A versatile strategy for gene trapping and trap conversion in emerging model organisms

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SUMMARY

Genetic model organisms such as Drosophila, C. elegans and the mouse provide formidable tools for studying mechanisms of development, physiology and behaviour. Established models alone, however, allow us to survey only a tiny fraction of the morphological and functional diversity present in the animal kingdom. Here, we present iTRAC, a versatile gene-trapping approach that combines the implementation of unbiased genetic screens with the generation of sophisticated genetic tools both in established and emerging model organisms. The approach utilises an exon-trapping transposon vector that carries an integrase docking site, allowing the targeted integration of new constructs into trapped loci. We provide proof of principle for iTRAC in the emerging model crustacean Parhyale hawaiensis: we generate traps that allow specific developmental and physiological processes to be visualised in unparalleled detail, we show that trapped genes can be easily cloned from an unsequenced genome, and we demonstrate targeting of new constructs into a trapped locus. Using this approach, gene traps can serve as platforms for generating diverse reporters, drivers for tissue-specific expression, gene knockdown and other genetic tools not yet imagined.

KEY WORDS: Parhyale, Gene trapping, fC31 integrase, Regeneration, Transgenesis, ITRAC

INTRODUCTION

Although established genetic models offer unmatched resources for genetic analysis, there is strong motivation to develop genetic tools in new species. This motivation stems from the diversity that is evident in development, morphology and physiology, which means that many questions cannot be addressed in the few well-established models. The development of transgenesis in emerging animal models, such as Nematostella vectensis (Renfer et al., 2010), Parhyale hawaiensis (Pavlopoulos and Averof, 2005), Tribolium castanum (Berghammer et al., 1999) and Ciona intestinalis (Sasakura et al., 2007), represents the first step for establishing sophisticated genetic techniques in these species. One such technique, gene trapping, captures gene expression at the site of transgene insertion. Transposon-mediated gene trapping allows the implementation of unbiased genetic screens to identify new genes and provides valuable markers for in vivo imaging and phenotypic characterisation (Bellen, 1999; Bellen et al., 1989).

In Drosophila, gene traps are also used to generate GAL4 drivers, powerful tools that exploit endogenous genes to direct gene expression with spatial and temporal specificity (Brand and Perrimon, 1993). In principle, gene trapping can be used to introduce a wide range of genetic tools, such as alternative expression drivers, recombinases, specialised markers and knockdown constructs, into a trapped locus. In practice, it is difficult to achieve because each application relies on a different transgene construct, and traps derive from unique insertions that cannot be reproduced with each construct. Replacing one type of construct with another, at a given locus, is possible but technically challenging and restricted to highly developed genetic models (Sepp and Auld, 1999).

We present a new approach termed integrase-mediated trap conversion (iTRAC) that allows primary gene traps to be adapted for diverse applications through transgene conversion. The approach uses a primary exon-trapping vector, based on the Minos transposon, that incorporates an attP docking site for the fC31 integrase. Once a trap has been generated and selected, a virtually unlimited range of secondary constructs carrying the cognate attB site can be integrated specifically into the docking site at the trapped locus (Fig. 1). As a proof of principle, we demonstrate iTRAC in Parhyale hawaiensis, a crustacean that has emerged as an attractive model for developmental studies (Browne et al., 2005; Extavour, 2005; Gerberding et al., 2005; Liubicich et al., 2009; Ozhan-Kizil et al., 2008; Pavlopoulos and Averof, 2005; Pavlopoulos et al., 2009; Price et al., 2010; Rehm et al., 2009; Vargas-Vila et al., 2010).

MATERIALS AND METHODS

Gene-trapping constructs

The 1.3 kb PhHsp70a fragment (accession FR749989) was isolated by inverse PCR from Parhyale genomic DNA using a previously described approach (Pavlopoulos and Averof, 2005; Pavlopoulos et al., 2009) and cloned upstream of the DsRed/SV40polyA reporter cassette to obtain plasmid pSIL(PhHsp70a-DsRed).

A 230 bp SpeI fragment containing the fC31 attP site from pTA-attP (Groth et al., 2000) was cloned into the SpeI site of pSIL(PhHsp70a-DsRed) to generate pSIL(PhHsp70a-DsRed). The attP-PhHsp70a-DsRed construct was cloned as an AseI fragment into the Minos vectors pMi(3xP3-DsRed) and pMi(3xP3-EGFP) (Pavlopoulos and Averof, 2005; Pavlopoulos et al., 2004), generating pMi(3xP3-DsRed;attP;PhHsp70a-DsRed) and pMi(3xP3-EGFP;attP;PhHsp70a-DsRed).

The transcription initiation and splice sites of PhHS and PhHsp70a were mapped by 5’ RACE from transgenic animals carrying stable insertions of PhHS-DsRed (Pavlopoulos and Averof, 2005), PhHS-DsRed (Pavlopoulos et al., 2009) and PhHsp70a-DsRed (DistalPhRed trap),...
Depending on its insertion site in the genome, the reporter might come (arrows), carrying a fragment containing SalI from populations of 20-30 heterozygous and homozygous DistalRed embryos, reverse transcribed using oligo(dT) primers and amplified in triplicate on the MJ Research Opticon real-time PCR machine; PCR efficiency with each set of primers was 1.98 and 1.72, respectively.

Trapped and normally spliced products were detected at a ratio of 0.09 (s.e.=0.18) in heterozygous embryos and 0.15 (s.e.=0.32) in homozygous embryos.

### RESULTS AND DISCUSSION

The gene-trapping vector

Our Minos gene-trapping vector carries the 3xP3-DsRed or 3xP3-EGFP transformation marker (Berghammer et al., 1999; Pavlopoulos and Averof, 2005), the 3C1 attP site, and a ‘trapping element’ (described below) upstream of the DsRed reporter and the early mRNA polyadenylation sequence of SV40. The two types of trapping strategies commonly employed, i.e. enhancer trapping and exon trapping, make use of a core promoter or a splice acceptor to capture the activity of cis-regulatory elements or splice donors of trapped genes, respectively. To create a trapping system that is widely applicable, we sought to identify promoters or splice acceptors capable of gene trapping in a range of species. First, we tested two core promoter elements, the *Drosophila hsp70* basal promoter and an artificial 'super core promoter' (which combines

respectively, using SMART-RACE (Clontech) and a reverse primer targeting the coding sequence of DsRed (5'-CTTGTTGCTACCTCGGCGGT-3'). Additional cDNA sequences from the Distal locus were obtained by 3' RACE on wild-type animals using forward primers targeting the sequences already determined (accession FR82133). In situ hybridisation using the Distal cDNA probe was carried out on wild-type embryos as described previously (Rehm et al., 2009). The DistalRed line carries additional transgene insertions that do not give visible traps.

Splicing to PhHsp70a-DsRed and normal splicing at the Distal locus were measured by quantitative RT-PCR using a common forward primer targeting the Distal 5' UTR (5'-TGCAGTCAGGGAGAAATG-3') and two reverse primers targeting DsRed (5'-GGGTGTTCCTGTAACCTTT-3') and a Distal 3' exon (5'-GTTCTGTCAGCTTCCTTGGC-3'). RNA was isolated from populations of 20-30 heterozygous and homozygous DistalRed embryos, reverse transcribed using oligo(dT) primers and amplified in triplicate on the MJ Research Opticon real-time PCR machine; PCR efficiency with each set of primers was 1.98 and 1.72, respectively.

Trapped and normally spliced products were detected at a ratio of 0.09 (s.e.=0.18) in heterozygous embryos and 0.15 (s.e.=0.32) in homozygous embryos.

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**Fig. 1. Gene-trap and trap conversion strategy.** (A) Primary gene-trapping construct based on a transposon vector (yellow inverted arrows), carrying a 3C1 attP recognition site (purple) and a trapping element (core promoter or splice acceptor) upstream of a reporter gene (DsRed). The vector may also include additional markers (not shown). Depending on its insertion site in the genome, the reporter might come to be expressed under the influence of nearby sequences (grey arrow). (B) Once a gene trap has been isolated, integrase-mediated trap conversion (iTRAC) utilizes attP as a docking site for integrating new constructs into the trapped locus. Constructs carrying the cognate attB site (magenta) are introduced into the locus by integrase-mediated site-specific recombination. A wide range of secondary constructs for different types of applications can be envisaged (see text); in this example, the DsRed trap is converted into one that expresses a different transgene X. (C) Integration mediated by single attP and attB sites results in duplication of the original trapping construct by the new construct. Complete replacement is also feasible using flanking pairs of attP and attB sites.
several core promoter motifs) (Juven-Gershon et al., 2006), for enhancer trapping activity in *Drosophila, Tribolium* and *Parhyale*, but neither was found to work across the species tested (Schinko et al., 2010) (data not shown). Next, we searched for core promoters and splice acceptors among sequences that lie upstream of *Parhyale hsp70* genes. Among the sequences tested, two elements were capable of efficient gene trapping in *Parhyale*: the heat-inducible element PhHS (Pavlopoulos et al., 2009) and a fragment named PhHsp70a. Both fragments could drive expression patterns specific to individual transgene insertions without any need for a heat shock (Fig. 2B-L). Using 5′ RACE on cDNA prepared from transgenic lines, we determined that PhHS contains a core promoter upstream of the transcription start site and a large intron within the 5′ UTR, whereas PhHsp70a is a truncated 5′ UTR sequence with a splice acceptor site that becomes spliced to the exons of trapped genes (see Fig. S1 in the supplementary material). We decided to focus on exon trapping mediated by PhHsp70a.

In our first experiment using the Minos[3xP3-DsRed;PhHsp70a-DsRed] vector, we recovered at least six independent traps from ~250 injected Parhyale embryos. Using the same vector, we also obtained four independent exon traps in *Drosophila* from ~350 injected embryos, suggesting that this construct can mediate exon trapping efficiently in diverse arthropods.

**Imaging of developmental and physiological processes**

A variety of exon traps have been generated to date using the PhHsp70a-DsRed trapping construct in *Parhyale* (Fig. 2). These include traps with expression in the central nervous system, mesoderm, appendages, mouthparts, gills and other patterns. Most lines have been propagated through many generations over 4-5 years, demonstrating that the transgenes are stable, with continuing activity and no detrimental effects on reproduction and survival.

In emerging model organisms, gene traps are likely to be first used as markers for visualising specific tissues or cell types, providing a means to follow dynamic cell behaviours, to study physiological processes in vivo and to assess phenotypes following experimental manipulations. Some of our traps mark well-recognised organs, such as the nervous system, gills or paragnaths (Fig. 2D,F,H,K), whereas others mark complex populations of cells and previously undescribed cell types. For instance, one trap marks a previously uncharacterised cell type on the dorsal epidermis of late embryos, juveniles and adults (Fig. 2C) that is associated with specific sensory or structural elements in the epidermis of *Parhyale* (Fig. 3A).

Another trap allows us to image cardiac function. *Parhyale* has a typical arthropod heart, consisting of a muscular tube with three pairs of lateral inflow valves and an anterior outflow valve (Fig. 3B). Using this trap, we were able to observe heart function and to visualise the opening and closing of valves in unprecedented detail (Fig. 3C and see Movie 1 in the supplementary material).

A third trap, which we named DistalDsRed, marks the distal part of all Parhyale limbs in embryos, larvae and adults (Fig. 2E,L). We have used this line to monitor limb regeneration following amputation in *Parhyale* (Fig. 3D,D′).

**Cloning trapped genes and mutagenic effects**

In emerging model organisms, transposon-based exon trapping is one of the most straightforward ways to isolate genes through unbiased genome-wide screens. The trapped gene of interest can be cloned easily by primer extension on cDNA from the trapped line, even when the genome is unsequenced. To demonstrate this, we cloned a cDNA from the gene trapped in the DistalDsRed line of *Parhyale*. The cDNA contains a long open reading frame with no similarity to known proteins. A corresponding probe revealed the same expression pattern as DistalDsRed in embryos (see Fig. S2 in the supplementary material). Similarly, we have cloned cDNAs of several other genes trapped by PhHsp70a-DsRed in Parhyale and Drosophila. Sequencing of these cDNAs led to our discovery of trans-splicing in Parhyale (Douris et al., 2010).
In exon-trapped genes, splicing of the endogenous transcript onto the gene-trap cassette generates a chimaeric mRNA that expresses, at most, the N-terminal portion of the endogenous protein. If splicing to PhHsp70a-DsRed were 100% efficient, traps would be mutagenic and we would expect to detect a loss-of-function phenotype in animals homozygous for the trap. However, the majority of exon traps obtained using PhHsp70a-DsRed do not cause a detectable phenotype in homozygous animals. We used quantitative RT-PCR to examine splicing to PhHsp70a-DsRed relative to normal splicing at the DistalDsRed locus. We found that only some transcripts are spliced to the PhHsp70a-DsRed trapping cassette, whereas a large proportion are still spliced onto the endogenous downstream exon. Thus, PhHsp70a-DsRed allows for sensitive trap detection with little disruption of endogenous gene function. This is helpful for maintaining stocks in the absence of balancer chromosomes. iTRAC, described below, provides the means to convert such traps into mutagenic insertions.

**Integrase-mediated trap conversion (iTRAC)**

The attP site in the trapping vector is a platform for integrating new constructs into trapped loci so as to generate new markers and tools for genetic analysis. If C31 integrase has never been used before in *Parhyale*, First, we devised a rapid PCR-based assay for if C31 integrase activity in vivo, which demonstrated efficient integrase-dependent recombination of attP and attB sites across two plasmids injected into early Parhyale embryos (Fig. 4A).

Next, we tested the ability of attB-bearing plasmids to integrate at attP sites inserted in the Parhyale genome in the presence of if C31 integrase mRNA. A plasmid carrying attB and a heat-inducible EGFP marker (PhHS-EGFP) integrated with high efficiency into a transgenic line carrying multiple copies of the attP sequence (104 of 207 injected embryos expressed EGFP after heat shock). No integration events were recovered by injection into wild-type embryos (0 out of 255 embryos). Integration of the PhHS-EGFP transgene also occurred in the germline, as judged from its transmission to the next generation.

Finally, we were able to demonstrate the conversion of a DsRed exon trap into one that expresses EGFP in the same pattern. A plasmid carrying attB-PhHsp70a-EGFP was injected with if C31 integrase mRNA into early embryos of the DistalDsRed transgenic line. A high proportion of these embryos (47 out of 90 survivors) showed EGFP fluorescence replacing DsRed fluorescence at least a subset of limbs (Fig. 4B). In ~20% of injected survivors, iTRAC also occurred in the germline and a stable DsRed to EGFP conversion was evident in subsequent generations (Fig. 4C). In DistalEGFP lines, DsRed expression could not be detected. These results indicate that iTRAC can be implemented with high efficiency in Parhyale.

**Conclusions**

Model organisms are defined by the experimental approaches they offer to address biological questions of broad interest. Candidate gene approaches have, until now, been the main avenue for applying knowledge gained in established models to other species of interest, but these approaches are biased and incomplete. The establishment of transgenesis in new species that span the phylogenetic tree of animals, from cnidarians to protostomes and chordates, sets the stage for developing forward and reverse genetics tools and approaches in a wide range of organisms. iTRAC provides a shortcut for implementing these approaches.

The versatility of iTRAC opens opportunities for a wide range of downstream applications: (1) the generation of markers for different types of microscopy, ranging from fluorescence-based live imaging to electron microscopy; (2) the implementation of binary systems for gene expression, such as the GAL4, LexA, tTA and Q systems; (3) the genetic marking and manipulation of clonal populations of cells using FLP or Cre recombinase; (4) the conversion of gene traps into gene knockouts, for instance by insertion of strong transcriptional terminators; (5) genetic cell ablation using cell-autonomously acting toxins; and (6) chromosome engineering. Conceivably, the same collections of traps could be used in the future to implement tools that are not yet...
The interplasmid assay for \( \text{\varphi}C31 \) integrase-mediated recombination involves injecting plasmids carrying an \text{attP} or an \text{attB} site (red and green, respectively) with \( \text{\varphi}C31 \) integrase mRNA into Parhyale embryos, and assaying recombination by PCR using primers F1, F2 and R. Each gel lane represents a single experiment, in which different combinations of plasmids and integrase mRNA were injected. The 1 kb band results from amplification of the \text{attP} plasmid fragment (red), whereas the 0.7 kb band results from amplification of a hybrid fragment created by recombination between \text{attP} and \text{attB} sites (green-red). The 0.7 kb fragment is strictly dependent on the presence of \( \text{\varphi}C31 \) integrase. The identity of this fragment was also verified by sequencing. (B) Ventral view of a Distal\text{\varphi}C31 embryo (G0) injected with \( \text{\varphi}C31 \) integrase mRNA and a plasmid carrying \text{attB} and \text{PhHsp70a-EGFP}. Integration at the 2-cell stage resulted in a mosaic in which the Distal\text{\varphi}C31 trap was converted to DistalEGFP on one side of the embryo; the other side retained Distal\text{\varphi}C31 expression. (C) Ventral view of DistalEGFP embryo, two generations after conversion (G2). DsRed fluorescence is not detectable in limbs; red autofluorescence of the yolk is shown for contrast.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
Z.K. established the \( \text{\varphi}C31 \) integrase system and implemented iTRAC in Parhyale; A.P. cloned the \text{hs}p70 sequences and established gene trapping in Parhyale; A.K. built and tested alternative trapping vectors; N.K. studied limb regeneration using gene traps; A.K. and N.K. determined promoter and splice sites in the \text{hs}p70 sequences; Z.K., Y.D. and A.K. cloned Distal and assessed splicing at that locus; M.A. conceived iTRAC, imaged gene traps, supervised the project and wrote the paper; all authors discussed the results and commented on the manuscript.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.066324/-/DC1

References


