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## Bacteriophage C31 Integrase Mediated Transgenesis in *Xenopus laevis* for Protein Expression at Endogenous Levels

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### Abstract

Bacteriophage  $\phi$ C31 inserts its genome into that of its host bacterium via the integrase enzyme which catalyzes recombination between a phage attachment site (*attP*) and a bacterial attachment site (*attB*). Integrase requires no accessory factors, has a high efficiency of recombination, and does not need perfect sequence fidelity for recognition and recombination between these attachment sites. These imperfect attachment sites, or pseudo-attachment sites, are present in many organisms and have been used to insert transgenes in a variety of species. Here we describe the  $\phi$ C31 integrase approach to make transgenic *Xenopus laevis* embryos.

### Keywords

*Xenopus*;  $\phi$ C31; integrase; transgenesis; fluorescence

### 1. Introduction

The frog, *Xenopus laevis*, has a long history of use for studies in embryonic development. Previously described *Xenopus* integration techniques often insert multiple copies of a transgene at random sites in the embryos genome (1–4). Though valuable for many experimental designs, these approaches are problematic for researchers who desire transgene expression to approximate endogenous gene expression levels. Rather, a site-directed integration approach that incorporates a regulated single copy of a transgene into the host genome would more closely match endogenous gene expression. The  $\phi$ C31 integrase approach is one way to accomplish this aim.

Bacteriophage  $\phi$ C31 encodes an integrase enzyme that inserts the phage genome into the genome of various *Streptomyces* bacteria (5,6). The integrase protein recognizes a 39-bp-long phage attachment site (*attP*) in its own genome and a 34-bp-long bacterial attachment site (*attB*) in the bacterium's genome and then catalyzes an integration event (7,8). The integrase enzyme does not require perfect sequence fidelity to recognize the *attP* site (7). Non-perfect *attP* sites, or pseudo-*attP* sites, may have as low as 24% sequence homology to endogenous *attP* sites and still allow recombination (7) although the recombination efficiency may be decreased.

Many groups have shown that integrase can insert plasmid DNA sequences that contain an *attB* site into pseudo-*attP* sites found in a variety of organisms. Utilizing this approach, transgenes have been inserted into the genomes of plant cells (9), mammalian cells (7,10–18), and *Drosophila* embryos (19). We used the pseudo-*attP* sites in the *Xenopus* genome and an *attB* site containing reporter plasmid to make transgenic *Xenopus* embryos (20) (Fig. 9.1). Surprisingly, the reporter genes are expressed in some expected tissues but not in

others. We recognized this as chromatin position effect and flanked the reporter gene with HS4 insulators. HS4 insulators stop the spread of chromatin silencing and also prevent distant enhancers from acting on a promoter (21,22). After making transgenic embryos with the new insulated reporter plasmid, we found that the transgenes expressed as expected (20) (Fig. 9.2). The techniques used to generate and recognize  $\phi$ C31 integrase mediated transgenic *Xenopus* embryos are described below.

## 2. Materials

### 2.1. Plasmids and Plasmid Preparation Reagents

1. *Plasmids*: The integrase plasmid pET11- $\phi$ C31poly(A) (23) can be obtained from Dr. Michelle Calos (calos@stanford.edu), and the *attB* reporter plasmids CMV-EGFP-DI-*attB* and CL-EGFP-DI-*attB* (20) can be obtained from Dr. Daniel Weeks (daniel-weeks@uiowa.edu). Permission to use HS4 insulator sequences and HS4 insulator constructs needs to be obtained from Dr. Gary Felsenfeld (gary.felsenfeld@nih.gov) (see Note 1).
2. *Plate and culture reagents*: Bacto-Yeast Extract, Bacto-Tryptone powder, and Bacto-agar (BD Biosciences, Sparks, MD).
3. *Antibiotics*: Ampicillin and Kanamycin (Sigma Aldrich, St. Louis, MO).
4. *Plasmid Purification Kits*: Qiaprep Spin Miniprep Kit, HiSpeed Plasmid Maxi Kit (Qiagen, Valencia, CA).
5. *In vitro RNA transcription*: T7 mMessage machine (Ambion, Austin, TX).
6. Agarose (RPI, Mt. Prospect, IL).
7. *10X TAE*: 0.4-M Tris-acetate, 0.01-M EDTA.
8. *10X Non-denaturing DNA loading buffer*: 50% glycerol, 60-mM EDTA, 1% SDS, and 0.05% bromophenol blue.
9. 1% agarose, 2.2-M formaldehyde MOPS gel.
10. 10X Denaturing RNA loading buffer.
11. Gel electrophoresis apparatus for flat-bed agarose gel electrophoresis (Owl Separation Systems, Portsmouth, NH).
12. *Spectrophotometer*: Nanodrop ND1000 (Nanodrop Technologies, Wilmington, DE).
13. UV-light transilluminator.

### 2.2. *Xenopus* Injection

1. *Xenopus laevis*: May be obtained from either *Xenopus I* (Dexter, MI) or Nasco (Fort Atkinson, WI).
2. Human Chorionic Gonadotropin (Sigma Aldrich).
3. Tricaine (3-aminobenzoic acid ethyl ester) (Sigma Aldrich).
4. 10X Marc's Modified Ringers Solution (MMR): 1-M NaCl, 20-mM KCl, 20-mM CaCl<sub>2</sub>, 10-mM MgCl<sub>2</sub>, 50-mM HEPES at pH 7.4.

<sup>1</sup>MTA agreements are required from Dr. Gary Felsenfeld (NIH) to use the HS4 insulator sequences and Dr. Daniel Weeks (Iowa) for the CMV-EGFP-DI-*attB* and CL-EGFP-DI-*attB* plasmids. An MTA is also required to obtain the plasmid pET11 $\phi$ C31poly(A) from Dr. Michele Calos (Stanford).

5. *Injection buffer*: 88-mM NaCl, 10-mM HEPES.
6. 0.3X MMR with 3% Ficol Type 400 (Sigma Aldrich).
7. 2% Cysteine (RPI) made in dH<sub>2</sub>O, pH 7.8–7.9 with NaOH. Should be made on the day of injection.
8. Microinjection needle puller.
9. Micromanipulator: Singer MK-1 (Singer instrument company, Somerset, England) or similar instrument.
10. Microinjector: Inject+Matic (Geneva, Switzerland) or similar instrument.
11. Glass capillary tubes (Singer).
12. 18°C incubator.

### 2.3. Microscopy

1. *Dissecting microscope*: Nikon SMZ (Nikon Instruments, Melville, NY) and Zeiss Stemi SV 11 fluorescent dissecting microscope (Zeiss MicroImaging, Thornwood, NY).
2. *Compound microscope with fluorescence*: Zeiss Axioplan 2 (Carl Zeiss MicroImaging) or similar instrument.
3. *Camera for photo documentation*: SPOT camera (Diagnostic Instruments, Sterling Heights, MI), Zeiss Axiocam (Carl Zeiss MicroImaging), or similar instrument.

### 2.4. Southern Blotting

1. *Genomic DNA purification*: Qiagen DNeasy (Qiagen).
2. *Restriction enzymes*: multiple suppliers.
3. Ethidium bromide (Sigma Aldrich).
4. Hybond-N+ nylon membrane (GE Healthcare, Chicago, IL).
5. Rediprime II DNA Labeling System (GE Healthcare).
6. Redivue  $\alpha$ -P<sup>32</sup> deoxycytidine (GE Healthcare).
7. RapidHyb solution (Ambion).
8. X-ray film, Kodak Biomax XAR (Eastman Kodak Co, Rochester, NY).
9. 20X SSC: 3.0-M NaCl and 0.3-M sodium citrate at pH 7.0.
10. *Depurination buffer*: 0.25-M HCl.
11. *Denaturation buffer*: 1.5-M NaCl with 0.5-M NaOH.
12. *Neutralization buffer*: 1-M Tris-HCl, pH 8.0, 1.5-M NaCl.
13. *Hybridization Oven*: Hybaid (Thermo Corporation, Waltham, MA).

## 3. Methods

### 3.1. Preparation of Plasmids and $\phi$ C31 Integrase mRNA

1. Transform the pET11- $\phi$ C31poly(A) plasmid and the *attB* reporter plasmids (CMV-EGFP-DI-*attB* and CL-EGFP-DI*attB*) into *Escherichia coli* and plate onto antibiotic selective plates using standard techniques. The pET11- $\phi$ C31poly(A)

plasmid contains an ampicillin resistance gene and the *attB* reporter plasmids contain a kanamycin resistance gene. We have found that insulated *attB* reporter plasmids frequently undergo rearrangements and thus recommend using Stbl2 cells (Invitrogen) which are designed to prevent recombination.

2. Select individual colonies on each plate and grow 3-ml cultures at 37°C overnight with agitation. Use 1 ml for mini-preps (Qiaprep mini-prep kit) and save the other 2 ml of culture at 4°C. Using the mini-prep-generated DNA, confirm plasmid size and insulator orientation by restriction enzyme digestion and gel analysis. Once plasmid size and insulator orientation are confirmed, inoculate 1 l of LB broth containing the appropriate antibiotic with the remaining 2 ml of culture. Grow at 37°C with agitation overnight.
3. Perform maxi-preps on the 1-l cultures following the manufacturer's instructions (Hi Speed Plasmid Maxi Kit). Do not add RNase to buffer P1.
4. Confirm maxi-prep-generated plasmid sequences by restriction enzyme digestion and gel analysis. Store the maxi-prep-generated DNAs at -20°C.
5. Linearize 5 µg of pET11-phiC31poly(A) maxi-prep DNA with either *Bam*HI or *Eco*RI restriction enzymes. Run approximately one-tenth of the digestion on a 1% agarose gel to ensure linearization. Heat inactivates the restriction enzyme in the remaining portion of digestion by heating reaction to 65°C for 20 min. Precipitate the DNA by adding one-tenth the volume of 5-M NH<sub>4</sub> acetate and 2 volumes of ethanol. Place solution at -20°C for 15 min and centrifuge at 10,000 g for 15 min. Remove the supernatant and resuspend the pellet in 10 µl of RNase-free TE buffer.
6. Synthesize the φC31 integrase mRNA using the T7 mMessage machine following the manufacturer's instructions. Because the protocol includes a DNase treatment step, the DNase needs to be removed or inactivated. We routinely follow the LiCl precipitation protocol described in the manufacturer's instructions. Resuspend the integrase mRNA in RNase-free water at a concentration of 1 mg/ml.
7. Run 1 µg of φC31 integrase mRNA on a formaldehyde MOPS gel in 1X MOPS buffer to ensure that the transcript is approximately 1.9 kilobases long.
8. Store φC31 integrase mRNA (for up to 1 month) at -80°C until it is needed.

### 3.2. Injection of *Xenopus* Embryos

1. Obtain approval from host institution to house and care for *Xenopus* adult frogs and embryos. If *Xenopus* experience is minimal, the book, *Early Development of Xenopus laevis – A Laboratory Manual* (24), may be a useful reference to consult.
2. Induce *Xenopus* females to lay eggs by injecting 1 ml (1000 IU) of human chorionic gonadotropin into the dorsal lymph sac the night before desired day of egg collection.
3. The next morning, the cloaca on the injected females should be swollen and there may be eggs in the water tank holding the frogs.
4. After eggs are present in the tank, inject a lethal dose of Tricaine (1 ml of a 10% solution) into the dorsal lymph sac of a *Xenopus* male and then surgically remove the testes. Store the testes in 1X MMR.
5. Induce egg laying into a dry petri dish by spreading apart the female's legs and gently squeezing and rubbing the pelvic region. Immediately fertilize the eggs by rubbing a small piece of testis (~one-sixth of a testis) through the eggs. Then crush the testis in ~1 ml of 0.3X MMR and spread this solution over the eggs. Let the

eggs sit in the sperm solution for about 1 min and then flood the eggs with 0.1X MMR.

6. Successful fertilization can be determined if the eggs align with the animal pole (pigmented half) facing up. Embryos should be spherical, uniform size, and have smooth even pigmentation of the animal hemisphere. At 30 min after fertilization, remove the 0.1X MMR and place the embryos in 2% cysteine, pH 7.8–7.9 for 2 min to remove the jelly coats.
7. Remove the cysteine and wash the embryos in three 5-ml washes of 0.3X MMR.
8. Place the embryos in 0.3X MMR, 0.3% ficol.
9. Inject single-cell embryos into the center of the animal hemisphere with either 10 nl of injection solution, 10 nl containing 5 pg of reporter plasmid resuspended in injection solution, or 10 nl containing 5 pg of reporter plasmid + 1 ng of  $\phi$ C31 integrase mRNA also resuspended in injection solution.

### 3.3. Monitoring Developing Embryos for Transgenesis

1. Allow injected embryos to develop at 18°C in 0.3X MMR, 0.3% Ficol for approximately 6–8 h after injection and then transfer to 0.3X MMR.
2. Remove delaminating or dead embryos and provide fresh 0.3X MMR at least twice a day for the first 3 days.
3. On the second day after fertilization, begin to monitor the embryos for GFP expression using a fluorescent microscope optimized to detect green fluorescence (*see* Note 2). Embryos injected with 1 ng integrase mRNA and 5 pg of CMV-EGFPDI-*attB* should express GFP uniformly (Fig. 9.2C) while embryos injected with 1-ng integrase mRNA and 5 pg of CL-EGFP-DI-*attB* should express GFP only in the lens of the eye (Fig. 9.2E). Embryos injected with 5 pg of reporter plasmid alone rarely give GFP expression (Fig. 9.2B). Digital photography with long exposure times may detect fluorescence before it is visible to the eye.
4. Once fluorescence is detected, photograph the embryos using a fluorescent microscope with a digital microscope. If embryo movement prevents photography, consider anesthetizing the embryos with 0.02% Tricaine. Embryos may be exposed to the Tricaine solution for approximately 5 min; longer exposure times may lead to the demise of the embryos. Embryos should be placed back into 0.3X MMR after photography.

### 3.4. Southern Blot Analysis to Confirm Integration

1. Isolate DNA from single-stage 46 embryos using the Qiagen DNeasy kit following the manufacturer's instructions. One-stage 46 embryo should yield approximately 50  $\mu$ g of DNA. DNA should be collected from non-injected embryos, embryos injected with reporter plasmid alone, and transgenic embryos determined by green fluorescence.
2. Digest 5–10  $\mu$ g of harvested DNA and 10–100 pg of reporter plasmid with a restriction enzyme that cuts the reporter plasmid at a single site overnight (for CL-EGFP-DI-*attB* or CMV-DI-EGFP *attB*, *Bam*HI is a suitable enzyme).

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<sup>2</sup>The amount of fluorescence produced from a single-copy transgene ( $\phi$ C31 integrase approach) may be significantly less than an embryo containing multiple copies of a transgene generated from the restriction enzyme mediated insertion (1,2) approach or the meganuclease (3,4) approach.

3. Run digested DNA on a 0.6% agarose gel. To allow for good separation, use a gel that is 10–15-cm long and run the gel until the bromophenol blue dye front is approximately 2 cm from the bottom of the gel. Stain the gel with ethidium bromide and then photograph the gel while on a UV transilluminator. Place a UV light visible ruler next to the gel while taking the photograph as it will be useful to approximate the size of the insertions.
4. Transfer the DNA onto a positively charged nylon membrane using standard southern blot protocols.
5. Following the transfer, rinse the membrane in 5X SSC and prehybridize in Ambion RapidHyb supplemented with 0.1 µg/ml herring sperm DNA at 45°C for 1–2 h with agitation.
6. Generate a P<sup>32</sup>-labeled EGFP probe. We digest the CLEGFP-DI-*attB* plasmid or CMV-EGFP-DI-*attB* plasmid with *Bam*HI and *Age*I, run the digested DNA on a 1% agarose gel, and then isolate the GFP fragment using the Qiagen Gel Extraction Kit. We then label the EGFP fragment using Rediprime II Random Prime Labeling System following the manufacturer's instructions. Free nucleotides are removed using the Qiagen nucleotide removal kit.
7. After prehybridizing the membrane in Ambion RapidHyb for 1–2 h, boil the labeled probe for 5 min and then place the probe on ice for 5 min. dd denatured probe to prehybridization solution and hybridize at 45°C overnight.
8. After hybridization, rinse the blot with 2X SSC, 0.1% SDS at room temperature and then wash the blot with two 68°C 100-ml washes of 2X SSC, 0.1% SDS (5 min each), two 100-ml washes using 1X SSC, 0.1% SDS (10 min each), and four 100-ml washes using 0.1X SSC, 0.1% SDS (30–60 min each).
9. Sandwich washed blot in plastic wrap and expose to Kodak MS film. We recommend using film cassettes with intensifying screens at –80°C. Develop the first exposure 12 h after placing the film. If the signal is weak, additional exposure time may be needed (48–120 h).
10. Analyze the southern blot for evidence of genomic integration. Southern blot insertion sites have ranged in size from 10 kb to 50 kb in size (Fig. 9.2F).

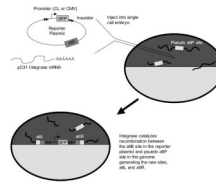
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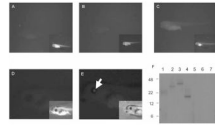
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**Fig. 9.1.**

Representation of  $\phi$ C31 integrase-mediated transgenesis in *Xenopus laevis*. The reporter plasmid containing an *attB* site and an insulated reporter gene is injected along with integrase mRNA into single-cell embryos. The chromosomes (*thick black lines*) contain numerous pseudo-*attP* sites. Inside the single-cell embryo, the integrase protein catalyzes recombination between the *attB* site in the reporter plasmid (*thin black line*) and a pseudo-*attP* site in the embryo's genome (*thick black line*). Recombination results in the formation of two new attachment sites, *attR* and *attL* flanking the integrated reporter plasmid.





**Fig. 9.2.**

$\phi$ C31 integrase-mediated transgenesis of insulated reporter plasmids generate *Xenopus* embryos with tissue-appropriate expression. In every case, the insert shows a brightfield image of the embryo. **(A)** Non-injected stage 42 embryo. **(B)** Stage 42 embryo injected with 5 pg of CMV-EGFP-DI-*attB* reporter plasmid. **(C)** Stage 42 embryo injected with 5 pg of CMV-EGFP-DI-*attB* reporter plasmid and 1 ng of integrase mRNA. **(D)** Stage 44 embryo injected with 5 pg of CL-EGFP-DI-*attB* plasmid alone. **(E)** Stage 44 embryo injected with 5 pg of CL-EGFP-DI-*attB* plasmid and 1 ng of integrase mRNA. GFP expression is indicated with the *white arrow*. **(F)** Southern blot demonstrating single integration events. Lanes 1 and 2 contains DNA harvested from single stage 46 CMV-EGFP-DI-*attB* transgenic embryos that expressed GFP uniformly. Lanes 3–4 contain DNA harvested from single stage 46 CL-EGFP-DI-*attB* transgenic embryos that expressed GFP in the lens of the eye. Lane 5 contains stage 46 non-injected DNA. Lane 6 contains DNA from a stage 46 embryo injected with CMV-EGFP-DI-*attB* plasmid alone. Lane 7 contains DNA from a stage 46 embryo injected with CL-EGFP-DI-*attB* plasmid alone. Lane 8 contains 10 pg of CMV-DI-EGFP-*attB* plasmid linearized with *Bam*HI. Markers in kilobase pairs are indicated to the left of the membrane.