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Transgene excision in zebrafish using the phiC31 integrase

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Abstract

Genome engineering strategies employing site-specific recombinases (SSRs) have become invaluable to the study of gene function in model organisms. One such SSR, the integrase encoded by the *Streptomyces* bacteriophage phiC31, promotes recombination between heterotypic attP and attB sites. In the present study I have examined the feasibility of the use of phiC31 integrase for intramolecular recombination strategies in zebrafish embryos. I report here that 1) phiC31 integrase is functional in zebrafish cells, 2) phiC31 integrase can excise a transgene cassette flanked by an attB and an attP site, analogous to a common use of the Cre/lox SSR system, 3) phiC31 integrase functions in the zebrafish germline, and 4) a phiC31 integrase-estrogen receptor hormone-binding domain variant fusion protein catalyzes attB-attP recombination in zebrafish embryos in a 4-hydroxytamoxifen-dependent manner, albeit less efficiently than phiC31 alone. These features should make this a useful approach for genome manipulations in the zebrafish.

The zebrafish *Danio rerio* has become well-established as a model for vertebrate developmental biology and is increasingly being used to study human disease (reviewed in Lieschke and Currie 2007). A vital area in which additional tools need to be developed for this organism is genome manipulation. In recent years, more efficient methods for obtaining germline integration of foreign genes have been described (Grabher and Wittbrodt 2008). Both lines expressing fluorescent markers to label particular cell populations, and to misexpress genes with spatial and temporal precision are needed to realize the full experimental potential of this system.

Site-specific recombinases such as the Cre/lox and Flp/FRT systems have been a powerful tool in mice for genome manipulation (reviewed in Branda and Dymecki. 2004). Both loxP and FRT sites comprise short inverted repeats around a core sequence, and recombination between two loxP sites or two FRT sites results in the exchange of sequences flanking each site; recombination between two loxP/FRT sites flanking a transgene in the presence of Cre/Flp causes the intervening sequence to be excised as a circular molecule. The phiC31 bacteriophage uses a distinct integrase to catalyze directed recombination between two different sequences, the attP site in the phage genome and the attB site in the *Streptomyces* chromosome. PhiC31 integrase is well-suited for the same types of intramolecular modifications for which Cre/lox and Flp/FRT are commonly used, namely conditional transgene deletion. Unlike Cre/lox and Flp/FRT, the attL and attR sites thus created cannot be acted upon by integrase alone, which requires additional, yet to be identified cofactors to

reverse the reaction. This unidirectionality makes the phiC31 system preferable for transgene insertion strategies such as recombinase-mediated cassette exchange (Bateman et al. 2006). Furthermore, because they recognize different target sequences, Cre, FLP and phiC31 recombinases can potentially be used in parallel to allow researchers to independently manipulate more than one transgene in a single organism. I therefore undertook to test the feasibility of using the phiC31 integrase in zebrafish.

Although its native environment is the *Streptomyces* bacteria, the integrase encoded by the phiC31 phage has been shown to function in a variety of eukaryotic systems, including *Drosophila* (Groth et al. 2004), mouse (Belteki et al. 2003), and frog embryos (Allen and Weeks. 2005) and human cells in culture (Groth et al. 2000). To verify that the integrase could function in zebrafish as well, mRNA encoding the integrase was coinjected into one-cell embryos with the plasmid pBCPB+, which contains a lacZ reporter flanked by an attB and an attP site (Groth et al. 2000). Recombination between these att sites excises the lacZ gene, but leaves the plasmid with ampicillin resistance and an origin of replication. Recombination was detected by recovering plasmid DNA from pooled injected embryos at 8-10 hours post-fertilization, transforming bacteria with the recovered plasmid, and plating on X-gal indicator plates for betagalactosidase activity. Transformation of bacteria with DNA from embryos co-injected with integrase mRNA and pBCPB+ produced a mixture of blue and white colonies (179 white/531 total, 33.7%). Restriction mapping of plasmid DNA from cultures of white colonies, followed by sequencing, confirmed precise excision of lacZ (n=10/10, data not shown). Similar analysis of blue colonies revealed no aberrant or partial recombination (n=10/10; data not shown), which has been observed in mouse (Sangiorgi et al. 2008). Co-injection of pBCPB+ with messenger RNA encoding a mouse codon-optimized integrase (PhiC31o; Raymond and Soriano 2007) gave over twice the frequency of recombination as the native integrase at the single dose tested (654/876; 74.7%). DNA injected from embryos injected with pBCPB+ plasmid alone gave a low background level of white colonies (8/968 total, 0.82%). PhiC31o was used for the remainder of experiments.

To observe recombination in living embryos, we constructed the plasmid pT2Kmin-XIpGbR, comprising GFP coding sequence and SV40 polyadenylation signal flanked by an attP site and an attB site, downstream of the *Xenopus* EF1a promoter and rabbit b-globin intron and followed by the DsRed-Express coding sequence and polyadenylation signal, all within terminal repeats from the Tol2 transposon (Figure 1a). The attP and attB sites in this plasmid were shortened from the native sequences to reduce the chance that they would interfere with expression of the GFP and DsRed ORFs. When injected alone into zebrafish embryos, this plasmid produces widespread green fluorescence, but no red fluorescence is observed (Figure 1b). However, when phiC31 integrase mRNA is co-injected, both green and red fluorescence can be seen (Figure 1d). Plasmid DNA recovered from these embryos and sequenced revealed the presence of both unrecombined and recombined plasmids, the latter with a precise excision between the attP and attB sites (data not shown).

To further characterize the activity of phiC31 integrase in zebrafish, stable transgenic lines were established by co-injecting embryos with the pT2Kmin-XIpGbR plasmid and Tol2 transposase mRNA (Figure 2a). Several sublines were bred out from a single founder with multiple transposon integrations. These lines display widespread expression of GFP at

varying levels, but no detectable red fluorescence even after several generations (data not shown), suggesting that the transgene is stable and not prone to recombination or gene conversion. Injection of transgenic embryos with mRNA encoding the phiC31 integrase, however, produced varying levels of red fluorescence (Figure 2b,c). Sequencing of PCR products obtained from genomic DNA with primers flanking the open reading frames indicated that the GFP cassette had been precisely excised (data not shown).

To determine if phiC31 integrase is active in the zebrafish germline, as has been demonstrated in the mouse germline (Belteki et al. 2003), pT2Kmin-XIpGbR transgenic fish that had been injected with PhiC31o mRNA were raised to adulthood. Individual transgenic fish were then bred to non-transgenic mates and their offspring examined for GFP and DsRed expression. DsRed expression was observed in offspring of 3 of 8 females and 6 of 14 males (Figure 3, Table 1), indicating that recombination can take place in the germline of either sex. Precise excision was confirmed by PCR and sequencing in two of these founders (Figure 3d and data not shown).

While spatial control of integrase expression can be achieved through the use of tissue-specific promoters, temporal control would also be highly desirable. Conditional promoters such as that of the heat shock protein 70 gene have been used to turn genes on and off at particular stages during zebrafish development, but at the cost of spatial control. In mouse, combined spatial and temporal control of recombination has been achieved through use of Cre recombinase fused to the ligand binding domain of the estrogen receptor. A recent report found that phiC31 integrase activity could also be regulated by fusion to this ER domain (Sharma et al. 2008). We therefore constructed and tested a PhiC31o-estrogen receptor fusion protein for inducible activity in zebrafish. Embryos from the pT2Kmin-XIpGbR transgenic line were injected with mRNA encoding PhiC31o alone or as an N-terminal fusion to the estrogen receptor variant ERT2. Embryos were treated with DMSO or 4-hydroxytamoxifen beginning 60-90 minutes after fertilization, and scored at approximately 30 hpf for the presence of DsRed-expressing cells. No recombination was observed with PhiC31o-ERT2 injection in the absence of 4-OHT, while nearly all embryos treated with 4-OHT had red cells (Table 2). Even in the presence of 4-OHT, however the fraction of cells within embryos that were DsRed-positive was much lower with PhiC31o-ERT2 than with PhiC31o alone (Figure 4). To what extent this difference was due to the delay in adding hormone versus potentially reduced activity of the fusion protein was not examined. However, these results indicate that optimization of parameters may be required to make conditional recombination an efficient approach with this system.

Several reports of successful application of the Cre/lox system in zebrafish have now been made (Dong and Stuart 2004, Langenau et al. 2005, Pan et al. 2005, Thummel et al. 2005, Feng et al. 2007, Wang et al. 2008), including recent successes with conditional approaches (Hans et al. 2009; Boniface et al. 2009). The demonstration here of intramolecular recombination in zebrafish mediated by the phiC31 integrase represents another tool now at hand for the zebrafish researcher. The XIpGbR transgenic line described here is analogous to the G2R Cre reporter described by Yoshikawa and colleagues (Yoshikawa et al. 2008) and allows a limited comparison between these two systems. While injection of Cre mRNA into G2R fish was sufficient to produce complete conversion of green to red (Yoshikawa et

al. 2008, Supplementary information), injection of PhiC31o mRNA into the XIpGbR line always produced embryos with a mix of green and red cells, suggesting that phiC31 integrase may be less efficient than Cre, due possibly to lower relative enzymatic activity or protein stability, or longer folding/maturation time. Although it remains to be seen how well expression of phiC31 integrase is tolerated in stable transgenic zebrafish, it is worth noting that injection of phiC31 mRNA had no obvious deleterious effect on embryonic development, and that transgenic Cre expression is apparently tolerated in the female germline (Liu et al. 2008). Moreover, many applications of a transgene excision approach will involve restricting recombinase expression with tissue-specific or inducible promoters, or restricting recombinase activity by fusion to a regulatory module such as a hormone-binding domain, and this should help to mitigate any issues of establishing and propagating phiC31 integrase transgenes. The success reported here suggests that additional applications of the phiC31 recombinase in zebrafish, notably intermolecular recombination for site-specific transgenesis (Groth et al. 2004, Bateman et al. 2006), should also be within reach.

Methods

Cloning

The phiC31 integrase plasmid pCS2P+int was created by inserting the integrase coding region from pCMVint as a XbaI/NheI fragment into the XbaI site of pCS2P+. The plasmid pCS2P+PhiC31o was created by excising the native phiC31 integrase sequence from pCS2P+int with EcoRI and XhoI and replacing it with an EcoRI-Sal I fragment from pPhiC31o (Raymond and Soriano 2007; Addgene plasmid 13794). pCS2P+PhiC31o-ERT2 was made by first replacing the CAG enhancer and Cre ORF from the plasmid pCAG-CreERT2 (Matsuda and Cepko 2007; Addgene plasmid 14797) with the PhiC31o ORF generated by PCR with the primers: forward, 5' GTC GAC ACC TAC GCC GGA GC 3'; and reverse, 5' CTC GAG CAC TTT CCG CTT TTT CTT AGG 3'. This was followed by subcloning of the entire PhiC31o-ERT2 ORF into pCS2P+. The Tol2 transposase plasmid pCS2+BkozTP was generated by optimizing the translational start of the transposase cDNA of pCS2+TP using the PCR primers: TPfor, 5' GGA TCC ACC ATG GAG GAA GTA TGT GAT TCA TCA 3'; and TPrev, 5' AGA TCT GTA GAG TTT CTT GTA GTG 3'. Messenger RNA for each integrase and the Tol2 transposase was synthesized from the corresponding pCS2P+ plasmid using the SP6 mMessage mMachine kit (Ambion) following linearization by restriction digest with Not I or BssHII.

The plasmid pT2K-XpGbR was constructed as follows: The plasmid pT2KXIG in (Urasaki et al. 2006) was digested with Xho I and Bgl II and ligated to an adapter formed by annealing the oligonucleotides: T2KlkrF, 5'-TCG ATA TCG TCG ACT CGA GCG GCC GCA-3'; and T2KlkrR, 5'-GAT CTG CGG CCG CTC GAG TCG ACG ATA-3'. This was followed by PCR with the primers Tol2F, 5'-GAG GAG TTC TTG ACA GAG GTG-3'; and Tol2R, 5'-GGG GTC AAG AAC CAG AGG TG-3'. The PCR product was cloned by TOPO blunt cloning into the vector pCR Blunt II TOPO (Invitrogen) and sequenced. The transposon sequence was excised with EcoRI and ends filled in with Klenow polymerase, then ligated into pBluescript SK- that had been cut with Pvu II and Ssp I to create the plasmid pT2Kmin. pT2Kmin-XIG was created by reinserting the Xho I-Bgl II fragment

from pT2KXIG into the Xho I and Bgl II sites of pT2Kmin. pT2KminXpGb was made by inserting the adapter attPfor, 5'-GAT CCC CAA CTG GGG TAA CCT TTG AGT TCT CTC AGT TGG GGG A-3'; + attPrev 5'-GAT CTC CCC CAA CTG AGA GAA CTC AAA GGT TAC CCC AGT TGG G-3'; into the BamHI site of pT2Kmin-XIG and the adapter attBfor, 5'-GAT CCG GGT GCC AGG GCG TGC CCT TGG GCT CCC CGG GCG CGA-3'; + attBrev 5'-GAT CTC GCG CCC GGG GAG CCC AAG GGC ACG CCC TGG CAC CCG-3' into the Bgl II site. Finally, a BamHI-BamHI fragment containing the coding region for the DsRed-Express gene and polyadenylation signal was cloned into the Bgl II site to give pT2K-XpGbR. Additional cloning details for all constructs are available upon request.

Embryo injections, recombination assay, and generation of transgenic lines

Zebrafish of the *AB strain were used for all experiments. Embryos were obtained by natural breeding of adults kept on a 14 hour/10 hour light/dark cycle. The recombination reporter plasmid pBCPB+ has already been described (Groth et al. 2000). 25 pg of this plasmid was injected into one-cell zebrafish embryos, alone or with 25 pg mRNA encoding phiC31 integrase variants. Plasmid DNA was recovered from pools of 10-25 embryos using the GeneJET miniprep kit (Fermentas). Competent bacteria were transformed and plated on agar containing chloramphenicol at 20 µg/ml and the indicator X-gal at 0.8 mg per plate. Plates were incubated overnight at 37°C and scored the next day for white and blue colonies resulting from recombined and non-recombined plasmids respectively.

Stable transgenic lines were established with the plasmid pT2K-XpGbR by co-injection of 25 pg plasmid per embryo with 25 pg of mRNA encoding the Tol2 transposase. Injected embryos were screened for GFP expression on the second and third days and those showing the strongest expression were selected to be raised to adulthood, at which point they were mated to non-transgenic animals and their offspring examined for GFP expression. To assess recombination of integrated transgenes, PCR was performed on genomic DNA with the following primers: forward (common), 5' CTG GTC ATC ATC CTG CCT TT 3'; EGFP reverse, 5' TGT GGC CGT TTA CGT CGC C 3'; DsRed reverse. 5' GCT TCT TGT AGT CGG GGA TG 3'.

For testing the PhiC31o-ERT2 fusion, 4-hydroxytamoxifen was dissolved in DMSO at a concentration of 300 µM, and added to embryos at 60-90 minutes post-fertilization to a final concentration of 300 nM. An equal volume of DMSO alone was added to the control dish.

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Figure 1. Recombination of injected plasmid

(a) Schematic of phiC31 reporter plasmid. (b) 22 hpf embryo injected with reporter plasmid alone, showing mosaic GFP expression (left) but no DsRed expression (right). (c) Schematic of reporter plasmid following recombination between *attP* and *attB* sites; GFP is excised and the DsRed ORF is brought under the *EF1 α* promoter. (d) 22 hpf embryo injected with reporter plasmid and phiC31 integrase mRNA, showing mosaic expression of both GFP (left) and DsRed (right).

Figure 2. Recombination of a chromosomally integrated transgene

(a) Schematic of the T2K-XpGbR Tol2 transgenic line. Recombination between attP and attB as a result of phiC31 integrase action will excise the GFP cassette and allow expression of the DsRed cassette. Small arrows indicate relative position of PCR primers used in Figure 4d. (Not to scale.) (b,c) A transgenic embryo that has been injected with PhiC31o (codon-optimized integrase) mRNA showing GFP (b) and DsRed (c) fluorescence at 24 hpf.

Figure 3. Recombination in the germline

Portion of a clutch of embryos from an outcross of a T2K-XpGbR transgenic adult that had been injected at the 1-cell stage with PhiC31o mRNA. (a) Brightfield; colored dots indicate the embryos that are expressing fluorescent protein as seen in b and c. (b) 7 of 15 embryos pictured express GFP. (c) 3 of the remaining GFP-negative embryos express DsRed, evidence of a recombination event in the parental germline. (d) Agarose gel showing results of PCR on genomic DNA obtained from F1 embryos, using a common forward primer and GFP- or DsRed-specific reverse primer. Expected product sizes: non-recombined locus with GFP-specific reverse primer, 330 basepairs; recombined locus with DsRed-specific primer, 507 basepairs. Lane 1, GFP-positive embryo with GFP-specific reverse primer; 2, GFP-positive embryo with DsRed-specific reverse primer; 3, DsRed-positive embryo with DsRed-specific reverse primer; 4,; M, markers (in basepairs).

Figure 4. Hormone-inducible transgene recombination

Pictured at approximately 30 hpf are T2K-XpGbR transgenic embryos injected with *PhiC31oER* mRNA, with addition of DMSO (a) or 4-hydroxytamoxifen (b), or injected with *PhiC31o* mRNA (c). DsRed expression resulting from transgene recombination is observed with PhiC31oERT2 only when 4-OHT is added, however fewer expressing cells are observed than with PhiC31o (rightmost panels).

Table 1
Germline recombination of a stable attP/attB transgene

	Males	Females	Total
F1 animals screened	14	8	22
F1's producing DsRed+ offspring	6	3	9
%	42.9	37.5	40.9
Avg.% of clutch	19.7	5.3	14.9
Range	0.0-31.8	0.0-7.9	

Table 2
Conditional recombination of a stable attP/attB transgene

mRNA/treatment	GFP+ embryos	DsRed+ embryos	Red/Green
4OHT only	52	0	0%
PhiC31o+DMSO	62	62	100%
PhiC31oERT2+DMSO	62	0	0%
PhiC31oERT2+4OHT	72	68	94%