Recommended method for chromosome exploitation: RMCE-based cassette-exchange systems in animal cell biotechnology

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Abstract

The availability of site-specific recombinases has revolutionized the rational construction of cell lines with predictable properties. Early efforts were directed to providing pre-characterized genomic *loci* with a single recombinase target site that served as an address for the integration of vectors carrying a compatible tag. Efficient procedures of this type had to await recombinases like Φ C31, which recombine *attP* and *attB* target sites in a one-way reaction – at least in the cellular environment of the higher eukaryotic cell. Still these procedures lead to the co-introduction of prokaryotic vector sequences that are known to cause epigenetic silencing. This review illuminates the actual status of the more advanced recombinase-mediated cassette exchange (RMCE) techniques that have been developed for the major members of site-specific recombinases (SR), Flp, Cre and Φ C31. In RMCE the genomic address consists of a set of heterospecific recombinase target (RT-) sites permitting the exchange of the intervening sequence for the gene of interest (GOI), as part of a similar cassette. This process locks the GOI in place and it is ‘clean’ in the sense that it does not co-introduce prokaryotic vector parts nor does it leave behind a selection marker.

Abbreviations: FRT – Flp-recombinase target site; HR – homologous recombination; RMCE – recombinase-mediated cassette exchange; RT – recombinase target; SR – site-specific recombination

Introduction

Genetic engineering strategies usually require permanent modification of the target genome. To this end great sophistication is invested for the design of the introduced genetic material. In striking contrast, much cruder methods still prevail for the placement of the introduced gene into the genome, random integration often being state

of the art. Lack of control over the position of introduced DNA results in unpredictable gene expression and potentially undesirable mutagenesis of important genes.

To change this situation, methods are actively developed that enable a targeted integration into stable and consistently expressed genomic *loci*. While homologous recombination (HR) can, in principle, provide great specificity to the integration

process, and while it is widely applied to stem cells, its efficiency for most biotechnologically relevant cell lines is much too low.

In this situation site-specific (SR) integration systems are of increasing relevance, as they provide targeting frequencies at least three orders of magnitude higher than those resulting from HR. Although mostly prokaryotic in origin or derived from yeast, some of these tools maintain their activity even in the environment of a higher eukaryotic cell. Site-specific recombinases can catalyze three basal and several more sophisticated reactions, most typically:

- *Integration (addition)* for instance of an entire vector sequence, by the crossover between two ‘recombinase target’ (RT) sites on separate DNA molecules (review: Bode et al. 2000);
- *Excision or resolution* between two identically, equally oriented RT sites (the so called ‘*flox-ing*’ or ‘*flrting*’ principles, depending on the recombinase system Cre/*loxP* or Flp/*FRT*). Excision is the formal, highly efficient (kinetically and thermodynamically favored) *reversion of the integration process* (review: Baer and Bode 2001);
- *Inversion* of a DNA segment that is flanked by two identical, but oppositely oriented RTs. The process leads to *equilibrium between the two possible orientations*;
- *RMCE (recombinase-mediated cassette exchange)* a genomically anchored *cassette* (typically a selection- and/or a reporter-gene) can be *exchanged for the ‘GOI’* (gene-of interest) bearing cassette that is presented as part of an ‘exchange vector’. We define as a ‘cassette’ a segment of DNA that is flanked by a ‘set’ of RTs, i.e., two heterospecific (non-compatible) sites (reviews: Bode et al. 2000; Baer and Bode 2001);
- ‘*Flexing*’ a strategic combination of the inversion and RMCE principles by which a cassette becomes *locked-in in its inverse orientation* (Schnutgen et al., 2003). In this case both flanks of the cassette are constituted by the same ‘doublet’ of heterospecific sites. The ‘doublets’ are oppositely oriented with regard to each other whereby inversion (two possibilities) is inevitably followed by the excision of one site leading to a unique end product. Inversion can be succeeded by standard RMCE steps (review: Branda and Dymecki 2004, and Figure 4).

The last two related subjects will form the core of this article.

Figure 1 presents an overview demonstrating the principle of RMCE in the framework of the classical ‘tag-and-exchange’ strategy (Seibler et al. 1998). The technique provides for the replacement of a genomically anchored cassette, which has either been established at a pre-defined genomic *locus* by HR or by random integration and selection. The genomic target (T′) thus formed is delimited by heterospecific RT sites (half arrows) and thereby permits site-specific recombination (SR) with an analogous replacement cassette that enters as part of the ‘exchange vector’ (e2 or e2′). This process leads to the exchange of the positive–negative selection marker (+/–) for a gene variant (Gene*) to yield a modified locus (T′′). These processes have been described in detail before (Schlake and Bode 1994; Seibler et al. 1998; reviews: Bode et al. 2000, 2003; Baer and Bode 2001).

In contrast to a simple integration reaction RMCE is an essentially irreversible process, as the exchange vector is presented at large molecular excess. Also in contrast to a simple integration, RMCE does not co-introduce the prokaryotic parts of the exchange vector and it can be performed such that no selection marker remains in the genome. Both features prevent a subsequent silencing of the transgene (Riu et al., 2005) due to epigenetic defense mechanisms like DNA methylation or due to promoter occlusion.

As for HR, the replacement of a gene by SR requires a double-reciprocal crossover, i.e., an event during which plasmid parts of the exchange vector are excised by crossover between two sections of homology (here: two sets of recombinase target sites located on T′ and e2, respectively). While these steps are usually separated in time for HR, for SR there is little if any evidence for long-lived intermediates (crossover at one or the other target site in SR, see Schlake and Bode 1994). This is the rationale for our simplified representation of RMCE (immediate replacement of one cassette for another by double-crossover) in all figures.

If we consider cell-type specific properties, the systematic modification of biotechnologically relevant cell lines such as CHO (Chinese hamster ovary) or BHK (baby hamster kidney) cells is a much more difficult task than the corresponding procedures for primary cells, especially embryonic stem (ES) cells. Central reasons are:

Figure 1. A 'tag-and-exchange' concept involving RMCE. The strategy enables the exchange of a pre-existing gene and was traditionally based on two successive homologous recombination (HR) steps. The 'tag' ('knock-out') step replaces the target gene (*T*) by a positive-negative selection marker (*T'*), which is subsequently replaced in the 'exchange' ('knock-in') reaction by a gene mutant to yield *T''*. The principle can be improved significantly if the tagging step introduces the selection marker in the form of a cassette that permits the exchange step to be done by site-specific recombination (SR). Here the second (RMCE-) step requires the double-reciprocal crossover between sets of heterospecific recombinase-target (RT-) sites, symbolized by the half-arrows marked ' F_m ' and ' F_n ', respectively. The reaction is driven by the stoichiometric excess of the exchange vector *e2*. If appropriately designed, it does not co-introduce prokaryotic (dashed line) vector parts. Since negative selection via HSV-Tk may cause difficulties in certain cases, positive selection has also been performed using a *neo*^r gene as part of a modified exchange vector (*e2'*). Since this positive marker has been 'floxed' (flanked by identical *loxP* sites, 'L'), it can later be removed by a pulse of Cre-recombinase (Roebroek et al. 2005).

- the genome of permanent cell lines is intrinsically unstable and therefore the long-term maintenance of a particular locus is not granted;
- permanent cell lines have been selected for the efficient uptake and integration of transgenes in multiple copies, which are mostly applied as part of a circular vector. While for ES cells the integration of linearized DNA is by far preferred over the integration of circular DNA, the opposite may be true for transformed cells. In other words: while ES cells will incorporate a circular plasmid preferentially by RMCE, this process may be accompanied by random integration events in the second case, especially at high doses of the exchange vector.

Therefore, to obtain a line with predictable expression characteristics, it will be necessary to tag a spectrum of integration sites at random,

determine their long-term characteristics via a reporter gene and select a suitable clone for re-use.

For a full appreciation of the options offered by RMCE, we will start with a brief description of the strategies that have already been realized for ES cells. Subsequent sections will be devoted to some properties of the major recombinase systems (Flp/*FRT*, Cre/*loxP* and Φ C31/*attP-attB*) and to refined strategies that are under present development. In the concluding part we will exemplify the use of these systems for the rational construction of production cell lines.

One step ahead: modification of murine ES cells

RMCE

After introducing the RMCE concept in 1994 (Schlake et al.) we reported its first application to

murine embryonic stem cells in 1998 (Seibler et al.). We isolated several clones for which we had introduced a positive/negative selection marker at *random* genomic locations and at the single copy level (situation T' in Figure 1). This marker is a fusion gene encoding both hygromycin (HYG) resistance and HSV-thymidine kinase (Tk), which provides a HYG^r state for the candidate clones. Since the selection cassette is flanked by a set of heterospecific *FRT* sites, in this case the wild type *FRT* site (F) and a mutant (F_3), it constituted a target (T') according to the RMCE principle. During such a reaction it was replaced by a matching cassette in which a *neo*^r gene is flanked by the same set of sites generating a potential new target (T'') for subsequent rounds of RMCE. As the *HSV-tk* function is lost by the T'–T'' conversion, the resulting clones are predicted to survive the presence of ganciclovir (GANC), which is otherwise converted, by HSV-tk, to a toxic nucleoside analogue ('negative selection' principle). Three out of the originally 10 characterized clones enabled targeting frequencies of 20–40%, for the others the efficiencies were lower due to integration at sites that were either less accessible or that mediated spontaneous resistance to GANC by loss, the mutation or epigenetic inactivation of *HSV-tk*. During this and the subsequent studies the following observations were made and accounted for:

- the choice of a fusion gene (*hygTk*) for positive/negative selection turned out to be appropriate: keeping clones under hygromycin up to the time point RMCE enforces the expression of a functional HygTk fusion protein. Thereby the phenomenon of spontaneous resistance is significantly reduced. Some of our later studies use GTN (a *egfp/tk/neo*^r fusion gene; see Unsinger et al. 2004) as an alternative positive/negative selection system enabling the use of GFP-expressing, G418 resistant cells for RMCE (Baer 2002);
- the proportion of clones that had undergone authentic RMCE can be increased significantly when the exchange vector is also provided with a *puro*^r gene (Goetze et al. 2005). Puromycin resistance develops already during the transient expression phase (Taniguchi et al. 1998) enabling a pre-selection for RMCE-candidates that have actually received the exchange plasmid. Other systems for a pre-selection have been developed since (see Figure 3 and below).

Using these improvements, we have injected RMCE-positive ES clones into blastocysts and implanted these into pseudopregnant foster mothers. Blood cells of the chimeric offspring were analyzed for GFP expression and surface-antigens were characterized to prove that a high percentage of such cells express a hematopoietic glycoprotein marker derived from the ES cells (E. Ernst GBF and D. Kioussis, MRC London, unpublished).

In a more advanced study we could realize the comprehensive tag-and-exchange strategy depicted in Figure 1: Cesari et al. (2004) have generated *Elk-1* null allele ES cells via HR knockout by a *hygTk* fusion gene. *Elk1*-deficient mice were viable and without phenotypical abnormalities, apart from male sterility, which was related to the *tk* function. This correlation was established by a subsequent Flp-mediated RMCE reaction, which replaced *hygTk* by a *neo*^r cassette. Altogether this report was the first successful application of RMCE for generating transgenic mice from ES cells.

More recently Roebroek's group chose Flp-RMCE to introduce mutations into the *Lrp-1* (LDL-receptor related protein) gene. To this end the endogenous locus was tagged by the *hygTk* cassette and exchanged for a cassette that – besides the LRP cDNA mutant – also contained a *neo*^r gene (exchange vector e2' in Figure 1). Pilot experiments demonstrated that a combination of G418-positive selection and ganciclovir-negative selection resulted in 100% efficiency, but the resulting ES cells grew poorly due to the presence of ganciclovir. Such a negative impact could be circumvented due to the high efficiency of G418 positive selection alone, which enabled exchange rates of 40–70%. To avoid negative effects of persistent *neo*^r expression the *floxed* gene could subsequently be removed by a pulse of Cre-recombinase. A remarkable control for the procedure's performance was a re-insertion of the *wt-Lrp-1*-gene, which restored the original phenotype (A. Roebroek/University of Leuven, personal communications and Roebroek et al. 2005). These results prove the efficiency of RMCE in murine ES cells even in the absence of negative selection. Another potential benefit of removing the positive selection marker is that, in principle, the second allele can be modified with the same targeting vector to create ES cells homozygous for the desired alteration.

Gene trap-mutagenesis

A standard gene-trap vector contains a promoterless reporter gene flanked by an upstream splice acceptor and a downstream polyadenylation signal. When inserted into an intron, transcriptional activation will result in a fusion transcript composed of upstream coding sequences and the reporter. The versatility of gene trap vectors could be improved by including sets of different recombinase-target sites that enabled the post-insertional modification, by RMCE, of the trapped genes. Along these lines Cobellis et al. (2005) constructed both 5'- and 3'- gene trap vectors based on heterospecific *FRT* sites (here: *FRT*_{wt} and its F5 mutant as reported by Schlake and Bode 1994). A library of ES cell clones was constructed and RACE-PCR applied to characterize about 10,000 insertion sites. The system was validated by demonstrating that inserted reporter transgenes had expression patterns identical to the endogenous genes.

'Flexing'

A recombinase-mediated '*flex*-switch' provides the stable inversion of a DNA insert. Such a strategy is employed to swap a coding sequence downstream from a promoter/enhancer element in a way that its expression is turned on while the expression of another one is concomitantly turned-off.

The *flex* procedure involves two recombination events, i.e., an inversion followed by an excision step. The principle is explained in the context of Figure 4 below: following inversion at either wild type or mutant target sites, a pair of homospecific sites will flank a heterospecific target site. Recombination between these directly oriented identical sites (which have to be separated by at least 82 base pairs) results in the excision of the intervening site whereby the inverted cassette becomes locked in place. After these steps the product remains flanked by heterospecific sites and it can hence serve as a substrate for subsequent rounds of RMCE.

An extended *flex* switch strategy has also been devised permitting these vectors to be inverted twice following their insertion into the genome

(review: Branda and Dymecki, 2004): the successive activation of two different SR systems (Cre and Flp in combination with the corresponding site-mutants) permits the reversion of the trap-induced modification.

Major tools: site-specific recombinases and their performance in RMCE

Recombinases belong either to the integrase or to the resolvase family. Members of the first class share similarities with the Int recombination system of bacteriophage λ in that they form a putative Holliday junction as the central recombinatorial intermediate. In contrast, the resolvase family introduces cuts close to the center of the crossover site and proceeds through a four strand cleaved intermediate. Here the protein-DNA linkage is a phosphodiester bond from the 5' end of DNA to a conserved serine (Stark et al. 1992).

Integrases like Cre and Flp carry out a tyrosine-mediated strand exchange by recombining two *identical* recognition-target sites. Basic reactions are grouped into intramolecular inversion, intramolecular excision, and intermolecular recombination (see: Introduction). During these processes the top and bottom strand cuts are fixed at points within the crossover site that are staggered by 6–8 bp. The process recreates the same sites and results in a product that can undergo subsequent rounds of recombination. This is why integration is usually immediately reversed by the close to 100% efficient excision reaction. Promoter- or ATG-trap procedures have been described for a recovery of the rare persisting integration events (see Schüebeler and Bode 1998) but they are comparatively inefficient in comparison to the related reactions catalyzed by the Φ C31-resolvase (below) or by reactions according to the RMCE principle (Figure 2a and b).

Both Cre- and Flp-integrases operate efficiently on both relaxed and supercoiled DNA although a preference of Flp for supercoiled substrates has been demonstrated *in vitro* (Umlauf and Cox 1988). Both enzymes conserve existing linking numbers during site-specific recombination – a fact that has opened intriguing possibilities for the study of supercoiling in genomic DNA (Kouzine et al. 2004).

Figure 2. Actual procedures to perform RMCE at pre-characterized genomic sites (a) Flp-RMCE. A single-copy insert consisting of a positive-negative selection marker (+/-) cassette (either *hyg/tk* or GTN) is established by standard electroporation. RMCE is driven by transfecting an exchange vector that carries the gene of interest (GOI) as part of a compatible cassette and can be enriched by 'negative selection' (survival of cells surviving the presence of ganciclovir due to their loss of the negative selection marker, usually *HSV-tk*). Recent versions of the exchange vector also encode Flp recombinase and an Rfp marker, which can be used to select successfully-transfected cells by fluorescence-activated cell sorting (FACS). As the exchange vector with the GOI will always be present at molar excess, its derivative (carrying the original positive/negative marker) will be lost during cell division or by degradation. (b) Φ C31 catalyzed RMCE between a genomic cassette (flanked by phage-derived attP-sites) and an exchange vector (containing the GOI flanked by bacterial attB sites). In the version described by Belteki and colleagues (2003), it is the genomic target, which encodes the recombinase in addition to a positive selection marker (*puro^r*). Φ C31 recombinase always recombines an attP-site with an attB-site. The product sites (*attL* and *attR*, respectively) are different from the educt sites and represent no substrates for the recombinase, at least in the absence of additional factors. In the author's original procedure the GOI was a *neo^r*-gene permitting the enrichment of successful RMCE-events by positive selection in the presence of G418. (c) Approaching the tag-and-exchange concept (Figure 1) by a two-step HR-based tagging procedure that involves an enhancer trap. The target of this procedure is the *C μ* locus in a hybridoma cell line, which is associated with its endogenous enhancer (*enh*). The first part of the tagging step (HR-1) consist in the crossover between homologous sequences (dashed lines). If the exchange vector is presented as linearized DNA, recombination at DNA ends will dominate. The HR-1 step prefers authentic recombination events to random integration since the *neo^r* gene carries a minimal promoter, which profits from the presence of the nearby enhancer such that the intermediate can be selected for G418. Properly targeted transformants can then be used to derive secondary recombinants (HR-2) that have properly excised the *tk*-containing vector parts (dotted line) and thereby become resistant, for instance to ganciclovir. Part of HR-2 events will re-create the initial situation such that selection for the presence of the GOI will be required. If the GOI has been flanked by SR-sites as for Figures 1, 2a and b, a re-usable cell line will result that lends itself to subsequent rounds of modification by RMCE.

Cre

The Cre/*loxP* system has evolved in bacteria for the conversion of dimeric phage P1 plasmids into their monomeric constituents by the phage-encoded integrase Cre ('causes recombination'). The Cre target site, *loxP* (locus of crossover in P1), is a 34 bp sequence consisting of two inverted 13 bp repeats separated by an 8 bp spacer. Improvements were reported for an NLS-tagged Cre recombinase (NCre; Nakano et al. 2005). Alternatively, fusion of Cre to a basic peptide from

HIV-TAT has been found to facilitate the cellular uptake of Cre-protein (Peitz et al. 2002) eliminating the need to electroporate cells with a Cre-expression plasmid.

For several years no combination of *loxP* sites was known that permitted RMCE. Initially, a mutant, *loxP511*, with a single-base substitution in the central eight-base region, has been used in conjunction with a *loxP_{wt}* site. This combination did not enable negative selection, however, due to its residual cross-recombination potential. As an intermediate solution this problem was overcome

by using two oppositely oriented *loxP_{wt}* sites in a system called ‘L1–1L’, which causes cassette inversion in addition to cassette exchange (review: Baer and Bode 2001). More recently, a mutant, *loxP V*, (previously called *loxP 2272*) with two-base substitutions was described whose recombination efficiency was as high as that of *loxP511*, but for which recombination with *loxP_{wt}* was undetectable. Consequently, *loxP V*, together with *loxP_{wt}*, was successfully applied in the RMCE reactions, yielding a high fidelity and efficiency by selecting for drug resistance (Nakano et al. 2005).

Problems. It has long been thought that the bacteriophage P1 origin of Cre ensures that it is inert in mammalian cells. Since 2000 (Schmidt et al.) a number of reports appeared suggesting that Cre recombinase causes chromosomal aberrations due to the occurrence of genomic pseudo-*loxP* sites. Since these *loxP*-like sites bear little resemblance to the phage *loxP* element, this ‘star activity’ may arise as a consequence of Cre-overexpression as it is commonly used in transgenic studies. The presence of palindromic DNA close to a *loxP* site also interferes with the homology search by Cre to an extent that heterospecific sites are no longer discriminated (Mlynarova et al. 2002). While the spacer double-mutant, *loxP V*, has permitted RMCE in conjunction with *loxP_{wt}*, additional sets of heterospecific, non-crossinteracting site variants would be required to permit multiplex-RMCE (see below). Efforts in this direction are now underway and a mutant L3 (three spacer positions mutagenized) has recently been introduced by Wong et al. (2005).

Flp

The Flp/*FRT* system provides for a highly efficient replication of the 2 μ plasmid in yeast by the inversion of a segment that is flanked by two identical, oppositely oriented 48 bp *FRT* (Flp-recombinase target) sites. Although Flp (‘flippase’) has been named for this ability, like the Cre/*loxP* system it also enables the excision of DNA segments depending on the relative orientation of the RT-sites. Also like for Cre but different from Φ C31 resolvase (discussed below) the formal reversal of excision (integration) can only occur against thermodynamic and kinetic barriers and it

is highly inefficient (Baer and Bode 2001). These findings led to the development of the Flp-RMCE concept that has first been realized in 1994 (Schlake and Bode). Although an RMCE-like protocol can also be performed by the simultaneous use of *loxP* and *FRT* sites in combination with Cre and Flp (the ‘froxing’ principle according to Lauth et al. 2002), the most convenient way still is the use of a single recombinase. To this end we have engineered the *FRT_{wt}* sequence in its 8 bp spacer and have obtained a series of *FRT_{mut}*-sites that recognize each other with maximum efficiency but show little or no cross-interaction with *FRT_{wt}* or a different mutant (reviews: Bode et al. 2000; Bode and Baer 2001). Considering the fact that each set of *FRT* sites represents a unique target in the genome, various pre-defined genomic *loci* can be addressed simultaneously in accord with the ‘multiplex RMCE’ concept (Bode et al. 2000 and Figure 5).

Problems. When compared with one another, Flp-integrase appears to be less efficient than Cre-integrase in recombining its substrates. Optimized versions of Flp such as the more thermostable Flp-L variant Buchholz et al. 1996) and the ‘enhanced’ Flp-e (Buchholz et al. 1998) have largely improved the potential of this system but even these modifications appear to have only 10% recombination activity on chromosomal targets compared to Cre or an optimized form of Φ C31 integrase (Andreas et al. 2002).

Lambdoid integrases

When integration alone is the goal, recombinases of the lambda-type have been expected to be superior to the above systems. Int-type enzymes can perform an isolated integration reaction as they require different accessory factors for its reversal: once integrated, the transgene is locked in place. Although this is the case for the well-known coliphage lambda integrase, this system could not live up to initial expectations.

In concert with other proteins (integration host factor, IHF and excisionase, Xis), λ -Int mediates phage integration into and excision out of the *Escherichia coli* chromosome. For integration, the two recombining sites are the short bacterial target *attB* site and the more extended phage *attP* site, which are identical in only 15 positions including

the locus of DNA exchange. Since the educt sites are different, recombination does not recreate *attB* and *attP* but it results in different products (the *attL* and *attR* hybrid sites).

Problems. Unfortunately, it was not possible to transplant this system into mammalian cells. This is due to the fact that in this milieu the required factors cannot be expressed in a biologically active form (review: Baer and Bode 2001). Since the 240 bp *attP*-site requires negative superhelicity in order to recombine with *attB*, this requirement has to be met by its representation as part of an intact plasmid. Even in the case of lambdoid phage HK022, for which integration in mammalian cells has been reported (Kolot et al. 1999), yields are rather low and could only be improved in case the necessary cofactors could be co-expressed in an active form.

Resolvases: Φ C31 recombinase, a new option

Recently, a new subgroup of the resolvase/invertase family has been identified consisting of high molecular mass (51–82 kDa) members with the catalytic domain in their N-terminal region. The Φ C31 recombinase from *Streptomyces* enables the phage to integrate into the bacterial chromosome via *attP/attB* site interaction analogous to the above Int enzymes (Thorpe and Smith 1998). The minimal extension of these sites has been determined to be only 34 and 39 bp, respectively (Groth et al. 2000). As for the Int protein, the product sites resulting from this crossover carry hybrid sequences refractory to further synapsis by the integrase and thereby the integrated state is locked.

The system properties have first been investigated in an *in vitro* assay containing only buffer, purified integrase, and DNAs encoding *attP* and *attB* where recombination occurred irrespective of whether the substrates were supercoiled or linear. The fact that the products of *attP/attB* recombination (*attL* and *attR*), were identical to those obtained after integration of the prophage in *S. ambifaciens* clearly demonstrated that the integration reaction requires no cofactors. No recombination was observed between *attB/attB*, *attP/attP*, *attL/attR*, or any combination of *attB* or *attP* with *attL* or *attR*, suggesting that excision of the prophage (*attL/attR* recombination) would

need additional phage- or *Streptomyces*-encoded factor(s).

It could be demonstrated that Φ C31 mediated integration works well in a mammalian cell milieu, consistent with its lack of cofactor requirements. The efficiency of the recombinase could be further enhanced by the C- but not N-terminal addition of a nuclear localization signal (NLS) whereby its efficiency became comparable to the widely used Cre/*loxP* system (Andreas et al. 2002). A Φ C31 recombinase-based RMCE system has recently been described (Belteki et al. 2003 and Figure 2b).

Problems. While in cell lines containing an inserted *attP* site integration occurs at frequencies 10- to 20-fold above the spontaneous background of random integration, a Φ C31-mediated recombination 5- to 10-fold above background was even observed in the absence of any inserted authentic *attP* sequence. This fact led to the detection of pseudo *attP* sites in the human and mouse genomes (Thyagarajan et al. 2001). In unmodified human cells, nearly 90% of the integration events are actually integrase mediated and sequencing the junctions of rescued integration events revealed a hierarchy of pseudo *attP* sites, some of which were used repeatedly. Since the only type of recombination occurs between *attB* and (pseudo-)*attP*, the product cassette cannot be subjected to other rounds of RMCE nor can multiplexing concepts be realized.

Improving RMCE efficiency

Auxiliary elements and their position

RMCE is based on the exchange of expression cassettes at a previously tagged site (Figure 1). Since the site of chromosomal integration remains the same in all targeted cells and since the copy number is reduced to one for common tandem head-to-tail insertions, the level of expression is highly predictable.

Conventional RMCE protocols involve the separate transfer of an exchange vector and a recombinase expression plasmid. Ideally, recipient cells that are candidates for cassette exchange are pre-selected to increase the proportion of authentic events in the population that resists negative selection (Figures 1 and 2). We have recently introduced an exchange vector comprising all these

functions in addition to the GOI as it encodes the FLP-e recombinase and a transfer marker (red fluorescent protein, RFP) that can be used for FACS-enrichment of successfully transfected cells (A. Oumard, unpublished and Figure 2a). Belteki et al. (2003) have applied a complementary RMCE approach using Φ C31 recombinase. In their method the nuclear target cassette encodes both the recombinase and a positive selection marker (Figure 2b). Since the cassette is flanked by *attP* sites, it is exchanged for the GOI and lost at a time where these functions are no longer needed.

Other refinements on the FLP-RMCE-concept have been summarized in Figure 3 (A. Oumard and J. Qiao, unpublished):

- using the *hyg^{tk}* positive/negative selection marker, spontaneous resistance to ganciclovir has been a recurrent problem (Seibler et al. 1998). One potential contribution to the loss of the *HSV-tk* function by processes other than RMCE is a minor cross-interaction between the F_{wt} and F_{mut} -sites, respectively. Such a contribution is effectively prevented by applying the *FRT*-variants in their inverse orientation (Seibler et al. 2005);
- the success of gene-trap vectors with the built-in option to perform subsequent modifications by RMCE has depended, in part, on the presence of ca. 1 kb regions of ‘neutral’ buffer DNA next to the *FRT* site of the cassettes (Cobellis et al. 2005). These buffers are thought to protect

Figure 3. Improved selection procedure for RMCE-competent genomic sites with appropriate expression characteristics. A positive-negative selection marker (+/-, i.e., *hyg^{tk}*) is anchored in the recipient cell in analogy to Figure 2a, but with the following modifications: (i) the heterospecific (*FRT*-) sites are inversely-oriented and (ii) the cassette is flanked by recombinogenic (S/MAR-) ‘buffer DNA’. The first measure would induce inversion but not excision in the rare event of crossover between equally-directed heterospecific *FRT*-sites. This is a precaution that eliminates the occurrence of ganciclovir-resistant (*tk*-) clones not due to RMCE. Like the genomic insert, the exchange vector is also flanked by S/MAR-DNA. After RMCE 1 highly (*e-gfp*-) expressing clones are recovered by FACS and subjected to a second RMCE-step in order to restore the original configuration for clones with highly expressed and RMCE-competent genomic sites. This property is anticipated for clones that lose their fluorescence during RMCE 2.

FRT-functions, for instance during the process of integration. Another argument might have been the presence of DNA elements interfering with the recognition function of the recombinase as reported for the Cre enzyme (see chapter on ‘Major tools: site-specific recombinases and their performance in RMCE: Cre). We have observed the beneficial properties of S/MAR sequences in the position of buffer DNA as the recombination efficiency was effectively increased in this case (J. Qiao, unpublished). In this context, several functions of S/MARs come into mind:

- S/MARs usually are AT-rich elements that do not contain nucleosome positioning signals and that are well suited to keep genomic *loci* open (review: Bode et al. 1998);
- S/MARs represent recombinogenic sequences with an increased propensity to undergo strand separation (Bode et al. 2005). The parameters listed by Umlauf and Cox (1988) regarding the role of GC content and supercoiling suggest that DNA unwinding facilitates recombination. It appears conceivable therefore that crossover reactions between identical FRTs are favored in this situation (Bode et al. 2005, in press).

Finally and in contrast to some previous approaches (Baer 2002) the selection for RMCE-competent, high and persistent expressing sites is performed only after two successive steps of RMCE where RMCE 2 restores the starting situation. Performance of all steps is monitored by FACS, which is the method to recover candidate clones via high fluorescence following RMCE 1 and via fluorescence loss via RMCE 2. Some aspects of this approach have been published (Goetze et al. 2005).

Extension of the flexing principle

The principle has been introduced in chapter on ‘flexing’ above. Figure 4 demonstrates its modification to express a GOI following site characterization by means of an *e-gfp* reporter. Involving two efficient, concerted recombinase-mediated steps, the procedure is appealing since it neither requires the secondary transfer of an exchange

vector nor negative selection for enrichment of the desired event. Although the coding region of the *e-gfp* reporter remains in place, the *flex*-switch uncouples it from the promoter in a way that no interference is to be expected.

The procedure resembles the concept in Figure 3 in that FACS-selection and FACS-counterselection steps are applied. While the first step serves to recover cells that underwent insertions, the second one is used to bring the GOI under the control of the promoter. This will only occur at sites that are accessible to the recombinase and will hence lead to clones that can be subjected to successive exchanges according to the standard RMCE protocol.

Use of retroviral integration sites

Retroviral vectors are known for their capability to anchor single copies of proviral DNA at accessible, stably transcribed matrix-associated genomic sites (Baer et al. 2000; Goetze et al. 2003). They have therefore been used for introducing sets of RT-sites into appropriate genomic *loci*. As usual, a reporter is introduced first and screening is then performed for cell clones with appropriate expression parameters. These clones are subsequently targeted by a modified RMCE procedure, which activates an ATG-free *neo^r* gene residing in the 3’LTR of the provirus (Wirth and Hauser 2004; review: Baer and Bode 2001). The procedure works with close to 100% efficiency but it inserts the GOI in the presence of a co-expressed selection marker and thereby it does not yet meet the stringent criteria of a ‘clean’ replacement (see: introduction).

Emerging applications

Antibody chain expression

Antibodies are the most important and fastest growing group of protein therapeutics. Currently 22 antibodies have been FDA approved and 260 are under clinical development.

So far only mammalian expression systems are applicable for the generation of fully functional IgG antibodies. Although there is considerable progress in the large scale production of

Figure 4. Applications of a flex-switch. Following inversion between equal components of the twin-sites, a set of identical (homospecific) sites flanks a heterospecific target site. Recombination between the now equally-oriented identical sites results in the excision of the intervening heterospecific site; this process locks the inverted cassette in place. The product remains flanked by heterospecific sites and can hence serve as a substrate for subsequent rounds of RMCE as for Figure 3.

recombinant antibodies in mammalian cells, the methods used to generate production cell lines are still conventional necessitating laborious screening routines. Since classical procedures mostly rely on the random insertion of mono-cistronic expression cassettes for both antibody chains into the genome of transfected host cell lines there is little control over the number of integrated copies, which trigger repeat-induced silencing (co-suppression) phenomena (Garrrick et al. 1998).

Although CHO gene amplification systems have been widely used for protein production (review: Wurm and Jordan 2003) these principles induce genomic instability as indicated above (Weidle et al. 1988). In addition, early work from this laboratory has already demonstrated that expres-

sion levels are more dependent on the characteristics of a genomic integration site rather than the number of integrated copies (Wirth et al. 1988). With the advent of RMCE-based strategies these techniques will become dispensable since they allow the architecture of the eukaryotic nucleus to be taken into account.

RMCE-multiplexing approach

The approach suggested in Figure 5 exploits the previously described RMCE-multiplexing principle (Bode et al. 2000) which relies on the availability of numerous heterospecific FRT sites that are available from the work of Schlake and Bode (1994) and on the fact that each set of heterospecific sites presents a unique address in the genome.

Step 1 introduces, by electroporation, a GTN fusion gene comprising *egfp*-, *HSVtk*- and *neo^r* functions. Suitable high- and long-term-stable clones are isolated by FACS as usual and the GTN cassette is subjected to RMCE, which serves to introduce a *hyg^r* cassette (step 2). This common concept, which has already been introduced in Figures 3 and 4, not only grants that we have to deal with a site that lends itself to cassette exchange but it also sets the stage for another round of transfection (step 3) which introduces a second cassette that is flanked by a different set of *FRT* sites (F4 and F5 in the Figure 5 example). Suitable clones are recovered as before. The final reaction is a common RMCE step (step 4) in which both targets are exchanged for the cassettes carrying the heavy and light chain antibody genes tagged by either an F3-F4- or an F4-F5 address.

Combination of HR and SR

Hybridoma cell lines are particular in that they contain recombinogenic genomic segments at their endogenous H- and L-chain *loci*. These sites make it possible to introduce variant H- and L-chains by homologous recombination. To this end Bautista

and Shulman (1993) and Ng and Baker (1998) have developed a two-step gene targeting procedure shown in a simplified form in Figure 2c. This representation has intentionally been aligned with RMCE concepts (Figure 2a and b) to suggest ways (in addition to the one shown in Figure 1) by which both technologies can be combined to generate an RMCE-competent cassette utilizing a strong, endogenous promoter/enhancer combination for the expression of any GOI. The HR 1 step of the procedure involves integration of the entire vector sequence into the *C μ* locus. As recombination is facilitated when the ends of the transferred DNA are homologous to the cognate chromosomal target, a strategically positioned cut will direct the primary site of crossover. Vector integration in the vicinity of the genomic μ -enhancer has been found to increase the expression of the *neo^r* gene that up to this point is barely expressed due to a minimal promoter. Specific integration creates G418 resistant cells for which the ultimate target and the inserted plasmid sequence with the *neo^r* and the *tk* genes can align. In the HR 2 step an intra-chromosomal crossover between vector-borne and chromosomal *C μ* regions (dashed lines)

Figure 5. Tag- and target reactions for two genomic sites. A four-step procedure according to the 'multiplex-RMCE' principle. The first genomic site is established and characterized by RMCE in step 2 (which resembles RMCE 2 in Figure 3). The second genomic site is established accordingly (step 3). The final RMCE reaction (step 4) serves to introduce both the light- and the heavy-chain genes, each at its pre-determined site. Clones surviving the presence of ganciclovir should have undergone exchange at both sites, they should be non-fluorescent, insensitive to ganciclovir but sensitive to both G418 and hygromycin.

eliminates the complete intervening vector sequence (dotted line) and this event can be enriched by Tk-counterselection according to negative selection principles. This second crossover might occur on either side of the aligned genes. While situation (A) replaces the $C\mu$ gene by the GOI, situation (B) would restore the starting situation.

Recent evidence for the actual performance of such a combined HR/SR strategy is found in a report by Winkler et al. (2005) on a heterohybridoma cell line. The original cell line produced IgM at levels of 30–100 $\mu\text{g/ml}$. It could subsequently be modified for the high level expression of recombinant proteins by targeting the human IgH locus on human chromosome 14, introduction of a set of heterospecific FRT sites and the subsequent use of the FLP-RMCE principle. A mother clone was thus established that lends itself to the rapid establishment of producer cells for recombinant proteins (see also <http://www.esact.org/newsletter/Sept2003.pdf>).

Viral vectors for gene therapy

Conventionally, the production of viral vectors for therapeutic use requires time-consuming cloning and screening steps to obtain packaging cells with high and stable production properties. In the following we describe two current RMCE-based concepts that have already proven their potential to improve the existing procedures. Much like in the examples above the first process relies on genomic sites with pre-determined properties for packaging retroviruses. The second procedure circumvents complicated cloning steps by RMCE-transfer of a GOI directly into an actively replicating adenovirus.

Retroviral packaging cell line

In the course of a current EU project human embryonal kidney cells (HEK293) have been screened for chromosomal loci with adequate expression properties. The procedure involves the transfer of an e-gfp transducing retroviral vector flanked by heterospecific FRT sites. Subsequent transfection of the helper genes led to the identification of suitable producer clones, which permit the integration and high titer production of any retroviral vector at the pre-selected locus. It has been confirmed that after specific integration the

therapeutic gene yields the same expression characteristics of marker gene as the original targeting vector (Coroadinha et al. 2005; Schucht et al. 2005, http://europa.eu.int/comm/research/quality-of-life/cell-factory/volume2/projects/qlk3-2002-01949_en.html).

Gutless adenoviral vectors

The Ad genome is a 36-kb double-stranded linear, episomal DNA that is tagged with a 55-kDa terminal protein (TP) at both ends. So far the most efficient production of Ad-vectors has relied on the adenovirus DNA-terminal protein complex in conjunction with a cosmid bearing the full-length virus genome (COS-TPC method): when transfected to permissive cells, a TPC-tagged Ad genome produces 100-fold more viral plaques than cloned viral DNA alone.

As the DNA-TP complex is abundantly present in infected host cells as part of the replicating viral genome, the direct RMCE-mediated introduction of a GOI into a replicating 'recipient' viral genome comes as a significant advance. To this end Nakano et al. (2005) used a constitutively recombinase expressing cell line (human embryonic kidney 293FNCre cells) performing Cre-RMCE between sets of non-compatible $loxP$ V and $loxP_{wt}$ sites (see chapter on 'Major tools: site-specific recombinases and their performance in RMCE: Cre). The productivity could be optimized with regard to several parameters, among these the MOI of the recipient virus and a maximization of the recipient virus cassette (which provides an advantage to the purpose vector in that the latter outgrows the recipient virus). An additional feature is the Cre-mediated excision of the *floxed* packaging signal from the recipient vector in 293FNCre cells, which contributes to the 'purpose vector' replicating faster than the recipient. As a consequence, the recipient could be eliminated by serial passaging. The method described here is considered first report describing the successful application of RMCE for the construction of viral vectors in mammalian cells.

Perspective

Since the first reports on the use of FLP and Cre for site-specific integration reactions in mammalian cells O'Gorman et al. 1991; Fukushima and Sauer

1992), these tools have become firmly established in the field. An important milestone has been the introduction of the cassette exchange (RMCE) principle for both systems (Schlake and Bode 1994; Bouhassira et al. 1997) and since this time the application of site-specific recombinases continues to increase exponentially. While the first applications were directed to solving fundamental questions and while they have found wide application for the systematic generation of ES cells and transgenic animals (review: Branda and Dymecki 2004) they are now at the verge of revolutionizing biotechnological production processes. Historically, to this end cells were selected for their growth characteristics in defined media and in suspension culture, as well as for the ease of transfection or the possibility of gene amplification – a traditional concept aimed at increasing the yields per cell. The CHO (Chinese-hamster-ovary) cell line was the first to meet these criteria. In cases where post-synthetic modifications like authentic glycosylation patterns had to be approached, the BHK (baby-hamster kidney) line cells has sometimes been the better choice (Grabenhorst et al. 1999).

While RMCE techniques are now actively applied to these systems, major obstacles have to be met which relate to genomic instability and to the fact that the mentioned cell lines are refractory to homologous recombination as elaborated in the introduction of this paper. In this situation it is anticipated that other cell types like hybridoma lines that were described in the chapter on ‘combination of HR and SR’ will be of increasing importance as they permit the systematic modification of known endogenous *loci* by the elaborate HR- and SR-based tag-and-exchange concept that is presented in Figure 1.

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