JOURNAL OF BACTERIOLOGY, Oct. 1977, p. 90-99
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Vol. 132, No. 1
Printed in U.S.A.

Replication of Thermosensitive Rts1 Plasmid Deoxyribonucleic Acid at the Nonpermissive Temperature

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Received for publication 15 April 1977

Replication of the thermosensitive drug resistance factor Rts1 was studied at the nonpermissive temperature (42°C). It was concluded from the following observations that replication of this plasmid takes place at 42°C without involving the covalently closed circular (CCC) form of deoxyribonucleic acid (DNA). (i) DNA-DNA reassociation kinetics studies with purified Rts1 DNA showed that Rts1 DNA increased several-fold during cell growth at 42°C while very little, if any, CCC DNA was synthesized. (ii) When *Escherichia coli* 20S0(Rts1) was labeled with [^{3}H]thymidine at 42°C, a significant amount of radioactive DNA hybridizable to Rts1 DNA was formed. This DNA was found in a fraction where DNA other than CCC DNA was expected in alkaline sucrose density gradient centrifugation analysis. When *E. coli* 20S0(Rts1) was labeled at 32°C, the labeled CCC DNA did not disappear during a chase period at 42°C. This indicates that preformed CCC DNA does not participate in replication at the nonpermissive temperature. These results are consistent with the hypothesis that there are two modes of replication of Rts1 DNA, one involving a CCC molecule and the other not involving this form, and that only the latter mode takes place at the nonpermissive temperature.

The drug resistance factor Rts1 is a plasmid that confers kanamycin resistance on its host bacterial cells. This plasmid was first found in *Proteus vulgaris* isolated in 1967 from a patient with a urinary tract infection (37) and has a molecular weight of 126×10^6 (9). In addition to conferring kanamycin resistance, this plasmid influences its host cell in two additional ways. First, at the nonpermissive temperature (42°C), the bacterial cell carrying this plasmid can divide, but no increase in viable cell number is observed, suggesting that after the cell division only one daughter cell is viable (7). Although the exact mechanism of this effect remains obscure, it has been observed that the cell envelope is damaged (10) and that the deleterious effect of Rts1 on the host is apparently mediated through cyclic adenosine 3',5'-monophosphate. When Rts1 was placed in *Escherichia coli* mutants that lack either adenylate cyclase or cyclic adenosine 3',5'-monophosphate receptor protein, the temperature-dependent effects of Rts1 were not observed (40, 40a). Another effect of this plasmid on the host is to restrict growth of T-even phages at the permissive temperature, but not at the nonpermissive temperature (10, 41). Furthermore, at 42°C an elongated cell shape develops (10), the rate of absorption of T4 phage changes, and permeability to antibiotics is altered.

In addition to its thermosensitive effects on the host, the replication, as well as the transfer, of the Rts1 plasmid appears to be strongly influenced by temperature. Thus, at high temperature, transfer of this plasmid is greatly reduced, and prolonged growth of Rts1-containing cells at 42°C results in a cell population that does not contain this plasmid (37). Studies on the replication of Rts1 itself by CsCl density gradient analysis suggested that replication of this plasmid may also be thermosensitive (36).

Recent studies suggest that these multiple thermosensitive characteristics may not be functions of one single plasmid gene. For example, the thermosensitive nature of plasmid transfer has been shown to be unrelated to its thermosensitive effect on the host (35, 42). Temperature-dependent restriction of T-even phages by Rts1 may be unrelated to the effect on host growth, although it may be controlled by the same gene as that for plasmid transfer (42). However, the thermosensitive nature of plasmid replication may be closely related to its influence on host growth. The close relationship between the effect on host growth and the

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thermosensitive block of plasmid replication may not be unique to Rts1. For example, the thermosensitive factor Col/VB*trp* recently isolated has an effect on the host growth as well as on its own replication. These two thermosensitive characteristics may be under the control of one gene, because a revertant of the thermosensitive block of replication of Col/VB*trp* was found to have also lost its effect on host growth (23, 24).

Although various thermosensitive plasmids with thermosensitive replication blocks have been reported (4, 19, 33), the mechanism of the thermosensitive replication block in these plasmids is not well understood. In our previous communications on the thermosensitive block of the replication of Rts1 deoxyribonucleic acid (DNA), we reported that an intermediate may accumulate at the nonpermissive temperature (42°C) that, upon shiftdown to the permissive temperature, may form circular Rts1 DNA (8). The fact that Rts1 DNA is synthesized at the nonpermissive temperature was further supported by the finding that each cell derived from division of elongated filamentous cells produced at the nonpermissive temperature in the presence of penicillin contained plasmids when penicillin was removed and the filaments were allowed to form individual cells (8). In this communication, we have used DNA-DNA reassociation kinetics to establish that Rts1 DNA replicates at the nonpermissive temperature. It was found that when the cells were exposed to 42°C for more than 1 h, there was at least a fivefold increase in Rts1 DNA, although very little, if any, of the covalently closed circular (CCC)-form Rts1 DNA was produced. During exposure to the nonpermissive temperature, no decrease in preformed CCC Rts1 DNA was observed, suggesting that the CCC form of Rts1 DNA does not participate in the replication of Rts1 DNA at the nonpermissive temperature. These observations suggest the possibility that there are two forms of the replication cycle, one involving the CCC form of Rts1 and a second that does not, and that only the latter occurs at the nonpermissive temperature.

MATERIALS AND METHODS

Bacterial strains and R factors. The properties of R factors, host strains, and conditions for culture have been described previously (7).

Preparation of ^{3}H-labeled Rts1 DNA for use as a probe in DNA-DNA hybridization. *E. coli* 20S0 cells harboring Rts1 DNA were grown overnight at 32°C, and the culture was diluted in 20 ml of M9 medium containing 25 μCi of [^{3}H]thymidine (200 μCi/μg) per ml to obtain a final cell concentration of 3×10^7 to 4×10^7 cells/ml. M9 medium contains (in grams per liter) Na_2HPO_4, 5.95; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1.8; $MgSO_4 \cdot 7H_2O$, 0.26; and glucose, 4.0. Since *E. coli* 20S0 requires thiamine hydrochloride, this was added to obtain a final concentration of 2 μg/ml. The cells were grown at 32°C for 4 to 5 h, and cell density at that time was approximately 5×10^8 to 6×10^8 cells/ml. The culture was then chilled, and cells were collected by centrifugation in a Sorvall SS34 rotor at 5,000 rpm for 5 min in the cold. The labeled cells were treated with sodium dodecyl sulfate and subjected to alkaline sucrose density gradient centrifugation in a Spinco SW27 rotor for 47 min at 24,000 rpm as described previously (8). After the centrifugation, the peak of radioactivity was pooled and concentrated. For further purification, the Rts1 DNA was subjected to neutral sucrose gradient (10 to 30%) centrifugation in 0.125 M ethylenediaminetetraacetate (pH 8.0)–1 M NaCl–0.25 M tris-(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) for 3.5 h at 25,000 rpm in a Beckman SW27 rotor.

The Rts1 DNA was pooled and sonically treated as described previously (11), suspended in 0.2 ml of 0.1 mM ethylenediaminetetraacetate, and kept at −20°C until used. The Rts1 probe prepared above was used in the experiments described in Fig. 1 through 4.

Preparation of ^{32}P-labeled Rts1 DNA probe. The overnight culture of *E. coli* 20S0(Rts1) was suspended in a medium containing 1 μCi of ^{32}P (100 μCi/2.5 μg) per ml, and ^{32}P-labeled Rts1 DNA was prepared as described in the preceding section. Labeling Rts1 with ^{32}P facilitates isolation and purification; it also permits one to estimate how much self-annealing of the probe takes place.

Preparation of a mixture of nonlabeled cellular and Rts1 DNA. Cellular DNA was prepared essentially according to Marmur (28). The ratios of the optical densities at 280 to those at 260 nm of these purified DNAs were between 0.52 and 0.53. For measurement of the total amount of DNA in a culture, 50 ml of each culture was saved, and DNA was measured in these samples by the diphenylamine method (13). DNA thus prepared was used in the experiments described in Fig. 1 through 4.

Estimation of DNA concentration in purified DNA samples. DNA concentrations in the purified samples were measured by either the optical density at 260 nm (20 = 1 mg/ml) or the fluorescence method (17). All DNA concentrations are expressed as optical density units at 260 nm.

Reassociation kinetics of Rts1 DNA. For studies of reassociation kinetics, DNA isolated from *E. coli* 20S0(Rts1) or *E. coli* 20S0 and probe Rts1 DNA in 0.1 mM ethylenediaminetetraacetate were mixed, heated to 100°C for 1 min, and rapidly cooled in ice-cold water. This mixture was combined with a buffer solution to obtain a final concentration of 0.3 M NaCl, 0.02 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.2), and 0.001 M ethylenediaminetetraacetate in a total volume of 10 μl. This buffer is called the annealing buffer. This solution was placed in a 30-μl capillary, and both ends were sealed. The sealed capillary was then heated for 5 min at 100°C and placed in a 68°C water bath. After

various incubation periods for the annealing of DNA, the capillary tube was placed at −20°C and stored until further analysis. After thawing, the reaction mixture (10 μl) was mixed with 2.2 ml of S1 buffer (0.03 M acetate buffer [pH 4.5]–0.3 M NaCl–0.003 M $ZnCl_2$) and 10 μg of denatured calf thymus DNA per ml. The mixture was divided into two parts of equal volume. One part received 2,000 U of S1 enzyme in S1 buffer, and the other portion (control) received an identical solution without S1 enzyme. For digestion by S1 enzyme, the mixtures were incubated at 50°C for 2 h, chilled at 0°C, and mixed with 0.2 ml of a solution containing 1 mg of calf thymus DNA per ml and 0.6 ml of 15% cold (0°C) trichloroacetic acid to precipitate the undigested DNA. The mixture was kept standing on ice for 15 min to complete the precipitation. The undigested DNA was pelleted by centrifugation for 15 min at 5,000 rpm in a Sorvall SS34 rotor at 4°C. The pellet was washed twice with 3 ml of cold 5% trichloroacetic acid and dissolved in 0.25 ml of a mixture of NCS and water (9:1, vol/vol). The solution was mixed with 4.75 ml of Omnifluor-toluene, and radioactivity was determined.

Analysis of labeled Rts1 DNA by PI-CsCl density gradient centrifugation. *E. coli* 20S0(Rts1) cells (10^8 cells/ml) were labeled at 32°C for 1 h in M9 medium (2 ml) containing 25 μCi of [^{3}H]thymidine per ml and 250 μg of 2′-deoxyadenosine per ml. The labeled cells were centrifuged to obtain cell pellets which were lysed according to Clewell and Helinski (3), with some modification. For the CsCl density gradient analysis, the DNA solution obtained above (1 ml) was mixed with 2 ml of a propidium diiodide (PI) solution (1.4 mg/ml in water) and 4 ml of a CsCl solution [130 g of CsCl in 70 ml of 0.02 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.0]. The total volume of the solution was 7 ml, and it had a refractive index of 1.3845, representing a density of 1.535 g/ml. The mixture was centrifuged at 41,000 rpm for 48 h at 20°C in a Beckman Spinco type 65 rotor. After the centrifugation, 4-drop fractions were collected from the bottoms of the tubes, and the radioactivity of each fraction was measured on Whatman 3MM filter paper disks in a Packard scintillation counter.

Chemicals. [^{3}H]thymidine (6 Ci/mmol) and inorganic [^{32}P]phosphate (carrier-free) were from New England Nuclear. PI monohydrate, CsCl (A grade), and Pronase were from Calbiochem. Ribonuclease A (bovine pancreas) and lysozyme (egg white) were from Sigma. Nuclease S1 was from Seikagaku Kogyo; NCS solubilizer was from Amersham/Searle. Antifoam A was from Dow Corning Corp. Sodium lauryl sarcosine (Sarkosyl) was from Schwarz/Mann.

RESULTS

Determination of the amount of Rts1 DNA in *E. coli* 20S0(Rts1) by DNA-DNA reassociation kinetics. Determination of the exact copy number of this plasmid in *E. coli* has so far not been possible because of difficulties in isolating the total amount of plasmid DNA from the host chromosome. In the preceding communication (8), the approximate amount of Rts1 DNA in *E. coli* 20S0(Rts1) was determined by alkaline sucrose gradient centrifugation, which separated the CCC form of Rts1 DNA from the host DNA. It is known that the isolation procedure for CCC DNA does not necessarily yield the total amount of Rts1 DNA in the cell because of possible breakage of circular DNA during the isolation procedure (12). In addition, it is quite possible that the linear or replicating form of the plasmid DNA may exist in a significant quantity in comparison with the total amount of CCC DNA. Since our preliminary results suggested that Rts1 DNA may replicate without going through the CCC form at the nonpermissive temperature (8), it appeared important to determine the exact number of Rts1 DNA molecules in *E. coli* 20S0(Rts1). To accomplish this, DNA-DNA reassociation kinetics with a purified ^{3}H-labeled Rts1 DNA probe was used. The reassociation kinetics of Rts1 DNA clearly indicated that there exists no appreciable homology between the *E. coli* 20S0 chromosome and Rts1 DNA (Fig. 1 and 2). From these data it was calculated that there is less than 0.0026 copy of an Rts1 DNA-like sequence in each host chromosome. This amount is almost negligible when compared to the amount of Rts1 DNA in *E. coli* 20S0(Rts1). To determine the exact amount of Rts1 DNA in *E. coli* 20S0(Rts1) grown at 32°C, a standard reassociation curve was constructed, using a known amount of purified unlabeled Rts1 DNA. There was a linear relationship between the amounts of added Rts1 DNA and the inverses of $C_0t_{1/2}$ values (Fig. 2). Using this standard curve and the reassociation kinetics of *E. coli* 20S0(Rts1) DNA with ^{3}H-labeled Rts1 DNA probe, the amount of Rts1 DNA in these cells was calculated to be approximately 6.6% that of chromosomal DNA. From the molecular weight of Rts1 DNA, 120×10^6 (9), and that of *E. coli* DNA, 2.5×10^9 (5), the copy number of Rts1 DNA per genome was estimated to be 1.4, which corresponds to approximately 2.24 copies per cell because about 1.6 copies of host genome are present per cell.

Net increase of Rts1 DNA at the nonpermissive temperature. Since previous work suggested that Rts1 DNA may replicate at the nonpermissive temperature (8), we determined the total amount of Rts1 DNA by DNA-DNA reassociation kinetics at various times after *E. coli* 20S0(Rts1) was exposed to the nonpermissive temperature. The total cell number increased linearly, and this was accompanied by a 28-fold increase in the total amount of DNA over a period of 9 h (Fig. 3). In confirmation of

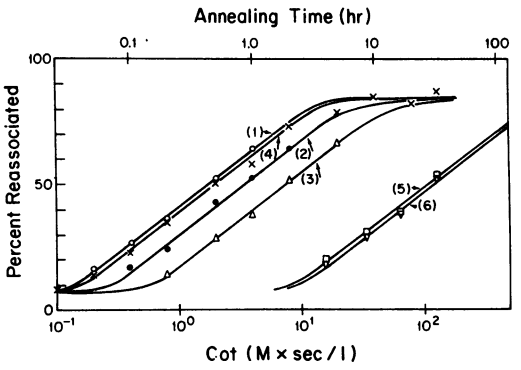

FIG. 1. *Determination of copy number of Rts1 DNA in E. coli 20S0(Rts1) grown at 32°C (permissive temperature). Reassociation kinetics of DNA with purified ^{3}H-labeled Rts1 DNA. The reaction mixtures contained optical densities (ODs) at 260 nm of 1.5 × 10^{-3} of tritium-labeled probe Rts1 DNA per ml (curves 1, 2, 3, and 4) or 7.5 × 10^{-3} of tritium-labeled Rts1 DNA per ml (curves 5 and 6). In addition, each reaction mixture contained the following, per milliliter: (1) 0.56 OD units of Rts1 DNA and 7.44 OD units of calf thymus DNA; (2) 0.28 OD of Rts1 DNA and 7.72 OD of calf thymus DNA; (3) 0.14 OD of Rts1 DNA and 7.86 OD of calf thymus DNA; (4) 8.0 OD of E. coli 20S0(Rts1) DNA (grown at 32°C); (5) 8.0 OD of E. coli 20S0 DNA (grown at 32°C); (6) 8.0 OD of calf thymus DNA. The total of radioactivities of probe Rts1 DNA in experiments 1 through 4 was 568 cpm, and that of 5 and 6 was 2,840 cpm. Annealing was stopped at various time intervals, and S1 enzyme-insensitive radioactivity was counted. Annealing observed in 5 and 6 represents self-annealing of the probe.*

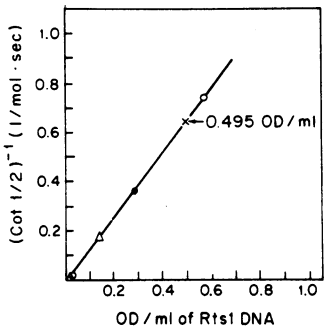

FIG. 2. *Standard curve for determination of Rts1 DNA. Since the reciprocal value of $C_0t_{1/2}$ is proportional to the amount of Rts1 DNA in the annealing mixture (2), reciprocal values of $C_0t_{1/2}$ of each curve (1, 2, and 3) in Fig. 1 were plotted against the corresponding amounts of Rts1 DNA added. The value at 46% reassociation in each curve shown in Fig. 1 was used as the $C_0t_{1/2}$ value, because in our experimental conditions 92% association was the maximum we could reach, no matter how long the incubation was carried out. Symbols used in this figure are the same as those of each DNA sample in Fig. 1. From these values, one can estimate that 8.0 OD of E. coli 20S0(Rts1) DNA (from cells grown at 32°C) contains 0.495 OD of Rts1 DNA.*

previous work (7), the viable cell count remained constant between 1 and 9 h of exposure to 42°C. The total DNA was isolated from this culture at various times, and the relative amount of Rts1 DNA was determined by reassociation kinetics with pure Rts1 DNA as probe (Fig. 4). As much as a sixfold increase in the amount of Rts1 DNA was observed during the 9-h exposure to 42°C. To determine how much CCC Rts1 DNA was synthesized in this experiment, a portion of the culture was exposed to radioactive thymidine, and the ratio of CCC Rts1 DNA synthesized to total DNA synthesis was determined. The ratio was found to be very small (0.26%). These results indicate that the absolute amount of Rts1 DNA increases during prolonged exposure to the nonpermissive temperature. It should be pointed out that the total cell count increased approximately 12-fold, whereas Rts1 DNA increased approximately 6-fold during this period. Since, on the average, more than two copies of the Rts1 genome exist per cell, one can conclude that each cell (viable or nonviable) still contains Rts1 DNA. It is possible that the remaining viable cells may have accumulated all the Rts1 DNA synthesized during this period, leaving all nonviable cells without Rts1 DNA. If so, the viable cells containing Rts1 must have many copies of this plasmid. This is unlikely because this plasmid is under stringent control, and not more than 2 to 3 copies of Rts1 exist in a cell.

Synthesis of labeled Rts1 DNA at the nonpermissive temperature in *E. coli* 20S0(Rts1). The preceding experiment indicated that the total amount of Rts1 DNA increased during the cells' exposure to the nonpermissive temperature. In that experiment, the total amount of Rts1 DNA, which included that which was synthesized at the permissive temperature (before the exposure to 42°C), was measured. To establish further that the Rts1 DNA is indeed synthesized at the nonpermissive temperature, formation of radioactive Rts1 DNA at 42°C was examined. In the experiment shown in Table 1, *E. coli* 20S0(Rts1) cells were first grown at the permissive temperature and equilibrated at the nonpermissive temperature for 1 h, and labeled thymidine was then added. After a 1-h exposure to the labeled medium at 42°C, cells were harvested and ^{3}H-labeled DNA was examined for the presence of Rts1 DNA by the DNA-DNA hybridization technique, using an excess of ^{32}P-labeled Rts1 DNA probe. To examine the possi-

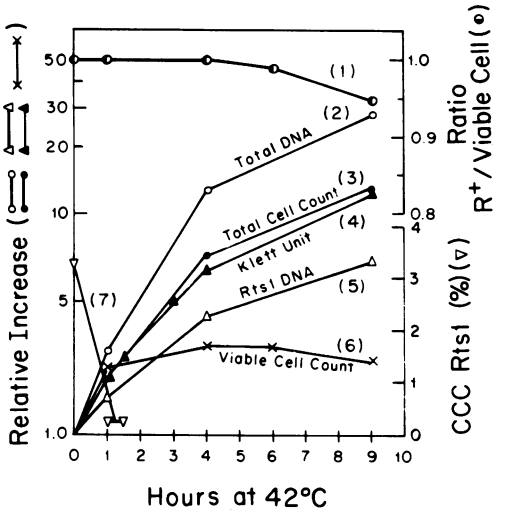

FIG. 3. *Time course of the change of (1) percentage of Rts1-containing cells, (2) total DNA, (3) total cell counts, (4) Klett units of the culture, (5) the amount of Rts1 DNA, (6) viable counts, and (7) the radioactive CCC Rts1 DNA of E. coli 20S0(Rts1) cells during growth at the nonpermissive temperature (42°C). Symbols: (◐) percentage of Rts1-containing cells determined by replica plating on MacConkey agar containing 50 μg of kanamycin per ml; (○) total amount of DNA in 50 ml of culture; (●) total cell counts per ml of the culture (total cell counts were determined microscopically with a Petroff-Hauser bacteria counter); (▲) Klett units of the culture (measured in a Klett colorimeter with a red filter); (△) amount of Rts1 DNA determined by reassociation kinetics as described in Fig. 4; (×) amount of colony-forming cells per ml of culture; (▽) the amount of labeled Rts1 CCC DNA formed during 0.5 h of exposure of a portion of culture to [³H]thymidine-containing medium. All the values except for the amount of labeled CCC Rts1 DNA were expressed as values relative to the amounts present at 0 time. The 0 time values are: (1) 5.8 × 10⁷ Rts1 cells per ml; (2) 0.57 μg of DNA per ml; (3) total cell counts, 5.8 × 10⁷ cells per ml; (4) 13 Klett units; (5) total Rts1 DNA measured by the C_0t curve, 0.036 μg/ml; (6) 5.6 × 10⁷ viable cells per ml.*

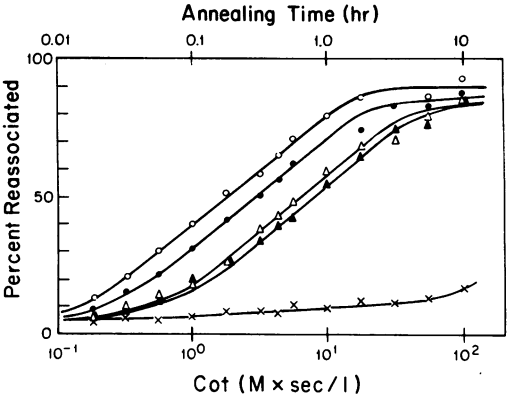

FIG. 4. *Determination of the total amount of Rts1 DNA in E. coli 20S0(Rts1) grown at the nonpermissive temperature; reassociation kinetics of total DNA with tritiated Rts1 probe DNA. Each annealing mixture (10 μl) contained 20 OD units of DNA per ml and 1.5 × 10⁻³ OD units of ³H-labeled Rts1 DNA (1,250 cpm) per ml. The $C_0t_{1/2}$ value was taken from the point where 46% of probe DNA was reassociated. Symbols: (○) DNA from E. coli 20S0(Rts1) cells grown at 32°C, ($C_0t_{1/2}$ value of 1.45); (●) from 20S0(Rts1) grown at 42°C for 1 h ($C_0t_{1/2}$ value of 2.35); (△) from 20S0(Rts1) grown at 42°C for 4 h ($C_0t_{1/2}$ value of 5.2); or (▲) from 20S0(Rts1) grown at 42°C for 9 h, ($C_0t_{1/2}$ value of 6.5); (×) DNA from E. coli 20S0 cells grown at 32°C.*

TABLE 1. *Evidence for the formation of ³H-labeled Rts1 DNA at 42°C – hybridization studies*[a]

Labeling conditions	Rts1 [³H]DNA (% of total DNA)[b]	CCC Rts1 [³H]DNA[c] (% of total DNA)
32°C	5.7	3.7
42°C, 1-2 h	1.7	<0.1

[a] For annealing of DNA, 0.1 μg of [³H]DNA from cultures in 8 μl of 0.1 mM ethylenediaminetetraacetate and 0.88 μg of ³²P-labeled Rts1 DNA in 10 μl of 0.1 M ethylenediaminetetraacetate were mixed, and 2 μl of 10-fold-concentrated annealing buffer was added. The mixture was placed in a 40-μl capillary, heated, and annealed at 68°C for 50 min.

[b] Under the experimental conditions, 63.7% of Rts1 DNA probe was annealed to itself. The values in this column were calculated on the basis of this information.

[c] From alkaline sucrose gradient analysis.

bility that small but significant amounts of CCC Rts1 DNA may be synthesized during the incubation at 42°C, a portion of each culture was subjected to alkaline sucrose gradient centrifugation. It is clear from this table that no detectable CCC Rts1 DNA was formed at 42°C. On the other hand, during exposure of these cells to [³H]thymidine between 1 and 2 h after temperature shift-up to 42°C, approximately 1.7% of the total DNA synthesized was hybridizable to Rts1 DNA (Table 1). This experiment strongly suggests that at the nonpermissive temperature, Rts1 DNA replicates but does not form CCC DNA. This replication in a noncircular form of Rts1 DNA was further substanby the experiment described in the next section.

Evidence for incorporation of [³H]thymidine into the noncircular form of Rts1 DNA at the nonpermissive temperature. To further estab-

lish the conclusion that Rts1 replicates at 42°C in a noncircular form, the experiment described in Fig. 5 was performed. In this experiment, cells were grown at the permissive temperature and incubated at the nonpermissive temperature for 1 h, and radioactive thymidine was added. After 1 h of exposure to radioactive thymidine, cells were harvested and the DNA was subjected to alkaline sucrose gradient centrifugation. Most of the labeled DNA remained at the top of the tube, and very little radioactivity (0.174% of total) was detected at the position where Rts1 CCC DNA was expected to sediment (Fig. 5B). When the radioactive DNA at the top of the tube was examined by hybridization with an excess amount of Rts1 DNA, 2.2% was found to be Rts1 DNA (shown as histogram). A control experiment on cells labeled at 32°C is shown in Fig. 5A. It can be seen from this figure that CCC Rts1 DNA synthesis was approximately 3.3% of total DNA synthesis. As one expects, all of this CCC Rts1 DNA peak radioactivity was hybridizable to the Rts1 DNA probe. The amount of Rts1 DNA found in the fraction where noncircular DNA is expected to sediment was 1.2% of the total DNA of this fraction (shown by the histogram). These experiments indicate that the synthesis of Rts1 DNA takes place at the nonpermissive temperature and that these DNAs are the noncircular form. Because of the possible breakdown of the circular form of Rts1 DNA during the isolation procedure, the values for noncircular Rts1 DNA at 32°C are probably larger than the actual amount that may exist in vivo at 32°C. Although it is not possible to estimate exactly how much Rts1 DNA is present in a noncircular form in vivo at 32°C, the data presented in Fig. 5A are consistent with the notion that some Rts1 DNA exists in this form even at the permissive temperature.

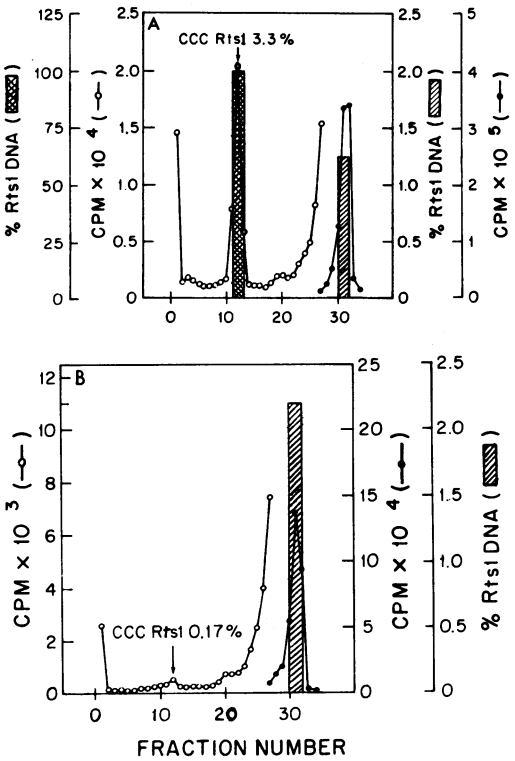

FIG. 5. *Incorporation of [³H]thymidine into the noncircular form of Rts1 DNA at 42°C. E. coli 20S0(Rts1) cells were grown, labeled, lysed, and subjected to alkaline sucrose gradient centrifugation. The fractions corresponding to CCC Rts1 DNA as well as noncircular DNA were pooled and used for the hybridization experiment. (A) Alkaline sucrose gradient centrifugation profile of Rts1 DNA labeled at 32°C; (B) alkaline sucrose gradient centrifugation behavior of labeled Rts1 DNA synthesized at the nonpermissive temperature (42°C). Symbols: (○) [³H]thymidine radioactivity per 30 μl of each fraction; (●) radioactivity representing noncircular DNA per 30 μl of each fraction; (histograms) percentages of radioactive Rts1 DNA as measured by annealing in the pooled fractions.*

Evidence for the increase of absolute amounts of noncircular Rts1 DNA at the nonpermissive temperature. To substantiate the notion that noncircular Rts1 DNA is accumulating at 42°C, it is desirable to estimate the net increase of noncircular Rts1 DNA during growth at 42°C. If one can measure the exact amount of noncircular Rts1 DNA at one point during exposure to 42°C and at a period later, the net increase of this DNA can be estimated. In the experiment described in Table 2, using a cell culture labeled for many generations and another labeled for 1.5 h at 42°C, an estimation of the net increase of noncircular Rts1 DNA was made. As shown in this table, the amount of noncircular Rts1 DNA present after 1 h of exposure to 42°C was 0.067 μg/ml. This value increased to 0.12 μg/ml during 1.5 h of growth at 42°C. This represents an approximately twofold increase of noncircular Rts1 DNA. The relatively high value for the amount of noncircular Rts1 DNA present at 1 h is due to the exposure of these cells to 42°C. In addition, as mentioned before, breakdown of CCC DNA during the isolation procedure may contribute to the high value of noncircular Rts1 DNA. For this reason, the actual increase of the noncircular form of Rts1 DNA is probably larger than indicated in this table. It is noted that at the end of 1 h of exposure to 42°C, the amount of

TABLE 2. *Increase in the net amount of noncircular Rts1 DNA at 42°C*[a]

Incubation at 42°C (h)	Total DNA (μg/ml)	Rts1 DNA (μg/ml) in:	
		CCC fraction	Non-CCC fraction
1	3.5	0.077	0.067
2.5	6.5	0.080	0.12

[a] *E. coli* 20S0(Rts1) cells were grown at 32°C in M9 medium, and the following two cultures were prepared from these cells. Culture 1 contained 5×10^7 cells/ml, 0.1 μg (25 μCi) of [^{3}H]thymidine per ml, 3 μg of thymidine per ml, and 250 μg of 2'-deoxyadenosine per ml in 15 ml of M9 medium. Culture 2 contained 5×10^7 cells/ml, 3.1 μg of thymidine per ml, and 250 μg of 2'-deoxyadenosine per ml in 15 ml of M9 medium. Cultures 1 and 2 were allowed to grow at 32°C for 4 h and were then shifted to 42°C for an additional hour. At this point, cells from culture 1 were harvested and subjected to alkaline sucrose gradient centrifugation. At the same time, culture 2 was poured onto a membrane filter (0.22-μm pore size, Millipore Corp.), and the cells were washed and suspended in 10 ml of prewarmed M9 medium. One-half of the suspension (5 ml) was mixed with 15 ml of prewarmed M9 medium containing 0.13 μg (33 μCi) of [^{3}H]thymidine per ml, 2.7 μg of thymidine per ml, and 333 μg of 2'-deoxyadenosine per ml. Cells were allowed to grow at 42°C for an additional 1.5 h and then were chilled. Cells from this culture were subjected to alkaline sucrose gradient centrifugation, and the DNA of each peak was used in the hybridization procedure. Total cell numbers at 1 and 2.5 h at 42°C (as shown in the table) were 2.7×10^8 and 4.9×10^8/ml, respectively.

CCC Rts1 DNA was 0.077 μg/ml, and this amount was almost identical to that present after 2.5 h of exposure to 42°C (0.08 μg/ml), indicating that no appreciable increase of CCC Rts1 DNA took place during this period. These experiments also showed that the total amount of Rts1 DNA (circular and noncircular) increased 40% during the exposure of the cells to 42°C for 1.5 h.

Evidence that preformed CCC Rts1 DNA does not participate in the replication of noncircular Rts1 DNA at the nonpermissive temperature. In the preceding communication, we reported that the CCC Rts1 DNA synthesized at the permissive temperature remains intact when cells are exposed to the nonpermissive temperature (8). In fact, the preformed CCC Rts1 DNA was so stable that one could detect labeled CCC Rts1 DNA by the alkaline sucrose density gradient centrifugation technique even after 3 h of exposure of the cells to the nonpermissive temperature. This suggested that the preformed CCC Rts1 DNA does not participate in the replication of Rts1 DNA at the nonpermissive temperature. However, the possibility existed that an intermediate form for the replication of circular DNA, such as a concatenated (25, 31) or open circular form (3), may exist and may participate in the replication of Rts1 DNA at the nonpermissive temperature. To examine this possibility, the experiment illustrated in Fig. 6 was performed. In this experiment, *E. coli* 20S0(Rts1) cells were grown at 32°C for 2 h in the presence of [^{3}H]thymidine. The cultures were then incubated with an excess amount of nonlabeled thymidine, and the temperature was shifted up to 42°C. The cells were harvested from time to time, and the amount of circular Rts1 DNA was measured by PI-CsCl density gradient centrifugation. The amount of preformed CCC Rts1 DNA did not change appreciably during the exposure to 42°C (Fig. 6B). The amount was approximately 4.5% of the total DNA, and this remained unchanged for as long as 4 h. When cells labeled at 32°C were subjected to this analysis, CCC Rts1 DNA was clearly separated from host DNA and noncircular Rts1 DNA (Fig. 6A).

If preformed CCC Rts1 DNA participates in replication of Rts1 DNA at 42°C, one would expect a decrease of this CCC Rts1 DNA because it will become a replicating or a concatenated form. As shown in Fig. 6, there was no sign of such intermediate formation or of a decrease in total amount of preformed CCC Rts1 DNA. To examine the possibility that one might be able to observe an intermediate such as a concatemer of Rts1 DNA at 42°C, the experiment illustrated in Fig. 7 was performed. In this experiment, cells were grown at 32°C, adjusted to the nonpermissive temperature, and [^{3}H]thymidine was given. After 1 h of exposure to the radioactive medium, the cells were harvested and analyzed by PI-CsCl density gradient centrifugation. It is clear from Fig. 7 that very little, if any, radioactivity was found in a position other than that of host DNA. This experiment suggests, therefore, that the noncircular form of Rts1 DNA that accumulates at 42°C is probably not a concatenated form of CCC DNA, which should be detectable by this analytical precedure (25). This is in agreement with the data of Fig. 6, where preformed CCC Rts1 DNA was shown not to decrease while the noncircular form of Rts1 DNA was accumulating at 42°C.

DISCUSSION

In the preceding communication (8) evidence was presented suggesting that a precursor to CCC Rts1 DNA appears to accumulate at the nonpermissive temperature (42°C). As an ex-

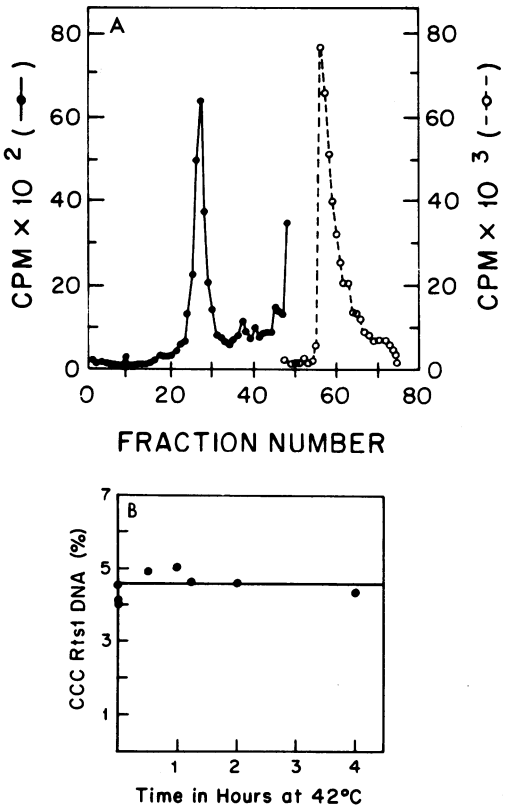

FIG. 6. *Evidence that preformed CCC Rts1 DNA does not participate in the replication of Rts1 DNA at the nonpermissive temperature. (A) Separation of CCC Rts1 DNA from host DNA by PI-CsCl centrifugation. E. coli 20S0(Rts1) were labeled, and lysates were prepared with Sarkosyl. The final extract (1 ml) was subjected to PI-CsCl density gradient centrifugation, and each fraction was counted. The CCC Rts1 peak represents 4.5% of the total DNA. (B) Presence of preformed CCC Rts1 DNA during the replication of Rts1 DNA at the nonpermissive temperature. E. coli 20S0(Rts1) cells (10^8/ml) were labeled with 50 μCi (0.6 μg) of [^{3}H]thymidine per ml. At the end of the 100-min incubation, 360 μg of the thymidine per ml (a 600-fold excess) was added to the culture, and the cells were allowed to grow for an additional 10 min. The labeled cells were then suspended to 7×10^7 cells/ml in M9 medium containing 90 μg of thymidine per ml. At the times indicated, approximately 10^8 cells were harvested and subjected to PI-CsCl density gradient centrifugation. The amount of CCC Rts1 DNA in each experiment is expressed as a percentage of the total radioactive DNA synthesized at 32°C. The total radioactivity incorporated into DNA did not change appreciably during the incubation period.*

tension of this work, the synthesis of Rts1 DNA at 42°C was further examined by the DNA-DNA hybridization technique. Data presented in this communication establish that DNA having complete homology to CCC Rts1 DNA is synthesized at 42°C. Thus, the actual amount of Rts1 DNA increased, and the shape of the C_0t curve indicated that the entire sequence of Rts1 DNA was replicated at 42°C (Fig. 4). Second, there was no appreciable formation of CCC Rts1 DNA, although the complete replication of noncircular Rts1 DNA occurred at 42°C. All the Rts1 DNA synthesized at 42°C was found in the noncircular DNA fraction in the alkaline sucrose gradient centrifugation analysis. A third point is that CCC Rts1 DNA formed at 32°C does not participate in the replication of Rts1 DNA at 42°C in a conventional concatenated ring form. In the experiment involving PI-CsCl density gradient centrifugation, it was found that the amount of prelabeled CCC Rts1 DNA did not decrease at all, and no accumulation of an intermediate form involving the prelabeled Rts1 DNA was detected at 42°C (Fig. 6). A fourth point is that during the exposure of *E. coli* 20S0(Rts1) to 42°C, nonviable cells are produced, but they probably contain Rts1. In other words, production of nonviable cells is not accompanied by an unequal distribution of Rts1 DNA among the progeny. This conclusion is based on the observation that Rts1 increases while viable cell counts remain the same, suggesting that nonviable cells must be receiving the copies of Rts1 DNA.

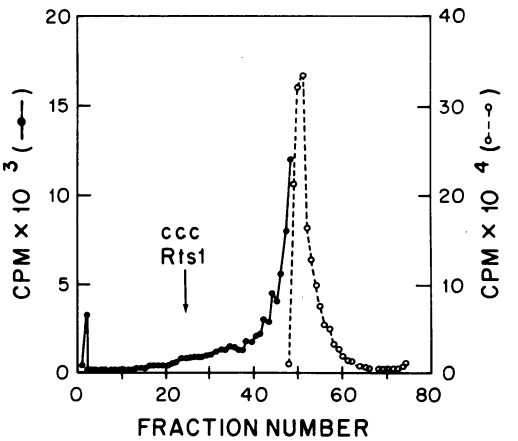

FIG. 7. *Absence of Rts1 DNA intermediate detectable by PI-CsCl density gradient centrifugation during the replication of Rts1 DNA at 42°C. E. coli 20S0(Rts1) cells grown at 32°C (10^8 in 2 ml of M9 medium) were brought up to 42°C for 1 h, and then 50 μCi of [^{3}H]thymidine and 500 μg of 2'-deoxyadenosine were added. Cells were then allowed to incorporate the radioactive material for 1 h at 42°C. The cells were harvested and subjected to PI-CsCl density gradient centrifugation.*

CCC DNA has been found almost ubiquitously in nature, and the mechanism of replication of this form of DNA has been extensively studied (15, 16, 20). A proposed model for replication of CCC DNA is shown in Fig. 8. In this scheme, step 1 represents the initiation of the CCC DNA replication. This step may involve, as shown in the replication of T4 phage DNA (32), binding of the CCC DNA to the cellular membrane. This possibility has been suggested from the studies of various plasmids that are incompatible with each other. It has been shown that CCC DNA combines with proteins to form a relaxation complex which may play an important role in the initiation of replication of the plasmid (14). One of the proteins that bind DNA appears to act as an endonuclease that introduces a nick into CCC DNA (14, 26, 27). Ribonucleic acid may be involved in this step and the elongation step (1, 15, 22, 34). Step 2 represents the elongation of the DNA chain and involves a concatenated form of CCC DNA (III) that will eventually become CCC DNA (31). Elongation of the DNA chain appears to take place either bidirectionally (6) or undirectionally (27). Step 3 represents the termination of chain elongation and separation of the two circular forms. For this step, as in the *E. coli* DNA termination step (29), protein may be involved in the final circularization of the elongated DNA chain, as reported for phage ϕ29 (30). If the synthesis of Rts1 DNA at 42°C occurs according to this scheme, one has to postulate that the temperature-sensitive block is at steps 1 and 3. This model is consistent with all the observations described here except the increase of Rts1 DNA at 42°C in the absence of CCC DNA formation (Fig. 2B). The apparent increase of Rts1 at 42°C during the 9-h period was approximately fivefold.

To account for this observation, we would like to suggest that Rts1 may be replicated at 42°C through a mechanism different from the conventional scheme given in Fig. 8. This hypothetical mode of replication does not involve CCC DNA. It must go on even at the permissive temperature because preformed CCC Rts1 DNA does not diminish at 42°C for a long period. This mode of replication must be in dynamic equilibrium with the regular mode of replication at 32°C, because the Rts1 formed at 42°C is rapidly converted to CCC Rts1 at 32°C. The exact physical nature of the Rts1 synthesized at 42°C is not understood at present. It may be a noncircular linear molecule that is so fragile that isolation procedure breaks it into smaller linear fragments. This unusual mode of replication may be peculiar only to Rts1, but the possibility exists that all plasmid DNA may have two modes of replication, as suggested in these studies.

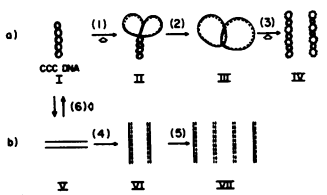

FIG. 8. *Possible mechanism of the thermosensitive block of Rts1 CCC DNA formation. I, CCC DNA; II and III, intermediate in replication involving CCC DNA; IV, newly formed, semi-conservatively replicated CCC DNA; V, linear form of Rts1 DNA; VI, semi-conservatively replicated linear-form Rts1 DNA; VII, newly formed linear Rts1 DNA involving those having both new strands.*

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-12337 from the National Institute of Allergy and Infectious Diseases.

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