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Replication of Plasmid RK2 In Vitro by a DNA-Membrane Complex: Evidence for Initiation of Replication and Its Coupling to Transcription and Translation

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The following results with an in vitro replication system utilizing a plasmid RK2 DNA-membrane complex indicate that the essential *trfA*-encoded replication protein of RK2 is present and active in the complex. (i) A complex extracted from a conditional replication mutant of RK2, which contains a temperature-sensitive mutation in *trfA*, displayed extensive DNA synthesis at the permissive temperature but little activity at the restrictive temperature. A control wild-type RK2 complex showed no inhibition of DNA synthesis at the restrictive temperature. (ii) Analysis of plasmid-encoded proteins revealed that the *trfA*-specified replication protein and other proteins which may be involved in the replication and maintenance of RK2 are located physically in the complex. Semiconservative plasmid DNA replication by the DNA-membrane complex was indicated by density shift experiments; DNA synthesized in the presence of a heavy-density precursor banded primarily in a heavier-density area of a neutral CsCl density gradient and consisted mostly of heavy- and light-density single-stranded DNA as determined by alkaline CsCl density gradient centrifugation. Plasmid RK2 DNA replication by the DNA-membrane complex appears to be coupled to transcription and translation as indicated by the following results: (i) the inhibitory effects of chloramphenicol on both DNA and protein synthesis by the complex; (ii) the stimulation of replication by components normally required for protein synthesis (tRNA and all the common amino acids); (iii) the synthesis of RNA and protein by the complex; and (iv) the synthesis of specific RK2-encoded proteins.

Although the DNA-membrane complex has been implicated as the site of DNA replication in the procaryotic cell (for a review, see reference 18), much of the evidence has been indirect. Only a few studies have attempted to determine directly whether DNA-membrane complexes can synthesize DNA in vitro (3, 4, 10–13); this demonstration is essential to provide an understanding of the role of the complex in DNA replication.

We have been working with the broad-host-range low-copy-number plasmid RK2 as a model system to investigate the significance of the DNA-membrane complex in DNA replication. RK2 has two genetic determinants that are absolutely required for its replication: *oriV*, the origin of replication (7, 19, 30, 34), and *trfA*, a gene which encodes a 33-kilodalton (kDa) protein (A2) needed for the initiation of replication at *oriV* (7, 16, 27, 28, 32, 33) (Fig. 1). In addition to these essential loci, the replication control system of RK2 contains two groups of genes (*kil* and *kor*) of which at least some interact to regulate the expression of *trfA* (for a review, see reference 9).

We have previously reported the development of an in vitro replication system which utilizes a plasmid RK2 DNA-membrane complex (11, 13). The complex, extracted from *Escherichia coli* minicells containing a miniplasmid derivative of RK2, was enriched for supercoiled DNA and was capable of synthesizing new plasmid DNA endogenously (i.e., without the addition of a template or enzymes) (13). Heavier-density newly synthesized DNA was detected after incubating the complex with a heavy-density precursor, and the reaction was inhibited by a number of drugs and antibiotics affecting the initiation and elongation steps of DNA replication (13). Evidence for RNA priming of initiation was obtained both biochemically (13) and ultrastructurally (11). Finally, of interest in these previous studies (13) was the inhibition of replication by chloramphenicol, an antibiotic which affects protein synthesis.

Although these results suggest that the DNA-membrane complex contains the replication apparatus, further evidence was needed for initiation of replication and the activity of specific RK2-encoded products in the complex. Also, it was important to investigate the effects of chloramphenicol and the possible requirement of new protein synthesis for RK2 replication in vitro. The new results presented in this paper provide additional support for the role of the plasmid DNA-membrane complex in the replication of RK2 and indicate that replication is coupled to transcription and translation of RK2-encoded products required for replication.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 YS1 and CSR603 were used in this study. YS1 is a minicell-producing strain (14). CSR603 (*recA1 uvrA6 phr-1*) (24) was the host strain utilized for radioactive labeling of plasmid-encoded proteins, using the maxicell method (23). Broad-host-range plasmid RK2 or derivatives of this plasmid were used. RK2 (56.4 kilobases [kb]) is a low-copy-number plasmid that confers resistance to kanamycin, ampicillin, and tetracycline (31) (Fig. 1). For most experiments, the mini-RK2 plasmid pRK2501 (11.0 kb) was utilized (Fig. 2). Plasmid pMR5 is a derivative of RP1 (shown to be identical to RK2 [5]) that contains a temperature-sensitive mutation in the essential replication gene *trfA* (22, 32).

Growth conditions and standard procedures. Media and

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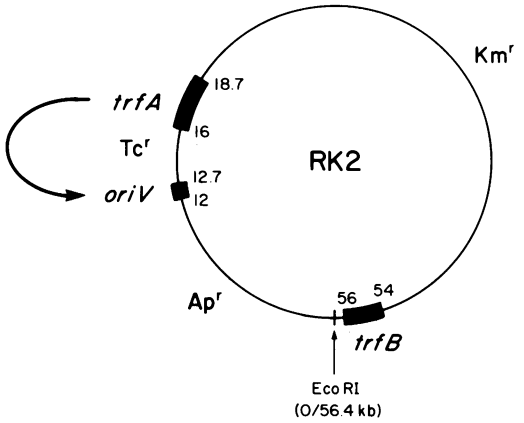

FIG. 1. Basic genetic and physical map of plasmid RK2. Coordinates of the RK2 map are defined by the distance in kilobases from the single *Eco*RI site. *oriV* and *trfA* are described in the text. The interaction of the essential *trfA*-encoded replication protein with *oriV* is indicated by the arrow. Solid boxes indicate replication and maintenance regions that comprise mini-RK2 plasmid pRK2501 (see Fig. 2). The *trfB* region encodes at least two genes (*korA* and *incC*) involved in replication and maintenance control (1, 8, 20, 26, 29, 33). Ap^r, Km^r, and Tc^r indicate genes for resistance to ampicillin, kanamycin, and tetracycline, respectively.

culture conditions for growth of bacteria have been described previously (13, 16). Isolation of plasmid DNA (15), transformation of *E. coli* with plasmid DNA (6), isolation of minicells (13), labeling of plasmid-encoded proteins in maxicells (16), and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (16) were performed by previously published procedures. SDS-polyacrylamide gels were prepared for autoradiography with Enlightning (New England Nuclear Corp., Boston, Mass.) by the recommended procedure of the manufacturer. Molecular weight standards were ^{14}C-labeled bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), lactoglobulin (18 kDa), and cytochrome *c* (12 kDa) from New England Nuclear Corp.

Extraction of a plasmid DNA-membrane complex from minicells. A significant modification of the original extraction procedure (13) employed in this study is described in detail. Minicells were isolated from a 6-liter late-log-phase culture of YS1 containing the plasmid under investigation. The minicells were suspended in 5 ml of 0.2 M Tris hydrochloride–0.25 mM EDTA–25% (wt/vol) sucrose (pH 8.0), and 0.6 ml of a fresh lysozyme solution (10 mg/ml) was added, followed immediately by 10 ml of H_2O. This dilution allowed a more efficient penetration of the lysozyme and a maximum yield of spheroplasts as shown by Witholt et al. (36). After incubation for 20 min at room temperature, 3.5 ml of Sarkosyl crystals (prepared by adding equal volumes of 0.1 M magnesium acetate and 3.0% [wt/vol] sodium lauroyl sarcosinate at 0°C) was added. The lysate was then distributed on top of four biphasic sucrose gradients (20% over 50%; 34 ml total) in TMK buffer (0.01 M Tris hydrochloride, 0.01 M magnesium acetate, 0.1 M KCl, pH 7.0) and centrifuged at 14,000 rpm for 30 min at 4°C in a Beckman ultracentrifuge with an SW28 rotor. The DNA-membrane complex (M-band) sedimented at the interface of the biphasic gradient, while the remaining cell extract remained near the top of the gradient (top fraction). The M-band was removed and dialyzed against a buffer consisting of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid), 50 mM KCl, 1 mM dithiothreitol, and 1 mM EDTA, pH 7.5. The dialysate was adjusted to a concentration of 4 mg of protein per ml. The total amount of plasmid DNA ranged from approximately 15 to 20 μg/ml.

Extraction of a plasmid DNA-membrane complex from maxicells. A 50-ml culture of CSR603(pRK2501) was grown, and plasmid-encoded proteins were selectively labeled with ^{14}C-amino acids by the maxicell method. One-fifth of the labeled cells were collected by centrifugation and suspended in 0.2 ml of sample lysis buffer (0.0625 M Tris hydrochloride, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, pH 6.8). This sample served as a whole-cell control. A plasmid DNA-membrane complex was extracted from the remaining labeled maxicells in a manner similar to that described above for minicells. The top fraction and M-band were each dialyzed for 24 h against protein sample buffer (0.0625 M Tris hydrochloride, 5% 2-mercaptoethanol, 10% glycerol, pH 6.8). After dialysis, each fraction was concentrated with polyethylene glycol (25) to a volume of 1.7 ml. The samples (50 μl of whole-cell extract, 100 μl of M-band, and 100 μl of top fraction) were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

It should be pointed out that although most of the bacterial DNA is degraded after being subjected to UV light in the maxicell procedure (23), a contaminating bacterial DNA-membrane complex was present in addition to the plasmid complex. However, this bacterial complex was minimal and did not interfere with the detection of plasmid-specific proteins which was the sole purpose for using this procedure. In addition, comparisons between plasmid complexes extracted from maxicells and minicells showed no difference in their capability of synthesizing plasmid DNA (unpublished data). It was necessary, however, to demonstrate newly synthesized plasmid DNA in maxicell-derived complexes by separating the plasmid DNA from contaminating bacterial DNA by ethidium bromide-CsCl density gradient centrifugation.

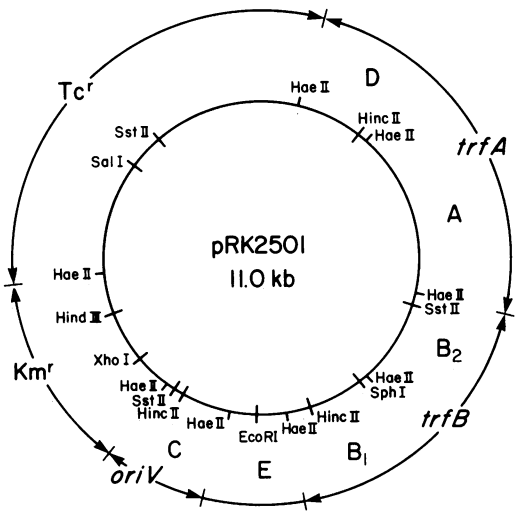

FIG. 2. Physical and genetic map of mini-RK2 plasmid pRK2501. The regions *oriV*, *trfA*, and *trfB* are described in the text and Fig. 1. Letters A, B_1, B_2, C, D, and E refer to *Hae*II restriction fragments. The E fragment is a nonessential RK2 region (56.0- to 0.3-kb region). The Tc^r region is from RK2 (12.7- to 16.0-kb region) and contains many *Hae*II restriction sites which are not shown. The Km^r region is a 1.4-kb *Hae*II fragment derived from Tn*903* (21). The construction of this plasmid has been reported previously (15).

DNA synthesis. The isolated plasmid DNA-membrane complex extracted from minicells was used as the basic component of an in vitro system as described in detail previously (13). In some cases, tRNA (0.2 mg/ml) (type XX; Sigma Chemical Co., St. Louis, Mo.) and all 20 common amino acids (0.01 M each) were added to the assay mixture. DNA synthesis was assayed by incorporation of [^{3}H]dTTP (2 μCi/0.1 ml) (20 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) into an acid-insoluble product over a period of 20 min at 30°C as previously described (13).

When replication products were to be analyzed for semiconservative DNA synthesis by equilibrium density centrifugation, the reaction was scaled up 20-fold, the incubation time was extended to 45 min, bromodeoxyuridine triphosphate replaced dTTP, and dATP was replaced by [^{3}H]dATP (2 μCi/0.1 ml) (25 Ci/mmol; ICN). In addition, because the results described in Fig. 6a showed that tRNA and the mixture of amino acids stimulated DNA synthesis, these components were also added at the same concentrations as that described above.

Equilibrium density centrifugation. Both neutral and alkaline CsCl density gradient centrifugations were used to analyze the details of semiconservative DNA replication. A plasmid DNA-membrane complex was extracted from pRK2501-containing minicells as described above except that the parental plasmid DNA was first prelabeled with [^{14}C]thymine (0.02 μCi/ml of culture) (5 mCi/mmol; New England Nuclear Corp.) during growth of the bacterial culture. After in vitro DNA synthesis by the DNA-membrane complex with bromodeoxyuridine triphosphate and [^{3}H]dATP as described above, plasmid DNA was extracted with buffered phenol as described previously (13). For neutral CsCl density gradients, the radioactive DNA samples were mixed with TES buffer (0.05 M Tris hydrochloride, 0.005 M EDTA, 0.05 M NaCl, pH 8.0), and the density of CsCl was adjusted to 1.725 g/cm^3 (total volume, 5 ml). After centrifugation to equilibrium at 40,000 rpm for 40 h at 20°C in a Beckman type 70.1 Ti rotor, fractions of the gradient were collected, and a portion of each sample was analyzed for density of CsCl by refractometry and detection of ^{3}H and ^{14}C acid-insoluble radioactivity by liquid scintillation techniques. The appropriate fractions from the heavier-density region were pooled (fractions 4 to 10; see Fig. 5), dialyzed to eliminate CsCl, and digested with *Eco*RI to generate unit-length linear molecules of pRK2501 (which has a single *Eco*RI site). The linear DNA molecules were then prepared for alkaline CsCl density gradient centrifugation by mixing the sample with a solution of 0.15 M NaCl–0.015 M sodium citrate–0.005 M EDTA–0.1 M NaOH–0.03% sodium lauroyl sarcosinate and adjusting the density of CsCl to 1.760 g/cm^3 (total volume, 5 ml). After centrifugation to equilibrium at 40,000 rpm for 40 h at 20°C in a type 70.1 Ti rotor, fractions were collected, densities were determined, and each fraction was analyzed for acid-insoluble radioactivity. Recovery of the radioactive counts from each gradient was approximately 85 to 90%.

Double-stranded ^{14}C-labeled light-density pRK2501 marker DNA was prepared as previously described (13). Single-stranded ^{14}C-labeled light-density marker DNA was formed by linearizing the double-stranded marker with *Eco*RI and centrifuging in the alkaline CsCl density gradient.

RNA and protein synthesis. The same assay conditions were used to detect RNA and protein synthesis by the plasmid DNA-membrane complex as described for DNA synthesis except that instead of adding a radioactive DNA precursor, either [^{3}H]UTP (2 μCi/0.1 ml) (20 Ci/mmol; ICN) or a mixture of ^{14}C-amino acids (0.2 μCi/0.1 ml) (1.87 mCi/mg; ICN) was added. After incubation, the samples were processed for detection of acid-insoluble radioactivity as previously described (13). However, in the case of ^{14}C-amino acid incorporation, the samples were first heated at 90°C for 20 min with 10% (wt/vol) trichloroacetic acid to extract nucleic acids. The remaining protein precipitate was washed with 5% trichloroacetic acid and ethanol and then assayed for acid-insoluble radioactivity.

For detection of specific radioactive plasmid-encoded proteins that may have been synthesized by the plasmid DNA-membrane complex in vitro, the reaction was scaled up 10-fold and included tRNA (0.2 mg/ml) and all 20 common amino acids (0.01 M each). After incubation of the complex for 30 min at 30°C with a mixture of ^{14}C-amino acids (8 μCi total) (1.87 mCi/mg; ICN), the reaction mixture was dialyzed overnight against protein sample buffer. The sample was concentrated with polyethylene glycol, and the in vitro-synthesized proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

RESULTS

Synthetic capability of a temperature-sensitive plasmid replication mutant. A derivative of RK2, pMR5, contains a temperature-sensitive mutation in *trfA* which allows replication at 30°C but not at 42°C (22, 32). The kinetics of [^{3}H]dTTP incorporation into acid-insoluble material by a pMR5 DNA-membrane complex (extracted from cells cultured at 30°C) and a control RK2 DNA-membrane complex (extracted from cells cultured at 37°C) was measured at 30 and 42°C (Fig. 3). With the pMR5 complex (Fig. 3a), there was significant incorporation of [^{3}H]dTTP at the permissive temperature, but little incorporation was observed at the restrictive temperature. In contrast, for the RK2 complex (Fig. 3b), there was not only no inhibition of synthetic activity at 42°C, but such activity was actually higher than that observed at 30°C. Although it is a general observation that in vitro replication systems are somewhat thermolabile above 30°C, it may be that the DNA-membrane complex protects the enzymatic environment to a greater extent (possibly because of its hydrophobic character) than do soluble extracts in which such temperature effects have been noted.

These results strongly suggest that the *trfA*-encoded replication protein is present and active under the appropriate conditions in the plasmid DNA-membrane complex and that replication is dependent on this protein. Furthermore, since this protein is involved in the initiation of RK2 replication, it appears that the DNA-membrane complex is involved in the initiation event.

Detection of plasmid-encoded proteins in the DNA-membrane complex. A pRK2501 plasmid DNA-membrane complex was extracted from UV-irradiated whole cells (maxicells) after selectively labeling pRK2501-encoded proteins by the maxicell method. The mini-RK2 plasmid pRK2501 was utilized since we had previously identified all the proteins specified by this plasmid (16). In addition, it expresses the proteins encoded by the *trfA* and *trfB* regions at a higher level than the parental RK2 plasmid (unpublished data), thus facilitating the detection of these proteins. The plasmid-encoded proteins located in the DNA-membrane complex (M-band) and the remaining cell extract (top fraction) are shown in Fig. 4. Most important, the essential A2 replication protein specified by *trfA* was a predominant protein in the DNA-membrane complex (lane 3) and was not

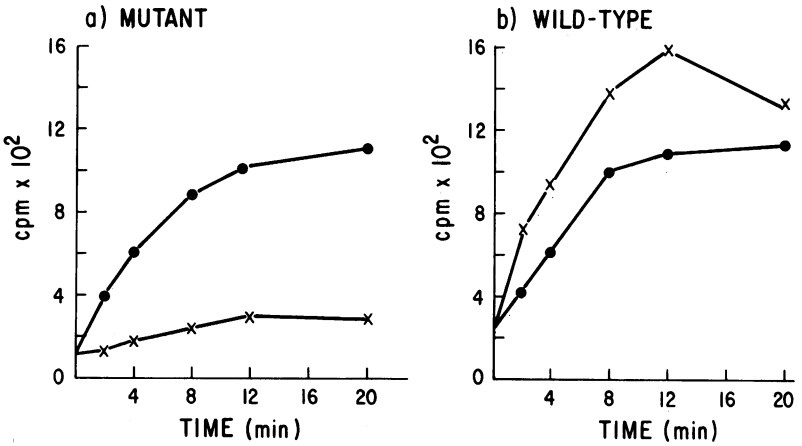

FIG. 3. DNA synthesis by a plasmid DNA-membrane complex. A plasmid DNA-membrane complex was isolated from plasmid-containing minicells and assayed for DNA synthesis by incorporation of [^{3}H]dTTP into an acid-insoluble product as described in Materials and Methods. (a) DNA synthesis by a pMR5 DNA-membrane complex. Plasmid pMR5 is a temperature-sensitive replication mutant of RK2 that contains a conditional mutation in the *trfA* gene (22, 32). Symbols: ●, permissive temperature, 30°C; ×, restrictive temperature, 42°C. (b) DNA synthesis by an RK2 DNA-membrane complex. Symbols: ●, 30°C; ×, 42°C.

detectable in the remaining cell extract (lane 4). Other RK2-specific proteins present in the complex include A1 (encoded by the *trfA* region), which may also have a replication function (16, 27, 28), B1 (encoded by the *trfB* region), which is associated with the expression of incompatibility by RK2 (2, 16, 29), and B2 (encoded by the *trfB* region), which has no known function (2, 16). The third *trfA* region-encoded protein, A3, which has no known function (16, 27, 28), was not present in the complex but instead was located in the remaining cell extract. The 13-kDa protein specified by *korA* (encoded by the *trfB* region), which is involved in the control of RK2 replication and maintenance (1, 2, 8, 26, 29), was not conclusively identifiable in this experiment because it is produced in relatively low quantities and comigrates with the abundantly produced A3 protein. The light protein band at about 13 kDa in the DNA-membrane complex fraction (lane 3) may be the *korA* protein, but we have no evidence for this.

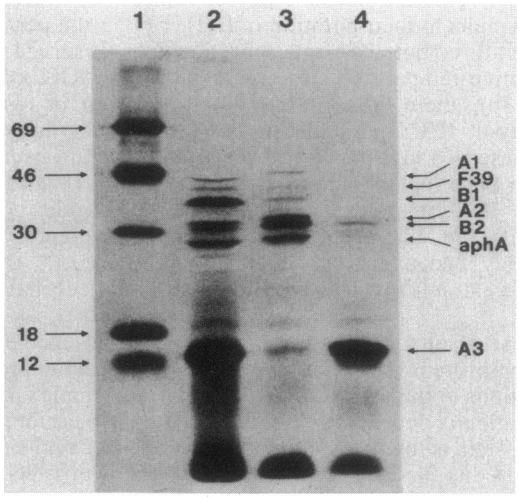

FIG. 4. Distribution of plasmid-encoded proteins in the DNA-membrane complex and remaining cell extract. Mini-RK2 plasmid pRK2501-encoded proteins were radioactively labeled in maxicells, and a DNA-membrane complex was isolated as described in Materials and Methods. The plasmid-specified proteins were separated by electrophoresis through a 12% SDS-polyacrylamide gel and visualized by autoradiography. Lanes: 1, markers; 2, whole-cell extract; 3, DNA-membrane complex (M-band); 4, remaining cell extract (top fraction). Numbers on the left refer to mass (in kilodaltons) of markers. RK2-encoded proteins are designated according to Kornacki et al. (16): A1 (43 kDa), A2 (33 kDa), and A3 (13 kDa) are encoded by the *trfA* region, and B1 (36 kDa) and B2 (30 kDa) are specified by the *trfB* region. F39 (39 kDa) is most likely a fusion product resulting from the juxtaposition of the *trfA* and *trfB* regions in pRK2501 (16). aphA is the aminoglycoside 3′-phosphotransferase encoded by the kanamycin resistance determinant *aphA* (16, 21).

It should be pointed out that this enrichment of plasmid-encoded proteins in the DNA-membrane complex is not due to the presence of more protein in the complex than in the remaining cell extract. Total protein determinations have shown that 70 to 75% is present in the remaining cell extract, whereas only 25 to 30% is associated with the complex (data not shown).

Further analysis of semiconservative DNA replication. Previous results demonstrated that a significant shift in density occurred when newly synthesized DNA extracted from a DNA-membrane complex after incubation with a heavy-density precursor (bromodeoxyuridine triphosphate) was centrifuged in a neutral CsCl density gradient (13). To confirm that this shift is representative of semiconservative DNA replication, it was necessary to determine the composition of the heavier-density product, as well as the percentage of parental molecules involved in the process. After extracting a pRK2501 plasmid DNA-membrane complex from minicells which had been incubated with [^{14}C]thymine to prelabel parental plasmid DNA, the complex was incubated in the usual assay solution with the heavy-density precursor and [^{3}H]dATP. The newly synthesized DNA was then extracted and centrifuged in a neutral CsCl density gradient (Fig. 5a). The appropriate fractions were pooled from the heavier-density region and centrifuged in an alkaline CsCl density gradient after linearizing the DNA with a one-cut restriction enzyme (*Eco*RI) to determine the composition of the heavy-density DNA (Fig. 5b).

The results show that newly synthesized [^{3}H]DNA bands at a heavier density in the neutral CsCl gradient than the bulk of parental [^{14}C]DNA (Fig. 5a). However, because of the probable presence of endogenous dTTP, the substitution of dTMP by bromodeoxyuridine monophosphate was not ex-

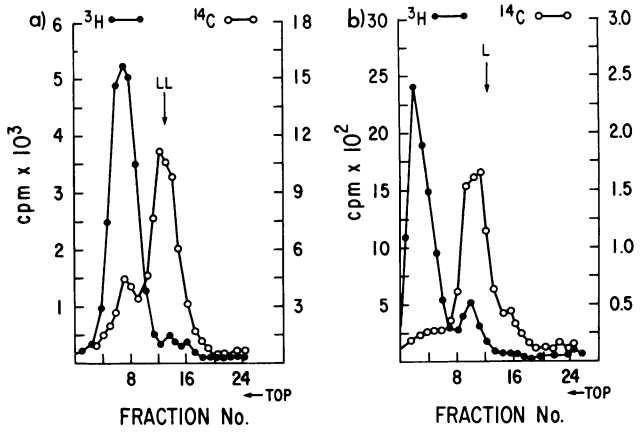

FIG. 5. Semiconservative DNA replication by the DNA-membrane complex. A plasmid DNA-membrane complex was extracted from pRK2501-containing minicells whose DNA had been prelabeled with [^{14}C]thymine. After the complex was incubated with bromodeoxyuridine triphosphate and [^{3}H]dATP in the usual assay solution, plasmid DNA was extracted and centrifuged in a neutral CsCl density gradient. The heavier-density product was removed, treated with *Eco*RI to linearize the DNA, and centrifuged in an alkaline CsCl density gradient. The details of these procedures are described in Materials and Methods. (a) Neutral CsCl density gradient. Symbols: ●, newly synthesized [^{3}H]DNA; ○, parental [^{14}C]DNA. LL indicates position of the light-density double-stranded DNA marker. (b) Alkaline CsCl density gradient. Symbols: ●, newly synthesized single-stranded [^{3}H]DNA; ○, parental single-stranded [^{14}C]DNA. L indicates position of the light-density single-stranded DNA marker.

tensive, and the density shift between the newly synthesized [^{3}H]DNA and light-density [^{14}C]DNA at their peaks was only 18 mg/cm^3 as determined by refractometry. Approximately 15% of the parental [^{14}C]DNA cosedimented with the [^{3}H]DNA in the heavier-density region, indicating that 15% of the parental molecules participated in the synthetic reaction.

After centrifugation of the pooled heavier-density fractions from the neutral gradient in an alkaline CsCl gradient, two major peaks of different densities were observed (Fig. 5b). The heavier-density DNA peak consisted mostly of newly synthesized single-stranded [^{3}H]DNA (90%) and a small shoulder of parental light-density [^{14}C]DNA which extended into the heavier-density area (10%). Similarly, the light-density DNA peak consisted mostly of single-stranded parental [^{14}C]DNA (85%) and a small shoulder of single-stranded [^{3}H]DNA extending from the heavier-density region (15%). Of great significance was the difference in density between the two peaks in the alkaline gradient (38 mg/cm^3 as determined by refractometry) in comparison with that observed in the neutral gradient (18 mg/cm^3). This twofold difference indicates that plasmid DNA synthesized in vitro consists of 85 to 90% heavy- or light-density single-stranded DNA molecules in which 90% of the newly synthesized heavier-density [^{3}H]DNA species is not covalently bound to parental DNA. In contrast, only 10 to 15% of the double-stranded heavier-density DNA represents newly synthesized [^{3}H]DNA covalently bound to parental DNA which is indicative of repair or nonspecific chain extension. These results support the occurrence of one round of semiconservative plasmid DNA replication by the plasmid DNA-membrane complex.

Protein and RNA synthesis by the DNA-membrane complex. Previous results have shown that in vitro replication by the plasmid DNA-membrane complex is inhibited by chloramphenicol, indicating a requirement for protein synthesis (13). To test this idea further, we examined the effects on DNA replication of adding exogenous components normally involved in protein synthesis such as tRNA and amino acids. (It should be pointed out that other well-known cofactors for protein synthesis such as GTP and cyclic AMP are already present in the assay medium.) In addition, assays for RNA and protein synthesis by the complex were also performed in the presence and absence of exogenous tRNA, amino acids, and chloramphenicol. The results are shown in Fig. 6.

It can be seen first (Fig. 6a) that the addition of tRNA and amino acids to the plasmid complex stimulated incorporation of [^{3}H]dTTP into an acid-insoluble product approximately 300% over the unsupplemented complex. Second, Fig. 6b

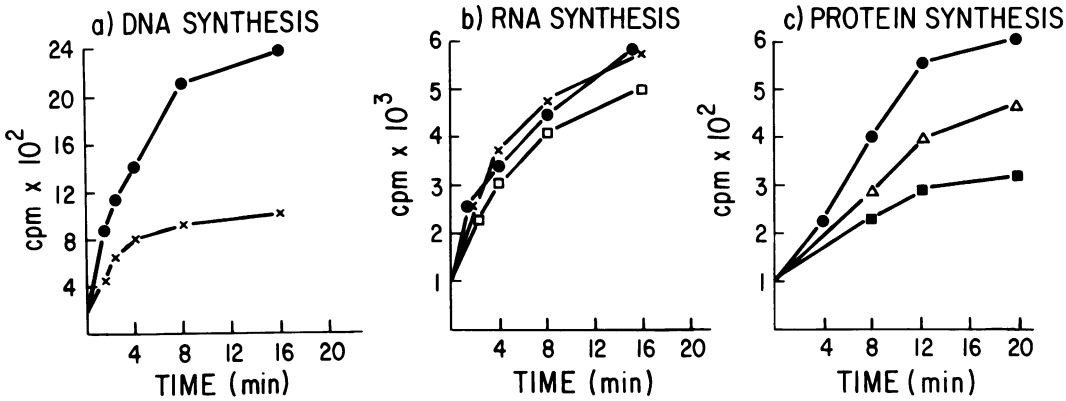

FIG. 6. Incorporation of DNA, RNA, and protein precursors into a pRK2501 DNA-membrane complex. Extraction of the complex, the assay solution, concentration of components, conditions of incubation, and detection of acid-insoluble radioactivity are all described in Materials and Methods. (a) Incorporation of [^{3}H]dTTP into the complex in the presence or absence of tRNA and amino acids. Symbols: ●, with tRNA and amino acids; ×, control. (b) Incorporation of [^{3}H]UTP into the complex in the presence or absence of tRNA, amino acids, and chloramphenicol. Control samples in which the assay solutions were first incubated with 0.3 N NaOH for 3 h at 37°C before acid precipitation showed no incorporation of radioactivity into an acid-insoluble product, indicating that the product was, in fact, RNA (data not shown). Symbols: ●, with tRNA and amino acids; □, with tRNA, amino acids, and chloramphenicol (100 μg/ml); ×, control. (c) Incorporation of a mixture of ^{14}C-amino acids into the complex in the presence or absence of tRNA, amino acids, and chloramphenicol. Symbols: ●, with tRNA and amino acids; △, with amino acids alone; ■, with tRNA, amino acids, and chloramphenicol (100 μg/ml).

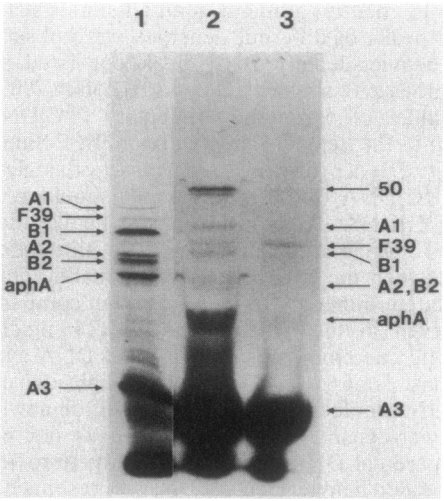

FIG. 7. Detection of plasmid-specific proteins synthesized in vitro by the DNA-membrane complex. A pRK2501 DNA-membrane complex was incubated with ^{14}C-amino acids in the presence or absence of tRNA, and the radioactive proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography as described in Materials and Methods. Lanes: 1, pRK2501-encoded proteins synthesized in maxicells; 2, proteins synthesized in vitro by the pRK2501 DNA-membrane complex in the presence of tRNA; 3, proteins synthesized in vitro by the pRK2501 DNA-membrane complex in the absence of tRNA. Although lanes 1 to 3 are from the same gel, lane 1 represents a shorter autoradiographic exposure time because this sample was more heavily labeled. The remaining two lanes were exposed for exactly the same amount of time. Plasmid pRK2501-encoded proteins are designated as in Fig. 4 (arrows on the left point to proteins synthesized in vivo [lane 1] and arrows on the right point to proteins synthesized in vitro [lane 2 and 3]). The protein bands in the in vitro-synthesized samples (lanes 2 and 3) are somewhat distorted. The middle of the band migrated faster than each of the extremities, thus producing a "smile" pattern. Although it is not certain what caused this distortion, it may be due to the detergent Sarkosyl which was present in the two samples.

shows that substantial acid-insoluble incorporation into the plasmid complex occurred when [^{3}H]UTP was used as the precursor and that the addition of tRNA plus amino acids had no detectable effect on such incorporation. Adding chloramphenicol did affect incorporation slightly, but not nearly as much as it did [^{3}H]dTTP incorporation for which the inhibition was substantial (13). Finally, Fig. 6c shows that incorporation of a mixture of ^{14}C-amino acids into the plasmid complex did occur in the presence or absence of tRNA but that the rate and extent of incorporation was greater in the presence of tRNA. The addition of chloramphenicol significantly inhibited ^{14}C-amino acid incorporation to below either of the above levels in a manner similar to that of the inhibition of [^{3}H]dTTP incorporation (13).

Synthesis of plasmid-encoded proteins by the DNA-membrane complex. A pRK2501 plasmid DNA-membrane complex was incubated with a mixture of ^{14}C-amino acids in the presence and absence of tRNA, and the in vitro-synthesized radioactive proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The results (Fig. 7) show that pRK2501-specified proteins were synthesized by the complex. In addition, the level of protein synthesis was much higher in the presence of tRNA than in the absence of tRNA (compare lane 2 with lane 3), in a manner similar to that observed with the incorporation of ^{14}C-amino acids into an acid-insoluble product (Fig. 6c). Most of the proteins synthesized in vitro were the same as those produced by the plasmid in vivo (compare lane 1 with lane 2). The in vitro-synthesized proteins that are clearly identifiable are A1, F39, B1, aphA, and A3. A broad band is observed in the region where the A2 and B2 proteins migrate, but it was not possible to resolve these proteins in this experiment. A 50-kDa protein that was synthesized in vitro was not observed in vivo. We do not know the nature of this protein. One possibility is that the protein represents a product that is subsequently processed in vivo and that this processing is inefficient in vitro. Nevertheless, these results demonstrate that the plasmid DNA-membrane complex is capable of in vitro synthesis of specific plasmid-encoded proteins.

DISCUSSION

Seven distinct observations support the conclusion that a plasmid RK2 DNA-membrane complex is capable of initiating and completing a round of plasmid DNA synthesis in vitro. This conclusion is based on the combined results of the present and previous studies (11, 13), because together they represent compelling evidence for the significance of the DNA-membrane complex in plasmid replication.

First, DNA synthesis by the RK2 DNA-membrane complex is dependent on the essential RK2 replication gene *trfA*. A complex extracted from a mutant of RK2 which contains a temperature-sensitive mutation in *trfA* displayed extensive DNA synthesis at the permissive temperature but little activity at the restrictive temperature. In contrast, a wild-type RK2 complex showed no inhibition of DNA synthesis at the restrictive temperature. These results indicate that the essential *trfA*-encoded replication protein, which is involved in the initiation of RK2 replication, is present and active in the complex. A minor complication with the results is the low level of synthesis by the mutant complex at the restrictive temperature. One possible reason for this synthesis is that the mutant complex was extracted from cells cultured at 30°C. Thus, at the time of extraction, some membrane-associated plasmid molecules could have been in various stages of chain completion. Consequently, a portion of the synthesis observed in vitro may represent elongation of preexisting replication forks. Additionally, the low level of synthesis at the restrictive temperature may reflect the small amount of DNA repair or nonspecific chain extension suggested by the present studies (Fig. 5). Nevertheless, the important point is that the majority of DNA synthesis was temperature sensitive which indicates that it is *trfA* dependent.

Second, the essential *trfA*-encoded A2 replication protein is located in the DNA-membrane complex, but not in the remaining cell extract. In addition, several other proteins which may be involved in the control of RK2 replication are also present in the complex. These include A1, which is specified by the *trfA* operon (16, 27, 28), and proteins B1 and B2, which are encoded by the *korA-korB* operon (2, 16, 29).

Third, specific plasmid RK2-encoded proteins are synthesized in vitro by the DNA-membrane complex that may be used directly for initiation of replication (see below). Although a question remains concerning the resolution of the *trfA*-encoded A2 replication protein, another protein synthesized in greater amounts than A2 (A1; Fig. 7, lane 2) may also be involved in the initiation of RK2 replication; both proteins are encoded by the *trfA* operon, and nucleotide sequencing studies have shown that the A1 and A2 proteins

are encoded by sequences which overlap in the same reading frame (16, 27, 28). Thus, although A2 is sufficient for RK2 replication in *E. coli* (16, 27), it is possible that A1 also acts to initiate replication.

Fourth, density-shift experiments with the DNA-membrane complex indicate that DNA synthesized in the presence of a heavy-density precursor undergoes one round of semiconservative DNA replication. However, because the substitution of the normal precursor by the heavy-density precursor was not extensive, and because multiple rounds of replication were not detected, further confirmation of this mode of synthesis is required. The lack of fully heavy-density DNA indicates that reinitiation did not occur preferentially after one round of replication. This may be due to, among other reasons, the instability of replication components over long incubation times in vitro, the presence of inhibitory components in the complex, the inability of the A2 replication protein to efficiently recognize an origin that may have dTMP substituted (albeit at a low level) by bromodeoxyuridine monophosphate, or the inability of the two progeny plasmids to segregate as a prerequisite for reinitiation.

Fifth, previous results (13) indicate that RNA polymerase and transcription are involved in DNA replication by the plasmid DNA-membrane complex. It was shown that replication by the complex is inhibited by rifampin in a rifampin-sensitive strain but not in a rifampin-resistant mutant. We have since found that streptovaricin will act in the same way (unpublished data). Both of these antibiotics affect the beta subunit of RNA polymerase (17). Therefore, the antibiotics may inhibit replication by (i) blocking initiation of replication by preventing primer RNA synthesis or (ii) inhibiting transcription of RK2-encoded products required for the initiation of replication (as suggested by the present experiments; see below), or both.

Sixth, plasmid DNA synthesis by the DNA-membrane complex was shown previously to require, in part, ribonucleoside triphosphates, the immediate precursors for RNA synthesis (13). This is consistent with the notion that RNA polymerase and transcription are involved in the DNA synthetic reaction.

Seventh, previous results demonstrated that a significant fraction of the plasmid DNA extracted from the DNA-membrane complex after incubation contains displacement or D loops in the *oriV* region which have properties of an early-replicating intermediate (11). The frequency of these loops was reduced significantly upon the addition of rifampin (11).

Some of the points discussed above have issues that remain to be clarified. Nevertheless, they all tend to support the conclusion that the plasmid RK2 DNA-membrane complex can initiate and complete the synthesis of plasmid DNA in vitro.

We have previously shown that in vitro replication by the plasmid RK2 DNA-membrane complex is inhibited by chloramphenicol, indicating a requirement for protein synthesis (13). Thus, since the complex is extracted by a procedure which may preserve polysomes and charged tRNA molecules (35), we postulated (i) that there may be a coupling of replication to transcription and translation in which the essential *trfA*-encoded replication protein and other plasmid proteins involved in RK2 replication are actually synthesized de novo and (ii) that protein synthesis inhibitors such as chloramphenicol inhibit replication by inhibiting the synthesis of these proteins. The following results of the present study support this hypothesis: (i) the inhibitory effects of chloramphenicol on both DNA and protein synthesis by the complex; (ii) the stimulation of replication by components normally required for protein synthesis (tRNA and all the common amino acids); (iii) the synthesis of RNA and protein by the complex; and (iv) the synthesis of specific RK2-encoded proteins. In addition, previous results showing inhibition of DNA synthesis by rifampin indicate that transcription is required for in vitro replication (13). Thus, these results suggest that in vitro replication of plasmid RK2 by the DNA-membrane complex is dependent on transcription and translation of RK2-encoded products. Although there are a number of problems that remain to be resolved, such as whether polypeptide chain completion is occurring rather than de novo protein synthesis, the important point is that specific RK2-encoded proteins are synthesized in vitro by the complex that may be used subsequently for initiation of replication.

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