Isolating Vector–insert Junctions from Yeast Artificial Chromosomes

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The physical mapping of complex genomes can be achieved by assembling contigs of yeast artificial chromosomes (YACs). In the early stages of map construction, randomly generated markers can be used to screen YAC libraries, order the clones, and assemble the contigs. However, as the maps increase in size, this process becomes less efficient, as randomly generated markers are less likely to add new clones to the ends of the contigs. This problem can be overcome by a more directed approach such as the generation of markers from the ends of YAC inserts. The purpose of this report is to consider the practical aspects of the methods used to isolate vector–insert junctions from YAC clones. Although many techniques are available, the high throughput and success rate of PCR-based strategies may prove to be the most efficient means for closing the gaps between contigs and completing the physical maps of complex genomes.

The advent of yeast artificial chromosome (YAC) cloning systems has facilitated greatly the mapping, cloning, and functional analysis of complex genomes (as reviewed).^(1–3) YACs spanning the euchromatic segments of the human Y⁽⁴⁾ and 21⁽⁵⁾ and extensive portions of the *Caenorhabditis elegans*⁽⁶⁾ and *Drosophila melanogaster*⁽⁷⁾ genomes attest to their importance in genome mapping. The positional cloning of disease-associated genes such as the neurofibromatosis type 1,⁽⁸⁾ adenomatous polyposis coli (APC),^(9,10) and the Huntington's disease genes^(11,12) also relied on cloning and mapping with YACs.

The physical map of a genomic region can be deduced by the assembly of contiguous clones (contigs). Contigs are a collection of cloned DNA segments with individual members that share overlap with at least one other member

of the set.⁽¹³⁾ By analyzing partial or complete restriction fragment patterns (fingerprints) of individual λ phage or cosmid clones, contigs spanning extensive portions of the *Escherichia coli*,⁽¹⁴⁾ *C. elegans*,⁽¹⁵⁾ and *Saccharomyces cerevisiae*^(13,16) genomes have been constructed. Contig construction using YACs has proved more arduous. The fingerprinting of YACs by restriction mapping, although feasible,^(17,18) is confounded by the large genomic insert, repetitive sequences, multiple YACs in a single cell, and chimeric fragments. As an alternative to fingerprinting methods, Green and Olson proposed the concept of sequence tagged site (STS)-content mapping.⁽¹⁹⁾ STSs are small, single-copy fragments in the genome that can be recovered or detected by unique PCR primers.⁽²⁰⁾ Conceivably, YAC contigs spanning entire chromosomes could be assembled by using STS-specific PCR assays to screen libraries and detect overlapping clones. This concept was supported by the use of 16 STSs to align 30 YACs into a 1.5-Mb contig spanning the cystic fibrosis gene region.⁽¹⁹⁾ On a larger scale, the generation of additional STSs should permit complete mapping of an entire genome. In the early stages of map construction, the random generation of STSs may prove to be the most efficient method to initiate contig assembly.^(21–23) However, if the probability of isolating a random STS is predicted by the Poisson distribution, then the efficiency of map construction will diminish greatly as the contigs expand to cover 60–80% of the genome.⁽²⁴⁾ Specifically, calculations based on the Poisson distribution predict that 60% of the total effort would be required to complete the final 13% of the map if a random approach is used exclusively. Palazzolo et al.⁽²⁴⁾ present computer simulations of contig building on

the basis of STS generation by random, single-end, clone-limited (isolation of an STS from one end of a clone), and double-end, clone-limited strategies (isolation of STSs from both ends of a clone). Clearly, the generation of STSs via the double-end, clone-limited strategy, especially in the latter stages of map construction, is the most efficient method for completing contig assembly.⁽²⁴⁾ In practical terms, however, the benefits of contig construction by a directed approach can be offset if methods for isolating STSs from the ends of the clones are inefficient or labor intensive relative to those used for generating random STSs.

The need to develop methods that easily and reliably yield terminal DNA sequences from the genomic insert was apparent early in the evolution of YAC cloning. This was prompted by the simple notion that the termini from YAC inserts will maximize a chromosomal walk by detecting clones in the library with the least amount of overlap. The purpose of this report is to review the utility of the techniques used to isolate YAC vector–insert junctions. The plethora of available techniques suggests that no preferred method has emerged. However, PCR-based techniques, rather than conventional plasmid or λ phage subcloning, have experienced the most widespread use (Table 1). All of the techniques provide the means to isolate STSs that can be used to build contigs and fill gaps. In addition, these end fragments can be used to assess the extent of overlap with other clones, determine telomeric–centromeric orientation of YACs, increase marker density for pulsed-field gel electrophoresis (PFGE) mapping, generate new restriction-fragment length polymorphisms, and identify chimeric YAC clones.

The techniques described in this re-

TABLE 1 Methods for Isolating YAC Vector–insert Junctions

Plasmid rescue
Plasmid rescue after vector arm modifications, in vivo
Conventional subcloning
λ phage plasmids
Genomic sequencing
PCR
Alu–vector anchor–vector inverse

port have been successful in isolating vector–insert junctions from the left or right arms of the pYAC series of vectors.⁽²⁵⁾ For reference, the left arms (LA) of pYAC vectors contain the *CEN4*, *ARS1*, and *TRP1* sequences, whereas the right arm (RA) contains the *URA3* gene. With some modifications, these methods will prove useful in isolating terminal fragments from DNA propagated in other cloning systems (e.g., Pls, BACs, PACs, and cosmids).

PLASMID RESCUE

The isolation of a YAC vector–insert junction by plasmid rescue was described by Burke et al.⁽²⁵⁾ YAC DNA is digested with a restriction enzyme that generates a telomere-free junction fragment. The fragments are self-ligated, and the resultant plasmids are used to transform *E. coli*. The advantages of plasmid rescue are its technical simplicity and the ability to obtain fragments as large as 20 kb. These fragments can be used as fluorescence in situ hybridization (FISH) probes, and their larger size also increases the likelihood of obtaining unique sequence information. Unfortunately, the original design of the pYAC series of vectors placed a *ColE1* origin of replication and ampicillin resistance gene only on the LA. Furthermore, the limited number of available restriction sites in the vector arm (*XhoI*, *NdeI*) limits the usefulness of this technique to isolating those genomic inserts that have either a *XhoI*–*SalI* or *NdeI* rare-cutting restriction site within ~20 kb of the LA cloning site.

Although the RA does not contain a *ColE1* origin of replication or an antibiotic resistance gene, vector–insert junctions can be rescued indirectly. YAC DNA, digested with an enzyme that yields vector–insert fragment containing the *URA3*

gene, is subcloned into a plasmid vector. The appropriate clone is isolated by *URA3* complementation of *E. coli* containing the *pyrF* mutation.⁽²⁶⁾

Newer YAC vectors such as pYAC-neo,⁽²⁷⁾ pJS97/98,⁽²⁸⁾ and the pGS966–999 series⁽²⁹⁾ permit plasmid rescue from both sides of the cloning site. They contain *ColE1* origins, antibiotic resistance genes, and several common restriction sites in both vector arms. As an added feature, some of these vectors contain T3 or T7 bacteriophage RNA polymerase promoters that permit riboprobe synthesis from either side of the cloning site.

The widely available Washington University,⁽³⁰⁾ CEPH,⁽³¹⁾ ICI,⁽³²⁾ and ICRF⁽³³⁾ human genomic YAC libraries were constructed using the pYAC4 vector. To facilitate plasmid rescue directly from these clones, Hermanson et al.⁽³⁴⁾ developed a set of integrating plasmids to retrofit the pYAC4 vector arms with elements present in the newer vectors: dual *ColE1* origins of replication, antibiotic resistance genes, and clusters of restriction sites (Fig. 1). These plasmids, pICL (integrating into *CEN* side with *LYS2*) and pLUS (*LYS2* integration into the

URA3 side) are linearized by endonuclease digestion and are used to transform yeast via a lithium acetate procedure. Transformants are selected for lysine prototrophy. pICL and pLUS integrate by homologous recombination into the ampicillin resistance gene of the LA and a segment of pBR322-related sequence in the RA, respectively. Integration site-specific PCR assays can be used to confirm that pICL and pLUS are inserted appropriately into the vector arms. By linearizing the plasmids before transformation, 70–100% of the lysine prototrophs contain integrating vectors at the appropriate pYAC4 site. As expected, transformation with nonlinearized plasmids dramatically reduces the percentage of *Lys*⁺ cells with correct insertions. Plasmid rescue of the YAC vector–insert junctions is achieved by digesting miniprep DNA of the *Lys*⁺ transformants with a restriction enzyme that cleaves in the polylinker, ligating the restriction fragments, transforming *E. coli* and selecting for ampicillin (pICL) or kanamycin resistance (pLUS) (Fig. 1). Over 50 end fragments, up to 20 kb in size, were rescued using this technique.

FIGURE 1 Plasmid rescue using integrating vectors. (A) Yeast are transformed with the integrating plasmids pICL or pLUS. These vectors contain a multiple cloning site (MCS), a yeast selectable marker (*LYS2*), and a segment that is homologous with sequences in either the left or right YAC vector arm. Integrating vectors are linearized before transformation to enhance homologous recombination. (B) Integration of pICL and pLUS into the left and right vector arms, respectively. (C) Plasmid rescue is achieved by digesting YAC DNA with a restriction enzyme that cleaves in the MCS and at an unknown position in the genomic insert (○). The DNA is self-ligated and used to transform *E. coli*. Adapted, with permission, from Hermanson et al.⁽³⁴⁾

The major disadvantage of this procedure may be the expenditure of time and effort necessary to complete this multi-step cloning process. However, the ability to isolate many large-size end fragments may justify this investment for some positional cloning experiments.

CONVENTIONAL SUBCLONING

Sublibraries of YAC DNA cloned into λ phage or plasmids can be used to isolate vector–insert junctions. Although these techniques are reliable, they are too time consuming and labor intensive for analysis of large numbers of clones. We have found that total yeast DNA partially digested with *MboI* and size fractionated by gel electrophoresis is suitable for ligation to replacement vectors such as λ DASHII (Stratagene) or EMBL3. We routinely screen 10^4 – 10^5 plaques by hybridization with a ^{32}P -labeled LA-specific (2.7-kb *Bam*HI–*Pvu*II pBR322 fragment) or RA-specific (1.7-kb *Bam*HI–*Pvu*II pBR322 fragment) probe to obtain several clones containing the pYAC4 vector–insert junction.

A scheme for directly subcloning into modified pUC19 plasmid vectors has been developed by Chaplin and Geraghty^(35,36) (Fig. 2A). Total yeast DNA is double digested with a rare-cutting restriction enzyme that cleaves in the vector arm and a frequent-cutting restriction enzyme that cleaves in the genomic insert. The frequent-cutting restriction endonuclease may also cleave in the vector arm but not in the interval between the rare-cutting restriction site and the *Eco*RI cloning site. For LA vector–insert junctions, the DNA is digested with *Cla*I and either *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, or *Sph*I. For the RA, *Sal*I is combined with either *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sph*I, or *Hind*III. The use of several frequent-cutting restriction enzymes increases the chances of obtaining a clonable fragment. After digestion, an aliquot of DNA is subjected to gel electrophoresis, blotted, and hybridized with either LA or RA probes (Fig. 2B). Subsequently, vector–insert fragments in the range of 2–7 kb are size selected by gel electrophoresis. The purified DNA fragments are ligated to a modified pUC19 vector that was double digested with the appropriate restriction enzyme. The original pUC19 vector was modified by subcloning a 475-bp *Taq*I stuffer fragment (derived from positions 653–1128

of pBR322) into the *Acc*I site of the poly-linker. This creates the vectors pUC19-ES and HS (Fig. 2A). In pUC19-ES, the *Acc*I site adjacent to the *Pst*I site, but not that adjacent to the *Xba*I site, is preserved. Conversely, in pUC19-HS, the *Acc*I site adjacent to the *Xba*I fragment is preserved, whereas that adjacent to the *Pst*I site is destroyed. The *Acc*I site is compatible with *Cla*I-digested DNA. The presence of the stuffer fragment after digestion and gel electrophoresis confirms that the vector has been prepared appro-

priately. After ligating the size-selected restriction fragments to the vector, *Rec*[−] *E. coli* are transformed and plated at a density of ~200 colonies per plate. Colony lifts are hybridized with either ^{32}P -labeled LA or RA probes (Fig. 2B). These probes can be prepared by PCR or by restriction endonuclease digestion and random priming of the isolated fragments. These pBR322 sequences are adjacent to the *SUP*4 region of pYAC4 and do not hybridize to pUC19. Approximately 1–4% of the colonies should hy-

FIGURE 2 (A) Modified pUC19 cloning vectors for isolating YAC vector–insert junctions. pUC19 vectors were modified by inserting, in both orientations, a 475-bp *Taq*I stuffer fragment. Appropriate double digestion of the vector is confirmed by gel electrophoresis and visualization of the stuffer fragment. (B) Reagents for plasmid cloning of YAC end fragments. pYAC vector arm probe. Map of pYAC vector demonstrating positions and sequences of left- and right-arm-specific probes and primers. The location of the pBR322 *Bam*HI sites that were destroyed during vector construction are shown for orientation. Adapted, with permission, from Chaplin.⁽³⁶⁾ These probes will not hybridize to the pUC vectors.

bridize with the probes. The high percentage of positive clones is caused by enrichment of vector–insert fragments afforded by double digestion with rare- and frequent-cutting restriction enzymes and subsequent size fractionation. Using this technique, Bronson et al.⁽³⁵⁾ constructed a YAC contig spanning a portion of the human major histocompatibility complex that contains the class I genes HLA-B and HLA-C.

GENOMIC SEQUENCING

Described by Coulson et al.,⁽³⁷⁾ this genomic sequencing technique employs the use of a vector-arm-specific primer and yeast miniprep DNA in a thermocycling dideoxy-sequencing reaction. Because this technique does not employ endonuclease digestion, ligation, or subcloning steps, it is a direct method for obtaining sequence information from YAC vector–insert junctions.

Primers specific for portions of *SUP4* gene inclusive of the *EcoRI* cloning site are synthesized for both the LA (pYACL2) and the RA (pYACR4) (Fig. 3). These primers are end-labeled in a kinasing reaction with [γ -³²P]dATP and then used directly in a 10-cycle (denaturation at 95°C for 1 min, annealing at 50°C for 2 min, elongation at 72°C for 2 min, dideoxy-sequencing reaction containing 1–2.5 μ g of genomic DNA. The amplification products are separated by electrophoresis through 6% denaturing acrylamide gels. Gels are dried and exposed to X-ray film that is backed with intensifying screens. After a 3-day exposure, ~150 bp of sequence can be read. Analysis of 180 YACs containing *C. elegans* DNA provided sequence from 275 ends. No sequence and double sequence ladders were obtained from <10% and <3%, respectively, of the reactions. These problems were secondary to inadequate minipreps and multiple transformants,

respectively. PCR primer pairs that amplified >100 bp fragments were developed successfully from ~250 of the DNA sequences. The failure of primers to amplify the correct fragment was caused by misreading of inadequate genomic sequence, misreading of good sequence, and synthesis of self-complementary oligonucleotides. Using the PCR fragments to probe DNA filters, 40 linkages were detected between the ~200 contigs spanning the *C. elegans* genome.

PCR-BASED METHODS

Alu–vector PCR

Approximately one million copies of the *Alu* repeat sequence are interspersed in the human genome.⁽³⁸⁾ Although they are present with an average spacing of 4 kb,⁽³⁹⁾ *Alu* elements tend to cluster in GC-rich, R bands.⁽⁴⁰⁾ The 300-bp *Alu* element consists of a head-to-tail dimer of

FIGURE 3 DNA sequence flanking the pYAC4 *EcoRI* cloning site. The location of various left arm (LA) and right arm (RA) vector primers used for different PCR applications (see text) are depicted by arrows. The vertical line demarcates LA from RA. Insertions and point mutations not predicted by the composite sequence (i.e., pBR322 and *SUP4*) are underlined. This sequence was obtained by standard dideoxy chain termination methods (G.A. Silverman, unpubl.).

two ~130-bp segments that share >70% homology with an original sequence.⁽³⁸⁾ The 3' end of each *Alu* monomer is AT rich, while the remaining portion is 62% GC rich.⁽³⁸⁾ In addition, there is a 31-bp insertion in the right-half monomer that is unique to primates.⁽³⁸⁾ Although different *Alu* families have evolved,⁽⁴¹⁾ Nelson et al.⁽⁴²⁾ developed primers, which permit inter-*Alu* amplification, from a consensus sequence⁽⁴³⁾ (Fig. 4A). Primers that direct DNA synthesis either 5' (#278) or 3' (TC65) of the *Alu* sequence are capable of generating specific products from somatic-cell hybrids, YACs, or λ phage containing human DNA.⁽⁴²⁾ Subsequently, Nelson et al.⁽⁴⁴⁾ extended their observations by demonstrating that YAC vector-insert junctions could be amplified by using either of the *Alu* primers in combination with either a LA (YAC4L) or RA (YAC4R) YAC vector primer (Figs. 3 and 4B). Products were detected using 100–500 ng of total yeast DNA and relatively routine PCR conditions (100 μ l volume: 1 μ M each primer,

50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, 300 μ M each dNTP, 2.5 units of *Taq* polymerase, and 35 cycles at 94°C for 1 min, 55°C for 45 sec, 68°C for 5 min). DNA sequence analysis of one PCR fragment confirmed that it was a vector-insert junction. Amplification products from both ends were obtained from 60% of the YAC clones. The lower success rate of *Alu*-vector PCR relative to the other PCR-based methods (see below) is likely caused by the length of the DNA segment between the *Alu* element and the vector arm being beyond the range that can be amplified by current PCR methods. Nonetheless, the simplicity and versatility of this technique (i.e., no endonuclease digestion or ligation steps) makes this method an indispensable adjunct to most large-scale walking projects. Indeed, 8.0-, 2.2-, and 1.2-Mb YAC contigs encompassing Xq26,⁽⁴⁵⁾ the Huntington's disease gene,⁽¹²⁾ and the iduronate sulfatase gene,⁽⁴⁶⁾ respectively, have been constructed, in part, using *Alu*-vector PCR.

The generation of *Alu*-vector PCR products may be enhanced by using different sets of *Alu* primers. Breukel et al.⁽⁴⁷⁾ detected PCR products in 14 of 21 YACs using different nondegenerate (*Alu3* or *Alu5*) or degenerate primers (PDJ33 or PDJ34) (Fig. 4A).

Anchor-vector PCR

Anchor, oligo-cassette, or ligation-mediated PCR methods can be used to isolate an unknown DNA fragment that is adjacent to a known sequence. This is achieved by ligating a known primer template to the unknown sequence. The presence of known flanking templates permits the intervening sequence to be amplified in a PCR assay. Riley et al.⁽⁴⁸⁾ adapted this technique to isolate vector-insert junctions from YAC clones. YAC DNA is digested with a restriction enzyme that cleaves at a known site in the vector arm and at an unknown location in the genomic insert. Restriction enzymes that cleave between the vector-

FIGURE 4 *Alu*-vector PCR. (A) Consensus human *Alu* sequence. The location of *Alu* primers for different PCR applications (see text) are depicted by arrows. The vertical line demarcates the left from the right *Alu* monomer. The 31-bp sequence unique to primates is in boldface type. Abbreviations: Y = C or T, R = G or A, W = T or A. (B) Example of *Alu*-vector vs. inter-*Alu* PCR. Adapted, with permission, from Nelson et al.⁽⁴²⁾

primer template sequence and the *EcoRI* cloning site must be avoided. For the isolation of LA fragments, the restriction enzymes *AluI*, *BglII*, *EcoRV*, *HincII*, *HinfI*, *PvuII*, and *RsaI* can be used. For the RA, *AccI*, *BglII*, *EcoRV*, *HincII*, *RsaI*, and *PvuII* can be used. Additional enzymes can be used if different vector-arm primers are selected (Fig. 3). The use of different restriction enzymes increases the chances of obtaining a vector-insert fragment that can be amplified by the PCR. After digestion, the DNA is ligated to a partial duplex DNA molecule or vectorette to form a library.

The vectorette consists of two oligonucleotides, a universal bottom strand that serves as an anchor template and a partially complementary top strand that serves as a linker (Fig. 5A). A similar type of cassette has been developed by Roux and Dhanarajan⁽⁴⁹⁾ (Fig. 5B). The vectorette oligonucleotides contain 11 or 12 bp of complementary sequence that flank 29 bp of noncomplementary sequence. By adding or deleting bases to the 5' end of the upper strand, vectorettes compatible with blunt- or sticky-ended genomic fragments can be constructed. This is a potential drawback because the cost of synthesizing an array of several upper strand oligonucleotides may be prohibitive. In practice, however, the blunt-end vectorette is satisfactory for many applications as it is compatible for ligation with DNA fragments digested with either *RsaI*, *HincII*, *PvuII*, or *EcoRV*.

The vector-insert junctions are amplified from the vectorette library using LA (#1089) or RA (#1091) vector primers (Fig. 3) and the universal vectorette primer (#224, Fig. 5A). The universal vectorette primer DNA sequence is identical to that of the "noncomplementary" region of the anchor template (bottom strand, Fig. 5A). The noncomplementary region of the vectorette confers selective amplification to this scheme. Because there is no template available for the universal vectorette during the first round of DNA synthesis, only YAC vector-insert-vectorette fragments will be amplified (Fig. 6). During this first round, YAC vector primers synthesize a new top strand that is complementary to the bottom strand. This generates a template for universal vectorette primer and permits subsequent logarithmic amplification of the vector-insert-vectorette fragment (Fig. 6). Because no first strand synthesis occurred, other DNA frag-

FIGURE 5 Anchors for ligation-mediated PCR. (A) Vectorette or bubble cassette. Adapted, with permission, from Riley et al.⁽⁴⁸⁾ (B) Anchor-adaptor. Adapted, with permission, from Roux and Dhanarajan.⁽⁴⁹⁾

FIGURE 6 Vectorette-YAC vector PCR. (A) YAC DNA is digested with a restriction endonuclease to yield a vector-insert fragment. (B) Vectorettes are ligated to restriction fragments to yield vectorette libraries. (C) Vectorette libraries are amplified using vector (open arrow in B) and universal vectorette (stippled arrow) primers. First-strand synthesis occurs only with the vector primer, as there is no template for the universal vectorette primer. (D) Only vectorette-vector fragments, extended by the vector primer, are available for logarithmic amplification with the universal vectorette primer.

ments ligated to the vectorette will not be amplified by the vectorette primers. PCR products generated by this technique can be reamplified or sequenced using a nested YAC vector (LA #1207 or RA #1208, Fig. 3) or vectorette primers (#368, Fig. 5A). DNA sequence analysis of amplified fragments confirmed that they contained vector–insert junctions.⁽⁴⁸⁾

The YAC vector–vectorette amplification technique has proved to be a robust method for isolating vector–insert junctions. Success rates exceed 90%. The construction of 2.6- and 1.5-Mb YAC contigs spanning the human dystrophin gene⁽⁵⁰⁾ and the region between D21S13 and D21S16,⁽⁵¹⁾ respectively, attest to the utility of this technique.

Other anchor–vector PCR methods have been developed to isolate vector–insert junctions from YAC clones. Kere et al.⁽⁵²⁾ adapted the method of Mueller and Wold⁽⁵³⁾ to isolate 14 insert ends from YACs that map to the human X chromosome. Success rates ranged from 80 to 85%. Capture PCR (CPCR) was developed by Langerstrom et al.⁽⁵⁴⁾ to facilitate end-fragment isolation using genomic DNA or yeast colonies. DNA is digested and ligated to a linker cassette. The ligation products are linearly amplified using a 5' biotinylated YAC vector arm primer. These extension products, now flanked with known template sequences, are purified by capture to streptavidin-coated beads or supports. After extensive washing, the extension products are logarithmically amplified using YAC vector and linker primers. Using only two restriction enzymes, *AluI* and *RsaI*, the success rate was ~85%.

Inverse PCR

Inverse PCR was developed also to isolate an unknown DNA sequence adjacent to a known sequence.^(55–57) Subsequently, this technique was modified and adapted for isolation of pYAC4 vector–insert junctions.^(58,59) First, sets of oligonucleotide primers in inverse orientation (i.e., 3' ← 5'/5' → 3') are synthesized for both the LA and RA (Fig. 3). Next, YAC DNA is digested with a restriction enzyme that cleaves in the vector arm and at an unknown location within the vector insert. *MboI*, *NlaIII*, *TaqI*, *NlaIV*, *HaeIII*, *EcoRV*, and *RsaI* can be used for the LA; and *NlaIV*, *HaeIII*, *NlaIII*, *HhaI*, *SphI*, *AccI*, and *HincII* for the RA (Fig. 3). The use of multiple restriction

enzymes enhances the probability of isolating a useful fragment. However, it may be useful to start with those enzymes that can be used on both vector arms. As with vectorette PCR, care must be taken to avoid the use of restriction endonucleases that cleave in the region between the two primer templates and the *EcoRI* cloning site. After digestion, the DNA is diluted to a concentration between 0.2 and 2.0 µg/ml. Ligation of DNA under these conditions favors the formation of monomer circles (Fig. 7). Circularization reorients the vector primers and permits amplification of the intervening genomic insert (Fig. 7). Confirmation of the appropriate terminal fragment can be verified by DNA sequencing or hybridization of the labeled fragment to blots of YAC DNA.^(58,59)

In comparison with the other PCR-based techniques used to isolate vector–insert junctions, this technique may be more difficult to perfect. However, once the technique is established, individual laboratories report success rates that rival or exceed those of the ligation-mediated methods. We have isolated YAC vector–insert junctions in 26 of 26 attempts. The fragments range in size from 26 to 1250 bp with a mean of ~500 bp. Al-

though a single set of PCR primers can be used for each vector arm, we have found that the combination of certain vector arm primers and restriction enzymes are more successful in yielding PCR fragments. For the LA, we use primer combinations #5 and #8 with DNA digested with *MboI* and *NlaIII* (Fig. 3). Primers #13 and #8 are used with *TaqI*-, *NlaIV*-, *HaeIII*-, and *EcoRV*-digested DNA. For the RA, primers #2 and #3 are used with *NlaIV*, *HaeIII*, *NlaIII*, *HhaI*, and *SphI* digests; and primers #11 and #3 with *AccI* and *HincII* digests.

Megabase-size YAC contigs spanning the *BCL2*,⁽⁵⁹⁾ Huntington's disease,⁽¹¹⁾ and *APC*^(9,10) genes have been constructed using inverse PCR. In addition, Gill and Somerville used a single restriction enzyme and inverse PCR to isolate 12 of 14 end fragments from a modified pYAC vector containing *Arabidopsis thaliana* DNA.⁽⁶⁰⁾ Collectively, these studies demonstrate that inverse PCR is an efficient method for isolating large numbers of vector–insert junctions.

CONCLUSIONS

Methods to isolate DNA fragments or sequences from the ends of the genomic

FIGURE 7 Inverse PCR (A) YAC DNA is digested with a restriction enzyme that cleaves at a known location in the vector arm and at an unknown location in the genomic insert (○). Templates for vector primers are oriented inversely and would direct DNA synthesis in opposite direction (arrows). (B) Restriction fragments are self-ligated to form monomer circles. (C) Circularized vector–insert junctions have reoriented primer templates that permit amplification of intervening genomic insert.

inserts are important adjuncts to YAC cloning. End fragments generated by these techniques facilitate positional cloning of disease-related genes, the closure of gaps between sets of clones, and the integration of contigs into high-resolution physical maps. Although numerous techniques have been developed to isolate end fragments with a high rate of success, no superior technique has emerged. In part, this is attributable to the task at hand. For example, if the goal is to isolate a relatively large DNA fragment for FISH studies, then plasmid rescue or conventional λ phage cloning, rather than PCR, is more likely to yield an end fragment of the appropriate size. In contrast, if the goal is to generate numerous STSs to complete the assembly of a contig spanning an entire mammalian chromosome, then PCR-based techniques may be more advantageous because of their high throughput.

The practical considerations of time and expense must be considered when developing strategies to isolate end fragments. Plasmid rescue and conventional cloning of end fragments can be initiated with reagents readily available to most molecular biology laboratories. However, these techniques may require weeks to isolate the desired fragment. Although time may not be a major consideration when isolating a few end fragments, it becomes a determining factor when large numbers of clones are involved. Using PCR-based techniques, an STS can be developed from the end of a YAC clone insert within a few days. However, when using PCR-based techniques, the cost of oligonucleotides and thermostable polymerases should be taken into account. Use of automation for the isolation of large numbers of end fragments can also help in reducing effort and costs expended on each STS.

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