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Replication of a Low-Copy-Number Plasmid by a Plasmid DNA-Membrane Complex Extracted from Minicells of *Escherichia coli*

WILLIAM FIRSHEIN,* PAUL STRUMPH, PATRICIA BENJAMIN, KERRY BURNSTEIN, AND JON KORNACKI

Biology Department, Wesleyan University, Middletown, Connecticut 06457

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A DNA-membrane complex was extracted from minicells of an *Escherichia coli* mutant harboring a "miniplasmid" derivative (11.2 kilobases) of the low-copy-number plasmid RK2 (56 kilobases). The complex contained various species of supercoiled and intermediate forms of plasmid DNA, of which approximately 20% was bound firmly to the membrane after centrifugation in a CsCl density gradient. The plasmid DNA-membrane complex synthesized new plasmid DNA without the addition of exogenous template, enzymes, or other proteins. DNA synthesis appeared to proceed semi-conservatively, was dependent on the four deoxynucleoside triphosphates, partially dependent on ribonucleoside triphosphates, and was sensitive to rifampin, an antibiotic known to inhibit initiation of replication. Novobiocin and nalidixic acid also inhibited synthesis, as did the omission of ATP. *N*-Ethylmaleimide, an inhibitor of DNA polymerase II and III activity, but not DNA polymerase I activity, also partially inhibited the synthetic reaction, as did chloramphenicol. The plasmid DNA synthetic product was analyzed by alkaline sucrose and dye-CsCl gradient centrifugation, as well as by agarose gel electrophoresis. In each case, the product consisted of parental and intermediate forms of plasmid DNA. Some chromosomal DNA was also synthesized by a contaminating bacterial DNA-membrane complex, but this synthesis was rifampin insensitive and could be separated from plasmid DNA synthesis.

Since the implication of the cell membrane in procaryote DNA replication was first proposed by Jacob et al. (12), a great deal of effort has been expended in investigating the interaction between these two cellular components. Complexes containing DNA and membrane have been isolated from a variety of bacteria and extrachromosomal DNA replicons (viruses and plasmids) (5–7, 9, 10, 12, 16–18, 20, 25, 26), and much evidence has accumulated to support the contention that such complexes may represent the site of DNA replication in vitro. Most important in this respect has been the observation that some complexes have been isolated that are capable of synthesizing DNA in vitro without the addition of exogenous template or enzymes (6, 7, 16, 20).

Nevertheless, the question of whether the DNA-membrane complex is involved in DNA synthesis has not been answered for a number of reasons. These include the fact that DNA replication entails several stages (initiation, elongation, termination), as well as ancillary reactions, such as repair and recombination. Additionally, contaminating components are probably present in all complexes thus far isolated, making it difficult to decide which components are integral to the functioning of the complex. Finally, there is a lack of sufficient variety of mutants for complementation assays to specifically relate a particular component to DNA replication.

If the complex could be reduced to an essential minimum with a small, defined template that initiates, replicates, and terminates its own replicon in a cellular environment which also was reduced in size and complexity, considerable progress might be forthcoming. Of great utility are the plasmid replicons that specify various antibiotic resistances (22). Also important is the evidence that during the process of duplication some plasmids have been associated with the cell membrane (17, 18, 26). A valuable property of plasmids is the ability to apply recombinant DNA techniques to reduce their size to "miniplasmids" containing only the region of replication origin (together with selective markers for identification purposes) (4, 11, 22–24).

We used such a miniplasmid (PRK-2501, a derivative of the broad-host-range low-copy-number plasmid RK2) to investigate (i) whether a DNA-membrane complex consisting primarily of plasmid DNA could be isolated and (ii) if so,

whether such a complex was capable of synthesizing plasmid DNA without added template or enzymes. This particular miniplasmid was chosen for several reasons. First, it contains only the essential regions of DNA replication, of which there are three, the origin of replication plus two others that act in "trans" to provide some factors necessary for the initiation of replication (22–24). Second, although a number of soluble systems have been derived from plasmid-containing *Escherichia coli* cells that replicate plasmid DNA (3, 4, 11, 19), none has been developed for low-copy-number plasmids. Of a number of reasons for this failure, two may be related to the lack of a proper configuration of the template or replication apparatus or both in the soluble extract. Under these conditions the use of a complex that may reflect the natural site of replication in vivo (the DNA-membrane complex) could be required to construct an appropriate replicating system.

MATERIALS AND METHODS

Bacterial and plasmid strains. *E. coli* K-12 strains YSI and KE-369 (a derivative of YSI), kindly supplied by M. Inuzuka, are minicell-producing mutants containing a number of markers (11). KE-369 is, in addition, End^- and Thy^-. Both strains were transformed with plasmid PRK-2501 DNA, using the procedure of Cohen et al. (2). PRK-2501 (11.2 kilobases)—containing the origin of RK2, two transacting regions (*trfA* and *trfB*), and two antibiotic markers (*kan* and *tet*) (24)—was maintained on LB agar plates under selective conditions (with 50 μg of kanamycin per ml), and the plasmid-free strains were maintained on the same medium under antibiotic-free conditions.

Growth conditions and isolation of minicells. In most experiments, antibiotic medium III (Penassay broth; Difco Laboratories) containing the appropriate concentrations of antibiotic and (if thymine requiring) 5 to 6 μg of thymine per ml were used. When preparing minicells, late-log-phase cultures (optical density at 600 nm = 0.45) were used as starting material. Unless otherwise specified, 8 to 10 liters was centrifuged, first at low speed (4,500 rpm for 5 min) to remove the bulk of the bacteria, and then the supernatant fluid was centrifuged at 10,000 rpm for 13 min to pellet the minicells. The minicells were enriched further by two successive sucrose gradient centrifugations (5 to 20%) in a solution consisting of 0.85% NaCl, 0.3% KH_2PO_4, and 0.6% Na_2HPO_4. The level of bacterial contamination (as determined by viable cell counts and microscopic analysis) ranged from 1 bacterial cell per 4×10^6 to 8×10^6 minicells.

In some experiments, M9 minimal medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.5% NaCl, 0.5% NH_4Cl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$) supplemented with 1% glucose, 0.5% Casamino Acids, 5 μg of thiamine hydrochloride per ml, 6 μg of thymine per ml, and 2 μCi of [^{3}H]thymine per ml (or 0.1 μCi of [2-^{14}C]thymine) was used to specifically label DNA (with strain KE-369 Thy^-).

Extraction of a plasmid DNA-membrane complex from minicells. The procedure was modified from that developed earlier (6, 25). Detergent crystals of Sarkosyl (sodium lauroyl sarcosinate) (prepared by adding an excess of 0.1 M magnesium acetate to 3% Sarkosyl) were added to spheroplasts of minicells prepared by treatment for 30 min at 0°C with lysozyme (1.3 mg/ml) and 4.4 mM EDTA in 0.05 M Tris (pH 8) buffer containing 25% (wt/vol) sucrose. The extract (2 to 3 ml) was placed on top of a 35-ml 20 to 50% (wt/vol) sucrose gradient (in 0.01 M Tris (pH 7)–0.01 M magnesium acetate–0.1 M KCl [TMK buffer]) and centrifuged for 30 min (4°C) at 13,000 rpm, using an SW27 rotor and a Beckman L-265B ultracentrifuge. The M-band, consisting of membrane associated with DNA and magnesium-Sarkosyl crystals, sedimented in the gradient at a level corresponding to 35 to 40% sucrose. It was removed with a wide-bore pipette and either dialyzed against appropriate buffers for use in enzyme analysis (see below) or the measurement of radioactivity. In some cases, the remaining cell extract (top fraction) was also analyzed. Microscopic observation of the M-band showed no intact minicells or whole cells.

Although the complex was in a crude state, several experiments were performed to minimize the possibility that this fraction was merely the result of nonspecific binding of DNA to membrane (data not shown). These include the observations that neither double- nor single-stranded radioactive purified plasmid DNA added to the minicells before extraction became associated with the complex. Second, the dialyzed complex could be rebanded in a similar sucrose gradient with only a minimum loss (20 to 25%) of DNA, suggesting that the various components do not redistribute themselves.

Sedimentation analysis and equilibrium density centrifugation. Linear 5 to 20% (wt/vol) alkaline sucrose gradients (in 0.3 M NaOH–0.01 M EDTA–0.9 M NaCl) were prepared in cellulose acetate tubes over a 60% sucrose shelf. Radioactive samples (200 to 400 μl) were centrifuged in a SW50.1 rotor at 44,000 rpm for 180 min at 4°C. Acid-insoluble radioactivity in various fractions (obtained by tube puncture) was determined by liquid scintillation techniques, using a Tracor (Mark III) counter.

For density gradient analysis, radioactive samples were added to cellulose acetate tubes containing a solution of CsCl in TES buffer (0.05 M Tris [pH 8], 0.005 M EDTA, 0.05 M NaCl), and the final density was adjusted to 1.600 g/cm^3. In some experiments, ethidium bromide (0.3 mg/ml) was added, and the final density was adjusted to 1.389 g/cm^3. The total volume was brought to 5.0 ml. After centrifugation to equilibrium at 40,000 rpm for 40 h at 25°C, either in an SW50.1 swinging bucket rotor or in a TI50 rotor, fractions were collected from the bottom of each tube, and acid-insoluble radioactivity was determined.

Recovery of the input counts from both the sucrose and CsCl gradients was usually greater than 80%.

Preparation of reference plasmid PRK-2501 DNA. Strain KE-369 Thy^- containing PRK-2501 was cultured in 1 liter of M9 glucose minimal medium containing 0.1 μCi of [2-^{14}C]thymine. Closed circular supercoiled DNA was prepared by ethidium bromide-CsCl density gradient centrifugation of Sarkosyl lysates of whole cells (1).

Isolation of a rifampin-resistant mutant. Large concentrations of cells of strain YSI containing the mini-

plasmid were plated on LB agar plates supplemented with various concentrations of rifampin (25 to 100 μg/ml). Colonies which appeared after 48 to 72 h on any of the plates were checked for their ability to incorporate [^{3}H]uridine (0.2 μCi/ml) into acid-insoluble material in the presence of rifampin. A number were found whose rates of incorporation were unaffected by rifampin, and one was used in the experiments.

Agarose gel electrophoresis. Agarose gel slabs (0.8 to 1.0%) (prepared in 0.09 M Tris-base, 0.09 M boric acid, and 0.025 M EDTA) were used to separate supercoiled and linear plasmid (and bacterial) DNA by vertical electrophoresis at 100 to 155 V/20 cm for 1.5 to 2 h. DNA bands were visualized by UV light after staining with ethidium bromide. Gels were sliced and processed for determination of radioactivity by melting them in a microwave oven and quickly adding Aquesol or Tritosol counting fluid (New England Nuclear Corp.) before the assay.

DNA synthesis. The standard reaction mixture was modified from that described by Inuzuka and Helinski (11). It contained (in 100 μl) 30 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfacid (pH 8); 30 mM KCl; 7.5 mM $MgCl_2$; 0.1 mM NAD; 7.5 mM creatine phosphate; 0.1 mg of creatine phosphokinase per ml; 0.1 mM cyclic AMP; 2 mM ATP; 0.5 mM each of GTP, CTP, and UTP; 0.02 mM each of dATP, dCTP, dGTP, and [^{3}H]dTTP; and 40 to 60 μl of the DNA-membrane complex extract. In some experiments bromodeoxyuridine triphosphate and [^{3}H]dATP (0.02 mM each) replaced [^{3}H]dTTP and dATP, respectively. The extract was previously dialyzed for 24 h (with two changes) against a buffer consisting of 10 mM HEPES (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, and 10% ethylene glycol (vol/vol). The dialysate was adjusted to a concentration of 1.75 to 2.5 mg of protein per ml (as determined by the Bio-Rad protein reagent). The total amount of DNA (as determined by the diphenylamine reagent) ranged from approximately 8 to 10 μg/ml. After incubation at 30°C, synthesis was terminated by the addition of 1 ml of ice-cold 5% trichloroacetic acid + 0.1 M sodium pyrophosphate solution. Acid-insoluble precipitates were collected on microfiber glass filters, washed successively with three portions of 5% trichloroacetic acid-pyrophosphate and twice with cold ethanol. Radioactivity was determined as described above.

Where replication products were to be characterized, the reaction was scaled up 20-fold. After incubation at 30°C, 20 mM EDTA was added, and the assay solution was treated with an equal volume of freshly distilled water-saturated neutralized phenol. After the aqueous and phenol layers were mixed, the aqueous layer was separated by centrifugation for 2 to 3 min in an Eppendorf microfuge, treated twice with ether to remove the phenol, and finally mixed with an equal weight of cold isopropanol. The precipitate that formed after storage of the extract for 2 h at −20°C was removed by centrifugation, lyophilized, and dissolved in a minimum amount of TES buffer for addition to CsCl or alkaline sucrose gradients and agarose gels.

When products were analyzed for semiconservative DNA synthesis, a modification of the procedure of Kado and Liu (13) was used to extract plasmid DNA. This procedure almost completely eliminates bacterial DNA without affecting plasmid supercoiled DNA. After incubation of the assay solution, an equal volume of 6% sodium dodecyl sulfate in 0.1 M Tris buffer (pH 12.6) was added, and the extract was heated at 55°C for 1 h. An equal volume of water-saturated phenol-chloroform was added, followed by centrifugation and treatment with ether and isopropanol, as described above, to remove phenol and precipitate the DNA.

Materials. Deoxyribonucleoside and ribonucleoside 5' triphosphates, bromodeoxyuridine 5' triphosphate, creatine phosphate and creatine phosphokinase, chloramphenicol, novobiocin, nalidixic acid, rifampin, NAD, cyclic AMP, pancreatic deoxyribonuclease, and HEPES buffer were purchased from Sigma Chemical Co. [*methyl*-^{3}H]dTTP (50 Ci/mmol), [^{3}H]dATP (60 Ci/mmol), [*methyl*-^{3}H]- and [2-^{14}C]thymine (40 Ci/mmol and 50 mCi/mmol, respectively), [5-^{3}H]uridine (25 Ci/mmol), 5-[2-^{14}C]bromouracil (15 mCi/mmol), and [2-^{14}C]glycerol (20 mCi/mmol) were purchased from ICN Corp. or New England Nuclear Corp.

RESULTS

Isolation of a plasmid DNA-membrane complex. Figure 1 shows the distribution of labeled DNA in a 20 to 50% sucrose gradient after extraction of a DNA-membrane complex from labeled (with [^{3}H]thymine) plasmid-containing and plasmid-free minicells by the Sarkosyl M-band technique. It can be seen first that a much larger amount of DNA was detected in the complex extracted from minicells with plasmid, and that there were two fractions of such DNA, one (comprising 60% of the total) sedimenting with a band of Sarkosyl crystals in the middle of the gradient (M-band) and the other sedimenting near the top of the gradient (top fraction). The DNA present in the plasmid-free minicell preparation was derived from bacterial contaminants. Based on comparisons of the total amounts of DNA detected in M-bands from plasmid-containing and plasmid-free minicells, the level of

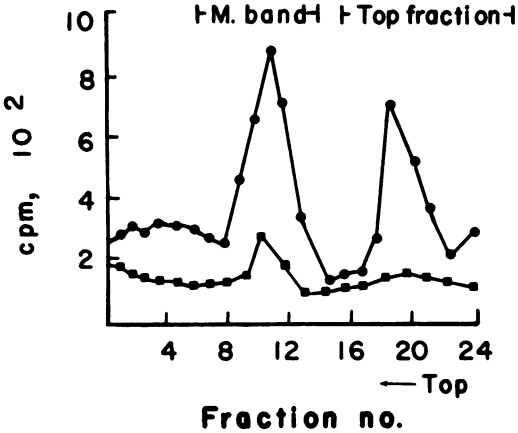

FIG. 1. Distribution of labeled DNA in an M-band and top fraction extracted from plasmid-containing (●) and plasmid-free (■) minicells.

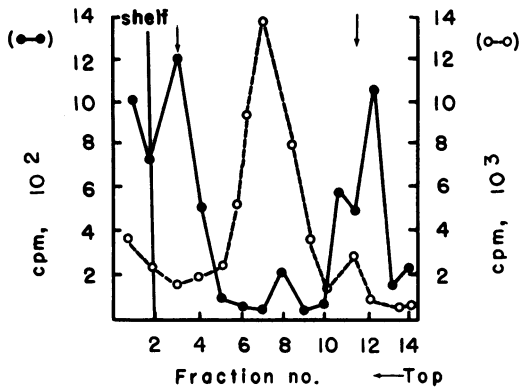

FIG. 2. Distribution of labeled DNA derived from a plasmid DNA M-band in an alkaline sucrose gradient. Symbols: ●, plasmid DNA from M-band; ○, bacterial DNA from M-band extracted from whole cells without plasmid. Arrows depict sedimentation of marker closed circular plasmid DNA (on the shelf) and nicked or linear plasmid DNA (near the top of the gradient.)

bacterial DNA contamination of the plasmid DNA M-band was approximately 8 to 10%.

Characterization of DNA in the complex. To determine the nature of the DNA present in the labeled M-band, samples were treated with alkali to dissolve membranes and denature the DNA present. These alkaline-treated preparations were layered on top of a 5 to 20% alkaline sucrose gradient over a 60% sucrose shelf and centrifuged together with marker plasmid DNA (Fig. 2). It can be seen that the M-band derived from the plasmid contained labeled DNA of two densities, one (about 55% of the total) sedimenting near the shelf with marker closed circular plasmid DNA and the other (about 35% of the total) sedimenting near the top of the gradient with open circular, linear, and nicked plasmid DNA. A small zonal peak was also observed sedimenting between the two plasmid DNAs at the position of the bacterial DNA marker derived from M-bands extracted from whole cells without plasmid.

A dialyzed M-band derived from plasmid-containing cells incubated with [^{14}C]glycerol and [^{3}H]thymine to label membrane phospholipids and DNA, respectively, was centrifuged in a neutral CsCl density gradient as described above. This procedure separates membrane-bound DNA (which floats to the top of the gradient) from dissociated or nonspecifically adsorbed DNA, which bands at the buoyant density of free plasmid or bacterial DNA. DNA not associated with the membrane (top fraction in the M-band preparation) and marker plasmid DNA were also centrifuged in similar gradients. It can be seen (Fig. 3A) that two zonal peaks of DNA were present from the M-band, one comprising about 17 to 20% of the total, remaining at the top of the gradient, and the other sedimenting at the buoyant density of free plasmid DNA. In contrast, DNA from the top fraction (Fig. 3B) sedimented at the buoyant density of free plasmid DNA only. The glycerol label from the M-band was also distributed in two zonal peaks, one with the DNA at the top of the gradient and one at a density heavier than that of free plasmid DNA. However, in the top fraction, the glycerol-labeled material was found only in the heavier density area, not at the top of the gradient. Presumably, the glycerol label at the top of the gradient in the M-band preparation represents lipid; but the identity of the glycerol-labeled material sedimenting in the lower part of the gradient in both the M-band and top-fraction preparations is not certain. Glycerol is known to be a precursor of phospholipids in gram-negative bacteria (15), but in minicell-producing mutants this pathway may not be the only one present.

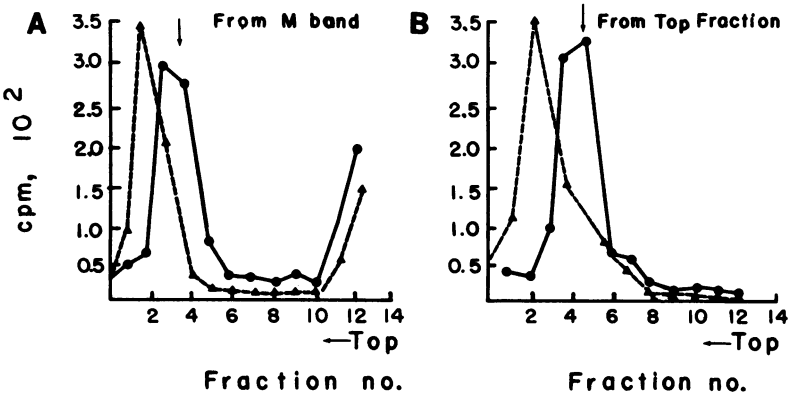

FIG. 3. Distribution of labeled DNA (●) and glycerol-labeled material (▲) from an M-band (A) and top fraction (B) derived from plasmid-containing minicells in a CsCl density gradient. The double label was [^{3}H]thymine and [2-^{14}C]glycerol.

For example, glycerol can enter the glycolytic cycle (21) and, at least in part, ultimately be incorporated into proteins (via conversion of pyruvate to amino acids). Such proteins, either by themselves, or complexed to RNA, could pellet in the gradient. Nevertheless, it is possible to conclude that a significant portion of the plasmid DNA is firmly associated with light-density material and resists dissociation with approximately 4 M CsCl. Finally, a separate peak of bacterial DNA could not be detected in the gradients, either because it was present in very low amounts or it sedimented too closely to free plasmid DNA.

Synthetic activity of the DNA-membrane complex. DNA synthesis by the membrane complex was assayed in the presence and absence of rifampin, an antibiotic known to inhibit initiation of DNA replication. Complexes extracted from both rifampin-sensitive and -resistant *E. coli* minicell-producing strains were assayed (Fig. 4). With the sensitive complex in the absence of rifampin, incorporation of [^{3}H]dTTP into an acid-insoluble product increased until 15 to 20 min, after which time further incorporation slowed. However, in the presence of rifampin, incorporation was inhibited after approximately 10 min of incubation, with the level of incorporation decreasing significantly after this time. Incorporation of precursors into rifampin-resistant complexes was not inhibited significantly in the presence of rifampin. Synthetic activity was not affected by the addition of exogenous plasmid or bacterial DNA at a concentration equal to that present in the complex (data not shown).

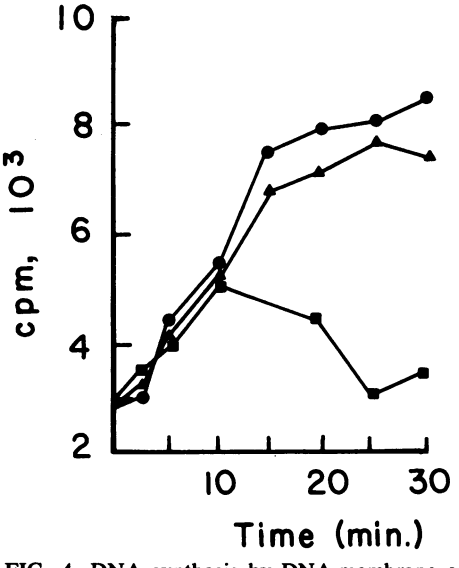

FIG. 4. DNA synthesis by DNA-membrane complexes extracted from rifampin-sensitive and -resistant plasmid-containing minicells in the presence and absence of rifampin. Symbols: ●, sensitive complex without rifampin; ■, sensitive complex with rifampin (100 μg/ml) added at time zero; ▲, resistant complex with rifampin (100 μg/ml).

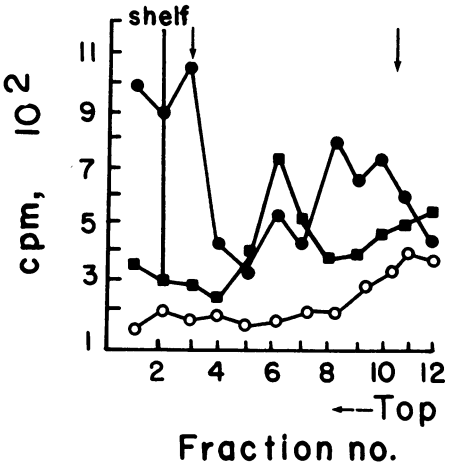

FIG. 5. Characterization of synthetic products by alkaline sucrose gradient centrifugation. Symbols: ●, incubation without rifampin; ■, incubation with rifampin for 30 min; ○, zero time control. Arrows indicate peak sedimentation of plasmid closed circular DNA (near the shelf of the gradient) and nicked and linear DNA (near the top of the gradient).

Characterization of synthetic product. (i) Alkaline sucrose gradients. The dialyzed DNA-membrane complex was incubated for 30 min in the presence or absence of rifampin, treated with alkali to dissolve membranes and denature the DNA, and centrifuged in alkaline sucrose gradients. Marker plasmid DNA was treated in the same way (Fig. 5). Three main zonal peaks were detected in the alkaline gradients after incubation of the complex without rifampin, one (approximately 35% of the total insoluble radioactivity) cosedimented with closed circular marker DNA near the bottom of the gradient; a second (approximately 45% of the total) sedimented near (but not at) the top of the gradient with nicked or linear plasmid DNA; and a third (approximately 20% of the total) sedimented in the middle of the gradient. By comparison with the data of Fig. 2, the middle peak represents bacterial DNA.

In the presence of rifampin, it can be seen that the formation of both closed circular and linear or nicked plasmid DNA was suppressed. However, the size of the bacterial peak was actually increased slightly over that seen without rifampin. Thus, it can be concluded that the DNA-membrane complex was synthesizing plasmid DNA but what may be bacterial DNA synthesis or chain extension was also occurring.

(ii) Agarose gel electrophoresis. The same ex-

periment was performed, but the DNA was subjected to agarose gel electrophoresis. Figure 6 shows that after 30 min of incubation of the complex in the absence of rifampin, a significant level of radioactivity was found to coincide with the plasmid DNA marker band visualized in the gel. However, perhaps because the gel slices were too broad, there was no separate peak of radioactivity that coincided with the bacterial DNA band, but rather a shoulder was detected. In the presence of rifampin, little radioactivity was observed (over the zero time control) coinciding with the plasmid DNA band in the gel, but a significant level of radioactivity did appear to coincide with the bacterial DNA band. Because of extended conformations, linear or nicked plasmid DNA molecules do not penetrate these gels as readily as supercoiled DNA during the relatively short electrophoresis time, but they can be visualized by UV light at the top of the gels. However, because the first gel slice encompassed an area below the top of the gel, radioactivity corresponding to these DNAs was not detected in the gel. In other experiments, we did detect radioactivity from gel slices when the top of the gels were included in the analyses (data not shown).

(iii) CsCl-ethidium bromide density gradients. Newly synthesized product DNA was extracted and centrifuged in CsCl-ethidium bromide gradients. These gradients separate supercoiled plasmid DNA from nicked and linear plasmid and bacterial DNA. It can be seen that supercoiled DNA was synthesized after incubation of the DNA-membrane complex derived from minicells containing plasmids (Fig. 7A). The product cosedimented exactly with marker plasmid DNA in the dye-CsCl gradient. Some nicked or linear plasmid DNA or both was also synthesized, as shown by the appearance of a peak in the vicinity of marker nicked plasmid DNA. However, as shown in Fig. 7C and D, this

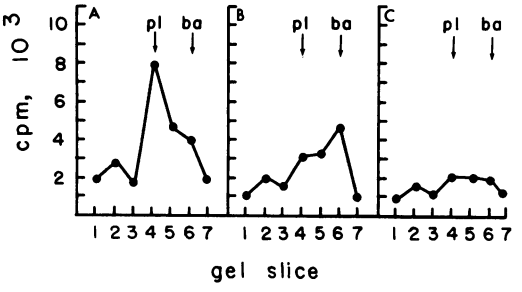

FIG. 6. Characterization of synthetic products by agarose gel electrophoresis. (A) Incubation without rifampin for 30 min; (B) incubation with rifampin for 30 min; (C) zero time control. Arrows indicate band of supercoiled plasmid DNA (pl) and bacterial DNA marker (ba).

second peak also contained bacterial DNA, which, from the results depicted in Fig. 7D, must be derived from whole cells contaminating the minicell preparation. In the presence of rifampin, the synthesis of the supercoiled DNA product was inhibited significantly (Fig. 7B), whereas the synthesis observed in the linear and nicked plasmid DNA peak was not affected to any great extent. Since rifampin probably also inhibited the formation of nicked and linear plasmid DNA, the DNA in the peak probably represents (primarily) rifampin-insensitive bacterial DNA synthesis. However, the amount of DNA detected in the lighter-density zonal peak in Fig. 7B is higher than that seen in Fig. 7D, which suggests that rifampin may not inhibit the formation of the nicked and linear plasmid DNA species completely (in Fig. 7B).

Semiconservative replication of plasmid DNA. Analysis of density-labeled PRK-2501 DNA synthesized in vitro by the complex in the presence of the heavy-density DNA precursor, bromodeoxyuridine triphosphate (Fig. 8), shows that after 45 min of incubation newly synthesized DNA bands at a density midway between the heavy- and light-density plasmid DNA markers added to the gradient. Since the DNA was extracted by a procedure that eliminates contaminating bacterial DNA (13), all of the newly synthesized DNA represents plasmid DNA. The shift in density from the light-density ^{14}C marker DNA and the newly synthesized [^{3}H]DNA at the peaks was approximately 8 mg/cm^3.

Effects of inhibitors on DNA synthesis by the DNA-membrane complex. Incorporation of [^{3}H]dTTP into DNA was inhibited significantly, but not completely by novobiocin and nalidixic acid (Fig. 9), known inhibitors of semiconservative replication and DNA gyrase activity (14, 19). *N*-Ethylmaleimide also exerted a strong but not complete inhibitory effect. Surprisingly, chloramphenicol also inhibited DNA replication significantly in these preparations. The inhibitory effects of novobiocin and nalidixic acid paralleled those of rifampin (Fig. 4), in that little inhibition was observed in the first 10 min, followed by an almost complete shutoff after that time. In contrast, the inhibitory effects of chloramphenicol were immediate, and the inhibition was proportional to the concentration of antibiotic used.

Requirements for DNA synthesis. Table 1 shows the effects of removing various components from the assay mixture. It can be seen that removal of those components required primarily for DNA chain extension, Mg^{2+} and three deoxyribonucleoside triphosphates, elicited the greatest inhibition of activity. Removal of the DNA template by DNase action also abolished most of the synthetic activity. Removal of com-

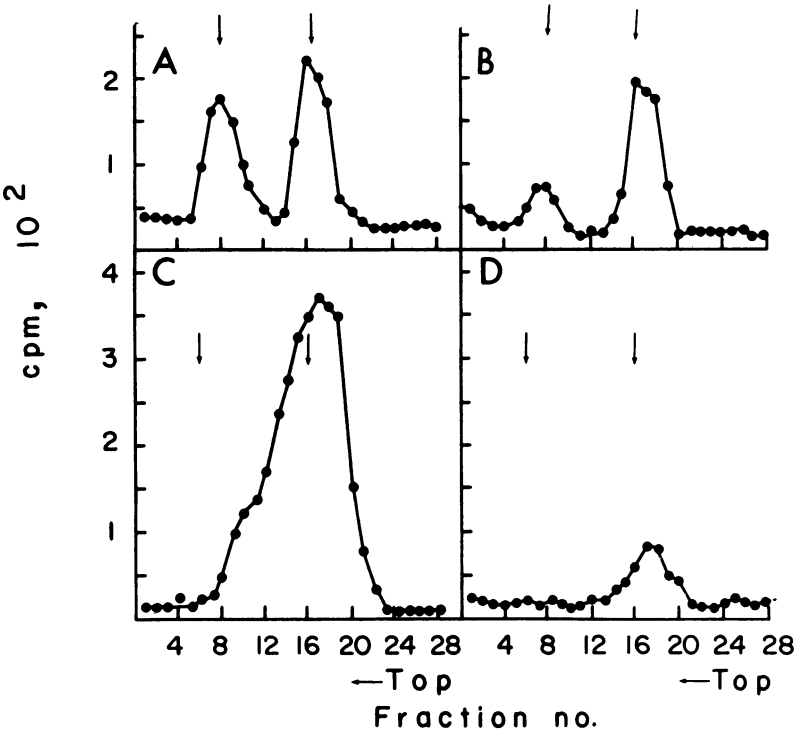

FIG. 7. Characterization of plasmid and chromosomal DNA products in CsCl-ethidium bromide density gradients. (A) DNA-membrane complex extracted from plasmid-containing minicells and incubated for 30 min without rifampin, (B) DNA-membrane complex extracted from plasmid-containing minicells and incubated for 30 min with rifampin, (C) DNA-membrane complex extracted from whole plasmid-free cells and incubated for 30 min without rifampin; (D) DNA-membrane complex extracted from plasmid-free minicells and incubated without rifampin for 30 min. Arrows indicate sedimentation of supercoiled plasmid DNA (closer to the bottom of the gradient) and nicked and linear plasmid DNA or bacterial DNA (closer to the middle of the gradient) markers.

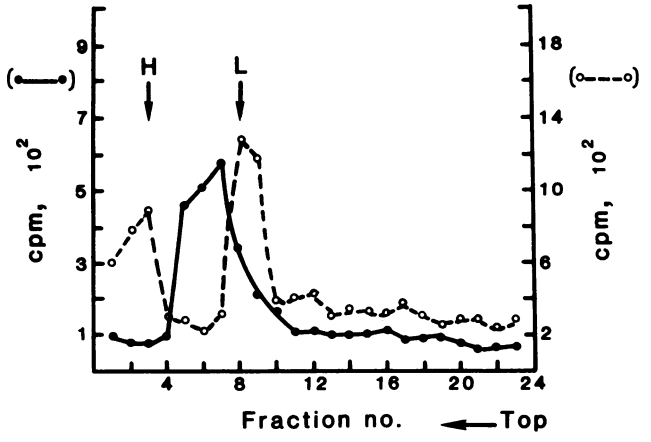

FIG. 8. Equilibrium CsCl density gradient centrifugation of plasmid DNA synthesized by the plasmid DNA-membrane complex in the presence of [^{3}H]dATP and bromodeoxyuridine 5'-triphosphate. Symbols: ●, newly synthesized [^{3}H]DNA; ○, heavy- and light-density ^{14}C-labeled marker DNAs. "H" and "L" stand for heavy and light density, respectively. The heavy-density marker was obtained by extraction of plasmid DNA from the plasmid-containing KE-369 Thy$^-$ strain subcultured three times in minimal medium containing 5-[2-^{14}C]bromouracil (0.1 μCi/ml) and 20% deuterium oxide.

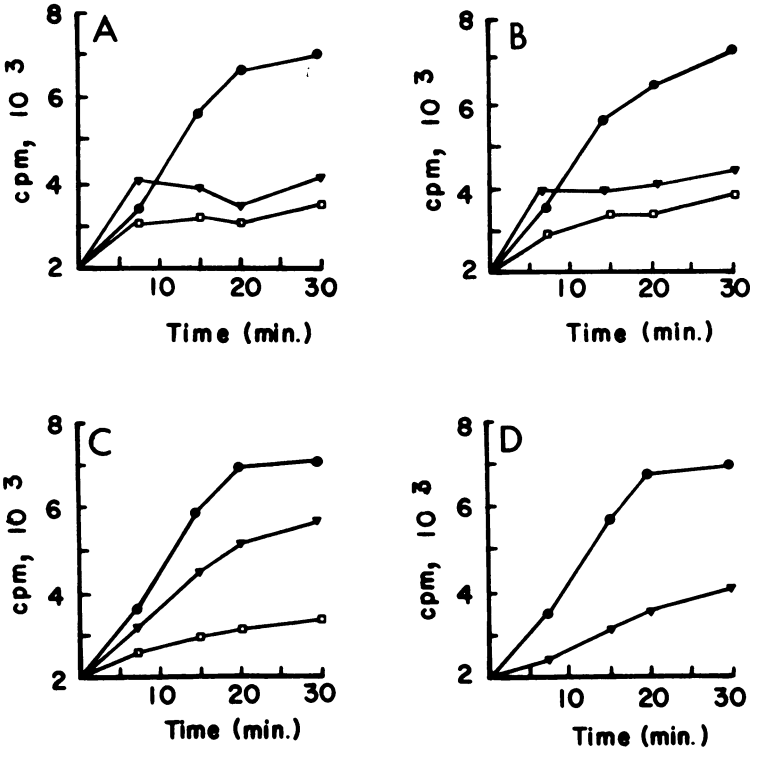

FIG. 9. Effects of inhibitors on DNA synthesis by the plasmid DNA-membrane complex. (●) controls; (A) supplemented with novobiocin at 20 μg/ml (▼) or 100 μg/ml (□); (B) supplemented with nalidixic acid at 100 μg/ml (▼) or 500 μg/ml (□); (C) supplemented with chloramphenicol at 20 μg/ml (▼) or 100 μg/ml (□); (D) supplemented with 20 mM *N*-ethylmaleimide (▼). Dithiothreitol was omitted when *N*-ethylmaleimide was added.

ponents required for the possible initiation of DNA synthesis, ATP and ribonucleoside triphosphates, inhibited activity, but not to the extent exhibited by Mg^{2+} and deoxynucleoside triphosphate depletion.

DISCUSSION

The results suggest that a DNA-membrane complex extracted from minicells of *E. coli* containing a derivative of the low-copy-number plasmid RK2 can initiate and complete the synthesis of new plasmid DNA in vitro without the addition of exogenous plasmid DNA or enzymes. The synthesis of a low-copy-number plasmid in vitro suggests that the complex can provide the proper configuration of the template or replication apparatus that may have been lacking in previously attempted soluble systems. However, the present experiments also suggest an additional property of the complex that could be of some importance in explaining its activity. This is the possibility that DNA synthesis depends upon new protein synthesis which may also be carried out by the complex in its relatively crude state (see below).

Rifampin, a known inhibitor of initiation, blocked most of the incorporation of precursors into DNA. The inability of rifampin to inhibit incorporation by complexes extracted from rifampin-resistant mutants suggests that its action in sensitive complexes is a result of a direct effect on initiation of inhibiting RNA polymerase activity (14).

TABLE 1. Requirements for DNA synthesis[a]

Addition	% Activity
Complete	100
+ DNase	4
− $MgCl_2$	4
− dATP, dCTP, dGTP	20
− UTP, CTP, GTP	50
− ATP	42
− cyclic AMP	65
− NAD	75

[a] DNA synthesis was carried out in the standard assay mixture. One hundred percent activity corresponded to a 6,800-cpm incorporation of [^{3}H]TTP into acid-insoluble material. For DNase treatment, the complex was treated with two successive portions of pancreatic DNase (50 μg/ml) for 15 min at 30°C before the assay.

Additional evidence for the initiation of plasmid DNA replication comes from (i) the probable occurrence of semiconservative DNA synthesis, (ii) the partial requirement for exogenous ribonucleoside triphosphates, and (iii) the inhibition of synthesis by novobiocin and nalidixic acid. The first observation indicates that initiation and completion of the entire plasmid DNA template occurred since newly synthesized DNA sedimented in the area of hybrid-density plasmid DNA in a CsCl gradient. However, the confirmation of this possibility must await an examination of the composition of the newly synthesized DNA by analysis of the separated single DNA strands. The second observation indicates nascent RNA synthesis. Since RNA synthesis involved in chain elongation by the discontinuous mechanism of DNA replication is insensitive to rifampin (14), it is possible that, at least in part, some origin primer RNA is being synthesized by the DNA-membrane complex. Inhibition by the antibiotics (and the partial requirement for high levels of ATP) suggests that DNA gyrase is present in the DNA-membrane complex (8) and involved in the synthesis of plasmid DNA, as observed in several other in vitro plasmid and viral replicating systems (4, 11, 19). This enzyme acts very early during initiation as part of a complex to nick and unwind the superhelical parental DNA molecules (14).

DNA synthesis is partially sensitive to *N*-ethylmaleimide, an inhibitor of DNA polymerase II and III activities, but not of DNA polymerase I activity (14). Since RK2 can proliferate in *polA* mutants that are defective in polymerase I activity and since DNA polymerase III has been found to be required for the synthesis of all plasmids thus far tested (23), it is possible that plasmid DNA synthesis by the DNA-membrane fraction is also carried out, at least in part, by DNA polymerase III.

The partial inhibition of plasmid DNA synthesis by deletion of NAD and cyclic AMP from the assay mixture has been observed by others (11, 19). Although NAD may act as a cofactor for DNA ligase activity in the DNA-membrane complex (9), the role of cyclic AMP, in view of its many effects on a variety of metabolic events, including membrane phenomena, is as yet unknown.

There are a number of complicating factors that must be explained for each of the points discussed above. Part of the complication comes from the presence of contaminating bacteria in the minicell preparations. Despite their relatively low levels, there is apparently sufficient material available to extract a chromosomal DNA-membrane complex together with the plasmid complex from the minicells (Fig. 1). This complex could also synthesize DNA (Fig. 7D) where a synthetic product extracted from plasmid-free minicells sedimented in the region of bacterial DNA. However, it is unlikely that bacterial DNA synthesis represents anything more than repair or nonspecific chain extension because it is rifampin insensitive (cf. Fig. 7B and D). It is of interest that as the size of the bacterial peak increased in the presence of rifampin, the sizes of the plasmid DNA peaks were reduced. One explanation for this phenomenon is that when rifampin is present to inhibit plasmid synthesis, rifampin-insensitive bacterial DNA synthesis can utilize the substrates and cofactors in the assay solution to a greater extent. It is this latter activity that can also explain, at least in part, (i) the inability of rifampin or the other drugs involved in inhibiting initiation reactions (novobiocin, nalidixic acid) to completely suppress the synthetic reactions, as measured by the incorporation of acid-soluble [^{3}H]dTTP into acid-insoluble material (Fig. 4, Fig. 9); and (ii) the results described in Table 1. Those components involved in DNA chain extension (Mg^{2+}, deoxynucleoside triphosphates) elicit the greatest inhibition of activity when they are deleted because all DNA synthesis (bacterial and plasmid DNA) is affected. However, the omission of components involved specifically in putative initiation reactions (ribonucleoside triphosphates, high concentrations of ATP) did not inhibit the synthetic reaction as completely.

An unexpected finding was the significant inhibition of DNA synthesis by chloramphenicol, an antibiotic which interferes with protein synthesis. A similar result was observed recently by Diaz, Nordström, and Staudenbauer (4) with an R1 plasmid soluble replicating system. Their interpretation was that replication of R1 is coupled to transcription and translation and that the protein synthesized may be an R1-specific initiator protein. In RK2, two regions, *trfA* and *trfB*, act in trans to supply proteins involved in RK2 DNA synthesis. However, only one region, *trfA*, is absolutely required for initiation (22). Since plasmid replication was obtained, we postulated that the *trf* initiating factors were already present in the complex, protected by some specific binding component. However, the results with chloramphenicol suggest that these factors may be synthesized by the crude DNA-membrane complex and used immediately for initiation. If this is so, one important question is how the DNA-membrane complex can carry out all of these reactions simultaneously. In the original study of the M-band, Tremblay et al. (25) observed that nascent RNA was present in the M-band, that it was bound to DNA in the complex, but that 10 to 15% of the pulse-labeled RNA remained even after removal of 99% of the DNA

with DNase. Subsequently, it was found by Tremblay et al. (25) that polysomes, possibly in the process of transcription, are also present in the M-band, as are ribosomal subunits. Thus, the M-band isolated from minicells containing the miniplasmid PRK-2501 may, in fact, represent a functional coupling of replication, transcription, and translation, as intimated by Diaz, Nordström, and Staudenbauer with R1 (4). However, further experimentation is required to substantiate this hypothesis, in particular whether or not the proteins are actually synthesized de novo or whether they are already in the process of being translated.

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