Replication of single-stranded plasmid pT181 DNA *in vitro*

(lagging strand replication/lagging strand origin)

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ABSTRACT   Plasmid pT181 is a 4437-base-pair, multicopy plasmid of *Staphylococcus aureus* that encodes tetracycline resistance. The replication of the leading strand of pT181 DNA initiates by covalent extension of a site-specific nick generated by the initiator protein at the origin of replication and proceeds by an asymmetric rolling circle mechanism. The origin of the leading strand synthesis also serves as the site for termination of replication. Replication of pT181 DNA *in vivo* and *in vitro* has been shown to generate a single-stranded intermediate that corresponds to the leading strand of the DNA. *In vivo* results have suggested that a palindromic sequence, *palA*, located near the leading strand termination site acts as the lagging strand origin. In this paper we report the development and characterization of an *in vitro* system for the replication of single-stranded pT181 DNA. Synthesis of the lagging strand of pT181 proceeded in the absence of the leading strand synthesis and did not require the pT181-encoded initiator protein, RepC. The replication of the lagging strand required RNA polymerase-dependent synthesis of an RNA primer. Replication of single-stranded pT181 DNA was found to be greatly stimulated in the presence of the *palA* sequence. We also show that *palA* acts as the lagging strand origin and that DNA synthesis initiates within this region.

A number of small, multicopy plasmids from *Staphylococcus aureus* such as pT181, pC221, pE194, pC194, and pUB110 share similar replication properties. Many of these plasmids have also been shown to replicate in *Bacillus subtilis* (1, 2). These plasmids encode replication initiator proteins, which have origin-specific nicking-closing activities (3, 4). These proteins are directly involved in the generation of a primer for the replication of the leading strand via an asymmetric rolling circle mechanism. A number of *in vivo* and *in vitro* studies have shown that single-stranded DNA (ssDNA) is an intermediate in the replication of these plasmids. The mechanism of replication of these plasmids is different from that of the more extensively studied plasmids of *Escherichia coli* and is in many ways similar to the replication of ssDNA bacteriophages of *E. coli* (5–7). These plasmids contain a palindromic sequence (*palA*) whose deletion results in plasmid instability, a decrease in the plasmid copy number, and the accumulation of large quantities of ssDNA *in vivo* (8, 9). On the basis of these observations, the *palA* sequence has been postulated to serve as the origin of lagging strand synthesis. The *palA* sequences of many plasmids have considerable homology (4, 9). This sequence has been shown to be orientation dependent, can function at different locations, and is interchangeable among many small *S. aureus* plasmids (4, 8, 9).

The replication properties of the pT181 plasmid of *S. aureus* have been extensively studied both *in vivo* and *in vitro* (4). An *in vitro* system for the replication of double-stranded, supercoiled pT181 DNA has been established and used to characterize the replication properties of this plasmid (10,

11). The pT181 plasmid has been shown to replicate unidirectionally from an origin of replication that is located within a 43-base-pair (bp) sequence (12, 13). The initiator protein of pT181, RepC, binds to the origin sequence and generates a nick in the bottom (leading) strand of the DNA between positions 70 and 71 on the pT181 map (3, 14). Replication of pT181 initiates by covalent extension of the DNA at the nick. The *palA* sequence of pT181 is contained within a 200-bp region near the leading strand termination site (8, 15, 16). The replication of pT181 DNA both *in vivo* and *in vitro* has been shown to generate circular, ssDNA corresponding to the leading strand of pT181 (8, 15). In this paper we describe an *in vitro* system for the replication of single-stranded pT181 DNA. ssDNA carrying the *palA* sequence replicated efficiently in this system, whereas ssDNA lacking the *palA* sequence replicated either poorly or not at all. The presence of the leading strand origin had no effect on ssDNA → double-stranded DNA (dsDNA) replication. The plasmid-encoded initiator protein was not required for the replication of ssDNA, showing that the replication of ss- and dsDNA can be uncoupled *in vitro*. Finally, our results also demonstrate that the start site of pT181 lagging strand synthesis is located within the *palA* sequence and that replication requires the synthesis of an RNA primer by the host RNA polymerase.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The restriction-deficient *S. aureus* strain RN4220 has been described (17). Plasmid pT181cop-608 is a copy mutant of pT181 that contains a 180-bp deletion of the sequences involved in the control of the plasmid copy number (18). This plasmid was used as the source of pT181 DNA in this study. Plasmid DNA was isolated by CsCl/ethidium bromide density gradient centrifugation (19).

Construction of Recombinant Plasmids and Preparation of ssDNA. To study the replication of single-stranded pT181 DNA, various restriction fragments of pT181 were cloned into *E. coli* JM109 by using the phagemid vector pGEM-3Zf (obtained from Promega). This vector replicates as a plasmid in *E. coli* and can generate ssDNA in the presence of a helper phage since it contains the phage f1 origin of replication (20). Plasmid pSK462 carrying the *palA* sequence was constructed by ligating a 331-bp *Mbo* I–*Hinf*I fragment (pT181 map positions 377–707) (18) from pT181cop-608, after filling in the ends, into the *Hinc*II site of pGEM-3Zf(−). Plasmid pSK464 carrying the leading strand origin was obtained by ligating a 128-bp *Mbo* I–*Hinf*I fragment (pT181 positions 31–158) into the *Hinc*II site of pGEM-3Zf(−) after filling in the sticky ends of the insert DNA. Plasmid pSK463 carrying both the leading and lagging strand origins was obtained by ligating a 1345-bp pT181cop-608 *Hind*III fragment (pT181 positions 3797–4437/0–885 with a 180-bp deletion) into the *Hind*III site of pGEM-

3Zf(−). Plasmid pSK467 containing the same 1345-bp HindIII fragment in opposite orientation with respect to the vector was generated by using the pGEM-3Zf(+) plasmid as the vector. It should be noted that the leading strand of pT181 contains both the RepC nick site as well as the *palA* sequence since it serves as a template for the lagging strand synthesis (4, 8). The orientation of the insert DNA with respect to the vector in various recombinant plasmids was determined by restriction enzyme analysis. Since only the plus strand of f1 phage is present in the phage particles, plasmid pSK462 was expected to generate a ssDNA that contained only the lagging strand origin, whereas pSK463 was expected to yield a ssDNA with both the leading and lagging strand origins. Plasmid pSK467 contained the insert in an orientation that is expected to produce a ssDNA that contains sequences complementary to the lagging and leading strand origins. Plasmid pSK464 is expected to produce ssDNA carrying only the leading strand origin. No recombinant plasmids were obtained that would generate ssDNA containing sequences complementary to only the leading or the lagging strands. ssDNA was isolated from the strains carrying the recombinant plasmids by infecting the cells with the helper phage M13KO7 as described (20). The phage was isolated by precipitation with PEG, and the ssDNA was isolated by extraction with phenol and chloroform (20). The purity of the ssDNA was checked by agarose gel electrophoresis.

Preparation of Cell-Free Extracts. Extracts were prepared from the restriction-deficient *S. aureus* strain RN4220, which was shown to accept DNA isolated from *E. coli* (17). Extracts were prepared essentially as described (10), except that three cycles of rapid freeze–thaw were included during cell lysis. Extracts were also prepared from the *S. aureus* strain RN1786 used previously for the replication of supercoiled pT181 DNA. The 0–70% saturated ammonium sulfate precipitate was collected, dialyzed, and used in the experiments. The protein concentration in the cell extracts was usually about 200 mg/ml.

In Vitro **Replication.** *In vitro* replication of single-stranded and supercoiled plasmid DNA using cell-free extracts was carried out as described (11). RepC protein was isolated by using an overproducing strain as described (11). Reaction mixtures (30 μl) contained 40 mM Tris·HCl (pH 8.0), 100 mM KCl, 12 mM Mg(OAc)$_2$, 1 mM dithiothreitol, 5% ethylene glycol (vol/vol), 2 mM ATP, 0.5 mM UTP, 0.5 mM CTP, 0.5 mM GTP, 50 μM dTTP, 50 μM dCTP, 50 μM dGTP, 20 μM [^{32}P]dATP (13,000 cpm/pmol), 50 μM NAD, 50 μM cAMP, 150 ng of single-stranded or 300 ng of supercoiled plasmid DNA, 1 mg of RN4220 protein extract (unless otherwise indicated), and 880 ng of RepC protein (where indicated). Incubations were carried out at 32°C for 1 hr (unless otherwise indicated). DNA was isolated by phenol/chloroform extraction and alcohol precipitation as described earlier (15), and the reaction products (with or without restriction enzyme digestion) were analyzed by electrophoresis through 1.2% agarose gels (3 V/cm for 16 hr) using Tris acetate/EDTA buffer containing ethidium bromide at 0.5 μg/ml. Reaction products were identified by autoradiography of dried gels. The positions of the various single-stranded and supercoiled plasmid DNA were identified by electrophoresing DNA markers on the same gels. In some cases, ssDNA replication was assayed by trichloroacetic acid precipitation, filtration through glass fiber filters, and determination of the radioactivity (11).

RESULTS

Replication of Single-Stranded pT181 DNA *in Vitro*. Various single-stranded and supercoiled DNA were tested for replication by using cell-free extracts made from strains RN1786 and RN4220 in the presence or absence of RepC protein.

Although RN1786 extracts replicated supercoiled pT181 DNA carrying the leading strand origin efficiently in the presence of RepC, no replication was observed with ssDNA templates (data not shown). As observed earlier with RN1786 extracts, supercoiled pT181cop-608 DNA replicated efficiently in RN4220 extracts in the presence of RepC protein (Fig. 1A, lanes 1 and 2). Single-stranded pSK462 DNA carrying the *palA* sequence replicated efficiently in this system in the absence of RepC protein (Fig. 1A, lane 4). Based on their migration in the gels, the replication products consisted primarily of supercoiled monomeric DNA and low levels of open circular DNA. This was also confirmed by the resistance of *in vitro* replication products to S1 nuclease treatment (data not shown). The extent of single-stranded pSK462 DNA replication was unchanged in the presence of RepC protein (data not shown). Single-stranded pSK463 DNA, which contains both the leading and lagging strand origins, also replicated efficiently (Fig. 1A, lane 6). As expected, in the presence of RepC protein, supercoiled pSK462 DNA did not replicate, whereas supercoiled pSK463 DNA replicated efficiently (Fig. 1A, lanes 3 and 5). Very little or no replication was observed with single-stranded pSK464
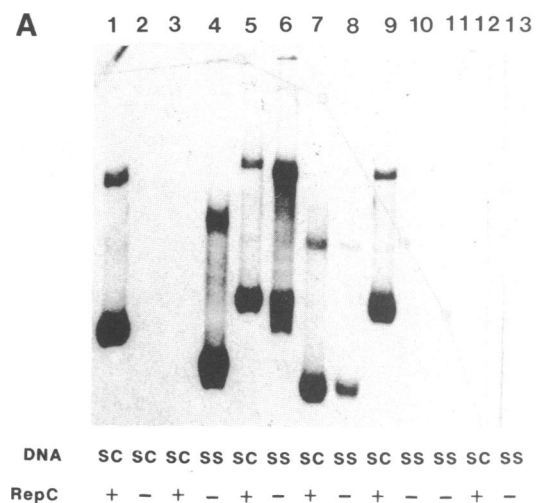

FIG. 1. Replication of single-stranded pT181 DNA *in vitro*. (A) Various single-stranded and supercoiled DNA were replicated *in vitro* in the presence or absence of RepC. Lanes 1 and 2, pT181cop-608 DNA; lanes 3 and 4, pSK462 DNA; lanes 5 and 6, pSK463 DNA; lanes 7 and 8, pSK464 DNA; lanes 9 and 10, pSK467 DNA; lane 11, ϕX174 DNA; lane 12, pGEM-3Zf(−) DNA; lane 13, pGEM-3Zf(+) DNA. In each lane the faster and slower migrating bands correspond to the supercoiled and open circular forms of dsDNA, respectively. The presence (+) or absence (−) of RepC in the reaction mixtures is indicated at the bottom. SC and SS indicate the supercoiled or single-stranded forms, respectively, of the added template DNA. (B) Treatment of ssDNA → dsDNA replication products with methylation-resistant and methylation-sensitive endonucleases. Single-stranded pSK462 DNA was replicated in the absence of RepC, and the reaction products were treated with the following restriction endonucleases: N, none; D, Dpn I; M, Mbo I; S, Sau3AI. Positions of supercoiled (SC), open circular (OC), and linear double-stranded (DS) pSK462 DNA are shown.

and pSK467 DNA lacking the *palA* sequence, respectively (Fig. 1*A*, lanes 8 and 10). As expected, the corresponding supercoiled DNA carrying the leading strand origin replicated efficiently in the presence of RepC protein (Fig. 1*A*, lanes 7 and 9). Single-stranded ϕX174 and pGEM-3Zf(+) DNA also did not replicate (Fig. 1*A*, lanes 11 and 13). Finally, supercoiled pGEM-3Zf(−) vector DNA did not replicate in

this system (Fig. 1*A*, lane 12). These results showed that the presence of *palA* greatly stimulates ssDNA replication *in vitro*.

To rule out the possibility of repair synthesis due to extensive priming of the template ssDNA isolated from *E. coli*, single-stranded pSK462 DNA was replicated *in vitro* and treated with *Dpn* I, *Mbo* I, or *Sau*3AI. The reaction products were subjected to agarose gel electrophoresis followed by autoradiography. The supercoiled and open circular forms of the DNA replication products were found to be mostly resistant to cleavage by *Mbo* I and *Dpn* I (although they were converted to the open circular and linear forms to varying extent) but were totally cleaved into smaller fragments by *Sau*3AI (Fig. 1*B*). Since the single-stranded template DNA was isolated from the *E. coli* strain JM109, it is expected to be methylated at the adenine residues within the GATC sequences. Conversion of this DNA to the double-stranded form *in vitro* should give rise to hemimethylated DNA, which would be resistant to cleavage by both *Mbo* I and *Dpn* I, since these enzymes cleave unmethylated and fully methylated DNA, respectively. The dsDNA replication products are expected to be sensitive to the endonuclease *Sau*3AI, which is insensitive to adenine methylation. These results showed that the *in vitro* system predominantly carries out replication of the input ssDNA with minimal repair synthesis.

Characterization of the ssDNA → dsDNA in Vitro Replication System. The rate of ssDNA → dsDNA synthesis was linear for up to about 60 min, after which a plateau was reached (Fig. 2*A*). DNA synthesis increased with increasing DNA concentrations, and maximum incorporation was obtained with 150 ng of the template DNA (Fig. 2*B*). In the presence of 150 ng of single-stranded template DNA, maximal replication was observed with 1 mg of the crude protein extract (Fig. 2*C*). Addition of larger amounts of extract resulted in an inhibition of ssDNA replication. The extent of ssDNA → dsDNA synthesis in the *in vitro* system in the presence of 150 ng of single-stranded template DNA and 1 mg
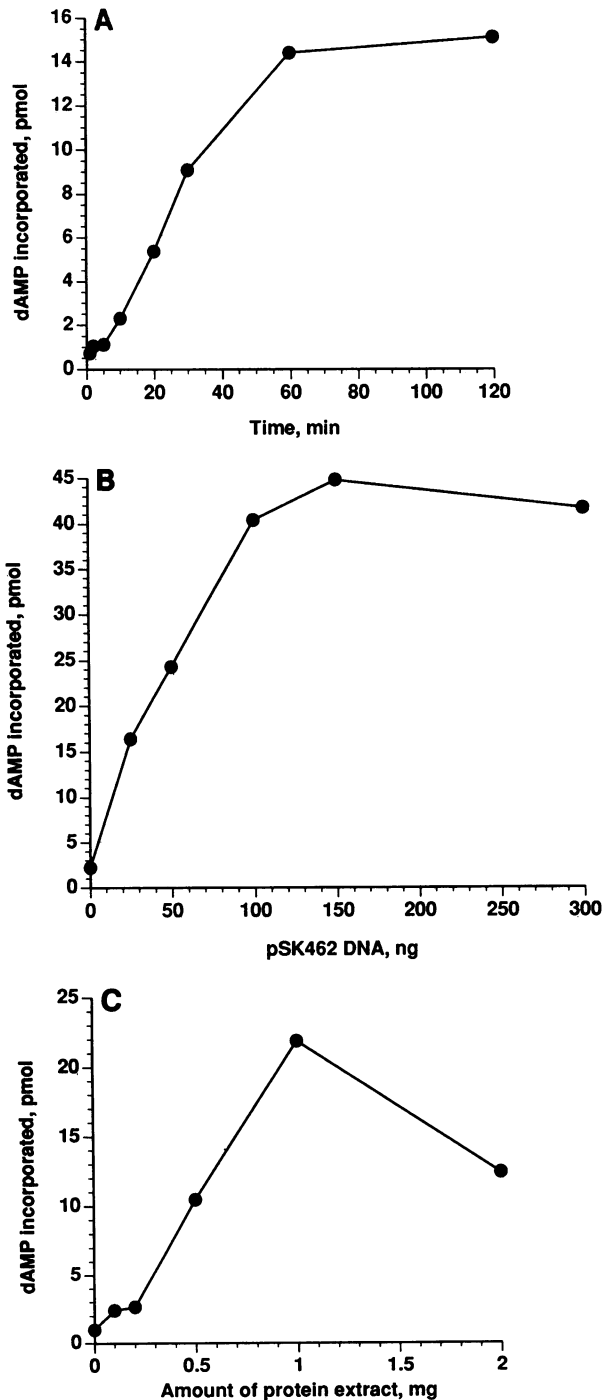

FIG. 2. Characteristics of the *in vitro* ssDNA → dsDNA replication system. (*A*) Rate of *in vitro* ssDNA → dsDNA replication. Reactions contained 150 ng of single-stranded pSK462 template DNA and 1 mg of the protein extract. (*B*) Effect of single-stranded template DNA concentration. Reactions contained 1 mg of the protein extract and variable amounts of single-stranded pSK462 DNA. (*C*) Effect of the amount of protein extract added. Reactions contained 150 ng of single-stranded pSK462 DNA and variable amounts of the protein extract. Reactions were carried out as described in *Materials and Methods*, and the acid-precipitable radioactivity was determined.
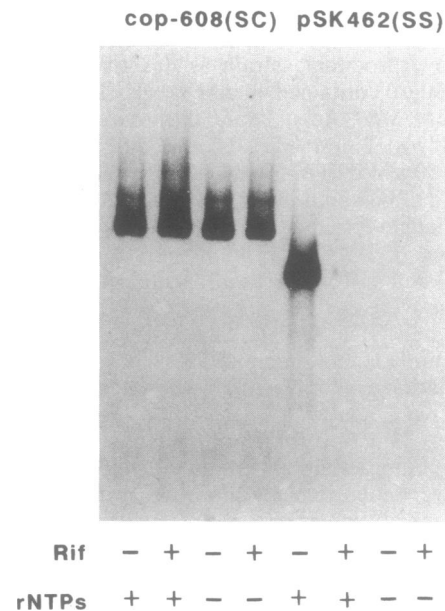

FIG. 3. Effect of rifampicin on single-stranded → double-stranded pT181 DNA synthesis *in vitro*. Supercoiled pT181cop-608 and single-stranded pSK462 DNA were replicated *in vitro* under standard conditions. pT181cop-608 and pSK462 DNA were linearized with *Kpn* I and *Eco*RI, respectively, to convert the supercoiled (SC) and open circular forms of the DNA into one band and were analyzed by agarose gel electrophoresis. The presence or absence of rifampicin (100 μg/ml) and rNTPs (GTP, CTP, and UTP) in the reactions is indicated at the bottom. Rif, rifampicin.

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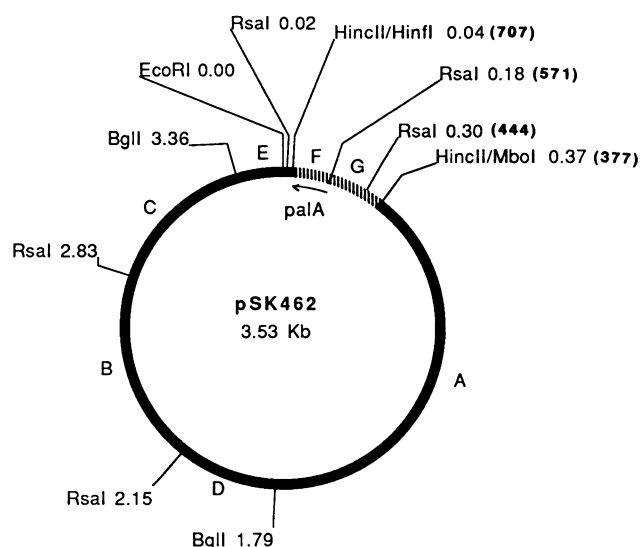
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FIG. 4. Restriction map of the pSK462 plasmid containing the *palA* region of pT181. The distances are shown in kilobase pairs (kb). The solid line indicates the vector pGEM-3Zf sequences, and the shaded line indicates a 331-bp *Mbo* I–*Hin*fI fragment of pT181 that contains the *palA* region. The pT181 nucleotide positions of some restriction sites in the *palA* region are given in base pairs and shown in boldfaced type in parentheses. The *Bgl* I and *Rsa* I sites are indicated, and the fragments expected to be generated upon treatment of the DNA with these enzymes (A–G) are indicated in decreasing size order. The arrow indicates the direction of lagging strand synthesis that initiates within the *Bgl* I–*Rsa* I F fragment. The *Hin*cII sites of pGEM-3Zf(−) vector used for the cloning of the *palA* region and the unique *Eco*RI site are also shown.

of RN4220 extract varied from about 15 to 45 pmol of dAMP depending on the DNA and extract preparation used. However, these amounts of DNA and protein extract in the reactions always yielded maximal synthesis.

Replication of Single-Stranded pT181 DNA Requires RNA Polymerase. Experiments were carried out to determine if the lagging strand synthesis requires an RNA primer and if primer synthesis is dependent upon the *S. aureus* RNA polymerase. As previously demonstrated (11), the *in vitro* replication of supercoiled pT181cop-608 DNA was inhibited only slightly in the presence of rifampicin and in the absence of CTP, GTP, and UTP (Fig. 3). However, the replication of single-stranded pSK462 DNA (carrying the *palA* region) was found to be almost totally inhibited in the presence of rifampicin and in the absence of rNTPs (Fig. 3).

Does the Lagging Strand Synthesis Initiate Within the *palA* Sequence? The results of experiments described above showed that the synthesis of the pT181 lagging strand is greatly stimulated in the presence of the *palA* sequence. We wished to determine if the lagging strand synthesis initiates within the *palA* region. *In vitro* replication experiments were carried out in the presence of various concentrations of the chain-terminating inhibitor dideoxy ATP (ddATP), in addition to the four dNTPs. After incubation, the DNA was digested with *Bgl* I and *Rsa* I, and the fragments were analyzed by polyacrylamide gel electrophoresis. The cleavage of double-stranded pSK462 DNA with these enzymes is expected to generate seven fragments of 1485 (fragment A), 676 (B), 538 (C), 360 (D), 186 (E), 157 (F), and 127 (G) bp (Fig. 4). Fragment E was found to be the most intensely labeled at the highest ddATP concentrations (Fig. 5) and hence the first to be replicated. At lower ddATP concentrations, fragments C, B, D, and A were replicated in that order. The replication of fragments F and G was observed only in the absence of ddATP (Fig. 5). The specific activity of the largest fragment (A) is lower than that of fragments B and C in the absence of
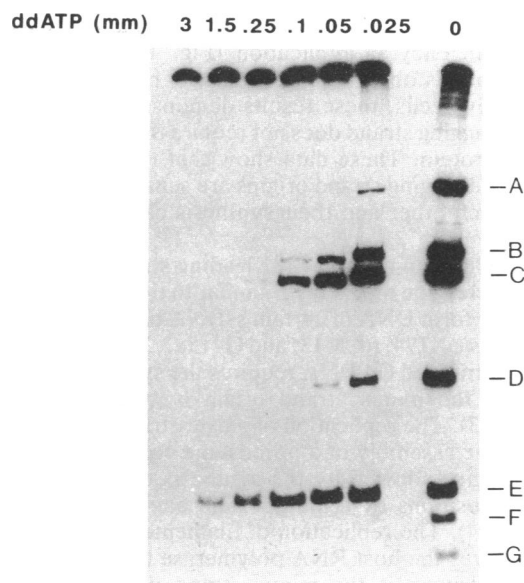

FIG. 5. Analysis of replicative intermediates synthesized *in vitro* in the presence of ddATP. Reactions were carried out as described in *Materials and Methods* using single-stranded pSK462 template DNA. The reactions contained 20 μM dATP and various concentrations of ddATP. The reaction products were digested with *Bgl* I and *Rsa* I, separated on a 5% polyacrylamide gel, and subjected to autoradiography. The positions of the various restriction fragments (A–G) are indicated. The concentrations of ddATP used are indicated at the top.

any ddATP, indicating that replication has not gone to completion in all the molecules that initiated replication.

DISCUSSION

In this report we have described a cell-free system for the replication of single-stranded pT181 DNA. Although *in vitro* systems have been described for the replication of ssDNA bacteriophages of *E. coli*, the current *in vitro* system is unique for the replication of single-stranded plasmids from Gram-positive bacteria. Extracts prepared from the restriction-deficient strain RN4220 were capable of replicating ssDNA (Fig. 1A). Replication of ssDNA in the *in vitro* system was greatly stimulated in the presence of the *palA* region. Template DNA lacking the *palA* region replicated either poorly or not at all (Fig. 1A). As expected, the synthesis of the lagging strand required an exposed single-stranded template strand since supercoiled template DNA was inactive for replication in the absence of RepC (Fig. 1A). *In vivo* studies have shown that the absence of the *palA* region causes plasmid instability, decreased copy number, and accumulation of large quantities of ssDNA (4, 8, 9). These results also suggested that, in the absence of *palA*, replication initiates at a low efficiency from other sites. Our *in vitro* results are consistent with the *in vivo* results since single-stranded pSK464 DNA (carrying the leading strand origin) replicated to a limited extent even though it lacks the *palA* region (Fig. 1A, lane 8). These results suggest that a weak, alternative signal for ssDNA → dsDNA conversion may be located within the 128-bp region of pSK464 DNA that includes the pT181 leading strand origin. The *palA* region has been shown to be strand-specific *in vivo* (4, 8, 21). Single-stranded pSK463 DNA carrying both the leading and lagging strand origins replicated efficiently in the *in vitro* system (Fig. 1A). However, no replication was observed with single-stranded pSK467 DNA carrying the complementary sequences. These results show that *palA* functions in a strand-specific manner during *in vitro* replication. The presence of the leading strand origin of pT181 in

single-stranded template DNA containing *palA* had no effect on the efficiency of replication (Fig. 1*A*). Since ssDNA replication was observed with extracts prepared from plasmid-negative cells, these results demonstrate that the replication of lagging strand does not require the plasmid-encoded initiator protein. These data show that the activities of the leading and lagging strand origins are separable and independent of each other and their synthesis can be uncoupled *in vitro*.

The replication of the pT181 leading strand proceeds by a rolling circle-type mechanism similar to that observed for the replicative form DNA of certain ssDNA bacteriophages of *E. coli* such as ϕX174, fd, M13, and G4 (5, 7, 22). The replication of single-stranded G4 DNA requires the synthesis of an RNA primer at the minus strand origin by the *dnaG*-encoded primase (23). The replication of single-stranded ϕX174 DNA requires the assembly of a primosome complex at the minus strand origin followed by the synthesis of RNA primers at multiple positions by a multiprotein complex involving the primase (24). The replication of filamentous phages such as M13 requires the host RNA polymerase for the synthesis of an RNA primer at the minus strand origin (25). Results presented here suggest that an RNA primer is involved in the replication of the lagging strand of pT181 since very little or no replication is observed in the absence of GTP, CTP, and UTP (Fig. 3). Since ssDNA → dsDNA synthesis is abolished in the presence of rifampicin, it is likely that the host-encoded, rifampicin-sensitive RNA polymerase is involved in the synthesis of the RNA primer. Thus, the lagging strand synthesis of pT181 appears to be most similar to that of the filamentous bacteriophages of *E. coli*.

We carried out experiments to directly test whether replication of single-stranded pT181 DNA initiates within the *palA* sequence. Using various concentrations of ddATP in the *in vitro* replication reactions, we observed that the order of replication of the various *Bgl* I–*Rsa* I fragments of pSK462 DNA was E, C, B, D, A, G, and F (Figs. 4 and 5). These results demonstrate that replication initiates within the F fragment (between positions 571 and 707 on the pT181 map), since it is the last to be replicated, and proceeds in a counterclockwise direction on the pSK462 map (Fig. 4). A region of ssDNA carrying the start site of replication is not expected to generate a restriction fragment until this region has been completely replicated and becomes susceptible to cleavage by restriction endonucleases that cleave only dsDNA. If replication initiates within the G fragment, fragment F should be labeled first. However, if replication initiates within the F fragment, fragment E should be replicated first. Since the E fragment was labeled first (Fig. 5), the lagging strand replication must initiate within the F fragment of pSK462, which contains nucleotides 571–707 of the pT181 plasmid. *In vivo* results have shown that the deletion of nucleotides downstream of pT181 map position 675 does not affect the activity of *palA* (8). Taken together, these results suggest that the lagging strand synthesis initiates between pT181 map positions 571 and 675. Intensive efforts to identify the exact start site of the pT181 lagging strand synthesis by analyzing the replication products obtained in the presence of ddATP on denaturing DNA sequencing gels have been unsuccessful (data not shown). Although unlikely, it is possible that the lagging strand synthesis initiates from multiple positions between nucleotides 571 and 675 of pT181.

The availability of a *palA*-dependent *in vitro* system capable of replicating single-stranded pT181 DNA should be useful in studies on the lagging strand replication, including the identification of the minimal origin sequence. Although we have not yet tested ssDNA carrying *palA* regions from different *S. aureus* plasmids, it is very likely that all such plasmids will replicate in this system. This assumption is based on the previous observations that the *palA* regions of several small, multicopy plasmids are interchangeable *in vivo*. Additionally, replication of the lagging strand of the pUB110 plasmid *in vivo* has been shown to require the synthesis of an RNA primer by the host RNA polymerase (21). Finally, the *in vitro* system should be useful in the isolation of proteins and enzymes required for the lagging strand synthesis.

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