-unwinding propensity (Bode et al. 1992) attracts sequence-specific proteins, which in turn fix separated strands. The positive and negative superhelicity, both caused by a tracking protein in adjacent DNA, can subsequently be released by introducing single strand (ss-) DNA breaks. While negative superhelicity (DNA unwinding) enables the immediate transcriptional initiation by the transcription machinery (Kay and Bode 1994), positive superhelicity (DNA over-winding) would stop the process. Unwinding can be supported by the addition of butyrate to the medium as this deacetylase inhibitor induces histone hyperacetylation, which causes unconstrained negative supercoils by releasing DNA from the protein core of the nucleosome (Bode et al. 1986)

Histone deacetylase inhibitors (HDACi) such as sodium butyrate (NaBu) were originally described for their ability to induce differentiation and cell cycle arrest in cultured cells. HDACi could effectively stop the growth of cancer cells at concentrations that cause limited toxicity in normal cells since inhibition of HDAC activity affects the expression only 2% of mammalian genes (de Ruijter et al. 2003). Consequently, derivatives have been developed to serve as anticancer agents in clinical trials for multiple types of cancers. In general, HDACi are capable of inhibiting HDACs with varying efficiency [e.g. nano-molar for trichostatin A (TSA) to milli-molar for NaBu]. Inhibition of HDACs enables acetyltransferases to hyperacetylate the lysine residues located in the N-terminal tails of the four nucleosomal core histones.

If transgenes are transfected as a part of an S/MAR-flanked artificial chromatin domain, the role of core histone hyperacetylation mediated by butyrate becomes obvious: Klehr et al. (1992) showed that transcription of the respective transgene was boosted 10-30 fold. Therefore HDACi and S/MARs synergize to create and stabilize a topology that is favorable for pre-activation of genes (Schlake et al. 1994).
1.7. Subject of this study

As introduced before, conventional transfection and selection strategies for recombinant protein production cause unstable and unpredictable expression profiles both due to uncontrollable integration sites and the number of integrated copies. Necessary screening procedures for suitable production clones are tedious and time-consuming. With the advent of site-specific recombination techniques, targeted integration of a transgene into a pre-tagged and pre-characterized transcriptional hotspot can be addressed. At the onset of this study a selection procedure like the conventional transfection and selection strategy was standard, and the co-integration of prokaryotic sequences was considered more or less unavoidable.

Even though recombinase-mediated cassette exchange (RMCE) strategies had been added to the scope of SSR activities, the identification of RMCE-competent transcriptional hotspots remained laborious as multiple randomly tagged single clones had to be screened. To circumvent this problem, the present study adds one more round of RMCE after tagged and targeted clones have been isolated (Fig 1.4). Only successfully re-targeted clones are then screened for members, which restore the initial situation at a single site and with a single-copy integration of the target. Both, the expression level and the targeting phenomena are monitored by the gain or loss of a fluorescent marker. Finally, the correctly targeted clones are chosen for the integration of various GOIs, even in the absence of a drug selection marker and a chemical agent. The well characterized S/MAR elements E and W are used to flank the target and their efficiency regarding transcriptional augmentation in the presence and absence of HDACis such as sodium butyrate (NaBu) or valproic acid (VPA) is investigated.
CHO-K1 wild type cells are tagged by a set of hetero-specific FRT sites, F and F3. The positive/negative (+/-) selection marker residing between the F and F3 sites is used to select the tagged cells. In the first round of RMCE (RMCE 1), a fluorescent reporter on an analogous exchange cassette replaces the +/- marker. The exchanged cells are enriched by negative selection and sorted for high expressers. In RMCE 2, the +/- marker is used once more to replace the fluorescent marker via a positive selection, which leads to restoration of the initial situation (non-fluorescence and resistant to positive selection). To this end, single clones are screened for presence of a unique targetable site. S/MAR elements E and W are used to flank the +/- selection cassette at the beginning.
2. Results

2.1. Aims of this study

Recombinase-Mediated Cassette Exchange (RMCE) techniques enable the targeted integration of a gene-of-interest (GOI) into a pre-defined genomic locus with desired expression properties. The application of RMCE for cell line developments aimed at biotechnological applications is of obvious advantage for the following reasons:

- the predictable expression of a GOI into a pre-characterized integration locus reduces the variability caused by position effect variegation (PEV)
- the re-use of a defined integration site ensures the homogeneity of cell growth properties
- the re-use of the same integration site will also reduce downstream processing workloads and simplify regulatory approvals.

So far, the bottleneck of RMCE for these purposes lies in the isolation of integration sites with both, the desired expression properties and a consistent exchangeability. In conventional RMCE, the identification of targetable sites relies on extensive screening procedures aimed at individual clones, which, though laborious and time-consuming, does not grant the ultimate success. Therefore the major topic of this study is the development of a new method to efficiently generate clones that bear single copy integration of a targetable genomic site with both a high expression level and a consistent exchangeability. Clones with these properties can form the basis of potential master cell lines. They will be further tested for their exchangeabilities in the absence of any drug selection. Our final goal is a production cell line for various GOIs, derived by a “clean” RMCE procedure (no drug selection, no auxiliary sequences besides the GOI) within a short period of time.
2.2. Strategy I

2.2.1. Overview of Strategy I

For conventional RMCE, drug selection has been the only way to enrich for authentic RMCE events. In our strategy, we introduce one additional round of RMCE to select for readily exchangeable targets. RMCE therefore is used both to distinguish exchangeable from non-exchangeable sites and to integrate the GOI into a suitable genomic target site with predictable expression properties.

These two rounds of consecutive RMCE steps are outlined in Fig 2.1. In this study, we define as a “cassette” a segment of DNA that is flanked by a set of Flp-Recognition Target (FRT) sites. This set consists of an FRT-mutant, “F3” (spacer sequence TTCAAATA) and an FRTwt-site “F” (containing the unchanged spacer sequence TCTAGAAA; Schlake and Bode 1994).

As a first step a so-called “parental cassette”, containing a hygromycin (hyg) thymidine kinase (tk) fusion gene (hygtk) under control of the HSV-thymidine kinase promoter (Ptk), is randomly integrated into CHO-K1 wild type (wt) cells to generate a Hygromycin (Hyg-) resistant strain by selection. This parental cassette can be supplemented, with advantage, by scaffold/matrix attachment regions (S/MARs): Being bordering elements S/MARs have been found to effect transcriptional augmentation by shielding against repressive actions at the integration loci (Bode et al. 2003; Abranches et al. 2005). Moreover, due to their strand-separation propensity (Bode et al. 1992), S/MARs were also found to increase the exchange potential (Qiao 2005). Therefore, in the parental cassette, the 2.2 kb human S/MAR element E (S/MAR-E) and the unrelated 1.3 kb plant S/MAR element W (S/MAR-W) are used as flanks, respectively (Goetze et al. 2005). A corresponding, non-S/MAR-flanked, parental cassette is used for comparison.

The choice of two vastly different S/MARs is dictated to avoid genome instability that might arise from recombination between identical sequences.

In the first exchange reaction (RMCE 1) the parental cassette is replaced by another cassette with the same set of FRT sites (F3 and F, respectively), carried by an “exchange vector”. This exchange vector contains, as a fluorescent marker, the d2egfp gene [a destabilized variant of enhanced green fluorescent protein (egfp) with a half-life of approximately two hours], driven
Results

by a *cytomegalovirus* promoter (P<sub>cmv</sub>). According to the RMCE principle, exchange of the *hyg*<sub>tk</sub> by the *d2egfp* cassette generates fluorescence, which serves as a traceable marker to evaluate the transcription level of targeted cells by FACS analysis (Kalejta *et al.* 1999). Selection is done by Ganciclovir (Ganc), which is a pro-drug excluding non-exchanged parental cells due to the viral suicide *tk* gene encoding the Thymidine Kinase (TK) from HSV [so called “negative-selection” principle (Seibler *et al.* 1998)]; upon the expression of the *tk* gene, Ganciclovir is converted into a phosphorylated nucleotide analog, which, after incorporation into the DNA of replicating eukaryotic cells, causes their death (Tomicic *et al.* 2001). For the population surviving Ganc selection, cells with high *d2egfp* expression can be collected by FACS. To this end, the expression of d2eGFP is ideally due to the RMCE-mediated integration of the exchange cassette. Unfortunately, the random integration of the exchange cassette into cells that acquired a spontaneous Ganc resistance due to either mutation or deletion (or a combination of these two situations) will also lead to fluorescent survivors (Seibler *et al.* 1998). In order to isolate those cells that owe their fluorescence exclusively to the desired RMCE-mediated integration, a second RMCE-step (RMCE 2) has been devised in order to restore the initial situation (non-fluorescence). This is done by replacing the *d2egfp* gene by an exchange cassette encoding a *hyg*<sub>tk</sub> fusion gene, driven by the *phosphoglycerate kinase* promoter (P<sub>pgk</sub>). Such an event causes the targeted cells to lose fluorescence again. Among the non-fluorescent population, clonal cells are screened and characterized by PCR and Southern blot analysis to identify those that in fact underwent two rounds of authentic RMCE. Clones with single copy integration at the unique target site can then be isolated to establish a master cell clone enabling the development of production cell lines for various GOIs. This process can again be supported by a Ganc counter-selection to enrich the authentic exchange events.
A “cassette” is defined as a segment of DNA that is flanked by a set of FRT sites - in this study the wild type FRT and the mutant FRT3 site. S/MAR elements E and W are used to flank the parental cassette to shield the target from heterochromatization and to increase RMCE potential. A parental cassette without S/MAR flanks is used for comparison.

The parental cassette containing the P\textsuperscript{tk}-driven hyg\textsuperscript{tk} fusion is electroporated into CHO-K1 wt cells to generate a Hyg-resistant strain. Following selection, a cassette with the fluorescent marker d\textsuperscript{2}egfp is used to replace the hyg\textsuperscript{tk} cassette via RMCE 1. Formally this means the “clean” exchange of two expression units without the co-introduction of unwanted vector sequences.

Cells with high expression levels can be enriched by FACS and are expected to contain a major RMCE-generated sub-population. Subsequently, in RMCE 2 the initial situation is restored by replacing the d\textsuperscript{2}egfp gene with a hyg\textsuperscript{tk} fusion – in this case driven by the pgk promoter (P\textsuperscript{pgk}). Cells undergoing authentic RMCE are expected to have lost fluorescence after RMCE 2 and can be screened and characterized accordingly. Finally, the clones with a consistent exchangeability and a single copy integration of the target are chosen to develop production cell lines for various GOIs.
2.2.2. Establishment of parental cells in Strategy I

The plasmids F3-hygtk-F and EF3-hygtk-FW (Fig. 2.2) were linearized by Scal or by Ascl for non-S/MAR- and S/MAR-flanked parental cassettes, respectively. After gel-purification, 3 μg linearized DNA of each was electroporated into 1E5 CHO-K1 wt cells. Following electroporation, an appropriate number of cells were seeded into T75 flask with media containing 650 U/ml Hygromycin. After colonies had appeared 7~10 days later, cells were pooled and passaged under Hygromycin selection until the transfection for the first round of RMCE. Continuous Hyg-selection anticipates the expression of an intact hygtk fusion gene and reduces spontaneous resistance phenomena (Seibler et al. 1998). The non-S/MAR- and S/MAR-flanked cell mixtures were named “C1” and “C2” respectively, throughout the following experiments.

![Figure 2.2: Schematic representation of the parental cassettes integrated into CHO-K1 wt cells by electroporation](image)

The Scal- or Ascl-restricted parental plasmids F3-hygtk-F and EF3-hygtk-FW was electroporated into CHO-K1 wt cells, respectively, to generate Hygromycin-resistant cell mixtures, designated as either “C1” (non-S/MAR-) or “C2” (S/MAR-flanked parental cells).

2.2.3. First round of RMCE in Strategy I

The first round of RMCE (RMCE 1) on “C1” (left) and “C2” (right) cells is presented in Fig 2.3A. 3 μg exchange plasmid F3-cmvd2egfp-F with an exchange cassette expressing d2egfp and 1 μg pFlpe ("enhanced Flp" according to Buchholz et al. 1998) were co-transfected into “C1” and “C2” cells respectively (Fig. 2.3A). One day post-transfection, 2.5 μg/ml of Puromycin was added to the culture medium to enforce the expression of Flpe based on the obligatory co-expression of the puromycin resistance gene that is co-expressed from the flpe-IRES-puromycin (flpe-pac) cassette (Taniguchi et al. 1998). Two days post-transfection,
20~30% of the highest d2eGFP expressing population was collected by FACS (Fig 2.3B). Since the S/MAR elements do not augment gene expression in the transient phase, no difference of the transfection efficiency between “C1” and “C2” was observed (Bode et al. 2000a). The sorted cells were named “10/06-C1” and “10/06-C2” for non-S/MAR- and S/MAR-flanked constructs, respectively. Following this step, 10 µg/ml Ganciclovir (Ganc) was added to the culture medium to exclude non-exchanged “C1” and “C2” cells. After 10~14 days, colonies had developed. After “10/06-C1” and “10/06-C2” cells had grown to confluence, the expression pattern of each population was determined by FACS analysis (Fig 2.3C). The fact that cells in “10/06-C2” expressed more d2eGFP than those in “10/06-C1” can, in principle, be due to several reasons:

1. S/MARs flanking the targeted cassette can alleviate silencing effects from the gene environment and thereby retain expression properties;
2. S/MARs can increase transfection efficiencies, i.e. the number of integrated transgene-copies, possibly due to their effect on single-strand DNA (ssDNA-) end formation (Klehr et al. 1991);
3. Due to their strand-separation potential, S/MARs have the potential to increase the rate of RMCE, which leads to a higher contribution of exchanged cells within the survivors.

Since only the cells with high expression level were of interest, the second sorting by FACS (Fig. 2.3C) was performed to collect the highest eGFP expressing cells from both “10/06-C1” (1%) and “10/06-C2” (10%), which were termed “10/06-C1-S” and “10/06-C2-S” (Fig 2.3D) accordingly. In order to verify the components of cell mixtures, several PCRs were performed on the genomic DNA both for “10/06-C1-S” and “10/06-C2-S”.

The first PCR was performed to trace the authentic exchange events within the high expressers that had been enriched by double sorting. Primer pairs were designed such that the forward (5’-) one was positioned upstream from the F3 site for the parental plasmid and the backward (3’-) one was placed within the exchanged cassette (F3-cmvd2egfp-F), as indicated (p2494/pGFP6 for “10/06-C1-S” and p2495/pGFP6 for “10/06-C2-S”) in Fig. 2.3A. Upon targeted integration by RMCE the amplified segment will be unique. PCRs on genomic DNA of CHO-K1 wt cells and on parental cells “C1” and “C2” were performed as negative controls. Authentic exchange events gave rise to a ~1.0 kb band and a ~900 bp band for non-S/MAR- and S/MAR-flanked cells respectively, as indicated in Fig 2.3E.
**Results**

Figure 2.3: First round of RMCE in Strategy I

**A:** An exchange plasmid containing a cassette (flanked by F3 and F sites) with the d2egfp gene under control of the cmv promoter (P\textsuperscript{cmv}) was co-transfected along with pFlpe into “C1” and “C2” cells (see Fig 2.2). Flpe is encoded as part of a bicistronic flpe-IRES-puromycin (flpe-pac) cassette. **B:** At the transient stage, 20~30% of highly expressing cells were sorted by FACS and then subjected to Ganc selection. **C:** Ganc-resistant cells were sorted once more to obtain the highest expressing cells (1% for non-S/MAR- flanked cells; 10% for S/MAR-flanked cells). **D:** Double-sorted cells showed stable fluorescent expression. **E:** PCR was performed to verify authentic exchange events in the fluorescent cells shown in “D”. The primer pairs used for PCR verification are indicated on the exchanged cassettes in “A”. PCRs on H\textsubscript{2}O and genomic DNA of CHO-K1 wt, “C1” or “C2” were performed as negative controls (-). M: 1.0 kb marker.
The second PCR was performed to detect whether the silencing of the \textit{tk} gene in the parental cassette led to the survival of non-exchanged cells among “10/06-C1-S” or “10/06-C2-S”. Therefore a primer pair was designed such that the forward one (5\textquotesingle-) was placed upstream from the F3 site for the parental plasmid while the backward one (3\textquotesingle-) was introduced into the parental cassette, F3-hygtk-F (p2494/p2471 for non-S/MAR-flanked cells and p1452/p2471 for S/MAR-flanked cells, see Fig 2.3A). PCRs on the genomic DNA of “C1” and “C2” were performed as positive controls. A band of \(~520\) bp and of \(~1.1\) kb for “10/06-C1-S” and “10/06-C2-S” respectively were found not only in the parental cells (positive controls) but also in the cell mixture after RMCE 1 (Fig 2.4), which indicated the silencing of the \textit{tk} gene caused the Ganc-resistance of at least some parental cells after RMCE 1. In addition, the loss or mutation of the \textit{tk} gene may have led to spontaneous Ganc-resistance as well.

\textbf{Figure 2.4: PCR analyses trace silenced \textit{tk} genes for “10/06-C1-S” and “10/06-C2-S” cells} 

PCR reactions on genomic DNA of “10/06-C1-S” and “10/06-C2-S” cells were performed with the primer pair indicated in Fig 2.3A. The same PCRs were performed with genomic DNA of “C1” and “C2” (Fig 2.2) as positive controls (+) for non-S/MAR- and S/MAR-flanked cells respectively. PCR reactions on H\textsubscript{2}O and genomic DNA of CHO-K1 cells were used as negative controls (-). The PCR signals indicate the existence of silenced \textit{tk} genes in Ganc-resistant “10/06-C1-S” and “10/06-C2-S” cells. M: 1.0 kb marker.

The third PCR was performed to detect whether random integration(s) of the exchange plasmid also contributed to the GFP expression of “10/06-C1-S” and “10/06-C2-S” cells. The primer pair p1230/p2484 is indicated on the exchange vector F3-cmvd2egfp-F (Fig. 2.3A), which led to a 900 bp amplified fragment as a positive control. PCR analyses were performed on the genomic DNA of “10/06-C1-S” and “10/06-C2-S”. As shown in Fig 2.5, a randomly integrated F3-cmvd2egfp-F construct was detected for both “10/06-C1-S” and “10/06-C2-S”
cells but not in parental cells “C1” and “C2” that served as negative controls.

![PCR gel image](image_url)

**Figure 2.5: PCR analyses to visualize random integration(s) of the exchange plasmid in RMCE 1**

PCR was performed as indicated in figure 2.3A on the exchange vector F3-cmvd2egfp-F. A 900 bp PCR band with genomic DNA of “10/06-C1-S” and “10/06-C2-S” showed that there were random integration(s) of the plasmid in the florescent cells after RMCE 1. PCRs on genomic DNA of CHO-K1, “C1” and “C2” for non-S/MAR- and S/MAR-flanks were performed as negative controls (-). PCR on the exchange plasmid F3-cmvd2egfp-F was done as a positive control (+). M: 1.0 kb marker.

The positive PCR results above underline the complexity of the cell mixture after RMCE 1 by Ganc counter-selection. First of all, not only RMCE-targeted d2egfp, but also randomly integrated d2egfp genes contributed the fluorescence of the cell mixture. Secondly, the counter-selection with Ganc was insufficient to kill all non-exchanged parental cells, which led to the spontaneous resistance of Ganc via epigenetic silencing. Therefore we have to deal with different integrations of exchange cassette contributing the fluorescence of the cell mixtures:

- only RMCE-targeted integration of the d2egfp gene, i.e. the expected targets
- only randomly integrated d2egfp gene into cells containing inactivated tk gene that causes Ganc-resistance
- both targeted and random integration of the d2egfp gene.

With the conventional RMCE strategy, intense screening has to be performed to recover the desired events, but both the complexity of the cell mixture and an unknown exchange rate will require tedious and time-consuming work to isolate the correct targets. Therefore, the second round of RMCE was introduced to circumvent these problems.
2.2.4. Second round of RMCE in Strategy I

The second round of RMCE (RMCE 2) is presented in Fig 2.6A for non-S/MAR- (left) and S/MAR-flanked cells (right) respectively. In order to isolate the RMCE-positive cells from the mixture, the hyg<sub>tk</sub> fusion gene was used once more to restore the initial situation during the second round of RMCE. The replacement of the <i>tk</i>- by a <i>pgk</i>-promoter on the exchange cassette was included to distinguish the exchanged <i>hyg<sub>tk</sub></i> cassette from the parental <i>hyg<sub>tk</sub></i> cassette. 3 μg exchange plasmid F3-<i>pgk</i><i>hyg<sub>tk</sub></i>-F and 1 μg pFlpe were co-transfected into “10/06-C1-S” and “10/06-C2-S” cells, respectively. In addition, a transfection without pFlpe was performed in parallel as a negative control. In this case, a neutral plasmid, BSpac-Δp (de la Luna et al. 1988), encoding Puromycin expression only was co-transfected to compensate for variations in the total amount of DNA and transfection agent. One day post-transfection the cells were transiently treated by 2.5 μg/ml Puromycin for the reasons delineated in chapter 2.2.3. Three days post-transfection, the cells were passaged into the medium containing 650 U/ml Hygromycin. After ~10-14 days, colonies that survived from Hygromycin selection were recovered.

The cells transfected without pFlpe (-Flp) grew as fast as those with pFlpe co-transfection (+Flp), which indicated that the phenomenon was mostly due to a randomly integrated exchange vector causing the Hygromycin resistance. After reaching confluence, cells were suspended for FACS analysis. The expression pattern for the non-S/MAR- and S/MAR-flanked cases with and without pFlpe co-transfection, respectively, is shown in Fig 2.6B. For non-S/MAR-flanked targets, there was no difference between +Flp cells and -Flp cells, which indicated that a consistent exchangeability for the cells that underwent RMCE 1 was not granted. Remarkably, however, for S/MAR-flanked targets, there was a population of non-fluorescent cells arising from the action of pFlpe, which indicated the continuous exchange of the same locus (<i>loci</i>) targeted in RMCE 1. In order to detect the authentic exchange events within this candidate population (L), PCR was performed with primer pairs p2494/p2822 for non-S/MAR-flanked targets and p1452/p2822 for S/MAR-flanked targets, which were directed towards the <i>pgk</i> promoter, as indicated in Fig 2.6A. As expected, only cells with S/MAR-flanked targets gave rise to a positive ~1.0 kb band on the PCR gel (data not shown), which was consistent with the results from FACS analysis.
Figure 2.6: Second round of RMCE in Strategy I

A: The exchange cassette containing the hyg′t′ gene under control of a pgk promoter (P^pgk) was co-transfected with pFlpe (+Flp) into “10/06-C1-S” and “10/06-C2-S” cells, respectively. A transfection without pFlpe (-Flp) was used as a negative control. B: FACS was used to evaluate the expression pattern of cells surviving Hyg selection. The S/MAR-flanked cells (right column), which were co-transfected with pFlpe (44/06+Flp), were split, by FACS, into a highly fluorescent population (H) and a non-fluorescent population (L). Clonal cells were isolated from the “L” population. C: PCRs were performed as for “A” on the genomic DNA of pFlpe co-transfected and Hyg-selected cell mixture (“44/06+Flp”, “H” and “L” population) and on isolated single clones. Negative controls were performed with H₂O, “C2” and non-pFlpe co-transfected and selected cells (“44/06-Flp”). Repetition of the same experiment with pFlpe co-transfection was performed as a positive control. M: 1.0 kb marker.
Therefore, after two rounds of consecutive RMCE, the exchangeable cells that gained fluorescence only by targeted integration of d2egfp through RMCE 1 have lost the fluorescence by a successful replacement of the d2egfp with a hygk fusion. The S/MAR-flanked cell mixture +Flp co-transfection was designated “44/06+Flp”, the one -Flp “44/06-Flp”. Consistently exchangeable cells were enriched and single clones from the non-fluorescent population of “44/06+Flp” were screened by FACS (Fig 2.6B).

2.2.5. Characterization of single clones screened after two consecutive RMCEs by PCR and Southern blot analysis

First of all, “44/06+Flp” cells were dissected, by FACS, into two populations, the non-fluorescent population (L) and fluorescent population (H; see Fig 2.6B). To verify authentic RMCE events, the PCR indicated in Fig 2.6A was performed on both populations and on the cell mixture (Fig 2.6C). PCR on genomic DNA of “44/06-Flp” was performed as a negative control. An authentic exchange band was found only for the non-fluorescent population (L) but not for the fluorescent population (H). The reason for the survival of population “H” as well as of cells without pFlpe co-transfection (“44/06-Flp”) from Hyg selection is probably due to the random integration of the exchange vector F3-pgkhygk-F, which has led to Hygromycin resistance. Among the non-fluorescent population (L), 59 single clones were screened by limited dilution. The same PCR was performed to detect the authentic exchange events in the screened single clones, among which 34 showed authentic exchange events. The PCR result of 5 representative clones is shown in Fig. 2.6C. PCRs on H2O and “10/06-C2-S” cells were performed as negative controls. A repetition of the same experiment (with pFlpe co-transfection) served to generate a positive PCR control.

In chapter 2.2.3, a silenced parental tk gene was found in the potentially targeted cells after the first round of RMCE. Therefore we wondered whether the screened single clones after RMCE 2 also bear copy(ies) of the parental hygk cassette. In order to clarify this point, a primer pair p2834/p2471 was designed such that the forward one (5’-) resided upstream of the F3 site in the parental cassette and the backward one (3’-) in the hygk gene, as shown in Fig 2.7A. Therefore we would be in the position to detect both, a silenced parental F3-hygk-F cassette and an exchanged F3-pgkhygk-F cassette. These situations can be distinguished
due to the different sizes of the \(tk\)- relative to the \(pgk\)-promoter by PCR amplification.

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**Figure 2.7 Characterization of single clones isolated from the “L” population of “44/06+Flp” by PCR and Southern-blot analyses**

**A:** Genomic DNA of single clones screened in Fig 2.6B was digested with \(Spel\). \(Spel\) introduces a single cut in the parental cassette but cuts twice in the exchanged cassette. **B:** PCRs were performed as for “A” on “44/06+Flp”, separated “L” and “H” populations and on isolated single clones. PCRs with \(H_2O\), “10/06-C2-S”, \(CHO-K1\) wt cells and “44/06-Flp” cells were performed as negative controls (-). **C:** Hybridization with a \(tk\)-specific probe (a) on \(Spel\)-digested genomic DNA of single clones 51 ~ 57 was used to confirm an authentic exchange reaction (2.6 kb) and a single copy of non-exchanged parental cassette (>5.1 kb, single band). **D:** Hybridization with an S/MAR-E-specific probe (b) served to detect a single copy integration of the exchanged cassette (>2.9 kb, single band) and to probe for a single copy of the parental cassette (>5.1 kb, single band) that might be left in each single clone.
Consistently, a band of 770 bp was found for the exchanged cassette in all the clones that showed authentic exchange (with primer pair p1452/p2822 in Fig. 2.6C). Surprisingly, however, a band of 405 bp for the parental cassette was also found in all single clones that showed an authentic exchange event. Figure 2.7B contains the PCR result for five examples of the single clones as analyzed in Fig 2.6C. PCRs on CHO-K1 cells, “10/06-C2-S” and “44/06-Flp” served as negative controls. Therefore, among the authentic exchange clones, there are silenced parental copies left in the genome, which might have caused non-anticipated recombination events that interfere with the expression of the targeted cassette due to the existence of multiple copies of the hygk gene.

So far, the characterization has concentrated on the existence of authentic exchange events in the screened single clones but not on the actual copy numbers of the target. PCR results showed that the exchanged hygk cassette and the silenced parental hygk cassette co-existed for 34 out of 59 single clones. In order to determine the copy numbers of the exchanged hygk and the parental hygk, Southern-blot analysis was performed on the genomic DNA of the single clones (Fig 2.7C and D). First, genomic DNA of the single clones were digested with SpeI: SpeI cuts once downstream of the F site on the parental hygk cassette but cuts twice in the exchanged hygk cassette (downstream of the F site and in the pgk promoter, as shown in Fig 2.7A). The first hybridization was performed with a tk-specific probe to determine the RMCE-mediated integration of the hygk cassette. Fig 2.7C shows the hybridization pattern of PCR positive clones 51,52,53,54,55,56,57. A defined 2.6 kb band indicates the exchanged hygk cassette but yields no information about the copy number. A single band >5.1 kb indicates a single copy integration of the silenced parental cassette left in the genome of each single clone. In order to determine the copy number of the exchanged cassette, a second hybridization with an S/MAR-E-specific probe was performed on the same membrane after stripping. As shown in Fig 2.7D, a single band >5.1 kb confirmed a single copy integration for the silenced parental cassette, while a single band >2.9 kb indicated a single copy integration of the exchanged cassette. Therefore, combining two hybridizations on the same DNA samples, a single copy of parental cassette and a single copy of exchanged cassette were found in all the authentically exchanged single clones. Nevertheless, the diversity among these clones was very low according to the similar hybridization pattern. Although ideal clones with a unique target site were not found, clones with at least one copy of
targetable site could be fully characterized. In order to use these lines to generate production lines, “clone 57” was chosen as a master cell line for further RMCE.

2.2.6. Third RMCE based on the characterized single clones

As anticipated, production cell lines could be derived from the master lines bearing a unique site that is targetable via RMCE. A negative selection with Ganc can eliminate the non-exchanged cells due to the expression of the tk gene. But from the results shown in RMCE 1, Ganc counter-selection is insufficient to kill non-exchanged cells; it rather leads to high ratio of silenced tk genes. Therefore, before exchanging for a GOI, a third RMCE is necessary to restore the fluorescent marker, after which the exchange of the GOI can be easily distinguished from non-exchanged cells by absence of the fluorescence after targeting.

2.2.6.1. Third RMCE with positive selection

Based on its comprehensive characterization in Fig 2.7C and D, “clone 57” was chosen for the third round of RMCE. The strategy is presented in Fig 2.8A.

The exchange cassette encoding a neomycin (neo) and d2egfp fusion gene (neo-d2egfp) under a cmv promoter (Pcmv) was co-transfected into “clone 57” along with pFlpe. In parallel, a transfection without pFlpe was performed as a negative control. After transient selection with 2.5 μg/ml Puromycin and stable selection with 500 μg/ml G418, only the pFlpe co-transfected (+Flp) but not the non-pFlpe co-transfected (-Flp) cells did survive. This indicated the targeted integration of neo-d2egfp fusion, which constituted the Neomycin-resistance for the surviving cells. FACS analysis was performed to evaluate the expression levels of surviving cells. As presented in Fig 2.8B, the majority of these cells showed eGFP expression. In order to verify authentic RMCE events, PCR was performed on the survivors with the primer pair p2834/pGFP6 shown in Fig 2.8A. As a negative control a PCR on “clone 57” was performed as well. For the survivors, a 1.5 kb band showed the authentic exchange (Fig 2.8C). Due to the death of cells without pFlpe co-transfection, in this case there should be very low ratio of randomly integrated exchange cassettes.
Results

2.2.6.2. Third RMCE in the absence of selection

Our final goal is to perform RMCE without any drug selection. Since “clone 57” can be exchanged under selection pressure, this clone was further exchanged in the absence of any drug selection. An experiment corresponding to the one in chapter 2.2.6.1 is presented in Fig 2.9A. The only difference is that there is no selection marker on the exchange cassette. The same control in the absence of pFlpe expression was also performed. 2 days post-transfection, the 10-20% highest expressers were collected by FACS, for both pFlpe transfected (+Flp) and non-pFlpe transfected (-Flp) cells (data not shown). After the sorted cells had grown to confluence, there was no difference between the expression level of +Flp
cells and -Flp cells. Therefore a second sorting was conducted to enrich 10% of the highest expressing cells again (data not shown). After that, the expression pattern of the double-sorted cells was evaluated by FACS analysis (Fig 2.9B). Unfortunately, there was still no difference between the +Flp cells and -Flp cells. PCR with the primer pair p2834/pGFP as designed in Fig 2.9A was performed on the +Flp cells and -Flp cells, respectively. PCR on “clone 57” served as a negative control. Surprisingly, a band of ~900 bp verified the authentic exchange event for +Flp transfected cells. Therefore, in the absence of drug selection, RMCE is still feasible at the consistently exchangeable site, but the random integration of the exchange construct is found to interfere with the authentic targeting event. Under this circumstance, the isolation of clones with only targeted integration still involves time-consuming, laborious screening processes.

Figure 2.9: Third RMCE on “clone 57” in the absence of selection
A: The exchange cassette with the d2egfp gene driven by a cmv promoter (Pcmv) was transfected into “clone 57” along with pFlpe (+Flp). A transfection without pFlpe (-Flp) was performed as a negative control. B: High expressers were enriched by two consecutive FAC-sortings for cells with and without pFlpe co-transfection. The expression pattern of the sorted cells was evaluated by FACS analysis. C: PCR with the primer pair designated in “A” was performed on the double sorted cells, depending on pFlpe co-transfection. PCR on “clone 57” was performed as a negative control (-). M: 1.0 kb marker.
2.3. Strategy II

2.3.1. Overview of Strategy II

Although Strategy I has been proven to be able to efficiently isolate clones bearing targetable sites, it has revealed a number of un-anticipated difficulties discouraging its further use:

1- the expression of targeted cassette was interfered with randomly integrated exchange cassette (Fig 2.5 and 2.9);

2- the negative selection strategy with Ganc caused too many spontaneous resistance events within the transfected and selected cells (Fig 2.3C);

3- among the cells with multi-copy integrations of the parental cassette, some copies turned out to be inactive regarding expression and exchange after counter-selection with Ganc. Therefore the selection process is expected to enrich only parental clones with a single target site (Fig 2.7);

4- due to the ineffective negative selection step, a third round of RMCE became necessary to mark the master cells with a fluorescent gene. Using this marker the targeted cells can be separated by FACS according to the loss of fluorescence after the targeted integration of a GOI (Fig 2.8 and 2.9).

In order to overcome these problems, our optimized Strategy II was developed. As for Strategy I, Strategy II is based on two consecutive RMCEs (Fig 2.10). S/MAR elements are used to flank the exchange cassette due to the anticipated RMCE-support indicated in Fig 2.3C and 2.6B. A parental cassette containing the d2egfp gene under control of the cmv promoter, with or without S/MAR-E and S/MAR-W elements, is electroporated into CHO-K1 wt cells. High expressers can then be collected by FACS.

Different from Strategy I, a straightforward “promoter trap” principle is employed in which the promoter driving the expression of the reporter gene is positioned outside the exchange cassette (Seibler and Bode 1997; Lauth et al. 2002), but upstream from the F3-d2egfp-F cassette. This situation guarantees that the exchange cassette can only be expressed upon targeted integration.
Results

Figure 2.10: Overview of Strategy II

A parental cassette encoding the d2egfp gene under control of the cmv promoter (P<sub>cmv</sub>) is microporated into CHO-K1 wt cells. Different from Strategy I, the promoter is positioned externally from the parental cassette, upstream from the F3 site. Thereby only RMCE-mediated integration of the exchange cassette can drive the reading frame of the targeted gene. S/MAR elements E and W are used to flank the parental cassette to shield the target from heterochromatization and to increase RMCE potential by its strand-separation propensity. The parental cassette without S/MARs is used for comparison. High d2eGFP expressers can be enriched by FACS multi-sorting. Following these steps, a promoter-less exchange cassette containing the hyg<sub>tk</sub> fusion is used to replace the d2egfp gene (RMCE 1). Potentially targeted cells can be collected by FACS due to the abolishment of fluorescence. In RMCE 2, a promoter-less exchange cassette containing the egfp-<i>tk-neo</i> (<i>GTN</i>) fusion is used to restore the initial fluorescence expression. High expressers can be screened via their fluorescent intensity. Finally, the clones with a single copy integration of the target and consistent exchangeability are chosen to develop production cell lines for various GOIs.

In the first round of RMCE, a promoter-less exchange cassette containing a hyg<sub>tk</sub> fusion is used to replace the d2egfp gene in the parental cells. Due to the simultaneous disappearance
of the fluorescence, the potentially targeted cells can then be collected by FACS for RMCE2. At the same time the cells bearing a single copy integration of the target will be enriched. Consequently, only the cells bearing a single copy of the parental cassette (or multiple copies integration, which are all replaced by F3-hygtk-F) will lose their fluorescence entirely. In the second round of RMCE, a promoter-less exchange cassette containing a fusion GTN gene encoding eGFP, TK and Neomycin protein is used to replace the hygtk fusion. The fluorescence is restored upon targeted integration of the GTN fusion by RMCE. To this end, the highest expressers can be screened and characterized to achieve variable master cell lines with the single copy integration at a unique targetable site that has undergone two rounds of RMCE. Master lines containing a single copy of the F3-GTN-F construct driven by the cmv promoter can then be used directly for the generation of production cell lines. The replacement of F3-GTN-F by various GOIs can be counter-selected by Ganc due to the tk gene in the master cells and/or collected by FACS due to the abolishment of fluorescence.

This optimized strategy was specifically designed to overcome the problems that had become apparent in Strategy I:

**Purpose 1: to overcome the interference from random integration(s) of the exchange plasmid.** In the initial strategy, random integration(s) of the exchange cassette F3-cmvd2egfp-F not only contributed to the fluorescence of the cells but could also constituted a secondary target site for further rounds of RMCE. This is why the “promoter trap” strategy (Fig 2.10) was employed, in which the promoter that originally resided between the F3 and F sites is positioned upstream from the F3 site. Therefore, using a promoter-less exchange plasmid and a Flp expression plasmid, the exchange construct will be expressed only upon correct RMCE-mediated integration (or the adventitious integration downstream from an endogenous promoter, which, however, is a rare event). Therefore a random integration of the exchange plasmid will impede the transcription of the contained cassette. In case of a random integration, the exchange cassette will in no case be strongly expressed, which reduce the interference with the expression of the targeted cassette.

**Purpose 2: to enrich the cells with single copy integration of the target.** For Strategy I, there was no selection step to enrich cells with a single copy target. For the existing studies, the selection of such clones had to rely on the screening of numerous individual clones derived by different gene transfer techniques (Baer et al. 2000), but even in this case the
Results

considerable efforts do not guarantee a success. With the present Strategy II, in the first round of RMCE the cells lose their fluorescence only upon total replacement of F3-d2egfp-F by F3-hygtk-F, regardless of whether we have to deal with single- or multi-copy integration of the parental cassette. Cells for which only some copies of the parental construct have been replaced will still keep the expression of the fluorescent marker and can be eliminated by FACS. Therefore the cells with a single copy integration of the exchangeable target or multi-copy integrations of exchangeable targets become strongly enriched lending themselves for further RMCE.

**Purpose 3: to generate master lines which can be directly used for the development of production cell lines through different means of selection processes.** For Strategy I, the master cell lines contain a F3-hygtk-F cassette, meaning that Ganc counter-selection is the only way to enrich RMCE-positive cells. However, as indicated in the first round of RMCE, Ganc counter-selection is insufficient to eliminate all non-exchanged parental cells - it rather leads to a high proportion of cells with a silenced tk gene. If the negative selection is omitted, the targeted cells cannot be distinguished from non-exchanged master cells in the absence of a drug selection. Therefore a third round of RMCE becomes necessary to mark the master cell lines with fluorescence once more for further exchange of GOIs, which can then be distinguished from non-exchanged master cells by FACS (Fig. 2.8). In order to circumvent this third round, we start the first round of RMCE with parental cells that are already marked with a fluorescent gene. In this case the targeted cells end up with the fluorescent marker gene already after two rounds of RMCE,. As a consequence, master cell lines can be screened and characterized at this stage and can directly be used for the generation of production cell lines. In addition, for the master cell lines persistence of the expression level can be continuously verified by the intensity of fluorescence. Finally production cell lines with targeted integration of the GOI can be directly collected by FACS due to their loss of fluorescence. As a security matter the egfp-tk-neo fusion gene provides the additional option to select the targeted cells by Ganc counter-selection due to the expression of tk gene.

In summary, Strategy II, along with two rounds of consecutive RMCEs, has the potential to enable the efficient isolation of clones with a high expression level and a consistent exchangeability.
2.3.2. Establishment of parental cells in Strategy II

In order to evaluate the expression level for different integration sites under natural conditions, the parental cells were generated in the absence of drug selection. 5 µg DralI linearized plasmid cmvF3-d2egfp-F or Ascl linearized EcmvF3-d2egfp-FW (Fig 2.11A) was transferred into 5E5 CHO-K1 wt cells by a Microporator. The microporated cells were expanded in T75 flask with normal medium. Two days after microporation, 2% of the highest d2GFP expressing cells were collected, by FACS, both for the non-S/MAR- and S/MAR-flanked situations (Fig 2.11B). In T75 flasks, confluence was reached within 5 days, after which the second sorting was performed to enrich for the top 10% of d2GFP expressing cells (Fig 2.11C). Sorted cells were designated “13/08-C1” and “13/08-C2” for non-S/MAR- and S/MAR-flanked cells, respectively (Fig 2.11D). The expression level of “13/08-C1” and “13/08-C2” was regularly evaluated by FACS analysis. For both cases an overlay analysis was performed at passages 1, 4 and 26 (Fig 2.11E). Since highly active transcriptional sites had been enriched by double-sorting, at early passages the expression levels were comparable due to the same enrichment process. However during long term culture, the S/MAR-flanked cells turned out to be more stable since fewer cells were silenced compared with the non-S/MAR-flanked cells. This confirms earlier observations that S/MARs can protect the transgene from the repressive effects of a heterochromatic environment and maintain the expression level during long term culture.
Figure 2.11: Establishment of parental cells in Strategy II

A: A DraIII-restricted non-S/MAR-flanked parental plasmid, cmvF3-d2egfp-F, or a related Ascl-restricted S/MAR-flanked parental plasmid EcmvF3-d2egfp-FW was microporated into CHO-K1 wt cells, respectively. B: Two days post-microporation 2% of the highest d2eGFP expressers were collected by FACS. C: After the sorted cells had reached confluence, the second sorting step was performed to enrich 10% of the highest expressing cells. D: The expression pattern of double-sorted cells is shown, (non-S/MAR- and S/MAR-flanked cells are designated “13/08-C1” and “13/08-C2”, respectively). E: The expression stability of “13/08-C1” and “13/08-C2” was evaluated by an overlay analysis of the expression level at different generations (passage 1, 4 and 26).
2.3.3. First round of RMCE in Strategy II

The first round of RMCE in Strategy II is presented in Fig 2.12A for non-S/MAR- and S/MAR-flanked parental cells, respectively. 3 μg of exchange vector F3-hygtk-F(w/p), containing a promoter-less hygtk fusion gene, was co-transfected together with 1 μg pFlpo (expressing “flpo-pac”) into “13/08-C1” and “13/08-C2” cells (Fig 2.12B). Flpo is a mouse-codon optimized Flp-variant, with recombination efficiencies similar to Cre (Raymond and Soriano 2007). A RMCE negative control (performed in the absence of pFlpo) was done in parallel, for which a neutral DNA only encoding Puromycin (“BSpac-Δp”) was co-transfected to adjust the same ratio of DNA and transfection agent. For the reason indicated in chapter 2.2.3., the cells were transiently selected with 2.5 μg/ml Puromycin for two days, starting one day post-transfection. 3 days post-transfection, an appropriate number of cells were passaged into medium containing 650 U/ml Hygromycin. After 5 days, colonies had grown for the pFlpo co-transfected cells (+Flp) but not for the non-pFlpo co-transfected cells (-Flp; see digital microscopic pictures in Fig 2.12C), indicating that the survivors were probably due to targeted integration of the hygtk gene downstream from the cmv promoter, which drives the expression of the hygromycin gene. The pooled non-S/MAR- and S/MAR-flanked cells were named “17/08-C1” and “17/08-C2”, respectively. The expression patterns of “17/08-C1” and “17/08-C2” were evaluated by FACS analysis. For both cell mixtures, a large population of non-fluorescent cells emerged (Fig 2.12D), which is the second point of evidence for RMCE-mediated integration. The survival of some fluorescent cells may be a consequence of only partial integration(s) of the hygtk gene into cells with multi-copies of parental cassettes. In theory, targeted integration of only one copy of exchange cassette is sufficient to constitute the Hyg-resistance, while the rest copies of parental cassette can keep the fluorescence expression. Therefore the non-fluorescent population “L” of each cell mixture was isolated by FACS (Fig 2.12D) and designated “17/08-C1-L” and “17/08-C2-L” respectively (Fig 2.12E). Meanwhile, the fluorescent population “H” was isolated as well.
Figure 2.12: First round of RMCE in Strategy II

A: Schematic presentation of RMCE 1 in Strategy II: The promoter-less exchange plasmid containing the hygtk fusion gene was co-transfected into non-S/MAR- and S/MAR-flanked cells ("13/08-C1" and "13/08-C2") along with pFlpo (+Flp). A transfection without pFlpo (-Flp) was used as a negative control. B and C: For cells surviving Hygromycin selection, photos were taken under a microscope. D: FACS analyses were performed on the survivors for "17/08-C1" and "17/08-C2", i.e. on pFlpo co-transfected cells. Populations of non-fluorescent cells (L) as well as high fluorescent cells (H) for "17/08-C1" and "17/08-C2" were separated by FACS and expanded under Hygromycin for PCR analyses and subsequent RMCE reactions.
In order to verify authentic exchange events in the Hyg-resistant cells, PCRs were performed with the genomic DNA of “17/08-C1” and “17/08-C2” cells, respectively. Primer pairs were designed such that the forward one (5’-) was positioned upstream from the cmv promoter and the backward one (3’-) within the exchange cassette, as indicated in Fig 2.12A for the primer pairs 2546/p2471 (“17/08-C1”) and p1515/p2741 (“17/08-C2”). PCRs on the genomic DNA of parental cells “13/08-C1” and “13/08-C2” were performed as negative controls. Unique bands of ~800 bp for non-S/MAR-flanked cells and of ~1.5 kb for S/MAR-flanked cells were found on the PCR gel (Fig 2.13). The same PCR was performed on the sorted sub-populations, “L” and “H”, for non-S/MAR- and S/MAR-flanked cells respectively.

Figure 2.13: Characterization of authentic exchange events after RMCE 1 by PCR analyses

PCR was performed as for Fig 2.12A to verify authentic exchange events for “17/08-C1”, “17/08-C2” and the “L” and “H” populations collected by FACS after RMCE 1. PCRs with H₂O and the parental cells (“13/08C1” and “13/08-C2”) were performed as negative controls (-). M: 1.0 kb marker.

Positive results from PCR indicated authentic exchange events among the non-fluorescent population (L) as well as partially targeted integration of the hyg tk gene in the fluorescent population (H). The non-fluorescent population “17/08-C1-L” for non-S/MAR-flanked cells and “17/08-C2-L” for S/MAR-flanked cells were expanded for the second round of RMCE.

In order to verify our initial expectation that RMCE can be performed in the absence of any selection pressure, the transfected cells were not further selected with Hygromycin after the transient selection step in Puromycin but were rather expanded directly into normal medium. PCR was performed as described above on genomic DNA of pFlpo co-transfected and
non-pFlpo co-transfected cells. Authentic exchange bands were neither found for unselected cells with pFlpo co-transfection nor without pFlpo transfection (data not shown). Therefore, if RMCE has been performed on cell mixtures, drug selection is still necessary in order to enrich for authentic events.

### 2.3.4. Second round of RMCE in Strategy II

The second round of RMCE in Strategy II is presented in Fig. 2.14A, again for non-S/MAR- and S/MAR-flanked cells. 3 μg of exchange vector containing a promoter-less egfp-tk-neo (GTN) fusion gene, “F3-GTN-F(w/p)”, was co-transfected along with 1 μg pFlpo (+Flp) into “17/08-C1-L” or “17/08-C2-L” cells (Fig 2.14B). The same negative control (-Flp) used for RMCE 1 was performed in parallel. After selection with 2.5 μg/ml Puromycin for 2 days, the transfected cells were pooled in medium containing 500 μg/ml G418. For +Flpo cells, colonies became apparent after 7 days under G418 pressure, while very few colonies survived in case of -Flp cells. This difference was documented by digital photos taken under the microscope (Fig 2.14C). The survival of a few colonies in -Flp cells was due to the fact that the cryptic promoter sequence in the ampicillin gene initiated some expression of the GTN fusion, which became evident from the transient expression of eGFP on F3-GTN-F(w/p) in CHO-K1 wt cells (data not shown). After the surviving cells had grown to confluence, the expression pattern was evaluated by FACS analysis, as shown in Fig 2.14D. Both for non-S/MAR- and S/MAR-flanked cells, a population of fluorescent cells was detected only for the +Flpo case, which indicated the RMCE-dependent targeted integration of the GTN fusion downstream from the cmv promoter. A few weak eGFP expressers were also detected for the -Flp case, which is likely due to random integration of exchange cassettes next to a cryptic promoter. Since only high expressers are of interest for the generation of production cells, 10% of the highest eGFP expressing cells (H) were selected by FACS. Some portion of the non-fluorescent population (L) was collected for control purposes. In order to further prove that expression of eGFP in the +Flpo case can be ascribed to the authentic RMCE-mediated integration of the GTN fusion, PCR was performed as indicated in Fig. 2.14A. Primer pairs were designed such that the forward one (5'-) was posed upstream from the cmv promoter and the backward one (3'-) within the exchange cassette as indicated (p2546/p1513 for non-S/MAR-flanked cells and p1452/p1513 for S/MAR-flanked cells). PCRs on “17/08-C1-L”,
“17/08-C2-L” and -Flp cells were used as negative controls both for non-S/MAR- and S/MAR-flanked cells respectively.

Figure 2.14: Second round of RMCE in Strategy II

A: Schematic presentation of RMCE 2 in Strategy II: The promoter-less exchange plasmid containing the GTN fusion gene was co-transfected into non-S/MAR- and S/MAR-flanked cells (“17/08-C1-L” and “17/08-C2-L”), along with pFlpo (+Flp), respectively. A transfection without pFlpo (-Flp) served as a negative control. B and C: For cells surviving G418 selection, digital photos were taken under a microscope. D: FACS analyses were performed on the survivors, both for cells with and without pFlpo co-transfection. The populations of highly fluorescent cells (H) and of non-fluorescent cells (L) were separated by FACS. Single clones were isolated from the “H”-populations both for non S/MAR- and S/MAR-flanked cells respectively.
Results

Fig 2.15 demonstrates that authentic exchange events could be detected for the +Flp cases as well as for the separated “H” and “L” populations, but not for the negative controls. The authentic exchange events in the “L” population were likely due to the integration of the GTN fusion, which was silenced. To verify this assumption, single clones from the “H” population of non-S/MAR- and S/MAR-flanked cells were screened and characterized.

**Figure 2.15: Characterization of authentic exchange events after RMCE 2 by PCR**
The primer pairs were designed as for Fig 214A. For both non-S/MAR- and S/MAR-flanked cells, PCRs were performed for pFlpo co-transfected cells (+Flp) surviving G418 selection. The populations were split into “H”- and “L”-fractions. PCR reactions on cells surviving G418 in the absence of pFlpo co-transfection (-Flp) as well as on “17/08-C1-L” and “17/08-C2-L” were used as negative controls. M: 1.0 kb marker.

### 2.3.5. Characterization of single clones screened after two consecutive RMCEs in Strategy II

#### 2.3.5.1. Characterization of the single clones by PCR and Southern-blot analyses

After RMCE 2, 24 single clones of each were isolated by FACS from the “H” population of non-S/MAR- and S/MAR-flanked cells. The non-S/MAR- and S/MAR-flanked single clones were numbered “C1-1” to “C1-24” and “C2-1” to “C2-24”, respectively. According to FACS analysis, each single clone showed homogenous eGFP expression. Evaluation of the expression stability in long term culture will be described in chapter 2.3.5.2 below. In order to verify an authentic exchange event within a single clone, the same PCR indicated in chapter 2.3.4 was performed. PCR on the cell mixture was performed as a positive control. PCR results of 11 representative clones for non-S/MAR- and S/MAR-flanked cells are presented in Fig 2.16A and prove that, in fact, all non-S/MAR- and S/MAR-flanked clones showed...
authentic exchange events.

In order to determine the copy number(s) of the target site(s), Southern-blot analyses on the genomic DNA of the single clones were performed in parallel. In all cases the genomic DNA was digested with \textit{NheI}, which cuts twice in the targeted cassette (within the \textit{cmv} promoter and within the \textit{GTN} fusion gene; see Fig 2.16B). \textit{NheI} also cuts once within the exchange plasmid F3-\textit{GTN}-F(w/p). The hybridization with a \textit{cmv}-specific probe was the first to be done. Since the \textit{cmv} promoter is derived from the parental cells, the number of unique hybridization bands reflects the number of targeted sites for individual clones. Fig 2.16C shows integration unique integration site for non-S/MAR-flanked clones, except for clones "C1-2" and "C1-13". For S/MAR-flanked clones, all proved a single integration site. Although the clone diversity of S/MAR-flanked clones appears to be very low according to the similar hybridization patterns among the 24 clones, this criterion is not sufficient to conclude that these clones are identical: S/MARs may have directed the respective vectors to a unique class of loci, for instance repetitive DNA. The hybridizations on parental cells, "13/08-C1" and "13/08-C2", the cells for RMCE 2, "17/08-C1-L" and "17/08-C2-L", and the non-pFlpo co-transfected cells in RMCE 2 were performed as negative controls for non-S/MAR- and S/MAR-flanked clones respectively.

Since after G418 selection there were some weakly eGFP-expressing cells detected for the non-pFlpo co-transfected population, randomly integrated exchange cassettes under control of cryptic promoter sequences for the pFlpo co-transfected cells might also exist. Therefore Southern blot analyses were conducted to define the contribution of RMCE-mediated or the random integration events (or both) to GFP expression. A \textit{gfp}-specific probe was used to hybridize the samples on the membrane after stripping. A defined 2.7 kb band indicates the RMCE-mediated integration of the exchange cassette. If there is extra random integration(s) of the exchange cassette, a band >2.7 kb would be detected. If there remained a silenced parental cassette (F3-\textit{d2egfp}-F) in the single clones (comparable to Strategy I), extra bands would be detected as well. In order to exclude the latter possibility, 24 single clones after RMCE 1, each for non-S/MAR- and S/MAR-flanked cells within "17/08-C1-L" and "17/08-C2-L", were screened. PCR with a primer pair specific for the parental cassette (F3-\textit{d2egfp}-F) was performed on these single clones. We conclude that none of the single clone contains any silenced parental cassette after RMCE 1 (F3-\textit{d2egfp}-F; data not shown).
Figure 2.16: Characterization, by PCR and Southern-blot analyses, of the single clones resulting from RMCE 2

A: PCR was performed on 11 single clones selected from non-S/MAR- or S/MAR-flanked populations as indicated in Fig 2.14A. PCR on the corresponding clone mixture was performed as a positive control. B: Single clones isolated after RMCE 2 were digested with NheI. C: The digested single clones were hybridized with a cmv-specific probe. D: The digested single clones were hybridized with a gfp-specific probe after stripping. Corresponding hybridizations on parental cells (“13/08-C1” and “13/08-C2”), cells for RMCE 2 (“17/08-C1-L” and “17/08-C2-L”) and on non-pFlpo co-transfected (-Flp) cells in RMCE 2 were performed as negative controls, both for non-S/MAR- and S/MAR-flanked clones.
Results

Data in Fig 2.16D show that, among 24 non-S/MAR-flanked single clones, a 2.7 kb band was found in all cases confirming the authentic exchange events that had already been concluded from the PCR results (Fig 2.16A). Moreover, only clone “C1-7” did contain one extra copy of a randomly integrated exchange cassette.

Among 24 S/MAR-flanked single clones, a 2.7 kb band was found throughout. However, clone “C2-4”, “C2-6” ~ “C2-9”, “C2-11”, “C2-15”, “C2-18” ~ “C2-24” harbored an extra copy of randomly integrated exchange vector in addition to the targeted site.

Combining with the first hybridization with the cmv-specific probe, a single copy has been integrated at the unique target site existing in all of screened single clones (except “C1-2” and “C1-13”). For these, loss of fluorescence (transition from d2egfp to hgytk in RMCE 1) and regain of fluorescence (transition from hgytk to GTN in RMCE 2) could be traced through two consecutive RMCEs. “C1-2” and “C1-13” contained two target sites. PCR with a primer pair specific for the F3-hgytk-F cassette was performed on each (data not shown). Neither of these two single clones still harbored the hgytk gene showing that both potential targets were addressed in RMCE 2.

Since our main interest concerns clones with a unique targetable site, all clones with multi-copy integrations of parental cassettes and random integrations of the exchange cassette were excluded for the generation of production cell lines. The remaining clones have been summarized in table 2.1.

Table 2.1: Characterization of single clones remaining after RMCE 2

<table>
<thead>
<tr>
<th></th>
<th>PCR+</th>
<th>Single copy integration of target site</th>
<th>Random integrations of exchange cassette</th>
<th>Clone diversity</th>
<th>Suitable clones (Master cell lines) for development of production cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-S/MAR-flanked</td>
<td>24/24</td>
<td>22/24</td>
<td>1/24</td>
<td>High</td>
<td>Except “C1-2”, “C1-7” and “C1-13”</td>
</tr>
</tbody>
</table>
2.3.5.2. Expression stability of the single clones in the presence and absence of G418

Since potential production cell lines for various GOIs are derived from the fully characterized master cell lines (chapter 2.3.5.1), the expression properties of production cell lines should reflect those of the master clones. Therefore both the expression level and stability of these clones are important criteria for choosing suitable candidates for further RMCE. Here we have FACS-evaluated the expression level of each 24 non-S/MAR- and S/MAR-flanked single clones during long term culture in the presence and absence of selection.

The expression properties of non-S/MAR- and S/MAR-flanked single clones at passage 2, 9 and 30 in the presence of G418 is shown in Fig 2.17A and Fig 2.18A. The data were evaluated as the mean level of GFP expressing cells. All clones had homogenous GFP expression properties - most of them maintained a stable expression level over 4-months culture in the presence of G418. An overlay analysis was also performed. Some representative data have been compiled in Figs 2.17B and 2.18B. For example, for non-S/MAR-flanked clones, “C1-1”, “C1-17” and “C1-20” maintain their expression level over 30 passages, while “C1-12” shows a shift to lower levels. In case of the S/MAR-flanked clones, “C2-2”, “C2-19” and “C2-23” keep the expression level stably but “C2-21” shows a decrease. Since the expression level of different single clones is mostly related to the genomic integration site, the variable expression properties of single clones confirm the presence of different integration sites.
Results

**Figure 2.17: Expression level of non-S/MAR-flanked single clones in the presence of G418**

A: The GFP expression level of 24 single clones was measured, by FACS analysis, at passages 2, 9 and 30. GFP expression was evaluated as the mean of GFP expressing cells.

B: The overlay analysis of clone “C1-1”, “C1-17”, “C1-20” and “C1-12” at passage 2 (black curve), 9 (green curve) and 30 (red curve).
Results

Figure 2.18: Expression level of S/MAR-flanked single clones in the presence of G418

A: GFP expression level of 24 single clones was measured by FACS analysis at passage 2, 9 and 30. The GFP expression was evaluated as the mean of the GFP expressing cells. B: Overlay analysis of clone “C2-2”, “C2-19”, “C2-23” and “C2-21” at passage 2 (black curve), 9 (green curve) and 30 (red curve).

As anticipated in chapter 2.1, our final goal is to integrate a GOI via RMCE in the absence of any drug selection, which means that after RMCE-mediated integration, the transgene is expressed under natural conditions. Therefore the expression properties of single clones in the absence of selection pressure are essential to choose suitable candidates for further modification. For this purpose, variable non-S/MAR- and S/MAR-flanked unique clones were selected based on the Southern-blot analyses (Fig.2.16C and D). For non-SMAR-flanked single clones, “C1-1, 3, 5, 10, 12, 17, 20, 21, 22” and for S/MAR-flanked clones, “C2-4, 9, 11, 14, 15, 17, 19, 20, 21, 23, 24” were chosen for continued culture in normal medium over 3 months. The mean level of the GFP expressing cells was used to evaluate the expression level for each single clone (see Fig 2.19A and Fig 2.20A). Different from the single clones
growing under selection pressure, not all of the clones showed homogenous expression in the absence of G418 over this period of time. Representative results of overlay analyses are shown in Fig 2.19B and Fig.2.20B. For instance, non-S/MAR-flanked clones “C1-5, 10, 20” did maintain consistent expression levels, but in case of clone “C1-12” a silenced population emerged that increased with time. The phenomenon is similar for S/MAR-flanked clones, e.g. stable clones “C2-4, 11, 14” and unstable clone “C2-9”.

Only the stably, homogeneously expressing clones will be chosen as master cell lines for further development of production lines.

**Figure 2.19: Expression level of non-S/MAR-flanked single clones in the absence of G418**

**A:** The GFP expression level of 9 single clones was measured, by FACS analysis, at passage 1, 15 and 25. GFP expression was evaluated as the mean of the GFP expressing cells. **B:** Overlay analysis of clone “C1-5”, “C1-10”, “C1-21” and “C1-12” at passage 1 (black curve), 15 (green curve) and 25 (red curve).
Figure 2.20: Expression level of S/MAR-flanked single clones in the absence of G418
A: The GFP expression level of 11 single clones was measured by FACS analysis at passage 1, 15 and 25. GFP expression was evaluated as the mean of the GFP expressing cells. B: Overlay analysis of clone “C2-4,” “C2-11”, “C2-14” and “C2-9” at passage 1 (black curve), 15 (green curve) and 25 (red curve).
2.3.6. Improvement of the transcription level of single clones by HDAC inhibitors

As described before, the expression properties of a given transgene in different single clones depends on the nature of the genomic integration site. This phenomenon, known as the position effect, is a major hurdle in the development of recombinant protein production in mammalian cells. In order to circumvent this effect, we used the targeted integration into previously characterized transcription-active sites. In addition, we introduced chromatin domain bordering elements (S/MARs) to shield the transcription process from the influences of surrounding chromatin structures (e.g. heterochromatization). During previous studies, it was found that the expression level of a reporter flanked by different S/MAR elements can be boosted, by adding a histone deacetylase inhibitor into culture medium (HDACi; Schlake et al. 1994). The S/MAR elements serve to organize the histone-acetylation state of an entire chromatin. Therefore, some representatives of non-S/MAR- and S/MAR-flanked single clones were treated with HDACi such as sodium butyrate (NaBu) or valproic acid (VPA). The non-S/MAR-flanked clones “C1-1, 3, 5, 10, 12, 17, 20, 21, 22” and the S/MAR-flanked ones “C2-4, 9, 11, 14, 15, 17, 19, 20, 21, 23, 24” were cultured with the medium supplied with 5 mM NaBu or 4 mM VPA for two days. The cells were rinsed with PBS and then suspended for FACS analysis. Expression levels were evaluated as the mean level for GFP expressing cells. For Fig 2.21A and B, the expression levels of untreated cells were normalized, which was set to 1. The factor for transcription augmentation is indicated on the bars both for NaBu and VPA. Comparison with the non-S/MAR-flanked clones clearly demonstrates that both agents have a significantly larger effect if the transgene is present in the form of an autonomously regulated unit (chromatin domain). For instance, NaBu augments expression 5-6 fold for most non-S/MAR-flanked clones, but 9-10 fold in case of S/MAR-flanked clones. HDACi treated cells also showed homogeneity regarding the expression level. As examples, overlay analyses are shown in Fig 2.21A and B for the situation before and after adding NaBu both for non-S/MAR-flanked clones (“C1-1” and “C1-21”) and S/MAR-flanked clones (“C2-4” and “C2-21”).
**Figure 2.21: Expression level of non-S/MAR- and S/MAR-flanked clones with NaBu or VPA treatment**

**A:** Expression levels of non-S/MAR-flanked clones “C1-1, 3, 5, 10, 12, 17, 20, 21, 22” without NaBu treatment were normalized, i.e. set to 1. The factor for NaBu or VPA is indicated on the bars. Overlay analyses on clones “C1-1” and “C1-21”, before and after NaBu treatment are also shown. **B:** The same experiments on S/MAR-flanked clones “C2-4, 9, 11, 14, 15, 17, 19, 20, 21, 23, 24”. Overlay analyses on clone “C2-4” and “C2-21”, before and after treating with NaBu are shown for comparison.
2.4. Development of production cell lines

2.4.1. The targeted integration of a GOI via RMCE 3

After full characterization of the single clones with target-ability and stable expression properties, suitable clones were chosen as master cell lines to establish production cell lines for various GOIs. Here, non-S/MAR-flanked clone “C1-23” and S/MAR-flanked clones “C2-1” and “C2-14” were selected for further development. “C1-23” and “C2-1” were chosen due to their high and homogenous expression level, while “C2-14” had an integration site different from that of other S/MAR-flanked clones (see Fig 2.16C). Following our Strategy II, a promoter-less bi-cistronic GOI-IRES-pac cassette was constructed on the exchange vector F3-GOI-IRES-pac-F. The nature of the GOI cannot yet be made public due to commercial reasons. This cassette was co-transfected into the selected master cell lines, together with the pFlpo expression construct (Fig 2.22A). This strategy provides three options to enrich for the authentic exchange events:

1 - negative selection with Ganc to exclude the non-exchanged cells;

2 - positive selection with Puro to obtain the exchanged cells;

3 - FAC-sorting of authentic RMCE-events according to the loss of fluorescence.

A negative control without pFlpo co-transfection was again performed in parallel, along with neutral DNA as described above (chapter 2.3.3). In order to test the exchangeability of these clones in the absence of drug selection, which is also the fastest way to obtain exchanged cells, the transfected cells were passaged into normal medium after transient selection with 2.5 μg/ml Puromycin for 2 days. FACS analyses were then performed on the pFlpo co-transfected cells (+Flp) as well as the non-pFlpo co-transfected cells (-Flp). From Fig 2.22B, the percentage of non-fluorescent cells in the presence of pFlpo is higher than the percentage in its absence. For instance, in clone “C1-23”, there are 4.15% non-fluorescent cells with co-transfection of pFlpo (1.24% non-fluorescent cells without co-transfection), which indicates that the exchanged (loss-of-fluorescence) cells contribute the higher percentage of the non-fluorescent population.
Figure 2.2: Third round of RMCE: Development of production cell lines

A: Schematic representation of an RMCE step to develop production cell lines: a promoter-less exchange cassette encoding the GOI-IRES-pac gene was transfected along with pFlpo (+Flp) to replace the GTN cassette in the master cell lines. A negative control without pFlpo co-transfection (-Flp) was performed in parallel. B: Master cell lines “C1-23”, “C2-1” and “C2-14” were chosen to derive GOI production cell lines. After transfection and one following passage, the difference between expression pattern of +Flp and -Flp transfected cells was evaluated by FACS analysis. The population of non-fluorescent cells was gated and sorted. The expression of GOI was only found in the sorted cells +Flp co-transfection, but not in the ones -Flp co-transfection. C: The PCR indicated in Fig 2.2A was performed on the sorted cells. PCR on the corresponding master line was performed as a negative control. Single clones from the sorted cells +Flp co-transfection were screened by FACS.
In order to prove this point, the non-fluorescent populations of +Flp cells and of -Flp cells were isolated by FACS. After expanding the sorted cells, supernatants were taken to measure the expression level of the GOI (performed by InVivo). Not surprising, the expression of GOI could only be monitored in the supernatants from +Flp cells. For the -Flp case, expression was below the detection level (data not shown). Meanwhile the authentic exchange events were also verified by PCR (Fig 2.22C) for the sorted, pFlpo co-transfected cells [the primer pair is indicated in Fig 2.22A for non-S/MAR- (p2546/p3073) and S/MAR-flanked cells (p1452/p3073)]. Sorted GOI expressing cells derived from “C1-23”, “C2-1” and “C2-14” were designated “39/08-23”, “37/08-1” and “37/08-14” accordingly. Subsequently, single clones were screened from “39/08-23”, “37/08-1” and “37/08-14” to find the high producers with the targeted integration of the GOI.

2.4.2. Screening and characterization of single clones with high GOI expression

In order to identify clones with high GOI expression, each 100 single clones of “39/08-23”, “3708-1” and “37/08-14” were screened by FACS. 11 of 100 (“a” ~ “k”) from “39/08-23”, 11 of 100 (“a” ~ “k”) from “3708-1” and 9 of 100 (“a” ~ “i”) from “3708-14” were found with high GOI expression (see Fig 2.23A). The remainder of single clones derived from the corresponding master cell lines either had an extremely low or an undetectable expression level (data not shown). The PCR shown in Fig 2.22A was performed on all GOI high expressers to verify authentic exchange events for single clones. Some representatives derived from 3 master lines are shown in Fig 2.23B. All randomly selected single clones showed RMCE-mediated integration events. In order to prove the GOI expression is exclusively due to RMCE, Southern-blots were performed on some of the single clones, which were selected for PCR analysis as indicated in Fig 2.22A. The genomic DNA of single clones was cut with NdeI (within the cmv promoter) and NotI (within the GOI; Fig 2.22A), which leads to a defined 950 bp band upon RMCE-mediated integration (randomly integrated GOI would give rise to additional bands). Since there is only one target site in the master cell lines, the RMCE-mediated integration of GOI is expected to be unique as well. After hybridization with a GOI-specific probe, it turned out that except for one clone (“clone c” derived from master line “C1-23”) all others showed a RMCE-mediated integration of the GOI exclusively (Fig.2.23C).
Results

Figure 2.23: Characterization of GOI expressing single clones derived from master cell lines

A: The GOI expression level of 11 single clones ("a" to "k") from "39/08-23", 11 single clones ("a" to "k") from "37/08-1" and 9 single clones ("a" to "i") from "37/08-14". B: PCRs on representatives of single clones were performed as designed in Fig 2.22A. PCRs with H2O and the corresponding master cell line from which the GOI single clones had been derived served as negative controls (-). A PCR on the pool from which the GOI single clones had been isolated was performed as a positive control (+). C: Southern-blot analysis was performed on some of the single clones as indicated in Fig 2.22A. NdeI/NotI digested single clones were hybridized with a GOI-specific probe. The corresponding master cell lines from which these single clones were derived were hybridized as negative controls.
Hybridizations on the corresponding master cell lines were performed as negative controls, clearly demonstrating that GOI expression can be ascribed to the targeted integration event of the GOI. The similar expression levels of GOI obtained for single clones from the same master line also shows that these are identical.

Analyses of the single clones derived from three master lines are summarized in Table 2.2.

Table 2.2: The summary of the analysis of GOI expressing clones derived from corresponding master lines

<table>
<thead>
<tr>
<th>Master cell lines</th>
<th>GOI high expressers</th>
<th>RMCE-mediated integration exclusively</th>
<th>Efficiency of screening for clones with targeted integration</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-23</td>
<td>11/100</td>
<td>4/5</td>
<td>8.8%</td>
</tr>
<tr>
<td>C2-1</td>
<td>11/100</td>
<td>4/4</td>
<td>11%</td>
</tr>
<tr>
<td>C2-14</td>
<td>9/100</td>
<td>5/5</td>
<td>9%</td>
</tr>
</tbody>
</table>

To summarize: with Strategy II, master cell lines with a unique target-able site can be efficiently isolated from randomly selected integration sites. These master cell lines show a stable and homogenous expression pattern with a consistent retarget-ability in the absence or presence of selection pressure. The production cell lines can be derived from the master lines in the absence of any drug selection. The efficiency of isolating high production clones with only targeted integration of the GOI is about 10%.
3. Discussion

Targeted integration of a GOI into a pre-identified transcriptional hotspot is a valuable approach to counteract the well known unstable and un-predictable expression patterns caused by position effects. This concept has enabled a production platform in which the transgenic cell line contains a single copy gene at a unique genomic location. Here we describe the benefits of a method consisting of two consecutive rounds of RMCE to isolate master clones with re usable hotspots that provide high and homogenous expression properties for any GOI. Relying on a “promoter trap” strategy and on FACS counter-selection, the production cell lines can be derived in the absence of drug selection. Such a combination of complementary selection procedures proves to be largely superior to the traditional processes.

The second approach to augment transcription level of the transgene relies on the use of chromatin bordering elements, e.g. S/MARs, to protect the integrated GOI from common repressive effects of the genomic environment. These are mostly caused by promoter-DNA methylation and histone methylation or hypoacetylation (Bode et al. 2000a; Bode et al. 2003). Histone deacetylase inhibitors (HDACi), e.g. sodium butyrate (NaBu) or valproic acid (VPA) were used to improve chromatin accessibility and thereby transcription rates by increasing histone acetylation levels (Davie 2003; Van Dyk et al. 2003).

3.1. How to improve RMCE efficiency?

3.1.1. The molecular basis of RMCE

The mechanism of RMCE is known to considerable extent (Schlake and Bode 1994; Bode et al. 2000b; Oumard et al. 2006) When RMCE was developed by Schlake and Bode (1994), the proposed mechanism still involved two possible intermediates that might arise from a single reciprocal crossover between identical FRT recombination targets (either F/F or F3/F3 pair) on parental and exchange cassettes, respectively (Fig 3.1). Intra-molecular recombination between the F/F or F3/F3 sites on the intermediate would then either restore the initial situation or lead to the desired exchange of the parental cassette by the GOI. A related mechanism was discussed for the Floxing system in which two site-specific recombinases,
Cre and Flp were used in combination (Lauth et al. 2002). For this two-step mechanism, the excision step would lead to an event corresponding to a net exchange or restore the educts reducing the efficiency.

![Diagram of the two-step mechanism of RMCE](Image)

**Figure 3.1: Two-step mechanism of RMCE (redrawn according to Fig 2 in Schlake and Bode 1994)**

Recombination between F/F or F3/F3 pair on the parental and exchange cassettes (according to the “Flp-in” principle) leads to two kinds of possible intermediates, which contain the insertion of the exchange cassette. According to a stepwise mechanism, the excision between F/F or F3/F3 on the intra-molecular intermediate yields the desired product of RMCE (solid line) – otherwise the educt-situation is restored (dashed line).

Ways to improve the rate and extent of RMCE have mainly relied on the improvement of recombinase activity, i.e. the increase of collision events between the parental and the exchange cassettes. In addition, elaborated selection principles were applied to enrich the desired RMCE product (Seibler and Bode 1997; Seibler et al. 1998)

Since intermediates were never detected for this mechanism (Seibler et al. 1998; Kim and
Lee 2008), at least for practical purposes the mechanism can be seen as a simultaneous
double-reciprocal crossover between the pair of hetero-specific FRT sets on parental and the
exchange cassettes. All efforts to improve RMCE rates based on the sequential type would
also apply for this mechanism.

3.1.2. Improvement of recombinase activity

There are two principal ways to improve the level of active recombinases, either by increasing
the amount of transfected enzyme or by creating an optimized enzyme version.

Initially the thermo-instability of Flp has limited its use for mammalian cells (Buchholz et al.
1996). After screening for thermo-stable mutants, Flpe was identified and shown to provide a
4 fold increase in recombination efficiency, which was still low if compared with Cre. Since, at
the onset of this study, Flpe was the only available variant, it was cloned into a bicistronic
expression construct flpe-IRES-pac. Transient selection with Puromycin could then serve to
isolate the cells receiving both, the exchange vector and the Flpe expression vector
(Taniguchi et al. 1998). In 2007, the de novo synthesis of a mouse codon-optimized Flp
variant (Flpo) was reported, the recombination efficiency of which resembles Cre (Raymond
and Soriano 2007). This gave raise to our flpo-IRES-pac vector variant. After transient
selection in Puro, the recombination efficiency of Flpo proved to be substantially higher than
Flpe in the same experimental setting (personal communication by S. Turan). Therefore
flpo-IRES-pac was used for Strategy II throughout.

Previous studies show that at high concentrations of the recombinase, recombination
proceeds very quickly and is complete within a few hours (Peitz et al. 2002). Therefore a
transient expression of Flp is sufficient to induce and complete the process. Due to the
cytotoxic effects of many transfected DNAs, the ratio of exchange vector and Flp expressing
vector was optimized and adjusted to 3:1.

3.1.3. The distance limitation between FRT sites

Site-specific recombination depends on random collision between two FRT sites. Since the
parental cassette is a single copy embedded in the host chromatin, increasing the amount of
the exchange cassette is the only way to raise the rate of random collision. Therefore, the
exchange cassette is commonly applied at a large molecular excess. Under these conditions RMCE becomes virtually irreversible, according to the mass action principle.

In case the two FRT sites are positioned on a single naked DNA molecule (e.g. the intermediates in Fig 3.1), the distance between the two FRT sites might also influence the rate of random collision. On one hand, two FRTs cannot randomly collide when the distance is too short (less than approximately 120 bp) because of the inherent stiffness of DNA. On the other hand, increased distance leads to a predictable and asymptotic decrease in recombination frequencies (Ringrose et al. 1999). Nevertheless, Wallace et al. (2007) described an extended strategy (recombinase-mediated genomic replacement, RMGR) that allowed the replacement of a large segment (>100 kb) of the mouse genome by the equivalent human synthetic region on a bacterial artificial chromosome (BAC) DNA. Since a stringent selection procedure was required to enrich the rare recombinants, the exchange of large DNA pieces appears to be difficult although recombination events can still be recovered by an appropriate selection procedure.

3.1.4. The position effect of FRT sites on recombination efficiency

Different integration sites in a genomic context can lead to variable expression levels for the same transgene ("position effect"). This phenomenon has also been reported to influence the recombination efficiency between two FRT sites (Vooijs et al. 2001). Similar to the incomplete understanding of position effects on gene expression, to what extent recombination is affected by chromatin environments is also unclear. The conventional way to identify integration sites with RMCE-competence is to tag them with a set of hetero-specific FRTs, and then test the exchangeability of the enclosed expression cassette (the called “tag and exchange” strategy according to Seibler et al. 1998). Along these lines, a panel of single clones with single copy integration of a parental cassette flanked by F and F3 sites at different loci was tested for the exchangeability under the same conditions (Qiao, master thesis, 2005). It proved to be true that not every integration locus can be targeted with a given exchange cassette. Therefore an identification process is standard and it has to rely on extensive screening.

In this situation, we developed a strategy consisting of two consecutive RMCEs: in RMCE 1
potential targetable sites were pre-screened at the clone-pool level; in RMCE 2, cells with targetable sites were recovered from the pool. Since the integration sites were thoroughly pre-screened, a smaller number of single clones were needed to derive a master cell line. By using a traceable fluorescent marker, the screening of integration sites with high and stable expression levels was accomplished at the same time. The process may be further supported by the addition, for instance of S/MARs. S/MAR elements alleviate position effects from variable integration sites and, in addition, they protect the FRTs within the same chromatin boundaries. Although the correlation between active transcription sites and RMCE-competence remains uncertain, it appears plausible that a high accessibility will provide both, high expression levels and Flp-mediated recombination rates.

The true bottleneck of RMCE is the unfavorable (since bi-molecular) mode of the recombinase-catalyzed reaction. Although the rate of RMCE can be improved by recombinase activity and the concentration of exchange partners, the cells with net replacement of exchange cassette will usually remain a minor portion among the transfected cells. Therefore the most crucial step is the rational isolation of RMCE-events, which will be discussed in the following.

3.2. How to efficiently isolate RMCE-competent loci with high expression level

3.2.1. How to isolate integration sites with stable and high expression level

Since a major aim of this study is the establishment of cell lines for protein production, stable and high expression levels of these lines are the predominant criteria. The GOI is targeted to a pre-characterized locus, the properties of which will determine the expression level of the GOI. In Strategy 1, cells with a high expression level of d2eGFP were selected after the first round of RMCE (Fig 2.3C and D). But the high expressers collected after this step did not consistently include the highly-expressed original targets. In the refined Strategy II, the expression level was directly evaluated after micorporation by the integrated fluorescent marker d2eGFP, which is a part of the parental vector. In this process potential
RMCE-competent clones were collected, by double sorting, only from the population, which had already proven an exceptional expression level. Expression of these selected cells could be analyzed in the absence of selection pressure, approaching the natural condition for a gene in its genomic environment. After two rounds of RMCE, clones bearing a consistently exchangeable site were further tested for their long-term stability in absence or (as a control) the presence of selection pressure. Only clones maintaining stable expression levels over 3-4 months were chosen to establish master cell lines for production purposes. Although all candidate clones harbor a single copy of the same reporter gene, they still show variable expression levels indicating the extent to which expression properties depend on the genomic environment. For example, for non-S/MAR-flanked clones (Fig 2.17B), “C1-1”, “C1-17” and “C1-20” had a stable expression level of d2eGFP, while “C1-12” underwent a decline. The situation was similar even for S/MAR-flanked clones (Fig 2.18B), e.g. clone “C2-2”, “C2-9” and “C2-23” were stable while “C2-21” was unstable. Even within the control series, the co-expression of a selection marker did not guarantee the stability of expression level for all different loci.

Fig 2.19 and Fig 2.20, shows that, nevertheless, there are some clones that are stably expressed in the absence of selection pressure. Only these stable expressers were candidates to yield master cell lines for further applications.

3.2.2. How to efficiently isolate RMCE-competent loci

3.2.2.1. The difficulties to isolate RMCE-competent loci

Usually, the identification of master clones with a unique exchangeable site relies on intensive screening of a huge variety of clones. To enable an efficient RMCE process, random integration(s) of the target should be stringently avoided since otherwise any extra integrated copy might constitute a secondary target and cause chromosomal recombination of homologous sequences. Therefore, an elaborate selection strategy was needed to select clones harboring a unique target site with dominant target-ability over random integration.

Selection strategies used for the RMCE-based engineering of embryonic stem (ES) cells provide useful examples. Seibler et al. (1998) applied RMCE in the framework of the classical “tag and exchange” strategy, in which a hyggtk positive/negative (+/-) selection marker flanked
by F and F3 sites was used to randomly tag different integration sites. Successful Flp-mediated replacement of *hyg* cassette in a clonal cell was then enriched by Ganc counter-selection to exclude non-exchanged cells. Targeting frequencies at different integration sites varied between 21% and 38%, which was still adequate. Later on a number of alternatives served to improve the efficiency by adding positive selection. As an example, an ATG-deficient *neomycin* gene was positioned downstream from the parental cassette. RMCE-mediated integration provided the missing translational start codon such that Neomycin could be expressed (Seibler 1999). A major disadvantage of this strategy is that the neomycin gene is left behind in the targeted host genome, which is the situation causing epigenetic silencing of the GOI (Artelt *et al.* 1991). In principle, this effect can be overcome if the selection marker is “floxed” (flanked by identical LoxP sites) enabling its removal by a pulse of Cre-recombinase once targeted integration events have been selected in G418. Roebroek *et al.* (2006) reported that targeting efficiencies of ~100% could be reached if positive and negative selection were used in conjunction and 63% if only positive selection was applied.

The application of RMCE for the development of production cell lines is just beginning. A HEK-293 derived cell line, Flp293A, has been described that integrates different retroviral vectors via RMCE using the ATG-supplementation RMCE-strategy mentioned above (Schucht *et al.* 2006). Unfortunately, the efficiency by which the specific cell line could be identified was not addressed as was its targeting efficiency. More recently, a CHO cell line producing erythropoietin was established by RMCE-mediated integration of the erythropoietin (EPO) expression cassette into a previously characterized locus with the appropriate characteristics (Kim and Lee 2008). Obviously, however, the targeting efficiency of the pre-selected loci was extremely low. Only one single completely exchanged clone was isolated using a co-expressed, independent positive selection marker.

In order to identify targetable loci, we started our study using the “negative selection strategy”, which had been successfully applied in ES cells. In Strategy I (Fig 2.3A), the parental cassette containing a *hyg* positive/negative selection marker was electroporated into CHO wt cells to obtain a high proportion of cells with single copy integration (Baer *et al.* 2000). In order to enrich the cells undergoing successful RMCE, Ganc counter-selection was performed to
Discussion

Excluding any non-exchanged parental cells. Originally unexpected, among the Ganc resistant cells the majority was non-fluorescent (>90% for non-S/MAR-flanked cells, >70% for S/MAR-flanked cells, Fig 2.3C), indicating that a silenced tk gene in the parental cells had caused Ganc resistance for the non-fluorescent population. After the high expressers had been enriched by FACS, authentic exchange events could be verified by PCR (Fig 2.3E), which supports the value of negative selection. However, using specific primer pairs silenced tk genes and randomly integrated exchange cassettes were also detected in the fluorescent cell population (Fig 2.4 and Fig 2.5). Such a random integration phenomenon has never been observed for ES cells. This can be ascribed to the fact that diploid cells (but not transformed cell lines) recruit circular constructs only by RMCE and not by random integration events.

As a consequence, one more selection step became necessary before clonal screening could be started, to pre-select cells that owe their fluorescence only to RMCE. To this end a second RMCE was added to restore the starting situation by replacing the fluorescent marker with the hygtk gene (Fig. 2.6). Only cells that received d2egfp via RMCE lost fluorescence and integrated a hygtk gene at the same locus (loci) again. These independent exchange events could be enriched by FACS counter-sorting. The randomly integrated hygtk gene(s) would lead to the survival of any other type of non-targeted cells, characterized by the continuous expression of d2eGFP. Fig 2.6B demonstrates that, for non-S/MAR-flanked cells, there was no notable exchange even for cells pre-screened after RMCE 1. But for S/MAR-flanked cells, a significant non-fluorescent population arose, as expected. This interpretation was validated by negative controls, performed in the absence of Flp. We can therefore safely conclude that the Flp activity induced an authentic RMCE event.

Subsequently, clonal cells were screened and characterized. PCR analyses (Fig 2.6C) showed that the majority (34 out of 59 isolated clones) had an authentic exchange of the single copy at the target site (Fig 2.7C and D). The efficiency of the second round of RMCE therefore was >56%, which substantially reduces the efforts of clonal screening and characterization. Since, for single clones, no random integration(s) of exchange cassette were detected, positive selection is sufficient to enforce and enrich targeted integration. This was confirmed by RMCE on the characterized single “clone 57” (Fig 2.8). Hyg-resistant “clone 57” was selected with G418 and shown to have undergone exchange for the neo-d2egfp cassette.
The death of the cells co-transfected without Fip expressing vector indicated very rare random integration of exchange cassette(s) in the targeted fluorescent cells.

Nevertheless, a number of non-anticipated properties or even drawbacks have to be mentioned:

1. The clonal diversity among the S/MAR-flanked clones is low. According to the similar hybridization pattern on Southern blot analyses, the seven clones shown in Fig 2.7C and Fig 2.7D could be related or even identical. The reason for such a phenomenon may lie in the dominant exchangeability of some loci which profit from the presence of S/MAR elements as chromatin borders. Alternatively, S/MARs have a targeting potential directing the associated transgenes to certain class of loci, e.g. regions of the genome with S/MAR-like sequences.

2. An extra copy of intact but silenced parental cassette was detected in all the screened single clones (Fig 2.7). This copy must have been integrated at the establishment phase of parental cells and silencing events might have been enriched by the counter-selection with Ganc in RMCE 1. Such a copy should be inactive both for expression and recombination. In theory, it should not influence the exchange of a GOI, but the unknown epigenetic background may have caused unexpected and uncontrollable alterations during the further development of this cell line. Therefore a clean exchange at a unique targetable site remains one prerequisite. Although Strategy I successfully improved the efficiency of isolating targetable sites, it still lacks the selection of clones with a unique targetable site. Besides that, the negative selection strategy with Ganc enriched for many spontaneous silencing events, which should be avoided.

3. For non-SMAR-flanked cells, there is no consistent exchangeability for the pre-screened clones after RMCE 1. Probably too few diverse cells were pre-screened due to the fact that less than 1% expressed d2eGFP at all. The second possibility is that the unknown epigenetic alterations may have mutated the F and/or F3 sites. Since no clonal cells could be screened, sequencing of these sites was not applicable. The addition of S/MAR elements on the other hand not only protected the expression of the targeted gene from silencing, it may also have served to protect the FRT sites (Cobellis et al. 2005).
Discussion

Because our ultimate goal was an RMCE system not needing drug selection, “clone 57”, which had been fully characterized after two rounds of RMCEs, was chosen for establishing a master cell line for further RMCE cycles. As described above, “clone 57” harbors a unique targetable site in addition to a silenced, recombination-inactive F/F3 site. After RMCE with a d2egfp gene and double sorting, authentic exchange events were detected by PCR analysis. But events of this type only represented a minimum of the whole population, which was concluded from similar expression patterns of cells transfected with or without Flp recombinase. Although it is feasible to have RMCE in the absence of drug selection, an even more stringent selection is needed to recover cells with only targeted integration from the mixture.

To summarize: consisting of two consecutive RMCEs, Strategy I could serve to efficiently isolate master clones with a high expression level and a consistent exchangeability. Nevertheless, the following points did remain for further refinement:

1- only clones with high expression level sites should be subjected to the selection of exchangeable sites;

2- the selection process should enrich clones with a unique targetable site;

3- the selection strategy should reduce random integration events of the exchange cassette, especially in case RMCE is performed in the absence of a selection pressure.

3.2.2.2. Refinements in Strategy II

- As discussed in chapter 2.3.1, high expressers were pre-selected by sorting for d2eGFP expression to ensure only active transcriptional sites to be tested for their exchangeability. The broad expression pattern of the sorted cells indicates variable integration sites (Fig 2.11D). The clones were not tested for expression stability because low expressers would overgrow the high expressers with time, especially in the absence of selection pressure. Fig 2.11E demonstrates that, after the cells were cultured for more than 2 months, a population of non-expressers emerged. Since d2eGFP expression was restored by RCME 2, the expression stability can be tested by FACS for single clones after screening with or without selection pressure. Only correctly targeted clones with high and homogenous expression were chosen for
master cell lines. The second advantage of this optimization is that master cell lines are marked by fluorescence. Cells for which a GOI has been introduced by RMCE can be easily collected and sorted by FACS due to the loss of fluorescent expression, which provides a fast and convenient way to enrich targeted clones other than by a drug selection.

- In Strategy II, parental cells were fluorescently marked. After exchange for the *hygtk* gene, targeted cells were enriched by sorting the non-fluorescent population (Fig 2.12D). In RMCE 1, the parental cells with either one or multiple integration sites lost fluorescence by replacing all *d2egfp* cassette(s). Cells with a partial replacement of *d2egfp* could, in principle, establish Hyg-resistance but they would still be fluorescent. This situation was verified by the proof of authentic exchange events in the fluorescent population via PCR (Fig 2.13). Some single clones were recovered from the non-fluorescent population after RMCE 1. In each of 24 authentically exchanged single clones, only 1 clone was detected with two integration sites in case of non-S/MAR-flanked cells (data not shown). For S/MAR-flanked cells, all clones contained a unique integration site (data not shown). Obviously, RMCE 1 not only pre-selects the potential targeted cells but also does enrich the cells with a unique targetable integration site.

- For Strategy II, a “promoter trap” principle was used to reduce interferences from randomly integrated cassette(s). In the absence of positive selection, random integration is favored over RMCE-mediated integration since RMCE can only take place at a single site whereas random integration can be widespread in the whole genome. Although authentic exchange events could be detected for Strategy I by PCR (“clone 57”) in the absence of positive selection, the exchanged cells cannot be easily distinguished from the mixture due to the high percentage of random integrations (Fig 2.9). But after the promoter was positioned outside of the parental cassette, the expression of a promoter-less targeted cassette can only be initiated by RMCE-mediated integration. This was demonstrated by selecting the targeted cells with a drug expressing gene (either *hygromycin* or *neomycin*): in Fig 2.12C and Fig 2.14C, only the targeted cells could survive the corresponding drug selection. In the
absence of selection pressure, 3 different master clones were exchanged for a GOI. 4 or 5 single clones derived from each master line were characterized for random integration of the GOI. Only a single case was found for which an extra random integration had taken place (Fig 2.23C). Therefore, the “promoter trap” strategy successfully trapped cells with only a targeted integration both in presence and absence of a selection pressure. There were very rare events leading to random integration(s) next to an endogenous promoter but in these cases expression was very weak. The fact that the “promoter trap” strategy does not permit the exchange of the pre-existent promoter for a different one is only an apparent one: since the expression of a transgene depends on a particular set of interactions between the promoter and its genomic environment, its exchangeability does not mean a realistic advantage. It has been shown by Seibler (Dissertation, 1999) that even the inversion of a transcription unit at a given locus can significantly alter the expression properties. Therefore this property does not limit the applications of Strategy II.

Considering the various optimization steps in Strategy II, a new method could be developed to efficiently isolate targetable clones with both a unique integration site and a high expression level. After two consecutive RMCEs, 21 of 24 screened clones showed the desired target-ability in the non-S/MAR approach; 10 of 24 screened clones showed an equivalent target-ability in the case of S/MAR flanks (Table 2.1). Some of these were tested for their expression stability in long term culture. Only the clones that showed stable and homogenous expression were chosen as a source of master cell lines.

The timeline was as follows:
- It took 10 days to pre-select the high expressers.
- The selection of targeted cells in two RMCEs required 10-15 days.
- The final step to screen clonal cells could be performed in another 20 days.

Therefore, it took approximately 2 months to identify targetable clones with a unique integration site and a high expression level. Compared to the traditional “integration and selection” strategy (6 months; Wurm 2004) or the conventional “tag-and-targeting” RMCE strategy (4 months; Schucht et al. 2006), our strategy is definite time-efficient (Fig 3.2).
Discussion

Traditional Process

Tag-and-Targeting

Parental Clones  →  Tagging and Targeting  →  Master Clones  →  Production Cell lines

4 months  1 months

Strategy II

Parental Clones  1st RMCE  2nd RMCE  3rd RMCE  →  Master Clones  →  Production Cell lines

2 weeks  2 weeks  2 weeks  1 month  1 month

Figure 3.2: Timeline comparison between different cell line development strategies

In traditional process, it takes minimum of 12 months to transfer the GOI and selection marker gene into the host, to screen high expresser and to develop large-scale production processes (Wurm 2004). In the tag-and-targeting strategy, isolation of master clones relies on the screening of numerous targetable single clones with high expression properties, which is still laborious and time-consuming (4 months). In our refined strategy, we speed up the isolation of master clones by two consecutive RMCEs. The efficiency is significantly improved to reduce the number of clones needed to be screened. For both of two latter strategies, development of production cell lines for various GOIs takes one month and the optimization of down-stream processing only needs to be done once.

The apparent low clone diversity in S/MAR-flanked clones observed for Strategy I (Fig 2.16) also appeared in Strategy II indicating that this phenomenon is not a coincidence. This low diversity was not introduced during the establishment of parental clones following microporation, since the broad and heterogeneous expression profile of the S/MAR-flanked cells was similar to non-S/MAR-flanked cells (Fig 2.11D). Therefore diversity must have been lost in the RMCE steps, which means that some locus has absolute exchange preference over others. The underlying is unknown. Its explanation and exploitation will need more genomic information about the particular characteristics of these sites.
3.2.3. The development of production cell lines from master cell lines

In this study, the non-S/MAR-flanked master line “C1-23”, and S/MAR-flanked master lines “C2-1” and “C2-14” were chosen for the development of production cell lines. The GOI was constructed as part of a bicistronic vector containing \textit{GOI-IRES-pac}, which provided the option to select the targeted cells with Puromycin. Since the potential targeted cells appeared right after co-transfection of the exchange vector and Flp expressing vector (Fig 2.22B), the positive RMCE-events were FACS-isolated from the mixture immediately in order to reduce the overgrowth by non-targeted cells. 9 or 11 single clones were detected with GOI expression from 100 screened clones that were derived from each of master line. The remainder showed extremely low or non-detectable expression, probably due to the background of transfected but non-exchanged master cells. Characterization of several GOI expressing clones proved a clean RMCE-mediated integration of GOI for most cases. Only in one case the single copy of a randomly integrated GOI was traced. Therefore the targeting efficiency of three master cell lines varies between 8.8% and 11% in the absence of drug selection (Table 2.2). If selection with Puromycin was also performed, a significantly larger population of non-fluorescent cells was observed in case the cells were co-transfected with exchange vector and Flp expressing vector (data not shown). This underlines the efficiency of positive selection for enriching targeted cells. Due to time restrictions clonal cells were not screened in this case.

At the time being, this is the first report describing the recovery of pure RMCE-mediated integration events in the absence of any drug selection. The average targeting efficiency for different integration sites is about 10%, which is reasonable for an efficient selection process. If really required, the efficiency can be much increased by a drug selection due to the high efficiency appeared in the two rounds of consecutive RMCEs. Previous studies with a positive selection strategy (Neo) showed much lower stringency and efficiency to select high producers, and it required more time for cell line development. In the strategy described here about 1 month is required to derive a suitable production cell line from a master cell line. This includes the screening characterization procedures for higher producers. Since various GOIs can be targeted to the same genomic locus, the downstream processes and regulatory
Discussion

approval can be simplified.

For yet unknown reasons the productivity of the isolated producers has so far remained behind expectations and there are indications that the production of this particular GOI in CHO cells is limited by an unknown mechanism. Since three different master lines with different integration sites were used to create production cell lines, the productivity among the GOI producers derived from different master lines would be expected to differ. This is obviously not true since all of the fully characterized clones with correct targeted integration agreed in their expression level (around 40 ng/ml, Fig 2.23A). Therefore there must exist steps apart from the transcription rate that govern the productivity of this GOI, e.g. the translation machinery, the endoplasmic reticulum machinery responsible for folding, post-translational modification, and secretion. If the productivity still remains below expectation, one would have to quantify both the intracellular and secreted portions of the GOI. Therefore, additional selection principles may become necessary covering the post-transcriptional steps, most notably the secretion capacity. If transcriptional silencing of the particular GOI is a matter, the use of DNA elements with chromatin opening function and anti-repressor elements could be considered (Benton et al. 2002; Kwaks et al. 2005).

3.3. How to improve and stabilize recombinant gene expression level

Recombinant gene expression in mammalian cells is a multistep process in which transcription is followed by posttranscriptional processing, translation and posttranslational modification. Subsequent steps involve protein folding under the control of chaperones.

After a transgene is stably integrated into a host genome, at a molecular level, the transgene is wrapped by histones to form nucleosomes, which are the basic building block of chromatin. The density of chromatin structure surrounding the integration site determines the accessibility of different transcriptional regulatory elements, which explains that the efficient expression of the transgene is highly dependent on the site of integration. Therefore enhancing gene transcription can be achieved by improving integration sites. One of the approaches towards this end is the introduction of artificial domain borders, i.e. S/MAR flanks which are able to prevent the surrounding heterochromatin from influencing the transcription
of the transgene.

During the past decade, much has become known about the role of chromatin modification on epigenetic gene regulation. The DNA methylation in the promoter sequence and modifications according to the histone code determine whether a gene is active or not. Since an open and active chromatin domain is generally associated with histone acetylation, histone deacetylase inhibitors (HDACi) can be used to augment the transcriptional potential as shown in this study, especially if applied in the context of S/MARs, which render the transgene amenable to the histone machinery (Bode et al. 1986; Klehr et al. 1992).

Both of these two approaches were applied to improve and stabilize the expression level of a targeted gene, which will be discussed in the following contest respectively.

3.3.1. The function of S/MARs

3.3.1.1. The function of S/MARs on gene expression

Scaffold/matrix attachment regions (S/MARs) are experimentally identified sequences associated with the protein/RNA backbone of the nucleus. Many reports have been published on the application of S/MARs to augment expression levels in different cell lines (Zahn-Zabal et al. 2001; Abranches et al. 2005; Goetze et al. 2005; Girod et al. 2005). Besides their role in structuring chromatin domains, S/MARs are involved in chromatin remodeling and subsequent transcriptional activation events (Bode et al. 2000a). S/MARs also act as “insulators” to block the spread of DNA methylation, which pose repressive effects on transgene expression (Bode et al. 2000a). When the expression cassette was linked to S/MARs, multi-copy tandem integrations could be observed (Klehr et al. 1991; Bode et al. 1996). In such cases, the expression level could be further increased due to arrays of several transgenes. These kinds of S/MAR functions are reflected by several observations in this study:

1. In Strategy I, after first round of RMCE, the S/MAR-flanked cells surviving from Ganc-selection showed higher expression of d2eGFP than the non-S/MAR-flanked cells (Fig 2.3C). Obviously S/MARs suppress chromosomal position effects, imparting position-independent expression. S/MARs directing multi-copy integration of d2eGFP is the second possible reason for the higher expression. Finally, being recombinogenic elements
S/MARs have the potential to increase the rate of RMCE due to their strand-separation potential, which leads to more RMCE-positive cells within the mixture (Fig. 2.3C).

2. In strategy II, after the d2egfp cassette was integrated into the CHO cell genome, the highest expressers were enriched twice by FACS. At the stable stage, the S/MAR-flanked cells only showed a slightly stronger expression than non-S/MAR-flanked cells (Fig 2.11D). But after the cells were cultured for extended times, especially in the absence of selection pressure, S/MAR-flanked cells showed a more stable expression, i.e. fewer cells were silenced than for non-SMAR-flanked cells. As anticipated, S/MARs have the function to protect and maintain transgene expression by alleviating the negative effects from chromatin modifications.

3. In Strategy II, after two rounds of RMCE, the expression of individual clones was evaluated by FACS analyses (Fig 2.18 and Fig 2.19). Obviously, there are more S/MAR-flanked high expressers than non-S/MAR-flanked ones (for instance, S/MAR-flanked clones “C4, C6, C8, C9, C11, C15, C19, C21”). Southern blot analyses proved that some of the clones contained multi-copy integrations (Fig 2.16D), which may be one reason for their higher expression. Repeat-induced silencing phenomena were also found in some of these cases (Fig 2.18), e.g. “C2-21” showed a decline of expression over long term culture (Garrick et al. 1998). Multi-copy integration phenomenon was rarely found in the non-S/MAR-flanked clones.

3.3.1.2. The function of S/MARs on RMCE rates

In this study the support function of S/MAR elements on the rate of RMCE became obvious. On one hand, acting as simple spacers S/MARs protect the FRT sites during the integration process from exonuclease degradation. For instance, in Strategy I, after RMCE 1, the pre-selected non-S/MAR flanked cells could not be further exchanged (Fig 2.3D), which is likely due to deletion or mutation of FRTs. On the other hand, S/MARs are recombinogenic sequences with an increased propensity to undergo strand separation (Bode et al. 2006). This property supports the crossover reactions between FRT sites, to increase the rate of RMCE. Therefore, in Strategy I, there were more S/MAR-flanked d2eGFP expressing cells than non-SMAR-flanked cells after the first round of RMCE (Fig 2.3C). This phenomenon was not
observed for Strategy II because of additional measures such as the “promoter trap” principle and exactly comparable sorting processes.

3.3.2. The function of HDACis

Compounds to create and maintain a favorable chromatin conformation are in wide use for bioprocess development (De Leon Gatti et al. 2007; Backliwal et al. 2008). Since an open and active chromatin domain is generally associated with histone acetylation, histone deacetylase inhibitors (HDACi) such as sodium butyrate (NaBu), valproic acid (VPA) are used to boost transcription by maintaining histone acetylation (Hunt et al. 2002; Backliwal et al. 2008). S/MAR-mediated transcriptional effects can be augmented by the addition of histone deacetylase inhibitors (such as NaBu), which in this context cause high levels of core-histone acetylation (Schlake et al. 1994). This is the case since S/MARs constructs approach the machinery involved in histone turnover (Hendzel et al. 1994). In a model study NaBu raised transcriptional levels < 2× for S/MAR-free constructs while its effect was ~4× for the same test gene with one flanking S/MAR or ~30× if elements “E” and “W” constituted the domain borders (cf. Fig. 1 in Schlake et al. 1994). In the present study, NaBu or VPA was added to the culture medium of single clones that were screened and characterized for a single copy integration, both for non-S/MAR- and S/MAR-flanked clones. An increase factor of 5~6 was found in most of non-S/MAR-flanked clones after a 48-hours treatment with NaBu (Fig 2.21A). In contrast, S/MAR-flanked clones showed an increase of more than 10 fold (Fig 2.21B). Some non-S/MAR-flanked clones e.g. “C1-3” and “C1-22” also boosted transcription similar to clones with authentic S/MAR-flanks. Since only integration sites with high expression level were selected, the notable (though somewhat smaller) response for non-S/MAR-flanked cassettes indicates that our pre-selected cells have incorporated the expression unit next to S/MAR-like elements pre-existing at the site of integration. NaBu was also added to selected single clones with a high productivity of the targeted GOI. An increase comparable to the corresponding master lines was observed (data not shown). VPA is a C5 short fatty acid like NaBu. Since the inhibition of histone deacetylase is not as strong as NaBu (Davie 2003), the function of VPA on transcription remains below that of NaBu (Fig 2.21).

Since HDACi arrests the cell cycle in G1 (and to some extent in G2), the available energy will be used for proteins other than those involved in replication. Both factors together explain the
beneficial action of these approaches.

3.4. Perspectives

3.4.1. Analyses of cis-regulatory elements on transgene expression

In this study, S/MAR elements showed the propensity to improve RMCE rate but this conclusion needs more evidence. For instance, S/MAR elements could be used to flank the exchange cassette as well. RMCE rate can be evaluated by the exchange between both S/MAR-flanked parental cassette and exchange cassette (Fig 3.3). The option to select targetable sites will then open the possibility to explore unknown functions of cis-acting elements in an identical genomic setting (Goetze et al. 2005).

![Diagram of strategy to analyze the function of cis-regulatory elements on transgene expression and/or targeting efficiency.](image)

*Figure 3.3 Strategy to analyze the function of cis-regulatory elements on transgene expression and/or targeting efficiency*

At a unique targetable site in host genome, variable cis-regulatory elements posed upstream and/or downstream of a reporter gene can be targeted into the exactly same integration site. Therefore the comparison of different cis-regulatory elements on transgene expression or RMCE rate can be performed within same genomic environment.
3.4.2. Multiplex RMCE

The synthesis and characterization of more FRT mutants that show cross recombination with themselves but not FRT\textsuperscript{wt} enable the multiplex RMCE principle (Turan, Diplom Thesis, 2007). The well characterized master clones in this study provide a host for the introduction of a second target site. The efficient screening strategy established here could quickly identify a new target site by integration of a second fluorescent marker (e.g. RFP; Fig 3.4). The expression ratio of two sites can be evaluated by the fluorescent intensity on FACS analysis. This provides the possibility, for instance, to optimize the expression level of H- and L-antibody chains. Last but not least the multi-target capacity could enable sophisticated mouse models for the analysis of not a single but multiple gene functions.

![Diagram of Multiplex RMCE Principle]

**Figure 3.4: Principle of Multiplex RMCE**

A second target site flanked by another pair of FRT sites can be identified as described in Strategy II. The light chain (LH) and heavy chain (HC) for an antibody can be simultaneously integrated into the target sites, which can be characterized by the loss of fluorescence. The ration of LC and HC can be optimized by the ratio of green and red intensity before targeting.
4. Material and Methods

4.1. Material

Chemicals used in this study were obtained from Amersham, Bayer, Biolabs, Biorad, Gibco, Merck, Roche and Sigma. Enzymes were purchased from Biolabs and Roche. Oligonucleotides were synthesized by MWG. Radioactive chemicals were purchased from Amersham-Buchler.

DNA was sequenced by the “Genome Analysis” (GNA-) group at the Helmholtz Centre for Infection Research (Braunschweig, Germany).

Plastic materials used in cell culture was bought from Gibco, Greiner, Nunc, Sarstedt and Seromed.

4.2. Computer programs

Analysis of DNA sequences, search for sequences, restriction sites for endonucleases and cloning simulation were done with Vector NTI 6.0 (Invitrogen). The analysis of Phosphorimager data was performed with the program ImageQuaNT from Molecular Dynamics. The processing of FACS analyses data was done with the program Cell Quest Pro and FACSDiva (Becton Dickinson). Processing and PC conversion of images was performed via Adobe Photoshop. This manuscript was written with the programs Word and Excel from Microsoft. Chromas Version 1.45 (Conor McCarthy, School of Health Science, Griffith University Queensland, Australia) was used for analyzing sequencing results.

4.3. Instrumentation

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<td>Leitz Labovert</td>
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<tr>
<td>Phosphor-Imager</td>
<td>Molecular Dynamics Storm 860</td>
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Material and Methods

Exposition Chamber
Molecular Dynamics Exposition Cassette for the phosphor-imager

FACS
FACSCalibur from Becton Dickinson
FACSAria from Becton Dickinson

Cell Culture Incubator
Labotect CO₂-inkubator C2000 from Labor-Technik-Goettingen

Clean Benches
Mecaplex, Sterilcard Hood VBM600 and SG400
Heraeus, HLB 2448
Heraeus, HSP 18

Thermo-Cycler
Biometra T3 Thermocycler

Vortexer
Scientific Industries Vortex Genie2

Electroporator
Biorad Gene Pulser and Pulse Controller

Microporator
MicroPorator MP-100 from NanoEnTek Inc.

Hybridization Incubator
Stuart Scientific Hybridization Oven

Milli-Q Water Purifier
Millipore Milli-Q

Power Supply
Bio-RAD Power PAC 300

Cell Counter
Schaerfe System CASY1

4.4. Medium and buffer solutions

All reagents were dissolved in “Millipore” water and sterile filtrated (pore size of 2 µm).

Penicillin/Streptomycin solution (100x)
1.212 g Penicillin/200 ml (10000 U/ml),
2 g Streptomycin/200 ml (10 mg/ml),
adjust to pH 7.0 with NaOH, stored at -20°C

Glutamine solution (100x)
29.23 g Glutamine dissolved to 1 l solution,
stored at -20 °C

PBS
140 mM NaCl, 27 mM KCl, 7.2 mM Na₂HPO₄,
14.7 mM KH₂PO₄ (pH 6.8 - 7.0), stored at 4°C

TEP
500 ml sterile PBS,
0.6 ml 0.5 M EDTA (final conc.: 6 mM),
15-20 ml Trypsin (0.1 - 0.2%; depending on the activity)

Puromycin
5 mg/ml in H₂O, stored at -4°C

G418
100 mg/ml in H₂O, stored at -20°C

Hygromycin
650 U/ml in H₂O, stored at -4°C

Ampicillin
50mg/ml in ethanol, stored at -20°C
Material and Methods

TE
10mM Tris/HCl, pH 8.0, 1 mM EDTA, pH 8.0

Medium for CHO-K1 cell
Dulbecco’s Modified Eagle’s Medium (DMEM)/Nut.Mix F12 (HAM) medium (1:1), with 10% fetal calf serum (FCS), 20 mM glutamine, 60 µg of penicillin/ml and 100 µg of streptomycin/ml

LB medium
10 g/l Bacto-Trypton, 5 g/l Bacto-yeast extract, 10 g/l NaCl, autoclaved

4.5. Molecular cloning technologies

4.5.1. Polymerase Chain Reaction (PCR)

PCR is used to amplify a defined DNA fragment with specific primers that recognize appropriate sequences in the leading and lagging strand of DNA. PCR will allow to amplify DNA stretch about a million-fold so that its size and nucleotide sequence can be determined. There are three major steps in PCR, which are repeated for 30~35 cycles:

1. Denaturation
   During the denaturation (the DNA double strand melts yielding single strands) all enzymatic reactions are stopped (for example, the extension from the previous step).

2. Annealing
   In this step primers anneal to the template DNA and the polymerase can combine with the template DNA to start copying a DNA strand.

3. Elongation
   The optimum elongation temperature is 68°C for the Expand Long Template Enzyme mix (containing thermostable Taq DNA polymerase and Tgo DNA polymerase, a thermostable DNA polymerase with proofreading activity) from Roche. The bases (complementary to the template) are coupled to the primer on the 3’ side (the polymerase adds dNTPs from 5’ to 3’, reading the template from 3’ to 5’; bases are added complementary to the template).

**Reaction components for a standard PCR reaction:**

10xPolymerase buffer

dNTPs mixture (25 mM for each nucleotide)

Primer mixture (10 pmol/µl)

Expand Long Template Enzyme mix from Roche (5 unit/µl)

DNA template (100 pg plasmid DNA or 100 ng HWM DNA)

The reaction is adjusted with water to 25 µl volume.
Material and Methods

A PCR program example:

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<tr>
<td>Denaturation</td>
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<td>Annealing</td>
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<td>Elongation</td>
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<tr>
<td>Cycling</td>
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<td>34 cycles</td>
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<tr>
<td>Ending</td>
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4.5.2. Enzymatic restriction of DNA

Standard DNA manipulations were carried out essentially according to Sambrook (Sambrook et al. 1989). In each restriction reaction, an amount of 0.5~1 µg DNA dissolved in 10 µl H₂O was digested with 0.5~2 units restriction enzyme over 1~2 hrs at the optimal reaction temperature for the enzyme. The reaction buffers (10x) were provided together with the restriction enzyme by the producing company. When required, BSA was added to the restriction reaction at a concentration of 100 µl/ml. The resulting DNA fragments were analyzed on an agarose gel.

4.5.3. Dephosphorylation of DNA-fragments

Alkaline Phosphatase (AP) from Roche

To avoid self-ligation of a DNA fragment digested with one restriction enzyme, the fragment was dephosphorylated with alkaline phosphatase. The restricted and purified fragment was mixed with 1 µl of alkaline phosphatase and 1 µl buffer and adjusted to 20 µl with H₂O. Dephosphorylation was done at 37°C for 30 mins. After that, 1 more µl alkaline phosphatase was pipetted in and incubated for another 30 mins.

4.5.4. Fill in reaction of DNA-fragment with sticky ends

1 µg of DNA with sticky ends was treated with 1 U Klenow enzyme (NEB) in the presence of 3 µl dNTP-mix (25 mM for each nucleotide) and 5 µl Buf II (NEB) in a total volume of 50 µl. The mixture was incubated for 15 mins at 25°C and terminated by heat inactivation (20 mins at 75°C).

4.5.5. Gel electrophoresis

The size of a DNA-fragment or entire plasmid was determined by analysis on 1-2% agarose gel. The required amount of agarose was dissolved in 1xTAE buffer, melted and poured into an electrophoresis chamber together with ethidium bromide solution (1g/ml). A volume of 10% v/v loading buffer was added to DNA samples at a ratio of 1/6. Samples were loaded onto the gel by using 1xTAE as running buffer. The electrophoresis was performed at 20-100 Volt/cm for about 30 mins. The agarose gel was photographed under UV light with wavelength at 260 nm.
4.5.6. DNA extraction from agarose gels

QIAquick Gel Extraction Kit (QIAGEN)

After separation on agarose gel, a DNA-fragment of interest was cut out from the gel with a lancet, weighted and treated by the means of the QIAquick Gel Extraction Kit (QIAGEN). The gel slice was weighted and put in an Eppendorf tube. 3 volumes of Buffer QX were added to 1 volume of gel (100 mg~100 µl). One volume of 100% isopropanol was added if the fragment was larger than 4 kb or smaller than 500 bp. The mixture was incubated at 50°C for 10 mins to dissolve the gel. To bind DNA, the completely dissolved DNA was pipetted onto the QIAquick Spin Column from the 2 ml collection tube and centrifuged for 1 min. 750 µl PE buffer was loaded onto the column. The mixture was incubated 5 mins at room temperature. After 1 min centrifugation, the column was put into a clean 1.5 ml Eppendorf tube. 25 µl H₂O was added to the center of the column directly to elute the DNA by 1 min centrifugation, 13,000 rpm.

4.5.7. Ligation

In each ligation reaction, an amount of 1 µl of template DNA and 3 µl of insertion fragment were mixed with 1 µl T4 DNA ligase (NEB). 10x ligase buffer was used and the whole solution was adjusted to 10 µl with H₂O. The reaction was run 2 hrs or overnight at room temperature. The ligation mixture was spread onto an agar plate supplemented with corresponding antibiotic, which was in turn put into 37°C room overnight.

4.5.8. Plasmid preparation

Mini preparation

STET: 8% Sucrose, 0.5% Triton 50 mM EDTA, 10 mM Tris (pH 8)

1. Pick up colonies from an agar plate and dissolve in 2 ml LB medium supplemented with corresponding antibiotic, e.g. 50 µg/ml ampicillin, shake at 180 rpm in 37°C room for 6 hrs; meanwhile, steak the related colony onto an agar plate with the same marker as the medium; place the plate in 37°C room overnight.

2. Take 2 ml medium to 2.0 ml eppendorf tube, centrifuge for 30 secs, 13,000 rpm.

3. Decant supernatant. Add 500 µl STET to re-suspend the pellet by vortexing.

4. Add 50 µl 10 mg lysozyme/1 ml TE solution (stored at -20°C) and mix by inverting the tube 10~15 times. Stand the tubes at room temperature for 2~3 mins.

5. The Eppendorf tubes are heated on a heat-block at 95°C for 90 secs, then centrifuged for 5 mins a 13,000 rpm.

6. The pellet is removed with toothpicks. Add 50 µl 8M NH₄Ac and 500 µl isopropanol. Mix by inverting the tubes. Then centrifuge for 5 mins, 13,000 rpm.

7. Decant the supernatant and wash the precipitation with 500 µl 70% v/v ethanol.

8. Centrifuge for 5 mins at 13,000 rpm and decant the supernatant. The tubes were dried by standing at room temperature for 10~15 mins.
Material and Methods

9. Elute the precipitation in 20~30 µl H₂O.

The mini prepared plasmid DNA was used to verify the correct cloning sites by enzymatic restriction.

**Maxi preparation**

Pick up corresponding positive colony into 250 ml LB medium and shake overnight at 180 rpm in 37°C room. Collect the medium and use QIAGEN Plasmid Maxi Kit to harvest the plasmid DNA.

1. Harvest the bacterial cells by centrifugation for 10 mins, 7,000 rpm with GSA rotor at 4°C.
2. Re-suspend the bacterial pellet in 10 ml Buffer P1.
3. Add 10 ml Buffer P2, mix gently but thoroughly by inverting 4-6 times, and incubate at room temperature for 5 mins.
4. Add 10 ml pre-chilled Buffer P3, mix immediately but gently by inverting 4-6 times, and incubate on ice for 20 mins.
5. Centrifuge for 30 mins at 4°C, 13,000 rpm with GSA rotor.
6. Equilibrate a QIAGEN-tip 500 by applying 10 ml Buffer QBT, and allow the column to empty by gravity flow.
7. Filter the supernatant with pre-wetted folded filter paper through QIAGEN-tip 500.
8. Wash the QIAGEN-tip 500 with 30 ml Buffer QC twice.
9. Elute DNA with 15 ml Buffer QF.
10. Precipitate DNA by adding 10.5 ml room-temperature isopopanol to the eluted DNA. Mix and centrifuge immediately for 30 mins at 4°C, 13,000 rpm with a karbon rotor.
11. Wash DNA pellet with 5 ml of room-temperature 70% ethanol, and centrifuge for 10 mins at 4°C, 13,000 rpm with a karbon rotor. Carefully decant the supernatant without disturbing the pellet.
12. Air-dry the pellet for 5-10 mins, and re-dissolve the DNA in a suitable volume of TE buffer.

**4.6. Use of Escherichia coli (E.coli)**

**4.6.1. E.coli strains**

DH10B: mcrA, mcrB, mrr, hsdR17, deoR, recA1, endA1, lacZDM15 (Gibco BRL)

**4.6.2. Preparation of agar plates**

To the LB-medium 15 g agar was added per liter. For preparation of agar plates containing Ampicillin, a sterile Ampicillin stock solution (50 mg/ml in ethanol) was diluted 1/1000 into the medium.
4.6.3. Preparation of electro-competent bacteria

5 ml overnight culture was added to 1 l LB medium. The cells were grown at 37°C under strong shaking until the cell suspension reached an OD600 of 0.5 - 1 and subsequently they were centrifuged for 10 mins in a cooled GS3-rotor at 3,000 rpm. The cell pellet was re-suspended in 1 l cold water and centrifuged as mentioned before. After this centrifugation step the pellet was re-suspended in 500 ml cold water and was centrifuged again as mentioned above. Then the cell pellet was re-suspended in 20 ml cold 10% glycerin (v/v), centrifuged and then re-suspended in 2-3 ml 10% glycerin. This cell suspension was divided into aliquots (50µl), shock frozen into liquid nitrogen and stored at -70°C.

4.6.4. Transformation of DNA into E.coli by electroporation

The electroporation-competent bacteria DH10B were thawed on ice. 2-3 µl ligation DNA was mixed with 50 µl of bacteria gently. The mixture was moved to pre-chilled electroporation 0.2 cm cuvette quickly, tapping the top of the cuvette until the mixture settled evenly to the bottom. The sample was pulsed once using 2.5 kV, 25 µF and 200 Ω. The time constant should range between 4 to 5 ms. Immediately after transformation, 1 ml LB medium was added to suspend the cells. The cells were incubated at 37°C, 180 rpm for 1 hr. After that, the mixture was spread on an agar petri plate and incubated over night at 37°C.

4.6.5. Conservation of bacterial colonies

For short term conservation bacteria were plated on agar plates, incubated at 37°C overnight and kept at 4°C.

For long-term conservation a 1 ml overnight culture was mixed with 87% glycerin in a 1/1 ratio and conserved at -70°C.

4.7. DNA analysis

4.7.1. Extract genomic DNA (high molecular weight DNA, HMW)

*Modified Bradleys*  
10 mM Tris/HCl, pH 7.5, 2 mM EDTA, 10 mM NaCl, 0.5% SDS, autoclaved

Confluent cells on 6-well plate were washed with 2 ml PBS. 500 µl modified Bradleys buffer. 1 mg/ml proteinase K was added on the cells and mixed gently. The viscous mixture was transferred to 1.5 ml Eppendorf tube and stored in 55°C incubator over night. The Eppendorf tube was spun down. 1 ml 75 mM NaAc in 100% ethanol (stored at -20°C) was added. After vortexing, the tube was incubated at room temperature for 2~3 hr, then centrifuged for 5 mins, 5,000 rpm. Supernatant was decanted. The pellet was washed with 0.5 ml 70% ethanol (stored at -20°C) and incubated at room temperature for 30 mins, twice. After 5 mins, 5,000 rpm centrifugation, the pellet was dried at room temperature for 10 mins. 20-30 µl TE was added to the pellet and incubated at 37°C for 30 mins. Finally, the DNA solution was sheared by pipetting and stored at 4°C.
4.7.2. Southern Blot analysis

Blotting
10 µg HMW DNA was digested overnight with an appropriate enzyme and run on 0.8% agarose gel in 1xTAE buffer. DNA was transferred to a positively charged Nylon membrane (Amersham) in 0.4 M NaOH. On a gel carrier plate 2 Whatman papers soaked in 0.4 M NaOH were placed, then a Whatman paper bridge was formed between two sides of buffer reservoir. On top of the bridge the gel was placed upside down and the nylon membrane, soaked in water was put onto the gel while eliminating air bubbles between the gel and the membrane. On top of the membrane 3 wet papers were positioned. A 10 cm stack of dry tissue paper was added to absorb the buffer. The blot apparatus was covered with plastic foil and a 3 kg weight was put on top. It took overnight for the transfer of DNA to the membrane. The nylon membrane was then neutralized 5 times in 2xSSC for 5 mins, backed at 80°C for 2 hrs before it was ready to use in the hybridization experiment. Hybridizations were done in buffer containing 0.5 M Na₂HPO₄ (1M Na₂HPO₄ and 1M NaH₂PO₄ were mixed in a ratio of 4/1), pH 7.2, 7% SDS and 2 mM EDTA. The membrane had to be pre-hybridized for at least 30 mins in 65°C incubator.

Labeling
Random priming with the “Redi-Prime DNA labeling system” (Amersham)
Labeling mix: dATP, dGTP, dTTP, exonuclease free Klenow enzyme, oligonucleotide primer (9-mer)

For the labeling reaction, a commercially available system from Amersham was used. The reaction mix was available as a freeze-dried pellet in an Eppendorf tube. First 2.5-100 ng DNA probe in 45 µl TE buffer was denatured in boiling water for 5 mins and put on ice immediately. The DNA and 5 µl of α[^32]P]dCTP were added to the labeling tube and mixed by pipetting. The mixture was incubated for 10 to 30 mins at 37°C. 2 µl of 0.5 mM EDTA was pipetted to the mixture to stop the reaction.

Hybridization
After labeling, the sample was separated from unincorporated nucleotides over a Sephadex G50 column. For this purpose, the ready to use stacked Micro-SpinTM G50 column from Pharmacia Biotech was used. Perfected packing of the column was reached by spinning the columns for 1 min at 3,000 rpm. The labeling mix was put on the column drop by drop and centrifuged through the column for 2 mins at 3,000 rpm. The labeled probe was denatured in boiling water bath for 2-5 mins, then put on ice immediately and added to the pre-hybridized membrane. Hybridization proceeded overnight in 65°C incubator.

Washing
After hybridization the membrane was washed with the following steps:

1. 250 ml 2xSSC/0.5% SDS 5 mins RT
2. 500 ml 2xSSC/0.5% SDS 30 mins 65°C
3. 250 ml 2xSSC/0.5% SDS 5 mins 65°C
Washing steps were performed until the signal on blot was lower than 40 ips with hand counter measurement.

**Exposition**

After washing, cover the blot with Whatman 3MM paper to remove most of excess moisture. Wrap moist blot in plastic film prior to and put into Molecular Dynamics Exposition Cassette for the phosphor-imager overnight. After that, the screen was scanned (Molecular Dynamics).

**Re-hybridization**

For re-hybridization, the signal was eliminated by washing several times in boiled 0.1% SSC/0.5% SDS. To check if the signal was completely removed, the stripped blot was exposed again, until there was no image on it. Re-hybridization was performed as described previously.

**4.8. Fluorescence-activated Cell Sorting (FACS)**

**4.8.1. Scanning by FACSCalibur (Becton Dickinson)**

*EPICS 2% inactivated FCS (heat FCS in 56°C water bath for 30 mins) in PBS*

The confluent cells on 6-well plate were washed with 2 ml PBS and collected by trypsinization with 500 µl TEP. The suspension was mixed with 500 µl PBS and centrifuged for 5 mins, 1,000 rpm at 4°C. The supernatant was removed. The pellet was re-suspended in 400 µl EPICS with 10 µg/ml propidium iodide (PI) to stain the dead cells. Fluorescence (GFP) expression was measured on FACSCalibur (Becton Dickinson). The excitation wavelength for GFP was 488 nm and emission was detected at 488 nm (on Fl-1). The PI emission at 620 nm was detected on Fl-3. Dead cells were excluded via an FSC-H vs. Fl-3 dot plot. The GFP expression was evaluated in an FSC-H vs. Fl-1 dot plot on the live cells. 1E5 events were collected. Data were acquired and analyzed by CellQuest™ Pro.

**4.8.2. Sorting by FACSARia (Becton Dickinson)**

The confluent cells on a 6-well plate were collected as described above. The suspended cells were passed through a 40 µm gauze to exclude cell clumps. Single cells or cell populations expressing GFP were separated by FACSARia (Becton Dickinson). GFP expressing cells can be detected and sorted at wavelength of 488 nm on Fl1-A. PI emission was detected at wavelength of 620 nm on Fl3-A. Doublets were excluded via an FSC-H vs. FSC-A dot plot. The dead cells were excluded via an FSC-A vs. Fl3-A dot plot. The sorting gate was the combination of the live cell gate, the doublet discrimination gate and the dot plot gate on FSC-A vs.Fl1-A. The sorted cells were incubated several days in the medium with 5 µg/ml Gentamycin. Data were acquired and analyzed by FACSDiva software.
4.9. Cell culture

4.9.1. Cell lines

CHO-K1: Chinese Hamster Ovary (ECACC Nr. 85051005)

4.9.2. Cell culture

4.9.2.1. Cell incubation

CHO-K1 cells were incubated at 37°C with 5% CO₂ in incubator.

4.9.2.2. Cell passage

When the cells have grown confluent on the container (dishes, plates or flasks), they were washed with PBS once. TEP was added to the cell container for trypsinization. After several minutes, cells were suspended by pipetting and a portion of 1/20-1/10 was transferred to fresh medium. The amount depended on the passage time and the size of cell container. Change medium every three days until the cells grow confluent for the next passage.

4.9.2.3. Freezing and Thawing cell

Freezing

Grow cells on a little flask until confluence (about 5x10⁶ cells). Wash cells with 5 ml PBS once and trypsinize them with 1 ml TEP. Suspend cells with 4 ml medium and then centrifuge for 5 mins, 1,000 rpm. After that, remove supernatant and re-suspend the cell pellet in 5 ml FCS with 5% DMSO. Aliquot the cells to 3 or 4 freezing vials (Bio-Freeze-Vials, Costar) and put them on ice immediately. After one hour on ice, package the vials with paper towels and freeze them into -70°C refrigerator. After 12 hrs, the freezing tubes can be moved to liquid nitrogen.

Thawing

Take the frozen vial out of liquid nitrogen and thaw it in 37°C water bath. Add 4 ml medium and centrifuge for 5 mins, 1,000 rpm. Remove supernatant in order to get rid of DMSO. The cell pellet was re-suspended in 5 ml medium and filled in a little flask.

4.9.3. Gene transfer methods

4.9.3.1. Electroporation

1E6 logarithmically growing, semi-confluent cells were washed with 5 ml PBS once and collected in 500 µl TEP. The pellet was re-suspended in 0.7 ml pre-chilled DMEM/HAM medium together with 3µg linearized DNA. The cell-DNA mixture was placed in 0.4 cm cuvette and pulsed at 360 V, 800 µF capacity and infinite resistance with a Biorad Gene Pulser and
Pulse Controller. The electroporated cells were plated onto a 10-cm plate containing 10 ml DMEM/HAM medium.

4.9.3.2. Microporation

5E5 logarithmically growing semi-confluent cells were collected by trypsinization and re-suspended in 100 µl Buffer R with 5 µg linearized DNA. The cell-DNA mixture was aspirated using Microporator 100 µl pipette and microporated at 1620 V with MicroPorator MP-100. The pulse width was 10 ms and the sample was pulsed three times. The microporated cells were seeded onto a 10-cm plate containing 10 ml DMEM/HAM medium.

4.9.3.3. Transfection for RMCE

One day prior to transfection, the cells were seeded in 6-well plate (1.2E5 cells/well). 4 hrs before transfection fresh medium was put on cells. Metafectene (Biontex) and DNA solution should have an ambient temperature and should be gently vortexed prior to use. Mix 3 µg of exchange DNA and 1µg of Flp expression DNA in 100 µl serum free medium. Incubate the mixture at room temperature for no longer than 5 mins. Mix 30 µl metafectene and 100 µl serum free medium. Combine the DNA solution and metafectene solution together and then incubate in room temperature for 15-20 mins. Add the mixture to one well drop by drop. One day post-transfection, the cells were treated with 2.5 µg/ml Puromycin for 48 hrs. For transient transfection, the cells were harvested 48 hrs after transfection. For stable transfection, PBS rinsed cells were passaged into the medium supplemented with corresponding antibiotic. Medium exchange was performed every 3 days. The selection was finished generally after 2 weeks.

4.9.3.4. Transfection for RMCE-negative control

Mimicking RMCE transfection, the neutral vector BSpac-Δp (de la Luna et al. 1988) only encoding Puromycin was used in place of the Flp expression for the control transfection: If integration happened, it was independent on Flp-recombinase.

4.10. Measurement of GOI expression

The measurement of GOI expression was accomplished by InVivo Biotech Services GmbH (Berlin). Due to commercial reasons, the details of the measurement cannot be revealed.
5. Plasmids and Oligonucleotides

5.1. Plasmids

5.1.1. Existing Plasmids used in this study

**pF3-hygtk-F** The *hygromycin thymidine kinase* fusion gene (*hygtk*) under control of the *HSV-thymidine kinase* promoter is flanked by FRT3 and FRT\^wt sites (Schlake and Bode 1994).

**pF3ESGTMWF** The *sv40* promoter driven *GTN* fusion gene (*Green fluorescent protein, Thymidine kinase and Neomycin resistant gene*) is linked to S/MAR-E (upstream) and S/MAR-W (downstream) elements; the whole cassette is flanked by FRT3 and FRT\^wt sites (Baer 2002).

**pd2egfp-basic** The *d2egfp* gene (a destabilized variant of *enhanced green fluorescent protein gene* with a half-life of approximately two hrs; Clontech, CA, USA).

**peGFPN1-mcs** The *enhanced green fluorescent protein gene* under control of the *cytomegalovirus* promoter (Clontech, CA, USA).

**pF3-pgkygltk-F** The *hygromycin thymidine kinase* fusion gene (*hygtk*) under control of the *phosphoglycerate kinase* (*pgk*) promoter is flanked by FRT3 and FRT\^wt sites (Fiering, S.; Seattle).

**pGFP-C1** The *neomycin resistant gene* (Clontech, CA, USA).

**pF3SGTNF** The *sv40* promoter driven *GTN* fusion gene is flanked by FRT3 and FRT\^wt sites (Baer 2002).

**pCR4TOPO-GOI** The synthetic GOI is cloned into TOPO vector (InVivo Biotech Services, Berlin).

**pmcsF3bpAFmcs** The bovine polyA signal (bpA) flanked by FRT3 and FRT\^wt sites sits between a multi-cloning site (Baer 2002).

**pFlpe** The enhanced FIp is under control of the CAGGS-promoter (Stewart, F.; EMBL).

**pFlpo-Puro** FIp is lined to the puromycin resistant gene via an IRES elements; the bicistronic construct is driven by the CAGGS-promoter (Turan, S.; HZI).

**pBSpac-Δp** The puromycin resistant gene lacks the prokaryotic promoter (de la Luna...
5.1.2. Plasmids constructed in this study

pESGTNW

The ~7.0 kb fragment including SMAR-E, GTN and SMAR-W elements was amplified with primer pair P2369 and P2423 on pF3ESGTNW as a template. The ~2.0 kb fragment containing amp gene and Ori element was amplified with primer pair P2427 and P2428 on the same template. Both of fragments were digested with BamHI and DralI and then ligated.

pEF3-hygkt-FW

The F3-hygkt-F cassette was amplified with primer pair P2433 and P2439 on pF3-hygkt-F as a template. The ~2.8 kb SalI/SpeI digested PCR fragment was inserted into SalI/SpeI restricted pESGTNW, in which the GTN fusion gene was replaced by the F3-hygkt-F cassette.

pcmvd2egfp-basic

The cmv promoter was amplified with primer pair P2472 and P2484 on template peGFPN1-mcs. The ~650 bp MluI/XhoI digested PCR fragment was inserted into MluI/XhoI digested pd2egfp-basic, in which the expression of d2eGFP was driven by the cmv promoter.

pF3-cmvd2egfp-F

The cmvd2egfp fragment was amplified with primer pair P2472 and P2496 on template pcmvd2egfp-basic. The ~1.7 kb KpnI/SpeI restricted PCR fragment was inserted into KpnI/SpeI digested pF3MCSF, in which the cmvd2egfp fragment was flanked by FRT3 and FRT" sites.

pF3-cmvneod2egfp-F

The neo gene was amplified with primer pair P2536 and P2539 on template pGFP-C1. The ~800 bp NheI/BspEII restricted PCR fragment was inserted into NheI/BspEII restricted pF3-cmvd2egfp-F, in which the neod2egfp fusion gene was driven by the cmv promoter.

pF3-d2egfp-F

The ~1.1 kb EcoRI/BamHI digested d2egfp gene from pd2egfp-basic was inserted into ~2.4
Plasmids and Oligonucleotides

kb EcoRI/BamHI restricted pF3MCSF, in which the d2egfp gene was flanked by FRT3 and FRT\textsuperscript{wt} sites.

**p\text{cmvF3-d2egfp-F}**

The F3-d2egfp-F cassette was amplified with primer pair P3077 and P3993 on pF3-d2egfp-F as a template. The ~1.2 kb Nhel/Sall digested PCR fragment was inserted into ~3.4 kb Nhel/Sall digested pcmvd2egfp-basic, in which the F3-d2egfp-F cassette was under control of the cmv promoter.

**pE\text{cmvF3-d2egfp-FW}**

The cmvF3-d2egfp-F fragment was amplified with primer pair P2084 and P2439 on pcmvF3-d2egfp-F as a template. The ~1.9 kb Sall/Spel digested PCR fragment was inserted into Sall/Spel restricted pEF3-hygtk-FW, in which the F3-hygtk-F was replaced by the cmvF3-d2egfp-F cassette.

**p\text{F3-hygtk-F(w/p)}**

pF3-hygtk-F was digested with MluI and Xhol and filled with Klenow. The blunt 5.3 kb fragment was self-ligated, in which the tk promoter was deleted (w/p).

**p\text{F3-GTNF(w/p)}**

pF3SGTNF was digested with Sall and PpuMI and filled with Klenow. The blunt 5.2 kb fragment was self-ligated, in which the sv40 promoter was deleted (w/p).

**p\text{F3-GOI-F}**

The ~480 bp SpeI/NotI fragment digested from pCR4TOPO-GOI was inserted into ~2.5 kb SpeI/NotI digested pF3MCSF, in which the GOI was integrated between FRT3 and FRT\textsuperscript{wt} sites.

**p\text{F3-cmvGOI-F}**

The ~620 bp EcoRV/Nhel digested GOI from pF3-GOI-F was inserted into ~3.0 kb EcoRV/Nhel digested pF3-cmvneod2egfp-F, in which the GOI replaced the neod2egfp fusion gene.
pF3-cmvGOI bpA-F

The cmvGOI fragment was amplified with primer pair P2084 and P3076 on pF3-cmvGOI-F as a template. The 1.1 kb SalI/SacI digested PCR fragment was inserted into SalI/SacI digested mcsF3bpAFmcs, in which a bovine polyA signal was insert into downstream of the cmvGOI fragment.

pF3-GOlbpA-F

pF3-cmvGOI bpA-F was digested with NheI and SalI and filled with Klenow. The blunt 3.0 kb fragment was self-ligated, in which the cmv promotor was deleted.

pF3-GOI-IRES-pac-F

The IRES-pac fragment was amplified with primer pair P3802 and P3803 on pFlpo-Puro as a template. The ~2.0 kb NotI digested PCR fragment was inserted into NotI digested and dephosphorated pF3-GOlbpA-F, in which a GOI-IRES-pac bicistronic construct was formed.

5.2. Oligonucleotides

p2369 5'-GCA GGA GTC GGA TCC TGA ATT CTA AGC CAG TAG TAC CTG C-3'
p2423 5'-GAC GCT GCC CAC TTG GTG AAT TCA GCA AGG TCG CCA CGC ACA AGA T-3'
p2427 5'-GAC GCT GCC CCG ATT TTA ATG AAT CGG CCA ACG CGC-3'
p2428 5'-GCA GGA GTC CAC CAA GTG GGC GCG CCC GAT CGC CGC GGT TAC T-3'
p2433 5'-GAC GCC CCT GCA TTA ATG AAT CGG CCA ACG CGC-3'
p2439 5'-GCA GGA GTC ACT AGT GCC AGG GGC TAC CAT GGA GAA GTT CCT ATT CCG AAG TTC-3'
p2472 5'-GAC GTC GCC AGC CGT GGT ACC CCG TAT TAC CGC CAT GCA T-3'
p2484 5'-CTC GAG ATC TGA GTC CGG TAG CGC TAG CGG ATC TGA CGG TT-3'
p2496 5'-GCA GGA GGC GCC CCA TCA CGC TGC TCA CAC TCC CCC CTG AAC CTG AA-3'
p2536 5'-GCC GAC ACC GCT AGC ATG ATT GAA CAA GAT GGA TT-3'
p2539 5'-GAC GCC ACC T CC GGA GGG GGG GGG GGA AGA ACT CGT CAA GAA GGC GAT AGA-3'
p3077 5'-GAC GCT GCT AGC AAG CTT GAA GTT CCT ATA CTA TTT GAA GAA TAG G-3'
p3993 5'-GAC GTC GTC GAC CGA CGG CCC CGA AGT TCC TAT TCC GAA GT-3'
p2084 5'-GAC GCT GCC GCT AGC GTC GAC GTT AGT TAT TAA TAG TAA TCA ATT ACG GG-3'
p3076 5'-GAC GTC GAG CTC GAG CGG CCC CGA ATT CGC CCT TAA GCT TAG-3'
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<td>5'-GAC GAC GCG GCC GCT ATA TGT TAT TTT CCA CCA TAT TGC-3'</td>
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<td>p3803</td>
<td>5'-GTG GAC GCG GCC GCT AAT GCA GCG GAT CCA GAC ATG ATA-3'</td>
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<td>p2471</td>
<td>5'-GTC GCG GTG AGT TCA GGC TT-3'</td>
</tr>
<tr>
<td>p1230</td>
<td>5'-</td>
</tr>
<tr>
<td>p2484</td>
<td>5'-CTC GAG ATC TGA GTC CGG TAG CGC TAG CGG ATC TGA CGG TT-3'</td>
</tr>
<tr>
<td>pGFP6</td>
<td>5'-ACACGCTGAACCTTGCGGCTTCCAGTCGC-3'</td>
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<td>p1452</td>
<td>5'-GAGTAGCATTAAACCGGCAAAGTCAATTTC-3'</td>
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6. Abbreviations

<table>
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<tr>
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<td>α</td>
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<td>cytomegalovirus promoter</td>
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<td>Cre</td>
<td>causes recombination (Cre-Recombinase)</td>
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<td>d2eGFP</td>
<td>destabilized variant of enhanced green fluorescent protein with a half-life of approximately two hours</td>
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<tr>
<td>d2egfp</td>
<td>destabilized variant of enhanced green fluorescent protein gene</td>
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<td>EDTA</td>
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<td>enhanced green fluorescent protein gene</td>
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<td>green fluorescent protein</td>
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<td>Description</td>
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<td>10^-9 meter</td>
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<td>nuclear localization signal</td>
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<td>origin of replication</td>
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<td>ubiquitous chromatin opening elements</td>
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<td>volt</td>
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<td>VPA</td>
<td>valproic acid</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volumen (volumen procent)</td>
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<tr>
<td>wt</td>
<td>wild type</td>
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<tr>
<td>Xis</td>
<td>excisionase</td>
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7. Literatures


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