Proc. Natl. Acad. Sci. USA
Vol. 78, No. 8, pp. 4902–4906, August 1981
Biochemistry

Replication of plasmid pT181 DNA *in vitro*: Requirement for a plasmid-encoded product

(*in vitro* replication/gel electrophoresis/copy mutants/plasmid-encoded function)

SALEEM A. KHAN, STEPHEN M. CARLETON, AND RICHARD P. NOVICK

Department of Plasmid Biology, The Public Health Research Institute of the City of New York, 455 First Avenue, New York, New York 10016

Communicated by Donald R. Helinski, May 15, 1981

ABSTRACT **PT181 is a naturally occurring 4.5-kilobase *Staphylococcus aureus* plasmid encoding resistance to tetracycline. The plasmid has a copy number of about 20 per cell; a mutant, *cop-608*, that has a copy number of 800–1000 has been isolated. A cell-free extract has been developed that carries out complete replication of this plasmid. Extracts made from a strain containing the mutant have much greater replication activity than do extracts of strains containing pT181. In an extract from which endogenous DNA has been removed, DNA synthesis is dependent upon the addition of exogenous plasmid DNA. The replication system is specific for pT181 and related plasmids but it is inactive with other *S. aureus* plasmids. Furthermore, pT181 DNA does not replicate in extracts made from plasmid-negative strains or strains containing other plasmids. The results suggest that a specific plasmid-encoded substance is required for the replication of pT181 DNA.**

The problem of how a nonessential replicon (plasmid) is maintained in a bacterial cell at a more or less constant level has been of considerable interest since the now-classic replicon hypothesis was proposed by Jacob *et al.* (1). As predicted by this hypothesis, most plasmids specify explicit functions that not only permit the plasmid to utilize the general replicative apparatus of the host cell but also determine the rate at which this occurs. Extensive molecular genetic analyses have revealed plasmid-encoded substances that are required for replication (positive effectors of replication) and others that act to keep the replication rate below some theoretical maximum (negative replicative control substances) (2, 3).

The precise molecular interactions by which such substances determine the rate of replication have not yet been determined for any plasmid. To this end, several groups of investigators have developed cell-free extracts of *Escherichia coli* in which certain plasmid are capable of semiconservative replication. These include three groups of plasmids—ColE1 and related plasmids (4–7), R6K (8, 9) and a derivative of R1 (10)—and there is substantial variability among the various plasmid systems. The ColE1 plasmid is known not to require plasmid-encoded protein for its replication either *in vivo* or *in vitro* (11, 12). pI258 (*in vivo*) and R6K (*in vitro*) do require a plasmid-encoded protein for replication (2, 9, 13, 14). For R6K, the replication protein π which is involved in the initiation of DNA replication, does not positively control the rate of initiation (A. Shafferman and D. R. Helinski, personal communication).

pT181 has been developed as the prototype of a common class of staphylococcal tetracycline resistance-plasmids (15, 16). pT181, 4.5 kilobases, specifies inducible resistance to tetracycline and has a copy number of about 20 per cell (15). Thermosensitive, replication-defective mutants have been isolated and have been shown to be *trans*-complementable by the wild-type plasmid (16). Mutants with increased copy numbers have also been isolated, by selection for increased tetracycline resistance and one of these, *cop-608*, has the highest copy number of any plasmid yet described (800–1000 per cell). This phenotype is due to a deletion of 179 base pairs (unpublished data).

We describe here a cell-free extract that catalyzes the complete semiconservative replication of pT181 plasmid DNA and show that the activity of this extract is dependent on a plasmid-determined product; the data further suggest that this product is rate-limiting for replication in wild-type extracts.

MATERIALS AND METHODS

Strains and plasmids used in these studies are listed in Table 1. Media and growth conditions were as described (19).

Materials. Nucleotide triphosphates, novobiocin, and rifampicin were from Sigma, [^{3}H]dTTP was from ICN (60 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels), and [α-^{32}P]dTTP was from New England Nuclear (30 Ci/mmol). SeaKem agarose was from Marine Colloids (Rockland, ME). All other chemicals were from standard commercial sources.

Preparation of Plasmid DNA and Gel Electrophoresis. Plasmid DNA was isolated by CsCl/ethidium bromide equilibrium centrifugation of cleared lysates (20, 21). Radioactively labeled products of *in vitro* replication were analyzed by electrophoresis on 0.8% or 1% vertical agarose slab gels with Tris/borate/EDTA buffer, pH 8.3 (22). Gels were run at 3.5 V/cm for 5 hr, stained with ethidium bromide, photographed, and dried on Whatman no. 1 filter paper at 37°C for 2 hr; autoradiographs were prepared by exposure to Kodak RP-Royal X-Omat x-ray film.

Preparation of Cell-Free Extracts. Bacteria were grown in 500 ml of brain heart infusion with shaking at 37°C and harvested at midexponential phase (5 × 10^8 cells per ml) at room temperature. Cells were converted to protoplasts in 10 mM Tris·HCl, pH 8/1 mM EDTA/1 mM dithiothreitol containing 200 μg of lysostaphin per ml (21) and were then lysed by freezing and thawing (6). Extracts were prepared essentially as described by Conrad and Campbell (7). Endogenous DNA was removed from the cell supernatant (crude extract) by streptomycin sulfate precipitation and the extracts were then subjected to ammonium sulfate precipitation (7). The 0–70% saturated ammonium sulfate precipitate from 5 g of cells was dissolved in 1 ml of 10 mM Tris·HCl, pH 8/1 mM EDTA/1 mM dithiothreitol/0.1 M KCl/10% (vol/vol) ethylene glycol and dialyzed against the same buffer for 3 hr in the cold. Small aliquots were frozen and stored at −70°C. This extract is referred to as "partially purified" extract.

Reaction Mixture and Conditions. Reaction mixtures (50 μl) contained 40 mM Tris·HCl (pH 8), 100 mM KCl, 12 mM magnesium acetate, dGTP, dCTP, dATP, and either [^{3}H]- or

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Table 1. *Staphylococcus aureus* strains and plasmids

Strain	Plasmid	Plasmid genotype	Source or derivation
RN3259	pT181	*tet, inc3*	(15)
RN3268	pRN8008	*tet, inc3 cop-608*	Copy number mutant of pT181
SA233	pE194	*ermC incII*	(15)
SA246	pSA0301	*tet, inc3 repCl*	(16)
DU4916	pSN1	*tet*	(17)
DU4916	pSN2	Unknown	(17)
RN453	pRN3038	pI258 *blaI443*	(2)
RN27	Plasmid negative	8325-3 (80α)	(18)

[α-^{32}P]dTTP each at 50 μM, 2 mM ATP, rCTP, rUTP, and rGTP each at 0.5 mM, 0.05 mM NAD, 0.05 mM cAMP, 0.5–1 μg of template plasmid DNA, and 20–25 μl of extract (added last). Incubations were at 30°C for various lengths of time as indicated. Reactions were terminated by the addition of cold 10% trichloroacetic acid/0.1 M PP_i; the acid-precipitable radioactivity was assayed on Whatman GF/C filters (8). For electrophoretic analysis of DNA, reaction mixtures after incubation were adjusted to 5% (vol/vol) glycerol, 1% $NaDodSO_4$, 1 mM EDTA, and 0.02% bromophenol blue and subjected to electrophoresis on 0.8% or 1% vertical agarose slab gels.

Sedimentation Analysis. Linear 5–20% sucrose gradients (5 ml) were prepared in cellulose nitrate tubes. Neutral sucrose gradients contained 50 mM Tris·HCl (pH 8.0), 5 mM EDTA, and 1 M NaCl. Alkaline sucrose gradients contained 0.3 M NaOH, 1 mM EDTA, and 1 M NaCl. Samples (200 μl) were centrifuged in an SW 50.1 rotor for 3 ¼ hr at 15°C. Gradients were fractionated and radioactivity was determined as described (8). Samples for neutral CsCl density gradient analysis were mixed with Tris/EDTA/NaCl buffer and adjusted to a density of 1.69 g/cm^3. Samples for alkaline CsCl density gradient analysis were mixed with 0.15 M NaCl/0.015 M sodium citrate/5 mM EDTA/0.1 M NaOH containing 0.03% sodium lauroyl sarcosinate. The density was adjusted to 1.71 g/cm^3 and the volume was adjusted to 2 ml. After centrifugation to equilibrium at 38,000 rpm for 48 hr at 15°C in a Ti 50 rotor, fractions were collected from the bottom and radioactivity was measured.

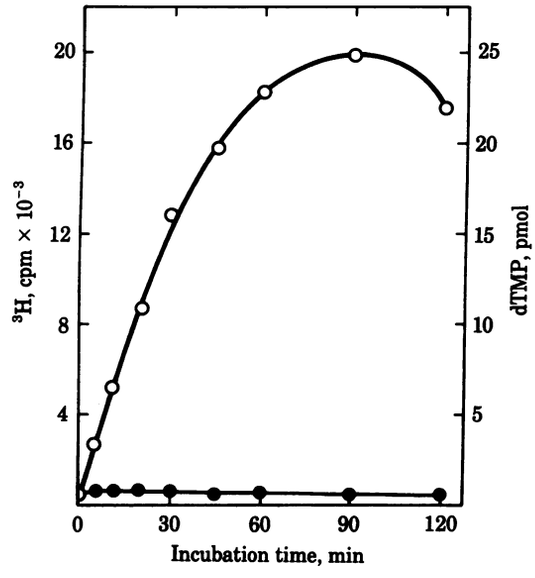

FIG. 1. Kinetics of incorporation of [^{3}H]dTMP in the presence (○) and absence (●) of *cop-608* DNA. Incubation (0.5 ml) was carried out under standard assay conditions in the presence of [^{3}H]dTTP, and 50-μl aliquots were withdrawn, acid-precipitated, and assayed.

RESULTS

Properties of Extracts. Both crude and partially purified extracts were tested for their ability to support plasmid DNA synthesis as shown by incorporation of radiolabeled deoxynucleotides into acid-precipitable material. However, all the experiments involving characterization of the plasmid replication properties were done with extracts from which endogenous DNA was removed by precipitation with streptomycin sulfate and fractionation by ammonium sulfate precipitation. In such extracts, incorporation of deoxyribonucleotides into acid-precipitable material was dependent on added plasmid DNA. The kinetics of [^{3}H]dTMP incorporation into DNA are shown in Fig. 1. The incorporation was linear for about 45 min and leveled off after 60 min.

Incorporation of labeled nucleotides into plasmid DNA was visualized by gel electrophoresis of the reaction mixtures followed by autoradiography. Three major ethidium-stained bands were seen (Fig. 2*A*): band 1 corresponds to plasmid dimers; band 2 is nicked or covalently closed relaxed plasmid monomer DNA; and band 3 represents supercoiled plasmid monomer DNA (17). A number of faint bands between bands 2 and 3 probably correspond to topoisomers. The autoradiogram (Fig. 2*B*) shows the appearance of newly synthesized plasmid DNA

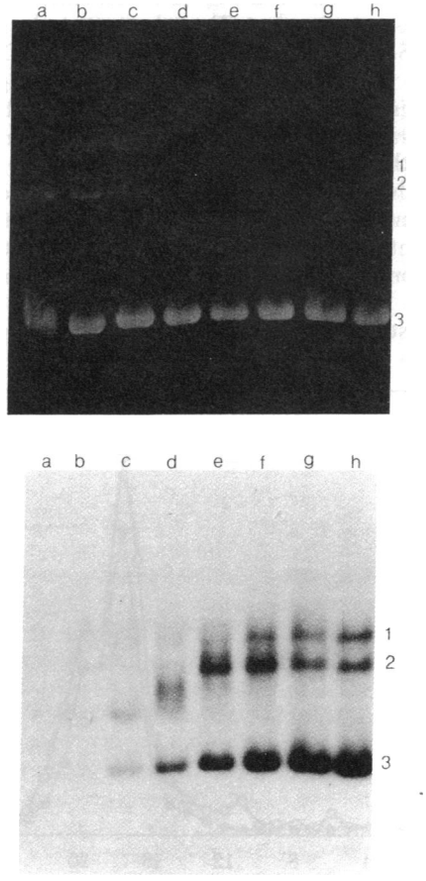

FIG. 2. Agarose gel electrophoresis (1% agarose gel) of *cop-608* DNA synthesized *in vitro* with [α-^{32}P]dTTP for various times. (*A*) Ethidium-stained. (*B*) Autoradiograph. a, 0 min; b, 2 min; c, 5 min; d, 10 min; e, 15 min; f, 30 min; g, 45 min; and h, 60 min.

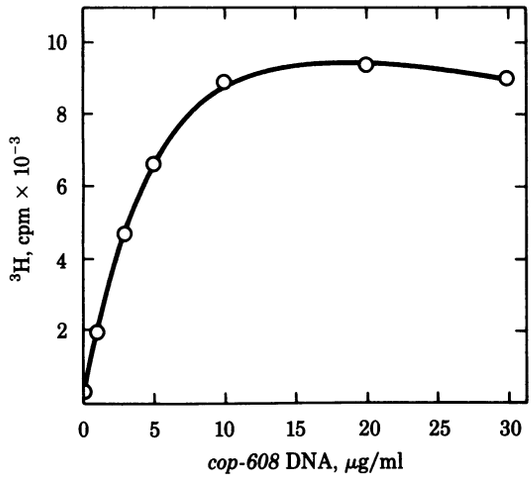

FIG. 3. Effect of DNA concentration on plasmid DNA synthesis. Standard reaction mixtures (50 μl) were incubated for 60 min at 30°C with *cop-608* DNA at various concentrations.

with time. A broad range of intermediates were synthesized first (between 0 and 5 min) and then, with time, most of the newly synthezied DNA was converted into supercoiled (band 3), relaxed circular forms (closed or open) (band 1), and a homogeneous species that is probably a set of replicative intermediates (band 2) (7, 23). (It should be noted that band 2 in Fig. 2A corresponds to band 1 in Fig. 2*B*; Fig. 2*A* has no prominent band corresponding to band 2 in Fig. 2*B*.) Monomeric supercoiled DNA was the major product. The gel pattern is the same when *cop-608* or pSA0301 DNAs are used as when pT181 DNA is used (not shown). Extracts made from strains containing pT181 or *cop-608* strains gave identical results except that the level of synthesis obtained in the *cop-608* extracts was always 5- to 10-fold higher than those with pT181 extracts. In all further experiments, therefore, RN3268 (*cop-608*) extracts were used.

The dependence of [^{3}H]dTMP incorporation on the plasmid DNA concentration is shown in Fig. 3. In these extracts, maximal incorporation was reached at a DNA concentration of 10 μg/ml.

Table 2 lists the requirements for *in vitro* DNA synthesis in these extracts. Incorporation was absolutely dependent upon K^+ and Mg^{2+} and on ATP as well as on exogenous DNA. The system was only partly inhibited by rifampicin (5–15 mg/ml); incorporation of precursors into supercoiled DNA clearly occurred in the presence of this inhibitor of RNA synthesis as demonstrated by gel electrophoresis and autoradiography (not shown). Novobiocin (1 μg/ml) inhibited incorporation to the extent of 50%, suggesting the involvement of DNA gyrase in this system.

Table 2. Requirements for plasmid DNA synthesis *in vitro*

Condition	Synthesis, %
Complete	100*
Lacking $Mg(OAc)_2$	5
40 mM KCl	83†
Lacking dNTPs	5
Lacking rCTP, rUTP, rGTP	67
Lacking rATP	5
Lacking DNA	7
Rifampicin (5 μg/ml)	69
Novobiocin (1 μg/ml)	51

* In the complete system, 20 pmol of [^{3}H]dTMP represents 100% incorporation.

† When the extracts were dialyzed against buffer not containing KCl, no synthesis was observed.

Characterization of the Plasmid DNA Products Synthesized *in Vitro*. Products of the *in vitro* DNA synthesis system were analyzed by neutral and alkaline sucrose gradients. In the neutral sucrose gradient (Fig. 4*A*), the ^{32}P-labeled newly synthesized DNA sedimented as 21 S (supercoiled) and 16 S (open circular) in proportions of 70% and 30%, respectively. Alkaline sucrose gradient analysis (Fig. 4*B*) of the products showed three peaks. The faster-sedimenting peak (supercoiled) constituted 75% of the total, the intermediate peak (possibly catenated plasmid molecules) was 7%, and the slowest sedimenting, broad peak (18%), corresponded to single-stranded circular monomers plus linear single strands of heterogeneous sizes. These experiments show that a major part of the DNA synthesized *in vitro* is supercoiled plasmid DNA and are consistent with the results obtained by agarose gel analysis (Fig. 2).

In a test for semiconservative replication, BrdUTP was added

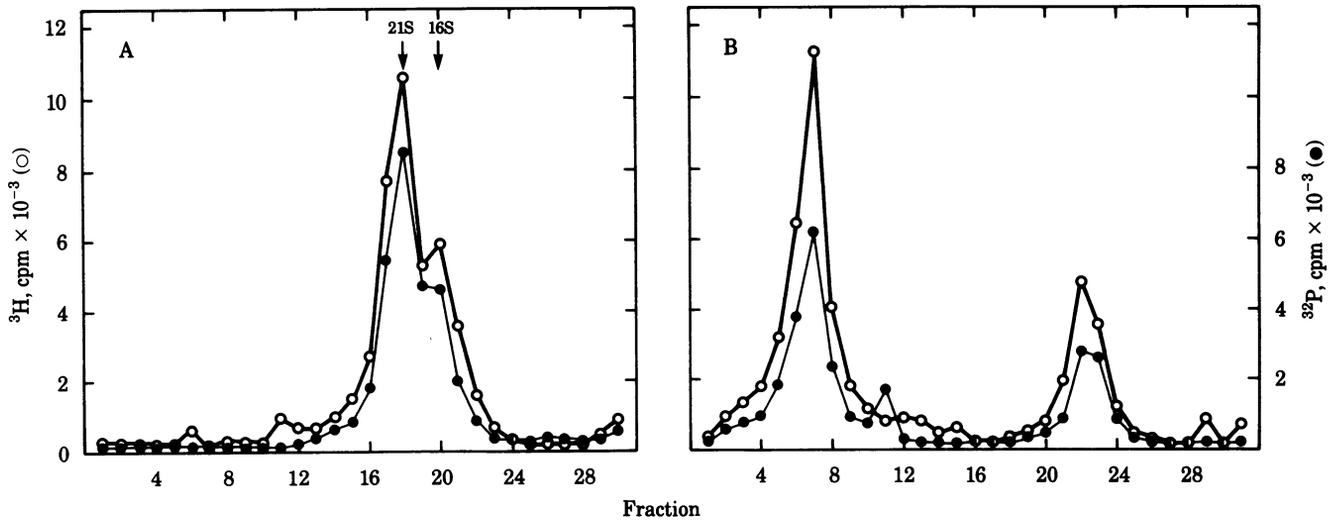

FIG. 4. Sedimentation analysis of *cop-608* DNA synthesized *in vitro*. A 200-μl standard reaction mixture was incubated in the presence of [α-^{32}P]dATP and ^{3}H-labeled *cop-608* DNA for 60 min. After phenol extraction and alcohol precipitation, the DNA was dissolved in 200 μl of Tris/EDTA/NaCl buffer and analyzed in a neutral (*A*) and an alkaline (*B*) sucrose gradient.

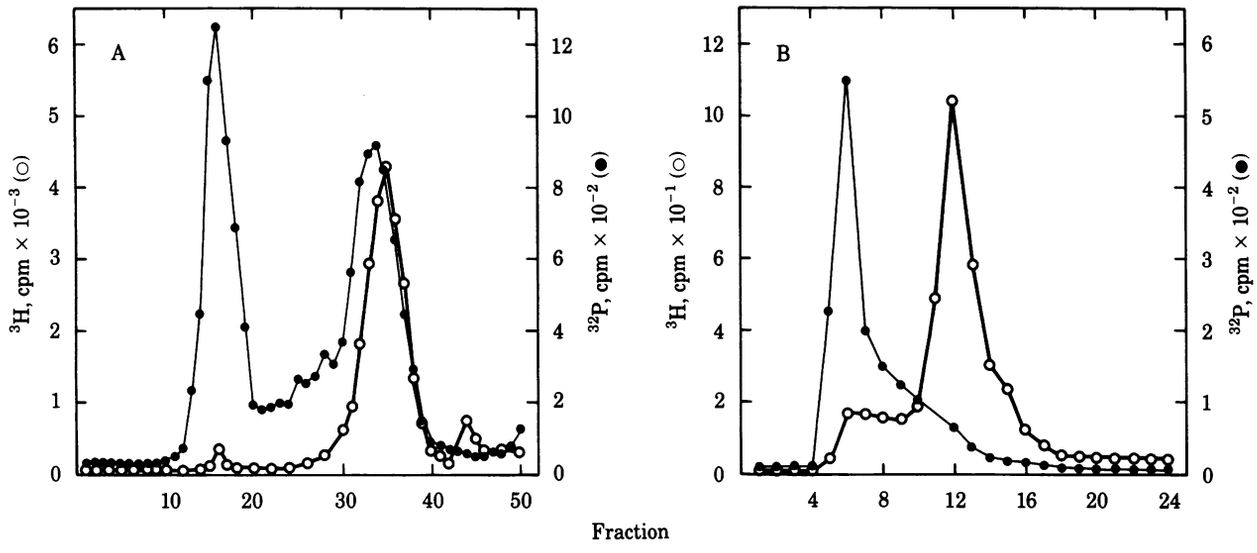

FIG. 5. Equilibrium CsCl density gradient centrifugation of BrdUrd-labeled *cop-608* DNA. Density labeling mixture (0.4 ml) containing [α-^{32}P]-dATP, BrdUTP, and ^{3}H-labeled *cop-608* DNA was incubated for 60 min under standard assay conditions. The labeled DNA was isolated and analyzed in a neutral CsCl density gradient (*A*). Aliquots (50 μl) of each fraction were assayed for acid-insoluble radioactivity. Fractions 14–19 were pooled and dialyzed against 5 mM Tris, pH 7.5/0.1 mM EDTA. After digestion with *Kpn* I, DNA was recovered by alcohol precipitation and samples were analyzed in a alkaline CsCl density gradient (*B*).

to the incubation mixture and the products were analyzed by neutral and alkaline CsCl density gradient centrifugation. The neutral density gradient separated two major bands (Fig. 5*A*), one consisting of completely replicated half-heavy molecules banding at a density of 1.74 g/cm^3, and the other of BrdUrd-labeled partially replicated molecules slightly heavier than the ^{3}H-labeled parental DNA (1.69 g/cm^3). The half-heavy peak fractions from Fig. 5*A* were pooled, dialyzed, alcohol precipitated, and then digested with *Kpn* I to generate unit-length linear molecules. In alkaline CsCl density gradients (Fig. 5*B*), this material separated clearly into a ^{32}P-labeled heavy and a ^{3}H-labeled light species, consistent with semiconservative replication.

Table 3. Specificity of *in vitro* DNA synthesis

Source of extract (strain)	Plasmid DNA	Acid precipitable cpm
RN3268 cop-608	None	952
	cop-608	9,750
	*cop-608**	8,237
	pT181	10,125
	pSA0301	10,522
	pSN1	9,356
	Relaxed* *cop-608*	3,789
	Linear *cop-608*	1,732
	pE194	1,179
	pI258	1,093
	pSN2	1,312
†	*cop-608*	9,813
RN3259 (pT181)	None	746
	pT181	2,321
	cop-608	2,512
	pE194	723
RN27 (no plasmid)	None	720
	pT181	1,017
	cop-608	1,183
	pE194	946

Incubations were for 40 min. Samples were precipitated with trichloroacetic acid and radioactivity was determined. *cop-608* DNA was linearized at the single X*ba* I site on pT181 DNA. The results shown here were confirmed by agarose gel analysis of the reaction products (not shown). Template DNA concentration, 10 μg/ml.

* Isolated by agarose gel electrophoresis.

† Preincubated at 30°C for 5 min with pancreatic RNase at 100 μg/ml.

Plasmid Specificity. Extracts were tested for incorporation of label into several different plasmid DNAs. The results were evaluated both by trichloroacetic acid precipitation and by agarose gel electrophoresis. The basic result was that, of the plasmids tested, only pT181 and its derivatives (*cop-608*, pSA0301) and a closely related tetracycline-resistance plasmid, pSN1 (17), were able to stimulate DNA synthesis in this system (Table 3). Plasmids pI258 and pE194, each of which specifies a *trans*-acting replication substance (ref. 2; D. Dubnau, personal communication), did not incorporate labeled DNA precursors in RN3268 extracts.

Substrate Configuration. Of the several isomeric forms of pT181 DNA, supercoiled monomers were by far the best substrate; *Xba* I-linearized DNA was virtually inactive (Table 3). A gel fraction containing relaxed circles had low but significant activity.

Although most of the RN3268 extracts prepared were specific for pT181 and closely related plasmids, occasional extracts were encountered that gave very high incorporation with all the plasmid DNAs tested. In such extracts, gel analysis showed that the substrate was largely degraded, indicating substantial nuclease activity; such activity was clearly minimal or absent from the "good" extracts. The reason for this variability is not known; however, this degradation provides a substrate for polymerase I, which is very active in these extracts, and possibly for other repair enzymes as well. In addition, this incorporation was insensitive to novobiocin but was partly sensitive to rifampicin.

Plasmid-Encoded Product. The involvement of a plasmid-encoded product in the *in vitro* replication of pT181 DNA was demonstrated by testing extracts prepared from the parental nonplasmid strain, RN27. These extracts failed to support DNA synthesis with pT181 DNA as well as with the other plasmids tested (Table 3). However, when these extracts were supplemented with an active RN3268 extract, pT181-specific DNA

synthesis was observed. To evaluate the possibility that the plasmid-encoded product is a RNA, extracts were pretreated with pancreatic RNase (100 μg/ml). No effect was observed, suggesting not only that the plasmid-encoded product is not an RNA but also that the replication process does not involve free single-stranded RNA.

DISCUSSION

On the basis of the results presented here it is concluded that cell-free extracts prepared from *Staphylococcus aureus* containing the plasmid pT181 *cop-608* (or its wild type parent) carry out a single round of semiconservative replication of exogenous pT181 plasmid DNA. Furthermore, a major part of the plasmid DNA synthesized is supercoiled. Because no fully heavy DNA was recovered in a light-to-heavy density transfer experiment, it appears that there was no reinitiation with the newly synthesized half-heavy molecules.

The *in vitro* system was specific for pT181 and closely related plasmids. DNA of other, unrelated, plasmids was inactive in extracts of pT181-containing cells and, moreover, extracts made from plasmid-negative strains or from strains carrying other plasmids were inactive in replication of pT181 DNA. Our interpretation of this specificity is that the extracts contain a plasmid-specified protein, repC, that is required for replication from the pT181 origin. This interpretation is supported by more recent results (unpublished data) in which deletions of a pT181::pE194 cointegrate that remove the pT181 origin abolished activity in these extracts.

Unlike plasmids ColE1 and R6K (4, 8), the *in vitro* replication of pT181 DNA was not completely sensitive to rifampicin: incorporation of labeled precursors into supercoiled monomers was inhibited only about 30% by rifampicin and to a similar extent in the absence of ribonucleotide triphosphates. Furthermore, rifampicin was also found to inhibit [^{3}H]dTMP incorporation by about 30% in extracts that were primarily active in repair synthesis (data not shown). These results suggest that the synthesis of an RNA primer is not required *in vitro* for the replication of pT181 DNA. We have not rigorously excluded the possibility that the molecules able to serve as substrate are "preprimed"; this would account for the lack of reinitiation and for the apparent limitation in the extent of substrate activity.

It was expected on the basis of genetic evidence (16) that pT181 would specify a product that is absolutely required for its replication and our results clearly substantiate this expectation. Experiments with RNase suggest that the product is not RNA, and so we predict that it is a protein. More recent experiments involving chromatographic fractionation of an active extract have further strengthened this conclusion (unpublished data). A fraction has been obtained after Bio-Gel and phosphocellulose column chromatography that, when added to extracts of plasmid-negative cells, permitted the specific incorporation of dNTPs into pT181 DNA. We regard it as highly probable but not proved that this product is actually encoded by pT181 rather than merely being controlled by the plasmid. Unlike the results with R6K (9), there is no apparent inhibition of DNA replication in the presence of excess pT181 DNA (30 μg/ml).

Finally, it can be concluded that, in strains containing the pT181 copy-number mutant *cop-608*, there is an impressive overproduction of the required product—extracts from cells carrying this mutant were 5- to 10-fold more active than extracts from cells containing the wild-type pT181 plasmid. An important but as yet unanswered question in this connection is that of whether the primary effect of the copy-number mutation is to increase the amount of the required product or the increase in product is a consequence of increased gene dosage secondary to a primary affect on some other stage of the replication pathway.

We thank Warren Rosenblum and Thomas Aldrich for their expert technical assistance. This work was supported by Grant VC-229 from the American Cancer Society (to R.P.N.).

1. Jacob, F., Brenner, S. & Cuzin, F. (1964) *Cold Spring Harbor Symp. Quant. Biol.* **28**, 329–348.
2. Novick, R. P. (1974) *Mol. Gen. Genet.* **135**, 131–147.
3. Nordstrom, K., Ingram, L. C. & Lumback, A. (1972) *J. Bacteriol.* **110**, 562–569.
4. Sakakibara, Y. & Tomizawa, J. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 802–806.
5. Sakakibara, Y. & Tomizawa, J. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 1403–1407.
6. Studenbauer, W. L. (1976) *Mol. Gen. Genet.* **145**, 273–280.
7. Conrad, S. E. & Campbell, J. L. (1979) *Nucleic Acids Res.* **6**, 3289–3303.
8. Inuzuka, M. & Helinski, D. R. (1978) *Biochemistry* **17**, 2567–2573.
9. Inuzuka, M. & Helinski, D. R. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5381–5385.
10. Bezanson, G. S. & Goebel, W. (1979) *Mol. Gen. Genet.* **170**, 49–56.
11. Donoghue, D. J. & Sharp, P. A. (1978) *J. Bacteriol.* **133**, 1287–1294.
12. Tomizawa, J., Sakakibara, Y. & Kakefuda, T. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1050–1054.
13. Wyman, L. & Novick, R. P. (1974) *Mol. Gen. Genet.* **135**, 149–161.
14. Kolter, R., Inuzuka, M., Figurski, D., Thomas, C., Stalker, D. & Helinski, D. R. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 91–97.
15. Iordanescu, S., Surdeanu, M., Della Latta, P. & Novick, R. P. (1978) *Plasmid* **1**, 468–479.
16. Iordanescu, S. (1976) *Arch. Roum. Pathol. Exp. Microbiol.* **35**, 257–264.
17. Shalita, Z., Hertman, I. & Sarid, S. (1977) *J. Bacteriol.* **129**, 317–325.
18. Novick, R. P. (1963) *J. Gen. Microbiol.* **33**, 121–136.
19. Novick, R. P. & Brodsky, R. (1972) *J. Mol. Biol.* **68**, 285–302.
20. Clewell, D. B. & Helinski, D. R. (1969) *Proc. Natl. Acad. Sci. USA* **62**, 1159–1166.
21. Novick, R. P., Murphy, E., Gryczan, T. J., Baron, E. & Edelman, I. (1979) *Plasmid* **2**, 109–129.
22. Greene, P. J., Betlach, M. C., Goodman, H. M. & Boyer, H. W. (1974) in *Methods in Molecular Biology—DNA Replication and Biosynthesis*, ed. Wickner, R. B. (Dekker, New York), Vol. 7, pp. 87–111.
23. Kreuger, K. N. & Cozzarelli, N. R. (1980) *Cell* **20**, 245–254.