*DNA ISOLATED FROM ESCHERICHIA COLI MINICELLS MATED WITH F+ CELLS**

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A mutant of *Escherichia coli* K-12 produces large numbers of small cell-like structures during growth in a variety of media. These "minicells" are enclosed by an apparently normal cell wall and membrane and contain protein and RNA. They do not contain detectable amounts of DNA.[1] We have asked if minicells can act as recipients of DNA during conjugation and, if so, what the properties of the transferred DNA are.

This report establishes that minicells do conjugate with F+ donors and acquire DNA. Properties of the transferred DNA are discussed.

Matsubara[3] suggested that the F episome exists in zygotes as a linear duplex with a molecular weight of 1.0×10^8 daltons. Freifelder and Freifelder[2] showed that, after replication in recipient cells, the F episome exists as a double-stranded circle with a molecular weight of 4.4×10^7 daltons. DNA isolated from minicells mated with F+ donors may represent stages in the conversion of transferred episome to vegetative episome.

Materials and Methods.—(a) *Materials:* DNA was isolated from *E. coli* P678-54, the minicell producing mutant, and χ15 cells by the Marmur procedure.[4] Egg-white lysozyme was purchased from Sigma Chemical Company (St. Louis, Mo.). Pronase was purchased from Calbiochem (Los Angeles, Calif.). Before use, a solution of 20 mg/ml pronase was incubated at 37°C for 60 min to destroy contaminating DNase. DNase was purchased from Worthington Biochemical (New York, N. Y.).

E. coli exonuclease I was a gift from Drs. Paul Sadowski and Jerard Hurwitz. The preparation used in the experiments described in this paper has a specific activity of 1700 units/mg protein and contains 10 mg/ml.[5]

(b) *Organisms and culture methods:* χ15 is a nonlysogenic, F+ prototroph;[6] P678-54 is a minicell-producing F− strain;[1] and χ696 is an F+ minicell producer which is a recombinant of χ15 and P678-54. The growth-and-mating medium was L-broth.[7] When bacteria were labeled with H^3-thymidine, 5 μc/ml of thymidine methyl-H^3 (6 c/mmole, Schwarz BioResearch, Inc., Orangeburg, N. Y.) and 100 μg/ml of adenosine[8] were added to the medium. To label cells with C^{14}-thymidine, 1 μc/ml thymidine-2-C^{14} (51.2 mc/mmole, Schwarz BioResearch) and 200 μg/ml adenosine were added to the medium. All incubations were at 37°C.

(c) *Mating procedure:* With 5 ml of a stationary phase P678-54 culture, 500 ml of L-broth were inoculated and incubated on a rotary shaker for about 3 hr. The culture was then centrifuged at 4000 rpm for 10 min in the GSA rotor of an RC-2B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The minicells were harvested from the supernatant fluid by centrifugation at 8000 rpm in the GSA rotor for 10 min and resuspended in medium. This procedure removes more than 99% of the normal-sized cells. Log phase χ15 cells were inoculated into 50 ml of medium containing H^3- or C^{14}-thymidine at a level of 10^7 cells/ml. After 2-hr incubation in standing cultures, the cells were harvested by centrifugation and resuspended in nonlabeled medium. Mating mixtures contained about 5×10^9 F+ cells and 10^{10} minicells in 10 ml of medium incubated in 125-ml microfernbach flasks without shaking.

(d) *CsCl density gradient centrifugation of DNA:* Reference C^{14} *E. coli* DNA was prepared from χ15 and P678-54 cultures. Since both preparations band at the same density in neutral or in alkaline CsCl, they were used interchangeably as references. Minicells were purified after they were mated with F+ cells and resuspended in 1 ml of buffer (0.1 *M* tris(hydroxymethyl)aminomethane (Tris), pH 8.0 with 0.05 *M* ethylenediaminetetraacetate (EDTA) and 0.2 *M* NaCl). The minicell suspension was lysed by incubation at 50°C for 10 min in 0.67% sodium dodecyl sulfate after prior incubation with 0.3 mg/ml lysozyme at 37°C for 30 min followed by incubation with 1 mg/ml pronase at 50°C for 2 hr. The lysate was then added to

9.4 ml of CsCl solution (saturated at 20°C), and enough water was added to bring the density of the solution to 1.70. For alkaline CsCl gradients, 1 ml of minicell lysate was added to 9.7 ml of saturated CsCl solution and 0.2 ml of 12 *M* CsOH solution, and enough water was added to bring the density to 1.74. The solutions were centrifuged in a Spinco 50 rotor at 33,000 rpm for 72 hr at 25°C,[9] fractions were collected on paper disks (Whatman 3MM, 2.3 cm), and the amount of trichloroacetic acid (TCA)-insoluble label was determined after the disks were washed in 10% TCA and ethanol and the label was counted in a liquid scintillation spectrophotometer. Density was calculated from refractive index measurements. When neutral CsCl gradient centrifugation was used for preparative separation of DNA fractions, the isolated fractions were dialyzed against two changes of 500 vol 0.01 *M* Tris-buffer (pH 8.0) for 48 hr.

(e) *Hydroxyapatite chromatography:* Hydroxyapatite (Hypatite C, Clarkson Chemical Co., Williamsport, Pa.) columns were prepared as described by Bernardi[10] and equilibrated with 0.05 *M* phosphate buffer pH 6.8. Columns (10-ml) were loaded with about 1.5 μg labeled DNA, washed with 50 ml of 0.05 *M* phosphate buffer, and eluted with a linear phosphate gradient (0.05–0.5 *M*). Total volume of eluent was 200 ml and was collected in about 40 fractions. TCA-insoluble label in the fractions was determined by adding carrier bovine serum albumin (final concentrations 1 mg/ml) to chilled fractions, precipitated by adding cold TCA (final concentration 5%). The count retained on Millipore filters was determined in the liquid scintillation spectrometer.

(f) *Fragmentation of DNA by sonic treatment:* H^3-labeled DNA extracted from minicells was mixed with native or denatured C^{14}-labeled *E. coli* DNA in Tris buffer, chilled to 0°C, and treated with the S-125 Branson sonifier (Branson Instrument, Inc., Danbury, Conn.) equipped with the microtip at 2.5 d-c amp for three 10-sec intervals. The solution was cooled in ice slurry during and between intervals of sonication. This treatment caused little or no denaturation as judged by the resistance of the C^{14}-labeled native *E. coli* DNA to digestion by *E. coli* exonuclease I.

(g) *Determination of percentage of hydrolysis of DNA to mononucleotides by E. coli exonuclease I:* Hydrolysis of DNA to mononucleotides was followed by measuring the TCA-insoluble label after incubation with the enzyme. A typical reaction mixture (0.2 ml) contained 1.0 μg DNA and 5 μl enzyme solution, 20 μmole glycine buffer pH 9.5, 0.5 μmole $MgCl_2$, 1 μmole mercaptoethanol, and 2.5 μg sRNA.[5] A control reaction mixture contained the same components except the enzyme. Reaction mixtures were incubated for 60 min at 37°C. Little or no additional hydrolysis is obtained upon further incubation.

Results.—(a) *Transfer of DNA from F⁺ cells to minicells:* Minicells were isolated from cultures of *E. coli* P678-54 by differential centrifugation and then mated with cells of *E. coli* χ15 labeled with H^3-thymidine. Mating was terminated after 30 min by vigorous agitation on a Vortex mixer for 30 sec, and most of the male cells were removed by differential centrifugation. The minicell suspension was further purified by sucrose gradient zonal centrifugation. Figure 1 shows the distribution of the TCA-insoluble label in the gradient. Phase-contrast microscopy shows that the major peak contains the residual F^+ cells and the minor peak consists of pure minicells. The minor peak is absent in experiments in which F^- cells were substituted for F^+ cells and also absent in experiments in which minicells derived from F^+ cultures (χ696) were substituted for minicells derived from F^- cultures (see Fig. 1).[11] The minicells containing TCA-insoluble label were pooled and subjected to another sucrose gradient centrifugation. The label in the minicell suspension could not be removed by repeated washing with Tris, EDTA-NaCl buffer, and the amount of TCA-insoluble label did not decrease after treatment of the intact minicells with DNase.

(b) *CsCl gradient centrifugation of DNA transferred from F⁺ cells to minicells:* Minicells which had been mated with F^+ cells labeled with H^3-thymidine were isolated and lysed together with *E. coli* (χ15) labeled with C^{14}-thymidine. The lysate was subjected to neutral CsCl density gradient centrifugation and the distribution of TCA-insoluble label determined (Fig. 2). The tritium-labeled

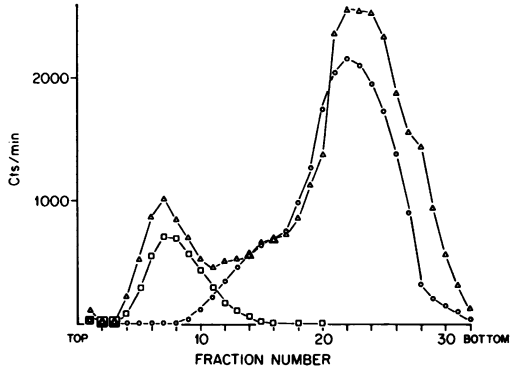

FIG. 1.—Sucrose gradient analysis of minicell suspensions after mating with H^3-labeled χ-15 donor cells. (Δ—Δ) Minicells derived from P678-54; (O-O) minicells derived from from χ696; (□-□) fractions 4–10 of P678-54 minicells subjected to a second sucrose gradient analysis.

DNA separated into two components: one with a buoyant density of 1.708 gm/cm^3 which contained, in different experiments, 20–40 per cent of the total label; and the other with a density of 1.724 gm/cm^3 which contained 60–80 per cent of the total label. When a similar lysate was subjected to alkaline CsCl gradient centrifugation (Fig. 3), the H^3-labeled material banded as a single component at a density of 1.756.

Heavy and light DNA were separated by neutral CsCl gradient centrifugation for denaturation studies. Each fraction was heated to 100°C for 10 min and rapidly cooled in ice; these samples were subjected to neutral CsCl density gradient centrifugation (Fig. 4). The density of the "light" DNA shifted to 1.724. The density of the "heavy" fraction was not affected by this treatment. Alkaline denaturation of both fractions gave results similar to heating and rapid cooling. The results described in this section suggest that the material extracted from minicells after mating with F^+ cells contains both single-stranded and double-stranded DNA.

(*c*) *Hydroxyapatite column chromatography:* The elution pattern of DNA from hydroxyapatite column with phosphate gradient has been shown to depend on its secondary structure rather than on chain length and base ratios.[10] Purified H^3-labeled "heavy" fraction was mixed with native or denatured C^{14}-labeled *E. coli* DNA and chromatographed on an hydroxyapatite column by the use of 0.05–0.5 *M* phosphate gradient. Figure 5 shows that the DNA in the "heavy"

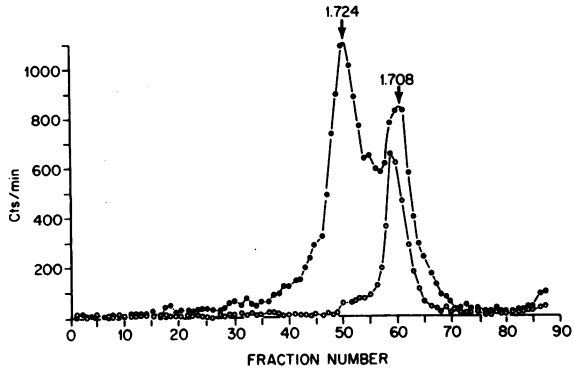

FIG. 2.—Density distribution in neutral CsCl of DNA isolated from minicells mated with H^3-thymidine-labeled χ15 cells (●-●). The lower curve (O-O) is a density marker of C^{14}-labeled χ15 DNA.

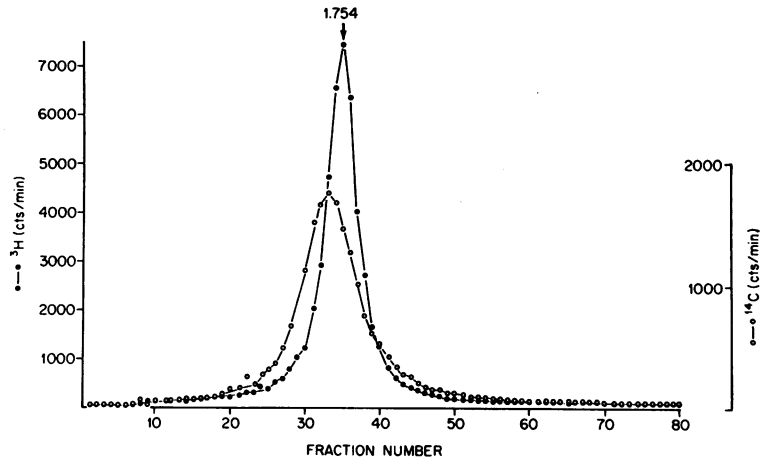

FIG. 3.—Density distribution in alkaline CsCl of DNA isolated from minicells mated with H^3-thymidine-labeled χ15 cells (●–●). The lower curve (O-O) is a density marker of C^{14}-labeled χ15 DNA.

fraction cochromatographs with denatured *E. coli* DNA at approximately 0.17 *M* PO_4 in contrast to native *E. coli* DNA which elutes at 0.23 *M* PO_4. These results further support the idea that the material contained in the "heavy" band consists of single-stranded DNA.

(d) *Sensitivity to E. coli exonuclease I:* Both fractions of the DNA extracted from minicells after mating with F^+ cells were tested for sensitivity to *E. coli* exonuclease I (Table 1). About half the total material associated with the heavy band was digested by exonuclease I. After sonication under conditions which did not increase the sensitivity of native *E. coli* DNA, 90 per cent of the "heavy"

TABLE 1. *Effect of E. coli exonuclease I on DNA extracted from minicells previously mated with F^+ cells.*

	Digestion by Exonuclease I (%)			
DNA preparation	Native	Sonicated	Heat-denatured	Sonicated and heat-denatured
"Heavy" fraction from minicells	44	82	95	97
"Light" fraction from minicells	7	35	90	98
E. coli DNA	<1	<1	80	98

fraction was susceptible to enzymatic digestion. Since exonuclease I is highly specific for single-stranded DNA,[5] these results indicate that at least 90 per cent of this fraction consists of single-stranded DNA with limited hydrogen-bonded structure.

Only 5 per cent of the light fraction is digestible by the enzyme before sonication, but after sonication 30 per cent is digestible. The effect of sonication on the susceptibility of the "light" fraction to digestion by exonuclease I suggests the possibility that it may have some single-stranded regions.

(e) *Synthesis of double-stranded DNA:* DNA which has been transferred soon after the initiation of mating may undergo changes before isolation from the

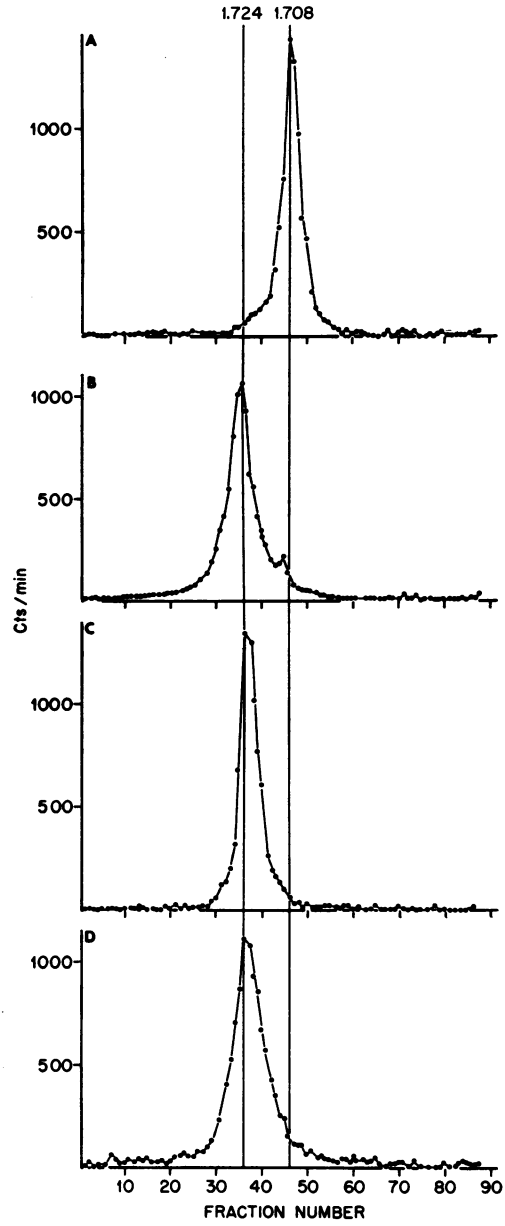

FIG. 4.—Effect of heat denaturation on the buoyant density of DNA from minicells. Neutral CsCl gradients of: (*A*) "light" fraction, (*B*) "light" fraction heated and quick-cooled, (*C*) "heavy" fraction, and (*D*) "heavy" fraction heated and quick-cooled.

minicells. We considered that one of the two fractions present at the time of extraction was transferred during mating and then served as a precursor for the synthesis of the other fraction. To explore this possibility, the following experiment was conducted: Minicells were mated with F^+ cells for 30 minutes, purified by differential centrifugation, and incubated at 37°C on a reciprocal shaker. Samples were taken before and after 30 minutes of incubation, lysed, and analyzed by neutral CsCl density gradient centrifugation. This experiment (Fig. 6)

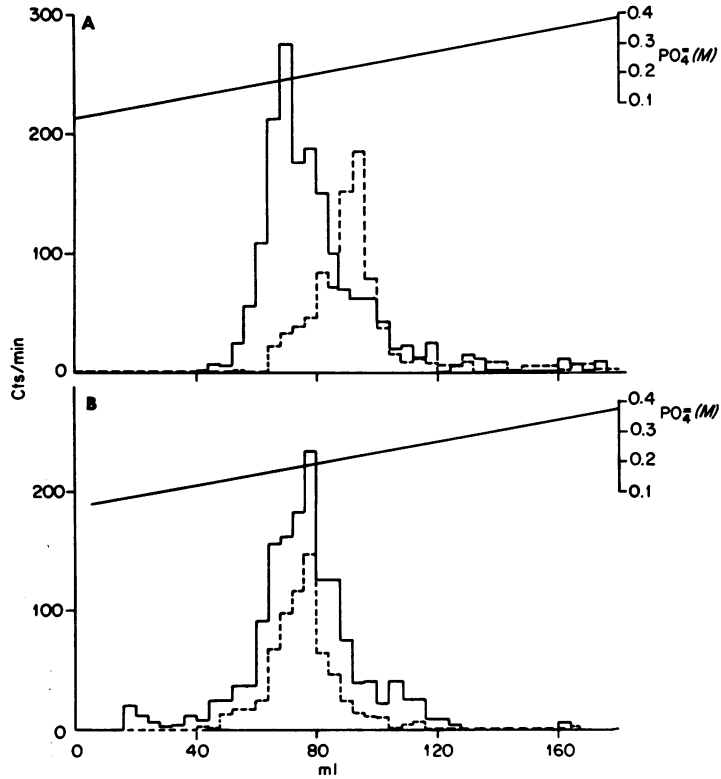

FIG. 5.—Hydroxyapatite chromatography of the "heavy" DNA from minicells with (*A*) native *E. coli* DNA and (*B*) heat-denatured *E. coli* DNA. (——) H^3-labeled DNA extracted from minicells; (----) C^{14}-labeled *E. coli* DNA.

shows that the metabolic activities of the minicells during the postmating incubation result in an increase in the amount of double-stranded material and a decrease in the amount of single-stranded material. In a similar experiment (Fig. 7), minicells were mated with F^+ cells prelabeled with C^{14}-thymidine, purified, and then incubated in the presence of H^3-thymidine. A control incubation mixture contained minicells similarly treated but not mated with F^+ cells. The experiment indicates that primer DNA is necessary for the incorporation of thymidine into TCA-insoluble material in minicells and that most of the label is incorporated into the double-stranded material. The results suggest that the single-stranded DNA present in minicells at the termination of mating serves as a template for synthesis of double-stranded DNA.

Discussion.—Our results show that minicells derived from F^- cultures of *E. coli* K-12 act as recipients for DNA in mating with F^+ cells. The absence of label from minicells incubated with labeled F^- cells (Fig. 1) and the inhibitory effect of nalidixic acid on incorporation of label from F^+ cells to minicells (unpublished results) indicate that the labeled DNA associated with minicells after incubation with F^+ cells was transferred by conjugation. It is very likely that

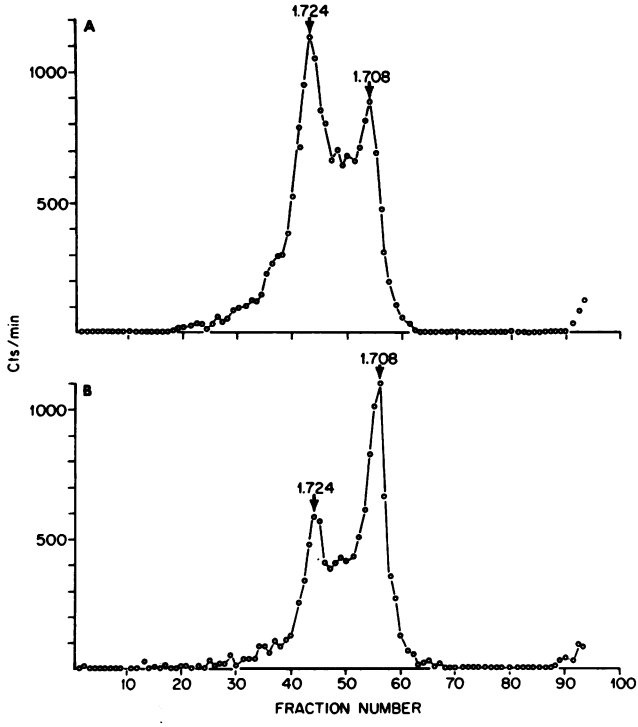

FIG. 6.—Effect of incubation of minicells on the relative amount of single- and double-stranded DNA. Neutral CsCl density gradient centrifugation of (*A*) H^3-labeled DNA extracted from minicells after termination of mating with χ15 cells, and (*B*) DNA extracted from minicells incubated for 30 min after separation from the donors.

this DNA is the F episome since it is the only element transferred at high frequency from F^+ donors.[12–14] The DNA extracted from minicells after mating consists of single-stranded and double-stranded fractions. Since DNA may undergo changes in the minicells, the isolation of single- and double-stranded DNA is consistent with at least two models: (1) DNA is transferred as a single strand and acts as a template for synthesis of the complementary strand; (2) DNA is transferred as a double-stranded structure which separates into single-stranded material.

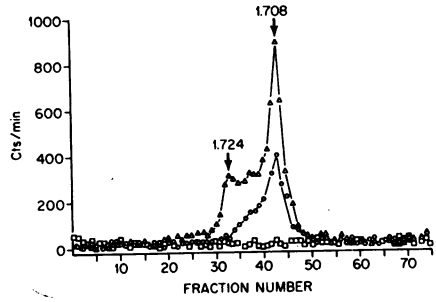

FIG. 7.—Incorporation of H^3-thymidine into minicells after mating with C^{14}-labeled χ15 cells. (Δ-Δ) TCA-insoluble C^{14} counts; (O-O) H^3-TCA-insoluble label incorporated after mating; (□-□) H^3-TCA-insoluble counts incorporated into minicells without mating.

The observation that single-stranded DNA is converted to double-stranded DNA after the termination of mating (Figs. 6 and 7) supports the first model. However, it does not exclude the possibility that part of the DNA is transferred as a double-stranded structure. The transfer of double-stranded DNA during conjugation is suggested by results by Ptashne[15] using F prime donors and by Gross and Caro[16] using Hfr donors. Preliminary experiments with Hfr donors and minicell recipients suggest that at least 95 per cent of the DNA isolated from the minicells is single-stranded.

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