Volume 6 Number 10 1979 Nucleic Acids Research

Characterization of an improved in vitro DNA replication system for Escherichia coli plasmids

Susan E.Conrad and Judith L.Campbell

Division of Biology and Department of Chemistry, California Institute of Technology, Pasadena, CA 91125, USA

Received 20 March 1979

ABSTRACT

A modified in vitro replication system has been characterized and used to catalogue the host proteins required for the replication of plasmid RSF1030. These extracts differ from systems described previously in that endogenous DNA is removed. Replication in vitro therefore requires an exogenouos RSF1030 template, even when extracts are prepared from cells carrying endogenous RSF1030. Synthesis in the in vitro system faithfully mimics in vivo replication with respect to the products synthesized, effects of specific inhibitors, and requirements for RNA polymerase and DNA polymerase I. In addition, we find that proteins encoded by dnaB, dnaC, dnaG, dnaI, dnaP and polC (DNA polymerase III), are required for in vitro plasmid synthesis. The product of dnaA is not required. Extracts prepared from E. coli mutants deficient in in vitro replication can be complemented by addition of purified proteins or of extracts carrying the wild type protein.

INTRODUCTION

Two in vitro systems have been described for the replication of plasmid DNA's, one by Sakakibara and Tomizawa (1), and another by Staudenbauer (2). These systems have been used for studying Col E1 and, more recently, RSF1030 replication (3; for summary see Refs. 4 and 5). These two plasmids have very similar replication properites. Both are present in multiple (20-30) copies per cell, and continue to replicate in the presence of chloramphenicol resulting in the accumulation of 1000-1500 copies per cell (4,6). In vivo replication of both plasmids requires DNA polymerase I and is inhibited by rifampicin (4,6,7). In the absence of DNA polymerase III, the rate of replication is decreased (7,8). In addition, both plasmids replicate unidirectionally from a unique origin (4,9). In spite of these similarities, plasmids Col E1 and RSF1030 appear to have evolved independently since they have no detectable sequence homology (6).

The two in vitro systems currently in use have several major flaws. In one (1), the rate and extent of synthesis is very low, and the extracts are

unstable. In the second (2), the extent of synthesis is increased, but there is endogenous plasmid DNA present in the extracts. It is desirable to use an in vitro system in which synthesis is dependent upon the addition of exogenous template in order to be sure that initiation is occurring in vitro and that synthesis does not reflect mere elongation of intermediates formed in vivo.

In the work presented here, we have extended the system of Staudenbauer by removing endogenous DNA and by concentrating extracts to allow increased levels of synthesis. The extent of synthesis we observe in vitro is almost three times greater than previously observed, making quantitative measurements and purification of products easier. Furthermore, the system is completely dependent upon the addition of exogenous, covalently-closed circular duplex DNA, allowing one to look at the replication of any desired added DNA or DNA's.

MATERIALS AND METHODS

Bacterial Strains and Plasmids--E. Coli W3110 thy^- and E. coli W3110 (RSF1030) were used as the standard strains for preparation of the in vitro system. Strains used to test for protein requirements are available on request. RSF1030 is a relaxed plasmid (4) that carries amp^R on the transposon Tn 2 (6,10).

Other Materials--Nalidixic acid, novobiocin and streptomycin sulfate were obtained from Sigma. Rifampicin was Rifampin from Calbiochem. Chloramphenicol was chloromycetin sodium succinate, Parke-Davis. [3H]dTTP (10 Ci/mmol) and [α-^{32}P]dTTP (300 Ci/mmol) were purchased from New England Nuclear. Agarose (Seakem, ME) was from Marine Colloids, Inc.

Growth of Cells--2 liters of E. coli W3110 (RSF1030) were grown at 37° to $O.D._{590}$=1.0 in L Broth supplemented with 0.1% glucose. Chloramphenicol (150 µg/ml) was added and the culture incubated for 4 h with shaking at 37° ($O.D._{590}$=3.6). Cells were harvested at room temperature and resuspended in 10 ml 0.025 M HEPES, pH 8.0/5 mM EGTA. The cells were frozen in liquid N_2.

Preparation of Extracts--Frozen cells were thawed by incubating at 0° for 20 min and then at 20°. The lysis procedure used was that of Staudenbauer (2). The cell suspension was brought to a final concentration of 0.15 M KCl and 0.2 mg/ml lysozyme. After incubation at 0° for 30 min the lysate was frozen in liquid N_2 for 2 min and then thawed at 20°. The lysate was centrifuged for 30 min at 100,000 χ g. The supernatant fluid, which will be referred to as Fraction I, contains about 17 mg per ml of protein and

has an A_{260} of 100-130.

Streptomycin Sulfate and Ammonium Sulfate Fractionation--To 2.0 ml of Fraction I, 0.2 ml of a 30 percent (w/v) solution of streptomycin sulfate was added. The solution was stirred for 30 min at 0°C. The precipitate was removed by centrifugation for 10 min at 20,000 χ g at 4°C. To 2.0 ml of supernatant, 0.944 g solid ammonium sulfate was added over a 10 min period. The mixture was stirred for 30 min at 0° and the precipitate was then removed by centrifugation at 20,000 χ g for 10 min. The pellet was dissolved in 1.0 ml 0.01 M HEPES, pH 8.0/1 mM DTT/1 mM EDTA/ 0.1 M KCl/10% ethylene glycol. The redissolved pellet was dialyzed against 100 ml of the same buffer for 3 h at 0°. The dialyzed material, Fraction II, was frozen and stored in liquid N_2. Fraction II contains about 11 mg per ml of protein.

Assay for DNA Synthesis--A standard assay for DNA synthesis has been established using Fraction II. Reactions (0.05 ml) contained 40 mM HEPES, pH 8.0, 100 mM KCl, 12 mM magnesium acetate, 50 μM each dCTP, dGTP, dATP, and [^{3}H]TTP, 2 mM ATP, 0.5 mM each rCTP, rGTP, rUTP, 1.5 nmol of RSF1030 DNA, and 20 μl extract. Incubations were carried out at 30°. Reactions were terminated by placing tubes at 0° followed by addition of 1 N HCl containing 0.1 M sodium pyrophosphate. The precipitate was collected on Whatman GF/C filters. The filters were washed, dried and radioactivity determined in a toluene-based fluid in a scintillation counter. The half-life of the DNA synthesizing activity was greater than 60 min at 30° in Fractions I and II. Fraction II has a half life of >60 min at 40°.

Preparation of Plasmid RSF1030 DNA--E. coli HMS 174 recA1 (RSF1030) was grown to $O.D._{590}$=1.0 in Vogel-Bonner medium (11). Chloramphenicol (250 μg/ml) was added and incubation continued for 17 h. Cells were harvested by centrifugation at 4°, and frozen and thawed one time. Cells (25 g) were lysed by the method of Clewell and Helinski (12), modified by the use of Triton X-100 in place of Brij 58 and sodium deoxycholate (13).

Supercoiled DNA was purified by two equilibrium bandings in CsCl (ρ=1.55)/EtdBr (100 μg/ml) gradients. In order to remove low molecular weight nucleic acid contaminants, the DNA was sedimented through 30 ml CsCl (ρ=1.3 gm/cc) containing 100 μg/ml EtdBr with a CsCl (ρ=1.7 gm/cc) cushion. EtdBr was removed by Dowex chromatography and CsCl was removed by dialysis.

Agarose Gel Electrophoresis--Vertical slab gels (7 5/8" x 6 1/2" x 4 mm) of 0.7% agarose were formed and run in E buffer (40 mM Tris, 5 mM NaAcetate, 1 mM EDTA, pH 7.4) as described previously (9). Samples were loaded in low salt buffer plus 2.5% Ficoll, and 0.02% bromophenol blue in a maximum volume of

50 µl for a ten slot gel. Neutral agarose gels were run at 40V for 12 hrs to resolve the different forms of circular DNA. For autoradiography, gels were first dried on a Slab Gel Dryer (Hoefer Sci. Instruments). Preflashed Kodak Xr-5 film was exposed at -70°C using an intensifying screen.

RESULTS

As pointed out in the Introduction, one goal of these studies was to develop an in vitro replication system dependent on the addition of exogenous DNA. Although one approach would be to prepare extracts from cells that do not carry plasmid, such extracts would not contain products specified by plasmid genes and one could not assess their role, if any, in plasmid replication. We therefore chose to prepare extracts from cells carrying the plasmid and to subsequently remove endogenous plasmid from the extracts. Since lability of the extracts made such fractionation difficult, we first established conditions for stabilizing the replicating activity. Sucrose and glycerol proved inhibitory (2,3). Ethylene glycol, however, proved an efficient stabilizing chemical and we have been able to develop a system (see Methods, Fraction II) that is totally dependent on addition of exogenous covalently closed, circular, duplex plasmid DNA.

In order to be sure that synthesis in Fraction II represented replication and not repair, Fraction II was compared to Fraction I with respect to the requirements for synthesis, the effect of inhibitors, and the nature of the products. Fraction I has been shown by others to represent replication (2).

Kinetics of Synthesis in Fraction I and Fraction II--The kinetics of RSF1030 DNA synthesis in Fraction I confirm the findings of Staudenbauer et al. (2). As shown in Figure 1, synthesis proceeds at a linear rate for 40 minutes and then plateaus at around 60 minutes. After an initial lag period of 10 minutes, the kinetics of synthesis in Fraction II, also shown in Fig. 1, are somewhat different. The rate is 30-50% higher than observed in Fraction I, with synthesis continuing at an almost linear rate for up to 90 minutes before a plateau is reached. The extent of synthesis obtained with Fraction II is almost three-fold that seen in Fraction I. As in Fraction I, however, a 10 minute lag period is observed before the synthesis increases at a linear rate. The initial rate of synthesis in Fraction II after the 10 minute lag is 180 pmols of dTMP incorporated per hour, or the equivalent of 2.6×10^{10} RSF1030 molecules per hour. Since the reaction mixture contains protein from 6×10^{9} cells, this rate represents 4.5

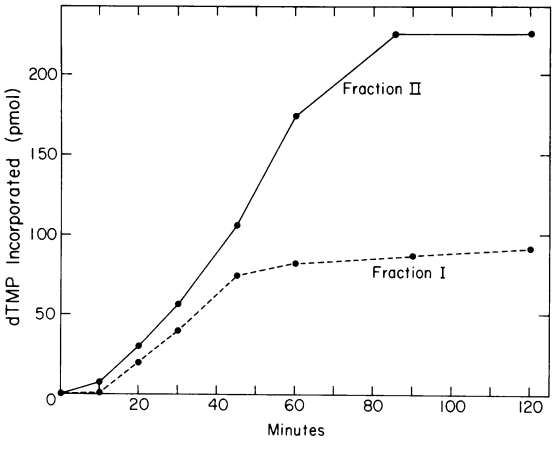

Figure 1. Kinetics of RSF1030 DNA Synthesis in Fractions I and II. Fractions I and II were prepared as described under "Materials and Methods." The kinetics of synthesis were then measured using standard reaction conditions described under "Materials and Methods."

RSF1030 molecules per hour per cell equivalent.

Requirements of the Reaction and Effects of Inhibitors--The requirements of the in vitro synthesis reaction in Fractions I and II were compared and are shown in Table I. Results with Fraction II are exactly the same as with Fraction I and are similar to those in other related in vitro

Table I

Requirements for DNA Synthesis in vitro

Conditions	Fraction I	Fraction II
	(%)	(%)
Complete	100	100
- MgAc	< 0.3	< 0.3
- dCTP, dGTP, dATP	< 0.3	< 0.3
- dATP	N.D.	66
- dCTP or dGTP	N.D.	34
- rCTP, rGTP, rUTP	< 0.3	< 0.3
- rATP	< 0.3	< 0.3
- DNA	60-80	< 0.3
40 mM KCl	80	80
Rifampicin (5 µg/ml)	1	1
Novobiocin (1 µg/ml)	1	1
Nalidixic Acid (50 µg/ml)	43	40

Table I. DNA synthesis activity was measured in the standard assay as described in "Materials and Methods," using either Fraction I or Fraction II prepared from E. coli W3110(RSF1030). In the complete system 120 pmol and 200 pmol of [^{3}H]TMP represent 100% incorporation of [^{3}H]TMP into acid-insoluble product using Fraction I and Fraction II, respectively.

systems (1,2). The most important similarities are the requirement for ribonucleotides and the inhibition of synthesis by rifampicin. Inhibition by rifampicin indicates that RNA polymerase is required for synthesis in Fraction II, and is strong evidence in itself that Fraction II carries out replication and not repair synthesis. The kinetics of rifampicin inhibition in Fraction II (Fig. 2) are the same as those reported by others (1) and show that, in Fraction II as in Fraction I, rifampicin inhibits only initiation. Inhibition of Fraction II by novobiocin and nalidixic acid further shows its similarity to Fraction I and implicates DNA gyrase (14,15) in synthesis in Fraction II (Table I, Fig. 2b).

Template Requirement in Fraction II--As shown in Fig. 3 and Table I, the amount of DNA synthesis in Fraction I in the absence of exogenous DNA is 63% of that observed in the presence of 10 µg/ml of added Form I RSF1030 DNA. In Fraction II, however, the amount of synthesis in the absence of DNA is <0.3% of the maximum observed when DNA is added to a final concentration of 10 µg/ml, indicating the complete dependence of synthesis in Fraction II upon exogenous DNA.

The initial rate of synthesis in Fraction II (after the 10 min lag) is proportional to the amount of DNA added to the reaction, although high concentrations are inhibitory (Fig. 3). Optimal synthesis is obtained when RSF1030 is added to a final concentration of 10 µg/ml, which represents a total of 5.3×10^{10} RSF1030 template molecules per reaction, or 9 molecules per cell equivalent of Fraction II added to the reaction. Since maximum

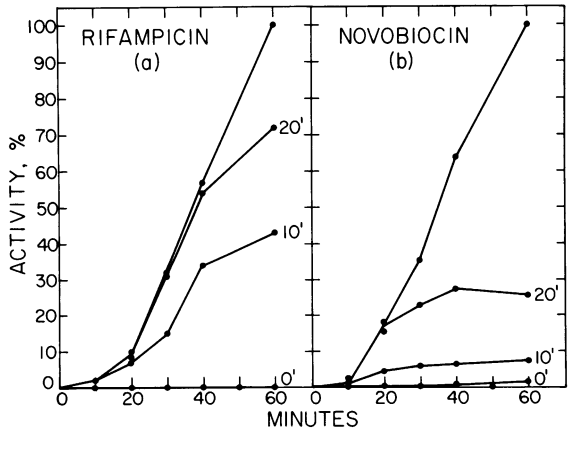

Figure 2. A. Inhibition of synthesis in Fraction II by Rifampicin. The kinetics of synthesis were measured in the standard reaction mixture. At times marked on the curves, rifampicin (10 µg/ml) was added to individual reaction mixtures and synthesis was allowed to continue in the presence of the drug. The extent of synthesis was determined at the times indicated on the abscissa. B. Inhibition of synthesis in Fraction II by novobiocin. The kinetics of inhibition of synthesis by novobiocin (1 µg/ml) were measured as described for rifampicin.

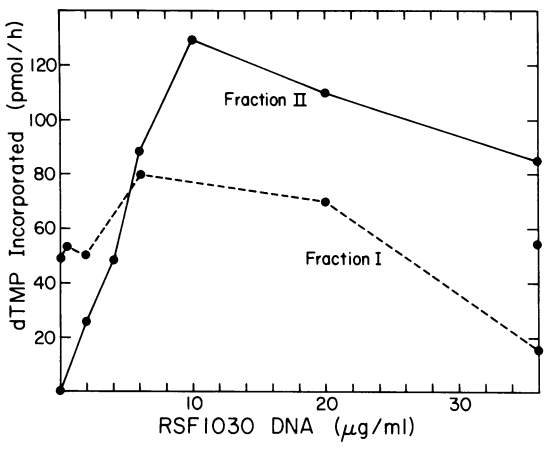

Figure 3. Effect of DNA concentration on the rate of DNA synthesis in Fractions I and II. Reactions were carried out in the standard reaction mixture except that the concentration of RSF1030 DNA was varied as shown.

synthesis ranges from 4.5 to 8 RSF1030 molecules produced per cell equivalent, depending on the extract used, 40-90% of the template, at most, is participating in replication. This is a more efficient use of template than in any other in vitro plasmid replication system.

The DNA requirement of Fraction II parallels the requirement for DNA observed in Fraction I extracts prepared from cells that carry no endogenous plasmid. However, the overall rate of synthesis in the latter extracts is always about three-fold less than the rate observed in extracts prepared from plasmid carrying cells.

COMPARISON OF THE PRODUCT IN FRACTIONS I AND II

Sucrose Gradient Analysis--RSF1030 DNA synthesized in an in vitro reaction containing covalently closed RSF1030 as template and [α-^{32}P]TTP has been analyzed by zone sedimentation through neutral and alkaline sucrose gradients (Fig. 4). Results using Fraction I are presented for comparison with Fraction II. These results clearly show that products are formed in approximately the same ratios in reactions catalyzed by either Fraction I or Fraction II. Although the proportion of newly synthesized DNA in each peak varies somewhat from experiment to experiment, the product distribution is identical to that found in Staudenbauer's Fraction I (2) and compatible with that found in the detergent lysis system (1), where synthesis is known to represent replication and not repair. In summary, distribution of product suggests that up to 90% of the molecules that initiate replication in vitro complete replication, while the remainder are present as partially replicated molecules.

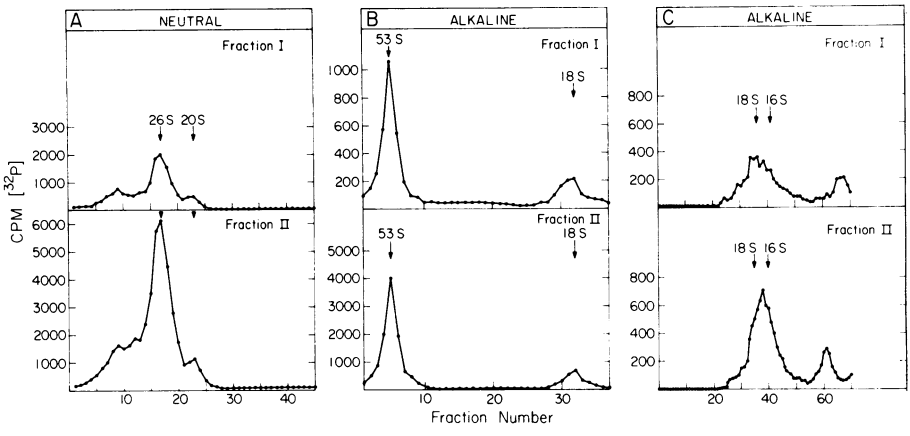

Figure 4. Sucrose gradient analysis of RSF1030 DNA synthesized in vitro. DNA was synthesized in an 0.1 ml reaction mixture containing [α-^{32}P]TTP (259 cpm/pmol) at 30° for 60'. The reaction was terminated by addition of EDTA and the reaction mixtures were extracted 1x with 0.2 ml phenol. 0.02 ml sodium acetate (10% w/v) was added and DNA precipitated by addition of 0.4 ml 95% ethanol followed by incubation at -70° for 10 min. The pellet was recovered by centrifugation, washed once with 70% ethanol (0°) and redissolved in 0.1 ml of 0.01 M HEPES-1 mM EGTA. An aliquot of each reaction was then analyzed by sedimentation through a neutral or alkaline sucrose gradient in an SW41 rotor at 35,000 rpm. A) neutral, 9 h centrifugation; B) alkaline, 9 h centrifugation; C) alkaline, 16 h centrifugation. Sedimentation is from the right to left. [^{3}H]RSF1030 and [^{3}H]SV40 Form I and Form II were included in each gradient at internal markers. Positions of [^{3}H]RSF1030 Form I and II are indicated by arrows and S values calculated from the positions of [^{3}H]SV40 markers.

Gel Electrophoresis--Despite the large number of similarities between Fractions I and II already shown we wished to demonstrate that newly synthesized DNA was not covalently attached to the template. Synthesis was carried out using an RSF1030 template and [α-^{32}P]TTP (200 cpm/pmol) in the standard reaction mixture for sixty minutes. The products were phenol extracted and ethanol precipitated and analyzed on 0.7% agarose gels (Fig. 5), prior to (Lanes 2 and 4) and after denaturing and renaturing (Lanes 3 and 5). Lanes 2 and 3 (EtdBr stained) give information about both template and product. Lanes 4 and 5 are autoradiograms of Lanes 2 and 3 and therefore only represent newly synthesized DNA.

Prior to denaturation, completely replicated molecules are seen at the position of Form I DNA (16) (Lanes 2 and 4). In addition, labeled DNA products are seen at the position of Form II and in two other bands, one migrating between Forms I and II, designated RI_{α}, and a second migrating

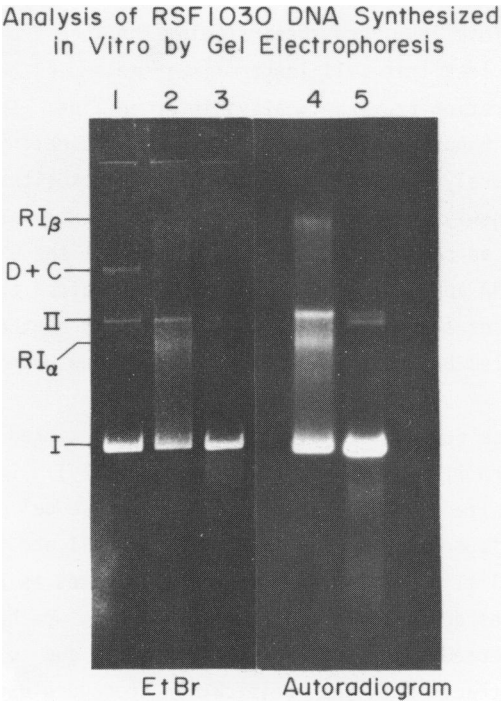

Figure 5. Agarose gel electrophoresis of RSF1030 DNA synthesized in vitro. RSF1030 DNA was synthesized in an 0.1 ml reaction mixture for 1 h at 30°. The reaction was terminated and DNA prepared for electrophoresis as in Fig. 4. One sample (0.05 ml) was heated at 90° for 90 sec before application to the gel. Samples were electrophoresed and gels autoradiographed as described previously (2) and in "Methods." Lane 1, RSF1030 Form I and Form II markers, D + C are dimers and catenated dimers; Lane 2, products of RSF1030 synthesis stained with EtdBr; Lane 3, DNA heated at 90° for 90 sec before electrophoresis; Lane 4, autoradiogram of Lane 2; Lane 5, autoradiogram of Lane 3.

slower than Form II indicated as RI_{β}. There is ^{32}P labeled material appearing througout the gel from the position of Form I DNA to the position of the most slowly migrating band. The material has been eluted from these regions of the gel, and analysis by electron microscopy indicates that they are circular replicative intermediates representing different extents of replication (9).

Upon denaturation of the product, Lanes 3 and 5, all of the labeled material above the Form II position disappears, as does the diffuse material between Forms I and II. A new band, which we have not characterized,

appears just below the Form II position. The amount of Form I material increases, and diffuse label now appears below the Form I position, probably representing less than full length linear molecules that have been released by denaturation from replicative intermediates. There are several very broad bands apparent in this region, indicating that replication may be prematurely terminated at several distinct sites. Thus, at least some of the newly synthesized DNA is not covalently attached to the template since it can be released by brief heating of the DNA. That the amount of Form I DNA appearing in the autoradiogram after the product is heated also increases suggests that some of the newly synthesized and therefore ^{32}P labeled DNA served as a template for new rounds of replication in vitro.

Neutral agarose gels do not distinguish between nicked and relaxed closed circles (Form II and Form I_o (16), respectively). In order to do this, newly synthesized DNA was analyzed on an agarose gel containing EtdBr, a system we found readily distinguishes Forms II and I_o (data not shown). There is little Form I_o DNA--most DNA migrates as Form I, II or the intermediates described above. We do not know whether the nicked forms are late intermediates in replication or arise due to random nicking activity in the extract or in the purification procedure used in preparing the DNA for gel analysis.

Further Characterization of Product--Because of the good correspondence between the requirements for synthesis and products of synthesis in Fractions I and II, and because the product was not all covalently attached to the template, no further product characterization by density gradients was considered necessary to prove that synthesis represented replication. This conclusion is supported below by the fact that a number of proteins known to be required for replication in vivo, but not thought to be repair enzymes, are absolutely required for DNA synthesis. Most importantly, however, we have shown by electron microscopy and gel electrophoresis that replication in Fraction II is initiated at a unique site and proceeds sequentially and unidirectionally (9). This result could only be due to replication and rules out the possibility that we are looking at repair synthesis, even in completely replicated molecules.

The above studies have been repeated with hosts not carrying endogenous plasmid. The results are identical, indicating that if any plasmid protein is involved in replication, a host protein can substitute for it.

Mutations Affecting DNA Polymerases--DNA polymerase I is required for

plasmid replication in vivo (4) and has been shown to be required for Col E1 and RSF1030 replication in vitro in Fraction I (2,7). In addition, DNA polymerase III is implicated in plasmid replication in vivo since in polC mutants synthesis slows down, although it does not cease completely. Staudenbauer has also shown that DNA polymerase III is required for plasmid replication in vitro (7,17). Fraction II was prepared from polA or polC mutants as a further test that Fraction II was actually carrying out DNA replication.

Fraction II was prepared from E. coli DK517', which contains a thermolabile DNA polymerase I. No replication was found whether the extract was first incubated at 30° or at 40°. Evidently the activity in strain DK517' is defective in vitro even at the temperature that permits RSF1030 replication in vivo. This confirms that DNA polymerase I is required in vitro in Fraction II as well as in vivo and, as Staudenbauer has already shown, in Fraction I (17). Identical results were obtained with a polA1 mutant.

Extracts of strain BT1026 polA1 $polC_{ts}$ supplemented with purified DNA polymerase I carries out synthesis at 30°, but not at 37° (17). Fraction II was prepared with E. coli dnaE486, which Livingston et al. (18) have shown contains no detectable DNA polymerase III activity in vitro. We found that there was no RSF1030 replication at any temperature in extracts of dnaE486 mutants, confirming that in Fraction II, as in Fraction I, DNA polymerase III is absolutely required for replication.

The usefulness of an invitro replication system lies in its ability to give new insight into the mechanism of DNA replication, and a great deal has been learned about the molecular biology of plasmid replication from in vitro replication studies. Requirements for DNA gyrase, RNA polymerase, DNA polymerase I and DNA polymerase III have recently been demonstrated (4). We have used Fraction II to investigate the additional protein requirements for plasmid replication.

A summary of the gene products required in vivo for plasmid replication is found in Ref. 4. We have prepared Fraction II from a number of mutants of E. coli defective in these required functions (dnaA, dnaB, dnaC, dnaE, and dnaG) and from several mutants defective in other functions not yet identified with plasmid replication in vivo, but that have phenotypes that suggest they might be (dnaZ, dnaP, dnaI). Extracts were prepared in most cases from strains that harbored RSF1030. Extracts from strains dnaG, dnaZ, and dnaI, however, did not contain endogenous plasmid. It remains to

be seen, therefore, if a plasmid gene product suppresses the requirement for any of these three activities. A summary of the results obtained using these mutants and corresponding revertants is shown in Table II. Staudenbauer has also demonstrated requirements for dnaB, dnaC, and dnaZ (19).

A complementation assay has been developed for the purification of dnaP protein based on the data in Table II. Addition of Fraction II prepared from E. colipolA1polB100polC$_{ts}$ stimulates synthesis in Fraction II from dnaP stains 3-fold, and the protein has been partially purified using this as an assay (unpublished data). In addition, purified RNase III added to Fraction II prepared from E. coli rnc restores DNA synthesis (20,21), indicating that Fraction II can be complemented by purified proteins.

Table II

Requirements for E. coli Proteins for RSF1030 DNA Synthesis in vitro

Gene	Activity	
	Parent or Revertant	Mutant
	(pmol)	(pmol)
dnaA	129	131
dnaB	129	1.0
dnaC	70	1.5
dnaE(polC)	N.D.	1.0
dnaG	28	2.8
dnaI	128	11.7
dnaP	81	12.5
dnaZ	N.D.	3.5
polA	N.D.	1.0
rnc	238	4.5

Table II. Fraction I and II were prepared from a strain carrying the mutation indicated on the left. A list of strains used is appended. For dnaA, dnaC, dnaI, and dnaP, extracts were prepared from isogenic parental strains as controls. For dnaG and dnaB, revertants were prepared by plating liquid cultures in soft agar and incubating at 42°. Synthesis was measured in Fraction I and Fraction II, but only results using Fraction II are presented. In all cases, 20 µl of Fraction II were incubated in the standard reaction mixture containing 0.1 mM DTT, but without DNA, for 0, 10, 20, 30 or 40 min at 40°. RES1030 DNA (10 µg/ml) was then added and synthesis measured for 60 min at 30°. The results with dnaA represent activity after 40 min at 40°. Values for all other strains represent synthesis after 0 min at 40°. Since 3 separate revertants of dnaB266 gave no activity, the result with the parental strain is used as control.

DISCUSSION

An improved in vitro system for studying plasmid DNA replication has been developed. The extent of synthesis observed in this system is approximately 4x that seen in the previous system. More importantly, synthesis is now completely dependent upon the addition of exogenous Form I DNA, although the cells from which the extracts are made contain endogenous plasmids. Evidence obtained in vivo using plasmids and chloramphenicol-treated cells indicates that no plasmid coded proteins are required for Col E1 replication (22,23), but does not discount the possibility that such gene products are normally involved in replication or can replace host proteins. Recently, Helinski has shown that in vitro replication of the transmissable, multicopy plasmid R6K does require an R6K encoded protein (24).

Analysis of the products synthesized in vitro both sucrose gradients and gel electrophoresis indicates that both fully and partially replicated molecules are formed during the reaction and that the newly synthesized DNA in the partially replicated molecules is not covalently attached to the template. Electron microscope studies of partially replicated molecules have, in fact, allowed us to map the origin and direction of RSF1030 replication (9). This system is dependent upon RNA polymerase, DNA polymerase I and III and DNA gyrase, as has been reported in earlier systems. In addition, the products encoded by the genes dnaB, dnaC, dnaG, dnaP, and dnaI appear to be required for plasmid DNA replication.

The increased amount of synthesis in this system makes it ideal for studying individual steps of the replication reaction. For example, many of the dna_{ts} mutants contain low residual levels of activity, that represent enough synthesis so that the replicative intermediates can be isolated. Analysis of these DNA molecules can provide information about the stage of the reaction at which the protein is required. Staudenbauer has shown that in dnaZ extracts, the product is 7S and can be elongated into completed 26S molecules by further indubation with polymerase III in the presence of rifampicin to prevent additional initiations (3). Although he has not analyzed this DNA, he has used it to study proteins required for its elongation. A similar approach will be helpful in analyzing residual activity in dnaI and dnaG mutants. In our hands, the levels of synthesis seen with polymerase III, dnaE, and dnaC mutants are probably too low to examine which DNA products are involved at which step. However, we can isolate intermediates and add them to these mutant extracts in order to observe their fate. Future studies will be aimed at separating the reaction into stages, and looking at

interactions of all the proteins involved.

One of the main advantages of the system has been to establish complementation assays for several proteins previously known from genetic evidence to be involved in E. coli replication, but not required in various in vitro phage replication systems. For instance, we have used the system to partially purify the dnaP protein (unpublished results), a protein thought to be involved in initiation of chromosomal DNA replication. We have also used complementation with purified RNase III to show that this enzyme, and therefore, perhaps RNA processing, is involved in DNA replication (20).

A novel use of the system, made possible by its dependence on exogenous DNA, has been its application as an in vitro transcription system. Using Fraction II in this capacity, we have shown that the coding sequence for a small 100 nucleotide RNA is involved in controlling plasmid copy number (20). Currently we are using this in vitro replication system to test complementation between self-replicating and non-self-replicating mutant DNAs missing the RNA coding sequence to study the mechanism by which the RNA exerts its effect. Complementation in vitro has the advantage that incompatibility phenomena that make in vivo results difficult to interpret are avoided.

ABBREVIATIONS

Form I, covalently closed circular duplex supercoiled DNA; Form I_o, covalently closed circular duplex relaxed DNA; Form II, nicked, circular duplex DNA; Form III, linear, duplex DNA; DTT, dithiothreitol; HEPES, N-2-hydroxyethylpiperazine-N'-2'ethane'sulfonic acid; EGTA, ethylene-glycol-bis-(β-aminorethyl ether)N, N'-tetra-acetic acid, N.D., not determined.

ACKNOWLEDGMENTS

This investigation was supported by grants from the United States Public Health Service: RR07003 and GM 25508. S.E.C. was supported by United States Public Health Service Training Grant No. 5 T32 GM 07616.

REFERENCES

1. Sakakibara, Y. and Tomizawa, J. (1974) Proc. Natl. Acad. Sci. USA 71, 802-806.
2. Staudenbauer, W.L. (1976) Molec. Gen. Genet. 145, 273-280.
3. _____. (1977) Molec. Gen. Genet. 156, 27-34.
4. _____. (1978) Curr. Topics Microbiol. Immunol. in press.
5. Wickner, S. (1978) Ann. Rev. Biochem. 47, 1163-1191.
6. Crosa, J.H., Luttrop, L.K., and Falkow, S. (1975) Proc. Natl. Acad. Sci. USA 72, 654-658.
7. Falkow, S. (1975) Infectious Multiple Drug Resistance, pp. 116-117. Pion Limited, London.

8. Collins, J.P., Williams, P.H., and Helinski, D.R. (1975) Molec. Gen. Genet. 136, 372-289.
9. Conrad, S.C., Wold, M. and Campbell, J.L. (1979) Proc. Natl. Acad. Sci. USA 76, 736-740.
10. Heffron, F., Sublett, R., Hedges, R., Jacob, A., and Falkow, S. (1975) J. Bacteriol. 122, 250-256.
11. Vogel, M.J. and Bonner, D.M. (1956) J. Biol. Chem. 218, 97-106.
12. Clewell, D.B. and Helinski, D.R. (1969) Proc. Natl. Acad. Sci. USA 62, 1159-1166.
13. Kuperszotch, Y.M. and Helinski, D.R. (1973) Biochem. Biophys. Res. Comm. 54, 1451-1459.
14. Gellert, M., O'Dea, M.H., Itoh, T., and Tomizawa, J. (1976) Proc. Natl. Acad. Sci. USA, 73, 4474-4478.
15. Gellert, M., Muzuuchi, K., O'Dea, M.H., Itoh, T., and Tomizawa, J. (1977) Proc. Natl. Acad. Sci. USA 74, 4772-4776.
16. Weil, R. and Vinograd, J. (1963) Proc. Natl. Acad. Sci. USA 50, 730-738.
17. Staudenbauer, W.L. (1976) Molec. Gen. Genet. 149, 151-158
18. Livingston, D.M. and Richardson, C.C. (1975) J. Biol. Chem. 250, 470-478.
19. Staudenbauer, W.L., Lanka, E., and Schuster, H. (1978) Molec. Gen. Genet. 162, 243-249.
20. Conrad, S.E. and Campbell, J.L. (1979) Cell, in press.
21. Dunn, J.J. (1976) J. Biol. Chem. 251, 3807-3814.
22. Donahue, D.J. and Sharp, P.A. (1978) J. Bacteriol. 133, 1287-1294.
23. Kahn, M. and Helinski, D.R. (1978) Proc. Natl. Acad. Sci. USA 75, 2200-2204.
24. Inuzuka, M., Helinski, D.R. (1978) Biochemistry 17, 2657-2574.

APPENDIX A

STRAINS USED

Strain Designation	Genotype	Source
CRT46	dnaA T46	Y. Hirota
PC5	dnaA	P. Carl
CR34/43	dnaB	Y. Hirota
PC1	dnaC	P. Carl
PC7	dnaC,D	P. Carl
PC2	dnaC	P. Carl
PC255	parent of PC1,2,5,7	P. Carl
BT1026	polA1 polC	H. Bonhoeffer
E613	polC T486	Y. Hirota
HMS121	polA1 polB100 polC1026	J. Campbell
E101	dnaF	Y. Hirota
PC3	dnaG	Y. Hirota
WM437	dnaI 208	W. Messer
WM301	$dnaI^{+}$	W. Messer
KY2750	dnaP 18	T. Yura
KY2053	$dnaP^{+}$	T. Yura
DK517'	$polA_{ts}$	D. Kingsbury
Lr107	rnc105	W. Studier