Gene xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Optimization of coliphage HK022 Integrase activity in human cells

Natalia Malchin [a,1], Janna Goltsman [a,1], Lital Dabool [a], Rena Gorovits [b], Qiuye Bao [c], Peter Dröge [c], Ezra Yagil [a], Mikhail Kolot [a,*]

[a] Department of Biochemistry, Tel-Aviv University, Tel-Aviv 69978, Israel
[b] Otto Warburg Center, The Hebrew University of Jerusalem, Rehovot 76100, Israel
[c] School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, 637551, Singapore

ARTICLE INFO

Article history:
Received 24 November 2008
Received in revised form 18 February 2009
Accepted 19 February 2009
Available online xxxx

Received by D.L. Court

ABSTRACT

The Integrase (Int) site-specific recombinase of coliphage HK022 catalyzes integrative and excisive DNA recombination between two attachment (*att*) sites in human cells without the need to supply the accessory proteins Integration Host Factor (IHF) and Excisionase (Xis). Previous work has shown that under these conditions, reactions in *cis*, i.e. both *att* sites are located on the same chromosome, can be detected without selection. However, recombination in *trans*, i.e. one *att* site positioned on a chromosome and the other on an episomal vector, was detected only after selection. Here we show that optimization of the *int*-HK022 gene for human codon usage according to the GeneOptimizer software algorithm, as well as addition of accessory proteins IHF and Xis improve the recombination efficiencies in human cells, such that recombinants in a *trans* reaction could be detected without selection.

1. Introduction

The Integrase (Int) recombinase of coliphage HK022 mediates integration and excision of the bacteriophage into and out of the chromosome of its *Escherichia coli* host, using a mechanism that is very similar to that of coliphage λ. In both phages, site-specific recombination reactions occur between two defined pairs of DNA attachment (*att*) sites. Integration results from recombination between the phage *attP* site and the host *attB*, and excision occurs between the recombinant *attR* and *attL* sites that flank the integrated prophage. In addition to Int, these reactions require DNA-bending accessory proteins. Integrative recombination requires the host-encoded integration host factor (IHF) and excisive recombination requires IHF and the phage-encoded excisionase (Xis). The latter can be replaced by the host-encoded factor for inversion specificity (Fis). IHF (22 kD) is a heterodimer of two small homologous peptides, IhfA and IhfB. Xis (8.6 kD) is a small monomeric protein (reviewed in Azaro and Landy, 2002; Weisberg et al., 1999).

The *int* genes of HK022 and λ were cloned and expressed in mammalian cells and were shown to be active in integrative as well as in excisive recombination when the proper *att* sites were supplied either on plasmids or on chromosomes, or on both. In contrast to the requirement of Int for IHF and Xis in its natural milieu, the wild type Int-HK022 is active in mammalian cells without the need to supply any accessory proteins (Harel-Levy et al., 2008; Kolot et al., 2003). Int-λ is active in mammalian cells only if it carries mutations which rendered it IHF-independent (Lorbach et al., 2000). However, when mammalian cells were supplied with IHF-bound plasmids as recombination substrates, or expressed an engineered single chain IHF, the inactive wild type Int-λ alleviated recombination on extrachromosomal substrates to the levels of the IHF-independent mutants (Christ et al., 2002; Corona et al., 2003). *In vitro* experiments with Int-λ have also indicated that mammalian chromatin-associated proteins HMG1 and HMG2 can substitute to some extent for the requirement of the prokaryotic accessory proteins (Segall et al., 1994).

The frequencies of Int-catalyzed recombination in *cis* (both *att* sites located in tandem on the same DNA molecule) on mammalian chromosomes are sufficiently high to be detected without selection (Christ and Dröge, 2002). In contrast, reactions in *trans* (one site on the chromosome, the other on a plasmid) are not detectable unless selection pressure is applied (Harel-Levy et al., 2008). If Int is to be developed as a tool for site-specific gene insertion, it is the chromosomal *trans* recombination reaction which is most important. Here we report improvements of the wild type Int-HK022 system through gene optimization and delivery of prokaryotic accessory proteins (IHF and Xis). This allowed us to detect chromosomal site-specific integration of an episomal vector without selection.

Abbreviations: att, attachment site; CMV, cytomegalovirus; FACS, fluorescent-activated cell sorting; Fis, factor for inversion specificity; GFP, green fluorescent protein; IHF, integration host factor; Int, Integrase; oInt, optimized Integrase; RMCE, recombinase-mediated cassette exchange; scIHF, single chain IHF; Xis, excisionase.

* Corresponding author. Tel.: +972 3 640 9823; fax: +972 3 6406834.
E-mail address: kolott@post.tau.ac.il (M. Kolot).

[1] Equal contribution.

0378-1119/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.gene.2009.02.013

2. Materials and methods

2.1. Cells, growth conditions, transfection, selection

Human embryonic kidney cell lines 293 or transgenic derivatives were used in this study. Cells ($\sim 4 \times 10^5$) were plated in a 6 well plate in Dulbecco's modified Eagle's medium, and 24 h later transfected with 1–10 μg DNA of each circular plasmid, using calcium phosphate as described before (Kolot et al., 1999). *E. coli* cells transformed with plasmids were grown in Luria–Bertani medium with the appropriate antibiotics.

2.2. Plasmids and oligomers

Plasmids and oligomers used as primers in PCR reactions are listed in Table 1A and B, respectively.

2.3. DNA extraction

Plasmid DNA from *E. coli* was prepared using a QIAprep Spin Miniprep Kit (Qiagen) or a GenElute™ HP plasmid Maxiprep kit (Sigma). Genomic DNA was extracted from human cells using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma).

2.4. Modification of the optimized int (oint) gene

The *int*-HK022 gene flanked with *HindIII* and *EcoRI* sites was modified for human codon usage by Geneart GmbH, Regensburg, Germany, according to GeneOptimizer software algorithm. It was supplied on vector pGA4.

2.5. Plasmid constructions

To construct plasmid pLD998, the *xis*-HK022 PCR fragment generated with plasmid pPG1 as template and oligomers oEY472 and oEY476 as primers was cloned into the EcoRI and NotI sites of pcDNA3. To construct plasmid pNA979, the HindIII–EcoRI o*int* fragment on pGA4 was cloned between the same sites on plasmid pcDNA3.

2.6. Immunoblots

Immunoblots were performed as previously described for Int (Kolot and Yagil 2003) and for Xis (Gottfried et al., 2004) using polyclonal antibodies.

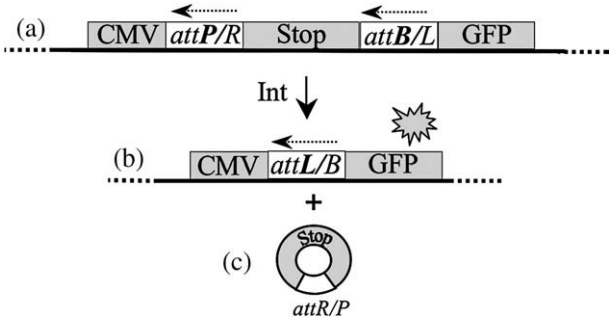

Fig. 1. Diagram representing the Int-catalyzed chromosomal reactions in *cis*. (a) chromosomal substrate. (b) and (c) reaction products. Dotted lines represent chromosomal DNA.

2.7. DNA sequencing

DNA sequences were obtained using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems and Hitachi).

2.8. Fluorescent-activated cell sorting (FACS) analysis

$\sim 2 \times 10^6$ cells from one well of a 6-well plate were collected 48 h post transfection. Following trypsin treatment 10^4 cells were selected by the FACS sorter. The GFP analysis was done with the FACSort and sorting was done with the FACSAria (Becton Dickinson Instrument) for fluorescent measurements. Data analysis was performed using the WinMDI2.8 program. Enrichment sorting was performed 72 h post transfection.

3. Results

3.1. In cis recombination assays

Site-specific recombination catalyzed by Int on human chromosomes in the *cis* configuration was monitored via expression of the green fluorescence protein (GFP). For integrative (*attP*×*attB*) reactions, we used a human cell line made transgenic with plasmid pMK218 as substrate for Int [Fig. 1(a)]. The recombination cassette is composed of a transcription terminator (Stop) flanked by tandem *attP* (P′OP) and *attB* (B′OB) sites. [Fig. 1(a) bold *att* sites]. Int-catalyzed *attP*×*attB* recombination removes the terminator sequence, thus allowing expression of GFP from the cytomegalovirus (CMV) promoter [Figs. 1(b) and (c)]. This can be easily quantified by

Table 1
A List of plasmids and oligomers that were used as primers for the PCR reactions.

A. Plasmid	Relevant genotype	Use	Source
pcDNA3	Neo^R oriSV40 vector	Cloning vector	Invitrogen
pMK52	*int*-HK022 on pcDNA3	Int expression	(Kolot et al., 1999)
pMK218	pCMV-*attP*-Stop-*attB*-GFP Neo^R	Chromosomal *cis* reaction	(Harel-Levy et al., 2008)
pMK189	pCMV-*attR*-Stop-*attL*-GFP Neo^R	Chromosomal *cis* reaction	(Harel-Levy et al., 2008)
pAM242	Stop-*attL*-GFP Neo^R	Episome in *trans* reaction	(Kolot et al., 2003)
pAM243	pCMV-*attR* Neo^R	Chromosomal *trans* reaction	(Harel-Levy et al., 2008)
pIHF2cP	*ihfA-ihfB* fusion	scIHF expression	(Corona et al., 2003)
pLD998	*xis*-HK022 on pcDNA3	Xis expression	This work
pNA979	o*int*- HK022 on pcDNA3	oInt expression	This work
pPG1	*xis* cloned on pETI1	PCR template	(Gottfried et al., 2000)
B. Primers	**Sequence**	**Location**	
oEY327	5′-CTAGAGTCGCGGCCGCTTTACTTGTACAGC-3′	GFP	
oEY398	5′-GGGAATAAGGGCGACACGGAAATGTTG-3′	Amp^R gene	
oEY472	5′-GCGGCCGCTCATGACTTCGCCTTCTTCCC-3′	*xis*	
oEY476	5′-GAATTCATGTACTTAACACTTCAGGAGTGGAAC-3′	*xis*	

Restriction sites are underlined.

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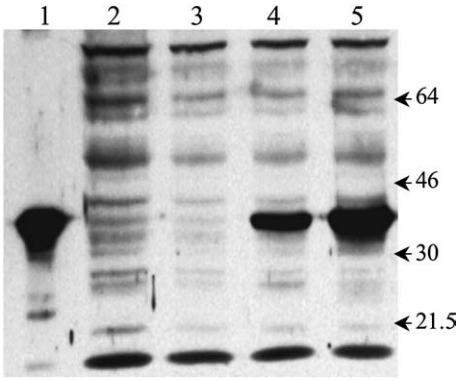

Fig. 2. Immunoblot of purified Int (Lane 1) and cell extracts of the following cell lines: Lane 2, untransfected 293 cells; Lane 3, cells transfected with vector (pcDNA3); Lane 4, cells transfected with the wild type Int-plasmid (pMK52); Lane 5, cells transfected with the oInt plasmid (pNA979). Arrows indicate location of molecular weight markers in kD.

fluorescent-activated cell sorting (FACS). To measure excisive recombination (*attR*×*attL*), we used a transgenic cell line made with plasmid pMK189 that carries *attR* (B′OP) and *attL* (P′OB) sites as direct repeats [(Fig. 1(a)]. Previous work from our laboratory with Int-HK022 has shown ~5% Int-catalyzed GFP-expressing cells in the integrative reaction and ~11% Int-catalyzed GFP-expressing cells in the excisive reaction (Harel-Levy et al., 2008).

3.2. Recombination activity of optimized Int in human cells

FLPe of yeast and Int of *Streptomyces* ϕC31 phage are two site-specific recombinases already used in mammalian genome engineering technologies (reviewed in Branda and Dymecki, 2004; Calos 2006; Sorrell and Kolb, 2005; Wirth et al., 2007). In order to increase recombinase expression levels and recombination efficiencies in mouse embryonic

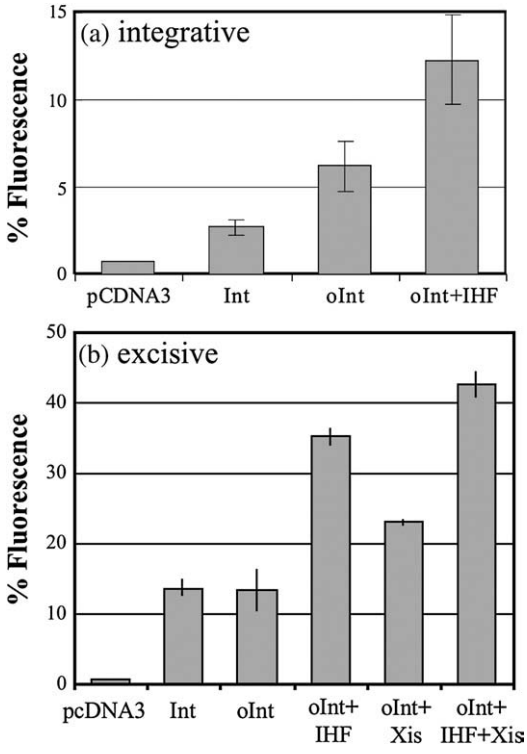

Fig. 3. (a) Fluorescence activity of cell lines transfected with the indicated plasmids in the *cis* integrative reaction. (b) Fluorescence activity of cell lines transfected with the indicated plasmids in the *cis* excisive reaction. Each result represents a mean of at least 3 experiments.

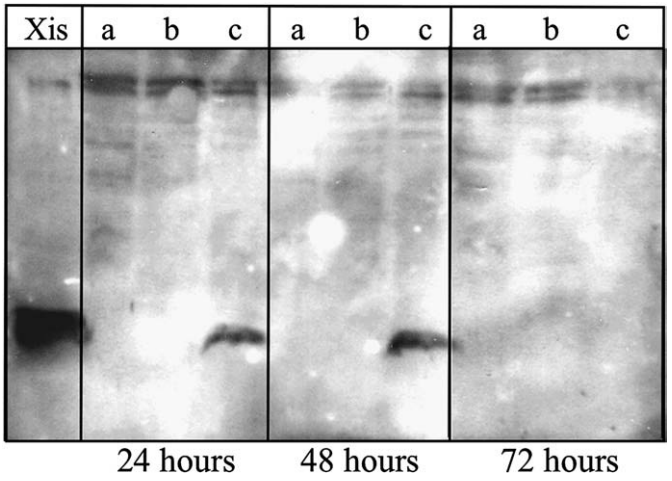

Fig. 4. Immunoblots of purified Xis (Lane Xis), and of the following cell extracts: Lanes a, untransformed cells (293); Lanes b, cells transfected with pcDNA3 vector (pcDNA3); Lanes c, cells transfected with the xis-expression plasmid (pLD998) Cells were harvested 24, 48 and 72 h after transfection with 10 μg plasmid.

stem (ES) cells, the corresponding recombinase genes *flp* and *int*-ϕC31 were optimized using Gene Optimizer software algorithm for mouse codon usage with a reduced number of CpG dinucleotides to avoid methylation. In addition, other elements like TATA boxes, ribosomal entry sites, AT and GC-rich stretches, cryptic splice sites, polyadenylation sites and potential RNA secondary structures were eliminated. Using stable transgenic lines that express these two optimized recombinases, their recombination activity on *cis*-designed substrates improved from a few percent to ~60–80% (Raymond and Soriano, 2007). The *int*-HK022 sequence was similarly modified for human-optimized codons (subsequently referred to as o*int*) and cloned into the eukaryotic expression vector pcDNA3. Expression of oInt was compared with that of wild type Int by an immunoblot prepared from human 293 cells 48 h after transfection with the respective plasmid (Fig. 2). Quantification indicates that oInt expression (lane 5) is about two fold higher than wild type Int (lane 4). Compared to the wild type Cre, a similarly optimized Cre (iCre) showed a 1.6 fold improved exression (Shimshek et al., 2002).

Next, we compared recombination efficiencies in cells transfected with either Int or oInt vectors. Transgenic cells carrying the integrative (*attP*×*attB*) reporter substrate were analyzed by FACS following transfection with equal amounts of Int expression vector (Fig. 3A, 2nd and 3rd columns). oInt improved the reaction ~2.5 fold. However, in a similar experiment using the transgenic cell line carrying the *attR*×*attL* substrate, the efficiency of recombination with oInt was similar to that observed with wild type Int (Fig. 3B, 2nd and 3rd columns). Transfection efficiencies using a GFP expression vector were usually over 90%.

3.3. Effects of prokaryotic accessory proteins on recombination in human cells

As mentioned above, the two genes *ihfA* and *ihfB* were recently used to engineer a single chain IHF (scIHF), which can be expressed in mammalian cells from a hybrid CMV and chicken β-actin promoter (plasmid pIHF2cP). The biological activity of scIHF was carefully analyzed and found to be undistinguishable from heteromeric IHF *in vitro* and in *E. coli*. Furthermore, it promoted recombination by wild type Int-λ in mammalian cells (Bao et al., 2007; Corona et al., 2003).

Transgenic cells carrying the *attP*×*attB* integrative substrate were co-transfected with the oInt expression plasmid and with plasmid pIHF2cP, followed by FACS analysis. The results (Fig. 3A) show that scIHF increased the recombination efficiency of oInt by a factor of 4.5. Altogether 12.6% of the cells treated with oInt and IHF were

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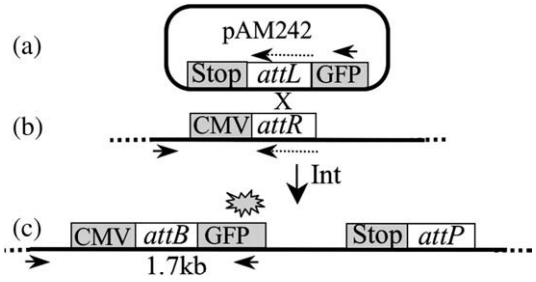

Fig. 5. Diagram representing the Int-catalyzed excisive reactions in *trans*. (a) plasmid substrate, (b) chromosomal substrate, (c) reaction product. Dotted lines represent chromosomal DNA, small arrows represent oligomers oEY327 and oEY398.

fluorescent which is more than 18 fold higher than the control, i.e. cells transfected with mock vector pCDNA3.

Since excisive (*attR*×*attL*) recombination requires both accessory proteins IHF and Xis, we cloned the *xis* gene of HK022 into the mammalian expression vector pcDNA3 under the control of the CMV promoter (plasmid pLD998). Xis expression was tested in human cell extracts prepared over a period of three days after transfection. Fig. 4 shows that Xis, which is known to be unstable in *E. coli* (Weisberg and Gottesmann, 1971), was indeed expressed 24 and 48 h post transfection, but was undetectable after 72 h.

Next, the effects of accessory factors were tested on excisive recombination. Cells were co-transfected with plasmids expressing oInt + scIHF, with oInt + Xis , or with oInt + scIHF + Xis (Fig. 3B columns 4–6). scIHF enhanced excision by a factor of 2.6, Xis by 1.7, and both factors together by 3.2. Altogether 42.6% of the cells treated with oInt + IHF + Xis were fluorescent which is over 80 fold higher than the mock control cells, i.e. cells transfected with mock vector pCDNA3. Identical experiments were carried out with the wild type Int and showed similar results, but, as expected, with markedly reduced overall efficiencies (not shown).

3.4. Reaction in trans

As mentioned above, in our previous study (Harel-Levy et al., 2008) we were unable to detect GFP expression in similar experiments designed to detect intermolecular recombination where one *att* site was on the chromosome and the other on a plasmid. Since we improved the performance of Int in human cells, we tested again excisive recombination in *trans* where *attL* was located on a plasmid flanked by the transcription terminator signal (Stop) and by the open reading frame of the GFP gene [plasmid pAM242, Fig. 5(a)]. The *attR* site downstream to the CMV promoter came from the chromosomally-inserted plasmid pAM243 [(Fig. 5(b)]. An *attR*×*attL* reaction results in

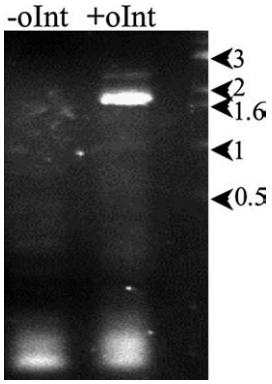

Fig. 7. PCR product using as template DNA of enriched fluorescent cells obtained in the Int-catalyzed excisive *trans* reaction, and of similarly transfected cells but with the pcDNA3 vector instead of Int. Primers were oligomers oEY327 + oEY398.

insertion of the episomal plasmid into the chromosome and leads to expression of GFP [Fig. 5(c)]. The chromosomal *attR* cells were co-transfected with plasmid pAM242 and plasmids expressing oInt, scIHF and Xis. FACS analysis revealed about 2.5% fluorescent cells compared to 0.2% in similar cells transfected with the episomal *attL* substrate and the accessory proteins in the absence of Int (Fig. 6). No significant fluorescence was obtained with wild type Int plus accessory factors (not shown). To verify that the fluorescent cells were products of an *attR*×*attL* reaction, they were enriched by two rounds of FACS, followed by PCR analysis using primers depicted as arrows in Fig. 5. The results (Fig. 7) show that the expected 1.7 kb PCR amplicon product appeared only in oInt-transfected cells and not in cells where oInt was omitted. The sequence of the PCR product (not shown) confirmed the presence of the expected recombinant *attB* site [(Fig. 5 (c)].

4. Discussion

The goal of this research was to improve the HK022 site-specific recombination system as a potential new tool for gene manipulations in mammalian cells. In reactions in *cis*, oInt improved integrative recombination when compared with wild type Int; an effect that was further enhanced by the presence of scIHF yielding over 12% cells that carried a recombinant product (Fig. 3A). In excisive recombination in *cis*, oInt alone did not improve recombination, but did so in the presence of scIHF and Xis (Fig. 3B), yielding over 40% cells that carried a recombinant product. As in our previous studies, excisive recombination is considerably more efficient than integrative recombination. A possible reason might be that in the excisive reaction the

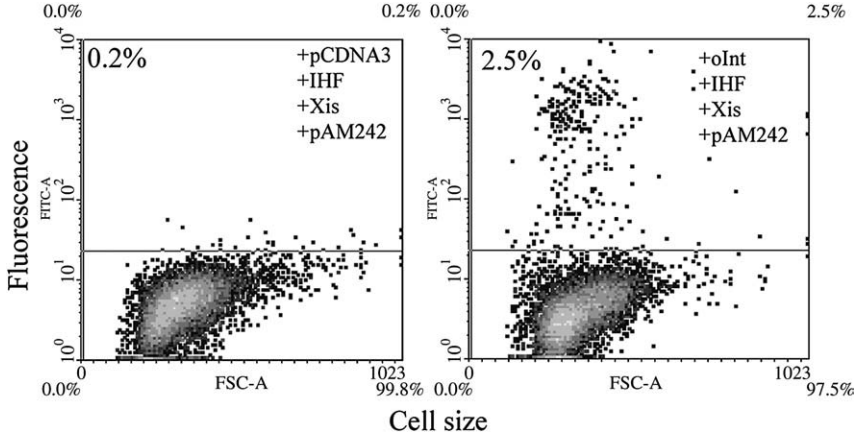

Fig. 6. Dot blot of FACS analysis of the Int-catalyzed excisive *trans* reaction.

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recombining sites (*attL* and *attR*) each carries one arm (P′ and P, respectively) that can tightly bind Int monomers and accessory proteins (Azaro and Landy, 2002). In integrative recombination, one of the substrates (*attB*) is armless and has to be recruited by the protein complex assembled on double-armed *attP* known as the intasome (Richet et al., 1988).

Similar experiments with substrates in *cis* configuration (intra-chromosomal recombination) done with the wild type Cre showed a maximum of 4%–6% recombination (Mao et al., 1999; Sauer and Henderson, 1988) and 6% for FLPe, a thermo-resistant mutant of FLP (Schaft et al., 2001). A comparative study between Int-ϕC31 and FLP recombinases relative to Cre, each engineered with a nuclear localization signal (NLS, Andreas et al., 2002), has demonstrated that Int-NLS of ϕC31 was as active as NLS-Cre and that NLS-FLPe was much less active, however, no recombination frequency data were given in this study. Altogether, these results indicate that the recombination efficiency of the optimized Int-HK022 does not lag behind those of the already practiced recombinases. A direct comparison with the activity of oInt with the recently optimized FLPe and Int-ϕC31 (Raymond and Soriano, 2007) is invalid because in the latters the substrates were supplied to cell lines that were transgenic for the relevant recombinase. However, the optimized iCre protein mentioned above improved recombination *in vitro* by 1.7 fold and was considerably more effective in transgenic mice (Shimshek et al., 2002).

A major goals of site-specific gene manipulations in higher organisms is the ability to insert genes either by recombination between a pair of target sites or, preferable, by the recombinase-mediated cassette exchange (RMCE) technology (Branda and Dymecki, 2004). Since these *trans* reactions are less frequent than the reactions in *cis* most of them are accomplished by selective methods. Our results have demonstrated that Int-HK022, in an excisive *trans* reaction, catalyzed by the oInt recombinase in the presence of IHF and Xis, could significantly improve the *trans* reaction at the chromosomal level. Chromosomal integration could now be detected without the use of selection. Since complex gene manipulations may require more that one site-specific recombination system (Sorrell and Kolb, 2005), the HK022 system may become a useful novel tool for genetic engineering in higher eukaryotes, and in particular for potential therapeutic applications of human embryonic stem cells (Zhou and Dröge, 2006).

Acknowledgments

Thanks to Dr. Orit Sagi-Asif for helping with the FACS analyses and to Michal Rosenzwaig for technical assistance. Research was supported by grant No. 2003394 to E.Y. and M.K from the United States–Israel Binational Science Foundation, Jerusalem, Israel.

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