A novel system for the rapid generation of precise DNA deletions

I. McCaffery, B. D. Williamson and C. L. Rutherford*

Molecular and Cellular Biology Program, Department of Biology, Derring Hall, Virginia Tech., Blacksburg, VA 24061-0406, USA

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ABSTRACT

To generate DNA deletions, a tandem array of class IIS restriction enzyme recognition sites was cloned into a plasmid. The recognition sites were arranged so that each enzyme cleaves at a different site within an adjacent target sequence. Digestion with both enzymes followed by end repair and ligation resulted in the deletion of DNA between the two sites of cleavage. Because both recognition sites are preserved following deletion, it was found that sequential deletions could be generated using cycles of restriction enzyme digestion, end repair and ligation. Therefore, this system represents a valuable tool in the definition of functional DNA sequences.

The analysis of transcriptional regulatory elements within a region of DNA typically involves deletion generation using the exonuclease III/mung bean nuclease system (1). Initially, large deletions are created and regulatory elements are mapped in vivo using a reporter gene system. Further deletions are often required to precisely define regulatory elements and this usually involves the generation of large numbers of deletions. While the exonuclease III/mung bean nuclease deletion system is a routine procedure, the deletions are cloned thus increasing the time required to map elements. Furthermore, following amplification, each of the multiple primers required often limits the number of deletions possible, thereby further reducing the resolution of regulatory element mapping. Moreover, following amplification, each of the deletions is cloned thus increasing the time required to map elements.

Here we describe a system that allows the rapid, sequential generation of small, predictable deletions of DNA sequences. The deletions generated using this system are not dependent on the sequence to be deleted or flanking sequences, and therefore do not suffer from the drawbacks of the PCR or exonuclease III/mung bean nuclease based systems. The system relies on the property of class IIS restriction enzymes of cleaving DNA at a specific site that is distant from the recognition sequence. We have exploited this property to generate DNA deletions by the insertion of a cassette into the plasmid. The cassette contains a tandem arrangement of the recognition sequences of two of these class IIS enzymes, BsgI and BsmBI (Fig. 1a). Each enzyme cleaves at a different distance from the recognition site, therefore, the sequence between the sites of cleavage is deleted upon digestion with both of the enzymes (Fig. 1b). Therefore, the DNA fragment to be deleted is (sub-)cloned 3′ to the deletion cassette. As depicted in Figure 1, this tandem array is organized such that the recognition sequence of both enzymes remains intact following the procedure, allowing further rounds of deletion. Various sizes of deletion can be produced depending on the method of repair of the resulting staggered ends prior to religation of the vector i.e. using mung bean nuclease or T4 DNA polymerase (see below).

The deletion vector (pNBL) was generated by the insertion of a double-stranded oligonucleotide into the multiple cloning site of pBluescript II SK+ as follows: two single-stranded oligonucleotides, NBL1 (5′-TCGACCGGTACCAGACTGGCTAC-3′) and NBL2 (5′-GGGCCCGTACGCTAC-3′), were combined in a total volume of 50 µl annealing buffer (1 M NaCl, 0.1 M Tris–HCl pH 7.8, 1 mM EDTA) and overlaid with mineral oil. The mixture of oligonucleotides was heated at 95°C for 5 min and allowed to gradually cool to room temperature. The resulting double-stranded oligonucleotide has an overhang at each end which are complementary to those produced by KpnI and SalI digestion. The plasmid pBluescript II SK+ was digested with KpnI and SalI and the linear vector was purified following agarose gel electrophoresis according to standard protocols (1). The double-stranded oligonucleotide was ligated to the linear vector and used to transform Escherichia coli. A recombinant clone was selected using a BsgI digestion (data not shown) because this site is unique to the inserted deletion cassette.

As discussed above, class IIS restriction enzymes, such as BsgI and BsmBI, cleave DNA at sites that are distant from their recognition sites. In the arrangement shown in Figure 1, digestion with BsgI followed by BsmBI would result in the excision of DNA sequences between the restriction sites of the two enzymes. In the case of these enzymes, staggered ends are produced (Fig. 1), therefore ends are repaired prior to religation. The size of the deletion resulting from these digestions depends on the method of repair of the resulting staggered ends prior to religation.

*To whom correspondence should be addressed. Tel: +1 540 231 8940; Fax: +1 540 231 9307; E-mail: Rutherford@vt.edu
the deletions depicted in Figure 1. This involved digestion with BsgI followed by BsmBI. Following gel purification and ligation of the digested pNBL plasmid, 100 ng of the ligation was used to transform E.coli. The remaining ligated DNA was purified by extraction with phenol/chloroform [using standard procedures (1)] and subjected to a further round of deletion by repeating the procedure outlined above. Once again, following ligation, 100 ng ligated DNA was used to transform E.coli and the remaining DNA was subjected to a further round of deletion. Again, 100 ng ligated DNA was used to transform E.coli. The vectors that resulted from these three rounds of deletion are known as pNBLA1, pNBLA2 and pNBLA3. As shown in Figure 1, it is predicted that these deletions would result in the destruction of restriction enzyme sites in the multiple cloning site and this was initially used to verify that the predicted deletions had occurred. This experiment was performed in duplicate and a representative experiment is presented here (Fig. 2).

As shown in Figure 1, the system described here is predicted to result in a 5 bp deletion including sequences that constitute the adjacent KpnI and SalI sites. The three rounds of deletion described above were performed and Figure 2 shows restriction enzyme analyses of deleted vectors. It is clear by comparison with the undeleted parent vector digestions (pNBL) that both the KpnI and SalI sites have been deleted in pNBLA1. Spaces in the sequence are used to represent cleavage of DNA. Only the positions of recognition sites that remain intact following relaxation of the deleted vector are boxed. (c) The sequence of pNBLA2. This construct was generated by performing an identical round of deletion as that depicted in (b) except pNBLA1 was used as a template. Similarly, (d) shows the sequence of pNBLA3, generated by a round of deletion using pNBLA2 as a template.

Figure 1. Generation of precise DNA deletions. (a) The sequence of a portion of the multiple cloning site of pNBL. Lowercase sequence is used to represent DNA sequences that were inserted as a cassette into pBluescript II SK+ to generate pNBL. Recognition sites for relevant restriction enzymes are boxed and labelled. The recognition sites for the class IIS enzymes use solid lines and other sites in the multiple cloning sites use dotted lines. The latter enzyme sites (boxed with dotted lines) are cleaved within the recognition site, however, the former (class IIS) enzyme cleavage sites are shown on each strand using solid arrows for BsmBI and open arrows for BsgI. (b) Schematic diagram to show the generation of a 5 bp deletion of pNBL to generate pNBLA1. Spaces in the sequence are used to represent cleavage of DNA. Only the positions of recognition sites that remain intact following relaxation of the deleted vector are boxed. (c) The sequence of pNBLA2. This construct was generated by performing an identical round of deletion as that depicted in (b) except pNBLA1 was used as a template. Similarly, (d) shows the sequence of pNBLA3, generated by a round of deletion using pNBLA2 as a template.

The marker used was lambda DNA digested with SylI. Only the sizes of relevant marker bands (bp) are shown.

Figure 2. Restriction enzyme mapping of deletions. For each of the deletions, a plasmid was selected for restriction mapping. Plasmid DNA was digested with restriction enzymes using the manufacturer’s recommended conditions. The marker used was lambda DNA digested with SylI. Only the sizes of relevant marker bands (bp) are shown.
allows deletions to be generated rapidly using techniques that are both routine and inexpensive. We envision that this technique would be used to generate small deletions to map regions of DNA that have been previously crudely mapped by the exonuclease/mung bean nuclease deletion procedure. One example of the use of this system is shown here. In this example, the DNA to be deleted would be (sub-)cloned downstream of the cloned deletion cassette in pNBL. However, where convenient, the class IIS restriction enzyme sites could also be incorporated into the 5′-end of a PCR primer that is used to amplify the DNA of interest, followed by cloning into a suitable vector. This DNA fragment can then be subjected to fine deletion analysis using the primer-encoded class IIS restriction enzyme sites. In any case, following the initial (sub-)cloning of the target DNA fragment into a vector the generation of multiple deletions can be rapidly performed without further time consuming sub-cloning.

In conclusion, we have demonstrated a novel deletion system that allows precise, sequential deletions of adjacent DNA sequence. This facilitates the analysis of transcriptional regulatory elements since it is easy to control the size and location of the deletion created. The use of other class IIS restriction enzymes would allow deletions of different sizes to be created. For example, the use of the BsgI and BbvI sites allows the creation of a maximum deletion of 16 bp. As other enzymes become available the deletion capacity of this type of deletion system would be greatly increased. The only constraint on class IIS enzyme sites that may be used is that they are not present on the both the vector and target DNA.

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REFERENCES