

piggyBac as a high-capacity transgenesis and gene-therapy vector in human cells and mice

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SUMMARY

The stable genomic integration and expression of a large transgene is a major hurdle in gene therapy. We show that the modified *piggyBac* (*PB*) transposon system can be used to introduce a 207 kb genomic DNA fragment containing the *RORy/yt* locus into human cells and mice. *PB*-mediated transgenesis results in a single copy of a stably inherited and expressed transgene. These results indicate that *PB* could serve as an effective high-capacity vector for functional analysis of the mammalian genome and for gene therapy in human cells.

INTRODUCTION

Gene therapy requires a safe and efficient way to introduce target DNA fragments with a defined orientation into cells to compensate for the deleterious effects of a mutated gene. A number of viral and non-viral gene delivery systems have been developed over the years (Atkinson and Chalmers, 2010; Thomas et al., 2003). Retroviral, lentiviral and adeno-associated viral vectors have been shown to be effective for gene introduction. In addition, stable integration of retroviral and lentiviral vectors benefit long-term expression of the transgene. However, limited cargo capacity prevents these vectors from being used to introduce large transgenes and genomic sequences. Adenoviral vectors are able to carry inserts of up to 30 kb, but they remain episomal after transduction and trigger immune responses easily, both leading to transient expression of the therapeutic DNA (Atkinson and Chalmers, 2010). In addition, virus-related risks are common concerns of using viral vectors because death and leukemia cases have been reported in clinical trials using adenoviral and retroviral vectors for gene therapy, respectively (Hacein-Bey-Abina et al., 2003; Raper et al., 2003).

As non-viral transgenic vectors, DNA transposons are attractive choices for gene therapy. We have previously shown that the modified *piggyBac* (*PB*) DNA transposon, which was originally identified in the cabbage looper moth *Trichoplusia ni* (Fraser et al., 1996), is highly efficient in mediating stable integration and expression of transgenes in human cells and in mice (Ding et al., 2005). Recently, *PB* has been shown to be able to carry fragments of more than 100 kb to hop in human and mouse embryonic stem

(ES) cells (Li et al., 2011; Rostovskaya et al., 2012). Here we analyzed the *in vivo* expression of an extra endogenous locus carried by *PB*-mediated BAC transgenes. Our data suggest *PB* as a potential candidate of high-capacity gene-therapy vector.

RESULTS

Construction of *PB/shuttle* and *PB[BAC]*

To test whether *PB* can mediate the integration of a large DNA fragment in human cells, we built a *PB* element carrying BAC genomic DNA based on a new *PB* transposon shuttle vector (*PB/shuttle*). *PB/shuttle* was developed by incorporating BAC homologous sequences (Box A and Box B) and a *PGK* promoter-driven *neomycin* resistance (*Neo*) gene into the transposable element (Sparwasser et al., 2004) (Fig. 1A). Any BAC clones can be introduced into *PB/shuttle* via homologous recombination resulting a transposable unit containing the entire BAC sequence and the *Neo* selection marker. A *diphtheria toxin A* expression cassette (*Dta*) was placed in the *PB/shuttle* vector outside of the *PB* end termini (PBL and PBR) for selection against random integration events of the *PB[BAC]* construct (Fig. 1A). We then generated a *PB[BAC]* clone with a 207.7 kb BAC, RP23-263k17, containing the *RORy/yt* locus of *C57BL/6J* mice (Fig. 1B). Correct recombinants were confirmed by PCR analysis with primers flanking each of the homologous sequences before further experiments (supplementary material Fig. S1).

PB-mediated BAC gene transfer in human 293 cells

We transfected *PB[BAC]* into human 293 cells with or without a *CMV-PBase* helper plasmid that ubiquitously expresses *piggyBac* transposase (PBase). Co-transfection with PBase generated on average sixfold more neomycin-resistant clones than the PBase negative control (Fig. 1C,D). Inverse PCR with primers on one of the *PB* termini was successfully used to isolate 15 integration sites from 13 clones derived from the co-transfection experiment (Fig. 2B and supplementary material Table S1). The TTAA target sequence of the *PB* transposition was found to be present in all 15 sites through inverse PCR. TTAA duplications were further confirmed in all five attempts by PCR analysis of the other *PB* terminus (Fig. 2A-C), demonstrating that these integrations occur through a clean *PB* transposition.

To examine whether the entire cargo is delivered into the genome after transposition, we examined the integrity of the

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TRANSLATIONAL IMPACT

Clinical issue

In gene therapy, DNA fragments are introduced into cells to override the deleterious effects of a disease-causing mutation, most commonly by replacing a dysfunctional or abnormal gene with a functional version. A number of viral and non-viral gene-transfer systems have been developed, but their clinical application is currently limited owing to associated risks and shortcomings. A major hurdle is the low 'cargo capacity' of existing vectors, which prevents them from being used to deliver full-length genes and genomic sequences. Adenoviral vectors can carry inserts of a large size; however, viral vectors are associated with a lack of copy number control, a propensity for deleterious integration and the elicitation of unwanted immunological responses in target cells. As non-viral transgenic vectors, DNA transposons represent a more reliable and safe agent for the delivery of large transgenes into human cells.

Results

It was recently shown that the *piggyBac* (*PB*) transposon can carry DNA fragments that are greater than 100 kb in size. In the present study, the authors demonstrate that a modified *PB* transposon system can efficiently introduce an intact mammalian gene of more than 200 kb into human and mouse cells *in vitro*. Moreover, they show that the gene is integrated with a defined orientation, and is stably integrated and inherited in mice *in vivo*. Finally, the authors report that expression of the transgene mimics that of the endogenous gene.

Implications and future directions

The modified *PB* transposon system facilitates the stable integration and inheritance of large DNA fragments, and allows controlled expression of transgenes *in situ*. The vector has the capacity to carry regulatory elements associated with a gene to overcome positional effects of expression, and to deliver genomic sequences encompassing wild-type alleles. Thereby, the system provides an efficient and reliable transgenic and gene-therapy tool that is particularly promising for the treatment of cystic fibrosis and Huntington's disease, where the affected gene and flanking regions cover long stretches of DNA.

genomic DNA in *PB[BAC]* transgenes. Ten pairs of primers were designed to detect 1-kb PCR fragments evenly spaced along the 200-kb BAC genomic insert. For 55% (5/9) of the clones carrying a single insertion of *PB[BAC]*, positive results were obtained in all PCR reactions (Fig. 2D,E). We further examined two clones by long-range PCR, amplifying 21 overlapping PCR fragments covering the entire length of the genomic insert. Both clones were positive for all PCR reactions (Fig. 2D,F and supplementary material Fig. S2). These results indicated that *PB[BAC]* vector could effectively mediate integration of a DNA fragment of more than 200 kb into the human genome in its entirety with a defined orientation.

PB-mediated BAC gene transfer in mice

We then examined whether *PB*-mediated BAC gene transfer could be stably inherited *in vivo*. To achieve this goal, we first introduced *PB[BAC]* into mouse W4/129S6 embryonic stem (ES) cells. Co-transfection with the *Act-PBase* helper plasmid produced 25 times more neomycin-resistant clones than the *PBase* negative control (Fig. 1E,F) (Ding et al., 2005). Inverse PCR detected a single band in 84% (31/37) of the clones tested, suggesting that most of the clones carried a single insertion of *PB[BAC]* (supplementary material Table S1). This outcome was consistent with the result from some clones tested by quantitative real-time PCR (data not shown). To further evaluate the integration events, we performed

fluorescence *in situ* hybridization (FISH) in two ES clones expected to carry a single insertion of *PB[BAC]*. Full-length RP23-263k17 BAC DNA was used as the probe to detect both endogenous loci (paternal and maternal alleles) and any transgenic copies present in the genome. FISH analysis of mitotically arrested cells identified three foci in each of the ES clones (Fig. 3A). In addition, analysis of an ES clone that gave two bands in inverse PCR (ES37) showed four hybridization signals in FISH (Fig. 3A).

We then performed blastocyst injection to produce transgenic animals. We generated three transgenic lines in total. Two of them were derived from the ES clone ES37 (supplementary material Table S2). We first confirmed the integrity of *PB[BAC]* in the progenies by genotyping four SNP markers in the BAC DNA (Fig. 3B and supplementary material Table S3). The transmission of *PB[BAC]* was then followed by PCR using *PB* and flanking genomic primers. In all cases the transgene was stably inherited.

PB-mediated BAC transgene expression in mice

Generation of transgenic animals allowed us to further study the expression pattern of the transgene. The *RORγ/yt* locus encodes two alternatively spliced transcripts differing in their 5'-terminal sequences. *RORγ* mRNA is expressed broadly in adult tissues. In contrast, *RORγt* mRNA can only be detected in CD4⁺CD8⁺ double-positive thymocytes (He et al., 1998; Kurebayashi et al., 2000). In transgenic animals, *RORγ* transcription is readily detected in kidney, liver and muscle by RT-PCR (Fig. 4A,D,E and supplementary material Fig. S3). In contrast, *RORγt* mRNA can only be detected in the thymus, but not the above tissues (Fig. 4A,C and supplementary material Fig. S3). These results suggest that *PB[BAC]* expression follows the same tissue specificity as the expression of endogenous alleles.

Transgenic expression is often affected by the surrounding genomic context. To evaluate the positional effects of *PB*-mediated transgene expression, we compared the expression of an *Act-RFP* transgenes in 101 *PB[Act-RFP]* lines. These randomly chosen transgenic lines were generated from mobilization of the same *PB[Act-RFP]* construct. Each line carries a single insertion at a different location in the genome. The *Act-RFP* transgene allows us to visually assess the level of gene expression in the transgenic animals. We have observed that the majority of the transgenic lines exhibit a comparable level of red fluorescence (68/101=67.3%), whereas 15.8% (16/101) and 16.9% (17/101) of the transgenic lines have either stronger or weaker signals, respectively (supplementary material Fig. S4A,B). Real-time RT-PCR analysis confirmed similar distribution of *RFP* expression levels in 18 randomly picked transgenic lines from this pool (supplementary material Fig. S4C). These data show that, similar to transposon-mediated transgenesis in *Drosophila*, *PB* can serve as a good transgenic tool in mammals despite some positional effects.

We next studied whether increasing the copy number of the *PB[BAC]* transgene would correlate with elevated of gene expression, by examining the expression level of the *RORγ* and *RORγt* transcripts in different *PB[BAC]* transgenic lines. We first measure the copy number of each transgenic integration by quantitative real-time PCR. Two of the transgenic lines carried a single copy transgene, while the other line (37-1) carried two closely linked *PB[BAC]* insertions (Fig. 4B). Real-time RT-PCR indeed detected a 66% increase in *RORγt* expression in line 37-1, and about

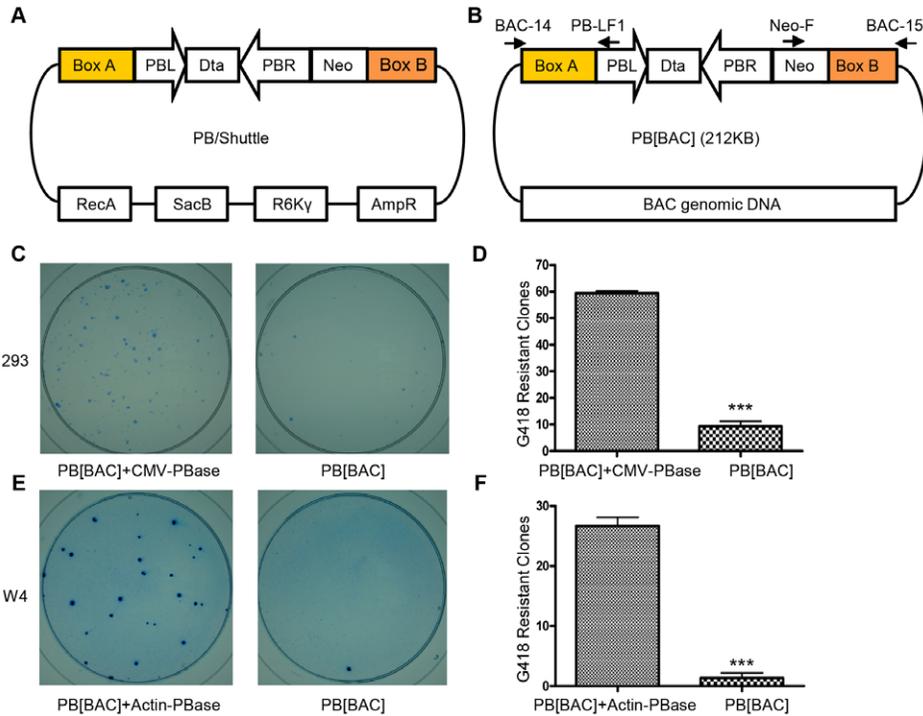


Fig. 1. PB-mediated BAC gene transfer in human and mouse ES cells. (A) Diagram of the *PB/shuttle* vector. The head-to-head *PB* termini (*PBR* and *PBL*) are separated by the negative selection marker *Dta*. This cassette, along with the positive selection marker *Neo*, is placed between the BAC homologous recombination sequences *Box A* and *Box B*. The vector also carries the *R6Ky* origin of replication, *RecA*, *AmpR* (ampicillin-resistance) and *SacB* genes are required for proper recombination and selection in *E. coli* cells (Sparwasser et al., 2004). (B) Diagram of *PB[BAC]*. Primers used to confirm the recombination events are marked with arrows. (C,D) An example of *PB[BAC]* integration in human 293 cells with *CMV-PBase*. Clones were stained with methylene blue after G418 selection (C) and the statistical results of three transfections plotted (D). (E,F) Similar experiments were repeated in murine W4 ES cells with *Act-PBase*. ****P*<0.001.

33% increase in expression in lines 37-2 and 27 (Fig. 4C). Similarly, we measured the *RORY* transcript in kidney and liver samples in two lines. Transgenic expression of the *RORY* transcript of line 37-1 is approximately twofold of that in line 37-2 (Fig. 4D,E). These data are consistent with the expectation that *PB*-mediated BAC transgene expression correlates with the copy number of the *PB[BAC]* transgene.

Our previous work suggested that the *PB* element allows transgene expression without significant silencing or epigenetic modification among generations (Ding et al., 2005). This feature also applied for *PB*-mediated BAC transgenesis. We analyzed *RORYt* expression in different generations of lines 37-1 and 37-2 by real-time RT-PCR. In both lines, the expression level remained stable among generations (supplementary material Fig. S5).

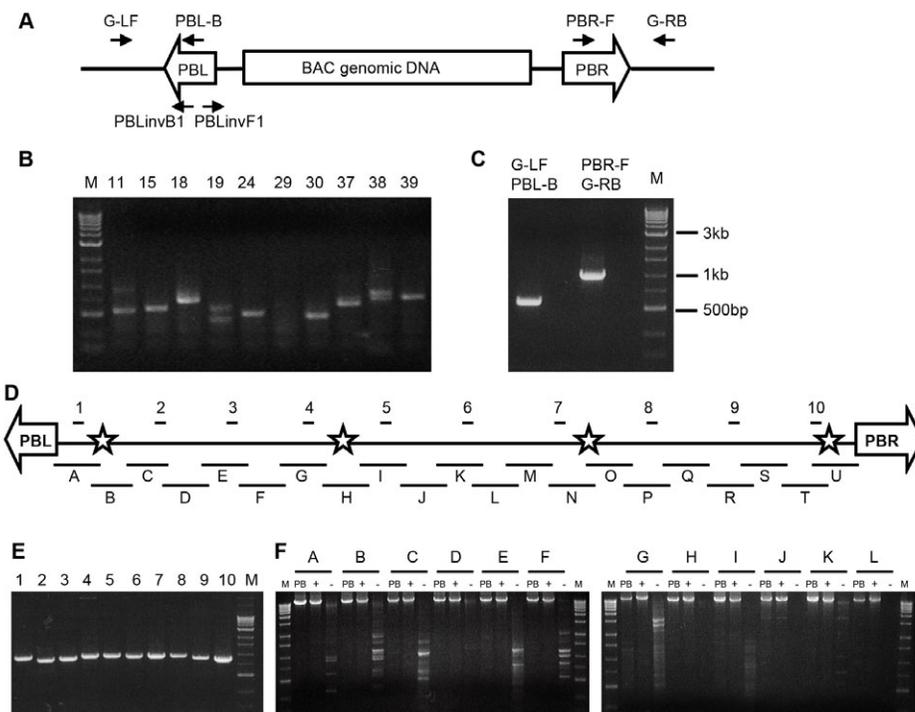


Fig. 2. Molecular analysis of *PB[BAC]* insertions in human 293 cells. (A-F) Inverse PCR with primers *PBLinvB1* and *PBLinvF1* (A) recovered single insertions in most of the clones (B). The insertion sites were confirmed by genotyping PCR using pairs of primers targeting both flanking genomic sequences (*G-RL* or *G-RB*) and *PB* terminal sequences (*PBL-B* or *PBR-F*), as shown in panel C. Integrity of *PB[BAC]* transgenes was analyzed by PCR using ten primer pairs evenly spaced along the BAC insert (fragments 1-10 in panel D). Two clones showed all positive results (E) and were further examined by long-range PCR using 21 pairs of overlapping primers covering the full length (fragments A-U in panel D), as shown in panel F and supplementary material Fig. S2. M, 1 kb ladder; +, positive control with *PB[BAC]* as the template; -, negative control with 293 genomic DNA as the template; stars mark location of SNPs used to verify BAC integrity in transgenic mice.

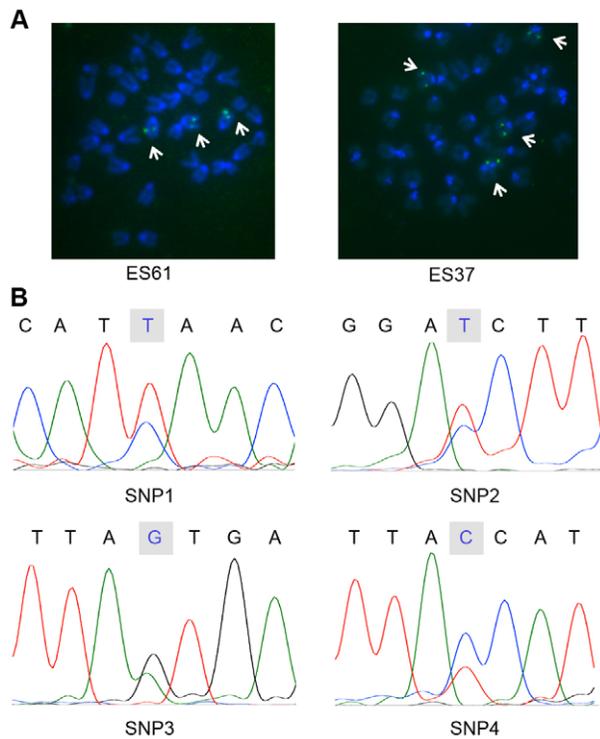


Fig. 3. Analysis of the *PB[BAC]* transgene in ES cells and in mice. (A) FISH analysis of two ES clones carrying one (ES61) or two (ES37) *PB[BAC]* insertions. (B) Sequencing results of four SNPs (shaded nucleotides with the position shown in Fig. 2D) in mice derived from the clone ES27.

DISCUSSION

A large cargo capacity is always desired for gene-therapy vectors. It allows the transgene to carry more regulatory elements to overcome the positional effect on expression. It also permits the vector to carry genomic fragments covering the wild-type alleles, which may reach ~180 kb in cases such as Huntington's disease and cystic fibrosis, to restore endogenous expression (Gusella et al., 1993; Kerem et al., 1989). Although the large *PB[BAC]* construct (207 kb) exhibits about 1.4% of transposition efficiency compared with the small *PB[PGK-neo]* construct (7.5 kb) (Ding et al., 2005), our results showed that the *PB* transposon-mediated transgenesis is efficient enough to introduce a DNA fragment of 207 kb into human cells and mice. This and previous reports make *PB* the transgenic vector that has the biggest cargo capacity thus far.

PB transgenes with such a gigantic insertion could transpose into the genome, resulting in single copy integrations with defined orientation of the transgene. These features are attractive for gene-therapy vectors because the insertion site could be easily mapped to recognize hazardous integration events before further use. Such a process is essential for BAC and other large transgenes, because rearrangements might occur during transgenic manipulations (Giraldo and Montoliu, 2001). In fact, we observed that 45% of the *PB[BAC]* 293 clones had deletions in the transgene.

We developed a shuttle recombinering vector that allows fast construction of *PB*-based BAC transgenes. The vector has both positive and negative selection markers that lead to fast enrichment of the clones carrying transposition events. It provides a convenient

tool for *PB*-based transgenesis with commonly used *pBACe3.6* BACs. With properly modified homologous arms, the design should also be applicable for other BACs.

An ideal gene-therapy vector allows stable expression of the transgene that preferably has the same tissue specificity of the endogenous allele. In our study, the *RORγ/γt* locus carried by *PB[BAC]* expressed *RORγt* thymus specifically in transgenic animals. The expression level of both *RORγt* and *RORγ* are position independent but copy number dependent. However, addition of each copy of the 129/Sv6 transgene produced a 66% increase in *RORγt* transcripts of an endogenous allele. This could be due to differential allelic expression (DAE), which is commonly observed in mammals (Cowles et al., 2002; Knight, 2004; Tang et al., 2011). Alternatively, the 200-kb BAC genomic sequences might not fully cover unknown remote regulatory sequences of the *RORγ/γt* locus. Nevertheless, the stable integration and expression of *PB*-mediated BAC gene transfer for DNA of more than 200 kb renders *PB* an attractive candidate vector for gene therapy of many known disease genes. It also provides a powerful tool for transgenesis in other mammals and for the functional dissection of the mammalian genome.

MATERIALS AND METHODS

PB/Shuttle construction

All primer sequences are listed in supplementary material Table S4. The *Sma*I fragment of *pBac-AB* (Ding et al., 2005) containing PBL was cut and cloned into the *Eco*RV site to form *pBac-BA*. Homologous arms Box A and Box B were PCR-amplified with primers BXA1/BXA2 and BXB1/BXB2, and cloned into T vectors to form *T-AB* and *T-CD*, respectively. The *Eco*RI-*Nde*I fragment of *T-AB* was then replaced by the *Eco*RI-*Nde*I fragment of *T-CD* to form *T-ABCD*. The *Bgl*II-*Eco*RI fragment of *T-ABCD* was further replaced by the *Bgl*II-*Eco*RI fragment of *pBac-BA* to form *T-ABCD-PB*. *Dta* and neomycin cassettes of *pGKNEO-F2L2-DTA* were cloned into the *Xba*I and *Sma*I sites of *T-ABCD-PB*, respectively, to form *T-ABCD-PB-Dta-Neo*. The *Not*I fragment of the shuttle vector *pDelsac* (Sparwasser et al., 2004) was finally replaced by the *Not*I fragment of *T-ABCD-PB-Dta-Neo* to form *PB/Shuttle*.

PB[BAC] construction

PB/Shuttle was used in a two-step RecA strategy for *PB[BAC]* construction as previously described (Sparwasser et al., 2004). Primer pairs BAC-14/*PB*-LF1 and BAC-15/*Neo*-F were used to identify the positive clones. BAC DNA was purified by using NucleoBond BAC 100 (Macherey-Nagel, cat. no. 740579).

Cell transfections

293 cells were cultured in DMEM (GIBCO-BRL) medium containing 10% fetal bovine serum at 37°C and 5% CO₂. For each well of a 24-well-plate, 2 × 10⁵ cells were seeded one day before transfection, transfected with 0.8 μg of circular *PB[BAC]* and 0.2 μg of circular *CMV-PBase* DNA as the test group, or the same amount of circular *PB[BAC]* with 0.2 μg of circular *pcDNA4/HisA* as the control. Transfection was performed with Lipofectamine 2000 following the standard protocol (Invitrogen). One day after transfection, cells were trypsinized and seeded onto one 10-cm plate. Drug selection with 500 μg/ml G418 (Gibco-BRL) was then continued for two weeks.

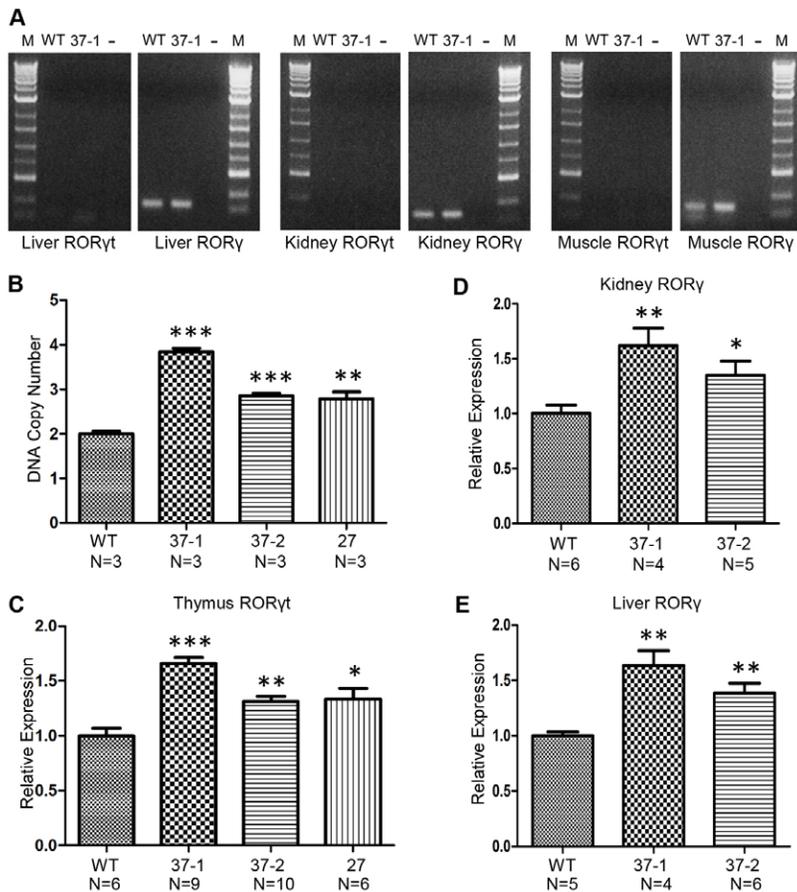


Fig. 4. PB-mediated BAC transgene expression in mice. (A) RT-PCR analysis showed *RORy* but not *RORYt* expression in the liver, kidney and muscle of wild-type (WT) and transgenic mice. (B) Quantitative real-time PCR with tail genomic DNA revealed different copy numbers of *PB[BAC]* in each of the transgenic lines. (C) Real-time RT-PCR showed elevated *RORYt* expression in the thymus of transgenic animals. (D,E) Similar levels of increased *RORy* expression were also observed in the kidney (D) and liver (E). All samples were taken from 20-day-old mice. Line numbers are indicated. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; -, cDNA-free control.

ES cells were cultured on 10-cm plates containing mitomycin-C-treated mouse embryonic fibroblast cells. 20 μg of *PB[BAC]* and 5 μg of *Actin-PBase* or *pCX* in the control group were used for electroporation of 10×10^6 cells. Immediately after electroporation, cells were divided onto three 10-cm plates. Selection with 200 $\mu\text{g}/\text{ml}$ G418 was initiated 24 hours after electroporation and lasted for one week. Cells were then fixed at room temperature with PBS containing 4% paraformaldehyde for 10 minutes and stained with 0.2% methylene blue for 1 hour.

PCR

To recover the flanking genomic sequences of *PB* insertions, *HaeIII* digests of the genomic DNA were self-ligated to serve as the template for inverse PCR. Primers used to recover the genomic sequences flanking the PBR terminus were PBRinvF3 and PBRinvB3.

To measure the gene expression level quantitatively, total RNA was isolated from tissues with TRIzol (Invitrogen) and treated with RNase-free deoxyribonuclease I (TaKaRa) to eliminate genomic DNA contamination. After reverse transcription (TaKaRa), real-time PCR were performed with Brilliant II Fast Sybr Green QPCR Master Mix (Stratagene) on an Mx3000P Quantitative PCR System (Stratagene). Expression of *GAPDH* was used as the baseline standard. PCR primers *RORYt*-F and *RORYt*-B were used for *RORYt*, *RORY*-F and *RORY*-B were used for *RORY*, and primers *RFP*-F and *RFP*-B were used for *RFP*.

To measure the copy number of BAC, genomic DNA extracted from mouse tails were used as the template for real-time PCR. Primers RT-BAC-F and RT-BAC-B were used for the amplification.

To examine the integrity of BAC transgenes, long-range PCR was carried out using the LongAmp *Taq* PCR kit (NEB).

Fluorescence *in situ* hybridization

We prepared metaphase chromosome spreads from ES cells. BAC DNA was probed with biotin by nick translation (Roche) and stained with Avidin-FITC (Roche). Chromosomes were identified by the banding patterns with DAPI staining. Photographs were taken with a Leica DMRXA2 camera and images prepared with Leica FW4000 and Adobe Photoshop.

Statistics

P-values were calculated with two-tailed unpaired Student's *t*-test on Prism 5 (GraphPad Software).

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

X.W. and T.X. designed the project. R.L. did the experiments and collected data; Y.Z., M.H., T.X. and X.W. analyzed the data. R.L., T.X. and X.W. prepared the manuscript. X.W. supervised all aspects of the work.

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SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at <http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.010827/-/DC1>

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