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Rescue of end fragments of yeast artificial chromosomes by homologous recombination in yeast

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ABSTRACT

Yeast artificial chromosomes (YACs) provide a powerful tool for the isolation and mapping of large regions of mammalian chromosomes. We developed a rapid and efficient method for the isolation of DNA fragments representing the extreme ends of YAC clones by the insertion of a rescue plasmid into the YAC vector by homologous recombination. Two rescue vectors were constructed containing a yeast *LYS2* selectable gene, a bacterial origin of replication, an antibiotic resistance gene, a polylinker containing multiple restriction sites, and a fragment homologous to one arm of the pYAC4 vector. The 'end-cloning' procedure involves transformation of the rescue vector into yeast cells carrying a YAC clone, followed by preparation of yeast DNA and transformation into bacterial cells. The resulting plasmids carry end-specific DNA fragments up to 20 kb in length, which are suitable for use as hybridization probes, as templates for direct DNA sequencing, and as probes for mapping by fluorescence *in situ* hybridization. These vectors are suitable for the rescue of end-clones from any YAC constructed using a pYAC-derived vector. We demonstrate the utility of these plasmids by rescuing YAC-end fragments from a human YAC library.

INTRODUCTION

The isolation of genes responsible for human hereditary diseases may be accomplished through a strategy known as positional cloning, the localization of genes by meiotic linkage mapping followed by their isolation by regional cloning and mapping (1). Yeast artificial chromosomes (YACs) have been widely used for the construction of genomic libraries (2,3) and for the isolation of extended regions of human chromosomes spanning regions containing human disease genes (4). The construction of overlapping sets of YAC clones, or contigs, is generally carried out through molecular 'walking', involving the isolation of end-specific probes which are then used for the detection and characterization of additional overlapping clones (5). Using this approach, YAC contigs spanning several hundred kilobases of DNA surrounding a unique DNA probe may be constructed. In many instances, the most time consuming step in the 'walking' procedure is the isolation of useful and reliable probes representing both ends of the human DNA insert carried by a YAC clone.

Recently, several different approaches have been utilized for the isolation of DNA fragments representing the extreme ends of YAC clones and the construction of contigs. YAC end clones may be isolated by directly subcloning the YAC DNA into bacteriophage, plasmid, or cosmid vectors (6,7) though this approach is extremely time consuming and labor intensive. Amplification of YAC-end-specific sequences using the polymerase chain reaction (PCR) has been accomplished with primers specific for an *Alu* repetitive element consensus sequence and a vector-specific sequence (8). This approach allows recovery of the end fragments of YACs only if an *Alu* repetitive element is located sufficiently close to the YAC-end for amplification to occur. 'Inverse PCR', in which an end-specific restriction fragment is circularized prior to amplification, has also been used but depends on the correct locations of appropriate restriction sites relative to the vector arm (9). The 'vectorette PCR' technique (10) utilizes mismatched linkers ligated onto the ends of digested YAC DNA to produce an amplifiable template. However, all PCR-based end-rescue methods usually result in probes of only several hundred base pairs in length which have limited usefulness for hybridization analysis or for localization using other mapping techniques (10). Also, PCR methods based on the random locations of repetitive sequences or restriction sites are frequently unsuccessful for isolating both ends of a given YAC clone thereby limiting their usefulness for bidirectional walking (8).

Most available YAC libraries are constructed using one of the pYAC series of vectors, or one of many derivatives, originally produced by Burke and Olson (2,3,11,12). The arm of pYAC4 containing the centromere (CEN) also contains a unique XhoI site, a bacterial origin of replication, and an antibiotic resistance gene. Therefore, the CEN end fragment of YAC clones constructed in pYAC4 can, in principle, be isolated simply by digesting the DNA with XhoI, circularizing, and transforming the DNA into *E.coli* (2,3). Though straightforward, we and others have found this approach to be very unreliable due to the scarcity of XhoI sites in human DNA (12). Furthermore, no such system is available for isolating the URA-side YAC-ends. To circumvent

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these problems, we have constructed two plasmid rescue vectors that can be used to 'retrofit' a YAC clone so that both YAC-ends can be efficiently and reliably isolated. The rescue plasmids incorporate a bacterial origin of replication, an antibiotic resistance gene, and several restriction sites in a polylinker. After targeted integration of the rescue plasmid by homologous recombination in yeast, the YAC-end fragment can be isolated by restriction enzyme digestion, circularization, and transformation into *E.coli*. We show this method to be extremely reliable for isolating end-specific clones containing several kilobases of DNA. These fragments can then be used directly as hybridization probes for Southern blot analysis, for fluorescence *in situ* hybridization to metaphase chromsomes and interphase nuclei, and for direct DNA sequence determination. Because this method is independent of the sequence or structure of the insert DNA, these vectors can furthermore be used to clone both ends of a YAC insert isolated from a genomic YAC library constructed in any pYAC derived vector using DNA from any species. Finally, this technique also provides a convenient method for the generation of DNA sequence landmarks, such as sequence tagged sites (STS) (13) directly from YAC clones.

MATERIALS AND METHODS

Construction of recombination vectors

pICL vector. The pICL vector was designed for rescue of the CEN YAC end fragment. The HindIII site in the polylinker of plasmid pHSS6 (14) was deleted by digesting with HindIII, blunt-ending with Klenow polymerase and recircularizing to yield pHSS6ΔH. The *LYS2* gene (15) from plasmid p1-L13 (kindly provided by S. Carl Falco) was cloned as a 5.0 kb EcoRI-ClaI fragment to produce pHSSLys. The 5.0 kb EcoRI-XbaI fragment from pHSSLys was cloned into pTZ18R (16) to produce p18Lys. A polylinker containing sites for the restriction enzymes KpnI, SphI, SacI, and BamHI was introduced into the EcoRI site of p18Lys utilizing EcoRI cohesive ends to yield the vector pICL (or plasmid for integrating into the CEN side with *LYS2*). The polylinker of pICL was formed using two complementary synthetic oligonucleotides:

5'-AATTGGTACCGCATGCGAGCTCGGATCC-3'
3'-CCATGGCGTACGCTCGAGCCTAGGTTAA-5'

The structure of the pICL vector is shown in Figure 1A.

pLUS vector. The pLUS vector was designed for rescue of the YAC URA end fragment. A polylinker containing a T7 RNA polymerase promoter and sites for the restriction enzymes HindIII, XbaI, SacI, KpnI, and BamHI was inserted between the SmaI and HindIII sites of pHSSLys. The polylinker consisted of two complementary oligonucleotides 40 and 44 bp long which were annealed to generate a duplex oligonucleotide with blunt ends on one side and HindIII cohesive ends on the other. The polylinker sequence of the resultant plasmid pHSSLysL is:

5'-AGCTTCTAGAGCTCGGTACCGGATCCCTATAGTGAGTCGTATTA-3'
3'-AGATCTCGAGCCATGGCCTAGGGATATCACTCAGCATAAT-5'

The 1800 bp SmaI-StuI URA side segment of pYAC4 (2,3) was cloned into pBluescript II (Stratagene, Inc.) and an EcoRI linker inserted into the BamHI site of the polylinker. The URA side segment was inserted into pHSSLysL as an EcoRI fragment and oriented by restriction analysis to yield pLUS (or plasmid for *Lys2* integration into the URA side). The structure of the pLUS vector is shown in Figure 1B.

Yeast transformation

Transfected plasmid DNA can be targeted for homologous recombination in yeast by linearizing the plasmid within a region of homology shared between the plasmid and the yeast genome. The pLUS vector was targeted to the URA arm of each YAC clone by linearizing with SalI which cuts within the 1800 bp region of homology between pLUS and pYAC4 (2,3). Similarly, pICL was targeted to the CEN arm of pYAC4 by linearizing with ScaI which cuts once in the plasmid β-lactamase gene.

Linearized pICL and pLUS vectors were transformed into yeast cells using 5−10 μg of plasmid DNA and a modified lithium acetate transformation procedure (18). The transformation mixture was plated on synthetic complete medium lacking lysine and uracil (19,20) and incubated at 30°C. A low concentration of adenine (10 mg/l) was employed in the synthetic medium to enhance the red color phenotype associated with the *ade2−1* allele. In the host yeast strain AB1380, both the *ade2−1* and *lys2−1* alleles are ochre mutations. The most frequent reversion to lysine prototrophy in AB1380 is due to forward mutation at a tRNA suppressor. Therefore, white (*ADE2 URA3 LYS2*) colonies represent ochre tRNA suppressor revertants, and red

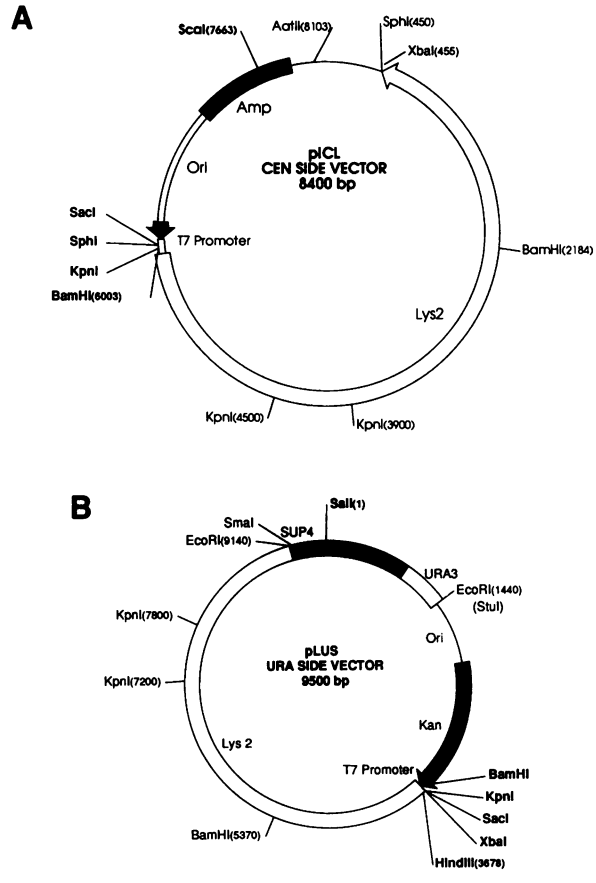

Figure 1. Structure of the YAC end fragment rescue vectors. **A.** pICL vector for rescuing the CEN-end YAC sequences. **B.** pLUS vector for isolating URA-side YAC end sequences. Amp refers to the ampicillin resistance (β lactamase) gene used to target CEN-end homologous recombination, Kan refers to the kanamycin resistance (neomycin/kanamycin phosphotransferase) gene, *ori* denotes the *E.coli* origin of replication, YAC4 refers to the excised region from the vector pYAC4 used to target URA-side homologous recombination, and *Lys2* denotes the α-aminoadipate reductase gene. The approximate locations of relevant restriction sites are shown and the numbering is shown according to the numbering system for pBR322 (20).

(*ade2 URA3 LYS2*) colonies contain integrated pICL or pLUS vector DNA. Approximately 2–10 red colonies per μg of plasmid DNA were observed on the plates after 3–4 days of growth. Red(*ade2*) Ura$^+$ Lys$^+$ colonies were picked and grown overnight in 2 mls of YPD medium (19,20). Yeast DNA was prepared and analyzed for homologous recombination of the vector sequences by PCR analysis. Positive colonies were streaked onto synthetic medium plates lacking lysine and uracil (19,20) for further use.

Assessment of homologous recombination by PCR amplification

CEN-side PCR. A PCR-based assay was designed to allow the red(*ade2*) Ura$^+$ Lys$^+$ colonies to be rapidly screened for integration of pICL by homologous recombination. The primer set consists of two 25-mer oligonucleotides. The first primer 5′-GCGCTTAATGCGCCGCTACAGGGCG-3′ is complementary to bases 2408–2432 of the f1 origin region of pTZ18R (16). The second primer 5′-GCTCACCGGCTCCAGATTTATCAGC-3′ corresponds to bases 3444–3468 of the ampicillin resistance gene of pBR322 (21). Using these primers, an amplification product of 870 bp is obtained either from YAC clones that have integrated pICL by homologous recombination into the CEN side of pYAC4 or the endogenous *lys2* gene. However, the 870 bp PCR fragment will be cleaved by PstI into two fragments 705 bp and 165 bp long only when pICL is targeted correctly to the homologous site in pYAC4. The parental YAC and the rescued YAC-end plasmid will not amplify with this primer set.

URA-side PCR. A PCR primer set was also constructed to screen for transformants where the pLUS vector had integrated into the YAC URA side arm by homologous recombination. The first primer 5′-CTTGAGATCGGGCGTTCGACTCGC-3′ is complementary to a sequence immediately proximal to the EcoRI cloning site located in the *SUP4* gene on the URA arm of pYAC4 (2,3). The second primer 5′-TGAACGGTGATCCCCACCGGAATTG-3′ is located in the origin region of pLUS immediately downstream of the pYAC4 fragment. A product of 1855 bp is produced by PCR amplification from YAC clones containing pLUS integrated by homologous recombination into the URA side vector arm, and from linearized YAC-end rescued plasmids. This primer set will not generate an amplification product from parental YAC clones or the pLUS vector DNA.

Rescue of YAC ends by transformation into *E.coli*

Individual red colonies that screen positive for homologous recombination by PCR are grown to saturation in two mls of YPD medium. DNA is prepared by either the glass bead (22) or spheroplast (23) method and digested to completion with one of the enzymes contained in the polylinker. We routinely obtain about 1 μg of DNA from a 2 ml yeast culture and use 0.5 μg for further analysis. Following digestion with restriction enzymes using conditions recommended by the manufacturers, the DNA is extracted with phenol/chloroform, precipitated with ethanol, and circularized by ligation at 16°C for 1–2 hours in a total

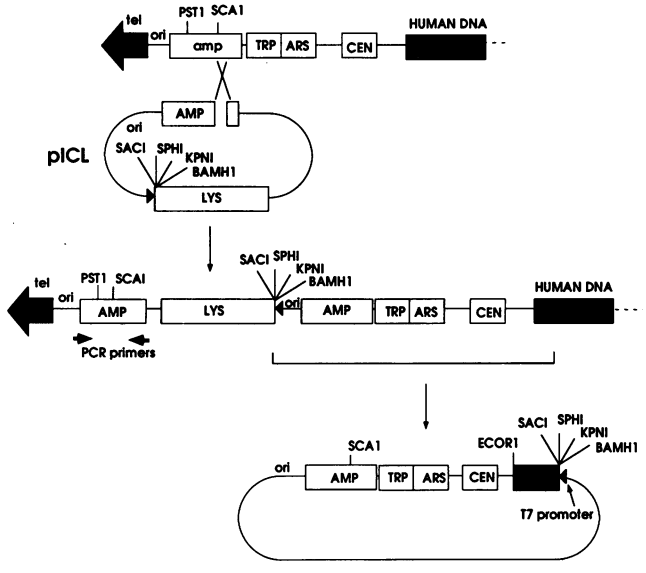

Figure 2. Strategy for cloning the CEN end of YAC inserts by homologous recombination in yeast followed by bacterial rescue with pICL. The pICL vector is linearized with ScaI at the unique restriction site located within the ampicillin resistance gene (AMP) and introduced into yeast cells carrying the appropriate YAC clone. Homologous recombination occurs between linearized pICL and the CEN side arm of the parental YAC clone resulting in a YAC carrying the *LYS2* selectable gene and in the insertion of pICL into the AMP gene of the YAC clone with duplication of the bacterial origin and the AMP gene. The YAC AMP gene carried by the YAC vector contains a PstI site which is used in a PCR-based assay to detect targeted homologous recombination as described in methods. Following selection on lysine deficient medium, yeast DNA is prepared, digested with an appropriate restriction enzyme, circularized by self-ligation, and electroporated into *E.coli* with subsequent selection on ampicillin-containing medium. The resultant bacterial plasmids carry the end-most restriction fragment from the YAC clone CEN end.

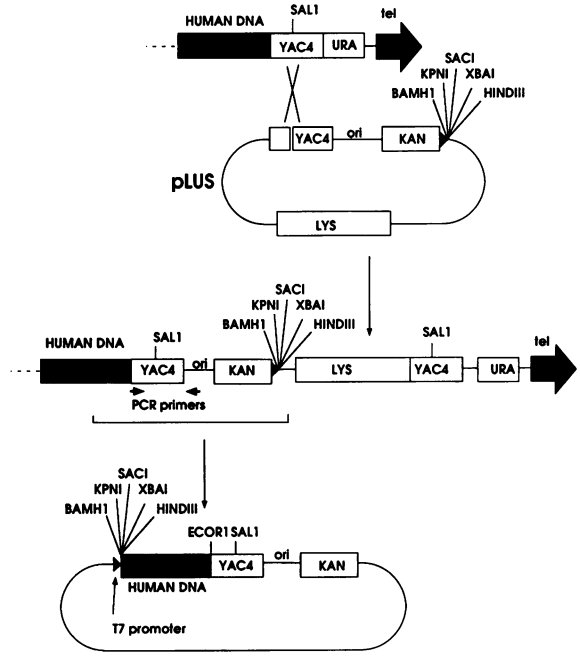

Figure 3. Strategy for cloning the URA end of YAC inserts by homologous recombination in yeast followed by bacterial rescue with pLUS. The pLUS vector is linearized with SalI at a unique site within the region of homology with YAC4 and undergoes homologous recombination between pLUS and the URA arm of the parental YAC clone. Recombination results in the insertion of pLUS into the YAC4 URA arm and a duplication of the SmaI-StuI region of pYAC4. The resulting YAC clone carries the *LYS2* gene and is selected on lysine deficient medium. Primers for amplification by PCR are located outside the YAC4 homology region and near the bacterial origin of replication and can be used to screen for homologous recombination as described in Materials and Methods. Following targeted recombination, yeast DNA is prepared and introduced into *E.coli* by electroporation. Selection on kanamycin containing medium results in the selection of plasmids containing the distal URA end of the YAC clone insert.

volume of 200μl. The DNA is then precipitated with ethanol and the pellet resuspended in 50 μl of electroporation-competent (24) XL-1 Blue *E.coli* cells (Statagene, Inc.), pulsed at 1.5kV, 200 Ω, and 25μF using a 0.1 cm cuvette and a BioRad Gene Pulser electroporation apparatus, and plated on LB agar containing 50 μg/ml ampicillin for pICL selection or 25 μg/ml kanamycin for pLUS selection. Electroporation routinely generated approximately 100−500 bacterial colonies per ml of starting yeast culture.

Oligonucleotide synthesis

All oligonucleotides were synthesized using an Applied Biosystems PCR-Mate automated oligonucleotide synthesizer and used for cloning or PCR amplification without further purification.

PCR amplification conditions

PCR amplification reactions were carried out in a final volume of 50 μl containing approximately 0.1μg of template DNA and 1U of Taq DNA polymerase in buffer supplied by Stratagene, Inc. DMSO was added to a final concentration of 10% for the pLUS PCR assay. Thirty-five cycles of amplification were carried out using parameters of 95°C for 30 seconds, 60°C for one minute, and 72°C for one minute in a Bellco thermal cycler. Amplification products were analyzed on 1% (pLUS) or 2% (pICL) agarose gels stained with ethidium bromide.

RESULTS AND DISCUSSION

The construction of overlapping YAC contigs plays an important role in many large scale mapping projects and is critically dependent on the ability to efficiently isolate end-probes for genome 'walking'. To address this problem, we constructed specialized plasmid vectors for the targeted integration by homologous recombination and rescue of YAC end fragments in bacteria. The pICL vector (Figure 1A) targets the YAC vector arm carrying the yeast centromere (CEN) and allows rescue by transformation into bacterial cells (Figure 2). The pLUS vector (Figure 1B) targets the opposite vector arm carrying the *URA3* selectable gene and allows rescue of the end fragment by a similar strategy (Figure 3). Site-specific targeting is achieved by linearizing the plasmids within the region of homology shared between the vectors and the YAC arms prior to introduction into yeast cells. The vectors integrate into the YAC arm introducing a *LYS2* selectable gene, a bacterial origin of replication, an antibiotic resistance gene, and a polylinker. Following transformation and selection for lysine prototrophy, yeast colonies are screened for targeted insertion of the vector by a PCR-based assay. To enable rescue of the YAC end fragment, total yeast DNA from a positive colony is then digested with any one of the several restriction enzymes whose site is present in the polylinker. The digested DNA is then circularized by ligation and transformed into bacterial cells to rescue the end fragment of the YAC clone as a bacterial plasmid. The rescued YAC-end plasmid can be used directly for DNA sequence analysis using a T7 RNA polymerase primer and for the preparation of hybridization probes.

To test this strategy, several yeast colonies carrying human yeast artificial chromosomes were isolated using PCR assays prepared from cosmid DNA landmark clones mapped to human chromosome 11 using high-resolution *in situ* suppression hybridization (26) or known gene sequences (in preparation). YAC clones were isolated from a human genomic library provided by M.Olson (25) using a PCR screening protocol and DNA pools (27). One to five YAC clones were isolated with each primer set and the YAC clones characterized by pulsed-field gel electrophoresis. Following characterization, the CEN and URA end fragments were isolated from each YAC clone using pICL and pLUS rescue vectors and the strategy described above.

To demonstrate end-fragment rescue, yeast cells carrying several different YAC clones were transformed with ScaI-linearized pICL vector DNA. Following transformation with pICL, 2−10 red (*ade2*, *LYS2*) colonies/μg of vector DNA were obtained and several of these were selected for further analysis. Ten red colonies were subjected to PCR analysis and 9 were shown to have the vector integrated into the YAC arm by

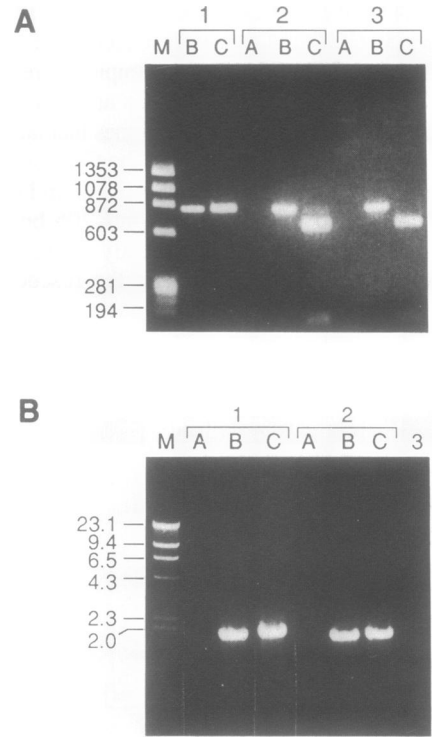

Figure 4. Detection of homologous recombination in YAC clones by PCR. **A.** PCR-based assay for homologous recombination between pICL and the CEN side of YAC clones. Yeast carrying a YAC clone were transformed with linearized pICL, selected on lysine deficient medium, and colonies screened by PCR for homologous integration of pICL. Following amplification, products were digested with PstI. An 870 bp product is amplified with pICL vector alone (lanes 1B) or using DNA from different pICL-transformed YAC clones (lanes 2B, 3B). The amplification product is cut by PstI into fragments of 705 bp and 165 bp when pICL is integrated by homologous recombination into the YAC4 CEN arm (lanes 2C, 3C). PstI does not digest amplification products from the pICL vector alone (lane 1C). This assay distinguishes between homologous recombination of pICL into the CEN side YAC arm and gene conversion or the integration of pICL into the endogenous *lys2* gene. The parental YAC DNA (lanes 2A, 3A) does not amplify with this primer set. Size marker (lane M) is HaeIII digestion products of bacteriophage φX174 DNA. **B.** PCR based assay for homologous recombination between pLUS and the URA arm of YAC clones. The URA side PCR primers described in Materials and Methods do not allow amplification using the pLUS vector as a template (lane 3) or using parental YAC DNA (lanes 1A, 2A). However, an 1850 bp fragment is obtained using YAC DNA with a pLUS vector introduced into the URA arm by targeted integration (lanes 1B, 2B). Amplification also occurs using linearized plasmid clones carrying the YAC ends rescued in *E.coli* (lanes 1C, 2C). Size marker (lane M) is HindIII digestion products of bacteriophage lambda.

homologous recombination (Figure 4A). PCR amplification of the rescue vector integrated into the CEN arm by homologous recombination yields an 870 bp amplification product containing an internal PstI site from the pYAC4 AMP gene. Cleavage of the PCR product with PstI yields two fragments 705 and 165 bp in length. If pICL integrates somewhere other than the YAC CEN arm, the 870 bp PCR amplification product will not cut with PstI since the pICL vector AMP gene does not contain a PstI site. The YAC end fragment was rescued by preparation of yeast DNA, digesting with SacI or BamHI, treatment with T4 DNA ligase, and transformation of XL-1 Blue bacteria by electroporation. The resulting transformation yielded 100 to 500 ampicillin-resistant colonies per 1 ml of yeast culture. DNA was prepared from bacterial colonies by a miniprep procedure and analyzed by restriction enzyme digestion (Figure 5). This analysis demonstrated the rescue of DNA fragments ranging up to 20 kb. Additional analysis by direct DNA sequencing, *in situ* hybridization analysis, and hybridization to YAC clones confirmed that these fragments represent the extreme end fragments of the initial target YAC clone.

Similarly, targeting vector pLUS was used for integration and rescue of the end fragment at the URA side of several YAC clones. Ten red colonies were selected following transformation with pLUS and analyzed for homologous recombination of pLUS in the YAC vector arm by PCR. Seven colonies demonstrated amplification of an 1850 bp product, corresponding to targeted integration into the URA YAC arm (Figure 4B). Rescue of plasmids from the yeast DNA, by digestion with BamHI, religation, and transformation of XL-1 blue bacteria by electroporation, yielded 100 to 500 kanamycin-resistant colonies per 1 ml of yeast culture. Analysis of a selection of these colonies by restriction enzyme analysis demonstrated the rescue of end-specific fragments (Figure 6). Additional analysis (not shown) confirmed the isolation of the end-specific fragment of each YAC clone.

Analysis of a series of YAC clones demonstrated that 70–100% of the colonies isolated following pLUS or pICL transformation test positive for targeted integration by PCR when linearized vector DNA is used. When transformation is carried out using circular plasmid DNA, or when the linearization was incomplete, a lower frequency of targeted recombination ranging from 0 to 60% is observed. Due to the large size of the *LYS2* gene, transformation with supercoiled pLUS and pICL vectors would be expected to preferentially target to the endogenous yeast *lys2* gene.

This 'end-rescue' approach has now been utilized for the successful isolation of both CEN and URA end fragments of over 50 human YAC clones. Targeting efficiency to the appropriate YAC arm using both the pLUS and pICL vector is routinely 80 to 100% and confirmation of homologous integration by PCR is an extremely rapid and efficient procedure. Because this method does not depend on the distribution of species-specific repetitive sequences near the vector arm, it is potentially useful for the isolation of end-specific fragments from any YAC clone and for YAC clones with insert DNA from any species. Though other

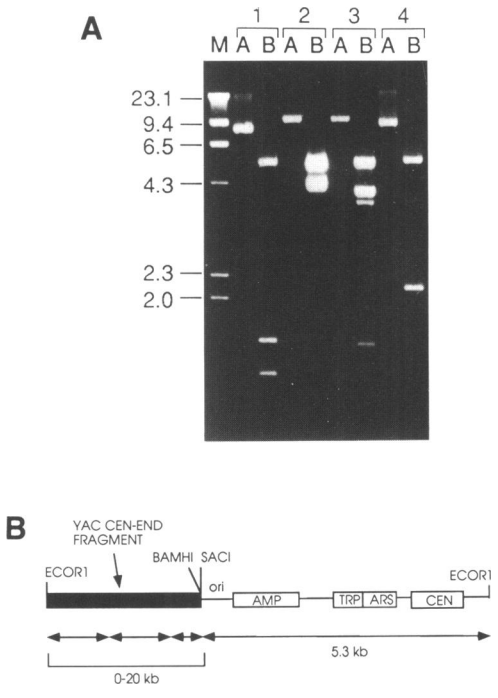

Figure 5. Characterization of rescued YAC CEN end clones obtained by pICL rescue. **A.** The end rescue protocol using pICL was applied to four different YAC clones and bacterial colonies selected with ampicillin. Minprep DNA was prepared, the DNA linearized with SacI (lanes 1A, 4A) or BamHI (lanes 2A, 3A) and digested with SacI and EcoRI (lanes 1B, 4B) or BamHI and EcoRI (lanes 2B, 3B) to release the insert band(s) from the vector. The vector band is present at 5.3 kb and additional bands represent rescued YAC end-fragments. The minor band in lane 3B is due to incomplete restriction enzyme digestion. B. Schematic diagram showing the structure of the rescued CEN-end plasmids. Size marker (lane M) is HindIII-digested lambda DNA.

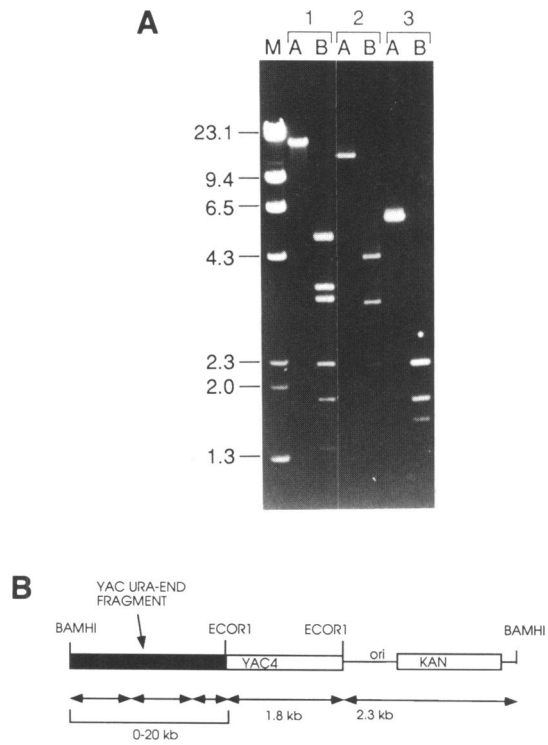

Figure 6. Characterization of rescued YAC URA end clones obtained with pLUS rescue. **A.** The end rescue protocol using the pLUS vector was applied to three different YAC clones and colonies selected with kanamycin. Miniprep DNA was prepared and linearized with BamHI (lanes 1A, 2A,3A) and digested with BamHI and EcoRI (lanes 1B, 2B, 3B) to release the insert band(s) from the vector. The vector appears as two fragments of 2.3 and 1.8 kb. **B.** Schematic diagram showing the structure of the rescued URA-end plasmids. Size markers (lane M) are HindIII digested lambda DNA mixed with HaeIII digested ϕX174.

methods for direct isolation or amplification of YAC end fragments have been described (8–10), we find that this method offers a number of distinct advantages over previous methods. In addition to the efficiency and reliability of this method relative to PCR based methods, and applicability of this strategy to YAC clones from any pYAC4-based library, there are significant advantages to the larger size of the YAC-end obtained by this procedure. While PCR-based methods result in amplification products of only several hundred base pairs, this method results in fragments of up to approximately 20 kb. These fragments are sufficiently large to be useful for mapping by fluorescence *in situ* hybridization to both metaphase chromosomes and interphase nuclei (L.Selleri, G.Hermanson, unpublished data). In addition, two color fluorescent *in situ* hybridization (28) allows the end-clones to be oriented in respect to a reference marker. This permits unidirectional 'walking' towards a desired chromosomal locus. Coupled with additional strategies for efficient YAC clone isolation and characterization, this plasmid rescue strategy is likely to be useful for the large scale mapping of the human genome and the isolation of disease genes by positional cloning.

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