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DNA Synthesis of Plasmids in *Escherichia coli*

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Introduction

GENERAL FEATURES OF DNA REPLICATION

DNA replication is a key step in duplication for all organisms and its initiation process is precisely regulated. Various biochemical and genetic studies on DNA replication have been done using as model systems phages and plasmids that are parasitic on *Escherichia coli*, and much evidence has been accumulated by investigating the duplication of these replicons (McMacken and Kelly, 1986; Kornberg and Baker, 1991). However, the complete mechanism of DNA replication is not yet known.

A *cis*-acting element that ensures replicon identity and is essential for the initiation of DNA replication is the definition of the replication origin, or *ori*. The specificity of replication initiation depends on the structure of the *ori*, which interacts with *trans*-acting factors such as initiator proteins specific for its replicon. In some replicons, with the aid of *in vitro* reconstituted systems of purified replication factors, the molecular mechanisms of the initiation event have been discovered (Bramhill and Kornberg, 1988a, b; Schnös *et al.*, 1988). Melting and unwinding of the duplex DNA in the *ori* region is done by initiator proteins and DNA helicases in a replicon-specific way. Such unwinding has also been found in the initiation of DNA replication of some eukaryotic replicons (Dean *et al.*, 1987; Umek and Kowalski, 1990). It may be that this process is one of the initiation processes common to almost all the replicons that replicate via Cairns-type intermediate molecules. On the other hand, little is known about the mechanism that introduces priming enzymes onto each template strand and initiates DNA strand synthesis with the concomitant establishment of the replication forks. It is certain that each

Abbreviations: ColE1, Colicin E1; IHF, integration host factor; $P_{RNA\ I}$, promoter for RNA I; $P_{RNA\ II}$, promoter for RNA II; RNase H, ribonuclease H.

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replicon has a unique mechanism to recruit priming enzymes and replication mechanisms.

In *E. coli, oriC*, the origin of chromosome DNA replication, is the site where the *E. coli* initiator protein (DnaA protein) specifically binds in the presence of some essential factors (Bramhill and Kornberg, 1988a; Kornberg, 1988). Through this initial event the initial complex is formed, which is then transformed into an open complex. In the open complex, complementary strands of the AT-rich regions are separated from each other. When the DnaB protein, a helicase, attaches onto a single-strand DNA region, the contiguous duplex is progressively unwound and the primer RNA synthesis is directed.

Initiation at the origins of DNA replication of plasmids requires specific initiator proteins encoded by themselves (Scott, 1984; Kües and Stahl, 1989). In some plasmids, duplexes in the origin regions are specifically opened by binding of the initiator proteins. After the opened areas are extended by helicases, the priming enzymes and enzyme complexes should attach onto the single-strand DNA templates, leading to the primer RNA syntheses.

In the *E. coli* system, three types of specific sequences on the template DNA have been shown to direct initiation of complementary strand DNA synthesis. This was shown by research on small single-strand DNA phages (Kornberg and Baker, 1991). The first type, the primosome assembly site, is found in bacteriophage ϕX174. In this case, synthesis of the complementary strand requires a multi-protein complex, primosome, which contains the *E. coli* proteins DnaB, DnaC, i, n, n′ and n″ in addition to DnaG primase (Masai and Arai, 1988; Masai, Nomura and Arai, 1990; Masai *et al.*, 1990; Nurse *et al.*, 1990; Kornberg and Baker, 1991). The primosome assembles under the direction of the primosome assembly site, a specific sequence on the viral strand of ϕX174. It then moves along the single-strand DNA template in its 5′ to 3′ orientation to direct the synthesis of multiple primers (Schlomai and Kornberg, 1980; Arai and Kornberg, 1981). It is believed that the DnaB protein, which is a DNA helicase, should function in the propulsion of replication forks, and that the primosome should direct the initiation of lagging-strand synthesis during replication of double-strand DNA (LeBowitz and McMacken, 1986; Baker, Funnell and Kornberg, 1987).

The second type of initiation sequence was found in bacteriophage G4. It requires only DnaG primase to synthesize the primer RNA in a defined region (Boucheé, Rowen and Kornberg, 1978; Rowen and Kornberg, 1978). The third type was found in the filamentous bacteriophage M13, which requires only the *E. coli* RNA polymerase to synthesize the primer RNA at a unique site (Schneck *et al.*, 1978).

DEFINITION OF THE SINGLE-STRAND DNA INITIATION SIGNAL

Each phage DNA described above contains a signal nucleotide sequence that is specifically recognized by the cognate enzyme or the enzyme complex and directs the priming for complementary DNA synthesis on the single-strand DNA template. This signal sequence is known as a single-strand DNA

initiation signal, or an *ssi* signal (Ray *et al.*, 1981; Sakai and Godson, 1985). In *E. coli*, a sequence non-specific priming mechanism has also been shown *in vitro* (van der Ende, Teerstra and Weisbeek, 1983; van der Ende *et al.*, 1983; Masai and Arai, 1988), but these non-specific systems are ineffective and insufficient for the normal level of DNA replication.

PLASMID DNA REPLICATION

Many bacterial plasmids of different incompatibility groups also have *ssi* signals in close proximity to the *oriV*s (Nomura and Ray, 1980; Nomura, Low and Ray, 1982; Bahk *et al.*, 1988; Bahk, Sakai and Komano, 1989; Honda *et al.*, 1989; Nomura *et al.*, 1991; Tanaka *et al.*, 1991). The priming mechanisms under the direction of the *ssi* signals of these plasmids include primosome-dependent mechanisms (Nomura and Ray, 1980; Zipursky and Marians, 1981; Nomura, Low and Ray, 1982), ABC-primosome-dependent mechanisms (Masai, Nomura and Arai, 1990) and plasmid-oriented mechanisms in a broad host-range plasmid, RSF1010 (Haring and Scherzinger, 1989; Honda *et al.*, 1989; Scholz *et al.*, 1989). Another broad host-range plasmid, RK2, also has at least two specific *ssi* signals in the *oriT* region (Yakobson *et al.*, 1990). Although some of these *ssi* signals are dispensable *in vivo*, they are essential for maintenance of wild-type copy numbers of the plasmids (van der Ende, Teerstra and Weisbeek, 1983). Furthermore, *in vitro* studies show that the *ssi* signals are also essential for initiation of the synthesis of the DNA strand in plasmids pBR322 and RI (Minden and Marians, 1985; Masai and Arai, 1988, 1989). Judging by the mode of function and the location of *ssi* signals in different plasmids, it is conceivable that these signals contribute to the specificity of plasmid-directed initiation events and to the establishment of the replication forks (Masai *et al.*, 1990; Honda *et al.*, 1991).

Replication of IncQ plasmids

STRUCTURAL AND FUNCTIONAL FEATURES OF THE IncQ PLASMID GENOMES

Broad host-range plasmids of the IncQ group in *Escherichia coli* are relatively small and non-self-transmissible. They are mobilizable by co-resident IncP plasmids (Willetts and Crowther, 1981; Frey *et al.*, 1983). RSF1010, R1162 and R300B are the best studied so far among the IncQ plasmids (Barth, 1979; Barth, Tobin and Sharpe, 1981; Meyer, Hinds and Brasch, 1982). Although these three plasmids have been independently isolated from different bacterial hosts, they are almost identical (Barth and Grinter, 1974; Guerry, Van Embden and Falkow, 1974; Grinter and Barth, 1976; Meyer *et al.*, 1982). They have high copy numbers of 10–12 or more per chromosome in *E. coli* and *Pseudomonas aeruginosa* (Barth and Grinter, 1974; Guerry, Van Embden and Falkow, 1974; Grinter and Barth, 1976; Meyer *et al.*, 1982; Scholz *et al.*, 1985; Lewington and Day, 1986). RSF1010 can propagate in nearly all the Gram-negative bacteria (Frey and Bagdasarian, 1989).

Genomes of plasmids RSF1010, R1162 and R300B are 8·6–8·7 kb in length (Barth and Grinter, 1974; Guerry, Van Embden and Falkow, 1974; Brasch and Meyer, 1986). The complete nucleotide sequence of 8684 bp in length is determined with RSF1010 (Scholz *et al.*, 1989). The RSF1010 genome can be roughly divided into three regions (*see Figure 1A*). In one region are *strA* and *strB* and the *sul* loci, which determine drug resistance against streptomycin and sulphonamide, respectively (Anderson and Lewis, 1965a, b; Anderson, 1968; Barth and Grinter, 1974; Smith, Humphreys and Anderson, 1975). In another region are contained genes essential to replication, *repA*, *repB(repB')*, and *repC*, which encode plasmid-specific helicase, primase, and initiation proteins, respectively (Diaz and Staudenbauer, 1982a; Kim and Meyer, 1985; Scherzinger *et al.*, 1984; Meyer *et al.*, 1985; Scholz *et al.*, 1985). While the RepB and RepB′ proteins are encoded by the same reading frame, the translation start codon of RepB′ is downstream from that of RepB. The 5′-terminal portion of *repC* overlaps with 14 nucleotides of the 3′-terminal part of *repA* (Haring and Scherzinger, 1989; Scholz *et al.*, 1989). The third region contains the *cis*-acting elements *oriV* and *oriT*, which are the origin of vegetative DNA replication and the origin for conjugative transfer of DNA, respectively (Nordheim, Hashimoto-Gato and Timmis, 1980; Meyer, Hinds and Brasch, 1982; Scholz *et al.*, 1985; Brasch and Meyer, 1986; Derbyshire, Hatfull and Willetts, 1987). In addition to *oriT*, the three genes *mobA*, *mobB* and *mobC*, which encode proteins of unknown functions, are required for the RSF1010 transfer.

THE *CIS*-ACTING ELEMENTS IN *oriV*

The three *rep* genes, *repA*, *repB′* and *repC*, and the *oriV* region, are the plasmid-specified factors essential for the vegetative DNA replication of RSF1010 (Diaz and Staudenbauer, 1982a; Scherzinger *et al.*, 1984; Kim and Meyer, 1985; Meyer *et al.*, 1985; Scholz *et al.*, 1985, 1989; Schmidhauser, Ditta and Helinski, 1988; *see Figure 1A*). The *oriV* region of R1162 can be divided into essential portions, which are adjacent 370 bp and 210 bp *Hpa*II fragments (Lin and Meyer, 1986; Kim, Lin and Meyer, 1987), corresponding to nucleotide positions 2181–2550 and 2561–2772 on the RSF1010 map, respectively (Scholz *et al.*, 1989; *see Figure 1A*). In the 370 bp segment there are three-and-a-half direct repeats of 20 bp (Meyer *et al.*, 1985; Scholz *et al.*, 1989); binding of RepC protein to these is essential for the origin function (Haring *et al.*, 1985; Haring and Scherzinger, 1989). The iterons exert incompatibility and are involved in plasmid replication (Lin and Meyer, 1986, 1987; Persson and Nordström, 1986). Although the nucleotide sequence of the iteron is homologous with those of IncP plasmids and the other replicons (Lin and Meyer, 1984; Meyer *et al.*, 1985; Kües and Stahl, 1989), its significance in plasmid evolution is not clear. A G+C-rich and an A+T-rich region flank the iterons in the proximal and distal positions, respectively. It is strongly suggested that the A+T-rich region is essential for the initiation of plasmid DNA replication (Kim and Meyer, 1991). In the 210 bp *Hpa*II segment is a large inverted repeat. The inverted repeat and the potential

cruciform structure may be important for replication initiation (Lin and Meyer, 1987; Scholz *et al.*, 1989). In the large inverted repeat region of RSF1010 are found two loci, *ssiA* and *ssiB*, the two plasmid-specific single-strand DNA initiation signals (Honda, Sakai and Komano, 1988; Haring and Scherzinger, 1989; Zhou and Meyer, 1990; Scherzinger *et al.*, 1991). Determinants of *ssiA* and *ssiB* are *cis*-acting specific nucleotide sequences in the *l*-strand and in the *r*-strand, respectively, directing the priming of their complementary strand syntheses in a RepB′ primase-dependent manner (Haring and Scherzinger, 1989; Honda *et al.*, 1989, 1991). Nucleotide sequences of the two initiation signals contain highly conserved 40 bp tracts which comprise inverted repeats. A start-point of the plasmid DNA synthesis is mapped in the 3′-flanking region of each inverted repeat (Honda, Sakai and Komano, 1988; Scholz *et al.*, 1989). The minimal *oriV* required *in vivo* and *in vitro* for replication initiation of the plasmid in *E. coli* is the 396 bp segment (nucleotide positions 2347–2742) (Haring and Scherzinger, 1989; Scholz *et al.*, 1989; Scherzinger *et al.*, 1991).

INITIATION OF THE PLASMID DNA REPLICATION

It has been shown *in vivo* and *in vitro* that vegetative DNA replication of plasmids RSF1010 and R1162 is dependent on *oriV* and three replication proteins RepA (essentially identical to RepIA of R1162), RepB′ (RepII of R1162), and RepC (RepIB of R1162) (Diaz and Staudenbauer, 1982a; Scherzinger *et al.*, 1984; Kim and Meyer, 1985; Meyer *et al.*, 1985; Scholz *et al.*, 1985, 1989; Schmidhauser, Ditta and Helinski, 1988; Scherzinger *et al.*, 1991; *see Figure 1A*). Vegetative DNA replication of IncQ plasmid initiates at *oriV* and proceeds unidirectionally in either direction, or bidirectionally (de Graff *et al.*, 1978; Scherzinger *et al.*, 1991). Initiation of vegetative DNA replication depends on the specific binding of RepC protein to the iterons in *oriV* (Haring *et al.*, 1985; Haring and Scherzinger, 1989). RepC protein is 31-kDa in its molecular mass, and is active as a dimer (Haring and Scherzinger, 1989). RepIB protein of R1162 (RepC of RSF1010) causes the localized melting of DNA at a site within the A+T-rich region in *oriV* (Kim and Meyer, 1991). It is also suggested that there is a specific interaction between RepIB and a site in the A+T-rich region about 60 bp away from the iterons, which are the primary binding sites of RepIB (Kim and Meyer, 1991). RepA protein, which has a molecular mass of 30 kDa as a monomer, is as hexamer in its active form, and has ATPase activity stimulated by non-specific single-strand DNA (Bagdasarian *et al.*, 1986; Haring and Scherzinger, 1989). RepA acts as a helicase in the unwinding of double-strand DNA in a reaction requiring ATP hydrolysis (Haring and Scherzinger, 1989). RepA acts as a helicase in the unwinding of double-strand DNA in a reaction requiring ATP hydrolysis (Haring and Scherzinger, 1989). RepB′ protein, which exists as monomers of molecular mass of 36 kDa, has primase activity on *ssiA* and *ssiB* as sole template sequences, directing the priming events for their complementary strand synthesis (Honda, Sakai and Komano, 1988; Haring and Scherzinger, 1989; Honda *et al.*, 1989, 1991). Neither the

double-strand *ssiA* and *ssiB* region nor a non-specific single-strand DNA can be used as a template by this enzyme (Haring and Scherzinger, 1989). It is strongly suggested that the RepB′ primase is directly responsible for the initiation of DNA synthesis on both strands of RSF1010, presumably by the synthesis of DNA primers (Haring and Scherzinger, 1989).

The RSF1010 replication is independent of *E. coli* DnaA, DnaB, DnaC and DnaG proteins, and RNA polymerase, while it is dependent on DNA polymerase III and DNA gyrase (Scholz *et al.*, 1985; Haring and Scherzinger, 1989; Scherzinger *et al.*, 1991). R1162 can integratively suppress the *dnaA46* temperature-sensitive mutation of *E. coli* (Brasch and Meyer, 1988). It is conceivable that such plasmid-specified functions described above confer the broad host-range property upon RSF1010 as a result of independence from many host replication proteins.

In the process of initiation of RSF1010 replication, the functions of the three *cis*-acting elements in *oriV*, the iterons, *ssiA* and *ssiB*, are independent of each other (Kim, Lin and Meyer, 1987; Lin and Meyer, 1987; Zhou and Meyer, 1990; Honda *et al.*, 1991, 1992; *see Figure 1B*). Binding of RepC protein to the iterons may alter higher structures of the *oriV* region (Haring and Scherzinger, 1989), leading to localized unwinding at a site in the A+T-rich region (Kim and Meyer, 1991). The melted area can serve as an entry site for RepA helicase followed by extended unwinding of the contiguous double-strand region in one direction away from the iterons until the nucleotide sequences of *ssiA* and *ssiB* are exposed as single strands (Zhou and Meyer, 1990; Honda *et al.*, 1991; Scherzinger *et al.*, 1991). The single-strand *ssiA* and *ssiB* sites are ready for specific recognition and priming of syntheses of the complementary single strands by RepB′ primase (Honda, Sakai and Komano, 1988; Haring and Scherzinger, 1989; Honda *et al.*, 1989). Formation of plasmid-specific replisomes during these processes would decide whether the plasmid replication proceeds unidirectionally or bidirectionally (Diaz and Staudenbauer, 1982a; Haring *et al.*, 1985; Bagdasarian *et al.*, 1986; Lin and Meyer, 1986; Scherzinger *et al.*, 1991). It can be assumed that the plasmid-specific replisome involves RepA, RepB′, and RepC. The bidirectional DNA replication of RSF1010 is significantly interpreted by the presence in *oriV* of *ssiA* and *ssiB*, each of which contains a start-point of DNA synthesis in opposite orientations (Lin and Meyer, 1987; Honda, Sakai and Komano, 1988; Zhou and Meyer, 1990). However, it is not clear yet what mechanism and which critical stage in the initiation processes are responsible for making a decision as to whether the RSF1010 replication proceeds unidirectionally or bidirectionally. With R1162, DNA chain elongation during the replication initiation starts from two sites, *ssiA*- and *ssiB*-analogues of R1162, and proceeds convergently (Lin and Meyer, 1987; Zhou and Meyer, 1990). One or both of *ssiA* and *ssiB* can be functionally replaced by primosome assembly sites from a plasmid and bacteriophage ϕX174, indicating that they are independent *cis*-acting elements (Honda *et al.*, 1991). They can be also functionally replaced by G4-type *ssi* signals, which, unlike primosome assembly sites, direct priming of leading-strand synthesis at a unique site (Honda *et al.*, 1992). This suggests that the RSF1010 DNA

replication proceeds in a strand displacement mode without involvement of an efficient mechanism of concomitant initiation of lagging-strand synthesis (Zhou and Meyer, 1990).

In addition to the above model interpreting the mechanism of replication initiation of RSF1010, another suggestion involves an important role for the large inverted repeat in *oriV*. After the RepC protein specifically binds to the iterons it may migrate to the large inverted repeat accompanied by the action of RepA protein, where a protein complex may be formed by assembly of RepA, RepB′ and RepC proteins (Lin and Meyer, 1986). After the recognition by the protein complex of the cruciform structure in the large inverted repeat region, or after the protein complex-mediated association of the two start-points of DNA synthesis by looping the large inverted repeat region, priming is done by RepB′ followed by initiation of the DNA synthesis with the replisome (Lin and Meyer, 1987).

CONTROL OF THE PLASMID REPLICATION

The *oriV* region of IncQ plasmids contains some elements involved in regulation of plasmid replication (Lin and Meyer, 1984). When the 20 bp iterons are cloned in a multi-copy vector, they are incompatible with IncQ plasmids because of titration of the RepC protein that binds to the iterons (Meyer *et al.*, 1985; Persson and Nordström, 1986). The intracellular amount of RepC protein affects the copy number of RSF1010 (Haring *et al.*, 1985; Frey and Bagdasarian, 1989). The RSF1010 promoter *p*1 is responsible for the transcription of *repB, repB′, repA* and *repC*, while the promoter *p*4 is for *repA* and *repC* (Bagdasarian *et al.*, 1986; Scholz *et al.*, 1989; *see Figure 1B*). Putative repressors are encoded immediately downstream of promoters *p*1 and *p*4, negatively regulating the action of the two promoters (Bagdasarian *et al.*, 1986; Frey and Bagdasarian, 1989). Consequently, deletions of the open reading frames for the putative repressors greatly elevates copy numbers in *E. coli*. Recently, the repressor protein for *p*4, the 7·7 kDa product of gene F, has been purified, and the gene product is demonstrated to regulate *p*4-directed expression of the *repAC* operon (Maeser *et al.*, 1990). These alterations in transcriptional regulation affect the host-range of RSF1010. All the copy number mutants carrying deletions in the repressor for *p*1 promoter lose the broad host-range property. They can propagate and stably maintain themselves in *E. coli*, but not in *Pseudomonas* species. The results are directly caused by the damage to the open reading frame encoding the repressor of *p*1. With the copy number mutants carrying deletions in the repressor of *p*4 promoter, elevation in the copy numbers in *Pseudomonas putida* is not so prominent as in *E. coli* (Frey and Bagdasarian, 1989). The two promoters are involved in the copy number control, and their physiological roles seem distinct from each other. Moreover, production of RepC protein, which is a primary limiting factor of RSF1010 replication (Haring *et al.*, 1985), is regulated in accordance with *repA* expression. Messenger RNA of the region where *repA* and *repC* overlap may form a stem-loop structure enclosing the ribosome binding site and start codon (GUG) of *repC*. Thus, it is suggested

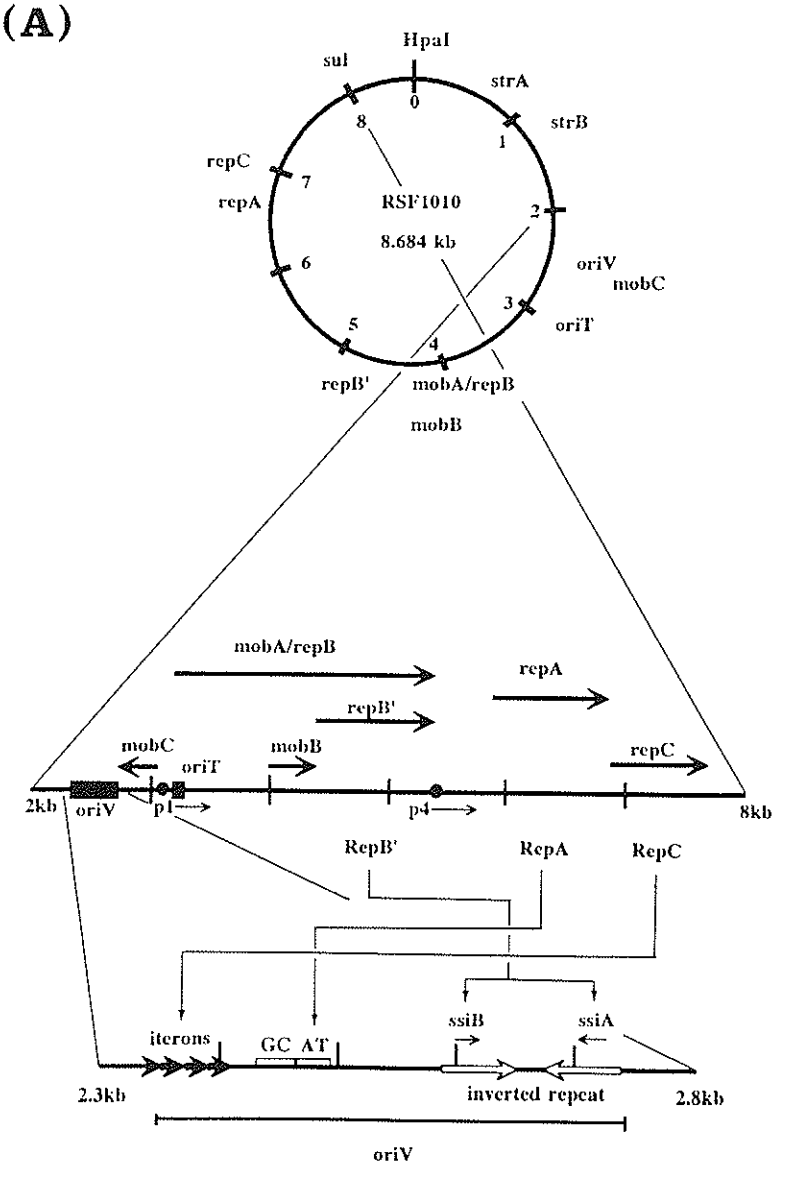

Figure 1. (A) Physical maps of plasmid RSF1010. The first A of the nucleotide sequence in the unique *HpaI* cleavage site is designated as the nucleotide number 1. The map coordinates are calibrated in kilobase pairs (kb). Thick solid arrows represent transcripts. Thin horizontal arrows with signs of p1 and p4 represent orientations of transcription under the control of promoters for the *rep* operons, *p*1 and *p*4, respectively. Positions of the promoters are indicated by solid circles. Thin horizontal arrows labelled ssiA and ssiB represent orientations of the initiation of DNA chain elongation under the control of *ssi* signals. Thick open arrows represent the large inverted repeat. The thick solid arrowheads represent iterons. Open boxes labelled GC and AT represent the G+C-rich and A+T-rich regions, respectively. Thin vertical arrows represent the action in *trans* of RepA, RepB' and RepC. Solid boxes represent the *oriV*- and *oriT*-determinants. The horizontal bar labelled *oriV* represents the area of *oriV*-determinant.

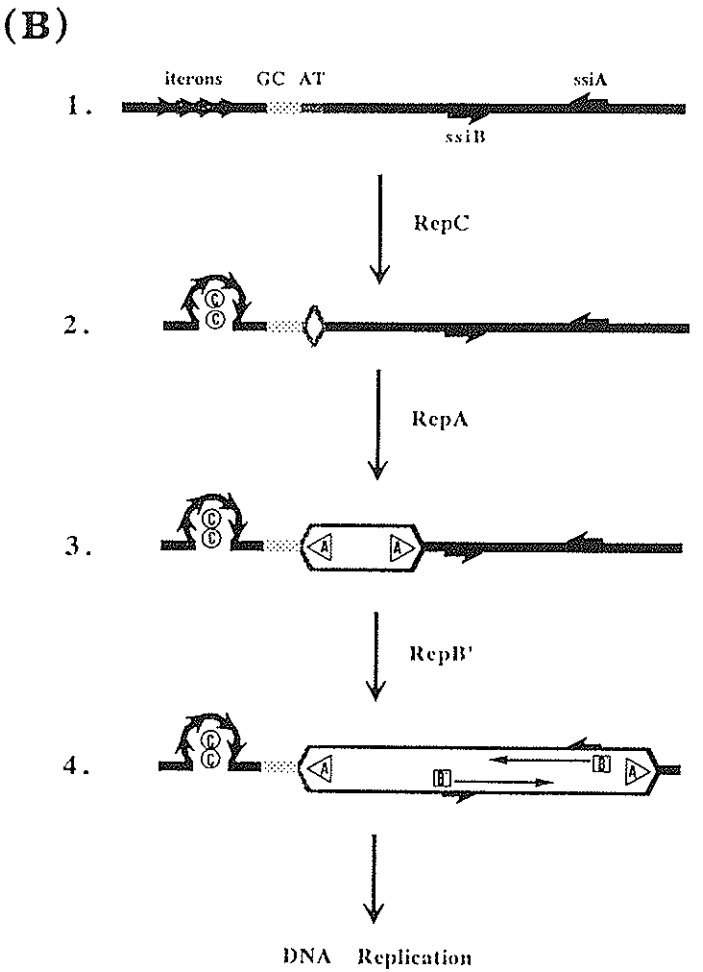

Figure 1. (B) Schematic representation of the events occurring in the early stages of initiation of DNA replication in the IncQ plasmids. (1) The duplex of the *oriV* region. (2) The RepC protein, the plasmid-specific initiator protein, binds specifically to the iterons, causing localized unwinding of the duplex in the A+T-rich region. (3) The locally melted region could serve as an entry site for the RepA protein, the plasmid-encoded helicase, the action of which unwinds the duplex in the flanking region. (4) As a result of the helicase action, regions of the two *ssi* signals are activated by being exposed as single strands. The activated *ssi* signals can be recognized by the RepB′ protein, the plasmid-specific primase, leading to the priming of syntheses of the two complementary DNA strands. Chain elongation of the two daughter strands progresses convergently. Solid arrowheads represent iterons. The stippled areas labelled GC and AT represent the G+C- and A+T-rich regions, respectively. Solid half-arrows labelled ssiA and ssiB represent determinants of the *ssi* signals. Thin horizontal arrows represent orientations of the daughter strand syntheses under the control of the *ssi* signals. The letter C in a circle, the letter A in a triangle and the letter B′ in a square represent the RepC, RepA and RepB′ proteins, respectively.

that, in addition to the transcription regulation, efficient translation of *repC* depends on the level of *repA* expression (Haring and Scherzinger, 1989). With R1162, the plasmid copy number depends on the amounts of two proteins encoded by the *repI* genes (Kim and Meyer, 1985). It has been demonstrated that expression of the *repI* genes is negatively regulated by a 75 nucleotide RNA complementary to the *repI* mRNA (Kim and Meyer, 1986).

CONJUGATIVE TRANSFER TO AND PROPAGATION IN UNCOMMON HOSTS

RSF1010 is not self-transmissible, but is mobilizable by co-resident conjugative plasmids. The four RSF1010-specified genes *mobA, mobB, mobC* and *oriT* are required for the transfer (Willetts and Crowther, 1981; Frey *et al.*, 1983). The two genes *mobA* and *repB* are identical to each other (Scholz *et al.*, 1989). Although *mobB* overlaps with *mobA*, it is translated in a reading frame distinct from that of *mobA* (Scholz *et al.*, 1989). The *oriT* region is localized to an 80–88 bp segment from the 800 bp *Hae*II fragment of RSF1010, containing the relaxation nick site and an inverted repeat probably involved in recognition and/or nicking by the mobilization proteins. The nucleotide sequence of *oriT* is homologous with those of plasmids ColE1 and RK2 (Nordheim, Hashimoto-Goto and Timmis, 1980; Derbyshire, Hatfull and Willetts, 1987). Moreover, it is suggested that *oriT* is involved in site-specific cross-over and ligation of the transferred plasmid strand (Meyer, 1989; Barlett, Erickson and Meyer, 1990). The *oriT* region of R1162 is localized to a 38 bp segment that contains an inverted repeat. The segment is involved in mobilization and conjugation-dependent recombination (Brasch and Meyer, 1986, 1987).

The host-range of IncQ plasmids extends beyond purple bacteria to cyanobacterium (Schmidhauser, Ditta and Helinski, 1988; Powell, Mergeay and Christofi, 1989). An IncQ plasmid can be conjugatively transferred to cyanobacterium from *E. coli*, and stably maintained as an autonomously replicating multicopy plasmid without structural alterations (Kreps *et al.*, 1990). RSF1010 can be conjugatively transferred from *E. coli* to Gram-positive bacteria *Streptomyces lividans* and *Mycobacterium smegmatis*. The plasmid is stably maintained in these bacterial cells (Gormley and Davies, 1991). Moreover, the *mob* and *oriT* functions of RSF1010 can mediate the plasmid transfer from *Agrobacterium* into plant cells (Buchanan-Wollaston, Passiatore and Cannon, 1987).

Replication of IncP plasmids

STRUCTURAL AND FUNCTIONAL FEATURES OF THE IncPα PLASMID GENOME

Broad host-range plasmids belonging to IncP in *Escherichia coli* are self-transmissible, and can be divided into two major subgroups, IncPα and IncPβ (Villarroel *et al.*, 1983; Yakobson and Guiney, 1983; Chikami *et al.*, 1985; Lanka *et al.*, 1985; Smith and Thomas, 1987, 1989; Thomas and Smith, 1987).

The IncPα plasmids RK2 and RP4 have been extensively studied. The plasmids RK2 and RP4, and other IncPα plasmids, RP1, R18 and R68, are indistinguishable from one another (Burkardt, Riess and Pühler, 1979; Currier and Morgan, 1981; Stockes, Moore and Krishnapillai, 1981).

The genome DNA of plasmid RK2 of 60 kb in length (Lanka, Lurz and Fürste, 1983; Thomas *et al.*, 1984; *see Figure 2*). Its copy number is five to seven per chromosome in *E. coli*, and three in *Pseudomonas* (Grinter, 1984; Itoh *et al.*, 1984). Determinants for resistance against ampicillin, kanamycin, and tetracycline are on the plasmid genome. Moreover, there is a cryptic resistance determinant against tellurite (Hedges and Jacob, 1974; Waters *et al.*, 1983; Pansegrau *et al.*, 1987). The *inc* and *cop* loci are involved in incompatibility and copy number control (Meyer, Hinds and Brasch, 1982; Thomas *et al.*, 1984; Thomas, 1986). The *kil* genes are determinants of the inhibition of host functions, with lethal effects on the host bacterial cells (Bechhofer and Figurski, 1983; Smith and Thomas, 1983; Smith, Shingler and Thomas, 1984; Young, Burlage and Figurski, 1987; Thomas, 1988; Goncharoff *et al.*, 1991). The *kor* genes suppress the effects of *kil* genes (Young, Bechhofer and Figurski, 1984; Bechhofer *et al.*, 1986; Young, Burlage and Figurski, 1987). The *trfA* genes encode *trans*-acting factors essential for the initiation of vegetative DNA replication of RK2 (Figurski and Helinski, 1979; Pohlman and Figurski, 1983; Thomas and Hussain, 1984). The gene products of *trfB*, as well as that of *korB*, repress the expression of *trfA* (Tardif and Grant, 1983; Thomas and Hussain, 1984; Young, Bechhofer and Figurski, 1984; Bechhofer *et al.*, 1986; Thomas, 1986; Young, Burlage and Figurski, 1987). The *oriV* region contains the origin of vegetative DNA replication of RK2 (Meyer and Helinski, 1977; Firshein and Caro, 1984; Thomas *et al.*, 1984). Genes involved in the conjugative transfer of IncPα plasmids belong to the three blocks of transfer loci, Tra1, Tra2 and Tra3 (Figurski *et al.*, 1976; Barth, Grinter and Bradley, 1978). The *oriT* region is a proposed origin of DNA transfer. The plasmid-specific primase is encoded by *pri*. The *fiwA* and *fiwB* loci are determinants of fertility inhibition of IncW plasmids (Goncharoff *et al.*, 1991; Yusoff and Stanisich, 1984).

CIS-ACTING ELEMENTS FOR VEGETATIVE DNA REPLICATION: THE ORIGIN FOR VEGETATIVE DNA REPLICATION (*oriV*)

Although two regions on the RK2 genome, *oriV* and *trfA*, are essential for the plasmid replication (Thomas, 1981), regulator genes are necessary for stable maintenance of the plasmid (Schwab, Saurugger and Lafferty, 1983; Schmidhauser and Helinski, 1985; *see Figure 2*). Unidirectional DNA replication is initiated at *oriV*, which is between the coordinates of 12 and 13 kb, and proceeds counterclockwise on the RK2 physical map (Meyer and Helinski, 1977; Firshein and Caro, 1984; Thomas *et al.*, 1984; Pinkney and Thomas, 1987). The DNA segment required in *cis* for the initiation of DNA replication is contained in a 750 bp *Hae*II fragment (Figurski and Helinski, 1979; Thomas *et al.*, 1979). This origin is further reduced in size to the 617 bp *oriV* region required for the replication in *Pseudomonas* species (Schmidhauser and

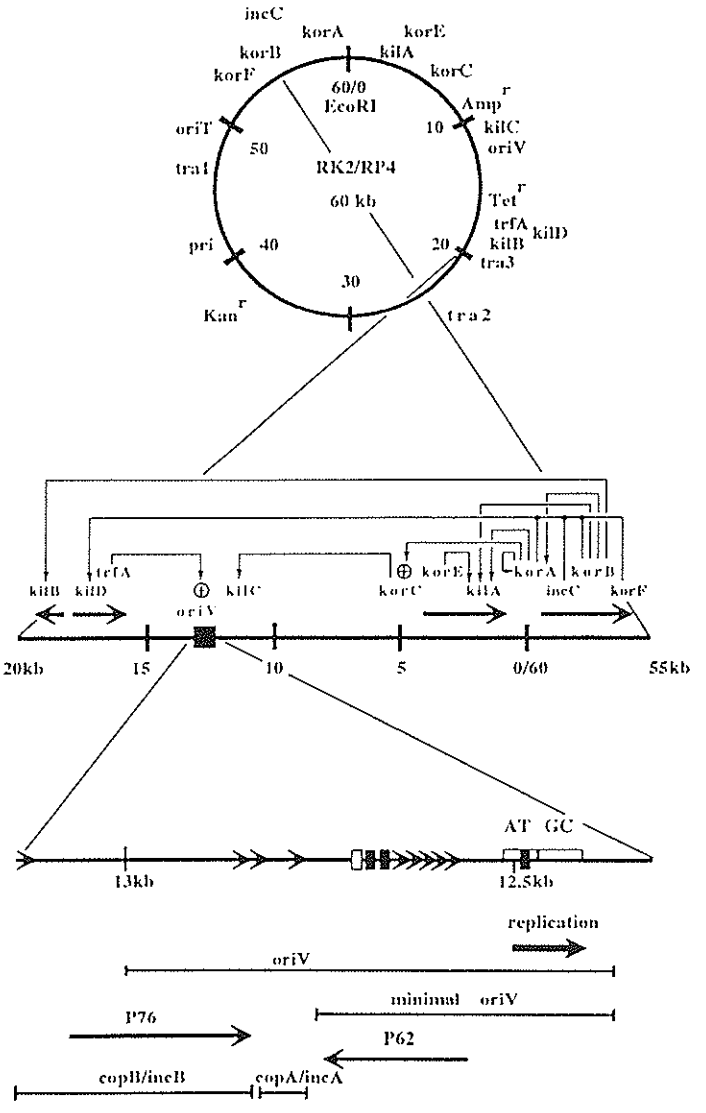

Figure 2. Physical maps of the IncPα plasmids. The map coordinates in kb are calibrated from the unique *EcoRI* site. Bold horizontal arrows represent transcripts. P76 and P62 indicate putative polypeptides encoded in the *oriV* region. The bold arrow labelled 'replication' represents the orientation of DNA replication. Thin vertical arrows represent repressive action in *trans* of the genes responsible for the plasmid maintenance and propagation. Thin vertical arrows labelled + represent positive actions in *trans*. A solid square represents the *oriV* determinant. Solid rectangular boxes represent *dnaA* boxes. An open rectangular box represents the region of an IHF binding site-like sequence. Arrowheads represent the iterons. The horizontal open boxes labelled AT and GC represent A+T- and G+C-rich regions, respectively. Horizontal bars labelled oriV, minimal oriV, copA/incA and copB/incA represent areas of determinants for *oriV*, minimal *oriV*, *copA/incA* and *copB/incB*, respectively.

Helinski, 1985). Eight 17 bp iterons (repeats) in the *oriV* region are divided into two clusters containing five and three iterons, which are about 120 bp apart from each other (Stalker, Thomas and Helinski, 1981; Smith and Thomas, 1985). Nucleotide sequences of these iterons are highly conserved and in a single orientation. The presence of direct repeats in replication origins is a characteristic structural feature seen in both narrow host-range and broad host-range plasmids of Gram-negative bacteria. Between the two clusters are two DnaA box homologous sequences (Stalker, Thomas and Helinski, 1981; Gaylo, Turjman and Bastia, 1987). Moreover, in each terminal part of the 617 bp *oriV* region there is a DnaA box homologous sequence (Pinkney *et al.*, 1988). A Pribnow box is present between the two central DnaA box (Stalker, Thomas and Helinski, 1981; Stalker, Kolter and Helinski, 1982). An IHF binding site-like sequence (a binding site for the integration host factor containing a highly AT-rich region, cf. *Figure 5*) is found in the central region of *oriV* (Stalker, Thomas and Helinski, 1981; Stalker, Kolter and Helinski, 1982; Kües and Stahl, 1989). A 49 bp A+T-rich region, which is flanked by a 67 bp G+C-rich tract, contains the right-most DnaA box (Smith and Thomas, 1985; Gaylo, Turjman and Bastia, 1987).

Some elements in *oriV* are non-essential, depending on the species of the host bacteria. The 617 bp *oriV* region for the replication in *Pseudomonas* species is further reduced in size to the 393 bp *Hpa*II fragment, a variant *oriV*, as a minimal *cis*-acting DNA region required for the initiation of RK2 DNA replication in *E. coli* (Stalker, Thomas and Helinski, 1981; Thomas, Stalker and Helinski, 1981; Schmidhauser, Filutowicz and Helinski, 1983; Cross, Warne and Thomas, 1986; *see Figure 2*). This minimal *oriV* lacks the cluster of three iterons. The plasmid DNA replication dependent on the minimal *oriV* results in higher copy numbers than usual (Thomas *et al.*, 1984). This is because the minimal *oriV* lacks targets for the *cop* functions acting in *trans*. Dependency of the RK2 replication in *E. coli* on DnaA has been confirmed both *in vivo* and *in vitro* (Gaylo, Turjman and Bastia, 1987; Pinkney *et al.*, 1988). Although disruption of the DnaA box in the A+T-rich region abolishes the plasmid replication, binding of DnaA protein at this site may not be essential for the replication (Gaylo, Turjman and Bastia, 1987).

Two ORFs in the *oriV* region encode two putative polypeptides, P62 and a part of P76 (Thomas *et al.*, 1984; *see Figure 2*). In the region upstream from the ORF encoding P76, there are a Shine–Dalgarno sequence and the two functional sequences conserved in prokaryotic promoters, –10 sequence and –35 sequence. These regulatory elements are outside the 750 bp *Hae*II segment. The –35 sequence overlaps with an extra 17 bp iteron outside the *oriV* region, and is thought to play a regulatory role. P76 may be associated with the *copB* function.

INITIATION OF VEGETATIVE DNA REPLICATION OF RK2

Action in *trans* of the initiation protein TrfA on *oriV* is essential for RK2 DNA replication (Figurski and Helinski, 1979; Thomas, 1981; Shingler and Thomas, 1984a; *see Figure 2*). Essential forms of the *trfA* products are

different according to the species of host bacteria (Kornacki, West and Firshein, 1984; Shingler and Thomas, 1984a, b; Smith and Thomas, 1984; Durland and Helinski, 1987). Transposon integration into the *trfA* gene and its promoter results in inhibition of the RK2 replication in *E. coli*, while replication of the plasmid derivatives in *Acinetobacter calcoaceticus, Rhizobium meliloti* and *Pseudomonas* species is not affected (Cowan and Krishnapillai, 1982; Krishnapillai, 1986; Krishnapillai *et al.*, 1987). Moreover, some *trfA* mutants of RK2 have temperature sensitivity of the plasmid replication in *E. coli*, but not in *Pseudomonas aeruginosa* or *Rhizobium meliloti* (Hooykaas, Dulk-Ras and Schilperoort, 1982; Tsuda, Harayama and Iino, 1984; Thomas and Smith, 1987). The other temperature-sensitive mutations introduced into *trfA* result in distinct types of alteration in the plasmid propagation (Valla *et al.*, 1991).

An operon containing *trfA* consists of *trfA/kilD* (*Figure 2*) and is designated the *trfA* operon. The *trfA* operon encodes three distinct polypeptides. One of these is a regulatory protein, KilD (Smith and Thomas, 1983); the other two are TrfA-43 (43 kDa) and TrfA-32 (32 kDa), which are products of the same reading frame (Kornacki, West and Firshein, 1984; Shingler and Thomas, 1984a, b; Smith and Thomas, 1984; Durland and Helinski, 1987). The translation start codon of TrfA-32 is downstream from that of TrfA-43. The in-frame overlapping of the *trfA* gene is important for copy number control, the broad host-range property, and stability in various bacterial hosts (Fang and Helinski, 1991), and expression of the *trfA* operon is negatively regulated by *korA*, *korB*, *korF* and *incC* in the *trfB* operon (Tardif and Grant, 1983; Smith and Thomas, 1984; Young, Bechhofer and Figurski, 1984; Bechhofer *et al.*, 1986; Thomas, 1986; Thomas and Smith, 1986; Kornacki, Balderes and Figurski, 1987; Theophilus and Thomas, 1987; Young, Burlage and Figurski, 1987; Ayres *et al.*, 1991). Intracellular levels of the four *kil* determinants (KilA, KilB, KilC, and KilD) are also regulated by *kor* (Young, Bechhofer and Figurski, 1984; Bechhofer *et al.*, 1986; Young, Burlage and Figurski, 1987). The broad host-range property of RK2 is achieved by the controlled expression of the *trfA* operon through the regulation network (*kil–kor* regulon) which consists of the five *kor* determinants (KorA, KorB, KorC, KorE and KorF) and the four *kil* determinants (KilA, KilB, KilC and KilD) (Figurski *et al.*, 1982; Bechhofer and Figurski, 1983; Smith and Thomas, 1983; Smith, Shingler and Thomas, 1984; Young, Burlage and Figurski, 1987; Thomas, 1988; *see Figure 2*). Modification or deletion of these regulator genes causes instability of the plasmid or changes in the host range (Thomas, Hussain and Smith, 1982; Thomas, 1983; Barth *et al.*, 1984; Schmidhauser and Helinski, 1985; Schreiner *et al.*, 1985; Theophilus *et al.*, 1985). TrfA-32 is essential for the RK2 replication in *E. coli, Pseudomonas putida, R. meliloti, Agrobacterium tumefaciens* and *Azotobacter vinelandii* (Kornacki, West and Firshein, 1984; Shingler and Thomas, 1984a; Durland and Helinski, 1987). In *P. aeruginosa* TrfA-43 is also essential for plasmid replication (Durland and Helinski, 1987). As suggested from the fact that amino acid sequences of TrfA-43 and TrfA-32 are homologous with those of double-stranded DNA binding protein, the two proteins specifically bind to

the 17 bp iterons in *oriV* (Smith, Shingler and Thomas, 1984; Krishnapillai, 1986; Durland and Helinski, 1987; Smith and Thomas, 1987; Pinkney *et al.*, 1988; Perri, Helinski and Toukdarian, 1991). As with the other plasmid replicons, a complex consisting of TrfA-43, TrfA-32 and replication proteins of the host bacterium is thought to bind to the *oriV* region, forming a replisome. However, it is not clear whether all the replisomes constructed for the RK2 replication in various Gram-negative bacteria are equivalent to one another. It is quite probable that distinct replisomes are formed depending on the species of the host bacteria, as suggested by the findings that TrfA-43 is essential for the RK2 replication in *P. aeruginosa* and that the DNA region necessary for functional *oriV*s varies according to the species of host bacteria (Shingler and Thomas, 1989).

In *E. coli*, DnaA protein, DnaB protein, DnaG primase, DNA gyrase, and DNA polymerase III are essential for the DNA replication of RK2, while DNA polymerase I is not essential (Thomas, Meyer and Helinski, 1980; Thomas, Stalker and Helinski, 1981; Gaylo, Turjman and Bastia, 1987; Pinkney *et al.*, 1988; Kostyal *et al.*, 1989). Binding of *E. coli* DnaA protein to the *oriV* region of RK2 is observed *in vivo* and *in vitro* (Gaylo, Turjman and Bastia, 1987; Pinkney *et al.*, 1988). *In vitro* studies suggest that there are two distinct mechanisms for the initiation of RK2 DNA replication. One is dependent on the *E. coli* RNA polymerase, and the other independent of the enzyme (Firshein *et al.*, 1982; Firshein and Caro, 1984; Kornacki and Firshein, 1986; Pinkney *et al.*, 1987; Thomas and Smith, 1987). During initiation of replication, simultaneous transcription of the genes of *trfA* and *trfB* operons may be necessary for DNA replication (Firshein *et al.*, 1982; Kornacki and Firshein, 1986).

CONTROL OF COPY NUMBER

The intracellular copy number of the RK2 plasmid is regulated by the level of expression of *trfA* (Durland *et al.*, 1990). The *incC* gene, which represses the expression of *trfA*, consequently has a negative effect on the copy numbers (Thomas and Helinski, 1989; *see Figure 2*). Some mutations in the *trfA* gene increase the copy number of the plasmid (Durland *et al.*, 1990). Since the expression of *trfA* is negatively regulated by KorB, mini-RK2 plasmids retaining *korB* have copy numbers equivalent to that of RK2, while copy numbers of those lacking *korB* are elevated to 10–11 (Thomas and Hussain, 1984). When the level of *trfA* expression is elevated under the control of a KorB-independent promoter, the repression by KorB is overcome and the plasmid copy numbers increase (Schreiner *et al.*, 1985). Under the physiological conditions in which the intracellular concentration of TrfA is not a limiting factor, the plasmid replication is regulated by *copA/incA* and *copB/incB* loci flanking the minimal *oriV* region. The *copA/incA* and *copB/incB* loci contain a 17 bp iteron and the cluster of three 17 bp iterons, respectively (*Figure 2*). Upon removal of these two elements, the plasmid copy number rises to 35–40 (Thomas *et al.*, 1984). It is supposed that titration of TrfA is not the sole function of *copA* and *copB* (Thomas and Hussain,

1984). Moreover, another copy number control mechanism is suggested, which is functional through origin coupling mediated by the TrfA protein bound to the iterons in *oriV* (Kittel and Helinski, 1991).

DNA SYNTHESIS AND ITS REGULATION IN CONJUGATIVE TRANSFER OF THE IncPα PLASMIDS

Plasmids of the IncP group mediate efficient DNA transfer between almost all the Gram-negative species (Guiney and Lanka, 1989). The conjugation system of RK2/RP4 belongs to IncPα. Three regions, Tra1, Tra2 and Tra3, identified on the plasmid genomes, are essential to the conjugative transfer (Figurski *et al.*, 1976; Barth and Grinter, 1977; Barth, Grinter and Bradley, 1978; Barth, 1979; Guiney and Yakobson, 1983; Lanka, Lurz and Fürste, 1983; Ubben and Schmitt, 1986; Guiney, Deiss and Simnad, 1988; Fürste *et al.*, 1989; Pansegrau *et al.*, 1990; *see Figure 2*). The origin of transfer, *oriT*, is included in a 250 bp segment that is at one end of Tral (Guiney and Helinski, 1979; Guiney, Deiss and Simnad, 1988). The *oriT* region contains characteristic 9 bp inverted repeats, partial destruction of which results in loss of the transfer function (Guiney and Yakobson, 1983; Guiney, 1984). With the plasmid RP4, the process of conjugative transfer is initiated by formation at *oriT* of a protein complex (relaxosome) involving the plasmid-encoded proteins TraH, TraI, and TraJ, followed by cleavage of the DNA strand to be transferred (Barth, 1979; Guiney, Deiss and Simnad, 1988; Fürste *et al.*, 1989; Pansegrau *et al.*, 1990). The cleavage occurs at the unique site (*nic*) in the *oriT* region, and transfer proceeds counterclockwise on the RK2 map (Grinter, 1981; Al-Doori, Watson and Scaife, 1982). The presence of the *nic* site and the inverted repeat in the *oriT* region is essential for these initiating events to be done (Guiney and Helinski, 1979; Guiney and Yakobson, 1983; Guiney, Deiss and Simnad, 1988).

The RK2 plasmid-specified primase is identified as one of the gene products essential for effective DNA transfer (Boulnois and Wilkins, 1979; Lanka *et al.*, 1979, 1984; Lanka and Barth, 1981). In both the complementary DNA strands of the *oriT* region there are some nucleotide sequences specifically recognized by the plasmid-encoded primase. The nucleotide sequences direct the priming of their complementary strand syntheses (Yakobson *et al.*, 1990). These observations suggest that the initiation of DNA syntheses is mediated by the plasmid-encoded primase in the donor and the recipient cells during the process of conjugal transfer of plasmid RK2. A nucleotide sequence in the RK2 *oriT* region is highly homologous with those in both the border junctions of the T-DNA of *Agrobacterium tumefaciens* tumour-inducing plasmids (Waters *et al.*, 1991). This suggests a relationship between conjugative transfer of the plasmid DNA with the IncPα system and T-DNA transfer to plants.

Structural feature of the replication origin region of Colicin E1 (ColE1) DNA and requirements of host factors

ColE1 is a narrow host-range *Escherichia coli* plasmid having a double-stranded circular structure and a molecular size of 6·6 kb, and replicates from a replication origin (*ori*) region unidirectionally (Bolivar *et al.*, 1977; Tomizawa, Ohmori and Bird, 1977). *In vivo* and *in vitro* studies of replication of ColE1 DNA show that *E. coli* factors, ribonuclease H (RNase H), DNA polymerase I, DNA gyrase, and topoisomerase are required (Tomizawa, Ohmori and Bird, 1977; Donoghue and Sharp, 1978; Itoh and Tomizawa, 1980; Hillenbrand and Staudenbauer, 1982; Minden and Marians, 1985). *Figure 3* shows the structural feature of origin region of ColE1. Two promoters are found upstream of the origin: one ($P_{RNA\ II}$) is 555 bp upstream, and the other ($P_{RNA\ I}$) is on the opposite strand at the 445 bp upstream from the origin. The first promoter is for the transcription of RNA II or precursor of primer RNA, and the second one is for RNA I or a regulator RNA, which participates the regulation of initiation of ColE1 DNA replication by forming an RNA II–RNA I hybrid (Morita and Oka, 1979; Itoh and Tomizawa, 1980).

An n′ recognition site, which is known to form a preprimosome (Kornberg and Baker, 1991), is found 150 nucleotides downstream from the replication origin (Zipursky and Marians, 1980; Böldicke *et al.*, 1981; Nomura, Low and Ray, 1982; Masai and Arai, 1988). The DnaA box is also found adjacent to the replication origin (Fuller, Funnell and Kornberg, 1984; Seufert and Messer, 1987; Seufert *et al.*, 1988). Replication of ColE1 DNA can start *in vivo* (Kogoma, 1984; Ogawa and Okazaki, 1984; Naito and Uchida, 1986) and *in vitro* (Casgupta, Masukata and Tomizawa, 1987; Masukata, Dasgupta and Tomizawa, 1987) in the absence of RNase H and DNA polymerase I.

SYNTHESIS OF PRIMER RNA IN INITIATION OF ColE1 DNA REPLICATION

Transcription of RNA II starts 555 bp upstream from the replication origin and ends heterogeneously about 240 bp downstream. The RNA transcript is nearly 700 bp long (Itoh and Tomizawa, 1980; Tomizawa *et al.*, 1981; Tomizawa and Masukata, 1987). This RNA transcript is called a precursor of primer RNA, or RNA II. The 3′-ends of approximately half of the nascent RNA II transcripts form a persistent DNA–RNA hybrid with template DNA near the replication origin (Itoh and Tomizawa, 1980; Selzer and Tomizawa, 1982; *see Figure 3A(1)*). In RNA II forming a persistent hybrid, the region about 265 nucleotide upstream of the replication origin can also form a persistent hybrid with the template DNA. This process is called coupling (Tomizawa and Itoh, 1982), and is dependent on the secondary structure of the 5′-end of RNA II (Masukata and Tomizawa, 1984, 1986; Wong and Polisky, 1985). The 5′-end of the transcribed RNA II molecule is released sequentially from the template DNA to form a unique secondary structure (Masukata and Tomizawa, 1984; *see Figure 3A(2)* and *Figure 3B*). The RNA II transcripts hybridized with template DNA (the coupled RNA II–

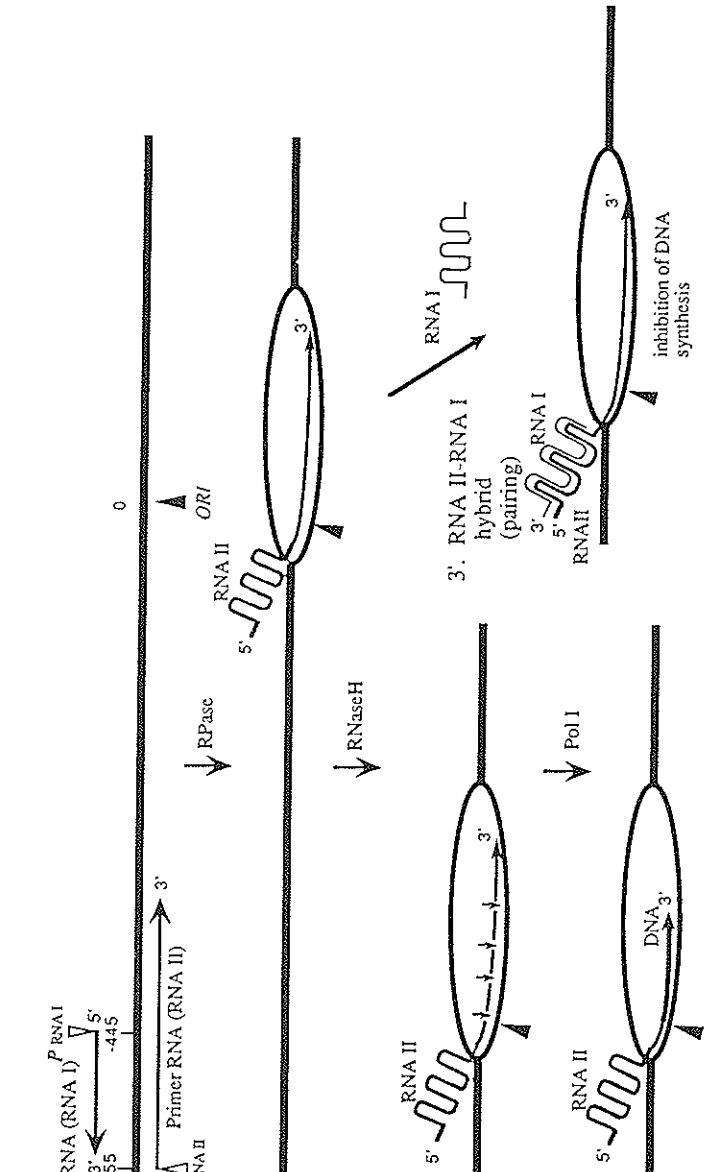

Figure 3. (A) Schematic illustration of the replication origin of plasmid ColE1 and the process of initiation of DNA synthesis. (1) Structural features of the 0·6 kb segment of ColE1 origin region including the upstream flanking region of the *ori*. The closed triangle represents the *ori*, where DNA synthesis starts. Thin horizontal arrows starting from open triangles labelled $P_{RNA\,I}$ and $P_{RNA\,II}$ represent orientations of transcription under the control of promoters for RNA I (antisense RNA) and RNA II (primer RNA), respectively. (2) Formation of RNA II-template DNA hybrid (coupling). The 5'-end of RNA II has a unique secondary structure. (3) RNA II is hybridized with DNA at the *ori* and its downstream region is cleaved by RNase H. (4) DNA synthesis starts from the 3'-end of the primer RNA formed by the cleavage of RNase H at the *ori*. The solid arrow shows the direction of DNA synthesis. (3'). When antisense RNA (RNA I) is hybridized with RNA II at the 5'-region (pairing), the inhibition of hybrid formation of RNA II with template DNA occurs. DNA synthesis is therefore inhibited (Masukata and Tomizawa, 1984, 1986; Dasgupta, Masukata and Tomizawa, 1987).

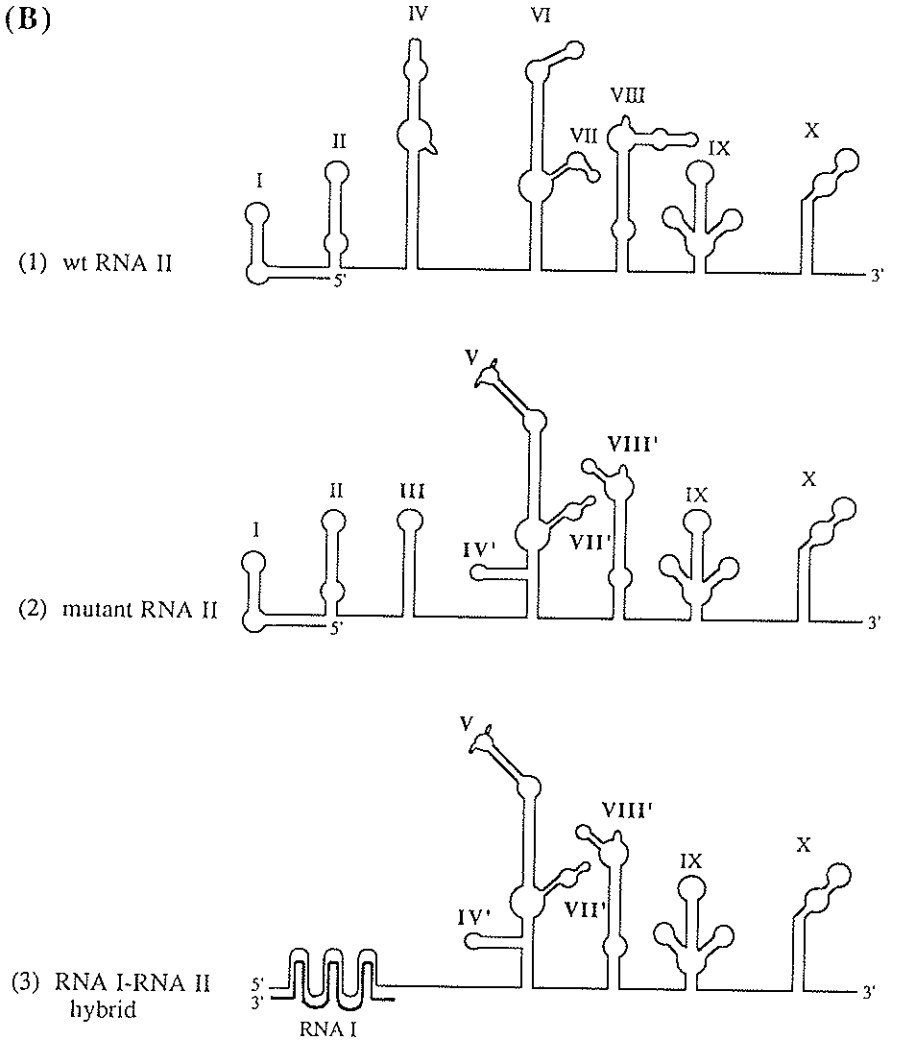

Figure 3. (B) Schematic illustration of the stem-loop structures of wild-type primer RNA (RNA II) of plasmid ColE1 and its inactive form. (1) The stem-loop structure of wild-type RNA II, which is the active form of RNA II for the initiation of DNA synthesis. (2) The inactive form of RNA II isolated from a mutant (*pri7*), which cannot make hybridization with DNA (pairing). (3) The RNA I-RNA II hybrid (pairing), which is inactive for initiation of DNA synthesis (Masukata and Tomizawa, 1986; Dasgupta, Masukata and Tomizawa, 1987).

DNA hybrid) are cleaved by RNase H at the *ori* and the downstream region of the replication origin to form a 555 bp mature RNA primer (Inoue and Uchida, 1991; *see Figure 3A(3)*). DNA synthesis by DNA polymerase I starts by adding deoxynucleotides to the 3′ OH ends of the primer RNA unidirectionally (*Figure 3A(4)*). The primer RNA–DNA hybrid is formed only when the nascent RNA II transcripts are present; no hybrid formation (coupling) is observed when RNA II is added exogenously (Masukata and Tomizawa, 1990).

Initiation of lagging-strand synthesis for pBR322 replication *in vitro* is dependent on the primosomal protein i encoded by *dnaT* (Masai and Arai, 1988). Early replicative intermediates with the newly synthesized leading strand immediately downstream of the replication origin accumulate in products synthesized in extracts from a *dnaT* strain that lacks primosomal protein i, or in wild-type extracts with anti-protein i antibody. An n′ site on the lagging strand is required for efficient replication of pBR322 DNA replication *in vitro*.

ColE1-type plasmids, including pBR322, do not require DnaA protein for replication (Hansen and Yarmolinsky, 1986; Kline *et al.*, 1986), but their rate of replication decreases in a host with a thermosensitive *dnaA* allele after a shift to the non-permissive temperature. These plasmids have at least one, and perhaps two, DnaA binding sites in the replication origin region. One site, 90 bp downstream of the origin of ColE1 and pBR322, is required for *in vitro* replication of pBR322 in the presence of DnaA protein and all primosome components except protein i. If protein i is included in the *in vitro* reaction mixture, DnaA protein is no longer required, but stimulates replication indirectly (Seufert and Messer, 1987; Ma and Campbell, 1988; Chiang, Xu and Bremer, 1991).

REGULATION OF INITIATION OF ColE1 DNA REPLICATION BY RNA I

Initiation of ColE1 DNA is mainly regulated by antisense RNA, or RNA I (Tomizawa, 1986). Synthesis of RNA I, consisting of 108 nucleotides, is initiated from the promoter for RNA I ($P_{RNA\ I}$) 445 bp upstream from the replication origin, proceeds in the opposite direction to RNA II, and ends a few bases before RNA II starts (Tomizawa, 1990; *see Figure 3A(1)*). The inhibition of hybrid formation of RNA II with DNA occurs by hybridization of RNA I with a complementary sequence in RNA II (Lacatena and Cesareni, 1981; Tomizawa and Itoh, 1981; Tomizawa *et al.*, 1981; Masukata and Tomizawa, 1986; *see Figure 3A(3′)*). Transcription of RNA I occurs more often than that of RNA II, because the promoter activity of the former, $P_{RNA\ I}$, is stronger than that of the latter, $P_{RNA\ II}$ (Lin-Chao and Bremer, 1987). Therefore a higher concentration of RNA I is present *in vivo* (Brenner and Tomizawa, 1991).

The hybridization (pairing) of RNA I and RNA II is dependent on the presence of the 5′ end of RNA I (Dooley, Tamm and Polisky, 1985; Tomizawa, 1986; Fitzwater *et al.*, 1988). When the 5′ end of RNA I is removed by RNase E, inhibition activity is reduced; that is replication of

ColE1 is stimulated (Tomcsanji and Apirion, 1985; Helmer-Citterich *et al.*, 1988). In an *E. coli relA* mutant, the level of RNA I seems to be regulated by interaction of complementary sequences of tRNAs, as the concentration of uncharged tRNAs increases in the cells (Hecker, Schroeter and Mach, 1986; Yavachev and Ivanov, 1988). The RNA I transcript has a tRNA-like conformation such as a clover-leaf structure composed of three stem-loops and a 5′ single-stranded tail of nine nucleotides (Morita and Oka, 1979; Tamm and Polisky, 1985; Yavachev and Ivanov, 1988). Similar palindrome structures (I, II and III in *Figure 3B*) are formed at the 5′-proximal region of the primer RNA transcript shortly after the start of the RNA II synthesis (Wong and Polisky, 1985; Tomizawa, 1986). The RNA I–RNA II hybrid is formed by interaction between specific bases of homologous single-stranded loops in RNA I and RNA II molecules (Lacatena and Cesareni, 1983; Tomizawa, 1986). This interaction brings together the single-stranded 5′-end of RNA I and its complementary RNA II sequence. The process of binding of RNA I to RNA II is a sequence of reactions producing a series of progressively more stable intermediates leading to the final product (Tomizawa, 1990).

HIGHER STRUCTURE OF PRIMER RNA (RNA II)

Palindrome structures of primer RNA or RNA II are shown (*Figure 3B*), and the secondary structure of the wild-type RNA II is compared to that of mutant (*pri7*) RNA II, which has no primer activity (Masukata and Tomizawa, 1986; *see Figure 3B(2)*). The essential difference between the two RNAs is that in the wild-type RNA II the structures III and V are absent, while in the mutant RNA II structure IV is absent, and structures III and V are formed instead. When RNA I is paired with the wild-type RNA II, the structure IV of RNA II cannot be formed (*Figure 3B(3)*). Pairing of RNA I to the binding region (5′-terminal region) of wild-type RNA II results in an alteration of the structures IV and VI (and probably VII and VIII) to form structure V (*Figure 3B(2,3)*). Thus, the structures VI and VII of wild-type RNA II play a role in hybridization with template DNA, and those I, II and IV (*Figure 3B(1)*) are not necessary.

The pairing of RNA I to RNA II and the inhibitory effect of RNA I are influenced by structural variations depending on the nascent primer transcript (Wong and Polisky, 1985; Ohmori, Murakami and Nagata, 1987). Growing RNA II transcripts of 110 to 360 nucleotides are sensitive to the inhibitory action of RNA I. Longer transcripts also interact with RNA I but the inhibition with it is not effective. As noted above, the structure IV of RNA II is altered by the hybridization with RNA I, but the secondary structures of the downstream region of RNA II are stable (Masukata and Tomizawa, 1986).

PROTEIN FACTOR ENHANCING RNA I–RNA II HYBRID FORMATION

Pairing of RNA I and RNA II is enhanced by a small protein dimer Rom (RNA of inhibition modulator) or Rop (repressor of primer) (Cesareni *et al.*,

1984; Lacatena *et al.*, 1984; Tomizawa and Som, 1984; Tomizawa, 1986). The Rom protein is encoded downstream from the replication origin of ColE1 DNA (Som and Tomizawa, 1983). The deletion of the non-essential *rom* gene as well as overproduction of Rom protein affects the plasmid copy number and incompatibility (Dooley, Tamm and Polisky, 1985; Nugent, Smith and Tacon, 1986). Rom protein enhances RNA I–RNA II binding, which depends on the length of the RNA II transcript. RNA I longer than 135 nucleotides binds strongly to RNA II in the presence of Rom protein (Perelson and Brendel, 1989). The binding starts with interaction between loops of RNA I and RNA II, which are fragments complementary to each other (Eguchi and Tomizawa, 1990). The target site of Rom protein is the stem-loop I of RNA I, where the structure III of RNA II hybridizes (Cesareni *et al.*, 1984; Dooley and Polisky, 1987; Eguchi and Tomizawa, 1990). Rom protein enhances complex formation by decreasing the rate of dissociation of the complex. Rom protein interacts with structures I, II and IV (*Figure 3B(1)*) of the RNA II transcript. The efficiencies of binding of Rom protein with RNA I and RNA II are similar (Helmer-Citterich *et al.*, 1988). The monomer of Rom protein consists of two α-helices, and the dimer forms an α-helix dipole by using a coiled-coil protein architecture (Banner, Kokkinidis and Tsernoglou, 1987; Castagnoli *et al.*, 1989). Owing to its symmetric structure, the dimer may function as an adaptor between RNA I and RNA II.

Replication of plasmid R1 (R100)

STRUCTURAL FEATURE OF THE REPLICATION ORIGIN REGION OF R1 GENOME

Plasmid R1 is a conjugative resistant low copy number IncII plasmid, has a molecular size of 9·0 kb, and is related to plasmid R100 or to the F factor of *Escherichia coli*. All genes required for replication and copy number control are within a 2·5 kb region (Nordström, Molin and Light, 1984; Rownd *et al.*, 1985). Three replication functions, *copA*, *copB* and *repA*, are encoded on the basic replicon of the R1 plasmid, and the R1 replication also relies on *E. coli* replication functions. The copy number of one or two per cell is determined by the availability of the RepA protein, which is rate-limiting for replication. The synthesis of this initiator protein is negatively controlled at both the transcriptional and post-transcriptional levels. RepA mRNAs are formed from two promoters, *CopB* promoter (P_{copB}) and *RepA* promoter (P_{rep}) (*Figure 4A*). The CopB RNA is constitutively transcribed. Its upstream region encodes the transcriptional repressor CopB, which shuts off the *repA* promoter almost completely. The main control system that is used to measure the concentration of the plasmid R1, and to adjust the replication frequency accordingly, acts post-transcriptionally (Light and Molin, 1983; Womble *et al.*, 1984). The key element is a small, unstable, untranslated RNA (CopA) which forms an RNA duplex with its complementary region in the RepA mRNA; the target for CopA binding (CopT) is upstream of the RepA coding

sequence. The kinetics of formation of the RNA duplex determines the copy number of the plasmid R1 (Givskov *et al.*, 1987; Perrson, Wagner and Nordström, 1988).

The formation of an RNA duplex leads to a decrease in the ability of the RepA mRNA to promote the synthesis of RepA protein. Computer simulation studies of RNA secondary structures indicate that the ribosome binding site of the RepA message is single-stranded in the free mRNA, but double-stranded (and therefore inaccessible to initiating ribosomes) when the upstream RNA duplex has been formed (Rownd *et al.*, 1985). However, *in vitro* studies have not provided any supportive evidence (Öhman and Wagner, 1989).

INITIATION OF REPLICATION OF PLASMID R1

R1 plasmid replicates through cairns-type intermediates and replication proceeds unindirectionally (Diaz and Staudenbauer, 1982b; Ohtsubo *et al.*,

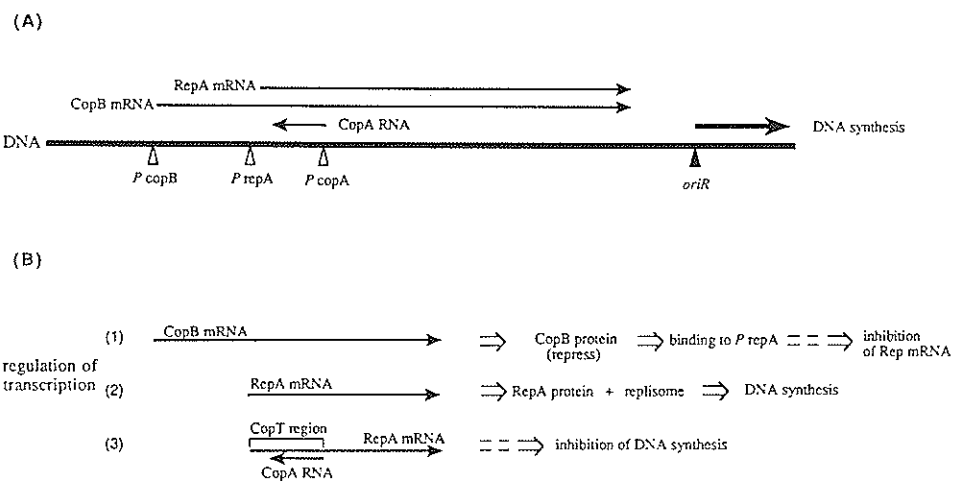

Figure 4. (A) Schematic illustration of the replication origin of R1 plasmid showing the structural features of the 2·5 kb segment of the R1 plasmid *oriR* region and the upstream flanking region of the *oriR*. The closed triangle represents the *oriR*, where DNA synthesis starts. The thick horizontal arrows show the direction of DNA synthesis. Thin horizontal arrows labelled with open triangles indicate the directions of transcription of CopA RNA, CopB mRNA and RepA mRNA, respectively. P_{copA}, P_{copB} and P_{repA} are promoters for CopA RNA, CopB mRNA and RepA mRNA, respectively.
(B) Regulation of transcription. (1) The CopB protein, the repressor, binds to the promoter of RepA (P_{repA}) to inhibit RepA mRNA synthesis. Consequently, initiation of DNA synthesis is inhibited. (2) The RepA protein together with host replisome results in initiation of DNA synthesis. (3) CopA RNA hybridizes with the CopT region of RepA mRNA. This RepA mRNA–CopA RNA hybrid cannot be used as a primer for initiation of DNA synthesis (Persson, Wagner and Nordström, 1988; Blomberg, Wagner and Nordström, 1990; Berzal-Herranz, Wagner and Diaz-Orejas, 1991).

1977). The replication origin of R1 plasmid is in a 188 bp sequence that is designated as *oriR* (Masai *et al.*, 1983). Replication of R1 plasmid starts about 400 bp downstream of *oriR*, proceeds unidirectionally *in vitro* (Masai and Arai, 1989), and depends on the plasmid-encoded RepA protein and host DnaA protein, both of which bind to sequences within *oriR* (Masai and Arai, 1987). *E. coli* DnaB, DnaC, DnaG and SSB proteins are also required for the plasmid R1 replication *in vitro* (Masai and Arai, 1987). It is possible that binding of RepA and DnaA proteins to *oriR* generates a conformational change of the *oriR*. This conformational change may allow the loading of the replisome, or the binding of the DnaB protein (helicase). Continuous leading-strand synthesis starts some 380 bp downstream of *oriR*. When a 418 base single-stranded DNA from position 1778 to 2195, derived from the leading-strand template, is used for the *in vitro* DNA template, the chimeric single-stranded DNA requires the single-strand DNA binding protein, primase, and DNA polymerase III holoenzyme. Furthermore, the priming occurs at a site identical to leading-strand initiation. These results suggest that leading-strand synthesis is primed by primase alone. Lagging-strand synthesis is specifically ended (at position 1515 or 1516) within *oriR*, preventing further leftward fork movement (Masai and Arai, 1989).

Plasmid R100, an R10related plasmid belonging to IncFII, has a replication origin region in a 284 bp fragment, which replicates unidirectionally as indicated for the R1 plasmid (Miyazaki *et al.*, 1988).

REGULATION OF INITIATION OF PLASMID R1 REPLICATION

The replication frequency of plasmid R1 is set by the availability of the rate-limiting protein RepA (Nordström, 1990). The expression of the *repA* gene, which is essential for replication of the R1 plasmid, is negatively controlled by an antisense RNA, CopA RNA (the *copA* gene transcript consisting of 91 nucleotides), which forms a duplex with the 5′-leader region of the RepA mRNA, the CopT RNA region (Berzal-Herranz, Wagner and Diaz-Orejas, 1991; *see Figure 4B*). The *copA* gene is contained within the DNA sequence coding for the RepA mRNA but has opposite polarity. Initially, the two molecules interact at their respective single-stranded loops. This contact is transient and facilitates initiation of hybridization at the 5′-end of CopA RNA and CopT RNA of plasmid R1 (Berzal-Herranz, Wagner and Diaz-Orejas, 1991). Binding between CopA RNA and CopT RNA proceeds in at least two steps, each of which has different requirements with respect to the structure and sequence of the RNAs involved. The rate of formation of the transient complex is rate limiting for the overall pairing reaction between CopA RNA and CopT RNA, both *in vitro* and *in vivo*. The initial transient interaction involves one stem-loop in each molecule. Complete duplex formation probably starts by base pairing between the single-stranded region located 5′ to the recognition loop in CopA, and its complementary region in CopT RNA. The binding reaction starts by an interaction between the single-stranded 5′-end of target RNA and its complementary sequence located in the loop domain of the antisense RNA in the insertion sequence

IS*10* of the composite transposon Tn*10* (Kittle *et al.*, 1989). However, in this case the site of initial interaction appears to be the same as the site at which stable duplex formation starts, and it is not known whether a transient complex is formed.

In plasmid R1, CopT RNA adopts a target stem-loop structure that permits CopA RNA to bind (Dong, Womble and Rowd, 1987). This binding affects the folding of the elongating CopT RNA such that the RepA SD region and the GUG start codon are sequestered. CopT RNA escaping the binding of CopA RNA is predicted to (a) refold into a CopA RNA-resistant conformation, and (b) have the ribosome binding region accessible in single-stranded loops. *In vitro* studies of the folding of CopA RNA shows that two loops of CopA RNA have their correspondence in CopT RNA and no major structural changes are found downstream of the duplex when CopA RNA is bound to its target RNA during transcription. Furthermore, a CopA/CopT RNA binding study indicates that the control region does not undergo drastic refolding (Öhman and Wagner, 1989; Perrson, Wagner and Nordström, 1988), in contrast to the case shown in plasmid NR1/R100 (Dong, Womble and Rownd, 1987).

A new regulatory mechanism has been postulated for plasmid pT181, which has a replication control region very similar to that of the IncFII plasmids (Novick *et al.*, 1989). In this case, the antisense RNA causes transcriptional attenuation, thereby preventing the synthesis of the RepC protein. The duplex between CopA RNA and CopT RNA is shown to be cleaved specifically by RNase III *in vivo* (Blomberg, Wagner and Nordström, 1990). Cleavage by RNase III seems to be a key event in the copy number control system of plasmid R1.

Regulation of replication is also affected by a protein, the product of the *copB* gene, that binds to the promoter of *repA* gene and represses its transcription (*Figure 4B*). Since the constitutively synthesized CopB is present at saturation levels, transcription from the *repA* promoter is normally completely repressed. Thus supplying additional copies of the *copB* gene on a high copy number vector has no effect on the *repA* miniplasmid copy number, hence the *copB* gene is not involved in IncFII incompatibility. The *copB* functions of plasmids R1, R100 and R6–5 have been compared (Nordström and Nordström, 1985), and it was found that deletion of the *copB* gene resulted in a 3·5-fold increase in the copy number of R100 and an eight-fold increase in the copy number of R1; these deletion mutants could be complemented only by homologous and not heterologous CopB. However, when the *copB* gene was left intact, R100 and R1 both had the same stringently controlled copy number. Thus, although there are two structurally different forms of the CopB protein, they show an analogous function in the RepA replication system. These alternate forms of the *copB* gene are therefore alleles, and will be referred to as *copBI* (the R1 allelic form) and *copBII* (the R100 and R6-5 allelic form). A RepA-like replication system, Rep1, in the IncFI plasmid ColV2-K94 or its kanamycin resistant derivative, has been identified (Weber, Mitra and Palchaudhuri, 1984). This replicon has been found to be structurally and functionally homologous to the RepA

replicon of R1, but differences in the nucleotide sequence of the *copA* gene have caused a loss of IncFII incompatibility in Rep1 (Weber and Palchaudhuri, 1986). The *copB* open reading frame of Rep1 may code for a protein essential for normal copy number control of pNS12. This *copB* mutant has an approximately 10-fold increase in copy number. The CopB-phenotype of ColV2-K94 could be complemented in *trans* by the *copB* gene of co-resident IncFII plasmids such as R1 and R538, but not R100, suggesting that ColV2-K94 and R1 or R538 contain the same *copB* allele (Banerjee, Weber and Palchaudhuri, 1990).

Replication of pSC101 DNA

STRUCTURAL FEATURE OF THE REPLICATION ORIGIN REGION OF pSC101 GENOME

Plasmid pSC101 is a low copy number R plasmid (formerly R6-5 or Tc6-5) and has a molecular size of 9·26 kb (Bernardi and Bernardi, 1984; Scott, 1984; Kües and Stahl, 1989; *see Figure 5A*). pSC101 DNA starts replication at the replication origin region unidirectionally (Yamaguchi and Yamaguchi, 1984a, b), and this region consists of 250 bp. For the pSC101 DNA replication a *trans*-acting initiation protein (37·5 kDa), RepA, is required (Churchward, Linder and Caro, 1983; Vocke and Bastia, 1983b; Armstrong *et al.*, 1984). The replication origin region contains a DnaA box, two 13-mer repeats (the same sequence found in *oriC* of *Escherichia coli*), and an 84% A+T-rich region that contains a binding site for the integration host factor (IHF) (Gamas *et al.*, 1986). Three 18 bp direct repeats, which are the binding sites for the RepA protein (Yamaguchi described Rep) encoded by the plasmid pSC101, are adjacent to the A+T-rich region (Armstrong *et al.*, 1984; Yamaguchi and Yamaguchi, 1984a). Three RepA protein binding sites are also found in front of the *repA* gene, which is the opposite side of the origin region (Vocke and Bastia, 1983a; Linder *et al.*, 1985; Yamaguchi and Masamune, 1985).

INITIATION OF REPLICATION OF pSC101 DNA

An initiator protein, RepA, binds to the three direct repeats in the replication origin region (Vocke and Bastia, 1983b; Vocke and Bastia, 1985; Yamaguchi and Masamune, 1985). This binding would be the start of a replisome formation (Funnell, Baker and Kornberg, 1987; Bramhill and Kornberg, 1988a), as it is considered that the RepA protein directs the formation of a complex with host proteins necessary for the initiation event of DNA replication. The host DnaA protein is essential for initiation of pSC101 DNA replication (Felton and Wright, 1979; Frey, Chandler and Caro, 1979; Hasunuma and Sekiguchi, 1979) as well as that of *E. coli* DNA (Fuller, Funnell and Kornberg, 1984; Messer *et al.*, 1991). The initiator protein, DnaA, interacts specifically with a 9 bp consensus sequence, the DnaA box, in *oriC* of the *E. coli* chromosome. This causes the local unwinding of an

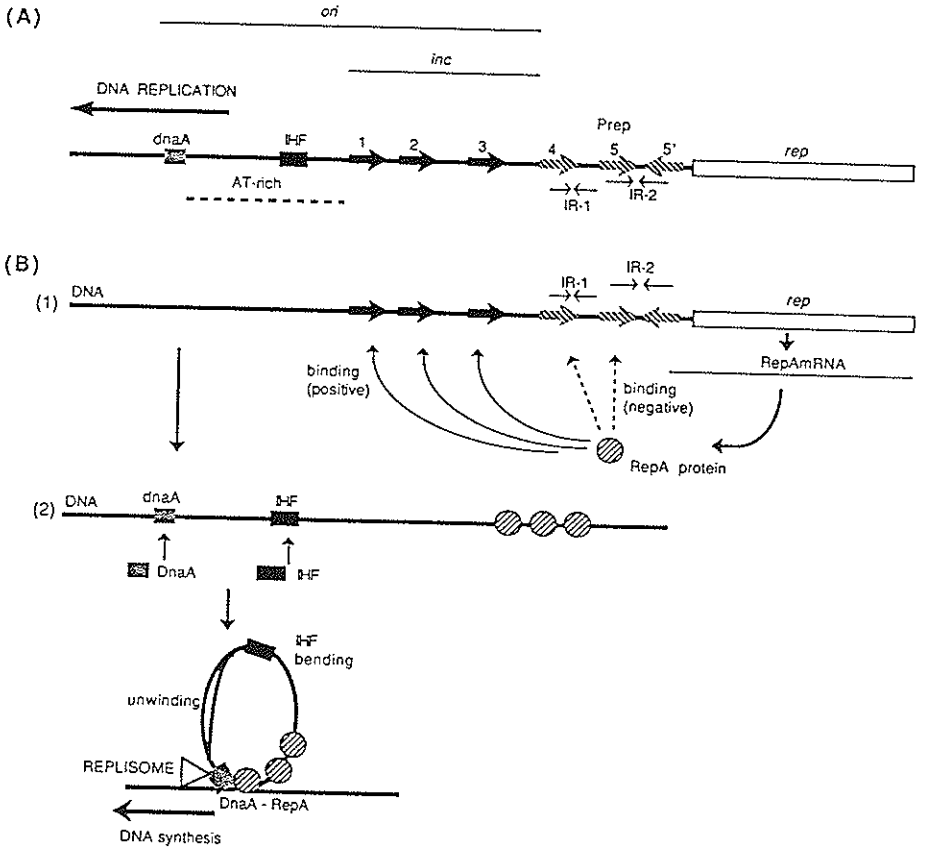

Figure 5. (A) Schematic illustration of the replication origin of plasmid pSC101. Thick horizontal open arrow represents the direction of DNA synthesis. Thin horizontal bars labelled *ori* and *inc* represent minimum origin and incompatibility, respectively. An open box labelled dnaA represents the DnaA protein binding sequence. IHF represents the integration host factor binding sequence. The horizontal broken line labelled 'AT-rich' represents an 84% A+T stretch. Thick closed arrows and thick hatched arrows with numbers are three direct repeat and additional repeat sequences, respectively. Thin horizontal arrows labelled IR-1 and IR-2 are the sequences with dyad-symmetry. A thin horizontal bar labelled P_{rep} is the putative promoter of the *repA* gene. The open box labelled rep represents the structural gene of the *repA* protein (Yamaguchi and Masamune, 1985).
(B) Autoregulation of initiation of plasmid pSC101 DNA replication. (1) Binding of the RepA protein to the three direct repeat sequences results in replication initiation (positive regulation), while binding of the RepA protein to the other repeat sequences reduces the frequency of initiation (negative regulation). (2) Binding of the IHF protein to the IHF sequences causes bending of the *ori* region, which enables the assembly of the protein factors, DnaA, DnaB and DnaC, essential for initiation of DNA synthesis (Linder *et al.*, 1985; Yamaguchi and Masamune, 1985).

A+T-rich region, which allows the binding of DnaG primase (Messer, 1987; Seufert and Messer, 1987; Gille and Messer, 1991).

The replication origin region of pSC101 DNA contains an AT-rich region which alters the DNA structure by bending (Koo, Wu and Crothers, 1987; Stenzel, Pastel and Bastia, 1987; Tan and Harvey, 1987). This bending is enhanced by binding of the IHF (Stenzel, Pastel and Bastia, 1987), which is an essential host factor in pSC101 DNA replication (Gamas *et al.*, 1986).

Therefore, DnaA and RepA proteins bound in corresponding recognition sites with respect to the origin region may be brought close enough to interact with each other to form a DNA–protein complex (*Figure 5B*). In this complex are assembled replisome components such as DnaB and DnaC proteins. These proteins are essential for pSC101 DNA replication (Frey, Chandler and Caro, 1984; Hasunuma and Sekiguchi, 1979). Bending at replication origins and the formation of DNA–protein complexes are also known in *E. coli oriC*, bacteriophage λ, and plasmid R6K, with unwinding of the double-stranded DNA (Dodson *et al.*, 1985; Mukherjee, Patel and Bastia, 1985; Echols, 1986; Zahn and Blattner, 1987; Bramhill and Kornberg, 1988a; Gille *et al.*, 1991).

The three tandem repeats of the 13-mer in the *oriC* region of *E. coli* near a DnaA box are the sites where the opening of the duplex is initiated. The DnaB, a helicase, assembled at the DnaA box, recognizes the unwound DNA region and moves in both directions along the DNA helix to separate the two strands (Bramhill and Kornberg, 1988a). In the replication origin region of pSC101 the same 13-mer repeat is found near the DnaA box. The unwinding by DnaB of the *ori* region from the 13-mer sequence might occur before the synthesis of primer RNA. It may be that for the synthesis of primer RNA, RNA polymerase is not used, because the promoter that has been found is oriented only in the opposite direction to replication (Churchward, Linder and Caro, 1983; Funnell, Baker and Kornberg, 1987). On the other hand, sequences homologous to the DnaG binding site of bacteriophage G4 (Sakai, Komano and Godson, 1985, 1987; Sakai *et al.*, 1988; Hiasa, Sakai and Komano, 1989; Hiasa *et al.*, 1989a, b, 1990) are found in both DNA strands (Churchward, Linder and Caro, 1983; Yamaguchi and Yamaguchi, 1984a). As it is reported that maintenance of plasmid pSC101 requires the host primase (Ely and Wright, 1985), it might be used for primer RNA synthesis in both leading-strand and lagging-strand syntheses.

REGULATION OF INITIATION OF pSC101 DNA

The RepA protein plays a positive role in the initiation of DNA synthesis by binding to the three direct repeats of the replication origin region. This allows the formation of a replisome in co-operation with the DnaA protein (Vocke and Bastia, 1985; Yamaguchi and Masamune, 1985; *Figure 5B*). The RepA protein has a function in the regulation of the transcription of the *repA* gene, that is, initiation of pSC101 DNA replication is regulated by the intracellular concentration of RepA protein. The repA protein exists as a dimer and binds preferentially to two nearly dyad-symmetric sequences overlapping the promoter of the *rep* gene (Linder *et al.*, 1985; Vocke and Bastia, 1985; Yamaguchi and Masamune, 1985). The replication origin of the pSC101 contains direct repeat sequences similar to the dyad-symmetric sequences, but one order of magnitude higher concentration of the RepA protein is required to bind to the origin repeats (Yamaguchi and Yamaguchi, 1984b). The RepA protein competes with RNA polymerase for the *repA* promoter sequence in dyad-symmetric sequences, and inhibits the transcription of the

repA gene. The *repA* gene is thus autoregulated by the RepA protein. The binding of RepA protein to the promoter precedes that to the direct repeat in the replication origin region; the concentration of RepA protein may be maintained at a critical level to keep the correct plasmid copy number (Sugiura *et al.*, 1990; Yamaguchi and Masamune, 1985). A protein factor that promotes binding of purified RepA to the direct repeat sequence is found in the *E. coli* extract (Sugiura *et al.*, 1990). In the presence of the factor, DNA fragments containing the direct repeat sequence can form a specific DNA–protein complex by the addition of low concentrations of the RepA protein. In contrast, the DNA containing an inverted repeat sequence in the promoter region loses its binding activity for the RepA protein upon incubation with the factor. The extensively purified factor is identified as exonuclease III, and this enzyme action is necessary for binding of RepA protein to the direct-repeat region of DNA. This binding of RepA protein to duplex DNA treated with exonuclease III is direct-repeat specific (Fueki and Yamaguchi, 1991a, b). Since the RepA protein cannot bind to the single-stranded direct-repeat sequence, partial single-stranded regions around the direct-repeat sequence are required for the binding of RepA protein (Vocke and Bastia, 1983b).

E. coli mutants defective in exonuclease III permit the replication of pSC101 (Vocke and Bastia, 1985). It is unlikely that creating the single-stranded region downstream of the direct-repeat sequences is necessary for plasmid replication, rather the structure produced by exonuclease III *in vitro* resembles the one required for binding of the RepA protein *in vivo*. Such a structure might be found by unwinding of the downstream region of the direct-repeat sequences.

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