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Origin and direction of replication of the bacteriocinogenic plasmid Clo DF13

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ABSTRACT

Cairn's type replicative intermediates of both the wildtype Clo DF13 plasmid and the copy mutant Clo DF13 cop3 were isolated by dye-buoyant density centrifugation. Replicative intermediates were linearized at the HpaI or SalI cleavage site, and examined with the electron-microscope. The data show that replication of both the Clo DF13 wild type plasmid and the Clo DF13 cop3 plasmid, initiates at about 2.8% on the physical map. Replication proceeds unindirectionally and counterclockwise on this map.

INTRODUCTION

Plasmids can be roughly divided into two groups, namely those with a relaxed replication control, resulting in a high number of plasmid copies per cell and those with a stringent replication control. Plasmids of the latter group are present in about 1-3 copies per cell. In our laboratory we study the replication and genetic constitution of plasmid Clo DF13, which originates from Enterobacter cloacae (1). This nonconjugative bacteriocinogenic plasmid has a molecular weight of 5.75 megadalton (2). With respect to its replication Clo DF13 behaves like other small bacteriocinogenic plasmids, such as Col E1. Both plasmid Clo DF13 and Col E1, are under relaxed replication control and are maintained at about 10-20 copies per cell; they require an active DNA polymerase I, whereas replication continues in the absence of de novo protein synthesis (2-5). Although a considerable number of reports on plasmid replication have been published in the past few years, still little is known about the control of plasmid replication.

Previously we have isolated conditional and nonconditional mutants of Clo DF13, with an altered replication control (6, 7). These mutants could be a powerfull tool to elucidate the molecular basis of the control of Clo DF13 replication. Studies on in vitro constructed deletion mutants of Clo DF13

have shown that a small part of the Clo DF13 genome, namely from 1.8 to 11.5% on the Clo DF13 physical map, contains the genetic information required for autonomous replication (8). Furthermore these experiments indicated that the cop3 mutation, which results in a seven times increase in the number of Clo DF13 copies per cell (6), is also located in this part of the Clo DF13 genome (8). In studies on the regulation of the plasmid replication process it is important to know where mutations resulting in an altered replication control, are located with respect to the location of the origin of replication.

In this study we present evidence that there is no difference between plasmid Clo DF13 cop3 and the wild type Clo DF13 plasmid with regard to the location of the replication origin and the direction of replication. The replication of both plasmids initiates at approximately 2.8% on the Clo DF13 physical map and proceeds unidirectionally.

MATERIALS AND METHODS

Materials

The restriction endonucleases HpaI and SalI were obtained from New England Biolabs. (Methyl-^{3}H) thymidine (specific activity 40-60 mCi/mmol) and (2-^{14}C) thymine (specific activity 50 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England. The sources of all other chemicals used, were as described previously (8, 9, 10).

Bacterial strains and Media

In this study we have used a thy derivative of the Escherichia coli minicell producing strain P678-54 (N3012), with the following markers: thr, ara, leu, azi, tonA, lacY, minA, gal, minB, rpsL, malA, xyl, mtl, thi, supE, strA (11). Plasmid Clo DF13 and the copy mutant of Clo DF13, Clo DF13 cop3, were introduced in this strain by conjugation. Cells were grown in M9 salts medium (12), supplemented with 0.2% glucose, 0.4% Casamino acids (Difco), 5 µg/ml thiamine, and 2.5 µg/ml thymine.

Isolation of Clo DF13 replicative intermediates

E. coli P678-54 thy, harbouring Clo DF13 or Clo DF13 cop3 was inoculated in one liter M9 medium, containing 0.5 µCi/ml ^{14}C thymine, and grown for several generations. At a density of about 2 x 10^{8} cells/ml, the cells were harvested, washed at 25°C and resuspended in 0.5 l fresh M9 medium without thymine.

After 30 min starvation for thymine at 37°C, the culture was shifted to 25°C and pulse labeled for 40 sec with ^{3}H thymidine (final conc. 20 µCi/ml). The incorporation was stopped by the addition of NaF (final conc. 0.1 M), and the culture was immediately frozen in a dry ice/ethanol bath. Cells were harvested at 0°C and lysed as described previously (13). The lysate was centrifuged at 48,000 x g for 30 min. The supernatant fluid was centrifuged to equilibrium in a CsCl-ethidium bromide gradient, as described by Veltkamp et al. (13). The gradient was fractionated by collecting 150 µl fractions from the bottom of the centrifuge tube and 5 µl of each fraction was assayed for radioactivity. The DNA, banding at a position intermediate between covalently closed circular and open circular/linear DNA, was pooled. Ethidium bromide was removed by extraction with CsCl-saturated iso-propanol, and the CsCl was removed by dialysis against TE buffer (0.05 M Tris, 0.005 M EDTA pH 8.0). Finally, the DNA was precipitated and dissolved in distilled water.

Cleavage of plasmid DNA with restriction endonucleases

Replicative intermediates of Clo DF13 were cleaved with HpaI or SalI endonuclease in reaction mixtures containing respectively 10 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 6 mM KCl, 1 mM dithiothreitol and 100 µg/ml gelatine or 6 mM Tris/HCl (pH 7.9), 6 mM $MgCl_2$, 100 mM NaCl and 6 mM 2-mercaptoethanol. The reactions were stopped by adding EDTA to a final concentration of 25 mM.

Electronmicroscopy of DNA

Purified plasmid DNA was prepared for electronmicroscopy by the method of Davis et al. (14). The protein nucleic acid film was picked up on parlodion coated copper grids and dried in ethanol and isopentane. The DNA was rotary shadowed with platinum-paladium alloy (80 : 20) and examined in a Philips EM-300 electronmicroscope. The images from electronmicrographs were projected onto tracing paper and relative lengths were measured with a Summagraphics digitizer coupled to the Hewlet Packard calculator 9825A.

RESULTS

Replicative intermediates of Clo DF13 were isolated by dye-buoyant density centrifugation, as described in materials and methods. As shown in Fig. 1, pulse labeled material sedimentated predominantly at a position between covalently closed circular, and open circular/linear DNA. Electronmicroscopic analysis revealed that about 5% of the open circular molecules, observed

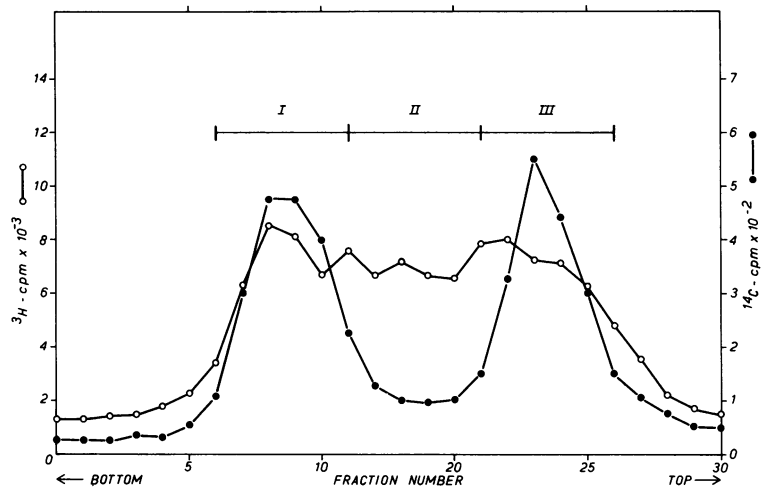

Figure 1. Isolation of Clo DF13 replicative intermediates by dye-buoyant density centrifugation. E. coli P678-54 thy harbouring plasmid Clo DF13 was prelabeled with ^{14}C thymine, and pulse labeled with ^3H thymidine for 40 sec as described in Materials and Methods. Cleared lysates, prepared as described previously (13), were subjected to CsCl-ethidium bromide equilibrium centrifugation. The gradient was fractionated, and the fractions were assayed for radioactivity (●-●) ^{14}C cpm x 10^{-2}, (o-o) ^3H cpm x 10^{-3}. Fraction I contains covalently closed circular Clo DF13 DNA, Fraction II contains replicative intermediates of Clo DF13, Fraction III is composed of open circular and linear plasmid DNA as well as chromosomal DNA.

in this fraction of the gradient, contained a symmetrical loop or "eye" of replicated DNA. Clo DF13 plasmid DNA contains single cleavage sites for both the restriction enzymes HpaI and SalI, located at respectively 0% and 66% on the Clo DF13 physical map (see Fig. 3; 8). To determine the mode of replication of the Clo DF13 plasmid, replicative intermediates were digested with either SalI or HpaI endonuclease and analysed with the electronmicroscope. Fig. 2 shows electronmicrographs of replicating Clo DF13 molecules which were digested with SalI (Fig. 2a-e) or HpaI (Fig. 2f-k) endonuclease. These molecules are arranged from top to bottom in the order of an increasing extent of replication. We observed that in all replicating Clo DF13 molecules (93) cleaved with HpaI, the replication fork had passed the HpaI cleavage site. In case of SalI cleaved molecules the replication fork had passed the SalI cleavage site in only 3 out of 90 examined molecules. These observations are schematically presented in Fig. 3. Cleavage of the replicating Clo DF13 molecule with HpaI will result in most cases in a linear molecule with four

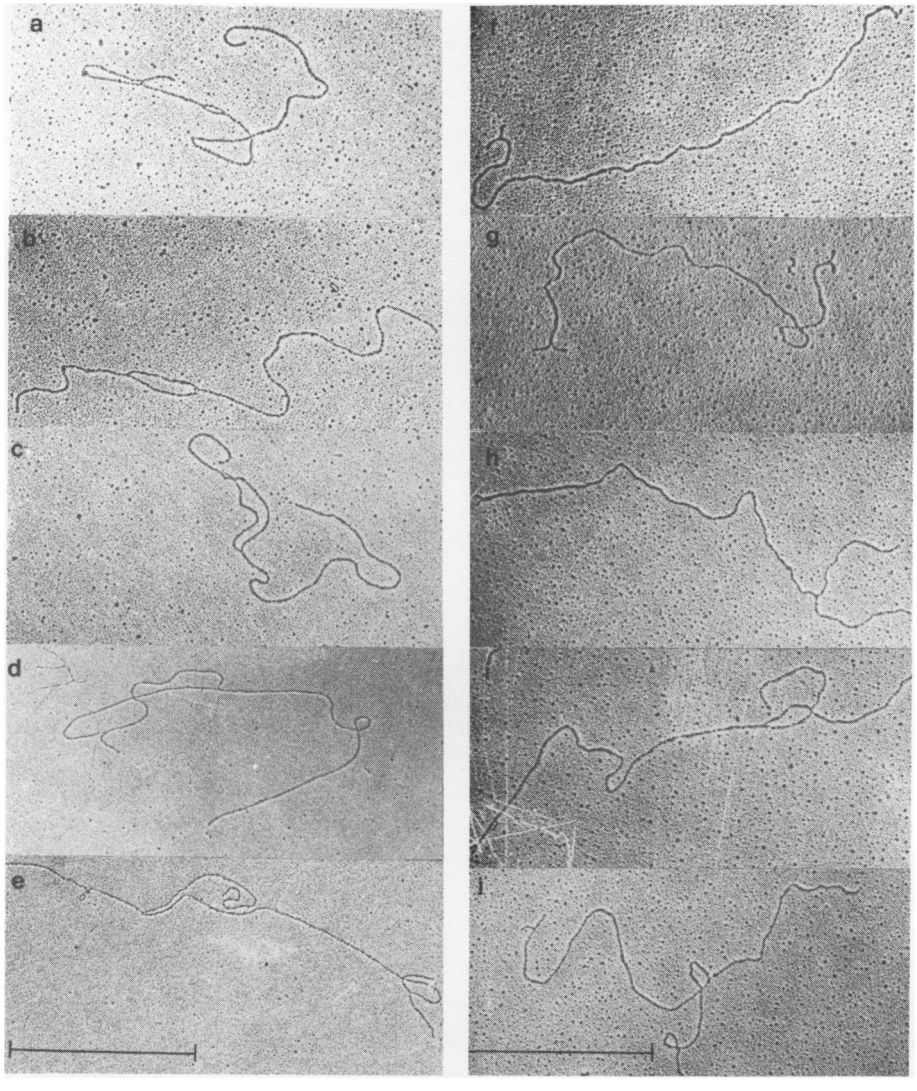

Figure 2. Electronmicrographs of replicating Clo DF13 molecules, cleaved with restriction endonucleases SalI (a-e) and HpaI (f-j). The molecules are ordered, from top to bottom, to an increasing extent of replication. The bar represents 1 μm.

replicated "arms", which are two by two of equal length. The replicated arms have arbitrarily been designated R1a, R1b, R2a and R2b (see Fig. 3).

In contrast, cleavage of a replicating Clo DF13 molecule with SalI will

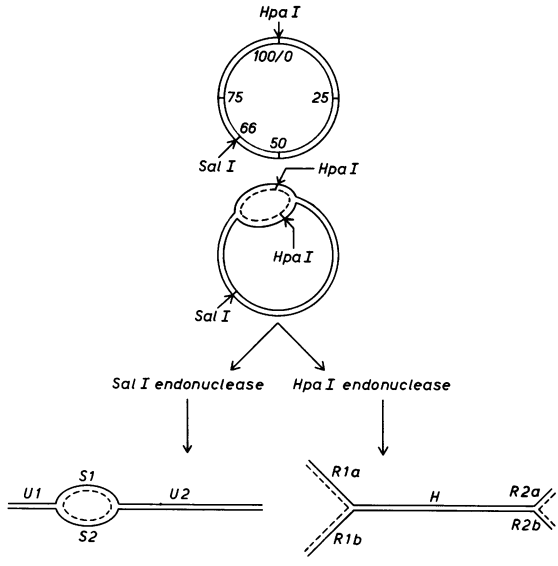

Figure 3. Example of the HpaI and SalI cleavage products of a replicating Clo DF13 molecule. U1 and U2 represent the unreplicated segments of a Clo DF13 molecule cleaved with SalI. The internal loop consists of two segments of replicated DNA called S1 and S2. Replicating Clo DF13 molecules cleaved with HpaI consist of an unreplicated segment H, branched by a pair of replicated arms of equal length on both ends, designated respectively R1a, R1b, and R2a, R2b.

predominantly result in a linear molecule, composed of a symmetrical loop or "eye" of replicated DNA bound by two unreplicated branches called U1 and U2. In Fig. 4 the relative lengths of the replicated "arms" R1 and R2 of HpaI cleaved Clo DF13 molecules are plotted versus the percentage of plasmid replication. This Figure shows that the relative length of one pair of replicated arms (R2) remains constant during replication. From the data in this figure it was deduced that Clo DF13 replication initiates at a unique site located at 2.8 $\pm$ 0.1% of the total genome size from the HpaI cleavage site. This result implicates that the origin is located at about 2.8 or 97.2% on the Clo DF13 physical map (the HpaI cleavage site is used as a reference point on this map; see Fig. 6). To discriminate between these possibilities we analyzed replicating Clo DF13 molecules cleaved with SalI.

In Fig. 5 we have plotted the relative lengths of the unreplicated arms U1 and U2 of SalI cleaved molecules, versus the percentage of plasmid replication. These data show a constant value of one unreplicated arm

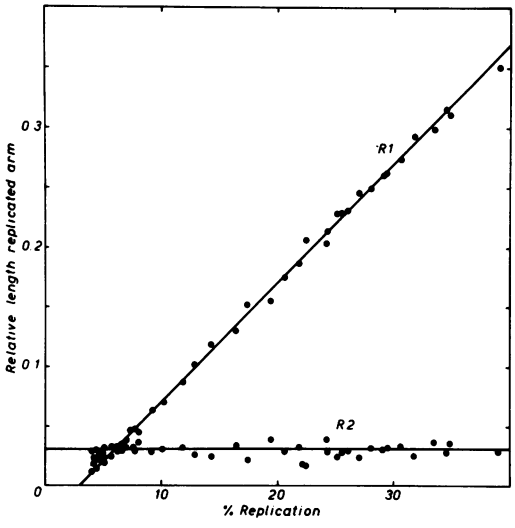

Figure 4. Analysis of the mode of replication of Clo DF13. HpaI cleaved molecules were photographed and measured. The relative length of the replicated arm R1 is defined as $R1 = \frac{R1a + R1b}{2T}$, where T represents the total length of the molecule $= \frac{R1a + R1b + R2a + R2b}{2} + H$. The percentage of replication $= (R1 + R2) \times 100\%$

(arbitrarily chosen as U2), which also indicates that Clo DF13 replicates unidirectionally. The mean relative value of U2 is 63.0 $\pm$ 0.2% of the total Clo DF13 genome size implicating that the origin of replication is located at either 3% or 29% on the Clo DF13 physical map. By combining the results obtained with HpaI cleaved molecules and SalI molecules (Fig. 4 and 5) it is obvious that Clo DF13 plasmid replication initiates at a unique site located at about 2.8% on the physical map. Replication proceeds

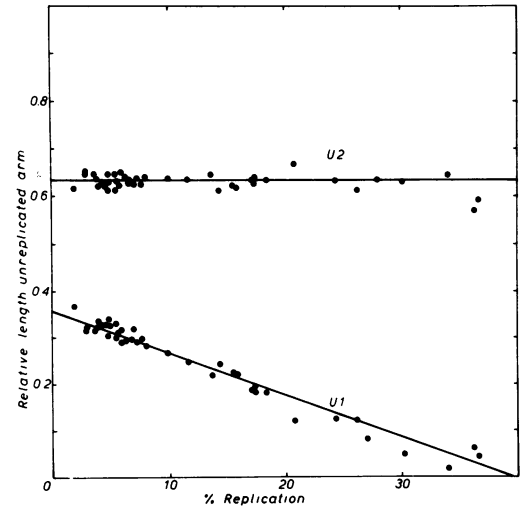

Figure 5. Analysis of the mode of replication of Clo DF13. SalI cleaved molecules were photographed and measured. The relative length of the unreplicated arm $U1,2 = \frac{U1,2}{T}$ where T represents the total length of the molecule $= U1 + U2 + \frac{S1 + S2}{2}$. The percentage of replication $= \frac{S1 + S2}{2T} \times 100\%$

unidirectionally and counterclockwise on this map (Fig. 6).

Using the same procedure as described above we examined the mode of replication of the copy mutant Clo DF13 cop3. From the analysis of replicative intermediates of Clo DF13 cop3, linearized with HpaI or SalI endonuclease, we found that there is no significant difference between Clo DF13 cop3 and the wild type Clo DF13 plasmid with respect to the origin and direction of replication (results not shown).

DISCUSSION

The electronmicroscopic analysis of Clo DF13 replicative intermediates, linearized at the HpaI or SalI cleavage site, revealed that the replication of plasmid Clo DF13 initiates at a unique site, located at approximately 2.8% on the Clo DF13 physical map. Furthermore, the data show that Clo DF13 replication proceeds unidirectionally, counterclockwise on this physical map (fig. 6). Although we did not obtain evidence that other sites on the Clo DF13 genome can also function as an origin of replication, it is still possible that specific conditions, mutations or deletions of particular parts of the Clo DF13 genome affects the plasmid replication process. For example

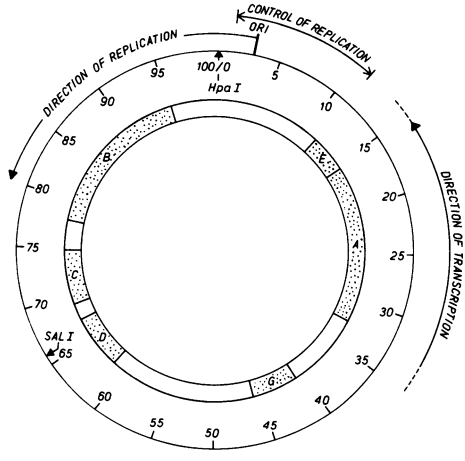

Figure 6. This figure shows the physical and genetic map of plasmid Clo DF13 with the HpaI cleavage site as reference point (set as 100/0%). Replication initiates at approximately 2.8% on this map, and proceeds unidirectionally and counterclockwise. The location of the genes A, B, C, D, E and G, based on the data obtained by Andreoli et al. (18), is indicated on the inner circle.

mutations or deletions might result in the utilization of other sites as replication origin or in a bidirectional mode of replication. Our studies indicated that the copy mutant of Clo DF13, Clo DF13 cop3, did not differ significantly from the wild type plasmid, with respect to the origin and mode of replication. This result implicates that the effect of the cop3 mutation, namely a seven times increased frequency of initiation of plasmid replication (6), is not caused by the utilization of another origin or multiple origins.

In a previous paper we have described the in vitro construction of deletion mutants of Clo DF13, using restriction endonucleases and T_4 ligase (8). The characterization of the Clo DF13 miniplasmids, revealed that all miniplasmids isolated by this procedure contained the Clo DF13 DNA region from 0 to 11.5%. It was also reported that the smallest miniplasmid, plasmid peV22, which was constructed with HaeII endonuclease, only contains the Clo DF13 DNA region from 5 to 11.5% (8). However, from the analysis of detailed cleavage maps which were constructed of different Clo DF13 miniplasmids, including peV22, we found that plasmid peV22 contains an additional small Clo DF13 HaeII fragment which is located from 1.8 - 5% on the physical map (Stuitje et al., manuscript in preparation). In view of these observations it is obvious that the origin of Clo DF13 replication (located at 2.8%) is situated on this small HaeII fragment. In this respect it is of interest to mention that the replication origin of plasmid Col E1, a plasmid that resembles Clo DF13 with respect to its replication (4, 5, 15, 16), is also located on a small HaeII fragment of similar size (17). We therefore suspect that the replication origins of these plasmids share homology.

The mapping of the replication origin on the Clo DF13 genome and the availability of a detailed cleavage map of the "replication region", enables us to elucidate the base sequence of the replication origin. Furthermore a comparison can be made between the base sequence of the replication region of the wild type plasmid and those of Clo DF13 mutants with an altered replication control. These studies, which are in progress, will give us more insight in the molecular basis of the regulation of plasmid replication.

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