Isolation of Catenated and Replicating DNA Molecules of Colicin Factor E1 from Minicells

(E. coli/sucrose gradients/ethidium bromide/CsCl/electron microscopy)

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ABSTRACT Various catenated and replicating molecules of colicin E1 isolated from minicells have been identified in regions of neutral sucrose density gradients or cesium chloride–ethidium bromide density gradients. These multimers have been found in those regions of the gradient where pulse-labeled DNA accumulates.

Adler et al. (1) isolated a mutant of Escherichia coli, P678-54, that produced significant numbers of small, DNA-less progeny ("minicells") under normal growth conditions. Subsequent work has shown that if plasmid or episomal DNAs are carried by that mutant, they can segregate into (2–6) and replicate in (2, 6, 7) the minicells. In this report minicells containing colicin factor E1 (Col E1) DNA have been used to examine DNA replication. Two principal advantages of studying DNA replication in minicells are first, the small size of the Col E1 DNA monomers [4 × 10^6 daltons (8)] and second, the absence of chromosomal DNA, which otherwise interferes with the identification of extra-chromosomal DNA. The study of the replication of Col E1 DNA in minicells has shown that (a) all of the observed DNA in minicells carrying Col E1 DNA is Col E1 DNA (2, 7); (b) the Col E1 DNA principally exists as covalently-closed circular and open circular molecules, though linear monomers are also found (2, 7), (c) the Col E1 DNA can undergo at least two complete rounds of replication (2); and (d) replicating structures identical to the forked circular molecules originally found by Cairns (9), as well as those similar to the structure of rolling circles as postulated by Gilbert and Dressler (10), can be identified (7). These observations have led to a further examination of Col E1 DNA replication, a part of which is the subject of this report.

MATERIALS AND METHODS

All experiments were performed with purified minicell preparations (1 viable bacterium per 10^9–10^10 minicells) isolated from log-phase cultures of E. coli strain P678-54 (Col E1) (2) grown in Tris–Casamino acid–glucose medium supplemented with 20 μg/ml of threonine and leucine and with 1 μg/ml of vitamin B12. The detailed procedures for minicell purification, [H]thymidine labeling of Col E1 DNA in the above medium supplemented with 250 μg/ml of deoxyadenosine and 0.5 μg/ml of thymidine, and DNA extraction from minicells treated with spheroplast medium [lysozyme–EDTA–pancreatic ribonuclease] are as described (2, 11). Pronase treatment (1 mg/ml of predigested Pronase for 15 min at 37°C) of minicells previously incubated in spheroplast medium was introduced into the DNA extraction procedure before the addition of Sarkosyl NL30 and subsequent phenol extraction (2, 11). Neutral sucrose density gradient sedimentation analysis (2) and cesium chloride–ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridium bromide) density-gradient analysis of DNA (2, 12) and electron microscopic techniques (7, 13, 14) were also described in detail. Buffer solutions used were Tris–saline (TS) 0.05 M Tris-0.05 M NaCl (pH 8.0); Tris–EDTA–Saline (TES), which is TS + 5 mM EDTA; and saline–sodium citrate solution (SSC), which contains 0.15 M NaCl and 0.015 M sodium citrate (pH 7.4).

RESULTS

It can be estimated that a Col E1 DNA molecule (4 × 10^6 daltons) (7, 8) should complete a round of replication in 5 sec if it replicates as rapidly as chromosomal DNA [the E. coli chromosome of 2.5 × 10^9 daltons (9) replicates in about 40 min (15)]. If the molecule did replicate at that rate in minicells, then by pulse-labeling of the replicating Col E1 DNA for about 5 sec, it should be possible to obtain as much as 50% of the total radioactively labeled DNA in a replicating form. Minicells carrying replicating Col E1 DNA were purified and labeled with [H]thymidine for different periods of time. The DNA was then extracted and either examined in a neutral sucrose density gradient or in a cesium chloride–ethidium bromide density gradient. The results of the neutral sucrose density-gradient analysis (Fig. 1) indicate that a broad peak of DNA, sedimenting more rapidly than the covalently-closed circular form of Col E1 (24 S) (2), was labeled during the 5-sec pulse of [H]thymidine and then became progressively less distinct as the labeling period was extended. Four separate experiments gave identical results. The fast-sedimenting peak accounted for 20–30% of the DNA that was pulse labeled in 5 sec. As the DNA was purified in the presence of Pronase, Sarkosyl, and phenol, the rapid sedimentation property should not be due to the binding of protein to DNA as has been described by Clewell and Helinski (17). The results of the cesium chloride–ethidium bromide density-gradient analysis, shown in Fig. 2, indicate that during a short pulse-labeling a significant amount of labeled DNA bands at an intermediate density between the positions of covalently-closed circular molecules (fractions 10–15) and of open circular and linear molecules (fractions 27–32) (12). On prolonged labeling, the DNA in the intermediate-
DNA, which maintains its sedimentation characteristics upon recentrifugation in an identical sucrose gradient, was further analyzed. DNA labeled for 1 hr was fractionated in a neutral sucrose-density gradient and divided into two fractions (b and c in Fig. 3b). Both fractions were examined in a cesium chloride-ethidium bromide density gradient. The result (Fig. 3b and c) shows that much of the fast-sedimenting DNA bands at the intermediate-density position.

Hudson and Vinograd (18, 19) have shown that several forms of catenated mitochondrial DNA from HeLa cells can sediment faster than covalently-closed circular molecules in a sucrose gradient, and can be found in cesium chloride-ethidium bromide gradients at a density between that of open and covalently-closed circular molecules. Tomizawa and Ogawa (20) and Levine et al. (21) have found that replicating, forked circular structures of bacteriophage lambda and SV40 virus, similar to those originally found by Cairns (9), also sediment in neutral sucrose at a position faster than covalently-closed circles. In view of these findings, and since a previous report indicated that a major replicating form of Col E1 DNA in minicells is a forked circular structure (7), it was expected that both replicating and catenated molecules might be present in the regions of the sucrose density or cesium chloride-ethidium bromide gradients where labeled DNA accumulated during a short pulse. Col E1 DNA density position decreases in relative amount, whereas the region containing covalently-closed circular molecules, as well as that containing both open circular and linear molecules, increases. In a pulse-chase experiment in which the DNA of minicells was labeled for 1 min, and was then chased for 40 min, a similar change in the banding properties of the DNA in the cesium chloride-ethidium bromide density gradient was observed.

It can be seen in Fig. 1 that the material labeled for 1.5 hr contains a small, broad band of fast-sedimenting DNA in addition to the 24S and 18S DNA. This fast-sedimenting

Fig. 1. Neutral sucrose density-gradient analysis of Col E1 DNA labeled for various times. Purified P678-54 (Col E1) minicells were incubated with [3H]thymidine (250 μCi/ml) for the 5-sec pulse, 100 μCi/ml for all other experiments) for the time indicated. Cold TES buffer containing 0.04 M NaCl was added to stop the labeling of the minicells, which were washed four times in TES containing 0.02 M NaCl. DNA was incubated in spheroplast medium, with successive additions of Pronase and Sarkosyl. Each DNA isolation step lasted about 15 min at 37°C. DNA was extracted with TES-saturated phenol at room temperature, dialyzed against 0.1 X SSC overnight at 4°C, and concentrated to 0.2 ml, using Carbowax 6000 when necessary. The DNA was layered on a 4.6 ml 5-20% TES-sucrose gradient and centrifuged at 84,000 X g in a Spinco SW50.1 rotor at 15°C for 4.5 hr. In a Spinco model L2 ultracentrifuge. The two principal peaks in the gradients are marked and are, from right to left, the 18S open and 24S covalently-closed circular forms of Col E1 DNA. A third broad peak is seen to the left of the 24S peak in the gradients containing 5-sec and 30-sec pulse-labeled DNA. The gradients of the 5-sec and 1.5-hr labeled DNA have been superimposed (a) to facilitate comparison. E. coli phage P1 [3P]DNA, with a reported sedimentation coefficient of about 43 S (2, 16), was added to each tube as a sedimentation marker and its position in the gradient is marked by an arrow. (a) 5 sec (●), total cpm 190, total fractions 30; 1.5 hr (○), total cpm 22,640, total fractions 30. (b) 30 sec, total cpm 390, total fractions 35. (c) 1.5 min, total cpm 630, total fractions 35. (d) 4 min, total cpm 1408, total fractions 30.

Fig. 2. Cesium chloride-ethidium bromide density-gradient centrifugation of Col E1 DNA labeled for various times. Col E1 DNA in minicells was labeled and purified as described in Fig. 1, dialyzed against 1 X SSC, added to a solution containing CsCl, 4.36 g; ethidium bromide, 1.9 ml of 700 μg/ml in sodium phosphate buffer, (pH 7.0) (11), and brought to a refractive index of 1.3860 as measured in an Abbe 3L refractometer. The solution was centrifuged in a Spinco 50 Ti fixed angle rotor at 105,000 X g for 48 hr at 5°C, and fractions were collected by drop collection from the bottom of the tube. Refractive indexes of selected fractions were always taken. E. coli [3P]DNA (2) was added to each gradient to mark the position of linear DNA. (a) 5 sec, total cpm 656, total fractions 50. (b) 1.5 min, total cpm 2875, total fractions 52. (c) 20 min, total cpm 1985, total fractions 52.
isolated from minicells was fractionated by either neutral sucrose density-gradient centrifugation or cesium chloride-ethidium bromide density-gradient centrifugation, and the pooled fractions of the sucrose gradient that sedimented just ahead of the position of the covalently-closed circular molecules and the pooled fractions obtained from the intermediate-density position of the cesium chloride-ethidium bromide gradient were examined by electron microscopy (Table 1). Examples of typical catenated Col El DNA molecules obtained from both fractions are shown in Fig. 4.

DISCUSSION

In previous work (7), replicating Col El DNA structures in minicells were detected by density labeling techniques. The DNA molecules found were principally in the form of replicating, Cairns type, double-forked circular structures. Several molecules in the form of open circles with long tails were found and tentatively identified as replicating "rolling circle" structures. The present work shows that replicating molecules of Col El DNA can be efficiently isolated (see Table 1) from the region of accumulation of pulse-labeled Col El DNA that sediments a little faster than 24 S, the position of covalently-closed circles in a neutral sucrose density gradient, or that band between the positions of open and covalently-closed circular DNA in a cesium chloride-ethidium bromide density gradient. Replicating, twisted circular DNA and replicating catenated molecules not previously reported were found together with the two replicating structures that had been described (7). These results

Fig. 3. A cesium chloride-ethidium bromide density-gradient analysis of Col El DNA obtained from a neutral sucrose density gradient. (a) Col El [3H]DNA labeled for 1 hr was isolated from minicells, prepared as described in Fig. 1, and fractionated in a neutral sucrose density gradient. P1 [32P]DNA was used as a sedimentation marker and its position in the gradient is indicated. Appropriate fractions of the gradient were pooled, dialyzed against 0.1 X SSC, and concentrated with Carbowax 6000. Material from pooled fractions b (9-14) and c (15-21) were analyzed by cesium chloride-ethidium bromide density-gradient centrifugation. (a) total cpm 18,781, total fractions 30. (b) total cpm 3,088, total fractions 54. (c) total cpm 11,917, total fractions 53.

Fig. 4. Electron micrographs of catenated Col El DNA obtained from neutral sucrose density gradients or cesium chloride-ethidium bromide density gradients.
TABLE I. Molecular forms of Col E1 DNA observed by electron microscopy

<table>
<thead>
<tr>
<th>Type</th>
<th>Number of molecules</th>
<th>From cesium chloride-ethidium bromide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twisted circle</td>
<td>332</td>
<td>93</td>
</tr>
<tr>
<td>Open circle</td>
<td>46</td>
<td>230</td>
</tr>
<tr>
<td>Linear fragment</td>
<td>29</td>
<td>67</td>
</tr>
<tr>
<td>Circular dimer</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Catenated (all types)</td>
<td>122</td>
<td>82</td>
</tr>
<tr>
<td>open-open</td>
<td>39</td>
<td>20</td>
</tr>
<tr>
<td>open-twisted</td>
<td>67</td>
<td>34</td>
</tr>
<tr>
<td>twisted-twisted</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>trimer</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Replicating (all types)</td>
<td>51</td>
<td>14</td>
</tr>
<tr>
<td>double-forked circle</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>circle (Cairns)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>circle with tail</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>twisted circle*</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>catenated (all types)</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td>585</td>
<td>486</td>
</tr>
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</table>

Twisted circle is synonymous with "covalently-closed circular" molecules. In the case of the twisted-circular replicating molecules (*), the covalently-closed circular nature of the molecules is uncertain. In a forthcoming paper a complete presentation will be made of the electron-microscopic evidence, demonstrating the variety of replicating and catenated Col E1 DNA structures reported in this table.

This work is supported (18, 19, 24, 25). The ability to isolate significant numbers of catenated structures from minicells should facilitate the resolution of the problem of their formation.

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