Volume 16 Number 19 1988 Nucleic Acids Research

Functional analysis of the leading strand replication origin of plasmid pUB110 in *Bacillus subtilis*

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Received July 22, 1988; Revised and Accepted August 30, 1988

Abstract: Supercoiled plasmid DNA is the substrate for initiation of pUB110 replication, and - by inference - for binding of its initiator protein (RepU) to the plasmid replication origin (oriU) in vivo. No hairpin structure is required for RepU-oriU recognition. RepH (the pC194 replication initiation protein) failed to initiate replication in trans at oriU.

The nucleotides that determine the specificity of the replication initiation process are located within oriU but termination is unefficient. Therefore the segment that forms the full recognition signal for termination is probably located 3' of the oriU recognition sequence. Two overlapping domains, one for initiation and one required for termination, compose the leading strand replication origin of plasmid pUB110.

INTRODUCTION

pUB110 is a 4.5 kb multicopy plasmid carrying neomycin (kanamycin) and phleomycin (bleomycin) resistance determinants (1, 2). The replication of pUB110 depends on a plasmid-encoded effector (3). It has been shown that pUB110 like the other Gram-positive plasmids pC194, pE194, pT181 exists intracellularly as double-stranded (dsDNA) and single-stranded ssDNA circles (4). The pUB110 copy number, determined for exponentially growing Bacillus subtilis rec^+ cells, is about 46 to 50 copies of the dsDNA form and 2 to 3 copies of the ssDNA form per cell (5). We have identified a 1.2 kb pUB110 segment that is necessary and sufficient for autonomous plasmid replication in B. subtilis rec^+ cells (3). This region can be divided into three functionally discrete segments: i) a 24 bp region that acts as the plasmid replication origin (oriU), ii) the 949 bp determinant of the essential replication initiation protein, repU, and iii) a 358 bp incompatibility region, incA, which overlaps with the repU gene (3). Outside the previously defined minimal replicon we have

identified an asymmetric 140 nucleotide region which functions as a lagging strand origin (oriL) and which is required for normal replication (5, 6). Analysis of plasmids lacking oriL suggests that pUB110 replicates via a rolling-circle mechanism with alternative mechanisms for priming lagging strand synthesis (5, 7).

We have previously shown that the RepU protein forms a specific complex with a pUB110 DNA fragment that contains the oriU region, which can then be trapped on nitrocellulose filters (3). In our assay, the binding occured with linear DNA. However, we would expect that the superhelical form is the natural substrate for RepU recognition.

Recently, it has been shown that the Staphylococcus aureus/ B. subtilis plasmids pT181 and pC194 also replicate via a rolling-circle mechanism (8, 6, 9). The latter plasmid is thought to belong to a family of plasmids of which pUB110 is the prototype. In vitro replication of plasmids pT181 and pC194 is only marginally inhibited by the DNA gyrase inhibitor novobiocin (10, 11, 12).

DNA gyrase is an ATP-dependent type II topoisomerase that is capable of introducing negative supercoils into relaxed, covalently-closed double-stranded DNA circles and removing them from covalently-closed double-stranded negatively supercoiled (CCC) DNA circles (13). The replication of CCC plasmid DNA following the theta mode of replication requires the elimination of torsional stress by the action of DNA gyrase. Very little information, however, is available about plasmids that replicate via a rolling circle mechanism as pUB110 in vivo.

In this report two questions are addressed: (a) what are the in vivo topological requirements for RepU-oriU recognition in B. subtilis and (b) do initiation and termination of leading strand DNA synthesis require identical domains?

MATERIALS AND METHODS

Strains and plasmids. Bacillus subtilis strains: YB886 (trpC2 metB5 amyE xin-1 attSPβ) and the recombination-deficient derivatives YB1015 (recE4) YB965 (polA5) and BG125 (addA5) were used (6).

The naturally occurring plasmid pUB110 (1) and the previously described plasmids pBG61, pBT33, pBT82, pBT86, pBD95(Ts), pBT88 (3) and pBT68 (14) have been used.

Transformation and DNA techniques. The method of Rottländer and Trautner (15) was used for transforming B.subtilis competent cells.

Plasmid DNAs were prepared on preparative and analytical scales as previously reported (16). Crude DNA lysates were prepared from exponentially growing cells essentially as described previously (6).

DNA sequencing was performed essentially as described by Sanger et al. (17) but the temperature of the polymerization reaction was varied from 28°C to 50°C.

Agarose DNA electrophoresis was carried out in a horizontal slab gel containing 0.7% (w/v) agarose, 0.4 μg ethidium bromide/ml and 50 mM-Tris-acetate (pH 8.0), 10 mM-EDTA was used as the electrophoresis buffer. Analysis of product topology was carried out by nicking the plasmids with DNAase I. Nicking reaction contain 2μg/ml DNAase I, 400μg/ml ethidiun bromide, and 0.3μg of precipitated DNA. After 15 min at room temperature the DNAs were separed by electrophoresis.

Southern hybridization, determination of the relative plasmid copy numbers and replication rates. For Southern analysis, the crude DNA lysates were separated by gel electrophoresis in 0.7% agarose (see above). When necessary, two gels were loaded with identical samples and run in parallel, and then transferred to GeneScreen nylon membranes (NEN Research products), either with or without prior depurination and denaturation, essentially as described previously (6). In non-denaturing conditions, only single-stranded DNA can be retained on the membrane. Twice CsCl-ethidium bromide purified and nick-translated pUB110 DNA was used as a probe.

Relative plasmid copy number and the replication rates were determined as previously described (3). Quantitative scans were integrated by using the LKB GelScan XL softward package.

For measuring the effect of novobiocin (Nv) the cells were grown at 37°C to OD_{560} = 0.1 (time zero). Aliquots of equal volume were removed at the time of addition (zero) and after 10,

30, 60 and 120 min, and crude DNA lysates were prepared as reported previously (6). A final Nv concentration of 10 μg/ml was chosen in order to block 99 % of the desired target (DNA synthesis), without substancial side effects.

Computer analysis. DNA secondary structures were predicted by using the computer software package of the University of Wisconsin Genetics Computer Group in a Vax computer (18).

RESULTS AND DISCUSSION.

Does pUB110 replication require supercoiled DNA ?

Maciag et al. (3) had defined the minimal region [coordinates 3118-4316 of McKenzie et al. (19)] that is necessary and sufficient for autonomous plasmid replication in Rec$^+$ cells. The RepU-dependent replication origin, termed oriU, has been

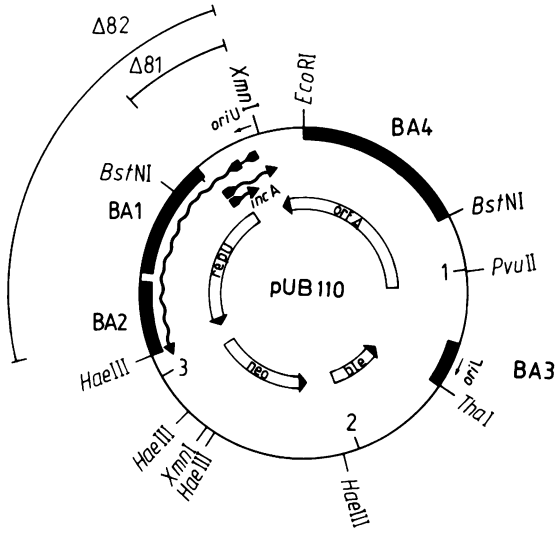

Fig. 1.
Physical structure of pUB110. The filled bars represent the membrane binding sites (BA), the wavy lines represent the putative RNA transcripts (repU and incA mRNAs). The arrows in the inner circle indicate the open reading frames (2, 3, 19). The small arrows outside the circle indicate the leading (oriU) and lagging (oriL) strand replication origins and the directionality of DNA synthesis. Nucleotide coordinates are given according to McKenzie et al. (19). Relevant restriction sites are depicted. Δ81 and Δ82 indicate the extension of the in vivo deletion in plasmids pBT81 and pBT82, respectively.

localized within a 24 bp segment at coordinates 4293-4316, upstream from the RepU coding region (see Fig. 1). Binding of RepU to the oriU region can be demonstrated by a filter binding assay (3) or by the retardation of electrophoretic mobility of restriction fragments carrying oriU. The normal mobility of those fragments is restored after incubation with KCl at a concentration greater than 260 mM or after treatment with SDS, suggesting a non-covalent association (3, Maciag and Alonso, unpublished).

The rate limiting protein, RepU, nicks a single strand of the DNA, within the oriU region, and replication proceeds by the rolling circle mechanism (3, 5, 7) formally analogous to that of bacteriophage ΦX174 (20).

Recently it was shown that in vitro replication of the plasmids pT181 and pC194 is inhibited only marginally by the DNA gyrase inhibitor novobiocin (10, 11, 12). From this observation

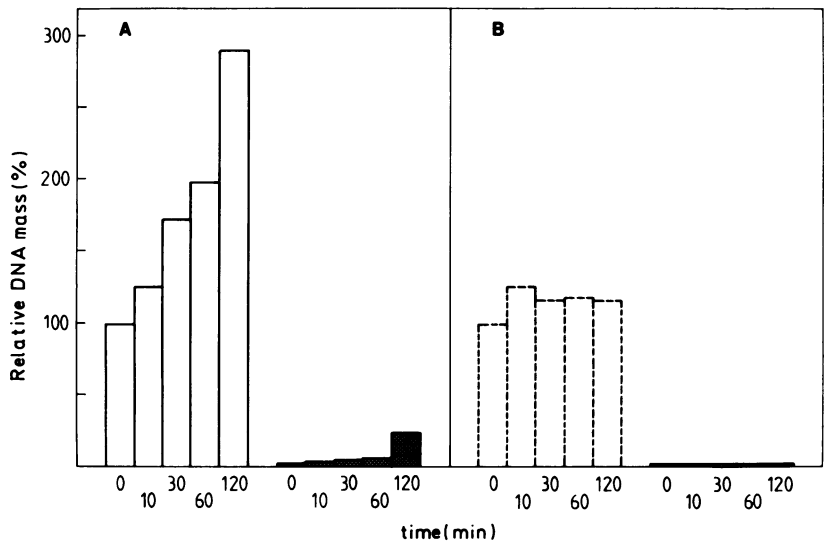

Fig. 2.
Time course of the relative DNA mass in the absence (A) or after novobiocin addition (B). Samples were withdrawn at time zero (the time of addition of the antibiotic) and after 10, 30, 60 and 120 min. The intensities of the DNA bands present at time zero (0) were set to 100 %. Open bars represent pUB110 dsDNA and stippled bars, ssDNA. Coordinates are shown only for plasmid pUB110.

we could assume that after one round of rolling circle replication, two unwound newly replicated molecules should be generated. However, two questions remain open: (a) are those newly replicated molecules catenated dimers or isolated monomeric rings? and, (b) are these relaxed molecules a substrate for initiation of plasmid replication? We have analyzed the situation by studying plasmid replication in the presence of novobiocin.

Sarachu et al. (21) had shown that 5 - 10 μg/ml of novobiocin (Nv) blocks DNA synthesis of SPO1-infected Bacillus subtilis cells whereas total RNA and protein syntheses are only inhibited 30 to 50%. Furthermore, it was shown that novobiocin (which blocks the ATP-dependent underwinding of DNA) affects DNA synthesis immediately upon addition (21).

As shown in Figure 2A, the amount of pUB110 dsDNA increased about 2.8- fold during 120 min. The addition of Nv 10 μg/ml (16μM) caused a marked decrease in the generation of pUB110 dsDNA form (Fig. 2B). After a 10 min period following the addition of Nv, the amount of dsDNA increased 1.3- fold like in the absence of Nv and this rise is statistically significant. The amount of dsDNA remained nearly constant at later times. On the other hand, the generation of ssDNA is blocked immediately after the addition of Nv. We failed to observe accumulation of catenated DNA dimers after Nv addition (data not shown).

Sheehy and Novick (22) have shown that the replication of plasmid pI258 in Staphylococcus aureus takes about 5 min. If this time is similar for B. subtilis, about 20% of the pUB110 molecules will be replicating at any moment in an unsynchronized culture, with cells growing with a mean generation time of 28 min. If initiation of plasmid replication is blocked, plasmid synthesis will stop after an increase of about 30%. The results described here, if Nv inhibits initiation, are consistant with these calculations.

From these results, we can infer that i) the in vivo replication of pUB110 requires an active DNA gyrase for initiation of replication, ii) elongation and termination of pUB110 replication seem to be insensitive to Nv and iii) two monomeric rings are formed after termination of the newly replicated molecules, as verified by our failure to observe

catenated DNA dimers. This finding also suggests that theta replication does not take place and that RepU like RepC of plasmid pT181 might have a topoisomerase-like activity (23).

The contradictory finding between the present *in vivo* and *in vitro* (see above) requirements for an active DNA gyrase can be reconciled, assuming that once the RepU protein has bound to its target (*oriU*), plasmid replication becomes insensitive to DNA gyrase inhibitors like Nv. Consistant with this hypothesis are the requirements of the streptococcal plasmid pLS1 for *in vitro* replication (24). The authors have shown that, in a plasmid-free extract, pLS1 replication is highly sensitive to Nv (24); whereas endogeneous plasmid replication, is inhibited by Nv to about 50 % in a plasmid-containing extract (delSolar and Espinosa, personal communication).

The question remains whether the topology of the template itself activates RepU recognition, or whether DNA gyrase unwinds the template and exposes it to action by DNA helicase and DNA polymerase III holoenzyme (see 25).

Secondary structure at the replication origin

Recently, it was hypothesized for pT181 and pC194 that a transient hairpin structure may exist and act as a recognition signal for initiation of plasmid replication (8, 9) by the plasmid-encoded RepC and RepH proteins, respectively (10, 14).

Two regions of hyphenated dyad symmetry of twelve (palindrome I) and five (palindrome II) base pairs can form hairpins within the pUB110 replication origin region (Fig. 3). These hairpins are imbedded in an 71 % A + T region and by standard thermodynamic calculations have free energies of formation of -46 kJ and -5 kJ per mol (26). Furthermore, if local melting to permit a fast extrusion of these structures can occur, the high A + T content would lower the energy required to separate the strands (see 27). Once the strands are separated, the stability of the hairpin would depend on the length and base composition of both the stem and the loop.

Are those predicted hairpins actually formed *in vivo*? In the absence of an *in vivo* assay in *B. subtilis*, as recently reported for *E. coli* (28), we took advantage of the fact that during traversal of hairpin templates, the large fragment of DNA

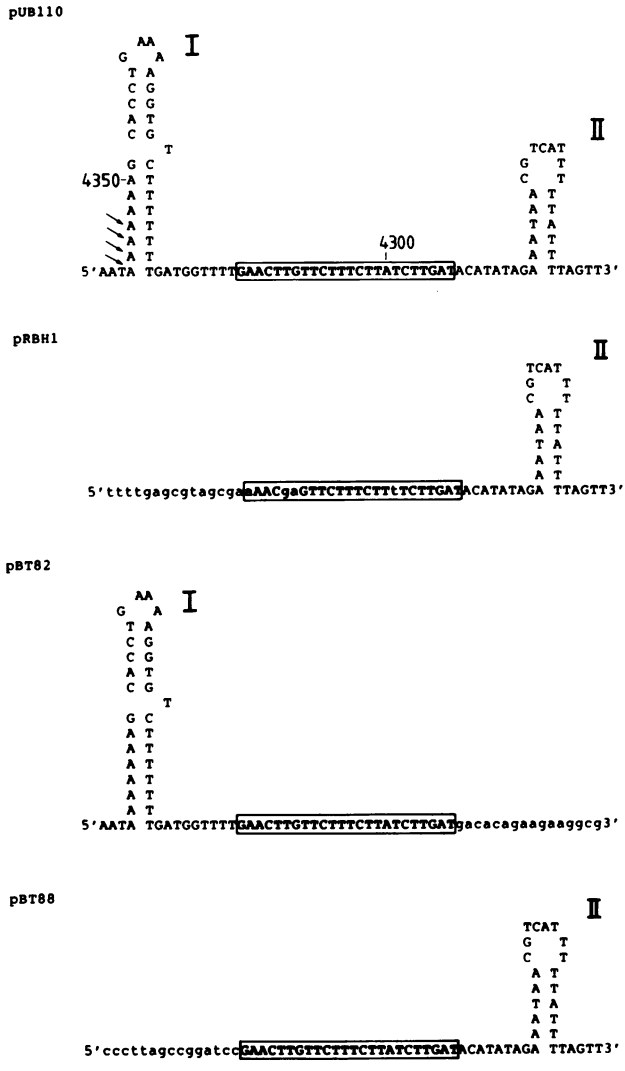

Fig. 3.
Hairpin structures in the oriU origin region. The potential palindromes I and II in pUB110, pRBH1 and pUB110 derivative plasmids are indicated. The boxes denote the oriU DNA sequence, the lower case bases corresponding to divergences with the pUB110 DNA sequence. The arrows, at the palindrome I, indicate the position of DNA polymerase I pausing.

polymerase I (Klenow enzyme) terminates just at the branch point of the stem in vitro. To test if palindromes I and II (see Fig. 3) may show this effect, the 294 bp DraI fragment [coordinates

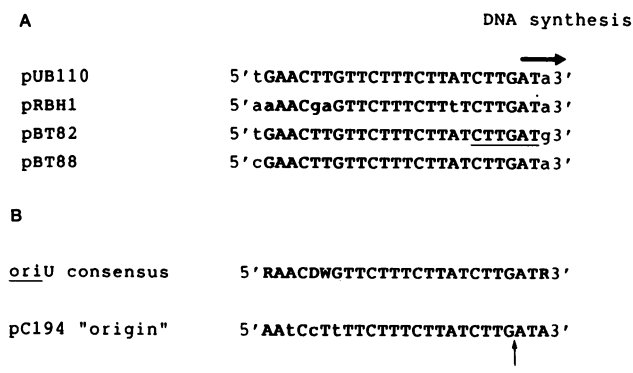

Fig. 4.
DNA sequence at the plasmid replication origin region. (A) The oriU region of pUB110 aligned with pRBH1 and pUB110 derivatives. Bold case letters indicate the oriU DNA sequence, in lower bold case bases divergent and lower case sequence outside oriU. The minimal termination origin is underlined for plasmid pBT82. The arrow indicates the directionality of DNA synthesis. (B) The oriU consensus sequence aligned with the pC194 "origin" sequence. The postulated nicking site is indicated by a vertical arrow. R = purine, W= adenine or thymidine and D= no cytosine (UIB).

4221-4515 of McKenzie et al. (19)] was cloned into M13 mp18, and primer extension by the Klenow enzyme was performed at various temperatures of incubation. The product of the polymerization reaction was then analyzed on a sequencing gel. For palindrome I, the position of the last nucleotides that can be properly used as template at 28°C are located at the base of the stem of the palindrome I (coordinates 4356-4354 of the pUB110 DNA sequence, see Fig. 3). This pausing effect, was reversed when the temperature of incubation was increased to 40°C. In contrast to palindrome I, the palindrome II was traversed, at any tested temperature, by the Klenow enzyme without apparent termination.

Does RepU-oriU interaction require a hairpin structure in the region of the replication origin? To test this hypothesis, we compared the oriU region of pUB110 with that of the pUB110-like plasmid pRBH1 (29) with pBT82 which is an in vivo deleted plasmid for palindrome II and with plasmid pBT88 which has been deleted in vitro for palindrome I (see 3, Fig.3).

The 12 base pair hairpin structure (palindrome I) is absent in plasmid pRBH1 which is, however, maintained in B. subtilis with the same copy number as pUB110 [43 and 47 copies per cell,

respectively (6, 29)]. Hence we assume that palindome I is dispensable for plasmid replication. Identical results were obtained with plasmid pBT88.

The function of palindrome II might be inferred from plasmid pBT82. In this plasmid palindrome II (rightward from the oriU sequence) is absent but it still can be replicated in B. subtilis. However a high accumulation of multimers (up to 16mer) was observed (see Fig. 5).

From these data we can conclude that: i) the palindrome I may be form in vivo, since in vitro the primer extension by Klenow enzyme is impaired at low temperatures, ii) palindromic DNA sequences by themselves are not recognized as signals for initiation of pUB110 replication, iii) the absence of palindrome I does not affect plasmid replication, iv) the palindrome II seems not to be required for initiation of plasmid replication and v) DNA sequence divergence leftward of the 24 bp oriU does not affect plasmid copy number.

Does pC194 replication initiation protein, RepH, act on the pUB110 replication origin?

Figure 4A shows the 24 bp oriU DNA sequence and the base substitutions within the oriU region. The nucleotide sequence of the pUB110 minimal replicon differs from that of pRBH1 by only five bases, four of those being located within the oriU region (oriU substitutions) and the fifth one, being located in the untranslated region of the repU gene (19, 29, and Fig. 3). pRBH1 is in all respects indistinguishable from pUB110. The fact that within oriU region nucleotides changes can occur indicates that not all the nucleotides of oriU are essential for replication.

Plasmids pUB110 and pC194, although belonging to different incompatibility groups, share a high degree of homology both within their replication origins (17 bp of homology out of 24bp, see Fig. 4B, 14, 9), at the Rep protein level (3), and they have similar overall functional organization at the replication level (3, 14).

In the case of a double-origin hybrid plasmid (see 30) it has recently been shown (9) that a round of replication initiated, "by a single-strand nick and 3' extension", at one of the origins (pC194 replication origin) might terminate at the other origin (pUB110 replication origin). It was concluded that the pC194

Table 1. Plating efficiency of recE4 cells carrying plasmid pBT86 or pBD95(Ts) in the presence or absence of a plasmid helper (pBT68 and pBG61).

plasmid[a]	temperature °C	Chloramphenicol resistant colonies per ml
pBD95(Ts)	42	$< 1.0 \times 10^3$
pBT86	30	2.1×10^8
pBT86	42	$< 1.0 \times 10^3$
pBT86[b]	42	$< 1.0 \times 10^3$
pBT86[c]	42	6.1×10^7

([a]) plasmid pBD95(Ts) and its derivative pBT86 are temperature sensitive for replication. ([b]) in the presence of a pC194 derivative plasmid (pBT68) and ([c]) in the presence of a pUB110 derivative plasmid (pBG61).

replication initiation protein, RepH (see 14) recognizes and terminates leading strand synthesis within the pUB110 replication origin region (9).

This observation raises the question of the interchangeability of the plasmid replication proteins. In other words, is the RepH protein of pC194 able to initiate plasmid replication at oriU ? It was shown that RepU, in an trans-complementation test, can activate a pUB110 replication origin present in pBT86, a thermosensitive replicon derived from pBD95(Ts) (3, Table 1). No activation, at the non-permissive temperature, of the cloned oriU region (plasmid pBT86) could be detected when the source of the replication protein was pC194 (see Table 1). Furthermore, the pUB110 RepU protein was not able to drive pC194 replication either *in vivo* or *in vitro* (3). Because, RepH can terminate leading strand synthesis of replication rounds initiated at the pC194 origin region, within 18 bp of the pUB110 replication region (30, 9), but RepH does not initiate plasmid replication at oriU we assume that there is not a complete overlap between the initiation and termination specific DNA sequences (see below).

From these data we can infer that oriU may be composed of two overlapping but distinct domains, one for initiation, 24 bp long (recognition domain) and the other 4 to 18 bp long (see below), for termination of leading strand synthesis.

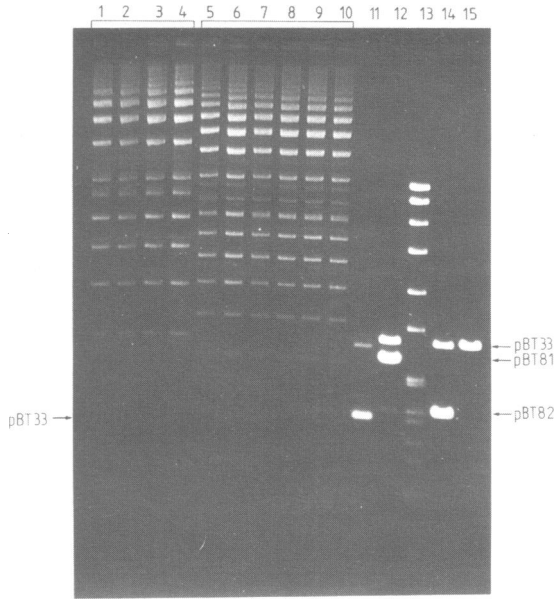

Fig. 5.
Electrophoretic analysis of the pBT33 derivatives in the addA5 genetic background. Individual transformants were inoculated and plasmid DNA was purified from each culture. Lanes 1 to 4 show heteroplasmid pBT81-pBT33, lanes 5 to 10 heteroplasmid pBT82-pBT33 and lane 11 plasmid pBT33. In lanes 12 a pBT81-pBT33 DNA digested with BglII, in lane 13 bacteriophage SPP1 DNA digested with EcoRI, in lane 14 and 15 heteroplasmid pBT82-pBT33 and pBT33 digested with BglII were shown.

Do initiation and termination of pUB110 leading strand synthesis show the same recognition target?

Recently, we have hypothesized that pC194 plasmid replication termination requires less sequence specificity than the replication initiation of the supercoiled plasmid form (see 31). This hypothesis was supported by the fact that rounds of replication initiated by RepH at its specific target (the pC194 replication origin) could terminate, with a subsequent reinitiation, at a 4 to 18 bp DNA sequence, that resemble the pC194 replication origin (see 30 - 32). In this work we extended the above hypothesis to plasmid pUB110. Basically, when a pUB110 derivative, which lacks the major lagging strand origin, pBT33, is introduced into the ATP-dependent DNase (addA5) mutant strain (see below), big and small transformant colonies were observed

(5). DNA preparations of ten big colonies were analyzed by gel electrophoresis, and in addition to pBT33 a second plasmid was observed in each transformed clone. Those plasmids accumulate a high amount of oligomeric plasmids forms and they can be grouped in two classes (see Fig. 5). Six of these were indistinguishable from the previously characterized and sequenced plasmid pBT82 (3). The remaining four are indistinguishable from each other and were termed pBT81 (Fig. 5). When a representative of each plasmid DNA is digested with restriction enzymes that cut DNA once all the DNA migrated with a mobility identical to that of the linear form of the plasmids (see Fig. 5). These results indicate that the plasmid DNA contained concatemeric molecules with the orientation of a head-to-tail configuration and that the heteroplasmid strain pBT81-pBT33 or pBT82-pBT33 bear two independent plasmid molecules.

Plasmid pBT81 contains a 316 bp deletion which has been mapped within the BstNI-XmnI restriction sites [coordinates 3871-4311 of McKenzie et al. (19)] when compared with the parental plasmid pBT33 (data not shown). pBT81 shares in commmon, with pBT82, the deletion end point that corresponds to the oriU region. In both plasmids a DNA segment coding for the incA and repU determinats is missing. Therefore they are virtually run-away replication plasmids and require the parental pBT33 as a helper.

Two questions remain open. First, how does deletion formation take place and second, why are the multimeric plasmid forms not resolved to monomers (see below)? A general assumption is that: (a) the specificity of initiation of pUB110 replication is due to an association of the RepU protein with oriU (non-covalent, see above), (b) after a single strand nick within oriU, the protein becomes covalently attached to the generated 5'-end (see 23), (c) the 5'-end of the displaced strand may travel along with the replication fork, and (d) aberrant termination with a subsequent reinitiation (31) may occur when certain host functions are exhausted. Since those aberrant terminations require DNA homology but no regions of dyad symmetry (see 31, this work) we conclude that hairpin structures are not required for termination of leading strand synthesis (see above).

We failed to observe both deleted plasmid forms, but the rationale to explain the generation of plasmids with proximal deletions is that i) plasmid replication initiated at oriU could terminate within short boxes of perfect homology with the 3' end of the oriU DNA sequence (underlined in figure 4A for plasmid pBT82) and ii) a subsequent reinitiation will finish now at oriU. Since, (a) a high amount of single-stranded linear and monomeric circular DNA forms (about 60 % of the total plasmid DNA) is accumulated by plasmid pBT33 in the addA5 mutant strains (see 5, 6), (b) single-stranded DNA is always complexed with a DNA-binding protein (as the E. coli SSB) and (c) there is, at least for E. coli, sufficient SSB protein in the cell to cover about 80 kb of single-stranded DNA (33), we hypothesized that plasmid pBT33 (which is 2.5 kb in size and has 45 copies per cell) may exhaust the SSB pool. In the absence of SSB proteins, RepU could cleave additional sites in vivo. This hypothesis is supported by the fact that, in vitro, the gene A protein of bacteriophage ΦX174 (34) and the RepC protein of plasmid pT181 (35) recognize additional cleavage sites in the absence of SSB protein.

The control mechanism of replication, in the heteroplasmid strain bearing either pBT81 and pBT33 or pBT82 and pBT33, is only provided by plasmid pBT33 (see above). Because the overall initiation rate in the strain containing pBT33 only is indistinguishable from the above heteroplasmid condition (data not shown) we conclude that i) RepU recognition site is located upstream of the deletion endjoint, ii) initiation of plasmid replication take place at a normal rate, and iii) termination is markedly affected when a DNA segment downstream of oriL is missing.

Are the oligomers formed in pBT82 recombination- or plasmid replication- dependent?

When B. subtilis wild type strain was transformed with plasmid pBT33 with low frequency a plasmid indistinguishable from pBT81 or pBT82 has been observed (5). Furthermore, when the wild type strain is co-transformed with either (pBT81-pBT33) or (pBT82-pBT33) DNAs a ladder of oligomeric plasmid forms indistinguishable of those presented in Fig. 5 was observed.

By electron microscopic analysis, we have found that the majority of the oligomeric forms of plasmid pBT81 (up to 15mers)

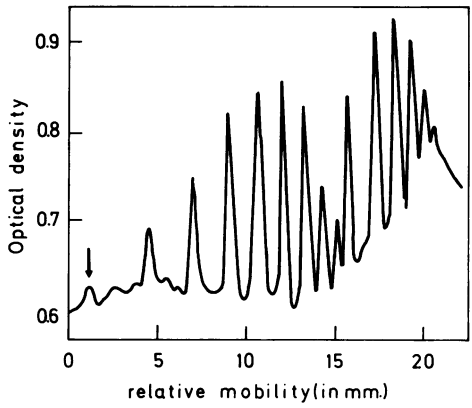

Fig. 6.
Quantitative analysis of pBT82 oligomeric forms. The peaks from left to right correspond to monomers, dimers etc. The amount of plasmid DNA measured as the intensity of the signal in the photographed negative is expressed in arbitrary units.

or pBT82 (up to 16mers) shown in Figure 5 are true multimers without catenated, 8-shaped molecules or knotted structures (5, Viret and Alonso unpublished). However, the presence of open circular forms render difficult the identification of multimers. Therefore plasmid DNA has been nicked with DNAase I in the presence of ethidium bromide and subsequently separated by agarose gel electrophoresis. Comparing these profiles with the ones of the untreated samples revealed that only a minor fraction of open circular particles and no catenate forms are present in the latter.

To quantify the rate of oligomerization we photographed the gels at a variety of exposures, and negatives were scanned. In Figure 6 the oligomeric profile of plasmid pBT82 is shown. pBT82 is a 1.5 kb non-autonomously replicating plasmid. One could explain oligomer formation by assuming that RepU requires a given plasmid size for an efficient cleavage and the size of the wild type pUB110 (4.5 kb) being optimal. A preferential resolution after reaching a plasmid size (3mers) that could correspond in size to the monomeric pUB110 has been observed in the polA5 strain (Leonhardt and Alonso, unpublished) but neither in the wild-type nor in the addA5 genetic backgrounds.

Obviously sequences downstream of oriU are required for

proper termination. The absence of these sequences reduces the efficiency of termination and leads to the generation of multimers. These multimers are not randomly distributed and show a Gaussian distribution with a maximum at 5mers and 10mers either in the wild-type or in the addA5 strains. This particular distribution could be explained as follows: the efficiency of termination in pBT82 is reduced to a probability of about 0.2 which would generate a multimer distribution with a maximum for 5mers. Whereas the second subpopulation (maximum for 10 mers) could be generated either if one of these multimers becomes a substrate for replication initiation or via intramolecular recombination. Since the second maximum at 10 mers is absent in the recE4 strain we favour the latter hypothesis.

We can assume that within the missing 316 bp in pBT81 [between oriU and the BA1 region (36, see Fig. 1)] a protein(s) may bind and provide additional information in order to assure termination. However, at present we cannot rule out that the base divergency at the 3' side of oriU (see Fig. 3) may account for the unefficient termination. Since plasmids pBT81 and pBT82 are present (in the wild-type strain) mainly as oligomers and their nucleotide sequence 3' end of oriU is different; we assume that RepU requires additional information in order to assure that termination by cleavage at oriU occurs. Indeed, by affecting the lagging strand gap filling reaction by DNA polymerase I (see 20) trimers are preferentially accumulated (see above). It is tempting to assume that the primosome may provide this "additional information" and by using the leaky polA5 mutant strain (see 37) this complex could became more stable.

Several lines of evidence suggest that pBT81 and pBT82 oligomeric formation is not induced either by homologous recombination or by site-specific rec-independent, replication-dependent recombination. First, it has been shown that none of the B. subtilis recombination-deficient strains is affected in plasmid interconversion from monomers to oligomers (38). Second, it has been observed in E. coli that recombination-induced oligomer formation from monomeric plasmids require the functions of the recB and recC genes (39). However, in B. subtilis pBT81 and pBT82 oligomeric formation takes place in the addA5 genetic background. The addA5 mutant strain is impaired in exonuclease V

activity, which is the B. subtilis counterpart to the E. coli RecBCD protein (see 37). Third, if the observed oligomers of pBT81 and pBT82 should be generated by intermolecular recombination, cointegrates between the parental pBT33 and pBT81 or pBT82 should be formed as well, which was however not visible in ordinary agarose gels. Finally, a single strand 5' terminus of an oligomeric replicating molecule might be capable of site-specific recombination by ligating its end with a 3' oriU end of another oligomeric replicating molecule as suggested for oriT mediated recombination (40). Contrary to oriT recombination, the pBT82 oligomerization process is not reversible: oligomers were not resolved to monomers on re-transfer in any genetic background tested so far.

CONCLUSIONS

Covalently closed, double-stranded, negatively supercoiled (CCC) pUB110 DNA circles are required for initiation of plasmid replication. In other words, CCC plasmid DNA is the in vivo substrate for oriU-RepU interaction.

Palindrome I within the origin region presumably assumes a cruciform structure but no transient hairpins seem to be required for initiation of pUB110 replication.

Initiation, but not termination, of replication by RepU protein is a pUB110 specific process. This could explain (a) our failure to show RepH initiation at oriU and, (b) that the pUB110 replication origin (oriU) or an 18 bp mini-derivative can function as a signal for termination of rounds of replication initiated at the pC194 replication origin by RepH (30, 9).

A DNA sequence comparison of the oriU region of plasmids pUB110 and pBT82 (see Fig. 3) allows the conclusion that the RepU recognition site (binding domain?) is located at the 5'-end of oriU (Fig. 3 and 4A), whereas the functinal termination site (nicking domain?) is located the 3'-end of oriU and may require DNA sequences further downstream. These facts suggest that there is partial overlapping of the sequences required for initiation and termination of leading strand DNA synthesis, as reported for the bacteriophages f1 (41) and ΦX174 (42).

ACKNOWLEDGEMENT

We are grateful to T.A.Trautner for helpful discussions and critical reading of the manuscript. We thank J.-F. Viret for the communication of unpublished results.

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