Structure and regulation of gene expression of a Clo DF13 plasmid DNA region involved in plasmid segregation and incompatibility


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ABSTRACT

The bacteriocinogenic plasmid Clo DF13 contains genetic information involved in the accurate partitioning of the plasmid (parA and parB) as well as in incompatibility phenomena (incA, B, C and D). In this paper we report on the primary structure and regulation of gene expression of the 29% - 50% part of Clo DF13, containing the DNA regions incA, incB and parB as well as genes K and L.

According to the results of our DNA sequence analysis, mapping of transposon insertions, RNA blotting and SI mapping experiments, we conclude that:

a) genes K and L are transcribed as one operon; transcription of this operon is initiated at a promoter (P2) located at 32.5% and proceeds in a clockwise direction.

b) treatment of cells with mitomycin-C, significantly enhances transcription from P2, although this promoter is probably not directly repressed by lexA protein.

c) Termination of transcription of this operon occurs between genes K and L, as well as distal to gene L.

The possible role of gene products and/or sites, located within the 29-50% DNA region, in plasmid incompatibility and segregation is discussed.

INTRODUCTION

The bacteriocinogenic plasmid Clo DF13 (9,600 bp; 1,2) is maintained in Escherichia coli at approximately 10 copies per cell (3). Copy control mutants of Clo DF13 have been isolated (4-7), such as the non-conditional mutant Clo DF13-cop3 with a plasmid copy number of about 70 (8). Clo DF13 encodes at least 9 proteins; the location of the genes encoding these proteins, and the functions of their gene products are presented in figure 1.

According to the functions encoded, Clo DF13 can be divided into at least 3 DNA regions, namely regions involved in: (a) initiation and regulation of DNA replication (1.8-9%; 6, 7), (b) bacteriocinogenity, immunity, lysis and transport of the bacteriocin (9-32%; 2, 9-14) and (c) mobilization (67-100%; 15, 16). For a detailed review, see reference 7.

Beside the genetic information involved in the functions mentioned above,
Figure 1. Genetic and functional map of bacteriocinogenic plasmid Clo DF13. The data presented in the outer circle are reviewed by Veltkamp and Stuitje (7). The inner circle shows the location of the DNA regions involved in incompatibility (incA, incB, incC and incD; 20) and stability (parA and parB; 18). For further explanation, see the text.

the Clo DF13 genome contains genetic information necessary for the stable maintenance of the plasmid (17). Recently Hakkaart, et al. localized 2 DNA regions on the Clo DF13 genome, parA and parB, which are involved in accurate partitioning of Clo DF13 plasmid molecules among daughter cells (see fig. 1; 18). Upon deletion of at least one of these par regions, mutants of Clo DF13 with an increased copynumber appeared to be unstable. Furthermore, these par- plasmids are isolated from E. coli mainly as multimeric molecules. Another phenomenon involved in plasmid maintenance is plasmid incompatibility (19). Recently, four different regions involved in this incompatibility phenomenon (incA through incD, fig. 1) were mapped on the Clo DF13 plasmid genome (20).

It is remarkable, that one Clo DF13 region (between 45% and 50%) appears to be involved in both, plasmid incompatibility (incB; 20) and partitioning (parB; 18). Van de Pol et al. (21) demonstrated that the Clo DF13 DNA region between 35% and 50% encodes at least two different proteins, namely protein K (m.w., 21 kD) and protein L (m.w., 10.5 kD). Furthermore, they presented evidence that the product of gene L inhibits the multiplication of double stranded DNA phages, like P1 and λ.

In this paper, we report on the primary structure and the regulation of gene expression of an operon, located between 32.5% and 49%, comprising
Table 1. Bacterial plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Transposon</th>
<th>Insertion of transposon</th>
<th>Affected gene/function</th>
<th>Source of reference</th>
</tr>
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<tbody>
<tr>
<td>Clo DF13-cop3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3, 5, 6</td>
</tr>
<tr>
<td>pVC22</td>
<td>Tn5</td>
<td>32.5</td>
<td>ND incA</td>
<td>20</td>
</tr>
<tr>
<td>pJN52</td>
<td>Tn901</td>
<td>36.5</td>
<td>L</td>
<td>21, 24, 25</td>
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<tr>
<td>pJN66</td>
<td>Tn901</td>
<td>37.0</td>
<td>R</td>
<td>&quot;</td>
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<td>Tn901</td>
<td>38.5</td>
<td>L</td>
<td>&quot;</td>
</tr>
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<td>Tn901</td>
<td>42</td>
<td>L</td>
<td>&quot;</td>
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<td>Tn901</td>
<td>44.5</td>
<td>R</td>
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</tr>
<tr>
<td>pJN56</td>
<td>Tn901</td>
<td>45</td>
<td>L</td>
<td>L, Dpi 21, 24, 25</td>
</tr>
</tbody>
</table>

Plasmids pVC22 and pJN52, 66, 63, 56 are derivatives of plasmid Clo DF13-cop3. pJN03 is a wild-type plasmid.

The sites of integration of transposons Tn901 and Tn5 are given in percentages of the Clo DF13 genetic map, as determined previously (see references) and/or during this study.

The orientation of the Tn901 insertion is indicated by capital L or R. L means that the left inverted repeat is orientated clockwise, whereas R indicates the opposite direction (24).

ND, not determined; Dpi, DNA phage interaction, stands for the inhibition of multiplication of DNA phages (21).

MATERIALS AND METHODS

Bacterial strains and plasmids

The plasmids used in this study are listed in table 1. The minicell producing strain P678-54 (22) was used as host strain for all Clo DF13 plasmids.

Media, Chemicals and Enzymes

Brain Heart Infusion medium (Oxoid Ltd., London) and Liquid Culture (LC) medium were normally used for growth in liquid and on agar plates. Except for Bam HI, all restriction enzymes used in this study were purchased from Boehringer Mannheim (FRG) and New England Biolabs. Bam HI was kindly...
provided by C. Pols and was isolated according to Greene et al. (23). T4 polynucleotide kinase, E. coli DNA polymerase I, the "Klenow" fragment of DNA polymerase I and Calf Intestine Alkaline Phosphatase (CIAP) were supplied by Boehringer, Mannheim (FRG). S1 nuclease was purchased from Sigma Chemical Co. α-32P-dNTPs (350 Ci/m mole) and γ-32P-ATP were obtained from the Radiochemical Centre, Amersham, England. Piperazine-1,4-bis (2-ethane sulfonic acid) (PIPES) was supplied by Merck, Darmstadt (FRG).

Isolation of plasmid DNA, restriction enzyme analysis and preparation of DNA restriction fragments

DNA of Clo DF13 and Clo DF13 transposon mutants was isolated as described previously (10). Analytical and preparative digestions of plasmid DNA with restriction endonucleases were performed in reaction mixtures recommended by New England Biolabs. Analytical digests were run on 1-2% agarose gels in 20 mM Tris-HCl pH 7.6, 10 mM NaAc, 1 mM EDTA, or on 3 mm 5% polyacrylamide gels (acryl: bisacryl, 40:1.36; 26). Preparative electrophoresis was carried out on 5% polyacrylamide gels (acryl:bisacryl, 38:2, 27), after which the DNA fragments were recovered from the gel by electro elution (10).

DNA sequence analysis procedures

DNA sequence analysis was carried out with the chemical degradation procedure of Maxam and Gilbert (27). DNA fragments were labeled at the 5'-end or the 3'-end with T4 polynucleotide kinase or "Klenow" polymerase respectively. In order to dephosphorylate the 5'-ends of DNA restriction fragments we incubated the DNA fragment during 30 min at 45°C in 10 mM Tris-HCl pH 9.5, 1 mM spermidine, 0.1 mM EDTA with 0.5 unit calves intestinal alkaline phosphatase (CIAP). CIAP was inactivated at 75°C during 20 minutes, after which kinase buffer, T4 polynucleotide kinase and γ-32P-ATP were added directly. Gel electrophoresis to separate single end labeled fragments or single strands, as well as elution of DNA and base modification reactions were carried out according to Maxam and Gilbert (27). Eight, twelve and twenty percent sequence gels were prepared as described previously (28).

Isolation of RNA, electrophoresis, transfer to DBM-paper, and hybridization

RNA was isolated from logarithmically growing cells as described previously (2). In order to prepare RNA blots, 650 µg RNA was layered on a 2.5% agarose gel (0.2 x 13 x 24 cm) containing 5 mM methylmercuric hydroxide (29). Electrophoresis was carried out for about 7.5 h at 100 V,
after which the gel was stained with ethidium bromide. Preparation of the
gel and the diazobenzyloxymethyl (DBM) paper for transfer of the RNA was
done as described previously (2). The RNA blots were hybridized with nick-
translated (30) Clo DF13 fragments, washed and autoradiographed according
to Wahl et al. (31).

Mapping of in vivo transcripts by S1 nuclease protection of DNA-RNA hybrids

5'- and 3'-ends of in vivo synthesized RNAs were mapped essentially as
described by Berk and Sharp (32). For this purpose we made use of DNA
restriction fragments, labeled at their 5'-ends or at their 3'-ends as
described above. RNA (25-200 μg), isolated from logarithmically growing
cells harbouring Clo DF13 (2), was precipitated with approximately 20 pmol
DNA probe and washed twice with 70% alcohol. The dried pellet was dissolved
in 15-20 μl S1-hybridization buffer (80% formamide; 40 mM PIPES, pH 6.4;
1 mM EDTA; 0.4 M NaCl). The RNA-DNA mixture was denatured at 85°C during
15 min, after which hybridization was carried out during 16 h at 53°C.
The hybridization was stopped by the addition of 10 volumes of cold S1
incubation buffer (0.25 M NaCl, 3 mM NaAc, pH 4.6, 1 mM ZnSO₄, 20 μg/ml
sonicated salmon sperm DNA) with 1000 units/ml S1 nuclease. After 1 h
incubation at 37°C the S1-resistant hybrids were precipitated by the
addition of ethanol and 1 μg/100 μl tRNA. After washing, the pellets were
dissolved in 5 μl 10 mM Tris-HCl pH 7.0, 1 mM EDTA, 0.1% SDS and
centrifuged during 5 min at 12,000 x g. 4.5 Microliter of the hybrids was
mixed with 4.5 μl 100% formamide (deionized), heated for 5 min at 100°C and
loaded on 5% or 8% sequence gels.

RESULTS

The nucleotide sequence of the Clo DF13 DNA region comprising genes K and L

The DNA region between 29% and 50%, comprising the genes K and L, also
contains part of the genetic information involved in incompatibility and
stability (20). Hakkaart et al., localized the incA region between 29% and
38%, whereas the parB and incB functions were assigned to the DNA region
between 45% and 50% (see fig. 1; 20). To get insight in the genetic
organization of this part of the plasmid genome, we analysed the DNA
sequence between 29% (BamHI site) and 50% (SmaI site) of the Clo DF13
genome. The strategy applied to analyse the DNA sequence by the chemical
degradation method of Maxam and Gilbert is represented in figure 2; both
dNA strands have been sequenced. The resulting nucleotide sequence is shown
in figure 3.
Figure 2. Sequencing strategy for the Clo DF13 DNA region between 29% and 50%. Indicated are: the N-terminal part of the cloacin gene, as well as the cloacin promoter (P; 10), the genes K, L and promoter P2. The direction and the length of the arrows indicate which strands were sequenced as well as the number of nucleotides read from the gels. Usually the label was introduced at the 5'-end, unless indicated by •.

Codon analysis of the entire DNA sequence between 29% and 50% (fig. 4), revealed the presence of several "open" reading frames of more than 100 nucleotides, in both directions. From previous studies it is known that gene K is transcribed in a clockwise direction (21). Evidence was also presented that gene L is located distal to gene K, probably in the same operon (see fig. 1; 21). From figure 4A it can be concluded that only 2 "open" frames (designated K and L) might represent genes K and L, according to their location, direction of transcription and the predicted molecular weight of the encoded proteins. The molecular weights of proteins K (188 aminoacids; 21,305 D) and L (87 aminoacids; 9,895 D), as predicted from the DNA sequence, fit very well with the molecular weights estimated from SDS-polyacrylamide gel electrophoresis (21 kD and 10.5 kD respectively; 21). The "open" frames N1, N2 and N3 might represent structural genes. It is unlikely that the "open frames" N4-N10 represent structural genes, since they are not preceded by Shine and Dalgarno sequences (35). Furthermore, in that case, transcription should proceed in a direction opposite to that of genes K and L (see also below).
Determination of integration sites of transposons Tn901 and Tn5

In order to confirm the location of genes K and L within the DNA sequence, and to get more insight in the location and nature of incA, incB and parB, we determined the integration sites of several transposon mutants (see table 1). It is known that some of these mutants are defective in the synthesis of protein K (pJN50, 66 and 63) or protein L (pJN56) (21).

The integration sites of pJN63 and pJN50 were mapped by restriction enzyme analysis on 5% polyacrylamide gels, using the restriction enzymes Hinfl, BglI and ClaI. All other integration sites were determined by DNA sequence analysis. For this purpose, we made use of restriction sites located within the inverted repeats of Tn5 (39) or Tn901. The inverted repeats of Tn901 are completely homologous with the inverted repeats of Tn3 (P. van den Elzen, unpublished results; 41). As an example, we present the determination of the integration site of Tn901 within plasmid pJN56 (fig. 5). In this case the DdeI restriction sites, located at 16 nucleotides from both ends of Tn901, were labeled at the 5'-end. As can be concluded from the 20% sequence gel, shown in figure 5, a duplication of 5 Clo DF13 nucleotides adjacent to the Tn901 inverted repeats has occurred. A similar 5 nucleotide duplication was observed in case of other Clo DF13::Tn901 mutants and was also reported for integrations of the related transposon Tn3 (40). In case of the Clo DF13::Tn5 insertion mutant pVC22, a 9 nucleotides duplication was found which is consistent with the observation of Auerswald et al. (39).

Based on the finemapping of the Tn901 integration sites within plasmids pJN56, 63, 60, 66 and 52 and their effects on the synthesis of proteins K and L (fig. 4; 21) we infer that the "open" frames K and L indeed encode the corresponding proteins. With respect to plasmid pJN66, we observed that integration of Tn901, in case of this mutant, has occurred proximal to the proposed structural gene K. However analysis of proteins encoded by this mutant in minicells, revealed that pJN66 does not synthesize protein K (21). This observation can be explained by polar effects of this transposon insertion, since transcription of the Tn901 specified ß-lactamase gene in this case proceeds in opposite direction of that of gene K (21).

Transcription of the Clo DF13 DNA region comprising genes K and L

To study the transcription of the Clo DF13 DNA region encoding proteins K and L, we analyzed the length and relative quantity of RNAs transcribed from this region by RNA blotting experiments. Furthermore, analysis of the location of promoter and terminator sites, as well as analysis of the
Figure 3. The nucleotide sequence of the 29%-50% DNA region of Clo DF13. The bases are numbered from the transcription initiation site (+1) of promoter P. The aminoacids of the cloacin gene, gene K and gene L are presented in Italics. The "open" frames N, N and N are indicated by (Met or Val + N) and by their stopcodon (N + *). Marked are: P, cloacin promoter; Pribnow boxes ("-10"; 33, 34); RNA polymerase recognition sites ("-35"; 33); potential ribosome binding sites according to Shine and Dalgarno (SD; 35); SOS box, presumed lexA binding site (SOS; 10); potential interaction sites for cAMP-cAMP Receptor Protein Complex (CI and CII; 10, 36-38), terminator sequence (T; 33); recognition sites for restriction endonucleases BgIII and KpnI. The integration sites of transposons Tn5 or Tn901 are indicated by the Clo DF13 nucleotides, duplicated upon insertion (-w; 39, 40). Wavy arrows indicate 5'-ends or 3'-ends of transcripts. Straight arrows indicate symmetric elements.

The effect of mitomycin-C on initiation of transcription, were performed using the Sl-nuclease protection method of Berk and Sharp (32).

RNA blotting experiments

RNA's, isolated from E. coli cells, harbouring Clo DF13 were run on a 2.5% agarose gel containing methylmercuric hydroxide. After electrophoresis and transfer of the RNAs to DE81-paper, strips were hybridized with Clo DF13 DNA or DNA fragments (specific activity of 20-30 x 10^6 dpm/pmol). The individual strips were autoradiographed during 1-2 days. The result of this experiment is shown in figure 6. From the hybridization pattern of Clo DF13 specific RNAs with Clo DF13 DNA fragments, it can be observed that the DNA regions located between 0% and 47% of the plasmid genome (fig. 6, lanes a-d) are transcribed relatively actively. The 47-70% DNA fragment only weakly hybridizes with 2 RNAs (lane e) whereas transcription of the mobility region of Clo DF13 seems to be repressed (lanes f, g). The RNAs encoded by the DNA regions comprising the origin of DNA replication and the bacteriocin operon have been the subject of earlier investigations (2, 7, 9, 10, 42) and will not be discussed in this paper.
Figure 4. A. Codon analysis of the Clo DF13 DNA region between 29% and 50%. Initiation and termination codons for translation are plotted on each frame, in both directions. Long and short angled lines represent ATG and GTG start codons respectively; vertical lines represent translation stop codons. Indicated are: the genes encoding cloacin DF13 (Clo) and the proteins K and L (K, L); reading frames longer than 100 nucleotides (N1 through N10).

B. Mapping of integration sites of transposons Tn5 (pVC22) and Tn901 (pJN plasmids) by DNA sequence analysis or restriction enzyme digestions. In the latter case --- indicates the maximum deviation of our localization. Transcripts: as localized by SI mapping (see fig. 7) are represented by thick arrows. Symbols used are: P1 and P2, promoter; T, terminator; IR, inverted repeat of 13 nucleotides; Dpi, DNA phage interaction.

C. Location of DNA regions involved in stability (parB) and incompatibility (incA + B), according to Bakkert et al. (18, 20).
Figure 5. Determination of the integration site of transposon Tn901 within plasmid pJN56 by DNA sequence analysis on a 20% sequence gel. The DNA sequence of restriction fragments containing part of the left and right inverted repeats of Tn901 (LIR and RIR) is read from the DdeI recognition sites present in both inverted repeats, in 5' → 3' direction. The 5 Clo DF13 nucleotides, duplicated upon integration of Tn901, and the direction within the Clo DF13 sequence (29% or 50%) in which the autoradiograph is read, are indicated.

e, after prolonged exposure, revealed that at least the 1200, 1250 and 1400 nucleotide RNAs hybridize weakly with the 30-35% DNA region (not shown). We infer that the coding regions of these RNAs are almost entirely confined to the 35-47% DNA region, but extend beyond the BglI site at 35%. From the RNA blotting

Figure 6. Hybridization patterns of RNA isolated from E. coli, harbouring Clo DF13-cop3. Isolation of total cellular RNA, electrophoresis, RNA transfer to DBM-paper and hybridization with 32P nick-translated DNA were performed as described in Materials and Methods. Different part of the Clo DF13 genome were used as probes, such as total circular Clo DF13 DNA (lane a), Clo DF13 BglI fragments (lanes b, d, e and f).
Figure 7. A. Strategy for mapping by S1 nuclease protection of 5'-ends of in vivo Clo DF13 transcripts. RNAs were isolated as described in the text. 32P label was introduced at 5'-ends (*) or 3'-ends (•) of the DNA fragments used as probes to map promoter and terminators respectively.

B. Mapping of the terminator between genes K and L (see text). M13 mp8 (43) x TaqI was used as marker.

C. Mapping of promoter P2 and analysis of the effect of mitomycin-C (Mit. C) on transcription from P1 and P2 (see text). pBR322 (44) x HpaII was used as marker.
experiment, using the 35-47% DNA region as probe (fig. 6,d), we conclude that no transcript is observed longer than 1400 nucleotides. We presume that some of the smaller transcripts are the result of degradation or premature termination.

Since transcription of genes K and L proceeds in a clockwise direction, these data indicate that initiation of transcription occurs between 35% (BglI site) and 31% (the cloacin promoter). In vitro transcription experiments (unpublished results) pointed to the existence of a promoter (designated P₂), at about 240 nucleotides upstream from the BglI site (at approximately 32.4%; figures 3, 7A).

S† protection experiments

The strategy, to localize the 5'-ends and the 3'-ends of in vivo RNAs, isolated from logarithmically growing cells is represented in figure 7A.

In a first approach we localized promoter P₂ by the use of probes a and b, which were labeled at the 5' end. An example of an S† experiment using probe a, overlapping with RNAs transcribed from P₁ (the cloacin promoter) and, P₂, is presented in figure 7c. Two major bands of about 590 and 108 nucleotides appear in lanes 4 and 5. These two DNA fragments are protected by RNAs transcribed from promoter P₁ (590 N) and P₂ (108 N) respectively. The minor bands of approximately 350 and 180 nucleotides probably are the result of S nuclease sensitive AT-rich stretches within the DNA sequence of the cloacin operon. Based on the length of the protected DNA fragment we concluded that promoter P₂ is located at 32.5% (fig. 7A). This result was confirmed by an identical experiment using probes b (data not shown). From these experiments and from a similar experiment using probe b we also concluded that no transcriptional activity is present in the region between promoter P₁ and P₂. Since treatment of cells with mitomycin-C results in enhanced transcription from promoter P₁ (the cloacin promoter), which is due to the recA dependent inactivation of the lexA repressor (10), we also determined the effect of mitomycin-C on transcription from promoter P₂. For this purpose, we performed the S† nuclease protection experiments, shown in figure 7C (lanes 4 and 5), with equal amounts of RNA, isolated from a mitomycin-C induced culture (lane 5) and from a control culture (lane 4). Mitomycin-C was added 60 min prior to RNA isolation. The results, shown in figure 7C, demonstrate that the addition of mitomycin-C induces transcription from the cloacin promoter as reported before (10). Strikingly, transcription from promoter P₂ is also enhanced upon the addition of mitomycin-C. However, analysis of the DNA sequence adjacent to promoter P₂ did not reveal sequences homologous to the so-called SOS boxes (lexA binding sites) observed in the case of lexA repressible genes (45). The presence of such a SOS box,
overlapping with the cloacin promoter (P₁), was reported previously (fig. 3, 10). We were able to exclude the possibility of an overall effect of mitomycin-C on transcription of Clo DF13, since S₁ protection experiments demonstrated that e.g. the quantity of Clo DF13 pre-primer RNA (6,46) is not affected by the addition of mitomycin-C (data not shown). In order to localize transcription termination sites, we made use of DNA fragments labeled at one 3'-end (probes c and d, fig. 7A). From minicell experiments it is known that protein K is synthesized in larger quantities than protein L. (52).

DNA sequence analysis of the region between genes K and L revealed a potential terminator structure, resembling ρ-independent types of terminators, located at position +1050 (see fig. 3;33). To obtain evidence that attenuation of transcription occurs between genes K and L, and to map the terminator involved in this process, we made use of probe c (fig. 7). As can be concluded from figure 7B, lane 4, not only complete protection of probe c occurs, but also partial protection resulting in a 360 nucleotide DNA band. From this result, we conclude that termination partially occurs between genes K and L, approximately at position +1069, distal to the terminator indicated in figures 3 and 7A. The total protection of probe c also indicates that transcription proceeds beyond this terminator into gene L. To investigate at which site(s) transcription terminates distal to gene L, and whether transcription proceeds beyond the SmaI site at 50%, we performed on S₁ protection experiment using probe d. As could be concluded from the length of the protected fragment, termination of transcription might occur approximately at position 1465. Proximal to position 1465 a terminator-like structure (33) can be observed in the DNA sequence (fig. 3). This termination site is located within the parB region as designated by Hakkaart et al. (18).

Although several minor bands were observed in this S₁ protection experiment, due to S₁ sensitive AT-rich stretches within this region (see fig. 3), no protection of the entire probe d occurred, indicating that transcription probably does not proceed beyond the SmaI site.

From the results of the S₁ protection experiments presented in this section, we concluded that transcription of the operon comprising genes K and L initiates at promoter P₂. Termination occurs partially at a terminator located between genes K and L, and within a region between gene L and the SmaI site at 50%.

DISCUSSION

In this paper, we present the nucleotide sequence of the Clo DF13 DNA region between 29% and 50%. Within this DNA sequence, we were able to identify
the genes K and L by transposon insertion mapping. Furthermore, we analyzed
the mode of transcription of this DNA region by RNA blotting and S1 nuclease
protection experiments.

From the data obtained so far, we conclude that:

a) The genes K and L are located in one operon; transcription of this operon
is initiated at 32.5% (promoter P2), and proceeds in a clockwise direction
on the Clo DF13 genetic map.

b) Attenuation of transcription occurs between genes K and L. Transcription
is terminated distal to gene L in a region involved in plasmid maintenance,
designated parB, and does not proceed beyond the SmaI site, located at 50%.

c) The genes K and L, as well as the potential genes N2 and N3, possess their
own ribosome binding sites (35). The ribosome binding site of gene K
shares about 60% homology with the optimal 47 nucleotide ribosome binding
site predicted by Scherer et al. (47), and is therefore probably very
efficient.

With respect to the regulation of transcription initiated at promoter P2,
three features can be distinguished:

1) Promoter P2 can be activated by mitomycin-C

2) A SOS box is not present in the vicinity of this promoter, indicating that
lexA protein is not directly involved. However, recently Brandsma et al. (52)
described a situation in which a lexA binding site located between two
promoters can be functional in both directions. Although promoter P1 and P2
are not overlapping, a similar role for the unique lexA binding site between
both promoters is possible. 3) A potential binding site for cAMP-cAMP Receptor
protein can be observed; this sequence (CI in fig. 3) shows a high degree of
homology with consensus sequences published before (36-38). This cAMP-CRP
binding site could be involved in negative regulation of transcription, as
reported for the galP2 promoter (48).

The elucidation of the nucleotide sequence of the DNA region between 29%
and 50% as well as the localization of transposon integration sites within
this region enabled us to define more precisely the genetic information
involved in incompatibility (incA and incB), stability (parB) as well as the
inhibition of the multiplication of DNA phages (20, 21). At this moment
there is no definite proof that the function of incA is correlated with a
particular site in the plasmid genome or with a plasmid encoded gene product.
In this respect, it is striking that integration of Tn5 within plasmid pVC22,
which integration affects incompatibility (incA;20), has occurred around the
transcription initiation site of promoter P2. Since integrations of
transposon Tn5 are highly polar (49), the possibility exists that this insertion has a polar effect on the expression of genetic information located downstream. The IncA region is located at about 32% (fig. 4c; 20). If the IncA phenotype is correlated with a particular gene product, the "open" frames N2 and N3 (fig. 4A) are the most likely candidates. Concerning the region between approximately 45% and 50% (SmaI), we know from previous (21,18) and recent (Hakkaart et al., manuscript submitted) experiments that this region is involved not only in the inhibition of the multiplication of DNA phages but also in plasmid incompatibility (IncB) and partitioning (parB). Recently, evidence was obtained that transcription into the parB region is essential for its functioning in plasmid maintenance. We suggest that termination of transcription in the parB region at position 1460 might be a crucial feature in the expression of the parB function. In the DNA region distal to gene L, symmetric elements are present; for instance, we observed a 13 nucleotide inverted repeat, separated by 36 nucleotides, around position 1490 (fig. 3). The role of these symmetric elements in plasmid maintenance will be discussed in a forthcoming publication (Hakkaart et al., manuscript submitted).

Further experiments will be focused on the role of cAMP-CRP complex, mitomycin-C and/or lexA on the expression of the operon containing genes K and L.

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