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USE OF VECTORETTE AND SUBVECTORETTE PCR FOR THE ISOLATION OF TERMINAL SEQUENCES FROM YEAST ARTIFICIAL CHROMOSOME (YAC) CLONES

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ABSTRACT

Development of yeast artificial chromosome (YAC) vectors, molecular cloning of large segments of chromosomal DNA, and their propagation in yeast cells has become feasible. Overlapping YAC provides a route to the development of physical maps of entire mammalian chromosomes. A rapid method was developed to isolate and sequence termini of YAC inserts quickly. The YAC clone is digested with a range of restriction enzymes, and ligated with a linker at its ends. The digested fragments were amplified using modified vector specific primers and a universal linker primer. PCR products were sequenced and the information used to drive new sets of primers for screening of YAC libraries to obtain overlapping clones and construct existing YAC contig.
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INTRODUCTION

The development of yeast artificial chromosome (YAC) cloning vectors[1] made it possible to clone large DNA segments of up to 1 megabase and has provided strong impetus to efforts of generating physical maps of large genomes. Long range physical maps are often obtained from large collections of YAC contigs, overlapping of YAC clones mostly isolated by a process known as chromosomal walking.[2] An important step in chromosome walking is isolation and sequence analysis of YAC termini. Such sequences can be used in restriction map construction of YACs or as sequence tagged sites (STS), landmarks for the mapping of complex genomes[3] or to screen YAC DNA libraries by polymerase chain reaction (PCR). The PCR products are in turn used as probes to identify individual YAC clones by filtration hybridization in the final step of screening.

Several methods have been described according to which terminal sequences of YAC inserts can be isolated. One approach would be to clone encompassing termini of YAC inserts into bacteriophage, plasmid or cosmid vectors through subcloning.[3,4] A major shortcoming of this approach is that it is a rather time consuming and labor intensive procedure. Others[6] have described a method in which the termini of YAC inserts can be isolated via insertion of rescue plasmids into the YAC vector. This method allows the isolation of DNA fragments up to 20 Kb. However, this technique also appears to be time consuming.

Several more rapid methods also exist. They are based on PCR technology. Inverse-PCR involves digestion of the insert with a restriction enzyme, ligation of the YAC-insert end into a circular double-stranded DNA and production of PCR products through amplification with primers. Primers

are designed in such a way as to direct the amplification in opposite direction from the vector region.[7-9]

Vectorette PCR is another method to retrieve an unknown sequence flanking a region of known sequence. The basic vectorette PCR strategy has been decribed previously.[10-20] Briefly, a vectorette-linker library is constructed from digested DNA for use in the PCR. The duplex vectorette linker consists of two annealed oligonucleotides of complementary sequence flanking a nonhomologous region resulting in a "bubble" in the DNA. The primer specific for the vectorette has identical sequence to the nonhomologous region of the linker. The specificity of the PCR is achieved by performing primer extension reactions from a sequence-specific primer to a vectorette linker in the adjacent unsequenced DNA (Figure 1). The synthesized sequence is complementary to the vectorette-specific primer in subsequent PCR cycles. Therefore, a specific DNA fragment can be amplified from a complex mixture of yeast genomic DNA and yeast artificial chromosome and recovered for use in sequencing reactions.

Variations of the vectorette PCR method have been proposed where the oligonucleotide linker sequence is designed to produce maximal amplification.[21,22]

We report a rapid and improved vectorette PCR method for the isolation of YAC-insert end and sequencing. The primers are constructed in such a way as to produce optimal PCR products. The method offers an improvement on previous reports and a large number of YAC ends can be characterized in a short time.

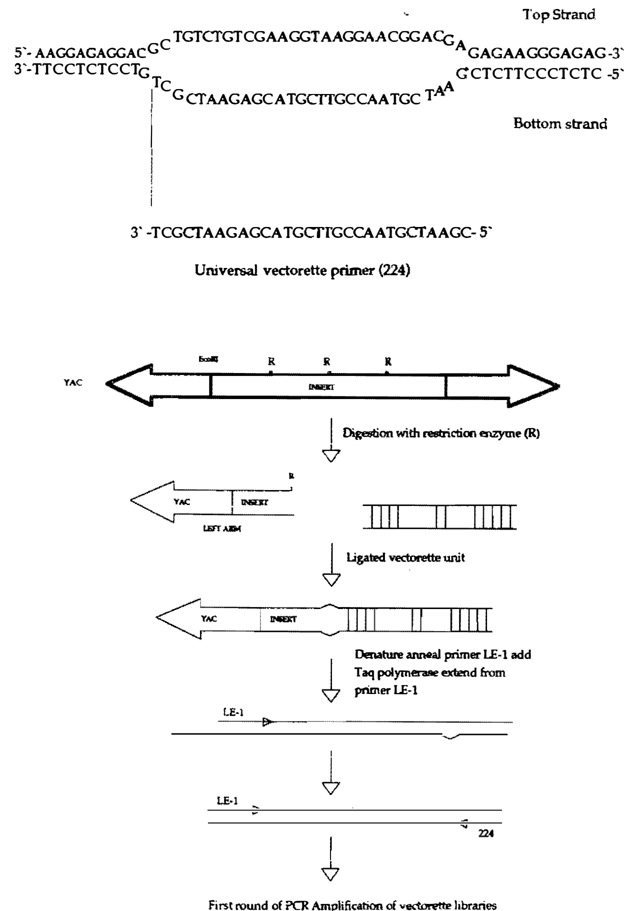

Fig. 1. Sequence of vectorette oligonucleotide showing the position of the universal amplification, and the schematic presentation of the first PCR reaction.

MATERIALS AND METHODS

Construction of vectorette libraries

The construction of mouse genomic YAC and isolation of YAC colonies has been described previously.[23,24] Individual YAC colonies were used to innoculate 5 mL aAHC medium (6.7g/L yeast nitrogen base without amino acid, 10g/L casein hydrolysate-acid, low salt, 40 mg/L adenine hemi-sulphate, pH 5.8), and shaken overnight at 200 rpm and 30°C. Yeast cells containing the YACs were harvested by centrifugation (3,000 rpm, 5min), and washed two times in 50 mM EDTA, pH 8.0. The cells were resuspended to 3.5×10^8 cells/mL density in solution A (1.2 M sorbitol, 20 mM EDTA pH 7.5, 14 mM β-mercaptoethanol) containing 20 μL of 2 mg/mL zymolyase (ICN, Paris, France). An equal volume of 2% Sea Plaque agarose (FMC Bio Products, Hess.- Oldendorf, Germany) in solution A at 40°C was added. The two solutions were mixed gently and 80 μL blocks cast into cleaned, prechilled block formers. The blocks were then placed at 4°C and allowed to set, before they were expelled into 2-3 volumes of solution A for 1 hour to allow spheroplast formation. The blocks were then transfered to solution B (0.1 M EDTA, 10 mM Tris-HCl, pH

Fig. 2. Nucleotide sequences flanking the EcoRI cloning site in the pYAC4 vector, which was used for the construction of the first generation of YAC library, as shown. The relative position of the primers LE-1, LE-2, RE-1, and RE-2 are indicated.

Table I. Sequences of the primers used for the vectorette PCR.

1.	Upper linker	5'-CTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGCTGTCCTCTTCCTTC-3'
2.	Lower linker	5'- GAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGAGAGAAGGGAGAG-3'
3.	224	5'-CGAATCGTAACCGTTCGTACGAGAATCGCT-3'
4.	RE-1	5'-GGTGATGTCGGCGCTCTCGGCGCCAGCAAC-3'
5.	LE-1	5'-GCTACTTGGAGCCACTATCGACTACGCGAT-3'
6.	RE-2	5'-TCGAACGCCCGATCTCAAGATTAC-3'
7.	LE-2	5'-TCTCGGTAGCCAAGTTGGTTTAAGG-3'
8.	Bubble sequencing primer	5'-CGCTGTCCTCTCCTT-3'

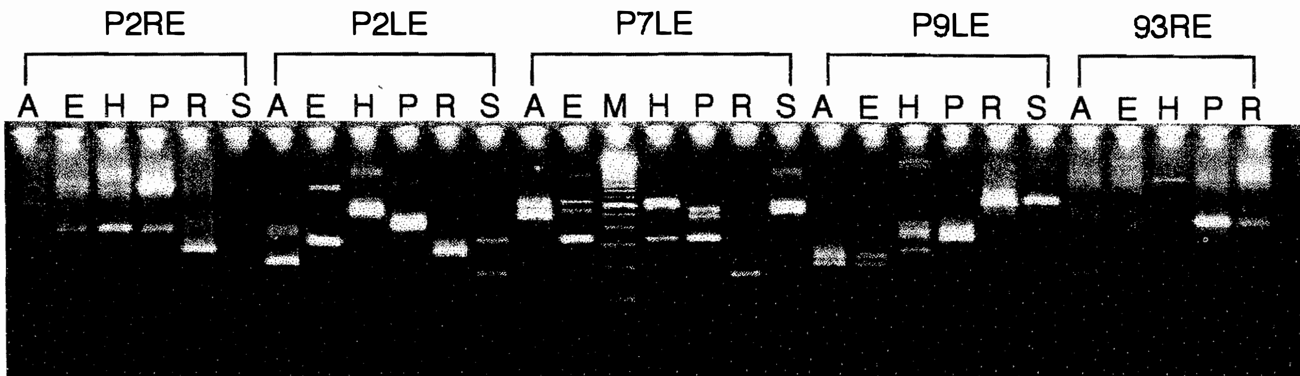

Fig. 3. Agarose gel electrophoresis of end fragments recovered by vectorette-PCR. End fragments are shown for 5 YAC end digested with six different enzymes (A: AluI; E: EcoRV; H: HincIII; P: PuvI; R: RsaI; S: ScaI; M: 100 bp ladder; BRL).

8.0, 1.0% lithium dodecyl sulfate) and incubated at 37°C for 1 hour. The agarose blocks were transfered into a fresh solution B and incubated at 37°C overnight. They were washed in 5 mL of 1X NDS (5X NDS solution: 186 g/L EDTA, 1.2 g/L Tris-base, pH 9.0, 10 g/L N-laurylsarcosine) for 2 hours at room temperature. The blocks were transfered into TE buffer and stored at 4°C. The agarose blocks are stable for over two years.

Each agarose block was cut into 3 equal slices. Prior to restriction digestion, blocks containing high molecular weight yeast/YAC DNA were incubated in H_2O for 20 minutes at 4°C to remove excess salt. Aqueous wash was repeated two more times. Subsequently, the agarose slices were placed in the appropriate 1X restriction enzyme digestion buffer (500 µL) and incubated for 30 minutes at the temperature at which digestion was supposed to be carried out. The restriction enzyme digestion buffer was removed and replaced with 200 µL of the fresh digestion buffer containing 25 units of the appropriate restriction enzymes. YAC miniprep DNA was digested with AluI, EcoRV, HincII, PvuII, RsaI, and ScaI (all from Gibco BRL, Gaithersberg, MD, USA). Digestion of YAC slices were carried out for 4 hours at the appropriate temperatures. At the end of incubation the digestion buffer was removed and the content of the tubes were heated to 65°C for 15 minutes to denature the restriction endonuclease activities and melt the agarose slices.

Two microliters of the melted agarose was added into ligation mixture containing 10 µL 5X ligation buffer (0.25 M Tris-HCl, pH 7.5, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% polyethylene glycol PEG-8000), 1µL T4 DNA ligase (5 U/µL), 1 µL of β-agarase (New England Bio Lab, Beverlym, MA, USA), and 2 µL of linker oligonucleotides (i.e., bottom strand + top strand). The final volume of the ligation mixture was adjusted to 50 µL with H_2O. Information concerning the linker sequences is provided in Table I. Following incubation for 1 hour at 37°C (or overnight at room temprature), the volume of the ligation mixture is adjusted to 200 µL with H_2O and stored at -20°C. All chemicals were of highest purity and purchased from Sigma Chemical Co. (St. Louis, MO, USA). Culture media were obtained from Difco Laboratories (Detroit, MI, USA).

Primers and linker oligonucleotides were purchased from Genset (Paris, France).

PCR amplification of vectorette libraries

PCR amplification was carried out on a Perkin Elmer Thermocycler (Norwalk, CT, USA). Primers RE1 and 224 (Table I) were used for amplification of the right end and LE1 and 224 were used for amplification of the left end. In 50 µL PCR reaction, containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dNTPs, 1.0 pmole of each primer and 1.0 unit of Taq DNA polymerase (Perkin Elmer), 100 ng of ligation mixture was added. In total, 35 cycles of PCR were performed with primary denaturation at 94°C for 9 minutes. Subsequent cycles consisted of denaturation for 1 minute at 94°C, annealing for 1 minute at 62°C and extension for 1 minute at 72°C. The PCR amplification reaction was completed by a final round of extension at 72°C for 7 minutes. Aliquotes of the reaction were analyzed on 2.0% agarose gel. One to 100-500 dilutions of the first PCR products were used as DNA templates for the second round of PCR amplification (nested PCR) using primer RE2 and 224 for the right end and LE2 and 224 for left end amplification. The annealing temperature for the second round of amplification was raised to 65°C. All the other PCR conditions were similar to the first round of amplification. The reaction mixture was analyzed by 2.0% agarose gel and stored at -20°C.

Direct sequencing of the amplified ends

For sequencing of the PCR products with fluorescent-labeled dideoxyterminator (Prism Ready Reaction, Applied Biosystem Kit, Foster City, CA, USA), 2 µL of the secondary PCR reaction products were added directly to a 18 µL volume of sequencing reaction mixture containing 0.5 µL (10 pmole) of the same specific primers, i.e. RE2 for the right end and LE2 for the left end, used for the PCR amplification of the ends, 8 µL of the dye terminator mix from the kit and 9.5 µL of H_2O. Cycle sequencing was carried out on a Perkin Elmer (Norwalk, CT, USA) geneAmp System 2400 thermocycler using 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Sequencing of the dideoxy products was performed on an automated DNA sequencer 373A (Applied Biosystem) according to the manufacturer's protocols and the sequences were analyzed with Sequence Navigator Program on an Apple MacIntosh computer.

RESULTS AND DISCUSSION

In order to facilitate and accelerate the isolation of a series of overlapping YAC clone sequential screening experiments, we sought a rapid and highly reliable procedure to isolate and sequence the terminal fragments of DNA inserts of YAC clones. Figure 1 provides the outline of the method. Yeast spheroplasts were prepared and immobilized in agarose blocks and equilibrated in buffer to remove impurities prior to restriction endonuclease digestion. Since the size of most YAC clones are fairly large, i.e., more than 100 Kb, it is important to avoid degradation of the YAC clones during the course of the reactions. For this reason, the YAC DNA was prepared in agarose blocks to reduce degradation.

After digestion with an appropriate restriction endonuclease, the resulting DNA fragments were ligated to synthetic blunt-ended oligonucleotide duplexes. Restriction endonucleases were chosen to produce blunt-ended cuts. Although with this method it is also possible to use cohesive-end producing restriction enzymes, various types of oligonucleotides are needed for given cohesive-end producing enzymes, whereas with the blunt-ended enzymes one oligonucleotide pair can be used for all the restriction endonucleases. The synthetic oligonucleotide duplexes are called vectorettes and the ligation production of restriction enzyme-digested DNA with vectorette duplex is known as vectorette library. Enzymes that cut at the cloning site (insertion site) or cut between the cloning site and the primer annealing region of the YAC vector (both in the right and the left arms) are not suitable for the generation of libraries.[25]

Primer 224 has been designed in such a way that it is identical to a non-complementary portion of the bottom strand of the duplex. Only terminal fragments containing vector sequences are produced from the yeast vector primer and primer 224. Products can only be synthesized from primer 224 if an initial round of synthesis has taken place from the yeast vector primer (Figure 1). Therefore, only specific amplification products can be amplified from the termini of the YAC inserts. The sequence of YAC vectors and the position of primers are shown in Figure 2. YACs assigned by PCR screening to the mouse chromosome 15 were tested in this report. The YAC clones were cut with AluI, EcoRV, HincIII, PvuII, RsaI, and ScaI.

For most YAC clones, the first round of PCR reaction did not provide enough DNA for sequencing, therefore a second round of PCR reaction with inter-vectorette primers was performed. The ability to recover specific vectorette PCR products depends on the purity of YAC DNA (i.e., stable YACs), the complexity of genomic DNA, the frequency of restriction sites in the DNA used to prepare the vectorette libraries, and the specificity of vectorette primers. The latter was optimized by designing primers with a relatively high GC content (54-58%), which permitted the use of a high annealing temperature (65°C) during PCR. Figure 3 shows the agarose gel electrophoretic separation of the second PCR reaction products of mouse YAC vectorette PCR products.

For most YAC clones more than one amplified reaction was obtained for a given end; therefore it was possible to

verify the result of DNA sequencing of one YAC end with the restriction digestion of at least two enzymes. The sequences obtained from the YAC ends were used to construct a new set of primers and screen the existing YACs for the overlapping region and/or screen YAC DNA libraries for new YAC clones.

The present vectorette amplification of the YAC-insert junction and construction of the vectorette library will facilitate construction of YAC contigs and speed up genomic walking which is very important for any genome study. With the protocol introduced in this report, it is possible to produce YAC-insert information in two days. The insert end sequences will be helpful to retrieve new YAC, BAC, P1 or cosmids from human and mammalian libraries.

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