Direct and crossover PCR amplification to facilitate Tn5supF-based sequencing of λ phage clones

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ABSTRACT

The 264 bp mini-transposon Tn5supF was constructed to sequence DNAs cloned in phage λ without extensive shotgun subcloning or primer walking. Unique sequences near each transposon end serve as primer binding sites, and a *supF* gene is used to select transposition to λ . We describe here PCR methods that facilitate Tn5supF-based sequencing. In a first pass, insertions are mapped relative to the ends of the cloned fragment using pairs of primers specific for vector DNA next to the cloning site and for a Tn5supF end. Most insertions not mapped in this step are near the center of the cloned fragment or in the vector arms, and are then mapped relative to the two innermost insertions by 'crossover' PCR. This involves amplification from primers on different DNA molecules, and generates hybrid DNA products whose lengths correspond to the distances between the two insertions. We routinely amplified more than 6 kb in direct PCR and 3 kb in crossover PCR; at the limit we amplified up to ~10 kb in direct PCR and ~6 kb in crossover PCR, but not reproducibly. Crossover PCR products were also obtained with insertions separated by only 200 bp, indicating that no rare sites are needed to switch templates. PCR products were purified by adsorption and then elution from glass slurry, and sequenced directly. Ladders of more than 400 bp were obtained from primer sites on each DNA strand; 2 kb was read from crossover PCR products, and showed that they were amplified with fidelity. In conclusion, direct and crossover PCR methods expedite transposon insertion mapping, and yield templates for accurate sequencing of both DNA strands.

INTRODUCTION

More efficient methods for preparing DNA for sequencing will be important in many areas, including analyses of gene structure and regulation, and large-scale genome sequencing. Strategies

in which bacterial transposons serve as mobile sources of universal primer binding sites are appealing as alternatives to shotgun subcloning (with its attendant redundant sequencing), and to primer walking, which is slow, costly, and inappropriate for segments of repetitive sequence (1–3). Tn5 is one of the transposons best suited for sequencing because it can insert into dozens of sites per kb of target DNA, and moves by a cut-and-paste conservative mechanism that generates simple insertions, not cointegrates (4,5). The 5.8 kb wild type Tn5 element itself is not convenient as a sequencing tool because it contains terminal 1.5 kb inverted repeats (the insertion sequence IS50), and thus does not provide unique leftward and rightward primer binding sites. An element with just 19 bp from each Tn5 or IS50 end will transpose, however, as long as transposase is provided in cis.

We constructed a 264 bp mini-Tn5 element called Tn5supF for analyses of λ phage clones. It contains a *supF* (suppressor tRNA) gene for selection, and the 19 bp ends of IS50 (3; Fig. 1). Tn5supF insertions in λ phage can be obtained by selecting plaques on a *dnaB*_{amber} strain (the DnaB protein is needed during λ replication; 6), a selection that does not require lysogenization or survival of infected cells. The small size of Tn5supF ensures that its insertion will rarely, if ever, make the resultant DNA too large for the λ phage head. The efficiency of transposon-based sequencing of λ clones had previously been limited by the effort of preparing many phage DNAs, and restriction mapping insertion sites before sequencing.

The polymerase chain reaction (PCR) has revolutionized much of molecular biology because it provides an easy means of amplifying and isolating any short DNA segment, once sequences bracketing the segment that can serve as primer binding sites have been defined (7–9). Although amplification usually proceeds from a single DNA species, hybrid products also arise due to priming at sites on different molecules ('crossover', 'biparental' or 'jumping' PCR. Crossover PCR can be ascribed to the relatively low processivity of commonly used thermostable DNA polymerases (e.g., Taq incorporates an average of 60–100 nt per single extension, but re-initiates synthesis many times from 3' ends of growing strands during single amplification cycles (10):

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the melting and reannealing steps allow DNA strands partially extended on a template from one parental phage to prime further synthesis on a template strand from the other parent, thereby generating hybrid products that can be further amplified (Fig. 2B). Although crossover PCR has sometimes been a nuisance to be avoided, as in the amplification of single genes from heterozygous diploid individuals (11–14), it has also been exploited to construct specific recombinant genes in vitro (15–20).

We describe here methods for mapping transposon insertions in λ clones by direct and crossover PCR on aliquots of phage plaques, and for using the PCR products as templates to sequence the DNA of interest. These methods are fast and efficient, and should be amenable to automation.

MATERIALS AND METHODS

The *E. coli* K-12 strains used in this study were: (i) DB4496, the host for Tn5supF transposition. It carries the single copy Tn5supF-donor plasmid pBRG1310, and is deficient in DNA adenine methylation due to a *dam::Tn9* chromosomal mutation [Tn5supF transposition is higher in *Dam*[−] than in *Dam*⁺ cells (3)]. (ii) DK21, the host for selecting Tn5supF insertions in λ by plaque formation. It is Sup^o and carries an amber mutant allele of *dnaB*, a gene which is essential for λ DNA replication, and a *ban* (*dnaB* analog) gene from phage P1, which allows bacterial replication, but not λ replication (6); and (iii) MC1061, a standard F[−] sup^o laboratory strain used for routine growth of λ phages, independent of whether they carried Tn5supF. The λ phages used contain cloned *E. coli* chromosomal DNAs (21, 22): λ 138 (10A6), λ 551(1C10); and a derivative of λ 656 (5B5) that carries a *kan* insertion at a central location (23). Tn5supF insertions in these phages were generated by growth on strain DB4496 and selection on DK21 as described (3), and the insertions used (Fig. 3) were mapped by direct and crossover PCR methods described below. Standard conditions were used for bacterial and λ phage growth (23–25). PCR amplification was carried out with thermostable polymerases Amplitaq (Perkin-Elmer Cetus, Norwalk, CT), Hot Tub (Amersham, Arlington Heights, IL) and Vent (New England Biolabs, Beverly, MA), using reaction buffers provided by the suppliers, after optimizing the Mg²⁺ concentrations as recommended. The following oligonucleotide primers were used for PCR and sequencing: (i) λ left arm cloning site (5'-GGCCATAGAGTCTTGACAGACAACTGC; underlined, λ coordinates 27928–27951); (ii) right arm cloning site (5'-GCGTAACGATCATATACATGGTCTCTCC; underlined, λ coordinates 34569–34544), (iii) Tn5supF I end (5'-TAGGAT-CCCGAGATCTGATC); (iv) Tn5supF O end (5'-TAGGAT-CCCCTACTTGTA; 3); (v) 5' upstream of the *kan* gene from pUC4K (5'-TGGTATGAGTCAGCAACACCTTCTT; 26); and (vi) 3' downstream of the *kan* gene from pUC4K (5'-ATCAGAGATTTTGAGACACAACGTG; 26). Electrophoretic conditions for agarose and polyacrylamide gels were as described

Fig. 1. Structure of the 264 bp mini-transposon Tn5supF. ▨ and ▩, divergent I and O ends that constitute the sites needed for transposition; → and ← primer binding sites.

(24). The 1 kb molecular weight standard (Life Technologies Inc., Gaithersburg, MD) was used as size standards in electrophoresis (fragment sizes: 12.2, 11.2, 10.2, 9.15, 8.15, 7.13, 6.1, 5.1, 4.1, 3.1, 2.0, 1.64, 1.02, 0.52, 0.40, 0.35, 0.30, 0.20, 0.13, 0.08 kb). PCR products were purified for sequencing by adsorption to a glass slurry (GeneClean kit; Bio101 Inc., La Jolla, CA), and then batch elution in distilled water or TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). In some cases the DNA was electrophoresed in an agarose gel, and a specific DNA band was purified with GeneClean. DNA sequences were obtained by the linear amplification (cycle) sequencing method (27–29), using dNTPs and ddNTPs from Boehringer-Mannheim (Indianapolis, IN) and Amplitaq (Perkin-Elmer Cetus), or the ds-DNA cycle sequencing kit (Life Technologies Inc.), and using purified PCR fragments or aliquots of λ phage plaques, as appropriate.

RESULTS

Mapping transposon insertions by direct PCR

If PCR amplification for half the length of DNA segments cloned in λ were routine, all Tn5supF insertions of interest in a λ clone could be mapped in one attempt using primers that match λ vector sequences next to the cloning site and that match Tn5supF ends. The appearance and size of an amplification product would indicate the orientation of the inserted transposon and its position in the cloned DNA (Fig. 2A). Because most current PCR protocols have been designed or optimized for amplification of,

A. DIRECT PCR

B. CROSSOVER PCR

Fig. 2. PCR amplification. (A) Direct PCR amplification on a single species of DNA molecule, using primers specific for λ arms and Tn5supF ends. (B) Crossover (biparental) PCR between phage containing Tn5supF insertions in same orientations.

at most, a few kb (9), and standard λ cloning vectors have a capacity of 13–20 kb, we began tests with Tn5^{supF}-insertion derivatives of several λ phage clones (Fig. 3) to find conditions for long distance amplification. The following protocol for PCR between Tn5^{supF} insertions and λ DNAs adjacent to the cloning site has given the longest distance amplification to date: (i) Suspend fresh plaques of Tn5^{supF}-containing phage from a lawn of strain DK21 or MC1061 in 100 μ l sterile distilled water, and use 5 μ l aliquots of the suspensions for PCR [the remainder can be stored at 4°C for one to two weeks for additional PCR, or phage growth, as needed]. (ii) Carry out PCR in a total volume of 25 μ l in a cocktail containing 1.5 mM MgCl₂ (for Amplitaq and Hot Tub DNA polymerases) or 2 mM MgSO₄ and 1 mg/ml BSA (for Vent DNA polymerase), 200 μ M dNTPs, 10 picomoles of each primer (one vector-specific and one Tn5^{supF}-specific), and 1 unit of the thermostable DNA polymerase (mixtures made at room temperature). (iii) Immediately place tubes in thermal cycler and amplify with the following cycling conditions: 1 cycle of 94°C, 1 min; 53°C, 30 sec; 72°C, 5–7 min; then 34 cycles of 94°C, 20 sec; 53°C, 30 sec; 72°C, 5–7 min; and finally 1 cycle of 94°C, 20 sec; 53°C, 30 sec; 72°C, 10 min in a model TC1 or TC480 thermal cycler (Perkin-Elmer Cetus). (iv) Electrophorese 5–10 μ l aliquots of each reaction mix in an agarose gel, and infer locations of Tn5^{supF} insertions from sizes of PCR products obtained. Using these conditions, up to 6 kb was usually amplified with Amplitaq, Hot Tub and Vent polymerases (more than 80% of attempts); on occasion amplification proceeded for up to 9 kb with Hot Tub, 10 kb with Amplitaq and 12 kb with Vent polymerase. Representative results are shown in Figs. 4 and 5. These best-case amplifications were not obtained reproducibly, however. We found that enzyme aliquots drawn from different batches, and also from different vials of the same batch of Amplitaq differed in ability to amplify DNA segments in the 6–10 kb length range, despite equivalent amplification of 1–2 kb. Variability was also seen in less extensive tests with Hot Tub and Vent polymerases. Use of high titer phage plate stocks or purified phage DNA gave results equivalent to those obtained with plaques; these more time-consuming preparation steps did not increase the length of DNA that we could amplify, decrease the variability in success of longest amplifications, or affect the accumulation of spurious fragments with Vent polymerase.

In studies with the best vials of Amplitaq, shortening the 72°C extension step to 3–4 min allowed amplification only up to about 5 kb (maximum), while lengthening the extension time to more than 10 min usually resulted in heterogeneous smears (30) instead of discrete bands. Attempts to amplify more than 8–10 kb sometimes resulted in spurious shorter DNA fragments (fast-migrating bands) (Fig. 5). These bands were more abundant with Vent than with Amplitaq or Hot Tub polymerases. Some may have resulted from false priming; others probably resulted from internal deletions generated by polymerase slippage during amplification from the intended primer binding sites, and then selection during PCR. Possible mismapping due to spurious long distance PCR products can be avoided by crossover PCR mapping against other known insertions and by use of other thermostable polymerases.

Further mapping of transposon insertions by crossover PCR

The crossover PCR method (Fig. 2B) was developed to map insertions near the center of cloned DNA fragments and beyond the reach of direct PCR from vector sites, and also to verify and refine maps made by long distance direct PCR. To map insertions near the center of a cloned fragment, aliquots of single plaques containing the innermost known insertions were mixed with aliquots of other λ ::Tn5^{supF} phage with unmapped insertions, and the DNA between known and unknown insertions was amplified with Tn5^{supF}-specific primers. Insertions were fine structure mapped similarly, using appropriate pairs of insertion-containing phage. Sites of insertion were inferred after electrophoresis in agarose gels from the sizes of crossover PCR products, as above.

Crossover PCR amplification was carried out as with direct PCR, but using mixtures of phage carrying different insertions and 5 pmoles each of the Tn5^{supF} O and I end primers. The cycling conditions found to be optimal for crossover PCR also differed slightly from those for direct PCR: 2–5 cycles of 94°C, 1 min; 53°C, 30 sec; 72°C, 30 sec; then 34 cycles of 94°C, 20 sec; 53°C, 30 sec; 72°C, 5–7 min; and one final cycle of 94°C, 20 sec; 53°C, 30 sec and 72°C, 10 min. Using this method, we obtained crossover PCR amplification for distance of up to 3 kb routinely, and up to 6 kb on occasion using Amplitaq polymerase (Fig. 6, left panel). At the other extreme, we reproducibly obtained crossover PCR products between sites as

Fig. 3. Sites of Tn5^{supF} insertions in λ phage used in present experiments. Sites of insertion were determined by direct and crossover PCR methods and verified by DNA sequencing in several cases, as described in the text. $\blacksquare$ and $\blacktriangle$, left and right arms, respectively, of λ vector. 33 of the 36 insertions shown here were inserted with the Tn5^{supF} I end closest to the left end of the cloned segment, such that *supF* could be read from the PL promoter in the λ vector; the three exceptions (in the opposite orientation) were insertions 4, 5 and 6 in λ 551.

close as 200 bp, and each of the 21 intervals of <1 kb tested gave crossover PCR products (Fig. 6, right panel). These outcomes indicate that no special sites are needed to trigger the jumping between templates that result in crossover PCR amplification. Similar (but no better) results were obtained in carrying out crossover PCR with high titer phage stocks or DNA purified from them.

DNA sequencing templates prepared by direct and crossover PCR

The products of direct PCR, which map *Tn5supF* insertions relative to vector sites, are equivalent to a nested deletion series (Fig. 2A), and allow one strand of target DNA to be sequenced using the appropriate *Tn5supF* primer. Those crossover PCR

Fig. 4. Products of direct PCR amplification by Amplitaq DNA polymerase. Numbers above each lane indicate sites of *Tn5supF* insertion in λ clones (see Fig. 3). The sizes in kb of largest fragments obtained in these representative amplifications (with phages λ 138-7, λ 551-6 and λ 138-9) are indicated at right of each panel. The smallest amplification products shown are 1.6, 0.4 and 9.5 kb with phages λ 138-1, λ 551-1 and λ 138-10, respectively.

Fig. 5. True and spurious products formed during long distance direct PCR amplification with Vent DNA polymerase. Numbers above lanes indicate sites of *Tn5supF* insertion in λ 138 (Fig. 3). The largest amplification products from the left and right ends of the cloned DNA (8.8 and 12.8 kb, respectively) are indicated. Other true amplification products from left end of segment in λ 138 were 6.4, 8.2 and 8.8 kb and from right end were 11.9, 10.1 and 9.5 kb. The predominant bands at ~ 5.9, 5.8, 5.0 and 3.2 kb in lanes 7 through 10, respectively (right panel), may correspond to internal deletion products; the bands at 2.2 kb in lanes 7, 8 and 9 (right panel) probably resulted from false priming.

products formed with *Tn5supF* insertions in the same orientation are even more useful because they have different left and right ends, corresponding to the different ends of *Tn5supF* (Fig. 2B). Both DNA strands of such products can be sequenced using the primers specific for the two *Tn5supF* ends. To prepare PCR products for sequencing, DNAs in the amplification mix were

Fig. 6. Crossover PCR amplification. Left panel, long distance crossover PCR amplification (up to 4.8 kb), using mixtures of *Tn5supF* insertion derivatives of λ 138; right panel, short distance crossover PCR amplification (down to 0.2 kb). The insertions in λ 138 used and product sizes in the left panel are (from left to right): 1 and 3 (1.5 kb), 1 and 4 (2.0 kb), 1 and 5 (2.8 kb), 1 and 6 (3.5 kb), 1 and 7 (4.1 kb), and 1 and 8 (4.8 kb). Similarly, the insertions in λ 656 *cysQ::kan* and product sizes in the right panel were: 8 and 6 (0.2 kb), 8 and 9 (0.3 kb), 8 and 5 (0.6 kb), 8 and 10 (0.8 kb), 8 and 4 (1.1 kb) and 8 and 11 (1.8 kb).

Fig. 7. Autoradiograph of a portion of linear amplification DNA sequencing gel following crossover PCR between λ 138::*Tn5supF* isolates 4 and 5. Sequencing was done with the *Tn5supF* I primer. Numbers indicate the distance in bp from the 3' end of the primer and correspond to *E. coli lacZ* positions 1064 (84), 935 (219), 964 (182) and 747 (399) (reference 31).

simply adsorbed to a glass slurry (GeneClean), and eluted with distilled water. Aliquots of ~5–50 ng (depending on DNA size) were then used with ³²P-end labelled PCR primers in a linear amplification (cycle) sequencing protocol (27–29). In a variant of this method, PCR products were first electrophoresed in agarose gels, and fractions containing DNAs of interest were excised, and DNAs were purified from the agarose with GeneClean as above. We tested crossover PCR products from *lacZ* as sequencing templates using those formed between insertions 4 and 5, 5 and 6, and 6 and 7 in phage λ 138. We routinely obtained readable sequence for about 400 bp (e.g., see Fig. 7). Electrophoresis prior to GeneClean treatment did not increase the quality of the sequence obtained, however, unless more than one band was present. The 2 kb read in these tests matched the already determined *E. coli lacZ* sequence (31) perfectly. This outcome indicates that the jumping between, and copying of, parental templates during crossover PCR occurs with a fidelity that is sufficient for sequencing pooled (uncloned) PCR products. Crossover PCR products were equivalent as sequencing templates to direct PCR products and highly purified λ DNA. Their use gave easy access to each DNA strand (Fig. 2B).

The efficient use of crossover PCR products made with transposon insertions as sequencing templates requires that the primers be different. This is accomplished if the two insertions are in the same orientation, which is expected with half the pairs of insertions on a random basis. We found, however, that more than 90% of insertions were in the same orientation, (the orientation that allowed *supF* transcription from the λ P_L vector promoter). This pattern means that the two ends of most crossover PCR products differ, and thus are immediately useful as sequencing templates.

DISCUSSION

Strategies in which transposon insertions in cloned DNAs serve as binding sites for primers hold great promise for DNA sequencing, and constituting attractive alternatives to the widely used shotgun subcloning and primer walking methods. To facilitate transposon-based sequencing, we developed methods to map insertions and prepare sequencing templates by PCR amplification from single phage plaques. Because PCR amplification for distances equal to half the 13–20 kb lengths typically cloned in λ phage is not yet routine, our methods were developed to allow insertion mapping in two stages. The first entails direct PCR amplification using primers specific for Tn5*supF* ends and for sites in vector DNA next to the cloning site. It generally located insertions within 6 kb of the ends of the cloned DNA segment in one attempt. The second stage entailed crossover PCR amplification between primer binding sites on different DNA molecules, and generated hybrid products. It allowed insertions near the center of cloned segments beyond the reach of direct PCR to be located, and the map positions of other insertions to be verified or refined. This simple two stage mapping allows the insertions needed for sequencing to be chosen efficiently. The PCR products used to map the insertions also serve as excellent templates for sequencing after a simple glass-adsorption purification. About 400 bp were usually read from each primer binding site using present sequencing conditions; the appearance of the ladders indicated that considerably more sequence would be read from such templates with improvements in the sequencing protocol and the resolution of products (32).

All 2 kb of *lacZ* sequence determined from crossover PCR products matched the known sequence precisely.

Although our experiments illustrate the great potential of combined PCR- and transposon-based mapping and sequencing, they also point to areas that are ripe for improvement. The sporadic amplification of more than 10 kb from phage plaques with the 'best' samples of thermostable polymerases is equivalent to the longest distance amplification recently reported by another group using purified DNAs (30), and would have been sufficient to map all insertions of interest in one attempt had it been reproducible. This capacity for such long distance PCR was not reproducible, however, even among tubes from the single batches of enzyme, despite uniformly excellent short range amplification. In addition, smaller spurious fragments which complicate interpretation of map positions were the dominant products in long distance amplifications with Vent polymerase, and were sometimes observed with Amplitaq and Hot Tub polymerases as well. Their formation is attributable to false priming or internal deletion, and their abundance probably reflects both the chance that they are formed, and the advantage they enjoy during DNA replication. Clearly, there is a need for commercially available 'mapping-grade' enzymes to increase the reach, reproducibility and length fidelity of amplification. This need may be met by new enzymes, or simply better conditions for the purification, storage or use of existing enzymes. Tn5*supF*-containing λ phage, such as used here, should be useful in the development of enzymes and protocols for reproducible long range amplification because they allow virtually any type and length of DNA sequence to be tested for amplification from a few universal primer binding sites, using conditions that are appropriate for the high throughput needs of large sequencing efforts.

Although this work has focused on the use of transposon insertions for analyzing λ phage clones, combined transposon and PCR methods should also be useful for generating DNA sequencing templates from larger DNAs cloned in cosmid and P1 phage vectors in protocols that are also highly amenable to automation.

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