A new vector for recombination-based cloning of large DNA fragments from yeast artificial chromosomes

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ABSTRACT

The functional analysis of genes frequently requires manipulation of large genomic regions embedded in yeast artificial chromosomes (YACs). We have designed a yeast–bacteria shuttle vector, pClasper, that can be used to clone specific regions of interest from YACs by homologous recombination. The important feature of pClasper is the presence of the mini-F factor replicon. This leads to a significant increase in the size of the plasmid inserts that can be maintained in bacteria after cloning by homologous recombination in yeast. The utility of this vector lies in its ability to maintain large fragments in bacteria and yeast, allowing for mutagenesis in yeast and simplified preparation of plasmid DNA in bacteria. Using PCR-generated recombinogenic fragments in pClasper we cloned a 27 kb region from a YAC containing the *Hoxc* cluster and a 130 kb region containing the entire *Hoxb* cluster. No rearrangements were seen when the recombinants in the shuttle vector were transferred to bacteria. We outline the potential uses of pClasper for functional studies of large genomic regions by transgenic and other analyses.

INTRODUCTION

For the functional analysis of many genes investigators need to isolate and manipulate large DNA fragments embedded in YACs. Researchers use fragmentation techniques to narrow down the region of interest in YACs (1,2). However, isolating sufficient quantities of YAC DNA from agarose gels for microinjection or electroporation remains cumbersome. Purification remains a problem when the YAC co-migrates with an endogenous chromosome. In addition, YACs may be chimeric or contain additional DNA regions that are not required for the particular functional study.

We have developed a new yeast–bacteria shuttle vector that allows investigators with experience in standard molecular biology techniques to selectively clone large regions from within a YAC. The resultant circular product can be transferred to bacteria for simplified preparation of large quantities of plasmid DNA. The shuttle vector makes mutagenesis techniques by yeast genetics accessible for the production of large reporter constructs.

The new vector, pClasper, combines the single-copy F factor replicon and chloramphenicol resistance gene for stable propagation of large circular DNA in bacteria with the *CEN6/ARS4* origin of replication and *LEU2* gene for maintenance and selection in yeast. The bacterial origin of replication we have chosen is based on that used in bacterial artificial chromosomes (BACs), previously shown to stably maintain at least 300 kb inserts (3). We have engineered 18 bp recognition sites for two intron-encoded endonucleases flanking a unique polylinker. We have used this vector to clone regions of 27 and 130 kb from YACs containing the mouse homeobox clusters, *Hoxc* and *Hoxb*.

MATERIALS AND METHODS

Construction of the shuttle vector

The vector pClasper (Fig. 1) was constructed as follows. The *Pml*I site in pRS415 (Stratagene) was cleaved to insert a linker with a *Sac*I site flanked on one side with a 10 bp reduplication of centromeric DNA from chromosome VI (*CEN6*) and with a random sequence on the other side to ascertain the orientation by PCR after cloning. The resultant plasmid was cleaved at the *Bam*HI site in the polylinker of pRS415 and at a single *Dra*III site between the polylinker and the β -isopropylmalate dehydrogenase gene (*LEU2*) to insert a 100 bp synthetic DNA fragment containing part of a newly designed polylinker. The new plasmid was digested with *Sac*I and *Bam*HI to isolate the *CEN6/ARS4* origin of replication, the *LEU2* gene and one half of the new polylinker. Plasmid pBCSK (Stratagene) was used as a substrate to generate by PCR a 1103 bp fragment encompassing the chloramphenicol acetyltransferase (*Cm^r*) gene from Tn9. The PCR primer for the 5'-end of the gene corresponded to the sequence 1901–1921 from pBCSK and contained a 40 bp linker with a *Sac*I restriction site, a *Bam*HI site and another fragment of the new polylinker. The downstream PCR primer corresponded to coordinates 2881–3001 of pBCSK and contained a linker with 20 bp of the 5'-end of the region of the mini-F gene between a *Bst*NI site in position 44.2 and a *Nsp*I site in position 44.3 of the coordinate map of the F plasmid (4). In a second round of PCR with 80 bp primers containing *Nsp*I sites at their 5'-end, the ends of the initial PCR product were extended to provide at the 5'-end of the *Cm^r* gene the other half of the new polylinker and at the 3'-end the full 100 bp *Bst*NI–*Nsp*I fragment necessary for stability

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Figure 1. Schematic representation of the pClasper vector. Relative positions of the yeast *LEU2* gene and *CEN6/ARS4* sequences and the bacterial chloramphenicol resistance (*Cm^r*) gene and mini-F factor origin of replications are shown. Unique restriction enzyme sites in the polylinker are indicated above. Unfilled lines are sequences carried from the parental vectors from which the above mentioned genes were cloned as described in Materials and Methods.

of the final mini-F fragment (4). A 5.3 kb *NspI*–*NspI* fragment was isolated from the pOF216 plasmid (5; ATCC). This fragment encompasses the mini-F region from position 44.3 to 49.4 and was ligated to the final *Cm^r* PCR product digested with *NspI*. To create pClasper the resultant plasmid was cleaved with *Bam*HI and *Sac*I and ligated to the *Bam*HI–*Sac*I *CEN6/ARS4*–*LEU2*–polylinker fragment derived from pRS415. pClasper is maintained in *Escherichia coli* strain DH10B (Gibco BRL).

Construction of targeting vectors

The YACs to be targeted were obtained from the Princeton mouse genomic YAC library (6). Isolation and characterization of the *Hoxb* and *Hoxc* cluster YACs have been previously described (7,8). The targeting vector designed to clone the *Hoxc*-8 gene, pCIC9C6, was made as follows. PCR primers for the *Hoxc*-6 gene (9) were designed to the 5' untranslated region of the gene. The 5' primer was 5'-TAGATCTGTTTGTCTCCCACATGCC-3' and contained a *Nru*I linker. The 3' primer was 5'-AGGTGGCAG-GATAAGGAAGGGTTAG-3' and contained a *Hind*III linker. The product was 706 bp. The PCR primers for the *Hoxc*-9 gene (10) were designed to amplify a 624 bp fragment of the 3' untranslated region. The 5' primer was 5'-CGACAAGGAAC-AAATCCTAAGCCC-3' and contained a *Bam*HI linker. The 3' primer was 5'-TGCATTTCAGCCTGATCCAGCCA-3' and contained a *Nru*I linker. All polymerase chain reactions (PCR) were carried out in a Hybaid Omnigene machine. Reactions contained 1× Boehringer Mannheim PCR buffer supplemented with 2.5 mM MgCl₂, 0.8 mM dNTPs and 0.5 U Taq polymerase (Boehringer Mannheim). The *Hoxc* YAC was used as template. Reaction conditions were 94°C 30 s, 55°C 30 s and 72°C 45 s for 30 cycles. PCR products were cloned into the T-tailed vector pCRII (Invitrogen Corporation). The *Hoxc*-9 *Bam*HI–*Nru*I PCR fragment was excised and ligated head-to-tail at the *Nru*I site of

the cloned *Hoxc*-6 PCR fragment. The 1.4 kb *Bam*HI–*Hind*III fragment containing the two PCR products was isolated and ligated to pClasper digested with the same enzymes to create pCIC9C6.

The targeting vector designed to clone the entire insert from a *Hoxb* cluster YAC, pCIYA, was made by overlap PCR as follows. PCR primers were designed to the YAC arms of pYAC4 (11). One set of primers were designed to amplify a region of 484 bp upstream of the single *Eco*RI site of pYAC4. The 5' primer was 5'-TCTCATGTGTTGACAGCTTATCA-3' (positions 1–23 of pYAC4 in GenBank, accession no. U01086) and contained a *Hind*III linker. The 3' primer was 5'-AGAGTATACTACATAAC-ATAACACA-3' (positions 460–484) and contained an *Nru*I linker and a 15 bp overlap with the 5' primer for the downstream pYAC4 sequence. The second set of primers were designed to amplify 494 bp of pYAC4 downstream of the *Eco*RI site. The 5' primer was 5'-TTCAAGGGAATTGATCCTCTACG-3' (positions 656–678) and contains an *Nru*I linker and 15 bp of sequence designed to overlap with the 3' upstream primer described above. The 3' primer was 5'-AAGATTCCGAATACCGCAAGC-3' (positions 1130–1150) and contained a *Bam*HI linker. The *Hoxb* YAC was used as template in PCR conditions which were as described above with the following modifications. After 10 cycles part of each reaction was diluted 1:10 and 1 µl of each was added to a new reaction containing primers for the 5' upstream and 3' downstream primers. PCR was continued for an additional 25 cycles. The 975 bp product containing both PCR fragments with an *Nru*I site in between was cloned into pCRII and subsequently digested with *Bam*HI and *Hind*III and ligated to pClasper to create pCIYA.

Preparation of DNA for yeast transformations

Since pClasper is maintained at single copy, the following procedures will facilitate the preparation of large quantities of plasmid DNA. *Escherichia coli* strain DH10B cells containing the targeting vectors were grown overnight in 1–2 l TB medium containing 20 µg/ml chloramphenicol. Each 1 l culture was divided into three centrifuge bottles to pellet. The Plasmid Maxi Protocol for Qiagen-tip 500 (Qiagen) was followed for each pellet with the following changes. After the addition of 10 ml each of buffers P1, P2 and P3 to each pellet the suspension was centrifuged at 4°C for 30 min at 15 000 g. The supernatant was removed, then re-centrifuged under the same conditions. The supernatants were pooled, passed through a 0.45 µm filter and loaded on a single Qiagen-tip 500 column following the supplier's directions. We generally obtained at least 20 µg vector DNA/cells, as determined by comparing both optical density and visual estimation on an agarose gel. The targeting vectors, pCIC9C6 and pCIYA, were both linearized for transformation by extensive overdigestion with *Nru*I.

Yeast strain construction

Saccharomyces cerevisiae strain AB1380 (MAT_a, Y⁺, *ura3*-52, *trp1*, *can1*-100, *lys2*-1, *his5*, *ade2*-1) carrying a YAC containing either the mouse *Hoxc* (440 kb) or *Hoxb* (150 kb) cluster was crossed with strain Y724 (MAT_α, *ura3*-52, *lys2*-801, *ade2*-101, *leu2*-Δ98, *trp1*Δ, *his3* Δ200, *cyh*^r, *can1*^r; M.Snyder, personal communication). Diploids were selected by plating on minimal media lacking leucine, histidine and tryptophan. Spores were isolated either by tetrad dissection or hydrophobicity (12) and

selected for *Leu*⁻, *Ura*⁺ and *Trp*⁺ phenotypes. The integrity of the YAC in the isolated spores was assessed by pulse field gel electrophoresis (PFGE) and Southern blotting. The resultant strains were YLC44-5 containing the *Hoxc* YAC and YLB15-20 containing the *Hoxb* YAC.

Yeast transformations

Linearized pCIC9C6 (7.5 µg) and pCIYA (4 µg) were transformed into strains YLC44-5 and YLB15-20, respectively, using a one-step LiAc transformation protocol (13) as follows. The yeast cells were grown overnight in 2 ml AHC medium to log phase. A sample of 1 ml cells was pelleted in a microcentrifuge. To the cell pellet was added 10 µl 10 mg/ml salmon sperm DNA sheared to an average size of 7 kb and denatured (14). To this was added 10 µl 1 M DTT and 1–10 µg linear targeting vector and the cell pellet was resuspended. One-Step buffer (100 µl, 0.2 N LiAc, 40% PEG 4000, pH 5.0) was added and cells were incubated at 45°C for 30 min. Sterile water (600 µl) was added and the cells were spread on drop-out medium plates lacking leucine.

Analysis of recombinants by PCR

Whole-cell PCR was performed on yeast colonies to identify recombinants. Individual yeast colonies were first replica plated by streaking in patches onto drop-out medium plates lacking leucine. A generous portion of each replica patch was resuspended in 100 µl sterile water. An aliquot of 10 µl resuspended yeast was used as template. The primers used to detect recombinants for pCIC9C6 were as follows. (i) A primer pair in which one primer is specific for the *LEU2* region of pClasper, 42 bp proximal to the start of the polylinker, and the second is specific to the *Hoxc*-6 5' untranslated DNA in the insert. Vector primer 5'-TTAAAGAACGTG-GACTCCAACG-3'; *Hoxc*-6 primer 5'-ACTGTGCTCT-GCAGTCTCATCCG-3'. (ii) Primers specific for the second exon of *Hoxc*-8. 5' primer 5'-CGCAGCGGTGACAACTTACA-3'; 3' primer 5'-CTCCTCTCTTTCTCCTCTTCTCCTA-3'. The primers used to detect recombinants for pCIYA were as follows. (i) The vector primer described above with a primer specific for pYAC4 in the insert. pYAC4 primer 5'-CAACTTGGCTAC-CGAGAGTA-3' (positions 501–520 in pYAC4 in GenBank). (ii) Primers specific for the *Hox*-2.9 gene (*Hoxb*-1; 7). PCR conditions were as described above except that a 5 min 94°C cycle was added prior to the amplification cycles to lyse the yeast cells and 45 amplification cycles were used. When total yeast DNA or bacterial minipreps were used as template PCR conditions were as described in the construction of the vector.

Electroporation of bacteria

Yeast carrying the recombined circular product from the pCIC9C6 transformation were grown overnight in liquid drop-out medium lacking leucine and total DNA was prepared by standard procedures (15). An aliquot of 0.5 µg total yeast DNA was mixed with 40 µl *E. coli* strain DH10B cells (Gibco BRL) and then transferred to an ice-cold 0.2 cm gapped cuvette. A BioRad Gene Pulser was used for electroporation with the settings 2.5 kV, 200 Ω, 25 µF. After electroporation 1 ml SOC medium was immediately added to the cells, which were then grown for 1 h at 37°C with shaking. Colonies were selected on plates containing 12.5 µg/ml chloramphenicol.

For the larger recombination product from the pCIYA transformation (130 kb) yeast DNA was prepared in 100 µl agarose plugs (16). Three plugs containing ~10 µg DNA each were digested with Gelase (Epicentre Technologies) and dialyzed overnight against 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. An aliquot of 5 µl dialyzed DNA was used for electroporation.

Field inversion gel electrophoresis (FIGE) and Southern analysis

Yeast DNA was prepared in agarose plugs as previously described (16). Bacterial DNA was prepared by standard alkaline lysis (17) except that DNA was gently resuspended to avoid shearing the DNA. Restriction enzyme-digested samples were run at 200 V for 18 h at 10°C with a 1–6 s pulse time and a 2:1 forward/reverse ratio using a Switchback Pulse Controller (Hoefer Scientific Instruments). For standard agarose gels yeast DNA was prepared by standard genomic DNA preparation protocols (15). Following electrophoresis the DNA was nicked by exposure to 254 nm UV light, denatured, neutralized and transferred to BAS NC supported nitrocellulose (Schleicher & Schuell Inc.).

The probe used for hybridization of *Hoxc* YAC-derived DNA was a 272 bp PCR product from the second exon of *Hoxc*-8. The probe used for hybridization of *Hoxb* YAC-derived DNA was a 250 bp PCR product from the first exon of *Hoxb*-1. Both probes were radiolabeled using the Megaprime random priming kit (Amersham). Hybridizations were done at a probe concentration of 10⁶ c.p.m./ml hybridization buffer (40% formamide, 4× Denhardt's, 4× SSC, 10% dextran sulfate, 0.1% SDS).

RESULTS

Outline of recombination-based cloning strategy

Figure 2 illustrates how a yeast–bacteria shuttle vector can be used to isolate specific regions from YACs. The procedure is an extension of yeast gap repair, where free ends of a linear vector recombine with homologous sequences in a chromosome (18). The result of recombination is a circular product with a copy of the sequences from the chromosome maintained between the recombinogenic ends. To clone specific regions from within a YAC we insert into the shuttle vector PCR-generated fragments homologous to sequences flanking the region to be cloned. The vector is linearized between the inserted fragments to create recombinogenic free ends. The linear vector is transformed into yeast carrying the YAC in the appropriate genetic background. The circular recombination product is selected for by acquisition of the *LEU2* gene carried on the shuttle vector. The specific recombination event is identified by PCR. The recombinant is then shuttled to bacteria for preparation and analysis of plasmid DNA.

Construction of a shuttle vector for isolation of specific YAC sequences by homologous recombination in yeast

We designed a new yeast–bacteria shuttle vector using as the bacterial origin of replication the F factor genes for replication and copy number control *oriS*, *repE*, *parA* and *parB*. This is the minimal region, previously referred to as the mini-F factor, required for stable maintenance of single-copy plasmids (19). The same regions of the F factor are used in bacterial artificial chromosomes (BACs; 3), which have been shown to maintain exogenous DNA inserts of up to 300 kb in bacteria. To this we added the *Cm^r* gene and the yeast *CEN6/ARS4* origin of replication and *LEU2* gene for

Figure 2. Schematic representation of homologous recombination between pClasper and a YAC. (A) The YAC (hatched lines) containing hypothetical gene X and two known sequences Y and Z (black boxes) recombines with linearized pClasper (unfilled lines) at the recombination ends (gray boxes) in the vector. Y and Z can be either sequences in the YAC flanking the region of interest or can be sequences in the YAC arms. (B) The result of homologous recombination is a circular product containing the homologous sequences Y and Z and gene X.

selection in yeast. We also added an extensive polylinker flanked by recognition sites for the intron-encoded endonucleases, *I-PpoI* and *I-SceI*. These endonucleases have 18 bp recognition sites and are therefore useful for removing vector sequence from very large inserts. The *I-SceI* recognition sites are internal to the *I-PpoI* sites and can be used to analyze the integrity of the transgene in mouse genomic DNA. The resultant vector, pClasper, is diagrammed in Figure 1.

The mitotic and structural stability of the new vector was found to be equivalent to the parental yeast vector, pRS415 (20), and to a similar bacterial vector, pBAC108L (3). This was determined by comparing the relative loss of the plasmid in yeast and bacteria in non-selective media (data not shown).

Construction of specific targeting vectors

To isolate regions from within a YAC, homologous sequences flanking the region to be targeted are cloned into the polylinker of pClasper with a unique restriction site in between for linearization of the vector. We generated these recombination ends by PCR using the YAC to be targeted as template. Two targeting vectors were generated with recombination ends of ~500 bp each (Fig. 3). The first was designed to isolate the *Hoxc-8* gene from a 440 kb YAC containing the entire *Hoxc* cluster (8). Recombination ends

440 kb *Hoxc* cluster YAC

150 kb *Hoxb* cluster YAC

Figure 3. Schematic representation of *Hox* cluster YACs. White boxes indicate genes contained on the YACs. Black rectangles represent YAC arms. Both *Hox* clusters are shown in a 3'→5' orientation as indicated for *Hoxc*. All *Hox* genes are transcribed in the same direction. Arrow heads indicate approximate location and orientation of homologous recombination ends. Recombination ends are 500 bp each, generated by PCR and cloned head-to-tail into pClasper to make the specific targeting vectors.

were designed to be homologous to genomic regions adjacent to the two genes flanking *Hoxc-8*: *Hoxc-9* and *Hoxc-6*. The targeting vector designed to isolate *Hoxc-8*, pCIC9C6, contained PCR-generated ends homologous to the 3' untranslated region of *Hoxc-9* and to the 5' untranslated region of *Hoxc-6* (see Materials and Methods).

The second targeting vector was designed to contain recombination sequences homologous to the arms of pYAC4 (11; Fig. 3). With this vector the entire insert of any pYAC4-based YAC could be circularized into pClasper. We used this targeting vector, pCIYA, to isolate a 130 kb region containing the entire *Hoxb* cluster from a 150 kb YAC (7). The PCR products for the recombination ends for both pCIC9C6 and pCIYA were inserted head-to-tail into the polylinker of pClasper and the vector was linearized at a *NruI* site designed between the two ends. Linearized DNA was used for LiAc transformation of yeast.

Recombination-based cloning of specific regions from within yeast artificial chromosomes

In order to select for the *LEU2* gene contained in pClasper the YAC needs to be in a yeast strain deficient for that gene. Most YAC libraries are constructed in the strain AB1380, which does not have the appropriate genetic background. The two yeast strains we used were produced by crossing AB1380 carrying the appropriate YAC with Y724 and isolating *LEU*⁻ spores that maintain the intact YAC (see Materials and Methods). After transformation of linearized recombinant plasmids into these strains yeast colonies were selected for the acquisition of the *LEU*⁺ phenotype and analyzed for loss of the YAC. The YAC was lost either by successive passaging in the absence of selection for the *URA3* and *TRP1* markers on the YAC arms (strain YLB15-20) or, when the YAC was very stable, by the addition of the uracil analog 5-fluoroorotic acid (5-FOA), which selects against the *URA3* marker (strain YLC44-5).

pCIC9C6 was transformed into yeast carrying a 440 kb mouse *Hoxc* cluster YAC (strain YLC44-5). pCIYA was transformed into yeast carrying a 150 kb mouse *Hoxb* cluster YAC (strain YLB15-20). After selection as described above, *LEU*⁺, *URA*⁻ yeast colonies carrying the expected circular recombination product were identified by whole-cell PCR using primers that were specific for the junction between the vector and the insert and primers specific for

Figure 4. Southern analysis of pCIC9C6 recombination products. Filters were hybridized with a *Hoxc-8* probe. Restriction enzymes used are shown. kb, position and size of 1 kb ladder size marker (Gibco BRL) bands are indicated. Lanes Y, yeast genomic DNA containing the *Hoxc* cluster YAC. Lanes 2, 3, 9 and 10, yeast genomic DNA containing two independent clones of the recombination product of pCIC9C6. Lanes 4, 5, 11 and 12, plasmid DNA from two independent clones of bacteria transformed with DNA from the yeast clone in lane 2. Lanes 6, 7, 13 and 14, plasmid DNA from two independent clones of bacteria transformed with DNA from the yeast clone in lane 3.

a gene in the insert. For the transformation with pCIC9C6 we obtained 13 LEU⁺ colonies, three of which were PCR positive for the circular recombination product. For the transformation with pCIYA three of 38 colonies obtained were positive.

Total genomic DNA was prepared from the PCR-positive colonies and reconfirmed by PCR to contain the recombination product. Yeast genomic DNA was used to transform *E. coli* strain DH10B by electroporation (21). When bacteria were transformed with the three recombinants of pCIC9C6 >100 chloramphenicol-resistant colonies/transformation were obtained. Plasmid DNA from two colonies from each transformation was prepared by a standard alkaline lysis procedure (17). Plasmid DNA was digested with either *I-PpoI* to release the insert or *EcoRI*. All six recombinants had the expected insert size (27 kb) and restriction pattern (data not shown). Southern analysis of two recombinants indicate that in yeast (Fig. 4, lanes 2, 3, 9 and 10) and in bacteria (Fig. 4, lanes 4–7 and 11–14) the recombinants are unrearranged when compared with the original *Hoxc* YAC (Fig. 4, lanes Y).

The recombination product for the pCIYA transformation was expected to be ~130 kb in size, since the recombinogenic ends were homologous to the arms of a 150 kb *Hoxb* YAC. When total yeast genomic DNA was prepared by standard alkaline lysis methods the DNA was PCR positive for the recombinant product, but failed to produce any chloramphenicol-resistant colonies upon transformation into bacteria. We suspect that the large recombination product was sheared during DNA preparation. To circumvent this problem we prepared yeast genomic DNA from one recombinant in agarose plugs (7). After Gelase digestion of the agarose plugs and dialysis the DNA was electroporated into bacteria, resulting in >50 chloramphenicol-resistant colonies/transformation. DNA was prepared by standard alkaline lysis from five colonies and analyzed by Southern blotting of a field inversion gel (FIGE). Digestion with *I-PpoI* produced a band of

Figure 5. Southern analysis of pCIYA recombination products. Restriction enzyme-digested DNA was run in FIGE as described in Materials and Methods. Filters were hybridized with a probe to *Hoxb-1*. (A) *I-PpoI*-digested plasmid DNA from five independent bacterial clones (lanes 1–5) of the recombination product. kb, position and size of Midrange PFG Marker II (New England Biolabs) bands are indicated. (B) Comparison of restriction digests of the original *Hoxb* YAC and a recombinant. Yeast genomic DNA containing the YAC was prepared as described in Materials and Methods. Plasmid DNA of the recombinant was prepared by standard alkaline lysis. The restriction enzyme used is indicated above each lane. kb, position and size of Midrange PFG Marker I (New England Biolabs) bands are indicated.

the predicted 130 kb that hybridized to a *Hoxb-1* probe (Fig. 5A). The insert from all five independent bacterial clones was the same size, indicating that the 130 kb insert is extremely stable in bacteria. Southern analysis using several restriction enzymes making rare cuts was used to compare the integrity of the recombinant with the original *Hoxb* YAC (Fig. 5B). When hybridized with a *Hoxb-1* probe, *NotI*, *SalI* and *EagI* produced identical sized bands of 70, 49 and 49 kb, respectively, from both the YAC and the recombinant. *SacII* digestion produced an 87 kb band from the YAC and a 97 kb band from the recombinant. The size difference is due to the lack of *SacII* sites in the pCIYA targeting vector, therefore the 10 kb vector is contained in the *SacII* band from the recombinant. These results indicate that we have stably captured an unrearranged 130 kb insert into the pClasper vector that contains the entire *Hoxb* cluster.

DISCUSSION

YACs have become important tools for cloning large genomic regions. Their large size allows analysis of large genes or gene clusters. However, it can be laborious to isolate enough YAC DNA from yeast to effectively undertake functional analyses. In addition, YACs frequently contain more genomic DNA than is necessary for function. We have designed a yeast–bacteria shuttle vector that circumvents these problems. The vector, pClasper, is used to clone specific regions from YACs. pClasper maintains large genomic inserts stably in both yeast and bacteria, allowing for mutagenesis in yeast and rapid DNA preparation in bacteria. We have used pClasper to isolate large subregions of YACs using homologous recombination in yeast. We have cloned a 27 kb

region containing the *Hoxc-8* gene from a 440 kb *Hoxc* YAC. We have also isolated the entire insert of 130 kb from a *Hoxb* YAC, producing a stable circular product containing the entire *Hoxb* cluster.

The procedure by which YAC sequences are isolated here is essentially an extension of yeast gap repair (18), in which free ends recombine with homologous regions in a yeast chromosome resulting in a circular product with the sequences from the chromosome contained between the recombinogenic ends. We have extended this general method to isolate large regions from YACs. Previous reports have also used a version of extended gap repair similar to ours. An early report on *Drosophila* YACs used recombination to rescue YAC end fragments (22). The recombinogenic ends were specific for both arms of the YAC and thus rescued the entire insert. Before bacterial transformation these authors digested the DNA and religated it to produce circular products with inserts of 13.5 kb and smaller. Recombination within a YAC has also been used to create a library of vectors for functional analysis (23). In this case one recombinogenic end was specific for a YAC arm, while the other end was created by random cloning from a library of cosmid fragments. After homologous recombination a library of circular YAC inserts up to 100 kb in length was recovered. These authors isolated the circular recombinants from yeast DNA by alkaline denaturation and phenol extraction, then microinjected them into *Caenorhabditis elegans* embryos to complement a vulval mutation.

In our extension of these methods we generated recombinogenic ends by PCR and recovered specific recombinants using 500 bp of homologous sequences. We have not attempted to use smaller recombinogenic ends, but theoretically sequences as small as 50 bp could be used, with a concurrent loss in specificity. We have transferred the circular vector containing the large YAC-derived insert directly from yeast DNA into bacteria. We have not detected any rearrangements of the DNA in bacteria. We have added 18 bp recognition sites for the intron-encoded endonucleases *I-PpoI* and *I-SceI* to the polylinker to facilitate the production of large amounts of linear DNA for generation of transgenic mice and analysis of the integrity and copy number of the transgenes in the mouse genome. In addition, the polylinker of pClasper allows for simplified cloning of recombinogenic end fragments and for mapping of the genomic insert.

We expect inserts between 1 and 300 kb to be efficiently cloned from YACs using pClasper. We have obtained stable large inserts in bacteria by using the F factor replicon in pClasper instead of a multicopy origin of replication. Unlike cosmid origins of replication, the F factor replicon maintains the plasmid as a single copy, thereby preventing recombination between identical copies of a large insert. We have also observed structural stability of the large inserts in yeast. We have observed no rearrangements in the 27 kb insert. We have observed similar stability in yeast with inserts of 40 and 60 kb (not shown). The 130 kb insert is also structurally stable when handled carefully by maintaining selection and limiting passaging of the cells. In the absence of these precautions we have experienced rearrangements of this recombinant. Larger fragments of exogenous DNA are generally believed to be more stable when replicating as linear instead of circular DNA (24,25). Others have reported stable centromere-based circular plasmids, similar to pClasper, with 25 kb–1.2 Mb inserts (26).

The methods outlined here are generally applicable to any laboratory with a general knowledge of molecular biology techniques. The only specialized technique we performed was

tetrad dissection to obtain yeast strains containing the YACs in the appropriate genetic background. Spencer *et al.* (27) have reported an alternative efficient method that takes advantage of the properties of yeast karyogamy mutants for transferring YACs to a new host. This technique requires replica plating rather than tetrad dissection to isolate YAC-containing strains of the appropriate genetic background.

Since pClasper is stably maintained in yeast, homologous recombination can be used to add reporter genes or for mutagenesis of the inserts (28). By transferring the regions of interest from a YAC to pClasper and selecting against the presence of the original YAC, the *URA3* and *TRP1* selectable markers can be used for targeted mutagenesis of the selected regions before transformation into bacteria. We have inserted a *lacZ* gene in the *Hoxc-8* gene in the pCIC9C6 recombinant by homologous recombination and generated β -galactosidase-expressing transgenic embryos that recapitulate the endogenous *Hoxc-8* expression pattern (Bradshaw, Shashikant and Ruddle, unpublished).

The vector can also be used to transfer DNA contained in other bacterial vectors, such as cosmids or P1, into yeast. It can be difficult to modify larger DNA inserts maintained in bacterial vectors when appropriate restriction sites are unavailable. If the region of interest could be transferred into yeast cells the researcher can easily introduce more than one specific change by genetic means. Others have shown that DNA can be cloned efficiently into yeast by co-transforming the DNA of interest with linearized vectors containing yeast replicons and appropriate regions of homology. Erickson *et al.* (29) have co-transformed λ clones and a yeast–bacteria shuttle vector to isolate specific genes by gap repair. Ketner *et al.* (30,31) have generated YAC clones with high efficiency by co-transforming the 36 kb adenovirus 2 genome with YAC arms.

The utility of the pClasper vector lies in its ability to make homologous recombination in yeast accessible for large genomic fragments cloned by either the methods described here or by conventional *in vitro* ligation and bacterial transformation. In addition to the generation of transgenic mice as described above, the vector can also be used to make targeting constructs for the production of mutant mice by homologous recombination in embryonic stem cells. The ability to introduce the desired changes in yeast will be especially useful when researchers want to move from the disruption of genes in the mouse germline (knock-out) to the introduction of specific mutations (knock-in) (32). Miller *et al.* (23) identified a gene responsible for a vulval mutant in *C.elegans* by rescue with transgenes made by recombinational cloning from YACs. Similarly, pClasper will facilitate the identification of new genes and gene functions by transgenic analysis in various organisms and by transfection of tissue culture cells.

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