Replication Termination for Staphylococcal Plasmids: Plasmids pT181 and pC221 Cross-React in the Termination Process

SERBAN IORDANESCU* AND STEVEN J. PROJAN

Department of Plasmid Biology, The Public Health Research Institute, New York, New York 10016

Received 10 December 1987/Accepted 13 May 1988

We present data which indicate that (i) the origin of replication of plasmids pT181 and pC221 can also function as termination signals; (ii) termination of replication occurs when a round of replication initiated either by RepC at the pT181 origin or by RepD at the pC221 origin reaches either of these origins, proving that the two plasmids cross-react for termination of replication; and (iii) the replication initiated at the origin of another staphylococcal plasmid, pE194, does not terminate at the origin of pT181 or pC221, indicating the existence of a specific relationship between the initiation and termination of a replication event.

The process of replication can be divided into three steps: initiation, elongation, and termination. The initiation process has been studied in great detail for many different bacterial replicons, since it represents the step that involves specific interactions which confer the property of autonomous replication; i.e., once it is initiated a round of replication proceeds until all the DNA covalently bound to the origin is duplicated. It is also the level at which most, if not all, regulatory mechanisms affecting replication act. Much less is known about the termination of DNA replication. For molecules with a bidirectional mode of replication, it is considered that termination occurs when the two replication forks meet, although for some replicons, such as the *Escherichia coli* chromosome or plasmid R6K, termination takes place preferentially in a specific region (22) which has the ability to stop, or at least to delay, replication rounds entering it from either side (19). For molecules replicating by the theta type of mechanism, termination involves mostly the separation of the interlocked daughter molecules (20, 32), a process in which the DNA gyrase is considered to play the main role (1). The process of replication termination has been studied in much more detail for molecules that use the rolling circle replication mechanism (5), such as the single-stranded DNA coliphages (10, 21). For these replicons it has been shown (3) that termination is an active process that involves the recognition of a sequence that may not completely overlap the sequence that is required for initiation.

pT181 is the best-characterized representative of a class of small staphylococcal plasmids that share a similar genome organization and mode of replication (28). Several lines of evidence suggest that pT181 and probably other related plasmids replicate via a rolling circle mechanism. These are summarized as follows. (i) The pT181-encoded protein RepC, which is known to be essential for plasmid replication (28), has been shown to possess site-specific nicking-closing activity *in vitro*. The nick occurs in the pT181 replication origin (16), at a specific site, between nucleotides 70 and 71 on the pT181 map (18). The 3' OH end generated by the cleavage is thought to be used for the initiation of a replication round by extension synthesis (17). (ii) A large asymmetrical palindrome, *palA*, is present in pT181 outside the region that is known to be required for replication. In the absence of *palA*, the plasmid is unstable, it has a lower copy number, and it accumulates large amounts of single-stranded DNA

(7). Only one strand, corresponding to the leading strand in replication, accumulates. Therefore, pT181 appears to replicate asymmetrically. First, the leading strand is synthesized by 3' extension from the nick introduced by RepC, and then the lagging strand synthesis is initiated at *palA*, which functions as a specific signal for this process (7). The *palA* signal functions inefficiently or not at all in *Bacillus subtilis*, in which pC194 and many other plasmids related to pT181 have been found to accumulate single-stranded circular DNA; only one strand, which, at least for the better-characterized plasmids of this group, corresponds to the leading strand, was accumulated (36).

The use of the origin of replication also as a termination signal is a characteristic of the rolling circle mode of replication (9). The approach used to test this aspect for plasmid pT181 was similar to that developed for phages f1 (2) and ϕ X174 (37), namely, the construction of hybrids carrying two origins of replication cloned in the same orientation. The ability of a heterologous plasmid origin to act as a signal in termination was also tested in this study. pC221, a plasmid with a similar genome organization and a high degree of sequence homology in the replication region with pT181 (34), was used. Plasmids pT181 and pC221 each codes for a protein (RepC for pT181 and RepD for pC221) that is essential for their autonomous replication. The results presented here indicate that under conditions in which RepC acts in initiation only on the pT181 origin and RepD acts only on the pC221 origin (i.e., each Rep protein acts only on the corresponding origin, which is normally embedded in the sequence encoding the Rep protein), the two Rep proteins can terminate a round of replication at either origin.

MATERIALS AND METHODS

Organisms. *Staphylococcus aureus* NCTC 8325 (wild type) was used as the host in all the experiments described here. The plasmids used in this study are listed in Table 1. Two plasmids were used to supply RepC (pT181) or RepD (pC221) *in trans*: (i) plasmids that used the respective Rep protein for their own replication and, therefore, that competed with a cloned homologous origin for utilization of the Rep protein, and (ii) plasmids that used another replicon for maintenance and that lacked any origin recognized by the Rep protein that they synthesized. Plasmids pSA0382 for RepC and pSA3320 for RepD belonged to the first class, while pSA6470 and pSA7420 belonged to the second class (Table 1). A more detailed description of these Rep donors

* Corresponding author.

TABLE 1. Plasmids used in this study

Plasmid	Phenotype or genotype	Derivation	Reference or source
pT181	Tc ^r Inc3	Naturally occurring	15
pC221	Cm ^r Inc4	Naturally occurring	34
RN5101	Em ^r Inc11 Tsr	Tsr mutant of pE194	29
pRN6321	Em ^r Inc11 Tsr; <i>TaqI</i> -C deleted	Deletion of <i>TaqI</i> C fragment from pRN5101	29
pE5	Em ^r Inc12	Naturally occurring	26
pSA0382	Tc ^r Inc3 <i>cop-652</i> ; <i>HindIII</i> -C deleted	Deletion of <i>HindIII</i> C fragment from pSA0342, a pT181 Cop mutant	12
pSA3320	Tc ^r Inc3 RepD ⁺ ori-pC221 ⁺	Deletion of <i>XbaI</i> B and <i>XmnI</i> B fragments from pCW48 (38)	This study
pSA6470	Km ^r Tc ^r Inc13 RepC ⁺ ori-pT181 ⁻	In vitro cointegrate between pUB110 and a pT181 derivative with an in vitro substitution inactivating the origin	This study
pSA7420	Km ^r Inc13 RepD ⁺ ori-pC221 ⁻	In vitro cointegrate between pUB110 and a pC221 derivative with a substituted pT181 origin	This study
pSA7600	Em ^r Cm ^r Inc11	See Fig. 1	This study
pSA7650	Em ^r Cm ^r Inc11	See Fig. 1	This study

will be presented elsewhere (S. Iordanescu, manuscript in preparation). Details of the construction of the hybrid plasmids that carried two cloned origins are presented in the text and Fig. 1.

Isolation and manipulation of DNA. Plasmid DNA was isolated by CsCl-ethidium bromide density gradient centrifugation of cleared lysates (31). Restriction enzymes were purchased from Boehringer GmbH (Mannheim, Federal Republic of Germany) and New England BioLabs, Inc. (Beverly, Mass.) and were used as specified by the producers. Restriction fragment isolation was done by extraction from polyacrylamide gels, as described previously (23). T4 DNA ligase was obtained from Collaborative Research Inc., and calf alkaline phosphatase was obtained from U.S. Biochemicals Corp. Sequencing was done by the dideoxy chain-termination method (35) by using either special primers for directly sequencing certain regions of the plasmid or by first cloning the restriction fragments of interest in M13 vectors (24). Since the fragments used for cloning the origins of replication of pT181 and pC221 carried asymmetric *HpaII* sites, the hybrids constructed were digested by this enzyme in order to determine the orientation of these fragments.

Genetic transfers. The methods used for *S. aureus* protoplast transformation and transduction have been described previously (6, 14).

Effect of Rep proteins on double-origin plasmids. The plasmid that was used as a source of Rep protein was transduced into a strain carrying the test plasmid by selecting only for the donor plasmid at the desired temperature. The transductant colonies were screened for plasmid content by agarose gel electrophoresis of whole-cell lysates (11) and were scored for antibiotic resistance.

RESULTS

Construction of hybrid plasmids containing two replication origins. Hybrid plasmids carrying two origins of replication cloned in the same orientation were used to test the ability of the origin to function also as a termination signal. The rationale was that if a round of replication that is initiated at one origin terminates at the second one, a plasmid deletion will result. To facilitate the identification and characterization of such deletion derivatives, a special vector was constructed to clone fragments carrying the origins of replication of pT181, pC221, or both.

The starting point in this construction was plasmid pRN5101, a temperature-sensitive replication (Tsr) mutant of plasmid pE194 (29). It has been shown previously that the

replication origins of pT181 or pC221 cloned into pRN5101 as *TaqI* fragments can support replication of the vector when a second plasmid encoding the corresponding Rep protein is present (29, 34). Deletion of the nonessential *TaqI* C fragment of pRN5101 gave rise to plasmid pRN6321. Cloning of the pC221 *TaqI* A fragment carrying the intact *cat* gene into the *TaqI* site of pRN6321 generated plasmid pSA7600 (Fig. 1). pSA7600 has unique *ClaI* and *AsuII* sites at which *TaqI* fragments can be cloned and carries the genes *ermC* and *cat*, which confer resistance to erythromycin (Em^r) and chloramphenicol (Cm^r), respectively, on either side of the two sites.

Neither the *TaqI* C fragment of pT181 (16) nor the *TaqI* F fragment of pC221 (34), which were used to clone the replication origins of the respective plasmids, contains a complete *rep* gene or the *pala* signal, which is required for lagging strand initiation (8). Therefore, efficient replication initiated from a cloned origin requires not only the supply of the corresponding Rep protein in *trans* but also the presence in *cis* of a *pala* structure in the appropriate orientation. As a consequence, the cloned origins can function efficiently only in that orientation in which they can use the *pala* of the pE194 replicon. To ensure that both derivatives which could potentially be generated by initiation at one cloned origin and termination at the other replicate efficiently, each of them had to carry a *pala* sequence. For this, a second vector, pSA7650, was constructed by substituting the *SacI*-*AsuII* fragment of pSA7600 with a *SacI*-*TaqI* fragment from pE5 (26), which carried the *pala* region of this plasmid. The construction restored the structural integrity of the *ermC* gene and the *AsuII* site and introduced the *pala* sequence of pE5 in an orientation with the same polarity as the *pala* of pE194 (Fig. 1). Therefore, in pSA7650 both regions between the sites that could be used for the insertion of the replication origins carried a *pala* sequence.

Both pSA7600 and pSA7650 were used in the construction of plasmids carrying the pT181 origin, the pC221 origin, or both (Table 2). All these plasmids carried three distinct origins of replication: that of the pE194 replicon, which was used as the vector in these constructs, in addition to the two cloned origins. However, for the purpose of this study, they are referred to as double-origin hybrids.

Properties of double-origin hybrids. In the absence of a source of RepC or RepD, the double-origin hybrids were found to have replication properties similar to those of pRN5101 and the original vectors. At 32°C they were maintained at about 50 copies per cell, while at 43°C they did not replicate at all. The presence of large direct repeats, as in the

FIG. 1. Derivation of vectors used in the construction of double-origin hybrids. The thin lines indicate pE194 DNA, the heavy lines indicate DNA coming from pC221, and the striped bar indicates pE5 DNA. rep represents the pE194 replicon. Only relevant restriction sites are presented. The drawing is not to scale.

case of two pT181 or pC221 origins in the same orientation, can potentially give rise to spontaneous deletions by homologous recombination. Recently, it has been reported that, at least in *B. subtilis*, replication initiated from a pE194 replicon that is present on the same structure as the direct repeats greatly stimulates this type of recombination (27). Therefore, the double-origin hybrids constructed in this study were

TABLE 2. Double-origin hybrids

Vector	Plasmid	Origin cloned at ^a :	
		<i>AseI</i>	<i>ClaI</i>
pSA7600	pSA7621	ori-pT181 I	ori-pT181 I
	pSA7622	ori-pT181 I	ori-pT181 II
	pSA7644	ori-pC221 I	ori-pT181 I
	pSA7633	ori-pC221 I	ori-pC221 I
pSA7650	pSA7655	ori-pT181 I	ori-pT181 I
	pSA7657	ori-pC221 I	ori-pT181 I

^a ori-pT181 is the origin of pT181 cloned as a 654-bp *TaqI* C fragment; ori-pC221 is the origin of pC221 cloned as a 278-bp *TaqI* F fragment; I represents the orientation of the origin in which replication was initiated clockwise, and the *palA* sequence of pE194 can be used; II indicates the opposite orientation.

tested for structural stability. The results obtained indicated that these hybrids are remarkably stable; after more than 100 generations of cell growth in nonselective medium at a temperature that was permissive for the pRN5101 vector, there was no loss of either of the two resistance markers and plasmid DNA bands of smaller size did not appear, as would be expected if any recombination had taken place (data not shown). Incidentally, these results also indicate that replication that is initiated by the pE194 replicon, which presumably uses a mode of replication like that of pT181 (36), does not terminate at either the pT181 or the pC221 origin.

Effect of Rep protein on hybrids carrying two identical origins. The hybrid plasmid pSA7655 carried two pT181 origins that were inserted as *TaqI* fragments at the *ClaI* and *AseI* sites of pSA7650 (Table 2 and Fig. 2). When the RepC protein was supplied in *trans* by another plasmid, replication was initiated at either of the pT181 origins of pSA7655. If the pT181 origin also served as a signal for termination, at least some of the replication rounds initiated at one origin would be terminated at the second one. This process would generate two smaller plasmids, each of which would carry only one pT181 origin and one of the other of the DNA segments delimited by the two origins (Fig. 2). One of the new plasmids would confer *Cm*^r and would carry the pE194 replicon and its *palA* sequence; the second one would express *Em*^r and would contain the *palA* from pE5. The two derivative plasmids would be mutually incompatible. At a permissive temperature, the *Cm*^r component would be expected to have a significant advantage over the *Em*^r component because of the presence of the active pE194 replicon (13); at a high temperature both components would be more or less equal in their ability to compete for maintenance.

The results obtained support these assumptions. When a plasmid supplying RepC was introduced by transduction into a strain harboring pSA7655, two new plasmids were generated. The process was practically complete by the time a transductant colony was formed. Gel electrophoresis of whole-cell lysates from such transductants demonstrated the presence of two plasmid bands with the sizes predicted by initiation at one pT181 origin and termination at the other. At a permissive temperature the *Em*^r component was maintained at a lower copy number than the *Cm*^r one, while at a high temperature the *Em*^r component was preponderant (compare lanes 2a and 2b in Fig. 3). The *Cm*^r and *Em*^r plasmids derived from pSA7655 were crossed from the original transductants into a plasmid-negative recipient and were found to have the expected replication properties. Both were complemented by RepC, indicating that they carried an active pT181 origin; the *Cm*^r plasmids replicated independently at the permissive temperature for the Tsr pE194 replicon but not at the restrictive temperature, while the *Em*^r plasmids were defective, requiring a source of RepC for maintenance. Because of the perfect homology between the two pT181 origins, it was not possible to determine precisely the sites involved in the generation of these derivatives.

Similar results were obtained when a source of RepD was introduced into a strain harboring an analogous hybrid with two copies of the pC221 origin (data not shown).

Behavior of hybrids carrying heterologous origins. Another set of experiments was performed to test for the ability of the pT181 origin to act as a termination signal for a replication round initiated at the pC221 origin, and vice versa. The hybrid pSA7657, which was derived from pSA7650 by cloning the pT181 and pC221 origins, each in the proper orientation (Table 2 and Fig. 2), was used for this approach.

When RepC was supplied in *trans*, pSA7657 gave rise to

FIG. 2. Structure of hybrids pSA7655 and pSA7657 and the generation of smaller derivatives from them in the presence of Rep proteins supplied in *trans*. Open bar, pT181 origin; filled bar, pC221 origin. The arrow above origin fragment indicates the direction of replication. The nicking site is located between the two *HpaII* sites.

an *Em^r* plasmid with a size expected for a plasmid whose replication was initiated at the pT181 origin and terminated at the pC221 origin (Fig. 2). No plasmid with the size of the other moiety of the hybrid molecule could be detected in this case. At a temperature permissive for the pE194 replicon, the newly generated *Em^r* plasmid was found to coexist with the original hybrid, while at a high temperature the hybrid could not be detected by gel electrophoresis (Fig. 3, lanes 5a and 5b), and its *Cm^r* marker was absent. It therefore appears that survival of the hybrid, even in the presence of RepC, requires the function of its pE194 replicon. This result can be explained by assuming that termination is a very efficient process so that all, or almost all, replication rounds initiated at the pT181 origin terminate at the pC221 origin. Only when replication was initiated at the pE194 origin was the entire molecule replicated.

In the presence of a RepD donor, a small plasmid carrying only the *Cm^r* marker of pSA7657 appeared (Fig. 2). In this case the original hybrid could not be detected (Fig. 3, lanes 6a and 6b). Since the functional pE194 replicon was expected to ensure the survival of the hybrid at a permissive temperature, it may be that, in this case, the hybrid was lost due to incompatibility with the *Cm^r* derivative, which might have some advantage in this relationship. If the reasoning on the efficiency of termination provided above is correct, it can be suggested that the advantage of the *Cm^r* plasmid over the original hybrid is due to the fact that it can use either the pE194 replication system or the initiation by RepD for its maintenance, while the hybrid can be replicated only by the pE194 replicon, with initiation by RepD at the pC221 origin generating new *Cm^r* derivative plasmids. The reason for the maintenance of the *Cm^r* derivative in a lower copy number than the original hybrid or the *Em^r* component (Fig. 3, lanes 6a and 6b) is not yet clear.

Some of the derivative plasmids were studied in more detail. The region of the origin in which pT181 and pC221 differed and which is considered to be responsible for the specificity of the origin in initiation (34) lies downstream from the nicking site, while the region upstream is identical in the two plasmids (Fig. 4). Therefore, it was expected that the derivative plasmids carried functional origins, with predictable initiation specificities. All eight derivatives obtained from pSA7657 in the presence of RepC or RepD had the expected replication properties. The *Cm^r* components carried a functional origin with pC221 specificity, while the *Em^r* components carried a functional origin with pT181 specificity. If, as discussed above, the derivative plasmids were generated by initiation at one origin and termination at the other, then, as shown in Fig. 2, the junctions produced by their formation should be located precisely in the positions where nicking takes place in the two origins. This prediction

FIG. 3. Agarose gel electrophoresis of whole-cell lysates. Strains carried plasmids pSA7655 (lane 1), pSA7655 and pSA6470 (lane 2), pSA6470 (lane 3), pSA7657 (lane 4), pSA7657 and pSA6470 (lane 5), pSA7657 and pSA7420 (lane 6), and pSA7420 (lane 7). Growth was at 32°C (lanes a) and 43°C (lanes b).

FIG. 4. Sequences of the origin regions of plasmids pT181 and pC221. Lowercase bases indicate divergence. Only the strand which was nicked by RepC in pT181 is presented.

was tested by sequencing this region. For all five plasmids tested, the junction was indeed found in a 44-base-pair (bp) sequence of perfect homology between pT181 and pC221 origins, a sequence that included the RepC nicking site (Fig. 4).

Similar studies were performed with pSA7644, a hybrid derived from pSA7600, which contained a single *palA* element. In pSA7644 the cloned pT181 and pC221 origins were in the same relative positions as they were in pSA7657. In the presence of RepD, pSA7644 gave rise to small derivative plasmids carrying only *Cm^r*, and the results were identical to those reported above for pSA7657. On the other hand, when RepC was supplied in *trans*, the initiation of replication at the pT181 origin cloned at the *ClaI* site was expected to generate an *Em^r* derivative without a *palA* sequence, if termination occurred at the pC221 origin cloned at the *AsuII* site. However, the results were different. (i) The *palA* mutant *Em^r* derivative plasmid carrying the pT181 origin could hardly be detected, if at all. This fact can be explained on the basis of the replication-defective phenotype of *palA* mutant plasmids (8). (ii) When selection was applied for the *Em^r* marker, the formation of cointegrates between the *Em^r* component and the plasmid providing the Rep protein was observed. (iii) Unexpectedly, derivative plasmids containing only the *Cm^r* marker appeared even in the absence of chloramphenicol selection. These derivative plasmids were replicated by the *Tsr* pE194 replicon, and therefore, the presence of a functional pT181 or pC221 origin could be tested by supplying RepC or RepD in *trans*. All 18 *Cm^r* derivative plasmids tested in this way were found to carry only a functional pT181 origin. Since the region responsible for the specificity of the pT181 origin is known to lie downstream from the nicking site of RepC (Fig. 2 and 4), these plasmids could not be generated by aberrant initiation by RepC at the pC221 origin and termination at the pT181 origin. Rather, they must have been the product of homologous recombination downstream from the nicking site. The precise site where the presumed crossover took place in the generation of these plasmids was determined by sequencing the fragment carrying the pT181 origin of two of them. The results obtained showed that the recombination events took place in short stretches of homology between the pT181 and pC221 origin fragments (about 70 and 120 bp, respectively) downstream from the replication nick. Such recombinants were not detected in the absence of RepC or RepD or, for pSA7657, in the presence of RepC. As a consequence, it is suggested that the generation of *Em^r* derivatives lacking *palA* from pSA7644 is involved, by an unknown mechanism, in the appearance of such recombinants.

Tests for the involvement of recombination in the dissociation of the double-origin hybrids. Two tests were conducted to evaluate the possible involvement of a Rep-promoted, site-specific recombination in the observed dissociation of double-origin hybrids.

(i) The presence of Rep-mediated inversions of the segment between two origins present in opposite orientations

was examined. The hybrid pSA7622, which carried two inverted pT181 origins, was used to test this possibility. Two fragments were generated when pSA7622 was digested with *BclI* (Fig. 1). The inversion of one of the two segments separated by the two pT181 origins should have given rise to two other fragments of different sizes. Such fragments were not detected in the digest of pSA7622 DNA isolated from a strain in which it coexisted with a plasmid supplying RepC (Fig. 5).

(ii) Both RepC and RepD led to the formation of smaller derivative plasmids from a hybrid carrying pT181 and pC221 origins. If this were the consequence of site-specific, Rep-mediated recombination, then RepC should have been able to generate such derivatives from a pC221 double-origin hybrid, and RepD should have been able to generate such derivatives from a pT181 double-origin hybrid. In this experiment plasmids pSA0382 and pSA3320 were used as the sources of RepC and RepD, respectively (Table 1). No new derivatives were generated from the pT181 double-origin hybrid pSA7655 in the presence of RepD provided by pSA3320, even after some 100 generations of coexistence of the two plasmids in the same cell; pSA0382 also had no effect on the structural stability of the pC221 double-origin hybrid pSA7633 (data not shown). These results prove that the ability of the Rep protein to initiate replication is required for generating derivative plasmids from a double-origin hybrid.

DISCUSSION

The results presented here indicate that hybrids carrying two origins of replication of plasmid pT181, plasmid pC221, or both in the same orientation generate smaller derivatives when RepC, RepD, or both are supplied in *trans*. Recombination between the direct repeats represented by the two cloned origins can be ruled out as a mechanism of dissociation on the basis of the structural stability of the double-origin hybrids in the absence of RepC, RepD, or both. Another possibility would be that the Rep proteins can promote homologous recombination. Recently, it has been shown that nicking at the origin of replication of plasmid pC194 in *E. coli* can stimulate a recombination event in

FIG. 5. Agarose gel electrophoresis of *BclI* digests of plasmid DNA isolated from cells carrying pSA7622 (lane 1), pSA7622 and pSA6470 (lane 2), and pSA6470 (lane 3).

which one of the crossovers takes place at the nick in the origin (25). There are several arguments against this possibility. (i) In the case of pT181-pC221 double-origin hybrids, each Rep protein generated only one component, which was the one expected if the Rep protein initiated a round of replication at the corresponding origin and terminated it at the other cloned origin. Recombination, however, would be expected to generate both components, regardless of which Rep protein was present. The absence of the second component was especially significant with respect to the dissociation of pSA7657 in the presence of RepC. In this case only an *Em^r* derivative was obtained, although the other potential component (*Cm^r*) should have been easy to detect, at least at a permissive temperature, since it would carry the replication system of pE194 (Fig. 2). (ii) No dissociations of a pT181 double-origin hybrid were observed in the presence of RepD, and alternatively, RepC had no effect on a pC221 double-origin hybrid. (iii) No Rep-mediated inversions could be detected.

Therefore, the results presented here can best be explained by assuming that the origins of replication of plasmids pT181 and pC221 also serve as signals for the termination of a round of replication initiated at one of these origins. This fact constitutes another argument in support of a rolling circle mechanism in the replication of these plasmids. In this context it should be mentioned that the pT181 DNA must be in the double-stranded form in order for the plasmid to be hereditarily stable. There is as yet no hint of a function for the single-stranded intermediate, which accumulates only in the absence of an efficient lagging strand initiation signal and, under these conditions, seems to have a detrimental effect on the host cell. This is in sharp contrast to the situation of the single-stranded phages, in which the generation of single-stranded DNA is essential in their life cycles. On the other hand, a rolling circle mechanism of replication might be important for these plasmids, which rely mostly on transduction for their transfer, by facilitating the production of long concatemers which are considered to be essential for efficient packaging by the bacteriophage during infection (30).

In the case of the double-origin hybrids used in this study, a round of replication initiated at one of the cloned origins and terminated at the other one was expected to give rise to a plasmid containing the DNA between the nicking sites of the two origins and to restore the original hybrid (Fig. 6). When the pE194 replication system present on the hybrid was turned off by raising the temperature, initiation of replication took place only at one of the cloned origins, depending on the Rep protein supplied. Under these conditions the efficiency of the termination process could be evaluated. If all or most replication rounds were terminated at the second origin, there would be no net increase in the number of copies of the hybrid, and as a consequence, it would be diluted during the growth of the cells. The results obtained, especially with pSA7657, indicate that this is the case. At a high temperature after some 25 cell generations, which are required for the growth of a colony that has received by transduction a plasmid supplying RepC or RepD, the original hybrid was no longer present, as tested by gel electrophoresis (Fig. 3, lanes 2b, 5b, and 6b). The absence of the original hybrid from most of the cells of such transductant colonies was also demonstrated by testing for the resistance markers that they expressed. Only the marker that was present on the derivative plasmid generated under the conditions of the experiment was expressed by these cells. Therefore, the termination process is quite efficient.

FIG. 6. Model for the generation of small derivative plasmids from double-origin hybrids in the presence of a Rep protein supplied in *trans*. The newly synthesized strand is presented as an interrupted line. The origin regions are presented as heavy lines. The generation steps were as follows. I, Initiation of replication at one of the two cloned origins; II, the replication fork reached the second cloned origin; III, a nick was presumably introduced by the Rep protein at the origin cleaving the displaced strand; the 5' end generated was joined to the 3' end of the newly synthesized strand, restoring the structure of the double-origin plasmid, while the 3' and 5' ends of the displaced strand were joined together; IV, synthesis of the lagging strand was initiated at the *palA* sequence on the single-stranded circle, generating finally a double-stranded plasmid carrying the DNA between the two origins in the original plasmid; there was no evidence whether this step could start before termination.

This conclusion is in agreement with the presence of only a small proportion of the DNA of these plasmids as dimers and higher multimers in *S. aureus* (33).

The two plasmids used in this study, pT181 and pC221, have a high degree of sequence homology in their replication regions (34). Both the origins of replication and the sequences encoding the Rep proteins of these plasmids have regions of perfect homology interspersed with regions where the sequences are quite different. Thus, the sequence of the origin of these plasmids can be divided into two distinct regions (Fig. 4): (i) 44 bp of perfect homology, which includes the nicking site and 11 bp upstream and 33 bp downstream of the nicking site, and (ii) a divergent region downstream which includes most of the RepC-binding site (17). Results of complementation studies conducted *in vivo* indicate that under normal conditions, as used in the experiments described here, each Rep protein initiates replication strictly at the origin of the corresponding plasmid (34; S. Iordanescu, manuscript in preparation). On the other hand, the results of this study indicate that both origins function as signals for termination for rounds of replication initiated by either Rep protein. This fact suggests that there is not a complete overlapping of the sequences that are required for initiation with those that are required for termination in the case of these plasmids, a situation similar to that reported previously for phage ϕ 1 (3). It is reasonable to assume that at least part of the region of perfect homology between the origins of pT181 and pC221 is involved in termination and

that this sequence is recognized by both Rep proteins. Deletion analysis of the pT181 origin has shown that 17 bp upstream from the nicking site is sufficient for the function of the origin in initiation (M. Gennaro, S. Iordanescu, R. P. Novick, R. W. Murray, T. R. Steck, S. A. Khan, manuscript in preparation). Studies are in progress to determine the minimum sequence of the pT181 origin that can still function in termination. The ability of two other staphylococcal plasmids, pUB110 and pC194, to cross-react in termination has been reported recently (7).

Another aspect worth emphasizing is the correlation that was observed between initiation and termination of a round of replication. Initiation at the origin of pE194, a plasmid that is considered to use the same replication mode as pT181 and pC221 (36), does not lead to termination at any of the other origins, while replication initiated at either the pT181 or pC221 origin has never been found to terminate at the pE194 origin. One way to explain this behavior is by suggesting that, as reported previously for phage ϕ X174 (4), at initiation the Rep protein becomes attached to the 5' end of the displaced strand, travels with the replication complex around the circle, and recognizes only its own specific termination signal. Some evidence has been obtained that supports a covalent binding of RepC to the 5' end of the generated nick (18). This interpretation may explain the ability of the Rep proteins of pT181 and pC221 to cross-react in termination but not initiation by assuming that the specificity at initiation is due to the binding step and that de novo binding is not required for termination. On the other hand, the specificity of termination can be understood if initiation at any one origin prevents subsequent initiations at any other origin while the round of replication is in progress (i.e., initiation requires a superhelical substrate), and as a consequence, an extrinsic Rep protein would not be able to cause termination by nicking the DNA at its own specific origin.

More experiments are needed to determine the precise way in which the Rep proteins of these plasmids act in termination. The fact that in the system described here two distinct Rep proteins each recognized two different origins as signals for termination, while they recognized only one origin for initiation, should facilitate the progress of these studies.

ACKNOWLEDGMENTS

This study was supported by Public Health Service research grants AI-24327 (to S.I.) and GM-33278 (to S.J.P.) from the National Institutes of Health.

We thank Richard Novick, David Dubnau, and Ellen Murphy for critically reading the manuscript. The technical assistance of Julie Lin-Breidt is gratefully acknowledged.

LITERATURE CITED

1. Cozzarelli, N. R. 1980. DNA gyrase and the supercoiling of DNA. *Science* **207**:953-960.
2. Dotto, G. P., and K. Horiuchi. 1981. Replication of a plasmid containing two origins of bacteriophage ϕ 1. *J. Mol. Biol.* **153**:169-176.
3. Dotto, G. P., K. Horiuchi, and N. D. Zinder. 1984. The functional origin of bacteriophage ϕ 1 DNA replication. Its signals and domains. *J. Mol. Biol.* **172**:507-521.
4. Eisenberg, S., J. Griffith, and A. Kornberg. 1977. ϕ X174 *cistron* A protein is a multifunctional enzyme in DNA replication. *Proc. Natl. Acad. Sci. USA* **74**:3198-3202.
5. Gilbert, W., and D. H. Dressler. 1968. DNA replication: the rolling circle model. *Cold Spring Harbor Symp. Quant. Biol.* **33**:473-485.
6. Gotz, F., S. Ahrne, and M. Lindberg. 1981. Plasmid transfer and genetic recombination by protoplast fusion in staphylococci. *J. Bacteriol.* **145**:74-81.
7. Gross, M. F., H. te Riele, and S. D. Ehrlich. 1987. Rolling circle replication of single-stranded DNA plasmid pC194. *EMBO J.* **6**:3863-3869.
8. Gruss, A. D., H. F. Ross, and R. P. Novick. 1987. Functional analysis of a palindromic sequence required for normal replication of several staphylococcal plasmids. *Proc. Natl. Acad. Sci. USA* **84**:2165-2169.
9. Horiuchi, K. 1980. Origin of DNA replication of bacteriophage ϕ 1 as the signal for termination. *Proc. Natl. Acad. Sci. USA* **77**:5226-5229.
10. Horiuchi, K., J. V. Ravetch, and N. D. Zinder. 1978. DNA replication of bacteriophage ϕ 1 in vivo. *Cold Spring Harbor Symp. Quant. Biol.* **43**:389-399.
11. Iordanescu, S. 1983. *Staphylococcus aureus* chromosomal mutation specifically affecting the copy number of Inc3 plasmids. *Plasmid* **10**:130-137.
12. Iordanescu, S. 1986. Effect of the deletion of a fragment dispensable for the autonomous maintenance of plasmid pT181 on the competition between incompatible plasmids. *Plasmid* **15**:191-198.
13. Iordanescu, S. 1987. The Inc3B determinant of plasmid pT181. A mutational analysis. *Mol. Gen. Genet.* **207**:60-67.
14. Iordanescu, S., and M. Surdeanu. 1980. Complementation of a plasmid replication defect by autonomous incompatible plasmids in *Staphylococcus aureus*. *Plasmid* **4**:1-7.
15. Iordanescu, S., M. Surdeanu, P. DellaLatta, and R. P. Novick. 1978. Incompatibility and molecular relationships between small staphylococcal plasmids carrying the same resistance marker. *Plasmid* **1**:468-479.
16. Khan, S. A., G. K. Adler, and R. P. Novick. 1982. Functional origin of replication of pT181 plasmid DNA is contained within a 168-base-pair segment. *Proc. Natl. Acad. Sci. USA* **79**:4580-4584.
17. Koepsel, R. R., R. W. Murray, and S. A. Khan. 1986. Sequence-specific interaction between the replication initiator protein of plasmid pT181 and its origin of replication. *Proc. Natl. Acad. Sci. USA* **83**:5484-5488.
18. Koepsel, R. R., R. W. Murray, W. D. Rosenblum, and S. A. Khan. 1985. The replication initiator protein of plasmid pT181 has sequence-specific endonuclease and topoisomerase-like activities. *Proc. Natl. Acad. Sci. USA* **82**:6845-6849.
19. Kolter, R., and D. R. Helinski. 1978. Activity of the replication terminus of plasmid R6K in hybrid replicons in *Escherichia coli*. *J. Mol. Biol.* **124**:425-441.
20. Kupersztoch, Y. M., and D. R. Helinski. 1973. A catenated DNA molecule as an intermediate in the replication of the resistance transfer factor R6K in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **54**:1451-1459.
21. Langeveld, S. A., A. D. M. van Mansfeld, P. D. Baas, H. S. Jansz, G. A. van Arkel, and P. J. Weisbeek. 1978. Nucleotide sequence of the origin of replication in bacteriophage ϕ X174 RF DNA. *Nature (London)* **271**:417-420.
22. Louarn, J., J. Patte, and J. M. Louarn. 1977. Evidence for a fixed termination site of chromosome replication in *Escherichia coli*. *J. Mol. Biol.* **115**:295-314.
23. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1983. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* **101**:10-89.
25. Michel, B., and S. D. Ehrlich. 1986. Illegitimate recombination occurs between the replication origin of the plasmid pC194 and a progressing replication fork. *EMBO J.* **5**:3691-3696.
26. Monod, M., C. DeNoya, and D. Dubnau. 1986. Sequence and properties of pIM13, a macrolide-lincosamide-streptogramin B resistance plasmid from *Bacillus subtilis*. *J. Bacteriol.* **167**:138-147.
27. Noirot, P., M.-A. Petit, and S. D. Ehrlich. 1987. Plasmid replication stimulates DNA recombination in *Bacillus subtilis*. *J. Mol. Biol.* **196**:39-48.
28. Novick, R. P., G. K. Adler, S. Majumder, S. A. Khan, S.

- Carleton, W. D. Rosenblum, and S. Iordanescu. 1982. Coding sequence for the pT181 *repC* product: a plasmid-coded protein uniquely required for replication. *Proc. Natl. Acad. Sci. USA* **79**:4108–4112.
29. Novick, R. P., G. K. Adler, S. J. Projan, S. Carleton, S. K. Highlander, A. Gruss, S. A. Khan, and S. Iordanescu. 1984. Control of pT181 replication. I. The pT181 copy control function acts by inhibiting the synthesis of a replication protein. *EMBO J.* **3**:2399–2405.
30. Novick, R. P., I. Edelman, and S. Lofdahl. 1986. Small *Staphylococcus aureus* plasmids are transduced as linear multimers that are formed and resolved by replicative processes. *J. Mol. Biol.* **192**:209–220.
31. Novick, R. P., E. Murphy, T. J. Gryczan, E. Baron, and I. Edelman. 1979. Penicillinase plasmids of *Staphylococcus aureus*. Restriction deletion maps. *Plasmid* **2**:109–129.
32. Novick, R. P., K. Smith, R. J. Sheehy, and E. Murphy. 1973. A catenated intermediate in plasmid replication. *Biochem. Biophys. Res. Commun.* **54**:1460–1469.
33. Projan, S. J., S. Carleton, and R. P. Novick. 1983. Determination of plasmid copy number by fluorescence densitometry. *Plasmid* **9**:182–190.
34. Projan, S. J., J. Kornblum, S. L. Moghazeh, I. Edelman, M. L. Gennaro, and R. P. Novick. 1985. Comparative sequence and functional analysis of pT181 and pC221, cognate plasmid replicons from *Staphylococcus aureus*. *Mol. Gen. Genet.* **199**:452–464.
35. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
36. te Riele, H., B. Michel, and S. D. Ehrlich. 1986. Single-stranded plasmid DNA in *Bacillus subtilis* and *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. USA* **83**:2541–2545.
37. van der Ende, A., R. Teertstra, and P. J. Weisbeek. 1982. Initiation and termination of the bacteriophage ϕ X174 rolling circle DNA replication *in vivo*: packaging of plasmid single-stranded DNA into bacteriophage ϕ X174 coats. *Nucleic Acids Res.* **10**:6849–6863.
38. Wilson, C. R., S. E. Skinner, and W. V. Shaw. 1981. Analysis of two chloramphenicol resistance plasmids from *Staphylococcus aureus*. *Plasmid* **5**:245–258.