JOURNAL OF BACTERIOLOGY, Oct. 1971, p. 300-308
Vol. 108, No. 1
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Physical and Functional Characteristics of R-Factor Deoxyribonucleic Acid Segregated into *Escherichia coli* Minicells[1]

STUART B. LEVY[2]

Laboratory of Biochemical Pharmacology, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland 20014; Blood Research Laboratory, New England Medical Center Hospitals, Boston, Massachusetts 02111; and the Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111

Received for publication 6 July 1971

Both fi^+ and fi^- type R factors function as intact replicative units when segregated into *Escherichia coli* minicells. Hybridization studies demonstrate that at least 95% of the deoxyribonucleic acid (DNA) in R^+ minicells is episomal in origin. About half of the DNA can be extracted in a closed circular form and about 75% of the DNA is membrane associated. DNA, ribonucleic acid, and protein synthesis proceeds in R^+ minicells in contrast to R^- minicell controls. The system offers a unique opportunity to study a relatively small replicative unit in a native cell environment and a simple means of isolating large quantities of episomal DNA.

In bacteria, cell activity may be dictated by extrachromosomal deoxyribonucleic acid (DNA) elements and chromosomal DNA. These elements, called episomes or plasmids, are replicated with chromosomal DNA and may act as sex factors enabling one bacterium to transfer genetic material to another. Resistance transfer factors (R factors; 22, 28) are such plasmids. Since these episomes represent only 1 to 2% of the total cell DNA, it has been difficult to study them in a large background of chromosomal DNA.

To circumvent this problem, R factors were introduced into an *Escherichia coli* mutant which produces minicells (2) during abnormal cell division. R factors residing in such a mutant segregate into the minicells which normally have no other DNA (S. B. Levy, Abstr. 10th Int. Congr. Microbiol., p. 60, 1970; 14, 19, 20), from which subsequent R-factor transfer to normal cells is as efficient as cell-to-cell transfer (19). Episomal segregation into minicells seems to be a general phenomenon. Kass and Yarmolinsky (15) have shown segregation and minicell transfer of the F′ gal sex factor. Inselburg has reported that colicin E1 will segregate into minicells and replicate there (13).

[1] Part of this work was presented at the Xth International Congress of Microbiology, Mexico City, Mexico, 9-15 August 1970.

[2] Present address: Blood Research Laboratory, New England Medical Center Hospitals, Boston, Mass. 02111.

We report here physical and functional characteristics of the DNA segregated into the minicells of R^+ minicell-producing strains. Analysis of the DNA in these minicells indicates that it is at least 95% pure R-factor DNA and at least half of it exists as a covalent circular molecule. This evidence supports preliminary studies which suggested that more than one R factor existed in a minicell (19). R^+ minicells will incorporate exogenous precursors into DNA, ribonucleic acid (RNA), and protein in contrast to R^- controls.

MATERIALS AND METHODS

Strains. *E. coli* minicell-producing strains, χ925 and χ984, were obtained from R. Curtiss, Oak Ridge National Laboratory. These were mated with other *E. coli* strains containing R factors to produce minicell strains containing R factors (Table 1). Rifampin-resistant strains were obtained by selecting resistant colonies from Penassay agar plates supplemented with 100 μg of rifampin (Ciba) per ml.

Media. L broth (17) was the standard growth medium. Synthetic media were prepared by using minimal salts media (9) to which appropriate nutrients were added.

Isotopes. Thymidine-*methyl*-^3H (19.5 Ci/mmole) and L-leucine-*4,5*-^3H (N) (58.2 Ci/mmole) were obtained from the New England Nuclear Corp. Uracil-*2*-^{14}C (54.8 Ci/mmole) and thymidine-*2*-^{14}C (47.9 Ci/mmole) were obtained from Schwarz BioResearch, Inc.

Liquid scintillation fluids. The following liquid scintillation fluids were used: (A) toluene, 3.8 liters; 2,5-

TABLE 1. *Bacterial strains*[a]

Escherichia coli strains	fi Type	R factor	Relevant characteristics	Origin	Source
χ925			Sm^R minicell producer	Adler P678-54 (reference 2)	R. Curtiss
χ984			Sm^R minicell producer	χ925	R. Curtiss
D1-1	+	222	$Cm^RSm^RSu^RTc^R$	CSH-2(222)	T. Watanabe
D1-7	+	222	$Cm^RSm^RSu^RTc^R$ minicell producer	D1-1 × χ984	
D1-9	+	R1-19 drd	$Cm^RKm^RAp^RSm^RSu^R$	Meynell strain RC 709 (reference 21)	A. Laskin
D1-12	+	R1-19 drd	$Cm^RKm^RAp^RSm^RSu^R$ minicell producer	D1-9 × χ984	
D2-2	−	64-11 drd	Sm^RTc^R	Meynell 2395 (reference 21)	R. Curtiss
D2-4	−	64-11 drd	Sm^RTc^R minicell producer	D2-2 × χ925	
D2-7	−	N-3	$Tc^RSu^RSm^R$	LT2(N-3) × CSH-2	
D2-9	−	N-3	$Tc^RSu^RSm^R$ minicell producer	D2-7 × χ984	

[a] All strains are F^-; drd, derepressed; Sm, streptomycin; Cm, chloramphenicol; Su, sulfonamide; Tc, tetracycline; Km, kanamycin; and Ap, ampicillin; fi, fertility inhibition (28).

diphenyloxazole (PPO), 16 g; and *p*-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP), 0.8 g; (B) Triton X-100, 1 liter; toluene, 1 liter; water, 224 ml; POPOP, 0.450 g; and POP, 16.5 g.

Minicell isolation. For analysis of the DNA, minicells were harvested by differential centrifugation and two sucrose gradients (2, 19). For the studies of macromolecular synthesis, minicells were purified further by substituting a penicillin treatment (18) for the second sucrose gradient. These highly purified preparations generally contained less than 1,000 contaminating bacterial cells per 10^{10} minicells. An abbreviated purification may also be used. After one sucrose gradient, the syntheses can be performed in the presence of penicillin (1500 units/ml) since any growing cells are sensitive and lyse, whereas minicells are resistant (18). The penicillin has no detrimental effect on DNA, RNA, or protein syntheses.

Isolation of labeled DNA. Cell DNA was labeled by growing a culture in L broth in the presence of thymidine-*methyl*-^3H (2.5 μCi/ml) and deoxyadenosine (250 μg/ml; 5, 19). The DNA was prepared according to the method of Berns and Thomas (3). The cells or minicells were washed twice in standard saline citrate [SSC (0.15 M sodium chloride-0.015 M sodium citrate)] and suspended in 27% sucrose solution in SSC. The preparations were lysed with 0.5% sodium dodecyl sulfate and heated for 10 min at 60 C. They were subsequently incubated overnight with Pronase (Calbiochem) at a final concentration of 1 mg/ml. These DNA preparations were then used for analysis of minicell DNA structure (*see below*). Care was taken to minimize shearing the preparations by using large-bore pipettes. The fact that all the acid-insoluble ^{3}H-thymidine was in DNA was confirmed by its resistance to Pronase, ribonuclease (RNase A), and T_1 (Worthington Biochemical Corporation) and its sensitivity to deoxyribonuclease (DNase I, Worthington Biochemical Corp.).

Isopycnic density gradient centrifugation. Cesium chloride gradients (4.5 ml) were prepared in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 6.8) at an average density of approximately 1.7 g/ml (refractive index of 1.4010). Samples of DNA preparations were centrifuged in a fixed-angle no. 40 rotor of a preparative ultracentrifuge (Spinco, model L-2) for 72 hr at 33,000 rev/min at 20 C. Fractions (0.2 ml) were collected from the bottom of the tube and counted in liquid scintillation fluid B in a Packard Tri-Carb Counter (19).

Alkaline sucrose gradient centrifugation. Alkaline 5 to 20% sucrose gradients (4.5 ml) were prepared in 1.0 N NaCl, 0.3 M NaOH, and 0.01 M ethylenediaminetetraacetic acid (EDTA) and adjusted to *p*H 12.5. Spinco polyallomer tubes were used to prevent surface sticking by DNA samples, and about 0.1% sarcosyl was added to allow even flow in the preparation of the gradient in these tubes. Samples were centrifuged in an SW no. 50 rotor in an ultracentrifuge (Spinco, model L-2) at 38,000 rev/min for 30 min at 23 C. Gradients were collected as drops from the bottom of the centrifuge onto Whatman no. 3MM paper discs pretreated with trichloroacetic acid and unlabeled thymidine. These discs were washed twice in cold 5% trichloroacetic acid, twice in ethanol, and then dried before counting (19).

DNA-DNA hybridization. These studies were performed for us by P. Guerry and S. Falkow by using hydroxyapatite chromatography (4, 6, 26).

Membrane binding of R-factor DNA. The degree of membrane bound R-factor DNA in the minicell was examined by using the M band technique (27). Cells and minicells containing radioactive labeled DNA (19) were separated, washed twice in 0.05 M Tris-hydrochloride (*p*H 8.0), and suspended at the same optical density in 1 ml of 10% sucrose in the same Tris buffer. EDTA was added to each to a final concentration of 3 × 10^{-4} M, followed 30 sec later by lysozyme (100

$\mu g/ml$). After 8 min at room temperature, $MgCl_2$ was added to 6×10^{-4} M to neutralize the EDTA. The samples were placed in ice, 0.5-ml samples were carefully layered on top of a 15 over 47% sucrose step gradient, and the preparation was then lysed by gently mixing with 0.15 ml of 5% sarcosyl and 0.1 M $MgCl_2$. Centrifugation and collection of M bands were as previously described (27).

Preparation of labeled protein. R^+ minicells were incubated in the presence of ^3H-leucine. The minicells were washed free of labeled media, suspended in 1 to 2 ml of 0.05 M Tris-hydrochloride buffer (*p*H 8.0) with 10^{-3} M EDTA, and lysed by sonic treatment in ice, using the Branson 20-kc sonifier at maximum output on no. 5 setting. The membranes and unlysed minicells were removed by low-speed centrifugation (1,500 × *g* for 10 min), and the proteins were separated from free ^3H-leucine by passage over Sephadex G75 or G100 equilibrated with 0.01 M phosphate buffer (*p*H 7.0).

Disc-gel electrophoresis. Analytic disc-gel electrophoreses were performed for us by L. D. Kohn in standard 7% gels at *p*H 9.5 (Canal Industrial Corp., Rockville, Md.). After electrophoreses, the gels were cut into 1.5-mm pieces and counted in liquid scintillation fluid after first solubilizing with 30% hydrogen peroxide (30).

RESULTS

Characterization of minicell DNA. Our previous studies suggested that more than one episome could be found in a minicell. The total DNA in the minicell represented more than 2% of bacterial DNA, and transfer was highly efficient (19). We assumed that all of the DNA was episomal DNA, but there remained the possibility that some pieces of bacterial DNA had segregated.

Hybridization studies were used to answer the question directly (Table 2). Over 84% of ^3H-labeled DNA extracted from D1-7 minicells hybridized with unlabeled DNA from *E. coli* harboring the R factor 222. Less than 4% of the same DNA hybridized with *E. coli* K-12 with no R factor. In terms of relative binding, these data indicated that at least 95% of the minicell DNA was episomal in origin. Hybridization of 222 DNA to the same *E. coli* strain containing a different R factor (N-3) showed about 15% binding relative to *E. coli* K-12 (222). This finding demonstrates the specificity of the binding for R factor 222 and suggests a greater homology between R factor N-3 and R factor 222 than between the latter and host chromosomal DNA.

Certain R factors, including 222, when transferred from *E. coli* into *Proteus*, separate into three distinct DNA species with different molecular weights and different buoyant densities in CsCl (10, 23, 25). To determine whether R factors segregated into minicells behave in a similar way, the buoyant density of minicell DNA in CsCl was examined. DNA preparations of different classes of R factors (F-type and I-type; 22) from minicells were examined. Only one peak of DNA was found for each (Fig. 1). The density of R factor 222 (fi^+, F-type) DNA is identical with bacterial DNA (Fig. 1A); R factor 64-11 (fi^-, I-type), however, is less dense than bacterial DNA. This latter result is consistent with the lighter density for some I-type R factors (Falkow, *personal communication*). These findings indicate that, in the minicell, R factors do not separate into different density classes as in *Proteus*. However, the possibility that there are DNA species with different weights but with the same buoyant density can still not be excluded.

In alkaline sucrose, covalently circular DNA sediments three to four times faster than open circular and linear DNA (11, 26). Analysis of DNA from minicells showed that over 45% of the label from R factor 222 (Fig. 2A) sedimented in a single peak three times faster than the rest of the DNA. Identical results were found with R factor N-3 in minicells (20). These findings mirror those reported by Silver and Falkow (26) who studied R factors in *E. coli* cells, and indicate the presence of a high percentage of covalently circular DNA molecules. This faster DNA peak is found in the total DNA extracted from whole cells of the mother R^+ minicell strain (D1-7) but it is not seen in the whole cells of the R^- minicell strain (χ984; Fig. 2B). Although only half of the minicell DNA is seen as closed covalent circles, the presence of a single sharp peak suggests that only one molecular-weight class exists.

The degree of binding of the minicell DNA to membranes was examined by the M band technique (27; Table 3). About 60 to 80% of the episomal DNA and over 90% of the cell DNA binds with the membrane-sarkosyl-magnesium crystals in the middle of the sucrose gradient. In cells in which both DNA and protein were labeled, about 30% of the total cell protein and 45% of the minicell protein were found associated with the DNA in the M band. In these experiments over 90% lysis of the preparations was achieved. Control samples using exogenous added labeled DNA at the time of lysis showed less than 4% binding to M bands. Whole unlysed minicells added to the gradients before centrifugation sedimented to the bottom. EDTA at 10^{-3} will give M bands, but the binding of DNA from both cells and minicells is greatly reduced. These findings rule against a nonspecific trapping of free DNA or whole minicells in the M band.

Macromolecular synthesis. The ability of R^+ minicells to incorporate exogenous precursors into DNA, RNA, and protein was examined

using radioactive labeled thymidine, uracil, and leucine or proline. Highly purified preparations of R^+ and R^- minicells were incubated with the labeled precursors. Only the R^+ minicells showed significant incorporation (Table 4, Fig. 3 and 4).

The rate of ^{3}H-thymidine incorporation into DNA per minicell (Fig. 3) represented 0.1 to 0.5% of the incorporation by a viable cell. To determine whether a significant amount of breakdown and repair of R-factor DNA was occurring in the minicell, R^+ minicells were prelabeled with ^{3}H-thymidine overnight or for 2 hr, washed, suspended, and incubated for 2 hr in the presence of exogenous unlabeled thymidine. In neither instance was a loss of counts noted, indicating stability of the DNA in the minicell (Fig. 3). Furthermore, minicells containing R factors can be mated twice with no loss of transfer efficiency, suggesting replication of the episome at least once (*unpublished data*).

RNA synthesis was studied in long-term and short-term labeling experiments (Table 4, Fig. 5). In both cases, minimal, if any, incorporation of ^{14}C- or ^{3}H-labeled uracil was found in R^- minicells. Table 4 shows the results of a 10-min labeling of both R^+ and R^- cells and minicells in which the RNA was extracted. Further characterization of this RNA will be discussed in a later communication. In general, there is 2 to 5% incorporation of the radioactive uracil per minicell as compared with a viable cell. In Fig. 5, the sensitivity of this uracil incorporation to rifampin, an inhibitor of RNA polymerase, is demonstrated.

Incorporation of labeled amino acids into proteins per minicell was about 2 to 5% of that found incorporated by a viable cell. The R^- controls repeatedly showed some, although minimal, amino acid incorporation. This incorporation was greatly reduced and often lost if the minicells were prepared at 0 C instead of at 23 C (18). To evaluate the contribution of even a small number of contaminating cells to the total incorporation by purified minicells, cells at a known viable count were added to a minicell preparation. The additional incorporation by the minicell preparation could be accounted for by this known quantity of added cells, and the incorporation per cell was the same whether in the presence or absence of minicells. It was thus determined that preparations of at least 10^4 minicells per viable cell would show no significant contribution by cell contamination. Protein synthesis in minicells was examined in the presence of chloramphenicol, rifampin, or penicillin (Fig. 4). Chloramphenicol did not inhibit protein synthesis in minicells from strain D1-7 (R factor 222) which have the chloramphenicol-acetylating enzyme (Fig. 4). The drug did depress protein synthesis in minicells from chloramphenicol-sensitive strains D2-4 and D2-9. Rifampin, added at least 10 min before the labeled amino acids, inhibited protein synthesis in all three R^+ minicells through its effect on the RNA polymerase (29). No effect on protein synthesis was noted by penicillin at 1,000 or 1,500 units/ml.

TABLE 2. *DNA-DNA hybridization of DNA extracted from* R^+ *(222) minicells*[a]

Source of unlabeled DNA	Relative binding of DNA from minicells (%)
Escherichia coli K-12 (222)	100[b]
E. coli K-12 (N-3)	15.4
E. coli K-12	4.6
None	0.8

[a] Study performed by P. Guerry and S. Falkow. ^{3}H-labeled DNA isolated from D1-7 minicells was diluted into 0.14 M phosphate buffer at *p*H 6.8. The preparation was sheared by sonic treatment to an approximate molecular weight of 250,000 daltons and heated at 100 C for 10 min in the presence of about a 1000-fold excess of unlabeled cell DNA. The denatured mixture was then incubated at 75 C for 16 hr. The DNA solutions were then transferred to hydroxyapatite columns equilibrated at 60 C. The column was washed with 100 ml of 0.14 M phosphate to remove all single stranded DNA. The reassociated DNA was eluted from the column at 95 C in 0.4 M phosphate.

[b] The absolute binding of labeled minicell DNA to unlabeled DNA from *E. coli* K-12 (222) was 84.4%.

The presence of multiple proteins was demonstrated by disc-gel electrophoresis (Fig. 4) of the soluble proteins synthesized. At least 15 different protein bands can be detected, indicating a wide distribution of label. Further purification of these proteins is being undertaken.

Origin of minicell RNA polymerase. The R^- minicells lack the RNA polymerase (7) which may explain why they cannot synthesize RNA and protein. The R^+ minicell is able to perform these functions which can be shown to be sensitive to rifampin, a specific inhibitor of the RNA polymerase (Fig. 4, 5A; reference 12). The RNA polymerase is found in the minicell which has received the episome. In several different experiments, the origin of the polymerase was examined. It appears to be bacterial in origin. Rifampin-resistant mutants were isolated from D1-7 and χ984. In the former, minicells from four different rifampin-resistant colonies were isolated and examined for RNA and protein synthesis in the presence and absence of rifampin (Fig. 5B). In all cases, both cells and minicells were equally resistant to the drug.

To examine further the possibility that the R factor might be determining the synthesis of its own RNA polymerase, R factors from the ri-

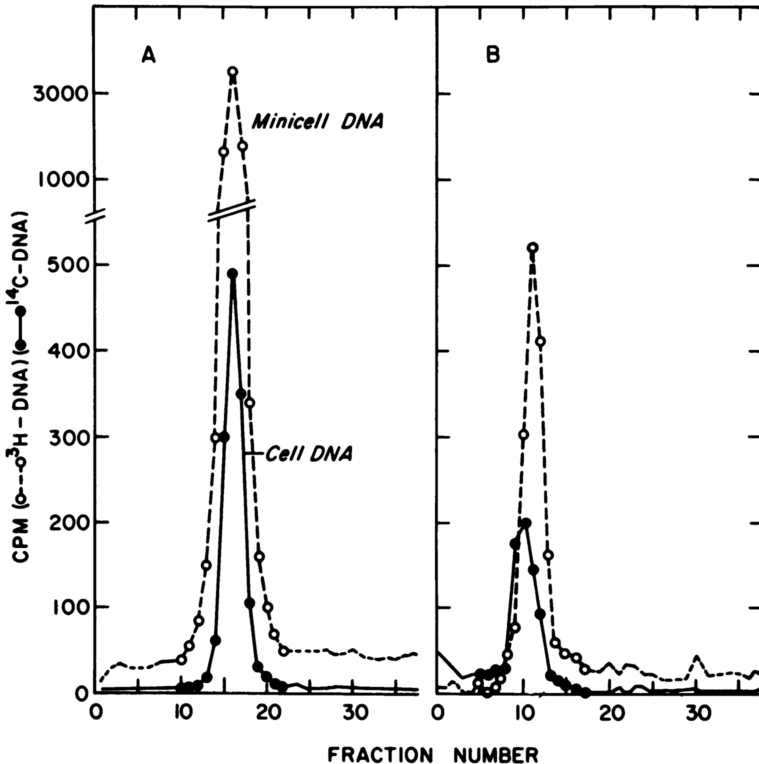

FIG. 1. *Isopycnic centrifugation of minicell DNA. The DNA in 200-ml cultures of E. coli D1-7 and D2-4 was labeled with thymidine-methyl-³H. The minicells were isolated, and the DNA was extracted as described in the text. A 10-ml culture of χ984 was labeled with thymidine-2-¹⁴C, and the DNA was extracted and treated similarly. DNA from either D1-7 (A) or D2-4 (B) minicells was mixed with DNA from χ984 and centrifuged to equilibrium in CsCl. Samples (0.2 ml) were collected from the bottom and counted.*

fampin-resistant D1-7 minicells were transferred by conjugation from minicells into a rifampin-sensitive minicell strain (χ925 which was made T4 resistant). The R$^+$ minicells isolated from this strain were now sensitive to rifampin, as was the host cell.

R factor 222 was next mated into the rifampin-resistant minicell strain, χ984. Four separate colonies of this rifampin-resistant χ984 (containing R factor 222) were tested for the resistance of the cells and minicells to rifampin. The results showed that, in every case in which the bacterial cells were completely resistant to the drug at 100 μg/ml, the minicells isolated from these cells were equally resistant. Other less resistant colonies of χ984 were also selected in which uracil incorporation in the presence of rifampin was inhibited to 50% of the control. When R factor 222 was introduced into these strains, the R$^+$ minicells were found to be more sensitive than the parent cell (Fig. 5C). In all instances, however, rifampin-resistant RNA polymerase was found in the minicells.

DISCUSSION

Falkow et al. (10) and Rownd (25) isolated R factors from *E. coli* chromosomal DNA by transferring them into *Proteus*, a strain with DNA of different buoyant density. In *Proteus*, R factor 222 exists in three independent replicative forms. Nisioka et al. (23) visualized these forms as distinctly different-size DNA circles using electron microscopy. In *E. coli*, the episome has only been found as a single unit with a buoyant density similar to chromosomal DNA (26). Cohen (8) demonstrated small amounts of a tiny circular DNA molecule in *E. coli* harboring the episome R1, suggesting that some separation of these units might be occurring in *E. coli* as well.

Having isolated the R factor in a minicell, we can conclude certain facts about its physical structure in vivo. As best determined, it is a single density molecular species, of which about half can be isolated as covalently circular molecules. Unlike studies in *Proteus* (10, 23, 25), only a single sharp peak was found on centrifu-

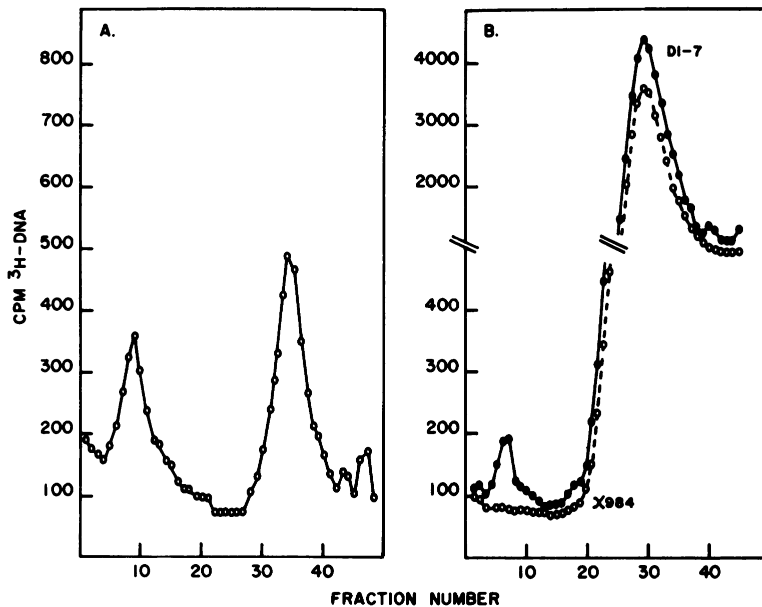

FIG. 2. *Alkaline sucrose gradient centrifugation of cell and minicell DNA. ³H-labeled DNA (0.2 ml) extracted from D1-7 minicells (A) and from χ984 and D1-7 cells (B) was layered onto three separate 5 to 20% alkaline sucrose gradients and centrifuged at 38,000 rev/min for 30 min at 23 C in an SW no. 50 rotor in an ultracentrifuge (Spinco, model L-2). Samples (0.1 ml) were collected from the bottom and counted.*

TABLE 3. *Membrane binding of cell and minicell DNA*[a]

	Expt	EDTA	Per cent in M band	
			DNA	Protein
Cells	1a	10^{-3}	35	
	1b	10^{-4}	85	
	2	10^{-4}	99	
	3	10^{-4}	93	29
	4	10^{-4}	95	
Minicells	1a	10^{-3}	7	
	1b	10^{-4}	55	
	2	10^{-4}	70	
	3	10^{-4}	63	45
	4	10^{-4}	75	

[a] Membrane binding of DNA from D1-7 cells and minicells was analyzed using the M band technique (27). In experiment 1, the preparations were divided: one half was treated with 10^{-3} M ethylenediaminetetraacetic acid (EDTA); the other half was treated with 10^{-4} M EDTA followed by neutralization with Mg^{2+}. The DNA was labeled with thymidine *methyl-³H* in experiments 1, 2, and 3, and with ¹⁴C-thymidine in experiment 4. Protein was labeled by growing cells overnight in the presence of ¹⁴C-leucine (0.5 μCi/ml).

gation of the purified R-factor DNA in cesium chloride. It has apparently retained its single structure despite its removal from bacterial DNA and the host cell. It seems reasonable to suppose, then, that some stable cytoplasmic substance retained by the *E. coli* minicells, but not found in *Proteus*, is responsible for the stable union of the different R factor units. It is still possible that the R-factor DNA has separated into DNA species of the same guanosine plus cytosine ratio and consequently the same buoyant density. This possibility seems unlikely in view of the previously cited studies on the

TABLE 4. *RNA synthesis by R^+ and R^- minicells and cells*[a]

Sample	Counts per min incorporated per 10^9 cell or minicell per 10 min
χ984 minicells (R^-)	10 ± 1
χ984 cells	8200 ± 30
D1-7 minicells (R^+)	140 ± 4
D1-7 cells	7200 ± 25

[a] Purified minicell cultures or their parent cells were incubated in 1 ml of L broth for 10 min in the presence of uracil-*2-¹⁴C* (30 μCi/ml). Incorporation was stopped by passing the culture over crushed ice. After thorough washing, the cells and minicells were lysed in ethylenediaminetetraacetic acid-lysozyme + 0.5% sodium dodecyl sulfate, and the RNA was extracted with phenol and precipitated with ethanol as described by Adesnik and Levinthal (1). Samples were counted in liquid scintillation fluid B.

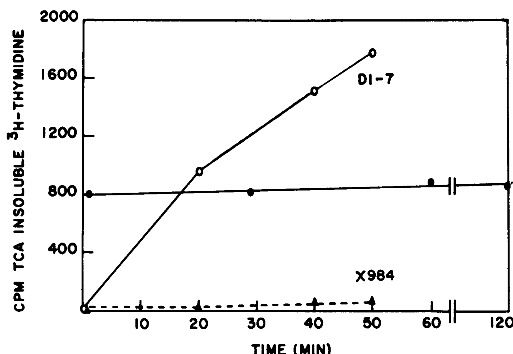

FIG. 3. *DNA synthesis by R^+ and R^- minicells. Highly purified minicell preparations (7 × 10^9 minicells/ml) were incubated in minimal salts media and 50% L broth in the presence of thymidine-methyl-^3H (50 μCi/ml). At different times, duplicate 50-μliter samples were removed and precipitated on paper discs pretreated with trichloroacetic acid and unlabeled thymidine. After washing and drying, the discs were suspended in liquid scintillation fluid B and counted (19). The results represent an average of the two samples. In a separate experiment, the DNA in R^+ minicells was labeled overnight with ^3H-thymidine, diluted into unlabeled media, and incubated for 2 hr. Samples were taken and precipitated in cold trichloroacetic acid* (●).

molecular nature of R factors in *E. coli* and *Proteus*. The possibility also exists that smaller circles, such as reported by Cohen (8), are present in the cell but did not segregate into the minicells.

The DNA isolated into minicells is at least 95% pure episomal DNA and may well be closer to 100%, since 4.6% probably represents the minimal degree of homology of R factor with *E. coli* DNA. The minicell method, therefore, offers an easy way to isolate pure episomal DNA without concern for bacterial DNA as in previously described isolation techniques (10, 11). By using a penicillin isolation (18), large fractions of episomal DNA can be obtained with relative ease.

DNA synthesis in the minicell is less than would be expected if all of the DNA were actively replicating. This amount of thymidine incorporation, however, is more than can be explained by repair, and indeed no breakdown of prelabeled DNA appears to be occurring in minicells. Since R factors transfer from minicells and, in fact, the mating efficiency seems unaltered after two matings, replication must be taking place, at least certainly at the time of mating. The lower incorporation by the total R-factor DNA in the minicell may, in fact, be indicative of its normal replication rate in the host cell. Since one episome is usually present per cell chromosome (25), the episome (being one-hundredth the size of chromosome DNA), must have to replicate at a slower rate or at a particular time in the cell cycle; i.e., during any short period in the culture, only a small number of R factors are replicating. Inselburg (14) by density labeling experiments showed replication of R factor 222 in minicells, but found evidence for no more than one replication in 2 hr of incubation. The possibility that mating stimulates this replication in the minicell is being examined.

Since there was good evidence that more than half of the R factor in *E. coli* was in a covalent circular form, Silver and Falkow postulated that this form was nonreplicating (26). Our results in minicells are strikingly consistent with theirs since we can repeatedly isolate about half of the R-factor DNA as covalent circular molecules. The actual fraction of covalent circles may be higher since some nicking and subsequent loss of

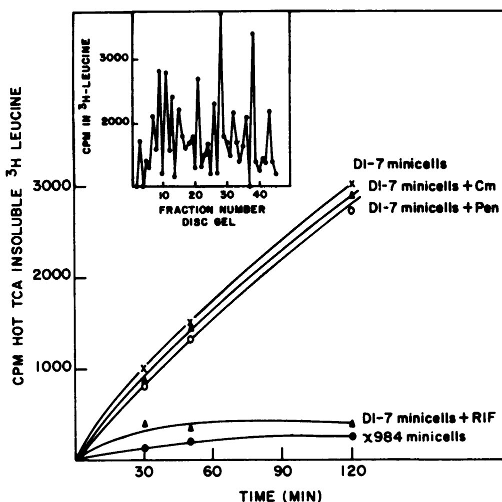

FIG. 4. *Protein synthesis by minicells. Equal quantities of minicells from D1-7 and χ984, purified by sucrose gradients, were washed and suspended in 3 ml of minimal salts media supplemented with histidine (100 μg/ml), pyridoxine (100 μg/ml), adenine (100 μg/ml), glucose (0.1%), and 10% L broth. Samples of D1-7 minicells were also suspended in similar media in the presence of chloramphenicol (25 μg/ml), rifampin (100 μg/ml), or penicillin (1,000 units/ml). After 20-min incubations at 37 C, L-leucine-4,5-^3H (100 μCi/ml) was added; at different times, duplicate 50-μliter samples were taken and precipitated on Whatman no. 3MM paper discs pretreated with trichloroacetic acid and unlabeled leucine. The discs were heated to 90 C for 15 min in 5% trichloroacetic acid and washed twice with trichloroacetic acid and then ethanol. After drying, these were counted in toluene-based scintillation fluid (19). The soluble proteins synthesized by minicell D1-7 were liberated by sonic treatment and isolated from free leucine by passage through a Sephadex G 100 column. The labeled proteins were separated by disc-gel electrophoresis (see insert).*

these circles may occur during the extraction and isolation procedure. Preparations of R-factor DNA left refrigerated for 24 to 48 hr do show reduced amounts of circular molecules, presumably due to single-strand breaks.

About 60 to 75% of the R-factor DNA is membrane associated. It is possible that only one form (circular or linear) is associated with the membrane. If, in fact, the circular form is nonreplicating, it might not be expected to be bound to the minicell membrane. Since up to 75% of the DNA has been found with M bands, and, since at least half of the DNA is circular, it is difficult to draw any conclusions. It is likely that all DNA is membrane bound, but perhaps the replicating and nonreplicating forms have a different association.

The segregated episome transforms an inactive anucleate minicell into one which can synthesize RNA and protein. The absence of such synthesis in the R^- minicell is ascribed to its lack of RNA polymerase. The presence of this enzyme in the R^+ minicells is evidenced by the sensitivity to rifampin. Studies with rifampin-resistant minicell strains indicate that the minicell polymerase is essentially bacterial in origin, but does not rule out the possibility that a small amount of polymerase (less than 10%) could also be synthesized by the R factor. Dominance studies with F′ merodiploids demonstrate that there is an interaction between sensitive and resistant enzyme pools in the cell which will determine the net level of sensitivity to the drug (16).

The finding of higher levels of sensitivity in R^+ minicells from partially resistant strains presents several possibilities. The host cell may have two enzyme pools, sensitive and resistant molecules, produced by a mutation which affects enzyme configuration or subunit structure and is incompletely expressed. The R-factor DNA may have a greater affinity for the more native, sensitive molecules and, therefore, take them into the minicell. The R factor might be synthesizing its own polymerase, but we have not found any evidence for this possibility in our studies transferring R factors from resistant minicells into sensitive cells and from sensitive cells into resistant minicell strains. It is more reasonable to suppose that resistance in these strains is related to a change in the molecular structure of the enzyme, making it less easy to bind to rifampin; however, some binding will occur. In the minicell, the R-factor DNA may compete with the drug for the enzyme. These possibilities are presently under investigation.

The bacterial polymerase appears to be carried into the minicells with the R factor. The high affinity of this enzyme for its DNA substrate may explain the absence of RNA polymerase in DNA-less minicells (7). The fact that the episome has picked up bacterial enzyme, despite the apparent close relationship of this enzyme with DNA, suggests either an excess of enzyme molecules in the bacterial cell or an easy availability of the enzyme to both episomal and chromosomal DNA. If in excess, the enzyme should be present in R^- minicells, but such activity has not been found (7). Attempts to find an inactive form of the enzyme in R^- minicells have also been unsuccessful.

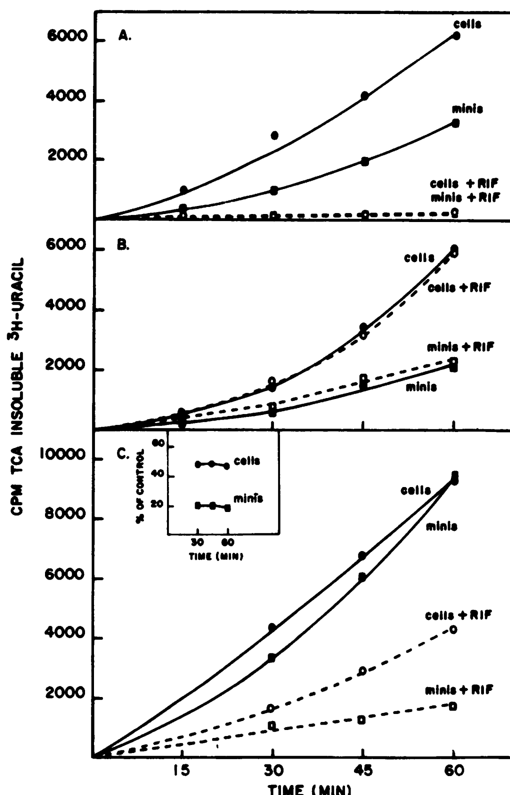

FIG. 5. *^{3}H-uracil incorporation into RNA by rifampin-sensitive and -resistant cells and minicells. Minicells and cells from rifampin-sensitive D1-7 (A), rifampicin-resistant D1-7 (B), and incompletely rifampin-resistant χ984(-222) (C) were harvested. ^{3}H-uracil incorporation (100 μCi/ml) into RNA by cells (at 3×10^7 to 5×10^7 viable counts/ml) and minicells (at 10^9 to 2×10^9/ml) was examined in L broth in the presence and absence of rifampin (100 μg/ml). Cells and minicells from χ984(-222) partially resistant to rifampin (100 μg/ml) were isolated, and incorporation studies were performed. The insert demonstrates the degree of incorporation as compared to the controls (in the absence of rifampin).*

The association of RNA polymerase with

DNA may differ at different times in a growth cycle. In *Proteus* for instance, R-factor synthesis and transcription is greatest during the stationary phase of the host bacteria (24). It is perhaps at this stage that bacterial RNA polymerase is more readily available to the plasmid.

The presence of more DNA per minicell than can be accounted for by the size of one R factor and the efficiency of transfer of these R factors from minicells to cells, as compared to cell-to-cell transfer (19), suggest strongly that at least one R factor is present in each minicell. These findings have led us to propose that the site of R-factor replication and the site of minicell division are adjacent geographically in the cell; thus, each time a minicell is formed it receives an R factor (20). The fact that 60 to 75% of the R-factor DNA is bound to minicell membranes (minicell M bands) is important additional evidence. The significance of these findings and their relation to certain segregated R factors which do not appear to replicate or transfer is being studied.

The R^+ minicell system offers the chance to focus on the plasmid alone in a cell environment. It may also facilitate the study of the mode of action of different chemicals on inhibition of plasmid replication or transfer, or both, where the only source of DNA is the plasmid itself.

ACKNOWLEDGMENTS

I thank K. Berns, M. Klagsbrun, and L. D. Kohn for their encouragement and help in the DNA, RNA, and protein studies, and P. Lynch and M. Schaechter for their help with the M band techniques. I deeply appreciate P. Guerry and S. Falkow for performing the hybridization studies and S. Falkow's helpful suggestions and advice.

This work was supported by Public Health Service grant no. AM 5210-10 from the National Institute of Arthritis and Metabolic Diseases.

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