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Regulation of Plasmid Replication

JUNE ROTHMAN SCOTT

Microbiology & Immunology, Emory University, Atlanta, Georgia 30322

INTRODUCTION

The regulation of cell division is a central problem for all biology. To comprehend regulation of cell division, an understanding of the control of DNA replication is necessary because the two are closely interdependent. It is difficult to study regulation of cellular replication at the molecular level since experimental perturbations of the system would be expected to be lethal to the cell. For this reason, bacterial plasmids, which are dispensable for cell viability but replicate independently of the bacterial chromosome, are popular as model systems for studies of regulation of DNA synthesis.

It is clear that plasmid-encoded antibiotic resistance among bacterial pathogens is a major problem worldwide in infectious diseases. Thus, in addition to serving as a possible model for host chromosome replication, plasmid replication is of practical medical importance. If we had a better understanding of the mechanisms that regulate the maintenance of plasmids in bacterial cells, it might be possible to reduce their prevalence.

Two factors are important in maintenance of plasmids in bacterial cell lines. One is their faithful replication at least once in each cell cycle and the other is their accurate partition so that each daughter cell receives at least one copy. In a given host under defined growth conditions, each specific plasmid is maintained at its individual characteristic number per cell, its "copy number." It is the system for regulation of replication that is primarily responsible for accurate copy number maintenance. Some plasmids have a high copy number and others may be maintained at about one per host DNA molecule. The latter class is inherited just as stably as the former, demonstrating the existence of an accurate mechanism, called partition, for distribution of the daughter plasmids to daughter cells at division.

The problems facing low- or unit-copy plasmids should be similar to those facing the bacterial chromosome, so one might expect this type of plasmid to be the best model system for host replication regulation. Since no single plasmid system is completely understood at the molecular level yet, the apparent differences in control of replication between high- and low-copy plasmids may be the result of the paucity of information currently available, and the two kinds of plasmids may turn out to be more similar than expected. On the other hand, multicopy plasmids such as ColE1 may actually have entirely different copy number regulation from low-copy plasmids such as P1 and F. Clearly, regulation need not be as precise for high-copy plasmids to achieve stable inheritance.

The presence or absence of plasmids in bacteria is a useful tool for epidemiology, and their classification into incompatibility groups is commonly used to distinguish among the different plasmid varieties (43, 44). An incompatibility group is composed of plasmids which are unable to persist in the same cell line (for review see reference 196). When two plasmids belonging to the same incompatibility group are present in the same cell, only one will be stably inherited. This is seen experimentally by introduction of a second plasmid into a plasmid-containing cell. When simultaneous selection for both is removed, incompatible plasmids segregate during cell division. Members of an incompatibility group are usually closely related and at least partially homologous. Although the molecular basis of incompatibility is not yet clearly understood, it is assumed that incompatible plasmids cannot be distinguished from each other at one or more of the stages at which plasmid maintenance is controlled. There are at least two such stages: DNA replication and segregation to daughter cells at cell division (partition).

Quantitatively, the regulation of replication is of primary importance in determining plasmid incompatibility. Therefore, diffusible factors that act to regulate plasmid replication are expected to, and do, affect plasmid incompatibility. If the system that controls replication recognizes two plasmids as identical, they will be incompatible. If we consider plasmids maintained at approximately one per cell, it is clear why this would happen. Once one plasmid has been replicated, the regulatory system prevents further replication rounds until after cell division. Thus, one of the daughter cells would inherit only one of the two kinds of plasmids. In this situation, the second plasmid would be eliminated from the cell line very rapidly.

The other system that affects incompatibility is the one that regulates accurate partition of the plasmids to the daughter cells at cell division. Again, if two plasmids are recognized as being the same, only one will be segregated accurately into the daughter cells (equipartitioned), and the other will be randomly partitioned between the daughters. This, too, will lead to loss of one plasmid from the cell line. Incompatibility determined by restricted partition is usually less severe than that determined by replication regulation.

In this review I will discuss only regulation of replication, not partition mechanisms, although the latter are also important for stable plasmid maintenance. In addition, I will only include discussion of plasmids whose replication control has been intensively studied. First, regulation of replication of each will be considered, and then general principles that emerge will be discussed.

ColE1

ColE1 is a member of a group of small plasmids that can replicate in the absence of de novo protein synthesis (reviewed in references 180, 215). In the presence of chloramphenicol or other drugs at levels that inhibit protein synthesis, the bacterial chromosome is prevented from undergoing new rounds of replication. However, since the ColE1 plasmid continues to replicate, it can be "amplified" (increased in number relative to the bacterial chromosome) by as much as 50-fold (26, 27, 49, 74, 86). This property results from the facts that ColE1 does not require any plasmid-encoded proteins for replication in vivo and that the necessary host proteins are stable. In addition, the inhibition of ColE1 replication responsible for maintenance of its normal copy number must be chloramphenicol sensitive. The ability to amplify ColE1-type plasmids has made them very popular vectors for in vitro cloning of genes which are desired in large quantities.

Host Functions Required

ColE1 replicates in vitro as well as in vivo in the absence of plasmid-specified proteins (207).

For replication, all of the members of the ColE1 group of replicons require the host-encoded enzymes DNA polymerase I (89) (product of the *polA* gene) and DNA-dependent

RNA polymerase (33, 36, 178, 195) as well as DNA polymerase III (the product of the *dnaE* gene) (179). The products of *Escherichia coli dnaB, -C, -G,* and *-Z* are also required. Plasmids belonging to this group include RSF1030, pMB1, CloDF13 (which encodes a cloacin), and p15A (a small cryptic plasmid found in *E. coli* 15).

Since it takes a small fraction of a cell cycle to replicate even a large plasmid, regulation of the number of replication events is expected to occur at the level of initiation of a new replication round (see review by Nordstrom et al. [K. Nordstrom, S. Molin, and J. Light, Plasmid, in press]). This has been demonstrated (214) for the ColE1-type plasmid CloDF13 by determining that the time it takes to replicate one copy of the wild type (90 s under growth conditions used) is the same as the replication time for a mutant with a sevenfold increase in copy number.

Origin and Direction of Replication

Replication of ColE1 is unidirectional from a specific origin site in vivo, as shown by electron microscopy (79, 114, 206). This is also true for ColE1 replication in vitro (207). The ColE1-type plasmids CloDF13 and RSF1030 (which were independently isolated from nature) have also been shown to replicate from a single origin in one direction (30, 187, 188). However, the miniplasmid pVH51, derived from ColE1 by in vitro deletion, replicates bidirectionally, although it utilizes the normal ColE1 origin site (74). This has not yet been explained.

Primer

For replication of the ColE1-type plasmids in vivo and in vitro, rifamycin-sensitive RNA synthesis is required (10, 28, 155). This rifamycin sensitivity suggests that the *E. coli* DNA-dependent RNA polymerase synthesizes the primer RNA for ColE1.

This deduction has been confirmed by much elegant work utilizing an in vitro replication system. In this system, with a mini-ColE1 template, DNA replication starts at any of three consecutive bases defining the origin of replication (206). The RNA primer promoter is located about 555 bases upstream of the replication origin. In the absence of DNA initiation, several sizes of transcripts that continue through the replication origin are synthesized (Fig. 1). However, some of the nascent transcripts hybridize with their template DNA near the origin. These RNA-DNA hybrids serve as the substrate for RNase H, which cleaves the hybridized preprimer RNA to produce the RNA primer (RNA II; 85). Deoxynucleotides are added directly to the primer by the host enzyme DNA polymerase I. The formation of a stable hybrid between the RNA preprimer and DNA is thus critical for initiation of DNA replication.

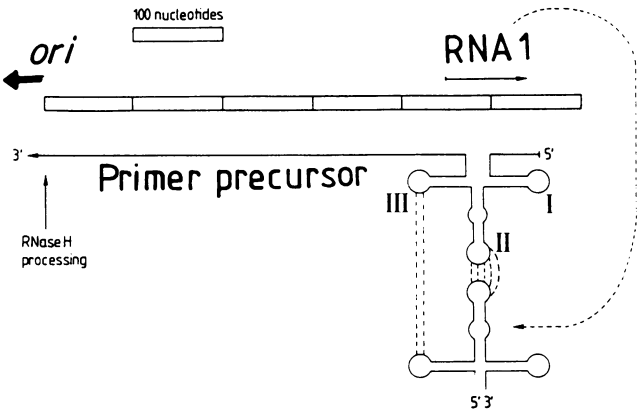

FIG. 1. Replication region of ColE1. The direction of DNA replication is indicated by the dark arrow, which starts from "*ori*." The open bar represents the DNA, and the vertical bars indicate distances of 100 nucleotides. The transcripts are indicated as arrows above the DNA. The cloverleaf structures for RNA I and the primer precursor are indicated; the postulated hydrogen bond interactions between them are shown as dotted lines. This figure is derived from one by Lacatena and Cesareni (103).

Negative Regulation by RNA I

The formation of the critical preprimer RNA-DNA hybrid is under negative control (70, 167) by a small RNA molecule called RNA I, whose presence leads to continuation of transcription through the replication origin instead of processing of the preprimer transcript by RNase H (203); RNA I is transcribed from the region shown genetically to be involved in copy number control (30, 73, 131, 133, 167, 186). It is about 100 nucleotides long (106, 144), starts 400 to 480 nucleotides upstream of the origin (21, 144), and ends near the start of the primer transcript (85) (see Fig. 1). Thus, the same DNA region that encodes primer RNA is used in the opposite direction to transcribe RNA I.

From their sequences, it can be deduced that both the inhibitory RNA I and the preprimer RNA II can form three stem-and-loop structures (6, 20, 103, 141, 144, 188, 203). As shown by the phenotype of mutants with high copy number (*cop* mutants; see below), these structures are important for processing of the primer and for interaction of the preprimer with RNA I (a diffusible molecule). The latter interaction is the basis for incompatibility in the ColE1 group of plasmids, as demonstrated by in vitro inhibition of formation of primer RNA (203). RSF1030, which is compatibile with ColE1, has a different nucleotide sequence for the primer-RNA I region from that of ColE1, but the molecules can be folded into analogous structures (173). In the region important for incompatibility, there are no protein reading frames common to the ColE1-type plasmids pST19, pBR322, and ColE1, so it can be concluded that there is no plasmid-specific factor other than RNA I encoded by this region that is involved in determination of incompatibility (173).

In vitro, RNA I inhibits formation of the primer (85), probably by inhibition of formation of the DNA-RNA hybrid between the primer precursor and its DNA template (205) which is required as the substrate for RNase H. That RNA I forms hybrids with the primer in vitro was demonstrated by the sensitivity of the double-stranded RNA product to RNase III (203). The RNA I molecule can be folded into a tRNA-like structure, with three loops and stems. Like a tRNA, there are seven base pairs in the middle loop (corresponding to the anticodon loop) (103). Lacatena and Cesareni believe that RNA I and the primer precursor interact in this loop to inhibit RNase H processing, probably by altering the structure of the primer precursor. By analyzing a large number of mutations that affect RNA-RNA primer interaction, Lacatena and Cesareni (103) found that the actual sequence of bases in this loop is not important for inhibition, but that the interaction of bases in this area determines the specificity of inhibition (incompatibility specificity). Enzymatic and chemical probes were used for a structural analysis which demonstrates that the RNA I's from ColE1 and RSF1030 (a member of a different incompatibility group) are very similar (191). This study also showed that a recessive *cop* mutant has an RNA I with an altered secondary struc-

ture, which supports the idea that such a structure is critical for function of this RNA molecule.

The importance of secondary structure for the conversion of preprimer to primer was shown in vitro by substituting inosine for guanine during the synthesis of RNA II. Inosine-cytosine pairs are less stable than guanine-cytosine pairs, so the secondary structure of the inosine-containing RNA II molecule was expected to be less stable than the cytosine-containing molecule. As expected, after the base substitution, primer formation was inhibited. This inhibition occurred even when the base substitution was 400 nucleotides upstream of the origin, demonstrating the importance of secondary structure in distant regions of the RNA II molecule (204).

To explain the finding that alteration of the sequence outside of the hybridized region also affects the initiation process (160), a regulatory model similar to that proposed for transcriptional attenuation has been envisioned. The model involves the relative probabilities of the pairing of regions within one RNA molecule. The intramolecular folding of either RNA I or RNA II may be altered so that the appropriate loops are not available in the single-stranded state for intermolecular pairing. When RNA I cannot pair with RNA II, the latter is free to hybridize to the DNA near the origin. Replication may then be initiated by an RNase H cleavage event.

In support of the deductions from in vitro experiments, mutations of ColE1 (133), pMB8 (30), CloDF13 (184), and RSF1030 (also called pNTP1 [67]) that are in the region specifying RNA I affect both copy number and incompatibility of the plasmid. Some of these copy number mutants are temperature sensitive (131, 135, 222). It is supposed that this temperature effect is caused by alterations in the secondary structure of the RNAs involved, since transcription from the mutant promoter is not temperature sensitive (131, 222; see below).

In support of the deductions from in vitro experiments, mutations of ColE1 (133), pMB8 (30), CloDF13 (186), and RSF1030 (also called pNTP1 [67]) that are in the region collaborators to isolate several kinds of copy mutants. Mutants in the target of the inhibitor (102) are defined by their insensitivity to inhibition by RNA I. All are altered in copy number (most have a higher number, but one has a lower number). Since the DNA that encodes the target of the inhibitor, RNA II, overlaps the DNA that encodes the inhibitor (see Fig. 1), single mutations in the target produce inhibitors unable to interact with a wild-type target (19).

On the basis of their interaction with a wild-type target, the ColE1 copy mutants are placed in one of two classes. Class A target mutants (22 of 41 target mutants isolated) produce an inhibitor active only on their own target and not on the wild-type target. Mutants are placed in this class when the presence of the wild-type plasmid in the same cell with the mutant does not alter the copy number of the mutant (102, 103). These target mutants define new incompatibility groups, showing that they are mutated in the RNA I inhibitor (102). Thus, a single class A mutation alters both the target and the inhibitor. The location of the target mutations defines the bases involved in the pairing of RNA I with the primer precursor (Fig. 1). By sequencing these mutations, Lacatena and Cesareni (103) were able to conclude that changes in loops 1 and 2 of the RNA primer (see Fig. 1) alter the incompatibility specificity but not the function of RNA I.

The rarer class B target mutants (14 of 41) are not able to interact either with wild-type or with their own cognate inhibitory elements (103). This class of mutant has a very high copy number (>2.5 times that of the wild type [102]), as expected if RNA I inhibition is no longer very effective. These mutations tend to produce destabilizations of the cloverleaf of RNA I and demonstrate that the sequence of stem 1 and of stem and loop 2 is very critical for this intramolecular interaction.

In another study, Tomizawa and Itoh (203) isolated eight independent mutants of the ColE1-type plasmid pNT7 that have lost incompatibility and simultaneously show an increase in copy number. These mutants define four base substitutions (i.e., duplicate mutants were isolated in four cases) which are single-base changes in the center of each of three palindromic sequences (equivalent to the loops in Fig. 1) in the region encoding primer and RNA I. These were shown in vitro to affect the rate of hybridization of RNA I to a homologous target, and there is a quantitative correlation between the hybridization and the copy number, suggesting the in vivo importance of this hybridization. The mutations of the two class A mutants (no longer incompatible with wild type, but still incompatible with themselves; see above) isolated by Tomizawa and Itoh (203) map at the same site in loops I and II (Fig. 1) as two of the Cesareni mutations. The two single mutants of Tomizawa and Itoh (203) that produce an inhibitor active on the wild-type target (class C) map in loop I (Fig. 1). Although Lacatena and Cesareni isolated mutants of this class (103), the mutations have not yet been located. These results together suggest that all three loops are involved in RNA I-primer interaction.

Temperature-sensitive copy mutants of ColE1 have lesions in the region encoding RNA I and the replication primer. These mutant promoters are not temperature sensitive for the activity of a galactokinase gene fused to them in vitro, so the copy mutant phenotype probably results from an effect on the secondary structure of the primer RNA (222).

Further in vivo support for this RNA I inhibition model of regulation of ColE1 replication comes from experiments that demonstrate that RNA I may be provided in *trans* to reduce the copy number of a copy mutant of a ColE1-type replicon (131, 133, 167). Furthermore, there appears to be an inverse relationship between the number of RNA I genes in the cell and the copy number of the ColE1-type plasmid (131).

A Second Negative Regulator

In addition to the copy mutants that have alterations in the RNA I-primer region of ColE1, Twigg and Sherratt (210) found a deletion of a distant nonessential region that also increases the copy number. This *cop* mutant is recessive and can be complemented by the plasmid ColK. Because ColE1 and ColK are mutually compatible (i.e., they are not in the same incompatibility group), the inhibitor defined by this mutation is not important in determining incompatibility.

The function defined by this mutation has been named Rop, for repressor of primer (20). Fusions to the β-galactosidase gene (*lac*) from the promoters for either primer RNA or RNA I show that transcription from the RNA I promoter is not influenced by the presence of wild-type ColE1 or the ColE1-type plasmid pMB1 in the cell, but that primer transcription is repressed by Rop, which is therefore the same in both of these plasmids. The *rop* mutation maps to a sequence that can encode a 63-amino acid protein and that is identical in pMB1 and ColE1 (20). When this fragment was cloned in vitro into another replicon, it was found to synthesize a 6,500 M_r polypeptide, as expected. These authors propose that the Rop protein modulates transcrip-

tion of the precursor for primer RNA and thus regulates copy number of the plasmid.

A 63-amino acid peptide encoded by the Rop region negatively regulates ColE1 replication in a crude in vitro extract, but not in a purified system. Furthermore, it does not protect the fragment of DNA containing the primer promoter and 40 bases downstream under conditions in which RNA polymerase protects. This suggests that another plasmid-coded factor may be required for Rop activity (R. M. Lacatena, D. W. Banner, and G. Cesarini, *in* N. Cozzerelli, ed., *Mechanisms of DNA Replication and Recombination*, in press); this factor has been recently identified as RNA I both in vivo and in vitro (Cesareni et al., manuscript in preparation).

Summary

Transcription of preprimer RNA begins about 500 bases upstream of the replication origin. To initiate DNA replication, the preprimer RNA must hybridize to its DNA template in the region of the origin. This RNA-DNA hybrid serves as a substrate for RNase H, which cleaves the preprimer RNA to form the primer onto which deoxynucleotides are added.

ColE1 replication is negatively regulated by RNA I, a small untranslated RNA molecule encoded within the DNA region that is used to transcribe the RNA primer, but in the opposite direction to the primer. Because RNA I is complementary to the preprimer RNA, the two can hybridize. When the preprimer RNA hybridizes to RNA I, it cannot hybridize to the DNA and thus cannot be used to form primer. An additional inhibitor of ColE1 replication is the 63-amino acid polypeptide called Rop, whose mode of action is still not clear. The *rop* gene maps 400 nucleotides downstream from the replication origin.

PLASMIDS OF THE FII INCOMPATIBILITY GROUP

The three members of incompatibility group FII whose replication has been studied in detail are R1, R6-5, and R100 (also called NR1 and R222) (124). They are extensively homologous to each other, as demonstrated by electron microscopic analysis of heteroduplexes (143, 165). All have a low copy number of about two per host chromosome and a size of about 100 kilobases (kb). Since they contain transfer (*tra*) genes which encode functions required for conjugal transfer of DNA, they are self-transmissible and are thus considered conjugative plasmids. Regulation of the replication of these plasmids was reviewed by Timmis et al. (200).

Host Functions Required

For replication of the FII group plasmids, the host functions *dnaB, -C, -E, -F*, and *-G* are required (152). Neither the *E. coli dnaA* function (65, 129, 136, 223) nor DNA polymerase I (encoded by the *polA* gene [93, 197]) is required.

Origin

Because these plasmids are so large, in vitro recombinant techniques were utilized to reduce their size (see below). The origin of replication of these plasmids was determined by electron microscopy of the miniplasmids (142, 190) in these experiments; replication was found to be unidirectional from a single origin. The miniplasmid origin is the one used primarily in vivo by the whole plasmid, as shown for R100 by partial denaturation mapping and autoradiography as well as by electron microscopy (170). When NR1 is transferred into *Proteus mirabilis*, additional origins, identified by denaturation mapping and electron microscopy, are activated (217). However, these techniques were not precise enough to locate the additional origin(s) specifically on the current restriction map and did not reveal how frequently the new origins are used.

Plasmid-Coded Functions

Two R-encoded functions, required for plasmid replication, were identified by Yoshikawa (223). An *E. coli* strain that has a temperature-sensitive defect in initiation of DNA replication (*dnaA* mutant) may be rescued at high temperature by formation of a cointegrate between the host chromosome and a plasmid. When the host chromosome becomes part of the plasmid replicon, the plasmid provides the origin for replication. Instead of requiring host replication functions, this cointegrate replicon requires plasmid functions. This phenomenon is called integrative suppression (136). Deletions of R100 unable to cause integrative suppression were mapped to two locations which define the two Rep functions of this plasmid. RepA (later called RepA1) was identified as being absolutely required for integrative suppression by R100.

To obtain smaller replicons, an *Eco*RI restriction fragment from a *Staphylococcus aureus* plasmid that encodes ampicillin resistance was ligated to incompatibility group FII plasmid DNA that had been digested with *Eco*RI (198). Subcloning from the resultant plasmid gave a 2.6-kb minireplicon that expresses normal incompatibility and approximately normal copy number and is able to replicate in a $PolA^-$ host (1, 41, 93, 125, 126, 128, 192, 197–199). The slight instability of the minireplicons was later determined to result from loss of a fragment encoding stability (*stb*) or partition (*par*) functions required for accurate partition of plasmids at cell division (125, 138). Electron microscopy of heteroduplexes shows that R100 and R1 share about 2.5 kb of DNA that is sufficient for autonomous replication, in agreement with the findings from subcloning experiments (143). This essential replication region includes a small nonhomology between R1 and R100 (142).

In addition to the origin, other plasmid DNA is required for replication of the FII group plasmids (92, 125, 140, 192). The RepA1 determinant, located earlier by Yoshikawa (223) as RepA, is within the minireplicon region and appears to be a plasmid function required for replication. The possibility has not been ruled out that additional plasmid-coded functions are also required.

From the DNA sequence of the minireplicon region in R1 and R100 (148, 154), a protein corresponding to RepA1 was deduced to have a molecular weight of 33,000. In chimeras containing the replication region of R100 and the ColE1 replicon (which requires $PolA^+$ for replication), mutants were constructed in vitro that are deleted for part of the *repA1* gene. These are unable to replicate in a *polA*$^-$ host (154). Minicells that contain an R100 miniplasmid or a cloned fragment from R100 contain a 33-kilodalton (kd) protein that is made from this region of the plasmid (2). Direct evidence that this is the RepA1 protein awaited development of an in vitro replication system (121; see below).

To approach the problem of regulation of replication genetically, mutants of these plasmids with increased copy number were isolated (40, 56, 68, 127, 130, 137, 192, 193, 211). In complementation tests with wild-type plasmids, some mutants were found to be *cis* specific and others to be recessive. Some of these mutants were conditional: suppressor sensitive or temperature sensitive (68). The existence of recessive and of conditional copy mutants suggests that

replication is under negative control. The mutations were mapped to two locations and were thus designated *copA* or *copB*. *copA* mutants had reduced incompatibility with wild-type plasmids, but *copB* mutants often showed normal incompatibility.

Promoters in the Replication Region

The sites at which RNA polymerase binds to the DNA of the replication region were determined by adding the polymerase to restriction nuclease-generated fragments of R6-5 or R100 DNA and examining the resulting complexes by electron microscopy (116) or filter binding (51). This identified three strong polymerase binding sites in the replication region (Fig. 2). Site 1 is probably the promoter for *copB* (transcript RNA-Cx; see below) and also can produce RepA1, site 2 is probably the major promoter for RepA1 (transcript RNA II; see below) and possibly for a primer RNA, and site 3 is that for *copA* (later shown to be RNA I = RNA-E; see below). RNA I and RNA II, which initiate from the polymerase binding sites, were identified by in vitro transcription (150). Promoters for these transcripts were also deduced from the DNA sequence (149) and confirmed by physical mapping, using fragments cloned in vitro (51). An additional transcript, named RNA-C, which is shorter than RNA-Cx, is also produced from the RNA-Cx promoter (51; D. D. Womble, V. A. Luckow, X. Dong, R. P. Wu, and R. H. Rownd, Proc. Natl. Acad. Sci. U.S.A., in press).

CopB = RepA2

The *copB* gene was defined by deletions and insertions in mini-R1 (127). These mutations increase the copy number of the plasmid by 10- to 15-fold. In the presence of the wild-type plasmid, a miniplasmid *copB* mutant had a low copy number, thus indicating that the mutant is recessive. In spite of the *copB* mutation, the R1 plasmid still expresses incompatibility, indicating that for this function in R1 the *copB* locus is not essential (127).

Because *copB* does not affect incompatibility, the region of sequence divergence observed between the different members of the IncFII group, R1 and R100, might be within the *copB* gene or its target or both. In support of this, R1 *copB* mutants are not complemented by wild-type R100. However, since removal of the N terminus of the presumptive coding sequence of *copB* generates mutants with the CopB phenotype, a product analogous to *copB* is also present in R100. As expected, such mutants are complemented by wild-type R100 in *trans* (111).

The *copB* product appears to be the 9.7-kd (84-amino acid) protein, called RepA2, that was predicted from the DNA sequence (149, 154, 185). This interpretation is consistent with the observation of such a protein in vitro (13) and in vivo (15, 40, 127), although the size of this protein seems to vary slightly from one FII group plasmid to another. The protein predicted from the sequence is basic and thus well adapted to binding to DNA. It is expected that in R100 this protein contains no tryptophan residues, and differential labeling in minicells confirms this (2). The sequence of the RepA2 protein differs in the mutually incompatible plasmids R1 and R100 (149), which further supports the finding that RepA2 is not involved in determination of incompatibility.

The direction of transcription of *copB* was deduced from identification of the restriction fragments encoding transcripts synthesized in vitro (51) and from in vitro constructions that mapped the promoter of this gene. In the latter study, use was made of two different vectors, each containing a selectable function whose gene had been deleted for its own promoter (108, 127). Therefore, for expression of the gene function from the vector, the *copB* promoter had to be present. The *copB* transcription direction is illustrated in Fig. 2.

To learn more about the effect of *copB* on its target, in vitro fusions (17) between *repA1*, the gene required for replication of the FII group plasmids, and *lacZ*, the gene encoding the easily assayed enzyme β-galactosidase, were constructed. Two types of fusions have been studied. In a "gene fusion" or "translational gene fusion," the N terminus of the *lacZ* gene is replaced by the *repA1* DNA so that a fused protein is produced. This protein, which retains galactosidase activity, is transcribed and translated from signals in the *repA1* DNA. In an "operon fusion" (18), the entire *lacZ* gene is placed under the promoter to be tested. The amount of galactosidase now reveals the activity of the promoter.

In a chimeric plasmid which lacks the $copB^+$ gene, the presence of $copB^+$ in *trans* inhibits expression of galactosidase from *repA1-lac* gene fusions (107, 109, 110; Womble et al., in press). Direct quantitation of transcripts synthesized in vivo was performed by filter hybridization to DNA probes isolated from constructed chimeric plasmids derived from different parts of the R plasmid (Womble et al., in press). These results, as well as those from in vivo data using fusion chimeras, indicate that the $copB^+$ product inhibits transcription of *repA1*. In both types of experiment, the target of the *copB* product was found to lie within the 60-base pair (bp) region containing the RNA II or RNA-A promoter (promoter 2 in Fig. 2) (107, 108; Womble et al., in press).

When a $copB^+$-containing plasmid that lacks the *copA* region is present, no effect on the copy number of a wild-

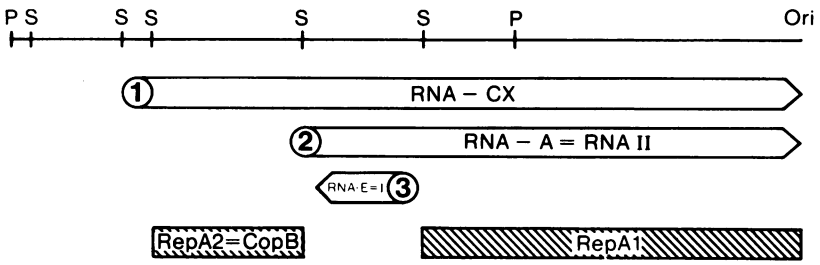

FIG. 2. Replication region of group FII plasmids. The upper line represents the DNA and shows the origin of replication. S indicates *Sau*3A sites (on NR1) and P is a *Pst*I site. Transcripts are indicated by open bars labeled with their names. Promoters 1, 2, and 3 are shown as circles at the beginning of their respective transcripts. Proteins are shown by shaded bars below their respective transcripts. The scale is taken from NR1 and based on Womble et al. (in press). The small *Sau*3A fragment is 60 bases long, and the one to its right is 332 bases.

type R1 plasmid is detectable, and there is no "switch off" of DNA replication in a host integratively suppressed by wild-type R1 (147). From this it was concluded that the addition of extra *copB*$^+$ product has no significant effect on the wild-type plasmid, presumably because the *copB* target is already saturated in the wild-type state. For this reason, *copB*$^+$ has no observable effect on incompatibility.

Expression of *copB*, determined by assaying β-galactosidase in a protein fusion strain (108), is not regulated by other *trans*-acting plasmid-coded functions. In addition, *copB* expression is proportional to growth rate between 0.4 and 2.0 doublings per h and appears to be gene dosage dependent. This leads to the view that, in the normal FII plasmid situation with about two copies per chromosome, *copB* plays no significant role in regulation of replication, in agreement with the results of Light and Molin (109) (see above).

Liu et al. (111) suggest that the *repA2/copB* function acts as a switch between low- and high-copy-number modes of replication and that the *copA* gene product acts to maintain a constant copy number in either mode. They view this as analogous to the establishment and maintenance modes in λ. From this model they predict a zygotic induction effect on replication of FII plasmids after conjugation: the plasmid should start to replicate rapidly until the *repA2/copB* product builds up. Since R100 and R1 have different *copB* products, they also predict that R100 should establish itself at the expense of a resident R1 plasmid, and vice versa. This type of model is also espoused by Womble et al. (in press), who find that the normal situation involves the presence of excess RepA2 (*copB* product).

CopA (RNA I = RNA E)

Action of *copA*. When the *copA*$^+$ region is provided from a chimeric plasmid in *trans*, it switches off DNA synthesis from a *dnaA* host that has been integratively suppressed by R1 (126). In agreement with this *trans*-acting inhibition of replication, *copA* mutants show reduced incompatibility, and physical mapping (by in vitro cloning of DNA fragments) indicates that incompatibility is determined by the *copA* region in all of the FII group plasmids. Cloning experiments have shown that *copA* acts independently of *copB*, although both inhibit expression of the *repA* gene (126).

Translational fusions of *repA1* to *lacZ*, in which β-galactosidase activity of a LacZ-RepA fusion protein is assayed, indicate that *copA* inhibits expression of the *repA1* gene (107). However, this is not a transcriptional inhibition, since transcription of the R100 *copA*-type mutant pRR12 is qualitatively and quantitatively indistinguishable from wild type, but shows stimulated *repA1* gene expression and increased copy number (50, 125; Womble et al., in press). This suggests that *copA* regulates RepA1 post-transcriptionally.

Product of the *copA* gene. From whole R1 in minicells, at least 10 low-molecular-weight RNAs were identified. Three of these are from the origin region (45a). Minicell experiments and in vitro transcription with R1 or chimeric miniplasmids indicate that the product of the *copA* gene is a small RNA, called RNA I (91 bp) or RNA-E (see Fig. 2; 51, 150, 184). Electron microscopic studies have identified an RNA polymerase binding site at the appropriate location for the *copA* promoter (116). This RNA is synthesized in the direction away from the replication origin. Mutants lacking *copA* function (inhibition of replication) do not express this RNA. From the DNA sequence, one of these mutants appears to have a base substitution in the promoter that precedes the *copA* gene.

In addition to the small RNA, the DNA sequence of this region could code for a 7.2-kd basic polypeptide of 65 amino acids (149, 185), but no such protein has yet been seen. The alterations produced in various *copA* mutants indicate that the polypeptide is not responsible for the CopA phenotype, but that the active *copA* product is untranslated RNA. For example, one copy mutant has a new termination site in the sequence for the polypeptide but does not alter the activity of the *copA* product (40). Also, two *cop* mutants that alter the polypeptide in the same way (i.e., have the same amino acid substitution) have different phenotypes (12), which supports the interpretation that the nucleotide sequence *per se* and not its protein product is important for the CopA phenotype.

In vitro, the transcript called RNA I (or RNA-E or CopA) is made from the 2.5-kb region of DNA common to R100 and R1 (150). The RNA I sequence is contained entirely within the coding region for the rightward transcripts that produce RepA1. These transcripts are, however, synthesized in the direction opposite to RNA I. Based on its sequence, RNA I can form two regions of stable secondary structure (150). Point mutants that have altered the inhibitor activity of CopA affect the 6-base loop attached to a 22-base stem that can be formed from this RNA (12, 149, 150, 184).

Target and interaction of CopA. Several lines of evidence indicate that the target of the CopA product, RNA I, is one of the cRNA molecules made from the other DNA strand in the same region (see Fig. 2). Some group FII plasmid *cop* mutants show reduced incompatibility with wild-type plasmids. These are located in the *copA* gene and are considered target-type mutants (40, 125). However, two differently marked plasmids with the same target-type mutation are mutually incompatible. Such mutants presumably alter both the *copA* product and its target, indicating that the target for CopA is within its own coding sequence (125).

That a point mutation can simultaneously affect the *copA*-RNA and the CopA target was confirmed by using translational fusions of Lac to RepA1. Activity of β-galactosidase was assayed from wild-type and mutant *copA* genes in the presence of extra copies of the respective *copA* gene located in *trans* (109).

When the DNA region containing the target for RNA I (designated *copT*) was provided in high copy number in *trans* on a chimeric plasmid, the copy number of a coresident mini-R6-5 replicon increased. Presumably, the many target copies compete with the R6-5 test plasmid for binding of RNA I (40). The *copT* site proved to be 1,600 bp upstream from the origin, within the *copA* gene. Danbara et al. (40) suggested that the target of RNA I is its DNA template.

However, the titration of *copA* RNA is dependent on the extent of transcription through the CopT target in the direction towards the *repA1* gene (110). This suggested that RNA I does not bind to a DNA strand, as conjectured by Danbara et al. (40), but rather to the *repA1* mRNA.

In a transcriptional fusion in which the *lacZ* gene is placed under the *repA1* promoter (promoter 2 in Fig. 2), little effect on *lacZ* expression is detected when the *copA*$^+$ region is introduced in *trans* (110). However, there is a strong effect of the *copA*$^+$ product on inhibition of translational RepA-LacZ gene fusions (107, 109; Womble et al., in press). Thus, it is concluded that there is no direct effect of RNA I on transcription of *repA1*, but that inhibition is a consequence of post-transcriptional regulation (110). Several alternatives have been suggested to explain the way in which RNA I expression is transmitted over a distance of more than 100 bases from *copT* to the start of the *repA1* structural gene.

These include an effect on secondary structure of the RNAs involved, a direct effect of RNA I on a (hypothetical) 63-amino acid polypeptide (deduced from the DNA sequence) which is encoded by the region containing the CopT site (184), or the direct involvement of a nuclease causing site-specific cleavage in RepA1 mRNA (110).

Womble et al. (in press) suggest that RNA I (RNA-E) hybridizes with rightwards transcripts to prevent efficient translation of *repA1* by altering the secondary structure of the transcript. The site of interaction is apparently within the six-base loop of RNA I since this is where the *cop* mutations are located by DNA sequence analysis (12). The postulated hybridization between RNA I and its mRNA target is similar to attenuation regulation in expression of many operons. Based on the nucleotide sequence, Womble et al. (in press) have drawn folded structures for the *repA1* transcript (RNA-Cx) in which the RepA1 ribosome binding region is single stranded. However, when RNA I pairs with RNA-Cx, the ribosome binding sequence is involved in base pairing to the loop of RNA I and is thus no longer in a single-stranded form.

In Vitro Replication

An extract of *E. coli* containing a mini-R1 derived from a *cop* mutant was able to incorporate radioactive nucleotides into DNA (46). Unlike the ColE1 in vitro replication system (207), the R1 system is stimulated more by endogenous plasmid DNA than by added R1 DNA. The system is specific in that added ColE1 DNA does not lead to incorporation. The product is closed covalent circular monomers, and therefore, as expected, incorporation is blocked by inhibitors of DNA gyrase.

An unusual feature of the R1 in vitro system is that it is inhibited by chloramphenicol, puromycin, streptomycin, and tetracycline, as well as rifampin. This indicates that it depends on DNA-directed protein synthesis (46). Endogenous plasmid DNA template replicates in the presence of rifampin, but exogenous plasmid does not. This suggests a requirement for a *cis*-acting RNA (46).

The origin and direction of replication in vitro, as determined by electron microscopy of linearized replication intermediates (48), are the same as those of R1 in vivo (94).

More recently, an in vitro R1 replication system similar to that developed by Staudenbauer (178) has been used (121). A fragment from R1 that contains *copA*, *repA1*, and the R1 replication origin in the vector pUC8 (which is unable to replicate in a *polA*$^-$ host) was used as the template. When a *polA*$^-$ host serves as the source of the extracts, the cointegrate replicon replicates from the R1 origin.

This in vitro system requires both the origin and the RepA1 product for replication (121). The origin was localized within 188 bp and separated from the *repA1* gene by in vitro construction. The requirement for the RepA1 protein can be provided in *trans* to a *repA*$^-$ *ori*$^+$ plasmid by adding a helper *repA*$^+$ *ori*$^-$ plasmid to the extract. The RepA1 protein is synthesized in vitro from the helper plasmid, and this synthesis is inhibited by chloramphenicol or rifamycin. The possibility that *repA1* message acts as a primer for DNA synthesis is excluded by the in vitro complementation result, since, in that case, the *repA1* gene is present only in *trans* to the origin being used (121).

The increase in the extent of replication of a *repA*$^-$ plasmid by complementation with a *repA*$^+$ *ori*$^+$ plasmid is very low in vitro; in vivo, this complementation fails completely, as previously observed (125, 154). However, in vitro complementation of a *repA*$^-$ plasmid by a *repA*$^+$ plasmid deleted for all or part of the origin is very effective. Thus, *repA1* complementation is most successful when the donor lacks all or part of the origin. From this, Masai et al. (121) concluded that RepA1 is probably inactivated after interaction with the origin and is therefore titrated out of the reaction mixture so that no free RepA1 protein is available for action in *trans*.

In vitro, when the *repA*$^+$ *ori*$^-$ cointegrate plasmid is used as template in a PolA-deficient extract (described above), after an initial period of RepA1 synthesis, DNA synthesis is no longer sensitive to rifampin. This suggests that the initiation of R1 replication does not require the *E. coli* RNA polymerase or any other rifampin-sensitive RNA polymerase. Perhaps the *dnaG* polymerase, normally required to initiate synthesis of Okazaki fragments of the lagging DNA strand (100), is used to make the RNA primer for DNA replication of the FII group plasmids (121).

Summary

In the normal low-copy-number state, the *copA* gene product (RNA I = RNA-E) hybridizes with the leader sequence of RNA-Cx mRNA that encodes RepA1, a protein required for replication. Usually, RNA II is folded so that the ribosome binding sites are exposed as single strands in looped-out regions. However, when RNA I interacts with this *repA1* mRNA, the message folds differently and these sites are no longer single stranded. This results in lack of translation of *repA1* and thus a deficit in this required protein. This is the primary regulatory mechanism for the FII group plasmids. The RepA1 protein appears to act predominantly in *cis* on the DNA region that includes the replication origin and is inactivated or otherwise removed by this binding.

Of the two promoter sites in this region, the one responsible for transcription of RNA-A (RNA II; promoter 2 in Fig. 2) is repressed by RepA2 (CopB). This means that normally (i.e., in the presence of RepA2) the promoter that transcribes RNA-C and RNA-Cx (promoter 1 in Fig. 2) is more active than that transcribing RNA-A (RNA II), so usually the longer transcript, RNA-Cx, is predominant. Both RNA-A and RNA-Cx encode RepA1. The left-most promoter is expressed constitutively, and the RNA-A promoter is repressed by the 11-kd protein product of the *copB* gene (RepA2).

If the copy number drops below the normal two per chromosome, the level of *copB* protein, which is proportional to the gene dosage, drops. This derepresses transcription of RNA-A, an additional message for RepA1, and leads to an increase in the amount of RepA1 and therefore of replication. Transcription rightwards (RNA-Cx and RNA-A) may also cause elongation of the *copA* product RNA I so that the inactive longer transcript is produced. Thus, initiation of additional rounds of replication is also fostered by a decrease in the amount of the replication inhibitor RNA I.

Little is known about the mechanism of action of RepA1, but there is now an in vitro assay system that should facilitate its purification. A potential primer RNA has been identified, but not studied further. Since in vitro replication is resistant to rifampin, it is probable that the RNA polymerase is not required.

pT181

Most of the plasmids whose replication is being studied intensively were isolated from *E. coli*. One of the few exceptions is the *S. aureus* plasmid pT181, which is very

similar to many other tetracycline-resistant staphylococcal plasmids, e.g., pT127 and pSN1 (S. A. Khan and R. P. Novick, Plasmid, in press). These plasmids are about 4.5 kb long, encode inducible resistance to tetracycline, and have a copy number of about 20 per cell (84).

Requirement for Plasmid-Coded Function

A function required for replication of pT181 is defined by thermosensitive plasmid mutants that are defective in replication. These mutants are complemented by a wild-type pT181 plasmid in the same cell (Iordanescu [1976], referred to in Khan et al. [88]). The five mutants studied all have mutations in the same cistron, called *repC* (83, 139). Using incorporation of radioactive thymidine, Novick et al. (139) demonstrated that these mutants stop replicating immediately after a shift up in temperature, and that for all five mutants the phenotype of lack of thymidine incorporation is complemented by the wild type.

Some deletion mutants constructed in vitro were determined to be in the *repC* gene because they can be complemented for replication (139). With these mutants, *repC* was located on the plasmid and was found to have one open reading frame for a 313-amino acid protein (139).

In Vitro Replication

A cell extract system that uses exogenous closed covalent plasmid DNA incorporates radioactive nucleotides mostly into monomeric supercoils by semiconservative replication (88). Only the DNAs of pT181 and its close relative pSN1 act as templates in this system. The extracts must be prepared from a strain carrying pT181, indicating that a plasmid-encoded product is required for in vitro replication (presumably this is RepC). Because replication is not sensitive to RNase in vitro, the required product is apparently a protein and replication does not involve free single-stranded RNA.

In density transfer experiments (unlabeled template in extracts containing heavily labeled precursors) no fully heavy DNA was obtained, suggesting that no reinitiation occurs in this in vitro replication system. Because replication in vitro is not completely sensitive to rifampin and because no reinitiation seems to occur, Khan et al. (88) suggest that the template may be preprimed before it is added to the system. An alternative interpretation is that, as in the case of FII (see above), pT181 utilizes a rifampin-resistant RNA polymerase to form the primer.

Origin and Direction of Replication

Initiation of replication in vitro was synchronized in the presence of the chain-terminating dideoxyTTP to determine the origin and direction of replication (87). By this technique, a 127-bp sequence that lies within the RepC coding region appears to contain the origin, and replication is unidirectional. This was confirmed by in vitro construction of deletion mutants which were tested for replication in vitro. Other replicons in which the origin lies within the region encoding an initiator protein include λ and φX174 (78, 122, 183).

Like the origin sequences of other replicons, the pT181 origin is rich in adenine-thymine pairs. It contains no tandem repeats or striking secondary structures (87).

RepC Protein

The entire pT181 plasmid DNA sequence contains only four open reading frames for polypeptides of more than 50 amino acids (Kahn and Novick, in press). All of the polypeptides are encoded by the same strand. The largest, which has a theoretical molecular weight of about 37,500 based on its DNA sequence, is the RepC protein.

The RepC protein itself binds to a region within the origin (W. Rosenblum and S. A. Khan, unpublished data [see Khan and Novick, in press]).

Negative Regulation of RepC

From the sequence, the mRNA for RepC may be attenuated by a stem-loop structure and, like IncFII group plasmids, may also be regulated at the level of translation since its ribosome binding site (168) is within a base-paired region (Khan and Novick, in press).

Within the long untranslated leader of *repC*, two small RNAs are encoded (C. Kumar and R. P. Novick, unpublished data). These appear to be negative regulators of replication since reduced amounts of these RNAs are produced by two of the pT181 *cop* mutants investigated. These *cop* mutants are recessive to wild-type pT181; i.e., when wild-type and *cop* mutant plasmids are in the same cell, the copy number of both is low. Sequence determination indicates that one mutant is in the −10 and the other in the −35 region of the small RNAs. A third *cop* mutant with a similar phenotype has an alteration in the termination site for one of the RNAs and a longer, apparently inactive RNA is produced by this mutant (S. J. Projan, C. Kumar, and R. P. Novick, unpublished data). These RNAs appear to be responsible for incompatibility of this plasmid on the basis of analysis of mutants. Furthermore, as with FII incompatibility group plasmids, the target of these RNAs is probably within the sequence that encodes the inhibitor RNAs. The negative regulation of replication appears to act by inhibiting transcription or translation of *repC* since, in an experiment in which the *repC*$^+$ donor plasmid is deleted for the target sequence, RepC protein can be provided in *trans* to overcome lack of replication due to incompatibility (R. P. Novick, G. K. Adler, S. J. Projan, S. Carleton, S. Highlander, S. Khan, and S. Iordanescu, submitted for publication).

The working model for regulation of replication of pT181 assumes that the RepC protein is rate limiting for replication. In support of this concept, when in vitro extracts from cells with different plasmids are mixed, their activity is additive. Furthermore, in vitro extracts prepared from cells containing the *cop-608* mutant (about 800 to 1,000 copies per cell) have 5 to 10 times more RepC activity than extracts from cells containing wild-type plasmid (88). In this model, the small RNAs encoded by the leader sequence for RepC bind to their target within the leader and alter its folding so that less RepC product is produced. The model is very similar to the mechanisms proposed for FII group plasmid replication regulation. Further experiments are required to determine whether these two plasmid groups, which appear otherwise to be very different, really have such similar replication controls.

PLASMIDS OF THE FI INCOMPATIBILITY GROUP

The F plasmid, a member of the FI incompatibility group, is best known as the prototype sex factor. It encodes functions that promote conjugational transfer both of itself and of any attached replicon (like the bacterial chromosome) and also of nonattached small plasmids that are incapable of self-transmission (e.g., ColE1). The copy number of F is unusually low: about one per chromosome equivalent in rapidly growing *E. coli* cells (29, 58). Thus, the mechanisms that regulate its replication are of particular interest because they must be very precise to prevent its loss from a strain.

Replication of F was recently reviewed by Lane (105) and by Kline et al. (91).

To define loci on F, a system of kilobase coordinates (166) is used. The designation 0 kb was given to the junction of F DNA with the bacterial chromosome in F100 (F'*gal*) and the numbering continues clockwise.

Origin and Direction of Replication

Because the F plasmid is so large (94.5 kb), a smaller F replicon was obtained by digestion with *Eco*RI and ligation to a nonreplicating drug resistance determinant. A self-replicating region of F was isolated in this manner. pML31 (113), a 9-kb plasmid also called mini-F, carries the kanamycin resistance fragment derived from the unrelated plasmid pSC105 (171). pML31 resembles F in copy number (29), stability, and incompatibility (113). Another mini-F plasmid, called pSC138, contains the same piece of F as does pML31 ligated to the ampicillin resistance determinant from an *S. aureus* plasmid (198). The isolation of these mini-F plasmids indicates that all genetic information needed for F replication is clustered in a small region. This has been called the "minimal replicon."

As determined by electron micrographic studies, replication of the mini-F plasmid pML31 is bidirectional and proceeds from a unique origin at 42.6 kb called *ori-1* or *oriV* (Fig. 3) (53, 69). Further deletions from the mini-F plasmid indicate that the minimal region required for replication can be reduced to only the 4 kb between 43 and 47 kb (119, 219).

When *ori-1* is deleted, the resulting plasmid is still replication proficient. A secondary origin (*ori-2* or *oriS*) is used in the deleted mini-F plasmid (57, 120). Replication from *ori-2*, which has been mapped to 45.1 kb (55), proceeds exclusively towards the right. It is not clear why *ori-1* is used preferentially for mini-F replication, and it is not known what combination of *ori-1* or *-2* or another origin is active in the wild-type F plasmid.

Required Host Factors

For replication of F, as for other plasmids, host functions are required. These include *dnaB* (66), *dnaC* (213, 225), and *dnaE* (polymerase III) (194). Although F can replace the host's *dnaA* function by integratively suppressing a *dnaA* mutant (136), after 1 h at nonpermissive conditions in a *dnaA* host, replication of F also stops (75, 225). Further evidence for the role of host functions in F replication comes from the isolation of *E. coli* chromosomal mutants that affect F copy number (34).

Required Plasmid Functions

In addition to the host-encoded functions required for F replication, it was recognized early that F-encoded functions are also required. Conditional mutants of F were isolated that are temperature sensitive for replication or replicate only in hosts containing a nonsense suppressor (39, 52, 54, 71, 89, 118).

Regulation Is Negative

Evidence has accumulated that regulation of F replication is negative. A chimeric plasmid containing both ColE1 and F replicons is maintained at 14 to 16 copies in a *polA*$^+$ host, in which ColE1 replication can occur. However, in a *polA*$^-$ host, replication of the chimera seems to be dependent on F replication since the copy number falls to one to two. When this chimeric plasmid was used in a *polA* host to measure F replication, Tsutsui and Matsubara (209) found inhibition of

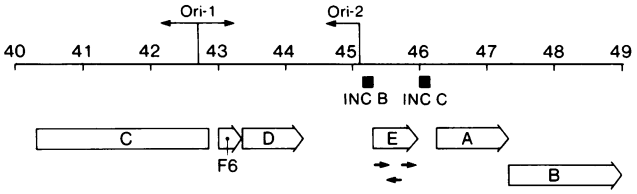

FIG. 3. Replication region of F. The line represents the F plasmid DNA. Numbers are kilobase coordinates, and the origins and directions of replication are indicated by the arrows. The incompatibility determinants are indicated by closed boxes. The open arrows indicate proteins from this region and their direction of transcription (where known). The small closed arrows under transcript E show the extent and direction of the open reading frames for the small hypothetical polypeptides.

replication in the presence of a second F replicon. Furthermore, since *cop* mutants of mini-F are recessive to wild type, it can be concluded that the wild type produces a diffusible product that inhibits F *cop* replication (91).

Products of the Minimal Replicon

With mini-F, much work has been done to identify the proteins, transcripts, and partial sequence of the region involved in replication control (see Fig. 3). By sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of products of subcloned fragments produced in minicells, proteins have been matched to their coding regions (see Fig. 3). The direction of transcription in these regions was deduced from electron microscopy of R loops (221), from constructs in which the F-region promoter is necessary for transcription of a gene whose expression can be measured (Trawick and Kline quoted in reference 91), from cloning the subfragments of the F region and observing appearance of the relevent protein in minicells (98), or, in a few cases, from the DNA sequence (134).

Because insertions of Tn*A* into the gene for protein E prevent replication (218), protein E is regarded as absolutely essential for F replication. In further confirmation of its critical role in F replication, this region of the F DNA is part of the segment that can act in *trans* to complement deleted F plasmids for replication (202). The E protein of the FI group plasmid R455 is similar enough to that of F to complement the F *ori-2* plasmid, albeit weakly, whereas the E proteins of R386 and R773 (other FI group plasmids) are unable to do so (202).

Mutants of F maintained at higher than normal copy number were isolated by Seelke et al. (159) after chemical mutagenesis. These mutants map to the coding region for protein E, between 45.35 and 45.88 kb. This further supports the conclusion that protein E is necessary and implies that availability of the E protein may be rate limiting for replication of mini-F.

In minicells, mutations in proteins A and B affect the amount of protein E detected (218). However, this is not confirmed by in vivo measurements of transcription of protein E made by assaying β-galactosidase produced by chimeras in which the promoter for E is fused to the *lacZ* gene (L. Sogaard-Andersen, L. A. Rokeach, and S. Molin, EMBO J., in press). In these experiments, the expression of protein E is not affected by any gene product other than E that is provided in *trans* from a compatible plasmid. However, the presence of one or more copies of the protein E gene itself represses expression of E-*lacZ*. Thus, Sogaard-Andersen et al. (in press) believe that E is autoregulatory: it

appears to repress its own synthesis. This autoregulation would provide the negative aspect of control of F replication discussed above. Furthermore, from the plasmids they constructed in vitro, they deduce that the operator site at which repression of E transcription occurs overlaps the promoter of the E gene itself.

The DNA sequence analysis (134) also identified reading frames for two 9- to 11-kd polypeptides in one direction and one in the opposite direction. These reading frames overlap that for protein D. There is no evidence at this time that these small polypeptides are actually synthesized. They have been shown not to be important for regulating synthesis of the E protein (Sogaard-Andersen et al., in press) and are insufficient when provided in *trans* to permit replication from *ori-2* (220).

Sequence Analysis of the Origin Region

Murotsu et al. (134) have determined the DNA sequence of the region between 44.1 and 46.35 kb, which contains *ori-2*, replicates like the parent F plasmid and also expresses incompatibility to F. They found two clusters of nine 19-bp direct repeats. One, oriented to the right, lies to the right of the coding frame for protein E, and the other, oriented in the opposite direction, is left of protein E (Fig. 3).

Incompatibility of the FI plasmid group is determined by these repeats. The left set corresponds to the IncB determinant and the homologous right set corresponds to IncC (201, 208). In vitro insertion of all or part of the repeat regions of F causes plasmids normally compatible with F to become incompatible. Use of cloning vectors with different copy number showed that, regardless of their orientation in the vector plasmid, the number of repeats is approximately proportional to the degree of F incompatibility (208).

Based on the sequence of Morotsu et al. (134), Sogaard-Andersen et al. (in press) point out that in the operator region to which the repressor for E transcription binds there is a 10-bp inverted repeat separated by 9 bp. Of the 10 bp, 8 bp are the same as eight consecutive bases in the repeats that compose IncB and IncC (see below). They therefore suggest that the E protein may bind to both IncB and IncC. Such binding will reduce the amount of free E protein available to participate in replication, for which its presence is essential, and might therefore result in incompatibility. (This type of model for incompatibility or regulation of replication is referred to as titration of a positively acting factor.) Within the repeated sequences, there is especially strong conservation of the bases that occur at positions separated by a single turn of the helix (10 bp), suggesting to Murotsu et al. (134) the possible importance of these repeats in binding to a protein.

The Sogaard-Andersen et al. (in press) hypothesis explains the incompatibility function of the repeat regions as due to their ability to bind the E protein. If the repeats provide alternative binding sites for protein E, an excess number of repeat sequences might reduce the concentration of the protein below that needed for replication, which would result in lack of replication of the F plasmid (incompatibility). However, if the E protein is autoregulated (Sogaard-Andersen et al., in press), the binding constant of E with each sequence will be important in determining the result expected for an excess of any component (repeat sequences or E protein). Since nothing is yet known about these binding affinities or about possible cooperative or inhibitory effects of E protein binding to nearby sites, it is difficult to predict the effect on replication expected from an excess number of repeat sequences.

The repeats also appear to be very important in regulating copy number. *copB* mutants, induced by Tn*3* insertional mutagenesis, map in a 100-bp region centered on 46.0 kb (90, 159), which is close to the carboxy terminus of the gene for protein E. These *copB* mutants complement the chemically induced *copA* mutants which are located within the gene encoding protein E (B. C. Kline and J. Trawick, Mol. Gen. Genet., in press). Therefore, *copB* mutants presumably produce active protein E and must be located to the right of its coding sequence, within the repeat region. *copA* mutants may be altered in the domain of protein E responsible for its binding to the operator to produce repression and therefore also for its binding to the repeats in IncB and IncC. This should lead to reduced incompatibility. In agreement with this, *copA* mutants are compatible with F*lac*, although both $IncB^+$ and $IncC^+$ determinants can be recovered intact by in vitro cloning of regions from these mutants (91, 120).

The *copB* region (which is the same as IncC) is composed of the five 19- to 22-bp direct repeats. Plasmids lacking two, three, or five of the repeats have a proportionally elevated copy number (9). If the high-copy phenotype of the *copB* mutants resulted from inactivation of a repressor, deletion and insertion mutations, all of which should completely inactivate a protein, would give the same copy number. This is not the case (Kline and Trawick, in press). Kline and Trawick suggest that the repeats may encode a small RNA repressor whose size determines its effectiveness. Although this is formally possible, the hypothesis currently favored is that in the cop^+ plasmid the *copB* repeats bind and remove something required for replication, probably protein E. In this model (Kline and Trawick, in press; Sogaard-Andersen et al., in press), the CopB phenotype results from an alteration of the repeat sequence so that it no longer binds protein E. This would also give an Inc^- phenotype, as observed.

Summary

The concentration of E appears to be rate limiting for replication of the F plasmid, although the mechanism of action of this protein has not been investigated. In a current model for regulation of F replication, the E protein has two domains, one of which is involved in positive regulation of replication and the other of which represses its own synthesis. Negative regulation of replication of F results from binding of the E protein to specific reiterated DNA sequences near the region that encodes E. One of these binding sites may be the operator at which E regulates its own synthesis, and the other sites, located in the IncB and -C determinants, compete with this operator for binding E. The possible role of the small protein molecules deduced from the DNA sequence to be encoded within the reading frame of the E protein has not yet been determined.

REPLICATION OF P1

P1, a large plasmid of about 90 kb (5, 224), is the prophage form of a temperate bacteriophage. It belongs to incompatibility group Y (72). Also in this group are P7 (formerly called ϕ amp, which is an independently isolated plasmid prophage carrying the ampicillin-resistant transposon Tn*902* [172, 224]), a cryptic plasmid from *E. coli* 15 (76), and pIP231 (a naturally occurring plasmid encoding tetracycline resistance and production of H_2S [14]). The P1 plasmid is nonconjugative, but appears to be mobilizable (11). Since it is a prophage, after induction it can spread to other hosts by

infection. P1 has a broad host range among gram-negative bacteria.

Like the F plasmid, P1 has a very low copy number of less than or equal to one per chromosome in rapidly growing cells (4, 77, 146, 157, 181). In spite of its extremely low copy number, this plasmid is very stably inherited (there is less than one plasmid-cured cell in 10^5 per generation [151]), indicating that its replication is tightly regulated. This may account for its extremely strong incompatibility (32).

Host Functions Required

Polymerase I is not required for P1 plasmid replication, and at this time it is not clear which polymerase is primarily responsible for replication of the P1 plasmid. Although extensive P1 plasmid replication occurs in *dnaE* (polymerase III-defective) hosts, the DNA synthesized is not in the closed covalent form (158). Therefore, polymerase III appears to be required to generate monomer-length circles of P1.

Replication of P1 does not require the initiation function of *E. coli* encoded by *dnaA*, since this plasmid can cause integrative suppression (22–25, 136). P1 requires *dnaC*$^+$ and *dnaG*$^+$ of the host, as well as *dnaB*$^+$ (158). Although P1 encodes a *dnaB* analog (42), this is normally repressed in the prophage state and is therefore not available for plasmid replication.

Isolation of Smaller Replicons

Before in vitro recombinant technology was available, the first mini-P1 replicons were isolated as specialized transducing particles produced spontaneously. One, called pIH1972, contains the P1 fragment called *Eco*RI-5 and P1 DNA to the left of this fragment (161, 162). In addition, two different classes of deletion plasmids, obtained in vivo in a λ vector, contain the *Eco*RI-5 fragment and P1 DNA either to its right (5R) or to its left (5L) (182). The only region that all miniplasmids have in common is *Eco*RI-5, but *Eco*RI-5 alone is not able to replicate (182). This led to the suggestion that there are two alternative "modes" of P1 plasmid replication involving genes to the right or left of fragment 5. These alternative replicons may utilize different replication origins like the additional replication origins revealed when fragments of other plasmids (e.g., F and FII) are cloned in vitro. It is not known at present which origin is used in vivo.

The 5L miniplasmid has a high copy number (about eight per chromosome) and is unstable in Rec$^+$ hosts (181). Although pIH1972 is also a 5L-type plasmid, it is maintained at a low copy number (A. Shafferman, personal communication), so the role of the region defined by 5L plasmids is still unclear. On the other hand, the 5R plasmids are fairly stable and have a low copy number. These are therefore considered to represent the normal mode of P1 plasmid replication (181). However, 5R plasmids may also be incomplete since they induce SOS functions in Rec$^+$ hosts (16), which is usually a sign of abnormal replication.

By analysis of deletions from λ P1:5R and chimeras constructed by addition of pieces of P1 DNA to high-copy-number vectors in vitro, the region required for replication was found to be <2.1 kb (3; A. L. Abeles, K. M. Snyder, and D. K. Chattoraj, J. Mol. Biol., in press). Within the replication region is the A incompatibility determinant, responsible for the very strong plasmid-specific incompatibility (3, 32). Adjacent to this region is a sequence encoding partition functions and containing the incompatibility B determinant, which is group Y specific and less severe than IncA (3).

The third method by which mini-P1 plasmids have been obtained is their spontaneous release from a *dnaA* host integratively suppressed by a P1 *cop* mutant when the temperature is dropped to that at which the mutant *dnaA* product can function (see below; J. R. Scott, B. J. Froehlich, K. McLain, and K. Tatti, manuscript in preparation). These are 5R-type miniplasmids and they are maintained at very high copy numbers, possibly because they are derived from *cop* mutants and possibly because they are very small replicons (see next section).

Evidence for Negative Regulation of Replication

Mutants of P1 maintained at high copy number were selected by requiring high antibiotic resistance (157). Two are thermosensitive for copy number, having a higher copy number at higher temperature. This suggests that they are defective in a negative regulator of replication.

For complementation tests with the P1 *cop* mutants, another Y-group replicon, wild-type P7, was used instead of P1 to supply Cop$^+$ (157) because P1-P1 incompatibility is too strong to permit isolation of colonies containing both plasmids (32). By use of these tests, the copy mutants were divided into two classes: *cis* specific and recessive. In a cell with both plasmids, the *cis*-specific mutants are maintained at a high copy number, whereas the wild-type plasmid is maintained at a low copy number. The recessive *cop* mutants, which include the thermosensitive ones, show a reduced copy number when present in the same cell with a wild-type plasmid, as expected for mutants defective in a replication inhibitor. Sequence determination (B. Baumstark, K. Lowery, and J. R. Scott, Mol. Gen. Genet., in press) has shown that the six *cop* mutants analyzed define only three base changes (i.e., each was isolated twice independently). This is similar to the situation with ColE1 *cop* mutants (see above). Unexpectedly, all of the P1 *cop* mutants lie within the reading frame of a protein required for replication (Abeles et al., in press; Baumstark et al., in press) (see below).

Plasmid-Encoded Function(s) Required for Replication

P1 *cop* mutants are usually maintained at about eight copies per chromosome. However, when a *dnaA* host that has been integratively suppressed by a P1 *cop* mutant is maintained at high temperature so that the host chromosome is replicated as part of the P1 plasmid replicon, the only P1 copy present is the integrated one (59). At low temperature, extrachromosomal P1 copies are again present, in addition to the integrated copy. The explanation suggested (59) is that there is a P1-specific factor required for replication that remains attached to the replicating molecule until (or after) termination of the replication round. The factor might be a plasmid-specific protein bound at the replication fork or a membrane site to which the fork remains attached until the replication round is completed. Normally, this factor is not rate limiting for replication, but when the P1-specific factor is utilized to replicate a large replicon (the *E. coli* chromosome in a suppressed *dnaA* strain at high temperature), the factor is not released for replication of the P1 plasmid during the entire cell cycle and therefore no plasmids are produced. However, when the chromosome can replicate on its own (low temperature), P1 plasmids are again found.

Mutant miniplasmids that are able to persist at high temperature in a P1 *cop*-suppressed *dnaA* host have been isolated (*rer* mutants; Scott et al., in preparation). Further study will tell whether these show the expected alterations in the quality or quantity of the function required for replication.

The extrachromosomal plasmid copies found in P1 *cop*-suppressed *dnaA* cells at low temperature are often minireplicons (Scott et al., in preparation). Some can be transformed to a P1-free strain and thus must contain a region sufficient for plasmid maintenance. Some miniplasmids cannot replicate in the absence of an additional piece of P1 DNA in *trans*, demonstrating their requirement for a diffusible P1-encoded factor.

From the DNA sequence of a minireplicon, an open reading frame was identified (Abeles et al., in press). In maxicells, the minireplicon produces a 32-kd protein corresponding to the size expected from the reading frame. A mutant of the 5R miniplasmid that fails to replicate in the absence of a nonsense suppressor produces this protein only in a strain carrying such a suppressor. This indicates that the protein is either the product of the mutated gene or is produced only when the wild-type form of this gene is present and suggests that the gene's function is required for replication of the P1 plasmid. The gene has been called *repA* (Abeles et al., in press). From the DNA sequence, the replication protein has a net positive charge, presumably enabling it to bind to DNA (or RNA).

Repeated Sequences

Outside of the *repA* gene, a series of 19-bp repeated sequences has been identified (Abeles et al., in press). As in the case of the F plasmid, these correlate with the location of IncC (five direct repeats) and IncA (nine direct repeats and six in the other orientation). Because the repeats face the same side of the helix, they may be involved in binding to a protein. The IncC determinant has a recognizable phenotype only when present in high copy number, which agrees with the idea that the repeated sequences are involved in titrating out a required protein. When the IncA repeats are ligated into a multicopy vector, they also interfere with maintenance of a P1 plasmid in the same cell, again suggesting competition for a required function.

Models for P1 Replication Regulation

All of the *cop* mutations investigated are very closely linked and lie within *repA* (Baumstark et al., in press). However, if the high-copy phenotype results from an alteration in the function of the RepA protein, the mutant protein should be more efficient than the wild type for replication. Unlike any of the mutants tested, this type of mutant would have a *trans*-dominant phenotype. We suggested (Baumstark et al., in press) two alternative explanations for this paradox.

In the first model, the *rep* coding frame may have a function other than encoding Rep. Functions encoded by overlapping DNA sequences are important in regulating other plasmids (e.g., ColE1 and the FII plasmids; see above) and such an overlapping region may also be utilized by P1. If this is correct, the phenotype of the recessive *cop* mutant may result from production of a defective inhibitor of replication, whereas the phenotype of the *cis*-specific *cop* mutants results from an alteration in the binding site of the inhibitor molecule.

In the second model, the P1 Rep protein is bifunctional, like the E protein of the F plasmid (Sogaard-Andersen et al., in press) (see above), and the high-copy phenotype results from an alteration in the *repA* gene. According to this model, there is a lack of identity between P1 and P7 in the domain of the Rep protein required for replication. Therefore, the more efficient Rep protein of the P1 *cop* mutant cannot influence the copy number of P7, so mutations that affect this domain appear to be *cis* specific. On the other hand, the recessive *cop* mutant lies in the domain of Rep that acts as a repressor of Rep synthesis, and this domain is functionally identical in both P1 and P7. Thus, P7 cop^+ is dominant to this P1 *cop* mutant.

Because of other similarities between the arrangement of the sequence of P1 and F in the replication region, this second alternative based on two domains of a protein required for replication seems very attractive. It is being tested directly by cloning the *rep* gene in the absence of incompatibility determinants.

R6K

Most plasmids are either small (<10 kb) and have a very high copy number, like ColE1, or large (around 100 kb) with a low or unit copy number (like plasmids of incompatibility groups FI and FII and P1). One example of a plasmid of intermediate size and intermediate copy number for which replication control has been investigated is R6K. This plasmid encodes ampicillin and streptomycin resistance and is conjugative (45). It is about 38 kb in size and has a copy number of 13 to 40 per cell, depending on the growth phase of the host (7). Originally R6K was called R_{TEM} because it was isolated from an *E. coli* strain with that name. It is a member of incompatibility group X.

Host Functions Required

R6K replication is independent of the host *dnaA* function since it causes integrative suppression (174). Further, it replicates in a $polA^- polB^-$ host, so it does not require DNA polymerase I or II (80). Mutants which are impaired in unwinding of DNA during replication (*rep* mutants) allow R6K DNA synthesis (145). Since initiation of replication of R6K is sensitive to rifampin, at least in vitro, it is concluded that this plasmid requires RNA polymerase for replication (80, 81). Because *E. coli* mutants that give a high copy number for R6K have been described (80, 117), there is at least one host function that may influence the rate of initiation of replication of plasmid R6K.

Origin and Direction of Replication

Electron microscopic studies of mini-R6K derivatives led to the identification of three origins of replication, called alpha, beta, and gamma, all of which are within a 3.9-kb region (35, 37, 38, 115). In vivo, *ori* alpha is used predominantly (35). Construction of R6K miniplasmids in vitro led to the identification of a 2.1-kb piece of DNA common to all clones (95). One or the other of the origins identified by electron microscopy is present in all clones, and all still show incompatibility. By complementation with a chromosomally integrated λ prophage containing the R6K *pir* gene (which is required in *trans* for replication; see below), a 392-kb fragment from R6K that contains a functional origin was isolated (97). This contains neither *ori* alpha nor *ori* beta, but only *ori* gamma (164).

The DNA sequence of the region containing *ori* gamma includes seven 22-bp direct repeats in tandem and another one just preceding the sequence for the required *pir* protein (175). Runs of adenine residues are present in the gamma origin and have been seen also in other origins, e.g., ColE1 (8, 206), λ (156), and the *E. coli ori* (123, 189).

The role of the adenine runs has not been investigated yet, but the direct repeats have been studied (96). A chimera containing two replicons, ColE1 and *ori* gamma, was muta-

genized with the transposon Tn*5*. To determine which insertions inactivate *ori* gamma, the plasmid was transformed to a *polA*$^-$ strain (in which the ColE1 replicon is not functional) lysogenic for a λ prophage containing the required R6K *pir* gene. Using these insertion mutants and deletion mutants recovered from them, Kolter and Helinski (96) found that loss of one repeat does not interfere with function of the origin, but loss of four repeats does. These studies also defined the 240-bp region that includes the seven 22-bp repeats as the maximum DNA essential for origin function.

Sequence analysis (169) shows that *ori* beta has some homology with *ori* gamma and a 15-bp homology with the *E. coli* origin. An adenine-thymine-rich region was also observed in the beta *ori* sequence, and several regions of dyad symmetry, at which regulatory proteins may act, were identified.

Replication Terminus and Direction of Replication

Replication of R6K is seen by electron microscopy to be sequentially bidirectional and assymmetric from either *ori* alpha or beta (115). From either origin, replication proceeds towards a terminus which is not located directly opposite the origin. Currently, this type of replication appears to be unique among plasmids. To learn about the role of the terminus, chimeras were constructed in vitro having both an R6K and a ColE1 origin. Both origins are functional, and the R6K terminus in both orientations delays replication forks that pass through it, even when they originate from the ColE1 origin (95). This function of the terminus occurs even in the presence of chloramphenicol, suggesting that no new protein synthesis is required for terminus activity. Chimeras in which the terminus is deleted still replicate, so the terminus is not essential for mini-R6K replication.

Replication intermediates of miniplasmids show transient accumulation of open circular molecules with the discontinuity near the terminus in either strand (8). The terminus was localized to a 2-kb region by isolation of these intermediates and filling in of the gap in vitro. The location of the label added in vitro was identified by restriction digestion and agarose gel electrophoresis. Starting with this information, further in vitro subcloning experiments limited the terminus to a 216-bp region (8). The gaps do not end precisely at the terminus, but overlap it, which suggested to Bastia et al. (8) that, similar to the role of the terminus in *E. coli* (101, 112), the R6K terminus delays but does not stop the replication forks. From the DNA sequence, the terminus contains no twofold rotational symmetric regions and has no significant open reading frame (9).

Required Plasmid Function

In addition to the origin, which is required in *cis*, another R6K region is required in *trans* for replication. A mutant of mini-R6K that is temperature sensitive for plasmid maintenance was isolated. This was done by mutagenizing a strain carrying R6K and looking for small colonies on selective media after plates were shifted from 32 to 42°C. The wild-type R6K plasmid complements the temperature-sensitive mutant for survival on selective plates, although this complementation is poor because of incompatibility (97).

From the mini-R6K, DNA fragments were subcloned into ColE1 to obtain a region that complements the temperature-sensitive mutant. A 1,370-bp region, which does not show incompatibility with R6K, performs this function (97).

The gene required in *trans* for replication of *ori* gamma was named *pir* (protein for initiation of replication) and the protein it codes for, identified by an in vitro assay (see below), was named Pi. In the sequence of a 1,565-bp fragment of R6K that contains both *pir* and *ori* gamma (95), the only coding frame for a polypeptide of more than 50 amino acids is that encoding Pi (176). From the sequence and from labeling of proteins synthesized in minicells, Pi is identified as a 35-kd basic protein (62, 176). Because it is basic, it should be suited to binding to DNA, which is presumably necessary for its role in replication of R6K.

The Pi protein is very labile, so it proved difficult to purify by standard techniques. It was purified (63) by constructing a gene fusion in vitro encoding the N-terminal polypeptide (alpha donor) of β-galactosidase fused to *pir*. The fusion protein produced in vivo has both activities, and it was purified by taking advantage of its β-galactosidase properties.

This protein binds to two sites on R6K DNA in the 5′ untranslated leader of the *pir* gene. This was determined by the filter binding technique (in which labeled R6K DNA is bound to nitrocellulose filters as a result of the attachment of the Pi protein to it) and by immunoprecipitation (with staphylococcal protein A and anti-β-galactosidase) of the complex of R6K DNA with the fusion protein. The Pi protein binds to the *ori* gamma region with the seven direct repeats (which are required for replication; see above) and to the lone repeat at the start of the *pir* gene. This is in agreement with the suggestion (177) that the latter repeat functions as an operator site for the *pir* gene which is autoregulated (repressed by its own product; see below). When the second (presumably autoregulatory) binding site is deleted, *ori* gamma still functions if Pi is provided in *trans* (95), so this interaction is not essential for replication. DNase I footprinting located the binding sites more precisely and showed that the seven tandem repeats are directly involved in binding Pi (64).

The Pi protein is required for replication of *ori* alpha and beta, as well as *ori* gamma, but alpha and beta are only active if the *ori* gamma region is present in *cis* (164). This might be because Pi must interact with the repeats in gamma to initiate replication anywhere (e.g., alpha or beta) on the strand of DNA.

ori alpha and beta are 2 and 12 kb, respectively, from the Pi binding site(s), indicating that Pi can act at a distance. The latter observation led Germino and Bastia (63) to suggest the concept of "initiator loading sites" at which the Pi protein enters the DNA and then moves along the DNA molecule to the origin.

Regulation of Pi Protein Production

The regulation of synthesis of the Pi protein is probably autogenous, as shown by experiments in which the *pir* promoter and coding sequences were cloned into separate vectors (163). The Pi protein provided in *trans* reduces expression of β-galactosidase from a *lacZ* gene fused to the *pir* promoter, indicating that Pi acts as a repressor for this promoter. Furthermore, a relatively small change in the concentration of Pi activity (as measured in the in vitro replication assay; see below) occurs in response to a 20-fold increase in the *pir* gene dosage. This again suggests that Pi represses its own synthesis.

The role of the Pi protein in R6K replication is still somewhat mysterious. Although it is absolutely required, Pi does not regulate the frequency of initiation of R6K replication and so must not be rate limiting. This was demonstrated (163) by measuring the copy number of an R6K origin

plasmid in vivo in a cell also containing the *pir* gene fused to each of several different promoters. Transcription through *pir* was varied over a wide range by repression or induction of the promoter, and, although the concentration of Pi varied 95-fold, no change in the copy number of the R6K replicon was detected.

In Vitro Replication System and Identification of Pi Protein

The discovery of the Pi protein, originally believed to be regulatory for R6K replication, depended on the development of an in vitro replication system for this plasmid. The first system described (80) utilized DNA template endogenous in the extract. The incorporation of radioactive nucleotides was sensitive to inhibitors of DNA-dependent RNA polymerase (streptolydigin and rifampin), so that RNA polymerase is considered necessary for R6K replication. Density transfer experiments demonstrated that in vitro replication is semiconservative, but since no completely heavy molecules were found it was concluded that molecules that complete one round of replication usually do not begin a second. The in vitro reaction is sensitive to arabinosyl-CTP, an inhibitor of DNA polymerases II and III. Since R6K does not require polymerase II, it was concluded that it probably requires polymerase III. Replication in vitro is also sensitive to novobiocin, so it probably requires DNA gyrase.

The in vitro system was further developed so that it can utilize exogenous closed covalent circular R6K DNA templates and can initiate new replication rounds (81). In this system also, R6K replication is semiconservative, and it is sensitive to inhibitors of DNA-dependent RNA polymerase. This suggested to the authors that synthesis of a plasmid-encoded protein is required for in vitro replication.

Extracts made with the R6K mutant that is temperature sensitive for replication show thermolability in vitro, so the mutant appears to be defective for an essential protein, called Pi (81). This protein is needed in vitro for replication from the alpha and beta origins, as well as from the gamma origin. This suggests a requirement for a functional gamma *ori* in *cis* to activate alpha or beta (164; see above). Excess R6K DNA in the in vitro system is inhibitory, apparently because it titrates out the Pi protein. This suggests the need for more than one protein molecule to form a single active initiation complex.

From preincubation experiments in vitro Inuzuka and Helinski (81) conclude that the Pi protein is involved in the initiation step of replication.

Origin Usage In Vitro

Electron microscopy of a mini-R6K replicated in vitro demonstrates that all three identified origins are used (82). However, the frequency of their usage differs from the in vivo situation. In vitro, alpha is used about 24% of the time; beta, about 43%; and gamma, about 37%. (In vivo, *ori* alpha is predominant; see above.) Furthermore, no bidirectional replication was observed in vitro, although *ori* beta and gamma can initiate replication in either direction. It was suggested that the preincubation step may lead to loss of selectivity of origins in vitro.

Terminus In Vitro

The replication terminus is not active in vitro in the above system (82), but, when cloned into pBR313 and pBR322, the R6K terminus delays in vitro replication (61). The effect of the terminus was detected by electron microscopic experiments and confirmed by constructing chimeras in which the terminus is located at different distances from the origin. Even if the extracts are produced from cells without a terminus on the plasmid, temporary arrest by the terminus of unidirectional replication is observed. This result indicates that neither an unstable plasmid-coded factor nor membrane association is required for terminus activity (61).

Negative Regulation of Replication

Recently, Stalker et al. (D. M. Stalker, M. Filutowicz, and D. R. Helinski, Proc. Natl. Acad. Sci. U.S.A., in press) isolated a cold-sensitive copy mutant of R6K that is recessive to a pir^+ plasmid present in *trans*. This suggests the existence of a negative regulatory loop for R6K replication. The mutant, which was isolated in a plasmid containing *pir* and *ori* beta and gamma, has been mapped to the *pir* gene. Sequence analysis was used to locate the mutation and show that it changes a glycine to an aspartic acid in the Pi protein. The DNA region that includes the mutant gene for the Pi protein acts in *trans* to increase the copy number of a pir^- replicon. If this is correct, the extra copies of Pi in the Shafferman et al. experiments (163; see above) should have reduced the copy number of R6K in *trans*. This was not observed. The contradiction in results in the two sets of experiments may be resolved by closer examination of the actual plasmids utilized to supply Pi protein and the replicons used to measure increased initiation of replication of R6K, since both sets of experiments were indirect.

Little is understood at this time about the apparent negative role of the Pi protein in replication of R6K (Stalker et al., in press) (see above). It may be caused by the inability of the mutant to repress its own synthesis, which would lead to overproduction of the Pi protein and a higher copy number. If the wild-type protein is present in the same cell with the mutant, both *pir* genes should be repressed, which would make the wild type dominant, as observed. It is also possible that there is a negative regulatory RNA molecule encoded from the DNA region that produces Pi.

The interpretation that Pi acts as a negative regulator of its own synthesis when it is present in excess instead of acting to initiate additional replication rounds implies that it binds more rapidly or with greater affinity to its operator site than to the origin. This may be because one Pi molecule can act at the operator to prevent further Pi synthesis whereas several molecules must interact to bind effectively to the seven copies of the repeated sequence at the origin.

Summary

R6K replication is complicated by the existence of three origins, all of which appear to be active in vivo. For its replication, an R6K-encoded protein called Pi is required. This protein appears to bind to a region of *ori* gamma that contains several 22-bp direct repeats, whose deletion inactivates plasmid replication. The Pi protein is autoregulated, but since the plasmid copy number does not increase when the gene dosage of Pi increases, the amount of Pi does not appear to be rate limiting in regulation of R6K replication. On the other hand, the DNA region encoding Pi acts in *trans* to repress R6K replication in vivo, so either Pi, or another function within the *pir* gene sequence, negatively regulates replication. Thus, although Pi is normally present in sufficient amount so that some other factor is rate-limiting for DNA initiation, it is possible that reducing the supply of the Pi protein will limit replication initiation of R6K. No plasmid factor that directly regulates R6K replication has yet been identified.

COMPARISONS AND CONCLUSIONS

The mechanisms that regulate plasmid replication show some striking similarities. In those cases that have been investigated, control of plasmid replication appears to involve an RNA folding mechanism or repeated DNA sequences or both which titrate out a protein required for replication. In addition, it appears that (i) the small DNA region that regulates replication is utilized very efficiently, (ii) most plasmids have alternative replication origins, and (iii) a secondary factor limits plasmid copy number when the primary regulatory pathway is disabled. I will discuss each of these in turn.

Importance of RNA Secondary Structure

The role of alternative configurations of RNA molecules is striking in regulation of replication of both the ColE1-type and FII incompatibility group plasmids. In both cases, refolding of a large RNA that has a positive regulatory function is induced by base pairing of this molecule with a small inhibitory RNA. The RNA-RNA interaction in both ColE1 and FII plasmids takes place far from the origin region, and the action of the inhibitor is transmitted over this distance because the large RNA target molecule is caused to fold differently by binding to the small RNA inhibitor.

In ColE1, the regulatory RNA is the preprimer which must hybridize to its template DNA to be converted by RNase H to primer. In FII, the regulatory RNA is the message for the required RepA1 protein. The positively acting RNA molecules of both ColE1 and FII can form specific complex secondary structures. The ColE1 preprimer RNA can be folded into a cloverleaf, which is similar to a tRNA molecule, and this exposes key sequences as single-stranded regions in the loops. The group FII mRNA can fold so that the ribosome binding site for the RepA1 protein is exposed in a single-stranded region. In both cases, a small RNA molecule plays an inhibitory role. In ColE1, the small RNA can also form a cloverleaf structure, and this structure has a single-stranded loop region that can interact with the single-stranded loop of the preprimer RNA (Fig. 1). This interaction prevents the preprimer from hybridizing to the DNA. In FII, the binding of the small RNA to the RepA1 messenger causes the mRNA to refold so that the site for ribosome binding is no longer single stranded and therefore is less available. Thus, in both cases, a negative regulatory RNA interacts with a required RNA to inhibit the action of the latter. The target for the inhibitor is at a distance from the site at which the direct interaction occurs. In both of these well-studied cases, the sequence encoding the negative regulatory RNA lies within that for the positively acting RNA on the opposite strand. The homology between the two RNA molecules causes their strong interaction.

Both of these plasmids have a positive regulator (preprimer RNA for ColE1 and RepA1 protein for FII plasmids) which is rate limiting for initiation. Both are negatively controlled by a *trans*-acting small RNA molecule.

This type of negative regulation by a small RNA molecule is apparently used also by pT181, but has not yet been found in the very low-copy-number plasmids F and P1. In these cases, it currently appears that the negative loop in regulation is provided by self-repression of the protein required for replication. However, it is still possible that a negatively acting small RNA (or possibly a peptide) may be found in the F and P1 cases as well.

Efficient Utilization of DNA

In all cases, it appears that the regulatory region of the plasmid DNA is utilized very efficiently, since the same DNA sequence frequently specifies more than one function. In ColE1-type plasmids, for example, one region encodes both the primer RNA and an inhibitor of DNA replication; in plasmids of incompatibility group FII (and probably also in pT181), the regulatory region encodes a protein essential for DNA replication as well as an inhibitor of its mRNA; and in the F factor, one region encodes a replication protein as well as several small RNA molecules that are potentially regulatory.

Because the conservation of "space" on the DNA molecule would seem unlikely to be of critical importance to a bacterium, there must be other reasons for this multifunctional utilization of a small DNA region. The overlapping coding sequences provide extensive homology between the two RNA molecules that are transcribed from them. The two RNAs can hybridize and thereby regulate the frequency of DNA initiation because they are transcribed from opposite strands. However, in the schemes proposed, only limited small regions of the two RNAs are involved in mutual base pairing, so their complete homology to each other is not necessary for their interaction. Why, then, is the sequence for one contained entirely within the sequence for the other? The overlapping of the regulatory functions on the DNA tends to prevent recombinational separation of DNA sequences that are functionally linked. Because they are so tightly linked genetically, regions encoding regulatory functions are likely to be inherited together. Furthermore, the overlap in the sequences encoding two replication functions tends to prevent genetic drift. Because the sequence encoding a regulatory function is likely to be part of the sequence that also encodes an essential function, a single mutation is likely to be lethal. Therefore, genetic drift (the accumulation of random mutations) within the regulatory gene(s) is less likely to occur.

Alternative Origins

When large plasmids are studied by molecular cloning techniques, several different DNA regions able to serve as origins of replication are found. For most plasmids, it is not clear which, if any, of these is utilized in vivo. Because the selective pressure for replication is so overwhelming, it is not surprising that when the predominant origin is deleted, replication will begin at another site. It seems likely that these secondary origin sites have sufficient sequence similarity to the primary origin to be recognized by the proteins of the replication complex and, in the absence of an origin sequence with a better "fit," to be utilized for initiation of DNA synthesis.

It is also possible that the secondary origins are used by plasmids in different hosts. This appears to be the case for the incompatibility group FII plasmid NR1 when it is transferred from *E. coli* to *P. mirabilis* (217).

In the case of the FII group plasmid R1, a minireplicon different from the one usually studied can be obtained by cloning fragments. This alternative minireplicon contains an otherwise unidentified replication function, RepD. The RepD miniplasmid is incompatible with the whole R1 plasmid; however, it is compatible with a mini-RepA1-containing plasmid (41, 92). No in vitro origin was seen in the RepD region (47), further suggesting that this miniplasmid is dissimilar to the parent R1 plasmid.

A miniplasmid derived from the F factor which is different from the classical mini-F has also been isolated. Like mini-F, this miniplasmid can also drive replication when ligated to a drug resistance determinant. However, unlike F, this replicon is not stably maintained at fast growth rates (104).

In addition, two minireplicons have been isolated from P1 by in vitro construction. Which, if either of these, is used in vivo is not yet known.

A clearer demonstration of alternative origin usage is that of the medium-sized plasmid R6K. In this case, electron microscopy has demonstrated that there are three origins that can be, and sometimes are, used in *E. coli*. Even in this case, though, one is much preferred for initiation in vivo.

Secondary Limitations on Plasmid Copy Number

Several cases have been noted in which the total copy number of a particular plasmid or group of related plasmids is limited even when mutations eliminate the normal negative regulatory circuit. In such mutants, the copy number depends inversely on the plasmid size. Although it is possible that all of these mutants retain a partially active replication inhibitor, this observation suggests that availability of some other factor has become rate limiting for replication of these mutants. All plasmids that share a requirement for this factor should form a pool whose total number is limited. Ruby and Novick (153) observed this for several staphylococcal plasmids and applied the term "plasmid space" to describe this phenomenon. Although few studies have directly tested this hypothesis, several observations lend it substantial support.

Because uncontrolled plasmid replication is likely to be lethal to the host (212), copy mutants can only be isolated if they are still regulated. If the mutation completely eliminates the normal regulatory system, the limitation in availability of another factor would be expected to determine the copy number of the plasmid. Thus, some copy mutants might still retain a partially active regulatory system and others which do not would be regulated by availability of some newly limiting factor. For copy mutants of both R100 and ColE1, the latter type of limitation is apparently the explanation for the size dependence of the copy number.

An example of this limitation in plasmid copy number is seen when pVH51, a miniplasmid derived from ColE1, is compared with its ColE1 parent. There are about five times as many copies of the miniplasmid as of ColE1, which means that the total amount of plasmid DNA per cell is the same in both cases (about 300 megadaltons in exponentially growing cells) (74). Warren and Sherratt (216) also found an indirect correlation between the size of a ColE1 derivative and its copy number. Another example was reported by Gelfand et al. (60), who found that the copy mutant of ColE1 which they isolated and a deletion derivative of it each comprise about 27% of the total cellular DNA. This indicates that the copy number of their deletion derivative is much higher than that of the parent plasmid. A limitation of this type on the total amount of ColE1 DNA per cell might be caused by limited availability of a factor required for replication of this plasmid.

Another case in which this type of explanation may hold is seen in integrative suppression of a bacterial *dnaA* mutant (which is temperature sensitive for initiation of replication) by a copy mutant of P1 (59). In this case, no extrachromosomal copies of the plasmid are present when the bacterial chromosome initiates replication from the plasmid origin (at high temperature). We have suggested (59) that this results from the lack of availability of a P1-specific factor required not just for initiation, but throughout the entire round of replication. Such a factor might be a subunit of the DNA polymerase, which is a highly processive enzyme. When the chromosome utilizes the P1-specific factor, the factor is not available for plasmid replication because it takes an entire cell cycle to replicate the chromosome. At low temperature, the chromosomal origin is used and extrachromosomal P1 DNA is replicated again.

Regulatory Role of Repeated DNA Sequences and Autoregulation of a Replication Protein

Another type of regulatory pathway that has been less completely elucidated occurs at the level of availability of a positively acting protein required for replication. These proteins (called E for the F plasmid and Rep for P1) are rate limiting for initiation of replication of these very-low-copy-number plasmids. Protein E is thought to have two domains: one catalyzes DNA replication in an unknown manner, and the other is autoregulatory. The autoregulatory domain recognizes a specific DNA sequence (the operator) and inhibits transcription. Because this sequence is reiterated on the DNA molecule at several sites other than the operator, the additional binding sites compete for binding of the protein both with the autoregulatory DNA site and with the replication initiation site. The proximity of the DNA binding sites suggests that positive or negative cooperativity or both may affect binding of the protein. The amount of protein bound at each DNA site is critical in determining how much protein is available for initiation of replication at any given time. Because no measurements of binding affinity have been made in any cases where autoregulation of a protein required for replication has been proposed, it is difficult to predict the effect (i.e., increase or decrease in initiation of replication) of an increase or decrease in effective concentration of the protein or of its DNA binding sites.

So far, no auxiliary factors (such as RNA or protein) that compete with or enhance the binding of the required protein to any of these sites have been identified, but it would not be surprising if they should be found in the future.

It is not clear whether this type of regulatory mechanism is utilized by any plasmids with a copy number higher than one per chromosome. In the multicopy plasmid R6K, it is possible that negative regulation is provided by the Pi protein. This protein is like E of plasmid F and Rep of P1 because it is required for replication of the plasmid. However, Pi differs from the other proteins because apparently it is not rate limiting for replication. Repeated DNA sequences are important for the positive action of Pi and may be involved in its autoregulation as well.

Prospects for the Future

A great deal of information on DNA replication has become available in recent years. However, in no case is the interaction of the regulatory molecules completely understood at the molecular level. Further detailed studies may reveal additional similarities between the plasmids or major differences.

On the one hand, it is possible that the concentration of free Rop protein of ColE1 is regulated in a manner like that of the E protein of the F plasmid: by binding to sequences of the DNA that are reiterated within small regions. On the other hand, further investigation may show that the small RNAs encoded within the reading frame for the E protein of the F plasmid interact with the mRNA for the E protein to

inhibit its synthesis, as is observed for the FII plasmids.

Only in the case of ColE1 has the RNA primer been identified. Synthesis of primer molecules in the larger plasmids may be regulated by mechanisms that have not yet been seen or by mechanisms similar to those used by ColE1.

The progress in the field of regulation of DNA replication has been enormous during the last 3 years, and, since many new techniques and mutants are currently available, there is every reason to expect this information explosion to lead to a much better understanding of plasmid replication in the very near future.

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