Replication Control and Other Stable Maintenance Mechanisms of Plasmids

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INTRODUCTION

The majority of gram-negative and gram-positive bacteria contain plasmids which are distinguished by their physical separation from the chromosome of the host cell as well as by their stable maintenance in this extrachromosomal state. As the result of a wide range of studies of plasmids in diverse bacteria, it is clear that these extrachromosomal elements determine a remarkable array of phenotypic traits in bacteria of medical, agricultural, environmental, and commercial importance. Thus, to fully understand the genetic and phenotypic properties of a bacterium, one must include an understanding of the one or more plasmid elements stably maintained in that organism. Because of both the similarities and differences in the mechanisms of initiation and regulation of replication of plasmids of gram-negative and gram-positive bacteria, a true understanding of the basic biology of plasmids is greatly enhanced by an examination of properties of plasmids from both major classes of bacteria. To keep within the space confines of this chapter, we have restricted ourselves to the initiation and regulation of plasmid replication and other mechanisms responsible for the stable maintenance of plasmids.

The early studies on plasmids, for the most part carried out in gram-negative bacteria, established two types of plasmids on the basis of overall genetic content. One type, designated nonconjugative or nontransmissible, has genes that are required for the initiation and regulation of autonomous replication but do not possess a functional set of genes that are required for conjugal transfer. If additional genes that provide a distinctive phenotype for the host are present, the plasmid is often given a specific designation. For example, a plasmid of *Escherichia coli* that carries a set of genes involved in colicin production is designated a Col plasmid. If the additional genes determine resistance to one or more antibiotics, then the element is usually designated an R plasmid. Col plasmids and R plasmids are broadly distributed, both geographically and with respect to their presence in a wide variety of nonpathogenic and pathogenic bacteria. The second type of plasmids is the conjugative or self-transmissible elements that are also designated sex factors. Plasmids of this class possess a functional set of genes that are involved in conjugal mating and DNA transfer as well as those required for autonomous replication. These two categories of plasmids are found in both gram-negative and gram-positive bacteria.

The classic early studies with the F sex factor of *E. coli,* in addition to establishing sexuality in bacteria, provided the basis for the development of fundamental concepts of molecular biology, including the role of repressors in the control of gene expression and the concept of an operon. Early studies with R plasmids underscored the importance of these extrachromosomal elements in the worldwide dissemination of antibiotic resistance in a wide range of bacteria, as well as the increased incidence of plasmid-specified multiple antibiotic resistance in response to increased use of antibiotics in human and veterinary medicine and in agriculture. Later work on R plasmids was key to the development of the concept of a transposable element and a mobile insertion sequence. There is also little question that R plasmids, along with several other plasmid elements including Col plasmids, provided one of the key factors in the development of gene cloning techniques.

Plasmid elements generally are in the form of double-stranded circular DNA molecules that are covalently intact and are isolated in the form of supercoiled DNA. As more attention has been given in the past few years to bacteria other than *E. coli,* linear double-stranded DNA plasmids also have been

discovered and characterized. Most plasmid elements of gram-negative bacteria are of the narrow host range type in that they are stably maintained in their natural host or closely related bacteria but fail to replicate or regulate their copy number in distantly related bacteria. Several well-characterized plasmids in gram-negative bacteria, however, have been found to have an extended host range, e.g., plasmid RK2 and other members of the IncP1 family and plasmid RSF1010. While these plasmid elements are capable of replicating in most gram-negative bacteria, their host range for controlled replication does not extend to the gram-positive bacteria. Many naturally occurring plasmids in gram-positive bacteria exhibit an extended host range within the gram-positive bacteria. With rare exceptions, a naturally occurring plasmid is incapable of controlled replication in both gram-negative and gram-positive bacteria.

Originally, plasmids were classified based on their incompatibility group (Inc), which is a property of the mechanisms by which they control replication initiation and stable inheritance. Plasmids of the same incompatibility group share these functions and as a consequence they cannot be stably coinherited. This is caused by two features of bacterial plasmid maintenance: random selection for replication and random assortment for partitioning. Classification of plasmids based on incompatibility is useful for practical purposes; e.g., in strain construction only compatible replicons can be stably introduced into the host. This chapter, however, will consider plasmids according to their structural features as well as their modes of replication. It will also include, where relevant, the plasmid form of the *E. coli* phages P1 and λ (λdv).

While plasmids vary considerably in size and copy number, in a particular bacterial host growing under specific conditions a given plasmid is maintained at a defined copy number. The key factor in the maintenance of a plasmid in the extrachromosomal state is the ability of the plasmid to control the frequency of replication initiation such that there is on the average one replication event per plasmid copy per generation. Studies of the regulation of plasmid replication involving a number of different replicons clearly show that plasmids employ different strategies to assure their maintenance in a host cell at a characteristic copy number, with similar mechanisms being used by both gram-negative and gram-positive bacteria. These different regulatory mechanisms have in common the ability to correct the stochastic fluctuations in plasmid copy number that occur during cell growth and division.

The responsiveness of a particular regulatory mechanism may on occasion fail in a particular cell to correct for a lower than normal copy number, with the net result that plasmidless daughter cells are produced through random segregation of the plasmid copies during cell division. To correct for this inefficiency in the regulation of plasmid copy number and for the relatively high probability of generating plasmidless cells by random segregation, particularly in the case of a low-copy-number plasmid, many plasmids encode genetic systems to assure stable retention in a growing bacterial population. For some plasmids a *cis*-acting partition mechanism ensures that each daughter cell receives at least one copy of a plasmid molecule after cell division. Advances in our understanding of the genetic elements that code for a plasmid partition mechanism have come largely from the analyses of plasmids of gram-negative bacteria; however, it is likely that similar mechanisms are specified by plasmids of gram-positive bacteria. Additionally, several plasmid-based toxin/antitoxin systems that cause the inhibition or death of plasmid-free segregants have been well characterized. These killing systems have been shown to involve the production of a protein toxin that acts intracellularly to cause the death of a plasmidless daughter cell. The mechanisms of plasmid-specified copy-number control, partitioning, and postsegregational cell killing that contribute to stable maintenance of plasmid elements in gram-negative and gram-positive bacteria are the major themes of this chapter.

Since it is not possible to adequately treat all aspects of plasmid maintenance in bacteria, we refer the reader to several recent reviews on various aspects of plasmid biology that will provide additional details and in some cases different perspectives on this subject (31, 49, 81, 86, 99, 114, 125, 155, 166, 185, 187, 195, 224, 228–230, 268, 296, 320, 338; G. del Solar, M. J. Ruiz-Echevarria, M. Espinosa, and R. Diaz, in press).

PLASMID REPLICATION AND ITS REGULATION IN GRAM-NEGATIVE BACTERIA

Structural Features of Plasmid Replicons

Regardless of plasmid size, the basic replicon of a plasmid generally consists of a contiguous set of information that includes a definable origin where DNA replication initiates and one or more adjoining controlling elements. This information is often contained within a segment that is 3 kb or less in size. The compactness of most plasmid replicons has certainly facilitated their isolation and characterization. The origin of replication (*ori*) has been delineated for most plasmids to within a several-hundred-base-pair segment. It contains recognition sites for those plasmid and host proteins involved in replication initiation. In addition, most replicons encode a structural gene, often designated *rep*, for a plasmid-specific protein that, in those cases studied, is required for the initiation of replication. Generally the Rep protein is capable of acting in *trans;* however, there are well-studied systems (e.g., plasmid R1) where this protein preferentially acts at an origin in *cis* to the *rep* gene. For most plasmids of gram-negative bacteria that have been characterized, replication proceeds from the origin in a unidirectional or bidirectional mode via a theta replication mechanism. However, a few plasmids of gram-negative bacteria have been shown to replicate via a rolling-circle (RC) mechanism (e.g., references 156 and 337). This is unlike the situation with gram-positive bacteria, where the plasmids characterized to date predominately replicate via an RC mechanism that involves the formation of a single-stranded circular DNA intermediate.

Most plasmids have a single origin of replication. However, in the well-studied case of the theta replicating plasmid R6K, three origins of replication have been defined under the control of a single replication initiation protein (54, 131). All three origins and the initiation protein gene are contained within a 4-kb segment within this 38-kb plasmid. In addition, as a consequence of more detailed studies of relatively large plasmids, an increasing number of plasmids of gram-negative bacteria have been shown to contain more than one replicon (53). For example, the various members of the IncFI family, including the classic F plasmid, contain either fully functional systems or remnants of three independent replication systems (22). The best-studied replicon in the F plasmid, designated FIA, has two origins of replication, with both origins controlled by the RepE initiation protein. An FIA-related replicon is also found on some IncH1 plasmids, while the *ori-2* origin of the FIA replicon is found on a naturally occurring plasmid of *Klebsiella pneumoniae* (58). This *Klebsiella* plasmid, pGSH500, also contains a pCU1-related replicon (58). A further demonstration of the variety and plasticity of plasmids is seen in the IncN plasmid, pCU1, which has a broad host range and contains three origins of replication clustered within a 1.2-kb region (15, 152).

A variety of plasmid replicons isolated from gram-negative and gram-positive bacteria have been characterized with respect to the key elements controlling the initiation of replication. In each case, a negative feedback control system has been identified which is responsible for maintenance of the plasmid at a steady-state copy number that is characteristic of the particular plasmid in a given host bacterium. In the case of broad-host-range plasmids this copy number may vary depending on the bacterial species. Any plasmid copy-control system must respond to upward or downward fluctuations in copy number during exponential growth of the bacterium and also allow for a relatively rapid rate of initiation of replication of a particular plasmid element after its entry into a cell via transformation, transduction, or conjugation. Despite the variety of replicons, two basic schemes for the regulation of replication of plasmid copy number have been elucidated. In one scheme the plasmid replicon encodes a small, diffusible RNA molecule that acts as an antisense transcript that negatively regulates a key step in the replication of the plasmid. The second basic mechanism is found in plasmids, for the most part in gram-negative bacteria, that contain a series of direct repeats, designated iterons, that are located within the functional sequence of nucleotides that make up the origin of replication and are essential for both the initiation of replication and its control. These direct repeats and the plasmid-specified replication initiation protein which binds to these iterons make up the essential elements for replication control of iteron-containing plasmids.

RNA-Regulated Plasmids

The ColE1-type and the IncFII-type plasmids of *E. coli* are the best understood replicons with respect to the regulation of initiation of replication (47, 74, 155, 166, 210, 228, 245, 329; del Solar et al., in press). In both cases the regulatory element is a small RNA molecule; in the ColE1-type plasmids this molecule regulates the functionality of an RNA primer that is necessary for replication initiation, while for the IncFII-type plasmids the regulatory RNA molecule controls the synthesis of the essential *cis*-acting replication initiation protein. In addition to the extensively studied plasmid ColE1, the ColE1-type family includes the naturally occurring plasmids p15A, RSF1030, pMB1, and CloDF13 as well as the cloning vectors pACYC184, pBR322, and related plasmids (166, 229). Unlike most plasmids in gram-negative bacteria, the ColE1 family of plasmids do not encode a replication initiation protein, and they require the host DNA polymerase I (Pol I) for early synthesis of the leading strand. Replication initiation involves the synthesis of a 700-base preprimer RNA, designated RNA II, whose transcription begins 555 bases upstream of the origin (defined as the transition point between primer RNA and DNA) (Fig. 1) (304, 305). The 3′ end of the RNA II molecule "couples" with the DNA molecule at the origin of replication by hybrid formation. The formation of a functional coupled complex is dependent on the generation of a specific secondary structure at the 5′ end of the RNA II molecule that in turn allows the formation of a stem-loop at the 3′ end of the RNA II-coupled complex that is recognized by RNase H (304, 305, 306). Cleavage of the RNA-DNA hybrid then occurs at the replication origin, releasing a 3′-hydroxyl group that serves as the primer for DNA synthesis catalyzed by Pol I. Replication then proceeds unidirectionally with the initiation of lagging-strand synthesis at specific ColE1 sequences. It has been further shown for ColE1 that there are two alternative modes of replication that allow for replication in the absence of either RNase H or both RNase H and Pol I (56). However, regardless of the replication mode, RNA II must be produced in the correct structural configuration to act as the primer for DNA replication.

Copy-number control of ColE1 is mainly dependent on the 108-bp RNA I transcript. RNA I binds to nascent RNA II by complementary base-pairing and thereby prevents the formation of the hybrid between RNA II and the DNA template that is recognized by RNase H. While the ColE1 plasmid does not encode a replication initiation protein, it does specify a protein (Rop) that lowers the copy number of the plasmid by stabilizing the RNA I-RNA II complex by binding to both RNA molecules (42, 121, 303, 306, 313). In addition to the role of the Rop protein in the regulation of ColE1 copy number, the rate of decay of RNA I clearly is a major factor in copy-number determination. It has been shown recently that the decay rate of RNA I is determined to a major extent by polyadenylation of the transcript (333) and by RNase E cleavage (179).

The requirement of Pol I for DNA replication, while infrequently found among plasmids, is not restricted to the ColE1 family. Plasmid pCU1 exhibits a requirement for Pol I for one (*oriV*) of the three functional origins of this plasmid; replication originating at the other two origins apparently is Pol I independent (152). Colicinogenic plasmids ColE2 and ColE3 also require this polymerase for replication (286). Interestingly, these relatively small plasmids, with the smallest defined origin sequence discovered to date (less than 50 bp), also specify a replication protein that is essential for replication (126, 223, 336). Finally, the naturally occurring gram-positive bacterial plasmid pAMβ1 also shows a requirement for Pol I (see below).

IncFII plasmids, including R1, NR1, and R6-5, are another well-studied group in which the key element in the regulatory apparatus for the initiation of replication is a small antisense RNA molecule (28, 227, 228, 261, 329). Unlike the ColE1 family, however, replication is controlled by the rate of synthesis of a plasmid-encoded replication protein (RepA). Plasmid R1 is maintained at a copy number of 1 to 2 per chromosome. The low copy number of this family of plasmids makes it especially important to have a regulatory control circuit that rapidly responds to changing copy number. As shown in Fig. 1, the minimal replicon (approximately 2.5 kb within the 100-kb plasmid) contains two promoters (*copBp* and *copAp*) that regulate transcripts of the *repA* gene initiating at a third promoter, *repAp*. The *copB* promoter is constitutively

active, while *repAp* is repressed by the small protein product of the *copB* gene. RepA protein production is also controlled by the 80-base antisense RNA product of *copA*. The homology between the RepA mRNA leader region, referred to as CopT, and CopA allows the two RNA molecules to form a specific RNA duplex. The formation of this duplex results in posttranscriptional control of RepA protein synthesis. Thus copy number of the R1 group of plasmids is determined by the rate of synthesis and degradation of RepA mRNA, the repressor activity of the CopB protein at the *repA* promoter, the concentration of the CopA RNA (which is determined by the rates of synthesis and decay of CopA RNA), the rate of formation of the RNA duplex of CopA and CopT, and the efficiency of translation of the RepA mRNA (228). Since it is estimated that the half-life of CopA is of the order of 1 to 2 min, the concentration of CopA is proportional to plasmid copy number. The interaction of these various regulatory elements results in the sensing of plasmid copy number during cell growth and provides for rapid adjustments in replication initiation frequency to reestablish the steady-state copy number. It is of interest that plasmid R1 is a case where a low copy number is corrected by an active mechanism through action of the *copB* promoter. This results in an increase in mRNA for RepA and a decrease in CopA RNA production because of convergent transcription. A detailed kinetic analysis of the interaction of these various elements in the control of replication of the R1 replicon has recently been carried out (228).

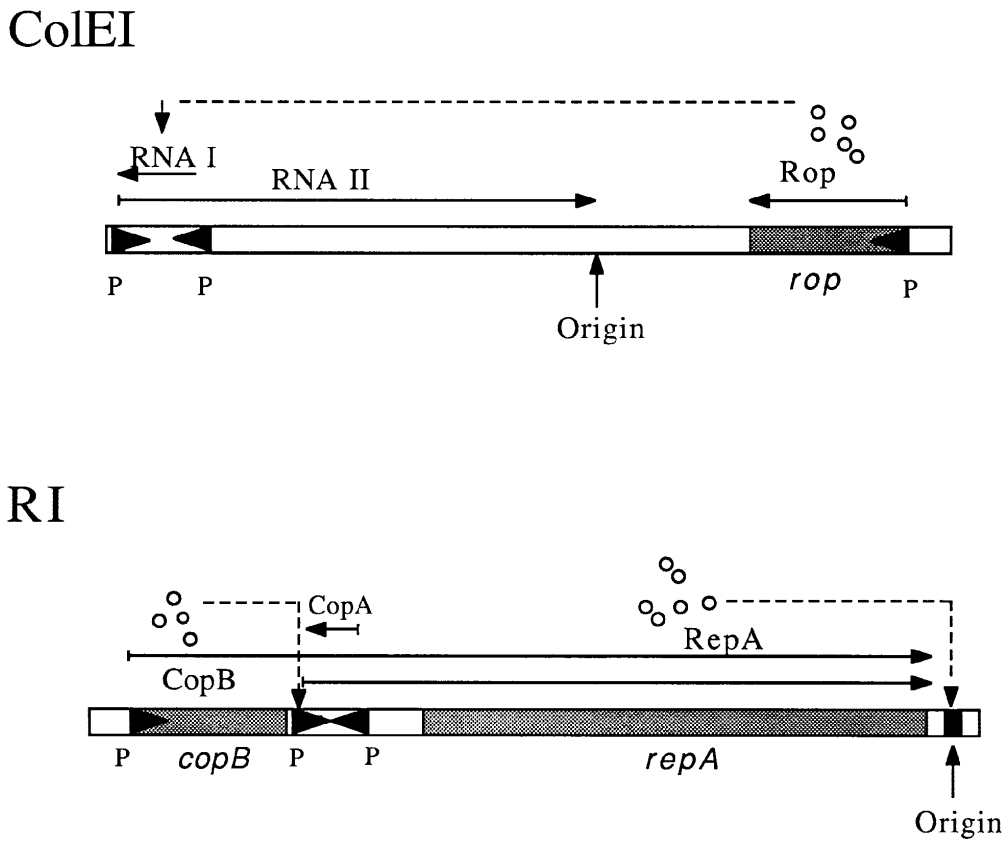


FIGURE 1 Replicons for two types of RNA-regulated plasmids. Bars represent the replication regions of plasmids Co1EI and RI. Promoters are noted by the letter P and the large arrowheads within the bars. The solid arrows above the bars are RNA transcripts. The stippled regions within the bars are protein-coding regions, and the proteins produced are depicted as circles. The dotted lines indicate the site of action for each protein. Some of the basic features of these models have been illustrated previously (47, 155, 228).

Iteron-Regulated Plasmids

The majority of plasmids in gram-negative bacteria that have been isolated and characterized to date contain one or more clusters of direct repeats within the replication origin region. These direct repeats, designated iterons, have been shown to provide specific multiple binding sites for the plasmid replication initiation protein. Additional direct and/or indirect repeats are also often found in the origin region of iteron-regulated plasmids (Fig. 2). These replicons invariably also encode a *trans*-acting replication initiation protein (often designated Rep), an A+T-rich region adjacent to the iterons, and generally one or more sites related to the consensus DnaA-binding site. The host-encoded DnaA protein is essential for chromosomal as well as plasmid replication. In *E. coli* it serves as the initiation protein for *oriC* by binding to the DnaA boxes in the origin (30). The iteron cluster in the minimal origin region consists of direct repeats generally of a size of 17 to 22 bp with a spacer nucleotide sequence between the smaller iteron sequences to provide a distance of approximately 22 bp between identical iteron sequence positions. This arrangement allows binding of the plasmid initiation protein to the iterons in the same phase of the DNA helix. The number of iterons in a fully functional origin varies depending on the plasmid element; e.g., plasmid pSC101 contains three iterons (52) while plasmid R6K contains seven (280). Deletion of two of the seven iterons within the R6K origin results in a significant loss of origin activity (160).

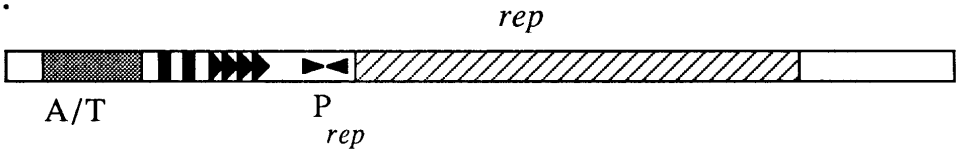
A number of iteron-containing plasmids contain a second cluster of direct repeats that is nonessential for replication but plays an important role in copy number control; i.e., deletion of the auxiliary cluster of repeats has been shown for several plasmid systems to result in a higher plasmid copy number and, in some cases, destabilization of the plasmid (5, 145, 219, 234, 297). The orientation of the repeats and the spacing between the repeats are variable in the auxiliary cluster, unlike the iterons within the origin.

The plasmid Rep protein gene is usually located adjacent to the replication origin (83). The Rep protein itself is generally of the size of 30 to 40 kDa and is usually found to be dimeric in solution (2, 45, 85, 176, 186, 190). As discussed below, however, for several plasmid initiation proteins the active form of the Rep protein for binding to the iterons has been shown to be the monomeric form (136, 186, 326). In the case of some plasmids, but not for all, the expression of the *rep* gene is autoregulated (51, 80, 259, 290, 318, 334). The binding site in the promoter is usually indirect repeats of sequences related to the plasmid iteron sequences. Although the Rep protein is required in a positive sense for the initiation of replication, in the case of plasmids R6K (84), P1 (239), and pSC101 (128), substantial overexpression of the Rep protein can result in the inhibition of replication. Mutants of the Rep proteins for plasmids R6K (130, 193, 279), RK2 (71, 76, 119), F (23, 122, 136, 270), P1 (277), Rts1 (339), R1 (209, 235a), ColV-K30 (94), and pSC101 (332) have been isolated which result in the plasmid being maintained at an increased copy number from severalfold to 20-fold or more. In several cases the copy-up phenotype has been shown to be suppressed by the presence of low levels of the wild-type Rep protein (71, 122, 192, 279). These observations have led to the proposal that the Rep proteins of iteron-containing plasmids exhibit both positive and negative activities. A model to account for these activities of the plasmid initiation protein is discussed below.

Plasmid Initiation Proteins

A general model for the initiation of DNA replication that is applicable to plasmid systems where replication occurs by the theta mode involves the following sequence of events (Fig. 3 and 4). The replicon-specific initiation protein recognizes and binds to specific sequences (e.g., iterons) at the replication origin and effects structural changes such as bending and/or localized melting of an adjacent A+T-rich region, unwinding of the duplex DNA, or both. The initiation protein, perhaps in concert with the host DnaA protein, guides host replication proteins into the open complex. This prepriming complex establishes the starting point for replication to proceed. The prepriming complex, designated as a specialized nucleoprotein complex, consists then of both plasmid-encoded and host-chromosomally encoded proteins in association with origin DNA sequences.

A.



B.

Iteron-containing plasmid	No. of iterons in minimal origin	No. of bp per iteron	Rep protein size (kDa)	Reference(s)
E. coli				
Narrow host range				
pSC101	3	18	37	9, 52, 317
R6K	7	22	35	281
F	4	9	29	219, 300
P1	5	19	32	5
Rts1	3	21	33	138, 144
pColV-K30	5	18	39	242
λdv	4	19	34	111, 309
pIP522	12	17	32	92
Broad host range				
R1162/RSF1010	3.5	20	31	118, 197
RK2	5	17	44/33	275, 282
pSa	2	13	36	236
RA1	13	19	33	181
Other bacteria				
Pseudomonas syringae pPS10	4	22	27	220
Chlamydia trachomatis pCTTI	4	22	30	278
Lactobacillus lactis pWV02	3.5	22	42	150
Neisseria gonorrhoeae pF3A	4	22	39	107
Clostridium perfringens pIP404	14+6	8+16	49	95

FIGURE 2 Iteron-containing plasmids. (A) Schematic of the replicon for an idealized iteron-containing plasmid. This diagram does not depict any specific origin; the replication regions of individual plasmids will vary. Within the minimal origin, there is an A+T-rich region (the shaded box), and a cluster of iterons (arrowheads). The *rep* gene sequence is often located near the origin (hatched box) and in the case of some plasmids is preceded by a pair of inverted repeats that serve as sites for autoregulation. (B) Compilation of iteron-containing plasmids. All plasmids listed have iterons within the origin; the requirement for these iterons for origin activity and/or binding of the Rep protein to the iterons has not been demonstrated in all cases. Those plasmids in which origin oterons have been shown to be involved only in copy-number control are not included (i.e., plasmid pCU1 or R27). The numbers and sizes of the iterons are given. The size of the Rep protein, in many cases, is deduced from the nucleotide sequence of the gene only. Plasmid pWV02 is representative of a family of related plasmids (see Table 1). Some of the basic features of this figure have been illustrated previously (155).

It is clear that all plasmid systems depend substantially or almost entirely on host DNA replication proteins (163). In most cases the plasmid specifies only the replication initiation protein. An extreme case is the ColE1-type plasmids, where replication initiation is totally dependent on host proteins (47, 74). In the case of plasmid RSF1010 and the related plasmid R1162, however, two proteins, in addition to the initiation protein RepC, are encoded by the plasmid and are required for replication (88, 118, 166). The RSF1010-specified proteins RepA and RepB exhibit helicase and primase activities, respectively. The encoding of these two additional enzymatic activities by the RSF1010 plasmid undoubtedly is of importance to the broad-host-range properties of this plasmid. Plasmid RK2, on the other hand, encodes two replication initiation proteins (TrfA-44 and TrfA-33) by using two translational start sites in the same reading frame within the *trfA* gene (162, 273, 275). While the 33-kDa TrfA protein appears to be sufficient for the initiation of replication of this plasmid in most gram-negative bacteria, the 44-kDa protein is essential for replication of this plasmid in *Pseudomonas aeruginosa* (69, 273). Thus, in this case the plasmid utilizes two forms of the initiation protein in order to extend its host range. It is remarkable that the 33-kDa TrfA protein alone is capable of initiating replication of this plasmid in a wide range of gram-negative bacteria, indicating an ability of this protein to adapt to the host replication machinery of a variety of distantly related bacteria.

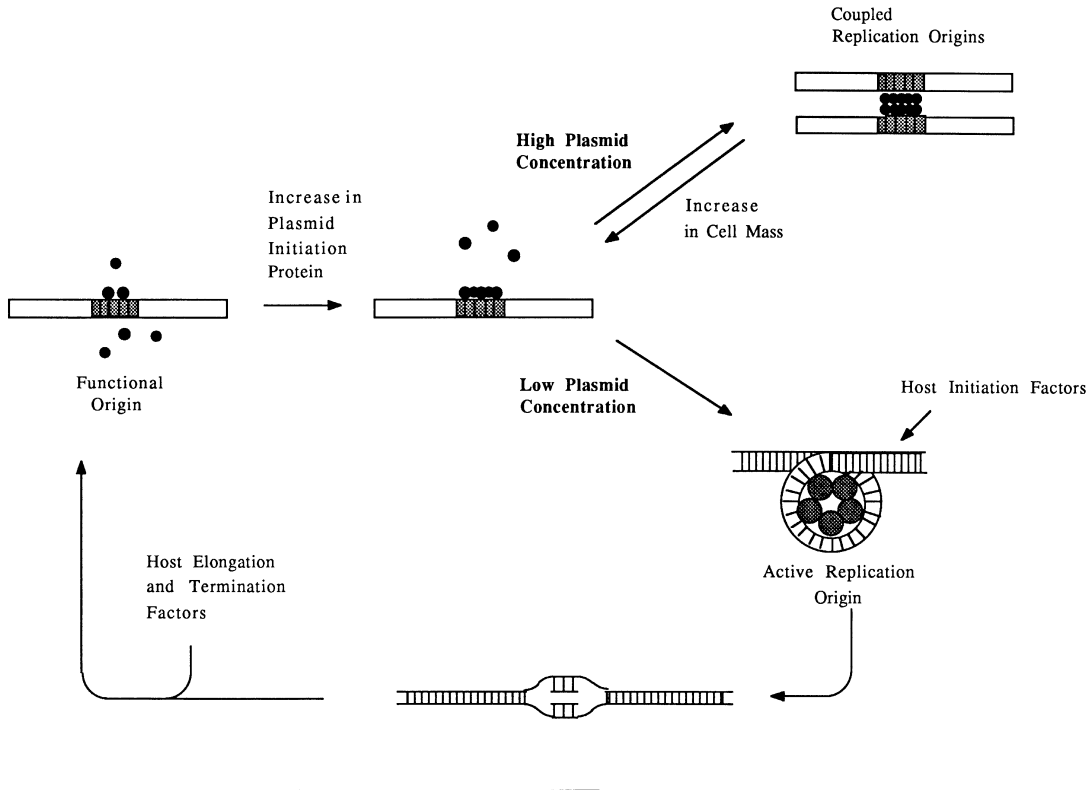


FIGURE 3 Model of steps involved in the initiation of replication and copy-number control in iteron-containing plasmids. Iteron-containing origin DNA is shown by horizontal ladders. Steps shown include binding of the plasmid Rep protein to iterons, nucleation and formation of a preinitiation complex (at low plasmid concentrations), and reversible intermolecular coupling of Rep protein-bound iterons (at high plasmid concentrations). Some of the basic features of this model have been illustrated previoysly (155).

Figures 3 and 4 illustrate several of the key steps in the initiation of replication of iteron-containing plasmids. While the major form of purified plasmid initiation proteins is a dimer in solution, it is now documented for plasmids P1 (326), F (136), and pSC101 (186) and indicated in the case of plasmid pPS10 (220) that the active form of the replication protein that binds to the iterons at the origin of replication is a monomer. *E. coli* heat shock proteins have been shown to promote the disassembly of the dimeric form of the RepA protein of plasmid P1 (326) in addition to activating the monomeric form for binding to the origin iterons (49, 57, 218). Evidence has been obtained for a similar role of the heat shock proteins in the case of the RepE initiation protein of the F plasmid. It has been suggested that the leucine-zipper motif of the RepA protein of pPS10 is involved in the dimerization of this protein (del Solar et al., in press). For several plasmids in which the expression of the initiation protein is autoregulated, it has been found that the dimer is the active form for binding to the promoter-operator region of the initiation protein gene (81, 136, 186, 220).

Binding of the plasmid Rep protein to the cluster of direct repeats at the origin has been readily demonstrated for a variety of iteron-containing plasmids by utilizing gel mobility shift electrophoresis or DNA footprinting. In the cases examined, the binding of Rep proteins to the iterons appears to be noncooperative. Interestingly, in the case of the replication initiation protein π of plasmid R6K, a single amino acid substitution changes the binding from a noncooperative to a cooperative mode (84a). There is no general pattern, however, with respect to an iteron-binding domain among the various Rep proteins. A mutational analysis of the RK2 TrfA protein resulted in the isolation of a number of DNA-binding mutants that had a single amino acid substitution distributed over a 20% region of the protein towards the N-terminal end of the protein (46, 176). In these same studies, however, it was found that the deletion of the two C-terminal amino acids of the TrfA protein resulted in a complete loss of DNA-binding activity.

Analyses of several plasmid systems clearly indicate the formation of a specialized nucleoprotein structure as a result of binding of the replication protein to the iterons, in some cases involving several host-encoded proteins. A structure, designated the O-some, consisting of eight λ O protein monomers wrapped by the origin DNA sequence, has been visualized by electron microscopy (68). This interaction results in bending of the DNA at the origin and helix destabilization (267). A similar complex has been shown to form in the case of the origin of replication of the pSC101 plasmid, but involving in addition to the RepA protein the host-encoded proteins DnaA and IHF (integration host factor) (283, 284). A key role for the IHF and DnaA proteins in the formation of a specialized nucleoprotein structure involving origin DNA and a plasmid Rep protein has also been demonstrated for the R6K plasmid (81, 82). While the DnaA protein plays a central role in the initiation of replication of the *E. coli* chromosomal origin of replication by forming an open replication initiation complex in the A+T-rich region of the origin (30), and also has been shown to be required for replication of a variety of plasmid systems (31, 163), it must work in concert with a plasmid Rep protein to activate a plasmid origin. We are only at the beginning stages in our understanding of the interplay between the DnaA and plasmid Rep proteins in activating a plasmid origin. Evidence has been obtained for RepA protein facilitation of DnaA protein binding to the origin of the R1 plasmid (189). In this system, binding of the R1 RepA protein also results in melting of the A+T-rich region at the origin (189, 237). The RepA-DnaA complex may play a role in guiding the DnaB/DnaC complex to a loading site within the open complex at the R1 origin. Recently, an interaction between the DnaA and DnaB proteins has been demonstrated in vitro (188). Unlike the situation with the λ (267), R1 (189, 237), and R6K (216) plasmid systems, where the plasmid replication initiation protein brings about strand opening at the origin, the P1 RepA protein alone does not melt the P1 origin region (218). With the P1 system the DnaA protein, binding to the DnaA boxes flanking the cluster of iterons, carries out this essential strand-opening step. The P1 RepA protein shows some stimulation of this activity of the DnaA protein. The P1 RepA protein, however, plays a key role in binding to the iterons and forming a nucleoprotein complex involving the wrapping of the DNA around the RepA protein monomers (217). Recent studies with plasmid RK2 have shown that both the DnaA protein and the replication initiation protein TrfA bind independently of each other to their specific binding sequences at the origin of

replication (A. Blasina and D. R. Helinski, submitted for publication). Thus, while the various iteron-containing plasmids require the interaction of the Rep protein and specific host proteins at the origin of replication, the precise role of these various proteins in the activation of the replication origin varies depending on the plasmid system.

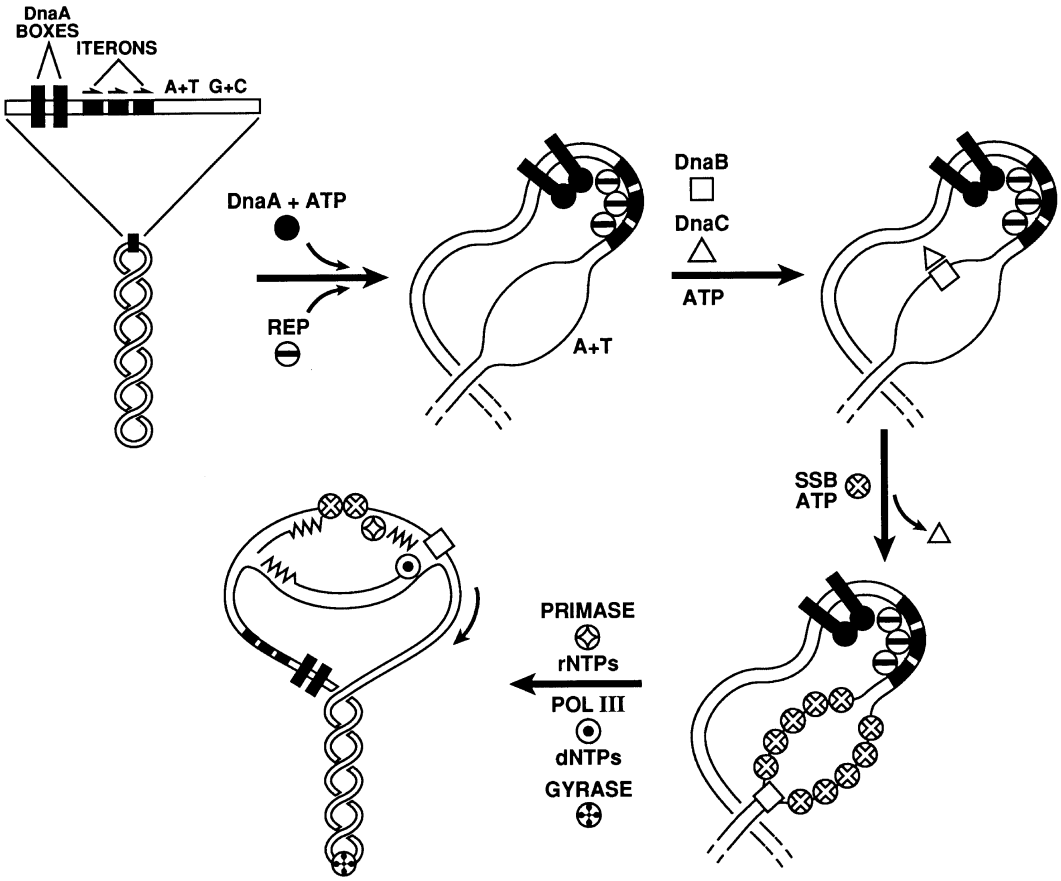


FIGURE 4 Initiation of DNA replication in an iteron-containing plasmid. In this model the various proteins indicated are host specified except for the plasmid-encoded Rep protein. Some of the basic features of this model have been illustrated in other review articles (14, 31, 163, 195).

In every case examined at the in vitro level, plasmids of gram-negative bacteria show a requirement for DNA Pol III for elongation of the replication fork. In the case of plasmid R6K, a specific region of the plasmid carries a replication termination signal (*ter*) that is not specific for the R6K replicon (17, 159). The *ter* sequence has been characterized and shown to interact specifically with an *E. coli* host protein designated Ter or Tus to form a functional termination complex (183).

Completion of the replication process for a theta-type plasmid further requires resolution of the catenated form of the two circular daughter DNA molecules that is generated by the elongation/termination process. The important role of the type 2 topoisomerase IV in the resolution of supercoiled catenates generated by duplication of plasmid molecules in *E. coli* has been demonstrated using an *E. coli* strain

carrying a mutation in the topoisomerase IV gene (6).

Role of Iterons in Copy Number Control

Direct repeats within the functional origin or as part of the auxiliary clusters adjacent to the origin, when inserted into a plasmid normally compatible with the iteron-containing plasmid, show strong incompatibility against the iteron plasmid (5, 145, 177, 194, 244, 299, 310, 335). In addition, the insertion of additional homologous iterons into an iteron-containing plasmid results in a reduction of copy number of the plasmid (192, 239). It was proposed initially that the incompatibility resulting from the insertion of iterons into a heterologous replicon was due to titration of a rate-limiting initiation protein (310). The fact that the initiation protein binds to the iterons and that the severity of incompatibility or copy-number reduction is proportional to the number of iterons inserted is consistent with this proposal (192, 239, 299). Furthermore, in the case of plasmid R1162 it was shown that increasing the concentration of the RepC initiation protein increased plasmid copy number and could also overcome iteron-induced incompatibility, again consistent with a titration model for the regulation of plasmid copy number (153). According to this model, replication initiates when sufficient replication protein is present in the cell to fully load the iteron-binding sites at the origin. This results in a duplication of iterons at the origin, with the consequence that there is an inadequate ratio of initiation protein to iterons for further initiation of replication until additional Rep protein is synthesized during the cell cycle.

Extensive studies carried out on the regulation of initiation of replication of the iteron-containing plasmids R6K (192, 207), P1 (3, 239), and RK2 (70, 154) have revealed properties of these plasmid systems that argue against a simple titration model for the regulation of plasmid copy number. For instance, in the case of many of these plasmid systems the expression of the *rep* gene is autoregulated (e.g., plasmids R6K [80], P1 [51], pSC101 [318, 334], and F [23, 190, 319]), while for plasmid RK2, regulatory elements outside the basic replicon region regulate the expression of the *trfA* initiation gene, thus dampening oscillations in the concentration of unbound replication protein during the cell cycle (79, 294, 295). Furthermore, increasing the concentration in vivo for the replication initiation protein for plasmids R6K, RK2, P1, pSC101, or Rts1 does not result in a proportional increase in plasmid copy number (70, 84, 128, 239, 291). For plasmid RK2, a 170-fold increase in the concentration of the TrfA protein results in only a 30% increase in copy number of a minimal RK2 replicon (70). Similarly, decreasing the level of the R6K π initiation protein to 1% of the normal level in *E. coli* (10,000 π dimers per cell) does not result in a decrease in the copy number of this plasmid (84). In response to these and other observations that are inconsistent with a simple titration model, several modifications of this model have been proposed. For example, the autoregulation paradox gave rise to the proposal that the Rep protein has a different affinity for iterons in the promoter-operator region of the *rep* gene than for the iterons at the origin, with the latter set of iterons possessing a lower affinity for the Rep protein (50). The higher affinity for the iterons controlling *rep* gene expression would result in a slower response of the *rep* promoter to decreasing concentrations of the Rep protein.

Trawick and Kline have proposed a two-form model for replication control of F (307). The autorepressor form of the Rep protein would only bind to the control locus. The form that is active in replication initiation would be generated by an irreversible conversion of a small amount of the autorepressor form to the initiation form and would bind to both the control locus and the origin. While this model has commanded some interest, it does not provide for a plasmid-specified element that controls the conversion of the autorepressor form to the initiator form in order to control the frequency of initiation of replication at the plasmid origin. As stated above, several Rep proteins have now been shown to exist as two forms, i.e., dimers and monomers. In the case of two Rep proteins, specified by the autoregulated plasmid systems F (136) and pSC101 (186), the dimer form functions as the autorepressor and the monomer form functions as the initiator protein. In these cases, however, the conversion of one form to the other is reversible.

In response to the data that have been obtained for several systems that are inconsistent with the original or a modified form of the titration model, an alternative regulatory mechanism has been proposed that involves a reversible pairing of plasmid molecules at their origin of replication, mediated specifically by the plasmid Rep protein and resulting in an inactivation of the origin. This model, called handcuffing or coupling, was first proposed for the iteron-containing plasmids R6K (192) and P1 (239). According to this model (Fig. 3), the Rep protein binds to the iteron cluster at the replication origin, and when the iterons are fully loaded, one of two events occurs, depending on the concentration of Rep-bound iterons. If the concentration of the protein-iteron complex is low, as for the case of a lower than normal plasmid copy number, replication is initiated at the origin by the plasmid Rep protein in concert with host replication proteins. However, when the copy number of the plasmid is at or above the normal level, the concentration of Rep protein-bound iterons is sufficiently high to reversibly couple all of the plasmid molecules to each other at the origin region. When the plasmid (iteron) concentration becomes sufficiently low, as in the case of cell growth without any initiation of plasmid replication, the coupled complexes dissociate into active origins. This mechanism of plasmid copy-number sensing is dependent on the iterons and the Rep protein and involves direct communication between plasmid molecules at their origin.

Both genetic and biochemical data have been obtained to support this model for the regulation of replication of plasmids R6K, P1, and RK2. The fact that increasing the concentration of Rep protein for each of these plasmid systems does not result in an increase in plasmid copy number, once the plasmid is established in the cell, is consistent with this model since it is the concentration of the iteron-Rep protein complex that is the determinant of copy number and not the Rep protein concentration alone. In addition, this model leads to the prediction that the Rep protein contains a function that is responsible for Rep-Rep association when complexed to the iterons and that this activity may be separable from the iteron-binding activity of the protein. In fact, copy-up mutants of the Rep proteins of plasmids R6K (85), P1 (218), and RK2 (46) have been obtained that are not altered in their affinity for the iterons at the replication origin, and at least in the case of the RK2 Rep protein, these mutants do not show a substantial difference in the ratio of monomer to dimer forms but are capable of maintaining the plasmid at a substantially higher copy number in *E. coli* (J. Cereghino and D. R. Helinski, unpublished data). In the case of R6K and RK2, providing a small amount of wild-type protein in *trans* suppresses this high-copy-number phenotype (71, 85, 279). Biochemical evidence in support of the handcuffing model includes the demonstration of the ability of the R6K, RK2, and P1 Rep proteins to couple plasmid molecules at their specific origins of replication (192, 215, 218; A. Blasina, B. L. Kittell, A. E. Toukdarian, and D. R. Helinski, submitted for publication). It has been further shown that some but not all copy-up mutants of the R6K Rep protein (206, 207) and all three copy-up mutants of the P1 RepA examined (218) were defective in this coupling activity in vitro. Finally, using an in vitro replication system, it was found for plasmids RK2 (154) and P1 (3) that the addition of iterons on a supercoiled heterologous plasmid would inhibit replication even in the presence of excess Rep protein. A copy-up replication initiation protein of plasmid RK2 showed substantially less sensitivity to iteron addition to the in vitro replication system, consistent with decreased activity in coupling iteron-containing molecules (154). A role for the auxiliary iterons of plasmid P1 which control plasmid copy number in the formation of coupled complexes has been demonstrated by electron microscopy (50, 239) and by iteron inhibition of plasmid replication in vitro (3). Examination of the P1 RepA protein-DNA complexes by electron microscopy showed looping between the auxiliary iteron region (*incA*) and the origin iterons. It was proposed that, like intermolecular coupling, the RepA-mediated looping can cause steric hindrance to the origin function by a similar mechanism (239).

While both genetic and biochemical data have been obtained in support of the coupling model of replication control, more direct proof for the formation of these complexes in vivo and a more consistent correlation between the copy-up phenotype and reduced activity of the replication initiation protein in the formation of paired complexes in vitro for the R6K (π) and RK2 (TrfA) Rep proteins must be obtained before this mechanism is established as the regulatory strategy for the three plasmid systems. Furthermore, it is likely that not all iteron-containing plasmids will satisfy the basic predictions of this model. A copy-up mutation in the

RepE protein of plasmid F has been shown to be altered in the proportion of monomer to dimer forms of this protein (136). Also, the concentration of the RepC initiation protein of plasmid RSF1010/R1162 in *E. coli* is limiting, and therefore, in this bacterium, plasmid copy number is increased in response to an increase in RepC protein (153). Even in the case of the three plasmid systems (R6K, RK2, and P1) where the data are most consistent with the coupling model, there is evidence supporting a role for other control elements in determining plasmid copy number (1, 81, 207, 223, 295, 331). Evidence has been obtained for a role of methylation (1) and the *E. coli* SeqA protein in the regulation of P1 replication (35a). Data also have been obtained for a role of membranes in the replication of plasmid RK2 (87, 198). It is clear that much remains to be learned before there is a clear understanding of all of the major factors controlling plasmid copy number and the mechanism of their interactions.

PLASMID REPLICATION AND ITS REGULATION IN GRAM-POSITIVE BACTERIA

Classification of Plasmids

Plasmids have been identified in most species of gram-positive bacteria, although in some important pathogens such as *Listeria monocytogenes* and *Streptococus pneumoniae* plasmids are quite uncommon. The vast majority of plasmids in gram-positive eubacteria are circular supercoiled DNA molecules ranging in size from 1.2 kb (149) to 100 kb (18, 41, 165). Linear plasmids have been identified thus far among the gram-positive bacteria only in *Streptomyces* and *Borrelia* spp.

Plasmids from gram-positive bacteria are classified primarily according to their structure and mode of replication and secondarily according to sequence homologies. Five replication types have been described. As noted, the first and commonest is the asymmetric RC (rolling-circle) type (110). Less common are plasmids known to use the theta mechanism, and these are of two very different types: the "conventional" theta, presumably using a short RNA primer, and a novel type using a long RNA primer and initiated by Pol I. The fourth and fifth classes are linear plasmids of two types, found in *Borrelia* sp. and *Streptomyces* sp., respectively (125). Although both of these genera contain circular as well as linear plasmids, the chromosomes of both are linear (178, 262). The linear plasmids of *Borrelia* have hairpin ends and are thought to replicate by a modified RC mechanism. The linear *Streptomyces* plasmids have covalently bound 5′-terminal proteins. It has been suggested that the chromosomes and linear plasmids of *Streptomyces* replicate by the same mechanism, and that this is also true for *Borrelia* sp. If these suggestions are correct, they have important implications for the long-standing question of whether plasmids primarily coevolved with the host chromosome or primarily evolved through horizontal spread from common ancestral sources.

The well-established principle that genes essential for plasmid replication and maintenance are clustered, forming a minimal replicon, has enabled the isolation and characterization of essential replication genes from a wide variety of plasmids. Sequence analysis of these has revealed homologies that can be used to define families of functionally related replicons. A compilation of these is presented in Table 1. One generalization that is immediately apparent is that RC plasmids are never very large (≤10 kb), presumably because of the structural instabilities inherent in large RC molecules (see below), whereas theta and linear plasmids have a very wide range of sizes, nearly up to that of the bacterial chromosome. Incompatibility groupings are of less utility in classification since they tend to identify only plasmids with virtually identical replicons, especially in the case of countertranscript regulation where a single base change can generate a new incompatibility type (304).

RC plasmids are widely dispersed among the gram-positive bacteria, and many families have members in very distant species, suggesting the occurrence of horizontal transfer. Additionally, many plasmids appear to have been assembled from cassettes, and these cassettes seem sometimes to have been reassorted across species lines. Thus a cassette containing a single-strand origin (SSO) that is functional in one set of species may be associated with a cassette containing a double-strand origin (DSO) (plus-strand origin) and

rep gene that have a different host range.

TABLE 1 Homologies among plasmids

Plasmid type	Family	Plasmid	Markers(s)[a]	Size (kb)	Native species	Reference
RC plasmids	pC194/pUB110	pC194	Cm	2.9	*Staphylococcus aureus*	271
		pUB110	Km	4.5	*S. aureus*	230
		pBAA1		6.8	*Bacillus subtilis*	269
		pBC16	Tc	4.6	*Bacillus cereus*	230
		p353–2		2.4	*Lactobacillus pentosus*	247
	pT181	pT181	Tc	4.4	*S. aureus*	230
		pC221	Cm *mob*	4.5	*S. aureus*	230
		pS194	Sm *mob*	4.4	*S. aureus*	230
	pE194/pMV158	pE194	MLS	3.7	*S. aureus*	230
		pMV158	Tc	5.5	*Streptococcus agalactiae*	169
		pWV01	Cryptic	3.3	*Lactobacillus lactis*	173
		pSH71	Cryptic	2.1	*L. lactis*	66
	pSN2	pSN2	Cryptic	1.2	*S. aureus*	230
		pIM13	MLS	2.1	*B. subtilis*	230
		pT48	MLS	2.1	*S. aureus*	230
		pE5	MLS	2.1	*S. aureus*	230
	pIJ101	pIJ101	*tra*	8.8	*Streptomyces lividans*	148
		pBL1	Cryptic	4.5	*Bacillus lactofermentum*	77
		pSN22	*tra*	11	*Streptomyces nigrifaciens*	147
		pSAM2	*tra*	11	*Streptomyces ambofaciens*	116
Theta plasmids	pWV02	pWV02	Cryptic	3.0	*L. lactis*	269
		pCI305		8.7		269
		pWV03				269
		pWV04				269
		pWV05				269
		pSK11L	*lac*	47		269
		pSL2A	*cit*	8		269
		pCI528				269
		pIL7				269
	pAMβ1	pAMβ1		26.5	*Enterococcus faecalis*	38
		pIP501		30	*S. agalactiae*	32
		pSM19035	MLS		*Streptococcus pyogenes*	20, 44
	pJH2	pJH2	*hly bcn tra*	56	*E. faecalis*	328
		pAD1	*hly bcn tra*	57	*E. faecalis*	328
		pCF10	*hly bcn tra*		*E. faecalis*	328
		pPD1	*bcn tra*	55	*E. faecalis*	328
	pI258	pI258	*bla* MLS ion	28	*S. aureus*	230
		pI524	*bla* ion	31	*S. aureus*	230
		pII147	*bla* ion	30	*S. aureus*	230
	pGO1	pGO1	Gm Sul Tm	65	*S. aureus*	230
		pIP404			*Clostridium perfringens*	274a
Linear plasmids	pSLA2	pSLA2	*tra*	17	*Streptomyces rochei*	48
		SCP1	*tra*	300	*Streptomyces coelicolor*	125
		pSCL	*tra*		*Streptomyces clavuligerus*	272
		SLP1	*tra*	17	*S. coelicolor*	275
	Hairpin			16	*Borrelia burgdorferi*	125

[a]Antibiotic resistance markers: Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; Sm, streptomycin; MLS, macrolides-lincosamides-streptogramin B; Gm, gentamicin; Sul, sulfamethoxazole; Tm, trimethoprim.

The theta mode of replication has been formally demonstrated for plasmids from gram-positive bacteria only recently (38). However, since the standard RC mode does not appear to be used for plasmids of >10 kb in size, it is very likely that all of the larger plasmids in gram-positive bacteria, as well as those in gram-negative bacteria, use the theta mode of replication. The linear *Borrelia* plasmids could represent an exception to this rule which will become apparent once the mode of replication of these plasmids has been

detailed. It is interesting that conjugative plasmids in eubacteria replicate by the theta mechanism even though conjugative transfer proceeds by a modified RC mechanism. That is, the RC process used for vegetative replication does not seem to be adaptable to conjugation, and vice versa. Indeed, there are several RC plasmids that encode RC mobilization functions that are separate and distinct from the RC functions used for vegetative replication. Indeed, the two sets of functions are unrelated and therefore of different ancestry, implying that they have been assembled by recombination. Three members of the pT181 family, namely pC221, pC223, and pS194 (230), exemplify this type of genome organization.

All linear plasmids thus far identified in gram-positive bacteria belong to a group of replicons known as invertrons, owing to their long terminal inverted repeat sequences (266). The majority of these, including the linear plasmids of *Streptomyces*, but not those of *Borrelia*, contain proteins covalently bound to their 5′ termini.

RC Replication

Leading-Strand Initiation: the DSO. RC replication in plasmids follows in essential details the pattern and strategy shown by the single-stranded coliphages (see the review by Baas and Jansz [13]). These plasmids always encode an initiator protein which introduces a site-specific nick in the leading- or plus-strand origin (now referred to as the DSO, or double-strand origin) (158), generating a 3′-OH priming terminus. In the pT181 family, the initiator protein becomes covalently attached to the 5′ site of the nick by a phosphotyrosine bond (293) and is very likely to remain attached throughout replication, as is the case with ϕX174. With pLS1 (a deletion derivative of pMV158), the initiator protein does not become covalently bound to the DNA (213), as is the case with the M13/fd group of single-stranded phages. Although it is widely assumed that all of the RC plasmid initiator proteins use a tyrosine-OH to form a permanent or transient protein-DNA bond during the nicking event, the only plasmids for which there is direct evidence for a tyrosine-DNA bond is the pT181 family, in which this linkage has been demonstrated biochemically (293). Although one or more conserved tyrosines are always found in predicted RC initiator proteins (161), it is probably unwise, at this juncture, to conclude that all RC replicons use a tyrosine hydroxyl for initiation. For the RC plasmids, aside from those of the pT181 family, the putative active tyrosine has been identified on the basis of motifs conserved in ϕX194 and in pC194. This identification is supported by site-directed mutagenesis experiments in which replacement of the putative active tyrosine of pC194 (Tyr-214) caused a 95% reduction in in vivo replication activity (222). However, replacement of either of two glutamate residues, one neighboring the putative active-site tyrosine and the other some distance away, had an even more profound effect on the activity of the protein. Noirot-Gros et al. (222) point out that carboxyl residues are “invariably present in the active sites of enzymes which cleave DNA” and suggest that Glu-210 (near Tyr-214) may be involved in the termination of replication (see below). This suggestion is supported by the presence of a glutamate residue at the corresponding position in the initiator protein of each of the 22 plasmids belonging to the pC194/pUB110 family. This conserved glutamate, however, is also present in the ϕX174 initiator protein, where any role in termination is more difficult to envision, given the well-established participation of a second active-site tyrosine in ϕX174 termination (117, 314a).

Two RC initiators (those of the pT181 and pMV158 families) have thus far been studied biochemically. Since they differ profoundly in several ways, their similarities may represent general properties of the class. The pT181 initiator proteins are dimeric (252), whereas those of pMV158 are hexameric (213). Primary sequences are unrelated. The pT181 initiators form a covalent tyrosine-DNA bond (293); the pMV158 initiator protein does not (213). Nevertheless, both types have site-specific nicking and topoisomerase I-like activities (158, 213). Both nick single-stranded DNA containing their recognition sequence and catalyze strand exchange (see Fig. 5) (213; C. Thomas, personal communication). The strand-exchange reaction is also characteristic of proteins that initiate RC conjugative DNA transfer (240). With two exceptions, all of these proteins in gram-positive bacteria, gram-negative bacteria, and RC viruses of eukaryotes as well as prokaryotes have recently been shown to constitute a vast and widespread single class

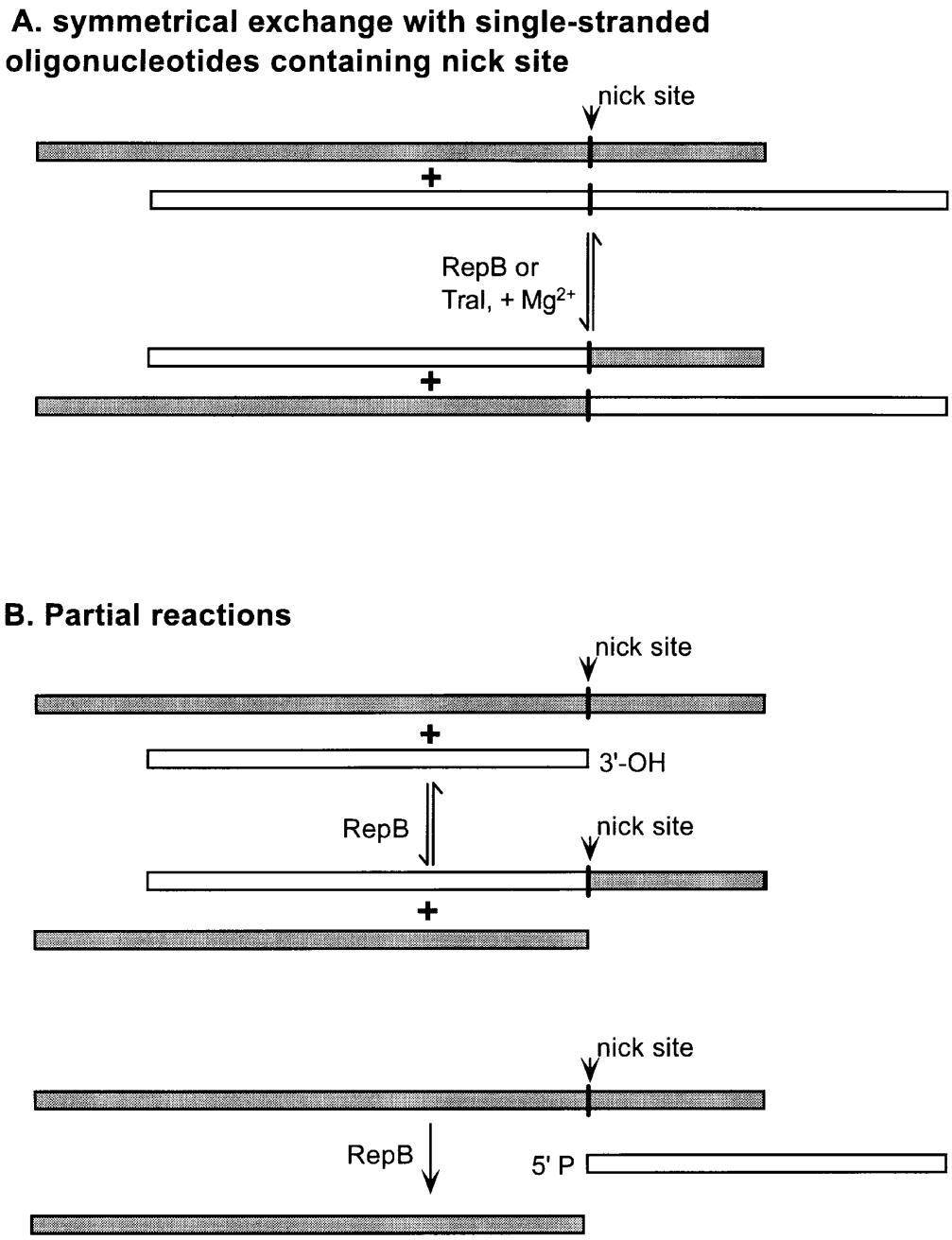


FIGURE 5 Strand transfer by initiator proteins. Both RC Rep proteins, such as RepB of pMV158, and conjugation-initiation proteins, such as TraI of RP4, can catalyze strand transfer between single-stranded oligonucleotides containing their specific recognition sequence(s). (A) Complete reaction, which is presumed to involve an intermediate in which the protein introduces a nick in one strand, becoming covalently linked to the 5' nick terminus. The 3'-OH, held in place by the protein, then attacks the corresponding sequence of another, intact, oligonucleotide, reversibly displacing its cognate. (B) Partial reaction, in which the 3'-OH half of the nicked substrate can displace its counterpart from the intact oligonucleotide, in the RepB-catalyzed reaction, whereas the 5' half is inactive.

characterized by certain shared amino acid motifs. The most striking of these are HUHUUU (where H = histidine and U = bulky hydrophobic residue) and YXX[KH](Y), where [KH] is lysine or histidine, X is any residue, and (Y) represents those few initiator proteins that have a second active tyrosine (161). The conserved tyrosine is assumed to be the residue that is joined to DNA during the nicking or topoisomerase I-like activities, and the HUH motif, which is also widely conserved among certain metalloenzymes, is likely to be involved in binding the divalent cations that are universally required for the function of these proteins. The two exceptions are the pT181 family and the pSN2 family (230). The pSN2 family initiator proteins have neither motif and are quite different from the others, in being only 16 kDa in size. The pT181 initiator proteins have a variant of the active tyrosine motif, YxxH, seen also with plasmids of the pE194 family, but lack the HUHUUU motif (127).

The topoisomerase I-like activity of RC initiator proteins is a side reaction since it not only decreases superhelix density, but also fails to generate a replicatable substrate. It is likely that it is an inevitable consequence of the site-specific nicking and strand-transfer (closing) activities necessary for the replication functions of these proteins. Indeed, RC replication may be viewed as a topoisomerase I reaction in which the nicking and closing components are separated by a round of replication. Thus, the well-established requirement for supercoiling for RC replication could simply represent the need to provide a single-stranded region which would allow the nicking reaction but prevent completion of the topoisomerase I-like reaction (closing) until after a round of replication has occurred. Several families of RC plasmids have an inverted repeat surrounding the nicking site (Fig. 6), and in one case this has been shown to form a cruciform in vivo, stimulated by binding of the initiator protein (221). In DSOs lacking a strong potential hairpin, one would presume that the single-stranded configuration is maintained during initiation by some other means.

In the pT181 family, the initiator proteins recognize the DSO using a recognition element consisting of six amino acid residues that is located some 80 residues C-terminal to the active tyrosine (230, 322). The corresponding regions of the DSO, however, are adjacent, indicating that the protein is folded so as to juxtapose the two regions. A similar organization has recently been described for the pMV158 initiator protein (63). The recognition region of the DSO represents the site of tight binding of the initiator protein, whereas the region surrounding the nick site is not affected by the initiator protein, at least by gel mobility shift analysis. Thus one may envision initiation as involving first a tight binding to the DSO recognition site, followed by a bending of the DNA (157) and possibly a deformation of the protein which brings the nicking site closer to the active tyrosine and promotes melting of the DNA. Binding efficiency is enhanced by a *cis*-acting sequence element, *cmp* (98), located about 1 kb from the DSO, which binds a specific 60-kDa cellular protein that is probably involved in its function (M. Gennaro, personal communication). The properties of this replication enhancer are quite similar to those of the pSC101 *par* locus (129, 204), which enhances replication by influencing the local supercoiling of the plasmid DNA and may also facilitate partitioning by enabling decatenation of plasmid molecules. Melting is facilitated by an A+T-rich region distal to the DSO hairpin (P.-Z. Wang and R. P. Novick, unpublished data), resulting in extrusion of the cruciform, which enables the active-site tyrosine of the initiator protein to attack the nicking target. The net result is an initiation complex in which the DSO is in an open configuration with the leading strand nicked and the initiator protein bound at its recognition site, on the downstream side of the nick (see Fig. 7).

It is interesting that the pMV158 and pT181 DSOs are organized similarly despite the lack of significant sequence homology between them (Fig. 6). Whether this represents a general plan for DSOs of RC replicons remains to be seen.

Following establishment of an initiation complex, the next step would have to be realignment of the sequences so that the 3′-OH priming site would once again pair with the template strand (Fig. 7) and the DNA configuration would serve as a suitable substrate for the replicase. The replicase is presumed to be Pol III, though the only direct evidence is inhibition by 5-hydroxy-*azo*-uracil (R. Novick, unpublished data; S. Khan, personal communication) and lack of any requirement for Pol I (in *Bacillus subtilis*) (S. Projan and R. Novick, unpublished data). *Staphylococcus aureus pcrA* mutants, defective in a helicase activity

similar to the Rep helicase of *E. coli*, accumulate the pT181 initiation complex (133), suggesting that the helicase is a key factor in converting this complex to a form that can be used by Pol III. Suppression of the defective replication seen in this helicase mutant occurs by mutations localized to an N-terminal subdomain of the initiator protein, presumably delineating the site of interaction between the initiator protein and the helicase (134).

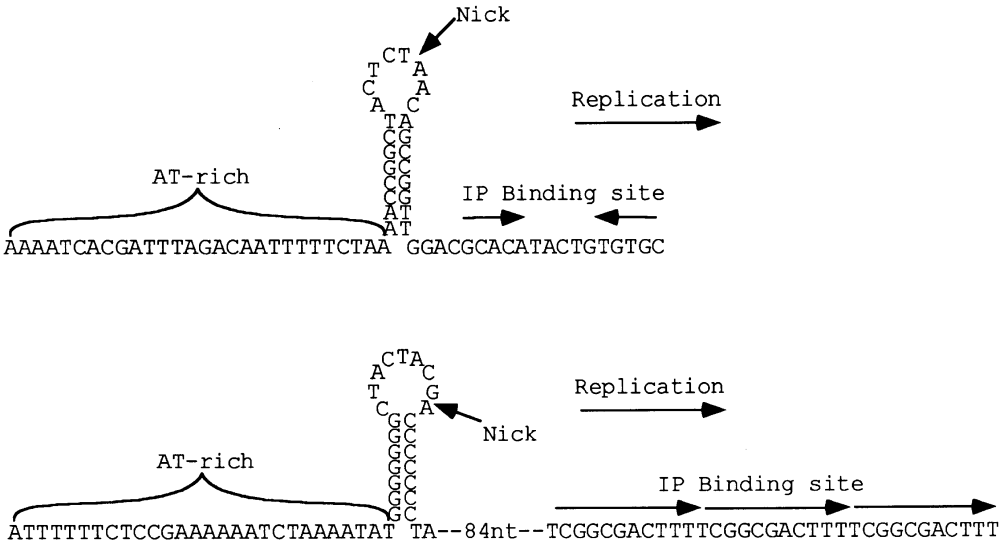


FIGURE 6 Organization of the DSOs of the pT181 and pMV158 plasmid families. Each of the DSOs contains a strong G+C-rich palindrome capable of forming a cruciform, in which the initiation nick site is within the single-stranded loop at the tip. Each also contains a highly A+T-rich region 5' to the cruciform and a site-specific initiator protein-binding region, separate from and 3' to the cruciform. The only significant different between the two is in the organization and placement of the initiator protein recognition sequences; those of the pT181 family comprise an inverted repeat adjacent to the cruciform, whereas those of the pMV158 family comprise a set of three direct repeats located some 80 nucleotides (nt) downstream. This organization may be related to the fact that the pT181 family initiator proteins are dimeric whereas the pMV158 initiator proteins are hexameric.

Mechanism of Termination. It is widely accepted that termination of RC replication is effected by a strand-transfer reaction catalyzed by the initiator protein (Fig. 5). Unlike ϕX174, whose initiator protein has two closely placed active tyrosines that alternate from cycle to cycle (117, 314a), most of the plasmid initiator proteins have only a single active tyrosine. With pT181, the initiator protein is a dimer and it is supposed that the hydroxyl of the active tyrosine of the second subunit would serve as the attacking group during termination (251). Alternatively, as proposed by Noirot-Gros et al. (222), the carboxyl group of a conserved neighboring glutamate could serve as the attacking group.

Direct evidence for a role of the initiator protein in termination consists of initiator protein mutations that cause the accumulation of multimers. Such mutations have been isolated for plasmids pIM13 (248), pT48 (43), and pUB110 (24). It is notable that as termination of RC plasmid replication is extremely efficient (135), multimers are very uncommon, and to date there is no evidence for any multimer resolution function among these plasmids (97). Thus, the accumulation of multimers is reasonable evidence for defective termination. As with the filamentous single-stranded coliphages, RC plasmids with two tandem origins show initiation at one and termination at the other. Constructs of this type have been used to show

that sequence requirements for termination are less stringent than for initiation. Thus, the pUB110 DSO can be used by the pC194 initiator protein for termination but not for initiation (199). The central hairpin of the pT181 DSO, containing the nick site (see Fig. 6), is sufficient for termination but not for initiation (135). An explanation for this difference may be that since the initiator protein is already bound to the DNA, specific recognition need not be required for termination; the nicking site and flanking sequences involved in any required structure are evidently sufficient.

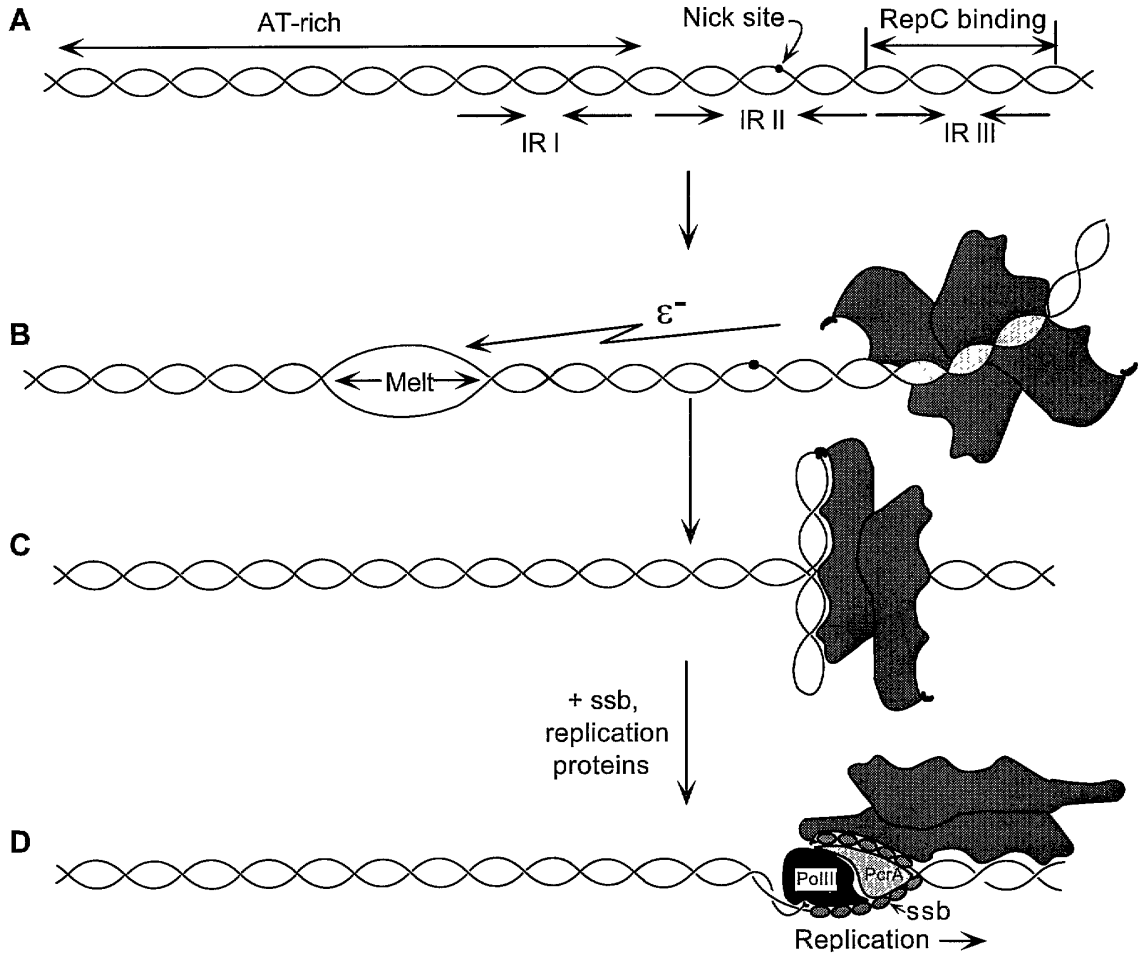


FIGURE 7 Model for the initiation of pT181 replication. (A) Organization of the DSO region. (B) Binding of RepC and melting of A+T-rich region driven by energy transfer (ε^-) or conformational distortion of the DNA. (C) Extrusion of the IR II cruciform with changing conformation of RepC and introduction of the nick. (D) Reorganization of nicked DSO, reestablishing template-primer pairing and allowing replication proteins to bind. The conformation at this stage may be stabilized by single-strand binding protein (ssb).

RC plasmids have been widely used as cloning vectors in gram-positive bacteria, and it has been repeatedly observed that increasing their size above 10 kb causes structural and hereditary instability. Structural instability is discussed below; in the present context, the accumulation of high-molecular-weight (HMW) multimers of plasmids containing large inserts has been repeatedly observed (112, 151, 316) and is thought to be a consequence of a failure to terminate replication (112). Perhaps, owing to the longer time required for polymerization, the initiator protein may dissociate from the displaced leading strand, a possibility that would seem to exist only where the initiator protein is not covalently bound. Alternatively,

the 5′ end of the leading strand may not be held in juxtaposition to the DSO if the displaced leading strand exceeds a certain length. In some studies (112) but not others (151), the base composition of the insert was important for the formation of HMW DNA. The presence of HMW DNA would be expected, by virtue of the copy control system, to reduce the monomer copy number proportionately and thus cause instability. This is sometimes but not always true (151). In fact, in some cases, the HMW DNA amounts to several times the normal unit copy number of the plasmid, indicating that any copy control expressed by this material is abnormal. Additionally, the accumulation of HMW DNA is inhibitory for cell growth and thus causes counterselection against the plasmid.

Minus-Strand Replication: the SSO. RC plasmid replication is asymmetric, as the leading strand is displaced as a single-stranded monomeric circle. Synthesis of the lagging strand on this single-stranded template generally begins at a specific, often palindromic site, the SSO. A variety of SSOs have been identified by deletion analysis on the basis of reduced double-strand copy number and the accumulation of circular single-stranded DNA corresponding to the displaced leading strand (65, 113, 184, 271). Four different classes have thus far been identified, SSO_A, SSO_U, SSO_T (114), and SSO_W (269; C. Bruand, S. D. Ehrlich, and L. Janniere, submitted for publication), which are distinguished on the basis of sequence, host range, and requirement for RNA polymerase (Rpo; see Table 2).

Several plasmids have been found to contain two or more SSOs, often with very different host specificities (314). Thus the SSOs may be important determinants of host range and host specificity among the RC plasmids. Cassette exchange involving SSOs would seem to have occurred frequently, since very different SSOs are found within families of closely related plasmids; in some cases, these “foreign” SSOs are closely related to SSOs from other plasmid families (113, 247, 271).

Complementary-strand synthesis is accomplished by a mechanism that relies wholly on host functions. With the exception of the SSO itself, all plasmid functions are dispensable and SSO-dependent complementary-strand synthesis can be carried out by cell-free extracts prepared from a plasmid-free strain (26). Interestingly, the efficiency of lagging-strand replication for a particular SSO is heavily host dependent. Thus some SSOs are quite host specific (113, 271), while others function effectively in several different species (29, 271). Furthermore, plasmids lacking any defined SSO can be constructed, and these replicate, often rather poorly, so long as the DSO is functional. In other words, single-stranded circular plasmid DNA can be used as a template for complementary-strand synthesis, with very variable efficiency, in the absence of any specific signaling or initiation sequence. This situation resembles that seen with ϕX174, which requires a full-scale primosome for lagging-strand initiation. Very possibly, a similar mechanism is used for lagging-strand synthesis by plasmids lacking a specific SSO.

TABLE 2 SSO classes

Class	Host range	Rpo required	Prototype plasmid
SSO_A	*Staphylococcus aureus*	+	pT181
SSO_U	*Staphylococcus aureus, Bacillus subtilis*	+	pUB110
SSO_T	*Staphylococcus aureus, Bacillus subtilis*	+	pBAA1
SSO_W	*Lactobacillus lactis*	Partial	pWV01

It is assumed that lagging-strand synthesis cannot begin until the SSO has been displaced by leading-strand extension and has become single stranded, allowing the formation of a hairpin (or other required secondary structure). Lagging-strand synthesis initiates within this structure (26) and most commonly requires the host Rpo, which is assumed to lay down an RNA primer, though this has yet to be demonstrated directly. As the SSOs generally lack consensus promoter sequences and as their secondary structure is imperfectly double stranded at best, RNA synthesis using such a template would have to involve a highly novel Rpo mechanism. Although single-stranded bubbles are preferential sites for the initiation of transcription by Rpo (7), the underlying substrate is basically double stranded, and fully single-stranded molecules are not used as templates. Certain SSOs function in the presence of rifampin, indicating

a lack of Rpo involvement (269). These SSOs could utilize primase in a manner analogous to that used by the single-stranded phage G4, or could utilize a full-scale primosome as does ϕX174. Sometimes a plasmid will have both an Rpo-dependent and an Rpo-independent SSO (269).

Theta Replication

Two major families replicating by the theta mode have been characterized, the pAMβ1 and pWV02 families (see Table 1). Many additional plasmids using the theta mode have been identified but not yet characterized. Unlike the RC plasmids, which differ in only minor ways from one another with respect to replication mechanisms, the two known theta classes are profoundly different, and their differences may parallel those between plasmids of the ColE1 and iteron families of the gram-negative bacteria.

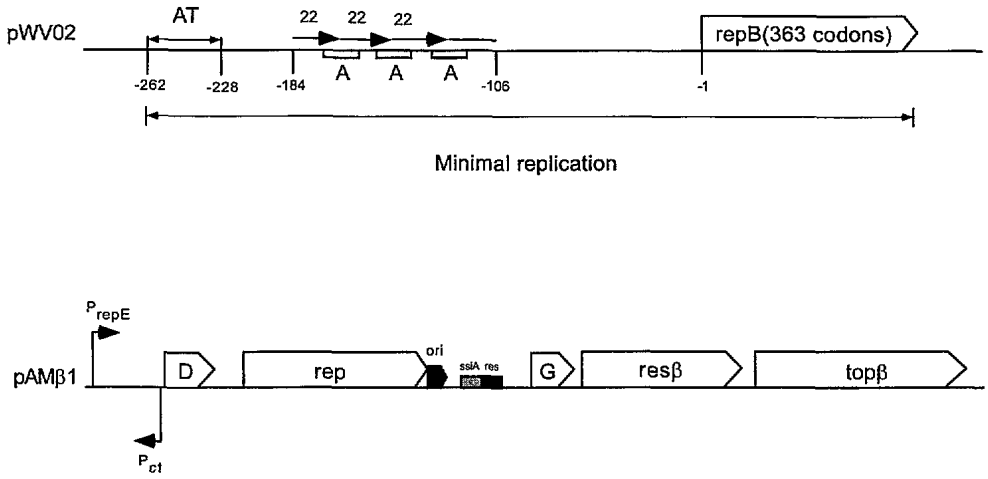


FIGURE 8 Typical replicons of plasmids using the theta mode of replication. pWVO2: the minimal replicon consists of the initiator protein gene (repB) and a~300-bp region upstream, which is essential and is presumed to contain the DSO. This region, highly conserved among members of the family, is marked by a set of 3 1/2 22-bp repeats containing segments resembling the DnaA box consensus (A) and a 34-bp A+T-rich region. pAMá1:the minimal replicon consists of the initiator protein gene (rep) and the DSO (ori), plus the rep promoter, Prep. The short open reading frames, D and G, are not required. The two downstream genes, res and top, encode a resolvase, involved in multimer resolution, and a type I topoisomerase, both of which are required for the switch from Pol I to Pol III (see text and Fig. 9). The copy-control region contains the repressor gene (cop) and a regulatory countertranscript (see text).

The replicon structure for pWV02, the prototype of the pWV02 class, is shown in Fig. 8. The major open reading frame, *rep,* is required for replication and presumably encodes an initiator protein. 5′ to the *rep* coding sequence is a highly conserved region of about 300 bp which is also essential for replication. Contained in this region is a 36-bp A+T-rich (~90% A+T) sequence, which is almost totally conserved among the nine plasmids studied, and a set of 3 1/2 22-bp repeats that could represent iterons comparable to those of the plasmids of gram-negative bacteria. Rather puzzling is the presence in pWV02 of a set of complicated and precisely conserved repetitive sequences 3′ to *rep,* covering about 1.8 kb and completely dispensable. It seems likely that

novel principles of plasmid DNA organization and function will be revealed by study of this fascinating set of plasmids.

Plasmids of the pAMβ1 family replicate by a unidirectional theta mechanism apparently quite similar to that used by ColE2 and ColE3 of *E. coli* (137). The organization of a typical replicon, that of pAMβ1, is diagrammed in Fig. 8. Initiation of replication utilizes a long primer, probably generated by Rpo from the P_{rep} promoter (see Fig. 8). This transcript also covers the initiator protein gene (*rep*), whose role is thought to be processing of the transcript to generate a primer terminus at *ori* (39). Unidirectional theta replication is initiated by Pol I and later switches to Pol III, possibly at or near a lagging-strand initiation site (SSI) that resembles the *n′*-primosome assembly sites (*pas*) of *E. coli* plasmids (Bruand et al., submitted).

Downstream of the leading-strand origin are located genes encoding a site-specific recombinase of the resolvase-invertase family and a replicon-specific topoisomerase I. The recombinase is utilized for multimer resolution (see below), and both enzymes are apparently involved in the switch from Pol I to Pol III (see Fig. 9). The Pol I replication complex is paused or halted by the bound resolvase, and this pause is a signal for the switch to Pol III. In the absence of the topoisomerase, long D-loops are then generated (L. Jannière, personal communication). It is not clear precisely how the topoisomerase prevents this.

As noted above, there are a large number of plasmids in gram-positive bacteria that probably utilize the theta mode of replication. For several of these, the functional organization of the replicon has been analyzed (see, for example, reference 324); however, details of the mode of replication and of the roles of the replication genes have not yet been determined.

Replication of Linear Plasmids

Thus far, the only true gram-positive bacteria in which linear plasmids have been identified are *Streptomyces* sp. and the related *Rhodococcus* spp. (reviewed in references 125 and 263). These plasmids, ranging in size from 9 to over 600 kb, belong to a large class of genetic elements known as invertrons, owing to their long terminal inverted repetitions (263), to which are covalently attached proteins that serve as primers for DNA synthesis. In the now classical mode of replication of these elements, de novo DNA synthesis is initiated at one or both ends of the linear molecule, primed by the terminal proteins rather than by RNA or DNA termini (see review [264]). Rather surprisingly, however, the two linear streptomyces plasmids that have been analyzed thus far, pSCL (272) and pSLA2 (48), have both been found to replicate bidirectionally from an internal origin, as shown in Fig. 10A, rather than from the ends. It has been proposed by Chang and Cohen (48) that the role of the terminal proteins in this mechanism is to prime the DNA synthesis required to fill in the 5′-terminal gap left on the lagging strand by the inability of the standard lagging-strand replication mechanism (RNA-primed Okazaki fragments) to complete a linear molecule. As suggested (48), this mechanism may be widespread in *Streptomyces* and may also be utilized for replication of the chromosome, which is linear in at least a few species (178). Spirochetes such as *Borrelia* sp. are considered here as they have a number of features in common with the gram-positive bacteria. As noted above, they have linear chromosomes and plasmids (125); the latter are characterized by hairpin telomeres in which the two strands are connected by a terminal loop. It is thought that this terminal loop is the site of initiation of replication and that this replication proceeds by a modified RC mechanism (a speculative model is shown in Fig. 10B).

Regulatory Mechanisms

All known plasmids of gram-positive bacteria use a plasmid-encoded protein, the initiator protein, for the initiation of replication at the DSO and control their initiation rate (copy number) primarily by controlling initiator protein synthesis. To maintain stable copy numbers, plasmids must thus also control the fate of the initiator protein following its use. There is, however, no evidence for any control of lagging-strand replication, nor is there evidence for any control of the function of the initiator protein, as is the case with the iteron plasmids of gram-negative bacteria.

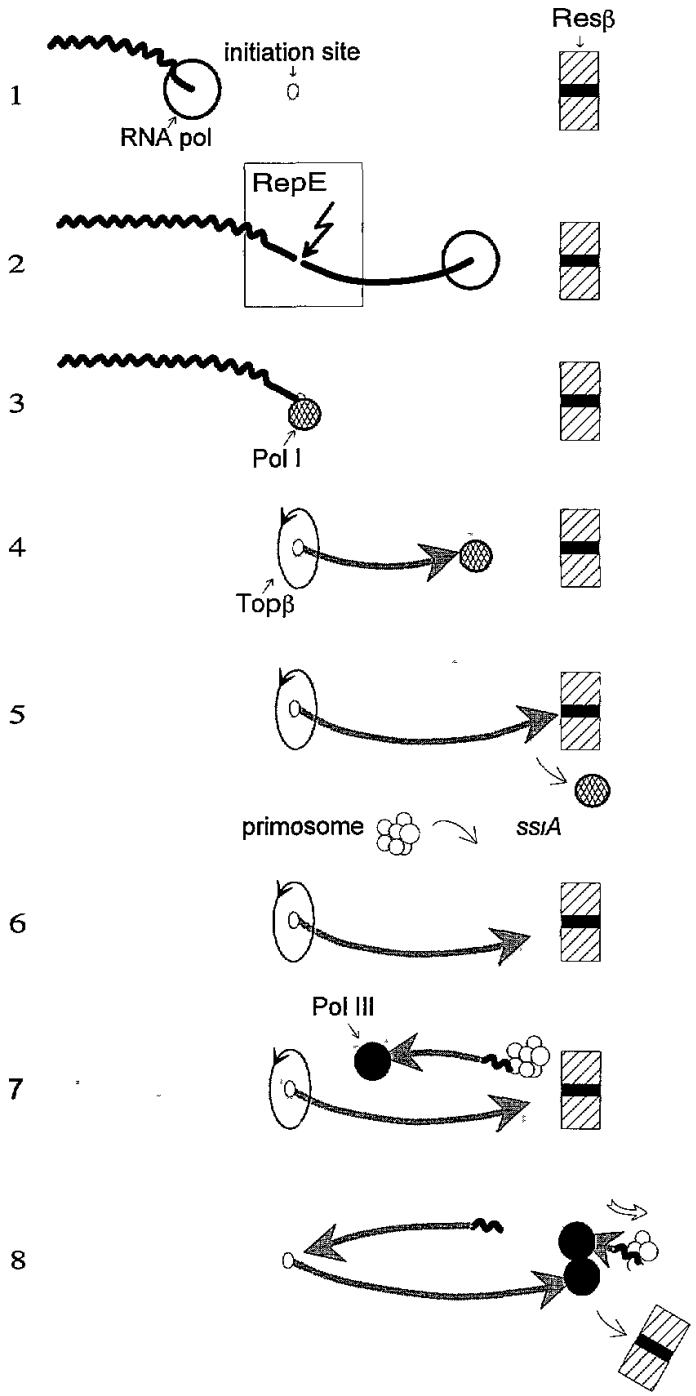


FIGURE 9 Initiation of pAMβ1 replication. (1) Generation of preprimer transcript by RNA polymerase. (2 and 3) Binding of RepE and processing of the preprimer transcript. (4 and 5) Attachment and early polymerization by Pol I. (6) Entry of Topβ and dissociation of Pol I at the site of bound Resβ. (7) Assembly of primosome at lagging-strand initiation site (*ssiA*). (8) Entry of Pol III and displacement of Resβ.

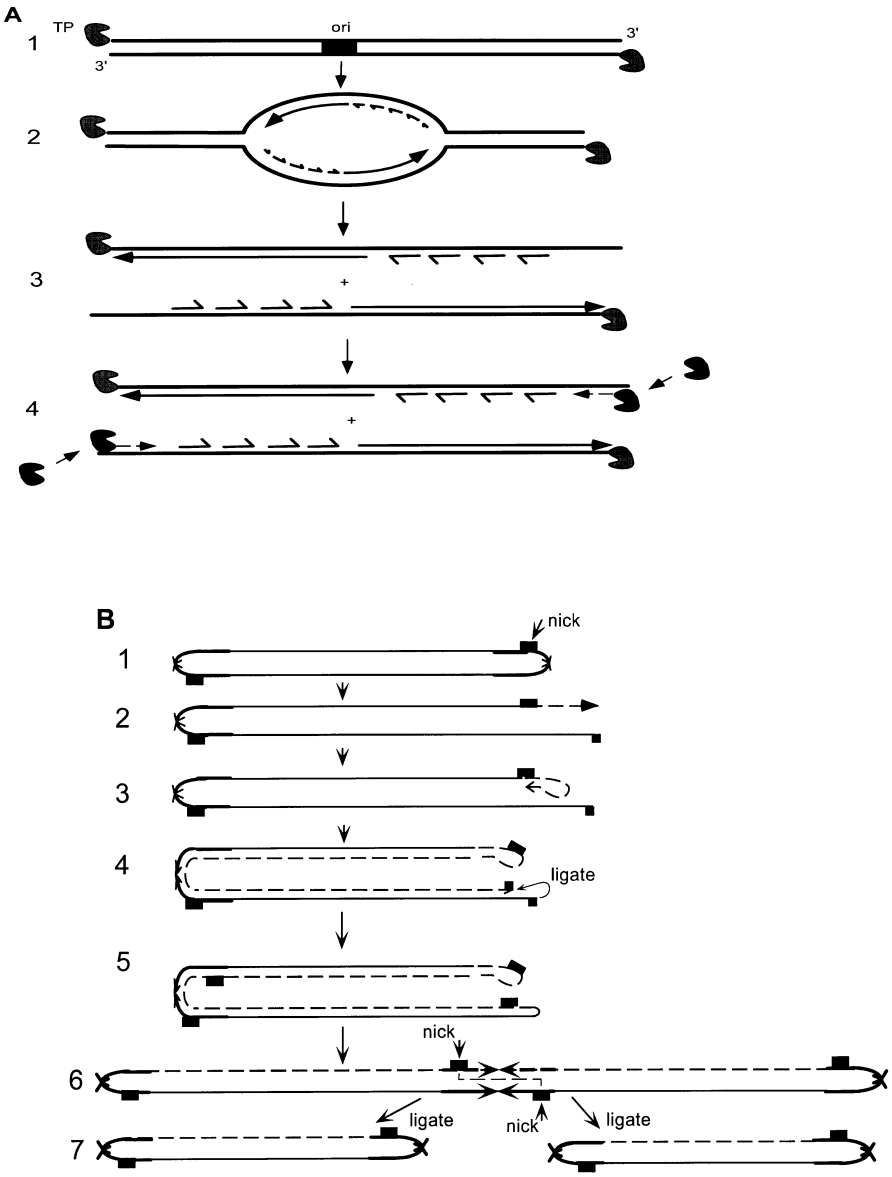


FIGURE 10 (A) Replication of a linear *Streptomyces* plasmid. (1) Resting plasmid with attached terminal proteins (TP). (2) Initiation of a typical bidirectional theta structure with Okazaki fragments, shown as short arrows. (3) Replication is complete except for short terminal gaps at the 5' ends of both strands. These are thought to be filled in by the terminal proteins, which bind and recognize these single-stranded regions as shown in step 4. (B) Speculative model for modified RC replication of a linear *Borrelia* plasmid with hairpin telomeres (patterned after the orthopoxvirus model of Moyer and Graves [214a]). (1) Replication is initiated by the nicking of an initiation site (shaded box) located within the telomeric inverted repeats (heavy arrows). Since the two telomeres are nearly identical, initiation could occur at either end. (2) A nick has been introduced and the 3' end is extended to the end (dashed arrow). (3) A short snapback loop then serves as the primer for replication, which proceeds (4) to the corresponding nick site at the other end of the molecule. (5) Ligation reestablishes continuity of the newly replicated strand, generating a head-to-head dimer with recreated nicking sites as shown (6), (7) These then serve as substrates for a second round of nicking ligation, generating a pair of monomeric progeny plasmids.

Regulatory mechanisms have thus far been analyzed in only a few cases, namely the pT181 family, the pUB110 family, the pMV158 family, and the pAMβ1 family, and an immediate generalization is that RC and theta plasmids share strategies for regulating initiator protein synthesis. Thus several families of RC plasmids use antisense RNAs (countertranscripts or countertranscript RNAs) to regulate initiator protein synthesis, and at least one family of theta plasmids, the pAMβ1 family, does so also. For the RC prototype, pT181, countertranscript RNAs act by causing translational attenuation of the initiator protein mRNA (233). A nearly identical mechanism has recently been described for two members of the pAMβ1 family, pIP501 (33) and pAMβ1 (172), and a similar mechanism is used by an RC plasmid from *Lactobacillus pentosus* (247). A schematic diagram of these parallel systems is presented in Fig. 11. In other families, such as the pMV158 and pUB110 families, countertranscript RNAs are also used (8, 64, 167), but their mechanism of action has yet to be determined. Many plasmids from gram-positive bacteria exhibit dual copy-number control, in which there is a repressor protein as well as a countertranscript RNA. Typical examples are the pMV158 family (64) and the pAMβ1 family (172). In both cases, the repressor gene is 5′ to the initiator protein gene and the repressor acts on the initiator protein gene promoter. In one case, that of pE194, this protein binds to a site upstream of the repressor gene so that the repressor gene is autoregulated (167). Although this type of autoregulatory circuit was proposed many years ago by Sompayrac and Maaløe (276), its effectiveness as a copy control mechanism is questionable. As mentioned above, since an autorepressor will maintain its own concentration at a constant level regardless of its gene dosage, it will not respond appropriately to fluctuations in plasmid copy number. The result will be a very broad distribution of copy numbers in individual cells and a consequent segregational instability of the plasmid. This has been seen experimentally and documented theoretically with λdv (330). The coupling of an autorepressor with a countertranscript RNA evidently obviates the disadvantages of the autorepressor, since these plasmids are perfectly stable. Nevertheless, it remains unclear whether the autorepressor confers any regulatory advantage.

Repeated nucleotide sequences are frequently found in or near the minimal replicon in all types of plasmids from gram-positive bacteria as well as in those from gram-negative bacteria. In the pWV02 family, as noted above, a set of 3 1/2 22-bp tandem direct repeats 5′ to the initiator protein gene is essential for replication, and it has been suggested that these may be iterons functionally similar to those of the gram-negative bacteria (269). No regulatory role has been ascribed to these sequences, however, and their remarkably high degree of conservation between pairs of compatible plasmids makes it unlikely that they function as plasmid-specific regulatory elements. For example, pWV04 and pWV05 are compatible, yet their iteron sets differ at only two positions. Additionally, and also unlike the iterons of plasmids from gram-negative bacteria, with one exception, pSL2A, all the copies in any one set are identical or differ at not more than one position. In contrast, the iterons of plasmids from gram-negative bacteria, which are the major determinants of incompatibility among these plasmids, differ considerably from plasmid to plasmid and among the members of any one plasmid's set. Since these iterons function as sequence-specific initiator protein-binding sites, and since such sites can often tolerate a substitution or two without a major change in specificity, if the pWV02 repeats are determinants of plasmid specificity (incompatibility) they must function by a novel mechanism. It is suggested, incidentally, that the term "iteron" be reserved for directly repeated sequences that have a well-defined role in the regulation of replication, as is the case for these sequence elements in the classical iteron plasmids of gram-negative bacteria.

Fate of the Initiator Protein. Most RC plasmids regulate their replication by controlling initiator protein synthesis in a hyperbolic manner. This means that synthesis of the initiator protein is not directly coupled to replication but is free to vary in response to the concentration of an inhibitor such as antisense RNA. This arrangement permits a rapid response to fluctuations, especially down fluctuations in copy number (124). Although such initiator proteins are maintained at a low, rate-limiting level, the maintenance of a stable copy number requires that they not be recycled, as is the initiator protein of ϕX174. This requirement has been confirmed by the demonstration of an inactive form of the initiator protein for the pT181 family

plasmids that is generated during replication (251). The inactivation is associated with the covalent linkage to one subunit of the initiator protein dimer of an oligonucleotide corresponding to the 10 to 12 nucleotides immediately 3′ to the DSO nick site (see Fig. 6). This oligonucleotide is probably attached to the active-site tyrosine and is probably generated during the termination step by a mechanism such as that shown in Fig. 12, in which replication proceeds a short distance past the DSO nick site, generating the DSO hairpin, which is cleaved by the free active tyrosine of the second subunit of the initiator protein. This would initiate a concerted strand transfer cascade, generating a single-stranded monomer corresponding to the displaced leading strand, circularizing the new leading strand, and releasing the initiator protein as an inactive heterodimer. Note that the replication-termination cycle as depicted here involves two nickings, one at initiation and the other at termination, and that termination involves two closings, one for the new and one for the old leading strand. The overall model, however, remains speculative.

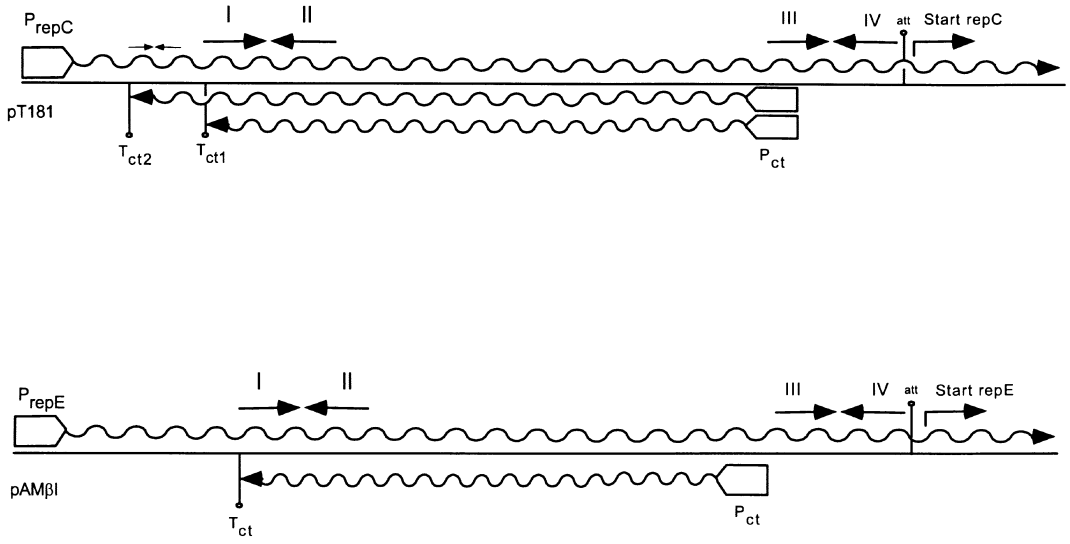


FIGURE 11 Regulation of plasmid replication by countertranscript-driven transcriptional attenuation. Two systems are diagrammed, pT181 at the top and pAMá1 below. Each system is characterized by a major rightward transcript that encodes the initiator protein and a countertranscript that blocks production of the initiator protein. The countertranscript is read from the untranslated leader region of the initiator protein gene mRNA and is complementary to it. In each system, there are two potential stem loops (I-II and III-IV) in the initiator protein mRNA. The III-IV loop is a ?-independent terminator, of which the proximal arm, III, is complementary to the distal arm, II pairs with III, preventing formation of the III-IV stem. In the presence of the countertranscript, complementary loops in the I-II hairpins "kiss," leading to the formation of a full-scale RNA duplex between the countertranscript and the untranslated complementary region of the initiator protein mRNA leader. As a result, the III-IV stem forms, causing termination (attenuation) of the initiator protein transcript immediately 5' to the translational start of the protein. Some members of the pT181 family have two countertranscripts, both of which are required for normal replication control; others have only one. It is probable that the countertranscript must interact with the nascent initiator protein mRNA leader; once transcription has proceeded past a critical point, the leader is predicted to fold into a configuration that is insensitive to the countertranscript.

Replication Control in Plasmid Cointegrates. Many bacterial plasmids and chromosomes contain one or more secondary replicons in addition to the one that normally drives replication. These secondary replicons have to be suppressed under normal circumstances. A current theory of negative regulation of replication predicts that if two replicons are joined, the one with the higher intrinsic copy number will drive the cointegrate and the one with the lower copy number will be suppressed because its negative regulator will be maintained at an elevated level. In practice, this prediction has been confirmed in only one case, that of F::ColE1 cointegrates (311). With some artificial cointegrates, the replicons are both used, at frequencies related to their intrinsic copy numbers. It is not known whether this ever occurs in naturally occurring plasmids. In other cointegrates, one replicon shuts off the other in a manner that has nothing to do with the negative regulatory function or the intrinsic copy number of either.

The classical case occurs where replication of the low-copy-number plasmid is controlled by an all-or-none switch. Function of both replicons is seen when the negative regulation is hyperbolic (initiation frequency is inversely proportional to the concentration of the regulatory molecule), and this, in retrospect, is quite predictable. The third situation, known as *cis* inhibition, has been seen in both prokaryotes and eukaryotes. In well-studied cases this inhibition turns out to be unrelated to any replication function of the inhibitory replicon but is rather caused by "poison" sequences carried by the latter (see, for example, references 78 and 182). A case in point is the inhibition of pT181 replication by pE194 sequences in *cis* (249). Here deletion of the pE194 SSO or of the pT181 countertranscript RNA promoter relieves the *cis* inhibition. The countertranscript RNA promoter is strong and reads away from the nearby pT181 DSO, which would increase negative supercoiling at the DSO (180). This, one might think, would promote rather than inhibit the initiation of replication, as it would favor DNA melting in the DSO region. A possible clue to this seeming paradox comes from the observation that the *cis*-inhibited origin cycles the pT181 initiator protein, converting it to the above-mentioned inactive form containing an oligonucleotide covalently attached to one subunit (A. Rasooly and R. Novick, unpublished data). This implies that replication is initiated at the *cis*-inhibited pT181 DSO, but is then aborted. One possible explanation is that the nicked initiation complex remains rotationally constrained and that the negative supercoiling stimulated by transcription from the countertranscript promoter forces the DSO cruciform to reextrude prematurely, immediately following the initiation of replication. This structure would then be recognized and cleaved by the second subunit, aborting the replication cycle. The role of the pE194 SSO would then have to be in prevention of the cancellation of these negative supercoils by the positives generated in front of the countertranscript. A naturally occurring example of this has recently been described by Pujol (C. Pujol, Ph.D. thesis, Université Paris-Sud, U.F.R. Scientifique d'Orsay, Paris, France, 1995) and coworkers, who have identified a *cis*-inhibited pT181-like plasmid within the pAMβ1 genome. As the initiator protein of this *cis* inhibited plasmid reacted with anti-RepC antibodies, Western immunoblotting showed that the modified form was produced (Rasooly and Novick, unpublished), suggesting that this type of *cis* inhibition may be common.

Many other natural plasmid counterparts have been described in both gram-negative and gram-positive bacteria (238, 243), and the bacterial chromosome is the repository of a large variety of integrated plasmids, all of which are unable to replicate in situ. Prophage-like repressors are of course possible; indeed, the integrating plasmids of *Streptomyces* spp. encode functions (*imp*) that specifically repress plasmid replication, quite like prophage repressors (108). Alternatively, integration may interrupt an essential replication function, such as the initiator protein gene (40, 191, 238). *cis* inhibition more or less like that described above may also be common.

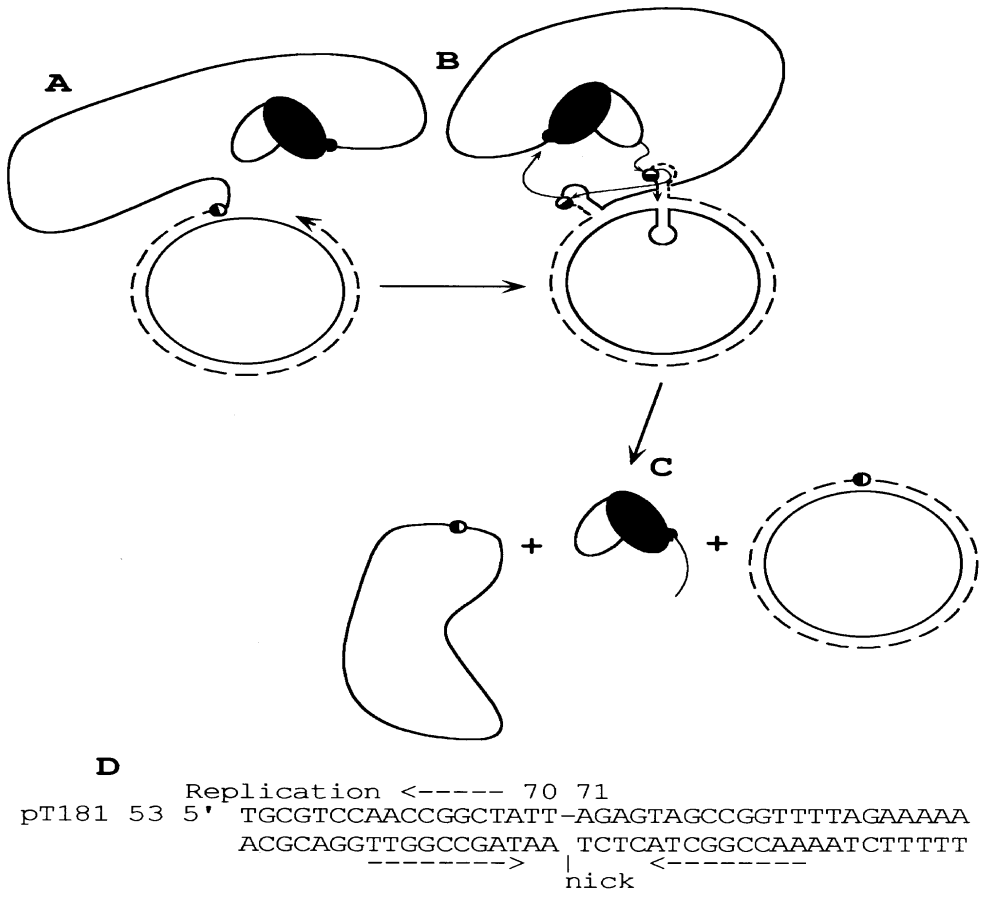


FIGURE 12 Possible mechanism of termination of pT181 replication with inactivation of the initiator protein. (A) Diagram of the expected configuration of a plasmid. (A) Diagram of the expected configuration of a plasmid DNA molecule nearing completion of a replication cycle. One subunit (hatched) of the RepC dimer is attached to the 5' end of the displaced plus strand, and the growing end of the nascent strand (arrow) is approaching the *ori* nick site. (B) The nick site has been replicated, the leading strand has extended a short distance further, and the *ori* hairpin has formed in both the new and displaced leading strands, as well as in the old template strand. The second subunit of RepC (open) initiates a concerted strand-transfer reaction (arrows) by nicking the newly synthesized ori and thus acquiring the observed oligonucleotide, which therefore consists of the -12-bp 3' half of the *ori* hairpin. The newly generated 3'-OH group then attacks the old *ori*, and the displaced 3'-OH attacks the old RepC-DNA bond, displacing RepC and restoring continuity of the displaced plus strand, which is released as a single-stranded circular monomer representing the displaced leading strand, a RepC heterodimer with the oligonucleotide attached to one subunit, and a double-stranded plasmid monomer containing the new leading strand. The single-stranded monomer is then replicated by an independent mechanism, not involving RepC, to complete the cycle. (D) The *ori* sequences surrounding the nick site are shown with the opposite-facing dashed arrows indicating the stem of the *ori* hairpin.

INCOMPATIBILITY

Our basic understanding of the major features of plasmid incompatibility has not changed significantly over the past decade (229). It is clear that the sharing of any function required for the regulation of plasmid replication by two plasmid elements is likely to result in incompatibility. This is true for plasmids of gram-negative and gram-positive bacteria. Random selection for replication causes disparities in the copy pools of two coresident isogenic plasmids, which cannot be corrected since a copy-number control system does not recognize the two as different (19, 115, 260). In addition, as a consequence of random assortment for partitioning, plasmids that use a common partitioning system show weak segregational incompatibility due to random selection for partitioning (11, 227, 232). This has been shown at least for plasmids of gram-negative bacteria. Analogous partitioning systems for plasmids of gram-positive bacteria have not been characterized to date.

Unidirectional or vectorial incompatibility is shown by pairs of coresident plasmids where one member contains a replicon function used by the other but not by itself. Thus, incompatibility has been seen with cloned copy control elements, a cloned DSO or origin iterons, and cloned partitioning elements. In addition, a disparity in the functionality of a replicon element between otherwise isogenic plasmids can result in asymmetric segregational incompatibility since the plasmid with the more efficient version of the element will outcompete the other. For example, this has been seen with pairs of pT181 derivatives differing in the replication enhancer, also known as the competitivity (*cmp*) locus. This locus effects the ability to bind and use the plasmid-specific initiation protein (98). Another interesting case is found in *Streptomyces,* where the *imp* replication repressor is an important cause of vectorial plasmid incompatibility (108).

Much of our understanding of the mechanistic details of incompatibility involving replication control elements has come from studies on the ColE1 plasmid (47, 74). Because RNA I of ColE1 is a diffusible negative replication regulator, it is the primary incompatibility determinant of the ColE1 plasmid. Consequently, a normally compatible coresident plasmid with a DNA insert that produces RNA I cannot coexist with a ColE1 replicon dependent on the identical RNA I for copy-number control (289). It also follows that single base changes in RNA I, with the complementary change in the overlapping primer (RNA II), can alter the specificity of the regulatory RNA I molecule and result in the creation of a new incompatibility group (168). The Rop protein of ColE1, although involved in copy-number control, is not an incompatibility determinant because it is not essential for replication even though it enhances the incompatibility exerted by RNA I (235, 306). Similarly, the ColE1 replication origin is not an incompatibility determinant because it does not titrate a diffusible inhibitor or activator molecule.

A similar set of observations have been made for the IncFII plasmids which, unlike the ColE1-type plasmids, encode a replication initiation protein. The CopA RNA, like RNA I, is the major incompatibility determinant of the IncFII plasmids (210, 261). As shown for ColE1, changes in CopA nucleotides that are critical for CopA-CopT interactions should change the incompatibility grouping of the plasmid. A divergence in the sequence of CopA is most likely responsible for the compatibility observed between the IncFII plasmids and several naturally occurring plasmids that apparently have a similar replicon structure and regulatory mechanism for replication (329). Because the RepA protein of the IncFII plasmids is *cis*-acting, the replication origin of these plasmids is not an incompatibility determinant (298).

It has been well established that the direct repeats at the origin and in the copy-control region of the iteron-containing plasmids are incompatibility determinants. This fact was initially established for the F plasmid, where it was shown that inserting a cluster of five 22-bp iterons into a compatible coresident plasmid resulted in incompatibility with the F replicon (299). As described above, this finding has been extended to a number of iteron-containing plasmids. In some cases it has been shown that this incompatibility, the severity of which is correlated directly with the number of iterons inserted, is not relieved in vivo by providing additional amounts of the plasmid-specific initiation protein. This latter observation has been used in support of the coupling model for plasmid replication control.

The major determinants of incompatibility in plasmids of gram-positive bacteria are the small RNA counter-transcript regulatory molecules and the origin of replication. It has been shown that the antisense RNAs of plasmid pT181, when cloned, exert strong incompatibility against pT181 replicons (231). Unlike the ColE1 and IncFII plasmids, however, the pT181 origin is also an incompatibility determinant (132, 231). This activity of the origin is consistent with its ability to bind the plasmid initiation protein and, therefore, to effectively titrate the rate-limiting, *trans*-acting RepC initiation protein. Interestingly, a comparative analysis of four naturally occurring plasmids of *Staphylococcus* that are related to plasmid pT181 showed that while each of the plasmids exhibited considerable structural similarity of their replicons, including considerable similarity in the sequences of their antisense RNAs and origin regions, there was sufficient nucleotide sequence divergence to place each of the plasmids in a separate incompatibility group (249).

It is likely that the phenomenon of incompatibility is operative in natural environments in placing limitations on plasmid combinations stably maintained in gram-negative and gram-positive bacteria. Plasmids that possess more than one functional replicon are likely to be less restricted in this regard.

STABLE MAINTENANCE MECHANISMS

Plasmid stability and instability can be divided into structural and hereditary types. Natural plasmids in their native hosts are hereditarily highly stable, and for the most part are also structurally stable, by virtue of having survived the forces of natural selection. Instabilities of both types arise primarily as a consequence of transfer to a non-native host or of in vitro manipulation.

Structural Stability

The streptococcal plasmid pAMβ1 is an example of host-specific structural instability in a natural plasmid. When transferred to *B. subtilis*, pAMβ1 undergoes site-specific excision of a 4.4-kb segment very closely related to the *Bacillus* plasmid pBC16, which replicates well in *B. subtilis* but not in *Streptococcus faecalis* (243). Here, the excision utilizes a pair of directly repeated sequences which are likely to have been used for formation of the cointegrate in the first place.

It has been observed frequently for artificial constructs that plasmids containing foreign DNA undergo deletions and other rearrangements. These rearrangements are often site specific and they usually cross a splice junction. In a number of cases, rearrangements can be determined to result from recombination between short direct repeats ranging from 3 to 20 bp in length (37, 75). Sometimes, however, repeated sequences are not found at the endpoints. Most of the studies detailing this have been done with *B. subtilis* (75). Of particular interest is the observation that plasmids of gram-positive bacteria that use the theta mode of replication are structurally much more stable than the RC plasmids (151). This difference has been attributed to the fact that single-stranded DNA is much more prone to replication errors than double-stranded DNA. For example, direct repeats in single-stranded DNA (i.e., in RC plasmids) are prone to slipped mispairing during lagging-strand replication, which causes deletions at least 1,000-fold more frequently than the same repeats in double-stranded DNA (i.e., for theta plasmids). In the latter case, for deletions caused by classical homologous recombination, slipped mispairing is also possible, but evidently occurs much less frequently in double-stranded DNA. The increasing probability of short direct repeats with increasing size is probably one of the reasons that RC plasmids of >10 kb are rare in nature and may account for the difficulty of constructing and stably maintaining large plasmids that replicate via the RC mechanism. These observations have led to the proposal that theta-replicating plasmids are much more useful as cloning vectors than RC plasmids (141, 151).

Hereditary Stability

A variety of changes, including transfer to a new host, mutations, deletions, rearrangements, and insertions of foreign DNA, may upset the delicate balance between plasmid and host that is required for hereditary stability, with a concomitant loss of the plasmid from the culture at some detectable frequency. It is often quite difficult to determine precisely the cause of this destabilization. However, one can discern at least three well-defined possibilities: (i) replication defects; (ii) partitioning defects; (iii) growth disadvantages for plasmid-containing cells. Genetic determinants involved in replication are not properly classed as stability determinants, though for practical reasons it may sometimes be useful to do so. For example, with respect to plasmids of gram-positive bacteria, defects in conversion from single stranded to double stranded, owing to SSO defects or poor SSO function in foreign hosts, usually cause plasmid instability, and SSOs have been referred to, probably incorrectly, as stability determinants.

Plasmids evidently ensure their hereditary stability either by high copy numbers or by specific genetic mechanisms. Among the latter are three: multimer resolution, equipartitioning, and postsegregational killing. Of these mechanisms, equipartitioning is generally used by low- or intermediate-copy-number plasmids. High-copy-number plasmids seem to rely primarily on random partitioning; multimer resolution systems are necessary if multimer formation occurs with significant frequency. In general, RC plasmids have extremely efficient replication termination mechanisms and consequently do not generally encode multimer resolution systems. Postsegregational killing is used by low- and intermediate-copy-number plasmids as a fail-safe mechanism. These specific genetic mechanisms responsible for plasmid hereditary stability have been the subject of a number of reviews (11, 12, 103, 208, 225, 229; del Solar et al., in press).

Multimer Resolution

The occurrence of plasmid multimers is often observed, with the individual units in the multimers generally oriented head-to-tail. The multimers likely originate by defects in the termination of replication or by homologous recombination of monomers. The formation of oligomers decreases the number of individual molecules for distribution to daughter cells at cell division and, thus, has an effect similar to that of a reduction of copy number. This can have serious consequences, particularly in the case of a normally low-copy-number plasmid. A number of different plasmids in gram-negative and gram-positive bacteria possess a multimer resolution system (*mrs*) that is site specific and will resolve dimers and higher multiple forms to monomers. In many cases the plasmid *mrs* system includes both a *cis*-acting site and an encoded recombinase that is specific for this site. This is the case in gram-negative bacteria for the *mrs* of plasmids P1 (285), F (170), RK2/RP4 (73, 106, 109, 253), and R46 (67) and a virulence plasmid of *Salmonella* (164). For ColE1 and related plasmids, however, multimer resolution involves a *cis*-acting site (*cer*) on the plasmid and four *E. coli* chromosomally encoded proteins, XerA(ArgR), XerB, XerC, and XerD (27). In addition to the plasmid-specific multimer resolution systems found for a variety of plasmids, many plasmids also carry functional transposons that also encode an *mrs* (e.g., the TnpR resolvases of the Tn*3* family). The role that these transposons play in reducing the number of plasmid multimers has not been systematically examined.

A multimer resolution system has been identified in the pAMβ1 family of plasmids from gram-positive bacteria. All of the well-studied members, pAMβ1 (250), pIP50 (250), and pSM19035 (257, 258), possess this system, which likely serves as a stability function. These plasmids encode proteins belonging to the resolvase-invertase family but differing from the classical Tn*3* resolvase in binding to only two rather than three separate sites in the target region. They mediate both resolution and inversion, but only in the presence of an unknown host factor (257). Interestingly, as noted above, they are also involved in replication. It has been proposed that the Res protein of plasmid pIP404 of *Clostridium perfringens* also is involved in the resolution of multimers of this naturally occurring plasmid (96).

Plasmid Partitioning

Unlike the partitioning of chromosomes of eukaryotic cells during cell division, the process of segregation of bacterial chromosomal or plasmid DNA to daughter cells is poorly understood. It is clear that for certain plasmid elements one can identify genes that act in *trans* on an in-*cis* site and these elements provide better than random segregational stability without affecting plasmid copy number or the growth properties of the host. On the basis of these properties, shared by several well-studied plasmid elements, it has been proposed that the stabilization observed is due to an active partitioning process and for this reason the region is designated *par* (for extensive reviews on plasmid partitioning systems see references 11, 12, 225, 327, and 329, and del Solar et al., in press). Most of our information comes from the work on plasmids F, P1 and R1/NR1. In the case of all three of these plasmid systems, genetic information includes, as a contiguous set, genes that encode two *trans*-acting proteins and an in-*cis* site that has been considered to be an analog of the centromere region of eukaryotic chromosomes. For those plasmids examined, the *par* region is not replicon specific but is effective when present on a heterologous replicon. In addition to these three extensively studied plasmids, stabilization elements on plasmids RK2/RP4 (73, 106, 253, 254) and pTar (93) and a *Salmonella* virulence plasmid (164) exhibit properties that suggest their functioning also as a partition system.

Partitioning defects are, broadly speaking, any defects that cause an increase in the probability of generating cells which lack the relevant plasmid (plasmid negatives) despite normal replication and copy number. The hallmark of a partitioning (or segregation) defect is a linear semilog survival curve. In practice, this is observed rather infrequently. Much more frequently, semilog survival curves for unstable plasmids show a downward convexity (acceleration), which is a likely indication that the plasmid negatives have a growth advantage, often too small to be detected in side-by-side measurements of growth rate (226). Misinterpretation of plasmid loss kinetics following the deletion of a region of a plasmid has on occasion resulted in the faulty assignment of a plasmid segment as a partitioning function. True segregation defects have two known causes, defects in partitioning and defects in self-correction of the plasmid copy pool.

The most extensively studied *par* systems are those of the plasmids F and P1. They also show a number of similarities in structure and function. Each system contains a *cis*-acting site and two *trans*-acting proteins: SopA (43.7 kDa) and SopB (35.4 kDa) for F (211), and ParA (44 kDa) and ParB (38 kDa) for P1 (4, 10). The ParA and ParB proteins of P1 show extensive homology to the corresponding SopA and SopB proteins of F (327). Despite this similarity in *trans*-acting proteins, the in-*cis* sites for P1 and F are quite different. This site for F (SopC) contains 12 43-bp direct repeats downstream of the *sopB* gene (123, 171, 211), while the corresponding centromere-like site for P1 contains a 20-bp inverted repeat (4). Further delineation of the in-*cis* site of F revealed that only one of the 43-bp repeats is necessary for *par* activity (25, 171). The SopA protein binds to and autoregulates its promoter (212); this repression is enhanced by the SopB protein (212). Similarly, the ParA protein regulates expression of the *parAB* operon by binding to its promoter (60); this binding is stimulated by the ParB protein (89). The amino acid sequence of both ParA and SopA contains an ATP-binding motif (214). ATPase activity has been demonstrated in ParA, and this activity is stimulated by ParB and DNA (60). The finding of this enzymatic activity for ParA has led to the speculation that ParA-catalyzed ATP hydrolysis may provide energy for a key step in the plasmid partitioning process. Both SopB (212, 323) and ParB (59) bind specifically to their corresponding in-*cis* sites, and in ParB this binding is enhanced by the IHF protein of *E. coli* (59, 90). IHF has also been shown to promote bending of the in-*cis par* region (91, 120). The properties of the F and P1 *par* systems have given rise to two different basic models for plasmid partitioning. Both models, which are an outgrowth of the original model of Jacob, Brenner, and Cuzin (139), invoke a role for the *trans*-acting proteins in translocating a partition complex consisting of a plasmid-DNA specialized nucleoprotein structure to a site on both sides of the cellular division plane during the segregation process. The basic difference in the two models is the proposal in one case that two plasmid molecules are paired at their specific in-*cis* sites before attachment to the host receptor site (10, 12), while in the alternative model freely diffusible plasmid

molecules associate individually with the specific receptors in the host (11, 139). Obviously, much remains to be done before we have an understanding of the mechanistic details of the plasmid partitioning process in bacteria.

The *par* systems of F and P1 are the prototypes for plasmid partitioning systems and bear a number of similarities to each other and the IncFII plasmids R1/NR1/R100 (55, 142, 329). Given the likelihood of partitioning systems in a number of other plasmids, it will be interesting to determine how much variation there is among different *par* systems. More recently, the stabilization region of the broad-host-range plasmid RK2 (designated *par*) has received considerable attention (106, 253, 266). This region consists of two operons within a 3.2-kb segment of DNA (106, 254). One of the operons (*parCBA*) encodes a site-specific resolution system that is capable of resolving multimers (73, 106, 109, 253), while the adjacent operon (*parDE*) encodes a toxin/antitoxin system that stabilizes by killing plasmidless cells (73, 109, 255, 256). The two adjacent operons are divergent in their arrangement and are autoregulated (61, 72). The *parA* gene encodes a 24-kDa resolvase that functions at a specific site that spans the promoter of the *parCBA* operon (73, 106, 253). The ParA protein belongs to the Tn*3* family of resolvases (106). The functions of the ParB or ParC proteins are largely unknown; however, the ParB protein displays nuclease activity (E. Grohmann and H. Schwab, submitted for publication). It has been speculated that the *parCBA* region specifies a nucleoprotein complex that contains both site-specific recombination and partitioning activities (73, 106, 254). Much work remains to be done before the mechanism of stabilization by the *parCBA* region is understood, particularly since it appears to function as a broad-host-range stabilization system. Another region of plasmid RK2 consisting of the *korA incC korB korF* and the *kfrA* operons has been proposed to stabilize by partitioning, on the basis of DNA sequence homology to known *par* systems and gene inactivation studies (214, 327). While this system alone is dispensable for stabilization of RK2 in *E. coli* (274), it may function as a partitioning system in certain other gram-negative bacteria.

Assuming random diffusion of plasmids at cell division, one can readily predict the frequency of appearance of plasmidless cells per generation for a plasmid maintained at a given copy number. Studies with plasmid pSC101 have raised the possibility that in some cases plasmid molecules are not freely diffusible but are compartmentalized, possibly in the form of plasmid aggregates. A region designated *par* in pSC101 substantially decreases the loss rate of this plasmid (196). In contrast to the F, P1, and IncFII plasmids, the pSC101 stabilization region does not encode a protein but consists of two direct repeats bordering an inverted repeat (196, 205, 312). One of the functions of this stabilization region may be to increase the random diffusion of the plasmid, perhaps by disaggregating plasmid molecules. The pSC101 *par* region possesses a binding site for *E. coli* DNA gyrase (321), and it has been shown that the loss of plasmid stability by deletion of the *par* region can be compensated for to a large extent by a *topA* mutation of *E. coli*, suggesting an important role of superhelicity in the stable maintenance of pSC101 and most likely other plasmid systems (203). Studies are continuing to determine whether there are multiple roles for this *par* region in the stable maintenance of pSC101.

For the gram-positive plasmid pADI, deletion of a region outside the minimal replicon region causes plasmid instability. It has been proposed that this region is a *par* locus and that the functional element consists of two small noncoding RNAs (325). Available data to date are insufficient to support this remarkable possibility convincingly: first, mutants with defects in this region show downwardly accelerating semilog survival curves, and one particular deletion is lethal, suggesting *kil/kor* or postsegregational lethality rather than a *par* system; second, plausible coding regions exist within the two RNAs and their translation has not been ruled out. Further experimentation is required to clarify the mechanism of stabilization by this system.

Finally, a gene outside the minimal replicon, encoding a 15-kDa protein, is required for incorporation of the plasmid pTH1030 into *B. subtilis* spores (175). Though this gene does not seem to affect hereditary stability in growing cells, it clearly qualifies as a plasmid stability determinant.

Killing of Plasmid-Free Segregants

A number of plasmid-encoded systems have evolved that, by virtue of their ability to kill cells which have have lost a specific plasmid element, stabilize the plasmid in a growing population of cells. These systems fall into two types, depending on whether the toxin responsible for the killing is excreted and works extracellularly or whether the toxin functions intracellularly on the cell that has lost the plasmid. Examples of the former type include both bacteriocins (e.g., colicins produced by colicinogenic plasmids of *E. coli* [241]) and microcins (16), both of which are antibiotically active polypeptides. In both cases, cells carrying plasmids that produce either a bacteriocin or a microcin are immune to the antibiotic that they produce, while cells in the population that have lost the plasmid lose their immunity to the toxin and are killed. Two types of stabilization systems that kill plasmidless cells from within have been described to date. In one case, both toxin and antitoxin proteins are specified by the plasmid but the toxin protein is more stable, or less susceptible to degradation, than is the antitoxin protein, while in the other case the synthesis of the toxin protein is prevented by inhibition of translation by a plasmid-specified antisense RNA. Both of these toxin/antidote systems have been described in some detail for plasmid systems (103, 208, 265).

Colicinogenic plasmids of *E. coli* encode protein toxins that are transported outside the host bacterium and act on other *E. coli* or closely related bacteria that do not carry that particular Col plasmid. While this mechanism of cell killing undoubtedly contributes to the stable maintenance of a Col plasmid, the activity of the toxin is dependent on close proximity of plasmid-containing and plasmid-free cells, unlike the toxin/antitoxin systems, where the toxin acts intracellularly. The first clear demonstration of stabilization by a toxin/antitoxin system came from studies with the *ccd* system of the F plasmid. It was observed that the loss of the F plasmid from an *E. coli* cell resulted in filament formation and ultimately cell death (140, 146, 202). The *ccd* operon, located near the *oriS* replication origin of the IncFIA replicon, consists of two genes (*ccdA* and *ccdB*) that encode a protein toxin (CcdB) and a protein (CcdA) that inhibits the activity of this toxin by association with the toxin protein (140, 146, 200). A third gene (*repD*) which specifies a resolvase that may play a role in site-specific recombination is also part of this operon (170). The CcdB protein autoregulates expression of the operon; the CcdA protein enhances the binding of the CcdB protein to its promoter (62, 287, 288). Stabilization of a plasmid carrying the *ccd* system is thought to be the result of greater stability of the toxin protein than the antitoxin and, therefore, the toxic activity of the CcdB protein is eventually released in cells that have lost the plasmid (315). In an elegant study, the target of the toxin activity of the CcdB protein was shown to be the GyrA protein (21, 201). The CcdA protein interacts with the CcdB-GyrA complex, and the resulting inactivation of CcdB by binding to CcdA releases GyrA activity. Studies with the F plasmid underscore the functioning of at least two mechanisms to assure stable maintenance of this low-copy-number plasmid: the F partitioning system and the backup *ccd* system. This is not unusual in that several plasmids have now been shown to carry multiple systems to stabilize their maintenance in a growing population of bacteria. In those plasmids examined, these systems function independent of the replicon that carries the system.

Plasmid R1 is another good example of a plasmid that carries more than one stabilization system. There are actually two toxin-antitoxin systems encoded by plasmid R1. The *parD* system of Rl shares the basic features of the F *ccd* system in that it consists of two genes in an operon that encode a toxin protein (Kid) and its antitoxin (Kis) (34, 35). The Kid protein has been shown in vitro to inhibit DNA replication, and this inhibition is prevented by the presence of the Kis protein, which forms a strong complex with the Kid protein (del Solar et al., in press). Interestingly, the Kis and CcdA proteins exhibit considerable homology to each other (35). The same system is found in the closely related plasmid R100. In this case the genes are designated *pemK* and *pemI* for the toxin and antitoxin, respectively (308). In addition to the *parD* system, plasmid R1 carries the *parB* operon that includes a toxin protein gene (*hok)* and an antitoxin sequence (*sok)* that specifies a relatively unstable antisense RNA (100, 104). Translation of the *hok* messenger is coupled to the synthesis of another structural gene in the *parB* operon, designated *mok,* and the expression of the *mok* messenger is inhibited by the *sok* RNA (102, 105, 292). Thus, the production of the Hok protein is indirectly inhibited by translational

inhibition of the Mok protein. The relatively rapid degradation of the *sok* mRNA in plasmid-free cells is thought to result in release of inhibition of the Mok protein and, consequently, the synthesis of the Hok protein toxin which is a membrane protein. Expression of the Hok protein results in the formation of "ghost cells" and a loss of cell viability (104). A system analogous to the *parB hok*/*sok* system, designated *flm* or *stm*, is also found on the F plasmid. Finally, genes with functional and structural homology to the *hok*/*sok* system have been identified on the *E. coli* chromosome (101, 103, 208, 246). The significance of these chromosomally located toxin/antidote systems, designated *gef* and *relF*, is not known.

It is now clear that plasmids often carry both a set of genes that have the properties of a partitioning system and a set of toxin/antitoxin genes, with the latter system correcting for errors in the regulation of replication that result in only a single copy of the plasmid in a dividing cell or a failure of the partitioning system. Recently, the well-studied P1 plasmid was shown to encode a toxin/antitoxin system (designated *phd/doc*) to complement the P1 partitioning system (174). In addition, the broad-host-range plasmid RK2, as described above, carries the *parDE* operon, which specifies antitoxin and toxin proteins and functions to kill plasmidless cells of *E. coli* and other gram-negative bacteria (254–256). As with the *ccd*, *parD*, and *parB* killing systems, the protein products of the RK2 *parDE* system are relatively small; their sizes are approximately 11 kDa (ParD) and 9 kDa (ParE) (254). Also, as with the *ccd* and *parD* systems, the ParD antitoxin protein forms a tight complex with the ParE toxin protein (E. P. Johnson, A. R. Strom, and D. R. Helinski, submitted for publication). In addition to the *parCBA* and *parDE* systems in RK2, evidence has been presented for the presence of a postsegregational arrest system (*pas*) that appears to inhibit the proliferation of plasmid-free cells (143). As with other plasmids carrying multiple systems for stabilization, much work remains to clarify the relative contributions of the individual systems to plasmid stability under different environmental conditions. Recent studies with RK2 have provided insight into the relative contributions of the *parCBA* and *parDE* systems toward stabilizing an intact RK2 plasmid in a variety of gram-negative bacteria (274; C. Easter, P. Sobecky, and D. R. Helinski, manuscript in preparation). The results of these studies suggest that the two systems differ in their contribution to stable maintenance depending on the host and the culture conditions. Finally, these studies further indicate a role for conjugal transfer in the stable maintenance of a conjugative plasmid in a growing population of bacteria.

CONCLUDING REMARKS

It is clear from detailed analyses of a variety of plasmid systems in gram-negative and gram-positive bacteria that highly ordered structures involving RNA (repressor) and RNA (target) molecules, or specialized nucleoprotein structures involving plasmid-specified initiation proteins and a specific replication origin sequence that often includes direct nucleotide sequence repeats, are key elements in the regulation of plasmid copy number. In every case it is the concentration of at least one of the components of these highly ordered complexes that determines the frequency of initiation of replication. Particularly for low- or intermediate-copy-number plasmids, mechanisms other than copy-number control are required to limit the number of plasmidless cells that are generated on the basis of simple random distribution of plasmids to daughter cells during cell division. One of these mechanisms appears to be active partitioning of plasmid molecules to assure that each daughter cell receives at least one copy of the plasmid. It is now clear that there are also a variety of plasmid-encoded toxin-antidote systems that act as a backup process to partitioning/regulatory failures. These systems kill those cells that fail to receive a specific plasmid molecule on cell division. Frequently, a particular plasmid will contain, in addition to a partitioning system, one or more toxin-antitoxin genes to provide for stable maintenance. The variety of replication control mechanisms and other stable maintenance systems underscores the ability of plasmids to evolve diverse strategies to assure their hereditary stability.

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