Replication of colicinogenic factor E 1 DNA: evidence for a discontinuous replication mechanism

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ABSTRACT

The mechanism of Col E 1 DNA replication was investigated in a plasmolysed cell system prepared from chloramphenicol-treated E. coli JC 411 (Col E 1). After pulse-labelling with ^3H -dTTP a considerable fraction of the newly synthesized DNA was recovered as single-stranded fragments. Upon alkali denaturation the pulse label was found in DNA chains sedimenting slower than unit length Col E 1 strands with a prominent peak at 5 S. During a chase with unlabeled precursors the label is transferred nearly completely into supercoiled Col E 1 DNA. DNA ligase appears to be required for the joining of the 5 S pieces since in the absence of NAD an accumulation of short fragments is observed.

INTRODUCTION

Colicinogenic factor E 1 is a small extrachromosomal DNA that directs the synthesis of an antibiotic protein. It is isolated from E. coli as a supercoiled covalently closed circular duplex with a molecular weight of 4.2×10^6 (1). Col E 1 DNA replicates extensively in the presence of chloramphenicol (2) an antibiotic which inhibits the initiation of chromosomal DNA replication. Replicating structures having the θ or "Cairns" form as well as σ shaped "rolling circles" have been observed by electron microscopy (3,4,5).

We have shown previously that phage M-13 infected E. coli cells plasmolysed by treatment with 2 M sucrose are capable of carrying out the semi-conservative replication of M-13 DNA from deoxynucleoside triphosphates (6,7). Plasmolysed cell systems seem therefore suited to study the replication of small DNA circles under quasi in vitro conditions. Recently we have prepared such a system from E. coli JC 411 (Col E 1) after blocking chromosomal DNA synthesis by growing the cells for several hours in chloramphenicol prior to plasmolysis. In

this system, incorporation of deoxynucleoside triphosphates into plasmid DNA requires ATP (2 mM), MgCl_2 (7.5 mM), and KCl (0.15 M). After a 30 min incubation at 30°C more than 80% of the incorporated label is found in supercoiled Col E 1 DNA. Density labelling experiments indicate that the supercoils are formed by a semi-conservative replication process (manuscript in preparation). Using this system we performed pulse-chase experiments to investigate whether Col E 1 DNA replication occurs by a continuous mechanism according to the "rolling circle" model (8) or by a discontinuous mode of DNA synthesis (9). The data presented in this paper seem to exclude a "rolling circle" like mechanism and provide evidence for a discontinuous replication of Col E 1 DNA.

MATERIALS AND METHODS

E. coli JC 411 (Col E 1) was grown in M-9 medium (10) at 37°C. ^{32}P -labeled reference Col E 1 DNA was prepared as described by Blair et al. (11). The sources of all of the reagents and assay of radioactivity have been described previously (7).

Preparation of plasmolysed cells. A 2 l culture of E. coli JC 411 was grown with aeration to a density of 4×10^8 cells/ml. Chloramphenicol (150 $\mu\text{g/ml}$) was added and aeration continued for 3.5 hours. The culture was then poured on an equal volume of ice and harvested by low speed centrifugation at 4°C. The pellet was washed twice with 30 ml of cold 20 mM HEPES pH 8.0 - 10 mM MgCl_2 - 0.1 mM EDTA. Plasmolysis was carried out by suspending the cells in 6 ml 20 mM HEPES pH 8.0 - 5 mM EGTA - 2 M Sucrose. Aliquots of the plasmolysed cell suspension (approximately 1×10^{11} cells/ml) were quickly frozen in an acetone-solid CO_2 bath and stored at -20°C.

Assay of DNA synthesis. Standard incubation mixtures (1 ml) contained 20 mM HEPES pH 8.0, 150 mM KCl, 7.5 mM MgCl_2 , 1 mM dithiothreitol, 2 mM ATP, 20 mM phosphoenolpyruvate, 50 $\mu\text{g/ml}$ pyruvate kinase, 0.01 mM NAD, 0.5 mM each of CTP, GTP and UTP, 0.05 mM each of dATP, dCTP, and

dGTP, and 0.01 mM ^3H -dTTP (specific activity 1000 cpm/pmole). 50 μl of the plasmolysed cell suspension (corresponding to 5×10^9 cells) were added and the mixture incubated at 30°C . Incubations were stopped by adding 1 ml of cold 0.1 M EDTA. To remove unincorporated radioactivity the cells were spun down by low speed centrifugation and washed once with cold 20 mM HEPES pH 8.0 - 5 mM EDTA.

Extraction of labeled DNA. Col E 1 DNA was isolated by the NaCl-SDS precipitation method described by Godson & Vapnek (12). The washed cell pellet was resuspended in 2 ml 20 mM HEPES pH 8.0 - 10% (w/w) sucrose and the cells converted to spheroplasts by adding 0.2 ml lysozyme (10 mg/ml) and 0.8 ml 0.25 M EDTA followed by a 30 min incubation at 0°C . Lysis was carried out by addition of 0.2 ml of 20% SDS and the lysate was kept on ice overnight. The precipitate was removed by centrifugation in a Servall SS 34 rotor at 15 000 rpm for 30 min. 0.8 ml 5 M NaCl were added to the supernatant and after 5 hours on ice the second precipitate was removed by centrifugation at 15 000 rpm for 15 min. Col E 1 DNA was precipitated from the supernatant by adding 2 volumes of ethanol and standing overnight at -20°C . The alcohol precipitate was collected by centrifugation and resuspended in 1 ml 50 mM Tris-HCl pH 8.0 - 5 mM EDTA - 0.1 M NaCl and further analysed by dye-buoyant density centrifugation.

Centrifugation techniques. Dye-buoyant density centrifugations were performed in a Spinco fixed-angle Ti 50 rotor at 40 000 rpm for 40 hours at 20°C . Tubes contained 4.0 g CsCl, 2.5 ml propidium diiodide (1 mg/ml), and 2.5 ml sample in Tris-EDTA-NaCl buffer. Velocity sedimentations in neutral and alkaline CsCl gradients were performed in a Spinco SW 56 rotor at 50 000 rpm for 120 min (neutral gradients) or 60 min (alkaline gradients) at 5°C . Linear CsCl gradients were prepared as described previously (13).

Benzoylated naphtoylated DEAE (BND) cellulose chromatography

Labeled DNA (in 6 ml 0.3 M NaCl - 0.01 M Tris-HCl pH 8.0 - 0.001 M EDTA) was adsorbed on a column (0.9 x 5 ml) of BND-cellulose (Boehringer) previously washed with the same buffer. The column was then eluted successively with 6 ml 0.3 M NaCl-

Tris-EDTA, 12 ml 1.0 M NaCl-Tris-EDTA, and a linear gradient (24 ml) of 0.0-1.8% caffeine in 1.0 M NaCl-Tris-EDTA. The flow rate was 0.5 ml/min. Chromatography was carried out at 37°C.

RESULTS

Synthesis of supercoiled Col E 1 DNA

Plasmolysed chloramphenicol-treated cells of E. coli JC 411 (Col E 1) were pulse-labeled with ^3H -dTTP for 2.5 min at 30°C and the pulse was followed by a chase with unlabeled dTTP as described in Fig. 1. The pulse length was chosen to be close to the estimated replication time of Col E 1 DNA (around 2 min at 32°C) (14). The chromosomal DNA was removed by precipitation with NaCl-SDS and the extracted plasmid DNA further analysed by dye-buoyant density centrifugation. This technique allows a separation of DNA depending on the superhelicity as well as the extent of single-strandedness of the molecules: Superhelical form I DNA binds less dye and bands at a heavier density than open circular form II DNA; replicative intermediates containing supercoiled regions as well single stranded DNA band at densities between form I and form II.

It can be seen in Fig. 1A that after a 2.5 min pulse only a small fraction of the incorporated label is found in the position of supercoiled DNA while most of the labeled DNA bands in an intermediate density region. If the pulse is followed by a 2.5 min chase a transfer of label from the intermediate density region to the positions of form I and form II DNA is observed (Fig. 1B). After a prolonged chase the label at intermediate density has practically disappeared and is now mostly found in the position of form I DNA (Fig. 1C). This material was further identified by velocity sedimentation as supercoiled Col E 1 DNA (data not shown).

A further characterization of the extracted DNA was attempted by BND-cellulose chromatography. This technique separates nucleic acids according to their secondary structure (15): Double-stranded DNA can be eluted by a salt gradient; single-stranded DNA or DNA containing single-stranded regions are eluted with a gradient of increasing caffeine

Fig. 1: Dye-buoyant density centrifugation of pulse-labeled DNA. Standard incubation mixtures were pre-incubated for 5 min at 30°C. The cells were then pulse-labeled with 50 μ C 3 H-dTTP for 2.5 min. Incorporation was either stopped by the addition of cold 0.1 M EDTA (A) or followed by a chase with 0.05 ml 10 mM dTTP for 2.5 min (B) or 25 min (C) resp.. Plasmid DNA was extracted and analysed by propidium diiodide-CsCl density centrifugation. A mixture of 32 P-labeled Col E 1 DNA form I and II was used as reference DNA. Density increases from right to left.

concentration in molar salt. The chromatographic pattern of the pulse-labeled DNA is shown in Fig. 2A. Since most of the label is eluted at a caffeine concentration higher than 1% we assume that this DNA is predominately single-stranded. After a 2.5 min chase a marked decrease in the amount of tightly bound label is observed concomitant with an increase in double-stranded material (Fig. 2B).

Fig. 2: BND-cellulose chromatography. Pulse-labeled DNA was prepared as described in Fig. 1 and analysed by BND-cellulose chromatography (see Methods). (A) 2.5 min pulse; (B) 2.5 min pulse - 2.5 min chase.

Fig. 3: Velocity sedimentation of pulse-labeled DNA. 0.2 ml portions of the pooled fractions from the gradients shown in Fig. 1A and 1B were analysed by velocity sedimentation in neutral and alkaline CsCl. The arrow indicates the position of a ^{32}P -labeled Col E1 DNA form II sedimentation marker. Sedimentation in this and all following gradients is from right to left.
 ○—○, DNA from gradient Fig. 1A; ●—●, DNA from gradient Fig. 1B.

Discontinuous chain growth

Corresponding regions (designated α , β , γ) of the gradients shown in Fig. 1A and 1B were pooled and analysed by velocity sedimentation in neutral and alkaline CsCl (Fig. 3). Neutral gradient centrifugation of the pulse-labeled DNA gave a rather unexpected result: A considerable fraction of the incorporated label is recovered in a broad band sedimenting at a much slower rate than form II DNA. Structures sedimenting faster than Col E 1 supercoils are also observed. A short chase results in a transfer of pulse label from both the fast and slowly sedimenting material to the positions of form I and form II DNA. In the alkaline gradients denatured supercoils (which cannot undergo strand separation and sediment around 50 S) are found in fraction α . However most of the pulse label is recovered in fragments which sediment slower than Col E 1 DNA strands of unit length.

Fig. 4: Alkaline CsCl velocity sedimentation. Pulse-labeled DNA from the intermediate density region of the gradients shown in Fig. 1 was layered on alkaline CsCl gradients and centrifuged in a Spinco SW 56 rotor at 50 000 rpm for 180 min at 5°C.

○—○, DNA from gradient Fig. 1A; ●—●, DNA from gradient Fig. 1B; x---x, ³²P-labeled Col E 1 DNA form II.

The sedimentation behaviour of the short fragments obtained by alkali denaturation was further analysed by extended velocity sedimentation in alkaline CsCl (Fig. 4). S-values were calculated by referring to the position of linear (17 S) and circular (19 S) Col E 1 DNA strands. The sedimentation pattern of the pulse-labeled material shows a prominent peak in the 5 S region with a shoulder of longer chains having a broad distribution up to 17 S. During a 2.5 min chase a change in the size distribution is observed leading to an increase in the proportion of label found in longer chains.

Fig. 5: Dye-buoyant density centrifugation. Standard incubation mixtures were incubated for 30 min at 30°C in the presence (A) or absence (B) of 0.01 mM NAD. The labeled plasmid DNA was extracted and analysed by propidium diiodide-CsCl density centrifugation. The arrow indicates the position of ³²P-labeled Col E 1 DNA form II. Density increases from right to left.

Involvement of DNA ligase

Incubations were routinely carried out in the presence of NAD which is required as a cofactor for *E. coli* DNA ligase (16). Under these conditions most of the plasmid DNA synthesized during a 30 min incubation is recovered as form I DNA (Fig. 5A). However if NAD is omitted from the incubation mixture a large portion of the newly synthesized DNA bands at an intermediate density (Fig. 5B). When this DNA is subjected to neutral and alkaline velocity sedimentation most of the label is found in slowly sedimenting fragments (Fig. 6). Furthermore if a pulse-chase experiment is performed in the absence of NAD only a small fraction of the pulse-labeled fragments can be chased into supercoiled DNA (data not shown). Concentrations of NAD or NADH as low as 1 μ M are sufficient for a normal yield of supercoiled molecules. NMN cannot substitute for NAD, whereas NADP or NADPH are effective only at much higher concentrations (0.1 - 1 mM).

Fig. 6: Velocity sedimentation of DNA synthesized in the absence of NAD. Neutral (A) and alkaline (B) CsCl velocity centrifugation was carried out as described in Methods. A mixture of ³²P-labeled Col E 1 DNA form I and II was used as sedimentation marker.

DISCUSSION

Plasmolysed chloramphenicol-treated E. coli cells carrying the Col E 1 factor incorporate a ^3H -dTTP pulse into short DNA chains. When analysed by alkaline velocity centrifugation the pulse-labeled DNA sediments largely as 5 S pieces. Assuming validity of the Studier equation (17) in the range of low S-values these pieces have a molecular weight around 100 000 daltons and are thus considerably shorter than the "Okazaki fragments" observed during chromosomal DNA replication. No pulse-labeled DNA chains longer than Col E 1 DNA strands of unit length were detectable. Therefore we have no evidence for a covalent attachment of newly synthesized DNA to the parental strands. During a chase with unlabeled dTTP the radioactive label is transferred from the short chains into open circular form II molecules (Fig. 3C) and further into covalently closed supercoils. A similar flow of radioactive label was observed previously during the in vivo replication of the minicircular DNA of E. coli 15, a small plasmid related to Col E 1 DNA (18).

A considerable portion of the pulse-labeled DNA was recovered as short fragments even under non-denaturing conditions. These fragments are not simply an artefact caused by extraction with SDS since they are also observed after lysis with non-ionic detergents in the presence of high salt (unpublished data). Chromatography on BND-cellulose indicates that this material consists largely of single-stranded DNA. Release of pulse-labeled DNA as single-stranded fragments has been observed in several systems (9,19,20) and has been interpreted as an instability (or absence) of the double-helical structure in the newly replicated DNA.

NAD is required for the synthesis of covalently closed circles and omission of NAD from the incubation mixture leads to the formation of short pulse-labeled fragments. Accumulation of "Okazaki pieces" has previously been observed in phage systems deficient in DNA ligase (21,22). This finding lends further support to a discontinuous mechanism of Col E 1 DNA chain growth and provides evidence that the joining of the

newly synthesized chains is carried out by the NAD-dependent DNA ligase. Experiments are in progress to clarify the structure of the replicative intermediates and to decide whether one or both strands are synthesized in a discontinuous fashion.

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