

Segregation into and Replication of Plasmid Deoxyribonucleic Acid in Chromosomeless Segregants of *Escherichia coli*

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The progeny cells of *Escherichia coli* strain P678-54, which normally do not contain deoxyribonucleic acid (DNA), were found to carry DNA when the parental cell carried colicin factor E1 (Col E1). The DNA found in the normally DNA-less segregants was shown to be Col E1 DNA, which is present primarily as a covalently closed circular molecule that can undergo more than one complete cycle of replication.

A variety of mutants of *Escherichia coli* have been isolated that segregate progeny cells which contain neither bacterial deoxyribonucleic acid (DNA; 1, 8, 9) nor the DNA of the episomal fertility factor, F, of *E. coli* (Abstract, Genetics, 56:550, 1967). One such mutant, P678-54 (1), normally segregates very small DNA-less progeny called "minicells," which, although containing normal amounts of many cellular components (1, 6) as do DNA-less segregants of other mutants (10), also show significant deficiencies in three other enzymes that normally interact with DNA: ribonucleic acid (RNA) polymerase, DNA methylase, and photoreactivating enzyme (6). As genetic (5, 13) and physical (2-4, 17) evidence exists which indicates that Col E1 DNA is not associated with the *E. coli* chromosome, an examination of the ability of Col E1 to segregate into and replicate in minicells was undertaken. The aim of this work is ultimately to provide insights into the compartmentation of DNA in bacteria and also to provide a way for examining the replication and expression of a replicon in the absence of the bacterial genome. This communication describes the successful demonstration of the segregation of Col E1 DNA into minicells and its subsequent replication.

MATERIALS AND METHODS

Bacterial and phage strains. Bacterial strains used were *E. coli* K-12 strains P678-54 of Adler (1) and its Col E1-carrying derivative P678-54 (Col E1); YS 40 and Hfr H (Col E1)/E1 obtained from D. Helinski; and W3110 obtained from J. Tomizawa. The colicinogenic derivative of P678-54 was obtained by mixing the recipient with Hfr H (Col E1)/E1, selecting strep-

tomycin-resistant survivors, and testing them for colicin production by using YS 40 as an indicator by the method of Ozeki et al. (14). P1 vir^s was obtained from June Scott (19) and used to prepare radioactive DNA for a sedimentation marker.

Media. TCG (Tris-Casamino Acids-glucose medium) contains 0.1 M tris(hydroxymethyl)amino-methane (Tris; pH 7.4), 0.4% Difco Casamino Acids or vitamin-free Casamino Acids for radioactive labeling, and 0.5% glucose. All TCG medium was supplemented with 20 µg each of threonine and leucine per ml and 1 µg of vitamin B₁ per ml. TCG low phosphate medium contained 5 µg of phosphate per ml and was used to prepare ³²P-phage or bacterial DNA. Saline citrate buffer (SSC) contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.4. TS buffer (Tris-saline) contains 0.05 M Tris and 0.05 M NaCl, pH 8.0. TES buffer is TS buffer that contains 0.005 M ethylenediaminetetraacetic acid (EDTA).

Growth of bacteria and purification of minicells. P678-54 or P678-54 (Col E1) grown overnight in TCG medium was inoculated 1/100 into fresh TCG medium and allowed to grow to about 2 × 10⁸ cells/ml. The cells were pelleted in the cold at 2,500 × g for 15 min in a Sorvall GS-3 head, and the supernatant fluid, which contains minicells, was then pelleted at 13,000 × g for 20 min. The 13,000 × g pellet was suspended in cold sterile TS buffer, washed once, and layered in 1.5 ml of TS buffer on a sterile 25-ml 5 to 20% TS-sucrose gradient. The gradient was spun for 20 min in an International table model centrifuge with a swinging-bucket rotor, and the band of minicells was removed, pelleted, resuspended in cold TS buffer, and rerun on a second sucrose gradient. The minicells obtained from the second banding were washed with TS buffer and used for further study. The final minicell preparations were tested for viable bacteria by plating on nutrient agar and for gross contamination with nonviable cells by staining smears

with methylene blue. A purified 10-ml suspension of minicells derived from a 4-liter culture routinely contained between 200 and 4,000 viable cells per ml and never more than $5\times$ that number after incubation for labeling. No normal-size cells were seen in stained smears in which more than 10,000 minicells were easily seen on a slide. The concentration of minicells was usually about 10^{11} /ml when purified from 4-liter batches.

Radioactive labeling of DNA and its isolation. Cells or purified minicells were incubated with shaking at 37 C in TCG medium that contained vitamin-free Casamino Acids, 250 μ g of deoxyadenosine per ml and, according to the specific experiment described in the legends of the appropriate figure, 0.2 to 1.0 μ g of thymine per ml, 10 μ g of 5-bromouracil per ml, 15 μ g of 5-fluorodeoxyuridine (FUDR) per ml (a gift of Hoffman-La Roche, Inc., Nutley, N.J.), and ^3H -thymidine to a final specific activity of 5 to 10 $\mu\text{C}/\mu\text{g}$ of thymine (New England Nuclear Corp.). Labeled cells were washed four times with cold TES buffer, 40 μ g of cold W3110 DNA per ml was added, and the cells were lysed exactly according to the lysozyme-sarkosyl method of Bazaral and Helinski (2). DNA to be examined by dye-bouyant density centrifugation was not treated further, whereas DNA to be examined by either cesium chloride density centrifugation or sucrose gradient velocity centrifugation was extracted with SSC-saturated phenol by using the slow-rotation method of Frankel (7), dialyzed overnight against cold SSC containing 0.01 M EDTA, and, if necessary, concentrated by using Carbowax 6000 (Union Carbide) before examination. ^{32}P -labeling of phage P1 and the extraction of its DNA were performed by the method of Ikeda and Tomizawa (12), whereas ^{32}P -labeling of W3110 DNA and its purification for use as density marker were performed as described previously by Inselburg (11).

Cesium chloride density gradient centrifugation. A 5-ml solution of cesium chloride of appropriate density prepared by adding DNA in SSC to solid cesium chloride was spun in a Spinco 40 fixed-angle rotor for 44 hr at $81,000 \times g$ at 15 C in a Spinco model L ultracentrifuge. Fractions were collected from the bottom of the tube by drop collection, and densities were determined by refractive index with an Abbe-3L refractometer.

Direct dye-bouyant centrifugation of sarkosyl lysates. Sarkosyl lysates of cells were added to cesium chloride-ethidium bromide solution prepared by the method of Bazaral and Helinski (2), with the exception that all components were reduced to one third and the gradients were centrifuged in a Spinco-65 fixed-angle rotor for 44 hr at $129,000 \times g$ at 20 C in a Spinco model L ultracentrifuge.

Sucrose gradient velocity centrifugation. Sucrose gradient centrifugation was performed in an SW39 swinging-bucket rotor at $74,000 \times g$ at 20 C for 2.5 to 5.5 hr by using a Spinco model L or L2 ultracentrifuge. Fractions were collected from the bottom of the tube by drop collection.

Counting of radioisotopes. Gradient fractions were generally collected onto filter-paper discs, dried, pre-

cipitated with 5% cold trichloroacetic acid, washed with cold absolute ethanol, dried, and counted in scintillation vials containing toluene with both 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]-benzene in a Mark I scintillation counter (Nuclear-Chicago Corp).

Alkali denaturation-renaturation of DNA. Alkali denaturation and renaturation was performed by the method of Roth and Helinski (17).

Deoxyribonuclease treatment of DNA. Limited treatment of DNA by purified pancreatic deoxyribonuclease (Worthington Biochemical Corp.) was performed by the method of Roth and Hayashi (16).

RESULTS

Segregation of Col E1 DNA into minicells. To demonstrate convincingly the segregation of an extrachromosomal DNA into the normally DNA-less minicells produced by strain P678-54, it is necessary (i) to exclude infectious transfer of the DNA, (ii) to distinguish the extrachromosomal DNA from cellular chromosomal DNA, and (iii) to demonstrate an enrichment of extrachromosomal DNA upon enrichment of minicells. It is known that Col E1 behaves as an autonomously replicating element which is not transmitted between F^- cells (5, 13). P678-54 (Col E1) did not transmit Col E1 to any of twenty thousand survivors of *E. coli* W3110 incubated with it in an overnight culture. Helinski and co-workers (2-4, 17) have shown that Col E1 DNA, which exists in *E. coli* as a covalently closed circular monomer with a molecule weight of 4.2×10^6 daltons, could be separated from cellular DNA by virtue of its higher relative density in a cesium chloride-ethidium bromide gradient (15). Taking advantage of this observation, P678-54 (Col E1) cells were grown in the presence of tritiated thymidine for approximately five generations, and either a sample of the whole culture (Fig. 1a) or of the purified minicells of the culture (Fig. 1b) was examined to determine whether DNA banding in the higher density position expected for a covalently closed circular molecule of Col E1 was obtainable and was enriched for in the minicell preparation. The satellite band of relatively denser DNA represents about 0.6% of the total counts in the whole-cell preparation and about 15% in the minicell preparation. No satellite DNA was obtained by using P678-54 cells or minicells as a DNA source.

Since the light DNA peak in (Fig. 1b) could represent either nicked circular Col E1 DNA, linear Col E1, or contaminating bacterial DNA, the DNA isolated from minicells derived from a culture of continuously labeled bacteria was examined by sucrose gradient centrifugation (Fig. 2) to determine whether a significant fraction of it sedimented as Col E1 DNA (2, 3).

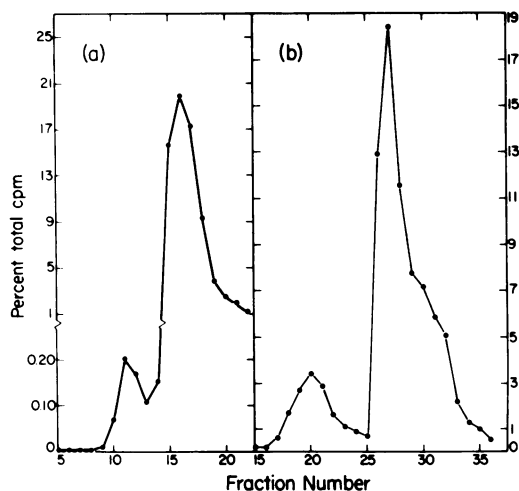


FIG. 1. Cesium chloride-ethidium bromide buoyant density centrifugation of DNA released from cells in a continuously labeled culture. (a) A 30-ml culture of P678-54 (Col E1) or (b) the minicells obtained from 30 ml of the same culture grown in the presence of ^3H -thymidine ($10 \mu\text{g}/\mu\text{g}$ of thymine) for approximately five generations were lysed. The lysate was added directly to a cesium chloride-ethidium bromide solution and centrifuged (see the text). Total fractions collected were (a) 48 and (b) 60. Total counts per minute were (a) 2.24×10^6 and (b) 2,731.

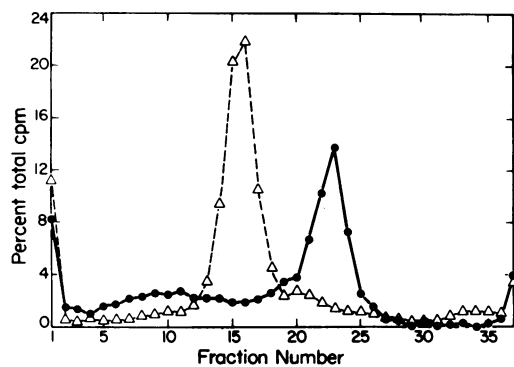


FIG. 2. Sucrose gradient analysis of phenol-extracted DNA isolated from minicells obtained from a continuously labeled culture. A 100-ml culture of P678-54 (Col E1) was grown for approximately five generations in the presence of ^3H -thymidine ($10 \mu\text{g}/\mu\text{g}$ of thymine) in TCG medium. DNA, extracted as described in the text, was concentrated with Carbowax 6000; (a) 0.2-ml amount was layered on a 4.5-ml 5 to 20% sucrose SSC gradient with ^{32}P -P1 DNA used as sedimentation marker and centrifuged at 20 C for 3 hr in a Spinco SW 39 rotor at 30,000 rev/min in a Spinco model L2 ultracentrifuge. Total fractions collected, 36.5. Total counts per minute were 2,502 for ^3H -DNA (●) and 962 for ^{32}P -DNA (Δ). The sedimentation coefficient of the ^3H peak is 26.8S compared to P1 DNA calculated to be 43.4S (19) from the reported molecular weight of 6×10^7 daltons (12).

The DNA obtained from continuously labeled minicells, although sedimenting slightly faster than the value of 23.4S reported for covalently closed circular Col E1 DNA, clearly sediments as if most of it were like the Col E1 DNA. The peak is not present in extracts of minicells derived from noncolicinogenic cells. If the per cent of DNA sedimenting in the P1 marker peak is assumed to represent 100% of the total P1 DNA, then roughly 80% of the ^3H -DNA in Fig. 2 is in what appears to be the Col E1 DNA peak.

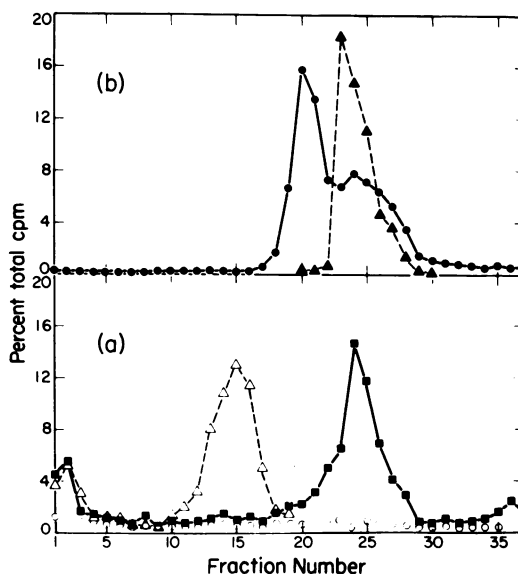


FIG. 3. Synthesis of DNA in minicells derived from P678-54 (Col E1) or P678-54. (a) Minicells from 4 liters of P678-54 (Col E1) grown to 2×10^8 cells/ml were suspended in 10 ml of TCG containing ^3H -thymidine ($5 \mu\text{g}/\mu\text{g}$ of thymine) and incubated for 2 hr at 37 C (viable count before lysis, 2×10^8 /ml). DNA was extracted from 2 ml of minicells with phenol after sarkosyl lysis, dialyzed overnight at 0 C against SSC-0.005 M EDTA, and concentrated with Carbowax 6000 to 0.2 ml. Sucrose gradient centrifugation was performed as described in the legend to Fig. 2. Total fractions collected, 36. Total counts per minute were 1,227 for ^3H -DNA (■) and 1,426 for ^{32}P -DNA (Δ). The sedimentation coefficient of the ^3H -peak is 24.6S. Radioactive materials obtained from P678-54 minicells, grown and treated as those obtained from P678-54 (Col E1), were examined on a different sucrose gradient. The results are superimposed here. Total counts per minute for ^3H -DNA (○), 225. (b) Two milliliters of a 10-ml suspension of minicells, incubated as described in 3a of this figure, was lysed with sarkosyl and centrifuged in a cesium chloride-ethidium bromide density gradient (see the text). ^{32}P W3110 DNA was used as a density reference. Total fractions collected, 50. Total counts per minute were 14,246 for ^3H -DNA (●) and 2,678 for ^{32}P -DNA (Δ). Viable count in minicell preparation before lysis, 2×10^4 .

Replication of DNA in P678-54 (Col E1) minicells and its characterization. Exposure of purified minicell preparations to ^3H -thymidine for from 10 min to 3 hr, with or without either chloramphenicol or mitomycin C present, leads to the labeling of DNA which usually sedimented as a 24.6S peak (Fig. 3a). In comparable preparations of minicells derived from P678-54, no significant DNA synthesis was found. Where

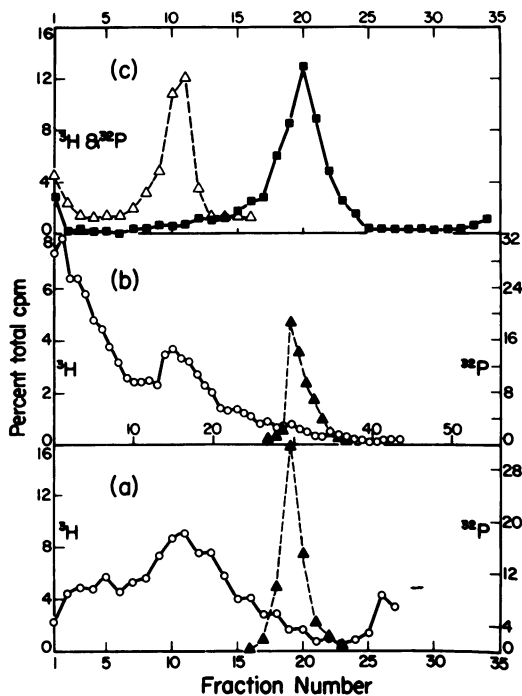


FIG. 4. Bromouracil density labeling of DNA in minicells. Minicells from 4 liters of P678-54 (Col E1) cells were purified (viable count less than 1,000/ml) and suspended in supplemented TCG containing 10 μg of 5-bromouracil per ml, 15 μg of FUDR per ml to inhibit the possible activity of thymidylate synthetase, 250 μg of deoxyadenosine per ml, 5 μC of ^3H -thymidine/ μg of 5-bromouracil, and shaken in the dark at 37 C for 3 hr. Samples were removed at 1 and 3 hr and lysed, and the DNA was extracted with phenol and dialyzed against SSC-EDTA. Figures 4a and b represent DNA from the 1- and 3-hr samples, respectively, banded in cesium chloride density gradients. ^{32}P -W3110-DNA was used as a density marker. Total counts per minute: (a) ^3H -DNA (\circ), 4,167 and ^{32}P -DNA (\blacktriangle), 2,124; (b) ^3H -DNA (\circ), 11,504 and ^{32}P -DNA (\blacktriangle), 2,060. Graph c represents a sample of the DNA used in the density gradient (b) which was examined in a sucrose density gradient as previously described in Fig. 2. ^{32}P -P1 marker DNA was used as a sedimentation marker. Total fractions collected, 34. Total counts per minute were 4,814 for ^3H -DNA (\blacksquare) and 6,562 for ^{32}P -DNA (\blacktriangle). The ^3H peak sediments at the 25S position.

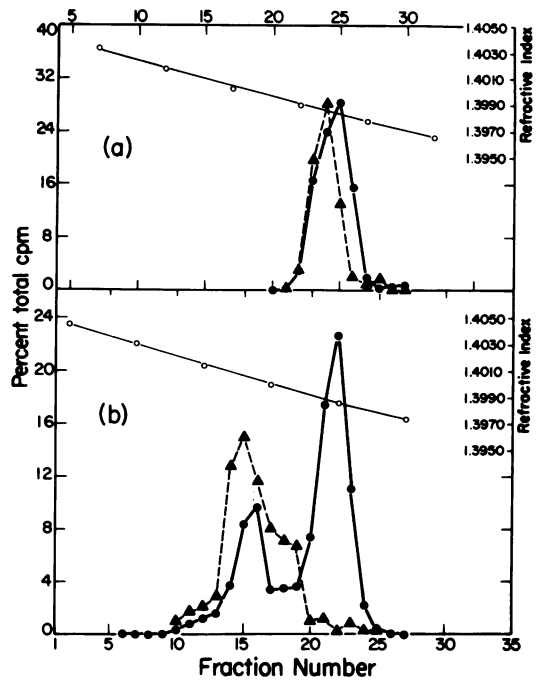


FIG. 5. The denaturation and renaturation of DNA synthesized in minicells. ^3H -DNA, obtained from minicells labeled with ^3H thymidine (5 $\mu\text{C}/\mu\text{g}$ of thymine) for 2 hr, was mixed with ^{32}P labeled W3110 bacterial DNA, dialyzed against 0.7 M NaCl and 0.05 M Na_2HPO_4 (pH 7.0), and divided into two portions: (a) no further treatment; (b) DNA alkali denatured and renatured by being brought to pH 12.2 with 0.1 N NaOH, held for 9 min at 0 C, and then brought to pH 8.5 with a solution containing 0.3 N HCl and 0.7 M Tris-hydrochloride. Both samples were banded in cesium chloride density gradients. The density marker was ^{32}P -W3110 DNA. Total counts per minute: (a) ^3H -DNA (\bullet), 1,268 and ^{32}P -DNA (\blacktriangle), 400; (b) ^3H -DNA (\bullet), 2,137 and ^{32}P -DNA (\blacktriangle), 374.

limited amounts of synthesis were seen, the DNA was found not to give the 24.6S peak found in the colicinogenic strain (Fig. 3a). In several experiments in which induction of increased Col E1 DNA synthesis by mitomycin C was looked for, it was not found. The failure to increase Col E1 DNA synthesis was probably not due to impermeability to mitomycin C, as colicin production was inducible in the P678-54 (Col E1) cells.

The newly replicated DNA from minicell preparations when analyzed by cesium chloride-ethidium bromide density gradient centrifugation gives two peaks, the denser one generally containing between 30 and 60% of the total counts in the gradient (Fig. 3b). The newly replicated DNA sediments in a manner similar to that described for the covalently closed circular molecules of Col E1 (2, 3). Other experiments in this

laboratory and a report of Clewell and Helinski (4) indicate that, during dye-bouyant density centrifugation, a conversion occurs in the Col E1 DNA from a supercoiled to a nonsupercoiled state.

Replication of DNA that has been introduced into minicells via conjugation has been reported (6); however, that, replication was only shown to involve one strand of a linear duplex molecule. That the replication of DNA demonstrated in Fig. 3 was not simply a DNA repair or strand completion process is shown in Fig. 4a and b, in which a density shift of replicating DNA through a half-heavy to a heavy position in 5-bromouracil medium is observed. That most of the replicated

DNA does in fact represent the Col E1 DNA can be seen from the sedimentation properties in Fig. 4c of a sample of the DNA examined in Fig. 4b.

Two demonstrations that the replicated DNA obtained from minicells is primarily in a twisted circular form are shown in Fig. 5 and 6. Figure 5 shows that, in a mixture of ^{32}P -labeled bacterial and tritiated minicell DNA, all the bacterial DNA is denatured during alkali denaturation-renaturation as demonstrated by an 0.017 g/cm^3 increase in density, whereas about 75% of the ^3H -DNA resists denaturation as would be expected of covalently closed circular molecules. Figure 6 shows the conversion with linear kinetics of the 24S form of replicated DNA isolated from minicells to an 18S form upon limited digestion with pancreatic deoxyribonuclease. This conversion is identical to that reported by Bazaral and Helinski for the conversion of covalently closed twisted circular Col E1 DNA to the open circular form (3).

DISCUSSION

From the results reported, it seems reasonable to conclude that Col E1 DNA is not prevented from segregating to otherwise DNA-less bacteria and can replicate normally in those cells in which it also exists primarily as a covalently closed circular molecule. The nature of the differences between the extrachromosomal state of the F factor and Col E1 DNA that causes their different segregation behavior remains to be seen.

The finding that extrachromosomal DNA can enter minicells by segregation suggests that DNA-less bacteria may be useful in examining a variety of extrachromosomal genetic elements.

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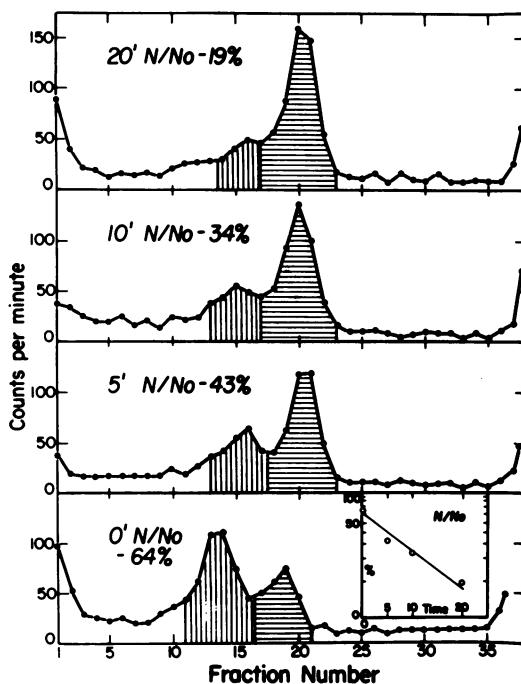


FIG. 6. Conversion of 24S to 18S (Col E1) DNA after exposure to pancreatic deoxyribonuclease. DNA extracted from P 678-54 (Col E1) minicells labeled with ^3H -thymidine ($5 \mu\text{g}/\mu\text{g}$ of thymine) for 2 hr was dialyzed against 0.03 M Tris and 0.01 M MgCl (pH 7.3) in the cold. To a 1.3-ml sample of that DNA, $0.001 \mu\text{g}$ of pancreatic deoxyribonuclease was added. The reaction occurred at 25 C , and 0.3-ml portions were removed at indicated intervals and added to an 0.05-ml solution containing 0.15 M NaCl and 0.3 M EDTA. A 0.3-ml amount of each sample was then layered on a 4.4 ml-5 to 20% sucrose-SSC gradient and centrifuged for 5.5 hr in an SW 39 rotor at 20 C and at $74,000 \times g$ in a Spinco model L2 ultracentrifuge. The inset scale represents a plot of the fraction of 24S DNA that has survived deoxyribonuclease degradation (N/No) versus time of enzyme exposure.

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