Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6214–6218, October 1983
Biochemistry

Initiation of DNA replication *in vitro* by a DNA–membrane complex extracted from *Bacillus subtilis*

(semiconservative DNA synthesis/initiation mutant/antibiotics/RNA precursors/transformation)

PATRICIA BENJAMIN AND WILLIAM FIRSHEIN

Department of Biology, Wesleyan University, Middletown, CT 06457

Communicated by Max Tishler, July 11, 1983

ABSTRACT **Initiation of DNA replication has been observed *in vitro* with a DNA–membrane complex extracted from *Bacillus subtilis*. Antibiotics known to interfere with various aspects of initiation inhibited DNA synthesis significantly *in vitro*, whereas a mutant resistant to one inhibitor failed to respond to its presence. The inhibitory effects occurred primarily when the immediate RNA precursors (ribonucleoside triphosphates) were present in the assay solution but not significantly when the precursors were omitted. Complexes extracted from a temperature-sensitive initiation mutant were almost incapable of synthesizing DNA at the restrictive temperature but displayed extensive synthesis at the permissive temperature. A strong indication of semiconservative DNA synthesis was obtained *in vitro* after density-shift experiments involving incubation of the complex with a heavy-density DNA precursor, followed by neutral and alkaline CsCl density gradient centrifugation. A significant amount of chain elongation or repair (or both) was also observed.**

A great deal of effort has been invested in designing *in vitro* replication systems in an attempt to elucidate factors controlling DNA replication in the bacterial cell. These experiments have focused mainly on bacterial viruses, naturally occurring plasmids, and *in vitro* constructed plasmids carrying the bacterial origin of replication (1–3). Such systems have the advantage of using well-characterized relatively simple templates that require many host proteins for replication. Nevertheless, understanding how initiation of bacterial DNA replication is controlled has remained elusive despite the advances made, possibly because heterologous or mismatched *in vitro* systems were used that did not or could not approximate the exact *in vivo* environment. The replication of plasmids containing the chromosomal origin of replication for *Escherichia coli* (*oriC*) is a case in point. Despite the elucidation of a number of factors involved in *oriC* replication (3–5), these minichromosomes do not behave the same as their normal chromosomal counterpart. Thus, Leonard *et al.* (4) showed that the initiation of the *oriC* plasmid and the bacterial chromosome were not coordinated in synchronous cultures. The *oriC* plasmid continued to replicate throughout the cell cycle, whereas the chromosome exhibited a strict synchronous pattern of replication. Additionally, the copy number of the minichromosome varied considerably from population to population (4), implying some abnormality in control mechanisms.

All of these studies suggest that the control a cell exerts over the replication of its genetic material involves a complex mechanism—possibly too complex to discern by studying replication of simplified replicons in soluble systems.

An alternate approach might be to study bacterial DNA replication by using the site of replication as the major component of an *in vitro* system. Such a system could preserve any structural interactions important in regulation that may be disrupted in soluble extracts. For example, it has been suggested that conformational changes in various components at the site of replication will result in an inhibition of initiation (6, 7). Thus, a unique DNA–protein complex (S-complex) involved in initiation was dissociated in germinating spores of *Bacillus subtilis* after the addition of the initiation inhibitor novobiocin (7). Further studies showed that the addition of ethidium bromide to germinating *B. subtilis* spores also inhibited initiation. In this case, however, both the S-complex and a DNA–membrane complex (M-complex) were dissociated (8). Finally, inhibition of initiation in temperature-sensitive initiation mutants at the restrictive temperature resulted in a decrease in membrane association near the chromosomal origin of replication (9).

That the cell membrane might be the site for DNA replication in the bacterial cell has been known for many years (9–14). We have shown that DNA–membrane complexes isolated from a *polA* mutant of *B. subtilis* by the M-band technique (14) have the ability to synthesize DNA *in vitro* endogenously—i.e., without the addition of exogenous DNA or enzymes (12). After further purification by CsCl density centrifugation and sucrose velocity gradient centrifugation, two subcomplexes were formed, both of which still retained the capability to synthesize DNA *in vitro* in an endogenous manner. *In vitro* synthesis by the subcomplexes was inhibited by hydroxyphenyl azo uracil, a specific inhibitor of DNA polymerase III in gram-positive bacteria (15). In addition, both subcomplexes were highly enriched for the *guaA* marker, which is located near the origin of replication. However, because our assay system did not contain some components that were required for the initiation of DNA replication, it was likely that the observed *in vitro* synthesis resulted from the elongation of previously initiated replication forks (12).

In the present study, the assay system was modified so that the ability of DNA–membrane complexes extracted from *B. subtilis* to initiate replication could be studied. The results indicate that DNA–membrane complexes have the potential to initiate DNA replication *in vitro*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *B. subtilis* 841 (*trp, thy, polA*), 841a (*trp, polA, thy, streptolydigin*^{r}), and 167 (*thy, dnaB19* were used. They were kindly supplied by N. Sueoka and N. Brown. The *dnaB* initiation mutant was first isolated by Karamata and Gross (16). It maps between the arginine and leucine loci near the middle of the *B. subtilis* genome. The cells were grown to midlogarithmic phase in Penassay (Difco antibiotic medium 3) broth with aeration at 37°C (an exception was

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Abbreviation: BrdUTP, bromodeoxyuridine triphosphate.

the strain carrying the *dnaB19* mutation, which was grown at 30°C). Frozen cell pellets were stored at −20°C.

Initial experiments were performed with the same *polA* strain used in previous experiments (12) for the same reason—i.e., to emphasize the major role of DNA polymerase III in the overall reaction (which is the primary polymerase in this strain because of the defect in DNA polymerase I activity). However, it was important to show that a strain normal for DNA polymerase I activity also would respond in the same way. This was accomplished by using the temperature-sensitive *dnaB19* mutant, whose DNA polymerase complement was complete and normal.

Preparation of DNA–Membrane Complexes. Cells were harvested from cultures in a Sorvall R26B centrifuge with the GSA rotor at 10,000 rpm (4°C) for 10 min. Cell pellets were washed twice with TMK buffer (0.01 M Tris·HCl, pH 7.5/0.01 M $MgCl_2$/0.1 M KCl) and incubated in the same buffer containing 25% sucrose and lysozyme (50 μg/ml) at 4°C for 20 min. The protoplasts that formed under these conditions were collected by centrifugation in the SS34 rotor (10,000 rpm at 4°C for 15 min) and resuspended in TMK buffer containing 1 mM phenylmethylsulfonyl fluoride and 0.3% Sarkosyl (sodium lauroyl sarkosinate; ICN). The lysate was incubated at 4°C for 30 min, layered onto biphasic 25%/60% sucrose solutions, and centrifuged at 17,000 rpm for 17 min at 4°C in an SW 28 rotor and L265B ultracentrifuge. A white band formed (the M-band) (14) at the sucrose interphase, which was collected and dialyzed against 8 liters of TK buffer (0.01 M Tris·HCl, pH 7.5/0.1 M KCl) containing 1 mM phenylmethylsulfonyl fluoride and 50% glycerol for 24 hr at 4°C. The percentage of cellular DNA in the M-band ranged from 75–82%. Microscopic examination revealed no intact cells in the M-band dialysate. No further purification of the complex was undertaken in order to preserve the putative site of replication in as "native" a state as possible.

***In Vitro* Replication Assay.** The standard reaction mixture, 100 μl, contained 50 μl of the dialyzed M-band preparation, 1.7 mM triethanolamine phosphate buffer (pH 7.5), 0.5 mM EDTA, 0.025% Triton X-100, 2 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 7.5 mM creatine phosphate, 100 μg of creatine phosphokinase per ml, 160 μM NAD, 25 μM dNTP [dATP, dCTP, dGTP, and [^{3}H]dTTP (73 Ci/mmol; 0.1 μCi per assay tube; 1 Ci = 37 GBq)], and 225 μM NTP (ATP, CTP, GTP, and UTP). Streptolydigin, streptovaricin (both kindly supplied by Upjohn), and nalidixic acid (Sigma) were used at 500 μg/ml, 500 μg/ml, and 1 mg/ml, respectively. These relatively high concentrations were necessary because of an adverse interaction between the antibiotics and the detergent used to extract the complexes (which could not be removed completely by dialysis). However, the results will show that the effective concentrations of the drugs were still capable of eliciting an appropriate response. Each assay tube was incubated at 30°C or at 41.5°C for various time intervals, and the reactions were terminated with the addition of 5% trichloroacetic acid/1% sodium pyrophosphate. Acid-insoluble radioactivity was determined by liquid scintillation techniques. In some experiments, the product was extracted in a different manner as described below.

Semiconservative Replication. Two-liter cultures of *B. subtilis* 167 (*thy*, *dnaB19*) were labeled with [^{14}C]thymidine (0.1 μCi/ml) and grown to the midlogarithmic phase at 30°C with aeration in Penassay broth. DNA–membrane complexes were isolated by the M-band technique as described above.

The reaction mixture (scaled up to 8 ml) was modified slightly to contain 5 ml of the M-band preparation, 9 mM triethanolamine phosphate buffer (pH 7.5), 2.5 mM EDTA, 0.125% Triton X-100, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 7.5 mM creatine phosphate, 100 μg of creatine phosphokinase (per ml), 160 μM NAD, 50 μM dNTP [dCTP, dGTP, bromodeoxyuridine triphosphate (BrdUTP), and [^{3}H]dATP (10 Ci/mmol; 1 μCi per assay tube)] and 225 μM NTP (ATP, CTP, GTP, and UTP). Each assay tube was incubated at 30°C or 41.5°C for 35 min, and the reaction was terminated by adding EDTA (pH 8.0) to a final concentration of 20 mM. The samples were then incubated with predigested RNase (10 μg/ml) at 37°C for 30 min, followed by incubation with predigested Pronase (100 μg/ml) for 60 min at 37°C. The DNA was extracted with chloroform/isoamyl alcohol, 24:1 (vol/vol) and dialyzed against 8 liters of TK buffer at 4°C. For neutral CsCl density gradient analysis, the samples were adjusted to a density of 1.707 gm/cm^3 with solid CsCl and centrifuged in a Beckman 70 Ti rotor for 72 hr at 40,000 rpm (23°C) in a Beckman L265B ultracentrifuge. Ten-drop fractions (250–300 μl) were collected from the bottom of each tube, and 25-μl aliquots of each of the fractions were tested for acid-insoluble radioactivity by liquid scintillation techniques. For alkaline CsCl density gradient analysis, the remaining volumes of the appropriate peak fractions from the neutral CsCl gradients were pooled and dialyzed against 8 liters of TK buffer at 4°C, and NaOH was added to a final concentration of 0.1 M. The density of each of the samples was adjusted to 1.745 gm/cm^3 with solid CsCl and centrifuged in a Beckman 70 Ti rotor for 46 hr at 38,000 rpm (23°C) in a Beckman L265B ultracentrifuge. Ten-drop fractions were collected from the bottom of each tube, and the entire sample was assayed for acid-insoluble radioactivity as described above.

RESULTS

Effect of Inhibitors on *in Vitro* Synthesis by the DNA–Membrane Complex. *In vitro* [^{3}H]dTTP incorporation by DNA–membrane complexes isolated from the *polA* strain was measured in the presence and absence of streptolydigin, streptovaricin, and nalidixic acid. Streptolydigin inhibits RNA chain elongation, whereas streptovaricin blocks the initiation but not the propagation of RNA chains (17). Both antibiotics have been shown to inhibit initiation of DNA replication by blocking RNA primer formation. Nalidixic acid inhibits DNA synthesis by blocking the NAL-A subunit of DNA gyrase, which induces negative supercoiling in DNA as a result of its nicking–ligating action (18–20). We found that synthetic activity was reduced severely by both streptolydigin and streptovaricin (Fig. 1 *a* and *b*) and was abolished completely by nalidixic acid (Fig. 1*c*).

The effects of these inhibitors on *in vitro* DNA synthesis was studied further by performing the same experiment in the presence and absence of the ribonucleoside triphosphates (immediate RNA precursors). Incorporation of [^{3}H]dTTP was reduced by 70.3% with streptolydigin in the presence of the precursors but only 12.7% when the precursors were absent (Table 1). Similarly, the results with streptovaricin inhibition were 66.7% and 12.6%, respectively. With nalidixic acid, despite the almost complete inhibition of [^{3}H]dTTP incorporation in the presence of the RNA precursors, there was much less of an inhibition (37.3%) when they were omitted.

To further investigate the role of the three inhibitors, a streptolydigin-resistant mutant was isolated from the *polA* mutant, and the ability of the DNA–membrane complex to replicate DNA in the presence and absence of the inhibitors was tested (Fig. 2). Streptolydigin had no effect on the incorporation of [^{3}H]dTTP by the complex, whereas a minimal amount of inhibition was detected in the presence of streptovaricin, probably because both streptolydigin and streptovaricin act on the β subunit of RNA polymerase. When nalidixic was present, a greater inhibition was observed in comparison to the other two antibiotics, but it was not nearly as severe as that observed in the streptolydigin-sensitive *polA* parent (compare Fig. 1).

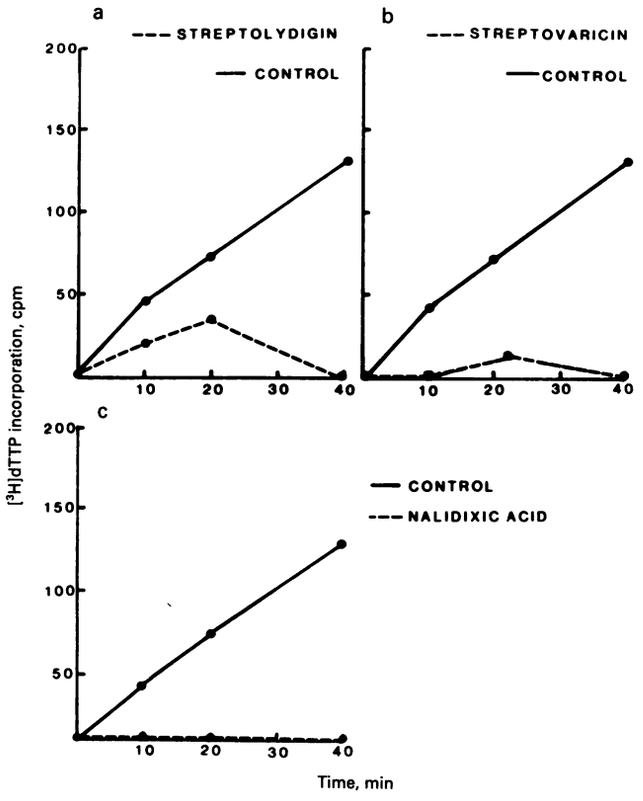

FIG. 1. Effects of various inhibitors on [^{3}H]dTTP incorporation by the DNA–membrane complex. The DNA–membrane complex was extracted and assayed in the presence of streptolydigin (50 μg/0.1 ml) (*a*), streptovaricin (50 μg/0.1 ml) (*b*), and nalidixic acid (100 μg/0.1 ml) (*c*).

Initiation of DNA Replication by DNA–Membrane Complexes Isolated from a Temperature-Sensitive Initiation Mutant. If DNA–membrane complexes have the ability to initiate DNA replication *in vitro*, then this potential should be most clearly demonstrated by comparing the synthetic capacity of complexes isolated from a temperature-sensitive initiation mutant at permissive and nonpermissive temperatures. Accordingly, the kinetics of *in vitro* [^{3}H]dTTP incorporation by the complexes isolated from the *B. subtilis* 167 (*thy dnaB19*) initiation mutant (16) were measured at permissive (30°C) and nonpermissive (41.5°C) temperatures (Fig. 3). Incorporation increased linearly at 30°C as a function of time, but at 41.5°C incorporation ceased after 5 min. To rule out the possibility of enzyme inactivation at 41.5°C, an identical experiment was performed with DNA–membrane complexes isolated from a wild-type strain. There was little or no difference in [^{3}H]dTTP incorporation by these complexes at either temperature (data not shown).

Table 1. Effect of inhibitors on DNA synthesis by the DNA–membrane complex

Inhibitor	Inhibition of DNA synthesis, %	
	With NTPs*	Without NTPs
Streptolydigin	70.3	12.7
Streptovaricin	66.7	12.6
Nalidixic acid	100	37.3

* NTPs included equimolar concentrations of GTP, UTP, CTP, and ATP. DNA synthesis was measured in the standard reaction mixture with or without the inhibitor as described over a period of 40 min at 30°C. That is, the extent of inhibition by each antibiotic in the presence and absence of NTPs after 10, 20, and 40 min of incubation were averaged together. Thus, for example, with streptolydigin, the acid-insoluble incorporation levels for [^{3}H]dTTP after 10, 20, and 40 min in the first control (i.e., in the absence of the antibiotic but in the presence of NTPs) were 470, 730, and 1,300 cpm/ml of assay solution, respectively. In the presence of streptolydigin and NTPs, the values were 200, 340, and 10 cpm/ml of assay solution, or an average inhibition value of 70.3% over the course of incubation. In the second control (i.e., in the absence of both the antibiotic and NTPs), the incorporation values were 2,310, 2,740, and 3,240 cpm/ml, whereas in the presence of streptolydigin only, the incorporation values were 2,240, 2,480, and 2,420 cpm/ml of assay solution, or an average inhibition value of 12.7% over the course of incubation. Although incorporation is actually greater in the absence (second control) rather than presence (first control), of the RNA precursors, the nature of the synthesis between the two controls is fundamentally different and should not be compared (see *Discussion*). Similar calculations were made for the two other inhibitors.

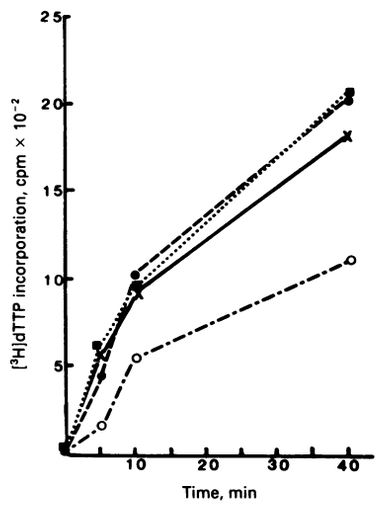

FIG. 2. Kinetics of [^{3}H]dTTP incorporation into a DNA–membrane complex extracted from a streptolydigin-resistant mutant in the absence (■) or presence of streptolydigin (●), streptovaricin (×), and nalidixic acid (○). See legend of Fig. 1 and text for details.

Semiconservative Replication by the DNA–Membrane Complex. To characterize the product synthesized by the DNA–membrane complex, heavy-density *B. subtilis* DNA was synthesized *in vitro* and analyzed by neutral and alkaline CsCl density gradient centrifugation. DNA–membrane complexes were isolated from prelabeled [^{14}C]DNA 167(*thy, dnaB19*) cells and incubated in modified assay solutions in which dTTP was replaced with bromodeoxyuridine triphosphate and [^{3}H]dATP was added in addition to unlabeled dATP. The reaction products

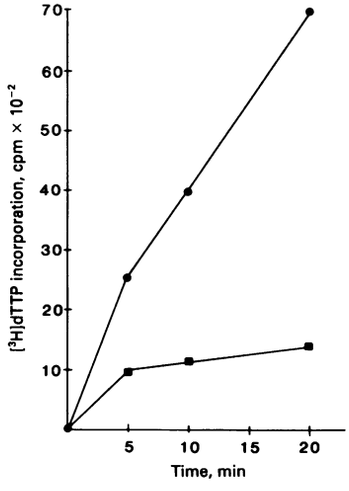

FIG. 3. Kinetics of [^{3}H]dTTP incorporation into a DNA–membrane complex extracted from a temperature-sensitive initiation mutant at the permissive and nonpermissive temperatures. The DNA–membrane complex was extracted and assayed at 30°C (permissive) (●) and 41.5°C (nonpermissive) (■) as described.

were extracted as described and centrifuged for 72 hr at 23°C in a neutral CsCl gradient with a mean density of 1.707 gm/cm^3.

Two peaks of radioactive DNA of different densities were present in gradients derived from complexes incubated for 35 min at 30°C (Fig. 4*a*). The DNA of one peak sedimented at a density of 1.740 gm/cm^3, whereas the DNA of the other peak sedimented at a density of 1.718 gm/cm^3. DNA synthesized at 41.5°C sedimented in one peak at a density of 1.712 gm/cm^3 (Fig. 4*b*). Bulk *B. subtilis* light DNA sedimented at a density of approximately 1.7007 gm/cm^3 as indicated by the arrows in the figures. Approximately 40% of the parental [^{14}C]DNA cosedimented in the heavier density peak with the newly synthesized [^{3}H]DNA. Because the difference in density between bulk *B. subtilis* DNA and the product synthesized by the complexes at 41.5°C was slight as compared with the products synthesized at 30°C, the possibility existed that a significant amount of semiconservative DNA replication had occurred in the DNA–membrane complex.

To further evaluate this possibility, two additional experiments were performed. This first involved an analysis of transformation frequencies of genetic markers near the origin of replication as described (12). DNA of the heavier-density peak resulting from *in vitro* synthesis at 30°C contained (*i*) 26% more *guaA*$^+$ transformants and 12% more *purA*$^+$ transformants than did the lighter-density peak species resulting from *in vitro* synthesis at 30°C (Fig. 4*a*) and (*ii*) 98% more *guaA*$^+$ transformants and 90% more *purA*$^+$ transformants than did the peak species resulting from *in vitro* synthesis at 41.5°C (Fig. 4*b*; transformation data not shown).

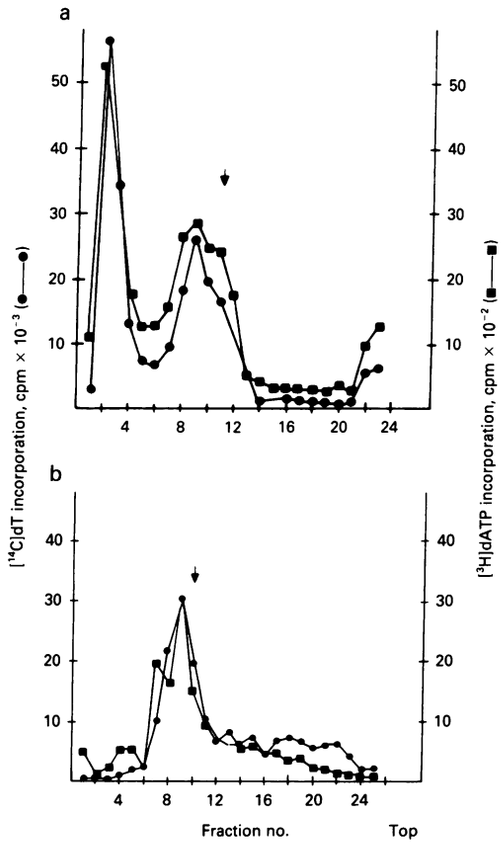

FIG. 4. Neutral CsCl density gradient centrifugation of DNA products synthesized *in vitro* by the DNA–membrane complex isolated from the *dnaB19* initiation mutant. Procedures for assay of the complex extracted from the temperature-sensitive initiation mutant with the heavy-density precursor bromodeoxyuridine triphosphate and CsCl density centrifugation are described. Incubation was for 35 min at 30°C (*a*) and 41.5°C (*b*). ●, ^{14}C-Prelabeled parental DNA; ■, ^{3}H-labeled newly synthesized DNA. The arrows indicate the position at which bulk light-density marker DNA sediments in the gradient.

The second experiment involved an analysis of the products in an alkaline CsCl density gradient. The peak fractions from the neutral CsCl gradients were pooled, dialyzed, and centrifuged in alkaline CsCl gradients as described. The heavier-density DNA peak derived from *in vitro* synthesis at 30°C consisted of two species of newly synthesized [^{3}H]DNA (Fig. 5). One species sedimented at a density of 1.804 gm/cm^3, and the other sedimented at a density of 1.756 gm/cm^3, which was near the mean density of ^{14}C-labeled single-stranded DNA (1.761 gm/cm^3) (Fig. 5, arrow). The lighter-density [^{3}H]DNA peak derived from *in vitro* synthesis at 30°C revealed one major species sedimenting at 1.775 gm/cm^3 and two minor species sedimenting at a density of 1.761 gm/cm^3 and 1.723 gm/cm^3, respectively. Analysis of the *in vitro* product synthesized at 41.5°C by the complexes revealed one species which sedimented at a density of 1.769 gm/cm^3.

The difference in density between the heavier-density single-stranded [^{3}H]DNA peak newly synthesized *in vitro* (derived from the heavy-density peak in the neutral CsCl gradient; Fig. 4*a*) and the single-stranded parental [^{14}C]DNA in the alkaline gradient was more than twice that in the neutral CsCl gradient (compare with Fig. 4*a*). This indicates that a significant portion (but not all; see below) of the newly synthesized bacterial DNA consists of ^{14}C-labeled light-density-strand and ^{3}H-labeled bromodeoxyuridine monophosphate-containing heavy-density-strand hybrid molecules. However, that nonspecific chain extension and/or repair of light-density parental DNA also occurred at the same time in these preparations was indicated by two observations. First, approximately 40% of the heavy density [^{3}H]DNA obtained from the neutral CsCl gradient actually consisted of molecules that sedimented in the alkaline CsCl gradient in the area of light-density single-stranded DNA. Second, a relatively large amount of parental [^{14}C]DNA (also about 40%) cosedimented with the newly synthesized [^{3}H]DNA in the neutral CsCl gradient. Although part of this [^{14}C]DNA represents hybrid-density DNA synthesized semi-

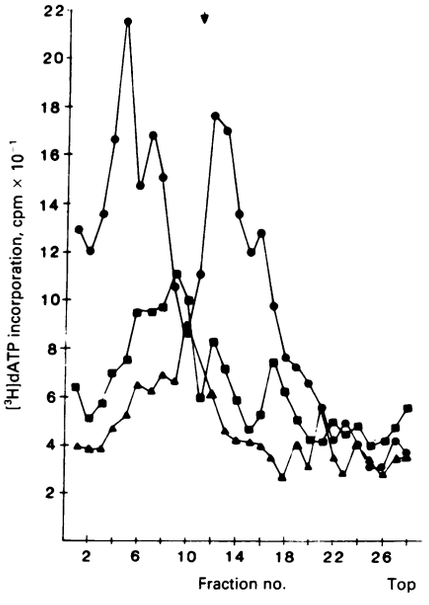

FIG. 5. Alkaline CsCl density gradient centrifugation of DNA products pooled from the neutral CsCl density gradient. Fractions 1–5 (●) and 8–12 (■) from Fig. 4*a* and fractions 6–11 (▲) from Fig. 4*b* were pooled, dialyzed, and recentrifuged in an alkaline CsCl density gradient. Arrow, position at which parental ^{14}C-prelabeled single-stranded DNA sediments in the gradient.

conservatively, some must represent light-density parental DNA elongated with the heavy-density precursor as well.

DISCUSSION

The experiments presented in this report demonstrate that DNA–membrane complexes have the potential to initiate the replication of bacterial DNA *in vitro*. Although elongation occurs in the absence of the immediate RNA precursors (12), there is a differential inhibitory effect of antibiotics that influences the initiation of DNA synthesis depending upon whether the precursors are present or not. In their absence, the adverse effects of two of the inhibitors (streptolydigin and streptovaricin) are severely reduced, which indicates that they are specifically involved with primer formation. This was supported further by the failure of these two antibiotics to affect replication in the streptolydigin-resistant mutant. The effects of the third inhibitor, nalidixic acid, are more difficult to evaluate. In the absence of ribonucleoside triphosphates, nalidixic acid inhibits overall DNA synthesis by only 33%, whereas in their presence, the drug was almost 100% inhibitory. This could be explained by the possibility that initiation is more sensitive to inhibitory effects on DNA gyrase activity than elongation but that elongation is also affected adversely. When initiation is inhibited, any elongation that would have proceeded from this reaction simply does not occur. However, in the absence of initiation, only nonspecific less-sensitive chain extension occurs. This interpretation presupposes that there is a competition for substrates and enzymes in the *in vitro* system between initiation (with RNA precursors) and nonspecific chain extension (without RNA precursors). Another puzzling observation is that there is a relatively strong cross-resistance to nalidixic acid in streptolydigin-resistant mutants (as compared to the wild type) even though the two inhibitors act on different enzymes (RNA polymerase and DNA gyrase) (Fig. 2). It is possible that there is an interaction between these two enzymes in initiation reactions that could be altered in the mutants.

Complexes isolated from cells that are defective in initiating DNA replication at a nonpermissive temperature fail to initiate replication at this temperature but do replicate well at 30°C. This result suggests strongly that the DNA–membrane complex is capable of initiating DNA replication under the proper conditions. Further support for initiation comes from experiments in which semiconservative DNA synthesis was demonstrated in complexes extracted from the temperature-sensitive initiation mutant and incubated at the permissive temperature. This was illustrated by the appearance of a heavier-density DNA peak in a neutral CsCl density gradient (Fig. 4*a*), which upon further analysis in alkaline CsCl gradients was determined to be composed, at least in part, of single-stranded newly synthesized heavy-density DNA and single-stranded light-density template DNA (see Fig. 5). One interesting point is that there is no second round of initiation because no completely heavy-density DNA was observed in the neutral CsCl density gradient (Fig. 4*a*). No significant change in density, from light-density *B. subtilis* DNA, was seen in the product synthesized by the complexes at 41.5°C after neutral CsCl density centrifugation (Fig. 4*b*). This observation indicates either that elongation of preexisting strands and initiation of new strands might be inhibited at this temperature or that elongation that would have occurred from the initiation of new strands does not occur at the restrictive temperature.

The relative percentages of *guaA*$^+$ and *purA*$^+$ transformants in the hybrid-density DNA population derived from neutral CsCl density gradients after centrifugation also supported *in vitro* initiation of new strands by the DNA–membrane complex. This data indicated that the *guaA* marker was replicated slightly ahead of the *purA* marker. The *guaA*$^+$ marker, which has been shown to be located very close to the origin (21, 22), lies adjacent to *Bam*HI fragment 7. This latter fragment is the first replicating fragment of the *B. subtilis* chromosome in synchronous cultures (23). In addition, Henckes *et al.* (22) have located a new ribosomal operon (rrnO) in *Bam*HI fragment 7. The promoter region of the operon has been proposed to play an important role in the regulation of DNA replication in *B. subtilis* (22, 24, 25).

Because the DNA–membrane complex preparation used in the present study is basically one step removed from whole cells, it is plausible that DNA replication is proceeding in a manner that is similar to *in vivo* conditions. All of the observed synthetic activity was endogenous, which implies that all of the enzymes and other replication factors were not only present but also active in the complexes as well. Whether these factors are localized in or near the membrane and whether the bacterial membrane is an absolute requirement in order for DNA replication to occur *in vivo* is not known at this time. Nevertheless, this report indicates that further study of the synthetic capabilities of DNA–membrane complexes will yield important information concerning the control of DNA replication in bacteria.

We thank Roger Mitty and Judith Hooper for their assistance. This work was supported in part by National Science Foundation Grant PCM 81-10082, and a Grant in Aid of Research from Sigma Xi awarded to P.B.

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