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Engineering *lacZ* Reporter Gene into an *ephA8* Bacterial Artificial Chromosome Using a Highly Efficient Bacterial Recombination System

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In this report, we describe an optimized method for generation of *ephA8* BAC transgenic mice expressing the *lacZ* reporter gene under *ephA8* regulatory sequences. First, we constructed a targeting vector that carries a 1.2 kb *ephA8* DNA upstream of its first exon, a *lacZ* expression cassette, a kanamycin cassette, and a 0.7 kb *ephA8* DNA downstream of its first exon. Second, the targeting vector was electroporated into cells containing the *ephA8* BAC and pKOBEGA, in which recombinases induce a homologous recombination between the *ephA8* BAC DNA and the targeting vector. Third, the FLP plasmid expressing the Flipase was electroporated into these bacteria to eliminate a kanamycin cassette from the recombinant BAC DNA. The appropriate structures of the modified *ephA8* BAC DNA were confirmed by Southern analysis. Finally, BAC transgenic mouse embryos were generated by pronuclear injection of the recombinant BAC DNA. Whole mount X-gal staining revealed that the *lacZ* reporter expression is restricted to the anterior region of the developing midbrain in each transgenic embryo. These results indicate that the *ephA*8 BAC DNA contains most, if not all, regulatory sequences to direct temporal and spatial expression of the *lacZ* gene *in vivo*.

Keywords: BAC, EphA8, Transgenics

Introduction

BACs (bacterial artificial chromosome) are large genomic DNA fragments, generally 100-300 kb in size, that are cloned into bacterial plasmids which can be propagated in large quantities in *E. coli* cultures (Shizuya *et al*., 1992; Kim *et al*., 1996; Tao and Zhang, 1998; Frengen *et al*., 1999). Since their genomic fragments are large and contain most regulatory sequences of a gene, BACs are most useful for inducing stable and tissue-specific expression of heterologous genes *in vivo* in BAC-transgenic mice.

Conventional transgenic techniques using plasmids or phage vectors with relatively short inserts (< 50 kb) have had many limitations because introns and essential regulatory elements are easily omitted in these constructs. In addition, the position-effect often influences foreign gene expression, depending on the chromosomal integration site (Abe *et al*., 2004). Due to these drawbacks, transgene expression often fails to match the expression patterns of the corresponding endogenous gene. These drawbacks can be overcome by BAC technology since large fragments of genomic DNA in BAC have a high probability of containing most regulatory elements and locus control regions. Currently, BAC-transgenic technology is ideally suited to analyzing gene expression *in vivo*, for example, for a large-scale project to create an atlas of CNS gene expression (Joyner *et al*., 2003). In addition, BAC technology represents a very powerful approach for spatial and temporal control of recombinase expression, which is essential for conditional gene knock-out technology.

BAC-transgenic technology requires modification of BAC DNA with a heterologous gene using a bacterial homologous recombination system. Several methods have been reported for insertion of genes or deletion of DNA segments in BACs using a bacterial homologous recombination system. In general, these methods can be classified into two categories, depending on whether RecA or the lambda recombination system is used. The first method is to use a shuttle vector carrying the gene of interest flanked by relatively short (<1 kb) homologous genomic fragments and the recombinase RecA, which directs homologous recombination between the shuttle vector and the BAC DNA in bacteria (Yang *et al*., 1997; Gong *et al*., 2002). The second method is to use the lambda recombination system, in which *exo*, *bet*, and *gam* are inducibly expressed from a plasmid (Muyrers *et al*., 1999; Datsenko and Wanner, 2000; Yu *et al*., 2000; Zhang *et al*., 2000). In this system, a linear DNA fragment carrying the

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gene modification flanked by very short genomic segments is introduced into BAC-containing bacteria, in which homologous recombination is directed by inducible lambda phage recombinases.

In this report, we established a BAC recombination technique, which is based on a lambda recombination system. Our approach allowed for reliable generation of *ephA8* BAC transgenic mice, in which the expression pattern of the *lacZ* reporter gene correlated well with that of the endogenous *ephA8* gene. This method should expedite the analysis of various *ephA8* regulatory elements and facilitate tissue-specific expression of heterologous genes *in vivo* for gene function study.

Materials and Methods

Construction of targeting vector. Homology arms A and B flanking mouse *ephA8* exon 1 were synthesized by PCR using the following primer sets: 5'-CATCTAGCTGGGGGACCTAGCGT-3' (forward primer for A arm), 5'-TGGCGCGCTCCTGCCTCTCCC GC-3' (reverse primer for A arm), 5'-CGCCTGTCCCCCGCTCTC TGGGT-3' (forward primer for B arm) and 5'-GAAGGGACCACC TGGCTTTGATT-3' (reverse primer for B arm). The targeting vector was constructed on the backbone of the pGEM11Z vector (Promega) as follows. First, pGEM11Z was digested with *Apa*I and then a homologous B arm inserted into this site. Second, the homologous arm A and *lacZ* containing the SV40 polyadenylation site were cloned into the *Xho*I site before the B arm in pGEM11Z, and then a FRT-Kana-FRT cassette was inserted into the *Spe*I site present in between the *lacZ*-pA and B arm. This targeting vector was digested with *Nsi*I/*Sfi*I followed by purification of the insert on a 0.8% low-melting agarose gel.

BAC modification by homologous recombination. For the first homologous recombination, BAC host cells were streaked on an agar plate supplemented with chloramphenicol and incubated overnight at 37°C. 5 ml of Luria broth (LB) supplemented with chloramphenicol was inoculated with a single colony and grown at 37°C overnight with shaking. The next day, 1 ml of the overnight culture (OD_{600} = 1.2) was transferred to 10 ml of LB and grown to OD_{600} = 0.7-0.8. The cells were then collected by centrifugation at 4,000 r.p.m. for 1 min in a 1.5 ml microtube at 4°C. The cell pellets were then resuspended in 888 μl of ice-cold water. Cells were transferred to a 1.5 ml microtube on ice and centrifuged using a cooling centrifuge for 1 min at 4°C. The tubes were placed on ice and the supernatant fluids aspirated. This process was then repeated two additional times. Finally, the cell pellet was resuspended in 50 μl of ice-cold water and transferred to a pre-cooled electroporation cuvette (BIO-RAD). Next, 10 ng of pKOBEGA plasmids were added and mixed (Depaepe *et al*., 2005). Electroporation was performed using a BIO-RAD electroporator and 1 ml of LB added to the cuvette, which was incubated at 30°C for 1 h. Cells were spread on plates containing chloramphenicol and ampicillin. The next day, a single chloramphenicol and ampicillin-resistant colony was selected and inoculated with 5 ml of LB supplemented with chloramphenicol and ampicillin, and grown to OD_{600} = 1.2. 1 ml of the overnight culture (OD_{600} = 1.2) was then transferred to 10 ml of LB and grown to OD_{600} = 0.05-0.1. Next, 10% L(+)arabinose (Sigma A-3256) in D.W. was added to the culture to a final concentration of 0.2% and shaken at 30°C for another hour. Cells were then collected, and the cell pellets washed as described above. Next, 1 μg of linear targeting vector was eletroporated into 50 μl of competent cells. Then 1.0 ml of LB was added to the electroporation cuvette and 10-100 μl of the cells subsequently plated onto a plate containing kanamycin and chloramphenicol and incubated at 37°C overnight.

For the second recombination, the first recombinant BAC clones were identified and inoculated with 5 ml of LB supplemented with chloramphenicol and kanamycin, and grown to OD_{600} = 1.2. 1 ml of the overnight culture (OD_{600} = 1.2) was then transferred to 10 ml of LB and grown to OD_{600} = 0.7-0.8. Cells were collected and the pellets washed as described above. Next, 10 ng of FLP plasmid (Gene Bridges) was eletroporated into 50 μl of competent cells. Then 1.0 ml of LB was added to the electroporation cuvette and 10 -100 μl of the cells subsequently plated onto a plate containing tetracycline, kanamycin and chloramphenicol and incubated at 30°C overnight. 5 ml of LB supplemented with chloramphenicol was inoculated with a single colony and incubated at 30°C with shaking. 1 ml of the overnight culture (OD_{600} = 1.2) was transferred to 20 ml of LB (OD_{600} = 0.05-0.1) and incubated for 2 h with shaking (OD_{600} = 0.5). Next, 10 ml of the cells were transferred to a new flask and shaken in a 42°C water bath for 15 min. The cells were put into wet ice and the flask shaken to make sure that the temperature dropped as rapidly as possible, then the flask was left in wet ice for another 5 min. 10-100 μl of cells were subsequently spread onto a plate containing chloramphenicol and incubated at 37°C overnight. We used the following antibiotic concentrations in our experiments: chloramphenicol, 12.5 μg/ml; ampicillin, 50 μg/ml; kanamycin, 20 μg/ml; tetracycline, 15 μg/ml.

Generation and analysis of BAC transgenic mice. Modified BAC DNA was prepared using the large-construct kit (Qiagen). The quality and concentration of the DNA was checked on a gel and the BAC DNA diluted to 10 ng/μl in injection buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA pH 8.0 and 100 mM NaCl). An aliquot of DNA was mixed with the same amount of 2X polyamine (50 : 50) 48 h to 1 week before the injection. The BAC DNA (5 ng/μl) was injected into 200 pronuclei of fertilized oocytes of C57BL/6 mice as described previously (Gong *et al*., 2003; Koo *et al*., 2003; Sparwasser *et al*., 2003). BAC transgenic mice and embryos were identified by PCR using the primers 5'-GCGGGAGAGGCAGGA GCGCGCCA-3' and 5'-CGGAAACCAGGCAAAGCGCCAT-3'.

Whole mount X-gal staining and Southern blot analysis. Embryos to be stained were dissected in phosphate buffered saline (PBS), fixed in 0.2% glutaraldehyde, and washed three times in washing buffer. These embryos were placed in β-galactosidase staining solution for 1-5 h at 37°C, and post-fixed in 4% PFA as described previously (Joyner *et al*., 1993; Park *et al*., 1997; Koo *et al*., 2003). Southern blot was performed as described previously (Sambrook *et al*., 2001).

Results

Modification of *ephA8* BAC DNA with a targeting vector in bacterial system. The EphA8 gene contains 17 exons and is about 28 kb in length (Fig. 1A and B). For analysis of the *ephA8* gene expression, a BAC clone, RP23-357K18, was selected for modification using the bacterial homologous recombination method. This BAC DNA contains the *ephA8* gene approximately in the center of its genomic insert, and is therefore likely to contain most of the regulatory elements and locus control regions (Fig. 1A). This BAC clone is about 287 kb in length and contains other genes, including *zbtb40*, *C1qa*, *C1qb*, *C11qc*, and *ephB2*.

For modification of the *ephA8* BAC DNA, we first transformed pKOBEGA plasmid carrying an inducible lambda phage recombinase system into the bacteria containing the *ephA8* BAC DNA, and the transformants were selected at 30°C in the presence of chloramphenicol and ampicilin, as previously described (Depaepe *et al*., 2005). Recombinases were then induced by incubating these bacteria in arabinose-containing medium. Next, an *ephA8* targeting vector containing a 1.2 kb DNA upstream of the first exon, a 3.5 kb *lacZ*-pA cassette, a 2.3 kb kanamycin resistance gene flanked by FRT sites, and a 0.7 kb DNA downstream of the first exon was constructed (Fig. 1B). This vector was electroporated into the bacteria containing the *ephA8* BAC DNA and pKOBEGA. In this system, the arabinose-induced recombinases mediate a homologous recombination between a targeting vector and the *ephA8* BAC DNA, and the recombinant BAC clones were further selected by incubation at 37°C in the presence of chloroamphenicol and kanamycin. The integrity and structure of this modified BAC DNA containing the targeting vector was assessed by Southern blot (Fig. 2). As expected, a 14.8 kb fragment of the *Hind*III-digested wild-type BAC DNA was hybridized by probe A and B (Fig. 2A and B, lane 1). When DNAs from the first BAC recombinant clones were digested with *Hind*III, the 5.6 kb fragment was hybridized with probe A (Fig. 2A, lanes 2-3) or *lacZ* (Fig. 2C, lanes 2-3), and a 14.4 kb fragment was hybridized with probe B (Fig. 2B, lanes 2-3), indicating that these clones carry cointegrates that occurred through the region of homology. Finally, FLP plasmid was electroporated into cells containing the first recombinant BAC DNA, and the transformants were selected at 30°C in the presence of choloramphenicol, kanamycin and tetracycline.

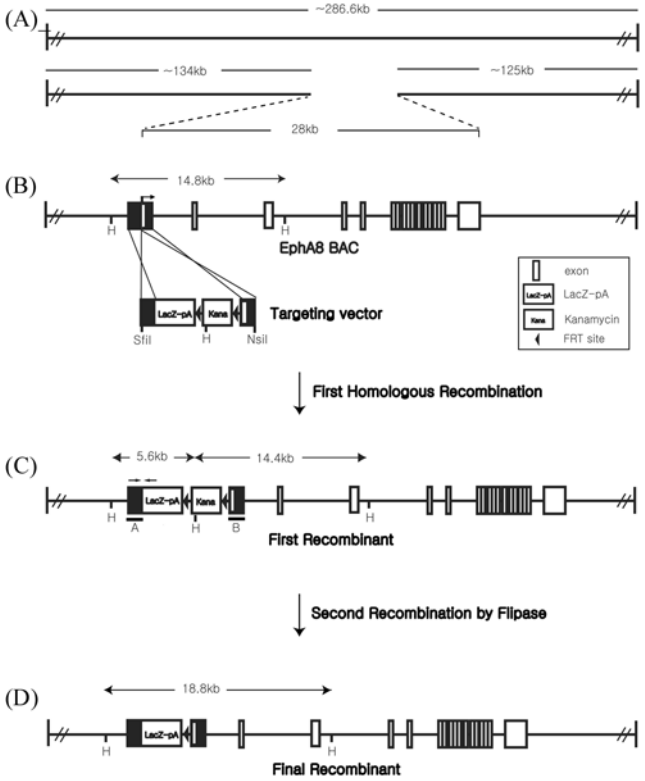

Fig. 1. Strategy for the *ephA8* BAC modification. (A) Location and size of the *ephA8* gene locus on the RP23-357K18 BAC clone. Note that the *ephA8* gene is approximately 28 kb long as represented by a thin line. (B) The first homologous recombination between the linearized targeting vector and the BAC DNA containing the *ephA8* gene. Note that the black boxes indicate the homologous genomic DNAs present in both the targeting vector and *ephA8* BAC. H, *Hind*III. (C) The genomic structure of the modified *ephA8* BAC DNA in which the targeting vector was integrated through the first homologous recombination. A and B represent the left and right homologous arms, respectively. (D) The genomic structure of the modified *ephA8* BAC DNA in which the FRT-Kana-FRT cassette was selectively removed by Flipase recombinase. This modified *ephA8* BAC is identical with the original *ephA8* BAC, RP23-357K18, except that it contains the *lacZ* gene in-framed with the first exon in *ephA8*.

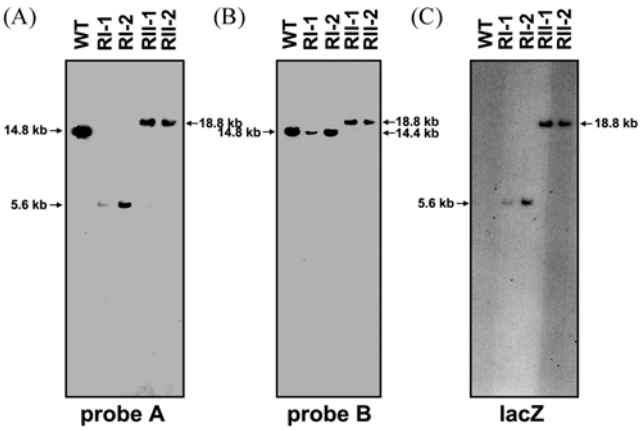

Fig. 2. Southern blot analyses of the modified BAC DNAs. The BAC DNAs from the indicated BAC clones were digested with *Hind*III and then analyzed by Southern blot using the indicated probes. WT denotes the original *ephA8* BAC clone, RP23-357K18. RI-1 and RI-2 represent two independent recombinant BAC clones, which were generated by the first homologous recombination. Likewise, RII-1 and RII-2 indicate two independent BAC clones induced by Flipase. The arrows represent the *Hind*III-digested BAC DNA fragments hybridized with the indicated probes.

Flipase was then induced by incubating at 42°C to eliminate a kanamycin cassette between two FRT sites, and the final recombinant BAC clones were cultured at 37°C in the presence of chloramphenicol. An appropriate structure of the final BAC DNAs was investigated by Southern blot, revealing that the same DNA fragment, which corresponded to appropriately 18.8 kb, was hybridized by probe A, B, and *lacZ*, respectively (Fig. 2A-C, lanes 4-5). Taken together, these results indicate that an *ephA8* BAC clone, RP23-357K18, was appropriately modified to contain a *lacZ* expression cassette inserted into the first exon of the *ephA8* gene through homologous recombination.

Production of ephA8 BAC transgenic mice and *in vivo* reporter expression analysis. To generate *ephA8* BAC transgenic mice, we used BAC DNAs of 5 ng/μl concentration for pronuclear injection into fertilized C57BL/6 mouse zygotes (Gong *et al*., 2003; Koo *et al*., 2003; Sparwasser *et al*., 2003). As shown in Fig. 3, seven embryos at embryonic day (E) 10.5 were obtained from a foster mother and subjected to PCR analysis to determine whether they contained the modified BAC DNA. One of these revealed an expected PCR product (lane 3), confirming that the *ephA8* BAC DNA was stably integrated into the mouse genome. We established at least three different transgenic lines after several rounds of microinjections. Two of these transgenic lines were crossed to generate transgenic embryos in order to investigate the temporal and spatial expression of *lacZ in vivo*. As shown in Fig. 4, *lacZ* expression patterns of *ephA8* BAC transgenic embryos derived from two different lines (panels C-F) were compared with those of the $ephA8^{lacZ/lacZ}$ embryo (panels A and B) using whole-mount X-gal staining (Park *et al*., 1997). The most prominent expression was observed in the anterior region of dorsal mesencephalon for both the *ephA8* BAC embryo and $ephA8^{lacZ/lacZ}$ embryo. Interestingly, *lacZ* expression was more laterally extended in the *ephA8* BAC transgenic embryo (panel D), possibly due to over-expression of *lacZ* in each transgenic embryo. In addition, ectopic expression was also observed in many other tissues of one particular *ephA8* BAC transgenic embryo shown in Fig. 4C and D, suggesting

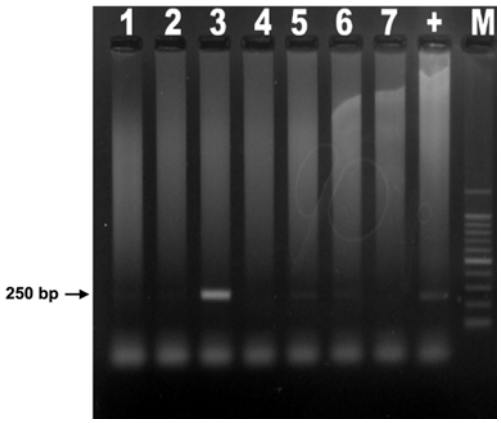

Fig. 3. Generation of the *ephA8* BAC transgenic line. PCR analysis was performed using tail genomic DNAs derived from seven littermates. One of these littermates showed the expected 250 bp PCR product, suggesting that this line is a potential transgenic line. Note that only 0.1 ng of BAC DNA was used as a positive control for PCR analysis. +, a positive control DNA derived from RII-1 BAC clone; M, a 100 bp marker.

that this ectopic expression pattern is likely due to the integration site of the BAC DNA. Nevertheless, these results strongly indicate that the reporter expression of the *ephA8* BAC transgenic embryo is very similar to that of the $ephA8^{lacZ/lacZ}$ embryo.

Discussion

In this report, *ephA8* BAC DNA was modified to contain a *lacZ* reporter downstream of the *ephA8* promoter through a bacterial homologous recombination system. This BAC DNA was stably integrated into the mouse genome and shown to express the reporter in the developing midbrain as the endogenous *ephA8* gene, indicating that BAC technology represents a powerful approach to study of the *ephA8* gene regulation and function *in vivo*.

To date, several methods have been described to use BAC

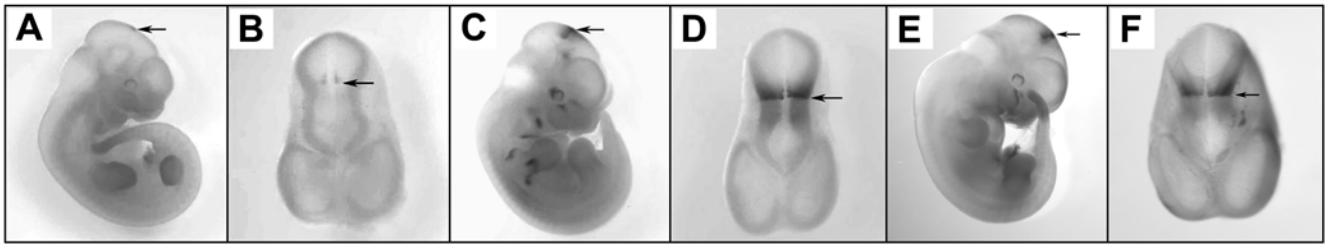

Fig. 4. Analysis of *lacZ* expression for the *ephA8* BAC transgenic embryo. EphA8 BAC transgenic and $ephA8^{lacZ/lacZ}$ embryos at embryonic day (E) 10.5 were analyzed for LacZ activity by whole mount X-gal staining. (A, B) Lateral and dorsal view of the $ephA8^{lacZ/lacZ}$ embryo, respectively. (C, D) Lateral and dorsal view of the *ephA8* BAC transgenic embryo, respectively. (E, F) Lateral and dorsal view of the *ephA8* BAC transgenic embryo derived from the different transgenic line, respectively. The black arrows indicate a boundary between diencephalon and mesencephalon.

DNA in introducing modification and transgenesis (Gong *et al*., 2002; Abe *et al*., 2004; Depaepe *et al*., 2005). The Heinz group reported a method in which BAC-containing bacteria are transformed with a shuttle vector carrying the gene modification flanked by BAC homologous DNA fragments and RecA bacterial recombinase (Gong *et al*., 2002). Although this method has been reported as a comprehensive and highly optimized method, we have encountered some difficulties, including subcloning of DNA fragments into the shuttle vector in pir2 competent cells, especially when manipulating large fragments. In addition, we found that the RecA-mediated recombination system did not work for certain genomic DNA fragments. The second method uses homologous recombination via an inducible lambda phage recombinase system encoded on a separate vector or in modified bacteria (Muyrers *et al*., 1999). In this system, the linear modification cassette can be generated by PCR without any cloning steps. However, in our study, PCR generated mutations on the amplified DNAs, especially in the FRT site, caused a serious problem for elimination of the kanamycin cassette through Flipase-mediated recombination. The method we describe here is highly optimized for generation of BAC-transgenic mice, which is modified from the approach developed by the Vanderhaeghen group (Depaepe *et al*., 2005). We have used a conventional subcloning method to generate a targeting vector, which was further induced to recombine with BAC DNA using a lambda phage recombinase system encoded on a pKOBEGA plasmid. Then, the kanamycin cassette was eliminated using a flipase recombinase system encoded on a FLP plasmid (Buchholz *et al*., 1996). Our method was very reliable and efficient since we used a specific bacterial recombination system and antibiotics selection to allow the specific recombinants to grow, although our approach may potentially be slow due to the many steps required for subcloning. We have not encountered any problems using the lambda recombination system that caused BAC instability due to its high recombination efficiency.

There is a high demand for the use of recombinases and inducible promoters for spatial and temporal control of gene expression *in vivo* in the study of gene function. BAC DNAs have a high probability of harboring most regulatory elements in the gene of interest as well as boundary elements of chromatin domains or locus control regions that insulate from regulatory influence by neighboring genes. This advantage makes BAC-transgenic mice ideal for analysis of gene expression and function *in vivo* (Depaepe *et al*, 2005; Jeong *et al*., 2006; Kim *et al*., 2006). Our BAC technology described here offers many potential uses including *in vivo* expression of dominant negative mutants and analysis of *cis*-acting regulatory elements.

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