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Control of pT181 replication I. The pT181 copy control function acts by inhibiting the synthesis of a replication protein

Richard P. Novick, Gail K. Adler[1], Steven J. Projan, Stephen Carleton[2], Sarah K. Highlander, Alexandra Gruss, Saleem A. Khan[3] and Serban Iordanescu[4]

Department of Plasmid Biology, The Public Health Research Institute of the City of New York, Inc., New York, NY 10016, USA and [4]Institute Cantacuzino, Bucharest, Roumania

[1]Present address: Deparment of Endocrinology, Brigham and Women's Hospital, Boston, MA 102115, USA
[2]Present address: Department of Developmental Biology and Cancer, Albert Einstein School of Medicine, New York, NY, USA
[3]Present address: Department of Microbiology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

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pT181 is a fully sequenced 4.4-kb 20 copy Tc^r plasmid from *Staphylococcus aureus*. Its replication system involves a unique unidirectional origin embedded in the coding sequence for a plasmid-determined protein, RepC, that is required for initiation. When joined to a 55 copy carrier plasmid, pE194, pT181 excludes autonomous isologous replicons by inhibiting their replication. Two types of spontaneous pT181 copy mutants have been isolated, one that eliminates sensitivity to this inhibition and another that does not. A spontaneous 180-bp deletion, Δ144, eliminates both the inhibitory activity and sensitivity to it. This deletion increases copy number by 50-fold and RepC production by at least 10-fold. It is located directly upstream from the *repC* coding sequence and the deletion-bearing plasmid supports the replication of inhibitor-sensitive plasmids in cells containing active inhibitor. This effect is probably due to the overproduction of RepC by the Δ144 plasmid. On the basis of these results, it is suggested that RepC synthesis is negatively controlled by an inhibitor that is encoded directly upstream from the *repC* coding sequence and acts as a tareget set in the same region. It is likely, therefore, that pT181 replication rate is determined by the level of RepC.

Key words: plasmid pT181/RepC synthesis/control function/replication rate

Introduction

The isolation of plasmid mutants with increased copy numbers (Nordström *et al.*, 1972) confirmed the prediction of Pritchard *et al.* (1969) that plasmid replication is controlled negatively and led to a series of investigations that have revealed a common mechanism involving the binding of a diffusible inhibitor to a specific target site. Plasmids that use the inhibitor-target mechanism include the ColE1 family and the IncFII plasmids. For both of these groups, the primary inhibitor is a small RNA species (RNA-I) whose target is an RNA transcript that is directly involved in the initiation of replication. In both cases, RNA-I at elevated gene dosage inhibits the replication of homologous plasmids in *trans* (Molin and Nordström, 1980; B. Polisky, personal communication). For the ColE1 group, inhibition is of the formation of the replication primer (Tomizawa *et al.*, 1981). For the IncFII plasmids, the synthesis of the *cis*-specific initiator protein, RepA is inhibited (Light and Molin, 1982). An important feature of these systems is that RNA-I and its target are overlapping divergent transcripts so that mutations occur that affect both simultaneously and in a complementary manner. Many of these mutations cause an increase in copy number and some alter the sequence specificity of the inhibitor-target interaction and establish new incompatibility types (Lacatena and Cesareni, 1981; Tomizawa and Itoh, 1981; Easton and Rownd, 1982).

pT181 is a 4.4-kb multicopy plasmid from *Staphylococcus aureus* that specifies tetracyline resistance and is the prototype of a widely dispersed family of staphylococcal plasmids. pT181 replicates unidirectionally from a unique origin (Khan *et al.*, 1982) embedded in the coding sequences for RepC, a freely diffusible protein required for replication of the plasmid (Novick *et al.*, 1982). This report is the first of three in which it is established that (I) pT181 also uses the inhibitor-target strategy for copy control; (II) the target of inhibition is expression of the *repC* gene; and (III) the functional organization of its control region is analogous to that of other inhibitor-target plasmids such as those of the ColE1 and IncFII groups. In this paper, the genetics and physiology of the inhibitor are described and it is established that the regulatory system controls RepC synthesis. The *trans* activity of the RepC protein means that pT181 replication is controlled indirectly in contrast to the other known inhibitor-target plasmids whose replication is controlled directly (cf. Grindley *et al.*, 1978). In the second paper (Carleton *et al.*, accompanying paper), we report the isolation and properties of copy mutants, many of which occur in a sharply defined cluster that presumably defines the primary site of the inhibitor-target interaction. In the third paper (Kumar and Novick, 1984), it is shown that the inhibitory activity resides in either or both of two short counter-transcripts read from the 5′ end of the *repC* mRNA leader region. The cluster of copy mutations is in the region where leader and counter-transcript overlap, suggesting that the control mechanism is based on complementary interactions between the leader and the counter-transcripts.

Results

Inhibition of pT181 by pT181::pE194 co-integrates

Elements of the pT181 copy control system were identified by the analysis of co-integrates between pT181 (22 copies/cell) and pE194 (55–60 copies). According to the theoretical predictions of the inhibitor-dilution model of replication control (Pritchard *et al.*, 1969), as demonstrated by Timmis *et al.* (1974), the replication of such hybrids should be controlled by pE194; any negative regulatory function expressed by the pT181 moiety would be present at an elevated copy number and the plasmid would therefore be hyper-repressed. Previous results with *in vivo* co-integrates between these two

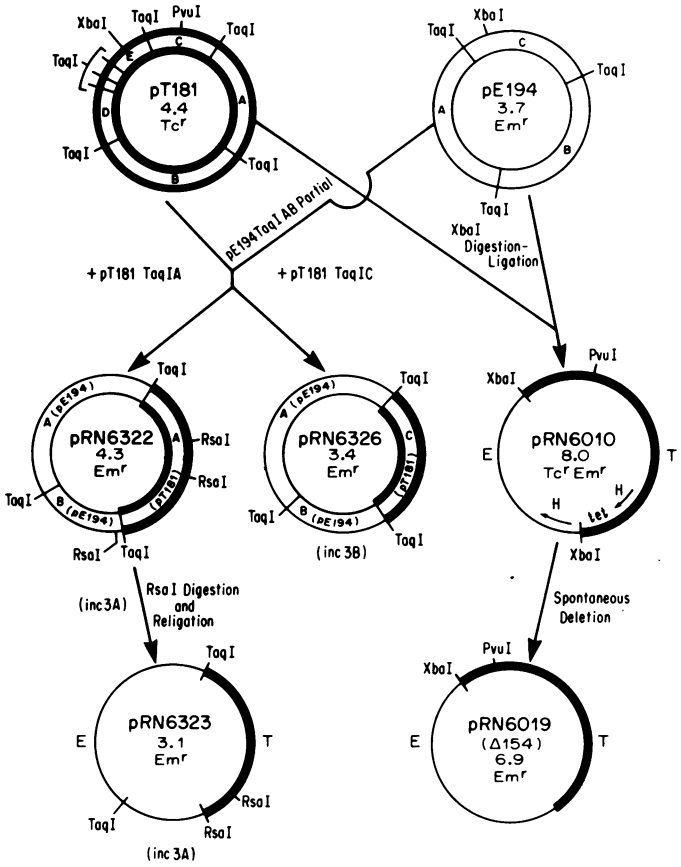

Fig. 1. Plasmid constructions. Only restriction sites involved in the cloning are shown. pT181 and its fragments are shown with heavy lines; pE194 and its fragments are drawn lightly. At right is shown the construction of pRN6010 by *Xba*I digestion and ligation. pRN6019 arose by the spontaneous deletion of 2 kb between the arrows marked 'H'. At left is shown the cloning of *inc3A (copA)* and *inc3B*. For *inc3A*, the largest *Taq*I fragment (A) of pT181 was ligated to a partial *Taq*I digest of pE194 and the resulting chimera was digested with *Rsa*I and re-ligated. The final subclone, pRN6323, lacks the *Rsa*I fragment between position 570 of pT181 and 611 of pE194 and has therefore lost a 23-bp stretch of pE194 DNA between the *Rsa*I (611) and *Taq*I (588) sites (Horinouchi and Weisblum, 1982) at 6 o'clock as shown. For *inc3B*, the third largest *Taq*I fragment of pT181 (C) was ligated to the same partial digest of pE194 (pRN6326). Both of these clones lack the non-essential *Taq*I-C fragment of pE194.

plasmids (Novick *et al.*, 1981) had suggested that they have copy numbers characteristic of the pE194 moiety (S. Projan, unpublished data) and that they interfere with the establishment of autonomous pT181 (Novick *et al.*, 1981). We therefore used such co-integrates to identify and map elements of the pT181 copy control system. For ease of manipulation, we constructed new co-integrates *in vitro* by ligation of *Xba*I-digested pE194 and pT181 DNAs followed by transformation of RN450, a plasmid-negative *S. aureus* strain, with joint selection for erythromycin (Em) and tetracycline (Tc) resistance. One example of each of the two orientations A and B, was chosen for further study, designated pRN6010 and pRN6050, respectively. The two plasmids had copy numbers of 50–60, characteristic of pE194, and as they had similar properties, the experiments described here were done with one only, pRN6010, and its derivatives. A diagram of the construction of pRN6010 and other plasmids used in this study is shown in Figure 1. Note that cloning into the unique pT181 *Xba*I site interrupts with *repC* coding sequence (see Figure 2), inactivating the pT181 replicon; however, as

Table I. Transductional analysis of pT181 incompatibility

Resident plasmid	Relevant characteristics	Donor plasmid	Relevant characteristics	RTF[a]
None		pT181	Inc3 Tc^r	1.0
None		pSA5000	Inc3 Cm^r	1.0
pRN6012		pT181		$<10^{-2}$
pRN6012		pSA5000		$<10^{-2}$
pRN6019	Δ154	pT181		$<10^{-3}$
pRN6038	Δ141	pT181		$<10^{-2}$
pRN6039	Δ242	pT181		1.2×10^{-2}
pRN6116	Δ142	pT181		$<10^{-2}$
pRN6146	Δ241	pT181		0.94
pRN6154	Δ243	pT181		0.86
pRN6157	Δ143	pT181		1.05
pRN6158	Δ240	pT181		0.91
pRN6264	Δ144 *cop-608*(D)[b]	pRN8018		7×10^{-2}
pRN6264		pRN8023	*cop-618*(D)	1.0
pRN6264		pRN8026	*cop-621*(R)	1.0
pRN6308	*cop-623*(D)	pRN8023	*cop-618*(D)	1.0
pRN6308		pRN8026	*cop-621*(R)	5×10^{-2}
pRN6012		pRN8008	*cop-608*(D)	1.0
pRN6012		pRN8023	*cop-618*(D)	1.0
pRN6012		pRN8026	*cop-621*(R)	5×10^{-2}
pRN6012		pRN8061	*cop-623*(D)	1.0
pRN6323	pE194::pT181 158-570 (*inc3A*)	pT181		$<10^{-2}$
pRN6323		pRN8023		1.0
pRN6323		pRN8026		5×10^{-2}
pRN6326	pE194::pT181 TaqI-C (*inc3B*)	pT181		10^{-1}
pRN6326		pRN8023		1.0
pRN6326		pRN8026		1.0

[a]RTF = relative transduction frequency (RTF for plasmid set at 1.0).
[b]D = dominant; R = recessive.

shown below and in the two companion papers (Carleton *et al.*, accompanying paper; Kumar and Novick, 1984) its replication control system is intact and functional.

The incompatibility between pT181::pE194 co-integrates and pT181 was evaluated by reciprocal transductions with wild-type pT181 and Tc^s derivatives of the co-integrate, pRN6010. Both ethyl methanesulphonate (EMS)-induced point mutants such as pRN6012 and spontaneous Tc^s deletions such as pRN6019 were utilized. These deletions, arising by intramolecular recombination involving a homologous 0.5-kb segment, indicated in Figure 1 as an arrow labeled 'H' (Novick *et al.*, 1981), do not detectably affect any replication function of either plasmid. In one direction, a Tc^{s}Em^r co-integrate plasmid was transduced into a strain containing pT181 and Em^r transductants were scored by replica plating for loss of the pT181 Tc^r marker. In the reciprocal crosses, the frequency with which Tc^r transductants could be obtained using recipients containing or lacking the co-integrate was determined. The results of both sets of crosses were consistent and demonstrated that it was impossible to establish autonomous wild-type pT181 in the presence of a pT181::pE194 co-integrate; the co-integrate always displaced the resident

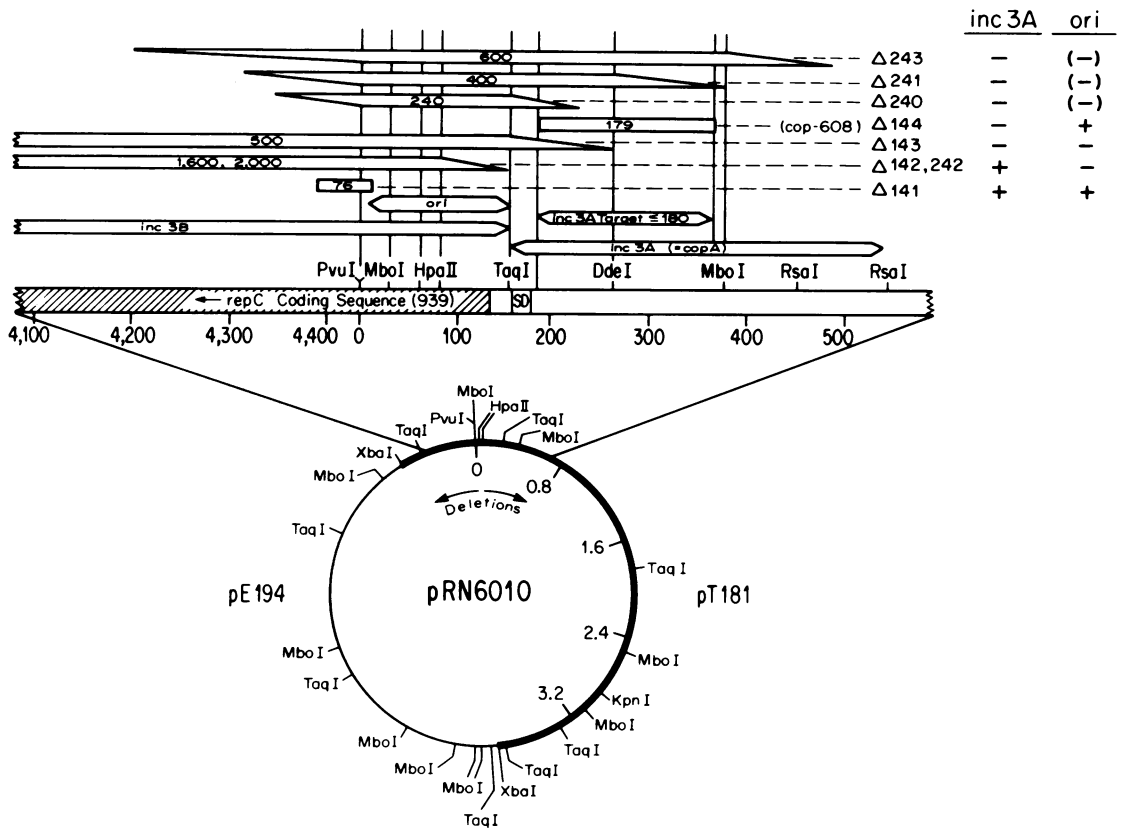

Fig. 2. Deletion map of the repC control region. Deletions 141-3 and 240-3 were generated by enzymatic digestion of *PvuI*-linearized pRN6010 DNA and were mapped by restriction analysis and scored for Inc3A activity (see text) and origin activity (Khan *et al.*, 1982). Numbers indicate estimated sizes of the deletions and tapering portions indicate the limits of uncertainty of the locations of the ends. The locations of Δ141 and Δ144 have been determined precisely by sequencing (Khan *et al.*, 1981; Khan and Novick, 1983). The locations of *inc3A* and *inc3B* were defined by cloning (see text). The Inc3A target was defined by Δ144.

pT181 and pT181 was unable to be introduced and maintained in a strain containing the co-integrate. In all cases, the few doubly resistant transductants that were obtained always contained recombinant plasmids. Typical results for the crosses in which the co-integrate was present in the recipient strain are listed in Table I. It is concluded from these experiments that the incompatibility exhibited by the co-integrate toward pT181, referred to as Inc3A, is uni-directional and absolute.

Mapping the Inc3A determinant

The *Inc3A* determinant(s) was mapped by testing the ability of enzymatically generated deletions of pRN6012 to prevent the establishment of pT181 by transduction. Bidirectional deletions starting at the unique *PvuI* site (see Figure 1), located within the *repC* coding sequence and ~100 bp from the replicative origin, were generated by digestion with exonuclease III (see Materials and methods), mapped by restriction analysis (Figure 2) and tested for their ability to prevent establishment of pT181. As summarized in Table I, the deletion derivatives were clearly divisible into two classes; deletions 141, 142 and 242 excluded pT181 as effectively as pRN6012, whereas deletions 143, 240, 241 and 243 showed no exclusion of pT181 and are therefore Inc3A$^-$. Agarose screening gels confirmed the existence of autonomous pT181 in strains containing these Inc3A$^-$ plasmids. As shown in Figure 2, all plasmids with deletions terminating to the left of the TaqI site at position 158 [*Taq* (n158)], are Inc3A$^+$, whereas all those with deletions extending beyond this point are Inc3A$^-$. These results place the left end of the *inc3A* determinant in the vicinity of *Taq*I (n158).

The *inc3A* gene was further localized by subcloning pT181 restriction fragments located within this region into a derivative of pE194, pRN4149, and scoring for Inc3A activity by transduction. The 412-bp *Taq*I-*Rsa*I subfragment located between positions 158 and 570 (see Figure 1) was the smallest fragment shown to express Inc3A activity (pRN6323). Additionally, the *Taq*I C fragment, located within the *repC* coding region, also exhibited a weak inhibition of pT181 establishment, which is referred to as *inc3B* (see Table I, pRN6326). It is noted that this fragment contains the N-terminal half of the *repC* coding sequence as well as the intact replication origin, either of which could be responsible for Inc3B activity. This activity was eliminated by all of the deletions analyzed that extended to the right of *Mbo*I (n31), and was expressed by plasmids such as pRN6264 which have deletions lying to the right of *Taq*I (n158).

The Inc3A target

Three of the copy mutants isolated by Carleton *et al.*, accompanying paper, were found to have small deletions (Δ144, Δ255 and Δ257), confined to the *Mbo*I-D fragment (n31 – 376). Sequence analysis revealed their precise locations as 183 – 362 (Δ144), 157 – 314 (Δ255) and 208 – 280 (Δ257) (Carleton *et al.*, accompanying paper). This property was analyzed. All three of the deletion-bearing plasmids (e.g., pRN8008, Table I), as well as several other copy mutants with point mutations (such as *cop-618* and *cop-623* (see Table I)) were able to co-exist stably with Inc3A$^+$ plasmids in non-selective media and had the same copy numbers under these conditions as when present singly (not shown). These mutants

Table II. Inhibition by pRN6287 of pT181 replication[a]

Plasmid	Time, min							
	−10	−5	0	5	10	15	20	25
pT181[b]	0.019	0.020	0.018	0.011	0.004	0.006	<0.0001	<0.0001
pRN8018[b]	0.18		0.18		0.16			0.16
pRN6287	0.012	0.012	0.014	0.021	0.050	0.10	0.16	0.16

[a]Cultures were grown in a turbidostat with a shift from 42°C to 32°C at time zero. 1.0 ml samples were removed at indicated times, pulsed for 5 min with β[^{3}H]thymidine (10 μCi/ml), then stopped with dry ice-ethanol and used to prepare sheared whole-cell minilysates. Equal sized samples were separated by agarose gel electrophoresis and the plasmid and chromosomal DNA bands excised and counted. Figures are ratios of incorporation into CCC plasmid and chromosomal DNAs for each time point.
[b]In the presence of pRN6287.

are therefore regarded as completely defective in the functional Inc3A target and are referred to as dominant. Since the three deletions lie within the *Taq*I-*Rsa*I fragment (158−570) previously shown to contain the *inc3A* determinant, it can be concluded that the two determinants are very closely linked, possibly overlapping. This close linkage was verified by cloning the *Taq*I-A fragment from PRN8008 (*cop-608* Δ144) to pRN4149, as shown in Figure 1, and testing the resulting plasmid for Inc3A activity. There was no reduction in the frequency of transduction of pT181 *cop-621*, a recessive copy mutant, to a strain containing this plasmid and transductants contained both plasmids at the same copy numbers as when present singly (not shown), indicating that Δ144 eliminates Inc3A activity as well as its target.

Inc3A inhibits pT181 replication in trans

If Inc3A is involved in copy control, it should inhibit pT181 replication *in trans*. To test directly for such inhibition of pT181 *in vivo*, one must use a plasmid that expresses Inc3A conditionally. This was accomplished by cloning pT181 to a derivative of pE194 with a thermosensitive replication defect (Tsr), pRN6149, and isolating a 2-kb Tc^s deletion. The resulting composite plasmids, pRN6287, had a copy number of ~3 at 42°C and it proved possible to construct and maintain heteroplasmid strains containing pRN6287 and pT181 at this temperature. Following a temperature shiftdown, the Tsr plasmid amplifies to its normal copy number and any predicted inhibitory effect on the replication of the test plasmid should be manifested during this amplification. A heteroplasmid containing pRN6287 and pT181, in which the two plasmids had copy numbers of 3 and 20, respectively, at 42°C, was shifted to 32°C and incorporation of tritiated thymidine into the two plasmid species was followed as a function of time. This experiment (see Table II) showed a rapid and complete inhibition of pT181 replication concomitantly with the amplification of pRN6287 after the temperature shift. Note that in the absence of an *inc3A*$^+$ plasmid, pT181 has a copy number of ~22 at 32°C. In an experiment of the same type, pT181 *cop-618* (a dominant copy mutant) showed no such inhibition, whereas pT181 *cop-621*, (a recessive copy mutant) was completely inhibited.

Since the *inc3A*$^+$ test plasmid used in this experiment, pRN6287, also contained the *inc3B* region, it was necessary to examine each separately. Accordingly, tests similar to that shown in Table II were performed with pRN6326 (pRN4149::pT181 *Taq*I-C) and with pRN6323 [pRN4149::pT181 (n158−n570)]. These tests indicate that the cloned *inc3A* region has the same inhibitory activity as did

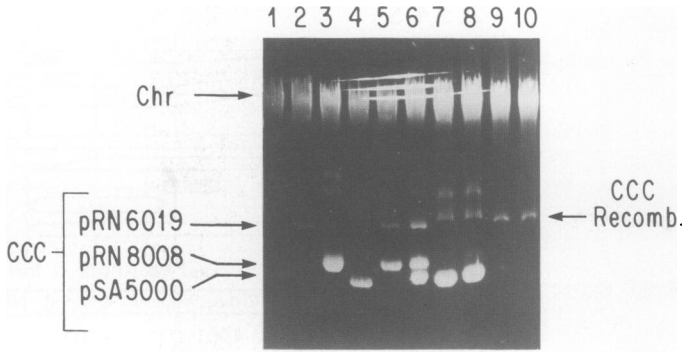

Fig. 3. Reversal of Inc3A inhibition of pSA5000 by pRN8008 (pT181 *cop-608*). Agarose gel electrophoretic analysis of whole-cell minilysates of strains containing the following plasmids is shown: **track 1**, pSA5000; **2**, pRN6019; **3**, pRN8008; **4**, pRN8028; **5**, pRN6019 + pRN8008; **6**, pRN6019 + pRN8008 + pSA5000; **7** and **8**, pRN8028; **9** and **10**, pSA5000 transductants of the strain shown in **tracks 2**, **7** and **8**. The single band seen is evidently a Cm^{r}Em^r recombinant plasmid.

the original co-integrate (pRN6019) and a derivative with a deletion of the origin region (pRN6116). The cloned *inc3B* region has an inhibitory activity similar to that of pRN6264 − it inhibits neither recessive nor dominant copy mutants but it interferes with the entry of the wild-type plasmid (Table I) and, in addition, reduces the copy number of the latter by ~50% (not shown). On the basis of these results, we conclude that *inc3A* and its target constitute the primary replication control elements of pT181 and refer to them henceforth as *copA* and *copT*, respectively. The significance, if any, of Inc3B in the regulation of pT181 replication remains unclear.

CopA acts by inhibiting expression of repC

Examination of the nucleotide sequence in the copy control region of pT181 (Khan and Novick, 1983) revealed that *copT* as defined by Δ144 (see Figure 2) is directly upstream from both the coding sequence for the *repC* protein, and from the replication origin. It therefore seemed probable that *copA* inhibits either the expression of *repC* or transcription of the origin − which could be required for activation, for primer formation, or both. We have shown previously that RepC is active *in trans*. Therefore, if *repC* expression is inhibited by *copA*, it should be possible to overcome the inhibition *in vivo* and permit the replication of a *copA*-sensitive plasmid in the presence of an active *copA* gene by introducing a dominant copy mutant. If the inhibition is of origin transcription, then it should not be possible to overcome it by any *trans*-active plasmid function. Accordingly, a strain containing both pRN6019 and pT181 *cop-608* Δ144 was constructed. The latter plasmid, as noted, is *copA*-insensitive and has been shown to overproduce *repC* activity by at least 10-fold as measured in extracts (Khan *et al.*, 1981). This two-plasmid strain accepted with *copA*-sensitive plasmid, pSA5000, and maintained it at a greatly elevated copy number (Figure 3, lane 6). This result is consistent with RepC-specific bypass of *copA* inhibition of pSA5000, and rules out origin transcription as the inhibited function. This conclusion was supported by two further experiments of the same type. In one of these, the plasmid used to supply *RepC* was pT181 *cop-623* (pRN8061), which was previously shown to express *copA* activity (see Table I). This plasmid overcame *copA* inhibition similarly to pRN8008 (see Figure 4, tracks 10−14), showing that titration of *copA* product cannot be the means by which the inhibition is overcome. In the second experiment, two test plasmids were used, pT181 *cop-632 repC3* (pSA0331) and pT181 *cop-*

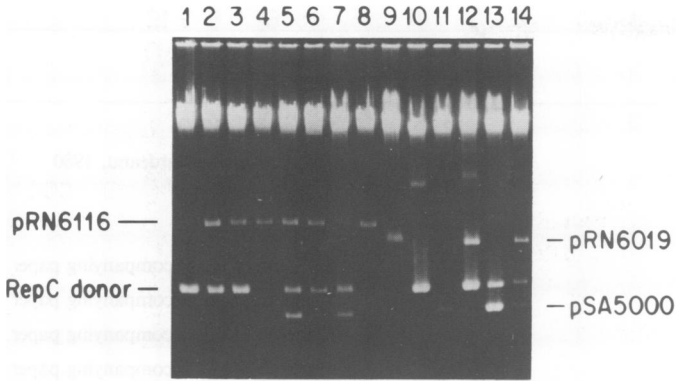

Fig. 4. Reversal of Inc3A inhibition of pSA5000 by pRN8061 (pT181 *cop-623*) and by *cop-632*. **Track 1**, pRN8032 (pT181 *cop-632*); **2**, pRN6116 + pRN8109, 43°C; **3**, pRN6116 + pSA0331, 32°C; **4**, same as **3**, 43°C; **5**, pRN6116 + pRN8061 + pSA5000, 32°C; **6**, pRN6116 + pSA0331 + pSA5000, 32°C; **7**, same as **5**, 43°C; **8**, same as **6**, 43°C; **9**, pRN6019; **10**, pRN8061; **11**, pSA5000; **12**, pRN6019 + pRN8061; **13**, pRN8061 + pSA5000; **14**, pRN6019 + pRN8061 + pSA5000. Note that pSA5000 cannot co-exist with either pRN6116 or pRN6019 alone.

632 (pRN8109). Both of these are dominant copy mutants with the same nucleotide change (position 316, G-T) (Carleton *et al.*, accompanying paper). pSA0331 carries in addition, the *repC3* allele, a temperature-sensitive mutation (Novick *et al.*, 1982). Both of these plasmids overcame *copA* inhibition of pSA5000 by pRN6116 at 32°C (Figure 4, tracks 5 and 6); however, at 43°C, the restrictive temperature for pSA0331, replication of both pSA0331 and pSA5000 were abolished in parallel (Figure 4, track 8), whereas replication of pRN8032 and pSA5000 continued normally (Figure 4, track 7). It is noted that pSA5000 ordinarily complements the *repC3* alelle of pSA0331 at the restrictive temperature (Novick *et al.*, 1982). These results suggest that all of the repC activity in the pSA0331 strain is temperature sensitive, i.e., the wild-type *repC* gene of pSA5000 is not expressed in the presence of an active *copA* gene.

Discussion

Here we have identified genetically two basic elements of the pT181 plasmid replication control system. The first of these was revealed as an incompatibility determinant that inhibits the replication of pT181 *in trans*. Because plasmid mutants resistant to this inhibition have elevated copy numbers, it was inferred that the inhibition is involved in copy control. Consistent with this inference is its gene dosage dependency in the physiological range. That is, the normal copy number of pT181 is 20−25; the wild-type plasmid and recessive copy mutants can replicate in the presence of a few extra copies of the inhibitor gene *in trans* but not in the presence of 50−60 copies (Table II). The quantitative stoichiometry of this gene dosage dependency is currently under study; results so far are consistent with a simple inverse proportionality between inhibitor gene dosage and replication rate.

The second element, defined by inhibitor-insensitive mutants, is the genetic target of the inhibition, which may or may not be equivalent to a physical binding site. Deletion mapping has shown that inhibitor and target determinants are quite closely linked and sequence analysis has shown that recessive and dominant copy mutations are interspersed (Carleton *et al.*, accompanying paper), suggesting that the two determinants overlap.

The direct function of this control system is evidently regulation of the synthesis of the replication protein RepC, which could be effected at the level either of transcription or of translation. The strongest evidence for this is the data presented in Figures 3 and 4: RepC production by pSA5000 is shut off by the pT181::pE194 co-integrate that expresses *copA* at an elevated gene dosage. This interpretation has two important implications: first, it requires that RepC be rate-limiting for pT181 replication. A direct test of this requirement is clearly critical for a definitive understanding of the system and is currently in progress. The fact that RepC is active *in trans* (Novick, *et al.*, 1982) means that its synthesis is not as tightly coupled to replication as appears to be the case for the R1 *repA* protein (Oertel *et al.*, 1979; Masai *et al.*, 1983). Second, the simplest interpretation of the fact that the inhibitor acts *in trans* to inhibit RepC synthesis is that the inhibitor is a diffusible substance.

Examination of the DNA sequence in the *copA* region has not revealed any reading frame capable of encoding a significant polypeptide, and results presented elsewhere (Kumar and Novick, 1984) have demonstrated two short transcripts encoded in this region whose presence is 100% correlated with CopA activity. Our overall working hypothesis, then, is that pT181 replication is negatively regulated by either or both of two short transcripts that act by controlling the rate of synthesis of a unique replication protein, RepC. pT181 is different from the two other known groups of inhibitor-target plasmids in an important respect: the product of the inhibited step in the pT181 replication control circuit is a *trans*-active protein, RepC, whereas with both of the other groups, the corresponding product is *cis*-specific.

Such *cis* specificity has several important physiological consequences: (i) the inhibition cannot be overridden *in trans*, (ii) dominant copy mutants show unilateral segregational incompatibilty with wild-type and recessive mutants (Uhlin and Nordström, 1975; Danbara *et al.*, 1981) and (iii) mutations changing the specificity of the inhibitor-target interaction generate new incompatibility groups. Plasmids such as pT181 differ from the ColE1 and IncFII types in all three of these properties; indeed, in the present paper it has been found that *copA* inhibition of pT181-like plasmids can be overridden *in trans* and in the accompanying paper (Carleton *et al.*), that dominant pT181 copy mutants show bilateral segregation incompatibility with wild-type and that mutational changes of inhibitor-target specificity do not generate new incompatibilty groups.

Materials and methods

Organisms and growth conditions

The plasmids used in this study are listed in Table III. The host strain was *S. aureus* RN450, a derivative of NTCC8325 cured of three prophages. Plasmid transfers were by transduction with phage ϕ11, (Novick, 1967) or by protoplast transformation (Chang and Cohen, 1979). Culture media and culture conditions were as described (Novick and Brodsky, 1972). *S. aureus* strains were stored in CY broth at −75°C and were grown in CY broth with vigorous aeration at the appropriate temperature. Antibiotics used for selection were erythromycin (gift of Eli Lilly and Co.), tetracycline (gift of Chas Pfizer and Co.), and chloramphenicol (gift of Parke Davis and Co.). Concentrations were as used previously (Novick *et al.*, 1982).

Plasmid inhibition kinetics were measured in CY broth in a turbidostat. Cultures were maintained at a Klett reading of 100 (540 nm filter), corresponding to 5 x 10^8 cells/ml, by manual adjustment of the flow rate. Generation times in the turbidostat were 50 min at 32°C and 35 min at 42°C. Samples (1 ml) were withdrawn and pulse-labeled with [^{3}H]thymidine (10 μCi/ml) for 5 min at intervals. These samples were used to prepare

Table III. Plasmids

Plasmid	Size, kb	Description	Copy no.[a]	Reference
pT181	4.4	Tc^r Inc3	22	Iordanescu, 1976b
pSA5000	4.0	Cm^r Inc3	22	Iordanescu and Surdeanu, 1980
pE194	3.7	Em^r Incll	55	Iordanescu, 1976a
pRN4149	3.7	Em^r Incll Tsr	Tsr^b	This paper
pRN8023	4.4	Tc^r Inc3 *cop-618*	200	Carleton *et al.*, accompanying paper
pRN8026	4.4	Tc^r Inc3 *cop-621*	200	Carleton *et al.*, accompanying paper
pRN8061	4.4	Tc^r Inc3 *cop-623*	400	Carleton *et al.*, accompanying paper
pRN8109	4.4	Tc^r Inc3 *cop-632*	180	Carleton *et al.*, accompanying paper
pSA0331	4.4	pT181 *cop-632 repC3*	Tsr^c	Carleton *et al.*, accompanying paper
pSA5502	8.2	Tc^sEm^r Inc3 Incll, *in vivo* co-integrate, pT181::pE194	55	Novick *et al.*, 1981
pRN6010	8.2	Tc^r EmR Inc3 Incll, *Xba*I clone, pT181::pE194, orient. A	55	This paper
pRN6012	8.2	Tc^s mutant of pRN6010	55	This paper
pRN6050	8.2	*Xba*I clone, orient. B	55	This paper
pRN6054	8.2	Tc^s mutant of pRN6050	55	This paper
pRN6019	6.2	Δ154 Tc^s - 2 kb del. of pRN6010	55	This paper
pRN6038	8.1	Δ141 *in vitro* del. of pRN6012	55	This paper
pRN6116	6.6	Δ142 *in vitro* del. of pRN6012	55	This paper
pRN6157	7.7	Δ143 *in vitro* del. of pRN6012	55	This paper
pRN8008	4.2	Δ144 spont. del. of pT181	800	Khan *et al.*, 1981
pRN6158	7.9	Δ240 *in vitro* del. of pRN6012	(60)	This paper
pRN6146	7.8	Δ241 *in vitro* del. of pRN6012	55	This paper
pRN6039	6.2	Δ242 *in vitro* del. of pRN6012	(60)	This paper
pRN6154	7.6	Δ243 *in vitro* del. of pRN6012	(60)	This paper
pRN8062	4.2	Δ255 spont. del. of pT181	340	Carleton *et al.*, accompanying paper
pRN8110	4.3	Δ257 spont. del. of pT181	280	Carleton *et al.*, accompanying paper
pRN6256	8.0	pRN8008::pE194 *Xba*I clone, orientation A	55	This paper
pRN6308	6.0	pRN8023::pE194 *Xba*I clone, orientation A, 2 kb deletion	55	This paper
pRN6264	6.0	2 kb deletion of pRN6256 Tc^s	55	This paper
pRN6287	6.2	pT181::pRN4149 *Xba*I clone, orientation A, 2 kb deletion	Tsr^b	This paper
pRN6322	5.1	pRN4149Δ*Taq*I-C::pT181*Taq*I-A *Taq*I clone, orient. A	Tsr^b	This paper
pRN6323	4.0	pRN4149Δ*Taq*I-C::pT181(158-570) - *Rsa*I del.	Tsr^b	This paper
pRN6326	4.2	*Taq*I clone pRN4149Δ*Taq*I-C::pT181 *Taq*I-C	Tsr^b	This paper

[a]Copies per cell. Figures in parentheses were approximated visually.
[b]55 copies/cell at 32°C, ~3 copies/cell at 42°C.
[c]180 copies/cell at 32°C, <1 copy/cell at 42°C.

sheared whole-cell lysates which were analyzed by agarose gel electrophoresis. Gels were scanned for copy number determination (see below) and bands were excised and counted for determinations of incorporation rates.

Isolation of plasmid mutants

Spontaneous copy mutants of pT181 were isolated by selection for increased tetracycline resistance as described (Carleton *et al.*, accompanying paper). Tetracycline-sensitive mutants were isolated by replica plating of EMS-treated organisms (Loveless and Howarth, 1959). Mutants with thermosensitive replication defects (Tsr) were isolated from nitrosoguanidine-treated cultures as previously described (Novick, 1974).

Plasmid DNA isolation and analysis

Plasmid DNA was isolated by two cycles of dye-cesium centrifugation of cleared lysates prepared as described previously (Novick *et al.*, 1979). Purified preparations were stored in 1 mM Tris, 0.1 mM EDTA, pH 7.8, at −20°C. Sheared whole-cell minilysates were prepared by the method of Eckhardt (1978) and analyzed on 1.0% horizontal agarose slab gels in Tris-borate-EDTA buffer. Plasmid DNA was quantitated by direct fluorescence densitometry of the ethidium bromide-stained gel patterns (Projan *et al.*, 1983) and copy numbers calculated from ratios of plasmid-to-chromosomal DNA as previously described (Projan *et al.*, 1983). In this method, total chromosomal DNA and total plasmid DNA are determined by integration of the scanner peaks with correction for the reduced ethidium bromide binding of supercoiled species as compared with relaxed DNA. Ratios of plasmid-to-chromosomal DNA are converted to copy numbers on the basis of predetermined figures for DNA per cell (Carleton *et al.*, accompanying paper) and plasmid mol. wt.

Enzymes

Restriction endonucleases were purchased from New England Biolabs (NBL) or Bethesda Research Laboratories (BRL) and were used according to the supplier's instructions. T4 DNA ligase was prepared by the method of Murray *et al.* (1979). *Escherichia coli* polymerase I and exonuclease III were from BRL, S1 nuclease was from Miles Laboratories.

Enzymatic reconstructions with DNA in vitro

Molecular cloning was performed by ligation of restriction fragments followed by protoplast transformation. Fragments were extracted from agarose by the freeze-squeeze method (Thuring *et al.*, 1975) and from acrylamide by homogenization (Maxam and Gilbert, 1980). DNAs to be joined were mixed in equimolar amounts at a concentration of ~1 μg/ml in ligase buffer (Murray *et al.*, 1979) plus one unit of T4 ligase per μg of DNA. Ligations were incubated overnight at 12.5°C, analyzed by agarose gel electrophoresis, and then used directly for transformation of protoplasts (Chang and Cohen, 1979).

For the enzymatic generation of deletions (Heffron *et al.*, 1977), a 2 μg sample of plasmid DNA was digested with a single-site restriction enzyme and the resulting linear DNA phenol extracted, ethanol precipitated and redissolved in 100 μl of buffer containing 67 mM Tris, pH 7.8, 90 mM NaCl, 4 mM $MgCl_2$, and 4 mM dithiothreitol (DTT). Exonuclease III (20 units) was then added and successive 18 μl samples were removed after various times (1 – 60 min) at 23°C. The exonuclease III reaction was terminated by the addition of 2 μl of 0.5 M acetate buffer, pH 4.5, containing 0.5 M NaCl, 60 mM $ZnSO_4$ and S1 nuclease (0.22 units). This reaction mixture was incubated for 2 h at 23°C and then phenol extracted, precipitated with ethanol and redissolved in 100 μl of 62.5 mM Tris, pH 7.7 plus 6.25 mM $MgCl_2$, 20 mM DTT, 66 mM ATP, 33 μM of each of the four deoxynucleotide triphosphates, and 1 unit of *E. coli* polymerase I. After incubation for 15 – 30 min at 37°C, 2.5 units of T4 ligase were added and the preparation was incubated at 12.5°C for 18 h. Such preparations were then used to transform protoplasts.

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