Volume 9 Number 19 1981 Nucleic Acids Research

ClaI, a new restriction endonuclease from Caryophanon latum L

H.Mayer*, R.Grosschedl+, H.Schütte* and G.Hobom+

*Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-3300 Braunschweig, and +Institut für Biologie III der Universität Freiburg, Schänzlestrasse 1, D-7800 Freiburg, GFR

Received 27 July 1981

ABSTRACT

From *Caryophanon latum* L a site specific restriction endonuclease (*Cla*I) has been purified, which recognises the DNA hexanucleotide palindrome 5'-A-T↓C-G-A-T-3'. Staggered cleavage generates DNA restriction fragments with 5'-terminal pCG extensions. A *Cla*I map of bacteriophage λ has been determined, which indicates cleavage inhibition due to adenine methylation at overlapping *Cla*I-GATC recognition sequences. Plasmid pBR322 is cut only once, in the tetracycline promoter region, and can, therefore, be used as a vector system for cloning fragments derived from *Cla*I digestions, and in addition for fragments generated by *Taq*I, *Hpa*II, and several other enzymes.

INTRODUCTION

From the large number of known restriction endonucleases (1) families can be discerned which cleave DNA substrates at closely related, though distinct recognition sites. In those cases where DNA fragments with the same type of terminal extensions are produced from staggered cleavages within a group of externally divergent, but centrally identical recognition sequences, the cloning of any fragment generated by such a family of restriction endonucleases is possible with a vector designed for any single member of this family. A versatile group of vector systems which has been missing for the co-cloning family of DNA restriction fragments with 5'-pCG single stranded ends is provided by cleavage of pBR322 and related derivatives with restriction endonuclease *Cla*I which is described in this article.

MATERIALS AND METHODS

Caryophanon latum L was isolated from cow dung by B. Rowenhagen and kindly given to us. *E.coli* 5K, obtained from S. Glover, was used for transformation, *E.coli dam*4 was provided by R. Eichenlaub. Bacterial alkaline phosphatase and DNaseI were purchased from Worthington, snake venom phosphodiesterase from Sigma, T4 polynucleotide kinase from Biogenics Research Corporation, and T4 DNA ligase from Miles. Plasmid DNAs were prepared following

a cleared lysate procedure in a previous description (2), λ DNAs were isolated from phage preparations obtained after temperature induction of λ*c*I857*S*7 or λ*c*I857*t*68 lysogenic strains or prepared by lytic infection of *E.coli dam*4. SV40 DNA was purchased from MRE, England, and the DNA restriction fragment λdvh93:*Taq*I-E was a gift from E. Schwarz.

Assay for *Cla*I restriction endonuclease activity: 0.1 - 0.3 µg of DNA were incubated in a total volume of 40 - 50 µl with 5 - 10 µl of activity containing fractions at 37°C for 60 min, either in 10 mM Tris HCl pH 8, 10 mM $MgCl_2$, or in 6 mM Tris-HCl pH 7.4, 6 mM $MgCl_2$, 50 mM NaCl, 6 mM 2-mercaptoethanol. The resulting fragment mixtures were separated by electrophoresis on agarose or polyacrylamide slab gels.

Analysis of the *Cla*I recognition sites: Two sets of DNA fragments were used for this analysis, 2 pmoles of *Cla*I digested pBR313 (3) DNA: 3 fragments, and an equimolar mixture (1 pmole each) of λdv1 (4) DNA: 3 fragments, λdv*imm*21AB5 (5) DNA: 3 fragments, and pHL81 (6) DNA: 4 fragments. The DNA fragment mixtures were dephosphorylated by incubation in 100 µl with 0.4 units of bacterial alkaline phophatase in 10 mM Tris HCl pH 9.3 for 1 h at 37°C, and the DNAs were deproteinised by phenol extraction, followed by ether extraction and ethanol precipitation. 5'-terminal labelling of the fragment mixtures was achieved using T4 polynucleotide kinase and γ-$[^{32}P]$-ATP following standard procedures (7). After ethanol precipitation most of the residual γ-^{32}P-ATP and ^{32}P-phosphate was removed through 1.5% agarose gel electrophoresis and re-isolation (8) of the labelled fragments.

For the analysis of the 5'-terminal nucleotide resulting from *Cla*I cleavage reactions both of the ^{32}P-labelled fragment mixtures were digested in 160 µl with 10 µg DNaseI in 10 mM Tris HCl pH 7.4, 10 mM $MgCl_2$ for 1 h at 37°C, and after raising the pH to 9.0 the incubations were continued for 1 h at 37°C in the presence of 2 µg snake venom phosphodiesterase. The resulting mononucleotides were separated by electrophoresis on Whatman 3 MM paper at pH 3.5 in pyridin : acetic acid : H_2O (1 : 10 : 159), identified under 254 nm UV illumination, and upon isolation measured for their ^{32}P-activity by liquid scintillation counting.

For the analysis of the 5'-terminal sequences of *Cla*I generated fragments, both of the ^{32}P-labelled fragment mixtures were digested partially, to oligonucleotides, by incubation first with DNaseI: 1 µg per 1.2 µg of total DNA in 60 µl for 30 min at 37°C as above, followed by additional treatment with 20 ng of snake venom phosphodiesterase. The reactions were stopped in one third each of the volumes after 0.5, 1, and 2 min at 37°C.

All three aliquots in both experiments were combined, lyophilised, dissolved in 10 µl H_2O, and separated in a two-dimensional fractionation procedure (9), using electrophoresis at pH 3.5 on cellulose acetate strips in the first, and homochromatography on 20% DEAE cellulose thin-layer plates at 65°C in the second dimension, with a 1 : 1 diluted 3% RNA homomix V solution.

In a second approach to determine the *Cla*I recognition site, DNA sequence analysis of the 5'-ends of *Cla*I generated fragments was used, following the Maxam Gilbert technique (7). For this purpose the *Cla*I generated fragments were dephosphorylated and labelled at their 5'-ends using γ-^{32}P - ATP and T4 polynucleotide kinase as above. DNA sequencing was performed using the G, A > C, C > T, and C + T reactions, the products were separated on 20% polyacrylamide gels in 7 m urea.

RESULTS

Isolation of restriction endonuclease *Cla*I

Caryophanon latum L was grown with shaking at 37°C in 30 ml medium prepared as follows: freshly collected cow dung mixed with the same volume of distilled water was homogenized and centrifuged at 25000 g for 1 h; the supernatant was added to the same volume of enriched nutrient broth (8 g nutrient broth (Difco), 5 g bacto peptone, 5 g NaCl, 3.5 g Na_2HPO_4, 1.5 g KH_2PO_4, 1 g glucose, 5 mg thiamine HCl per 1000 ml, adjusted to pH 7, and autoclaved). Fast growing cells show linear trichome chains (10) of about 3 x 15 µ in size, which at stationary phase collapse very rapidly. Large, log-phase cells (1-3 x 10^7 cell units/ml, controlled by light microscopy) were harvested by centrifugation, resuspended in 6 ml buffer A (10 mM potassium phosphate, 1 mM EDTA, 0.1% 2-mercaptoethanol, pH 7.5) and sonicated at 0°C using a Branson sonifier at 60 watts until the OD_{578nm} had dropped to 50%. The homogenate was centrifuged at 45000 g for 20 min at 2°C, and the supernatant was applied to a DEAE-cellulose column (2.5 x 12 cm) equilibrated by buffer A. After washing with buffer A the adsorbed protein was eluted in three 100 ml steps of 0.2, 0.5, and 1 M NaCl in buffer A. Fractions of 5 ml were collected and tested for endonuclease activity; most of this activity was contained in part of the 0.5 M NaCl step. The enzyme preparation was judged to be free of contaminating non specific nuclease activities, since a 5-fold excess of the enzyme at 15 hours incubation with λ-DNA still yielded the characteristic pattern of *Cla*I fragmentation unchanged, and an incubation with a 5'-[^{32}P]-labelled DNA fragment for 15 hours resulted in only

insignificant amounts of nonprecipitable ^{32}P-activity in several enzyme preparations. The enzyme was stored in 10 mM potassium phosphate, pH 7.5, 100 mM KCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 50% (v/v) glycerol at -20°C with only minor loss of activity after 6 months. The total yield was about 1300 U/per 3 g of cells, wet weight (1 U of activity digests 1 µg λ-DNA in 1 h at 37°C to completion).

Determination of the recognition site of *Cla*I

In a first approach the recognition site was characterised by determining the 5'-terminal nucleotide and following positions of various DNA fragments produced by cleavage with *Cla*I, both from individual fragments, and from fragment mixtures to show the specific and degenerate positions (if present) within the *Cla*I generated fragment ends.

After complete digestion with DNaseI and snake venom phosphodiesterase of the mixture of three fragments (six 5'-$[^{32}P]$-labelled fragment ends) generated by *Cla*I cleavage from pBR313 DNA, 98.7% of the radioactivity was found in the cytidylic acid spot, indicating that C is the 5'-terminal nucleotide in all of these fragments. The products of a partial digestion with DNaseI and snake venom phosphodiesterase of the same fragment preparation were separated two-dimensionally by electrophoresis on cellulose acetate at pH 3.4 (first dimension) and by homochromatography on DEAE-cellulose (second dimension). From the autoradiogram (Fig. 1) it can be concluded that all fragments of this mixture terminate in a 5'-C-G-A-T-N-sequence. Confirming data for both results were obtained from a second mixture with ten fragments (twenty 5'-$[^{32}P]$-labelled fragment ends), generated by *Cla*I cleavage of λdv1 DNA : 3 fragments, λdv*imm*21AB5 DNA : 3 fragments, and pHL81 DNA : 4 fragments. For these experiments 5'-$[^{32}P]$-labelled fragments were prepared individually, mixed in equivalent amounts according to their specific activities, and treated as described above (results not shown).

A second approach to determine the recognition site of *Cla*I was based on DNA sequencing using the Maxam-Gilbert technique (7). For this purpose the very small *Cla*I-generated fragment which extends to the right of the *Eco*RI site in pBR313 (pBR322) DNA was chosen for individual DNA sequencing. The ^{32}P -labelled fragment pBR313 : *Cla*I-B was cleaved by *Eco*RI, yielding two subfragments of 2.32 kb and 24 bp, respectively. The latter was used for determining the distance of its ^{32}P -labelled 5'-end relative to the *Eco*RI generated 3'-end, and was run on a sequencing gel in between two fragments with known DNA sequences. When the sequencing bands of equal mobility from the two standard fragments were used as a ladder for back extrapolation down

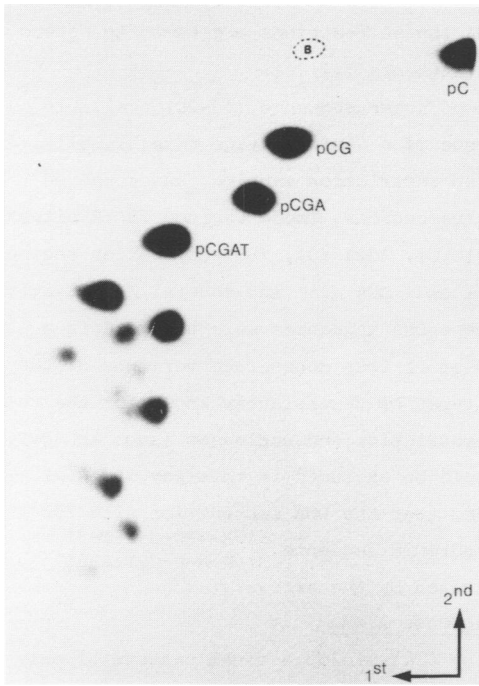

Figure 1. Autoradiogram of a two-dimensional fractionation of oligonucleotides resulting from a partial DNaseI/snake venom phosphodiesterase digestion of 5'-[^{32}P]-labelled fragments produced by cleavage of pBR313 DNA with *Cla*I. Directions of electrophoresis in the first (1st), and of homochromatography in the second (2nd) dimension have been indicated and the position of Xylene Cyanol FF(B) has been marked. The sequences deduced for the series of unique 5'-terminal oligonucleotides from their locations in this analysis have been indicated in the figure, degeneracy is beginning after four positions of unique sequence.

to the 5'-end we concluded that the *Cla*I end would be located at position +26 relative to the *Eco*RI end at position +3, which again points out a Cytosin located in the lower strand of a $\begin{matrix}5'\text{-A-T-C-G-A-T-}3'\\3'\text{-T-A-G-C-T-A-}5'\end{matrix}$ palindrome in the known pBR322 (11) sequence (results not shown). Several other *Cla*I fragments have also been used for DNA sequencing in the same way, including those derived from the *Cla*I sites at λ:37999, λ:42160, and λ:42800 (see Fig. 3). In every case their 5'-ends could be determined at the location of a C residue in a palindromic sequence identical to the one above. DNA sequencing of an equal mixture of all three fragments generated from pBR313 DNA by *Cla*I cleavage indicated confirming results, with degeneracy starting at the fifth position, above a unique GAT sequence at positions two to four from the 5'-end. - The two additional *Cla*I restriction sites in pBR313 DNA at 1.8 kb and 6.7 kb are not located in regions of known DNA sequence.

As is known from other type II restriction endonucleases cleavage by *Cla*I appears to result in fragments with 5'-phosphate bearing ends. This conclusion was drawn from the ability to religate *Cla*I linearised pBR322 DNA back into circular molecules and linear concatemers, and to ligate foreign *Cla*I fragments as well as *Taq*I or *Hga*II fragments into that linear vector

DNA as observed on the corresponding gel electrophoreses and in consecutive cloning experiments (see below). T4 DNA ligase reactions are known to depend on 5'-phosphate ends of substrate DNA fragments (12).

All plasmid, phage and virus DNAs of known sequence (13-20) available to us have been analysed for the presence of a *Cla*I cleavage site(s), all of which were then localized by detailed restriction mapping, and compared with the known sequence of the respective regions. These include fd (2 sites), ϕX174 (0), SV40 (0), λdvh93 (0), λdv021 (0), λdv1 (3), ϕ80 replication region (1), 21 replication region (1), as well as λ DNA (15) and several λ derivatives as described below. Finally, all of these DNA sequences were analysed in a computer-aided search for the presence of various degenerate versions of the unique *Cla*I recognition sequence. All types of degeneracies known for the recognition sites of several other (1) restriction endonucleases (such as *Acc*I, *Acy*I, *Ava*I, *Hae*II, *Hgi*AI or *Hin*dII) could be excluded in this way for *Cla*I.

From these data it can be concluded that the DNA recognition site for endonuclease *Cla*I consists of the palindromic sequence $\begin{matrix}5'\text{-A-T}\downarrow\text{C-G-A-T-3'}\\ 3'\text{-T-A-G-C}\uparrow\text{T-A-5'}\end{matrix}$ which is cleaved by the enzyme as indicated by the arrows.

Determination of a *Cla*I map of bacteriophage λ DNA

Digestion of bacteriophage λ DNA by *Cla*I yields a final pattern of only partial cleavage at several of its recognition sites while the majority of fragmentation reactions is complete, see Figure 2, lanes 3 and 4. The nature of the *Cla*I recognition sequence suggested to us that partial resistance against *Cla*I hydrolysis might result from an occasional overlap with a *Sau*3A (*Mbo*I) restriction site, i.e. might depend on the occurence of an ATCGATC or GATCGAT sequence in λ DNA at the respective positions. GATC sequences are known to be fully or nearly fully methylated at their adenine positions in plasmid or chromosomal DNA molecules obtained from *E.coli* cells (21), but phage DNAs isolated after induction or lytic infection might only be partially methylated. Accordingly, bacteriophage λ and several of its derivatives were grown on an *E.coli dam*$^-$ strain and the unmethylated phage DNAs obtained indeed yielded *Cla*I digestion patterns with no partial fragmentation remaining, such as displayed in Fig. 2, lane 2 for λ*b*2 DNA as an example.

In the course of our analysis of the aberrant digestion pattern of λ DNA a complete *Cla*I map of λ DNA has been obtained which is presented in Fig. 3. This map has been based on *Cla*I single, and on *Cla*I double digestions together with various other enzymes, of both λ$^+$ DNA and a number of λ DNA deletion and substitution derivatives. These included λ*b*2 (see Fig. 2, lanes 2 and 3), λ*nin*5, λgt·λC, λ*h*434, λ*h*80, λ*imm*80, λ*imm*21, λ*imm*434, λ*imm*21*b*2, λdv1,

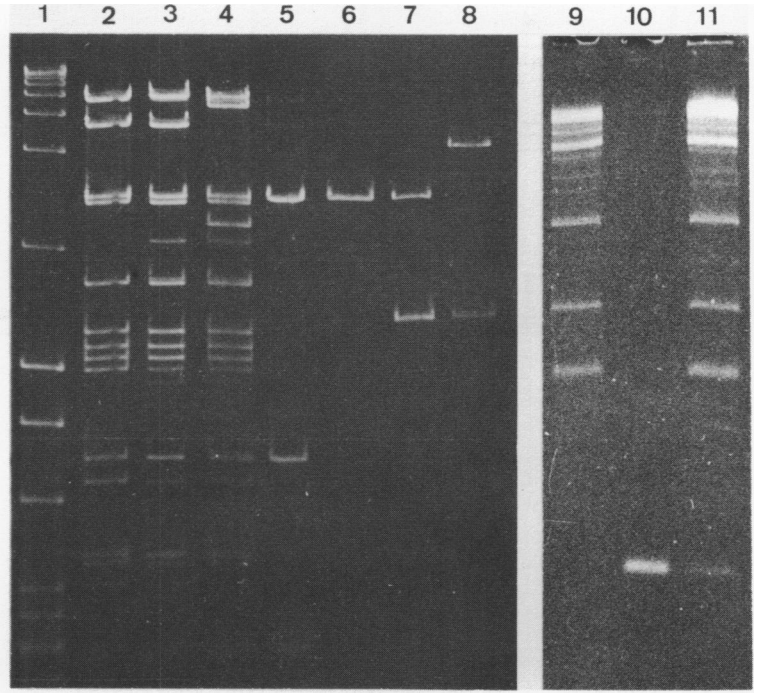

Figure 2. Restriction analysis of bacteriophage λ DNA and hybrid plasmid DNAs obtained after insertion of λ DNA fragments into the pBR322 *Cla*I site by 0.8% (lanes 1-8) and 2% (lanes 9-11) agarose gel electrophoresis. (1) Marker lane, composed of partially *Eco*RI digested λdv021 octomer DNA (top eight fragments, multimers of 3152 bp) and *Hae*III digested λdv1 DNA (bottom eight fragments: 1670, 1310, 890, 534, 460, 362, 344 and 270 bp). (2) and (3) *Cla*I digestion of λb_2 DNA grown on *E.coli dam*$^-$ and *E.coli dam*$^+$, respectively. (4) *Cla*I cleavage pattern of λcI857S7 DNA grown on *E.coli dam*$^+$. Submolar bands at 12.0 and 11.5 kb as well as 3.22, 2.60, 1.56 and 1.66, 0.94, 0.62, 0.54 kb are due to partial cleavage of λ DNA grown in *E.coli dam*$^+$ cells; trace amounts at 6.25 kb result from partial addition of the left and right terminal fragments. (5) *Cla*I re-cleavage of hybrid plasmid pHL76 DNA isolated from one of the tetracycline sensitive colonies after λ DNA : *Cla*I shotgun cloning into pBR322 : *Cla*I. λ DNA fragments 11 + 16 (1.11 + 0.35 kb) have been cloned. (6) *Cla*I recleavage of hybrid plasmid pHL75 DNA isolated from one of the tetracycline resistant colonies of the same cloning reaction. λ DNA fragment 3 (4.3 kb) has been cloned into pBR322 (4.3 kb), as concluded from its further characterization by (7) *Eco*RI + *Cla*I double digestion (4.3 + 2.15 + 2.15 kb), and (8) *Eco*RI single digestion (6.5 + 2.2 kb). (9) Electrophoretic separation on a 2% agarose gel of the *Taq*I fragments of pBR322 DNA. (10) Analysis of the isolated λdvh93:*Taq*I-E fragment. (11) *Taq*I cleavage of hybrid plasmid DNA obtained from a clone (tetracycline sensitive) of a λdvh93:*Taq*I-E/pBR322:*Cla*I ligation and transformation experiment.

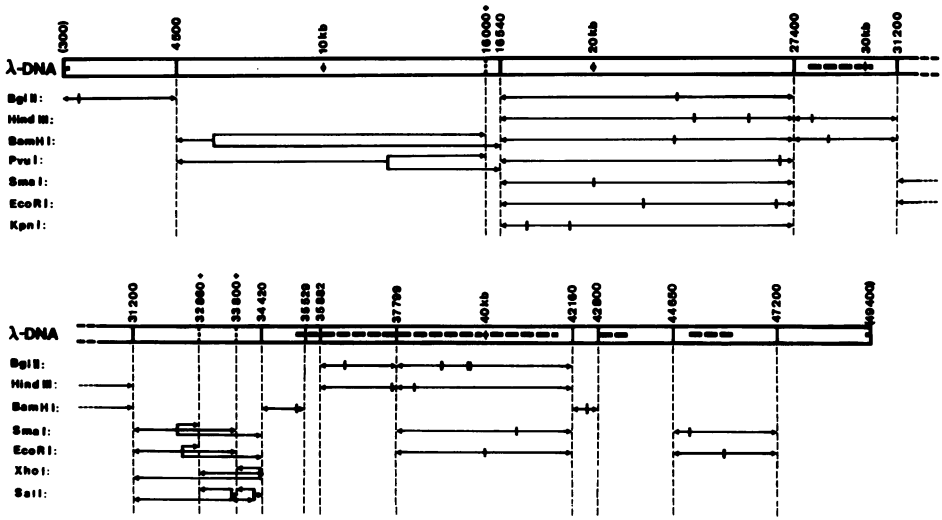

Figure 3. *Cla*I map of bacteriophage λ DNA. *Cla*I cleavage positions are indicated by vertical bars (which are hatched for the three positions of partially inhibited reaction, see text), and by numbers above the line that refer to the standard *Eco*RI cut in the λ origin of replication = 40000. Segments of known λ DNA sequence are indicated by fat horizontal bars. Double headed arrows below the main line refer to *Cla*I fragments of λ^+ DNA which are cleaved by a second enzyme at the position(s) marked on that arrow. The resulting subfragments have been used for mapping the *Cla*I recognition sites relative to the cleavage positions known for the various enzymes listed on the left. Subfragmentations on groups of two or three correlated λ DNA:*Cla*I fragments that result from incomplete cleavage (see Fig. 2) have been drawn accordingly, e.g. double digestion with *Xho*I (which has a single recognition site in λ DNA, λ:34360) will convert three λ DNA-*Cla*I partial fragments of 2600, 1560, and 620 bp into 2540, 1500, and 560 bp, respectively, and will yield a common 60 bp subfragment in stoichiometric ammounts.

λdv*imm*21AB5, λdv*imm*434 phage or plasmid DNAs, and also several isolated or cloned DNA fragments that had been obtained from these phage or plasmid DNAs (compare ref. 6). Only the fragment size measurements referring to double digestions of wildtype λ^+ DNA or λ^+ DNA fragments have been indicated in the lower part of Fig. 3, they are indirectly supported by the measurements which have been done in the other fragmentation series but are not shown here. Out of the fifteen recognition sites in λ^+ DNA five occur in segments that have been sequenced to date (18,22).

Because of the differences observed in the digestion of $\lambda \cdot dam^+$ versus $\lambda \cdot dam^-$ DNAs we conclude that *Cla*I hydrolysis is inhibited at A-methylated recognition sequences. While the final relative yields of the respective

λ·*dam*$^+$ DNA fragments did not change in several experiments when using the same phage DNA preparation, some variability in the *Cla*I cleavage yield was observed among different such phage DNA preparations. It appears that the different extent of methylation at these phage DNA GATC sites may be reflected in the partial inability of *Cla*I cleavage, but no effort has been made for an independent determination of that specific level of methylation. Out of the three sites on λ DNA with inefficient *Cla*I digestion (see Fig. 3) two are next to each other in the right arm and are cleaved with similar relative yields of 60 to 70% each, while a third one is located in the left arm and is hydrolysed only at 10 to 20% fractional yields. It is not known whether this difference results from a GATCGATC sequence occurring at λ:16000 or from regional variations in the GATC methylation reaction under limiting conditions.

*Cla*I linearised pBR322 DNA as a vector system

In pBR322 the single *Cla*I recognition site at position 23-28 is immediately to the left of the *Hin*dIII site located at position 29-34. This suggested that pBR322 might be a suitable vector for *Cla*I generated restriction fragments, and that insertion of fragments into this site should result in tetracycline sensitivity, as is generally observed on cloning *Hin*dIII fragments into pBR322. This expectation was indeed fulfilled when a mixture of λ DNA fragments obtained after *Cla*I digestion was ligated to *Cla*I linearised pBR322 DNA, which then was used for transformation of *E.coli* 5K. Most of the resulting ampicillin (100 µg/ml) resistant, tetracycline (20 µg/ml) sensitive clones contained a single λ:*Cla*I fragment integrated into the vector, while some of them carried two such fragments (see Fig. 2, lane 5).

Among a similar number of ampicillin and tetracycline (50 µg/ml) resistant colonies analysed, several carried the λ DNA fragment of 4.3 kb integrated which is known to cover the λp_R promoter at λ:38855 (λ:37799-42160, see Fig. 3). Part of the corresponding hybrid plasmid restriction analysis is shown in Fig. 2, lanes 6-8. In further digestions with *Hin*dIII (4.8 + 3.8 kb), *Pst*I (7.8 + 0.8 kb), and *Bgl*II + *Bam*HI (5.1 + 2.9 + 0.65 + 0.06 kb) the insert was determined to be oriented left to right, i.e. with λp_R able to transcribe into the tetracycline region. Tetracycline resistant colonies have also been obtained upon cloning promoter fragments with λp_{lit}, $\phi 80 p_{lit}$, or $\phi 80 p_L$ in direct orientation relative to the tetracycline genes, while invertedly oriented fragment inserts resulted in tetracycline sensitive clones (to be published in more detail elsewhere).

Two exceptional pBR322:*Cla*I clones have been obtained with inserts

known not to carry a promoter signal, but nevertheless turned out to be tetracycline resistant (with limiting concentrations of 50 µg/ml and 20 µg/ml, respectively). Upon comparison of the two DNA sequences resulting across the *Cla*I-*Hin*dIII junction in these two fusion reactions with published promoter consensus sequences (23,24) it became evident that in both experiments promoter-similar sequences had been reconstituted. Since the *Cla*I recognition sequence is located at positions -22 to -17 of the tetracycline promoter signal an exchange of the upstream -35 region for a similar sequence inserted by cloning may yield a result comparable to integration of a complete promoter site. In general therefore, insertion of a DNA fragment into the *Cla*I site of pBR322 will usually, but not in either of the two cases mentioned, lead to inactivation of the tet^R phenotype. Equivalent results have been described for the integration of fragments into the adjacent *Hin*dIII site in pBR322 (25,26).

Restriction endonuclease *Cla*I generates fragments containing 5'-terminal pCG-dinucleotide extensions, and identical single-stranded termini are also found at the fragment ends of several other enzymes such as *Taq*I (27) and *Hpa*II (28). In order to test whether any of the fragments produced by a different member of this family of restriction endonucleases could also be cloned into the pBR322:*Cla*I restriction site, a *Taq*I fragment obtained from λdvh93 (17) was chosen for this purpose. λdvh93:*Taq*I-E (λ:39230-39396, comprising the central section of the λ-*c*II gene) was isolated from gel electrophoresis, and ligated *in vitro* to pBR322 DNA cleaved by *Cla*I. All of the ampicillin resistant and tetracycline sensitive clones analysed contained an extra *Taq*I fragment in their hybrid plasmid DNAs, which corresponded to the inserted λdvh93 DNA segment (see Fig. 2, lane 11). Several other *Taq*I and *Hpa*II fragments of λ, φ80 and IS*5* DNA have also been cloned into the *Cla*I site of pBR322 or one of its derivatives, and the clones will be described elsewhere.

DISCUSSION

The determination of the recognition sequence for endonuclease *Cla*I and the occurrence of a single *Cla*I restriction site in the tetracycline promoter sequence of plasmid pBR322 (adjacent to the pBR322 *Hin*dIII site) allows to use this vector DNA as a cloning vehicle for any DNA fragment with 5'-pCG protruding ends. Fragments of this character are generated by *Cla*I digestion, but also by cleavage with *Taq*I, *Hpa*II, *Acy*I, *Hgi*DI, *Sci*NI, *Nar*I and *Asu*II (1) or combinations thereof. Because of their usually rather high num-

ber of cleavage sites no suitable vector system had been developed previously for any of these enzymes. The resulting rather small fragments quite often do not carry a complete coding sequence for any gene, but are useful in the minimization analysis of DNA regions carrying a transcription signal, and for reconstructing synthetic chains of such signal elements. It is important in this regard that the *Cla*I recognition site is located in the tetracycline promoter sequence rather than the tetracycline coding region, because tetracycline resistance can now be used for monitoring the presence, orientation and also strength and regulatory modulations of a promotor sequence located on such a proximal fragment. The very close spacing of the single *Eco*RI-*Cla*I-*Hin*dIII restriction sites all located in the tetracycline promoter region further adds to the versatility of this system, because *Eco*RI-*Taq*I fragments and similar segments can also be inserted.

In the way outlined above the position and orientation of promoters λ-p_{lit}, $\phi 80p_R$, $\phi 80p_{rm}$, $\phi 80p_L$, and $\phi 80p_{lit}$ has been determined using several steps of a fragment minimization procedure (R. Grosschedl and G. Hobom, unpublished results). The same system has, however, also been used for screening transcription terminator carrying segments. This was made possible by inserting a p_{lacUV5} promoter fragment to the left of the *Eco*RI site, in rightward direction, and several terminator signals have been determined using this modified system (pHL200; M. Kröger and G. Hobom, submitted for publication). Several other modifications of the basic pBR322 system have also been constructed. Finally, synthetically arranged chains of transcription signals using two or more of the co-cloning 5'-pCG (e.g. promoter-attenuator) fragments have been achieved in the pBR322:*Cla*I or modified system, both in our laboratory and elsewhere (29).

Although a specific, *Cla*I-related modification enzyme has not yet been isolated from *Caryophanon latum* at least one prerequisite for a true restriction enzyme has been fulfilled for *Cla*I: the enzymatic DNA cleavage reaction is inhibited by specific methylation of its recognition sequence. The observed inhibition of *Cla*I cleavage at *Cla*I-GATC overlapping recognition sites is reminiscent of the similar inhibition of *Taq*I cleavage at the very same sites (30, and our unpublished observations). From this result it is not yet possible to conclude whether methylation at positions 1 and 6 or 2 and 5 (or both) would be critical for inhibition of *Cla*I cleavage. It is not yet known whether C-methylation at positions 3 and 4 will not inhibit the cleavage reaction as has been observed for *Taq*I (30). Similar to the presumptive *Taq*I modification enzyme, however, an adenine methylation reaction may be expec-

ted for the *Cla*I modification system present in *Caryophanon latum*. Inhibition of cleavage due to overlapping 'foreign' modification sites has also been observed for *Eco*RII/*Asu*I sequences: $CC^{A}_{T}\downarrow GGNCC$ (M. Kröger, personal communication) and can be similarly interpreted.

ACKNOWLEDGEMENT

We thank Mrs. G. Schlingmann for expert technical assistance, B. Rowenhagen for *Caryophanon latum* L, E. Schwarz for preparing the λdvh93:*Taq*I-E fragment, J. Gross for computer-programming, J. Collins for a critical reading of the manuscript, and H. Kössel for many helpful suggestions. This work has been supported by a grant from the Deutsche Forschungsgemeinschaft to G.H.

REFERENCES

1. Roberts,R.J. (1978) Gene 4, 183-193
2. Collins,J. and Brüning,J. (1978) Gene 4, 85-107
3. Bolivar,F., Rodriguez,R.L., Betlach,M.C. and Boyer,H.W. (1977) Gene 2, 75-93
4. Matsubara,K. and Kaiser,A.D. (1968) Cold Spring Harbor Symp.Quant.Biol. 33, 769-775
5. Matsubara,K. and Otsuji,Y. (1978) Plasmid 1, 284-296
6. Lusky,M. and Hobom,G. (1979) Gene 6, 137-172; 173-197
7. Maxam,A. and Gilbert,W. ((1977) Proc.Natl.Acad.Sci.USA 74, 560-564
8. Wienand,U., Schwarz,Z. und Feix,G. (1979) FEBS Letters 98, 319-323
9. Brownlee,G.G. and Sanger,F. (1969) Eur.J.Biochem. 11, 395-399
10. Trentini,W.C. (1978) Ann.Rev.Microbiol. 32, 123-141
11. Sutcliffe,J.G. (1979) Cold Spring Harbor Symp.Quant.Biol. 43, 77-90
12. Lehman,I.R. (1974) Science 186, 790-794
13. Beck,E., Sommer,R., Auerswald,E.A., Kurz,C., Zink,B., Osterburg,G. and Schaller,H. (1978) Nucleic Acids Res. 5, 4495-4503
14. Sanger,F., Coulson,A.R., Friedman,T., Air,G.M., Barrell,B.G., Brown,N.L., Fiddes,J.C., Hutchison III,C.A., Slocombe,P.M. and Smith,M. (1978) J.Mol.Biol. 125, 225-246
15. Fiers,W., Contreras,R., Haegeman,G., Rogiers,R., Van de Voorde,A., Van Heuverswyn,H., Van Herreweghe,J., Volkaert,G. and Ysebart,M. (1978) Nature 273, 113-120
16. Reddy,V.B., Thimmappaya,B., Dhar,R., Subramanian,K.N., Zain,B.S., Pan,J., Ghosh,P.K., Celma,M.L. and Weissman,S.M. (1978) Science 200, 494-502
17. Schwarz,E., Scherer,G., Hobom,G. and Kössel,H. (1980) Biochemistry International 1, 386-394
18. Landsmann,J. (1980) thesis, Univ. Köln
19. Grosschedl,R. (1978) thesis, Univ. Freiburg
20. Schwarz,E. (1979) thesis, Univ. Freiburg
21. Vovis,G.F. and Lacks,S. (1977) J.Mol.Biol. 115, 525-538
22. Franklin,N.C. and Bennett,G.N. (1979) Gene 8, 107-119
23. Rosenberg,M. and Court,D. (1979) Ann.Rev.Genet. 13, 319-353
24. Siebenlist,U., Simpson,R.B. and Gilbert,W. (1980) Cell 20, 269-281
25. Tait,R.C., Heyneker,H.L., Rodriguez,R.L., Bolivar,F., Covarrubias,A., Betlach,M.C. and Boyer,H.W. (1979) in Microbiology-1979, D.Schlesinger, Ed., pp. 174-176

26. Widera,G., Gautier,F., Lindenmaier,W. and Collins,J. (1978) Molec.Gen. Genet. 163, 301-305
27. Sato,S., Hutchison,C.A. and Harris,J.I. (1977) Proc.Natl.Acad.Sci.USA 74, 542-546
28. Garfin,D.E. and Goodman,H.M. (1974) Biochem.Biophys.Res.Comm. 59, 108-116
29. Edman,J.C., Hallewell,R.A., Valenzuela,P., Goodman,H.M. and Rutter,W.J. (1981) Nature 291, 503-508
30. Streeck,R.E. (1980) Gene 12, 267-275