Gene, 80 (1989) 151–154
Elsevier

GENE 03013

A cassette with seven unique restriction sites, including octanucleotide sequences: extension of multiple-cloning-site plasmids

(Recombinant DNA; vector; *Not*I; *Sfi*I; oligodeoxyribonucleotides; plasmids)

Jörg D. Hoheisel

Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz (F.R.G.)

Received by G. Wilcox: 3 January 1989
Accepted: 27 January 1989

SUMMARY

A 27-bp synthetic DNA cassette was constructed which contains the restriction sites of the two rare-cutter enzymes *Not*I and *Sfi*I and, in an overlapping arrangement, those of five enzymes with 6-bp recognition sequences: *Apa*I, *Bal*I, *Nde*I, *Sac*II, *Xma*III. The protruding termini of the fragment allow its insertion into any *Eco*RI-cut DNA creating a new *Eco*RI site at one side of the cassette only.

This fragment was integrated into the pUC18-like multiple-cloning-site (MCS) plasmids pTZ18R and pTZ19R, producing a set of vectors which carry seven additional unique restriction sites (giving a total of 17) within their MCS. They still provide the capabilities of simple recombinant selection by blue/white coloured colonies, creation of single-stranded DNA in the presence of a helper phage, and in vitro transcription of cloned DNA using T7 RNA-polymerase. Plasmids with two copies of the DNA cassette inserted into their MCS were also constructed. Beside the advantages they provide in some cloning procedures, these latter plasmids, which carry a tandem repeat, are valuable sources of related 27-bp fragments, with features similar to the original but with different cloning termini.

INTRODUCTION

Vectors are a fundamental tool in molecular biology and genetic engineering. A prerequisite of their application is the presence of unique restriction sites. The usefulness increases with the accumulation of such sites in short MCS. That of the plasmid pUC18 (Yanisch-Perron et al., 1985), which provides a simple selection of recombinant clones due to its positioning inside the *lacZ*α-gene, is in common use (Pouwels et al., 1987). The pTZ-vector family (Mead et al., 1986) derives from the pUC plasmids

Correspondence to: Dr. J.D. Hoheisel at his present address: Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, (U.K.) Tel. (1)242-0200, ext. 3308; Fax (1)405-1556.

Abbreviations: Ap, ampicillin; bp, base pair(s); dNTP, deoxyribonucleotide triphosphate; ds, double strand(ed); MCS, multiple cloning site(s); nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; ss, single strand(ed); T_m, melting temperature; USB, United States Biochemical Corporation, Cleveland, OH; XGal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

but has been improved by insertion of the promoter sequence of the T7 RNA polymerase immediately adjacent to the MCS and by introduction of the bacteriophage f1 origin of DNA replication.

During mapping of protein-binding sites, unidirectional deletions in DNA, cloned in pTZ18R and pTZ19R, using exonuclease III (Henikoff, 1984; Hoheisel and Pohl, 1987) had to be isolated. Therefore, two restriction sites were necessary for linearization: one to protect the vector DNA itself from digestion, after the end was filled-in with α-thiophosphate nt (Putney et al., 1981), the other to produce the substrate for exonuclease III. To reduce the frequency of undesired cleavage inside the recombinant DNA, a short synthetic DNA cassette was constructed which allows double digestion with the two rare-cutting enzymes *Not*I and *Sfi*I and does not affect the features of the pTZ plasmids after insertion into their *Eco*RI site. Careful design of the sequence permitted the introduction of five additional restriction sites which are absent in pTZ as well as in pUC vectors.

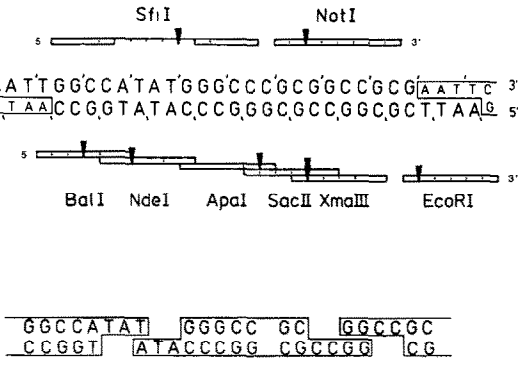

Fig. 1. Sequence of the 27-bp synthetic oligo cassette containing the restriction sites of *Sfi*I, *Not*I, *Bal*I, *Nde*I, *Apa*I, *Sac*II, and *Xma*III. The 5′-protruding termini are complementary to the ss ends of *Eco*RI-cut DNA (framed sequence). However, insertion creates one new *Eco*RI site only, distal to the *Not*I site. The boxes above and below the oligo show the location of each restriction site. Arrowheads mark the exact position of the cuts in the upper strand (5′–3′). The tick marks indicate codons in the reading-frame of the *lacZ*α-gene for both 5′–3′ MCS orientations in the pTZ18R. The lower part of the figure shows the termini which are produced by double digestion with *Sfi*I + *Not*I.

EXPERIMENTAL AND DISCUSSION

(a) Design of the cassette

The 27-bp ds fragment, which is outlined in Fig. 1, was assembled by annealing two synthetic oligos. Their sequences were designed as to meet several requirements.

(1) They are complementary to each other in a core region of 23 bp. Therefore, their hybridization produces a 23-bp ds fragment with 4-nt ss protrusions at the 5′ termini, which complement the ss ends of *Eco*RI-cleaved DNA. An insertion, however, creates only one new *Eco*RI site distal the *Not*I site.

(2) Independent of the cassette's orientation, neither frameshift nor any terminator or Cys codon occurs in any reading frame.

(3) An overlapping arrangement of the *Not*I and *Sfi*I sites, which would be possible to design on account of the interrupted recognition sequence of *Sfi*I, was avoided so as to permit two separated cuts with these rare-cutting enzymes. If the DNA is linearized by such a double digestion, a fill-in reaction catalysed by PolIk in the presence of dATP, dCTP, and labeled (or substituted) dGTP results in selective labelling of the *Not*I terminus only.

(4) A single additional bp, to give a total size of 27 bp to maintain the reading frame, is located between the *Sfi*I and *Not*I sites (Fig. 1). Its choice as well as the 5 bp which split the recognition sequence of *Sfi*I provided an opportunity to create additional restriction sites. The final arrangement presented in Fig. 1 contains the maximal number of such restriction sites which are absent in the sequences of pTZ18/19 and pUC18/19.

(b) Extension of the MCS of pTZ18R and pTZ19R

A 25-μM solution of the synthetic, unpurified oligos, both in equimolar amounts, was boiled for 3 min and immediately incubated at 68°C for 20 min, and 61°C for 2 h (13°C and 20°C, respectively, below the estimated T_m of the complementary 23 nt; Hames and Higgins, 1986), followed by slow cooling to room temperature. The ds fragment was phosphorylated with T4 polynucleotide kinase (USB), and subsequently a 1000-fold molar excess of the segment was ligated with *Eco*RI-linearized pTZ18R or pTZ19R (Maniatis et al., 1982). $CaCl_2$-treated *Escherichia coli* JM83 (Yanisch-Perron et al., 1985)

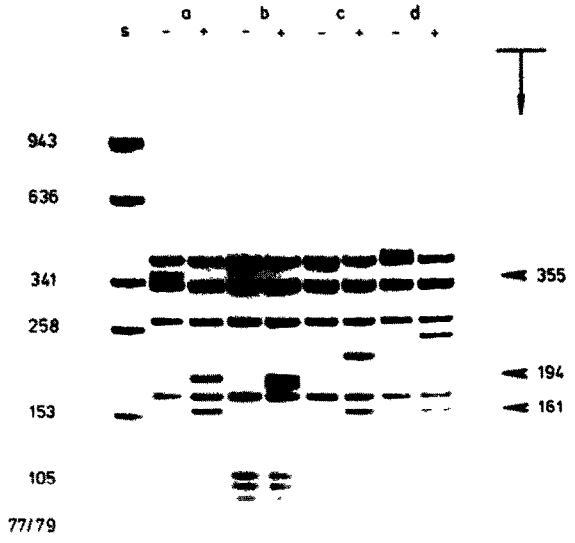

Fig. 2. Restriction analysis. Analysis of vector pTZ18R (lane **a**) and of three extended derivatives, containing the cassette once (lane **b**, pJDH118; lane **c**, pJDH218; see Fig. 4) or twice (lane **d**, pJDH618). Electrophoresis was in low-melting-point 3% agarose. For analysis, the plasmid DNA was digested with *Cfo*I (−), producing in pTZ18R a 355-bp fragment which contains the MCS; therefore, a subsequent cleavage with *Eco*RI (+) specifically cut that fragment. Since an insertion of the cassette creates a new *Eco*RI site at one end only, the 161-bp or the 194-bp fragment of pTZ18R is extended by 27 bp (**b**, **c**) or 54 bp (**d**), indicating the number and the orientation of inserts. Standard (**s**) is a *Sau*3A digest of pUC18. Sizes (in bp) are indicated on the margins.

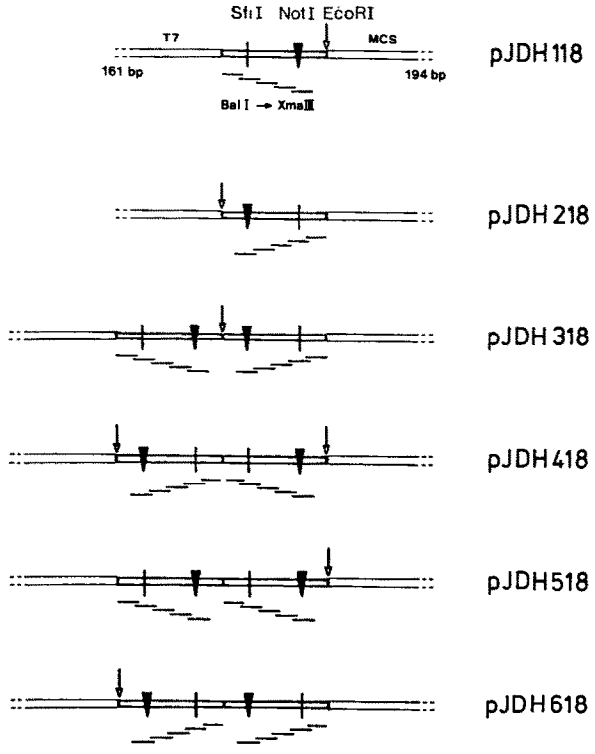

Fig. 4. The set of pTZ18R derivatives carrying one or two cassettes. In the uppermost graph, the symbols for the *Eco*RI, *Not*I, and *Sfi*I sites are shown. Staggered short lines (top-to-bottom) represent the *Bal*I, *Nde*I, *Apa*I, *Sac*II, *Xma*III sites, respectively. The orientation of the vector is indicated by the positions of the T7 promoter and the MCS valid for all derivatives as shown for PJDH118, as well as by the sizes of the adjacent fragments (161 bp, 194 bp) created by a *Cfo*I + *Eco*RI double digestion of pTZ18R. The derivatives of pTZ19R, which contain the insert in the same orientation to the remaining MCS, are named accordingly (e.g., pJDH119).

was transformed and plated onto 100 μg Ap/ml and XGal (30 μl of a 2% solution per plate; Sigma). The plasmid DNA of some blue colonies was isolated and characterized by restriction analyses (Fig. 2). About 50% of the plasmids analyzed carried the MCS cassette. Of these, 50% contained one copy of the cassette, 30% two copies, and 20% more than two copies.

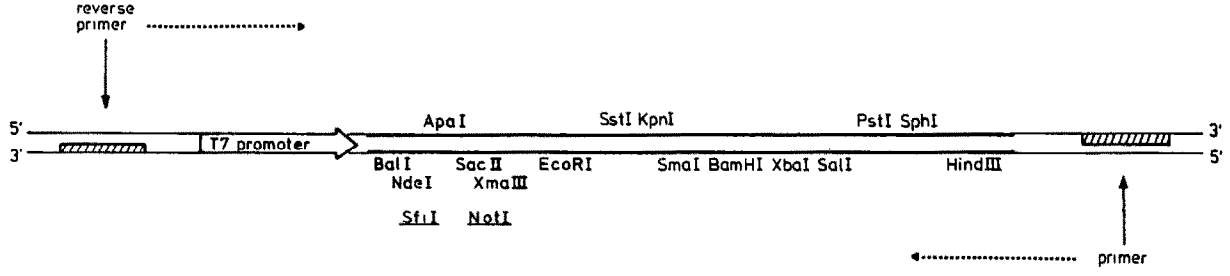

Fig. 3. Arrangement of the 17 unique restriction sites in the MCS of the plasmid pJDH118. The five enzymes marked above the DNA produce 4 nt 3′-protrusions, which are not digested by exonuclease III (Henikoff, 1984). The T7 promoter is represented by an arrow, indicating the direction of transcription. The hatched boxes show sequences complementary to the commercial primer and reverse-primer oligos.

The successful cloning into the *Eco*RI-cut vector, the activity of the α-complementation peptide of the β-galactosidase in clones carrying an extended MCS, and the results of the characterization by gel electrophoresis, as shown in Fig. 2, indicate an insertion of the intact cassette. This was confirmed for each isolate by digestions with all the eight enzymes whose restriction sites cover the entire fragment (data not presented). Fig. 3 shows all the 17 unique restriction sites of an extended MCS, with the cassette in an orientation that does not change the position of the *Eco*RI-site.

A set of plasmids containing the cassette as a repeat was also isolated (Fig. 4). They allow directional cloning into the overlapping restriction sites of the cassette by partial digestion of the vector DNA with one enzyme, followed by complete cleavage with the other one. Inverted MCS repeats provide several restriction sites in different combinations, in particular those of the rare-cutting enzymes, to excise an entire inserted DNA fragment.

The clones pJDH518/618, containing a direct repeat (Fig. 4), are a valuable source of some related 27-bp segments. Cleavage with *Apa*I or *Sac*II produces fragments with both sites of the rare-cutters, while digestion with *Bal*I, *Nde*I, *Xma*III, or *Not*I, creates fragments containing either the *Sfi*I or the *Not*I site. All of these carry four of the 6-bp recognition sequences.

(c) Conclusions

The synthetic 27-bp fragment allows the extension of any vector containing an *Eco*RI site. In addition to its five 6-bp restriction sequences, the introduction of the *Not*I and *Sfi*I rare restriction sites (Qiang and Schildkraut, 1987) can simplify the application of many vectors in the cloning, characterization, and handling of DNA. For instance, the technique of plasmid rescue (Perucho et al., 1980) might be developed into a cosmid-rescue methodology by the insertion of the cassette into an appropriate vector. Also using the plasmid derivatives described here, it may be very well possible to linearize recombinant clones without cleaving the inserted DNA; this is helpful for cloning blunt-ended DNA fragments in a fast and efficient way (Upcroft and Healey, 1987).

ACKNOWLEDGEMENTS

I am very grateful to Dr. F.M. Pohl for stimulating discussions and his support. I also thank Dr. K.H. Scheit, MPI für Biophysikalsche Chemie, Göttingen, for the synthesis of the oligos and Drs. Alister G. Craig and Robin Smith for helpful comments on the manuscript. This work was supported by the European Community, Grant No. BAP-0135-D.

REFERENCES

Hames, B.D. and Higgins, S.J. (Eds.): Nucleic Acid Hybridization, A Practical Approach. IRL Press, Oxford, 1986.

Henikoff, S.: Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28 (1984) 351–359.

Hoheisel, J.D. and Pohl, F.M.: Searching for potential Z-DNA in genomic *Escherichia coli* DNA. J. Mol. Biol. 193 (1987) 447–464.

Maniatis, T., Fritsch, E.F. and Sambrook, J.: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.

Mead, D.A., Szczęsna-Skorupa, E. and Kemper, B.: Single-stranded DNA 'blue' T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. Prot. Engin. 1 (1986) 67–74.

Perucho, M., Hanahan, D. and Wigler, M.: Genetic and physical linkage of exogenous sequences in transformed cells. Cell 22 (1980) 309–317.

Pouwels, P.H., Enger-Valk, B.E. and Brammar, W.J.: Cloning Vectors. Elsevier, Amsterdam, 1987.

Putney, S.D., Benkovic, S.J. and Schimmel, P.R.: A DNA fragment with an alpha-phosphorothioate nucleotide at one end is asymmetrically blocked from digestion by exonuclease III and can be replicated in vivo. Proc. Natl. Acad. Sci. USA 78 (1981) 7350–7354.

Qiang, B.-Q. and Schildkraut, I.: *Not*I and *Sfi*I: restriction endonucleases with octanucleotide recognition sequences. Methods Enzymol. 155 (1987) 15–21.

Upcroft, P. and Healey, A.: Rapid and efficient method for cloning of blunt-ended fragments. Gene 51 (1987) 69–75.

Yanisch-Perron, C., Vieira, J. and Messing, J.: Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene 33 (1985) 103–119.