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The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers

(Recombinant DNA; multiple cloning sites; restriction sites mobilizing element; dideoxy sequencing)

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SUMMARY

A series of plasmid vectors containing the multiple cloning site (MCS7) of M13mp7 has been constructed. In one of these vectors a kanamycin-resistance marker has been inserted into the center of the symmetrical MCS7 to yield a restriction-site-mobilizing element (RSM). The drug-resistance marker can be cleaved out of this vector with any of the restriction enzymes that recognize a site of the flanking sequences of the RSM to generate an RSM with either various sticky ends or blunt ends. These fragments can be used for insertion mutagenesis of any target molecule with compatible restriction sites. Insertion mutants are selected by their resistance to kanamycin. When the drug-resistance marker is removed with *Pst*I, a small in-frame insertion can be generated. In addition, two new MCSs having single restriction sites have been formed by altering the symmetrical structure of MCS7. The resulting plasmids pUC8 and pUC9 allow one to clone doubly digested restriction fragments separately with both orientations in respect to the *lac* promoter. The terminal sequences of any DNA cloned in these plasmids can be characterized using the universal M13 primers.

INTRODUCTION

Unique restriction sites occurring within the structural region of easily testable genetic markers, such as the site for *Pst*I in the β-lactamase gene in pBR322, have been used as cloning sites (Bolivar et al., 1977). A new type of cloning vector based on a single-stranded DNA phage containing a segment of the *Escherichia coli lac* operon as a selectable marker gene did not have many useful restriction sites for cloning DNA into this marker (Messing et al., 1977). Therefore, unique sites for cloning had to be introduced into the appropriate region of the vector by mutagenesis without the inactivation of the function of the gene product. (Gronenborn and Messing, 1978). Insertion of restriction fragments into the newly created cloning site of M13mp2, however, can disrupt the coding information, and then leads to the loss of a functional gene product. This inactivation can be tested

Abbreviations: APH, aminoglycoside 3′-phosphotransferase; bp, base pairs; MCS, multiple cloning site; RSM, restriction-site-mobilizing element.

by a histochemical reaction on agar plates and does not require replica plating like in the case of a drug-resistance marker. Thus, insertion of DNA into the newly created cloning site can be screened for by a one-step procedure (Gronenborn and Messing, 1978; Messing and Gronenborn, 1978).

Insertions into the *Eco*RI site do not necessarily disrupt the coding region of the *lac* DNA. In-frame insertions may allow the synthesis of an altered protein that is still functional. This flexibility in protein structure may be a special case, since function of the gene product is tested by its ability to complement a defective β-galactosidase encoded by the host and not to exhibit enzyme activity by itself. Altered protein structure that does not cause the loss of function leads mostly to a weaker interaction with the defective β-galactosidase and a less intense histochemical reaction. It is therefore appropriate to state that any insertion into the *Eco*RI site of M13mp2 leads rather to a reduction rather than to an inactivation of *lac* function (Gronenborn and Messing, 1978; Messing, 1979). This property of the *lac* DNA of M13mp2 has been the rationale to introduce 14 additional codons containing an array of different cloning sites. The resulting vector M13mp7 allows the cloning of a larger variety of restriction fragments using a histochemical screen for recombinant molecules (Messing et al., 1981).

Placing several cloning sites within a unique position of a desirable marker offers two new advantages to recombinant technology. First, a versatile primer constructed for the *Eco*RI vector M13mp2 (Heidecker et al., 1980) can be used to sequence DNA fragments cloned in any of the multiple cloning sites (MCS7) of M13mp7 (Messing et al., 1981). Second, if a drug-resistance marker is inserted into the center of these restriction sites, this array of restriction sites, a multiple of single codons, can be conveniently transferred to any other genetic system.

Pursuing these applications, a series of vectors have been constructed that were derived from the shotgun sequencing vector M13mp7. Since some of these characteristics are useful for plasmid as well as M13 vectors, plasmids with the pertinent pBR322 segment were constructed before transferring the modified sequence to phage M13. A pBR322 derivative resistant to *Acc*I, *Pst*I, *Hin*cII was used as a recipient for the MCS from M13mp7. In these plasmids, the β-lactamase function can be used for the selection of the plasmid and the *lac* DNA for the insertional inactivation. In addition, a 1430-bp fragment conferring kanamycin resistance has been inserted into the *Pst*I site of the MCS7 by GC-tailing to generate a vehicle for the mobilization of the MCS. In addition, two new MCSs have been constructed with these modified plasmids that contain a series of single restriction sites rather than the series of symmetrical sites. The two new vectors, pUC8 and pUC9, have this array of sites in the two different polarities. They can be used to clone doubly digested DNA fragments in both orientations. All pUC plasmids allow the use of the chain-terminator nucleotide sequencing method (Sanger et al., 1977) in the presence of the single-stranded M13 master primer (Messing et al., 1981).

MATERIALS AND METHODS

(a) Strains

The plasmids pML21 (Hershfield et al., 1976) and pUR2 (Ruther, 1980) have been kindly provided by J. Collins and U. Ruther, respectively. Phage M13mLL2 (Rothstein et al., 1980) has been kindly provided by R. Wu. M13mp7 and JM103 have been described (Messing et al., 1981). Plasmids pUC3, pUC4, pUC5, pUC6, pUC7, pUC71K, pUC8, pUC9, pUC81, pUC91 are described in this paper and their genealogy is given in Fig. 1. The host for these plasmids is the *E. coli* K-12 strain JM83 which carries the *lacZ* ΔM15 on a φ80 integrated into the chromosome (*ara*, Δ*lac-pro*, *strA*, *thi*, φ80d*lacZ* ΔM15) (Messing, 1979).

(b) Single base changes and deletions

Single base changes in the plasmid DNA were induced by treating growing cells with EMS to alter the *Pst*I site and with HA to alter the *Hin*cII site. Conditions of the mutagenesis were the same as described in Miller (1972).

Deletions were introduced using either BAL31 to alter the *Acc*I site in pUC5 or S1 nuclease to remove the cohesive ends of one of the *Eco*RI sites

in pUC71K. Both enzymes were obtained from Bethesda Research Laboratory and applied as recommended by the supplier. In both reactions, blunt ends were generated and used to circularize the molecule (pUC5) or to add *Hin*dIII linker molecules (pUC71K) as described below.

Plasmids having lost a unique restriction site were enriched by the differential transformation of host cells with mutant molecules as described recently (Messing et al., 1981).

(c) Plating of host cells

JM83 and JM103 were transformed with the pUC plasmids using calcium chloride treatment (Cohen et al., 1972). After transformation, cells were allowed to grow for at least 40 min before plating. The plates contained 2YT (Miller, 1972) and 50 μl of 50 mg/ml ampicillin and 50 μl of 2% Xgal (Miller, 1972) were added to the plates just before use. Since JM103 contains the *lacI*^{q} mutation (Muller-Hill et al., 1968), 10 μl of 100 mM IPTG was added as an inducer for this strain (Messing et al., 1981).

(d) Insertion of the kanamycin-resistance marker into the MC8

Plasmid pML21 (Hershfield et al., 1976) was cleaved with *Hae*II to excise the 1430-bp fragment bearing kanamycin-resistance gene (Oka et al., 1981). Plasmid pUC4 (Fig. 1) was digested with *Pst*I and both were treated with terminal transferase (Bethesda Research Laboratories) in the presence of dCTP (for pML21) or in the presence of dGTP (for pUC4). The homopolymer extension was performed as recommended by the supplier to allow the appropriate hybridization of the *Hae*II fragments and pUC4. This corresponds to the standard procedure for regenerating the *Pst*I site (Villa-Komaroff et al., 1978). After hybridization, JM83 cells were transformed and selected in the presence of 100 μg/ml kanamycin. Single colonies were saved and further purified by growing on selective plates.

(e) Addition of linkers

The *Hin*dIII linker (CCAAGCTTGG) was used as described for M13mp5 (Messing, 1979) and was originally obtained from K. Itakura. The *Pst*I linker (GCTGCCAGC) was purchased from Bio Logicals (Toronto). Both linkers were obtained in the phosphorylated form. Plasmid pUC71K was cleaved with *Eco*RI under conditions that allow cleavage of the molecules in only one of the two sites (Messing et al., 1977). The linearized DNA was treated with S1 nuclease as described above and purified by gel electrophoresis (Messing et al., 1977). Then *Hin*dIII linkers were added by blunt end ligation with T4 DNA ligase purified as described by Tait et al. (1980). In a second blunt end ligation, the *Pst*I linkers were added. The reaction products were treated with *Pst*I to remove the excess *Pst*I linkers, half of the parental MCS and the insert conferring kanamycin resistance. After purification by gel electrophoresis, the molecules with *Pst*I ends were circularized via their cohesive ends with T4 DNA ligase and amplified in JM83. The total batch was used to prepare plasmid DNA which was cleaved with *Hin*dIII to remove the excess *Hin*dIII linkers. The linearized products were recircularized after diluting the DNA solution and used to transform JM83 again. Single blue colonies were saved and selected again by a repeated plating.

(f) Shuttle experiments between M13 and plasmids

Transfer experiments between M13 and pUC plasmids are rather simple because M13 is detected as a plaque or by its transducing properties and pUC by conferring ampicillin resistance. Since the pUC plasmids without the *lac* DNA contain two *Hae*II sites, they were treated with *Hae*II under conditions that allow only one cut (see also map in Fig. 5). The M13 DNA was cleaved with *Hae*II giving rise to 5 fragments, one of them containing the *lac* DNA. Ligation of the *lac Hae*II fragment into the pUC plasmids was monitored by transforming JM83 cells and scoring blue ampicillin-resistant colonies.

The RSM1 (pUC4K, Fig. 1) was cleaved with *Bam*HI and inserted into the *Bam*HI site of M13mLL2 RF giving rise to RSM2 which has two *Sma*I sites in addition to the other sites of the MCS7. The insertion of this fragment was selected by the transduction of kanamycin resistance by the M13mLL2K phage. Since the *Bam*HI site in

M13mLL2 was flanked by the *Sma*I and the *Eco*RI sites, the RSM2 was cleaved with *Eco*RI and inserted into the *Eco*RI site of pUC7. The product, pUC71K, was identified by growth of recipient cells on plates containing ampicillin and kanamycin.

(g) Plasmid sequencing with chain terminators

About 500 ng of pUC81 were cleaved with *Bgl*I and mixed with 10 molar excess of the reverse primer (New England Biolabs) in the same buffer used for M13mp7 single-stranded DNA (Messing et al., 1981). Instead of heating the mixture to 55°C as described, it was boiled for 3 min and chilled in ice water. The primer extension was carried out immediately as previously described (Messing et al., 1981).

Enzymes, chemicals, and media and procedures such as agarose and polyacrylamide gel electrophoresis, ethidium bromide staining, plasmid preparations, etc. are described in detail elsewhere (Messing, 1982b).

RESULTS AND DISCUSSION

(a) Plasmid mutants selected with restriction enzymes

The plasmid pUR2 consists of the 2297-bp *Pvu*II/*Eco*RI fragment of pBR322 (Sutcliffe, 1979) and the *lac* *Hae*II fragment from M13mp2 (Gronenborn and Messing, 1978). The *Pvu*II/*Eco*RI fragment was circularized with the *lac* DNA inserted into one of the *Hae*II sites flanking the pBR322 origin (Ruther, 1980). The part of the *lac* operon required to function in intracistronic complementation, therefore, was reduced in size from the 789-bp *Hin*cII fragment in M13mp1 (Messing et al., 1977) to a 433-bp *Hae*II fragment in pUR2 (Ruther, 1980). Since the region of pBR322 conferring tetracycline resistance (Bolivar et al., 1977) was removed, only ampicillin can be used for plasmid selection.

The part of the pBR322 remaining in pUR2 contains a *Pst*I, a *Hin*cII and an *Acc*I site which would interfere with the use of the multiple cloning sites in the *lac* DNA of the MCS7. As shown in our earlier work (Gronenborn and Messing, 1978; Messing, 1979; Messing et al., 1981), restriction endonucleases in combination with a transformation of *E. coli* cells can be used to enrich for plasmid mutants with alterations in restriction endonuclease cleavage sites. A 1000-fold enrichment can be obtained per transformation cycle (Messing et al., 1981). Since this enrichment is based on the differential transformation efficiency of circular and linear DNA molecules, the restriction enzymes used for the selection must have a unique recognition on the wild-type DNA molecule. Therefore, the mutagenesis and selection to remove restriction site was carried out for plasmid pUR1 before introducing the *lac* *Hae*II fragment from M13mp7 to generate pUC4 (Fig. 1).

The cleavage sites for *Pst*I and *Hin*cII reside within the structural gene for β-lactamase, a func-

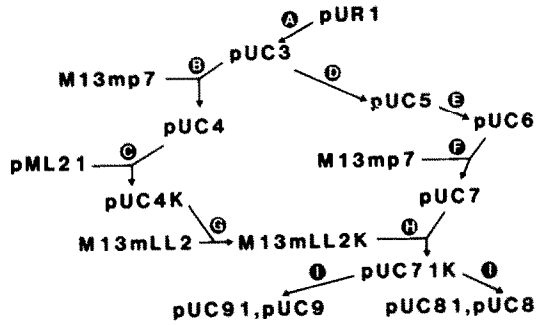

Fig. 1. The genealogy of the pUC plasmids. The plasmid constructions are derived from pBR322 (Bolivar et al., 1977). The first derivative is pUR1 (Ruther, 1980), a mini pBR322 containing nucleotides 2067 to 4362 map position and the ampicillin-resistance gene. The *Pst*I site CTGCAG (3612) was changed to CTACAG to yield pUC3 (A). The *Hin*cII site GTCAAC (3908) was changed to GTCAAT to yield pUC5 (D). The *Acc*I site (2246) was deleted by BAL31 treatment of yield pUC6 (E).

The *lac* *Hae*II fragment (804 to 1237) from M13mp7 (Messing et al., 1981) was inserted into the *Hae*II sites (2352) of pUC3 to yield pUC4 (B) and of pUC6 to yield pUC7 (F). The *Hae*II fragment from pML21 containing the APH I activity was cloned into pUC4 to yield pUC4K (C). The APH I sequence was cleaved out of pUC4K with *Bam*HI and inserted into M13mLL2 to yield M13mLL2K (G). The APH I sequence was then cleaved out of M13mLL2K with *Eco*RI and inserted into pUC7 to yield pUC71K (H). The replacement of one of the two *Eco*RI, *Sma*I, *Bam*HI, and *Sal*I sites by the *Hin*dIII site yields the two frame shift mutants, pUC81 and pUC91, and the two in-frame derivatives pUC8 and pUC9 (I). The designated map positions relate either to the pBR322 sequence (Sutcliffe, 1979) or to the M13mp7 sequence (Messing et al., 1981).

tion, which is required for plasmid selection. To avoid the loss of this function, these sites were altered by single base changes. Since the *Acc*I site resides within a nonessential region, it was removed by BAL31 treatment as described in MATERIALS AND METHODS. All intermediates pUC3 (*Pst*I-resistant), pUC5 (*Hin*cII-resistant) and pUC6 (*Acc*I-resistant) give normal plasmid yield and produce ampicillin-resistant colonies. Each mutational change was confirmed by analysing the reaction with the corresponding restriction endonuclease and agarose gel electrophoresis (not shown). The genealogy of these plasmid mutants as well as the other ones described in this paper is illustrated in Fig. 1.

(b) Plasmids pUC4 and pUC7 derived from M13mp7

Plasmid pUC3 lacking the *Pst*I site was partially digested with *Hae*II and the *lac Hae*II fragment from M13mp7 was cloned into pUC3. Recombinants were screened in JM83 for the formation of blue colonies as described in MATERIALS AND METHODS. The resulting plasmid pUC4 served as the starting plasmid to construct an RSM element as described in the next section.

To use the *Hin*cII and the *Acc*I sites in the *lac* MCS as cloning sites, the *lac Hae*II fragment was transferred from M13mp7 to pUC6. A blue colony was selected from this transformation experiment and its plasmid designated pUC7. In both cases, pUC4 and pUC7, the *lac Hae*II fragment has been inserted into the *Hae*II site between the amino-terminus of the β-lactamase gene and the plasmid origin. Transcription of the *lac* DNA occurs in the same direction as the transcription of the β-lactamase gene as demonstrated by the *Taq*I restriction pattern (not shown). A map of these plasmids is given in Fig. 5.

(c) A restriction-cleavage-sites-mobilizing element (RSM)

Random insertion of the MCS into the coding region of any gene can be used to study the structure of gene products. Codon insertions allow one to screen protein domains for their function. Furthermore, the insertion of such a MCS in the appropriate region of a gene can be used to design operon fusions in vitro which depend on the availability of certain restriction cleavage sites.

In M13mp7, these cloning sites serve for the insertion of restriction fragments produced by frequently cutting restriction enzymes like *Sau*3A, *Taq*I, *Msp*I, *Alu*I, etc. (Messing and Seeburg, 1981). The concept can be reversed in the following way. (1) These enzymes which cut frequently can be used to linearize a plasmid molecule by a limited digestion to produce random acceptor sites in the same way as has been done with M13RF (Messing et al., 1977) or with other double-stranded DNA molecules (Heffron et al., 1979). (2) The MCS is cut with the appropriate enzyme to produce compatible ends (Messing and Seeburg, 1981). (3) The MCS is inserted into the different acceptor sites leading to an in-frame insertion in different parts of the parent molecule. The restriction cleavage patterns of recombinant molecules can be used to map rapidly individual mutants. To enrich for the successful insertion of a MCS into a receptor molecule a restriction cleavage sites mobilizing element has been constructed. This element resembles a transposon, except that transposition is directed by cloning.

The drug-resistance marker Tn*903* which has been inserted into the central *Pst*I site of the MCS7 has been prepared as a *Hae*II fragment from pML21 (Hershfield et al., 1976). It encodes type I APH, aminoglycoside 3′-phosphotransferase, which confers kanamycin resistance and does not duplicate any site in the MCS7 (Oka et al., 1981). After the directed transposition and selection of transformed cells with kanamycin, the drug-resistance marker is removed by *Pst*I to create an in-frame insertion. To facilitate *Pst*I cleavage, the *Hae*II fragment containing the APH activity was cloned into pUC4 by GC-tailing (Villa Komaroff et al., 1978). JM83 host cells harboring the new plasmid pUC4K (Fig. 1) grow on medium containing kanamycin, but form colorless colonies when tested for β-galactosidase activity as described in MATERIALS AND METHODS. If the plasmid is cleaved with *Pst*I, two fragments of the size of pUC4 and the APH fragment conferring kanamycin resistance are produced (Fig. 2). If after cleavage the DNA fragments are ligated again and used to transform JM83, formation of blue col-

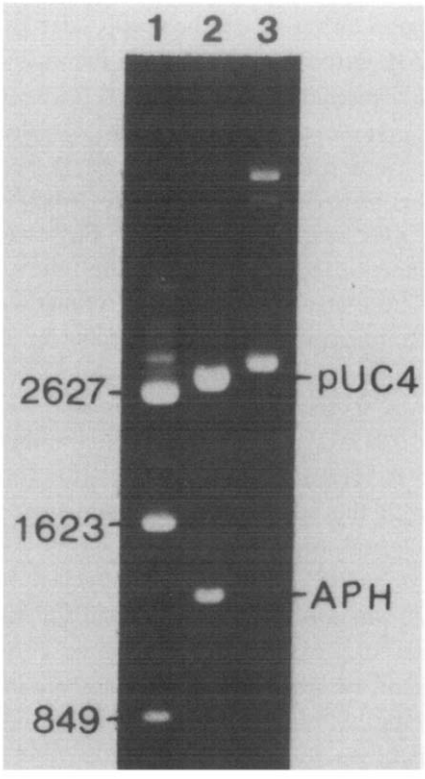

Fig. 2. Electrophoretic analysis of the RSM. The *Hae*II fragment containing the APH I activity was cloned into the *Pst*I site of pUC4 as described in MATERIALS AND METHODS. Since the GC-tailing regenerates the *Pst*I site, this fragment can be cleaved out again with *Pst*I as shown in lane 2. Positions of the APH and the pUC4 fragments are as indicated. Lane 3 contains a sample of uncut pUC4K and lane 1 a size marker. The same result is obtained when pUC4K is cleaved with *Eco*RI, *Bam*HI, *Sal*I, *Acc*I, or *Hin*cII (not included in this electrophoresis). Electrophoresis was performed with a 1% agarose gel in Tris-borate buffer (MATERIALS AND METHODS).

onies can be observed (not shown) indicating the proper release of the drug resistance from the RSM. The RSM has been used to study the structural part of the β-lactamase gene in pBR322. A number of different insertion mutants have been obtained showing different effects on function in respect to the altered structure (Vieira, J., Gardner, R.C. and Messing, J., manuscript in preparation).

(d) Plasmid vectors for "double-digest" cloning

The insertion of the APH fragment into the MCS7 of pUC4 serves not only as a RSM but also as an intermediate in constructing a new type of MCSs which play an important role in "double-digest" cloning. If the MCS in pUC4 or pUC7 (Fig. 5) is cleaved with a restriction endonuclease except *Pst*I, a small synthetic insert of a multiple of single codons is released. To produce a molecule with two different ends, a restriction cut has to be made in only one of the two possible sites. Since a limited digest is rather hard to detect and cannot physically be separated from the final cleavage product, "double-digest" cloning into this MCS is difficult and the separation of the two possible orientations cannot be directed. Having an insert of 1430 bp in the *Pst*I site of the MCS, however, all sites flanking the *Pst*I sites are set far apart. This approach has facilitated the isolation of a single size class of linear DNA molecules representing both intermediates produced by the limited digestion with *Eco*RI. They were then treated with S1 nuclease to remove the cohesive ends of the *Eco*RI site. A double synthetic linker consisting of a *Hin*dIII decamer and a *Pst*I octamer has been added by blunt end ligation. After cleavage with *Pst*I the redundant sites and the "kanamycin fragment" were removed and blue colony formers were selected as described in MATERIALS AND METHODS. This approach led to two different plasmids having the symmetry of the MCS removed and having a *Hin*dIII site added on to the *Pst*I site. Plasmids with all these sites in the two possible orientations were obtained.

Following this scheme the second GTCGAC in the MCS was lost and only one of the three enzymes recognizing this sequence can be used at a time (Fig. 5). Since it would be important to clone for instance *Hpa*II/*Hae*III or *Taq*I/*Alu*I fragments a second blunt end cloning site besides the *Hin*cII site has to be introduced. As the junction between the *Eco*RI site and the *Bam*HI site resembles a *Sma*I site this sequence was considered for the appropriate mutation.

The single steps of this construction were as follows. The APH fragment was released from pUC4K using *Bam*HI restriction endonuclease and transferred to the RF of M13mLL2 to yield M13mLL2K. M13mLL2 is a derivative of M13mp2 containing an *Eco*RI/*Sma*I/*Bam*HI/*Sma*I/*Eco*RI adapter putting the *lac* sequence out of phase (Rothstein et al., 1980). The APH fragment from M13mLL2K was then transferred as an *Eco*RI fragment to pUC7 to yield pUC71K

(Fig. 1). The symmetry was then removed and the *Hin*dIII site added as described above.

Individual transformants forming a blue colony were saved and plasmid DNA prepared. The orientation and the presence of the cleavage sites were checked by testing the appropriate cutting pattern by polyacrylamide gel electrophoresis. The analysis of the two examples designated pUC8 and pUC9 representing both orientations is given in Fig. 3.

(e) Frameshift correction of the *lac* DNA

Since the *Bam*HI/*Eco*RI adapter in M13mLL2 puts the *lac* sequence in a reading frame +2, the net addition of nucleotides in pUC8 and pUC9 should be as follows. Four nucleotides were removed with S1 nuclease. In addition, half of the MCS, a 25-nucleotide-long *Eco*RI fragment including one of the *Sma*I sites was replaced with a *Hin*dIII/*Pst*I adapter, 15 nucleotides long. This is a deletion of three codons and a reading frame

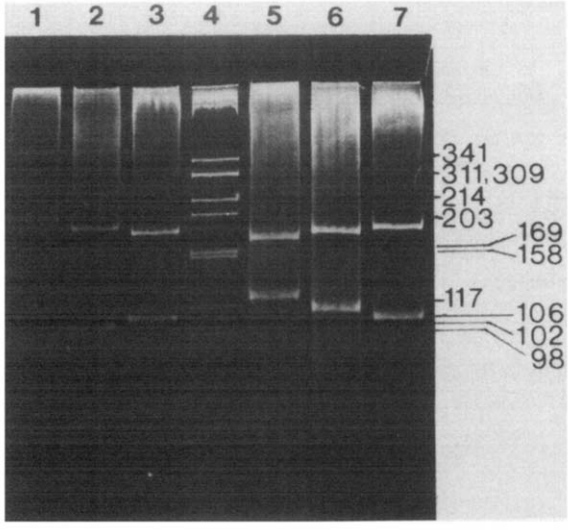

Fig. 3. Electrophoretic analysis of the MCS8 and MCS9. The constructions of the plasmids pUC8 and pUC9 are described in the text. The positions of the various sites in the MCSs were determined by double-digestion experiments. Both plasmids were cleaved with *Pvu*II which cleaves in M13mp7 at position 873 and 1183 in respect to the *Pst*I site at position 986. Aliquots of both cleaved plasmids were then cleaved with one of the enzymes recognizing one of the sites in the MCS (lanes 1–3, pUC8, lanes 5–7, pUC9, lane 4 size marker, lanes 1 and 5, *Hin*dIII, lanes 2 and 6, *Pst*I, lanes 3 and 6, *Sal*I). Electrophoresis was performed with a 6% polyacrylamide gel in Tris-borate buffer.

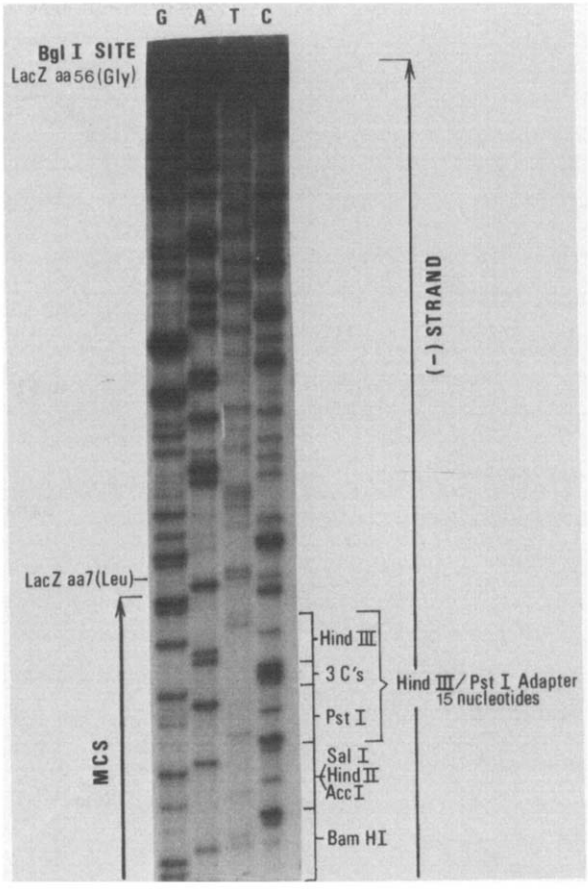

Fig. 4. DNA sequencing with pUC plasmids. The strategic differences of M13 versus plasmid sequencing are discussed in the text. The above sequence is derived from pUC81 to demonstrate the potential of sequencing the complementary strand and the structure of the new *Pst*I/*Hin*dIII adapter. Since this example was not used to sequence a piece of cloned DNA, the unique *Bgl*I site was used to linearize pUC81 instead of *Hin*dIII. Both sites are positioned distal to the synthetic primer in relation to the MCS. After cleavage the annealing and sequencing reaction was carried out as described in MATERIALS AND METHODS. The reactions were loaded in the order GATC. The pertinent part of the MCS containing the *Hin*dIII/*Pst*I adapter with the three GC base pairs in the middle is labeled. The sequence of the (−) strand of the *lacZ* gene from amino acid residue 7 (Leu) to the *Bgl*I site amino acid residue 56 (Gly) was marked accordingly. Electrophoresis was performed with a 8% polyacrylamide gel (MATERIALS AND METHODS).

+1. Products of this predicted nature have been indeed isolated, but do no longer produce blue colonies (pUC81 and pUC91). Because of the powerful screening using the blue color assay, products with a 1-bp deletion putting the *lac* sequence in-frame again have been identified. The presence of the predicted sites has been assured by

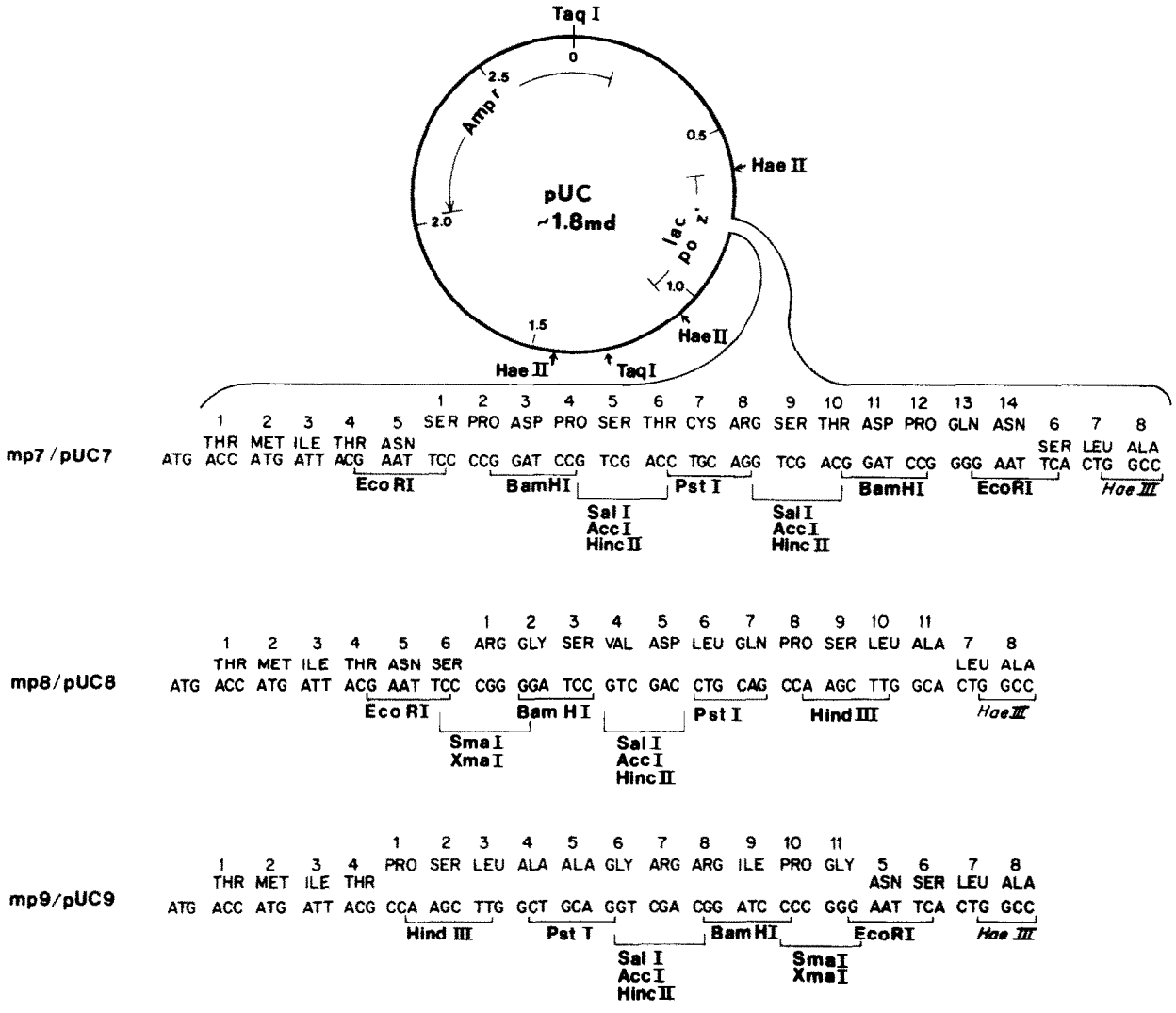

Fig. 5. Genetic maps of pUC plasmids. The *Taq*I site (4018 bp on the pBR322 map) serves as a reference for the maps of the pUC plasmids. Besides the *Taq*I sites in the MCS as part of the *Sal*I sites, only one other *Taq*I site (2574) located between the two *Hae*II sites (2352 and 2722) was derived from the pBR322 sequence. The different MCSs are presented with their recognition sequences in the reading frame of the amino terminus of β-galactosidase starting with the ATG codon at the left and ending with the *Hae*III site and codon 8, the alanine codon to the right. The additional codons of the modified *lac* sequence in the MCSs were set up and counted separately.

restriction cleavage analysis as shown in Fig. 3. The exact nature of the deletion was revealed by sequencing both, MCS8 and MCS9 in M13mp8 and M13mp9 with the universal sequencing primer (Messing and Vieira, 1982). From these data it can be assumed that possibly during the ligation of the two linkers one of the external Gs has been deleted. The pertinent sequence of pUC8 and pUC9 is compared with pUC7 (Fig. 5).

(f) DNA sequencing of pUC plasmids using the M13 master primer

Since the plasmid DNA is double-stranded as the RF of the M13 vectors, both strands can be sequenced with the appropriate single-stranded primer (Smith et al., 1979; Wallace et al., 1981). Instead of preparing the complementary strand in vitro and determining its sequence with the reverse

primer (Hong, 1981), this primer can be applied directly with the pUC plasmids. Fig. 4 shows the sequence of the *lac* (−) strand of the *lacZ* gene, the strand which is not packaged into the viral coat of M13 phage vectors and therefore not available in single-stranded form.

If plasmid sequencing and M13 sequencing are compared, it is still faster to prepare M13 templates in pure form than plasmid DNA (Howarth et al., 1981; Hu and Messing, 1982; Messing, 1982a). Nevertheless, once a plasmid clone is prepared, the border sequences of a cloned fragment can be rapidly determined with both universal primers. In general, one of the unique sites in the MCS8 or MCS9 distal to the primer used can be cleaved to linearize the recombinant plasmid on one side of the insertion. Strand separation of the linear double-stranded molecule can be achieved by boiling (Fig. 4) or by treatment with exonuclease III (Smith, 1979; Heidecker et al., 1980). The single-stranded primer allows DNA synthesis to occur only complementary to the cloned DNA. Since the two universal primers are used in combination with the array of single sites, the sequences of both ends of a cloned DNA fragment are readily accessible with a standard procedure.

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Note added in proof

In the meantime, a new pUC vector pair has been constructed. They have two additional cloning sites for *Sst*I and *Xba*I in their polylinker. pUC12 is derived from pUC8, and pUC13 from pUC9.