A Novel Method for Generating Nested Deletions Using the in Vitro Bacteriophage T3 DNA Packaging System

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Abstract

To sequence a DNA segment inserted into a cosmid vector under the directed sequencing strategy, we established a simple and rapid method for generating nested deletions which uses the in vitro packaging system of bacteriophage T3 DNA. The principle is based on the previous finding that this system can translocate any linear double-stranded DNA up to 40 kb into the phage capsid in a time-dependent manner and the encapsulated DNA becomes DNase-resistant. For this purpose, we constructed a cosmid vector that carries two different antibiotic selection markers at both sides of the multiple cloning site, and after insertion of a DNA segment, the clone was linearized by A-terminase at the cos site. After the packaging reaction in vitro followed by DNase treatment, the encapsulated DNA was introduced into Escherichia coli cells to give clones with unidirectional deletions by differential antibiotic selection. Restriction and sequence analyses of deletion clones demonstrated that an ordered set of clones with nested deletions, ranging from less than 1 kb to 25 kb, was created from either the end of the DNA segment. Thus, nested deletion clones that cover the entire region of a ~40-kb cosmid insert can be obtained by a single packaging reaction, and its restriction map can be simultaneously obtained.

Key words: T3 bacteriophage; in vitro DNA packaging; nested deletion; DNA sequencing; cosmid

1. Introduction

Recent progress in DNA technology has been greatly increasing the speed and the ability of reading DNA sequences. However, improvement in the acquisition efficiency of raw DNA sequence data alone does not accelerate DNA sequencing projects as a whole; it requires more convenient and efficient strategies which minimize the assembling process of raw sequence data to a long contiguous DNA sequence. The strategies currently adopted are classified into random (shotgun) and directed types. The random strategy is suitable for automation but demands a high degree of redundant sequencing to complete assembling of a long DNA sequence. The larger the target DNA is, the more serious this drawback. On the other hand, the directed strategy makes it possible to perform assembling more efficiently, and in fact, various approaches for directed DNA sequencing have been reported. Nevertheless, application of most of these approaches is limited for relatively short DNA segments.

In this study, we established a simple and rapid method for generating nested deletions from a DNA segment with the size of cosmid inserts (~40 kb) by the use of the in vitro packaging reaction of bacteriophage T3 DNA. In this packaging system, any linear double-stranded DNAs up to 40 kb can be efficiently translocated into the phage capsid. These features of the packaging reaction urged us to examine whether or not this system can be used for constructing an ordered set of nested deletions of a cosmid clone. The present results demonstrate that this system can be adaptable for producing an ordered set of nested deletions.

2. Materials and Methods

2.1. Strains, plasmids, and enzymes

Escherichia coli strains XL1-blue MRF' and DH10B were used for generation of cosmid clones and for cloning of deletions, respectively. Plasmids, pBluescript SK+ and pHSG398, were obtained from Stratagene (La Jolla, CA) and Takara Shuzo Co., respectively. Adenosine-5'-O-(3-thiotriphosphate) (ATP-γ-S) and RNase-free DNase I were purchased from Boehringer-Mannheim Yamanouchi.
Co. DNA-modifying and restriction enzymes were the products of Takara Shuzo Co.

2.2. Construction of a cosmid vector pAT2

For isolation of the rightward and leftward deletion clones, a new 4.4-kb cosmid vector, pAT2, was constructed (Fig. 1). A half of this vector carrying the chloramphenicol-resistance (Cm') gene flanked by HindIII sites was derived from pHSG398: the SacI-HindIII sequence carrying the multiple cloning site of pHSG398 was deleted, and a synthetic oligonucleotide linker containing the EcoRI-NotI-BamHI-NotI-EcoRI sites was inserted into the unique EcoRI site of pHSG398. The remaining half of pAT2, which carries the cos site and ampicillin-resistance (Ap') gene, was constructed from the pBluescript SK+ (Stratagene). The original multiple cloning site of pBluescript SK+ vector was replaced by the 108-bp R4-cosN-R3 sequence in A phage DNA (nucleotide positions -40 to +68 of the A phage map). A small SsiI fragment of pBluescript SK+ vector was removed, and the original SsiI sites were converted to the HindIII sites with a synthetic linker.

Figure 1. The structure of a new cosmid vector, pAT2. pAT2 carries EcoRI, NotI and BamHI sites as unique cloning sites. The two HindIII sites indicated are junction points of two parental vectors, pHSG398 and pBluescript SK+. Two small arrows denoted as T3 and T7 indicate promoters for T3 and T7 bacteriophage RNA polymerase, respectively. ApT' and CmT' genes are shown as open arrows. The cos site, plasmid origin of replication, and fl filamentous phage origin of replication are denoted as cos, ori, and fl IG, respectively.

2.3. Preparation of cosmid clones

For assessment of the system, cosmid clones were prepared by ligation of the NotI digests of Synechocystis PCC6803 genomic DNA with dephosphorylated NotI-digested pAT2 vector. The ligation product was introduced into E. coli XL1-blue MRF' with an in vitro λ phage packaging kit (Gigapack II Gold, Stratagene), and E. coli cells carrying cosmids were selected on an agar plate containing ampicillin (Ap). A cosmid clone, named pKY11, which had no NotI site within the insert (∼45 kb) was used in this study.

2.4. Generation of nested deletions by T3 packaging system

pKY11 DNA (2 μg) was completely digested by λ-terminase (8 units, Takara Shuzo Co.) in 10 μl of a reaction mixture consisting of 13 mM Tris-HCl (pH 8.0), 3 mM MgCl₂, 75 mM KCl, 0.1 mM ATP-γ-S, 0.05 mM ethylenediaminetetraacetic acid (EDTA), 6 mM β-mercaptoethanol and 5 mM spermidine for 3 h at 30°C. The digest was added to 96 μl of the defined bacteriophage T3 DNA packaging system containing 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM MgCl₂, 7 mM β-mercaptoethanol, 1 mM spermidine, 5% (w/v) polyethylene glycol #6000, 0.02 mM EDTA, 0.01 mM ATP-γ-S, 20 μg of T3 phage proheads, 3 μg of gp19 and 4 μg of gp18. The proheads and a T3 packaging protein, gp18, were purified as described previously and the other packaging protein, gp19, was prepared as described by Kimura and Fujisawa. After pre-incubation for 30 min at 30°C, the packaging reaction was initiated by the addition of 4 μl of 5 mM ATP and allowed to proceed for 15 min at 30°C. After the reaction, the mixture was treated with DNase I (10 units/μl) for 10 min on ice to digest unpackaged DNA. The mixture was then incubated for 10 min at 65°C in 0.2% sodium dodecyl sulfate and 40 mM EDTA, and then treated with 80 μg of proteinase K for 15 min at 55°C. After phenol-chloroform extraction followed by ethanol precipitation, the termini of the recovered DNA were blunt-ended by T4 DNA polymerase (3.2 units) under the conditions recommended by the supplier. After heat-inactivation of the polymerase for 5 min at 70°C, one-fifth of the resulting mixture (5 μl) was subjected to self-ligation in 50 μl with a DNA ligation kit (Takara Shuzo Co.). After incubation for 60 min at 16°C, the DNA was extracted with phenol-chloroform, precipitated with ethanol, suspended in 18 μl of distilled water, and introduced into E. coli cells using Electromax DH10B competent cells (Gibco BRL, Grand Island, NY) and a Cell-Porator E. coli pulser (Gibco BRL). The transformants were selected on agar plates containing either Ap or chloramphenicol (Cm), and plasmid DNAs were isolated using an automatic plasmid isolation system (Pl-100S, Kurabo).
Figure 2. The schematic illustration of nested deletion strategy based on the bacteriophage T3 DNA packaging system. Cosmid DNA derived from pAT2 is linearized with λ-terminase and then packaged into the phage capsid with the in vitro packaging system. Digestion of uncapsuled cosmid DNA with DNase I generates a set of DNAs deleted unidirectionally. The resulting DNase I-resistant DNA is self-circularized with T4 DNA ligase after blunt-ending and then introduced into E. coli cells by electroporation. ApF and CmF clones provide unidirectional clockwise and counterclockwise deletions, respectively. Portions of the insert DNA shown as a filled bar is numbered to indicate the direction of the insert.
2.5. Sequencing analysis

Plasmid DNA prepared with PI-100Σ was treated with ribonuclease A and then precipitated with polyethylene glycol for removal of contaminating RNAs. The cycle DNA sequencing reaction was performed with Taq polymerase by CATALYST800 (Applied Biosystems, Mountain View, CA) using a kit from Applied Biosystems, and sequence analysis was performed with the ABI 373 STRETCH sequencer (Applied Biosystem) and the ABI sequence analysis system, INHERIT (Applied Biosystem).

3. Results and Discussion

3.1. Construction of a cosmid vector suitable for nested deletion system using the in vitro DNA packaging reaction

To adapt the In vitro packaging system of bacteriophage T3 DNA for generation of nested deletions, we constructed a new cosmid vector, pAT2, as shown in Fig. 1. This vector carries two antibiotic selection markers (Ap and Cm genes) which facilitate differential selection of direction (rightward or leftward) of deletions from the cos site, and the cos site serves as a unique cleavage site for linearization of cosmid DNAs. T3 and T7 RNA polymerase promoters are present at both sides of the cos site so that T3 or T7 sequencing primer can be used for sequencing deletions.

3.2. in vitro T3 DNA packaging reaction of a cosmid DNA

Overall procedures of the deletion system are schematically illustrated in Fig. 2. Since any linear double-stranded DNA can be efficiently encapsulated by a defined in vitro system for packaging T3 phage DNA as previously reported, we first examined the efficiency of translocation of the linearized pKY11 DNA into the phage capsid. For linearization of the cosmid clone, we used λ-terminase since it is very unlikely that the insert DNA carries a nucleotide sequence similar to the cos site. ATP-γ-S was included in the digestion mixture as a non-hydrolyzable ATP analog in place of ATP, since the packaging reaction mixture also required the same ATP analog. Translocation of the DNAs into the phage head was monitored by examining the sizes and the amounts of DNAs protected from DNase I digestion, as only the encapsulated DNAs are resistant to DNase I under the degradation conditions employed. As shown in Fig. 3, the sizes and amounts of the protected DNAs increased with the packaging reaction time, which proved that translocation of the linearized cosmid DNA proceeded as expected. Sequestration of cosmid DNA into capsid seemed to cease after 2-min incubation and further extension of the reaction time did not change the electrophoretic patterns of DNase I-resistant DNAs any further. Although most of the linearized cosmid DNA molecules appeared to be packaged into the capsid, the sizes of DNAs translocated into the capsid were not uniform, since the DNase I-resistant DNAs appeared as a smear band (centered at larger than 20 kb) on an agarose gel even after the longest incubation (Fig. 3). We previously anticipated that the length of the deletion is adjustable by the reaction time, but the above observation suggests that encapsulation does not proceed in a completely synchronous manner.

3.3. Isolation and analyses of nested deletions

The separation of rightward and leftward deletions from the cos site was conducted by growing transformed E. coli cells on a plate containing either Ap or Cm; we isolated Cm clones as leftward deletions (i.e., deletions from Ap gene side) and Ap clones as rightward ones (i.e., deletions from Cm gene side) (Fig. 2). For characterization of clones, the plasmid DNAs were recovered and digested with either Ncol or EcoRI. More than 90%
Figure 4. Length distribution of deletion plasmids generated by the in vitro packaging system. Plasmid DNAs of randomly isolated 46 and 45 clones in the Ap\textsuperscript{r} (A) and Cm\textsuperscript{r} (B) deletion libraries, respectively, were digested with Not\textdagger and fractionated on 0.8% agarose gels. The positions of marker HindIII digests of λ DNA are at both sides of the patterns. The band intensities roughly reflect the yields of plasmids from E. coli cells. The lane number increases with decreasing insert sizes of the plasmid DNAs.
Figure 5. Characterization of deletion clones by restriction enzymes and the deduced EcoRI restriction map of the insert in pKY11. Panel A: Plasmid DNAs isolated from Ap<sup>+</sup> clones were digested with NotI and fractionated on 0.8% agarose gels. Lanes 1 to 16, plasmids with large-sized inserts (25 kb-12.5 kb); lane 17 to 24, plasmids with middle-sized inserts (10 kb-8.7 kb); lane 25 to 32, plasmids with small-sized inserts (7.5 kb-6 kb); lane m, HindIII fragments of A DNA. Panel B: Gel electrophoretic patterns of the same set of plasmids as in panel A, but digested with EcoRI on 0.8% agarose gels. All the EcoRI fragments generated from pKY11 are shown in lane O and their lengths are given in kb. Panel C: The deduced EcoRI restriction map of pKY11. EcoRI sites are shown by vertical arrows and the molecular sizes of fragments are indicated above each fragment in kb. Large and small open boxes indicate the antibiotic-resistant genes (Ap and Cm) and the cos sequences divided by λ-terminase, respectively.
of clones were found to harbor plasmids whose restriction maps were consistent with those expected for unidirectional deletions. Furthermore, the sizes of plasmid DNAs were varied widely. The result of a typical analysis is shown in Fig. 4. In this experiment, 50 clones were randomly selected from each of the Apf and Cm′f libraries, and the discrete DNA bands yielded from 46 Apf clones and 45 Cm′f clones are shown in order of decreasing size. Apparently, the band sizes randomly distribute in the range of approximately 2.3 kb to 25 kb, although the yields of plasmid DNA somewhat varied with clones. By increasing the clone number analyzed, the size distribution of DNA bands became more contiguous. Note that the deletion clones carry the vector moiety (~2 kb) at the distal end of deletion, so that the size of the actual deletions in the cosmid insert should be about 2 kb shorter than the observed fragment size.

The observed size distribution of deletion plasmids was apparently shorter than that of DNase I-resistant DNA fragments analyzed before transformation (Fig. 3). The apparent difference in the size distributions before and after transformation may be due to low efficiencies of self-circularization and transformation of longer DNAs.13~15 The loss of longer DNAs during the purification step after circularization and transformation of longer DNAs.13~15 For further assessment of our method, we analyzed the sequences of nested deletion clones. In this experiment, 150 clones were randomly selected from each of the Apf and Cm′f deletion libraries. When analyzed their restriction patterns, 138 Apf and 140 Cm′f clones gave discrete bands suitable for DNA sequencing, and could be contiguously aligned in the size range from approximately 2 kb to 25 kb. Thus, we arbitrarily selected a set of 10 clones that were predicted to span 2–3 kb from each of the Apf and Cm′f deletion libraries, and confirmed that these clones indeed yielded a contiguous sequence by sequence comparison (data not shown).

The cosmid vector carried the EcoRI site at both sides of the cloning site, so that it was possible to construct the restriction map of the insert by analysis of the set of deletion clones from both directions. In Fig. 5, the EcoRI digests of the Apf clones that carried large-size inserts (Nos. 1 to 16), middle-size inserts (Nos. 17 to 24) and small-size inserts (Nos. 25 to 32) are shown (panel B), in comparison with the patterns of the NotI digests of the corresponding clones (panel A). The order of the EcoRI site from the left end of the insert can be predicted by comparison of the restriction patterns. By combining the data for the Cm′f and Apf clones, therefore, the complete restriction map of the insert in a cosmid clone (~45 kb) can be constructed. The EcoRI restriction map of the cosmid clone pKY11, that was deduced by analysis of deletion clones obtained by a single packaging reaction, is shown in Fig. 5C.

For generation of nested deletions from cosmids-size DNAs, a transposon-based approach termed the “deletion factory” strategy has been reported.16,17 Compared with this transposon-mediated method which includes in vivo processes, our method is relatively simple and a contiguous set of nested deletions up to 25 kb can be generated from both ends of a ~45-kb cosmid insert by a single packaging reaction. Yet only the sequencing clones for one strand can be obtained, except in the region covered by both of the unidirectional deletions, by the present system. The packaging capacity of bacteriophage T3 is about 40 kb, so that it would be possible to extend the deletion size further by modification of the reaction conditions. This should make it possible to obtain the sequence information for both strands of the cosmid insert. The modification of the reaction system according to this line of reasoning is in progress.

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