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Rapid Generation of Nested Deletions by Differential Restriction Digestion

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ABSTRACT

A method was devised for generating nested deletions in DNA that exploits the difference in frequency of restriction sites recognized by compatible restriction endonucleases. A cloning vector was constructed that contains no common blunt-end or *RsaI* restriction sites and two 8-bp blunt-end restriction sites flanking a commodious multiple cloning site. DNA fragments are cloned into the multiple cloning site using blue-white selection, and nested deletions are generated by digesting the resulting plasmid with either *SwaI* or *PmeI* and partially digesting the insert DNA with *RsaI*. The DNAs are ligated and transformed, producing a family of plasmids with different-sized deletions. The DNA sequence of these inserts can be rapidly determined, and the overlapping sequences can be assembled in silico to produce a large DNA contig. Nested deletions generated in this manner can also be used for the structure-function analysis of proteins.

INTRODUCTION

To sequence the large amounts of DNA required for genomic research, it is currently standard practice to obtain DNA sequences by random "shotgun" sequencing. Shotgun sequencing of random DNA fragments can be used to generate sequence data quickly; unfortunately, this strategy requires a labor-intensive assembly of contiguous sequences. Furthermore, because redundant DNA segments are sequenced repeatedly, this strategy is inefficient in terms of time, effort, and money. Similarly, a primer walking strategy allows for the simple assembly of contiguous segments, but is slow, as new sequencing primers cannot be designed until the DNA sequence generated from the previous sequencing primer is analyzed.

Several methods have been described in the literature that are designed to re-

duce the cost and time involved in sequencing long DNA fragments. These methods typically involve the production of a series of nonrandom nested deletions that can be sequenced simultaneously and later assembled using a computer into one continuous DNA sequence. Methods used to construct nested deletions include DNA sonication (2,9), DNase I digestion (6,11,15), exonuclease III digestion (7,10,16), and restriction endonuclease digestion (14,18). Recently, several nested deletion strategies utilizing PCR have been reported (5,8,12). In addition, a number of in vivo (1,13) or in vitro (19) transposon-mediated methods have been developed and marketed (Epicentre, Madison, WI, USA) that utilize random transposon insertions as a binding site for sequencing primers. These transposon insertions can be used to generate nested deletions. Unfortunately, many of these methods are time consuming, require several steps using different DNA-modifying enzymes, or involve intramolecular cloning reactions. This report describes

an alternative method that overcomes many of the limitations often associated with the construction of nonrandom nested deletions in cloned DNA.

Utilizing the difference in frequency between "rare" restriction enzyme sites in DNA (8-bp recognition sites) and those with 4-bp recognition sites, we have developed a technique that rapidly produces deletions in a cloned DNA fragment with only a few simple steps. Complete digestion of the cloned DNA with a rare-cutting restriction enzyme followed by partial digestion with a frequent-cutting restriction enzyme produces a series of cuts in the target DNA at different distances from the rare restriction site situated in the cloning vector. Provided that the rare- and frequent-cutting enzymes produce complementary DNA ends, the different-sized DNA molecules can be ligated to produce a series of nested deletions. The difference between this technique and other multiple restriction endonuclease methods (14,18) is that these nested deletions are generated in a con-

Figure 1. Deletion strategy and restriction map of p824. (A) Model for differential restriction digestion to generate nested deletions. The DNA to be deleted is cloned into restriction site X in the MCS of the plasmid. The insert DNA contains many common (C) 4-bp restriction sites; the cloning vector has no common restriction sites. The resulting plasmid is digested to completion with either of two rare-cutting 8-bp restriction enzymes situated at one end of the plasmid's MCS (R1 and R2). A partial restriction digest is then performed on the linearized DNA with a complementary frequently cutting restriction enzyme, generating deletions in the cloned DNA. The linear DNAs are then self-ligated, forming plasmids with different-sized deletions. Overlapping sequence data can be obtained from each deleted plasmid by sequencing into the deleted DNA from a primer binding site (P) near the plasmid MCS. (B) Specialized vector p824 for differential restriction digestion. The cloning vector p824 consists of an ampicillin resistance gene (*bla*), the ColE1 origin of replication (*oriR*), and a unique MCS situated within the *lacZ* peptide gene. The plasmid backbone does not contain any restriction sites for *RsaI* (GTAC). The MCS does not contain any sites for commonly used blunt-end cutting restriction enzymes and is flanked by rare-cutting blunt-end restriction enzyme sites for *PmeI* (GTTTAAAC) and *SwaI* (ATTTAAAT). SP6 and T7 sequencing primers can be used to obtain sequence from deletions generated from either direction in a cloned DNA insert.

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tiguous, colinear, cloned DNA fragment, eliminating any intermolecular ligation steps. Provided that the cloning vector does not contain restriction sites for the utilized frequent-cutting restriction enzyme, the deleted DNA can be converted back into plasmid form through intramolecular ligation. The DNAs are then transformed into an *E. coli* cloning host to generate a family of different-sized deletions. Here we have exploited the difference in frequencies between complementary 8-bp and 4-bp restriction sites in DNA to generate a set of nested deletions.

MATERIALS AND METHODS

Bacterial Strains, DNA, and Media

E. coli strain DH5 α [F⁻ ϕ 80d/*lacZ* Δ M15, *endA*1, *recA*1, *hsdR*17 (r_K⁻, m_K⁻), *supE*44, *thi*-1, *gyrA*96, *relA*1, Δ (*lacZYA-argF*), U169, λ] (Invitrogen, Carlsbad, CA, USA) was used as a plasmid host. Plasmid pGEM-5Zf(-) (Promega, Madison, WI, USA) has been described previously. All PCR primers, sequencing primers, and oligonucleotides used in this study were purchased from Invitrogen. LB media (Invitrogen) was used for plasmid propagation with 100 μ g/mL ampicillin. X-gal (Invitrogen) was added when appropriate at a concentration of 30 μ g/mL. Cloning, ligation, and transformation of DNA followed standard molecular biological protocols (17).

Deletion Vector Construction-p824

pGEM-5Zf(-) was chosen as the basis for cloning vector p824 (Figure 1A) because of the paucity of *RsaI* restriction sites in the plasmid DNA. To remove the *ScaI/RsaI* restriction site from the β -lactamase gene in pGEM-5Zf(-), the primers d(GACTTGGTTGAATATCACCAGTCAC) and d(ATTCTGA GAATACCGCGCCC) were designed and utilized in a PCR mutagenesis strategy with *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) as previously described (3). The standard PCR contained 1 $\times$ native *Pfu* buffer [10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.5), 2 mM MgSO₄, 0.1% Triton[®] X-100, 100 μ g/mL BSA], 200 μ M

each dNTP, 0.5 μ M each primer, 5–10 ng template, 5 U *Pfu* DNA polymerase, and was performed as follows: initial template denaturation at 96°C for 3 min, followed by 30 cycles of denaturation at 96°C for 1 min, primer annealing at 55°C for 1 min, extension at 72°C for 6 min (2 min/kb as recommended by the manufacturer), followed by a final 7-min extension at 72°C. The DNA products were purified using Gene-Clean[®] (Bio 101, Vista, CA, USA), self-ligated using T4 DNA ligase (Invitrogen), and transformed into competent *E. coli* DH5 α (Invitrogen). Ampicillin-resistant transformants were checked by restriction digestion for loss of the *ScaI* site and verified by DNA sequence analysis. This procedure eliminated the *ScaI/RsaI* restriction site in plasmid pGEM-5Zf(-).1 while retaining the original β -lactamase amino acid sequence. To introduce a *SwaI* restriction site at one end of the multiple cloning site (MCS) next to the *NsiI* site, PCR was again performed with *Pfu* DNA polymerase and the primers d(ACTA TAGAATACTCATTTAAATATGCAT-CCAACGC) and d(GTCACCTAAAT-AGCTTGCGTAATCATGG). The resulting blunt end DNA fragment was self-ligated and transformed into *E. coli* DH5 α , the correct clone was selected following digestion with *SwaI* (Roche Applied Science, Indianapolis, IN, USA), and named pGEM-5Zf(-).2. To introduce a *PmeI* restriction site at the opposite end of the MCS and replace the *AatII* and *ApaI* sites, *Pfu* DNA polymerase PCR was performed with the primers d(TAGTGAGTCGTATTACA ATTCACTGGCCGTCG) and d(TAGGGCGAATTGGGTTTAAACTCGCATGCTC). Similarly, the correct clone was selected based on test digests with *PmeI* (New England Biolabs, Beverly, MA, USA) and named pGEM-5Zf(-).3. In both cases, the addition of 6 bp to either end of the MCS did not significantly affect β -galactosidase activity. This allows blue-white screening to be used during the initial cloning of a DNA fragment into the MCS, before nested deletions are constructed. By including one rare-cutting blunt-end restriction site at either end of the MCS, nested deletions can be made in the cloned DNA in either direction with a blunt-end frequent-cutting restriction enzyme

such as *RsaI*. The DNA sequence can be obtained from deletions made from either end of the MCS by utilizing T7 and SP6 sequencing primers.

Not only can the 4-bp *RsaI* restriction site be used to produce blunt-ended deletions, but other less frequently cutting 6-bp restriction endonucleases that produce blunt-ends in DNA can also be used to generate additional deletions. To remove blunt-ended restriction sites from the MCS, and at the same time introduce more useful restriction sites, several additional modifications were made to the vector. The oligonucleotides d(TGGATCCCCATGGGAGCT) and d(CCCATGGGGATCCATGCA) were annealed together and ligated into the plasmid previously digested with *NsiI* and *SstI*. This eliminated the *BstXI* site and added a new *BanHI* site and *NcoI* site, forming pGEM-5Zf(-).4. Similarly, the oligonucleotides d(GGCCGCAAG CTTGGGCCCGAATTCAGATCTGCG-ATG) and d(CAGATCTGAATTCGG GCCCAAGCTTGC) were annealed together and ligated into the plasmid previously digested with *NotI* and *SphI*. This eliminated *SpeI*, *EcoRV*, *SacII*, and *NcoI* sites and introduced new *HindIII*, *ApaI*, *EcoRI*, and *BglII* sites. The final version of the new MCS of p824 was verified by DNA sequencing. Overall, the modified MCS of p824 has seven new restriction sites and is 9 bp shorter than the original pGEM-5Zf(-) MCS (Figure 1B). The complete nucleotide sequence of the nested deletion plasmid p824 has been deposited in GenBank[®] under the accession number AF305698.

Production of *RsaI* Partial Digests

After cloning a target DNA fragment into the MCS of p824, the resulting plasmid is digested to completion with either *PmeI* or *SwaI* for 1 h at 37°C. After thus linearizing the plasmid, *RsaI* partial digests are performed. One microliter (10 U) of either *PmeI* or *SwaI* is added to 50 μ L sterile distilled water to make a stock solution containing approximately 0.2 U enzyme/ μ L. To reactions containing 1 μ g plasmid DNA and 2 μ L 10 $\times$ reaction buffer, the diluted enzyme is added to the tubes in volumes of 2–10 μ L, and the final volume is raised to 20 μ L with sterile distilled water. The reactions are mixed and incu-

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bated for exactly 20 min at 37°C. The reactions are stopped by adding gel loading buffer and incubating them at 4°C. One-third of the reaction is electrophoresed on an agarose gel to identify the reaction containing the restriction enzyme dilution that gives appropriate deletion products of the desired size(s). While digestion of more than one *RsaI* site in a given clone is a possibility, the rapid ligation conditions do not favor the incorporation of these blunt-ended fragments into the final deleted plasmid. The saved original reaction is purified using Gene-Clean, diluted to half the original volume with sterile distilled water (20 µL), and the DNAs are ligated using conditions that favor intramolecular interactions rather than intermolecular reactions (3 h at 25°C). One microliter of ligated reaction products is transformed into a suitable *E. coli* host such as DH5α. Mini-plasmid preparations are prepared from the resulting clones and sized on agarose gels, and plasmids with different sizes of deletions are selected for DNA sequencing. Although this procedure does not purify small *RsaI* restriction fragments away from the larger plasmids, we have found that under the conditions described, no intermolecular blunt-end ligation occurs. A similar deletion strategy has been employed for the deletion of DNA flanking a plasposon's site of insertion, except that only *RsaI-SwaI* deletions can be constructed (4). In this instance, this general deletion technique allows the rapid production of a large number of sequencing templates from a plasposon rescued as a plasmid.

RESULTS AND DISCUSSION

Nested deletions can be generated in target DNA in the following manner. A target DNA fragment is cloned into the p824 MCS at any of the 6-bp "sticky-end" restriction sites and transformed into a recipient *E. coli* host. DNA fragment insertions can be detected by the lack of β-galactosidase production on plates containing X-gal via blue-white screening. Once the desired DNA fragment is inserted in the p824 cloning vector, the resulting plasmid is purified and subjected to two different blunt-end restriction digestions. The first restric-

tion digestion with either *SwaI* or *PmeI* is carried out to completion and serves to linearize the plasmid at one end of the MCS. The second restriction digestion is performed with *RsaI*, a frequent-cutting restriction enzyme that does not cut the plasmid backbone. By decreasing the concentration of enzyme and/or time normally used to digest DNA, the enzyme can be manipulated to digest the target DNA at only one of many possible *RsaI* restriction sites. Typical reaction conditions for this *RsaI* partial digestion are 1 U/1 µg DNA in 20 µL at 37°C for 20 min. This partial digestion produces a set of DNAs with blunt ends that can be self-ligated to form plasmids with different sizes of deletions (Figure 1A). Such nested deletion plasmids can then be used to delineate the location of genes on a DNA fragment, to examine the structure-function relationships of encoded proteins, or to generate templates for sequencing reactions. DNA sequence data can be easily obtained from each deleted plasmid by using the SP6 (*SwaI-RsaI* digestions) or T7 (*PmeI-RsaI* digestions) primer binding sites that are designed to read into the MCS. By performing *SwaI-RsaI* digestions and *PmeI-RsaI* digestions on the same DNA clone, there is no need to clone the original DNA fragment into p824 in both orientations, as the DNA sequence of both strands can be obtained through deletions from either end of the cloned fragment.

In this study, we have exploited the difference in frequencies between 8-bp and 4-bp restriction sites in DNA to generate nested deletions. Assuming that all bases occur with equal frequency, a 4-bp restriction site will occur every 4⁴ or 256 nucleotide pairs, and an 8-bp restriction site will occur every 4⁸ or 65536 nucleotide pairs. In our experience, *RsaI* restriction sites (GTAC) occur with less frequency than expected, with an average of one *RsaI* site per kilobase of approximately 65% GC-rich bacterial DNA. As anticipated, some DNA regions have more or less *RsaI* sites than this. It is probable that more *RsaI* sites will occur in target DNA with a GC content closer to 50%. The p824 plasmid backbone originated from pGEM-5Zf(-), with modifications to remove an *RsaI* site from the β-galactosidase gene and add additional

restriction sites, including the *PmeI* and *SwaI* sites flanking the MCS. DNA fragments up to approximately 20 kb have been cloned into this vector using blue-white screening on LB plates containing ampicillin and X-gal, and we have successfully produced nested deletions in DNA fragments up to 15 kb in length. Figure 1A shows deletion mutagenesis of a typical DNA fragment. It is important to note that nested deletions can be obtained in both orientations from one p824 clone, eliminating the need for additional subcloning steps.

To demonstrate the utility of this nested deletion system, a 9-kb DNA fragment was cloned into p824, resulting in a 12-kb plasmid (named p824.9) (Figure 2, lane 2). To eliminate some of the extraneous DNA insert before generating nested deletions and sequencing, the plasmid p824.9 was digested to completion with *SmaI* and *PmeI*, thereby removing 4 kb from the DNA insert, ligated and transformed into *E. coli* DH5α to produce the 8-kb plasmid p824.5 (Figure 2, lane 3). This plasmid containing a 5-kb DNA insert served as the substrate for the generation of nested deletions. Five microcentrifuge tubes containing equal quantities of this plasmid were digested to completion with *SwaI*, followed by partial digestion with *RsaI* (0.4, 0.8, 1.0, 1.2, and 1.6 U), and incubated at 37°C for 20 min. After the reactions were stopped, one-third of the digestion products from each tube were gel electrophoresed, and the reaction showing the widest range of digested products was selected for further manipulation. The remaining DNA from this reaction was purified, self-ligated, and transformed into *E. coli* DH5α. Plasmids isolated from colonies obtained from this transformation were purified and electrophoresed, as shown in Figure 2, lanes 4–10. Generally, we have found that, although the number of *RsaI* recognition sites (GTAC) varies depending upon the DNA source, there is on average at least one *RsaI* site within each kilobase of DNA.

Although this technique is a simple and efficient method for generating nested deletions, there are special instances when problems can be encountered. If there are *PmeI* and/or *SwaI* sites within the cloned DNA, deletions will only be generated starting at the second

rare restriction site. Fortunately, the occurrence of either of these restriction sites in DNA is rare; therefore, sequence data can be obtained from a clone containing a *PmeI* site by making *SwaI*-*RsaI* deletions, or vice-versa. Furthermore, because there are no restriction sites in p824 for enzymes that recognize 6-bp sites and produce blunt-end cleavage products such as *EcoRV*, *HpaI*, *NruI*, *PvuII*, *ScaI*, *SmaI*, and *StuI*, these enzymes can be used to generate additional deletions in the target DNA as demonstrated (p824.5; Figure 2, lane 3).

The procedures described here for generating overlapping deletions in cloned DNAs should provide a rapid and efficacious strategy for obtaining large amounts of DNA sequence data. Nested deletions generated from plasmid p824 insertions can also serve as substrates for open reading frame localization or protein structure/function experiments. Nested deletions can be generated in a cloned DNA fragment and templates prepared for DNA sequencing in approximately two days. Using p824 is more economical than shotgun cloning and sequencing in terms of

number of subclones that need to be constructed and more economical than primer walking in terms of the time and money required for the synthesis of sequencing primers.

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Figure 2. Size determination of deletion products using p824. A plasmid consisting of p824 containing a 9-kb insert (p824.9, lane 2) was deleted first with *SmaI*-*PmeI* (p824.5, lane 3), followed by digestion with *SwaI*-*RsaI* (lanes 4-10), and the resulting isolates were screened for size on a 0.7% agarose gel (100 V for 3 h at 25°C). The faint larger band in each lane is a plasmid dimer of the deleted plasmid. Lane 1 contains 0.2 µg supercoiled DNA ladder with bands at 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, and 16 kb.