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

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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 fl (2) and φX174 (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 replication origin 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.

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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 CiaI and AsuII sites at which TaqI fragments can be cloned and carries the genes ermC and cat, which confer resistance to erythromycin (Em') and chloramphenicol (Cm'), 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 cis but also the presence of 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 the 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.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Phenotype or genotype</th>
<th>Derivation</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>pT181</td>
<td>Te' Inc3</td>
<td>Naturally occurring</td>
<td>15</td>
</tr>
<tr>
<td>pC221</td>
<td>Cm' Inc4</td>
<td>Naturally occurring</td>
<td>34</td>
</tr>
<tr>
<td>pRN5101</td>
<td>Em' Inc11 Tsr</td>
<td>Tsr mutant of pE194</td>
<td>29</td>
</tr>
<tr>
<td>pRN6321</td>
<td>Em' Inc11 Tsr; TaqI-C deleted</td>
<td>Deletion of TaqI C fragment from pRN5101</td>
<td>29</td>
</tr>
<tr>
<td>pE5</td>
<td>Em' Inc12</td>
<td>Naturally occurring</td>
<td>26</td>
</tr>
<tr>
<td>pSA0382</td>
<td>Te' Inc3 cop-652; HindIII-C deleted</td>
<td>Deletion of HindIII C fragment from pSA0342, a pT181 Cop mutant</td>
<td>12</td>
</tr>
<tr>
<td>pSA3320</td>
<td>Te' Inc3 RepD' ori-pC221'</td>
<td>In vitro cointegrate between pUB110 and a pT181 derivative with an in vitro substitution inactivating the origin</td>
<td>This study</td>
</tr>
<tr>
<td>pSA6470</td>
<td>Km' Te' Inc3 RepC' ori-pT181'</td>
<td>In vitro cointegrate between pUB110 and a pC221 derivative with a substituted pT181 origin</td>
<td>This study</td>
</tr>
<tr>
<td>pSA7420</td>
<td>Km' Inc13 RepD' ori-pC221'</td>
<td>In vitro cointegrate between pUB110 and a pC221 derivative with a substituted pT181 origin</td>
<td>This study</td>
</tr>
<tr>
<td>pSA7600</td>
<td>Em' Cm' Inc11</td>
<td>See Fig. 1</td>
<td>This study</td>
</tr>
<tr>
<td>pSA7650</td>
<td>Em' Cm' Inc11</td>
<td>See Fig. 1</td>
<td>This study</td>
</tr>
</tbody>
</table>

TABLE 1. Plasmids used in this study
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 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 *AsuII* 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 or the other of the DNA segments delimited by the two origins (Fig. 2). One of the new plasmids would confer Cm" and would carry the pE194 replicon and its *palA* sequence; the second one would express Em" and would contain the *palA* from pE5. The two derivative plasmids would be mutually incompatible. At a permissive temperature, the Cm" component would be expected to have a significant advantage over the Em" 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" component was maintained at a lower copy number than the Cm" one, while at a high temperature the Em" component was preponderant (compare lanes 2a and 2b in Fig. 3). The Cm" and Em" 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 Em" plasmids replicated independently at the permissive temperature for the Ts* Em" pE194 replicon but not at the restrictive temperature, while the Em" 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

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**TABLE 2. Double-origin hybrids**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Plasmid</th>
<th>Origin cloned at*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AsuII</td>
</tr>
<tr>
<td>pSA7600</td>
<td>pSA7621</td>
<td>ori-pT181 I</td>
</tr>
<tr>
<td></td>
<td>pSA7622</td>
<td>ori-pT181 I</td>
</tr>
<tr>
<td></td>
<td>pSA7644</td>
<td>ori-pC221 I</td>
</tr>
<tr>
<td></td>
<td>pSA7633</td>
<td>ori-pC221 I</td>
</tr>
<tr>
<td>pSA7650</td>
<td>pSA7655</td>
<td>ori-pT181 I</td>
</tr>
<tr>
<td></td>
<td>pSA7657</td>
<td>ori-pT181 I</td>
</tr>
</tbody>
</table>

* 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.
an Em′ 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′ 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′ 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′ 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′ 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′ 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′ derivative plasmids. The reason for the maintenance of the Cm′ derivative in a lower copy number than the original hybrid or the Em′ 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′ components carried a functional origin with pC221 specificity, while the Em′ 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...
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 homolo-
gous 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 dissocia-
tion 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 seg-
ment 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 exper-
niment 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.

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.

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 Emr derivative was obtained, although the other potential component (CmR) 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 concatamers 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 pAI 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; 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 T1 (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 pC314, 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 4X174 (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 Rep C 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.

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LITERATURE CITED


