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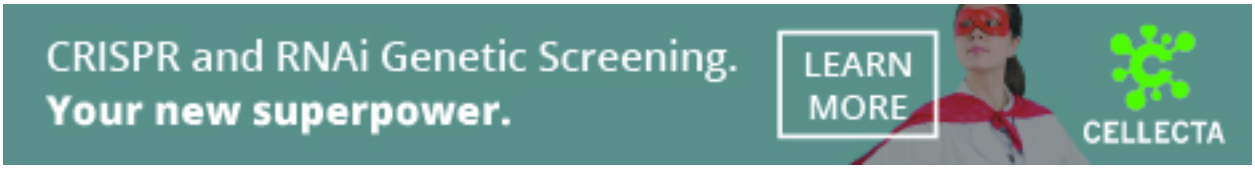

Rapid and Efficient Cloning of Alu-PCR Products Using Uracil DNA Glycosylase

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By incorporating dUMP residues into the 5′ end of PCR primers, one can generate products which, after treatment with uracil DNA glycosylase (UDG), contain 3′ overhangs. These overhangs can be annealed to vector molecules with complementary overhangs generated in a similar fashion and transformed directly into *Escherichia coli* without the need for ligase. We have tested this method of ligation-independent cloning by using UDG to create complementary single-stranded sticky ends between vector and Alu-PCR products generated from cosmid clones containing DNA from human chromosome 21. Using a single primer, Alu-PCR amplifies the sequence between appropriately oriented, repetitive (Alu) sequences in human DNA that are no more than 2 to 3 kb apart. Nineteen Alu-PCR products were observed in four human chromosome 21 cosmids. Thirteen of these products were detected among 48 subclones picked at random after cloning of the Alu-PCR products using UDG. The size or abundance of an Alu-PCR product did not appear to affect significantly the efficiency of cloning. Eight of the subclones were tested and all hybridized to human chromosome 21 DNA. UDG cloning should prove to be a general PCR cloning method that allows one to rapidly subclone small fragments from human genomic DNA.

Cloning of PCR products can be accomplished in several ways; however, all contain drawbacks. Although blunt-end ligation can be used to clone DNA generated by PCR,[1] this method is not as efficient as one that first generates overhangs by restriction enzyme digestion. The latter method requires that restriction enzyme sites be included in the PCR primers and on the absence of the particular restriction site within the fragment to be cloned. This method also requires that the enzyme cut close to the end of a DNA duplex; some enzymes do this better than others. Recently a procedure was reported that avoids these pitfalls by generating a 12-nucleotide 5′ overhang in the PCR products, which are then annealed to a vector with complementary overhangs and transformed into *Escherichia coli* without using restriction enzymes or ligase.[2] This method of ligation-independent cloning uses the 3′ to 5′ exonuclease activity of T4 DNA polymerase to create overhangs, but first it requires purification of the PCR products.

To improve upon this method, we have introduced a dUMP-containing tail to the 5′ end of a PCR primer and used uracil DNA glycosylase (UDG) to generate 3′ overhangs on PCR products. UDG is expressed by organisms ranging from bacteria to human cells and is an important part of the uracil-excision pathway. UDG cleaves the *N*-glycosylic bond between the deoxyribose moiety and uracil in single- and double-stranded DNA.[3,4] This cleavage results in the formation of abasic sites that destabilize base-pairing in double-stranded DNA. We have utilized the property of this enzyme to degrade the dUMP residues for the generation of 3′ overhangs in PCR products. These molecules can then be cloned by annealing to a vector that contains complementary overhangs (generated by the same approach; Fig. 1). We demonstrate that cloning of Alu-PCR products using UDG is both rapid and efficient. Alu-PCR products ranging in size from 0.25 to 1.7 kb were subcloned from four human chromosome 21 cosmids.

METHODS

Enzymes and Reagents

Taq DNA polymerase was purchased from Perkin-Elmer Cetus; dNTPs were from Boehringer Mannheim. Com-petent bacteria (DH10B), proteinase K, and restriction enzymes were from BRL. Oligonucleotides were synthesized using an ABI-380A DNA synthesizer and purified by Sephadex G-25 chromatography. The following primers were used: vector primers 5′-(UAG)4 ACC ATC GTC GAC CTG CAG, and 5′-(UAG)4 ACC ATC GGA TCC CCG GGT; Alu primer 5′-(CUA)4 GCC ACT GCA CTC CAG CC.

Amplification of Vector and Human Cosmid DNA

All PCR reactions were 50 μl covered with mineral oil using the following final buffer concentration: 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, and 0.2 mM of each dNTP. A Perkin-Elmer Cetus thermal cycler was used to generate Alu-PCR products as well as to analyze the inserts from subclones. After an initial 5 min at 93°C, 35 cycles of 1 min at 60°C, 1 min at 72°C, and 1 min at 93°C were used. An additional 5 min at 72°C was used for the last cycle. Twenty to 30 ng of each

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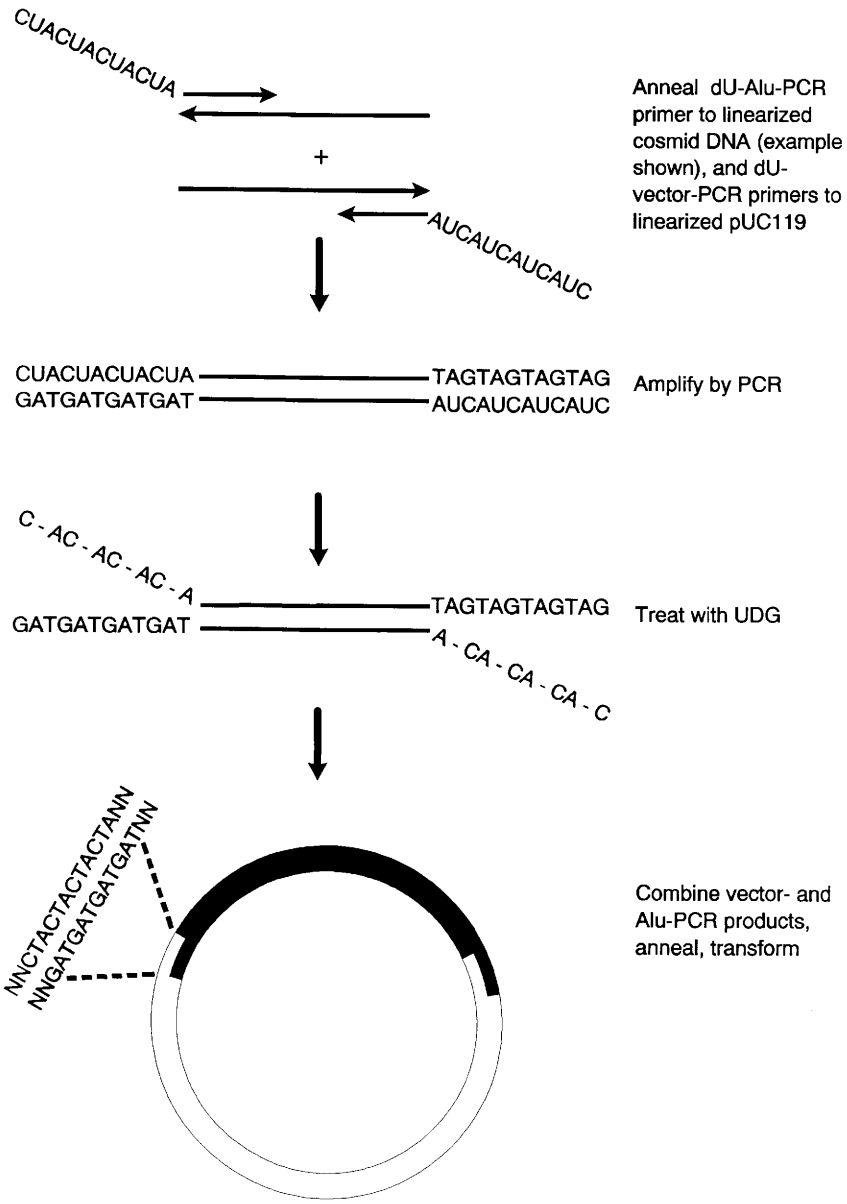

FIGURE 1 Schematic representation of uracil DNA glycosylase cloning of Alu-PCR products.

of the four *Not*I-linearized cosmids was amplified; 1 ng of *Xba*I-linearized pUC119[5] was amplified as described above. Products from PCR reactions were analyzed by 1% agarose gel electrophoresis in TAE buffer with ethidium bromide.

UDG Treatment

The vector and Alu-PCR products were precipitated with ethanol and dissolved in the following buffer (25 mM Tris-HCl [pH 7.8], 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, 0.4 mM ATP). Single-stranded 3′ overhangs consisting of 10 nucleotides in the vector and 11 nucleotides in the Alu-PCR products were made by treating vector (225 ng) and Alu-PCR products (110–212 ng) each separately with *E. coli* UDG (BRL) in a final volume of 10 μl for 10 min at 37°C. Initial experiments used 16 units of UDG; however, as little as 1 unit has been found to be sufficient. A 10-min treatment at 65°C was used following the UDG treatment.

Cloning and Transformation

UDG-treated vector (45 ng) was combined with UDG-treated Alu-PCR reaction products (45–106 ng) in a final volume of 20 μl in the above Tris, $MgCl_2$, β-mercaptoethanol, ATP buffer for 1 hr at room temperature. A 5-μl amount from each combination was transformed into 50 μl of DH10B competent cells (BRL) following the manufacturer's recommendations, and plated onto LB plates containing ampicillin, X-gal, and IPTG.

PCR Analysis of Transformants

Subclones were analyzed by PCR using the Alu primer. Single white colonies were dispersed into 12 μl of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 μg/ml proteinase K and incubated at 55°C for 15 min, 80°C for 15 min, and chilled on ice. PCR components including the Alu primer were added and amplified for 30 cycles using the above protocol. A 5-μl amount of each analysis was run on an agarose gel for sizing.

Southern Blot Analysis

One hundred to 200 ng of DNA from the PCR analysis of individual transformants was labeled by random primer extension (BRL),[6] and preannealed with human Cot-1 DNA (BRL) following the manufacturer's recommendations. The preannealed probes were hybridized to nylon filters containing 5 μg per lane of *Eco*RI-digested DNA from a human lymphoblastoid cell line, mouse (3T6) cells, Chinese hamster (CHO-K1) cells, and two somatic cell hybrids that contain human chromsome 21 as the only human chromosome: WAV-17 (human x mouse) and 153E7b (human x hamster). The filters were hybridized in modified Church and Gilbert solution, 0.25 M NaPi (pH 7.2), 7% SDS, 1 mM EDTA,[7] overnight at 65°C, washed to 0.3x SSC at 65°C, and autoradiographed.

RESULTS

To generate PCR products containing dUMP, DNA sequence from four human chromosome 21 cosmids was amplified with an Alu primer that contained (CUA)4 at its 5′ end. Linearized vector DNA, pUC119, was amplified using primers with (UAG)4 at the 5′ ends. Treatment of such PCR products with UDG resulted in removal of the dUMP residues and generated single-stranded 3′ overhangs (Fig. 1). The

cleaved products were then allowed to anneal at room temperature and used directly to transform *E. coli*. Forty-eight independent transformants from the four cosmids were picked and insert DNA prepared by PCR. The DNA from single colonies was prepared by proteinase K digestion, followed by addition of Alu-PCR reaction components and amplification to regenerate the insert. These products were run side-by-side with the products of the original PCR reaction containing the entire cosmid DNA on an agarose gel (Fig. 2). One prominent and several less abundant Alu-PCR products were observed for cosmid 611 (Fig. 2A). Nine out of 12 pUC119 subclones contained the most abundant 0.25-kb product. Three other subclones were obtained from cosmid 611, including one subclone (lane 8) which gives a faintly visible band of 1 kb in the lane containing cosmid 611 DNA (lane A). For cosmid 702 (Fig. 2B), three prominent Alu-PCR products were observed (lane B), and all were represented among the subclones analyzed. Of the five visible bands in the original Alu-PCR of cosmid 706 (Fig. 2C, lane C), three different clones were identified in the 12 that were selected for analysis. Finally, two of the three Alu-PCR products from cosmid 708 (Fig. 2D, lane D) were represented in the panel of 12 subclones analyzed, including two clones containing inserts that are barely detectable in the original Alu-PCR. From the four cosmids examined, 48

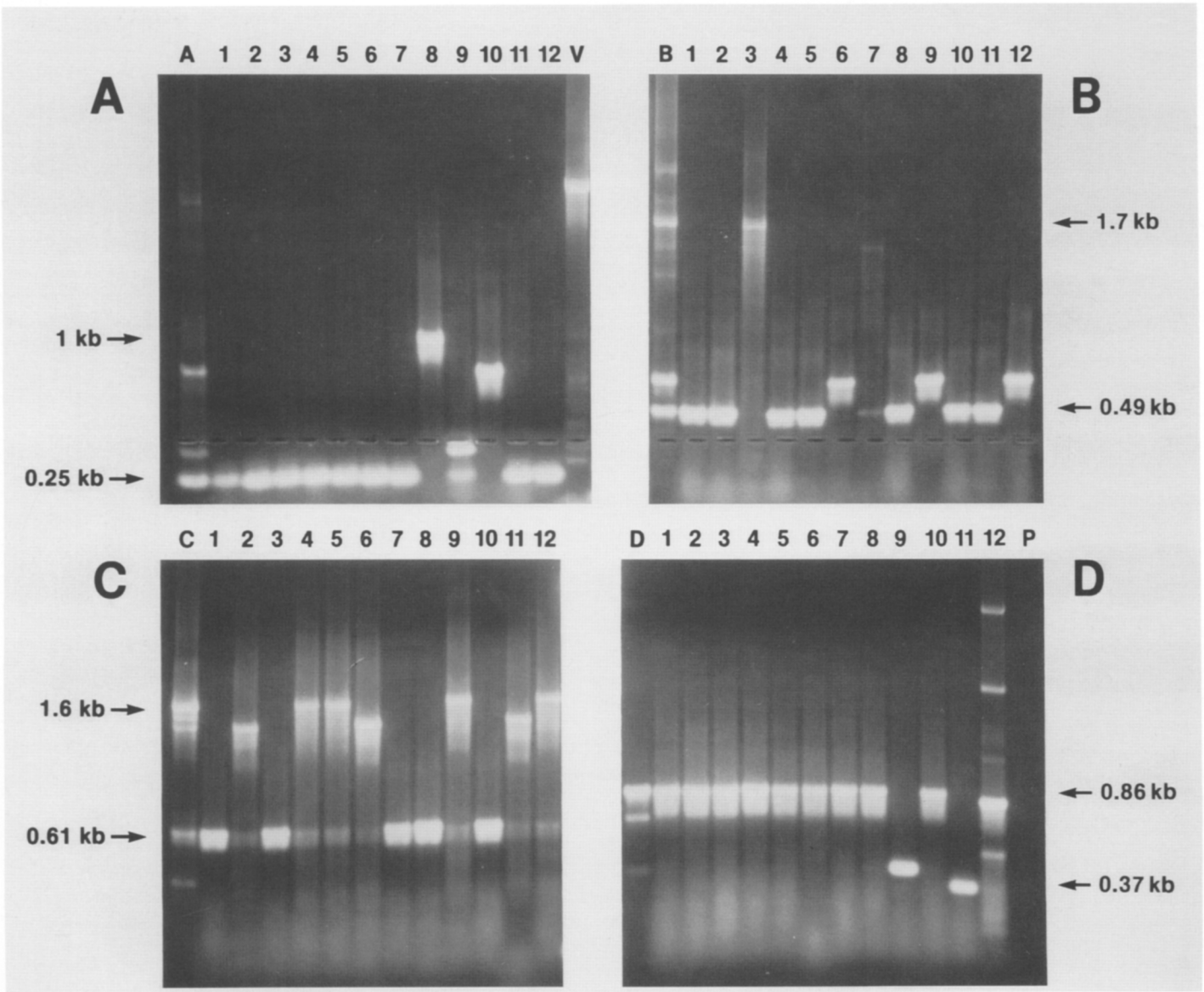

FIGURE 2 Alu-PCR of cosmids and DNA miniprep analysis of Alu-PCR subclones. Alu-PCR products from 4 human chromosome 21 cosmids are displayed side by side with the inserts generated by Alu-PCR from 12 subclones for each cosmid. (*A*) Cosmid 611; (*B*) cosmid 702; (*C*) cosmid 706; (*D*) cosmid 708. (Lanes *A*, *B*, *C*, *D*) Alu-PCR reaction products of cosmid DNA; (lanes *1–12*) individual subclones (colonies) from cosmid analyzed by Alu-PCR. (*A*; lane *V*) Linearized pUC119 DNA. (*D*; lane *P*) Supercoiled pUC119 DNA. Molecular size markers are the 123-bp and 1-kb ladders from BRL (not shown).

white colonies were analyzed and all contained inserts. From transformations performed from each cosmid, approximately 6000 colonies were obtained. Less than 1% of the total colonies were blue, or pUC119 vector without insert. Faint blue colonies accounted for less than 5% of the total number of colonies and were most likely due to in-frame cloning events.[2]

The cosmids used in this report were isolated from libraries made from somatic cell hybrids that contain human chromosome 21. The chromosome 21 origin of the Alu-PCR products was confirmed by ^{32}P-labeling DNA from individual subclones by random primer extension, and preannealing with human Cot-1 DNA to suppress repetitive DNA sequences. Probes were then hybridized to total human, hybrid, mouse, and hamster DNA (Fig. 3). Probes from clones A8, B1, B3, B6, C1, C2, D1, and D9 gave hybridization results consistent with their human chromosome 21 origin. Figure 3 shows a representative autoradiograph of probe C2, confirming the chromosome 21 origin of this subclone.

Although the products of the Alu-PCR reaction would be expected to contain small amounts of Alu sequence (Alu primers are at the extreme 3′ end of the Alu consensus sequence[2,8]), all of the subclones required repetitive DNA sequence suppression with Cot-1 DNA.

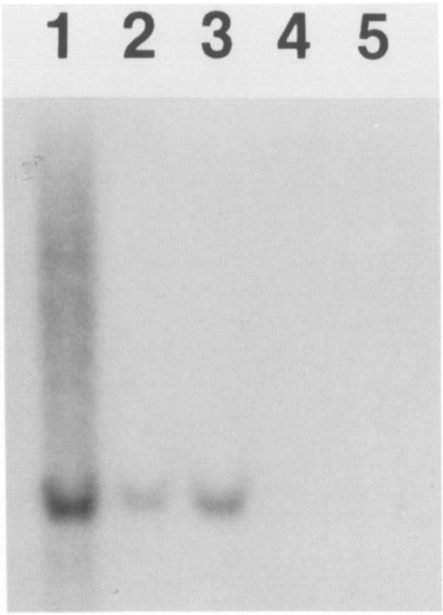

FIGURE 3 Southern blot of genomic DNA hybridized to an Alu-PCR subclone from human chromosome 21 cosmid 706. Subclone C2 insert DNA was labeled by random primer extension and hybridized to *Eco*RI-digested (lane *1*), human lymphoblastoid DNA, 153E7b (lane *2*), WAV-17 (lane *3*), NIH3T6 (lane *4*), CHO-K1 DNA (lane *5*).

DISCUSSION

We have developed an efficient and rapid method for the cloning of PCR products. By incorporating dUMP into the 5′ end of PCR primers and subsequently treating the reaction products with uracil DNA glycosylase (UDG), 3′ overhangs were generated. These molecules were annealed to a vector containing complementary ends and transformed directly into *E. coli*. Using this procedure, 13 of 19 Alu-PCR products from four human chromosome 21 cosmids were recovered after a rapid analytical screening of 48 subclones. All subclones tested hybridized to chromosome 21 DNA. In contrast to conventional PCR cloning procedures, this method does not require restriction enzymes or ligase, and there is no need to purify the PCR reaction products prior to cloning. In addition, rapid screening of single colonies to confirm the presence and size of insert can be performed. This method has been used to clone the amplification products from a single primer hybridized to appropriately oriented Alu repetitive elements in human DNA (Alu-PCR)[9]; however, it should be of general use for cloning other PCR products.

We have recently extended UDG cloning to more complex DNAs such as yeast artificial chromosomes (YACs). This method has also been successfully employed to generate libraries of chromosome-specific DNA sequences from somatic cell hybrids that contain single human chromosomes. Library complexity may be improved by using an additional Alu primer as well as primers homologous to other highly repetitive DNA elements such as L1, THE, or other moderately repetitive sequences.[10]

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