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Rolling-Circle Replication of Bacterial Plasmids

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INTRODUCTION

Studies on bacterial plasmids have contributed significantly to our understanding of the mechanisms and regulation of DNA replication, gene expression, and recombination, as well as other areas of prokaryotic biology. Studies on plasmid replication in both gram-positive and gram-negative bacteria have identified important *cis*- and *trans*-acting regions which are involved in replication and its regulation, partitioning, and stability of these extrachromosomal genetic elements. Most of the early studies on plasmid replication were done with *Escherichia coli* plasmids such as ColE1, pSC101, R6K, R1, RK2, RSF1010, and F, which were all found to replicate by a theta-type mechanism. More recently, a large number of small multicopy plasmids in different bacteria have been found to replicate by a rolling-circle (RC) mechanism originally observed for a number of single-stranded DNA (ssDNA) bacteriophages of *E. coli* (3, 4, 36, 127, 133, 166). This mode of replication involves the generation of a site-specific nick by the plasmid-encoded initiator (Rep) proteins at the plasmid leading-strand origin, followed by covalent extension of the 3′ hydroxyl end by a DNA polymerase. RC replication has also been observed for some animal parvoviruses (7, 20) and mitochondrial DNA in higher plants (5). The picture that has emerged from these studies suggests that RC plasmids are much more widespread than was originally thought and that they share considerable structural and mechanistic similarities in their genomic organization and replication properties. Although the RC plasmids also share many replication properties with the ssDNA bacteriophages of *E. coli*, there are significant differences as well. These include differences in the biochemical activities and mechanism of action of their initiator (Rep) proteins and in the regulation of their DNA replication.

Previous reviews on RC replication of plasmids that may be of interest to readers have been published (27, 38, 49, 64, 72, 112). This review will emphasize the biochemical and mechanistic aspects of RC replication and deal with the classification of RC plasmids into various groups based on the homologies in their leading-strand origins and initiator proteins, the functional domains of the leading- or double-strand origin (DSO) that are required for initiation and termination of replication, the structure of single-strand origins (SSOs) and the mechanism of lagging-strand replication, the domain structure and biochemical activities of the initiator proteins, in vitro replication systems for RC plasmids, the mechanism of regulation of replication and copy number, including the nature of inactivation of the RC plasmid initiator proteins after their utilization in replication, and our current knowledge of the role of host proteins in RC replication of bacterial plasmids.

DISTRIBUTION OF ROLLING-CIRCLE PLASMIDS

RC plasmids were originally discovered in *Staphylococcus aureus* and have now been described in a large number of other gram-positive bacteria such as *Bacillus subtilis*, *Clostridium butyricum*, *Brevibacterium lactofermentum*, *Streptococcus agalactiae*, *Lactococcus lactis*, *Leuconostoc lactis*, and *Streptomyces*. More recently, a number of RC plasmids have been isolated from gram-negative organisms such as *Actinobacillus actinomycetemcomitans*, *Bacteroides*, cyanobacteria, *Helicobacter pylori*, *Selnomonas ruminantium*, *Shigella sonnei*, and *Zymomonas mobilis*. In addition, RC plasmids have been observed in other prokaryotes such as *Mycoplasma mycoides* and the spirochete *Treponema denticola*. The diversity of RC plasmids is also evident from recent findings that some plasmids in the domain

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Archaea also replicate by an RC mechanism. The various RC plasmids can be classified into at least five families based on homology in their initiator proteins and DSOs. A list of various RC plasmid families and a partial list of their members are shown in Table 1. Plasmids that do not belong to any of these families are shown under "other RC plasmids" in Table 1. It should be noted that some plasmids within this category are related to each other, and it is anticipated that as more RC plasmids are discovered and characterized, they will form additional plasmid families based on their homology.

FUNCTIONAL ORGANIZATION

Most RC plasmids are relatively small and range in size from 1.3 to approximately 10 kb. They have a very compact structure, and in most cases all the plasmid genes are transcribed in one direction. The structural organization of one plasmid from each of the five RC plasmid families is shown in Fig. 1. In most RC plasmids, transcription of the initiator gene and leading-strand replication proceed in the same direction. However, it appears that in pSN2 and related plasmids, transcription of the *rep* gene and replication proceed in opposite directions. An interesting feature of the organization of some RC plasmids is that their structural genes encoding the initiator proteins also contain the DSOs, such that their initiators can bind to their own encoding sequences (86, 144). While the significance of this structural organization is not clear, it may become more obvious as studies on RC plasmids move to a more mechanistic level. For example, it is possible that initiator-origin interactions in such cases interfere either directly or indirectly with the synthesis of the Rep protein and play a role in the regulation of plasmid replication and copy number.

MODEL FOR REPLICATION OF ROLLING-CIRCLE PLASMIDS

Our current understanding of the various steps during the RC replication of bacterial plasmids is shown in Fig. 2. This model is based predominantly on the results of studies obtained with the plasmids of the pT181 family, and it is anticipated that other plasmid families may have variations in the individual steps shown in Fig. 2. The first step in RC replication is an interaction of the plasmid DSOs with their initiator proteins. In some cases, such as the plasmids of the pT181 family, the initiator-DNA interaction results in the extrusion of a cruciform structure in which the Rep nick site is present in the loop of the hairpin region (88, 109). The Rep proteins then nick at their specific cleavage sites and become covalently attached to the 5′ phosphate end of the DNA through a phosphotyrosine linkage. This is the case for plasmids of the pT181 and possibly pC194/pUB110 families. However, in the case of the pLS1 family, it is likely that the Rep proteins do not become covalently attached to the DNA (101, 102). While the nicking and nicking-closing reactions occur efficiently in vitro in the presence of the Rep proteins and their cognate DNAs, it is more likely that the nicking event to generate a primer for leading-strand synthesis in vivo is tightly regulated and occurs only when the appropriate replication proteins have been assembled at the origin. This probably involves recruitment of the host replication proteins such as DNA helicase, single-stranded-DNA binding protein, and a DNA polymerase to the origin. DNA polymerase III then extends the Rep-dependent nick, and the replication fork proceeds around the circle until the nick site has been regenerated. An interesting twist to the classical RC mode of replication, at least for plasmids of the pT181 family (and possibly the pUB110 plasmid), is that the replication fork proceeds approximately 10 nucleotides (nt) beyond the Rep nick site and the hairpin structure surrounding the nick site is regenerated. At this stage, interaction of the Rep protein with the regenerated origin sequence presumably stalls the replication fork. This step could involve a termination protein that may facilitate the pausing of the replication fork. The Rep protein (which may act as a dimer or multimer) then cleaves at the regenerated nick site, and a series of concerted cleavage/joining reactions occur that result in the release of a circular, leading-strand ssDNA and a nicked open-circular DNA containing the newly replicated leading strand. The nick is then sealed by the host DNA ligase and the DNA is subsequently converted to the supercoiled form with DNA gyrase. The newly replicated SC DNA can then rejoin the plasmid pool undergoing replication. The Rep protein in the case of plasmids of the pT181 family (and possibly pUB110) is inactivated by the attachment of an approximately 10-nt oligonucleotide to its active tyrosine residue. The 10-nt stretch corresponds to the sequence immediately downstream of the Rep nick site. The leading-strand DNA released by displacement synthesis is converted to the double-stranded DNA (dsDNA) form by using the SSO. This step involves synthesis of RNA primers by the host RNA polymerase followed by short extension synthesis by DNA polymerase I and subsequent replication by DNA polymerase III. The dsDNA is then sealed with DNA ligase and supercoiled with DNA gyrase. The events summarized above will be addressed in more detail in this review.

LEADING-STRAND REPLICATION

Double-Strand Origin

Replication of the leading strand of RC plasmids initiates from its leading-strand origin (DSO) and proceeds in a unidirectional manner. The fully functional DSOs of RC plasmids usually consist of less than 100 bp. The DSOs of some RC plasmid families are embedded within the coding sequence of their Rep proteins, while those of other families are located upstream of their initiator genes (10, 11, 22, 44, 48, 74, 80, 123, 137, 160). Some DSOs such as those of plasmids pT181, pLS1, and pKYM contain a static bend (85, 114, 115). In the case of the plasmids of the pT181 family, binding of the Rep protein to the DSO results in extrusion of a cruciform structure in which the Rep nick site is located within an ssDNA region (85, 109). These structural changes presumably facilitate nicking of the DNA by the initiator protein and assembly of the replisome at the origin. The DSOs are highly specific for their cognate initiators and contain domains that are involved in the initiation as well as termination of leading-strand replication. Generally, the DSOs contain two regions, one which is involved in sequence-specific, noncovalent binding to the initiator protein, and one which contains the Rep nick site. These two regions can either be adjacent to each other, as in plasmids of the pT181 and pC194/pUB110 families, or be separated by a spacer region of 13 to 91 nt, as with pLS1 and related plasmids (86, 88, 101–103, 142, 144, 148, 155). A comparison of the origin sequence among plasmids of the same family reveals that while their nick regions are highly conserved, the Rep binding sequences are not well conserved (27, 38, 49, 64, 72, 112, 129). A typical example of the DSOs with adjacent Rep binding and nicking sequences as exemplified by plasmids of the pT181 family is shown in Fig. 3. The Rep nick site is generally located in the loop of a hairpin region, as exemplified by plasmids of the pT181, pC194/pUB110, and pE194/pLS1 families (Fig. 3) (10, 48, 84, 88, 101, 123, 137). The Rep binding

TABLE 1. RC plasmids of bacteria

Plasmid[a]	Size (kb)	Resistance gene[b]	Original host	Reference
pT181				
pT181	4.4	Tc	*Staphylococcus aureus*	79
pC221	4.6	Cm	*Staphylococcus aureus*	121
pC223	4.6	Cm	*Staphylococcus aureus*	121
pCW7	4.2	Cm	*Staphylococcus aureus*	112
pHD2	2.1	Cryptic	*Bacillus thuringiensis*	98
pLUG10	3.1	Cd	*Staphylococcus lugdunensis*	16
pOg32	2.5	Cryptic	*Leuconostoc oenos*	14
pS194	4.4	Sm	*Staphylococcus aureus*	121
pT127	4.4	Tc	*Staphylococcus aureus*	112
pTZ12 (pTZ10)	2.5	Cm	*Corynebacterium xerosis*	2
pUB112	4.1	Cm	*Staphylococcus aureus*	121
pE194/pLS1				
pE194	3.7	Em	*Staphylococcus aureus*	112
pA1	2.8	Cryptic	*Lactobacillus plantarum*	146
pC1305	8.7	Cryptic	*Lactococcus lactis*	54
pCI411	2.9	Cryptic	*Leuconostoc lactis*	18
pFX2	2.5	Cryptic	*Lactococcus lactis*	149
pKMK1	1.9	Cryptic	*Mycoplasma mycoides*	81
pLS1 (pMV158)	5.5	Tc	*Streptococcus agalactiae*	27
pSH71	2.1	Cryptic	*Lactococcus lactis*	33
pWV01	3.3	Cryptic	*Lactococcus lactis*	92
pC194/pUB110				
pC194	2.9	Cm	*Staphylococcus aureus*	112
pAMα1	9.6	Tc	*Streptococcus faecalis*	117
pBAA1	6.8	Cryptic	*Bacillus subtilis*	32
pBC1	1.6	Cryptic	*Bacillus coagulans*	31
pBC16	4.6	Tc	*Bacillus cereus*	6
pBP614	5.6	Cryptic	*Bacillus popilliae*	93
pBS2	2.3	Cryptic	*Bacillus subtilis*	21
pC30il	2.1	Cryptic	*Lactobacillus plantarum*	135
pCA2.4	2.4	Cryptic	*Synechocystis* strain PCC 6803	154
pCB101	6.0	Cryptic	*Clostridium butyricum*	13
pCB2.4	2.3	Cryptic	*Synechocystis* strain PCC 6803	151
pCC5.2	5.2	Cryptic	*Synechocystis* strain PCC 6803	150
pFTB14	8.2	Cryptic	*Bacillus liquefaciens*	105
pGT5	3.4	Cryptic	*Pyrococcus abyssi*	37
pJDB21	2.5	Cryptic	*Selenomonas ruminantium*	161
pKYM	2.1	Cryptic	*Shigella sonnei*	156
pLAB1000	3.3	Cryptic	*Lactobacillus hilgardii*	67
pLo13	3.9	Cryptic	*Leuconostoc oenos*	40
pLP1	2.1	Cryptic	*Lactobacillus plantarum*	12
pOX6	3.2	Cd	*Staphylococcus aureus*	112
pRF1	4.2	Cryptic	*Plectonema* sp. strain PCC 6402	116
pRBH1(pTB19)	1.75	Km	Bacilli[c]	104
pSH1451	6.1	Salt resistance	*Bacillus pumilus*	53
pSN1981	4.9	Tc	*Bacillus subtilis*	132
pTA1060	8.6	Cryptic	*Bacillus subtilis*	15
pTD1	2.7	Cryptic	*Treponema denticola*	94
pTHT15	4.5	Tc	Bacilli[c]	57
pUB110	4.5	Km	*Staphylococcus aureus*	99
pUH1	5.7	γ-Glut[d]	*Bacillus subtilis*	51
pVA380-1	4.2	Cryptic	*Streptococcus ferus*	91
pWC1	2.8	Cryptic	*Lactococcus lactis*	119
pWGB32	2.4	Smr	*Staphylococcus aureus*	46
p353-2	2.4	Cryptic	*Lactobacillus pentosus*	120
pSN2				
pSN2	1.3	Cryptic	*Staphylococcus aureus*	78
pBI143	2.7	Cryptic	*Bacteroides fragilis*	136
pE5	2.1	Em	*Staphylococcus aureus*	122
pE12	2.2	Em	*Staphylococcus aureus*	122
pIM13	2.1	Em	*Bacillus subtilis*	122
pNE131	2.1	Em	*Staphylococcus epidermidis*	122
pT48	2.1	Em	*Staphylococcus aureus*	112
pTCS1	1.3	Cryptic	*Staphylococcus aureus*	112
pZMO2	1.9	Cryptic	*Zymomonas mobilis*	145

Continued on following page

TABLE 1—*Continued*

Plasmid[a]	Size (kb)	Resistance gene[b]	Original host	Reference
pIJ101/pJVI[e]				
pIJ101	8.6	Cryptic	*Streptomyces phaeochromogenes*	71
pBL1	4.5	Cryptic	*Brevibacterium lactofermentum*	39
pJV1	10.5	Cryptic	*Streptomyces phaeochromogenes*	130
pSG5	3.3	Cryptic	*Streptomyces ghanaensis*	107
pSN22	11.0	Cryptic	*Streptomyces nigrifaciens*	69
Other RC plasmids				
pC1305	8.7	Cryptic	*Lactococcus lactis*	54
pG12	9.7	Cryptic	*Bacillus thuringiensis*	96
pGRB1	1.7	Cryptic	*Halobacterium* strain GRB	134
pHK2	10.5	Cryptic	*Halobaferax* sp. strain Aa2.2	56
pHPK255	1.5	Cryptic	*Helicobacter pylori*	82
pTX14-1	5.4	Cryptic	*Bacillus thuringiensis*	17
pTX14-3	7.6	Cryptic	*Bacillus thuringiensis*	1
pVT736-1	2.0	Cryptic	*Actinobacillus actinomycetemcomitans*	41

[a] Plasmids in bold represent those after which the particular families are named; in some cases, two plasmids represent a particular family.
[b] Cm, chloramphenicol resistance; Cd, cadmium resistance; Em, erythromycin resistance; Km, kanamycin resistance; Sm, streptomycin resistance; Smr, staphylococcal multidrug resistance; Tc, tetracycline resistance.
[c] Thermophilic bacilli.
[d] γ-Glut, γ-glutamyltranspeptidase.
[e] Plasmids of this family show some homology to plasmids of the pC194/pUB110 family.

sequence may also include inverted repeat (IR) sequences such as the IRIII region in the pT181 family (Fig. 3). Plasmids of the pE194/pLS1 family contain two or three directly repeated sequences, or iterons, within their DSOs (101), although iterons are generally not present in the origins of most RC plasmids.

Initiation and Termination of RC Replication

Experiments with plasmids containing two copies of the DSOs have shown that both the Rep binding and nicking sequences are required for the initiation of replication (44, 47, 63, 106, 157). On the other hand, a subregion of the origin containing the nick region alone is sufficient for efficient termination of replication (47, 63, 137, 157, 164, 165). It has been shown with the pC194 and pT181 plasmids that synthetic oligonucleotides (18-mers) containing the nick sites of their respective initiators are sufficient for the termination step (47, 164). Thus, the initiation domain of the origin is much larger than the termination domain and the specific Rep binding sequence is required for initiation but is dispensable for the termination step. This is consistent with the observation that termination of replication of a particular plasmid can be promoted at the origins of most plasmids belonging to the same family. For example, replication of pT181 can be terminated by the RepC protein at the origin of pC221, a plasmid of the same family (59). Similarly, pC194 replication can terminate at the origin of the related plasmid pUB110 while replication initiated from the pE194 origin can terminate at the origin of the related pMV158 plasmid (47, 137). The ability of a Rep protein to promote termination at a heterologous origin of the same family is not surprising due to the conservation of the nick region in plasmids of the same family. So far, no sequence has been identified within the origin that is required for termination but not the initiation step. The above observations reveal that the Rep protein must interact stably with the origin to assemble a replication initiation complex. Once replication has initiated, it is likely that Rep is a component of or in close proximity to the replication fork (with or without covalent attachment to the nick site). Thus, when the replication fork reaches the regenerated origin sequence, it is likely that the nick region alone is sufficient for recognition by the replisome-associated Rep protein, which then initiates the process of termination. However, it is obvious that a specific, albeit weak interaction between Rep and the nick region is required for appropriate termination of replication to occur. It has been shown that nicking at the origin by Rep is required but not sufficient for the initiation of replication (44, 47, 164, 165). Termination of replication also requires nicking by Rep, and current evidence suggests that the efficiency of nicking of the DNA is not directly related to the efficiency of termination (165). Our laboratory has found that the IRII region of the pT181 origin containing the RepC nick site is sufficient for the termination step (164). However, mutational analysis of IRII

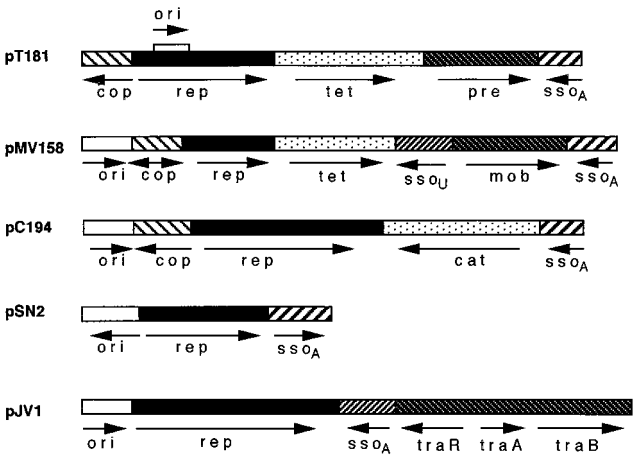

FIG. 1. Functional organization of RC plasmids belonging to five different families. The maps are not drawn to scale. The direction of transcription of various genes and the direction of replication from the origins (DSOs) are shown. Plasmid pMV158 encodes two copy control genes (an antisense RNA and a repressor), which are transcribed in opposite directions. Plasmid pMV158 contains both sso_A and sso_U sequences, whereas the other plasmids contain only one SSO. ori, double-strand origin; sso, single-strand origin; rep, initiator gene; cop, copy control gene(s); pre, recombinase gene; mob, mobilization function; tra, DNA transfer genes; tet, tetracycline resistance gene; cat, chloramphenicol resistance gene.

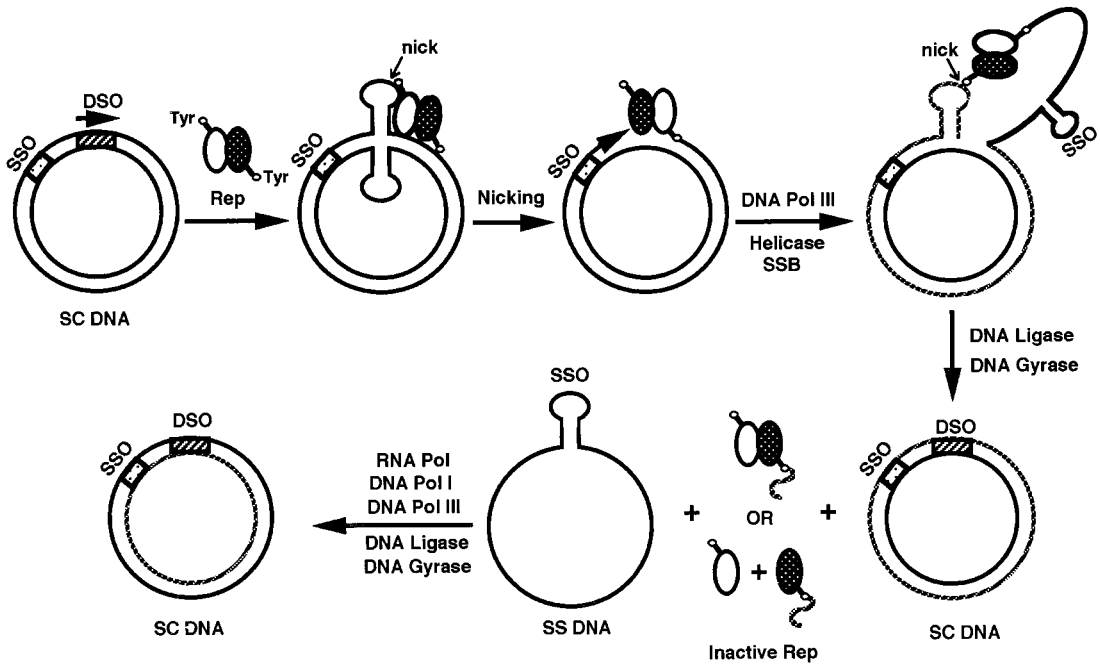

FIG. 2. Model for the replication of RC plasmids. For details, see the text. The Rep protein is shown as a dimer in the model, but different initiators may function as monomers or oligomers. Similarly, the inactivated Rep protein may be released as a monomer, dimer, or oligomer. SC, supercoiled.

showed that while some nucleotides in this region are more important for nicking by RepC, other nucleotides are more important in religation of the DNA by the initiator protein (165).

The role of sequences required for nicking and religation by Rep has been studied in some detail for various RC initiators in vitro. Such experiments involved the treatment of defined SS oligonucleotides containing the Rep nick site with the Rep proteins and analysis of the product (84, 102, 143, 165). For plasmids in which Rep becomes covalently attached to the DNA, such as those of the pT181 family, Rep attached to the 5′ phosphate of the oligonucleotide downstream of the nick site can be isolated and used to study the religation of upstream oligonucleotides containing a 3′-OH end. Regeneration of the original, full-length oligonucleotide in these cases reveals the sequence requirements for the religation step. By using this approach, sequences in the left arm of IRII in the pT181 origin were found not to be critical for RepC nicking, since an oligonucleotide containing the right arm and loop of IRII was nicked by RepC as efficiently as was the full IRII (165). On the other hand, the left-arm sequence of IRII was critical for religation by RepC (163). Since our results show that the full IRII region is much more efficient than the right arm and loop of IRII in termination (165), it is highly likely that the efficiency of religation of the DNA by Rep is directly related to the efficiency of termination of replication.

Studies with plasmids of the pT181 and pC194 families have provided more detailed information on the sequences important in the initiation and termination steps (44, 47, 48, 63, 164, 165). Mutational analyses have shown that the structure of the IRII region of the pT181 family is critical for the initiation step but is not absolutely required for termination (165). These studies suggest that the interaction between Rep and the origin is inherently different for these two steps. It should be noted that the DNA structures during the initiation and termination steps are likely to be different. Initiation occurs on SC DNA in which the Rep-ori interaction is critical and may involve the generation of a cruciform structure at the origin, as is the case for plasmids of the pT181 family (109). On the other hand, the DNA is likely to be in a more relaxed form during the termination event, and both relaxed and ssDNA can presumably be recognized by Rep.

For plasmids of the pT181 family (and possibly that of pUB110), termination of replication is followed by inactivation of the Rep protein due to the attachment of an oligonucleotide (10-mer) to the active tyrosine residue, which is responsible for nicking-closing of the DNA (103, 124, 125). This probably results from the passage of the replication fork to approximately 10 nt beyond the regenerated Rep nick site in the DNA. At this stage, the progress of the replication fork is arrested and the Rep protein cleaves the DNA at the regenerated nick site with the concomitant attachment of the oligonucleotide to the tyrosine residue. Why does replication proceed beyond the Rep nick site after completion of the synthesis of the leading strand? Studies with the pT181 system provide a plausible explanation for this event. The loop of the IRII hairpin in the pT181 origin contains the RepC nick site (Fig. 3). Footprinting and electrophoretic mobility shift assay studies have shown that RepC interacts weakly with the right arm of IRII in addition to its major binding site (IRIII) located immediately downstream of IRII (Fig. 3) (86, 163). However, no specific interaction between the left arm of IRII and RepC has been reported. Once replication is initiated from the nick located in the loop of IRII, it proceeds in a rightward direction. When the leading strand has been almost completely replicated, the replication fork approaches the origin from the left and encounters the left arm of IRII and the RepC nick site. Since the left arm and loop of IRII neither bind nor are nicked by RepC (86, 165), replication may continue beyond the nick site until the right-arm sequence has been synthesized. A specific interaction between the right-arm sequence and RepC, followed by cleavage in the loop of IRII, may then occur. This would release the leading strand of the DNA with the concomitant attachment of the oligonucleotide representing the right arm of IRII to RepC. This prediction is consistent with our dem-

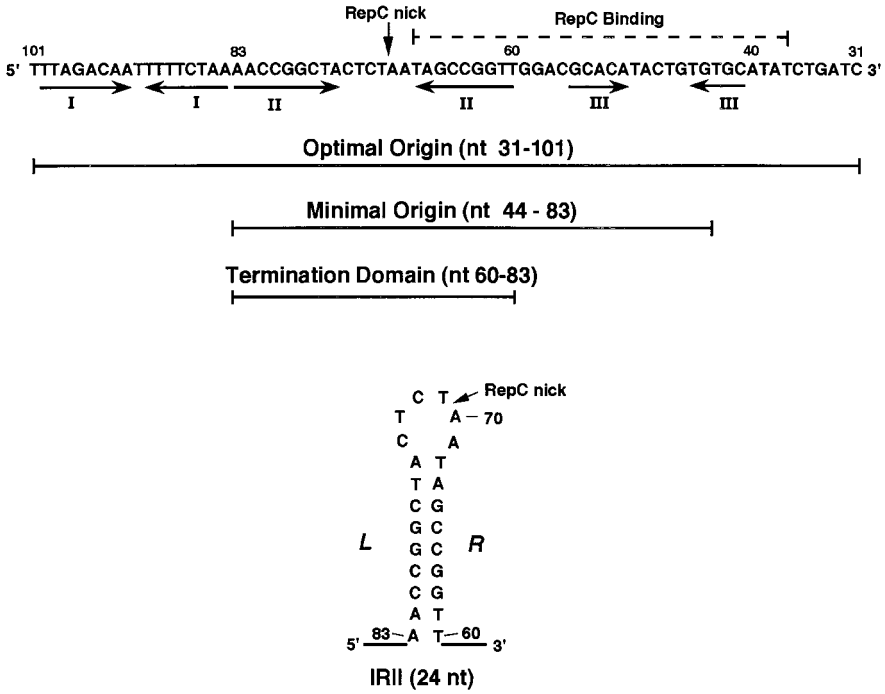

FIG. 3. Nucleotide sequence of the pT181 origin. Only the leading strand of the DNA is shown, and replication of the plasmids of the pT181 family proceeds in a rightward direction. The IR sequences IRI, IRII, and IRIII are shown by horizontal arrows. The initiator binding sequence, including the IRIII region that determines the specificity of initiator-origin interactions, is shown by a dotted line. The nick site of the initiator present within the conserved IRII region is indicated by a vertical arrow. The optimal origin, the minimal origin required for initiation and termination, and the region sufficient for termination alone is shown. The predicted secondary structure and sequence of the IRII region, which is sufficient for termination of replication and is conserved in the plasmids of the pT181 family, is shown at the bottom. Numbering is according to the published sequence of pT181 (79).

onstration that an ssDNA oligonucleotide containing the right arm and loop of IRII is efficiently cleaved by RepC (165). For the pC194 plasmid, termination of replication involves transesterification promoted by the Glu-210 residue of RepA and hence no oligonucleotide is attached to the active tyrosine residue (110).

While the sequence requirements for termination have been studied in some detail, our understanding of the molecular events during the termination step is very limited. For example, it is not known whether a termination protein is involved in RC replication. The function of such a protein may be to block the helicase activity once the replication fork has displaced the full-length leading strand and regenerated the Rep nick site. However, this protein has to be different from the sequence-specific replication terminator protein of *B. subtilis*, which is unable to block RC replication of the pT181 plasmid containing the replication terminator protein binding sites (70). It will also be of interest to investigate whether specific interactions occur between the Rep protein, helicases, and the putative termination protein and the role of origin sequences in this interaction. Studies on the DSOs of RC plasmids have so far failed to identify any regions or nucleotides that are required for termination but are dispensable for initiation (44, 165). Thus, it appears that regions of the DSOs that interact with the Rep proteins and/or the replication machinery during termination are also critical for the assembly of the replication initiation complex.

Replication Enhancer

Some RC plasmids contain an enhancer sequence that stimulates replication. The best studied of these is the *cmp* element of plasmid pT181 (19, 43, 45). The *cmp* element of pT181 functions up to a distance of 1 kb from the DSO (45). This region consists of approximately 100 bp and contains a locus for DNA bending (55). The *cmp* element functions by increasing the interaction between the Rep protein and the DSO (43). Recently, a host factor called CBF1, which binds to the *cmp* region of pT181, has been isolated (162). Its precise function is not known, and it will be interesting to determine whether it interacts with Rep and/or the DSO.

LAGGING-STRAND REPLICATION

Replication of the lagging strand of RC plasmids initiates from their SSO. Although plasmids with the SSOs deleted can replicate to some extent, they become unstable and have a decreased copy number (26, 50). The SSOs usually have extensive secondary structure, and unlike the DSOs, their sequences are generally not homologous among plasmids belonging to the same family. The SSOs are sequence and orientation specific and are generally located a short distance upstream of the DSOs (50). The SSOs function only when they have been exposed in an ssDNA form, and therefore replication of the lagging strand of RC plasmids does not initiate until the leading strand has been almost fully synthesized. Thus, most RC plasmids usually replicate by an asymmetric RC mechanism and ssDNA corresponding to the leading strand is routinely observed in vivo as well as in vitro (106, 118, 139, 140). Indeed, the presence of ssDNA in a cell is a hallmark of RC plasmids, and the presence of plasmid ssDNA provides a strong suggestive evidence that a particular plasmid replicates by the RC mechanism.

Several types of SSOs, such as sso_A, sso_U, sso_T, and sso_W, have been identified based on their secondary structures (10, 50, 83, 95, 100, 128, 159). The SSOs of a particular type usually have only minimal sequence homology. Plasmids such as pT181, pE194, pLS1, and pIJ101 contain a sso_A-type origin (26,

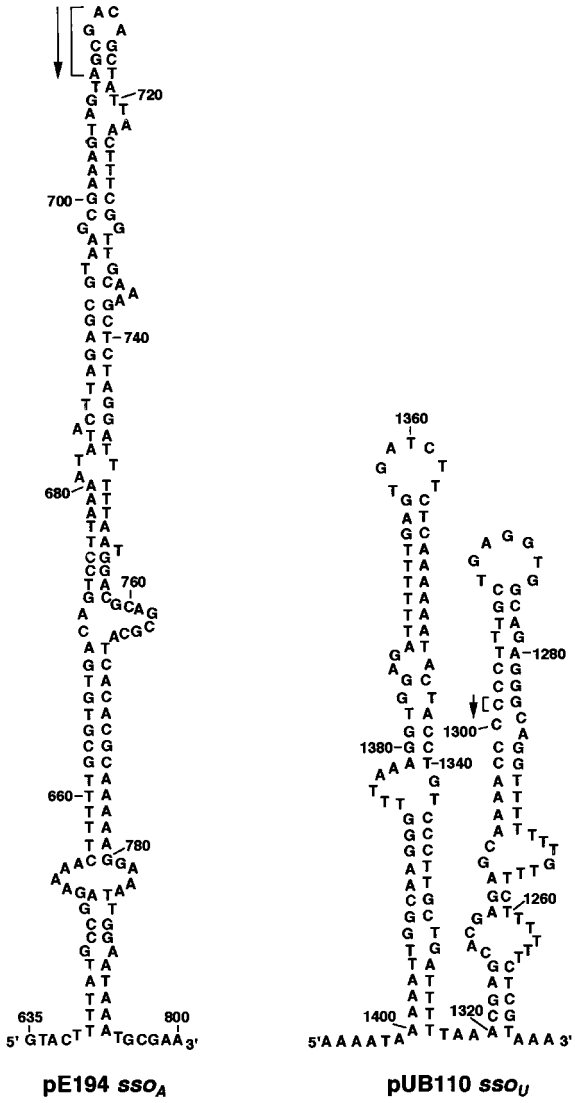

FIG. 4. Folded structures of sso_A (exemplified by pE194) and sso_U (exemplified by pUB110). The parentheses and arrows indicate the major start sites of lagging-strand replication from these SSOs. The bracket in the pE194 sso_A also corresponds to the conserved CS_6 sequence. The first stem-loop structure located at the bottom of the pE194 sso_A corresponds to RS_B, which is the binding site for RNA polymerase.

50, 131), pUB110 contains sso_U (10), pWVO1 contains sso_W (128), and pBAA1 and pTA1060 contain sso_T (100). Some plasmids such as pMV158 contain two SSOs, sso_A and sso_U (89), while the *Streptomyces* plasmid pSN22 appears to contain three SSOs (138). The predicted secondary structures of the sso_A of pE194 and the sso_U of pUB110 are shown in Fig. 4. While sso_A-type origins appear to function only in their natural hosts, sso_U of pUB110 has been shown to be functional in both its natural host, *Staphylococcus aureus*, and *B. subtilis* (10). Thus, the ability of an SSO to function in various hosts may contribute to plasmid promiscuity and horizontal transfer among related bacteria.

The function of SSOs in lagging-strand replication of RC plasmids has recently been studied in some detail. Both in vivo and in vitro studies have shown that host proteins are solely responsible for lagging-strand synthesis and that the host RNA polymerase synthesizes RNA primers from both sso_A- and sso_U-type origins (9, 10, 30, 89, 128). Some SSOs such as the sso_W of pWV01 appear to support replication of the lagging strand in both an RNA polymerase-dependent and an RNA polymerase-independent manner (128). In vitro replication studies have shown that synthesis of the lagging strand supported from the sso_U of pUB110 initiates at a single, fixed position (30). Recently, the structure-function relationship of the sso_A-type origins has been studied. In contrast to sso_U, synthesis of RNA primers from sso_A appears to initiate at multiple positions (30). The sso_A-type origins contain two conserved sequences, a 6-nt sequence 5′TAGCGA/T3′ (CS_6) located in the central loop of their secondary structures and a recombination site, RS_B, that is known to be involved in plasmid recombination (Fig. 4). RNA polymerase has been shown to bind to ssDNA containing the RS_B sequence, which is expected to be present in a dsDNA form due to the secondary structure of sso_A (90). RNA polymerase then synthesizes an approximately 20-nt RNA primer in the vicinity of RS_B. The conserved CS_6 sequence of sso_A serves as the termination site of RNA primer synthesis and the transition point from RNA to DNA synthesis (30, 90). The host DNA polymerase I then initiates DNA synthesis by using the RNA primer, and, later, DNA polymerase III continues lagging-strand synthesis (90). The above results reveal the significance of the extensive secondary structures found in most SSOs. Since RNA polymerase is known to synthesize RNA primers from most SSOs, the palindromic structure present in these origins is likely to play an important role in polymerase binding. Some SSOs such as those present in plasmids pLS1 and pT181 contain sequences that resemble the −35 and weak −10 regions (50, 90). It is likely that intrastrand pairing in the SSOs is critical for their recognition by the host RNA polymerase. Since the predicted folded structures of sso_A and sso_U are quite different, there appears to be a good deal of diversity in the structures and sequences that can be used by host RNA polymerase for RNA primer synthesis. In spite of considerable new information on the functions of SSOs, many important questions remain to be answered. Foremost among these is why some SSOs function only in their natural hosts while others are able to function in two or more bacterial hosts (68). One possibility is that some SSOs such sso_A are recognized only by their cognate RNA polymerase while other SSOs such as sso_U of pUB110 may be recognized by the RNA polymerase of different hosts. However, this possibility seems unlikely in view of recent results obtained with the sso_A of the streptococcal plasmid pLS1. In this study, the *B. subtilis* RNA polymerase was found to bind to the sso_A of the streptococcal plasmid pLS1 and synthesized an RNA primer from this origin (90). However, the pLS1 sso_A is inactive in *B. subtilis*. Therefore, it is more likely that some other host enzyme(s) (DNA polymerase I?) may determine the ability of a particular SSO to support lagging-strand replication in various hosts.

INITIATOR PROTEINS

Biochemical Activities

The replication initiator protein of plasmid pT181, RepC, was the first RC plasmid initiator to be isolated and shown to contain origin-nicking activity (87, 88). This observation, along with the demonstration that in vitro replication of the leading strand of this plasmid did not require an RNA primer and the presence of ssDNA in strains carrying various plasmids in *S. aureus* and *B. subtilis* provided the first indication that many plasmids replicate by an RC mechanism (86, 87, 139, 140). Many RC initiator proteins have now been isolated and their

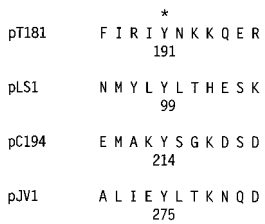

FIG. 5. Alignment of the amino acid sequence at the active site of the initiator proteins of the four RC plasmid families. The active tyrosine residues involved in the nicking of the DNA are shown by an asterisk, and their location within each protein is indicated.

biochemical activities have been studied. These include RepC and RepD (encoded by pT181 and pC221, respectively), RepB (pLS1), RepU or RepH (pUB110), and RepK (pKYM) (22, 87, 103, 114, 142). All RC initiator proteins have been shown to nick the DSO present in their cognate plasmids and also to bind noncovalently to the DSO (22, 87, 101, 103, 114, 142, 144, 167). The Rep proteins of the plasmids of the pT181 family also bend the origin DNA and induce a cruciform structure within the DSO (85, 109). The active tyrosine residue involved in DNA nicking as well as adjacent amino acids are conserved in initiators belonging to the same plasmid family (58, 144). However, the Rep proteins are usually highly specific for the replication of their cognate plasmid. This is because the Rep binding sequences are not well conserved among members of the same plasmid family. As a result, Rep fails to interact stably with origins of other plasmids of the same family (28, 59, 144, 167). It is, however, known that in the absence of its cognate origin, a Rep protein can drive replication from a heterologous origin of the same family if it is expressed at high levels in vivo and in vitro (19, 28, 59, 167). These results suggest that a weak interaction between the nick region and Rep can support replication to a limited extent. Replacement of the active tyrosine residue in the RepC protein of pT181 with serine destroyed its nicking-closing activity, demonstrating that the hydroxyl group of serine cannot catalyze DNA nicking in this case (29). An extensive alignment of the proteins involved in nicking-closing of the DNA has been published (58). The nicking domain of initiators belonging to four RC plasmid families is shown in Fig. 5. Currently, this information is not available for plasmids of the pSN2 family.

Studies with purified Rep proteins of RC plasmids have shown that supercoiled plasmids containing the nick region but lacking the Rep binding region are relaxed by the Rep proteins (101, 102, 164). Similarly, ssDNA oligonucleotides containing the nick region but lacking the binding region are nicked by the Rep proteins (65, 84, 101, 102, 165). It is also known that secondary structure is not important for Rep nicking activity when the DNA is ssDNA (165). There are some differences in the nicking activities of the various RC Rep proteins as well. For example, while the RepC protein of pT181 can nick linear dsDNA containing the nick site, the RepB protein of pLS1 is unable to nick linear dsDNA (86, 102). However, the dsDNA that is nicked by RepC included its binding site (86). Whether dsDNA containing the RepC nick site but lacking its binding site is also nicked by RepC remains to be determined. In vitro and in vivo studies have shown that the ability of an initiator to interact with the DSO is the sole determinant of their replication specificity (28, 147). The Rep protein of pLS1 (similar to the gene 2 protein of coliphage fd) appears not to be covalently attached to the DNA after nicking, in contrast to the initiators of the pT181 and pC194/pUB110 plasmid families (88, 102, 103, 141). Since the pLS1 DSO is also known to contain iterons, it is possible that the nature of Rep-origin interactions is different in the pLS1/pE194 family from that in pT181, pUB110, and possibly other RC plasmids. The evidence that the Rep protein of pLS1 exists as a hexamer while the Rep proteins of other RC plasmids that have been studied to date exist either as a monomer or as a dimer in solution may also be responsible for the different arrangement of the binding and nick regions within the DSOs (22, 103, 126). This issue needs further investigation, and the availability of Rep-DNA crystal structures in the future should provide more detailed information on their interactions.

Functional Domains

Recently, intensive efforts have been directed toward an understanding of the domain structure of the RC initiator proteins. The initiator proteins of RC plasmids contain at least two domains, one involved in origin nicking and the other involved in specific recognition of the DSO sequence (28, 29, 144, 147). Since the initiators belonging to a particular plasmid family are highly homologous yet are generally specific for the replication of their cognate DNA, this leads to the prediction that the variable regions in the initiators of closely related plasmids must contain their replication specificity and sequence-specific DNA binding domains (28, 147). This was found to be the case for plasmids of the pT181 family.

The domain structure of the initiators of the pT181 family has been examined in some detail. The active tyrosine is located at position 191 for RepC encoded by the pT181 plasmid, while amino acids 265 to 270 are critical for its sequence-specific DNA binding activity (28, 29, 141, 147). Mutational analysis has shown that the sequence-specific DNA binding and DNA relaxation activity of RepC can be uncoupled (29). Thus, RepC mutants have been described that can nick at the origin but do not bind the DNA ($nick^+$ $bind^-$) as well as those that can bind to the DNA but not nick it ($bind^+$ $nick^-$) (29). These mutants were used to demonstrate that both the nicking-closing and sequence-specific DNA binding activities of the RepC protein are absolutely required for its DNA replication activity (29). A variable region of the initiator proteins of the pT181 family is located near their carboxyl-terminal end and is involved in their DNA binding activity (Fig. 6). Hybrid proteins were generated in which six amino acids in RepC (positions 265 to 270) were exchanged with the corresponding amino acids of the closely related RepD and RepE proteins (encoded by the pC221 and pS194 plasmids, respectively). These studies showed that the above six amino acids switched the DNA replication specificity of the hybrid initiator proteins to the source of these particular amino acids (28, 147). These amino acids were also shown to determine the DNA binding specificity of the initiators of the pT181 family (28). However, a

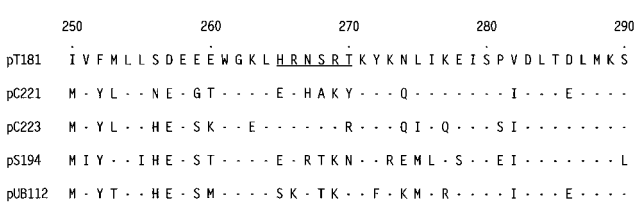

FIG. 6. Amino acid sequence of the DNA binding domains of the initiator proteins encoded by plasmids of the pT181 family. Numbering corresponds to the amino acids of the pT181 initiator, RepC. Identity of amino acids to those of RepC is indicated by dashes. Amino acids that determine the specificity of the initiators in origin binding and replication are underlined.

synthetic peptide containing 26 amino acids including the above 6-amino-acid region was found to be inactive in DNA binding (73). Thus, the initiators of the pT181 family contain a modular structure in which the DNA binding domain is located at a similar position in the various initiators of this family. While only a few amino acids within this region are responsible for determining the specificity of DNA-protein interactions, a larger region is required for the ability of the initiators to bind to the DNA. It is highly likely that most RC initiator proteins contain a modular arrangement with well-defined regions involved in sequence-specific DNA binding and origin nicking.

Oligomeric State

The oligomeric state of RC Rep proteins has recently been investigated. The Rep proteins of pUB110 and pKYM appear to be present as a monomer, while the RepB protein of pLS1 may be a hexamer in solution (22, 103, 114). Studies with initiators of the plasmid pT181 family suggest that their Rep proteins exist as a dimer in solution (126, 144). However, recent evidence indicates that RepC encoded by pT181 may be a monomer in solution and dimerize on the DNA (163). In addition to the initial nick at the origin by the Rep proteins during the initiation of RC replication, other concerted Rep-mediated nicking and closing events occur during the termination step. These include nicking of the leading strand present in dsDNA after it has been fully replicated and ligation of the released leading strand. The gene A protein of ϕX174 acts as a monomer and contains two closely spaced tyrosine residues at its active site (3, 36, 127, 166). This protein carries out two successive transesterification reactions during the termination step, which results in the release of the leading-strand DNA to finish one round of replication and reinitiation of another round. However, reinitiation of replication generally does not occur in RC plasmids (111), and their initiator proteins contain only one active tyrosine residue. How do plasmid initiators carry out the above steps with a single active tyrosine residue? There appear to be at least two ways in which this can be accomplished. The active site of the pC194 RepA protein contains two closely spaced amino acids, Tyr-214 and Glu-210, that are involved in the initiation and termination steps. Tyr-214 is involved in nicking of the DNA during initiation, while Glu-210 activates a water molecule to mediate a nucleophilic attack of a phosphodiester bond in the newly replicated DNA during the termination step (110). Subsequently, transesterification generates a circular ssDNA corresponding to the leading strand and the RepA protein is released from the DNA (110). Thus, reinitiation in this case is prevented by utilization of a glutamate residue in place of a tyrosine during the termination step. This model of RepA function has been elegantly confirmed in a recent study in which replacement of Glu-210 by a tyrosine residue was shown to promote reinitiation after one round of replication (111). The plasmids of the pT181 and possibly other RC plasmid families may carry out initiation-termination steps in a different manner. Studies with the RepC protein of pT181 have shown that an initiator dimer is assembled on the DNA (either as a preexisting dimer or Rep dimerization on the DNA) (126, 163). Thus, the active Tyr-191 residues of both the subunits probably participate in the transesterification reactions during the termination step.

Inactivation

The replication and copy number of RC plasmids are tightly regulated, and the initiator proteins are unable to catalyze multiple rounds of DNA synthesis. As discussed above, the Rep proteins of the plasmids of the pT181 family are inactivated after supporting one round of replication. Current evidence suggests that the initiator protein encoded by pT181 plasmid exists as a dimer, termed RepC/RepC (126). Upon attachment of an oligonucleotide to the Tyr-191 residue of one subunit, a heterodimer termed RepC/RepC* is generated. Although RepC/RepC* is able to bind to DNA, it has little or no nicking activity and is unable to melt the origin region that is necessary to expose the nick site in an ssDNA form (66). This may explain why RepC/RepC* is defective in the initiation of replication. It should be noted that in most of the above studies, RepC/RepC* was purified from pT181-containing cells and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed that these preparations contained an approximately equal mixture of the RepC and RepC* forms (65, 66). Recently, our laboratory has generated RepC* in vitro by incubating purified RepC with ssDNA oligonucleotides containing the RepC nick site. These preparations contained approximately 90% RepC* and 10% unmodified RepC as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (163). These results suggested that either an oligonucleotide was attached to the active tyrosine residue of both the RepC subunits or RepC was present as a monomer under the conditions used. The in vitro-generated RepC* was shown to lack DNA replication, DNA relaxation, and ssDNA cleavage activity but contained DNA binding activity (163). Our recent fast protein liquid chromatography analysis and electrophoretic mobility shift assays suggest that RepC may exist as a monomer in solution but dimerizes on the DNA (163). Interestingly, RepC* is defective in dimerization on the DNA. Further studies are required to analyze the nature and activities of RepC*. RepC/RepC* has also been shown to inhibit RepC-supported in vitro replication, suggesting a possible regulatory role for the inactivated initiator in replication (65). However, there is no evidence that RepC* plays a regulatory role in replication in vivo. This issue needs to be further investigated. Preliminary studies with the RepU/RepH initiator protein of pUB110 suggest that this initiator may also be inactivated by attachment of an oligonucleotide (103). However, the related RepA protein of pC194 does not appear to be inactivated by this mechanism, since the initiation and termination reactions supported by this protein are different, as discussed above. Since the pUB110 and pC194 initiator proteins as well as their nicking sequences are highly homologous, additional studies are required to resolve the issue of their Rep protein inactivation. Further studies are also needed to evaluate whether attachment of oligonucleotides to the initiator proteins is a widespread mechanism for inactivation of RC initiator proteins. Since the Rep proteins of the pLS1/pE194 family do not appear to attach to the DNA covalently (102), it is very likely that additional mechanisms to inactivate the Rep protein (for example, by protease degradation or monomer-dimer interconversion) exist and have yet to be discovered.

The Tyr-237 residue of the RepK protein encoded by pKYM (belonging to the pC194/pUB110 family) is involved in DNA nicking (129, 156). It has been shown that the host HU protein is involved in pKYM replication (158). In the absence of HU, RepK appears to bind weakly to the origin, resulting in low-level replication. Two mutants of RepK, with W179Y and D277L mutations, were found to bind stably to the origin and initiated replication in the absence of HU (158). Thus, it appears that HU stimulates the binding of RepK to the origin (158).

An issue that requires further investigation is whether the initiation and termination activities of the Rep proteins are mutationally separable. While it is likely that some regions of the Rep proteins are required for both the initiation and ter-

mination steps, certain domains may be required for only one of these steps. For example, a particular domain of a Rep protein that interacts with the helicase or other enzymes involved in the initiation and elongation steps may be required only for initiation (and elongation). Similarly, a domain of the Rep protein that interacts with a putative termination protein may be required only for the termination step. So far, there is only a single published study that suggests that the initiation and termination activities of the Rep proteins may be separable. The G148E mutant of the pUB110 RepU (RepH) protein was found to be competent in initiation but was defective in termination (8). In the presence of this mutant, the copy number of pUB110 was elevated and the additional plasmid copies were present as multimers (8). This presumably occurred due to readthrough replication at the regenerated origin (8). The specific step(s) in which the G148E mutant protein is defective remains to be elucidated. To identify the specific molecular events and DNA-protein interactions that occur during the initiation and termination steps, it will be important to attempt to isolate Rep mutants that are defective in each of these steps and investigate their biochemical activities.

TABLE 2. Elements involved in the copy number control of RC plasmids

Plasmid	*cop* product(s)	Mode of action
pT181	Antisense RNAs; 85 and 150 nt	Transcriptional attenuation of Rep mRNA
p353-2	Antisense RNAs; 75 and 250 nt	Transcriptional attenuation of Rep mRNA
pLS1	Antisense RNA, 50 nt	Inhibition of Rep mRNA translation
	CopG repressor; 5.1 kDa	Inhibition of *rep* gene transcription
pVT736-1	Antisense RNA, 76 nt	Inhibition of Rep mRNA translation
pUB110	Antisense RNA	Inhibition of Rep mRNA translation?
	RepU/RepU* complex	Inhibition of *rep* gene transcription?

IN VITRO SYSTEMS FOR REPLICATION OF RC PLASMIDS

The first in vitro replication system for RC plasmids was developed with cell extracts from *S. aureus* for the pT181 plasmid (75). The ability of plasmid pT181 DNA to replicate in extracts made from plasmid-containing cells but not from cells lacking this plasmid showed the involvement of a plasmid-encoded product in pT181 replication (75). Subsequent purification of the pT181 initiator protein, RepC, directly demonstrated its requirement in pT181 replication (87). Also, a lack of a requirement of RNA primers for leading-strand replication and the minimal inhibitory effect of RNA polymerase and DNA gyrase inhibitors on in vitro replication in earlier studies suggested that such plasmids may replicate by an RC mechanism (75, 76, 87). Several in vitro systems have now been developed and used to study the properties of RC plasmids. In addition to the system for the plasmids of the pT181 family (87), these systems include extracts derived from *E. coli* that can replicate the pKYM and pMV158 plasmids (25, 52) and from the cyanobacterium *Synechocystis* strain 6803 for the replication of cyanobacterial plasmids (152, 153). In vitro replication systems have also been developed from *S. aureus* and *Streptococcus pneumoniae* for the replication of plasmid ssDNA (9, 30, 90). In general, the in vitro systems mimic the replication properties of plasmids in vivo, and these systems have provided considerable insight into the mechanism of replication of RC plasmids. In vitro replication of RC plasmids has also been shown to generate ssDNA, as observed in vivo (77, 106, 164, 165). These systems have also been very useful in the study of sequence requirements for initiation and termination of RC replication by using plasmids containing two copies of the origin (164, 165). Also, in vitro studies have demonstrated that sequence-specific, noncovalent interaction between the origin and the Rep proteins is critical for replication (28, 29). Finally, in vitro systems have begun to shed some light on the host proteins involved in RC plasmid replication. The ultimate goal of these studies would be to develop in vitro replication systems with purified proteins, unlike the current systems that utilize crude cell extracts. This not only would enhance our understanding of the proteins that are required for RC replication but also should reveal the specific protein-DNA and protein-protein interactions that occur during the initiation and termination of replication. Another goal would be to identify proteins that are present in the replication initiation complex that assembles at the origin.

HOST PROTEINS INVOLVED IN RC REPLICATION

Although considerable information has accumulated on RC plasmids in recent years, very little is known about the involvement of host proteins in this mode of replication. Replication of the leading strand of pT181 has been shown to require the PcrA helicase and DNA polymerase III (60, 62, 112). The DNA binding protein HU is involved in the replication of the pKYM plasmid in *E. coli* (158). The pKYM DSO contains a core and an adjacent enhancer region. The HU protein binds to the replication enhancer region and facilitates the binding of RepK to the origin (158). Lagging-strand replication of several RC plasmids containing either the sso_A- or sso_U-type origin has been shown to require RNA polymerase for the synthesis of RNA primers (10, 30, 89, 90, 128). In addition, DNA polymerase I is required for lagging-strand replication initiating from the sso_A of plasmid pLS1 in *Streptococcus pneumoniae* (34, 89, 90). Replication from some SSOs appears not to require primer RNA synthesis by RNA polymerase and may require a primosome-type assembly, although this issue needs to be further investigated (128). It is likely that plasmid RC replication also requires the host single-stranded-DNA binding protein, DNA ligase, and DNA gyrase.

REGULATION OF REPLICATION OF RC PLASMIDS

The replication of most well-studied RC plasmids has been shown to be regulated indirectly by controlling the synthesis of their Rep proteins. The Rep proteins encoded by several RC plasmids are known to be rate-limiting for the replication (38, 97, 112). Three general mechanisms have been identified for the copy number control of RC plasmids, namely, transcriptional attenuation of Rep mRNA, inhibition of Rep mRNA translation, and inhibition of *rep* gene transcription (Table 2). The first two of these mechanisms involve an antisense RNA (countertranscript). In plasmids of the pT181 family, p353-2 (pC194/pUB110 family), and pVT736-1, one or more antisense RNAs cause premature termination of Rep mRNA transcription (35, 42, 113, 120). The regulatory antisense RNAs encoded by these plasmids are complementary to the leader

regions of their respective Rep mRNAs, and both of these RNAs can assume extensive secondary structures containing hairpin regions (113). The countertranscripts and the Rep mRNAs initially interact through base pairing between the unpaired regions present in the loop of their hairpin structures. This is presumably followed by the generation of linear duplexes in a zipper-like manner. The pairing of the countertranscripts with the leader region of the Rep mRNAs as they are being transcribed leads to the generation of hairpin structures in the mRNAs that resemble transcription terminators (113). This results in premature termination of transcription of the Rep mRNAs. Since the antisense RNAs are constitutively synthesized in a gene dosage-dependent manner, only a small amount (usually less than 5%) of full-length Rep mRNA is synthesized, resulting in limited levels of the Rep protein (113). Due to sequence and structure conservation, it is likely that many members of the above plasmid families also regulate the synthesis of their Rep proteins by a transcriptional attenuation mechanism.

The copy number of the streptococcal plasmid pLS1 is synergistically regulated by an antisense RNA and a repressor protein (23, 24). The first mechanism involves inhibition of translation of the Rep mRNA by an antisense RNA (24). The antisense RNA encoded by pLS1 is complementary to the 5′ upstream region of the RepB mRNA. It is likely that pairing between these two RNAs masks the ribosome binding site of the RepB mRNA which interferes with the formation of a translation initiation complex (24). Additional studies, including the use of in vitro translation systems, are required to directly demonstrate the translational inhibition model for the regulation of Rep synthesis. The second level of regulation in the case of the pLS1 plasmid involves transcriptional repression of RepB mRNA synthesis by the small, 5.1-kDa CopG protein (23). The CopG protein has been purified and shown to bind to the promoter region of the *repB* gene (23). Other plasmids of the pLS1 family such as pE194, pADB201, and pLB4 can also potentially encode antisense RNAs and repressor proteins, and the synthesis of their Rep proteins may also be regulated in a manner similar to that of the pLS1 plasmid. Host proteins may also be involved in the regulation of replication of RC plasmids. For example, the HU protein appears to regulate the replication of the pKYM plasmid by controlling the synthesis of the initiator protein through its binding to the promoter of the *repK* gene (108).

For plasmid pUB110, preliminary studies suggest that the initiator RepU protein is converted to an inactive RepU/RepU* oligomeric form after one round of replication (103). DNase I footprinting studies have shown that the RepU/RepU* complex binds to an extended region, including the DSO and the upstream region of the *repU* gene, and is able to autoregulate RepU synthesis (103). Thus, autoregulation by the inactivated Rep protein may be another mechanism by which RC plasmids can fine-tune the levels of their initiator proteins. The overexpression of the RepC protein of plasmid pT181 has also been shown to inhibit plasmid replication (61). It has been postulated that this inhibition is due to the formation of inactive RepC-origin complexes. RepC overexpression also decreased chromosome replication, probably due to the sequestering of the PcrA helicase that is required for both pT181 and chromosome replication (61). Thus, while the initiator proteins are normally rate limiting for replication, regulation of replication by the initiators may also occur under conditions in which the Rep proteins are expressed at very high levels.

CONCLUSION AND FUTURE DIRECTIONS

RC plasmids have been extensively studied in recent years. These studies have shown a strong evolutionary relationship between RC plasmids and ssDNA bacteriophages of *E. coli*. While there are many similarities in the replication mechanisms of RC plasmids and ssDNA coliphages, there are significant differences as well. The major difference lies in the tight regulation of replication in the RC plasmids. The RC plasmids have acquired mechanisms that regulate the synthesis of their initiator proteins, which are normally rate limiting for replication. Furthermore, reutilization of the Rep proteins in plasmid replication is prevented by their inactivation. This has not been observed in the case of the initiators of the ssDNA coliphages. Much is now known about the structure-function relationship of RC plasmid initiators. These proteins have well-defined domains that are involved in their sequence-specific DNA binding and nicking-closing activities. Similarly, the DSOs of RC plasmids have a domain for binding the initiators and a region that is required for nicking. The nick region is conserved between the initiators of the same plasmid family, while the variable binding region provides the specificity for replication. The similarity between the initiator proteins and DSOs of RC plasmids found in diverse bacteria as well as members of the *Archaea* suggests a common evolution of RC plasmids. The SSOs of RC plasmids have been characterized, and it appears that the ability of a particular type of SSO to be recognized by the replication proteins and enzymes of various hosts may play an important role in the horizontal transfer of RC plasmids. In spite of the impressive progress that has been made in the study of RC plasmids in recent years, much remains to be done. The composition of the replication initiation complex and the precise role of the initiator protein in this assembly are not well understood. Many host replication proteins required for RC replication remain to be identified. Future studies are needed to identify specific protein-protein and protein-DNA interactions that occur during the initiation and termination of RC replication. Although one mode of inactivation of the Rep proteins by the attachment of an oligonucleotide to their active tyrosine residues has been identified, details of this event have to be investigated. Also, whether this is a common mechanism for the inactivation of RC initiators or whether other inactivation mechanisms are also operational remains to be determined. It is anticipated that future studies, especially those at the biochemical and structural levels (X-ray crystallography) should greatly enhance our understanding of the molecular details of the replication of RC plasmids and its regulation.

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