Synthesis of Ribonucleic Acid and Protein in Plasmid-Containing Minicells of *Escherichia coli* K-12

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Unlike the deoxyribonucleic acid (DNA)-deficient minicells produced by F⁻ parents, minicells produced by plasmid-containing strains contain significant amounts of plasmid DNA. We examined the ability of plasmid-containing minicells to synthesize ribonucleic acid (RNA) and protein. In vivo, minicells produced by F⁻ parents are unable to incorporate radioactive precursors into acid-insoluble RNA or protein, whereas minicells produced by F', R⁺, or Col⁺ parents are capable of such synthesis. Using a variety of approaches, including polyacrylamide gel analysis of the RNA species produced and electron microscope autoradiography, we demonstrated that the synthesis observed in minicell preparations is a property of the plasmid-containing minicells and not a result of the few cells (approximately 1 per 10^6 minicells) contaminating the preparations. That the observed synthesis is of biological importance is suggested by the ability of plasmid-containing minicells to yield viable phage upon infection with T4.

Minicells are small, spherical, anucleate bodies continuously produced during growth of a mutant strain of *Escherichia coli* K-12, originally isolated and described by Adler et al. (1). Minicells purified from F⁻ parental cells by sucrose gradient sedimentation contain normal amounts of protein and ribonucleic acid (RNA) but are deficient in deoxyribonucleic acid (DNA) and several enzymes that use DNA as a template or substrate [e.g., DNA-dependent RNA polymerase, DNA methylase, and the photoreactivating enzyme (6)], and are thus unable to synthesize DNA, RNA, and protein.

Since minicells produced by F⁻ strains are capable of consuming oxygen at a constant rate for up to 72 hr (J. W. Black, Masters Thesis, Univ. of Tennessee, 1967) and extracts prepared from F⁻ minicells are capable of synthesizing polyphenylalanine when supplied with polyuridylic acid, an artificial messenger RNA (13), we hypothesized that such minicells would be able to synthesize RNA and protein in vivo if they could be supplied with DNA and the DNA-dependent RNA polymerase. In the first attempt to supply the transcription apparatus, Cohen et al. (6) demonstrated that single-stranded F and F'*lac⁺* DNA could be transferred to F⁻ minicells by conjugation. Although the single-stranded DNA was converted to double-stranded DNA in the

F⁻ minicells that contain DNA polymerase (6), the minicells were still incapable of synthesizing RNA and protein. These studies indicated that some necessary synthetic component, perhaps the DNA-dependent RNA polymerase, is not transferred with DNA during conjugation. We therefore sought another method of providing minicells with DNA.

Rownd (29) and Kontomichalou et al. (20) have demonstrated that, in some bacterial strains containing plasmids, the number of plasmids per chromosome increases as the culture enters the stationary phase of growth. We thought it important, therefore, to determine whether plasmid DNA, and possibly the associated DNA-dependent RNA polymerase, would be segregated into minicells produced by plasmid-containing strains, and, if so, to test these minicells for DNA, RNA, and protein synthesis.

Several laboratories have recently reported the segregation of DNA into minicells during growth of plasmid-containing strains (17, 19, 23, 28; Abstract, Proc. Xth Int. Congr. Microbiol. p. 60, 1970; Abstract, Genetics **64**:s54, 1970). Inselburg (17) has used a variety of physical techniques to show that the nontransmissable Col E1 plasmid segregates into and is capable of replication in minicells produced by Col E1⁺ strains. Electron micrographs of replicating Col E1 DNA from

minicells have been presented by Inselburg and Fuke (18). Genetic evidence for the segregation of plasmid DNA into minicells produced by plasmid-containing strains has been presented by Levy and Norman (23) and Kass and Yarmolinsky (19). They used minicells produced by R⁺ and F'gal⁺ strains, respectively, as donors of plasmid genetic markers in conjugal matings between minicells and suitable recipients.

Previous reports from our laboratory (28; Abstract, Genetics **64**:s19, 1970; Abstract, Genetics **64**:s54, 1970) have presented (i) physical and genetic evidence demonstrating the segregation of plasmid DNA into minicells produced by F⁺, F', R⁺, and Col⁺ strains; (ii) data on the replication of these plasmids in minicells; and (iii) preliminary information on the ability of plasmid-containing minicells to synthesize RNA and protein. This communication reports data from experiments in which we assessed the ability of plasmid-containing minicells to synthesize RNA and protein and examined the type of RNA synthesized in plasmid-containing minicells. Also presented are results of experiments establishing that the observed synthesis of RNA and protein is actually occurring in the minicells, and demonstrating that plasmid-containing minicells are capable of producing viable phage particles.

MATERIALS AND METHODS

Bacterial strains. The genotypes and sources of the strains used in these experiments are listed in Table 1. The minicell-producing strain carrying R64-11 was prepared by adding 0.5 ml each of overnight cultures of the R-factor donor and minicell-producing recipient strains to 9.0 ml of L broth (21). The mixture was incubated for 3 hr at 37 C, and appropriate dilutions were plated onto minimal media (9) containing 25 µg of oxytetracycline (Chas. Pfizer & Co., Inc., Brooklyn, N.Y.) per ml and 200 µg of streptomycin sulfate (E. R. Squibb and Sons, New York, N.Y.) per ml. Recombi-

nant colonies were removed and purified after incubation at 37 C. Colicinogenic minicell-producing strains were prepared by using donors containing Col factors and the same conjugation conditions used for R-factor transfer. After overnight incubation of appropriate dilutions of the mating mixture plated on Penassay agar (9) plus 200 µg of streptomycin sulfate per ml to contraselect against donor cells, the plates were replica plated (10) to similar plates. The master plates were then treated with chloroform vapors and subsequently overlaid with 2.5 ml of 0.7% Penassay agar containing a colicin-sensitive, streptomycin-resistant derivative of E. coli B. After 5 to 8 hr of incubation at 37 C, the plates were examined for zones of inhibition; colonies inhibiting the growth of the indicator bacterial strain were then picked from the replicate plates for purification. All R⁺ and Col⁺ minicell-producing strains were tested for sensitivity to the appropriate donor-specific phage, ability to transfer the plasmid, and plasmid and chromosome genotype. The F pili-specific phages MS-2 and f1 (see reference 38) and the I pili-specific phage If2 (26) were used in these tests.

Isolation of minicells. A mineral salts solution (7) supplemented with 0.5% Casamino Acids (Difco), 2 µg of thiamine hydrochloride per ml, and either 3% glycerol (w/v) or 0.5% glucose (w/v) was used for growth of the minicell-producing strains. Additional growth requirements were supplied at the appropriate concentrations (9). Cultures were incubated overnight in a New Brunswick rotary shaker at 37 C. Cells and minicells were sedimented by centrifugation at 10,000 rev/min in a Sorvall SS34 rotor at 4 C for 10 min. (All subsequent steps were conducted at 4 C or ice bath temperatures.) By use of a Vortex mixer for 2 min, the pellet was suspended in one-tenth the original culture volume in sterile buffered saline with gelatin (BSG; see reference 7). Samples of 2.5 to 3 ml were then layered over 35 ml of sterile 5 to 20% (w/v) linear sucrose in BSG gradients and centrifuged at 5,000 rev/min for 15 min with a Spinco SW27 swinging bucket rotor in a Beckman L2 or L3 ultracentrifuge. Each gradient could thus be loaded with cells and minicells from about 30 ml of the original culture. When larger quantities were required, the proportion of minicells on the

TABLE 1. *Bacterial strains*ᵃ

Strain	Plasmid genotype	Chromosome genotype	Derivation and source of plasmid
χ925ᵇ		thr⁻leu⁻lacY⁻minA⁻T6ˢgal⁻minB⁻strʳthi⁻ ᵈ	P678-54 from H. I. Adler
χ1009	Rfi⁻TcSm drd	thr⁻leu⁻lacY⁻minA⁻T6ˢgal⁻minB⁻strʳthi⁻	R64-11 from G. Meynell into χ925
χ1041	FColVMB trp⁺ ᶜ	thr⁻leu⁻lacY⁻minA⁻T6ˢgal⁻minB⁻strʳthi⁻	Fredericq factor from C. Berg into χ925
χ1058	ColB drd	thr⁻leu⁻lacY⁻minA⁻T6ˢgal⁻minB⁻strʳthi⁻	ColB drd from R. C. Clowes into χ925

ᵃ The nomenclature follows the proposals of Demerec et al. (11), with exceptions noted below and by Curtiss (8). All mutations conferring auxotrophic requirements are listed, but some that confer inability to use carbon sources or resistance to drugs and phages are omitted for the sake of brevity. R-factor drug-resistance abbreviations include: Tc, tetracycline; Sm, streptomycin.

ᵇ Same as P678-54 of Adler et al. (1).

ᶜ Referred to as Col trp⁺.

ᵈ Genetic mapping experiments indicate that two loci may be involved in the minicell phenotype (K. J. Roozen and R. Curtiss, *unpublished data*).

gradient could be increased by preceding the first su- crose gradient with a differential centrifugation of the overnight cultures. In these cases, 400 ml of culture was centrifuged in a Sorvall GSA rotor at 1,500 to 2,500 rev/min for 5 min. The supernatant liquid, which was greatly enriched for minicells, was then centrifuged at 8,500 rev/min in the GSA rotor for 15 min. The pellet was suspended in 9 ml of BSG and layered over three of the 35-ml, 5 to 20% sucrose gradients and cen- trifuged as above. Minicell bands were withdrawn from the gradients with a syringe, diluted with cold BSG, and centrifuged at 15,000 rev/min for 15 min in a Sor- vall SS34 rotor. The pellet was then suspended in BSG, layered over a second 35-ml, 5 to 20% sucrose gra- dient, and centrifuged as described above. After the minicell band had been removed and the minicells pel- leted, the minicell fraction was suspended in the appro- priate growth medium at an absorbancy of 0.2 at 620 nm and assayed for contaminating bacterial cells by plating on complete media or direct counting with a Petroff-Hauser counting chamber, or both. The puri- fied minicell fraction usually contained less than 1 cell per 10^6 minicells.

Incorporation of radioactive precursors. All radioac- tive precursors were purchased from Schwarz Biore- search, Inc. (Orangeburg, N.Y.). [^{3}H]uridine, [^{3}H]ura- cil, and [^{3}H]leucine had specific activities of 28, 7, and 58 Ci/mmole, respectively, and were used at 5 μCi/ml unless otherwise specified. [^{14}C]tryptophan with a spe- cific activity of 34 mCi/mmole and the labeled recon- stituted protein hydrolysate ([^{14}C]RPH) were used at 1 μCi/ml. The specific activity of the radioactive precur- sors was not diluted in these experiments to facilitate detection of low levels of incorporation.

RNA synthesis was assayed by the incorporation of either [^{3}H]uridine or [^{3}H]uracil into cold trichloroa- cetic acid-insoluble material. Purified minicells or cells (obtained from the pellet of the first sucrose gradient) were suspended to an absorbancy at 620 nm of 0.2 (ap- proximately 8 $\times$ 10^8 minicells or 1 $\times$ 10^8 cells per ml) in a mineral salts solution (7) containing 0.5% glucose, 2 μg of thiamine hydrochloride per ml, plus either 0.5% Casamino Acids or a mixture of the individual amino acids at the appropriate concentrations (9). The cul- tures were incubated for 10 min at 37 C before addi- tion of the label. When used, the RNA synthesis inhibi- tor, rifampin (Ciba Pharmaceutical Co., Summit, N.J.), was added to 100 μg/ml at the beginning of the preincubation. Samples were collected at various times onto Whatman 3MM filter discs (W and R Balston, Ltd., England), and the discs were immersed in cold 10% trichloroacetic acid within 1 min after sampling.

Protein synthesis was assayed by the incorporation of [^{3}H]leucine, [^{14}C]tryptophan, or labeled amino acids of the [^{14}C]RPH into hot acid-insoluble material. Min- icell and cell densities were similar to those described above. The media used were varied depending on which radioactive precursor was used. When [^{14}C]tryptophan was used as the labeled precursor, the mineral salts, glucose, thiamine, and Casamino Acids medium was used. When [^{3}H]leucine was the precursor, the incuba- tion mixture used was composed of mineral salts, glu- cose, thiamine, and a mixture of all individual amino acids except leucine. When the [^{14}C]RPH was used to label proteins, the nine unlabeled amino acids missing from the mixture plus threonine and leucine were added to the mineral salts-glucose-thiamine medium. Cultures were preincubated and sampled as described above. Where specified, chloramphenicol, from Parke, Davis & Co., Detroit, Mich., and cyclic 3'5'-adenosine monophosphate (cAMP), from Schwarz Bioresearch, Inc., Orangeburg, N.Y., were used at 100 μg/ml and 10^{-3} M, respectively.

Cell and minicell cultures treated with 50 μg of am- picillin trihydrate/ml (Bristol Laboratories, Syracuse, N.Y.) were incubated for 70 min at 37 C in the mineral salts, glucose, thiamine medium with either Casamino Acids or a mixture of amino acids before addition of the radioactive precursor.

Isolation of RNA. RNA was extracted from cells and minicells by a modification of the method of E. Volkin (*personal communication*). The cells were lysed by adding 2.5 ml of 0.1 M tris(hydroxymethyl)amino- methane (Tris)-hydrochloride (pH 7.6), 0.5 M NaCl, 5% sodium dodecyl sulfate, and 14% diethyl pyrocar- bonate (K & K Laboratories, Hollywood, Calif.) per 10 ml of culture. The mixture was shaken for 15 min at room temperature. An equal volume of liquid phenol that had been equilibrated with 0.1 M Tris-hydrochlo- ride (pH 7.2) was then added, and the mixture was shaken for at least 30 min at room temperature before being centrifuged for 30 min at 10,000 rev/min in the Sorvall SS34 rotor. The aqueous phase was removed, and 2.5 volumes of ethanol was added to precipitate the RNA. After overnight storage at −20 C, the pre- cipitate was collected by centrifugation and dissolved in 0.1 M NaCl. This solution was then dialyzed at 4 C against 1 M NaCl for at least 2 hr and then against 0.1 M NaCl overnight.

Polyacrylamide gel electrophoresis. The various spe- cies of RNA were separated on polyacrylamide gels by the method of Loening (25). The separations were car- ried out with a Canalco model 12 electrophoresis appa- ratus for 100 min (unless otherwise indicated) at 5 ma per gel in glass tubes (10 cm by 6 mm inside diameter). Two milliliters of gel was used per tube, giving a gel length of about 6.7 cm. About two A_{260} (absorbancy at 260 nm) units of nonlabeled cellular RNA was used as an optical marker and carrier. The gels were scanned at 260 nm in a Gilford 240 spectrophotometer and then frozen at their original length over dry ice. The frozen gels were sliced into 1-mm transverse sections with a gel slicer (Mickle Laboratory Engineering Co.)

Scintillation counting. All radioactive samples except those from the acrylamide gels were placed on Whatman 3MM filter discs and precipitated with cold 10% trichloroacetic acid. Filters containing DNA and RNA were washed twice with 5% cold trichloroacetic acid and twice with 100% ethanol and were then air- dried and placed into vials. Approximately 3 ml of 5% (w/v) 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]-thiophene (Packard Instrument Co., Downers Grove, Ill.) in tol- uene was then added to the vials, and the samples were counted in a Packard TriCarb scintillation spectrom- eter. Filters with protein samples were treated in the same manner except for the addition of a 30-min wash in 10% trichloroacetic acid at 85 C before the 5% cold trichloroacetic acid and ethanol washes. Gel samples

were prepared for counting by the technique of Weinberg and Penman (35).

T4 bacteriophage infection. The production of bacteriophage was monitored by a modification of the one-step growth experiment of Ellis and Delbruck (12). Cells taken from the pellet of the first sucrose gradient and purified minicells were suspended in L broth at an A_{620} of 0.2. Where indicated, the minicell preparation was incubated at 37 C for 90 min in the presence of 50 μg of ampicillin per ml to further reduce the level of cell contamination. At 10 min after the addition of ferrous cyanide to a concentration of 10^{-2} M, phage T4 was added at multiplicities of infection ranging from 0.4 to 2.1. After allowing 15 min for adsorption, a sample of the culture was diluted into T4 antiserum. Dilutions of each culture to concentrations convenient for sampling were carried out for 5 min after the addition of antiserum. Phage titers were determined by the soft agar overlay technique with *E. coli* B used as the indicator strain. All incubations were carried out at 37 C. The analytical sucrose gradient and fractionation techniques have been described previously (28).

Electron microscope autoradiography. Purified minicell preparations were suspended to an A_{620} of 0.2 in mineral salts supplemented with 0.5% glucose, thiamine, and a mixture of unlabeled amino acids. Leucine was not included in the amino acid mixture. The appropriate radioactive precursor was then added to a concentration of 25 μCi/ml, and the suspension was incubated for 60 min at 37 C. After incubation, the minicell preparations were washed three times in BSG containing 2 mg of unlabeled leucine and uridine per ml and stored in 1% Formalin. Electron microscope autoradiographs were prepared with Ilford L-4 (Ilford Ltd., Essex, England) emulsion as described by Caro (4) and Caro and van Tubergen (5). Microdal-X (Eastman-Kodak, Rochester, N.Y.) was used for developing the autoradiographs.

RESULTS

RNA synthesis in plasmid-containing minicells. Minicells produced by F⁻ strains are deficient in DNA and DNA-dependent RNA polymerase (6) and are therefore incapable of synthesizing RNA. Since this implies that RNA polymerase remains closely associated with its DNA substrate, one might predict that minicells inheriting plasmid DNA by segregation would also inherit the RNA polymerase and thus become capable of synthesizing RNA. We have examined this possibility by purifying minicells produced by plasmid-containing parents and assessing their ability to incorporate [³H]uridine into acid-insoluble material.

The abilities of F⁻ (χ925), Col *trp*⁺ (χ1041), and R64-11⁺ (χ1009) cells to incorporate [³H]-uridine are compared in Fig. 1A. Figures 1B and C show that minicells produced by parents hosting the R64-11 or Col *trp*⁺ plasmid are also capable of incorporating the radioactive precursor into trichloroacetic acid-insoluble material

under the same conditions. In contrast, minicells produced by the F⁻ strain (χ925) do not show significant levels of incorporation (Fig. 1B).

Rifampin has been shown to inhibit the incorporation of radioactive RNA precursors into acid-insoluble material by inhibiting the initiation of RNA synthesis (32). To show that this finding extends to the strains used in our experiments, we compared the incorporation of [³H]-uridine in the presence and absence of rifampin (Fig. 1A). The low initial incorporation of the labeled precursor can be attributed in part to the presence of RNA molecules whose synthesis was initiated before the addition of the antibiotic. As shown in Fig. 1B and C, the presence of rifampin in the incubation mixture reduced the incorporation of [³H]uridine into minicells containing R64-11 and Col *trp*⁺ to the background level observed in F⁻ minicells without rifampin. This antibiotic has no effect on the level of incorporation in F⁻ minicells (Fig. 1B).

Although cells usually incorporate [³H]uridine into RNA very rapidly, we were somewhat concerned when, in our initial incorporation with plasmid-containing minicells, a plateau for [³H]-uridine incorporation was reached after only a few minutes, at a level representing only a very small percentage of the added isotope (Fig. 1B and C). To determine whether this was due to a lack of additional synthesis or to a lack of available label, we conducted a standard incorporation experiment in which the normal amount of [³H]uridine was added at time zero and an additional amount after 30 min of incubation at 37 C. The results (Fig. 1C) indicate that the Col *trp*⁺-containing minicells are still capable of incorporating the radioactive RNA precursor into acid-insoluble material after 30 min of incubation and that the lack of additional isotope was responsible for the incorporation reaching a plateau so rapidly. We then considered the possibility that not all minicells produced by plasmid-containing parents are capable of RNA synthesis and that such minicells took up [³H]uridine from the medium, making it unavailable for incorporation into acid-insoluble material. To test this hypothesis, we added an equal number of minicells produced by F⁻ parents (those not synthesizing RNA) to minicells produced by χ1041 and added the normal amount of [³H]uridine. We found that the level of [³H]uridine incorporation was reduced approximately 40%, which suggests that minicells unable to synthesize RNA may be reducing the level of [³H]uridine available for incorporation by minicells capable of synthesizing RNA.

Since minicell preparations contain a number of contaminating cells, it is important to demon-

strate that the observed incorporation of radioactive precursors is a property of the minicells and not simply a result of the contaminating cells. This was done by assaying the ability of various cell concentrations to incorporate [³H]uridine into acid-insoluble material under the same conditions used in the minicell experiments. The results (Fig. 1D) show insignificant amounts of incorporation at initial cell concentrations below 10^5/ml. As previously stated, the contaminating cell titer is usually no more than 10^3 cells/ml at the start of the incubations. It is also pertinent that, although minicells prepared from R⁺ and Col⁺ parents are capable of incorporating [³H]-uridine into RNA, and F⁻ minicells are not, the numbers of contaminating cells in these preparations are approximately equal.

More direct evidence for the incorporation of [³H]uridine into trichloroacetic acid-insoluble material by plasmid-containing minicells was obtained by electron microscope autoradiography. In the preparations examined, significant numbers of silver grains were observed over plasmid-containing minicells.

From these results, we conclude that preparations of plasmid-containing minicells are capable of synthesizing RNA and that the observed incorporation of [³H]uridine into acid-insoluble material is a property of the minicells.

Protein synthesis in plasmid-containing minicells. Fralick et al. (13) reported that, although F⁻ minicells supplied with DNA by conjugation are incapable of synthesizing protein, extracts prepared from purified F⁻ minicells can incorporate phenylalanine into hot trichloracetic acid-insoluble material when polyuridylic acid is added as an artificial messenger RNA molecule. From these results, it might be predicted that minicells capable of synthesizing RNA should also be able to synthesize protein. We examined this possibility by assessing the ability of minicells produced by R⁺ and Col⁺ parental cells to incorporate radioactive amino acids into hot acid-insoluble material.

The ability of F⁻ cells (χ925) to synthesize protein in the presence and absence of cAMP and in the presence of cAMP and chloramphenicol, a protein synthesis inhibitor (15), is shown in Fig. 2A. Although χ925 cells readily synthesize protein and show a marked reduction in synthesis when chloramphenicol is present, it is apparent from the data in Fig. 2B that minicells derived from this strain are essentially incapable of protein synthesis and, as expected, their incorporation of amino acids into hot acid-insoluble material is unaffected by the presence of chloramphenicol. On the other hand, minicells produced by Col⁺ or R⁺ strains incorporate ¹⁴C-
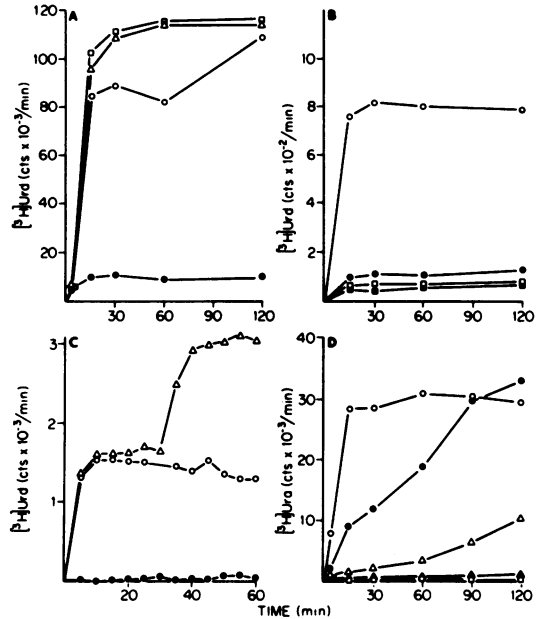

FIG. 1. *Incorporation of [³H]uridine ([³H]Urd) into cold trichloroacetic acid-insoluble material by cells and minicells. Cells or purified minicells were suspended in the mineral salts solution supplement with glucose, thiamine, and either Casamino Acids or a mixture of amino acids to an A_{620} of 0.2. After 10 min of preincubation at 37 C, [³H]Urd or [³H]uracil ([³H]Ura) was added to 5 μCi/ml, and samples were taken at intervals to measure the amount of acid-insoluble radioactive material. Rifampin, when present, was added at the start of the preincubation. (A) Comparison of the incorporation of [³H]Urd by cells of χ925 (△), χ1009 (□), and χ1041 in the presence (●) and absence (○) of rifampin. (B) Incorporation of [³H]Urd by purified minicells produced by χ925 in the presence (■) and absence (□) of rifampin; χ1009 in the presence (●) and absence (○) of rifampin. (C) Comparison of the incorporation of [³H]Urd by minicells derived from χ1041 in the presence (●) and absence (○) of rifampin. Open triangles indicate incorporation by χ1041 minicells when label was added at time zero and an additional 5 μCi/ml was added after 30 min of incubation. (D) Comparison of [³H]Ura incorporation by χ925 at initial cell concentrations of 10^8 (○), 10^7 (●), 10^6 (△), 10^5 (▲), and 10^4 (□) cells/ml.*

labeled amino acids into such material (Fig. 2B and C) at a rate similar to that of χ925 cells (Fig. 2A), although at a lower level. In the presence of chloramphenicol, incorporation by the plasmid-containing minicells is reduced to the level of F⁻ minicells (Fig. 2B and C). Our results also indicate that cAMP, which has been shown to stimulate synthesis of several inducible enzymes in *E. coli* but not to affect total RNA and protein synthesis (27), has no effect on plasmid-containing minicells (Fig. 2B and C).
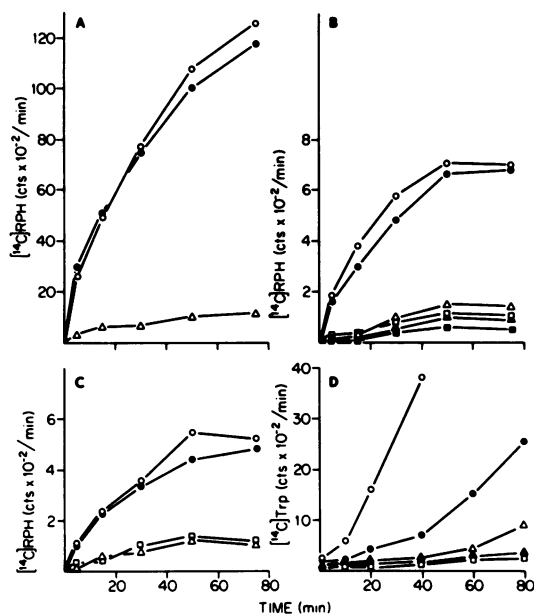
FIG. 2. *Incorporation of radioactively labeled amino acids into hot trichloroacetic acid-insoluble material by cells and minicells. Cells or purified minicells were suspended in mineral salts solution supplemented with glucose, thiamine, and either Casamino Acids or a mixture of unlabeled amino acids to an A_{620} of 0.2. After 10 min of preincubation at 37 C, the labeled precursor was added to 1 $\mu Ci/ml$, and samples were taken at intervals to measure the amount of hot acid-insoluble radioactive material. Where indicated, cyclic 3'5'-adenosine monophosphate (cAMP), rifampin, and chloramphenicol were added at the start of the preincubation. (A) Comparison of the incorporation of labeled reconstituted protein hydrolysate ($[^{14}C]RPH$) amino acids by $\chi 925$ cells taken from the pellet of the first sucrose gradient. No cAMP present (●), cAMP present (○), cAMP and chloramphenicol present (△). (B) Comparison of the incorporation of $[^{14}C]RPH$ amino acids by purified minicells of $\chi 1041$ and $\chi 925$: $\chi 1041$ with no cAMP presemt (●), $\chi 1041$ with cAMP present (○), $\chi 1041$ with cAMP and chloramphenicol present (△), $\chi 925$ with no cAMP present (□), $\chi 925$ with cAMP and chloramiphenicol (■), and $\chi 925$ with cAMP and rifampin present (▲). (C) Incorporation of $[^{14}C]RPH$ amino acids by purified minicells from $\chi 1009$. With no cAMP present (●), with cAMP present (○), with cAMP and chloramphenicol present (△), and with cAMP and rifampin present (□). (D) Comparison of the incorporation of $[^{14}C]Trp$ by $\chi 925$ at initial cell concentrations of 10^8 (○), 10^7 (●), 10^6 (△), 10^5 (▲), and 10^4 (□) cells/ml.*

Interestingly, the presence of rifampin in the incubation media with plasmid-containing minicells prevents the incorporation of both RNA (Fig. 1B and C) and protein (Fig. 2B and C) precursors into trichloroacetic acid-insoluble material, which suggests that endogenous RNA syn-

thesis is a prerequisite for protein synthesis in plasmid-containing minicells, that is, translation is from messenger RNA synthesized in the minicells and not from messenger RNA segregated into minicells when they are produced. There is no significant difference in the level of protein synthesis in F⁻ minicells in the presence and absence of the RNA synthesis inhibitor, rifampin (Fig. 2B), suggesting that there is little if any messenger RNA carried over into minicells isolated from stationary-phase cultures.

Again, the level of contaminating cells is approximately equal in all minicell preparations. To show that the amount of protein synthesis by the low number of contaminating cells is insignificant, we conducted an experiment in which the amount of incorporation by various cell concentrations was assessed under the conditions used in the minicell experiments. As illustrated in Fig. 2D, the incorporation of $[^{14}C]$tryptophan into protein is insignificant when the initial concentration of cells is below $10^5/ml$.

These results, combined with electron-microscope autoradiographic observations of silver grains over plasmid-containing minicells that have been incubated in the presence of $[^3H]$leucine and prepared as described above, indicate that plasmid-containing minicells are capable of protein synthesis.

Effect of ampicillin on macromolecular synthesis in minicells. Ampicillin, like penicillin, is a bacteriocidal antibiotic that has its primary effect on dividing cells (2). Since minicells do not divide, we incubated a number of minicell preparations in the presence of ampicillin to reduce the level of cell contamination. If RNA and protein synthesis due to contaminating cells is insignificant, as suggested by the experiments described in the previous sections, the level of incorporation by minicell preparations should be unaffected by preincubation in ampicillin. The effect of a 70-min preincubation on the ability of cell and minicell preparations of $\chi 1041$ to incorporate $[^3H]$uridine is presented in Fig. 3A and B, respectively. Preincubation of the cell and minicell preparations reduced the viable cell titer by factors of 1,000 and 100, respectively. Although the level of $[^3H]$uridine incorporation in the cell fraction was greatly reduced by ampicillin treatment (Fig. 3A), the level of incorporation in the minicell fraction was unaffected by this treatment (Fig. 3B). This result supports our conclusion that plasmid-containing minicells have significant RNA-synthesizing capabilities.

Similar experiments were conducted to assess the effect of ampicillin treatment on protein synthesis in cell and minicell preparations (Fig. 3C and D). Again, treatment of cell preparations

with ampicillin greatly reduced the level of [³H]-leucine incorporation into protein (Fig. 3C), but the results with the minicell preparations were unexpected. Treatment of the χ1041 minicell preparation with ampicillin caused an approximately 60% reduction in the incorporation of [³H]leucine into protein. This reduction might have been the result of a secondary effect of the antibiotic, but, since the primary action of ampicillin on normally dividing cells is lethal, such an effect would not have been observed. Gale and Folkes (14) reported the reduction of β-galactosidase synthesis in cell-free extracts of *Staphlococcous aureus* in the presence of penicillin.

Species of RNA synthesized in plasmid-containing minicells. The plasmids used in this study (R64-11, Col B, Col *trp⁺*) probably do not carry any ribosomal RNA cistrons that are present in multiple copies but map together on the bacterial chromosome (37). Thus, the RNA synthesized from the plasmid template should have a composition that is distinguishable from the RNA synthesized with the chromosome as a template. Preliminary results (28) from sucrose gradient analyses of RNA synthesized by cell and minicell preparations of R⁺ and Col⁺ strains indicated that, unlike the cellular preparations, the majority of RNA synthesized in plasmid-containing minicells ranges in size from 4*S* to 14*S*. The 16*S* and 23*S* ribosomal subunits were not synthesized in the minicells. We have now examined the species of RNA synthesized in plasmid-containing minicells more carefully by polyacrylamide gel electrophoresis. Figures 4A and C show the electrophoretic profiles on 2.6% polyacrylamide gels of [³H]uridine-labeled RNA from cells containing the R64-11 and Col *trp⁺* plasmids. As expected, the major peaks of radioactivity are in the 23, 16, and 4 to 5*S* regions. In comparison, the profiles obtained from RNA synthesized in plasmid-containing minicells are quite different (Fig. 4B and D). A large portion of the radioactively labeled RNA from plasmid-containing minicells migrates as heterodisperse RNA in the region between 6 and 16*S*, as expected for messenger RNA. There is no detectable peak of labeled RNA in the 16 or 23*S* regions, which supports the hypothesis that contaminating cells are not contributing significantly to the RNA synthesis observed in our experiments.

We examined the rapidly migrating 4 to 5*S* material more carefully by electrophoresis on 5% polyacrylamide gels. As seen in Fig. 5A and B, RNA synthesized in colicin-containing minicells contains two peaks of radioactive material in this region—one migrating slightly faster than the 4*S* optical marker and the other slightly faster than the 5*S* optical marker. The RNA from minicells
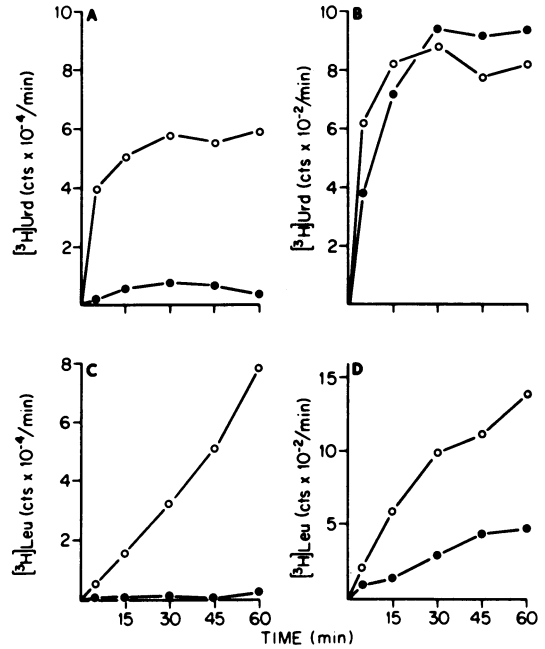

FIG. 3. *Effect of ampicillin treatment on the ability of plasmid-containing minicells to incorporate [³H]uridine ([³H]Urd) [³H]leucine ([³H]Leu) into trichloroacetic acid-insoluble material. The χ1041 cell fraction was prepared from the pellet of the first sucrose gradient. Minicells and cells were adjusted to an A₆₂₀ of 0.2, and the samples were preincubated at 37 C in the presence or absence of ampicillin for 70 min. Label was added at time zero, and samples were taken to determine the radioactivity insoluble in cold trichloroacetic acid (for [³H]Urd) or hot trichloroacetic acid (for [³H]-Leu). Incorporation of [³H]Urd by cells (A) and purified minicells (B) in the presence (●) and absence (○) of ampicillin preincubation. Incorporation of [³H]Leu by cells (C) and minicells (D) under similar conditions.*

containing R64-11 (Fig. 5C) has the peak that migrates slightly faster than the 4*S* optical marker but lacks the peak migrating faster than the 5*S* marker.

To make sure that the peaks of the minicell RNA described above actually migrate faster than the marker and that their positions were not due to an artifact of the freezing and slicing of the gels, we coelectrophoresed the [³H]uridine-labeled minicell RNA with [¹⁴C]uridine-labeled cellular RNA. Our findings showed that the relative positions of the peaks were the same as when the optical marker was used.

Since in polyacrylamide gel electrophoresis the distance migrated is proportional to either the molecular weight or *S* value of the RNA (24), one can estimate these parameters by comparison with a standard. When the mobilities of the
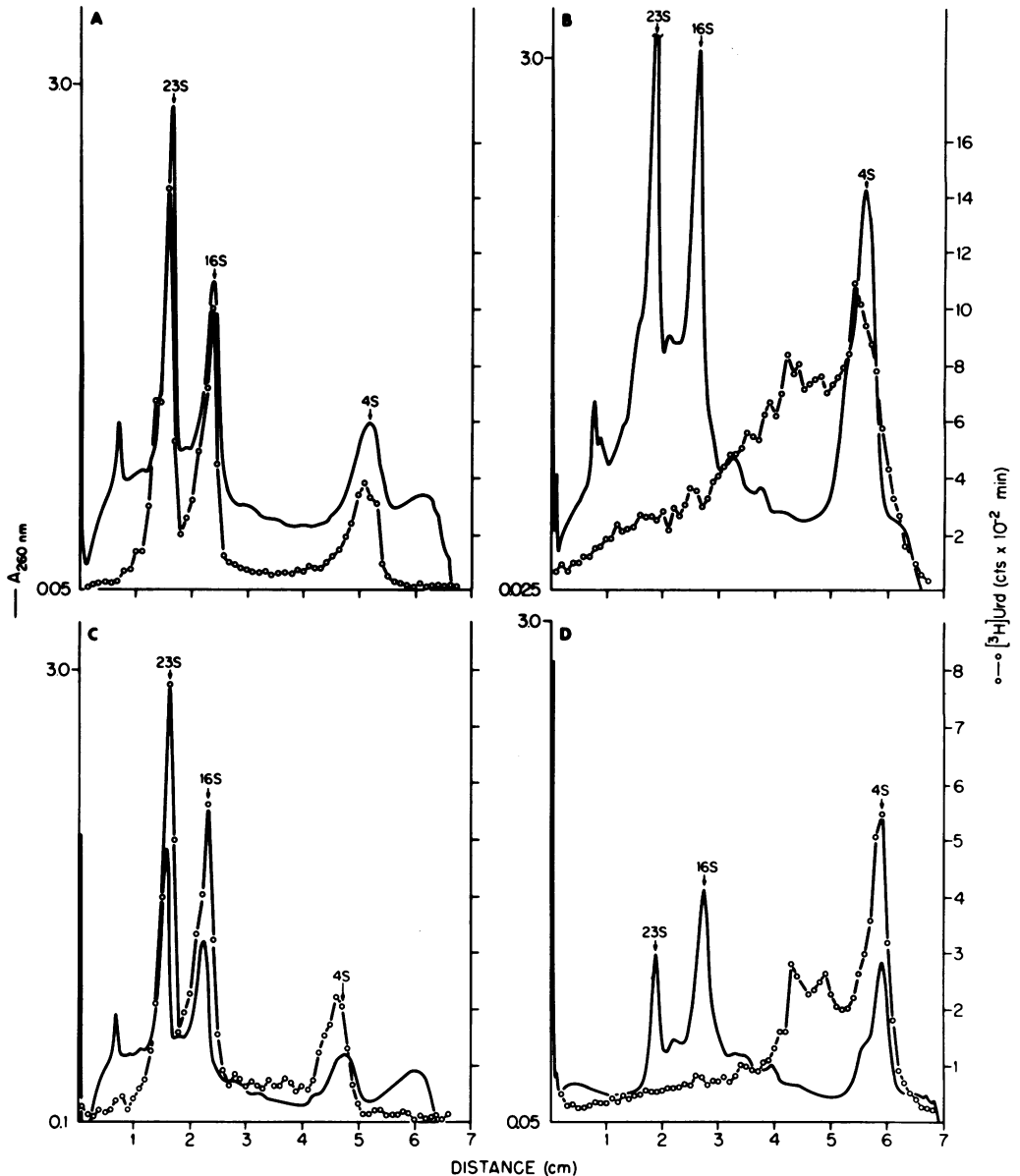
FIG. 4. *Electrophoresis on 2.6% polyacrylamide gels of RNA labeled in and extracted from cells or purified minicells. (A) Strain χ1041 Col trp+ cellular RNA extracted after 1 hr of incubation with 5 μCi of [³H]uridine [(³H]Urd)/ml. (B) Strain χ1041 Col trp+ minicell RNA extracted after 12 min of incubation with 50 μCi of [³H]-Urd/ml. (C) Strain χ1009 R64-11 cellular RNA extracted after 1 hr of incubation with 5 μCi of [³H]Urd/ml (electrophoresed for only 85 min). (D) Strain χ1009 R64-11 minicell RNA extracted after 70 min of incubation with 10 μCi of [³H]Urd/ml. The optical marker (—A₂₆₀ nm) is unlabeled χ925 cellular RNA; radioactive RNA (O).*

RNA peaks relative to the 4S optical marker are plotted against S value and molecular weight (Fig. 6), the fast-moving peak of each of the three RNA preparations analyzed has a mobility equivalent to about 3.8S, or a molecular weight of 22,000 daltons. These values are slightly lower than the average values usually cited for transfer

RNA molecules (3). In the RNA profiles from Col factor-containing minicells, the peak that migrates faster than 5S material has a value of 4.6S, or about 34,000 daltons. The RNA from minicells containing R64-11 is of interest because of the well-defined peaks that migrate between the 5 and 16S markers (Fig. 5C). The average S
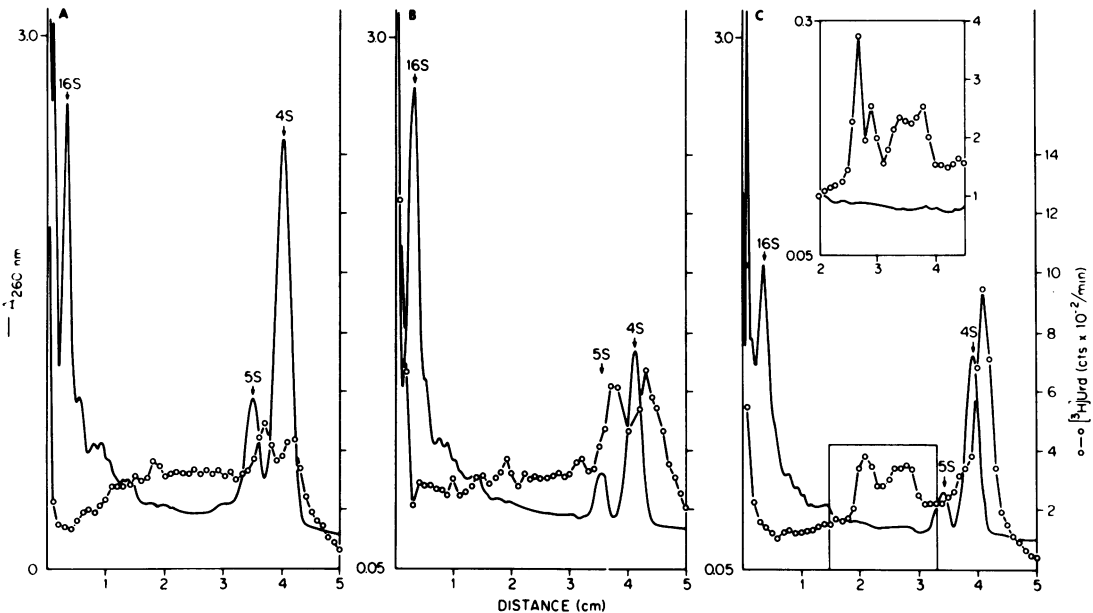
FIG. 5. *Electrophoresis on 5.0% polyacrylamide gels of RNA labeled in and extracted from purified minicells.* (A) Strain χ1041 Col trp⁺ minicell RNA extracted after 12 min of incubation with 50 μCi of [³H]uridine ([³H]-Urd)/ml. (B) Strain χ1058 ColB minicell RNA extracted after 12 min of incubation with 50 μCi of [³H]Urd/ml. (C) Strain χ1009 R64-11 minicell RNA extracted after 70 min of incubation with 10 μCi of [³H]Urd/ml. (Inset is boxed region of C after 135 min of electrophoresis.) *The optical marker* (—A $_{260 nm}$) *is unlabeled* χ925 *cellular RNA; radioactive RNA* (O).

values of these two peaks, which were determined from the plot in Fig. 6, are 6.4 and 8.6 — which correspond to molecular weights of 73,000 and 125,000 daltons, respectively. Longer electrophoresis of these peaks on 5% gels resolves each into at least two components (*see* Fig. 5C, insert).

These results suggest that a major portion of the RNA synthesized in minicells is a heterodisperse RNA and that, as expected, the ribosomal RNA genes are not available for transcription.

Production of viable T4 phage by plasmid-containing minicells. To determine whether the synthetic activities of plasmid-containing minicells result in a biologically functional product, we examined the ability of the minicells to produce viable T4 phage. Cell and minicell preparations were infected at low multiplicities with T4 phage and then assayed for infective centers and viable phage production. The results of several experiments with cells and F⁻, R⁺, and Col trp⁺ minicells are summarized in Table 2. From these results one can see that the infection of a culture of χ925 yielded a normal amount of progeny per infective center, whereas similar cells removed from the pellet of the first sucrose gradient yielded a much lower number of progeny phage after infection. This indicated that purification of

cells on sucrose gradients was somewhat deleterious to phage production. T4 infection of minicell preparations from F⁻ parents resulted in a low number of infective centers, but no significant change in the viable phage titer during the incubation period was observed. On the other hand, minicells derived from R⁺ or Col⁺ parents showed a higher number of infective centers when infected with T4 and a 9- to 36-fold increase in viable phage titer after incubation. Although the burst size is small, one must keep in mind that minicells are much smaller than normal *E. coli* cells and are isolated by sucrose gradient centrifugation, which appears to lower the burst size in the control experiments. Possible explanations for the low infectivity observed will be discussed later in this communication.

To show that the phage are being produced in the minicells and not in the few contaminating cells, we infected a purified minicell preparation with T4 and then layered the preparation onto a 5 to 20% sucrose density gradient. The gradient was then spun, and fractions were collected and assayed for viable phage titer. The peaks for infective centers and final phage titers coincided with the optical density peak for the slowly sedimenting minicells, indicating that the T4 is produced by material sedimenting as minicells in the sucrose gradient.
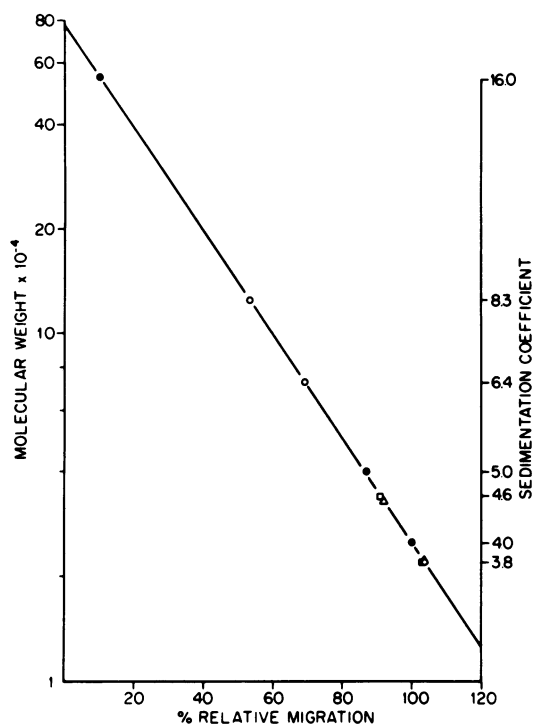
FIG. 6. *Plot of relative migration of peaks in Fig. 5 against both molecular weight and sedimentation coefficients. The optical marker transfer RNA peak was used as the 100% migration standard with the migration of the other peaks plotted relative to it. The straight line along which the experimental points were placed was established by plotting and connecting the relative migrations of the optical 16S and 5S ribosomal and 4S transfer RNA peaks against either their molecular weight or sedimentation coefficients. Optical marker (●), χ1009 R64-11 minicell RNA (○), χ1041 Col trp minicell RNA (△), χ1058 ColB minicell RNA (□).*

DISCUSSION

Minicells are a potentially useful system for studying a variety of cellular processes. Our demonstration that plasmid-containing minicells are capable of synthesizing RNA and protein should greatly enhance the value of minicells as an investigative tool. That the de novo macromolecular synthesis we observed in these experiments is a property of the plasmid-containing minicells and not the result of contaminating cells is shown in Fig. 1 to 4. The ability of these minicells to produce viable T4 bacteriophage after infection (Table 2) is taken as an indication that their synthetic activities are of biological interest.

Minicells produced by F⁻ parents are deficient in DNA (1), and lysates prepared from these minicells show little DNA-dependent RNA polymerase activity (6), whereas minicells produced by plasmid-infected parents contain DNA and are capable of RNA synthesis. It therefore appears that the DNA-dependent RNA polymerase "segregates" with its substrate into minicells. The absence of this enzyme in F⁻ minicells and its presence in plasmid-containing minicells suggests some compartmentalization of enzymes within the cytoplasm of *E. coli*. Several other enzymes that use DNA as a substrate (i.e., DNA methylase and the photoreactivating enzyme) have also been shown to be absent in F⁻ minicells (see reference 6). The presence of these enzymes in plasmid-containing minicells is under investigation.

In addition to the properties already described, the minicell system has a number of unusual characteristics that might have some bearing on its usefulness as an experimental tool. Several of these are discussed below.

TABLE 2. *T4 infection of cells and minicells[a]*

Strain	Fraction	Multiplicity of infection	Infective centers	T4 titer after burst	Burst size	Cell titer[b]	Special conditions
χ925	Cells	1.1	1.1×10^8	1.5×10^{10}	136	2.0×10^8	Broth-grown culture containing minicells; not purified
χ925	Minicells	2.1	7.5×10^3	8.0×10^3	1	4.0×10^2	Preincubation with ampicillin
χ1041	Cells	1.7	3.9×10^7	2.0×10^9	51	1.0×10^8	Cells from pellet of first sucrose gradient
χ1041	Minicells	2.1	2.3×10^5	8.1×10^6	35	1.9×10^3	Preincubation with ampicillin
χ1041	Minicells	0.4	1.9×10^5	2.7×10^6	14	1.9×10^3	Preincubation with ampicillin
χ1041	Minicells	0.2	2.8×10^5	2.5×10^6	9	1.9×10^3	Preincubation with ampicillin
χ1009	Minicells	2.1	6.5×10^4	8.0×10^5	12	1.3×10^3	Preincubation with ampicillin
χ1009	Minicells	0.4	6.5×10^4	7.1×10^5	11	4.2×10^3	Incubation in minimal media
χ1009	Minicells	0.2	2.0×10^4	7.3×10^5	36	4.2×10^3	

[a] Cells or minicells were suspended in L broth at an A_{620} of 0.2, and the T4 infection was carried out as described in Materials and Methods.

[b] After incubation in ampicillin.

In an earlier paper from this laboratory (28), it was reported that DNA is also segregated into minicells produced by F$^+$- and F'-containing strains and that the minicells produced by cells carrying F' KLF-1 or F'*lac*$^+$ are efficient donors of the plasmid markers in conjugation experiments. In these experiments, 10 to 20% of the minicells from F' cultures were capable of conjugal transfer of the F' to normal F$^-$ strains. Similar genetic results were reported by Kass and Yarmolinsky (19) for minicells produced by F'*gal*$^+$ parents.

In spite of the efficient donor ability of minicells produced by F'-containing strains, we have observed a very low level of RNA and protein synthesis in preparations of these minicells (about 30 to 50% of the incorporation found in the same number of R- or Col-containing minicells). This result is surprising when one considers that the F factor is a derepressed plasmid. In contrast to R- and Col-containing minicells, we have been unable to demonstrate an enrichment for closed circular molecules in DNA prepared from F'-containing minicells. Perhaps some of these inconsistencies will become understandable when more is known about the molecular nature of F', R, and Col plasmids in vivo and when we complete electron microscope autoradiographic studies to determine (i) the proportion of minicells produced by various strains inheriting DNA by segregation and (ii) the proportion of these minicells capable of DNA, RNA, and protein synthesis. Evidence to suggest that strains carrying different types of plasmids segregate different amounts of DNA into minicells has been presented (28).

Minicells isolated from cultures of R$^+$ and Col$^+$ strains in the log phase of growth are less productive in terms of RNA and protein syntheses than minicells isolated from cultures in the stationary phase of growth. A point bearing on this observation is the unanswered question about the proportion of minicells that contain plasmid DNA in minicells produced at various times during the growth of a culture. Another factor that could be important in the lower RNA and protein-synthesizing capabilities of minicells isolated from log-phase cultures is the considerably higher activity of ribonuclease I in minicell extracts prepared from log-phase cultures compared to those prepared from stationary-phase cultures (Heppel, cited reference in 13). Fralick et al. (13) used this argument to explain why the concentration of polyuridylic acid required to direct polyphenylalinine synthesis in minicell extracts prepared from log-phase cultures is higher than that for extracts from stationary phase-cultures. Another difference between minicells from

log-phase and stationary-phase cultures is that the level of contaminating parental cells in purified minicell preparations obtained from log-phase cultures is usually 10- to 100-fold higher than in preparations obtained from stationary-phase cultures.

We have also observed, but not extensively investigated, apparent differences in the ability of plasmid-containing minicells to synthesize macromolecules, depending on the method of isolation. As indicated in Materials and Methods, we routinely isolate minicells by a series of sucrose gradient sedimentations in linear 5 to 20% gradients. This method has the obvious disadvantage of exposing the minicells to an osmotic change resulting in plasmolysis. The reduced synthetic capabilities of plasmolyzed cells of *E. coli* were recently reported by Rubenstein et al. (30; see also Table 2), and Gros et al. (16) reported alterations in cell permeability in plasmolyzed cells. In a few experiments, therefore, we isolated the minicells in glycerol gradients (10 to 30% glycerol in BSG); glycerol is freely diffusible between the extra- and intracellular regions. Plasmid-containing minicells isolated in this manner had at least two undesirable properties. First, the synthetic capabilities were severely impaired; and, second, the level of cell contamination was a factor of 10 to 100 greater than minicells isolated by sucrose gradient sedimentation.

Another method of minicell isolation that would circumvent the use of sucrose was suggested by Levy (22). Penicillin, a bacteriocidal agent that acts only on dividing cells, is used to lyse cells in cultures containing cells and minicells. Although this method may be valuable for some studies with plasmid-containing minicells, our results with a closely related compound, ampicillin, indicate that protein synthesis in minicells treated with this antibiotic is reduced by about 60%. The use of such a treatment, therefore, may not be advisable.

Perhaps the most unexpected result in these studies was the observation that a substantial portion of the radioactively labeled RNA isolated from plasmid-containing minicells had an S value of 3.8, which is close to the value of transfer RNA molecules. In view of the similarity between these S values and the reports from a number of laboratories that some viruses specify transfer RNA molecules (31, 33, 34, 36), we are investigating the possibility that R and Col factors may also code for transfer RNA molecules. An alternative explanation for the appearance of radioactivity in transfer RNA molecules isolated from minicells may involve the conversion of radioactively labeled uridine to

cytosine and the subsequent incorporation of cytosine into the transfer RNA molecule during the rapid turnover of the terminal nucleotides of the transfer RNA molecule. Similarly, studies are also in progress to determine the significance of the 4.6*S* RNA species observed in RNA synthesized in Col-factor-containing minicells. The observation that RNA from some minicell preparations that migrates as heterodisperse RNA can be resolved into definite peaks suggests that it may be possible to isolate specific messenger RNA species from plasmid-containing minicells.

In conclusion, it appears that plasmid-containing minicells are capable of synthesizing DNA, RNA, and protein and will therefore provide a valuable system for studying replication, transcription, or translation.

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