Replicating DNA: Structure of Colicin Factor E1

Abstract. *Replicating circular molecules of colicin factor E1 have been isolated from minicells produced by a colicinogenic derivative of* Escherichia coli *P678-54. The replicating structures observed in electron micrographs are circular molecules with two branched points. Several structures found among the replicating molecules are interpretable as replicating rolling circles.*

Replicating circular molecules of bacterial (*1*), viral (*2–4*), and mitochondrial (*5*) DNA have been visualized. We report here the isolation of forked circular molecules of colicin factor E1 (Col E1) DNA from *Escherichia coli* which have properties that are consistent with their being a replicating structure of that extrachromosomal genetic element.

Col E1 DNA segregates into and replicates in minicells (*6*) of the colicinogenic derivative of *E. coli* strain P678-54 (*7*). Minicells derived from the noncolicinogenic strain do not contain DNA.

We have isolated Col E1 DNA from minicells (*7*) and have examined the molecules by electron microscopy (*2*, *8*). The general procedures for purification and labeling of minicells containing Col E1 DNA and the purification and density gradient analysis of that replicated DNA have been described (*7*). The DNA was found on electron microscopic examination to exist principally as small, twisted circular and open circular molecules. Measurements of 50 randomly selected open circular molecules gave an average length of 2.31 ± 0.06 μm, which closely corresponds to the value 2.33 ± 0.06 μm previously reported for Col E1 DNA molecules (*9*). This finding also confirms the previous observation that highly purified Col E1 DNA can be obtained from minicells (*7*).

Table 1. Measurements and analysis of lengths (*L*) of molecules in Fig. 2. Electron micrograph projections were measured with a map measure. The unit circumferences calculated by $[(L1 + L2)/2] + L3$ were in the range of 2.23 to 2.37 μm, with a 6 percent deviation. The lengths of the two replicating strands coincided with an 8 percent error.

Labels in Fig. 2	Replicated regions (μm)		Unreplicated region (μm)	Unit circumference (μm)	Replication (%)
	L1	*L2*	*L3*	$\left(\frac{L1+L2}{2}\right)+L3$	$\frac{L1+L2}{2}\Big/\left(\frac{L1+L2}{2}+L3\right)$
a	0.11	0.12	2.25	2.37	5
b	0.42	0.43	1.88	2.31	18
c	0.50	0.59	1.71	2.26	24
d	0.86	0.89	1.44	2.32	38
e	0.92	0.93	1.38	2.31	40
f	1.27	1.40	1.00	2.34	58
g	1.52	1.53	0.83	2.36	65
h	1.54	1.69	0.61	2.23	72
i	1.97	2.19	0.25	2.33	89
j	2.03	2.11	0.17	2.24	92
k	2.28	2.37	0	2.33	99+

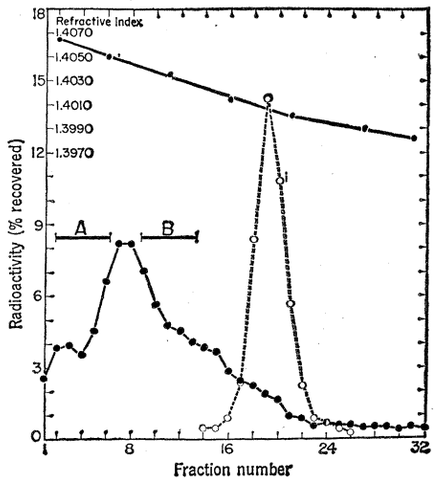

Fig. 1. Separation of replicating Col E1 DNA isolated from minicells of Col E1 colicinogenic *Escherichia coli* strain P678-54. The minicell purification and DNA labeling procedure has been described (*7*) except that minicells obtained from 16 liters of medium were quick frozen in 7.5 percent glycerol in tris glucose and stored at −70°C for up to 2 days prior to being thawed for simultaneous treatment with 5-bromouracil and tritiated thymidine (*7*) for 45 minutes. The DNA was released by the sarkosyl-lysozyme method (*7*, *11*) except that no DNA was added as carrier, transfer RNA (30 μg/ml) was added prior to lysis, and the mixture was incubated for 1 hour more at 37°C in the presence of self-digested pronase (1 mg/ml); previously incubated at 37°C for 4 hours. The digest was added to solid cesium chloride, brought to a density of 1.720 g/cm³ with TES (0.05*M* tris, 0.005*M* EDTA, and 0.005*M* NaCl, *p*H 8.0) and centrifuged at 38,000 rev/min for 48 hours. [^{32}P]DNA from *E. coli* was a density marker. The density of gradients were measured by refractive index and radioactivity in the fraction precipitable by cold trichloroacetic acid (*7*). Fractions in A and B, on either side of the peak of half-heavy DNA, which is denser by 0.039 g/cm³ than the *E. coli* marker, were respectively pooled and dialyzed against 2*M* ammonium acetate, 0.15*M* sodium chloride, and 0.015*M* sodium citrate and observed by electron microscopy. (Solid circles) [^{3}H]DNA from minicells; (open circles) [^{32}P]DNA from *E. coli*, which was the density marker.

Col E1 DNA molecules that are in the process of being replicated are obtained by labeling the replicating Col E1 DNA in minicells with 5-bromouracil and separating the partially replicated DNA from either the unreplicated or completely replicated molecules in a cesium chloride density gradient (*3*) (Fig. 1).

Fractions A and B (Fig. 1), taken from regions of the density gradient expected to contain partially replicated molecules, were examined by electron microscopy. In another experiment not shown, further purification of 5-bromouracil-labeled replicating DNA was attempted by centrifugation in cesium chloride of selected fractions of a density gradient. The results of electron microscopic observations made on those samples were similar to those made on material shown in Fig. 1.

While most of the DNA molecules observed in fractions A and B were open circular and twisted circular forms that represent molecules which had completed one cycle of replication, 3 percent of all molecules were forked open circles with two branch points. Scanning of fraction A shows molecules of various kinds as follows: open circle, 60 percent; twisted circle, 29 percent; linear monomer, 8 percent; forked circle, 3 percent. Some forked circular molecules are shown in Fig. 2. No forked circular molecules were found among 640 molecules observed in the light region of the gradient where unreplicated Col E1 DNA is found.

The total length of the molecules of the type shown in Fig. 2 is always greater than the length of a unit open circular molecule. Two of the three portions of the molecules delimited by the

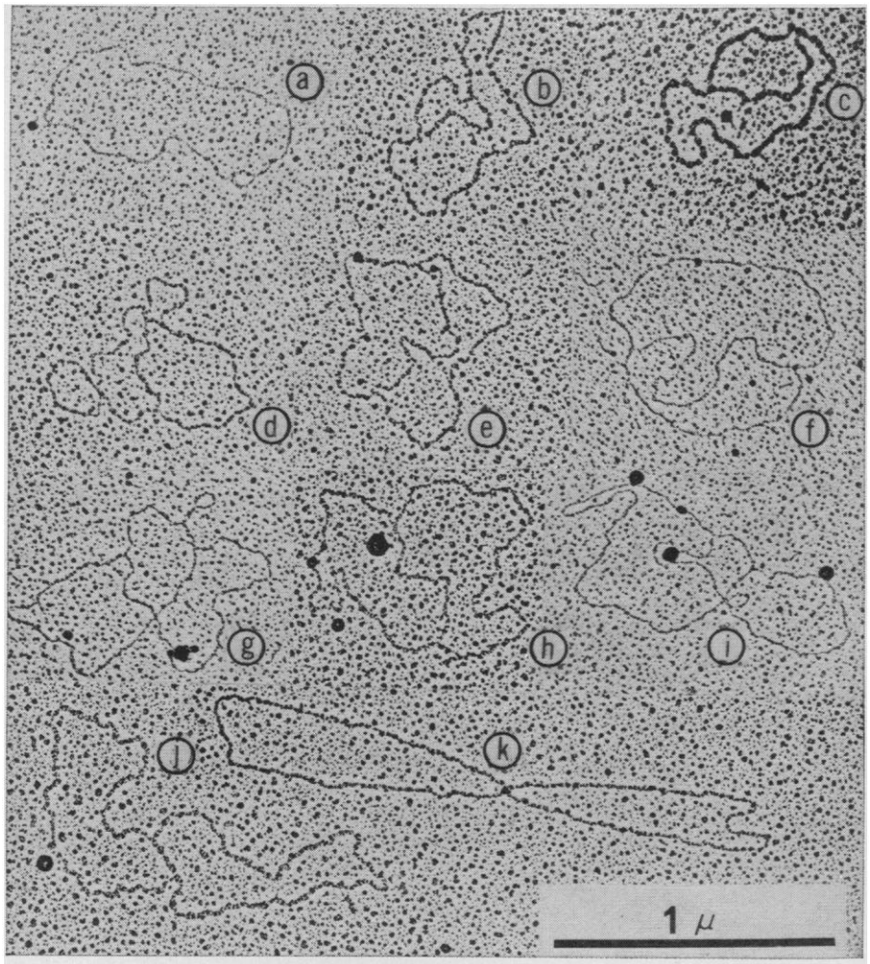

Fig. 2 (top left). Electron micrographs of replicating molecules of Col E1 DNA arranged according to the percentage of replication. As described for Fig. 1, 0.15 ml of DNA solution was mixed with 0.05 ml of 0.04 percent cytochrome c solution and spread on the surface of twice-distilled water. A carbon film was used for a supporting film. The samples were shadow-cast rotationally with platinum-palladium (80 : 20). A Hitachi HS-7S electron microscope was used. Photographs were taken at a magnification of 8100 [magnification standard: latex spheres (Dow Chemical)]. Letters correspond to those in Table 1.

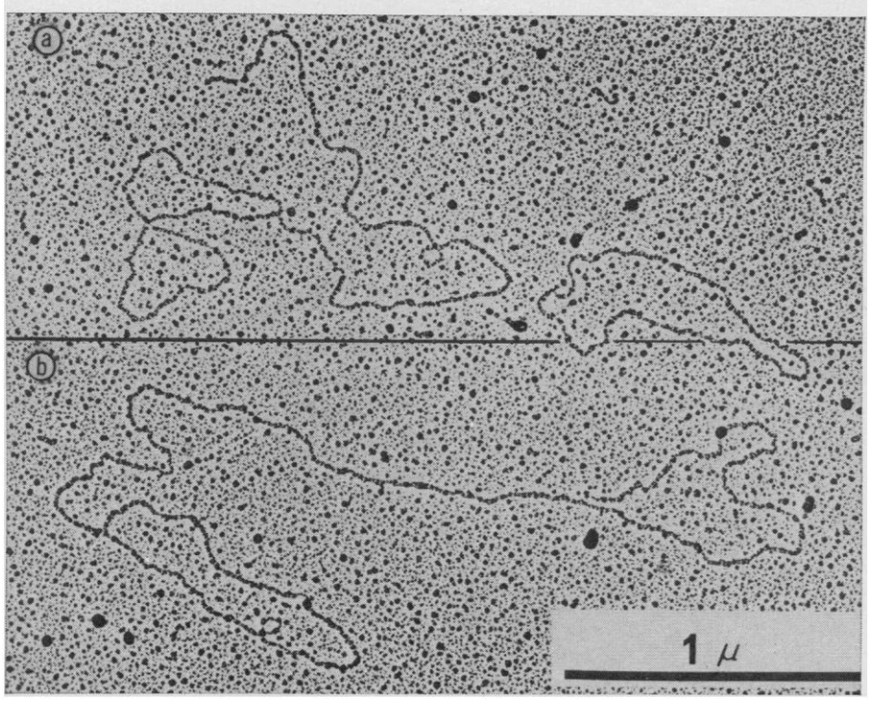

Fig. 3. Electron micrographs of Col E1 DNA molecules which may be "rolling circles" (found in fraction A, Fig. 1). The dimensions of the molecules are (a) a 2.37-μm circle attached to a 2.96-μm linear portion; (b) a 2.37-μm circle (lower left) and an attached 4.76-μm linear portion.

forks were always of almost identical lengths. The sum of the length of one of the equal branches and the unequal branch of these molecules was constant and equal to the unit circumference of a normal open circle. The measurements of 11 molecules shown in Fig. 2 are given in Table 1. These results are taken to indicate that these molecules represent replicating circular forms of Col E1 DNA, the replicated portion being represented by the regions of identical length.

Among the replicating DNA molecules isolated in fractions A and B were a small number of open circular molecules that had one short linear strand of DNA attached. Two molecules in fraction A had an attached linear strand that was significantly longer than the unit open circle (Fig. 3). The possibility that the structures in Fig. 3 are superimposition artifacts seems small, inasmuch as linear molecules exceeding the length of the Col E1 unit circle were rarely observed and the concentration of DNA in the samples was very low. While these structure resemble models proposed for replicating DNA (*10*) it is not now possible to state whether these circles with tails have any role in Col E1 DNA replication. Their presence, however, should be taken into account in the construction of models of DNA replication.

That only three open circular molecules of Col E1 of twice the normal length of the unit circle were seen in samples examined confirms the report of their low incidence in *E. coli* (*11*).

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12. We thank Dr. C. A. Thomas for assistance and comments, and Mrs. E. Jenkins for technical help. Supported by NIH grants AI 08937 (to J.I.) and 2 RO1-AI-0818602 (to Dr. C. A. Thomas).

3 March 1970; revised 2 June 1970 ■

Phenotypic Reversion of Flacca, a Wilty Mutant of Tomato, by Abscisic Acid

Abstract. *The tomato mutant* flacca *wilts rapidly under water deficit because its stomata resist closure. Application of abscisic acid to intact mutant plants changes their morphology toward the phenotype of the control normal variety, Rheinlands Ruhm. The treated mutant plants do not show wilting symptoms, and the resistance to closure of their stomata decreases with hormone treatment.*

Stomatal mechanism is affected by the four groups of plant growth substances. Kinetin, and possibly gibberellin, stimulates stomatal opening (*1*), whereas auxins and abscisic acid induce closure (*2–5*). The concentration of abscisic acid was reported to increase in wilting, detached leaves of wheat (*6*) and in tobacco plants subjected to osmotic root stress (*7*). The authors suggested that endogenous abscisic acid regulates water loss, apparently by reducing stomatal opening.

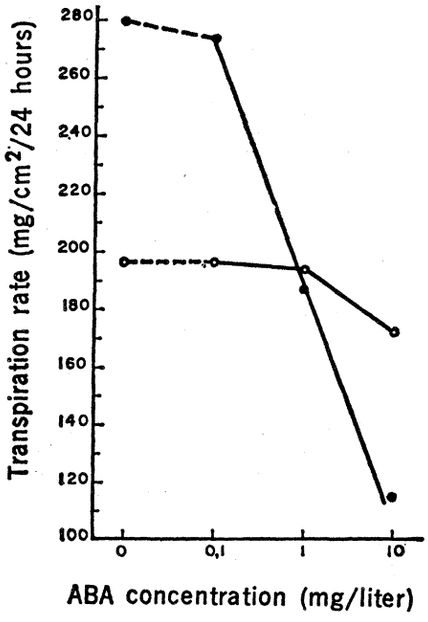

Fig. 1. Water loss from detached leaves from mutant (filled circles) and normal (open circles) plants treated with abscisic acid. Each point represents the mean value of 15 to 20 third leaves from the apex.

We now provide additional support for this suggestion, from results with a wilty mutant of tomato in which the metabolism of abscisic acid appears to be defective (*8*).

The tomato mutant, *flacca* (*flc*), wilts rapidly under water stress due to abnormal stomatal behavior. The stomata of the mutant resist closure under conditions which induce closure in the normal variety, Rheinlands Ruhm (RR), in which the mutation was induced. These conditions include —darkness, wilting, plasmolysis of guard cells, and treatment with phenylmercuric acetate (*9*). The mutant plant is also distinguished from the normal variety by its growth habit; it is much thinner and shorter. In addition, the *flacca* mutant develops, at maturity, symptoms characteristic of auxin excess, namely, strong rooting along the stem, swelling of the upper part of the stem, and epinasty of the leaves (*9*).

When *dl*-abscisic acid (ABA) (*10*) was applied to the mutant seedlings, either by foliage spray (1.0, 10.0 mg/liter) given once a day or in root solution (1.0 mg/liter), a change in the growth habit of the mutant was evident after several days. The mutant plants looked very much like the normal ones in stem thickness and height, and in size, shape, and turgidity of their leaves. In addition, guttation appeared in the morning on the edges of the leaves of the plants treated with ABA, as in normal control plants. Guttation is completely absent in the control mutant plants. Similarly, the symptoms characteristic of an excess of auxin did not appear in those mutant plants sprayed daily with ABA (10.0 mg/liter).

Stomatal opening of mutant and normal plants that were sprayed daily with ABA for 14 days starting from seedling stage was determined indirectly by measuring the transpiration rate of detached leaves (Fig. 1). Cut petioles were inserted in distilled water in sealed beakers, and water loss was determined after 24 hours. Tested *flc* leaves were kept in darkness, since detached mutant leaves of nontreated plants wilt quickly in the light. Those of the normal variety remained in the greenhouse.

The stronger response of *flc* leaves to external ABA as compared with leaves of RR might indicate that the internal concentration of this hormone is much lower than optimum. It should be noted that no full reversion of the mutant phenotype occurred even at 10.0 mg ABA per liter, at least with respect to transpiration rate. Water loss from mutant leaves treated with 10 mg ABA per liter was still higher than that of the control normal leaves when both were put under similar light conditions. When leaves were left in the greenhouse, the mean water loss from ABA-treated *flc* leaves was 256.76 mg/cm²/24 hours, whereas it was 196.76 mg/cm²/24 hours for nontreated RR leaves. Direct observation of stomatal opening (Table 1) with the silicon rubber technique (*3*, *11*) confirmed the above results.

Although the size of stomatal aperture was not measured, it is obvious from the percentages of open stomata that ABA did not change the frequency of open stomata in the light, whereas it induced stomatal closure of *flc* leaves in the dark. It seems, therefore, that ABA in the intact plant ensures normally regulated stomatal behavior.

Three observations indicate that normal stomatal behavior is dependent upon the continuous presence of

Table 1. Percentage of open stomata in leaves of tomato plants. Leaf disks were floated on water for 3 hours either under light (about 33,000 lu/m²) or in the dark before leaf impressions were made.

Variety	Treatment			
	Control		ABA (10 mg/liter)	
	Light	Darkness	Light	Darkness
flc	100	92.48	100	14.56*
RR	100	0.0	100	2.4*

* The stomata were open only to a narrow slit.