Nucleic Acids Research, Vol. 20, No. 3 ***395–400***

Copy number control of the streptococcal plasmid pIP501 occurs at three levels

S.Brantl and D.Behnke*
Institute for Microbiology and Experimental Therapy, Beutenbergstraße 11, 0-6900 Jena, FRG

Received December 9, 1991; Revised and Accepted December 31, 1991 EMBL accession no. X62150

ABSTRACT

Transcriptional analysis of the replication region of plasmid pIP501 has revealed three active promoters. The *rep*R gene which is essential for pIP501 replication was transcribed from promoter p_{II}. A small antisense RNA (136 nt, RNAIII) generated from promoter p_{III} was complementary to the leader region of the *rep*R mRNA. Introduction of either point mutations or deletions into promoter p_{III} or RNAIII resulted in a 5–20fold increased plasmid copy number suggesting a negative regulatory function for RNAIII. The *cop*R gene, the complete DNA and amino acid sequence of which is reported, was dispensable for pIP501 replication. However, deletion of the *cop*R promoter p_I and/or the *cop*R coding sequence led to a 10–20fold increase in plasmid copy number. This effect was also observed when a –1 frameshift mutation was introduced into the CopR coding region. Mutations in *cop*R and p_{III}/RNAIII were not additive. It is, therefore, proposed that both components act at the same level of copy number control most likely in a sequential way. A second level of copy number control was found to involve an inverted repeat structure upstream of and overlapping with promoter p_{II}. Destruction of this repeat sequence by deletion caused an increase in copy number 2–3fold higher than that observed for either RNAIII or *cop*R mutations. A working model is proposed how different components of pIP501 interact to regulate its copy number.

INTRODUCTION

Maintenance of a plasmid in a bacterial cell requires the successful interaction of plasmid and host-encoded replication functions. The segregational stability of a plasmid may, thus, be severely influenced by the quality of this interaction. Plasmids that are stably propagated in a large range of bacterial hosts must have developed properties and regulatory mechanisms which ensure successful interaction with the peculiarities of the specific replication functions of different hosts. A molecular analysis of the regulatory strategies realized by such plasmids appears, therefore, to be particularly interesting.

A group of broad host range plasmids represented by pSM19035 (1, 2), pIP501 (3, 4) and pAMβ1 (5) has recently become attractive for use in *B. subtilis*. These plasmids which were originally isolated from various species of streptococci have subsequently been found to replicate in other Gram-positive hosts as well (6–11). In contrast to the frequently used cloning vectors derived from small staphylococcal replicons (12–14) these plasmids are segregationally stable and replicate via a theta-type mechanism (15, 16). Plasmids pSM19035, pIP501 and pAMβ1 which are incompatible share extensive similarities in the DNA sequence and organization of their replication regions (17–20). All plasmids code for an essential Rep protein and a smaller nonessential Cop protein. Analysis of the transcriptional units within the replication region of pIP501 both *in vitro* and *in vivo* has revealed the presence of three active promoters (Brantl et al., submitted). Two of them—promoters p_I and p_{II}—directed transcription of the *cop*R and *rep*R genes while a noncoding countertranscript of 136 nt in length (RNAIII) originated from the third promoter (p_{III}). This countertranscript had a high secondary structure potential and was complementary to a major part of the leader region of the *rep*R mRNA. RNAIII, thus, was likely to be a regulatory antisense RNA. Fig. 1 summarizes the transcriptional organization of the replication region of pIP501 and lists the DNA sequences of promoters p_I–p_{III}.

Here we present evidence that RNAIII in fact is a regulatory antisense RNA involved in copy number control of pIP501. In addition, the CopR protein was also found to influence the copy number of pIP501. Both CopR and RNAIII acted at the same control level. A second level of copy control was identified which involved an inverted repeat sequence upstream of promoter p_{II}.

MATERIALS AND METHODS

Bacterial strains and plasmids

The plasmids used in this study to construct new derivatives of pIP501 have previously been published (20). Plasmids were propagated in *B. subtilis* DB104 *his*, *npr*R2, *npr*E18, *apr*A3 (21) and *E. coli* strain TG1 and its *rec*A derivative TG2 (22) were used for subcloning and mutagenesis experiments.

* To whom correspondence should be addressed

DNA preparation and manipulation

The isolation of plasmid DNA from *B. subtilis* has been reported (20). DNA manipulations like restriction enzyme cleavage, ligation, filling-in reactions with Klenow fragment of DNA polymerase I or treatment with T4 DNA polymerase, exonuclease III and S1 were either carried out at the conditions specified by the manufacturer or according to standard protocols (22). Polymerase chain reactions were carried out with a GenAmp PCR Kit from Perkin Elmer/Cetus.

DNA sequencing of the *cop*R gene

A 3.2 kb KpnI/EcoRI fragment of plasmid pGB3631 comprising the missing 5'end of the *cop*R gene was cloned into pUC118. The sequence of the initial 300 nucleotides 5' of the KpnI site was determined by the dideoxy chain termination method (23) with the universal sequencing primer. The data were confirmed by sequencing the opposite strand with the help of a specific oligonucleotide primer. The *cop*R DNA sequence was analysed on a Macintosh IIci computer and MacMolly software.

Construction of mutant plasmids

Three basic plasmids were initially constructed which were subsequently used to supply components for various recloning steps and PCR experiments. A KpnI/EcoRI fragment of pUC118-F (20) encompassing the replication region of pIP501 except for the 5' end of the *cop*R gene was subcloned into the shuttle vector pBT48 (20) to yield plasmid pPR1 (Fig. 3). The 5' part of the *cop*R gene was separately cloned on plasmid pCOP2 (not shown) which was obtained by inserting a 765 bp HincII/KpnI fragment of plasmid pGB3631 into pUC19 cleaved with BamHI (filled-in) and KpnI. From this plasmid the 5' end of the *cop*R gene together with its expression signals could be recovered as a XbaI/KpnI fragment. Plasmid pCOP4 was constructed by inserting this XbaI/KpnI fragment into pPR1 thus restoring a functional *cop*R gene. Plasmid pCOP8 was generated by cleaving pCOP4 with KpnI and removing the 3' overhanging ends with T4 DNA polymerase. Deletion of a KpnI/HpaI fragment from pPRI yielded plasmid pPR2. Treatment of plasmid pUC118-F linearized with BamHI/PstI with exonuclease III and nuclease S1 followed by recloning of the truncated *rep*-fragments into pBT48 gave rise to plasmids pPR3/4. The deletion mutants pPR8, pPR11, pPR12 and pPR13 were all constructed by single PCR reactions using pUC118-F as a template, the reverse sequencing primer and the following mutagenic oligonucleotides:

5' GGGAGATCTGGATCCTGAATAAAGAATAC (pPR8)
5' GGTCTAGAGGATCCACGAAATCATTGC (pPR11)
5' GTCTAGAGGATCCTTGCTTATTTTT (pPR12)
5' GGTCTAGAGGATCCTTATTTTTTTAAAAAGCG (pPR13).

The amplified fragments were cleaved with BamHI and EcoRI and inserted into pBT48 linearized with the same pair of enzymes. Point mutations and deletions present on plasmids pPR5,6,7,9,10 were generated by a two step PCR reaction. Again pUC118-F served as the template. Initially one of the mutagenic oligonucleotides listed below and either the universal or the reverse sequencing primer were used to amplify the fragment proximal and distal to the deletions. In the second step the PCR products of the first reactions were joined and amplified as a single fragment by using the universal and reverse sequencing primer only. The following oligonucleotides were used:

5' GTAAACCCATTCTATCGGGTTT/ 5' AAACCCGATAGAATGGGTTT-AC (pPR5)
5' GAGGGGATTTAAAGTCTTTAAG/ 5' CTTAAAGACTTTAAATCCCC (pPR6)
5' ATATCTGTAAACCCTCGGGTTTTTGAGG/5' CCTCAAAAACCCGAG-GGTTTACAGATAT (pPR7)
5' CCCAAAATTTGGTGTCTTTAAGAAGAT/5' ATCTTCTTAAAGACACC-AAATTTTGGG (pPR9)
5' GAGCCACGACCAGGTCTTTAAGAAG/5' CTTCTTAAAGACCTGGT-CGTGGCTC (pPR10)

The amplified fragments were cleaved with BamHI and EcoRI and inserted into pBT48. Plasmids pCOP5-pCOP10 were then obtained by insertion of the XbaI/KpnI cassette from pCOP2 which reconstituted a functional *cop*R gene on all plasmids. All deletions and mutations were confirmed by DNA sequencing. After establishing the mutant plasmids in *E. coli* they were transformed into *B. subtilis*.

Determination of plasmid copy number

B. subtilis DB104 strains containing the different mutant plasmids were grown to the same optical density in the late logarithmic phase. Cells were harvested from 1.5 ml aliquots, plasmids were isolated as described (20) and redissolved in 12μl of distilled water. Aliquots of these preparations were linearized with EcoRI and separated on 1% agarose gels. Differences in plasmid copy number were estimated by diluting the plasmid preparations until the intensities of the DNA bands were approximately identical to that of the wild type plasmid pCOP4. For all plasmids four prepartions were run in parallel and each estimation was confirmed at least by five independent experiments. The increase in copy number was thus reflected by the average dilution factor obtained in these parallel experiments.

RESULTS

CopR is involved in copy control

We have previoulsy reported the DNA sequence of the replication region of plasmid pIP501 and identified a gene, *rep*R, which was essential for plasmid replication (20). Further analysis of the DNA sequence located upstream of *rep*R revealed a second open reading frame the 5' end of which was however missing. Fig. 2 presents the complete DNA sequence of this open reading frame which we designated *cop*R. The *cop*R gene coded for a putative small protein of 11.4 kdal in size. It was preceded by a typical translational initiation region and a σ^A promoter (p_I) which has

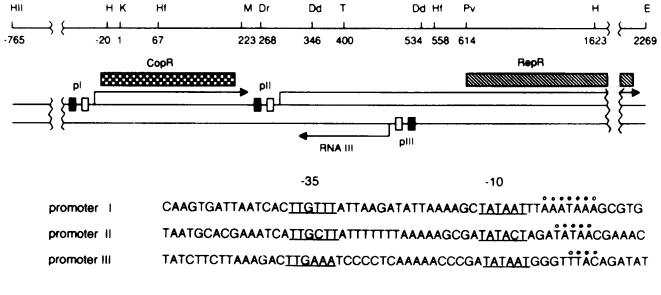

Fig. 1. Schematic representation and DNA sequences of the promoters mapped within a HincII-EcoRI fragment encompassing the replication region of plasmid pIP501 (Brantl et al. submitted). The −35 and −10 boxes are shown as filled and open rectangles, respectively. Transcriptional start sites are indicated above the DNA sequences. Abbreviations of restriction sites: Dd−DdeI; Dr-DraI; E−EcoRI; HII−HincII; H−HindIII; Hf−HinfI; K−KpnI; M−MboI, Pv−PvuII, Sn−SnaBI; T−TaqI. Nucleotides were numbered according to the originally published sequence of the pIP501 replication region (20).

been shown to be active both *in vitro* and *in vivo* (Fig.1, Brantl et al., submitted). A comparison of the *cop*R DNA and protein sequence with sequences deposited at the EMBL data bank revealed only homologies to the respective regions of the related streptococcal plasmids pSM19035 and pAMβ1.

Although the *cop*R gene was obviously not essential for plasmid replication previous observations with a deletion derivative (pGB354, 4) of pIP501 in *Streptococcus sanguis* suggested its involvement in copy number control. To substantiate this observation we constructed a set of deletions removing promoter p_I and increasing segments of the *cop*R coding sequence. The deletion present on plasmid pPR1 removed all of the *cop*R expression signals and in addition the initial 39 nucleotides of the *cop*R coding sequence (Fig. 3). On plasmids pPR2 and pPR3 these deletions extended further into the *cop*R gene removing 159 or 219 nucleotides of the coding sequences. (Fig. 3). The copy number of all three deletion derivatives was found to be increased 10−20 fold over that observed for pCOP4 carrying the intact *cop*R region (Fig. 5B). No differences in copy number were noted between the three deletion derivatives. Since no direct evidence was available for the existence of a CopR protein a frameshift mutation was generated within the *cop*R reading frame by cleaving pCOP4 with KpnI, trimming off the single stranded ends with T4 DNA poymerase and subsequent religation. The deletion, which was confirmed by DNA sequencing, caused a −1 frame-shift resulting in a new putative protein of 23 amino acids (vs 92 for CopR) only the initial 13 of which were identical with CopR. Determination of the copy number of this plasmid (pCOP8, Fig. 3) revealed the same increased copy number as seen for plasmids pPR1−3 (Fig. 5B). We, therefore, conclude that the *cop*R gene in fact codes for a protein which, although being nonessential for replication, acts as a negative regulator in controlling the copy number of pIP501.

Mutations in promoter pIII cause increased copy numbers

Transcriptional analysis of the pIP501 replication region revealed the presence of a short noncoding RNA which was 136 nucleotides long and originated from promoter p_{III} (Fig. 1). Both the location and the secondary structure potential of RNAIII were reminiscent of the regulatory antisense RNAs known to control the replication initiation in other plasmids. In order to study the functional role of RNAIII in pIP501 replication various

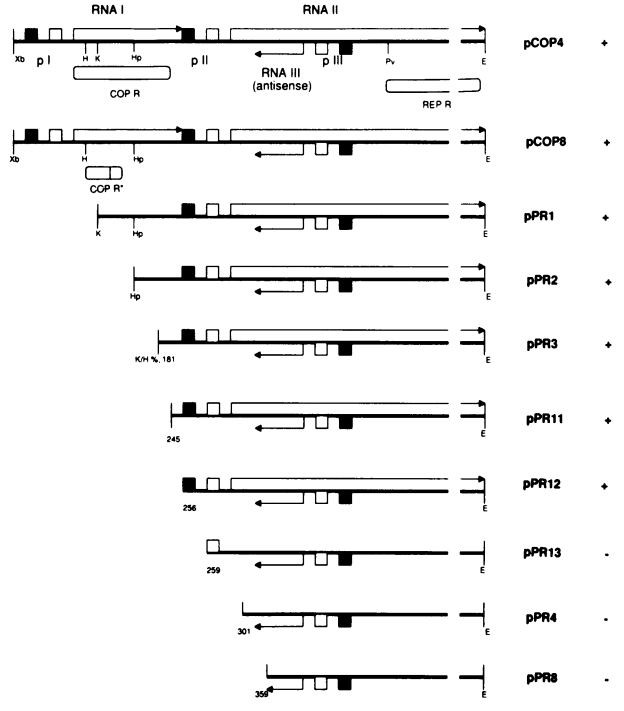

Fig. 3. Deletional analysis of the region upstream of the essential *rep*R gene of pIP501. Arrows indicate transcripts and their directions. Black and empty boxes denote −35 and −10 regions of promoters p_I−p_{III}. RepR,CopR and CopR* refer to the native or truncated proteins coded for by the *rep*R and *cop*R genes or resulting from a frameshift mutation on pCOP8. The +/− signs at the right hand panel indicate the ability of the mutant plasmids to replicate in *B. subtilis*. Nucleotide numberings refer to the previously published sequence (20). Hp−HpaI; Xb−XbaI, for abbreviations of other restriction sites see legend to Fig. 1.

```
                                        -35
-140 ATCACAAATC ACAAGTGATT AATCACTTGT TTATTAAGAT ATTAAAAGCT-91
       -10                          SD
 -90 ATAATTTAAA TAAAGCGTGA ATTTTATGAC ACAAAAAGAG GGGGAGAAA-41

 -40 C TTG GAA CTA GCA TTT AGA GAA AGC TTA AAA AAG ATG AGA  -1
       Met Glu Leu Ala Phe Arg Glu Ser Leu Lys Lys Met Arg
     KpnI
   1 GGT ACC AAA TCA  AAA GAA AAA TTC TCC  CAA GAA TTA GAA  39
     Gly Thr Lys Ser  Lys Glu Lys Phe Ser  Gln Glu Leu Glu

  40 ATG AGT AGA TCA  AAT TAT TCA CGA ATA  GAA TCA GGA AAA  79
     Met Ser Arg Ser  Asn Tyr Ser Arg Ile  Glu Ser Gly Lys

  80 TCA GAT CCA ACC  ATA AAA ACA CTA GAA  CAA ATT GTA AAG 119
     Ser Asp Pro Thr  Ile Lys Thr Leu Glu  Gln Ile Val Lys

 120 TTA ACT AAC TCA  ACG CTA GTA GTG GAT  TTA ATC CCA AAT 159
     Leu Thr Asn Ser  Thr Leu Val Val Asp  Leu Ile Pro Asn

 160 GAG CCA ACA GAA  CCA GAA CCA GAA ACA  GAA CAA GTA ACA 199
     Glu Pro Thr Glu  Pro Glu Pro Glu Thr  Glu Gln Val Thr

 200 TTG GAG TTA GAA  ATG GAA GAA GAA AAA  AGC AAT GAT TTC 239
     Leu Glu Leu Glu  Met Glu Glu Glu Lys  Ser Asn Asp Phe

 240 GTG TGA  ATAATGCACG AAATCATTGC TTATTTTTTT  AAAAAGCGAT 286
     Val  *

 287 ATACTAG 293
```

Fig. 2. Nucleotide and amino acid sequence of the *cop*R gene of pIP501. The −35 and −10 boxes of promoter p_I as well as the putative SD-sequence are boxed. The KpnI site was used to create a −1 frameshift on pCOP8 by removing the single stranded ends with T4-DNA polymerase. Nucleotides are numbered in accordance with the previously published sequence (20). EMBL accession number: X62150.

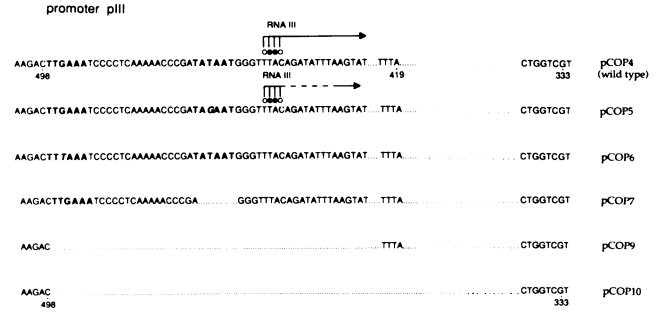

Fig. 4. Compilation of the point mutations and deletions introduced into promoter p_{III} and the region encoding RNAIII. The wild-type sequence is represented by pCOP4. Nucleotides changed in the point mutations are indicated in italics. DNA sequences are shown in a 5′→3′ orientation and numbering refers to the previously published sequence (20). The startpoint of RNAIII mapped *in vivo* is indicated above the DNA sequences.

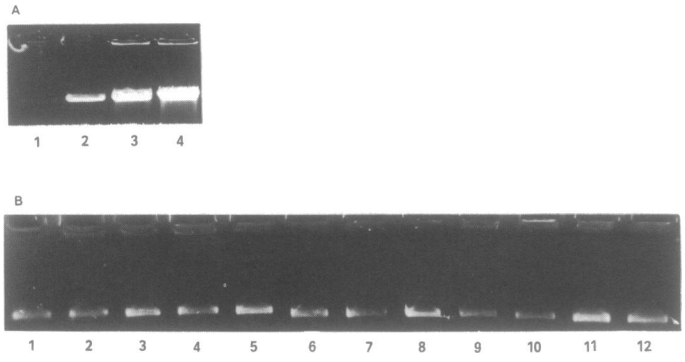

Fig. 5. Estimation of the copy numbers of mutant derivatives of plasmid pIP501 as compared to pCOP4 carrying wild-type replication and copy control functions. **A**: Separation on 1% agarose gels of EcoRI linearized aliquots of undiluted plasmid DNAs prepared from 1.5 ml culture volumes of *B. subtilis* strains grown to the same optical density in the late logarithmic phase. Lanes: 1−pCOP4, 2−pCOP5, 3−pCOP6, 4−pCOP7. **B**: Separation on 1% agarose gels of dilutions of plasmid preparations. 1−pCOP4 undiluted, 2−pCOP5 5 fold dilution, 3−pCOP6, 4−pCOP7, 5−pCOP8, 6−pPR1, 7−pPR5, 8−pPR6, 9−pPR7, 10−pPR9, 11−pPR11 all at 20 fold dilution, 12−pPR11 40 fold dilution.

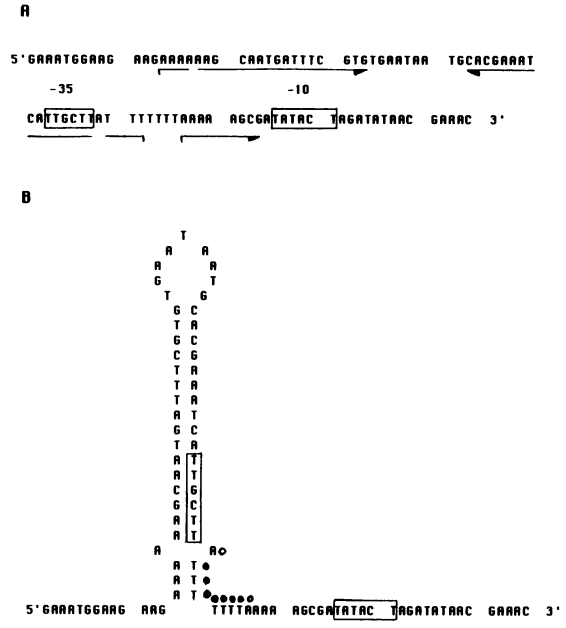

Fig. 6. DNA sequence of the region upstream of promoter p_{II} (**A**) and its potential secondary structure (**B**). Arrows indicate direct and inverted repeats. The −10 and −35 regions of promoter p_{II} are boxed. Open and closed circles denote weak and strong termination signals for *cop*R mRNA (Brantl et al. submitted).

mutations were introduced into promoter p_{III} as outlined in the Materials and Methods section. They are summarized in Fig. 4. On plasmid pCOP5 a single point mutation changed the consensus-like −10 box of promoter p_{III} into the less favourable sequence TAGAAT. This change was expected to cause a mild promoter-down mutation. In contrast, conversion of the −35 box of p_{III} into TTTAAA (plasmid pCOP6, Fig. 4) as well as the complete deletion of the −10 box (plasmid pCOP7, Fig. 4) were expected to severely reduce or abolish transcription from p_{III}. In addition two larger deletions were constructed removing p_{III} and the RNAIII coding region either partially (pCOP9) or almost completely (pCOP10). Comparison of the copy numbers of plasmid pCOP4 (wild-type pIII and RNAIII) with those of pCOP5−9 revealed significantly increased copy numbers for all plasmids carrying mutations or deletions in RNAIII and/or pIII (Fig. 5A/B). It was interesting to note that the point mutation on pCOP5 in fact caused a less pronounced increase in copy number. However, the fivefold elevation as compared to pCOP4 was nevertheless a significant effect (Fig. 5A/B). The increase in copy number was identical for all other plasmids no matter whether a point mutation in the −35 box of p_{III} or progressive deletions of pIII and RNAIII were introduced (Fig. 5). In all these cases the increase was estimated to be approximately 10−20 fold. This was comparable to the effect observed upon inactivation of copR. We, therefore, conclude that RNAIII in fact is a regulatory element which in addition to CopR is involved in controlling pIP501 replication. As already noted for CopR the presence of RNAIII is, however, also not essential for pIP501 replication to occur.

In order to find out whether CopR and RNAIII act on independent steps of replication control a second series of plasmids (pPR6−9) was examined which were identical to the pCOP5−9 counterparts except for a deletion of promoter p_I and the initial 39 nucleotides of the *cop*R gene. This deletion was equivalent to the one present on plasmid pPR1 (see above).The plasmids designated pPR5−9 had, however, the same 10−20 fold elevated copy numbers as pCOP5−9. This lack of an additive effect of *cop*R and p_{III} mutations suggested that both components either independently affected the same step in copy number control or alternatively were part of a sequence of control events. Thus, copy number mutations in p_{III}/RNAIII can only be detected in the presence of a functionally intact *cop*R gene.

While the mutations and deletions present on pCOP5−9 still allowed replication of these plasmids to occur we failed to establish pCOP10 in *B. subtilis*. The deletion present on pCOP10 removed virtually all of the RNAIII sequence. This included the 3′ terminal part which has the capacity to form a stable secondary structure (Brantl et al., submitted). As plasmid pCOP9 was still able to replicate in *B. subtilis* we conclude that DNA sequences between positions 337 and 417 may be essential when *rep*R expression is directed by its native promoter p_{II}.

Deletions upstream of promoter pII affect the copy number

Transcription of the *rep*R gene was directed by promoter p_{II} (Fig. 1). Examination of the DNA-sequence located immediately upstream of this promoter revealed the presence of a long inverted repeat sequence and a shorter direct one (Fig. 6A). The large inverted repeats had the potential to form a stem-loop structure which caused termination of p_I directed transcription (Fig. 6B, Brantl et al., submitted). On the other hand the peculiar overlap of the IR structure with p_{II} and the presence of an additional direct repeat within the spacer of promoter p_{II} suggested that these sequences may serve an additional regulatory function. We, therefore, constructed two deletions removing either one half of the large IR structure or all of it exactly up to the −35 region of p_{II} (pPR11 and pPR12, Fig. 3). Surprisingly, both plasmids had a significantly increased copy number which was even 2−3fold higher than that found for mutations in *cop*R or p_{III}/RNAIII (Fig. 5B). To assure that this effect was not caused by a switch to an unidentified promoter located further upstream or downstream of p_{II} a third deletion was constructed extending just 3 more nucleotides. This deletion inactivated the −35 box

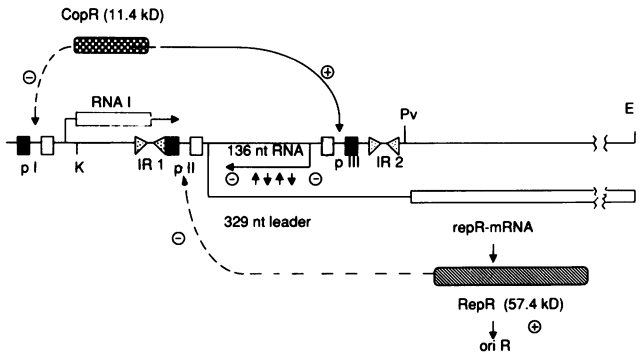

Fig. 7. Working model on the regulatory interactions of components controlling the replication initiation of plasmid pIP501. Hypothetical interactions are indicated by broken lines. IR1/IR2 – inverted repeat sequences, p_I, p_{II}, p_{III} – promoters I, II, III, K – KpnI, Pv – PvuII, E – EcoRI.

of promoter pII (Fig. 3). The corresponding plasmid pPR13 failed to replicate in B. subtilis as did two other mutants which carried deletions of promoter pII and 40 or 90 downstream nucleotides (pPR4, pPR8, Fig. 3). The inverted repeat structure overlapping promoter pII, therefore, in fact serves a dual function. On the one hand it terminates transcription from the upstream promoter pI and on the other hand it is apparently involved in down regulation of pII activity.

DISCUSSION

Knowledge of the transcriptional units within the replication region of pIP501 has facilitated a targeted mutational analysis of functions likely to be involved in copy number control of this plasmid. Based on this analysis two levels of copy number control have been identified which involve at least three components—the CopR protein, a 136 nt long antisense RNAIII and repeat sequences overlapping promoter p_{II}. Both mechanisms are directed to control the amount of the RepR protein which thus appears to be the rate-limiting factor for pIP501 replication. These data together with the results from DNA sequencing (20) and transcriptional studies (Fig. 1) allow to propose a working model on the regulatory interaction of plasmid components controlling the replication of pIP501. This model is presented in Fig. 7. The *rep*R gene which codes for a 57.4 kDal essential protein (RepR) is transcribed from promoter p_{II}. The *rep*R mRNA includes a 329 nt long leader sequence part of which is complementary to a small antisense RNA (RNAIII, 136 nt) which is transcribed from promoter p_{III} at the opposite strand. The antisense RNAIII as well as its counterpart sequence in the *rep*R mRNA leader possess a high capacity for secondary structure formation. The single-stranded loops of the folded RNAIII are particularly GC-rich which would facilitate initial interaction with the target sequence on the *rep*R mRNA (Brantl. et al., submitted). Since mutations in promoter p_{III} or deletions of the RNAIII coding sequence lead to an increase in plasmid copy number we propose that RNAIII in fact functions as an antisense RNA negatively regulating the expression of *rep*R. This is also consistent with our observation that variations in the amount of RNAIII caused by a moderate down mutation in p_{III} led to only a gradual increase in copy number. The region of complementarity between RNAIII and *rep*R mRNA does not include the translational start signals of *rep*R. The exact mechanism how RNAIII affects *rep*R expression thus remains to be resolved. RNAIII may either act at a posttranscriptional level to control translation of the *rep*R mRNA or alternatively by a transcriptional attenuation type of mechanism involving the terminator-like structure found upstream of p_{III} (IR2, Fig. 6). Small antisense RNAs have been described to regulate the frequency of replication of several well-studied plasmids (24 – 30). While in ColE1 the antisense RNA acts in a direct manner by preventing the maturation of a replication primer (31, 32) its role in the control of plasmids pT181 or R1 replication is indirect and similar to the alternatives proposed for RNAIII action of pIP501. In pT181 the antisense RNA has recently been shown to act by inducing premature termination of *rep*C transcription via a novel transcriptional attenuation-like mechanism rather than by preventing translation of the *rep*C mRNA (24, 33). In contrast, the *cop*A antisense RNA of R1 prevents translation of the *rep*A gene by an as yet unresolved mechanism. The kinetics of *cop*A interaction with its target has recently been studied in great detail (34, 35). The initial reaction appears to be the formation of a 'kissing complex' between ss loops of *cop*A and its target *cop*T. Subsequently both RNAs anneal completely in a zipper-like reaction to form a stable duplex which somehow is subject to a processing event involving RNaseIII. This processing event seems to be essential for *cop*A action (36). Since in pIP501 the sequence around the translational start site of *rep*R does not contain regions with secondary structure potential RNAIII may act by a mechanism similar to that of *cop*A of plasmid R1.

In contrast to plasmid R1, however, the CopR protein of pIP501 acts at the same control level as RNAIII. This conclusion is supported by the absense of an additive effect of mutations in RNAIII and *cop*R. While in R1 mutations in both the antisense RNA and CopB result in runaway replication (37) the same increase in copy number of pIP501 is observed no matter whether mutations inactivate RNAIII, *cop*R or both functions. We, therefore, propose that CopR of pIP501 acts either by enhancing transcription from promoter p_{III} or alternatively by promoting the interaction of RNAIII with its target sequence in the *rep*R mRNA leader. Examination of the *cop*R sequence has revealed neither a helix-turn-helix DNA binding motif nor any significant sequence homology with the ColE1 Rom protein which has been shown to stimulate hybrid formation between the antisense RNA and its target (38 – 40). Gene fusion experiments are currently underway to discriminate between these alternatives.

The second mechanism of copy control obviously affects transcription at promoter p_{II}. This mechanism involves a region containing direct and inverted repeat sequences which partially overlap promoter p_{II}. Deletion of this structure caused a dramatic increase of plasmid copy number which was higher than that obtained by mutations in either *cop*R or RNAIII. The data presented exclude an involvement of CopR in this mechanism and rule out interference between termination of p_I directed transcription and p_{II} promoter utilization as the underlying mechanism. We, therefore, propose that the repeat structures overlapping p_{II} constitute a target site for autoregulation of *rep*R expression. Autoregulation of a rate-limiting Rep-protein has been found to be involved in copy number control of plasmid pSC101 (41 – 43). Like in pIP501 the promoter transcribing the *rep*A gene of pSC101 overlaps with inverted and direct repeat sequences. These sequences have been shown to be the target of negative autoregulation of the RepA protein of pSC101. It is interesting to point out that on pSC101 the repeat sequences overlapping the *rep*-promoter share a high degree of similarity with repeat

sequences found in the origin of replication of pSC101 – the second target of the RepA protein (44). A similar situation may hold true for pIP501. Recently the origin of replication has been mapped for plasmid pAMβ1—a plasmid closely related with pIP501 (16). The origin maps immediately downstream of the *rep* gene of pAMβ1 and encompasses an inverted repeat which shares considerable similarity with the one overlapping promoter p_{II} of pIP501. The DNA sequences of pIP501 and pAMβ1 are identical in this region and we do have experimental evidence confirming the location of the replication origin of pIP501 at this location (unpublished data). Gene fusion experiments are currently underway to probe for the proposed autoregulation of RepR.

ACKNOWLEDGEMENTS

We would like to thank E. Birch-Hirschfeld for synthesizing and supplying the oligonucleotides. The excellent technical assistance of Ina Poitz is gratefully acknowledged. This work was supported by a grant from the Max Planck Society to D.B.

REFERENCES

1. Behnke, D., Malke, H., Hartmann, M. and Walter, F. (1979) Plasmid **2**, 605–616.
2. Behnke, D. and Ferretti, J.J. (1980) Plasmid **4**, 130–138.
3. Horodniceanu, T., Bouanchaud, D.H., Bieth, G. and Chabbert, Y.A. (1976) Antimicrob. Agents Chemother. **10**, 795–801.
4. Behnke, D., Gilmore, M. and Ferretti, J.J. (1981) Mol. Gen. Genet. **182**, 414–421.
5. Clewell, D.B., Yagi, Y., Dunny, G.M. and Schultz, S.K. (1974) J. Bacteriol. **117**, 283–289.
6. Gibson, E.M., Chace, N.M., London, S.B. and London, J. (1979) J. Bacteriol. **137**, 614–619.
7. Engel, H.W.B., Soedirman, N., Rost, J.A., van Leeuwen, W.J. and van Embden, J.D.A. (1980) J. Bacteriol. **142**, 407–413.
8. Schuberg, D.R., Clewell, D.B. and Glatzer, L. (1982) Antimicrob. Agents Chemother. **22**, 204–207.
9. Gonzalez, C.F. and Kunka, B.S. (1983) Appl. Environ. Microbiol. **46**, 81–89.
10. Buu-Hoi, A., Beth, G. and Horaud, T. (1984) Antimicrob. Agents Chemother. **25**, 284–291.
11. Oultram, J.D. and Young, M. (1985) FEMS Microbiol. Lett. **27**, 129–134.
12. Gruss, A. and Ehrlich, S.D. (1989) Microbiol. Rev. **53**, 231–241.
13. Alonso, J.C. (1989) Microbiologia (SEM) **5**, 5–12.
14. Bron, S. (1990) In Harwood, C.R. and Cutting, S.M. (eds), Molecular Biology Methods for *Bacillus*, John Wiley & Sons Ltd., pp. 75–138.
15. Janniere, L., Bruand, C. and Ehrlich, S.D. (1990) Gene **87**, 53–61.
16. Bruand, C., Ehrlich, S.D. and Janniere, L. (1991) EMBO J. **10**, 2171–2177.
17. Sorokin, A. and Khasak, V. (1989) In Butler, L.O., Harwood, C. and Moseley, B.E.B. (eds) Genetic Transformation and Expression, Intercept Ltd. Andover, U.K., pp. 269–281.
18. Swinfield, T.J., Oultram, J.D., Thompson, D.E., Brehm, J.K. and Minton, N.P. (1990) Gene **87**, 79–90.
19. Brantl, S., Nowak, A., Behnke, D. and Alonso, J.C. (1989) Nucl. Acids Res. **17**, 1011–1011.
20. Brantl, S., Behnke, D. and Alonso, J.C. (1990) Nucl. Acids Res. **18**, 4783–4790.
21. Kawamura, F. and Doi, R.H. (1984) J. Bacteriol. **160**, 442–444.
22. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, New York.
23. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467.
24. Novick, R.P. (1987) Microbiol. Rev. **51**, 381–395.
25. Kumar, C. and Novick, R.P. (1985) Proc. Natl. Acad. Sci. USA **82**, 638–642.
26. Light, J. and Molin, S. (1983) EMBO J. **2**, 93–98.
27. Stougaard, P., Molin, S. and Nordström, K. (1981) Proc. Natl. Acad. Sci. USA **78**, 6008–6012.
28. Lacks, S.A., Lopez, P., Greenberg, B. and Espinosa, M. (1986) J. Mol. Biol. **192**, 753–765.
29. Alonso, J.C. and Tailor, R.H. (1987) Mol. Gen. Genet. **210**, 476–484.
30. Maciag, I.E., Viret, J.-F. and Alonso, J.C. (1988) Mol. Gen. Genet. **212**, 236–240.
31. Tomizawa, J. and Itoh, T. (1981) Proc. Natl. Acad. Sci.USA **78**, 6096–6100.
32. Tomizawa, J. (1984) Cell **38**, 861–870.
33. Novick, R. P., Iordanescu, S., Projan, S.J., Kornblum, J. and Edelman. J. (1989) Cell **59**, 395–404.
34. Persson, C., Wagner, E.G.H. and Nordström, K. (1990) EMBO J. **9**, 3767–3775.
35. Persson, C., Wagner, E.G.H. and Nordström, K. (1990) EMBO J. **9**, 3777–3785.
36. Blomberg, P., Gerhart, E., Wagner, E.G.H. and Nordström, K. (1990) EMBO J. **9**, 2331–2340.
37. Nordström, K., Molin, S. and Light, J. (1984) Plasmid **12**, 71–90.
38. Cesareni, G., Muesing, M.A. and Polisky, B. (1982) Proc. Natl. Acad. Sci. USA **79**, 6313–6317.
39. Twigg, A.J. and Sherratt, D. (1980) Nature **283**, 216–218.
40. Som, T. and Tomizawa, J.-I. (1983) Proc. Natl. Acad. Sci. USA **80**, 3232–3236.
41. Lindner, P., Churchward, G., Guixian, X., Yi-Yi, Y. and Caro, L. (1985) J. Mol. Biol. **181**, 383–393.
42. Vocke, C. and Bastia, D. (1985) Proc. Natl. Acad. Sci. USA **82**, 2252–2256.
43. Yamaguchi, K. and Masamune, Y. (1985) Mol. Gen. Genet. **200**, 362–307.
44. Yamaguchi, K. and Yamaguchi, M. (1984) Gene **29**, 211–219.