Highly Efficient Modification of Bacterial Artificial Chromosomes (BACs) Using Novel Shuttle Vectors Containing the R6Kγ Origin of Replication

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Bacterial artificial chromosome (BAC) mediated transgenesis has proven to be a highly reliable way to obtain accurate transgene expression for in vivo studies of gene expression and function. A rate-limiting step in use of this technology to characterize large numbers of genes has been the process with which BACs can be modified by homologous recombination in *Escherichia coli*. We report here a highly efficient method for modifying BACs by using a novel set of shuttle vectors that contain the R6Kγ origin for DNA replication, the *E. coli RecA* gene for recombination, and the *SacB* gene for negative selection. These new vectors greatly increased the ease with which one can clone the shuttle vectors, as well as screen for co-integrated and resolved clones. Furthermore, we simplify the shuttle vector cloning to one step by incorporation of a "built-in" resolution cassette for rapid removal of the unwanted vector sequences. This new system has been used to modify a dozen BACs. It is well suited for efficient production of modified BACs for use in a variety of in vivo studies.

Bacterial artificial chromosomes (BACs) and P-1 derived artificial chromosomes (PACs) have several advantages over the traditional large DNA cloning system, the yeast artificial chromosomes (YACs). These include large carrying capacity (~100–300 kb), high clonal stability, low rate of chimerism, and the ease with which they can be handled (Shizuka et al. 1992; Ioannou et al. 1994; Marra et al. 1997; Kelley et al. 1999). BACs have served as the primary source of archived genomic DNA for a variety of genome mapping and sequencing projects (Mozo et al. 1999; Hoskins et al. 2000; Osoegawa et al. 2000; McPherson et al. 2001). Indeed, physical contigs are now available for the entire human genome and the mouse genome, as well as a variety of other experimental organisms (Mozo et al. 1999; Hoskins et al. 2000; Osoegawa et al. 2000; McPherson et al. 2001).

Transgenic mice are important tools for in vivo genetic studies. The conventional transgenic constructs with <20 kb of the genomic sequence often result in strong position effects, leading to lack of expression or incorrect expression. Because the genomic DNA inserts in BACs are sufficiently large in most instances to carry an entire transcription unit and its associated regulatory regions, BACs can be used for a variety of functional studies that could not be accomplished by using conventional transgenic approaches. An important advance in the use of BACs for functional studies came with the development of methods for precise manipulation (i.e., marker/gene insertion, deletion, point mutation, etc.) of the genomic DNA carried in the BAC (Yang et al. 1997). This ability to manipulate BAC constructs made possible strategies for gene expression and function studies, as well as cell mark-

ing and isolation experiments, that had previously been widely used only in invertebrate systems (Heintz 2001).

The first and, so far, most widely used method (Yang et al. 1997) for manipulation of BAC DNA relies on a temperature-sensitive pSV1.RecA shuttle vector carrying the *Escherichia coli RecA* gene, which restores to the recombination-deficient BAC host the ability to undergo the homologous recombination steps required to generate precise modifications at a desired position within the genomic DNA insert. This system has been successfully applied to generate a series of murine BAC transgenes with accurate expression of marker genes in vivo, including *Zipro1* (Yang et al. 1997, 1999), *Calbindin* (X.W. Yang, W. Jiang, and N. Heintz, unpubl.), *Acha9* (Zuo et al. 1999), *Rag1* and *Rag2* (Yu et al. 1999a,b), *Renin-1* (Mullins et al. 2000), *NPY* (DeFalco et al. 2001), *Myf4/Myf5* (Carvajal et al. 2001), P57 (Kip2) (John et al. 2001a), and *Neuronatin* (John et al. 2001b). Several other systems for BAC modification protocols have subsequently been developed, by using transient introduction of *RecE/T* or λ *Red* and *Gam* genes to achieve proper targeting of the desired modification within the BAC (Jessen et al. 1998; Zhang et al. 1998; Imam et al. 2000; Lee et al. 2001).

Here we report a novel RecA-based system for BAC modification that is simple, highly efficient, and suitable for large-scale studies. This system relies on a new shuttle vector containing an *E. coli* replication origin (R6kγ) that can not replicate in the BAC host strain DH10B (Filutowicz and Rakowski 1998), a *RecA* gene to support homologous recombination, and a highly efficient negative selectable marker (*sacB*) to enhance the removal of unwanted vector sequences from the manipulated BAC. By using this new BAC modification method, we have prepared BAC transgenic constructs for a dozen central nervous system (CNS) expressed genes. This new BAC modification method can be readily applied for large-scale studies of gene expression and function in vivo.

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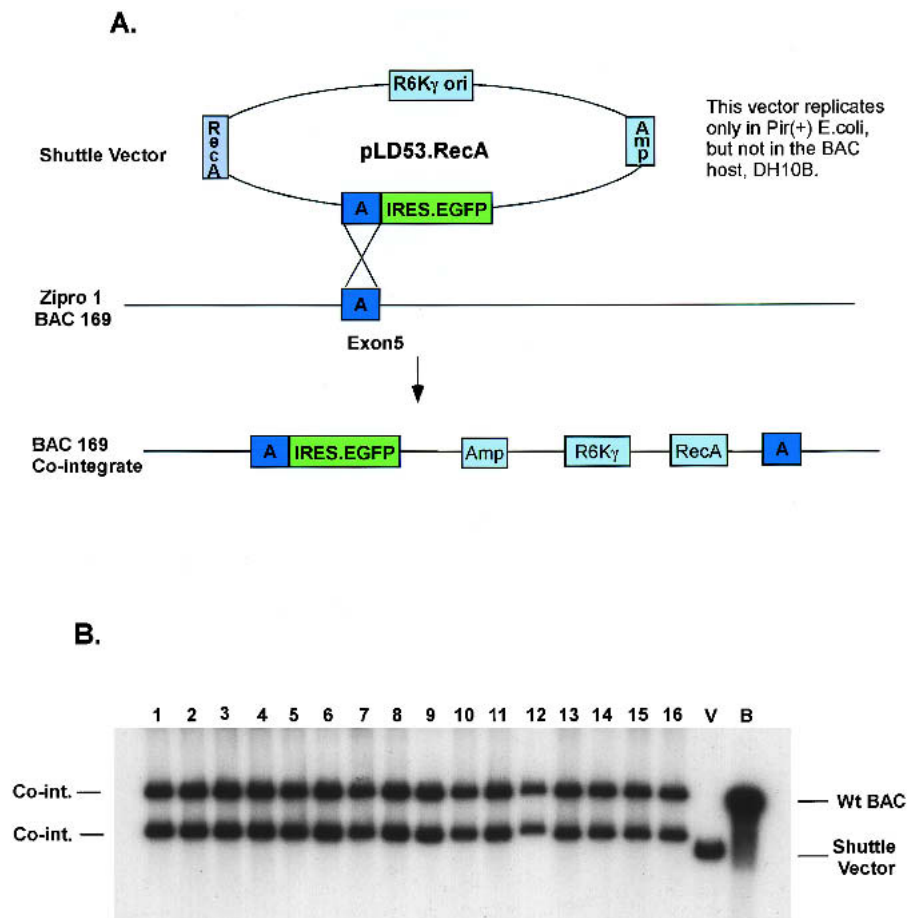**E-MAIL Heintz@rockvax.rockefeller.edu; FAX (212) 327-7878.**

RESULTS

An Improved Shuttle Vector for BAC Modification Based on the R6kγ Origin of Replication

BAC libraries are maintained in the *RecA⁻* bacterial strain (DH10B), which is deficient in homologous recombination (Schizuya et al. 1992). The first step in manipulating BACs involves restoration of the competence for homologous recombination by introduction into the host cell of an enzyme or enzymes that can complement its deficiency for homologous recombination, and selection against freely replicating shuttle vector to identify cells in which the shuttle vector had co-integrated into the BAC by homologous recombination. In our original BAC modification system, the temperature-sensitive pSV1.RecA shuttle vector may replicate in the BAC host cells at a low level to generate a background of false positives, which may reduce the co-integration efficiency (defined as the number of correct co-integrates among the total number of colonies tested).

To search for vectors that are simple to be manipulated and can achieve high efficiency of BAC modification, we tested the pLD53 vector described by Metcalf et al. (1996). This vector contains an R6kγ origin of replication, which is absolutely dependent on the π protein encoded by the *pir* gene for replication (Filutowicz et al. 1998). It can be grown at relatively high copy number in *pir⁺* cells (such as the Pir2 cells from Invitrogen) to obtain sufficient DNA for cloning. Because the BAC host cells are *pir⁻*, this vector can not freely replicate in these cells and therefore could confer very lower background. We hypothesized that a shuttle vector containing the R6kγ origin, the *RecA* gene and a recombination cassette (Fig. 1A), when transformed into BAC host bacteria, may express sufficient RecA protein to support homologous recombination between the shuttle vector and the BAC. And the efficiency with which we recovered correct co-integrates could be relatively high because there should be few background colonies containing free shuttle vectors.

To test this strategy, we constructed a shuttle vector based on pLD53 (Metcalf et al. 1996) that carried the *E. coli RecA* gene, an *IRES.EGFP* marker gene, and a 500-bp homology arm "A" corresponding to the last exon of the *Zipro1* gene (Yang et al. 1999), as shown in Figure 1. The vector was electroporated into a *Zipro1* containing BAC cells, and the transformants were grown in liquid LB containing chloramphenicol (Chlor) and ampicillin (Amp) overnight. The co-integrates were further selected by two additional rounds of liquid growth in Amp and Chlor before plating on LB plates containing these selectable markers. DNA was prepared from 16 individual colonies and analyzed for the presence of the correct co-integrates. As illustrated in Figure 1, all 16 independent colonies contain the correct co-integrates, and the co-integration efficiency in this case was 100%. Furthermore, DNA fingerprinting showed no gross rearrangements or deletions in these modified BACs (data not shown). This result demonstrates that the transient expression of *RecA* from a nonreplicating R6kγ-RecA shuttle vector is sufficient to support homologous recombination in the recombination-deficient BAC host, and that this selection protocol greatly facilitates the identification of the desired co-integrates.

Figure 1 (*A*) Efficient modification of a *Zipro1* BAC by using R6kγ RecA-based shuttle vector. A 500-bp fragment corresponding to the last exon of the *Zipro 1* gene (Yang et al. 1997) and an *IRESEGFP* marker gene were cloned into pLD53-RecA vector. This shuttle vector was then electroporated into *Zipro1* containing BAC169 cells (Yang et al. 1997). Co-integrates were formed via homologous recombination and selected by chloramphenicol and ampicillin. (*B*) Southern blot analysis of the co-integrate BAC clones. Co-integrates BAC DNA were digested with *Spe*I and separated by electrophoresis on a 1% agarose gel. The DNA was transferred to nylon membrane, and the blot was analyzed by using the "A Box" as a probe: co-integrate BAC clones (lanes *1–16*), wild-type BAC (lane *B*), and shuttle vector (lane *V*).

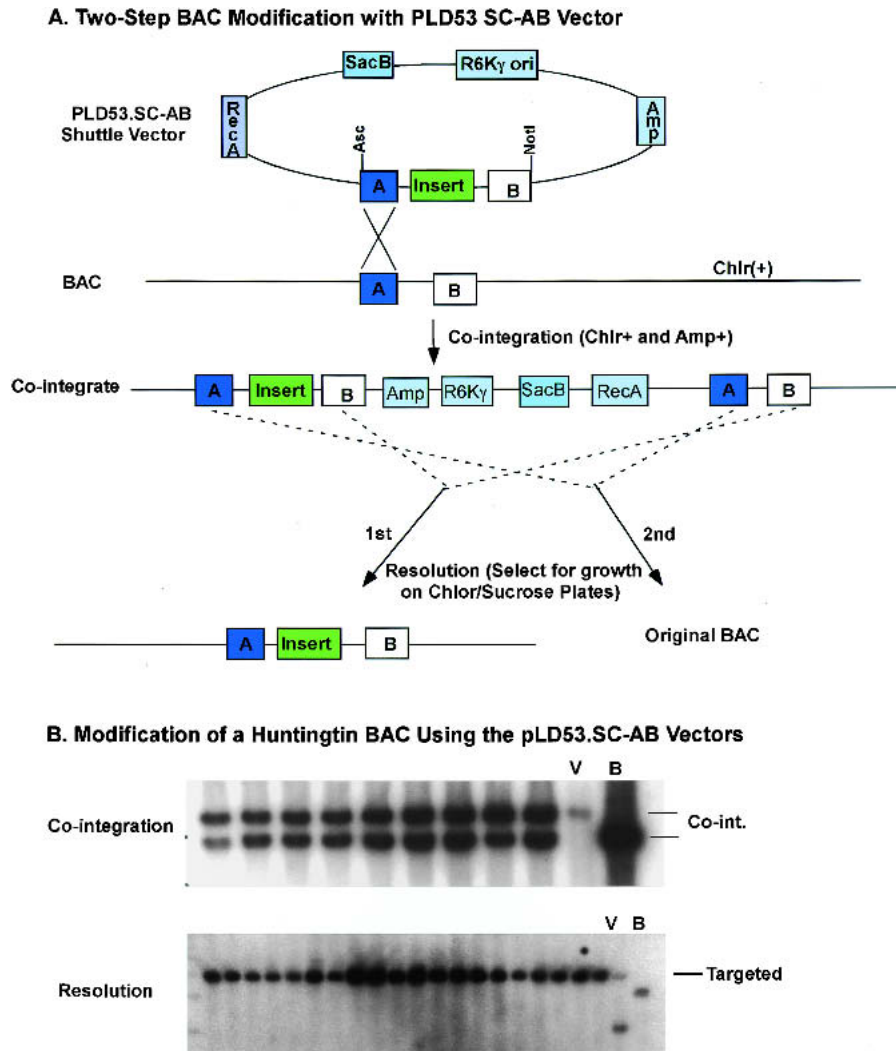### The Use of pLD53.RecA Vectors for the Two-Step BAC Modification

Our original pSV1.RecA-based BAC modification system uses the two steps of homologous recombina-

tion—a co-integration step, which results in complete integration of the shuttle vector into the BAC, and a resolution step, which results in excision and subsequent loss of the shuttle vector and precise placement of intended modification in the chosen site on the BAC (Yang et al. 1997). Such a two-step modification protocol is highly desirable for transgenic studies because it results in precise modification of the BAC without leaving any unwanted sequences behind, unlike the RecET- or λ Red–based modification protocols in which at least an extra LoxP site or an extra FRT site is left behind in the modified BAC (Zhang et al. 1998; Lee et al. 2001). These extra sequences may limit one's option of using Cre or Flp recombinase in further genetic manipulations of these BAC transgenic mice.

In order to perform the two-step BAC modification, we designed the pLD53.SC-AB shuttle vector (Fig. 2A). Besides the *R6Kγ* origin, the *E. coli RecA* gene, and Amp-resistant gene, it also has two unique cloning sites, *Asc*I and *Not*I, for the subcloning of the recombination cassette. As in our original protocol, the recombination cassette has two homology arms of ~500 bp each (termed "A" and "B" homology arms), in between which a modification (i.e., insertion, deletion, point mutation) is to be introduced. The shuttle vector also has *SacB* gene as a negative selection gene that can be used to select for resolved BACs. The *SacB* gene product, levansucrase, converts sucrose to levan, which is highly toxic to the host cells (Gay et al. 1985). Therefore, bacteria containing the *SacB* gene are unable to grow on LB plates containing 5% to 6% sucrose and can be selected against using this simple plating step. In the original pSV1.RecA-based method, the *Tet* gene is used for the negative selection step by growing on fusaric acid plates. In the current study, we found that there are several advantages of the *SacB* gene over the *Tet* gene as a negative selection marker gene. First, the preparation of sucrose/Chlor plates (only two chemicals besides LB agar) is much simpler than the preparation of the fusaric acid plates (~10 chemicals). Second, sucrose appears much less toxic to the bacteria than does fusaric acid; therefore, the selected colonies grew much faster on sucrose . Third, in our experience, the efficiency of negative selection with the *SacB* is better than that of the fusaric acid when using the pLD53 vector system to modify BACs (data not shown).

The outline for the two-step protocol of BAC modification incorporating these features is diagramed in Figure 2A. In the first step, shuttle vector plasmids carrying the recombination cassette ("A" homology arm, a modification, and "B" homology arm) were electroporated into the BAC host, and the correct co-integrates were selected

Figure 2 A schematic representation of the two-step BAC modification system with pLD53.SC-AB vector. (*A*) The pLD53.SC-AB vector is based on the backbone of the plasmid pLD53 (Metcalf et al. 1996). A *Sal*I-*Not*I fragment containing a *SacB* gene and a *RecA* gene was subcloned into the pLD53 vector. This shuttle vector carries a R6kγ origin, an ampicillin-resistant gene, and *Asc*I and *Not*I sites as unique cloning sites for subcloning the recombination cassette, which contains a modification (insertion, deletion, or point mutation) flanked by two homology arms of ~500 bp each. The two BAC modification steps are illustrated: (1) The co-integration of the shuttle vector pLD53.SC-AB into the BAC via homologous recombination through Box A; and (2) the resolution of the co-integrant involves a second homologous recombination event (either through two homology A or two homology B) to eliminate the pLD53 vector and other unnecessary sequences from the co-integrate. The resolved BACs were selected by growth on sucrose owing to the loss of *SacB* gene. (*B*) Southern blot analysis of a Huntington BAC clone modified by two-step BAC modification method. Co-integrates and resolved BAC candidates were first screened by PCR (data not shown). Positive clones were selected, and their DNA was digested with *Eco*RI, separated by electrophoresis on a 1% agarose gel, and transferred to nylon membrane. The blots were analyzed using the "A Box" as probes. Controls used were wild-type BAC DNA (B) and shuttle vector (V).

Table 1. Summary of BAC Modification Efficiency by Using the pLD53.SC-AB and pLD53.SC1 Shuttle Vectors

Gene name	Co-integrate efficiency	Resolution efficiency
Two homology arms (using pLD53.SC-AB vector)		
Ht (BAC866-103Q)	10/20 (50%)	7/26 (27%)
Ht (BAC866-260Q)	9/20 (45%)	17/25 (85%)
Ht (BAC399-260Q)	3/20 (15%)	20/20 (100%)
One homology arm (using pLD53.SC1 vector)		
BF1	9/12 (75%)	10/20 (50%)
Pro-Cadherin	16/17 (94%)	14/17 (82%)
Leap1	4/10 (40%)	20/20 (100%)
RORα	16/16 (100%)	12/14 (86%)
RORβ	20/20 (100%)	15/17 (88%)
Ziprol/Ru49	16/16 (100%)	20/35 (57%)
Smoothen	19/19 (100%)	7/20 (35%)
α Synuclein	11/20 (55%)	9/20 (45%)
Ht (BAC 866-iEGFP)	20/20 (100%)	11/20 (55%)
Ht (BAC 399-iEGFP)	20/20 (100%)	3/40 (7.5%)

by growth in Chlor and Amp. The co-integrates, through either A or B homology arms, were identified through simple colony PCR, and the positive clones were confirmed by Southern blot analysis. In the second step, resolved clones were selected by direct plating on LB plates with 6% sucrose and Chlor. Colonies growing on these plates were tested by colony PCR, and the positive clones were confirmed by Southern blot analysis. The modified BACs were fingerprinted by two or three different restriction digestions and were compared with that of the wild-type BACs to ensure that there was no rearrangement or deletion.

Another optional screening step can be incorporated into the protocol to maximize the recovery of the correctly resolved BACs. Because resolved BACs have lost the shuttle vector carrying the *SacB*, *RecA*, and *Amp* genes, higher resolution efficiency can be achieved by adding a screen for the loss of the *RecA* gene and/or the loss of the *Amp* gene. The loss of *RecA* gene confers resistance to UV light (Rupp et al. 1971). Those colonies that are UV sensitive (*RecA⁻*) and/or Amp sensitive are chosen from the master plate for further analysis. This simple added screen improves the recovery of the desired resolved products to >80% in the majority of cases.

By using the pLD53 SC-AB plasmid, we have modified two different human BACs containing the 170-kb Huntingtin gene. In three separate modifications, we have inserted two different disease-causing mutations, one encoding 103 glutamine repeats and another encoding 260 glutamine repeats into the Huntingtin BACs. In all cases, the co-integration efficiency was between 15% and 50%, and the resolution efficiency was between 27% and 100% (Table 1; Fig. 2B).

The Use of a "Built-In" Resolution Cassette for Two-Step BAC Modification

To further simplify the shuttle vector cloning process, we next developed an improved strategy for shuttle vector preparation based on a "built-in" resolution cassette. The logic for this design was that if we provide two copies of the marker gene cassette (i.e., IRES.EGFP or EGFP alone) properly positioned in the shuttle vector, those duplicate copies can be used for highly efficient removal of the vector sequences during the resolution step of BAC modification (Fig. 3). This design has

two obvious advantages. First, provision of this built-in resolution cassette obviates the need to clone separate homology arms "A" and "B" into the shuttle vector to provide independent sites for the co-integration and resolution steps of the BAC modification protocols. In this case, a simple cloning step involving ligation of a PCR-amplified "A" box into the precut pLD53.SC1 shuttle vector is sufficient to produce the targeting construct. In our hands, this simple cloning step was achieved at ~100% efficiency (data not shown). Second, because the recombination efficiency positively correlates with the homology length, the desired resolution through the built-in recombination cassettes (in this case two 1.4-kb IRES.EGFP sequences) is much more efficient than that through the "A" homology arms (~0.5 kb). The fact that resolution occurs through the same cassette in all constructs ensures uniformly high efficiencies of resolution in nearly all experiments. One consequence of the built-in resolution strategy is that the final resolved product contains a duplicated "A" homology adjacent to the polyA sequence in the resolved BAC (Fig. 3). We believe such small duplication will not affect the transgene expression, because it is a small endogenous sequence and is not transcribed.

The built-in resolution strategy also results in very high co-integration and resolution efficiency. We have modified six different CNS-expressed genes (*BF1*, *pro-Cadherin*, *Leap1*, *RORa*, *RORb*, *Zipro1*). As shown in Figure 4 and Table 1, the co-integration efficiency in these experiments was between 40% to 100% (average, 84%; median, 94%), and the resolution efficiency using the built-in *iEGFP* marker genes was between 50% to 100% efficient (average, 77%; median, 82%). In summary, the use of built-in resolution cassette with the PLD53.RecA shuttle vectors has greatly simplified the shuttle vector construction and maintained high co-integration and resolution efficiency.

DISCUSSION

BACs are important large insert vectors used widely in positional cloning studies (Shizuya et al. 1992; Osoegawa et al. 2000). The ability to modify BACs for transgenic studies enables BACs to be used increasingly in functional studies (Heintz 2001). Although several different methodologies are now available for manipulating BACs by homologous recombination, the current study represents a significant improvement on the simplicity and efficiency of the RecA-based BAC modification system. A major finding of the current study is that the R6kγ-based shuttle vectors, although they can not replicate in the BAC host bacteria, can express sufficient RecA protein to support efficient co-integration of the shuttle vector.

Several features of the current R6kγ vector-based shuttle vectors enable the significant improvement in the RecA-based BAC modification process. First, these plasmids can readily be manipulated in pir(+) bacteria in terms of cloning and DNA preparation. Second, because the nonintegrated shuttle vec-

tor plasmids could not replicate at all in the BAC host, they are lost automatically. This obviates the need for temperature shifting in the original modification system. Third, the *SacB* gene as a negative selection marker gene also has advantages over the *Tet* gene: It significantly simplifies the plate preparation step (much fewer ingredients in the sucrose plates than in the fusaric acid plates) and shortens the incubation time from 3 d to overnight. Fourth, the use of UV and/or Amp selection can further increase the resolution efficiency. Finally, the current system enables one to perform colony PCR to identify with high reliability both the co-integrates or resolved BACs. Such colony PCR with the pSV1.RecA system may produce false positives owing to the low persistence of the free shuttle vectors. Therefore, in the current protocol, no DNA preparation is necessary during the modification process. Overall, the current BAC modification system greatly shortens the total time needed for each modification cycle, and one may be able to modify multiple BACs during each cycle. Our data documented that a single investigator can easily use the built-in resolution cassette system to manipulate up to six different BACs simultaneously in each cycle, and each cycle usually takes ~2 wk to complete.

A highly efficient method for BAC modification allows one to fully explore the advantage of the BAC system for functional studies in the postgenomic era (Magdaleno et al. 1999; Heintz 2000). It is already clear that preparation of BAC transgenic mice can provide an important complement to other studies of gene expression and function such as gene targeting (Magdaleno et al. 1999; Heintz 2000). With the completion of the human genome project and a variety of other genome projects (Adams et al. 2000; Arabidopsis Genome Initiative 2000; Lander et al 2001; Venter et al. 2001), and the increasing ease of identification of appropriate BACs from the existing databases (Mozo et al. 1999; Hoskins et al. 2000; Osoegawa et al. 2000; McPherson et al. 2001; Tao et al. 2001), we believe that the highly efficient BAC modification system presented here can be applied to in vivo large-scale BAC-based studies of gene expression and function.
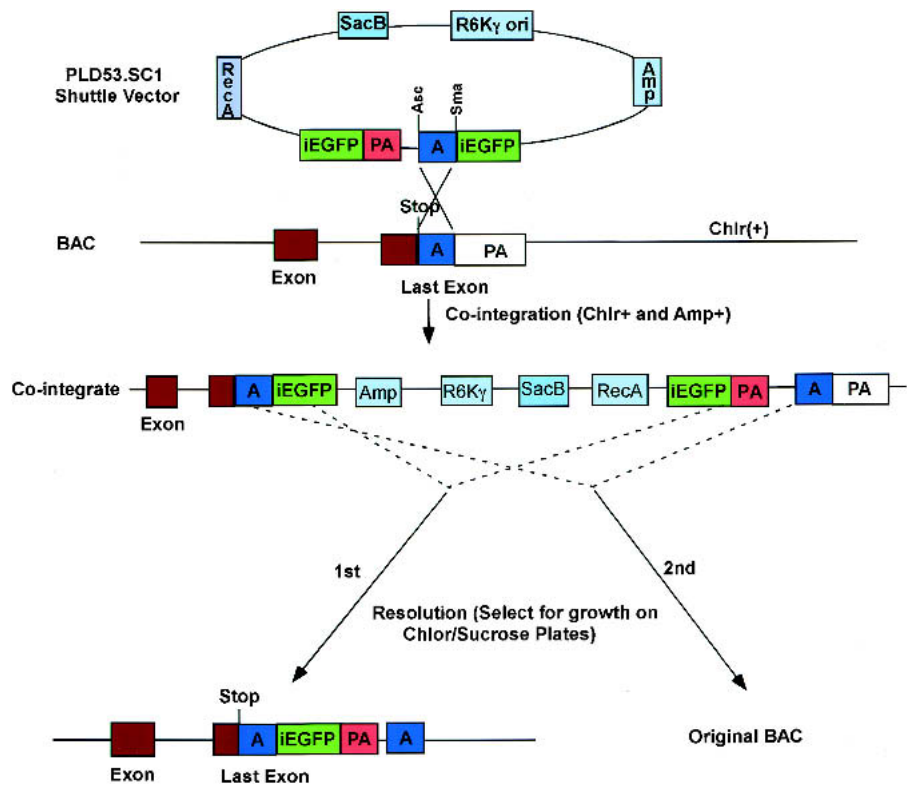
METHODS

Genes Used for BAC Modification

The accession nos. for genes used for modification are listed below: Zipro1/RU49, U41671; BF1, U36760; Pro-cadherin, NM_007767; Leap1, AF220501; ROR α, NM_013646; ROR β, NM_006914; Smoothen, AF089721; Huntingtin, NM002111; and α-synuclein, NM007308. BACs were screened either with a PCR probe from the CITB mouse BAC library or with the RPCI-23 library

(purchased from Research Genetics) by using standard protocol. Huntingtin and α-synuclein BACs were screened from the RPCI-11 human BAC libraries.
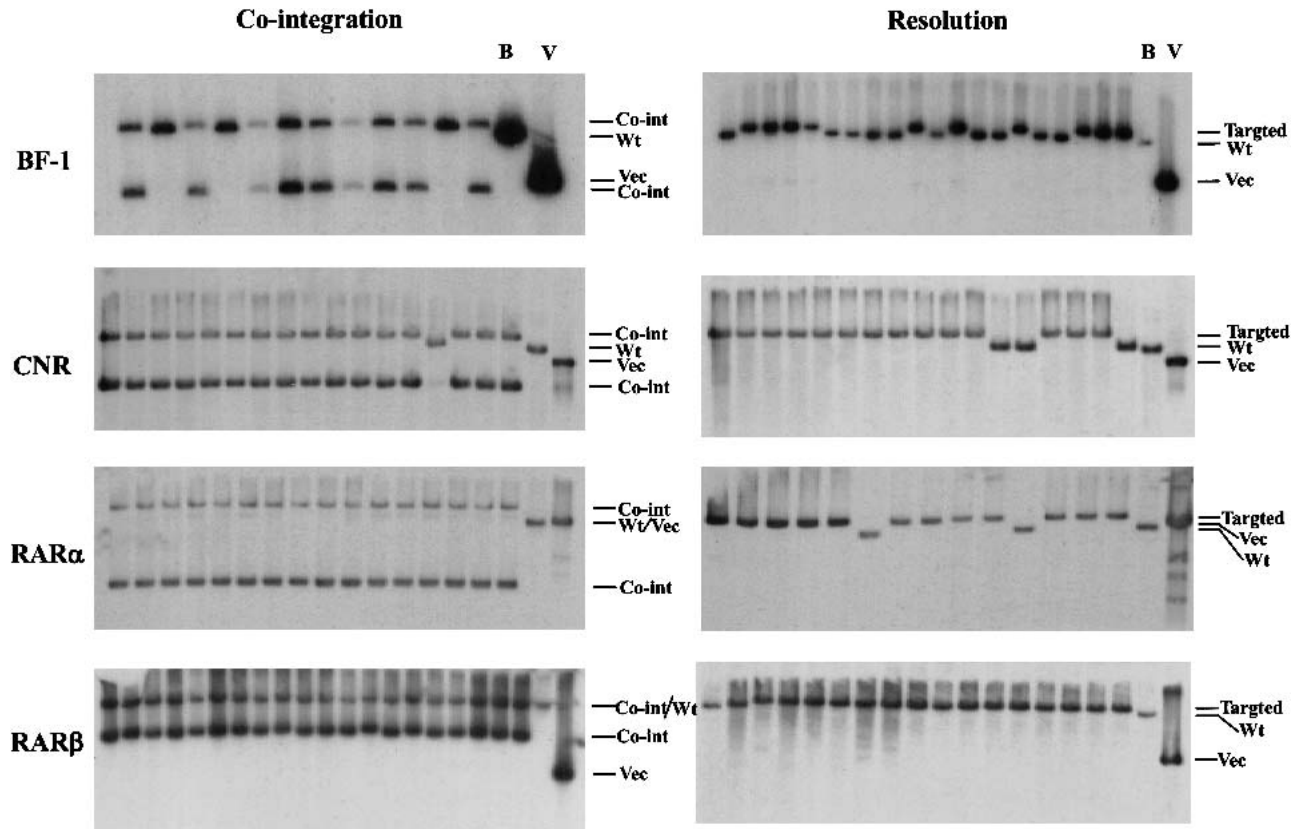
Construction of the Vectors

The plasmids (pLD53.SC-AB and pLD53 SC1) are derived from the pLD53 (Metcalf et al. 1996). pLD53 was digested with *Bam*HI and *Sac*I to replace the *tetAR* and *oriT* origin with a *Not*I-*Sal*I-*Spe*I adaptor. The building vector was based on the pBluescript SK vector (Stratagene). A 2.3-kb *Sal*I-*Eco*RV fragment containing the *SacB* gene from *Bacillus subtilis* was cloned into *Sal*I and *Eco*RV sites of the pBS.SK vector. A 1.7-kb *Bam*HI fragment containing *RecA* from pSV1.RecA (Yang et al. 1997) was subcloned into *Bam*HI site. For pLD53.SC-AB shuttle vector, this cassette was then cloned into *Not*I-*Sal*I sites of the modified pLD53 vector. Besides the R6Kγ origin, the *E. coli RecA* gene, a *SacB* gene, and an Amp-resistant gene, this vector also has two unique cloning sites, *Asc*I and *Not*I, for the subcloning of the "A" and "B" homology arms. For pLD53-SC1, besides the *SacB* gene and *RecA* gene, a *Spe*I fragment of *IRESEGFP* was further cloned into *Spe*I site of pBluescript SK, and a second *IRESEGFP* was cloned into *Asc*I site located upstream of the first *IRESEGFP*; the 5′ end *Asc*I site was knocked out. A PGK.poly A fragment with 3′ end *Asc*I-*Not*I-*Swa*I-*Sma*I multiple cloning site was then cloned into *Asc*I and *Sma*I sites located upstream of the first *IRESEGFP*. The whole

Figure 3 A schematic representation of the two-step BAC modification system with pLD53.SC1 vector. This vector is based on the backbone of the plasmid pLD53 (Metcalf et al. 1996). A *Sal*I-*Not*I fragment containing a *SacB* gene, a *RecA* gene, two *IRESEGFP* genes, and multiple cloning sites was subcloned into a mutated pLD53. This shuttle vector carries a R6kγ origin, an ampicillin-resistant gene, and *Asc*I and *Sma*I as unique cloning sites for subcloning of Box A. The two BAC modification steps are illustrated: (1) The co-integration of the shuttle vector pLD53.SC1 into the BAC via homologous recombination through Box A, and (2) the resolution of the co-integrant involves a second homologous recombination event (either through two homology A or two IRESEGFP) to eliminate the pLD53 vector and other unnecessary sequences from the co-integrate. The resolved BACs are selected by growth on sucrose owing to the loss of *SacB* gene.

Figure 4 Southern blot analysis of clones modified by the pLD53.SC1 shuttle vector with the built-in resolution strategy. Co-integrates and resolved BAC DNA were digested with *Spe*I, separated by electrophoresis on a 1% agarose gel, and transferred to nylon membrane. The blots were analyzed using the "A Box" as probes. Controls used were wild-type BAC DNA (lane *B*) and shuttle vector (lane *V*).

recombination cassette was then cloned into *Not*I-*Sal*I sites of the modified pLD53 vector. This shuttle vector was digested with *Asc*/*Sma*1 to subclone the 300- to 500-bp "A-box" fragment, which was PCR amplified and digested with *Asc*I before cloning.

Preparation of the Competent Cells for Electroporation and Selection for Co-integrates

Overnight culture of BAC host bacterial was diluted 1000-fold in 100 mL of LB medium supplemented with Chlor (12.5µg/mL), grown to an OD_{600} of 0.7–0.8, and chilled on ice for 15 min. Bacteria were collected by centrifugation at 3000 rpm for 10 min at 4°C and resuspended in equal volume of ice-cold 10% glycerol. This step was repeated twice, and the bacteria pellet was resuspended in 400 µL of ice-cold 10% glycerol. Aliquots were stored in −80°C. Before the electroporation, cells were thawed on ice, and 2µL of DNA (0.5µg/µL) was added. Electroporation was performed using BioRad Gene Pulser II and ice-cold cuvettes as follows: 40 µL of competent cells were mixed with 2 µL of plasmid DNA (0.5µg/mL) on ice for 1 min. DNA and cells were transferred to a cold 0.1-cm cuvette. Electroporation conditions were 25 µF, 1.8 kV, and 200 Ω. After electroporation, 1 mL of SOC was immediately added to the cuvette, and the contents were then transferred to a 17 × 100-mm polypropylene tube. After recovery for 1 h at 37°C with shaking, the cells were selected in LB medium containing proper antibiotics for overnight. Then the culture was diluted 1:1000 and incubated for ~14 h at 37°C. This culture was further diluted 1:5000 and incubated for ~8 h at 37°C. Then, cells were diluted and spread onto LB plates con-

taining Chlor (12.5 µg/mL) and Amp (50 µg/mL). Individual colonies were picked to analyze for presence of co-integrates.

Identification and Characterization of Co-integrates and Resolved BACs

First, the co-integrates were identified by double selection for Chlor (12.5 µg/mL) and Amp (50 µg/mL). Selected colonies were picked, grown in 3 mL of LB containing Chlor and Amp and were characterized by PCR or Southern blot analysis. DNA was prepared and digested with *Spe*I. Correct co-integrates were identified by Southern blot by using "A box" as a hybridization probe.

Identification and Characterization of Resolved BACs

Once the co-integrates were identified, two positive colonies were picked from the Chlor/Amp plates, inoculated with 5mL of LB supplemented with Chlor (12.5µg/mL) and 5%–6% sucrose, and incubated for 8 h at 37°C for CITB-BACs and for 1 h for RPCI23-BACs. Culture was diluted 1:5000, spread onto LB plates containing Chlor (12.5µg/mL) and 5%–6% sucrose, and incubated overnight at 37°C.

To enhance the resolution efficiency, exposure to UV light may be used as a selection step. Colonies were picked and plated on two agar plates: The master plate was incubated at 37°C directly; the other plate was exposed with UV light for 30 sec and incubated at 37°C. UV-sensitive colonies (hence, RecA⁻) were picked from the master plate and inoculated with 3 mL of LB supplemented with Chlor (12.5µg/mL) only. The resolved BACs were screened by PCR or Southern blot.

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