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Replication of Antibiotic Resistance Plasmid R6K DNA in Vitro†

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ABSTRACT: A soluble extract prepared from cells of an *Escherichia coli* strain carrying the antibiotic resistance plasmid R6K is capable of carrying out the complete process of R6K DNA replication. DNA synthesis in vitro is dependent on the four deoxyribo- and ribonucleotide triphosphates and is sensitive to rifampin and streptolydigin, inhibitors of DNA-dependent RNA polymerase. The incorporation of deoxyribonucleotides into R6K DNA also is sensitive to actinomycin D, novobiocin, arabinofuranosyl-CTP, and *N*-ethylmaleimide. Kinetics of synthesis are linear for 60 to 120 min. Replication proceeds semiconservatively and supercoiled closed-circular DNA molecules are synthesized. Analysis by alkaline sucrose gradient centrifugation indicated that the early R6K DNA products contain DNA fragments of approximately 18 S in size, corresponding to the length between the R6K α origin of replication and the terminus of replication observed in vivo. Addition of exogenous supercoiled R6K DNA is inhibitory to the in vitro system, whereas the addition of R6K DNA in the form of relaxation complex stimulates R6K DNA synthesis to a small extent.

Bacterial plasmids, including the antibiotic resistance (R) plasmids, are circular duplex DNA molecules that are present stably in the extrachromosomal state in host cells. These elements have the fundamental character of autonomous replication as covalently closed circular DNA molecules and segregation to the daughter cells of the host bacterium (Clowes, 1972; Helinski, 1973, 1976). Plasmid DNA replication is a complex process that proceeds through several successive steps: initiation of DNA replication, semiconservative DNA synthesis, termination and segregation of daughter strands. An important approach to the study of the complex process of DNA replication is the systematic analysis of a replication system in vitro (Kornberg, 1974; Geider, 1976). With regards to plasmid replication, soluble systems have been derived from *Escherichia coli* cells that are capable of carrying out the replication of the plasmid ColE1 DNA (Sakakibara & Tomizawa, 1974a; Tomizawa et al., 1975; Staudenbauer, 1976). In these systems, cell extracts are made from chloramphenicol-treated *E. coli* cells and no plasmid-coded protein is required for ColE1 replication.

Plasmid R6K is a naturally occurring conjugative plasmid that determines resistance to the antibiotics ampicillin and streptomycin (Kontomichalou et al., 1970). The molecular weight of R6K is 25×10^6, and it exhibits a relaxed mode of replication in *E. coli* cells in that it is present as 11 to 13 copies per chromosome in log-phase cells (Kontomichalou et al., 1970). The mode of replication is unique in that it is bidirectional and proceeds sequentially to an asymmetric terminus from either of two possible origins (α and β) (Lovett et al., 1975; Crosa et al., 1975, 1976). It has also been reported that catenated molecules of this DNA are replicative intermediates (Kupersztoch & Helinski, 1973) and replicating R6K DNA molecules are found preferentially associated with the folded chromosome of *E. coli* (Wlodarczyk & Kline, 1976). Of the known host replication functions, at least the *dnaB, dnaC, dnaD* and *polC* (*dnaE*) genes are required for R6K DNA replication (Arai & Clowes, 1975). Finally, a large proportion of R6K DNA molecules can be isolated from cells in the form of a relaxation complex (Kupersztoch-Portnoy et al., 1974). The unique bidirectional mode of replication in vivo and other replication properties make R6K especially attractive for studying the molecular mechanism and regulation of replication of plasmid DNA in vitro. In this paper the properties of a cell-free extract system derived from *E. coli* cells and capable of replicating supercoiled R6K DNA are described.

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Materials and Methods

Bacterial Strains. E. coli KE346 was derived by transformation of strain YS1 (Tomizawa et al., 1975) with plasmid R6K DNA using the procedure of Cohen et al. (1972). YS1 is *thr leu tonA lacY minA supE gal minB end str thi* and was kindly supplied by Y. Sakakibara. KE348 is a *thyA deo* derivative of KE346 obtained by trimethoprim selection (Miller, 1972).

Materials. Deoxyribonucleoside and ribonucleoside, 5′-triphosphate (dNTPs and rNTPs), novobiocin, nalidixic acid, streptolydigin, chloramphenicol, *ara*-CTP,[1] NEM, NAD, cAMP, EGTA, and Brij 58 were purchased from Sigma. Bromodeoxyuridine 5′-triphosphate (BrdUTP) was obtained from P-L Biochemicals, Inc. [*methyl*-^{3}H]dTTP (50 Ci/mmol), [α-^{32}P]dTTP (50 Ci/mmol), [α-^{32}P]dATP (140 Ci/mmol), and [*methyl*-^{3}H]- and [*methyl*-^{14}C]thymine were from New England Nuclear Corp. Rifampin, actinomycin D, Pronase, and Hepes buffer were obtained from Calbiochem. *Bam*H1 endonuclease was purchased from New England BioLabs.

Preparation of Extracts. Extracts were prepared according to a modified procedure of Staudenbauer (1976). Cells carrying the R6K plasmid were grown at 37 °C with shaking in 750 mL of Penassay broth (Difco) to a cell density of about 2×10^8 cells/mL. The culture was centrifuged at 7000 rpm for 5 min in a Sorval GSA rotor at room temperature, washed with 25 mM Hepes (pH 8.0)–5 mM EGTA buffer, and then resuspended with 2.6 mL of the same buffer and quickly frozen in a liquid nitrogen bath. The frozen cells were thawed first at 0 °C and then at 7 °C, and 0.26 μL of 2 M KCl and 88 μL of 15 mg/mL egg white lysozyme (Sigma) were added successively to the cells. The mixture was then incubated at 0 °C for 30 min. The suspension was frozen in a liquid nitrogen bath, thawed at 10 °C, and immediately put in ice. The lysate was centrifuged at 135 000*g* for 30 min in an SW 50.1 rotor at 0 °C. The supernatants of the centrifugation, designated cell extracts, contained approximately 15 mg/mL of protein and 50 μg/mL of DNA. The cell extracts were divided into small portions and stored in a liquid nitrogen bath for at least 2 weeks without loss of R6K DNA synthetic activity. This amount of DNA corresponded to approximately 20% of the total amount of covalently closed circular (ccc) R6K DNA, as determined by ethidium bromide–CsCl density gradient centrifugation of sarkosyl lysates of KE348. This cell extract was used for the in vitro replication studies unless otherwise stated. In some experiments, cell extracts were prepared according to the procedure of Sakakibara & Tomizawa (1974a).

Assay of DNA Synthesis. The standard reaction mixture (50 μL) contained 30 mM Hepes buffer (pH 8.0), 28 mM KCl, 7.5 mM $MgCl_2$, 0.1 mM NAD, 0.1 mM cAMP, 2 mM rATP, 0.5 mM each of rGTP, rCTP, and rUTP, 0.02 mM each of dATP, dGTP, dCTP, and [^{3}H]dTTP or [α-^{32}P]dTTP, and 30 μL of the cell extract. The cell extract contained approximately 10 mM Hepes buffer and 52 mM KCl. After the incubations were carried out at 30 °C for a suitable time, synthesis was terminated by the addition of 2 mL of cold 10% trichloroacetic acid–0.1 M sodium pyrophosphate (PP_i) solution. Acid-insoluble precipitates were collected on a Whatman GF/C glass filter and washed successively with 5 mL of cold trichloroacetic acid–PP_i (two times), cold 0.01 N HCl, and cold ethanol. Radioactivity on the dried filters was determined in a toluene-based solvent in a liquid scintillation counter. Density labeling mixtures were standard reaction mixtures except that the four dNTPs were replaced by 0.05 mM each of BrdUTP, dGTP, dCTP, and [α-^{32}P]dATP.

In those experiments where the replication products were characterized, the reaction was stopped by the addition of 20 mM EDTA and 1.5 mg/mL of self-digested Pronase was added to inactivate relaxation complex of R6K (Kupersztoch-Portnoy et al., 1974). The mixture was incubated at 30 °C for 15 min followed by the addition of 0.25% sodium dodecyl sulfate and further incubation at 30 °C for 10 min. After the addition of 0.1 volume of 0.5 M Tris (pH 8.0)–0.05 M EDTA–0.5 M NaCl, the reaction mixture was gently inverted with an equal volume of chloroform–isoamyl alcohol (24:1 v/v) and centrifuged at 12 000 rpm for 10 min in an SS34 rotor. The aqueous phase was then dialyzed against TES buffer consisting of 0.05 M Tris (pH 8.0)–0.005 M EDTA–0.05 M NaCl unless otherwise stated.

Preparation of Reference R6K DNA. Strain KE346 or KE348 was cultured in M9-glucose medium supplemented with 0.5% casamino acid, 5 μg/mL of thiamine, 2 μg/mL of thymine, and 6 μCi/mL of [^{3}H]thymine or -thymidine, or 0.5 μCi/mL of [^{14}C]thymine. For KE346, deoxyadenosine was added at the concentration of 250 μg/mL to stimulate the incorporation of labeled thymidine. Closed circular R6K DNA was prepared by ethidium bromide–CsCl density gradient centrifugation of sarkosyl lysate of the cells (Bazaral & Helinski, 1968; Figurski et al., 1976).

Sedimentation Analysis. Linear 5 to 20% sucrose gradients (4.4 mL) were prepared in cellulose nitrate tubes on the top of 0.3 mL of a CsCl-saturated 20% sucrose shelf. Neutral sucrose gradients contained 0.05 M Tris (pH 8.0), 0.005 M EDTA and 0.5 M NaCl and alkaline sucrose gradients contained 0.3 M NaOH, 0.001 M EDTA, and 1.0 M NaCl. Samples of 100 to 250 μL were layered on each gradient and centrifuged in an SW 50.1 rotor at 45 000 rpm for 55 min at 15 °C or for 35 min at 15 °C for neutral or alkaline sucrose gradients, respectively, unless otherwise indicated.

Equilibrium Centrifugation. For neutral CsCl density gradient analysis, samples were mixed with TES buffer and the density was adjusted to 1.728 g/cm^3. For alkaline CsCl density gradient analysis, the sample was first digested with *Bam*HI restriction enzyme at 37 °C for 4 h in 0.006 M Tris (pH 7.5)–0.05 M NaCl–0.006 M $MgCl_2$–0.006 M β-mercaptoethanol to generate unit length linear molecules of R6K DNA. *Bam*HI cleaves R6K DNA at a single site (R. Kolter & D. Helinski, manuscript submitted for publication). The linear molecules were then mixed in 0.15 M NaCl–0.015 M sodium citrate–0.005 M EDTA containing 0.1 M NaOH and 0.03% sodium *N*-lauroylsarcosinate. The density was adjusted to 1.76 g/cm^3 and the total volume was brought to 4.5 mL. After centrifugation to equilibrium at 36 000 rpm for 40–60 h at 15 °C in an SW 50.1 rotor, fractions were collected from the bottom of the tube and acid-insoluble radioactivity was determined as described above. Recovery of the input counts from each gradient was usually greater than 80%.

Preparation of R6K Relaxation Complex. KE348 cells were grown in 250 mL of M9 medium containing 0.5% casamino acid, 0.1% glucose, 5 μg/mL thymine, and [^{3}H]thymine (6 μCi/mL). The cells were harvested after the cell density reached 3×10^8 cells/mL. Lysis of the cells was carried out with Triton X-100 (Kupersztoch-Portnoy et al., 1974) and a

[1] Abbreviations used: ccc, covalently closed circular; oc, open circular; NAD, nicotinamide adenine dinucleotide; cAMP, cyclic adenosine 3′,5′-monophosphoric acid; BrdUTP, bromodeoxyuridine 5′-triphosphate; *ara*-CTP, 1-β-arabinofuranosylcytosine 5′-triphosphate; NEM, *N*-ethylmaleimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; sarkosyl, sodium *N*-lauroylsarcosinate; TES, 0.05 M Tris (pH 8.0)–0.005 M EDTA–0.05 M NaCl.

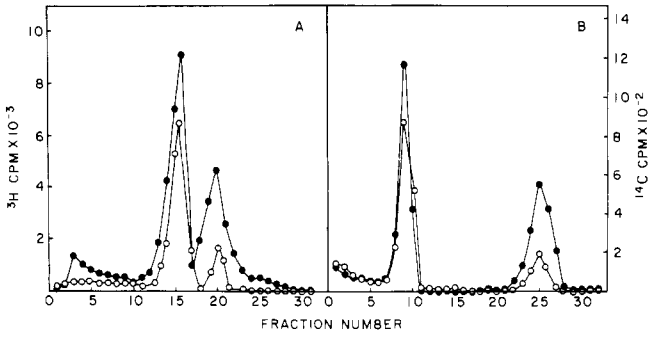

FIGURE 1: Characterization of DNA in the cell extract. Strain KE346 carrying the R6K plasmid was grown at 37 °C with [^{3}H]thymidine (0.5 μCi/mL) and deoxyadenosine (250 μg/mL) to 2 × 10^8 cells/mL. The cell extract was prepared as described in Materials and Methods. Fifty microliters of the cell extract was pretreated with 2 mg/mL Pronase at 30 °C for 10 min in the presence of 10 mM EDTA. This was centrifuged in a neutral (A) and an alkaline (B) sucrose gradient with ^{14}C-labeled R6K DNA. The fractions were collected from the bottom of the gradient and acid-insoluble counts were determined. Recovery of ^{3}H and ^{14}C counts was more than 80% in each case. (● — ●) ^{3}H-labeled DNA; (○ — ○) ^{14}C-labeled reference R6K DNA.

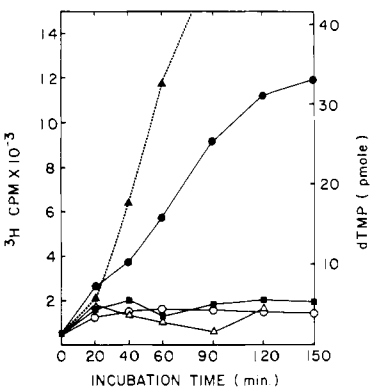

FIGURE 2: Kinetics of incorporation of [^{3}H]dTMP and the effect of the addition of rifampin, ccc-R6K DNA, ccc-ColE1 DNA, and rATP. DNA synthesis was assayed by incubating 0.3 mL of the standard reaction mixture containing [^{3}H]dTTP (● — ●) in the presence of rifampin (10 μg/mL) (○ — ○), ccc-R6K DNA (15 μg/mL) (■ — ■), or ccc-ColE1 DNA (15 μg/mL) (▲ - - - ▲), and in the absence of rATP (△ — △). At the times indicated, 50-μL portions were withdrawn.

cleared lysate was prepared to remove the bulk of chromosomal DNA by spinning at 16 000 rpm for 20 min in an SS34 rotor. The cleared lysate was layered on a linear gradient of 15 to 50% sucrose in 0.01 M Tris (pH 8.0)–0.005 M EDTA–0.06 M KCl and centrifuged at 22 500 rpm for 240 min at 4 °C in an SW 27 rotor. The fast sedimenting portion of the R6K DNA peak was collected and dialyzed against TES. The complex was concentrated by precipitation with 0.3 M potassium acetate and 2.5 volume of ethanol which were added together to prevent the dissociation of relaxation protein from R6K DNA with high salt. This purified complex showed about 30% relaxability after treatment with 0.25% sodium dodecyl sulfate at 25 °C for 10 min.

Results

Properties of the R6K DNA Synthesis System. DNA in cell extracts prepared from cells of the KE346 strain carrying the R6K plasmid was found to consist almost entirely of covalently closed circular (ccc) molecules and open circular (oc) molecules of R6K DNA, and of no chromosomal DNA fragment when analyzed by neutral and alkaline sucrose gradient centrifugation (Figure 1). Similar results were obtained by ethidium bromide–CsCl density gradient centrifugation analysis of the cell extract. The kinetics of DNA synthesis in vitro utilizing this cell extract are shown in Figure 2. The rate of DNA synthesis was approximately 0.6 pmol of dTMP per min per mg of protein at 30 °C and the incorporation continued for 60 min to 150 min. Magnesium ion was required for R6K DNA synthesis in vitro. In the absence of added $MgCl_2$, no detectable incorporation was observed (Table I). A maximum rate of the synthesis was obtained with 7.5 mM $MgCl_2$. More than 15 mM $MgCl_2$ was inhibitory, with the addition of 20 mM $MgCl_2$ resulting in an 85% reduction in the DNA synthesis. Optimal synthesis was observed in the range of 80 to 100 mM KCl. When 200 mM KCl was added to the reaction, the incorporation was almost completely blocked. The addition of 0.1 mM NAD increased DNA synthesis approximately 1.3 times; however, dithiothreitol (up to 5 mM) or cAMP (0.1 mM) had no detectable stimulatory effect.

DNA synthesis required rATP (Figure 2 and Table I) and was inhibited 84% at 10 μg/mL and completely at 100 μg/mL of novobiocin, a potent inhibitor of semiconservative DNA replication in vitro (Staudenbauer, 1975) and DNA gyrase (Gellert et al., 1976) (Table II). These results indicated that the incorporation of dTMP was not ATP-independent DNA repair or ATP-dependent DNA repair as after UV irradiation, but represented semiconservative DNA replication.

Replication required deoxyribonucleotide and ribonucleotide triphosphates (Figure 3). Minimum concentration of the four dNTPs was 20 μM each and minimum concentration of rGTP, rCTP, and rUTP was 0.5 mM each in the presence of 2 mM rATP. Bovine serum albumin was added to stabilize the system

TABLE I: Requirements for R6K DNA Synthesis in Vitro.[a]

Additions	Activity (%)
Complete	100
—$MgCl_2$	2
—KCl[b]	8
—dATP, dGTP, dCTP	15
—rATP	5
—rGTP, rCTP, rUTP	22
—NAD	78

[a] DNA synthesis was measured in the standard reaction mixture (50 μL) described in Materials and Methods. One hundred percent activity corresponded to 10.4 pmol of dTMP incorporation for 60 min. [b] Although the reaction mixture is not supplemented with KCl in this case, 30 mM KCl is provided by the cell extract.

TABLE II: Effect of Inhibitors on R6K DNA Synthesis.[a]

Inhibitor	[^{3}H]TMP Incorp (cpm)	Rel %
None	2127	100
Rifampin, 20 μg/mL	336	16
Rifampin, 100 μg/mL	95	4
Streptolydigin, 100 μg/mL	441	21
Actinomycin D, 2.5 μg/mL	293	14
Novobiocin, 10 μg/mL	331	16
Novobiocin, 100 μg/mL	51	2
Nalidixic acid, 100 μg/mL	2115	99
Nalidixic acid, 500 μg/mL	1336	63
ara-CTP, 0.25 mM	416	20
NEM, 10 mM	178	8
Chloramphenicol, 0.2 mM	1616	76

[a] DNA synthesis was carried out in the standard reaction mixture with or without the inhibitor for 60 min.

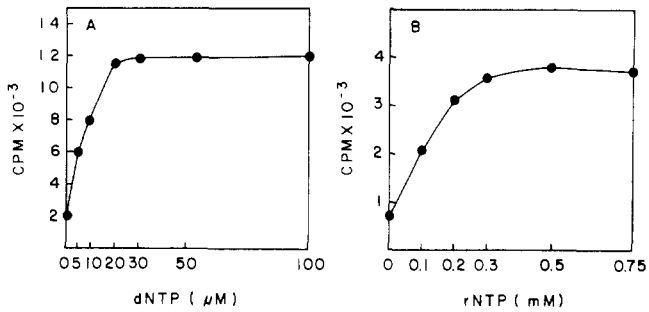

FIGURE 3: Effect of concentration of dNTP and rNTP on the activity of the in vitro system. Reactions were carried out for 60 min in the standard reaction mixture (50 μL) except that the concentrations of dATP, dGTP, dCTP, and [^{3}H]dTTP were varied as indicated (A), and the concentrations of rGTP, rCTP, and rUTP were varied as indicated in the presence of 2 mM rATP (B).

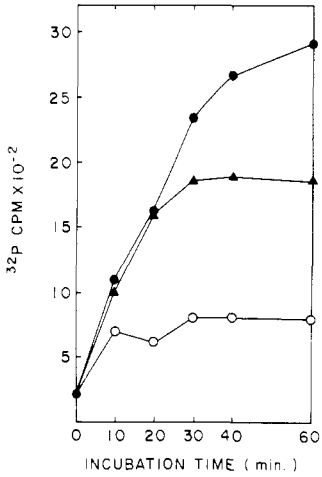

FIGURE 4: Effect of rifampin on R6K DNA synthesis. The reaction conditions were as described in the legend to Figure 2 except that [α-^{32}P]dATP was used instead of [^{3}H]dTTP. Rifampin (20 μg/mL) was either not added (●) or added at 0 (○) and 10 min (▲) after incubation.

and to minimize the adherence of enzymes and DNA to the wall of the reaction tube.

DNA synthesis was markedly stimulated by the addition of ccc-ColE1 DNA. On the other hand, it was almost completely inhibited by the addition of 15 μg/mL ccc-R6K DNA as shown in Figure 2. In contrast, the addition of 10 μg/mL of ccc-R6K DNA in the form of relaxation complex, prepared by sedimentation in a sucrose gradient, stimulated the level of R6K DNA synthesis 1.3-fold.

Effect of Inhibitors of Macromolecular Synthesis. The effect of various agents on R6K DNA synthesis is shown in Table II. Incorporation of [^{3}H]TMP was strongly inhibited by rifampin and streptolydigin which act on DNA-dependent RNA polymerase. Actinomycin D, which inhibits the template activity of DNA (Hartman et al., 1968), also showed a marked inhibitory effect. Novobiocin, a potent inhibitor of semiconservative DNA replication (Staudenbauer, 1975) and DNA gyrase (Gellert et al., 1976), blocked R6K DNA synthesis. High concentration of nalidixic acid (500 μg/mL) had only a partial effect on synthesis. Arabinosyl CTP, which inhibits the activity of DNA polymerase II and III of *E. coli* (Reddy et al., 1971), and *N*-ethylmaleimide, which is an SH-blocking agent, exerted a strong inhibitory effect. Chloramphenicol addition resulted in only a small reduction of R6K DNA synthesis.

It had been reported that spermidine and glycerol enhanced

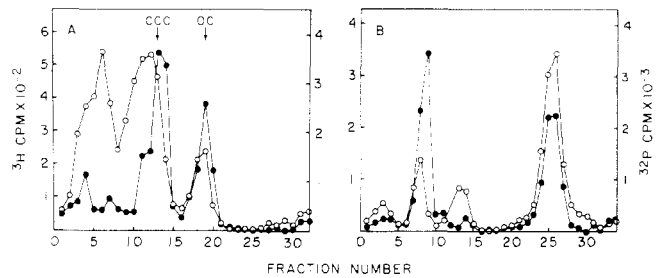

FIGURE 5: Sedimentation analysis of R6K DNA synthesized in vitro. DNA was synthesized for 120 min in the standard assay condition with [α-^{32}P]dATP and ^{3}H-labeled R6K DNA in the cell extract prepared as described in the legend of Figure 1. After extraction of DNA as described in Materials and Methods, 0.2 mL of sample was analyzed in a neutral (A) and an alkaline (B) sucrose gradient. (● — ●) ^{3}H-labeled parental DNA; (○ -- ○) ^{32}P-labeled newly synthesized DNA in vitro. The arrows indicate the positions of the peaks corresponding to ccc (51 S) and oc (38 S) R6K DNA.

the formation of early replicative intermediates of ColE1 DNA in vitro (Sakakibara & Tomizawa, 1974b). Spermidine stimulated R6K DNA synthesis 1.2- to 1.5-fold at a concentration of 0.3 mM while more than 2 mM was inhibitory. On the other hand, glycerol at concentrations of 2 to 10% showed a strong inhibitory effect.

Effect of Rifampin on R6K DNA Synthesis. As described above, R6K DNA synthesis in vitro requires nascent RNA synthesis, since synthesis requires four rNTPs and is susceptible to rifampin, streptolydigin, and actinomycin D which interfere with RNA synthesis (Figures 1 and 3 and Tables I and II). The results in Figure 4 show that the amount of incorporation of TMP was dependent on the time of addition of rifampin to the reaction mixture. When rifampin was added at 0 time, residual synthesis, possibly representing DNA chain elongation of initiated molecules in the cell extract, was observed. When rifampin was added at 10 min, DNA synthesis continued for more than 10 min at almost the same rate as that without rifampin followed by a decreased rate of incorporation and cessation. These results indicate that in this system the rifampin-sensitive step is completed for a number of molecules within the 10-min period. Analysis of the plasmid DNA products synthesized in the presence of rifampin indicated an increased ratio (approximately two times) of completely replicated molecules of plasmid DNA upon comparison with the results in the absence of rifampin.

Characterization of the Plasmid DNA Product Synthesized in vitro. ^{32}P-labeled DNA, synthesized in the reaction mixture containing ^{3}H-labeled R6K DNA as a template, was analyzed by neutral and alkaline sucrose gradient centrifugations. In a neutral sucrose gradient (Figure 5A), the ^{32}P-labeled DNA sedimented as 65 to 75S molecules in the size, and a broad peak of 53S DNA and a peak corresponding to oc-R6K DNA were in the proportions of 39%, 49%, and 12%, respectively. The broad peak of DNA sedimenting as 65S to 70S possibly contained the replicative intermediates observed in vivo that have been characterized as 69S molecules (Kupersztoch & Helinski, 1973; Kline, personal communication). In an alkaline sucrose gradient (Figure 5B), the DNA product showed three main peaks. The faster sedimenting peak (16% of the total) cosedimented with ^{3}H-labeled closed circular R6K DNA. The proportion of ccc-DNA in the total was up to 30% in other experiments. The sedimenting property of the intermediate peak DNA (14% of the total) is characteristic of an R6K catenated molecule composed of a circular single-stranded monomeric subunit interlocked with a denatured, covalently closed monomeric unit (Kupersztoch & Helinski, 1973). The slowest

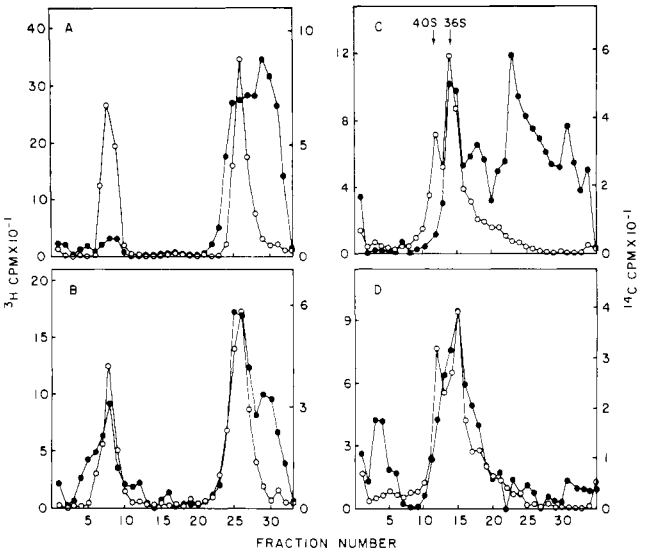

FIGURE 6: Alkaline sucrose gradient analysis of R6K DNA synthesized for 20 min or 60 min in vitro. The standard reaction mixture (0.15 mL) was incubated with [^{3}H]dTTP for 20 min (A and C) or 60 min (B and D). DNA was centrifuged in alkaline sucrose gradient at 45 000 rpm for 35 min (A and B) or 95 min (C and D). In C and D, 40S and 36S molecules of ^{14}C-labeled reference represent circular and linear single strands of R6K DNA, respectively. (● — ●) ^{3}H-labeled DNA; (○ — ○) ^{14}C-labeled reference DNA.

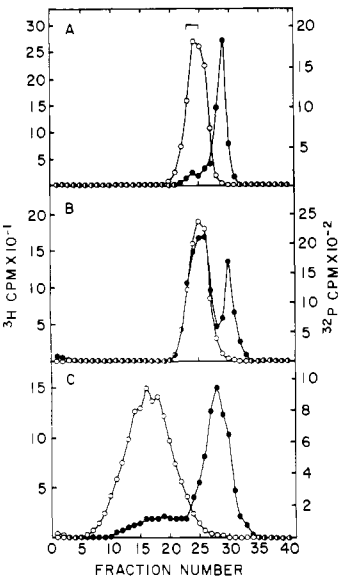

FIGURE 7: Equilibrium CsCl density gradient centrifugation of bromodeoxyuridine-labeled R6K DNA. The extract was prepared from cells labeled with [^{3}H]thymidine as described in the legend to Figure 1. A density labeling mixture (1.2 mL) containing [α-^{32}P]dATP and BrdUTP, as described in Materials and Methods, was incubated at 30 °C for 60 min. The labeled DNA was isolated and analyzed in a neutral CsCl density gradient (A). Fraction numbers 23 to 25 were pooled and dialyzed against 6 mM Tris (pH 7.5)-0.1 mM EDTA. After treatment with a restriction enzyme, *Bam*HI, this DNA was recentrifuged in a neutral (B) and an alkaline (C) CsCl density gradient. Approximately 50 fractions were collected and the radioactivity was measured. The density differences between the light ^{3}H-labeled DNA and the ^{32}P-labeled DNA at their peaks were approximately 28 mg/cm^3 in B and 54 mg/cm^3 in C. (● — ●) ^{3}H-labeled parental DNA; (○ — ○) ^{32}P-labeled newly synthesized DNA in vitro.

sedimenting DNA (63% of the total) cosedimented with the denatured strand of the monomer unit size of R6K DNA. A minor peak (7% of the total) was observed in the bottom of the gradient and may represent catenated molecules consisting of two interlocked closed circular monomeric units. These results indicated that most of the DNA synthesized in this in vitro system corresponds to R6K DNA.

In order to investigate further the physical characteristics of the replicative intermediates, DNA products were extracted after incubation for 20 and 60 min and analyzed in alkaline sucrose gradients. As shown in Figure 6A, ccc-R6K DNA molecules composed only 5% of the total [^{3}H]DNA counts in a short incubation. The main products were characteristic of replicative intermediates that contained the linear molecules of unit length of R6K DNA and smaller DNA sedimenting approximately as an 18S fragment (Figure 6C). These smaller DNA molecules were not observed in a neutral sucrose gradient. When the reaction mixture was incubated for 60 min, the amount of lower molecular weight DNA decreased. In addition, the level of covalently closed circular R6K DNA molecules increased to 17% of the total ^{3}H-labeled products (Figure 6B). Single-stranded circular and linear molecules of unit size of plasmid DNA also were present, presumably derived from open circular DNA molecules (Figure 6D).

Semiconservative Replication of R6K DNA. In order to clarify the replication mode of plasmid DNA in vitro, density-labeled R6K DNA was synthesized in the in vitro system and analyzed. A cell extract was prepared from [^{3}H]thymine-labeled cells carrying the R6K plasmid and was incubated for 60 min in the reaction mixture in which TTP and dATP were replaced with BrdUTP and [α-^{32}P]dATP. The sedimentation profile of the synthesized ^{32}P-labeled DNA in a neutral and an alkaline sucrose gradient was similar to that of the DNA synthesized in the presence of TTP (Figure 5). Figure 7A shows the profile of the DNA products in a neutral CsCl density gradient centrifugation. The majority of newly synthesized ^{32}P-labeled DNA in vitro banded at a density of approximately 1.74 g/cm^3. Because of the presence of endogenous dTTP, the substitution of dTMP by BrdUMP was not extensive. It was shown in this experiment that 15% of ^{3}H-labeled template DNA was density transferred after 60 min incubation, while 28% was density transferred after 120-min incubation in another experiment. The DNA in the fractions indicated in Figure 7A was centrifuged again in a neutral CsCl density gradient and in an alkaline CsCl density gradient after treatment with *Bam*H1 restriction endonuclease to generate linear molecules from closed circular or open circular DNA molecules (*Bam*H1 cleaves R6K at a single site). The difference in density between ^{32}P-labeled DNA newly synthesized in vitro and ^{3}H-labeled, template DNA in the alkaline CsCl density gradient (Figure 7C) was almost twice that in the neutral CsCl density gradient (Figure 7B). This indicates that plasmid DNA synthesized in vitro consists of ^{3}H-labeled light strand and ^{32}P-labeled BrdUMP-heavy strand, hybrid molecules, and therefore that DNA synthesis proceeds semiconservatively. The broad peak of ^{32}P-labeled DNA and the level of ^{3}H-labeled DNA in the region of the ^{32}P-labeled DNA in the alkaline CsCl density gradient indicate that elongation synthesis also occurred in ^{3}H-labeled DNA strand of some molecules.

Discussion

The data presented demonstrate the initiation, elongation, and termination of replication of the antibiotic resistance plasmid R6K in a cell extract prepared from *E. coli* cells carrying this plasmid. The replication of the plasmid was shown to proceed semiconservatively with 15% of template plasmid DNA in the reaction mixture density transferred after 60-min incubation (Figure 7). The absence of fully heavy DNA indicates that reinitiation did not preferentially occur in the

molecules that had completed the first round of replication. It is also possible that essential protein(s) for R6K replication may not be stable under long incubation conditions. It was further shown that initiation of replication of R6K DNA required nascent RNA synthesis, since it required four rNTPs and was sensitive to the RNA polymerase inhibitors, rifampin and streptolydigin. The activity of the *dnaG* protein which is necessary for RNA synthesis in the chain elongation of DNA replication is refampicin resistance (Bouché et al., 1975). The rifampin sensitivity of the initiation of replication and the kinetic studies on R6K DNA synthesis after the addition of rifampin at various times suggest that the RNA required is origin primer RNA. The likelihood that ccc-R6K DNA serves as a template for replication in vitro is supported by the recent observation that exogenous ccc-R6K DNA, added to a reaction mixture containing proteins partially purified from the cell extract, can be used as a template for rifampin-sensitive synthesis (manuscript in preparation). Protein synthesis in the cell extract is probably not required for the replication of R6K DNA since cell extracts were prepared by centrifugation at 13 500*g* and the incorporation of deoxyribonucleotides is not inhibited substantially by a high concentration of chloramphenicol.

Replicative intermediates that contained a single-stranded segment of DNA of approximately 18S DNA were synthesized under short incubation conditions. It should be noted that the size of this DNA fragment corresponds to the approximate length of the segment of R6K DNA from the α origin of replication to the terminus (Lovett et al., 1975). Under longer incubation conditions, fewer of these molecules were observed and the proportion of circular and linear molecules of unit length of R6K DNA increased (Figure 6). These data indicate that the initiation step was more labile under the in vitro conditions than the elongation steps in R6K DNA synthesis, and its cessation resulted in the decrease of molecules carrying short DNA fragments and an increase of ccc and oc molecules of R6K DNA. Recent electron microscope analysis of the replicative intermediates has demonstrated that both origins (α and β) and terminus of this plasmid are active in the in vitro DNA synthesis system (manuscript in preparation).

Of the products of R6K DNA synthesis, the amounts of ccc molecules observed in alkaline sucrose gradients were approximately 16 to 30% of the total. This amount appears to be less than the proportion of ccc-R6K DNA found in neutral sucrose gradients (Figure 5). These results may be accounted for by the synthesis in this system of RNA containing R6K DNA molecules that are sensitive to alkaline conditions analogous to the synthesis of RNA-containing ColE1 DNA in whole cells in the presence of chloramphenicol (Blair et al., 1972). Furthermore, the accumulation of catenated molecules suggests that the final process of separation of two daughter molecules to form covalently closed monomers is partially defective in this system.

R6K DNA synthesis was sensitive to arabinosyl-CTP, an inhibitor of the activity of DNA polymerase II and III (Reddy et al., 1971). Since the R6K plasmid replicates in a *polA polB* strain that is defective in DNA polymerase I and II (Inuzuka & Helinski, unpublished results), it is likely that DNA polymerase III is the essential enzyme for incorporation of deoxyribonucleotides into R6K DNA in vivo and in vitro. To confirm the above results, studies are in progress using a temperature-sensitive *polC* mutant.

R6K DNA synthesis was sensitive to novobiocin. This result suggests that DNA gyrase is involved in the replication of R6K plasmid as an essential enzyme for duplex DNA replication as reported for several other DNA replication systems including ColE1 and phage λ DNA (Gellert et al., 1976), $\phi\chi174$ RFI DNA (Marians et al., 1977) and T7 DNA (Itoh & Tomizawa, 1977).

Exogenous addition of ccc-R6K DNA (10 to 20 μg/mL) markedly inhibited R6K DNA synthesis in this system, whereas the addition of ccc-ColE1 DNA stimulated total incorporation. This observation may result from competition between endogenous R6K DNA in the cell extracts and exogenously supplied ccc-DNA for an essential protein(s) for their replication that is present at a limiting concentration in the cell extract. A requirement for an R6K plasmid-coded protein that is present in limiting concentrations in the cell extract recently has been observed (manuscript in preparation).

Addition of ccc-R6K DNA in the form of relaxation complex stimulated R6K DNA synthesis to a small extent. This result might indicate that the relaxation complex contains as one of its components an essential R6K plasmid-coded protein. Alternatively, an essential protein for DNA replication is adventitiously associated with the purified R6K relaxation complex. A more detailed analysis of the biochemical properties of this in vitro system for R6K DNA synthesis hopefully will provide novel insights to the regulation of replication of this plasmid.

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Antibody Nucleic Acid Complexes. Immunospecific Retention of N^6-Methyladenosine-Containing Transfer Ribonucleic Acid†

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ABSTRACT: Antibodies specific for N^6-methyladenosine (m^{6}A) were immobilized on Sepharose and the resulting immunoadsorbent was tested for its ability to retain those *Escherichia coli* tRNAs containing the antigenic hapten, i.e., m^{6}A. Results obtained with [^{32}P]PO_4- and [*methyl*-^{3}H]-methionine-labeled tRNAs indicated that approximately 3 to 5% of the radioactive RNA was retained by the immunoadsorbent. Under identical conditions, but in the presence of m^{6}A (1 mg/mL), less than 0.2% of the radioactivity was retained. Subsequent characterization of the retained tRNA via (a) analysis of *methyl*-^{3}H-labeled, methylated nucleosides, (b) two-dimensional gel electrophoresis, and (c) analysis of the retention of [^{3}H]aminoacyl-tRNA species led to the conclusion that the anti-m^{6}A/Sepharose adsorbent quantitatively and exclusively retained a single tRNA species containing m^{6}A, namely, tRNA^{Val}.

Antibodies that immunospecifically recognize a variety of modified constituents present in nucleic acids have been characterized (Erlanger & Beiser, 1964; Karol & Tanenbaum, 1967; Levine et al., 1971; Sawicki et al., 1971, 1976; Hacker et al., 1972). Recent utilization of such antibody preparations, e.g., anti-m$_2$6A antibodies, has confirmed the presence or absence of N^6,N^6-dimethyladenosine (m$_2$6A)[1] residues in the 23S rRNA species of Kasugamycin-sensitive and -resistant strains of bacteria (Politz & Glitz, 1977), while anti-5-methylcytidine (m^{5}C) antibodies have been used to study the distribution and arrangement of 5-methylcytosine residues in human chromosomes (Lubit et al., 1976). Other investigations have employed antibodies which specifically recognize inosine and the Y nucleoside to isolate those tRNAs containing the corresponding antigenic hapten (Inouye et al., 1973; Fuchs et al., 1974).

In view of the above and other recent findings which indicate that mammalian RNAs (tRNA, rRNA, mRNA, low molecular weight nuclear RNA, and heterogeneous nuclear RNA) possess a variety of methylated nucleosides (Perry & Kelley, 1974; Desrosiers et al., 1974, 1975; Shatkin, 1976; Ro-Choi & Henning, 1977; Weinberg & Penman, 1968), we became interested in examining the possibility that antibodies specific for such minor constituents could be employed as a means of isolating and characterizing nucleic acid populations on the basis of their composition of methylated nucleosides. Our attention has been focused upon characterizing antibodies that specifically recognize N^6-methyladenosine (m^{6}A) and 7-methylguanosine (m^{7}G). Recently, we have demonstrated that immunoadsorbents containing the above antibodies can quantitatively and exclusively retain mono- and oligonucleotides possessing m^{6}A and m^{7}G (Munns et al., 1977a,b). The results described herein demonstrate the ability of anti-m^{6}A immunoadsorbents to retain a specific tRNA population containing m^{6}A, namely, tRNA^{Val} from *Escherichia coli*.

Experimental Procedures

All radioactive isotopes employed in this investigation were obtained from Amersham/Searle. They included carrier-free [^{32}P]PO_4, [*methyl*-^{3}H]methionine (8 to 10 Ci/mmol), [2,3-^{3}H]valine (36 Ci/mmol), [4,5-^{3}H]leucine (58 Ci/mmol), and [2,3-^{3}H]phenylalanine. X-ray film (XM-2), for autoradiographic purposes (Figure 3), was obtained from Eastman Kodak; thin-layer chromatographic plates (Anasil GF, 20 × 20 cm, 0.25 mm thickness), for separating methylated nucleosides, were from New England Nuclear; and aminoacyl-tRNA synthetases were from Miles Laboratories. Enzymes used in the digestion of tRNA were obtained from P-L Biochemicals (*Penicillium* nuclease) and from Worthington (alkaline phosphatase). Conditions for enzymatic digestion of tRNA have been described as have the procedures for sep-

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[1] Abbreviations used: m^{6}A, N^6-methyladenosine; m$_2$6A, N^6,N^6-dimethyladenosine; m^{1}A, 1-methyladenosine; m^{2}A, 2-methyladenosine; A^m, 2′-*O*-methyladenosine; A, adenosine; m^{7}G, 7-methyladenosine; m^{1}G, 1-methylguanosine; G^m, 2′-*O*-methylguanosine; G, guanosine; m^{5}U, 5-methyluridine; U^m, 2′-*O*-methyluridine; U, uridine; m^{5}C, 5-methylcytidine; C^m, 2′-*O*-methylcytidine; C, cytidine; NaCl–P, phosphate-buffered saline, pH 7.4; NaDodSO$_4$ (SDS in figures), sodium dodecyl sulfate; PCA, perchloric acid; RNase, ribonuclease; anti-m^{6}A/Sepharose, rabbit anti-m^{6}A antibody-coupled Sepharose; BSA, bovine serum albumin.

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