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Optimization of coliphage HK022 Integrase activity in human cells

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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.

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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.

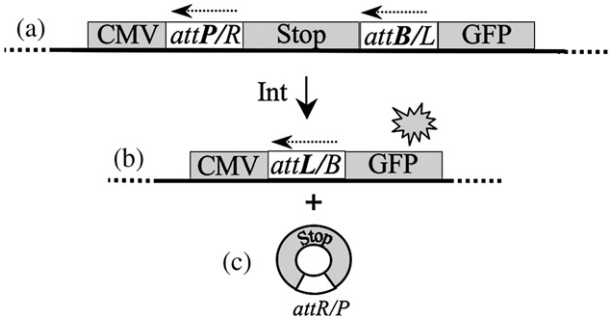

Fig. 1. Diagram representing the Int-catalyzed chromosomal reactions in *cis*. (a) chromosomal substrate. (b) and (c) reaction products. Dotted lines represent chromosomal DNA.

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.