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Biochemistry

Requirement of a plasmid-encoded protein for replication *in vitro* of plasmid R6K

(*Escherichia coli*/antibiotic resistance/initiation of replication/temperature-sensitive mutants)

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ABSTRACT **Conditions are described for the replication of exogeneous R6K DNA in an *in vitro* system prepared from *Escherichia coli* cells. Replication of plasmid DNA in this system is semiconservative and sensitive to actinomycin D, novobiocin, arabinofuranosyl-CTP, *N*-ethylmaleimide, and inhibitors of DNA-dependent RNA polymerase. An ammonium sulfate fraction prepared from cells carrying the R6K plasmid is required for replication. A direct role in replication for a plasmid-encoded protein, designated π, in this fraction is indicated by the inactivity of this fraction when prepared from cells carrying a temperature-sensitive mutant plasmid and the thermolability of this fraction when prepared from cells carrying a partial revertant of the mutant plasmid. This plasmid-encoded protein is necessary for the initiation of R6K DNA replication and functions before or during the formation of nascent RNA in the initiation process. The results of titration assays of this protein using various template DNAs suggest that the protein interacts with the plasmid DNA at the region essential for DNA replication.**

A plasmid element coexists as an independent replicon with the host chromosome of a bacterial cell. Replication of plasmid DNA in most cases requires plasmid-specified products in addition to gene products encoded by the host chromosome (1, 2). Although temperature-sensitive replication mutants of plasmids have been isolated (1–5), studies *in vivo* with these mutants have yielded only limited information on the nature and mode of action of plasmid-specified products that regulate plasmid replication. In the case of bacteriophage, considerable information on the nature and function of proteins involved in the regulation of replication of phage DNA has been derived from the analysis of *in vitro* replication systems (6, 7). A comparable level of understanding of the regulation of plasmid replication requires the development of analogous systems for plasmid DNA replication *in vitro*. Extracts of *Escherichia coli* cells have been shown to be capable of replicating the low molecular weight, nonconjugative plasmids ColE1 (8), CloDF13, and RSF1030 (9). Replication of plasmid DNA in these systems does not require a plasmid-encoded protein (9, 10).

The ampicillin- and streptomycin-resistant plasmid R6K is a naturally occurring conjugative plasmid with a molecular weight of 25×10^6 (11). The relaxed mode of replication of R6K is unique in that it is bidirectional and proceeds sequentially to an asymmetric terminus from either one of two functional origins (12, 13). In a previous report, we described a cell-free system that is capable of carrying out the replication of endogeneous R6K plasmid DNA (14). With this system it was shown that initiation of replication of covalently closed circular (CCC) R6K DNA molecules present in cell extracts prepared from *E. coli* cells carrying the plasmid was by a process that required nascent RNA synthesis. The addition of CCC-R6K DNA to this system, however, resulted in an inhibition of replication. In this paper, conditions for the replication of exogenous CCC-R6K DNA molecules in a cell-free system are described. Evidence is presented for the role of an R6K-encoded protein in the initiation of replication of the R6K DNA.

MATERIALS AND METHODS

Bacterial Strains. All strains used were derivatives of *E. coli* K-12 YS1 (10). KE346(R6K) and KE348(R6K) have been described in a previous paper (14). KE357 [a *thyA deo* Δ*trpE5* derivative (KE354) of YS1 carrying plasmid pRK353], KE356, KE370, and KE371 (YS1 strains carrying pRK419, pMI22, and pMI24, respectively), and KE372 (a mutant strain carrying an elevated copy number of R6K) were used as plasmid-containing strains. pRK353 and pRK419 are self-replicating, low molecular weight derivatives of R6K constructed by R. Kolter (15). pRK353 contains a 3.1-megadalton segment of R6K that includes an R6K replication origin and the *trpE* gene of *E. coli*. pRK419 contains 1.3-megadalton segment of R6K that includes an R6K replication origin plus the kanamycin resistance segment of plasmid pMK20. pMI22 is a temperature-sensitive replication mutant of pRK419, obtained by nitrosoguanidine mutagenesis, that can be maintained at 32°C but not at 37°C or 43°C. pMI24 was obtained spontaneously as a partial revertant of pMI22 that can replicate at 37°C but not at 43°C. YS1 and KE354 were used as strains free of plasmid DNA.

Materials. Most of the materials used have been described in a previous paper (14). Buffer A contains 0.05 M Tris·HCl (pH 7.5) and 10% sucrose. Buffer B contains 0.01 M Tris·HCl (pH 7.5), 10% sucrose, 0.001 M EDTA, 0.001 M dithiothreitol, and 0.05 M KCl.

Preparation of Extracts. Cells carrying or lacking plasmid were grown at 37°C in 1.5 liters of Penassay broth (Difco) to approximately 2×10^8 cells per ml and collected at room temperature. After one washing with buffer A, the cells were resuspended in 4 ml of buffer A and quickly frozen in a liquid nitrogen bath. No loss of the activity for R6K DNA replication was seen in lysate prepared from the cells stored at −70°C for at least two months. Frozen cell suspension (5 ml) were thawed at 4°C and then placed at 0°C. Lysozyme (0.25 ml of 8 mg/ml in buffer A) and 0.25 ml of 2 M KCl were then added. After 30 min, the lysate was centrifuged at 40,000 rpm for 20 min in a Beckman SW 50.1 rotor at 0°C. If cell lysis was incomplete, an additional step of freezing in liquid nitrogen and thawing was added before centrifugation. The clear amber supernatent was made 0.001 M EDTA and 0.001 M dithiothreitol and was stored in liquid nitrogen. Cell extracts contained protein at 15 to 20 mg/ml.

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Abbreviations: CCC, covalently closed circular; AS(0–40), ammonium sulfate precipitate fraction obtained by adding to cell extracts ammonium sulfate to 40% saturation; AS(40–60), ammonium sulfate precipitate fraction of supernatant of AS(0–40) after the addition of ammonium sulfate to 60% saturation; R^+ and R^-, carrying or free of R6K plasmid, respectively.

Ammonium Sulfate Precipitation. Cell extracts prepared from R6K-containing (R^+) cells or cells free of the plasmid (R^-) were adjusted to a final concentration of 40% saturation with saturated, neutralized, ammonium sulfate in 0.05 M Tris·HCl (pH 7.5)/0.001 M EDTA at 0°C. After 30 min, the precipitate was collected, dissolved in 1/20th vol of extract in buffer B, and then dialyzed against buffer B at 0°C [fraction AS(0–40)]. If DNA was precipitated along with the protein, the AS(0–40) was passed through a DEAE-cellulose column. The supernatant was adjusted to a final concentration of 60% ammonium sulfate saturation at 0°C. The precipitate was collected, dissolved, and dialyzed as described above [fraction AS(40–60)]. These fractions were stored in small portions at −70°C for at least two months without loss of R6K DNA synthesis activity. The AS(0–40) and AS(40–60) fractions contained 5–10 mg and 50 mg of protein per ml respectively.

Assay of DNA Synthesis. The standard reaction mixture (50 μl) contained 40 mM Hepes buffer (pH 8.0), 80 mM KCl, 7.5 mM $MgCl_2$, 0.1 mM NAD^+, 2 mM rATP, 0.5 mM each of rGTP, rCTP, and rUTP, 0.02 mM each of dATP, dGTP, dCTP, and [^{3}H]dTTP (200–500 cpm per pmol of dTTP), bovine serum albumin at 50 μg/ml, CCC-R6K DNA at 10 μg/ml and 2.5 μl each of AS(0–40)-R^+ and AS(40–60)-R^- fractions. After incubation at 30°C, acid-insoluble radioactivity was determined as described in a previous paper (14).

RESULTS

Requirement of an R6K-Encoded Product for R6K DNA Replication. Conditions have been described for the replication of endogenous R6K DNA by a cell extract prepared from R^+ cells (14). In order to investigate the requirement of a plasmid-encoded product for R6K DNA replication, a cell extract was prepared from cells of a strain that does not carry the R6K plasmid. As shown in Fig. 1, R6K DNA synthesis was not observed in the reaction mixture containing R^- cell extract and CCC-R6K DNA, whereas ColE1 DNA synthesis proceeded in this reaction mixture, in agreement with the results of previous reports (10).

In an attempt to overcome the observed inhibition of plasmid DNA replication by the addition of CCC-R6K DNA to a reaction mixture containing a cell extract prepared from R^+ cells (14), an ammonium sulfate fraction, AS(0–40), was prepared from a cell extract of cells carrying the R6K plasmid. This procedure was carried out in order to remove endogeneous plasmid DNA and concentrate any plasmid-encoded protein. An AS(40–60) fraction was prepared from R^- cells as an additional source of host-specified proteins that may be required for plasmid replication.

As shown in Fig. 2, R6K DNA synthesis in this system required added CCC-R6K DNA, AS(0–40)-R^+, and AS(40–60)-R^- fractions. The AS(0–40)-R^+ fraction could not be replaced with the AS(0–40)-R^- fraction. The activities of both the AS(0–40)-R^+ and the AS(40–60)-R^- fractions were heat labile (10 min at 65°C). Incorporation of [^{3}H]dTMP was also dependent on the four deoxyribo- and ribonucleotide triphosphates, $MgCl_2$, KCl, and nicotinamide-adenine dinucleotide as previously described (14).

To demonstrate directly the requirement of a plasmid-encoded protein for R6K DNA replication, a mutant plasmid, pMI22, temperature sensitive for replication, that can be maintained at 32°C but not at 37°C or 43°C was isolated from a mini-R6K derivative, pRK419. The AS(0–40) fraction prepared from cells carrying this mutant plasmid did not have any detectable activity for R6K DNA synthesis even at the low temperature of 25°C. A partial revertant (pMI24) of the mutant plasmid that can replicate at 37°C but not at 43°C was isolated from pMI22. As shown in Fig. 3, DNA synthesis using the AS(0–40) fraction from cells carrying the pMI24 mutant plasmid occurred at 25°C but not at 35°C. By contrast, an AS(0–40) fraction prepared from a high copy number R6K mutant showed considerably more DNA synthesis at 35°C than at 25°C. In another experiment, the AS(0–40) fraction prepared from cells carrying pRK419 showed the thermostable activity characteristic of the wild-type R6K. Addition of the AS(0–40) fraction prepared from wild-type cells to that of the temperature-sensitive mutant gave essentially the same kinetics of DNA synthesis as that of the wild-type fraction alone. These results indicate that the temperature-sensitive mutant plasmid specifies a thermolabile protein essential for R6K DNA replication. This essential R6K-encoded protein in the AS(0–40)-R^+ fraction is designated π.

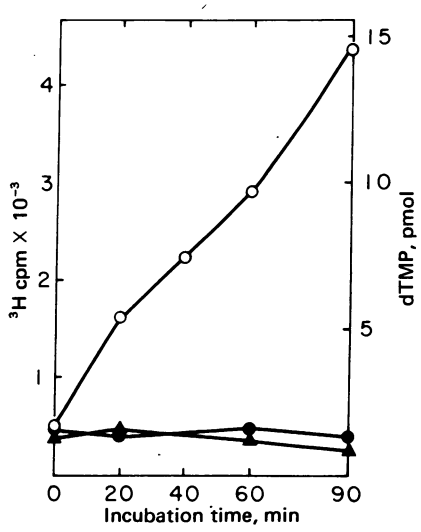

FIG. 1. Replication of R6K and ColE1 DNA in an extract prepared from cells lacking the R6K plasmid. CCC-R6K or ColE1 DNA (10 μg/ml) was added to the standard reaction mixture (50 μl) containing 30 μl of cell extract of YS1 as previously described (14). The incorporation of [^{3}H]dTMP into DNA was determined as described (14). ●, CCC-R6K DNA added; ○, CCC-ColE1 DNA added; ▲, no addition of DNA.

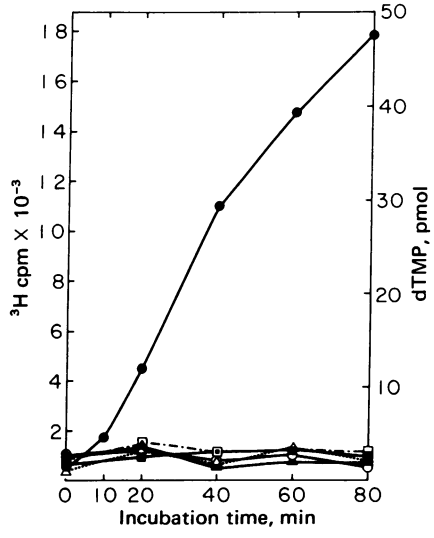

FIG. 2. Kinetics of incorporation of [^{3}H]dTMP into DNA and requirements for the reaction. The standard reaction mixture (50 μl) contained the AS(0–40)-R^+ and AS(40–60)-R^- fractions described in *Materials and Methods*. The reaction was carried out at 30°C. ●, Standard reaction mixture; □, with the addition of rifampicin (20 μg/ml); ■, less CCC-R6K DNA; ▲, less AS(0–40)-R^+; △, less AS(40–60)-R^-; ○, with AS(0–40)-R^- in place of AS(0–40)-R^+.

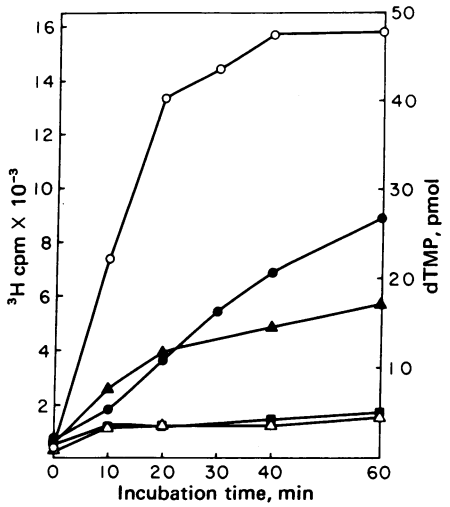

FIG. 3. Activity of fractionated cell extracts prepared from cells carrying a temperature-sensitive replication mutant of a mini-R6K. KE371, carrying a high copy number mutant of R6K, and KE372, carrying the partial revertant pMI24 derived from the mutant plasmid pMI22, were cultured at 30°C. DNA synthesis was carried out in the standard reaction mixture except that the AS(0–40)-R$^-$ fraction was also added. The AS(0–40) fraction of KE371 was assayed at 25°C (●) or 35°C (○), and that of cells carrying the temperature-sensitive mutant plasmid was assayed at 25°C without (▲) or with rifampicin (20 μg/ml) (■), or at 35°C (△).

Effect of Inhibitors of Macromolecular Synthesis on R6K DNA Synthesis. As shown in Table 1, rifampicin, streptolydigin, and actinomycin D strongly inhibited R6K DNA synthesis when CCC-R6K DNA was used as a template DNA. These results indicate that nascent RNA synthesis is necessary for replication of exogenous as well as endogenous R6K DNA (14). Chloramphenicol, an inhibitor of protein synthesis, did not affect R6K DNA synthesis *in vitro*. A high concentration of nalidixic acid (500 μg/ml) showed little effect on the synthesis. Novobiocin, an inhibitor of DNA gyrase (16), blocked R6K DNA synthesis. Arabinofuranosyl-CTP, which inhibits the activity of DNA polymerases II and III of *E. coli* (17), and *N*-ethylmaleimide, which inhibits DNA polymerases II and III (6) and the *dnaC* gene product (18), also showed strong inhibitory effects.

Properties of the DNA Products Synthesized *In Vitro*. To demonstrate that plasmid DNA replication proceeds semiconservatively in this system, ^{3}H-labeled CCC-R6K DNA was incubated in the reaction mixture containing BrdUTP and [α-^{32}P]dATP. Density-labeled DNA was analyzed by neutral CsCl density gradient centrifugation (Fig. 4*a*). Under these conditions, approximately 15% of template DNA was density-transferred and no significant amount of fully heavy ^{32}P-labeled DNA was observed. The DNA in the ^{32}P-labeled peak fraction was then centrifuged in a neutral CsCl and an alkaline CsCl density gradient after treatment with *Bam*HI restriction endonuclease to generate unit length linear molecules of R6K DNA. As shown in Fig. 4 *b* and *c*, half-heavy DNA consisting of a ^{32}P-labeled BrdUMP-heavy strand and a ^{3}H-labeled light strand was synthesized, indicating that R6K DNA synthesis proceeded semiconservatively from exongenous CCC-R6K DNA.

The half-heavy DNA (A) and middle density DNA (B) indicated in Fig. 4*a* were further analyzed by sucrose gradient centrifugations. In a neutral sucrose gradient (Fig. 5*a*), ^{32}P-labeled half-heavy DNA (A) showed three main peaks at the positions of dimer and/or catenated CCC, monomer CCC, and

Table 1. Effect of macromolecule synthesis inhibitors on R6K DNA synthesis

Addition	Concentration	pmol of dTMP incorporated	Relative %
None		10.6	100
Rifampicin	2 μg/ml	0.2	2
Streptolydigin	100 μg/ml	1.8	17
Actinomycin D	2.5 μg/ml	0.1	1
Chloramphenicol	250 μg/ml	10.9	103
Nalidixic acid	100 μg/ml	9.2	87
	500 μg/ml	7.0	66
Novobiocin	100 μg/ml	0.5	5
Arabinofuranosyl-CTP	250 μM	1.2	11
N-Ethylmaleimide	10 mM	3.3	31

DNA synthesis was carried out for 60 min in the standard reaction mixture containing 2.5 μl of the AS(0–40) fraction prepared from KE357 and R6K DNA at 10 μg/ml with or without an inhibitor.

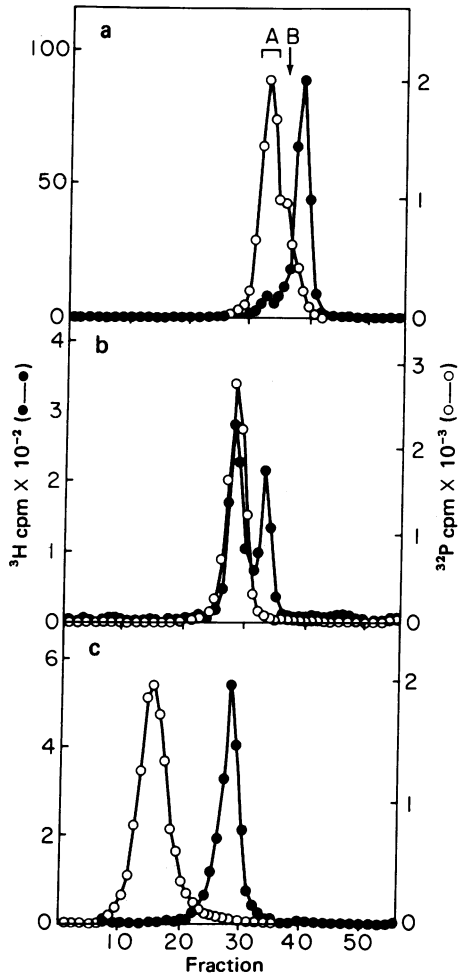

FIG. 4. Equilibrium CsCl density gradient centrifugation of BrdU-labeled R6K DNA. ^{3}H-Labeled CCC-R6K DNA was incubated at 30°C for 80 min in the standard reaction mixture (1.2 ml) containing the AS(0–40) fraction of KE357, except that four dNTPs were replaced by 0.05 mM each of BrdUTP, dGTP, dCTP, and [α-^{32}P]-dATP. The labeled DNA was analyzed in a neutral CsCl density gradient (*a*) as previously described (14). Fraction A (fractions 32–34) was collected and dialyzed against 6 mM Tris·HCl (pH 7.5)/0.1 mM EDTA. After digestion with *Bam*HI restriction endonuclease, this DNA was recentrifuged in neutral (*b*) and alkaline (*c*) CsCl density gradients. The density differences between the ^{3}H-labeled DNA and the ^{32}P-labeled DNA at their peaks were approximately 27 mg/cm^3 in *b* and 54 mg/cm^3 in *c*. ○, ^{32}P-labeled newly synthesized DNA *in vitro*; ●, ^{3}H-labeled parental DNA.

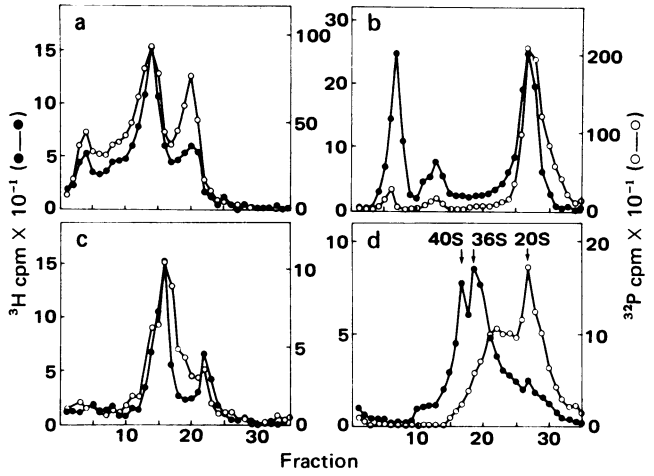

FIG. 5. Sedimentation analysis of newly synthesized BrdU-labeled R6K DNA. The half-heavy DNA (fraction A in Fig. 4*a*) was analyzed in neutral (*a*) and alkaline (*b*) sucrose gradients as described previously (14). DNA of fraction 36 of Fig. 4*a* was also sedimented in neutral (*c*) and alkaline (*d*) sucrose gradients. In alkaline gradients, the DNA was centrifuged at 45,000 rpm for 35 min (*b*) or 95 min (*d*) in a Beckman SW 50.1 rotor. In *d*, 40S and 36S molecules are circular and linear single strands of R6K DNA, respectively. The same symbols are used as in Fig. 4.

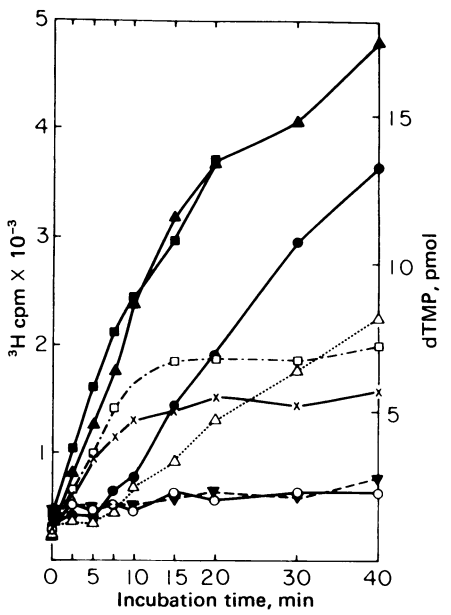

FIG. 6. Involvement of the π protein in the initiation process of R6K DNA replication. A standard reaction mixture (300 μl) containing an AS(0–40) fraction of KE357 was incubated with (○) or without (●) rifampicin (20 μg/ml), and 30 μl was withdrawn at indicated times. A reaction mixture without dNTPs was preincubated for 10 min (▲, X) or 20 min (■, □) and dNTPs containing [^{3}H]dTTP were added at 0 time with (X, □) or without (▲, ■) rifampicin. The reaction mixture also was preincubated for 10 min in the absence of dNTPs and the AS(0–40) fraction of KE357, and these components were added at 0 time with (▼) or without (△) rifampicin.

open circular DNA molecules in the proportion of 18%, 54%, and 28%, respectively.

In the alkaline sucrose gradient (Fig. 5*b*), the ^{32}P-labeled half-heavy DNA sedimented as CCC-molecules (6%), catenated molecules (6%), and open circular and/or linear molecules (83%) of R6K DNA. It was also observed that ^{32}P-labeled DNA of middle density (fraction B) consisted of DNA that sedimented in an alkaline sucrose gradient (Fig. 5*d*) as 20S to 30S fragments. The smaller than unit length fragments were not observed in a neutral sucrose gradient (Fig. 5*c*). These results suggest that the DNA at the middle density position consists of partially replicated molecules of R6K.

R6K-Encoded Protein Is Involved in the Initiation of Plasmid DNA Replication. In order to investigate at what step π is involved in replication of the plasmid, the following experiments were performed. When all reactants, including CCC-R6K DNA, AS(0–40)-R$^+$, AS(0–40)-R$^-$, AS(40–60)-R$^-$, rNTPs, and dNTPs, were mixed and incubated, a lag of 5–7.5 min in the incorporation of [^{3}H]dTMP was observed (Fig. 6). Addition of rifampicin completely inhibited R6K DNA synthesis in this reaction mixture. However, when a standard reaction mixture was preincubated in the absence of dNTPs for 10 min or 20 min, DNA synthesis began without a lag upon the addition of dNTPs. A significant portion of this synthesis is resistant to rifampicin. When the standard reaction mixture lacking both AS(0–40)-R$^+$ and dNTPs was preincubated for 10 min, and AS(0–40)-R$^+$ and dNTPs were supplied at 0 time, the delay in initiation of plasmid DNA synthesis was again observed. DNA synthesis did not occur when rifampicin was added along with the AS(0–40)-R$^+$ fraction and dNTPs. These results indicate that nascent RNA synthesis, most likely primer RNA synthesis, is required for the initiation of R6K DNA replication, and the plasmid-encoded π protein functions before or during the synthesis of the nascent RNA.

Functional Binding of R6K Replication Protein to the Replication Region of R6K DNA. As described in a previous paper (14), exogenous R6K DNA markedly inhibited DNA synthesis in the *in vitro* system containing a cell extract of R$^+$ cells. It was suggested that this might result from competition between endogenous R6K DNA in the cell extract and exogenous CCC-DNA for an essential replication protein(s) that is present at a limiting concentration in the cell extract. Because a plasmid-encoded protein is indispensable for R6K DNA replication, this essential replication protein might be titrated by an assay using various concentrations of R6K and mini-R6K DNA. R6K, pRK353, and pRK419 DNA were used as template DNA. pR353 and pRK419 contain only 3.1 and 1.3 megadaltons, respectively, of R6K DNA. DNA synthesis was assayed by using various concentrations of template DNA in the reaction mixture in the presence of a constant amount of the AS(0–40)-R$^+$ fraction and an excess amount of the AS(0–60)-R$^-$ fraction. As shown in Fig. 7, the optimal concentration of each template DNA for DNA synthesis was 1.4 to 1.5 × 10^{10} molecules per 50 μl of the reaction mixture. Higher concentrations of plasmid DNA were inhibitory. Furthermore, the amount of DNA synthesized at the optimal concentration of template DNA was essentially proportional to the molecular weight of template DNA. The effect of concentration of plasmid DNA did not depend on the concentration of the AS(0–60)-R$^-$ fraction. Thus, the essential R6K-encoded protein is present at a limiting concentration in the AS(0–40)-R$^+$ fraction and its activity is dependent on the concentration of the replication region of the plasmid rather than the total DNA concentration. These results suggest that the R6K-encoded protein functionally binds to template plasmid DNA at the essential region for DNA replication. The effect of the concentration of the AS(0–40)-R$^+$ fraction on DNA synthesis was also examined. The amount of plasmid DNA synthesis was found to increase proportionally with the amount of the AS(0–40)-R$^+$ fraction added until a protein concentration of 40 μg/ml was reached, at which point no further increase in DNA synthesis occurred. Significant DNA synthesis did not occur until protein of this fraction was added at approximately 14 μg/ml.

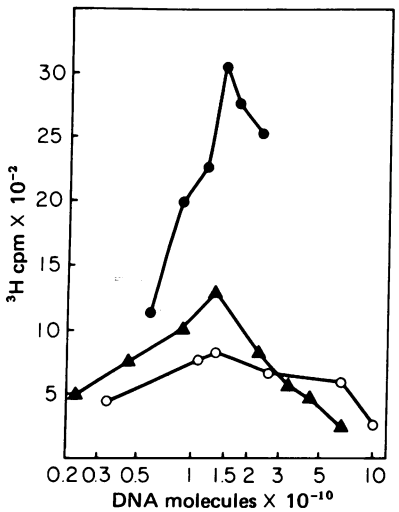

FIG. 7. Effect of DNA concentration on plasmid DNA synthesis. Standard reaction mixtures (50 μl) containing 2.5 μl of AS(0–40) of KE357 were incubated for 60 min at 30°C with various concentrations of R6K (●), pRK353 (▲), and pRK419 (○). R6K, pRK353, and pRK419 DNA have molecular weights of 25, 6.7, and 2.2 × 10^6, respectively.

DISCUSSION

The development of a replication system *in vitro* for exogenous CCC-R6K DNA has permitted a direct demonstration of the initiation of R6K DNA replication *in vitro* on a CCC-DNA template by a process that requires nascent RNA synthesis. The properties of this system and the product of the replication were essentially the same as those of the replication system described earlier (14) in which endogenous DNA in cell extracts was used as the template. Unlike ColE1 plasmid DNA, in which replication does not require a plasmid-encoded protein *in vitro* (10) or *in vivo* (19, 20), R6K requires a plasmid-encoded protein for replication *in vitro*. The thermolability of the activity in the AS(0–40) fraction prepared from a temperature-sensitive replication mutant of pRK419 also indicates that the plasmid-encoded protein is directly involved in replication of the plasmid.

The results of preincubating the reaction mixture in the presence or absence of the AS(0–40)-R^+ fraction indicated that the R6K-encoded protein functions at the level of initiation of CCC-R6K replication. In addition, the activity of this protein is dependent on the concentration of the replication region of the R6K plasmid. Additional studies with smaller fragments of the R6K replication region obtained by restriction enzyme cleavage should reveal whether the R6K protein associates with the R6K plasmid at or near one or both origins of replication. The addition of higher concentrations of plasmid DNA to the replication system was inhibitory for DNA synthesis in the presence of a constant amount of the AS(0–40)-R^+ fraction and no DNA synthesis was found at lower concentrations (less than 14 μg of protein per ml) of this fraction in the presence of a constant plasmid DNA concentration. One possible explanation for these results is that two or more molecules of the R6K protein are required to associate with the origin for the formation of an active complex in the initiation of R6K replication.

The genetic locus (*pir*) of this R6K-encoded protein, designated π, was determined to be on a 1300-base-pair segment located near the replication origin of the R6K genome by assaying the R6K DNA synthesis activity of AS(0–40) fractions prepared from mini-R6K derivatives (M. Inuzuka and R. Kolter, unpublished observations). The π protein has been specifically labeled in minicells with [^{35}S]methionine and partially purified, and its molecular weight has been estimated as approximately 35,000 by sodium dodecyl sulfate/polyacrylamide gel analysis (unpublished observations). Assuming no overlapping genes, the coding information in the *pir* locus is essentially equivalent to that required for the π protein. This suggests that R6K encodes for a single protein essential for replication of the plasmid.

While the mechanism of action of the R6K protein remains to be elucidated, its properties described in this study suggest a role as a positive regulatory protein that functions in the initiation of R6K DNA replication. It will be necessary to obtain this protein in pure form in order to define the biochemical nature of its role in the initiation of R6K replication.

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