

Use of a linear multicopy vector based on the mini-replicon of temperate coliphage N15 for cloning DNA with abnormal secondary structures

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ABSTRACT

A new cloning vector pN15L is described. It is a linear 13.8 kb plasmid based on the coliphage N15 mini-replicon. The vector capacity exceeds 50 kb and the copy number is 250 per *Escherichia coli* chromosome. We show that some artificial and natural palindromes and ~5% of human DNA *Bgl*II fragments can be cloned effectively in linear vector pN15L, whereas they either sharply reduce the copy number of circular vector pUC19 or cannot be cloned at all. We conclude that pN15L may be usefully employed to clone large imperfect palindromes and some abnormal sequences of human DNA.

INTRODUCTION

The *Escherichia coli* temperate bacteriophage N15 was discovered and investigated by V. Ravin *et al.* (1–3). The N15 prophage is not integrated into the bacterial chromosome but exists as a low copy number plasmid (4). Later it was shown that prophage N15 (plasmid N15) is a linear double-stranded DNA with covalenly closed ends (5,6). The analysis of linear and circular mini-plasmids containing different fragments of N15 DNA allowed the location of regions essential for replication (mini-replicon) and plasmid maintenance (7). The sequence of the 46.3 kb phage DNA is available in the GenBank database (accession no. AF064539). In the present article we describe a multicopy linear vector based on the mini-replicon of N15.

MATERIALS AND METHODS

Standard methods

Restriction enzymes and T4 DNA ligase were purchased from Promega and were used as specified by the vendor. DNA extraction and *in vitro* recombinant DNA manipulations were carried out according to standard methods (8). Dr D. Mukha (Institute of General Genetics, Moscow) kindly provided *Tetrahymena pyriformis* DNA. Bacterial strain DH5α was used as the host strain for cloning in pUC19, DH5α(N15) for cloning in pN15L.

Construction of linear vector pN15L

N15 phage DNA was partly digested by *Sau*3A and ligated with the kanamycin resistance gene excised from plasmid pUC4K with *Bam*HI. Several dozen plasmids of various copy numbers and lengths were obtained after transformation of *E.coli* C and *E.coli* C(N15). One of the highest copy number plasmids was used to create a linear vector by inserting a DNA fragment containing the SP6 and T7 promoters and *Bgl*II and *Not*I restriction sites into its *Bgl*II site. The vector obtained (named pN15L) has the same end structures and the same replicon as plasmid N15 (Fig. 1).

RESULTS AND DISCUSSION

Vector pN15L is a linear 13.8 kb plasmid (Fig. 1). pN15L can only be maintained in the N15 lysogenic strain and is stable only under selective conditions. The role of resident N15 prophage in the maintenance of pN15L is as yet unknown. Under selective conditions (50 mg/ml kanamycin) plasmid pN15L has a very high copy number, 250 per bacterial chromosome. The efficiency of DH5α(N15) transformation by pN15L is 3-fold lower than that by pUC19. Insertion of foreign DNA fragments into the *Bgl*II site of pN15L decreases both the efficiency of transformation and copy number, but the total amount of plasmid DNA per cell remains constant, even in the case of a 50 kb insertion.

Cloning of palindromic sequences in commonly used *E.coli* vectors poses some specific problems due to the tendency of palindromes to form cruciform structures in circular supercoiled DNA (9,10). Problems are connected with hairpin formation during lagging strand replication (11,12). These hairpin structures may then be cleaved by SbcCD nuclease (13). Maintenance of palindromes essentially depends on the replicon (9). Vector pN15L is very different from the usual pUC-like circular vectors. First, it is a linear and, probably, not supercoiled plasmid; second, pN15L has a unique set of genes responsible for DNA replication ('the replication machinery'). Both factors may be important for propagation of palindromes. This prompted us to compare the cloning fidelity of artificial and natural palindromes in linear vector pN15L and in the typical circular vector pUC19.

We cloned in pUC19 artificial palindromes that contained two 5.5 kb *Bam*HI–*Bgl*II λ DNA fragments oriented head-to-head and

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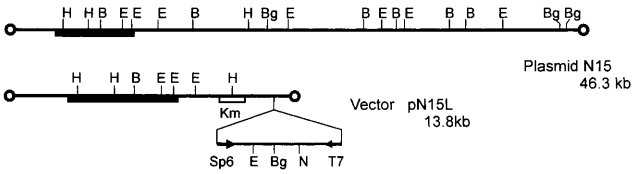

Figure 1. Restriction maps of plasmids N15 and pN15L. Open circles, ends of plasmids having hairpin structure. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; N, *Not*I. Black box, the mini-replicon; open box, kanamycin resistance gene.

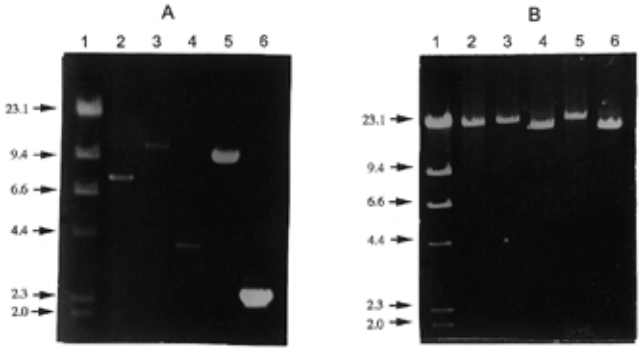

Figure 2. Some fragments of human genomic DNA decrease the copy number of recombinant pUC19 but not pN15L plasmids. (**A**) Recombinant pUC19 plasmids. Lane 1, *Hin*dIII-digested λ DNA; lanes 2–4, low copy number plasmids; lanes 5 and 6, normal copy number plasmids. (**B**) Recombinant pN15L plasmids containing inserts from recombinant pUC19 plasmids shown in (A) lanes 2–6. Lane 1, *Hin*dIII-digested λ DNA; lanes 2–6, pN15L with inserts from pUC19 shown in (A) lanes 2–6, respectively. Arrows indicate the positions and sizes (in kb) of the λ *Hin*dIII marker fragments.

divided by a central spacer of different length. Among 72 recombinant clones analysed, four contained very low copy number plasmids. Restriction analysis of 'abnormal' insertions showed that these palindromes had spacers of lengths 40–60 bp, whereas the same analysis for several 'normal' insertions showed that they had spacers of 100 bp or more. The insertions in these low copy number plasmids were excised with *Bam*HI and cloned in the *Bgl*II site of pN15L. All pN15L recombinants containing these 'abnormal' insertions were of normal pN15L copy number. We conclude that use of a linear vector is preferable for cloning of large imperfect palindromes.

As an example of a natural palindrome we used a palindrome from *T.pyriformis* in cloning experiments. Extrachromosomal DNA of this organism exists as a large imperfect palindrome ~20 kb long with a central spacer of 24 bp (14). The central 12.5 kb *Bam*HI fragment was isolated and cloned in pUC19 and pN15L. Insertion of this fragment into the *Bgl*II site of pN15L did not affect the efficiency of transformation or the copy number. In contrast, insertion of this fragment into pUC19 decreased the copy number more drastically than a non-palindromic insert of the same size. Moreover, in some clones we observed deletions in the cloned fragment. Similar results were obtained using the N15 lysogen as a host strain. We conclude that this palindrome can be cloned correctly in the linear vector but not in pUC19.

It is known that human DNA comprises palindromes and palindrome-like sequences (see for example 15). On the other hand, some cosmid clones are unstable and their propagation can result in rearrangements of inserted sequences (16). It is possible that one of the reasons for such instability is inverted repeats and other abnormal sequences. Because our results show that the linear vector pN15L is preferable to a circular one for cloning the palindromes tested, we decided to compare the cloning fidelity of human DNA in the linear and circular vectors. We chose pUC19 as an example of a circular vector because the most common cosmid vectors usually employed for cloning eukaryotic genomic DNA are based on the same replicon.

Two sets of experiments were carried out.

DNA from human lymphocytes was treated with *Bam*HI and cloned in pUC19. Seventy randomly chosen recombinant clones were analysed. Plasmids in 67 clones had a normal copy number but three plasmids had a very low copy number (Fig. 2). The low copy numbers of these plasmids was not due to very long inserts and was independent of orientation of insertion. When these abnormal inserts and several control inserts were recloned in pN15L, all the pN15L recombinant plasmids had a normal copy number (Fig. 2). We can conclude that some abnormal sequences exist in human DNA.

In the second set of experiments we estimated the frequency of human DNA sequences which are clonable in the linear but not in the circular vector. DNA from human lymphocytes was treated with *Bgl*II and cloned in pN15L. Two hundred randomly chosen clones were analysed. All clones had recombinant plasmids with a normal copy number. Insertions from these clones were excised with *Bgl*II and recloned in the pUC19 *Bam*HI site. In most cases insertions could be cloned normally in pUC19 but in seven cases the plasmids in recombinant clones were of very low copy number. Moreover, in three cases (clones 1–3) we did not obtain any pUC19 recombinant. Analysis of these 10 abnormal insertions showed that clone 1 contains a palindrome with a 270 bp stem and a 190 bp central spacer. The spacer consists of two 95 bp direct repeats. The inverted repeats were also observed in clone 2.

In summary, we believe that use of the linear vector pN15L gives some advantages for cloning of large imperfect palindromes and some abnormal sequences present in human DNA. The vector pN15L is very different from pUC-like circular vectors. The most important distinctions are the form of the vectors and the DNA replication machinery. Which of these properties of pN15L is responsible for the advantages in cloning of 'abnormal' DNA sequences is as yet unknown.

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