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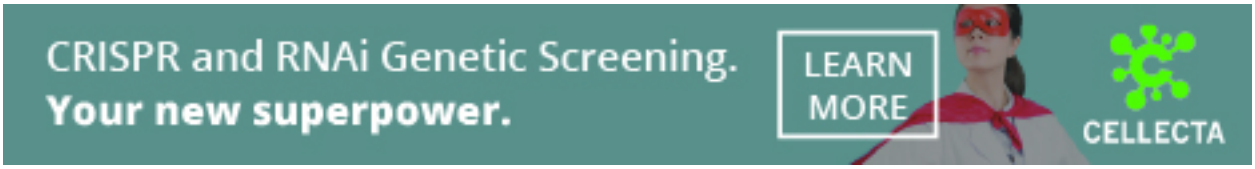

# Linker-Mediated Recombinational Subcloning of Large DNA Fragments Using Yeast

Christopher K. Raymond,[1] Elizabeth H. Sims, and Maynard V. Olson

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**The homologous recombination pathway in yeast is an ideal tool for the sequence-specific assembly of plasmids. Complementary 80-nucleotide oligonucleotides that overlap a vector and a target fragment were found to serve as efficient recombination linkers for fragment subcloning. Using electroporation, single-stranded 80-mers were adequate for routine plasmid construction. A cycloheximide-based counterselection was introduced to increase the specificity of cloning by homologous recombination relative to nonspecific vector background. Reconstruction experiments suggest this counterselection increased cloning specificity by 100-fold. Cycloheximide counterselection was used in conjunction with 80-bp linkers to subclone targeted regions from bacterial artificial chromosomes. This technology may find broad application in the final stages of completing the Human Genome Sequencing Project and in applications of BAC clones to the functional analysis of complex genomes.**

In recent years there has been a massive increase in the amount of available DNA sequence information. Consequently, most DNA subcloning efforts involve joining DNA fragments whose sequences are known. Additionally, there is an increasing need to build recombinant DNA molecules that feature precise fusions of sequences (e.g., in the creation of chimeric protein-encoding open reading frames, in the juxtaposition of DNA regulatory elements to coding DNA, or in the generation of site-directed mutants). This requirement favors cloning methods that rely on homologous sequence overlaps for the generation of recombinant clones.

Yeast cells are an ideal "reagent" in which to conduct sequence-specific cloning. In the yeast *Saccharomyces cerevisiae*, homologous recombination is the favored pathway for DNA double-strand break repair (Orr-Weaver and Szostak 1985). Moreover, linear DNA strands that share sequence overlaps readily undergo recombination when cotransformed into yeast cells (Botstein and Shortle 1985; Kunes et al. 1985, 1987; Ma et al. 1987). These observations serve as the foundation for a wide variety of yeast-based DNA cloning and manipulation methods that rely on homologous recombination between overlapping DNA segments. Of particular relevance to this work are methods used to subclone large DNA inserts into yeast–*Escherichia coli* shuttle vectors. In one method, a targeting plasmid was used to transfer entire inserts of λ clones from a physically ordered yeast genomic library into a vector suitable both for complementation tests of mapped yeast mutations and for complete sequencing (Erickson and Johnston 1993). In another set of applications, yeast targeting plasmids were used to rescue entire genomic inserts from PAC and P1 clones for subsequent manipulation (Criswell and Bradshaw 1998; Bhargava et al. 1999). These and other reports provided a clear demonstration of the feasibility of manipulating large, exogenously introduced DNA fragments in yeast.

When homologous recombination is used to capture entire inserts present in a specific cloning vector, a universal capture vector can be designed for this purpose. In contrast, to subclone a specific region of a BAC or PAC clone by existing methods, a specific targeting plasmid must be built for each targeted subregion of each clone. We describe here an approach to targeting the capture of specific subregions of any cloned insert that bypasses the need for customized construction of targeting plasmids.

The motivation for this research derives in part from the final stages of the Human Genome Sequencing Project where many small sequence gaps (10–50 Kb) are spanned by large bacterial artificial chromosomes (BACs; ~175 Kb; Lander et al. 2001; Osoegawa et al. 2001). Our goal was to develop a single-step, sequence-specific cloning method to extract the unsequenced portions from such BACs. To achieve this goal, we combined the use of recombination linkers that provide the necessary targeting specificity with a counterselection against unwanted background clones. Recombination linkers are short segments of DNA that promote recombinational joining of unrelated DNA fragments (Raymond et al. 1999; DeMarini et al. 2001). They take advantage of the observation that the extent of DNA sequence overlap required for efficient recombination is a relatively modest 40 base pairs (Kunes et al. 1987, Hua et al. 1997; Oldenberg et al. 1997). Linkers, which are created from synthetic oligonucleotides, provide sequence overlaps with each of two sequences that are to be joined. In the context of large-fragment subcloning, cotransformation of the donor BAC DNA, a universal acceptor vector, and a pair of linkers that specify the two vector–fragment junctions is used to generate the desired subclone. The strength of the approach is that the linker sequences are used to target recombinational cloning to specific sites in the target BAC. A cycloheximide-based counterselection was also introduced to enrich for recombinant clones. In mouse knock-out genetics, counterselection has been used with great success to differentiate between background, nonspecific chromosomal integration and the desired gene-replacement events that occur by homologous recombination (Mansour et al. 1988; Zijlstra et al. 1989; Hasty et al. 1991). As reported here, this strategy has a demonstrable benefit in yeast recombinational cloning as well. The methods described here for large-fragment subclon-

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ing are broadly applicable to diverse strategies involving sequence-specific recombinational cloning in yeast.

## RESULTS

### Tests of Recombination Linkers

Forty-base-pair overlaps are adequate to ensure efficient recombination in yeast (Hua et al. 1997; Oldenberg et al. 1997). Hence, we considered it likely that 80-bp recombination linkers, with 40 bp of vector overlap and 40 bp of fragment overlap, would be adequate to stimulate efficient fragment subcloning. A plasmid-assembly assay, consisting of a chloramphenicol-resistant ($Cm^R$), PCR-generated donor fragment and an ampicillin-resistant ($Amp^R$), *URA3*-marked ($Ura^+$) acceptor vector was used to quantify cloning efficiency. Linkers (Fig. 1A) were made by simple mixing of complementary 80-nucleotide oligonucleotides that had been resuspended in TE buffer. No effort was made to facilitate annealing of complementary strands as gel analysis of linker mixtures suggested they rapidly and spontaneously formed 80-bp double-strand DNA molecules (data not shown). Yeast transformations were performed using the lithium acetate method (Geitz and Woods 2001) or electroporation (Meilhoc et al. 1990; Faber et al. 1994). Plasmid repair was measured by the recovery of $Ura^+$ yeast cells. $Ura^+$ transformants were then scraped en masse from the transformation plates, plasmid DNA prepared from these cells was used to transform *E. coli* to $Amp^R$, and the fragment subcloning efficiency was monitored (by replica plating) as the percent of $Amp^R$ colonies that were also $Cm^R$. As is evident in Figure 1B, 80-bp linkers were very effective in promoting fragment subcloning.

Earlier reports demonstrated that yeast can be successfully transformed with single-stranded plasmid or fragment DNAs and with oligonucleotides (Burgers and Percival. 1987; Simon and Moore 1987; Moerschell et al. 1991). These accounts motivated us to test the efficiency of single-stranded

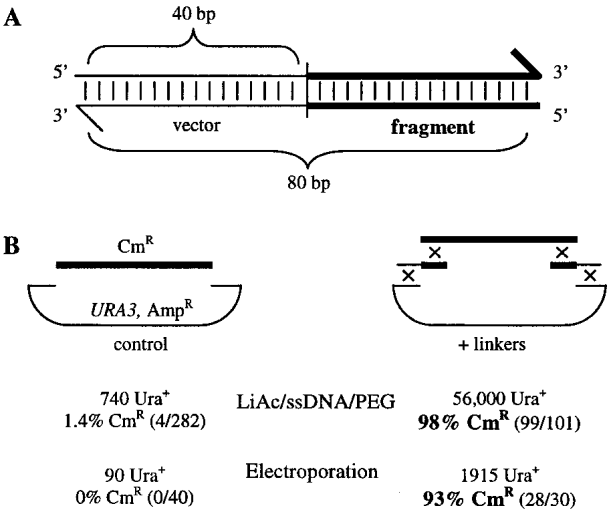


**Figure 1** Self-assembling 80-bp yeast recombination linkers. (*A*) Complementary 80-nucleotide oligonucleotides overlap the vector and fragment by equal, 40-bp segments. (*B*) Using either of the two common intact-yeast-cell transformation methods, 80-bp linkers support efficient plasmid repair as monitored by the recovery of $Ura^+$ colonies. Direct measurements of fragment subcloning, expressed as a percent of $Amp^R$ plasmids that had acquired $Cm^R$, revealed that the majority of linker-repaired plasmids harbor the targeted insert DNA.

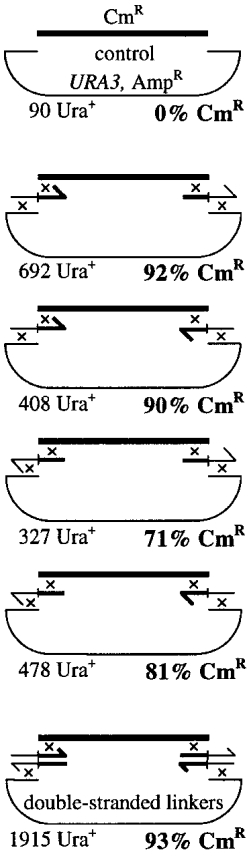


**Figure 2** Single-stranded 80-nucleotide linkers support fragment subcloning. Single-strand linkers are depicted 5′ to 3′, with arrowheads at their 3′ ends. Plasmid repair was scored as $Ura^+$ colonies, and fragment subcloning as the % $Cm^R$ plasmids.

80-nucleotide recombination linkers in promoting fragment subcloning. Using the electroporation transformation method, we found single-stranded linkers to be nearly as effective as double-stranded molecules in facilitating fragment subcloning (Fig. 2). The orientation of the single strands appeared to have little influence on the cloning efficiency. The simplicity of electroporation coupled with the ease of designing and obtaining 80-nucleotide oligonucleotides make this an appealing scheme for routine fragment subcloning in yeast. Single-stranded linkers failed to support fragment subcloning using the lithium acetate transformation method (data not shown). We reasoned that the vast excess of single-stranded carrier DNA used in this method might competitively inhibit uptake of linker segments, so we conducted the transformation without carrier. Under these conditions, double-stranded 80-bp linkers elevated plasmid repair >100-fold, yet single-stranded linkers were without effect (data not shown). These observations underscore the fundamental biological differences between these transformation methods.

### Cycloheximide Counterselection

The primary source of background in recombinational cloning experiments occurs when vector circularizes on itself via illegitimate end-joining (Boulton and Jackson 1996; Raymond et al. 1999). In the experiments described above, this background was insignificant because of high concentrations

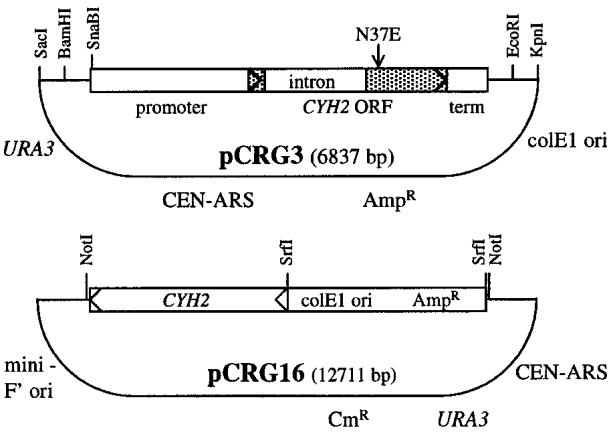


**Figure 3** Plasmids used in this study. In plasmid pCRG3, the 1.95-Kb *CYH2* gene, recombinationally cloned into the *Sma*I site of pRS316, is depicted as the promoter, open reading frame (ORF), and terminator that are drawn to scale. The ORF consists of two exons (shaded) separated by a 510-bp intron. Reference restriction enzyme sites from the pRS316 polylinker are indicated. In plasmid pCRG16, the high-copy-number colE1 ori, Amp$^R$ "stuffer fragment" is liberated from the BAC recombination vector by digestion with *Srf*I.

and modest sizes of input DNA fragments. However, under less optimal conditions, illegitimate end-joining can become a significant source of background. Previous work with mouse gene knock-out strategies (Mansour et al. 1988; Zijlstra et al. 1989; Hasty et al. 1991) suggested that the introduction of a counter-selectable marker would increase the proportion of recombinant clones over end-joined plasmids. The wild-type yeast *CYH2* gene (Fig. 1) was chosen for this purpose. Although the yeast strain used in this study, CRY1, is cycloheximide-resistant (*cyh2$^R$*), the introduction of the wild-type *CYH2* allele into this strain confers dominant sensitivity to the drug (Kaufer et al. 1983; Sikorski and Boeke 1991). By virtue of its position in cloning vectors relative to the sites at which recombination is targeted, the *CYH2* gene is lost in recombinant clones but retained in most end-joined vectors. Hence the population of cells harboring recombinant plasmids is greatly enriched in the presence of cycloheximide.

The *CYH2* counterselection strategy was validated by the reconstruction experiment shown in Figure 4. The efficiency of plasmid repair was measured for a control vector and a *CYH2*-containing vector, with or without an overlapping DNA fragment, and in the presence or absence of cycloheximide. The data reveal three important points. First, in a control vector that lacks the *CYH2* gene, the absolute and relative numbers of Ura$^+$ transformants in the samples with and without overlapping fragment were essentially unaffected by cycloheximide. This result demonstrates that the drug does not significantly alter the efficiency of transformation or plasmid recombination. Second, the addition of the *CYH2* gene to the vector did not appreciably change the efficiency of plasmid repair relative to the control vector. This result indicates that the addition of the 1.95-Kb *CYH2* gene cassette does not sterically interfere with recombination to any appreciable extent. Third and most significantly, the end-joining background of the *CYH2*-containing vector in the presence of cycloheximide was reduced 100-fold relative to the control sample. Overall, the data indicate that *CYH2* counterselection increased the absolute specificity of recombination over end-joining to ~100,000-fold.

## Subcloning from BACs

The linker-mediated cloning strategy was combined with *CYH2* counterselection to subclone targeted regions from BACs (Fig. 5). The cloning vector, pCRG16, is a single-copy mini F′ plasmid derived from the pBAC series of plasmids (Shizuya et al. 1992; Frengen et al. 1999; see Methods). It was retrofitted with yeast replication and selection sequences, the *CYH2* counter-selectable marker, and a high-copy-number colE1 ori, Amp$^R$ "stuffer fragment" (Fig. 3). The stuffer fragment, which facilitates the preparation of the pCRG16 vector, is released prior to use of the vector by digestion with *Srf*I endonuclease. Two 25-Kb regions from human chromosome 7 BAC RPCI-11–98D12 and one 42 Kbp region from human chromosome 7 BAC RPCI-11–100L10 (Osoegawa et al. 2001) were targeted in these experiments. The clone ends were chosen from DNA sequence regions that were determined to be unique by the `RepeatMasker` program (A.F.A. Smit and P. Green, unpubl.; http://reapeatmasker.genome.washington.edu).

BAC vectors and the cloning vector pCRG16 both contain mini-F′ replication sequences and therefore share extensive homology that runs the risk of leading to undesired recombination events. To eliminate this potential artifact, target BAC DNA was digested separately with two different restriction enzymes, *Asc*I or *Not*I, which recognize eight-base-pair sequences. The digests were pooled prior to transformation into yeast. These enzymes cleave at the vector-insert junction in BAC clones, thereby liberating the genomic insert from vector sequences. The limitation in this strategy is that DNA fragments that contain both *Asc*I and *Not*I recognition sites will be difficult to subclone by the protocol we describe. To subclone BAC DNA, *Srf*I-linearized pCRG16, 80-bp targeting linkers, and linearized BAC DNA were cotransformed into yeast spheroplasts that were subsequently plated on Ura-deficient media containing cycloheximide (Fig. 5B). Individual transformant colonies were screened by whole-cell PCR (Ling et al. 1995) for the expected vector-to-fragment se-

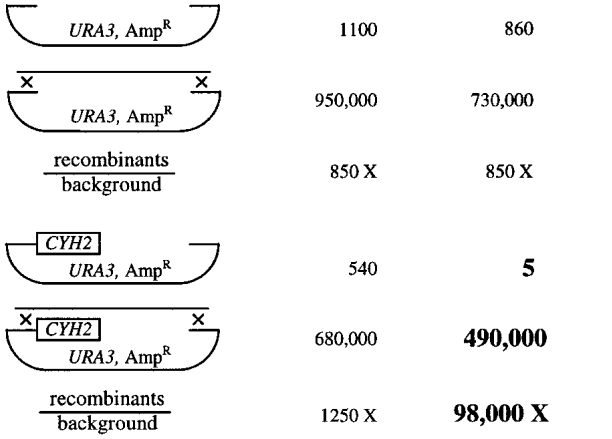


**Figure 4** Validation of the *CYH2*/cycloheximide counterselection. Plasmid repair was monitored as the recovery of Ura$^+$ colonies for the control plasmid pRS316 (*Sma*I) or the test plasmid pCRG3 (*Sna*BI) in the presence or absence of an overlapping repair fragment. The cells were transformed by the LiAc/ssDNA/PEG method. Cycloheximide-resistant colonies were selected in the presence of 2.5 μg/mL of the drug.

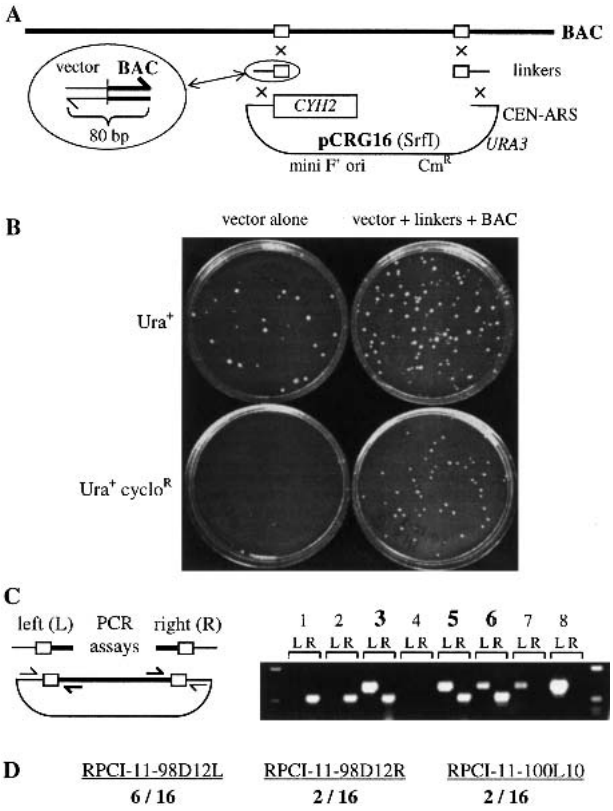


**Figure 5** Subcloning of large DNA regions from BACs. (*A*) BAC subregions were subcloned into a common acceptor vector using targeting 80-bp linkers (*inset*). The common vector, pCRG16, is a yeast ↔ *E. coli* shuttle plasmid that carries the yeast *CEN6/ARSH4* segregation/replication functions and the yeast *URA3* selectable marker. For replication and selection in *E. coli*, the plasmid contains the single-copy mini F′ replication/segregation functions and the chloramphenicol-resistance marker, respectively. For BAC subcloning, 100 ng of *Srf*I-digested pCRG16 acceptor vector, 1 µg (20 pmole) of each linker, and 1 µg BAC DNA digested with *Asc*I or *Not*I and combined in equal amounts were cotransformed into yeast spheroplasts. (*B*) Transformation plates of spheroplasts transformed with vector alone or vector + linkers + BAC DNA and plated on Ura-deficient media or Ura-deficient media containing 2.5 µg/mL of cycloheximide. The RPCI-11–98D12L cotransformation is shown. (*C*) Schematic of left and right target-insert PCR assay strategy and whole-yeast-cell PCR data for eight representative colonies from the RPCI-11–98D12L subcloning. Double-positive clones are shown highlighted in boldface type. (*D*) Yield of PCR double-positive yeast colonies from three representative BAC subcloning experiments.

quence on both ends of the recombinant clone (Fig. 5C). In all three experiments, clones with both recombinant ends were recovered (Fig. 5D). Further characterization by restriction enzyme analysis demonstrated that all of these clones yielded identical restriction digests consistent with that predicted if they harbored the specific region that had been targeted (data not shown). We have not characterized incorrect clones extensively, but note that clones with only one recombinant end are encountered rather frequently. On two occasions where we did explore the source clones that appeared to have a single correct vector-insert junction, the result was a consequence of false-negative PCR results. Subsequent transfer of PCR single-positive clones to *E. coli* and rescreening revealed that these clones had both recombinant ends and gel analysis showed they possessed the expected inserts (data not shown). These observations underscore our experience that the PCR assay is the least reliable part of the BAC subcloning process.

## DISCUSSION

Linker-mediated recombinational subcloning in yeast is particularly well suited to DNA targets that cannot be readily amplified by PCR. Previously reported methods for creating recombination linkers were cumbersome (Raymond et al. 1999) or yielded relatively inefficient results (DeMarini et al. 2001). We find that 80-bp linkers created by simple mixing of complementary 80-nucleotide oligonucleotides promote efficient plasmid assembly using any one of three different yeast transformation methods. Using electroporation, single-stranded 80-nucleotide linkers were adequate for routine subcloning; for example, recent use of this method for generating a vector construct yielded the desired plasmid with 80% efficiency (16/20 constructs; C. Raymond, unpubl.). We are currently extending design features of recombination linkers. In one application, the overlap regions of 80-nucleotide linkers are staggered by 40 bp, similar to the design concept introduced by DeMarini et al. (2001), but with a longer overlap region (40 bp) between linkers (Fig. 6A). This design provides 40 nucleotides of overlap with both the vector and target fragment while providing 40 bp in the central region for the introduction of novel sequences. This may be particularly useful for introducing restriction enzyme recognition sites or for creating peptide tags on recombinant proteins. In addition, we are exploring the use of tailed-PCR to generate linkers for BAC subcloning (Fig. 6B). This method creates linkers with longer overlaps with the target BAC. We are evaluating whether these linkers enhance the recovery of recombinant BAC subclones.

The use of a counter-selectable marker to discriminate between nonspecific events and homologous recombination has been used with great success in mouse genetics (Mansour et al. 1988; Zijlstra et al. 1989; Hasty et al. 1991). We employed a similar strategy by incorporating the *CYH2*/cycloheximide counterselection into the yeast recombinational cloning schemes. In principle, any marker that confers dominant sensitivity to a selective agent could be used (e.g., *URA3* and 5-fluoroorotic acid, *LYS2* and α-amino adipic acid,

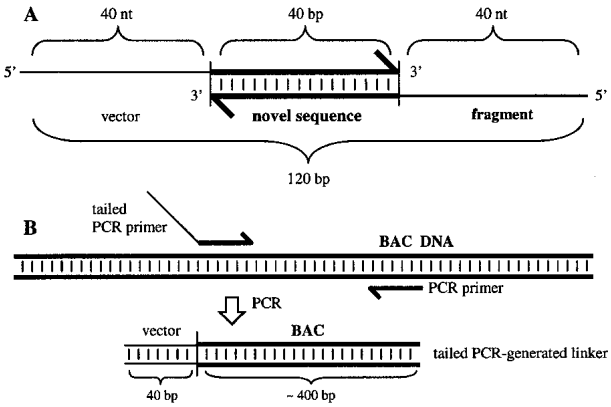


**Figure 6** Alternative methods for generating recombination linkers. (*A*) Overlapping 80-mer oligonucleotides provide overlap with vector and fragment while facilitating the introduction of additional sequences. (*B*) Linkers created by a tailed-PCR reaction possess longer stretches of overlap with target BACs.

*CAN1* and canavanine). In practice, the coupling of selection for uracil prototrophy and cycloheximide resistance ($Ura^+$ $cyclo^R$) yielded dramatic increases in recombinational cloning specificity in our hands.

As the Human Genome Sequencing Project proceeds from the rough-draft phase toward completion of the finished sequence (Lander et al. 2001), two related inefficiencies emerge. First, there is often extensive overlap between adjacent BAC clones that define chromosomal tiling paths, and second, there are numerous small, unsequenced gaps that are likely to be spanned by large BACs following targeted screening of BAC libraries (McPherson et al. 2001). Methods to trim the redundancy from these clones could substantially reduce the cost and complexity of finishing the genome sequence. BAC trimming using bacterial recombination systems has been reported (Hill et al. 2000). In this system, subcloning would require two sequential deletions of the redundant BAC segments. In contrast, we report here a method that combines the simplicity of recombination linkers with the robustness of *CYH2* counterselection to create an efficient technology for BAC subcloning. The subcloning efficiency of the method compares favorably with previously reported methods that rely on custom targeting plasmids (Criswell and Bradshaw 1998; Bhargava et al. 1999). We have used yeast recombinational subcloning methods to "trim" 10 BACs in our chromosome 7 sequencing project, reducing the sequencing burden roughly fivefold from 1650 Kb to 350 Kb (C. Raymond, R. Kaul, and M. Olson, unpubl.). To date, restriction enzyme fingerprint comparison between the parental BACs and the recombinational subclones has not revealed detectable rearrangements, hence we believe that yeast subcloning will be a tenable method for large-scale trimming of BACs.

The methods described here have more general application. Many experiments in functional genomics involve the fusion of large DNA sequence blocks, frequently derived from BACs, with reporter genes and selectable markers. Linker-mediated, yeast-based subcloning technology will facilitate the assembly of these large, complex plasmid constructs. Moreover, the increases in recombinational cloning specificity brought about by the *CYH2* counterselection opens the possibility of cloning sequences directly from whole genomes. The most successful examples to date rely on the recovery of cryptic yeast autonomously replicating sequence elements in the targeted genomic fragments (Larionov et al. 1996; Kouprina and Larionov 1999). We have used specific targeting vectors in conjunction with *CYH2* counterselection to recover targeted genomic sequences from the *Pseudomonas aeruginosa* bacterial genome, and have one example of successful capture of a targeted segment from total human genomic DNA (C. Raymond, E. Sims, and M. Olson, unpubl.). The breadth of application, ease of implementation, and modularity of the techniques described here suggest that they have the potential to evolve into standard tools for analyzing and manipulating large segments of genomic DNA.

# METHODS

## Strains and Media

Yeast strain CRY1 (*MATα*, *ura3*Δ, *cyh2*$^R$) was created from the S288C derivative BY4709 (Brachmann et al. 1998) by cotransformation with overlapping 1-Kb PCR products amplified from the *CYH2* locus with primers that incorporate the cycloheximide resistant N37E allele (Table 1). Cycloheximide-resistant strains were selected on media containing 2.5 µg/mL cycloheximide (Spectrum Chemicals, Gardena, CA) made up as a 10 mg/mL stock solution in 100% ethanol. Standard yeast media were used (Sherman 1991). The *E. coli* strain used in this study was DH10B (Hanahan et al. 1991). Antibiotics were from Sigma Chemical (St. Louis, MO) used at concentrations of 100 µg/mL for ampicillin and 3 µg/mL for chloramphenicol.

## Oligonucleotides, Plasmids, Fragments

Oligonucleotides used in this study are listed in Table 1. They were obtained from Invitrogen (Carlsbad, CA) as desalted, lyophilized preparations, and they were suspended in TE (10 mM Tris at pH 8.0, 1 mM EDTA) for use as linkers or water for use as PCR primers, both at 100 µM. *Srf*I restriction enzyme was from Stratagene (La Jolla, CA); all other restriction enzymes were from New England Biolabs (Beverly, MA). Plasmid pRS316 has been described (Sikorski and Heiter 1989). All plasmid assembly was done using yeast recombination. A 100-µL aliquot of electrocompetent cells was cotransformed with 100 ng of vector (containing CEN-ARS and *URA3*) and 1 µg of fragment(s). Total DNA was prepared from yeast transformants for transfer to *E. coli* as described by Hoffman and Winston (1987). Briefly, all of the $Ura^+$ colonies on the surface of a transformation plate were resuspended in 2–5 mL of water (depending on number and size of colonies). Slurry (1 mL) was transferred to a microfuge tube and cells were pelleted at one-half maximal speed in a microfuge for 2 min. The cell pellet was resuspended in 500 µL of yeast lysis buffer (2% Triton X100, 1% SDS, 100 mM NaCl, 10 mM Tris at pH8.0, 1 mM EDTA) and transferred to a tube containing 300 µL of glass beads (Sigma G-8772) and 300 µL of phenol–chloroform (Invitrogen). The sample was vortexed vigorously for 2 min and spun at top speed in a microfuge, and the DNA in 400 µL of the aqueous layer was precipitated with 800 µL of 100% ethanol. The pellet was resuspended in 100 µL of water and 2 µL was used to transform electrocompetent *E. coli* cells to antibiotic resistance. Plasmid DNA from individual colonies was analyzed by restriction digests to confirm that the anticipated construct had been obtained.

Plasmid pCRG3 carries a subclone of the wild-type *CYH2* gene amplified from BY4709 and recombined into the *Sma*I site of pRS316 (Fig. 3). The mini F′ yeast ↔ *E. coli* shuttle plasmid pCRG16 was made by recombining a PCR amplified CEN-ARS and *URA3* segment from pRS316 into the unique *Srf*I site in pBeloBAC11, which itself was derived from pBAC108L (Shizuya et al. 1992). The intermediate plasmid, pCRG4, was linearized with *Not*I and recombined in a four fragment plasmid assembly with overlapping PCR fragments carrying the yeast *CYH2* gene, the ampicillin resistance and colE1 origin of replication regions amplified from pRS316, and an 80-bp double-strand linker to connect the $Amp^R$-colE1 ori segment to the vector. The final construct, pCRG16, is shown in Figure 3.

The chloramphenicol resistance ($Cm^R$) PCR fragment used in the plasmid assembly assay was amplified from pBeloBAC11 (Shizuya et al. 1992). Cotransformations were performed with 100 ng of acceptor vector DNA, 1 µg of PCR-generated donor fragment DNA, and 1 µg (20 pmole) of double-stranded linker DNA. For single-stranded linkers, 1 µg (40 pmole) of 80-bp oligonucleotide was used. The PCR fragment used in the validation of cycloheximide counterselection is a 450-bp fragment amplified from pRS316. This fragment overlaps the double-strand break in pRS316 (*Sma*I) and pCRG3 (*Sna*BI) by 200 base pairs on one end and 250 base pairs on the other. As above, 100 ng of acceptor vector and 1 µg of donor fragment were used in cotransformations. Generic versions of the 80-nucleotide oligonucleotides that constitute the recombination linkers used to subclone BAC segments are shown in Table 1. The BAC-specific sequences are available upon request.

**Table 1. Primers Used in This Study**

| 5′ to 3′ primer sequence | Application |
|---|---|
| ATGCTACGTACCTGTTTAACTCTTC | Amplification of *cyh2*$^R$ (N37E) allele, set 1. |
| GTTAATTCTGTGGTGATGTTCACCACCGGCCATACCTCTAC | Amplification of *cyh2*$^R$ (N37E) allele, set 1. |
| TATGTTTATATATGGATTTTGAAA | Amplification of *cyh2*$^R$ (N37E) allele, set 2. |
| GTAGAGGTATGGCCGGTGGTGAACATCACCACAGAATTAAC | Amplification of *cyh2*$^R$ (N37E) allele, set 2. |
| CCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCATGCTACGTA CCTGTTTAACTCTTC | Amplification of *CYH2* gene for recombinational subcloning into *SmaI* site of pRS316. |
| CGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCCTATGTTTAT ATATGGATTTTGAAA | Amplification of *CYH2* gene for recombinational subcloning into *SmaI* site of pRS316. |
| TTATTTTTATAGCACGTGATGAAAAGGACCGCGGCCGCAAGGGGTTCGCG TTGGCCGATT | Amplification of the CEN-ARS, *URA3* region of pRS316 for recombinational subcloning into *SrfI*-digested pBeloBAC11. |
| TTCAATTTAATTATATCAGTTATTACCCTGCGTCGACCAATTCTCATGTT TGACAGCTTA | Amplification of the CEN-ARS, *URA3* region of pRS316 for recombinational subcloning into *SrfI*-digested pBeloBAC11 |
| CGATTCATTAATGCAGGCCCGGGCATGCTACGTACCTGTTTAACTCTTC | Amplification of *CYH2* for recombinational cloning into *NotI*-digested pCRG4. |
| TATAGCATACATTATACGAAGTTATATTCGATGCGGCCGCCTATGTTTAT ATATGGATTTTGAAA | Amplification of *CYH2* for recombinational cloning into *NotI*-digested pCRG4. |
| AACAGGTACGTAGCATGCCCGGGCCTGCATTAATGAATCGGCCAACGCG | Amplification of the Amp$^R$, colE1 ori region of pRS316 for recombinational subcloning into *NotI*-digested pCRG4. |
| GCGGCCGCGCCCGGGCACGTCAGGTGGCACTTTTCGGGGAA | Amplification of the Amp$^R$, colE1 ori region of pRS316 for recombinational subcloning into *NotI*-digested pCRG4. |
| TTTCCCCGAAAAGTGCCACCTGACGTGCCCGGGCGGCCGCGGTCCTTTTC ATCACGTGCTATAAAAATAATTATAATTTA | Top strand of recombination linker used to join the Amp$^R$, colE1 fragment to *NotI*-digested pCRG4. |
| TAAATTATAATTATTTTTATAGCACGTGATGAAAAGGACCGCGGCCGCCC GGGCACGTCAGGTGGCACTTTTCGGGGAAA | Bottom strand of recombination linker used to join the Amp$^R$, colE1 fragment to *NotI*-digested pCRG4. |
| TCATCGAATTTCTGCCATTCATCCG | Amplification of Cm$^R$ fragment from pBeloBAC11. |
| ACTTTCACCATAATGAAATAAGATC | Amplification of Cm$^R$ fragment from pBeloBAC11. |
| CCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCAATTTCTGCC ATTCATCCGCTTATTATCACTTATTCAGGC | Top strand of linker set 1 used for recombinational subcloning of the Cm$^R$ fragment into pRS316. |
| GCCTGAATAAGTGATAATAAGCGGATGAATGGCAGAAATTGGGGGATCCA CTAGTTCTAGAGCGGCCGCCACCGCGGTGG | Bottom strand of linker set 1 used for recombinational subcloning of the Cm$^R$ fragment into pRS316. |
| TCCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGGGCTGCAGG AATTCGATATCAAGCTTATCGATACCGTCG | Top strand of linker set 2 used for recombinational subcloning of the Cm$^R$ fragment into pRS316. |
| CGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCACTACCGGGC GTATTTTTTGAGTTATCGAGATTTTCAGGA | Bottom strand of linker set 2 used for recombinational subcloning of the Cm$^R$ fragment into pRS316. |
| GGTGCGGGCCTCTTCGCTATTACGC | Amplification of 450 bp "repair fragment" from pRS316. |
| CCGCGCGTTGGCCGATTCATTAATG | Amplification of 450 bp "repair fragment" from pRS316. |
| CGAGCTCATCGCTAATAACTTCGTA | Vector-specific PCR primer used to detect recombinant BAC subclones. |
| TATAGCACGTGATGAAAAGGACCGC | Vector-specific PCR primer used to detect recombinant BAC subclones. |
| TATAGCATACATTATACGAAGTTATATTCGATGCGGCCGCNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNNNNNNNNN | Top strand of "generic" linker set 1 (left side) used to subclone BACs (N=BAC-specific sequence). |
| NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGCGGCCGCAT CGAATATAACTTCGTATAATGTATGCTATA | Bottom strand of "generic" linker set 1 (left side) used to subclone BACs (N=BAC-specific sequence). |
| NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGCGGCCGCGG TCCTTTTCATCACGTGCTATAAAAATAATT | Top strand of "generic" linker set 2 (right side) used to subclone BACs (N=BAC-specific sequence). |
| AATTATTTTTATAGCACGTGATGAAAAGGACCGCGGCCGCNNNNNNNNNN NNNNNNNNNNNNNNNNNNNNNNNNNNNNN | Bottom strand of "generic" linker set 2 (right side) used to subclone BACs (N=BAC-specific sequence). |
| CAAAAAGTTCTGTTGCTTCTGCAGC | RPCI-11-98D12L-specific primer used to detect recombinant BAC subclones. |
| AATCTCTAGTACCAAAGAGAATTAT | RPCI-11-98D12L-specific primer used to detect recombinant BAC subclones. |
| TGAAAGGGATCACTTGTATGATCTG | RPCI-11-98D12R-specific primer used to detect recombinant BAC subclones. |
| CTTTTATTACCCTGAGTTCAGAATT | RPCI-11-98D12R-specific primer used to detect recombinant BAC subclones. |
| AAAAGTATTTTACTAAGGCCAGAAC | RPCI-11-100L10-specific primer used to detect recombinant BAC subclones. |
| TCTACATCCTCCAAGAGATTCACCT | RPCI-11-100L10-specific primer used to detect recombinant BAC subclones. |

The chromosome 7 human BACs used in this study were RPCI-11–98D12 (accession no. AC008154) and RPCI-11–100L10 (accession no. AC087070) (Osoegawa et al. 2001). Plasmid and BAC DNA used in yeast transformations was prepared by growth of *E. coli* cells harboring these plasmids in 20 mL of Terrific broth + antibiotic. Cell pellets were resuspended in 4 ml of 10 mM EDTA (pH 8.0), lysed in 8 mL of 0.2 N NaOH, 1% SDS, and neutralized with 6 mL of ice cold 4M K-acetate (pH 4.5). The debris was removed by centrifugation and the nucleic acids precipitated from the supernatant by the addition of 10 mL of isopropanol. The pellet was washed with 70% ethanol and resuspended in 1 mL of TE containing

100 µg/mL RNAase (Qiagen, Valencia, CA) at 37°C for 30 min. Solid material formed during the incubation was pelleted for 5 min in a microfuge, and the clarified supernatant was transferred into 9 mL of QBT (Qiagen). The DNA was then purified on a Qiagen midi-column as recommended by the manufacturer. BAC DNA was linearized by digestion with *Asc*I or with *Not*I, precipitated with ethanol, and the separate digests were combined in equal amounts to a final concentration of 1 µg/µL prior to transformation. BAC DNA was recombinationally subcloned by cotransforming 100 ng of pCRG16 (*Srf*I) acceptor vector, 1 µg (20 pmole) of double-stranded linker, and 1 µg of digested BAC DNA into yeast spheroplasts, as described below.

### Transformations

Electroporation of yeast cells was performed essentially as described (Meilhoc et al. 1990; Faber et al. 1994). Briefly, freshly grown yeast cells were used to inoculate 200 mL of YEPD broth to $OD_{600}$ of 0.25. The culture was shaken at 30°C for 4–6 h. Cells were harvested at 3500*g* for 1 min and resuspended in 40 mL of KD (50 mM $KPO_4$ at pH 7.5, 25 mM freshly added dithiothriotol) at 30° C for 15 min. Harvested cells were then washed in 200 mL of ice-cold STM (270 mM sucrose, 10 mM Tris at pH 7.5, 1 mM $MgCl_2$), washed in 100 mL of cold STM, and resuspended in 3–5 mL of STM. Aliquots (100 µL) were frozen at −80°C. For transformations, DNA was added to an aliquot of cells and the mixture was transferred to a 2-mm electroporation cuvette (BioRad, Hercules, CA). Cells were pulsed in a BioRad Gene Pulser at 0.75 kV, infinite ohms, and 25 µF, and 600 µl of ice-cold 1.2 M sorbitol was used to resuspend the cells. Aliquots (300 µL) were spread on selective media. The 'LiAc/ssDNA/PEG' lithium acetate transformation protocol was performed exactly as described (Agatep et al. 1998). Spheroplast transformations were performed by using freshly grown yeast cells to inoculate 200 mL of YEPD broth to $OD_{600}$ of 0.25. The culture was shaken at 30°C for 4–6 h. The culture was harvested at 3500*g* for 1 min, washed in 20 mL of sterile water, washed in 20 mL of 1.2 M sorbitol, and resuspended in 20 mL of SCE (1.2 M sorbitol, 100 mM NaCitrate at pH 5.8, 10 mM EDTA). Two hundred microliters of 1 M dithiothriatol and 20 µl of 10 mg/mL Zymolyase 100T (ICN Biomedicals, Aurora, OH) dissolved in 10 mM $NaPO_4$ (pH 7.5) were added and the cells were spheroplasted at 30°C for 30 min. Spheroplasts were harvested at 200–300*g*, washed in 20 mL of 1.2 M sorbitol, washed in 20 mL of STC (1.2 M sorbitol, 10 mM Tris at pH 7.5, 10 mM $CaCl_2$), and resuspended in 20 mL of STC. DNA (<10 µL volume) was added to 100 µL of spheroplasts and incubated at room temperature for 10 min. One milliliter of freshly prepared PEG 8000 (20% PEG Sigma P2139, 10 mM Tris at pH 7.5, 10 mM $CaCl_2$) was added and the incubation was continued another 10 min. The spheroplasts were collected at 200–300*g* and resuspended in 150 µL of SOS (25% YEPD, 1.2 M sorbitol, 7 mM $CaCl_2$) and incubated at 30°C for 30–45 min. Cells were plated in 2.5 mL of top agar (standard drop out media with 1.2 M sorbitol and 2.5% agar) onto selective media plates (containing 1.2 M sorbitol) that were prewarmed to 50°C. No cycloheximide was added to the top agar.

### Detection and Analysis of Recombinant Plasmids

Whole-yeast-cell PCR was used to detect yeast transformants harboring BAC subclones (Ling et al. 1995). Yeast colonies were touched with a yellow tip and the cells were resuspended in 2 µL of spheroplast solution (2.5 mg/mL zymolyase, 1.2 M sorbitol, 100 mM sodium phosphate at pH 7.4). The PCR reaction mixture for 2 µL of spheroplasts in 50 µL of total volume was 0.5 µL of TAQ polymerase (Roche, Indianapolis, IN), 1× manufacturer supplied buffer, 250 µM dNTPs (Roche), and 1.0 µM of each primer. Thermocycler parameters were 95°C for 5 min, 30 cycles of 94°C for 30 sec, 50°C for 60 sec and 72°C for 60 sec, 72°C for 5 min, and then 10°C. PCR reactions were performed in an Applied Biosystems 2700 thermocycler (Foster City, CA). Subsequent restriction analysis on purified BAC and subclone DNA was performed using multiple complete digest (MCD) technology (Wong et al. 1997).

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